Golgi-resident TRIO regulates membrane trafficking during neurite outgrowth

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Neurite outgrowth requires coordinated cytoskeletal rearrangements in the growth cone and directional membrane delivery from the neuronal soma. As an essential Rho guanine nucleotide-exchange factor (GEF), TRIO is necessary for cytoskeletal dynamics during neurite outgrowth, but its participation in the membrane delivery is unclear. Using co-localization studies, live-cell imaging, and fluorescence recovery after photobleaching analysis, along with neurite outgrowth assay and various biochemical approaches, we here report that in mouse cerebellar granule neurons, TRIO protein pools at the Golgi and regulates membrane trafficking by controlling the directional maintenance of both RAB8 (member RAS oncogene family 8) and RAB10-positive membrane vesicles. We found that the spectrin repeats in Golgi-resident TRIO confer RAB8 and RAB10 activation by interacting with and activating the RAB GEF RABIN8. Constitutively active RAB8 or RAB10 could partially restore the neurite outgrowth of TRIO-deficient cerebellar granule neurons, suggesting that TRIO-regulated membrane trafficking has an important functional role in neurite outgrowth. Our results also suggest cross-talk between Rho GEF and Rab GEF in controlling both cytoskeletal dynamics and membrane trafficking during neuronal development. They further highlight how protein pools localized to specific organelles regulate crucial cellular activities and functions. In conclusion, our findings indicate that TRIO regulates membrane trafficking during neurite outgrowth in coordination with its GEF-dependent function in controlling cytoskeletal dynamics via Rho GTPases.

After receiving developmental signals, post-mitotic neurons differentiate into mature neurons and establish specific functional structures, including neurites. The area of the plasma membrane of a developing neuron is estimated to increase by 10,000-fold because of the formation of axons and dendrites (1, 2). During this period, a large number of intracellular processes are initiated, including protein and lipid synthesis, cytoskeletal dynamics, membrane production, and trafficking. Cytoskeletal rearrangements and membrane trafficking are both required for neurite outgrowth, with the former providing the driving force for growth cone turning and elongation (3, 4) and the latter providing membrane lipids and proteins at sites located far from the Golgi network (2, 5). Within these highly regulated processes, Rho family GTPases, such as RAC1, CDC42, and RHOA, have been found to regulate cytoskeletal rearrangements (6–8). RAC1 and CDC42 regulate neurite elongation and branch formation, and RHOA induces neurite retraction (9–12). On the other hand, Rab family GTPases regulate membrane trafficking processes (2, 13–15), in which RAB6, 8, 10, 13, and 33 function in trans-Golgi network (TGN)-related vesicles; RAB5, 7, 21, and 22 regulate early and late endosomes; and RAB4, 11, and 35 regulate recycling endosomes (13, 16). The GTPase activities of both Rho and Rab are controlled by GTP-/GDP-bound cycles, which are regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins, respectively (8, 13). The orchestrated interactions of these two families of small GTPases are thus essential for neurite development.

As a member of the Dbl family of GEFs, the triple functional domain (TRIO) is a large GEF with multiple functional domains that regulate Rho family GTPases, including RAC1, CDC42, and RHOA (17), and plays a key role in axon growth and guidance in Caenorhabditis elegans and Drosophila (18–23). Deletion of TRIO in mice abolishes neurite outgrowth and axon guidance and produces multiple abnormalities, such as deformities in the skeletal muscle, disorganization of neuronal tissues, and defects in learning and memory (24–28). TRIO also serves a role in excitatory synaptic transmission and long-term potentiation (29). In particular, several TRIO mutants have been identified in individuals with intellectual disability, schizophrenia, and autism (30–33). Biochemically, the N-terminal

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This article contains Figs. S1–S7 and Movies S1–S4.

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3 The abbreviations used are: TGN, trans-Golgi network; CDS, coding sequence; CGN, cerebellar granule neuron; FRAP, fluorescence recovery after photobleaching; GEF, guanine nucleotide exchange factor; GST, glutathione S-transferase; PCC, Pearson’s correlation coefficient; EGF, enhanced green fluorescent protein; MCS, multiple cloning site; IPTG, isopropyl β-D-thiogalactopyranoside; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DAPI, 4′,6′-diamino-2-phenylindole; DIV, day(s) in vitro; PMSF, phenylmethylsulfonyl fluoride; ANOVA, analysis of variance.

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GEF domain is responsible for the activation of the Rho GTPases RAC1 and RHOG (further activates CDC42), whereas the C-terminal GEF domain contributes to RHOA activation (34). The third functional domain is the C-terminal protein serine/threonine kinase domain, whose function remains unknown. The full-length protein containing this kinase domain is expressed at relatively low levels in the nervous system (31, 35, 36). The TRIO N terminus also contains spectrin repeats and a SEC-14 motif, whose functions are as yet undetermined. Because spectrin is associated to the Golgi apparatus, here we have determined the possible involvement of TRIO in the Golgi apparatus and the Golgi-derived membrane vesicles. The result shows that TRIO is located in the Golgi apparatus through the spectrin repeats in a complex with RABIN8, a common GEF for both RAB8 and RAB10. TRIO facilitates RABIN8 phosphorylation via a direct interaction with RABIN8. TRIO deletion leads to an increase in the frequency of the directional switch of RAB8- and RAB10-positive vesicles and hence a decrease in the traffic distance of the vesicles. However, the velocities of the vesicles are not affected. This observation revealed a novel role of TRIO in membrane trafficking, which is instructive to understand the process coupling of membrane trafficking and cytoskeleton rearrangement during neurite outgrowth.

Results

TRIO is able to localize in the Golgi apparatus

Because TRIO contains spectrin repeats, which are tightly associated with the Golgi apparatus (37), we speculate that TRIO may have the ability to localize at the Golgi apparatus, thereby potentially regulating Golgi-derived membrane trafficking. First, we prepared subcellular fractions of mouse cerebellum using OptiPrep density gradient. The result showed a novel role of TRIO in membrane trafficking, which is instructive to understand the process coupling of membrane trafficking and cytoskeleton rearrangement during neurite outgrowth.

Figure 1. TRIO localizes to the Golgi apparatus in CGNs. A, P10 mouse cerebellum was homogenized, and the postnuclear supernatants were subjected to 2.5–30% OptiPrep density gradient for subcellular fractionation. Fractions were subjected to Western blotting with TRIO, GM130, BIP, EEA1, and RAB5 antibodies. B, relative distribution of blotted proteins in A, with the strongest intensity of each protein considered as 1. C, CGNs isolated from WT mice were cultured for 24–48 h and then stained with the antibody against Golgi markers GM130, TGN46, VAMP4, and RCAS1, as well as the TRIO-N antisemur and DAPI. The scale bar in the left panel represents 20 μm, and the scale bars in the right magnified panels represent 5 μm. Scatter plots and the PCCs of the fluorescence intensities of red and green channels are also shown. D, scatter plots and bar graph showing the PCC of TRIO and Golgi markers fluorescence intensities quantified from 15 to 20 neurons in each group, from three independent experiments. MW, molecular weight.
studied the subcellular localization of TRIO in the mouse neuroblastoma Neuro-2a cells. Ectopic expression of EGFP-fused TRIO9S and TRIO8, the two main isoforms expressed in cerebellum (35, 36), displayed a strong EGFP fluorescence in the perinuclear region, whereas EGFP alone did not (Fig. S1). Staining the transfected Neuro-2a cells with Golgi marker GM130 or TGN38 suggested that the enriched EGFP fluorescence in the perinuclear region was highly associated with Golgi apparatus (Fig. S1).

To determine the endogenous TRIO localization in neurons, we prepared a TRIO-specific antiserum targeting the N-terminal spectrin repeats (Fig. S2A). The specificity of this antiserum was determined using TrioNKO (Trioflox/flox;Nestin-Cre) (27) lysates by Western blotting (Fig. S2B) and cultured TrioWKO (Trioflox/flox;Wnt1-Cre, see below) CGNs by immunofluorescence (Fig. S2C). In addition, fluorescence intensity derived from this antiserum was highly correlated with EGFP intensity in COS-7 cells overexpressing EGFP-TRIO9S or EGFP-TRIO8 (Fig. S2D). We then immunostained endogenous TRIO in cultured CGNs using this antiserum and found that endogenous TRIO was also enriched in the perinuclear region, with lower but still detectable intensity along the neurite and in the growth cone (Fig. 1C). This enrichment of TRIO fluorescence was correlated with GM130 (Fig. 1C), suggesting that the endogenous TRIO also localizes in Golgi apparatus in CGNs. To confirm this observation, co-localization analysis of TRIO with other Golgi markers, including TGN46, VAMP4, and RCAS1, was performed (Fig. 1D). Quantification of the co-localization indicated that TRIO protein was highly correlated with Golgi apparatus, especially trans-Golgi networks (Fig. 1D). To investigate the amino acids required for TRIO’s localization in Golgi, we constructed a series of plasmids encoding truncated TRIO proteins fused with EGFP (Fig. 2A). TRIO(1–230) encodes the SEC-14 domain; TRIO(208–673), TRIO(446–909), and TRIO(672–1295) encode the fragments of different spectrin repeats; and TRIO(1296–1909) encodes the N-terminal GEF1 domain. After transfection with the plasmids, the COS-7 cells expressing EGFP-TRIO(208–673) displayed EGFP fluorescence signals highly restricted in the GM130-positive region (Fig. 2B), whereas the COS-7 cells transfected with other plasmids, including the control EGFP plasmid, showed diffused EGFP signals in the cytosol or in the nuclei (Fig. 2B). EGFP-TRIO(208–673) was also enriched in regions positive for other Golgi markers, including TGN46 (Fig. 2C), VAMP4 (Fig. 2D), and RCAS1 (Fig. 2E). Thus, TRIO is able to localize in the Golgi apparatus through the first spectrin fragment.

Figure 2. TRIO spectrin repeats localizes to the Golgi apparatus. A, schematic diagram of TRIO (1–230), (208 – 673), (446 –909), (672–1295), and (1296 –1909) sequences. B, COS-7 cells transfected with pEGFP-C3, pEGFP-TRIO(1–230), pEGFP-TRIO(208 – 673), pEGFP-TRIO(446–909), pEGFP-TRIO(672–1295), and pEGFP-TRIO(1296–1909) were stained with the Golgi marker GM130. EGFP-TRIO(208 – 673) fluorescence was highly correlated with GM130 signal. The scale bar represents 20 μm. C–E, COS-7 cells transfected with pEGFP-TRIO(208 – 673) were stained with Golgi markers TGN46 (C), VAMP4 (D), and RCAS1 (E). The scale bars represent 10 μm.
TRIO regulates membrane trafficking during neurite outgrowth

To study the function of TRIO during neurite outgrowth, we prepared TRIO-deficient CGNs by crossing Trio floxed mice with Wnt1-Cre (27, 38). Wnt1-Cre was expressed in the cerebellum and in cultured CGNs, as illustrated by the Rosa-mTmG reporter (39) (Fig. S2, A and B). Unlike the Trio<sup>NKO</sup> mice we reported previously, the resultant Trio<sup>WKO</sup> (Trio<sup>flox/flox</sup>;Wnt1-Cre) mice live to adulthood, although the body weights were apparently smaller than control mice. We then performed a fluorescence recovery after photobleaching (FRAP) analysis to determine the potential functions of TRIO in membrane trafficking. EGFP-tagged human transferrin receptor (EGFP–hTfR) was transfected to neurons to label newly synthesized membranes (40, 41). Because the membrane recovery from photobleaching reflects the processes of membrane trafficking and synthesis, there are investigators using this method to assess the membrane dynamics during hippocampal axonal growth (41). After photobleaching, Trio<sup>WKO</sup> neurites showed a slower recovery rate of the fluorescence intensity than the control neurites (p < 0.01) (Fig. 3, A and B). Quantification of the area under curve after bleaching showed that the recovery percentage of TRIO-deficient CGNs was significantly decreased compared with the control neurites (p < 0.01) (Fig. 3C). Thus, the TRIO-deficient CGNs exhibited abnormal membrane recovery during the neurite growth.

Because TRIO is associated with Golgi apparatus, we then determined the role of TRIO in the directional trafficking of TGN-derived membrane vesicles. It has been reported that the directional trafficking of these membrane vesicles to the neuronal growth cone is primarily mediated by RAB8 and RAB10 during axonal growth (42, 43), in which tdTomato fluorescent protein–fused RAB10 is applied to label RAB10-positive membrane vesicles and monitor the trafficking behaviors (42). We here used the tdTomato to label RAB8A and RAB10 to investigate whether TRIO affects RAB8- and RAB10-positive membrane trafficking. We observed the labeled membrane vesicles using time-lapse microscopy, generated the kymographs, and analyzed the trafficking behaviors using KymoAnalyzer (44). TRIO knockout in CGNs decreased the average distance traveled by RAB8A-positive vesicles in both the anterograde and retrograde directions, but the switch frequencies were significantly increased (Fig. 4, A–C, and Movies S1 and S2). However, the average velocities of vesicles traveling in both directions, and the percentages of time spent in motion states were not altered (Fig. 4, D and E, and Movies S1 and S2). In addition, RAB10-positive vesicles in TRIO-deficient CGNs showed a similar behavior to RAB8-positive vesicles in terms of the decreased distance traveled and increased switching frequency and comparable average velocities and percentage of time spent in motion states (Fig. 4, F–J, and Movies S3 and S4). This observation suggested an essential role of TRIO in regulating the switching frequency of the membrane vesicles.

**RAB8/RAB10 activation is required for TRIO-mediated neurite outgrowth**

The abnormal trafficking of RAB8- and RAB10-positive vesicle prompted us to hypothesize that RAB8 and RAB10 activities were altered in TRIO-deficient neurons. Three siRNAs specifically targeting Rab8a or Rab10 were introduced to knockdown these two GTPases (Fig. S4, A and E). The result showed that knockdown of Rab8a or Rab10 significantly inhibited neurite outgrowth (Fig. S4, B–D and F–H) as in previous reports (43, 45), suggesting a required role for RAB8 and RAB10 in neurite outgrowth in CGNs. We then measured the levels of GTP-bound RAB8 and RAB10 in the developing cerebella. After pulldown with the purified GST–MICAL-L2-C protein (46), the GTP–RAB8 and GTP–RAB10 proteins from the cerebellum were measured by Western blotting. The results showed that the GTP-bound forms of both RAB8 and RAB10 were decreased in TRIO-deficient cerebellar tissues (Fig. 5, A and B), suggesting a requirement of TRIO for RAB8/RAB10 activation. The observation from Neuro-2a cells overexpressing EGFP–TRIO9S or EGFP–TRIO8 displaying increased levels of GTP–RAB8 (Fig. 5, C and D) also supports this conclusion. The activation of RAB10 could not be determined because of the low expression of RAB10 in Neuro-2a cells (data not shown).

In parallel, we also performed GST–FIP3–RBD11 pulldown assay (47, 48) and GST–Rabaptin5–R5BD pulldown assay (49) to determine the GTP-bound levels of RAB11 (a recycling endosome-associated Rab) and RAB5 (an early endosome-associated Rab), in TRIO-deficient cerebella or TRIO-overexpressing Neuro-2a cells (Fig. S5). No alteration of either GTP–RAB11 or GTP–RAB5 was observed, implying that TRIO activated RAB8/RAB10 in a selective manner. We then assessed the roles of RAB8 and RAB10 in the neurite outgrowth defect induced by TRIO deletion. After the introduction of RAB8A(Q67L), an active form of RAB8, into TRIO-deficient CGNs, the neurite...
length was significantly restored (56.9 ± 4.4 μm versus 40.5 ± 2.1 μm, p < 0.05) (Fig. 5, E and F). We also transfected the CGNs with RAB8A(T22N), a dominant-negative RAB8, and analyzed neurite growth. The dominant-negative RAB8 did not decrease the neurite length in TRIO-deficient CGNs further (40.5 ± 2.1 μm versus 44.2 ± 0.7 μm). For control CGN, however, the introduction of the dominant-negative RAB8 led to a significant decrease in neurite length (52.8 ± 6.9 μm versus 67.7 ± 5.8 μm, p < 0.05) (Fig. 5, E and F), and an increased percentage of the neurons with neurites less than 50 μm (Fig. 5G). Introduction of constitutive-active RAB8 to TRIO-deficient neurons led to a decreased percentage of the neuron with neurite less than 50 μm (Fig. 5G). We also introduced RAB10(T23N) into the control neurons and found that this dominant-negative RAB10 protein dramatically inhibited neurite outgrowth (Fig. 5, H–J), whereas the constitutive-active RAB10 rescued the growth defect of TrioWKO CGN neurites (Fig. 5, H–J). Of noted, these dominant-negative and constitutive active Rab mutants did not modulate the activity of Rho family GTPases, because overexpressing these mutants in Neuro-2a cells did not alter RAC1 activity (Fig. S6). Based on these results, we conclude that both RAB8 and RAB10 are required for neurite outgrowth in CGNs, and these two Rab GTPases may be regulated by TRIO.

**TRIO recruits RABIN8 to traffic vesicles and hence facilitates RABIN8 activation**

Because RABIN8 has been identified as the major GEF for RAB8 and RAB10 in neurite outgrowth (46, 50), we hypothesized that TRIO might be necessary for RABIN8 activation. We first measured active RABIN8 (phosphorylated RABIN8) in the protein lysates from the fresh cerebellar tissues from TRIOWKO mice and their control littermates at postnatal day 10. The phosphorylated RABIN8 was immunoprecipitated and blotted with an anti–phospho-mitogen-activated protein kinase/cyclin-de-
ependent kinase substrate antibody (51) (Fig. 6A). The levels of phosphorylated RABIN8 were dramatically decreased in TrioWKO cerebella (Fig. 6, A and B) and CGNs in culture (Fig. 6, C and D). Because TRIO has no kinase domain for RABIN8 phosphorylation, the effect of TRIO on RABIN8 phosphorylation may be mediated through ERK1/2 (51). We thus measured the ERK1/2 protein in the fractions of membrane vesicles and expectedly found that ERK and RABIN8 co-existed in the subcellular fractions that were positive for RAB8 and RAB10 (Fig. 6E).

To understand the interplays of TRIO and RABIN8, we respectively characterized RABIN8 localization in TGN and membrane vesicles. We immunostained COS-7 cells with anti-RABIN8 and anti-TGN38 antibodies. The RABIN8 signal was observed in the cytoplasm and was enriched in the TGN38-positive region (Fig. S3A). In cultured CGNs, the RABIN8 signal was also enriched in the TGN38-positive TGN. This localization was consistent with the observation from cultured hippocampal neurons (52), and the localization pattern of RABIN8 in TGN was not affected apparently by TRIO knockout (Fig. S3B).

We then examined the localization of RABIN8 in membrane vesicles by immunostaining. RABIN8 was detected in the RAB8-positive vesicles along the neurite from control CGN (Fig. 6F), but TRIO knockout resulted in a significantly decreased portion of RABIN8 overlapping with RAB8 (0.44 ± 0.03 versus 0.28 ± 0.01 of Pearson’s correlation coefficient, and 0.82 ± 0.02 versus 0.65 ± 0.03 of Manders’ coefficient 2, p < 0.05 (Fig. 6, F and G), suggesting that TRIO deletion reduced the accessibility of RABIN8 to RAB8-positive vesicles. This result indicated that TRIO was necessary for RABIN8 recruitment to the membrane vesicles where RABIN8 phosphorylation might be possibly catalyzed by ERK1/2.

Figure 5. RAB8/RAB10 activation is required for TRIO-mediated neurite outgrowth. A, GST–MICAL-L2-C pulldown assay to determine GTP-RAB8 and GTP-RAB10 level in cerebella isolated from P10 TrioWKO mice and littermate control mice. B, quantification of GTP-RAB8 and GTP-RAB10 levels in A. The error bars indicate S.E. (Student’s t test). *, p < 0.05; n = 4. C, GST-JFC1 pulldown assay to determine GTP-RAB8 levels in Neuro-2a cells transfected with either the pEGFP-TRIO9S, pEGFP-TRIO8, or pEGFP-C3 plasmid. D, quantification of GTP-RAB8 levels in C. The error bars indicate S.E. (one-way ANOVA with Bonferroni’s test). *, p < 0.05; n = 4. E, CGNs isolated from control or TrioWKO mice were transfected with either pcDNA–EGFP, pcDNA-RAB8(A(T22N))-P2A-EGFP, or pcDNA-RAB8A(Q67L)-P2A-EGFP. Transfected neurons were cultured for 2 DIV, followed by staining with DAPI, EGFP, and Tau. The scale bar represents 20 μm. F, quantification of the neurite length in the images shown in E. The error bars indicate S.E. (two-way ANOVA with Tukey’s test). *, p < 0.05; n = 4. Numbers of measured neurons are indicated in the columns. G, quantification of neurite length distribution in E. The error bars indicate S.E.; n = 4. H, CGNs isolated from control or TrioWKO mice were transfected with either pEGFP-C3, pEGFP–RAB10(A(T23N)), or pEGFP–RAB10(Q68L) and then cultured for 2 DIV, followed by staining with DAPI, EGFP, and Tau. The scale bar represents 20 μm. I, quantification of the neurite length in the images shown in H. The error bars indicate S.E.; n = 4. A.U., arbitrary units; n.s., not significant; MW, molecular weight.
The spectrin repeats of the TRIO N terminus are required for the interaction with RABIN8

To test whether the recruitment of RABIN8 by TRIO is mediated by physical interaction, we performed a series of binding assays. The P21 cerebellar lysates that expressed TRIO9S and TRIO8 at equivalent levels were subjected to immunoprecipitation using a FLAG antibody covalently conjugated to agarose beads. Western blots of these immunoprecipitated samples showed that TRIO8 was detected in the immunoprecipitates of the cells expressing FLAG-RABIN8 (Fig. 7B). This observation was also supported by GST pulldown assay for P21 cerebellar lysates by using purified GST and recombinant GST-RABIN8 fusing protein. Both main isoforms of TRIO were detected in the samples containing GST-RABIN8, but not in the samples containing GST alone (Fig. 7D).

To identify the region of TRIO required for RABIN8 interaction, we prepared two truncated TRIO fragments: TRIO(1–1295) containing the spectrin repeats and TRIO(1296–1909) containing the GEF1 domain. Immunoprecipitation using a FLAG antibody covalently conjugated to agarose beads. Western blots of these immunoprecipitated samples showed that TRIO8 was detected in the immunoprecipitates of the cells expressing FLAG-RABIN8 (Fig. 7B). This observation was also supported by GST pulldown assay for P21 cerebellar lysates by using purified GST and recombinant GST-RABIN8 fusing protein. Both main isoforms of TRIO were detected in the samples containing GST-RABIN8, but not in the samples containing GST alone (Fig. 7D).
cipitates, whereas EGFP-TRIO(1296–1909) did not produce any signal (Fig. 7C). Thus, the N-terminal region rather than the GEF domain of TRIO is required for the interaction with RABIN8. This conclusion was also supported by the result from the application of TRIO GEF1 inhibitor ITX3 (53), which showed no alteration of RABIN8 phosphorylation level in the ITX3-treated CGNs, whereas in which, the GTP-RAC1 levels were decreased (Fig. 7, E and F). To determine whether TRIO directly bind with RABIN8, we next expressed and purified the His6-tagged RABIN8 and then incubated with GST or the GST-fused TRIO variants: TRIO(1–230), TRIO(446–909), and TRIO(672–1295) (Fig. 2A). We also expressed TRIO(208–673) but failed in the purification because of the formation of a tight insoluble inclusion body. The result showed that the spectrin-containing variants were able to strongly bind to His6-RABIN8, and TRIO(1–230) containing SEC-14 domain bound weakly to His6-RABIN8, whereas GST alone did not show any binding (Fig. 7G). These results suggested that TRIO was able to physically bind with RABIN8 through the spectrin repeats. We then transfected COS-7 cells with either plasmid of pEGFP-C3, pEGFP-TRIO(1–230), pEGFP-TRIO(208–673), pEGFP-TRIO(446–909), pEGFP-TRIO(672–1295), or pEGFP-TRIO(1296–1909), and were lysed and subjected to immunoprecipitation. RABIN8 phosphorylation level was determined. I, quantification of the pRABIN8 level in H. The error bars indicate S.E. (one-way ANOVA with Bonferroni’s test). *, p < 0.05; **, p < 0.01; n = 6. n.s., not significant; A.U., arbitrary units; IP, immunoprecipitation. MW, molecular weight; WB, Western blot.

Figure 7. TRIO directly interacts with RABIN8. A, co-immunoprecipitation of endogenous TRIO and RABIN8 in P21 mouse cerebellum. B, co-immunoprecipitation of EGFP-TRIO8 and FLAG-RABIN8 in Neuro-2a cells. C, co-immunoprecipitation of FLAG-RABIN8 with EGFP-TRIO(1–1295) and TRIO (1296 –1909) in COS-7 cells. D, GST-RABIN8 pulldown assay of P21 mouse cerebellum lysates. E, RABIN8 phosphorylation assay to CGNs treated with ITX3, RAC1 activity was decreased. F, quantification of RABIN8 phosphorylation in E. The error bars indicate S.E. (Student’s t test); n = 3. G, direct binding assay of His6-RABIN8 to GST–TRIO variants. Coomassie Blue staining of GST–TRIO variants are shown at the bottom. H, COS-7 cells were transfected with FLAG-RABIN8, together with either pEGFP-C3, pEGFP-TRIO(1–230), pEGFP-TRIO(208–673), pEGFP-TRIO(446–909), pEGFP-TRIO(672–1295), or pEGFP-TRIO(1296–1909), and were lysed and subjected to immunoprecipitation. RABIN8 phosphorylation level was determined. I, quantification of the pRABIN8 level in H. The error bars indicate S.E. (one-way ANOVA with Bonferroni’s test). *, p < 0.05; **, p < 0.01; n = 6. n.s., not significant; A.U., arbitrary units; IP, immunoprecipitation. MW, molecular weight; WB, Western blot.

TRIO regulates membrane trafficking
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Discussion

In this report, we reveal that Golgi-resident TRIO essentially regulates the directional membrane trafficking of the developing CGNs. At the trans-Golgi network and membrane vesicles, the pooled TRIO interacts with and activates RABIN8, which is necessary for RAB8 and RAB10 activation. RAB8 and RAB10 activation are key for the membrane vesicles trafficking from neuronal soma to the growth cone, and constitutively active RAB8 and RAB10 restored the impaired outgrowth of TRIO-deficient neurites. Based on these results, we concluded that TRIO mediated neurite growth by coordinating at least two processes: cytoskeletal rearrangement and membrane trafficking. This coordination was established by different pools of TRIO protein. We propose a working model of the role of TRIO in neurite growth. In this model, extracellular signals activate growth cone-resident TRIO and lead to the activation of Rho GTPases, which regulates cytoskeletal dynamics in the growth cone, thereby regulating neurite elongation. Simultaneously, Golgi-resident TRIO recruits RABIN8 to membrane vesicles for activation through a physical interaction of the spectrin repeats within the TRIO N terminus, and the activated RABIN8 drives membrane transport through the activation of Rab GTPases such as RAB8 and RAB10. The cytoskeletal rearrangements mediated by TRIO-activated Rho GTPases provide the driving force for neurite growth, and the membrane vesicles transport lipid cargoes to enable new membrane formation. This working model is further supported by multiple lines of evidence: (1) Deletion of TRIO leads to an impaired response to various signaling molecules, such as Netrin-1 and Semaphorin-6A (27). This implies a common regulatory scenario shared by these signals, similar to the membrane trafficking process required for neurite outgrowth. (2) A point mutation within the spectrin repeat also causes autism, schizophrenia, and intellectual disability (30–33). Because several Dbl family proteins, such as Kalirin, contain spectrin repeats, we predict that these proteins might also follow the similar working model to achieve their corresponding functions. Importantly, because many proteins contain multiple domains and some of them are capable of binding to subcellular structures, the subcellular pool formation might be a general regulatory mechanism for the functional protein machines.

Recently, TRIO mutations were identified in patients with autism, schizophrenia, and intellectual disability (30–33), and the mutations may occur both in TRIO GEFD1 and the spectrin repeat (N1080I) (32). These observations strongly suggest a critical role for TRIO in learning and memory. Based on our result, in addition to the altered cytoskeleton rearrangement mediated by TRIO GEF domains, the altered membrane trafficking mediated by the spectrin repeat may also essentially contribute to the phenotypes of these diseases. This may be instructive in developing therapeutic interventions for this condition. For example, we may use reagents to activate membrane trafficking to restore the impaired neurite growth in subjects carrying a mutation in the spectrin repeat.

In summary, we revealed a function of TRIO in regulating membrane trafficking during neurite outgrowth, in addition to the GEF-dependent function in regulating cytoskeletal dynamics by Rho GTPases. This function is accomplished by Golgi and the Golgi-derived vesicle pool of TRIO, whereby interaction with RABIN8 via the non-GEF domain. TRIO recruits and activates RABIN8 and subsequently activates RAB8 and RAB10 so as to regulate membrane trafficking by controlling the membrane vesicle switches frequency. Our findings enabled us to propose a molecular mechanism depicting the coordination of cytoskeletal rearrangements and membrane-trafficking during neurite growth.

Materials and methods

DNA constructs

The DNA fragments composing the mouse Trio CDS (1–6998) were PCR-amplified from mouse genomic DNA, mouse cerebellar cDNA, and IMAGE clones 6406413 and 6306998 (Thermo Fisher Scientific) and were inserted into the multiple cloning site (MCS)–modified pUC19 vector to generate pUC19–EcoRI–TRIO (1–6998)–XhoI–NotI using a ClonExpress MultiS one-step cloning kit (C112, Vazyme, Nanjing, China) and T4 DNA ligase (TaKaRa), following a series steps of molecular cloning. Trio9S (6984–7110) was PCR-amplified from IMAGE clone 6406413 (Thermo Fisher Scientific) and inserted into XhoI- and NotI-linearized pUC19–EcoRI–TRIO (1–6998)–XhoI–NotI using a ClonExpress II one-step cloning kit (C112, Vazyme) to construct pUC19–TRIO9S. The resulting pUC19–TRIO9S was then cloned into the EcoRI- and NotI-linearized MCS-modified pEGFP-C3 vector to construct the pEGFP–TRIO9S plasmid.

The mouse Rab8a CDS was PCR-amplified from mouse cerebellar cDNA and cloned into pMD19T vector (TaKaRa). The Rab8a(T22N) and Rab8a(Q67L) CDS were then cloned into the EcoRI- and NotI-linearized MCS-modified pEGFP-C3 vector to construct the pEGFP–RAB8A plasmid.

The mouse Rab10 CDS was PCR-amplified from mouse cerebellar cDNA and cloned into pMD19T vector (TaKaRa). The Rab10 CDS were then cloned into the EcoRI- and NotI-linearized MCS-modified pEGFP-C3 vector to construct the pEGFP–RAB10 plasmid.

In summary, we revealed a function of TRIO in regulating membrane trafficking during neurite outgrowth, in addition to the GEF-dependent function in regulating cytoskeletal dynamics by Rho GTPases. This function is accomplished by Golgi and the Golgi-derived vesicle pool of TRIO, whereby interaction with RABIN8 via the non-GEF domain. TRIO recruits and activates RABIN8 and subsequently activates RAB8 and RAB10 so as to regulate membrane trafficking by controlling the membrane vesicle switches frequency. Our findings enabled us to propose a molecular mechanism depicting the coordination of cytoskeletal rearrangements and membrane-trafficking during neurite growth.
Animals

The mice used in this study were *Tri**o** floxed mice (27), B6.Cg-Tg(Nes-cre)1Kn/J (Nestin-Cre mouse, 003771, The Jackson Laboratory), Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4)11Rth/J (Wnt1-Cre mouse, 003829, The Jackson Laboratory), Tg(Wnt1-Cre, 007676, The Jackson Laboratory), Tg(Wnt1-cro)11Rth Tg(Wnt1-GAL4)11Rth/J (Wnt1-Cre mouse, 003829, The Jackson Laboratory), and Nes-Cre, WKO) to delete TRIO in the cerebellum. Wnt1-Cre mice were isolated and trypsinized in TrypLE (catalog no. 12604-013, Life Technologies), according to the manufacturer’s instructions. COS-7 cells overexpressing FLAG-RAB10 were transfected by *Rab10*-specific siRNAs or control siRNA using Lipofectamine RNAiMAX (catalog no. 32011, Sudgen Biotech, Nanjing, China), according to the manufacturer’s instructions. COS-7 cells overexpressing FLAG-RAB10 were transfected by *Rab10*-specific siRNAs or control siRNA using Lipofectamine RNAiMAX (catalog no. 32011, Sudgen Biotech, Nanjing, China), according to the manufacturer’s instructions.

Cell culture and transfection

Neuro-2a cells were cultured in minimum essential medium (catalog no. 12571-063, Life Technologies), and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, catalog no. 12800-017, Life Technologies), both of which were supplemented with 10% fetal bovine serum (FBS, catalog no. 10099-141, Life Technologies), 100 units/ml penicillin, and 100 µg/ml streptomycin, in an atmosphere of 5% CO₂. Before transfection, the medium was replaced with fresh culture medium. Plasmid transfections were performed using Lipofectamine LipoMAX (catalog no. 32011, Sudgen Biotech, Nanjing, China), according to the manufacturer’s instructions. COS-7 cells overexpressing FLAG-RAB10 were transfected by *Rab10*-specific siRNAs or control siRNA using Lipofectamine RNAiMAX (catalog no. 13778-030, Life Technologies), according to the manufacturer’s instructions.

Neuronal culture

Cerebellar granule neurons were prepared from mice before postnatal day 8, as previously described (27). Briefly, cerebella were isolated and trypsinized in TrypLE (catalog no. 12604-013, Life Technologies) at 37 °C for 10 min and were passed through a fire-polished glass pipette to obtain a single cell suspension. Approximately 4–5 × 10⁴ CGNs were plated onto poly-d-lysine (100 µg/ml, P0899, Sigma–Aldrich)–coated 8-mm × 8-mm glass coverslips in wells of a 24-well plate in DMEM, 10% FBS at 37 °C and incubated for 4 h. The medium was then replaced with Neurobasal medium (catalog no. 21003-049, Life Technologies) supplemented with 2% B-27 (catalog no. 17504-

Generation of *TRIO* N terminus-specific antiserum

The CT233 antigen was selected, which was previously used as rat spectrin 5 and 6, to raise a rabbit anti-TRIO polyclonal antibody (35). The codon-optimized DNA sequence encoding mouse TRIO spectrin repeats 5 and 6, from amino acids Val⁶⁷⁴ to Arg⁹⁰⁰, was synthesized (Genscript, Nanjing, China) and cloned into the pGEX-5X-1 vector (GE Healthcare). The plasmid was expressed in DH5α cells, production of the GST fusion protein was induced with 0.1 mM IPTG, and the recombinant protein was purified using the batch purification method with GSH–Sepharose 4B (catalog no. 17-0756-01, GE Healthcare), according to the manufacturer’s instructions. The eluted protein was then dialyzed against PBS overnight and quantified using a Bio-Rad protein assay kit (catalog no. 500-0006, Bio-Rad). GST was not cleaved, and the fusion protein was diluted to 1 mg/ml in PBS. It was mixed well with an equal volume of Freund’s complete adjuvant (F5881, Sigma–Aldrich) by vortexing for 30 min at room temperature. 200 µl of the mixture (100 µg of fusion protein) were used to immunize BALB/c mice via intraperitoneal injections. These mice were immunized a second and third time by repeating the injections of 100 µg of fusion protein mixed with an equal volume of Freund’s incomplete adjuvant (F5506, Sigma–Aldrich) after 14 and 28 days, respectively. The mice were sacrificed 40 days after the first injection, and sera were collected, followed by the characterization of each antiserum by Western blotting. Of all the antiseras collected, the most specific antiserum was selected and used in the present study.

siRNAs

Double-stranded oligonucleotides targeting mouse *Rab8a* and *Rab10* were synthesized (GenePharma, Shanghai, China) with the following sequences: siRab8a#1, 5’-GCCUUCAACUCGAGCUUAAC-3’; siRab8a#2, 5’-CUCGAUGGCAAGGUGAUAA-3’; siRab8a#3, 5’-GGAUUUGGAUGCGCACUAU-3’; siRab10#1, 5’-UGAGGAUGUGGAAGAAGUG-3’; siRab10#2, 5’-UUGCGGCAUGCCUCAAU-3’; siRab10#3, 5’-GCAUCAUGUCAUGUAGA-3’; and control siRNA, 5’-UUCUCGGAACGGUGACUG-3’.

Animals

The mice used in this study were *Tri**o** floxed mice (27), B6.Cg-Tg(Nes-cre)1Kn/J (Nestin-Cre mouse, 003771, The Jackson Laboratory), Tg(Wnt1-cro)11Rth Tg(Wnt1-GAL4)11Rth/J (Wnt1-Cre mouse, 003829, The Jackson Laboratory), and B6.129(Cg)-Gt(Rosa)26So5(CAG-EGFP)Lne/J (Rosa-mTmG mice, 007676, The Jackson Laboratory). *Tri**o** floxed mice were crossed with Nestin-Cre mice (*Tri**o***floxed*, Nes-Cre, or *Tri**o***NKO*) to delete TRIO in the cerebellum. Wnt1-Cre mice were also crossed with Rosa-mTmG mice to examine Cre expression in the cerebellum and cultured CGNs. All animal procedures were performed according to the animal protocol approved by the Institutional Animal Care and Use Committee of the Model Animal Research Center of Nanjing University (Nanjing, China).
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044, Life Technologies), 1× GlutaMAX (catalog no. 35050-061, Life Technologies), and 1× penicillin/streptomycin (catalog no. 15140-122, Life Technologies) and cultured for the indicated times. To inhibit GEF1 activity, 100 μM ITX3 (HY-16663, MedChemExpress) (from 10 mM stock solution in DMSO) was used for 48 h, and its vehicle control was 1% DMSO.

Neuronal nucleofection

CGNs were prepared from P6-8 mice as described above, because a sufficient number of neurons can be obtained from mice at this age for nucleofection. CGNs from each cerebellum were suspended in 200 μl of nucleofection buffer, and each 100-μl cell suspension (~2 × 10⁷ neurons) was used for a nucleofection reaction using an Amaxa Nucleofector 2b device (Lonza) with program O-005. For the nucleofection of plasmids, 5 μg of each plasmid were used in a single reaction. The cells were then pipetted out of the cuvette in 1 ml of DMEM containing 10% FBS and aliquoted into three wells of a 24-well plate on poly-D-lysine–coated 8-mm wells, 5% CO₂, and 1% penicillin/streptomycin (catalog no. 15140122, Life Technologies) for 24–30 h and observed under a GE del taVision Elite microscope (GE healthcare) at 37 °C and 5% CO₂. Time-lapse images were acquired every 100 ms for a total of 30 s using a 60× objective lens (PlanApo NA1.42, oil immersion, Olympus). A pixel resolution of 0.108 μm/pixel was generated using this system, and deconvoluted images were generated. Kymographs of RAB8A- and RAB10-positive vesicles were generated using the KymoResliceWide plugin in ImageJ software, with a line width setting of 10 pixels, and the movement parameters, including distance traveled, switch frequencies, velocities, and percentage of time in motion, were semiautomatically analyzed using the KymoAnalyzer macro (44) in ImageJ software.

Live-cell imaging and kymograph analysis

Nucleofected neurons were cultured in phenol red-free Neurobasal medium (catalog no. 12348-17, Life Technologies) supplemented with 2% B-27 (catalog no. 17504-044, Life Technologies), 1× GlutaMAX (catalog no. 35050061, Life Technologies), and 1× penicillin/streptomycin (catalog no. 15140122, Life Technologies) for 24–30 h and observed under a GE del taVision Elite microscope (GE healthcare) at 37 °C and 5% CO₂. Time-lapse images were acquired every 100 ms for a total of 30 s using a 60× objective lens (PlanApo NA1.42, oil immersion, Olympus). A pixel resolution of 0.108 μm/pixel was generated using this system, and deconvoluted images were generated. Kymographs of RAB8A- and RAB10-positive vesicles were generated using the KymoResliceWide plugin in ImageJ software, with a line width setting of 10 pixels, and the movement parameters, including distance traveled, switch frequencies, velocities, and percentage of time in motion, were semiautomatically analyzed using the KymoAnalyzer macro (44) in ImageJ software.

FRAP analysis

Neurons grown in 35-mm glass-bottomed dishes for 1 DIV were transfected with pEGFP–hTfR with the calcium method using the CalPhos kit (Clontech), according to a previously described procedure (58), and the cells were allowed to express the fusion protein for another 24–30 h. FRAP experiments were performed using a Zeiss LSM880 confocal microscope with a controlled temperature of 37 °C in a humid chamber with a 5% CO₂ atmosphere. Five scans of prebleaching images were initially acquired to obtain a baseline intensity measurement. Bleaching was performed on selected neurites expressing EGF–hTfR using the 488-nm argon laser line at 100% power with 20 iterations. The intensity during fluorescence recovery was measured by acquiring 150 additional scans at 2-s intervals with a 2% laser power at a resolution of 512 × 512 pixels and an optical zoom of 2. Background correction and data normalization were performed using previously described methods (41). Briefly, a group of bleached neurites, a nonbleached region, and background region were first determined, and the intensity of the background region was subtracted from the intensity of the bleached neurites and nonbleached region at each time point for background correction. The corrected intensity of the bleached neurites was divided by the intensity of the nonbleached region and normalized to the average intensity of the five prebleaching scans.
**Subcellular fractionation**

Subcellular fraction was prepared using OptiPrep Axis-
Shield density gradient medium according to the procedure of the application sheet S24 (Alere Technologies AS, Oslo, Norway) with modifications. Briefly, the cerebellum was iso-
lated from P10 mouse and homogenized in 1 ml of homogeni-
ization medium (0.25 M sucrose, 1 mM EDTA, and 10 mM
HEPES-NaOH, pH 7.4) by Dounce tissue grinder and five passes
through a fine syringe needle. Postnuclear supernatant was pre-
bred by centrifuging the homogenate at 1500 × g for 10 min. Discon-
tinuous OptiPrep gradient was prepared using under-
layering technique by layering 2 ml of the following solutions:
2.5, 5, 10, 15, 20, and 30% iodixanol in homogenization
medium. Approximately 700 µl of postnuclear supernatant was
uploaded to the gradient and centrifuged at 88,000 × g at 4 °C
for 16 h using SW32 rotor (Beckman, German). Twenty-eight
fractions (500 µl each) were collected from the top, supple-
mented with 4 × Laemmli buffer, boiled at 95 °C for 10 min, and
subjected to Western blotting.

**Purification of the GST fusion protein**

GST, GST–RABIN8, GST–JFC1, GST–MICAL-L2-C, GST–
RABAPTIN5–R5BD, GST–FIP3–RBD11, and GST–PAK–
GBD were expressed in DH5α cells that had been induced with
0.1 mM IPTG at 37 °C for 4 h, and GST–TRIO (1–230), GST–
TRIO (446–909), and GST–TRIO (672–1295) were expressed
in BL21(DE3) cells that had been induced with 0.1 mM IPTG at
30 °C for 4 h. All GST fusion proteins were purified with GSH–
Sepharose 4B (catalog no. 17-0756-01, GE Healthcare) using
the gravity flow method, according to the manufacturer’s
instructions. Eluted GST–TRIO variants were dialyzed
against buffer containing 20 mM HEPES-NaOH, pH 7.4, and
150 mM NaCl; other GST proteins were dialyzed against 50
mM Tris-HCl, pH 7.4, overnight; and protein concentrations
were determined using a Bio-Rad protein assay kit (catalog
no. 500-0006).

**GST pulldown assay**

The P21 mouse cerebellum was lysed in lysis buffer (50 mM
Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton
X-100, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml apro-
tin). The lysate was cleared by centrifugation at 12,000 rpm
for 10 min, and equal amounts of cleared lysate were incubated
with 40 µl of a 50% slurry of GST–Sepharose 4B and 20 µg of
purified GST or GST–RABIN8 overnight at 4 °C. The beads
were then washed three times with 500 µl of lysis buffer prior to
the addition of 2 × Laemmli buffer. Samples were boiled at
95 °C for 10 min and subjected to Western blotting.

**Purification of the His6–RABIN8 and direct binding assay**

His6–RABIN8 were expressed in BL21(DE3) cells that had
been induced with 1 mM IPTG at 37 °C for 4 h. Bacteria pellets
were then sonicated in PBS with 1 mg/ml lysozyme. Proteins
were then pelleted by centrifugation at 9000 rpm for 10 min and
dissolved in PBS with 8 M urea. Cleared supernatant were then
incubated with nickel–nitrotriacetic acid beads (Life Technol-
ologies) at room temperature for 1 h. The beads were first washed
by gravity flow with 100 mM Tris-HCl, pH 8.0, 6 M urea, and 20
mM imidazole and then washed another three times by the same
wash buffer in which the urea concentration was 4, 2, and 0 M.
The purified His6–RABIN8 was eluted with 500 mM imidazole
in 100 mM Tris-HCl, pH 8.0, and dialyzed against buffer con-
taining 20 mM HEPES-NaOH, pH 7.4, and 150 mM NaCl. The
concentration was determined using a Bio-Rad protein assay kit
(catalog no. 500-0006, Bio-Rad). Direct binding of His6–
RABIN8 to GST–TRIO variants was assayed by diluting 10 µg
of purified His6–RABIN8, 20 µg of purified GST–TRIO vari-
ants, and 40 µl of a 50% slurry of GST–Sepharose 4B (catalog
no. 17-0756-01, GE Healthcare) in 600 µl of binding buffer (20
mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.1% Triton X-100).
The samples were incubated overnight at 4 °C. The superna-
tants were collected, and the beads were washed three times
with binding buffer and boiled at 95 °C for 10 min after the
addition of Laemmli buffer. For Western blotting, 1/10 of
pellet samples and 1/100 of supernatant samples were loaded to
SDS-PAGE.

**Immunoprecipitation**

Immunoprecipitation was performed to determine the inter-
action of RABIN8 and TRIO, and RABIN8 phosphorylation.
Mouse cerebella, Neuro-2a cells, COS-7 cells, or CGNs cul-
tured in 60-mm dishes were lysed in lysis buffer (50 mM
Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton
X-100, 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml apro-
tin). For RABIN8 immunoprecipitation to determine the phos-
phorylation level, 1× PhosSTOP phosphatase inhibitor mixture
(catalog no. 04906845001, Roche) was included in the lysis
buffer. Lysates were cleared by centrifugation at 12000 rpm for
10 min; some cleared supernatants were retained as the total
input, and the remaining lysates were incubated with 20 µl of a
50% slurry of Anti-FLAG M2 affinity gel (A2220, Sigma–
Aldrich) or 40 µl of a 50% slurry of protein A–Sepharose
(catalog no. 17-0974-01, GE Healthcare) plus the rabbit anti-
TRIO antibody (H120, sc-28564, Santa Cruz Biotechnology), the rab-
bit anti-RABIN8 antibody (12321-1-AP, Proteintech), or equal
amount of the normal rabbit IgG (sc-2027, Santa Cruz Biotech-
nology) overnight at 4 °C. Bound proteins were eluted as
described above and subjected to Western blotting.

**Rab GTPases activation assay**

GST–MICAL-L2-C, GST–Rabaptin5–R5BD, GST–FIP3–
RBD11, and GST–JFC1 were purified as described above.
Rab GTPases activation assays were performed as previously
described (51). For the GST pulldown, cerebella were lysed in
600 µl of lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 5
mM MgCl2, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, 10
µg/ml leupeptin, and 10 µg/ml aprotinin), and the Neuro-2a
cells cultured in wells of a 6-well plate that had been transfectioned
with pEGFP-C3, pEGFP-TRIO9S, and pEGFP-TRIO8 were
serum-starved overnight at 36 h after transfection and were
then lysed with 300 µl of lysis buffer. An aliquot of the cleared
lysates (60 µl of cerebellar lysates or 30 µl of Neuro-2a lysates)
was retained as the total protein sample, and the remaining
lysates were incubated with 20 µg of GST–JFC1 and 40 µl of a
50% slurry of GST–Sepharose 4B (catalog no. 17-0756-01, GE
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Healthcare) at 4 °C for 4 h, followed by three washes with 400 μl of lysis buffer. The bound GTP-Rabs was eluted by boiling with Laemmli buffer and was detected by Western blotting.

**RAC1 activation assay**

RAC1 activation assays were performed using a previously described procedure (59), with minor modifications. Neuro-2a cells were lysed in lysis buffer (50 mM Tris·HCl, pH 7.2, 1% Triton X-100, 500 mM NaCl, 10 mM MgCl₂, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and the cleared lysates were incubated with 20 μg of GST-PAK-GBD and 40 μl of a 50% slurry of GSH–Sepharose 4B (catalog no. 17-0756-01, GE Healthcare) at 4 °C for 45 min. The beads were then washed three times with 400 μl of lysis buffer and were boiled to elute the bound GTP-RAC1. For experiment in Fig. 7E, the supernatant was collected and directly used for RABIN8 immunoprecipitation to detect phosphorylated RABIN8 level.

**Protein extraction and Western blotting**

The mouse cerebellar tissue was lysed at the indicated time points in modified RIPA buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 1 × protease inhibitor mixture (catalog no. 04693132001, Roche). Western blotting was performed to detect protein expression, GST pulldowns, direct binding assay, immunoprecipitations, RAB8/RAB10 activation assay, and RAC1 activation assay. The antibodies used were TRIO (1:200, H120, sc-28564, Santa Cruz Biotechnology), the homemade TRIO antiserum (1:2000), GM-130 (1:1000, clone 4/Rab8, 610844, BD Biosciences), RAB8 (1:1000, clone 4/Rab8, 610844, BD Biosciences), RAB10 (1:1000, 11808-1-AP, Proteintech), phospho-mitogen-activated protein kinase/cyclin-dependent kinase substrates (1:500, clone 40/BiP, 610978, BD Biosciences), RAB1 (1:1000, clone 47/Rab11, 610656, BD Biosciences), RABIN8 (1:1000, 12321-1-AP, Proteintech), phospho-mitogen-activated protein kinase/cyclin-dependent kinase substrates (1:1000, 34B2, catalog no. 2325, Cell Signaling), FLAG (1:1000, clone M2, F1804, Sigma–Aldrich), RAC1 (1:1000, clone 23A8, catalog no. 05–389, Millipore), ERK1/2 (1:1000, BS3627, BioWorld), and β-actin (1:5000, clone AC-15, A1978, Sigma–Aldrich). For blotting RABIN8 immunoprecipitations, a mouse secondary antibody specific to rabbit IgG light chain was used (1:5000, ab99697, Abcam).

**Statistical analysis**

All quantification analysis of Western blotting was performed using ImageJ software. In all experiments using mice, n represents the number of mice used. The data are presented as the means ± S.E. The statistical analysis was performed using Prism 6.05 software. As indicated in the figure legends, Student's t test or one-way ANOVA with Bonferroni's test was used for tests with only one variable, and a two-way ANOVA with Tukey's test was used for tests with two independent variables. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

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