Chapter 10
ARF-Like (ARL) Proteins

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Abstract The ARF-like (ARL) proteins, within the ARF family, are a collection of functionally diverse GTPases that share extensive (>40 %) identity with the ARFs and each other and are assumed to share basic mechanisms of regulation and a very incompletely documented degree of overlapping regulators. At least four ARLs were already present in the last eukaryotic common ancestor, along with one ARF, and these have been expanded to >20 members in mammals. We know little about the majority of these proteins so our review will focus on those about which the most is known, including ARL1, ARL2, ARL3, ARL4s, ARL6, ARL13s, and ARFRP1. From this fragmentary information we extract some generalizations and conclusions regarding the sources and extent of specificity and functions of the ARLs.

Keywords ADP-ribosylation factor-like (ARL) • ARL1 • ARL2 • ARFRP1 • ARL13B • ARL GAP • ARL3 • ARL4 • ARL6

10.1 Introduction

A review of the ARL proteins today really requires one to review the ARF family as a whole and reflect upon the functions and mechanisms that are shared between the ARFs and ARLs and perhaps highlight those that are not. Because we are discussing a gene family we assume a common origin and functionality, with paralogs diverging in sequence and increasingly in functions, with time. Phylogenetic analyses revealed the presence of at least six members of the ARF family in the last eukaryotic common ancestor: ARF, ARL1, ARL2, ARL8, ARFRP1, and SAR1 (Li et al. 2004). Preliminary phylogenetic data coming from a greatly expanded set of proteomes suggest that this is likely an underestimate (M. Elias,

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J. Dacks, and R.A. Kahn; unpublished observation). Nomenclature in the family is still a bit confusing, despite attempts along the way to clarify (Kahn et al. 1992, 2006). ARF1, the founding member of the family, was purified based upon its activity as cofactor for the cholera toxin-catalyzed ADP-ribosylation of its pathophysiological substrate Gs_{α}, the activator of adenylyl cyclase. Thus, the *A*DP-*r*ibosylation *f*actor was termed ARF (Kahn and Gilman 1984). This in vitro assay and later the ability to complement the essential function of ARF1 in the yeast *S. cerevisiae* (Kahn et al. 1991) were completely consistent and became the functional standards of a *bona fide* ARF. Those proteins that fell within the ARF family but lacked these defining activities were deemed ARLs. As a result, they are far more functionally diverse than are the ARFs. Molecular cloning by a number of means, and now genome sequencing, has identified far more genes that encode paralogs in every eukaryotic species; with >20 in humans (Li et al. 2004). All ARLs lie within the ARF family so it is incorrect to ever refer to the ARL family.

The ARFs are described in detail in other chapters so will not be further discussed here beyond the major role they play in the regulation of membrane traffic, through the recruitment of adaptor proteins to membranes (e.g., COPI, GGAs, Mints), and both their regulation of and responsiveness to changes in local phospholipid composition (D'Souza-Schorey and Chavrier 2006; Gillingham and Munro 2007; Inoue and Randazzo 2007; Donaldson and Jackson 2011). This information is important to any discussion of the ARLs for a number of reasons. Not least is that we know far less about any of the ARLs, and virtually nothing about several of them. This has resulted in instances in which reports describing the identification of a new ARL discuss it in terms of how it might work in membrane traffic, based upon homology to the ARFs, but without any experimental support. We now have several examples in which ARF family members regulate essential cellular functions that have little or nothing to do with membrane traffic.

The role of ARFs and ARLs as regulators of the assembly of multi-protein complexes is likely widespread. It also seems likely that the localized regulation of the biosynthesis of distinct lipids, particularly phosphatidylinositol phosphates (PIPs), and responsiveness of ARL signaling to those changes will be common among a large number of ARF family members. But the GTP-dependent, N-terminal myristoyl switch that is also a hallmark of the ARF proteins is *not* shared by many of the ARLs (perhaps only ARL1 and the ARL4's). The myristoyl switch is essential to the translocation of ARFs from the cytosol to membranes to initiate recruitment of other proteins and assembly of a protein coat on a nascent budding vesicle. But while N-myristoylation is rare among the ARLs, the amphipathic N-terminal extension of about 14 residues (when compared or aligned to other members of the RAS superfamily) is a highly conserved and a very distinctive feature of ARF family members. The conformation of this N-terminal helix is sensitive to the guanine nucleotide bound, so effectively serves as a third switch region, additional to the canonical switch I and II in all regulatory GTPases. The other key structural feature that distinguishes ARFs is the use of an "interswitch toggle," which was identified by homology in all ARF family members save ARL4 and ARL6 (Pasqualato et al. 2002). The interswitch toggle involves a shift in

register along the length of the protein to connect the N-terminal helix to the guanine nucleotide binding site. However, a number of ARLs have very divergent guanine nucleotide-binding properties (e.g., ARL2 (Hanzal-Bayer et al. 2005) and ARL13B (Miertzschke et al. 2013), which suggests that caution is warranted before assuming too much about whether the conservation of some of these canonical properties of ARFs hold true among the ARLs. Thus, one of the challenges ahead is to decipher the extent to which the models emerging for ARFs will hold true for the entire family and which ARLs have evolved distinctive actions and mechanisms for achieving their own cell functions.

A few common themes are important to be kept in mind in order to understand the actions of the ARF family. (1) Effectors lack a defined ARF- or ARL-binding domain and thus no common structural domain has been conserved that allows one to search protein databases for predicted binding partners or effectors. Perhaps the closest thing we have is the GRIP domain, some of which bind ARL1-GTP (Munro and Nichols 1999a; Jackson 2003; Lu and Hong 2003; Panic et al. 2003; Derby et al. 2004; Lu et al. 2004; Wu et al. 2004; Short et al. 2005). (2) Effectors bind specific ARFs and ARLs in a GTP-dependent manner but often with relatively low affinity and a dependence on lipids/detergents, making many commonly used co-purification strategies inefficient. (3) This is not to suggest that high-affinity interactions are not involved; rather simply to point out that effectors of ARFs or ARLs lack a common domain. (4) Each GTPase in the family appears to bind and regulate more than one downstream effector, making the regulation of localization and sources of specificity key questions to address in assessing biological roles of each family member. (5) To date, each ARL is found to localize and act in more than a single cellular location. This emphasizes the central issue of the means of localization, but we also believe this commonly provides essential cross talk between organelles. (6) Finally, the shared or distinctive specificities of ARF family members with regulators, notably the guanine nucleotide activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), are critical determinants of the temporal and spatial regulation of the signals generated, and also provide cross talk between pathways.

10.2 ARL1

ARL1 is the most ARF-like of the ARLs and arguably should have been named ARF7, based upon similarities in localization and apparent cellular actions. ADP-ribosylation factor-like 1 (ARL1) was discovered in a genomic study of *Drosophila melanogaster*, where it was found to be an essential gene for development (Tamkun et al. 1991) and later shown also to be essential for viability in trypanosomes (Price et al. 2005). The mammalian isoform that was later identified (Schurmann et al. 1994) shares 57 % sequence identity with human ARF1, more than any other ARL. Typical of the ARF family, ARL1 contains an amphipathic, N-terminal α-helix, which is myristoylated (Lee et al. 1997), and is required for

membrane recruitment (Lu et al. 2001; Price et al. 2005). Several ARL1 orthologs have been shown to bind and hydrolyze GTP (Tamkun et al. 1991; Lee et al. 1997), an activity influenced by the presence of lipids and detergent (Tamkun et al. 1991). Unfortunately, we know precious little about the regulators of ARL1, with only a single yeast ARL1 GEF, Syt1 (Chen et al. 2010), or ARL1 GAP, GCS1 (Liu et al. 2005), reported.

The structural and nucleotide-binding properties of ARL1 are reminiscent of the ARFs. Similarities extend to functions, as ARL1 is a membrane-associated protein involved in the regulation of membrane traffic at the Golgi apparatus (Munro 2005). Indirect immunofluorescence studies of ARL1 in normal rat kidney (NRK) cells showed it to be Golgi associated, based upon co-localization with the Golgi markers p28 and mannosidase II (Lowe et al. 1996). Electron microscopy with immunogold labeling revealed that ARL1 is specifically localized to the *trans*-Golgi (Lu et al. 2001). Treatment of cells with the microtubule destabilizing drug, nocodazole, which causes Golgi complex fragmentation, resulted in a scattered, punctate staining pattern of ARL1 that co-localized with p28 and mannosidase II (Lowe et al. 1996). Upon disruption of Golgi structure through treatment with brefeldin A, an ARF GEF inhibitor, ARL1, redistributed to the cytosol, as opposed to mannosidase II, which redistributed to the endoplasmic reticulum. ARL1 redistribution into cytosol after brefeldin A treatment differed from that of ARFs, requiring 5 min and 2 min, respectively. Brefeldin A is membrane permeable and binds directly to a subset of ARF GEFs and inactivates them, leading to the loss of activated ARFs in cells, particularly at the Golgi, with consequent release of ARF-dependent adaptors and ARFs themselves into the cytosol within the first couple of minutes of treatment. These data suggest that the slower release of ARL1 from membranes in response to brefeldin A may be a secondary effect of the drug; e.g., perhaps an ARF is involved in recruitment of an ARL1 GEF.

Regulation of ARL1 activity is important for maintaining the structural integrity of the Golgi apparatus, though again the physiologically relevant modulators of ARL1 activity are not known. Expression of the constitutively inactive ARL1 mutant, ARL1[T31N], thought to act by tight association and inactivation of ARL1 GEFs (which remain undefined), results in fragmentation of the Golgi apparatus (Lu et al. 2001). This is the same phenotype observed in cells treated with brefeldin A (Klausner et al. 1992) and cells expressing ARF1[T31N] (Dascher and Balch 1994). The localization of γ-adaptin, a component of the AP-1 coat complex, to the Golgi is disrupted in cells expressing ARL1[T31N] (Lu et al. 2001). Expression of the constitutively active ARL1 mutant, ARL1[Q71L], which lacks the glutamine required for GTP hydrolysis, also disrupts normal Golgi structure. Chinese hamster ovary (CHO) cells expressing ARL1[Q71L] displayed expanded Golgi structures, as marked by the Golgi SNARE protein, GS28 (Lu et al. 2001). Golgi expansion upon ARL1[Q71L] expression is similar to the phenotype observed from ARF1[Q71L], though not as dramatic (Van Valkenburgh et al. 2001). Expression of ARL1[Q71L] also resulted in increased Golgi localization of γ-adaptin and β-COP, a component of the COPI protein complex that associates with non-clathrin-coated transport vesicles (Lu et al. 2001). Increased

localization of ARFs to the Golgi was also observed in cells expressing ARL1 [Q71L]. This effect appeared to be specific to the Golgi as localization of COPII at the endoplasmic reticulum and α-adaptin at the plasma membrane was unaltered by ARL1[Q71L] expression. Brefeldin A treatment typically results in the loss of Golgi-associated AP-1, COPI, and ARFs; however, these proteins were retained at the Golgi in ARL1[Q71L]-expressing cells treated with brefeldin A (Lu et al. 2001). These data suggest a role for ARL1 in the recruitment of AP-1, COPI, and ARFs to the Golgi, though this recruitment appears to be indirect as the brefeldin A-induced cytosolic redistribution of these proteins does not coincide with ARL1 (Lowe et al. 1996).

The list of ARL1 effectors continues to increase and includes the GRIP domain containing Golgins, the ARF GEFs BIG1/2, and interesting but confusing links to the yeast phospholipid flippase DRS2. It is quite possible that the brefeldin A sensitivities of ARL1, which again are quite similar but temporally distinct from those of ARFs, result from the binding and role of ARL1 in recruiting BIGs to membranes. ARL1 recruits the ARF GEFs, BIG1 and BIG2, to the *trans*-Golgi (Christis and Munro 2012), thereby acting upstream of ARF activation at the Golgi. This direct binding of ARL1 to an ARF GEF is conserved in yeast as it also was found to bind the ortholog of the BIGs, GEA2 (Tsai et al. 2013). ARL1 also interacts with and recruits several other proteins to the Golgi apparatus. Yeast two-hybrid screens using the GTPase-defective mutant of ARL1 identified multiple ARL1 binding partners, including PDEδ, SCOCO, Arfaptin 2/POR1, pericentrin, and MLKP1, a subset of which are shared effectors for ARF1 (Lu et al. 2001; Van Valkenburgh et al. 2001). ARL1 directly interacts with a glutamate receptor-interacting protein (GRIP) domain to facilitate the recruitment of Golgi-associated proteins, namely, members of the Golgin family (Munro and Nichols 1999b; Van Valkenburgh et al. 2001; Lu and Hong 2003; Lu et al. 2005). Structural studies revealed that the C-terminal GRIP domain of Golgin-245 forms a homodimer that interacts with two molecules of ARL1-GTP (Panic et al. 2003; Wu et al. 2004), leaving the N-terminal coiled-coil region to project into the cytosol and function in effector recruitment. And analogous in a very general way to the important role that phospholipids play in ARF activities, Lu et al. (2006) found that lipids are also an essential component in the ARL1-dependent recruitment of Golgins. Knockdown of ARL1 or Golgin-97 in HeLa cells inhibited retrograde transport from the endosome to the Golgi, suggesting that ARL1 and Golgin-97 act as a tethering complex in endosome-to-Golgi trafficking (Lu et al. 2004). Importantly, members of two laboratories discovered that ARL1 acts in close conjunction with another family member, ARFRP1, in what may ultimately prove to be a hallmark of the family: GTPase cascades or pathways involving two or more ARF family members.

10.3 ARFRP1

ADP-ribosylation factor-related protein 1 (ARFRP1) was first discovered through PCR-based screenings of cDNA libraries (Schurmann et al. 1995). ARFRP1 shares only 33 % sequence identity with ARF1 and only slightly more with other ARLs, at 39 % identity with ARL3. ARFRP1 exhibits a low level of GAP-independent hydrolysis of GTP, and thus may contrast to ARFs which have none. It also lacks the N-terminal myristoylation site (glycine 2) found in ARFs and ARL1. Instead, it was shown in yeast that N-acetylation of ARFRP1 is required for its localization to the Golgi apparatus and that this is accomplished through interaction with the integral membrane protein, Sys1p (Behnia et al. 2004; Setty et al. 2004). Note that the *S. cerevisiae* ortholog of ARFRP1 continues to be named Arl3p, despite the willingness of curators of other genomes to remedy this confusion. No GEFs or GAPs have been identified for ARFRP1.

ARFRP1 localizes to the *trans*-Golgi, where its activity is required for ARL1 association with the same membranes (Setty et al. 2003; Behnia et al. 2004). Knockdown of ARFRP1 in HeLa cells or knockout in mouse embryos abrogated the Golgi association of ARL1 and its effectors, including Golgin-245 (Zahn et al. 2006). Recruitment of these proteins to the Golgi was dependent upon the GTP-bound, active state of ARFRP1. However, these conclusions may be in contention as a later study found that ARFRP1 is not required for the Golgi localization of ARL1 and its effectors (Nishimoto-Morita et al. 2009). This same study showed distinct roles for ARFRP1 and ARL1 in Golgi-mediated anterograde and retrograde transport, respectively. Export of vesicular stomatitis virus glycoprotein G (VSV-G) from the *trans*-Golgi was inhibited by siRNA-mediated knockdown of ARFRP1 in HeLa cells, but was unaffected by depletion of ARL1. ARFRP1 is required for export of the planar cell polarity signaling protein, Vangl2, through its interaction with AP-1, despite the fact that its traffic was unaffected by ARL1 (Guo et al. 2013). The simplest explanation to reconcile the different dependencies on ARL1 is simply that ARFRP1 uses multiple effectors and only a subset depends upon ARL1 acting downstream.

ARFRP1 is an essential gene and is required for the proper sorting and traffic of diverse signaling proteins. ARFRP1-null mice are embryonically lethal (Mueller et al. 2002), likely resulting from defects in cell–cell adhesion, as the loss of ARFRP1 leads to disruption of E-cadherin transport from the Golgi to the plasma membrane (Zahn et al. 2008). Hepatocyte-specific knockout of ARFRP1 in mice led to reduced expression and impaired transport of insulin-like growth factor 1 (IGF1) and the glucose transporter, GLUT2 (Hesse et al. 2012). These effects led to reduced glycogen uptake and glucose storage in the liver, which ultimately resulted in growth retardation at an early age. The absence of ARFRP1 in liver cells also caused a reduction in lipidation and assembly of very low-density lipoproteins (VLDLs), impairing the ability of the liver to recycle and redistribute lipids to other organs in the body (Hesse et al. 2014).

Emerging roles for ARFRP1 have been described in the formation of lipid droplets (adipocytes) and chylomicrons (enterocytes) (Hesse et al. 2013). Lipid droplets are dynamic organelles that store neutral lipids and are involved in the regulation of lipid metabolism (Martin and Parton 2006). Adipocyte-specific knockout of ARFRP1 in mice resulted in loss of triglyceride storage, decreased lipid droplet size, and increased lipolysis (Hommel et al. 2010). Chylomicrons are lipid-carrying organelles in intestinal cells that transport dietary fat and fat-soluble vitamins to the bloodstream via the lymphatic system (Xiao and Lewis 2012). Enterocyte-specific knockout of ARFRP1 resulted in reduced triglyceride content of chylomicrons and decreased association of lipoproteins, such as apolipoprotein A-I (Jaschke et al. 2012). Through regulating the maturation and lipidation of lipid droplets and chylomicrons, ARFRP1 is essential for the proper regulation of lipid traffic and metabolism.

10.4 ARL2

ARL2 is ubiquitously expressed and highly conserved throughout eukaryotic evolution. It is an ancient protein dating back to the last eukaryotic common ancestor (Li et al. 2004; Logsdon and Kahn 2004) and is essential in all model organisms where it has been studied [including *S. pombe, A. thaliana,* and *C. elegans* (Hoyt et al. 1990; McElver et al. 2000; Radcliffe et al. 2000; Antoshechkin and Han 2002)], with the exception of *S. cerevisiae* (*CIN4*; (Hoyt et al. 1990; Stearns et al. 1990). It has arguably become the most studied ARL to date, both because it is essential and because it has been implicated in a variety of human diseases, including cancer, heart disease, and retinal degeneration (Mori et al. 2006; van Rooij et al. 2006; Beghin et al. 2008, 2009; Nishi et al. 2010). ARL2 acts in at least four different locations within cells: cytosol, mitochondria, centrosomes, and nuclei. No ARL2 GEFs have been described to date and its distinctive nucleotide-binding properties, at least within the ARF family, and tight binding to the tubulin-specific co-chaperone, cofactor D, suggest either that it does not use a GEF or that the mechanism of activation will be different from other members of the ARF family.

ARL2 appears to be capable of functioning as a tumor suppressor with potentially important clinical relevance for chemotherapy. Levels of ARL2 expression in breast cancer cells are directly linked to three-dimensional growth in vitro and tumor growth in vivo. Stable knockdown of ARL2 in MCF-7 cells increased tumor size compared to control MCF-7 cells when injected into adult mammary fat pads of immunodeficient (SCID) mice. Conversely, stable upregulation of ARL2 expression decreased tumor size. The ARL2 expression level was also correlated with sensitivity of MCF-7 cells to several different chemotherapeutics, including taxol, gemcitabine, and doxorubicin (Beghin et al. 2008), with higher levels of ARL2 resulting in greater sensitivity to these agents. There was also a correlation between ARL2 protein levels in primary tumors with tumor size and metastasis in human

breast cancer patients although the analysis was done with a limited sample size (Beghin et al. 2009).

The levels of ARL2 expression also appear to be important to, and therefore tightly regulated in, the heart, with correlations between ARL2 expression levels and heart disease. Falling ATP levels and changes in mitochondrial morphology are indicative of heart disease and immediately precede heart failure (Abel and Doenst 2011; Verdejo et al. 2012). ARL2 is essential for the maintenance of both mitochondrial morphology and ATP levels (Nishi et al. 2010). In the adenine nucleotide transporter-1 (ANT1) knockout mouse, a model for cardiomyopathy (Graham et al. 1997), ARL2 levels in mitochondria drastically increase in heart and skeletal muscle, but not other tissues examined (brain, liver, lung), providing a strong correlative link to the disease (Sharer et al. 2002). Four microRNAs targeting ARL2 are also highly expressed in the heart and two of these microRNAs, miR-15b and -195, are upregulated in cardiac hypertrophy (van Rooij et al. 2006; Sayed et al. 2007; Nishi et al. 2010). These links between ARL2 and heart function are currently thought to result from the required role of ARL2 in mitochondria and in sustaining cellular ATP levels (see below), but this link has not been proven.

Although not implicated directly, ARL2 appears to play important role(s) in normal retinal function, likely as a regulator of its effector proteins: Binder of ARL2 (BART) and HRG4. Mutations in each of these ARL2 binding partners have been linked to retinal degeneration, a disease state closely linked to ciliary dysfunction (Kobayashi et al. 2000; Davidson et al. 2013). The mutation in BART resulted in reduced binding to ARL2 and the loss of BART from the basal body of the cilium in NIH-3 T3 cells. Depletion of ARL2 similarly caused a loss in BART staining at the basal body (Davidson et al. 2013). These data suggest that proper localization of BART to the basal body is facilitated by ARL2 and thereby links each protein to retinal cell function. In a transgenic model of retinal degeneration in which HRG4 was replaced with a truncation mutant, a progressive loss of up to 50 % of ARL2 in retinas accompanied degeneration over the course of 20 months. The truncated HRG4 also displayed a threefold increase in affinity for ARL2, likely leading to a nonfunctional sequestration of ARL2 in retinal cells (Kobayashi et al. 2003; Mori et al. 2006). Thus, each of these tissue-specific clinical manifestations of defects in ARL2 signaling highlights the importance of this ancient cell regulator and compels more basic studies of its biology in eukaryotes in order to provide molecular models of its actions.

ARL2 localizes to several different compartments within the cell and regulates a number of essential cellular processes. ARL2 is not myristoylated like ARFs and ARL1 and displays much more rapid nucleotide exchange, so much so that one may question the need for a canonical ARL2 GEF in cells (Shern et al. 2003; Hanzal-Bayer et al. 2005). It is estimated that ~90 % of the cellular ARL2 is found in cytosol where it is tightly bound to the tubulin co-chaperone, cofactor D. After purification from bovine testes the ARL2/cofactor D complex displayed free exchange of GDP but was unable to bind GTP (Shern et al. 2003). Thus, binding to cofactor D was thought to prevent the activation of ARL2 by keeping it in its GDP-bound state, perhaps analogous to the actions of a guanine nucleotide

dissociation inhibitor (GDI) on members of the RHO family (Sasaki and Takai 1998; DerMardirossian and Bokoch 2005; Cherfils and Zeghouf 2013). Both ARL2 and cofactor D are also found at the centrosome (Cunningham and Kahn 2008). Although it is not known whether they interact at that site, this seems likely based on functional overlap between these two proteins (Bhamidipati et al. 2000; Zhou et al. 2006).

ARL2 and cofactor D are key regulators of microtubule dynamics (for a recent review of cofactor D, see Tian and Cowan 2013). However, while cofactor D was first identified and consistently found to be one of five cofactors required for in vitro folding of functional tubulin heterodimers (Tian et al. 1996), no clear role for ARL2 in tubulin folding has been documented. Rather, it appears to be involved in the regulation of microtubule dynamics through its binding to, and regulation of the activities of, cofactor D and its ability to bind native tubulin dimers and likely microtubules and drive them to an inactive state (Bhamidipati et al. 2000; Tian et al. 2010). Expression of a constitutively active mutant of ARL2, ARL2[Q71L], resulted in an overall loss in microtubule density in Chinese Hamster Ovary cells, with ~35 % of cells exhibiting a complete loss of microtubules. These effects were prevented by the treatment of cells with the microtubule stabilizing drug taxol. ARL2[Q71L] expression resulted in the loss of the ability to polymerize new microtubules (aster formation) after washout of the microtubule depolymerizing drug nocodazole (Zhou et al. 2006). Thus, ARL2[Q71L] appears to be preventing the polymerization of microtubules, perhaps instead of stimulating catastrophe. Expression of ARL2[Q71L] also caused fragmentation of the centrosome and cell cycle arrest during M phase (Zhou et al. 2006). These data suggest that ARL2 [Q71L] binds and sequesters a component(s) of the centrosome, e.g., the γ-tubulin ring complex, that is essential for the polymerization of microtubules.

In MCF-7 cells stably transfected with ARL2 siRNA or expression vectors to modify cellular levels of ARL2, there was a correlation between ARL2 content and the rates of both microtubule growth and catastrophe (Beghin et al. 2007). The proposed mechanism of ARL2 in regulating these processes is by regulating the amount of polymerizable αβ-tubulin heterodimers present in cells as there was also a correlation between these levels and ARL2 content in cells. This role for ARL2 is further supported by the finding that overexpression of ARL2 prevents the loss of microtubules resulting from overexpression of cofactor D (Bhamidipati et al. 2000; Tian et al. 2010). ARL2 and cofactor D have also been implicated in the dissociation of the apical junctional complex through an unknown mechanism that appears to be independent of their roles in the regulation of microtubule dynamics (Shultz et al. 2008).

Examination of ARL2 in the unicellular parasite *Trypanosoma brucei* revealed roles in cytokinesis consistent with its roles in regulating microtubule dynamics in mammalian cells (Price et al. 2010). Depletion or overexpression of TbARL2 inhibited cleavage furrow formation resulting in cell cycle arrest and multinucleated cells. Taken together, studies of the roles of ARL2 and cofactor D in microtubule dynamics provide the most detailed models of a central function of ARL2 in cells that is certainly highly conserved. However, this is also unlikely to

explain all the clinical or disease-related data. Rather, we believe other sites of action, particularly the mitochondria, may play an important and perhaps central role in ARL2 actions and may be its essential function in a broad array of cells and tissues.

A small fraction (5–10 %) of ARL2 localizes to mitochondria, where it is essential for the maintenance of cellular energy metabolism, mitochondrial motility, and mitochondrial morphology (Sharer et al. 2002; Nishi et al. 2010). Depletion of ARL2 by siRNA in HeLa cells reduces cellular ATP levels by ~50 %, an effect rivaling the strongest ATP poisons. ARL2 has been localized to the inner membrane space (Sharer et al. 2002; Rhee et al. 2013) but is also likely present in the matrix. ARL2 was found in an in vitro assay to bind the adenine nucleotide transporter-1 (ANT1), which resides in the inner mitochondrial membrane and exchanges matrix ATP for ADP in the intermembrane space (Sharer et al. 2002; Bowzard et al. 2005). Interestingly, targeted knockout of ANT1 in mice resulted in a dramatic redistribution of cytosolic ARL2 to mitochondria in heart and skeletal muscle (Sharer et al. 2002), suggesting that the import of ARL2 into mitochondria is a regulated process. Although this led to the model that deficiencies in ATP transport may explain the loss of ATP in ARL2 siRNA cells, reconstitution assays of ANT failed to show any effects of adding ARL2 and BART. Thus, the mechanism of ARL2 in the regulation of ATP levels and the functional consequence of the ARL2/ANT interaction remain unknown. Depletion of ARL2 with siRNAs or expression of ARL2[T30N] also caused mitochondrial fragmentation and clustering of mitochondria around the nucleus. Clustering of mitochondria around the nucleus is indicative of a defect in mitochondrial motility. Thus, ARL2 is required for at least three aspects of mitochondrial function, but the molecular mechanism (s) are unknown. Dissection of these closely related functions in mitochondria and clearly resolving them from effects in other parts of the cell are challenging and a central goal of current research.

Despite the list of essential cell functions linked to ARL2 there are surprisingly few effectors identified. Cofactor D is likely one of those, although early data on the ARL2/cofactor D complex purified from bovine testes implicated it as a GDI, or inhibitor of ARL2 activation, rather than a likely effector. BART was the first ARL2 effector identified, and was purified based on its high-affinity and GTP-dependent binding to ARL2, though it also binds its closest paralog, ARL3 (Sharer and Kahn 1999). A structure of the ARL2-GTP/BART complex has been obtained by x-ray crystallography and was the first to document a direct role for residues in the N-terminal, amphipathic, α-helix in effector binding (Zhang et al. 2009). Like ARL2, a fraction of BART localizes to mitochondria but appears not to mediate any of the three functions described above as its knockdown by siRNA failed to phenocopy any of them. Currently, the only functional information regarding the ARL2/BART interaction is in the nucleus, where BART and ARL2 facilitate STAT3-mediated transcriptional activation, as depletion of BART decreased the levels of phosphorylated STAT3 and transcription of STAT3 target genes following stimulation of STAT3 signaling (Muromoto et al. 2008). Depletion of ARL2 or BART by siRNA or expression of ARL2[T30N] decreased the amount

of STAT3 accumulated in the nucleus upon activation. Thus, the likely mechanism of ARL2 in STAT3 signaling is as a nuclear retention signal for STAT3 as part of a complex with BART and STAT3. It is unfortunate that more is not known about the actions of ARL2 in the nucleus as indirect immunofluorescence staining of cells in culture with ARL2-specific antisera shows a very strong signal there. The small size of BART (19 kDa) and the permeability of the nuclear pore have made accurate determination of the size of the nuclear pool of ARL2 by cell fractionation impossible.

Finally, ARL2 has been linked to the transport of farnesylated GTPases outside of the ARF family. The ARL2 binding partner PDEδ binds to farnesylated GTPases and sequesters the hydrophobic moiety to increase solubility and promote transport between membranes (Williams 2011). ARL2 binds PDEδ directly and in a GTP-dependent manner to stimulate the release of farnesylated Rheb or Ras from PDEδ (Hanzal-Bayer et al. 2002, 2005; Ismail et al. 2011). Several structural studies of ARL2 and PDEδ suggest an allosteric mechanism of ARL2-stimulated release of cargo from PDEδ (Renault et al. 2001; Hanzal-Bayer et al. 2002; Ismail et al. 2011; Watzlich et al. 2013).

Interestingly, though less directly, ARL2 also has been linked to actin-based cilia and functions in the inner ear. After the discovery of ELMOD2 as the first ARL2 GAP (Bowzard et al. 2007; East et al. 2012) and description of its paralogs, ELMOD1 and ELMOD3, as sharing GAP activity for members of the ARF family it was recently discovered that mutations in each of these paralogs are linked to deafness in mice (Johnson et al. 2012) and humans (Jaworek et al. 2013), respectively. Though these GAPs were first discovered through actions as ARL2 GAPs there is currently no evidence demonstrating a role for ARL2 in stereocilia or that defects in ARL2 signaling contribute to deafness. Indeed, a recent study found that ELMODs are very active as GAPs directed against a number of different members of the ARF family (Ivanova et al. 2014). However, the fact that ELMODs are single domain proteins, and that domain has been shown to be the GAP domain (East et al. 2012), is strongly suggestive that one or more members of the ARF family will be found to regulate key aspects of stereocilia development or biology.

10.5 ARL GAPs

A wealth of information is currently available regarding ARF GEFs and ARF GAPs and regulators of the two SARs (Casanova 2007; Gillingham and Munro 2007; Kahn et al. 2008; Miller and Barlowe 2010; East and Kahn 2011; Schlacht et al. 2013). Considerably less is known about the regulators of the ARL proteins despite there being far more ARLs than ARFs. To date, only five GAPs for any of the 22 mammalian ARLs have been identified and there are no known GEFs. The five mammalian ARL GAPs include the three paralogous ELMO domain containing proteins ELMOD1-3, cofactor C, and retinitis pigmentosa 2 (RP2) (Bowzard et al. 2007; Veltel et al. 2008a; Jaworek et al. 2013). In the yeast

S. cerevisiae the ARF GAP Gcs1p is also active as an ARL1 GAP (Liu et al. 2005). Although the evidence that Gcs1p is a bona fide ARL1 GAP is convincing (Liu et al. 2005), there is currently no evidence that this activity is conserved in the human ortholog of Gcs1, ARFGAP1. It is also difficult to differentiate the function(s) of Gcs1p as an ARL1 or ARF GAP, given the spatial and functional overlap in the GTPase substrates. We focus here primarily on the mammalian ARL GAPs. ELMODs have been implicated in deafness, idiopathic pulmonary fibrosis (IPF), and a diverse array of cellular functions. Cofactor C is involved primarily in tubulin polymerization. RP2 facilitates traffic of proteins to the cilium and is mutated in the most severe form of retinitis pigmentosa. Each of these activities and links to disease are discussed in detail below. Because these five known GAPs have been studied most extensively or were first discovered as ARL2/ARL3 GAPs we have positioned this section between these two GTPases. As described below, the specificities of these GAPs are incompletely characterized, but such data are likely to reveal more specific roles in multiple signaling pathways.

ELMODs are ancient proteins with promiscuous specificity for ARF family GTPases (East et al. 2012; Ivanova et al. 2014). The three ELMODs make up one-half of the ELMO family of proteins in humans, named for the functions of ELMO1 and ELMO2 in regulating *e*ngu*l*fment and cell *mo*tility. The only region of homology shared by ELMODs and ELMOs is within a single domain termed the ELMO domain and there is currently no evidence that ELMO1-3 possess GAP activity for any ARF family GTPase. Phylogenetic analysis of the ELMO family of proteins confirmed that ELMODs and ELMOs represent two phylogenetically distinct groups of proteins (East et al. 2012). ELMODs were found in a wide sampling of eukaryotes and were likely present in the last eukaryotic common ancestor, suggesting some function of the ELMODs that is certainly ancient and likely essential. In contrast, the ELMOs arose later in evolution, contain within them additional domains, and lack the conserved, putative catalytic arginine required for GAP activity (East et al. 2012). ELMOD1 and ELMOD2 were the first mammalian ARL GAPs to be reported and exhibited GAP activity against ARL2 (Bowzard et al. 2007). Though initially reported to lack ARL2 GAP activity, we later found that ELMOD3 does have such activity, but its specific activity is very low, compared to its paralogs ELMOD1 and ELMOD2 (Ivanova et al. 2014). ELMOD1-3 possess highly variable levels of GAP activity against ARL1, ARL2, ARL3, ARF1, and ARF6 but are inactive as GAPs for ARL13B (Jaworek et al. 2013; Ivanova et al. 2014). These results are intriguing because ELMODs lack the canonical ARF GAP domain present in every other known ARF GAP that includes a four cysteine zinc finger and conserved, catalytic arginine (Schlacht et al. 2013). The GAP domain of ELMODs lies within the ELMO domain and a highly conserved arginine residue was found to be essential for efficient GAP activity for both ARL2 and ARF1, suggesting a single, novel GAP domain (East et al. 2012). Thus, ELMODs and Gcs1p represent the first evidence for cross talk between ARFs and ARLs at the level of GAPs. Each of the three ELMODs also binds the non-opioid sigma-1 receptor (SigmaR1). When co-expressed and purified as a complex with GST-ELMOD1 or GST-ELMOD2, SigmaR1 almost completely

ablated their ARL2 GAP activity (Ivanova et al. 2014). SigmaR1 is an incompletely understood receptor but implicated in an impressive array of human diseases and brain functions (Bourrie et al. 2004; Marrazzo et al. 2005; Hashimoto 2009, 2013; van Waarde et al. 2011; Kourrich et al. 2012; Niitsu et al. 2012; Bhuiyan et al. 2013). A number of agonists and antagonists are known for SigmaR1 and many are in clinical use today, making its binding to ELMODs of particular interest. Currently the best characterized molecular function of Sigma1R is as a chaperone for regulators of calcium signaling and cell survival pathways (Hayashi and Su 2007; Mori et al. 2013). Thus, ELMODs, or the ARF family members they act upon, may play important regulatory roles in one or more of these functions linked to SigmaR1.

ELMOD2 has the highest levels of GAP activities against ARF family members in vitro and has been linked in different ways to human biology or disease. Genetic mapping of six families with familial idiopathic fibrosis (IPF) identified a single haplotype associated with the disease containing ELMOD2 and another uncharacterized gene (Hodgson et al. 2006). Of the two genes only ELMOD2 mRNA expression levels were reduced in the lungs of IPF patients compared to a control group, identifying ELMOD2 as a candidate gene for IPF. Overexpression and knockdown studies of ELMOD2 in A549 cells revealed a potential link between ELMOD2 and antiviral gene expression (Pulkkinen et al. 2010). ELMOD2 localizes to lipid droplets, based upon its presence in several proteomics studies of lipid droplets (Hodges and Wu 2010; Bouchoux et al. 2011) and the finding that epitope-tagged ELMOD2-HA was present on lipid droplets in HeLa cells stimulated with oleic acid (East et al. 2012). There is currently no information regarding the function of ELMOD2 at lipid droplets.

Studies in HeLa cells also revealed a role for ELMOD2 as an ARL2 effector in mitochondria. ELMOD2 localized to the mitochondrial matrix and knockdown of ELMOD2 resulted in mitochondrial fragmentation and clustering around the nucleus, phenocopying depletion of ARL2 (L. Newman, C. Zhang, and R.A. Kahn, unpublished observations). It may appear counterintuitive that a GAP, often viewed as acting antagonistically to its target GTPase, is found to mimic or phenocopy its substrate. However, we have argued previously (East and Kahn 2011) that many, perhaps all, ARF family GAPs function as both temporal regulators of GTPase signaling and effectors of their substrate GTPases. As effectors they become key downstream components in propagation of the relevant biological signal.

ELMOD1 and ELMOD3 have been linked to deafness in mice and humans, respectively, with proposed roles in the proper function and/or maintenance of stereocilia. Two randomly occurring, allelic mutations resulting in deafness in mice have both been linked to ELMOD1 (Johnson et al. 2012). Scanning electron microscopy of cochlear hair cells revealed progressively worsening stereocilia abnormalities including degeneration, fusion, elongation, and complete loss. Stereocilia are actin-based cilia essential to the mechanoelectrical transduction process in hearing. In newborn mice, stereocilia were normal, indicating that stereocilia development was not affected. Thus, ELMOD1 appears to have some

function that is essential for the maintenance of stereocilia. A genetic linkage analysis in a human family revealed a mutation in ELMOD3 linked to nonsyndromic deafness (Jaworek et al. 2013). Immunolocalization of ELMOD3 in the mouse organ of Corti revealed expression in inner ear hair cells and specific localization to stereocilia. ELMOD3 also associates with the actin cytoskeleton. When expressed in MDCK cells GFP-ELMOD3 co-localized with actin at the plasma membrane and this localization was lost when the actin cytoskeleton was disrupted with cytochalasin D. GFP-ELMOD3 was also recruited to actin bundles when co-expressed with the actin bundling protein espin (Jaworek et al. 2013). Proper regulation of actin dynamics is essential for the maintenance of stereocilia structure and to their mechanosensory functions. Localization of ELMOD3 to stereocilia and its close association with actin structures are suggestive of a role for ELMOD3 in the regulation of actin dynamics in that organelle. Because ELMODs contain no domains other than the GAP domain, we currently interpret their roles in stereocilia in the inner ear as implicating roles for one or more ARF family GTPases at that site. Unfortunately, with the broad specificity of the ELMODs as GAPs we cannot currently predict which GTPase(s) is most likely to be involved.

The strongest evidence to date that ELMODs can act as ARF GAPs in cells, and thereby provide potential cross talk between ARF and ARL biologies, comes from studies in cultured cells. When expressed in HeLa cells, ELMOD1-HA localizes to the Golgi at early time points and causes dramatic changes to Golgi morphology at later time points that are reminiscent of the effects of overexpression of ARFGAP1 or of the ARF GEF inhibitor brefeldin A (Yu and Roth 2002; Randazzo and Hirsch 2004; East et al. 2012). This phenotype was interpreted as evidence that ELMOD1 is acting as an ARF GAP at the Golgi because of the correlative decrease in levels of activated ARFs in affected cells (East et al. 2012). ELMOD1-HA also localized to lipid droplets in HeLa cells, similar to ELMOD2, but has not yet appeared in any proteomics datasets from that organelle. We also reported localization of endogenous ELMOD1 at nuclear speckles (East et al. 2012), though the functional significance of this finding is unclear. Roles of ELMOD1 at the Golgi and at lipid droplets are solely based on overexpression of tagged proteins and require additional support.

RP2 and cofactor C are ARL3 GAPs that share structural homology within their GAP domains but not to ELMO domains. RP2 was originally identified as an effector of ARL3 (Bartolini et al. 2002) but was later found to possess GAP activity for ARL3 (Veltel et al. 2008a). Extensive studies of the specificity of RP2 for other ARF family GTPases have not been reported, but RP2 lacks GAP activity for ARFs and has very low activity against ARL2 (Veltel et al. 2008a). The crystal structure of RP2 bound to ARL3 supports a catalytic arginine mechanism for ARL3 GAP activity, as previously described for RAS and ARF GAPs, and mutation of that arginine ablated ARL3 GAP activity (Kuhnel et al. 2006; Veltel et al. 2008a). RP2 shares some structural homology and functional overlap with the tubulin folding chaperone cofactor C (Bartolini et al. 2002; Veltel et al. 2008a). Each protein can stimulate the GTPase activity of tubulin (Bartolini et al. 2002) and cofactor C has

very low GAP activity for ARL3 relative to RP2 (Veltel et al. 2008a). Four residues important for GAP activity of RP2, including its catalytic arginine, are also conserved in cofactor C, suggesting a similar GAP domain and mechanism (Veltel et al. 2008a). That cofactor C exhibits such weak activity for ARL3 and no activity for ARL2 may suggest that ARL3 is not the biological substrate of cofactor C in cells or that those assays omitted important cofactors or co-regulators. These findings are also intriguing because whereas ELMODs are GAPs with dual specificity for ARFs and ARLs, cofactor C and RP2 display dual specificity for ARLs and tubulin. Better quantification of specific activities of each GAP against a broad array of substrates would certainly help researchers assess the likely biological significance of each GAP–GTPase pairing.

RP2 has been linked to the most severe form of retinitis pigmentosa and contributes to the maintenance of photoreceptor function, most likely through its role in regulating the traffic of proteins to the cilium. Several studies have reported mutations in RP2 linked to retinitis pigmentosa in human families. Depletion of RP2 in zebrafish or the targeted knockout of RP2 in mice resulted in retinal degeneration (Schwahn et al. 1998; Miano et al. 2001; Shu et al. 2011; Li et al. 2013). RP2 is ubiquitously expressed and localizes primarily to the plasma membrane (Chapple et al. 2000). RP2 also localizes to the basal body and periciliary ridge of the cilium and to the Golgi complex (Evans et al. 2010). RP2 and ARL3 facilitate vesicular traffic of proteins between the Golgi and the primary cilium (Evans et al. 2010). Co-expression of RFP-RP2 and ARL3[Q71L] resulted in a redistribution of both proteins from the plasma membrane and cytosol, respectively, to co-localize on intracellular vesicles. Using the well-studied cargo VSVG-GFP as a marker for intracellular vesicles, knockdown of RP2 reduced both the number of vesicles in cells and their average distance from the Golgi. Expression of ARL3[Q71L] phenocopied this effect. Finally, RP2 and ARL3 are essential to the maintenance of Golgi morphology as depletion of either by siRNA resulted in the loss in Golgi morphology and dispersion throughout the cell (Zhou et al. 2006; Evans et al. 2010). These data were taken as evidence that RP2 and ARL3 are involved in regulating membrane traffic, though effectors or other details of mechanisms by which they do so are currently lacking.

RP2 is also involved in the transport of lipid-modified cargos to the cilium through the HRG4 pathway discussed in the ARL2 and ARL3 sections of this review. This transport is mediated by a ternary complex between RP2, ARL3, and HRG4 (Veltel et al. 2008b) and at least one cargo has been identified to use this machinery (Wright et al. 2011). RP2 and ARL3 are also necessary for the proper localization of the G_{β} subunit of transducin to the plasma membrane which may also use this transport mechanism (Schwarz et al. 2012b). That a stable complex forms including ARL3 and RP2 is consistent with its functioning as an effector of ARL3 in this transport pathway.

10.6 ARL3

ARF-like 3 (ARL3) was first identified as an expressed sequence tag and through PCR screening in human and rat tissues, respectively (Cavenagh et al. 1994). ARL3 is 43 % identical to ARF1, but lacks activity in the defining ARF assays, described above. Similar to other ARLs (particularly ARL2) and quite different from ARFs, ARL3 possesses a relatively low affinity for guanine nucleotides (Linari et al. 1999), which is only minimally influenced by lipids/detergents or the concentration of Mg^{2+} (Cavenagh et al. 1994). ARL2 and ARL3 exhibit 53 % identity and share many structural features (Hillig et al. 2000; Hanzal-Bayer et al. 2002). Thus, it is not surprising that ARL2 and ARL3 share several GAPs and effectors. Despite the overlap in binding partners, ARL2 and ARL3 participate in distinct, though often related, cellular processes. ARL3 is most clearly shown to be important for the proper regulation of centrosomal/microtubule processes, ciliogenesis, and transport of signaling proteins to the primary cilium. Like other members of the ARF family, it likely plays other roles, e.g., at the Golgi. ARL3 localizes to the Golgi and to intracellular vesicles in cells in culture (Zhou et al. 2006; Evans et al. 2010) and it has been suggested that proper regulation of ARL3 activity is required for vesicle traffic from the Golgi to the primary cilium (Evans et al. 2010). Additionally, expression of the constitutively active ARL3 mutant, ARL3[Q71L], resulted in Golgi fragmentation (Evans et al. 2010). However, we currently lack any models to explain its role at the Golgi or in vesicular traffic.

ARL3 is a microtubule-associated protein that localizes to centrosomes and is required for proper cell cycle progression (Zhou et al. 2006). Indirect immunofluorescence in cultured cells revealed that ARL3 is found on several microtubule-dense structures, including mitotic spindles, midzones, and midbodies. Knockdown of ARL3 by siRNA resulted in altered cell morphology, increased levels of acetylated α-tubulin, and an increase in the number of binucleated cells. Cell cycle defects were also prevalent in ARL3 siRNA-treated cells, as evidenced by a delayed metaphase and failed cytokinesis, which likely explains the increase in binucleated cells. ARL3 also associates with the manchette, a microtubule-based structure required for proper sperm development (Qi et al. 2013). Consistent with this, knockdown of ARL3 in mouse testes caused abnormal sperm development.

In a process similar to the abscission of a dividing cell, ARL3 is also important for tracheal branch fusion in developing *Drosophila* embryos (Jiang et al. 2007; Kakihara et al. 2008). In both processes, membrane deformation is preceded by recruitment of the exocyst complex to the plasma membrane, specifically to the midbody of dividing cells (Gromley et al. 2005), and to the site of tubule formation in tracheal fusion cells (Kakihara et al. 2008). ARL3 was required for the transport of Sec5, a component of the exocyst complex, to the fusion site at the plasma membrane of tracheal fusion cells. ARL3 protein-null *Drosophila* embryos failed to properly form tracheal tubules, likely resulting from inhibited traffic of the exocyst complex to the site of membrane deformation (Kakihara et al. 2008). These studies of the *Drosophila* ARL3 ortholog may provide insight into the mechanism

underlying the cytokinesis defects observed in ARL3-depleted mammalian cells, but further investigations are required to test such a model.

Better understood, or perhaps just more thoroughly studied, is the role of ARL3 in the regulation of ciliary processes, including ciliogenesis and cilia signaling. A phylogenetic study found that ARL3 was present only in ciliated organisms throughout eukaryotic evolution and this provides strong evidence for its role in that organelle (Avidor-Reiss et al. 2004). In a study of the ARL3 ortholog of the protozoan *Leishmania donovani* (*Ld*ARL-3A), it was shown that expression of a constitutively active mutant, *Ld*ARL-3[Q70L], resulted in decreased flagellum length in the extracellular promastigotes (Cuvillier et al. 2000). It was later shown that ARL3 localizes to the connecting cilium of human photoreceptor cells and the primary cilium of cultured mouse embryonic fibroblast (NIH3T3) cells (Grayson et al. 2002; Zhou et al. 2006). A functional link to ciliary processes was established with the development of an ARL3-null mouse model (Schrick et al. 2006) as these mice exhibit several phenotypes that were typical of cilia defects, including cyst formation in the kidney, liver, and pancreas, and impaired photoreceptor development. Mice lacking ARL3 also had severe physical deformities, and all died within three weeks of birth. Interestingly, cilia in the ARL3-null mice appeared to be structurally normal, which suggests that the observed phenotypes are a result of impairment(s) in cilia function rather than in ciliogenesis. This conclusion was further supported by studies of ARL3 in *C. elegans* (Li et al. 2010) in which ARL3-null worms displayed normally structured cilia. However, similar to the study in *Leishmania*, expression of the constitutively active mutant, ARL3 [Q72L], resulted in impaired ciliogenesis. In a fascinating finding that is likely to lead to real insights in some ARL functions, depletion of ARL3 partially rescued the ciliogenesis defects that result from deletion of the worm ARL13 gene (Li et al. 2010). These data provide consistent evidence for the role of ARL3 as a negative regulator of ciliogenesis and provide intriguing functional links between ARL3 and ARL13.

Though not required for cilia formation, ARL3 plays pivotal roles in the regulation of cilia signaling. In *C. elegans*, ARL3 acting in concert with ARL13 is required for the proper regulation of intraflagellar transport through HDAC6-mediated regulation of the association of subcomplex B with the kinesin motor, KIF17 (Li et al. 2010). Through interaction with specific effectors, including PDEδ and HRG4 (also named UNC119), ARL3 is also involved in the targeting of lipid-modified cargos to the primary cilium (Ismail et al. 2011, 2012; Wright et al. 2011; Watzlich et al. 2013). A yeast two-hybrid screen initially identified ARL3 as a binding partner of PDEδ (Linari et al. 1999). PDEδ is responsible for the membrane targeting of a subset of prenylated cargo to the primary cilium (Zhang et al. 2012) with ARL3 functioning as a release factor for the farnesylated cargo (Ismail et al. 2011; Watzlich et al. 2013). This role is further supported by the involvement of the Retinitis Pigmentosa GTPase Regulator (RPGR) in the PDEδ/cargo/ARL3 mechanism (Watzlich et al. 2013). RPGR localizes to the cilium where it regulates ciliary traffic (Brunner et al. 2010). RPGR binds PDEδ directly and is proposed to serve as a scaffolding protein to facilitate ARL3-mediated release of cargo from

PDEδ into cilia (Watzlich et al. 2013). HRG4, a homolog of PDEδ, binds and recruits myristoylated proteins to the ciliary membrane (Constantine et al. 2012) and like PDEδ, ARL3 binding to HRG4 causes the release of bound cargo, such as the ciliary protein nephronophthisis 3 (NPHP3) (Wright et al. 2011; Ismail et al. 2012). ARL3-mediated release of lipid-modified cargo from HRG4 is also regulated by RP2, an ARL3 GAP (Wright et al. 2011). Formation of a ternary complex consisting of ARL3, RP2, and HRG4 precedes the RP2-stimulated inactivation of ARL3 (Veltel et al. 2008b), thereby releasing HRG4 and allowing it to bind and continue transporting additional myristoylated cargo to the primary cilium. It has also been proposed that RP2, HRG4, and PDEδ may coordinate the traffic and association of the heterotrimeric G protein, transducin (Schwarz et al. 2012a). RP2 interacts with the Gβ1 subunit of transducin, and binding to RP2 could be competed with ARL3 (Schwarz et al. 2012b). HRG4 facilitates the membrane targeting of the N-acylated Gα1 subunit (Zhang et al. 2011a). Finally, as the Gγ1 subunit is farnesylated (Lai et al. 1990), it is feasible that PDEδ could be involved in its transport. ARL3 binding to RP2, HRG4, and PDEδ would release the transducin subunits, thus facilitating assembly of the complete heterotrimer (Schwarz et al. 2012a).

10.7 ARL4

The ARL4 subgroup consists of three proteins ARL4A, ARL4C, and ARL4D [previously named ARL4, ARL7, and ARF4L/ARL9, respectively (Kahn et al. 2006)] based on their high level of sequence conservation. ARL4 proteins display rapid guanine nucleotide exchange properties in vitro and all three proteins are believed to exist in a constitutively GTP-bound state in cells (Jacobs et al. 1999). Each ARL4 is N-myristoylated and possesses a poly-basic region at the C-terminus that functions as a nuclear localization signal when fused to GFP (Jacobs et al. 1999). However, only ARL4A has been shown to localize to the nucleus and no function has been identified for any ARL4 protein within the nucleus (Jacobs et al. 1999; Lin et al. 2000). Each of the three ARL4s can recruit the ARF6 GEF, ARNO, to the plasma membrane through direct binding, and both ARL4A and ARL4D can facilitate remodeling of the actin cytoskeleton through activation of Rac1 (Hofmann et al. 2007; Li et al. 2007; Patel et al. 2011). ARL4A also recruits the unconventional Rac1 GEF complex, ELMO1/DOCK180, to the plasma membrane to promote Rac1 GEF activity. Expression of ARL4A or a constitutively active mutant was sufficient to activate this pathway and cause changes to the actin cytoskeleton resulting in cellular protrusions and the loss of actin stress fibers (Patel et al. 2011). Thus, ARL4 proteins are likely important regulators of the ARNO/ELMO/Rac1 signaling pathway to regulate actin dynamics at the plasma membrane, a property shared by ARF6 (Santy and Casanova 2001; Santy et al. 2005). Though how these two GTPases may be coordinated in this regard is unknown.

ARL4A mRNA expression is developmentally regulated and the message is highly expressed in adult testes, consistent with a role for ARL4A in spermatogenesis (Jacobs et al. 1998; Lin et al. 2000; Schurmann et al. 2002; Buttitta et al. 2003). The only reported phenotypes resulting from the targeted knockout of the ARL4A gene in mice were reductions in testis weight and sperm count (Schurmann et al. 2002). However, ARL4A$^{-/-}$ male and female mice remained viable and fertile and there was no reduction in litter size (Schurmann et al. 2002). Thus, ARL4A has some role in spermatogenesis that is not yet fully understood. In cells, ARL4A localizes to the Golgi where it is essential for the maintenance of Golgi morphology and endosome-to-Golgi vesicular traffic. In the proposed mechanism, ARL4A binds the Golgin GCC185 to facilitate the recruitment of cytoplasmic linker-associated proteins 1 and 2 (CLASP1 and CLASP2) that are essential for the maintenance of Golgi structure (Lin et al. 2011).

ARL4C has also been implicated in the vesicular traffic of cholesterol in the reverse cholesterol transport pathway (Engel et al. 2004; Wei et al. 2009; Hong et al. 2011; Sun et al. 2012). Expression of ARL4C is induced by activation of cholesterol export pathways in human macrophages and ARL4C overexpression increased the rate of intracellular cholesterol transport to the plasma membrane (Engel et al. 2004; Hong et al. 2011). Loss of ARL4C activity by expression of a dominant negative mutant also inhibited cholesterol efflux (Engel et al. 2004). In another study, expression of a dominant active mutant of ARL4C, but not wild-type ARL4C, promoted the transport of transferrin from early endosomes to recycling endosomes (Wei et al. 2009). The mechanism of ARL4C in the regulation of vesicular traffic has not been determined but may act similarly to ARL4A, as Golgi morphology was not studied in any of the ARL4C studies. Thus, both ARL4A and ARL4C may play important roles in the regulation of vesicular traffic and Golgi morphology.

ARL4D partially localizes to mitochondria where it has been implicated in the regulation of mitochondrial morphology and membrane potential (Li et al. 2012). A dominant negative mutant of ARL4D, but not the wild-type protein, predominantly localized to mitochondria, resulted in reduced mitochondrial membrane potential, and caused mitochondrial fragmentation (Li et al. 2012). Overexpression of ARL4D also suppressed adipogenesis and lipid storage in 3T3-L1 cells (Yu et al. 2011). Given the theme of overlapping effectors and regulators it will be interesting to compare for example ELMOD2 actions on ARL2 and ARL4D in mitochondria.

10.8 ARL6

ARL6 (also named BBS3) is best known for its link to Bardet–Biedl Syndrome (BBS), a genetic disease resulting from ciliary dysfunction. Several studies have identified mutations in ARL6 linked to BBS in humans, and targeted knockout of ARL6 in mice results in classic BBS phenotypes consistent with other in vivo

models of BBS and with severe ciliary defects (Chiang et al. 2004; Fan et al. 2004; Zhang et al. 2011c; Khan et al. 2013). Indeed, functional studies of ARL6 reveal a role in cilia and the targeting of proteins to cilia. Knockdown of ARL6 in the unicellular organism *Trypanosoma brucei* resulted in the shortening of the motile cilium (Price et al. 2012), and in *C. elegans* ARL6 is specifically expressed in ciliated cells (Fan et al. 2004). Overexpression of ARL6 or constitutively active or inactive mutants of ARL6 in ciliated mammalian hTERT-RPE cells caused defects in cilia length and number (Wiens et al. 2010). Endogenous ARL6 partially localizes to and along the length of the cilium and to basal bodies (Fan et al. 2004; Jin et al. 2010; Wiens et al. 2010; Zhang et al. 2011c). ARL6 is essential for the proper localization of several ciliary proteins, including melanin and Smoothened (Jin et al. 2010; Pretorius et al. 2010, 2011; Zhang et al. 2011c). The proposed mechanism for ARL6 in the targeting of proteins to cilia models it as a regulator of the BBSome coat complex, which traffics membrane proteins specifically to cilia. ARL6/BBS3 binds the BBSome directly and is essential for both the targeting of the BBSome to cilia in cells and for the recruitment of the BBSome to membranes in vitro (Jin et al. 2010). Thus, ARL6 is an essential member of the ciliary transport machinery through its effector, the BBSome. ARL6 has also been implicated in Wnt signaling, but those effects may be secondary to its role in ciliary transport (Wiens et al. 2010). A longer variant of ARL6, BBS3L, was identified in humans, mouse, and zebrafish and has been linked to proper visual function (Pretorius et al. 2010). Specific knockdown of BBS3L in zebrafish or knockout of the BBS3L variant in mice results in visual impairment and retinal degeneration, but none of the other classic BBS phenotypes were observed with loss of ARL6 (Pretorius et al. 2010, 2011). Expression of BBS3L, but not the shorter ARL6 variant, was also sufficient to rescue the visual defects (Pretorius et al. 2010). Thus, this long BBS3L variant has some function that is important for retinal function (s) that is distinct from the shorter and better characterized ARL6 variant.

10.9 ARL13s

ARL13s arose early in eukaryotic evolution and are absent from non-ciliated organisms (Elias, M, Dacks, J, and Kahn, RA; unpublished observations). The single ARL13 gene seen in lower eukaryotes (e.g., flies and worms) diverged into two in mammals: ARL13A and ARL13B. Nothing is currently known about ARL13A but there has been a high level of interest recently in ARL13B as a result of its roles in cilia biology and links to Joubert syndrome in patients.

ARL13B was first identified in an insertional mutagenesis screen in zebrafish that identified mutants resulting in cystic kidneys (Sun et al. 2004) and in mammals in a genetic screen for recessive mutations with abnormal neural tube patterning (Garcia-Garcia et al. 2005; Caspary et al. 2007). The causative point mutation in mice resulted in aberrant splicing leading to loss of exon 2 and consequently of the ARL13B protein. ARL13B is highly enriched in cilia, and as many as 7 phenotypes

related to cilia biology have been described in MEFs for this original allele, termed *Arl13b*^{hnn}. In *Arl13b*^{hnn} embryos, cilia occur in normal numbers, yet are short; in *Arl13b*^{hnn} MEFs, only 10 % of cells are ciliated, compared to 70 % in their wild-type counterparts (Caspary et al. 2007; Larkins et al. 2011). Shorter cilia are likely due to a structural defect, incomplete B tubules in the outer doublets of the axoneme. *Arl13b*^{hnn} MEFs also display constitutive low-level activation of the Shh pathway. Normally, upon Shh ligand stimulation the cell surface Shh receptor Patched 1 (Ptch1) exits cilia, and the obligate transducer of the pathway, Smoothened (Smo), a G protein-coupled receptor (GPCR), becomes enriched on ciliary membranes and localizes evenly along the entire length (Riobo et al. 2006; Polizio et al. 2011; Corbit et al. 2005; Rohatgi et al. 2007). The regulation of this critical dynamic movement is lost in *Arl13b*^{hnn} cells; Smo is present in the cilia of *Arl13b*-deficient cells independently of Shh stimulation, and it displays a patchy distribution along cilia (Larkins et al. 2011). These data argue that ARL13B is required for at least 2 steps in Shh signaling: Shh-dependent Smo import and Smo distribution within the cilium. Other GPCRs display identical defects in distribution within cilia in *Arl13b*^{hnn} (Hamon et al. 1999; Handel et al. 1999; Brailov et al. 2000; Schulz et al. 2000; Berbari et al. 2008; Higginbotham et al. 2012). In addition, the entry of soluble proteins is highly regulated at the transition zone (Anderson 1972; Gilula and Satir 1972; Rohatgi and Snell 2010). Septin 2 is a critical component of this diffusion barrier (Hu et al. 2010) and is itself mislocalized in *Arl13b*^{hnn} cells, making it possible that the Shh-independent enrichment of Smo within the cilium reflects an abnormal diffusion barrier. Shh signaling is mediated by the traffic of Gli transcription factors from cilia to the nucleus, where they activate or repress target genes (Briscoe and Therond 2013).

The number of distinct actions of ARL13B in cilia is uncertain because some of the phenotypes are expected to be secondary to the loss of cilia or major structural defects in the organelle itself. Recently, a non-ciliary role for ARL13B in endocytic recycling was reported (Barral et al. 2012). While it has become the norm for any ARF family GTPase to possess multiple functions in cells (Caster and Kahn 2010), these findings highlight the likelihood that a subset of ARL13B actions may be in traffic to cilia, perhaps comparable to ARL6 with the BBSome (Jin and Nachury 2009; Jin et al. 2010; Nachury et al. 2010; Wei et al. 2012) or ARF4 and the ARF GAP, ASAP1, at the *trans*-Golgi network (Deretic et al. 2005; Ward et al. 2011; Deretic and Wang 2012; Wang et al. 2012). Dissection of distinct ARL13B actions will be a critical step in the development of molecular models of its functions.

Structurally, ARL13s are readily distinguished in the ARF family as having extensions from the C-termini of up to 247 additional residues beyond the canonical ~180 residues of the "ARF domain" that defines the family and includes all canonical GTP-binding motifs. ARL13s lack the site of N-myristoylation (Glycine 2) and instead have a conserved cysteine, the likely site of palmitoylation (Cevik et al. 2010). The 2 paralogs in mammals arose from a common ancestor, but one cannot readily assign the single ARL13 ortholog in lower species as an ARL13A or ARL13B. Human and mouse ARL13A are 256 and 372 residues in length, respectively, and share 62 % identity. Human and mouse ARL13B

share >80 % identity. The human and mouse paralogs share only 31 % identity within each species. For comparison, human ARL13B and ARL13A share with the single *Chlamydomonas* ARL13 31 % and 25 % identity, respectively, and have been found to be functionally homologous. These values are low among ARF family members, though quite high considering the time involved in evolutionary terms, and are skewed to lower values in large part as a result of the relatively low sequence conservation in the C-terminal half of the protein, termed the C-terminal domain or CTD.

The ARL13B protein is made up of 2 domains: the N-terminal canonical GTP binding (G) domain and a C-terminal domain (CTD) that has no clear homology to other proteins or domains (Sun et al. 2004; Caspary et al. 2007; Duldulao et al. 2009; Cevik et al. 2010). The G domain possesses all the hallmarks of a GTPase, including 4 consensus nucleotide-binding motifs and the nucleotide-sensitive switch 1 and switch 2 loops that mediate interaction with effectors and modulators (Joneson et al. 1996; Kuai and Kahn 2000). ARL13B must employ highly unusual, possibly unique, mechanisms for achieving temporal regulation in signaling due to the absence of the conserved glutamine in the second consensus nucleotide-binding motif: WDVGG*Q* in ARFs, FDLGG*G* in ARL13B. This single Q-to-G change is predicted to be critical to ARL13B acting as a GTPase because the homologous Q in ARF (and RAS and heterotrimeric Gα) proteins directly controls both intrinsic and GAP-stimulated GTP hydrolysis; the homologous Q is commonly mutated to generate a dominant-activating GTPase. Thus, ARL13B was predicted to have a very slow rate of inactivation, or to use a different mechanism of GTP hydrolysis, or both (Miertzschke et al. 2013). There is a precedent: RAP1 also lacks the catalytic Q, so uses a distinct mechanism of GTP hydrolysis (Brinkmann et al. 2002; Daumke et al. 2004; Scrima et al. 2008).

Three different missense mutations have been found in patients with Joubert syndrome (Lee et al. 1997; Cantagrel et al. 2008; Thomas et al. submitted). Two of the 3 point mutants in ARL13B that cause Joubert syndrome are in switch 2, R79Q and Y86C, while the third is in the CTD (R200C). *Arl13b*^{hnn} mice are embryonic lethal, yet Joubert patients reach adulthood with specific neural, ocular, and renal defects, consistent with each mutation affecting a subset of *Arl13b* phenotypes (Caspary et al. 2007; Parisi and Glass 2012). Thus, we expect that each of these mutants is a hypomorph and affects a subset of ARL13B effectors. Such mutants offer outstanding tools that often prove invaluable in dissecting roles for ARL13B in cilia and cell biologies. Testing these mutations in model systems has confirmed the importance of those residues to ARL13 functions across species (Duldulao et al. 2009; Cevik et al. 2010). These three residues and mutations have been analyzed structurally using the crystal structure of the ARL13B ortholog from *Chlamydomonas reinhardtii* to generate a number of conclusions and predictions regarding their effects on the protein's structure and functions (Miertzschke et al. 2013). Finally, all three of these mutations are conserved in ARL13As, making it likely that there is some level of functional redundancy between the paralogs in each species that has two genes.

10.10 Summary

Throughout this review we have attempted to highlight both the currently understood key functions of different members of the ARF family as well as the fact that most, likely all, function at more than one location in cells and in different signaling pathways. This paradox, that location is so important and at the same time one ARL acts at multiple locations, is a central aspect of regulatory GTPases that we believe provides the cells with vital cross talk between signaling pathways but also offers challenges in our dissection of specificity in signal output. We summarize the locations of ARF family members in Fig. 10.1. But note that some of these location assignments are based on functional consequences of (over)expression of mutant and/or epitope-tagged proteins. The dangers of tagging ARFs have been well documented (Jian et al. 2010) and certainly extend to at least some (and we should assume all) ARLs. The need for better, specific antibodies that allow localization of endogenous proteins in several different cell and tissue types, and in response to different conditions or stressors, is obvious and should be a goal of the community of researchers working with ARLs. As highlighted in Fig. 10.1 there are multiple members of the ARF family in just about every organelle shown and we have very incomplete information regarding their regulators or extent of functional overlap. This remains one of the key challenges in developing better, more complete, models of ARF family mechanisms.

Not shown in Fig. 10.1 is the fact that each of the members of the ARF family is also found in the cytosol. In fact, in most cases it is likely the majority of the GTPase that resides there and only transiently binds to membranes or proteins there. We mentioned in our introduction the key role of translocation onto a membrane that is thought to be coincident with activation of ARFs and ARL1. This is less of a hallmark of most ARLs, some of which contain nuclear localization signals (ARL4s) or are inside organelles (e.g., ARL2 in mitochondria and the nucleus; ARL13B in cilia). ARL2 is currently the most promiscuous member of the family as far as localization, with most in the cytosol bound to cofactor D, but also pools specifically localizing to mitochondria, the nucleus, centrosomes, and cilia. The extent to which these different pools of a single GTPase interact or, stated differently, the effect of increasing one of those pools at the expense of another creates opportunities for higher order signaling but we suspect also is contributing to some misinterpretation of data (our own and that of others) resulting from protein overexpression. We believe this idea of higher order signaling in the ARF family is an important one and is based in part on the following logic, using ARL2 as the example. (1) Expansion in each of the families of GTPases in the RAS superfamily has been commonplace. (2) Yet there is only one ARL2 found in eukaryotes that has been preserved during the same evolutionary distance that ARFs have expanded from one to six in mammals. (3) ARL2 is present in and performs important/essential functions in multiple cellular locations. (4) We conclude that by maintaining these functions linked to a single GTPase there is consequent cross

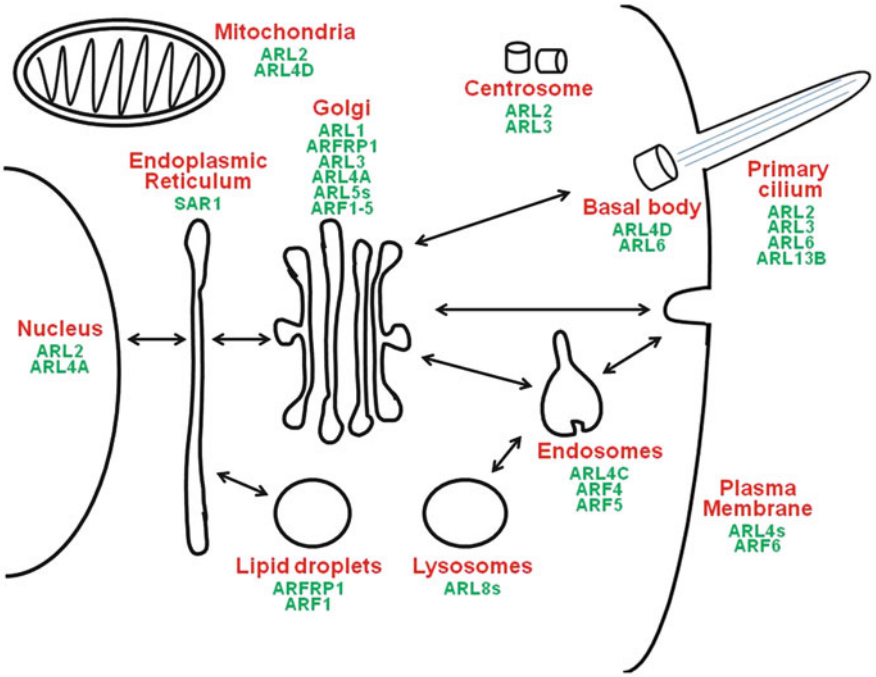

Fig. 10.1 Summary of the cellular locations of members of the ARF family of regulatory GTPases. Organelles (labeled in *red text*) and GTPases (*green text*) are shown, based upon findings described in the text. ARFs are included to highlight their co-localization at several organelles with ARLs but are not described in the text

talk between those locations and functions in cells that is essential to the maintenance of cell homeostasis or signaling.

Finally, we note a number of publications suggesting roles for different ARF family members in aspects of viral or bacterial pathogenesis (Matto et al. 2011; van der Linden et al. 2010; Yang et al. 2011) as well as human cancers (Louro et al. 2004; Beghin et al. 2008, 2009; Taniuchi et al. 2011) and inherited diseases (Parisi and Glass 1993; Chiang et al. 2004; Fan et al. 2004; Cantagrel et al. 2008; Cevik et al. 2010; Zhang et al. 2011b; Thomas et al. submitted). In other RAS superfamily GTPases, both the GTPase and its modulators (particularly GEFs and GAPs) are linked to cancer or other human diseases (e.g., (Shannon et al. 1994; Bollag et al. 1996). Similarly, as the modulators of ARLs are increasingly studied they too are quickly being linked to human disease states (Chapple et al. 2001; Hodgson et al. 2006; Veltel et al. 2008a; Johnson et al. 2012; Jaworek et al. 2013). The assay used to purify the first ARF used its role in cholera toxin action in vitro (Schleifer et al. 1982; Kahn and Gilman 1984) and this was later found to extend also to the closely related *E. coli* heat-labile toxin (Zhu and Kahn 2001; Zhu et al. 2001). The use of ARF activity by bacterial toxins extends to *Legionella pneumophila*, which encodes the RalF protein that contains the ARF GEF Sec7

domain, and this has been shown to be involved in establishment of the replicative organelle (Nagai et al. 2002; Amor et al. 2005; Alix et al. 2012). Thus the ARLs and their interaction partners have already proven to be a rich source of proteins implicated in human diseases and therefore a clinically important group of cell signalers. The fact that several members of the family are ancient regulators of essential cell functions has made them targets for use by opportunistic pathogens that exploit such pathways and mutations in nonessential GTPases or their modulators can impair the normally highly regulated signals into ones more conducive to human pathologies. The facts that ARLs typically perform multiple functions in all cells and that protein–protein interactions are challenging drug targets might dampen enthusiasm for pursuit of ARLs as chemotherapeutic targets. The utility of drugs like Brefeldin A (Misumi et al. 1986; Ishii et al. 1989; Sausville et al. 1996) and Golgicide A (Saenz et al. 2009) in the research setting offer cause for real optimism that drugs of enormous value in the clinic may be within sight (Viaud et al. 2007; Vigil et al. 2010). But for now there are many, many important and unanswered questions to be addressed about the roles and mechanisms of this important family of cell regulators of basic cell function and their links to human diseases.

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