

Scoring a backstage pass: Mechanisms of ciliogenesis and ciliary access

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Cilia are conserved, microtubule-based cell surface projections that emanate from basal bodies, membrane-docked centrioles. The beating of motile cilia and flagella enables cells to swim and epithelia to displace fluids. In contrast, most primary cilia do not beat but instead detect environmental or intercellular stimuli. Inborn defects in both kinds of cilia cause human ciliopathies, diseases with diverse manifestations such as heterotaxia and kidney cysts. These diseases are caused by defects in ciliogenesis or ciliary function. The signaling functions of cilia require regulation of ciliary composition, which depends on the control of protein traffic into and out of cilia.

Introduction

Ciliated organisms are found in each of the existing eukaryotic clades, including Excavata, such as euglenids, Rhizaria, such as amoeboflagellates, Chromalveolata, such as dinoflagellates, Amoebozoa, such as pelobionts, Plantae, such as green algae, and Opisthokonta, such as the fungus *Batrachomyces dendrobatidis* and animals. Thus, the eukaryotic ancestor must have had a cilium, which performed both motor and sensory functions (Satir et al., 2008; Cavalier-Smith, 2010). In multicellular organisms, many cilia have become specialized cellular antennae, known as primary cilia, which regulate processes such as embryogenesis, tumorigenesis, feeding behavior, kidney function, vision, and smell (Davenport et al., 2007; McEwen et al., 2008; Han et al., 2009; Wong et al., 2009; Goetz and Anderson, 2010; Hildebrandt et al., 2011). Unlike primary cilia, motile cilia and flagella are restricted to a handful of human tissues, in which they propel sperm, regulate embryonic left–right patterning, clear airway mucus, and participate in cerebrospinal fluid movement (Bloodgood, 2010).

Cilia are not fully encompassed by the membrane (Fig. 1); yet, their composition is distinct from that of the surrounding

cytosol and plasma membrane. Other specialized subcellular domains, such as neuronal axons, generate their unique composition through multiple mechanisms (Winckler and Mellman, 2010). Some proteins are delivered directly to axons by means of specific targeting signals and pathways (selective targeting). Other proteins accumulate in axons by being endocytosed from somatodendritic, but not axonal, membranes (selective removal). Yet, other proteins concentrate inside axons by specifically interacting with axonal components (selective retention), whereas still others cannot enter axons because they are anchored to cytoskeletal or extracellular matrix elements elsewhere in the cell (selective exclusion). These mechanisms act in concert with diffusion barriers at the base of the axon, which restrict entrance and exit of both membrane and soluble proteins (Winckler and Mellman, 2010). Emerging evidence suggests that, similar to axons, selective targeting, exclusion, retention, and diffusion barriers also control ciliary composition (Mazelova et al., 2009a; Dishinger et al., 2010; Hu et al., 2010; Francis et al., 2011).

Building the cilium: Where the centriole meets the membrane

The ciliary axoneme is nucleated from the mother centriole, the older of the two centrioles in the centrosome (Bornens, 2012). Because mother centrioles are part of spindle poles during cell division, cilia must disassemble before mitosis and form again only upon entry into G1 (Kobayashi and Dynlacht, 2011).

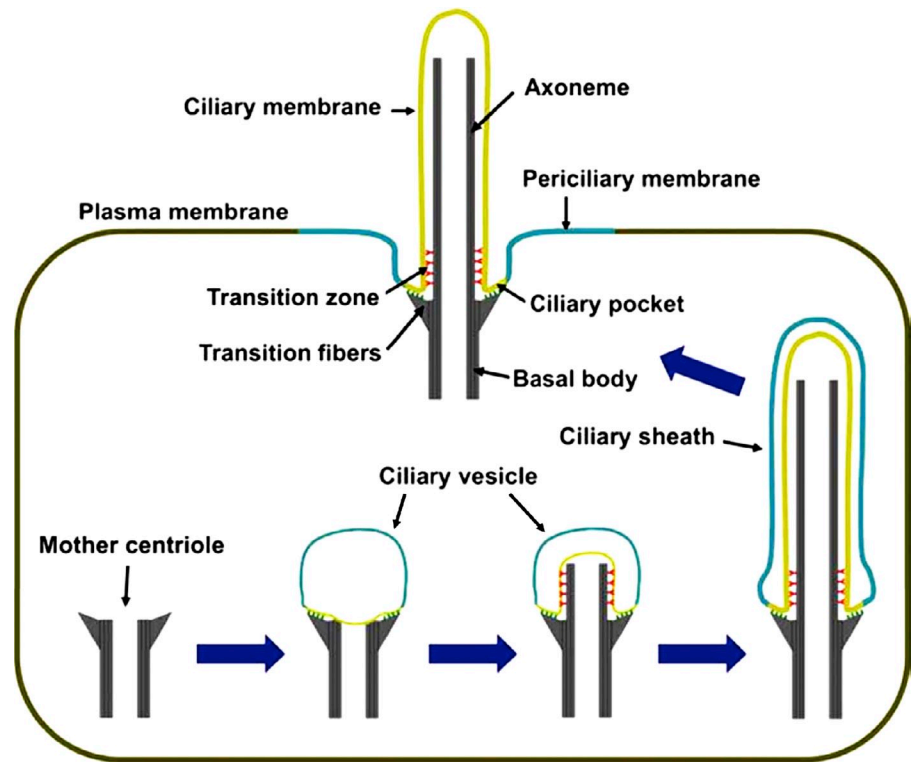
Ciliogenesis begins with the attachment of the distal end of the mother centriole to a vesicle (Fig. 1; Sorokin, 1962, 1968). This attachment is mediated by the centriolar distal appendages, also called transition fibers when they are associated with a cilium (Anderson, 1972; Deane et al., 2001). After docking, a bud emerges from the mother centriole, bending the membrane (Sorokin, 1962). This bud elongates from its tip to form the axoneme, but the base remains structurally distinct and will become the transition zone (Rosenbaum and Child, 1967; Boisvieux-Ulrich et al., 1989). The transition zone starts where the nine microtubule triplets in the basal body become doublets and is characterized by Y links, champagne glass-shaped structures that connect each doublet to the overlying membrane (Gilula and Satir, 1972). This overlying membrane

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Abbreviations used in this paper: CLS, ciliary localization sequence; CNG, cyclic nucleotide gated; GPCR, G protein–coupled receptor; IFT, intraflagellar transport; JBTS, Joubert syndrome; MKS, Meckel syndrome; NES, nuclear export sequence; NPHP, nephronophthisis.

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Figure 1. **Ciliogenesis.** Cilium formation starts when a mother centriole contacts a ciliary vesicle. Axonemes elongate at their tips and so are constructed from proximal to distal, with the most proximal region giving rise to the transition zone. The ciliary vesicle grows with the axoneme and gives rise to the ciliary sheath, whose fusion with the plasma membrane externalizes the cilium and transforms the outer sheath into the periciliary membrane.



contains the ciliary necklace, a circumferential set of intramembranous particles (Fig. 2, A–C; Gilula and Satir, 1972; Sattler and Staehelin, 1974; Menco, 1980; Hufnagel, 1983; Fisch and Dupuis-Williams, 2011).

The transition zone houses a network of ciliopathy proteins that play important roles in Y link and axoneme formation (Fig. 2, D–G; van Reeuwijk et al., 2011). One of these complexes spans the membrane and contains many of the proteins implicated in Meckel syndrome (MKS) and Joubert syndrome (JBTS), two ciliopathies characterized by brain, kidney, and limb defects (Dowdle et al., 2011; Garcia-Gonzalo et al., 2011; Sang et al., 2011; Chih et al., 2012). Another transition zone complex contains Nphp1, 4, and 8, three proteins encoded by genes mutated in nephronophthisis (NPHP), a cystic kidney ciliopathy (Winkelbauer et al., 2005; Fliegauf et al., 2006; Vierkotten et al., 2007; Jiang et al., 2008, 2009; Sang et al., 2011; Won et al., 2011). In *Caenorhabditis elegans*, homologues of the MKS–JBTS and NPHP complexes have overlapping functions in forming both Y links and transition fibers (Williams et al., 2008, 2010, 2011; Huang et al., 2011; Warburton-Pitt et al., 2012). Nphp8, mutations in which can also cause MKS or JBTS, functionally interacts with members of both *C. elegans* modules and is required for the transition zone localization of MKS–JBTS proteins (Huang et al., 2011; Williams et al., 2011). Thus, Nphp8 connects both modules, functionally if not structurally. In other organisms, a similar role may be played by Cep290, a protein that is absent from *C. elegans* but is involved in human NPHP, MKS, and JBTS and is part of the MKS–JBTS complex (Garcia-Gonzalo et al., 2011; Sang et al., 2011). In the green alga *Chlamydomonas reinhardtii*, Cep290 is essential for Y link formation, indicating that, despite their evolutionary

conservation, Y link assembly has species-specific requirements (Craige et al., 2010).

From the transition zone, the axoneme elongates until it reaches a stable length (Ishikawa and Marshall, 2011). As no protein synthesis occurs within the cilium, intraflagellar transport (IFT) must deliver axoneme components to the ciliary tip for assembly (Rosenbaum and Child, 1967). IFT is a bidirectional axoneme trafficking system driven by microtubule motors (Kinesin-2 and cytoplasmic Dynein propel the anterograde and retrograde directions, respectively) that are associated with two subcomplexes, IFT-A and -B (Pedersen and Rosenbaum, 2008). Although Kinesin-2 and IFT-B subunits are essential for axoneme formation, cytoplasmic Dynein and many IFT-A subunits are not, but disruptions in either cause cilia to become short and bulbous (Marszalek et al., 1999; Huangfu et al., 2003; Huangfu and Anderson, 2005; May et al., 2005; Pedersen and Rosenbaum, 2008; Tran et al., 2008; Ocbina et al., 2011; Qin et al., 2011). These data suggest that the IFT-B complex is required for anterograde IFT, which traffics tubulin subunits and other building blocks to the ciliary tip and is thus required for ciliogenesis, and that IFT-A complexes participate in retrograde IFT. These results do not exclude roles for IFT-B in retrograde trafficking or for IFT-A in the anterograde trafficking of some cargo.

Transition fibers may promote ciliogenesis by recruiting IFT components to the ciliary base (Deane et al., 2001; Ishikawa et al., 2005; Graser et al., 2007; Singla et al., 2010). Transition zone proteins are also required for ciliogenesis in some cell types of both *C. elegans* and mice (Garcia-Gonzalo et al., 2011; Williams et al., 2011). Because some transition zone proteins interact with IFT components, they might also help recruit them

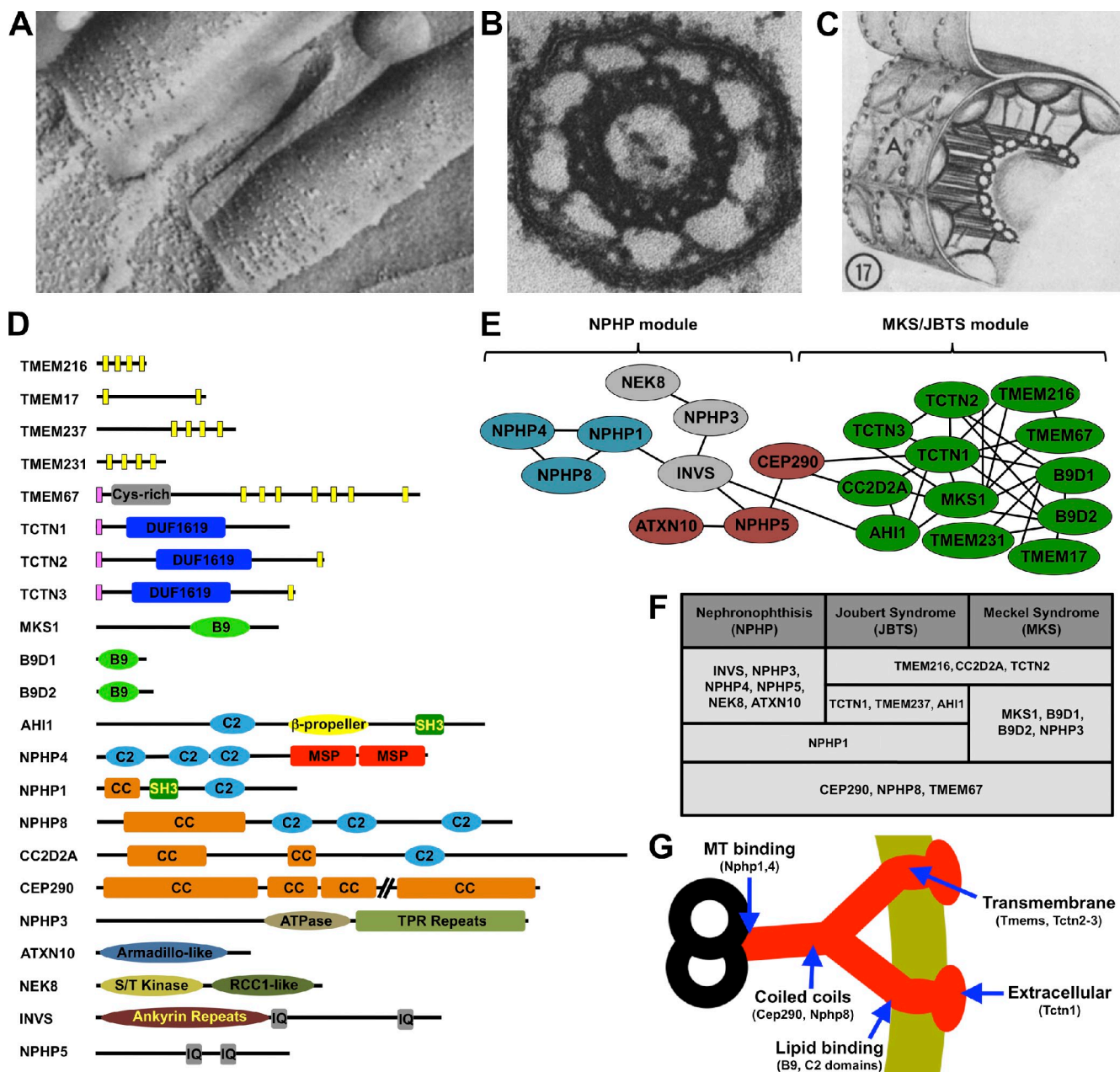


Figure 2. The transition zone. (A) Freeze-etch electron micrograph of tracheal epithelial cilia. The ciliary necklaces are at the ciliary base. (B) Electron micrograph of a cross section through a transition zone of a mollusk gill cilium, showing the microtubule doublets connected to the ciliary membrane by nine Y links. (C) Diagram of the transition zone, showing the Y links connecting the microtubules to the ciliary necklaces. The A indicates the convex freeze-fracture face of the membrane. A–C are obtained from Gilula and Satir (1972). (D) Domain structure of transition zone components. The Tectonic proteins (TCTN1–3) share a signal peptide (pink bars) and a cysteine-rich Tectonic domain. Transition zone transmembrane proteins (TMEMs) include TCTN2, TCTN3, TMEM17, TMEM67, TMEM231, and TMEM237 (predicted transmembrane helices are shown as yellow bars). MKS1, B9D1, and B9D2 share B9 domains related to lipid-binding C2 domains. Several transition zone proteins contain C2 domains, coiled-coil (CC) domains, or both. Inversin (INVS) and NPHP5 have calmodulin-binding IQ motifs. NPHP4 contains two major sperm protein (MSP) domains, NPHP3 is an ATPase with tetratricopeptide repeat (TPR) domains, NEK8 is a serine/threonine kinase, and ATXN10 contains Armadillo repeats. (E) The transition zone protein interaction network. Genetic experiments in *C. elegans* reveal two main functional modules in this network. The first module is mostly comprised of genes, the human homologues of which are implicated in NPHP, whereas the second contains genes associated with MKS and JBTS. These functional modules closely match the results of biochemical experiments in mammalian cells. According to these, proteins in the MKS–JBTS module (green) mostly interact with other proteins within the same module and only rarely with those in the NPHP module (each line designates a reported protein–protein interaction). The NPHP module consists of several interconnected complexes, shown in different colors (Sang et al., 2011; van Reeuwijk et al., 2011). (F) Most known transition zone proteins are encoded by genes mutated in at least one of three related ciliopathies, NPHP, JBTS, and MKS. (G) Schematic of a Y link with a model of its composition. Nphp1 and Nphp4 bind microtubules, so they may connect Y links to microtubule doublets (Mollet et al., 2005). Cep290 and other coiled-coil proteins may form the central portion Y links. C2 and B9 domain-containing proteins are predicted to bind lipids, so they may be membrane proximal. Tctn1 also interacts with transmembrane proteins but is predicted to be on the extracellular face of the ciliary necklaces, as it contains a signal peptide.

to the ciliary base (Zhao and Malicki, 2011). However, cell types that grow axonemes in the absence of specific transition zone components display normal levels of IFT proteins and normal IFT rates, indicating that this may not be the case (Garcia-Gonzalo et al., 2011; Williams et al., 2011).

The elongation of the axoneme requires a parallel expansion of the ciliary membrane. This expansion takes place at the plasma membrane in epithelial cells but occurs intracellularly in mesenchymal cells (Sorokin, 1962, 1968). In the latter case, the ciliary vesicle fuses with secondary vesicles, creating a ciliary sheath with an inner and an outer membrane (Fig. 1). In epithelial cells, ciliary membrane expansion may result from both vesicle fusion at the ciliary base and the lateral diffusion of lipids and proteins from the contiguous plasma membrane. Consistent with the intimate involvement of vesicle trafficking in ciliogenesis, several proteins involved in vesicle budding (AP-1 and Clathrin), targeting (Rab8, Rab11, and TRAPP), tethering (Exocyst), and fusion (SNAREs) participate in ciliogenesis (Yoshimura et al., 2007; Mazelova et al., 2009b; Zuo et al., 2009; Kaplan et al., 2010; Westlake et al., 2011).

Fusion of the ciliary sheath with the plasma membrane exposes the cilium to the extracellular space (Fig. 1). Because this process is topologically equivalent to other forms of exocytosis, externalizing the cilium likely requires exocytic machinery. Upon fusion, the outer membrane of the ciliary sheath becomes the periciliary membrane, a domain that continues to act as the docking region for cilium-bound vesicles and thus plays an important role in the homeostasis of mature cilia (Bouck, 1971; Peters et al., 1983; Papermaster et al., 1985; Nachury et al., 2010).

How are ciliary membrane and axoneme extension coordinated? One regulator of this coordination may be Broad minded (Bromi; Ko et al., 2010). A *Bromi* mouse mutation causes an expansion of the ciliary membrane, within which the axoneme is curled, consistent with miscoordination of axoneme-membrane attachment or growth. Bromi acts via cell cycle-related kinase (CCRK), whose disruption recapitulates the *Bromi* phenotype. Interestingly, mutations in the *C. reinhardtii* CCRK orthologue lead to one flagellum being longer than the other (Tam et al., 2007). Thus, CCRK may have a conserved role in controlling the size of the ciliary axoneme and membrane.

Ciliary protein trafficking

Trafficking of receptors and signal transducers to primary cilia is critical for ciliary function and is disrupted in ciliopathies (Berbari et al., 2008a; Garcia-Gonzalo et al., 2011; Lancaster et al., 2011). Furthermore, ciliary signaling often involves regulated trafficking of select proteins into and out of cilia. A good example is vertebrate Hedgehog signaling. Sonic hedgehog binds to its receptor Patched, causing it to exit the cilium and allowing Smoothened to enter, which in turn affects the ciliary accumulation and activity of Gli transcription factors (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007; Kim et al., 2009). Similarly, regulated trafficking of signaling proteins into or out of *C. reinhardtii* flagella is required for mating and phototaxis (Pan et al., 2003; Wang et al., 2006; Huang et al., 2007; Lechtreck et al., 2009). In addition, ciliary protein trafficking can modulate signaling sensitivity. For example, retinal

photoreceptors adapt to darker environments by increasing Transducin and decreasing Arrestin in their outer segments, which are specialized cilia (Calvert et al., 2006). Thus, understanding how proteins reach the ciliary base and how they enter and accumulate in the cilium is essential to understanding cilium-based intercellular communication.

Trafficking of soluble proteins to the ciliary base. Cytosolic proteins should be able to reach the ciliary base by diffusion. However, transport to *Xenopus laevis* centrioles is at least threefold faster than diffusion, and several centriolar components require microtubules and the Dynein-Dynactin complex to reach centrioles (Young et al., 2000; Dammermann and Merdes, 2002; Quintyne and Schroer, 2002; Guo et al., 2006; Kodani et al., 2010). Furthermore, the ciliary localization of Gli2 requires cytoplasmic microtubules, the minus ends of which anchor to the subdistal appendages of the basal body (Mogensen et al., 2000; Kim et al., 2009).

Interestingly, some of the proteins involved in this microtubule-dependent pathway, including PCM-1 and Par6- α , accumulate in centriolar satellites, electron-dense granules that surround centrioles (Kubo et al., 1999; Dammermann and Merdes, 2002; Kodani et al., 2010). These satellites are also observed in the vicinity of ciliary basal bodies (Kubo et al., 1999; Mogensen et al., 2000; Kunimoto et al., 2012). Several ciliopathy proteins, including Odf1, Cep290, and the BBSome complex, localize to centriolar satellites as well as the cilium (Nachury et al., 2007; Kim et al., 2008, 2009; Lopes et al., 2011). Collectively, these data suggest a role for minus end-directed microtubule traffic of soluble proteins toward the ciliary base and the use of satellites as way stations for some (Fig. 3 A).

Trafficking of soluble proteins into the cilium. Two large complexes involved in flagellar motility, the outer Dynein arms and the radial spoke complex, both require IFT to enter flagella, suggesting that there is a size limit for entry into the cilium that is overcome using IFT (Fig. 4, A and B; Qin et al., 2004; Hou et al., 2007). However, the molecular mass barrier to ciliary entry may be high; in rods, single (27 kD), tandem (54 kD), and triple GFP (81 kD) all reach the outer segments, and mathematical modeling suggests they diffuse freely through the connecting cilia (Calvert et al., 2010; Najafi et al., 2012).

Alternatively, different cell types may have different requirements for ciliary entry. Although photoreceptor connecting cilia structurally resemble the transition zones of other cilia, connecting cilia may be optimized for fast protein exchange to adapt to changes in illumination (Calvert et al., 2006). Dextrans of 40 kD and larger fail to enter cilia of nonphotoreceptor cells, whereas dextrans 10 kD or smaller readily enter, suggesting that the cilia of some cell types have low size exclusion limits (Kee et al., 2012).

Given that the basal body lumen is filled with electron-dense material, the only path available for soluble proteins to access the cilium may be between adjacent transition fibers (Fisch and Dupuis-Williams, 2011; Brito et al., 2012). These spaces, ~60 nm at their widest, could theoretically fit large protein complexes but not vesicles (Nachury et al., 2010). However, the actual exclusion limit for these spaces may be lower and remains unknown. A size exclusion barrier might

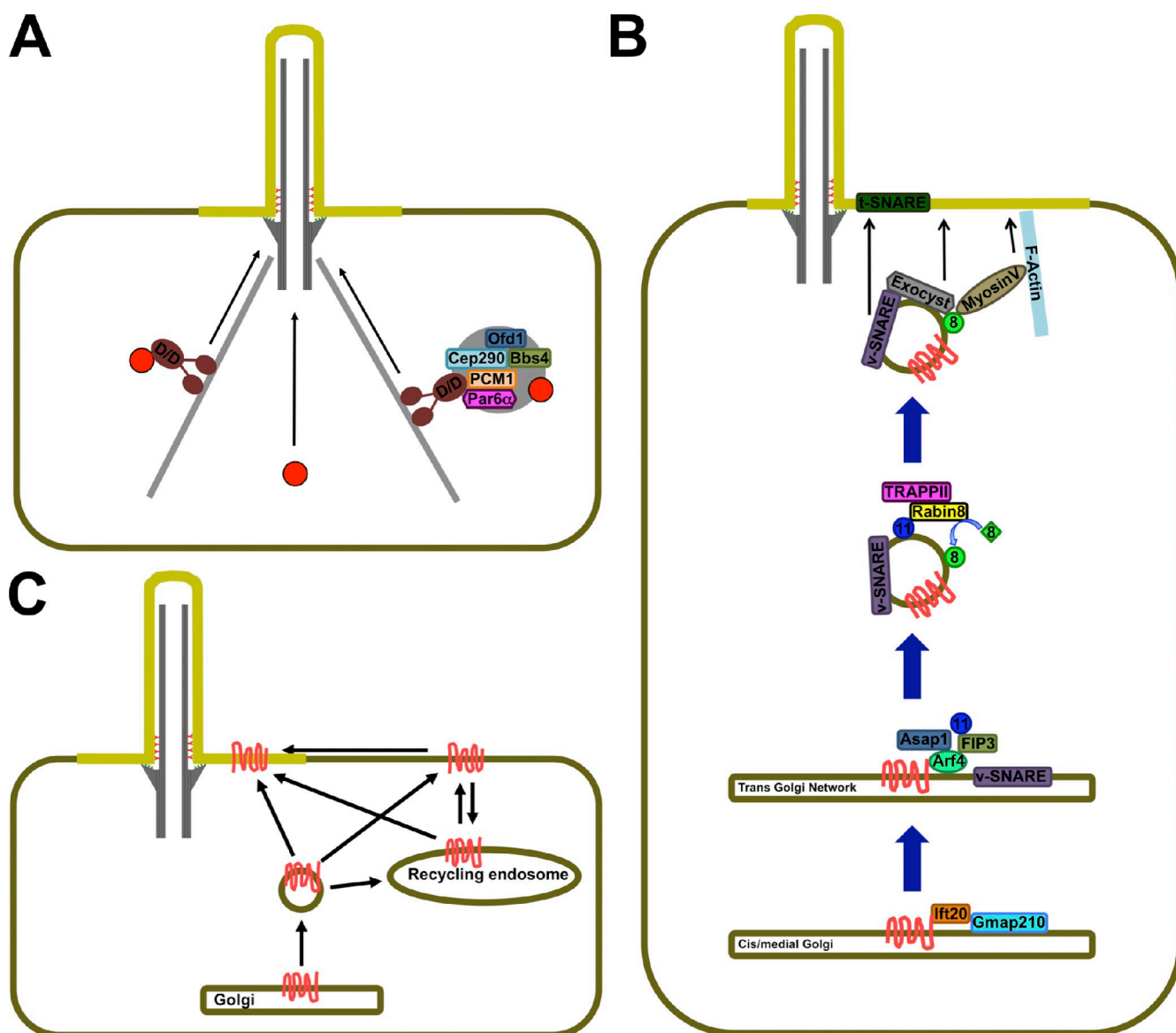


Figure 3. Trafficking to the ciliary base. (A) Soluble proteins (red circles) may reach the ciliary base by diffusion (center) or travel as cargo on minus end-directed microtubule motors, such as the Dynein–Dynactin complex (D/D; left). (right) Trafficking of soluble cargo may also involve centriolar satellites, large protein aggregates that may serve as assembly points, and way stations for cilium-bound proteins. (B) Trafficking of some transmembrane proteins (red) along the Golgi may be aided by IFT20, which may then hand them off to Arf4. Arf4 orchestrates the formation of cargo-containing cilium-bound vesicles that contain active Rab11 (blue), whose effector Rabin8 recruits active Rab8 (green) to the vesicle surface. Rab8 in turn recruits effectors that mediate the vesicle’s approach, tethering, and fusion with the periciliary membrane. (C) Transmembrane proteins (red) may reach the cilium laterally from the plasma membrane or aboard vesicles that fuse with the periciliary membrane. Cilia-bound vesicles may derive from the Golgi or from recycling endosomes, which themselves may receive input from the Golgi and plasma membrane.

also reside at the transition zone, where Y links may regulate soluble protein entry (Menco, 1980). In addition to acting as size exclusion filters, these physical barriers may also function as “smart” filters, allowing passage of large proteins only if they contain ciliary localization sequences (CLSs).

Recently, it has been suggested that ciliary and nuclear entry share several characteristics (Fig. 4 B; Dishinger et al., 2010; Kee et al., 2012). The nuclear pore excludes proteins greater than ~30 kD but allows passage of larger proteins if bound to importins or exportins (Cook et al., 2007). Importins and exportins directly interact with and transiently displace the phenylalanine-glycine repeat nucleoporins, whose natively

unfolded repeats form a meshwork that occludes the nuclear pore lumen (Hoelz et al., 2011). In this way, importins and exportins catalyze the nucleocytoplasmic transport of their cargos, which they recognize via NLSs or nuclear export sequences (NESs), respectively. Giving direction to the process is a concentration gradient of the GTP-bound form of the small GTPase Ran, which accumulates inside the nucleus where it stimulates cargo dissociation from importins and cargo association with exportins. The Ran-GTP gradient is in turn generated by the asymmetric distribution of RCC1, the chromatin-associated exchange factor that generates Ran-GTP, and RanGAP, the cytosolic enzyme that generates Ran-GDP (Cook et al., 2007).

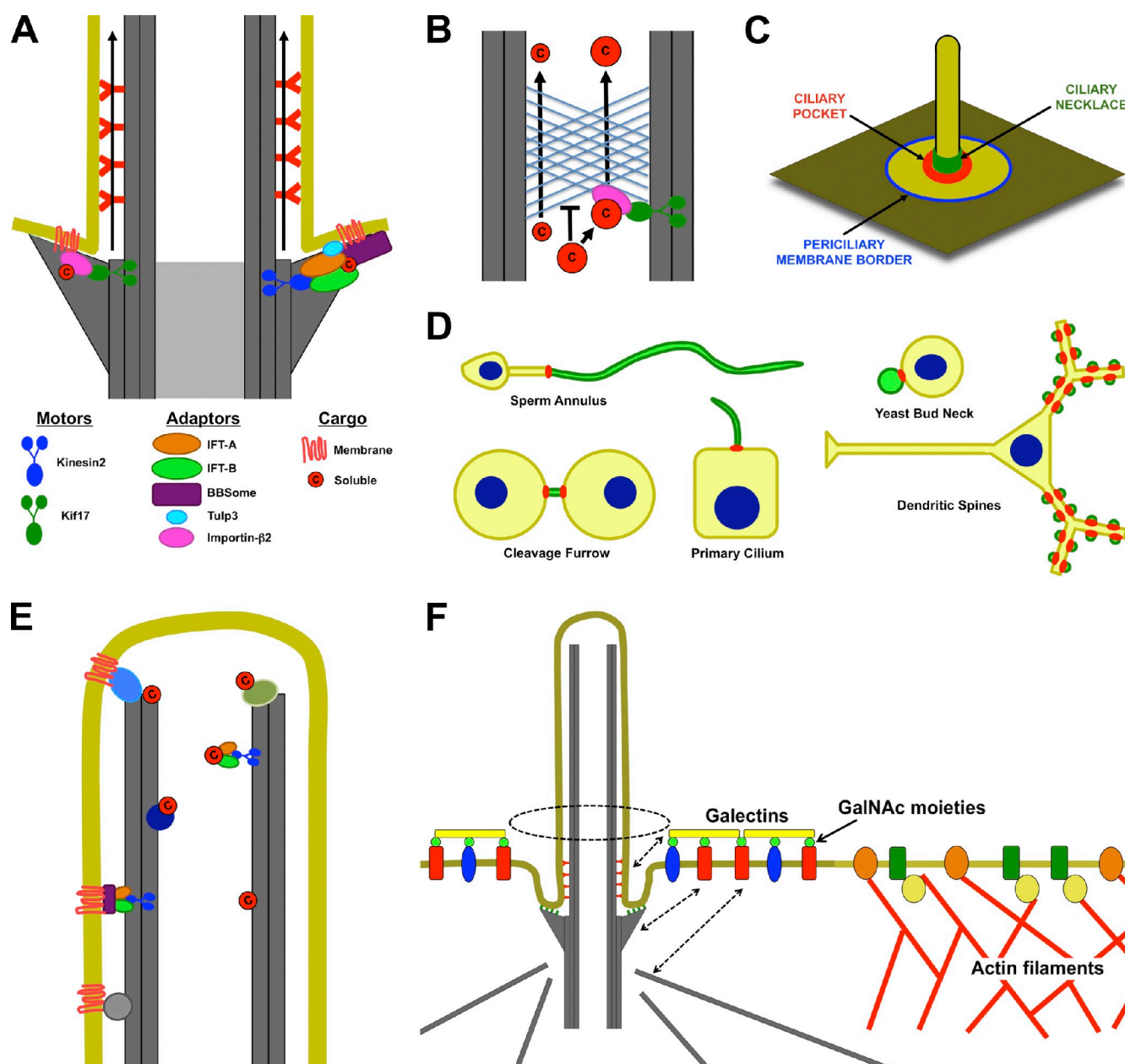


Figure 4. Access into and maintenance of distinct ciliary compartments. (A) Both soluble and transmembrane proteins may need to associate directly or indirectly with plus end-directed microtubule motors (Kinesin-2 or Kif17) to enter the cilium. The entrance may be between adjacent transition fibers. (B) Like the nuclear pore, the ciliary base can exclude proteins on the basis of their size. Thus, small proteins may diffuse into the cilium, but large proteins may need to associate with transport machinery to enter the cilium. (C) Potential locations for diffusion barriers preventing entrance of membrane proteins into cilia include the border between the plasma and periciliary membranes (blue), the bottom of the ciliary pocket where transition fibers anchor the basal body to the membrane (red), and the ciliary necklace (green). (D) Septin rings (red) form membrane diffusion barriers that define specific membrane compartments (green), including those of cilia, flagella, the midbody, dendritic spines, and yeast buds. (E) Soluble and membrane proteins may be retained inside cilia by directly or indirectly interacting with microtubules. (F) Selective exclusion and retention can account for differences in periciliary and plasma membrane composition. The cortical actin cytoskeleton is excluded from the region under the periciliary membrane. As a result, membrane proteins that directly or indirectly interact with actin filaments are excluded from the periciliary region. Conversely, certain proteins, such as Galectin-3, that accumulate in the periciliary region may be retained by extracytosolic interactions with the ciliary membrane or by interactions with the basal body or its associated microtubules (dashed arrows). c, cargo; GalNAc, N-acetyl-galactosamine.

Surprisingly, importin binding is also required for some proteins to accumulate inside cilia (Fan et al., 2007; Dishinger et al., 2010; Hurd et al., 2011). One such protein is the kinesin Kif17, whose ciliary localization depends on importin-β2 binding to an NLS-like CLS in Kif17 (Dishinger et al., 2010). Indeed, replacing the CLS in Kif17 with a bona fide NLS supports its ciliary entry, and Kif17 mutants containing the CLS, but lacking

the kinesin motor domain, accumulate in the nucleus instead of the cilium (Dishinger et al., 2010). Furthermore, grafting the Kif17 CLS onto a nonciliary kinesin causes the resulting fusion protein to go to the cilium (Dishinger et al., 2010). Therefore, ciliary localization of Kif17 requires both importin binding and an additional activity present in its motor domain, possibly plus end-directed microtubule motility. Kif17 is in turn required for

the olfactory cyclic nucleotide-gated (CNG) channel to accumulate in cilia, supporting the hypothesis that kinesin–importin complexes transport other proteins into cilia (Jenkins et al., 2006).

Like importin- β 2, Ran is implicated in ciliary trafficking. A GTP-locked form of Ran causes Kif17 to move from the ciliary tip to less distal ciliary positions until it disappears from the cilium altogether (Dishinger et al., 2010). Interestingly, knockdown of RanBP1, a RanGAP cofactor present in the cilium, leads to an accumulation of ciliary Ran-GTP and also causes Kif17 to leave the ciliary tip (Fan et al., 2011). These results can potentially be explained by effects of Ran-GTP on the balance between anterograde and retrograde trafficking of Kif17 along ciliary microtubules or by changes in microtubule dynamics, which Ran-GTP is known to regulate (Keryer et al., 2003; Mishra et al., 2010; Fan et al., 2011; Halpin et al., 2011). Together with the fact that a GDP-locked Ran mutant that disrupts nucleocytoplasmic transport does not affect the ciliary localization of Kif17, these data suggest that the ciliary functions of Ran differ from those in nucleocytoplasmic transport (Dishinger et al., 2010). Future experiments that address how Ran-GTP levels in the cilium are controlled, the function of nucleoporins at the ciliary base (Kee et al., 2012), and whether exportins regulate ciliary exit will clarify the extent to which trafficking through the ciliary base and nuclear pores resemble each other.

Intraciliary retention of soluble proteins. The steady-state accumulation of a protein within a cilium can be achieved by either promoting its entry or blocking its exit. Analogous to ciliary entry, a protein's ciliary exit rate reflects two parameters: (1) how frequently it reaches the ciliary base from within the cilium, and (2) how efficiently it crosses the ciliary base. Many ciliary components directly or indirectly interact with microtubules, limiting their access to the ciliary base and retarding their exit (Sloboda and Howard, 2007). Also, a slow rate of exit across the ciliary base may cause some proteins to be retained at the transition zone or in the compartment above it where Inversin localizes (Shiba et al., 2009; Sang et al., 2011).

Trafficking of membrane proteins to the ciliary base. Transmembrane proteins start their lives in the endoplasmic reticulum, from which they travel to the Golgi apparatus. After reaching the TGN, they are sorted into vesicles bound to different subcellular destinations. The plasma membrane acts as the default destination of TGN-derived vesicles, but targeting sequences on the transmembrane cargo proteins can drive vesicles to other compartments (Gu et al., 2001; Baker et al., 2008). In particular, CLSs on transmembrane proteins help recruit ciliary trafficking components that guide these vesicles to the periciliary membrane. For example, a VxPx motif in the cytosolic tail of Rhodopsin, a light-activated G protein-coupled receptor (GPCR), recruits the small GTPase Arf4 to the TGN, leading to the formation of a complex that mediates Rhodopsin incorporation into cilium-bound vesicles (Fig. 3 B). This complex includes ASAP1, a protein that may couple cargo recruitment and vesicle formation, Rab11, which participates in ciliary vesicle trafficking, and Arfophilin-1/FIP3, which is a dual Arf/Rab11 interactor and may hence allow Rab11 recruitment to Arf4-containing vesicles (Mazelova et al., 2009a).

Like Rhodopsin, ciliary targeting of Polycystin-1 and -2, two transmembrane proteins involved in polycystic kidney disease, is dependent on Arf4-binding CLSs that contain VxPx motifs and, at least for Polycystin-1, also depends on ASAP1 and Rab11 (Geng et al., 2006; Ward et al., 2011). Similarly, the CNGB1b subunit of the olfactory CNG channel contains a VxPx CLS (Jenkins et al., 2006). Therefore, Rhodopsin, Polycystins, and the CNG channel appear to use a common ciliary targeting mechanism. Formation of these cilium-bound vesicles may also involve clathrin coats and AP-1 adaptors, as these are required to move ODR-10, another GPCR, to *C. elegans* sensory cilia (Dwyer et al., 2001; Kaplan et al., 2010). As clathrin and AP-1 are essential for budding of endosome-bound vesicles from the TGN, endosome- and cilium-bound vesicles may form by similar mechanisms, or some ciliary proteins may travel to cilia via endosomes (Fig. 3 C; Bonifacino and Traub, 2003; Kaplan et al., 2010).

Like Clathrin and AP-1, other proteins may have specialized roles in ciliary trafficking. For example, IFT20, a component of the IFT-B complex involved in ciliogenesis, also localizes to Golgi cisternae, post-Golgi vesicles, and basal bodies (Follit et al., 2006, 2008; Sedmak and Wolfrum, 2010). This localization suggests that IFT20 may have roles in moving proteins from the Golgi to the basal body, and indeed, moderate knockdown of IFT20 inhibits the ciliary localization of Polycystin-2 (Follit et al., 2006). GMAP210, a protein required for the Golgi but not basal body localization of IFT20, also promotes the ciliary localization of Polycystin-2, consistent with a trafficking role for IFT20 in the Golgi (Follit et al., 2008). Similarly, IFT20 is required in rod cells for the efficient transport of Rhodopsin out of the Golgi (Keady et al., 2011). IFT20 interacts with the cytosolic tail of Rhodopsin but not through the VxPx motif (Keady et al., 2011). It will be interesting to see whether IFT20 binds to other ciliary membrane proteins, such as Polycystin-1 and -2, to target them to cilia.

Intriguingly, IFT20 localizes predominantly to the cis- and medial-Golgi cisternae and not the TGN, where most transmembrane proteins are sorted for delivery to post-Golgi compartments (Follit et al., 2006). One possibility is that IFT20 accompanies its cargo as it moves through the cis- and medial-Golgi and hands it off to Arf4 at the TGN (Fig. 3 B). Alternatively, IFT20 might facilitate transport from the cis-Golgi to the cilium, bypassing the TGN, as has been proposed for Polycystin-2 (Hoffmeister et al., 2011).

Post-Golgi trafficking of cilium-bound vesicles involves Rab11, whose active form recruits and activates the Rab8–guanine nucleotide exchange factor Rabin8, which in turn recruits Rab8 and TRAPPII, a vesicle-trafficking complex (Knödler et al., 2010; Westlake et al., 2011). Active Rab8 then recruits effectors, including the Exocyst complex and possibly MyosinV, that tether the vesicle to the periciliary membrane (Fig. 3 B; Deretic et al., 2004; Ishikawa et al., 2005; Roland et al., 2007; Omori et al., 2008; Mazelova et al., 2009b; Jin et al., 2011). The Exocyst complex facilitates the pairing of cognate SNARE proteins, leading to vesicle fusion with the periciliary membrane (Mazelova et al., 2009b). In addition to delivering membrane proteins to the ciliary base, this same pathway drives ciliary membrane expansion during ciliogenesis, indicating that both processes rely on the

same machinery (He and Guo, 2009; Mazelova et al., 2009b; Zuo et al., 2009; Follit et al., 2010; Kaplan et al., 2010; Hoffmeister et al., 2011; Ward et al., 2011; Westlake et al., 2011).

Vesicles containing cilium-bound membrane proteins may not always travel directly from the Golgi to the periciliary membrane (Fig. 3 C). A possible way station is recycling endosomes, which contain Rab8, Rab11, and other cilia regulators (Finetti et al., 2009; Kaplan et al., 2010; Kim et al., 2010a). In other instances the cilium is reached via the plasma membrane, as is the case of Smoothed (Milenkovic et al., 2009; Wang et al., 2009).

Trafficking of membrane proteins into the cilium. One way for peripheral membrane proteins to enter the cilium is by associating with proteins that mask their hydrophobic moieties, thereby allowing them to behave like their cytosolic counterparts. For example, two N-myristoylated proteins, Nphp3 and Cystin, rely on Unc119b, a soluble myristoyl-binding protein, for transport to the cilium (Wright et al., 2011). Interestingly, Unc119b cannot enter the cilium unless bound to its cargo (Wright et al., 2011; Zhang et al., 2011; Nakata et al., 2012). Upon ciliary entry, Arl3 dissociates the Unc119b–cargo complex, allowing the myristoylated cargo to associate with the ciliary membrane (Wright et al., 2011). Similarly, Pde6d, an Unc119b-related Arl3 effector, binds and helps bring prenylated proteins into photoreceptor outer segments (Zhang et al., 2004, 2007). Therefore, recognition and masking of lipid modifications may be a general mechanism for transporting peripheral membrane proteins to cilia (Cevik et al., 2010; Emmer et al., 2010; Evans et al., 2010; Follit et al., 2010; Maric et al., 2011).

Unlike peripheral membrane proteins, transmembrane proteins must enter cilia by moving laterally from the periciliary membrane, as membrane extraction or vesicle entry into cilia seems unlikely. Membrane diffusion barriers separate the ciliary and periciliary membranes in at least some cell types, indicating that lateral transport between these two membranes must be facilitated by machinery that engages CLSs in the cargo (Hu et al., 2010; Chih et al., 2012). For example, ciliary GPCRs, including Mchr1, Sstr3, and Htr6, rely on a CLS in their third intracellular loop to enter cilia (Berbari et al., 2008a). Ciliary entry of these GPCRs requires direct binding of this CLS to the BBSome, a complex including most Bardet–Biedl syndrome–associated proteins (Nachury et al., 2007; Berbari et al., 2008b; Jin et al., 2010; Seo et al., 2011). The BBSome also interacts with the IFT machinery, whose microtubule motors may provide the driving force needed for GPCRs to enter cilia (Ou et al., 2007; Lechtreck et al., 2009).

More generally, direct or indirect association with microtubule motors may be the sine qua non for transmembrane proteins to cross the ciliary base. If so, different combinations of motors, adaptors, and CLSs may control ciliary entry and exit (Fig. 4 A). As motors, Kinesin II and Kif17 mediate ciliary entry, whereas cytoplasmic Dynein 2 functions during exit (Jenkins et al., 2006; Ishikawa and Marshall, 2011). Adaptors for these motors may include IFT proteins, the BBSome, and Tulp3 (Ou et al., 2007; Lechtreck et al., 2009; Mukhopadhyay et al., 2010). Tulp3 interacts with IFT-A

components and, like the BBSome, is required for the ciliary accumulation of Sstr3 and Mchr1 (Berbari et al., 2008b; Mukhopadhyay et al., 2010). However, Tulp3 is not known to interact with the CLSs of these receptors, and its role in repressing Hedgehog signaling indicates that it has additional functions (Cameron et al., 2009; Norman et al., 2009; Patterson et al., 2009).

The main adaptor for Kif17 may be importin- β 2, which binds to and is required for the ciliary targeting of several membrane proteins (Fan et al., 2007; Hurd et al., 2011). Another Kif17 adaptor may be Ankyrin G, which interacts with a CLS on CNGB1b, the ciliary targeting of which is blocked by dominant-negative Kif17 (Jenkins et al., 2006; Kizhatil et al., 2009). The presence of two separate CLSs in CNGB1b, the VxPx-containing motif and the Ankyrin-binding sequence, raises the possibility that packaging into cilium-bound vesicles and entry from the periciliary membrane represent separate decisions. Thus, cilium-bound cargo proteins and vesicle-trafficking components may be separated at the periciliary membrane, with the nonciliary vesicular components being retrieved from the periciliary membrane by endocytosis. Accordingly, the periciliary membrane, which when invaginated is called the ciliary pocket, is an active site of clathrin-dependent endocytosis (Gadelha et al., 2009; Molla-Herman et al., 2010).

What is the nature of the membrane diffusion barrier at the ciliary base? Two recent studies have identified a Septin ring and members of the MKS–JBTS transition zone complex as components of this barrier (Hu et al., 2010; Chih et al., 2012).

Septins are small GTPases that interact with membranes and can polymerize into filaments and rings (Weirich et al., 2008). These rings form barriers to membrane diffusion in many contexts, from the yeast bud neck to dendritic spines (Fig. 4, C and D; Caudron and Barral, 2009). In sperm, the Septin4-based annulus forms a barrier that controls flagellar composition (Ihara et al., 2005; Kissel et al., 2005; Kwitny et al., 2010). Similarly, Septins 2 and 7 are part of a ring and restrict membrane protein diffusion at the base of primary cilia (Hu et al., 2010; Kim et al., 2010b). Interestingly, the sperm annulus assembles from the flagellar pocket (Molla-Herman et al., 2010; Shang et al., 2010). Hence, the ciliary pocket, or more generally the periciliary membrane, may play an analogous role in the formation of the ciliary Septin ring. Consistent with this hypothesis, the ciliary pocket is a docking site for actin filaments, which interact with Septins and modulate Septin ring formation (Weirich et al., 2008; Molla-Herman et al., 2010; Kim et al., 2011).

Similar to Septins, components of the MKS–JBTS transition zone complex also restrict diffusion of membrane proteins across the ciliary base, thus allowing ciliary membrane proteins to accumulate in cilia while keeping plasma membrane proteins out (Dowdle et al., 2011; Garcia-Gonzalo et al., 2011; Chih et al., 2012). In *C. elegans*, defects in ciliary composition have only been detected upon disruption of both MKS–JBTS and NPHP complex members (Williams et al., 2011). However, whether these complexes collaborate to form a membrane diffusion barrier at the transition zones of other organisms is not yet clear.

The Septin barrier and the transition zone barrier may be interdependent or even one and the same, as Septin2 is required for the transition zone localization of several MKS–JBTS complex proteins, including B9d1, Cc2d2a, and Tmem231 (Chih et al., 2012). Because Tectonic1, a core MKS–JBTS complex component, is not required for Septin2 to form a ring at the ciliary base, the membrane diffusion barrier may assemble sequentially, with the Septin ring forming first (Garcia-Gonzalo et al., 2011). Where exactly this barrier lies is not yet clear, but it is likely to be in the region of the transition fibers and the Y links. Consistent with this hypothesis, *C. elegans* cilia lacking Y links and transition fibers fail to exclude plasma membrane proteins from cilia, and *C. reinhardtii* flagella of *Cep290* mutants lack Y links and fail to accumulate flagellar membrane proteins (Craig et al., 2010; Williams et al., 2011). In addition, *Cep290* mutant flagella possess increased levels of IFT and BBSome components, suggesting a role for Y links in controlling the ciliary localization of soluble proteins as well (Craig et al., 2010).

Intraciliary and extraciliary retention of membrane proteins. As with soluble proteins, intraciliary retention is one means by which membrane-associated proteins can accumulate in cilia (Fig. 4 E). For instance, the ciliary levels of CEACAM1, a transmembrane protein, increase when the protein is conjugated to a microtubule-binding domain (Francis et al., 2011). However, the extent to which similar mechanisms operate in vivo is not yet clear.

Analogously, extraciliary retention may explain how some membrane proteins are excluded from cilia. This may be especially important in photoreceptors, where the outer segment acts as the default destination for transmembrane proteins, perhaps reflecting the massive amount of transport through connecting cilia, each of which traffics ~1,000 Rhodopsin molecules per second (Besharse et al., 1977; Baker et al., 2008; Gospe et al., 2010). For example, nonciliary transmembrane proteins, such as Glut1 or Syntaxin3, use targeting sequences to be kept outside of the outer segment (Baker et al., 2008; Gospe et al., 2010). These results raise the question of why Rhodopsin requires its CLS. Perhaps this CLS increases the efficiency of Rhodopsin transport to the cilium, rather than being strictly required for it.

Another example of ciliary exclusion is the transmembrane protein Podocalyxin, which is restricted from reaching the ciliary base of MDCK cells by its association with the cortical actin cytoskeleton (Francis et al., 2011). When its ties to the actin cytoskeleton are severed, Podocalyxin readily enters the periciliary and ciliary membranes, bringing into question the existence of diffusion barriers at the ciliary base (Francis et al., 2011). One possible explanation is that, in these experiments, Podocalyxin reached the nascent ciliary membrane before diffusion barrier formation, as Podocalyxin was expressed before cilia formed (Breslow and Nachury, 2011). Alternatively, it is possible that Podocalyxin contains a CLS that allows it to enter cilia when detached from actin. Regardless, these data raise the possibility that ciliary barriers depend on the cellular differentiation state or the cell type.

Similar retention mechanisms may also account for why some proteins, such as Galectin-3, localize to the periciliary membrane but are excluded from the ciliary and plasma membranes

(Vieira et al., 2006). Because Galectin-3 is secreted from cells and cross-links glycosylated proteins on the cell surface, its retention to the periciliary region may depend on interactions with unidentified periciliary transmembrane proteins, whose localization may in turn depend on associations with structures at the ciliary base (Fig. 4 F; Partridge et al., 2004; Ohtsubo et al., 2005; Huang 2010). Therefore, the distinct periciliary membrane identity could arise from a combination of retention and exclusion mechanisms even in the absence of a diffusion barrier separating it from the plasma membrane (Fig. 4 C). Additional mechanisms, such as selective removal, may also aid in the generation of a distinct periciliary membrane. For instance, Galectin-3–mediated cross-linking can prevent endocytosis of cell surface proteins, which might facilitate their accumulation in the periciliary membrane (Partridge et al., 2004; Winckler and Mellman, 2010).

How to reconcile the evidence for selective exclusion, retention, and diffusion barriers, all of which can control ciliary protein localization? Each of these mechanisms may apply to some, but not all, ciliary proteins, and some of these proteins may be subject to multiple mechanisms of ciliary localization control. This diversity of mechanisms may allow the cilium to maintain a unique composition despite not being bounded by the membrane.

Conclusion

The cilium is a specialized organelle whose function critically depends on its composition. To control which proteins enter and exit cilia, cells regulate protein entrance across its base, the only region not surrounded by a membrane. The ciliary base acts as a selective filter, allowing passage of proteins with specific biophysical properties or that associate with transporters. Cells also control ciliary composition by controlling which proteins reach the ciliary base. Although soluble proteins do so by diffusion or by traveling along microtubules, transmembrane proteins must incorporate into cilium-bound vesicles leaving the Golgi or enter indirectly via the plasma membrane or recycling endosomes. In addition to these selective targeting mechanisms, selective exclusion and retention also control the composition of cilia and may allow the periciliary membrane to maintain its unique identity. Thus, the complex architecture of the cilium allows for multiple independent regulatory mechanisms that control its composition and allow its function to emerge from its form.

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