Molecular mechanisms of protein and lipid targeting to ciliary membranes

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Summary

Cilia are specialized surface regions of eukaryotic cells that serve a variety of functions, ranging from motility to sensation and to regulation of cell growth and differentiation. The discovery that a number of human diseases, collectively known as ciliopathies, result from defective cilium function has expanded interest in these structures. Among the many properties of cilia, motility and intraflagellar transport have been most extensively studied. The latter is the process by which multiprotein complexes associate with microtubule motors to transport structural subunits along the axoneme to and from the ciliary tip. By contrast, the mechanisms by which membrane proteins and lipids are specifically targeted to the cilium are still largely unknown. In this Commentary, we review the current knowledge of protein and lipid targeting to ciliary membranes and outline important issues for future study. We also integrate this information into a proposed model of how the cell specifically targets proteins and lipids to the specialized membrane of this unique organelle.

This article is part of a Minifocus on cilia and flagella. For further reading, please see related articles: 'The primary cilium at a glance' by Peter Satir et al. (*J. Cell Sci.* **123**, 499-503), 'Sensory reception is an attribute of both primary cilia and motile cilia' by Robert A. Bloodgood (*J. Cell Sci.* **123**, 505-509), 'The perennial organelle: assembly and disassembly of the primary cilium' by E. Scott Seeley and Maxence V. Nachury (*J. Cell Sci.* **123**, 511-518) and 'Flagellar and ciliary beating: the proven and the possible' by Charles B. Lindemann and Kathleen A. Lesich (*J. Cell Sci.* **123**, 519-528).

Key words: Cilia, Intraflagellar transport, Lipid rafts, Palmitoylation, Targeting

Introduction

Cilia and flagella are ancient organelles found in organisms spanning the eukaryotic lineage. The basic structure and biogenesis of cilia and flagella are highly conserved. With few exceptions, these organelles consist of a microtubule-based axonemal complex that is assembled on a basal body and projects out from the cell surface, ensheathed in membrane. Because there are no fundamental differences between cilia and flagella, we use the term 'cilium' to refer to both structures.

Cilia can be motile or immotile. Motile cilia have a readily observable function, whether it be the propulsion of the cell through fluid (e.g. the protists) or the sweeping of material along the cell surface (e.g. respiratory epithelial cells). By contrast, since their discovery over 100 years ago (Zimmermann, 1898), immotile cilia have been mostly thought to be vestigial organelles. More recently, however, the finding that dysfunction of immotile cilia is linked to a variety of human diseases (collectively known as ciliopathies) has led to a resurgence of interest in investigating the physiological functions and biogenesis of cilia (Tobin and Beales, 2009). For example, the proteins in which mutations cause autosomal dominant polycystic kidney disease (European Polycystic Kidney Disease Consortium, 1994; Mochizuki et al., 1996) have been shown to function in ciliary signaling (Pazour et al., 2000; Torres and Harris, 2006). Renal tubular epithelial cells sense lumenal urine flow through deflection of their primary cilia. This in turn causes an intraciliary calcium flux, regulated by the transmembrane proteins polycystin-1 and polycystin-2, that modulates downstream STAT6 and Wnt cellular proliferation pathways. Dysregulation of this pathway as a result of mutations in the genes encoding the polycystins leads to the hallmark pathologic features of autosomal dominant polycystic kidney disease.

At the same time that investigations of the primary cilium illuminated its important sensory functions, studies of motile cilia revealed a surprising complexity in these ancient organelles. Putative signaling proteins, including protein kinases and phosphodiesterases, localize to motile cilia in a variety of cell types, indicating the potential for signal transduction. The best-understood signaling pathways that have been characterized in cilia include the guanylyl-cyclase-mediated chemotactic response of sea urchin spermatozoa (Kaupp, 2003) and an adenylyl-cyclase- and phosphorylation-dependent mating process activated by flagellar adhesion of *Chlamydomonas* (Pan and Snell, 2000). In humans, airway epithelial cilia have recently been shown to regulate their beat frequency in response to extracellular noxious stimuli through a calcium-regulated pathway (Shah, 2009). Phylogenetic analysis of the structural components of the eukaryotic cilium has led to a model whereby a common eukaryotic ancestor contained a single 9 + 2 cilium, probably derived from the microtubule intracellular transport system, with combined sensory and motor functions (Mitchell, 2007). In certain cell types, including vertebrate photoreceptors and olfactory neurons, cilia then further evolved to perform tissue-specific functions.

The recent increase in our understanding of the importance of ciliary function in physiology has led to renewed interest in the molecular mechanisms that underlie ciliary biogenesis and maintenance. Although the current understanding of protein and lipid targeting is not as advanced for the cilium as for organelles such as the endoplasmic reticulum or lysosome, several recent reports have begun to elucidate the mechanisms involved. In this Commentary, we draw on recent findings to present a general model for the transport of ciliary membrane proteins and lipids from the Golgi complex to the ciliary membrane. We focus on molecules known to play specific

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roles in ciliary targeting, rather than on those molecules that are generally involved in establishment of cell polarity or in mediating vesicular transport. We draw on examples from diverse organisms and assume that key themes, if not exact molecules, are probably common to ciliary biogenesis and targeting in all eukaryotes.

Axoneme assembly

Nearly all ciliary axonemes contain microtubules in either the 9 + 2 arrangement, in which 9 outer doublets surround a central pair, or the 9 + 0 arrangement, in which the central pair of microtubules is absent. Motility is driven by dynein-mediated sliding between these microtubules. In either arrangement, axoneme assembly is driven by a process known as intraflagellar transport (IFT) (Kozminski et al., 1993). The ciliary axoneme and its associated cytoskeletal elements are not elongated by the addition of subunits to the base, but by extension from the growing tip (Johnson and Rosenbaum, 1992; Song and Dentler, 2001; Stephens, 2000). IFT is responsible for the delivery of structural subunits to the ciliary tip, as well as for their recycling back to the cell body (Qin et al., 2004) (Fig. 1). These processes are mediated by two distinct

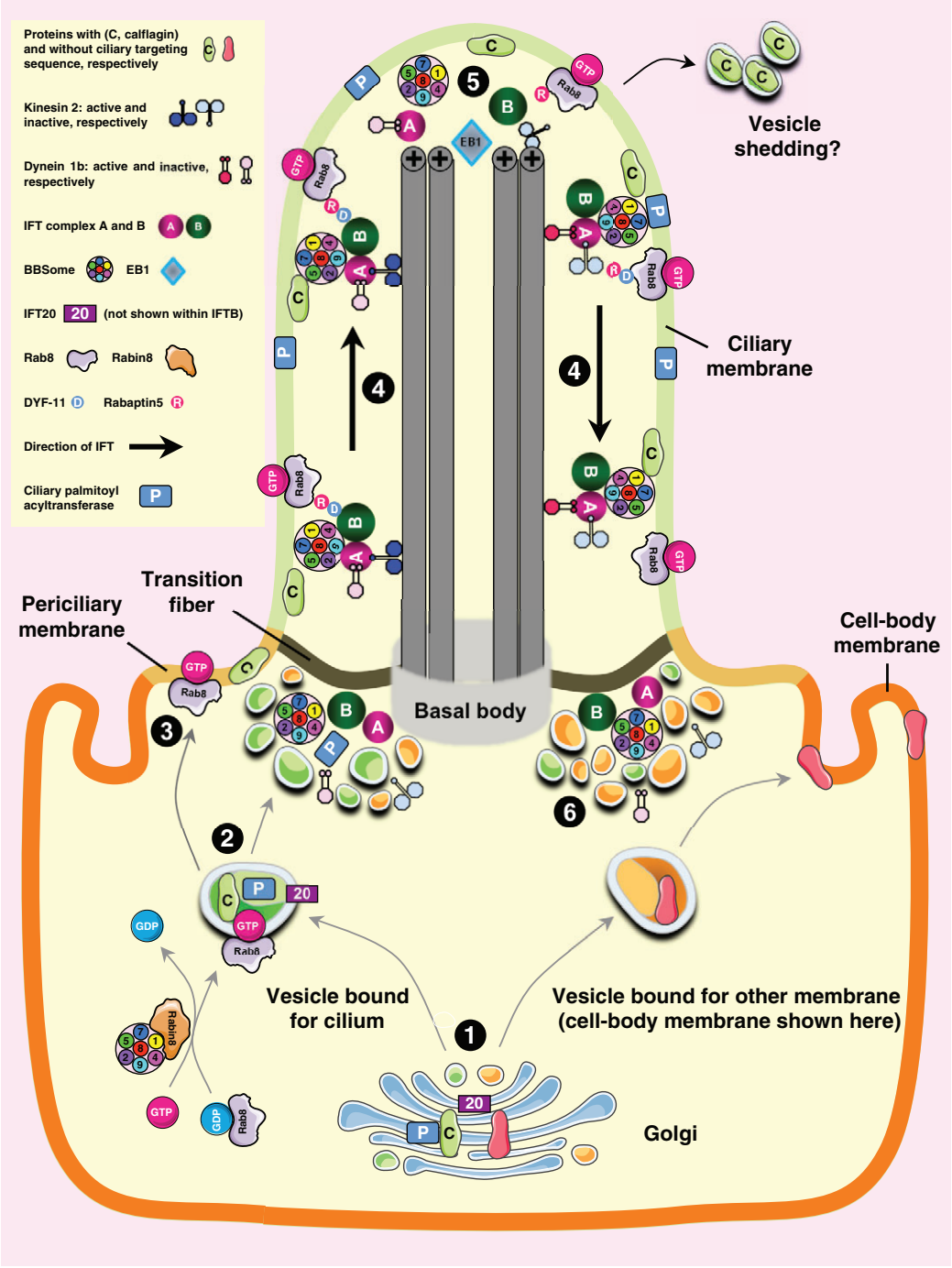

Fig. 1. See next page for legend.

multiprotein complexes, known as the A and B complexes, whose movement is powered by microtubule motors of the kinesin 2 and dynein 1b families for anterograde and retrograde movement, respectively (Kozminski et al., 1995; Pazour et al., 1999; Porter et al., 1999; Signor et al., 1999). Immunogold electron microscopy experiments localize the IFT component IFT52 to the periphery of transition fibers, which suggests that IFT complexes assemble and dock at the base of the cilium before entry into the organelle. These fibers connect the basal body to the membrane at the base of the cilium and create an environment that restricts free entry of diffusible molecules (Deane et al., 2001). This implies that active mechanisms, currently uncharacterized, transport ciliary proteins through or around this barrier. An interesting candidate that might participate in this process is importin-β, a protein with a known role in the transit of nuclear proteins through nuclear pore complexes. Importin-β directly binds to the ciliary protein Crumbs3-CLPI (CRB3-CLPI), and the expression of a dominant-negative importin-β disrupts ciliogenesis, possibly by blocking the entry of proteins into the cilium (Fan et al., 2007).

Fig. 1. Protein targeting to the ciliary membrane. The eukaryotic cilium is a distinct organelle that is separated from the cytoplasm by transition fibers that connect the basal body to the membrane and separate the ciliary membrane (green) from the periciliary (pale orange) (Reiter and Mostov, 2006) and cell-body membranes (dark orange) (Sloboda and Rosenbaum, 2007). Although cilia in certain cell types differ in the fine details of their structures, functions, mechanisms of assembly and regulation, some general principles have emerged in recent years; this figure attempts to integrate these general concepts. The ciliary membrane has a lipid composition that is distinct from that of the periciliary and cell-body membranes because it is highly enriched in sterols, glycolipids and sphingolipids (Tyler et al., 2009). This specialized composition is probably formed in the Golgi (Ejsing et al., 2009; Schuck and Simons, 2004). Various cell membranes have unique complements of membrane proteins, and one mechanism that might contribute to this specialized composition is the association of certain types of proteins with vesicles of specific lipid composition. The model shown is based on the current literature and recent findings from our own laboratory and outlines six stages: (1) Vesicles of different lipid composition and containing specific cargos form in the Golgi (Klemm et al., 2009). For example, those vesicles rich in sterols and sphinoglipids might load certain lipid raft-associated proteins such as the calflagins. One or more cilium-specific palmitoyl acyltransferases might reside in these vesicles to confer lipid-raft association to their substrates in order to sort them into these vesicles. These ciliary vesicles also load with other proteins that contain ciliary targeting sequences, whereas vesicles of other compositions might load other types of cargo (e.g. proteins bound for the cell-body membrane, shown here as one example). IFT20 also loads with the ciliary-bound vesicles and might serve as an adaptor to recruit other ciliary cargo. (2) Vesicles destined for the cilium interact with GTP-Rab8, which is produced by GDP-GTP exchange catalyzed by Rabin8 and the BBSome. This facilitates movement of the vesicles to the base of the cilium near the transition fiber (Hao and Scholey, 2009; Jin and Nachury, 2009), although the molecular mechanism for transport is not known. (3) Vesicles fuse with the periciliary membrane, and ciliary lipids and proteins enter the cilium. Some vesicles accumulate at the base of the cilium, giving rise to the ciliary necklace structure. The periciliary membrane and transition fiber can take different forms in different cell types. For example, the trypanosome flagellar pocket is a unique dynamic structure through which all endocytosis and exocytosis occurs, and which is much larger than the periciliary membrane of most cells. (4) IFT complexes, which consist of protein subcomplexes IFT-A and IFT-B and are possibly associated with the BBSome (Ou et al., 2005), move cargo along the length of the cilium. The BBSome might also interact directly with the ciliary membrane (Hao and Scholey, 2009). IFT is driven in the anterograde direction by kinesin 2 and in the retrograde direction by dynein 1b. A key component of IFT-B is DYF-11, which might promote a separate membrane association of the IFT complex through Rabaptin5 and GTP-Rab8 (Omori et al., 2008), although these interactions need further investigation because they were not confirmed in other studies (Follit et al., 2009). Ciliary membrane proteins might also be associated with the IFT complexes. (5) IFT complexes disassociate from their cargo at the ciliary tip, where anterograde motors become inactivated and retrograde motors become active. (6) Turnover products are recycled back to the base of the cilium. Note that not all components of IFT are included in this model, and specific elements are not drawn to scale.

Ciliary membrane trafficking

To form a fully functional organelle, IFT-mediated axonemal assembly must be coordinated with the delivery of membrane lipids and proteins. The ciliary membrane is not simply a homogeneous extension of the plasma membrane; extensive data support the notion that the ciliary membrane has a lipid and protein composition that differs from that of the cell-body membrane [see Tyler et al. (Tyler et al., 2009) and references therein]. In theory, the distinct composition of proteins and lipids of the plasma membrane versus the ciliary membrane could be achieved through multiple mechanisms. In a targeted delivery model, the molecules destined for either location would be sorted at an earlier point and then delivered separately to the specific membrane. Alternatively, in a diffusion-retention model, transported molecules would be delivered by a common pathway and freely exchange among different domains, but differ with respect to their selective retention at each target membrane. Experimental evidence has long favored the former model. Early ultrastructural studies of the ciliary membrane showed a ‘ciliary necklace’ in close proximity to axonemal transition fibers that appeared to form a diffusion barrier for entry into the cilium (Gilula and Satir, 1972). Instead, ciliary membrane proteins and lipids are probably delivered to the periciliary membrane directly adjacent the base of the cilium. The earliest ultrastructural studies also support this view; vesicles containing ciliary membrane proteins were visualized fusing with the periciliary membrane in algae (Bouck, 1971). Subsequent experiments in photoreceptors revealed that vesicles accumulate at the base of the connecting cilium before entry, suggesting that vesicles are specifically targeted to this membrane (Papermaster et al., 1985). Therefore, we propose a pathway for ciliary membrane trafficking based on the targeted delivery model, in which vesicle transport is crucial. The steps in this pathway include cargo selection and vesicle budding from the Golgi, vesicle targeting and fusion at the periciliary membrane, and finally active transport of membrane components into the ciliary membrane (Fig. 1).

Cargo sorting and vesicle budding at the Golgi

A principal mechanism by which eukaryotic cells establish distinct compositions for different membrane-bound organelles is through selective incorporation of export cargo at the trans-Golgi into vesicles that are then targeted to specific compartments. This process is regulated by the assembly of several distinct multiprotein complexes on vesicle membranes (Guo, 2000). In particular, small guanosine triphosphate hydrolases (GTPases) of the Arf, Arl and Rab families play central roles in cargo sorting at the Golgi. These proteins cycle between active and inactive states that are determined by whether they are bound to GTP or GDP, as modulated by the activity of GTPase-activating proteins (GAPs) and guanine-nucleotide-exchange factors (GEFs). When GTP-bound, these GTPases recruit effector proteins that regulate membrane-membrane and membrane-cytoskeleton interactions.

Targeting of proteins to different cellular locations can be mediated by recognition of targeting sequences within the cargo by specific

receptors. These receptors might be the GTPases themselves or the coat protein complexes whose assembly they promote. The benefits of identifying organelle-specific targeting sequences are twofold. First, it enables prediction of localization for other proteins that have the same the targeting sequence. Second, it provides a means of identifying the molecular interactions that cause this targeting (e.g. binding of ER-resident proteins to the KDEL receptor). A variety of ciliary targeting sequences have been identified that contain diverse polypeptide and acylation motifs (Pazour and Bloodgood, 2008). However, the manner in which these sequences confer ciliary targeting remains largely obscure. An exception is the direct binding of the GTPase Arf4 by the rhodopsin VxPx motif, a ciliary targeting sequence that is common to the ciliary proteins polycystin-2 in renal tubule epithelial cells (Geng, 2006) and cyclic-nucleotide-gated channel β1 (CNG-β1) in olfactory neurons (Jenkins et al., 2006). In the Golgi, Arf4 binding to rhodopsin via this motif promotes the assembly of a complex that includes Rab11, ASAP1 (ArfGAP with SH3 domain, ankyrin repeat and PH domain 1) and FIP3 (Rab11-family-interacting protein 3) and in turn triggers vesicle budding from the trans-Golgi network (Inoue et al., 2008; Mazelova et al., 2009a). ASAP1 seems to be a key player in these events because it possesses a Bin-Amphiphysin-Rvs (BAR) domain that binds to curved membranes and promotes further membrane deformation (Nie et al., 2006). ASAP1 can actually promote tubulation of vesicles in vitro (Krauss et al., 2008), suggesting that it can facilitate vesicle budding from the trans-Golgi network. Therefore, this multiprotein complex might be involved in a mechanism for the selective sorting of ciliary proteins into vesicles and vesicular budding. Further investigation of the mechanisms by which ciliary targeting sequences confer localization will undoubtedly elucidate additional molecules involved in ciliary targeting. It should be noted that the divergence of ciliary targeting sequences probably reflects coevolution with binding partners; the nature of the sequence itself might not be as important as the physiological interaction that facilitates proper trafficking.

Another binding partner for a ciliary targeting motif has also recently been described. CNG-β1 was shown to both colocalize and coimmunoprecipitate with the membrane adaptor ankyrin-G through its C-terminal domain. This interaction is both necessary and sufficient for the targeting of human CNG-β1 to the ciliary membrane when expressed in *Xenopus* photoreceptors (Kizhatil et al., 2009). However, the stage of transport at which this interaction confers targeting to the ciliary membrane remains unclear.

In addition to transport mediated by the direct binding of vesicle membrane proteins, ciliary cargo might be recruited into ciliary-bound vesicles indirectly through interactions with other ciliary proteins that serve as adaptors. A promising candidate for such an adaptor is the IFT protein IFT20. In contrast to all other known IFT components, which localize to the cilium and/or basal body, IFT20 also localizes to the Golgi (Follit et al., 2006). The pools of Golgi-localized IFT20 and IFT20 localized to the basal body and/or cilium are in dynamic exchange. Total inhibition of IFT20 expression prevents ciliogenesis, whereas partial inhibition that depletes the amount of IFT20 in the Golgi pool but not the ciliary pool results in defective transport of polycystin-2 to the ciliary membrane. This suggests that IFT20 serves as an adaptor or chaperone for the targeting of polycystin-2 to the ciliary membrane.

IFT20 is anchored to the Golgi through its interaction with GMAP210 (also known as thyroid hormone receptor interacting protein 11, TRIP11) (Follit et al., 2008), a member of the golgin family of proteins. GMAP210 contains both an ArfGAP1 lipid-packing sensor (ALPS) domain that preferentially binds to curved membranes (Drin et al., 2008) and GRIP-related Arf-binding (GRAB) domains that bind to small GTPases (Short et al., 2005). Notably, although cells from GMAP210-null mice exhibit normal Golgi architecture, they have reduced levels of polycystin-2 in their ciliary membranes. Both IFT20 and GMAP210 therefore have clear roles in the trafficking of polycystin-2 to the ciliary membrane, and are ideally positioned to mediate cargo sorting within the Golgi. Given these findings, it will be interesting to know whether these proteins perform a similar function in the transport of other ciliary cargo, and whether they do so through their interaction with specific GTPases.

Vesicle targeting and fusion at the periciliary membrane

Following cargo sorting and vesicle budding from the Golgi, vesicles are delivered to their target membranes. This is a multistep process involving transport, tethering and fusion. The molecular mediators that govern these steps for vesicles bound for ciliary locations are only recently coming to light. It appears that a key player in targeting vesicles to their sites of fusion is the exocyst, a multiprotein complex whose components were originally discovered in yeast for their roles in secretion (Novick, 1980; TerBush, 1996). In polarized epithelial cells, the exocyst localizes to basolateral membranes, where it mediates the delivery of vesicles. An analogous function in ciliary protein targeting was suggested by the observation that exocyst components were localized to the membrane of the primary cilium in MDCK cells (Rogers et al., 2004). The exocyst is an effector of the GTPase Rab11 (Guo, 1999), whose recruitment to rhodopsin-containing vesicles at the Golgi was discussed above. During cytokinesis in yeast, the exocyst guides the tethering of Rab11- and FIP3-containing vesicles to the cleavage furrow (Fielding, 2005). An analogous role in ciliated cells might therefore promote the targeting of vesicles to the ciliary membrane. Consistent with this idea, knocking down the expression of the exocyst protein Sec10 in MDCK cells abolishes ciliogenesis (Zuo et al., 2009).

In addition to Rab11, several other GTPases play a role in ciliary protein targeting. Rab8 associates with ciliary-bound vesicles soon after they bud from the Golgi. Expression of dominant-negative Rab8 impairs rhodopsin delivery in *Xenopus* photoreceptors (Moritz et al., 2001), as well as the delivery of membrane to the primary cilia of immortalized human retinal pigment epithelial cells (Nachury et al., 2007). The work by Nachury and colleagues also showed that the activity of Rab8 requires the expression of a GEF (Rabin8) that interacts with a multiprotein complex called the BBSome. The exact function of the BBSome is unclear, although it is known that mutations in the proteins that it comprises cause Bardet-Biedl syndrome (BBS), a pleiotropic disease associated with multiple ciliary signaling defects (Blacque and Leroux, 2006). One of the BBSome proteins is Arl6, a GTPase that was previously found to be involved in ciliary trafficking (Fan et al., 2004). Other GTPases implicated in ciliary targeting include IFT27 in *Chlamydomonas* (Qin et al., 2007) and RabL5 in *Trypanosoma brucei* (Adhiambo et al., 2009). Finally, systematic screening of 46 human Rabs demonstrated a requirement for Rab23 and Rab17, in addition to Rab8, in ciliogenesis (Yoshimura et al., 2007). Therefore, multiple GTPases have been identified that probably contribute to ciliary membrane trafficking through the assembly of multiprotein complexes on vesicle coats.

Vesicle delivery to target membranes is guided by the cytoskeleton. Very little is known, however, about the cytoskeletal interactions that guide vesicle delivery to the ciliary membrane. In photoreceptors, Rab8 colocalizes with actin filaments near the vesicle fusion site, and this colocalization seems to be mediated by ezrin and/or moesin

and Rac1 (Deretic et al., 2004). In addition, it has been shown that inhibition of the actin-based motor myosin-VIIa disrupts rhodopsin delivery (Liu, 1999). The actin network thus might be involved in transporting vesicles to the ciliary membrane. More research is needed to clarify the extent of actin involvement in guiding vesicle transport to the cilium, and how this process might intersect with microtubule-based transport.

Once vesicles are tethered to target membranous compartments, soluble NSF attachment protein receptor (SNARE) proteins on vesicle (v-SNARE) and target (t-SNARE) membranes drive membrane fusion. Although the pairing of specific v-SNAREs with cognate t-SNAREs does not confer specificity to vesicle targeting, particular SNARE pairings are associated with specific fusion events (Bethani et al., 2007; Brandhorst et al., 2006; Guo, 2000; ter Beest et al., 2005). In vertebrate photoreceptors, the membrane at the base of the cilium is enriched in the t-SNARE syntaxin 3 in a manner that depends on microtubules (Mazelova et al., 2009b). Modulation of the cell lipid composition that promotes the association of syntaxin 3 with another t-SNARE, SNAP-25, correlates with increased delivery of rhodopsin to the ciliary membrane. Therefore, syntaxin 3 and SNAP-25 are promising candidates for mediating the fusion of vesicles at the base of the ciliary membrane and, together with the GTPases, multiprotein complexes and cytoskeletal elements discussed above, are probably key players in this crucial step of ciliary targeting.

Intraciliary movement

Following vesicle fusion at the periciliary membrane, lipids and membrane proteins enter the cilium. As discussed above, it appears that a diffusion barrier restricts passive diffusion into the cilium, indicating that an active means of transport into the cilium probably occurs. An obvious candidate for this function is the IFT complex. However, although electron micrographs of IFT complexes show a tightly apposed overlying membrane, none of the IFT proteins discovered to date possesses transmembrane domains, lipid modifications or lipid-binding domains. Moreover, investigation of an IFT-membrane interaction has been hindered by the detergent-based treatments that are involved in most biochemical purifications of IFT-complex proteins (Shogomori and Brown, 2003).

Despite the difficulties in detecting an interaction between IFT complexes and membrane proteins, several lines of evidence suggest that IFT plays a role in the transport of ciliary membrane proteins. Cyclic-nucleotide-gated channels in mammalian cells (Jenkins et al., 2006) and a membrane-bound aurora kinase in *Chlamydomonas reinhardtii* have both been shown to require IFT for their transport into the cilium (Pan and Snell, 2003), and polycystin-2 accumulates in cilia when retrograde IFT is inhibited in a variety of cell types (Huang et al., 2007; Pazour et al., 2002). Perhaps most convincingly, ciliary transient receptor potential vanilloid (TRPV) channels have been visualized translocating within *Caenorhabditis elegans* cilia at speeds that are characteristic of IFT (Qin et al., 2005).

Evidence of a biochemical association between IFT complexes and ciliary membrane proteins has also emerged. For example, IFT complexes co-purify with DnaJ chaperones and the membrane-associated proteins rhodopsin and guanylyl cyclase 1 in photoreceptors (Bhowmick et al., 2009). In addition, an indirect association between Rab8 and IFT20 through Elipsa and rabaptin 5 has recently been reported, and the inhibition of Elipsa expression causes defective ciliogenesis in zebrafish (Omori et al., 2008). In *C. elegans*, the Elipsa homolog DYF-11 translocates within sensory cilia via IFT (Kunitomo and Iino, 2008), supporting the link between IFT and this putative binding partner of Rab8. Therefore, both functional and biochemical evidence implicating IFT in the transport of membrane proteins within the cilium is accumulating.

If ciliary membrane proteins indeed traffic into cilia with IFT complexes, an equally important consideration becomes how they disassociate from these complexes, lest they recycle back out of the organelle. The logical place to look for molecular regulators of IFT disassociation is at the tip of the cilium because this is where axonemal subunits are incorporated. The plus-end microtubule-binding protein EB1 localizes to the tip of the cilium (Pedersen, 2003), where it interacts with the retrograde complex protein IFT172 (Pedersen et al., 2005). The ciliary tip is probably also the site where anterograde and retrograde IFT transport are coordinated. This coordination might involve the BBSome because inhibiting the expression of BBS proteins, which transit with IFT complexes in *C. elegans*, leads to uncoupling of the anterograde and retrograde movement (Ou et al., 2005).

Composition and sorting of ciliary membrane lipids

Early studies of ciliary membrane lipid composition indicated that the lipids contain a high content of sterols (Chailley and Boisvieux-Ulrich, 1985; Kaneshiro, 1990; Souto-Padron and de Souza, 1983; Tetley, 1986), glycolipids (Bloodgood et al., 1995) and sphingolipids (Kaneshiro et al., 1984; Kaya et al., 1984), which now are known to be components of canonical lipid-raft microdomains. This suggested to us (Tyler et al., 2009) that cilia might be enriched in lipid rafts and that this special lipid composition might serve to recruit or retain ciliary membrane proteins, such as the dually acylated flagellar calcium-binding protein (FCaBP) in *Trypanosoma cruzi* (Godsel and Engman, 1999) and its homolog in *Trypanosoma brucei*, calflagin (Emmer et al., 2009). These proteins require both myristoylation and palmitoylation for lipid-raft association and ciliary-membrane targeting. Microscopic and biochemical analysis of the trypanosome flagella showed that they are highly enriched in sterols, sphingolipids and some dually acylated proteins, and that they possess high liquid order as determined by laurdan two-photon microscopy (Tyler et al., 2009) [which involves the intercalation of laurdan into membranes and its fluorescence according to the molecular freedom of the water molecules surrounding it (Gaus et al., 2003)]. Interestingly, mutation of the palmitoylation site of calflagin, or inhibition of the acyltransferase that palmitoylates calflagin (both of which lead to the production of a protein that has only a myristoyl group), leads to protein mislocalization to the cell-body membrane (Emmer et al., 2009). Thus, although myristoylation is sufficient for cell-body-membrane targeting, palmitoylation serves as a secondary signal for calflagin lipid-raft association and flagellar targeting. Disruption of lipid rafts also leads to protein mislocalization (Tyler et al., 2009).

An association between lipid rafts and many other cilia-targeted proteins has also been described in other systems, including vertebrate photoreceptors (Senin et al., 2004), *Chlamydomonas reinhardtii* (Iomini et al., 2006), mammalian spermatozoa (Travis et al., 2001) and *Leishmania major* (Tull et al., 2004). Similarly, certain proteins that are thought to be involved in targeting proteins to ciliary membranes have been shown to require protein-lipid interactions for their localization in non-ciliated cells. For example, the SNARE protein syntaxin 3, whose involvement in rhodopsin trafficking was discussed above (Mazelova et al., 2009b), requires cholesterol for its clustering in lipid rafts at the plasma membrane of epithelial cells (Low, 2006). In a similar way, the localization of

the exocyst in MDCK cells is mediated by a direct interaction with phosphatidylinositol (4,5)-bisphosphate (Liu et al., 2007). Whether these same protein-lipid associations are involved in localizing syntaxin 3 and the exocyst to ciliary membranes remains to be determined.

Collectively, these findings illustrate the distinct lipid composition of the eukaryotic ciliary membrane relative to the plasma membrane and provide a potential mechanism for downstream protein recruitment. However, the mechanisms by which the cell establishes and maintains the discrete lipid composition of the ciliary membrane are unknown. It should also be noted that, although it is tempting to speculate that lipid-raft enrichment occurs upstream of protein sorting, it is equally plausible that ciliary proteins drive lipid-raft coalescence and their enrichment in ciliary membranes. These two models are not mutually exclusive, however, and perhaps the most likely scenario is that protein and lipid sorting have reciprocal effects on each other during the trafficking of these molecules to and within cilia. A major advance in this area was the finding that a transmembrane lipid-raft protein is sorted in the Golgi complex specifically into vesicles that are enriched in sphingolipids and sterols and that exhibit high liquid order by laurdan microscopy (Klemm et al., 2009). This work indicates that lipid sorting and formation of vesicles with distinct lipid composition can occur in the trans-Golgi network. It is therefore reasonable to hypothesize that lipid-raft vesicles containing specific cargo can form here as well, a subset of which might be destined for the ciliary membrane. This notion is consistent with a targeted delivery model for the delivery of ciliary components.

Concluding remarks

In this Commentary, we have attempted to trace the synthesis, assembly, packaging and trafficking of ciliary proteins and lipids from their site of initial assembly to their final destination in ciliary membranes. The key steps in this process are illustrated in Fig. 1. One interesting aspect, illustrated in this figure but not discussed above, is the possibility that the cilium is a secretory organelle in addition to being a motility and sensory organelle. This is a notion that we have had for some time – since the discovery nearly two decades ago that *T. cruzi* FCaBP can be found in vesicles shed from the cell (Ouaissi et al., 1992). This possibility is eloquently described in a recent review by Baldari and Rosenbaum (Baldari and Rosenbaum, 2009), which integrates information from various fields and posits that vesicle secretion could be a natural and important extension of normal ciliary processes. Clearly, it is important to determine the mechanisms regulating this exocytosis and the functions of shed vesicles in intercellular communication and host-pathogen interactions. It will also be interesting to know how the unique lipid composition of the ciliary membrane is established and maintained, and how vesicles of different lipid composition and containing specific cargo are formed in the Golgi. Where are the enzymes involved in the synthesis and assembly of these unique lipid vesicles located, and how are they regulated? In addition, the molecular mechanisms underlying the differential trafficking of specific vesicle populations from the Golgi to their intracellular destinations are still largely unknown. As the known functions of cilia in basic cell physiology and regulation, as well as in health and disease, increase each month, it will be fascinating to follow this field as it evolves in the coming years.

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