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ABSTRACT **Detailed structural, biochemical, cell biological, and genetic studies of any gene/protein are required to develop models of its actions in cells. Studying a protein family in the aggregate yields additional information, as one can include analyses of their coevolution, acquisition or loss of functionalities, structural pliability, and the emergence of shared or variations in molecular mechanisms. An even richer understanding of cell biology can be achieved through evaluating functionally linked protein families. In this review, we summarize current knowledge of *three* protein families: the ARF GTPases, the guanine nucleotide exchange factors (ARF GEFs) that activate them, and the GTPase-activating proteins (ARF GAPs) that have the ability to both propagate and terminate signaling. However, despite decades of scrutiny, our understanding of how these essential proteins function in cells remains fragmentary. We believe that the inherent complexity of ARF signaling and its regulation by GEFs and GAPs will require the concerted effort of many laboratories working together, ideally within a consortium to optimally pool information and resources. The collaborative study of these three functionally connected families (≥70 mammalian genes) will yield transformative insights into regulation of cell signaling.**

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Abbreviations used: ARFRP1, activated ARF-related protein 1; ARL, ARF-like; BRAG, Brefeldin A–resistant ARF GEF (now IQSec); EFA6, exchange factor for ARF6; ER, endoplasmic reticulum; ERES, ER exit sites; FA, focal adhesion; GAP, GTPase-activating protein; GEEC, glycophosphatidyl-inositol–enriched endosomal compartment; GEF, guanine nucleotide exchange factor; LECA, last eukaryotic common ancestor; PIP, phosphoinositide; PM, plasma membrane; PSD, pleckstrin homology and Sec7 domain; TBCD, tubulin cochaperone cofactor D; TGN, *trans*-Golgi network.

INTRODUCTION

Members of the family of regulatory GTPases that include ARFs, ARF-like (ARLs), and SARs have emerged as key regulators of cellular signaling involved in almost all aspects of cell biology (Tables 1–3, Figure 1, and Supplemental Tables I–III) (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011; Jackson and Bouvet, 2014). Their importance is underscored by findings showing that complete or conditional deletions or mutations result in embryonic lethality or organ-specific defects, with links to a variety of diseases (Table 4) (Seixas *et al.*, 2013; Zhang *et al.*, 2013). ARF family GTPases control key cellular processes, including bidirectional membrane trafficking (secretion and endocytosis), ciliogenesis, lipid metabolism, energy use, motility, division, apoptosis, and transcriptional regulation. Like all regulatory GTPases, ARF family GTPases operate

	GTPase	Localization	Function(s)	Interactors
1	Arf1	Cytosol, Golgi	Recruitment of coat complexes, activation of PLD, PI kinases	COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs
2	Arf3	Cytosol, Golgi	Recruitment of coat complexes, activation of PLD, PI kinases	COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs
3	Arf4	Cytosol, Golgi, endosomes		COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs
4	Arf5	Cytosol, Golgi, endosomes	Recruitment of coat complexes, activation of PLD, PI kinases	COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs
5	Arf6	PM, endosomes, RE, cortical actin	Cortical actin rearrangement, endocytosis, PLD activation	β-arrestin, POR1, PLD, Cytohesins, MKLP1, FilGAP
6	Arl1	Golgi, TGN	Endosome–Golgi secretory traffic, LD formation	Arfaptin, MKLP1, PDEd, HRG4, Golgins, GRIP-domain proteins
7	Arl2	Cytosol, mitochondria, centrosomes, basal bodies, cilia, RRs	Tubulin heterodimer assembly, mitochondrial fusion, Prenyl-protein traffic	TBCD/β-tubulin, TBCD, ELMOD1-3, BART/ARL2BP, PDEd, HRG4/UNC119
8	Arl3	Cytosol, centrosomes, cilia, mitotic spindle, midbody, Golgi	Cytokinesis, Prenyl- and Myr-protein traffic	PDE6δ, HRG4/UNC119, Golgins, ARL13B, BART/ARL2BP
9	Arl4a	Cytosol, nucleus, TGN, endosomes, PM	Endosome–Golgi traffic, actin remodeling, cell migration	ELMO, GCC185, Robo1, Cytohesin2
10	Arl4c	Cytosol, nucleus, PM	Cholesterol traffic, filopodia, cell migration, tumorigenesis	α-Tubulin, filamin-A, Cytohesin2
11	Arl4d	Cytosol, mitochondria, nucleus, PM, actin	Actin remodeling, neurite outgrowth	HP1, importin-α, Cytohesin2,
12	Arl5a	Nucleus	Endosome–Golgi traffic	HP1α, GARP, Ragulator
13	Arl5b	Nucleus	Endosome–Golgi traffic	HP1α, GARP, Ragulator
14	Arl5c			
15	Arl6	Cilia		BBSome, Sec61β
16	Arl8a	Lysosomes, phagolysosomes	Lysosomal traffic and fusion	SKIP-kinesin1b, HOPS complex
17	Arl8b	Lysosomes, phagolysosomes	Lysosomal traffic and fusion	SKIP-kinesin1b, HOPS complex
18	Arl9			
19	Arl10	Nuclei, mitochondria		
20	Arl11			p-ERK
21	Arl13a			
22	Arl13b	Cilia, EE, CDRs, centrosomes	Regulation of ciliary formation/maintenance, axoneme, Hh signaling, EEs	ARL3, INPP5E, PDE6δ, tubulin, FIP5, UBC9, MYH9
23	Arl14			
24	Arl15	Cytosol, Golgi	Genetic links to adiponectin levels and type 2 diabetes	ASAP2
25	Arl16	Cytosol, mitochondria, nucleus, cilia		RIG-I
26	Arfrp1	*trans*-Golgi	Recruitment of Arl1 and Golgin-97/245 to *trans*-Golgi	Sec7-1, Cytohesin
27	Sar1a	ER		
28	Sar1b	ER		
29	Trim23	Lysosomes, Golgi, autophagosomes	Ubiquitin ligase, viral infection, membrane trafficking	UBE2D2, TBK1, Cytohesin1

National Center for Biotechnology Information (NCBI) gene names are listed, along with cellular localization, identified functions, and protein interactors. Abbreviations used include CDR, circular dorsal ruffles; EE, early endosomes; PLD, phospholipase D; PM, plasma membrane; RE, recycling endosomes; RRs, rods and rings. Additional information is included in Supplemental Table I.

TABLE 1: Human ARF family GTPases.

	GEF	Localization	Function(s)	Interactors
1	Arfgef1/BIG1	TGN, sorting endosomes	Activation of Arf1/3, recruitment of AP1/AP3, myelination	Arl1, Arf1/3, ARF4/5
2	Arfgef2/BIG2	TGN, sorting endosomes	Activation of Arf1/3, recruitment of AP1/AP3	Arl1, Arf1/3, ARF4/5
3	Cyth1	PM	Cell adhesion/migration, integrin regulation	GRASP/tamalin, CNKSR1-3, CASP, Arl4A, Arl4D, Arf6
4	Cyth2/ARNO	PM, REs, Ruffles	Cell adhesion/migration, integrin regulation, actin remodeling, endosome traffic	GRASP/tamalin, CNKSR1-3, CASP, Arl4A, Arl4D, Arf6, paxillin, RLIP76, β-arrestin, pallidin
5	Cyth3/ARNO3/GRP1	PM, (Glut4-positive) endosomes	Glut4 exocytosis, cell migration	GRASP/tamalin, CNKSR1-3, CASP, Arl4A, Arl4D, Arf6
6	Cyth4			
7	GBF1	Golgi	Membrane traffic at *cis*-Golgi	p115, Rab1b, COG4, γ-COP, GGA1-3, ATLG, Gmh1
8	Iqsec1/BRAG2/GEP100	PM	Integrin endocytosis/cell adhesion regulation of AMPA receptor traffic	Calmodulin, MAP4K4, Arf5, Arf6
9	Iqsec2/BRAG1	PSDs	Regulation of AMPA receptor traffic	Calmodulin, PSD95, IRSp53, Arf6
10	Iqsec3	PSDs	Regulation of GABAergic synapse formation	Calmodulin, gephyrin, Arf6
11	Psd/EFA6	PM, tight junctions, axons, PSDs, endosomes	Tight junction formation, epithelial lumen formation	α-Actinin-1, 4, Arf6
12	Psd2/EFA6C			
13	Psd3/EFA6D			
14	Psd4/EFA6B	PM, epithelial tight junctions	Tight junction formation, epithelial lumen formation	α-Actinin-1, 4, Arf6
15	Fbox8			

NCBI gene names are listed, along with cellular localization, identified functions, and protein interactors. Abbreviations used include PM, plasma membrane; PSD, postsynaptic densities, RE, recycling endosomes; TGN, *trans*-Golgi network. Additional information is included in Supplemental Table II.

TABLE 2: Human ARF GEFs.

as "molecular switches" by interconverting between inactive (GDP-bound) and active (GTP-bound) conformations. Upon binding GTP, the activated GTPases alter their conformations, which increases their affinity for effectors and can alter their localization in cells, each of which contributes to the generation of a specific biological output. Activated (GTP-bound) ARF family GTPases propagate their effects through a specific redistribution of effectors (e.g., recruitment to a membrane), allosteric activation of effector enzymatic activity, conformational changes within the effector resulting in increased affinity for other cellular components (proteins, lipids, etc.), or a combination of such changes. As a consequence, the signal output of these GTPases is tightly controlled by the regulated binding of GTP and the half-life of the activated state. These are in turn controlled by the stimulation of the release of bound GDP (to allow GTP to bind spontaneously) by guanine nucleotide exchange factors (GEFs) and of their intrinsic GTPase activity by GTPase-activating proteins (GAPs) (Casanova, 2007; Inoue and Randazzo, 2007; Randazzo *et al.*, 2007; Bui *et al.*, 2009; Spang *et al.*, 2010; East and Kahn, 2011; Wright *et al.*, 2014; Vitali *et al.*, 2017). Thus, the triad of GEF–GTPase–GAP can be viewed as a minimal component in signaling pathways that alter a large fraction of cellular behaviors. Yet, despite their clear importance in cell biology and links to human pathologies, our understanding of the pathways involved and molecular mechanisms remain fragmentary. In this review, we briefly summarize the known roles of the ARF family GTPases, their GEFs and GAPs, their localization in cells, and their interactors. Rather than describing in detail any one of the many pathways in which they operate, we instead emphasize the extensive overlap in specificities and actions between family members, as this represents the largest challenge to achieving a deep understanding of their mechanisms of action. Because every pathway requires the minimum GEF–GTPase–GAP triad, we argue for a systematic approach to study each family and the three families together. We end our review by highlighting some key questions and challenges in ARF signaling, and hope that it inspires more collaborative efforts to address the large, complex, but vitally important area of ARF signaling.

ARF FAMILY GTPASES

Families of ARF GTPases and their cellular functions

Included within the ~30 members of the mammalian ARF family are the six "true ARFs" (humans lack ARF2, thus the discrepancy between this number and Table 1), the 21 ARF-like (ARL) proteins, two SARs, and Trim23 (Table 1; additional information included in Supplemental Table I) (Li *et al.*, 2004; Kahn *et al.*, 2006). The six mammalian ARFs are highly conserved, sharing > 65% sequence identity, and perform similar and/or overlapping functions. ARLs are more divergent, sharing typically 40–60% identity, and largely perform distinct cellular functions. The two mammalian SARs share ~90%

	GAP	Localization	Function(s)	Interactors
1	Arfgap1	Golgi	ER protein retrieval	γ-Adaptin (AP-1), KDEL receptor/ERD2, p24
2	Arfgap2	Golgi		
3	Arfgap3	TGN, EEs	EE–LE transport of M6PR and EGFR	γ-COP (COPI), GGA1/2
4	Acap1/CENTB1	Rab11 REs	Integrin and TfnR recycling	β1-Integrin, TfnR, clathrin heavy chain
5	Acap2/CENTB2	PM, phagocytic cup, ARF6 endosomes	Neurite outgrowth, FcγR-mediated phagocytosis	Rab35
6	Acap3/CENTB5		Neurite outgrowth, neuronal migration	
7	Adap1/CENTA1	Membrane ruffles, mitochondria, dendrites, synapse	*Salmonella* invasion, beta2-AR internalization, dendritic differentiation	Kif13b
8	Adap2/CENTA2			
9	Agap1	AP-3 endosomes	Endosome–lysosome transport	AP-3, Kif2A
10	Agap2/PIKE	FAs, Rab4/AP-1endosomes	Cell migration, neurite outgrowth, invasion, TfnR recycling	FAK, RACK1, Akt, Homer, AP-1
11	Agap3	Endosomes		
12	Agfg1/HRB, RIP	Clathrin/AP-2/EPS15 vesicles	TfnR endocytosis, HIV-1 replication	Rev
13	Agfg2			
14	Arap1	EEs, CDRs, podosomes	EGFR endocytosis, macropinocytosis, secretory lysosomes	CIN85, AP-3
15	Arap2	FAs, APPL EEs	FA turnover, SF formation, integrin endocytosis	RhoA, Arf6, APPL1
16	Arap3	Podosome-like adhesions	Cell migration, invasion, RhoGAP stimulation	Rap1, RhoGAP
17	Asap1	PM, FAs, podosomes/invadopodia, CDRs	Cell migration, invasion, SF formation, integrin and EGFR recycling	FAK, Crk, CrkL, Src, cortactin, NM2A, PRKD2, CIN85, CDAP
18	Asap2	Cell periphery, phagocytic cup	Cell migration, FcγR-mediated phagocytosis	Selenoprotein K
19	Asap3	PM, CDRs	Cell migration, integrin recycling, invasion	Grb2
20	Git1	FAs, SNX27 endosomes, REs, EEs	Cell migration, invasion, EGFR traffic/degradation	PIX, Arf6, paxillin, MEK1, FAK, SNX6
21	Git2	PM, FAs	Cell migration, invasion, beta2-Adrenergic R down-regulation	Vav2, paxillin, GRKs
22	Smap1	PM	TfnR endocytosis	Clathrin heavy chain
23	Smap2	EE, TGN	EE–TGN transport	Clathrin heavy chain, CALM
24	ELMOD1	Golgi, nuclear speckles, LDs		
25	ELMOD2	ER, mitochondria, LDs, centrosomes, RRs	Mitochondrial fusion	ARL2, other ARF family GTPases
26	ELMOD3	PM, actin, lagging edge		
27	RP2	PM, microtubules, nucleus	Ciliary traffic	ARL3, UNC119, G protein β1

NCBI gene names are listed, along with cellular localization, identified functions, and protein interactors. Abbreviations used include CDR, circular dorsal ruffles; EE, early endosomes; EGFR, epidermal growth factor receptor; FA, focal adhesions; LD, lipid droplets; LE, late endosomes; PM, plasma membrane; RE, recycling endosomes; RRs, rods and rings; SF, stress fibers. Additional information is included in Supplemental Table III.

TABLE 3: Human ARF GAPs.

primary sequence identity (but < 30% to any other family member), and have a specialized role in traffic from the endoplasmic reticulum (ER) to the Golgi. The ARF family GTPases are distinct from the other families of small, regulatory GTPases (RAS, RHO, RAB) in having an N-terminal extension of ~14 amino acids and covalent modifications at or near this end. All six ARFs are N-myristoylated, while ARLs are

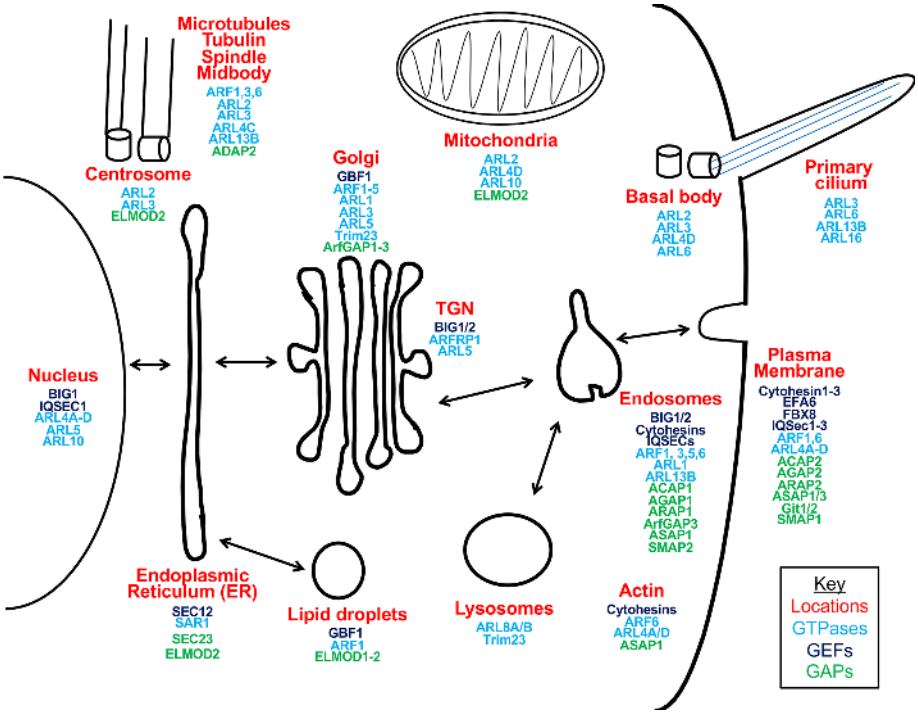

FIGURE 1: Subcellular localization of the ARF family GTPases, ARF GEFs, and ARF GAPs. A schematic cell with organelles (in red) showing the localization of the GTPases (in light blue), GEFs (in purple), and GAPs (in green). More detailed information for these localizations is provided in references cited in the text.

myristoylated (e.g., ARL1), palmitoylated (e.g., ARL13B), or acetylated (e.g., ARFRP1), with each modification critical for activity. In this section, we summarize briefly the actions of the different ARF GTPases. This should not be taken as an exhaustive description of their actions, and we apologize to the many researchers whose work is not included in the interest of space.

The ARFs are best known for their roles in recruitment of coat proteins/complexes and initiation of vesicle formation in membrane trafficking, particularly at the Golgi. However, a brief glance at Figure 1, which depicts localizations for all ARF family members, as well as the known GEFs and GAPs, reveals far more complexity in locations and presumed functions. ARFs are found not only in all regions of the Golgi, but also at the plasma membrane (PM), endosomes, lipid droplets, and midbodies. ARFs often show overlapping distribution (e.g., ARF1, 4, and 5 localize to the *cis*-Golgi), and one ARF can localize to multiple sites (e.g., ARF1 can be recruited to the Golgi, lipid droplets, and the PM; however, it is likely that a large cytosolic pool remains). ARFs have both redundant and distinct functions, as seen in studies showing that small interfering RNA–mediated knockdown of any one ARF1/3/4/5 yielded no obvious phenotypes, while each double knockdown of distinct pairs of these GTPases resulted in specific phenotypes (Volpicelli-Daley *et al.*, 2005). ARFs affect cellular processes by recruiting effectors. The term "effector" is used herein to indicate a protein that binds preferentially to the activated (GTP-bound) form, resulting in a signal or output that changes some aspect of cell biology. There are more than 20 known ARF effectors and most of these are essential components in membrane traffic in all cells (Table 1). The best-characterized ARF effectors are adaptors for coat proteins or the coat proteins themselves, including both monomeric (e.g., GGAs or MINTs) (Boman *et al.*, 2000; Dell'Angelica *et al.*, 2000; Hill *et al.*, 2003) and oligomeric complexes (e.g., COPI, AP-1, AP-3, and AP-4) (Serafini *et al.*, 1991; Stamnes and Rothman, 1993; Traub *et al.*, 1993; West *et al.*, 1997; Ooi *et al.*, 1998; Hirst *et al.*, 1999; Drake *et al.*, 2000; Donaldson and Jackson, 2011). ARFs also recruit non-coat proteins to membranes (e.g., golgin-160, GCC88) (Derby *et al.*, 2004; Gilbert *et al.*, 2018) and can activate lipid-modifying enzymes (e.g., phospholipase D, PI(4)5-kinase) (Brown *et al.*, 1993; Cockcroft *et al.*, 1994; Honda *et al.*, 1999; Jones *et al.*, 2000) and/or lipid transporters (e.g., FAPP1) (Godi *et al.*, 2004). ARF1, ARF3, and ARF6 are implicated in cell division and/or cytokinesis, but the effectors involved remain to be identified (Altan-Bonnet *et al.*, 2003; Hanai *et al.*, 2016; Nakayama, 2016). ARF6 appears to act predominantly in the cell periphery, where it regulates both endosomal recycling and cortical actin dynamics, which are also linked to RAC GTPase signaling (Donaldson, 2003; Schweitzer *et al.*, 2011).

ARLs localize to and have functions in processes involving tubulin/microtubules at centrosomes, spindles, midbodies, basal bodies, and cilia (Figure 1). ARLs are much more divergent in action, with some ARLs functioning in parallel with those of the ARFs in membrane traffic, while others regulate completely different processes. For example, ARL1 is most closely related to ARFs in primary sequence, localizes to the *trans*-Golgi network (TGN), and acts in membrane trafficking through its ability to recruit proteins to that site (Yu and Lee, 2017). At the TGN, ARL1 effectors include BIG1 (an ARF GEF), Golgin-97, and Golgi-245. ARL1 also localizes to sorting endosomes, where it recruits BIG1 to activate ARF1 and ARF3 (D'Souza *et al.*, 2014). In yeast, GTP-bound ARL1 is located at the Golgi complex and facilitates the exit of vesicles from the TGN (Munro, 2005). In mammalian cells, ARL1 is involved in cell polarity (Lock *et al.*, 2005); innate immunity (Murray and Stow, 2014); and the secretion of insulin (Gehart *et al.*, 2012), chromogranin A (Cruz-Garcia *et al.*, 2013), and matrix metalloproteinases (Eiseler *et al.*, 2016). In marked contrast, ARL2 localizes to multiple cellular sites to perform surprisingly distinctive functions. ARL2 is found in 1) cytosol, as a 1:1:1 trimer with the tubulin cochaperone cofactor D (TBCD) and β-tubulin, and is required for αβ-tubulin biogenesis (Francis *et al.*, 2017a,b); 2) the mitochondrial intermembrane space, where it promotes mitochondrial fusion (Newman *et al.*, 2014, 2017); 3) at centrosomes (Zhou *et al.*, 2006); 4) in the nucleus; and 5) at rods and rings, which are implicated in guanine nucleotide metabolism (Schiavon *et al.*, 2018). Despite its involvement in such disparate activities, ARL2 is ubiquitously expressed in eukaryotes and has not duplicated into paralogues that might allow separation of functions (Francis *et al.*, 2016). One intriguing hypothesis born from these observations is that the use of a shared cell regulator at multiple locations might serve as a means of communication between those sites, a phenomenon termed "higher-order signaling" (Francis *et al.*, 2016).

ARL2 and ARL3 display both overlapping and distinct actions and interactions (Van Valkenburgh *et al.*, 2001; Zhou *et al.*, 2006). Both are linked to microtubules, localize to centrosomes, and share the ability to bind PDEδ in a GTP-dependent manner and promote

GTPase	Conventional knockout	Conditional knockout	Reference
ARF1	Embryonically lethal (E5.5)	—	Hayakawa *et al.* (2014)
ARF4		*Postnatal deletion (Arf4^{flox}/CagCreER)*: Reduced viability; reduced size of the pancreas; yellowish feces in lower intestine; hair turned from black to gray	Pearring *et al.* (2017)
		Photoreceptor cells (Arf4^{flox}/iCre75): Normal rhodopsin localization; no retinal degeneration	Pearring *et al.* (2017)
		Kidney (Arf4^{flox}/HoxB7Cre): No cystic disease	Pearring *et al.* (2017)
		Pancreas (Arf4^{flox}/CagCreER): Degeneration of exocrine pancreas; infiltration of adipocytes in exocrine pancreas; normal islet size and organization	Pearring *et al.* (2017)
ARF6	Embryonically lethal (midgestation); smaller liver with progressive apoptosis; defective hepatic cord formation	—	Suzuki *et al.* (2006)
ARF6		*Endothelial cells (Arf6^{flox}/Tie2-Cre)*: Reduced tumor angiogenesis via impaired HGF-induced endothelial β1-integrin recycling	Hongu *et al.* (2015)
		Neuronal cells (Arf6^{flox}/Nestin-Cre): Smaller size of fimbria of hippocampus and corpus callosum; impaired oligodendrocyte myelination in the hippocampal fimbria and the corpus callosum during development, due to reduced secretion of fibroblast growth factor-2	Akiyama *et al.* (2014)
		Platelets (Arf6^{flox}/PF4-Cre): Impaired αIIbβ3-integrin trafficking resulting in reduced fibrinogen uptake and storage	Huang *et al.* (2016)
		Podocyte specific (Arf6^{flox}-Nphs2-Cre): Normal kidney development In model of acute podocyte effacement: protection from podocyte effacement In model for immune complex–mediated injury: aggravated proteinuria	Lin *et al.* (2017)
ARL3	Early death (3 wk of age); abnormal development of renal, hepatic, and pancreatic epithelial tubule structures; abnormal epithelial cell proliferation and cyst formation; photoreceptor degeneration (at P14)	—	Schrick *et al.* (2006)
		Retina specific (Arl3^{flox}/Six3-Cre): Impaired ciliogenesis; no formation of connecting cilia and outer segments; degeneration of retina at 2 mo; inability of retina to respond to light rapidly	Hanke-Gogokhia *et al.* (2016)
		Rod photoreceptor specific (Arl3^{flox}/iCre75): Photopic responses started to decline at the age of 1 mo; degeneration of rods and cones at 2 mo; decline of retinal thickness Trafficking deficiencies of lipidated phototransduction proteins, e.g., farnesylated rhodopsin kinase (GRK1)	Hanke-Gogokhia *et al.* (2016)

TABLE 4: Phenotypes of mice with mutations/deletions of ARF family GTPases.

Continues

GTPase	Conventional knockout	Conditional knockout	Reference
ARL4	Reduction of testis weight (30%) and sperm count (60%) without affecting fertility	—	Schurmann *et al.* (2002)
ARL6 (BBS3)	Development of BBS-associated phenotypes: retinal degeneration, male infertility, loss of sperm flagella, severe hydrocephalus, thinning of the cerebral cortex; reduced size of hippocampus and corpus striatum, reduced number and misshaping of ependymal cell cilia, increased body fat	—	Zhang *et al.* (2011)
ARL13B (a GEF of ARL3)	Hennin (hnn) mutation (ENU-induced mutation) corresponding to Arl3 null allele: Embryonically lethal; at ED 9.5, open neural tube in the head, caudal spinal cord, and randomized heart looping; at ED 14, abnormal eyes and axial polydactyly Nodal cilia half the normal length; abnormal structure of the axoneme Specific disruption of the Sonic hedgehog (Shh) signaling pathway		Caspary *et al.* (2007)
		Kidney specific (Arl13B^{flox}-Ksp-Cre): Defective cilia biogenesis and rapid kidney cyst formation due to an overproliferation followed by fibrosis; increased kidney-to-body weight ratio; mutant mice dead at around P60	Li *et al.* (2016)
		Retina specific (Arl13B^{flox}/Six3-Cre): Absence of outer segments of the retina (starting at P6); photoreceptor rhodopsin: failure to form mature transition zones and outer segments and rapid degeneration; normal docking of basal bodies of photoreceptors to cell membranes	Hanke-Gogokhia *et al.* (2017)
		Tamoxifen-inducible Cre/loxP recombination (Arl13B^{flox}-CAG-CreER) at 1 mo of age: Destabilization of axonemes and transition zones, leading to progressive photoreceptor degeneration; impairment of anterograde intraflagellar transport (IFT) due to marked reduction of IFT88 protein at basal bodies; impaired retinogenesis, including early postnatal proliferation of retinal progenitor cells, development of photoreceptor cilia, and morphogenesis of photoreceptor outer segment; mislocalization of rhodopsin, prenylated phosphodiesterase-6 (PDE6), and IFT88	Hanke-Gogokhia *et al.* (2017); Dilan *et al.* (2018)
		Tamoxifen-inducible Cre/loxP recombination (Arl13B^{flox}-CAGG-CreER) at postnatal day 4: Mutant mice smaller than the control littermates; ~two-thirds dead from cystic kidneys between P27 and P51 Normal cerebellar size and foliation	Bay *et al.* (2018)
ARFRP1	Embryonically lethal; apoptotic epiblast cells within ectoderm at ED 6.0 and 7.0 Mistargeting of E-cadherin to intracellular compartments		Mueller *et al.* (2002); Zahn *et al.* (2008)

TABLE 4: Phenotypes of mice with mutations/deletions of ARF family GTPases.

Continues

GTPase	Conventional knockout	Conditional knockout	Reference
		Intestine specific (Arfrp1^{flox}/villin-Cre): Lower abundance of E-cadherin at the lateral membrane of the cell surface of crypts and villi (E-cadherin is associated with intracellular membranes); marked growth retardation due to impaired lipid uptake; impaired chylomicron lipidation and reduced release of ApoA-I	Zahn *et al.* (2008); Jaschke *et al.* (2012)
		Liver specific (Arfrp1^{flox}/alb-Cre): Early growth retardation due to reduced secretion of hepatic insulin-like growth factor 1 (IGF1); decreased glucose transport and glycogen storage; intracellular retention of glucose transporter GLUT2 Impaired VLDL lipidation resulting in reduced plasma triglyceride levels in the fasted state	Hesse *et al.* (2012)
		Adipocyte specific (Arfrp1^{flox}/ap2-Cre): Nearly abolished triglyceride storage in adipocytes, smaller lipid droplets, impaired lipid droplet fusion, and enhanced lipolysis Impaired sorting of the glucose transporter GLUT4 to intracellular storage compartment	Hommel *et al.* (2010); Hesse *et al.* (2010)
		Inducible adipocyte specific Tamoxifen-inducible Cre/loxP recombination Arfrp1^{flox}/CreERT2): Impaired secretion of adiponectin and recycling of insulin receptor; decreased insulin signaling in adipose tissue and liver.	Rodiger *et al.* (2018)

Indicated are the ARF and ARL proteins deleted either as whole-body knockout (conventional knockout) or in a cell-type or tissue-specific manner, including inducible deletions (conditional knockout). E, embryonic day; ENU, *N*-ethyl-*N*-nitrosourea; HGF, hepatocyte growth factor; P, postnatal day; VLDL, very low density lipoprotein.

TABLE 4: Phenotypes of mice with mutations/deletions of ARF family GTPases. Continued

the release of isoprenylated cargoes (Van Valkenburgh *et al.*, 2001; Ismail *et al.*, 2011). While they both also bind HRG4, only ARL3 binding results in the release of N-myristoylated cargoes from this carrier (Ismail *et al.*, 2012). ARL2 is involved in tubulin heterodimer biogenesis, while ARL3 functions in cytokinesis (Zhou *et al.*, 2006). ARL3 also appears to act at the Golgi, though how such action may be integrated with its other roles remains unclear (Zhou *et al.*, 2006). ARL3 is essential in photoreceptor cells that employ an elaborate variation of cilia in outer segments (Panic *et al.*, 2003; Zhou *et al.*, 2006; Hanke-Gogokhia *et al.*, 2018) (Table 4). An unexpected finding is that ARL3 is activated by ARL13B (Gotthardt *et al.*, 2015; Ivanova *et al.*, 2017), which raises the possibility that other ARLs may also serve as ARL GEFs, particularly as so few ARL GEFs have been identified to date.

Primary cilia are a major site of action for four different ARLs: ARL3, 6, 13B, and 16 (Figure 1). Each of these GTPases plays a critical role(s) in ciliary biology, with details and mechanisms still under investigation. Their activity is particularly important during development (Zhang *et al.*, 2013), as mutations in these genes cause ciliopathies and developmental disorders in mammals (Chiang *et al.*, 2004; Caspary *et al.*, 2007; Wiens *et al.*, 2010; Liew *et al.*, 2014; Alkanderi *et al.*, 2018) (Table 4). Although best known for its role in cilia and in Hedgehog signaling, ARL13B also functions outside the cilium by interacting with a kinesin to facilitate axon guidance (Higginbotham *et al.*, 2012; Casalou *et al.*, 2014). ARL13B is also distinctive, as it is nearly twice the size of other GTPases in the ARF family, having a C-terminal domain as large as the GTPase domain. ARL6 also goes by the name BBS3, as its mutation is linked to Bardet-Biedl syndrome and defects in Wnt signaling (Chiang *et al.*, 2004; Wiens *et al.*, 2010; Zhang *et al.*, 2011). ARL6 acts in ciliary trafficking through recruitment of the BBSome complex (Jin and Nachury, 2009; Jin *et al.*, 2010; Ye *et al.*, 2018).

The theme of ARF GTPases sharing a similar function is evident in the recruitment of Golgins to the Golgi. As mentioned earlier, ARFs recruit Golgin-160 and GCC88 to the Golgi, while ARL1 recruits Golgin-97 and Golgin-245 (Derby *et al.*, 2004). This is just one of several examples in which ARLs and ARFs act in a common pathway. However, ARL4A, which performs a similar function by interacting with GCC185 at the TGN to modulate the integrity of the Golgi, also has a completely distinct function in endosome-to-Golgi transport (Lin *et al.*, 2011). ARL4A also plays a role in actin cytoskeleton rearrangement involving ELMO/DOCK180-induced RAC signaling (Patel *et al.*, 2011). ARL4C and ARL4D modulate actin remodeling and cell migration through their interacting partners, filamin-A and Cytohesin 2, respectively (Li *et al.*, 2007; Chiang *et al.*, 2017). Expression of ARL4C in normal epithelial cells promotes migration and proliferation, indicating a role in epithelial morphogenesis (Matsumoto *et al.*, 2014). Each ARL4 paralogue can recruit the ARF GEF Cytohesins to the PM (Hofmann *et al.*, 2007; Li *et al.*, 2007). ARL5 localizes to the Golgi and influences endosome–Golgi traffic through interactions with the GARP complex (Rosa-Ferreira *et al.*, 2015), while ARL8 also acts in vesicular trafficking, predominantly at lysosomes, where it can influence the motility of this organelle (Khatter *et al.*, 2015). With the exceptions of ARL2 and ARL3, which act in cytosol to affect the assembly and dynamics of microtubules and centrosomes, almost all ARFs and ARLs act at membranes. (Though nothing is currently known about the locations and functions of ARLs 9, 11, 14, or 15.)

Activated ARF-related protein 1 (ARFRP1) also localizes to the TGN and has been implicated in vesicular trafficking of vesicular stomatitis virus G protein (Shin *et al.*, 2005; Nishimoto-Morita *et al.*, 2009), glucose transporters (Hesse *et al.*, 2012), and other PM

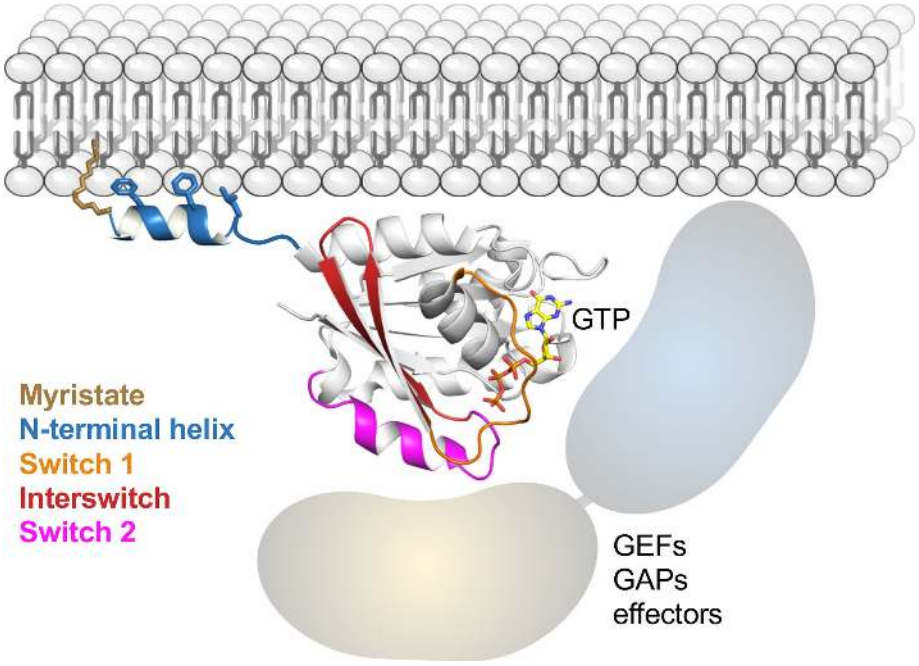

FIGURE 2: Structural determinants of ARF association with membranes and interactors. ARFs have four regions that change conformation between GDP- and GTP-bound forms: the canonical switch 1 (in orange) and switch 2 (in magenta) that directly sense the nature of the bound nucleotide; the myristoylated N-terminal helix (in blue), which is autoinhibitory in ARF-GDP and binds the membrane in ARF-GTP; and the interswitch (in red) that functions as a push button to ensure allosteric communication between the membrane- and the nucleotide-binding sites. GEFs, GAPs, and effectors generally bind to switch 1, switch 2, and/or the interswitch by one domain (in light yellow) and carry other domains that bind to the membrane (in light blue). The membrane bilayer is denoted in gray.

proteins. ARL1 and its effectors Golgin-97 and Golgin-245 are recruited to the TGN by ARFRP1 (Setty *et al.*, 2003; Zahn *et al.*, 2006). In addition to acting in membrane trafficking, ARFRP1 also plays roles in regulating metabolism, especially lipid and fat storage. Knockout of *Arfrp1* in different mouse tissues causes severe metabolic defects (Hommel *et al.*, 2010; Jaschke *et al.*, 2012; Rodiger *et al.*, 2018) (Table 4). Despite their similarities to ARFs and ARL1, neither GEFs nor GAPs for ARFRP1 have been identified, making descriptions of pathways challenging.

Two members of the ARF family are much larger than the 20- to 25-kDa norm, ARL13B with 428 residues and Trim23/ARD1 with 574 residues (Supplemental Table I). Homologues of the C-terminal domain of ARL13B are not found in other proteins, and we have little information on its function, save the presence of a VXPX ciliary localization motif. Trim23 is a multidomain protein having a RING finger, two B-boxes, and a coiled-coil motif (thus a tripartite motif [TRIM]) (Vichi *et al.*, 2005). These domains have polyubiquitination activity and act in the antiviral defense system and adipocyte differentiation (Arimoto *et al.*, 2010; Watanabe *et al.*, 2015; Sparrer *et al.*, 2017).

The functions and mechanisms of SARs are well characterized. SAR1 is activated by the Sec12 GEF, leading to its recruitment to the site of protein export from the ER, ERES (ER exit sites) (Figure 1). Activated SAR1 recruits the Sec23/24 complex and later the Sec13/31 complex to form the COPII coat necessary to generate COPII vesicles. The Sec13/31 complex promotes the GAP activity of Sec23 to "inactivate" SAR that serves to recycle all components. Sec12 and Sec23 are an atypical GEF and GAP pair as they lack a canonical GEF or GAP domain, and thus this regulatory system is of limited use in modeling mechanisms of the ARF/ARL GEFs and GAPs. Consequently, SARs are omitted from further discussion in this review.

Structural insight into the actions of ARF GTPases

While all ARF family members share the canonical G domain with nucleotide-sensitive switch 1 and 2 loops (Amor *et al.*, 1994), they display structural signatures that strikingly distinguish them from other small GTPase families (Figure 2) (Pasqualato *et al.*, 2002). The hallmark of members of the ARF family is an allosteric structural feature, which allows their nucleotide-binding site to communicate with regions located on the other side of the GTPase. It is based on an interswitch region (as it connects switch 1 and switch 2), that toggles like a push button between the inactive and the active conformations (Yu *et al.*, 2012). The ability of the interswitch to toggle is encoded in a conserved sequence signature at the beginning of the switch 2 (Pasqualato *et al.*, 2002). In toggling between these two positions, the interswitch simultaneously modifies the conformation of both the nucleotide-binding site and the other side of the protein. In ARF and related GTPases, the rearrangement of the interswitch is coupled to a variable N-terminal extension, which is autoinhibitory in the GDP-bound form and swings out to facilitate activation. In ARF GTPases, this region is a myristoylated amphipathic helix that interacts with membranes through the myristoyl group and the neighboring residues (Antonny *et al.*, 1997; Liu *et al.*, 2009, 2010). This is a prerequisite for their activation by GTP, thus coupling the activation of the GTPase to its interaction with the membrane bilayer (see also the *ARF GEFs* section).

Because of these major differences compared with classical RAS-like GTPases, caution is needed when using mutants and fusions to manipulate the activation state of ARF GTPases. The glutamine at the beginning of switch 2 is generally critical for GAP-stimulated GTP hydrolysis (Cherfils and Zeghouf, 2013), and this is also the case for ARF GTPases (e.g., Q71 in ARF1 or ARL1, Q70 in ARL2) (Zhang *et al.*, 1994; Van Valkenburgh *et al.*, 2001). However, another classical mutation, a P-loop serine/threonine to asparagine substitution, which gives rise to a dominant-negative version in many small GTPases by reducing their affinity for guanine nucleotide and titrating their GEFs, may not function the same in all ARF GTPases (Macia *et al.*, 2004). Alternatively, mutation of another threonine, located in switch 1, trapped ARF6 in a GDP-bound form, a mutation that could in principle also function in related ARF GTPases, many of which share the same structural feature. Another important aspect is that, given the regulatory role of the N-terminus and the need for lipid modifications, ARFs should be tagged only at the C-terminus, and even then only with caution (Jian *et al.*, 2010).

ARFs function by binding effectors, and structural studies of many ARF/ARL–effector complexes show that ARFs have similar conformations in all complexes and bind most effectors in the

same area centered on an invariant triad of aromatic residues in the switch/interswitch regions (Khan and Menetrey, 2013). In contrast, the *effectors* bind ARF-GTP through binding sites that are distinct in primary, secondary, ternary, and quaternary structures (Cherfils, 2014). Thus, the solved structures of known ARF–effector complexes do not inform on structural determinants that could be used to predict the binding of other effectors to ARF/ARLs. Interestingly, at least one ARF effector (i.e., coatamer) enhances the GTP hydrolytic activity of an ARF GAP, suggesting the formation of a ternary ARF-effector-GAP complex. Supporting evidence for formation of such a ternary complex is provided by a composite structural model for the ARF1/coatamer/ArfGAP1 complex (Yu *et al.*, 2012) and by the recent cryoelectron microscopy study of ARF1/COP1/ArfGAP complex reconstituted on a lipid vesicle (Dodonova *et al.*, 2017). A common feature of ARF–effector interactions is that they are predicted to position effectors in precise orientations in apposition to the membrane (DiNitto *et al.*, 2007; Liu *et al.*, 2009, 2010; Cherfils, 2014). This "solid phasing" will impart orientation constraints for effector interactions that are important to produce signals. As a consequence, biochemical assays used to determine affinities for and activities of effectors should incorporate membranes and are subject to changes in response to different lipid components of those membranes.

Regulating ARFs

ARFs are N-myristoylated (a cotranslational covalent modification that is not reversible), but this modification, while critical to activity, is unlikely to be regulatory. Other posttranslational modifications (e.g., phosphorylation, ubiquitination) occur on the GTPases, GEFs, and GAPs, but have been largely underexplored. The details of ARF activation and deactivation are discussed in the *ARF GEFs* and *ARF GAPs* sections.

ARF GEFS

Families of ARF GEFs and their cellular functions

ARFs require GEFs to accelerate nucleotide exchange. This is likely true of the ARLs as well, though relatively weak affinity for guanine nucleotides by ARL2, ARL13B, and perhaps others suggests the possibility of other means of regulating their activation process. As no GEFs for ARLs have been identified, except that of ARL13B for ARL3 (Gotthardt *et al.*, 2015), this section is limited to GEFs that act on mammalian ARFs. The human genome encodes 15 ARF GEFs divided into six families based on sequence relatedness, domain organization, and phylogenetic analyses: GBF, BIGs, Cytohesins, EFA6/Psd, BRAG/IQSec, and FBX (Table 2, Figure 3A, and Supplemental Table II). All ARF GEFs share a common catalytic Sec7 domain (Sec7d) and a mechanism of action, but display diversity in their actions and regulation in cells (Peyroche *et al.*, 1996; Cherfils and Chardin, 1999; Jackson and Casanova, 2000; Casanova, 2007). The locations of ARF GEFs in cells parallel those of ARF1-6 at the Golgi, endosomes, and the PM (Figure 1). In this section, we briefly describe the actions of human ARF GEFs.

GBF1 and BIG1/2 are key regulators of membrane traffic within the secretory and endosomal pathways (Wright *et al.*, 2014). GBF1 preferentially localizes to the Er-Golgi intermediate compartment (ERGIC) and the *cis*-Golgi, where it mediates ARF activation required for COPI vesicle formation. GBF1 likely activates ARF1 and ARF4, as only the simultaneous removal of these, but not any other pair of ARFs, inhibits COPI traffic (Zhao *et al.*, 2002, 2006; Volpicelli-Daley *et al.*, 2005; Szul *et al.*, 2007; Manolea *et al.*, 2008). GBF1 also acts

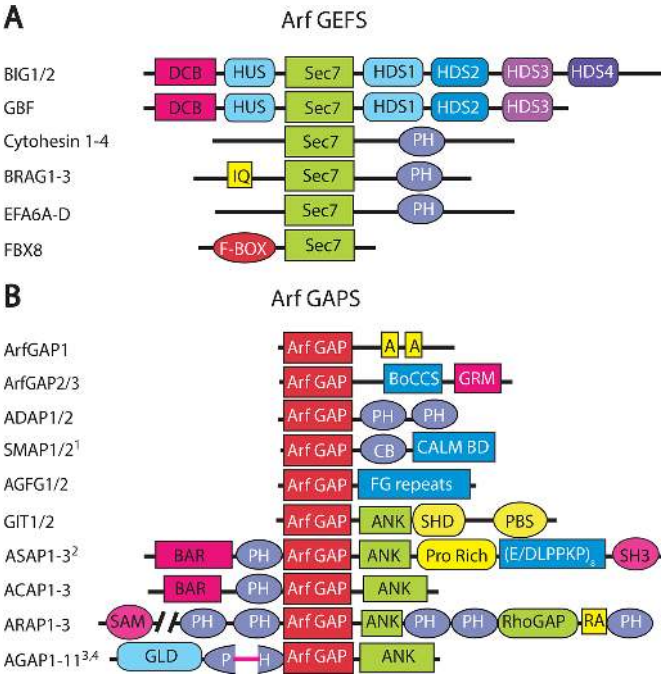

FIGURE 3: Domain organization of ARF GEFs and ARF GAPs. A schematic of the domains present in each subfamily of the ARF GEFs (A) and ARF GAPs (B). The defining ARF GEF/Sec7 domain and the ARF GAP domain are aligned. Protein lengths are not drawn to scale. Abbreviations (in alphabetical order): A, ARF GAP lipid-packing sensor (ALPS); ANK, ankyrin repeat; BAR, Bin/Amphiphysin/Rvs; BoCCS, binder of coatomer, cargo, and SNARE; CALM BD, calm binding domain; CB, clathrin box; DCB, dimerization and cyclophilin binding; $E/DLPPKP_8$, 8 repeats of this primary sequence (single letter code); F-BOX, cyclin F protein interaction motif; FG repeats, multiple copies of XXFG repeated; GLD, GTP binding protein–like domain; GRM, Glo3 regulatory motif; HDS(1-4), homology downstream of Sec7; HUS, homology upstream of Sec7; IQ, isoleucine/glutamine calmodulin-binding motif; PBS, Paxillin binding site; PH, pleckstrin homology; Pro-rich, proline rich; RA, Ras association; Rho GAP, Rho GTPase-activating protein; SAM, sterile α motif; SHD, Spa homology domain.

at the TGN to support the recruitment of BIG1 and BIG2 through activating ARF4 and ARF5 (Lowery *et al.*, 2013). GBF1 facilitates lipid droplet formation (Ellong *et al.*, 2011; Takashima *et al.*, 2011; Bouvet *et al.*, 2013), is detected at PM sites involved in active migration and chemotaxis (Mazaki *et al.*, 2012; Busby *et al.*, 2017), and in some cells facilitates traffic through the glycophosphatidyl-inositol–enriched endosomal compartments (GEECs) pathway (Gupta *et al.*, 2009), but the ARFs activated for these functions are unknown. BIG1 and BIG2 localize to the TGN and endosomes, where they mediate ARF activation required for endosome–PM recycling, TGN–PM recycling, TGN–late endosome transport, and in some cells TGN–granule transport (Shinotsuka *et al.*, 2002a; Zhao *et al.*, 2002). BIG1 and BIG2 facilitate the recruitment of the clathrin adaptors AP1 and AP3 through activation of ARF1 and ARF3 (Pacheco-Rodriguez *et al.*, 2002; Shinotsuka *et al.*, 2002a,b; Ishizaki *et al.*, 2008). BIG1 seems to play additional nontrafficking roles, as it is detected in the nucleus of serum-starved cells (Padilla *et al.*, 2004, 2008).

The Cytohesins localize to and regulate endosomal trafficking (Figure 1), including the stimulated recycling of the glucose transporter GLUT4, integrins, and other proteins (Caumont *et al.*, 2000; Oh and Santy, 2010, 2012; Li *et al.*, 2012; Salem *et al.*, 2015).

Cytohesins can be recruited to the PM in response to insulin, epidermal growth factor (EGF), or nerve growth factor (NGF) (Venkateswarlu *et al.*, 1998a,b), where they are required for signaling by these hormones (Li *et al.*, 2003; Fuss *et al.*, 2006; Hafner *et al.*, 2006; Lim *et al.*, 2010; Attar and Santy, 2013; Hahn *et al.*, 2013; Pan *et al.*, 2013; Reviriego-Mendoza and Santy, 2015). Cytohesins also stimulate Rac activation and actin polymerization at the cell periphery (Frank *et al.*, 1998; Santy and Casanova, 2001; Santy *et al.*, 2005; Li *et al.*, 2007; White *et al.*, 2010; Reviriego-Mendoza and Santy, 2015), resulting in increased cell migration (Santy and Casanova, 2001; Santy *et al.*, 2005; Torii *et al.*, 2010; Attar and Santy, 2013). Cytohesins perform these functions by activating ARF1 and/or 6. However, all ARF isoforms are efficiently activated by Cytohesins in vitro, raising the question of how specific ARF isoforms are selected in vivo.

The BRAGs (Brefeldin A–resistant ARF GEF, later renamed IQSecs) contain a calmodulin-binding IQ motif in the N-terminal third of each protein (Figure 3A). IQSec1 and 3 are highly enriched in the central nervous system, while IQSec2 is ubiquitous. IQSecs localize to endosomes and the PM in nonneuronal cells, and to postsynaptic densities in neurons (reviewed in D'Souza and Casanova, 2016) (Figure 1), In general, IQSecs appear to control the internalization of adhesive and/or signaling molecules. Examples include the adhesion proteins dumbfounded (Duf), roughest (rst), and N-cadherin in myoblasts (Bach *et al.*, 2010); the semaphorin, Sema3E, and its cognate receptor plexin D1 in endothelial cells (Sakurai *et al.*, 2011); synaptic AMPA receptors in neuronal pathfinding (Charych *et al.*, 2004; Scholz *et al.*, 2010; Elagabani *et al.*, 2016); and β1 integrins in metastasizing breast cancer cells (Moravec *et al.*, 2012). IQSecs perform these functions through the activation of ARF5 and/or ARF6. Like Cytohesins, IQSecs efficiently activate all ARFs in vitro (Peurois *et al.*, 2017), and the mechanisms that determine their selectivity in cells remain elusive. IQSec1 also acts in the nucleus (Dunphy *et al.*, 2007), raising the question of how its various functions are integrated and regulated.

The EFA6 (exchange factor for ARF6)/PSD (pleckstrin homology and Sec7 domain) proteins activate ARF6 and regulate actin cytoskeleton dynamics at the cell surface (Franco *et al.*, 1999; Macia *et al.*, 2001) (Figure 1). They appear to support distinct functions in specific cells and during development, as suggested by their varied tissue distribution (all EFA6 proteins except EFA6B are abundant in brain but are differentially distributed within different brain regions, with EFA6C showing the most selective localization to only Purkinje cells and the choroid plexus; Matsuya *et al.*, 2005) and changing expression levels during development (Sakagami *et al.*, 2006). EFA6 has been implicated in dendritic branching and spine formation (Inaba *et al.*, 2004) and might regulate endocytosis of neurotransmitter receptors (Decressac *et al.*, 2004). It also has been implicated in clathrin-mediated endocytosis through a regulatory interaction with endophilin (Boulakirba *et al.*, 2014).

FBX8 is the sole representative of the last GEF subfamily and is the least understood (Table 2). The role of the N-terminal F-box domain is unclear (Figure 3B). Paradoxically, though serving as a GEF for ARF6 at the cell surface, it also exhibits a suppressive effect on ARF6 activity, perhaps through a poorly described effect on mono-ubiquitination of ARF6 (Yano *et al.*, 2008). Interestingly, FBX8 shows no GEF activity in vitro, raising uncertainty as to whether it is a bona fide GEF.

Structural insight into the mechanisms of ARF activation by GEFs

All ARF GEFs use the same mechanism to promote nucleotide exchange through the highly conserved, catalytic ~200-residue Sec7 domain (Sec7d), so named based on homology to the domain in the *Saccharomyces cerevisiae* Sec7 protein (Peyroche *et al.*, 1996). To be activated by a Sec7d, ARF-GDP must be primed by membranes that displace the autoinhibitory N-terminal helix, thus allowing the GEF to promote the toggle of the interswitch and secure ARF-GDP on the membrane before GDP dissociation (Renault *et al.*, 2003). Next, the Sec7d inserts an invariant glutamate (also called the "glutamic finger"; Beraud-Dufour *et al.*, 1998; Renault *et al.*, 2003) into the active site, which competes with the phosphates of GDP to promote its dissociation and the formation of a nucleotide-free complex that can bind GTP (Goldberg, 1998). Charge-reversal mutation of the glutamic finger renders a Sec7d catalytically inactive (Beraud-Dufour *et al.*, 1998). Thus, in a manner that is unique to ARF GEFs, stimulation of GDP/GTP exchange has an absolute requirement for a membrane, which can be likened to a cofactor. Interestingly, the Sec7d of GBF and BIG is generally, although not always, the target of the fungal toxin Brefeldin A, which traps an abortive ARF–GDP–BFA–Sec7 complex (Peyroche *et al.*, 1999; Mossessova *et al.*, 2003; Renault *et al.*, 2003).

Regulating GEFs

ARF signaling is tightly regulated in cells, implying that the activating GEFs are catalytically active only at specific times and places. GEFs are regulated by multiple molecular mechanisms that impact their membrane association and/or catalytic activity. All inactive ARF GEFs are cytosolic, but activate ARF only on membranes, suggesting that membrane recruitment represents a regulatory step. Recruitment strategies differ among the GEFs, albeit some commonalities are emerging. GBF1 and BIG1/2 share a common domain architecture composed of domains located upstream and downstream of the Sec7d, coined HUS and HDS domains, respectively (Mouratou *et al.*, 2005). These proteins are recruited through an interaction of their N-terminal regions (up to the Sec7d) with a small GTPase: Rab1b for GBF1 (Alvarez *et al.*, 2003; Monetta *et al.*, 2007) and ARL1 (Christis and Munro, 2012; McDonold and Fromme, 2014) and ARF4/5 (Lowery *et al.*, 2013) for BIG1 and BIG2. Such a system is reminiscent of the "cascade" of Rab GTPases working at several stages of membrane trafficking (Jones *et al.*, 1999; Stalder and Antonny, 2013). The C-terminal regions of GBF1 and BIG1/2 are also important, as intact HDS domains are required for their membrane association (McDonold and Fromme, 2014; Chen *et al.*, 2017; Gustafson and Fromme, 2017; Meissner *et al.*, 2018; Pocognoni *et al.*, 2018). It is likely that multiple domains position these GEFs on the membrane, but how such interactions are ordered and whether or not they display cooperativity is unknown. The catalytic activity of GBF1, BIG1, and BIG2 appears to be regulated through allosteric mechanisms. The activity of the yeast Sec7p (orthologue of BIG1/2) is stimulated through conformational changes induced by binding of Ypt (yeast Rab orthologues) GTPases to its N- and C-terminal domains (McDonold and Fromme, 2014). In addition, binding of ARF-GTP also stimulates activity in a forward loop where the generated product further activates Sec7d. There are three allosteric binding sites on Sec7p, two for Ypts and one for activated ARF, leaving open the catalytic GEF site for binding ARF-GDP. Such a regulatory/stimulatory effect may ensure a concentrated burst of activated ARFs to locally recruit a plethora of effectors. However, mammalian BIG1/2 and GBF1 do not show an analogous regulatory mechanism. Instead, the catalytic activity of GBF1 may be stimulated by a HDS1-phosphoinositide (PIP) interaction (Meissner *et al.*, 2018), analogous to the PH domain regulating the catalytic activity of BRAG/IQSecs.

Cytohesins, BRAG/IQSecs, and EFA6/PSDs contain a PH domain downstream of their Sec7 domain (Figure 3A) that facilitates membrane recruitment by interacting with PIPs and other anionic

phospholipids and, in some cases, the active forms of ARF/ARL GTPases. The binding properties and structural modalities, however, diverge between the families. The PH domains of Cytohesins play multiple roles, including specific recognition of PIP_2 and PIP_3 by the canonical lipid-binding site (DiNitto *et al.*, 2003; Cronin *et al.*, 2004), autoinhibition of the Sec7 active site (DiNitto *et al.*, 2007), and implementation of a positive-feedback loop by binding to ARF-GTP or ARL4-GTP (Cohen *et al.*, 2007; Hofmann *et al.*, 2007; Malaby *et al.*, 2013; Stalder and Antonny, 2013). An important determinant is the polybasic region located immediately downstream of the PH domain, which contributes both to autoinhibition and recruitment to the membrane. Other layers of regulation have been described. One of these is an autoinhibitory interaction mediated by the N-terminal coiled coil, a domain involved in cytohesin dimerization. Autoinhibition is relieved by AKT-dependent phosphorylation of a threonine residue in the PH domain, which allows the recruitment of Cytohesins to membranes (Li *et al.*, 2012; Hiester and Santy, 2013). Phosphorylation of protein kinase C (PKC) sites in the polybasic regions of Cyth1 and Cyth2/ARNO also stimulates their GEF activity, presumably by destabilizing the autoinhibited state (DiNitto *et al.*, 2007). Grp1 lacks these PKC sites but can be phosphorylated by AKT on a serine near the catalytic site in the Sec7d and a threonine in the β1/β2 loop in the PH domain, thereby influencing GEF activity and PIP affinity/specificity, respectively (Li *et al.*, 2012).

The PH domains of BRAG/IQSecs differ from those in Cytohesins in that they do not autoinhibit the Sec7 domain (Jian *et al.*, 2012; Aizel *et al.*, 2013) and they bind several anionic lipids instead of recognizing a single phosphoinositide with high specificity (Karandur *et al.*, 2017). PIP_2 binding increases their catalytic activity, likely by positioning the GEF in an optimal membrane-based orientation with respect to the ARF GTPase (Karandur *et al.*, 2017). In contrast to Cytohesins, ARF-GTP has no effect on BRAG/IQSec activity. In addition, BRAG/IQSecs are unique among the GEFs in their sensitivity to calcium due to the noncanonical IQ motif in the N-terminus (Figure 3A), which fits the consensus for binding to calcium-free calmodulin. BRAG1/IQSec2 binds to Ca^{2+}-free calmodulin in vitro, and addition of Ca^{2+} causes its dissociation from membranes (Aizel *et al.*, 2013; Roy *et al.*, 2016). Whether this dissociation is due to a calmodulin-based regulation, to competition of Ca^{2+} with phospholipids for binding to the PH domain, or both acting in synergy remains to be established but raises the question of possible crosstalk between Ca^{2+} and ARF signaling.

EFA6/PSD is also recruited to anionic membranes by its PH domain and a polybasic element in its C-terminus, but its activity is inhibited by ARF-GTP, indicating negative-feedback regulation (Padovani *et al.*, 2014). Its GEF activity is enhanced by direct interaction with endophilin in clathrin-mediated endocytosis (Boulakirba *et al.*, 2014).

ARF GAPS

Families of ARF GAPs and their cellular functions

ARF GAPs are defined by the presence of the conserved, catalytic GAP domain (Figure 3B), first identified in ArfGAP1, which binds to ARF-GTP to promote hydrolysis of GTP to GDP. The human genome encodes at least 28 proteins containing an ARF GAP domain or having GAP activity (Gillingham and Munro, 2007; Kahn *et al.*, 2008; Donaldson and Jackson, 2011) (Table 3; additional information included in Supplemental Table III). There are eight additional ARF GAP genes on chromosome 10, but it is not known whether these are expressed. ARF GAPs are divided into 10 subtypes based on sequence similarity and shared domain structure (Figure 3B) (Randazzo and Hirsch, 2004; Inoue and Randazzo, 2007; Spang *et al.*, 2010). Each GAP subtype, and even members within a particular subtype, display distinct localizations (Figure 1) and functions, and those can be either ARF dependent or ARF independent (Gillingham and Munro, 2007; Spang *et al.*, 2010; Donaldson and Jackson, 2011; Vitali *et al.*, 2017). An exception is the ELMOD family proteins that lack the ARF GAP domain, yet have in vitro activity against a wide range of ARF family GTPases, including both ARFs and several ARLs (Bowzard *et al.*, 2007; East *et al.*, 2012; Ivanova *et al.*, 2014). The three mammalian proteins, ELMOD1-3, share an ELMO domain and an apparent catalytic arginine (East *et al.*, 2012). Their cellular locations are shown in Figure 1, but are not discussed further.

With the well-established role of ARFs in membrane trafficking, most studies of ARF GAPs focused in this area, and specifically in coat/adaptor recruitment to membranes, predominantly at the Golgi and PM (Gillingham and Munro, 2007; Spang *et al.*, 2010; Donaldson and Jackson, 2011; Shiba and Randazzo, 2012; Vitali *et al.*, 2017). At least six subtypes of GAPs are involved in the recruitment of ARF-dependent adaptors, including the COPI coatomer, GGAs, and clathrin and its adaptor AP-3. Because GAPs can inactivate ARFs, the early models posited that GAPs function exclusively as terminators of ARF signaling (Weimer *et al.*, 2008). However, the role of GAPs is far more complex. Compelling evidence for function of GAPs in supporting ARF activities, rather than solely acting as signal terminators, initially came from a screen for high-copy suppressors of ARF insufficiency in yeast that showed that all ARF GAPs in that organism could compensate for the ARF deficiency (Zhang *et al.*, 1998). The idea of GAPs being involved in propagation of an ARF signal was further supported by the finding that a number of GAPs drive coat assembly and cargo selection during the formation of transport vesicles (Yang *et al.*, 2002; Lee *et al.*, 2005; Spang *et al.*, 2010; Bai *et al.*, 2011; Shiba *et al.*, 2011). These observations suggest that GAPs can serve as ARF effectors, or that ARF activity requires multiple rounds of inactivation/activation cycles that require GAPs, or both.

ARF GAPs also regulate the actin cytoskeleton and associated adhesive structures (Hoefen and Berk, 2006; Randazzo *et al.*, 2007; Ha *et al.*, 2008; Casalou *et al.*, 2016; Zhou *et al.*, 2016; Luo *et al.*, 2017; Vitali *et al.*, 2017); for example, at least seven GAPs (GIT1, GIT2, ASAP1-3, ARAP2, and AGAP2) associate with focal adhesions (FAs) and function therein (Figure 1; listed under PM in this figure to save space). GAP effects are mediated in part by regulating traffic of FA components to the nascent structures and through effects on RHO GTPase signaling, including acting as scaffolds for components in the Rho family GTPase pathways (Zhao *et al.*, 2000; Lamorte *et al.*, 2003; Yin *et al.*, 2005; Frank *et al.*, 2006) and directly binding to and altering the functions of actin, non-muscle myosin 2 (Chen *et al.*, 2016), and Kif2A (Luo *et al.*, 2016). Some effects on the cytoskeleton can be propagated by GAP mutants lacking catalytic activity but able to bind ARF-GTP (Randazzo *et al.*, 2000), again supporting the role of GAPs as effectors rather than simply signal terminators. Some GAPs (e.g., ARAPs) contain both ARF GAP and RHO GAP domains, with functions that can be attributed to either activity (Miura *et al.*, 2002; Krugmann *et al.*, 2002; Stacey *et al.*, 2004; Nishiya *et al.*, 2005; Yoon *et al.*, 2006, 2008; Gambardella *et al.*, 2011, 2012; Chen *et al.*, 2013, 2014; Luo *et al.*, 2018).

ARF GAPs also affect the activities of protein kinases (e.g., AGAP2 binds and activates AKT; Liu *et al.*, 2007; while ARAP2 reduces AKT phosphorylation, and thereby its activity, by an unknown mechanism; Luo *et al.*, 2018). Thus, a single ARF GAP can affect multiple signaling pathways, and multiple ARF GAPs may impinge

on a single pathway. Unfortunately, our knowledge of the many functions in signaling and integration of multiple signaling pathways to elicit distinct phenotypic responses is still fragmentary.

Structural insight into the mechanisms of ARF GAPs

Soon after the discovery of the first ARF GAP (Cukierman *et al.*, 1995), the role of the catalytic arginine (aka an "arginine finger"; Ahmadian *et al.*, 1997; Scheffzek *et al.*, 1998; Cherfils and Zeghouf, 2013) in the hydrolysis of the β-γ phosphate bond by the ARF was established (Randazzo *et al.*, 2000). The use of a highly conserved, catalytic arginine in GAP-stimulated GTP hydrolysis is also present in RHO GAPs (Barrett *et al.*, 1997; Amin *et al.*, 2016). Similar to many GAPs, the ARF GAP domain inserts the arginine finger into the nucleotide-binding site to stabilize the transition state of the reaction, and this requires the conserved glutamine in the switch 2 region (Ismail *et al.*, 2010; Cherfils and Zeghouf, 2013). Loss of GAP activity upon mutation of the arginine finger is fully consistent with its catalytic function. The arginine finger mechanism appears to be shared by both the ARF GAPs and ELMOD1-3, despite their disparate structures (East *et al.*, 2012). Calcium stimulates the in vitro GAP activity of ASAP but not of other GAPs, while also competing with its association to the membrane, again raising the intriguing issue of cross-talk between Ca^{2+} and ARF signaling (Ismail *et al.*, 2010).

Regulating ARF GAPs

Membranes play a central role in regulating ArfGAPs, by restricting both their activities to specific subcellular locations and allosteric control of their GAP activity. Recruitment to membranes and allosteric activation of GAPs is commonly conferred by their PH domains, which are N-terminal to the GAP domains; this is true for the ASAP, ACAP, ARAP, and AGAP subfamilies (Kam *et al.*, 2000; Nie *et al.*, 2002; Campa *et al.*, 2009; Jian *et al.*, 2015) (Figure 3B). Other domains also contribute to regulating GAP activity. The curvature-sensing BAR domain of ASAP1 positions an autoinhibitory motif to contact the PH and GAP domains, thus inhibiting GAP activity (Jian *et al.*, 2009), while a BAR domain binding partner, NM2A, stimulates ASAP1 activity, perhaps by relieving the autoinhibition (Chen *et al.*, 2016). In a landmark study of ArfGAP1, recognition of membrane curvature by an ALPS motif, a peptide that folds as a helix to bind curved membranes, was shown to stimulate its GAP activity (Bigay *et al.*, 2005). The catalytic activity of ArfGAP1 and ArfGAP2 also can be allosterically regulated by coatomer and cargo binding (Goldberg, 2000; Luo and Randazzo, 2008; Luo *et al.*, 2009). In another example, nonmuscle myosin 2A stimulates ASAP1 activity, perhaps by relieving autoinhibition (Chen *et al.*, 2016).

For several ARF GAPs that function in FAs, including GITs, ASAP1, and AGAP2, targeting is achieved by binding to specific FA components (Turner *et al.*, 2001; Randazzo *et al.*, 2007; Vitali *et al.*, 2017). ARF GAPs that regulate the Golgi and endocytic compartments also are targeted by binding to vesicle coat proteins, including SMAPs binding through clathrin boxes to clathrin heavy chain and ArfGAP1 binding the δ-COP component of COPI coatomer (Tanabe *et al.*, 2005; Natsume *et al.*, 2006; Weimer *et al.*, 2008; Spang *et al.*, 2010; Suckling *et al.*, 2015). These studies highlight the general principle, in which membrane and protein features that define the environmental conditions are coupled to the regulation of the GAP activity. They also show that specific mechanisms are remarkably diverse, likely to allow diverse ARF functions, and the need to unravel these mechanisms to allow a clear understanding of ARF GAP functions in cells.

EVOLUTIONARY PARALLELS BETWEEN ARF GTPASES AND THEIR GEFS AND GAPS

The complexity of human ARF GTPases and their GEFs and GAPs presents a major challenge in defining their functionalities. An evolutionary approach can help by categorizing the proteins based on their evolutionary history and presence or absence in different organisms with diverging cell biologies (Figure 4A). It can also help to connect the human complement to that of other model (and nonmodel) organisms by detailing how the diversity of the human proteins arose. Functional diversity can most easily be conceptualized as arising at three levels: 1) vertebrate-specific machinery that arose in the lineage giving rise to animals, 2) machinery present in the common ancestor of all eukaryotes, and 3) machinery present in the archaeal contributor to the origin of eukaryotes. The human complement of proteins in these three families includes a large number of subfamilies, shared among vertebrates, for example, humans, mice, rats, and fish. These are largely explained by the series of whole-genome duplications that took place at the dawn of vertebrates and gave rise to ARFs 1-5 (Manolea *et al.*, 2010), 2-3 paralogues in all ARF GAPs (except for ArfGAP1; Schlacht *et al.*, 2013), and 2-4 paralogues in almost all ARF GEFs (Figure 4B and Tables 2 and 3). The human complement also partly reflects expansion of the families in the lineage that gave rise to animals, whether at the ancestor of all animals (i.e., holozoan) or of animals plus fungi (i.e., opisthokont). Examples include the duplication that gave rise to class I versus class II ARFs in holozoans (Manolea *et al.*, 2010), the emergence of the GAP ASAP (Schlacht *et al.*, 2013), or that of the GEF EFA6, each of which arose in the opisthokonts (S. V. Pipaliya, A. Schlacht, C. M. Klinger, R. A. Kahn, and J. Dacks, personal communication). These are quite ancient (arising around a billion years ago; Eme *et al.*, 2014), but still reflect ARF and regulatory machinery that is restricted to a relatively limited subset of eukaryotes, later expanded in vertebrates. We share these proteins with basal animal lineages and fungi, meaning that molecular cell biological data from model organisms (e.g., *Drosophila melanogaster*, *Caenorhabditis elegans*, and *S. cerevisiae*) can meaningfully be applied to understand these proteins in human cells. However, there are no orthologues of these proteins in other eukaryotes, including plants, which likely reflects important functional differences.

To understand ARF signaling and regulatory biology common to all eukaryotic organisms, we need to look for proteins that arose before the common ancestor of all eukaryotic life (around 2 billion years ago; Eme *et al.*, 2014) and contributed common machinery in its descendent lineages. We know that this last eukaryotic common ancestor (LECA) was sophisticated, possessing a complement of membrane trafficking machinery that rivals that found in many eukaryotes today. LECA contained 16 ancient ARF GTPases, two of which were true ARFs (R. Petrželková and M. Eliáš, personal communication). It also had six ARF GAPs (SMAP, AGFG, ArfGAP1, ArfGAP2, ACAP, ArfGAP_C2; Schlacht *et al.*, 2013) (Figure 4A). This last protein is absent from human and yeast, but present in other eukaryotic lineages like plants and plant pathogens such as Phytophthora. The existence of ArfGAP_C2 highlights the fundamental eukaryotic cell biology left to be discovered, especially that not present in mammals. The LECA also possessed at least two ELMOD GAPs, which work on both ARFs and ARLs (East *et al.*, 2012) (Figure 4A). GBF1 and BIG were also present in the LECA (Bui *et al.*, 2009), as was Cytohesin (S. V. Pipaliya *et al.*, personal communication). Clearly, the complexity of ARF signaling had already been well developed at the dawn of eukaryotes. Recently, it has been possible to dig even deeper into the origins of ARFs with the discovery of

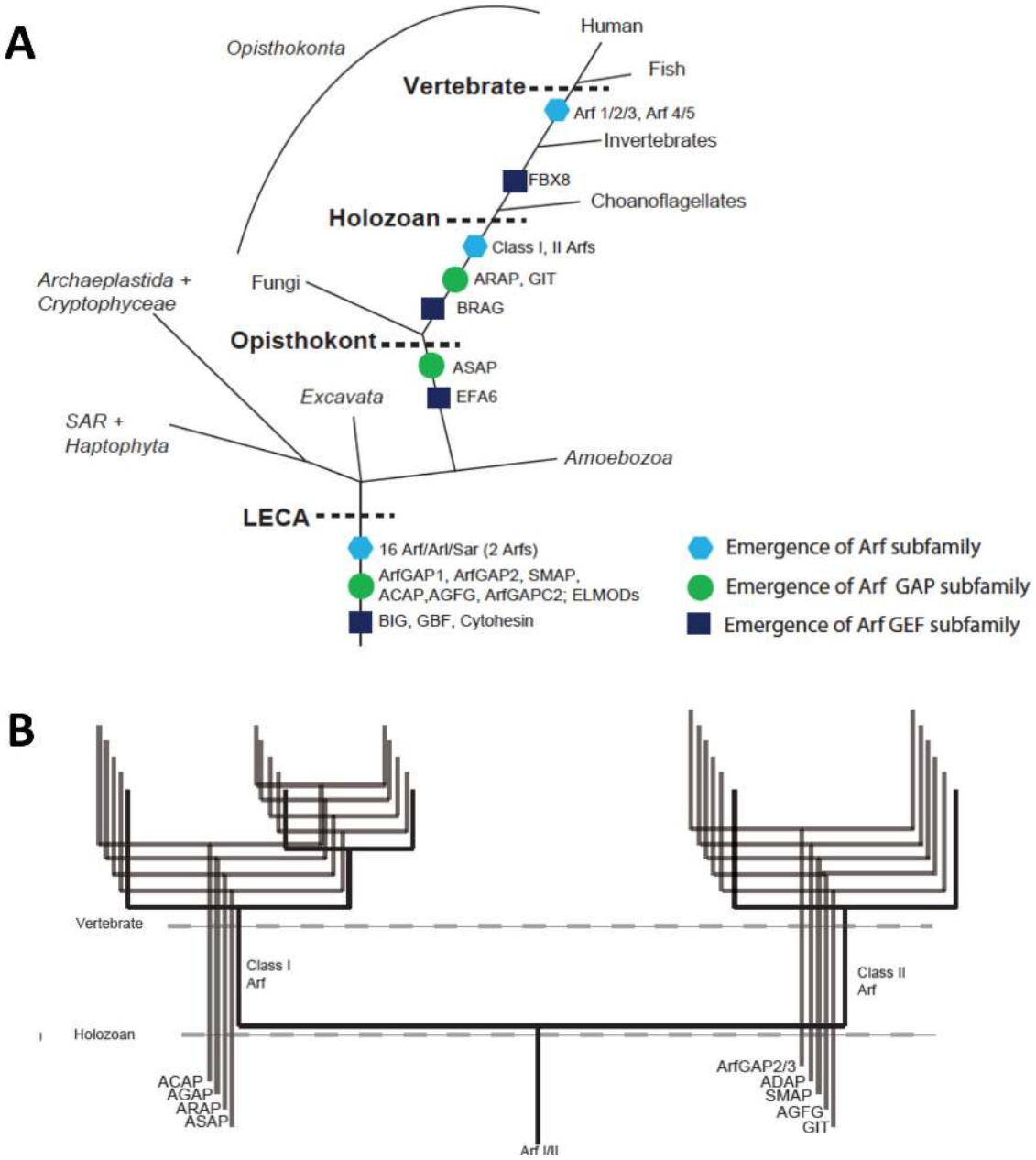

FIGURE 4: Evolution of the ARF family and its regulators. (A) The timing of the emergence of the relevant protein subfamilies is shown mapped on a simplified tree of eukaryotes. The polygons, circles, and squares denote the latest point by which the ARF GTPases, GAPs, and GEFs must have evolved, respectively, with the names of the subfamilies given to the right. The names of the eukaryotic supergroups are in italics, while the relevant "reconstructed ancestor" discussed in the text are in bold and noted by a dashed line. (B) Overlay of ARF1-5 evolution with that of the nine ARF GAP subfamilies that possess multiple paralogues. ARF evolution is depicted in black and Arf GAP in gray, with duplications at the base of Holozoa and Vertebrata. Relevant evolutionary transitions are illustrated by dashed lines.

the Asgard archaea, metagenomic assemblies of what appear to be the closest living descendants of the archaeal lineage that contributed to the birth of eukaryotes (Spang *et al.*, 2015; Zaremba-Niedzwiedzka *et al.*, 2017). Within these genomes can be found GTPases that are not ARFs, ARLs, or SARs, but are clearly close relatives of the GTPases from which the ARF family later arose (Spang *et al.*, 2015; Klinger *et al.*, 2016). Examples of some proteins with domains similar to possible ARF GAPs and GEFs were also found, in some cases fused to the GTPases themselves.

Defining evolutionary patterns for the ARF, GEF, and GAP families individually is extremely informative, but comparisons among the three families can be even more powerful. Human GAPs show very similar duplication patterns to the ARFs. Class I ARFs gave rise to ARFs 1-3, while class II ARFs gave rise to ARF4 and 5 (Manolea *et al.*, 2010) (Figure 4B). The ARF GAPs show the same breakdown of two (ADAP, AGFG, GIT, SMAP) or three (ARFGAP, ACAP, AGAP, ARAP, ASAP) paralogues within these subfamilies (Schlacht *et al.*, 2013). This is a correlative observation, but raises testable models of coordinated activity of these proteins for which the substrate

specificity and biological functions are poorly and incompletely understood. Whether the expansions in the GEFs reflect a coevolutionary or functional relationship with the ARFs and GAPs is an open but exciting question. Interestingly, during the progressive expansion of the ARFs and their regulatory machinery from the LECA to humans, some protein families have resisted expansion. There is only a single GBF1 (but two orthologues, Gea1 and Gea2, in yeast) and a single ArfGAP1, both of which act in the early secretory pathway, suggesting a selective constraint on the plasticity of this pathway, as compared with the late secretory and endocytic systems. Similarly, there is a single ARL2 that acts at multiple sites in distinct pathways but has resisted segregating those functions via duplication with paralogues having distinct localization and actions (Sharer and Kahn, 1999; Bowzard *et al.*, 2005; Zhou *et al.*, 2006; Newman *et al.*, 2014). Evolutionary cell biology of cofunctioning families of proteins can reveal unexpected aspects of diverse organelle functions (for examples, see Dacks and Robinson, 2017) and provide crossover insights into possible mechanisms of action and regulatory networks for each family. While the ARF family is typically described as being one of four large families within the RAS superfamily, the evolutionary analysis (Klinger *et al.*, 2016), together with the unique and unifying structural mechanism employed by ARFs (Pasqualato *et al.*, 2002; see also earlier discussion), argue that ARF GTPases form their own superfamily.

ARF GTPASES/GEFS/GAPS IN CANCER AND OTHER DISEASES

As for most small GTPases, ARFs (and their GEFs and GAPs) have been associated with human diseases, and many more roles in pathologies continue to be discovered. A number of GTPases in the RAS superfamily have strong links to cancers, with RAS being the most commonly found mutated oncogene in human cancers and a RAS GAP, NF1, prominently altered in neurofibromatosis (Downward, 2003). We asked whether such links might be found within the human ARF families described herein. Interrogation of available next-generation sequencing data in the Cancer Genome Atlas (via http://cbioportal.com) reveals that ARF signaling is altered in cancer (albeit less commonly than is RAS or RHO signaling). Importantly, the mechanisms by which ARF GTPase/GEF/GAP signaling is genetically altered in cancers differs markedly from those seen in RAS and RHO families. RAS signaling is most commonly altered by missense mutation or amplification of the GTPases, deletion of RAS GAPs, and/or mutation of RAS effectors (Downward, 2003). Similarly, RHO signaling is most often altered by missense mutation or amplification of the GTPase, overexpression of GEFs, loss of GAPs, alterations in posttranslational modifications, and/or alternative splicing (Porter *et al.*, 2016). In contrast, missense mutations that render ARF GAPs inactive are largely not observed, and the most common genetic alterations observed are amplification events, particularly of the GTPases ARF4 and ARL14 and the ARF GAPs AGAP2 and ASAP1. ARF4 is commonly amplified in prostate cancer (20%) and is an important regulator of breast cancer cell migration (Jang *et al.*, 2012). ARL14 has yet to be studied in the lab, but its high amplification rate in squamous cell lung cancer (23%), esophageal cancer (13%), and ovarian cancer (11%) merits further investigation. AGAP2 is often coamplified with CDK4 and promotes cancer cell survival, migration, and invasion in glioblastoma models (Qi *et al.*, 2017). ASAP1 expression correlates with metastatic potential in uveal melanoma, colon cancer, prostate cancer, and laryngeal squamous cell carcinoma (Ehlers *et al.*, 2005; Muller *et al.*, 2010; Li *et al.*, 2014) and is associated with increased motility and invasiveness of uveal melanoma and breast cancer cells (Ehlers *et al.*, 2005; Onodera *et al.*, 2005). Furthermore, AGAP2 and ASAP1 amplification is associated with decreased overall and progression-free survival (Ehlers *et al.*, 2005). In addition, a number of reports implicate ARF4 (Jang *et al.*, 2012) and ARF6 (Hashimoto *et al.*, 2004; Hongu *et al.*, 2015; Li *et al.*, 2017) in cancer cell migration, invasion, and metastasis. However, the molecular mechanisms through which the changes in ARF GTPases and their GAPs elicit pathology remain to be defined. In addition to these amplification events, genomic deletions of at least one GTPase are observed. ARL11 (aka ADP-ribosylation factor-like tumor suppressor gene 1 [ARLTS1]), is commonly deleted in prostate cancer and sarcoma, and the expression of this gene in lung cancer is down-regulated due to promoter hypermethylation (Yendamuri *et al.*, 2008). Likewise, the ARF GEF BRAG2 has been implicated in breast cancer and uveal melanoma (Morishige *et al.*, 2008; Yoo *et al.*, 2016).

Mutations in ARF GEFs have been identified as causes of human neurological disease. For example, a large number of mutations in BRAG1/IQSec2 have been identified and implicated in nonsyndromic X-linked intellectual disability (Mignot *et al.*, 2018), a subset of which occur within either the IQ motif or the Sec7d. These mutations alter the trafficking of AMPA receptors in hippocampal neurons, suggesting a molecular basis for the deficits in learning and memory associated with this disease. Schwann cell–specific deletion of BIG1 prevents myelination (Miyamoto *et al.*, 2018), while mutations in BIG2 cause periventricular heterotopia with microcephaly (Sheen *et al.*, 2004; Lu *et al.*, 2006; Grzmil *et al.*, 2010). In both cases, we lack an understanding of the underlying mechanisms causing the GEF dysfunction.

TISSUE-SPECIFIC FUNCTIONS OF ARF GTPASES/GEFS/GAPS

There is growing evidence that at least some of these GTPases/GEFs/GAPs (especially those arising late in evolution) show differential tissue expression patterns and act in a tissue-specific manner or are expressed and function during specific stages of development. This is evident from studies in which specific GTPases have been mutated/deleted in mice (either total or tissue-specific knockout) and cause a variety of phenotypes (Table 4) (Mueller *et al.*, 2002; Schurmann *et al.*, 2002; Schrick *et al.*, 2006; Suzuki *et al.*, 2006; Caspary *et al.*, 2007; Zahn *et al.*, 2008; Hesse *et al.*, 2010, 2012; Hommel *et al.*, 2010; Zhang *et al.*, 2011; Jaschke *et al.*, 2012; Akiyama *et al.*, 2014; Hayakawa *et al.*, 2014; Hongu *et al.*, 2015; Hanke-Gogokhia *et al.*, 2016, 2017; Huang *et al.*, 2016; Li *et al.*, 2016; Lin *et al.*, 2017; Bay *et al.*, 2018; Dilan *et al.*, 2018; Pearring *et al.*, 2017; Rodiger *et al.*, 2018). As might be expected for ancient and highly conserved proteins, several GTPases are essential, and their deletion results in embryonic lethality. However, the use of tissue-specific deletions provides a wealth of new information and highlights the importance of these proteins in cells, tissues, and whole organisms, as exemplified by deletions of ARF6 in endothelial and neuronal cells as well as in platelets and podocytes and of the essential gene *ARFRP1* in liver, adipocytes, or intestine (Table 4). Tissue-specific expression of designer mutations in GTPases/GEFs/GAPs is another approach yielding novel insights (e.g., expression of the dominant active [Q70L]ARL2 in photoreceptor cells; Wright *et al.*, 2018). There are also large efforts underway to systematically knock out each mouse gene, and these will add both key reagents and important information on the biology of the three families of proteins discussed herein. We did not include such data, but they can be found at the following sites: National Institutes of Health (NIH) Knockout Mouse Project (www.komp.org), Mouse Genome

Informatics (www.informatics.jax.org), and International Mouse Phenotyping Consortium (www.mousephenotype.org).

In addition, many ARF GTPase/GEF/GAP genes give rise to multiple splice isoforms, yet we know little or nothing about how the expression of such isoforms is regulated in different tissues, whether the isoforms have distinct cellular localizations, perform distinct actions, or are regulated through different mechanisms. For example, Cytohesin 1 isoforms differing by the inclusion of a three-nucleotide glycine-coding microexon in the PH domain display differential affinity for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ and localize either to the PM (triglycine isoform) or to the leading edge (diglycine isoform) (Ratcliffe *et al.*, 2018). Whether they perform different functions at those sites is unknown.

KEY QUESTIONS AND CHALLENGES

We reviewed key facets of current knowledge of ARF GTPases and their regulatory GEFs and GAPs. Here, we highlight what we consider the most glaring deficiencies that, if addressed experimentally, will advance our understanding of the underlying mechanisms and regulation of a broad array of essential cell processes.

1. *Functionalities of ARF family GTPases in cells:* We are largely ignorant of how many different functions a single GTPase can perform in a cell, which GTPases support which cellular functions, and the extent to which functional redundancy between different GTPases occurs. In some cases, these functions may be very similar (e.g., ARF1 regulating multiple steps of membrane trafficking), while in others they may be distinct (e.g., ARL2 acting from the intermembrane space to regulate mitochondrial fusion and from the cytosol to regulate αβ-tubulin assembly). When a single GTPase performs multiple functions at distinct intracellular sites, how is its distribution regulated, and how are the distinct functions coordinated to achieve integrated cellular homeostasis?
2. *What subset of ARFs, GEFs, and GAPs is used in a given cellular response?* It is well accepted that, if a regulatory GTPase is involved in a specific pathway, it will need an upstream GEF and a downstream GAP/effector to serve that regulatory role. In vitro studies using purified components reconstituted on membranes provide a powerful means to decipher complex regulatory properties at the molecular level, determine affinities and specificities, and generate testable hypotheses to interrogate these mechanisms in the cell. However, in vitro conditions are poor mimetics of those in a cell, and it is challenging to identify how such mechanisms are mobilized, altered, or combined by the cell to generate a specific response.
3. *ARF/GEF/GAP effectors/interactomes:* We are largely ignorant of the proteins that bind to each GTPase/GEF/GAP and how such interactions influence their activity and/or downstream events. Do the effectors/interactomes differ depending on location, and what defines the order, hierarchy, and cooperativity of such interactions? For example, do GEFs participate in the selection of effectors, that is, do GEFs both activate ARFs and bind ARF effectors/GAPs to promote the specificity of the downstream event, perhaps serving as a scaffold, as shown for GBF1 binding the γ-COP component of the coatomer (Deng *et al.*, 2009)? Our fragmentary knowledge of ARF family effectors and the downstream actions they perform is largely due to three technical difficulties. First, many GTPase-GAP/effector interactions occur within the constricted diffusion of effectors "solid phased" on the membrane surface and have relatively weak affinities in solution, making many common techniques of interactor identification (e.g., coimmunoprecipitation, affinity chromatography, or copurification) of limited utility. Second, ARFs often work in concert with phospholipids in so-called coincidence detection mechanisms, in which the interactions may require a particular lipid composition or membrane curvature. One example of this is the recruitment of the AP-1 clathrin adaptor complex to endosomal membranes, which requires its simultaneous binding to both ARF1 and the phosphoinositide PI(4)P (Ren *et al.*, 2013). Identification of new ARF effectors may therefore require affinity isolation approaches that incorporate lipids. Just such an approach recently identified a lipid-dependent interaction between ARF1 and the actin regulatory WAVE complex (Koronakis *et al.*, 2011). Third, most of these protein are cytosolic and may only transiently and incompletely associate with membranes to perform their key regulatory function(s), making it common for databases designed to catalogue localizations of proteins in cells or interactomes to miss important sites of action (e.g., compare our Figure 1 with data in the Human Protein Atlas: www.proteinatlas.org).
4. *Posttranslational modifications:* ARF GTPases/GEFs/GAPs are subject to posttranslational modifications that include phosphorylation and ubiquitination. These modification are transient and are likely to play important roles in localization, activation, selection of binding partners, and biological outputs. However, very few studies have analyzed the consequences of posttranslational modifications on protein function(s) or identified the responsible kinase or other modifiers. We also are ignorant of how the functional or metabolic status of a cell influences phosphorylation of specific proteins to evoke the appropriate functional response.
5. *Identification of ARL GEFs and GAPs:* This review focuses on the ARF GEFs and GAPs, largely because so little is known about the identity of ARL GEFs, GAPs, or effectors. Although ARLs comprise the largest group of the ARF family, we know the least about them and their regulators/interactors. We believe that the identification and characterization of each new GAP/GEF will provide important new insights into the regulation of essential cell processes. The finding that ELMODs, purified from mammalian tissues based on their GAP activity toward ARL2, also act on ARFs, showcases our ignorance of important and unexpected means of regulating ARFs as well as ARLs. Such studies increase the complexity and the challenges in sorting out specificities and pathways, but without such missing information, we risk fundamentally misinterpreting a lot of what we think we know about signaling by the ARF family.

SUMMARY

Surprisingly, despite decades of accumulated knowledge on ARF GTPases and their GEFs and GAPs, including an atomic understanding of the GDP/GTP exchange and GTP hydrolysis reactions, we remain ignorant of fundamental and key aspects of their action and regulation. Defining the answers posed here for even a single protein is a daunting task for any investigator. Yet, we argue that studying the entire ARF family of GTPases together and in concert with the families of their GEFs and GAPs will provide substantially more information and is critical to our understanding of 1) sources of specificity and functional redundancies, 2) complexities resulting from one protein acting at multiple sites, 3) enigmas of coordination between multiple GTPases to perform a single function, 4) the interconnections between ARF signaling and other cellular functions, and 5) how the actions of the GTPases/GEFs/GAPs are integrated with cellular physiology and/or contribute to pathology when gone awry. No one laboratory can hope to make more than a small dent in the black box before us. Thus, we hope that this review might serve as

an argument in support of more collaborative efforts to address this large, complex, but vitally important field of ARF signaling.

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