An endocytic pathway as a target of *tubby* for regulation of fat storage

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The *tubby* loci provide a unique opportunity to study adult-onset obesity. Mutation in either mammalian *tubby* or its homologue in *Caenorhabditis elegans*, *tub-1*, results in increased fat storage. Previously, we have shown that TUB-1 interacts with a new Rab GTPase-activating protein, RBG-3, for the regulation of fat storage. To understand further the molecular mechanism of TUB-1, we identified the Rab GTPase downstream of RBG-3. We found that RBG-3 preferentially stimulates the intrinsic GTPase activity of RAB-7 in both human and *C. elegans*. Importantly, either mutation or RNA interference knockdown in *rab-7* reduces stored fat in wild type and *tub-1* mutants. In addition, the small GTPase *rab-5* and genes that regulate Rab membrane localization and nucleotide recycling are required for the regulation of fat storage, thereby defining a role for endocytic recycling in this process. We propose that TUB-1 controls receptor or sensory molecule degradation in neurons by regulating a RAB-7-mediated endocytic pathway.

Keywords: *tubby*; *Caenorhabditis elegans*; RAB-7; fat storage; endocytosis

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INTRODUCTION

Adult-onset obesity is reaching epidemic proportions worldwide and is a leading cause of type II diabetes, heart disease and hypertension. Mutation in the gene *tubby* provides one of the few defined models of adult-onset obesity and results in mice with adult-onset obesity, insulin resistance and progressive neuro-sensory deficits (Carroll *et al*, 2004). *Tubby* is also an important candidate gene that influences body weight in humans (Shiri-Sverdlov *et al*, 2006). Several studies have suggested the following possible roles for *tubby*: a transcription factor (Boggon *et al*, 1999; Santagata *et al*, 2001); an adaptor molecule (Kapeller *et al*, 1999);

or a regulator of vesicular transport (Ikeda *et al*, 2002; Mukhopadhyay *et al*, 2005); however, the actual molecular mechanism of *tubby* is unclear.

The function of *tubby* as a regulator of fat storage is conserved. Deletion of its homologue in *Caenorhabditis elegans*, *tub-1*, leads to increased storage of triglycerides, the main type of fat in worms (Ashrafi *et al*, 2003; Mukhopadhyay *et al*, 2005). Therefore, with its amenability for genetic and molecular analysis, *C. elegans* provides a powerful system for deciphering the molecular mechanism of *tubby*. We have recently shown that *tub-1* interacts with a new Rab GTPase-activating protein (Rab GAP), RBG-3, for the regulation of fat storage (Mukhopadhyay *et al*, 2005). Here, we show that RBG-3 uses RAB-7 preferentially as a substrate, and that *rab-7* is required for normal fat storage in wild type and also *tub-1* mutants. In a manner similar to *tub-1*, we found that *rab-7* function is also required for normal chemotaxis. In addition, we identified *rab-5* as another important regulator of fat storage. Importantly, involvement of *rab-7* and *rab-5* indicates that endosomal transport is a functional regulatory component of fat storage. Furthermore, we show that genes that regulate both nucleotide cycling and membrane localization of Rabs are also required for normal fat storage. Together, we propose that in *C. elegans* TUB-1 regulates fat storage by modulating receptor or sensory molecule degradation through a RAB-7-mediated endocytic mechanism.

RESULTS

RBG-3 is a GTPase-activating protein for RAB-7

We have shown that TUB-1 functions upstream of a new Rab GAP, RBG-3 (Mukhopadhyay *et al*, 2005). To explain the downstream signalling mechanism of *tub-1*, we first profiled the substrate specificity of RBG-3 with respect to a panel of purified mammalian Rab GTPases (Pan *et al*, 2006), which corresponded to 16 out of the 18 annotated rab genes in the *C. elegans* database (www.wormbase.org). By using an optical GAP assay that continuously monitors the release of inorganic phosphate resulting from GTP hydrolysis (Fig 1A), we found that the catalytic efficiency (k_{cat}/K_m) of RBG-3 relative to the intrinsic rate constant in the absence of RBG-3 (k_{intr}) was highest for Rab7, 5- to 10-fold lower for Rab18 and Rab33, and 10- to 1,000-fold lower for other Rab GTPases (Fig 1B). RBG-3 also showed a 3- to 100-fold higher

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Fig 1 | Identification of RAB-7 as a preferred substrate of RBG-3. (A) Diagrammatic representation of the GTPase-activating protein (GAP) assay. (B) Profile of catalytic efficiency (k_{cat}/K_m) relative to the intrinsic rate constant for GTP hydrolysis of mammalian Rab GTPases, *Caenorhabditis elegans* RAB-7 and ARL-6. (C) Kinetics of GTP hydrolysis for *C. elegans* RAB-7 in the absence and presence of the indicated concentrations of RBG-3. Solid lines represent a simultaneously fitted pseudo-first-order Michaelis-Menten model function. (D) Kinetics of GTP hydrolysis for *C. elegans* RAB-7 in the presence and absence of 250 nM wild-type RBG-3 and the RBG-3(Arg128Ala) mutant. GEF, guanine nucleotide exchange factor; MESG, 2-amino-6-mercapto-7-methylpurine riboside; PNP, purine nucleoside phosphorylase.

catalytic efficiency for Rab7 compared with other mammalian Rab GTPases (supplementary Fig S1 online). Thus, RBG-3 shows a preference for human RAB7 as a substrate. When *C. elegans* RAB-7 was used, we found that RBG-3 could accelerate its GTP hydrolysis (Fig 1B). Kinetic analysis of GTP hydrolysis for RAB-7 in the absence and presence of RBG-3 showed that the catalytic efficiency (k_{cat}/K_m) of RBG-3 for RAB-7 was $3,500 \text{ M}^{-1} \text{ s}^{-1}$ (Fig 1C). Therefore, we find that RBG-3 has equivalent GAP activity for RAB7 and RAB-7, and significantly weaker activity for other mammalian Rab GTPases.

The phenotype of *tubby* mutant mice resembles human syndromes such as Alstrom and Bardet-Biedl (BBS; Naggert et al, 1997). Several loci that lead to BBS have recently been cloned in humans (Beales, 2005; Leroux, 2007) and mutation in ARL6, a small GTPase, was identified as the underlying cause of BBS3 (Fan et al, 2004). Therefore, ARL-6 was considered to be a potential target of RBG-3. When assayed, RBG-3 failed to increase the intrinsic GTPase activity of *C. elegans* ARL-6 (Fig 1B). These observations suggest that RBG-3 might function as a GAP in a TUB-1/RAB-7 pathway rather than the BBS pathway.

Initially, we suggested that RAB-3 might be a target for RBG-3, on the basis of the following facts: (i) both RAB-3 and TUB-1 are selectively expressed in the neurons (Backberg & Meister, 2004; Mukhopadhyay et al, 2005); (ii) both *rab-3* and *tub-1* mutants are

aldicarb-resistant (Nonet et al, 1997; A.M. & H.A.T., unpublished data); (iii) both *rab-3* and *tub-1* mutants show behavioural defects (Nonet et al, 1997; Mukhopadhyay et al, 2005); and (iv) mutations result in similar changes in vesicle morphology in neurons (Nonet et al, 1997; Backberg & Meister, 2004). However, on the basis of a GAP assay, we found that RAB-3 is not a substrate for RBG-3. Furthermore, *rab-3(y250)* or *rab-3(y251)* (data not shown), and also *rab-3* RNA interference (RNAi) worms (Table 1), do not show any defects in fat storage. Therefore, RAB-7 is a preferred substrate of RBG-3 and might be a target of the TUB-1 pathway.

To test whether the observed increase in phosphate production requires the catalytic activity of RBG-3, as would be expected for an authentic RAB-7 GAP, we mutated an arginine residue that constitutes the 'arginine finger' of RBG-3 to alanine (Arg128Ala) and determined its effects on the catalytic activity of RAB-7. Rab GAPs with the TRE2/BUB/CDC16 domain have been shown to catalyse GTP hydrolysis by supplying two catalytic residues in *trans*, an arginine finger and a glutamine finger (Pan et al, 2006). Importantly, this Arg128Ala mutant protein markedly lost its ability to accelerate the hydrolysis of RAB-7 (Fig 1D). The catalytic efficiency of RBG-3(Arg128Ala) decreased to $3 \text{ M}^{-1} \text{ s}^{-1}$, which is 1,000-fold lower than that of the wild-type RBG-3. These results show that the catalytic arginine is required for the activity of RBG-3 towards RAB-7.

Table 1|Effect of RNA interference knockdown of different *rab* and rab-associated protein genes

CGC name	Sequence name	Alternative name	Effects on growth	Effect on fat storage	
				Wild type	<i>tub-1(nr2004)</i>
<i>Rab-1</i>	C39F7.4	<i>Rab-1</i>	S, L, A	ND	ND
<i>Rab-2</i>	F53F10.4	<i>Unc-108</i>	E, S, L, A	NC	NC
<i>Rab-3</i>	C18A3.6	<i>Rab-3, rbl-3</i>	None	NC	NC
<i>Rab-5</i>	F26H9.6	<i>Rab-5</i>	E, L, A	Decrease/ mislocalize	Decrease/ mislocalize
<i>Rab-6</i>	T25G12.4	<i>Rab-6.2</i>	None	NC	NC
<i>Rab-7</i>	W03C9.3	<i>Rab-7</i>	E	Decrease	Decrease
<i>Rab-11</i>	F53G12.1	<i>Rab-11.1</i>	None	NC	NC
<i>Rab-14</i>	K09A9.2	<i>Rab-14</i>	None	NC	NC
<i>Rab-18</i>	Y92C3B.3	<i>Rab-18</i>	L, A	ND	ND
<i>Rab-19</i>	Y62E10A.9	<i>Rab-19</i>	None	NC	NC
<i>Rab-27</i>	Y87G2A.4	<i>Aex-6</i>	A	NC	NC
<i>Rab-28</i>	Y11D7A.4	<i>Rab-28</i>	None	NC	NC
<i>Rab-30</i>	Y45F3A.2	<i>Rab-30</i>	None	NC	NC
<i>Rab-33</i>	F43D9.2	<i>Rab-33</i>	None	NC	NC
<i>Rab-35</i>	Y47D3A.25	<i>Rab-35</i>	None	NC	NC
<i>Rab-39</i>	D2013.1	<i>Rab-39</i>	None	NC	NC
—	T08G5.5	Guanine nucleotide exchange factor	E	Decrease	Decrease
—	T05E11.3	GRP94, endoplasmin, HSP90	L, A, S, E	Decrease	Decrease
—	M57.2	Protein geranylgeranyltransferase type II, α -subunit	L, E	Decrease	Decrease
—	B0280.1	Protein geranylgeranyltransferase type II, β -subunit	None	NC	NC
<i>Obr-1</i>	Y47D3A.17	Oxysterol binding protein related	None	NC	NC
<i>Obr-2</i>	F14H8.1	Oxysterol binding protein related	E	NC	NC
<i>Obr-3</i>	ZK1086.1	Oxysterol binding protein related	None	NC	NC
<i>Obr-4</i>	C32F10.1	Oxysterol binding protein related	None	NC	NC

A, larval arrest; CGC, Caenorhabditis Genetics Center; E, embryonic lethal; HSP90, heat-shock protein 90; L, larval lethal; NC, no change in fat store; ND, not determined; RNAi, RNA interference; S, sterile.
Effect on growth was observed when worms (either wild type or *tub-1(nr2004)* mutants) were transferred to RNAi at L4 and subsequent generation scored for phenotypes. The *rab-1* and *rab-18* RNAi effect on fat storage could not be determined owing to the severity of larval lethal/arrest.

***rab-7* is essential for the regulation of fat storage**

tub-1 and *rbg-3* regulate fat storage as shown by Nile Red staining (Mukhopadhyay *et al*, 2005). Therefore, if *rab-7* is a target of the *tub-1* pathway, disruption of *rab-7* should also affect fat storage. RNAi knockdown of *rab-7* or *rab-7(ok511)* mutants resulted in decreased fat storage in both wild type and *tub-1(nr2004)* mutants (Fig 2A–C). Thus, RAB-7 is an essential component of the machinery that regulates fat storage in worms.

***rab-7* is expressed ubiquitously**

tub-1 and *rbg-3* are expressed exclusively in the amphid neurons and control fat storage in the intestine (Mukhopadhyay *et al*, 2005). Therefore, we examined next whether *rab-7* was expressed in similar tissues. We expressed the *rab-7* gene fused to green

fluorescent protein (GFP) in *C. elegans*. As reported in other systems (Nahm *et al*, 2003; Verhoeven *et al*, 2003), RAB-7::GFP is expressed ubiquitously (Fig 3A), including the amphid neurons where both TUB-1 and RBG-3 are expressed. Expression of the human and mouse homologue of RAB-7 was also found in neurons (Verhoeven *et al*, 2003; Saxena *et al*, 2005). Thus, our data show how tissue-specific factors—TUB-1 and RBG-3—regulate fat metabolism by controlling a ubiquitous factor, RAB-7.

***rab-7* is required for chemotaxis**

tub-1(nr2004) mutants are compromised in chemotaxis towards volatile chemoattractants sensed by the AWA and AWC (amphid wing cell A and C, respectively) neurons (Mukhopadhyay *et al*, 2005). As *rab-7* is a potential target of the *tub-1* pathway, we

Fig 2 | Effect of *rab-7* and *rab-5* RNA interference on wild-type and *tub-1(nr2004)* fat storage. (A) Homozygous (*rab-7/rab-7*) or heterozygous (*rab-7/+*) worms were identified by the presence or absence of GFP expression using the *rab-7(ok511)* strain (middle panel). Heterozygous worms are wild type with semidominant expression of GFP in the pharynx (indicated by the arrows), whereas homozygous worms lack GFP. The worms were stained with Nile Red to visualize fat storage (bottom panel) and subsequently observed using a rhodamine filter (emission 560–590 nm). (B) Wild-type or (C) *tub-1(nr2004)* animals were grown on control, *rab-7* or *rab-5* RNAi and fat staining was visualized using Nile Red (top panel). The corresponding differential interference contrast image is on the right. The Nile Red fluorescence was quantified (bottom panel) using 50 worms. GFP, green fluorescent protein; RNAi, RNA interference.

determined whether *rab-7*-knockdown worms are also defective in chemotaxis. Worms were assayed on pyrazine (10 mg/ml), isoamyl alcohol (1:200) and benzaldehyde (1:100), after being fed *rab-7* RNAi or control RNAi bacterial food. Similarly to *tub-1* mutants, RNAi of *rab-7* resulted in defective chemotaxis to volatile chemoattractants sensed by both the AWA and AWC neurons (Fig 3B). Thus, defects in the TUB-1/RAB-7 pathway result in defective fat storage and defective chemotaxis.

Other RAB proteins in fat storage regulation

To determine the contribution of other *C. elegans* RABs to fat storage, we used RNAi knockdown of 16 *rab* genes. Surprisingly, we found that apart from *rab-7*, only *rab-5* significantly affects fat storage. Similar to *rab-7*, *rab-5* knockdown reduced fat storage in both wild type and *tub-1(nr2004)* mutants (Fig 2B,C). RAB-5 is involved in the early stages of endocytosis, whereas RAB-7 is required for steps leading to fusion with lysosomes (Stein et al,

Fig 3 | *Rab-7* is required for normal chemotaxis. (A) Head region of a *rab-7::gfp*-expressing transgenic worm visualized using an FITC filter (left panel) showing expression of GFP. The same worm was dye-filled with DiI and visualized under a rhodamine filter (middle panel) to show the amphid neurons. The two photos were merged (right panel) to show that *rab-7* is also expressed in the amphid neurons. (B) Chemotaxis assay of wild-type worms on control RNAi or *rab-7* RNAi. Aliquots (1 μ l) of benzaldehyde (1:200 diluted), isoamyl alcohol (1:100) or pyrazine (10 mg/ml) were used for the assay. DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine iodide; FITC, fluorescent isothiocyanate; GFP, green fluorescent protein; RNAi, RNA interference.

2003). Therefore, these data suggest that TUB-1 might regulate the degradation of unknown receptors or sensory molecules in neurons, by controlling the endocytic pathway at the level of RAB-7.

Proteins that modify RAB are essential for fat storage

RAB proteins are tightly regulated at the level of membrane association and nucleotide recycling (Stein et al, 2003). The newly synthesized RABs associate with the RAB escort protein (REP), which maintains the complex in a soluble form. This is recognized by RAB geranylgeranyl transferase (RABGGTase) that catalyses the addition of geranylgeranyl groups, facilitating its delivery to the membrane. The RAB-GDP dissociation inhibitor (RAB-GDI) binds to RAB to recycle GDP-RAB to the donor membrane. A heat-shock protein 90 (HSP90) chaperone complex is involved in recognizing the RAB-GDI::GDP-RAB complex (Sakisaka et al, 2002). We found that several of the RAB regulatory proteins are required for normal fat storage (Fig 4; Table 1). RNAi knockdown of RABGGTase (M57.2), guanine exchange factor (T08G5.5) and HSP90 homologue (T05E11.3) markedly reduced fat storage. We found no defect with the knockdown of Y67D2.1 (REP), B0280.1 (GGTase β -subunit) or oxysterol-binding proteins (*obr-1*, *obr-2*, *obr-3* and *obr-4*), whereas the knockdown of Y57G11C.10

(RAB-GDI) was lethal. Together, we found that proteins that mediate correct RAB function are required for normal fat storage.

DISCUSSION

Although the *tubby* gene was cloned a decade ago, the molecular nature of the protein has remained elusive. Using *C. elegans*, we have recently shown that TUB-1 interacts with RBG-3 to regulate fat storage, implicating vesicular transport in this process (Mukhopadhyay et al, 2005). Here, we have shown that RAB-7 is a downstream target of *tubby*, thus identifying the endocytic pathway as an important regulator of fat storage.

TUB-1 and RBG-3 are expressed exclusively in the neurons of *C. elegans* (Mukhopadhyay et al, 2005). However, fat storage occurs only in the intestinal cells of worms. This suggests that there is a neuroendocrine component involved in signalling between the *tub-1*-expressing neurons and the intestinal cells. The nature of the neuroendocrine signal is largely unknown. However, as the forkhead transcription factor *daf-16*—the target of the *C. elegans* insulin-like signalling pathway—fails to suppress the lipid accumulation of *tub-1* mutant (Mukhopadhyay et al, 2005), the signal is unlikely to be the *C. elegans* insulin genes. The endocytic pathway that TUB-1 regulates might be instrumental in secretion of the neuroendocrine signal that is important for the regulation of fat storage.

Our findings suggest that, biochemically, a mutation in *tub-1* might activate RBG-3 and shift the balance of nucleotide-bound RAB-7 towards the inactive GDP-bound state (Fig 5). The ratio of GTP to GDP-bound RAB-7 might essentially modulate the neuroendocrine signal that is secreted from the neurons to increase fat storage in a *tub-1* mutant. However, mutation or RNAi of *rab-7* will eliminate both the inactive and active form, resulting in complete abrogation of the neuroendocrine signal and depletion of fat storage in *tub-1* mutant and wild-type worms. Similarly, mutations that effectively disrupt the endocytic process, such as depletion of *rab-5* (by RNAi), also reduce the fat storage of *tub-1*, although RAB-5 is not a biochemical target of TUB-1 pathway.

We have identified RAB-7 as a target of *tub-1* signalling. RAB-7 is proposed to regulate the transport of G-protein-coupled receptors (GPCRs) or other chemosensory receptors from early endosomes to late endosomes and lysosomes (Seachrist & Ferguson, 2003). In one model, which is consistent with our findings, the sensory receptors in *C. elegans* neurons might need to be periodically replaced with other receptors depending on the sensory inputs, for example, changes in environment. The TUB-1/RBG-3/RAB-7 pathway might regulate recycling and degradation of these receptors in the neurons to control fat storage systemically. Accordingly, *Rab5* and *Rab7* have recently been found to control endocytic sorting specifically in the neurons (Deinhardt et al, 2006). However, it is still possible that this process might occur in another part of the worm, such as the intestine. Studies on overexpression of *rab-7* might help to address this issue; however, overexpression of *rab-7* is lethal in *C. elegans* (A.M. & H.A.T., unpublished data). Our findings are most consistent with a neuronal function for this pathway, as we found that both TUB-1 and RBG-3 are exclusively neuronal, and RBG-3 preferentially uses RAB-7 as a target.

Our study implicates the endocytic pathway in fat storage regulation. We found that apart from *rab-7*, RNAi knockdown of

Fig 4 | Effects of RAB-associated proteins on normal fat storage. Wild-type or *tub-1(nr2004)* worms were grown on RNAi constructs for T05E11.3 (*hsp-82*), T08G5.5 (GEF) or M57.2 (GGTase) and fat storage was visualized by using Nile Red. The fluorescence was quantified using 35 worms. Arrows indicate the position of the pharynx. GEF, guanine nucleotide exchange factor; hsp, heat-shock protein; RNAi, RNA interference.

rab-5 also affects fat storage. RAB5 is involved in segregation of cargo into clathrin-coated vesicles and facilitates early endosome fusion (Stein *et al*, 2003). We found that some proteins that are important for RAB membrane localization and nucleotide recycling are also required for normal fat storage. The RNAi knockdown of these genes, similar to *rab-5* and *rab-7*, results in varying degrees of embryonic lethality (more than 10%; see Table 1). Therefore, the fat storage phenotype might simply be due to sickness in the treated worms, rather than a specific fat storage defect. To address the specificity of our results, first we fed RNAi bacteria to L1 worms and visualized Nile Red staining in the adults, thus circumventing the embryonic lethality. Second, following RNAi treatment, only healthy-looking worms were used for the Nile Red assays. Our results indicate that the decreased fat storage, as a result of *rab-7* or *rab-5* RNAi knockdown, is a specific fat storage phenotype and not due to a general sickness.

Tubby is an important locus for the regulation of fat storage in humans (Shiri-Sverdlov *et al*, 2006). Understanding the molecular mechanism of how *tubby* controls fat storage will help in the evolution of drug targets to adult-onset obesity. Beginning with the identification of RAB7, we have defined early and late endocytic processes in the regulation of fat storage and as a target of *tubby*. Identifying the proteins that are degraded and recycled is an important next step and might define additional new targets to

combat human obesity. Taken together, we have moved one important step forward in understanding the function of *tubby* in the regulation of fat storage by identifying the vesicular pathway that it regulates.

METHODS

Cloning and purification of protein. The cloning and purification of RAB proteins were reported previously (Pan *et al*, 2006). The GAP domain of RBG-3 (amino acids 7–450) was subcloned into PET24b (Amersham Biosciences, Piscataway, NJ, USA). The RBG-3(Arg128Ala) mutant version was created using the Quickchange Mutagenesis kit (Qiagen-Valencia, CA, USA). BL21(DE3) competent cells transformed with the RBG-3 construct were grown in 2 × YT with 50 mg/l kanamycin at room temperature (23–25 °C) to OD₆₀₀ of 0.3–0.4. Isopropyl-β-D-galactopyranoside was added at a final concentration of 0.05 mM to induce protein expression. After 16–18 h of induction, cells were spun down and resuspended in lysis buffer (50 mM Tris, 100 mM NaCl (pH 8.0), 2 mM MgCl₂, 0.1% mercaptoethanol, 0.2 mg/ml lysozyme). Cells were disrupted by sonication and cleared lysates were obtained by centrifugation. The supernatant was loaded on a Ni-NTA agarose column (Qiagen). After the column was washed with a wash buffer (50 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole, 2 mM MgCl₂, 0.1%

Fig 5 | Mechanism of action of the *tub-1* pathway. TUB-1, expressed exclusively in the neurons, regulates RBG-3, a Rab GAP. RBG-3 promotes the GTPase hydrolysis of RAB-7, an important effector of endocytosis. Together, this pathway might regulate the degradation of sensory molecules or receptors in the neurons. Neuroendocrine signals generated in the neurons then regulate fat storage in the intestine. RAB-5 is also an important regulator of endocytosis and fat storage, but is regulated by a mechanism independent of the TUB-1 pathway. Rab GAP, Rab GTPase-activating protein.

mercaptoethanol), the protein was eluted with elution buffer (250 mM imidazole, 50 mM Tris, 100 mM NaCl (pH 8.0), 2 mM MgCl₂, 0.1% mercaptoethanol). RBG-3 protein was further purified by gel filtration on Superdex-75 (Amersham Biosciences). **GAP assay.** RAB proteins were first loaded with GTP. RAB (2–3 mg) protein was incubated with 25-fold molar excess of GTP at 25 °C for 1 h in exchange buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol). Free nucleotides were removed by a D-Salt™ dextran desalting column (Pierce Biotechnology, Rockford, IL, USA) pre-equilibrated with reaction buffer (20 mM HEPES (pH 7.5), 150 mM NaCl). GTP hydrolysis by the RAB GTPases was measured by using the EnzChek Phosphate Assay Kit (Invitrogen, Carlsbad, CA, USA) modified to a 96-well format, which continuously monitors the release of free inorganic phosphate by a spectrophotometric method. Briefly, in each well, a 75 µl solution containing GTP-preloaded RABs was mixed with a 25 µl solution containing enzyme, buffer and GAPs by using a Precision 2000 automated

microplate pipetting system (Bio-Tek Instruments Inc., Winooski, VT, USA). The final solution contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.15 mM 2-amino-6-mercapto-7-methylpurine riboside (MESG), 7.5 U of purine nucleoside phosphorylase, 10 mM MgCl₂, 20 µM GTP-preloaded RABs and the indicated amount of GAPs. The time courses of absorbance change at 360 nm were recorded on a monochromator-based Safire² microplate detection system (TECAN, Durham, NC, USA). Kinetic data were analysed by simultaneous fitting to the pseudo-first-order Michaelis–Menten equation

$$A(t) = (A_{\infty} - A_0)(1 - \exp(-k_{\text{obs}}t)) + A_0$$

where

$$k_{\text{obs}} = k_{\text{intr}} + (k_{\text{cat}}/K_m)[\text{GAP}]$$

The catalytic efficiency (k_{cat}/K_m) and intrinsic rate constant for GTP hydrolysis (k_{intr}) were treated as global parameters.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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