

# Rab22 controls NGF signaling and neurite outgrowth in PC12 cells

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**ABSTRACT** Rab22 is a small GTPase that is localized on early endosomes and regulates early endosomal sorting. This study reports that Rab22 promotes nerve growth factor (NGF) signaling-dependent neurite outgrowth and gene expression in PC12 cells by sorting NGF and the activated/phosphorylated receptor (pTrkA) into signaling endosomes to sustain signal transduction in the cell. NGF binding induces the endocytosis of pTrkA into Rab22-containing endosomes. Knockdown of Rab22 via small hairpin RNA (shRNA) blocks NGF-induced pTrkA endocytosis into the endosomes and gene expression (VGF) and neurite outgrowth. Overexpression of human Rab22 can rescue the inhibitory effects of the Rab22 shRNA, suggesting a specific Rab22 function in NGF signal transduction, rather than off-target effects. Furthermore, the Rab22 effector, Rabex-5, is necessary for NGF-induced neurite outgrowth and gene expression, as evidenced by the inhibitory effect of shRNA-mediated knockdown of Rabex-5. Disruption of the Rab22–Rabex-5 interaction via overexpression of the Rab22-binding domain of Rabex-5 in the cell also blocks NGF-induced neurite outgrowth, suggesting a critical role of Rab22–Rabex-5 interaction in the biogenesis of NGF-signaling endosomes to sustain the signal for neurite outgrowth. These data provide the first evidence for an early endosomal Rab GTPase as a positive regulator of NGF signal transduction and cell differentiation.

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## INTRODUCTION

Nerve growth factor (NGF) is a neurotrophin that promotes neuronal survival and differentiation, via phosphorylation/activation of the TrkA receptor and signal transduction (Huang and Reichardt, 2003). NGF signaling is known to induce neurite outgrowth, a hallmark of cell differentiation, in PC12 cells (a rat pheochromocytoma cell line; Greene and Tischler, 1976). Endocytosis of the NGF-TrkA complex into signaling endosomes is necessary for sustained signaling and

neurite outgrowth (Zhang *et al.*, 2000; Delcroix *et al.*, 2003; Kuruvilla *et al.*, 2004). The signaling endosomes contain early endosomal markers but appear to be long-lived and are diverted from transport to late endosomes and lysosomes. Indeed, NGF signaling inhibits Rab5 activity that is essential for early endosome fusion and entry to the degradative endocytic pathway (Liu *et al.*, 2007). Along this line, inhibition of Rab5 or Rab7 by dominant negative mutants enhances NGF signaling and neurite outgrowth (Saxena *et al.*, 2005; Liu *et al.*, 2007).

The biogenesis of signaling endosomes is not well understood, especially their difference from normal early endosomes destined for late endosomes/lysosomes and degradation. The inhibitory effect of Rab5 suggests that other early endosomal Rab(s) may facilitate the biogenesis of signaling endosomes in the cell. Early endosomal Rab GTPases, such as the Rab5 subfamily members, control early endosomal fusion and sorting (Stenmark, 2009). The Rab5 subfamily contains Rab5, Rab21, Rab22, and Rab31 (also known as Rab22b; Pereira-Leal and Seabra, 2000). Except for Rab31, which is mainly localized to the TGN and is involved in transport from TGN to endosomes (Rodriguez-Gabin *et al.*, 2001), the other three Rab5 subfamily members are associated with early endosomes. Rab5 is the prototype; it is best characterized and is known for its role in

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Abbreviations used: ANOVA, analysis of variance; DsRed, *Discosoma* sp. red fluorescent protein; EET, early endosomal targeting; EGF, epidermal growth factor; eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FL, full-length; GEF, guanine nucleotide exchange factor; NGF, nerve growth factor; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcriptase PCR; RFP, red fluorescent protein; shRNA, small hairpin RNA; WT, wild-type.

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promoting early endosome fusion (Bucci *et al.*, 1992; Li *et al.*, 1994; Ohya *et al.*, 2009), which is essential for concentrating endocytosed ligands and receptors, as well as for subsequent conversion/maturation to late endosomes (Rink *et al.*, 2005; Nordmann *et al.*, 2010). Thus Rab5 activity is crucial for entry to the degradative late endosomes and lysosomes. Rab21 shows similar function in promoting early endocytosis of transferrin and epidermal growth factor (EGF; Simpson *et al.*, 2004). Consistently Rab5 and Rab21 interact with some of the same effectors, including EEA1, Rabaptin-5, APPL1 (Zhu *et al.*, 2007), and  $\beta_1$  integrins (Pellinen *et al.*, 2006), and share a common guanine nucleotide exchange factor (GEF), Rabex-5 (Horiuchi *et al.*, 1997; Delprato *et al.*, 2004).

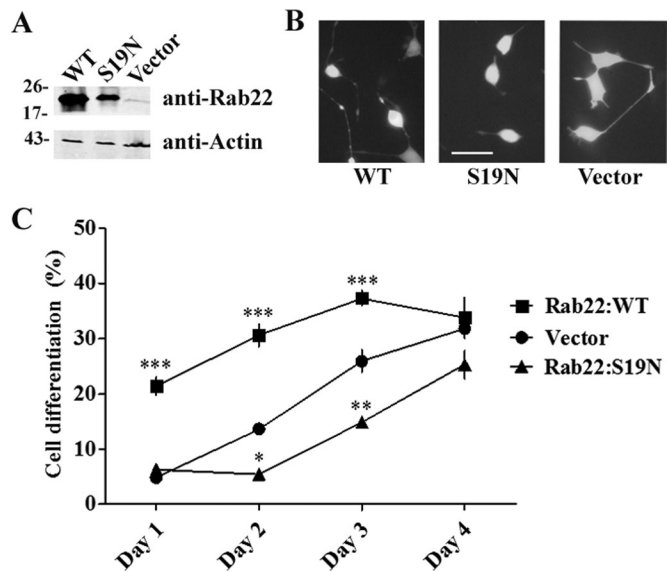
Rab22, however, exhibits a distinct function and promotes the formation of a specialized population of early endosomes that facilitate the endocytosis and recycling of both clathrin-dependent and clathrin-independent ligands, such as transferrin (Magadan *et al.*, 2006) and MHC-I (Weigert *et al.*, 2004). Rab22 also interacts with a subset of Rab5 effectors, such as EEA1 and Rabenosyn-5 (Kauppi *et al.*, 2002; Mishra *et al.*, 2010), suggesting a role in early endosome fusion, but a critical difference between Rab22 and Rab5 is that Rab22 utilizes Rabex-5 as an effector rather than a GEF (Zhu *et al.*, 2009). In the current study, we find that Rab22 and Rab22–Rabex-5 interaction are essential for the biogenesis of NGF-signaling endosomes and NGF signaling-dependent processes, including gene expression and neurite outgrowth in PC12 cells. The Rab22 membrane domain, via Rabex-5, may link to and regulate the Rab5 and Rab21 domains to establish NGF-signaling endosomes and facilitate NGF-induced cell differentiation.

## RESULTS

### Rab22 is essential for NGF-induced neurite outgrowth in PC12 cells

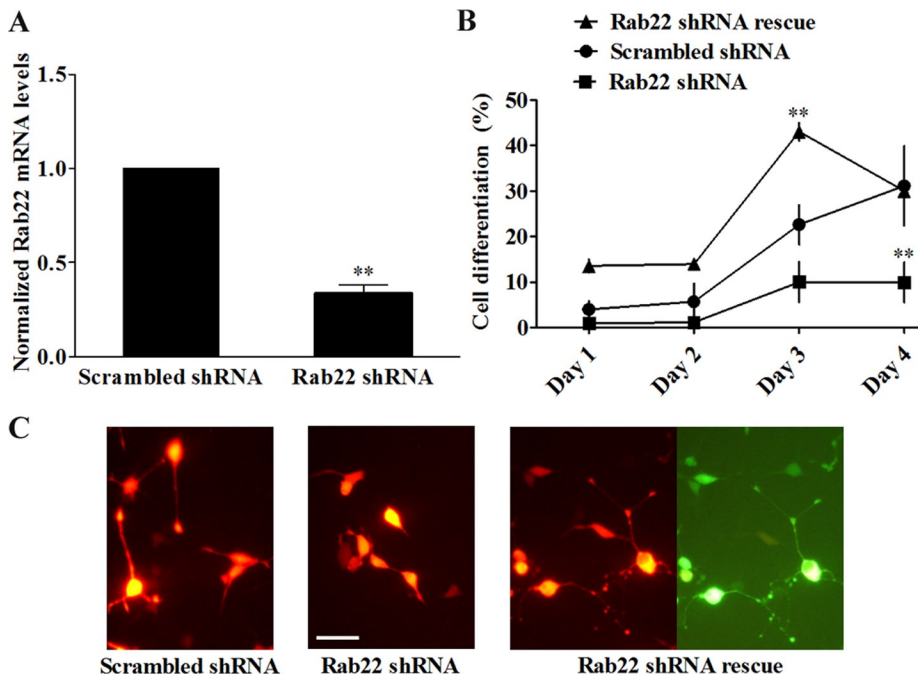
It is known that overexpression of Rab5 to enhance early endosomal sorting to late endosomes/lysosomes and degradation can reduce NGF signaling and block neurite outgrowth in PC12 cells, while inhibition of Rab5 function by dominant negative mutants enhances NGF-induced neurite outgrowth (Liu *et al.*, 2007). The existing data suggested a critical role for early endosomal sorting in the function of NGF-signaling endosomes, and prompted us to investigate the other Rab5 family members associated with early endosomes, including Rab21 and Rab22. To this end, we overexpressed wild-type (WT) as well as a dominant negative mutant (Rab21:T33N or Rab22:S19N) of the Rabs in PC12 cells via a bidirectional expression vector (pBI/eGFP) that simultaneously expressed enhanced green fluorescent protein (eGFP) for identification of transfected cells by fluorescence microscopy. On NGF treatment (100 ng/ml), neurite outgrowth was determined for up to 4 d. The effect of Rab21 was essentially the same as that of Rab5, that is, overexpression of WT inhibited neurite outgrowth, while the Rab21:T33N mutant enhanced the process (Supplemental Figure S1), suggesting that Rab21 and Rab5 promote the same early endosomal sorting pathway destined for late endosomes/lysosomes and degradation that reduces NGF signaling and neurite outgrowth. In contrast, overexpression of Rab22 significantly enhanced NGF-induced neurite outgrowth, while the dominant negative mutant (Rab22:S19N) inhibited the process (Figure 1). The data suggest that Rab22 is a positive regulator of NGF signaling and neurite outgrowth in PC12 cells. Rab22 is the only Rab5 subfamily member that promotes NGF-induced neurite outgrowth; we also tested Rab31 in the same assay and it showed neither positive nor negative effect (Figure S2).

To ascertain the positive role of Rab22 in NGF-induced neurite outgrowth and to confirm the inhibitory effect of the Rab22:S19N



**FIGURE 1:** Effects of overexpression of Rab22:WT and Rab22:S19N on NGF-induced neurite outgrowth in PC12 cells. (A) Immunoblot showing overexpressed Rab22:WT and Rab22:S19N, as well as endogenous Rab22 (Vector) in the cell lysates, as detected with the anti-Rab22 antibody. The actin level in each sample is also shown as a loading control. Molecular mass standards (in kDa) are indicated on the left. (B) Fluorescence microscopy images comparing NGF-induced neurite outgrowth at day 2 among cells overexpressing Rab22:WT and Rab22:S19N, and control cells (Vector). Scale bar: 50  $\mu$ m. (C) Quantification of cell differentiation as percentage of transfected cells containing long neurites. On NGF treatment (100 ng/ml), 500 transfected cells (with GFP expression) were counted each day in each case, and the percentage of cells containing neurites was determined by fluorescence microscopy as shown in (B). Two-way analysis of variance (ANOVA) was performed (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$ , vs. the vector control); error bars indicate SEM of three independent experiments.

mutant in this process, a rat Rab22-specific small hairpin RNA (shRNA) was used to knock down endogenous Rab22 in PC12 cells and was tested for any inhibitory effect on NGF-induced neurite outgrowth. The Rab22 shRNA and a scrambled control shRNA were expressed, respectively, via a vector that simultaneously expresses monomeric *Discosoma* sp. red fluorescent protein (DsRed) for identification of transfected cells by fluorescence microscopy or fluorescence-activated cell sorting (FACS). The transfected cells were isolated by FACS and subjected to quantitative reverse transcriptase PCR (qRT-PCR) to determine endogenous mRNA levels of Rab22. The results indicated that the Rab22 shRNA effectively reduced endogenous Rab22 mRNA level by 75%, in comparison to control cells expressing the scrambled shRNA (Figure 2A). Importantly, the Rab22 knock-down blocked NGF-induced neurite outgrowth, as determined by fluorescence microscopy (Figure 2, B and C), consistent with the inhibitory effect of Rab22:S19N (Figure 1). To rule out possible off-target effects of the rat Rab22 shRNA, human Rab22 was coexpressed via the pBI/eGFP vector to determine if it can rescue the inhibitory effect of the Rab22 shRNA on NGF-induced neurite growth. Indeed, overexpression of human Rab22 in cells expressing the Rab22 shRNA, as evidenced by the expression of both DsRed and GFP, completely reversed the block and actually enhanced NGF-induced neurite outgrowth, compared with control cells expressing the scrambled shRNA (Figure 2, B and C), suggesting the inhibitory effect of the Rab22 shRNA is due to reduction of Rab22 expression.



**FIGURE 2:** Inhibitory effect of Rab22 knockdown on NGF-induced neurite outgrowth in PC12 cells. (A) qRT-PCR results comparing the mRNA levels of Rab22 between control cells expressing the scrambled shRNA and cells expressing the Rab22 shRNA, with the Rab22 mRNA level in control cells normalized as 1. \*\* $p < 0.01$  (Student's *t* test). (B) Quantification of cell differentiation as percentage of transfected cells containing long neurites. Cells were transfected with the constructs expressing the scrambled shRNA, the Rab22 shRNA, or both Rab22 shRNA and human Rab22 (rescue), as indicated. The expression vector for the shRNAs simultaneously expresses DsRed, while the expression vector for human Rab22 simultaneously expresses eGFP. On NGF treatment (100 ng/ml), 500 transfected cells (with the expression of DsRed or both DsRed and GFP) were counted each day in each case, and the percentage of cells containing neurites was determined. Two-way ANOVA was performed (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ , vs. the vector control); error bars indicate SEM of three independent experiments. (C) Fluorescence microscopy images comparing NGF-induced neurite outgrowth at day 4 among cells expressing the scrambled shRNA, the Rab22 shRNA, or both Rab22 shRNA and human Rab22 (rescue), as indicated. Shown are representative images of the cells used for quantification of NGF-induced neurite outgrowth described in (B). Scale bar: 50  $\mu$ m.

### Rab22 essential for intracellular NGF signal transduction on endosomes

Neurite outgrowth in PC12 cells depends on NGF-mediated binding and activation/phosphorylation of the TrkA receptor and downstream signaling events leading to new gene expression. Endocytosis of the NGF-pTrkA complex into endosomes and continuous signaling on the endosomes is necessary for NGF-induced neurite outgrowth (Zhang *et al.*, 2000). When TrkA was overexpressed in PC12 cells, there was some degree of autoactivation but the activated pTrkA remained mostly at the plasma membrane, with little endocytosis and no intracellular localization on the early endosomes labeled by red fluorescent protein (RFP)-Rab22 (Figure 3A). In the absence of NGF, there was little neurite outgrowth (Figure S3). NGF treatment induced endocytosis of pTrkA into large endosomes that contained RFP-Rab22 (Figure 3, B and C), which correlated with a dramatic increase in neurite outgrowth (Figure S3). In contrast, EGF treatment had no effect on pTrkA endocytosis and neurite outgrowth (Figure S3).

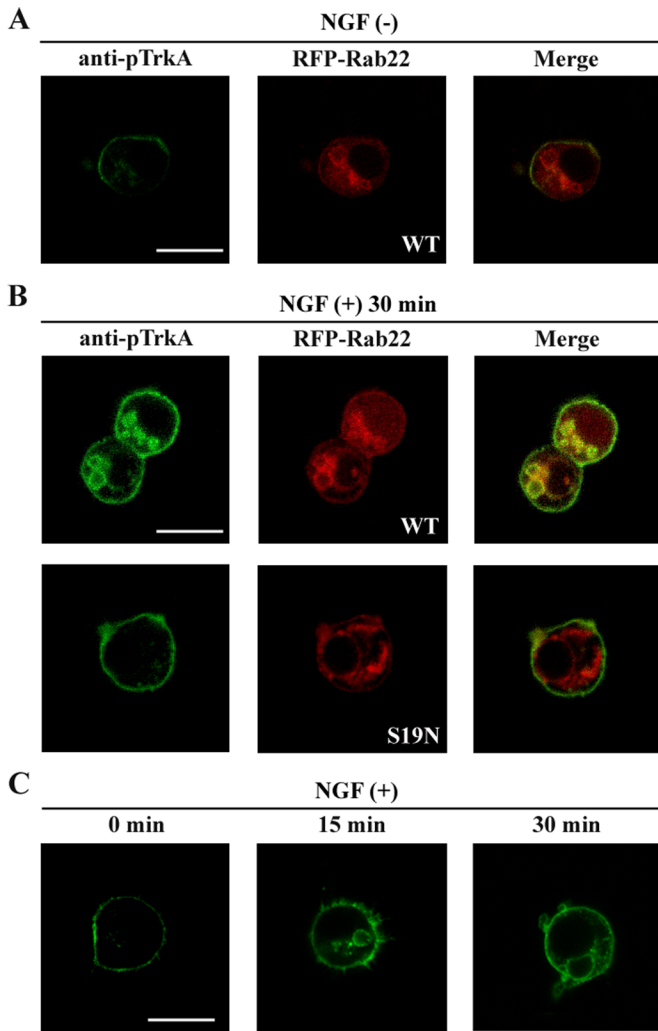
Inhibition of Rab22 function in cells expressing the dominant negative RFP-Rab22:S19N blocked NGF-induced endocytosis of pTrkA, which remained on the plasma membrane and was not found on the large endosomes (Figure 3B). There was no colocalization between pTrkA and RFP-Rab22:S19N in the cell (Figure 3B). Indeed,

RFP-Rab22:S19N inhibited pTrkA internalization into endosomes upon NGF treatment at all time points, including 5, 10, and 20 min as well as 30 min (Figure S4), consistent with a block of pTrkA endocytosis rather than faster recycling. Taken together with the inhibitory effect of Rab22:S19N on NGF-induced neurite outgrowth (Figure 1), the data suggested that the pTrkA level on Rab22-containing endosomes is important for intracellular signaling leading to neurite outgrowth. This contention was further supported by Rab22 knockdown experiments. Rab22 shRNA strongly inhibited endocytosis of pTrkA into the large endosomes, compared with control cells expressing a scrambled shRNA (Figure 4, A and B). The control cells showed increased pTrkA endocytosis into the endosomes over the time course of NGF treatment (up to nearly 30% of the cells; Figure 4), which correlated with the percentage of cells growing neurites in response to NGF (Figures 1 and 2). In contrast, Rab22 shRNA cells showed no response to NGF-induced pTrkA endocytosis, and the percentage of cells containing intracellular pTrkA staining remained at a low background level (<5%) over the time course of NGF treatment (Figure 4). On the other hand, the pTrkA staining was prominent on the plasma membrane in all cells upon NGF treatment (Figure 4A). The data from Rab22 knockdown are consistent with those from expression of the dominant negative Rab22:S19N mutant.

Furthermore, Rab22 knockdown essentially abolished NGF-induced VGF (nonacronymic) expression (Figure 5), a well-known NGF-inducible gene (Levi *et al.*, 1985). In this regard, cells transfected with the constructs expressing scrambled and Rab22 shRNAs were isolated by FACS based on the simultaneous expression of DsRed and used to determine the mRNA levels of VGF by qRT-PCR. Indeed, NGF treatment strongly stimulated VGF expression by over sixfold in control cells, but the NGF-stimulated VGF expression was blocked in cells expressing the Rab22 shRNA (Figure 5).

### Rab22-Rabex-5 interaction facilitates NGF-induced neurite outgrowth

Although Rab22 is closely related to Rab5 and Rab21, with nearly 50% amino acid sequence identity, Rab22 is distinguished from the latter two Rabs by its unique interaction profile with Rabex-5. Rabex-5 is a potent GEF for activation of Rab5 and Rab21, but not for Rab22 (Delprato *et al.*, 2004), instead it is an effector for Rab22 and favors binding to Rab22-GTP, which is responsible for recruiting Rabex-5 to the endosomes (Zhu *et al.*, 2009). To determine if the Rab22-Rabex-5 interaction is important for the Rab22 activity in NGF signaling and neurite outgrowth, we expressed full-length (FL) Rabex-5 and several truncation mutants of Rabex-5 in PC12 cells (Figure 6A) via the pBI/eGFP vector; the effects on NGF-induced neurite outgrowth were determined by fluorescence microscopy (Figure 6, B and C). Overexpression of FL Rabex-5 significantly



**FIGURE 3:** NGF-induced endocytosis of pTrkA into Rab22-containing early endosomes. (A) Confocal fluorescence microscopy showing the localization of pTrkA and RFP-Rab22 in PC12 cells, in the absence of NGF. Cells overexpressing TrkA and RFP-Rab22 were immunostained with the anti-pTrkA polyclonal antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 488. Scale bar: 12  $\mu$ m. (B) Confocal fluorescence microscopy showing colocalization of pTrkA with RFP-Rab22 on large early endosomes in PC12 cells, upon NGF treatment. Cells overexpressing TrkA and RFP-Rab22 (either WT or S19N, as indicated) were treated with NGF (100 ng/ml) for 30 min and then immunostained with the anti-pTrkA polyclonal antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 488, followed by confocal fluorescence microscopy. Scale bar: 12  $\mu$ m. (C) Confocal fluorescence microscopy showing NGF-induced endocytosis of pTrkA into large endosomes in PC12 cells. Cells overexpressing TrkA were treated with NGF for different times as indicated, and then immunostained with the anti-pTrkA polyclonal antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 488, as described above. Scale bar: 12  $\mu$ m.

enhanced neurite outgrowth, suggesting Rabex-5 plays a positive role in the process (Figure 6, B and C). Furthermore, the stimulatory activity of Rabex-5 in NGF-induced neurite outgrowth was independent of its GEF activity for Rab5 and Rab21, since the point mutant (D314A), which lacks the GEF activity (Delprato *et al.*, 2004), showed the same stimulatory effect as the FL Rabex-5 (Figure 6, B and C).

In contrast, the N-terminal fragment (residues 1–399), which contains the early endosomal targeting (EET) domain, which binds to Rab22, associates with early endosomes (Zhu *et al.*, 2009), and po-

tentially blocks endogenous Rab22–Rabex-5 interaction, inhibited NGF-induced neurite outgrowth (Figure 6, B and C). Indeed, overexpression of the EET domain (residues 80–231) itself dramatically inhibited the neurite outgrowth by 70% in comparison to the vector control (Figure 6, B and C), suggesting the EET domain may disrupt endogenous Rab22–Rabex5 interaction necessary for neurite outgrowth. In support of this contention, the C-terminal fragment (residues 135–492), which lacks an intact EET domain and the ability to bind Rab22 and is cytosolic (Zhu *et al.*, 2009), had no significant effect on NGF-induced neurite outgrowth in comparison to the vector control (Figure 6, B and C).

### Rabex-5 essential for NGF signaling and NGF-induced neurite outgrowth

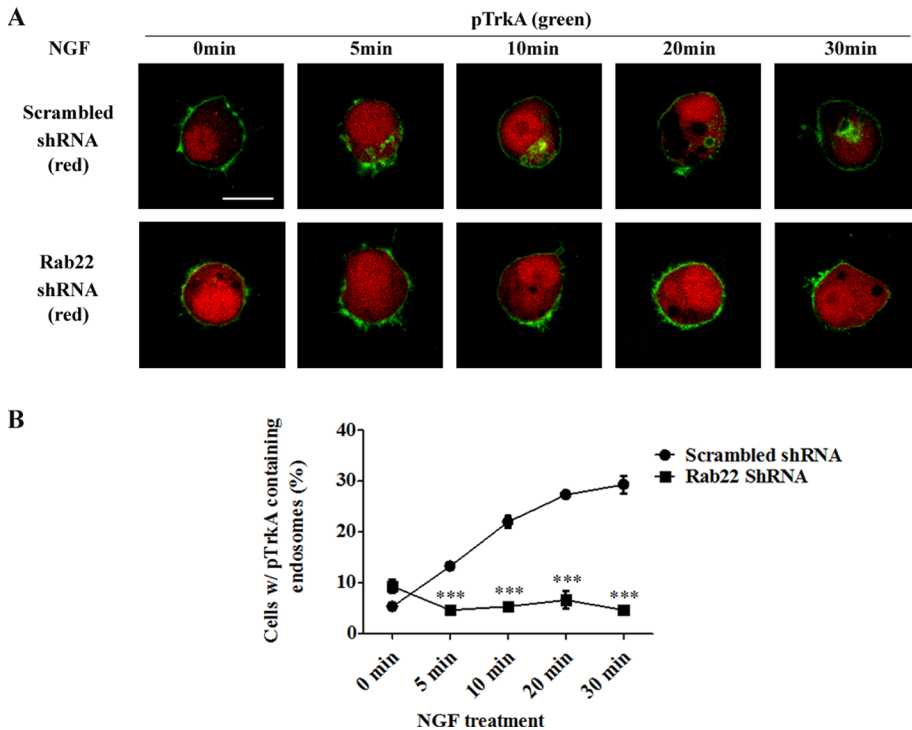
We further determined whether Rabex-5 knockdown via shRNA inhibits NGF-induced neurite outgrowth and gene expression in PC12 cells. The same vector for expressing Rab22 shRNA (Figure 2) was used to express a rat Rabex-5–specific shRNA and a control scrambled shRNA (Figure 7). To this end, the transfected cells were isolated by FACS based on their expression of DsRed, and mRNA levels of endogenous Rabex-5 were determined by qRT-PCR. The Rabex-5 shRNA effectively reduced endogenous Rabex-5 mRNA level by ~70%, in comparison to the control shRNA (Figure 7A). Another set of transfected cells was directly observed by fluorescence microscopy for neurite outgrowth upon NGF treatment. Like Rab22 shRNA, the Rabex-5 shRNA inhibited NGF-induced neurite outgrowth (Figure 7, B and C). Importantly, overexpression of bovine Rabex-5 completely rescued the inhibitory effect of the rat Rabex-5 shRNA and increased NGF-induced neurite outgrowth (Figure 7, B and C), suggesting the shRNA effect was due to specific Rabex-5 knockdown, rather than off-target effects.

Furthermore, additional cells expressing the control shRNA or the Rabex-5 shRNA were either treated with NGF or not treated, which was followed by FACS and qRT-PCR for analysis of the VGF mRNA levels in these cells. The NGF treatment strongly induced VGF expression in control cells expressing the scrambled shRNA, but this NGF-inducible VGF expression was significantly reduced in cells expressing the Rabex-5 shRNA (Figure 8).

### DISCUSSION

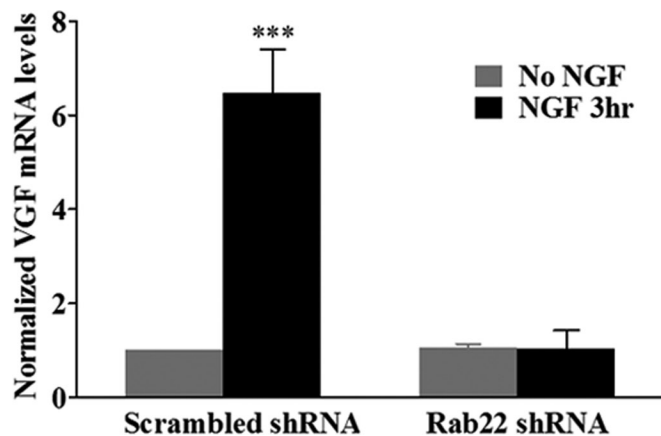
NGF initiates signal transduction at the plasma membrane via binding to the TrkA receptor and activating its tyrosine kinase activity, leading to phosphorylation of Tyr490 and Tyr785 in the cytoplasmic domain of TrkA that recruits signaling proteins to the membrane to start the signal transduction processes (Huang and Reichardt, 2003). However, the plasma membrane events are not sufficient to induce neurite outgrowth in PC12 cells (Zhang *et al.*, 2000). The NGF–pTrkA complex is endocytosed into signaling endosomes on which pTrkA continues to recruit signaling molecules to sustain signal transduction and gene expression, leading to neurite outgrowth and cell differentiation (Delcroix *et al.*, 2003; Kuruvilla *et al.*, 2004). Our data support this concept. Overexpression of TrkA leads to autophosphorylation, but the resulting pTrkA stays on the plasma membrane without ligand (NGF), and there is no endocytosis and no neurite outgrowth.

Our finding that NGF binding induces pTrkA endocytosis into Rab22-containing endosomes suggests an important role of Rab22 in the biogenesis and function of NGF-signaling endosomes. Indeed, Rab22 inhibition by shRNA or dominant negative mutant blocks the endocytosis of NGF–pTrkA into endosomes, which correlates with reduced neurite outgrowth and VGF expression. Consistently, overexpression of Rab22 facilitates NGF signaling and



**FIGURE 4:** Inhibitory effect of Rab22 knockdown on the endocytosis and localization of pTrkA on endosomes. (A) Confocal fluorescence microscopy showing pTrkA endocytosis in PC12 cells expressing the Rab22 shRNA (top panels) or the scrambled shRNA (bottom panels), in response to NGF treatment (100 ng/ml) for the indicated times. The vector expressing the shRNAs also expressed DsRed, and the cells were immunostained with the anti-pTrkA polyclonal antibody and goat anti-rabbit IgG conjugated to Alexa Fluor 488. Scale bar: 12  $\mu$ m. (B) Quantification of the effect of Rab22 shRNA on pTrkA endocytosis into intracellular endosomes. Fifty cells expressing the scrambled shRNA or the Rab22 shRNA described in (A) were counted to determine the percentage of cells containing pTrkA-positive endosomes. Two-way ANOVA was performed (\*\**p* < 0.001); error bars indicate SEM of triplicate samples.

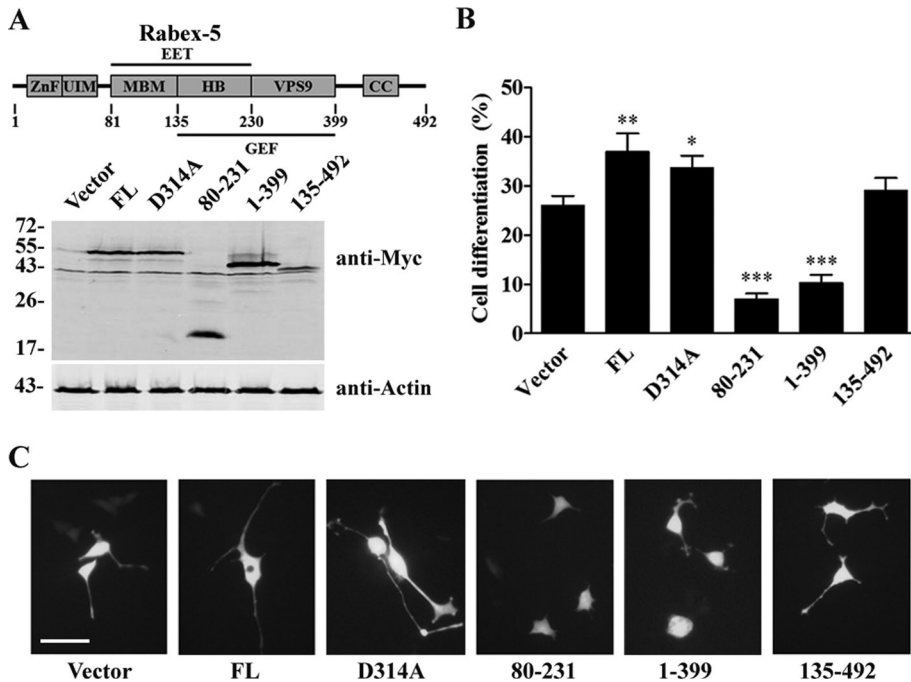
enhances the NGF-induced processes. The positive regulatory function of Rab22 in promoting the formation of pTrkA-containing signaling endosomes and NGF-induced neurite outgrowth and gene



**FIGURE 5:** Inhibitory effect of Rab22 knockdown on NGF-induced expression of VGF. Transfected cells expressing the scrambled shRNA or the Rab22 shRNA were either treated or not treated with NGF (100 ng/ml) for 3 h, as indicated, which was followed by isolation by FACS and measurement of VGF mRNA levels by qRT-PCR. The data are from triplicate samples and the VGF mRNA levels in control cells (scrambled shRNA) without NGF treatment were set as the base values. Two-way ANOVA was performed (\*\**p* < 0.001); error bars indicate SEM.

expression is in contrast to the negative effects of the other early endosomal Rab5 subfamily members (Rab5 and Rab21). Overexpression of Rab5 (Liu *et al.*, 2007) or Rab21 (this report) blocks NGF-induced neurite outgrowth, while inhibition of either Rab by dominant negative mutants enhances the NGF signaling and neurite outgrowth. Indeed, unlike Rab22:S19N, the dominant negative Rab5 mutant (Rab5:S34N) does not block NGF-induced pTrkA endocytosis (unpublished data), suggesting a Rab22-specific pathway for pTrkA endocytosis. Rab GTPases, in their active, GTP-bound form, are known to recruit fusion factors and organize membrane domains for fusion. The Rab5 membrane domain, in particular, concentrates PI3P, EEA1, and Rabenosyn-5 and promotes soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent early endosome fusion (Ohya *et al.*, 2009), which facilitates the transport of ligands and receptors along the endocytic pathway to late endosomes/lysosomes and degradation (Rink *et al.*, 2005). Like Rab5, Rab21 promotes the same pathway to late endosomes and lysosomes (Simpson *et al.*, 2004), and our data indicate that the Rab5- and Rab21-mediated endocytic pathway abrogates NGF-signaling endosomes and reduces neurite outgrowth.

While Rab5 is the prototype of the Rab5 subfamily in evolution and exists in all eukaryotes from yeast to man, Rab22 is the latest addition to the Rab5 subfamily and appears in metazoa and higher eukaryotes (Pereira-Leal and Seabra, 2001), consistent with its function in signaling endosomes for NGF and possibly for other factors regulating cell differentiation and development. Rab22 is closely related to Rab5 and Rab21, with nearly 50% amino acid sequence identity. Rab22 also interacts with the fusion effectors, such as EEA1 and Rabenosyn-5 (Kauppi *et al.*, 2002; Mishra *et al.*, 2010), which indicates Rab22 may compete with Rab5 for these fusion factors to form functional Rab22 membrane domain and promote early endosome fusion. However, the Rab22-mediated endosome fusion is functionally distinct from that of Rab5 and appears to sort endocytosed cargoes from both clathrin-dependent and clathrin-independent pathways to a slow recycling pathway (Weigert *et al.*, 2004; Magadan *et al.*, 2006), rather than to late endosomes and lysosomes. Indeed, Rab22 exhibits different effector interaction profiles from Rab5 and Rab21, in that Rab22 does not interact with APPL1 (Zhu *et al.*, 2007), which is a Rab5 effector along the endocytic pathway that promotes EGF signal transduction and cell growth (Miaczynska *et al.*, 2004), but utilizes Rabex-5 as an effector and recruits Rabex-5 to the Rab22-containing endosomes (Zhu *et al.*, 2009). Rabex-5 is a potent GEF for activation of Rab5 and Rab21, but not Rab22 (Horiuchi *et al.*, 1997; Delprato *et al.*, 2004). Although the Rab22-recruited Rabex-5 may contain GEF activity for Rab5 and Rab21, our data indicate the stimulatory activity of Rabex-5 in NGF-induced neurite outgrowth does not require GEF activity, as the GEF-deficient D314A mutant is fully active in this process. We speculate that Rab22-recruited Rabex-5 may sequester Rab5 on the membrane and converge the Rab domains;



**FIGURE 6:** Disruption of Rab22–Rabex-5 interaction blocks NGF-induced neurite outgrowth in PC12 cells. (A) Schematic structure of Rabex-5 and immunoblot showing the expressions of the Myc-tagged Rabex-5 proteins. The immunoblot was probed with anti-Myc and anti-actin antibodies. The actin level in each sample serves as a loading control. Molecular mass standards (in kDa) are indicated on the left. ZnF, Zinc finger; UIM, ubiquitin-interacting motif; MBM, membrane-binding motif; HB, helical bundle; Vps9, Vps9 domain; CC, coiled-coil. The EET and GEF domains are also indicated. (B) Percentage of differentiated cells with long neurites after NGF treatment for 4 d. The cells expressing the indicated Rabex-5 proteins were identified by simultaneous GFP expression from the vector via fluorescence microscopy, and the percentage of cells containing long neurites was determined from 500 cells in each case. One-way ANOVA was performed (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ); error bars indicate SEM of three independent experiments. (C) Fluorescence microscopy images of NGF-induced neurite outgrowth in cells expressing the indicated Rabex-5 proteins. Shown are representative images of the cells used for quantification of NGF-induced neurite outgrowth described in (B). Scale bar: 50  $\mu$ m.

the resulting complex likely exhibits distinct functions from the Rab5 domain itself. Furthermore, Rabex-5 as an effector is likely to recruit new signaling molecules to the Rab22 domain to facilitate NGF signaling and neurite outgrowth.

We find that the Rab22–Rabex-5 interaction is critical for NGF-induced neurite outgrowth and gene expression. Disruption of this interaction by overexpression of the Rabex-5 EET domain, which binds to Rab22 (Zhu *et al.*, 2009), blocks NGF-induced neurite outgrowth. Although our results do not rule out the possibility that the EET domain may affect other Rab22 interactions, the importance of Rabex-5 is substantiated by the shRNA-mediated knockdown data, indicating that Rabex-5 is necessary for NGF-induced neurite outgrowth and gene expression. The mechanism by which the Rab22–Rabex-5 interaction may contribute to the biogenesis and function of NGF-signaling endosomes is not immediately clear. An attractive possibility is linkage to Rab5 and Rab21 for sequestration of these two Rabs, which retards the entry to late endosomes and lysosomes or sorting to the Rab4-mediated fast-recycling pathway. As an effector for Rab22 and a GEF for Rab5, Rabex-5 is a linker between active Rab22-GTP and inactive Rab5-GDP and is necessary for the colocalization of Rab22 and Rab5 on the same endosomes, since Rabex-5-deficient NF73 cells from Rabex-5 knockout mice (Kalesnikoff *et al.*, 2007) exhibit separation of Rab22 and Rab5 into distinct populations of early endosomes (Zhu *et al.*, 2009). Early endosomes are

heterogeneous compartments marked by Rab5, Rab21, Rab22, or Rab4 or combinations of these Rabs, and the extent of colocalization of these Rab membrane domains depends on linker proteins such as Rabex-5, Rabaptin-5, and Rabenosyn-5 (de Renzis *et al.*, 2002). Our data suggest that the temporal convergence of the Rab22, Rab5, and Rab21 domains on early endosomes mediated by Rabex-5 may facilitate the biogenesis of NGF-signaling endosomes and NGF-induced cell differentiation.

## MATERIALS AND METHODS

### Recombinant protein expression

Rab22, Rab5, Rab21, and Rab31 used in this study were human Rabs with the National Center for Biotechnology Information (NCBI) accession numbers NM\_020673, NM\_004162, NM\_014999, and NM\_006868. Rabex-5 and TrkA were bovine and rat proteins, respectively, with the NCBI accession numbers NM\_174591 and NM\_021589.

### Antibodies

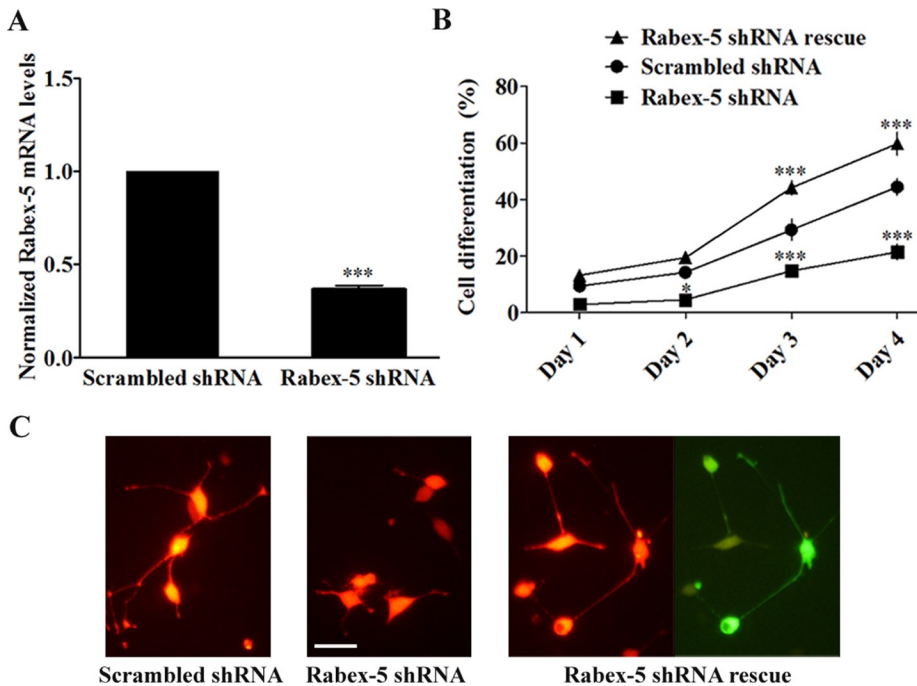
Monoclonal antibodies for actin and Myc were purchased from Sigma-Aldrich (St. Louis, MO), whereas polyclonal antibodies for Rab22 and phospho-TrkA (at Tyr490) were from ProteinTech Group (Chicago, IL) and Cell Signaling Technology (Beverly, MA), respectively.

### Cell cultures and transfection

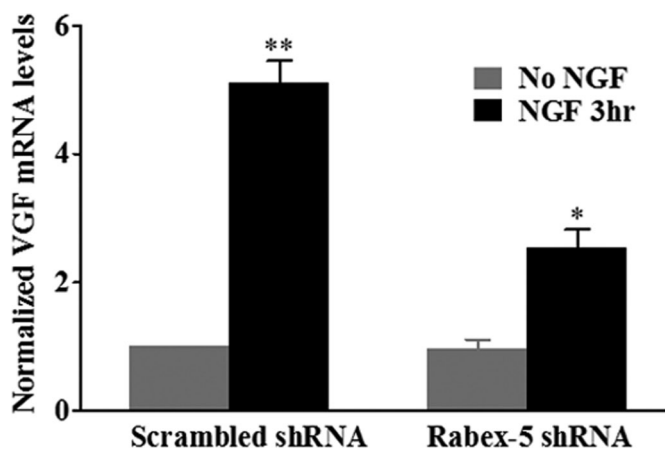
Tet-Off PC12 cells (Clontech, Mountain View, CA) were grown and transfected as described previously (Liu *et al.*, 2007). Briefly, cells were grown in 35-mm poly-lysine-coated culture dishes in DMEM containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 20 U/ml penicillin/streptomycin, 1 mM L-glutamine, and 200  $\mu$ g/ml Geneticin (G-418; Invitrogen, Carlsbad, CA). Cells were seeded at a density of  $2 \times 10^5$  cells/dish and incubated at 37°C in a humidified incubator with 10% CO<sub>2</sub> for 24 h unless otherwise indicated. Transfection of the cells with plasmid constructs expressing the recombinant proteins was conducted by using Lipfectamine 2000 (Invitrogen). All media and reagents for the cell culture were purchased from Invitrogen.

### Neurite outgrowth assay

The assay was slightly modified from a previous procedure (Liu *et al.*, 2007). After transfection, the cells were allowed to recover in full growth medium and to express the recombinant proteins for 24 h unless otherwise indicated. The growth medium was then replaced with DMEM containing only 1% horse serum and 100 ng/ml NGF. The cells were maintained at 37°C for 4 d, with NGF replenishment at day 2. Neurite outgrowth in transfected cells, which expressed GFP or RFP, was observed each day with an inverted fluorescence microscope (Nikon Daphot 300, Tokyo, Japan) and the images were stored in a connected computer and analyzed with Nikon ACT-1 software. Differentiated cells were defined as those containing at least one neurite twice as long as the cell body diameter and quantified as percentage of the transfected cells. In each case,



**FIGURE 7:** Inhibitory effect of Rabex-5 knockdown on NGF-induced neurite outgrowth in PC12 cells. (A) qRT-PCR results comparing the mRNA levels of Rabex-5 between control cells expressing the scrambled shRNA and cells expressing the Rabex-5 shRNA, with the Rabex-5 mRNA level in control cells normalized as 1. \*\*\* $p < 0.001$  (Student's *t* test). (B) Quantification of cell differentiation as percentage of transfected cells containing long neurites. Cells were transfected with the constructs expressing the scrambled shRNA, the Rabex-5 shRNA, or both Rabex-5 shRNA and bovine Rabex-5 (rescue), as indicated. The expression vector for the shRNAs simultaneously expresses DsRed, while the expression vector for bovine Rabex-5 simultaneously expresses eGFP. On NGF treatment (100 ng/ml), 500 transfected cells (with the expression of DsRed or both DsRed and GFP) were counted each day in each case, and the percentage of cells containing neurites was determined by fluorescence microscopy. Two-way ANOVA was performed (\* $p < 0.05$ , \*\*\* $p < 0.001$ , vs. the vector control); error bars indicate SEM of three independent experiments. (C) Fluorescence microscopy images comparing NGF-induced neurite outgrowth at day 4 among cells expressing the scrambled shRNA, the Rabex-5 shRNA, or both Rabex-5 shRNA and bovine Rabex-5 (rescue), as indicated. Shown are representative images of the cells used for quantification of NGF-induced neurite outgrowth described in (B). Scale bar: 50  $\mu$ m.



**FIGURE 8:** Inhibitory effect of Rabex-5 knockdown on NGF-induced expression of VGF. (A) Transfected cells expressing the scrambled shRNA or the Rabex-5 shRNA were either treated with NGF (100 ng/ml) for 3 h or not treated, as indicated, which was followed by FACS and qRT-PCR for measurement of VGF mRNA levels. The data are from triplicate samples, and the VGF mRNA levels in control cells (scrambled shRNA) without NGF treatment were set as the base values. Two-way ANOVA was performed (\* $p < 0.05$ , \*\* $p < 0.01$ ); error bars indicate SEM.

500 transfected cells were counted and the SEM was calculated based on the results of at least three independent experiments.

### Confocal fluorescence microscopy

We used a Leica SP2 MP confocal laser scanning microscope (Buffalo Grove, IL) in the Flow and Image Cytometry Laboratory (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Tet-Off PC12 cells were grown on poly-lysine-coated coverslips in 35-mm culture dishes for 24 h, and then transfected with the expression constructs as indicated. At 48 h posttransfection, the cells were treated with 100 ng/ml NGF for different lengths of time and were processed for confocal fluorescence microscopy. To this end, cells were rinsed three times with phosphate-buffered saline (PBS) and fixed for 15 min with 4% paraformaldehyde (wt/vol in PBS) at 37°C, and then permeabilized with 0.05% saponin (wt/vol in PBS) for 15 min at room temperature. The cells were then stained with the anti-pTrkA antibody that specifically recognizes the cytoplasmic domain of activated TrkA and a fluorescent secondary antibody (goat anti-rabbit IgG conjugated to Alexa Fluor 488 [Invitrogen]). The coverslips were then mounted on glass slides in anti-fade reagent (Invitrogen) and viewed with the confocal microscope.

### Immunoblot analysis

Cells were lysed in 1% SDS (125  $\mu$ l per 35-mm dish) and the lysates were sheared to reduce stickiness by passing through a 26-gauge needle five times with a 1-ml syringe, followed by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA), probed with the indicated primary antibodies, and incubated with IRDye 800CW secondary antibodies, after which they were visualized and quantified with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), according to the manufacturer's instructions.

### shRNA-mediated inhibition of Rab22 and Rabex-5

The expression of Rab22 shRNAs using the pSIREN-RetroQ-DsRed-Express vector (Clontech) was previously described (Zhu *et al.*, 2009). We used the same vector to express rat-specific shRNAs to knock down endogenous Rab22 or Rabex-5 in the PC12 cells. The simultaneous expression of DsRed from the vector allowed identification of the transfected cells by fluorescence microscopy or FACS. A number of targeting sequences were identified for rat Rab22 and Rabex-5 by the Clontech RNAi designer website (<http://bioinfo.clontech.com/rnaidesigner/frontpage.jsp>), and two of these were selected for each gene: 5'-AGACAGCCGTCGGAGCCAAAG-3' and 5'-AAGGACTACGCCGATTCCATT-3' for Rab22; 5'-GGTGTAGCAAGCACATCTT-3' and 5'-GGGGAATTCATGTGGATCA-3' for Rabex-5. Accordingly, complementary pairs of oligonucleotides for corresponding shRNAs were made, annealed, and cloned into the

vector for expression of shRNAs targeting the aforementioned Rab22 and Rabex-5 sequences. A pair of oligonucleotides designed against a scrambled sequence (5'-GCAGAGCAACCA-GAGTTCTAC-3') were also annealed and cloned into the vector for expression of a negative control shRNA. The resulting constructs expressing the shRNAs were then transfected into the PC12 cells and tested for their effectiveness in reducing the levels of Rab22 and Rabex-5; this was accomplished by isolation of the transfected PC12 cells via FACS and analysis of the mRNA levels of Rab22 and Rabex-5 via qRT-PCR. The two shRNAs for each gene were both effective in knocking down the respective gene expression, although only the results for one of the shRNAs (the first one) for each gene are shown in Figures 2, 4, 5, 7, and 8.

### qRT-PCR

Using FACS, transfected PC12 cells were isolated based on their expression of GFP or RFP or both. To this end, the cells were trypsinized, resuspended in the growth medium, centrifuged at  $400 \times g$  for 5 min. The cell pellets were resuspended in Hank's balanced salt solution containing 0.1% bovine serum albumin without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and phenol red, and the transfected cells were isolated with an Influx Cell Sorter in the Flow and Image Cytometry Laboratory. Total RNAs were extracted from the sorted cells by using the PureLink RNA kit (Invitrogen) and were used as the template for the synthesis of cDNAs by using the Advantage RT-for-PCR kit (Clontech). This was followed by two-step real-time PCR to determine relative mRNA levels of the indicated proteins using an Eppendorf RealPlex-4 Mastercycler and Power SYBR Green PCR Mastermix (Applied Biosystems, Bedford, MA), with specific sets of oligonucleotide primers for rat GAPDH, VGF, Rab22, and Rabex-5. The mRNA levels of GAPDH served as internal controls for normalization; statistical analysis was performed with triplicate samples in at least two independent experiments (Pfaffl, 2001). The results were confirmed with a second pair of primers for each of the genes.

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