

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; Pfeiffer 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

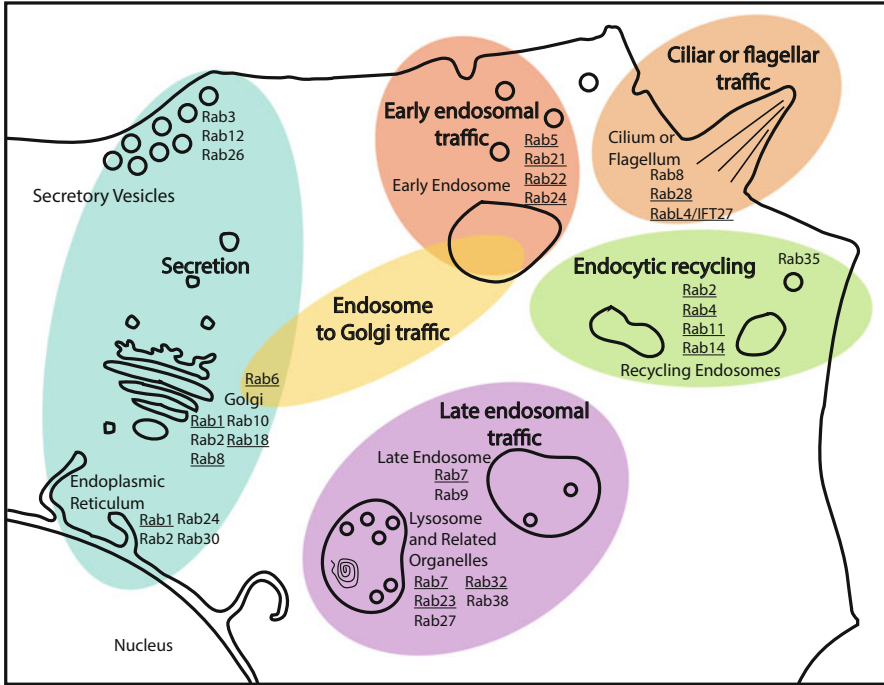


Fig. 2.1 Compartmentalization of Rab GTPases within eukaryotic cells. Rab GTPases are localized to specific organelles. Six Rab supergroups that were defined by Klöpper (Klöpper et al. 2012) are highlighted as functional groups. Underlined Rab GTPases were identified to be present in the LECA in both 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

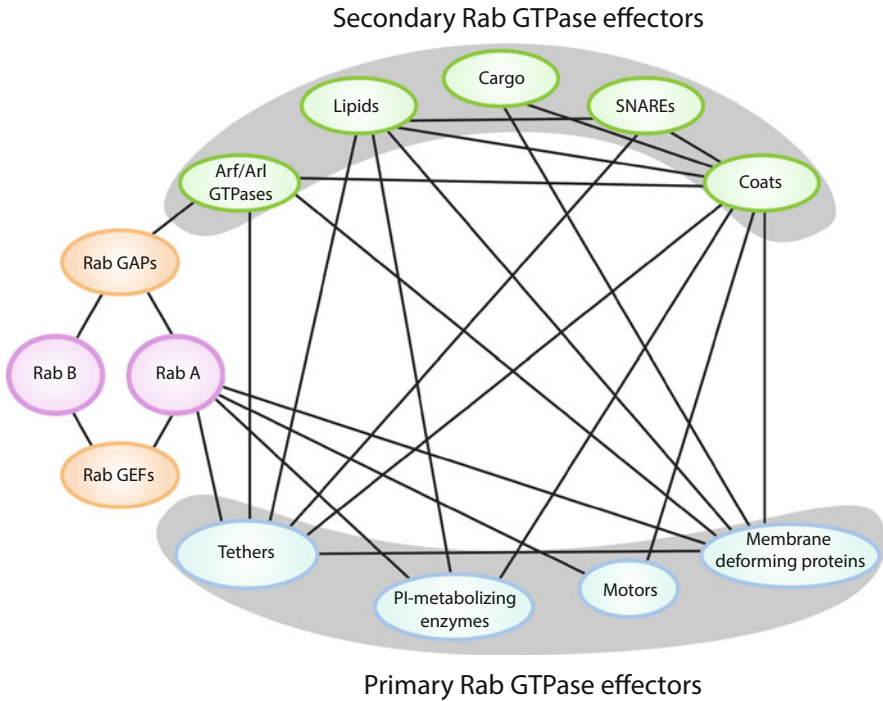


Fig. 2.2 Rab GTPases play a central role in the formation of membrane domains. Here we describe a Rabcentric model summarizing the known possible interactions (*lines*) of Rabs GTPases with other membrane-defining factors. Rab proteins (*purple*) directly interact with GAPs and GEFs (*orange*), which 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

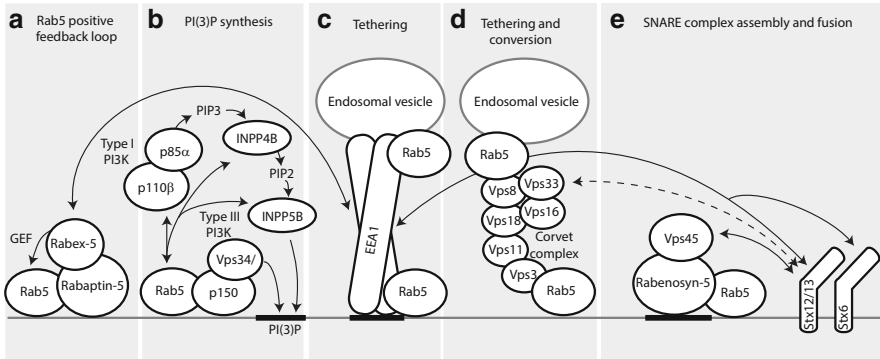


Fig. 2.3 Multiple steps in Rab5 membrane domain formation on endosomes. (a) The establishment of a Rab5 membrane domain involves the binding of Rab5 to a complex of Rabaptin-5 and Rabex-5. As Rabex-5 is a GEF for Rab5, a positive feedback loop is created, resulting in the recruitment of more Rab5. (b) Rab5 directly binds Type I and Type III PI3K 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 p85 α -p110 β (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 knock-down 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 PI4K β 1 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 Zn²⁺-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 Myosin Vb (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; Pfeiffer 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

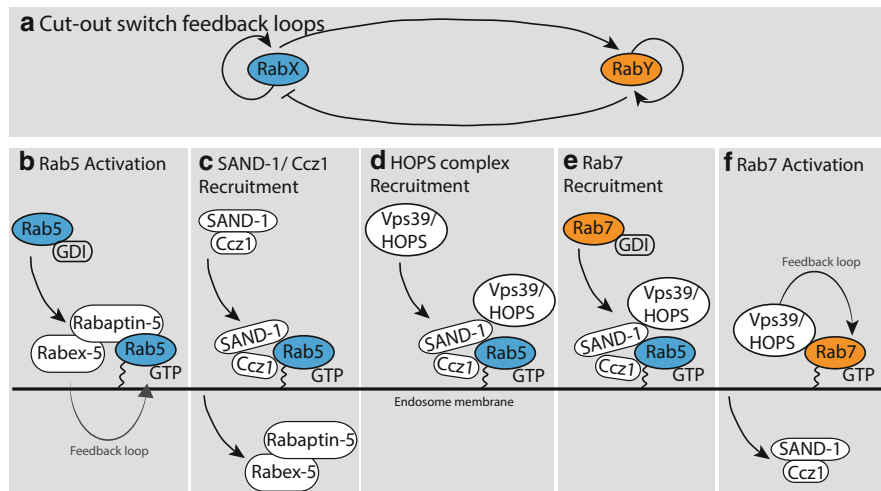


Fig. 2.4 Conversion model of early Rab5 endosomes to late Rab7 endosomes. The conversion of an endosome is a systematic series of events involving Rab5 and Rab7, their respective GEFs and GAPs, and the switch protein, SAND-1/Mon1. **(a)** Cutout switch feedback loops. Both Rab GTPases are under the control 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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