

# Multiple regions contribute to membrane targeting of Rab GTPases

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## Summary

Small GTPases of the Rab family are key regulators of membrane trafficking. Each Rab shows a characteristic subcellular distribution, and may serve as an important determinant of organelle identity. The molecular mechanisms responsible for targeting Rabs to specific intracellular compartments, however, remain poorly understood. The divergent C-terminal hypervariable region was postulated to contain Rab targeting information. We generated a series of hybrid Rab proteins by exchanging the hypervariable domains of Rab1a, Rab2a, Rab5a, Rab7 and Rab27a, and analysed their subcellular localisations. We found that the various hybrid proteins retained their targeting to the parent organelle and were functionally active. We conclude that the hypervariable region does not contain a general Rab targeting signal. Furthermore, we identified other regions within the RabF and RabSF motifs that are required for

specific targeting of Rab27a to secretory granules or melanosomes, and Rab5a to endosomes. We observed only partial overlap between targeting-determining regions in the Rab proteins examined, suggesting that Rab recruitment may be complex and at least partially Rab-specific. Mutations in these targeting-determining regions induced localisation to the ER, an observation that further strengthens our previous finding that ER/Golgi membranes serve as the default location for Rabs that have lost targeting information.

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## Introduction

Rab GTPases are key regulators of most if not all vesicular trafficking events between the various subcellular compartments within the eukaryotic cell (Barrowman and Novick, 2003; Goud, 2002; Pfeffer, 2003; Seabra et al., 2002; Segev, 2001; Zerial and McBride, 2001). Rabs have been implicated in regulating the formation of vesicles at the donor membrane, as well as the movement, tethering and docking of vesicles, and their fusion with the acceptor membrane. The regulatory capacity of Rab proteins is dependent on their ability to cycle between GTP-bound 'active' and GDP-bound 'inactive' states. Activation of a Rab is coupled to its association with intracellular membranes, allowing it to recruit downstream effector proteins to the cytoplasmic surface of a subcellular compartment.

More than 60 Rab proteins have been described in humans, some of which are ubiquitous while others are subject to tissue-specific and developmentally regulated expression (Pereira-Leal and Seabra, 2001; Seabra et al., 2002). Each Rab shows a characteristic subcellular distribution. In fact, Rabs may serve as important determinants of organelle identity and membrane organisation (Munro, 2002; Pfeffer, 2001; Seabra and Wasmeier, 2004; Zerial and McBride, 2001). Through the recruitment of specific effector molecules, Rabs can give rise to membrane subdomains within a compartment. Rab5a and Rab4,

although both localised to early endosomes, are largely present on distinct domains (De Renzis et al., 2002; Sonnichsen et al., 2000). Similarly, Rab7 and Rab9 occupy separate domains within late endosomal membranes (Barbero et al., 2002).

Surprisingly, the molecular mechanisms regulating the specific association of Rabs with cellular membranes remain obscure. Geranylgeranylation of one, or more commonly two, cysteine residues at the C terminus of Rab proteins, catalysed by the Rab escort protein (REP)/Rab geranylgeranyl transferase complex (Pereira-Leal et al., 2001; Pylypenko et al., 2003), is required for insertion into and stable association with cellular membranes. The appropriate double-geranylgeranyl modification in yeast and mammalian Rab proteins with double-cysteine prenylation motifs is also essential for their correct intracellular localisation (Calero et al., 2003; Gomes et al., 2003). However, geranylgeranylation is a common feature of all Rabs and thus cannot in itself account for their organelle-specific targeting.

The C-terminal hypervariable region of 35 to 40 amino acids shows the highest level of sequence divergence between Rab family members, and was postulated to act as a signal for subcellular targeting. Over a decade ago, Zerial and co-workers showed that replacing the C-terminal 35 residues of Rab5 with the equivalent C-terminal region of Rab7 resulted in re-

localisation of the hybrid Rab to Rab7-positive late endosomal structures (Chavrier et al., 1991). Conversely, the C terminus of Rab5 targeted a chimeric Rab6/Rab5 (Stenmark et al., 1994) to early endosomes. In *Saccharomyces cerevisiae*, the hypervariable domain of Ypt1p reportedly re-localised Sec4p, and vice versa (Brennwald and Novick, 1993). However, complementation studies showed that the hybrid Ypt1p/Sec4p proteins retained their original function indicating that at least a proportion of the protein reached its original compartment (Brennwald and Novick, 1993; Dunn et al., 1993).

Targeting of Rab proteins via the hypervariable region is a widely accepted model, but the underlying molecular mechanisms have not been elucidated further. In this study we analysed the structural requirements for targeting of Rab GTPases, with a focus on the specific determinants present in the C-terminal hypervariable domain. Surprisingly, we found that the hypervariable region did not represent a general targeting domain. We identified other sequence elements within Rabs that appear to be necessary for specific localisation, suggesting a model where multiple regions contribute to subcellular targeting.

## Materials and Methods

### Plasmid constructs

Rab hybrid proteins were initially generated by mutating the conserved ETSAK motif to introduce an *Eco47III* site and silencing the internal *Eco47III* site present within the pEGFP vector using the Stratagene Quickchange site-directed mutagenesis system (SDM), as described previously (Gomes et al., 2003). The C termini of selected Rabs were exchanged by digesting the respective pEGFP-Rab plasmid with *Eco47III* and *NdeI*, then ligating the desired *Eco47III-NdeI* fragment from a different Rab. The length of the hypervariable domain on the hybrid proteins was adjusted by SDM where desired. *Trypanosoma brucei* cDNAs encoding Rab5a orthologues (Pal et al., 2002) were a gift from Mark Field (Imperial College, London, UK) and subcloned into pEGFP-C2. *S. cerevisiae* cDNAs encoding Ypt51, Ypt52 and Ypt53 in pEGFP-C3 were a gift from Ruth Collins (Cornell University, NY). Full details for each plasmid constructed are described in the supplementary material.

### Antibodies

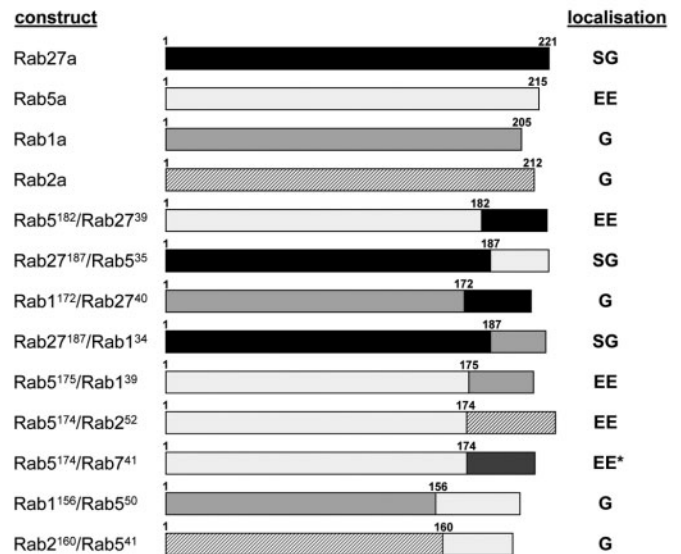
The monoclonal antibodies were used at the following dilutions: anti-transferrin receptor (1:100; Zymed Laboratories), anti-human Golgin-97 (1:200; Molecular Probes), anti-myc Ab-1 (1:100; Oncogene), anti-Golgi58k (1:50; Sigma) and anti-ACTH (1:10,000; a gift from A. White, University of Manchester, UK). The anti-PDI polyclonal antibody (StressGen Biotechnologies) was used at 1:2,000 dilution.

### Mice and melanocyte cell line

C57BL6/J mice homozygous for *ashen* (*Rab27a<sup>ash</sup>*) (Barral et al., 2002) were crossed with C57BL6/J mice carrying an *Ink4a-Arf* exon 2 deletion (Serrano et al., 1996), at Texas A&M University. Litters homozygous for both mutations were obtained at the third generation, and trunk skins from these neonatal mice were used at St. George's Hospital Medical School for preparation of melanocyte cultures, as described previously (Sviderskaya et al., 2002).

### Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G and 100 U/ml streptomycin at 37°C with 10% CO<sub>2</sub>. AtT20 mouse anterior pituitary cells were grown in



**Fig. 1.** Schematic representation of wild-type and C-terminal hybrid Rab proteins used in this study. The numbers above each bar are residue numbers indicating the total length of each wild-type Rab, or the point at which the hypervariable region from another Rab was fused to generate a hybrid protein. On the right, the subcellular localisations of the proteins used are summarised: SG, secretory granule; EE, early endosome; G, Golgi; \*denotes partial mislocalisation.

DMEM/F-12 (3:1 ratio) supplemented with 15% fetal bovine serum and antibiotics. Melan-ink4a-ashen (Melan-ash2) melanocytes were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin G, 100 U/ml streptomycin, 200 nM phorbol 12-myristate 13-acetate (PMA; Calbiochem) and 200 pM cholera toxin at 37°C with 10% CO<sub>2</sub>. For immunofluorescence experiments, cells were grown on glass coverslips for 24 hours, transfected and fixed after 24 hours (HeLa cells) or 48 hours (AtT20 and melan-ash2 cells). HeLa cells were transfected with GeneJuice [CN Biosciences (UK) Ltd., Nottingham, UK], AtT20 and Melan-ash2 cells were transfected using LipofectAMINE 2000 (Invitrogen), according to the manufacturer's recommendations.

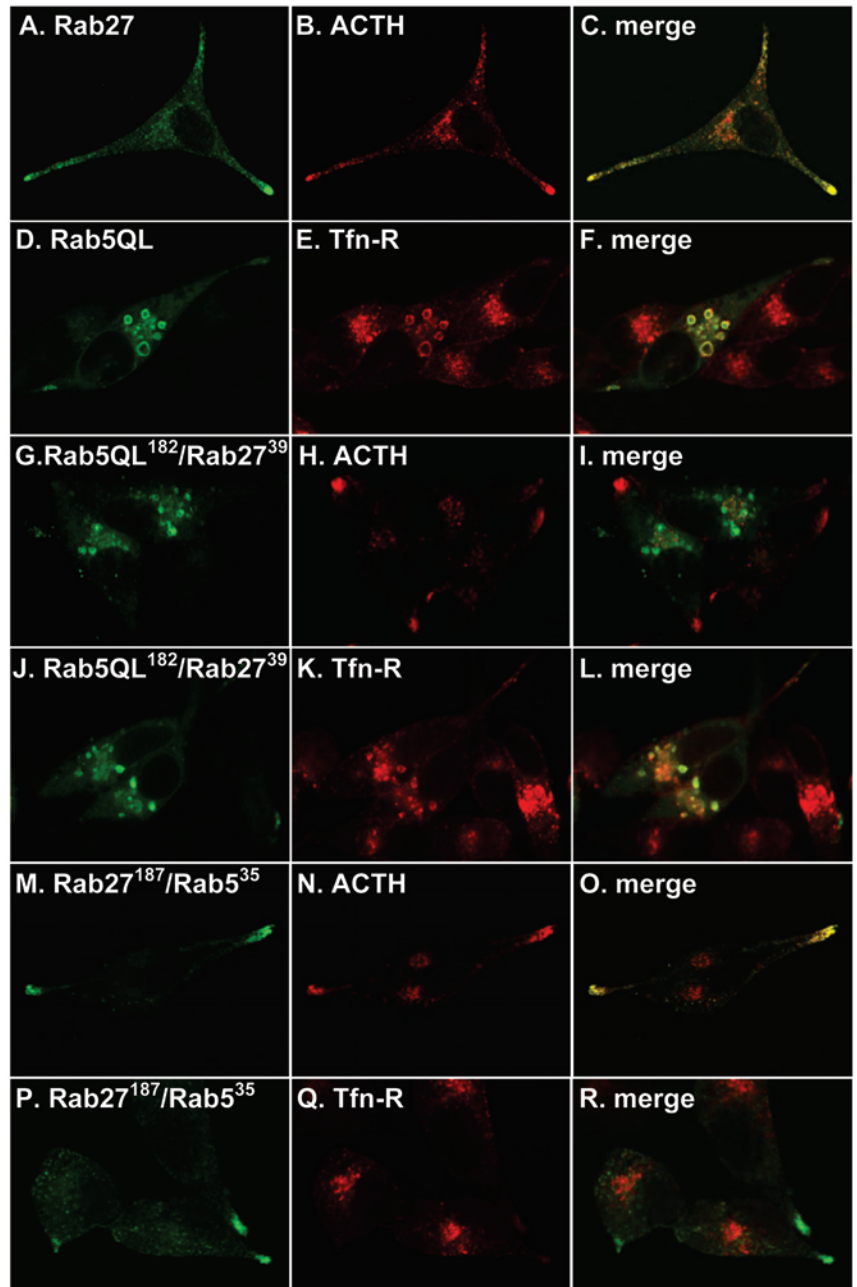
### Immunofluorescence and confocal microscopy

Cells were washed with PBS, fixed with 3% paraformaldehyde for 15–30 minutes, washed three times with PBS and once with 50 mM NH<sub>4</sub>Cl in PBS, then permeabilised in PBS containing 0.05% saponin and 1% bovine serum albumin. Permeabilised cells were incubated with primary antibody followed by Alexa568-conjugated secondary antibody (Molecular Probes). All incubation and washing steps were carried out in PBS/BSA/saponin. Coverslips were mounted in ImmunoFluor medium (ICN, Basingstoke, Hants, UK) and fluorescence was visualised using a Leica DM-IRBE confocal microscope (Leica, Wetzlar, Germany). Images were processed with TCS-NT software (Leica) and Adobe Photoshop 5.5 software. All images presented are single section in the z-plane and representative of at least 80% of the transfected cells.

## Results

### Targeting of a tissue-specific Rab protein in a specialised cell type

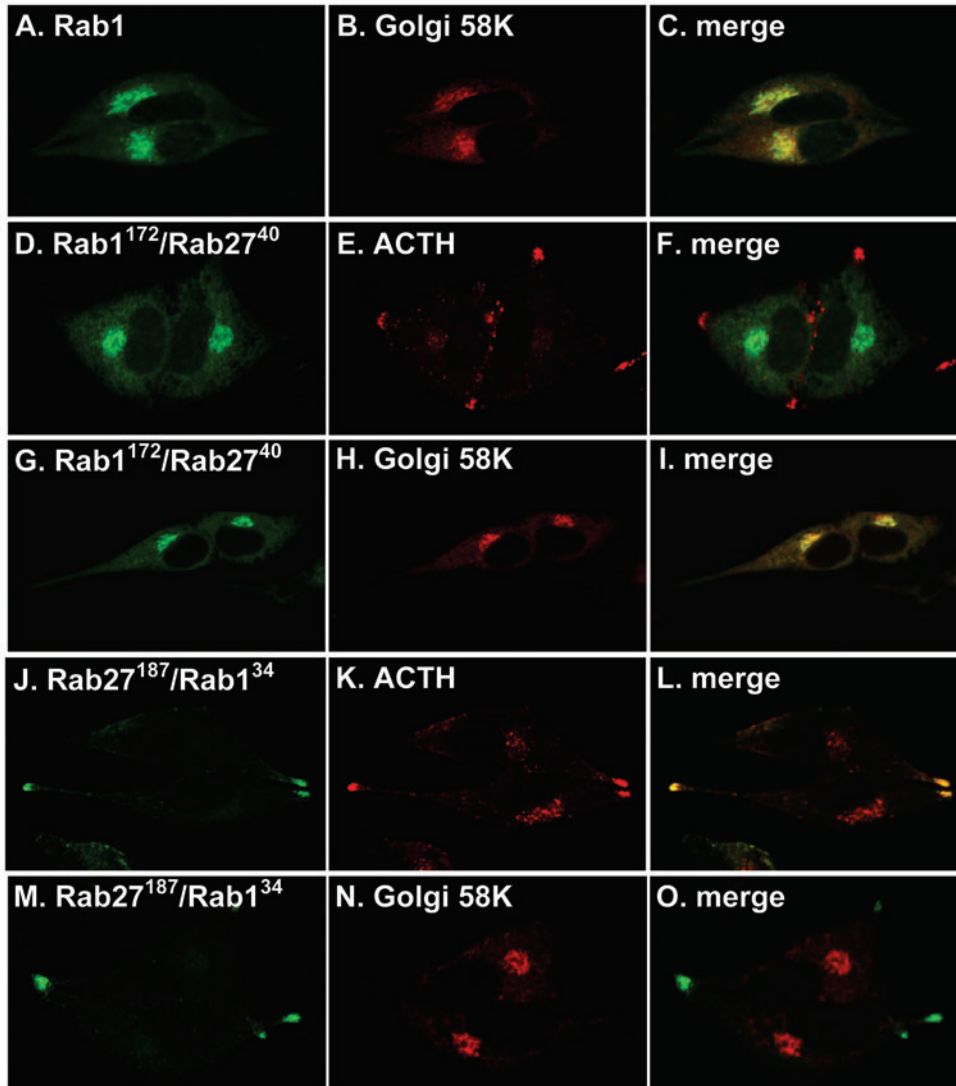
In order to study the targeting determinants present in the C-terminal hypervariable domain, we produced a series of



**Fig. 2.** Expression of EGFP-Rab27/Rab5 fusion proteins in AtT20 cells. Cells were transiently transfected with EGFP-Rab27 (A-C), EGFP-Rab5Q79L (D-F), EGFP-Rab5Q79L<sup>182</sup>/Rab27<sup>39</sup> (G-L) or EGFP-Rab27<sup>187</sup>/Rab5<sup>35</sup> (M-R). After fixation, cells were permeabilised, and immunolabelled with antibodies to ACTH (B,H,N) or Tfn-R (E,K,Q). Panels on the left (A,D,G,J,M,P) show EGFP fluorescence and on the right (C,F,I,L,O,R) are the merged images.

chimeric Rabs containing exchanged C termini (Fig. 1). One of the model Rabs used in this study was Rab27a, which is expressed in a tissue-specific manner, and is localised to melanosomes in pigmented cells (Bahadoran et al., 2001; Hume et al., 2001; Wu et al., 2001) and dense-core secretory granules in neuroendocrine cells (Desnos et al., 2003; Tolmachova et al., 2003; Zhao et al., 2002). In AtT20 anterior pituitary-derived cells, EGFP-Rab27a co-localised with the secretory granule marker ACTH, particularly at the tips of extended cell processes, which are enriched in mature secretory granules (Fig. 2A-C). Little Rab27a was detected in a perinuclear ACTH-positive compartment representing hormone precursor in transit through the secretory pathway (Wasmeier and Hutton, 2001). For the construction of Rab27 hybrid proteins (Fig. 1), the N-terminal 187 residues of Rab27

had to be retained to allow membrane association of the proteins, perhaps reflecting the fact that Rab27a is naturally an inefficient substrate for prenylation and is thus more sensitive to structural rearrangements (Larijani et al., 2003). The C-terminal 35 amino acids were proposed to be the minimal sequence necessary and sufficient for targeting of Rab5 and Rab7 (Chavrier et al., 1991), therefore we fused the hypervariable regions to a residue 35 amino acids away from the C terminus of Rab27, located just downstream of helix  $\alpha$ 5. The Rab27/Rab5 hybrids (Rab27<sup>187</sup>/Rab5<sup>35</sup> and Rab5QL<sup>182</sup>/Rab27<sup>39</sup>) and Rab27/Rab1 hybrids (Rab27<sup>187</sup>/Rab1<sup>40</sup> and Rab1<sup>172</sup>/Rab27<sup>40</sup>) were expressed in AtT20 cells. Neither the Rab5a hypervariable region (Fig. 2M-R) nor the Rab1a hypervariable region (Fig. 3J-O) resulted in the re-targeting of Rab27a from secretory granules to either the early endosome



**Fig. 3.** Expression of EGFP-Rab27/Rab1 fusion proteins in AtT20 cells. Cells were transiently transfected with EGFP-Rab1 (A-C), EGFP-Rab1<sup>172</sup>/Rab27<sup>40</sup> (D-I) or EGFP-Rab27<sup>187</sup>/Rab1<sup>34</sup> (J-O). After fixation, cells were permeabilised and immunolabelled with antibodies to ACTH (E,K) or the Golgi 58K protein (B,H,N). Panels on the left (A,D,G,J,M) show EGFP fluorescence and panels on the right (C,F,I,L,O) are the merged images.

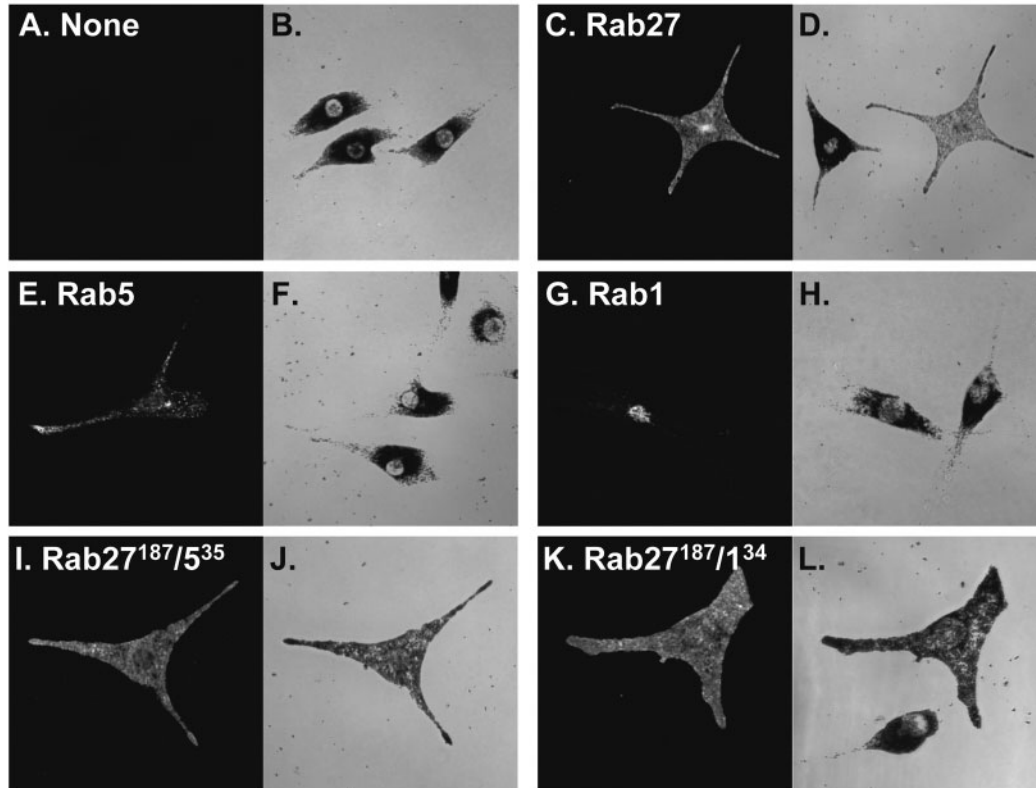
(Fig. 2) or the Golgi apparatus (Fig. 3). Conversely, the hypervariable C terminus of Rab27a was not sufficient to redirect Rab5aQL (Fig. 2G-L) or Rab1a (Fig. 3D-I) to secretory granules. Similar results were obtained using cultured melanocytes (our unpublished results). These findings suggest that the hypervariable region of Rab27a does not represent a sufficient signal for the subcellular targeting of this Rab protein.

AtT20 cells expressing the dominant active form of Rab5a, Rab5aQ79L, showed characteristically enlarged endosomes, positive for the endosomal marker transferrin receptor (Tfn-R). Similarly, enlarged endosomes were observed in cells expressing Rab5QL<sup>182</sup>/Rab27<sup>39</sup>, suggesting that the hybrid protein retained functional activity. To test whether the Rab27a C terminus was important for function, we used melanocytes derived from the *ashen* (Rab27a null) mouse, which characteristically accumulate pigment granules in the perinuclear region (Fig. 4B). Transfection with EGFP-Rab27a (Fig. 4C,D), but not EGFP-Rab5a (Fig. 4E,F) or EGFP-Rab1a (Fig. 4G,H), led to the reversal of the mutant phenotype, with melanosomes distributed throughout the cytoplasm and enriched at the tips of dendrites, as in wild-type cells. Similarly,

expression of Rab27<sup>187</sup>/Rab5<sup>35</sup> or Rab27<sup>187</sup>/Rab1<sup>40</sup> in *ashen* melanocytes resulted in the redistribution of melanosomes to the cell periphery, indicating that the hybrid proteins retained Rab27a activity irrespective of the identity of the hypervariable region (Fig. 4I-L).

#### Rab5 orthologues are targeted to endosomes in mammalian cells

To define conserved determinants for endosomal targeting of the ubiquitously expressed Rab5 protein, we used the Clustal W sequence alignment algorithm to analyse the C-terminal hypervariable domains of several Rab5 orthologues in lower eukaryotes. Apart from the cysteine residues in the prenylation motif at the extreme C terminus and a phenylalanine residue in helix  $\alpha_5$ , there was no significant sequence conservation at the amino acid level between mammalian and lower eukaryote Rab5 sequences (see Fig. S1 in supplementary material). We expressed EGFP-tagged Rab(Ypt) proteins representing Rab5 homologues from the lower eukaryotes *Saccharomyces cerevisiae* (Ypt51, Ypt52 and Ypt53) and *Trypanosoma brucei* (TbRab5A and TbRab5B) in HeLa cells. Using confocal



**Fig. 4.** Expression of EGFP-Rab proteins in *ashen* melanocytes. Melan-ash2 cells were transiently transfected with EGFP-Rab27 (C,D), EGFP-Rab5 (E,F), EGFP-Rab1 (G,H), EGFP-Rab27<sup>187</sup>/Rab5<sup>35</sup> (I,J) or EGFP-Rab27<sup>187</sup>/Rab1<sup>34</sup> (K,L). A and B show non-transfected cells; A,C,E,G,I,K show EGFP fluorescence, and B,D,F,H,J,L show the corresponding phase-contrast images.

microscopy, we observed localisation of all five Rab5-like proteins to dispersed punctate structures positive for the Tfn-R, an endosomal marker (Fig. 5), suggesting that Rab5 orthologues are correctly targeted to early endosomes in HeLa cells. In addition, some Ypt52p, TbRab5A and TbRab5B were also observed in the perinuclear region, consistent with a recycling endosome and/or Golgi localisation (Fig. 5). Despite their divergent hypervariable domains, it appears that all Rab5 proteins examined contained the necessary targeting information for localisation to early endosomes.

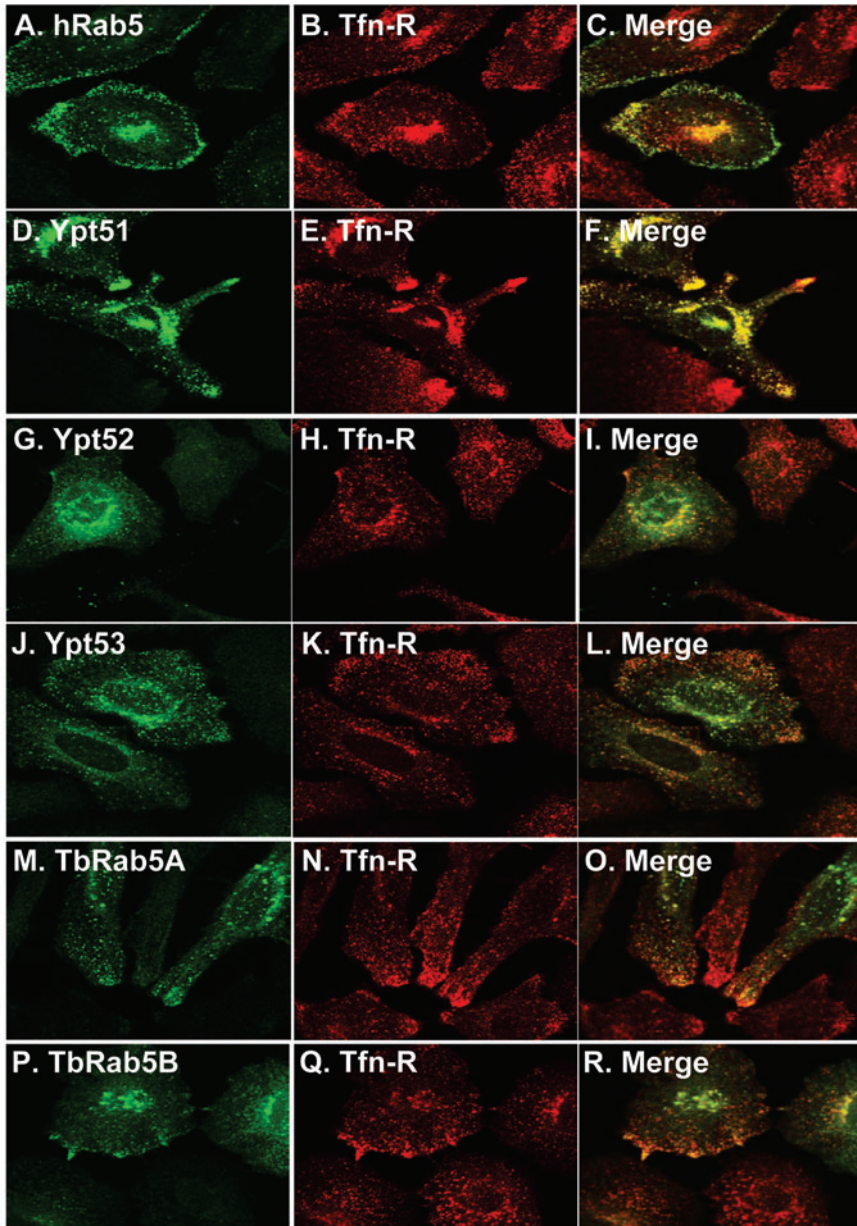
Membrane targeting of the related GTPase K-Ras is dependent on basic residues within the C-terminal region (Hancock et al., 1991; Hancock et al., 1990). We replaced the six basic residues in the hypervariable domain of Rab5a with non-charged residues (Rab5 $\Delta$ BR). These mutations were introduced into the dominant active form of Rab5a, Rab5aQ79L (Bucci et al., 1992). Transiently expressed EGFP-Rab5QL $\Delta$ BR associated with enlarged structures that also contained the endosomal marker EEA1, suggesting that the mutant protein was both correctly targeted to early endosomes and functionally active (our unpublished results).

The C-terminal hypervariable domains of Rab1, Rab2 and Rab7 did not alter the endosomal localisation of Rab5

To extend our analysis of the role of the hypervariable region in Rab targeting, we chose other well-characterised Rabs with

distinctive cellular distributions in mammalian cells. We generated a series of hybrid Rab proteins bearing the N-terminal 174 residues of Rab5a attached to the corresponding C terminus of another Rab (Fig. 1). All hybrid Rabs described in this study, except where noted, were prenylation-competent as evidenced by three criteria. Firstly, they partitioned into the detergent phase in Triton X-114 extraction experiments (see Fig. S2 in supplementary material); secondly, they did not accumulate within the nucleus of transfected cells, unlike soluble EGFP-tagged Rabs lacking a prenylation motif; thirdly, they resisted cell permeabilisation prior to fixation. Prenylation competence of a hybrid or mutant Rab protein is strong evidence that the protein has retained its overall Rab structure. This is because of the high stringency and specificity of the REP/Rab geranylgeranyl transferase system, such that only correctly folded Rab proteins undergo geranylgeranylation (Seabra, 2000).

EGFP-tagged Rab5a hybrid proteins were transiently expressed in HeLa cells, and their subcellular localisation was analysed by confocal light microscopy. Wild-type Rab5a decorates Tfn-R-positive early endosomes (Fig. 5A-C) and regulates early endosome dynamics (Zerial and McBride, 2001). The constitutively active mutant, Rab5aQ79L, induces the formation of enlarged endosomes which tend to cluster in the perinuclear region (Fig. 2 and data not shown) (Zerial and McBride, 2001). The hypervariable regions of Rab1a, Rab2a or Rab7 did not disrupt the targeting of Rab5a to early endosomes, as shown by co-localisation of the hybrid proteins



**Fig. 5.** Transient over-expression of EGFP-hRab5a and lower eukaryote (*S. cerevisiae* and *T. brucei*) homologues in HeLa cells. HeLa cells were transfected with EGFP-hRab5a (A-C), EGFP-Ypt51 (D-F), EGFP-Ypt52 (G-I), EGFP-Ypt53 (J-L), EGFP-TbRab5A (M-O) or EGFP-TbRab5B (P-R). After 24 hours, cells were fixed, permeabilised and immunostained with an anti-Tfn-R antibody (B,E,H,K,N,Q) as described in Materials and Methods. The panels on the left (A,D,G,J,M,P) are EGFP fluorescence and those on the right (C,F,I,L,O,R) are the merged fluorescent signals.

with myc-tagged wild-type Rab5a (Fig. 6A-C, G-I and M-O, respectively) or Tfn-R (not shown). Whilst most of these hybrids were only observed on endosomes labelled by the co-transfected marker myc-Rab5, a proportion of the Rab5<sup>174</sup>/Rab7<sup>41</sup> hybrid showed a perinuclear pattern, which could represent either ER/Golgi and/or late endosomal localisation (Fig. 6M). Furthermore, constitutively active forms (Q79L) of the Rab5a hybrids formed enlarged endosomes that co-localised with the Rab5 effector EEA1 (Fig. 6D-F, J-L, P-R) and Tfn-R (data not shown), suggesting that the hybrid proteins

retained Rab5a activity. These results suggest that the hypervariable regions of Rab1a and Rab2a are not sufficient to target Rab5a to their cognate compartments. Furthermore, the Rab5a hypervariable domain itself is not necessary for targeting to early endosomes, or function as measured by endosome fusion.

#### The C-terminal hypervariable region of Rab5a did not re-target other Rab proteins to early endosomes

To extend the above findings, we generated the converse hybrid proteins by fusing the C-terminal hypervariable domain of Rab5a to the N-terminal region of Rab1a or Rab2a (Fig. 7). Wild-type Rab1a and Rab2a localised to the Golgi apparatus, as previously reported (Fig. 7A-C, G-I) (Chavrier et al., 1990; Goud et al., 1990). The Rab1<sup>156</sup>/Rab5<sup>50</sup> and Rab2<sup>160</sup>/Rab5<sup>41</sup> hybrids associated with perinuclear membranes where they co-localised with Golgin-97, a Golgi marker (Fig. 7D-F and J-L, respectively). Neither of these hybrids was present in Tfn-R-positive endosomes (Fig. 7M-O; our unpublished results).

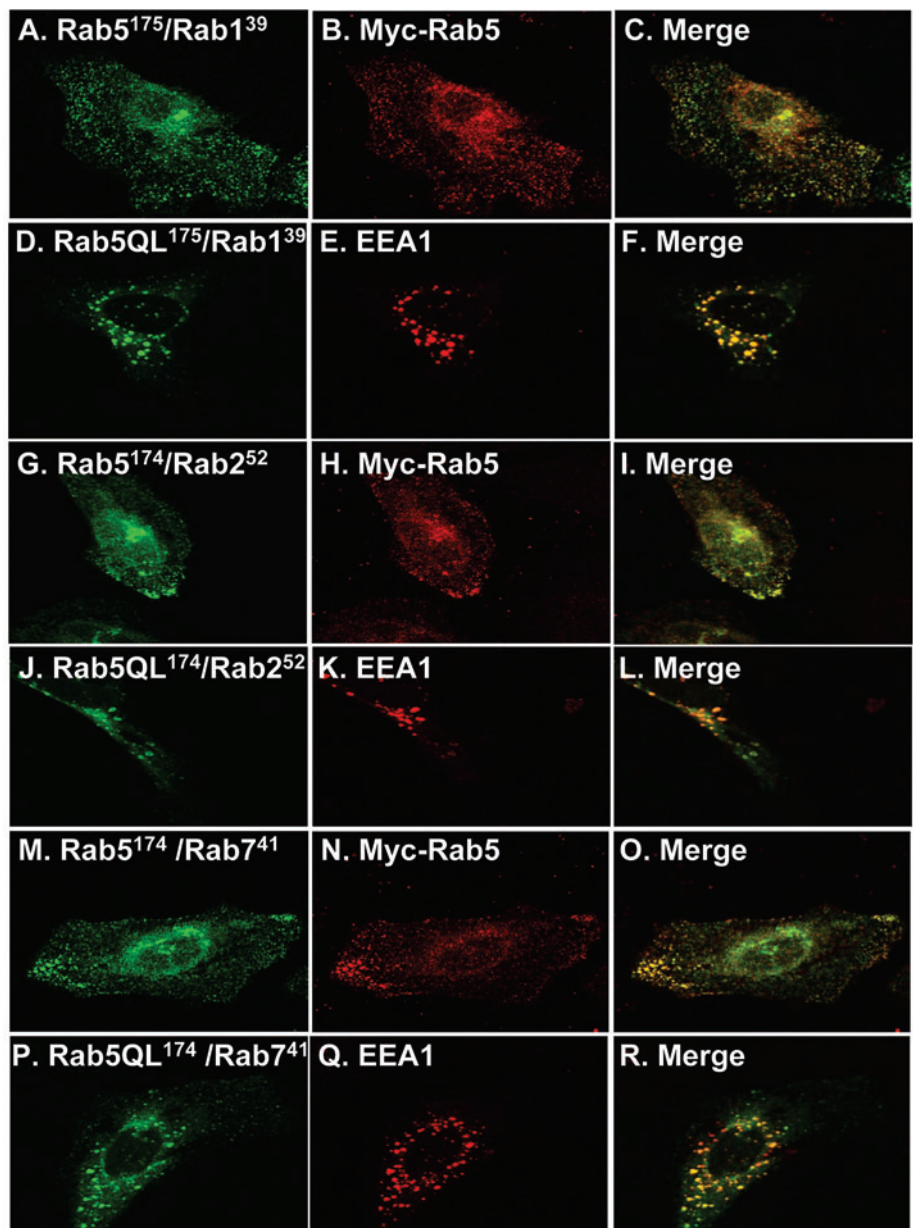
All Rab chimeras described in the above studies had been fused to EGFP. There is evidence in the literature suggesting that EGFP fusion does not alter localisation or indeed function (Tolmachova et al., 2003). Furthermore, some EGFP-Rab5 chimeras were found to co-localise with co-expressed myc-Rab5 (Fig. 6). Nevertheless, to exclude the possibility that targeting or function of the hybrid Rabs could have been altered by the presence of the EGFP tag in our studies, we replaced it with the much smaller myc-tag in a number of our constructs. Myc-tagged Rab27<sup>187</sup>/Rab5<sup>35</sup> was localised to secretory granules in AtT20 cells (see Fig. S3D-F in supplementary material), similar to the EGFP-tagged version (Fig. 2M-R). Myc-Rab5QL<sup>182</sup>/Rab27<sup>39</sup>, however, localised to Tfn-R-positive enlarged endosomes (see Fig. S3J-L in supplementary material). Similarly, myc-Rab5<sup>175</sup>/Rab1<sup>39</sup> localised to early endosomes in HeLa cells (see Fig. S3M-O in supplementary material), and myc-Rab5QL<sup>175</sup>/Rab1<sup>39</sup> caused the formation of enlarged endosomes (see Fig. S3P-R in supplementary material), consistent with our observations based on EGFP-tagged proteins.

#### Identification of domains in Rab5a and Rab27a required for subcellular targeting

The above results prompted a search for Rab targeting

determinants N-terminal of the hypervariable domain. In recent studies, we performed large-scale bioinformatics analyses on Rab sequences from a range of species where we identified Rab family-specific (RabF) and Rab subfamily-specific (RabSF) sequences (Pereira-Leal and Seabra, 2000; Pereira-Leal and Seabra, 2001). Five short sequence motifs that distinguish mammalian Rab proteins from other classes of small GTPases were defined as RabF regions, while four larger non-contiguous segments specifically conserved between subfamily members were designated RabSF regions. To test the role of RabF and RabSF regions in membrane targeting, we created hybrid proteins based on either Rab5a or Rab27a by exchanging residues within the RabF and RabSF regions between the two proteins. In most cases, targeting was not affected (our unpublished results). One example is the introduction of the Rab27a SF1 (residues 17-21) plus SF4 (residues 183-202) into Rab5a (Fig. 8M-O). Substitution of the RabSF2 region resulted in a mutant protein unable to associate with membranes (Fig. 8P-R), most likely because of defects in prenylation, given the lack of hydrophobicity of the transfected protein (see Fig. S2 in supplementary material). Unfortunately, the cytosolic distribution of this fusion protein did not allow us to assess its role in targeting. However, certain changes resulted in dramatic effects on targeting of membrane-associated Rabs. Introducing the RabSF3 region of Rab27a (residues 109-132) into Rab5a led to a redistribution of the mutant Rab5QL/27<sup>SF3</sup> protein, resulting in the partial co-localisation with the ER marker PDI and the Golgi marker Golgin-97 (Fig. 8A-I). Substitution of the RabF4 region of Rab5a (residues 86-93; Rab5QL/Rab27<sup>F4</sup>) with that of Rab27a had a similar effect (Fig. 8J-L).

Mutations were also introduced into Rab27a and analysed in AtT20 cells. Substituting part of the RabSF2 region of Rab27a (amino acid residues 35-40) with equivalent residues present in Rab5a led to a loss of secretory granule targeting and the redistribution of the hybrid Rab27/5<sup>SF2</sup> to other cellular membranes, most prominently in the perinuclear region where it co-localised with the Golgi marker Golgi-58K (Fig. 9A-F). Similarly Rab27/5<sup>SF3</sup> (substitution of residues 110-116) which contained part of the Rab5 SF3 region, was observed on ER and Golgi membranes instead of secretory granules (Fig. 9G-L). Unlike Rab5QL/Rab27<sup>F4</sup>, the substitution of the RabF4 region in

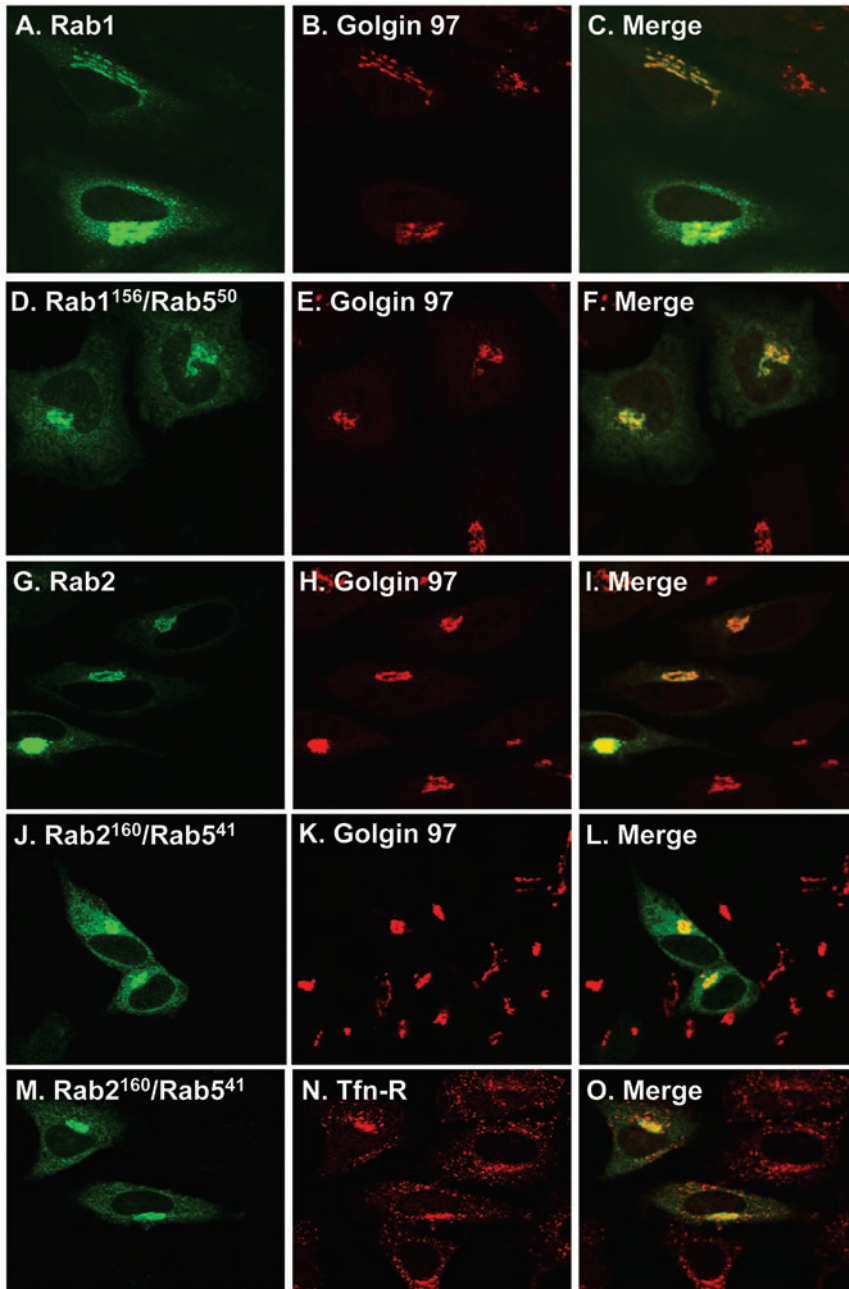


**Fig. 6.** Transient over-expression in HeLa cells of EGFP-Rab5 fusion proteins containing the hypervariable domain of other Rab proteins. HeLa cells were co-transfected with C-myc-Rab5a and EGFP-Rab5<sup>175</sup>/Rab1<sup>39</sup> (A-C), EGFP-Rab5<sup>174</sup>/Rab2<sup>52</sup> (G-I) or EGFP-Rab5<sup>174</sup>/Rab7<sup>41</sup> (M-O). Cells transfected with the constitutively active forms of the hybrid proteins EGFP-Rab5QL<sup>175</sup>/Rab1<sup>39</sup> (D-F), EGFP-Rab5QL<sup>174</sup>/Rab2<sup>52</sup> (J-L) and EGFP-Rab5QL<sup>174</sup>/Rab7<sup>41</sup> (P-R) were immunostained with anti-EEA1 antibody (E,K,Q).

Rab27a did not affect the localisation of Rab27/Rab5<sup>F4</sup>, which was seen exclusively on ACTH-positive granules (Fig. 9M-O). Altogether, our studies suggest that selected regions other than the hypervariable region are required for membrane targeting, including RabF4 and RabSF3 in Rab5a, and RabSF2 and RabSF3 in Rab27a.

## Discussion

We present evidence suggesting that the Rab hypervariable region is not a general targeting domain for Rab GTPases.



**Fig. 7.** Transient over-expression in HeLa cells of several EGFP-Rab fusion proteins containing the hypervariable domain of Rab5a. HeLa cells were transfected with EGFP-Rab1a (A-C), EGFP-Rab1<sup>156</sup>/Rab5<sup>50</sup> (D-F), EGFP-Rab2 (G-I) or EGFP-Rab2<sup>160</sup>/Rab5<sup>41</sup> (J-O). After 24 hours, cells were fixed, permeabilised and immunostained with an anti-Golgin97 antibodies (B,E,H,K), or Tfn-R antibody (N) as described in Materials and Methods. The panels on the left (A,D,G,J,M) show the EGFP signal and on the right (C,F,I,L,O) are the merged fluorescent signals.

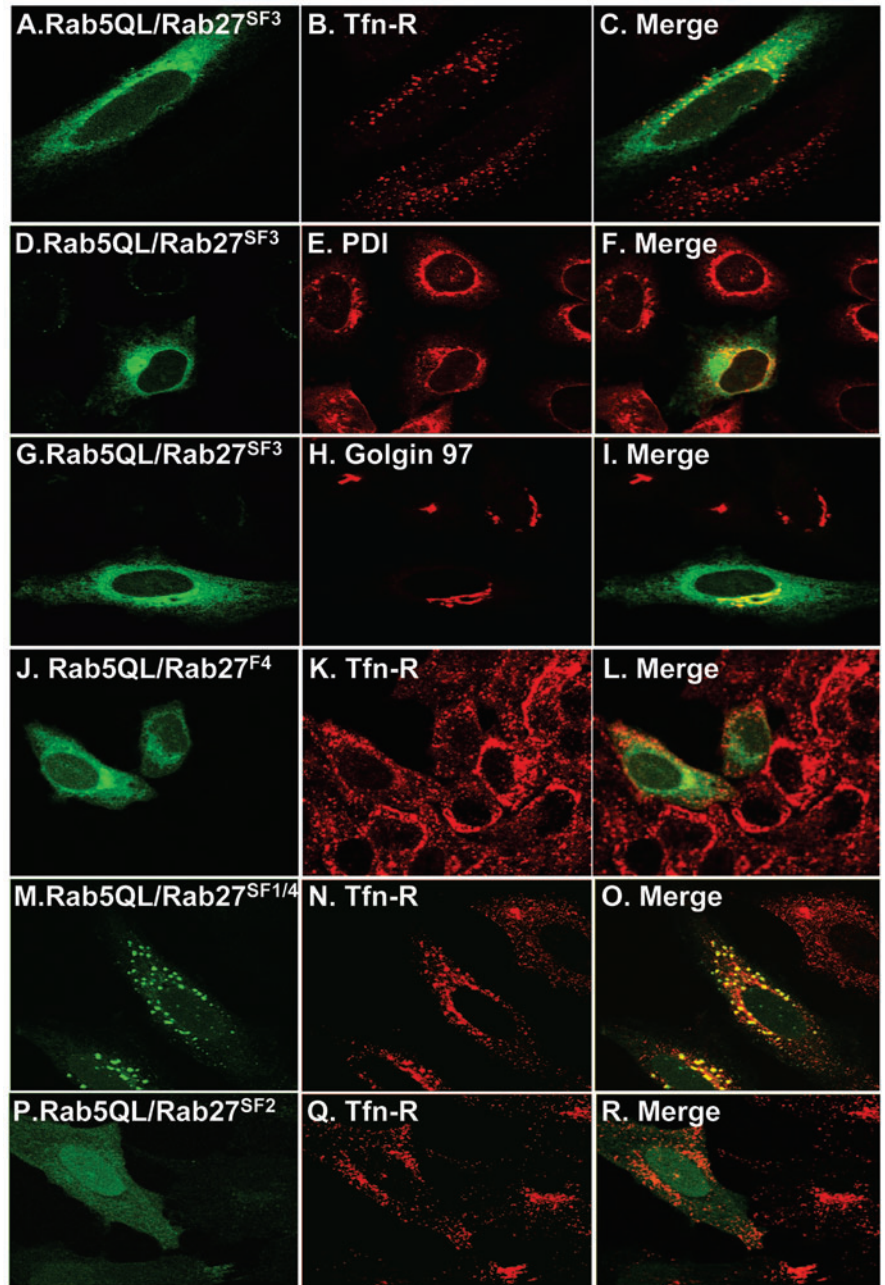
Instead, we identified other regions in the RabF and RabSF motifs that are necessary for specific targeting. We observed only partial overlap between regions determining the targeting of the different Rab proteins examined, indicating that Rab recruitment may be complex and partially Rab-specific. Our studies further suggest that the ER/Golgi membranes serve as the default location for Rabs that have lost targeting information.

### The C-terminal hypervariable region is not a general targeting determinant

Early studies suggested that the highly variable C-terminal end of Rab proteins was the main structural determinant for their specific targeting to distinct cellular membranes (Chavrier et al., 1991; Stenmark et al., 1994). Surprisingly, our present studies do not support this model. Reciprocal exchanges of the hypervariable domains of Rab1a, Rab2a, Rab5a, Rab7 and Rab27a failed to re-direct the hybrid proteins away from their original compartment to the new compartment designated by the hypervariable region. Furthermore, lower eukaryote (*S. cerevisiae* and *T. brucei*) homologues of Rab5 fused to EGFP are correctly targeted to early endosomes in mammalian cells, as previously described for *S. cerevisiae* Rab5 orthologues (Singer-Kruger et al., 1995), despite little conservation of the hypervariable domain at the amino acid level. We also show here that a hybrid Rab27a with a Rab5a hypervariable domain retained Rab27 function, as determined by its ability to rescue the melanosome distribution defect observed in melanocytes derived from the *ashen* (Rab27a null) mice (Fig. 4). Our present findings are consistent with a previous study which suggested that the Ras hypervariable region in the context of Rab6 did not affect targeting of the fusion protein to the Golgi apparatus, the location of wild-type Rab6 (Beranger et al., 1994). In yeast, reciprocal exchanges of the hypervariable domains between Ypt1 and Sec4 had no effect on their function. Ypt1p containing the hypervariable domain of Sec4p fully complemented a deletion of *YPT1* in yeast (Brennwald and Novick, 1993; Dunn et al., 1993) and similarly Sec4p with the hypervariable domain of Ypt1p complemented the *SEC4* deletion (Brennwald and Novick, 1993). These complementation studies suggest that a significant proportion of the hybrid proteins were correctly targeted to their original site of action and that they were indeed functional.

The difference between our conclusions and earlier results proposing a key role for the hypervariable C-terminal domain in targeting remains puzzling. However, it should be noted that while the current study examines Rab proteins acting in very distinct cellular locations and pathways (such as melanosomes, endosomes and Golgi), the main focus in previous work had been on Rabs functioning within the endosomal system. Given that targeting appears to involve multiple and partially Rab-specific regions, it is possible that Rabs with an 'endosomal character' share some localisation determinants, but possess



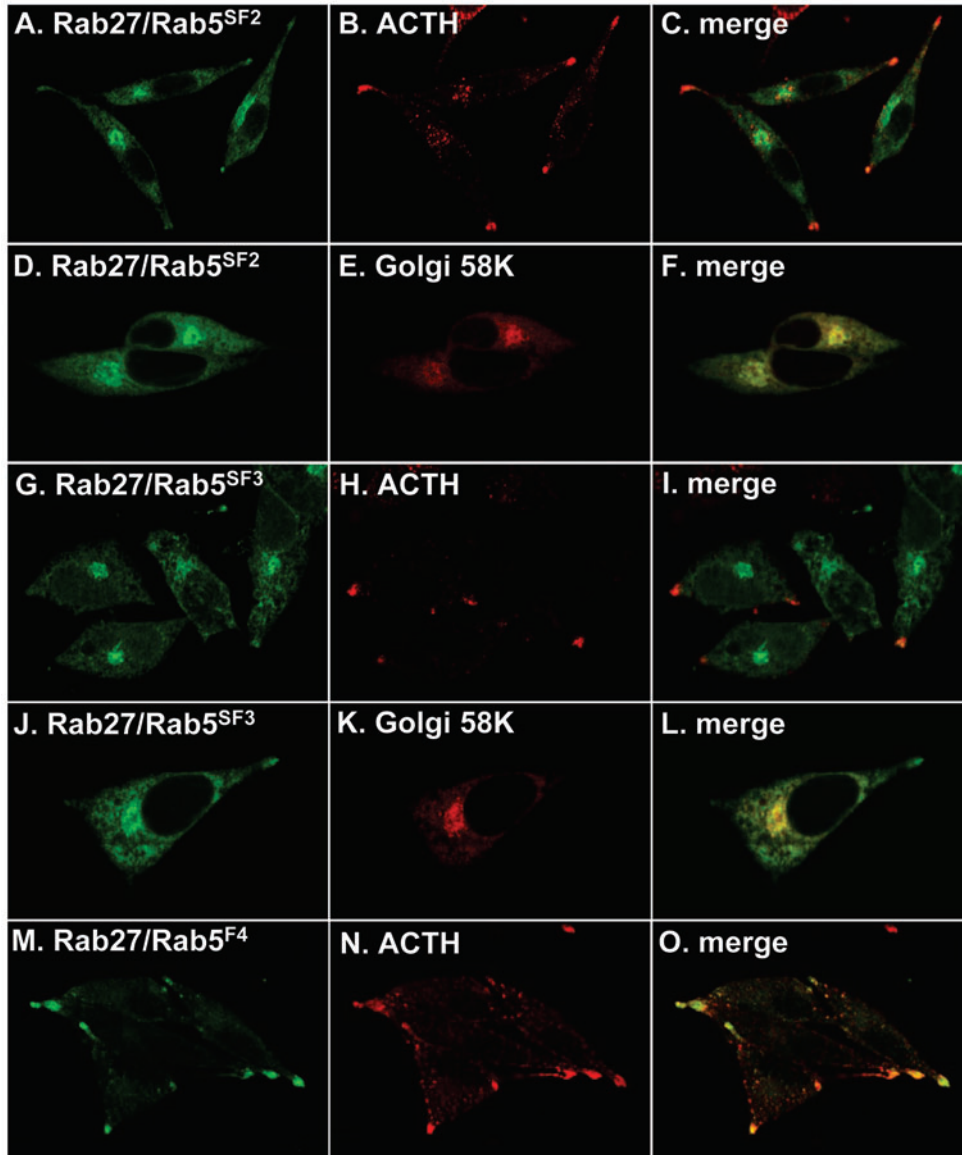


**Fig. 8.** Transient over-expression in HeLa cells of EGFP-Rab5Q79L with the RabF or RabSF domains of Rab27a. HeLa cells were transfected with EGFP-Rab5QL/Rab27<sup>SF3</sup> (A-I), EGFP-Rab5QL/Rab27<sup>F4</sup> (J-L), EGFP-Rab5QL/Rab27<sup>SF1-SF4</sup> (M-O) or EGFP-Rab5QL/Rab27<sup>SF2</sup> (P-R). After 24 hours, cells were fixed, permeabilised and immunostained with an anti-Tfn-R antibody (B,K,N,Q), with anti-PDI antibodies (E) or anti-Golgin97 antibodies (H). The panels on the left (A,D,G,J,M,P) are EGFP signal and on the right (C,F,I,L,O,R) are the merged fluorescent signals.

additional targeting information in their hypervariable domains, which contributes to their accurate localisation within the endosomal system. It may be significant that the only construct in our studies that was similar to one used in the early studies, the Rab5/Rab7 hybrid, partially exhibited an aberrant localisation. Whilst it is impossible to rule out late endosomal localisation, we found this aberrant localisation more consistent with ER mistargeting (Fig. 6). Furthermore, it remains possible that the GFP and myc-tags may have influenced our results, although the overwhelming evidence suggests that the tags do not introduce such artifacts. Finally, the expression systems may have influenced the results as the vaccinia system used previously allowed observation at earlier time points after transfection.

#### The ER and Golgi as a default localisation for Rab proteins that have lost targeting information

We had previously observed that the ER and Golgi are preferential sites of membrane translocation for mistargeted Rabs, i.e. Rabs that have lost targeting information. Rabs with mutations in the prenylation motif that resulted in singly rather than doubly geranylgeranylated proteins showed mistargeting to the ER and loss of function (Gomes et al., 2003). Furthermore, the expression of Rab27a in undifferentiated cultured cell types such as HeLa or HEK-293 cells led to ER localisation (Hannah et al., 2003). Thus, the absence of specialised compartments such as secretory granules and/or lysosome-related organelles in these cells appears to result in the association of Rab27a with the ER by default (Hannah et



**Fig. 9.** Expression of chimeric EGFP-Rab27 containing RabSF or RabF domains of Rab5 in AtT20 cells. Cells were transiently transfected with EGFP-Rab27/Rab5<sup>SF2</sup> (A-F), EGFP-Rab27/Rab5<sup>SF3</sup> (G-L) or EGFP-Rab27/Rab5<sup>F4</sup> (M-O). After fixation, cells were permeabilised and immunolabelled with antibodies to ACTH (B,H,N) or the Golgi 58K protein (E,K). Panels on the left (A,D,G,J,M) show EGFP fluorescence and panels on the right (C,F,I,L,O) are the merged images.

al., 2003; Hume et al., 2001; Tolmachova et al., 2003; Yi et al., 2002). Here we show several examples of ER/Golgi targeting upon domain swapping and we suggest that this localisation reveals mistargeting.

#### Regions necessary for targeting

Mistargeting to the ER was thus used as an assay to search for regions necessary for Rab targeting. We focused our mutagenesis studies on the previously characterised RabF and RabSF regions (Pereira-Leal and Seabra, 2000; Pereira-Leal and Seabra, 2001). The RabF and surrounding sequences form the switch regions, which change conformation upon GTP-binding, and the RabSF regions are areas of greater conservation between subfamily members, such as the Rab5/21/22 subfamily and the Rab27/3 subfamily. Thus, targeting information could potentially reside in these regions. We observed clear mistargeting and possibly loss of function when swapping certain internal elements between Rab5a and Rab27a including RabSF2, RabF4 and RabSF3. The RabF4

region, typically YYRGA, is generally highly conserved between Rabs but Rab27a possesses a significantly different F4 region, FFRDA. This may have caused local structural changes resulting in loss of targeting information. The RabSF2 region, more precisely loop 2, had previously been implicated in Rab6 targeting to the Golgi (Beranger et al., 1994) and our results are consistent with this observation as they suggest that Rab27 SF2 (loop 2) is necessary for targeting to secretory granules. The RabSF2 region may also be involved in targeting other members of the Rab family, but in some cases (such as Rab5) mutagenesis in this region resulted in soluble proteins, making it difficult to analyse its contribution. Finally, our results suggest that the RabSF3 region may play an important role in targeting.

It is noteworthy that two of the candidate targeting regions reported here partially overlap with the switch regions (RabSF2 with switch I and RabF4 with switch II), and are expected to participate in effector binding. This raises the possibility that the interaction with effectors may promote Rab targeting. Taken together, our data suggests that Rab targeting

is determined by multiple sequence motifs within Rab proteins that are not necessarily localised in the same vicinity.

### Models for Rab membrane targeting

The molecular mechanisms regulating Rab membrane delivery by REP/GDI proteins remain unclear (Pfeffer, 2001; Seabra and Wasmeier, 2004). At least three activities appear to be necessary: recognition of the correct intracellular target membrane, dissociation of the Rab:REP/GDI complex through a GDI displacement factor (GDF) and nucleotide exchange to prevent membrane extraction. Recently, Pfeffer and co-workers demonstrated that PRA1/Yip3 possesses GDF activity (but not exchange activity) towards endosomal Rabs (Sivars et al., 2003). Yip proteins, a family of Rab-binding integral membrane proteins, could aid in Rab targeting but may not represent the main targeting determinant since they display broad specificity towards different Rabs (Sivars et al., 2003). Another interesting model suggested that a Rab protein may regulate the activation of another Rab acting in a downstream transport step by recruiting its nucleotide exchange factor (GEF), as proposed for the recruitment by Ypt32 of Sec2p, a GEF for Sec4p (Ortiz et al., 2002). Certain Rab effectors, once recruited, may also serve to expand the Rab-specific membrane domain through generation of a positive feed-back loop (Zerial and McBride, 2001). This would argue for a direct link between Rab targeting and Rab function, as discussed above. Taken together, it seems likely that multiple factors contribute to the specificity and regulation of Rab recruitment.

The present studies identifying targeting determinants within Rabs represent the first step towards testing these models. Future experiments will be aimed at elucidating the role of these targeting-determining regions in Rab localisation. This should provide further mechanistic insights into the regulated delivery of Rab GTPases to specific intracellular membranes and into the mechanisms regulating the organisation of intracellular compartments.

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## Supplementary Materials and Methods

### Rab5a/Rab27a internal domains mutants

Construct: **Rab5aQL/Rab27<sup>SF2</sup>**

Made by three consecutive rounds of site-directed mutagenesis (SDM).

Primers used:

- (1) SF2-F2 GGCCAATTCATGAAAAGCTTATCACTACCATGGGGCTGCTTTTC
- (2) SF2-F3 CCTAGTGCTTCGTTTTATCGATGGCAAATTTAATTCAAAGTTTATCACTACCATGG
- (3) SF2-F4 GGCAAATCAAGCGTACTGTATCAGTATACCGATGGCAAATTTAATTCA

Sequence changed from LVLRFVKGQFHEFQES to VLYQYTDGKFNSKFIT

Template used: EGFP-Rab5aQL

Construct: **Rab5aQL/Rab27<sup>SF3</sup>**

Made by three consecutive rounds of SDM.

Primers used:

- (1) SF3-F2 GGCAAGCAAGTCCTAACCTGATATCGTTTTATGCGGAAACAAGGCCG
- (2) SF3-F3 GGTAAAGAACTTCAGATGCATGCATATTGTGAGAACCCTGATATCG
- (3) SF3-F4 GCATGCATCTGAAGTTGGCTGATCCAATTTCTTACGTTAAAAAGGACTCCTC

Sequence changed from ARAKNWVKELQRQASPNIVIALS to

LNVRNWSQLQMHAYCENPDIVLC

Template used: EGFP-Rab5aQL

Construct: **Rab5aQL/Rab27<sup>SF4</sup>**

Made by three consecutive rounds of SDM

Primers used:

- (1) SF4-F2 GCTAAAAAATTGCCAAAGGTCGACAAATCATGGAATCCAGGAGCAAATTCGCC
- (2) SF4-F3 GGCAAATCAAGCGTACTGTATCAGTATACCGATGGCAAATTTAATTCA
- (3) SF4-F4 GTCGACAAATCATGGATCCCAGAAGGAGTTGTTTCGACGCGAAGGAGTAGAC

Sequence changed from KNEPQNPGANSAR to KRMERCVDKSWIPEGVVRS

Template used: EGFP-Rab5aQL

Construct: **Rab5aQL/Rab27<sup>SF4E</sup>**

Made by two consecutive rounds of SDM.

Primers used:

- (1) SF4D-F GGAGTTGTTTCGAGCAACGGACATGTAGACCTTACCG
- (2) SF4E-F CGCAGCAACGGACATGCAAGCACTGACGAACCCACACAACC

Sequence changed from KNEPQNPGANSAR RGRGVDLT to

KRMERCVDKSWIPEGVVRSNGHASTD

Template used: EGFP-Rab5aQL/Rab27<sup>SF4</sup>

Construct: **Rab5aQL/Rab27<sup>SF1-SF4</sup>**

Made by SDM

Primer used: 27X5aSF1-F1

GGCCAAATACTGGAAATTACGACTACCTGATCAAACACTAGTACTTCTGGGA

Sequence changed from KICQF to YDYLI and KNEPQNPGANSAR to

KRMERCVDKSWIPEGVVRS

Template used: EGFP-Rab5aQL/Rab27<sup>SF4</sup>

Construct: **Rab5aQL/Rab27<sup>F4</sup>**

Primer: A2-L5-F CGATACCATAGCCTAACACAGCGTTCTTCAGAGATGCACAAGCAGCC

Sequence changed from APMYYRG to TTAFFRD

Template EGFP-Rab5aQL

### Rab27a/Rab5a internal domains mutants

Construct: **Rab27a/5a<sup>SF2</sup>**

Created by two consecutive rounds of SDM.

Primers used:

- (1) Rab27/SF2-A:5 ACTGATGGGAAGTTCCACGAATTCTTCATCACCCACAGTG
- (2) Rab27/SF2-B:5 AAGTTCCACGAATTCAGGAATCCACAGTGGGCATTG

Sequence changed from NSKFIT to HEFQES

Template used EGFP-Rab27a

Construct: **Rab27/Rab5<sup>SF3</sup>**

Created by two consecutive rounds of SDM.

Primers used:

(1) Rab27/SF3-b1:5 ATGTCCGAAACTGGGTAAAGGAGCTACAGATGCAC  
 (2) Rab27/SF3-a2b1:5 AGTTTCCTCAATGCCAAAACTGGGTAAAG  
 Sequence changed from VRNWISQ to AKNWVKE  
 Template used: EGFP-Rab27a

Construct: **Rab27/Rab5<sup>F4</sup>**  
 Made by one round of SDM.  
 Primer used: CCTGACCACGGCATACTACCGCGGCGCTATGGGTTTCC  
 Sequence changed from FFRDA to YYRGA  
 Template used: EGFP-Rab27a

#### Hypervariable domain swap constructs

Construct: **Rab1a<sup>156</sup>/Rab5<sup>50</sup>**  
 Obtained by ligating the N-terminus fragment of pEGFP-Rab1a with C-end fragment of pEGFP-Rab5a cut at the *Eco47III*-mutated ETSAK motif (Gomes *et al.*, 2003) with *NdeI* and *Eco47III*

Construct: **Rab2a<sup>153</sup>/Rab5<sup>49</sup>**  
 Obtained by ligating the N terminus fragment of pEGFP-Rab2a with C-end fragment of pEGFP-Rab5a cut at the *Eco47III*-mutated ETSAK motif with *NdeI* and *Eco47III*.

Construct: **Rab2a<sup>160</sup>/Rab5<sup>41</sup>**  
 Obtained by SDM using EGFP-Rab2a<sup>153</sup>/Rab5<sup>49</sup> as template  
 Primer used: 2-5PrimerSak-F AGCGCTAAAACAGCATCGAATGTAGAGGAAGCTTTTC

Construct: **Rab5a<sup>165</sup>/Rab1<sup>48</sup>**  
 Obtained by ligating the N terminus fragment of pEGFP-Rab5a with C-end fragment of pEGFP-Rab1a cut at the *Eco47III*-mutated ETSAK motif with *NdeI* and *Eco47III*.

Construct: **Rab5aQL<sup>165</sup>/Rab1<sup>48</sup>**  
 Obtained by SDM using EGFP-Rab5a<sup>165</sup>/Rab1<sup>48</sup> as template.  
 Primer used: Rab5QL3' ACAGCTGGTCTAGAACGATAC

Constructs: **Rab5a<sup>175</sup>/Rab1<sup>39</sup> and Rab5QL<sup>175</sup>/Rab1<sup>39</sup>**  
 Obtained by SDM  
 P r i m e r u s e d : 5 - 1 S a k - F  
 GAGACAAGCGCTAAGACCTCAATGAATGTAAATGAGATCTTCATGACGATG  
 Templates used: EGFP-Rab5a<sup>165</sup>/Rab1<sup>48</sup> and EGFP-Rab5aQL<sup>165</sup>/Rab1<sup>48</sup>, respectively.

Construct: **Rab5a<sup>166</sup>/Rab2<sup>60</sup>**  
 Obtained by ligating the N terminus fragment of pEGFP-Rab5a with C-end fragment of pEGFP-Rab2a cut at the *Eco47III*-mutated ETSAK motif with *NdeI* and *Eco47III*.

Construct: **Rab5aQL<sup>166</sup>/Rab2<sup>60</sup>**  
 Obtained by SDM using Rab5a<sup>166</sup>/Rab2<sup>60</sup> as template  
 Primer used: Rab5QL3' ACAGCTGGTCTAGAACGATAC

Constructs: **Rab5a<sup>174</sup>/Rab2<sup>52</sup> and Rab5QL<sup>174</sup>/Rab2<sup>52</sup>**  
 Were obtained by SDM.  
 Primer: 5-2Sak-F CAAGCGCTAAGACTTCTATGAATGTAAATGAGATCTTTATTAATACAGC  
 Templates used: Rab5a<sup>166</sup>/Rab2<sup>60</sup> and Rab5aQL<sup>166</sup>/Rab2<sup>60</sup>, respectively.

Construct: **Rab5aQL<sup>182</sup>/Rab27a<sup>39</sup>**  
 Generated through four consecutive rounds of SDM.  
 Primers used:  
 (1) Rab5/SF4-F:27 CATGCAAGCACTGACCAACTCTCAGAACCAACCAGGAATC  
 (2) Rab5/SF4-G:27 GACCAACTCTCAGAAGAAAAGGAGAAACAGTGTGTAGTAAC  
 (3) Rab5/SF4-H1:27 AAGAAAAGGAGAAAAGGGGCTTGTAAGTAAG  
 (4) Rab5/SF4-H2:27 GAAAGGGGCTTGTTGCTAACTGACGGT  
 Template used: EGFP-Rab5aQL/Rab27<sup>SF4E</sup>

Construct: **Rab27a<sup>187</sup>/Rab5a<sup>35</sup>**  
 Created through several consecutive rounds of SDM.  
 Primers used for individual rounds:  
 (1) Rab27/SF4-A:5 CTCCTGGACCTGATCCCGAAGGACAAATCCTGGATTC  
 (2) Rab27/SF4-B:5 ACCTGATCCCGAAGAACGAACCCAGATTCCAGAGGGGG  
 (3) Rab27/SF4-C:5 AGAACGAACCCAGAAATCCAGGGGCGAATGTGCGGTCCAATGCC  
 (4) Rab27/SF4-H1:5 AATCCAGGGGCGAATTCGGCGCGCGGTGGCCATACTTCTAC  
 (5) Rab27/SF4-H2:5 AGAAGGAGAAGGGGTGTTGCAGCAATTGAGGATCCACCGG

(6) Rab27/SF4-H3:5 AATTCGGCGCGCGGTTCGCGGTGTTGATACAGATCAACTCAG  
 (7) Rab27/SF4-H4:5 AACTCAGTGAGGAGACGCGGAATCAGTGTTGCAGCAATT  
 (8) Rab27/SF4-H5:5 GTCGCGGTGTTGATTAACTGAACTCAGTGAGGAG  
 (9) Rab27/SF4-H6:5 TTGATTAACTGAACCCACTCAGCCGACGCGGAATCAGTG  
 (10) Rab27/SF4-X2:5 CCTGGACCTGATCATGAAGCGAATGGAAAGATTGCCGAAGAACGAAC  
 Template used: EGFP-Rab27a.

Construct: **Rab27<sup>187</sup>/Rab1<sup>34</sup>**

The N terminus of Rab27a was excised from pEGFP-Rab27a with *NdeI* and *Eco47III* and was inserted into pEGFP-Rab1a cut with the same enzymes. This was used as the initial template for consecutive rounds of SDM with the following primer in each round:

(1) 27:1Mmod1 TTGAAACCAGCGCTGCGAACGGAACGAATATATCACAGTCTTTCATGA  
 (2) 27:1Mmod2 ACGAATATATCACAGGCTATCGAGATGCTGTTAGATGAGATTAATAAAGCG  
 (3) 27:1Mmod3 AGATGCTGTTAGATCTGATTATGAAAAGGATGGAAAGAATTAATAAAGCGAAT

Construct: **Rab1a<sup>172</sup>/Rab27a<sup>40</sup>**

The N terminus of Rab1a was ligated to the C terminus of Rab27a using the *NdeI* and *Eco47III* restriction sites. This served as the template for two consecutive rounds of SDM using the following primers:

(1) 1:27Mmod1 TGGAAACCAGCGCTAAAAATGCGACAAACGTAGAACCAAGCAATTGAGAT  
 (2) 1:27Mmod2 ACAAACGTAGAACCAATCATTATGACGATGGCGGCCGAGATCATGAAGCGGAT

Other constructs

**pEGFP-Rab5aQL-<sub>BR</sub>** (R209S, R195A, R197A, K179A, K180A, K183A)

Lysine residues at positions 179, 180 and 183 were mutated to alanines using the following primer.

Rab5aBR-3F CATGGCAATAGCTGCAGCATTGCCAGCGAATGAACCACAAAATCC

Arginine residues 195 and 197 were mutated to alanines using the following primer

Rab5aBR-2F GGAGCAAATCTGCAGCAGGAGCAGGAGTAGACCTTACCG

Arginine 209 was mutated to Serine using

Rab5aBR-1F CCCACACAACCAACTAGTAATCAGTGTTGTAGTAAC

**EGFP-TbRab5a** and **EGFP-TbRab5aQL**. TbRab5a was amplified from pXSS-TbRab5a (from M.C. Field) and cloned into pEGFP-C2 using the following primers

TbRab5a-F AAGCTTCGAATTCATGTTCGGTGTTCAGCGACACC

*EcoRI*

TbRab5a-R GGGCCCCGGGATCCTCAGCAGGCACACCCGCTTCC

*BamHI*

**EGFP-TbRab5b** and **EGFP-TbRab5bQL** were made by amplifying the cDNA from pXSS-TbRab5b (from M.C. Field) and cloned into pEGFP-C2 using the following primers:

TbRab5B-F CGCACTCAGATCTCGAGCATGTCTGTGAAGACCGTTGCC

*XhoI*

TbRab5B-R ACTGGCCCCGGGATCCTCAACACAGCAACCGGAAC

*BamHI*

**pEGFP-Ypt51(VPS21)**, **pEGFP-Ypt52**, **pEGFP-Ypt53** in pEGFP-C3 vector were obtained from Dr. Ruth Collins (Cornell University, NY).