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Permeability barriers for generating a unique ciliary protein and lipid composition

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Abstract

Cilia (and flagella) are microtubule-based protrusions that are found in single or multiple copies on the surface of most eukaryotic cells. Defects in cilia formation and/or function have now been correlated with an expanding spectrum of human genetic diseases termed ciliopathies. Recent work indicates that cilia are indeed a *bona fide* organelle with a unique protein and lipid content that enables specific cellular functions. Despite the physiological and clinical relevance of cilia, our understanding of how a unique protein and lipid composition is generated for this organelle remains poor. Here we review recent work on the mechanisms that determine the protein and lipid content, and thus the functional outputs, of this unique organelle.

Graphical abstract

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Introduction

Cilia (and flagella) are microtubule-based protrusions that are found in single or multiple copies on the surface of most eukaryotic cells. When present in multiple copies, cilia are generally motile organelles that beat to move fluid over the surface of the cell or to move the cell itself (review, [1]). When present as a single protrusion, cilia are generally sensory organelles that play a large number of essential roles in eukaryotic biology ranging from regulating embryonic development to sensing the extracellular environment to tissue homeostasis (reviews, [2-4]). Defects in cilia formation and/or function have now been correlated with an expanding spectrum of human genetic diseases termed ciliopathies (reviews, [5-7]). Since cilia are found on the surface of nearly all cells in the human body, most ciliopathies occur as syndromic disorders that affect many systems during development and/or adult life including the skeletal, kidney, central nervous, olfactory, and visual systems. Recent work indicates that the cilium is indeed a *bona fide* organelle with a unique protein and lipid content that enables specific cellular functions. Here we review recent work on the mechanisms that determine the protein and lipid content, and thus the functional outputs, of this unique organelle.

Cilium form and function

Cilia contain a microtubule core, the axoneme, which consists of a ring of nine doublet microtubules without (9+0) or with (9+2) two single microtubules in the center (Figure 1; for a review of cilium structure, see [8]). The axonemal microtubules serve not only a structural role but are also the “tracks” for intraflagellar transport (IFT), a transport process driven by kinesin and cytoplasmic dynein motors that delivers building blocks and signaling molecules within the cilium (review, [9]). The doublet microtubules of the axoneme are extensions of the triplet microtubules of the mother centriole (the basal body).

The base of the cilium contains a specialized region called the transition zone (TZ) that contains structures that appear Y-shaped by electron microscopy (thus called Y-links) (Figure 1). Although the protein composition of the Y-links is not known, they serve to connect the doublet microtubules to the ciliary membrane. The Y-links may also organize a structure within the ciliary membrane called the ciliary necklace [10] (Figure 1). Proximal to the TZ are the transition fibers (TFs, derived from the distal appendages) that anchor the mother centriole to the periciliary membrane (the border between the plasma membrane and the ciliary membrane). The periciliary membrane is frequently infolded to produce a ciliary pocket that is an active site for exocytosis and endocytosis of ciliary membrane proteins [11,12].

Cilia require a unique protein complement for their function as motility-generating organelles, including axonemal dyneins, nexin/dynein regulatory complexes, and radial spoke complexes [13,14]. Cilia also require a unique protein complement for their function as sensory organelles, including receptor tyrosine kinases, ion channels, G-protein coupled receptors, and receptors for growth factors, Notch, Hedgehog, and extracellular matrix proteins (reviews, [15-18]). Understanding how these proteins are targeted to the ciliary compartment has implications for understanding a large number of cellular signaling

pathways and the resultant diseases of their dysfunction. Unlike other organelles, the cilium is not entirely enclosed by a membrane barrier but rather is open to the cytoplasm at the base. The ciliary base thus needs to control the selective entry of ciliary proteins and the TZ and the TFs are candidate gating complexes.

Generating a unique luminal protein composition

The prototype for an organelle that is not entirely enclosed by a membrane barrier is the nucleus where nuclear pore complexes (NPCs) embedded in the nuclear envelope provide the permeability barrier (reviews, [19-21]). NPCs are comprised of ~30 different nucleoporins (Nups) that assemble into defined subcomplexes such as the scaffold, the central channel, and the nuclear basket. The NPC has the remarkable property of being freely permeable to small molecules but becomes increasingly restrictive as the size of the molecule approaches or exceeds a diameter of ~5 nm, thus limiting the passage of proteins in a size- and/or mass-dependent manner. Critical for this permeability is the presence of a specific class of nucleoporins (Nups) that contain intrinsically disordered domains rich in repeats of the dipeptide Phe-Gly (FG repeats). Large proteins and protein complexes containing nuclear import or export sequences are escorted through NPCs by karyopherins (importins and exportins, respectively) that interact with FG-containing Nups within the central channel. The association and dissociation of karyopherin-cargo complexes are regulated by a concentration gradient of RanGTP/GDP across the nuclear envelope.

The base of the cilium contains a permeability barrier with properties similar to that of the NPC. Small molecules are able to pass freely between the cytosolic and ciliary compartments but the access of larger molecules is restricted [22]. Like the NPC, the ciliary barrier is a “kinetic barrier”, meaning that increasingly larger objects take progressively longer to pass through ciliary pores [23,24]. It is not just overall molecular weight but the shape and thus the diameter of the molecule that determines its ability to enter the cilium by diffusion. For example, the increased mass of a GFP fusion does not impact the ability of soluble proteins to enter cilia [25-27], presumably because the GFP moiety does not alter the diameter of the molecule. Like the NPC, the ciliary barrier uses importins and a RanGTP/GDP gradient to regulate entry of large molecules [28-30] (Figure 1). Further work is needed to understand the roles of specific karyopherins in ciliary import/export and how the RanGTP/GDP gradient is generated. While the GTPase activating protein RanGAP generates a high cytosolic concentration of RanGDP that impacts both nuclear and ciliary barriers [29], it is not known whether these compartments utilize the same exchange factor (RCC1 in the nucleus) or whether the cilium contains a unique exchange factor.

The cilium's permeability barrier is also characterized by the presence of Nups, including FG-containing Nups [22,31], and has thus been termed the ciliary pore complex (CPC, Figure 2). To study the function of FG-containing Nups in gated entry, Takao et al. developed a “pore clogging assay” by rapid and specific crosslinking of the central channel nucleoporin Nup62 [32**]. Crosslinking of Nup62 blocked the entry of a variety of soluble proteins into the cilium, including the kinesin-2 motor KIF17, the transcription factors Gli2 and Gtl3, and the IFT component IFT88 [32**]. Crosslinking of Nup62 did not, however, block the entry of a variety of transmembrane or membrane-associated proteins into the

cilium [32**]. This work indicates that an FG-Nup permeability barrier regulates the entry of soluble proteins into the cilium, and that the passage of membrane proteins requires a different gating mechanism.

Before their passage through the CPC, large protein complexes such as IFT particles appear to be anchored at the TFs [33]. In a mutagenesis screen for worms with abnormal ciliogenesis, Wei et al [34*] identified *dyf-19* (homolog of human *FBF1*) as essential for the ciliary entry of IFT complexes. The DYF-19 deficiency did not affect TF assembly but resulted in the accumulation of assembled IFT particles below the TFs. Using bimolecular fluorescence complementation (BiFC) to visualize interactions at the ciliary base, the authors showed that DYF-19/FBF1 interacts with the IFT-B component DYF-11 (homolog of human IFT54) at the TFs [34*]. Thus, the TFs appear to be the site for assembly and/or docking of IFT particles before their entry into the cilium.

Generating a unique membrane protein content

The ciliary membrane appears continuous with the adjacent plasma membrane but the exchange of components between these environments is hindered. The idea of a permeability barrier for membrane proteins was first suggested by work in *Chlamydomonas* where the agglutinin proteins are restricted to the cell body until ciliary adhesion during fertilization [35,36]. The restricted passage of membrane proteins between the ciliary and plasma membranes has now been demonstrated using fluorescence recovery after photobleaching (FRAP) assays in cilia across species [37-40].

Early work using freeze-etching identified membrane particles organized into strands at the base of motile and primary cilia [10]. Termed the ciliary necklace, these particles were proposed to form a physical barrier between the plasma and ciliary membranes (Figure 2). While the molecular identity of the ciliary necklace remains unknown, two protein complexes have been identified that contribute to restricted passage of membrane proteins at this location. First, in mammalian cells, septins localize to the base of primary and motile cilia (Figure 2) [37,38,41-43] and between the middle and principal pieces of the sperm flagellum [44,45]. Septins are membrane-associated GTPases that assemble into filaments or spirals that restrict protein diffusion along the plane of the membrane (reviews, [46,47]). Importantly, FRAP analysis showed that loss of septin function enables the movement of fluorescently-tagged ciliary proteins from the plasma membrane into the cilium [38]. However, septin function may indirectly alter the passage of membrane proteins due to effects on the localization of TZ proteins [37] and septins have been found to localize along the entire cilium in some cells and tissues [41,48].

Second, a large number of ciliopathy gene products localize to the TZ at the base of the cilium across species, including Meckel Syndrome (MKS) and nephronophthisis (NPHP) gene products (Figure 2). Biochemical and genetic analyses place these proteins into three functional modules: MKS, NPHP1-4-8, and NPHP5-6 [49,50]. The MKS module is conserved across ciliated organisms whereas the NPHP1-4-8 and NPHP5-6 modules may coordinate with the MKS module in a species- or cell-specific manner [51,52*].

Knockout or knockdown of MKS module components results in alterations to ciliary membrane protein composition. In some cases, non-ciliary membrane proteins were found to abnormally enter the cilium (e.g. [37,53**,54**,55]) whereas in other cases, ciliary proteins were no longer restricted to the ciliary membrane (e.g. [42,56-59*]). Thus, MKS proteins and septins may work together to create a permeability barrier that restricts the passage of membrane proteins at the base of the cilium (Figure 2). As the CPC regulates the passage of soluble but not membrane proteins [32**], the MKS module and CPC appear to play distinct but likely overlapping roles in ciliary gating.

Proteins in the MKS module contain transmembrane or lipid-binding domains, suggesting that this module could comprise or regulate the ciliary necklace. Consistent with a barrier function, members of the MKS module are largely immobile at the TZ [54**]. Furthermore, recent superresolution imaging studies have localized MKS proteins in up to nine clusters arranged in a ring whose diameter corresponds to that of the ciliary membrane [54**,60*,61**]. Thus, the dynamics and localization of MKS module proteins supports their functional role as a barrier to membrane protein passage at the ciliary necklace region.

Proteins in the NPHP1-4-8 and NPHP5-6 modules contain structural domains and thus may form the Y-links and/or regulate their assembly (Figure 2). In *C. elegans*, single mutations in NPHP4 or NPHP8 (MKS-5) can cause a reduction in the number and/or thickness of the Y-links [53**,54**,59*]. In *Chlamydomonas*, CEP290 (NPHP6) localizes to the Y-links by immunoelectron microscopy and loss of CEP290 (NPHP6) function results in defects in microtubule-to-membrane linkages [62]. Furthermore, superresolution imaging has localized components of the NPHP1-4-8 and NPHP5-6 complexes to a ring that is interior to the ciliary membrane and adjacent to the microtubule doublets [54**,61**]. In addition to a structural role, the NPHP complexes are also involved in gating at the TZ. Loss of NPHP4 function in *C. elegans* had no effect on the ciliary localization of channel proteins but caused mislocalization of IFT components [63] whereas loss of NPHP4 function in *Chlamydomonas* caused alterations to both membrane and soluble ciliary protein content [56]. As components of the Y-links, the NPHP module proteins may therefore impact entry of both membrane and soluble proteins. The arms of the Y-links may organize the MKS module proteins and regulate the entry/exit of membrane proteins whereas the stalks of the Y-links may organize the CPC proteins to provide a barrier adjacent to the microtubule doublets that regulates the entry/exit of soluble proteins (Figure 2).

Generating a unique ciliary lipid content

Organelle identity and function rely not only on a unique protein complement, but also on a unique lipid content. In particular, phosphoinositide content is critical for dictating organelle identity (reviews [64,65]) and recent work indicates that cilia are no exception. The first clue to a unique ciliary phosphoinositide composition was the findings that the inositol 5-phosphatase INPP5E localizes to primary cilia (Figure 3) and mutations in INPP5E cause the ciliopathy Joubert syndrome [66,67]. At the same time, work in *C. elegans* identified the inositol 5-phosphatase CIL-1 as regulating the ciliary localization of the transmembrane channels TRPP and PKD-2, likely due to its effects on phosphoinositide distribution [68]. Three recent papers have now characterized INPP5E in mammalian cells. Chavez et al and

Garcia-Gonzalo et al showed that phosphoinositide 4-phosphate [PI(4)P] is present throughout the ciliary membrane whereas PI(4,5)P₂ is enriched at the cilium base but absent from the ciliary membrane [69**,70**] (Figure 3). Both groups showed that localization of active INPP5E to the cilium is required to generate a membrane environment enriched in PI(4)P and that this lipid environment restricts the ciliary localization of two negative regulators of Hedgehog signaling, Tulp3 and Gpr161 [69**,70**]. Xu et al. showed that the 5-phosphatase function of INPP5E is counteracted by the PI4P 5-kinase PIPKIγ and that enrichment of PI(4,5)P₂ at the base of the cilium is critical for ciliogenesis [71**]. A PI(4,5)P₂-enriched zone at the base of the cilium has also been found in *T. brucei* and *C. elegans* [53**,72]. Since PI(4,5)P₂ effector proteins include the exocyst, septins, tubby family proteins, and clathrin adaptor proteins [73-76], a PI(4,5)P₂-enriched domain could mark the ciliary pocket as a specialized site for vesicle exocytosis and endocytosis (Figure 3).

The base of the cilium also contains a region of condensed lipid that could provide a barrier to lipid movement between the ciliary and plasma membranes (Figure 3). This region of condensed lipid was identified using Laurdan [77], a probe whose fluorescence emission is sensitive to the degree of condensation (ordering) of its lipid bilayer environment. This ring of condensed lipid does not appear to regulate the passage of membrane proteins as knockdown of MAL, a membrane protein that partitions into condensed membranes, disrupted the formation of the ring of condensed lipid but did not cause the missorting of non-ciliary proteins to the ciliary compartment [78]. The region of condensed lipid may thus contribute to establishing and/or maintaining unique lipid environments for the ciliary and plasma membranes.

Future Directions

Despite the physiological and clinical relevance of cilia, our understanding of how a unique protein and lipid content is generated for this organelle remains poor. A critical question concerns the functional relationships between the TZ, CPC, TFs, and condensed lipids in terms of gated entry and exit of both proteins and lipids. And although some of the components of each of these gating complexes have been identified, much work is needed to delineate the mechanisms by which they function to restrict the passage of some molecules and permit the entry of others. For the CPC, it will be important to determine whether the mechanism of barrier formation observed for Nups of the CPC is similar to that of Nups within the NPC. For the TZ, the relationships between the MKS and NPHP modules are fairly established but the mechanisms by which these proteins form a permeability barrier, together with the septins, remain unclear. And while exciting recent work has established the cilium as a PI(4)P-enriched organelle, the ciliary functions of other ciliary phosphoinositide kinases and phosphatases are unknown. Finally, a complete understanding of ciliary gating will require generating an architectural map of the ciliary base that marries the structural components seen in electron microscopy with the functional components identified by biochemical and genetics approaches. Recent superresolution microscopy approaches have begun to address this question but more work is needed.

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Highlights

- nucleoporins comprise a permeability barrier for the entry of soluble proteins
- MKS proteins regulate the entry of membrane proteins at the transition zone
- NPHP proteins are structural components of the transition zone and regulate protein entry
- the inositol 5-phosphatase INPP5E creates a PI(4)P environment in the cilium
- extraciliary PI(4,5)P₂ may regulate exocytosis and endocytosis adjacent to the cilium

Figure 1. Cilium structure

The structural basis of the cilium is the axoneme which consists of a ring of nine doublet microtubules (blue) without (9+0) or with (9+2) two single microtubules in the center. The doublet microtubules also function as the tracks for intraflagellar transport (IFT) with anterograde IFT driven by kinesin motors (red) and retrograde IFT driven by cytoplasmic dynein (purple) motors. The transition zone (TZ) contains the ciliary necklace (black spots), Y-links (red), and septins (orange). The transition fibers (TFs, purple triangles) link the triplet microtubules of the mother centriole to the periciliary membrane. The cilium contains high levels of RanGTP whereas the cytosol contains high levels of RanGDP.

Figure 2. Permeability barriers that generate a unique ciliary protein composition

A cross-section through the TZ shows a putative arrangement of doublet microtubules (blue), the CPCs (yellow), and the Y-links (red). The CPCs contain Nups, particularly FG-repeat Nups (green squiggles), and form a permeability barrier for the entry of soluble proteins. The MKS module proteins are membrane-localized and form a permeability barrier, together with the septins (orange), for the entry of membrane proteins. The NPHP module proteins localize to the Y-links and may organize both the CPC and the MKS module.

Figure 3. Permeability barriers that generate a unique ciliary lipid composition

Phospholipid phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P₂] is absent from the ciliary membrane but enriched at the cilium base and plasma membrane (red) where it could provide specialized sites for vesicle exocytosis and endocytosis. In contrast, phosphatidylinositol 4-phosphate [PI(4)P] is generated in the ciliary membrane by the ciliary-localized 5-phosphatase INPP5E. A PI(4)P-enriched membrane is critical for the signaling functions of the organelle. A region of condensed lipid at the base of the cilium may restrict the passage of lipids between the plasma and ciliary membranes.