RAB PROTEINS AS MEMBRANE ORGANIZERS

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Cellular organelles in the exocytic and endocytic pathways have a distinctive spatial distribution and communicate through an elaborate system of vesiculo-tubular transport. Rab proteins and their effectors coordinate consecutive stages of transport, such as vesicle formation, vesicle and organelle motility, and tethering of vesicles to their target compartment. These molecules are highly compartmentalized in organelle membranes, making them excellent candidates for determining transport specificity and organelle identity.

How do transport vesicles or tubular structures generated in one compartment encounter their target organelle and engage in membrane fusion? A typical transport reaction can be viewed as a four-step process that consists of formation of a vesicle or tubular intermediate, movement of the vesicle towards its target compartment, tethering/docking with the acceptor membrane and, ultimately, fusion of the lipid bilayers. The specificity of membrane tethering and fusion is critical to preserve organelle identity and the proper flow of cargo within the cell. The first specific event is the Rab-mediated tethering of an incoming vesicle to the correct target organelle. Following this, the specific topological pairing of COGNATE SNARES (soluble NSF attachment protein receptor, where NSF stands for *N*-ethylmaleimide-sensitive fusion protein) between the two bilayers (SNARE pins) ensures precision in the fusion event[1–4] (see the review by Chen and Scheller on page 98 of this issue). SNAREs are enriched in certain organelles, which helps to identify the correct target and to limit nonspecific fusion events. However, during vesicular transport, SNAREs inevitably spread throughout many cellular compartments. Any given organelle will contain SNARE complexes that must remain inactive until they return to their specific place of function. An additional layer of regulation is therefore inevitable to ensure that *trans*-SNARE complexes fuse membranes only at the appropriate time and in the correct place.

In this review, we discuss how Rab GTPases and their EFFECTORS fit the criteria for a regulatory system that provides the complementary specificity to SNARE complexes during membrane tethering and fusion. The discovery of molecular interactions between Rab effectors and components of the SNARE machinery provides a new understanding of how Rab proteins directly regulate SNARE function. We begin by summarizing the role of Rab proteins and the identification of some of their effectors. We then describe recent evidence indicating that Rab effectors are not randomly distributed on the organelle membrane but are clustered in distinct functional domains. The model emerging from these observations is that, rather than being mere regulators of SNARE protein complexes, Rab GTPases and their effectors are primary determinants of compartmental specificity in the organelles of eukaryotic cells.

COGNATE SNARES
SNAREs on opposite membranes that are destined to form *trans*-SNARE complexes to mediate fusion.

NSF
Molecular chaperone involved in recycling SNAREs after one round of fusion.

EFFECTOR
A protein or protein complex that binds the GTPase directly and in a GTP-dependent manner and is required for the downstream function determined by that GTPase.

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Heterogeneity of Rab effectors

Rab proteins constitute the largest family of monomeric small GTPases. Eleven Rab (Yptp/Sec4p) proteins are expressed in the yeast *Saccharomyces cerevisiae* but there might be as many as 63 family members in humans (J. Schultz and P. Bork, personal communication) as estimated from expressed-sequence tags (ESTs) and the sequenced human genome. This increased complexity throughout evolution reflects a greater need for cell organization and intracellular transport in the different cell types of multicellular organisms. Numerous studies have established that Rab proteins are distributed to distinct intracellular compartments and regulate transport between organelles (reviewed in REF. 5, see FIG. 1 and TABLES 1,2) (see extra online material). The regulatory principle of Rab proteins, as for other GTPases, lies in

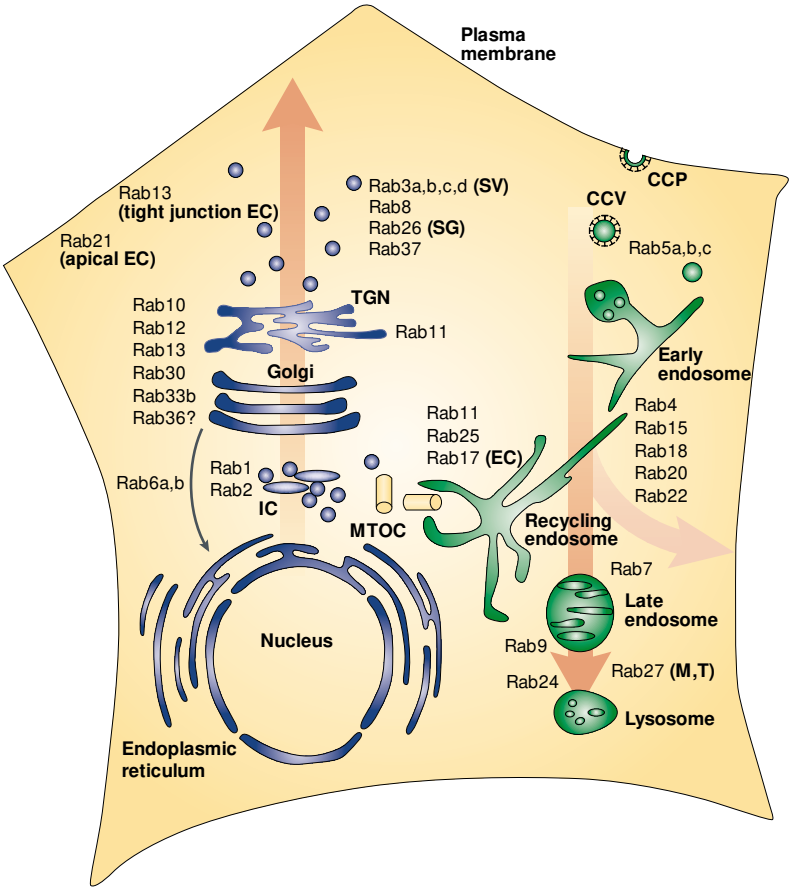


Figure 1 | **Map of intracellular localization of Rab proteins.** Summarizes the intracellular localization of Rab proteins in mammalian cells. Some proteins are cell- (for example, Rab3a in neurons) or tissue-specific (for example, Rab17 in epithelia) or show cell-type-specific localization (for example, Rab13 in tight junctions). (CCV, clathrin-coated vesicle; CCP, clathrin-coated pit; EC, epithelial cells; IC, ER–Golgi intermediate compartment; M, melanosomes; MTOC, microtubule-organizing centre; SG, secretory granules; SV, synaptic vesicles; T, T-cell granules; TGN, *trans*-Golgi nextwork.)

their ability to function as molecular switches that oscillate between GTP- and GDP-bound conformations. The GTP-bound form is considered the 'active' form. However, with respect to the physiology of the regulated process, the most important feature is the ability of GTPases to cycle regularly between GTP- and GDP-bound states. This cycle imposes temporal and spatial regulation to membrane transport, with the Rab proteins acting like timers whose clocks are set depending on the (intrinsic and catalysed) rates of nucleotide exchange and hydrolysis. Their on/off regulatory function is restricted to the membrane compartments where they are localized.

Each transport step requires that the activated Rab proteins bind to soluble factors that act as 'effectors' to transduce the signal of the Rab GTPase in the transport mechanism. Many established or putative effector proteins and regulators have been identified and characterized (TABLES 1,2). Given the structural conservation of Rab GTPases and SNAREs, one might expect that Rab effectors could also be grouped in a family of structurally conserved proteins. This does not seem to be the case. The structural heterogeneity shown by Rab effectors implies that these are highly specialized molecules whose activities are exclusively tailored for individual organelles and transport systems. However, some Rab effectors do share structural features. For example, p115/Uso1p, Rabaptin-5 and early endosome antigen 1 (EEA1), all contain predicted coiled-coil regions, and Rab3-interacting molecule (Rim1), EEA1 and Rabenosyn-5 contain zinc-fingers. A more comprehensive analysis of Rab effectors, taking into account their structural and functional properties, will be necessary to categorize these molecules.

Regulation of intracellular transport

It is well established that Rab proteins function in the tethering/docking of vesicles to their target compartment, leading to membrane fusion. However, Rab proteins have also been implicated in vesicle budding and, more recently, in the interaction of vesicles with cytoskeletal elements. The finding that Rab proteins have several functions suggests that all steps of vesicle transport could be coordinated by the same regulatory machinery.

Membrane tethering. Over the past few years, several studies have shown that membrane tethering is a conserved mechanism that depends on Rab effectors, rather than on SNARE complexes. Depending on the system, the recruitment of Rab and tethering effector proteins can be either symmetrical or asymmetrical between donor and acceptor membranes. In the yeast secretory pathway, tethering of endoplasmic reticulum (ER)-derived vesicles to the Golgi complex depends on the membrane recruitment of Uso1p by Ypt1p but not on SNAREs[6]. Whereas no direct interaction between Uso1p and the Rab protein could be detected in the latter study, the mammalian homologue of Uso1p, p115 was recently shown to bind directly to Rab1 (REF. 7). However, there are mechanistic differences between mammalian and yeast ER-to-Golgi transport. Whereas Rab1 recruits p115 onto COPII (coat protein 2) VESICLES already at the budding step, in yeast the requirement for Ypt1p might be only at the level of the target membrane[8]. Beside these proteins, a multi-protein complex called TRAPP (transport protein particle) also targets ER-derived vesicles to the Golgi apparatus[9]. The TRAPP complex accelerates nucleotide exchange on Ypt1p, probably in the Golgi[10] where this GTPase is essential[8]. The precise function of the complex and its tethering role in relation with Ypt1p activation awaits further analysis.

Delivery of post-Golgi vesicles to the plasma membrane in yeast depends on Sec4p and the tethering factor that interacts with this Rab protein is also a multi-protein complex — the exocyst. The exocyst was identified by Novick and co-workers as a complex of seven proteins that are specifically required for exocytosis[11]. An equivalent complex exists in mammals[12]. One of its subunits, Sec3p, marks the sites of exocytosis on the plasma membrane in yeast[13]. The exocyst mediates vesicle targeting and, through the Sec15p subunit, interacts specifically and directly with Sec4p in a GTP-dependent manner[14]. These data raise several interesting questions. How

EEA1
The antigen involved in a human autoimmune disease.

COPII VESICLES
Coated vesicles involved in transport from the endoplasmic reticulum to the Golgi.

Table 1 | **Rab proteins and their effectors**

Rab	Rab function	Direct effector	Effector function	Rab specificity	Effector partners	Partner features
Rab1	• ER–Golgi transport	p115	• Tethering • Sequestering SNAREs into budded vesicles	Rab1–GTP	Giantin GM130	• Tethering of COPI-coated vesicles to Golgi
		PRA1	• Rab receptor (proposed)	Rab1 Rab3 Rab4b Rab5a Rab5c	VAMP2	• v-SNARE involved in bilayer fusion
Rab3	• Rab3a: synaptic vesicle and chromaffin granule secretion • Rab3b, c, d: regulated secretion	Rabphilin-3	• Potentiates fusion	Rab3–GTP	α-actinin Rabaptin-5	• Crosslinks actin filaments into bundles • Stimulated by Rabphilin-3 interactions • Also binds Rabaptin-5, an effector of Rab5 and Rab4
		RIM1 RIM2	• Membrane fusion	Rab3–GTP	RIM–BP1	• Contains fibronectin type III repeats and SH3 domains
		Calmodulin	• Confers calcium sensitivity to protein interactions	Rab3	Many	• Multiple functions
Rab4	• Localized to early/recycling endosomes • Role in sorting/recycling in early endosomes	Rabaptin-5, Rabaptin-5β Rabaptin-4	• Activates Rab5 through complex with Rabex-5 • Implicated in protein sorting and recycling	Rab4–GTP Rab5–GTP	Rabex-5	• Nucleotide exchange factor
Rab5	• Ligand sequestration at plasma membrane • CCV–EE and EE–EE fusion • Endosome motility	Rabaptin-5 Rabaptin-5β	• Stabilizes Rabex-5 recruitment	Rab5–GTP Rab4–GTP	Rabex-5 Rabphilin-3	• Nucleotide exchange factor
		EEA1	• Tethering, core fusion component	Rab5–GTP	Syntaxin13 Syntaxin6	• t-SNAREs essential for bilayer fusion
		p150	• Class III PI(3)K regulatory subunit	Rab5–GTP	hVps34	• Class III PI(3)K catalytic subunit
		p110β	• Class I PI(3)K catalytic subunit	Rab5–GTP	p85-α	• Class I PI(3)K regulatory subunit
		Rabenosyn-5	• Required for CCV–EE and EE–EE fusion	Rab5–GTP Rab4–GTP	hVps45	• Regulates SNARE complex formation or disassembly
Rab6	• Retrograde Golgi–ER and intra-Golgi transport	Rabkinesin-6	• Vesicle motility • Cytokinesis	Rab6–GTP	Microtubules	
Rab8	• TGN–plasma membrane traffic (basolateral in epithelial cells)	Rab8IP	• Stress-activated protein kinase	Rab8–GTP		
Rab9	• Late endosome to Golgi	p40	• Stimulates fusion	Rab9–GTP		
Rab11	• Recycling through perinuclear recycling endosomes • Plasma membrane–Golgi traffic	Rab11BP	• Unclear	Rab11–GTP	mSec13	• Coat component of COPII vesicles
Rab13	• Involved in the formation of the tight junction	δ-PDE	• Extracts Rab13 from membrane	Rab13		
Rab33b	• Intra-Golgi transport	Rab33b-BP	• Probably regulates motility of Rab33 vesicles	Rab33b–GTP		
Ypt1p	• ER–Golgi	Uso1p	• Tethering of ER-derived vesicles	Recruitment regulated by Ypt1p		
		Yp1p–Yif1p complex	• Ypt GTPase binding to the Yip1p–Yif1p complex essential for vesicle docking and fusion	Ypt1p, Ypt31p Sec4p		• Integral membrane protein • Possible receptor and GDI-releasing factor
Sec4p	• Delivery of TGN-derived vesicles into the bud	Sec15p	• Tethering through interaction of vesicular Sec15p and Sec10p with target complex in the bud	Sec4p–GTP	Exocyst	• Marks the site for docking or fusion
Ypt51p/ Vps21p	• Golgi–endosome and plasma membrane–endosome transport	Vac1p	• TGN–Golgi transport	Ypt51p–GTP	Vps45p Pep12p	• Regulates SNARE complex formation or disassembly • Pep12p is a t-SNARE
Ypt7p	• Vacuole fusion	Vam2p–Vam6p	• Tethering and nucleotide exchange activity	Ypt7p–GTP	HOPS complex Vam3p SNARE complex	• Links SNAREs with Ypt activation • Marks the site for tethering/fusion
			• Budding of AP3 vesicles?		δ-adaptin	• AP3-vesicle formation at the Golgi

(CCV, clathrin-coated vesicle; EE, early endosome; ER, endoplasmic reticulum; PDE, phosphodiesterase; PI(3)K, phosphoinositol-3-OH kinase; SH3, Src homology region 3 domain;TGN, *trans*-Golgi network; Vamp, vesicle-associated membrane protein.)

Table 2 | **Regulatory proteins**

Rab/Ypt	GAP	GEF
Rab1	?	Mss4
Rab3	Rab3-GAP	Mss4, possibly Rabin 3
Rab5	Tuberous sclerosis 2 (?) RN-Tre	Rabex5
Rab6	GAPcenA	?
Rab8	?	Mss4
Rab10	?	Mss4
Ypt1p	?	TRAPP* Dss4p
Ypt51p/ Vps21p	Gyp1p, Gyp3p	Vps9p
Ypt6p	Gyp2p Gyp3p, Gyp4p, Gyp6p	Ric1p–Rigp1p
Ypt7p	Gyp4p, Gyp7p	HOPS‡
Sec4p	Gyp1p, Gyp2p/Mdr1p, Gyp3p, Gyp4p/Msb4p	Sec2p Dss4p

*TRAPP complex: Trs20p, Trs23p, Trs31p, Trs33p, Trs65p, Trs85p, Trs120p, Trs130p, Bet3p, Bet5p. ‡HOPS complex: Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, Vps41p.

and where is assembly of this complex regulated? What is the role of each subunit and that of Sec4p in this process? What determines localization of Sec3p?

In the early ENDOCYTIC PATHWAY, Rab5 regulates clathrin-coated-vesicle-mediated transport from the plasma membrane to the early endosomes as well as homotypic early endosome fusion[15,16]. EEA1 is the Rab5 effector that mediates tethering/docking of early endosomes[17]. EEA1 is a largely coiled-coil protein that contains two zinc-fingers and two Rab5-binding domains at the amino and carboxyl termini[18]. Considering that Rab5 is also required on both donor and acceptor membranes for fusion to occur[19,20], EEA1 could bridge two membranes that bear Rab5 (see below).

A symmetrical requirement for a Rab protein in vesicle docking and fusion has also been determined for Ypt7p in yeast vacuole fusion[21,22]. Ypt7p binds to and regulates the membrane localization of a multi-protein complex (HOPS, which stands for homotypic fusion and vacuole protein sorting; also referred to as Class C Vps protein complex[23]) which includes the Vam2p/Vps41p and Vam6p/Vps39p proteins[24,25]. As these proteins have been implicated in vacuolar membrane docking[26], HOPS seems to be the effector that mediates Ypt7p-dependent tethering. Consistent with the symmetrical requirement for Ypt7p, the HOPS complex is also needed on each vacuole partner undergoing homotypic fusion[26]. Furthermore, HOPS stimulates nucleotide exchange on Ypt7p[27]. So, as shown earlier for the Rabaptin-5–Rabex-5 complex of Rab5 (REF. 28), HOPS couples nucleotide exchange on a Rab protein to effector recruitment and function.

Vesicle budding. More elusive is the role of Rab proteins in budding, for which there is contradictory evidence depending on the experimental system. For example, *in vivo* studies have indicated a possible role for Rab1 in budding of vesicles from the ER[29] and for Rab9 from endosomes directed to the *trans*-Golgi network (TGN)[30]. More recently, however, Rab1 has been shown not to be essential for COPII vesicle formation *in vitro*, although it commits the vesicles to targeting and fusion[7]. In yeast, Ypt1p (Rab1) is also not needed for COPII vesicle formation but is exclusively required on target Golgi membranes[8,31].

A function in vesicle formation has also been attributed to Rab5. Rab5, which modulates the half-life of clathrin-coated pits (CCV) on the plasma membrane *in vivo*[16], is required for vesicle formation *in vitro*[32]. Consistent with this finding, overexpression of RN-Tre, a newly identified Rab5 GAP (GTPase-activating protein), downregulates Rab5 and inhibits receptor internalization[33].

One component of the Ypt7p (Rab7)-tethering complex HOPS, Vam2p/Vps41p[24], has also been implicated in the budding of vesicles from the Golgi[34], although Ypt7p itself has not yet been directly involved in Golgi budding events[26].

Thus, depending on the specific transport event, Rab proteins might directly or indirectly influence vesicle budding. They could regulate the concentration and/or assembly of coat components or help to incorporate cargo molecules selectively into the nascent vesicles. Alternatively, their presence in an active state might function as a checkpoint that ensures the delivery of a vesicle to its appropriate target compartment.

Vesicle motility. The task of Rab proteins is not restricted to membrane budding and fusion. More recently, these GTPases have been shown to determine the distribution of cellular compartments by regulating the movement of vesicles and organelles along cytoskeletal filaments. A role for Rab6 in microtubule-dependent transport has been inferred from the discovery that this GTPase interacts with a kinesin-like protein, Rabkinesin-6 (REF. 35), which is important for cytokinesis[36]. Rab5 regulates both the attachment of early endosomes to, and the motility along, microtubules[37]. There are also functional connections between Rab proteins and motors of the actin cytoskeleton. Genetic interactions have been uncovered in yeast between Sec4p and the myosin heavy chain Myo2p, indicating a possible mechanism whereby vesicles are propelled by motor proteins along polarized actin cables towards the site of exocytosis[38,39]. Another potential link between Rab and myosin proteins can be deduced from studies of a human disease, Griscelli syndrome. This is a rare, autosomal recessive disorder that is characterized by defective pigmentation of the skin and hair due to an aberrant accumulation of melanosomes in melanocytes. Mutations in two human genes have been associated with the disease, one in the *MYO5A* gene and the second in the *RAB27A* gene[40]. Interestingly, the same proteins are lost in the mouse mutants *dilute* (*myosin-VA*) and *ashen* (*Rab27a*), which are defective in pigment granule transport[41,42]. As Rab27a and myosin-VA

CCP
Area of the plasma membrane where receptors and the clathrin machinery are concentrated, preparing to form a vesicle.

function in the same pathway of melanosome transport in melanocytes, it will be interesting to see whether Rab27a and myosin-VA can directly interact.

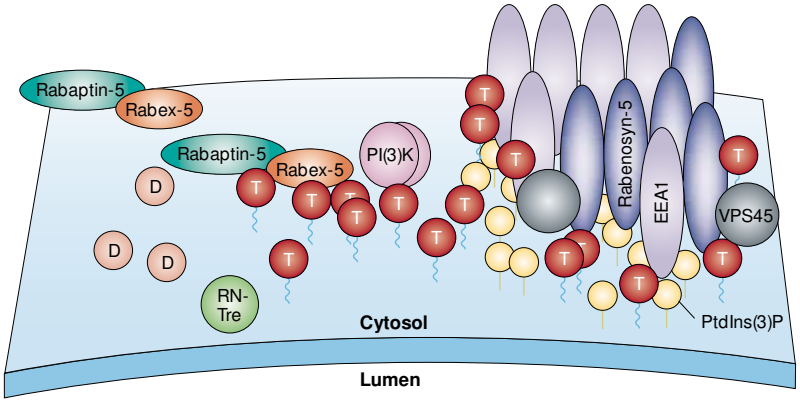
Multiplicity of Rab5 effectors

The identification of interacting molecules has revealed an extraordinary complexity of the machinery downstream of Rab5. Using an affinity-chromatography procedure, it was possible to identify >20 polypeptides from bovine brain cytosol that interact directly or indirectly with the GTP-bound form of Rab5 (REF. 17). An important principle emerging from this analysis is that Rab5 effectors function in a cooperative fashion (BOX 1).

The first Rab5 effector identified and found to be essential for early endosome fusion was Rabaptin-5 (REF. 43). Rabaptin-5 forms a complex with another protein, Rabex-5, which catalyses nucleotide exchange on Rab5 (REF. 28). Upon activation of Rab5 by Rabex-5, the Rabaptin-5–Rabex-5 complex induces its own membrane recruitment through Rabaptin-5 (Lippe *et al.* manuscript in preparation). This positive-feedback loop counteracts GTP hydrolysis[44] and is thought to create a microenvironment that is enriched in active Rab5 on the membrane where other Rab5 effectors are recruited[28].

How does this local clustering of activated Rab5 proteins regulate the tethering machinery? The carboxy-terminal end of the tethering factor EEA1 contains two structural elements that are essential for targeting to the early endosome membrane[45]. One is the FYVE finger, a zinc-finger that specifically binds to phosphatidylinositol-3-phosphate, PtdIns(3)P[46,47], and a Rab5-binding site located immediately upstream of the FYVE finger[18]. The discovery that phosphatidylinositol-3-OH kinases (PI(3)Ks) are Rab5 effectors[48] is

Box 1 | Rab5 effectors cluster in a Rab5 domain



The complex network of Rab5 regulators and effectors involves positive feedback loops and, according to the model presented in the figure, is designed to generate a local amplification of active Rab5 and the clustered recruitment of Rab5 effectors on the early endosome membrane.

Rab5-GTP (T in the figure) is unstable on the early endosome where it undergoes continuous cycles of GTP hydrolysis (Rab-GDP is shown as D in the figure), catalysed by RN-Tre[33] and nucleotide exchange[44]. The first feedback loop is due to the Rabaptin-5–Rabex-5 complex that activates Rab5 through the nucleotide-exchange activity of Rabex-5 and gets recruited on the early endosome membrane through Rabaptin-5. In this case, the product of the reaction (Rab5-GTP) recruits the enzyme. A second feedback loop is due to the cooperativity between effectors. Active Rab5 interacts with the hVPS34-p150 phosphoinositol-3-OH kinase (PI(3)K), thus coupling phosphatidylinositol-3-phosphate (PtdIns(3)P) production to Rab5 localization. The concomitant presence of Rab5 and PtdIns(3)P allows the recruitment of the Rab5 FYVE effectors early endosome antigen 1 (EEA1) and Rabenosyn-5. It is also known that EEA1, Rabaptin-5 and Rabex-5 form high-molecular-weight oligomers on the early endosome membrane. Because the oligomers also incorporate the nucleotide-exchange factor for Rab5, Rabex-5, the effectors themselves feedback on the recruitment and clustering of Rab5 within a limited area of the early endosome.

But what determines the specific targeting of Rab5 to the early endosome to initiate a Rab domain? Candidates for Rab receptors and GDI-releasing factors have been found[97–101] but, eventually, their localization also needs to be explained. Similarly, what determines the targeting of hVPS34/p150? Important factors for the generation of a Rab domain might be the cooperativity and the self-organization properties of its components described above. We have seen in fact that, through positive feedback loops, the localization of one component depends on the recruitment of the other. None of the individual components (Rab5 or PI(3)K) is sufficient to form a domain but it is the combinatorial use of all components that creates the specificity of that particular membrane environment. For example, EEA1 is absent from the plasma membrane and clathrin-coated vesicles[20,102], consistent with the idea that hVPS34 produces PtdIns(3)P on early endosomes[48,55]. On the plasma membrane, therefore, Rab5 alone is not sufficient to recruit EEA1 and the other FYVE effectors, arguing that clusters of Rab5/hVPS34/PtdIns(3)P/EEA1/Rabenosyn-5 are present only on a subcompartment of the early endosome[57]. In addition to these interactions, generation of a Rab domain will most probably require associations between Rab5 effectors and additional membrane proteins.

So the Rab5 machinery can be viewed as a typical modular system[103], in which specific biochemical interactions between Rab5 effectors and regulators as well as other endosomal proteins create spatial segregation. By regulating the assembly of a specific membrane domain, these molecules contribute to the compartmental specificity, robustness and dynamic properties of the early endosome.

another example of cooperativity between effector molecules. PI(3)Ks function in various cellular processes[49,50]. Distinct types of PI(3)K have unique functions in signal transduction and mitogenesis, cytoskeletal organization and membrane transport[51]. Rab5 interacts directly with two types of PI(3)Ks. The first is p85α/p110β, a type I kinase that mainly phosphorylates PtdIns(4)P and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), generating PtdIns(3,4)P_2 and phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) (REF. 49). The function of p85α/p110β with respect to Rab5 is not known, but, given that type I PI(3)Ks are established components of the signal-transduction machinery, the interaction between p85α/p110β and Rab5 might reveal new properties of Rab5 in response to intracellular signalling. The second PI(3)K is hVPS34/p150, the mammalian homologue of yeast Vps34p/Vps15p[52,53]. This kinase preferentially phosphorylates phosphatidylinositol to PtdIns(3)P, which is necessary for the recruitment of FYVE finger proteins on the early endosome[48,51,54]. Rab5 is therefore coupled to the generation of PtdIns(3)P by the recruitment of hVPS34/p150 (BOX. 1), a result that is consistent with the enrichment of PtdIns(3)P on early endosomes[55]. This mechanism is not limited to the membrane recruitment of EEA1. Rabenosyn-5 is another FYVE finger Rab5 effector that, similar to EEA1, is recruited in a Rab5- and PtdIns(3)P-dependent fashion to early endosomes, where it functions in docking and fusion[56]. This mechanism could also indirectly regulate the recruitment of FYVE finger proteins other than Rab5 effectors to the early endosome.

Purified clathrin-coated vesicles (CCV) fail to recruit EEA1, despite the presence of Rab5 (REF. 20,57). EEA1 (and probably Rabenosyn-5) can be exclusively attached to early endosomes and this asymmetrical recruitment between transport vesicles and their target organelle directly correlates with the asymmetrical distribution of hVPS34 (REF. 48). Binding of EEA1 to activated Rab5 through its amino-terminal site[18] is therefore not sufficient for membrane recruitment. However, a patch of EEA1 molecules[58] could provide several (low affinity) binding sites that are sufficient to tether an incoming CCV to the early endosome, thus providing directionality to the transport process.

So far, less than half of the Rab5-interacting proteins have been identified and several other molecules still need to be integrated in the Rab5 scheme. In addition, if other Rab GTPases interact with effector proteins with a complexity similar to that of Rab5, it means that the number of total Rab effectors is likely to increase considerably with the functional characterization of other Rab proteins.

Rab domains

The diversity of biochemical reactions that are regulated by Rab proteins raises the question of how these processes are coordinated. We propose that Rab5 and its effectors are not randomly recruited and distributed on the early endosome membrane but are spatially segregated in a defined membrane domain or Rab domain.

There is increasing evidence that membrane-bound molecules are not randomly distributed in the membrane bilayer but are enriched in membrane domains of varying lipid composition[59]. However, the membrane arrangement that is regulated by Rab5 is very different from that of other membrane domains such as LIPID RAFTS, which primarily depend on the intercalation of sphingolipids with cholesterol[60]. First, protein–lipid interactions are a central factor in the generation of the Rab5 domain. The localized synthesis of PtdIns(3)P not only allows the specific recruitment of FYVE effectors in conjunction with Rab5, but also contributes to their clustering. The dynamic properties of a Rab5 domain probably include a spatial and temporal control over PtdIns(3)P synthesis and turnover. As membrane flows through the early endosome, phosphoinositides other than PtdIns(3)P (for example, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3)[48,61], could be either converted to PtdIns(3)P or excluded from the Rab5 domain. Conversely, the PtdIns(3)P that is generated in the Rab5 domain and not retained by Rab5 effectors could be used in other endosomal sub-compartments, undergo further modifications or be degraded[55,62–64]. PtdIns (3)P is also enriched in the internal vesicles of multivesicular endosomes[55], suggesting that this phospholipid could also be generated at later stages of the endocytic pathway to function in combination with other Rab proteins.

A second important factor in the formation of a Rab5 domain is the effector cooperativity. The local generation of lipids and recruitment of individual effectors is not sufficient to maintain a membrane domain. In the absence of lateral interactions between the proteins within the domains, these lipid–protein complexes would rapidly diffuse throughout the plane of the membrane, filling the whole endocytic pathway. One possible mechanism to avoid this is protein oligomerization. On the membrane, EEA1, Rabaptin-5 and Rabex-5 form dynamic oligomeric complexes with NSF[58]. However, oligomerization still might not be sufficient and a scaffold, such as the actin framework or one that is analogous to the spectrin/ankyrin system[65], or to the Golgi stacking machinery[66], might be needed to stabilize the local membrane composition of Rab effectors and arrange them in a precise architectural layout. Cytoskeletal tracks could also be connected with this scaffold to increase the efficiency of vesicle delivery to a Rab domain.

Last, the generation and maintenance of the Rab5 domain depends on energy. The hydrolysis of GTP regulates the kinetics and limits the extent of effector recruitment. Through PtdIns(3)P production, PI(3)K uses ATP to recruit and, more importantly, to cluster these molecules (BOX 1). The ATPase activity of NSF regulates the dynamics of the hetero-oligomers[58]. The integration between GTPase and ATPase cycles therefore ensures a dynamic state between assembly and disassembly of oligomeric complexes of proteins and lipids and, consequently, confers a specific control on the size of that membrane domain.

Visualization of Rab domains

Morphological studies have lent support to the proposal

CCV
Coated vesicles involved in the endocytosis of receptors at the plasma membrane.

LIPID RAFTS
Lipids including cholesterol and sphingomyelin aggregated laterally to form membrane microdomains.

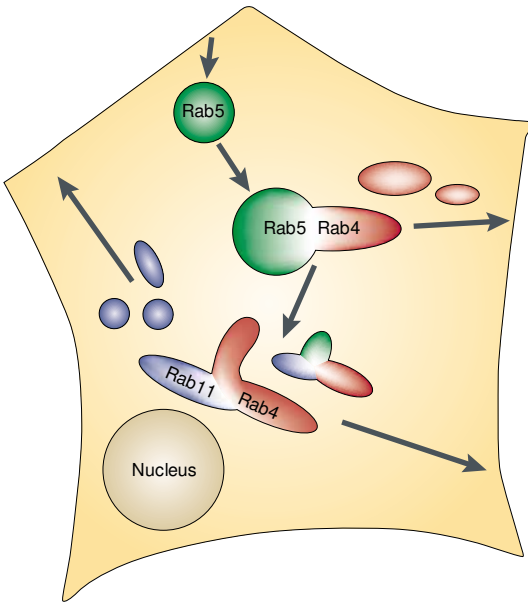


Figure 2 | **Model of Rab domains on endosomes.** Studies of Rab5, Rab4 and Rab11 tagged with green fluorescent protein (GFP) have shown that these Rab proteins are compartmentalized within the membrane of early endosomes[68]. Cargo flows sequentially through these domains as indicated by the arrows. The Rab domains also have a specific distribution and different pharmacological properties[68]. We propose that, similarly to the Rab5 effectors, Rab4 and Rab11 effectors are clustered in defined areas of the endosome membranes that are linked to each other through bifunctional Rab effectors.

that Rab proteins and/or Rab effectors are clustered in defined membrane domains. Upon expression of the activated Rab5Q79L mutant of Rab5, Rab5 and EEA1 concentrate in brightly fluorescent spots on the enlarged endosomal membrane[58,67]. Rab5, and probably its effectors, particularly accumulate at the interface between fusing vesicles and persist in a discrete spot for minutes after endosome fusion[67]. Immunofluorescence and video microscopy studies conducted on cells that express Rab5, Rab4 and Rab11 tagged with GREEN FLUORESCENT PROTEIN (GFP) have also revealed compartmentalization of these three GTPases within the endosomal membrane[68]. Three main populations of endosome could be distinguished: one that contains primarily Rab5, a second that contains both Rab5 and Rab4, and a third one that harbours Rab4 and Rab11. This distribution is consistent with biochemical and ultrastructural studies[57,69,70]. The former two would correspond to early/sorting endosomes and the latter to perinuclear RECYCLING ENDOSOMES[71]. TRANSFERRIN internalized as endocytic tracer was found first to enter the Rab5 domain and then to move sequentially through Rab4- and Rab11-positive structures. Given the function of these Rab proteins[16,72,73], the Rab5 domain would be the gateway into the early endosome, whereas the Rab4 and Rab11 domains would contain the machinery that is necessary for sorting and recycling of transferrin to the cell surface. Endosomes can therefore be viewed as a mosaic of Rab4, Rab5 and Rab11 (as well as other Rab) domains that dynamically interact but keep a relatively stable distribution over time (FIG. 2). These Rab domains could be connected through tethering molecules without intermixing. Molecules such as Rabaptin-5, which interacts with Rab5 and Rab4 through two distinct regions (REF. 74), might functionally and structurally link the Rab5 and Rab4 domains. Other molecules should then link other domains together (that is, Rab4 with Rab11). When combined with other types of lipid microdomains that have been shown to exist on endosomes[75,76], the multiplicity of membrane platforms could reach a high level of complexity.

This membrane compartmentalization is not limited to the Rab5 system, but has been observed for other Rab effectors that are involved in vesicle tethering. Docking sites could be inferred from the fluorescent morphology of discrete punctate sites around the yeast vacuole that contain at least two proteins required for vacuolar protein sorting and morphology, Vam2/Vps41p and Vam6/Vps39p[77]. Striking fluorescent images of patching were also seen in budding yeast for Sec3p, a component of the exocyst, which is restricted to the bud site[13]. The exocyst localizes to APICAL JUNCTIONAL COMPLEXES in polarized mammalian cells[78], the apical pole of pancreatic acinar cells[79], and the tips of growing NEURITES, growth cones and synapse-assembly sites in developing neurons[80]. Highly dynamic transport carriers bearing Rab6 and containing specific cargo molecules were observed to translocate from the Golgi to the ER[81], supporting the idea that this retrograde transport pathway involves a subdomain of the Golgi complex. Thus, patches of Rab effectors have been observed within both endocytic and biosynthetic organelles, indicating that these molecules might generally be restricted spatially on the membrane of organelles.

Rabs link to SNAREs and motor proteins

A crucial factor in vesicular transport is the coordination between Rab-dependent membrane tethering, docking and SNARE-dependent membrane fusion. Upon successful tethering and priming, the SNAREs engage in *trans*-interactions between vesicle- and target-SNAREs. This interaction leads to closely apposed membranes, resulting in fusion. We propose that the selective incorporation of *CIS*-SNARE COMPLEXES within a Rab domain is a prerequisite for the cognate SNAREs to pair *in trans* upon tethering/docking (BOX 2). Molecular interactions with Rab effectors within the Rab domain might result in the selective enrichment of *cis*-SNARE complexes at their site of function.

Several recent studies have reported direct molecular links between Rab effector proteins and components of the SNARE machinery (BOX 2). Sec1p and related proteins, Vps33p and Vps45p, regulate SNARE pairing by sequestering syntaxin molecules[82,83]. Interactions between Rab effectors and Sec1 family members have been observed both in mammalian cells[56] and in yeast[25,84–86]. These interactions might function to retrieve the inhibitors from the SNAREs and might result in conformational changes that allow

GREEN FLUORESCENT PROTEIN
Autofluorescent protein originally identified in the jellyfish *Aequorea Victoria*.

RECYCLING ENDOSOME
About 90% of endocytosed receptors are recycled to the plasma membrane. At least part of this traffic occurs through recycling endosomes.

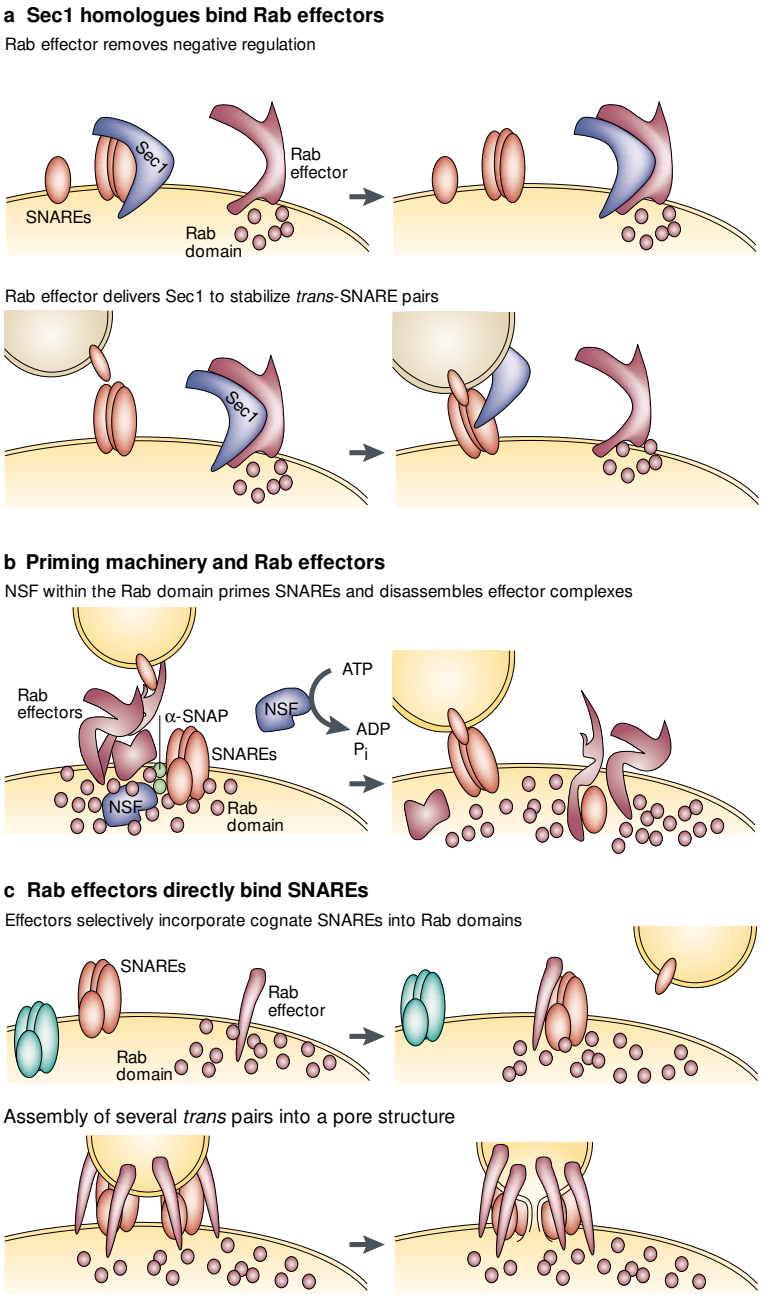
TRANSFERRIN
Protein involved in ferric ion uptake into the cell. The pathway followed by transferrin bound to its receptor defines the recycling pathway.

APICAL JUNCTIONAL COMPLEX
Desmosomes, adherens junctions and tight junctions make up the apical junctional complex.

NEURITE
Process extended by a nerve cell that can give rise to an axon or a dendrite.

CIS-SNARE COMPLEX
SNARE pairing occurring within the same membrane.

Box 2 | **Molecular interactions showing cooperativity between Rab and SNARE machineries**



a | The Rab effectors Vac1p, Rabenosyn-5 and the HOPS complex can bind Sec1 homologues directly[25,56,84–86]. The functional importance of this interaction can be seen as two distinct, but not mutually exclusive, possibilities. First (top), the Rab effector might remove Sec1 from the primed *cis*-SNARE complex to free the SNAREs for interactions *in trans*. Second (bottom), the Rab effector/Sec1 complex might stabilize *trans*-SNARE pairs after docking[104].

b | In two independent systems, the hydrolysis of ATP by *N*-ethyl-maleimide-sensitive fusion protein (NSF) and α-SNAP could regulate Rab effectors–SNARE interactions and oligomeric assembly of Rab effectors . The mechanistic consequences of these reactions are not clear but they might mediate the cyclical assembly/disassembly of protein complexes during membrane transport. In the early endosome, EEA1, Rabaptin-5 and Rabex-5 exist in oligomeric complexes with NSF[58]. The presence of NSF within the oligomers could function either to locally prime SNAREs or to regulate interactions among Rab effectors or between effectors and SNAREs, ultimately leading to *trans*-SNARE pairing. Sec18p (NSF homologue) activity might be required at several stages during vacuole fusion in yeast[24–26], in which hydrolysis of ATP by Sec18p releases the Ypt7p (Rab7 homologue) HOPS effector complex from the Vam3p SNARE complex. HOPS can then bind and activate[27] Ypt7p leading to vacuole tethering (b, right). In addition, the Ypt7p effector complex can also bind to primed Vam3p, which might aid assembly of *trans*-SNARE complexes[87]. Both data from mammalian and yeast systems therefore support the idea that vesicle tethering and SNARE priming are spatially and temporally coupled.

c | Multiple direct links have been established between Rab effectors and SNAREs. Complexes formed between EEA1 and two t-SNAREs, syntaxin13 and syntaxin6 (REFS 58,105) within the Rab5 domain might mediate fusion between endosomes, or between *trans*-Golgi-network-derived vesicles and endosomes, respectively. The Ypt7p effector proteins have also been found in complexes with the SNARE Vam3p complex[24,25,87]. Through directly binding to syntaxin-5 complexes, the Rab1 effector p115 might ensure their incorporation into COPII vesicles[7]. The concentration of Rab effectors within a restricted membrane area would increase the apparent affinity and enhance the rate of Rab effectors–SNARE and SNARE–SNARE complex formation. In this way, only the correct SNAREs would be selectively incorporated within the Rab domain , at the target site for fusion (top). These interactions might also contribute to the steady-state localization of SNAREs, despite their regular cycle between organelles. In addition, effector–SNARE interactions could be functionally required for fusion[58,87], possibly for the architectural arrangement and bridging of *trans*-SNARE pairs (bottom).

SEC18P (Sec18p)
Saccharomyces cerevisiae homologue of NSF.

HAEMAGGLUTININ
Spike protein of the influenza virus. HA is the best-understood fusion protein.

SNAREs to be primed or stabilized in a *trans*-paired conformation (BOX 2).

A second set of interactions has been observed between the Rab effectors and the SNARE priming machinery. For example, Vac1p could be precipitated together with Pep12p and SEC18P in the presence of a *sec18-1* mutant[84]. The Rab5 effector proteins EEA1 and Rabaptin-5–Rabex-5 complex form oligomers on the endosome whose assembly is dependent on the ATPase activity of NSF[58]. Interactions between the Ypt7p effector HOPS and SNAREs also depend on the ATPase activity of Sec18p (REF. 24). Finally, in both systems there are specific links to the SNARE proteins themselves. EEA1 binds directly to syntaxin 13 and this interaction is functionally required for endosome fusion[58]. The Ypt7p effector proteins have also been found in complexes with the SNARE Vam3p complex[24,25,87].

The compartmentalization of Rab effectors and the SNARE machinery within specifically assembled domains explains how productive, fusion-competent SNARE pairing would occur only within the membrane environment that has been selected for vesicle tethering, despite the presence of a cycling population of SNAREs at any given time and despite promiscuous pairing. In this context, it is interesting to consider the mechanistic links between the tethering machinery and the activation of SNAREs. The transition from membrane tethering to fusion implies that a relatively long distance must be bridged after activation of the SNARE machinery within the Rab domain. Using electron microscopy, tethered vesicles (50–100 nm in diameter) have been observed ~75–150 nm away from the target[88]. This is consistent with the length of the rod-like tethering factors. Ultrastructural analysis has shown that EEA1, for example, is associated with filamentous material that extends from the cytoplasmic surface of the endosome[57]. The current hypothesis is that the tethered vesicle swings through the highly viscous cytosol, attached by its 'string', until it collides with the surface of the target. Once the vesicle lands, the activated SNAREs engage for close docking, which closes the gap between the two bilayers. This would suggest that the tethering factors can communicate across the ~100-nm distance the fact that a vesicle has been 'trapped' to signal the priming of SNAREs. An alternative possibility is that tethering factors might undergo a large conformational change upon binding a vesicle at the free end, which would reduce the distance gap and result in the vesicle being pulled into close proximity to its target. There are many proteins that undergo extensive conformational changes that are important for their function. For example, HAEMAGGLUTININ (HA) of the influenza virus drastically changes its shape upon pH reduction[89], the light-chain-(calmodulin)-binding domain of myosin swings to generate movement in response to conformational changes in the motor domain[90,91], and the flagellar protein spasmin extends from a tightly coiled spring to a long rod (a distance difference of microns) to propel the *Vorticella velogiines* protozoa[92]. Interestingly, calmodulin is required for endosome fusion[93] and EEA1 contains a putative calmodulin-binding IQ motif, which might potentially function in such a conformational change.

Functional coordination is not restricted to vesicle tethering and fusion but is also important for vesicle motility. Strikingly, similarly to endosome membrane docking and fusion, Rab5-dependent endosome movement along microtubules depends on hVPS34 PI(3)K. So, Rab5 functionally links regulation of membrane transport, motility and intracellular distribution of early endosomes. The higher the levels of Rab5–GTP, the higher the recruitment of Rab5 effectors, including hVPS34, and the higher the movement, tethering and fusion activity of endosomes. This implies that a microtubule motor or a molecule that regulates its recruitment and/or activity might be a PtdIns(3)P-binding protein. Rab5 effectors implicated in microtubule-dependent early endosome motility have not yet been identified, although it is possible that a minus-ended kinesin might be involved[37]. Rabkinesin-6 is a Rab6 effector[35] and a second kinesin-related protein, Rab33b-BP, binds to Rab33b[94]. How these interactions result in vesicle motility remains to be determined.

Perspectives for membrane biology

We are beginning to understand the fundamental principles of membrane transport from one intracellular compartment to another. However, a more complex task will be to define an organelle in molecular terms, determine its boundaries, explain how its transport machinery is specifically localized and what controls its size and intracellular distribution. Understanding the mechanisms of membrane compartmentalization and organelle biogenesis will therefore be an important challenge for the future.

We have argued that the compartmentalization of Rab proteins and their effectors contributes to the formation of membrane domains and hence to the structural and functional properties of organelles. It will be important to find out whether this mode of function is limited to the early endosomal system or can be generalized to other Rab proteins of different organelles. Moreover, the idea of Rab domains needs to be integrated with other functional modules. For example, functional interactions of Rab proteins with Rho and ARF GTPases and their effectors[95,96] could link vesicle formation and targeting with the role of the cytoskeleton in organelle structure and function. However, beside protein machineries, biological membranes contain different kinds of lipid microenvironments[59,74,75]. It will be interesting to see how the physico-chemical properties of these membrane platforms participate in the membrane compartmentalization of organelles and act on the location and function of the transport machinery.

Morphological techniques will become increasingly important to assess the distribution and, most importantly, the dynamics of membrane compartments. In addition to studies *in vivo*, to elucidate the mechanisms that underlie the compartmentalization of biological membranes it will be necessary to develop biophysical methods (for example, artificial membrane systems) that recapitulate the clustering of molecules and their

TRANSCYTOSIS PATHWAYS
Transport of macromolecules across a cell, consisting of endocytosis of a macromolecule at one side of a monolayer and exocytosis at the other side.

APICAL
Plasma membrane surface of an epithelial cell that faces the lumen.

BASOLATERAL
Plasma membrane surface of an epithelial cell that adjoins underlying tissue.

behaviour *in vitro*. These systems should also help us understand the principles that govern the size of cellular organelles and their sub-compartments. Why are endosomes scattered in comparison with the Golgi complex? What determines the geometry of these compartments? It will be interesting to see, in relation to the regulation of homotypic endosome fusion by Rab5 (REF. 44), whether the timer function of Rab GTPases could have a general function in regulating organelle dynamics.

The disclosure of new protein sequences from the different genome projects provides an extraordinary number of novel tools with which to explore the organization of biological membranes. We will also learn a great deal from the generation and expansion of protein families throughout evolution. How has the basic transport machinery of yeast evolved from this simple eukaryote to multicellular organisms? Physiological processes typical of tissues and organs, such as neurotransmitter release, insulin-regulated traffic, recycling and transcytosis, require cell-type specific modifications of the intracellular pathways for the transport of particular cargo molecules. How is transport regulated in polarized epithelial cells and neurons? Different Rab domains containing different sets of Rab effectors could govern protein sorting and transport in the secretory, endocytic/recycling and TRANSCYTOTIC PATHWAYS of polarized cells. Efforts in this direction are expected to provide clues into, for example, what makes APICAL and BASOLATERAL early endosomes functionally distinct in polarized epithelial cells. How is their spatial distribution controlled? It will be fascinating to see how the basic molecular principles that account for membrane compartmentalization and organelle polarity can contribute, at larger scale, to an integrated understanding of cell polarity and complex tissues in multicellular organisms.

Links

DATABASE LINKS p115 | Rabaptin-5 | EEA1 | Rabenosyn-5 | Ypt1p | Rab1 | TRAPP | Sec4p | Exocyst | Rab5 | Ypt7p | HOPS | Vps41p | Vps39p | Rabex-5 | Rab9 | Rab6 | Rabkinesin-6 | Griscelli syndrome | *MYO5A* | *RAB27A* | *myosin-VA* | *Rab27a* | FYVE finger | p85α | p110β | hVPS34 | p150| Rab4 | Rab11 | Sec1p | Vps33p | Vps45p | Vac1p | Pep12p | syntaxin 13 | Vam3p | calmodulin | calmodulin-binding IQ motif | Rab33b | syntaxin 6
FURTHER INFORMATION Zerial lab home page | University of Ottawa Heart Institute

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RAB1

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http://www.ottawaheart.ca/hcwelcome.htm