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REVIEW

Role of small GTPases in polarized vesicle transport to primary cilium

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Abstract: Small GTPases play crucial roles in regulating polarized vesicle trafficking in a cell. These molecules are involved in diverse pathways and regulate cell shape, organelle integrity, and cell signaling. One of the most important cellular signaling centers is the primary or sensory cilium. Small GTPases orchestrate a diverse set of events to not only generate these structures but also mediate their function and maintenance during the life of the cell. In this review, we will discuss the various small GTPases involved in regulating cilia biogenesis and function and their involvement in human diseases.

Keywords: ciliopathies, RPGR, RP2, retina, kidney, photoreceptor, neuronal cilia

Introduction

The cilium is an evolutionarily conserved microtubule based structure that protrudes from the cell surface of almost all cell types.[1] There are two types of cilia that are structurally fairly similar but show distinct functional involvement: motile cilia and immotile cilia. Motile cilia (other than flagella) are involved in mucociliary transport and are composed of 9 outer doublets of microtubules and a pair of central microtubules (9+2 arrangement).[2] On the other hand, nonmotile or primary (sensory) cilium is usually present as a single entity per cell and is involved in sensing extracellular environment. It is also composed of 9 microtubule doublets but lacks the central pair (9+0 arrangement).[3] A stringently controlled and elaborate program of coordinated trafficking of membrane and microtubule assemblies regulates early ciliogenic events, which are orchestrated by a process called intraflagellar transport (IFT). Elegant studies and review articles have covered their role and involvement in cilia formation.[4–7] The aim of this article is to discuss the mechanisms involved in regulating trafficking of membrane proteins to the cilia, specifically focusing on the role of small GTPases involved in polarized trafficking of vesicles from the Golgi network to the base of cilia for entry and incorporation into the ciliary membrane.

Primary cilia are involved in dynamic cellular processes such as signaling cascades (sonic hedgehog signaling, planar cell polarity signaling) and sensory transduction (olfaction, mechanosensation, and photoreception).[8–13] Cilia carry out such processes by concentrating key receptor and signaling moieties in the ciliary membrane while excluding others.[6] It is a considerable feat on the part of cilia to regulate their membrane composition given the fact that the ciliary membrane is continuous with the plasma membrane. This suggests that cilia maintain a barrier-like structure called membrane diffusion barrier, first characterized in the unicellular green alga *Chlamydomonas*.[14,15]

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However, membrane diffusion barriers have been described in other cell types (eg, those between apical and basolateral membranes of polarized epithelial cells and in neurons).[16,17]

Delivery of membrane proteins to cilia

Polarized vesicle trafficking has emerged as a fundamental mechanism to deliver proteins and membranes to their proper cellular compartments. Abnormalities in this process can lead to significant impacts on normal cell function.[18–21] Trafficking to the cilium consist of two steps: 1) sorting of the proteins destined to the cilium and 2) transport to the cilium. Both these steps require a coordinated action of multiple protein assemblies. In the first step, protein sorting from the trans-Golgi network (TGN) is based upon the presence of a ciliary targeting sequence (CTS). Such a sequence was first described in the G-protein-coupled receptor rhodopsin present in rod photoreceptor cilia.[22,23] It was shown that disruption of the CTS of rhodopsin results in its mistrafficking and severe retinal degeneration.[24,25] Following these studies, additional ciliary membrane proteins were found to possess functional CTS.[26–29] A notable observation in the case of photoreceptors is that due to immense load of protein trafficking to cilia, a majority of the cellular machinery is dedicated to sorting of proteins to outer segment (OS), which results in the ciliary targeting being a default pathway in photoreceptors. Proteins that do not possess a targeting signal seem to piggyback on rhodopsin transport machinery and are targeted to the OS.[30] Such observations reinforce the need to critically evaluate protein-targeting mechanisms in a cell-type specific manner. It is possible that multiple mechanisms are involved in ciliary targeting in different cell types depending upon the load of cilia function.[19]

The next step in ciliary targeting is the machinery that recognizes the CTS and targets those vesicles to the cilium. Although vesicular trafficking is essential for several cellular functions, the regulation of vesicular trafficking is still poorly understood. Small GTPases are highly conserved proteins that play an important role in regulating polarized vesicle trafficking. These GTPases include proteins belonging to the RAB family, along with the Arf/Arl family of small GTPases, which regulate the vesicle formation, movement, and fusion.[31–35] Here, we will discuss the role of these small GTPases in vesicular transport.

Small GTPases in ciliary protein trafficking

Much of the information about polarized protein trafficking to cilia has come from elegant studies using vertebrate photoreceptors. Photoreceptors are polarized and one of the highly metabolically active cell types, second only to cancer cells.[36] The polar distribution of key proteins is maintained by stringently regulated trafficking of proteins from their site of synthesis in the inner segment to the sensory ciliary compartment called the OS.[37] In addition, photoreceptors periodically shed their distal ciliary tips containing the bleached photopigment.[38] Such shedding triggers replenishment of the ciliary membrane and associated proteins at the proximal end. It is estimated that approximately 2,000 molecules of rhodopsin are transported per minute in a normal human retina.[39–41] Post-Golgi vesicles containing the most abundant photoreceptor protein rhodopsin fuse with the plasma membrane near the base of the cilium.[42] Several GTPases have been identified that participate in the trafficking of rhodopsin.[43]

On the basis of sequence and function, small GTPases can be divided into five major subfamilies: Ras, Rho, RAB, Arf/Arl, and Ran.[44] These enzymes undergo cycling reactions between the active GTP-bound and the inactive GDP-bound states. Guanine nucleotide exchange factors (GEFs) stimulate GDP (guanosine diphosphate) to GTP (guanosine triphosphate) conversion whereas GTPase activating proteins (GAPs) mediate GTP hydrolysis.[44] Elegant analyses have revealed the involvement of Arf/Arl, RAB, and Ran GTPases in ciliary function (Table 1).[31]

Arf/Arl GTPases

Arf (ADP ribosylation factor) proteins were originally identified as cofactors for cholera toxin-catalyzed ADP-ribosylation.[45]

Table 1 Role of GTPases in cilia function

GTPase	Function	GAP/GEF
ARL3	Negative regulator of ciliogenesis. Required for targeting proteins such as NPHP3 to the ciliary membrane by releasing myristoylated NPHP3 from UNC119B cargo adapter into the cilium	RP2/NI
ARL6	Involved in membrane protein trafficking at the base of the ciliary organelle. Plays an important role in regulation of BBSome ciliary trafficking	NI
ARL13B	Involved in ciliogenesis by regulating stability of IFT complex	NI
ARF4	Involved in Golgi to cilia transport. Regulates rhodopsin trafficking	ASAP/NI
RAB8A	Regulates ciliary membrane sorting or trafficking, cilia formation	NI/RPGR, RABIN8
RAB11	Acts upstream of RAB8, ciliary membrane assembly	NI
RAB10	Membrane transport	NI
RAB23	Regulates hedgehog signaling	Evi5-L/NI
RAB28	Regulates photoreceptor protein trafficking	NI

Abbreviations: IFT, intraflagellar transport; GAP, GTPase activating protein; GEF, Guanine nucleotide exchange factor; NI, not identified.

There are 6 types of mammalian Arfs (Arf1–Arf6).[46] Of these, Arf4 is the only known Arf family GTPase to function in ciliary targeting.[24] Recent studies have shown that these proteins regulate vesicular trafficking pathways. The CTS of rhodopsin recruits Arf4 at the Golgi/TGN in inner segment of photoreceptors.[43] Arf4 activity is modulated by its GEF GBF1 and GAP ASAP1. ASAP1 is also involved in ciliary targeting of rhodopsin and in the coordinated action of Arf and RAB GTPases (see "RAB GTPases" section). In addition to Arfs, there are Arf-like GTPases called Arls. There are >20 members in the Arl family of proteins.[47,48] A notable feature of Arls is a characteristic lack of biochemical activity.[49] Nonetheless, Arl proteins play key roles in membrane trafficking and human ciliopathies.[34] Three Arl proteins, Arl3, Arl6, and Arl13b have been implicated in ciliary targeting.[50]

Arl3

The involvement of Arl3 in cilia development and function was revealed in studies using *Leishmania donovani* and *Caenorhabditis elegans*.[51] In these investigations, it was revealed that Arl3 likely acts by modulating the integrity of the IFT complex thereby regulating cilia formation. Although its exact role in cilia formation is still unclear, Arl3 has been shown to modulate cilia-dependent signaling events. This is achieved by participating in the trafficking of ciliary receptor proteins, such as polycystin-1 and polycystin-2.[52] Interestingly, Arl3$^{-/-}$ mice exhibit ciliopathy phenotypes, including cystic kidney disease and photoreceptor development.[53] Consistently, rhodopsin mistrafficking was observed in Arl3$^{-/-}$ mouse retina. Further involvement of Arl3 in photoreceptor function came from studies indicating that Arl3 activity could be modulated in vitro by RP2 (retinitis pigmentosa 2), which is mutated in X-linked retinitis pigmentosa.[54–57] It was found that RP2 acts as a GAP for Arl3, which is predicted to trigger the release of lipid-modified cargo, such as PDE6d and UNC119 in the cilia.[57] Arl3 interacts with these proteins in GTP-bound form. According to the current model, Arl3-GTP binds to UNC119 complexed with myristoylated NPHP3 (nephrocystin-3) to traffic to cilia. When inside cilia, RP2 acts to release UNC119 and NPHP3 from Arl3 by converting Arl3-GTP to Arl3-GDP.[58,59] Although such a hypothesis is attractive, additional experimental support is necessary to further test this model. For example, although RP2 is involved in retinal degeneration there was no evidence of mislocalization of NPHP3 in the *Rp2*^{null} mice.[60] Moreover, RP2, Arl3, and NPHP3 have not been found in a complex in the retina. Such discrepancies underscore the need to interpret results in a cell-type dependent manner.

Arl6

Also known as BBS3, Arl6 was the first small GTPase protein that was linked to the human ciliopathy, Bardet–Biedl syndrome (BBS).[61,62] Using a protein pull-down assay with homogenized bovine retina, Jin et al[63] showed that Arl6 bound a ciliary membrane protein complex containing 7 BBS proteins (BBSome) and BBIP10. Ciliary localization of BBSome is dependent on ARL6 in its GTP bound form. Studies have shown that SSTR3 (somatostatin receptor 3) is a cargo for BBSome and is lost from cilia in hippocampal neurons of *Bbs2*$^{-/-}$ and *Bbs4*$^{-/-}$.[64] Arl6 and the BBSome bind to the CTS of SSTR3 to transport to the ciliary membrane. This finding supports the hypothesis that the abnormal Arl6 activity leads to the compromised ciliary entry of critical cargo proteins essential for cilia assembly and signaling and, thus, causes the variety of BBS symptoms. A recent study by Su et al,[65] showed that polycystin-1 interacts with the BBSome complex and expression of a mutant form of Arl6 results in loss of ciliary localization suggesting that polycystin-1 may be a cargo for the BBSome.

Arl13b

Arl13b is another small GTPase protein connected to the human ciliopathy, Joubert syndrome, an inherited neurodevelopmental disorder with midbrain–hindbrain malformations, retinal dystrophy and, occasionally, nephronophthisis.[66] Duldulao et al,[67] first demonstrated that Arl13b is a protein that is highly enriched in the cilium and is required for cilia formation in multiple organs in zebrafish, and that loss of A*rl13b* leads to multiple cilia-associated phenotypes and thus ciliary localization is crucial for the in vivo function of Arl13b. Arl13b localizes to a proximal ciliary compartment, where it associates with ciliary membranes via palmitoylation modification motifs. Defects in ciliary morphology and ultrastructure and destabilization of IFT complex have been observed in *C. elegans* Arl13 mutants. Li et al[68] observed shortened cilia with various ultrastructural deformities and a disrupted association between IFT subcomplexes A and B in Arl13 mutants and that these abnormalities were eliminated by depletion of Arl3, another ciliary small GTPase. Recently, it was shown that SUMOylation of Arl13 is crucial for proper ciliary targeting of various sensory receptors such as polycystin-2 suggesting SUMOylation modification of GTPase ARL13b regulates proper ciliary targeting of various sensory receptors.[69] Humbert et al[28] identified that Arl13b is in complex with other proteins and helps regulate ciliary trafficking of phospholipid phosphatase INPP5E, which is mutated in Joubert Syndrome.

RAB GTPases

RAB GTPases represent a large family of small GTPases, and in humans more than 60 members have been identified.[70] Several RAB proteins have been shown to be involved in regulation of ciliary functions and these include RAB8, RAB11, RAB10, RAB17, and RAB23.[33] Identification of RAB8 in photoreceptors was the first study to link cilia and RAB proteins.[42] In that study, it was demonstrated that a small fraction of RAB8 is associated with post-Golgi vesicles. Microscopic analysis showed that post-Golgi vesicles, having rhodopsin, were localized at the base of the OS where colocalization of RAB8 and actin were observed suggesting that RAB8 may be involved in trafficking of rhodopsin. Later, it was shown that RAB8 was also involved in trafficking of several other proteins, such as fibrocystin (involved in autosomal recessive polycystic kidney disease), to cilia.[26] Nachury et al,[71] showed that the BBSome associates with the RAB8 GEF RABIN8 and promotes docking and fusion of vesicles to the ciliary membrane. Elegant live-cell imaging experiments by Westlake et al,[72] revealed the earliest steps in RAB8 membrane assembly during cilia formation. RAB8 is targeted to the primary cilium during early ciliogenesis followed by a gradual loss from the cilium as the organelle matures. RAB8 is activated by its GEF RABIN8, which is recruited to the centrosome and activated by a mechanism involving RAB11 and homologs of the yeast transport protein particle II (TRAPPII) complex subunits.[73] RABIN8 binds to the TRAPPII complex and this interaction is essential for RABIN8 localization to centrosome, trafficking, and ciliogenesis. In addition to specific proteins, cell cytoskeleton is also known to play a role in modulating RAB8–RAB11 activity during cilia formation. It was found that branched actin network negatively regulates RAB8 activation likely by inhibiting the association of RAB8 with RAB11-containing vesicles.[74] Knödler et al,[75] showed a functional connection between RAB11, RABIN8, and RAB8. RAB11 binds directly to RABIN8 and stimulates the GEF activity of RABIN8 toward RAB8. In another study it was shown that RAB8, RAB5, and RAB23 have distinct functions in ciliary transport.[76] RAB8 is involved in the transport of Smo, EB1, and kim1, but only RAB5 plays a role in the trafficking of the apical-membrane-localized Kim1, whereas RAB23 is localized in the cilia and regulates ciliary entry of Smo. It was also shown that full length RABIN8 has a self-inhibitory sequence.[77] RAB11 binds to this region and activates RABIN8 toward RAB8. In its activated conformation, RABIN8 also interacts with Sec15, a subunit of the exocyst and downstream effector of RAB8. Expression of constitutively activated RAB8 promotes the association of Sec15 with RABIN8. Using immunofluorescence microscopy, it was shown that Sec15 colocalized with RAB8 along the primary cilium and that localization was necessary for ciliary functions. Hence, the RABIN8–RAB8–Sec15 interaction may couple the activation of RAB8 to the recruitment of the RAB8 effector and is involved in the regulation of vesicular trafficking for primary cilium formation.[77]

Small GTPases and motor proteins in vesicle trafficking

Primary cilia are built and maintained by IFT, whereby the two IFT complexes, IFT-A and IFT-B, carry cargo via kinesin and dynein motors for anterograde and retrograde transport, respectively.[78] The interactions between IFT motors and IFT complexes are essential for the transport of ciliary cargo.[79] IFT complexes likely function as adaptors that mediate interactions between anterograde/retrograde motors and ciliary cargoes, facilitating cargo transport between the base and tip of the cilium.[79]

The microtubule and actin network plays an important role in motor protein driven ciliary trafficking. Actin based myosin motors are usually involved in slower and short range local transport events.[80] Kinesin and dyneins mediate microtubules based long-range transport. Kinesin motors transport cargo toward the plus end of the microtubules and have a domain structure relatively similar to myosins. Kinesin motors consist of a heavy chain and a light chain.[81] There are two dynein motor isoforms, cytoplasmic dynein 1 and cytoplasmic dynein 2. Dyneins are minus end-directed motor proteins which mediate cargo transport toward the centrosome.[82] Dynein motors are huge multimeric complexes composed of two heavy chains and associate with intermediate, light intermediate, and several light chains. Motor domains are located in the heavy chain, whereas the accessory subunits are involved in the interaction with the cargo and regulatory proteins.[83]

Small GTPases play an important role in regulating the interaction of cargo with actin- and microtubule-based motor proteins.[84] ARL13 regulates the binding of IFT-A and IFT-B, while ARL3 acts antagonistically with ARL-13 to regulate IFT integrity and ciliogenesis.[68,85] Defects in *Arl13* mutants are rescued by depletion of ARL3 through an HDAC6-dependent pathway suggesting that ARL13 acts antagonistically with ARL3 in cilia formation.[68] Like ARL3, activation of HDAC6 was also found to promote cilia disassembly while loss of HDAC6 activity selectively stabilizes cilia in human epithelial cells.[86] Interestingly, HDAC6 was shown to interact with

BBIP10, a subunit of the BBSome that binds to both IFT-A and IFT-B, suggesting a potential functional crosstalk between IFT, ARL3, ARL13, and ARL6-BBSome in cilia.[87]

Recent studies have shown that a kinesin family member KIF13A binds to the active form of RAB11 to regulate endosomal sorting and recycling of endosomal cargo.[88] In photoreceptors, actin cytoskeleton at the base of the ciliary OS is involved in the targeted delivery and fusion of RAB8-positive vesicles. This is organized by a systematic and concerted process involving RAB8, Sec8 (part of the exocyst complex), actin motors, and the actin cytoskeleton.[89–91]

Working model of GTPase-regulated ciliary protein trafficking

As evident from the aforementioned discussion, there are significant parallels between different cell types for regulating ciliary biogenesis and membrane protein trafficking. The basic machinery for vesicle sorting, targeting, delivery, and fusion are shared among different cell types. Figure 1A depicts a brief illustration of the basic model of ciliary membrane protein trafficking. RAB GTPases and its effectors, the BBSome and IFT are involved in cargo vesicle targeting to the ciliary membrane as well as exit from the cilium. Post-Golgi cargo vesicles are coated with RAB8-GDP and trafficked to the base of the cilium. RABIN8, a GEF for RAB8A, trafficked independently by RAB11 activates RAB8 to RAB8-GTP, which results in the fusion of the cargo vesicles with the ciliary membrane. The BBSome complex is involved in the fusion as well as tethering of the cargo to the IFT for trafficking. This is mediated by the action of BBS3/ARL6. Figure 1B demonstrates the working model of the targeting of rhodopsin to the photoreceptor OS. First step in rhodopsin sorting involves the action of Arf4 at the TGN (Figure 1). These rhodopsin containing post-Golgi vesicles are called rhodopsin transport carriers. At the TGN, activated Arf4 interacts directly with the rhodopsin ciliary targeting signal VxPx.[92] Arf4 dependent budding of the vesicles are regulated by ASAP1, RAB11, and RAB11-Arf effector. ASAP1 selectively binds RAB11a and the RAB11-Arf effector FIP3. Following GTP hydrolysis and dissociation

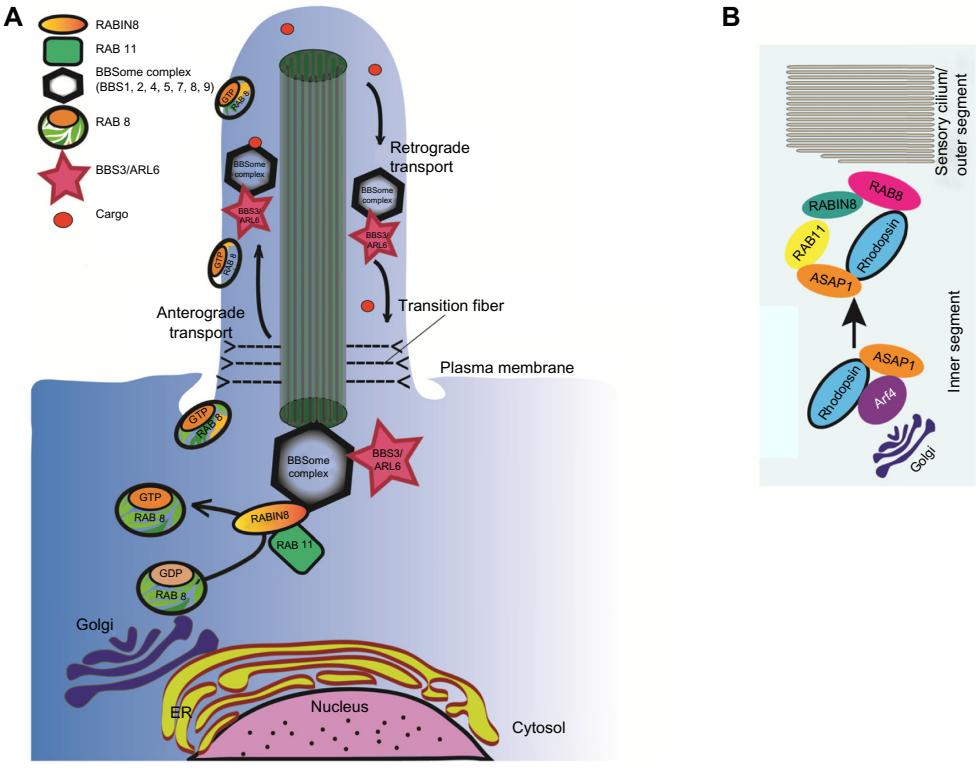

Figure 1 A working model of GTPase-regulated cellular trafficking.

Notes: (**A**) Proposed model of involvement of RAB GTPases in cargo transport to primary cilia. Cargo vesicles sorted at the Golgi are trafficked to the base of cilia in a RAB8 dependent manner. The RAB8-GEF RABIN8 activates RAB8 resulting in vesicle fusion and cargo delivery. This process is orchestrated by the BBSome complex and activity of small GTPase BBS3/ARL6. The cargo is trafficked in anterograde manner by IFT-B complex and returned to the base by retrograde IFT-A complex. Transition fibers at the base of the cilia act as a diffusion barrier to restrict entry of cargo inside the cilium. (**B**) A model of rhodopsin trafficking in photoreceptors. Rhodopsin transport carriers bud from Golgi in the presence of ARF4/ASAP1 complex. This complex is then loaded with RAB8 and its activators for delivery to the base of the OS and fusion with the apical inner segment membrane.

Abbreviations: ER, endoplasmic reticulum; OS; outer segment; GDP, guanosine diphosphate; GTP, guanosine triphosphate; IFT, intraflagellar transport.

of Arf4, ASAP1 and RAB11a remain associated at the TGN where they recruit RAB8 and its GEF RABIN8. RAB8 then regulates the final stages of polarized membrane trafficking at the cilium.[43] Some ciliary and centrosomal proteins involved in retinal degeneration, such as RPGR, CEP290, PCM-1, and OCRL bind to RAB8 and likely affect its localization and/or function in cilia assembly or maintenance.[93–95] Of these, RPGR is the only protein other than RABIN8, which possess a GEF activity toward RAB8.[93] The molecular mechanisms underlying such effects are under investigation.

Future directions

Owing to their biochemical activities, small GTPases are an attractive candidate for drug design to treat disorders associated with them. However, a roadblock in such studies is the ubiquitous and developmentally crucial role of these GTPases in organisms. Therefore, wide-range targeting of such GTPases is not relevant to devise therapeutic paradigms. Tissue and cell-type specific investigation of GTPases in cilia related functions and the involvement of compensating role of GTPases are critical to develop a detailed understanding of a context-dependent function of these proteins. Given that the load of ciliary function is different in distinct cell types depending upon their function, such investigations have a high probability of success and should provide novel information on their activity which can then be utilized to develop cell-type specific therapeutic intermediates.

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