ARF GTPases and their GEFs and GAPs: concepts and challenges

Elizabeth Sztul
University of Alabama, Birmingham

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ARF GTPases and their GEFs and GAPs: concepts and challenges


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.

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...
<table>
<thead>
<tr>
<th>GTPase</th>
<th>Localization</th>
<th>Function(s)</th>
<th>Interactors</th>
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<tbody>
<tr>
<td>Arf1</td>
<td>Cytosol, Golgi</td>
<td>Recruitment of coat complexes, activation of PLD, PI kinases</td>
<td>COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs</td>
</tr>
<tr>
<td>Arf3</td>
<td>Cytosol, Golgi</td>
<td>Recruitment of coat complexes, activation of PLD, PI kinases</td>
<td>COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs</td>
</tr>
<tr>
<td>Arf4</td>
<td>Cytosol, Golgi, endosomes</td>
<td></td>
<td>COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs</td>
</tr>
<tr>
<td>Arf5</td>
<td>Cytosol, Golgi, endosomes</td>
<td>Recruitment of coat complexes, activation of PLD, PI kinases</td>
<td>COP-I, GGAs, MINTs, Cholera toxin, Arfaptin, MKLP1, ARF GEFs, ARF GAPs</td>
</tr>
<tr>
<td>Arf6</td>
<td>PM, endosomes, RE, cortical actin</td>
<td>Cortical actin rearrangement, endocytosis, PLD activation</td>
<td>β-arrestin, POR1, PLD, Cytohesins, MKLP1, FilGAP</td>
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<tr>
<td>Arf7</td>
<td>Golgi, TGN</td>
<td>Endosome–Golgi secretory traffic, LD formation</td>
<td>Arfaptin, MKLP1, PDEd, HRG4, Golgins, GRIP-domain proteins</td>
</tr>
<tr>
<td>Arf7</td>
<td>Cytosol, mitochondria, centrosomes, basal bodies, cilia, RRs</td>
<td>Tubulin heterodimer assembly, mitochondrial fusion, Prenyl-protein traffic</td>
<td>TBCD/β-tubulin, TBCD, ELMOD1-3, BART/ARL2BP, PDEd, HRG4/UNC119</td>
</tr>
<tr>
<td>Arf8</td>
<td>Cytosol, centrosomes, cilia, mitotic spindle, midbody, Golgi</td>
<td>Cytokinesis, Prenyl- and Myr-protein traffic</td>
<td>PDE66, HRG4/UNC119, Golgins, ARL13B, BART/ARL2BP</td>
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<tr>
<td>Arf9</td>
<td>Cytosol, nucleus, TGN, endosomes, PM</td>
<td>Endosome–Golgi traffic, actin remodeling, cell migration</td>
<td>ELMO, GCC185, Robo1, Cytohesin2</td>
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<tr>
<td>Arf10</td>
<td>Cytosol, mitochondria, nucleus, PM, actin</td>
<td>Actin remodeling, neurite outgrowth</td>
<td>HP1, importin-α, Cytohesin2</td>
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<tr>
<td>Arf11</td>
<td>Nucleus</td>
<td>Endosome–Golgi traffic</td>
<td>HP1α, GARP, Regulator</td>
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<tr>
<td>Arf12</td>
<td>Nucleus</td>
<td></td>
<td>HP1α, GARP, Regulator</td>
</tr>
<tr>
<td>Arf13</td>
<td>Nucleus</td>
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<tr>
<td>Arf14</td>
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<tr>
<td>Arf15</td>
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<tr>
<td>Arf16</td>
<td>Cilia</td>
<td></td>
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<tr>
<td>Arf17</td>
<td>Lysosomes, phagolysosomes</td>
<td>Lysosomal traffic and fusion</td>
<td>SKIP-kinesin1b, HOPS complex</td>
</tr>
<tr>
<td>Arf18</td>
<td>Lysosomes, phagolysosomes</td>
<td>Lysosomal traffic and fusion</td>
<td>SKIP-kinesin1b, HOPS complex</td>
</tr>
<tr>
<td>Arf19</td>
<td>Lysosomes, phagolysosomes</td>
<td>Lysosomal traffic and fusion</td>
<td>SKIP-kinesin1b, HOPS complex</td>
</tr>
<tr>
<td>Arf20</td>
<td>Lysosomes, phagolysosomes</td>
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<td>Arf21</td>
<td>Lysosomes, phagolysosomes</td>
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<td>Arf22</td>
<td>Lysosomes, phagolysosomes</td>
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<td>Arf23</td>
<td>Lysosomes, phagolysosomes</td>
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<td>Arf24</td>
<td>Lysosomes, phagolysosomes</td>
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<tr>
<td>Arf25</td>
<td>Lysosomes, phagolysosomes</td>
<td></td>
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<tr>
<td>Arf26</td>
<td>Lysosomes, phagolysosomes</td>
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<tr>
<td>Arf27</td>
<td>Lysosomes, phagolysosomes</td>
<td></td>
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<tr>
<td>Arf28</td>
<td>Lysosomes, phagolysosomes</td>
<td></td>
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<tr>
<td>Arf29</td>
<td>Lysosomes, Golgi, autophagosomes</td>
<td>Ubiquitin ligase, viral infection, membrane trafficking</td>
<td>UBE2D2, TBK1, Cytohesin1</td>
</tr>
</tbody>
</table>

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.
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%
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

<table>
<thead>
<tr>
<th>GAP</th>
<th>Localization</th>
<th>Function(s)</th>
<th>Interactors</th>
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<tbody>
<tr>
<td>Arfgap1</td>
<td>Golgi</td>
<td>ER protein retrieval</td>
<td>γ-Adaptin (AP-1), KDEL receptor/ERD2, p24</td>
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<td>Arfgap2</td>
<td>Golgi</td>
<td></td>
<td></td>
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<tr>
<td>Arfgap3</td>
<td>TGN, EEs</td>
<td>EE–LE transport of M6PR and EGFR</td>
<td>γ-COP (COPI), GGA1/2</td>
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<tr>
<td>Acap1/CENTB1</td>
<td>Rab11 REs</td>
<td>Integrin and TfnR recycling</td>
<td>β1-Integrin, TfnR, clathrin heavy chain</td>
</tr>
<tr>
<td>Acap2/CENTB2</td>
<td>PM, phagocytic cup, ARF6 endosomes</td>
<td>Neurite outgrowth, FcγR-mediated phagocytosis</td>
<td>Rab35</td>
</tr>
<tr>
<td>Acap3/CENTB5</td>
<td>Neurite outgrowth, neuronal migration</td>
<td></td>
<td></td>
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<tr>
<td>Adap1/CENTA1</td>
<td>Membrane ruffles, mito-chondria, dendrites, synapse</td>
<td>Salmonella invasion, beta2-AR internalization, dendritic differentiation</td>
<td>Kif13b</td>
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<tr>
<td>Agap1</td>
<td>AP-3 endosomes</td>
<td>Endosome–lysosome transport</td>
<td>AP-3, Kif2A</td>
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<td>Agap2/PIKE</td>
<td>FAs, Rab4/AP-1-endosomes</td>
<td>Cell migration, neurite outgrowth, invasion, TfnR recycling</td>
<td>FAK, RACK1, Akt, Homer, AP-1</td>
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<tr>
<td>Agap3</td>
<td>Endosomes</td>
<td></td>
<td></td>
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<tr>
<td>Agfg1/HRB, RIP</td>
<td>Clathrin/AP-2/EPS15 vesicles</td>
<td>TfnR endocytosis, HIV-1 replication</td>
<td>Rev</td>
</tr>
<tr>
<td>Agfg2</td>
<td>EGFR endocytosis, macropinocytosis, secretory lysosomes</td>
<td>CIN85, AP-3</td>
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<td>Arap1</td>
<td>EEs, CDRs, podosomes</td>
<td>Cell migration, invasion, RhoGAP stimulation</td>
<td>Rp1, RhoGAP</td>
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<td>Arap2</td>
<td>FAs, APPL EEs</td>
<td>FA turnover, SF formation, integrin endocytosis</td>
<td>RhoA, Arf6, APPL1</td>
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<td>Arap3</td>
<td>Podosome-like adhesions</td>
<td>Cell migration, invasion, Fas, EGFR recycling</td>
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</tr>
<tr>
<td>Asap1</td>
<td>PM, FAs, podosomes/invasion, CDRs</td>
<td>Cell migration, invasion, SF formation, integrin and EGFR recycling</td>
<td>FAK, Crk, CrkL, Src, cortactin, NM2A, PRKD2, CIN85, CDAP</td>
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<tr>
<td>Asap2</td>
<td>Cell periphery, phagocytic cup</td>
<td>Cell migration, FcγR-mediated phagocytosis</td>
<td>Selenoprotein K</td>
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<tr>
<td>Asap3</td>
<td>PM, CDRs</td>
<td>Cell migration, integrin recycling, invasion</td>
<td>Grb2</td>
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<tr>
<td>Git1</td>
<td>FAs, SNX27 endosomes, REs, EEs</td>
<td>Cell migration, invasion, EGFR traffic/degradation</td>
<td>Pix, Arf6, paxillin, MEK1, FAK, SNX6</td>
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<td>Git2</td>
<td>PM, FAs</td>
<td>Cell migration, invasion, beta2-Adrenergic R down-regulation</td>
<td>Vav2, paxillin, GRKs</td>
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<tr>
<td>Smap1</td>
<td>PM</td>
<td>TfnR endocytosis</td>
<td>Clathrin heavy chain</td>
</tr>
<tr>
<td>Smap2</td>
<td>EE, TGN</td>
<td>EE–TGN transport</td>
<td>Clathrin heavy chain, CALM</td>
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<td>ELMOD1</td>
<td>Golgi, nuclear speckles, LDs</td>
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<tr>
<td>ELMOD2</td>
<td>ER, mitochondria, LDs, centrosomes, RRs</td>
<td>Mitochondrial fusion</td>
<td>ARL2, other ARF family GTPases</td>
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<tr>
<td>ELMOD3</td>
<td>PM, actin, lagging edge</td>
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<td></td>
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<td>RP2</td>
<td>PM, microtubules, nucleus</td>
<td>Ciliary traffic</td>
<td>ARL3, UNC119, G protein β1</td>
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</tbody>
</table>

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.**

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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 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.

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![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.](image-url)
<table>
<thead>
<tr>
<th>GTPase</th>
<th>Conventional knockout</th>
<th>Conditional knockout</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>ARF1</td>
<td>Embryonically lethal (E5.5)</td>
<td>—</td>
<td>Hayakawa et al. (2014)</td>
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<tr>
<td>ARF4</td>
<td>Postnatal deletion (Arf4&lt;sup&gt;flox&lt;/sup&gt;/CagCreER): Reduced viability; reduced size of the pancreas; yellowish feces in lower intestine; hair turned from black to gray</td>
<td>—</td>
<td>Pearring et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>Photoreceptor cells (Arf4&lt;sup&gt;flox&lt;/sup&gt;/iCre75): Normal rhodopsin localization; no retinal degeneration</td>
<td></td>
<td>Pearring et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>Kidney (Arf4&lt;sup&gt;flox&lt;/sup&gt;/HoxB7Cre): No cystic disease</td>
<td></td>
<td>Pearring et al. (2017)</td>
</tr>
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<td></td>
<td>Pancreas (Arf4&lt;sup&gt;flox&lt;/sup&gt;/CagCreER): Degeneration of exocrine pancreas; infiltration of adipocytes in exocrine pancreas; normal islet size and organization</td>
<td></td>
<td>Pearring et al. (2017)</td>
</tr>
<tr>
<td>ARF6</td>
<td>Embryonically lethal (midgestation); smaller liver with progressive apoptosis; defective hepatic cord formation</td>
<td>—</td>
<td>Suzuki et al. (2006)</td>
</tr>
<tr>
<td>ARF6</td>
<td>Endothelial cells (Arf6&lt;sup&gt;flox&lt;/sup&gt;/Tie2-Cre): Reduced tumor angiogenesis via impaired HGF-induced endothelial β1-integrin recycling</td>
<td></td>
<td>Hongu et al. (2015)</td>
</tr>
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<td></td>
<td>Neuronal cells (Arf6&lt;sup&gt;flox&lt;/sup&gt;/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</td>
<td></td>
<td>Akiyama et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Platelets (Arf6&lt;sup&gt;flox&lt;/sup&gt;/PF4-Cre): Impaired αIIbβ3-integrin trafficking resulting in reduced fibrinogen uptake and storage</td>
<td></td>
<td>Huang et al. (2016)</td>
</tr>
<tr>
<td>ARL3</td>
<td>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)</td>
<td>—</td>
<td>Schrick et al. (2006)</td>
</tr>
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<td></td>
<td>Retina specific (Arl3&lt;sup&gt;flox&lt;/sup&gt;/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</td>
<td></td>
<td>Hanke-Gogokhia et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>Rod photoreceptor specific (Arl3&lt;sup&gt;flox&lt;/sup&gt;/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)</td>
<td></td>
<td>Hanke-Gogokhia et al. (2016)</td>
</tr>
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</table>

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

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<thead>
<tr>
<th>GTPase</th>
<th>Conventional knockout</th>
<th>Conditional knockout</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARL4</td>
<td>Reduction of testis weight (30%) and sperm count (60%) without affecting fertility</td>
<td>—</td>
<td>Schurmann et al. (2002)</td>
</tr>
<tr>
<td>ARL6</td>
<td>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</td>
<td>—</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>ARL13B</td>
<td>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</td>
<td>—</td>
<td>Caspary et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Kidney specific (Arl13B&lt;sup&gt;fl&lt;/sup&gt;-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 Retina specific (Arl13B&lt;sup&gt;fl&lt;/sup&gt;/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 Tamoxifen-inducible Cre/loxP recombination (Arl13B&lt;sup&gt;fl&lt;/sup&gt;-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 Tamoxifen-inducible Cre/loxP recombination (Arl13B&lt;sup&gt;fl&lt;/sup&gt;-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</td>
<td>—</td>
<td>Li et al. (2016); Hanke-Gogokhia et al. (2017); Dilan et al. (2018); Bay et al. (2018)</td>
</tr>
<tr>
<td>ARFRP1</td>
<td>Embryonically lethal; apoptotic epiblast cells within ectoderm at ED 6.0 and 7.0 Mistargeting of E-cadherin to intracellular compartments</td>
<td>—</td>
<td>Mueller et al. (2002); Zahn et al. (2008)</td>
</tr>
</tbody>
</table>

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

Continues
Conditional knockout

Reference

Zahn et al. (2011). ARL6 acts in ciliary trafficking

Conventional knockout

Reference

Jaschke et al. (2012) E. Sztul (E-cadherin is associated with intracellular membranes);

Zahin et al. (2011; 2010; Zhang et al. 2010).

Intestine specific (Arfrp1+/villin-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

Liver specific (Arfrp1+/alb-Cre):

Nearly abolished triglyceride storage in adipocytes, smaller lipid droplets, impaired lipid droplet fusion, and enhanced lipolysis

Inducible adipocyte specific Tamoxifen-inducible Cre/loxP recombination Arfrp1+/ap2-Cre):

Impaired secretion of adiponectin and recycling of insulin receptor; decreased insulin signaling in adipose tissue and liver.

ENU,

N-ethyl-N-nitrosourea

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

Indicated are the ARF and ARL proteins deleted with or without body knockout (conditional knockout or in all tissues), respectively. (L.D., very low density lipoprotein.

EN1, N-Myc-nearby HGF, hepatocyte growth factor; P, postnatal day. (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 Golgi 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
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 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 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).

ARF's 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

![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.](image-url)
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., coatomer) 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/coatomer/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 COP1 vesicle formation. GBF1 likely activates ARF1 and ARF4, as only the simultaneous removal of these, but not any other pair of ARFs, inhibits COP1 traffic (Zhao et al., 2002, 2006; Volpicelli-Daley et al., 2005; Szu1 et al., 2007; Manolea et al., 2008). GBF1 also acts 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 glycosphosphatidylinositol–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; Tori 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 dumboffound (Dufl), 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 (Duphny 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 (Boulakiba 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 (Renaut et al., 2003). Next, the Sec7d inserts an invariant glutamate (also called the “glutamatic finger”; Beraud-Dufour et al., 1998; Renaut 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 glutamatic 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; Renaut 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., 2003). 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 PI(3,4,5)P₃ and PI(4,5)P₂ 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). Gpr1 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 PI(4,5)P₂ 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 (Karan dur et al., 2017). PI(4,5)P₂ binding increases their catalytic activity, likely by positioning the GEF in an optimal membrane-based orientation with respect to the ARF GTPase (Karan dur 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 Ca²⁺-free calmodulin in vitro, and addition of Ca²⁺ 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 Ca²⁺ with phospholipids for binding to the PH domain, or both acting in synergy remains to be established but raises the question of possible cross-talk between Ca²⁺ 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 (Boula kirba 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 coaterom, 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 Ki2A (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; Nishiyama 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 intrin 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 Ca²⁺ 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 coatamer 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 β-COP component of COPI coatamer (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 archaean 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; Emé 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; Emé 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. Petreková and M. Eliás, 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...
the Asgard archaea, metagenomic assemblies of what appear to be the closest living descendants of the archaean 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

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 superfamilies 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.
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 or amplification or 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 [ARLT51]), 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 knock-out) 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; Pearing 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
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 α-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/effect 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/effectector 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., communoprecipitation, 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 Ptd(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 modifications 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 ARF 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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