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Chapter 6

STRUCTURE AND FUNCTION OF THE PRIMARY CILIUM

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ABSTRACT

Primary cilia are microtubule-based organelles that extend from the apical surface of most mammalian cells. They have evolved to function as cellular antennas that receive and transmit signals from the extracellular environment such as mechanical stimuli, light, proteins and chemicals. The sensory functions of the primary cilia are highlighted by their link to the human genetic diseases termed ciliopathies, which are characterized by a multitude of symptoms including cystic kidney disease, neurodevelopmental abnormalities, obesity, retinal degeneration among other developmental ones. Over the past decade, the earlier ultrastructural description of the cilia has been dissected at the molecular level. Importantly the underlying mechanisms of the assembly and disassembly of cilia, maintenance of the ciliary composition and the disease mechanisms of ciliopathies have started to emerge with unexpected

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complexity. In this chapter, we will present an integrative viewpoint on the structural features of the cilium that contribute to its function, discuss the components and interactions that assemble and maintain a functional cilium and conclude with its signaling functions and disease links. For each section, we summarize the mechanisms and describe the current status of our understanding by focusing on the recent discoveries and the emerging technologies in the field.

THE PRIMARY CILIUM STRUCTURE

The primary cilium is a non-motile microtubule-based structure that protrudes from the apical surface of cells during growth arrest. Sorokin and colleagues first described primary cilia on fibroblasts and smooth muscle cells. Since then, these structures have been observed on almost all mammalian cells (Sorokin, 1962). Ultrastructural studies of the primary cilium have revealed unprecedented complexity for its structure and morphology (Fisch and Dupuis-Williams, 2011). The primary cilium size usually varies in length from 1 to 5 μm with a diameter of approximately 0.2 μm that gets narrower towards its tip (Scherft and Daems, 1967). Very short potentially mechanosensitive primary cilia were described in osteocytes (Uzbekov et al., 2012; Uzbekov and Benhamou, 2014). Their small length (below 300 nm) was probably associated with the hard bone environment of these cells.

At the core of the primary cilium is a microtubule-based axoneme composed of nine radially arranged doublet microtubules **(Figure 1)**. Surrounding the ciliary axoneme is the ciliary membrane that is continuous with the plasma membrane, but molecularly distinct from it in terms of protein and lipid content. The organization of the ciliary microtubules is traditionally abbreviated as (9x2 + 0) axoneme, referring to nine peripheral doublet and no central singlet microtubules. This organization is distinct from the (9x2 + 2) axoneme of motile cilia. A microtubule doublet consists of a complete A-tubule of 13 protofilaments and an adjacent incomplete B-tubule of 10 protofilaments (Pedersen et al., 2012). The protofilaments are polymerized from alpha-beta-tubulin heterodimers longitudinally in the

presence of GTP. Ciliary tubulins are modified by a combination of various posttranslational modifications including acetylation, glutamylation and detyrosination, which together regulate ciliary stability (Janke and Bulinski, 2011).

The ciliary axoneme is nucleated by the older of the two centrioles of the centrosome, the mother centriole, which transforms into a basal body during primary cilium formation. The interphase centrosome is composed of two orthogonally arranged centrioles that are connected by 2-4 striated rootlets at their proximal ends (Figure 1) (Bettencourt-Dias et al., 2011; Kim and Dynlacht, 2013). The centrioles are evolutionarily conserved symmetrical cylindrical structures of nine triplet microtubules. At the onset of ciliogenesis, the centrosome migrates to the cell surface and the 9-fold symmetrical radial microtubule triplets of the mother centriole template the ring of the nine axonemal microtubules. Interestingly, the microtubule triplets of the basal body transition to the microtubule doublets at the distal end of the basal body, which forms the core of the ciliary axoneme (Figure 1). Why and how this transition from microtubule triplets to doublets happens during ciliogenesis is not known. Moreover, in most cilia, the B-tubules of the microtubule doublets terminate towards the distal part of the cilium, which leaves singlet A-tubules. The length of this singlet region varies between cell types (Bertelli and Regoli, 1994; Satir, 1965; Satir, 1968). Distal to the singlet region of microtubules is the ciliary tip, which has been implicated in regulation of ciliary length, IFT remodeling and Hedgehog signaling.

Despite their highly conserved size and structures, the two centrioles of the centrosome differ in structural details, age and function (Nigg and Stearns, 2011). The older (mother) centriole, but not the younger (daughter) centriole, has unique structural components that contribute to the primary cilium structure (Figure 1). First, the mother centriole bears nine distal appendages that interact with the apical plasma membrane, and nucleates the formation of the ciliary axoneme that pushes the plasma membrane outward to form the primary cilium as it grows.

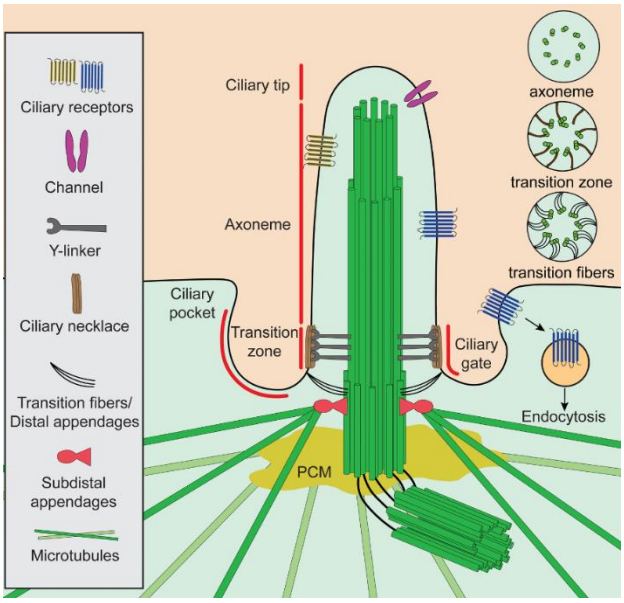

Figure 1. Primary cilium structure. The primary cilium protrudes from the apical side of quiescent cells and is composed of a basal body, an axoneme and a ciliary membrane. The centrosome is composed of two centrioles and the associated pericentriolar material (PCM). Mother centrioles are the older of the two centrioles that bear distal and subdistal appendages. Basal bodies are mother centrioles that have been modified by the addition of the accessory structure and are connected to the younger daughter centrioles by striated rootlets at their proximal ends. During ciliogenesis, the 9-fold symmetrical microtubule triplets of the basal body nucleate the microtubule doublets, which transitions to singlet microtubules towards the distal part of the cilium. Distal to the singlet microtubules is the ciliary tip, which is specialized compartment for regulating ciliary length, trafficking and signaling. The ciliary gate is the most proximal region of the primary cilium and is composed of the transition fibers and the transition zone. Transition fibers originate from the distal appendages of the basal body and connect the outer surface of the triplet microtubule barrel to the plasma membrane. Distal to the transition fibers is the transition zone, which is composed of the Y-shaped linkers and ciliary necklace, and functions as a diffusion barrier and selective gate for the transport of soluble and membrane-bound components into and out of cilia. Y-shaped linkers connect the outer doublet microtubules of the axoneme to the ciliary membrane. The ciliary necklace consisting of circumferential parallel strands of intramembrane particles encircles the base of the axoneme. The proximal region of the cilium is surrounded by a cytoplasmic invagination of the periciliary membrane, termed the ciliary pocket, which exhibits endocytic activity and interacts with the actin cytoskeleton.

Second, proximal to the distal appendages of the mother centriole are 1 to 13 (usually 2-5) subdistal appendages (Uzbekov and Alieva, 2013), that project laterally from the sides of the triplet microtubule barrel. The basal feet of the basal bodies in the ciliary epithelia are homologues of the subdistal appendages. Transmission Electron Microscopy (TEM) studies identified microtubule ends embedded in the ends of the basal feet, which suggests a function for these structures in anchoring a subset of microtubules to the base of the cilium (Bornens, 2002).

Ciliary Compartments

Several distinct compartments in the primary cilium have been defined based on morphology and localization of specific proteins and here we will discuss the structure and function of three major compartments, which are ciliary gate, ciliary pocket, ciliary tip.

The "ciliary gate" is the most proximal region of the primary cilium and is composed of two distinct structural domains: the transition fibers and the transition zone (Figure 1). The transition fibers originate from the distal appendages of the basal body and connect the outer surface of the triplet microtubule barrel to the plasma membrane (Anderson, 1972). In TEM cross-sections, transition fibers are visualized as a pinwheel-like structure. Although the precise function and composition of transition fibers is not known, they are proposed to serve as a pore complex for regulating transport of vesicles and macromolecules in and out of cilia or as a docking platform for the transport factors of the ciliary building blocks (Deane et al., 2001; Rosenbaum and Witman, 2002). Distal to the transition fibers is the transition zone, where the microtubule triplets of the basal body transitions to the microtubule doublets of the axoneme. The transition zone is composed of the "Y-shaped linkers" that connect the outer doublet microtubules of the axoneme to the ciliary membrane, and the ciliary necklace consisting of circumferential parallel strands of intramembrane particles that encircle the base of the axoneme (Goncalves and Pelletier, 2017; Szymanska and Johnson, 2012). Like transition fibers,

the transition zone is proposed to regulate ciliary composition by regulating intracellular trafficking to and from the cilium.

The proximal region of the cilium is surrounded by a cytoplasmic invagination of the periciliary membrane, termed "ciliary pocket" in many cell types and organisms (Figure 1) (Ghossoub et al., 2011; Molla-Herman et al., 2010). Ciliary pocket is reminiscent of the flagellar pocket, a plasma membrane invagination at the base of the flagellum specialized in vesicular trafficking to and from the flagella in Trypanosomatids (Field and Carrington, 2009). In mammalian cells, the ciliary pocket is an endocytic membrane compartment that interacts with the actin cytoskeleton. Importantly, clathrin-coated vesicles form around the ciliary pocket, suggesting that this compartment is important for the docking of the vesicles originated from the secretory pathway or from endosomes (Molla-Herman et al., 2010).

The distal region of the cilium is a specialized compartment called the "ciliary tip" (Figure 1). This compartment has recently emerged as an important regulator of cilium assembly by adding and removing tubulin subunits at the plus ends of axonemal microtubules, ciliary trafficking by remodeling IFT and ciliary signaling by recruiting the Hedgehog signaling components Gli2/3 and SuFu upon Hedgehog pathway activation (Satish Tammana et al., 2013). Despite its implication in these important processes, the ultrastructure, composition and dynamics of the ciliary tip remain poorly understood.

PRIMARY CILIUM ASSEMBLY AND DISASSEMBLY

The key steps of ciliogenesis have been described by numerous ultrastructural studies that date back to 1959 to the description of this pathway in vertebrate ciliary photoreceptors (Tokuyasu and Yamada, 1959). Based on the model suggested by Sorokin and colleagues in 1962, the first step in ciliogenesis is the localization of a large Golgi-like vesicle, ciliary vesicle, to the distal end of the mother centriole (Sorokin, 1962). This vesicle flattens to form a double membrane sheath around the

axoneme and later fuses with the plasma membrane. Subsequent studies added more steps to this pathway and identified the molecular components and dynamics of these different steps.

Two distinct ciliogenesis pathways have been described in mammalian cells, which are distinguished by whether the basal body migrates to the cell surface or not at the onset of ciliogenesis (Figure 2). In the "extracellular" pathway, which is described for epithelial cells, the basal body migrates and docks to the apical surface of the plasma membrane. This precedes the assembly of the axoneme that pushes the plasma membrane out to form the cilium. In the "intracellular" pathway, which is described for mesenchymal cells, fibroblasts and neuronal precursors, ciliary vesicles associate with the distal appendages of the mother centrioles and axoneme elongates concomitantly with the recruitment and fusion of ciliary vesicles with the plasma membrane, which protrudes the cilium from the cell surface.

The cilium assembly pathways are highly ordered processes composed of distinct phases (Figure 2) (Pedersen et al., 2012; Sanchez and Dynlacht, 2016). Cilium assembly initiates when cells exit the mitotic cycle in response to mitogen deprivation and differentiation cues. In the intracellular cilium assembly pathway, several Golgi-derived small periciliary vesicles accumulate around the distal appendages of the mother centriole at the onset of ciliogenesis. A recent study showed that dynein mediates Myosin Va-associated periciliary vesicle transportation to the pericentrosomal region along microtubules, followed by myosin Va-mediated periciliary vesicle transportation to the distal ends of the mother centrioles along the centrosome-associated branched network (Wu et al., 2018). Periciliary vesicles fuse to form the "ciliary vesicle" on the distal end of the mother centriole followed by elongation of the axoneme underneath this cap. As vesicles fuse with the ciliary vesicle, the growing nascent axoneme is encased in a double membrane containing the ciliary sheath. Finally, this nascent cilium docks to the plasma membrane through fusion with the ciliary sheath to form a cilium protruding from the cell surface.

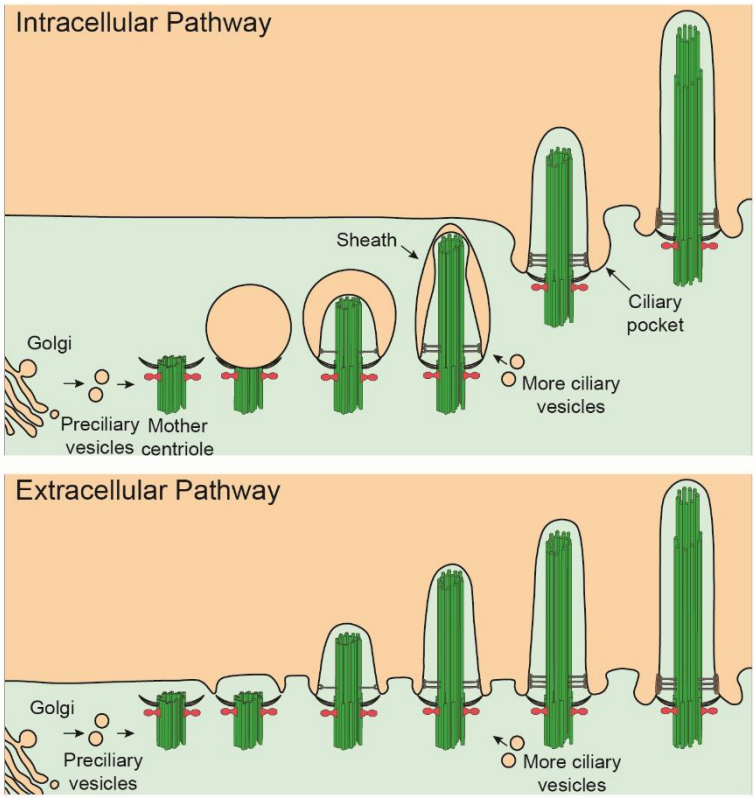

Figure 2. Primary Cilium Assembly Pathways. There are two distinct ciliogenesis pathways described in mammalian cells. The cilium assembly pathway is a highly ordered process composed of distinct phases and is divided into two different pathways based on whether or not the basal body migrates to the cell surface or not at the onset of ciliogenesis. In the intracellular cilium assembly pathway, at the onset of ciliogenesis, Golgi-derived small periciliary vesicles accumulate around the distal appendages of the mother centriole. Periciliary vesicles fuse to form the ciliary vesicle and concomitantly the axoneme elongates from the mother centriole through microtubule polymerization. The growing nascent axoneme is encased by a double membrane structure termed the ciliary sheath. When nascent axoneme migrates to the plasma membrane, the ciliary sheath fuses with the plasma membrane for extending the cilium from the cell surface towards the extracellular environment. In the extracellular pathway, the basal body migrates and docks to the apical surface of the plasma membrane followed by the assembly of the axoneme that pushes the plasma membrane out to form the cilium.

In contrast to the intracellular pathway, the extracellular pathway starts with docking of the mother centriole to the plasma membrane, where the cilium grows directly towards the extracellular space through axoneme elongation (Figure 2).

Cilium disassembly is initiated by mitogen stimulation that results in entry into cell cycle. Studies in mammalian cells identified two distinct phases of the cilium assembly pathway, the first one that occurs in G1 phase shortly after cells exit cell cycle and the second one that occurs prior to mitosis (Sanchez and Dynlacht, 2016). The scaffolding protein HEF1 and calcium-calmodulin activate Aurora A kinase, which then phosphorylates and stimulates the histone deacetylase HDAC6. This results in cilium disassembly through two major activities (Plotnikova et al., 2012; Pugacheva et al., 2007). First, it deacetylates modified, stabilized tubulin within the ciliary axoneme. Second, it deacetylates cortactin that then enhances actin polymerization (Ran et al., 2015). Complementing the HDAC6-mediated destabilization, two members of the Kinesin 13 family of the depolymerizing kinesins Kif2a and Kif24 were also implicated in depolymerization of axonemal microtubules (Kobayashi et al., 2011; Miyamoto et al., 2015). In contrast to cilium assembly, much less is understood about the mechanism of cilium disassembly. Future studies on the high-resolution spatiotemporal imaging of cilium disassembly will be critical in visualizing the dynamics of this process. Moreover, development of genetic tools or chemical inhibitors will be necessary in order to conclude whether cell cycle reentry is the cause or consequence of cilium disassembly.

Parts List for the Primary Cilium

Precise understanding of the structure, function and regulation of the primary cilium requires the identification and characterization of the ciliary proteome. Once the complete ciliary proteome is identified, time-resolved interaction maps between the ciliary proteins are required to elucidate the underlying mechanisms of the dynamic ciliary processes like ciliary

trafficking and signaling. A combination of bioinformatics, genomic and proteomics approaches and genetic screens over the years identified over 800 proteins associated with primary cilium.

Majority of the ciliary proteins were identified using multiciliated and flagellated cells and tissues as starting material using a combination of approaches. First, the proteomic analysis of cilia purified from human bronchial epithelial tissue culture (Ostrowski et al., 2002) and *Tetrahymena thermophile* cells (Smith et al., 2005), and flagella purified from *Chlamydomonas reinhardtii* (Pazour et al., 2005) and *Trypanosoma brucei* (Broadhead et al., 2006) together helped define the ciliary parts list and identified the genetic causes of ciliopathies. Second, transcriptomic profiling of cells (Blacque et al., 2005) and bioinformatics analysis of the *Caenorhabditis elegans* genes regulated by Regulatory Factor X (RFX)-type transcription factor or harboring X-box consensus sequence in their promoter identified a catalogue of ciliary genes (Efimenko et al., 2005; Swoboda et al., 2000). Finally, comparative genome analysis of ciliated and non-ciliated organisms (Avidor-Reiss et al., 2004; Li et al., 2004) identified the list of proteins that are required for cilia assembly and function independent of their localization to cilia. Although the comparative genomics of ciliated and non-ciliated organisms were successful in identifying the highly conserved structural proteins of the cilia, they were not able to identify many signaling components including the Hedgehog pathway components, as these pathways are not conserved beyond animals. A recent study by Sigg and colleagues overcame this problem by identifying and comparing the ciliary proteomes from sea urchins, sea anemones and choanoflagellates, which are organisms at the key phylogenetic nods of animal evolution (Sigg et al., 2017). This evolutionary proteomics study identified a core, shared ciliary proteome that illuminated the ancestry of ciliary signaling proteins including G protein Coupled Receptor (GPCR), Hedgehog and Transient Receptor Potential (TRP) channel signaling pathways. While these studies together contributed significantly to the components of the primary cilium that are shared with the motile cilia such as the structural components of the

axoneme, they were not sufficient to identify the primary cilium-specific components that are required for its specialized sensory functions.

Identification of the mammalian primary cilia proteome studies faces two significant challenges: First, due to the low abundance of ciliary material, biochemical purifications of the intact primary cilium in sufficient quantities and purity for mass spectrometry analysis have been difficult. Second, cilium is a very dynamic structure and most of the ciliary interactions are transient and insoluble. These interactions cannot be detected with standard biochemical approaches. The first proteomic analysis of the primary cilia from mammalian cells was reported only after primary cilia from mouse kidney cells were purified by developing a purification approach based on a previously published calcium-shock method (Ishikawa et al., 2012; Mayer et al., 2008; Rhein and Cagan, 1980). Multidimensional protein identification technology (MudPIT), protein correlation profiling (PCP) and subtractive proteomic analysis of the purified cilia identified 195 proteins as part of the ciliary proteome (Ishikawa et al., 2012). The mammalian ciliary proteome was complemented with the proteomic analysis of specialized sensory cilia from the mouse photoreceptor cells (Liu et al., 2007) and rat olfactory cells (Mayer et al., 2009; Mayer et al., 2008), and the unique, multiple (9x2 + 0) cilia in choroid plexus epithelial cells (CPECs) of the juvenile swine (Narita et al., 2012) to comprehensively identify over 2000 candidate proteins as part of non-motile cilia. Interestingly, the proteome of CPECs and photoreceptor cilia had a significant overlap with each other, but not with the proteome of motile cilia, indicating that this group might represent sensory cilia-specific proteins. Among the proteins identified by proteomics studies of the sensory cilia and the mammalian primary cilia, the signaling factors and membrane proteins of the cilia including the BBSome complex components, polycystin-1 and polycystin-2 were underrepresented, suggesting that this approach had sensitivity problems in detecting transient and insoluble ciliary components and that ciliary components had been lost during biochemical purifications. The challenges in identification of such interactions were in part overcome by the

application of the innovative proximity-labeling approaches for the identification of the ciliary proteome.

Proximity labeling is an emergent technology that makes use of enzymes that generate free biotin radicals for temporally and spatially labeling the proximity partners of proteins of interest fused with these enzymes (Chen and Perrimon, 2017). The mutant biotin ligase BirA* and the engineered ascorbate peroxidase APEX2 are two of these enzymes that have been successfully applied for proximity mapping of different cellular compartments. APEX2-catalyzed proximity labeling has been successfully used for identifying the ciliary proteome by two different studies (Kohli et al., 2017; Mick et al., 2015). Both studies made use of cilia-targeted labeling enzymes, the fusions of ciliary targeting sequences of the G-protein-coupled receptor Htr6 or NPHP3 with the APEX2 enzyme. Biotinylation of the ciliary proteins by activation of cilia-targeted-APEX2 with H_2O_2 and biotin-phenol incubation followed by streptavidin pulldowns and mass spectrometry identified about 200 candidate ciliary proteins (Figure 3).

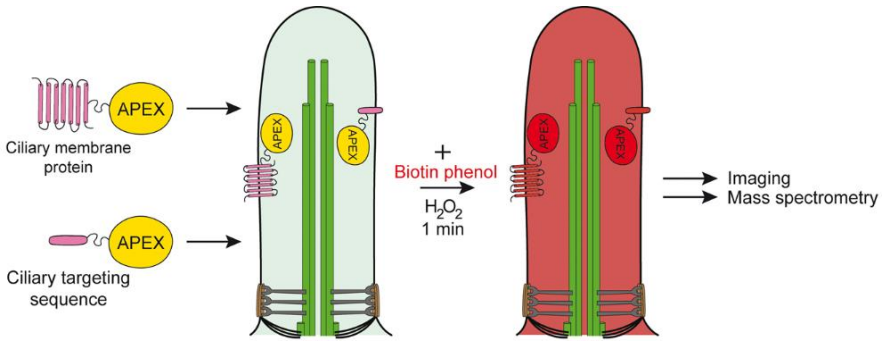

Figure 3. Identification of the ciliary proteome by APEX2-based proximity labeling. The engineered ascorbate peroxidase APEX2 is fused to a ciliary targeting sequence or a ciliary membrane protein and targeted to the cilium. Biotinylation of the ciliary proteins is activated in cells expressing this fusion protein by incubation with H_2O_2 and biotin-phenol for one minute, which is followed by streptavidin pulldowns and mass spectrometry.

While the APEX2-fused ciliary targeting sequence of NPHP3 probed both the ciliary membrane and the shaft during biotinylation, APEX2-fused

Htr6 specifically probed the interactions at the ciliary membrane, and thus these two proteomes are valuable resources for compiling the dynamic ciliary proteome. The ciliary proteome identified by proximity labeling include the stable or transient ciliary proteins, including components of the intraflagellar transport (IFT) and Bardet Biedl Syndrome (BBSome) complexes that mediate intraciliary trafficking, structural components and ciliary membrane proteins, as well as previously unrecognized interactions. One of the novel ciliary signaling mechanisms revealed by cilia-APEX studies is the intraciliary AC6/cAMP/PKA signaling axis, where PKA regulates Hedgehog activity by phosphorylating Gli3 in the primary cilium (Mick et al., 2015). Another novel mechanism revealed by cilia-APEX studies is the identification of a number of actin-binding proteins in the cilium, which might be required to stabilize cilia and the ciliary membrane during dynamic changes in ciliary length (Kohli et al., 2017). Moreover, these approaches proved to be successful in the quantitative analysis of the proteomic composition of the cilium in Ift27$^{-/-}$ mouse kidney cells and in cells treated with actin depolymerization drugs. These analyses revealed new mechanisms for the pathways regulated by Ift27-mediated trafficking and actin-regulated ciliary processes (Kohli et al., 2017; Mick et al., 2015). Taken together, proximity-based proteomics of the cilia has provided to be highly efficient and sensitive, and holds great promise for future studies in screening for changes in the ciliary interior upon stimulation with different signals and in affected ciliopathy tissues.

MECHANISMS FOR CILIARY COMPARTMENTALIZATION

Primary cilium play important roles in dynamic cellular processes and its proper function in these processes require tight spatiotemporal regulation of the ciliary content. The unique ciliary content likely enables efficient molecular interactions, which is critical for initiation of signal transduction in response to extracellular stimuli.

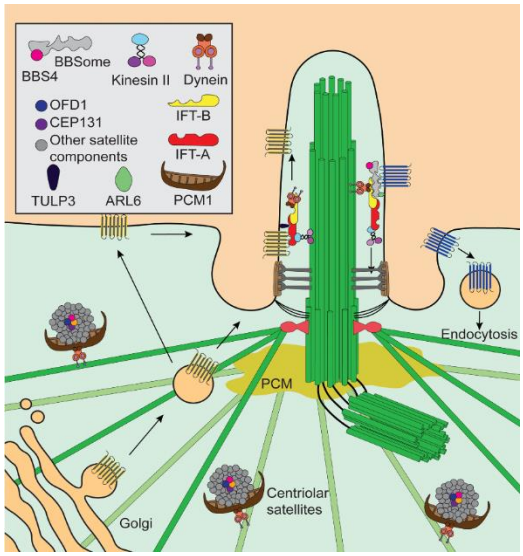

Figure 4. Overview of Trafficking Pathways involved in Ciliary Compartmentalization. The assembly, maintenance and disassembly of the primary cilium require integration of multiple pathways that mediate entry of proteins to the cilium exit of proteins from the cilium and retention of proteins at the cilium. Most ciliary proteins are recruited to the periciliary membrane by vesicular trafficking, recycling endosomes and lateral diffusion. Vesicular trafficking delivers vesicles from the trans Golgi network (TGN) to the ciliary base, where vesicles fuse with the periciliary membrane in a SNARE-dependent manner. Recycling endosomes serve as an intermediate sorting compartment and segregates proteins to the ciliary pocket of to the apical plasma membrane. Finally, some proteins are recruited to the ciliary membrane through lateral diffusion from the plasma membrane to the periciliary membrane. When proteins are at the ciliary base, they enter the cilium through intraflagellar trafficking (IFT). IFT pathway starts with binding of ciliary cargoes to anterograde IFT-A/TULP3 trains near the basal body, their movement to the ciliary tip by IFT-A/TULP3 and release from the IFT-A/TULP3 at the tip and ends with the trains reorganizing at the tip and returning to the cell body by retrograde IFT-B with new cargo. The eight subunit BBSome complex functions in regulated exit from cilia. Activated GPCRs lead to accumulation of BBSome at the ciliary tip and processive retrograde BBSome trains form in an Arl6-dependent manner. Activated GPCRs are removed from the cilium by a BBSome-mediated retrieval pathway. The least understood ciliary recruitment pathway is mediated by the centriolar satellites, an array of granules that localize and move around the centrosome/cilium complex in a microtubule and molecular motor-dependent manner. Satellites are macromolecular complex assemblies that are scaffolded by PCM1 and they function as intermediate sites in delivering proteins such as BBS4 to the cilium and ODF1 to the centrosome.

The ciliary membrane is continuous with the plasma membrane, yet the cilium has a unique protein and lipid composition that dynamically changes in response to mechano- and chemosignals. Moreover, protein synthesis does not occur inside the cilium and thus active protein trafficking into and out of the cilium is required for assembling and maintaining a functional cilium. Since the identification of the cilium as a sensory organelle, there has been a lot of effort in elucidating the mechanisms that make the primary cilium a specialized compartment. The current picture is highly complex that integrates coordination of multiple mechanisms for protein entry into the cilium, protein exit out of the cilium and protein retention at the cilium, which are discussed above.

Transition Zone

Transition zone functions as a diffusion barrier and selective gate for the transport of soluble and membrane-bound components into and out of the cilia (Figure 4). The first evidence for the gatekeeper function of the transition zone for the cilium was reported by Spencer and colleagues, whose work aimed to study the dynamic behavior of rhodopsin between the inner and outer segment of the vertebrate photoreceptor cells (Spencer et al., 1988). Although rhodopsin normally functions at the outer segment and is restricted there, it freely diffused to the inner segment when photoreceptor cells were mechanically disrupted to lose their separation by the connecting cilium, demonstrating that an intact transition zone limits this diffusion. This result was corroborated by the studies on the *Chlamydomonas* gametes, which adhered to each other through the active agglutinins in their flagella, but not through the ones found on the body (Hunnicutt et al., 1990). The presence of two different agglutinin pools in *Chlamydomonas* is the classical evidence for the presence of a functional barrier at the base of the cilium. Supporting these results are the subsequent studies in different model systems including the fluorescence recovery after photobleaching (FRAP) analysis of ciliary protein dynamics

that identified no fluorescence recovery for these proteins when the whole cilium is bleached in contrast to their fast recovery when part of the cilium is bleached (Chih et al., 2011; Hu et al., 2010), as well as altered ciliary protein content when the transition zone proteins were disrupted in mammalian cells by and *C. elegans* by RNAi and knockouts (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Williams et al., 2011). The crucial function of the transition zone as a selective diffusion barrier, which was convincingly demonstrated by various experiments, has led to questions on the underlying molecular mechanism of this function and also how disruption of this function causes ciliopathies.

MKS, NPHP and CEP290 are the three main modules of the transition zone that were identified by genetic studies in *C. elegans* and the biochemical characterization of protein-protein interactions (Chih et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011). These modules are composed of multiple soluble and membrane proteins that interact and depend on each other for localization to the transition zone for assembling and maintaining a functional transition zone. Adding to the list of transition zone components are the proteomics studies of the transition zones isolated from cell walls of *Chlamydomonas* after axoneme disassembly by biochemical purifications (Diener et al., 2015) and from Trypanosome by affinity purifications (Dean et al., 2016). *Chlamydomonas* and Trypanosome transition zone proteomes identified proteins specific to these organisms as well as orthologs of candidate ciliopathy genes. Finally, the Biotin Identification (BioID) proximity screens performed in non-ciliated and ciliated human embryonic kidney epithelial cells with transition zone proteins as baits identified extensive proximity interactions for these proteins with components of other centrosomal and ciliary structures. This screen showed that some of these interactions were detected independent of cilia formation and interactions between the three transition zone modules increased in ciliated cells (Gupta et al., 2015). The identification of a nearly complete parts list for the transition zone through these studies has provided the leads for the research on elucidating how

these components come together through spatial and temporal interactions to assemble a functional transition zone.

Emergent technologies in high-resolution imaging have proven to be very important in visualizing the structures like transition zone that are below diffraction limit of conventional microscopes; thus, their application to the known transition zone components revealed their spatial organization in different cell types. Spatial locations of CEP290, RPGRIP1L, MKS1, TCTN2, CBY and TMEM67 relative to each other were determined to generate an architectural map of the transition zone using stimulated emission depletion (STED) superresolution microscopy combined with electron microscopy in RPE1 cells (Yang et al., 2015) and 3D superresolution structured illumination microscopy (3D-SIM) in *Drosophila* spermatocytes (Pratt et al., 2016). Analysis of the Trypanosome transition zone by fluorescence microscopy and electron microscopy determined the presence of distinct domains within the transition zone (Dean et al., 2016), in agreement with the different transition zone complexes revealed by the BioID proximity mapping studies (Gupta et al., 2015). Future studies are required to determine how these interactions change temporally in response to signals, in ciliated cells of different tissues and in ciliopathy patient samples.

Intraflagellar Transport

Intraflagellar transport (IFT) is the movement of material into and out of the cilium and is a critical process for assembling, maintaining and disassembling cilia. IFT is highly conserved among a wide range of eukaryotes. Defects in IFT structure and function disrupt biogenesis of cilia and flagella. IFT was first observed as the bidirectional movement of granule-like particles along the *Chlamydomonas* flagellum using differential interference contrast (DIC) microscopy (Kozminski et al., 1993) and subsequent work using various imaging approaches identified IFT in other model systems including *C. elegans* (Hao et al., 2009),

Tetrahymena (Jiang et al., 2015), *Trypanosoma* (Santi-Rocca et al., 2015) and mammalian cells (Williams et al., 2014). Through electron microscopy studies, "trains" of IFT particles were observed as linear arrays of granules move between the ciliary membrane and the underlying axonemal outer doublet microtubules. A combination of fluorescence and electron microscopy analysis of *Chlamydomonas* flagella showed that IFT particles move anterogradely along B-microtubules of the outer doublets, whereas retrogradely along A-microtubules, explaining in part why these complexes simultaneously traffic inside cilia without crashing into each other (Stepanek and Pigino, 2016).

The components of the IFT machinery were identified mostly using mutants in various model organisms, such as the flagellar assembly (fla) mutants in *Chlamydomonas* (Adams et al., 1982; Huang et al., 2013), chemosensory (che), osmotic avoidance (osm), dye-filling (dyf) and dauer-formation (daf) mutants in *C. elegans* (Inglis et al., 2007). Recent work on biochemical mapping and the structural studies of the complex interactions has contributed to an emerging model for how the complex subunits interact to assemble functional trains (Nakayama and Katoh, 2018). IFT is composed of two distinct multisubunit subcomplexes termed IFT-A and IFT-B (Figure 4). IFT-B is a complex of 16 subunits and mediates anterograde trafficking from the ciliary base to the tip by heterotrimeric kinesin-2 motor proteins. IFT-A is a complex of 6 subunits plus TULP3 and mediates retrograde trafficking by cytoplasmic dynein-2 complex. Although the emerging model for IFT pathway is becoming more complex with each new advance in the field, the basic IFT pathway starts with binding of ciliary cargoes to IFT near the basal body, their movement to the ciliary tip by anterograde IFT and release from IFT at the tip, and ends with the trains reorganizing at the tip and returning to the cell body by retrograde IFT with new cargo.

Protein transport by IFT can be divided into four steps, which are cargo loading, admission into the cilium, translocation along the shaft, and unloading. IFT cargoes are defined as proteins that move to cilia by IFT but are not required for IFT assembly and function themselves (Figure 4). They include proteins that traffic from the cell body to their ciliary

assembly sites including tubulin, radial spoke proteins, inner- and outer-dynein arms, IFT motor proteins including dynein and kinesin, and proteins associated with ciliary signaling. Given that anterograde transport is critical in supplying the proteins required for cilia biogenesis, disruptions of IFT-B complex proteins result in cilia formation defects. Moreover, defects in IFT proteins that function in the transport of signaling molecules are associated with developmental defects. For example, IFT88 and IFT21 mice mutants have Hedgehog-related phenotypes due to defects in ciliary localization of Smoothened, Gli2 and Gli3 proteins. Also IFT122 mutants are defective in Opsin transport in mouse photoreceptors that results in retinal defects (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). While cargo adaptors, factors stabilizing cargoes on the trains and projection domains have been implicated in loading of specific cargo (Lechtreck, 2015), our current understanding of IFT and cargo interactions is incomplete. IFT-cargo interactions need to be studied for each specific cargo given the underlying complexity and heterogeneity of these interactions.

Finally, a number of IFT subunits such as IFT88 and IFT27 has been described for their cilia-independent functions during cell-cycle regulation, mitosis, cell migration, microtubules stability and orientation (Yuan and Sun, 2013). Future studies are required to dissect the molecular mechanism of the nonciliary functions of IFT that will reveal exciting avenues for uncovering the complexity of IFT.

BBSome Complex

Bardet-Biedly syndrome (BBS) is a ciliopathy characterized by multiple symptoms including retinal degeneration, polydactyly, cystic kidneys, obesity and is linked to mutations affecting 22 reported genes to date. BBSome complex was first purified from bovine retina and identified as an obligate complex of eight different BBS syndrome proteins using tandem affinity purification of BBS4 from mammalian cells (Figure 4) (Nachury et al., 2007). The octameric BBSome complex was later purified

from various sources including bovine retina (Jin et al., 2010), *Chlamydomonas* (Lechtreck et al., 2009), *Paramecium* (Valentine et al., 2012) and *Trypanosoma* (Langousis et al., 2016), indicating that this complex is highly conserved in ciliated organisms that use their cilia for sensory functions. The eight subunit BBSome complex is very stable as it does not separate into subcomplexes when treated with harsh buffers and disassembles only when any of its subunits is disrupted (Jin et al., 2010).

Mutations in BBSome components in different organisms are all associated with ciliary signaling defects, rather than structural defects in the cilium assembly reported for IFT mutants. In agreement with such sensory function, BBSome is lost in ciliated organisms that use cilia only for motility (Carvalho-Santos et al., 2010). Among the signaling defects of BBSome mutants are the defects in Hedgehog signaling pathway, which is the most studied and understood signaling pathway associated with the cilia to date. For example, mutations in genes encoding BBSome proteins exacerbate the patterning defects like polydactyly and exencephaly of cilia-mutant mice (Goetz et al., 2017; Yee et al., 2015) and result in skeletal patterning abnormalities in zebrafish (Tayeh et al., 2008). Two recent genome-wide CRISPR screens for Hedgehog regulators both identified all BBSome subunits as high scoring hits (Breslow et al., 2018; Pusapati et al., 2018), which supports the previous studies that showed defects in Hedgehog-induced transcriptional response in mammalian cells (Zhang et al., 2012). Finally, disrupted BBSome function results in inability to mount an infection and evade the host immune system in *Trypanosome* and *Leishmenia* and swimming defects away from high light dosages in *Chlamydomonas* (Langousis et al., 2016; Price et al., 2013). The regulatory functions of the BBSome complex in Hedgehog signaling and other yet unidentified sensory signaling pathways in part must be the defective mechanisms underlying the developmental symptoms of BBS.

BBSome complex moves both in retrograde and anterograde directions in *Chlamydomonas*, mammalian cells and *C. elegans*, suggesting possible roles in ciliary import and/or exit (Nigg et al., 2014). It was initially proposed to function in ciliary import of proteins based on experiments where the Somatostatin Receptor 3 (SSTR3) and Melanocortin

concentrating hormone receptor 1 (MCHR1), Polycystin 1 (PC1), and Smo were lost from cilia in cells defective for the BBSome function or Arl6 function (Berbari et al., 2008; Jin et al., 2010; Zhang et al., 2011). This function was further supported by the direct interaction between the BBSome complex and these ciliary targets (Klink et al., 2017; Seo et al., 2011; Su et al., 2014). The ciliary import function of the BBSome complex was challenged by further studies that identified its role in the ciliary exit of the membrane-associated phospholipase D (PLD) from cilia in *Chlamydomonas* (Lechtreck et al., 2013; Lechtreck et al., 2009) and dopamine receptor (Drd1), Sstr3, GPr161, Smo and the Hedgehog receptor Patched 1 in mammalian cells (Klink et al., 2017; Seo et al., 2011; Su et al., 2014; Ye et al., 2018; Zhang et al., 2012).

Recent studies has convincingly limited BBSome function to ciliary exit, instead of a dual function in both ciliary entry and exit, and identified IFT-A/TULP3 complex as the major mediator of ciliary entry for membrane proteins (Figure 4). Real-time imaging of the ciliary receptors showed that activated GPCRs were lost as packed in ectosomes from ciliary tips when BBSome-mediated ciliary exit was inhibited. These results suggested that decreased ciliary levels of signaling receptors reported in BBS mutants is likely due to increase in ectocytosis rather than import defects (Ye et al., 2018). Moreover, a wide variety structurally diverse ciliary membrane proteins including sixteen different GPCRs, the polycystin complex PC1/PC2, the cytoprotein pKDH1, Smo was shown to physically interact with IFT-A complex components and to be recruited to cilia by the tubby family protein TULP3 that functions as a general adapter for ciliary trafficking (Badgandi et al., 2017; Fu et al., 2016; Hirano et al., 2017). Given that BBSome moves both in retrograde and anterograde directions, it is proposed to effectively capture and bind to its cargoes only in the anterograde direction either constitutively as in the case of PLD or in a regulated manner as in the case of Gpr161 and Sstr3. Future studies dissecting the molecular machinery and the signals that regulate the affinity of the BBSome to its different cargoes are required to reveal a more complete picture of the intraciliary trafficking.

Centriolar Satellites

Centriolar satellites are an array of 70-100 nm membrane-less granules that localize around the centrosomes and cilia in animal cells (Figure 4) (Barenz et al., 2011). They move along microtubules in a molecular-motor dependent manner and have been implicated in various centrosome and cilium-mediated cellular processes. The best-characterized function of satellites is their role in cilium assembly as many satellite proteins including their scaffolding protein PCM1 were shown to inhibit or interfere ciliogenesis. Despite their well-established role in cilium assembly, the molecular mechanism of action for satellites in this process is not known. Given their movement along microtubules, satellites have been proposed to regulate transport of proteins to or away from the centrosome/cilium complex. While this function has been reported for a wide variety of proteins with functions ranging from cell cycle progression to pericentriolar material recruitment and assembly, such function for satellites has only started to be discovered. The function of satellites in regulation of ciliary targeting has most been studied for the BBSome component BBS4, which localizes to centriolar satellites in unciliated cells and redistributes to the primary cilium in ciliated cells (Figure 4) (Nachury et al., 2007). Cells depleted for satellites components PCM1, CCDC66, Cep72 and Cep290 have significant defects in the ciliary recruitment of BBS4 (Conkar et al., 2017; Nachury et al., 2007; Stowe et al., 2012). Two models have been proposed to explain the function of satellites in ciliary recruitment of BBS4. The first model is the molecular motor-based trafficking of satellite-BBS4 complex to the cilium. Supporting this model, BBS4 interacts with PCM1 and $p150^{Glued}$ subunit of dynein/dynactin complex, identifying BBS4 as a bridging factor between satellites and dynein (Kim et al., 2004). The second model is the possible sequestration function for satellites that stores BBS4 in unciliated cells and releases it to form the BBSome complex in ciliated cells (Stowe et al., 2012). Future studies are required to distinguish between these models and to determine the exact function of satellites in regulation of the ciliary composition.

Vesicular Trafficking

Vesicular transport of lipids and proteins from the trans-Golgi network (TGN) and recycling endosomes to the ciliary base is an important mechanism of assembling and maintaining the cilium (Figure 4). Ciliary membrane biogenesis and ciliary targeting of membrane proteins are both regulated by components of the vesicular trafficking pathways such as Rab GTPases, their cognate GTPase-activating proteins (GAPs) and ADP-ribosylation factor (ARF)-like small GTPases (ARLs) (Sung and Leroux, 2013). The small G protein ARF4 for rhodopsin and fibrocystin trafficking, the GGA1 adapters for PC1/2 trafficking and the Tulp3 and tubby adapters for trafficking of multiple GPCRs are among the well-characterized components of the secretory pathway with functions in ciliary trafficking (Pedersen et al., 2016).

Vesicles budding from the TGN are directed to a specific docking site at the periciliary base where coordinated interactions between the Rab family small GTPases related to the exocyst complex tether the vesicles to the periciliary membrane. The small GTPases Rab11 and Rab8 mediate vesicular transport to the ciliary based where the guanine nucleotide exchange factor Rabin 8 binds to the exocyst complex, which then tethers vesicles to the periciliary membrane. Following tethering, vesicle fusion with the periciliary membrane is likely to be mediated by v-SNARE and t-SNARE interactions. Once the membrane proteins are incorporated to the periciliary membrane, they then cross the ciliary gate and enter the ciliary membrane. In addition this pathway, lateral diffusion or transport of proteins from the apical plasma membrane into the ciliary membrane were reported for Smo, but since this report, there has not been other proteins shown to be transported through this lateral pathway, indicating that it might be a specific transport pathway for Smo.

Ectosomes

Ciliary proteins are expelled from the tip of the cilium via extracellular vesicles termed ectosomes, which are now recognized as an important

pathway for ciliary trafficking and function (Figure 5). While the precise role of ectosomes is not known, recent studies have assigned functions for ectosomes in maintaining ciliary composition by disposing selected proteins from the cilium, mediating intercellular communication through exchange of proteins and genetic material, and remodeling of the extracellular space (Wood and Rosenbaum, 2015). Ectosomes were first described in *Chlamydomonas* by electron microscopy as membrane-bound vesicles budding from the flagella that function in budding (Bergman et al., 1975). Subsequent studies defined these vesicles as bioactive for they contain lytic enzymes required for digesting the mother cell wall and allowing post-mitotic hatching of the daughters (Wood et al., 2013). In *C. elegans*, ciliary ectosomes are shed and released by ciliated sensory neurons, which play a role in modulating animal-to-animal communication (Wang and Barr, 2016). Ectosomes were recently identified for their function in forming the light-sensitive discs of the specialized photoreceptor cilium. In photoreceptor cells, the cilium has an innate ability to produce massive amounts of ectosomes. The release of these ectosomes are blocked by the membrane protein peripherin that retains them at the cilium and shapes them into the photoreceptor discs of the outer segment through elongation and flattening (Molday and Goldberg, 2017; Salinas et al., 2017). In mammalian cilia, activated GPCRs that fail to undergo BBSome-mediated retrieval from the cilia concentrate into membranous buds at the ciliary tip and then are released into ectosomes. Interestingly, actin/myosin 6/drebrin mediates the scission of ectosomes from the cilia tips (Figure 5). Moreover, Hedgehog signaling fails when both ectocytosis and retrieval are blocked by inhibition of the actin-regulated machinery (Nager et al., 2017).

Given the emerging functions of ectosomes in ciliary processes, there has been significant interest in dissecting the underlying molecular mechanism of biogenesis, shedding and release of ectosomes. In addition to the actin-regulated machinery identified in mammalian cells, studies in *C. elegans* and *Chlamydomonas* identified IFT for its role in these processes.

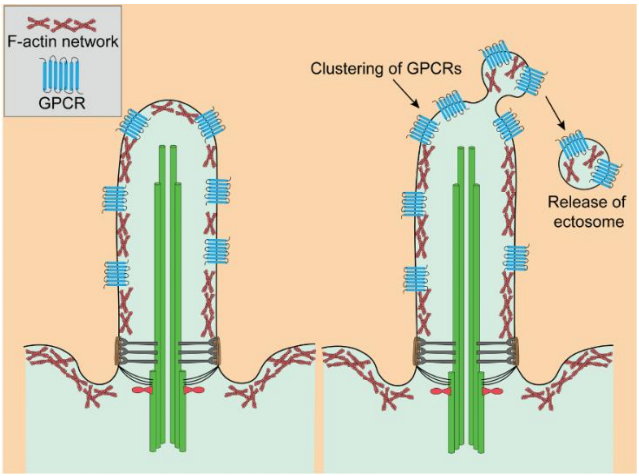

Figure 5. Ectosome release from the ciliary tip. Ciliary proteins are expelled from the tip of the cilium via extracellular vesicles termed ectosomes. Ectosomes function in maintaining ciliary composition by disposing selected proteins from the cilium, mediate intercellular communication through exchange of proteins and genetic material, and remodel the extracellular space. In mammalian cilia, activated GPCRs that fail to undergo BBSome-mediated retrieval from the cilia concentrate into membranous buds at the ciliary tip, which are released into ectosomes. Scission of ectosomes from the ciliary tips is mediated by an actin-regulated machinery that is composed of actin, myosin 6 and drebrin.

The proteome of the ectosomes purified from *Chlamydomonas* flagella is different from the flagellar membrane proteome. Ectosomes comprise a subset of flagellar membrane proteins, the endosomal sorting complex required for transport (ESCRT) proteins, small GTPases and ubiquitinated proteins, corroborating the function of ectosomes in cell signaling and turnover of ciliary proteins (Long et al., 2016). Ciliary ectocytosis is an emerging new field and future work on these structures has potential to reveal their function and mechanism of action in physiological and pathological contexts.

PRIMARY CILIUM AS THE SENSORY ORGANELLE

Primary cilium is a specialized compartment for localization of components linked to sensory perception and multiple signaling pathways;

thus is considered as the cellular antenna. The first signaling pathway associated with cilia is the one that induces gamete activation and cell-cell fusion in *Chlamydomonas* during fertilization through interactions between agglutinin on the cilia of the plus and minus gametes (Wang et al., 2006). Since then, many important signaling pathways have been linked to the primary cilium. About 25 rhodopsin-family GPCRs, proteins linked to polycystic kidney disease including TRP-channel family proteins PC/2, the single-pass transmembrane protein fibrocystic and sonic hedgehog (Shh) family components including Smoothened (Smo) and the orphan GPCR Gpr161 localize to the primary cilium in a dynamic manner (Wheway et al., 2018). In addition to the Hedgehog signaling, a diverse array of other signaling pathways including Hedgehog, Wnt, Notch, Hippo, GPCR, PDGF (and other receptor tyrosine kinases including FGF), mTOR, and transforming growth factor beta have been linked to the cilium. The key functions of the primary cilium in various signaling pathways is highlighted by the diseases including ciliopathies and cancer that are linked to defects in the structure and function of the cilium (Reiter and Leroux, 2017).

The best-characterized signaling pathway mediated by the primary cilium is the vertebrate Hedgehog (Hh) pathway **(Figure 6)** (Bangs and Anderson, 2017). Unlike other developmental signaling pathways linked to the primary cilium, Hh signaling is completely dependent on the primary cilium in mammalian cells, which therefore fail to respond to Hh in the absence of primary cilia. The key discovery that linked the primary cilium to Hedgehog signaling came from genetic screens for mouse embryonic patterning mutants that identified IFT proteins among the mutated genes (Huangfu et al., 2003). The regulation of Hh signaling is complex where multiple factors traffic into and out of cilia in response to pathway activation by secreted ligands, which are Sonic hedgehog (Shh), Indian (Ihh) and Desert (Dhh) in mammalian cells (Figure 6). In the absence of ligand, the Shh receptor Patched (Ptch1) localizes to the cilium and it inhibits the activity of the seven-pass transmembrane protein Smoothened (Smo), which is stored in vesicles in the cytoplasm. The main effectors of the Hedgehog signaling Gli2 and Gli3, kinesin-4 motor protein Kif7 and

Suppressor of Fused (SuFu) are present in low levels at the ciliary tip, where Gli2/3 are processed to their repressor forms and Hedgehog target gene transcription is turned off (Pedersen and Akhmanova, 2014). When cells receive the Hh signal, Shh binds to Ptch1, causing the Ptch1 to exit the cilium concomitantly with Smo ciliary entry and accumulation of Kif7, Gli2/3 and SuFu at the ciliary tip. Gli2/3 are processed at the ciliary tip by posttranslational modifications to their full-length activator forms, which exit the cilia and enter the nucleus to activate transcription of downstream Hh target genes. The switch between the repressor and activator forms of Gli2/3 has been shown to be tightly regulated by sequential phosphorylation and dephosphorylation events by kinases PKA, GSK-3β and CK1. Regulation of dynamic ciliary localization of the Hh pathway components is very important for the pathway activity and IFT complex mediates the movement of these components into and out of the cilium.

Although Hh pathway has been a major focus of many studies, biochemical steps leading from ligand binding to target gene activation in Hh signaling are still far from clear. Identification of Hedgehog regulators in an unbiased way was recently achieved by two different genome-wide functional CRISPR screens, which both identified positive and negative Hedgehog regulators with high sensitivity. The single guide RNA lentiviral library was combined with an engineered Hh-pathway-sensitive reporter for antibiotic-based selection by Breslow and colleagues (Breslow et al., 2018) and with a fluorescence-based transcriptional reporter for phenotypic enrichment by Pusapati and colleagues (Pusapati et al., 2018). These screens generated datasets that serve as a valuable resource for the discovery of new components for Hh signaling and consequently for the associated diseases such as ciliopathies and other developmental ones. While the function of the primary cilium in Hedgehog signaling has been addressed by many studies, there are still many remaining questions regarding the role of primary cilium in specialized cells and different organisms. Importantly, the function of the primary cilium in signaling pathways other than Hedgehog signaling is poorly understood and future studies are required to address this question.

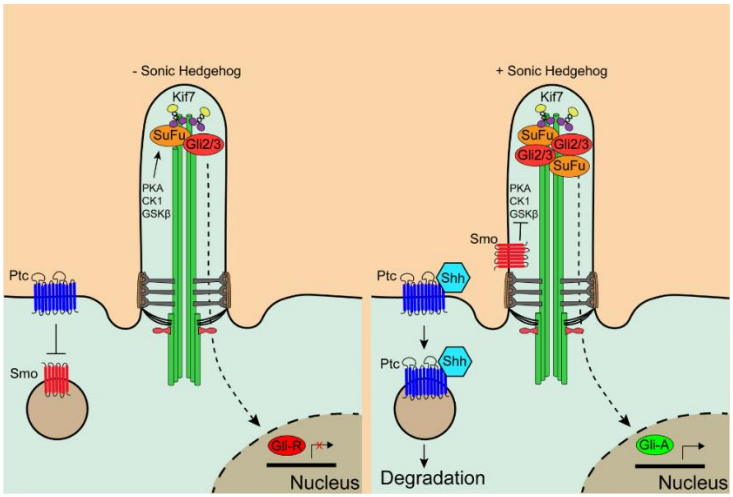

Figure 6. Hedgehog Signaling Pathway. The core components of the Hedgehog signaling pathway localize to the primary cilium in a tightly controlled and activation-dependent manner. In the absence of Hedgehog ligand Sonic Hedgehog (Shh), the G protein coupled receptor Patched (Ptc1) localizes at the ciliary membrane and inhibits the localization of the transmembrane receptor protein Smoothened (Smo) to the cilium. Transcription factors Gli2 and Gli3 along with Sufu and Kif7 accumulate at the ciliary tip, where they are processed by PKA, CK1 and GSKβ into a "repressor" form (Gli-R). Gli-R enters the nucleus and represses transcription of downstream Hedgehog target genes. When cells receive the Shh, Shh binds to Ptc1, which then internalized and is targeted for degradation. Activated Smo can now localize to the cilium, where it inhibits PKA, CK1 and GSKβ. This allows Gli2 and Gli3 to be processed into "activator" form (Gli-A), which enters the nucleus and activates transcription of downstream Hedgehog target genes.

CONCLUSION

Research over the past several decades has elucidated the broad, diverse and complex ways of primary cilium assembly, maintenance and function using a combination of genetics, proteomics, developmental biology and cell biology approaches. The primary cilium has turned out to be fascinating, complex and directly linked to human diseases including the ciliopathies and cancer. It has become clear that almost all major signaling pathways in vertebrates converge on the primary cilium, leading to the adoption of the term "the cell's antenna" for describing primary

cilium. A deeper understanding of the mechanisms that control cilia formation *in vivo* and the dissection of biochemical steps of the Hedgehog pathway in future will provide insight into the development diagnostic and therapeutic tools in both ciliopathies and cancer.

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