Alfred Wittinghofer *Editor*

Ras Superfamily Small G Proteins: Biology and Mechanisms 2

Transport

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Part I
Rab Subfamily

Chapter 1
Prenylation of RabGTPases, Their Delivery to Membranes, and Rab Recycling

Roger S. Goody, Yaowen Wu, and Aymelt Itzen

Abstract Rab proteins, in common with many other Ras superfamily members, need to be lipidated (in the case of Rab geranylgeranylated) at their C-terminus to be able to interact with membranes. Here, we review the basic mechanism of geranlygeranylation and then address the still unsolved question of how prenylated Rabs are delivered to specific membranes. The evidence reviewed leads to a model in which specifically localized guanine nucleotide exchange factors are an essential component of the targeting mechanism, and that additional stabilizing interactions are important in a number of cases, including effector interactions and positively charged residues in the C-terminus in at least one case examined. Finally, there is evidence that in some cases additional still unidentified factors are involved.

Keywords RabGTPases • Prenylation • Delivery • Localization • GEF • GDI • GDF

1.1 Introduction

Intracellular vesicular transport is an important and essential aspect of the functioning of eukaryotic cells. Transport between membrane-bound compartments occurs via vesicles or tubular structures in a highly complex and tightly regulated

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manner. While many different classes of proteins are involved in the overall process, a crucial regulatory role is played by members of the Rab family of small GTPases (Hutagalung and Novick 2011; Stenmark 2009). In order to fulfill their role, the 60 members (in humans) have to be modified by addition of geranylgeranyl groups (one, or in most cases two) to the C-terminus in a process referred to as prenylation. These prenyl groups endow the Rab proteins with a high affinity for membranes and allow them to play various roles, for example connecting vesicles to systems of motor-proteins or recognizing specific tethering factors that guide vesicles to a specific membrane or membrane compartment as a first step in the fusion process. While the principles involved in these events are quite well understood in a number of cases, one essential but unclarified property that Rab proteins must have in order to play the role envisaged for them is localization to a specific membrane or membrane domain. In this review, we will first discuss the events leading to prenylation, then discuss the principles involved in delivery of Rabs to membranes, and finally summarize the status of our knowledge on the targeting of Rab proteins to specific membranes.

1.2 Prenylation of Rab Proteins

Rab proteins differ from Ras and Rho proteins, which are also prenylated near their C-terminus, by not having a specific prenylation recognition sequence motif. Ras and Rho proteins have the so-called CaaX box at their very C-termini, with the nature of the X residue determining whether farnesylation (Ras proteins) or geranylgeranylation (Rho proteins) occurs. In Rab proteins, the C-terminal motifs are commonly CC, CXC, or CCXX, but in a few cases CXXX motifs occur (Rab8, Rab13, Rab18, Rab23, Rab38). The explanation for the lack of a specific prenylation motif is that specificity is in fact generated via an accessory protein, the Rab escort protein (REP), which interacts with all RabGTPases (but not other members of the Ras superfamily) and with Rab geranylgeranyl transferase (RabGGTase), presenting the Rab C-terminus to the active site of the prenylating enzyme. This leads to the curious situation of having a specific enzymatic modification of a group of substrates with similar overall structure but differently structured modification sites, with the added peculiarity that the modifying enzyme has little or no (measurable) affinity for its substrates. The latter point is put into perspective if the REP:Rab complexes are regarded as the substrates, and these do indeed have affinity and specificity for RabGGTase. In the presence of the substrate geranylgeranyl pyrophosphate, i.e., the lipid donor, the affinity of RabGGTase for REP:Rab complexes is in fact very high [K_d in the nM range (Thoma et al. 2001a, b)].

The Rab prenylation reaction has been investigated in detail in a number of publications, and many of the data obtained are summarized in Fig. 1.1 (Wu et al. 2009). The importance of the CIM (CBR interacting motif, where CBR means C-terminus binding region) region of Rab proteins was noticed in

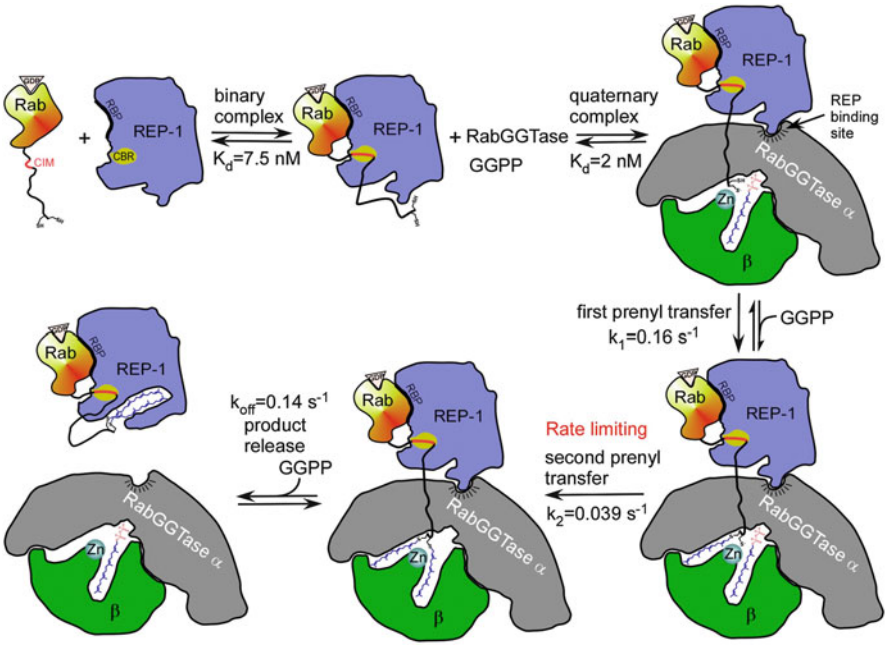

Fig. 1.1 The mechanism of Rab prenylation in the Rab:REP:RabGGTase complex. After an initial relatively weak association of the globular domain of Rab with REP, interaction of the CIM (C-terminal interaction motif) of Rab with the CBR (C-terminal binding region) of REP leads to tightening of this interaction. This is followed by a high-affinity interaction of the Rab:REP complex with RabGGTase and geranylgeranyl pyrophosphate (GGPP) to form the high-affinity quaternary complex. The first geranylgeranyl group is transferred to the most C-terminal cysteine of Rab with the help of a catalytic zinc ion. The second prenyl group is then transferred more slowly (in the case of Rab7) to the preceding cysteine, after which the prenylated Rab:REP complex is released under the influence of a further lipid substrate molecule. The constants given apply to Rab7 and REP-1. Figure modified from the publication of Wu et al. (2009)

structural investigations on a complex between prenylated Rab proteins and REP (Rak et al. 2004) or GDI (GDP dissociation inhibitor) (Rak et al. 2003). This short motif, consisting of a polar residue sandwiched between two hydrophobic residues, is essential for high-affinity binding to REP and for efficient prenylation. The positioning of the cysteine groups with respect to this motif is critical. In wild-type Rab7, there are 12 residues downstream of this motif before the first prenylatable cysteine, and this can be shortened by a further 3 residues without drastic effects on the prenylation reaction, but shortening to 7 residues leads to only residual activity and with 6 residues or less there is no detectable activity (Wu et al. 2009). Interestingly, it has recently been shown that the region between the CIM and the cysteines can be replaced by a non-peptidic structure and that cysteine can be replaced by a simple thiol group and still retain full prenylation

properties both in vitro and in vivo (Li et al. 2014). These results are discussed later in connection with the role of the hypervariable C-terminus in Rab localization.

The discussion so far has implicitly assumed that the Rab protein harbors a GDP molecule, since the GDP form is prenylated more efficiently than the GTP form (Seabra 1996). This is probably partially related to the fact that the GTP form of Rab proteins is bound a factor of ca. 10 more weakly than the GDP form to REP (Alexandrov et al. 1998). A conceptual problem that arises at this point is the fact that freshly synthesized Rabs in the cytoplasm will mainly have GTP bound, since the cellular concentration of GTP is ca. tenfold higher than that of GDP. The stronger binding of REP to the GDP form of Rabs will tend to compensate for the imbalance in the GTP/GDP concentrations, and taking the data available at their face value, this could lead to similar amounts of Rabs in the two different forms bound to REP (depending on the relative concentrations of REP and Rab molecules), and therefore to production of prenylated Rabs in both nucleotide-bound forms. Even if the rate of prenylation is slower for the GTP-bound form, this means that significant amounts of prenylated Rabs with bound GTP can be produced. As discussed in detail below, Rabs that associate with membranes in the GTP form are resistant to extraction by GDI, meaning that if association with a wrong membrane occurs initially, this will be essentially irreversible as long as GTP hydrolysis does not occur. This is an unlikely scenario, since production of activated (i.e., GTP-bound) Rabs at their appropriate membrane location needs to be strictly regulated. As described below, prenylated Rab:GTP is bound much more weakly than prenylated Rab:GDP to REP, increasing the probability of Rab release from its complex with GDI leading to membrane association. In this situation some additional factor that is responsible for targeting would be needed. Again, this is a theme that will arise in later discussions.

1.3 Delivery of Prenylated Rab Proteins to Membranes

An interesting question that has been touched upon but not examined extensively is whether direct delivery of Rabs from their complexes with REP to their specific membrane occurs, or whether they first locate to a default membrane, possibly the ER and/or Golgi. Rabs ending with CXC are carboxymethylated, as are some mono-prenylated Rabs (Leung et al. 2007), and the enzyme that performs this (isoprenylcysteine carboxymethylase, or Icmt) is ER localized. The authors conclude that Rabs with a CC motif, which are not carboxymethylated, are located directly to their target membrane, but that CXC Rabs first have to visit the ER, but evidence against initial localization to ER for all Rabs was not provided. Moreover, the ER and Golgi appear to be the default site of mistargeted Rab proteins arising from the loss of targeting elements on Rab proteins per se or the depletion of GEFs (Ali et al. 2004; Cabrera and Ungermann 2013; Li et al. 2014). However, if some are still in the GTP form, there would still be a problem for GDI to extract them

again to allow delivery to the correct target membrane, unless there is a generic RabGAP at the ER and Golgi that acts on them, for which there is no evidence.

After prenylation of Rab proteins in the complex between REP, Rab, and RabGGTase, a further incoming lipid substrate molecule (GGPP) is able to dissociate the RabGGTase and leave the Rab protein as a soluble complex with REP (Thoma et al. 2001b) (Fig. 1.1). This high-affinity complex [K_d in the nM range (Wu et al. 2007)] has to be disrupted to allow Rabs to be attached to membranes. A similar situation arises with recycling of Rabs by GDI, which has similar properties to REP in terms of binding and solubilizing prenylated Rab proteins. In both cases, there is a stable interaction that has to be overcome to allow insertion of the prenyl groups into the membrane.

It has been suggested that the attachment of Rabs to membranes is catalyzed by a GDF (GDI displacement factor) that is able to disrupt the stable GDI:Rab complexes (Dirac-Svejstrup et al. 1997). A molecule that appears to have the desired properties is Pra-1 (Yip3 in yeast), an intrinsic membrane protein reportedly localized in the late Golgi and early endosomes with activity towards Rab9 and other endosome-associated Rabs (Rab5, Rab7) (Sivars et al. 2003). Although there is no convincing evidence for their mode of action, other members of the Yip family are Rab interacting proteins thought to be candidate GDFs. However, recent evidence has been presented indicating that the activities of these proteins are not required for correct localization of yeast Rabs (Cabrera and Ungermann 2013).

The lack of definitive evidence on the existence and properties of GDFs for Rabs was a reason for the enthusiastic reception of publications suggesting that one of the over 250 proteins injected by *Legionella pneumophila* into infected cells is a *bona fide* GDF as well as being a GEF towards host cell Rab1 (Ingmundson et al. 2007; Machner and Isberg 2007). While the GEF properties of DrrA/SidM have been amply confirmed and extensively characterized, it was also shown that the apparent GDF activity of this protein was actually a product of its GEF activity (Schoebel et al. 2009), and in fact all Rab GEFs will have this apparent activity towards their cognate Rabs. The source of this effect is the preference of GDI for Rab:GDP rather than for Rab:GTP. It was shown that replacement of GDP by GTP in prenylated Rabs leads to a loss of affinity of about three orders of magnitude towards both GDI and REP (Wu et al. 2010). Because of this, Rab:GDP that dissociates from its complex with GDI or REP spontaneously will undergo nucleotide exchange in the presence of a cognate GEF, thus preventing rebinding to GDI and possibly allowing membrane insertion. These considerations lead to the notion that the presence of a GEF at a specific membrane location might lead to trapping of its activated cognate Rab at the same location (Schoebel et al. 2009; Wu et al. 2010).

Since molecules with genuine GDF activity towards Rab:GDI complexes have so far proven elusive, with the exception of Yip3, the question of their necessity for the process arises. Since Rab:GDI and in particular Rab:REP complexes dissociate slowly, it appears that acceleration over the rate seen in vitro is likely to be required in vivo. One possibility that has not yet been considered is the role of membranes in this process, except in the very general sense that interaction with a membrane will make a thermodynamic contribution to the process by providing a trap, whose

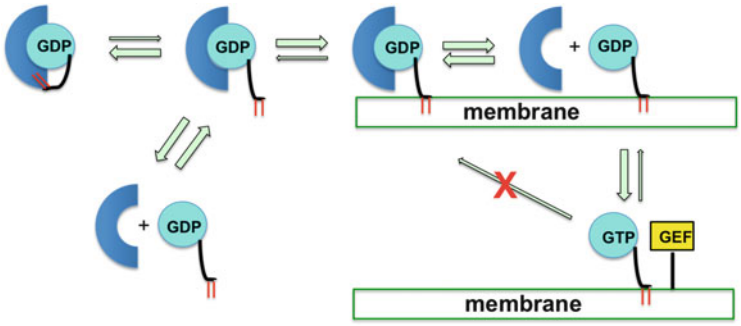

Fig. 1.2 Model for Rab delivery to membranes without a GDF. Assuming that there is a certain tendency for the lipid moieties at the C-terminus to dissociate from their binding site on Rab, this state might be temporarily trapped by the proximity of a membrane. If a cognate GEF is present at this membrane, membrane attachment will be made essentially irreversible because of generation of the GTP bound state of the Rab protein, which reduces the affinity to GDI dramatically

properties are enhanced by the influence of concerted or sequential GDP/GTP exchange on the Rab:GDI affinity.

A hint towards a possible active role of membranes is suggested by comparing the properties of Rab:GDI or Rho:GDI complexes with those of complexes of farnesylated Ras family proteins with PDEδ (Ismail et al. 2011). In this case, the interaction appears to be almost exclusively with the prenyl group, and the structure of farnesylated Rheb:GDP in complex with PDEδ complex confirms that there is little or no interaction between PDEδ and the body of the GTPase. In the case of Rab or Rho complexes with GDI, the main contribution to affinity and therefore specificity appears to come from the specific interaction of GDI with the GTPase domain. The idea therefore arises that whereas there is a relatively strong interaction of the farnesyl group with the lipid binding site of PDEδ, the interaction of geranylgeranyl groups with the lipid binding site of GDI or REP is weak, leading to a dynamic equilibrium between a state in which both the GTPase domain and lipid are bound to GDI, and a state in which the lipid group is transiently free (see Fig. 1.2). If this occurs in close proximity to a membrane, insertion of the lipid group could then lead to complete dissociation of Rab:GDP from GDI, which would again be reversible, unless a membrane-localized GEF can facilitate GDP replacement by GTP, thus preventing the reverse reaction (i.e., rebinding of Rab to GDI). These arguments are summarized in Fig. 1.2.

Interestingly, a genuine GDF-type mechanism appears to operate in the case of interaction of farnesylated proteins with PDEδ (Chandra et al. 2012; Ismail et al. 2011). This protein binds farnesyl groups in a manner that is independent of the GTPase core domain of Ras-family members, as already discussed. It appears to act as a chaperone for farnesylated proteins in a similar manner to Rab and Rho GDIs, with the important exception that there is no dependence of the interaction on the nucleotide state of the GTPases. Intriguingly, PDEδ interacts with other

Ras-family GTPases, namely Arl2 and Arl3, in a GTP-dependent manner, and this interaction leads to active displacement of bound farnesyl groups by an allosteric competitive mechanism. This is therefore a genuine GDF-like mechanism and avoids the thermodynamic dead end that would be encountered with a GDF mechanism that involves a specific strong interaction with the chaperoned GTPase, as envisaged in the original Rab-GDF concept. In the PDEδ case, the interaction of the GDF (i.e., Arl2 or Arl3) is with the chaperone itself and is in fact reversible on hydrolysis of GTP at the active site of the Arl protein, resulting in loss of affinity to PDEδ. This process is therefore ultimately driven by GTP hydrolysis, although the directly relevant factor is the GTP/GDP concentration gradient, as for the hypothetical model for GEF-driven Rab localization. In both cases, the relative concentrations of GTPase:GTP and GTPase:GDP complexes will be regulated by the interplay of GEF and GAP activities in their various locations.

1.4 Testing the GEF Targeting Mechanism for Rab Proteins in Cells

If the arguments summarized above on the role of GEFs in Rab targeting are valid, it should be possible to manipulate the location of Rab proteins by controlling the localization of their cognate GEFs. This has been addressed by deliberate mislocalization of GEFs to the outer mitochondrial membrane in an acute fashion taking advantage of a mitochondrial binding domain (i.e., the *Listeria monocytogenes* ActA mitochondria-targeting sequence) fused to the FRB protein (FKB-rapamycin binding protein) (Blumer et al. 2013). Rab-GEFs fused to FKBP (FK506 binding protein) can then be recruited from their normal location to mitochondria on addition of a rapamycin analog, and in the three cases tested (Rab5/Rabex5, Rab1/DrrA, and Rab8/Rabin8), this is followed by relocation of the corresponding Rab proteins from their normal location to mitochondria. A similar approach in which the Rab32/Rab38 GEF BLOC-3 was mislocalized to mitochondria also resulted in the mislocalization of Rab32 (Gerondopoulos et al. 2012). This is strong evidence that GEFs can regulate the localization of their cognate Rabs. Obviously, this targeting mechanism can only function if Rab GEFs are localized to specific membranes. While known Rab GEFs are not integral membrane proteins, they appear to be recruited by a number of mechanisms to distinct membrane locations in practically all cases that have been sufficiently well investigated (Table 1.1) (Barr 2013; Blumer et al. 2013) excluding the atypical GEF Mss4 (Itzen et al. 2006; Wixler et al. 2011) (Dss4 in yeast) that is probably not a bona fide GEF but a type of chaperone for a number of nucleotide-free Rabs.

Table 1.1 Overview of Rab-GEFs, their sites of localization and their localization mechanism

GEF	Rab	Localization	Localizer(s)
TRAPP (Cai et al. 2007; Wang et al. 2000)	Rab1	ER-Golgi	Sec23 (CopII)
Ric1/Rgp1 (Pusapati et al. 2012)	Rab6	Golgi, cytosol	Rab33b:GTP
Sec2 (Ortiz et al. 2002; Walch-Solimena et al. 1997)	Sec4	Secretory vesicles	Ypt32:GTP, Sec15 (exocyst)
Rabin8 (Chiba et al. 2013; Hattula et al. 2002; Knodler et al. 2010)	Rab8	Cilia, cortical actin	Rab11:GTP, Sec15 (exocyst), Phosphatidylserine
Rabex5 (Horiuchi et al. 1997)	Rab5	Early endosomes	Rabaptin5, ubiquitinylation
DrrA/SidM (Brombacher et al. 2009; Machner and Isberg 2007; Murata et al. 2006)	Rab1	Legionella containing vacuole/plasma membrane	PI(4)P
Mon1/Ccz1 (Lachmann et al. 2012; Nordmann et al. 2010)	Ypt7/Rab7	Late endosomes	Vps21(Rab5):GTP
DENND1A (Allaire et al. 2010; Yoshimura et al. 2010)	Rab35	Clathrin coated pits, (plasma membrane)	AP2
DENND5A/5B (Miserey-Lenkei et al. 2007; Recacha et al. 2009; Yoshimura et al. 2010)	Rab39	Golgi	Rab6:GTP
DENND2 (Yoshimura et al. 2010)	Rab9	Actin filaments lysosomal trafficking	
MADD/Rab3Gep (Figueiredo et al. 2008; Yoshimura et al. 2010)	Rab27	Melanosomes	
Bloc-3 (Gerondopoulos et al. 2012)	Rab32/38	Melanosomes	
Crag (Xiong et al. 2012)	Rab11	Rhabdomeres	
FAM116 (Linford et al. 2012)	Rab14	Recycling endosomes, cytosol	
MTMR5/13 (Yoshimura et al. 2010)	Rab28		
DENND1C (Yoshimura et al. 2010)	Rab13		
DENND3 (Yoshimura et al. 2010)	Rab12		
DENND1B (Yoshimura et al. 2010)	Rab35		

In the case of recently discovered GEFs, this information is incomplete or absent

1.5 Do Additional Factors Contribute to Rab Targeting?

Although the evidence described is strong support for a decisive role of GEFs in Rab targeting, over the years other factors have been suggested to play an important part. The original hypothesis that the hypervariable C-terminal region alone acted as the sole determining factor (Chavrier et al. 1991) has been challenged and at least partially disproved, suggesting that multiple regions in Rabs contribute to membrane targeting (Ali et al. 2004). However, further testing of the role of the

C-terminus appeared appropriate, and recent experiments have addressed this question again (Li et al. 2014). Semisynthetic Rabs were prepared that contained C-termini in which much of the C-terminus was replaced by polyethylene glycol, except for a three amino acid motif known from earlier work to be essential for interaction with GDI and REP (CIM, or C-terminal interaction motif) (Rak et al. 2003, 2004; Wu et al. 2009). In some constructs, the unstructured region N-terminal to the CIM was replaced by a flexible Gly-Gly-Ser (GGS) repeat structure. In all constructs in which the appropriate lengths between the GTPase core domain and the CIM, as well as between the CIM and C-terminal cysteines, were maintained, the constructs could be geranylgeranylated both in vitro and in vivo (as judged by the evidence that membrane localization occurs). In the case of Rab1 and Rab5, such microinjected proteins localized in the same manner as wild-type constructs fused to fluorescent proteins, suggesting that here the exact structure of the C-terminus is not important. However, the targeting of C-terminally modified Rab7 and Rab35 is compromised in certain constructs, suggesting a role of parts of the C-terminus in correct localization. For Rab35, this is probably due to a requirement for a polybasic region near the C-terminus to interact with the highly negatively charged plasma membrane (Fig. 1.3). In Rab7, the sequence N-terminal to the CIM appears to be partially important, probably because of its known involvement in interaction with the effector RILP (Wu et al. 2005). This is reminiscent of evidence that effector binding is essential for the correct localization of Rab9 (Aivazian et al. 2006).

The notion that effector interactions are needed for localization of Rabs is not immediately attractive based on the accepted paradigm of Rab action, which is that recruitment to specific membranes in the active GTP-bound form is required to allow interaction with a cognate effector molecule, for example, to connect to transport systems or to engage tethering factors. If the interaction with one effector is required for localization, this interaction is presumably necessary for the continued residence of the Rab protein at its correct membrane localization. Effector interactions are typically not of very high affinity, and are rapidly reversible, so that it could be imagined that the initial localizing interaction might be replaced competitively by interaction with a different effector (that is not membrane localized, or at least not to the same membrane), but this would lead to destabilization of Rab in the membrane, which is probably not a desired consequence. Perhaps the essential equilibrium concepts behind this argument are too naïve. Thus, if the rate of spontaneous release of a Rab protein from the membrane is slow on the timescale of the events triggered by the second effector interaction, it is possible that the Rab molecule could fulfill its function before spontaneous or GAP/GDI-induced loss from the membrane, for example, after a fusion event.

A different situation can be envisaged with effectors that form heterotetrameric complexes with their cognate Rabs. Thus, it is possible that the dimeric structure of the Rab7 effector RILP is of importance in stabilizing the membrane-bound form of Rab7 (see Fig. 1.4). A complex with the stoichiometry of 2:2 (effector:Rab) will be stabilized in the membrane compared to Rab alone or to the situation in which there is only a 1:1 complex (unless there is an additional interaction of the effector with

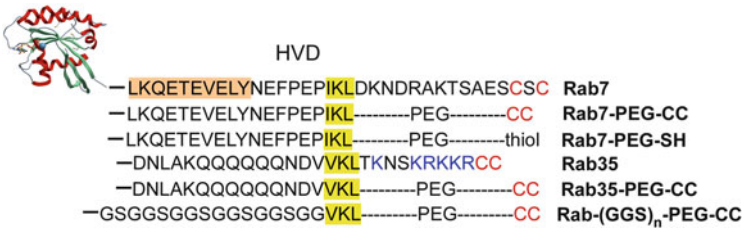

Fig. 1.3 Substitution of the Rab HVD with different C-terminal structures. The CIM is highlighted in *yellow*, the RILP interaction sequence of Rab7 in *orange*, while the basic residues in Rab35 are in *blue* and the prenylatable cysteines are in *red*

the membrane or another membrane component). This is because in the heterotetrameric complex there are four rather than two prenyl groups that interact with the membrane. It is conceivable that this is the reason for the importance of the Rab7 effector interaction for membrane stabilization, as described above. This is perhaps of more general significance, since several other Rab-effector interactions involve this dimeric type of interaction, including Rab5:Rabaptin5 (Zhu et al. 2004), Rab6:GCC185 (Burguete et al. 2008), Rab11:FIP2 (Jagoe et al. 2006), and Rab11:FIP3 (Eathiraj et al. 2006; Shiba et al. 2006). In the absence of additional interactions of the effector with the membrane or other membrane-bound components, this cannot be a targeting mechanism, but could be a mechanism to stabilize a Rab protein at a specific membrane after the initial targeting step, in which GDP/GTP exchange by a specifically localized GEF probably plays the most important role. Thus, a dimeric or better divalent effector would be able both to stabilize the membrane-bound form of the Rab protein and to interact with a further partner, for example, the dynein–dynactin complex in the case of Rab7 (Tan et al. 2011). It will be of interest to determine whether Rab effectors that also interact with the C-terminal region, but in a monovalent manner, also contribute to Rab targeting. Known examples are Rab3:Rabphilin (Ostermeier and Brunger 1999) and Rab27:melanophilin (Kukimoto-Niino et al. 2008).

In the case of targeting of Rab27a to melanosomes, it has been shown that impaired effector binding does not affect membrane localization, whereas the nonredundant GEF activity of Rab3GEP/MADD is essential (Tarafder et al. 2011). However, mutants of Rab27a were found that were substrates for the GEF activity of Rab3GEP in vitro but were not correctly localized, suggesting that an additional factor or activity (i.e., not just GEF activity) is required for targeting. However, it is not clear what the effect of these mutations on Rab27 cycling is. In a similar vein, it was recently shown that the Ypt7 GEF Mon1:Ccz1 is required for correct localization of wild-type Ypt7, but not for a Ypt7 mutant that showed facilitated (i.e., GEF-independent) nucleotide exchange (Cabrera and Ungermann 2013). This again suggests that an additional factor or factors are required for targeting and even suggests that the exact site of the exchange reaction is not important.

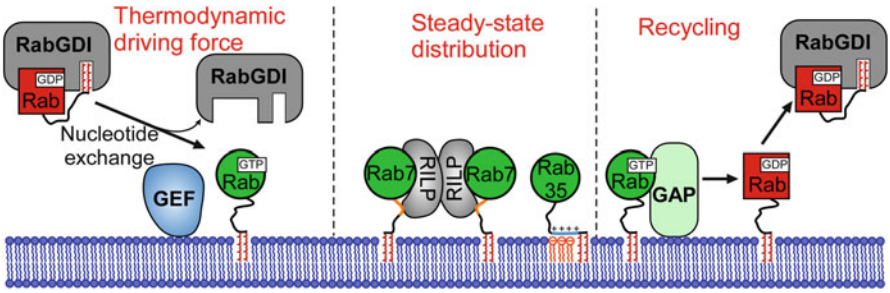

Fig. 1.4 Overview of important factors and principles in Rab targeting. GEF-mediated nucleotide exchange appears to be the major factor for Initial insertion of Rab proteins into membranes. Further interactions are probably of importance in determining the steady-state distribution between membrane and GDI. Note that univalent interaction with an effector will not increase stability unless there is an additional membrane-anchoring interaction of the effector (direct or indirect). A bivalent effector interaction of the type depicted could lead to stabilization of the Rab-membrane interaction, but the targeting step would still be dependent on GEFs. [Figure modified from Li et al. (2014)]

1.6 Conclusion

The work described briefly here leads to the conclusion that GEFs are major determinants in Rab targeting, but numerous observations suggest that a model in which GEFs are the sole determining factor is not correct. In some situations, there is evidence that effector interactions are important, while other observations demand another, additional targeting factor or principle. As a working model, we suggest that GTP/GDP exchange at a specific location plays a crucial role in targeting, but that additional stabilization of the membrane-bound state occurs in at least some cases, possibly including formation of a heterotetrameric complex containing two effector and two Rab molecules. In general, it is appears to be the interplay of GEFs, GAPs, effectors, and possibly still unrecognized principles that determines localization in a highly dynamic and complex process.

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Chapter 2
Rab Proteins and the Organization of Organelle Membrane Domains

Marnix Wieffer, Marisa P. McShane, and Marino Zerial

Abstract Many critical cellular processes, like vesicular transport and signaling, rely on the establishment and maintenance of membrane domains. Membrane domains consist of a cluster of specific lipids and proteins that provide membranes with a distinct molecular identity. Rab GTPases are one of the main coordinators of membrane domain formation and dynamics. In this chapter, we will give a brief introduction into Rab GTPases and focus on how they create and coordinate membrane domains. This will include an in-depth look into the Rab effectors and binding partners that define membrane domains. Throughout we will highlight how these proteins are regulated such as via feedback and feed-forward loops to create cascades. Finally, we propose that signaling domains on organelles are also coordinated by Rab GTPases.

Keywords Rab GTPase • Endosome • Rab effector • Membrane domain • Signaling

2.1 Introduction

It is becoming increasingly clear that cellular membranes are not homogeneous but highly compartmentalized into membrane domains of different size, formed by lipid–lipid, protein–protein, and protein–lipid interactions. Such membrane domains are key to the formation of morphologically and functionally distinguishable features like vesicular coats, tubules, and signaling platforms (Gould and Lippincott-Schwartz 2009; Bonifacino and Glick 2004). Other examples of membrane nanodomains are lipid rafts and Ras nanoclusters (Lingwood and Simons 2010; Abankwa et al. 2008; Hancock and Parton 2005).

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One of the purposes of a membrane domain is to establish and maintain membrane identity to thereby ensure intracellular transport directionality. Intracellular transport is a multistep process, which includes cargo selection, coated-vesicle formation, directed vesicular movement, target membrane recognition, and fusion. For example, cargo from the extracellular environment is internalized into early endosomes where it is sorted for recycling to the plasma membrane or degradation in lysosomes. Clearly, such steps need to be coordinated in time and space. The work on Rab GTPases has revealed molecular features and functional properties that fit with such a spatiotemporal coordination (Zerial and McBride 2001; Pfeffer 2013b; Barr 2013). First, Rab GTPase localization is highly compartmentalized and organelles often have a unique complement of Rab proteins (Fig. 2.1) (Galvez et al. 2012; Hutagalung and Novick 2011; Stenmark 2009). Second, Rab GTPases are tunable as they can take an inactive GDP-bound or active GTP-bound conformation and shuttle between the cytosol and the membrane. Third, in GTP-bound form membrane-localized Rab proteins recruit diverse effector proteins that carry out different functions in vesicle tethering, fusion, and organelle motility (Stenmark 2009; Grosshans et al. 2006b) (Fig. 2.2). In particular, biochemical studies of Rab5, a key regulator of early endocytic trafficking (Zeigerer et al. 2012), have uncovered a unique complexity of regulators and provided important insights into the membrane compartmentalization of this GTPase (Christoforidis et al. 1999a). Rab5 has the largest interactome of any individual small GTPase family member described so far (Christoforidis et al. 1999a). Indeed, several of these effector molecules act coordinately and cooperatively with other components of the transport machinery. For example, the localized synthesis of phosphatidylinositol 3-phosphate PI(3)P by PI3 kinases is regulated by Rab5 and required for the recruitment of PI(3)P-binding and Rab5-binding proteins that function in membrane tethering and fusion (further discussed below). As these proteins function sequentially or concomitantly, it makes sense that they are localized to a Rab5 membrane domain where these activities can be enriched.

Which mechanisms account for such compartmentalization? First, Rab5 recruitment and activation is under the control of a positive feedback loop to guarantee the localized enrichment of Rab5 (Horiuchi et al. 1997; Stenmark et al. 1995). Second, Rab5 regulates the generation of PI(3)P through the interaction with PI3 kinases (Christoforidis et al. 1999b; Murray et al. 2002). Third, the Rab5/PI(3)P effectors oligomerize into higher molecular weight complexes, stabilizing the Rab5 membrane domain (McBride et al. 1999). Oligomerization occurs through low-affinity multivalent interactions that result in the assembly of multi-protein complexes on the endosomal membrane where the local concentration is sufficiently high. Energy is required to regulate the dynamics of these oligomers in the form of GTP (Rab5) and ATP (through *N*-ethylmaleimide-sensitive factor, NSF). The molecular details of these interactions are known only in part. Therefore, more work is required to elucidate how the various proteins contribute to the formation of the oligomers.

In this book chapter, we will first briefly discuss the conservation of Rab GTPases. Next, we will review the role of the Rab GTPase family and the broad spectrum of effectors in membrane domain formation and organelle remodeling

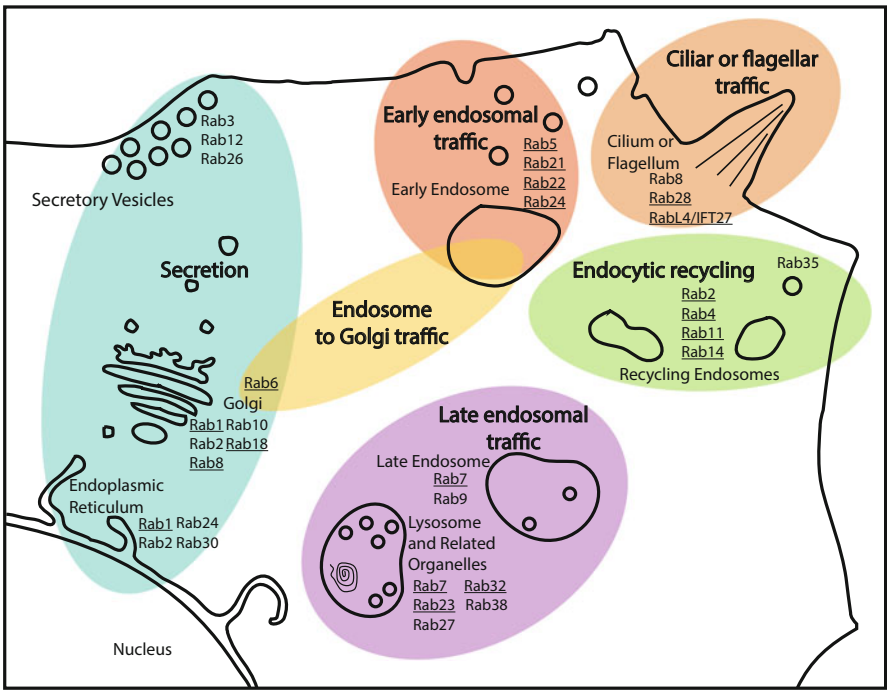

Fig. 2.1 Compartmentalization of Rab GTPases within eukaryotic cells. Rab GTPases are localized to specific organelles. Six Rab supergroups that were defined by Klöpper (Klöpper et al. 2012) are highlighted as functional groups. Underlined Rab GTPases were identified to be present in the LECA in both the studies of Elias et al. (2012) and Klöpper et al. (2012). Figure adapted from Stenmark (Stenmark 2012). Rab GTPase localizations are derived from (Galvez et al. 2012; Stenmark 2009; Hutagalung and Novick 2011)

during cargo transport. We will end by discussing the contribution of Rab proteins to the regulation of signaling.

2.2 Evolution of the Rab GTPase Family

The importance of Rab GTPases is illustrated by the fact that they are evolutionarily conserved. This strongly suggests they played a critical role in endomembrane evolution (Gurkan et al. 2007). Interestingly, the number of Rab GTPases dramatically changed between species with both gains and losses. In mammalian cells, more than 60 Rab GTPases have been identified whereas in fungi, there are between 8 and 12 depending on the species (Pereira-Leal 2008; Klöpper et al. 2012; Stein et al. 2012). The loss can in some instances be correlated with changes in cellular organization, such as the concomitant loss of cilia and ciliary Rabs proteins, but this is not always the case (Klöpper et al. 2012). Recently, several groups proposed that

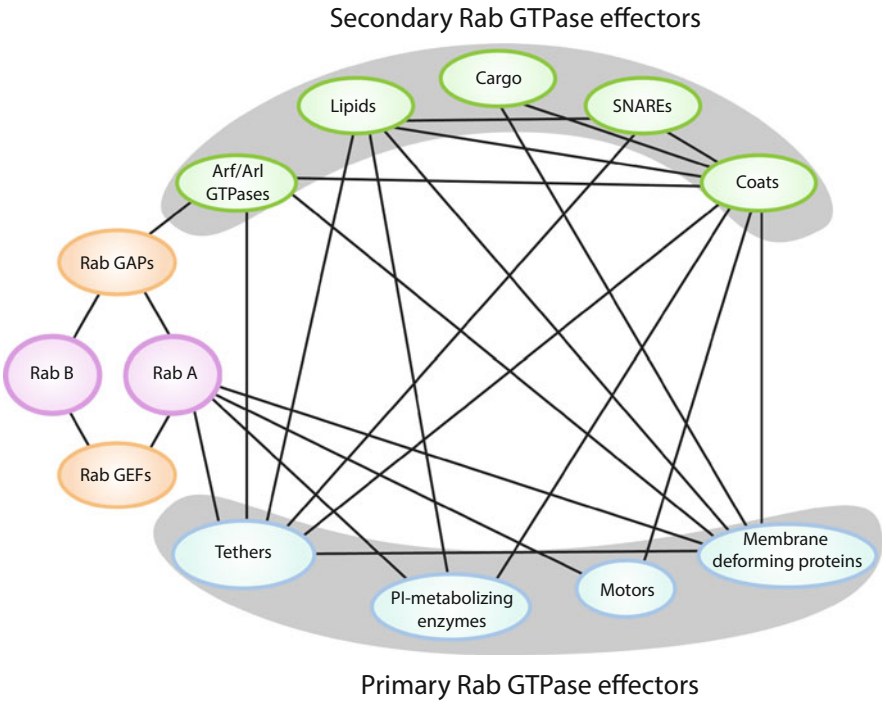

Fig. 2.2 Rab GTPases play a central role in the formation of membrane domains. Here we describe a Rabcentric model summarizing the known possible interactions (*lines*) of Rabs GTPases with other membrane-defining factors. Rab proteins (*purple*) directly interact with GAPs and GEFs (*orange*), which often show activity towards up or downstream Rab GTPases. Thereby Rab cascades are created, ensuring vectorial flow of membranes and proteins within the cell. In addition, Rabs GTPases directly interact with tethers, PI-metabolizing enzymes, motors, and membrane-deforming proteins (*blue*). Through these primary effectors, Rab GTPases indirectly bind to, amongst others, other small GTPases, lipids, cargo, SNAREs, and coats (*green*). Through cooperative interactions between Rab GTPases and primary and secondary effectors, defined membrane domains are formed with critical roles in membrane trafficking and signaling

the last eukaryotic common ancestor (LECA) expressed between 20 and 23 Rab paralogues (Elias et al. 2012; Klöpper et al. 2012) (Fig. 2.1, underlined). The LECA was probably highly advanced as it contained Rab proteins important for pathways like endocytosis, exocytosis, and ciliary sorting (Fig. 2.1, highlighted and labeled by the functional group) (Elias et al. 2012; Klöpper et al. 2012). In addition to Rab proteins, many other components of the transport machinery, such as coat and tethering proteins, are also highly conserved (Field et al. 2011; Yu and Hughson 2010). Therefore, the basic mechanisms of membrane transport were established early during eukaryotic evolution and Rab GTPases likely played a defining role in this process.

2.3 A Rabcentric View of Domain Formation

Obviously, Rab GTPases are not the only membrane-defining factors as they are just one of the cogwheels that make up the molecular clockwork of membrane traffic (Fig. 2.2). However, due to their tunable nature (GDP/GTP) and their direct and indirect connections to many other factors determining membrane functional identity, like phosphoinositides, tethers, and Soluble NSF Attachment protein Receptors (SNAREs), they do represent essential components of membrane domains (Fig. 2.2). In the next section, we would like to discuss how Rab GTPases functionally cross talk with other membrane factors and how these cooperatively define membrane domains and identity. Due to space limitations, we focus on the general concepts based on our current knowledge of the well-studied mammalian and yeast Rab proteins.

2.3.1 Rab GTPases Create Positive Feedback Loops

Rab GTPases behave both as soluble cytosolic and membrane-bound proteins and are post-translationally modified by the addition of prenyl (geranyl–geranyl) lipids (Lane and Beese 2006). Cytosolic GDP-bound Rab GTPases are in complex with Rab GDP-dissociation inhibitor (GDI) to keep them soluble. They can bind to the membrane after removal of GDI by a GDI displacement factor (GDF) (Dirac-Svejstrup et al. 1997; Ullrich et al. 1994; Sivars et al. 2003). After membrane binding, the Rab GTPase can be rapidly re-extracted from the membrane by Rab GDI (Wu et al. 2010). Therefore, Rab GDI maintains the equilibrium between membrane association and dissociation of Rab proteins. This equilibrium can be shifted in opposite directions by Guanine nucleotide Exchange Factors (GEFs) which catalyze the exchange of GDP for GTP (Barr and Lambright 2010; Cherfils and Zeghouf 2013) and GTPase-activating proteins (GAPs) which stimulate GTP hydrolysis (Frasa et al. 2012; Barr and Lambright 2010). Upon GTP binding, Rab proteins undergo a conformational change. This protects them from removal by Rab GDI and allows for Rab effector protein binding. Within this model, the subcellular localization of the GDF and especially of GEFs dictates the membrane localization of Rab GTPases (Blümer et al. 2013; Barr 2013). This, however, raises the question of what dictates the localization of GEFs.

Some Rab proteins are known to recruit their own GEF. In this way, a simple positive feedback loop is created, which contributes to the amplification of active Rab proteins and the establishment of a Rab membrane domain. Such a constellation exists at the level of endosomes where Rab5 recruits the Rab5 GEF Rabex-5 (also referred to as RabGEF1) through the interaction with the effector Rabaptin-5 (Horiuchi et al. 1997; Stenmark et al. 1995) (Fig. 2.3a). This is similar to the Cdc42p-positive feedback loop required for bud assembly in budding yeast, where Cdc42p recruits its own GEF complex (Kozubowski et al. 2008; Freisinger

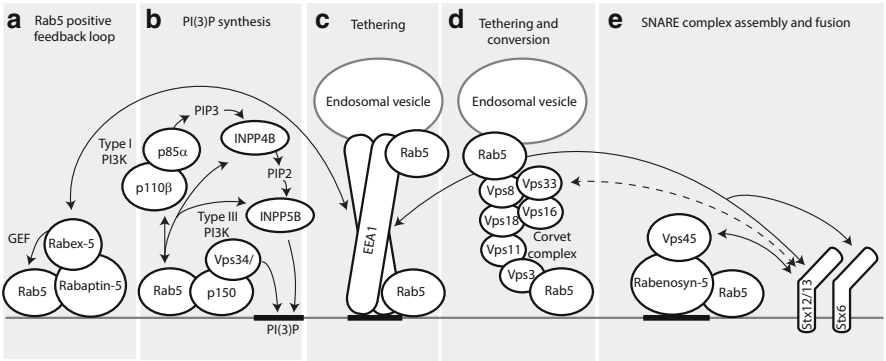

Fig. 2.3 Multiple steps in Rab5 membrane domain formation on endosomes. (**a**) The establishment of a Rab5 membrane domain involves the binding of Rab5 to a complex of Rabaptin-5 and Rabex-5. As Rabex-5 is a GEF for Rab5, a positive feedback loop is created, resulting in the recruitment of more Rab5. (**b**) Rab5 directly binds Type I and Type III PI3 kinase (PI3K) complexes. Type I PI3K synthesizes PI(3,4,5)P3 which, through the sequential action of Rab5-interacting phosphoinositide phosphatases INPP4B and INPP5B, can be hydrolyzed to PI(3)P. Type III PI3K synthesizes PI(3)P directly from PI. (**c**) PI(3)P and Rab5 combined function as binding platform for the coiled coil tether EEA1, which binds incoming endosomal vesicles. EEA1 forms a high molecular weight oligomeric complex with Rabex-5/Rabaptin-5 and endosomal SNAREs Syntaxin 6 and Syntaxin 12/13. (**d**) In yeast, and possible in mammals, Ypt51p/Vps21p (Rab5) is also involved in tethering of endosomal vesicles through the multi-subunit tether CORVET. CORVET subunit Vps33 is a member of the Sec1/Munc18 (SM) family and potentially interacts with endosomal SNAREs (*dotted line*). (**e**) SM protein Vps45 regulates the activity of endosomal SNAREs. Vps45 resides in a complex with Rabenosyn-5, which is recruited to membranes through an interaction with PI(3)P and Rab5

et al. 2013). Also in yeast, the late-endosomal Ypt7p (mammalian Rab7) recruits the HOPS complex that binds to the Mon1p–Ccz1p dimer. Mon1p–Ccz1p shows GEF activity towards Ypt7 and thereby enhances Ypt7 recruitment and endosomal maturation (Nordmann et al. 2010). It should be noted that Rab GTPases can also recruit GAPs and GEFs acting on upstream or downstream Rab GTPases, creating a Rab cascade that supports the vectorial flow of membrane within intracellular transport (see below *Rab cascades and conversion*).

In summary, many Rab GTPases can directly or indirectly interact with their activators, Rab GEFs. Thereby a positive feedback loop is established resulting in the recruitment of more Rab molecules, and consequently the binding of further downstream effector proteins. Therefore, the interaction between Rab GTPases and GEFs plays a seeding role in the establishment and maintenance of membrane domains.

2.3.2 Rab GTPases Cross talk with Phosphoinositides

Phosphoinositides (PIs) are key lipids in the specification of cellular organelles (Di Paolo and De Camilli 2006). PIs can be phosphorylated by PI kinases at the 3, 4, and 5 position on the inositol head group, giving rise to 7 PI species. Each species shows specific enrichment on subcellular compartments and membrane microdomains (Simonsen et al. 2001; Di Paolo and De Camilli 2006; Krauß and Haucke 2007). For example, phosphatidylinositol 4-phosphate PI(4)P is highly enriched at the Golgi complex, whereas PI(3)P is abundant at early endosomes. Phosphoinositide metabolism and Rab GTPase function are tightly interconnected and Rab proteins can directly bind PI kinases and phosphatases to generate lipid domains enriched in specific PIs (Jean and Kiger 2012). This is exemplified by the interaction of Rab5 with two PI3 kinases and two PI phosphatases. First, Rab5 binds the Type I PI3 kinase p85α–p110β (Kurosu and Katada 2001; Christoforidis et al. 1999b), which produces PI(3,4)P2 and PI(3,4,5)P3, and is required for cell motility, autophagy, and various signaling cascades initiated at the plasma membrane (Dou et al. 2013; Vanhaesebroeck et al. 2010; Yoo et al. 2010) (Fig. 2.3b). Second, Rab5 binds the Type III PI3 kinase Vps34–p150, (Christoforidis et al. 1999b; Murray et al. 2002; Backer 2008), thereby producing PI(3)P, an essential lipid for endosome function (Li et al. 1995; Jones and Clague 1995; Ohya et al. 2009) (Fig. 2.3b). PI(3)P, together with Rab5, forms a binding platform for downstream effector proteins containing a FYVE domain, like Early Endosome Antigen 1 (EEA1), Rabankyrin-5, and Rabenosyn-5. It should be noted that knockdown of Vps34 in human Glioblastoma cells did not prevent binding of EEA1 to membranes suggesting that other sources of PI(3)P might be available (Johnson et al. 2006). This could be provided by the hydrolysis of the signaling lipid PI(3,4,5)P3. Rab5 can recruit both the inositol polyphosphate-5-phosphatase B (INNP5B), which hydrolyzes PI(3,4,5)P3 to PI(3,4)P2, and the 4-phosphatase INNP4B, which hydrolyzes PI(3,4)P2 to PI(3)P, on endocytic vesicles (Shin et al. 2005). It is likely that both enzymes function sequentially to produce PI(3)P on endosomes in a Rab5-dependent manner (Fig. 2.3b) as suggested by in vitro studies (Shin et al. 2005).

Other examples of a direct link between PI-metabolizing enzymes and Rab proteins are the recruitment of the PI4 kinase "Four wheel drive" to Golgi membranes by Rab11 in Drosophila melanogaster (Polevoy et al. 2009) and that of the PI4Kβ1 by RabA4b in plants (Preuss et al. 2006). Also, the 5-phosphatase Oculocerebrorenal syndrome of Lowe protein OCRL (INPP5F) is recruited by Rab5 to endocytic vesicles and by Rab1 and Rab6 to the Golgi (Hyvola et al. 2006).

At the Golgi complex in yeast, we find an example of a "triangular" relationship between Rab proteins, GEFs and PIs. Exocytic traffic between the Golgi complex and the plasma membrane depends on the Rab family member Sec4p. Activation of Sec4p requires the GEF Sec2p, which binds both PI(4)P and the Rab11 homologue Ypt31p and its paralogue Ypt32p (Mizuno-Yamasaki et al. 2010).

In conclusion, Rab proteins control the formation of membrane domains by acting through both Rab GEFs and PI metabolism. Rab GEFs and PIs are directly

and indirectly interconnected and thereby a cooperative interaction mesh is created. This sustains membrane domain formation and further downstream PI/Rab GTPase effector recruitment.

2.3.3 *Rab Effector Proteins Include Membrane Tethers*

Molecular tethers guide the specific recognition and binding (tethering) of two opposing membranes and are thereby a prerequisite for membrane fusion. A key function of Rab proteins is to recruit such tethers on their respective membrane domain. Almost all tethers fall into two categories: multi-subunit complexes or rod-shaped extended coiled coil proteins. Examples of multi-subunit tethers are the transport protein particle (TRAPP), conserved oligomeric Golgi (COG), dependence on SLY1–20 (Dsl1) and Golgi-associated retrograde protein (GARP) complexes at the Golgi complex, Exocyst at the plasma membrane, and the "class C core vacuole/endosome tethering" (CORVET) and "homotypic fusion and protein sorting" (HOPS) complex at early and late endosomes respectively (Bonifacino and Hierro 2011; Bröcker et al. 2010). Examples of extended coiled coil tethers are EEA1 at endosomes and the Golgin family at the Golgi (Munro 2011). Membrane recognition domains are located on both sides of the tethers to provide membrane specificity by interaction with, amongst others, PIs and Rab GTPases (Bröcker et al. 2010). For example, Rab5 recruits a variety of tethers, including EEA1 and the CORVET complex (Christoforidis et al. 1999a; Simonsen et al. 1998; Peplowska et al. 2007).

EEA1 is an essential component of the endosomal docking and fusion machinery (Mills et al. 1998; Christoforidis et al. 1999a) and forms parallel coiled coil homodimers. On the N-terminus, EEA1 contains a Zn^{2+}-finger domain through which it binds to Rab5 and to a lesser extent, the related Rab22 (Kauppi et al. 2002; Simonsen et al. 1998; Lawe et al. 2002). The EEA1 C-terminus also has a Rab5 binding site and a FYVE domain for binding both Rab5 and PI(3)P (Fig. 2.3c) (Merithew et al. 2003; Simonsen et al. 1998). This topology allows for a more stable recruitment to the PI(3)P-enriched endosomal membrane via the C-terminus and, therefore, makes it suitable for tethering of incoming Rab5-positive endocytic vesicles onto endosomes. It should be noted that mutations within the C-terminal Rab5 binding site did not inhibit EEA1 membrane binding but induced enlarged endosomes suggesting that the interaction with Rab5 is required for proper endosome function (Lawe et al. 2002).

The multi-subunit endosomal tethering complex CORVET has only been studied in yeast so far. The complex consists of four core class C subunits Vps11p, Vps16p, Vps18p, and Vps33p, which are shared with the late endosomal/lysosomal tethering complex HOPS, and two CORVET-specific subunits Vps3p and Vps8p (Fig. 2.3d) (Peplowska et al. 2007). Both Vps3p and Vps8p can interact with Ypt51p/Vps21p, the yeast homologue of Rab5 (Horazdovsky et al. 1996; Plemel et al. 2011; Markgraf et al. 2009). By extending recent structural insight into HOPS

complex organization to the CORVET complex, we can assume that Vps3p and Vps8p are located on opposing ends of the complex and therefore ideally positioned for tethering Vps21p-positive membranes (Bröcker et al. 2012).

Other examples of Rab proteins binding to multi-subunit tethering complexes are Rab1/Ypt1p binding to TRAPP, Dsl, and COG complexes at the Golgi, Rab6 binding to GARP complex at the *trans*-Golgi network (Bröcker et al. 2010), and yeast Sec4p to its effector Sec15p, a subunit of the plasma membrane tethering Exocyst complex (Guo et al. 1999). Interestingly, Sec15p binds to Sec2p, the GEF for Sec4p, creating another elegant feed-forward system for Rab membrane domain generation (Stalder et al. 2013). Therefore, the general principle of the recruitment of tethers to Rab domains is conserved throughout evolution. Importantly, tethers are not just merely recruited, but a critical layer in the establishment of Rab membrane domains through interactions with GEFs, SNAREs, and PIs (Figs. 2.2 and 2.3).

2.3.4 *Rab GTPases Regulate SNAREs Through Tethers and SM Proteins*

Membrane fusion within the endocytic and exocytic pathways critically depends on the family of SNARE proteins. SNAREs on the donor and target membranes form a *trans*-SNARE complex that leads to membrane fusion. After fusion, the SNARE complex is disassembled with the help of NSF and Soluble NSF attachment protein (SNAP) so the SNAREs can be reutilized for new fusion events (Südhof and Rothman 2009). As SNARE pairing is rather promiscuous (Brandhorst et al. 2006), tight control of SNARE membrane localization and the fusogenic activity is essential. Rab GTPases are ideal candidates to perform such a function as they interact through their tethering effectors with SNAREs and Sec1/Munc18 (SM) priming factors (Fig. 2.2). The human genome encodes four classes of SM type of proteins, each restricted to a specific intracellular trafficking domain. Sly1 functions predominantly at the ER and Golgi, Munc18 at the plasma membrane, Vps45 at early endosomes, and Vps33 at the late endosomes and lysosomes (Rizo and Südhof 2012).

Vps45 is recruited to early endosomes through interaction with the Rab5 effector and PI(3)P-interacting protein Rabenosyn-5 (Nielsen et al. 2000) (Fig. 2.3e). Although the underlying molecular mechanism is not well understood, both Rabenosyn-5 and Vps45 are indispensable for endosomal fusion (Nielsen et al. 2000; Ohya et al. 2009). Most likely Vps45 controls the open/closed conformational state of endosomal SNAREs. The SM family member Vps33 is a subunit of both the CORVET and HOPS tethering complexes and binds Rab5 and Rab7, respectively. In the context of the CORVET complex, Vps33 could regulate endosomal SNARE activity. Although this has not been shown experimentally, this notion is supported by the fact that as a part of the HOPS complex, Vps33p

controls vacuolar fusion by priming the vacuolar SNAREs Vam3p, Vti1p, and Vam7p (Stroupe et al. 2006).

An additional way for Rab proteins to influence SNARE pairing is through interactions with molecular tethers. For example, the Rab6/Ypt6p-binding tethering complex GARP interacts with a variety of SNAREs, including the Golgi t-SNARE Tlg1p (Conibear et al. 2003; Siniossoglou and Pelham 2002), an evolutionary conserved interaction (Pérez-Victoria et al. 2010). Similarly, in yeast Sec4p interacts with Sro7p, an Lgl family member shown to regulate exocytic SNARE function (Grosshans et al. 2006a). At endosomes, EEA1 interacts directly with the endosomal SNAREs Syntaxin 6 and Syntaxin 13 (McBride et al. 1999; Simonsen et al. 1999) (Fig. 2.3e). Importantly, Syntaxin 13 is a part of a high molecular weight oligomer, whereby formation depends on Rab5 and EEA1 (McBride et al. 1999). This clustering is required for SNARE-driven fusion activity. These data argue that SNAREs alone have only limited membrane fusion activity. However, through enrichment and clustering of SNAREs, SNARE regulators, and tethers in Rab membrane domains, increased efficiency and specificity of membrane fusion can be achieved.

2.3.5 Motors Proteins Directly and Indirectly Interact with Rab GTPases

Both exocytic and endocytic membrane traffic depend on the transport of cargo-containing vesicles by molecular motors. Rab domains present ideal regulators of motor recruitment and activity as they define the identity and thereby the destination of the vesicle. A wide variety of actin and microtubule-dependent motors have been localized to early endosomes (Hunt and Stephens 2011). Although early endosome motility depends on Rab5 (Nielsen et al. 1999), no direct interaction between Rab5 and an endosomal molecular motor is currently known. However, the endosomal Kinesin Kif16B contains a PX domain via which it interacts with endosomal PI(3)P (Hoepfner et al. 2005). Also, Kif16B was recently shown to interact with the Rab4-related Rab14 (Ueno et al. 2011). Another example of a direct Rab/motor interaction is the binding of Rab4 to Kif3, important for the transport of GLUT4 vesicles (Imamura et al. 2003). Rab11 regulates plasma membrane recycling through a direct interaction with MyosinVb (Lapierre et al. 2001) and indirectly via the linker Rab11–FIP2 (Hales et al. 2002). In yeast, the Golgi-localized Rab Ypt31p/Ypt32p facilitates the recruitment of the Myosin V type motor Myo2p to exocytic vesicles, whereas the downstream GTPase Sec4p binds directly to Myo2p to coordinate transport of exocytic vesicles along the actin cytoskeleton (Jin et al. 2011; Lipatova et al. 2008). Interestingly, these processes are regulated by PI(4)P (Santiago-Tirado et al. 2011). In conclusion, Rab membrane domains function as a landmark for the binding of downstream effectors like motor proteins necessary to control the specificity of organelle and vesicular transport.

2.3.6 *Membrane-Deforming Proteins*

The sorting of protein and lipid cargo requires the formation of membrane-enclosed transport carriers, either vesicle or tubules. The formation of such transport carriers has to be coordinated in space and time to ensure correct cargo selection and targeting. Transport carrier formation requires the recruitment and assembly of coats and membrane-deforming proteins. Rab GTPases interact indirectly with coat complexes through interaction with tethers. Indeed, at the yeast *cis*-Golgi, Ypt1p binds to the coiled coil tether Uso1p (p115 in mammals), which recognizes incoming COPII-coated vesicles from the ER (Cao et al. 1998). Thereby it contributes to correct vesicle targeting in the exocytic system. Ypt1p is also required for retrograde Golgi trafficking by binding the multi-subunit tethering complex COG, which tethers COPI-coated vesicles (Suvorova et al. 2002).

Recycling of cargo from endosomes to the *trans*-Golgi network depends on the formation of cargo-containing membrane tubules at endosomes. This process relies on the Retromer, which consists of the tripartite complex of Vps26, Vps29, and Vps35, responsible for cargo recognition, and a Sorting nexin (SNX) dimer (Seaman 2012; Pfeffer 2013a). Members of the SNX family contain a BAR domain that binds to curved membranes (Carlton et al. 2004), and a PX domain which binds to PI(3)P. The binding of SNX to PI(3)P is essential for retromer function. Interestingly, synthesis of this pool of PI(3)P likely depends on the activity of Rab5 (Rojas et al. 2008). Furthermore, membrane recruitment of retromer depends on the direct binding of the Vps subcomplex to GTP-Rab7 (Rojas et al. 2008), a process in which the late endosomal Rab9 could play a similar or complementary role (Carroll et al. 2001; Dong et al. 2013). Thus, the sequential activity of Rab5 (PI(3)P synthesis) and Rab7 (Vps complex binding) is required for retromer function.

There are other examples of Rab GTPases recruiting membrane-deforming proteins. At early endosomes, Rab35 forms a tripartite complex with MICAL-L1 and ACAP2 to serve as a scaffold for recruitment of EHD1 to endosomal recycling tubules (Kobayashi and Fukuda 2013; Kouranti et al. 2006). EHD1 belongs to the Dynamin-like EHD family and contributes to the scission of the tubule. Interestingly, ACAP2 also functions as a GEF for the GTPase Arf6. In return, Arf6 binds to the Rab35 GAP TBC1D10 (Chesneau et al. 2012), generating cross talk between Arf and Rab GTPase families.

Above, we provide an overview of some of the possible interactions that Rab GTPases have with membrane-defining factors (Fig. 2.2). Through the consecutive action of Rab GTPases, GEFs, PI kinases and tethers, membrane domain formation is initiated (Fig. 2.3). Membrane domains function as a binding platform for further downstream effector proteins. This concept of membrane domain formation is mainly based on research on the well-studied Rab5 and its effectors. Nonetheless, due to the conservation of Rab GTPase and effectors, it is conceivable that similar molecular principles apply to the membrane domains formed of other Rab GTPases.

2.4 Directional Flow of Cargo via Rab Cascades and Conversion

Cargo flow relies on both cargo transport in vesicles and tubules and the conversion of the identity of organelles, for example, from early to late endosome (Vonderheit and Helenius 2005; Rink et al. 2005). Up to this point, two semi-related mechanisms have been described to explain cargo flow. The first is Rab conversion whereupon a particular Rab domain on an organelle is transformed to another Rab domain thus changing the membrane identity of the organelle. The second involves Rab coupling by Rab effectors leading to Rab cascades. In this section, we describe the requirements and the ordered series of transitions in cascades and conversion events necessary for the directional transport of cargo.

For both mechanisms, a distinct localization of the Rab proteins on membranes in a specific trafficking pathway is necessary. It was first shown for the recycling pathway that Rab proteins are indeed enriched on distinct areas of the endosome (Sonnichsen et al. 2000). By studying Rab4, Rab5, and Rab11, three dynamic populations of endosomes were identified: $Rab5^+$, $Rab5^+/Rab4^+$, and $Rab4^+/Rab11^+$. These different populations displayed differential sensitivity to pharmacological agents supporting the existence of distinct membrane domains. A similar compartmentalization exists on late endosomes where Rab7 and Rab9 localized to distinct membrane domains (Barbero et al. 2002). Interestingly, a few Rab effectors can also bind two different Rab GTPases simultaneously. For example, Rabaptin-5 and Rabenosyn-5 functionally couple Rab4 and Rab5 (Vitale et al. 1998; De Renzis et al. 2002). Another divalent Rab effector is the Rab Coupling Protein which binds to both Rab4 and Rab11 (Lindsay et al. 2002). The coupling of Rab GTPases either by localization of Rabs in a similar domain or by divalent Rab effectors is important for proper cargo sorting.

In addition to Rab GTPases localizing primarily to distinct membrane domains, the activation of the two Rab GTPases during membrane conversion must be coordinated. This can occur via a toggle switch where one Rab GTPase is activated at a time or a cutout switch where the activation of one Rab GTPase increases until a threshold value when the activation of the second Rab GTPase then occurs. A cutout switch was proposed to occur for LDL transport from Rab5 early endosomes to Rab7 late endosomes by combining mathematical models with experimental data (Del Conte-Zerial et al. 2008). In this cutout switch, a positive feedback loop from Rab5 to Rab7 exists with a negative feedback loop from Rab7 to Rab5 (Fig. 2.4a). These feedback loops control activation of the downstream Rab GTPase and deactivation of the upstream Rab GTPase.

Feedback loops are regulated by GEFs and GAPs. For the transition, GAP proteins are recruited as effectors for the upstream Rab GTPase and GEFs are recruited for the downstream Rab GTPase. For example in yeast, GEF cascades and GAP cascades at the Golgi are necessary to respectively activate the Rab GTPase during the conversion process and inactivate the counter Rab GTPase (Rivera-Molina and Novick 2009; Ortiz et al. 2002; Suda et al. 2013). The Golgi-localized

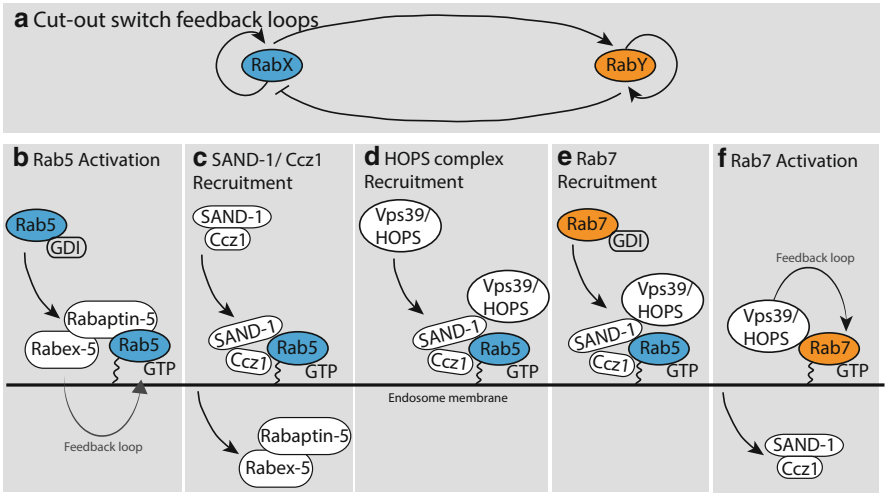

Fig. 2.4 Conversion model of early Rab5 endosomes to late Rab7 endosomes. The conversion of an endosome is a systematic series of events involving Rab5 and Rab7, their respective GEFs and GAPs, and the switch protein, SAND-1/Mon1. (**a**) Cutout switch feedback loops. Both Rab GTPases are under the control of positive feedback loops, which amplify their own activation. In addition, the upstream Rab GTPase (RabX) will activate the downstream Rab GTPase (RabY), whereas the downstream Rab GTPase inhibits the upstream Rab GTPase. (**b**) Rab5 recruits the Rabaptin-5–Rabex-5 complex. Rabex-5 is a Rab5 GEF and therefore a positive feedback loop is initiated. This will lead to the recruitment of (**c**) SAND-1/Mon1–Ccz1 resulting in displacement of the Rabaptin-5–Rabex-5 complex and interruption of the positive feedback loop. At a certain unknown threshold, SAND-1/Mon1–Ccz1 recruits the HOPS complex to Rab5 endosomes (**d**), which in turn recruits Rab7 (**e**). This switch then inhibits Rab5 activation and leads to Rab7 activation and a positive feedback loop (**f**)

Ypt31p/Ypt32p matures into secretory vesicles labeled with Sec4p. This switch is initiated by the recruitment of the Sec4 GEF by GTP-bound Ypt31p/Ypt32p (Ortiz et al. 2002). GAP cascades have been shown to occur between the Golgi and the endosome and within the Golgi from maturation of early to late Golgi (Suda et al. 2013; Rivera-Molina and Novick 2009). In both instances, the downstream Rab GTPase recruits the Rab GAP. The GEF and GAP cascades explain how Rab GTPases and Rab domains can dynamically interact and convert. However, additional mechanistic details are needed to better understand the process.

In addition to the GEFs and GAPs, additional proteins control the switch and the feedback loops. Recently, C. elegans SAND-1 and its mammalian homologue Mon1 were found to interfere with the binding of a Rab5 GEF to Rab5 by binding to Rab5–GTP in the conversion of Rab5–Rab7. Thereby, the Rab5 activation feedback loop is interrupted and the recruitment of Rab7 to the endosomal membrane is enhanced (Poteryaev et al. 2010). Initially in this model, Rab5 is activated by a positive feedback loop (Fig. 2.4b–f). Next, SAND-1/Mon1 in a dimer with Ccz1 is recruited to the Rab5 endosomes. The SAND-1/Mon1-Ccz1 complex recruits the HOPS complex whereupon HOPS functions as a Rab7 GEF. This

results in the activation of Rab7 and subsequent loss of Rab5. The recruitment of Rab7 may occur with the HOPS complex (Wurmser et al. 2000; Price et al. 2000), but there is too little evidence to support this as SAND-1/Mon1 binds to the common components of the HOPS and CORVET complexes. In addition, it is still unclear what exactly dictates the switch from Rab5 to Rab7 in time and space. This dual role of SAND-1/Mon1 by regulating two GEFs highlights how the coordination can efficiently occur and suggests that additional proteins may play similar roles for other membrane trafficking events.

Overall, Rab GTPases and their effectors are coordinated spatially and temporally via GEF and GAP cascades and conversion events to ensure a directional flow of cargo in the cell.

2.5 Signaling Functions Linked to Rab GTPases

Domains formed by Rab GTPases not only function in trafficking, they also are hypothesized to have a role in signaling. Many signaling events utilize similar proteins despite different downstream effects and a major question in the field is how is this controlled. Similar to conversion, signaling platforms are necessary for a signal to be spatially and temporally coordinated (Brandman and Meyer 2008; Grecco et al. 2011). Multiple proteins are known to link endocytosis and signaling (Sorkin and von Zastrow 2009; Platta and Stenmark 2011; Numrich and Ungermann 2014; Palfy et al. 2012). Signaling from endosomes via these proteins can be controlled in various ways, including altered receptor sorting or by creating specificity of the signaling. Due to space limitations and recent reviews, we will focus on two examples, Hepatocyte growth factor-regulated tyrosine kinase Substrate (Hrs) and Adaptor Protein containing Pleckstrin homology domain, Phosphotyrosine binding domain, and Leucine zipper motif (APPL), which control signaling events in disparate manners.

By controlling the sorting of the signaling receptor, Hrs alters endocytic signaling. Hrs is recruited to early endosomes via binding of its FYVE-finger domain to the PI(3)P-enriched Rab5 early endosomes. Here, Hrs interacts with Endosomal Sorting Complexes Required for Transport (ESCRT) components and ubiquitinated signaling receptors and thereby regulates the sorting of ubiquitinated cargo into intraluminal vesicles (Lloyd et al. 2002; Bache et al. 2002; Raiborg et al. 2002). Knockdown of Hrs results in sustained Epidermal Growth Factor (EGF) signaling increased EGF receptor recycling and a subsequent reduction in EGF receptor degradation (Raiborg et al. 2008; Malerød et al. 2007). Hrs is one example of how controlling EGF receptor sorting alters signaling events.

A different manner in which signals are controlled at the endosome is by creating signaling specificity. APPL1 and APPL2 are multi-domain proteins (here referred to as APPL) that bind directly to Rab5–GTP to mediate signaling from a specific endosomal membrane population (Miaczynska et al. 2004). In addition, APPL binds directly to the signaling proteins Akt and Adiponectin receptor (Mitsuuchi

et al. 1999; Mao et al. 2006). By binding to these various proteins, APPL coordinates signaling events spatially at the endosomal membrane. More interestingly, the localization of APPL and Akt at the endosome results in signaling specificity. This is exemplified by the fact that only a subset of downstream Akt effectors are regulated by APPL or localized on endosomes, respectively, shown in zebrafish and HeLa cells (Schenck et al. 2008; Miaczynska et al. 2004). This specificity is important for survival during the development of the zebrafish. This illustrates that the control of signaling at endosomes is of crucial importance and justifies further investigation into the relation between endosomes and signaling.

These are just two examples that illustrate how signaling can be controlled at endosomes. Overall, these events likely occur in defined membrane domains, but this so far has not been explicitly shown and requires further investigation.

2.6 Concluding Remarks and Future Perspective

Membrane domains are critical to ensure directional flow of membrane trafficking. In this chapter we discussed how Rab GTPases contribute to the establishment and conversion of membrane domains through the direct and indirect interactions with other membrane-defining factors like GAPs/GEFs, PI-metabolizing enzymes, tethers, SNAREs, motors, and membrane-deforming proteins (Fig. 2.2). Despite this knowledge, several key aspects still warrant further support and investigation.

An outstanding question is why there are so many Rab proteins including the related isoforms. For example, Rab5 has 3 paralogues in mammalian cell, Rab5a, Rab5b, and Rab5c (Gurkan et al. 2005) and all localize to early endosomes. Additionally, endosomal-localized Rab21, Rab22, and Rab31 are structurally and evolutionarily highly related to Rab5 (Stein et al. 2012). The functional significance of this redundancy is currently unknown. Some have tissue-specific expression patterns (Diekmann et al. 2011; Gurkan et al. 2005; Chan et al. 2011). Additionally, highly related Rab GTPases could have subtle differences in their binding affinities for the same effector protein. It would be informative to perform a systematic analysis of affinities of an effector protein for a set of related GTPases, for example, as performed for determining the binding of Rab4 and Rab5 to Rabenosyn-5 (Eathiraj et al. 2005). Also, a detailed analysis of knockdown phenotypes could reveal different phenotypes (Chen et al. 2009). Through the better understanding of the individual functions of related Rab GTPases, we might gain valuable insight into the fine-tuning of membrane domain formation.

Although the existence of Rab membrane domains is commonly acknowledged, we poorly understand the underlying molecular concepts. For example, how membrane tethers functionally control SNAREs and membrane fusion remains an unanswered question. Due to the complexity of cellular membranes, it is hard to extract such basic principles from a whole cell system. To address these core problems, it is necessary to further explore biochemical and biophysical approaches that allow us to reconstitute membrane domains ex vivo (Ohya et al. 2009).

Whereas most membrane domain models are derived from the study of individual protein–protein interactions and crude biochemical approaches, little is known how Rab GTPases and Rab effectors are organized spatially. Research in this direction is hampered by the technical limitation of standard light microscopes. Both the size of membrane domains and the distance between two distinct membrane domains are below the 200 nm diffraction limit of fluorescent microscopes and can therefore not be resolved. Fortunately, the recent advances in super-resolution microscopy and correlative light-electron microscopy (CLEM) (Hensel et al. 2013) provide a means to study this problem.

Most cell biological research on Rab domain localization and dynamics is performed in mammalian dedifferentiated tissue culture cells. Combined with the biochemical approaches discussed above, tissue culture cells have been extremely useful for the identification of basic aspects of domain formation; however, they poorly represent the complex nature of physiological cells in three-dimensional tissue. Therefore, future research on Rab GTPases and their membrane domains should increasingly focus on cells in a three-dimensional tissue context and living vertebrate model system (Weigert et al. 2013).

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Chapter 3
Effectors of Rab GTPases: Rab Binding Specificity and Their Role in Coordination of Rab Function and Localization

Lena Katharina Oesterlin, Olena Pylypenko, and Bruno Goud

Abstract Effector proteins interact with GTPases in their active/GTP-bound state. To achieve this specificity, all effector proteins interact with Rab proteins via a similar Rab surface area which shows the largest conformational changes upon nucleotide exchange. This surface is also involved in the interaction with other Rab interacting proteins. In spite of the high structural similarity of the Rab proteins, the Rab binding domains of effector proteins display a large structural diversity. Structural comparison of uncomplexed activated Rab proteins and Rab:effector complexes reveals two distinct binding mode, that can be defined as key-lock or induced-fit mechanism (depending on conformational change upon binding). Generally, the specificity of Rab:effector interactions seems to be determined by surface residues on the Rab protein. In addition, the structural plasticity and conformational stability of the Rab proteins are crucial for their specificity.

Rab:effector interactions exhibit a large diversity in binding affinities. The μM equilibrium dissociation constants (K_ds) of some complexes are most likely crucial for a fast regulation of Rab-regulated processes as they allow fast access of GAP proteins to the active Rab GTPase. Fast dissociation rates would also be necessary for a better regulation of effector recruitment as only a combination of the interaction of the effector protein with a Rab on the one hand and the interaction of another domain of the modular effector protein with the membrane or a membrane-associated protein on the other hand would lead to efficient membrane recruitment of an effector protein and, therefore, to signal transduction.

Keywords Rab effectors • Rab GTPases • Rab:Rab effector structure

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3.1 Introduction

Rab proteins are key players for the regulation of intracellular trafficking events. The regulation by Rab proteins occurs through their interaction with effector proteins, which bind specifically to the active, GTP-bound form of the GTPase (Hutagalung and Novick 2011). Intracellular trafficking events are composed of many different steps [reviewed in Bonifacino and Glick (2004)], including budding and fission of transport vesicles from donor membranes, their movement along cytoskeletal tracks, and their docking/fusion to acceptor membranes. Rab GTPases regulate virtually all these steps. As these processes are very diverse in nature, it is not surprising that Rab effectors are also very diverse. In the first step of vesicular transport, the budding and fission of vesicles, the cargo recruitment needs to be tightly regulated. The Rab9 effector TIP47 for instance has been implicated in this process as it changes its affinity for its cargo Mannose-6-phosphate receptor upon Rab binding (Carroll et al. 2001). The transport to the correct target membrane is regulated via the interaction with different types of motor proteins that are effectors of Rab GTPases such as the actin-binding motors Myosin Vb (MyoVb), an effector of Rab11 (Lapierre et al. 2001), and MyoVI, which binds via Optineurin to Rab8 (Sahlender et al. 2005). Rab6 was the first Rab protein for which a direct interaction with a microtubule binding motor (Rabkinesin-6) was demonstrated (Echard et al. 1998). Rab6 is also linked to the Dynein/Dynactin complex through a direct interaction with the Dynactin subunit p150^{Glued} (Short et al. 2002) or indirectly via Bicaudal D1/2 (Matanis et al. 2002). A tight regulation of the interaction with motor proteins as well as an exact recognition of the target membrane is essential to achieve correct vesicle targeting. After delivery of the vesicle to the target membrane, the vesicle has to be tethered to allow subsequent fusion. Several interactions between Rab GTPases and tethering factors have been reported such as the interaction between Rab1 and the *cis*-Golgi tethering protein GM130 (in complex with GRASP65) and the tethering factor p115 (Allan et al. 2000; Moyer et al. 2001). Furthermore the large multicomponent tethering complexes COG and Exocyst have also been demonstrated to be Rab effectors [reviewed in Beard et al. (2005)]. After correct tethering of a vesicle, the vesicle coat needs to be disassembled to allow vesicle fusion, the last and final step of a transport event. In comparison to the other steps, the influence of Rab proteins on this last step seems to be more indirect [reviewed in Grosshans et al. (2006)].

Until today more than 100 Rab effector proteins have been identified [reviewed in Hutagalung and Novick (2011)]. However, if one takes as an example the extensively studied Rab6, about 20 putative effector proteins have been described (Goud and Akmanova 2012). This makes it likely that for many other Rab GTPases new effector proteins will be identified in the future.

In this review we focus on explanations for the specificity of Rab:effector interactions, the role of effectors in the coordination of Rab function, and their localization to specific membranes.

3.2 Structural Features of Rab:Effector Interactions

3.2.1 Diversity of Rab Effectors

Rab effector proteins are multi-domain proteins containing at least one domain that is responsible for the interaction with the Rab protein. To date, 22 structures of these Rab binding domains (RBDs) of effectors in complex with Rab proteins (representing 16 different Rab:effector complexes) have been published (Table 3.1, Fig. 3.1). In spite of the very high structural similarity of different Rab proteins, the minimal effector RBDs significantly vary both in size (from 3.7 kDa for EAA1 to 45.6 kDa for MyoVb) and in structural organization. Most RBDs exhibit a primarily α-helical structure, forming either coiled-coils (Rabaptin-5, FIPs, GCC185, RILP), α-hairpins [Rabenosyn-5 Helical Domain 2 (HR2) and Heptad Repeat Domain (HepR)], or α-helical bundles (R6IP1, MyoVb). Some of the RBDs contain Zn^{2+} binding subdomains that play either a scaffolding role supporting the effector structure (Slac-2, Rabphilin-3), or they directly interact with the Rab (EEA1). β-sandwich subdomains can also directly interact with a Rab (OCRL1) or play a scaffolding role (R6IP1).

As can be seen from the summary in Table 3.1 and in Fig. 3.1, 30 % of the effector structures display a 2:2 stoichiometry. In all these cases, an effector dimer interacts with two Rab proteins that do not contact each other directly. The interactions occur via a dimeric symmetrical coiled-coil, which forms two symmetrical Rab binding sites. The monomeric RBDs, however, generate only a single Rab binding site (Fig. 3.1).

In Fig. 3.1, representative Rab:effector structures have been grouped according to their interacting Rab protein (one group, for example, is formed by Rabaptin-5, EEA1, and the Helical Region 2 (HR2) of Rabenosyn-5 as they can all interact with Rab5). The recently published structures of LidA in complex with Rab1/Rab8 are not represented in the figure and are not further discussed in this review. This protein indeed represents a very unusual bacterial Rab effector, also termed Rab supereffector, because of its high affinity for Rab:GTP and Rab:GDP and its extensive promiscuity in Rab interactions (Schoebel et al. 2011; Cheng et al. 2012). When comparing the native Rab:effector structures, it can be clearly seen that the RBDs interacting with the same Rab protein have large structural differences. Rab5-specific effector structures are represented by three different folds, a C_2H_2 zinc finger for EAA1, a parallel homodimeric coiled-coil for Rabaptin-5, and an α-helical hairpin for Rabenosyn-5 (HR2), that exhibit structural similarity to an antiparallel coiled-coil. The Rab6 binding golgin GCC185 is a parallel coiled-coil. In contrast, the R6IP1 RBD is composed of two subdomains: an α-helical bundle supported by a β-sandwich. Rab11 effector RBDs are represented by the highly homologous parallel coiled-coils of FIP2 and FIP3, and a two subdomain unit composed of two α-helical bundles for MyoVb. For some effector proteins, like the Rab27 effectors Slp2-a, Slac-2a/melanophilin, and Rabphilin-3a, a clear structural homology can be observed. All three RBDs consist of two

Table 3.1 Structurally characterized Rab:effector interactions and their stoichiometry

Rab	Effector	PDB Id	Complex stoichiometry	Reference
Rab1a:GDP	LidA	3SFV	1:1	Cheng et al. (2012)
Rab1a:GTP	LidA	3TKL	1:1	Cheng et al. (2012)
Rab3aQ81L: GTP	Rabphilin-3a	1ZBD	1:1	Ostermeier and Brunger (1999)
Rab4aQ67L: GTP	Rabenosyn-5 (HepR)	1Z0K	1:1	Eathiraj et al. (2005)
Rab5aQ79L: GTP	EEA1 (C_2H_2 Zn finger)	3MJH	1:1	Mishra et al. (2010)
Rab5a:GppNHp	Rabaptin-5	1TU3	2:2	Zhu et al. (2004)
Rab6aQ72L: GTP	GCC185	3BBP	2:2	Burguete et al. (2008)
Rab6aQ72L: GTP	Rab6IP1	3CWZ	1:1	Recacha et al. (2009)
Rab7aQ67L: GTP	RILP	1YHN	2:2	Wu et al. (2005)
Rab8a:GppNHp	LidA	3TNF	1:1	Schoebel et al. (2011)
Rab8a:GppNHp	OCRL1	3QBT	1:1	Hou et al. (2011)
Rab11a: GPPNHP	FIP2	4C4P	2:2	Lall et al. (2013)
Rab11aQ70L: GTP	FIP2	2GZH	2:2	Jagoe et al. (2006)
Rab11aQ70L: GTP	FIP2	2GZD	2:2	Jagoe et al. (2006)
Rab11aQ70L: GTP	FIP3	2D7C	2:2	Shiba et al. (2006)
Rab11aQ70L: GTP	FIP3	2HV8	2:2	Eathiraj et al. (2006)
Rab11a:GTP	MyoVb	4LX0	1:1	Pylypenko et al. (2013)
Rab11a: GDP	MyoVb	4LWZ	1:1	Pylypenko et al. (2013)
Rab22a Q64L: GTP	Rabenosyn-5 (HR2)	1Z0J	1:1	Eathiraj et al. (2005)
Rab25:GTP	FIP2	3TSO	2:2	Lall et al. (2013)
Rab27aQ78L: GTP	Slp2-a	3BC1	1:1	Chavas et al. (2008)
Rab27bQ78L: GTP	Slac2-a/ melanophilin	2ZET	1:1	Kukimoto-Niino et al. (2008)

α-helical regions linked either directly (Slp2-a) or via a Zn^{2+} binding subdomain (Slac2-a and Rabphilin-3a). This is consistent with the fact that the Slp2-a, Slac2-a, and Rabphilin-3a effector domains exhibit significant sequence similarity. Structural homology, however, does not necessarily lead to binding of the same Rab proteins. The two α-helical hairpin RBDs of Rabenosyn-5 for example are highly homologous in structure, but they do recognize distinct Rab proteins.

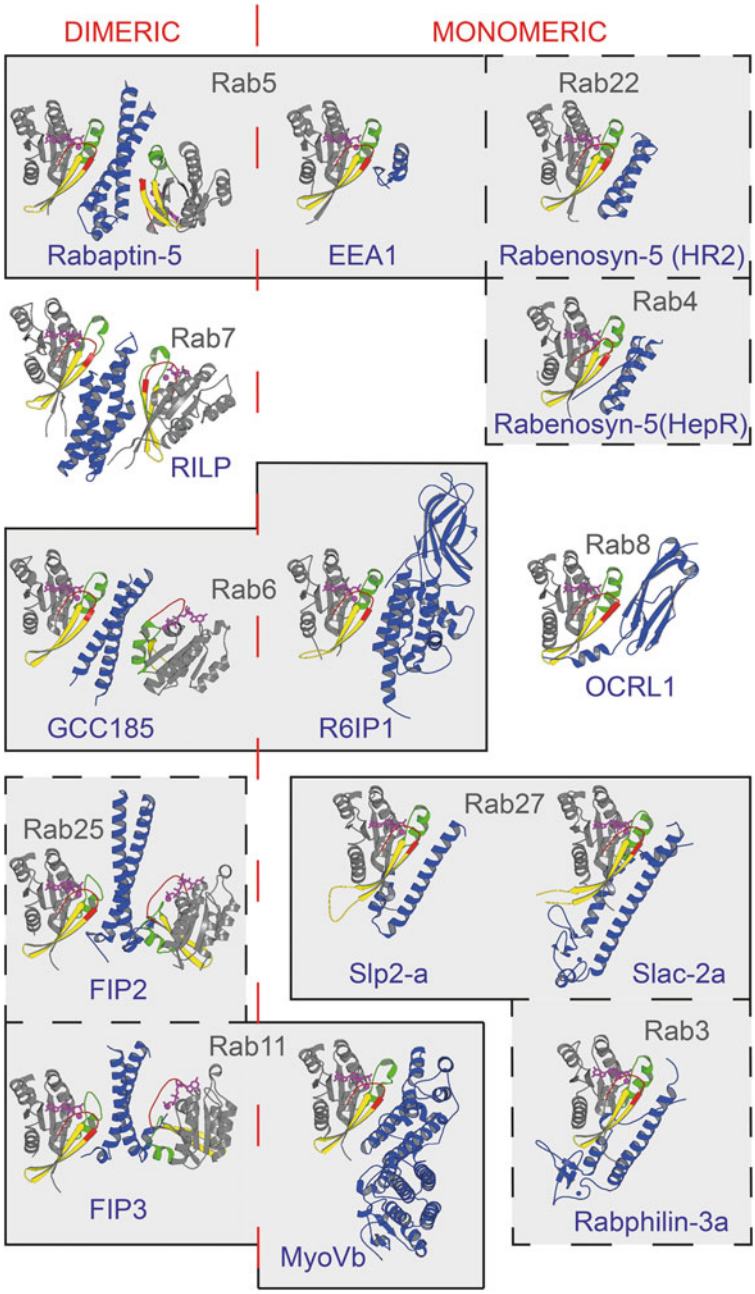

Fig. 3.1 Representative Rab:effector structures shown in *ribbon representation*. The structures are grouped according to the Rab protein with which they interact. All Rab proteins are depicted in *light gray*. Their switch regions are colored in *red* (switch I) and *green* (switch II); the interswitch region is *yellow*. Effector proteins are colored in *dark blue*. The Rab proteins have been superimposed to allow a comparison of effector orientation. Monomeric (1:1) complexes are

3.2.2 Conformational Switching of Rab Proteins

An underlying feature of all GTPases is a large conformational difference between the two nucleotide binding states (Vetter and Wittinghofer 2001). As effector proteins should bind specifically to the active/GTP-bound form of the Rab protein, it is not surprising that all effectors interact mainly with the switch regions of the Rab protein that show the largest conformational changes upon nucleotide exchange (Fig. 3.2). In the GDP-bound state, the Rab switches are conformationally flexible, whereas in the GTP-bound state the γ-phosphate moiety of GTP mediates intermolecular interactions stabilizing the switches in a conformation that can be recognized by specific effectors. The first published structure of a Rab:effector complex (Rab3:Rabphilin3a) demonstrated that, besides the switch and interswitch regions, Rab3 interacts with the effector via three regions termed complementary determining regions (CDRs, illustrated in Fig. 3.2) (Ostermeier and Brunger 1999; Lee et al. 2009). CDR1 and CDR2 are localized at the beginning of the first β-sheet (β1) and in a loop region between the α3 helix and the β5 sheet, respectively. The CDR3 region is localized in the C-terminal region of the protein in the immediate extension of the α5 helix. The CDRs exhibit low sequence homology among different Rabs and were proposed to determine effector recognition specificity in Rab GTPases (Ostermeier and Brunger 1999). Furthermore, a structural comparison of this first Rab:effector structure with the structure of unbound Rab5 (Merithew et al. 2001) suggested that the conformations of three conserved aromatic residues forming a hydrophobic triad in Rab proteins are relevant in achieving effector specificity: a phenylalanine and a tryptophan in the interswitch region and a tyrosine/phenylalanine in the switch II region (illustrated in Fig. 3.2). These residues exhibit different conformations among Rab GTPases of different subfamilies, which impact their surface complementarity to the specific effectors (Merithew et al. 2001).

Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) need to access the nucleotide binding site to regulate the nucleotide binding status of Rab proteins. The guanine nucleotide dissociation inhibitor proteins (GDIs) as well as the Rab escort protein (REP) sense the nucleotide state of the Rab proteins (Wu et al. 2010). Therefore, it is not surprising that the binding sites of these proteins largely overlap with that of effector proteins as shown in Fig. 3.3. To our knowledge, simultaneous binding of REP, GDI, GEFs, GAPs, and effectors has therefore never been observed.

The intimate involvement of the switch regions in the interaction with many different binding partners makes it difficult to find mutations in Rab proteins that target only one effector recognition without impairing Rab activation/deactivation and/or recycling/prenylation. Therefore, the dissection of Rab effector networks in

Fig. 3.1 (continued) represented on the *right*, and dimeric (2:2) complexes are shown on the *left side* of the figure. PDB IDs and corresponding references for the structures are listed in Table 3.1

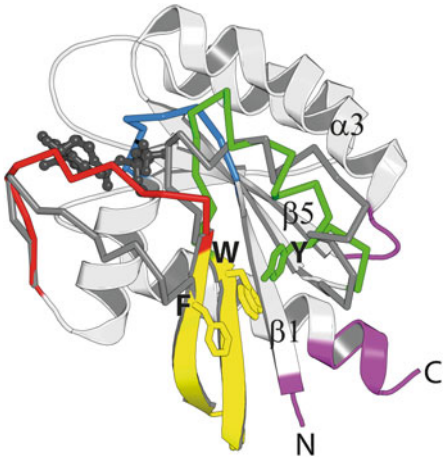

Fig. 3.2 Structural features of Rab proteins and their conformational changes upon nucleotide exchange. The comparison of the GTP and the GDP-bound structure of Sec4p is shown (PDB ID 1G16/1G17, Stroupe and Brunger (2000)). In the GTP-bound structure switch I is colored in *red*, switch II is colored in *green*, the p-loop is *blue*, the interswitch regions are *yellow*, and the CDRs (complementary determining regions) are shown in *pink* (numbered 1–3 from N to C-terminus). The bound GTP is depicted in *dark gray*. Aromatic residues forming the Rab hydrophobic triad are shown in *sticks* and labeled with one letter amino acid code. The largest conformational changes occur within the switch regions

cell biology experiments is challenging, as off-target effects could lead to results that are difficult to interpret. Generally, mutations in the CDRs might have the highest potential to only influence Rab:effector interactions as these regions are not significantly involved in the interaction with other proteins and also seem to be partially responsible for effector specificity.

3.2.3 Rab:Effector Binding Sites

The large amount of available structures of Rab proteins in complex with effectors makes it possible to compare the interaction sites of different effector proteins. In Fig. 3.4, Rab:effector structures are depicted in which the interacting residues are represented by spheres. The structures have been sorted according to the size of the interacting surface area on the Rab proteins (as listed in Table 3.2). The surface area on the Rab protein that is buried upon effector complex formation ranges from 560 $Å^2$ to 1860 $Å^2$. The smallest interaction area is observed in the Rab5:EAA1 complex, which also represents the smallest RBD structure solved to date. However for the other effectors, the buried area is not correlated to the size of the RBD.

To evaluate the conservation of the residues involved in Rab:effector interactions, we have performed a sequence alignment of all Rab proteins that are present

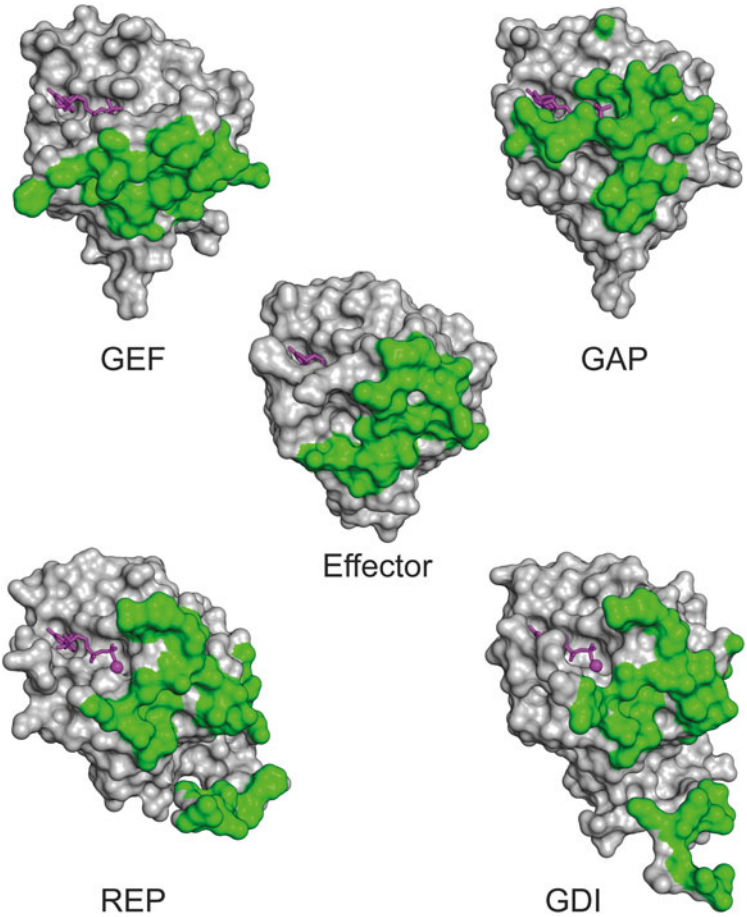

Fig. 3.3 Interaction sites of different Rab binding proteins. Surface representation of Rab proteins illustrating the interacting residues within complex structures. The interaction sites of a Rab: effector, Rab:GEF, and Rab:GAP interaction and of GDI and REP are shown in *green*. All Rab residues that contain atoms that are within 5 Å of an effector atom were considered to be part of the interaction site. The Rab8:Rabin8 structure was chosen as an example of a Rab:GEF structure [PDB ID 4LHZ (Hou et al. 2011)]. The GAP interaction was taken from the Rab1b:TBC1D20 structure [PDB ID 4HLQ (Gavriljuk et al. 2012)]. The Rab7:REP [PDB ID 1VG0 (Rak et al. 2004)] and the Ypt1:GDI structure [PDB ID 2BCG (Pylypenko et al. 2006)] were used to represent the interactions of REP and GDI. In comparison to the other interactions the interactions with REP and GDI show a second interaction area that represents the C-terminal region of the Rab proteins

in the published complexes [performed with ClustalX (Larkin et al. 2007)]. In Fig. 3.4, the interacting residues on the Rab proteins are colored according to their conservation within this alignment. It can be seen that all Rab proteins recognize

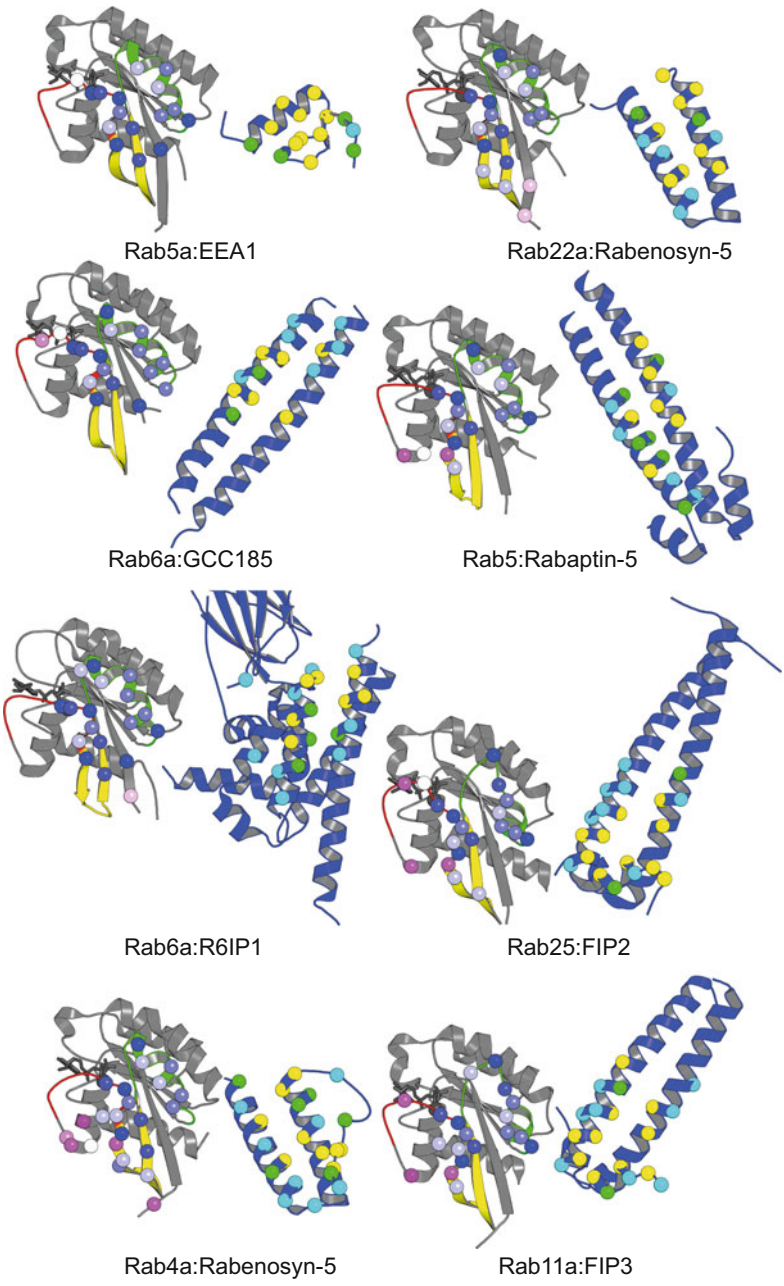

Fig. 3.4 (continued)

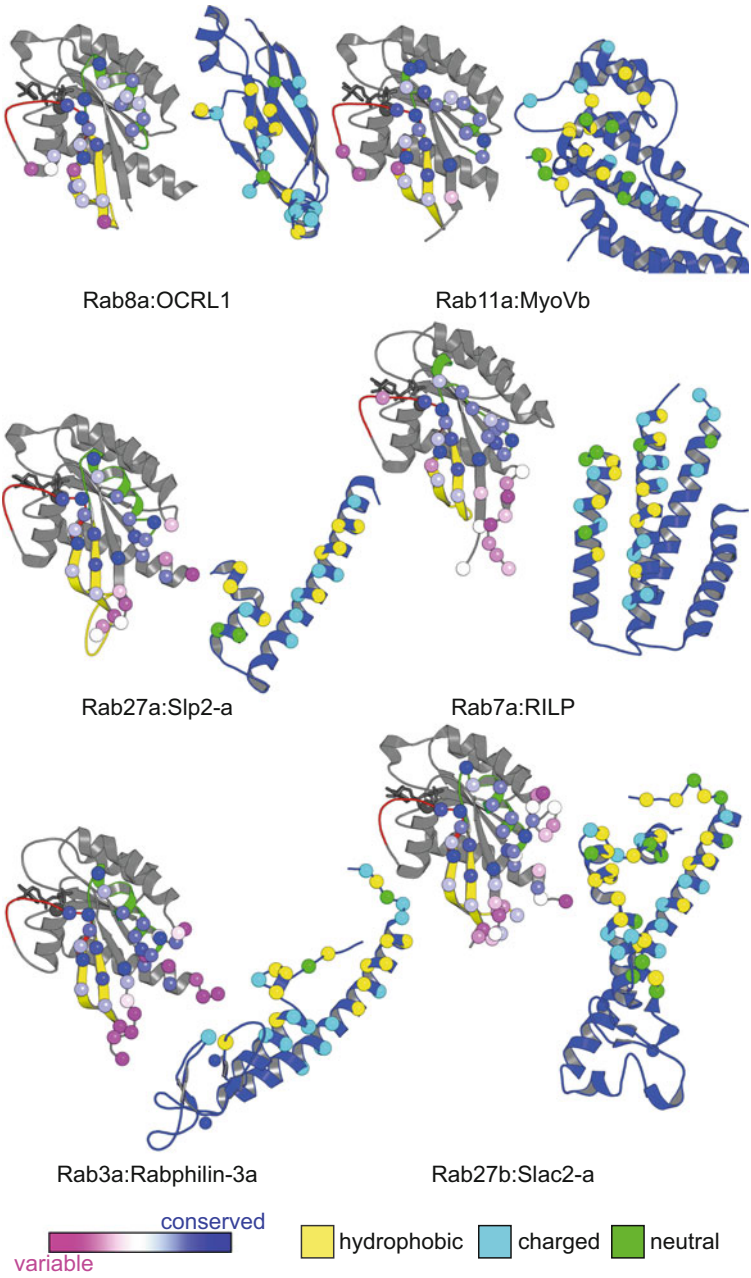

Fig. 3.4 Conservation of Rab:effector interaction sites. Ribbon representation of Rab proteins taken from the Rab:effector structures summarized in Table 3.1. In comparison to Fig. 3.1, the Rab proteins and their effectors have been rotated by 90° in opposite directions to allow a visualization of the binding sites. All Rab residues containing atoms that are within 5 Å of an effector atom are

Table 3.2 Comparison of Rab:effector interaction surfaces and affinities

Rab	Effector	PDB ID	Buried area[b]/Å^2	# of H bonds[b]	# of salt bridges[b]	K_d/nM
Rab5a	EEA1	3MJH	560	7	0	2,400
Rab22	Rabenosyn-5 (HR2)	1Z0J	670.5	10	3	9,700
Rab6a	GCC185	3BBP	675.1	9	4	2,300
Rab5a	Rabaptin-5	1TU3	727.8	8	1	
Rab6a	R6IP1	3CWZ	749.4	9	10	
Rab11a	FIP2	4C4P	774.5	5	7	250/(40)[a]
Rab25	FIP2	3TSO	840.4	8	3	650
Rab4a	Rabenosyn-5 (Heptad repeats)	1Z0K	893.8	14	6	6,200
Rab11a	FIP3	2HV8	944.1	13	6	290
Rab8a	OCRL(539-901)	3QBT	954.9	16	7	900
Rab11a	MyoVb	4LX0	979.6	7	0	254
Rab27a	Slp2-a	3BC1	985.2	9	6	(13.4)[a]
Rab7a	RILP	1YHN	1,265.6	13	10	
Rab3a	Rabphilin-3a	1ZBD	1,426.5	9	6	
Rab27(a)/b	Slac2-a/melanophilin	2ZET	1,860.2	21	9	(112)[a]

[a]Brackets mark affinities that were not described in the same publication; for the Rab27:Slac2-a interaction, the affinity was determined for Rab27a while the structure of Rab27b and Slac2-a was determined.

[b]Calculated using PISA v1.48 (http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver) (Krissinel and Henrick 2007).

their effectors using the most conserved parts of the protein—switch I, switch II, and the interswitch region. The largest interaction areas are observed in the complexes where the interaction between Rab and effector protein is extended towards the less conserved CDRs. The effector binding specificity is defined by the Rab amino acid composition of the binding site. The strictly conserved residues within the Rab effector binding sites contribute to Rab-family specific effector recognition. Each RBD binds to its specific subset of Rab proteins. This subset recognition is achieved by residues that are only conserved within the subset of Rab proteins that are recognized by a specific effector (Eathiraj et al. 2005). Non-conserved residues within the interaction site support the recognition of individual Rabs. Rab4 and Rab5 for example selectively bind to the two distinct

Fig. 3.4 (continued) depicted as *spheres*. The switch regions are colored in *red* (switch I) and *green* (switch II), and the interswitch regions are shown in *yellow*. Interacting residues on Rab proteins are colored according to their conservation determined by sequence alignment of all Rab proteins in published Rab:effector structures [performed with The ConSurf Server (Glaser et al. 2003; Landau et al. 2005)]. Interacting residues of the effector proteins are colored according to the properties of the amino acid (hydrophobic, charged, neutral). The structures were sorted according to the size of the interacting area on the Rab protein as summarized in Table 3.2

Rab effector domains of Rabenosyn-5. Structure-based point mutations of Rab5 to the corresponding residues in Rab4 switch I and switch II significantly reduce its affinity to the Rabenosyn-5 HR2 domain, and Rab5-mimicking point mutations in switch I of Rab4 reduce the affinity towards the Rabenosyn-5 HepR domain (Eathiraj et al. 2005). Another example is the highly specific interaction of Rab27 with Slac2-a, an effector protein that does not interact with Rab3 that is highly homologous to Rab27. Replacement of only four CDR1 and switch II residues in Rab3a by the corresponding residues of Rab27b led to a significant Slac2-a binding activity (Kukimoto-Niino et al. 2008), demonstrating that selectivity within highly homologous Rab proteins can be achieved by only a couple of residues. However, this is not the case for phylogenetically distinct Rab GTPases. Mutations of the Rab5 switch region that exchange amino acids with their homologues in Rab4 lead to a reduced affinity for Rab5 effectors. However, the Rab5-mimicking switch region mutant of Rab4 is not able to interact with these effectors. Additional substitutions in the proximal protein core are necessary to achieve binding of these Rab4 mutants to Rab5-specific effectors (Mishra et al. 2010). This demonstrates that, besides the surface residues, structure and conformational stability of the switch regions (and therefore of the protein core) are important for specific effector recognition (Mishra et al. 2010).

A comparison of different structures of Rab:effector complexes reveals that, even though several effectors interact with Rab proteins via α-helical domains, the orientation of the α-helices on the surface of the Rab is significantly different (Fig. 3.4). The coiled-coil GCC185 and the Rabaptin-5 effector structures show, for example, a rotation of approx. 90° of the axis drawn through the longitude of the α-helices. Even effectors binding to the same GTPase exhibit absolutely different structures (i.e., the Rab5 binding effectors EAA1 and Rabaptin-5). However, the Rab binding sites of most of the effectors exhibit a common feature, which can be seen in Fig. 3.4 where we colored the interacting residues of the effector proteins according to their properties (hydrophobic, charged, neutral). All Rab effectors clearly display a hydrophobic patch on the surface that is involved in Rab interaction. In the complexes, the hydrophobic patches interact with a complementary hydrophobic surface on the Rab proteins, which is formed by nonpolar residues of switch I and switch II and the conserved hydrophobic triad. This confirms the proposed crucial role of the hydrophobic triad in specific recognition of binding partners, as these residues can achieve specificity by structural conformation as discussed earlier. This observation may help to identify potential Rab binding sites for effectors of known ternary structure without determining the complex structures as solvent-exposed hydrophobic residues have a high potential to be part of the Rab binding interface.

3.2.4 *Rab Structural Plasticity Contributes to Effector Binding Specificity*

A comparison of the structure of uncomplexed GTP-bound Rab proteins and their structure within the Rab:effector complex reveals two different binding modes for Rab:effector interactions. Most of the Rab effectors show little or no conformational change upon binding to Rab proteins. This binding mechanism could be characterized as key-lock mechanism. For example, comparison of the structures of unbound Rab3 and Rab3 bound to Rabphilin-3 demonstrates that the Rab3 structure is preformed for Rabphilin-3 recognition and binding (Ostermeier and Brunger 1999) (Fig. 3.5). In contrast, Rab11 exhibits a significant structural change upon effector binding that is expressed in a conformational rearrangement in switch II upon FIP2/3 binding (Eathiraj et al. 2006; Shiba et al. 2006; Lall et al. 2013) and is even more pronounced in the MyoVb:Rab11 complex (Pylypenko et al. 2013). In this complex, the conformation of both switches and of the hydrophobic triad residues is changed in the Rab:effector structure in comparison to uncomplexed Rab11:GTP. This demonstrates the importance of structural plasticity of Rab proteins for binding of some effectors, and it indicates a possible induced-fit mechanism of the binding. Of note, Rab6 binds to its effectors with different binding modes. R6IP1 recognizes and binds Rab6 without a conformational change (Recacha et al. 2009), whereas GCC185 binding requires remodeling of the hydrophobic triad residues that is also associated with a shift of switch II (Burguete et al. 2008). These observations demonstrate that not only the amino acid composition but also their conformational flexibility contribute to Rab:effector binding specificity.

3.3 Affinities of Rab Effector Interactions

The affinities between active Rab GTPases and their cognate effectors span a wide range from 40 nM to 16 μM (Table 3.3). Even though some interactions are of moderately high affinity, the interaction between the molecules remains dynamic on a physiologically relevant timescale. Likely, dynamic binding of effectors is required to permit the efficient binding of GAPs at the end of the Rab cycle in order to allow its enzymatic deactivation. Since effectors and GAPs share overlapping binding sites on Rabs, dynamic binding ensures access of the GAP to the Rab due to the rapid spontaneous dissociation of the effector. It could be already demonstrated with the high-affinity binding protein LidA from *Legionella pneumophila* that a high-affinity Rab binding protein (with a pico- to nanomolar K_d) can act as a competitive inhibitor for GAP binding (Schoebel et al. 2011; Neunuebel et al. 2012). This demonstrates that the dissociation rate of the effector protein can largely influence the accessibility of the Rab protein for a GAP protein and can therefore indirectly regulate the speed of deactivation. Especially in processes that

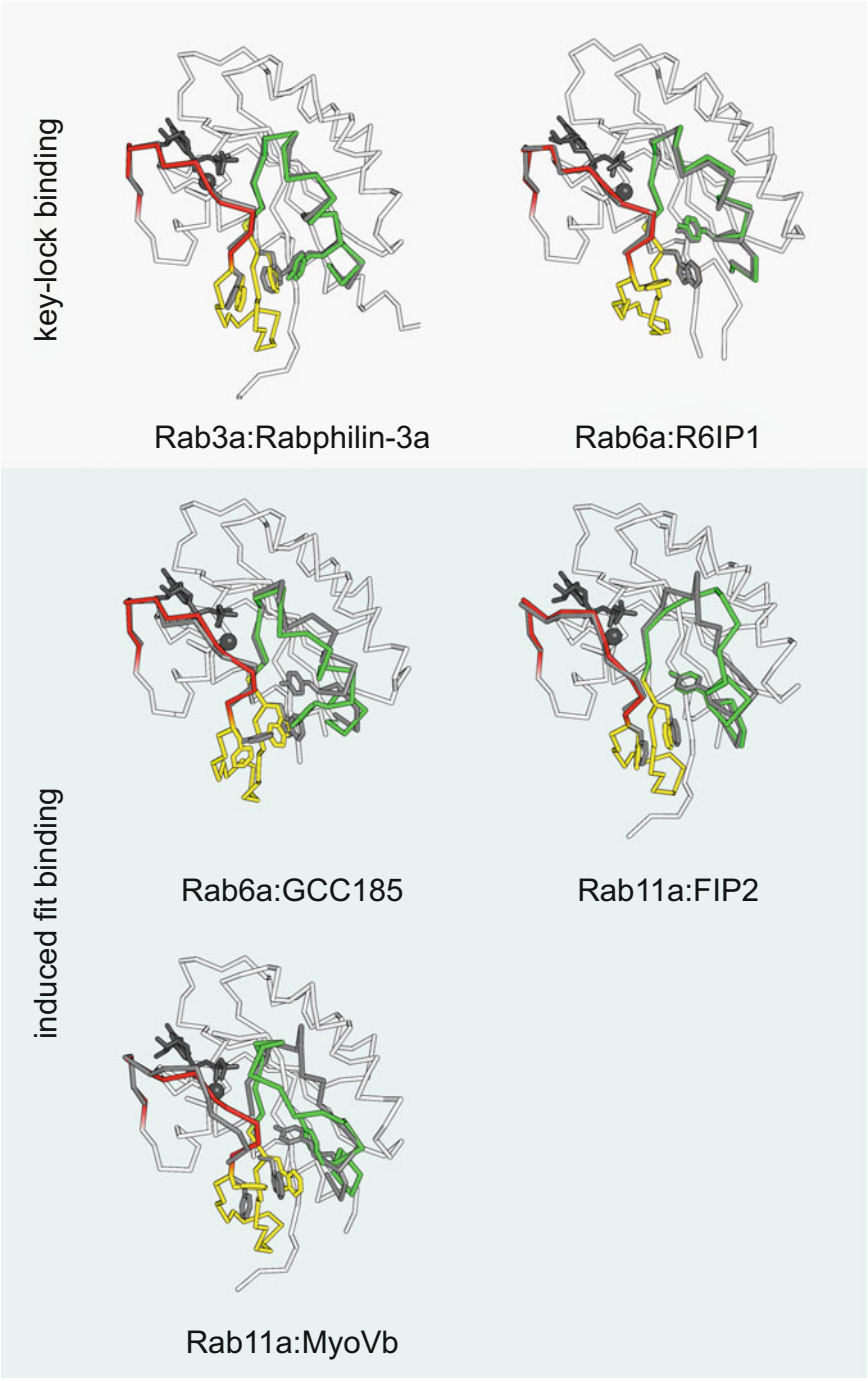

Fig. 3.5 Comparison of bound and unbound Rabs. Superimposition of Rab proteins in a complex with an effector (switch I *green*, switch II *red*, interswitch *yellow*) on the corresponding unbound

need to be regulated in a very short time frame, such as for example neurotransmitter release at the synapse, a fast dissociation rate of the effector protein (which in most cases would be correlated to a low affinity) would ensure a fast deactivation of the Rab protein.

It is also important to keep in mind that Rab effector proteins are usually multidomain proteins and that the known Rab:effector affinities (Table 3.3) were mostly determined using only the isolated RBD and all the measurements were performed in the absence of membranes. The other domains of the Rab effector proteins, however, will contribute to the localization of the Rab effector to the membrane. For example, the PI(3,4,5)P binding C2 domains of FIP proteins are necessary to target these effectors to the plasma membrane (Lindsay and McCaffrey 2004). Also, the EEA1 FYVE domain recognizes PI(3)P and mediates PI(3)P-dependent recruitment of this effector to early endosomes (Dumas et al. 2001). The interaction with other membrane-bound proteins will also contribute to membrane recruitment such as Arf/Arl GTPases that recruit Golgins containing a GRIP domain to Golgi/trans-Golgi network (TGN) membranes (Goud and Gleeson 2010). Another mechanism that increases the effective affinity of an effector for the membrane is their dimerization as one effector dimer can interact with two Rab proteins. Of note, this is not only achieved by dimerization of RBDs—also effector proteins with monomeric RBDs can be dimerized in vivo via other domains of the effector protein. MyoVb, for example, is dimerized via its central stalk region and EEA1 via coiled-coil formation (Callaghan et al. 1999; Pylypenko et al. 2013). This modularity of the membrane interaction will probably ensure a better signal integration as only a combination of different membrane interactions will lead to an efficient recruitment of an effector and therefore to signal transduction. This could ensure that single mislocalized Rab proteins do not initiate signal transduction cascades.

To correlate the available structural information for Rab:effector interactions with the determined affinities, we have added the determined affinities to Table 3.2, which shows the calculated area of Rab surface that is involved in the Rab:effector interactions as well as the number of H bonds and salt bridges involved in the interactions. A direct correlation between the buried surface area and the affinities between the Rab protein and its effectors does not become apparent. However, it seems to be apparent that the lower (μM) affinities, with the exception of the Rabenosyn-5(HepR):Rab4 interaction, can be observed in cases where the surface area is slightly smaller. As can be seen in Table 3.3, several RBDs are not very

Fig. 3.5 (continued) Rabs (the entire structure is shown in *gray*) demonstrates Rab conformational change upon effector binding. Unbound Rab3 structure [PDB ID 3RAB (Dumas et al. 1999)] is superimposed on the Rab3 structure from the Rab3:Rabphilin-3 complex structure [PDB ID 1ZBD (Ostermeier and Brunger 1999)], Rab6 [PDB ID 1YZQ (Eathiraj et al. 2005)] is superimposed on Rab6:R6IP1 [PDB ID 3CWZ (Recacha et al. 2009)] and Rab6:GCC185 [PDB ID 3BBP (Burguete et al. 2008)], and Rab11 [PDB ID 1YZK (Eathiraj et al. 2005)] is superimposed on Rab11:FIP2 [PDB ID 2GZD (Jagoe et al. 2006)] and Rab11:MyoVb [PDB ID 4LX0 (Pylypenko et al. 2013)]

Table 3.3 Rab:effector affinities

Rab	Effector	K_d	Meth.[a]	Reference
Rab1b	OCRL (539-901)	3.7 μM	FS	Hou et al. (2011)
Rab3a/c/d	Rim1/ELKS	1–2 μM	SPR	Wang et al. (2001)
Rab4a	Rabenosyn-5 (HepR)	6.2 μM–7.7 μM	SPR	Eathiraj et al. (2005)
Rab5	Rabenosyn-5 (C_2H_2 Zn finger)	0.9 μM–1 μM	SPR	Eathiraj et al. (2005)
Rab5a	Rabenosyn-5 (C_2H_2 Zn finger)	4.8 μM	SPR	Mishra et al. (2010)
Rab5	Rabenosyn-5 (HR2)	2.7 μM–4.6 μM	SPR	Eathiraj et al. (2005)
Rab5a	OCRL (539-901)	3.6 μM	FS	Hou et al. (2011)
Rab5a	EEA1	2.4 μM	SPR	Mishra et al. (2010)
Rab6a	OCRL(539-901)	3.7 μM	FS	Hou et al. (2011)
Rab6a	Bicaudal	1.5 μM	FS	Bergbrede et al. (2009)
Rab6a	PIST	2.7 μM	FS	Bergbrede et al. (2009)
Rab6a	$p150^{Glued}$	2.4 μM	FS	Bergbrede et al. (2009)
Rab6a	Rim1/ELKS	16 μM	SPR	Wang et al. (2001)
Rab6	GCC185	2.3 μM	ITC	Burguete et al. (2008)
Rab8a	OCRL (539-901)	900 nM	FS	Hou et al. (2011)
Rab9	GCC185	4.5 μM	ITC	Burguete et al. (2008)
Rab11a/b	Evi5	55 nM/50 nM	SPR	Westlake et al. (2007)
Rab11a/b	FIP1/RCP	107 nM/173 nM	ITC	Junutula et al. (2004)
Rab11a/b	FIP2	40 nM/44 nM	ITC	Junutula et al. (2004)
Rab11a/b	FIP5/Rip11	54 nM/46 nM	ITC	Junutula et al. (2004)
Rab11a	FIP2	250 nM	ITC	Lall et al. (2013)
Rab11a	FIP3	290 nM	FS	Eathiraj et al. (2006)
Rab11a	MyoVb	254 nM	SPR	Pylypenko et al. (2013)
Rab14	Rabenosyn-5 (HepR)	13 μM	SPR	Eathiraj et al. (2005)
Rab14	Rabenosyn-5 (HR2)/(fl without C_2H_2 Zn finger)	54 μM/9.6 μM	SPR	Eathiraj et al. (2005)
Rab21	Rabenosyn-5	63 μM	SPR	Mishra et al. (2010)
Rab22a	EEA1	14 μM	SPR	Mishra et al. (2010)
Rab22	Rabenosyn-5 (HR2)	9.7 μM	SPR	Eathiraj et al. (2005)
Rab24	Rabenosyn-5 (HR2)	10 μM	SPR	Eathiraj et al. (2005)

(continued)

Table 3.3 (continued)

Rab	Effector	K_d	Meth.[a]	Reference
Rab25	FIP2	650 nM	ITC	Lall et al. (2013)
Rab25	MyoVb	537 nM	SPR	Pylypenko et al. (2013)
Rab27a	Slp2-a	13.4 nM	SPR	Fukuda (2006)
Rab27a	Slp4-a	19.2 nM	SPR	Fukuda (2006)
Rab27a	Slac2-a/melanophilin	112 nM	SPR	Fukuda (2006)

[a]Methods used to determine the K_d values: *FS* fluorescence spectroscopy, *SPR* surface plasmon resonance, *ITC* isothermal titration calorimetry.

specific in their interactions and bind to several Rab proteins. The impact of this will be discussed in the following chapter.

3.4 Promiscuity of Rab Effector Proteins

A systematic screening for novel Rab effectors based on the yeast two-hybrid assay performed by the Fukuda group revealed that many effectors exhibit, at least in vitro, a broad Rab binding specificity (Fukuda et al. 2008). In most cases, such effectors contain two or more Rab binding sites (see below). There are however a few examples of effectors that are able to bind many Rab proteins through the same domain. The best known are the two functionally related inositol 5-phosphatases OCRL and INPP5B. Most studies focused on OCRL. OCRL can interact, at least in vitro, with a subset of sixteen Rab GTPases (including isoforms), i.e., Rab1, Rab3, Rab5, Rab6, Rab8, Rab13, Rab14, Rab22b, and Rab35 (Hyvola et al. 2006; Fukuda et al. 2008). The Rab binding site of OCRL has been mapped to a region between the phosphatase and the Rho-GAP domains (the ASH domain) (Hyvola et al. 2006). It consists of a relatively short domain of about 130 amino acids. Possible explanations for the binding of such a large variety of different Rab proteins can be deduced from the structure of the Rab binding domain of OCRL in complex with Rab8a (Hou et al. 2011). This study shows that the hydrophobic triad appears to have only a minor role in OCRL binding, which could be one reason for the capacity of OCRL to interact with many Rabs. Furthermore, the interaction between OCRL and the Rab protein occurs mostly via polar interactions, and most of the intermolecular hydrogen bonds formed involve the main chain atoms of the Rab molecule and side chains that are conserved between different Rab proteins. This interaction is less sequence sensitive compared to other Rab:effector interactions, and may explain the promiscuity of OCRL in Rab recognition.

Another example of promiscuity in Rab binding is the Rab5 effector Rabenosyn-5. Rabenosyn-5 has distinct Rab4 and Rab5 binding sites (see below); however the minimal Rab4 binding domain also binds with similar affinity to Rab14 (subset-1) (Eathiraj et al. 2005). In addition, the Rab5 binding domain

binds as well to Rab22 and Rab24 (subset-2) (Table 3.3, Eathiraj et al. (2005)). The Rab-subset key residues interacting with the effector domains, identified by structural analysis, exhibit clear conservation within the subset, and vary between different subsets (Eathiraj et al. 2005). Structure-based mutational analysis demonstrated that Rab-subset sequence conservations can account for the selective recognition of distinct effector domains (Eathiraj et al. 2005).

Other examples include RILP, a well-characterized Rab7 effector that interacts with Rab34 and Rab36 (Matsui et al. 2012), and MyoVa that interacts with three subsets of Rab proteins using three distinct RBDs (Lindsay et al. 2013). The first subset, consisting of Rab6 and Rab14, binds to the central stalk of MyoVa. The second subset (Rab8 and Rab10) interacts (dependent on exonD) in a region localized between the globular tail domain (GTD) and the central stalk (Roland et al. 2009; Lindsay et al. 2013). The Rab11–Rab3–Rab39b subset interacts with the MyoV GTD (Lindsay et al. 2013). Of note, the interaction between the MyoVa central stalk and Rab6/Rab14 can be greatly impaired by mutation of a single MyoVa amino acid. The finding that this amino acid is a tyrosine suggests that phosphorylation events could be involved in the selective recognition of Rab GTPases binding to the same domain of an effector (Lindsay et al. 2013). The interaction between the MyoVa/b GTD and the Rab11–Rab3–Rab39b subset is abolished by point mutations of three MyoV residues (Lindsay et al. 2013), which are involved in Rab11a binding (Pylypenko et al. 2013). However, another MyoV Gln residue mutation, which also disrupts the interaction with Rab11a, does not affect Rab3a and Rab39b binding (Lindsay et al. 2013). This may indicate that the MyoV GTD binds to different Rab GTPases using different but overlapping binding sites or that a different subset of residues is involved in the interaction at the same binding site. Further structural studies are necessary to understand how MyoV can bind phylogenetically diverse Rab proteins using the same binding site.

It is likely that many other Rab effectors that can bind several Rab GTPases via the same domain remain to be discovered. This will help to understand the physiological relevance of promiscuity observed in some Rab:effector interactions. One hypothesis is that promiscuous effectors fulfill generic functions. For instance, the ability of OCRL to bind different Rab proteins could reflect the significance of the Rab and OCRL-dependent remodeling of lipid compositions for various stages of intracellular transport (Mehta et al. 2014) as well as for the completion of cytokinesis (Dambournet et al. 2011). For other Rab effectors though, the promiscuity in in vitro experiments might not be of physiological relevance. In these cases, specificity might simply be unimportant as other factors (such as the correct membrane composition and other interaction partners, as discussed earlier) are necessary to achieve correct targeting of the effector protein to a certain membrane. The “off-target” Rab and the effector would not be present on the same membrane in vivo and therefore there was no evolutionary necessity to develop a clear binding specificity for a certain Rab protein.

3.5 Effectors and the Coordination of Rab Function

3.5.1 Effectors and Rab Cascades

To ensure unidirectionality of intracellular transport, Rab function needs to be coordinated. Effectors of upstream Rab GTPases with GEF domains for downstream Rab proteins play a key role in this process also called Rab cascade (Fig. 3.6). The first cascade to be identified involved the yeast Rab protein Ypt32 that recruits the Sec4p nucleotide exchange factor Sec2p (Ortiz et al. 2002). This cascade has also been demonstrated to exist with the homologous proteins in mammalian cells. Rabin8, the GEF protein of Rab8, is also a direct effector protein of Rab11, and binding of Rab11 to Rabin8 even enhances the nucleotide exchange activity of the GEF protein (Knodler et al. 2010). Novick and colleagues then demonstrated that this cascade is complemented by a GAP cascade (Fig. 3.7), in which an active Rab protein (Ypt32p) recruits an effector protein that contains a GAP domain for an upstream Rab protein—in this case Gyp1p that deactivates Ypt1p (Rivera-Molina and Novick 2009).

Two other cascades involve protein complexes as GEF effector units. The first cascade involves the Ric1–Rgp1 complex that is an effector protein of Rab33b and a nucleotide exchange factor for Rab6 (Pusapati et al. 2012). Rab6IP1, an effector protein of Rab6 and Rab11 (Janoueix-Lerosey et al. 1995; Miserey-Lenkei et al. 2007), then likely continues the cascade on a second level as its DENN domain has been demonstrated to have GEF activity towards Rab39 in vitro (Yoshimura et al. 2010). The second GEF cascade that was discovered in recent years involves the BLOC-3 complex consisting of Hps1 and Hps4. Hps4 binds to the GTP-bound form of Rab9 (Kloer et al. 2010), and the complex is therefore recruited to membranes on which active Rab9 is localized. The Hps1–Hps4 complex activates Rab32/38 (Gerondopoulos et al. 2012) which then recruits Varp (Tamura et al. 2009) that has a GEF domain to activate Rab21 (Zhang et al. 2006). Surprisingly, Rab9 was also demonstrated to participate in a GAP cascade to deactivate Rab32 by RUTBC1 (Nottingham et al. 2011). It remains to be determined how these two activities can lead to regulation of Rab9 activity in vivo.

In addition to the classical cascades, Rab proteins also create positive effector loops to enhance their activation. The best studied example of this is the feedback loop between Rabex5, Rab5, and Rabaptin5. In this feedback loop, the GEF protein of Rab5, Rabex5, is recruited by the effector protein Rabaptin5, which in turn is recruited by the active Rab5 (Stenmark et al. 1995; Horiuchi et al. 1997). A similar feedback loop has been demonstrated to exist in yeast where the Sec4p nucleotide exchange factor Sec2p (Walch-Solimena et al. 1997) is recruited by the Sec4p effector complex Exocyst (Guo et al. 1999; Medkova et al. 2006).

Effector binding itself can also be part of a feedback loop that involves membrane remodeling. The Rab5 effector hVPS34, for example, catalyzes the synthesis of phosphatidylinositol 3-phosphate (PI3P) from phosphatidylinositol (Christoforidis et al. 1999). This in turn is important for the membrane binding of

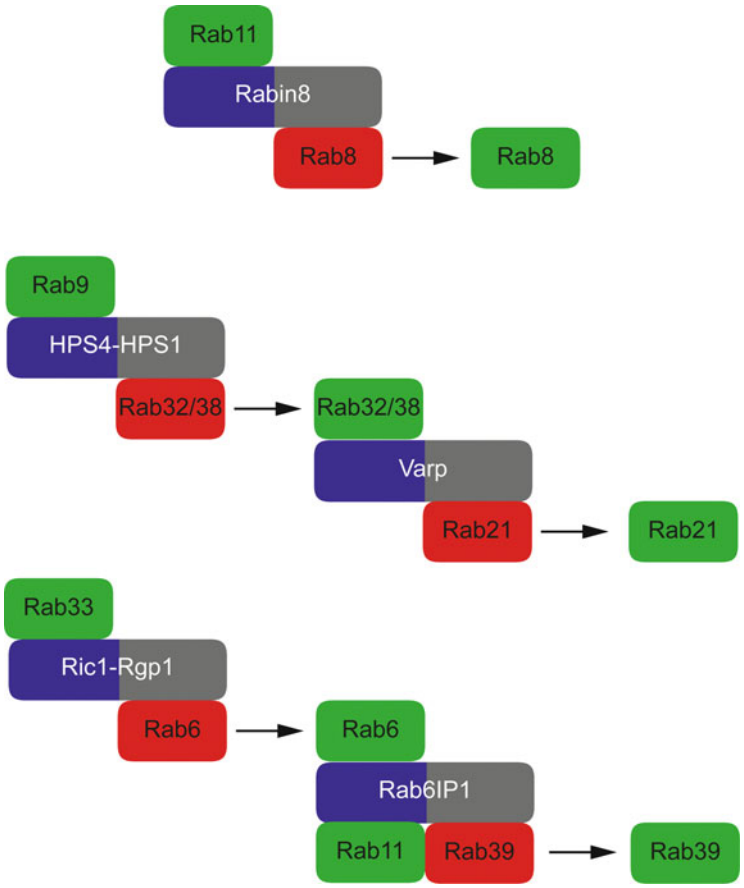

Fig. 3.6 Schematic representation of Rab-GEF cascades. Active/GTP-bound Rab proteins are shown in *green*, and inactive/GDP-bound Rab proteins are colored in *red*. The effector proteins/complexes are depicted in *blue* (RBD) and *gray* (GEF domain)

EEA1, another Rab5 effector, which binds PI3P via its FYVE domain (Stenmark et al. 1996). Rabenosyn-5 recruitment also requires this feedback via PI3P binding (Nielsen et al. 2000).

The combination of feedback loops with cascades is very essential as certain threshold concentrations of Rab proteins on the membrane will have to be reached to achieve efficient effector recruitment. After the first recruitment of a Rab protein to its target membrane feedback, loops (in combination with the already present GEF protein) will ensure the accumulation of this Rab protein on the membrane domain. Only after a certain threshold is reached, the effector protein of this Rab protein will be recruited efficiently due to the relatively low affinity of the effector proteins as discussed earlier. This effector protein will in turn recruit, via its GEF

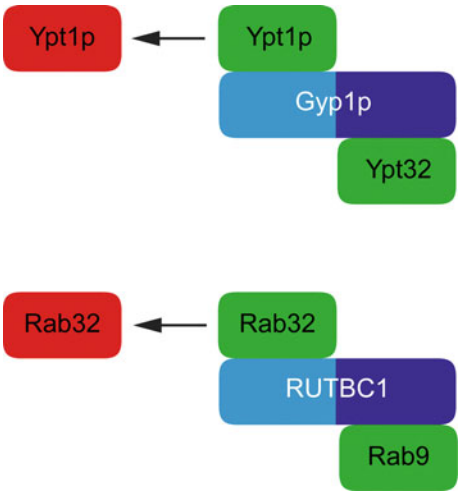

Fig. 3.7 Schematic representation of Rab-GAP cascades. Active/GTP-bound Rab proteins are shown in *green*, and inactive/GDP-bound Rab proteins are colored in *red*. The effector proteins are depicted in *dark blue* (RBD) and in *light blue* (GAP domain)

domain, a downstream Rab protein. Again, a positive feedback loop will ensure the accumulation of this Rab protein on the membrane domain, which will then, provided a certain threshold is reached, recruit an effector protein. This will deactivate the first Rab protein, thereby ensuring its release from the membrane domain.

3.5.2 Effector Coupling

Divalent and multivalent effectors directly participate in the coordination of Rab function. To our knowledge, the first divalent Rab effector to be described was Rabaptin-5, demonstrated to bind to both Rab4 and Rab5 (Vitale et al. 1998). Later on, the Rab4- and Rab5-binding sites were shown to interact with other Rab GTPases (see above). Several other proteins that bind to both Rab4 and Rab5 through separate domains have been identified, such as Rabaptin-5 and Rabip4′ (de Renzis et al. 2002; Fouraux et al. 2004). These effectors link Rab4- and Rab5-regulated trafficking and signaling pathways. For instance, the overexpression of Rabenosyn-5 increases the overlap between Rab4 and Rab5 domains on early endosomes and stimulates transferrin recycling (de Renzis et al. 2002).

Another example of a divalent effector is Rab6IP1/DENND5 that interacts with Rab6 and Rab11 (Miserey-Lenkei et al. 2007). Rab6IP1/DENND5 binds to Rab6a, but not to Rab11a, via its C-terminal RUN domain whereas Rab11a recognition required the full-length protein (Miserey-Lenkei et al. 2007; Recacha et al. 2009). In addition, Rab6IP1/DENND5 shows a GEF activity in vitro towards Rab39, indicating that it likely interacts in vivo with Rab39 via its N-terminal DENN domain (Yoshimura et al. 2010).

In addition to the effector proteins that comprise two Rab binding domains, there are effectors that have the capacity to bind to even more Rabs through separate domains. Among them are proteins of the Golgin family, which are large, mostly coiled-coil proteins present on Golgi and TGN membranes (Goud and Gleeson 2010). They were proposed to act as tentacles to catch transport vesicles moving to or through the Golgi complex (Sinka et al. 2008). For instance, the Drosophila golgin-97, which is recruited to Golgi membranes by interaction of its GRIP domain with Arl1, binds through different sites located across its entire length to dRab6, dRab19, and dRab30 (Sinka et al. 2008). Similarly, the human golgin GCC185/GCC2 was demonstrated to interact through five different sites with Rab1a/b, Rab2a/b, Rab6a/b, Rab9a/b, Rab15, Rab27b, Rab30, Rab33b, Rab35, and Rab36 (Hayes et al. 2009). The interaction with Rab6 may help in the recruitment of GCC185 to Golgi membranes (Burguete et al. 2008), but this remains to be confirmed (Houghton et al. 2009). In addition, the interactions between Golgins and Rab GTPases located on adjacent compartments, such as perinuclear recycling endosomes (Rab11) or pre-Golgi compartments (Rab1 and Rab2), could play an important role in defining the spatial relationship between Golgi/TGN membranes and membranes of these compartments as they would bridge the different compartments (Goud and Gleeson 2010).

Another example of an effector that is likely involved in the coordination of Rab function is the actin-based motor MyoVa recently shown to interact directly with thirteen Rabs (including isoforms), i.e., Rab3, Rab6, Rab8, Rab10, Rab11, Rab14, Rab25, and Rab39b (Lindsay et al. 2013) via three distinct Rab binding domains (see above). The physiological role of multiple interactions between MyoVa/b and Rab GTPases is still poorly understood. Only Rab10 and Rab11, dependent on the MyoVa splice variant, are involved in MyoVa recruitment to intracellular membranes. One tentative hypothesis is that MyoVa, once recruited to membranes by Rab10 or Rab11, relieves a MyoVa autoinhibition to allow its participation in Rab cascades involving the other Rab GTPases that interact with MyoVa. These cascades could then regulate both remodeling and maturation of endocytic/recycling compartments and post-Golgi secretory pathways (Lindsay et al. 2013).

Finally, one should mention the case where a direct interaction takes place between two effectors of the same Rab GTPase. For instance, FIP2 can bind to Rab11a and to MyoVb, and evidence exists that the tripartite association of Rab11 with FIP2 and MyoVb is implicated in regulating the dynamics of Rab11-positive membranes and recycling events (Hales et al. 2002; Wang et al. 2008; Gidon et al. 2012).

3.5.3 Effectors and the Localization of Rab GTPases

The mechanisms by which Rab GTPases are targeted to specific membranes are not yet fully understood, although growing evidence indicates that GEFs are key players in these processes (Schoebel et al. 2009; Wu et al. 2010; Blumer et al. 2013). However, Rab effectors could also play a direct role. The first example

reported is TIP47 (tail-interacting protein of 47 kDa), a protein involved in the biogenesis of lipid droplets (Bulankina et al. 2009). TIP47 was shown to stabilize Rab9 on late endosomal membranes (Carroll et al. 2001; Ganley et al. 2004). Recently, Wu and colleagues demonstrated, using an elegant approach (replacement of the C-terminal hypervariable region (HVD) of several Rabs by a polyethylene glycol linker), that the interaction between Rab7 and its effector RILP is essential for correct localization of Rab7 (Li et al. 2014). Depletion of RILP by siRNA indeed leads to a cytosolic distribution of Rab7. Of note, both RILP and TIP47 interact with the Rab7 and Rab9 C-terminal regions, respectively (Wu et al. 2005; Aivazian et al. 2006).

Rab:effector interactions that involve the CDR regions of the protein usually show a larger buried surface area upon interaction and therefore seem to have a tendency to have higher affinities. These lower K_d values will most likely be reflected, as discussed earlier, in a lower dissociation rate that might inhibit the access of the GAP protein. Therefore, specifically effector proteins that have a larger interaction surface would likely be involved in the stabilization of Rab membrane localization. As Rab membrane localization is a process involving several equilibria (binding of GEF, GAP etc.), it is likely that other Rab effector proteins will take part in Rab membrane localization.

3.6 Conclusions

Rab effector proteins display a large functional diversity, and their Rab binding domains show a large structural diversity. Their binding occurs via two different binding modes that can be described as key-lock and induced-fit mechanisms. Besides surface residues, structural plasticity and conformational stability of the Rab proteins are crucial for effector specificity.

In addition to their function in the regulation of cellular trafficking effects via downstream signalling, growing evidence indicates that Rab effector proteins are also involved directly in the regulation of Rab localization via different cascades and feedback loops. As Rab proteins are emerging as key determinants of organelle specificity, a tight regulation of their localization as well as the localization of their effector proteins that transmit their signal is crucial for organelle identity. Further biochemical, structural, and cell biological studies are crucial to get a more complete picture of Rab effector specificity and localization.

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Chapter 4
A Rab Effector Called the Exocyst and Related Vesicle Tether Complexes

Peter Jay Novick

Abstract Vesicular transport between membrane-bounded organelles requires, in most cases, a large multi-subunit protein complex that serves in the initial recognition of the target compartment by the vesicle, a step commonly termed tethering. Four different tethers that act at four different stages of membrane traffic exhibit a common architecture: at least some of their subunits form rod-shaped structures from helical bundles that are stacked in series. These subunits are often linked to each other through coiled-coil domains at their amino termini to form a multi-armed complex. In three cases these tethers are recruited to the membrane by binding to the activated form of a Rab GTPase and in two cases interactions with vesicle coat proteins are thought to play a role in vesicle recognition. Interactions with SNARE proteins on both the vesicle and target membranes suggest possible roles in directing SNARE complex assembly in preparation for membrane fusion. Here, we will review in depth the first of these tethers, the exocyst complex that is required for the exocytic fusion of vesicles with the plasma membrane, and then discuss various parallels in both structure and function with the related tethers, COG, GARP, and Dsl, that act on other transport pathways.

Keywords Rab effector • Vesicle tether

4.1 Backstory of the Exocyst

Most subunits of the exocyst were first identified in a genetic screen for temperature-sensitive (ts) yeast secretory (*sec*) mutants blocked in general protein secretion (Novick et al. 1980). Of the ten *sec* complementation groups blocked at the post-Golgi stage of the secretory pathway, six were ultimately shown to be

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defective in one of the genes encoding an exocyst subunit. These include *SEC3, SEC5, SEC6, SEC8, SEC10*, and *SEC15* (TerBush et al. 1996). The connection between the exocyst and Rab GTPase regulation emerged very early through an effort to clone the *SEC15* gene (Salminen and Novick 1987). A low copy number plasmid library of yeast genomic fragments was introduced into the ts *sec15-1* strain and a plasmid was recovered from a transformant that exhibited nearly wild-type growth at the restrictive temperature. Despite the nearly complete restoration of growth and secretion by the plasmid, integration by homologous recombination established that the genomic fragment was not actually derived from the *SEC15* locus, but rather from *SEC4*, another gene required for post-Golgi transport (Salminen and Novick 1987). Duplication of *SEC4* was found to restore growth and secretion to most of the post-Golgi-blocked *sec* mutants at or close to their restrictive temperature. Conversely, combining *sec4*-8, a ts allele of *sec4,* with ts alleles of the same set of post-Golgi-blocked *sec* mutants proved to be lethal at all temperatures (Salminen and Novick 1987). These two genetic phenomena, later termed multi-copy suppression and synthetic lethality, suggested a model in which the *SEC4* gene product acts as a master regulator of the final stage of the secretory pathway. A model for this regulatory mechanism began to come into focus when *SEC4* was sequenced and found to encode a member of the *Ras* superfamily of GTP-binding proteins (Salminen and Novick 1987). By this model, Sec4p, in its GTP-bound form would promote the function of a downstream effector or effectors that act on the secretory pathway. Increasing the level of Sec4p-GTP by duplication of *SEC4* would help to compensate for a partial defect downstream, while decreasing the level of Sec4p-GTP, such as in the *sec4-8* mutant, would make partial defects in those downstream components even more severe. This model highlighted a key question, which of the *SEC* gene products serves as a Sec4p effector?

4.2 Sec15p Responds to Sec4-GTP

Early studies on the physiological effects of *SEC15* overexpression offered some important clues to the identity of one Sec4p effector and its role on the secretory pathway. Overexpression of *SEC15* from the strong, inducible *GAL1* promoter blocked cell growth and led to the formation of a cluster of secretory vesicles and a patch of Sec15 protein (Salminen and Novick 1989). Immuno-electron microscopy established that the overexpressed Sec15 protein was associated with the abnormal vesicle patch (Walch-Solimena et al. 1997). Importantly, ts mutations in *sec4* and *sec2*, but not the other *sec* genes required late in the pathway, prevented Sec15p patch formation without affecting the level of Sec15p expression (Salminen and Novick 1989). Based on these observations we speculated that, at normal levels of expression, Sec15p responds to Sec4-GTP to tether secretory vesicles to their fusion partner, the plasma membrane, while at high levels of Sec15p expression, the vesicles aggregate with each other, so long as Sec4p is active. Two hybrid, chemical cross-linking and pull-down studies subsequently established that Sec15p is in fact

a direct binding partner of Sec4p-GTP (Guo et al. 1999b). Sec2p, we later found, encodes the exchange factor that activates Sec4p (Walch-Solimena et al. 1997), in agreement with the model. As we will discuss below, a wide variety of experiments have since helped to establish that Sec15p, together with the other components of the exocyst, serves to link secretory vesicles carrying Sec4-GTP to the plasma membrane in preparation for exocytic fusion.

4.3 Enter the Exocyst

As a growing number of post-Golgi *SEC* genes were sequenced and the gene products analyzed, a pattern emerged: Sec6p, Sec8p, and Sec15p are all large hydrophilic proteins, lacking trans membrane domains or any strong homology to known proteins. In fractionation studies, they all exhibited the properties of peripherally associated membrane proteins, yet even their soluble forms could be pelleted at very high speeds (Bowser et al. 1992). In time, these three proteins, together with Sec3p, Sec5p, Sec10p, Exo70p, and Exo84p, were found to be components of the same large oligomeric complex that came to be known as the exocyst to denote its essential role in exocytosis (Guo et al. 1999a; TerBush et al. 1996; TerBush and Novick 1995). The complex contains one copy of each of the eight subunits. Subsequent studies have shown that all components of the exocyst are conserved among eukaryotes, although the sequence identities are generally quite low. As in yeast, these components associate with each other in a large complex (Hsu et al. 1998).

4.4 Exocyst Localization Suggests a Role as a Tether

Localization studies in yeast proved to be quite informative regarding exocyst function. All subunits follow a cell cycle-dependent pattern of localization that precisely parallels that of cell surface growth and protein secretion (Boyd et al. 2004; Finger et al. 1998). At the start of the cell cycle, the exocyst localizes to a spot at the cell cortex. From this site the bud subsequently emerges and grows. The exocyst persists as a tightly localized spot at the tip of small buds, yet at the time of the G2/M transition, the spot abruptly disperses around the cortex of the bud and then rapidly re-concentrates at the neck between the large bud and the mother cell. The neck localization persists through cytokinesis and cell separation.

Although all exocyst subunits display this characteristic pattern of localization, different subunits were found to respond differently to disruption of actin-dependent polarized delivery of secretory vesicles. The localization of most exocyst subunits was lost very rapidly following a block in vesicle formation or disruption of the actin cytoskeleton; however, the localization of Sec3p and Exo70p was found to be substantially resistant to these treatments (Boyd et al. 2004; Finger

et al. 1998). These results suggested a model in which six of the subunits, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo84p, were delivered to exocytic sites by riding on secretory vesicles as they are transported along actin cables, while the other two subunits, Sec3p and Exo70p, directly recognize these sites on the cell cortex independent of ongoing vesicle delivery. Fluorescence recovery after photobleaching experiments added support to this model: Sec3p exhibited much slower recovery times than the six actin-dependent subunits, suggesting a distinct, more stable mechanism of localization, while Exo70p exhibited biphasic recovery consistent with localization by both mechanisms (Boyd et al. 2004). Tagging of the subunits with three tandem GFP moieties allowed, in all cases except Sec3p, direct visualization of the rapid, vectorial movement of small puncta, presumably secretory vesicles, to exocytic sites (Boyd et al. 2004).

The more stable, actin-independent localization of Sec3p and Exo70p suggested an interaction with a component of the polarity establishment machinery that acts upstream or independent of polarized actin assembly. The amino-terminal region of Sec3p binds as an effector to members of the Rho family of GTPases, including Cdc42p, and Sec3p localization requires Cdc42p function (Guo et al. 2001; Zhang et al. 2001). Sec3p also binds to phosphatidyl inositol 4, 5 bisphosphate (PI4,5P2), a lipid primarily associated with the inner leaflet of the plasma membrane, and this interaction is also required for Sec3p localization (Zhang et al. 2008). Exo70p similarly binds to members of the Rho GTPase family as well as to PI4,5P2 (He et al. 2007). Mutations in either *SEC3* or *EXO70* that block their actin-independent localization have little effect on growth or secretion on their own, yet these mutations are synthetically lethal in combination with each other (Guo et al. 2001; Hutagalung et al. 2009). In fact this combination is lethal even in the presence of high copy number suppressors that can bypass a complete deletion of either *SEC3* or *EXO70*. These results imply that it is essential that either Sec3p or Exo70p interact with polarity determinants at the cell cortex, yet in this regard they are redundant in their function (Hutagalung et al. 2009).

4.5 Rab and SNARE Interactions Are Needed for Exocyst Recruitment to Vesicles

The interaction of the exocyst with secretory vesicles involves several distinct vesicle components. Sec4p is highly concentrated on secretory vesicles, as is its exchange protein Sec2p, suggesting that Sec4p is associated with vesicles in its GTP-bound form (Goud et al. 1988; Walch-Solimena et al. 1997). Since Sec15p binds to Sec4-GTP (Guo et al. 1999b), this interaction is likely to contribute to the recruitment of the exocyst to vesicles. The loss of Sec15p patch formation when overexpressed in *sec4* or *sec2* mutants supports this proposal (Salminen and Novick 1989). However, a recent study suggests that another interaction contributes as well. A directed screen of the yeast deletion library for mutants defective in Sec15-

GFP localization implicated a number of components of clathrin-coated vesicles; however, the lack of co-localization between clathrin and Sec15p suggested an indirect role for clathrin in exocyst localization (Shen et al. 2013). Clathrin is required to internalize the redundant vesicle SNARE proteins, Snc1p and Snc2p, from the plasma membrane for reutilization on the exocytic pathway (Lewis et al. 2000) and *snc1* mutants specifically defective in internalization exhibit a defect in exocyst localization (Shen et al. 2013). Direct interaction of Snc2p with Sec6p was observed in binding studies. The Sec6p binding site mapped to a small region just before the trans membrane domain of Snc2p, within its SNARE motif. Mutants of *snc2* defective in Sec6p binding, but still functional for in vitro liposome fusion, exhibit an exocyst localization defect as well as a ts block in growth and secretion (Shen et al. 2013). In total, the results suggest that exocyst recruitment to vesicles is mediated by the Sec4p–Sec15p interaction and the Sec6p–Snc1/2p interaction, working in parallel.

4.6 The Exocyst as a Vesicle Tether: And More

The association of certain exocyst components with secretory vesicles and others with the plasma membrane at exocytic sites led to the proposal of the exocyst as a tether that serves to link incoming vesicles to the target membrane in preparation for exocytic fusion. Additional studies of the exocyst suggest that it likely does more than just passively link the vesicle to the target membrane. Analysis of the assembly state of the exocytic SNARE complex established that exocyst function is required prior to SNARE complex assembly (Grote et al. 2000). Furthermore, direct interactions of Sec6p with the tSNARE, Sec9 (Sivaram et al. 2005), the vSNARE, Snc1/2 (Shen et al. 2013), and the SNARE interacting protein, Sec1p (Morgera et al. 2012), suggest a possible role in SNARE complex assembly. In addition, Exo84p was shown to bind to Sro7p (Zhang et al. 2005), a protein that binds to the tSNARE, Sec9p (Lehman et al. 1999), and controls the level of SNARE complex assembly (Williams and Novick 2009). In total, these results, as well as more extensive studies on related tethering complexes, discussed in a later section, support a role for the exocyst in the regulation of SNARE complex assembly. Nonetheless, no stimulation of SNARE complex assembly has yet been demonstrated in vitro using the soluble domains of the exocytic SNAREs and either the purified exocyst complex or isolated Sec6p (Sivaram et al. 2005). Recent analysis of the kinetic localization of Sec8p in MDCK cells supports the notion that it is brought to the plasma membrane on carrier vesicles and remains at exocytic sites even after membrane fusion, consistent with roles in both SNARE complex assembly and fusion pore opening (Rivera-Molina and Toomre 2013).

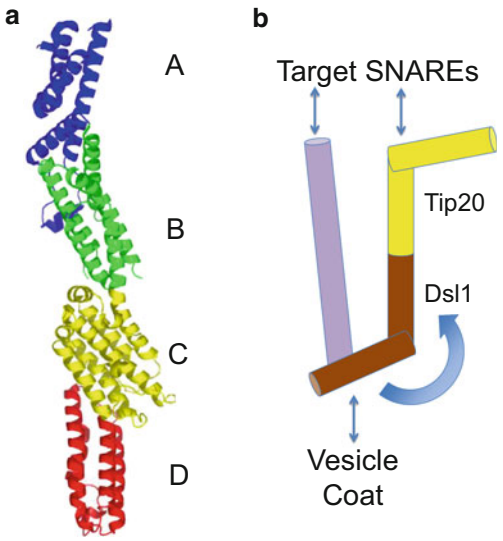

Fig. 4.1 (**a**) Yeast Exo70p, lacking the first 62 amino acids forms a rod, 160 Å long and 30–35 Å wide, consisting of four helical bundles arranged in series (Dong et al. 2005). The four domains are indicated with different colors and are labeled A–D. (**b**) The Dsl1 complex from yeast forms a "Y"-shaped structure from rodlike subunits Dsl1p, Sec39p, and Tip20p. Flexibility in the Dsl1p subunit allows variability in the angle of the "Y". Sec39p and Tip20p can bind to the SNAREs on the target membrane, while Dsl1p can bind to the COP I coat on the incoming vesicles (Ren et al. 2009)

4.7 Structural Studies of the Exocyst

While high resolution structural analysis of the fully assembled, intact exocyst complex has not proven to be feasible, individual subunits, or parts of subunits, have been crystallized. The structure of nearly full length yeast Exo70p was solved (Dong et al. 2005) (Fig. 4.1a). It forms a rod consisting of four helical bundles, termed domains A–D, arranged in series. The final helical bundle, domain D, contains a conserved polybasic patch that binds to PI4,5P2 (He et al. 2007) and therefore presumably represents a site of attachment to the plasma membrane. Interestingly, domain C can be completely deleted with little effect on growth or secretion; however, the actin-independent localization of Exo70p is lost suggesting an interaction between domain C and the polarity establishment machinery (Hutagalung et al. 2009).

The structure of the carboxy terminal third of yeast Exo84p was also solved (Dong et al. 2005). Its structure consists of two helical domains arranged in tandem. This structure is nearly superimposable on first two domains of Exo70p, despite very low sequence similarity. This architectural pattern continued with the solution of the structures of the carboxy terminal regions of yeast Sec6p (Sivaram et al. 2006) and Sec15p from *drosophila* (Wu et al. 2005). Sec6p is also formed

of stacked helical bundles, but in a more curved overall conformation (Sivaram et al. 2006) and the carboxy terminal region of Sec15p consists of two helical bundles (Wu et al. 2005). Only small segments of some of the other subunits have been solved to date; however, the high probability of helical structure predicted by their sequences supports the possibility that these subunits could also share a similar architecture, forming rods of stacked helical bundles. This prediction is consistent with electron micrographs of the rotary-shadowed, intact mammalian exocyst, which show flowerlike structures in which the "petals" which are of appropriate dimensions to represent individual subunits are opened to varying extents (Hsu et al. 1998).

4.8 Tethering Complexes Related by Structure

Initial sequence analysis suggested that each exocyst subunit is unique within the yeast genome and not a member of a family, in contrast to the Rab, SNARE, and Sec1/Munc18 families, in which different members of each family act at different stages of membrane traffic. Subsequent analysis, however, detected small regions of weak sequence similarity between certain exocyst components and members of the Conserved Oligomeric Golgi (COG) and Golgi-Associated Retrograde Protein (GARP) tethering complexes (Whyte and Munro 2001). These regions were primarily but not exclusively confined to short regions near the amino termini that are predicted to be in a coiled-coil conformation. Based on these similarities COG, GARP, and the exocyst were given the name quatrefoil tethering complexes (Whyte and Munro 2001). Subsequent structural analysis has established the architectural similarity of at least some subunits of these tethering complexes as well as the Dsl1 tethering complex and the term Complexes Associated with Tethering Containing Helical Rods (CATCHR) has been applied to this group of four complexes (Yu and Hughson 2010). It seems likely based on the structural similarities and patterns of protein interactions, discussed below, that all four complexes play analogous roles on different traffic pathways. Nonetheless, the involvement of CATCHR complexes is not universal since unrelated proteins, such as the long coiled protein p115, tether membranes at other stages of transport.

4.8.1 COG

The COG complex acts to tether COPI vesicles in retrograde, intra-Golgi transport. It contains eight subunits: four (COG 1–4) are essential for yeast viability, while the other four (COG 5–8) are dispensable. Portions of the COG2 and COG4 structures were solved and shown to consist of helical bundles similar to those of the exocyst subunits Exo70, Exo84, Sec6, and Sec15 (Cavanaugh et al. 2007; Richardson et al. 2009). A complex of Cog1–4 was analyzed by electron microscopy and

found to form a "Y"-shaped structure. The subunits are held together by a predicted four-helix bundle comprised of the amino-terminal coiled-coil regions of each of the four subunits. One leg of the "Y" formed by COG1 acts as a link to the remaining subunits, COG 5–8 (Lees et al. 2010).

4.8.2 DSL1

The Dsl1 complex, required for tethering of Golgi-derived COP1 vesicles to the ER, is the simplest of the CATCHR family, having only three subunits, Dsl1, Sec39, and Tip20. Dsl1 and Tip20 fit the CATCHR architecture of rods formed from stacked helical bundles (Tripathi et al. 2009). Sec39 is also predominantly alpha helical, but the helices are arranged perpendicular to the long axis in a continuous ribbon rather than in bundles. Dsl1 links Sec39 to Tip20 generating a "Y"-shaped overall structure (Ren et al. 2009). The inherent flexibility of Dsl1 allows for great variability in the angle of the "Y" (Fig 4.1b).

4.8.3 GARP

The GARP complex is required for tethering of endosome-derived vesicles to the trans Golgi network. GARP consists of four subunits, Vps51, Vps52, Vps53, and Vps54. Partial structures of two of them indicate structural features similar to that of the exocyst . The carboxy terminal region of Vps53 consists of two helical bundles in tandem (Vasan et al. 2010), while the carboxy terminal region of Vps54 consists of three helical bundles (Perez-Victoria et al. 2010). The amino termini are involved in assembly of the GARP complex, suggesting the possibility of higher order parallels with the COG and Dsl1 structures.

4.9 From Structures to Functions

Understanding the functions of these four tethering complexes at a detailed mechanistic level has proven to be quite challenging. Many interactions with other components of the vesicular traffic machinery have been noted and some common themes have emerged; nonetheless, major questions still remain. What is lacking is a coherent model describing how all of the different interactions work in concert to direct vesicle traffic. Below some of the interactions that are common among the different tethers are summarized together with some current ideas regarding the roles of these interactions in the transport reactions.

4.9.1 Interactions with Rabs

Three of the four CATCHR complexes have been shown to act as direct effectors of a Rab GTPase. In yeast the exocyst binds to Sec4-GTP through the Sec15p subunit and this interaction is thought to help recruit the exocyst to secretory vesicles (Guo et al. 1999b), as described in an earlier section. In animal cells Sec15 interacts with Rab11, a GTPase that controls export from recycling endosomes (Wu et al. 2005). In both cases the exocyst is implicated in promoting the interaction of Rab-associated vesicles with the plasma membrane in preparation for fusion. It is not known if the Rab interaction has any role beyond recruitment of the exocyst, although an unassembled pool of Sec3p was found to develop in a *sec4-8* mutant upon a shift to its restrictive temperature, suggesting a possible role for Sec4p in exocyst assembly (Guo et al. 1999b).

The COG complex interacts with Ypt1-GTP in yeast, while in mammalian cells it interacts not only with the Ypt1 homologue, Rab1-GTP, but also a number of other Golgi-localized Rab proteins (Miller et al. 2013). This interaction is needed for the normal Golgi localization of COG. The GARP complex interacts with Rab6 (Liewen et al. 2005), or its yeast homologue Ypt6 (Siniossoglou and Pelham 2002), through its Vps52 subunit. The Dsl1 complex is the only member of this group not known to interact with a Rab protein. As in the case of the exocyst, these tethers are recruited to specific membranes by their interactions with Rabs; however, additional roles for the Rabs in tether function have not been excluded.

4.9.2 Interactions with SNAREs

All four CATCHR complexes have been shown to interact with at least a subset of the SNAREs that catalyze fusion of the tethered transport vesicle to the acceptor compartment. Rather than list them all, I refer readers to a very recent review (Hong and Lev 2014). These include interactions with both the SNAREs on transport vesicles as well as SNAREs on the acceptor compartments. Furthermore, loss of tether function leads to a failure to assemble the SNAREs into a fusion-competent, four-helix bundle. However, this could potentially reflect either a direct role in catalyzing SNARE complex assembly or an indirect role in bringing the vesicle into sufficient proximity to the target compartment to permit SNARE complex assembly at the intrinsic rate. To date the only direct, active role in SNARE complex assembly has been shown for the Dsl1 complex. A modest acceleration of assembly was shown in vitro using the soluble, cytoplasmic domains of the relevant SNAREs (Ren et al. 2009).

An additional level of complexity arises from the observation that these tethers interact not only with SNAREs, but also with other SNARE interacting proteins, such as the Sec1/Munc18 family members that have themselves been implicated in SNARE assembly (Laufman et al. 2009; Morgera et al. 2012). A more

comprehensive analysis of the role of CATCHR tethers in SNARE assembly must include all components in a more physiological setting using membrane-anchored SNAREs embedded in liposomes.

4.9.3 Interactions with Coats

The initial recognition of a vesicle by CATCHR tethers may, in some cases, be through a vesicle coat protein. Dsl1 binds to the COPI coat on retrograde Golgi-ER vesicles (Andag et al. 2001) (Fig 4.1b) while COG recognizes the COPI coat on retrograde intra-Golgi vesicles (Suvorova et al. 2002). Since both tethers bind to the same coat even though the vesicles are destined to different target compartments, recognition of the appropriate vesicles must presumably involve other components as well. There are no reports to date of GARP interacting with a vesicle coat. Furthermore, the secretory vesicles tethered by the exocyst apparently do not have a protein coat, so a different recognition mechanism must be at work in this case.

For a vesicle to fuse with its target membrane any coat must first be removed. The DSl1 tether was suggested to promote removal of the COPI coat. An unstructured loop of the Dsl1p subunit interacts with the COPI coat at a site needed for coat assembly and thus the interaction with Dsl1p could disrupt coat stability (Zink et al. 2009). This model has been questioned since mutations within the loop that interfere with coat interactions have little effect on transport and the mammalian homologue of Dsl1p, ZW10, lacks the COPI-binding region. Interactions between the Tip20p subunit and the COPI coat have also been noted (Diefenbacher et al. 2011), possibly accounting for these results.

4.10 Making Way for Fusion?

Perhaps one of the most perplexing questions is how a vesicle membrane and target membrane can fuse while a large, multi-subunit complex of proteins is positioned between them. Fusion requires intimate contact between the opposing lipid bilayers. It would seem that having a large CATCHR complex separating the two membranes would act as an impediment to intimacy in this reaction. Several possible resolutions to this apparent conundrum come to mind. It is possible that the complex disassembles or is in some other way displaced from the site of contact, making way for bilayer fusion. However, recent imaging of the exocyst by TIRF microscopy suggests that it remains in place throughout the fusion reaction and during the expansion of the fusion pore (Rivera-Molina and Toomre 2013). Another possibility is that inherent flexibility within the CATCHR complexes allows the two membranes to approach each other with the complex in place. The Dsl1 complex has been shown to be flexible with a highly variable angle in its "Y"-shaped structure (Ren et al. 2009) (Fig 4.1b). Current evidence is consistent

with the possibility that the other CATCHR tethers are also flexible. Such flexibility would allow the two membranes to initially recognize one another at some distance, but then approach each other as the complex bends.

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Chapter 5
Rab GEFs and GAPs: The Enigma Variations

Francis A. Barr

Abstract Rab GTPases are key regulators of membrane traffic activated on the surface of organelle and vesicle membranes during vesicle trafficking events, cell polarisation and autophagy. Rabs undergo a cycle of activation involving GTP binding and inactivation involving GTP hydrolysis in response to cellular regulators. Each Rab has a cognate GDP–GTP exchange factor (GEF) promoting release of GDP and subsequent binding of GTP, and a GTPase activating protein (GAP) stimulating the slow intrinsic GTP hydrolysis. Together these GEF and GAP regulators determine when and where a specific Rab is activated, and how long its activity will persist. Rab GEFs fall into a number of discrete families, the largest of which are the Vps9 domain, DENN and DENN domain-related proteins. Other Rab GEF families, including TRAPP, Ric1-Rgp1, Mon1-Ccz1 and Hps1-Hps4, are comprised of two or more polypeptide chains. By contrast, almost all known Rab GAPs possess a TBC1 domain. Here I will discuss the mechanisms by which these GEFs and GAPs regulate Rab GTPases, highlighting common themes and points of difference, and briefly outlining the cellular processes they regulate.

Keywords Vesicle traffic • Rab GTPase • GEF • GAP • TBC1 domain • Longin domain

5.1 Rab Function and the Need for Regulation

Rab GTPases form a large and highly conserved subfamily of the Ras-related GTPase in eukaryotic cells. Rabs are key regulators of membrane traffic activated at the surface of organelle and vesicle membranes during vesicle trafficking events, cell polarisation and autophagy (Zerial and McBride 2001; Pfeffer and Aivazian 2004).

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Careful analysis of Rab evolution indicates that the last common eukaryotic ancestor possessed 12 Rabs that we know to be involved in ER–Golgi trafficking, endocytosis, endocytic recycling, lysosome-related organelle formation, lipid storage and cilium function (Elias et al. 2012; Klopper et al. 2012). The size of the Rab family differs between species, but generally increases in metazoan lineages and is reduced in some single cell eukaryotes such as yeasts. Human cells express a subset of 66 Rabs, while budding yeast express 11 Rab/Ypts. The increased number of Rabs in higher eukaryotes is thought to reflect the greater number of cellular compartments, and increased complexity of cell polarisation in metazoans compared to single cell eukaryotes. Like other Ras superfamily proteins, Rabs switch between two states: an inactive GDP-bound state and an active GTP-bound state (Wittinghofer and Vetter 2011). This cycle of activation and inactivation is under the control of cellular regulators promoting GDP–GTP exchange during Rab activation and GTP hydrolysis during Rab inactivation (Barr and Lambright 2010; Barr 2013). Accordingly, each Rab has a cognate GDP–GTP exchange factor (GEF) promoting release of GDP and subsequent binding of GTP, and a GTPase activating protein (GAP) stimulating the slow intrinsic GTP hydrolysis. This cycle of activation and inactivation is linked to a cycle of membrane binding and release. Rabs are prenylated at the C-terminus and this modification is required for targeting to membrane surfaces (Khosravi-Far et al. 1991; Peter et al. 1992). When in the GDP-bound inactive form Rabs rapidly partition between a cytoplasmic pool bound to a protein termed GDI (guanine nucleotide dissociation inhibitor) and a membrane-associated pool that is accessible to GEFs (Soldati et al. 1993; Wu et al. 2010). GDI interacts with the GDP-bound form of the Rab nucleotide-binding domain, and also shields the hydrophobic prenylated tail of the Rab from the aqueous environment of the cytoplasm (Rak et al. 2003). Rabs accumulate at the membrane surface where their cognate GEF is located following GDP–GTP exchange, because GTP-bound Rabs have greatly reduced affinity for GDI and therefore do not partition into the cytoplasm (Soldati et al. 1993; Rak et al. 2003; Blumer et al. 2013). In this chapter I will focus on the mechanisms by which GEF and GAP regulatory factors act on their target Rabs.

5.2 Rab GEF Families and Mechanism

Rab GEFs initiate a kinetic proofreading system for vesicle and target organelle membrane surfaces by promoting accumulation of active Rabs only at the required sites (Barr 2013). Rab GEFs and their target Rabs are therefore key determinants of membrane trafficking. As for many other components of the membrane trafficking machinery the first candidate Rab GEFs were initially identified by means of genetic screens in budding yeast. Later biochemical characterisation then matched the GEFs to their targets Rab GTPases, and cell biological studies have confirmed these functional relationships (Mizuno-Yamasaki et al. 2012; Hutagalung and Novick 2011). As a result the identity of GEFs for the major Rabs controlling secretory and endocytic trafficking is known (Barr and Lambright 2010).

High-resolution X-ray crystallography has resulted in the elucidation of structures for five different Rab-GEF pairs. The cellular regulatory pairs Rab1-TRAPP, Sec4-Sec2 (equivalent to Rab8-Rabin8), Rab21-Rabex, Rab35-DENND1B and the Rab1-DrrA pair created upon infection by the intracellular pathogen *Legionella* (Delprato and Lambright 2007; Delprato et al. 2004; Cai et al. 2008; Dong et al. 2007; Sato et al. 2007b; Schoebel et al. 2009; Suh et al. 2010; Wu et al. 2011). These structures define different classes of Rab GEF in complex with a magnesium and nucleotide free target Rab, and therefore represent likely intermediates in the nucleotide exchange reaction. Details of how specificity in Rab interactions may be achieved have been discussed elsewhere (Lee et al. 2009), and here I will focus on the mechanism by which these different groups of GEF promote GDP–GTP exchange and contrast this with the canonical mechanism used by Ras.

Before explaining the mechanism of nucleotide exchange it is first important to explain some details of the mode of GDP/GTP binding in Rab GTPases. Here, Rab1 in complex with the non-hydrolysable GTP analogue GMP-PNP (Fig. 5.1) is taken as representative of Rabs in general, and the role of three regions termed switch I, switch II and the phosphate interaction site or P-loop will be explained. In Rab1 a magnesium ion makes direct contact with serine 22 in the GKS motif of the P-loop and threonine 40 in the conserved TIGVD RabF1 motif at the end of the switch I region. The beta- and gamma-phosphates of the bound GTP contact the metal ion. All these interactions fall in one plane around the magnesium ion. In addition the conserved lysine in the P-loop GKS motif interacts with the gamma-phosphate of GTP. Finally, there are two water molecules positioned above and below the metal ion. One of these water molecules interacts with the alpha-phosphate of the bound nucleotide. Aspartate 63 in the switch II region may influence the environment of and possibly contact these water molecules as well as the P-loop serine 22. The same water molecule is also predicted to interact with the main chain carbonyl oxygen of threonine 65 in the switch II region. Many studies of Ras superfamily GTPases use a serine to asparagine mutation in the P-loop, S22N in Rab1, since this prevents magnesium binding, and hence stable GDP or GTP binding. As well as these contacts to the beta- and gamma-phosphates of the nucleotide, a conserved aromatic residue in the switch I region, tyrosine 33 in Rab1, makes a potential ring stacking interaction with the guanine ring. Finally, aspartate 124 makes end on interactions with the guanine ring and is important for nucleotide specificity, while lysine 122 contacts the ribose sugar forming the guanosine moiety of the bound nucleotide. Following hydrolysis of GTP to GDP and release of inorganic phosphate, this interaction network is altered (Stroupe and Brunger 2000; Huber and Scheidig 2005; Dumas et al. 1999). First, the interactions of the threonine in the TIGID Rab F1 motif with the gamma-phosphate are lost, resulting in large conformational change in the switch I region. The P-loop lysine now interacts with the beta-phosphate of the bound nucleotide. As in other GTP-binding proteins in the GDP-bound form, water molecules replace the interactions of this conserved threonine and the gamma-phosphate with the magnesium ion. Depending on the Rab, one of these water molecules may be coordinated by an interaction with the

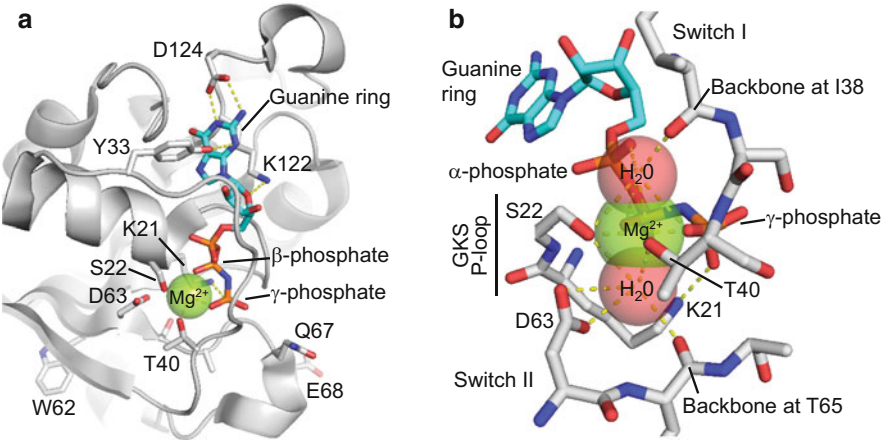

Fig. 5.1 The Rab1 nucleotide-binding site. (**a**) The structure of Rab1 bound to Mg^{2+} GMP-PNP (PDB: 1YZN) is shown in the figure. The polypeptide backbone is drawn in *grey* ribbon form, and only amino acid side chains involved in interaction with the nucleotide or magnesium ion are displayed. The switch II region W62 to E68 runs from left to right at the bottom of the structure. Switch I Y33 to T40 runs from top to bottom. The guanine ring, beta and gamma-phosphates of GMP-PNP are highlighted. (**b**) An enlarged view of the magnesium and phosphate-binding region of Rab1 is shown. In this view the two water molecules sitting above and below the magnesium ion are shown

conserved switch II glutamate. This has been observed for Rab4a, but not Sec4 GDP structures (Huber and Scheidig 2005; Stroupe and Brunger 2000).

Rab GEFs interact with Mg^{2+} and GDP-bound Rabs promoting conformational rearrangements in the switch I and II regions and nucleotide exchange. However, the order of events and intermediates in the exchange reaction cannot be precisely defined based on current data, and further studies of reaction intermediates for multiple Rab-GEF pairs are needed. GDP release occurs because switch I and II rearrangements displace the conserved aromatic residue making contacts with the guanine ring and disrupt the Mg^{2+} and phosphate-binding site. Previously it has been suggested that rearrangement of switch II and disruption of the Mg^{2+} binding site occurs only after or in concert with GDP release, whereas switch I displacement occurs during formation of the initial GEF-Rab-GDP intermediate. More recent evidence is consistent with the view that disruption of the Mg^{2+} binding site and rearrangement of switch II may be a primary event in nucleotide release (Langemeyer et al. 2014; Uejima et al. 2010). For Ras, Ran, Rho and Arf GTPases a highly conserved acidic residue typically glutamate intrinsic to the switch II region contacts the P-loop lysine (Gasper et al. 2008; Wittinghofer and Vetter 2011). This is thought to stabilise the GEF-bound nucleotide-free form of the GTPase (Boriack-Sjodin et al. 1998). However, this glutamate does not play the same role in Rab GEFs suggesting there are crucial mechanistic differences in the activation mechanism (Langemeyer et al. 2014; Gasper et al. 2008). The details are described in more detail for each of the Rab-GEF pairs in the subsequent sections.

5.2.1 *Vps9 Family GEFs for the Rab5/21 Subfamily*

Rab5 subfamily GTPases, including Rab5A-C, Rab17, Rab21 and Rab22A/B, regulate trafficking in the early endocytic pathway. Vps9/Rabex the founder member of the Rab5 GEF family was identified through biochemical characterization of mammalian Rab5 and budding yeast screens for defects in vacuolar protein sorting (Vps) (Burd et al. 1996; Hama et al. 1999; Horiuchi et al. 1997). A family of GEF proteins with the Vps9 domain exists in higher eukaryotes, and humans (Carney et al. 2006). The best characterised is the mammalian Vps9 orthologue Rabex which is thought to be a major activator of Rab5 during trafficking into early endosomes from the plasma membrane (Horiuchi et al. 1997). Screens in *C. elegans* have shown that a second Vps9 domain GEF receptor-mediated endocytosis defective 6 (RME-6) is also involved in Rab5 regulation in the clathrin-mediated endocytic pathway, and this has been confirmed in mammalian cell lines (Sato et al. 2005; Semerdjieva et al. 2008). Interestingly, Vps9 domain GEFs can act on multiple although not necessarily all Rab5 subfamily members. Rabex activates Rab5 and Rab21 with equal efficiency, k_{cat}/K_m 2.3 and $3.2 \times 10^4\ M^{-1}\ s^{-1}$, but shows two orders of magnitude lesser activity towards Rab22 (Delprato et al. 2004). While there is no crystal structure for Rab5 in complex with a Vps9 domain GEF, a high-resolution X-ray crystal structure of a complex of the closely related GTPase Rab21 together with the Vps9 domain of Rabex has been solved (Delprato and Lambright 2007). This structure shows that a conserved aspartate finger residue in the Vps9 domain is inserted into the Mg^{2+} and phosphate-binding site where it contacts the P-loop lysine. The Vps9 domain engages the Rab switch regions stabilising an open conformation for switch I. More details of the exchange mechanism have come from the *Arabidopsis thaliana* Rab5 family GTPase ARA7 structure in both nucleotide-free and nucleotide-bound forms in complex with its Vps9 domain exchange factor (Uejima et al. 2010). These structures indicate that GEF interaction and insertion of the aspartate finger promote movement of the P-loop lysine away from the beta-phosphate of GDP and towards the conserved aspartate residue at the base of switch II. This would lead to release of the magnesium ion. It is proposed that these changes result in deprotonation of the beta-phosphate of GDP and destabilise GDP binding due to repulsion between this oxygen anions and the aspartate finger (Uejima et al. 2010). Once magnesium and GDP have left the binding pocket the aspartate finger of the Vps9 GEF also contacts the P-loop lysine and stabilises the nucleotide-free form of the Rab-GEF complex. Other evidence supporting this picture comes from analysis of the human Rab5-Rabex GEF system. Mutation of the conserved switch II aspartate leads to a form of Rab5 refractory to Rabex-stimulated GDP release (Langemeyer et al. 2014). Expulsion of the magnesium and GDP as a consequence of GEF binding inevitably results in a loss of defined switch I structure and movement to a more open position. Vps9 domain GEFs may therefore promote nucleotide exchange primarily though interactions with the P-loop lysine and switch II rather than promoting rearrangements in switch I.

5.2.2 Sec2/Rabin Is the GEF for Sec4/Rab8

Groundbreaking genetic screens in budding yeast generated the first protein secretion (Sec) mutants (Novick et al. 1980; Salminen and Novick 1987). Sec4, Rab8 in humans, was one of the first Rab family GTPases linked to vesicle transport as a result of such screens. Sec4 was shown to function in the delivery of Golgi-derived transport vesicles to the bud tip, the terminal step of the secretory pathway in budding yeast (Salminen and Novick 1987; Goud et al. 1988). Another of the Sec mutants, Sec2, was then shown to encode the exchange factor for Sec4 (Walch-Solimena et al. 1997). Sec4 cannot be activated and recruited to secretory vesicles in the absence of Sec2 function. Structural studies show that Sec2 forms an asymmetric coiled coil that interacts with and distorts the Sec4 switch regions (Dong et al. 2007; Sato et al. 2007a). A hydrophobic segment or platform formed by Sec2 leucine 104/108 and phenylalanine 109 interacts with hydrophobic residues in the Sec4 switch I region, and two aromatic residues phenylalanine 57 and tryptophan 74 (Dong et al. 2007; Sato et al. 2007a). Phenylalanine 57 lies immediately after the TIGID RabF1 motif, while tryptophan 74 falls at the start of the WDTAGQE switch II sequence. GDP release may be primarily due to the altered Sec4 switch I structure (Dong et al. 2007; Sato et al. 2007b). It is notable that in this structure Sec4 adopts a radically different P-loop conformation where lysine 33 interacts with a serine at position 161 (Dong et al. 2007). However in one of the Sec2-Sec4 structures the electron density for the P-loop is poorly defined, and there is insufficient density to be entirely certain of the P-loop lysine side chain position (Dong et al. 2007). In a second structure a phosphate occupies the P-loop site, which may not be present during the normal exchange process (Sato et al. 2007a). Because of these caveats it is difficult to draw any firm conclusions about the precise mechanism employed by Sec2/Rabin family GEFs. In summary, while both these known structures support the view that GDP release is promoted by switch I and II rearrangement more work is needed to define the intermediates leading to GDP release.

5.2.3 Multisubunit TRAPP Complexes form GEFs for Rab1

TRAPP I is a multisubunit GEF for Rab1/Ypt1 GTPases that function primarily in the COP II pathway of ER to Golgi trafficking (Plutner et al. 1991; Allan et al. 2000; Segev et al. 1988; Wang et al. 2000; Sacher et al. 2001; Kim et al. 2006; Cai et al. 2008). Rab1 has also been implicated in autophagy together with TRAPP III, a form of TRAPP I containing an additional targeting subunit Trs85 (Kakuta et al. 2012; Lynch-Day et al. 2010; Montpetit and Conibear 2009; Tan et al. 2013; Taussig et al. 2013). The catalytic core of the TRAPP I complex is comprised of five subunits: Trs31, Bet5, Trs23 and two copies of Bet3 (Cai et al. 2008). Trs23, Bet5 and one copy of Bet3 make contact with the Rab1 switch

regions and amino-terminus (Kim et al. 2006; Cai et al. 2008). Bet3 and Bet5 are both longin domain proteins and form a dimeric platform for Rab binding (Kinch and Grishin 2006; Levine et al. 2013) (Fig. 5.2). Similarly to Sec2, TRAPP makes contacts with residues in its target Rab in and adjacent to the TIGID RabF1 motif, and the conserved tryptophan at the start of the WDTAGQE switch II sequence (Cai et al. 2008). In addition, the extreme C-terminus of Bet3 has a highly conserved di-acidic motif, DE or EE that inserts into the phosphate-binding site. The last residue of Bet3 E192 interacts with the Rab1 P-loop lysine 21 and therefore perturbs the interactions needed for magnesium ion and phosphate binding. In the GEF-bound conformation the switch II region of Rab1 is restructured and the conserved glutamine residue at position 67 rotates and also makes contact with the P-loop lysine 21. Mutational analysis confirms that both the Bet3 acidic finger residue E192 and Rab1 Q67 in switch II are required for TRAPP-stimulated GDP release from Rab1 (Cai et al. 2008; Langemeyer et al. 2014). The nucleotide-free Rab1 switch I region is only partially structured in the TRAPP complex, and the density between residues 31 and 37 is lost (Cai et al. 2008). It is important to note that switch I is not physically displaced from its normal position by TRAPP. Rather Bet3 perturbs the interactions needed for magnesium ion and phosphate binding. This suggests that loss of stabilisation of switch I position occurs after magnesium ion and GDP release. Additional support for this view comes from the behaviour of the Rab1 Q67A mutant. This shows normal GDP and GTP-binding properties, but cannot release GDP in response to TRAPP (Langemeyer et al. 2014). If switch I displacement occurred prior to switch II rearrangement, then GDP release should have already occurred before the switch II glutamine 67 made contact with the P-loop lysine 21.

5.2.4 DrrA: A Pathogen-Encoded Rab1 GEF

DrrA/SidM is a *Legionella* protein with Rab1 GDP–GTP exchange activity required for Rab1 recruitment to the pathogen-containing intracellular vacuole (Machner and Isberg 2006; Murata et al. 2006; Ingmundson et al. 2007; Machner and Isberg 2007). Like the cellular GEFs discussed here, DrrA interacts with the switch regions and P-loop of its target Rab, and therefore promotes nucleotide exchange (Schoebel et al. 2009; Suh et al. 2009). Interestingly, there are obvious differences in the conformation of Rab1 in Rab1-DrrA complexes when compared to Rab1-TRAPP complexes. In the Rab1-DrrA complex the Rab1 P-loop lysine 21 contacts the Rab switch II aspartate 63 and glutamine 67. This is different to the Rab1-TRAPP complex where no contacts between the Rab1 switch II region and the P-loop lysine 21 are observed. It also diverges from the geometry seen in the Ras-SOS GEF complex where the Ras P-loop lysine interacts with a conserved glutamate intrinsic to the Ras active site switch II region (Wittinghofer and Vetter 2011). Mutation of this glutamate in Ras therefore reduces GEF-stimulated GDP release (Gasper et al. 2008) In Rab1, an equivalent switch II glutamine mutation

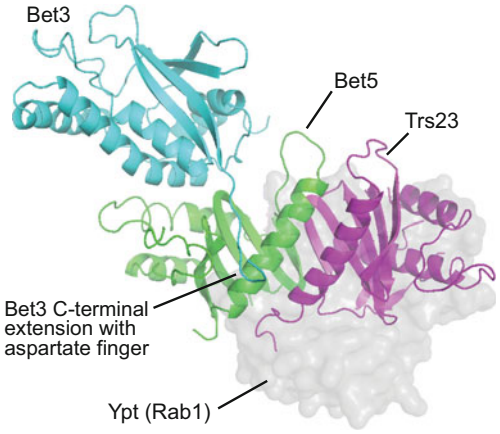

Fig. 5.2 The TRAPP longin core domains and Bet3 aspartate finger subunit. The polypeptide backbones of the Bet5 (*green*) and Trs23 (*magenta*) longin domain core subunits of TRAPP are shown in ribbon form. The longin domain is formed by two alpha-helices that form a hairpin overlaid by 5 beta-sheets. The final long alpha-helix lies over this platform and forms the interaction with the adjacent longin subunit which is related by a 180° rotation. The target Rab, Ypt1, sits on top of this platform slightly offset towards Trs23. The Bet3 subunit (*cyan*) contributes an extended C-terminal region carrying the aspartate finger residue needed for GEF activity

does not result in a reduction in either TRAPP- or DrrA-mediated GDP-release (Langemeyer et al. 2014). The defining feature of the Rab1-DrrA complex therefore appears to be a ternary interaction involving the Rab P-loop lysine 21 and switch II aspartate 63 and glutamine 67. Mutational analysis indicates that glutamine 67 although not required for nucleotide binding is important for DrrA-stimulated GDP release. This mutation has no effect on TRAPP-stimulated GDP release consistent with the structural data suggesting that the P-loop lysine interacts with the aspartate finger residue in the Bet3 C-terminus (Cai et al. 2008). DrrA and TRAPP therefore promote different conformations in Rab1, both of which lead to GDP release. This indicates there is considerable plasticity in the Rab nucleotide-binding domain, and that Rab GEFs can promote exchange by mechanistically separable pathways (Langemeyer et al. 2014).

5.2.5 DENN and DENN-Related Proteins Act on Diverse Rabs

DENN (differentially expressed normal versus neoplastic) domain proteins form the largest group of Rab GEFs (Levivier et al. 2001; Marat et al. 2011; Sato et al. 2008; Allaire et al. 2010; Yoshimura et al. 2010). In humans this family comprises seven subgroups DENND1–5, myotubularin-related proteins 5 and 13 (MTMR5/13) and MAP-kinase activating death domain protein (MADD), and

a divergent group of DENN-related proteins with four subgroups FAM116A/B, KIAA1147, AVL9 and FAM45A (Sato et al. 2008; Allaire et al. 2010; Yoshimura et al. 2010; Linford et al. 2012). DENN domain proteins were initially implicated as Rab GEFs when the protein MADD was purified as a Rab3 GEF from brain and subsequently the *C. elegans* orthologue AEX-3 found to act as a regulator of Rab3 in vivo (Wada et al. 1997; Brown and Howe 1998; Iwasaki et al. 1997; Figueiredo et al. 2008). Wider appreciation that DENN domain proteins were Rab GEFs came once it was shown that the *C. elegans* receptor-mediated endocytosis defective mutant rme-4 encoded a DENN domain protein homologous to mammalian DENND1A and regulated the Rab35 family GTPase RME-5 (Sato et al. 2008). Both RME-4 and DENND1A have specific Rab35 GEF activity (Sato et al. 2008; Allaire et al. 2010; Yoshimura et al. 2010). A family wide study of the human DENN domain proteins then defined the substrate specificity of this entire family of Rab GEFs. This defines the following Rab-GEF pairs: Rab35-DENND1A/B, Rab13-DENND1C, Rab2-DENND2A-D, Rab12-DENND3, Rab10-DENND4A/B, Rab39-DENND5A/B, Rab28-MTMR5/13 and Rab3/27A/B-MADD, respectively (Yoshimura et al. 2010). The best evidence confirming these biochemical assignments has been obtained for Rab3-MADD, Rab35-DENND1 and Rab10-DENND4. As already mentioned Rab35 and DENND1 are required for endocytic uptake in *C. elegans* and other work in human cells suggests they act at an early stage in the endocytic trafficking pathway and are needed for transport from the cell surface to the Golgi (Sato et al. 2008; Allaire et al. 2006, 2010; Yoshimura et al. 2010). Studies in *Drosophila melanogaster* provide compelling evidence that Rab10 and the DENND4 orthologue CRAG are required for polarised traffic of collagen and other cargo to the basolateral surface in epithelial cells (Denef et al. 2008; Lerner et al. 2013). These defects are reminiscent of those seen when Rab10 is inactivated in human cells (Denef et al. 2008; Schuck et al. 2007). Less is known about the function of the DENN-related GEFs and a defined Rab target and function has been found for only one subfamily member. FAM116A/B have been shown to be specific GEFs for Rab14, and to act in endocytic recycling by activation of Rab14. This pathway is important for the recycling of cell surface ADAM proteases and the control of cadherin family cell–cell adherens junctions in migrating cells (Linford et al. 2012).

A high-resolution X-ray crystal structure has been obtained for only one DENN domain protein, human DENND1B with Rab35 (Wu et al. 2011). This structure was a major advance, since it provided crucial new information about general themes in Rab GEF architecture and mechanism. Despite sharing no sequence similarity this structure revealed that the DENND1 has a longin fold similar to the Bet3 and Bet5 subunits of TRAPP, and a unique C-terminal domain (Levine et al. 2013; Cai et al. 2008; Wu et al. 2011). Both these domains make contacts with the Rab35 TIGID RabF1 motif, and the conserved tryptophan and polypeptide backbone of the WDTAGQE switch II sequence. The specificity of DENND1 for the GDP form of Rab35 may be explained by the availability of the TIGID RabF1 motif, since the threonine residue in this motif makes interactions with the magnesium ion in the GTP form of Rabs. Unlike TRAPP and Vps9 there are no insertions from the GEF

into the Rab nucleotide-binding pocket. Instead GEF binding promotes a conformation related to that seen in Rab1-DrrA complexes where the P-loop lysine 21 interacts with the switch II aspartate 63 and glutamine 67 (Schoebel et al. 2009; Suh et al. 2009). DENND1 fails to promote efficient nucleotide release from Rab35 glutamine 67 to alanine mutants (Langemeyer et al. 2014).

These observations suggest that switch II rearrangement is a primary cause of GDP release. Further support for this view comes from the analysis of mutations in the conserved aromatic residue phenylalanine 33 in switch I. Mutation of this residue to alanine gives rise to a form of Rab35 that shows rapid nucleotide release properties even in the absence of its DENND1 GEF (Langemeyer et al. 2014). If switch I rearrangement occurred as a primary consequence of GEF binding, then GDP release would still occur with Rab35 switch II glutamine mutant. Since GDP release is greatly reduced in the Rab35 glutamine 67 mutant, switch II rearrangement is likely to occur before that of switch I. The most parsimonious conclusion is that GEF binding promotes switch II rearrangement and release of magnesium and GDP, which then results in a loss of defined switch I structure and movement to a more open position.

5.2.6 HerMon GEFs for Rab7 Family GTPases

The different members of the Rab7/32/38 subfamily of GTPases are required for traffic to lysosomes and different classes of lysosome-related organelle such as the melanosomes and platelet granules. However, for a long time it was unclear how these Rabs are activated. Genetic screens for endocytic traffic defects revealed that yeast Mon1 and its *C. elegans* orthologue SAND-1 are important for Ypt7 and RAB-7 function (Poteryaev et al. 2010; Wang et al. 2003). Subsequently it was shown that the budding yeast Mon1-Ccz1 complex has Rab7 GEF activity consistent with its function in traffic to the vacuole and Ypt7-dependent vacuole tethering (Hoffman-Sommer et al. 2005; Wang et al. 2003; Nordmann et al. 2010). This was confirmed for the human Mon1-Ccz1 complex, which has specific activity towards Rab7 and does not act on the other closely related Rabs 32 and 38 (Gerondopoulos et al. 2012). Both Mon1 and Ccz1 subunits are required for this GEF activity, and neither subunit alone can activate Rab7. The Rab7-related Rabs Rab32 and Rab38 function in trafficking to lysosome-related organelles including the melanosome (Wasmeier et al. 2006). The Rab32/38 GEF is biogenesis of lysosome-related organelles complex-3 (BLOC-3), a complex of two proteins Hps1 and Hps4 distantly related to the Rab7 GEF Mon1-Ccz1 (Gerondopoulos et al. 2012). Both Hps1 and Hps4 are mutated in the human pigmentation and blood clotting disorder Hermansky Pudlak Syndrome, while Rab38 is altered in the ruby rat pigmentation mutant (Chiang et al. 2003; Martina et al. 2003; Nazarian et al. 2003; Oiso et al. 2004). Like Mon1-Ccz1, BLOC-3 has specific Rab GEF activity requiring the presence of both subunits (Gerondopoulos et al. 2012). Sequence analysis of the related Hps1 and Mon1 subunits of the HerMon GEFs suggest that these proteins

may have an N-terminal longin fold (Gerondopoulos et al. 2012). Despite this potential longin region there is no sequence similarity with TRAPP or DENND1, the GEFs known to contain longin domains from X-ray structure determination. Initial structural analysis has been performed on the BLOC-3 complex, which forms a 1:1 heterodimer, but it has not proven possible to obtain a structure possibly due to the presence of a long unstructured loop in the central region of Hps4 (Kloer et al. 2010). Mon1-Ccz1 and Hps1-Hps4 therefore form the founding members of the *Her*mansky Pudlak Syndrome and *Mon*1-Ccz1 (HerMon) GEF family acting on Rab7 and related GTPases.

5.2.7 Ric1-Rgp1 Is a Binary GEF Complex for Rab6

Rab6 family GTPases function in retrograde trafficking at or to the Golgi apparatus (White et al. 1999; Girod et al. 1999; Martinez et al. 1994). The best understood family member in terms of its regulation is budding yeast Ypt6, which functions in recycling of the exocytic SNARE Snc1 from endosomes back to the late Golgi where it is packaged into secretory vesicles targeted to the bud tip (Siniossoglou et al. 2000). Genetic screens in budding yeast identified a complex formed from Ric1 and Rgp1 that has Ypt6 GEF activity in vitro (Siniossoglou et al. 2000). Both Ypt6 and Ric1 are needed more generally for recycling of membrane proteins to the late Golgi in yeast (Bensen et al. 2001). In vivo both Ric1 and Rgp1 are required for correct localisation of Ypt6, consistent with the idea they act as its GEF. In human cells, the Ric1-Rgp1 complex acts as the GEF for Rab6 at the Golgi, and interestingly it is targeted via interactions with another Golgi Rab GTPase Rab33B (Pusapati et al. 2012). This is reminiscent of the BLOC-3 that interacts with, and may be targeted by, Rab9 (Kloer et al. 2010). It is tempting to speculate that the properties and dimeric form of Ric1-Rgp1 might indicate some relationship to the HerMon GEFs. However, Ric1-Rgp1 displays no homology to other Rab GEFs and lacks any conserved domains. It may therefore represent a unique class of Rab GEF for which structural data would be valuable.

5.2.8 Structural Themes in Rab Regulators

Based on the available evidence, Rab GEFs fall into one of two classes. Those that act by inserting an acidic finger residue into the Rab magnesium and phosphate-binding site, and those that induce restructuring of Rab switch I or II without insertion into the nucleotide-binding site. In both cases altered coordination of the P-loop lysine displacing the bound magnesium is likely to be central to the mechanism of GDP release. Likewise, specificity for the Rab GDP form may in part be due to the availability of the RabF1 TIGID motif in GDP-bound but not GTP-bound Rabs. Despite the lack of, or only limited sequence homology,

structural biology and highly sensitive sequence alignment tools based on hidden Markov modelling show that a number of Rab GEFs are united by longin/roadblock domains (Yoshimura et al. 2010; Wu et al. 2011; Levine et al. 2013). TRAPP, DENN family GEFs and the HerMon GEFs for Rab7-related GTPases all contain longin domains. These domains may therefore act as general platforms for GTPase regulation by promoting conformational change in the switch I and II regions, which represents a common theme in Rab activation. The longin/roadblock platform has also been found in other GTPase regulatory systems involving vesicle coat protein complexes, the ER signal sequence receptor, and a bacterial cell polarity MglA/MglB (Sun et al. 2007; Levine et al. 2013; Miertzschke et al. 2011). Therefore these domains may represent an ancient regulatory module used to control diverse GTPases and not only Rabs.

5.3 Rab GAPs: The TBC Domain Proteins

TBC1 (Tre-2/Cdc16/Bub2) domain GTPase activating proteins (GAPs) were originally identified in genetic screens for modulators of yeast Rab/Ypt function (Strom et al. 1993; Du et al. 1998; Albert et al. 1999; Vollmer et al. 1999; Albert and Gallwitz 2000; Eitzen et al. 2000). Because of this they are referred to as GAPs for Ypts (Gyp) in yeasts. In higher eukaryotes, they are typically referred to as TBC1 domain proteins. Analysis of the evolutionary history of Rabs and Rab GAPs has failed to create a clear picture of their relationship (Gabernet-Castello et al. 2013). This type of investigation has suggested that there is an ancient complement of ten TBC domain proteins, yet matching this to the cognate Rabs has proven difficult. This difficulty may be in part due to the presence of other domains in addition to the Rab GAP activity encoding TBC domain. Because of this it is important to biochemically define the target specificity of each Rab GAP using purified proteins. Expression of Rab GAPs has been relatively widely used as a tool to inactivate Rabs in cellular trafficking pathways (Hsu et al. 2010; Yoshimura et al. 2010; Fuchs et al. 2007; Longatti et al. 2012). Once a specific GAP or set of GAPs blocking a transport pathway is known, then it is relatively simple to identify the target Rabs using biochemical assays for GTP hydrolysis. This methodology has been used to identify Rabs involved in endocytic and secretory trafficking, as well as cell polarisation during cilium formation. Caution is required when interpreting such screens, since in some cases the effect of the GAP may not always correlate with catalytic activity (Haas et al. 2007; Longatti et al. 2012). A counterscreen with a mutated catalytically inactive Rab GAP can be used to test for this. One complication that has arisen from such approaches is the promiscuous nature of some TBC domain proteins in terms of their Rab target specificity.

5.3.1 Rab GAP Mechanism

Pioneering structural studies of Rab GAPs have revealed that the catalytic mechanism for GTP hydrolysis differs from that used by Ras and Rho GAPs (Pan et al. 2006; Gavriljuk et al. 2012). For Ras, GTP hydrolysis requires a glutamine residue intrinsic to the Ras switch II DxxGQ sequence and a catalytic arginine finger residue from the GAP inserted into the Ras nucleotide-binding site. By contrast the TBC1 domain contains two signature motifs IxxDxxR and YxQ (Neuwald 1997), and both these are required for catalytic activity (Pan et al. 2006). Structures of Rab33 GDP-AlF_3 with the Gyp1 TBC1 domain and Rab1 GDP-BeF_3 with TBC1D20 reveal a dual finger mechanism involving the conserved arginine of the IxxDxxR motif, and the conserved glutamine of the YxQ motif (Pan et al. 2006; Gavriljuk et al. 2012) (Fig. 5.3). While the TBC1 domain arginine adopts an equivalent position to that seen for Ras GAPs, the Rab switch II glutamine plays no direct role in (GAP stimulated) GTP hydrolysis. Instead this function is taken by the conserved glutamine of the YxQ motif in the TBC1 domain. The Rab switch II glutamine projects away from the nucleotide-binding site and contacts the backbone of the GAP. Fitting with the idea that the switch II glutamine plays little direct role in GTP hydrolysis, mutation in this residue had little effect on the GAP activity of RUTBC3 towards Rab5 or RUTBC1 towards Rab33B (Nottingham et al. 2011). In the case of Rab5 the switch II glutamine resulted in a greater than 5-fold reduction in basal GTP hydrolysis (Langemeyer et al. 2014). This indicates that for some Rabs basal and GAP-stimulated GTP hydrolysis may occur via different mechanisms. Analysis of two other Rab-GAP pairs complicates this simple picture. Rab35 and Rab1 switch II glutamine mutants showed only a slight decrease in basal GTP hydrolysis, while GTP hydrolysis was greatly reduced when stimulated by TBC1D10A or TBC1D20, respectively (Langemeyer et al. 2014). By contrast, mutations of the catalytic arginine or glutamine residues in the TBC domain greatly reduced the catalytic efficiency of the respective GAPs (Pan et al. 2006; Haas et al. 2005; Haas et al. 2007). Thus, the major determinants of GAP-stimulated GTP hydrolysis are the catalytic residues contributed by the GAP, while the Rab switch II glutamine plays a greater or lesser role depending on the Rab under scrutiny.

As already described, unlike Ras the Rabs don't always require the conserved glutamine for hydrolysis, but may need it for the GEF reaction in some instances. This suggests there is a fundamental difference in the conformational plasticity in the switch regions of Ras and Rabs that has consequences for regulatory mechanisms.

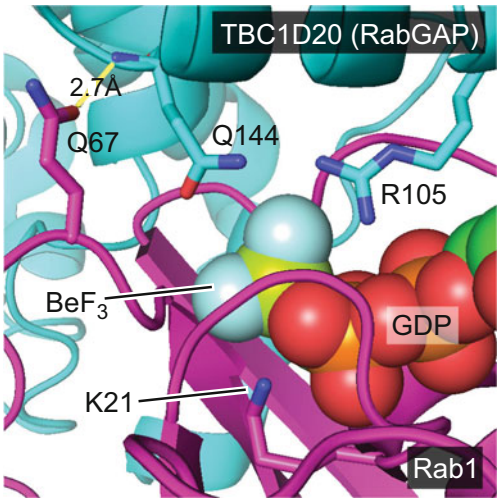

Fig. 5.3 Rab GAP mechanism and structure. TBC domain Rab GAPs utilise an arginine/glutamine dual finger mechanism. The interface between Rab1 (*magenta*) and its GAP TBC1D20 (*cyan*) shows the Rab1 switch II glutamine 67 residue contacting the GAP backbone. Glutamine 144 and arginine 105 finger residues contributed by the GAP are in close proximity to the gamma-phosphate position of the bound GTP, mimicked by GDP:berylium fluoride in this structure

5.3.2 Defining Cellular Functions for Rab GAPs

Although it has been relatively simple to identify the Rab GAP complement due to the presence of the conserved TBC1 domain in these proteins, elucidating specific cellular functions for these proteins has proven more difficult. In yeast the Gyps are not essential for growth under standard laboratory conditions, and biochemical analysis has provided conflicting reports on specificity. Here I will describe the functions of a subset of Rab-RabGAP pairs in secretion and cell polarisation, and endocytic trafficking and autophagy.

TBC1D20 and its yeast orthologue Gyp8 are ER-localised RabGAPs For Rab1 and Ypt1, respectively (De Antoni et al. 2002; Haas et al. 2007). Human TBC1D20 shows equivalent biochemical activity towards both Rab1 and Rab2, and some weak activity towards Rab18 (Haas et al. 2007). Cell biological studies have implicated both Rab1 and Rab2 in ER to Golgi trafficking (Tisdale et al. 1992; Schwaninger et al. 1992), while Rab18 plays a role in lipid droplet formation from the ER (Ozeki et al. 2005; Martin et al. 2005). Both overexpression and genome-wide siRNA screening support a physiologically relevant role for TBC1D20 in the regulation of secretion (Haas et al. 2007; Wendler et al. 2010), but have not definitively confirmed whether Rab1, 2 or 18 or a combination of these GTPases is the crucial cellular target. Loss-of-function mutations in TBC1D20 are found in the blind sterile mouse, and cause the human neurodegenerative and developmental

disorder Warburg Micro Syndrome (Liegel et al. 2013). TBC1D20 has also been implicated in other forms of neurodegenerative disorder (Gitler et al. 2008). This suggests that TBC1D20 function is essential for normal neuronal cell function, but may be less critical in other cell types. In budding yeast Gyp1 acts as a GAP for Ypt1 at the Golgi. In contrast to TBC1D20/Gyp8 which is an ER transmembrane protein, Gyp1 is a peripheral membrane protein found at the Golgi (Du and Novick 2001; De Antoni et al. 2002; Haas et al. 2007). Rab1/Ypt1 may therefore be regulated by a combination of both ER and Golgi GAPs.

Four TBC1 domain GAPs Gyp3/Msb3, Gyp4/Msb4, Gyl1/App2 and Gyp5 have been implicated in the regulation of Sec4 in polarised growth of budding yeast (Albert and Gallwitz 2000; Gao et al. 2003; Prigent et al. 2011; Chesneau et al. 2004; Chesneau et al. 2008). Why so many GAPs are required for Sec4 regulation during polarised growth is unclear. Biochemical characterisation paints a confusing picture of Gyp specificity, and Gyp3/Msb3 shows strong activity towards both Sec4 and Rab5-related GTPases. Furthermore, Gyp3/Msb3 has also been shown to act as a GAP for yeast Rab5-related GTPases during endosomal maturation (Nickerson et al. 2012; Lachmann et al. 2012). In this case the BLOC-1 complex recruits Gyp3/Msb3 to the yeast vacuole and promotes its activity towards Rab5 family GTPases (Nickerson et al. 2012; Lachmann et al. 2012). Therefore, in budding yeast it appears that Gyps may have more than one target GTPase and the specific site of action may be determined by additional regulatory factors. This may be due to the need to integrate regulation of different trafficking pathways, for example polarised growth with endocytosis; however this notion is speculative at best.

Overexpression screening and subsequent biochemical analysis have provided a clearer picture of the function of some human Rab-RabGAP pairs involved in formation of polarised cell structures. The primary cilium is an important site of signalling formed at the apical surface of many epithelial cell layers, and on fibroblast cell lines in culture. A series of Rab-RabGAP pairs was identified for the process of primary cilium formation in human cell lines (Yoshimura et al. 2007). TBC1D30 is a GAP for Rab8 enriched at primary cilia, while EVI5-like is the GAP for Rab23 known to modulate the Hedgehog signalling pathway at cilia during development (Yoshimura et al. 2007). A second GAP for Rab8, TBC1D17, has also been reported (Vaibhava et al. 2012). Intriguingly, TBC1D17 forms a complex with optineurin, and glaucoma-associated mutants in optineurin appear to potentiate TBC1D17 inhibition of Rab8 (Vaibhava et al. 2012). In addition to Rab8, Rab10 is an important regulator of transport in polarised cells and is controlled by two GAPs TBC1D1 and TBC1D4 (Peck et al. 2009; Miinea et al. 2005). These proteins are best characterised in terms of their function in the glucose transporter recycling pathway, and have different regulatory properties. TBC1D4 and TBC1D1 are regulated by the Akt and AMP-activated kinase signalling pathways, respectively (Peck et al. 2009; Miinea et al. 2005). Therefore in this instance multiple Rab GAPs are used so that specific signalling inputs can modulate the activity of the target GTPase, rather than to create redundancy.

Endocytic trafficking involves a complex network of trafficking between the cell surface, endocytic compartments and the Golgi apparatus. Accordingly, multiple Rabs are required for normal endocytic traffic including Rab5, Rab7 and Rab35. A series of studies have implicated different TBC1 domain Rab GAPs in the regulation of Rab5 during the plasma membrane to early endosome traffic. RabGAP-5/RUTBC3 has a high specificity for Rab5, and is required for growth factor receptor trafficking (Fuchs et al. 2007; Haas et al. 2005). Another GAP, RN-tre, was initially found as a GAP important for Rab5 inactivation during endocytosis and uptake of beta1-integrins at focal adhesion sites (Lanzetti et al. 2000; Palamidessi et al. 2013). RN-tre is also implicated in Rab43 regulation during endocytic trafficking of the Shiga toxin from the cell surface to the Golgi apparatus (Fuchs et al. 2007). In terms of its biochemical specificity, Rn-tre has some activity towards Rab5A-C, but shows a greater activity towards Rab43 (Fuchs et al. 2007; Haas et al. 2005). It may therefore act as a GAP for both Rab5 and Rab43 in vivo, and could be regulated by different regulatory signals depending on the tissue type of growth state of the cells. A further Rab5 GAP, TBC-2, has been identified from genetics screens for phagocytosis mutants in *C. elegans* (Li et al. 2009). However, the related mammalian protein TBC1D2/Armus appears to act as a GAP for Rab7 and regulate E-cadherin degradation (Frasa et al. 2010). Rab7 is inhibited by a second GAP TBC1D5 which binds to the retromer coat complex involved in the endosome to Golgi retrieval of the mannose-6-phosphate receptor (Seaman et al. 2009). In addition to the Rab5-Rab7 pathway, Rab35 is an important regulator of endocytosis in metazoa (Sato et al. 2008; Marat and McPherson 2010 ; Allaire et al. 2010). As well as the DENND1 GEF regulator already described, a Rab35 GAP TBC1D10A-C has been identified. TBC1D10A-C have been implicated in Rab35 regulation in trafficking from the cell surface to the Golgi as well as in the Rab35-dependent pathway for exosome formation (Fuchs et al. 2007; Patino-Lopez et al. 2008; Hsu et al. 2010). In *Drosophila melanogaster*, the TBC1D10 homologue whacked and Rab35 are important for the formation of cell surface protrusions called seamless tubes (Schottenfeld-Roames and Ghabrial 2012). These cell–cell connections become elaborated if whacked is lost or Rab35 dominant active mutations are expressed (Schottenfeld-Roames and Ghabrial 2012), suggesting whacked is important for inactivating Rab35 in this system. A neuronal TBC1 domain GAP for Rab35 has also been found in *Drosophila melanogaster* (Uytterhoeven et al. 2011). The *skywalker* protein has been implicated together with Rab35 in endocytic trafficking at the synapse (Uytterhoeven et al. 2011); however the biochemical specificity of this protein for Rab35 still needs to be confirmed.

TBC1 domain Rab GAPs have also been implicated in the regulation of endocytic traffic during autophagy (Popovic et al. 2012; Longatti et al. 2012; Carroll et al. 2013). A diverse set of 14 TBC1 domain proteins could be pulled down using GST-tagged LC3, and mapping experiments showed this is due to the presence of LC3 interaction (LIR) motifs in these GAPs (Popovic et al. 2012). TBC1D5 was found to possess two LIR motifs, and the LC3 interaction titrated out the interaction of TBC1D5 with the retromer complex. This suggests that TBC1D5

and RabGAPs more generally play roles in reprogramming membrane trafficking following starvation-induced autophagy (Popovic et al. 2012). Other evidence links Rab GAPs to the regulation of autophagy. TBC1D2/Armus may promote transient Rab7 inactivation during the early stages of autophagy (Carroll et al. 2013). TBC1D14 localises to recycling endosomes where it binds to Rab11 rather than acting as a Rab11 GAP (Longatti et al. 2012). TBC1D14 also interacts with the ULK1 autophagy kinase on recycling endosomes, and when overexpressed blocks autophagy. Under starvation conditions TBC1D14 relocalises from the recycling endosomes to the Golgi apparatus, while ULK1 localises to autophagosomes (Longatti et al. 2012). Together these findings indicate that transport from recycling endosomes is a key step in autophagy, and suggest that TBC1D14 is an important regulator of this process.

Finally, there are a number of Rab GAPs for which there is currently no specificity data, but evidence that suggests important physiological functions and are therefore worthy of some mention. TBC1D24 mutations are associated with epilepsy and deafness, and result in severe neurodegeneration (Afawi et al. 2013; Falace et al. 2010; Guven and Tolun 2013; Rehman et al. 2014). The target Rab regulated by TBC1D24 remains mysterious; however these data suggest that it functions in a trafficking pathway required for proper neuronal function. TBC1D23 was isolated by comparative genomic screens of mouse and *C. elegans* searching for regulators of innate immunity (De Arras et al. 2012; Alper et al. 2008).

5.4 Closing Remarks

Rabs are similar to but diverge from Ras in key aspects. Despite high levels of sequence homology in the regions needed for nucleotide binding there are crucial differences in the mechanism by which both GEF and GAP regulators control Rab activation and inactivation. While good evidence exists for the function of GEFs in driving Rab activation on specific organelles to promote specific transport events, the evidence relating to Rab GAP function is less clear. It seems most probable that combinations of Rab GAPs work in synergy in response to specific signalling inputs, rather than single GAPs acting in isolation. Further studies on the cell biological functions of Rab and their regulators, as well as careful analysis of the mechanism and structures of these proteins, are clearly required.

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Part II
Ran

Chapter 6
Ran in Nucleocytoplasmic Transport

Murray Stewart

Abstract The Ran GTPase is a crucial component of all nucleocytoplasmic transport pathways except those that mediate the nuclear export of mRNAs and ribosome subunits. The nucleotide state of Ran provides a means by which the cytoplasmic and nucleoplasmic compartments are recognized as a consequence of RCC1, the Ran guanine nucleotide exchange factor (RanGEF), being located in the nucleus, whereas the Ran GTPase activating protein, RanGAP, is located in the cytoplasm. This spatial arrangement of RanGEF and RanGAP results in nuclear Ran being primarily in the GTP-bound state whereas cytoplasmic Ran is in the GDP bound state. This distribution of Ran nucleotide state in turn mediates the binding and release of cargo macromolecules in the donor and target compartments, respectively. Generally the nucleocytoplasmic transport of macromolecules is mediated by transport factors of the karyopherin-β family that change their conformation depending on whether RanGTP is bound or not, and this conformational change in turn mediates the affinity of the transport factors for their cargoes.

Keywords Ran • Nuclear transport • GTPase

6.1 Introduction

The small Ras-family GTPase Ran (Gsp1 in yeast) is a crucial component of karyopherin-based nucleocytoplasmic transport pathways. Selective transport between the nuclear and cytoplasmic compartments is a fundamental cellular function of *Eukaryotes*. The separation of eukaryotic cells into nuclear and cytoplasmic compartments by the nuclear envelope enables transcription to be separated from translation and so facilitates the myriad of processing steps that enable the

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gene expression pathway to be so much more complex and sophisticated than that seen in *Prokaryotes*. In this context, the crucial function of the nuclear envelope is to keep transcripts and ribosomes separated until the nuclear processing of nascent RNA has been completed, but this function needs to be balanced against the need to transfer macromolecules between the nuclear and cytoplasmic compartments. For example, when the nuclear phase of their production has been completed, both mature transcripts and ribosome subunits need to be exported to the cytoplasm. Conversely, the proteins involved in replication, transcription, and processing that are synthesized in the cytoplasm need to be imported into the nucleus. This exchange of macromolecules between the nuclear and cytoplasmic compartments takes place through nuclear pore complexes (NPCs) that form a selectively permeable barrier between the compartments. Except for the nuclear export of mature mRNAs and ribosomal subunits, transport through NPCs is mediated by transport factors of the karyopherin-β superfamily, including importin-β, CAS, and CRM1, that are able to overcome the barrier generated by the crowding of the disordered regions of NPC proteins that occupy the central transport channel of the pore. In all of these karyopherin-mediated pathways, the karyopherin binds its cargo in the donor compartment and, after diffusing through a NPC transport channel, releases the cargo in the donor compartment (reviewed by Stewart 2007). Generally the passage of cargo:carrier complexes through NPCs is mediated by diffusion and is bidirectional. Directionality of transport is established by the binding and release of the cargo in the appropriate compartments, and so nucleocytoplasmic transport can be conveniently considered as a thermal ratchet where Brownian (thermal) motion is rectified by the energy released by GTP hydrolysis on Ran (Stewart 2007). Thus, although nucleocytoplasmic is an active, energy-requiring, transport process, metabolic energy is not used to actually move the material through the NPCs, but is employed instead to distinguish between donor and acceptor compartments. Consequently nucleocytoplasmic transport of macromolecules can be thought of as analogous to a biological manifestation of Maxwell's daemon (Maxwell 1872; Fig. 6.1).

6.2 Structural Changes in Ran Associated with Bound Nucleotide State

Although the structure of Ran is based on the core common to all Ras-family GTPases, it also has an acidic "tail" domain that is crucial to its function in nucleocytoplasmic transport (Scheffzek et al. 1995). In common with other Ras-family small GTPases, Ran undergoes a considerable conformational change between the GTP-bound and GDP-bound states (Figs. 6.2 and 6.3) that is associated with its function. However, in addition to the usual changes in the conformation of the switch I and switch II loops that is analogous to that seen in Ras, Ran also undergoes a considerable conformational change at its C-terminus where it has an

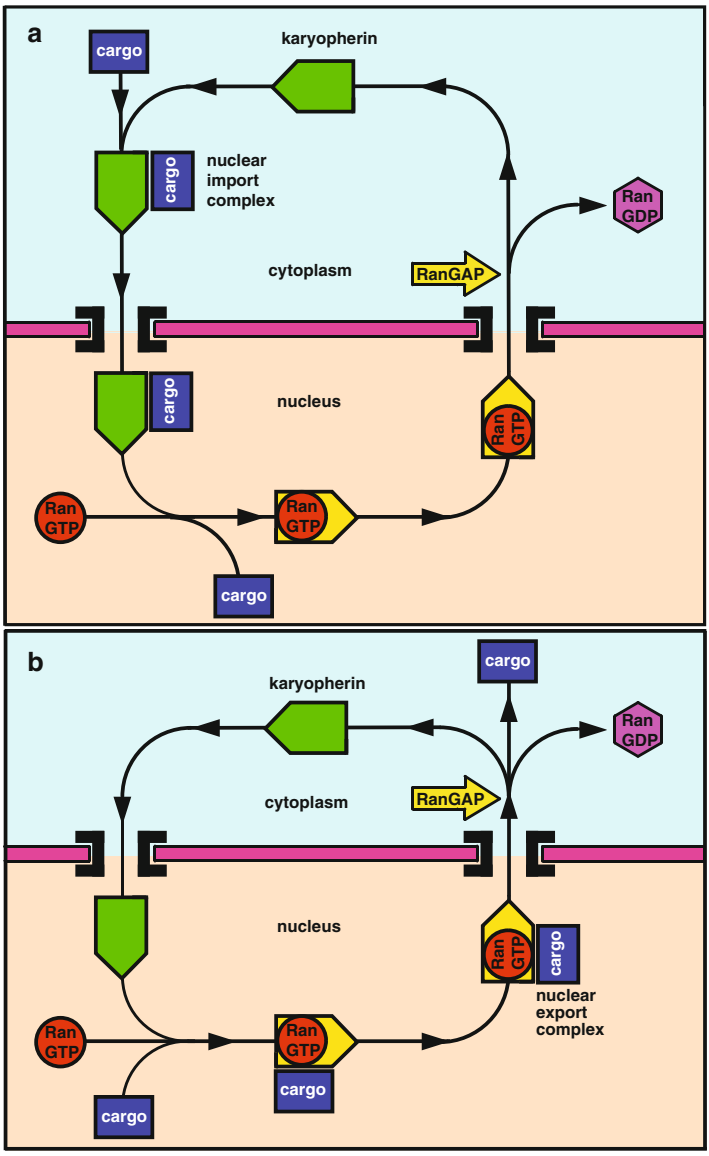

Fig. 6.1 Overview of karyopherin-mediated nuclear import and export cycles. (**a**) In nuclear import cycles, a karyopherin such as importin-β binds its cargo in the cytoplasm, often using importin-α as an adapter. The cargo:carrier complex then equilibrates between the cytoplasm and the nucleus by diffusing through nuclear NPCs via weak interactions with nucleoporins that contain a large number of repeating FG sequence motifs. When the karyopherin:cargo complex reaches the nucleus it is dissociated by RanGTP binding to the karyopherin. The karyopherin: RanGTP complex then diffuses through nuclear pores back to the cytoplasm where RanGAP hydrolyzes the GTP leading to the dissociation of RanGDP and freeing the karyopherin to participate in a further import cycle. (**b**) An analogous cycle is employed to export macromolecules such as proteins, t-RNA and pre-microRNAs from the nucleus. Here the cargo, RanGTP, and karyopherin interact to for a ternary complex that diffuses through NPCs to the cytoplasm, where

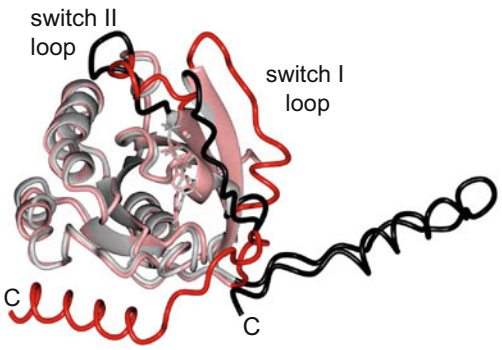

Fig. 6.2 Conformational changes in Ran associated with different nucleotide-bound states. Crystal structures of Ran in the GTP-bound (*gray*, *black*; PDB accession code 1RRP, Vetter et al. 1999b) and GDP-bound forms (*pink*, *red*; PDB accession code 1BYU, Stewart et al. 1998b). In common with other Ran-family GTPases, there are major conformational changes in the switch I and switch II loops between the GTP-bound form (*black*) and the GDP-bound form (*red*), but in Ran there is also a major conformational change at the C-terminus following residue 176. In the GDP-bound form, the C-terminal region binds to a positive patch centered on residue 150, whereas in the GTP-bound form, the position of the C-terminus differs depending on the protein to which Ran is bound. The structure shown here is from the complex between RanGTP and a Ran-binding domain of RanBP2 (Vetter et al. 1999b)

unstructured linker followed by a 16-residue α-helical extension followed by an acidic DEDDDL motif that is not observed in other Ras-family members (Scheffzek et al. 1995). This C-terminal region is crucial in orchestrating the interaction of Ran with karyopherin family transport factors and is essential to defining the structure of GDP-bound Ran (Nilsson et al. 2002). Remarkably the GDP-bound form of Ran in which this domain has been removed by truncation at residue 176 has the properties and structure of the GTP-bound form (Nilsson et al. 2002; Matsuura and Stewart 2004). In the GDP-bound state, the C-terminal region is in contact with the Ras-like core of Ran (Scheffzek et al. 1995), whereas in the GTP-bound state it extends away from this core (Vetter et al. 1999a, b), probably as a result of a steric clash between the switch I loop and the unstructured linker region (Nilsson et al. 2002). Because of the way in which it mimics the GTP-bound state, Ran truncated at residue 176 has been invaluable for obtaining crystal structures of its complexes with karyopherin family transport factors (see, for example, Matsuura and Stewart 2004; Lee et al. 2005; Cook et al. 2009; Monecke et al. 2009; Okada et al. 2009; Koyama and Matsuura 2010).

Fig. 6.1 (continued) the cargo is released following GTP hydrolysis and the karyopherin recycled to the nucleus. In both import and export pathways, the RanGDP generated in the cytoplasm is recycled to the nucleus by NTF2 where RanGTP is regenerated by the RanGEF, RCC1

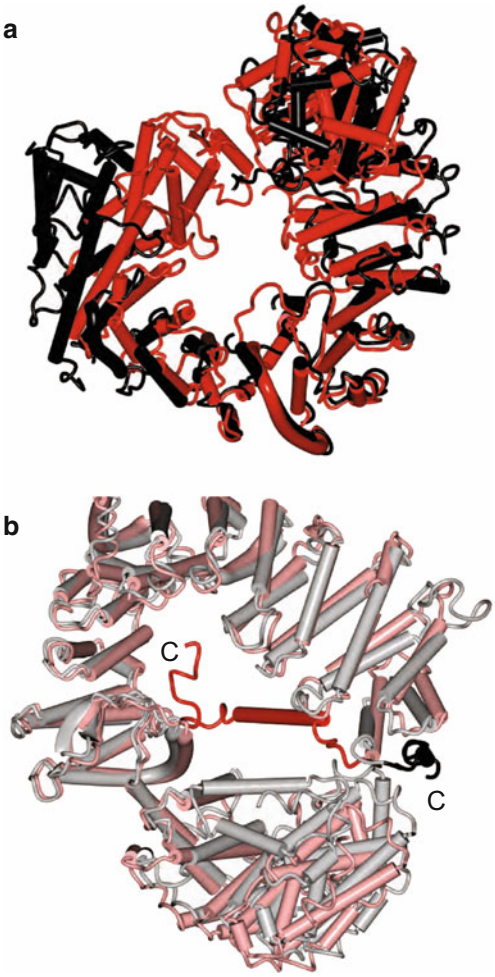

Fig. 6.3 Conformational changes in karyopherin structures associated with RanGTP binding. (**a**) The helicoid of the importin-α nuclear export factor, Cse1, undergoes a considerable conformational change between the isolated molecules (*red*, PDB accession code 1Z3H, Cook et al. 2005) and when bound to RanGTP and yeast importin-α (*black*, PDB accession code 1WA5, Matsuura and Stewart 2004). (**b**) Although yeast CRM1 (Xpo1) shows less change in the helicoid pitch between the isolated molecule (*pink*/*red*, PDB accession code 3VYC, Saito and Matsuura 2013) and when bound to RanGTP and cargo (*gray*/*black*, PDB accession code 3M1I, Koyama and Matsuura 2010), there is a major change in the position of the C-terminal helix that, in the RanGTP:cargo complex (*red*), takes up a position immediately below the NES-binding site, whereas in the isolated molecule it binds near the N-terminus (*black*)

6.3 The Spatial Distribution of Ran Nucleotide State

The nucleotide state of Ran is determined by the action of a guanine nucleotide exchange factor (RanGEF or RCC1, Prp20 in yeast) and a Ran GTPase activating protein (RanGAP, Rna1 in yeast). RCC1 mediates nucleotide exchange on Ran, whereas RanGAP stimulates the Ran GTPase reaction. In both cases these accessory proteins stimulate their respective processes by roughly five orders of magnitude (Klebe et al. 1995a). The asymmetric distribution of these proteins is crucial for imposing directionality on karyopherin-based nuclecytoplasmic transport pathways (Izaurralde et al. 1997).

The RanGEF, RCC1 (yeast Prp20), has a 7-bladed β-propeller structure (Renault et al. 1998) and catalyzes the exchange of nucleotides on Ran (Bischoff and Ponstingl 1991) by stabilizing the nucleotide-free transition state (Klebe et al. 1995b; Renault et al. 2001). Although Ran has a slightly higher affinity for GDP than GTP, because the nucleotide in cells is almost entirely GTP, RCC1 essentially results in RanGDP rapidly being converted into RanGTP (Klebe et al. 1995b). Moreover, because RCC1 is bound to chromatin (Ohtsubo et al. 1989), nuclear Ran is primarily in the GTP-bound form.

Conversely, RanGAP, in conjunction with Ran-binding protein 1 (RanBP1), stimulates the Ran GTPase activity resulting in the conversion of RanGTP to RanGDP (Bischoff and Görlich 1997). In metazoans, RanGAP binds to RanBP2 that is present in cytoplasmic fibrils emanating from NPCs in an interaction that requires RanGAP to be modified with the small ubiquitin-related protein, SUMO (Mahajan et al. 1997; Matunis et al. 1996, 1998). Because RanGAP is located in the cytoplasm, cytoplasmic Ran is primarily in the GDP-bound form. In common with many other Ras-family GTPases, the enzymatic activity of Ran is impaired by mutation of a key glutamine (Q69L mutation), albeit this mutation also introduces changes into the structure of Ran switch II loop in the GDP-bound state (Stewart et al. 1998b). Although most GAPs for Ras-family GTPases employ an arginine finger to catalyze GTP hydrolysis (Ahmadian et al. 1997), RanGAP has a novel fold (Hillig et al. 1999) and employs a novel mechanism in which the correct positioning of the catalytic machinery on Ran itself and its shielding from the solvent are sufficient (Hillig et al. 1999; Seewald et al. 2002).

6.4 NTF2 Facilitates Return of RanGDP to the Nucleus

Following RanGAP-mediated GTP hydrolysis in the cytoplasm, it is necessary for RanGDP to return to the nucleus to be recharged with GTP by RCC1. Although RanGDP is sufficiently small to be able to diffuse through the NPC transport channel, it appears that this simple diffusion is not sufficiently rapid to maintain adequate levels of nuclear RanGTP and so the nuclear import of RanGDP is expedited by a specialized import factor, NTF2 (Wong et al. 1997; Ribbeck

et al. 1998; Smith et al. 1998), that binds RanGDP with ~100 nM affinity but does not bind RanGTP. The ~100 nM affinity of NTF2 for RanDGP is sufficient for it to remain bound during the ~100 ms required to traverse the pore after which it can dissociate in the nucleus to enable the Ran nucleotide to be exchanged (Bayliss et al. 1999; Chaillan-Huntington et al. 2000). Like the karyopherins, NTF2 is able to surmount the NPC barrier function by binding to FG nucleoporins, albeit in this case only those that have a FxFG core motif (Bayliss et al. 1999, 2002b).

The selectivity exhibited by NTF2 for binding RanGDP but not Ran-GTP derives from NTF2 binding primarily to the Ran switch II loop (Stewart et al. 1998a) that changes its conformation dramatically between the different nucleotide states (Fig. 6.2). In RanGDP, the conformation taken up by the switch II loop enables the aromatic side chain of Phe72 of Ran to insert into the central hydrophobic cavity of NTF2 while also facilitating formation of a salt bridge between Lys71 of Ran and Asp92, Glu93, and Asp94 of NTF2 (Stewart et al. 1998a; Kent et al. 1999). Mutations in NTF2 that impair its binding to Ran result in Ran mislocalization and growth defects in yeast (Paschal and Gerace 1995; Clarkson et al. 1997; Quimby et al. 2001) underlining the functional importance of this interaction in vivo. Interestingly, NTF2 null mutants can be suppressed by overexpression of Ran, indicating that, although RanGDP is sufficiently small to be able to diffuse unaided through NPCs, its import needs to be accelerated in living cells because of the high rate at which Ran is employed in nucleocytoplasmic transport (Paschal et al. 1997). The switch II loop in the GDP-bound form of the Ran Q69L mutant, in which the GTPase activity is impaired and which is used extensively in functional studies, takes on a different conformation to wild type which may complicate studies in which it is used in vivo (Stewart et al. 1998b).

In addition to binding RanGDP, NTF2 is also able to bind to FG nucleoporins and so facilitate the transport of RanGDP from the cytoplasm to the nucleus through NPCs. Mutation of the FG-nucleoporin binding site impairs Ran nuclear import and leads to growth defects in yeast (Bayliss et al. 1999; Quimby et al. 2001).

Mog1 is another Ran-binding protein that has a vague structural homology to NTF2 and which binds to Ran in either nucleotide state (Stewart and Baker 2000). Although NTF2 and Mog1 compete for binding to RanGDP, the binding sites do not appear to be identical. The precise function of Mog1 remains somewhat speculative, but the ability of Mog1 overexpression to suppress a number of temperature-sensitive variants of the yeast Ran homologue Gsp1 would be consistent with formation of the complex contributing to the thermal stability of Ran (Oki and Nishimoto 1998).

6.5 Nuclear Import Pathways

Proteins are imported into the nucleus by karyopherin-family (Chook and Blobel 2001) transport factors such as importin-β and transportin (Kap95 and Kap104, respectively, in yeast). These transport factors bind their cargoes in the cytoplasm,

where Ran is GDP bound, and, after passage through NPCs, release the cargo in the nucleus following a conformational change induced by the binding of RanGTP to the karyopherin (reviewed by Conti et al. 2006; Stewart 2007). In the cytoplasm, transportin binds its cargoes directly (Lee et al. 2006), whereas, although importin-β does bind some cargoes (such as SREBP-2—Lee et al. 2003) directly, it frequently employs the importin-α adaptor that binds classical nuclear localization sequences (NLSs—see Lange et al. 2007) on cargo proteins and to importin-β through its importin-β binding domain (IBB—Görlich et al. 1996). The resultant cargo:carrier complexes are able to overcome the NPCs' barrier function that is generated by the disordered regions of NPC proteins or nucleoporins (Denning et al. 2003; Frey et al. 2006; Lim et al. 2006), because of specific interactions between the karyopherins and sequence motifs that contain phenylalanine and glycine (FG) cores present in multiple copies in the nucleoporins (Bayliss et al. 1999, 2000; Ribbeck and Görlich 2001). The interaction energy liberated by the binding of the karyopherins is sufficient to overcome the barrier function generated by these proteins and enable the cargo:carrier complex to diffuse back and forth through the pore's transport channel (reviewed by Stewart 2007).

When the cargo:carrier complex reaches the nucleus RanGTP binds, leading to a conformational change that reduces the affinity of the carrier for its cargo, or importin-α if it has been employed as an adaptor (Lee et al. 2005; Conti et al. 2006). This process may be facilitated by importin-β having a higher affinity for nucleoporins located at the nucleoplasmic face of the pore, such as Nup1 in yeast (Gilchrist et al. 2002; Liu and Stewart 2005). This higher affinity will result in the cargo:carrier complexes being concentrated in this region of the transport channel and so increase the likelihood that they will be dissociated by RanGTP binding. Following cargo release mediated by RanGTP binding, the karyopherin:RanGTP complex can then diffuse back to the cytoplasm through the pore. When the karyopherin:RanGTP complex encounters RanGAP and RanBP1, the Ran GTPase activity is stimulated, resulting in GTP hydrolysis and the generation of RanGDP that dissociates from the karyopherin, leaving it free to bind another cargo molecule and enter another import cycle. Cargo bound to importin-α is released though the concerted action of an autoinhibitory mechanism, whereby the IBB domain binds to the NLS-binding site on importin-α (Kobe 1999). This process is facilitated by nucleoporins, such as Nup2 (yeast) and Nup50 (metazoans), that are located at the nuclear face of the NPC together with the importin-α nuclear export factor, CAS (Cse1 in yeast) complexed with RanGTP (Gilchrist et al. 2002; Matsuura et al. 2003; Matsuura and Stewart 2005).

Crystal structures of the karyopherins (reviewed by Conti et al. 2006; Stewart 2007; Cook and Conti 2010; Chook and Süel 2011) have shown that they are helicoidal molecules that are constructed from a series of ~20 HEAT repeat motifs that are based on two α-helices that resemble the coil of a spring (Stewart 2003). Generally cargoes and the importin-α IBB domain bind to the inner concave face of the helicoid (Cingolani et al. 1999; Lee et al. 2003), whereas nucleoporin FG repeat cores bind to the exterior, convex, surface (Bayliss et al. 2000, 2002a). As discussed in greater detail below, RanGTP binding results in a change in the pitch of the karyopherin helicoid that alters its affinity for cargo (reviewed by Conti et al. 2006).

6.6 Karyopherin-Based Nuclear Export Pathways

The nuclear export of both proteins and small RNAs (such a tRNA and microRNA) is mediated by karyopherin-based export factors such as CRM1 (Xpo1 in yeast), CAS (Cse1 in yeast), Exportin-t (Los1 in yeast), and Exportin-5. In contrast to the nuclear import cycle, these transport factors bind their cargoes in the nucleus in conjunction with RanGTP. The cargo:carrier:RanGTP complex then diffuses back and forth through the pore transport channel until it is dissociated following RanGAP-stimulated GTP hydrolysis in the cytoplasm. CRM1 serves as a general protein nuclear export factor, whereas CAS is a specific export factor for importin-α (Kutay et al. 1997). Exportin-t mediates the export of tRNAs (Kutay et al. 1998) and Exportin-5 that of microRNAs (Lund et al. 2004). Crystal structures of these nuclear export factors bound to representative cargos have established the basis for their molecular recognition (reviewed by Conti et al. 2006; Stewart 2003, 2009; Cook and Conti 2010; Chook and Süel 2011). Although most cargoes bind on the inner concave face of the transport factors, CRM1 is an exception where specific hydrophobic nuclear export sequences (NESs) instead bind on the exterior, convex, surface in a groove formed between two of the α-helical HEAT repeats from which the molecule is constructed (Dong et al. 2009a, b; Monecke et al. 2009; Okada et al. 2009; Cook and Conti 2010; Güttler et al. 2010; Koyama and Matsuura 2010; Saito and Matsuura 2013).

6.7 RanGTP Binding Alters Karyopherin Conformation and Thus Affinity for Cargoes

The crystal structures of a considerable number of karyopherins alone or bound to cargoes and/or RanGTP have shown that the conformation of their HEAT repeat helicoid is sensitive to the presence of RanGTP and that these conformational states alter the affinity of the karyopherin for its cargo (reviewed by Conti et al. 2006). In the case of import transport factors such as importin-β and transportin, RanGTP binding appears to increase the helicoid pitch (Chook and Blobel 1999, 2001; Lee et al. 2005), whereas the converse appears to be true for export transport factors such as Xpo1 (Monecke et al. 2009; Dong et al. 2009a; Güttler et al. 2010; Koyama and Matsuura 2010), Cse1 (Matsuura and Stewart 2005; Cook et al. 2005), and Los1 (Cook et al. 2009), where RanGTP binding makes the helicoid more compact. In the case of CRM1, these conformational changes appear to be augmented by the binding of the tip of a long α-helix to a region on the convex underside of the helicoid that lies under the NES-binding site (Dong et al. 2009b; Fox et al. 2011). It has been proposed that in all of these transport factors energy stored by distorting the helicoid in the presence of RanGTP generated a “spring-loaded” molecule that facilitates dissociation of complexes in the cytoplasm following GTP hydrolysis on Ran (Matsuura and Stewart 2004; Lee et al. 2005; Conti et al. 2006).

RanGTP binding to karyopherins is able to mediate a change in the helicoidal pitch by interacting with generally two or three sites dispersed along the molecule so that the karyopherin tends to be wrapped around RanGTP. Generally all karyopherins bind RanGTP at a site near their N-terminus and which involves residues from the first three HEAT repeats of the karyopherin helicoid (Vetter et al. 1999a; Chook and Blobel 1999; Matsuura and Stewart 2005; Lee et al. 2005; Cook et al. 2009; Monecke et al. 2009; Dong et al. 2009a; Okada et al. 2009) interacting primarily with the switch II loop. The other areas of interaction are not so well conserved and tend to vary somewhat between different karyopherins. In the yeast Cse1:Kap60:RanGTP complex, for example, the switch I loop interacts with both the N-terminal region of Cse1 as well as a flexible loop emanating from near its C-terminus, while residues centered on Ran Lys152 also interact with the importin-α analogue, Kap60 in the complex (Matsuura and Stewart 2004). In the importin-β:RanGTP complex, in addition to the interaction with HEAT repeats 1–4 at the importin-β N-terminus, basic residues (Lys 134, Lys139, Arg140, and Lys141) on the opposite face of RanGTP bind at HEAT repeats 7 and 8 and a third site, centered on Lys152 (analogous to the second Ran-binding site on Cse1), binds to HEAT repeats 12–15. RanGTP binding at these three sites bends the importin-β into a more rigid, compact conformation in which the distortion of the helicoid disrupts the conformation of the extensive IBB-binding region on its inner, concave, surface and thereby facilitates the release of importin-α (Lee et al. 2005). RanGTP binding also induces a considerable change in the conformation of Exportin-t (Los1 in yeast) that results in its somewhat open conformation in solution becoming more compact and bringing its N- and C-termini closer together to form a binding site for tRNAs (Cook et al. 2009). This mode of binding explains how Xpo-t can recognize all mature tRNAs in the cell and yet distinguish them from those that have not been properly processed, thus coupling tRNA export to quality control (Cook et al. 2009). An analogous conformational-change linked molecular recognition was also seen in the crystal structure of pre-miRNA complexed with Exportin-5 and RanGTP (Okada et al. 2009). This crystal structure shows that Exportin-5:RanGTP recognizes the 2-nucleotide 3′ overhang and the double-stranded stem of the pre-miRNA, shielding it from degradation, whereas a tunnel-like structure of Exportin-5 interacts strongly with the 2-nucleotide 3′ overhang through H-bonds and ionic interactions. Interestingly, RNA recognition in this complex does not depend on RNA sequence, implying that Exportin-5:RanGTP can recognize a variety of pre-miRNAs (Okada et al. 2009). However, RanGTP binding appears to cause a much smaller structural alteration in the helicoid in some other karyopherins, including transportin and CRM1 (Xpo1 in yeast). For CRM1 binding either NES-containing cargoes or the snurportin adaptor is cooperative with RanGTP binding and a major determinant in modulating the NES-binding site appears to be the position adopted by a long α-helical extension located at the CRM1 C-terminus that, in the presence of RanGTP, is placed so that a group of charged residues at its C-terminus is located immediately below the NES-binding site and so is ideally placed to influence the affinity of CRM1 for NESs (Dong et al. 2009a, b; Monecke et al. 2009; Fox et al. 2011). Comparison with the

structures of the CRM1–NES–RanGTP complexes with a 2.1 Å resolution crystal structure of unliganded yeast CRM1 (Xpo1p) shows how RanGTP binding alters the conformation of CRM1 to facilitate NES binding (Saito and Matsuura 2013). An internal loop of CRM1 (referred to as HEAT9 loop) is primarily responsible for maintaining the NES-binding cleft in a closed conformation, preventing NES binding in the absence of RanGTP and also shows that the C-terminal tail of CRM1 stabilizes the autoinhibitory conformation of the HEAT9 loop, reinforcing this auto-inhibition (Saito and Matsuura 2013).

6.8 Ran Binding to Other Components of the Nucleocytoplasmic Transport Machinery

In addition to its interactions with karyopherins that are crucial for establishing the direction of transport between the nuclear and cytoplasmic compartments, Ran also interacts with Ran-binding protein 1 (RanBP1) and several nucleoporins that have putative Ran-binding motifs, including Nup358 (RanBP2), Nup153, Nup1, and Nup2. RanBP1 functions in the GTP hydrolysis step associated with the release of Ran from karyopherins in the cytoplasm and it is possible that the Ran-binding domains of Nup358 have an analogous function (Yaseen and Blobel 1999). The Ran-binding domains of the nucleoporins located on the nucleoplasmic face of NPCs (Nup153, Nup1, and Nup2) may function in accelerating the binding to Ran to karyopherins as they reach the nucleus, albeit there does not appear to be direct experiments evidence to support this hypothesis.

RanBP1 (yeast Yrb1) is a small, 23 kDa protein that binds Ran in the GTP-bound state but not in the GDP-bound state. RanBP1 by itself does not activate RanGTPase, but functions to increase the GTP hydrolysis induced by RanGAP1 by an order of magnitude (Bischoff et al. 1995). However, although RanBP1 functions to increase the efficiency of GTP hydrolysis on Ran, the structure of the Ran–RanBP1–RanGAP complex (Seewald et al. 2002) shows that RanBP1 is located away from the active site of Ran and detailed kinetic analysis shows that RanBP1 does not influence the rate-limiting step of the reaction, which is the cleavage of GTP and/or inorganic phosphate release. Instead RanBP1 influences the dynamics of the Ran–RanGAP interaction, resulting in a 20-fold stimulation of the already very fast association rate (Seewald et al. 2003). In yeast, RanBP1 forms a complex with the nuclear protein export factor CRM1, RanGTP, and NES-containing cargoes that is exported to the cytoplasm (Künzler et al. 2000). In addition to promoting the dissociation of Ran from karyopherins in the cytoplasm, RanBP1 also promotes the dissociation of NES-containing cargos from CRM1 in the cytoplasm (Kehlenbach et al. 1999). The 2.0 Å resolution crystal structure of yeast CRM1:RanBP1:RanGTP complex, which is an intermediate in the disassembly of the CRM1 nuclear export complex, shows that the Ran-binding domain of RanBP1 in conjunction with the acidic C-terminal tail of Ran generates a

large movement of the intra-HEAT9 loop of CRM1 that repositions it to a region on the CRM1 inner surface immediately behind the NES-binding site. This, in turn, causes conformational rearrangements in HEAT repeats 11 and 12 so that the hydrophobic NES-binding cleft on the CRM1 outer surface closes, squeezing out the NES cargo (Koyama and Matsuura 2010). RanBP2, a constituent of the fibrils that emanate from the cytoplasmic face of NPCs, contains four Ran-binding domains that are homologous to that of RanBP1. The structure of Ran bound to a non-hydrolyzable GTP analogue in complex with the first Ran-binding domain (RanBD1) of human RanBP2 showed that RanBD1 has a pleckstrin-homology domain fold and that the C-terminus of Ran is wrapped around RanBD1, contacting a basic patch on RanBD1 through its acidic end (Vetter et al. 1999b). Sequence analysis indicates that Nup1 and Nup2 also have domains with an analogous fold. In addition to these pleckstrin-homology Ran-binding domains, Nup358 and Nup153 also have a series of Zn-finger domains that bind Ran. Crystal structures of each of the four Nup153 Zn-fingers in complex with Ran show that each finger binds one Ran molecule in apparently non-allosteric fashion and that the affinity for RanGDP is higher than for RanGTP (Schrader et al. 2008; Partridge and Schwartz 2009). The structure of Nup153 Zn-finger 2 binding to Ran shows that its binding site overlaps with the site to which importin-β binds, suggesting the Nup153 Zn-fingers could generate a Ran reservoir at the nucleoplasmic face of NPCs that could facilitate nucleotide exchange through the formation of a ternary Nup153–Ran–RCC1 complex to ensure efficient termination of nuclear import cycles (Schrader et al. 2008).

6.9 Concluding Remarks

The function of Ran in nucleocytoplasmic transport is relatively well understood where its nucleotide state functions primarily to identify the nuclear and cytoplasmic compartments and thereby orchestrate the assembly and disassembly of karyopherin:cargo complexes in the donor and acceptor compartments, respectively. In this way the energy liberated by GTP hydrolysis is employed to rectify the Brownian motion of the karyopherin:cargo complexes so that there is a net transport of material from one compartment to the other. In some respects, the use of energy to sort molecules like this is somewhat analogous to Maxwell's demon (Maxwell 1872). The overall recognition is frequently based on the binding of RanGTP generating a considerable difference in the conformation of the helicoidal karyopherin transport factors. The difference in the nucleotide state of Ran between the cytoplasmic and nuclear compartments is maintained by the Ran GEF, RCC1, being confined to the nucleus whereas RanGAP is cytoplasmic. This distribution of GEF and GAP results in nuclear Ran being almost entirely in the GTP-bound state whereas in the cytoplasm it is GDP bound. The nuclear transport of Ran itself is determined by complex formation whereby RanGTP is exported from the nucleus bound to karyopherins and RanGDP is imported bound to NTF2. The difference in the affinity of Ran for its partners results from the dramatic change in the

conformation of the switch I and switch II loops between GTP- and GDP-bound forms and is augmented by the behavior of the acidic C-terminal region that is not found in other Ras-family GTPases.

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Chapter 7
Ran in Mitosis

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Abstract A direct function of the small GTPase Ran in mitosis was established clearly in 1999 when several groups investigated the influence of recombinant Ran variants on mitotic microtubule functions using cell extracts from unfertilised Xenopus eggs. Since then, the knowledge of Ran's functions in mitosis or meiosis (M-phase) has developed rapidly. This chapter summarises the logic of discoveries that led to our current knowledge of both mechanisms and targets of Ran in M-phase and focuses on the main principles.

Keywords Ran • RCC1 • importins • exortin • RanGAP • RanBP1/BP2 • mitosis • cell division • microtubules • microtubule-associated proteins • spindle • spindle poles • kinetochores • chromatin • nucleus • nuclear envelope • nuclear pore complex

7.1 Introduction: A Mysterious Link Between RCC1 and Cell Cycle Progression

In the late 1970s, systematic analyses of the eukaryotic cell division cycle in yeasts inspired several laboratories to search for cell cycle regulatory genes in mammalian cells using forward genetics. Takeharu Nishimoto and Claudio Basilico isolated temperature-sensitive (ts) baby hamster kidney (BHK) cells, which entered mitosis ahead of time at restrictive temperature. The cell clone termed *tsBN2* showed premature chromosome condensation, which phenocopied induced mitotic chromosome compaction seen in fusions of interphase and mitotic cells (Nishimoto et al. 1978). Several years later the Nishimoto lab succeeded to identify the mutant

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allele using an isolated DNA fragment that efficiently restored wild-type behaviour in *tsBN2* cells at restrictive temperature (Kai et al. 1986). The cDNA complementing the ts allele encoded a 421 amino acids open reading frame termed *regulator of chromosome condensation*, RCC1. RCC1 turned out to be highly conserved from yeast to man consistent with a function in a basic cellular process such as cell cycle control. The way that RCC1 was identified strongly suggested it had an important role as a cell cycle regulator or, at least, as an effector of mitotic entry and chromosome condensation. Consistent with being a key regulatory component of the cell cycle, RCC1 function seemed to act upstream of Cdc25 phosphatase and Cyclin-dependent kinase 1 (Cdk1) (Seki et al. 1992), two master regulators of mitosis. Only later was the molecular function of RCC1 identified as being the guanine nucleotide exchange factor for the small GTPase Ran (Bischoff and Ponstingl 1991), and the identification of Ran-regulated nuclear transport receptors provided a consistent explanation for the initially observed phenotype of *tsBN2* cells. The balance of nuclear import and export of key regulators for mitotic entry, such as Cdc25, probably changed following the reduction of RCC1 activity in *tsBN2* cells at the restrictive temperature. Nuclear accumulation of Cdc25 in *tsBN2* nuclei was proposed to account for premature entry into mitosis as it dephosphorylated, and therefore activated, Cdk1 ahead of time. The exact nature of nuclear transport defects in *tsBN2* cells stays unclear until now, but ever since the characterisation of the primary functions of RCC1 and Ran, the connection of Ran components, nucleocytoplasmic transport and the cell cycle fertilised and stimulated research in area of cell division. Important principles of mitosis and many molecular details were worked after the initial observations had been made. Our current knowledge is summarised in a number of excellent reviews, which I recommend for further reading (Dasso 2001; Moore 2001; Weis 2003; Clarke and Zhang 2004; Di Fiore et al. 2004; Gruss and Vernos 2004; Zheng 2004; Ciciarello and Lavia 2005; Ciciarello et al. 2007; O'Connell and Khodjakov 2007; Clarke and Zhang 2008; Kalab and Heald 2008; Rensen et al. 2008; Sato and Toda 2010; Kalab et al. 2011).

7.2 Uncoupling Interphase and Mitosis

Conceptually and experimentally, the intimate interdependence between cell cycle progression and nucleocytoplasmic transport was very difficult to analyse in intact mammalian cells. Chronic, incomplete loss of RCC1 activity in *tsBN2* cells would certainly cause accumulating defects in nucleocytoplasmic transport over time. Whether these primary defects were responsible for all cell division defects observed or if the loss of RCC1 would also affect mitosis independently of nucleocytoplasmic transport could not be dissected in intact cells. An idea to solve this problem was to use cell-free extracts from amphibian eggs that were naturally arrested in the second metaphase of meiosis and could proceed through the cell cycle in a strictly defined manner. This biochemically accessible system

enabled the RCC1 function in guanine nucleotide exchange to be interfered with by using recombinant Ran variants [T24N, (Klebe et al. 1995)], which efficiently blocked exchange function at defined time points during cell cycle progression (Clarke et al. 1995). Addition of recombinant RanT24N inhibited Cdk1 activity independently of nucleocytoplasmic transport, suggesting that the Ran system may function directly in mitosis independently of its role in nucleocytoplasmic transport. The idea to use cell-free extracts from unfertilized amphibian eggs in combination with recombinant Ran variants further stimulated key observations that elucidated direct functions of Ran in mitotic microtubule (MT) assembly, M-phase spindle formation as well as nuclear re-formation after mitosis.

7.3 A Direct Function of Ran in Mitosis Uncoupled from Its Role in Nucleocytoplasmic Transport

Cell-free extracts of Xenopus eggs preserve the natural arrest in metaphase II of meiosis, which features unfertilised vertebrate eggs in general (Masui and Markert 1971). However, these extracts can recapitulate minimal embryonic cell cycles ex vivo when released from their arrest. Released extracts show a short interphase with low cyclin B and Cdk1 activity levels, after which constantly accumulating cyclin B accelerates Cdk1 activity up to maximum in proceeding M-phase. Cdk1-stimulated rapid degradation of cyclin B then leads to a sharp drop in Cdk1 activity. Cyclin B levels oscillate in that way in cell-free egg extracts independently of nuclear structures. However, nuclei added from the beginning of the reaction will follow the cell cycle, replicate their DNA at low Cdk1 activity and build up bipolar spindles when the Cdk1 activity rises (Murray 1991; Sawin and Mitchison 1991). Inducing the cycling reaction in the presence of male sperm, for instance, results in sperm chromatin decondensing, duplicating, re-condensing and finally aligning on the metaphase plate of the bipolar spindle. The female cytoplasm also turns back the basal body of the sperm into a functional centrosome, which was lost from the female cytoplasm during the long growth phase of the oocyte. This first centrosome duplicates concomitant with DNA replication to ensure that one centrosome can localise to either of the two poles of the spindle. The formally meiotic egg extracts recapitulate general principles of MT assembly seen during both meiotic spindle formation in oocytes and mitotic spindle assembly in embryos and somatic cells. Very dynamic M-phase (meiotic and mitotic) MTs in egg extracts exclusively but strongly nucleate form the spindle poles and the chromatin to finally build up a bipolar spindle.

When several groups addressed a possible role of Ran in M-phase MT assembly using egg extracts of the African clawed frog *Xenopus laevis*, they consistently observed that the sole addition of any stable form of RanGTP, e.g. the hydrolysis-deficient Ran variants RanQ69L (Klebe et al. 1995), Ran G19V or Ran L43E (Carey et al. 1996) loaded with GTP, or Ran (wild-type or G19V) preloaded with

GTPγS, induced MT assembly in M-phase extracts (Carazo-Salas et al. 1999; Ohba et al. 1999; Wilde and Zheng 1999; Zhang et al. 1999a). Microtubules did not assemble in the absence of exogenous Ran, upon adding wild-type Ran loaded with GDP, or upon addition of nucleotide-free Ran (Ran T24N). Importantly, RanGTP triggered MT assembly independent of any particular source of MT nucleation, such as the centrosomes or the chromatin of sperm nuclei, and assembled a range of structures including pseudo-spindles. In these experiments, Ran was added into M-phase extracts long after entry into M-phase, i.e. long after nuclear envelope (NE) and nuclear pore complex (NPC) breakdown, and the cessation of nucleocytoplasmic transport. These observations strikingly demonstrated a direct function of Ran in M-phase MT assembly independent of nucleocytoplasmic transport. They also showed that the Xenopus cell-free system allows spindle formation by a complete self-assembly process. Size and shape of pseudo-spindles seemed to be determined entirely by the pattern of factors activated by RanGTP in this system. These results, although intriguing, immediately posed several questions. Why, for instance, did extracts that contain high concentrations of endogenous Ran not assemble MTs on their own? What was the function of Ran in regular spindle assembly around sperm nuclei?

The answer to the first question turned out to be rather straightforward. Addition of recombinant RCC1, which rapidly increases guanine nucleotide exchange in Ran and therefore elevates endogenous RanGTP levels, also stimulated M-phase MT assembly (Carazo-Salas et al. 1999; Kalab et al. 1999; Ohba et al. 1999). This clearly indicated that endogenous Ran remains primarily GDP bound in M-phase egg extract and suggested that Ran's hydrolysis stimulating proteins RanGAP1, RanBP1 and RanBP2 (Zhang and Hawley 1990) dominate over the activity of cytosolic RCC1.

Answering the second question, however, was more complicated because it required inhibition of any possible RanGTP production in reactions that assembled spindles in the presence of sperm nuclei. Addition of recombinant RanT24N to block RCC1 activity, or addition of an excess of RanBP1 to counteract RanGTP production, interfered with proper spindle formation. Still MT production was not completely reduced, questioning a universal role of Ran in spindle formation (Kalab et al. 1999; Zhang et al. 1999a; Carazo-Salas et al. 2001). In contrast, interfering with, or counteracting of, RanGTP production using the same methods completely abolished spindle formation around artificial chromosomes in form of simple chromatin beads (Carazo-Salas et al. 1999). Spindle formation around chromatin beads recapitulates the pathway of centriole-free meiotic spindle formation in vertebrate oocytes. In this experiment, chromatin was the sole source of MTs, whereas chromatin cooperates with centrosomes in spindle formation around sperm nuclei.

Together both experiments suggested an intriguing hypothesis: RanGTP production may be rate-limiting for the activity of M-phase chromatin in MT production. No MT assembly would occur in the absence of RanGTP while increasing RanGTP concentrations readily assemble MTs (Carazo-Salas et al. 1999; Kalab et al. 1999). More recently, it could be shown that single 10 μm glass bead bearing

immobilised RCC1 assembles spindles in the Xenopus cell-free system adding further evidence for this hypothesis (Halpin et al. 2011).

It is now widely accepted that RanGTP plays a key role for chromatin-driven processes in spindle formation. These include MT nucleation as well as MT organisation generating a bipolar structure. However, Ran stimulates additional functions in M-phase MT assembly. Although inhibition of RanGTP production by RanT24N had no influence on MT nucleation from isolated centrosomes (Carazo-Salas et al. 1999), it greatly stimulated the nucleation of a radial MT array ("aster") formed around both isolated centrosomes (Gruss et al. 2002) and sperm centrioles that stay in the vicinity of chromatin (Kalab et al. 1999; Wilde and Zheng 1999; Zhang et al. 1999b; Carazo-Salas et al. 2001): Moreover, RanGTP strongly influenced MT dynamics in Xenopus cell-free extracts (Kalab et al. 1999; Carazo-Salas et al. 2001; Wilde et al. 2001). All these data show that RanGTP mediates a variety of different processes in spindle formation during M-phase. This concept even expanded when Ran variants were coupled to beads and tested in extracts at late stages of M-phase that would readily return to interphase. Remarkably, these beads assembled pseudo-nuclei with an intact NE and incorporated NPCs allowing regular nucleocytoplasmic transport (Zhang and Clarke 2000). Although these experiments strikingly demonstrated a second important function of Ran in M-phase, they made it even more difficult to explain how Ran mechanistically acts in these diverse and complicated processes. Moreover, a formal proof to show if and how RanGTP was generated in M-phase in the absence of an intact nucleus was still missing.

7.4 Production of RanGTP in Mitosis: A Diffusible Gradient Around Chromatin

In interphase, the asymmetric distribution of RanGTP between the nucleoplasm and the cytoplasm maintains the directionality of nucleocytoplasmic transport. RanGTP, present at high concentrations in the nucleoplasm, dissociates import complexes and assembles export complexes. In contrast, low cytoplasmic RanGTP concentration allows import complex assembly and promotes disassembly of export complexes after hydrolysis of GTP in Ran. An intact NE greatly increases the efficiency of both processes. It dramatically limits diffusion of free RanGTP out of the nucleus and maintains RanGTP concentration differences of almost 3 orders of magnitude in between the nucleus and the cytoplasm (Görlich et al. 2003). Moreover, it enables strict separation of RCC1 and the combination of RanGAP and RanBP1/BP2. This situation changes in open mitosis of metazoan cells after NE breakdown. However, the accessory components of Ran stay active throughout the cell cycle. In particular, RCC1 continues to work as a chromatin-bound guanine nucleotide Ran exchange factor in M-phase (Fig. 7.1), which is well supported by several detailed biochemical and structural studies on RCC1 binding to chromatin.

The structure of RCC1 resembles a seven-bladed propeller (Renault et al. 1998), whose N-terminus seems to mediate chromatin docking by promoting interaction with the DNA (Chen et al. 2007), while other sequence elements bind to the Histone 2A/B dimer (Nemergut et al. 2001). A recent structural analysis of two RCC1 molecules on a nucleosome shows insights into the mode of interaction with nucleosomes: Two Arginines in an unstructured region in RCC1 named the "switchback loop" mediate binding to an acidic patch in the H2A/B dimer. A motif next to the switchback loop further contributes to RCC1 DNA binding directly and promotes contacts with the negatively charged DNA phospho-backbone. Although not visible in the crystal structure, the model also supports an important function in DNA binding of the unstructured N-terminal RCC1 tail (Makde et al. 2010). Consistently, mitotic phosphorylation of RCC1 in the N-terminal tail at Serine 11 by Cdk1 modulates chromatin binding (Hutchins et al. 2004; Li and Zheng 2004; Hitakomate et al. 2010), a mechanism that targets in particular one (γ) out of three human RCC1 isoforms (α, β, γ) to mitotic chromatin. The γ-isoform shows high mitotic phosphorylation levels, interacts tightly with mitotic chromatin and, in the form of a phosphomimetic mutant, efficiently rescues the temperature-dependent *tsBN2* phenotype of premature chromosome condensation (Hood and Clarke 2007). Furthermore, N-terminal tri-methylation after trimming the starting methionine of RCC1 promotes direct DNA binding, which is essential for proper mitosis in intact human cells (Chen et al. 2007). Strong chromatin binding of RCC1 in mitosis is also coupled to Ran's nucleotide exchange. The binary RCC1–RanGDP complex stably binds to chromatin, while RCC1–RanGTP quickly dissociates. This mechanism ensures local, effective RanGTP production on mitotic chromatin (Li et al. 2003) and is consistent with the structural data of RCC1 on nucleosomes (Hondele and Ladurner 2010; Makde et al. 2010). Although recent data question the role of N-terminal phosphorylations of RCC1 in chromatin binding, they confirm, using fluorescence correlation spectroscopy, that the interaction of RCC1 with chromatin is positively regulated in mitosis. Tight binding also increases the enzymatic activity in nucleotide exchange (Bierbaum and Bastiaens 2013), which validates the idea that the generation of RanGTP not only continues in mitosis but that RCC1 on mitotic chromosomes produces more RanGTP than during interphase (Fig. 7.1).

To ensure efficient mitotic RanGTP production, Ran also undergoes modifications in mitosis, in particular at Serine 135 (S135), which is targeted by *p21 activated kinase 4* (PAK4). PAKs best understood function lies in the regulation of exit from G1 and G2 phases of the cell cycle, but recent data indicate that it also regulates proper chromosome alignment and spindle function in mitosis (Bompard et al. 2013). Phosphorylation of Ran at S135 makes the GTP conformation more resistant to GTP hydrolysis, thus increasing the effective RanGTP concentration in a PAK4-dependent manner. Interestingly, colocalisation studies in human cells indicate that active PAK4 and Ran colocalize not only on mitotic chromosomes but also at centrosomes and the spindle midzone (Bompard et al. 2010). This further suggests a complex, non-uniform pattern of RanGTP activity that regulates key activities in spindle formation locally even at a distance from chromatin.

Although indirect evidence and computational models (Görlich et al. 2003) strongly suggested a diffusible gradient of RanGTP around chromatin, direct proof only came from the visualisation of RanGTP by specific biosensors using Förster Resonance Energy Transfer (FRET). In 2002, Petr Kalab pioneered this approach when he engineered a FRET sensor comprising EYFG and ECFP as donor and acceptor for FRET, connected by a high-affinity RanGTP binding site derived from yeast RanBP1 (Kalab et al. 2002). The conformational change in the RanBP1 peptide upon RanGTP binding diminished FRET and allowed imaging RanGTP specifically. Strikingly, the sensor detected a spherical gradient of RanGTP around mitotic sperm chromatin in Xenopus cell-free extracts. Importantly, measurable size (17 μm) and steepness of the gradient matched computational simulation using the known biochemical parameters of the Ran system (Kalab et al. 2002; Görlich et al. 2003). Spindle size (ca. 30 μm), however, seemed to exceed the size of the gradient.

7.5 Targets of the Ran System in M-Phase

The considerable complexity of Ran functions in M-phase strongly suggested the presence of a variety of different molecular targets in spindle formation and nuclear reformation. However, it initially remained unclear how Ran would activate these downstream activities.

After being produced and diffusing away from chromatin in M-phase, RanGTP will meet its most abundant interaction partners as a function of time, among them the Ran binding proteins RanBP1 and RanBP2. They, in concert with RanGAP, promote GTP hydrolysis in Ran. These interactions seem to be non-productive. When RanGTP, however, meets importin β-like transport receptors (importins and exportins), it changes their binding properties in the same way as in the interphase nucleus. For example, RanGTP binding to importin β generally releases importin-bound protein. Several experiments in Xenopus cell-free extracts using importin fragments and an excess of nuclear localisations signal (NLS)s to compete importin–substrate interactions initially revealed that the release of spindle assembly factors from importin α/β or importin β is a key mechanism of Ran-dependent spindle formation (Gruss et al. 2001; Nachury et al. 2001; Wiese et al. 2001) (Fig. 7.2). In support of this model, it could be shown recently that a small molecule inhibitor of importin β, importazole, not only blocks nuclear import into semi-permeabilised human cells but abolishes spindle formation in intact cells and Xenopus cell-free extracts (Soderholm et al. 2011). Interestingly, the idea of Ran's mechanism in M-phase was also directly confirmed by FRET probes to monitor the activity of RanGTP in M-phase. In these probes, a conformational change in importin β, leading to the dissociation of the N-terminus of importin α (importin β binding domain, IBB), was turned into an increasing FRET signal. This strongly indicated dissociation of endogenous NLS proteins from importins and suggested the presence of free spindle assembly factors. The range of the gradient

of free IBB nicely matched with measurements using fluorophore-labelled Ran and importin β as FRET pair. Both approaches detected a 25–35 μm large gradient around mitotic sperm chromatin in Xenopus cell-free extracts, in intact cells and in amphibian oocytes (Caudron et al. 2005; Kalab et al. 2006; Dumont et al. 2007). The gradient of free substrates therefore exceeds the size of the RanGTP gradient and matches the actual size of the spindle. Even at a large distance from chromatin and the virtual absence of RanGTP, importin-free substrates may therefore mediate functions in spindle formation such as the organisation of spindle poles.

It is now generally accepted that RanGTP activates a variety of MT regulators using the conformational switches on two nuclear transport receptors—importin β and exportin1/CRM1—to locally activate (importin β) or to specifically target (exportin 1) downstream activities (Figs. 7.1 and 7.2) in spindle formation. Among these, the release from importin β is the predominant mechanism of substrate activation in M-phase. Identification of most of the substrates regulated by RanGTP and the transport receptors importin β and exportin1/CRM1 again relied on the use of Xenopus egg extracts. The strong activity of RanGTP to induce formation of MT assemblies in M-phase independently of any particular source for nucleation provided a homogenous system, which could be further fractionated by simple biochemical methods or specifically depleted of candidate proteins to identify targets of the Ran system in spindle formation. Understanding the predominant mechanism of the action of RanGTP in spindle formation, i.e. to release NLS-containing proteins from importins, suggested that Ran targets are generally nuclear proteins in interphase and bind to MTs in M-phase. The first proteins identified as Ran targets matched these predictions. The MT-associated proteins TPX2 (targeting protein for XKlp2) (Gruss et al. 2001) and NuMA (nuclear protein of the mitotic apparatus) (Nachury et al. 2001; Wiese et al. 2001) localise to the spindle in M-phase and are nuclear proteins in interphase of human or Xenopus somatic cells (Hetzer et al. 2002). The interaction of TPX2 with importin α is remarkable as the key NLS in TPX2 contacts the minor binding site in importin, while most other NLS proteins interact with importin α's major binding side. This special binding mode may enable competitive sequestration of TPX2 even in the presence of many free NLS-containing proteins and ensure proper spatial regulation of TPX2 in response to RanGTP (Giesecke and Stewart 2010).

Although not all details of TPX2 function are understood, it has been shown in cell-free extracts and with purified components that TPX2 promotes MT assembly (Gruss et al. 2001; Schatz et al. 2003; Brunet et al. 2004). Moreover, Importin-Free TPX2 associates with other MT-associated proteins and the MT minus-end motor dynein (Wittmann et al. 1998; Ma et al. 2010) to form complexes with new MT modulating functions, in particular to establish proper organisation of spindle poles (Wittmann et al. 2000). Under control of the dimeric E3 ubiquitin ligase BRCA1/BARD1 (breast cancer 1/BRCA1-associated ring domain protein 1), free TPX2 interacts with the MAPs XRHAMM (Xenopus hyaluronan-mediated motility receptor) and NuMA (Merdes et al. 1996), which supports a complicated bundling and spindle pole organisation activity that could not be executed by the single entities of the complex. One of the most abundant nuclear proteins that localises to the spindle in mitosis of somatic cells is poly-ADP-ribose polymerase [PARP

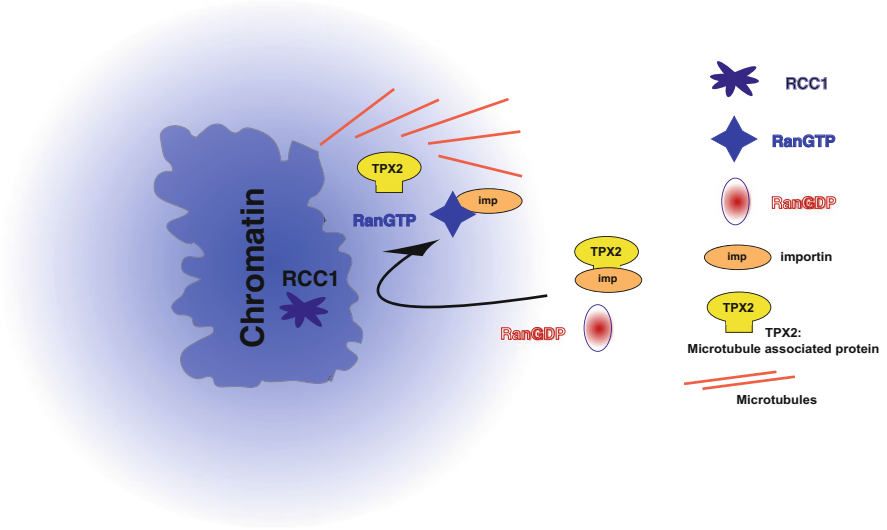

Fig. 7.1 Tight binding and ongoing activity of RCC1 on M-phase chromatin generate RanGTP from RanGDP in the vicinity of chromatin. As it diffuses away from chromatin, with time, the probability for GTP hydrolysis on Ran by RanBP1/BP2 and RanGAP increases. As a result, a diffusible gradient of RanGTP becomes established around M-phase chromatin. RanGTP, by a mechanism analogous to its function in the interphase nucleus, releases microtubule-associated proteins, such as TPX2 (targeting protein for Xklp2), microtubule motors or other spindle assembly factors from importins (importin α, importin β or importin 7). Factors released from importins can then function in, for example, microtubule nucleation as shown for TPX2

(Tegha-Dunghu et al. 2008)], which is required for proper mitosis in human cells (Chang et al. 2004, 2005). Interestingly, also PARP functions together with NuMA (Chang et al. 2009).

Bundling of MT in the vicinity of chromatin requires the activity of chromatin-bound NuSAP (Ribbeck et al. 2006, 2007; Vanden Bosch et al. 2010), a nucleolar protein in interphase nuclei of somatic cells, which, consistently, is under the control of RanGTP in the Xenopus system. Interestingly, NuSAP functions could be modulated by binding to importins α, β and 7 (Ribbeck et al. 2006, 2007) indicating a complicated set of regulatory inputs. In a cell cycle-dependent manner, the chromatin protein ISWI dissociates from mitotic chromatin, binds MTs and stabilises spindle MTs in particular during anaphase. Apart from regulation by the cell cycle machinery, ISWI's MT binding stays under the control of RanGTP to ensure high local activity of ISWI on spindle MTs and proper chromosome segregation (Yokoyama et al. 2009). In contrast, it is the release from importin β that mediates proper association of the chromokinesin motor protein Xkid on chromatin (Tahara et al. 2008).

The activity of RanGTP to form complete spindles also seems to trigger the association of TPX2 with the Xenopus chTOG (colonic and hepatic tumour overexpressed gene) ortholog XMAP215 (Xenopus MT-associated protein of

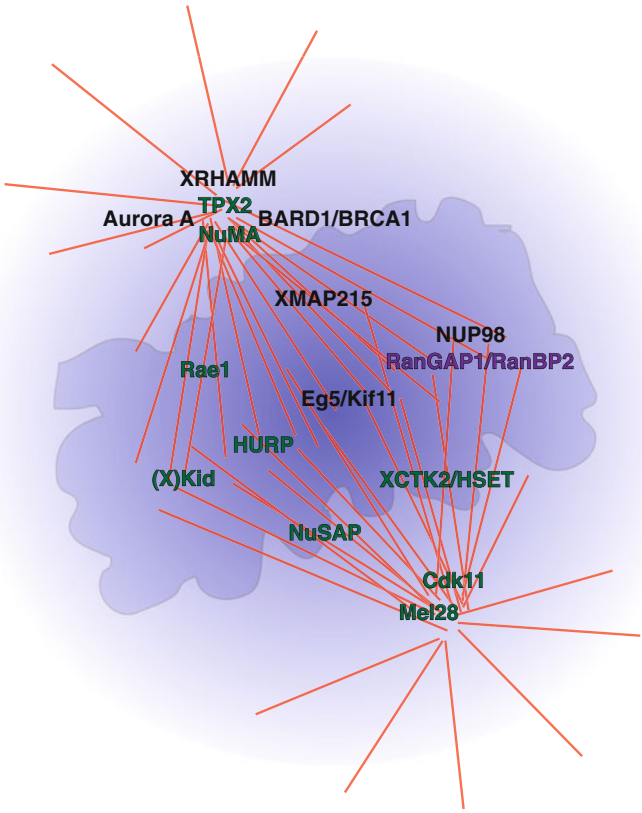

Fig. 7.2 The Ran system controls the activity of a variety of different factors required in spindle formation. The majority of them dissociate from importins (see Fig. 7.1) and are shown in *green*. They often cooperate with additional regulators of spindle formation that are not directly controlled by importins or exportins and which are themselves not subject to control by the Ran system (*black*). RanGTP promotes binding of the nuclear export receptor CRM1 to the RanGAP/RanBP2 (*purple*) complex and promotes its functional association with kinetochores

215 kDa), one of the most potent MT stabilisers in the vertebrate cytoplasm, with the MAP HURP (hepatoma upregulated protein), the tetrameric mitotic motor protein Eg5/Kif11 and Aurora A kinase. Inhibition of HURP function by selective partial immunodepletion from Xenopus egg extracts abolishes formation of this complex. Under these conditions, RanGTP nucleates MTs, but the asters initially assembled do not organise into pseudo-spindles (Koffa et al. 2006). Interestingly, HURP is also regulated by RanGTP through direct binding to importin β. Its complete immunodepletion strongly reduces RanGTP-mediated MT assembly around chromatin beads (Casanova et al. 2008), and siRNA mediated depletion of HURP from human cells weakens bundling and stabilisation of kinetochore (K) fibres MTs (Koffa et al. 2006; Sillje et al. 2006) by modulation of the activity of KIF18A, an MT depolymerising kinesin (Ye et al. 2011). Knock-down of the

supposed fly ortholog of HURP, *Mars*, results in strikingly similar mitosis defect phenotypes (Yang and Fan 2008; Zhang et al. 2009).

Although the precise function of the HUPRP/TPX2/Eg5 complex in the Xenopus cell-free system remains controversial, the tetrameric motor Eg5 has been suggested to acquire higher activity in the presence of RanGTP (Wilde et al. 2001), possibly cooperating with directly Ran-regulated activities such as TPX2 (Ma et al. 2010). In vitro studies and experiments in Xenopus extracts suggest that RanGTP also activates the C-terminal, minus-end directed kinesin motor protein XCTK2 (HSET/kinesin14 in humans) for functions in MT bundling and MT sliding, presumably at MT minus ends to organise spindle poles (Ems-McClung et al. 2004), or to control spindle size (Cai et al. 2009). In human cells, the main interaction partner of TPX2 is the mitotic kinase Aurora A, whose targeting to spindle MTs depends on TPX2 (Kufer et al. 2002). Detailed structural analysis shows that the very N-terminus of TPX2 drives a conformational change in the activation segment of the kinase that makes the t-loop phosphorylation less accessible for dephosphorylation and kinase inactivation (Bayliss et al. 2003, 2004). Aurora A modifies not only TPX2 itself, but also Eg5, TACC (transformed acidic coiled-coil protein) and other centrosomal proteins to modulate their functions in centrosome maturation and spindle pole organisation (Eyers and Maller 2004; Barr and Gergely 2007). The release of TPX2 from importin α/β triggers a whole variety of different activities of the chromatin acting on spindle MTs. Consistently, chromatin-driven, RanGTP-mediated spindle formation entirely depends on free TPX2 as shown by immunodepletion from Xenopus egg extracts (Gruss et al. 2001). However, its depletion did not reduce the capacity of centrosomes to nucleate MTs; on the contrary, it allowed strong Ran-dependent stimulation of centrosomal nucleation (Gruss et al. 2002). These data also account for the previous observations of RanGTP-dependent stimulation of centrosome function and enabled the biochemical identification and characterisation of Cdk11 as the key effector in Ran-regulated increase in MT nucleation from centrosomes (Yokoyama et al. 2008).

Ran-regulated activities in spindle formation even involve a structural role of RNA. RanGTP activates, by the release from importin β, an RNA containing complex with the MT-associated and RNA binding protein Rae1. Immunodepletion of the complex from Xenopus egg extracts inhibits spindle formation. Purified Rae1 ribonucleoprotein particles in turn strongly stimulate MT assembly in vitro in an RNA-dependent manner (Blower et al. 2005). Interestingly, Rae1 works as a nuclear-pore-associated mRNA export factor in interphase nuclei (Pritchard et al. 1999) providing a possible link between nuclear pore complex proteins and mitosis (Hofmann et al. 2010). The Rae1 interaction partner at NPCs, Nup98, directly contributes to spindle formation as an MT-bound protein modulating the activity of the MT depolymerising kinesin MCAK (Cross and Powers 2011). NPCs generally disassemble in mitosis, but NPC proteins can take over mitotic functions as subcomplexes with new features. Targeting of the RanBP2/RanGAP-SUMO-Ubc9 complex via RanGTP provides the best understood example of how RanGTP in conjunction with the export receptor exportin 1 regulates the specific localization

of proteins in mitosis. The specific kinetochore localisation of RanBP2/RanGAP-SUMO-Ubc9 requires RanGTP and exportin 1, which form an export-like complex with RanBP2/RanGAP-SUMO-Ubc9. The specific localisation mediates key mitotic functions of the complex in proper MT to kinetochore attachment (Joseph et al. 2002, 2004; Salina et al. 2003; Swaminathan et al. 2004; Arnaoutov et al. 2005).

Interestingly, regulation by Cdk1 overlays the mitotic function of exportin 1: Cdk1 phosphorylates exportin 1 on Serine 391 to strengthen association with the RanGAP–RanBP2 complex and thus integrates RanGTP and Cdk1 regulation of spindle formation (Wu et al. 2013). Recent data suggest that kinetochore localization of RanGAP1 also depends on RanGTP through release of factors from importin β (Roscioli et al. 2012), indicating that both principal mechanisms to regulate Ran functions in mitosis cooperate on the same protein complex. The Nup107 complex that in human cells consists of seven proteins also localises to kinetochores in human cells (Belgareh et al. 2001; Loiodice et al. 2004). Immunodepletion of this complex from Xenopus egg extracts leads to almost complete loss of MTs around sperm nuclei (Orjalo et al. 2006) and siRNA knock-down to deplete the complex from mitotic cells causes chromosome alignment failure (Zuccolo et al. 2007). Impaired localisation of the chromosome passenger complex including Aurora B kinase after depletion of SEH1, a critical component of the complex, contributes to alignment defects (Platani et al. 2009) as well as the Nup107-dependent kinetochore targeting of the γ-tubulin ring complex for proper nucleation of kinetochore MTs (Mishra et al. 2010). Consistently, previous data had shown that RanGTP accumulation supports local MT nucleation at kinetochores (Torosantucci et al. 2008). The Nup107 complex in general and its recently identified nuclear-associated protein ELYS/Mel28 in particular pioneer the re-assembly of NPCs after mitosis (Rasala et al. 2006; Franz et al. 2007; Doucet et al. 2010). ELYS/Mel28 acts as a Ran-regulated scaffolding factor for further NPC protein association (Fernandez and Piano 2006; Franz et al. 2007). This opens up an intriguing licensing mechanism, which couples interphase and mitosis. At the onset of mitosis, the Nup107 complex needs to be released from NPCs to allow spindle formation. In turn, dissociation of the complex from kinetochores after mitosis is a prerequisite for postmitotic NPC assembly. Very recently, we could show that ELYS/Mel28 also carries out an essential function in spindle formation as a Ran-regulated MAP. Specific immunodepletion of ELYS/Mel28 leaves most of the Nup107 complex in Xenopus egg extracts but completely abolishes any Ran-driven MT assembly around chromatin and Ran-dependent stimulation of centrosomal nucleation (Yokoyama et al., in revision). ELYS/Mel28 therefore not only is the key factor to control timely, apparently mutually exclusive spindle formation and NPC assembly, but also puts both processes under the strict control of the Ran system. Experimental evidence from Xenopus cell-free extracts documents that nuclear lamins, which stabilise the nuclear structure during interphase, may support mitotic functions by wrapping a shell-like matrix around the spindle under the control of RanGTP (Tsai et al. 2006).

While most Ran targets in M-phase were identified in Xenopus cell-free extracts, experiments from flies and even yeasts contributed to our understanding of Ran functions in M-phase and confirmed the evolutionary conserved key role of Ran in cell division. In Drosophila syncytial embryos, injection of Ran variants not only interferes with proper spindle formation but also affects chromosome segregation, possibly by interfering with the function of kinesin motor activities (Silverman-Gavrila and Wilde 2006). Later in cell division, timely activation of the actin bundling protein Anillin, a nuclear protein during interphase, in the fly embryo gets under the control of the Ran system. Targeting of the septin peanut to pseudocleavge furrows requires importin-free anillin and thus RanGTP (Silverman-Gavrila et al. 2008). Adenomatous Polyposis Coli (APC) protein is part of the β-catenin degradation complex, which regulates wnt signalling but in complex with EB (end binding) protein family members tracks the plus end of MTs. Its MT assembling activity in vitro is strongly reduced by importin β binding (Dikovskaya et al. 2010).

Timely regulated import of spindle proteins under the control of the Ran system allows spindle assembly in eukaryotes managing chromosome segregation in closed nuclei. Specifically, the SPB protein Alp7, the yeast ortholog of vertebrate TACC, needs to be released from importins in the nucleoplasm to enable spindle formation (Sato and Toda 2007, 2010).

The strict cell cycle-dependent synthesis of many nuclear Ran targets in somatic cells adds an additional layer of regulation to Ran functions in M-phase. Initially the epitope to the human autoantibody MSA-35, which was shown to be expressed only at late stages of the cell cycle, turned out to be the mitotic motor Eg5. TPX2 was initially found as the "restricted expressed proliferation-associated antigen 86" in human cells (Heidebrecht et al. 1996) and starts to accumulate during S-phase (Heidebrecht et al. 1996; Gruss et al. 2002) and quickly disappears after mitosis (Gruss et al. 2002; Stewart and Fang 2005). The cell cycle controls expression of the Ran targets HURP (Tsou et al. 2003) and NuSAP (Raemaekers et al. 2003) in a similar manner as for TPX2 and Eg5.

The N-terminus of TPX2, comprising a KEN box motif and a second unusual degradation signal, undergoes polyubiquitination by the Anaphase Promoting Complex at mitotic exit in somatic cells to ensure its proper degradation after spindle disassembly (Stewart and Fang 2005). In addition, the Ran targets XRHAMM/HMMR, NuSAP, HURP and BARD1 show cell cycle-dependent degradation in human cells. Interestingly, release from importin β triggers degradation, at least in parts, already during metaphase. Coupling release and degradation before anaphase not only prepares the system for efficient down-regulation of Ran-regulated spindle assembly proteins after mitotic exit, but also implies a potential negative feed-back regulation to balance the levels of mitotic Ran targets during spindle formation (Song and Rape 2010). In general, most targets of RanGTP in M-phase are expressed in a cell cycle-dependent manner. These observations make the mitosis-specific targets of Ran GTP excellent markers for actively or hyperactively proliferating cells (Morgan-Lappe et al. 2007; Rensen et al. 2008). Consistent with this observation, antibodies against TPX2 termed "KI-2" have

served as markers for transformed cells in several cancer models. Malignancy was correlated with the levels of TPX2 expression in malignant lymphoma (Heidebrecht et al. 1997; Rudolph et al. 1998, 1999), mantle cell lymphoma (Brizova et al. 2010; Cordes et al. 2010), malignant nevocellular tumours (Heidebrecht et al. 1997), salivary gland sarcoma (Shigeishi et al. 2009), laryngeal cancer (Cordes et al. 2010), non-small cell lung cancer (Kadara et al. 2009), meningioma (Stuart et al. 2011), cervical cancer (Chang et al. 2012) and breast cancer (Colak et al. 2013). TPX2 is also a proliferation-associated protein that was found to be overexpressed in BPDE-transformed human bronchial epithelial (16HBE-C) cells (Zhang et al. 2008), is overexpressed in liver, lung, prostate and pancreatic cancers (Wang et al. 2002), and has been identified as a candidate oncogene on 20q11.2 showing copy number-driven overexpression in non-small-cell lung cancer and PDAC (Tonon et al. 2005). TPX2 targeting siRNAs consistently cause cell cycle arrest and apoptosis in cancer cell lines (Warner et al. 2009). A siRNA library-based screening revealed that TPX2 is one of the three genes that significantly reduced the survival of multiple human tumour cell lines (Morgan-Lappe et al. 2007). It is tempting to speculate that small molecule inhibitors or inhibitory antibodies against TPX2 function as potent cytostatic agents directed specifically against proliferating cells. In human HeLa cells, inhibiting the function of TPX2 leads to a very strong cell cycle arrest followed by apoptosis (Garrett et al. 2002; Gruss et al. 2002), indicating that cancer cells essentially rely on the MT polymerising activity of TPX2. Recently, withanone, a small molecule inhibitor of cell proliferation from *Withania somnifera*, was suggested to target TPX2 in intact cells and may thus give a perspective as a novel anti-cancer drug (Widodo et al. 2010; Grover et al. 2012). Interestingly, modelling of a complex of Aurora A, TPX2 and withanone predicts that the compound interferes with proper Aurora A-TPX2 interaction (Widodo et al. 2010) and could thus selectively influence a very defined event upon entry into mitosis. However, the exact molecular basis of withanone's anti-proliferative potential in intact cancer cells remains to be determined and further characterisation of the compounds is required to validate the use of TPX2 as a selective anti-cancer target.

7.6 Reading the RanGTP Gradient

The complex pattern of proteins and protein complexes released from importin and localised via exportin, their dynamic interactions with MTs and other spindle assembly proteins in space and time may help to envision how mitosis orchestrates the complicated self-assembly program driving spindle formation upon induction by RanGTP. Combinatorial interactions of more and more Ran-regulated factors activated with time correlate with the exponential production of MT mass after RanGTP addition to Xenopus cell-free extracts (Clausen and Ribbeck 2007; Petry et al. 2013).

The shape of the RanGTP gradient will, in first approximation, be turned into a gradient of importin β-free and exportin 1-targeted spindle regulators that, in turn, promote key functions in the spindle formation process. The fact that a gradient and not a concentration switch (like in interphase) defines Ran functions in mitosis is a striking consequence of the behaviour of the system after NEB. Nevertheless, the molecular mechanisms underlying the response to the Ran system (i.e. changing the conformation of importin-β-like transport receptors) are the same in interphase and mitosis. There are several principles whereby the RanGTP gradient may be translated into information for spindle assembly. In the context of individual substrates, the mitotic cytoplasm may generate a graded response. It uses the actual RanGTP concentration as a direct measure of the distance to chromatin; in other words: the farther away from chromatin, the weaker the response. That does not exclude, however, that different factors can react differently to the same actual RanGTP concentration. Release by Ran and association with importins depend on the nature of the substrate and the absolute concentration of the downstream factors. For example, 90 % released substrate A may promote a weak response in comparison to 10 % released substrate B, if the activity of substrate B exceeds A by more than tenfold. Importantly, several substrates released simultaneously may potentially cooperate with each other and thus generate positive feed-back loops that enable switch-like behaviour, such as MT nucleation, to be triggered. Therefore, the mitotic Ran gradient and its downstream responses are designed in a way that can build up a complex machine such as the mitotic spindle with different activities required at different locations at large distances from each other. Such a mechanism contrasts to the behaviour seen in the more homogenous nuclear compartment, where RanGTP tends to switch quantitatively all import substrates to their free states and associate all exportin substrates to complexes destined for nuclear export, independently of their subnuclear localisation.

Although clear evidence for a functional coordination of all nuclear proteins working in spindle formation in open mitosis is missing, it is very likely that mitosis must tightly regulate the spatio-temporal activity pattern of these factors. In large oocytes or early embryos, nuclear proteins are stockpiled in the cytoplasm to quickly assemble several hundreds of nuclei with little de novo synthesis. Upon initial spindle assembly during meiosis, or, after fertilisation, during the first cleavage divisions, the vast majority of stored nuclear proteins reside at large distances from chromatin. RanGTP restricts spindle formation to the vicinity of chromatin and centrosomes, while importins ensure sequestration and inactivation of nuclear proteins away from chromosomes. In contrast, in rather small somatic cells, the temporal regulation provided by cell cycle-driven NE breakdown may be even more important than the spatial regulation by the RanGTP gradient in the mitotic cytoplasm as it spans almost the entire cell.

The concept of a gradient generated by the local production of a diffusible component is, particularly in mitosis, not restricted to RanGTP. It also relies largely on gradients of differentially phosphorylated mitotic effector proteins and is generated, in case of Aurora B, by the localisation of the kinase to mitotic chromatin. Being part of the chromosome passenger complex, Aurora B accumulates at the

centromeric chromatin region until anaphase onset. The widely accepted model of Aurora B function implies that it phosphorylates several outer kinetochores proteins in the vicinity of the kinase to modulate their MT binding behaviour. Stable, end-on MT binding to kinetochores, however, generates tension and drags away the substrates from the source of phosphorylation, which further stabilises MT kinetochore interaction (Liu et al. 2009). Recent FRET experiments in intact cells suggest that the Aurora B and RanGTP gradients cooperate to ensure efficient spindle formation (Lee et al. 2012) and it will be interesting in the future to unravel the molecular details about this communication.

7.7 Evolution of the Ran System

Evolution of the nuclear compartment marks the development of eukaryotic cells. A diffusible gradient of RanGTP interacting with Ran responsive receptors around chromosomes, however, may have evolved prior to the closed nucleus. Like todays transport receptors, these receptors could have served as chaperones or regulators for the function of bound substrates. Efficient binding of Ran's exchange factor to chromatin and strictly localised Ran guanine nucleotide exchange on chromatin generated a compartment defined by high RanGTP concentration and active nuclear proteins without the boundary of the NE. After release from their receptors, actual binding to destined structures on chromatin may have restricted diffusion and may have allowed specific functions of active nuclear proteins. The system certainly became much more efficient when the NE closed and NPCs evolved together with FG repeats and a new set of transport receptors, in which chaperone activity for nuclear proteins, Ran responsiveness and solubility in the hydrophobic environment of NPCs were combined. From this perspective, open mitosis might possibly be viewed as a cell biological fossil, overcome by many simple eukaryotes. A compact, comparatively small genome and an evolutionary optimization through countless generations could have allowed the development of closed mitosis, which enabled efficient separation of cytoplasmic and nuclear functions throughout the cell cycle. Interestingly, the highly conserved nucleoporin Mel28/ELYS displays a complicated primary structure in metazoan cells with, e.g., 2,275 amino acids in humans, whereas a minimal version of this nucleoporin with 298 amino acids in *S. pombe* is bearing only the motif required for incorporation into NPCs (i.e. ELY domain) but misses all additional sequence elements whose evolution correlates with open mitosis (Yokoyama et al. 2014).

7.8 Ran and Importins in Primary Cilia Formation

The molecular analysis of nucleocytoplasmic transport started with the characterization of nuclear protein import as an active, i.e. energy-dependent, saturable and signal (i.e. NLS)-mediated process (Goldfarb et al. 1986). Later, it turned out that nuclear protein import requires soluble transport receptors (Adam et al. 1990), which directly recognise the NLS (Görlich et al. 1994; Weis et al. 1995). A similar concept was recently suggested to work for the selective import of proteins, which are destined for primary cilia. The vast majority of human cells assemble primary cilia to function as cellular "antennae" in sensing environmental cues and to convert respective stimuli to the cytoplasm and the nucleus. The structure of primary cilia reflects their specific function. Cilia are generated around the unique microtubule array of the axoneme, which initially assembles and elongates from the centriole-based basal body. The ciliary (axonemal) MTs are surrounded by a specialised ciliary membrane. Both the membrane and the interior of the cilium comprise a unique set of proteins, which are targeted there by selective, signal-mediated transport processes. Consistently, primary cilia seal themselves from the cytoplasm by transitional elements that assemble from the basal body to reach the plasma membrane and to limit diffusion of large molecules into cilia. Signals recognised as "ciliary targeting signals (CLS)" often contain short stretches of basic amino acids and are in some cases remarkably similar to NLSs. They can even function in nuclear targeting if other important ciliary determinants in the primary structure are deleted (Dishinger et al. 2010). Indeed, targeting of ciliary motor protein Kif17 turned out to rely on importin β2/transportin and, apparently, RanGTP, which seems to accumulate inside the primary cilium (Dishinger et al. 2010). These observations open up the intriguing possibility that nuclear import and ciliary targeting may share similar principles and use an overlapping set of proteins for targeting (Gruss 2010). Consistent with that, the permeability barrier made up of transitional elements at the base of primary cilia was suggested to contain NPC proteins (Kee et al. 2012). However, experimental evidence is lacking for several key features of the model, such as proof for an active nucleotide exchange factor for Ran, which constantly produces RanGTP to maintain the necessary high RanGTP concentration inside the ciliary compartment. Moreover, although some NPCs were found accumulating at transitional elements, there is no indication for any regular structure similar to NPCs at the ciliary transition zone. It will thus be a very interesting topic in the future to understand parallels and specific differences between nuclear import and ciliary protein targeting and transport.

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Part III
Arf Subfamily

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

Catherine L. Jackson

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

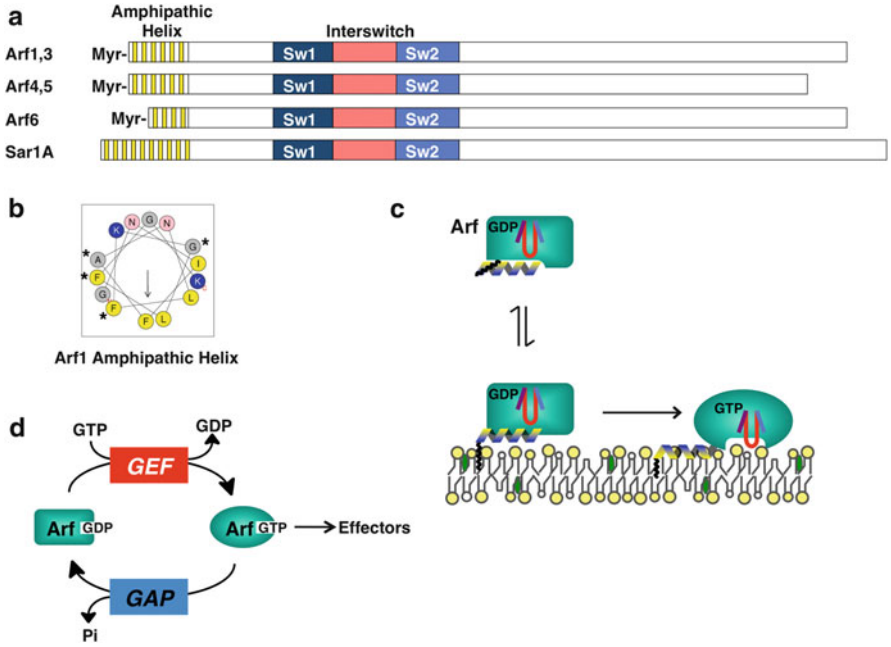

Fig. 8.1 The domain structure and regulation of Arf proteins. (**a**) A schematic representation of the three classes of Arf proteins in humans (Class I: Arf1,3; Class II: Arf 4,5; Class III: Arf6), and the Arf-related protein Sar1 for comparison, showing the conserved amino-terminal amphipathic helix, 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	–	–	–

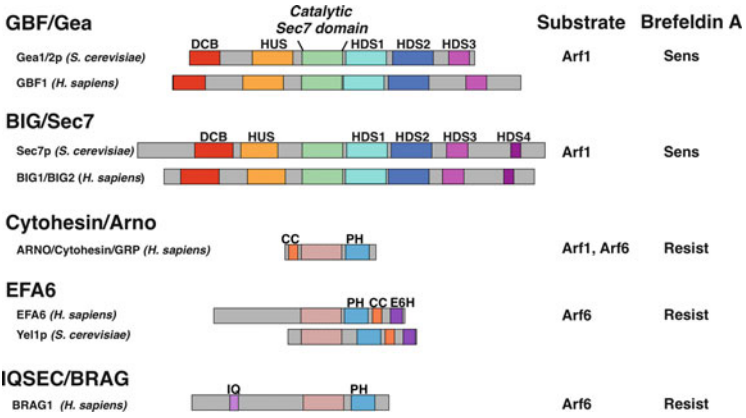

Fig. 8.2 The domain structure of the major cellular families of Arf GEFs. The domains of the five major families of Arf GEFs are shown, approximately to scale. Representative members shown are from humans and budding yeast. The well-characterized Arf substrates for each family are listed, and the sensitivity (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

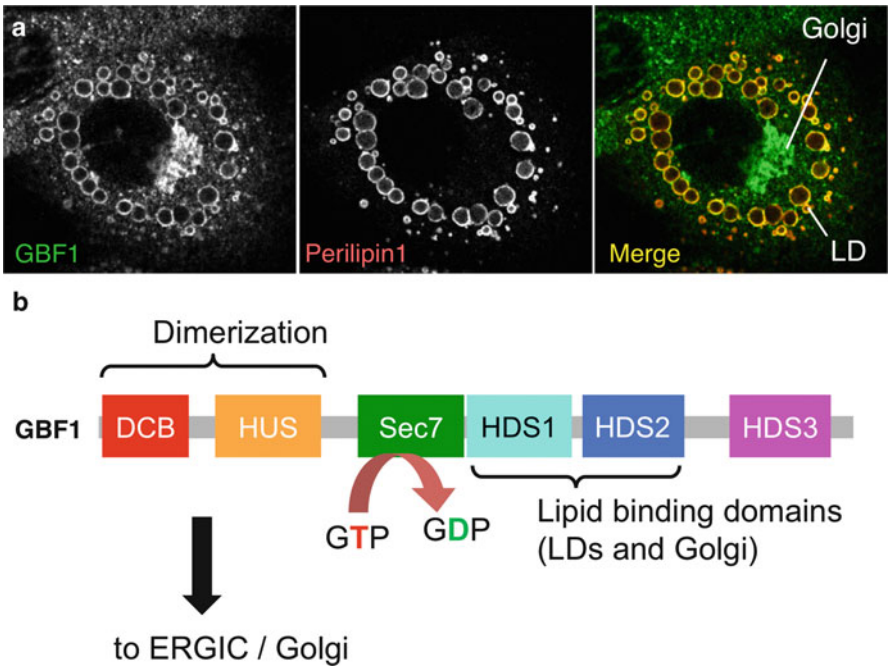

Fig. 8.3 Localization of GBF1 to both lipid droplets and the Golgi apparatus. (**a**) 3T3L1 adipocytes differentiated for 1 week were processed for immunofluorescence using GBF1 and perilipin 1 antibodies (image courtesy of K. Soni). LD, lipid droplet. (**b**) Mechanism of localization of GBF1. The domains 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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Chapter 9
Small G Proteins: Arf Family GTPases in Vesicular Transport

Frank Adolf and Felix T. Wieland

Abstract Small GTP-binding proteins of the ADP-ribosylation factor (Arf) family are key components of trafficking vesicles. In the past three decades a number of vesicular carriers, whose formation depends on members of the Arf family were identified, and general molecular mechanisms how these transport carriers form and operate were established. Here we describe discovery and roles of the Arf-dependent carriers of the early secretory pathway, the COPI and COPII vesicles. We will discuss their function with regard to molecular mechanisms in coat recruitment, selection of cargo proteins, vesicle membrane budding/scission, and vesicle uncoating.

Keywords Biosynthetic membrane transport • Coated transport vesicles • Membrane scission catalyzed by small GTPases

9.1 Introduction

A hallmark of eukaryotic cells is the presence of an elaborate endomembrane system. Transport of secretory and endocytic cargo between the different organelles to control and maintain protein and lipid homeostasis is a major task in eukaryotic cells. The discovery of a secretory pathway and the transport of secretory proteins along a series of different organelles, in pioneering electron microscopic studies in exocrine pancreatic cells (Caro and Palade 1964; Jamieson and Palade 1967), led to formulation of the vesicle transport hypothesis (Palade 1975). According to this hypothesis transport between the different organelles of the biosynthetic-secretory and endocytic pathways is achieved by means of directed and regulated vesicular transport. Today we know that the biogenesis of transport vesicles is driven by

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recruitment and polymerisation of cytosolic coat proteins. Coat proteins (with the help of auxiliary proteins) (1) help to incorporate the right set of cargo proteins, and at the same time (2) they physically curve the donor membrane into a vesicle bud, (3) help to pinch off the nascent transport vesicle from its donor membrane, and (4) finally must detach from the released vesicle membrane to allow the transport vesicle to fuse with its target membrane.

The small GTP-binding proteins of the Arf family play a core role in most types of transport vesicles. Although the small GTP-binding protein Arf was initially identified as a cofactor in the post-translational cholera toxin-mediated ADP-ribosylation of the $G_s\alpha$ subunit of heterotrimeric G proteins (Kahn and Gilman 1984), the major biological function of Arf proteins is in intracellular vesicular transport. Formation of several transport carriers in the secretory and endocytic pathways directly depends on small GTP-binding proteins of the Arf family. In the formation of such carriers the Arf proteins operate as molecular switches (Bourne et al. 1990, 1991), which cycle between a GDP-bound, 'inactive', mainly cytosolic and a GTP-bound, 'active', exclusively membrane-associated conformation. Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) regulate these switches. 'Activated' small GTP-binding proteins then recruit coat protein and help to mediate coat polymerisation, whereas GTP hydrolysis leads to their release from the vesicle membrane, prerequisite for shedding off the coat proteins to prepare the vesicle for docking to and fusion with its target membrane (see Jackson 2014).

Mammalian cells possess several Arf proteins (Tsuchiya et al. 1991). Based on sequence homology Arf proteins can be divided into three classes: class I (Arf1, Arf2, and Arf3), class II (Arf4 and Arf5), and class III (with Arf6 is the sole member. *Saccharomyces cerevisiae* has only three Arf proteins: Arf1p and Arf2p belong to class I, and Arf3p belongs to class III. Arfs of class I and II are localised to membranes of the early secretory pathway (e.g. the ERGIC and the Golgi apparatus), but can be also found on compartments of the late secretory/endocytic pathway (e.g. endosomes). In contrast Arf6 is primarily localised at the plasma membrane (see Jackson 2014).

Several coat proteins are well defined that characterise various types of coated vesicles. The best-characterised vesicular transport carriers are COPI and COPII vesicles, which serve trafficking routes in the early secretory pathway, as well as clathrin-coated vesicles (CCVs), which operate with various clathrin adaptor proteins in the late secretory pathway.

Arf1 is the central regulator in the formation of COPI vesicles (Serafini et al. 1991a). Most recently Arf1, Arf4, and Arf5 but not Arf3 and Arf6 were found on COPI-coated vesicles generated form Golgi-enriched membranes in vitro (Popoff et al. 2011b). In addition, Arf1 is involved in the formation of CCVs that operate with the tetrameric adaptor protein complexes AP1 (Stamnes and Rothman 1993; Traub et al. 1993), AP3 (Ooi et al. 1998), (Boehm et al. 2001) or the monomeric adaptor protein complexes GGA1–3 (Boman et al. 2000; Dell'Angelica et al. 2000; Puertollano et al. 2001), as well as in membrane recruitment of the clathrin-independent adaptor protein complex AP4 (Boehm et al. 2001). The small

GTP-binding protein Arf6 is indirectly involved in the recruitment of the tetrameric endocytic adaptor protein complex AP2 to the plasma membrane (PM). Arf6 activates phosphatidylinositol-4-phosphate-5-kinase (PIP5K), which leads to a local accumulation of phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P_2) and hence to the recruitment of endocytic adaptor proteins with PtdIns(4,5)P_2 binding domains (Krauss et al. 2003; Zheng and Bobich 2004). In addition, a direct interaction between Arf6 and AP2 was reported (Paleotti et al. 2005).

In contrast to Arf proteins with their various effector molecules, the sole function of the small GTP-binding protein Secretion-associated, Ras-related protein 1 (Sar1) is to control the formation of COPII-coated vesicles (Barlowe et al. 1993, 1994). Sar1 was initially identified in a screen for multicopy suppressors of the temperature-sensitive ER-to-Golgi transport mutant *SEC12* (Nakano and Muramatsu 1989). Mammalian cells possess two Sar1 isoforms, Sar1a and Sar1b, with ~90 % sequence identity (Kuge et al. 1994), whereas yeast has one isoform Sar1p. Interestingly, despite this remarkably high degree of conservation, individual mammalian Sar1 isoforms are associated with distinct human pathologies. The isoform Sar1b is required for packaging of chylomicrons into transport carriers and hence mutations in this isoform are linked to lipid absorption disorders (Jones et al. 2003). Sar1b is uniquely sensitive to mutations in Sec23A, which provoke cranio-lenticulo-sutural dysplasia (CLSD) (Boyadjiev et al. 2006; Fromme et al. 2007).

In this review we will discuss discovery, molecular composition, and roles of the Arf family GTP-binding protein-dependent vesicular carriers of the early secretory pathway, with a focus on the molecular mechanisms in coat recruitment, incorporation of cargo proteins, vesicle budding/scission, and vesicle uncoating. We will describe the COPI and II systems separately and highlight similarities as well as differences in molecular mechanisms in their formation.

9.2 COPI Vesicles and Their Machinery

COPI-coated vesicles were initially identified by electron microscopy of Golgi-enriched membranes after incubation with cytosol and ATP (Orci et al. 1986). Up to this point it was believed that CCVs are mediators of all intracellular vesicular transport pathways. Based on the presence of vesicular stomatitis virus G glycoprotein (VSV-G) in buds and vesicles, it was proposed that a class of new vesicles mediate vesicular transport within the Golgi stack, which were initially termed ‘Golgi-derived-coated vesicles’ (GCV) or ‘non-clathrin-coated vesicles’ (Orci et al. 1986). Incubations of enriched Golgi membranes with cytosol, ATP, and the poorly hydrolyzable GTP analogue GTPγS revealed an irreversible block in intra-Golgi transport, at a stage between vesicle tethering and fusion, pointing to an involvement of a GTP-binding protein in transport through the Golgi complex. Consistently, ‘non-clathrin-coated vesicles’ and buds were accumulated fivefold (Melancon et al. 1987). Large-scale purification of ‘non-clathrin-coated vesicles’ by differential and sucrose gradient centrifugation (Malhotra et al. 1989) led to the

identification of the four large coat proteins (COPs) α-COP, β-COP, γ-COP, and δ-COP (Duden et al. 1991; Serafini et al. 1991b) and the small GTP-binding protein ADP-ribosylation factor 1 (Arf1) (Serafini et al. 1991a) as stoichiometric components of these coated vesicles. Subsequently a cytosolic complex referred to as 'coatomer' (for Golgi coat promoter) consisting of the four large COPs and several smaller subunits was isolated (Waters et al. 1991), which eventually led to the identification of three other coatomer subunits: β′-COP (Harrison-Lavoie et al. 1993; Stenbeck et al. 1993), ε-COP (Hara-Kuge et al. 1994), and ζ-COP (Kuge et al. 1993). Finally, 'non-clathrin-coated vesicles' (COP-coated vesicles) were renamed to COPI vesicles after the identification of a new class of coated vesicles formed by *SEC* proteins, which were designated COPII vesicles (Barlowe et al. 1994).

In a living cell coatomer is mainly localised at membranes of the Golgi apparatus (Duden et al. 1991; Griffiths et al. 1995; Serafini et al. 1991b), the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) (Griffiths et al. 1995), coated vesicles surrounding the Golgi apparatus (Duden et al. 1991; Griffiths et al. 1995; Serafini et al. 1991b), and the endoplasmic reticulum (ER) (Orci et al. 1994), highlighting a function of COPI vesicles in the early secretory pathway at the ER–Golgi interface.

Even though COPI-coated vesicles are known for more than 25 years, it is still contentious in which trafficking steps these vesicles are directly involved. Undisputed is the role of COPI vesicles in the retrograde transport of escaped ER proteins or cycling membrane proteins from the ERGIC and *cis*-Golgi membranes back to the ER (Cosson and Letourneur 1994; Letourneur et al. 1994). In addition, COPI vesicles are implicated in anterograde IC-to-Golgi transport (Aridor et al. 1995; Scales et al. 1997; Shima et al. 1999), as well as in retrograde (Lanoix et al. 1999, 2001; Love et al. 1998) and anterograde intra-Golgi transport (Orci et al. 1997; Pellett et al. 2013). A more comprehensive review on additional functions of COPI-coated vesicles or membrane-bound coatomer, e.g. in the breakdown of the Golgi apparatus during mitosis, in positioning of the Golgi apparatus, in lipid droplet homeostasis, or in the lysosomal/endosomal pathway, can be found elsewhere (Popoff et al. 2011a).

9.2.1 The Heptameric Coat Complex Coatomer

The COPI coat complex coatomer is a 550-kDa heteromeric complex composed of the seven subunits α-COP (140 kDa), β-COP (107 kDa), β′-COP (102 kDa), γ-COP (97 kDa), δ-COP (57 kDa), ε-COP (35 kDa), and ζ-COP (24/20 kDa). The heptameric coat complex can be dissociated by high salt treatment in vitro into two functionally distinct subcomplexes, a tetramer composed of β/γ/δ/ζ-COP and a trimer composed of α/β′/ε-COP (Lowe and Kreis 1995; Pavel et al. 1998). More recently the heptamer as well as both subcomplexes were also expressed and purified utilising a mammalian expression system (Sahlmuller et al. 2011). The

subunits of the tetramer show weak but significant sequence homology to subunits of the clathrin adaptor protein complexes (APs). The two large subunits β-COP and γ-COP are built of an N-terminal trunk domain and a C-terminal appendage domain connected by an unstructured linker region, and share homology with the large subunits of clathrin adaptor protein complexes (e.g., α-adaptin and β-adaptin) (Duden et al. 1991; Serafini et al. 1991b). Likewise δ-COP and ζ-COP share homology with the medium (μ-adaptins) and the small (σ-adaptins) subunits, respectively (Cosson et al. 1996; Faulstich et al. 1996). Identification of a common ancestor of subunits of the terameric subcomplex and APs reaffirms this view (Schledzewski et al. 1999).

A related domain organisation between the subunits of the β/γ/δ/ζ-COP subcomplex and APs was revealed by yeast two-hybrid analysis (Eugster et al. 2004; Faulstich et al. 1996). X-ray crystallisation analysis of the γ-COP appendage domain showed a striking structural similarity with the corresponding domains of α-adaptin and β2-adaptin, the large subunits of the AP2 complex (Hoffman et al. 2003; Watson et al. 2004). In addition, ζ-COP shows striking resemblance to σ2-adaptin, the small subunit of AP2 (Yu et al. 2009). Taken together, the overall structure and the mode of membrane binding of the β/γ/δ/ζ-COP subcomplex and APs are related. Similar interactions between Arf1 and subunits of coatomer and APs revealed by site-specific cross-linking (Austin et al. 2000, 2002; Sun et al. 2007; Zhao et al. 1997, 1999), and by a most recently solved X-ray crystal structure of Arf1 bound to the γ/ζ-COP subcomplex of coatomer, together with biochemical characterisation of the Arf1 binding site in the β/δ-COP subcomplex (Yu et al. 2012), corroborate this view.

Two isoforms, each, were identified for the γ-COP (γ1-COP and γ2-COP) and ζ-COP (ζ1-COP and ζ2-COP) subunit, which allow the assembly of four heptameric coatomer isoforms (γ1ζ1, γ1ζ2, γ2ζ1, and γ2ζ2). γ1-COP and γ2-COP are ~80 % identical, whereas ζ1-COP and ζ2-COP share ~75 % identity, and differ largely by a 30 amino acids extension at the N-terminus of ζ2-COP (Futatsumori et al. 2000; Wegmann et al. 2004). Quantitative analysis revealed different amounts of coatomer isoforms in mammalian cells. Furthermore, significant differences in the localisation of coatomer isoforms were observed. Whereas γ1ζ1 and γ1ζ2 were preferentially localised at the ERGIC and the *cis*-Golgi, the majority of γ2ζ1 was localised to the *trans*-Golgi. Taken together, existence and differential localisation within a cell point to different roles of coatomer isoforms, similar to the various roles of distinct APs in the secretory and endocytic pathways (Moelleken et al. 2007; Wegmann et al. 2004). This view is further supported by identification of different subpopulations of COPI vesicles containing distinct sets of cargo proteins in living cells (Orci et al. 1997) and in vitro (Lanoix et al. 2001; Malsam et al. 2005).

9.2.2 COPII Vesicles and Their Machinery

The most gene products required for the formation of COPII-coated vesicles were initially identified by a screen in *S. cerevisiae* for temperature-sensitive secretion mutants (*SEC*) with a defect in protein secretion and cell growth (Novick et al. 1980). Subsequent studies revealed striking similarity of the secretory pathways in budding yeast and mammalian cell (Novick et al. 1981). Morphological analysis classified a set of mutants causing accumulation of excess ER membranes (class I mutants) (*SEC12*, *SEC13*, *SEC16*, and *SEC23*), and another class that led to the accumulation of ER-derived vesicles (class II mutants) (*SEC17*, *SEC18*, and *SEC22*) (Kaiser and Schekman 1990).

Combination of mutant cell lysates deficient in ER-to-Golgi transport with in vitro ER-to-Golgi transport assays allowed characterisation of proteins involved. Hereby, a new class of vesicular carriers was identified as ER-to-Golgi transport intermediates (Baker et al. 1988; Groesch et al. 1990; Rexach and Schekman 1991; Ruohola et al. 1988). Biochemical characterisation revealed that Sec23p (Hicke and Schekman 1989) together with Sec24p (Hicke et al. 1992) operates in a heterodimeric complex required for the formation of ER-derived transport vesicles (Hicke et al. 1992). Subsequently, Sec13p was found to operate in a heterooligomeric complex with Sec31p (Pryer et al. 1993; Salama et al. 1993). Eventually, it became clear that the five cytosolic proteins Sar1p, the Sec23/24p, and the Sec13/31p subcomplexes are required and sufficient for the formation of ER-derived vesicles in vitro (Barlowe et al. 1994; Salama et al. 1993).

The presence of proteins involved in vesicle fusion (Sec22p and Bet1p) and the absence of ER-resident proteins (Kar2p/BiP) in vesicles generated in vitro (Rexach et al. 1994; Salama et al. 1993), combined with the competence of these vesicles to fuse with Golgi-membranes (Barlowe et al. 1994; Rexach et al. 1994) established that this minimal set of proteins is capable to form functional ER-to-Golgi transport intermediates. Electron microscopy revealed 60–65 nm vesicles with an electron-dense coat, morphologically distinct from Clathrin-coated and Golgi-derived 'non-clathrin-coated' vesicles. Consequently, the latter were renamed to COPI vesicles and the vesicles formed by the five cytosolic proteins Sar1p, Se23/24p, and Sec13/31p were termed COPII vesicles (Barlowe et al. 1994).

9.2.3 The COPII Coat Subcomplexes Sec23/24 and Sec13/31

The Sec23/24 complex is a heterodimeric complex. Single-particle EM analysis revealed a bow-tie-shaped complex, of 17 nm in length, comprising two globular domains of similar type (Lederkremer et al. 2001). X-ray crystallographic analysis disclosed a positively charged concave membrane-proximal surface of the Sec23/24 complex, which seems to match the curvature of a COPII vesicle with a typical diameter of 60 nm. The subunits Sec23 and Sec24 have striking similar folds with

five distinguishable domains: (1) a β-barrel domain, (2) a zinc finger domain, (3) a trunk domain, (4) an all-helical region, and (5) a carboxy-terminal gelsolin-like domain. The trunk domains of Sec23 and Sec24 form the interface of the heterodimer (Bi et al. 2002).

The Sec23/24 complex is recruited to ER membranes by direct interaction of Sar1 with the Sec23 subunit (Bi et al. 2002), which is the Sar1-specific GTPase activating protein (Bi et al. 2002; Yoshihisa et al. 1993). Mammals possess two isoforms Sec23A and Sec23B, with ~85 % sequence identity. Interestingly, although these proteins are highly related, mutations in individual Sec23 isoforms are linked to various human genetic diseases. Mutations in Sec23A result in cranio-lenticulo-sutural dysplasia (CLSD) (Boyadjiev et al. 2006), while congenital dyserythropoietic anaemia type II (CDAII) is caused by mutations in the Sec23B isoform (Bianchi et al. 2009; Schwarz et al. 2009).

On the other hand, the Sec24 subunit is responsible for binding and thus concentration of cargo proteins into nascent COPII vesicles (Miller et al. 2002, 2003; Mossessova et al. 2003). In *S. cerevisiae* two non-essential Sec24 homologues: Iss1p/Sfb2p (Kurihara et al. 2000) and Lst1p/Sfb3p (Roberg et al. 1999) exist, which are ~55 % and ~23 % identical with Sec24p, respectively. Database search and cloning of the corresponding mammalian cDNAs revealed the existence of four homologues Sec24A, Sec24B, Sec24C, and Sec24D. The mammalian homologues can be grouped into two classes: Sec24A/B and Sec24C/D. Within a class, the isoforms are ~50 % identical, whereas Sec24A/B and Sec24C/D share ~20 % identity (Pagano et al. 1999; Tang et al. 1999).

The Sec13/31 subcomplex is a heterotetramer, consisting of two Sec13 and two Sec31 subunits. Single-particle electron microscopy analysis revealed a structure with an elongated shape of 28–30 nm length, comprising five distinguishable globular domains connected by flexible joints (Lederkremer et al. 2001). The molecular architecture of the complex was solved by X-ray crystallography. In the complex the C-terminal α-solenoid structures of two Sec31 subunits dimerise tail to tail to form a rod-like structure, whereas the N-terminus of Sec31 forms a seven-bladed β-propeller. One Sec13 subunit each is wedged between the β-propeller and α-solenoid domain of one Sec31 subunit and forms a second 7-bladed β-propeller structure, in which one blade is contributed by the Sec31 subunit (Fath et al. 2007). The Sec13/31 subcomplex together with the Sec23/24 subcomplex (Antonny et al. 2003; Stagg et al. 2008), as well as the Sec13/31 subcomplex alone (Stagg et al. 2006) can self-assemble into COPII cage-like particles similar to 'empty cages' of clathrin triskelia. Cryo-electron microscopic analysis of self-assembled cages revealed that the COPII coat can adopt various geometries (Stagg et al. 2006, 2008). Fitting of the Sec13/31 crystal structures into an electron microscopy density map revealed that the N-terminal β-propeller domains of Sec31 subunits form the contact points of the vertices. A hinge in the central part formed by interlocked α-solenoid structures might allow the coat to adapt to various sizes (Fath et al. 2007).

Taken together the structural features and their location in cage structures explain how the coat confers stability and at the same time functional flexibility

to accommodate cargo of various sizes. Like for the inner layer Sec23/24, isoforms exist of the outer layer protein Sec31 (Shugrue et al. 1999; Stankewich et al. 2006; Tang et al. 2000).

9.3 Molecular Mechanism of COPI Vesicle Biogenesis

9.3.1 Coat Recruitment

The initial step in the formation of COPI vesicles is recruitment and activation of the small GTPase Arf1 from the cytosol to the correct donor membrane. In vitro cross-linking experiments revealed a specific interaction of a carboxy-terminal site of Arf1-GDP with dimeric cytoplasmic tails of the type-I transmembrane proteins p23 and p24 (Gommel et al. 2001), suggesting a role of p24 proteins as an Arf-GDP membrane receptor. This interaction was subsequently confirmed by FRET measurements in living cells (Majoul et al. 2001) and pull-down experiments (Contreras et al. 2004).

In addition, recruitment of Arf1 to the *cis*-Golgi apparatus was reported to involve the Golgi SNARE membrin/GS27. Mutation of an MxxE motif within Arf1 impairs recruitment of the small GTPase to the *cis*-Golgi, whereas recruitment to the *trans*-Golgi apparatus is unaffected (Honda et al. 2005). Thus additional signals are likely to exist to ensure specific Arf-GDP recruitment to the trans side of the Golgi apparatus.

Several lines of evidence indicate that GBF1 in mammals and its homologues Gea1p and Gea2p in yeast are the GEFs involved in Arf activation. GBF1 is primarily localised at the ERGIC and the *cis*-Golgi apparatus, where it shows a substantial colocalisation with the COPI coat (Claude et al. 1999; Garcia-Mata and Sztul 2003; Zhao et al. 2002). Overexpression of the catalytically inactive variant GBF1 E794K (Garcia-Mata et al. 2003), injection of antibodies directed against GBF1 (Zhao et al. 2006), or siRNA knock-down of GBF1 (Manolea et al. 2008; Szul et al. 2007) causes dissociation of COPI from ERGIC/Golgi membranes. The exact mechanism of recruitment of GBF1 to membranes of the early secretory pathway is not completely understood, but seems to involve the small GTPase Rab1b. Overexpression of a dominant negative mutant of Rab1 causes COPI dissociation from membranes, an effect which can be reversed by overexpression of Arf1 or GBF1 (Alvarez et al. 2003). Furthermore knock-down experiments of Rab1b suggest that the small GTPase is required for GBF1 membrane association, and a direct interaction was mapped to a N-terminal fragment (aa 1-380) of the GEF (Monetta et al. 2007). PtdIns(4)P was also reported to be required for recruitment of GBF1, and it was proposed that local concentration of PtdIns(4)P might be regulated by Rab1-dependent activation of phosphatidylinositol 4-kinase (PtdIns(4) KIIIalpha) (Dumaresq-Doiron et al. 2010).

On the membrane, the GEF interacts with Arf via a highly conserved Sec7 domain (Cherfils et al. 1998; Mossessova et al. 1998). Insertion of a conserved glutamate residue (also referred to as 'glutamic finger') in this domain (exposed at the tip of a hydrophilic loop between helices 6 and 7) into the nucleotide binding pocket of Arf triggers displacement of GDP by electrostatic competition with the β-phosphate of the nucleotide (Beraud-Dufour et al. 1998; Renault et al. 2003). Nucleotide exchange causes a conformational change of the switch I and switch II domains of Arf, affecting the relative position of the interswitch domain. In its GDP-bound form the myristoylated, amphipathic N-terminal helix of Arf is buried in a hydrophobic pocket. GTP binding causes a 7–8 Å shift of the interswitch region, which concomitantly displaces the N-terminal helix from its pocket (Amor et al. 1994; Goldberg 1998). As a result, the myristoylated, amphipathic N-terminal helix is shallowly inserted into the lipid bilayer and anchors the GTPase to the membrane (Antonny et al. 1997; Franco et al. 1995, 1996). In the prevailing view the amphipathic N-terminal helix of Arf is oriented parallel to the lipid bilayer when bound to membranes (Antonny et al. 1997; Davies et al. 2003), whereas the myristoyl-anchor is inserted perpendicular into the lipid bilayer (Harroun et al. 2005). In a more recent NMR study it was reported that the myristoyl-anchor is oriented horizontal to the membrane and partially folds back onto the N-terminal helix of Arf1 (Liu et al. 2010). Due to the use of highly curved bicelles to mimic natural membranes an altered orientation of membrane-bound Arf cannot be excluded.

In contrast to the stepwise mode of recruitment of two coat layers in CCVs and COPII vesicles, activated, membrane-bound Arf recruits the heptameric coat complex coatomer en bloc, (Donaldson et al. 1992; Hara-Kuge et al. 1994; Palmer et al. 1993). Interestingly, GBF1 seems to interact also directly with γ-COP, providing a molecular explanation how specificity of COPI coat recruitment by GBF is achieved (Deng et al. 2009).

Several interactions between activated Arf1 and coatomer have been described. Site-directed photo-cross-linking experiments revealed a specific interaction of Arf1 with the trunk domains of γ-COP and β-COP, as well as with β′-COP and the longin domain of δ-COP (Sun et al. 2007; Zhao et al. 1997, 1999).

More recently the crystal structure of Arf1 bound to a γ/ζ-COP subcomplex was solved. Structure-guided biochemical analysis of Arf1 binding to a β/δ-COP subcomplex revealed common Arf1 binding sites in the γ-COP and β-COP subunit. Furthermore the γ-COP and β-COP binding sites are related to the PtdIns(4,5)P_2 binding site in AP2, indicating a similar mechanism of membrane recruitment for coatomer and APs (Yu et al. 2012). This view is further corroborated by a crystal structure of Arf1 in complex with AP1 (Ren et al. 2013). Consistent with multiple Arf-coatomer interfaces, the ratio of the small GTPase to coatomer was determined to 3–4 Arf per coatomer (Beck et al. 2009; Serafini et al. 1991a). Binding of coatomer to the cytoplasmically exposed tail of p24 proteins is believed to further stabilise the coat on membranes. Binding of dimeric, cytoplasmic tails of p23 and p24 induces a conformational change within the γ-COP subunit, which is

transmitted to the α-COP subunit and leads to coatomer polymerisation. Based on these results a mechanism was forwarded by which an induced conformational change of coatomer results in coat polymerisation and thus likely provides the energy to bend the donor membrane (Langer et al. 2008; Reinhard et al. 1999).

9.3.2 Sorting of Cargo and Cycling Membrane Proteins

Incorporation of cargo proteins and cycling membrane proteins (e.g. cargo receptors or SNAREs) above their bulk concentration of the donor membrane is achieved by direct or indirect signal-mediated interaction with vesicle coat proteins. The first signals that had been identified to be involved in the retention/retrieval of membrane proteins in/to the ER were dilysine motifs with the consensus sequence KKxx or KxKxx (Jackson et al. 1990; Nilsson et al. 1989). Membrane proteins bearing such motifs at their extreme C-terminus (with lysine at position -5 and -3, or -4 and -3) are retrieved from post-ER compartments (Gaynor et al. 1994; Jackson et al. 1993). Coatomer interacts directly with dilysine-containing proteins and is essential for their retrieval (Cosson and Letourneur 1994; Letourneur et al. 1994). Coatomers with α-COP (RET1-1) and β′-COP (SEC27-1) mutations but not γ-COP (SEC21) mutants have lost their ability to bind dilysine-containing proteins (Letourneur et al. 1994). Accordingly, dilysine-containing proteins were reported to interact with the α/β′/ε-COP subcomplex (Lowe and Kreis 1995), implying that α-COP and β′-COP recognise these motifs. Emp47p, which contains a KxKxx motif (KTKLL), is unaffected by the α-COP (RET1-1) mutant, whereas KKxx containing proteins are mislocalised in these mutants, indicating that α-COP discriminates between the different dilysine motifs (Schroder et al. 1995; Schroder-Kohne et al. 1998). Functional and yeast two-hybrid analysis identified the N-terminal β-propeller (comprising WD40 repeats) of α-COP and β′-COP to be responsible for binding to dilysine motifs, with a preference of α-COP for KKxx and β′-COP for KxKxx motifs. Deletion of the β-propeller domains from either α-COP or β′-COP is tolerated, whereas deletion of both β-propeller domains is lethal in yeast indicating at least partially redundant functions (Eugster et al. 2000, 2004). The preference of β′-COP for KxKxx motifs was confirmed by isothermal titration calorimetric (ITC) measurements, and an X-ray crystal structure of the β-propeller domain of β′-COP bound to a KxKxx cargo peptide, which also offered an explanation for the requirement of the two-residue spacing between the C-terminus and first lysine residue (Jackson et al. 2012). Another study reported that α-COP and β′-COP bind with equal affinity to KKxx and KxKxx motifs, although the crystal structures of the β-propellers from α-COP and β′-COP with bound peptides revealed distinct binding modes of these motifs (Ma and Goldberg 2013).

In contrast to dilysine-containing cargo proteins, members of the p24 family bind via their conserved FFxxBB(x)$_n$ motifs (with B either K or R, and $n \geq 2$) to two independent sites in the trunk and appendage domains of γ-COP, exclusively in their dimeric form (Bethune et al. 2006; Harter and Wieland 1998). The different

modes of binding of dilysine-containing proteins and p24 proteins are further highlighted by the fact that binding of p24 proteins to coatomer is dependent on the diphenylalanine motif and is modulated by the di-basic motif (Dominguez et al. 1998; Fiedler et al. 1996; Sohn et al. 1996). Cycling of the p24 proteins between Golgi and ER (Nickel et al. 1997) and their binding sites on coatomer different from cargo binding sites, as well as induction of polymerisation of coatomer upon binding of p23 or p24 dimers (Langer et al. 2008; Reinhard et al. 1999), qualifies these type-I transmembrane proteins as membrane machinery of COPI vesicles. This view is further supported by studies in yeast (Aguilera-Romero et al. 2008; Stamnes et al. 1995).

Arginine-based motifs are another class of ER-retrieval signals with the consensus sequence Φ/Ψ/RRxR. These sorting signals are involved in the quality control of multimeric membrane proteins by maintaining unassembled subunits in the ER, and become inactivated upon correct assembly to allow export from the ER (Margeta-Mitrovic et al. 2000; Zerangue et al. 1999). Binding of coatomer to different arginine-based sorting signals implies that ER localisation can be achieved due to COPI-dependent retrieval (Brock et al. 2005; O'Kelly et al. 2002; Yuan et al. 2003). More recently β-COP and δ-COP were identified as coatomer subunits involved in the recognition of arginine-based motifs (Michelsen et al. 2007).

Additional less well-characterised sorting signals include the δL-motif, which was identified to bind to δ-COP (Cosson et al. 1998), and a motif FxxxFxxxFxxLL found in Dopamine 1 receptor, which interacts with δ-COP (Bermak et al. 2002). More recent studies imply that correct localisation of Golgi glycosyl-transferases (lacking known COPI binding motifs) is achieved by binding to cytosolic Vps74p in yeast and its mammalian homologue GOLPH3, which links Golgi enzymes via a conserved arginine motif to coatomer (Schmitz et al. 2008; Tu et al. 2008, 2012).

Soluble ER-resident proteins contain the C-terminal motif KDEL (HDEL in yeast), which prevents their secretion (Munro and Pelham 1987) by an ER-retrieval mechanism from post-ER compartments (Dean and Pelham 1990; Pelham 1988). The integral membrane protein Erd2p was initially identified as receptor mediating retrieval of proteins with the motif HDEL (Semenza et al. 1990). Vertebrates have three homologues of Erd2p (Lewis and Pelham 1990, 1992b; Raykhel et al. 2007). At steady state the KDEL receptors (KDELr) are localised to the ERGIC and *cis*-Golgi (Griffiths et al. 1994). Binding to KDEL signal-containing protein induces its redistribution to the ER (Lewis and Pelham 1992a). A higher pH in the ER is believed to dissociate the cargo and to allow recycling of the free KDELr (Wilson et al. 1993). Colocalisation of KDELr with coatomer and its presence in purified COPI vesicles (Griffiths et al. 1994; Sonnichsen et al. 1996), a requirement for Arf and coatomer in an in vitro retrieval assay (Spang and Schekman 1998), and inhibition of ER targeting of endocytosed cholera toxin by antibodies against β-COP and p23 (Majoul et al. 1998) together imply a role of COPI in the retrograde transport of the KDELr.

Efficient incorporation of vectorially transported proteins into COPI vesicles was proposed to require GTP hydrolysis (Lanoix et al. 1999; Malsam et al. 1999;

Nickel et al. 1998; Pepperkok et al. 2000), whereas uptake of the cycling membrane proteins such as p23, p24, or KDELr is independent of GTP hydrolysis (Malsam et al. 1999; Nickel et al. 1998). The mechanism underlying this process is currently not known [reviewed in Antonny (2011), Popoff et al. (2011a)].

9.3.3 Scission of COPI Vesicles

Arf1 and coatomer are the cytosolic components essential for in vitro formation of COPI vesicles from Golgi-enriched membranes (Orci et al. 1993), chemically defined large unilamellar vesicles (LUVs) (Bremser et al. 1999; Spang et al. 1998), or giant unilamellar vesicles (GUVs) (Faini et al. 2012). Consistent with a role of p24 proteins as membrane machinery, COPI vesicle formation from LUVs is stimulated by the cytoplasmic peptide of p23 linked to lipid (Bremser et al. 1999). Taken together, the ability to deform the membrane into a COPI bud and to promote COPI vesicle scission depends exclusively on these two cytosolic proteins.

Activated Arf1 alone is capable to induce liposome deformation by shallow insertion of its N-terminal amphipatic helix (Beck et al. 2008, 2011; Krauss et al. 2008; Lundmark et al. 2008), i.e. the small GTPase exerts *membrane curvature potentiating activity* (Faini et al. 2013). Further scrutiny indicated that Arf1, but not a point mutant Arf1 Y35A, forms GTP-dependent dimers, when recruited to Golgi-enriched membranes. Unlike Arf1 wild type, the variant Arf1 Y35A does not exhibit membrane curvature potentiating activity and is unable to promote COPI vesicle formation in vitro (Beck et al. 2008). On the other hand, this variant is fully functional in the recruitment of coatomer, resulting in coated liposomes curved into buds, arrested in a state before membrane scission. Thus, the ability to form a COPI-coated bud depends on the recruitment and polymerisation of coatomer and is independent on the membrane curvature potentiating activity of the small GTPase. On the other hand this membrane curvature potentiating activity is prerequisite for COPI vesicle scission (Beck et al. 2011).

COPI vesicles were originally identified under conditions where GTP hydrolysis was blocked (Barlowe et al. 1994; Malhotra et al. 1989). Subsequently, in a variety of studies GTP hydrolysis was either described to be necessary or not needed for the formation of these vesicles. This controversy has most recently been addressed in an in vitro reconstitution assay based on semi-intact cell. In this study formation of COPI vesicles was confirmed to be independent of GTP hydrolysis (Adolf et al. 2013).

9.3.4 Uncoating of COPI Vesicles

Uncoating of a vesicle is a prerequisite for membrane fusion. In the prevailing model GTP hydrolysis by Arf, triggered by ArfGAPs, results in the release of Arf and coatomer from the vesicle membrane (Reinhard et al. 2003; Tanigawa et al. 1993). ArfGAP1 and its yeast homologue Gcs1p, as well as ArfGAP2/3 and their yeast homologue Glo3p, were proposed to participate in COPI vesicle transport with partially redundant functions. Yeast is viable after deletion of either Gsc1p or Glo3p; however, efficient retrograde Golgi-to-ER transport requires both proteins (Poon et al. 1999). In mammalian cells only a triple knock-down of ArfGAP1–3 had a phenotypic effect, an increased level of membrane-bound Arf1, and accumulation of ERGIC53, GM130, and coatomer in the ER-Golgi intermediate compartment. As a consequence retrograde Golgi-to-ER transport was blocked, similar to experiments where β-COP was knocked down (Saitoh et al. 2009).

Surprisingly, the non-catalytical domain controls the recruitment of ArfGAP1 and ArfGAP2/3 by different mechanisms. ArfGAP1 is recruited to the membrane in a curvature-sensitive manner (Bigay et al. 2003), based on the presence of an ArfGAP1 lipid packing sensor (ALPS) motif (Bigay et al. 2005). ArfGAP2/3 lack this ALPS motif and consistently do not display curvature sensitivity. In contrast, ArfGAP2/3 recruitment seems to be strictly dependent on a direct interaction with coatomer (Frigerio et al. 2007; Kliouchnikov et al. 2009; Pevzner et al. 2012; Weimer et al. 2008).

The exact functions of the different ArfGAP proteins are still elusive. Based on the data described above it was proposed that the coatomer-independent ArfGAP1 might be involved in GTP hydrolysis-dependent uptake of cargo proteins into COPI vesicles, whereas the coatomer-dependent ArfGAP2/3 might be the GAPs involved in COPI vesicle uncoating (Popoff et al. 2011a). Likewise, based on their properties a role of ArfGAP1 in vesicle uncoating, and of ArfGAP2/3 in cargo sorting, was proposed (Antonny 2011).

The tethering complex Dsl1, comprising Dsl1p, Dsl3p, and Tip20, is required for Golgi-to-ER transport in yeast (Andag et al. 2001). Dsl1p directly interacts with α-COP and δ-COP (Andag et al. 2001; Reilly et al. 2001). The finding that Dsl1p utilises binding sites identical to those involved in interactions between coatomer subunits, and the observation that cells defective in the Dsl1 complex accumulate COPI-coated vesicles, led to the suggestion that this tethering complex plays an additional role in vesicle uncoating (Zink et al. 2009).

Individual steps in the formation and uncoating of COPI-coated vesicles are depicted in Fig. 9.1.

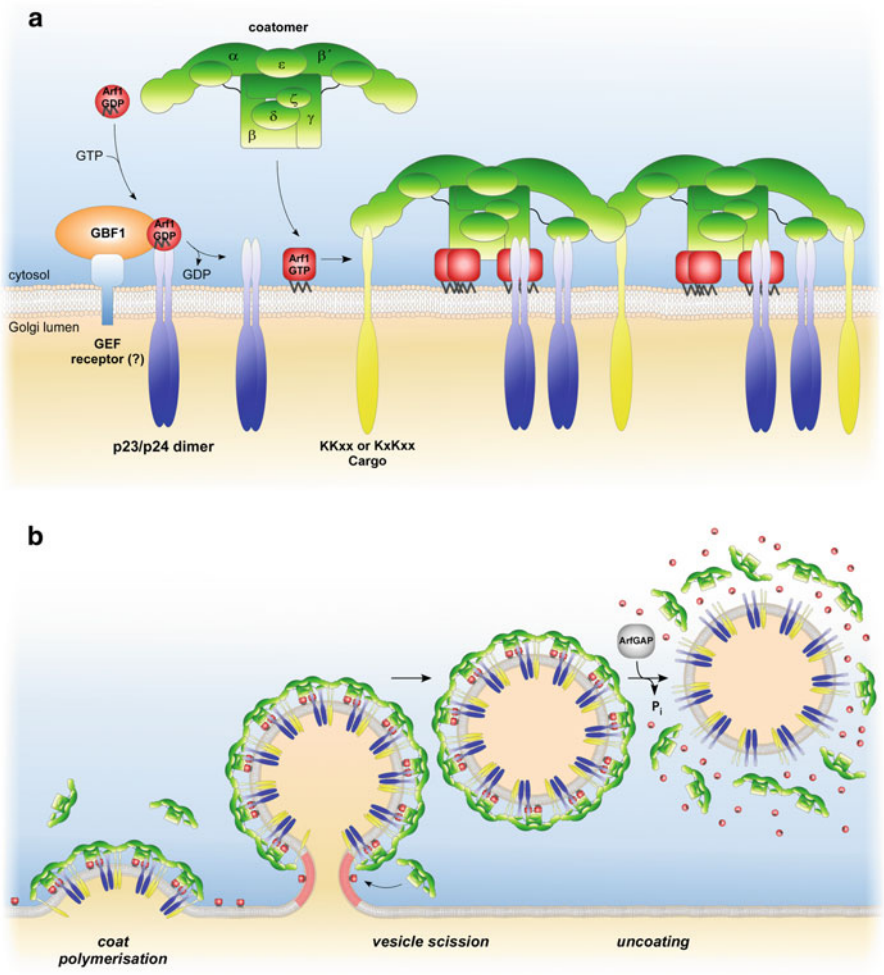

Fig. 9.1 Individual steps in the life cycle of COPI vesicles. Arf is recruited to donor membranes by interaction with dimeric cytoplasmic tails of membrane machinery proteins (e.g. p23 or p24). Subsequently, Arf is anchored to the membrane by activation via the GEF GBF1. On membranes, Arf1-GTP can form a homodimer. Efficient cargo uptake (but not uptake of machinery proteins) requires GTP hydrolysis by an unknown mechanism. Membrane-bound Arf recruits en bloc the coat complex coatomer. Dimeric p24 proteins bind to sites within the trunk and appendage domains of γ-COP. Cargo proteins bind to distinct binding sites in α-COP and β′-COPs. Binding of dimeric p24 proteins to the trunk domain of γ-COP induces a conformational change of coatomer, which is transmitted to α-COP and triggers coat polymerisation. Vesicle scission requires membrane curvature potentiating activity of the (oligomerised) small GTPase Arf1. ArfGAP-stimulated GTP hydrolysis leads to release of the small GTPase and subsequent release of the coat

9.4 Molecular Mechanism of COPII Vesicle Formation

9.4.1 COPII Coat Recruitment

As in the formation of COPI vesicles, the initial step in the biogenesis of COPII vesicle is recruitment and activation of a small GTPase, here Sar1. Unlike in the COPI system, this is accomplished by a type II integral membrane glycoprotein, the Sar1 GEF Sec12 (Barlowe and Schekman 1993; d'Enfert et al. 1991; Nakano et al. 1988). Localisation of Sec12 to the ER is achieved by two independent mechanisms and thereby restricts Sar1 activation and concomitant recruitment of the COPII coat proteins to this organelle. The N-terminal cytoplasmic domain of Sec12 is involved in static retention in the ER. In addition, Sec12, when escaped from the ER, is actively retrieved via a Rer1-dependent mechanism (Boehm et al. 1994; Nishikawa and Nakano 1993; Sato et al. 1995, 1996). The integral membrane protein Rer1 recognises signals in the transmembrane domain of Sec12 (and other proteins) and links the GEF to the COPI coat for retrograde transport via a dilysine motif (Boehm et al. 1997; Sato et al. 2001, 2003).

Nucleotide exchange causes a conformational change within Sar1 similar to Arf1, which in turn causes the exposure of its N-terminal amphipathic (non-myristoylated) helix, by which the small GTPase is anchored to the ER membrane (Bi et al. 2002; Huang et al. 2001). The COPII coat is successively assembled by recruitment of the inner coat subcomplex Sec23/24 and the outer coat subcomplex Sec13/31 (Matsuoka et al. 1998). Activated Sar1 recruits the Sec23/24 heterodimer to the ER membrane by interaction with the Sec23 subunit (Bi et al. 2002). The membrane-bound Sar1/Sec23/24 complex then recruits the Sec13/31 subcomplex to the ER membrane (Matsuoka et al. 1998). Interaction of Sec23/24 with Sec13/31 involves a 50-residue fragment of the unstructured proline-rich region of the Sec31 subunit. This fragment is required and sufficient to stimulate the rate of GTP hydrolysis in the Sar1/Sec23 complex. A crystal structure uncovered how a single point mutation in human Sec23A linked to cranio-lenticulo-sutural dysplasia (CLSD). Mutation of the phenylalanine residue F380 (F382L), which is placed within the binding pocket of the Sec31 active fragment, disrupts interaction of Sec13/31 with Sec23/24 and therefore nucleation of COPII vesicles (Bi et al. 2007).

The Sec13/31 subcomplex is believed to act as a structural scaffold, which clusters adjacent pre-budding complexes into a COPII coat lattice. This idea is supported by various observations. The Sec13/31 heterotetramer has the ability to self-assemble into COPII cage-like structures in the absence of membranes, Sar1, and the Sec23/24 subcomplex (Stagg et al. 2006). In addition, recruitment via his-tagged NΔ23-Sar1 to artificial membranes of Sec23/24 plus Sec13/31 (but not Sec23/24 alone) causes the formation of typical COPII-coated buds (Lee et al. 2005). A cryo-electron microscopy study of COPII cages formed by Sec23/24 and Sec13/31 uncovered an asymmetric assembly of four Sec23/24 units underneath the vertices of the outer COPII coat, which would require oligomerisation of

the inner coat before Sec13/31 recruitment (Stagg et al. 2008). This idea is supported by the observation that pre-budding complexes can oligomerise in artificial planar bilayers (Tabata et al. 2009).

9.4.2 Cargo Sorting into COPII Vesicles

Efficient ER export of cycling machinery proteins and many secretory proteins relies on signal-mediated sorting into COPII vesicles. Initially a complex of Sar1 and Sec23/24 was identified to bind selectively to secretory cargo and SNARE proteins. This complex is an elementary functional unit of the COPII coat, also referred to as pre-budding complex (Aridor et al. 1998; Kuehn et al. 1998; Springer and Schekman 1998).

A variety of genetic, biochemical, and structural studies revealed multiple independent cargo binding sites on Sec24 (Buchanan et al. 2010; Miller et al. 2002, 2003; Mossessova et al. 2003). The capacity to bind to diverse ER export motifs is further expanded by Sec24 isoforms, with overlapping but also distinct functions (Kurihara et al. 2000; Pagano et al. 1999; Peng et al. 2000; Roberg et al. 1999; Tang et al. 1999).

The motif DxE was identified to be required for efficient ER export of VSV-G (Nishimura and Balch 1997; Nishimura et al. 1999). Although this motif is in general referred to as diacidic motif (D/ExD/E), additional neighbouring residues are required for efficient ER export of VSV-G and other proteins (Sevier et al. 2000). A series of structural studies led to the identification of several distinct binding sites on Sec24, also referred to as A-, B-, and C-site. The motif YxxxNPF found in the yeast SNARE Sed5p binds to the A-site, Sys1p containing an ExE motif, as well as Bet1p containing an LxxLE motif, utilises the B-site, and the SNARE Sec22 binds to a third independent binding site, also referred to as C-site, (Mossessova et al. 2003). Crystallographic analysis and biochemical characterisation of the mammalian proteins revealed various Sec24 isoform-specific interactions with ER-to-Golgi SNARE proteins. The SNAREs syntaxin5 and membrin/GS27 bind to Sec24C and Sec24D via the motif IxM, whereas the SNARE Sec22b binds to Sec24A and Sec24B via a conformational epitope (Mancias and Goldberg 2007, 2008).

Another type of ER export signals are dihydrophobic motifs (FF, YY, LL, or FY) found in the cytoplasmic tails of the transmembrane cargo adaptors ERGIC53/Emp47p (Kappeler et al. 1997; Sato and Nakano 2002), p24 family proteins (Dominguez et al. 1998; Fiedler et al. 1996), and the Erv41/46p complex (Otte and Barlowe 2002). ERGIC53/Emp47p is described as a cargo adaptor for multiple high mannose containing glycoproteins (Appenzeller et al. 1999; Moussalli et al. 1999; Nichols et al. 1998; Vollenweider et al. 1998). Members of the p24 family are directly required for COPII-mediated export of glycosyl-phosphatidylinositol-anchored proteins (GPI-APs) in both *S. cerevisiae* (Belden and Barlowe 1996; Marzioch et al. 1999; Muniz et al. 2000; Schimmoller

et al. 1995) and mammalian cells (Takida et al. 2008). Recognition of GPI-AP by p24 requires GPI-anchor remodelling (Castillon et al. 2011; Fujita et al. 2011). Release of GPI-APs from its adaptor is triggered by the more acidic pH in post-ER compartments (Fujita et al. 2011). Individual Erv family proteins seem to be involved in transport of the soluble cargo protein glycosylated pro-alpha-factor (Belden and Barlowe 2001; Otte and Barlowe 2004), the plasma membrane glycoprotein Axl2p (Powers and Barlowe 1998, 2002), vacuolar alkaline phosphatase, and the *cis*-Golgi localised manosyl transferase Ktr3p (Bue and Barlowe 2009; Bue et al. 2006).

Secretion of collagen, which is too large to fit into a typical COPII vesicle, relies on the cargo adaptors TANGO1 and its homologue cTAGE5. These accessory proteins concomitantly promote the formation and loading of collagen carriers, but do not enter the nascent vesicle (Saito et al. 2009, 2011).

Although an overwhelming number of studies support a primary role of the Sec24 subunit in cargo recognition, other coat subunits might also contribute to this process (Giraudo and Maccioni 2003; Quintero et al. 2010).

More comprehensive reviews on cargo sorting into COPII vesicles can be found elsewhere (Dancourt and Barlowe 2010; Gillon et al. 2012; Kinoshita et al. 2013; Malhotra and Erlmann 2011; Venditti et al. 2014).

9.4.3 Scission of COPII Vesicles

Like Arf1, Sar1 exerts membrane curvature potentiating activity by shallow insertion of its N-terminal amphipathic helix. Addition of purified Sar1 to artificial membranes in the absence of the inner and outer COPII coat caused conversion of liposomes into long tubules (Bielli et al. 2005; Lee et al. 2005). The extent of tubulation is dramatically reduced when bulky hydrophobic residues of the amphiphatic helix are mutated. Concomitantly, formation of free COPII vesicles from native membranes is reduced, although the COPII coat proteins are recruited to membranes. Likewise, a his-tagged NΔ23-Sar1 variant did not exhibit membrane curvature potentiating activity when recruited to liposomes via nickel-chelating lipids. Addition of Sec23/24, Sec13/31, and GMP-PNP to this system caused the formation of COPII-coated buds, which fail to detach from the donor membrane. Thus, like in the COPI system, a lack of membrane curvature potentiating activity causes a scission arrest (Lee et al. 2005), and vesicle scission does not require GTP hydrolysis (Adolf et al. 2013).

9.4.4 COPII Coat Stabilisation and Vesicle Uncoating

In the prevailing view GTP hydrolysis leads to a conformational change of the small GTPase and thus retraction of its N-terminal amphipathic helix, dissociation

from the membrane, and concomitant coat disassembly (Antonny et al. 2001; Oka and Nakano 1994). GTP hydrolysis as prerequisite for COPII vesicle uncoating is supported by the observation that COPII vesicles formed under conditions when GTP hydrolysis by Sar1 is blocked fail to fuse with their acceptor membrane (Aridor et al. 1995; Barlowe et al. 1994; Oka and Nakano 1994; Rowe et al. 1996).

In contrast to COPI vesicles, in the COPII system the GTPase activating protein required for GTP hydrolysis, Sec23, is a component of the COPII coat (Yoshihisa et al. 1993). Sec23 inserts a catalytically active arginine residue into the Sar1 nucleotide binding pocket, which results in stimulation of GTP hydrolysis (Bi et al. 2002). Binding of the Sec13/31 subcomplex further accelerates the GTPase activity (Antonny et al. 2001). An extended unstructured loop of Sec31 (also referred to as active fragment) inserts amino acid side chains in the vicinity of the Sar1 active site, which causes reorientation of the catalytic arginine and thus leads to an elevated GTP hydrolysis rate (Bi et al. 2007).

This organisation raises the question how the COPII coat is stabilised on the ER membrane to promote vesicle formation, while its built-in GAP activity is maximal when the coat is fully assembled, and thus concomitantly triggers its own disassembly (Antonny et al. 2001). Several additional factors might contribute to prevent premature coat disassembly. Whereas formation of vesicles from chemically defined liposomes with purified Sar1, Sec23/24, and Sec13/31 is only possible in the presence of the non-hydrolysable GTP analogue GMP-PNP (Matsuoka et al. 1998), addition of sub-stoichiometric amounts of Sec12 to this minimal reconstitution system allowed GTP-dependent budding of COPII vesicles (Futai et al. 2004).

The peripheral ER membrane protein Sec16 is another factor that contributes to regulate the assembly and stability of nascent COPII vesicles. Sec16 was initially identified in the classical screen for secretion mutants in *S. cerevisiae* (Novick et al. 1980), and subsequently shown to be required for vesicle formation from the ER (Kaiser and Schekman 1990). Biochemical and genetic analysis revealed direct interactions of Sec16 with Sar1, Sec12, Sec23, Sec24, Sec13, and Sec31 (Espenshade et al. 1995; Gimeno et al. 1996; Montegna et al. 2012; Nakano and Muramatsu 1989; Shaywitz et al. 1997). A screen for mutants in *Pichia pastoris* identified Sec16 as a major player in maintaining ERES integrity upstream of the assembly of the COPII coat, (Connerly et al. 2005). The mammalian Sec16 homologues have features common with *Pichia pastoris* proteins. Knock-down of Sec16 causes ERES fragmentation and blocks ER export (Bhattacharyya and Glick 2007; Iinuma et al. 2007; Ivan et al. 2008; Watson et al. 2006). Sec16 does not modulate the GAP activity of Sec23 directly (Supek et al. 2002), however reduces GTP hydrolysis by competing with binding of the Sec13/31 heterotetramer to the Sec23 subunit (Kung et al. 2012; Yorimitsu and Sato 2012). Taken together the multi-domain protein Sec16 has multiple functions. It acts as a scaffold for ERES formation upstream of the COPII coat, promotes COPII coat assembly by direct recruitment of COPII coat components, and further prevents premature coat disassembly by interfering with the GTP hydrolysis activity of the fully assembled COPII coat.

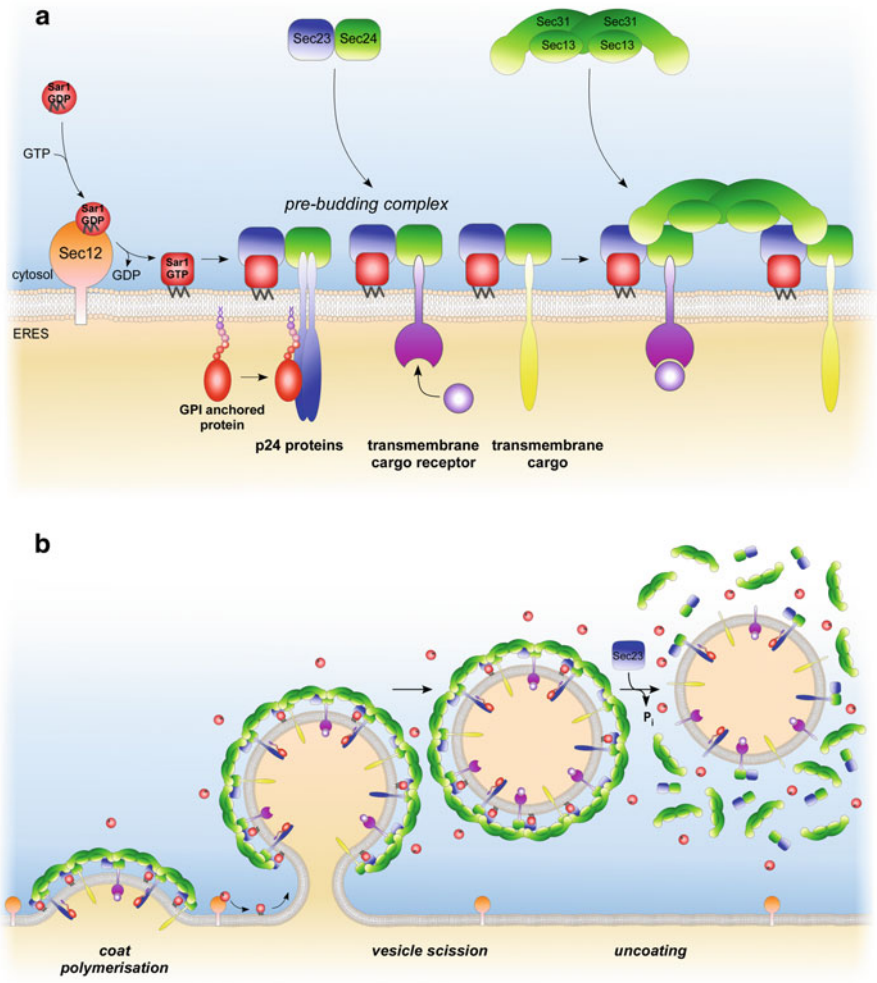

Fig. 9.2 Individual steps in the live cycle of COPII vesicles. Sar1 is recruited and anchored to the ER membrane by activation via the GEF Sec12. Membrane-bound Sar1 recruits the inner COPII coat subcomplex Sec23/24 by binding to the Sec23 subunit. The cargo binding subunit Sec24 binds ER export motif-containing transmembrane cargo proteins and trafficking proteins (e.g. SNAREs), as well as cargo adaptors (e.g. ERGIC53 and p24 proteins). The outer COPII subcomplex Sec13/31 binds to the pre-budding complexes and drives coat polymerisation. Vesicle scission requires the membrane curvature potentiating activity of the small GTPase Sar1. Sec23-stimulated GTP hydrolysis by Sar1 leads to release of the small GTPase and concomitant release of the coat

In addition, membrane cargo seems also to contribute to COPII coat stability by increasing the membrane association time of the COPII coat during multiple cycles of GTP hydrolysis (Forster et al. 2006; Sato and Nakano 2005). This may represent

the molecular basis for a GTPase-driven kinetic proofreading mechanism (Sato and Nakano 2007).

Individual steps in the formation and uncoating of COPII-coated vesicles are depicted in Fig. 9.2.

9.5 Conclusions and Perspectives

A unifying hallmark in the process of transport by coated vesicles is the multitasking of a small GTPase: Arf and Sar1p have various roles in the cycle of coated carriers. They catalyse steps that require hydrolysis of GTP (cargo uptake and uncoating) and steps independent of GTP hydrolysis (coat recruitment and scission). Whereas the mechanism of GTP hydrolysis dependent uptake of cargo is still elusive, our knowledge on the mechanisms of coat recruitment and coat release is more advanced, as outlined above.

From investigations on both the COPI and the COPII systems a simple mechanistic model emerges for the step of membrane separation, i.e. the scission of the vesicle membrane from the donor membrane (Beck et al. 2011; Faini et al. 2013). Membrane scission relies upon the membrane curvature potentiating activity of the activated small GTPase. Shallow insertion of the G protein's amphiphilic helix into the cytoplasmic leaflet stabilises positive curvature of the bilayer (in liposomes with transmembrane proteins lacking, shallow insertion may even convert the bilayer into tubules, a read-out for membrane curvature potentiating activity). It is then the coat protein recruited by the small GTPase that sculpts the membrane into the shape of a bud. As the bud matures, a neck is formed between bud and donor membrane, with an area of negative curvature. Accommodation in negatively curved layers is energetically highly unfavourable for an amphipatic helix (McMahon and Gallop 2005). Therefore, when forced to stay in such areas of negative curvature by multiple interactions of the small GTPase with the covering inner layer of the coat, the high-energy state in these zones is released by fusion of the closely juxtapositioned membranes in the neck (Beck et al. 2011).

Such a mechanism could hold for all scission steps of vesicles that operate with small GTPases, COPI, COPII, and CCVs with AP1, AP3, and GGA1–3, as well as clathrin-independent AP4 vesicles. The model differs from those for endocytosis based on CCVs with AP2, where the large GTPase dynamin plays a role, and where hydrolysis of GTP is thought to be prerequisite for the scission step proper.

Although our insight is quite advanced into molecular mechanisms that involve small GTPases in vesicular transport, there are fascinating questions left, mechanistic ones and more general ones, e.g. how is GTP hydrolysis mechanistically linked to the uptake of cargo? How is the soluble, non-aggregated state of coatomer regained after uncoating? What are the specific roles of the various ArfGAPs? Are all proteins actively sorted into the secretory pathway by interaction of motifs with receptors? What is the role of the isoforms of COPs? What is the role of the various Arf family members? Do isoforms of coat proteins create uniformly coated

vesicles? How exactly are Arf molecules arranged within the coat to allow efficient uncoating? What is the role of lipid sorting in COPI vesicles? Are lipids also sorted during formation of COPII vesicles? Are the lipid compositions identical of the membranes of isoformic vesicles?

Likewise, more general questions are still pending: why do principally different mechanisms exist of coat recruitment, one step in the COPI, and two steps in the CCV and COPII systems? To what extent does vesicular transport contribute to overall membrane transport within a eukaryotic cell? Answers to such questions will finally help to solve a core question pending now for more than 100 years: why does the Golgi apparatus possess its characteristic structure, a staple of flat cisternae?

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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

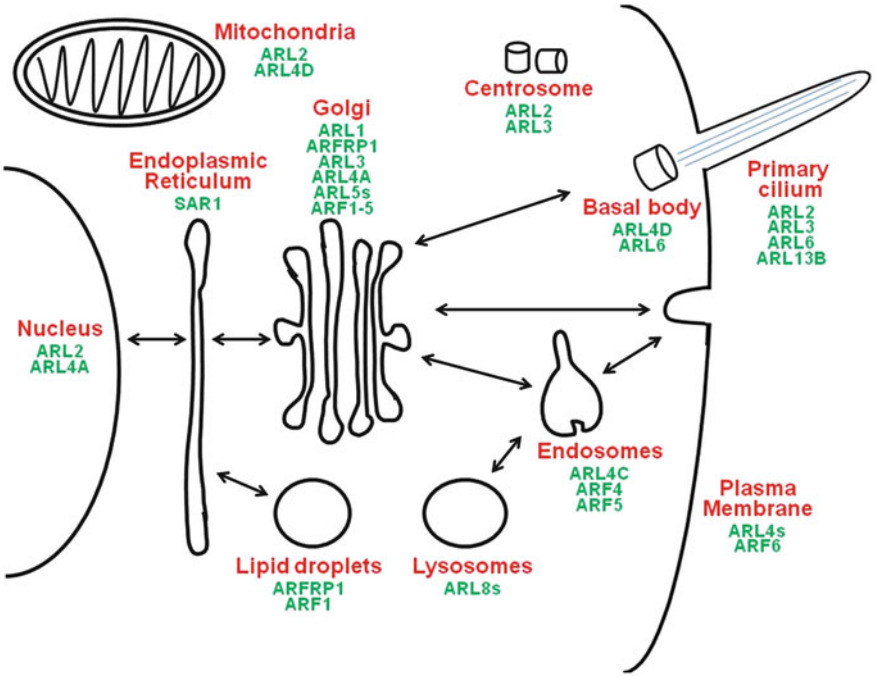

Fig. 10.1 Summary of the cellular locations of members of the ARF family of regulatory GTPases. Organelles (labeled in *red text*) and GTPases (*green text*) are shown, based upon findings described in the text. ARFs are included to highlight their co-localization at several organelles with ARLs but 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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Chapter 11
ArfGAPs: Not Only for the Termination

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Abstract While Arf-family small GTPases (Arf-GTPases) consist of 5 members in humans, 31 human genes have been identified that encode proteins bearing the GTPase-activating protein (GAP) domain for Arf-GTPases. Interestingly, Arf1, the first identified Arf, was shown to substantially lack intrinsic GTPase activity, which other Ras-superfamily members of small GTPases generally bear. Likewise, ArfGAP domains primarily consist of zinc-finger structures, and do not resemble GAP domains for other small GTPases. Arfs primarily function in intracellular vesicle/membrane trafficking. A general model shows that Arfs play roles in membrane budding, in which GTP-Arfs recruit coatomer proteins to generate and maintain membrane curvature to initiate the budding. Coatomers are thought to be separated from Arf-mediated vesicles before they reach the target membrane, while this separation may or may not be coupled with the GTP hydrolysis activity. We have shown that several ArfGAPs, such as AMAP1 and AMAP2, have the ability to bind stably to GTP-Arf6, without immediate GTP hydrolysis. They each contain a BAR domain and hence may act as coatomers for Arf-mediated vesicles. These ArfGAPs moreover act to recruit their binding proteins to sites of Arf6 activation, which are not coatomer components. These findings have amended the classical, general model of the functions of ArfGAPs, as well as Arf-GTPases. In this review, we will describe the recent information revealed about ArfGAPs, with the aim to decipher and discuss their fundamental roles.

Keywords ArfGAP • Arf family • Membrane traffic

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11.1 Introduction

Arfs play pivotal roles in the trafficking of membrane vesicles, which may be involved in carrying cargos as well as in membrane remodeling. In mammals, the Arf family consists of 6 members, Arf1–6, while Arf2 is missing in humans. These members are subclassified into class I (Arf1–3), class II (Arf4 and 5), and class III (Arf6). Class I Arfs primarily function at perinuclear areas and mediate transport from the *cis*-Golgi network to the endoplasmic reticulum, in which they primarily function in the initiation of membrane budding by recruiting coat proteins to sites of Arf activation, while the fission of vesicles and their transport to target membranes are controlled by other proteins. Arf6 primarily functions at the cell periphery, mediating the recycling of plasma membrane components and some cell surface receptors. The function of the class II Arfs is still largely unknown (D'Souza-Schorey and Chavrier 2006).

Arf1, the first isolated Arf, was found to substantially lack intrinsic GTPase activity, unlike the other small GTPases (Kahn and Gilman 1984). Perhaps related to this, ArfGAP domains primarily consist of a zinc-finger structure, and do not resemble the GAP domains for other small GTPases. It has moreover been shown that the ArfGAP-binding site does not overlap with the coatomer-binding site on the Arf1 molecule (Goldberg 1999), while GAP-binding sites largely overlap with effector-binding sites in many other small GTPases, including Ras. Two different models have been proposed with regard to the mechanism by which the Arf/ArfGAP complex hydrolyzes GTP. Goldberg (1999) has proposed that Arf1-ArfGAP1 constitutes only part of the usual protein binding interface and that the key residue(s) required for maximal rates of the GTP hydrolysis, like the "arginine finger," may not be provided by ArfGAP1, but further association of the coatomer with the Arf1-ArfGAP1 complex may be required for the maximal GTP hydrolysis activity. In contrast, structural analysis of Pap/ASAP suggests that the critical residues for hydrolysis of GTP bound to Arf1 may actually be provided by this ArfGAP molecule (Mandiyan et al. 1999). This model is consistent with a result that Pap/ASAP exhibits efficient catalytic GAP activity for GTP-Arf1 in vitro (Andreev et al. 1999).

Several members of ArfGAPs do not act as simple GAPs for Arf-GTPases. We have shown that AMAP1 and AMAP2 (also called ASAP1 and ASAP2, see later) have the potential to bind stably to GTP-Arf6, without immediately hydrolyzing GTP in the presence of Mg^{2+} (Hashimoto et al. 2004), while these ArfGAPs exhibit GAP activity toward Arf1 and Arf5 (Brown et al. 1998). We showed that through this property, AMAP1 acts to recruit β1 integrin to sites of Arf6 activation (i.e., the plasma membrane) to promote the recycling of β1 integrin (Onodera et al. 2012). Interestingly, ASAP3, an isoform of AMAP/ASAP, was recently shown to exhibit Ca^{2+}-dependent GAP activity toward Arf-GTPases (Ismail et al. 2010). These findings collectively challenge the classical view of the functions of ArfGAPs, as well as Arfs. In this review, we will first summarize the basic information on the

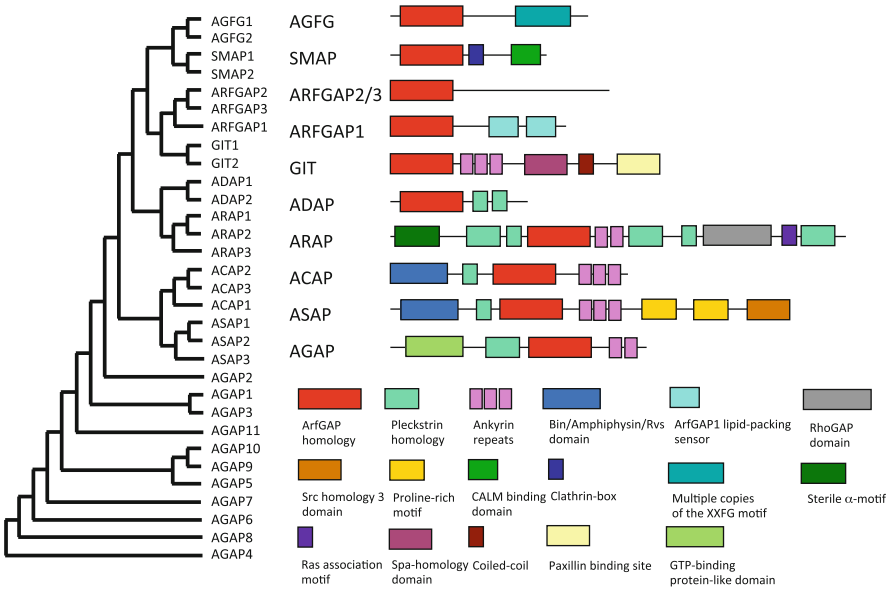

Fig. 11.1 Phylogenetic tree of ArfGAP proteins in the human genome. Thirty-one human genes have been identified that encode proteins bearing the GAP domain for Arf-GTPases

structure and function of ArfGAP-family members (see Fig. 11.1), and discuss possible revised models of the functions of some ArfGAPs.

11.2 ArfGAP1 Subfamily

In the budding yeast *Saccharomyces cerevisiae*, six proteins possess the conserved ArfGAP domain, and four of these family members have proven ArfGAP functions (Poon et al. 1996, 1999; Zhang et al. 2003). The Gcs1 and Glo3 members of the yeast ArfGAP family are the most extensively characterized. These ArfGAP proteins have partially overlapping functions in retrograde transport from the Golgi to the endoplasmic reticulum (ER) (Poon et al. 1999).

ArfGAP1 is the mammalian ortholog of yeast Gsc1p (Cukierman et al. 1995). ArfGAP1 functions at the Golgi by promoting the hydrolysis of GTP bound to Arf1 (Liu et al. 2005). ArfGAP has a zinc-finger structure that is required for GAP activity, near its N-terminus (Ireland et al. 1994). A region C-terminal to the ArfGAP domain contains two ArfGAP1 Lipid Packing Sensor (ALPS) motifs. This motif forms an amphipathic α-helix that differs from the classical one and contains abundant serine and threonine residues on its polar surface, and thereby appears to be able to act as a sensor that recognizes degree of the membrane curvatures in order to determine the timing of hydrolysis of GTP-Arf1 on budding

membranes, where vesicles are being formed by the accumulation of the COP-I complex to sites of GTP-Arf1 (Bigay et al. 2005). When the Arf1/ArfGAP1 complex starts to hydrolyze GTP bound to Arf1, uncoating of the COP-I complex from the Arf1 vesicles is initiated, which is necessary to fuse the vesicles with the target membrane (Orci et al. 1993; Rothman and Wieland 1996; Spang et al. 1998). On the other hand, the above model of the ALPS motifs alternatively suggests that ArfGAP1 may bind to the COP-I-coated areas, as the underlying membrane becomes curved, and gradually eliminates Arf1-GTP from these areas (Bigay et al. 2003; Ambroggio et al. 2010).

The clathrin-AP-2 (adaptorprotein-2) complex is a well-characterized coat protein. ArfGAP1 also interacts with AP-2, and promotes AP-2-dependent transferrin receptor (TfR) endocytosis (Bai et al. 2011). Interestingly, during the onset of TfR endocytosis, AP-2 can stimulate the GAP activity of ArfGAP1 toward Arf6, which directly binds to the cytoplasmic domain of TfR in the GTP-bound form (Bai et al. 2011).

Leucine-rich repeat kinase 2 (LRRK2), which possesses a GTPase domain and kinase activity, is also a binding partner of ArfGAP1, which then promotes the kinase activity of LRRK2 (Stafa et al. 2012). Mutations in the LRRK2 gene are the most common cause of Parkinson's disease. Some mutations of LRRK2 found in familial Parkinson's disease may enhance the interaction of LRRK2 with ArfGAP1. Such an abnormal interaction appears to dysregulate the GTPase activity of LRRK2 and may hence cause neuronal toxicity, while the pathogenic effects of familial LRRK2 mutations are still not completely understood (Stafa et al. 2012).

11.3 ArfGAP2 Subfamily

ArfGAP2 and 3 are mammalian orthologs of yeast Glo3p (Frigerio et al. 2007). ArfGAP2/3 are closely related to each other in structure, but show little similarity to ArfGAP1 except for the GAP domain. ArfGAP2/3 are localized to peripheral punctate structures besides the Golgi (Frigerio et al. 2007). Like ArfGAP1, ArfGAP2/3 promote hydrolysis of GTP bound to Arf1, which is followed by dissociation of the COP-I coat (Frigerio et al. 2007). Unlike ArfGAP1, however, ArfGAP2/3 lack the ALPS motif (Watson et al. 2004; Frigerio et al. 2007) but instead possess the Glo3 motif at the C-terminus (Yahara et al. 2006), which interacts with the COP-I coat (Frigerio et al. 2007). The COP-I coat is required for the recruitment of ArfGAP2/3 to the Golgi membrane, and their GAP activity is dependent on the association with the COP-I coat (Weimer et al. 2008), while such a dependence of GAP activity on coatomers is not observed with ArfGAP1 (Szafer et al. 2000). It has also been shown that ArfGAP2/3, once recruited to the Golgi membrane by its interaction with the COP-I coat, exhibit GAP activities that are even higher than that of ArfGAP1 (Weimer et al. 2008). It has hence been proposed that accumulation of the COP-I coatomer on the budding membrane, when COP-I recruits ArfGAP2/3, determines the timing of hydrolysis of GTP-Arf1 (Weimer

et al. 2008), while the curvature of the COP-I-containing membranes, when ArfGAP1 recognizes the curvature, determines the timing of hydrolysis of GTP-Arf1, as earlier mentioned.

11.4 ADAP Subfamily

ADAP1 (ArfGAP with dual pleckstrin homology (PH) domains, also known as centaurin α1, $p42^{IP4}$, or PIP_3BP) was purified from rat brain using a $PI(3,4,5)P_3$- or $Ins(1,3,4,5)P_4$-affinity matrix (Hammonds-Odie et al. 1996). ADAP1 demonstrates GAP activity toward Arf6, but not Arf1 (Venkateswarlu et al. 2004). ADAP2 was identified as a protein closely related to ADAP1 and contains the same domain structure (Whitley et al. 2002). ADAP1/2 possess a GAP domain at the N-terminus, followed by two PH domains. The PH domains of ADAP1 bind to $PI(3,4)P_2$ and PIP_3 (Venkateswarlu and Cullen 1999), while those of ADAP2 preferentially bind to $PI(3,4)P_2$ (Whitley et al. 2002). These PH domains act to recruit ADAP1/2 to the plasma membrane from the cytosol in a PI3 kinase-dependent manner (Venkateswarlu et al. 2004, 2007). It has been reported that such PI3 kinase-dependent recruitment of ADAP1 to the plasma membrane is necessary to block Arf6-dependent actin remodeling (Venkateswarlu et al. 2004).

ADAP1 is mainly expressed in the brain, whereas ADAP2 has a broad tissue distribution, including the heart, skeletal muscle, and adipocytes but not the brain (Whitley et al. 2002). The restricted distribution and high expression of ADAP1 in the brain indicates that ADAP1 is vital for neuronal functions.

Regarding their involvement in neurological disease, ADAP1 expression is increased in the neuritic plaques of Alzheimer's disease patients (Reiser and Bernstein 2002), while ADAP2 gene deletion has been found in a majority of neurofibromatosis type I patients (Jenne et al. 2000).

11.5 SMAP Subfamily

Stromal membrane-associated protein-1 (SMAP-1) was identified in mice as a type II membrane protein (Sato et al. 1998). SMAP1 preferentially exhibits GAP activity toward Arf6, while its isoform SMAP2 acts on Arf1 (Natsume et al. 2006). SMAP1/2 contain a GAP domain and a clathrin interaction motif (LLGLD), which binds both clathrin heavy chains and CALM, a clathrin assembly protein. SMAP1 is diffusely located within the cytoplasm and colocalizes with AP-2, while SMAP2 is found bound to endosomes and colocalizes with AP-1 and EpsinR, which are adaptor proteins responsible for selecting cargo proteins. SMAP1 is involved in Arf6-specific vesicle trafficking, but not Arf6-mediated actin remodeling, and regulates clathrin-dependent endocytosis of the transferrin receptor and E-cadherin. SMAP2 regulates Arf1-dependent retrograde transport of TGN38/46

from early endosomes to the trans-Golgi network (Kon et al. 2007). SMAP1 deficiency is involved in oncogenesis, especially in leukemia (Kon et al. 2013; Sangar et al. 2013). c-Kit is a receptor for stem cell factor (SCF), and its signaling is essential for hematopoiesis. SMAP1 deficiency in mice was shown to affect the intracellular sorting of c-Kit of mast cells from multivesicular bodies to lysosomes, when c-Kit is internalized upon ligand binding, thus causing intracellular accumulation of undegraded c-Kit, accompanied by enhanced Erk activation and increased cell growth (Kon et al. 2013).

11.6 GIT Subfamily

The GIT subfamily, namely Git1 and Git2, were originally identified as ArfGAPs that interact with G protein-coupled receptor kinase 2 (GRK2) (Premont et al. 1998). Git1/2 exhibit GAP activity against Arf1, Arf2, Arf3, Arf5, and Arf6 in vitro (Vitale et al. 2000). On the other hand, in vivo analyses showed that Git1 acts as the GAP toward Arf6 (Di Cesare et al. 2000), and Git2 and its short form show GAP activity for Arf1 (Mazaki et al. 2001, 2006). Git1/2 consist of five domains: the N-terminal GAP domain, three Ankyrin (ANK) repeats, a Spa2-homology domain (SHD), a coiled-coil domain, and a paxillin-binding site. The SHD binds to p21-activated kinase interacting exchange factor (PIX), which is a guanine nucleotide exchanger (GEF) for Rac1 and Cdc42. Through binding to PIX, Git1/2 interact with p21-activated kinase (PAK). The coiled-coil domain of Git1 was recently shown to interact with GRK6, in the GRK6-mediated engulfment of apoptotic cells (Nakaya et al. 2013).

Git1/2 are found at various sites within cells, including the Golgi and focal adhesion plaques (Vitale et al. 2000; Mazaki et al. 2001). Perhaps by regulating the class I Arfs, Git1/2 is involved in Golgi architecture and function. Overexpression of Arf1 has been shown to affect the Golgi. Likewise, overexpression of Git2 affected the morphology of the Golgi, in which the intact GAP domain was necessary (Mazaki et al. 2001). Moreover, overexpressed Git1 was shown to no longer co-localize with the Golgi, but was found to co-localize with some endosomal structures, as well as membrane ruffles and focal adhesions (Di Cesare et al. 2000; Loo et al. 2004).

Git1/2 are employed by various types of cell surface receptors, including integrins and G protein-coupled receptors (GPCRs), and engage in various cellular functions. Git2 is involved in the formation of focal adhesion complexes (Mazaki et al. 2001), which are formed upon the activation of integrins, and are hence involved in cell spreading onto extracellular matrices and cell migration (Hynes 1992; Lauffenburger and Horwitz 1996; Sheetz et al. 1998). Git1/2 play integral roles not only in the formation of focal adhesions, but also in integrin signaling. The ability of Git1/2 to interact with the focal adhesion protein paxillin as well as with PIX generates a robust pathway regulating actin-based cytoskeletal remodeling, namely the paxillin-Git-PIX-PAK pathway, which regulates the activities of Rac1/

Cdc42 and the dynamics of actin fibers under integrins (Brown et al. 2002; Premont et al. 2004). Under GPCRs, Git2 is integral for chemotaxis and the suppressive control of superoxide production in neutrophils, by forming the Gβγ-Pak1-Pixα-Git2 signaling axis upon GPCR activation (Mazaki et al. 2006). Git1 functions under ephrin receptor A2 (EphA2) in epithelial cells, in which Git1 suppresses Arf6 activity in order to block the endocytosis of E-cadherin during cell–cell contacting, and hence contributes to the maturation of adherence junctions as well as the tight junction (Miura et al. 2009). Likewise, Git1 also plays a role in the negative regulation of clathrin-dependent endocytosis. Arf6 has been shown to play a key role in the formation of clathrin-coated pits, in which Arf6 is activated by ARNO (Claing et al. 2001).

Suppression of Arf6 activity by Git1 was shown to suppress the internalization of GPCRs, such as β1 and β2 adrenergic receptors and the M1 muscarinic receptor, and ligand-activated EGFR, all of which are otherwise thought to be internalized via clathrin-coated pits (Claing et al. 2000, 2001).

Slit2-Roundabout4 (Robo4) signaling is known to inhibit the migration of cells toward a chemoattractant gradient (Park et al. 2003; Seth et al. 2005). In endothelial cells, Slit2-Robo4 signaling blocks Arf6 and Rac activation in response to VEGFR and integrin activation. Concomitant initiation of Slit2-Robo4 signaling stimulates the recruitment of the paxillin and Git1 complex to the cytoplasmic tail of Robo4, which induces the inactivation of Arf6 and Rac. Blocking of this protrusive activity is necessary for vascular stability (Jones et al. 2009).

Blocking of Arp2/3-dependent actin polymerization by PICK1 is one of the main mechanisms for AMPA receptor (AMAPR) internalization and long-term depression (LTD) (Rocca et al. 2008; Nakamura et al. 2011). PICK1 was shown to be an Arf1 effector. GTP-Arf1 inhibits the PICK1-mediated inhibition of Arp2/3-dependent actin polymerization. NMDA receptor activation leads to the activation of Git1, which inhibits Arf1 activity and then activates PICK1. The Git1-Arf1-PICK1-Arp2/3 pathway appears to regulate LTD via AMPAR internalization (Rocca et al. 2013).

In bone metabolism, Git2, but not Git1, was reported to be involved in the suppression of Arf6 activity, for the proper maintenance of the polarity of osteoclasts (Heckel et al. 2009).

Pathologically, Gits are involved in Huntington's disease. Git1 interacts with the N-terminal region of huntingtin (HTT), which contains a polyglutamine domain, and this interaction results in the augmentation of HTT levels (Blagoveshchenskaya et al. 2002; Goehler et al. 2004). Git1 was moreover implicated in attention deficit hyperactivity disorder (ADHD) in humans (Won et al. 2011) and the infection of HIV (Hoefen and Berk 2006).

11.7 AGFG Subfamily

The ArfGAP domain and FG repeat-containing proteins (AGFG) subfamily consists of AGFG1 and AGFG2. AGFG1 was originally identified using yeast two-hybrid screens with the HIV Rev protein (Bogerd et al. 1995; Fritz et al. 1995), and is therefore called HIV Rev-binding protein (Hrb), Rev/Rex activation domain-binding protein (Rab), or Rev-interacting protein (RIP). AGFGs have an ArfGAP domain at the N-terminus, and phenylalanine-glycine (FG) repeats at the C-terminus. The GAP domain of AGFG possesses homology with nucleoporins and is thought to be involved in the RNA transport function of HIV-1 Rev, possibly together with the nuclear export receptor CRM1 (Fritz et al. 1995; Farjot et al. 1999). However, the ArfGAP activity of AGFG has not yet been reported. The FG repeats of AGFG possess homology with that of nucleoporins, which mediate nucleocytoplasmic transport. AGFG1 was shown to be an essential cofactor for the Rev protein, which enhances the release of incompletely spliced HIV-1 RNAs from the perinuclear region (Sánchez-Velar et al. 2004), and also an adaptor protein that seems to be involved in some types of clathrin-mediated membrane trafficking, via interacting with Eps15 (Salcini et al. 1997), AP-2 (Schmid et al. 2006), or VAMP-7 (Chaineau et al. 2008; Pryor et al. 2008).

11.8 ASAP Subfamily

This subfamily of ArfGAPs has been called by many different names, and unification of the nomenclature was proposed more than once (Kahn 2003; Kahn et al. 2008). ASAP1 was also called KIAA1249, DEF1, DDEF1, centaurin β4, and PAG2 and once proposed to be unified as AMAP1 (Kahn 2003). ASAP2 was called PAG3, and PAPα, and once proposed to be unified as AMAP2 (Kahn 2003). ASAP3 was called ACAP4 and DDEFL1. ASAP1 was originally named when it was purified from bovine brain as a PI(4,5)P_2-dependent GAP against Arf1 (Brown et al. 1998). PAPα was originally named as a protein that exhibits PI(4,5)P_2-dependent GAP activity toward Arf1 in vitro (Andreev et al. 1999). The specific activity of ASAP1 toward Arf1 was also demonstrated in vivo, in which overexpressed ASAP1 was shown to reduce cellular GTP-Arf1, but not GTP-Arf6 (Furman et al. 2002).

The ASAPs contain the Bin/Amphiphysin/RVS (BAR) domain, the PH domain, the GAP domain, the ANK repeat domain, a proline-rich region (PRR), and the Src homology 3 (SH3) domain (Brown et al. 1998).

The PH domain is essential for the PI(4,5)P_2-dependent GAP activity (Kam et al. 2000). GAP activity toward Arf1 or Arf5 was detectable using a truncated recombinant protein of ASAP1, which was composed of the PH domain, the GAP

domain, and the ANK repeat domain, while its PH domain-deleted construct exhibited much less GAP activity (Kam et al. 2000).

The BAR domain was originally recognized as a conserved domain in Bin1, amphiphysins, and the yeast proteins Rvs167p and Rvs161p (David et al. 1994). The crystal structure of the amphiphysin BAR domain revealed a decade later demonstrated that BAR domains are dimers (Peter et al. 2004). The BAR domain forms a bundle of three α-helices, and self-dimerizes to form a crescent-shaped structure that binds acid phospholipid and other proteins. The inner curved surface of the BAR domain has a positively charged membrane-binding interface that interacts with negatively charged membranes, and appears to be involved in the tubulation of membranes in intracellular trafficking processes (Gallop and McMahon 2005). The BAR domain also contributes to the membrane scission of budding vesicles (Dawson et al. 2006).

The BAR domain of ASAP1, together with the PH domain, dimerizes and binds to large unilamellar vesicles containing acidic phospholipids. The recombinant protein composed of the BAR, PH, and ArfGAP domains efficiently induced tubular structures. Consistently, in vivo studies showed that ASAP1 induces tubular formation, which was dependent on the presence of the BAR domain, GTP-Arf1, and phospholipids, similar to those of large unilamellar vesicles (Nie et al. 2006). These results suggest that ASAP1 functions as an effector of Arf1-dependent membrane bending via its BAR domain.

The BAR domain of ASAP1 also binds to the FIP3 protein, which is a Rab11 effector. Formation of a ternary complex composed of ASAP1, FIP3, and Rab11 was shown to enhance the GAP activity of ASAP1 against Arf1 (Inoue et al. 2008). In the Golgi/trans-Golgi network (TGN)-to-cilia trafficking, ASAP1 binds GTP-Arf4, and subsequently interacts with FIP3 and Rab11 to form a complex that initiates membrane curvature and regulates the budding of post-TGN carriers targeted to primary cilia (Mazelova et al. 2009). The BAR domain was also shown to be involved in the formation of podosomes in NIH3T3 cells (Bharti et al. 2007).

The PRR of ASAP1 binds to numerous proteins containing the SH3 domain, such as Src-family proteins, Crk, CrkL, paxillin, CIN85, CD2AP, cortactin, and PRKD2, while the SH3 domain of AMAP1 binds to proline-rich motifs of Fak, Pyk2 and POB1 (Onodera et al. 2005, 2012; Nam et al. 2007; Inoue and Randazzo 2007).

Phosphorylation of ASAP1 by Src-family kinases was shown to regulate invadopodia formation in NIH 3T3 cells (Bharti et al. 2007). ASAP1 was also shown to localize to focal adhesions, and to regulate cell migration through cytoskeleton remodeling (Kondo et al. 2000; Randazzo et al. 2000). At focal adhesions, Pyk2, an isoform of FAK, also binds to ASAP1, and phosphorylation of Tyr308 and Tyr782 of AMAP1 by Pyk2 was shown to reduce its GAP activity toward Arf1 (Kruljac-Letunic et al. 2003).

ASAP1 also seems to be involved in the recycling of EGFR (Kowanetz et al. 2004). The Cbl family of ubiquitin ligases, including Cbl, Cbl-b, and Cbl-3, are known to be involved in the downregulation of RTKs (Thien and Langdon 2001). ASAP1 can bind to CIN85, which binds to Cbls, and it was shown that

through this binding, AMAP1 is involved in EGFR recycling (Kowanetz et al. 2004).

We have originally identified ASAP1 as PAG2 (Kondo et al. 2000), and now call it AMAP1 following the proposal by Kahn (2003). We have shown that AMAP1 is overexpressed frequently in malignant and invasive breast cancers, and plays essential roles in cancer invasion (Onodera et al. 2005). Overexpression of the AMAP1 protein was also observed in colorectal cancers, and correlated with metastasis and poor prognosis of the patients (Müller et al. 2010). AMAP1 was found to bind to PRKD2 (protein kinase D2) and to form a complex with β1 integrin in breast cancer cells; and hence promotes the recycling of β1 integrins upon EGF stimulation (Onodera et al. 2012). The AMAP1 and CIN85 complex was also found to localize to the invadopodia of breast cancer cells, and CIN85-mediated monoubiquitination of AMAP1 by Cbl was shown to be crucial for invasion activity (Nam et al. 2007). GEP100 acts as a GEF for Arf6 in cancer invasion (Morishige et al. 2008). Co-overexpression of AMAP1and GEP100 was found to correlate with rapid local recurrence in breast cancer patients after breast conservative therapy (Kinoshita et al. 2013).

11.9 ACAP Subfamily

ACAP1 and ACAP2 were identified using cDNA databases, by searching for proteins with predicted structural similarity to ASAPs (Jackson et al. 2000). They possess the BAR domain, the PH domain, the GAP domain, and three ANK repeats. ACAP is an acronym for ArfGAP proteins with coiled-coil, ANK repeat and PH domain. ACAPs were shown to exhibit GAP activity preferentially toward Arf6 in vitro and in vivo (Jackson et al. 2000). ACAP1/2 primarily function at the cell periphery, and colocalize with Arf6 (Jackson et al. 2000).

ACAP1 is a key component of the clathrin coat complex, in which Arf6 regulates the recycling of the transferrin receptor (TfR) (Dai et al. 2004), integrin (Li et al. 2005), and Glut4 (glucose transporter type 4), which is required for glucose homeostasis (Li et al. 2007). ACAP1 was shown to promote the sorting of TfR, its cargo, through recognition of the sorting signals present in the cytoplasmic domain of TfR (Dai et al. 2004), and ACAP1 may hence promote TfR recycling from recycling endosomes.

Regulation of β1 integrin recycling by ACAP1 was shown to require the phosphorylation of ACAP1 by Akt (Li et al. 2005). A linker region between the GAP and ANK domains acts to prevent β1 integrin binding, and phosphorylation at Ser554 by Akt within the linker region relieves this autoinhibition (Bai et al. 2012). Signaling from different growth factors enhances the Ser554 phosphorylation by Akt and promotes binding of ACAP1 to the sorting signal of β1 integrin, which results in enhanced integrin recycling (Li et al. 2005; Bai et al. 2012).

Integrins are involved in axon growth and regeneration. Suppression of Arf6 activation by ACAP1 was shown to increase the recycling of β1 integrins to the cell

surface of neurons, which then increases their anterograde transport (Eva et al. 2012). Arf6 also stimulates phospholipase D-mediated generation of phosphatidic acids (Massenburg et al. 1994). Such phosphatidic acids are required for the recruitment of ACAP1 to endosomes, in order to enhance endosomal membrane recycling (Jovanovic et al. 2006). ACAP2 interacts with GTP-Rab35 via the ANK repeats, in order to regulate Rab35-dependent neurite outgrowth via suppression of Arf6 activity (Kanno et al. 2010).

Rab10 is involved in recycling endosome function (Chen et al. 2006). CNT1, a binding partner of Rab10 in *C. elegans*, is an ortholog of mammalian ACAP. GTP-Arf6 promotes the production of $PI(4,5)P_2$ through activation of phosphatidylinositol-4-phosphate 5-kinase (Honda et al. 1999). Rab10 recruits CNT-1 to membranes of the recycling endosomes, where CNT-1 colocalizes with Rab10 and Arf6 (Shi et al. 2012). Therefore, it seems likely that the Rab10-CNT-1-Arf6 pathway controls the levels of $PI(4,5)P_2$ in recycling endosomes, in a manner reciprocal to Arf6 activation (Shi et al. 2012). Certain $PI(4,5)P_2$ binding proteins are implicated in membrane bending and membrane fission. Therefore, the Rab10-CNT-1-Arf6 pathway is likely to be important for evolutionally conserved recycling endosome functions.

11.10 AGAP Subfamily

Arf GAP with GTP binding protein-like, ANK repeat, and PH domain 1 (AGAP1) was originally identified using cDNA databases as ASAP-type ArfGAPs containing the PH domain, the GAP domain, and ANK repeat domains (Nie et al. 2002). In humans, there are 11 genes predicted to encode AGAP proteins in chromosome 10q, as well as a number of pseudogenes (Gillingham and Munro 2007). AGAP1 and AGAP2 have $PI(4,5)P_2$-mediated GAP activity against Arf1, Arf5, and Arf6, and function in the endocytic system (Nie et al. 2002). AGAP1, via its PH domain, directly binds to AP-3, which is a coat protein involved in trafficking in the endosomal–lysosomal system, and regulates AP-3-dependent trafficking (Nie et al. 2003). AGAP2 was shown to form a complex with AP-1, another coat protein involved in the TGN and endosomes, and regulates AP-1/Rab4-based recycling endosomes, which recycle proteins such as the transferrin receptor (Nie et al. 2005).

Amplification of the *AGAP2* gene (also known as *PIKE*) and overexpression of its gene product are observed in glioblastoma, prostate carcinoma, and other cancers (Liu et al. 2007). Cancer cells overexpressing AGAP2 show resistance to apoptosis. AGAP2 expression was also shown to enhance the proliferation, foci formation, and tumor progression of prostate cancer cells (Cai et al. 2009). As for the mechanism, ectopic expression of AGAP2 in glioblastoma cells was shown to activate Akt, which may block the apoptosis (Ahn et al. 2004). It was also shown that Akt phosphorylates AGAP2 at Ser629, which enhances GTP binding by downregulating the GTPase activity of the GAP domain, and that phosphorylated AGAP2 then binds to the p50 subunit of NF-κB to enhance its transcription activity.

Therefore, aberrantly high expression of AGAP2 appears to contribute to both the initiation and progression of carcinogenesis (Cai et al. 2009).

11.11 ARAP Subfamily

ARAP1 and ARAP2 were identified by their homology with other ArfGAPs (Miura et al. 2002). ARAP3 was identified using a phosphoinositide-affinity matrix, as a phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$)-binding protein (Krugmann et al. 2002). ARAPs are the most structurally complex members of the ArfGAPs, and contain the SAM (sterile α motif) domain, 5 PH domains, the Rho-GAP domain, the Ras-association domains, and ANK repeats, besides the GAP domain. ARAPs show $PI(3,4,5)P_3$-dependent GAP activity toward Arf6 (Miura et al. 2002; Krugmann et al. 2002; Yoon et al. 2006).

ARAP1 localizes to the plasma membrane, the Golgi complex, and endosomal compartments, such as recycling endosomes, late endosomes/multivesicular bodies (MVBs), and lysosomes. The subcellular localization of ARAP1 appears to be controlled by its phosphorylation, and also by its interaction with 3- and 4-phosphorylated phosphoinositides through its 5 PH domains (Daniele et al. 2008). It was shown that EGF stimulation causes the translocation of ARAP1 to endocytic compartments to colocalize with ligand-bound EGFR at endosomes in order to regulate the attenuation of EGFR signal transduction (Daniele et al. 2008; Yoon et al. 2008). ARAP2, which is different from ARAP1 and ARAP3, lacks RhoGAP activity, and likely functions as a Rho effector. ARAP2 localizes to the cell periphery and on focal adhesions composed of paxillin and vinculin, and contributes to the assembly of both stress fibers and focal adhesions (Yoon et al. 2006). ARAP3 is mostly localized in the cytoplasm (Krugmann et al. 2002). Addition of EGF to PC12 cells caused a rapid translocation of some of the GFP-ARAP3 to the plasma membrane, and addition of PDGF to PAE cells caused some translocation of GFP-ARAP3 to the ruffling edges of lamellipodia (Krugmann et al. 2002).

11.12 How Is the Timing of GTP Hydrolysis by ArfGAPs Controlled?

As already mentioned above, one of the representative classical model of vesicle formation, which was mostly based on the possible interaction of Arf1 and ArfGAP1, is as follows (Rothman and Wieland 1996; Schekman and Orci 1996; Roth 1999). Arf1, when activated, initiates membrane budding by recruiting the COP-I complexes. Assembly and accumulation of the COP-I complexes then generate curved membranes. ArfGAP1 possesses the ALPS motif, which binds to

curved membranes. The binding site for COP-I on the Arf1 molecule does not overlap with that of ArfGAP1 (Goldberg 1999). Via the ALPS motif, ArfGAP1 is then recruited by GTP-Arf1 to the COP-I-coated curved membranes, and hydrolyzes GTP-Arf1. This hydrolysis was previously thought to release the COP-I coat from vesicles (Tanigawa et al. 1993). However, it was shown later on that COP-I remains on the membrane even after GTP-Arf1 is hydrolyzed and dissociated from the membrane (Presley et al. 2002). Therefore, dissociation of COP-I from vesicles may not necessarily be linked to the hydrolysis of GTP-Arf1 and hence the release of Arf1 from the vesicles. Consistently, a proteomic analysis of purified COP-I-coated vesicles showed that Arf-GTPases are rarely found within the COP-I vesicles (Gilchrist et al. 2006) (Fig. 11.2). Therefore, it is likely that some ArfGAPs may inactivate their cognate Arfs at each location of membrane curvature, even before the overall vesicular structures are formed and vesicles are separated from the donor membrane.

On the other hand, AMAP/ASAP- and ACAP-family members contain the BAR domain, which may sense the membrane curvature, as well as may generate, stabilize, and stably bind to curved membranes, as also mentioned earlier. It was proposed that ASAP1 plays a role in the formation of curved membranes: GTP-Arf1 is anchored to the membrane via its myristoylation, and the binding of ASAP1 to GTP-Arf1 then vents the Arf1-containing membrane (Nie et al. 2006). Consistent with this notion, the BAR domain of ASAP3/ACAP4 was recently demonstrated to have the potential to tubulate liposomes in vitro (Zhao et al. 2013). Therefore, it is plausible that ASAP1 has a dual function: one is to promote vesicle formation mediated by GTP-Arf1, and the other is to hydrolyze GTP-Arf1. The GAP activity of ASAP1 toward Arf1 requires phosphatidylinositol-phosphates, such as $PI(4,5)P_2$ (Kam et al. 2000). Therefore, it is plausible to assume that ASAP1 hydrolyzes GTP-Arf1 to release Arf1 from the vesicles, only after sufficient amounts of modified lipids are accumulated.

A recent study on the co-crystal structure of the ASAP3 and Arf6 complex revealed that Ca^{2+} promotes the GAP activity of ASAP3 toward GTP-Arf6 (Ismail et al. 2010). ASAP3 is an AMAP/ASAP-family member; and Gln479 of ASAP3, crucial for Ca^{2+} binding, is conserved among the AMAP/ASAP-family members and also in ARAP3. This notion was intriguing because we have shown that AMAP1 and AMAP2, members of the ASAP family, bind stably to GTP-Arf6, even in the presence of Mg^{2+} without hydrolyzing GTP, and have proposed that these ArfGAPs act to recruit proteins, other than coatomers, to the sites of Arf6 activation, as mentioned earlier. It can hence be hypothesized that these ArfGAPs, as far as Ca^{2+} is not supplied, act as (1) binding proteins to GTP-Arf6 in order to recruit certain proteins to the sites of Arf6 activation, and simultaneously act as (2) coatomer components for Arf6-mediated vesicles to vent the membrane; and that Ca^{2+} spikes, perhaps induced by various extracellular stimuli, then trigger hydrolysis of GTP-Arf6 and hence strip the vesicles from these ArfGAPs (Fig. 11.3). In this regard, it should be noted that GDP-Arf6 is known to remain bound to membranes primarily via its myristoylation and its N-terminal basic helix, while GDP-Arf1 is no more bound to membranes even when it is still myristoylated

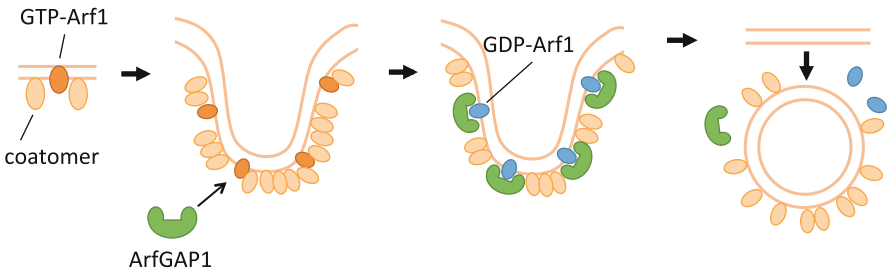

Fig. 11.2 A classical model of the activity of ArfGAPs, primarily based on the possible interaction of ArfGAP1 with GTP-Arf1. GTP-Arf1 recruits the COP-I complexes to initiate membrane budding, in which assembly and accumulation of the COP-I complexes generate curves in the membranes. ArfGAP1 is then recruited to these curved membranes via its ALPS motif, to be localized in proximity to GTP-Arf1, in order to hydrolyze GTP and hence release Arf1 from the vesicle. On the other hand, the dissociation of COP-I from vesicles appears to be unnecessary for the hydrolysis of GTP and the release of Arf1

(Donaldson and Jackson 2011). This property of Arf6 may be crucial for its role in membrane "recycling," in order to memorize which compartments of membranes should be brought back to the recycling processes. On the other hand, we have previously discussed a possible reason why AMAP1 and AMAP2 nevertheless exhibit GAP activity toward other Arfs, like Arf1 and Arf5, in the presence of Mg^{2+} (Sabe 2003).

Dysregulation of Ca^{2+} homeostasis has long been highly implicated in the development of cancer malignancy, including invasiveness and metastatic potentials (Prevarskaya et al. 2011). The AMAP1 protein is overexpressed in most malignant breast cancers, and constitutes a core component of the Arf6-based invasion machinery (Onodera et al. 2005, 2012). Expression of the *ASAP3*/*DDEFL1* gene is also upregulated in hepatocellular carcinomas (Okabe et al. 2004), and overexpression of the *AMAP1* gene was shown to be associated with malignant phenotypes of colorectal cancer (Müller et al. 2010), uveal melanomas (Ehlers et al. 2005), prostate cancer (Lin et al. 2008), and pancreatic carcinoma and adenocarcinoma (Harada et al. 2009). These bodies of information support the idea that the above hypothesis deserves further experimental scrutiny to clarify the following: (1) what kinds of the stimuli that induce intracellular free Ca^{2+} to activate the GAP activity of AMAPs/ASAPs, and (2) whether a Mg^{2+} ion, if it preoccupies ArfGAPs, can be replaced by a Ca^{2+} ion within the same ArfGAP molecule.

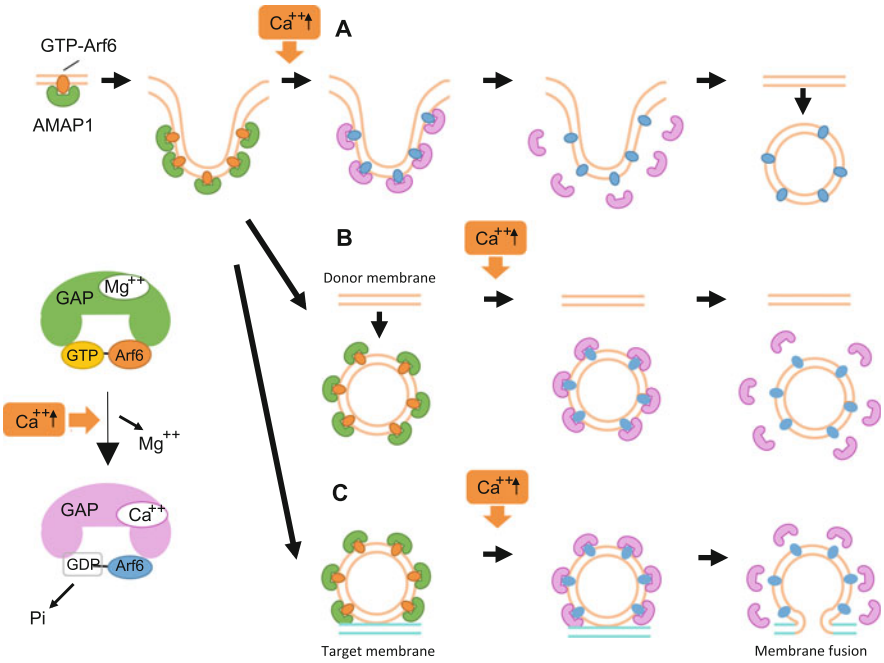

Fig. 11.3 A proposed model of the actions of AMAPs/ASAPs toward GTP-Arf6. AMAPs bind stably to GTP-Arf6, even in the presence of Mg^{2+} without causing immediate hydrolysis of GTP. Through this property, AMAPs may play a role in recruiting their binding partners that are not coat components to sites of Arf6 activation. AMAPs have a BAR domain and may also act to vent Arf6-containing membranes. Ca^{2+} spikes then trigger the GAP activity toward GTP-Arf6. This may occur during vesicle formation (**a**), after the fission of vesicles from the donor membrane (**b**), and after the vesicles reach the target membranes (**c**). In (**c**), membrane fusion and cargo release are directly regulated by Ca^{2+}, in which Ca^{2+} strips AMAPs from the vesicles. Of note, unlike Arf1, myristoylated Arf6 can remain bound to membranes even in its GDP-bound form, a property that is likely to be important for Arf6-mediated recycling

11.13 Perspectives

ArfGAPs are characteristic for their possession of different kinds of functional modules, besides the GAP domain. We discussed here several examples in which ArfGAPs play roles not only in hydrolyzing GTP-Arfs: ArfGAPs appear to act as coatomer components to vent membranes, and also act to recruit proteins, which are not coatomers, to the sites of Arf activation. These data indicate that besides vesicle formation, Arfs definitely have roles to recruit proteins not directly involved in vesicle formation. Whether such proteins then become cargos of vesicles is still an open question. In general, moreover, Arfs on their own do not determine the destination of vesicles (i.e., target membranes) but need other small-GTPases, like Rabs, while mechanisms that link Arfs with certain Rabs are largely unknown. Moreover, actin- or tubulin-based cytoskeletal remodeling often requires

membrane remodeling, and, indeed, it has been shown that Arf6 activity is a prerequisite for the activation of Rho-family GTPases, such as Rac and Cdc42 (Donaldson 2003). Intriguingly, moreover, it has been pointed out that Arfs are conserved throughout eukaryotes, and are present in the earliest eukaryotes, such as *Giardia lamblia*, in which no Ras GTPases nor hetero-trimeric G-proteins are found (http://smart.embl-heidelberg.de/smart/do_annotation.pl?DOMAIN=Arf&BLAST=DUMMY&EVOLUTION=Show#Evolution; Kahn 2003). Formation of vesicles and their cargo selection, trafficking, and recycling, as well as the processes of endocytosis and the exocytosis therein involved, are crucial for cell integrity and function, including neuronal transmission and communication. Therefore, further studies on the detailed functions and regulatory mechanisms of ArfGAPs bearing multiple different modules, as well as how the timing of GAPing is controlled, will definitely push molecular cell biology, as well as medicine and neuroscience, into new frontiers.

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Part IV
RAGs

Chapter 12
Rag GTPases

Jenna L. Jewell and Kun-Liang Guan

Abstract The Rag GTPases appear to reside on the lysosome and link amino acid stimulation to mTOR complex 1 (mTORC1) activation. mTORC1 couples nutrient availability to cell growth. Dysregulation of mTORC1 is implicated in a number of human diseases, including cancer and diabetes. In response to amino acid availability the Rag GTPases are regulated by the Ragulator and GATOR complexes, which are a guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP), respectively. Here we review the current knowledge of Rag GTPases, with emphasis on amino acid-dependent regulation of mTORC1.

Keywords Rag GTPases • mTORC1 • amino acids • Ragulator • Gator • v-ATPase • lysosome

12.1 Discovery and Unique Properties of Rag GTPases

Rag GTPases have recently been identified as key components of the amino acid signaling pathway to activate mTOR complex 1 (mTORC1). Rag GTPases belong to the Ras superfamily; however, they have unique characteristics that distinguish them from other small GTPases. For example, Rag GTPases have a long carboxyl-terminal domain, lack a membrane-targeting sequence, and can form heterodimers (Nakashima et al. 1999; Sekiguchi et al. 2001). There are four Rag proteins in mammals: RagA and Rag B, which have high sequence similarity and are functionally redundant; and RagC and RagD, which are also highly related in sequence and are functionally equivalent. RagA or RagB forms a heterodimer with either RagC or RagD, with the possibility of forming four distinct complex arrangements (Sekiguchi et al. 2001). In *Saccharomyces cerevisiae*, the RagA/B orthologue Gtr1

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was the first member of the Rag GTPase family to be identified (Bun-Ya et al. 1992), with mammalian RagA/B being described later (Hirose et al. 1998). Initially, Gtr1 was implicated in phosphate uptake and the Ran/Gsp1-GTPase pathway (Bun-Ya et al. 1992; Hirose et al. 1998). Subsequent studies identified Gtr2 in yeast and the mammalian orthologues RagC/D due to their ability to form heterodimers with Gtr1 and RagA/B, respectively (Nakashima et al. 1999; Sekiguchi et al. 2001). Like yeast, *Drosophila melanogaster* only have one RagA-like and one RagC-like gene (Kim et al. 2008; Li et al. 2010).

The yeast Gtr1–Gtr2 heterodimer crystal structure was recently solved, presenting a pseudo twofold symmetry resembling a horseshoe. The structure revealed an amino-terminal GTPase domain on each protein where GTP binding and hydrolysis occur, and a carboxyl-terminal domain on each protein identifying multiple contacts required for Gtr1 (bound to GTP) and Gtr2 (bound to GDP) dimerization (Gong et al. 2011; Jeong et al. 2012). Dimerization of the Rag GTPases is important for mTORC1 activation and Rag protein stability, since depletion of RagA/B also abolishes RagC/D protein levels (Kim et al. 2008; Sancak et al. 2008). More structural elements of the Rag GTPases are discussed below, and how they play a role in amino acid-induced mTORC1 activation.

12.2 Regulation of Amino Acid Signaling to mTORC1 by Rag GTPases

Mammalian target of rapamycin (mTOR, also referred to as mechanistic TOR) is a conserved serine/threonine kinase that is the catalytic subunit of mTOR complex 1 (mTORC1), a master regulator that couples amino acids and cell growth. Dysregulation of mTORC1 is common in a number of human diseases, including cancer and diabetes. Multiple stimuli regulate mTORC1 kinase activity, such as growth factors, stress, energy status, and amino acids. The molecular mechanisms involved in mTORC1 regulation by growth factors and cellular energy levels have been studied extensively (Laplante and Sabatini 2012; Zoncu et al. 2011b). Although amino acid signaling is essential for mTORC1 activation (Hara et al. 1998; Wang et al. 1998), few mechanistic details have been known until recently. In addition, specific amino acids like leucine (Hara et al. 1998; Nicklin et al. 2009; Sancak et al. 2008), glutamine (Duran et al. 2012; Kim et al. 2013; Nicklin et al. 2009; van der Vos et al. 2012), and arginine (Bauchart-Thevret et al. 2010; Hara et al. 1998) have been implicated in mTORC1 activation, but the mechanistic details of how these amino acids are sensed are still obscure. In 2008, Sabatini's group and we identified the Rag GTPases as crucial for amino acid signaling to mTORC1. Our lab discovered the Rag GTPases through the screening of small GTPases using RNA interference (RNAi) in *Drosophila melanogaster*, whereas Sabatini and colleagues identified the Rags as Raptor (a component of

mTORC1) interacting proteins through an immunoprecipitation-mass spectrometric analysis approach (Kim et al. 2008; Sancak et al. 2008).

Similar to other small GTPases, the activation state of the Rag GTPases is controlled by their guanine nucleotide state, and this is proposed to be regulated by amino acids (Sancak et al. 2008). Specifically, in the presence of amino acids, the heterodimeric Rag complex is active, where RagA and RagB are GTP bound and RagC and RagD are GDP bound. Alternatively, in the absence of amino acids, RagA and RagB are GDP bound and RagC and RagD are GTP bound. Consistently in yeast, amino acid-rich conditions promote the active form where Gtr1 GTP bound is in complex with Gtr2 GDP bound (Fig. 12.1) (Binda et al. 2009). It appears that RagA/B may play a more dominant role over RagC/D in the activation of mTORC1 by amino acids in mammals. RagA/B binds directly to mTORC1 (Gong et al. 2011), and overexpression of constitutively active RagA/B (GTP bound) alone renders mTORC1 insensitive to amino acid starvation (Kim et al. 2008; Sancak et al. 2008). Unlike mammals, active Gtr1 (GTP bound) can only partially activate TORC1 (Binda et al. 2009). Co-expression of RagC/D enhances mTORC1 activation, in part because RagC/D stabilizes RagA/B. In fact, the depletion of RagA/B significantly diminished RagC/D levels, consistent with the notion that RagA/B stabilizes RagC/D by forming heterodimers (Sancak et al. 2008; Sekiguchi et al. 2001). Furthermore, the inactive mutant Rag complex (RagA/B GDP bound and RagC/D GTP bound) fails to respond to amino acids and restrains mTORC1 activity even under amino acid sufficiency (Kim et al. 2008; Sancak et al. 2008). These findings suggest that the presence of amino acids determines the guanine nucleotide state of the Rag GTPases and ultimately mTORC1 activation. However, it is worth noting that a new study using different biochemical methods revealed that amino acids activate mTORC1 without altering the guanine nucleotide loading of the Rag GTPases (Oshiro et al. 2014). Thus, whether amino acids regulate the guanine nucleotide loading of the Rag GTPases is currently under debate.

12.2.1 Rag GTPases at the Lysosome

Rag GTPases appear to reside at the lysosome in order to activate mTORC1. Whether or not the Rags can localize to other cellular compartments in response to stimuli is unknown. The active Rag complex (RagA/B GTP bound and RagC/D GDP bound) in amino acid sufficient conditions binds directly to mTORC1 via interaction with raptor (an essential subunit of mTORC1) and recruits it to the lysosome. How mTORC1 translocates to the lysosome is unknown. Some speculate that the Rag GTPases may come off at some point to retrieve mTORC1, but there is no data to support this. When amino acids are limiting, mTORC1 is scattered throughout the cell at undefined locations, but it is quickly redistributed to vesicles containing lysosome-associated membrane protein 2 (LAMP2; a lysosome marker) and RAB7 (late endosome marker) in response to amino acid stimulation (Sancak

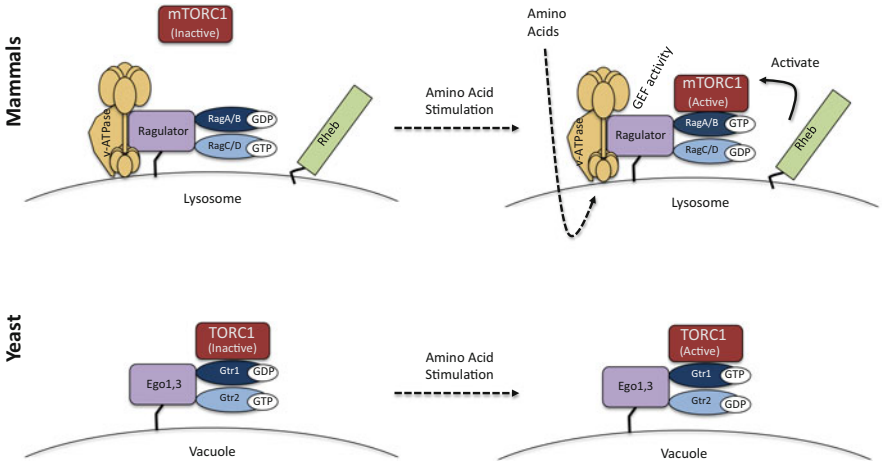

Fig. 12.1 Amino acid-induced Rag GTPase mTORC1 activation in mammals and yeast. Amino acids activate mTORC1 in both mammals and yeast. In mammals, amino acids activate mTORC1 at the lysosome (*top*). Under amino acid starvation, mTORC1 is distributed throughout the cell at undefined locations. The heterodimeric Rag complex is inactive at the lysosome, where RagA/B is GDP bound and RagC/D is GTP bound (*top*, *left*). In the current model, it is thought that amino acids accumulate in the lysosomal lumen, first communicating to the v-ATPase (*top*, *right*). The v-ATPase through binding interactions with the Ragulator somehow activates the GEF activity of the Ragulator, towards RagA/B exchanging GDP for GTP. RagA/B GTP bound and RagC/D GDP bound interact with and activate mTORC1 at the lysosome. In yeast, TORC1 remains at the vacuole (the lysosome equivalent) under both amino acid starvation and stimulation (*bottom*). Under amino acid starvation Gtr1 (RagA/B orthologue) is GDP bound and Gtr2 (RagC/D orthologue) is GTP bound (*bottom*, *left*). The interaction between Gtr1–Gtr2 is guanine nucleotide dependent, whereas TORC1 binds to the active form. However, whether TORC1 is anchored to vacuole by other components is not known. Upon amino acid stimulation the Gtr1–Gtr2 complex becomes active, where Gtr1 is GTP bound and Gtr2 is GDP bound, and TORC1 interacts with the Gtr1–Gtr2 and becomes activated (*bottom*, *right*)

et al. 2008). As expected, knockdown of the Rag GTPases blocks the amino acid-induced lysosomal localization of mTORC1 (Sancak et al. 2008). It is envisioned that, once at the lysosome, mTORC1 encounters Rheb, a potent mTORC1 activator that is controlled by growth factor signaling. The tuberous sclerosis complex (TSC) tumor suppressor is also thought to reside at the lysosome, which negatively regulates mTORC1 by acting as a GTPase activating protein (GAP) for Rheb (Dibble et al. 2012). Thus, the lysosome serves as an mTORC1 activation hub where mTORC1 can sense multiple stimuli, such as amino acids through the Rag GTPases and growth factors through Rheb.

Unlike in mammals, yeast TORC1 resides at the vacuole (the yeast equivalent of the lysosome) regardless of amino acid availability (Fig. 12.1). Therefore, TORC1 shuttling from an undefined location to the lysosome in response to amino acids appears to have evolved in higher eukaryotes (Binda et al. 2009). Nevertheless, consistent with the active Rag complex in mammals (RagA/B GTP bound and RagC/D GDP bound), the active yeast complex (Gtr1 GTP bound and Gtr2 GDP

bound) physically interacts with and contributes to TORC1 activation. This interaction is dependent on amino acid availability and guanine nucleotide loading (Binda et al. 2009).

12.2.2 Ragulator Complex

Rag GTPases reside on the lysosomal surface but lack a lipid-anchoring motif, implying that Rag binding proteins must anchor them there. The Ragulator complex was characterized shortly after identification of the Rags as a scaffold for the heterodimeric Rag complex at the lysosome. The pentameric Ragulator complex is composed of p18, p14, MP1, c7orf59, and HBXIP (encoded by LAMTOR1–5, respectively) (Bar-Peled et al. 2012; Sancak et al. 2010). The heterodimeric Rag GTPases and four Ragulator components are tethered to the lysosomal surface by p18, which associates with the membrane via its myristoylated and palmitoylated residues (Fig. 12.1). Ablation of specific Ragulator components abolishes Rag and mTORC1 lysosomal localization, thus preventing activation of mTORC1 (Bar-Peled et al. 2012; Sancak et al. 2010). Moreover, the Rag GTPase–Ragulator interaction is sensitive to amino acid availability. Rag GTPase–Ragulator binding strengthens under amino acid deprivation and weakens with amino acid stimulation (Bar-Peled et al. 2012). Thus, the Ragulator is essential in maintaining the heterodimeric Rag complex along with mTORC1 at the lysosome.

Ragulator orthologues in yeast have not yet been identified. However, in yeast TORC1 has been identified as a component of the vacuolar localized Ego complex (EGOC). EGOC contains Ego1, Ego3, Gtr1, and Gtr2. The Ragulator and the EGOC lack sequence similarity, but the structural conservation is noticeable. Studies have demonstrated that, like p18, Ego1 is palmitoylated and myristoylated and maintains the Gtr1–Gtr2 complex and TORC1 on the vacuolar membrane (Ashrafi et al. 1998; Kogan et al. 2010). Ego3 is structurally similar to the Ragulator components p14 and MP1 (Kogan et al. 2010; Zhang et al. 2012). Furthermore, roadblock domains have been identified in the high-resolution crystal structures of p14, MP1, HBXIP, and Gtr1–Gtr2 (Garcia-Saez et al. 2011; Kurzbauer et al. 2004; Lunin et al. 2004). Roadblock domains also appear in the C-terminal region of the Rag GTPases and C7orf59 based on structural predictions (Bar-Peled et al. 2012; Gong et al. 2011). Thus, the Rag GTPases and each component of the Ragulator complex, aside from the anchoring protein p18, appear to contain a roadblock domain. The functional significance of this domain is unknown, but it may be important for mediating protein–protein interactions among these proteins in the amino acid signaling cascade to mTORC1.

12.2.3 Vacuolar H^+-ATPase

The vacuolar H^+-ATPase (v-ATPase) controls mTORC1 activation in response to amino acids in both *Drosophila melanogaster* and mammalian cells through interaction with Rag GTPases and Ragulator on the lysosome surface (Zoncu et al. 2011a). The v-ATPase pumps protons into lysosomes to maintain the acidic lysosomal pH and function. Two domains comprise the v-ATPase: the peripheral cytosolic V_1 domain (containing eight subunits: A–H) and the integral membrane V_0 domain (has five subunits: a, d, c, c′, c″). The V_1 domain hydrolyzes ATP to drive proton translocation from the cytosol through the V_0 channel into the lysosome, acidifying it (Nishi and Forgac 2002).

Both the V_1 and V_0 domains interact with the Ragulator, whereas only the V_1 domain binds to the Rag GTPases. These interactions are thought to be sensitive to amino acid availability, like Rag GTPase-Ragulator binding. For example, the interaction between v-ATPase V_1 domain and the Ragulator and Rag GTPases is regulated by amino acids, strengthened by amino acid starvation, and weakened by amino acid stimulation. Chemical inhibition of the v-ATPase with Salicylihalamide A renders the V_1 domain–Rag and V_1 domain–Ragulator associations insensitive to amino acids (Zoncu et al. 2011a). In addition, chemical inhibition of the v-ATPase also desensitizes Rag GTPase–Ragulator binding to amino acid stimulation, suggesting that the v-ATPase is upstream of the Ragulator (Fig. 12.1) (Bar-Peled et al. 2012). Unlike the Ragulator, knockdown of v-ATPase subunits or v-ATPase chemical inhibitors do not affect Rag lysosomal localization. Despite having no role on lysosomal Rag localization, the knockdown of v-ATPase subunits precludes mTORC1 lysosomal localization and activation. ATP hydrolysis by the v-ATPase V_1 domain is important for Rag–mTORC1 interaction; however, there is currently no mechanistic explanation as to why this is the case (Zoncu et al. 2011a).

Although many unanswered questions remain, a recent model has emerged which proposes that amino acids within the lysosomal lumen communicate to the v-ATPase through an "inside-out" mechanism (an increase of amino acids inside the lysosomal lumen signals to and activates mTORC1, which resides on the outside of the lysosome; Fig. 12.1). The v-ATPase, through contact interactions, controls the Ragulator that anchors the Rag GTPases to the lysosome. When the Rag complex is activated (RagA/B GTP bound and RagC/D GDP bound) mTORC1 is recruited to the lysosome and activated. Multiple studies have identified additional Rag GTPase interacting proteins implicated in this pathway (Table 12.1). Amino acids are thought to communicate to the Rag-GTPases, which directly bind to and somehow redistribute mTORC1 to the surface of lysosomes. Rheb, a potent mTORC1 activator mediating growth factors, is also thought to reside at the lysosome. Thus, the lysosome serves as a hub coordinating multiple stimuli to achieve optimal mTORC1 activation and cell growth.

Table 12.1 Components that bind Rag GTPases involved in amino acid signaling to mTORC1

Component	Mammals (M) or Yeast (Y)	Function	mTORC1 activation	References
EGOC	Y	Consists of Ego1 and Ego3, anchors the Gtr1–Gtr2 complex to the vacuole	↑	Ashrafi et al. (1998), Kogan et al. (2010)
Folliculin	M	RagC/D GAP	↓	Tsun et al. (2013), Petit et al. (2013)
GATOR	M and Y	Multicomponent GATOR complex, thought to be a GAP for RagA/B (and yeast Gtr1)	↓	Bar-Peled et al. (2013), Panchaud et al. (2013)
LeuRS	M and Y	Cytosolic enzyme, charges leucine to cognate tRNA, reported as a RagD GAP in mammals	↑	Bonfils et al. (2012), Han et al. (2012)
mTORC1	M and Y	Conserved serine/threonine kinase, TORC1 in yeast	–	Laplante and Sabatini (2012), Zoncu et al. (2011a, b)
p62	M	Targets cargo for autophagy, binds to Rag complex	↑	Duran et al. (2011)
(PAT1)/ SLC36A1	M	Proton-assisted amino acid transporter	↑↓	Zoncu et al. (2011b), Ogmundsdottir et al. (2012)
Ragulator	M	Acts as a GEF for RagA/B, anchors the Rag complex to the lysosome	↑	Sancak et al. (2010), Bar-Peled et al. (2012)
TFEB	M	Transcription factor	↑	Martina and Puertollano (2013)
SH3BP4	M	Regulates endocytosis of transferrin receptor	↓	Kim et al. (2012)
Vam6	Y	Gtr1 (RagA/B) GEF	↑	Binda et al. (2009), Valbuena et al. (2012)
v-ATPase	M	Maintain PH homeostasis and lysosomal function	↑	Zoncu et al. (2011b)

12.3 Rag GTPase GEFs and GAPs

Identification of the guanine nucleotide exchange factors (GEFs) and the opposing GTPase activating proteins (GAPs) that modulate Rag GTPase is crucial to fully understand amino acid sensing by mTORC1. GEFs usually activate GTPases by

promoting the exchange of guanosine nucleotides, GDP to GTP. In contrast, GAPs act antagonistically to deactivate GTPases by increasing their intrinsic rate of GTP hydrolysis (Bos et al. 2007). As previously noted, the active Rag heterodimeric complex consists of RagA/B bound to GTP and RagC/D bound to GDP. Thus, the orchestration of the GEFs and GAPs for Rags GTPases will supply a better understanding of mTORC1 regulation by amino acids and cell growth.

12.3.1 Ragulator

Shortly after the Ragulator complex was identified as an anchor for the Rag GTPases at the lysosome, it was discovered to also be a GEF for RagA/B, promoting the exchange of GDP for GTP (Bar-Peled et al. 2012). RagA/B GTP bound is essential for the activation of the Rag complex and for mTORC1 lysosomal localization and activation (Kim et al. 2008; Sancak et al. 2008). The identification of the Ragulator as a GEF for RagA/B provided more mechanistic detail for the amino acid signaling cascade to mTORC1 and Rag guanine nucleotide loading. All five subunits of the Ragulator complex are required for RagA/B GEF activity (Bar-Peled et al. 2012). However, it appears that none of the Ragulator components contain any GEF-like catalytic domains. Guanine nucleotide-free RagA/B favors association with the Ragulator over guanine nucleotide-bound RagA/B, which is a common characteristic found in other GEF–GTPase interactions (Bos et al. 2007; Feig 1999). The Ragulator does not appear to have any GEF activity towards RagC/D (Bar-Peled et al. 2012). This is perhaps due to differences in the switch I and switch II regions between RagA/B and RagC/D. These switch regions are known to act as recognition motifs for GEF and GTPase interactions (Goldberg 1998).

12.3.2 Vam6

There are no identified orthologues of the Ragulator components in yeast. However, Vam6 has been reported to function as a Gtr1 (RagA/B orthologue) GEF in *Saccharomyces cerevisiae* (Binda et al. 2009; Valbuena et al. 2012). In addition to Vam6 promoting nucleotide exchange of Gtr1, Gtr1 interacts with Ego1 in a manner dependent on the GTP loading of Gtr1. Moreover, the association between Gtr1 and Ego1 was dramatically decreased in a Vam6-deleted strain (Binda et al. 2009). The mammalian orthologue of Vam6, VPS39, is involved in promoting late endosome to lysosome fusion. VPS39 does not appear to bind to or function as a GEF for RagA/B in mammals, possibly suggesting that the GEF for Gtr1 in yeast and RagA/B in mammals has diverged (Bar-Peled et al. 2012). There are other homologues of VPS39 in mammalian cells that have not been assessed as potential GEFs for RagA/B, such as TGFβRAP1 (Messler et al. 2011). Given the high sequence similarity between Gtr1 and RagA/B, and the importance of guanine

nucleotide binding in regulating TORC1, it is surprising that the GEF is not also conserved among yeast and mammals.

12.3.3 Leucyl-tRNA Synthetase

Two parallel, independent studies identified leucyl-tRNA synthetase (LeuRS) as a direct sensor for the amino acid leucine in regulating mTORC1 activation. LeuRS is an enzyme in the cytosol that is required for protein synthesis and catalyzes the ATP-dependent ligation of leucine to its corresponding tRNA. One study was done in yeast and the other in mammalian cells, with considerable differences in molecular details between the two studies (Bonfils et al. 2012; Han et al. 2012). In mammals, LeuRS was shown to translocate to the lysosome and directly interact with and function as a GAP for RagD in response to leucine stimulation (Han et al. 2012). Surprisingly, LeuRS could not function as a GAP for RagC, despite the high degree of sequence similarity. As previously mentioned, the active heterodimeric Rag complex consists of RagA/B GTP bound and RagC/D GDP bound. Thus, by LeuRS serving as a GAP for RagD, it positively regulates mTORC1 activation and cell growth in response to leucine stimulation. LeuRS was reported to bind to the C-terminus of RagD, which has been shown to be critical for contact–contact interactions of Gtr1–Gtr2 binding via the crystal structure and TORC1 activation in yeast (Han et al. 2012). One would speculate that LeuRS binding to the C-terminus of RagD would disrupt Rag heterodimerization, protein stability, and function. Importantly, the arginine residue in human LeuRS that is typically necessary for GAP activity is not conserved in *Drosophila melanogaster*. This is rather concerning because the activation of mTORC1 by amino acids occurs in all eukaryotes.

In yeast, LeuRS was shown to regulate Gtr1 (the RagA/B orthologue) and not Gtr2 (the RagC/D orthologue). Specifically, LeuRS was shown to bind to the active form of Gtr1 (Gtr1-GTP bound), preventing GTP hydrolysis and locking Gtr1 in its active form, thereby promoting TORC1 activation (Bonfils et al. 2012). Leucine stimulation is thought to cause a conformational change in LeuRS, in which it associates with Gtr1 bound to GTP at the vacuole. In addition, LeuRS was reported to interact with a different domain on Gtr1 than the domain suggested to mediate the LeuRS–RagD interaction in mammals. It was purposed that LeuRS binding to Gtr1 bound to GTP blocked an unidentified GAP for Gtr1. Future work is needed to better work out the precise details and role of LeuRS in terms of mTORC1 activation. Although the role of LeuRS is unclear, both studies suggest that LeuRS promotes mTORC1 activation and could potentially be a direct sensor for leucine. How LeuRS sensing by leucine in the cytoplasm integrates into the model described above, where amino acids accumulate within the lysosomal lumen (Fig. 12.1), and how other amino acids are sensed are unknown. This work suggests that multiple amino acid sensing/signaling pathways to mTORC1 may exist.

12.3.4 GATOR Complex

The multimeric GAP activity towards Rags (GATOR) complex promotes GTP hydrolysis of RagA/B. GATOR interacts with the Rag GTPases and consists of eight proteins divided into two subcomplexes, referred to as GATOR1 and GATOR2 (Fig. 12.2) (Bar-Peled et al. 2013). GATOR1 displays GAP activity and contains DEPDC5, Nprl2, and Nprl3. However, it is unclear which component in the GATOR1 complex is responsible for the GAP activity. Interestingly, DEPDC5 and Nprl2 are frequently mutated in human cancer (Lerman and Minna 2000; Li et al. 2004; Seng et al. 2005). GATOR2 consists of Mios, Sec13, Seh1l, WDR24, and WDR59 (Bar-Peled et al. 2013), and functions to inhibit GATOR1. Inactivation of GATOR1 renders mTORC1 resistant to amino acid deprivation, consequently leaving mTORC1 in a constitutively active state. In contrast, knockdown of GATOR2 inhibits mTORC1, and epistasis analysis reveals that GATOR2 negatively regulates DEPDC5.

In parallel, yeast studies revealed similar complexes which regulate TORC1 called SEACIT and SEACAT (Fig. 12.2) (Panchaud et al. 2013). SEACIT (similar to GATOR1 in mammals) contains Iml1, Npr2, and Npr3. SEACAT (similar to GATOR2 in mammals) contains Sea4, Sec13, Seh1, Sea2, and Sea3. Similar to the mammalian studies, the GATOR1-like complex SEACIT has GAP activity towards Gtr1 (RagA/B orthologue) in *Saccharomyces cerevisiae*. DEPDC5 yeast homologue Iml1 interacts with Gtr1, stimulating its GTPase activity at the vacuole under amino acid starvation and thus leaving TORC1 inactive. Iml1 contains a conserved arginine that is a critical catalytic residue of GAPs. Although this arginine does not reside within a domain containing recognizable homology to GAPs for other small GTPases. The yeast homologues of Nprl2 (Npr2) and Nprl3 (Npr3) have previously been described to regulate TORC1 through genome-wide screening (Neklesa and Davis 2009).

Noteworthy, Npr2 and Npr3 contain a N-terminus longin domain, the structure of which is closely related to the roadblock domains and may serve as a platform for Rag GTPase interactions (Levine et al. 2013). Like the roadblock domains, additional repeated domains emerged when closely analyzing the GATOR complexes. Proteins in the GATOR2 subcomplex contain WD repeats. mLST8, a component of mTORC1, also contains WD repeats. Also, a negative regulator of mTORC1 called Deptor (originally called DEPDC6) contains DEP domains like DEPDC5. Although, the GATOR complexes are a very important discovery for better understanding the Rag GTPases, how GATOR senses amino acids is unknown. Furthermore, the mechanistic detail of how GATOR2 regulates GATOR1 is undetermined.

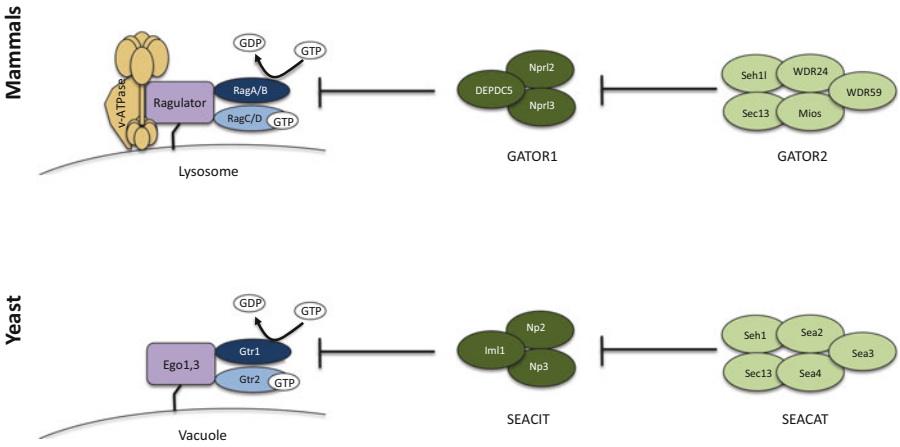

Fig. 12.2 Rag GTPase regulation by the multimeric GATOR complex. Under amino acid starvation conditions the multimeric GATOR complex negatively regulates the Rag GTPases in mammals and yeast. In mammals, the GATOR complex is comprised of two subcomplexes called GATOR1 and GATOR2 (*top*). GATOR1 acts as a GAP for RagA/B exchanging GTP for GDP. GATOR1 inactivates the Rag complex and thus mTORC1 activation. GATOR2 is thought to inhibit the GATOR1 complex, although the mechanistic details are obscure. In yeast, SEACIT (similar to GATOR1) acts as a GAP for Gtr1 (RagA/B orthologue) exchanging GTP for GDP, inhibiting TORC1 (*bottom*). SEACAT (similar to GATOR2) negatively regulates SEACIT. How amino acids specifically regulate these complexes is not known

12.3.5 Folliculin

Two recent studies identified folliculin (FLCN) as an important component involved in amino acid signaling to mTORC1. A human disease referred to as Birt–Hogg–Dubé syndrome arises from loss-of-function mutations in the FLCN gene. Birt–Hogg–Dubé syndrome is characterized by fibrofolliculomas (benign hair follicle tumors) and a strong predisposition towards the development of pneumothorax, pulmonary cysts, and renal carcinomas (Birt et al. 1977; Nickerson et al. 2002). Interestingly, FLCN is evolutionarily conserved, but its precise molecular function is unknown (Schmidt 2013; van Slegtenhorst et al. 2007). Both studies revealed FLCN to be critical for mTORC1 activation and lysosomal localization (Petit et al. 2013; Tsun et al. 2013). Moreover, under amino acid starvation conditions, FLCN was recruited to the lysosome where it directly interacted with the Rag GTPases. A differing detail between the two studies is that one study found that FLCN, promoted by FNIP1 (a folliculin binding protein), bound to the GTPase domain of RagA (Petit et al. 2013), whereas the other study identified folliculin-FNIP as a GAP for RagC/D (Tsun et al. 2013). There is evidence in yeast that FLCN plays a role in the amino acid signaling cascade to TORC1. Deletion mutants for the budding yeast orthologues of FLCN (LST7) and the Rags (Gtr1 and Gtr2) exhibited similar growth sensitivities to various environmental and chemical insults, using a chemical genomic screen (Hillenmeyer et al. 2008).

12.4 TFEB Regulation by Rag GTPases

Rag GTPases have been documented to modulate the transcription factor EB (TFEB) activity. As a member of the basic helix-loop-helix leucine zipper family of transcription factors, TFEB controls lysosomal biogenesis by positively regulating genes belonging to the CLEAR (coordinated lysosomal expression and regulation) network (Palmieri et al. 2011; Sardiello and Ballabio 2009; Sardiello et al. 2009). The first 30 amino acids of TFEB interact with and are recruited to the lysosome by the switch regions of the GTPase domain of the Rags (Martina and Puertollano 2013). At the lysosome, in the presence of amino acids it has been purposed that mTORC1 inhibits TFEB by phosphorylating it on several residues including serine 211. Phosphorylation of serine 211 creates a recognition site for the cytosolic chaperone 14-3-3, sequestering TFEB in the cytosol. In contrast, amino acid withdrawal results in mTORC1 inactivation and the dissociation of the TFEB-14-3-3 complex. This dissociation allows TFEB to be transported back into the nucleus, resulting in TFEB-dependent transcription of numerous genes, including lysosomal genes (Martina and Puertollano 2013; Roczniak-Ferguson et al. 2012). Taken together, the Rag GTPase may also play a role in the control of gene expression to modulate lysosome biogenesis.

12.5 Concluding Remarks and Future Directions

Rag GTPases have unique characteristics that separate them from other GTPases and have been characterized as important components involved in the amino acid signaling cascade to mTORC1. Increased mTORC1 activation occurs in many human cancers, and inhibition of mTORC1 has proven efficacious in several clinical therapies. Multiple groups have made significant advancements in detailing the structural and functional features of the Rag GTPases. In addition, Rag GTPase GEFs and GAPs have been identified, as well as multiple Rag-interacting proteins. Future studies focused on the molecular details involved in Rag-induced mTORC1 activation will lead to a better understanding of cell growth and also clarify the role of this pathway in disease. Identifying precise amino acid sensors that control Rag guanine nucleotide binding could shed light onto the role of this signaling pathway in human pathogenesis, in particular cancer. Furthermore, we know that Rag GTPases are essential in mTORC1 activation, but whether they have additional roles besides regulating mTORC1 will be of great interest.

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