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The ciliary diffusion barrier: the gatekeeper for the primary cilium compartment

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Abstract

The primary cilium is a cellular antenna that detects and transmits chemical and mechanical cues in the environment through receptors and downstream signal proteins enriched along the ciliary membrane. While it is known that ciliary membrane proteins enter the cilium by way of vesicular and intraflagellar transport, less is known about how ciliary membrane proteins are retained in, and how apical membrane proteins are excluded from the cilium. Here, we review evidence for a membrane diffusion barrier at the base of the primary cilium, and highlight the recent finding of a septin cytoskeleton diffusion barrier. We also discuss candidate ciliopathy genes that may be involved in formation of the barrier, and the role of a diffusion barrier as a common mechanism for compartmentalizing membranes and lipid domains.

Keywords

primary cilium; ciliary membrane proteins; ciliopathy; diffusion barrier; basal body; ciliary pocket; ciliary necklace; transition zone; septins; GTPases

Introduction

Cilia are rod-like membrane projections of several microns length on the apical surface of many, if not all cell types in vertebrates (Pazour and Bloodgood 2008). They are composed of a cylindrically organized microtubule axoneme that emanates from a centriole-derived structure called the basal body (Marshall 2008; Satir and Christensen 2007). The ciliary membrane surrounds the axoneme and is contiguous with the surrounding plasma membrane (Fig. 1).

Cilia are assembled and maintained through a bidirectional transportation system called intraflagellar trafficking (IFT) mediated by IFT complexes and molecular motors moving along axonemal microtubules (Fig. 1) (Ishikawa and Marshall 2011; Pedersen and Rosenbaum 2008; Pigino et al. 2009; Rosenbaum and Witman 2002). IFT regulates cilia assembly, resorption and signaling, and defects in IFT proteins are found in a variety of cilium-related diseases (Pazour and Rosenbaum 2002; Pedersen and Rosenbaum 2008; Scholey and Anderson 2006; Snell et al. 2004; Wang et al. 2006).

Cilia are categorized into motile or multi-cilia (9 + 2 pattern of microtubule structure), and immotile or primary cilia (9 + 0 pattern of microtubule structure) based on the mobility and number of cilia, and the organization of axonemal microtubules (Fig. 1). Motile cilia in the

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respiratory airway clear mucus by beating constantly in coordinated waves (Shah et al. 2009), and nodal cilia generate flow to specify left-right asymmetry (Basu and Brueckner 2008; Nonaka et al. 1998). Sperm has a specialized cilium that is motile and drives sperm motility. This class of motile cilia will not be discussed further, and the reader is directed to several a recent review of this field (Salathe 2007).

The primary cilium in mammals was first identified over 100 years ago (Zimmermann 1898). However, it was considered a vestigial appendage of little importance and was largely ignored until the last decade when studies began to link ciliary dysfunction with genetic diseases such as polycystic kidney disease (PKD) (Bloodgood 2009; Pazour et al. 2000).

Recent studies have revealed that the primary cilium is the cell's antenna which receives and transmits extracellular signals through specific receptors on the ciliary membrane that initiate cell signaling cascades critical for normal development and homeostasis (Eggenschwiler and Anderson 2007; Gerdes et al. 2009; Lancaster and Gleeson 2009; Marshall and Nonaka 2006; Pazour and Witman 2003; Singla and Reiter 2006; Sloboda and Rosenbaum 2007). Also mediated by the primary cilium (Eggenschwiler and Anderson 2007; Pazour and Bloodgood 2008) are mechano-reception (Malone et al. 2007; Nauli et al. 2003; Praetorius and Spring 2001; Schwartz et al. 1997), chemo-reception (McEwen et al. 2008; Mombaerts 1999), photo-reception (Besharse and Horst 1990), and extracellular signaling by Sonic Hedgehog (Shh) (Huangfu et al. 2003; Rohatgi et al. 2007; Wong and Reiter 2008) and Wnt (Gerdes and Katsanis 2008), Planar Cell Polarity (PCP) (Jones and Chen 2008; Ross et al. 2005), and PDGF-AA (Schneider et al. 2005).

Heritable diseases, called ciliopathies, are associated with ciliary dysfunction. They present clinically with a complex combination of phenotypes including cystic kidneys, retinal degeneration, hearing loss, situs inversus, and other defects: for example, PKD (Nauli et al. 2003; Pazour et al. 2000; Yoder et al. 2002), Bardet-Biedl syndrome (BBS) (Kulaga et al. 2004; Mykytyn and Sheffield 2004), Nephronophthisis (NPHP) (Hildebrandt et al. 2009), Meckel-Gruber syndrome (MKS) (Delous et al. 2007; Kyttala et al. 2006), Joubert syndrome (JBTS) (Baala et al. 2007; Parisi et al. 2007), Usher syndrome (Yan and Liu 2010). Each syndrome is caused by mutations in a number of genes, and in general the normal proteins encoded by those genes localize to the primary cilium or basal body (Badano et al. 2006; Fliegauf et al. 2007; Sharma et al. 2008).

Distinct protein and lipid compositions of the ciliary membrane and surrounding plasma membrane

The ciliary membrane is contiguous with the surrounding plasma membrane but retains a distinct composition of lipids and proteins required for cilia-mediated sensing/signaling events, ciliary membrane trafficking and ciliogenesis. However, mechanisms for retaining these proteins and lipids in the primary cilia are not clear.

In tissue culture cell lines, many proteins and signaling pathways are concentrated in the primary cilium including: the polycystic kidney disease-causing proteins polycystin-1, polycystin-2, cystin and polaris (Nauli et al. 2003; Pazour et al. 2002; Yoder et al. 2002); Shh signaling components Smoothened (Smo), Patched1 (Ptc1), Gli2, Gli3 and β-arrestin (Corbit et al. 2005; Haycraft et al. 2005; Kovacs et al. 2008; Rohatgi et al. 2007); platelet-derived growth factor receptor α (PDGFRα) (Schneider et al. 2005); the angiopoietin receptors tyrosine kinases Tie-1 and Tie-2 (Teilmann and Christensen 2005); and, melanin-concentrating hormone receptor 1 (Mchr1) (Berbari et al. 2008b). Specialized membrane signaling proteins are also found in olfactory cilia such as membrane and olfactory transduction proteins (Mayer et al. 2008), in the olfactory sensory neurons of *C. elegans*

such as the odorant receptor cyclic nucleotide-gated channel CNGB1b (Jenkins et al. 2006), in motile cilia in airway epithelial such as sensory bitter taste receptors (Shah et al. 2009), and in the outer segment, a specialized cilium of rod photoreceptors that contain the photosensor, rhodopsin (Tam et al. 2000).

Small GTPases that mediate trafficking and biogenesis of ciliary membrane are also enriched in the primary cilium. Rab8a is present in primary cilia of cultured cells and coordinates with BBS proteins to promote ciliary membrane growth (Knödler et al. 2010; Nachury et al. 2007; Westlake et al. 2011). Proteomic analyses of photoreceptors and *Chlamydomonas reinhardtii* revealed that Rab subfamily members, ARF subfamily members, RAN, and SNARE proteins are present in the sensory cilium and flagellum, respectively (Kwok et al. 2008; Liu et al. 2007a; Pazour et al. 2005). The ADP ribosylation factor-like (ARL) family of small GTPases of the Ras superfamily are enriched in the cilium and mutations in Arl13b gives rise to PKD phenotype in Zebrafish (Duldulao et al. 2009). BBS proteins are present in primary cilium and assemble as a coat on vesicles that deliver membrane proteins to the cilium (Jin et al. 2010; Nachury et al. 2007). Some polarity protein complexes such as the transmembrane protein Crumbs3 localize to cilia of cultured MDCK cells, and are required for ciliogenesis (Fan et al. 2007; Sfakianos et al. 2007).

The ciliary membrane also maintains a lipid composition different from that of the apical plasma membrane. In quail oviduct, a high concentration of cholesterol was found on the shaft of ciliary membrane but not in the ciliary necklace enriched in intramembrane particles (Chailley and Boisvieux-Ulrich 1985). The trypanosome flagellar membrane is enriched in sterols and saturated fatty acids (Tyler et al. 2009). In addition, lipid-raft associated proteins such as palmitoylated and myristoylated proteins are targeted to the ciliary membranes (Emmer et al. 2010; Emmer et al. 2009; Janich and Corbeil 2007). Several studies have linked ciliopathy with defective phosphotidylinositol (PtdIns) signaling by inositol polyphosphate-5-phosphatase E (INPP5E), which mediates PtdIns metabolism and localizes in the primary cilia. Mutations of INPP5E were found in ciliopathy patients and impaired INPP5E phosphatase activity (Bielas et al. 2009; Jacoby et al. 2009). Therefore, cilia should be enriched in INPP5E product PI(4)P and PI(3,4)P2. A number of cilium-related proteins have been shown to bind phospholipids including Tubby-like protein 3 (TULP3) and BBSome proteins (Jin et al. 2010; Mukhopadhyay et al. 2010; Nachury et al. 2007).

Lipid rafts and lipid micro-domains may organize a micro-environment for signal transduction complexes (Simons and Toomre 2000). It remains to be determined if the primary ciliary membrane contains lipid rafts and if so, whether they play roles in cilia sensory functions. Difficulties in purifying primary cilia and the lack of tools to detect or manipulate lipids have impeded our understanding the function of lipids in the ciliary membrane (Mitchell et al. 2009). Nevertheless, the concentration and restricted distribution of proteins and lipids in the ciliary membrane indicate that the contents of the ciliary membrane and the surrounding plasma membrane are physically and functionally separated.

Early evidence of a membrane diffusion barrier in the primary cilium

Early studies suggested that the presence of a membrane diffusion barrier at the base of the primary cilium that physically and functionally separated the surrounding apical plasma membrane and ciliary membrane.

In *Chlamydomonas reinhardii*, glycoprotein agglutinins which mediate the adhesion of two algae during mating are segregated into two pools comprising an active fraction on flagella and an inactive fraction on the plasma membrane. However, cell body agglutinins move into the flagellum in response to mating signal, demonstrating that the function barrier can be opened by regulatory signals (Hunnicutt et al. 1990). In *Chlamydomonas eugametos*, the

agglutination antigens present on the cell body are unable to diffuse into the flagellar/ciliary membrane, suggesting a physical barrier at the base of the ciliary membrane (Musgrave et al. 1986). In Madin–Darby canine kidney (MDCK) cells, Laurdan staining showed that the ciliary membrane has a condensed lipid zone of high lipid order at the base of primary cilium, regarded as the periciliary membrane domain (Pazour and Bloodgood 2008) and glycosylphosphatidylinositol (GPI) anchored proteins, while can diffuse in the surrounding plasma membrane, were excluded from the ciliary membrane in fixed cells (Vieira et al. 2006) although a recent study using live cell microscopy indicated that GPI-GFP is in the ciliary membrane of MDCK cells (Francis et al. 2011).

The outer segment of photoreceptor cells in the retina is a specialized primary cilium that concentrates the membrane protein rhodopsin (Besharse et al. 1977). In photoreceptor rod cells, rhodopsin is compartmentalized in the outer segment yet diffuses into the inner segment after breaching the connecting cilium, suggesting that the connecting cilium serves as a membrane diffusion barrier between the inner and outer segments (Spencer et al. 1988). Interestingly, in retinal rod photoreceptors the small soluble protein GFP is able to diffuse between the outer and inner segments across the connecting cilium, but it remains to be determined if larger soluble proteins have the same property (Calvert et al. 2010). It is possible, therefore, that mechanisms involved in retaining membrane proteins and soluble proteins in primary cilium are different. Nevertheless, these data suggest the presence of a membrane diffusion barrier surrounding the ciliary membrane.

Direct test of a membrane diffusion barrier in the primary cilium of mammalian cells

Ciliary membrane proteins can be targeted to the ciliary membrane through ciliary targeting sequences (CTS), and they become enriched in the cilum (Berbari et al. 2008a; Follit et al. 2009; Geng et al. 2006; Nachury et al. 2010; Pazour and Bloodgood 2008; Rohatgi et al. 2007; Tam et al. 2000; Tao et al. 2009). Several hypotheses have been put forward to explain the retention and enrichment of ciliary membrane proteins: active transport, binding-retention, and a diffusion barrier (Fig. 2) (Emmer et al. 2010; Nachury et al. 2010). The active transport hypothesis posits that newly synthesized proteins are actively transported into the primary cilia to offset the constant, free diffusion of proteins out of the primary cilia (Fig. 2A). In the binding-retention hypothesis, proteins once transported into the primary cilia are “fixed” in the primary cilia possibly by binding to a ciliary matrix or the microtubule axoneme (Fig. 2B). In diffusion barrier hypothesis, proteins are retained by a physical barrier that prevents proteins from diffusing from the ciliary membrane into the surrounding apical plasma membrane (Fig. 2C).

The diffusion of ciliary membrane proteins in the ciliary membrane and surrounding apical plasma membrane of polarized epithelial cells were measured directly using fluorescence recovery after photobleaching (FRAP) (Hu et al. 2010). Photobleaching of the whole cilium or plasma membrane pool revealed that four membrane proteins in the apical plasma membrane and ciliary membrane are mobile, but do not exchange, indicating the presence of a physical barrier that blocks the free diffusion of those membrane proteins between these two adjacent plasma membrane compartments. It should be noted, however IFT88 shows high turnover and mobility suggesting IFT complexes may adopt the active-transportation mechanism to enter and be retained within the primary cilium (Hu et al. 2010). Interestingly, in *Chlamydomonas*, only a small portion of polycystic kidney disease 2 (PKD2) is mobile within the flagella suggesting that distinct mechanisms may be involved in retaining different ciliary membrane proteins in the ciliary membrane (Huang et al. 2007). *Chlamydomonas* flagellar proteome showed that a subset of membrane proteins are more enriched in the axonemal fraction than in the membrane and matrix fraction suggesting their anchorage to the axoneme (Pazour et al. 2005). Taken together, these results indicate that at least some membrane proteins are retained in the ciliary membrane by a diffusion barrier,

and not by the active transportation or binding-retention hypothesis, whereas a subset of membrane proteins such as PKD2 may utilize the binding-retention mechanism to maintain ciliary localization.

Septins as a component of the membrane diffusion barrier in the ciliary membrane

Septins comprise a large, conserved family of GTPases that form linear heterotrimers (heterotetramers in budding yeast) which in turn assemble into apolar filaments, bundles and rings (Bertin et al. 2008; Sirajuddin et al. 2007; Versele and Thorner 2005). They play important roles in mitosis, cell migration, and cell morphogenesis by forming scaffolds and diffusion barriers (Caudron and Barral 2009; Hu et al. 2008; Joo et al. 2007; Kinoshita et al. 2002; Kremer et al. 2007; Oh and Bi 2011; Spiliotis et al. 2008; Spiliotis et al. 2005).

Studies in a several biological systems indicate that septins assemble into structures that regulate the distribution of membrane proteins between different compartments of cells, and hence have characteristics of a diffusion barrier. In budding yeast, septins assemble into hourglass shape rings and ordered protein “gauzes” at the mother-daughter neck. There, septins act as a scaffold to restrict the distribution of polarity and exocytosis factors (Faty et al. 2002; Gladfelter et al. 2001; Rodal et al. 2005) and form a diffusion barrier between the mother and daughter cells for plasma membrane proteins, the nuclear envelop and the endoplasmic reticulum (ER) to maintain asymmetric cell division (Barral et al. 2000; Dobbelaere and Barral 2004; Luedeke et al. 2005; Shcheprova et al. 2008). Disruption of the septin rings results in mislocalization of cortical proteins at the bud neck, and therefore a failure of cytokinesis (Dobbelaere and Barral 2004; Oh and Bi 2010; Versele and Thorner 2005). In mitotic mammalian cells, septins surround the midbody, and have been proposed to be a cortical barrier between the two daughter cells (Schmidt and Nichols 2004). In sperm, septin filaments encircle the cortical membrane between the middle and principle piece of sperm tail; septin gene knockout causes a defect in sperm motility due to cortical disorganization and dispersion of the membrane protein Basigin due to a loss of the septin-based diffusion barrier (Ihara et al. 2005; Kissel et al. 2005; Kwitny et al. 2010; Steels et al. 2007). In hippocampal neurons, septins localize at the membrane and at the base of dendrite spines; depletion of septins affects dendritic branch morphogenesis (Tada et al. 2007; Xie et al. 2007). In mouse epithelial cells, septins localize to the base of the primary cilia at the boundary between ciliary membrane and plasma membrane, and between the axoneme and distal/subdistal appendage proteins of basal body (Hu et al. 2010) (Fig. 3). In *Xenopus* epidermis, septins form ring-like structures at the base of cilia in multi-ciliated cells while exogenous, over-expressed SEPT2 localizes along the shaft of cilia suggesting septins may play a role in the axonemal matrix for ciliary function (Kim et al. 2010).

Depletion of septins in both mouse epithelial cells and *Xenopus* impairs the formation and organization of cilia. In epithelial cells with reduced levels of SEPT2, ciliary membrane proteins can diffuse across the barrier as measured using FRAP (Hu et al. 2010). Similarly, the enrichment of ciliary membrane proteins involved in Shh signaling is partially lost, resulting in a reduced Shh signaling. Thus, SEPT2 contributes to the formation of the diffusion barrier at the base of the primary cilium. In *Xenopus*, septins interact with PCP signaling proteins and Fritz (Kim et al. 2010). Septins and Fritz are in the same pathway to control convergent-extention and ciliogenesis in early *Xenopus* development. In addition, depletion of septins or Fritz impairs Shh signaling. Finally, mutations in the *Fritz* gene are found in patients with Meckel-Gruber and Bardet-Biedl syndromes, although it is not known whether septin mutations also exist in patients with those ciliopathies and whether mutations in *Fritz* in those patients are pathogenic. The finding that a septin cytoskeleton is localized to sites of many of these barriers for membrane compartmentalization indicates an evolutionarily conserved mechanism to organize and compartmentalize membrane structure.

Despite these recent advances, several important questions remain. First, it remains unknown how septins contribute to the formation of the membrane diffusion barrier; there are no data to explain how septins are specifically recruited to the diffusion barrier at the base of primary cilia, sperm annulus, bases of dendritic spins or midbody of mitotic cells. Septins can interact with phospholipid membranes and mediate their tubulation (Tanaka-Takiguchi et al. 2009; Zhang et al. 1999), and phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) promotes the assembly of yeast septins *in vitro* (Bertin et al. 2010). It is possible that septins locally organize membrane lipids which in turn restrict the mobility of membrane proteins. Alternatively, septins may bind transmembrane proteins which serve as the diffusion barrier, although transmembrane proteins that interact with septins have not been identified.

Second, it is unknown whether the septin-mediated membrane diffusion barrier has selectivity for different membrane proteins, soluble (cytoplasmic) proteins of different molecular sizes, or peripheral membrane proteins that bind to different lipids.

Third, it is unknown whether and how ciliary diffusion barriers are regulated. For example, ciliary membrane proteins can be targeted and transported to the ciliary membrane during ciliogenesis indicating that the barrier either has not formed, or is permissive to the diffusion of ciliary membrane proteins. In another example, Shh binding to Ptc1 results in Ptc1 leaving the ciliary membrane causing Smo enrichment in the cilia (Corbit et al. 2005; Milenkovic et al. 2009; Rohatgi et al. 2007; Wong and Reiter 2008). How the ciliary barrier selectively gates these receptors remains unknown. In this context it is interesting to note that nuclear transport components RanGTP and importin-β2 mediate the shuttling of cytoplasmic kinesin-2 motor KIF17 into cilia (Dishinger et al. 2010; Hurd et al. 2011), but it is unknown how this complex by-passes the ciliary barrier.

Ultrastructure of the ciliary base

The diffusion barrier is localized at the boundary of apical plasma membrane and ciliary membrane (Hu et al. 2010) (Fig. 1–3). Ultrastructural studies of the ciliary base shed some light on the structural nature of the diffusion barrier. Freeze-fracture electron microscopy (EM) revealed a “ciliary necklace” surrounding the membrane at the base of cilia (Fig. 1) (Gilula and Satir 1972). The necklace is composed of rows of particles associated with the membrane that are connected to the basal body by appendages, and was proposed to form the membrane diffusion barrier or organize lipids at the transitional zone between the basal body and axonemal microtubules (Satir and Christensen 2007) (see Fig. 1). However, the molecular identity of the ciliary necklace remains to be determined.

Another membrane structure at the base of the ciliary membrane comprises the ciliary membrane pocket (Fig. 1). Cross sections of *Elliptio* lateral cilium revealed a pocket structure at the base of ciliary membrane with the ciliary necklace localized on the inner side of the pocket (Gilula and Satir 1972; Sorokin 1962). In Trypanosomatid, a protozoan parasite which uses a single flagellum as an invasion tool, a ciliary/flagellar pocket also exists and appears as a site for protein endocytosis and exocytosis (Gadelha et al. 2009; Gull 2003; Kohl et al. 2005; Overath et al. 1997). A cytoskeleton protein BILBO1 at the ciliary pocket was identified and plays an important role in ciliary pocket biogenesis (Bonhivers et al. 2008). A membrane pocket was also identified in *Xenopus* rod photoreceptor at the base of connecting cilum (Papermaster et al. 1985). Additional ultrastructural studies showed a similar structure in human retinal pigment epithelial (RPE) cells, mouse 3T3 cells and some mouse kidney epithelial (IMCD3) cells (Molla-Herman et al. 2010; Rohatgi and Snell 2010). Actin filaments were observed in the vicinity of ciliary pocket, possibly mediating the position of cilium. Remarkably, clathrin-coated pits and vesicles were also found

exclusively at the ciliary pocket, further indicating that they are sites of endocytic targeting and recycling of ciliary membrane proteins (Molla-Herman et al. 2010).

Taken together, a ciliary diffusion barrier protein complex may be localized at the transition zone (TZ) consisting of the basal body, its accessory appendages (also called transition fibers) connected to the ciliary pocket, the proximal region of the axoneme, the ciliary necklace membrane region and the Y-connector between axonemal microtubules and ciliary necklace membrane (Fig. 1). This complex may be the main site for trafficking in and out of ciliary membrane and, as a diffusion barrier, regulate membrane and cytosolic protein diffusion (Nachury et al. 2010).

Additional candidates for the ciliary diffusion barrier

Several proteins, in addition to septins, have been identified at the ciliary diffusion barrier complex/transitional zone (Sharma et al. 2008) and some may constitute the diffusion barrier. Interestingly, many of them are proteins encoded by ciliopathy genes. In *C. elegans* and cilia of different mammalian tissues, the Nephronophthisis disease gene product NPHP1, NPHP2/Invesin, NPHP4/Nephroretinin, NPHP6/CEP290/MKS-4, NPHP8/RPGRIP1L/MKS-5, NPHP9/Nek8 and NPHP11/TMEM67/MKS-3/Meckelin are localized mainly at the transitional zone at the ciliary base (Delous et al. 2007; Fliegauf et al. 2006; Mollet et al. 2005; Otto et al. 2005; Otto et al. 2003; Otto et al. 2008; Sayer et al. 2006; Shiba et al. 2010; Simons et al. 2005; Valente et al. 2006; Winkelbauer et al. 2005). Remarkably, in *Chlamydomonas reinhardtii*, CEP290 is an integral component of Y-shape connector that links the microtubule doublets to the ciliary necklace at the TZ; significantly, *cep290* mutant causes a loss of the Y-shape link and therefore the association between axonemal microtubules and ciliary membrane. Loss of CEPT290 leads to a reduction of IFT-associated and membrane proteins in the flagella (Craige et al. 2010). Thus, CEP290 appears to function as a gatekeeper to regulate the delivery and exit of flagellar proteins (Betleja and Cole 2010). Meckel-Gruber syndrome (MKS) proteins MKS-1, MKS-2/TMEM216, MKS-3/Meckelin/TMEM67 and their related proteins are also localized to the ciliary base and are required for ciliogenesis (Bialas et al. 2009; Dawe et al. 2009; Valente et al. 2010; Williams et al. 2009; Williams et al. 2008). A recent study systematically characterized the localization and function interaction of MKS and NPHP proteins at the TZ in *C. elegans* and demonstrated they establish the attachment between basal body/TZ and ciliary membrane and ciliary gating function (Williams et al. 2011). It would be of great interest to determine their ultrastructure localization and protein binding profiles. In *Xenopus* photoreceptors, the Usher syndrome gene products SANS (USH1G), Whirlin (USH2D), USH2b and VLGR1b are in a complex that forms a bridge between the connecting cilium and periciliary membrane (Liu et al. 2007b; Maerker et al. 2008); Usher syndrome is a disorder that causes combined deafness and blindness characterized with degeneration of retinal photoreceptors. Retinitis pigmentosa GTPase regulator (RPGR) localizes at the connecting cilium and maintains the polarized distribution of rhodopsin in the photoreceptor cells (He et al. 2008; Hong et al. 2001; Roepman et al. 2005); mutations in RPGR are a frequent cause of retinitis pigmentosa (RP), a retinal degeneration disease. Basal body distal appendage protein Cep164 forms a donut-shaped structure at the base of primary cilium (Graser et al. 2007). Oral-facial-digital type I (OFD1) syndrome gene product OFD1 has basal body and centrosomal localizations (Romio et al. 2004).

Although these proteins are candidates for forming the diffusion barrier, details of the fine structure localization and functions of these proteins networks at the transitional zone and at the base of the cilia remain to be determined. Immuno-gold labeling and super-resolution microscopy could be used to further pinpoint their localization at the ciliary base. Tandem affinity purification system coupled with sequential mass spectrometry has been used successfully to identify BBSomes and their associated proteins (Nachury et al. 2007), and

this might be an application useful for the purification of protein complex in the transitional zone. Protein interactions could be verified subsequently by biochemical methods and genetic interaction studies. *In-vitro* purification, reconstitution, high resolution EM and crystal structures of those protein complexes would be helpful to study their structure-function relationships.

Conclusions and Perspectives

The ciliary diffusion barrier maintains the specific concentration of ciliary membrane proteins and associated signaling complexes within the cilium compared to the surrounding (apical) plasma membrane. The diffusion barrier appears to be localized to the TZ at the base of the ciliary membrane, and consist of ciliary necklace, the Y-link connecting ciliary necklace and axonemal microtubules, a septin cytoskeleton, and may include a complex protein network involving proteins encoded by ciliopathy genes.

Many important aspects of the diffusion barrier are poorly understood. First, selectivity of the diffusion barrier to different proteins remains to be determined. The diffusion of additional integral membrane proteins, peripheral proteins and IFT complex and associated proteins could be tested using FRAP. Soluble proteins of different sizes could be tagged with fluorophores and injected into or expressed in cells and their distribution observed in cilia. Interestingly, 10kDa fluorescently labeled dextrans can enter mammalian primary cilia while dextrans of 40kDa or larger are excluded from the ciliary compartment suggesting a size exclusion mechanism controlling the ciliary entry of soluble proteins (Kee et al. 2010). The underlying mechanisms involved in selectivity should be addressed. Second, the signaling cascades regulating the assembly, disassembly and permeability of the diffusion barrier are unknown. Third, the biochemical composition and structure of the diffusion barrier are unknown. Protein complexes encoded by ciliopathy genes at the TZ could be purified and identified, and their localization at the ciliary base could be pinpointed using high-resolution immuno-gold EM. Protein depletion or genetic knock-outs of single or multiple components are needed to test the structural and functional importance of specific proteins and combinations of proteins at the TZ of the diffusion barrier. Once the molecular nature of the diffusion barrier is defined, animal models can be established to explore the physiological relevance of the ciliary diffusion barrier in development, homeostasis and diseases.

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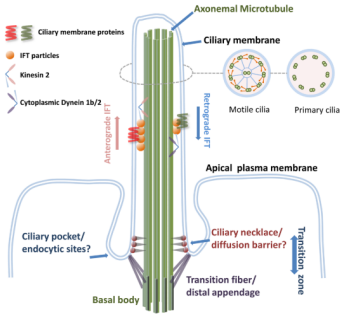

Fig. 1. Scheme of a fully assembled primary cilium and ciliary base structures

IFT particles move with their cargos (eg. ciliary membrane proteins) within the cilia by the molecular motors kinesin 2 (anterograde trafficking) and cytoplasmic dynein 1b/2 (retrograde trafficking). At the ciliary base, transition fiber/distal appendages link the basal body to the ciliary base, and together with ciliary necklace they form the transition zone and a diffusion barrier for ciliary proteins. Plasma membrane invagination (ciliary pocket) at the ciliary base appears to be sites for endocytosis. The illustration is adapted from (Rosenbaum and Witman 2002) and EM images from (Gilula and Satir 1972).

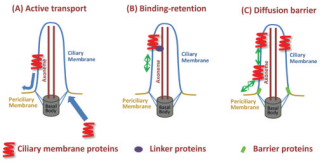

Fig. 2. Scheme of three hypotheses to retain ciliary membrane proteins

A: Active transportation hypothesis. Ciliary membrane proteins are actively targeted and transported into the ciliary membrane and they are able to diffuse out of the ciliary membrane into plasma membrane or endocytosed from the plasma membrane into cytoplasm. The transportation rate into ciliary membrane is faster than the diffusion rate out of the cilium, thereby resulting in the enrichment of proteins in the cilium. **B:** Binding-retention hypothesis. Ciliary membrane proteins, once transported into the ciliary membrane, bind stably to the axoneme and are retained. **C:** Diffusion barrier hypothesis. Ciliary membrane proteins, once transported into the ciliary membrane are prevented from diffusing from the ciliary membrane by a physical barrier but may actively move within ciliary membrane.

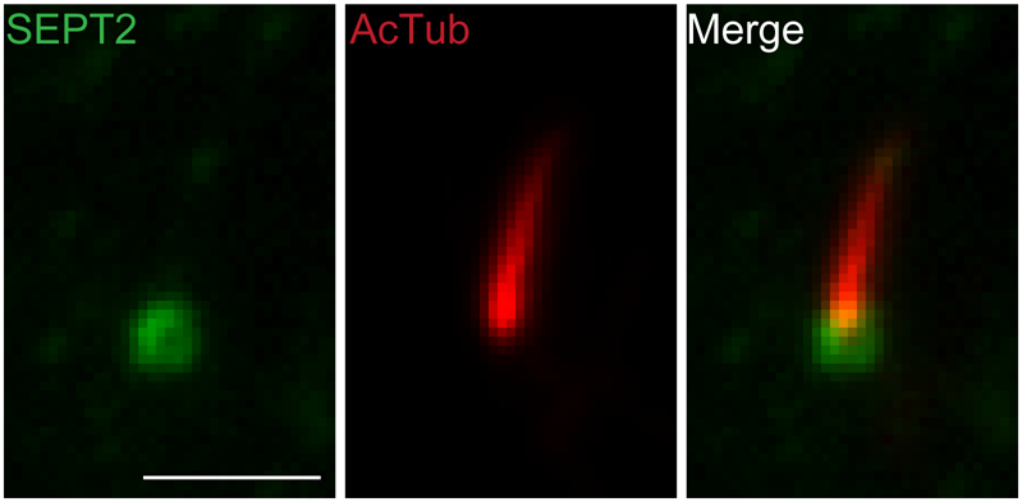

Fig. 3. SEPT2 forms a ring-like structure at the ciliary base of IMCD3 cells

IMCD3 cells were fixed and stained with anti-SEPT2 antibody (green) and anti-acetylated tubulin antibody (red). The image shows the primary cilium on the apical membrane. Scale bar, ~2μm.