

## Review

# How can mammalian Rab small GTPases be comprehensively analyzed?: Development of new tools to comprehensively analyze mammalian Rabs in membrane traffic

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**Summary.** Small GTPase Rabs constitute the largest family of membrane trafficking proteins that are conserved in all eukaryotic cells. The number of different Rab isoforms in multicellular organisms is usually greater than that in unicellular organisms (e.g., approximately 60 different Rabs in each species of mammals investigated versus approximately 10 Rabs in yeasts). The expansion of Rab isoforms in mammals is often regarded as due to the acquisition of specialized membrane trafficking events in the specialized cell types of higher eukaryotes. However, because of their large numbers the precise function of most mammalian Rab isoforms is still unknown. The recent development of new tools for comprehensive analysis (e.g., Rab panels) has paved the way for systematic investigation of the involvement of mammalian Rab isoforms in specialized membrane trafficking events. The tools include collections of enhanced green fluorescent protein (EGFP)-tagged mouse and human Rabs, FLAG-tagged Rabs, glutathione S-transferase (GST)-tagged Rabs, Gal4-binding domain (GBD)-tagged Rabs, Tre-2/Bub2/Cdc16 (TBC) domain-containing Rab-GTPase-activating proteins (GAPs), and small interfering RNAs. EGFP-Rabs are used to screen for Rabs that are localized on specific organelles and regulate their transport, and GST-Rabs and GBD-Rabs are used to screen for novel Rab effectors by GST pull-down assays and yeast two-hybrid assays, respectively. Combined use of these tools now makes it possible to efficiently

determine the function of mammalian Rab isoforms in membrane traffic. This article reviews the development of new tools for systematic analysis of Rab proteins and their application to Rab-mediated membrane traffic.

**Key words:** Comprehensive screening, Membrane traffic, Small GTPase, Rab effector

### Introduction

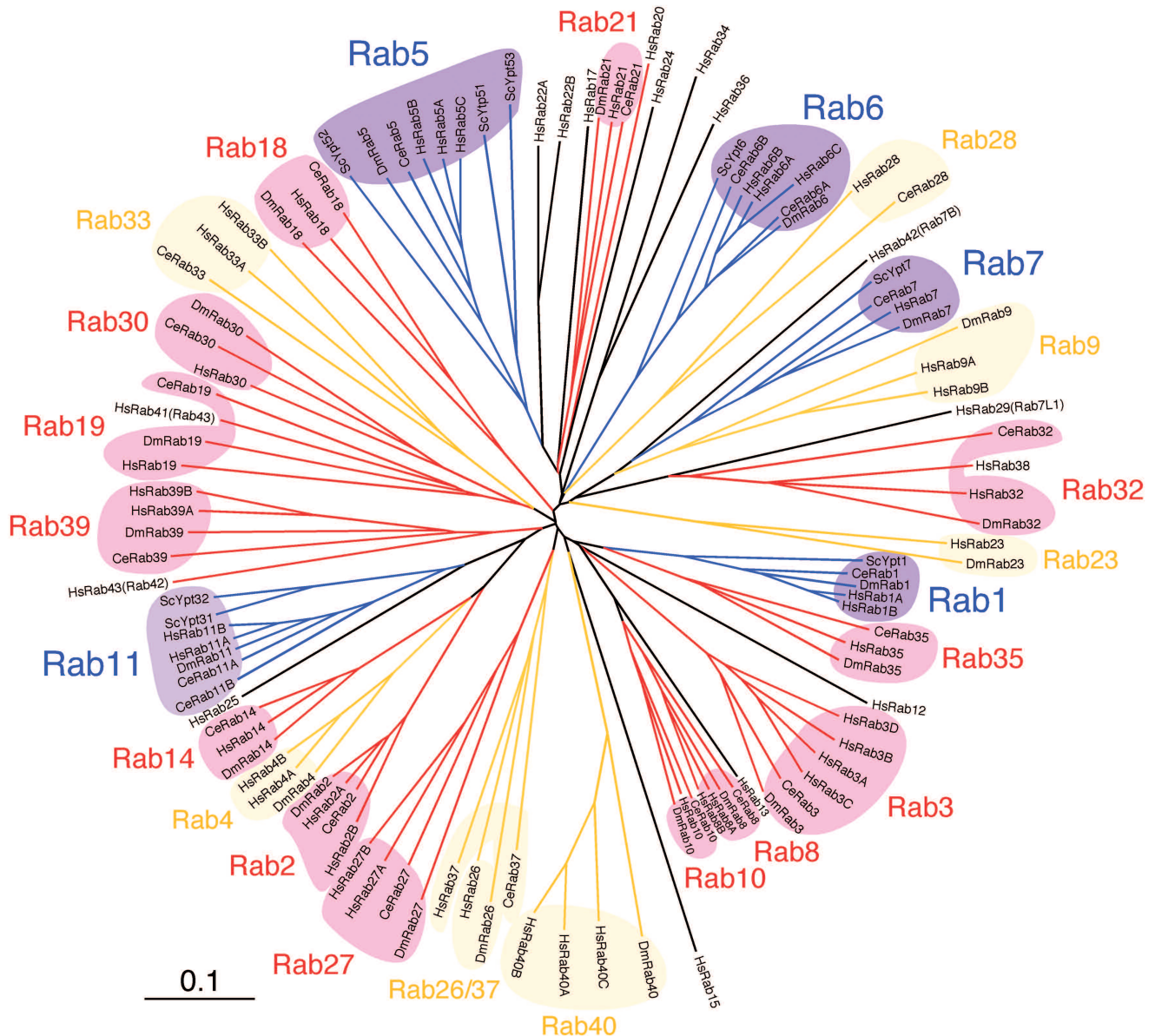
Small GTPase Rab functions as a molecular switch by cycling between two nucleotide-bound states, a GTP-bound active state and a GDP-bound inactive state. Two key regulatory factors are involved in this cycling: guanine nucleotide exchange factor (GEF), which activates Rab by accelerating the exchange of GDP for GTP, and GTPase-activating protein (GAP), which inactivates Rabs by promoting the GTPase activity of Rab. The GTP-bound form of Rab is generally thought to target specific organelles/vesicles and to promote their transport through specific interaction with its binding partner, called a "Rab effector" (reviewed in Grosshans et al., 2006; Schwartz et al., 2007; Fukuda, 2008; Stenmark, 2009). The number of Rab isoforms per organism varies from species to species (Pereira-Leal and Seabra, 2001), e.g., budding yeasts contain only eleven Rab isoforms, whereas human and mouse cells contain approximately 60 Rab isoforms (Bock et al., 2001; Pereira-Leal and Seabra, 2001; Itoh et al., 2006), only five Rab isoforms have been conserved during evolution (Fig. 1, blue). The higher number of Rabs in humans and mice seems to be related to the complexity of their tissues and the cell specialization in higher eukaryotes. However, the precise function of most

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mammal-specific or vertebrate-specific Rabs largely remains unknown.

The literature on Rab research shows that four methods have often been used to investigate the function of specific Rab isoform in membrane traffic. The first, and most commonly used method, has been overexpression in cells of a constitutive active (CA)

mutant that mimics the GTP-bound form or of a constitutive negative (CN) mutant that mimics the GDP-bound form (Fig. 2(a)). The second method, which has come into use recently, is knockdown of a specific Rab by RNA interference technology (Fig. 2(b)). The third method is based on a dominant negative approach in which a specific Rab effector domain is overexpressed in



**Fig. 1.** Molecular dendrogram of human (Hs, *Homo sapiens*), fruit fly (Dm, *Drosophila melanogaster*), nematode (Ce, *Caenorhabditis elegans*), and budding yeast (Sc, *Saccharomyces cerevisiae*) Rab families. The phylogenetic tree was drawn by using the ClustalW program set at the default parameters. Rab isoforms common to all four species (Rab1, Rab5, Rab6, Rab7, and Rab11) are shown in blue. Rab isoforms that are conserved in animals (Rab2, Rab3, Rab8, Rab10, Rab14, Rab19, Rab21, Rab27, Rab30, Rab32, Rab35, and Rab39) are shown in red. Rab isoforms that are conserved between human and *D. melanogaster* (or *C. elegans*) (Rab4, Rab9, Rab23, Rab26, Rab28, Rab33, Rab37, and Rab40) are shown in orange. For the sake of clarity, *S. cerevisiae*-specific, *C. elegans*-specific, and *D. melanogaster*-specific Rabs have not been included in the phylogenetic tree. Rab nomenclature is according to Pereira-Leal and Seabra (2001) and Itoh et al. (2006).

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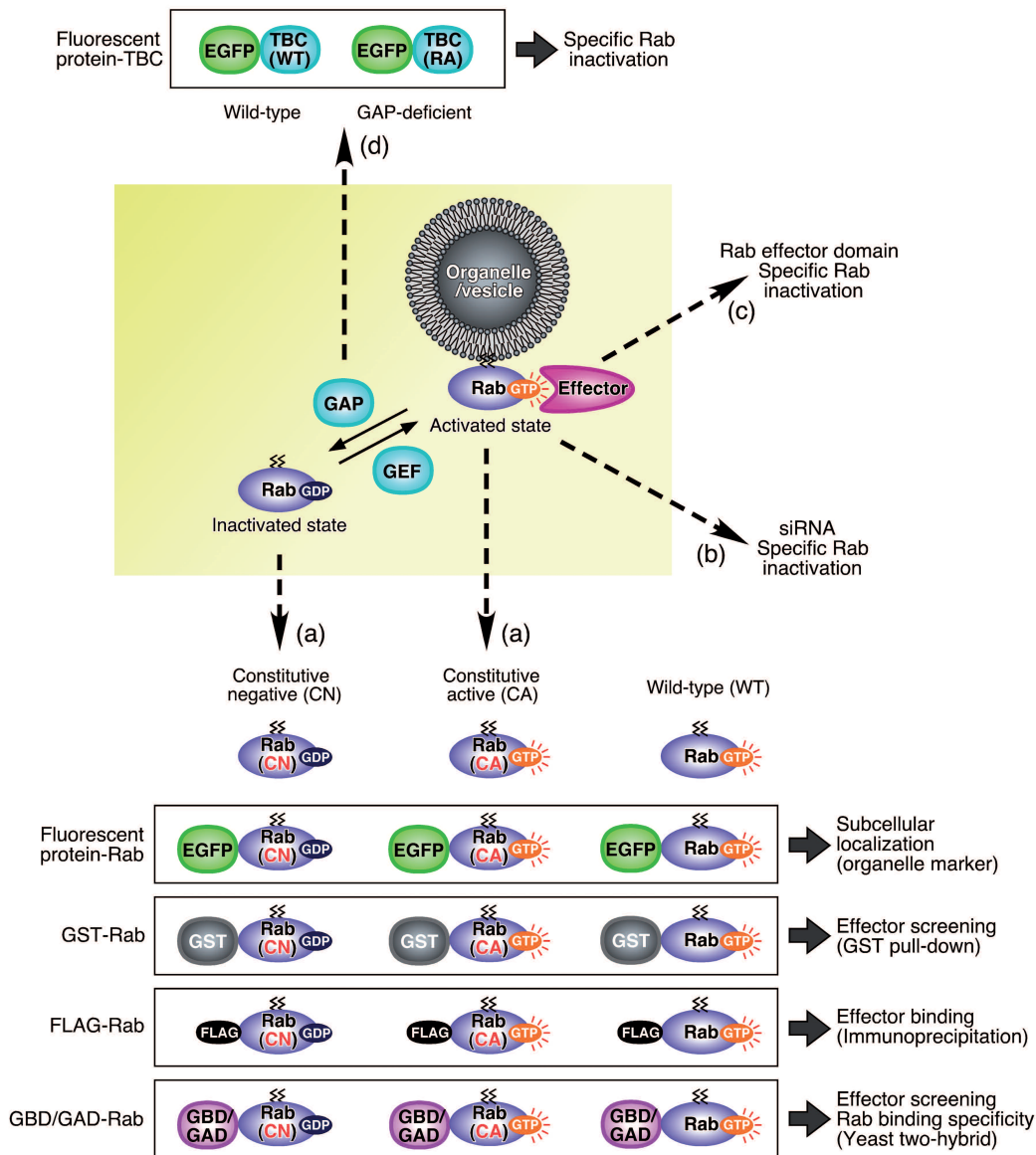
cells (Fig. 2(c)). Although the dominant negative effect relies on the Rab binding specificity of the Rab effector domain used, examination of the literature shows that the Rab binding specificity of most Rab effector domains has never been thoroughly investigated. The last of the four methods is inactivation of a specific Rab by overexpressing Tre-2/Bub2/Cdc16 (TBC) domain-containing Rab GAP in cells (Fig. 2(d)). Several different types of tools for analysis of mammalian Rabs in membrane traffic have recently been developed based on these methods, and attempts have been made to use them to systematically and comprehensively analyze all the Rab isoforms in humans and mice. The types of tools include tools for studying the function of Rab in specific

organelle/vesicle transport (see section I below) and tools for screening for novel Rab effectors and determining their Rab binding specificity (see section II below) (Fig. 2). In this article, I review the literature on the development of each tool and its application to elucidation of the molecular mechanism of Rab-mediated membrane traffic.

### Tools for investigating the function of Rabs in specific organelle transport

#### Fluorescent protein-tagged Rabs

Rab isoforms are generally thought to be targeted to



**Fig. 2.** Tools for comprehensively studying Rab functions (boxed; see text for details). The GDP-bound, inactivated form of Rab is activated by specific GEF, and the GTP-bound, activated form of Rab is recruited to a specific type of organelle/vesicle. GTP-Rab promotes the transport of the organelle/vesicle by interacting with specific effector molecules. GTP-Rab is inactivated by specific GAP.

distinct subcellular compartments (or organelles) and to regulate a specific type of membrane trafficking event (Stenmark, 2009). The targeting of a specific organelle by each Rab isoform makes it possible to visualize specific membrane compartments by using fluorescently tagged Rabs. As an example, EGFP (enhanced green fluorescent protein)-tagged Rab5A, Rab7, and Rab11A are widely used as markers for early endosomes, late endosomes, and recycling endosomes, respectively (Stenmark, 2009), and these endosomes are spatially segregated in some cell types, e.g., COS-1 cells (Misaki et al., 2007). In other words, it is possible to screen for Rabs that are involved in specific membrane trafficking events by expressing each of the mammalian Rabs with an EGFP tag. This tool was first applied to screening for Rab isoforms involved in dense-core vesicle exocytosis by neuroendocrine PC12 cells (Tsuboi and Fukuda, 2006). Using 40 EGFP-Rab isoforms for screening revealed that only four Rabs, i.e., Rab3A, Rab27A, Rab33A, and Rab37, are predominantly localized on dense-core vesicles in the distal portion of the neurites of nerve growth factor-differentiated PC12 cells (Fig. 3, 1st screening). At least two of them, Rab3A and Rab27A, were found to be endogenously expressed on dense-core vesicles in PC12 cells (Fig. 3, 2nd screening) and to cooperatively regulate the docking step of dense-core vesicle exocytosis. Knockdown of either Rab3A or Rab27A with specific small interfering RNA (siRNA) causes a reduction in the number of plasma membrane-docked dense-core vesicles in PC12 cells (Fig. 3, 3rd

screening). Similarly, screening for Rabs involved in phagosome maturation has been performed by expressing 48 GFP- or CFP (cyan fluorescent protein)-tagged Rabs (Smith et al., 2007). A drawback of this screening method is that not all Rabs are always involved in the transport of the specific organelles where they are localized. As an example, EGFP-tagged Rab33A and Rab37 are clearly localized on dense-core vesicles in PC12 cells, but Rab37 is not endogenously expressed in PC12 cells, and knockdown of endogenous Rab33A by specific siRNA had no effect on dense-core vesicle exocytosis (Tsuboi and Fukuda, 2006). Thus, fluorescent protein-tagged Rabs need to be used in combination with other tools to determine the true function of Rab isoforms in specific membrane trafficking events.

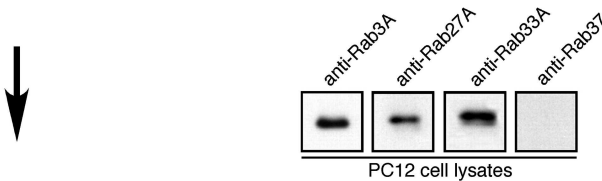
Constitutive active (CA) and constitutive negative (CN) Rab mutants

Since, as with other small GTPases, CA mutants and CN mutants of Rab can be easily produced by a single amino acid substitution of the conserved Gln for Leu in the switch II region and of the conserved Thr/Ser for Asn in the  $\alpha 1$  helix (or conserved Asn for Ile in the  $\beta 5$  strand), respectively, fluorescently tagged CA or CN mutant Rabs may also be a useful screening tool for Rabs that affect specific membrane trafficking events or cell activities. Heo and Meyer were the first to report the large-scale screening for small GTPases that affect cell

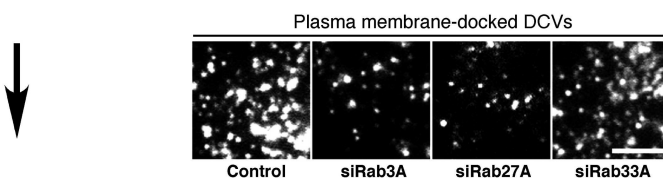
### 1st screening: Screening for EGFP-Rabs specifically targeted to DCVs in PC12 cells



### 2nd screening: Endogenous expression of candidate Rabs in PC12 cells



### 3rd screening: Effect of knockdown of candidate Rabs with siRNA on DCV exocytosis



### Rab3A & Rab27A

**Fig. 3.** Scheme for the comprehensive analysis of Rab isoforms involved in dense-core vesicle exocytosis in PC12 cells. In the first screening, Rab isoforms specifically targeted to dense-core vesicles (DCVs) in the neurite of PC12 cells were selected by expression of EGFP-tagged Rabs (Rab3A, Rab27A, Rab33A, and Rab37). In the second screening, endogenous expression of candidate Rabs was investigated by immunoblotting with specific antibodies (Rab3A, Rab27A, and Rab33A). In the final screening, the effect of knockdown of candidate Rabs with specific siRNA on DCV exocytosis was investigated by total internal reflection microscopy. Knockdown of Rab3A, Rab27A, or both, reduced the number of plasma membrane-docked DCVs and exocytotic events, whereas knockdown of Rab33A had no effect (adapted with permission from Tsuboi and Fukuda, 2006).

morphology by expressing GFP- or CFP-tagged CA mutants of Ras superfamily members, including Rab family members (Heo and Meyer, 2003). Among the Rab family members tested, Rab8A and Rab8B had the most dramatic effect on cell morphology, because they induced large branched structures with local lamellipodia and filopodia. Collections of CA mutants of Ras superfamily members have also been used to screen for plasma membrane-localized Rab isoforms, and Rab35 has been shown to be targeted to the plasma membrane through polybasic-geranylgeranyl motifs, i.e., polybasic sequence is located immediately before the geranylgeranylation site) (Heo et al., 2006). Since some Rab(CA)s promote membrane traffic, whereas others inhibit membrane traffic (e.g., Rab5A(CA) promotes homotypic fusion of early endosomes (Stenmark et al., 1994), whereas Rab27A(CA) inhibits melanosome transport (Bahadoran et al., 2003)), effects of Rab(CA)s on membrane traffic should be carefully evaluated.

Although the dominant negative forms of other small GTPases (e.g., Ras, Rho, Rac, and Cdc42) and some Rab isoforms (e.g., Rab5A and Rab11A) have often been used for cell analyses in studies reported in the literature, no attempt has ever been made to use mammalian Rab(CN)s for comprehensive screening, presumably because the level of Rab(CN) expression is generally much lower than that of wild-type Rabs or Rab(CA)s (M.F., unpublished data), and Rab(CN)s seem to be rapidly degraded within the cell (Ramalho et al., 2002). Recently, however, *Drosophila* transgenic lines that express a wild-type, CA mutant, and CN mutant of 31 *Drosophila* Rabs have been established (Zhang et al., 2007), and Rab35(CN) has been found to affect bristle morphology by inhibiting actin bundling in *Drosophila* (Zhang et al., 2009). Thus, fluorescent protein-tagged Rab(CN)s with a highly efficient and stable expression system may also be useful tools for analyzing mammalian Rab function, the same as fluorescent protein-tagged Rab(CA)s are. However, the data obtained by overexpression of fluorescent protein-tagged Rab(CA)s or Rab(CN)s need to be carefully evaluated, because Rab(CA)s or Rab(CN)s may affect organelle transport even if the candidate Rabs are not endogenously expressed. Endogenous expression and knockdown of candidate Rabs are necessary to reveal the true function of Rab isoforms in specific membrane trafficking events.

TBC (Tre-2/Bub2/Cdc16) domain-containing Rab-GAPs

Since Rab-GAP is able to inactivate its substrate Rab by promoting GTPase activity, overexpression of Rab-GAP in cells should result in specific inactivation of its substrate Rab, which, in turn, would inhibit specific organelle transport. Although the specific Rab-GAP of most mammalian Rabs has yet to be identified, a TBC domain is generally thought to function as a Rab-GAP

domain, and more than 40 different TBC domain-containing proteins (referred to as TBC/Rab-GAP proteins below) have been found to be present in humans (Bernards, 2003; Ishibashi et al., 2009). Therefore, expression of an EGFP-tagged TBC/Rab-GAP protein and its catalytically inactive mutant (RA or RK; catalytic Arg residues mutated to Ala or Lys) as a negative control (Lanzetti et al., 2000; Gao et al., 2003; Pan et al., 2006) makes it possible to evaluate the function of endogenous Rab proteins in specific membrane trafficking events. As an example, expression of EPI64/TBC1D10A/Rab27A-GAP $\alpha$ , but not its catalytically inactive EPI64(R160K) mutant, in cultured melanocytes causes peri-nuclear melanosome clustering through inactivation of Rab27A on melanosomes (Itoh and Fukuda, 2006). Similarly, expression of 39 different TBC/Rab-GAP proteins in telomerase-immortalized retinal pigment epithelial (hTERT-RPE1) cells has revealed the involvement of 3 TBC/Rab-GAP proteins (TBC1D7, TBC1D30, and Evi5-like) in primary cilium formation (Yoshimura et al., 2007). Further determination of their target Rabs has indicated that three Rabs, Rab8A (a target of TBC1D30), Rab17 (a target of TBC1D7), and Rab23 (a target of Evi5-like), are involved in primary cilium formation (Yoshimura et al., 2007). Involvement of Rab isoforms in Shiga toxin uptake (Fuchs et al., 2007) and maintenance of the Golgi complex (Haas et al., 2007) has been revealed by the same screening method. Although TBC/Rab-GAP proteins are useful for inactivating the function of endogenous Rab proteins, the results need to be evaluated cautiously, because the literature is inconsistent with regard to Rab-GAP activity and specificity of some TBC/Rab-GAPs (e.g., GAPCenA; Cuif et al., 1999; Haas et al., 2005; Itoh et al., 2006; Fuchs et al., 2007; Kanno et al., 2010).

#### Small interfering RNAs (siRNAs)

A collection of siRNAs against Rab family members is another powerful tool for investigating the involvement of Rab isoforms in specific membrane trafficking events. As an example, siRNA-mediated knockdown of 57 Rabs has revealed that Rab3B/3C-positive recycling vesicles are involved in antigen cross-presentation in dendritic cells (Zou et al., 2009). Screening in a similar manner with RNA interference (RNAi) for 59 human Rabs has revealed that two closely related isoforms, Rab27A and Rab27B, regulate different steps of the exosome secretion pathway in HeLa cells by interacting with different effectors, i.e., Slp4-a and Slac2-b, respectively (Ostrowski et al., 2010). Although siRNAs for Rabs are now commercially available, and RNAi screening is easy to perform, it is recommended that an siRNA approach is used in combination with other approaches (e.g., rescue by siRNA-resistant Rabs and endogenous expression of Rabs by specific antibody) to avoid off-target effects of siRNAs.

### Screening tools for novel Rab effectors and determination of their Rab binding specificity

#### GST (glutathione S-transferase)-tagged or His-tagged Rabs

Since, as noted above in the Introduction, the GTP-bound active form of Rab is generally thought to promote membrane traffic through interaction with specific effector molecules, identification of specific Rab effector molecules becomes a crucial step in understanding the molecular basis of Rab-mediated membrane traffic. The method most widely used to identify Rab effectors in studies reported in the literature on Rab research has been GST-Rab (or His-Rab) pull-down assay from tissue or cell lysates in combination with mass spectroscopic analysis, and a variety of Rab effector candidates have already been identified by this method. Although, with the exception of a few Rab effectors, e.g., Rab27 effectors (Fukuda, 2003, 2006), the Rab binding specificity of Rab effector candidates identified thus far has never been thoroughly investigated, it is important to evaluate their Rab binding specificity, because some Rab effector candidates have been found to bind other Rabs that had never been tested in the initial pull-down assay. One example is rabphilin, which was originally described as a Rab3 effector (Shirataki et al., 1993) but was later found to also function as a Rab27 effector in regulated secretion (Fukuda et al., 2004; Tsuboi and Fukuda, 2005). Similarly, Rabenosyn-5 was originally described as a Rab5 effector (Nielsen et al., 2000) but is now known to bind multiple Rabs (Eathiraj et al., 2005). More recently, GST pull-down assays with 60 different mouse or human Rabs (i.e., Rab1~Rab43) have been employed as a means of large-scale screening for novel Rab effectors, and the Rab binding specificity of Rab effector candidates was determined (Itoh et al., 2008; Kanno et al., 2010). Such large collections of 60 GST-Rabs are very powerful tools for determining the Rab binding specificity of Rab effectors. Actually, Atg16L1, an essential component of autophagosome formation, has been identified as a potential Rab33B effector (Itoh et al., 2008), and centaurin  $\beta$ 2, an Arf6-GAP, as a potential Rab35 effector (Kanno et al., 2010).

#### GBD (Gal4 DNA-binding domain)- and GAD (Gal4 activation domain)-tagged Rabs

Another method widely used to identify Rab effectors is yeast two-hybrid assay with the wild-type or CA form of Rab (or prenylation-deficient Rab) as bait. The same as when screening for Rab effectors by the GST pull-down assay described above, however, careful evaluation of the Rab binding specificity of Rab effector candidates is necessary to understand the molecular mechanism of Rab-mediated membrane traffic. A panel of human or mouse Rab(CA)s or Rab(CN)s fused to GBD (Gal4 DNA-binding domain) or GAD (Gal4 activation domain) has recently been developed (Haas et

al., 2005; Itoh et al., 2006; Tamura et al., 2009), and the panels have been used to thoroughly investigate Rab binding specificity of Rab effector candidates (Fukuda et al., 2008). A panel of Rab(CA)s fused to GBD or GAD constructed in the yeast expression system was first applied to screening for specific TBC/Rab-GAPs (Haas et al., 2005; Itoh et al., 2006), and the target of some TBC/Rab-GAPs was actually determined by this method, although not all TBC/Rab-GAPs stably interact with their substrate Rabs (Itoh et al., 2006). Similarly, the target of GEFs could be determined by using a panel of Rab(CN)s fused to GBD or GAD constructed in the yeast expression system. Actually, Varp, a VPS9 domain-containing Rab21-GEF (Zhang et al., 2006), has been found to specifically interact with its substrate Rab21(CN) and not to interact with 59 other Rab(CN)s (Tamura et al., 2009). Panels of Rab(CA)s fused to GBD or GAD are also very powerful screening tools for novel Rab effectors and for determining their Rab binding specificity. Interestingly, a recent large-scale screening for novel Rab effectors with Rab1~30 as bait showed that more than half of the Rab effector candidates identified bind more than two Rab isoforms (Fukuda et al., 2008). In addition, GCC185, a trans-Golgi network-localized protein with multiple coiled-coil domains, has been shown to contain multiple Rab binding sites by yeast two-hybrid assays (Hayes et al., 2009). These observations strongly indicate the importance of determining the exact Rab binding specificity of Rab effector domains.

#### FLAG-tagged Rabs

Since Rab effector candidates identified by the *in vitro* GST pull-down assay and yeast two-hybrid assay described above sometimes contain pseudo-positive binding partners that do not bind Rabs *in vivo* or in mammalian cultured cells, it is important to confirm candidates' interactions in mammalian cells. Epitope-tagged Rabs and Rab effectors are often co-expressed in mammalian cultured cells and their associations are evaluated by co-immunoprecipitation assay as a method of confirmation. Collections of epitope-tagged Rabs are also useful tools for determining the Rab binding specificity of Rab effector candidates, and the Rab binding specificity of Rab27 effectors, Slps (synaptotagmin-like proteins) and Slac2s (Slp homologue lacking C2 domains), has been determined by co-immunoprecipitation assay with 60 different FLAG-Rabs (Kuroda et al., 2002; Fukuda, 2003, 2006). However, since simultaneous co-immunoprecipitation of 60 different FLAG-Rabs is much more laborious than the yeast two-hybrid assay, the Rab binding specificity of Rab effectors has rarely been determined by this method in studies reported in the literature.

#### Future Rab research

Since the first Rab isoform associated with human disease (i.e., Rab27A and type 2 Griscelli syndrome)

was reported in 2000 (Ménasché et al., 2000), increasing numbers of Rabs and their regulators have been shown to be associated with human and/or murine diseases (Seabra et al., 2002; Stenmark, 2009; Giannandrea et al., 2010). Because Rabs seem to be involved in a variety of membrane trafficking events, which underlie many important cellular events in a variety of cell types, including neurons, immune cells, and cancer cells, a great deal of attention will be directed to Rabs in many other fields outside the classical membrane traffic field. I am therefore convinced that comprehensive analysis of mammalian Rabs by means of the tools described above will become a trend in the attempt to understand Rab-mediated cellular events. Several Rab research tools are now available from commercial suppliers and non-profit organizations, e.g., the Missouri S&T cDNA Resource Center (<http://www.cdna.org/index.html>), and the availability of such tools will enable significant advances in our understanding of the molecular basis of Rab-mediated membrane traffic in the near future.

Potential involvement of specific Rab isoform(s) in specific membrane trafficking events can be revealed by using a single tool, but use of combinations of different tools is recommended to reliably identify true function of candidate Rab proteins, because each tool has a drawback, e.g., an off target effect in the case of siRNA and endogenous expression in the case of CA/CN mutants. An example of combined use of Rab tools is shown in Fig. 3, where three different tools, EGFP-Rabs (1st screening), specific antibodies (2nd screening), and siRNAs (third screening), were used to identify Rab isoforms involved in dense-core vesicle exocytosis in PC12 cells (Tsuboi and Fukuda, 2006). Although the availability of specific antibodies against each Rab is very limited at present, their use is an ideal means of demonstrating endogenous expression of Rab proteins in cells. Further development of specific antibody against each Rab isoform, which can be used for both immunoblotting and immunostaining, is anticipated in the future.

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