Small GTPases 4:2, 70–77; April/May/June 2013; © 2013 Landes Bioscience

Crosstalk of Arf and Rab GTPases en route to cilia

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Keywords: Arf GTPases, Golgi, Rab GTPases, cilium, membrane trafficking

Abbreviations: TGN, Trans-Golgi Network

Small GTPases are versatile temporal and spatial regulators of virtually all cellular processes including signal transduction, cytoskeleton dynamics and membrane trafficking. They function as molecular switches, aided by a multitude of regulatory and effector proteins that link them into functional networks. A picture is beginning to emerge whereupon scaffold proteins with many functional domains perform the regulatory and effector functions, thus allowing the ordered recruitment and activation of small GTPases. This leads to the formation of scaffolding patches that coordinate cargo concentration and capture, with the recruitment and activation of the membrane tethering complexes and fusion regulators. This review will focus on the crosstalk of Arf and Rab GTPases at the Golgi complex and the scaffolds that facilitate their activation during trafficking of sensory receptors to primary cilia. The evolutionary conservation of the GTPase cascades in ciliogenesis and yeast budding will be discussed.

Introduction

Small GTPases of the Ras, Rho, Rab and Arf families form functional networks that coordinate their actions to control membrane outgrowth, cell shape and motility. The Golgi complex represents a nexus that promotes communication between different families of small GTPases.[1] This review will focus on the cross talk between Arf and Rab family GTPases that regulate membrane trafficking from the Golgi and the trans-Golgi network (TGN) to primary cilia, the specialized organelles that are present on nearly all eukaryotic cells. Primary (non-motile) cilia function as cellular antennas, capturing extracellular and intracellular signals through sensory receptors and signal transduction complexes that are specifically targeted to and highly concentrated in the ciliary membranes.[2]

The extreme case of ciliary receptor trafficking involves the light receptor rhodopsin, which is expressed exclusively in retinal rod photoreceptor cells. In these cells primary cilia elaborate the light sensing organelles, the rod outer segments that are filled with a large number of membranous disks containing rhodopsin and associated phototransduction machinery. Rhodopsin is a prototypic G-protein coupled receptor (GPCR) synthesized in the ER and processed in the Golgi complex, both of which are localized in the rod photoreceptor cell body, or the rod inner segment. Upon exiting the Golgi cisternae, rhodopsin reaches the TGN where it is targeted to the base of the cilium and the rod outer segment through direct interactions with a specific ciliary-targeting complex.[3] The ciliary targeting complex acts as an effector of the small GTP-binding protein Arf4, which directly interacts with the highly conserved rhodopsin C-terminal VxPx targeting motif, mutations in which cause Autosomal Dominant Retinitis Pigmentosa (ADRP) and blindness.[4,5] The VxPx motif that is recognized by Arf4 is also present and functional in other ciliary receptors, such as polycystins 1 and 2 and the cyclic nucleotide-gated channel CNGB1b subunit.[6-8]

The Arf4-based ciliary targeting complex includes three additional proteins: (1) the Arf GTPase activating protein (GAP) ASAP1,[9-11] (2) the small GTPase Rab11a, which is also a component of the Rab ciliogenesis cascade[12,13] and (3) the Rab11/Arf effector FIP3.[14,15] During budding of ciliary-targeted carriers from the TGN, the Arf GAP ASAP1 forms a tripartite complex with activated Arf4 and rhodopsin. ASAP1 recognizes the second ciliary targeting signal of rhodopsin, the FR motif, which lies within its cytoplasmic helix H8.[16] The FR motif is also conserved among several ciliary-targeted GPCRs.[17] The rhodopsin FR-AA mutant is defective in ASAP1 binding and does not localize to primary cilia.[16]

Model for the Assembly of Cilia Targeted Trafficking Complexes

The Arf4-regulated complex bridges the Arf and Rab GTPase pathways at the Golgi/TGN.[16] To better understand the communication between different families of small GTPases in the directed membrane transport to primary cilia, we began spatiotemporal modeling of the complexes that facilitate crosstalk between Arf and Rab GTPases. The ciliary cargo rhodopsin and the relevant GTPases have been crystallized alone, in complexes with their regulatory proteins, or with their effectors.[18-26] We used the available structural information to help generate a spatiotemporal model of signaling junctions at the Golgi/TGN that mediate communication between small GTPases during ciliary targeting. This model is presented in **Figure 1**.

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Submitted: 01/24/13; Revised: 03/18/13; Accepted: 03/21/13
http://dx.doi.org/10.4161/sgtp.24396

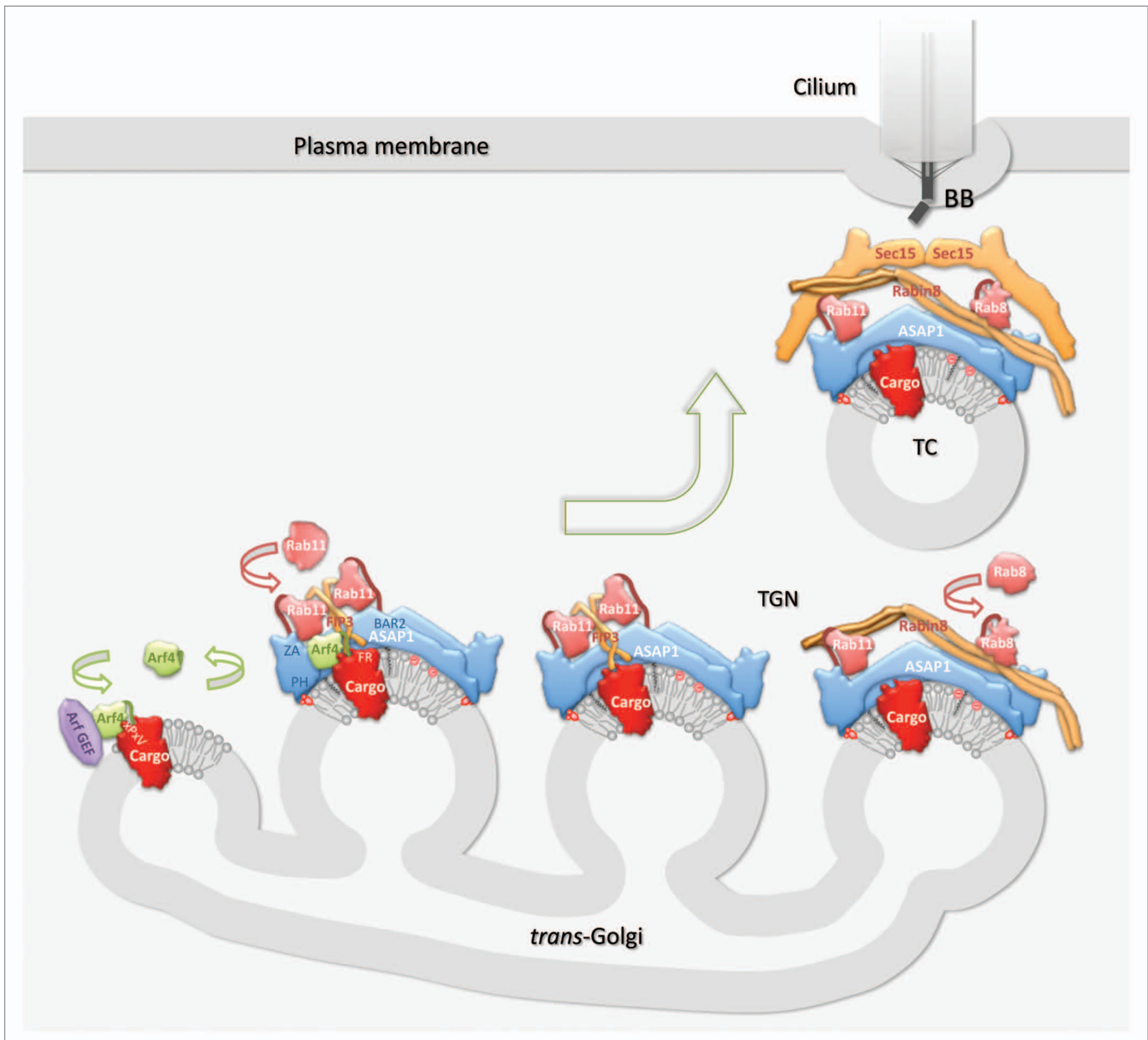


Figure 1. Model of the signaling junctions that mediate communication between Arf and Rab GTPases in Golgi/TGN-to-cilia trafficking. The model is based on the available data on protein-protein and protein-lipid interactions, as well as the crystal structures of rhodopsin and the small G-proteins that have been crystallized alone, or in complexes with their interacting proteins.[18-26] Crystal structures were superimposed and traced. Only the portions of interacting proteins for which the structures are known are included, except for the extension of Rabin8. Arf4 is modeled on the structure of Arf6,[25] Rab11 is from Eathiraj et al.[19] and Rab8 is based on Sec4 and Rab8.[20,83] ASAP1 is represented by its N-terminal half, also called the BAR-PZA domain, which encompasses the BAR, PH, GAP and ankyrin repeat domains (AA 1–724).[22,25,26,50] BAR-PZA is sufficient to control membrane tubulation and stimulate carrier budding in vitro.[3,28,53] FIP3 is represented by its C-terminal domain (AA 695–756),[19] which contains the Rab11 and Arf4 binding sites. The ASAP1 binding site is immediately N-terminal to this portion of FIP3.[29] Rabin8 is represented by the N-terminal residues 17–167 of the yeast homolog Sec2p.[20] The extension of Rabin8, which contains the Rab11 and Sec15 binding sites[37,65] is outlined in the model. Sec15 is as shown by Munson and Novick.[21] TC, transport carriers.

Our model predicts that during ciliary membrane trafficking scaffold proteins provide platforms for the activation and inactivation of small GTPases at the Golgi/TGN. Current data support the notion that Arf4 is activated at the Golgi by a specific GEF, likely GBF1.[27] At the Golgi/TGN, activated Arf4 binds the VxPx targeting signal of the ciliary cargo, such as rhodopsin.[3] ASAP1 is recruited to the TGN by a combination of activated Arf4, $PI(4,5)P_2$ and acidic phospholipids (indicated by red head groups in **Fig. 1**). There, ASAP1 recognizes the FR ciliary targeting motif of rhodopsin and forms a tripartite complex with rhodopsin and Arf4.[16] Because ASAP1 acts as a dimer,[28] it can potentially capture 2 molecules of cargo while simultaneously initiating membrane bending. Next, a complex is formed between Arf4, ASAP1, FIP3 and Rab11.[3] At this point the GAP activity of ASAP1 is

likely activated by FIP3[29] and possibly by other regulators localized at the TGN. The GTP hydrolysis promoted by ASAP1 inactivates Arf4[3] causing its dissociation from the TGN membranes.

At the TGN budding sites, Rab11a and ASAP1 bind and recruit the Rab8 nucleotide exchange factor Rabin8[30] and Rab8, a crucial regulator of ciliogenesis.[31-35] Rab11a associated with the ASAP1 dimer could potentially bring 4 molecules of Rabin8, which would activate four molecules of Rab8, thus enriching these membrane domains in GTPases that provide specificity for ciliary transport.

The conserved octameric membrane-tethering complex, the exocyst,[21,36] is likely recruited to the post-TGN transport carriers (**Fig. 1**, TCs). The Sec15 component of the exocyst recognizes Rab11a, Rabin8 and Rab8. In our model we extended the coiled-coil domain of Rabin8 to represent the region responsible for the binding to activated Rab11a and Sec15[37] (**Fig. 1**, the outlined extension of Rabin8). On transport carriers, the conformation of Rabin8 likely changes and its binding specificity for Sec15 increases.[38] It is possible that two molecules of Sec15 could be engaged simultaneously, one binding to Rab11a and/or Rabin8 and the other to activated Rab8, thus concentrating exocyst complexes around activation platforms to facilitate tethering of the ciliary transport carriers in preparation for fusion with the periciliary plasma membrane. Multiple lines of experimental evidence support this model, as detailed sequentially below.

Arf4

Arf4 is a crucial regulator of ciliary targeting that interacts with the cargo at the Golgi/TGN. Arf4 belongs to the Arf family of small GTP-binding proteins that includes Arf, Arf-like (Arl) and Sar proteins.[39] In a mechanism common to all GTP-binding proteins, the active state of Arfs is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Arf4 is likely activated by the GEF GBF1.[27] In their GTP-bound conformation Arfs are membrane-associated as a result of an N-terminal "myristoyl switch" that tightly couples activation and membrane binding. Arfs regulate membrane trafficking, lipid metabolism, organelle morphology and cytoskeleton dynamics in a highly coordinated fashion.[40-42] In membrane targeting, the downstream effectors of Arfs include the canonical coat complexes and protein adaptors.[39,43] Due to the low intrinsic GTPase activity of Arf proteins, Arf GAPs are essential for Arf inactivation and are often incorporated into protein coats.[44] The GAP for Arf4 is ASAP1, an essential regulator of ciliary targeting.[3]

The mammalian Arfs consist of six isoforms (Arf1-Arf6), and all but Arf6 are associated with the Golgi. Current experimental evidence points to the broad and partially redundant function for Arf1-Arf5.[45,46] Arf4 and several of the Arl proteins are also strongly implicated in specialized membrane transport, particularly in membrane targeting to primary cilia.[3,33,43] In addition to the already described Arf4-regulated ciliary targeting complex, another protein complex involved in ciliary targeting, named the BBSome, functions as an effector of Arl6 and its dysfunction causes the Bardet-Biedel Syndrome.[33,47] Arl3 and Arl13b are also involved in ciliogenesis and their impaired function is responsible for Retinitis Pigmentosa 2 (RP2) and Joubert syndrome.[48,49]

ASAP1

A common feature of several regulatory proteins involved in ciliary targeting are their scaffolding properties, owing to a multitude of functional domains involved in protein-protein or protein-lipid interactions. The centerpiece of the Arf4-based ciliary targeting complex is a multifunctional scaffold protein, the Arf GAP ASAP1.[3,16] The large size of ASAP1 (~130 kDa), its dimeric nature, ability to link Arf and Rab signaling at the TGN and multiple protein and lipid interactions that link Arf activation to complex assembly make it the first of several essential scaffolds in ciliary trafficking. ASAP1 regulates traffic to the plasma membrane as well as actin remodeling, likely through trafficking of actin regulators.[10,11] The GAP activity of ASAP1 on Arf4 is essential for the budding of ciliary-targeted post-TGN carriers, a process in which ASAP1 also functions as an Arf4 effector.[3] In addition to the Arf GAP domain, ASAP1 contains the N-terminal BAR (Bin/amphiphysin/Rvs)[50] domain, pleckstrin homology (PH), SH3 and a proline-rich domain, which provide binding modes for the proteins and phospholipids with which ASAP1 interacts. Membrane recruitment of ASAP1 is mediated by a coincidence detection of activated Arfs, acidic phospholipids and PI(4,5)P_2, which also regulates its GAP activity.[9,51] BAR domains, which recognize acidic phospholipids, are involved in sensing and/or inducing membrane curvature.[52] The BAR domain of ASAP1 mediates membrane tubulation and homodimerization and acts as an autoinhibitor of its GAP activity.[28,53] BAR domains have also been identified as the recognition site for the binding of small GTPases of the Rab, Arf and Rho families.[54-56] Interestingly, structural analysis has revealed a flexible linker between the BAR and PH domains of ASAP1,[53] suggesting that its BAR domain may change conformation and switch between lipid binding and binding to small GTPases and their regulators during ciliary targeting. The expression of mutant ASAP1 that prolongs Arf activation increases membrane recruitment of Rab11, suggesting its role in the activation of Rab11.[28] ASAP1 regulates multiple steps in membrane targeting through a ternary complex with Rab11a and the Arf/Rab11 effector FIP3.[3,29] ASAP1 may oligomerize with FIP3 to form a protein coat that regulates specialized TGN-to-plasma membrane trafficking.

FIP3

FIP3 is a member of the family of Arf- and Rab11-interacting proteins.[14,15] Like ASAP1, FIP3 also forms a homodimer and potentially acts as a scaffold protein. FIP3 forms a functional ternary complex with Rab11 and ASAP1 and can also simultaneously bind Rab11 and an Arf.[29,57] The affinity of FIP3 for Arf4 is low; however Rab11 and ASAP1 enhance their direct binding.[3] Thus, FIP3 is probably the last component recruited to the ciliary targeting complex already containing Arf4, ASAP1 and Rab11. FIP3 is ~82 kDa protein that can potentially link all other

components of the complex and may serve as a spatiotemporal regulator of GTP hydrolysis on Arf4. The C-terminal domain of FIP3 contains both the Arf and Rab11 binding sites.[19,58] N-terminal to these, FIP3 binds to the BAR domain of ASAP1, and as a result of this interaction the Arf GAP activity of ASAP1 is stimulated.[29] This may be pertinent to the FIP3 function within the ciliary targeting complex, where it may serve to elicit ASAP1 GAP activity on Arf4 during carrier budding from the TGN. The resulting inactivation of Arf4 promotes its dissociation from the membrane and the ciliary targeting complex, likely allowing conformational changes among the remaining components that further support growth of nascent, budding carriers.

Rab11-Rabin8-Rab8

Rab11, which is a component of the Arf4-based ciliary targeting complex, belongs to the larger family of Rab GTPases. Rab GTPases are specifically localized to different membrane domains where they cooperate to regulate intracellular membrane traffic through diverse mechanisms that include Rab GEF and Rab GAP cascades, common Rab effectors and positive feedback loops involving Rab interacting proteins.[59] In addition to Rab11, Rab8a also regulates ciliogenesis and controls the final stages of polarized membrane traffic, carrier fusion and lumenogenesis.[31-35,60] Rab11a is essential for the activation of Rab8 during ciliogenesis.[12,13] It has been established that Rab11a activates Rab8 through the Rab guanine nucleotide exchange factor (GEF) Rabin8,[30] in a cascade of molecular interactions known as the Rab11-Rabin8-Rab8 ciliogenesis cascade.[12,13,16,37] During initiation of ciliogenesis, Rabin8 relocalizes to Rab11-positive membrane carriers that are transported to centrosomes, at the base of the cilia.[13] Furthermore, the Rab11a-Rabin8 complex is already formed at the Golgi/TGN, concomitant with the formation of ciliary targeted post-TGN carriers.[16]

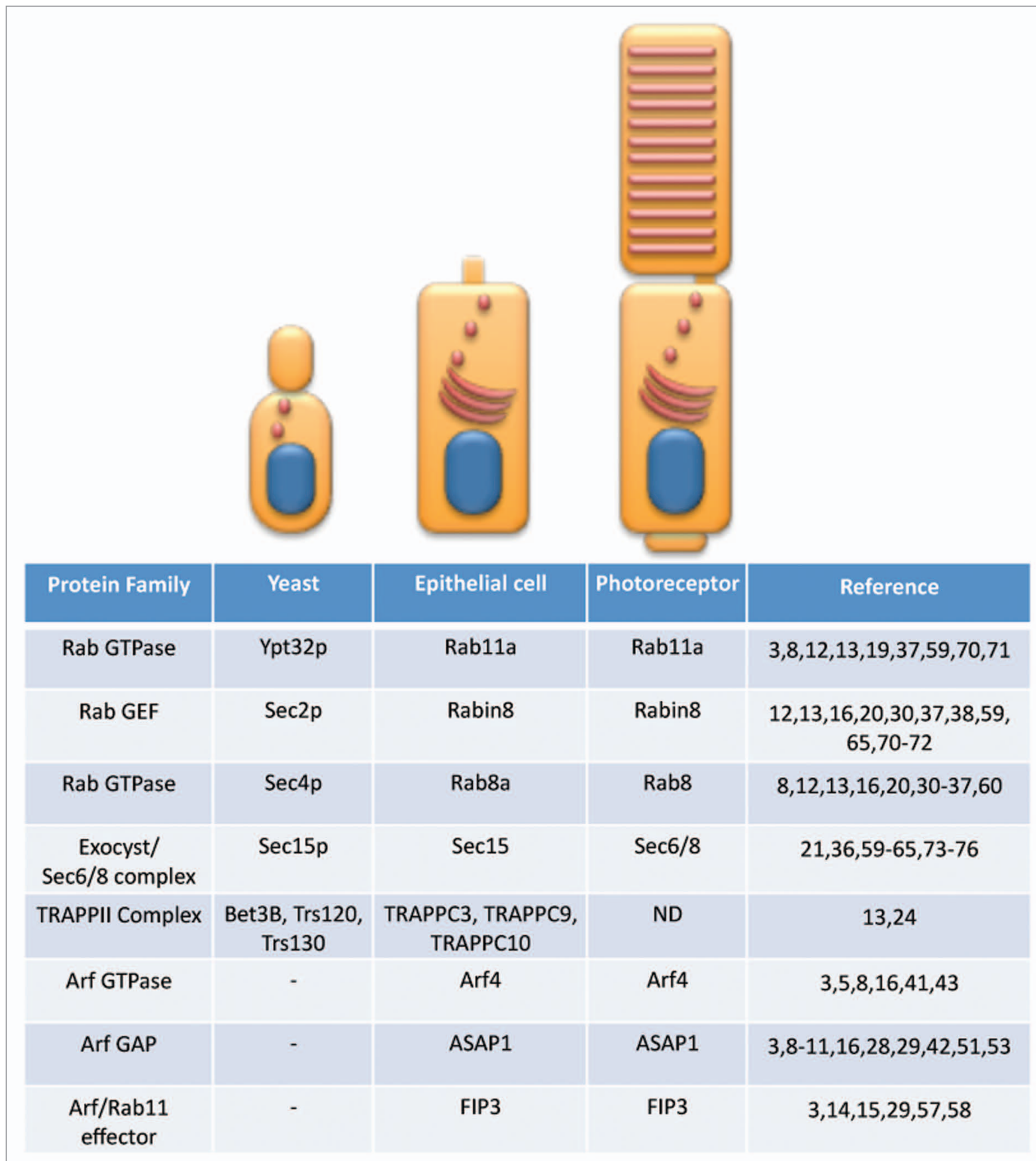
Rabin8 forms a physical bridge between Rab11a and Rab8. Surprisingly, Rabin8 binds ASAP1 directly and independently of Rab11, forming a complex with ASAP1 and the inactive (GDP-bound) form of Rab8.[16] Rabin8 probably forms a homodimer, by analogy to its yeast homolog Sec2p.[20] As Rabin8 is predominantly an extended coil-coil, it could associate with ASAP1 through the helical bundles of the BAR domain. Rabin8 also interacts directly with the Sec15 component of the exocyst (or Sec6/8 complex),[37] which tethers Rab8a-positive vesicles to the plasma membrane.[61-64] It likely binds to Rab11 and Sec15 sequentially, similar to Sec2p, which changes its binding affinities during the forward progression of membrane carriers.[37,65] It is possible that the reciprocal activation of Rab8 and Rab11 may also take place during the regulation of ciliogenesis because Rab11 can be activated by the Rab8 effector huntingtin.[66,67] Several sensory receptors directly bind Rab11a and/or Rab8a,[8,16,68] possibly increasing the fidelity of ciliary targeting, although the mechanism of recognition of the ciliary-targeted cargo by Rab GTPases is presently not clear.

Rabin8 is ~53 kDa protein that acts as a scaffold for the Rab11a-Rab8 succession. ASAP1 serves as a platform upon which this cascade functions to ensure the spatially restricted activation of Rab8 during ciliary targeting. The direct interactions between ASAP1 and Rabin8[16] could potentially allow them to remain associated independently of the regulatory GTPases. Thus, it is possible that scaffolding complexes persist and that activation/inactivation of small GTPases take place on these templates allowing them to communicate in a coordinated and cooperative manner that is also spatially and temporally restricted. Our data provide two pieces of evidence that the activation of Rab11 may follow its recruitment into the ciliary targeting complex and association with scaffold proteins. First, ASAP1 interacts and coimmunoprecipitates with either the GDP or GTP-bound forms of Rab11.[16] Second, Rab11 accumulates in the cytosol when the budding of post-TGN carriers is prevented by inhibitory antibodies, indicating that the activation and membrane attachment of Rab11 may be coupled with carrier budding.[3]

Our model predicts that the scaffold proteins that interact with Arf and Rab GTPases envelop the membranes in layers that start with ASAP1, which binds the cargo and the phospholipids directly, and culminate with Sec15, which later participates in tethering to the plasma membrane. As ASAP1 and Rabin8 persist on the periciliary carriers,[16] which likely subsequently acquire Sec15, it appears that the layers of scaffold proteins remain on the fusion competent carriers. Although we have omitted multiple or alternative complexes from our model for clarity, multiple types of interactions with small GTPases could clearly lead to the formation of scaffolding patches that can coordinate cargo concentration and capture at the TGN, with the recruitment and activation of the membrane tethering and fusion regulators such as Rab8 and the exocyst. This is in line with the membrane-organizing properties of Rab GTPases that are also conserved in the endocytic pathways.[69]

The Evolutionary Conservation of the GTPase Cascades

The evolutionary conservation of the Rab GTPase regulatory circuits is remarkable. The grouping of small GTPases and their interacting proteins presented in **Figure 2** reveals a notable succession of Rab GTPases and their regulators, which are used for two different but related functions: the Rab11-Rabin8-Rab8-Sec15 ciliogenesis cascade, which parallels the Ypt32p-Sec2p-Sec4p-Sec15p cascade that regulates the yeast budding pathway.[36,65,70-72] As already mentioned, the yeast counterpart of Rabin8 is Sec2p, a specific GEF for the Rab8 counterpart Sec4p.[20,65,70,71] Sec2p is cooperatively recruited to the Golgi by the Rab11 counterpart Ypt32p and phosphatidylinositol 4-phosphate (PI4P), which regulates the conformation and binding partners of Sec2p.[71] Although Sec2p is primarily associated with the secretory vesicles, its mutants that block secretion are localized in the cytoplasm.[72] Interestingly, Sec2p directly associates with the Sec4p effector, the exocyst, through the Sec15p subunit.[65] This Rab8 GEF-effector interaction network is also conserved in membrane trafficking to the cilium, as Sec15 interacts with Rabin8, co-localizes with activated Rab8 in the cilium and regulates ciliogenesis.[37,63] In addition to Rab8a, Rab11a also interacts with the exocyst in epithelial cells



Protein Family	Yeast	Epithelial cell	Photoreceptor	Reference
Rab GTPase	Ypt32p	Rab11a	Rab11a	3,8,12,13,19,37,59,70,71
Rab GEF	Sec2p	Rabin8	Rabin8	12,13,16,20,30,37,38,59, 65,70-72
Rab GTPase	Sec4p	Rab8a	Rab8	8,12,13,16,20,30-37,60
Exocyst/ Sec6/8 complex	Sec15p	Sec15	Sec6/8	21,36,59-65,73-76
TRAPPII Complex	Bet3B, Trs120, Trs130	TRAPPC3, TRAPPC9, TRAPPC10	ND	13,24
Arf GTPase	-	Arf4	Arf4	3,5,8,16,41,43
Arf GAP	-	ASAP1	ASAP1	3,8-11,16,28,29,42,51,53
Arf/Rab11 effector	-	FIP3	FIP3	3,14,15,29,57,58

Figure 2. The evolutionary conservation of the Arf and Rab GTPase regulatory circuits. Small GTPases, their regulators and effectors are grouped, revealing the high conservation of the Rab11a/Rabin8/Rab8a complex, the exocyst (Sec6/8 complex) and the TRAPPII complex. These ancient complexes are conserved from yeast to mammals. Arf4, ASAP1 and FIP3 are absent from yeast. However, they have conserved roles in ciliogenesis in epithelial cells and photoreceptors of higher eukaryotes. ND, not determined.

and photoreceptors, where the exocyst is localized at the base of the cilium.[60,73-76] This differs from the mode of action of yeast Ypt32p, which does not associate with the exocyst.

Like Sec2p, Rabin8 is also recruited to the membrane by the Ypt32p counterpart Rab11a and by specific phospholipids, although Rabin8 preferentially binds phosphatidylserine (PS) and phosphatidic acid (PA).[38] Interestingly, modifications that increase the TGN membrane content of PS and PA dramatically enhance budding of post-TGN carriers that are incompetent for membrane fusion, similar to those carrying mutant Rab8.[32,77] This phenotype could be due to the interference of PS and PA with the switch in binding specificity of Rabin8 from PS to Sec15, which is regulated by NDR2-mediated phosphorylation of Rabin8.[38] Mutation in NDR2 causes canine retinal degeneration,[78] indicating that the switch in binding partners of Rabin8 has a crucial role in ciliogenesis.

During ciliogenesis, Rabin8 interacts directly with the transport protein particle (TRAPP)II complex.[13] Mammalian TRAPPII is a conserved nonameric complex involved in intra-Golgi transport.[24] It has been reported that the TRAPPC3, 9 and 10 subunits of the TRAPPII complex are involved in ciliation through interactions with Rabin8, but it appears that they may act independently of the entire TRAPPII complex that is involved in intra-Golgi traffic.[13] Bet3B, a subunit of all 3 yeast TRAPP complexes, associates directly with a component of the COPII coat Sec23, which acts as a GAP for the Arf family GTPase Sar1.[79] A striking possibility is that TRAPPC3, a mammalian counterpart of Bet3B may directly interact with ASAP1. We have already indicated that some functions of ASAP1 resemble those of Sec23,[16] which controls the forward direction of vesicle traffic.[80] Furthermore, ASAP1 also fulfils similar functions as Sec24, which binds and captures transmembrane cargo.[81] Thus, the proofreading of cargo incorporation into membrane carriers may involve analogous molecular interactions performed either by individual molecules or by large scaffolds with multiple functional domains. ASAP1, Rabin8 and Rab11 could simultaneously interact with separate subunits of the large TRAPPII complex. The identity of the GEF for vertebrate Rab11 is unclear, but it might be the TRAPPC10 subunit of TRAPPII,[24] a homolog of the yeast Rab GEF Trs130, and ASAP1 may play a role in TRAPPII recruitment and/or activation. TRAPPII may additionally function in a positive feedback loop to activate Rab1 and its downstream effector GBF1, which is a GEF for Arf GTPases, including Arf4.[24,27,82]

Unlike the Rabs involved in ciliary trafficking, which are conserved from yeast to mammals, Arf4 is absent from yeast[43] and so are ASAP1 and FIP3. They arose later in evolution, probably reflecting a need for more complex cell organization. ASAP1 and FIP3 likely evolved through the accrual of many functional domains present in individual proteins that cooperatively function in membrane trafficking. In ciliated cells, Arf4 regulates the assembly of the specific complex containing ASAP1 and FIP3, which acts as a scaffold that links the ciliary-targeted post-TGN carriers to the evolutionary conserved Rab11a-Rabin8-Rab8 complex to ensure their proper delivery to the cilium.

Concluding Remarks

Interaction networks of small GTPases are typically shaped by their regulatory proteins, scaffolds and membrane tethers. The multifunctional scaffold proteins that provide interaction modules for the multitude of small G-proteins organize the signaling junctions allowing communication between Arf and Rab GTPases. In Golgi/TGN-to-cilia trafficking, these complexes include the conserved machinery involved in the selection, packaging and the delivery of ciliary cargo. Similar regulatory platforms likely form flexible hubs for cross-talk of small GTPases at multiple stages of trafficking to regulate the directional movement of membrane cargo. Our model should provide basis for further experiments that will scrutinize the existence of the transient complexes that are predicted here. Future investigation will reveal the level of conservation of molecular complexes that coordinate actions of small GTPases in homologous trafficking pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

I thank past and present lab members and collaborators for their valuable contributions and many colleagues for their stimulating discussions. Support was provided by the NIH grant EY-12421.

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