Rabin8 regulates neurite outgrowth in both GEF activity–dependent and –independent manners

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ABSTRACT **Many aspects of membrane-trafficking events are regulated by Rab-family small GTPases. Neurite outgrowth requires massive addition of proteins and lipids to the tips of growing neurites by membrane trafficking, and although several Rabs, including Rab8, Rab10, and Rab11, have been implicated in this process, their regulatory mechanisms during neurite outgrowth are poorly understood. Here, we show that Rabin8, a Rab8-guanine nucleotide exchange factor (GEF), regulates nerve growth factor (NGF)–induced neurite outgrowth of PC12 cells. Knockdown of Rabin8 results in inhibition of neurite outgrowth, whereas overexpression promotes it. We also find that Rab10 is a novel substrate of Rabin8 and that both Rab8 and Rab10 function during neurite outgrowth downstream of Rabin8. Surprisingly, however, a GEF activity–deficient isoform of Rabin8 also promotes neurite outgrowth, indicating the existence of a GEF activity–independent role of Rabin8. The Arf6/Rab8-positive recycling endosomes (Arf6/Rab8-REs) and Rab10/Rab11-positive REs (Rab10/Rab11-REs) in NGF-stimulated PC12 cells are differently distributed. Rabin8 localizes on both RE populations and appears to activate Rab8 and Rab10 there. These localizations and functions of Rabin8 are Rab11 dependent. Thus Rabin8 regulates neurite outgrowth both by coordinating with Rab8, Rab10, and Rab11 and by a GEF activity–independent mechanism.**

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INTRODUCTION

In eukaryotic cells, various proteins and lipids are distributed to their proper subcellular locations by an intracellular transport system, the so-called membrane trafficking system, many aspects of which are regulated by Rab-family small GTPases (Fukuda, 2008; Stenmark, 2009; Hutagalung and Novick, 2011; Wandinger-Ness and Zerial, 2014). As with other small GTPases, Rabs switch between an active, GTP-bound state and an inactive, GDP-bound state with the help of guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In their active form, Rabs are localized on specific intracellular membranes and recruit their effector proteins there to regulate various steps in membrane trafficking, such as budding, transport, tethering, and fusion of vesicles or organelles with target membranes. In the final step of membrane trafficking, Rabs must be inactivated and detached from the vesicle/organelle membrane and from their effectors, and they are then recycled back to the cytosol. Thus knowing where and when Rabs are activated by GEFs and inactivated by GAPs is crucial to better understanding the functions of each Rab family member.

One of the >60 mammalian Rabs, Rab8 (an orthologue of yeast Sec4p) regulates post-Golgi trafficking and endocytic recycling (Peränen, 2011). Rabin8 is known to be a Rab8-GEF that interacts with GDP-Rab8 through an evolutionarily conserved Sec2 domain and then activates it (Hattula *et al.*, 2002). Rabin8 also binds to GTP-Rab11 through its C-terminal region (Knödler *et al.*, 2010; Vetter *et al.*, 2015) and, by so doing, is believed to mediate the conversion from Rab11-positive membranes to Rab8-positive membranes. Rabin8 has been shown to regulate endosomal trafficking during primary ciliogenesis and epithelial polarization through such a "Rab cascade" mechanism (a cascade from Rab11 to Rab8; Nachury *et al.*, 2007; Knödler *et al.*, 2010; Bryant *et al.*, 2010), but it is unknown whether such a Rab cascade mechanism operates in all Rab8-dependent membrane-trafficking events, such as, for example, neurite outgrowth (Huber *et al.*, 1995).

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Abbreviations used: CA/CN, constitutively active/negative; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; KD, knockdown; MeOH, methanol; NGF, nerve growth factor; RE, recycling endosome; RT, reverse transcription; shRNA, short hairpin RNA; siRNA, small interfering RNA; SR, siRNA/shRNA-resistant; TCA, trichloroacetic acid.

When neurons differentiate, they extend long neurites from their cell bodies that connect with each other and ultimately establish neuronal networks. Because this process requires a great expansion of the plasma membrane area that depends on a continuous supply of membrane proteins and lipids into the neurites, in addition to remodeling of the cytoskeleton (Pfenninger, 2009), regulation of membrane trafficking must be closely coupled to neurite outgrowth, and, in fact, several Rab family members have been implicated in neurite outgrowth (reviewed in Villarroel-Campos *et al.*, 2014). For example, Rab10, together with its effectors myosin Vb and JIP1, regulates post-Golgi trafficking of plasmalemmal precursor vesicles that are eventually added to neurite tips (Wang *et al.*, 2011; Liu *et al.*, 2013; Deng *et al.*, 2014). Rab8, Rab13, Rab35, and Rab36 localize on recycling endosomes (REs) and are believed to regulate membrane trafficking through this compartment (Huber *et al.*, 1995; Sakane *et al.*, 2010; Kobayashi and Fukuda, 2012; Kobayashi *et al.*, 2014). Rab11 also regulates endocytic recycling through REs and is particularly implicated in the targeting of integrins and Trk receptors to the neurite tips, which is required for adhesion and signal transduction during neurite outgrowth (Ascaño *et al.*, 2009; Eva *et al.*, 2010). However, how these Rabs are regulated by upstream signals and how they cross-talk with each other during neurite outgrowth are not well understood.

In this study, we investigated the involvement of Rabin8 in neurite outgrowth and especially focused on its GEF activity and Rab11-binding activity. The results showed that knockdown of Rabin8 resulted in inhibition of NGF-induced neurite outgrowth of PC12 cells, whereas overexpression of Rabin8 promoted it. We also discovered that Rab10 is a novel substrate for the GEF activity of Rabin8 and that both Rab8 and Rab10 function in neurite outgrowth downstream of Rabin8, even though they are differently distributed in NGF-stimulated PC12 cells. However, we were surprised to find that a GEF activity–deficient isoform of Rabin8 that is endogenously expressed in mouse brain had the ability to promote neurite outgrowth and that the GEF activity–independent role of Rabin8 requires its Rab11-binding activity. Our findings indicate that Rabin8 regulates neurite outgrowth not only by coordinating with Rab8, Rab10, and Rab11 but also by a Rab11-dependent and GEF activity–independent pathway.

RESULTS

Rabin8 has GEF activity toward both Rab8 and Rab10

To investigate the role of Rabin8, we first performed a comprehensive assay for binding between Rabin8 and 60 different mammalian Rabs by a yeast two-hybrid system that we had established previously (Fukuda *et al.*, 2008, 2011). Consistent with the findings in previous studies, the results showed that Rabin8 binds to a constitutively active (CA) form of Rab11 and to constitutively negative (CN) forms of Rab3 and Rab8 (Figure 1B). Despite its interaction with both GDP-Rab3 and GDP-Rab8 through its Sec2 domain, Rabin8 has been shown to activate Rab8 alone and not to activate Rab3 (Hattula *et al.*, 2002). Because the results of our assay also revealed a previously uncharacterized Rabin8–Rab interaction, that is, a Rabin8–Rab10(CN) interaction (Figure 1B), we next attempted to determine whether Rabin8 actually has GEF activity toward Rab10, as well as to Rab8, by performing GEF activity assays in cells. In brief, after coexpressing FLAG-Rab8A or FLAG-Rab10 and green fluorescent protein (GFP) or GFP-Rabin8 in COS-7 cells, we pulled down the GTP-bound FLAG-Rab8A or FLAG-Rab10 with a C-terminal fragment of MICAL-L2 (named MICAL-L2 C), which preferentially binds to the active forms of Rab8, Rab10, Rab13, and Rab15 (Fukuda *et al.*, 2008). When coexpressed with GFP-Rabin8, FLAG-Rab8A was pulled down more than when coexpressed with GFP alone (Figure 1C), confirming that the level of GTP-Rab8A was dramatically increased by Rabin8. Similarly, we detected robust activation of Rab10 in cells when coexpressed with Rabin8 (Figure 1D), indicating that Rabin8 activates both Rab8 and Rab10 in living cells. The activation of Rab10 by Rabin8 is unlikely to occur as a side effect of Rab8 activation because Rabin8 can activate Rab10 even in Rab8-depleted cells (Figure 1E).

To determine whether the Rab10-GEF activity of Rabin8 is attributable to its Sec2 domain, we used a splicing isoform of mouse Rabin8 that lacks 32 amino acids (corresponding to exon 4) in the Sec2 domain (hereafter referred to as Rabin8 ΔGEF; Figure 1A) as a negative control. The results of reverse transcription (RT)–PCR and immunoblot analyses indicated that this isoform was naturally and abundantly expressed in the mouse brain (Supplemental Figure S1). In contrast to full-length Rabin8, Rabin8 ΔGEF was unable to interact with Rab3(CN) and Rab8(CN), although it still exhibited normal binding activity toward Rab11(CA) (Figure 1B). Moreover, no increase in the level of GTP-Rab8A or GTP-Rab10 was observed when Rabin8 ΔGEF was overexpressed (Figure 1, C and D), indicating that an intact Sec2 domain is essential to exerting of GEF activity toward Rab8 and Rab10.

Both Rab8 and Rab10 are involved in neurite outgrowth of PC12 cells

Because Rab8 and Rab10 are required for neurite outgrowth in rat hippocampal neurons (Huber *et al.*, 1995; Wang *et al.*, 2011) and Rabin8 is capable of activating both Rabs (Figure 1, C and D), we hypothesized that Rabin8 was also involved in this process. We used NGF-differentiated PC12 cells as a model of neurite outgrowth (Kobayashi and Fukuda, 2012) to test our hypothesis. In brief, PC12 cells were exposed to 100 ng/ml NGF for 36 h, and after fixation with paraformaldehyde, total neurite length in each cell was measured. The average neurite length of the cells that had been transfected with control vectors or a control small interfering RNA (siRNA) was usually ~50 μm, whereas the cells treated with siRab8A/B or siRab10 had significantly shorter neurites (Figure 2A). The effects of Rab8A/B depletion and Rab10 depletion were clearly rescued by reexpression of siRNA-resistant (SR) Rab8A and Rab10, respectively (Supplemental Figure S2A), although the rescue effect by GFP-^{SR}Rab10 was relatively weak. This weak rescue effect was likely to have been caused by N-terminal GFP fusion, which partially disturbs the function of Rab10 in neurite outgrowth, because nontagged ^{SR}Rab10 almost fully rescued the phenotype (Supplemental Figure S2B). We also investigated the effect of overexpression of Rab8 and Rab10 mutants. When a CN form of Rab8A (Rab8A(T22N)) or Rab10 (Rab10(T23N)) was expressed in PC12 cells, neurite outgrowth was significantly inhibited. The opposite effect (i.e., promotion of neurite outgrowth) was observed when a CA form of Rab8A (Rab8A(Q67L)) was expressed, but expression of Rab10 (Rab10(Q68L)) had no effect (Figure 2B). These results indicated that both Rab8 and Rab10 are involved in neurite outgrowth of PC12 cells, just as they are in rat hippocampal neurons.

Next we attempted to identify the membrane compartment on which Rab8 and Rab10 localize. We previously demonstrated differing distributions of Arf6-positive REs, on which Rab8 also localizes (named Arf6/Rab8-REs), and Rab11-positive REs (named Rab11-REs) in NGF-stimulated PC12 cells (Kobayashi and Fukuda, 2013): the Arf6/Rab8-REs are concentrated in an area close to the centrosome (Figure 2C, top left, red) and surrounded by the Rab11-REs, and there is only partial colocalization (Figure 2C, top left, green). Intriguingly, the same segregation was also seen between Rab8 and

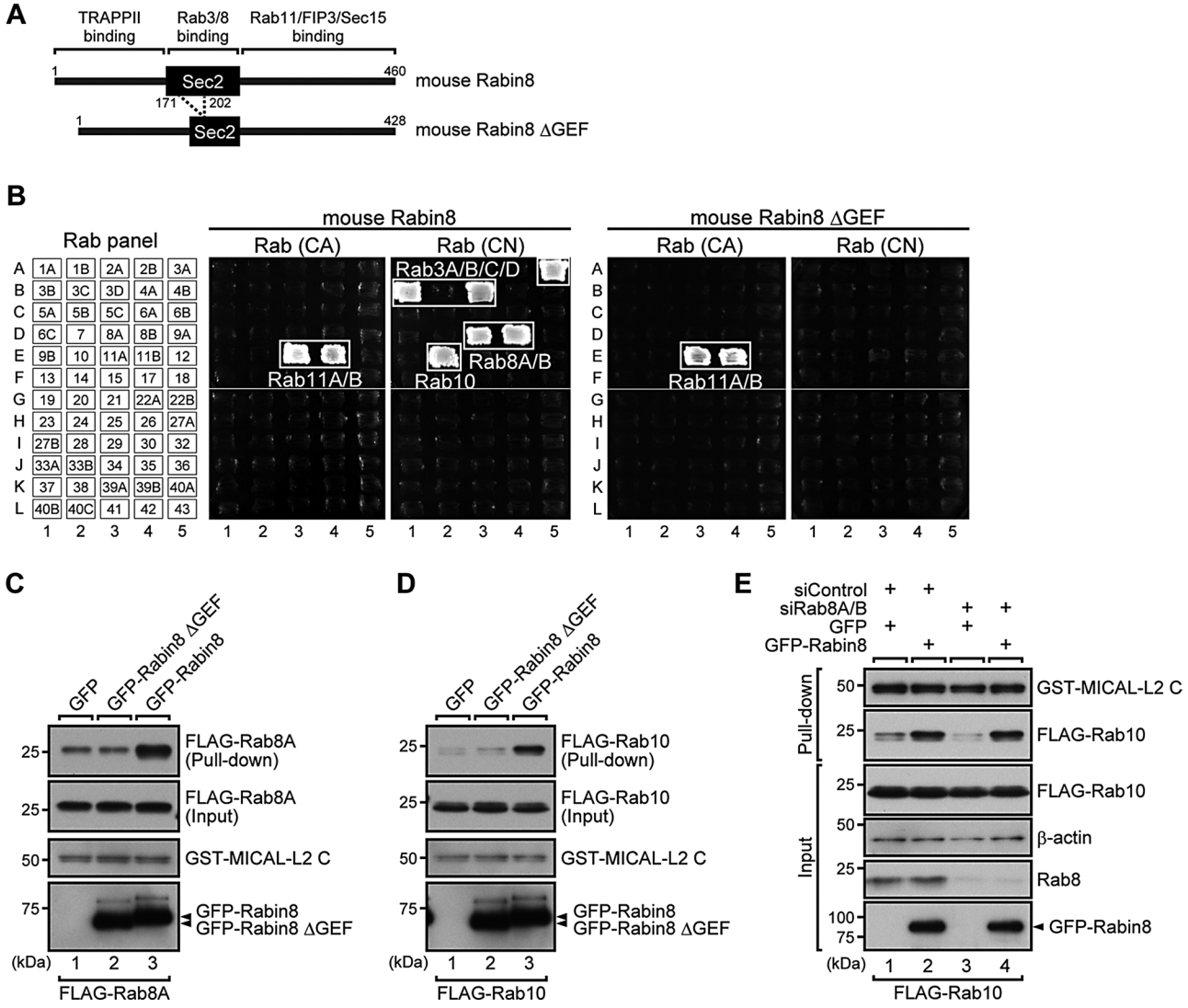

FIGURE 1: Rabin8 has GEF activity toward Rab8 and Rab10. (A) Domain organization of mouse Rabin8 protein. Rabin8 contains a Sec2 homology domain (black boxes) in the middle of the molecule. Molecules that have been shown to bind to Rabin8 (TRAPPII, Rab3/8/11, FIP3, and Sec15) are summarized at the top. Rabin8 ΔGEF is a mouse brain–specific alternative splicing isoform of Rabin8 (Supplemental Figure S1). (B) Rab-binding specificity of Rabin8 as revealed by yeast two-hybrid panels (Fukuda *et al.*, 2008, 2011). Yeast cells containing pGBD-C1 plasmid expressing CA (constitutively active; mimics a GTP-fixed form) or CN (constitutively negative; mimics a GDP-fixed form) mutants of Rab (positions indicated on the left) and pGAD-C1 plasmid expressing mouse Rabin8 or mouse Rabin8 ΔGEF were streaked on SC-AHLW and incubated at 30°C for 1 wk. Positive patches are boxed. (C, D) Overexpression of Rabin8, but not Rabin8 ΔGEF, activates both Rab8A and Rab10 in living cells. COS-7 cells were transfected with plasmids encoding GFP, GFP-Rabin8 ΔGEF, or GFP-Rabin8 in combination with pEF-FLAG-Rab8A or -FLAG-Rab10. The cells were lysed 24 h after transfection, and GTP-bound FLAG-Rab8A or FLAG-Rab10 was pulled down with the GST-tagged C-terminal region of MICAL-L2 (GST-MICAL-L2 C; Fukuda *et al.*, 2008). The precipitated samples were analyzed by immunoblotting with the antibodies indicated. (E) Overexpression of Rabin8 activates Rab10 even in Rab8A/B-depleted cells. 293T cells were transfected with siControl or human siRab8A/B and cultured for 24 h. The cells were then replated and cotransfected with plasmids encoding GFP or GFP-Rabin8 and pEF-FLAG-Rab10. The cells were lysed 24 h after transfection, and GTP-bound FLAG-Rab10 was pulled down with GST-MICAL-L2 C. The precipitated samples were analyzed by immunoblotting with the antibodies indicated.

Rab10, that is, Rab10 surrounded a Rab8 area just as Rab11 did (Figure 2C, right). Because Rab10 colocalized well with Rab11 (Figure 2C, bottom left), Rab10 seemed likely to reside on the Rab11-REs rather than on the Arf6/Rab8-REs. The fact that inhibition of either Rab8 or Rab10 alone can impair neurite outgrowth and that they reside on different populations of REs suggested that these two Rabs play independent roles during neurite outgrowth.

Rabin8 localizes on both Arf6/Rab8- and Rab11-positive REs

We next investigated the localization of Rabin8 by performing an immunofluorescence analysis. Consistent with the results of a previous study that showed GFP-Rabin8 localization on Rab11-positive endosomes (Westlake *et al.*, 2011), endogenous Rabin8 was present in the perinuclear region and colocalized with Rab11 (Figure 3A, top). However, although Rab11 exhibited a ring-like distribution,

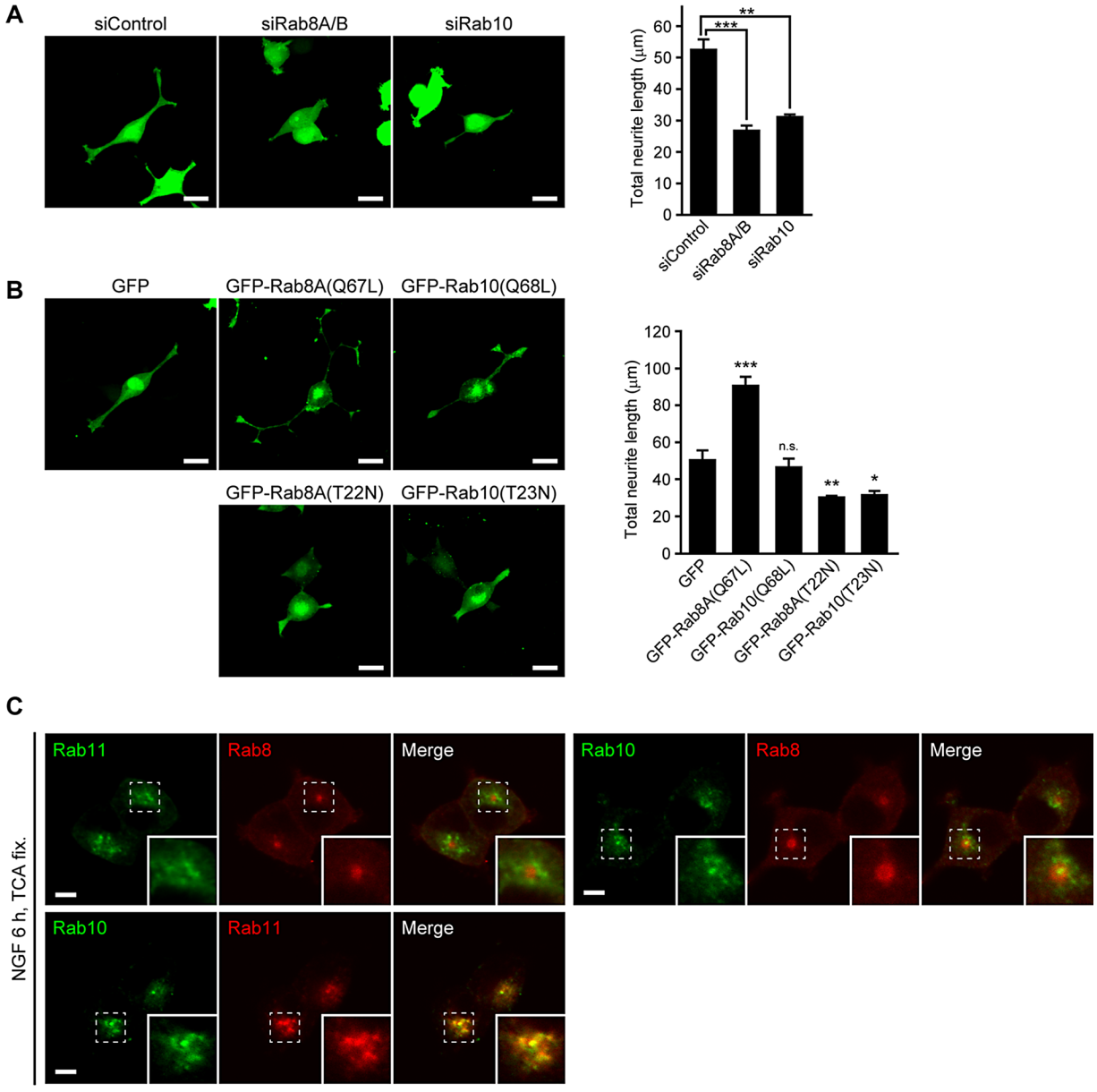

FIGURE 2: Involvement of Rab8 and Rab10 in neurite outgrowth of PC12 cells. (A) Knockdown of Rab8 and Rab10 resulted in inhibition of neurite outgrowth. PC12 cells were transfected with siControl, siRab8A+8B, or siRab10 in addition to pEGFP-C1 as a transfection marker. The cells were cultured for 48 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Left, typical images of the GFP-positive cells. Scale bars, 20 μm. Right, graph comparing the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{**}p < 0.01$; $^{***}p < 0.001$ ($n = 3$; Dunnett's test). (B) Effect of overexpression of CA and CN forms of Rab8 and Rab10 on neurite outgrowth. PC12 cells were transfected with plasmids encoding GFP, GFP-Rab8A(Q67L), GFP-Rab10(Q68L), GFP-Rab8A(T22N), or GFP-Rab10(T23N). The cells were cultured for 24 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Left, typical images of the GFP-positive cells. Scale bars, 20 μm. Right, graph comparing the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s. (not significant), $p > 0.05$ ($n = 3$; Dunnett's test). (C) Localization of endogenous Rab8, Rab10, and Rab11 in NGF-stimulated PC12 cells. PC12 cells were exposed to NGF for 6 h, and after fixation with TCA, they were immunostained with anti-Rab8 (red), anti-Rab10 (green), and anti-Rab11 (green, top left; red, bottom left) antibodies. Insets, magnified views of the boxed area. Scale bars, 5 μm.

being mostly absent from the Arf6/Rab8-RE–positive area, Rabin8 was also found in the area where Rab11 was excluded. When Rabin8 was costained with Rab8, Rabin8 staining overlapped well with Rab8 staining in the Arf6/Rab8-RE area but was also observed in the surrounding area (Figure 3A, bottom). These results indicated that Rabin8 localizes on both RE populations, those of Rab11-RE and those of Arf6/Rab8-RE.

During the course of testing different fixation methods in an attempt to achieve clear immunostaining of Rabin8 in PC12 cells, we discovered that methanol (MeOH) fixation emphasized Rabin8 signals at Arf6/Rab8-REs but that no Rabin8 signals were seen at the Rab11-REs (Figure 3B). We do not know why the Rab11-RE–localized Rabin8 signals in the MeOH-fixed cells failed to appear. However, because the Rab10 and Rab11 signals were also dramatically reduced

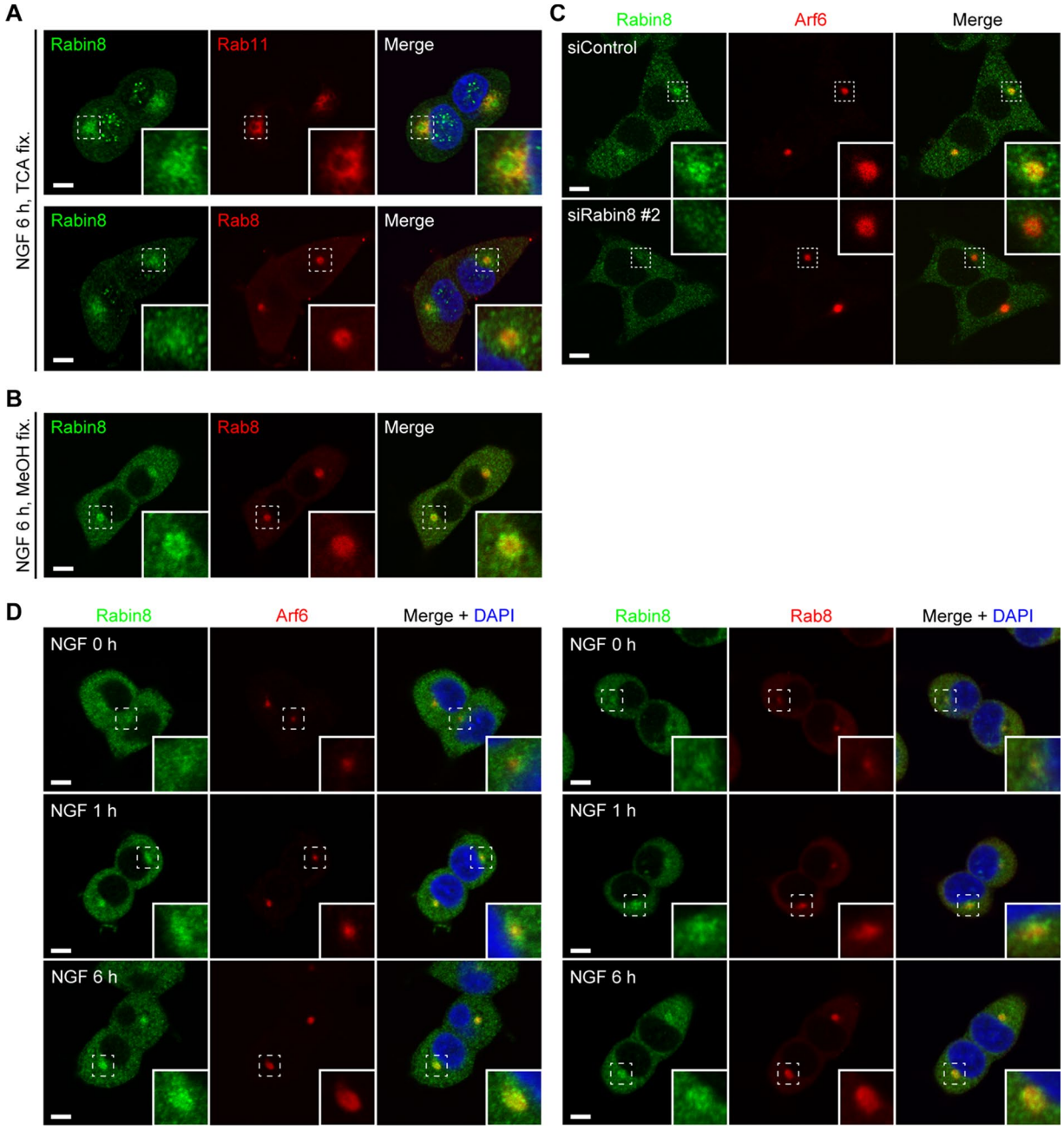

FIGURE 3: Endogenous Rabin8 localizes on both Arf6/Rab8- and Rab11-positive REs in PC12 cells. (A) Rabin8 colocalized with both Rab8 and Rab11 in TCA-fixed cells. PC12 cells were exposed to NGF for 6 h, fixed with TCA, and then immunostained with anti-Rabin8 (green), anti-Rab8 (red, bottom), and anti-Rab11 (red, top) antibodies. Scale bars, 5 μm. (B) Rabin8 colocalized with Rab8 in the MeOH-fixed cells. PC12 cells were exposed to NGF for 6 h, fixed with MeOH, and then immunostained with anti-Rabin8 (green) and anti-Rab8 (red) antibodies. In contrast to TCA fixation, MeOH fixation was followed by weaker Rabin8 signals, which overlapped with Rab11 in the TCA-fixed cells (A), and as a result, the pericentrosomal Rabin8 signals, which overlapped with Arf6 (C), were more prominent in the MeOH-fixed cells. Scale bars, 5 μm. (C) Rabin8 colocalized with Arf6 in the MeOH-fixed cells. PC12 cells were transfected with siControl or siRabin8. The cells were then cultured for 72 h, exposed to NGF for 6 h, and, after fixation with MeOH, immunostained with anti-Rabin8 (green) and anti-Arf6 (red) antibodies. Scale bars, 5 μm. Note the dramatically weaker perinuclear Rabin8 signals in the siRabin8-treated cells. (D) Rabin8 accumulated at the Arf6/Rab8-REs in an NGF-stimulation-dependent manner. PC12 cells were cultured for 24 h and exposed to NGF for 0, 1, or 6 h. The cells were fixed with MeOH and then immunostained with anti-Rabin8 (green) and anti-Rab8 (red, left) or anti-Arf6 (red, right) antibodies. Scale bars, 5 μm. Insets, magnified views of the boxed areas.

in the MeOH-fixed cells (unpublished data), one possible explanation is that the structure of Rab11-RE is not preserved by MeOH fixation, whereas another may simply be the nature of the polyclonal antibody against Rabin8 that we used (e.g., because a certain epitope of Rabin8 at the Rab11-REs may be more sensitive to MeOH). However, the latter possibility is most unlikely because immunostaining for

GFP-Rabin8 using an anti-GFP antibody also exhibited an immunostaining pattern similar to that of endogenous Rabin8 (Supplemental Figure S3A). In any case, we decided to use the MeOH fixation method to specifically monitor Rabin8 signals in the pericentrosomal area. Consistent with our findings described earlier, Rabin8 colocalized with Arf6 even in MeOH-fixed cells and the Rabin8 staining was dramatically reduced in the siRabin8-treated cells (Figure 3C), indicating that the staining is not nonspecific. In addition, no Rabin8 localization was seen in the control, non–NGF-treated, MeOH-fixed cells (Figure 3D, top, NGF = 0 h). Rabin8 gradually accumulated in response to NGF stimulation (Figure 3D, middle and bottom, NGF = 1 and 6 h, respectively), just as Arf6 and Rab8 do (Kobayashi and Fukuda, 2012; Kobayashi *et al.*, 2014). We therefore concluded that Rabin8 localizes on both Rab11-REs and Arf6/Rab8-REs and that its accumulation at Arf6/Rab8-REs is NGF dependent.

Rabin8 regulates neurite outgrowth in both GEF activity–dependent and –independent manners

The NGF-dependent accumulation of Rabin8 at the Arf6/Rab8-REs further prompted us to investigate its functional involvement in neurite outgrowth of PC12 cells. We constructed two independent short hairpin RNAs (shRNAs) targeting rat Rabin8 (shRabin8 #2 and #3) to evaluate the effect of Rabin8 knockdown on neurite outgrowth. When endogenous Rabin8 was depleted by the transfection of these shRNAs, the average neurite length of Rabin8-knockdown (KD) cells was much shorter than in the shControl-transfected cells (Figure 4, A and B), and this effect was almost completely rescued by reexpression of shRNA-resistant (SR) Rabin8 (Supplemental Figure S3B). We also took a gain-of-function approach. In contrast to the results of the knockdown experiment, neurite outgrowth was significantly increased by overexpressing GFP-Rabin8 (Figure 4C). These results indicated that Rabin8 is functionally involved in neurite outgrowth of PC12 cells.

Because Rabin8 possesses GEF activity toward Rab8A and Rab10 (Figure 1, C and D), we wondered whether its GEF activity is actually required for neurite outgrowth. To determine whether it is, we first adopted a dominant-negative approach in which we used the dominant-negative Rab8A(T22N) and Rab10(T23N) mutants. As shown in Figure 4D, the promoting effect of Rabin8 on neurite outgrowth was clearly attenuated by coexpression of Rab8A(T22N) or Rab10(T23N). Similar attenuation was also observed when we knocked down endogenous Rab8A/B or Rab10 instead of coexpressing their dominant-negative mutants (Supplemental Figure S3C). Next we evaluated the effect of GEF activity–deficient Rabin8 ΔGEF on neurite outgrowth, which lacks amino acids in the Sec2/GEF domain. If the promoting effect of Rabin8 overexpression depended solely on its GEF activity, Rabin8 ΔGEF would not promote neurite outgrowth. However, neurite outgrowth was also significantly enhanced when GFP-Rabin8 ΔGEF was overexpressed (Figure 4C). Although the difference was not statistically significant, the promoting effect of GFP-Rabin8 tended to be stronger than that of GFP-Rabin8 ΔGEF, suggesting that the GEF activity of Rabin8 is partly responsible for neurite outgrowth. In addition, Rabin8 ΔGEF was able to promote neurite outgrowth even in Rab8A/B- or Rab10-depleted cells (Figure 4E). These results, taken together, indicated that Rabin8 plays both GEF activity–dependent and –independent roles during neurite outgrowth.

Rab11 binding is required for Rabin8 localization and function during neurite outgrowth

As noted, Rabin8 is one of the Rab11 effectors, and its localization and function are regulated by Rab11 during primary ciliogenesis (Knödler *et al.*, 2010; Westlake *et al.*, 2011; Feng *et al.*, 2012). To determine whether this is true in regard to neurite outgrowth, we constructed two Rab11-binding-deficient Rabin8 mutants (Figure 5A). The Rabin8 Δ(300-305) mutant lacks six amino acids (300-SLYNEF-305) located after the Sec2 domain, and their absence results in inability to bind to Rab11 without affecting Rab3/8/10 binding activity (Feng *et al.*, 2012; Supplemental Figure S4). When this mutation was introduced in Rabin8 ΔGEF (shown as a Rabin8 ΔGEF/Δ(300-305) mutant), binding activity toward both Rab3/8/10 and Rab11 was completely abrogated (Supplemental Figure S4). We then evaluated the effect of overexpression of the two Rab11-binding-deficient mutants on neurite outgrowth. In contrast to the effect of Rabin8 or Rabin8 ΔGEF, Rabin8 Δ(300-305) did not promote neurite outgrowth and instead had a dominant-negative effect (Figure 5B). The other mutant, Rabin8 ΔGEF/Δ(300-305), inhibited neurite outgrowth to the same extent that Rabin8 Δ(300-305) did. Furthermore, the promoting effects of both Rabin8 and Rabin8 ΔGEF on neurite outgrowth were markedly suppressed by knocking down endogenous Rab11A/B (Figure 5E), indicating that Rab11-binding is crucial for both the GEF activity–dependent and –independent functions of Rabin8.

We also investigated the localizations of the Rabin8 Δ(300-305) and Rabin8 ΔGEF/Δ(300-305) mutants. Consistent with the results showing that endogenous Rabin8 localizes on the Rab11-REs (Figure 3), exogenously expressed GFP-Rabin8 and GFP-Rabin8 ΔGEF also localized on Rab11-REs (Figure 5C). However, neither Rabin8 Δ(300-305) nor Rabin8 ΔGEF/Δ(300-305) localized on the Rab11-REs and instead dispersed into the cytoplasm (Figure 5C, right). We then investigated whether Rab11 is required for Rabin8 localization by performing a knockdown experiment. When endogenous Rab11A/B were depleted by specific siRNAs, there were significantly fewer Rabin8 signals in the perinuclear region of the Rab11-KD cells in comparison with the non-KD cells (Figure 5D; see *Materials and Methods* for the detailed procedure). Taken together, these results indicate that Rab11-binding is required for both the localization and function of Rabin8 during neurite outgrowth.

DISCUSSION

The data obtained in this study revealed previously unknown roles of Rabin8 during neurite outgrowth, and, based on these new roles, we propose a new model of how Rabin8 regulates NGF-induced neurite outgrowth of PC12 cells in both GEF activity–dependent and –independent manners (Figure 6). Before NGF stimulation, weak or no Arf6/Rab8-REs are visible, and most of the Rabin8 is present on the Rab11-REs (Figure 6, left). In response to NGF stimulation, Arf6/Rab8-REs emerge beside the centrosome and are accompanied by accumulation of Rabin8 there (Figure 6, right). Rabin8 activates Rab8 at the Arf6/Rab8-REs (Figure 6(1)) and Rab10 at the Rab11-REs (Figure 6(2)), and their activation leads to the promotion of membrane trafficking toward neurites. Rabin8 also promotes neurite outgrowth independently of its GEF activity but in a Rab11-dependent manner (Figure 6(3)). These Rabin8-dependent membrane-trafficking routes cooperatively supply proteins and lipids to neurite tips for successful neurite outgrowth.

Rab10 as a novel substrate of Rabin8

Rabin8 was originally identified as a Rab3-interacting protein but was subsequently found to exert GEF activity toward Rab8 instead of Rab3 (Brondyk *et al.*, 1995; Hattula *et al.*, 2002). Another study further confirmed by means of in vitro GEF activity assays that Rabin8 activates Rab8A/B but not Rab3A or Rab10 (Yoshimura *et al.*, 2010). In the present study, however, we clearly showed that

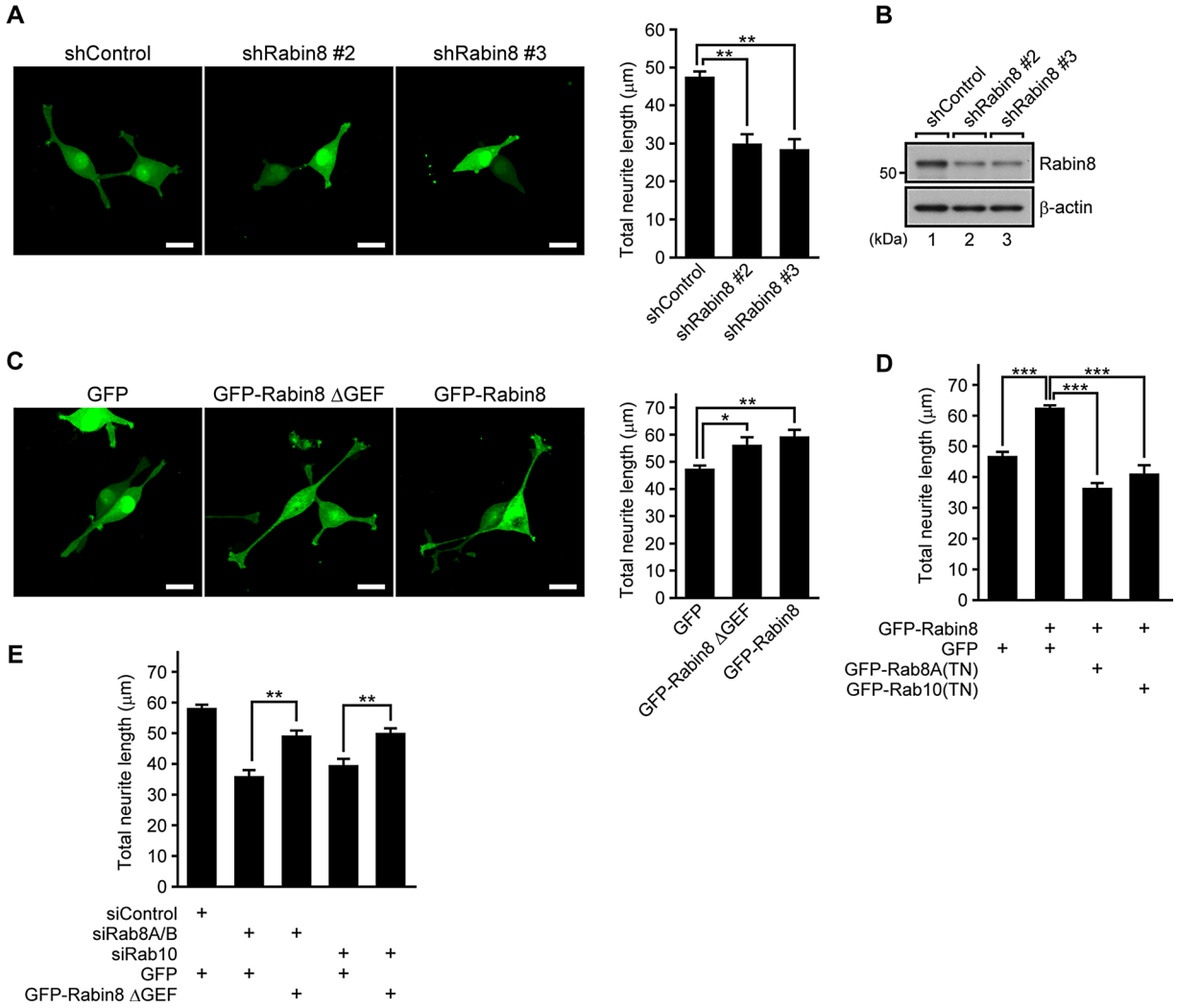

FIGURE 4: Effect of knockdown and overexpression of Rabin8 on neurite outgrowth of PC12 cells. (A) Knockdown of Rabin8 resulted in inhibition of neurite outgrowth. PC12 cells were transfected with shControl or shRabin8 (#2 and #3) together with pEGFP-C1 as a transfection marker. The cells were cultured for 24 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Left, typical images of the GFP-positive cells. Scale bars, 20 μm. Right, graph comparing the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{**}p < 0.01$ ($n = 3$; Dunnett's test). (B) Knockdown efficiency of Rabin8 as revealed by immunoblotting. PC12 cells were transfected with shControl or shRabin8 (#2 and #3) and cultured for 48 h. The cell lysates were analyzed by immunoblotting with anti-Rabin8 and anti–β-actin antibodies. (C) Effect of overexpression of Rabin8 on neurite outgrowth. PC12 cells were transfected with plasmids encoding GFP, GFP-Rabin8 ΔGEF, or GFP-Rabin8. The cells were cultured for 24 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Left, typical images of the GFP-positive cells. Scale bars, 20 μm. Right, graph comparing the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{*}p < 0.05$; $^{**}p < 0.01$ ($n = 8$; Tukey's test). (D) The promoting effect of Rabin8 on neurite outgrowth was canceled by coexpression of a constitutively negative form of Rab8 and Rab10. PC12 cells were transfected with plasmids encoding GFP, GFP-Rabin8, GFP-Rab8A(T22N), and GFP-Rab10(T23N) in combination as shown by the plus signs. The cells were cultured for 24 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Comparison of the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{***}p < 0.001$ ($n = 8$; Tukey's test). (E) Overexpression of Rabin8 ΔGEF promoted neurite outgrowth even in Rab8A/B- or Rab10-depleted cells. PC12 cells were transfected with siControl, siRab8A/B, or siRab10 in combination with plasmids encoding GFP or GFP-Rabin8 ΔGEF as shown by the plus signs. The cells were cultured for 48 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Comparison of the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{**}p < 0.01$ ($n = 3$; Tukey's test).

Rabin8 activates both Rab8 and Rab10 in living cells (Figure 1). Although we do not know the exact reason for this discrepancy, it might be attributable to the difference between the assays. Because all of the previous studies on the GEF activity of Rabin8 were performed in vitro with bacterially purified materials, an effect of posttranslational modifications and/or other interacting partners on the GEF activity of Rabin8 cannot be ruled out. In fact, it has been shown that phosphorylation by ERK1/2 or binding to Rab11

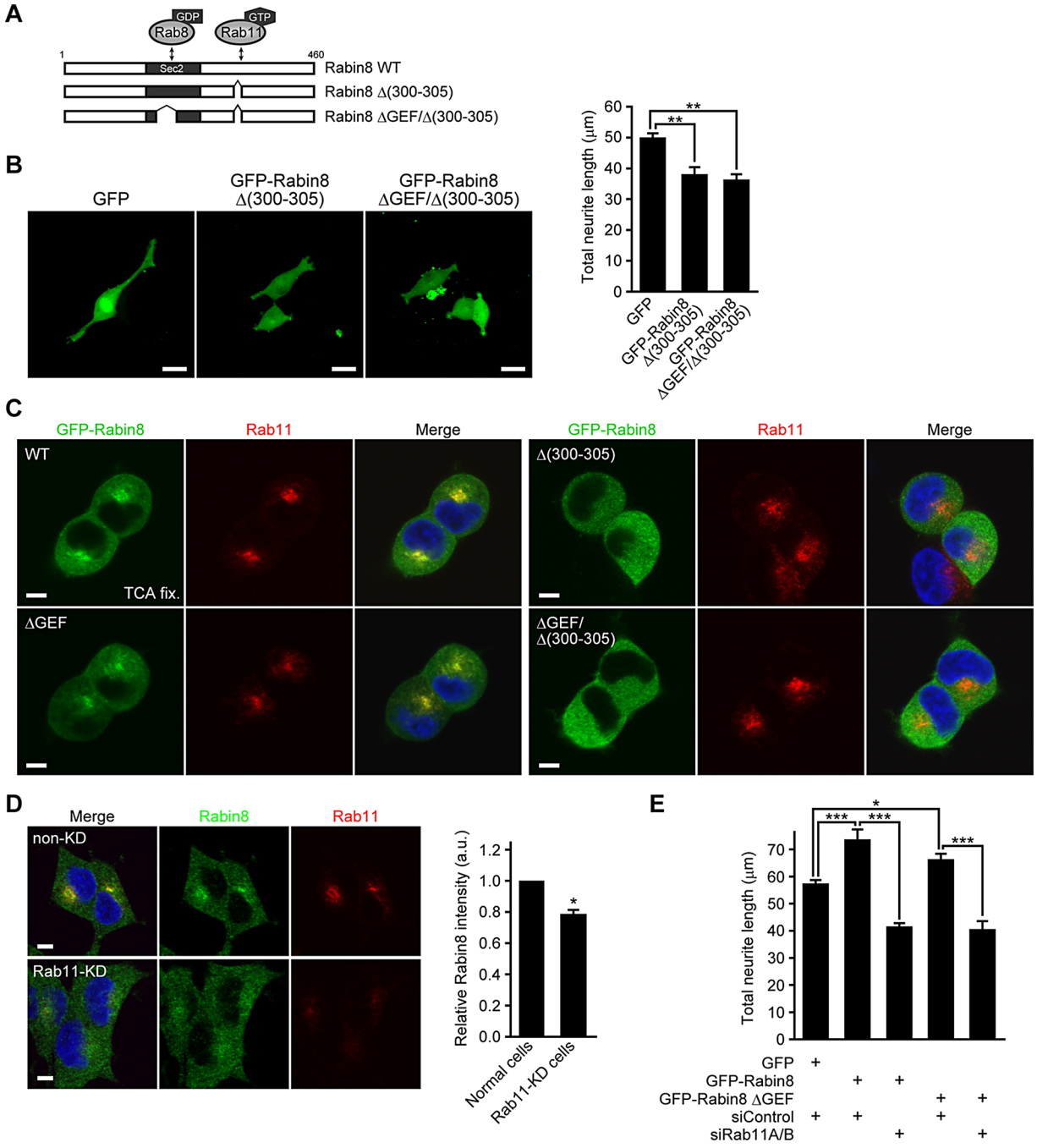

FIGURE 5: Rab11 binding is required for Rabin8 function in neurite outgrowth. (A) The Rabin8 mutants used. The Δ(300-305) mutant lacks six amino acids (300-SLYNEF-305), and the result is inability to bind to Rab11 (Feng *et al.*, 2012). The ΔGEF/Δ(300-305) mutant has the same deletion plus the absence of exon 4 in the Sec2 domain. Rab-binding properties of these mutants are shown in Supplemental Figure S4. (B) Overexpression of the Rabin8 Δ(300-305) mutant resulted in inhibition of neurite outgrowth. PC12 cells were transfected with plasmids encoding GFP, GFP-Rabin8 Δ(300-305), or GFP-Rabin8 ΔGEF/Δ(300-305). The cells were cultured for 24 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Left, typical images of the GFP-positive cells. Scale bars, 20 μm. Right, graph compares the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. $^{**}p < 0.01$ ($n = 3$; Dunnett's test). (C) Rabin8 Δ(300-305) mutants are unable to localize on REs. PC12 cells were transfected with plasmids encoding GFP, GFP-Rabin8 ΔGEF, GFP-Rabin8 Δ(300-305), or GFP-Rabin8 ΔGEF/Δ(300-305). The cells were cultured for 24 h, exposed to NGF for 6 h, and, after fixation with TCA, were immunostained with anti-GFP (green) and anti-Rab11 (red) antibodies. Scale bars, 5 μm. (D) Rabin8 localization on REs decreased as a result of knocking down Rab11. PC12 cells were transfected with siRab11A/B. The cells were cultured for 60 h, exposed to NGF for 6 h, and, after fixation with TCA, immunostained with anti-Rabin8 (green) and anti-Rab11 (red) antibodies. Left, typical images of the non-KD cells and Rab11-KD cells. Scale bars, 5 μm. Right, graph comparing Rabin8 localization at REs based on measurements of signal intensity in the perinuclear region of >10 cells in each experiment. Data are means and SEM. $^{*}p < 0.01$ ($n = 3$; unpaired *t* test). (E) The promoting effects of both Rabin8 and

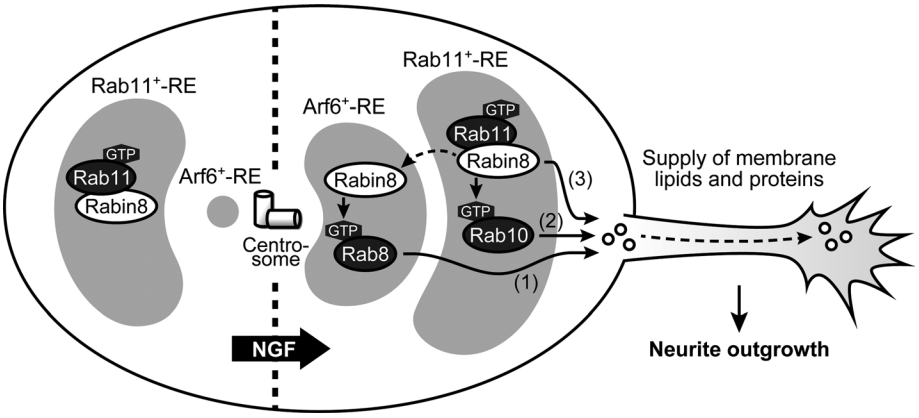

FIGURE 6: Proposed model of the three functions of Rabin8 during neurite outgrowth of PC12 cells (see *Discussion* for details).

enhances the GEF activity of Rabin8 (Knödler *et al.*, 2010; Wang *et al.*, 2015). These factors might explain why we detected the GEF activity of Rabin8 toward Rab10 in mammalian cells by our effector pull-down assay.

A Rab cascade on REs and its role during neurite outgrowth

Accumulating evidence suggests that the membrane trafficking through REs plays important roles in neuronal functions, including neurite outgrowth (Shirane and Nakayama, 2006; Li and DiFiglia, 2012). Not only do REs act as a source of membrane supply, but they also mediate the transcytosis of certain specific cargoes, including α9/β1 integrins and Trk receptors. During neurite outgrowth, such cargoes are endocytosed at the soma surface and transported toward the neurite tips, thereby establishing proper adhesion and signal transduction for neurite extension (Ascaño *et al.*, 2009; Eva *et al.*, 2010). REs are also known to serve as intermediate stations for post-Golgi vesicles in the exocytic pathway (Ang *et al.*, 2004), which is also likely to participate in neurite outgrowth.

Sec2p, Sec4p, and Ypt32p are the yeast homologues of mammalian Rabin8, Rab8, and Rab11, respectively. Ypt32p is mainly present at the Golgi membrane and is believed to regulate export of post-Golgi vesicles. Sec2p is recruited to Ypt32p-positive vesicles as an effector and then activates another Rab, Sec4p. Finally, Sec4p recruits the tethering/fusion regulators, including Sec15p and Sro7p, to complete exocytosis. This sequential Rab functioning is known as a Rab cascade (Ortiz *et al.*, 2002; Novick *et al.*, 2006). Although the Rab11 and Rab8 in mammalian cells mainly localize on REs rather than on the Golgi, existence of a similar Rab cascade (from Rab11 to Rab8) has been shown in the regulation of primary ciliogenesis and apical lumen formation of epithelial cells (Nachury *et al.*, 2007; Knödler *et al.*, 2010; Westlake *et al.*, 2011; Bryant *et al.*, 2010). We speculated that the same cascade is used to regulate RE functions during neurite outgrowth. We demonstrated that Rabin8 activates not only Rab8 but also Rab10 and observed that Rab8 and Rab10 were associated with different populations of REs in NGF-stimulated PC12 cells (Figure 2). Rabin8 localized on both REs in a Rab11-dependent manner (Figures 3 and 5) and is likely to activate downstream Rabs—Rab8 and Rab10—and thereby promote neurite outgrowth. In addition to the known Rab11–Rabin8–Rab8 cascade, a Rab11–Rabin8–Rab10 cascade also likely participates in ciliogenesis because Rab8 was found to be insufficient for ciliogenesis and Rab10 was found to be needed (Sato *et al.*, 2014).

The GEF activity–independent role of Rabin8

We found that the Rabin8 ΔGEF isoform, as well as the full-length Rabin8, promotes neurite outgrowth. Owing to the lack of the amino acids in the Sec2 domain, Rabin8 ΔGEF did not exhibit GEF activity toward Rab8 or Rab10. Thus far, only a few reports have argued that Rabin8 has a GEF activity–independent role. Discoidal/fusiform-shaped vesicles in bladder umbrella cells were shown to be exocytosed in a Rab8- and Rab11-dependent manner (Khandelwal *et al.*, 2013), and that study demonstrated that overexpression of Rabin8(F201A) (a putative GEF activity–deficient mutant of Rabin8), as well as of wild-type Rabin8, promotes this process. Furthermore, suppression of autophagosome formation by overexpression of Rabin8 in hTERT-RPE1 cells was shown to be dispensable for its GEF activity (Amagai *et al.*, 2015). In the present study, we obtained evidence of GEF activity–independent function of Rabin8 during neurite outgrowth as well. The precise mechanism of the GEF activity–independent role of Rabin8 is unclear, but we were able to demonstrate that it requires Rab11 binding (Figure 5B). Because the Rabin8 ΔGEF isoform is specifically and abundantly expressed in mouse brain (approximately half of the Rabin8 protein is the Rabin8 ΔGEF isoform; Supplemental Figure S1C), the GEF activity–independent, Rab11-dependent function of Rabin8 is likely to be physiologically relevant in the brain. Because Rabin8 specifically interacts with active Rab11 (Figure 1), we speculate that Rab11 is not just a scaffold that recruits Rabin8 to REs and that a Rab11–Rabin8 complex itself may have a Rab–Rab effector function that promotes certain membrane trafficking during neurite outgrowth. In addition to Rab11, several other interacting partners of Rabin8, including a TRAPPII complex, Sec15, phosphatidylserine, and FIP3, have been identified (Westlake *et al.*, 2011; Feng *et al.*, 2012; Chiba *et al.*, 2013; Wang and Deretic, 2015). Investigation of involvement of these molecules in the GEF activity–independent function of Rabin8 in neurite outgrowth is now underway in our laboratory. In our preliminary data, overexpression of an N-terminal–deleted mutant of Rabin8 (Rabin8 Δ(1-140)), which has lost its ability to bind to the TRAPPII complex, still promoted neurite outgrowth but did so less efficiently than wild-type Rabin8 (Supplemental Figure S5), suggesting that the binding to the TRAPPII complex is also required for Rabin8 function in neurite outgrowth. Further in-depth analysis of this mutant should clarify the function of the Rabin8–TRAPPII complex during neurite outgrowth.

Rabin8 ΔGEF on neurite outgrowth were canceled by knockdown of endogenous Rab11A/B. PC12 cells were transfected with siControl or siRab11A/B in combination with plasmids encoding GFP, GFP-Rabin8, or GFP-Rabin8 ΔGEF as shown by the plus signs. The cells were cultured for 48 h, exposed to NGF for 36 h, and then fixed with paraformaldehyde. Comparison of the means of the total length of the neurites of each of the GFP-positive cells (>50 cells) in each experiment. Data are means and SEM. *$p < 0.05$; ***$p < 0.001$ ($n = 3$; Tukey's test).

In summary, the results of the present study demonstrate that Rabin8 regulates neurite outgrowth by activating Rab8 and Rab10 and that Rabin8 also has a GEF activity–independent role in neurite outgrowth. Our findings shed light on a previously unknown Rab11-depdendent mechanism of Rabin8 action beyond the well-known Rab11–Rab8 cascade mechanism.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant human β-NGF was purchased from Merck (480275; Darmstadt, Germany). A rabbit polyclonal anti-Rabin8 antibody and a guinea pig polyclonal anti-GFP antibody were raised against glutathione *S*-transferase (GST)–Rabin8 and GST-GFP, respectively, as antigens and were affinity purified as described previously (Fukuda and Mikoshiba, 1999). The other antibodies used in this study were anti-Arf6 antibody (3A-1, sc-7971) and horseradish peroxidase (HRP)–conjugated anti-GST antibody (Z-5, sc-459) from Santa Cruz Biotechnology (Dallas, TX); anti–α-tubulin antibody (DM1A, T9026) and HRP-conjugated FLAG tag antibody (M2, A8592) from Sigma-Aldrich (St. Louis, MO); β-actin (G043) from Applied Biological Materials (Richmond, Canada); anti-Rab8 antibody (610845; reacting with both Rab8A and Rab8B) and anti-Rab11 antibody (610656; reacting with both Rab11A and Rab11B) from BD Biosciences (San Jose, CA); anti-Rab10 antibody (D36C4, 8127) from Cell Signaling Technology (Danvers, MA); and anti-Rab11 antibody (715300) and Alexa Fluor–labeled secondary antibodies from Life Technologies (Carlsbad, CA).

Plasmid construction and RNA interference

The cDNAs of mouse Rabin8, mouse Rabin8 ΔGEF (registered in the National Center for Biotechnology Information database as NM_001003950.2), mouse Rab8A, and mouse Rab10 were amplified from Marathon-Ready mouse brain and/or testis cDNA (BD Biosciences) by PCR with specific primers. Rabin8 Δ(300-305), Rab8A(Q67L)/(T22N), Rab10(Q68L)/(T23N), ^{SR}Rabin8, ^{SR}Rab8A, and ^{SR}Rab10 were prepared by standard mutagenesis techniques. These cDNAs were subcloned into the pEGFP-C1 vector (Clontech, Mountain View, CA), pIRES2-AcGFP1 vector (Clontech), and pEF-FLAG vector (pEF-BOS vector with FLAG-tag sequence at the upstream of the cloning site; Fukuda *et al.*, 1999). shRNA expression plasmids were constructed by inserting two independent target sequences against rat Rabin8 into the pSilencer-neo vector (Applied Biosystems, Waltham, MA). The shRNA target sequences were shRabin8 #2 (5′-AGCAGCTGAAGGAAGCTCAAG-3′) and shRabin8 #3 (5′- GTACAAGCAGTGCTATGAGTG-3′). siRNAs against rat Rab8A (5′-TCACGACAGCCTACTACAG-3′), rat Rab8B (5′-ATCCTTTGACAATATTAAA-3′), human Rab8A (5′-CCATAGGAATTGACTTTAA-3′), human Rab8B (5′-TGACAAAACTCAACAGAAA-3′), rat Rab10 (5′-GTGGCTTAGAAACATAGAT-3′), rat Rab11A (5′-TCTGGAAAGCAAGAGTACC-3′), and rat Rab11B (5′-GCATTCAAGAACATCCTCA-3′) were chemically synthesized by Nippon Gene (Toyama, Japan) as described previously (Matsui and Fukuda, 2013; Aizawa and Fukuda, 2015). Stealth siRNAs against rat Rab11A (target site, 5′-TGTCCTTATTGGAGATTCTGGTGTT-3′) and rat Rab11B (target site, 5′-GACGACGAGTACGACTACCTATTCA-3′) were purchased from Life Technologies.

Cell culture and transfection

PC12 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 10% horse serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. COS-7 cells and 293T cells were cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. One day after plating, plasmids and siRNAs were transfected into cells by using Lipofectamine 2000 (Life Technologies) or Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions.

RT (reverse transcription)-PCR analysis

Mouse first-strand cDNAs from various tissues were obtained from Clontech (Mouse MTC panel I). The primers used for amplification of Rabin8 and Rabin8 ΔGEF fragments were 5′-GGATCCATGGCTAACGACCCCTTGGA-3′ (forward) and 5′-CTCACCATCTTGTGAGCTTC-3′ (reverse). PCR was performed by using ExTaq DNA polymerase (Takara, Shiga, Japan) according to the manufacturer's instructions.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed as described previously (Kobayashi *et al.*, 2015).

GEF activity assay in COS-7 cells and 293T cells

FLAG-tagged Rab8A or Rab10 and GFP-tagged Rabin8 were coexpressed in COS-7 cells or 293T cells as described. Cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 100 mM $MgCl_2$, 1 mM dithiothreitol, 1× protease inhibitor [1873580; Roche, Basel, Switzerland]) and centrifuged at 17,400 × *g* for 10 min at 4°C. The supernatants were incubated with 5 μl of glutathione Sepharose 4B (GE Healthcare, Little Chalfont, UK) and 1 μg of GST-tagged C-terminal fragment of MICAL-L2 (Fukuda *et al.*, 2008) for 1 h at 4°C. The beads were washed with the lysis buffer three times and boiled in a sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.02% bromophenol blue). Samples were subjected to SDS–PAGE and analyzed by immunoblotting with appropriate antibodies.

Purification of GST fusion proteins

GST-Rabin8, GST-GFP, and GST-MICAL-L2 C were expressed in *Escherichia coli* JM109 and purified as described previously (Kuroda and Fukuda, 2005).

Neurite outgrowth assay

PC12 cells were plated on a poly-L-lysine–coated, 35-mm, glass-bottomed dish at 3×10^4 cells/dish. For knockdown experiments, cells were cotransfected with 1.0 μg of shRNA plasmids or 20 nM siRNAs and 0.5 μg of pEGFP-C1 as a transfection marker and then cultured for 48 h. For overexpression experiments, cells were transfected with cDNAs that had been cloned into the pEGFP-C1 vector and then cultured for 24 h. Cells were exposed to 100 ng/ml NGF for 36 h and fixed with 4% paraformaldehyde. Images were acquired of at least 50 GFP-positive cells in each sample, and the total length of all of each cell's neurites was measured with MetaMorph software (Molecular Devices, Sunnyvale, CA). Experiments were repeated at least three times, and the means of the data obtained from each sample were analyzed statistically.

Fluorescence microscopy

For immunostaining, PC12 cells were fixed with either ice-cold MeOH for 5 min or 10% trichloroacetic acid (TCA) for 10 min and then permeabilized with 0.3% Triton X-100/phosphate-buffered saline (PBS) for 2 min. Next the cells were blocked with 1% bovine serum albumin/PBS for 30 min and incubated with appropriate antibodies diluted in Can-Get-Signal solution (NKB-501; Toyobo, Osaka, Japan). Fluorescence images were obtained by using a

confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan) equipped with a Plan-Apochromat 63×/1.4 oil immersion objective lens and an electron-multiplying charge-coupled device camera (C9100; Hamamatsu Photonics, Shizuoka, Japan).

Quantification of perinuclear signals of Rabin8 in Rab11-knockdown cells

PC12 cells were plated on a poly-L-lysine–coated, 35-mm, glass-bottomed dish at 3×10^4 cells/dish and transfected with siRab11A+B (20 nM each). Cells were cultured for 60 h, fixed with 10% TCA, and then immunostained with anti-Rabin8 and anti-Rab11 antibodies. Under our experimental conditions, about half of the siRab11-treated cells were depleted of endogenous Rab11 (Rab11-KD cells), and the rest were not depleted of endogenous Rab11 at all (non-KD cells). We therefore compared the levels of perinuclear Rabin8 signals in the Rab11-KD cells and non-KD cells in the same dish by the measuring average intensity of the Rabin8 immunofluorescence signals in their perinuclear region.

Statistical analysis

All quantitative data are expressed as the means and SEM. Tukey's test, Dunnett's test, and Student's *t* test were performed on R software to evaluate the statistical significance of differences between the samples.

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