Chapter 8
Arf Proteins and Their Regulators: At the Interface Between Membrane Lipids and the Protein Trafficking Machinery

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Abstract The Arf small GTP-binding (G) proteins regulate membrane traffic and organelle structure in eukaryotic cells through a regulated cycle of GTP binding and hydrolysis. The first function identified for Arf proteins was recruitment of cytosolic coat complexes to membranes to mediate vesicle formation. However, subsequent studies have uncovered additional functions, including roles in plasma membrane signalling pathways, cytoskeleton regulation, lipid droplet function, and non-vesicular lipid transport. In contrast to other families of G proteins, there are only a few Arf proteins in each organism, yet they function specifically at many different cellular locations. Part of this specificity is achieved by formation of complexes with their guanine nucleotide-exchange factors (GEFs) and GTPase activating proteins (GAPs) that catalyse GTP binding and hydrolysis, respectively. Because these regulators outnumber their Arf substrates by at least 3-to-1, an important aspect of understanding Arf function is elucidating the mechanisms by which a single Arf protein is incorporated into different GEF, GAP, and effector complexes. New insights into these mechanisms have come from recent studies showing GEF–effector interactions, Arf activation cascades, and positive feedback loops. A unifying theme in the function of Arf proteins, carried out in conjunction with their regulators and effectors, is sensing and modulating the properties of the lipids that make up cellular membranes.

Keywords ADP ribosylation factor (Arf) • membrane • vesicular trafficking • lipid trafficking • membrane contact site (MCS) • lipid kinase • vesicle coat protein • guanine nucleotide-exchange factor (GEF) • GTPase activating protein (GAP)

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8.1 Introduction

Arf proteins are low molecular weight GTP-binding (G) proteins that are regulated through a cycle of GTP binding and hydrolysis, in which binding of GTP activates and GTP hydrolysis inactivates the G protein (Donaldson and Jackson 2011; Gillingham and Munro 2007b) (Fig. 8.1). In their active GTP-bound form, Arf proteins are tightly associated with the membrane surface. Hence they bring their effectors, proteins that bind specifically to the GTP-bound form, into close contact with the lipid bilayer. These Arf effector proteins include coat complexes that deform membranes and promote cargo sorting, enzymes such as the phosphatidylinositol kinases that alter membrane lipid composition, and actin cytoskeletal components (Table 8.1). Arf1 is the founding member of the family, and was originally identified as a protein factor required for the ADP-ribosylation of the adenylate cyclase activator Gsα by cholera toxin (Schleifer et al. 1982). Although subsequent studies led to the discovery that the major cellular function of Arf1 is regulation of membrane trafficking, its name comes from this initial finding. The original discovery that Arf1 has an essential function in the secretory pathway at the level of the Golgi came from studies in yeast (Stearns et al. 1990a, b). Soon thereafter, the role of Arf1 in recruiting the COPI coat complex to membranes of the early secretory pathway to mediate COPI vesicle budding was demonstrated both in vitro and in cells. The reconstitution in vitro of COPI vesicle budding provided important mechanistic insights (Rothman and Wieland 1996), and the function of Arf1 in cells was greatly aided by use of the specific inhibitor of Arf1 activation, brefeldin A (Klausner et al. 1992).

Mammalian Arf proteins can be divided into three classes based on sequence homology: Class I (Arfs1–3), Class II (Arfs 4–5), and Class III (Arf6). Class I Arfs are highly conserved and are present in all eukaryotes, whereas the Class II Arfs arose during animal cell evolution, diverging from the Class I Arfs in the animal lineage after fungi separated, but before choanoflagellates did (Manolea et al. 2010; Schlacht et al. 2013). Consequently, in all invertebrates, such as *D. melanogaster* and *C. elegans*, there is one member of each of the three Arf classes. In certain lineages, some Arf classes have undergone expansion. For example, vertebrates have multiple members of Class I and II Arf proteins (Li et al. 2004). Although yeast lack Class II Arfs, they have two highly similar Class I Arf proteins and a single Class III member. Plants have numerous Class I Arfs (six in Arabidopsis) that are all more closely related to each other than to Class I Arfs of other eukaryotic supergroups, as well as highly diverged Arfs with some characteristics of mammalian Class III Arf6 (Gebbie et al. 2005). The Arf proteins are part of a larger family that also includes the Arf-like (Arl) proteins. There are more than 20 Arl proteins, with a wide range of functions including membrane trafficking, targeting of proteins to cilia, microtubule regulation, and lysosome function (Donaldson and Jackson 2011; Gillingham and Munro 2007b). The most divergent Arl protein is Sar1, an evolutionarily ancient small G protein that shares a highly related function

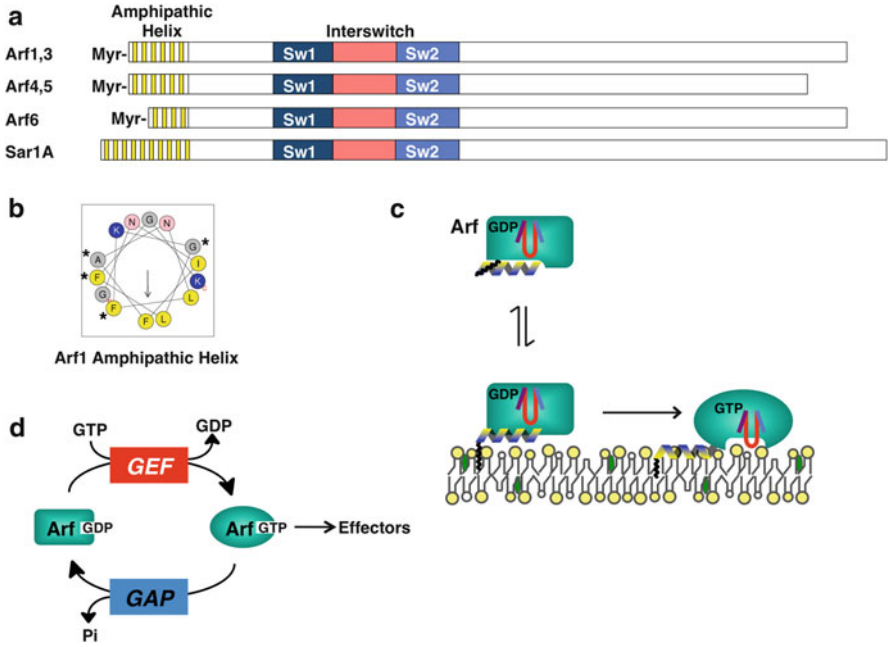

Fig. 8.1 The domain structure and regulation of Arf proteins. (**a**) A schematic representation of the three classes of Arf proteins in humans (Class I: Arf1,3; Class II: Arf 4,5; Class III: Arf6), and the Arf-related protein Sar1 for comparison, showing the conserved amino-terminal amphipathic helix, present in all Arf and Arf-related proteins. The myristoyl (Myr) lipid modifications at the amino terminus of Arf proteins ensure tight membrane association of the GTP-bound form; note that the amphipathic helices of Arf proteins are shorter than the non-myristoylated Sar1 N-terminal helix. The effector regions of the G protein, called switch I (Sw1) and switch II (Sw2), and the interswitch region between them, are depicted. These regions change conformation upon exchange of GDP for GTP, and are involved in interactions with effectors. (**b**) A helical wheel plot of the N-terminal amphipathic helix of Arf1. The α-helical properties were calculated using Heliquest software (http://heliquest.ipmc.cnrs.fr/). The four residues marked with an (*asterisk*) are those that differ between *cis*-Golgi-localized Arf1 and *trans*-Golgi-localized Arf3 (Manolea et al. 2008), and are important for specific localization of each Arf protein. (**c**) Arf-GDP reversibly associates with the membrane surface, and the myristoylated amino-terminal helix ensures tight membrane association of Arf-GTP. The switch and interswitch regions are also shown, which undergo a conformational change upon GTP binding to enter the hydrophobic pocket which the N-terminal amphipathic helix occupies in the GDP-bound form. (**d**) Arf family G proteins undergo a cycle of GTP binding and hydrolysis, mediated by GEFs and GAPs, respectively. The GTP-bound form carries out functions through interaction with effectors, including vesicle coat proteins and enzymes that can modify membrane lipid composition. The spatiotemporal regulation of Arf activation is mediated by the GEFs and GAPs through their interactions with membrane lipids and specific protein partners

to that of the Class I Arfs, the recruitment of a coat complex to membranes to mediate vesicle formation (Lee et al. 2004).

Studies of the evolution of membrane trafficking protein families, including Arf, coat, adaptor, Rab, and SNARE proteins, have revealed a high level of complexity and sophistication of the endomembrane system in the early ancestors of modern

Table 8.1 Arf effectors

Effector	Arf	Location	Arf interacting region
Coat complexes			
COPI	Arf1	Golgi, ERGIC	γ-COP β-COP
	Arfs 4, 5	Golgi, ERGIC	ND
AP1/clathrin	Arfs 1, 3	TGN, endosomes	γ-AP-1, β-AP-1
GGA1, 2, 3/clathrin	Arfs 1, 3	TGN, endosomes	GAT domain
AP3	Arfs 1, 3	Endosomes, TGN	Endosomes, TGN
AP4	Arfs 1, 3	TGN	ε-AP-4, μ-AP-4 (also binds GDP-bound form)
Lipid-modifying enzymes			
FAPP1, FAPP2	Arf1	*trans*-Golgi	PH domain
CERT	Arf1	*trans*-Golgi	PH domain
OSBP	Arf1	*trans*-Golgi	PH domain
PI 4 kinase	Arf1	*trans*-Golgi	*trans*-Golgi
PI4P-5 kinase	Arfs1–6	PM (Arf6)	PM (Arf6)
Phospholipase D	Arfs1–6	PM (Arf6)	PM (Arf6)
Tethers			
GMAP-210	Arf1	*cis*-Golgi	C-terminal GRAB domain
Golgin160	Arf1	Golgi	N-terminus
Exocyst	Arf6	PM	Sec10
G protein regulators			
ARHGAP21	Arf1, 6	Golgi, PM	PH domain, C-terminal helix
Cytohesin/Arno	Arf6, Arl4	PM	PH domain
Scaffolding proteins			
JIP3, JIP4	Arf6	Endosomes, intercellular bridge	Leucine zipper domain (LZII)
FIP3, FIP4	Arfs 5, 6	Recycling endosomes, midbody	CC
Nm23-H1	Arf6	PM, cell junctions	ND
Arfaptin1, arfaptin2	Arf1	Golgi, TGN	BAR domain
Cargo			
Rhodopsin	Arf4	TGN	VxPx targeting motif

AP adaptor protein, *BAR* Bin/Amphiphysin/Rvs, *CC* coiled-coil, *COP* coatomer protein, *ER* endoplasmic reticulum, *ERGIC* ER-Golgi intermediate compartment, *GAT* GGA (Golgi-localized, γ-adaptin homologous, ADP-ribosylation factor-binding protein) and TOM1 homologous, *GRAB* GRIP (golgin-97/RabBP2α/Imh1p/p230)-related Arf binding, *PM* plasma membrane, *TGN trans*-Golgi network; ND, not determined

eukaryotic cells (Dacks and Field 2007). Intriguingly, the last eukaryotic common ancestor (LECA) likely possessed only one Arf family member, in contrast to having already nearly 20 Rab proteins (Koumandou et al. 2013). However, multiple Arf GEFs and GAPs existed in this ancient eukaryotic ancestor, supporting the idea that a key feature of Arf function is a single Arf protein participating in multiple GEF and GAP regulatory complexes (Koumandou et al. 2013; Schlacht et al. 2013). This feature is conserved in modern organisms, for example, in humans, which have only 5 Arf proteins, yet at least 15 GEFs and 31 GAPs. Perhaps a clue as to the

nature of the primordial Arf protein comes from the protozoan parasite *Trypanosoma brucei*, which expresses a single Arf protein that has characteristics of both Class I and Class III mammalian Arfs. TbArf1 is a basic protein with a high pI similar to that of human Arf6, but contains the Golgi-targeting motif MxxE (Price et al. 2007), found in human Arf1 and Arf3 (Honda et al. 2005). TbArf1 localizes to the Golgi, and functional studies indicate that it has roles in both endocytosis and in Golgi–lysosome trafficking (Price et al. 2007).

The spatiotemporal control of Arf protein function is mediated by regulators of Arf GTP binding and GTP hydrolysis (Fig. 8.1d). The Arf guanine nucleotide-exchange factors (GEFs) catalyse GDP release from their substrate Arf, allowing GTP, which is more abundant in cells, to bind. This nucleotide-exchange activity is carried out by the Sec7 domain, a highly evolutionarily conserved domain first identified as a homology domain in the yeast Sec7p protein, and whose function was first identified in the yeast Gea1p protein (Peyroche et al. 1996). A Sec7 domain is present in all Arf GEFs identified to date. The Arf GTPase activating proteins (GAPs) catalyse the hydrolysis of GTP on their substrate Arf, a function carried out by a conserved GAP domain, characterized by the presence of a zinc finger. The Arf GAPs are essential because Arf proteins have negligible intrinsic GTP hydrolysis activity (Kahn and Gilman 1986). The primary sequence homology of the catalytic domains of the GEFs and GAPs facilitated their identification, but their Arf substrate specificity has not been fully elucidated. A recent study of the Arf GAPs has revealed that they have likely co-evolved with their Arf substrates (Schlacht et al. 2013). These results support the conclusion that the Arfs function in a tightly coordinated manner with their regulators.

There are seven subfamilies of Arf GEFs in eukaryotic cells (Cox et al. 2004) (Table 8.2, Fig. 8.2). The GBF/Gea and BIG/Sec7 GEFs are localized to the Golgi, and use Arf1 as a substrate (Donaldson and Jackson 2011). In animal cells and yeast, these GEFs act sequentially, with GBF/Gea proteins functioning at the early Golgi, and BIG/Sec7 proteins at the *trans*-Golgi and *trans*-Golgi network (TGN) (Franzusoff et al. 1991; Peyroche et al. 2001; Zhao et al. 2002). The cytohesin/Arno, EFA6, and IQSEC/BRAG subfamilies function primarily in endosomal–PM trafficking pathways at the cell periphery, and primarily act on Arf6 (Casanova 2007; Cox et al. 2004; Gillingham and Munro 2007b). The EFA6 GEFs regulate endocytosis, endosomal membrane recycling, and actin cytoskeleton remodelling (Casanova 2007; Franco et al. 1999). Yel1p is the orthologue of EFA6 in budding yeast, *Saccharomyces cerevisiae*, exhibiting exchange activity in vitro on Arf3p (the yeast Class III member) (Gillingham and Munro 2007a), which is involved in endocytosis (Smaczynska-de Rooij et al. 2008). Yel1p has a homology domain in the C-terminus resembling that of EFA6 family members (Gillingham and Munro 2007a). Syt1p has a Sec7 domain that most closely resembles that of the IQSEC/BRAG family in mammalian cells, although it lacks an IQ motif (Cox et al. 2004), and functions with Arl3p and Arl1p at the *trans*-Golgi in yeast (Chen et al. 2010). The FBXO8 Arf GEFs contain an F-box in addition to the Sec7 domain, and are present in vertebrates, and at least one invertebrate (Gillingham and Munro 2007b), but are not present in yeast, worms, or flies. Little is known of its function, although

Table 8.2 Arf guanine nucleotide exchange factors

Common protein name (human)	Human gene name	Human uniprot name, accession number	*S. cerevisiae* name, uniprot accession #	*C. elegans*	*D. melanogaster*
GBF1	GBF1	GBF1, Q92538	Gea1p, P47102 Gea2p, P39993	C24H11.7 (gbf-1)	CG8487 (garz)
BIG1	ARFGEF1	BIG1, Q9Y6D6	Sec7p, P11075	Y6B3A.1	CG7578 (sec71)
BIG2	ARFGEF2	BIG2, Q9Y6D5			
EFA6A	PSD1	PSD1, A5PKW4	Yel1p P34225	Y55D9A.1 (efa-6)	CG31158 (Efa6)
EFA6B	PSD4	PSD4, Q8NDX1			
EFA6C	PSD2	PSD2, Q9BQI7			
EFA6D	PSD3	PSD3, Q9NYI0			
IQSEC1/BRAG2/GEP100	IQSEC1	IQEC1, Q6DN90	Syt1p Q06836	M02B7.5	CG32434 (siz, loner, schizo)
IQSEC2/BRAG1	IQSEC2	IQEC2, Q5JU85			
IQSEC3/BRAG3/ synArfGEF	IQSEC3	IQEC3, Q9UPP2			
Cytohesin1	PSCD1	CYH1, Q15438	none	K06H7.4 (grp-1)	CG11628 (Grp1, step/steppke)
Cytohesin2/Arno	PSCD2	CYH2, Q99418			
Cytohesin3/Grp	PSCD3	CYH3, O43739			
Cytohesin4	PSCD4	CYH4, Q9UIA0			
FBXO8	FBXO8	FBX8, Q9NRD0	–	–	–

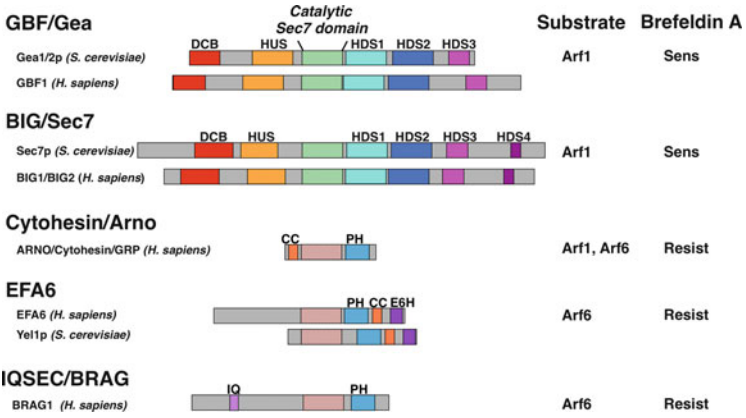

Fig. 8.2 The domain structure of the major cellular families of Arf GEFs. The domains of the five major families of Arf GEFs are shown, approximately to scale. Representative members shown are from humans and budding yeast. The well-characterized Arf substrates for each family are listed, and the sensitivity (Sens) or resistance (Resist) of their exchange activity to brefeldin A is indicated. *DCB* dimerization and cyclophilin binding, *HUS* homology upstream of Sec7, *HDS* homology downstream of Sec7, *CC* coiled coil, *PH* pleckstrin homology, *E6H* EFA6 homology, *IQ* isoleucine–glutamine motif

recently a form of bilateral cleft lip has been associated with a deletion of a chromosomal region including the FBXO8 gene (Calcia et al. 2013). RalF is the founding member of a family of bacterial Arf1 GEFs, first identified in *Legionella pneumophila* and *Rickettsia prowazekii* (Amor et al. 2005; Cox et al. 2004). These GEFs do not have a prokaryotic origin, but rather were incorporated into bacterial genomes through horizontal transfer from their eukaryotic hosts (Nagai et al. 2002). The Sec7 domain of RalF is autoinhibited by the capping domain of the protein, which is relieved by interaction with a specific lipid environment (Alix et al. 2012; Folly-Klan et al. 2013).

There are 11 subfamilies of Arf GAPs, 10 of which are found in humans (Schlacht et al. 2013) (Table 8.3). A recent phylogenetic study has indicated that six Arf GAP families (ArfGAP1, ArfGAP2/3, SMAP, ACAP, AGFG, and the newly identified ArfGAPC2 family) are ancient, probably existing in the LECA prior to separation of the eukaryotic supergroups. On the other hand, ASAP, ARAP, and GIT families arose more recently in evolution, being found only in animals. Through the course of evolution, the Arf GAPs have undergone extensive domain shuffling, losing and gaining different domains in a complex pattern to give rise to the proteins existing today (Schlacht et al. 2013). The PH, BAR, RhoGAP, ankyrin repeat, and C2 domains of Arf GAP proteins are conserved across eukaryotes, and therefore were probably present in the primordial Arf GAPs, whereas other domains such as GOLD, CALM, and SH3 domains are restricted to specific lineages (Schlacht et al. 2013).

The first Arf GAP identified was ArfGAP1 (Cukierman et al. 1995), which has two ALPS motifs in its C-terminal region that mediate specific binding to the highly

Table 8.3 Arf GTPase activating proteins

Common protein name (other name)	Human gene name (other Names)	Human uniprot name, accession number	*S. cerevisiae*, uniprot accession (other fungi)	*C. elegans*	*D. melanogaster*
ArfGAP1	ARFGAP1	ARFG1, Q8N6T3	Gcs1p, P35197	K02B12.7	CG4237 (*Gap69C*)
ArfGAP2	ARFGAP2 (ZNF289)	ARFG2, Q8N6H7	Glo3p, P38682	F07F6.4	CG6838
ArfGAP3	ARFGAP3	ARFG3, Q9NP61			
ARFGAPC2	–	–	–	–	–
SMAP1	SMAP1	SMAP1, Q8IYB5	Age2p, P40529	W09D10.1	CG8243
SMAP2	SMAP2 (SMAP1L)	SMAP2, Q8WU79	Gts1p, P40956		
AGFG1 (Hrb)	AGFG1 (HRB, RAB, RIP)	AGFG1, P52594	(F4NRR3_BATDJ, *B. dendrobatidis*)	–	CG3365 (*drongo*)
AGFG2 (HrbL)	AGFG2 (HRBL, RABR)	AGFG2, O95081			
ACAP1 (Centaurin β1)	ACAP1 (CENTB1, KIAA0050)	ACAP1, Q15027	(I1C4D2_RHIO9, *Rhizopus delemar*)	Y17G7B.15 (*cnt-1*)	CG6742 (*cenB1A*)
ACAP2 (Centaurin β2)	ACAP2 (CENTB2, KIAA0041)	ACAP2, Q15057			
ACAP3 (Centaurin β5)	ACAP3 (CENTB5, KIAA1716)	ACAP3, Q96P50			
AGAP1 (Centaurin γ2)	AGAP1 (CENTG2, KIAA1099)	AGAP1, Q9UPQ3	(I1CMQ2_RHIO9, *Rhizopus delemar*)	Y39A1A.15 (*cnt-2*)	CG31811 (*cenG1A*)
AGAP2 (Centaurin γ1)	AGAP2 (CENTG1, KIAA0167)	AGAP2, Q99490			
AGAP3 (Centaurin γ3)	AGAP3 (CENTG3)	AGAP3, Q96P47			

AGAP4-11	AGAP4 (CTGLF1, MRIP2); AGAP5-10 (CTGLF2-7); AGAP11 (KIAA1975)	AGAP4, Q96P64; AGAP5, A6NIR3; AGAP6, Q5VW22; AGAP7, Q5VUJ5; AGAP8, Q5SRD3; AGAP9, Q5VTM2; AGA10, Q5T2P9; AGA11, Q8TF27;			
ADAP1	ADAP1 (CENTA1)	ADAP1, O75689	–	–	–
ADAP2	ADAP2 (CENTA2)	ADAP2, Q9NPF8			
GIT1	GIT1	GIT1, Q9Y2X7	–	F14F3.2 (*git-1*)	CG16728
GIT2	GIT2 (KIAA0148)	GIT2, Q14161			
ASAP1	ASAP1 (DDEF1, KIAA1249)	ASAP1, Q9ULH1	–	–	CG30372 (*Asap1*)
ASAP2	ASAP2 (DDEF2, KIAA0400)	ASAP2, O43150			
ASAP3	ASAP3 (DDEFL1, UPLC1)	ASAP3, Q8TDY4			
ARAP1	ARAP1 (CENTD2, KIAA0782)	ARAP1, Q96P48	–	F23H11.4 (Rho-GAP only)	CG4937 (Rho-GAP only)
ARAP2	ARAP2 (CENTD1, KIAA0580)	ARAP2, Q8WZ64			
ARAP3	ARAP3 (CENTD3)	ARAP3, Q8WWN8			

curved membranes of COPI vesicles (Bigay et al. 2003, 2005; Mesmin et al. 2007). In an elegant self-organizing mechanism, COPI vesicles are generated through activation of Arf1 at the Golgi, which recruits COPI to deform the membrane into a highly curved vesicle, which then recruits ArfGAP1 through its ALPS motif to hydrolyse the GTP on Arf1, releasing the coat and allowing fusion of the vesicle with its target membrane (Bigay et al. 2003). ArfGAP2 and 3 also bind to COPI vesicles, but do so through direct interaction with the COPI coat, and hence can also bind to COPI-coated regions that are not highly curved (Kliouchnikov et al. 2009; Weimer et al. 2008). ArfGAP1, 2, and 3 and their yeast orthologues Gcs1p and Glo3p function at the early Golgi (Spang et al. 2010), whereas most of the other Arf GAPs function at the cell periphery in TGN–endosomal–plasma membrane trafficking and actin cytoskeleton remodelling (Inoue and Randazzo 2007; Sabe et al. 2006). The following chapter in this volume will describe the latter class of Arf GAPs in depth.

8.2 Localization and Functions of Arf Proteins

A major distinguishing feature of the Arf proteins is the presence of a myristoylated amino-terminal amphipathic helix that is necessary for membrane binding (Fig. 8.1a, b, c). Myristoylation is a cotranslational modification required for the essential functions of Arf1 in vivo (Kahn et al. 1995). In cells, myristoylation is required for correct localization of Arf proteins, including Golgi localization of Arf1 and PM localization of Arf6 (Donaldson and Jackson 2011). In vitro, the myristoyl group of the GDP-bound form of Arf1 is available for membrane insertion, permitting a weak association of the inactive form of Arf1 with membranes (Franco et al. 1995). Upon GTP binding, the N-terminal amphipathic helix of Arf1 is released and inserts into the membrane, resulting in tight membrane association (Antonny et al. 1997) (Fig. 8.1c). Structural studies revealed the details of this change in conformation, showing that the interswitch region changes position to occlude the hydrophobic pocket that harbours the amphipathic N-terminal helix in the GDP-bound form of Arf1 (Goldberg 1998). NMR studies of N-myristoylated Arf1-GTP further confirmed this mechanism (Liu et al. 2010). Thus, in addition to changes in the effector binding regions upon exchange of GDP for GTP, Arf proteins undergo a second change in conformation that brings them into very close contact with the membrane (Fig. 8.1c) (Antonny et al. 1997; Chavrier and Menetrey 2010). This property distinguishes them from other small G proteins of the Ras superfamily, including the Ras, Rho, and Rab Families, which have a long C-terminal linker to which their lipid membrane anchor is attached (Gillingham and Munro 2007b). Arf effectors are thus constrained to a position close to the membrane, in contrast to those of Rab and Rho, which can be located at a distance from the membrane surface (Gillingham and Munro 2007b; Khan and Menetrey 2013).

Another distinction between Arf proteins and those of the Rab and Rho families is that no guanosine diphosphate dissociation inhibitors (GDI) have been identified. All Arfs are tightly membrane bound in their active GTP-bound conformation because of nucleotide regulation of the position of the N-terminal amphipathic helix, and simply hydrolysing the GTP on Arf proteins is sufficient to render them soluble in vitro. Indeed, Arf1 and Arf3 appear to be released from membranes into the cytosol upon GTP hydrolysis in cells. However, Arf6 remains bound to membranes in cells in its GDP-bound conformation. There is also evidence that Arf4-GDP and Arf5-GDP remain membrane bound in cells, in the latter case, to the ER–Golgi intermediate compartment (ERGIC) (Chun et al. 2008; Duijsings et al. 2009). This membrane association is likely due to interaction of the GDP-bound forms with membrane-associated proteins. For Arf6, members of the Kalirin family of Rho GEFs have been shown to bind specifically to the GDP-bound form through their spectrin-like repeat domain (Koo et al. 2007). Arf6-GDP recruits Kalirin to the membrane where it subsequently activates Rac and RhoG to regulate actin dynamics (Koo et al. 2007). Arf6-GDP binds several TBC (Tre-2/Bub/Cdc16) domain-containing proteins, which have Rab GAP activity (Haas et al. 2007), including TBC1D24, a protein mutated in familial infantile myoclonic epilepsy (Falace et al. 2010), and the TRE17 oncogene (Martinu et al. 2004). Hence interactions with the GDP-bound form of a G protein could provide a mechanism for a single Arf protein to trigger alternative signalling pathways depending on the nucleotide bound, which could have important implications in human disease (Donaldson and Jackson 2011).

Following activation on membranes, GTP-bound Arfs recruit coat proteins, lipid-modifying enzymes, tethers, and other effector molecules that modulate the properties of membranes and mediate vesicle trafficking (Table 8.1). The first function of the Arf proteins to be identified was their ability to recruit cytosolic coat proteins to membranes. In the early secretory pathway, Arf1 recruits coatomer complex I (COPI), which sorts cargo proteins into COPI-coated vesicles as it curves the membrane to form the vesicle (Beck et al. 2009). Arf1 at the *trans*-Golgi network (TGN) also recruits the heterotetrameric clathrin adaptor proteins (AP), AP-1, AP-3, and AP-4 and the three monomeric Golgi-localized γ-ear-containing, ADP-ribosylation factor-binding proteins (GGAs 1–3) (Bonifacino and Lippincott-Schwartz 2003). These various coat proteins specifically bind to cargo proteins and incorporate them into forming vesicles for sorting and transport to their correct destination.

Arf proteins can also recruit and activate enzymes that alter membrane lipid composition. The first of these enzymes to be identified was phospholipase D (PLD), which hydrolyses phosphatidylcholine to generate phosphatidic acid (Brown et al. 1993; Cockcroft et al. 1994). PLD is activated by all Arf proteins and also by Arl1 (Hong et al. 1998). PLD activation by Arf6 is involved in a number of processes at the cell periphery, including regulated endocytosis and cell migration (D'Souza-Schorey and Chavrier 2006).

Another major function of Arf proteins is regulation of phosphoinositide levels in cells. All Arf proteins can both recruit to membranes and stimulate the activity of

phosphatidylinositol 4-phosphate, 5-kinase (PIP5K), an enzyme that phosphorylates inositol 4-phosphate (PI4P) at the 5-position to generate phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) (Honda et al. 1999). In cells, it is primarily Arf6 that colocalizes with PIP5K at the PM to generate $PI(4,5)P_2$, which in turn stimulates PM ruffling (Honda et al. 1999). At the Golgi, Arf1 recruits and stimulates the activity of phosphatidylinositol 4-kinase, forming PI4P, an important membrane lipid for Golgi function (De Matteis and Godi 2004a).

8.3 New Insights into Coat Recruitment by Arf Proteins

As described above, Arf1 can recruit the COPI coat to early Golgi membranes, as well as the heterotetrameric clathrin adaptor complexes (AP-1, AP-3, AP-4) and the three monomeric adaptors (GGA1, GGA2, and GGA3) to *trans*-Golgi, TGN, and endosomal membranes. How a single Arf protein can recruit multiple coats to different membrane sites in cells is still not fully understood, but one important contribution to specificity comes from the Arf GEFs. In both mammalian and yeast cells, GBF1 and Gea1/2, respectively, interact directly with COPI (Deng et al. 2009), and knockdown of GBF1 inhibits COPI recruitment to membranes in mammalian cells (Deng et al. 2009; Ishizaki et al. 2008; Manolea et al. 2008; Szul et al. 2007). In contrast, knockdown of BIG1 and BIG2 Arf1 GEFs inhibits AP-1 and GGA recruitment to the *trans*-Golgi (Ishizaki et al. 2008; Manolea et al. 2008). In yeast, a single class I Arf is responsible for recruiting COPI, AP-1, and GGA coats to membranes in cells, and hence mechanisms such as Arf GEF-mediated specificity are required. In mammalian cells, Arfs 3–5 could contribute an additional layer of specificity. It has been shown recently that a specific subset of Arf family members (Arf1, Arf4, and Arf5, but not Arf3 or Arf6) are incorporated into COPI vesicles reconstituted using cytosol (Popoff et al. 2011). A full understanding of the mechanisms determining the specificity of coat recruitment by Arf family members and their regulators is an important open question in the field.

Important insights into the recruitment of coats by Arf1 have come from recent structural studies (Ren et al. 2013; Yu et al. 2012). The structure of Arf1-GTP bound to a subcomplex of the COPI coat revealed a binding site on the γ subunit, which would position the entire COPI complex on the membrane surface in a conformation very similar to the membrane-bound AP-2/clathrin complex (Yu et al. 2012). COPI, AP-2/clathrin, and AP-1/clathrin complexes share a remarkable level of structural similarity, although AP-2 is recruited to membranes by plasma membrane $PI(4,5)P_2$ rather than an activated Arf protein (Jackson et al. 2010). All of these coats have two large subunits that are symmetrically located within the complex, which in the case of COPI are the β and γ subunits. Biochemical studies confirmed a second Arf1-GTP binding site on the β subunit of COPI (Yu et al. 2012). The structure of Arf1 in complex with the entire central trunk region of the AP-1 adaptor complex revealed only one of the two Arf1-GTP binding sites on AP-1, on the β1 subunit (Ren et al. 2013). This Arf1–AP-1 structure

is almost identical to the active, cargo-bound AP-2 complex (Jackson et al. 2010), and reveals a second interaction, involving the back side of Arf, opposite the switch regions, to a second site on the AP-1 γ subunit. Biochemical and cell biological analyses showed that recruitment of the AP-1 adaptor to membranes requires two binding sites for Arf1-GTP (one on each of the symmetrical β1 and γ subunits), in a manner analogous to those found for COPI. However, the conformation of the active Arf1-GTP–AP-1 complex docked to a membrane bilayer is not compatible with binding of Arf1 molecules at both recruitment sites (Ren et al. 2013). Hence the proposed model based on these results is recruitment of a closed conformation of AP-1 by two Arf1 molecules, a change in conformation to the open cargo-bound form of the adaptor with one molecule of cargo engaged, and then a large conformational change mediated by binding of the trunk of the γ subunit to the back side of Arf1-GTP, concomitantly releasing the γ recruitment site (Ren et al. 2013). Given the structural similarities, this model will likely apply to other Arf1-GTP–coat complexes, including COPI.

8.4 New Functions of Golgi-Localized Arfs

The majority of initial studies on the Arf proteins focused mainly on Arf1 at the Golgi and Arf6 at the cell periphery (D'Souza-Schorey and Chavrier 2006; Donaldson and Jackson 2011). This exclusivity was warranted, as Arf1 is the most highly expressed of the Arf proteins in cells, and is essential for viability both in mammalian cells (Reiling et al. 2013) and in budding yeast, *S. cerevisiae* (Stearns et al. 1990a). Arf6 has functions at the cell periphery in cell adhesion and motility that are involved in numerous human pathologies such as cancer, and is the only member of the class III Arfs in mammalian cells. Arf3, Arf4, and Arf5, similarly to Arf1, all localize to internal membranes, including the Golgi. As described above, phylogenetic studies support the conclusion that these other Class I and II Arfs arose late in animal cell evolution, so may be involved in more specialized functions, or in increasing the spatial or temporal resolution of Arf1-mediated processes (Manolea et al. 2010). Interestingly, partial depletion experiments show that under conditions where single knockdowns have little if any phenotypic effects, siRNA depletion of pairs of Arf1–Arf5 has effects on specific membrane trafficking steps (Volpicelli-Daley et al. 2005). For example, the double knockdown of Arf1 and Arf4 affects transport in the early secretory pathway, inhibiting COPI coat recruitment, but has little effect on several other trafficking pathways (Volpicelli-Daley et al. 2005). Consistent with this observation, Arf4 localizes to the ERGIC and *cis*-Golgi (Chun et al. 2008), and together with Arf1 at the *cis*-Golgi, it organizes trafficking between these compartments (Ben-Tekaya et al. 2010).

Arf1 and Arf3 differ only at seven amino acid positions in their amino- and carboxy-terminal regions and previously they were thought to function and localize identically in cells. However, a Golgi-targeting sequence contained within the α-3

helix of Arf1 and Arf3 targets a chimaera between Arf3 and Arf1 to the early Golgi (Honda et al. 2005). Furthermore, Arf3 localizes specifically to the *trans*-Golgi and TGN and this localization depends on four Arf3-specific amino acids contained in the N-terminal amphipathic helix, which are conserved among Arf3 homologues (Manolea et al. 2010) (Fig. 8.1b). Arf3, but not Arf1, becomes cytosolic at 20 °C, the temperature at which exit from the TGN is blocked (Manolea et al. 2010). These results support the conclusion that Arf3 has a critical role during exit from the Golgi.

Recently, Class II Arfs have been shown to participate in novel functions at the TGN. D. Deretic and colleagues have found that Arf4 specifically recognizes the VxPx cytosolic targeting motif in retinal rhodopsin to facilitate its transport into the rod outer segment, a specialized cilium (Deretic et al. 2005). This ciliary targeting complex includes Rab11, FIP3 (a dual Arf and Rab11 effector), and ASAP1, an Arf GAP (Mazelova et al. 2009), in addition to Arf4. The mechanism by which this complex facilitates the packaging of rhodopsin into post-Golgi carriers has not yet been determined, but it is known that rhodopsin itself initiates complex formation by recruiting Arf4. The rhodopsin-binding site of Arf4 has been mapped to the α-3 helix (Deretic et al. 2005), which corresponds to the region of Arf1 that binds the SNARE protein membrin to mediate targeting to the early Golgi (Honda et al. 2005). Hence, the α-3 helix might generally allow Arf protein binding to membrane receptors. Arf4 and Arf5 can also directly bind to the calcium-dependent activator protein for secretion (CAPS), which regulates exocytosis of dense core vesicles from nerve terminals (Sadakata et al. 2010). Arf1 and Arf4 together have been shown to play a role in endosome–TGN trafficking; the double knockdown causes tubulation of the recycling endosome and an inhibition of TGN38 and mannose-6-phosphate receptor trafficking from there back to the TGN (Nakai et al. 2013). How these roles of Arf4 and 5 at the TGN in cells can be reconciled with findings of Arf4 localization to, and Arf4 and 5 functioning at, the early Golgi (Ben-Tekaya et al. 2010; Chun et al. 2008; Popoff et al. 2011) is not known.

A recent study has found a specific role for Arf4 in mediating brefeldin A-induced apoptosis, a pathway used by human pathogens such as *Chlamydia trachomatis* and *Shigella flexneri* (Reiling et al. 2013). In response to brefeldin A and other Golgi-disrupting agents, cells upregulate expression of Arf4 at the transcriptional level, through proteolytic activation of the basic leucine zipper transcription factor CREB3 at the Golgi (Reiling et al. 2013). Interestingly, a previous study found that knockdown of GBF1 caused an upregulation of the unfolded protein response and apoptosis (Citterio et al. 2008), possibly through this newly defined CREB3/Arf4 pathway. These results suggest that Golgi stress might trigger a response through Arf4 aimed at re-establishing Golgi function, and if this fails, mediate a switch to induction of apoptosis (Reiling et al. 2013).

8.5 Arf1 in Lipid Trafficking

A novel function for Arf1 in lipid droplet (LD) metabolism has been uncovered recently (Beller et al. 2008; Guo et al. 2008; Soni et al. 2009). Lipid droplets are well known for their function in storage of energy in the form of triglycerides (Ducharme and Bickel 2008; Londos et al. 2005). More recently, their dynamic structure and integration with membrane trafficking pathways have revealed that they are in fact *bona fide* organelles (Walther and Farese 2012). LDs are distinct from other organelles in having a neutral lipid core surrounded by a phospholipid monolayer (Tauchi-Sato et al. 2002), rather than a bilayer encompassing an aqueous interior. In addition to triglycerides, which are the major energy storage molecules of eukaryotic cells, the neutral lipid core of LDs also contains esterified cholesterol. These two classes of neutral lipids serve as storage precursors of the two major lipid components of cellular membranes. Arf1, along with its GEF GBF1 and effector COPI, associates with LDs (Fig. 8.3) and is required for recruitment of a subset of lipid droplet associated proteins to the LD surface (Soni et al. 2009). In mammalian cells, these LD components include a triglyceride lipase (ATGL) and a perilipin family member (PLIN2) (Soni et al. 2009), and for ATGL at least, this function is conserved in Drosophila (Beller et al. 2008). GBF1 itself is recruited to LDs via HDS1, the domain just downstream of the catalytic Sec7 domain, which binds both liposomes and artificial droplets directly in vitro (Bouvet et al. 2013). This domain and the downstream HDS2 domain are both required for localization of GBF1 to the Golgi. However, when expressed alone as GFP fusions in cells, HDS1 and HDS2 are targeted only to LDs, not to the Golgi (Ellong et al. 2011). The Sec7 domain regulates HDS1 association with membranes, acting as an inhibitor of localization, and the N-terminal DCB and HUS domains are required in addition to target GBF1 to the Golgi (Bouvet et al. 2013).

B. Antonny and colleagues have proposed a novel mechanism of protein recruitment to the unique surface of LDs. In a recent study, they demonstrated that the density of phospholipids in the monolayer surrounding the neutral lipid core of an artificial LD could be decreased, leading as expected to an increase in surface tension (Thiam et al. 2013). Hence in contrast to a bilayer, which would tear with even a small decrease in phospholipid density in one leaflet, the phospholipids of the LD surface can be spread apart, albeit with the unfavourable effect of exposing the hydrophobic core to the aqueous environment of the cytosol. This increase in surface tension could favour the recruitment of cytosolic proteins to the LD, which would compensate for the lower density of phospholipids by helping to cover the LD surface. B. Antonny and F. Pincet further hypothesized that GBF1, Arf1, and COPI might function to recruit proteins to LDs by decreasing phospholipid density, as a result of their removal by budding of small droplets from the surface of a large LD. Indeed, Arf1-GTP and COPI were sufficient to cause budding of 60–100 nm diameter droplets from an artificial droplet surface in vitro (Thiam et al. 2013). In support of their hypothesis, these authors then found that the binding of α-synuclein, known to associate with LDs in cells (Cole et al. 2002), was promoted

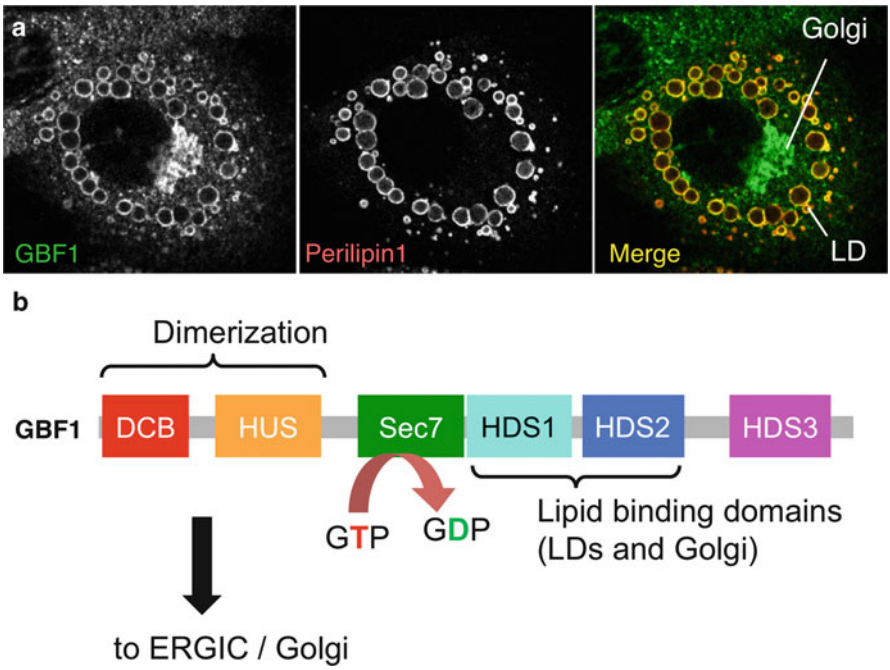

Fig. 8.3 Localization of GBF1 to both lipid droplets and the Golgi apparatus. (**a**) 3T3L1 adipocytes differentiated for 1 week were processed for immunofluorescence using GBF1 and perilipin 1 antibodies (image courtesy of K. Soni). LD, lipid droplet. (**b**) Mechanism of localization of GBF1. The domains shown are as in Fig. 8.2. HDS1 and HDS2 domains are required for Golgi localization, but these domains on their own are targeted to LDs, not the Golgi. The Sec7 domain acts as a negative regulator of the localization of HDS1, rendering this lipid binding domain soluble both in vitro and in cells. The upstream DCB and HUS domains are required for targeting of GBF1 to the Golgi

when the phospholipid density of artificial droplets decreased (Thiam et al. 2013). This result supports the hypothesis that LDs can become more "reactive" when they experience a decrease in phospholipid density on their surface, because of the unfavourable increase in surface tension. Together, these findings provide an elegant explanation for the function of GBF1, Arf1, and COPI in recruitment of proteins to the LD surface.

In addition to its function in lipid metabolism of LDs, Arf1 has an important function in lipid trafficking at the Golgi that is distinct from its role in vesicle formation. A number of lipid transfer proteins are recruited by Arf1 to membranes at sites of close contact between the ER and other organelles, known as membrane contact sites (MCS) (Levine and Rabouille 2005; Stefan et al. 2013). FAPP2, CERT, and OSBP transfer glucosylceramide, ceramide, and sterol, respectively, and all three possess a PH domain that requires both Arf1 and PI4P in order to bind to *trans*-Golgi membranes (De Matteis and Godi 2004b) (Table 8.1). The first clue that these proteins might bridge two different organelles came from studies

indicating that in addition to a late Golgi-specific PH domain, they also carry a motif mediating binding to the ER-localized protein VAP (Lev 2010; Levine and Loewen 2006). A recent study showed that these two localization regions of OSBP are sufficient to mediate contact between the ER and Golgi, and moreover, that OSBP activity regulates this association (Mesmin et al. 2013). FAPP2-mediated delivery of glucosylceramide to the *trans*-Golgi has been shown to be dependent on Arf1 and also to be required for vesicular trafficking from the Golgi to the PM (D'Angelo et al. 2007). Hence Arf1, by recruiting proteins that mediate transfer of sphingolipid precursors and sterol, plays an essential role in establishing the characteristic lipid environment of the *trans*-Golgi, PM, and endosomal system of the cell, an important feature of cellular organization (Bigay and Antonny 2012). A key question regarding these transfer proteins is how they transport lipids such as sterols up their concentration gradient. For OSBP, B. Mesmin and colleagues showed that sterol is exchanged for PI4P at the *trans*-Golgi, where it is returned to the ER and the phosphate hydrolysed by the ER-resident PI4P phosphatase Sac1p. The energy from hydrolysis of phosphate on PI4P drives the transport of sterol up its concentration gradient (Mesmin et al. 2013).

8.6 New Insights into Arf6 Function

Arf6 is localized to the plasma membrane and regulates both the cortical actin cytoskeleton and endosomal membrane recycling. At the plasma membrane, Arf6 modulates membrane lipid composition through activation of PI4P 5-kinase (PIP5K) and PLD, resulting in production of $PI(4,5)P_2$ and PA. These phospholipids can influence the sorting of membrane proteins within the PM, and are important for the formation of clathrin-coated pits during endocytosis. They are also required for the recruitment and activation of Rho family G proteins such as Rac to induce actin polymerization. Arf6 is associated with endosomal membranes derived from clathrin-independent forms of endocytosis, where it functions to recycle membrane components back to the plasma membrane (Grant and Donaldson 2009). Recycling through endosomal compartments by Arf6 is required for the polarized delivery of Cdc42, Rac, and the Par6 complex to the leading edge of migrating cells (Osmani et al. 2010), and for recycling of proteins involved in cell adhesion (Balasubramanian et al. 2007). Arf6 is also associated with clathrin-coated vesicles, where it mediates the rapid recycling of transferrin receptor back to the plasma membrane through interaction with the microtubule motor adaptor protein JIP4 after clathrin uncoating (Montagnac et al. 2011). Arf6 can interact with adaptor protein 2 (AP-2) (Paleotti et al. 2005) and with AP-2 and clathrin during G-protein coupled receptor cell signalling (Poupart et al. 2007). Hence Arf6 has a general function in mediating recycling of components back to the PM after endocytic internalization via different routes.

Studies of the Arf6 homologues in model organisms have illustrated the evolutionarily conserved nature of Arf6 function. Arf3p, the yeast Arf6 homologue,

affects polarization events such as bud site selection in *S. cerevisiae* (Huang et al. 2003), and the switch in cellular growth from monopolar extension to bipolar extension in fission yeast (Fujita 2008). Budding yeast Arf3p contributes to PM PI (4,5)P_2 levels like its mammalian homologue (Smaczynska-de Rooij et al. 2008), and is involved in uncoating of clathrin-coated endocytic vesicles along with its specific GAP Gts1p (Toret et al. 2008). In the filamentous fungus *Aspergillus nidulans*, the Class III ArfB localizes to both the plasma membrane and endomembranes, and regulates endocytosis and polarity establishment during hyphal growth (Lee et al. 2008). Arf6 in Drosophila is also involved in endocytic recycling, which is required for cytokinesis in spermatocytes (Dyer et al. 2007). In mammalian cells, Arf6 is involved in cytokinesis through interaction with JIP4 (Montagnac et al. 2009). The crystal structure of Arf6 in complex with JIP4 shows that residues adjacent to the switch regions are structural determinants for the specific binding of JIP4 to Arf6 (Isabet et al. 2009).

In mammals, Arf6 is not required for early embryonic development, as demonstrated by the fact that homozygous knockout mice can develop to mid-gestation or even to birth, although they die shortly thereafter (Suzuki et al. 2006). Hence, the critical physiological roles of Arf6 in cell adhesion and cell migration appear to be more important for functions that become crucial late in development of the organism, such as wound healing and metastasis, rather than functions necessary for early development. Detailed descriptions of the functions of Arf6 in these processes have been shown in a large number of studies, and the reader is referred to recent excellent reviews on the subject (D'Souza-Schorey and Chavrier 2006; Schweitzer et al. 2011).

8.7 Arf GEFs: Cascades and Positive Feedback Loops

Of the seven subfamilies of Arf GEFs in eukaryotic cells, probably the most intensively studied are the cytohesin/Arno proteins. This subfamily was the first one to be identified in mammalian cells (Chardin et al. 1996), and in addition to a wide range of crucial physiological functions, its members have proved to be highly amenable to biochemical and structural characterization. The cytohesin/Arno GEFs function in plasma membrane–endosomal membrane trafficking routes, in cytoskeleton regulation, as well as in signal transduction pathways important for cell proliferation, immune response, and growth control (Casanova 2007; Kolanus 2007). Members of this GEF family can catalyse exchange on both Arf1 and Arf6 in vitro and in cells, although in vitro they are more efficient GEFs for Arf1 (Casanova 2007; Macia et al. 2001). Cytohesin activation is spatially regulated through relief of autoinhibition, positive feedback loops, and activation cascades. At the plasma membrane, the PH domains of cytohesin family members interact with PM-specific phosphoinositides and with the GTP-bound forms of Arf6 (Cohen et al. 2007) and Arl4 (Hofmann et al. 2007; Li et al. 2007), leading to cytohesin recruitment and further activation of Arf6 or Arf1 at the plasma membrane.

A crystal structure of the autoinhibited Sec7 domain in tandem with the PH domain of cytohesin3/Grp1 revealed that the C-terminal helix that follows the PH domain and the linker between the Sec7 and PH domains block the catalytic site (DiNitto et al. 2007). Interaction of the PH domain with Arf6-GTP and phosphoinositides, either PI(4,5)P_2 or PI(3,4,5)P_3, as well as the interaction of the polybasic C-terminus of cytohesin/Arno with acidic phospholipids, all contribute to relieving this autoinhibition (DiNitto et al. 2007). Reconstitution of the cytohesin/Arno exchange assay on liposomes, in the presence of both activating Arf6-GTP and substrate Arf1, revealed that mutations in the PH domain of cytohesin/Arno that abolished interaction with Arf6-GTP were completely inactive (Stalder et al. 2011). Hence interaction of the PH domain with an activating Arf protein is an absolute requirement for relief of cytohesin/Arno autoinhibition on membranes. A recent structure of the Sec7 and PH domains of Arno in complex with Arf6-GTP, when compared to the autoinhibited structure, reveals a large conformational change upon Arf6-GTP binding. Binding of Arf6-GTP creates grooves at the Arf6–PH domain interface into which the autoinhibitory elements bind, thus uncovering the binding site for the substrate Arf (Malaby et al. 2013). Together these studies demonstrate how precise spatial regulation of cytohesin/Arno activation is achieved: a specific phosphoinositide (PIP_2 and/or PIP_3), additional acidic phospholipids, and an active Arf localized to the plasma membrane must all coincide to relieve autoinhibition, thus restricting the membrane domain at which these GEFs can become active.

The activation of cytohesins by a GTP-bound Arf family member raises the question of whether they can engage in a positive feedback loop whereby the product of the reaction can stimulate exchange. Indeed, such a positive feedback loop has been demonstrated (Stalder et al. 2011). Cytohesin/Arno is one of the most efficient GEFs in vitro on its myristoylated Arf1 substrate ($k_{cat}/K_m \sim 10^6\ M^{-1}\ s^{-1}$), and this is due in part to the stimulation of exchange by interaction of Arf1-GTP with the PH domain (Stalder et al. 2011). Given the high level of efficiency of this GEF, it would be reasonable to expect a tight regulation of its activity. As described above, one such mechanism is found in the absolute requirement for an activating Arf protein to relieve autoinhibition (Stalder et al. 2011). This study also showed that other Arf effectors are able to compete with the cytohesin/Arno PH domain for available Arf-GTP, suggesting that the activating Arf protein (Arl4-GTP or Arf6-GTP) must be present in excess of other effectors to reach a level sufficient to stimulate this GEF (Stalder et al. 2011). Hence, in order to activate cytohesin/Arno, a burst of Arl4- or Arf6-GTP must be produced to overcome autoinhibition, but once in its active conformation, this GEF has a high capacity to stimulate exchange on its Arf substrates due to the effect of positive feedback. Interestingly, stimulation of exchange activity by the product of the reaction has been demonstrated for the *trans*-Golgi localized Sec7p Arf1 GEF (Richardson et al. 2012), as well as for the Ras GEF Sos (Boykevisch et al. 2006). Hence, this type of positive feedback regulation may be a general property of small G proteins.

In the case of Sec7p, the HDS1 domain downstream of the catalytic Sec7 domain binds to membranes and to Arf1-GTP both in vitro and in cells, and like the PH

domain in the cytohesin proteins, is responsible for mediating relief of autoinhibition and the positive feedback effect (Richardson et al. 2012). For Sec7, the HDS1–Arf1-GTP interaction is required to maintain Golgi localization of Sec7p (Richardson et al. 2012). Interestingly, reduction of Arf1 levels in yeast cells is particularly detrimental to *trans*-Golgi function (Gall et al. 2000), a result that could potentially be explained by the need for Sec7 to have a high level of Arf1 for its autocatalytic activity. One consequence of this mechanism could be to maintain directionality in trafficking through the Golgi. As described above, the GBF/Gea family of GEFs act early in the secretory pathway, at the *cis*-Golgi, and Sec7/BIG GEFs act later at the *trans*-Golgi. Interestingly, the HDS1 domain of GBF1, also immediately downstream of the Sec7 domain, is a direct lipid binding domain that does not require Arf1-GTP for membrane binding like Sec7p does (Bouvet et al. 2013). These results taken together suggest that one mechanism to drive trafficking forward through the Golgi is initial recruitment of GBF1/Gea GEFs to produce Arf1-GTP, which then can recruit the later-acting Sec7/BIG GEFs to Golgi membranes.

Whether Arf6, Arf1, or both are the primary substrates for the cytohesin GEFs has been a long-standing controversy and is still an open question. However, Arf6-GTP is more efficient at relieving autoinhibition of cytohesins than Arf1-GTP, both in vitro and in cells (Cohen et al. 2007; DiNitto et al. 2007). This fact, combined with the requirement for acidic phospholipids in cytohesin membrane binding, would restrict activation of cytohesins to PM or endosomal membranes. These results illustrate the complexity of Arno activation and shed light on this long-standing debate over the physiological substrate of cytohesins in cells. The fact that Arf6-GTP can activate cytoshesin/Arno, that the capacity to activate this GEF is highly dependent on relative levels of cytohesin/Arno and effectors, and that both Arf6 and Arf1 positive feedback loops exist, all need to be taken into consideration in evaluating in vivo results. Arf1 is required for specific processes at the PM such as recruitment of proteins to focal adhesions and in phagocytosis, and Arf1-GTP localizes to these sites (Beemiller et al. 2006; Furman et al. 2002; Kruljac-Letunic et al. 2003; Norman et al. 1998). In the forming phagocytic cup, Arf6-GTP is recruited early, followed by Arf1-GTP, at a stage that requires rapid insertion of new membrane. These results support the idea that the Arf6–cytohesin–Arf1 cascade may play an important role in processes that require a high level of Arf protein. Arf6 is less abundant than Arf1 in cells, and since both Arf1 and Arf6 can recruit effectors such as PI4P 5 kinase and PLD, processes requiring an acute activation of such effectors may rely on the more abundant Arf1 to provide an adequate supply. Another process in which this mechanism may operate is in the insulin signalling pathway, where both Arf1 and Arf6 were shown to contribute to activation of PI4P 5 kinase and PLD by cytohesin-2/Arno (Lim et al. 2010).

8.8 Arf Proteins and Their Regulators in Human Disease

The implication of Arf proteins and their GEFs and GAPs in human pathologies is a rapidly expanding area. Arfs and their regulators have been linked to neurodevelopmental disorders, neurodegenerative diseases, cancers, and both viral and bacterial infections. Here, I will describe examples of their roles in human disorders, and the reader is referred to other sources for a more comprehensive description of specific topics in this growing field (Dani et al. 2013; Lovrecic et al. 2010; Poincloux et al. 2009; Sabe et al. 2006, 2009; Seixas et al. 2013; Stafa et al. 2012; Tan and Evin 2012).

Mutations in the BIG2 Arf1 GEF have been linked to autosomal recessive periventricular heterotopia (ARPH), a disorder of neuronal migration that leads to severe malformation of the cerebral cortex (microcephaly) and developmental delay (Sheen et al. 2004). Two mutations in BIG2 have been identified in ARPH patients, one of which is a frame shift mutation that results in truncation of the majority of the protein (Sheen et al. 2004). The disease symptoms are a result of the failure of a specific class of neurons to migrate from their point of origin in the lateral ventricular proliferative zone to the cerebral cortex (Ferland et al. 2009; Sheen et al. 2004). This defect arises from a defect in vesicular trafficking that alters the adhesion properties of these neurons (Ferland et al. 2009). In addition to BIG2, mutations in the gene encoding filamin A also cause ARPH. A recent study has found a mechanistic connection between these two proteins in a mouse model of the disease (Zhang et al. 2013).

The IQSEC/BRAG Arf GEFs are highly expressed in the postsynaptic density of the central nervous system (Casanova 2007), and play important roles in signalling during synaptic transmission (Myers et al. 2012). This family of Arf GEFs use Arf6 as a substrate, but can also act on Arf5 (Moravec et al. 2012). BRAG1/IQSEC2 is mutated in X-linked nonsyndromic intellectual disability, a form of mental retardation. Three point mutations isolated from patients map to the Sec7 domain and result in proteins that cannot activate Arf6 normally (Shoubridge et al. 2010a, b). BRAG2 has been linked to alterations in synaptic content during long-term depression (LTD). Signalling through AMPA-type glutamate receptors facilitates LTD, and donwregulation of activated AMPA receptors is normally regulated by AMPA receptor-mediated recruitment of BRAG2, which in turn activates Arf6 and endocytosis (Scholz et al. 2010).

The GEFs and GAPs for Arf1 and Arf6 at the cell periphery have roles which are becoming more clearly defined in the progression of numerous cancers (D'Souza-Schorey and Chavrier 2006; Muller et al. 2010; Peng et al. 2013; Sabe et al. 2006, 2009; Sangar et al. 2014; Yoo et al. 2012). Cytohesin/Arno GEFs affect signalling through epidermal growth factor (EGF)/ErbB, promoting conformational changes that increase *trans*-phosphorylation of EGFRs upon ligand-induced dimerization (Bill et al. 2010). Importantly, these GEFs are specifically inhibited by SecinH3, which holds promise as anti-cancer drug, since treatment of an EGF receptor-

dependent lung cancer cell line with SecinH3 resulted in reduced proliferation (Bill et al. 2010).

Arf proteins and their regulators are hijacked by numerous bacterial and viral pathogens (Dautry-Varsat et al. 2005; Goody and Itzen 2013; Hsu et al. 2010; Humphreys et al. 2013; Matto et al. 2011). The Arf GEF GBF1 is required for the replication of numerous viruses, including enteroviruses, hepatitis C virus, and coronaviruses (Belov et al. 2008; Goueslain et al. 2010; Lanke et al. 2009; Winchester et al. 2008). These viruses remodel ER and early secretory pathway membranes to form replication complexes, for which they subvert the function of GBF1. Interestingly, a major requirement for replication of several of these viruses is gaining control of host lipid trafficking and metabolism pathways (Alvisi et al. 2011; Hsu et al. 2010; Ilnytska et al. 2013).

8.9 Conclusions and Perspectives

The activity of Arf proteins is regulated in a spatiotemporal manner by their GEFs and GAPs, highlighting the importance of precise localization of these regulators. The mechanisms of this spatiotemporal control are now beginning to emerge. Coincidence detection mechanisms involving binding to specific lipids and protein partners play an important role, and for the cytohesins, a fairly complete description of how relief of autoinhibition is coupled to precise spatial cues has been obtained. Arf activation cascades have been demonstrated for the cytohesins, but may play an important role in the activation of other families of GEFs as well. Such cascades, similarly to those demonstrated for Rab G proteins, could be involved in transforming one membrane domain into another during highly dynamic membrane trafficking maturation events. These transformations involve coordinate changes in both the lipid and protein composition of each membrane domain, a specific function of the Arf family members, which recruit both lipid-modifying enzymes and protein effectors such as coats and tethers. The signature feature of Arf family proteins, their N-terminal membrane-binding amphipathic helix, ensures that they are closely associated with the lipid bilayer in their GTP-bound form. Future studies on how Arf family proteins function will therefore continue to require in vitro reconstitution on model membranes.

Among the small G proteins, the Arf family is perhaps the most enigmatic. Why so few? What is the unifying principle in the broad range of functions defined for each one? The small number of Arf proteins in a given eukaryotic cell is an ancient property of the Arf proteins, since phylogenetic analyses indicate that the last common ancestor of the eukaryotes had only one Arf protein, despite a significant level of complexity, and numerous distinct Arf regulators and effectors (COPI, *trans*-Golgi clathrin-adaptor proteins, coiled-coil tether, and phospholipase D). One obvious consequence of having a single protein with different functions is that the various processes can be integrated in a simple manner. For example, the single pool of Arf1 must be distributed among all of the different GEF, GAP, and effector

complexes in the cell, which could provide a type of global regulation of all of the functions that these different complexes carry out. In particular, Arf1 is involved in the recruitment of coat complexes and membrane tethers at the Golgi that mediate vesicular trafficking, and also in lipid droplet metabolism and in recruitment of at least three lipid transfer proteins that mediate non-vesicular lipid trafficking in cells. Hence a fundamental role of the Arf proteins may be in coordinating both vesicular and non-vesicular lipid trafficking pathways in the cell.

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