From the cytoplasm into the cilium: Bon voyage

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The primary cilium compartmentalizes a tiny fraction of the cell surface and volume, yet many proteins are highly enriched in this area and so efficient mechanisms are necessary to concentrate them in the ciliary compartment. Here we review mechanisms that are thought to deliver protein cargo to the base of cilia and are likely to interact with ciliary gating mechanisms. Given the immense variety of ciliary cytosolic and transmembrane proteins, it is almost certain that multiple, albeit frequently interconnected, pathways mediate this process. It is also clear that none of these pathways is fully understood at the present time. Mechanisms that are discussed below facilitate ciliary localization of structural and signaling molecules, which include receptors, G-proteins, ion channels, and enzymes. These mechanisms form a basis for every aspect of cilia function in early embryonic patterning, organ morphogenesis, sensory perception and elsewhere.

In an idealized round cell, with a radius of 10 μm, the membrane of a primary cilium (typically 2 μm long) occupies approximately 1/1000 of the total cell surface. Similarly, the cytoplasmic volume of the cilium (the cilioplasm) is a very small fraction of the cell’s cytoplasm. Yet many proteins are highly enriched in this area. This is accomplished by a combination of transport and barrier mechanisms that operate at cilia base. Transport into cilia involves two phases: first ciliary proteins are transported through the cytoplasm to the cilium base, and then, in the second phase, they are translocated inside the ciliary shaft by a mechanism known as intraflagellar transport (IFT) (Fig. 1).¹⁻⁴ The transition from cytoplasmic to intraflagellator transport occurs at cilia base, which is also the area that features selective barrier mechanisms that restrict the free movement of proteins into and out of the cilium.

For transmembrane proteins, transport through the cytoplasm into the cilium involves a mechanism that concentrates ciliary proteins in a specific class of exocytotic vesicles, another mechanism that translocates these vesicles toward the ciliary axoneme and, finally, machinery that mediates vesicle fusion at the base of the cilium (see examples in refs. 5–8). This final step must be spatially coordinated with a diffusion barrier(s) at the cilium base. A variation of this transport pathway has been also described and involves rapid lateral translocation of a protein, which is initially deposited in the plasma membrane, into the ciliary membrane.⁹

In the case of cytosolic proteins, translocation into the ciliary compartment does not require vesicle formation and fusion. Similar to transmembrane proteins, however, cytoplasmic proteins must cross a size-selective diffusion barrier at the ciliary base.¹⁰ The nature of this barrier appears to be very different from that which restricts the movement of transmembrane proteins. Inside the ciliary compartment, both transmembrane and cytosolic proteins appear to be actively transported, which implies that they interact with ciliary motors, such as kinesins and dyneins, and with adaptor complexes of these motors, such as the IFT particle.¹¹⁻¹²

Although some similarities exist between transport in the cytoplasm and intraflagellar transport, in general these two mechanisms are considerably different. This is particularly obvious for ciliary transmembrane proteins, which are embedded in vesicle membranes during cytoplasmic transport. In contrast to this, the same proteins translocate in the plane of the lipid bilayer of the ciliary membrane during intraflagellar transport in the cilium.¹³⁻¹⁴ Transport in the cytoplasm and intraflagellar transport also rely on different adaptor proteins. Intraflagellar transport requires the so-called IFT particle—a protein complex of over 20 proteins. In the absence of IFT particle components, intraflagellar transport does not occur and cilia do not form.¹⁵⁻¹⁸ In contrast, IFT proteins play only a limited role in cytoplasmic transport. Although exceptions exist and are discussed below, the majority of IFT proteins do not seem to be required to transport ciliary proteins in the cytoplasm.

An additional important difference between transport in the cytoplasm and intraflagellar transport of ciliary proteins is that these two transport modes appear to rely on different motors. The plus ends of intraflagellar microtubules are directed toward cilia tips (Fig. 1). Consistent with this, the anterograde intraflagellar transport that translocates proteins along the ciliary axoneme is driven by microtubule-dependent, plus end-directed motors (recently reviewed in ref. 19). These motors are homodimeric and heterotrimeric kinesin 2 family members, which also function in some other transport processes in the cell. The directionality of cytoplasmic microtubules in many cells is, however, opposite to that observed in cilia: their minus ends point toward the apical surface of the cell, which is where the ciliary centriole is located.²⁰⁻²² This makes it improbable that kinesin 2 family motors move vesicles all the way from the Golgi apparatus to the ciliary centriole. As a consequence, to travel from the Golgi apparatus to ciliary tips, transmembrane proteins have to switch...
their motors. This motor exchange most likely occurs at the cilial base.

The movement of ciliary proteins is restricted by diffusion barriers at the cilial base. These barriers are selectively permeable to both transmembrane and cytoplasmic proteins and regulate trafficking in both directions to keep some proteins outside and others inside the cilium.29-35 These characteristics suggest that barriers at the cilial base or transport mechanisms that move proteins across these barriers employ intricate recognition mechanisms to assure proper selectivity. Failure of barrier function has
damaging consequences, which include cell death.26-28

Below, we review transport mechanisms that are thought to deliver protein cargo to the base of cilia and are likely to interact with ciliary gating mechanisms. Given the immense variety of ciliary cytosolic and transmembrane proteins, it is almost certain that multiple pathways mediate this process. We focus on the cilial base and comment on transport in the cytoplasm and intraflagellar transport only to the extent to which these mechanisms are related to protein translocation across diffusion barriers at the interface of the ciliary compartment and the cytoplasm. Mechanisms that are discussed below facilitate ciliary localization of structural and signaling molecules, which include receptors, G-proteins, ion channels, and enzymes. The ciliary localization of these proteins is essential for cilia function in many aspects of development and physiology, such as organ formation, growth, morphogenesis, and function.

**The Cilium Base is a Transport Hub**

The base of the cilium is located in the cytoplasm, near the cell surface, and functions as a hub for protein transport into and out of the cilium. It consists of a centriole and membrane-associated structures: the septin ring, transition fibers, and the transition zone. A specialized membrane area, called the ciliary pocket, is found at the base of some cilia.29,30 The centriole’s main role is to provide a template for the extension of the ciliary axoneme and to give rise to transition fibers, which are anchored to the distal end of the cilium (Fig. 1). The membrane-associated structures are thought to be involved in regulating ciliary transport. The septin ring and transition fibers appear to concentrate and/or confine ciliary membrane proteins and components of ciliary transport machinery to the base of the cilium. In this area, intraflagellar transport (IFT) proteins appear to bind cargo proteins and, subsequently, transport them across the transition zone into the cilium. The transition zone is thus thought of as the gate into the cilium.

**Septin Ring**

Septins are a family of self-polymerizing GTPases. They are implicated in a variety of cellular processes and generally form membrane barriers. In the yeast bud neck, for example, septins separate the mother cell and the budding daughter cell into distinct membrane compartments (reviewed in ref. 31). In yeast, septin polymers appear to accomplish this by binding directly to the plasma membrane.32 A septin ring is also observed at the base of cilia in IMCD3 cells and in airway epithelia.23,32 In addition, septins are observed in the annulus found at the base of the mammalian sperm flagellum.33,34 The annulus is thought to be homologous to the ring cilium found at the base of the insect sperm cilium35 and it is, therefore, likely that Septin association with cilia is conserved between vertebrates and invertebrates.

The physical connection between the septin ring and other structures at the base of the cilium, such as the transition zone, transition fibers and the ciliary pocket, is not well understood. Septin 2 forms a ring of 500 nm in diameter, which is consistent with ciliary pocket dimensions.36 It is therefore tempting to speculate that the septin ring is associated with the cytoplasmic face of the ciliary pocket. However, Septin 2 localization in IMCD3 cells suggests that it is positioned between the axoneme and cilium appendage proteins Cep164 and Odf2.23 More detailed ultrastructural studies will help to determine precisely where the septin ring is found.

In tissue culture, Septin 2 is required to localize transition zone proteins B9D1, CC2D2A, and TMEM231, which suggests that septins function upstream in the assembly of at least some transition zone structures.24 Nevertheless, Septins are currently not known to interact directly with transition zone proteins. They are, however, essential for creating a diffusion barrier between the ciliary membrane and the plasma membrane.23,37 Evidence for this is provided by photo-bleaching experiments, which demonstrated that partial depletion of SEPT2 in IMCD3 cells results in an abnormally short cilium that displays an increase in the diffusional mobility of several proteins from the plasma membrane to the cilium.23 In addition, in SEPT2-depleted cells, ciliary membrane proteins were also found in the plasma membrane surrounding the cilium.23 These observations indicate that Septins limit protein movement between the plasma membrane and the ciliary membrane.

**Transition Fibers and Distal Appendages of Centrioles**

Transition fibers are fibrous links that extend from the distal sides of the mother cilium (the older of the two centrioles found in a centrosome, characterized by the presence of appendages at its distal terminus) to the plasma membrane (Fig. 1), and feature 9-fold symmetry that mirrors the symmetry of the cilium. They have been proposed to form a meshwork that carries out two important functions. First, it provides a large capacity-docking site for IFT proteins.38,39 Second, vesicles and large protein complexes may be blocked from entering the cilium by the transition fiber barrier. As a consequence, vesicles that carry ciliary membrane proteins may have to fuse with the plasma or ciliary membrane (the boundary between the two is not clearly defined) at the periphery of the transition fibers.40 One model hypothesizes that transition fibers and Septins function together to delimit a “landing strip” around the base of the cilium, an area that is isolated by a diffusion barrier from...
Figure 1. For figure legend see page 141.
the rest of the plasma membrane. In this area, cytoplasmic vesicles that carry membrane proteins destined for the cilium fuse to the target membrane. Once membrane cargo proteins "land" in this area, they interact with IFT transport machinery to cross the transition zone.

The precise composition of transition fibers is not clear. They do, however, appear to include ODF2/Cenexin and, therefore, may be related in some way to distal appendages of the mother centriole. ODF2 and other distal appendage proteins, such as Cep164 and Cep83, function in centriole docking. Docking is a process that results in the attachment of the mother centriole to the plasma membrane and is essential for cilia formation. The presence of ODF2/Cenexin in transition fibers suggests that they may be assembled as an extension of distal appendages and anchor the centriole to the plasma membrane after the initial docking event.

It was suggested some time ago, and confirmed by recent studies, that IFT proteins accumulate near transition fibers. In support of the idea that distal appendages and transition fibers are related, a distal appendage protein C2cd3 is critical for both centriole docking and the recruitment of the intraflagellar transport proteins Ift88 and Ift52 to the mother centriole. Furthermore, a distal centriole protein, CCDC41, appears to act in concert with IFT20, to support vesicle-centriole association. These functional similarities between distal appendages and transition fibers suggest that distal appendages differentiate into transition fibers during ciliogenesis.

The Transition Zone

The transition zone is found just distal to the ciliary centriole (i.e., basal body) and is the most proximal part of the membrane-covered cilium (Fig. 1). The structural hallmark of the transition zone is the presence of attachments, seen on electron micrographs, between the axoneme and the ciliary membrane, referred to as "Y links." The location and structural features of the transition zone led to the idea that it provides a gating mechanism that controls transport into the cilium and the ciliary membrane. Support for this is provided by several observations. First, biochemical analysis of isolated Chlamydomonas flagella revealed that mutations in Cep290, a transition zone protein, result in abnormal flagellar protein content. Second, the trans-membrane TRAM proteins, which normally localize to the dendrite plasma membrane and are excluded from the cilium, are found inside the cilium in certain C. elegans transition zone mutants. Third, knockdown of multiple transition zone proteins in primary hippocampal neurons by RNAi reduces the amount of somatostatin receptor (SSTR3) in the cilium and, likewise, transition zone proteins are required to restrict non-ciliary-membrane proteins from cilia in IMCD3 cells. Finally, in the mouse, the transition zone protein, Tectonic, is required to localize the membrane-associated and trans-membrane proteins Arl13b, AC3, Smoothed, and Pkd2 to cilia. These gating functions of transition zone proteins allow the cilium to function as a distinct subcellular compartment that executes numerous signaling functions with high efficiency and without interference with metabolic and enzymatic processes in the cytoplasm.

To date, multiple transition zone proteins have been identified (Fig. 2). The domain content of these proteins, such as the presence of coiled-coil regions, is suggestive of a structural role. Transition zone proteins also feature transmembrane domains or membrane association motifs, such as B9/C2, which suggests that they may attach the multi-component ciliary gate complexes to the membrane. A major structural role for transition zone proteins is to form Y-links. Studies in tissue culture, mouse, and Chlamydomonas have shown that Cep290 contributes to Y-link formation. This is a large coil-coiled domain protein, which theoretically could span the space between the axoneme and ciliary membrane. This would be the case if the Cep290 C-terminal microtubule binding domain associated with axonemal microtubules and its N-terminus with the membrane. In addition, Cep290 contains many coil-coiled regions and is predicted to assume an elongated conformation. Overall, Cep290 may act as a large scaffold to organize the transition zone and assist in the formation of the Y-links. Similar to Cep290, mutations in several Nephrocystins and Meckel-Gruber syndrome proteins affect Y-link formation. As discussed below, these proteins form functionally redundant modules.

Given the diversity of ciliary proteins and changes of ciliary content that occur under different physiological conditions, one would expect that transport across the transition zone is regulated by proteins with enzymatic activity. Surprisingly however, only one group of transition zone proteins, NIMA-related kinases, or NEKs, displays enzymatic activity. This group includes NEK8 in mammals and Fa2p in Chlamydomonas. In mammals, complete loss of NEK8 disrupts the localization of polycystin-1 and...
polycystin-2, as well as the function of multiple signaling pathways. In contrast, the Chlamydomonas NIMA family-related kinase, Fa2p, localizes to the transition zone and is implicated in microtubule severing during deflagellation. The Fa2p protein may, therefore, have a role that is distinct from the regulation of ciliary transport. It is noteworthy that in tissue culture cells Nek2 plays a role in cilium disassembly. This kinase is found, however, in the distal portion of the centriole and is not known to be a transition zone component. More recently, the cell cycle-regulated Polo-like kinase (Plk1) was found to localize to the transition zone and bind the transition zone protein NPHP1. Also in this case, Plk1 function is implicated in ciliary disassembly. Given the paucity of transition zone proteins that display enzymatic activity and have the potential to regulate ciliary transport, regulatory roles are likely to be performed by other groups of proteins. Small GTPases are good candidates for such regulators and Ran, Rabs, and Arls are implicated in ciliary transport.

Mutations in transition zone proteins result in a broad spectrum of diseases, collectively known as ciliopathies. Remarkably, mutations in a single transition zone protein can cause a wide range of disease symptoms of varying severity. For example, mutations in CEP290 produce phenotypes ranging from relatively mild disorders, such as Retinitis Pigmentosa and Leber’s Congenital Amaurosis, to progressively more severe diseases, which include Nephronophthisis/Senior-Loken syndrome, Joubert syndrome (JBTS), and Meckel-Gruber syndrome (MKS). Why a particular mutation in a transition zone protein results in one phenotype but not another remains unclear.

**Modularity of transition zone components**

Systematic genetic analyses of transition zone proteins, performed in *C. elegans*, mammalian cells, and the mouse, suggest that they are organized into at least two groups, referred to as the NPHP and MKS modules (Fig. 2). Defects in human NPHP and MKS module proteins cause mainly Nephronophthisis (NPHP) and Meckel-Gruber Syndrome (MKS), respectively. The MKS phenotype is more severe (perinatal lethality), compared with the NPHP phenotype (kidney disease and retinal degeneration), which suggests that the MKS module may play a more fundamental role. For example, MKS module proteins may have an important structural role in the transition zones of all cilia, whereas NPHP module proteins may function in transition zones of selected, specialized cilia. With few exceptions, which are discussed below, functional differences in transition zones of cilia in different tissues have not yet been characterized.

In *C. elegans*, where cilia are found only in sensory neurons, the NPHP and MKS modules are largely redundant and strong ciliary phenotypes are observed when mutations in genes belonging to both modules are combined. For example, in a dye-filling assay, which is commonly used to evaluate cilia differentiation in this model organism, *mks-6* or *nphp-4* mutations alone display...
mild phenotypes, whereas the phenotypes of mks-6; nphp-4 double mutants are much more severe.\textsuperscript{25} In contrast, only mild dye-filling is observed in mks-6 mutants combined with other MKS mutants, such as mks-1 and mks-3.\textsuperscript{25}

In agreement with genetic studies that indicate the presence of functional modules, biochemical analyses of transition zone proteins reveal that they form complexes. The Mks1, Mks6, and Tectonic proteins form a complex that is important for neural tube closure.\textsuperscript{74} Likewise, all three B9-domain proteins, B9D1, B9D2, and MKS1, which functionally belong to the MKS module, co-immunoprecipitate with any epitope-tagged B9 protein.\textsuperscript{25} In C. elegans, the localization of any one of the B9 proteins is disrupted by mutations in the other two.\textsuperscript{76} Within the NPHP module, it was proposed that NPHP1, NPHP4, and NPHP8 bind each other at the transition zone.\textsuperscript{74} Finally, NPHP proteins NPHP2/Inversin, Nphp3, Nek8/NPHP9, and ANKS6 also form a complex, which defines the so-called Inversin compartment found at the base of the cilium, above the transition zone.\textsuperscript{77-79} The significance of the modular structure of the transition zone is not clear. Perhaps it is related to multiple, evolutionarily conserved functions mediated by this part of the cilium. It will be interesting to examine the evolution of these modules and determine whether redundancy results in module elimination or, alternatively, adaptation to new functions in certain phyla.

**Evolutionary considerations**

Transition zone proteins are conserved in the eukaryotic tree of life, which indicates that they are ancient components of the cilium and likely play important roles in its biology. For example, the transition zone protein Cep290 is found in both main branches of eukaryotic evolution: the Unikonts (eukaryotes whose ancestors had a single cilium), and Bikonts (eukaryotes whose ancestors had two cilia) (Fig. 2D).\textsuperscript{80-82} To date, only two transition zone proteins (Cep290 and a NIMA-related kinase Fa2p) have been studied in Bikonts. The aforementioned study of Cep290 in *Chlamydomonas* suggests that it has a conserved role in regulating ciliary compartmentalization.\textsuperscript{51} In contrast, the analysis of *Chlamydomonas* Fa2p points to a role in microtubule severing during deflagellation and it is not clear whether Fa2p function is conserved.\textsuperscript{58}

Little is known about the role of other transition zone components in unicellular organisms and to what extent transition zone proteins have changed their function during evolution. For example, in *Chlamydomonas*, Cep290 is found only in the transition zone,\textsuperscript{51} whereas mammalian Cep290 is also present in centrosomal and centriolar satellites.\textsuperscript{83,84} According to one hypothesis, the original role of centrioles is to form cilia and centriole involvement in centrosome and cell division is a later metazoan innovation.\textsuperscript{85} Therefore, it is possible that Cep290 localization outside of the ciliary transition zone is a more recent evolutionary adaptation.

**Specialized transition zones**

Although ciliary transition zones are structurally very similar in diverse cells and tissues of organisms as different as *Chlamydomonas*, *C. elegans*, and *Homo sapiens*, in some cases transition zones appear to develop adaptations to specific tasks. Two examples of this have been characterized in sensory neurons and sperm cells. Transition zones in sensory neurons are longer compared with those in most cells and are referred to as “connecting cilium” (Fig. 3A) (reviewed in ref. 86). The exceptional length of these transition zones is presumably a structural requirement that reflects the unusual morphology of cilia in sensory cells. Another special case has been described in sperm cells, where the centriole is attached to the nucleus, the proximal end of the axoneme localizes to the cytoplasm and only the distal part of the axoneme appears to be confined in a separate membrane compartment (Fig. 3B and C) (reviewed in ref. 87). This unique cilium configuration appears to satisfy two special needs. The first is to produce a hydrodynamically streamlined shape that supports effective sperm swimming. The second is to position mitochondria, which provide an energy source, close to the axoneme, presumably, to facilitate flagellar motility. Since mitochondria are too bulky to enter the cilium, it appears that sperm cells place the proximal end of the axoneme in the proximity of mitochondria in the cytoplasm.

**Sensory neurons**

The so-called “connecting cilium” is found in insect sensory neurons and in vertebrate photoreceptors.\textsuperscript{50,88} In contrast to the transition zone of most cilia, the connecting cilium is formed between two bulky compartments, referred to as the “inner” and

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**Figure 3.** Specialized transition zones. (A) Vertebrate photoreceptors and *Drosophila* sensory neurons share similar cellular architecture and an atypical transition zone, known as the connecting cilium. The outer segments of vertebrate photoreceptors measure 20–25 um in rods and 10–15 um in cones of most species.\textsuperscript{202-203} Outer segments of fly sensory neurons are approximately 5–50 um long.\textsuperscript{203,204} Modified from Avidor-Reiss et al., 2004. (B and C) Illustration of compartmentalized (B) and cytoplasmic (C) cilogenesis, featuring transition zone and ring centriole/annulus, respectively.
“outer” segment, which display much greater width and volume, compared with the transition zone itself (Fig. 3A). This contrasts with the majority of cells in which the transition zone is as narrow as the rest of the cilium (Fig. 3). The high volume of the outer segment and the remarkably active protein traffic in connecting cilia is likely to impose unusual structural requirements, such as increased mechanical strength. This may explain why connecting cilia are longer than a typical transition zone: approximately 1 μm in vertebrate photoreceptors and 0.6 μm in Drosophila mechanosensory neurons while typical transition zones in non-sensory cells are 0.2 μm long. This length difference is reflected in transition zone ultrastructure, which features 2–7 rows of Y links in most cilia and 30–40 rows in photoreceptors.

**Sperm cells**

Cilia in most cells assemble and maintain their axoneme within a membrane compartment that projects from the surface of the cell. In these cilia, the transition zone forms at the base of the membrane projection, close to the distal end of a centriole, which is located next to the cell’s plasma membrane. In contrast, in cytoplasmic cilia, found in mammalian and Drosophila sperm cells, as well as in the microgametes of the malarial parasite Plasmodium, the axoneme localizes, at least partially, in the cytoplasm and the centriole is not associated with a membrane-enclosed axoneme (Fig. 3B and C).

In the sperm cells of most animals, the centriole is anchored to the nuclear membrane and only the tip of the axoneme is located inside a cap-like membranous structure that is morphologically similar to a typical cell surface ciliary compartment. Candidate Drosophila transition zone proteins, such as Uncordinated (thought to be homologous to OFD1), Chibby, and Dilatory (CEP131), localize to the ciliary cap base in sperm cells. These observations suggest that the base of the membrane-compartment in sperm axoneme is analogous to the transition zone in typical cilia.

**Transition Zone Interactions with Ciliary Transport Mechanisms**

How proteins targeted to the cilium are transported across the transition zone remains unclear. Several scenarios are possible and these are not necessarily mutually exclusive. One idea is that cytosolic proteins are first targeted to a site near the centriole or transition fibers. These proteins bind, sometimes as complexes, to IFT machinery and become its cargo. For example, radial spokes, which are large axonemal complexes that consist of 22 polypeptides, are partially assembled in the cytoplasm before being transported to the flagellar tip by anterograde IFT. In this model, the IFT machinery, which is licensed to cross the transition zone, delivers the cargo into the cilium, and the capacity of transition zone proteins to interact with the IFT machinery is essential for ciliary entry.

Specificity is determined by the ability of the cargo to interact with IFT proteins. The key element of this model is thus the molecular basis of interactions between IFT proteins and the ciliary cargo molecules that are being transported into the cilium. In some cases, cargo-IFT interactions are direct, while other cargo proteins may require the involvement of additional cargo-specific adapters.

Complexes of IFT proteins are very large. In Chlamydomonas, anterograde IFT trains fill the gap between the axoneme and the ciliary membrane, and are 700 nm long. They are unlikely to cross the transition zone without hindrance due to the presence of structures such as transition fibers and Y-links. Therefore, IFT proteins may have unique characteristics that allow them to interact with the transition zone. Indeed, at least some transition zone proteins interact with IFT particle components. This may facilitate the transport of IFT-associated cargo proteins into the cilium. The nature of interactions between transition zone proteins and IFT particles remains largely unknown. One can imagine, however, that IFT proteins induce conformational changes in the transition zone, which then open a path for IFT trains to go through.

In some circumstances, the regulated entry of cargo into cilia can occur independently from IFT. In Chlamydomonas, the flagellar adhesion-induced 65-kDa-membrane protein, SAG1-C65, can translocate into the cilium under the restrictive conditions of the temperature-sensitive kinesin-2 mutant, flat10, which blocks IFT. This IFT-independent ciliary entry requires cytoplasmic microtubules but it is not clear whether it occurs due to active transport or a temporary relaxation of the ciliary diffusion barrier.

Active transport is not the only manner in which proteins can enter the cilium. There is evidence that small proteins can diffuse into the ciliary shaft, which may be limited mainly by steric hindrance that is imposed by the narrow distance between the axoneme and ciliary membrane. In an in vitro assay, using cells in which the plasma membrane was made permeable but the ciliary membrane remained intact, it was found that proteins >9 nm in diameter (100 kD) are restricted from entering cilia. Similarly, a newly developed methodology, which relies on intact cells and has been termed “chemically inducible diffusion trap at cilia,” has provided estimates that proteins up to 7.9 nm in diameter can diffuse into the cilium. Consistent with the idea that the ciliary base acts as a gate, mathematical modeling in this study suggested that a molecular sieve acts as a passive barrier at the cilium base. Another study, which relied on fluorescently labeled dextrans and recombinant proteins of various sizes, found that 40K and 70K dextrans and the 67K bovine serum albumin were significantly restricted from entering the cilium, while smaller dextran molecules and recombinant proteins could freely enter the cilioplasm. In photoreceptor sensory cilia, limits on the speed of diffusion appear to be imposed by the size of the protein relative to the highly constrained spaces between outer segment disc membranes. This study compared the entry of GFP monomers, dimers and trimers into the frog outer segment and concluded that ciliary entry may be controlled by a steric volume exclusion mechanism that does not require a diffusion barrier.

Estimates of barrier permeability vary, which may be due to the different shapes of tested cargo molecules (globular vs. rod-like) or disparate physical parameters of the transition zone in different cells. The photoreceptor cilium appears to be more permeable, compared with cilia of RPE cells. This is somewhat counterintuitive as the photoreceptor transition zone is far from the other transition zones.
organized to cilia in other organisms. However, whether the findings of this analysis can be extrapolated to cilia in other organisms.

Ciliary Targeting Sequences

How are the proteins involved in cilia formation and function identified as cargo by ciliary transport machinery? Studies of cilia proteins have revealed specialized signal sequences, known as ciliary targeting sequences (CTS), which direct them to the ciliary compartment. In transmembrane proteins, CTSs are found on the cytoplasmic side of the membrane, both in intracellular vesicles and in the ciliary shaft. The CTSs most often localize to the C-termini of proteins, less frequently to N-termini and in some cases to intracellular loops of multi-pass transmembrane proteins (Table 1). The cilia targeting sequences are quite diverse, which suggests that they interact with different pieces of the transport machinery. This is not unexpected as multiple pathways are likely to mediate protein targeting to cilia.

The most common molecular feature of ciliary targeting sequences is the VxP motif, characterized only in transmembrane and membrane-associated proteins so far, which include G protein-coupled receptors (GPCRs), polycystins, CNG channels, and retinol dehydrogenase (Table 1). In experimental conditions, the VxP motif is frequently necessary for ciliary targeting and its mutations abolish ciliary localization (references in Table 1). Human mutations in the VxP motif of rod opsin cause rapid rod photoreceptor degeneration (reviewed in ref. 109), most likely due to the need to prevent retrograde diffusion of opsin, which is highly concentrated in the cilium of these cells. Despite being exceptionally long, the photoreceptor transition zone may need to be permeable to cytosolic proteins in order to accommodate machinery that supports massive anterograde opsin transport, which, at least in some species, is estimated to occur at 1000 molecules/s.107

Another potential ciliary gating mechanism has emerged from electron microscopic analysis of Tetrahymena flagella.108 This study described a structure at the ciliary base, termed the “ciliary partitioning system” (CPS), which appears to block the ciliary entry of cytoplasmic proteins. It features nine openings, one near each of the axoneme doublet microtubules, that could allow cargo-loaded IFT particles to enter the cilium. It is not clear, however, whether the findings of this analysis can be extrapolated to cilia in other organisms.

| Table 1 | Targeting Sequences in Various Proteins |
|---------|----------------------------------------|
| Opaline | C-terminal sequences (C-termini)       |
| CNG     | C-terminal sequences (C-termini)       |
| Retinol | C-terminal sequences (C-termini)       |
| Dihydro | C-terminal sequences (C-termini)       |
| Opsin   | C-terminal sequences (C-termini)       |

The VxP motif is present in many GPCRs (Table 1 in ref. 117). In contrast to opsin, however, it is not positioned at the very C-terminus of the protein sequence and its importance has not been experimentally tested. Many GPCRs feature an additional targeting sequence in the 3rd intracellular loop (hereafter I3-CTS) (Table 1). For example, a somatostatin receptor, SSTR3, contains the AxxxQ motif in this region. This motif is absent in five other SSTR receptors that are expressed in the rat CNS but do not localize to cilia.117

The importance of the I3-CTS has been demonstrated by domain swap experiments. Although the wild type SSTR5 receptor does not localize to cilia, a chimeric version of SSTR5, which contains the 3rd intracellular loop from SSTR3, does. Mutating the AxxxQ motifs in the SSTR3 I3-CTS abolishes the ciliary localization of this chimeric SSTR5/3.117 Similar experiments have been performed for HTR6 and also demonstrated the importance of the AxxxQ motif in the 3rd intracellular loop. Not all I3-CTS motifs of GPCRs are, however, related to each other. The neuropeptide Y receptor, NPY2R, contains two I3-CTS motifs that show no similarity to those in SSTRs.118

In contrast to opsin, another abundant membrane protein of photoreceptor cilia, peripherin, does not feature the VxP motif.119 Peripherin CTS is unrelated to that found in opsin. This dissimilarity of opsin and peripherin CTS is consistent with genetic studies. Mutations that affect opsin transport do not affect

longer, compared with that in most other cell types.10 The length of the transition zone in photoreceptors may, however, reflect its mechanical role in supporting the bulky outer segment or the need to prevent retrograde diffusion of opsin, which is highly concentrated in the cilium of these cells. Despite being exceptionally long, the photoreceptor transition zone may need to be permeable to cytosolic proteins in order to accommodate machinery that supports massive anterograde opsin transport, which, at least in some species, is estimated to occur at 1000 molecules/s.107

Further analysis of photoreceptors has revealed that transmembrane proteins are generally targeted to photoreceptor cilia unless they contain signals that direct them elsewhere.112 This finding is, however, more than likely a peculiarity of vertebrate photoreceptors as it has not been observed in other cells.112 The unusual behavior of photoreceptors is believed to result from the exceptionally active transport of membrane material from the cell body into the ciliary compartment, necessitated by the continuous shedding of membrane in the distal region of photoreceptor cilia at the rate of approximately 10% per day.113,114 It is this unusual feature that results in the photoreceptor cilia being a default destination for the trafficking of transmembrane proteins. In other cells, membrane association of proteins appears to be neither necessary nor sufficient for ciliary targeting.

In addition to the VxP sequence and palmitoylation sites, opsinC44 contains the FR motif that was originally identified in a nematode olfactory GPCR, ODR-10.115 This juxtamembrane motif consists of a hydrophobic amino acid followed by a basic amino acid and is also found in cytoplasmic tails of other GPCRs.116 The importance of this motif has not been tested in opsin but its ablation eliminates ciliary targeting of ODR-10 and Smoothened.115,116

The VxP motif is present in many GPCRs (Table 1 in ref. 117). In contrast to opsin, however, it is not positioned at the very C-terminus of the protein sequence and its importance has not been experimentally tested. Many GPCRs feature an additional targeting sequence in the 3rd intracellular loop (hereafter I3-CTS) (Table 1). For example, a somatostatin receptor, SSTR3, contains the AxxxQ motif in this region. This motif is absent in five other SSTR receptors that are expressed in the rat CNS but do not localize to cilia.117

The importance of the I3-CTS has been demonstrated by domain swap experiments. Although the wild type SSTR5 receptor does not localize to cilia, a chimeric version of SSTR5, which contains the 3rd intracellular loop from SSTR3, does. Mutating the AxxxQ motifs in the SSTR3 I3-CTS abolishes the ciliary localization of this chimeric SSTR5/3.117 Similar experiments have been performed for HTR6 and also demonstrated the importance of the AxxxQ motif in the 3rd intracellular loop. Not all I3-CTS motifs of GPCRs are, however, related to each other. The neuropeptide Y receptor, NPY2R, contains two I3-CTS motifs that show no similarity to those in SSTRs.118

In contrast to opsin, another abundant membrane protein of photoreceptor cilia, peripherin, does not feature the VxP motif.119 Peripherin CTS is unrelated to that found in opsin. This dissimilarity of opsin and peripherin CTS is consistent with genetic studies. Mutations that affect opsin transport do not affect
| Gene | Sequence | Location | Function | Reference |
|------|----------|----------|----------|-----------|
| **Transmembrane** | | | | |
| Rhodopsin (human)  
other opsins similar | …FR…TTGCGK…QVAPA-c | C-terminus | 44 C-terminal aa. sufficient; underlined residues necessary (based on experimental work and human mutations); palmitoylation sites in blue | 109, 111, 192 |
| | …AAAAQQ… | 3rd intracel. loop | Not necessary | |
| SSTR3 (mouse)  
similar in HTR6, MCHR1, D1, others | …APSCQ…  
…AxxxxQ… consensus | 3rd intracel. loop | Underlined residues necessary, intracellular loop 3 sufficient in the context of non-ciliary GPCR | 117, 131 |
| | …LLP…17aa;  
…[V/L]xP consensus | C-terminus | Not tested | |
| NPY2R (human)  
similar in GPR83, PGR15L | …RIW… | 3rd intracel. loop | Underlined residues necessary | 118 |
| | …RRQK… | 3rd intracel. loop | Underlined residues necessary | |
| ODR-10 (C. elegans)  
similar in smo, opsins, SSTR3, HTR6, others | …FR…30aa  
…hydrophobic/basic… | C-terminal half, juxta-membrane | Underlined residues necessary | 5, 115, 116 |
| Pkd2 (human) | n-MVNSSRVQPO… | N-terminus | Underlined residues necessary 15 N-term aa. sufficient | 123 |
| Pkd1 (human) | …KVHPSST-c | C-terminus | Underlined residues necessary | 193 |
| CNGB1b (rat)  
similar in CNGA2 | …RVSP…  22aa | C-terminus | Underlined residues necessary; RVSP not sufficient | 194 |
| Fibrocystin (mouse) | …CLVCCWFKKSTRKIKP… 174aa | C-terminus, juxta-membrane | 17 juxtamembrane aa. sufficient | 195 |
| Peripherin (Xenopus) | …KSSWELVKSMEKLNKVTAG… 10aa | C-terminus | aa 307–336 and 317–346 sufficient in the presence of opsin membrane targeting sequence | 119 |
| **Membrane-associated** | | | | |
| Cystin (mouse) | n-MGSGSS…21aa…TASEGGTA… | N-terminus | Underlined residues necessary, myristoylation site is in blue, aa. 28–35 sufficient when fused to exogenous myristoylation sequence | 196 |
| INPP5E (human) | …VRPG…10aa…  
FDRELYL…25aa…CSVS | C-terminus | underlined residues necessary; 66 C-terminal aa. not sufficient, upstream VxP-like motif not necessary, CaaX motif is in blue | 110 |
| NPHP3 (mouse)  
similar in Gnat1, ODR-3, GPA-13, others | n-MGTASSLVSPTG…  
aa. 96–201 | N-terminus | underlined residues necessary, myristoylation site is in blue, aa. 96–201 necessary and sufficient for base of cilia targeting, VxP not necessary | 186, 188 |
| Retinol dehydrogenase (cattle) | …LRCLASCFCRTPYWPR-c | C-terminus | 16 C-terminal aa. sufficient; underlined residues necessary; presumptive palmitoylation sites in blue | 197 |
| RP2 (human) | 85 aa…CTNCIIFLPGVK… | N-terminus, NLS-related | M9-related, underlined residues necessary | 183 |
| | n-MGCFFSKRRKAD KES… | N-terminus, NLS-related | 15 N-terminal aa. sufficient; lipidation sites and a presumptive nuclear localization signal are in blue | |
| **Cytoplasmic** | | | | |
| Gli2, (human)  
similar in Gli3 | aa. 852–1183 | central region | necessary but not sufficient for ciliary localization | 198 |
| Kif17 (human) | …KRKK…10aa | C-terminus, NLS-related | necessary, C-terminal 228 aa. sufficient to target non-ciliary kinesin | 63 |
peripherin. Based on these observations, it has been suggested that these two transmembrane proteins are translocated into the same ciliary compartment by different pathways.

The CTSs of cytoplasmic proteins are less well characterized. The targeting sequences characterized thus far in Gli and Kif17 are unrelated to one another (Table 1). The CTS of the Kif17 kinesin is a stretch of basic amino acids that is related to nuclear localization signals (see below). Whether similar signals function in other cilia-targeted cytosol-soluble proteins remains to be determined.

**CTS-interacting Proteins**

Several CTSs are well conserved in orthologous proteins throughout vertebrate evolution, at the level of the primary sequence. This suggests that they mediate interactions with the components of transport machinery. Indeed, binding partners have been described for CTSs in GPCRs and a ciliary phosphoinositide phosphatase, INPP5E.

To determine which proteins bind opsinC44 and target opsin to the photoreceptor cilium, several groups have used proteomic approaches. This included cross-linking and yeast two-hybrid experiments that used opsin C-terminal sequences as the bait. Molecular analyses of proteins identified via these approaches suggest that they function sequentially at several stages of opsin transport. It is believed that opsin transport from the Golgi to the cilium is initiated by a small GTP-ase, Arf4, which binds the opsin CTS and appears to mediate the budding of opsin-carrier vesicles from the trans-Golgi network. It has been recently proposed that Arf4 enhances the binding of an Arf GAP, ASAP1, to the FR motif in opsinC44. This, in turn, initiates the assembly of the Rab11/Rabin8/Rab8 complex in the cytoplasm and is thought to trigger opsin translocation to cilia (recently reviewed in refs. 3, 5, and 126).

Once formed at the Golgi, opsin carrier vesicles are translocated to the base of the cilium. Based on results of a yeast two-hybrid screen, which revealed that the opsin C-terminus interacts directly with cytoplasmic dynein, it has been suggested that microtubule-dependent transport accounts for opsin translocation through the cytoplasm. Support for this idea is also provided by observations that cytoplasmic Dynein-I heavy chain (DYNC1H1) co-purifies with IFT complex A proteins. As discussed below, in addition to their role in ciliary IFT, complex A proteins are thought to function at the base of cilia to facilitate the transport of GPCRs. Evidence has also been presented, however, for the transport of opsin by microtubule-independent mechanisms. The contribution of microtubule-dependent motors to the transport of GPCRs in the cytoplasm requires further investigation.

In contrast to opsin, many GPCRs require I3-CTS for their ciliary localization. The function of the I3-CTS appears to be mediated by BBS proteins. Evidence for this has been provided by the observations that mouse knockouts of BBS2 and BBS4 abolish the ciliary localization of SSTR3 and MCHR1. Similarly, interference with BBS3 and BBS4 in tissue culture disrupts ciliary targeting of a chimeric transmembrane receptor that contains SSTR3 I3-CTS. In protein interaction tests, I3-CTS of SSTR3 pulls down all BBsome components tested, while the I3-CTS of the dopamine receptor 1 appears to interact directly with BBS5. A likely function of I3-CTS is thus to integrate GPCRs in membrane coats formed by BBS proteins.

Finally, in INPP5E, CTS is also found at the C-terminus and mediates direct interactions with a small GTP-ase, Arl13b. Mutations in the sequence of this CTS abolish Arl13b binding. The studies of ciliary targeting sequences have been highly informative and are likely to continue to generate valuable insights. The binding partners of most CTSs remain unknown.

**The Rab8/Rab11 Cassette: A Central Component of the Ciliary Transport Hub?**

A number of recent studies have revealed the importance of a cassette of two small GTPases, Rab8, and Rab11, in cilia-directed traffic (Fig. 1). The first indication that Rab8 may play a central role in ciliary transport came from the observation that the GDP-locked variant of Rab8 causes a striking accumulation of vesicles around the ciliary centriole when overexpressed in photoreceptor cells. Later studies revealed that Rab8 localizes to the ciliary membrane and is a major component of a mechanism that drives cilia formation in general. The overexpression of GTP-locked mutant Rab8 results in cilia elongation and uncoupling between cilia length and the extent of the acetylated region on the ciliary axoneme. These observations suggest that Rab8 acts as a potent activator of vesicle fusion to membranes at the ciliary base.

Further analysis demonstrated that Rab8 is a downstream component of a pathway that involves Rabin8 and Rab11. The Rabin8 protein functions as a Rab8 guanine nucleotide exchange factor (GEF) and is found at the ciliary base. In agreement with its role in stimulating Rab8 activity and cilia formation, Rabin8 presence at the ciliary centriole is transient and correlates with ciliogenesis. The Rabin8 protein localizes Rabin8 to vesicles at the ciliary base and in the GTP-bound state stimulates its GEF activity. All three proteins are required for normal ciliogenesis in tissue culture conditions.

Several additional studies showed that the Rab11/Rabin8/Rab8 pathway is functionally related to vesicle trafficking by the exocyst and TRAPP vesicle tethering complexes, and BBS protein membrane coats. In addition, Rab8 and Rabin8 bind centriolar appendage proteins, ODF2 and Cep164, respectively, which indicates that they may function in vesicle docking in the basal body area. In agreement with this, CEP290 is also necessary for Rab8 localization to the ciliary membrane. Finally, Rab8 binds a Rab effector protein, Rabaptin5, which in turn appears to interact with Elipsa/IFT54, an IFT particle component. These observations place the Rab8/Rabin8/Rab11 complex at the center of several molecular events that are likely to facilitate vesicle delivery to the cilia base: vesicle coat formation, recognition of the vesicle target site, tethering at the target site, and fusion to the plasma membrane.
and, potentially, interaction with transport machinery that will carry vesicle cargo further into the ciliary shaft.

The picture of Rab8/Rabin8/Rab11 function is still incomplete however. Although several cell culture studies suggest that Rab8 is a key regulator of ciliogenesis, a double knockout of both Rab8 genes (two are found in the mouse genome) does not affect cilia in the mouse. A knockdown of a related gene, Rab10, in the Rab8α−/−;Rab8β−/− double mutant background does, however, cause cilia defects. This result reveals an unexpected level of redundancy in ciliary Rab function, an area that requires further investigation. Likewise, as discussed below, factors that function upstream of Rab11, its GEFs in particular, remain to be identified.

The exocyst

The exocyst is a vesicle tethering complex that is closely related to the Rab8/Rab11 ciliogenic pathway (Fig. 1). In both yeast and mammals, the exocyst consists of eight proteins that mediate exocytosis. Intriguing similarities exist between ciliary GTPases and Rabs that regulate yeast exocyst function. Yeast Rabs, Ypt31 and Ypt32, interact with an effector, Sec2p, which, in turn, functions as a GEF for Sec4p. It has been proposed that Ypt31/32, Sec2p, and Sec4p are functionally homologous to Rabin8, Rab11, and Rab8, respectively, and thus related Rab cascades may function in ciliogenesis and in the budding of yeast cells.

The Rab8/Sec4p protein, in the GTP-bound state, interacts with an exocyst constituent, Sec5, both in yeast and in higher eukaryotes. This led to the conclusion that the exocyst complex functions as a Rab effector and mediates Rab8/Sec4p function. Is this also the case in ciliogenesis? Indeed, the exocyst does seem to play some role in cilia formation as several of its components, such as Sec 6, 8, and 15, localize to cilia and the knockdown of some others, Sec10 and Sec15 in particular, inhibits cilia formation in tissue culture conditions. The role of human Sec15 in ciliogenesis is further supported by observations that it binds Rabin8, an interaction that is enhanced by the phosphorylation of Rabin8 by NRD2, a kinase known to function in cilia. Based on knockdown results, exocyst function is not confined to a specialized subset of ciliary membrane cargo proteins, but rather appears to have a more general role in cilia formation. By analogy to yeast exocyst function, it is likely to act downstream in the Rab11/Rab8 ciliogenic pathway, possibly facilitating vesicle fusion with periciliary membranes.

TRAPP II complex in ciliary trafficking

Another Rab8/Rab11-related mechanism in the delivery of cilia-bound vesicles appears to involve TRAPP II, a vesicle tethering complex that consists of 10 cytoplasmic proteins and is best known for its role in trafficking to Golgi membranes (reviewed in ref. 148) (Fig. 1). Evidence for its relatedness to the Rab8/Rab11 pathway is provided by TAP-tag experiments showing that all TRAPP II complex constituents co-purify with Rabin8. Moreover, RNAi depletion of three TRAPP II complex components, TRAPPC3, TRAPPC9, and TRAPPC10, leads to a loss of Rabin8 localization from the pericentrosomal area and impaired ciliogenesis. These experiments strongly suggest that TRAPP proteins contribute to cilia formation.

The idea that the TRAPP II complex functions in the Rab8/Rab11 pathway has been recently strengthened by the observation that TRAPP C10 contains a longin domain, which is a protein interaction module frequently found in Rab GEFs. This has led to the proposition that a dimerization of longin domains from TRAPP C10 and TRAPP C2 provides a surface for Rab11 binding. As TRAPP complexes are known to display GEF activity toward Rab GTPases, including Ypt31/32 (putative yeast homologs of Rab11), ample precedent exists for a potential role of the TRAPP II complex as a Rab11 GEF. No other candidate Rab11 GEF has been identified thus far and so the mechanism of Rab11 activation remains unknown. The TRAPP II complex could function upstream of the Rab11/Rab8 pathway by activating Rab11, and thereby trigger a series of events that eventually result in vesicle fusion. This idea appears less convincing, however, in the light of observations that only a subset of TRAPP II complex components display knockdown phenotypes that affect ciliogenesis.

BBS proteins as membrane coats

A major contributor to ciliary GPCR transport appears to be a complex of proteins encoded by genes originally identified as defective in a human ciliopathy, the Bardet-Biedl Syndrome (BBS) (Fig. 1). Quite remarkably, seven of these proteins co-purify in tandem-affinity purification (TAP) experiments. This led to the realization that they form a complex, which was named the BBSome. As the number of BBS genes exceeds the number of BBS proteins that co-purify in TAP assays, some BBS proteins may be less tightly associated or form separate complexes. Indeed, three chaperonin-related BBS proteins, BBS6, BBS10, and BBS12, are thought to form another complex that mediates BBSome assembly.

Genetic studies have shown that BBS complex components are required for the ciliary localization of opsins, somatostatin receptor 3 (SSTR3), melanin concentrating hormone receptor (MCHR1), neuropeptide Y receptor (NPYR2) and potentially other GPCRs. These observations are complemented by intriguing cell biological and protein-protein interaction data showing that, at least in vitro, BBS proteins form coats on the surface of lipid vesicles. Formation of BBS membrane coats requires a non-core BBS protein, Arl6. This is a small GTP-ase, also known at BBS3, which interacts with membrane lipids via an N-terminal amphipathic helix. The idea that BBS proteins form vesicle coats also finds support from bioinformatic analyses, which suggest a similarity between ciliary transport proteins and vesicle coat components that function in ER to Golgi transport (COPI, COP II) or the internalization of surface proteins (Clathrin coated pits).

By analogy to COPI, COP II, and Clathrin coated pits, one function of BBS membrane coat complexes could be to trap GPCRs in vesicles and direct their translocation into the cilium. As discussed above, experimental support for this idea comes from genetic and biochemical analyses of interactions between GPCR I3-CTS sequences and BBS proteins. These experiments provide strong evidence that BBS proteins participate in the ciliary targeting of GPCRs. The exact mechanism of how this occurs remains, however, unclear. Some insight into this process...
has come from observations that the absence of BBS proteins does not affect cell surface targeting of cilia-bound cargo but rather shifts its localization from the ciliary membrane to plasma membrane.\(^8\) Nevertheless, the existing data does not allow one to conclusively discriminate between several models. In one scenario, BBS coats could assemble on membranes of intracellular vesicles in a CTS-dependent manner to target vesicles to the ciliary compartment. However, direct evidence for the presence of such coated vesicles is missing, as BBS proteins have not been detected on intracellular membranes thus far.\(^8\) Alternatively, BBS proteins could assemble membrane-associated complexes, referred to as planar coats, on the cell surface outside cilia, bind GPCRs and then translocate them across the diffusion barrier into the ciliary compartment. This model finds support in the realization that IFT trains, which contain BBS proteins, can be thought of as planar coats.\(^15\) Yet another possibility is that BBS vesicle coats.\(^8\) Importantly, Exo70 appears to provide positional information in polarized exocytosis (reviewed in refs. 163 and 164). This suggests a speculative hypothesis that perhaps a complex of BBS proteins and exocyst components defines the site of cargo delivery during cilia formation.

**Tubby and IFT Proteins at the Gate to the Ciliary Compartment**

Another group of genes with a role in trafficking to the cilium is the Tubby family, which consists of Tubby and several Tubby-like (TULP) genes (reviewed in ref. 165) (Fig. 1). These proteins are not directly related to the Rab8/Rab11 pathway, but nonetheless display functional similarities to BBS proteins. Similar to BBS mutations, loss of Tubby family genes does not cause pronounced structural cilia defects but impairs ciliary localization of selected GPCRs, which include opsins, SSTR3, MCHR1, Gpr161 and NPY.\(^118,120,166,167\) Mutations in the human TULP1 gene cause retinal degeneration, which is consistent with a role in opsin trafficking.\(^168\) The Tubby family proteins share a conserved C-terminal region that folds into a 12 stranded β-barrel and interacts with phosphoinositides.\(^169\) N-terminal regions, on the other hand, are variable and thought to play diverse roles. The N-terminus of Tubby-like 3 (TULP3), for example, binds IFT complex A poly-peptides.\(^6\) Based on the TULP3 requirement for GPCR transport and its potential involvement in dual binding interactions with membrane phosphoinositides and IFT poly-peptides, TULP3 has been proposed to link peri-cilia vesicles (i.e., vesicles that accumulate at the cilia base and are likely to transport GPCRs) with cytoplasmic IFT A protein complexes. This could bring GPCR-carrying vesicles into the proximity of transition fibers, the transition zone and other ciliary gating mechanisms.

Many questions regarding the function of Tubby family genes remain to be answered. For example, it is not clear why their role is selective for only some GPCRs and Smoothened is not affected by the loss of TULP3.\(^6\) Given their interactions with phosphoinositides, Tubby proteins could contribute to vesicle coat formation. This idea is supported by genetic data from vertebrates and nematodes alike, which suggests that Tubby-family and BBS genes play related roles.\(^118,170\) One indication of this relatedness is that both BBS and Tubby-family gene mutants share obesity as a phenotype.\(^154,171-173\) This phenotype correlates with the role of TULP3 and BBS18/BBIP10 in the ciliary targeting of a Neuropeptide Y receptor, NPY2R.\(^119\) Molecular underpinnings of this functional relatedness remain obscure.

**IFT Complex A Function at the Cilia Base**

A set of experiments has suggested that IFT complex A functions in concert with Tubby proteins. First, TAP-tag co-purification analysis revealed that TULP3 binds IFT complex A components.\(^6\) Furthermore, functional studies showed that depletion of three IFT complex A proteins, Ift122, Ift140 and Ift144, does not cause ciliogenesis defects but decreases the ciliary content of TULP3.\(^6,166\) Finally, both Tubby and IFT complex A proteins are necessary for the ciliary entry of GPCRs.\(^6\) In agreement with this, we (Avidor-Reiss lab) also observed that, in fly *osg1/ift122* mutants, the CNG channel is no longer transported into the cilium and accumulates at its base (Fig. 4). Taken together, these observations indicate that the role of IFT complex A in ciliary compartment-directed transport extends to a fairly broad spectrum of cargos.

In agreement with the idea that the function of IFT complex A proteins extends beyond intraflagellar transport, IFT complex A and Tubby mutants are phenotypically similar to one another but differ from mutants in the intraflagellar dynein, DYNC2H1 (reviewed in refs. 6 and 174). These results led to the idea that three IFT complex A “core” proteins function at the base of the cilium to facilitate ciliary access of certain proteins.\(^6\) The molecular basis of this ciliary access mechanism remains unknown. It could enhance the fusion of cytoplasmic vesicles. Alternatively, IFT complex A-vesicle interactions at the cilia base could initiate the loading of vesicle cargo, GPCRs and CNG channels, for example, onto the axonemal IFT particle trains, which also include IFT complex A proteins.
Some information has also become available on the mechanisms that drive the translocation of IFT complex A proteins toward the cilium. Proteomic experiments revealed that Ift140 physically interacts with cytoplasmic dynein, DYNC1H1, which led to the idea that this motor may transport complex A proteins to the cilia base.6 Another player in the cytoplasmic transport of IFT proteins may be a planar cell polarity effector gene, fuzzy. Interference with fuzzy function causes loss of Ift43 from the basal body area.175 Whether fuzzy is also required for the targeting of other IFT complex A proteins remains to be investigated. The Fuzzy protein does not seem to function in transporting the complex B component, IFT20.173 As a side note, Fuzzy appears to play multiple roles in ciliogenesis because it has also been recently reported to recruit Rab8-positive vesicles to the basal body.176 Although data from several studies have yet to be assembled into a coherent model, evidence is accumulating that IFT complex A proteins function in the ciliary centriole area to mediate transport into the ciliary compartment.

**IFT20 in Transport from Cytoplasm to Cilia**

In addition to IFT complex A proteins, a complex B component, Ift20, also appears to facilitate cilia-directed trafficking in the cytoplasm. This has been suggested by the observation that a mild knockdown of Ift20 does not affect ciliary length but does decrease the ciliary content of Pkd2, a ciliary transmembrane protein.177 The Ift20 protein has also been found to localize to the Golgi apparatus, which led to suggestions that it may play a role in Golgi to cillum transport. This function does not appear to extend to all IFT complex B constituents because Ift88 knockdown does not produce a similar effect.177

Consistent with the above experiments, two Golgi-associated proteins, Golgin and CCDC41, have been shown to be functionally related to Ift20. When Golgin is mutated in mouse cells, it causes loss of Ift20 from the Golgi apparatus, cilia shortening and a partial reduction of ciliary Pkd2 expression.178 These observations suggest that Golgin and Ift20 function in the same pathway, to localize Pkd2 to cilia. The second protein, CCDC41, is necessary for Ift20 localization to the ciliary centriole but not to the Golgi apparatus.179 The role of CCDC41 in Pkd2 targeting has not been investigated.

**Lateral Transport into the Cilium from the Plasma Membrane**

Although most models of ciliary targeting mechanisms invoke vesicle delivery from cytoplasmic pools located at the cilia base, an important alternative involves translocation of transmembrane proteins from the plasma membrane that surrounds the cilium. This mechanism has been best characterized in the case of Smoothened (Smo), a hedgehog cascade component that rapidly translocates to the cilium following hedgehog pathway stimulation (recently reviewed in ref. 179). Sequential labeling of SNAP-tagged Smo with fluorescent and non-fluorescent SNAP substrates made it possible to distinguish membrane and intracellular pools of Smo and led to the conclusion that Smo rapidly translocates into the ciliary membrane from the surrounding plasmalemma.2 The contribution of Smo from the plasma membrane is pronounced during the first hour after hedgehog pathway stimulation. Beyond this, the intracellular pools become the principal source of ciliary Smo. Transport from the plasma membrane to the cilium has been also reported in Chlamydomonas following earlier studies of agglutinins.180 Little is known, however, about its importance in other contexts. It is possible, but remains to be evaluated, that other transmembrane receptors, such as GPCRs, are also transported to cilia from the plasma membrane.

**Transport of Cytosolic Proteins**

The majority of structural and motility-related ciliary proteins, such as tubulin, radial spoke components and dynein arms, are soluble in the cell’s cytoplasm and thus do not require vesicle transport for their delivery to the ciliary compartment.179,181 Whether an active transport mechanism is involved is currently unclear. As discussed above, the transport of cytoplasmic-soluble proteins into the cilium appears to be restricted by mechanisms that limit diffusion. The nature of diffusion barriers that limit the movement of cytoplasmic proteins appears to be very different from these that restrict transmembrane proteins.

**Is the base of the cilium similar to the nuclear pore?**

It has been noted that some ciliary proteins, such as the Kif17 kinesin, which is the best studied in this regard, feature sequences related to nuclear localization signals (Table 1).63 This observation led to the intriguing idea that the base of the cilium, also referred to as the ciliary pore, functions in a similar manner to the nuclear pore. In the context of the cell nucleus, nuclear import signals of cargo polypeptides interact with Importins, which are proteins that mediate nuclear import (reviewed in ref. 182).
Importins bind cargo polypeptides in the cytoplasm and translocate them through the nuclear pore into the nucleus. Inside the nucleus, a Ras superfamily GTPase, Ran, binds Importins to induce a conformational change that leads to cargo release. The function of Ran in the nucleus is potentiated by a GEF, RCC1, which contributes to the high intranuclear concentration of Ran-GTP. In the cytoplasm, Ran is inactivated by interactions with GAP proteins, RanGAP1, RanBP1, and RanBP2, which results in a low level of GTP-bound Ran.\(^{182}\)

Several studies have provided evidence that many components of the nuclear import system function in ciliary targeting. First, deletion of nuclear localization signal-related sequences from Kif17 impairs its transport into the ciliary compartment.\(^{53}\) Second, consistent with the nuclear import model and the ciliosome behaving like the nucleolus, overexpression of GTP-locked Ran in the cytoplasm also impairs ciliary localization of Kif17, presumably as a result of premature Importin-cargo dissociation prior to ciliary import. In agreement with this, Kif17 binds Importin β2, an interaction that is also dependent on the presence of CLS and is disrupted by GTP-locked Ran.\(^{63}\) Another study has also revealed that RanBP1 (a protein that stimulates Ran GAP) localizes to the ciliary centriole, and the cilium itself, and its knockdown promotes ciliogenesis.\(^{183}\) Although this observation can be interpreted in several ways, it also supports the idea that nuclear pore-related pathways function at the cilium base. Finally, nuclear pore components, nucleoporins, including NUP37, NUP35, NUP62, NUP93, NUP133, and NUP214, are found in cilia, where they co-localize with a transition zone protein, CEP290.\(^{18}\) This raises an intriguing possibility that nuclear pore proteins are integrated into the structure of the transition zone.

Kif17 is not the only protein that requires Importin for its ciliary localization. An Arl3 GAP, RP2, also binds Importin β2 and its ciliary localization is impaired in Importin-deficient cells.\(^{183}\) Importin β2 knockdown appears, however, to have a limited impact on cilia size and morphology, which suggests that this pathway regulates only a subset of cargos.\(^{18}\) It is not clear whether the transport of major ciliary structural elements, such as tubulin, radial spokes or dynein arms, also requires nuclear pore-related transport machinery. If so, it most likely relies on alternative Importins. As the ciliary proteome contains numerous cytosol-soluble proteins, one has to wonder what fraction of these polypeptides relies on a system related to nuclear import for their ciliary localization.

**Delivery of lipidated proteins from cytoplasm to cilia**

A unique transport mechanism exists for a group of lipidated proteins that are targeted to cilia. Despite hydrophobic characteristics of their lipid tails, these proteins are thought to be solubilized in the cytoplasm by binding to another polypeptide. Lipid-modifications are fairly common in ciliary proteins\(^{10,184}\) and lipid attachment sites are frequently associated with CTSs (Table 1). A specialized mechanism exists that renders at least some of N-myristoylated ciliary proteins soluble in the cytoplasm. These proteins are thus likely to be subject to transport barrier mechanisms that are typical of cytoplasmic, but not transmembrane, polypeptides.

The key player in the transport of N-myristoylated ciliary proteins is UNC119, a protein that is structurally related to Guanine Nucleotide Dissociation Inhibitors (GDIs), which maintain prenylated small GTPases in a soluble form (reviewed recently in ref. 185). The UNC119 proteins have been shown to bind a Nephrocystin, NPHP3, and Gα subunits of several heterotrimeric ciliary G-proteins.\(^{186,187}\) It has been proposed that UNC119 interactions with NPHP3 and a ciliary Transducin solubilize these polypeptides in the cytoplasm by providing a hydrophobic environment for their acyl tails. The UNC119-solubilized Transducin is thought to translocate into the photoreceptor cilium via diffusion.\(^{186}\) Whether diffusion also drives the transport of UNC119 complexes into cilia of other cells is unclear. In the ciliosome of RPE cells, UNC119b interacts with GTP-bound Arl3, a small GTPase, which is thought to dissociate UNC119b from NPHP3 and release NHPH3 into the ciliary membrane.\(^{187}\) Following this, the Arl3-UNC119 complex is, in turn, dissociated by an interaction with RP2, which functions as an Arl3 GAP.\(^{187}\)

Does UNC119 target N-myristoylated proteins to cilia or act solely as a solubilizing factor? The N-terminal myristoylation motif of NPHP3 is necessary for its ciliary localization.\(^{188}\) This sequence is, however, insufficient for ciliary targeting, which indicates that additional signals in the NPHP3 polypeptide contribute to ciliary localization.\(^{188}\) An additional layer of complexity in the ciliary targeting of NPHP3 has been revealed by observations that an N-terminal coiled-coil domain of NPHP3, which does not include the myristoylation site, is both necessary and sufficient for targeting to the cilium base. This observation led to the idea that the ciliary targeting of NPHP3 is a two-step process. The first step is driven by the N-terminal CTS but does not require myristoylation, which is required only in the second step.\(^{188}\)

UNC119 myristoyl-binding mutant does not localize to cilia, which suggests that UNC119 ciliary localization requires interaction with NPHP3.\(^{187}\) The most plausible interpretation of these results is that UNC119 does not provide a CTS for ciliary targeting and serves as a solubilization factor that facilitates the transport of myristoylated proteins across the transition zone. In photoreceptor cells, it may also function as a solubilization factor during cytoplasmic transport of the ciliary Transducin.\(^{186}\) Finally, NPHP3 transport seems to proceed independently from the ciliary pore Ran/Importin system.\(^{188}\) Thus, lipidated proteins appear to follow their own unique traffic rules during cilia-directed transport.

Protein-protein interaction tests have revealed that UNC119 binds the N-termini of many proteins, which include Src-type tyrosine kinases and small Arl-like GTPases (reviewed in ref. 184). This suggests that the role of UNC119 in cilia-directed trafficking may extend beyond the transport of NPHP3 and G-proteins that has been characterized so far. Genetic analysis of this issue in vertebrates has so far been confounded by potential functional redundancy of UNC119 genes (two are found in vertebrate genomes).\(^{186}\) A role for UNC119 in the transport of diverse cargos is also suggested by the observation that it regulates Lck kinase localization during immunological synapse formation, a process related to ciliogenesis.\(^{189,190}\)
In contrast to the myristoylated proteins discussed above, the CaaX prenylation motif found in a ciliary phosphatidylinositol phosphate, INPP5E, is not necessary for ciliary targeting. The CTS of INPP5E is well characterized and does not include the CaaX motif. It mediates direct interactions with a small GTPase, Arl3b, which localizes to the ciliary membrane. Surprisingly, Arl3b binding to the CTS of INPP5E releases its interaction with PDE6D, a prenyl-binding protein. It is not clear how the lipid tail of INPP5E is shielded during cytoplasmic transport. Perhaps as yet unidentified factors interact with lipid tails of prenylated proteins during their transport to cilia.

Some Closing Remarks

The targeting of proteins to cilia is of fundamental importance for the formation and function of these fascinating cell surface structures. Given the diversity of signaling events that are mediated by cilia and the corresponding variety of signal transduction components that localize to the cilopilem and the ciliary membrane, it is not surprising that the transport pathways that deliver ciliary proteins are also diverse. Separate pathways appear to handle the transport of transmembrane, membrane-associated and cytoplasmic proteins and, even within each of the above three categories, the transport of structurally disparate proteins is likely to involve somewhat different mechanisms. Although this has not been investigated in sufficient detail thus far, ciliary transport mechanisms are likely to modulate ciliary signal transduction by regulating, for example, interactions between transport machinery and essential signaling proteins, such as GPCRs.

Ciliary transport pathways have to be closely integrated with the function of diffusion barriers that operate at the boundary of the ciliary compartment. Such barriers affect the movement of both transmembrane and cytoplasmic proteins, although transport of the latter appears to be less stringently controlled. The diversity of ciliary transport pathways and their cargos is most likely paralleled by similarly diverse ciliary gating mechanisms. It is usually assumed that transition zones are largely the same in all cilia. This view is most likely incorrect, as cilia of many tissues are likely to feature specialized gating mechanisms to accommodate similarly specialized ciliary proteins.

The transport of even fairly generic ciliary protein complexes poses some difficult questions as it remains unclear how large protein conglomerates, the IFT particle for example, navigate through gating mechanisms at cilia base. Such large complexes may induce conformational changes in transition zone components. Visualizing such changes is technically difficult but perhaps can be achieved with the help of recent advances in microscopy. Efforts to purposely manipulate ciliary transport to achieve practical benefits have not been undertaken to any significant degree so far. Along with an increased understanding of ciliary transport pathways, it will become possible to manipulate cilia function by designing artificial ciliary cargo molecules. Such cargo could be used to modify ciliary microtubules and thereby affect cilia stability or to change cilia responsiveness to ligands by engineering ciliary receptors. Engineered cilia could be used to manipulate cell proliferation or to direct cell migration. Cells with engineered cilia could be used for therapeutic purposes to stimulate tissue repair in many organs, such as lungs, oviducts, brain and the spinal canal. Such goals are relatively far off at this time but may become accessible in the long run. In the shorter term, many questions regarding the function of transport mechanisms and ciliary diffusion barriers remain to be solved. We need to address these questions before we wish cilia-directed cargo proteins bon voyage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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