Multiple Types of Guanine Nucleotide Exchange Factors (GEFs) for Rab Small GTPases

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ABSTRACT. Rab small GTPases are highly conserved master regulators of membrane traffic in all eukaryotes. The same as the activation and inactivation of other small GTPases, the activation and inactivation of Rabs are tightly controlled by specific GEFs (guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins), respectively. Although almost all Rab-GAPs reported thus far have a TBC (Tre-2/Bub2/Cdc16)/Rab-GAP domain in common, recent accumulating evidence has indicated the existence of a number of structurally unrelated types of Rab-GEFs, including DENN proteins, VPS9 proteins, Sec2 proteins, TRAPP complexes, heterodimer GEFs (Mon1–Ccz1, HPS1–HPS4 (BLOC-3 complex), Ric1–Rgp1 and Rab3GAP1/2), and other GEFs (e.g., REI-1 and RPGR). In this review article we provide an up-to-date overview of the structures and functions of all putative Rab-GEFs in mammals, with a special focus on their substrate Rabs, interacting proteins, associations with genetic diseases, and intracellular localizations.

Key words: guanine nucleotide exchange factor (GEF), Rab small GTPase, longin domain, Rab cascade, autophagy

Introduction

Rab small GTPases constitute the largest family in the Ras-related small GTPase superfamily, and they are thought to be master regulators of intracellular membrane traffic in all eukaryotes (Diekmann *et al.*, 2011). More than 60 different Rabs, each of which localizes to a distinct intracellular membrane compartment(s) where it regulates a specific membrane trafficking pathway(s), have been identified in humans. Like other small GTPases, Rabs function as switch

molecules by cycling between a GTP-bound active state and a GDP-bound inactive state. In the GTP-bound active state, Rabs localize on the surface of a specific vesicle/membrane and regulate its trafficking by recruiting a specific effector protein(s) to the surface membrane (Fukuda, 2008; Zhen and Stenmark, 2015).

The same as the activation and inactivation of other small GTPases, the spatiotemporal activation and inactivation of Rabs are tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively (Barr and Lambright, 2010). All Rab-GAPs identified thus far, except Rab3GAP, a heterodimer complex of Rab3GAP1 and Rab3GAP2 (Fukui *et al.*, 1997; Nagano *et al.*, 1998), contain the same catalytic TBC (Tre-2/Bub2/Cdc16) domain, which stimulates the intrinsically sluggish GTP-hydrolysis activity of Rabs (Fukuda, 2011). On the other hand, at least six different types of Rab-GEFs, i.e., DENN (differentially expressed in normal and neoplastic cells)-domain-containing proteins (simply referred to as DENN proteins below), VPS9 (vacuolar protein sorting 9)-domain-containing proteins (VPS9 proteins), Sec2-domain-containing proteins (Sec2 proteins), multi-subunit TRAPP (transport protein particle) complexes, heterodimer GEF complexes, and other GEFs, have been reported (Barr and Lambright, 2010). Interestingly, several additional Rab-GEF-related proteins have been identified during the past five years, and some of them are associated

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Abbreviations: ALS, amyotrophic lateral sclerosis; ANKR, ankyrin-repeat; BLOC, biogenesis of lysosome-related organelles complex; DENN, differentially expressed in normal and neoplastic cells; FLCN, folliculin; FRET, fluorescence resonance energy transfer; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GRAB, guanine nucleotide exchange factor for Rab3A; HVR, hypervariable region; HOPS, homotypic fusion and vacuole protein sorting; HPS, Hermansky-Pudlak syndrome; KO, knockout; mTORC1, mammalian/mechanistic target of rapamycin complex 1; Rabin8, Rab8-interacting protein; REI, Rab-11-interacting protein; SH3BP5, SH3-binding protein 5; RPGR, retinitis pigmentosa GTPase regulator; TBC, Tre-2/Bub2/Cdc16; TRAPP, transport protein particle; Varp, VPS9-ankyrin repeat protein; VPS9, vacuolar protein sorting 9.

with human genetic diseases. In this review article we summarize the properties of putative Rab-GEFs identified thus far and provide an up-to-date overview of studies on these Rab-GEFs (unless otherwise specified, GEF means Rab-GEF hereafter). We also discuss the relationships between the intracellular localizations of GEFs and Rab-activation as well as emerging roles of longin domains as general interacting motifs of small GTPases including Rabs (Zhang *et al.*, 2012; Levine *et al.*, 2013b).

Principles of the GEF-accelerated GDP-GTP exchange reaction

GDP dissociation from a Rab is usually very slow, and the process is accelerated by a GEF. The same as in the activation of other small GTPases, the GDP dissociation from a Rab is thought to be initiated by the formation of a low-affinity complex between a GDP-bound Rab and a GEF, with the low-affinity complex converting into a high-affinity nucleotide-free Rab-GEF complex after dissociation of GDP. The higher cytosolic concentration of GTP (~1 mM) relative to GDP ensures GTP-binding to the Rab as soon as GDP has been dissociated, which eventually displaces the GEF from the Rab to yield the active form of the Rab (Fig. 1) (Cherfils and Zeghouf, 2013). The interaction between a nucleotide-free Rab and a GEF, which is an intermediate step in the activation process, is usually stable, and thus allows their crystal structures to be determined. Thus far, at least five crystal structures of Rab-GEF complexes, i.e., of Rabex5-Rab21, Sec2p-Sec4p, Rabin8-Rab8, TRAPP I-Ypt1p, and DENND1B-Rab35, have been determined, and they provide the structural basis for Rab activation by these GEFs (Delprato and Lambright, 2007; Dong *et al.*, 2007; Sato *et al.*, 2007; Cai *et al.*, 2008b; Wu *et al.*, 2011; Guo *et al.*, 2013). The stable interaction between GEFs and their substrate Rabs can be applied to identifying the substrate specificity of GEFs, e.g., the Rab21-specific GEF Varp specifically interacts with a constitutively negative form of Rab21 and does not interact with constitutively negative forms of 59 other Rabs (Tamura *et al.*, 2009; Mori *et al.*, 2013).

Substrates and functions of Rab-GEFs

Thus far, more than 40 putative Rab-GEFs have been reported in humans (summarized in Table I), but GEFs that have not yet been identified must be present, because about half of the Rabs are still “orphans” in terms of identification of their GEFs (Fig. 2). Based on the similarity of the primary sequences of the GEFs that have been reported, at least three independent conserved Rab-GEF domains have been identified, i.e., a DENN domain, a Sec2 domain, and a VPS9 domain (Fig. 3). Most GEFs contain one of these

Fig. 1. Schematic model of GDP-GTP exchange of a Rab protein by a GEF. In general, a GDP-bound form of Rab is present in the cytosol through interaction with GDI (top). A GEF, a Rab activation enzyme, forms a high-affinity complex with a nucleotide-free form of the Rab (bottom right), and such complexes are often used for crystallographic analysis because of their stability (Delprato and Lambright, 2007; Dong *et al.*, 2007; Sato *et al.*, 2007; Cai *et al.*, 2008b; Wu *et al.*, 2011; Guo *et al.*, 2013). When the Rab binds to GTP, the GEF is released from the Rab (bottom left). The resulting GTP-bound form of the Rab localizes to a specific membrane compartment, where it recruits its specific effector molecule and thereby promotes membrane traffic. Finally, the bound GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the Rab assisted by a GAP, and the Rab returns to the GDP-bound state.

GEF domains, but the rest lack such domains and often exhibit a unique subunit composition (Fig. 3). More specifically, while DENN (or VPS9) proteins and Sec2 proteins are thought to be monomers and homodimers, respectively (Cherfils and Zeghouf, 2013), most other GEFs are composed of at least two subunits, e.g., there are heterodimer GEFs (Mon1-Ccz1, HPS1-HPS4, Ric1-Rgp1, and Rab3GAP1-Rab3GAP2) and multi-subunit GEFs (TRAPP complexes). Although DENN proteins, Mon1-Ccz1 and HPS1-HPS4 heterodimer GEF complexes, and the TRAPP complexes are clearly classified into three different groups based on their subunit composition as well as sequence similarity, all share a longin-like fold consisting of five antiparallel β strands sandwiched between α helices that often plays a crucial role in interaction with Rab (Kinch and Grishin, 2006; Levine *et al.*, 2013b). Longin domains were originally characterized as an N-terminal conserved domain of a vesicle SNARE, e.g., VAMP7 (also called TI-VAMP) (Filippini *et al.*, 2001), and some of them are now recognized as an interacting domain for small GTPases (e.g., Rabs and Rags). Interestingly, a number of uncharacterized longin-domain-containing proteins (named longin proteins) have been identified in eukaryotes (reviewed in De Franceschi *et al.*, 2014; Daste *et al.*, 2015). It is therefore possible that such uncharacterized longin proteins (e.g., DENN-related protein; see next section) function as

novel GEFs. Below we provide an overview of six different types of GEFs identified thus far: DENN proteins, VPS9 proteins, Sec2 proteins, the TRAPP complexes, heterodimer GEF complexes, and other GEFs.

DENN and DENN-related proteins

DENN-domain-containing GEFs and related proteins (DENN-related proteins) constitute the largest class of Rab-GEFs identified thus far (Table I). Although VPS9-domain-containing GEFs (see next section) have been characterized as activators of Rab5 family members, all of which belong to the same phylogenetic group (Fig. 2), no clear phylogenetic relationship between DENN proteins and their substrate Rabs has been observed; i.e., the substrate Rabs of the DENN proteins are varied and phylogenetically different. DENN (also called MADD and Rab3GEP) and its *Caenorhabditis elegans* homologue AEX-3 are the first DENN proteins to have been characterized as GEFs for Rab3 and Rab27 (Wada *et al.*, 1997; Iwasaki *et al.*, 1997; Mahoney *et al.*, 2006; Figueiredo *et al.*, 2008). A subsequent bioinformatic analysis has revealed the existence of a number of DENN homologous (referred to as “classical DENN proteins”) in mammals, and their homologous regions (i.e., DENN domains) are divided into three subdomains, upstream DENN (u-DENN), core DENN (c-DENN), and downstream DENN (d-DENN) (Levivier *et al.*, 2001). The crystal structure of DENND1B has demonstrated that the u-DENN domain and an N-terminal region of the c-DENN domain constitute a longin fold, which contributes an interaction surface for its substrate Rab35 (Wu *et al.*, 2011). Interestingly, DENN-related domains, whose secondary and 3-dimensional structures are quite similar to classical DENN domains despite the two domains showing little conservation at the primary sequence level, have also been identified in several DENN-related proteins (Table I) (Nookala *et al.*, 2012; Zhang *et al.*, 2012; Levine *et al.*, 2013a). Whether DENND6/FAM116 is classified as a classical DENN protein or a DENN-related protein is a matter of controversy in the literature (Marat *et al.*, 2011; Linford *et al.*, 2012). We have included DENND6 in the classical DENN group in Table I in this review article, because DENND6 shows the highest degree of sequence similarity to DENN proteins (Linford *et al.*, 2012). In addition, DENND6 has actually been shown to function as a GEF for Rab14, which regulates a recycling pathway required for cell migration (Linford *et al.*, 2012). The substrate specificity, major interacting proteins, and relationships to human diseases (or animal models of human diseases) of the classical DENN proteins are summarized in Table I. Since the details of the classical DENN proteins, including their domain organization, have already been well summarized in Marat *et al.* (2011), in this section we particularly focus on recent advances in DENN research (after 2011).

One of the most important recent advances has been the

discovery of phospho-regulation of DENN proteins. For example, a serine and threonine kinase Mst1 has been found to be capable of phosphorylating DENND1C *in vitro*, and coexpression of Rab13 with DENND1C in COS-7 cells increases the amount of GTP-bound Rab13 (Nishikimi *et al.*, 2014). Two serine residues outside the DENN domain of DENND1A/connectin 1 can also be phosphorylated, and when they are phosphorylated, the amount of GTP-Rab35 increases in an Akt-dependent manner (Kulasekaran *et al.*, 2015). Starvation-induced ULK1-dependent phosphorylation of two serine residues outside the DENN domain occurs in DENND3 and results in activating endogenous Rab12 and thereby directly regulating macroautophagy through interaction between Rab12 and LC3 in H1299 cells (human lung carcinoma) (Xu *et al.*, 2015). However, a different mechanism by which Rab12 and DENND3 indirectly regulate macroautophagy by modulating the activity of mTORC1 (mammalian/mechanistic target of rapamycin complex 1), a well-known master regulator of cell growth and metabolism, through promoting degradation of amino-acid transporter PAT4 (proton-coupled amino-acid transporter 4) has also been reported (Matsui and Fukuda, 2013; Matsui *et al.*, 2014). Although the phospho-regulation mechanism of DENN proteins is worth noting, because the phosphorylation-dependent enhancement of GEF activity is measured indirectly by quantifying GTP-Rabs by means of effector pull-down assays (MICAL-L2 for Rab13 and RILP-L1 for Rab12) or by quantifying the interaction between DENND1A and its substrate GDP-Rab35, whether phosphorylation of these DENN proteins directly enhances their GDP release activity from respective substrate Rabs remains unknown. *In vitro* GEF assays performed with purified phosphorylated and non-phosphorylated GEFs will be necessary to resolve this issue.

Several DENN-related proteins have been suggested to function as regulators of small GTPases other than Rabs. FLCN (folliculin) is the most studied DENN-related protein, and FLCN deficiency causes Birt-Hogg-Dubé hereditary cancer syndrome (Schmidt and Linehan, 2015). Although FLCN has been shown to exhibit GEF activity toward Rab35 *in vitro*, whether it functions as an actual GEF for Rab35 *in vivo* remains to be determined (Nookala *et al.*, 2012). Instead, FLCN has recently been shown to function as a GAP for RagC/D GTPases through forming a complex with either FNIP1 or FNIP2 (Tsun *et al.*, 2013). Rags are atypical members of Ras-related GTPases and function as obligate heterodimers composed of RagA or RagB (A/B) and RagC or RagD (C/D). A combination of GTP-RagA/B and GDP-RagC/D is thought to activate mTORC1 by recruiting it to lysosomes (reviewed in Eltschinger and Loewith, 2016). Actually, RagC/D inactivation by the FLCN-FNIP2 complex has clearly been shown to promote mTORC1 binding to Rags *in vitro*, although the spatiotemporal regulatory mechanism by which FLCN-FNIP1/2 inactivates RagC/D within cells is

Table I. RAB-GEFs IN HUMAN

	Synonyms	Substrate Rabs	Major interacting proteins	Human diseases and their animal models
DENN (Gene ID)				
DENND1A (57706)	connecdenn 1	Rab35 (Allaire <i>et al.</i> , 2010) ^{2,3} (Yoshimura <i>et al.</i> , 2010) ¹ (Fukuda <i>et al.</i> , 2011) ⁴	α -adaptin, clathrin heavy chain, endophilin A1, intersectin, NECAP (Marat <i>et al.</i> , 2011)	<i>C. elegans rme-4</i> mutant (Sato <i>et al.</i> , 2008)
DENND1B (163486)	connecdenn 2	Rab35 (Marat and McPherson, 2010) ^{2,3} (Yoshimura <i>et al.</i> , 2010) ¹	α -adaptin, AP2 β 2, clathrin heavy chain (Marat <i>et al.</i> , 2011)	<i>Dennd1b</i> KO mice (Yang <i>et al.</i> , 2016)
DENND1C (79958)	connecdenn 3	Rab35 (Marat and McPherson, 2010) ^{2,3} Rab13 (Yoshimura <i>et al.</i> , 2010) ¹	α -adaptin, clathrin heavy chain (Marat <i>et al.</i> , 2011)	
DENND2A (27147)		Rab9 (Yoshimura <i>et al.</i> , 2010) ¹		
ST5 (6764)	DENND2B	Rab9 (Yoshimura <i>et al.</i> , 2010) ¹ Rab13 (Ioannou <i>et al.</i> , 2015) ^{3,4,5}		
DENND2C (163259)		Rab9 (Yoshimura <i>et al.</i> , 2010) ¹		
DENND2D (79961)				
DENND3 (22898)		Rab12 (Yoshimura <i>et al.</i> , 2010) ¹ Rab12 (Matsui <i>et al.</i> , 2014) ⁴		
DENND4A (10260)	IRLB/MYCPBP			<i>Drosophila Crag</i> mutant (Denef <i>et al.</i> , 2008)
DENND4B/C (9909/55667)		Rab10 (Yoshimura <i>et al.</i> , 2010) ¹		
DENND5A (23258)	Rab6IP1	Rab39 (Yoshimura <i>et al.</i> , 2010) ¹	Rab6, Rab11A (Marat <i>et al.</i> , 2011) SNX1 (Fernandes <i>et al.</i> , 2012)	
DENND5B (160518)	Rab6IP-like			
DENND6A/B (201627/414918)	FAM116A/B	Rab14 (Linford <i>et al.</i> , 2012) ¹		
SBF1 (6305)	MTMR5	Rab28 (Yoshimura <i>et al.</i> , 2010) ¹	MTMR2 (Marat <i>et al.</i> , 2011)	<i>Sbf-1</i> -null mice (Firestein <i>et al.</i> , 2002)
SBF2 (81846)	MTMR13			<i>Sbf-2</i> -null mice (Tersar <i>et al.</i> , 2007) Charcot-Marie-Tooth disease type 4B (Baets <i>et al.</i> , 2014)
MADD (8567)	DENN/Rab3GEP	Rab3 (Wada <i>et al.</i> , 1997) ¹ (Figueiredo <i>et al.</i> , 2008) ² Rab27 (Mahoney <i>et al.</i> , 2006) ¹ (Figueiredo <i>et al.</i> , 2008) ² (Yoshimura <i>et al.</i> , 2010) ¹	Rab3, Kif1a, Kif1b β , DR4, DR5, TNFR1, JNK3, LRDD, IA-2 (Marat <i>et al.</i> , 2011) Rabconnectin-3 (Kawabe <i>et al.</i> , 2003)	<i>Madd</i> -null mice (Tanaka <i>et al.</i> , 2001) <i>C. elegans aex-3</i> mutant (Iwasaki <i>et al.</i> , 1997)
DENN-related				
FLCN (201163)	folliculin	Rab35 (Nookala <i>et al.</i> , 2012) ¹	GAP for RagC/D GTPase (Tsun <i>et al.</i> , 2013)	Birt-Hogg-Dubé syndrome <i>Fln</i> -, <i>Fnip1</i> -, <i>Fnip2</i> -null mice (Schmidt and Linehan, 2015)
FNIP1 (96459)		ND		
FNIP2 (57600)				
AVL9 (23080)		ND	Rho3p (Ito <i>et al.</i> , 2001) GTR2 (yeast RagC/D homologue) (Zhang <i>et al.</i> , 2010)	
KIAA1147 (57189)	LCHN/ANR2	ND		
FAM45A (404636)	ANR3	ND		
C9ORF72 (427370)	DENNL72	ND	Rab1, Rab5, Rab7, Rab11, ubiquilin-1/2, hnRNPA1, hnRNPA2/B1, actin (Farg, <i>et al.</i> , 2014)	<i>C9orf72</i> -null mice (Panda <i>et al.</i> , 2013; Koppers <i>et al.</i> , 2015) Fronto-temporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (Lattante <i>et al.</i> , 2015)
SMCR8 (140775)		ND		
NPR2 (4882)	GATOR1 complex	ND	GAP for RagA/B GTPase (Bar-peled <i>et al.</i> , 2013)	
NPR3 (4883)				
TRAPP complex				
TRAPP I core components				
TRAPPC1 (58485)	Bet5/MUM-2	Ypt1p (Wang <i>et al.</i> , 2000) ^{1,2,3} Ypt31/32p (Jones <i>et al.</i> , 2000) ^{1,2} Rab1 (Yamasaki <i>et al.</i> , 2009) ²		
TRAPPC2 (6399)	Trs20/Sedlin		CLIC1/2, PAM14, MBP1, PITX1, SF1 (Yu and Liang, 2012) TANGO1 (Brunet and Sacher, 2014)	Spondyloepiphyseal dysplasia tarda (Brunet and Sacher, 2014)
TRAPPC2L (51693)	Tca17		TECPR-1 (Yu and Liang, 2012)	
TRAPPC3 (27095)	Bet3		Sec23, TECPR-1 (Yu and Liang, 2012)	
TRAPPC4 (51399)	Trs23/Synbindin		ERK2, Syndecan-2, TECPR-1 (Yu and Liang, 2012)	
TRAPPC5 (126003)	Trs31		TECPR-1 (Yu and Liang, 2012)	
TRAPPC6A/B (79090/122553)	Trs33/TPC6			<i>myhp</i> mice (Brunet and Sacher, 2014)
TRAPP II subunits				
TRAPPC9 (83696)	Trs120/NIBP		p150 ^{Gluad} , NIK, IKK β , γ 1COP (Yu and Liang, 2012)	autosomal-recessive mental retardation (Brunet and Sacher, 2014)
TRAPPC10 (7109)	Trs130/TMEM1		Rabin8, γ 1COP (Yu and Liang, 2012)	
TRAPP III subunits				
TRAPPC8 (22878)	Trs85/GSG1		TECPR-1 (Yu and Liang, 2012) TBC1D14 (Lamb <i>et al.</i> , 2016)	

Rab-GEFs and Their Target Rabs

Table I. (continued)

	Synonyms	Substrate Rabs	Major interacting proteins	Human diseases and their animal models
TRAPPC11 (60684)			TECPR-1 (Yu and Liang, 2012)	Spectrum of limb girdle muscular dystrophy and myopathy (Brunet and Sacher, 2014)
TRAPPC12 (51112)	TRAMM/TTC15		CENP-E (Milev <i>et al.</i> , 2015) TECPR-1 (Yu and Liang, 2012)	
TRAPPC13 (80006)	Trs65		Gea2 (Yu and Liang, 2012)	
Heterodimer type				
MON1A (84315)	SAND-1	Ypt7p (Nordmann <i>et al.</i> , 2010) ¹ Rab7 (Gerondopoulos <i>et al.</i> , 2012) ¹	Rab5 (Kinchen and Ravichandran 2010) VPS21 (Nordmann <i>et al.</i> , 2010) VPS11, VPS18, VPS39 (Wang <i>et al.</i> , 2003; Nordmann <i>et al.</i> , 2010) Rabex-5, VPS33A (Poteryaev <i>et al.</i> , 2010)	
CCZ1 (51622)				
MON1B (22879)			Rabex-5, VPS11, VPS16A, VPS18, VPS33A, VPS41 (Poteryaev <i>et al.</i> , 2010)	
HPS1 (3257)	BLOC-3 complex	Rab32, 38 (Gerondopoulos <i>et al.</i> , 2012) ¹		Hermansky–Pudlak syndrome (HPS) (Wei and Li, 2013)
HPS4 (89781)			Rab9 (Kloer <i>et al.</i> , 2010)	
Rab3GAP1 (22930)		Rab18 (Gerondopoulos <i>et al.</i> , 2014) ¹	GAP for Rab3 (Fukui <i>et al.</i> , 1997) VAP-B (Hantan <i>et al.</i> , 2014)	Micro and Martsolf syndromes (Handley and Aligianis, 2012)
Rab3GAP2 (25782)				
RIC1 (57589)		Ypt6p (Siniosoglou <i>et al.</i> , 2000) ^{1,3} Rab6 (Pusapati <i>et al.</i> , 2012) ^{2,3}	Rab33B (Pusapati <i>et al.</i> , 2012)	
RGP1 (9827)				
VPS9 domain				
RABGEF1 (27342)	Rabex-5	Rab5 (Horiuchi <i>et al.</i> , 1997) ^{1,2,3} Rab21, 22A (Delprato <i>et al.</i> , 2004) ¹ Rab17 (Yoshimura <i>et al.</i> , 2010) ¹ (Mori <i>et al.</i> , 2013) ³	Rabaptin-5 (Horiuchi <i>et al.</i> , 1997) Rabaptin-5β (Gournier <i>et al.</i> , 1998) Rab22 (Zhu <i>et al.</i> , 2009)	<i>Rabgef1</i> KO mice (Tam <i>et al.</i> , 2004)
GAPVD1 (26130)	Gapex-5/hRME-6	<i>C. elegans</i> RAB-5 (Sato <i>et al.</i> , 2005) ³ Rab22B/31 (Lodhi <i>et al.</i> , 2007) ^{3,4} Rab5 (Kitano <i>et al.</i> , 2008) ⁵	α-adaptin (Sato <i>et al.</i> , 2005) CIP4 (Lodhi <i>et al.</i> , 2007) EB1 (Kitano <i>et al.</i> , 2008)	<i>C. elegans rme-6</i> mutant (Sato <i>et al.</i> , 2005)
RIN1 (9610)		Rab5 (Tall <i>et al.</i> , 2001) ^{1,2,3} Rab22B/31 (Kajiho <i>et al.</i> , 2011) ⁴	EGFR, H-Ras, ABL2 (Carney <i>et al.</i> , 2006), STAM2 (Kong <i>et al.</i> , 2007) EphA4 (Deininger <i>et al.</i> , 2008)	<i>Rin1</i> KO mice (Dhaka <i>et al.</i> , 2003)
RIN2 (54453)		Rab5 (Saito <i>et al.</i> , 2002) ² Rab22B/31 (Kajiho <i>et al.</i> , 2011) ⁴	amphiphysin II (Carney <i>et al.</i> , 2006) R-Ras (Sandri <i>et al.</i> , 2012)	MACS syndrome (Basel-Vanagaite <i>et al.</i> , 2009)
RIN3 (79890)		Rab5 (Kajiho <i>et al.</i> , 2003) ^{1,2} Rab22B/31 (Kajiho <i>et al.</i> , 2011) ^{2,4}	amphiphysin II (Carney <i>et al.</i> , 2006) BIN2 (Janson <i>et al.</i> , 2012) CD2AP (Rouka <i>et al.</i> , 2015)	
RINL (126432)		Rab5, 22A (Woller <i>et al.</i> , 2011) ^{1,3} Rab21, 22B/31 (Kajiho <i>et al.</i> , 2012) ⁴	EphA8, Odin (Kajiho <i>et al.</i> , 2012)	
ALS2 (57679)	Alsin	Rab5 (Otomo <i>et al.</i> , 2003) ^{1,2,3} (Topp <i>et al.</i> , 2004) ^{1,3} Rab22B/31 (Kajiho <i>et al.</i> , 2011) ⁴	Rac1 (Kunita <i>et al.</i> , 2007) GRIP1 (Lai <i>et al.</i> , 2006) Neurocalcin α (Masutani <i>et al.</i> , 2008)	ALS and <i>ALS2</i> KO mice (Cai <i>et al.</i> , 2008a)
ALS2CL (259173)		Rab5 (Hadano <i>et al.</i> , 2004) ^{1,3} Rab22B/31 (Kajiho <i>et al.</i> , 2011) ⁴	ALS2 (Suzuki-Utsunomiya <i>et al.</i> , 2007)	
ANKRD27 (84079)	Varp	Rab21 (Zhang <i>et al.</i> , 2006) ^{2,3} (Tamura <i>et al.</i> , 2011) ³	Rab32 (Tamura <i>et al.</i> , 2009) Rab38 (Wang <i>et al.</i> , 2008) VAMP7 (Burgo <i>et al.</i> , 2009) VPS29 (Hesketh <i>et al.</i> , 2014) VPS35 (McGough <i>et al.</i> , 2014) Rab40 (Yatsu <i>et al.</i> , 2015) RACK1 (Marubashi <i>et al.</i> , 2016b)	
VPS9D1 (9605)	C16orf7	ND		
Homodimer				
Rab3IP (117177)	RABIN3/RABIN8/ Gtpat12	Sec4p (Walch-Solimena <i>et al.</i> , 1997) ^{1,2,3} Rab8 (Hattula <i>et al.</i> , 2002) ^{1,2,3}	Ypt31/32p (Ortiz <i>et al.</i> , 2002) Rab11 (Knödler <i>et al.</i> , 2010) Sec15 (Guo <i>et al.</i> , 1999; Feng <i>et al.</i> , 2012) mTRAPP II (Westlake <i>et al.</i> , 2011) BBS1 (Nachury <i>et al.</i> , 2007) FIP3 (Vetter <i>et al.</i> , 2015; Wang <i>et al.</i> , 2015a)	<i>Pat12</i> mutant mice (Yoshida <i>et al.</i> , 1995)
Rab3IL1 (5866)	GRAB	Rab8 (Yoshimura <i>et al.</i> , 2010) ¹ Rab3 (Luo <i>et al.</i> , 2001) ^{1,2,3}	Ip6k1 (Luo <i>et al.</i> , 2001) Rab11 (Horgan <i>et al.</i> , 2013)	
Others				
SH3BP5 (9467)	REI-1	Rab11 (Sakaguchi <i>et al.</i> , 2015) ^{1,3}		
SH3BP5L (80851)		ND		
RPGR (6103)		Rab8 (Murga-Zamalloa <i>et al.</i> , 2010) ^{2,3}		X-linked retinitis pigmentosa (Jacobson <i>et al.</i> , 1997)

All putative GEFs and their substrate Rabs, major interacting proteins, and association with diseases, including animal models, are listed. The numbers at the upper right of each reference in the “Substrate Rabs” column indicate the methods by which GEF activities were investigated (i.e., 1, GDP-release assays performed with [³H]GDP or MANT-GDP; 2, GTP-loading assays performed with [³⁵S]GTPγS, [³²P]GTP, or MANT-GTP; 3, binding assays between GEFs and GDP-bound or nucleotide free Rabs; 4, quantification of GTP-bound Rabs by effector pull-down assays or “*in cell*” GEF activity assays” (Kajiho *et al.*, 2011); and 5, monitoring Rab activity in cells with FRET biosensors). More detailed information about DENN-protein interacting proteins and the TRAPP-complex interacting proteins is summarized in Marat *et al.* (2011) and Yu and Liang (2012), respectively. ND, not determined.

Fig. 2. Relationships between human Rab isoforms and their respective GEFs. A simple phylogenic tree of human Rabs ("A" isoforms alone) was generated by using the MUSCLE alignment and DrawTree online tools. See Diekmann *et al.* (2011) for more precise Rab family phylogenic trees. The nomenclature of the human Rabs is according to Itoh *et al.* (2006). Rabs whose GEFs have already been determined are shown against a color background, and the names of their respective GEFs are shown next to them. Based on their sequence similarity and/or subunit composition, in this article these GEFs have been classified into six groups (the members of each group are shown against a different color background) (see text for details). Note that the targets of VPS9 proteins belong to the same Rab5 family (blue background), whereas the targets of the GEFs in the other groups are distributed differently in the phylogenetic tree.

not completely understood (Tsun *et al.*, 2013). Interestingly, a GATOR1 complex, which is also composed of two DENN-related proteins (NPRL2 and NPRL3) and DEPDC5, has been reported to exhibit GAP activity toward RagA/B, even though the DENN-related domains of both NPRL2 and NPRL3 contain a u-DENN (longin) domain alone and lack c-DENN and d-DENN domains (Zhang *et al.*, 2012; Bar-Peled *et al.*, 2013). The yeast (*Saccharomyces cerevisiae*) protein Afi1p has also been identified as a

DENN-related protein (Zhang *et al.*, 2012; Levine *et al.*, 2013a) that specifically binds GTP-bound Arf3p, but no Af1p homologues have ever been identified in animals (Tsai *et al.*, 2008). Based on these findings together with the fact that sequence conservation between classical DENN and DENN-related domains is very low, DENN-related proteins are likely to function as regulators of other small GTPases, e.g., Rags and Arfs, rather than as Rab-GEFs.

Fig. 3. Domain organization of representative members of various human GEF families. DENND1A, DENND6A, MADD (DENN proteins), Rabex-5, Rin1 (VPS9 proteins), Mon1A/Ccz1, Rab3GAP1/2 (heterodimer GEF), Rabin8 (homodimer GEF), SH3BP5, and the mTRAPP II complex (multi-subunit GEF) are shown (their gene IDs are listed in Table I). Their domain structures, except for the longin domains of Mon1A and Ccz1, were identified by using the SMART program. The longin domains of Mon1A and Ccz1 are depicted according to Kinch and Grishin (2006) and are presumably involved in heterodimer formation between Mon1A and Ccz1, the same as the longin domains of yeast Mon1 and Ccz1. The TRAPP I core complex within the mTRAPP II complex is shown in green, and mTRAPP II-specific subunits are shown in blue. Subunits that contain a longin domain are indicated by arrows. Amino acid numbers are shown on both sides. GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; RA, Ras association; SH2, Src homology 2; VPS9, vacuolar protein sorting 9; and Zn²⁺, zinc finger domain.

VPS9 protein

The VPS9 domain was originally identified in yeast Vps9p and subsequently characterized as a GEF domain for one or several members of the Rab5 subfamily (Fig. 2) (Burd *et al.*, 1996). Ten different VPS9 proteins have been identified

in humans and based on their domain organization they have been classified into six subgroups (i.e., Rabex-5, Gapex-5, Varp, Als2 family members, Rin family members, and VPS9D1) (Fukuda, 2016). Most VPS9 proteins contain a variety of protein motifs in addition to the VPS9 domain, and they enable VPS9 proteins to play multiple roles in

Rab5-mediated endosomal trafficking (Table I and Fig. 3) (see also Carney *et al.*, 2006; Fukuda, 2016, for a summary of the domain organization of each VPS9 protein except VPS9D1, a previously uncharacterized protein with a C-terminal VPS9 domain). The best characterized VPS9 protein is Rabex-5 (also known as RABGEF1), which exhibits *in vitro* and/or *in vivo* GEF activity toward several Rab5 subfamily members, including Rab5, Rab17, Rab21, and Rab22A (Horiuchi *et al.*, 1997; Delprato *et al.*, 2004; Yoshimura *et al.*, 2010; Mori *et al.*, 2013). In mammalian cells, Rabex-5 is recruited to early endosomes through its N-terminal ubiquitin-binding domain, where Rabaptin5, a Rabex-5-binding partner, relieves autoinhibition of Rabex-5 thereby yielding an active form of Rab5 (Mattera and Bonifacio, 2008; Zhang *et al.*, 2014). Although a feedback mechanism in which Rab5 recruits Rabaptin5 that is associated with Rabex-5 and enhances Rab5 activation has been suggested, a recent study has shown that the membrane localization of Rabaptin5 depends on Rab4 and Rabex-5, not on Rab5 (Kälin *et al.*, 2015). On the other hand, Gapex-5 (also called hRME-6), which was originally identified in *C. elegans rme-6* mutants and exhibits GEF activity toward Rab5 and Rab22B/31, localizes on clathrin-coated vesicles and regulates Rab5-mediated uncoating of AP2 from clathrin-coated vesicles in mammalian cells (Sato *et al.*, 2005; Lodhi *et al.*, 2007; Semerdjieva *et al.*, 2008). In addition, use of a FRET (fluorescence resonance energy transfer) sensor for Rab5 called “Raichu-Rab5” has shown that Gapex-5, not Rabex-5 or Rin1, is required for activation of Rab5 when milk-fat-globule epidermal growth factor 8 (MFG-E8) is engulfed by apoptotic thymocytes (Kitano *et al.*, 2008). Thus, the existence of multiple types of VPS9 proteins with different characters is likely to ensure multiple roles of Rab5 in endocytic trafficking.

Several VPS9 proteins have been implicated in the regulation of other small GTPases, including Ras, Rho, and Ran, in addition to activation of Rab5 family members. For example, although RIN family proteins (i.e., RIN1–3 and RINL) share GEF activity toward several Rab5 subfamily members *in vitro* (Table I), only RIN1–3, not RINL, contain an additional RA (Ras association) domain, which functions as an effector domain of GTP-Ras (reviewed in Carney *et al.*, 2006). Actually, RIN2, whose deficiency has been shown to cause MACS (macrocephaly, alopecia, cutis laxa, and scoliosis) syndrome, is localized on early endosomes together with Rab5 and R-Ras and regulates endothelial cell adhesion (Basel-Vanagaite *et al.*, 2009; Sandri *et al.*, 2012). Another example is ALS2 (amyotrophic lateral sclerosis 2, also called *alsin*), a well-known causative gene for a juvenile autosomal recessive form of motor neuron disease. ALS2 protein contains two other putative GEF domains of small GTPases in addition to the VPS9 domain: an RCC1 (RanGEF)-like domain with weak Ran-GEF activity and a DH/PH domain that is a hallmark of Rho family-GEFs (Otomo *et al.*, 2003). However, a subsequent

study has shown that ALS2 acts as a Rac1 effector rather than a Rac1-GEF (Kunita *et al.*, 2007). The molecular and cellular roles of ALS2 in endosomal trafficking are summarized in detail elsewhere (reviewed in Hadano *et al.*, 2007). Moreover, Rabex-5 contains an A20-type zinc finger domain with E3 ubiquitin ligase activity for H-Ras and N-Ras, which promotes endosomal localization of Ras and attenuates Ras-mediated ERK activation (reviewed in Colicelli, 2010), and Gapex-5 contains an evolutionarily conserved Ras-GAP-like domain in its N-terminal region, but its physiological role is unclear (Sato *et al.*, 2005). Taken together, these findings suggest that these VPS9 proteins may link Rab5-mediated membrane traffic and other cellular events, including cell proliferation and differentiation.

Varp (VPS9-ankyrin repeat protein, also known as Ankrd27) is another interesting VPS9 protein, because it functionally links to other membrane trafficking proteins, including v-SNARE and the retromer complex. Varp is composed of an N-terminal VPS9 domain and C-terminal two ankyrin-repeat (ANKR) domains, and it was originally characterized as a specific GEF for Rab21 (Zhang *et al.*, 2006). Rab21-activation by Varp and its interaction with VAMP7 cooperate to regulate neurite outgrowth of both PC12 cells and mouse hippocampal neurons and dendrite outgrowth of melanocytes (Burgo *et al.*, 2009, 2012; Ohbayashi *et al.*, 2012). Varp is also involved in melanogenic enzyme transport to melanosomes, which are known as lysosome-related organelles, through interaction with Rab32 and Rab38 via the ANKR1 domain (Tamura *et al.*, 2009, 2011) and with Rab40C via the ANKR2 domain (Yatsu *et al.*, 2015). Moreover, Varp interacts with two subunits of the retromer complex (VPS29 and VPS35) and is involved in the endosome-to-plasma-membrane recycling of certain transmembrane proteins (Hesketh *et al.*, 2014; McGough *et al.*, 2014). Thus, rather than simply being a Rab21-GEF, Varp plays multiple roles in endosomal trafficking through interaction with other membrane trafficking regulators (Fukuda, 2016).

Sec2 protein

SEC2 was originally identified in *S. cerevisiae* as a gene essential for cell growth and vesicle transport from the Golgi to the plasma membrane. Genetic and biochemical analyses of SEC2 mutants have demonstrated that the product of the wild-type SEC2 gene, Sec2p, is a GEF for Sec4p, a yeast homologue of Rab8 (Walch-Solimena *et al.*, 1997). Two mammalian Sec2p homologues, Rabin8 (Rab8-interacting protein; also called Rabin3) and GRAB (guanine nucleotide exchange factor for Rab3A), have subsequently been shown to exhibit GEF activity toward Rab8 (Hattula *et al.*, 2002; Yoshimura *et al.*, 2010). As their names indicate, Rabin8 is capable of binding to GDP-Rab8, and GRAB exhibits GEF activity toward Rab3 (Brondyk *et al.*, 1995; Luo *et al.*, 2001. Figueiredo *et al.*,

2008). However, recent studies have shown that GRAB is a specific GEF for Rab8 rather than for Rab3 (Yoshimura *et al.*, 2010; Guo *et al.*, 2013), and the physiological significance of the interaction between GDP-Rab3 and Rabin8 or GRAB remains completely unknown. Crystallographic studies have shown that the conserved GEF domain of Sec2p (i.e., Sec2 domain in the middle of the molecule; see Fig. 3) homodimerizes into a long parallel coiled-coil which provides a surface for interaction with Sec4p (Dong *et al.*, 2007; Sato *et al.*, 2007).

One of the best characterized physiological functions of Rabin8 is in the polarized membrane trafficking from the *trans*-Golgi network (or recycling endosomes) to the plasma membrane that is required for ciliogenesis and epithelial lumenogenesis. Interestingly, Rabin8 is thought to function as a Rab11 effector, i.e., it directly interacts with GTP-Rab11 at its C-terminal domain (Fig. 3), and that the interaction is required for Rabin8 recruitment to Rab11-positive vesicles/organelles, where Rab8 is activated. Thus, the Rab11–Rabin8–Rab8 axis is likely to form a Rab cascade that was originally proposed in yeasts (Ypt32p–Sec2p–Sec4p; see Ortiz *et al.*, 2002 for details) (Fig. 4B). Rabin8, GTP-Rab8, and GTP-Rab11 further interact with their downstream effector Sec15, a component of the exocyst tethering complex, and their interactions facilitate tethering of Rab8 and/or Rab11-positive carriers to polarized compartments (reviewed in Das and Guo, 2011). The same as the DENN proteins described above, Rabin8 is phosphorylated by several kinases, and phosphorylation alters the biochemical properties of Rabin8, e.g., results in increased GEF activity (Wang *et al.*, 2015b), decreased affinity for phosphatidylserine, and increased affinity for Sec15 (Chiba *et al.*, 2013). Interestingly, although effector binding to Rab is generally thought to be mutually exclusive, in addition to binding to Rabin8, Rab11 is able to simultaneously bind to its effector FIP3 (Vetter *et al.*, 2015). The Rab11–Rabin8–FIP3 complex may establish the platform required for Rab8 activation. Moreover, the N-terminal domain of Rabin8 interacts with the TRAPP II complex (Fig. 3), a multi-subunit GEF for Rab1 (see next section below), but the physiological role of this interaction is poorly understood (Westlake *et al.*, 2011).

TRAPP complexes

The TRAPP complexes are a multi-subunit GEF (Fig. 3) whose GEF activity was originally demonstrated toward Ypt1p in yeast and later toward Rab1 (a Ypt1p homologue) in mammals (Wang *et al.*, 2000; Yamasaki *et al.*, 2009). Because there have been several recent reviews of the literature on the architecture and cellular functions of TRAPP complexes as well as their association with human genetic diseases (Sacher *et al.*, 2008; Barrowman *et al.*, 2010; Yu and Liang, 2012; Brunet and Sacher, 2014), in this section we will only briefly summarize their basic cellular func-

tions with a special focus on their GEF activity.

Yeast contains three forms of the TRAPP complex, each of which has a distinct function, the TRAPP I complex, in ER-Golgi traffic; the TRAPP II complex, in intra-Golgi and endosome-Golgi traffic; and the TRAPP III complex, in autophagy. The TRAPP I complex forms a stable core that is responsible for Ypt1p GEF activity (Fig. 3) (Kim *et al.*, 2006; Cai *et al.*, 2008b), whereas the TRAPP II complex and TRAPP III complex each contains an additional individual set of subunits in addition to the TRAPP I core (Yu and Liang, 2012). All three TRAPP I–III complexes are capable of activating Ypt1p (Brunet and Sacher, 2014). The minimal set of subunits required for Ypt1p-GEF activity is: Bet3p, Trs23p, Trs31p, and Bet5p (TRAPPC3, TRAPPC4, TRAPPC5, and TRAPPC1, respectively, in mammals), and two of them, Trs23p and Bet5p, contain a longin domain that interacts with Ypt1p (Kim *et al.*, 2006; Cai *et al.*, 2008b). One of the controversial issues in the area of TRAPP research is whether the TRAPP II complex acts as a GEF for the yeast Rab11 homologues Ypt31p and Ypt32p. In fact, several groups have shown that the TRAPP II complex functions as a specific GEF for Rab11 homologues in *S. cerevisiae* and *Aspergillus nidulans* (Morozova *et al.*, 2006; Cabrera and Ungermann, 2013; Pinar *et al.*, 2015).

Unlike yeasts, no small-core TRAPP complex equivalent to the yeast TRAPP I complex appears to be present in mammals, suggesting that TRAPP complexes play differently roles in early secretory trafficking in yeasts and mammals (Scrivens *et al.*, 2011). On the other hand, mammals contain two functionally different TRAPP complexes, named mTRAPP II and mTRAPP III, that are broadly similar to but clearly different in terms of their subunit composition from the yeast TRAPP II complex and TRAPP III complex, respectively. For example, TRAPPC13, a mammalian homologue of the yeast TRAPP II-specific subunit Trs65p, is not contained in the mTRAPP II complex but appears to be present in the mTRAPP III complex, and the mTRAPP III complex contains additional mammal-specific subunits (TRAPPC11 and TRAPPC12) (Bassik *et al.*, 2013). In contrast to the yeast TRAPP II complex, the mTRAPP II complex (TRAPPC10-containing complex) exhibits GEF activity toward Rab1, but not toward Rab11 (Yamasaki *et al.*, 2009). Because mutations in genes encoding different subunits of mTRAPP complexes result in different pathologies, mammalian TRAPP complexes appear to regulate a variety of membrane trafficking events (reviewed in Brunet and Sacher, 2014).

Heterodimer GEF complexes

Four different heterodimer GEF complexes, each of which shows completely different substrate specificity, have been reported. The Mon1A–Ccz1 complex, HPS1–HPS4 complex (also known as BLOC-3, biogenesis of lysosome-related organelles complex-3), Ric1–Rgp1 complex, and

Fig. 4. Schematic models of “Rab GEF cascades”. Two well characterized (A, B) and two hypothetical (C, D) Rab GEF cascades are shown. (A) Rab5–(Mon1–Ccz1)–Rab7 cascade. The Mon1–Ccz1 complex is recruited to Rab5-positive endosomes through direct interaction between Mon1 and Rab5, and the recruited complex activates Rab7 there. Both Mon1 and Rab7 interact with the HOPS tethering complex. The Rab5-to-Rab7 conversion promotes early-to-late endosome maturation. (B) Rab11–Rabin8–Rab8 cascade. Rab11 recruits Rabin8 to a Rab11-positive *trans*-Golgi network and/or recycling endosomes, where Rabin8 activates Rab8. Rab11 simultaneously binds both Rabin8 and FIP3, and so doing may establish a platform for Rab8 activation. (C) Rab9–(HPS1–HPS4)–Rab32/38 cascade. Rab9A has been proposed to recruit the BLOC-3 (HPS1–HPS4) complex to melanogenic enzyme-positive endosomes, where the complex activates Rab32 and Rab38 (Marubashi *et al.*, 2016a). (D) Rab33B–(Ric1–Rgp1)–Rab6 cascade. *cis*-Golgi-resident Rab33B has been proposed to recruit the Ric1–Rgp1 complex to *medial*-Golgi compartments, where Rab6 is activated (Pusapati *et al.*, 2012). The latter two models (C, D) have not yet been demonstrated experimentally.

Rab3GAP1–Rab3GAP2 complex function as GEFs for Rab7, Rab32/38, Rab6, and Rab18, respectively. Although weak sequence similarity has been found between the Mon1A–Ccz1 complex and the HPS1–HPS4 complex, no clear homology has been found between any of the other GEF complexes. However, dimerization of two subunits is required for the GEF activity of each of them, and neither subunit alone accelerates GDP release from Rabs.

Mon1–Ccz1 and HPS1–HPS4 (BLOC-3) complexes

Genetic and biochemical analyses of Mon1 and Ccz1 in *S. cerevisiae* and metazoan cells have indicated that they form a heterodimer complex that is linked to activation of Rab7 (or Ypt7p in *S. cerevisiae*) on late endosomes. The Mon1–Ccz1 complex was subsequently clearly demonstrated to function as a specific GEF for Ypt7p/Rab7 and to play a crucial role in the Rab5-to-Rab7 conversion on endosomes, which leads to promotion of the early-to-late endosomal

transition and endosomal maturation (reviewed in Balderhaar and Ungermann, 2013). Interestingly, Mon1 is recruited to Rab5 (VPS21 in yeast)-positive endosomes through direct interaction with GTP-bound Rab5 (i.e., Rab5 effector) in *S. cerevisiae* and *C. elegans* (Kinchen and Ravichandran, 2010; Nordmann *et al.*, 2010), indicating that Rab5 and Rab7 constitute a cascade from Rab5 to Rab7 through the Rab7-GEF Mon1–Ccz1 complex (Fig. 4A). Mon1 has also been shown to be capable of displacing Rabex5 (Rab5-GEF) from endosomes when Mon1 is overexpressed in metazoan cells (Poteryaev *et al.*, 2010). Mon1 and Ccz1 bind each other via their longin domains (Fig. 3), and the resulting heterodimer complex actually functions as a specific GEF for Ypt7p on late endosomes, and thereby promotes fusion of late endosomes with vacuoles by recruiting a HOPS (homotypic fusion and vacuole protein sorting) tethering complex (Nordmann *et al.*, 2010; and reviewed in Solinger and Spang, 2013) (Fig.

4A). In addition, the Mon1–Ccz1 complex preferentially binds to negatively charged phospholipids, including to phosphatidylinositol 3-phosphate (PI3P), which mainly localizes on early endosomes, and its interaction with the PI3P may facilitate encounters between Ypt7p and the Mon1–Ccz1 complex in specific endosomal compartments (Poteryaev *et al.*, 2010; Cabrera *et al.*, 2014). Similarly, the mammalian Mon1A–Ccz1 complex functions as the GEF for Rab7 on late endosomes, but not on lysosomes (Gerondopoulos *et al.*, 2012; Yasuda *et al.*, 2016). Another Mon1 isoform, Mon1B, which is homologous to Mon1A, is present in vertebrates. However, Mon1A and Mon1B appear to have overlapping but different rather than redundant functions, because, even though both isoforms interact with HOPS subunit VPS33A, only Mon1B has the ability to bind other HOPS subunits, i.e., VPS11, VPS16A, VPS18, and VPS41 (Poteryaev *et al.*, 2010).

On the other hand, VPS39, a subunit of the HOPS complex, has also been reported to be a GEF for Ypt7p/Rab7 (Wurmser *et al.*, 2000). However, VPS39 is unlikely to be a Rab7-GEF, because another group has demonstrated that VPS39 (or the HOPS complex) does not exhibit any Ypt7p-GEF activity. This discrepancy may be explained by contamination by the Mon1–Ccz1 complex during the GEF assays, because VPS39 strongly interacts with Mon1 (Nordmann *et al.*, 2010). Since different results of assays of binding between HOPS subunits and Mon1 appear to have been obtained in previous studies, a more detailed characterization of the ability of Mon1 to bind to HOPS subunits will be necessary in the future (Wang *et al.*, 2003; Nordmann *et al.*, 2010; Poteryaev *et al.*, 2010).

HPS1 and HPS4 also form a heterodimer (BLOC-3) and are well-known causative gene products of Hermansky-Pudlak syndrome (HPS), which is characterized by deficient biogenesis of lysosome-related organelles, including melanosomes in melanocytes and dense granules in platelets (Dell'Angelica, 2004; Wei and Li, 2013). Because of these defects, both HPS1 patients and HPS4 patients exhibit oculocutaneous albinism and a bleeding tendency. Interestingly, bioinformatic analysis has shown that both HPS1 and HPS4 contain longin-like domains, the same as Mon1 and Ccz1 do (Kinch and Grishin, 2006). Consistent with this information, BLOC-3 has been shown to be a GEF for two closely related Rabs, Rab32 and Rab38, whose deficiency causes the diluted coat color of *chocolate* mice and *Ruby* rats (an HPS model). Both Rabs are known to redundantly regulate melanogenic enzyme transport to melanosomes through interaction with their specific effector Varp (Wasmeier *et al.*, 2006; Tamura *et al.*, 2009). In the absence of either HPS1 or HPS4, Rab32 and Rab38 are unable to localize on melanogenic-enzyme-positive vesicles, and the result is inhibition of melanosome biogenesis (Gerondopoulos *et al.*, 2012). Although Varp contains the VPS9 domain and also functions as a Rab21-GEF as described above, the GEF activity of Varp is not essential

for transport of melanogenic enzymes, suggesting that, at least in melanocytes, Rab21 and Rab32/38 function in independent pathways rather than constitute a cascade from Rab32/38 to Rab21 (Tamura *et al.*, 2011; Ohbayashi *et al.*, 2012). In addition to the Rab32/38-GEF activity of BLOC-3, its subunit HPS4 has been suggested to function as an effector for Rab9 (Kloer *et al.*, 2010). Actually, involvement of Rab9A in melanogenic enzyme transport has very recently been reported by two independent groups (Mahanty *et al.*, 2016; Marubashi *et al.*, 2016a), but whether the HPS4–Rab9A interaction (or a cascade from Rab9A to Rab32/38) is actually required for this process has never been determined experimentally (Fig. 4C).

Ric1–Rgp1 complex

The Ric1–Rgp1 heterodimer complex was originally identified as a GEF for Rab6 by genetic analysis of *YPT6*, a yeast homologue of the *Rab6* gene, and subsequent study has shown that the mammalian Ric1–Rgp1 complex also possesses GEF activity toward Rab6 in mammalian cells (Siniossoglou *et al.*, 2000; Pusapati *et al.*, 2012). The Ric1–Rgp1 complex is present in mammalian cell Golgi compartments, where Rab6 is activated, and is required for Rab6-mediated retrograde transport of mannose 6-phosphate receptors from late endosomes to the Golgi. Interestingly, mammalian Ric1 protein has been shown to directly interact with medial Golgi-resident Rab33B and possibly contribute to a “Rab cascade” (Pusapati *et al.*, 2012) (Fig. 4D).

Rab3GAP1–Rab3GAP2 complex

As indicated by their names, Rab3GAP1 and Rab3GAP2 were originally identified as subunits of a Rab3-GAP complex that stimulates GTP hydrolysis by Rab3 (Fukui *et al.*, 1997; Nagano *et al.*, 1998). Subsequent human genetic analyses have indicated that the Rab3GAP complex is functionally linked to Rab18, rather than to Rab3 (i.e., the Rab3GAP complex and Rab18 function in the same pathway), because mutations in either *Rab18*, *Rab3GAP1*, or *Rab3GAP2* cause the same Warburg Micro syndromes, rare autosomal recessive disorders characterized by ocular and neurological abnormalities and hypothalamic hypogonadism (Handley and Aligianis, 2012). Actually, it has recently been shown that the Rab3GAP complex localizes on a subdomain of the endoplasmic reticulum (ER) and functions as a specific GEF for Rab18 and thereby maintains appropriate ER structures in mammalian cells, although the precise relationship between the pathology of the syndromes and subcellular ER structures remains elusive (Friedman and Voeltz, 2011; Gerondopoulos *et al.*, 2014). In addition, the disease-associated mutations in the Rab3GAP complex result in abolition of its Rab18-GEF activity and dissociation of Rab18 from the ER, which induces disruption of the ER tubular network as well as the spread of ER sheets in cells.

However, the same point mutations have no effect on its Rab3-GAP activity, indicating that reduced GTP hydrolysis by Rab3 is not a major cause of Warburg Micro syndromes. More recently, involvement of the Rab3GAP complex in autophagy has been reported (Spang *et al.*, 2014; Zirin *et al.*, 2015), but nothing is known about the direct relationship between Rab18-GEF activity of Rab3GAP (or Rab18 itself) and autophagy. Nevertheless, since the ER is thought to be a major membranous source of autophagosomes (Lamb *et al.*, 2013), it is tempting to speculate that the Rab3GAP complex and its target substrate Rab18 contribute to autophagosome formation by modulating ER morphology. Alternatively, since both Rab18 and the Rab3GAP complex are involved in ER-derived lipid drop formation (Ozeki *et al.*, 2005; Spang *et al.*, 2014), and since lipid droplets have been suggested to regulate autophagosome biogenesis (Shpilka and Elazar, 2015), Rab18 and the Rab3GAP complex may modulate autophagy by regulating lipid droplet biogenesis. How Rab18 regulates ER morphology, lipid droplet formation, and autophagy is one of the most important issues to be clarified in the future.

Warburg Micro syndromes and their mouse model *blind sterile* (*bs*) are also caused by mutations in *TBC1D20* (Liegel *et al.*, 2013), whose gene product localizes on the ER and exhibits strong GAP activity toward both Rab1 and Rab2 *in vitro* (Haas *et al.*, 2007; Sklan *et al.*, 2007). Interestingly, the causative mutation in *bs* mice is located in the TBC/Rab-GAP domain of *TBC1D20* and impairs its Rab1/2-GAP activity, suggesting that there is a functional link between inactivation of Rab1/2 and activation of Rab18 (e.g., a cascade from Rab1/2 to Rab18) on the ER membrane. Alternatively, in living cells *TBC1D20* may simply function as a Rab18-GAP rather than as a Rab1/2-GAP, because the specificity of GAP activity *in vitro* and in living cells sometimes differs (Marubashi *et al.*, 2016a). Actually, Rab18 has been shown to be more stably associated with the ER membrane in *TBC1D20*-deficient cells (Handley *et al.*, 2015), suggesting that active Rab18 had increased in these cells. When and where Rab18 is activated by the Rab3GAP complex and inactivated by certain Rab-GAPs (e.g., *TBC1D20*) are other important issues to be clarified in order to achieve a better understanding of the pathogenesis of Warburg Micro syndromes.

Other GEFs, including REI-1

Although the yeast TRAPP II complex exhibits GEF activity for Ypt31/32p (yeast homologues of mammalian Rab11) and the fruit fly DENND4 homologue CRAG weakly activates Rab11, no common specific GEFs for Rab11 in metazoans had ever been identified until recently (Morozova *et al.*, 2006; Xiong *et al.*, 2012). In 2015, *C. elegans* RAB-11-interacting proteins (REI-1 and REI-2) were identified as a unique family of specific GEFs for Rab11 (Sakaguchi *et*

al., 2015). Notably, REI family proteins are evolutionarily conserved in metazoans and contain an SH3-binding protein 5 (SH3BP5) domain (Fig. 3). *C. elegans* REI-1 and a human homologue, SH3BP5, exhibit specific GEF activity toward Rab11 *in vitro*. REI-1 co-localizes with RAB-11 on the late-Golgi membranes of *C. elegans* embryos, and loss of REI-1 specifically impairs the targeting of RAB-11 there. Interestingly, however, deletion of both *rei-1* and *rei-2* does not disrupt RAB-11 localization in oocytes, and the effect of the *rei-1* and *rei-2* double mutations on viability is milder than that of *rab-11.1* knockdown, suggesting the existence of other GEFs for RAB-11 in *C. elegans* oocytes.

A product of the *RPGR* (retinitis pigmentosa GTPase regulator) gene, in which mutations cause X-linked retinitis pigmentosa, has been proposed to be another GEF for Rab8. RPGR contains an RCC1-like domain, which is homologous to Ran-GEF RCC1, and functional ablation of RPGR in hTERT-RPE1 cells inhibits ciliary localization of Rab8A and results in shorter primary cilia (Murgazamalloa *et al.*, 2010). However, since GEF activity for other Rabs and Ran has yet to be investigated, the possibility that RPGR acts as a GEF for other small GTPases cannot be ruled out.

Although DrrA/SidM is a protein from the bacterial pathogen *Legionella pneumophila*, it has been shown to possess specific GEF activity toward Rab1 in host mammalian cells (Machner and Isberg, 2006; Murata *et al.*, 2006). Subsequent kinetic analysis of DrrA GEF activity toward Rab1 and crystallographic analysis of the structure of DrrA in complex with nucleotide-free Rab1 have shown that DrrA is a highly efficient GEF that catalyzes nucleotide exchange based on the same biochemical mechanism as other GEFs do, even though the GEF domain of DrrA is structurally unrelated to other GEF domains (Schoebel *et al.*, 2009). Interestingly, DrrA/SidM is necessary for recruitment of host Rab1 to the intracellular *Legionella*-containing vacuole (LCV), a process that is considered important for bacterial replication in mammalian host cells (reviewed in Aktories, 2011).

MSS4 (Dss4 in yeast) is the first candidate Rab-GEF protein that actually exhibits weak GEF activity toward Rab1, Rab3, Rab8, and Rab10 (Burton *et al.*, 1994). However, because biochemical experiments and a crystal structure analysis have revealed an atypical interaction between nucleotide-free Rab8 and MSS4, MSS4 may function as a form of chaperone for nucleotide-free Rabs, rather than as an actual GEF (Nuoffer *et al.*, 1997; Itzen *et al.*, 2006).

Relationships between localization of GEF and Rab recruitment

How Rabs are targeted to specific membranes/organelles is one of the perplexing questions in the field of research on

Rab-mediated membrane traffic. Recent accumulating evidence has indicated that the intracellular localization of GEFs is one of the major determinants of the specific localization of Rabs (Gerondopoulos *et al.*, 2012; Blümer *et al.*, 2013; Gerondopoulos *et al.*, 2014). When certain GEFs (i.e., BLOC-3, Rabex-5, DrrA, Rabin8, and Rab3GAP) are forcibly targeted to the mitochondrial outer membrane by utilizing a mitochondria-targeting tag, their substrate Rabs (i.e., Rab32/Rab38, Rab5, Rab1, Rab8, and Rab18, respectively) also become mislocalized on the mitochondrial outer membrane, indicating that the membrane localization of these GEFs can determine the membrane specificity of their substrate Rabs. However, since not all GEFs localize on specific membrane structures, additional as yet unidentified determinants must exist. For example, DENN/MADD/Rab3GEP does not exhibit any membrane localization (presumably present in the cytosol), whereas its substrate Rab27A clearly localizes on mature melanosomes in melanocytes and on amylase-containing secretory granules in parotid acinar cells (Figueiredo *et al.*, 2008; Imai *et al.*, 2013). Moreover, a Ypt7p mutant with a unique mutation (K127E), which may increase the efficiency of nucleotide exchange of Ypt7p, is able to localize on vacuoles even in GEF (Mon1-Ccz1)-deficient cells, suggesting that additional factors contribute to the vacuole localization of Ypt7p in budding yeasts (Cabrera and Ungermann, 2013). An in depth analysis of the relationship between the localization of each GEF and its substrate Rab(s) will be necessary to determine whether GEF localization is the major determinant of the localization of most Rabs.

Another possible determinant of specific Rab localization is the C-terminal hypervariable region (HVR) of Rabs, because the HVRs of Rab isoforms differ considerably. Actually, it has been suggested that the dual number of cysteine residues for prenylation in the HVR (e.g., Rab5, Rab27, Ypt1p, and Sec4p), the binding ability of HVRs to effector proteins (in the cases of Rab7 and Rab9), and/or the polybasic residues in the HVR of Rab35 are crucial for specific membrane targeting of these Rabs (Calero *et al.*, 2003; Gomes *et al.*, 2003; Aivazian *et al.*, 2006; Li *et al.*, 2014). Post-translational modification of Rabs may also be a determinant of specific Rab localization, because recent studies have shown that phosphorylation of Rabs impairs their interaction with GEFs. For example, PINK1 (PTEN-induced kinase 1) indirectly regulates phosphorylation of Rab8, and the phosphorylation inhibits GDP release by Rabin8 because of impairment of the interaction between Rab8 and Rabin8 (Lai *et al.*, 2015). Phosphorylation of Rab7, which is dephosphorylated by PTEN (phosphatase and tensin homolog deleted from chromosome 10), also decreases its affinity for GDI, Ccz1, and RILP, and as a result inhibits its late endosomal-lysosomal localization (Shinde and Maddika, 2016). Thus, Rab targeting to their cognate membranes is likely to be regulated by multiple factors besides their GEF localization.

Concluding remarks and perspectives

As reviewed above, a number of structurally unrelated GEFs have been identified thus far and their substrates, cellular roles, structural architectures, and post-translational modifications are gradually being characterized. Nevertheless, the GEFs for about half of the mammalian Rabs remain to be determined, and there must be many as yet unidentified GEFs in higher eukaryotes (Fig. 2). In contrast to the GEFs for other small GTPases, there are many structurally unrelated Rab-GEFs, which makes it difficult to identify novel GEFs by means of conventional homology search techniques alone (Cherfils and Zeghouf, 2013). Actually, Rab-GEFs have often been discovered by analyzing genetic interactions between yeast mutants and/or between mammalian diseases. However, the longin-like fold has recently been shown to be a common feature of several GEFs, including DENN proteins, the BLOC-3 (Mon1-Ccz1) complex, and the TRAPP complexes). Thus, future investigations of as yet uncharacterized longin proteins may yield important clues that will enable identification of novel GEFs (Levine *et al.*, 2013b; De Franceschi *et al.*, 2014; Daste *et al.*, 2015).

Because of the existence of several atypical Rabs with a unique mutation in the switch region that is responsible for their interaction with guanine nucleotides, whether all Rabs require their respective GEF to be activated is an open question. For example, Rab20, Rab24, and Rab25 lack a conserved glutamine residue in the switch II region and instead contain arginine, serine, and leucine, respectively, at the corresponding position, and glutamine-to-leucine and glutamine-to-serine substitutions are known to cause deficient Rab GTPase activity (Erdman *et al.*, 2000). Another example is Rab40C, which lacks a conserved serine/threonine residue in the switch I region and contains glycine in its place. Since no GEFs (and no GAPs) have been reported for these atypical Rabs (Fig. 2), it is uncertain whether these atypical Rabs function as molecular switches like ordinary Rabs.

Great care is needed in regard to GEF assays, because different results have been reported for several GEFs in the literature. The discrepancies may be attributable to differences in the experimental conditions of the GEF assays, e.g., recombinant Rabs from bacteria as opposed to recombinant Rabs from eukaryotic cells, and *in vitro* GEF assays as opposed to *in vivo* GEF assays. For example, some studies have shown that DENN/MADD/Rab3GEP actually promotes GDP release of both Rab3 and Rab27, while others have shown that it specifically promotes GDP release of Rab27, not Rab3 (Wada *et al.*, 1997; Figueiredo *et al.*, 2008; Yoshimura *et al.*, 2010). The reason for the discrepancy may be the use of an unprenylated Rab3 protein as a substrate for the GEF assays, because C-terminal prenylation of Rab3 appears to be crucial for its recognition by DENN. Thus, how Rabs are purified, i.e., purified from

bacteria (without prenylation) or eukaryotic cells (with prenylation), is a critical factor in GEF assays for certain Rabs, at least for Rab3 (Wada *et al.*, 1997; Yoshimura *et al.*, 2010). The presence of an additional co-factor would also affect the GDP releasing activity of certain GEFs. For example, immobilization of Rabs on certain kinds of liposomes in GDP release assays could dramatically enhance GEF activity. Actually, the GDP release activity of the Mon1–Ccz1 complex for Ypt7p has been reported to increase 1600-fold when Ypt7p is immobilized on vesicles containing NTA (nickel-nitrilotriacetic acid) lipids (Cabrera *et al.*, 2014), and a similar result was obtained for the Rab11-GEF REI-1 (Sakaguchi *et al.*, 2015). Different GEF assay methods, e.g., an *in vitro* GEF assay as opposed to *in vivo* GEF assay, may also yield different results (GEF assay methods used to determine GEF activity are summarized in Table I). All DENND2 isoforms were originally identified as Rab9-GEFs by *in vitro* GEF assays (Yoshimura *et al.*, 2010), while DENND2B was subsequently identified as a Rab13-GEF by using a biosensor for Rab13 (Ioannou *et al.*, 2015). Thus, the most appropriate GEF assay method should be carefully chosen when attempting to characterize a novel GEF protein. Ideally, several independent assay methods, including *in vitro* and *in vivo* GEF assays, should be performed to avoid misleading results. Further identification and characterization of Rab-GEFs in the next decade is certain to reveal the mechanisms responsible for the spatiotemporal regulation of Rab small GTPases during membrane traffic.

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The authors declare no competing financial interests.

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