Rufy3 is an adapter protein for small GTPases that activates a Rac guanine nucleotide exchange factor to control neuronal polarity

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Small GTP-binding proteins (also known as small GTPases) form a central large group that act as transducers in various cellular events (1). GTPases are classified into the Ras, Rho, and Rab subfamilies with each playing a specific role in signaling pathways, cytoskeletal rearrangement, and intracellular trafficking. These proteins share several common biochemical properties; for example, their activated (GTP-bound) form is highly motile at the tips of the extending axon. Accurate synaptogenesis requires formation of growth cones with highly motile activity at the tips of the extending axon. We previously conducted proteomic analysis of rodent growth cone proteins and showed that these proteins from more than 30 rodent species are small G proteins (4, 8). Of these, Rap2 was the most abundant membrane-bound Ras family protein, and Rufy3 was the most abundant adapter protein specific to small G proteins (4, 8). We recently confirmed that these two proteins form a complex and are colocalized in the growth cone (9). This complex is formed prior to axon growth in the first step of the determination of neuronal polarity (9) during which time a single axon grows in a neuron (10, 11). Complex formation during this step is dependent upon an abundant membrane protein, glycoprotein M6A (GPM6a) (4, 8). Using GPM6a knock-out (KO) mice, we recently demonstrated that GPM6a is responsible for neuronal polarity via the Rufy3-Rap2 complex (9). This protein complex accumulates in lipid rafts and activates T-cell lymphoma invasion and metastasis 2 (Tiam2/STEF), a Rac1 activator (9). Rufy3 and Tiam2/STEF were previously known as RUN and FYVE domain–containing 3 (Rufy3; also known as Single1/Rap2-interacting protein X), lacks an FYVE domain, which interacts with phosphatidylinositol 4,5-bisphosphate, and therefore cannot directly associate with the cell membrane (2). The analysis of Rufy3 using X-ray crystallography has shown that Rap2, a Ras family member, is tightly and stably bound to Rufy3 in a GTP- and RUN domain–dependent manner (3).

Small G proteins are critical in various steps of neuronal development in the mammalian brain (4–7). For example, accurate synaptogenesis requires formation of growth cones with highly motile activity at the tips of the extending axon. A group of such adapter proteins was recently shown to contain a RUN domain which is involved in binding small G proteins (2) of which one, RUN and FYVE domain–containing 3 (Rufy3; also known as Singar1/Rap2-interacting protein X), lacks an FYVE domain, which interacts with phosphatidylinositol 4,5-bisphosphate, and therefore cannot directly associate with the cell membrane (2). The analysis of Rufy3 using X-ray crystallography has shown that Rap2, a Ras family member, is tightly and stably bound to Rufy3 in a GTP- and RUN domain–dependent manner (3).

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Here, we further investigated the mechanism underlying Rufy3-dependent processes by producing Rufy3-KO mice and examining the properties of Rufy3-deficient neurons. We found that Rufy3 is recruited via GPM6a to detergent-resistant membrane domains, which are biochemically equivalent to lipid rafts. We also showed that Rufy3 is a component of a ternary complex that induces the assembly of Rap2 in the growth cone. In the absence of Rufy3, the accumulation of a Rac guanine nucleotide exchange factor, Tiam2/STEF, is inhibited downstream of Rap2. In addition, Rufy3 regulates the localization of Rap2 and Tiam2/STEF. Taking these findings together, we conclude that Rufy3 is a physiological adapter for Rap2 and activates Tiam2/STEF for polarity determination under the regulation of GPM6a.

### Results

**Rufy3 deficiency leads to multiple-axon formation irrespective of the localization of GPM6a in cortical neurons**

To further examine the physiological functions of Rufy3 in the determination of polarity, we generated KO mice deficient in Rufy3 using C57BL/6N strain mice (Fig. 1A; also see Fig. S1, A and B). Deficiency in Rufy3 was confirmed using Southern and Western blot analyses (Fig. 1B; also see Fig. S1, A and B). Rufy3-KO mice died just after birth. These mice did not show macroscopic brain abnormalities or microscopic defects in major neural networks, and therefore we first verified the formation of neuronal polarity in their embryonic cortical neurons. Consistent with the effects of Rufy3 RNAi (13), significant multiple-axon formation was observed in the *rufy3*−/− neurons at 2 days *in vitro* (Fig. 1, C–G). Overexpression of EGFP-Rufy3 in KO neurons suppressed excessive axon formation (Fig. S2, A and B), indicating that Rufy3 is involved in the determination of neuronal polarity. We previously reported that the determination of neuronal polarity is induced within a few hours of plating neurons on laminin (LN) (9) (summarized in Fig. 1C). To examine the effects of Rufy3 deficiency on GPM6a-regulated signaling, we confirmed neuronal polarity in wild type (WT) and *rufy3*−/− neurons 30 min after plating on LN (Fig. 1D). Multiple neurites protruded from the *rufy3*−/− neurons, whereas single protrusions were observed for WT neurons (Fig. 1, E and F). As we demonstrated previously (9), this neurite protruded from the GPM6a-assembled membrane area in the WT neuron, whereas the multiple neurites from *rufy3*−/− neurons and *gpm6a*−/− neurons (9) were positioned irrespective of GPM6a localization (Fig. 1, H–J). These results suggested that Rufy3 is involved in the regulation of neuronal polarity determination by GPM6a.

**Rufy3 is recruited to detergent-resistant membrane domains via GPM6a**

We recently reported that Rufy3 directly binds to GPM6a (9), and the complex colocalizes in the peripheral (P-) domain of the axonal growth cone (Fig. 2A; also see Fig. S3A). Previously, Rufy3 was reported to interact with fascin, an actin-bundling protein (14); however, our immunoanalysis showed that Rufy3 is localized at the edge of the growth cone rather than colocalized with F-actin bundles such as fascin (Fig. S3) (15). We further examined the interactions between Rufy3 and GPM6a in pulldown experiments using EGFP-tagged Rufy3 fragments and FLAG-GPM6a. The binding of Rufy3 to GPM6a required the Rufy3 RUN domain (Fig. 2B) as previously observed for the binding of Rufy3 to Rap2 (3, 9).

According to our proteomics data, Rufy3 is an abundant cytosolic protein in the axonal growth cone (8), and thus we investigated the effects of Rufy3 binding to GPM6a on the localization of the complex using a reconstitution assay. The results showed that EGF-Rufy3 was originally distributed in the cytosol and then was transferred to the tips of the protrusions by colocalization with mCherry-GPM6a (Fig. 2C). As shown previously (9), we confirmed that detergent-resistant membrane (DRM) even prepared from embryonic brains represents the lipid rafts as described previously (16). DRM analysis showed that EGFP-Rufy3 coexpressed with GPM6a was partially distributed in the DRM fractions, although proteins expressed individually were exclusively localized in non-DRM fractions (Fig. 2, D and E). Similarly, endogenous Rufy3 was distributed in the DRM fractions of embryonic WT brains but not in brains from *gpm6a*−/− embryos (Fig. 2, F and G), indicating that Rufy3 is captured by GPM6a and accumulates in lipid rafts through Rufy3-GPM6a interactions.

**Rufy3 mediates between GPM6a and GTP-bound Rap2**

We previously reported that the active form (GTP-bound; i.e. V12G constitutively active mutant) of Rap2 specifically binds to Rufy3, and GPM6a forms a ternary complex with Rufy2 and Rufy3 (9). In coimmunoprecipitation experiments, the active form (V12G), but not the inactive form (S17N), of Rap2 was selectively coprecipitated with GPM6a via Myc-Rufy3 in a dose-dependent manner (Fig. 3A). Because the ternary complex asymmetrically accumulates at the origin sites of neurites (9), we hypothesized that Rufy3 intermediated between GPM6a and Rap2 to assemble the ternary complex and that this complex participates in the downstream signaling of GPM6a. To examine this possibility, we compared the localization of Rap2 at the growth cone in both WT and *rufy3*−/− neurons.

Immunofluorescence analysis indicated that Rap2 is not specifically localized at the growth cone and is independent of the developmental stage of *rufy3*−/− neurons (Fig. 3, B–D). In contrast, GPM6a accumulated at the growth cone despite the deficiency of Rufy3, indicating that Rufy3 is required for Rap2, its downstream molecule, but not for its upstream molecule, GPM6a. Taken together with our previous report (9), we conclude that Rufy3 is required for the recruitment of Rap2 to form a ternary complex downstream of GPM6a-dependent signaling. Live imaging analysis of EGFP-Rap2 revealed that Rap2 stably remained in the growth cone in WT but not *rufy3*−/− neurons (Fig. 3, E–H). These results indicate that Rufy3 induces formation of a GPM6a-Rufy3-Rap2 ternary complex, acts as an adapter between GPM6a and Rap2, and assembles active GTP-bound Rap2 in the axonal growth cone.

**Deficiency of Rufy3 inhibits the accumulation of Tiam2/STEF downstream of Rap2**

We previously identified Tiam2/STEF as an active Rap2 partner and demonstrated its colocalization with GPM6a-Rufy3-Rap2 ternary complex in the cortical neuron (9). Tiam2/STEF,
Abnormal polarity in Rufy3-KO neurons

A. Rufy3-KO neurons

B. WT vs. Chimera

C. Plating on LN

D. Phalloidin DII

E. Neurons (%)

F. Neurons (%)

G. Number of Neurites

H. EGFP-GPM6a

I. EGFP-GPM6a
Abnormal polarity in Rufy3-KO neurons

To confirm that Rap2 and Tiam2/STEF affect the same pathway controlling polarity downstream of Rufy3, we performed an inhibition assay with Rufy3−/− neurons using domain-negative mutations of Rap2 or Tiam2/STEF. The ratio of Rufy3−/− polarized neurons was significantly decreased compared with control (WT) neurons on LN (Fig. S5, A–D), and neither overexpression of dominant-negative Rap2 nor overexpression of STEF significantly affected the reduced ratio of polarized Rufy3−/− neurons (Fig. S5, A–D). These results are consistent with the idea that Rap2 and STEF are downstream of the Rufy3 in the signaling pathway for polarity determination.

STEF was reported to be associated with the Par6-Par3-atypical PKC polarity complex through its direct interaction with Par3 (12). We examined whether localization of the Par complex in the growth cone was also regulated downstream of Rufy3. To show whether Rap2 and Tiam2/STEF act as positive regulators for neuronal polarity by activating Rac1 (18), we assessed the GEF activity of Tiam2/STEF by its interaction with active Rap2 (9). We examined whether suppression of Rap2 accumulation in Rufy3-KO mice affected Tiam2/STEF localization, as previously characterized as a regulator of neuronal cell polarity (20), Rufy3 is already known as one of the cell-autonomous polarity determinants; however, using Rufy3-KO mice, we confirmed that Rufy3 is also physiologically involved in the determination of neuronal polarity (Figs. 1, C–I, 3, C–H, and 5) and axon growth at a later stage (Fig. 4, D–F). Consistent with other molecules involved in neuronal polarity, deficiency of Rufy3 alone in vivo did not result in large defects in the network, similar to several other cell-autonomous polarity determinants (10). We also demonstrated the importance of Rufy3 in its role in controlling its upstream partner protein, GPM6a, with its downstream partner, Rap2, in lipid rafts. With Rufy3, GPM6a signaling was effectively transduced to activate STEF/Tiam2 as demonstrated previously (9), whereas in the absence of Rufy3, Rac activation by STEF/Tiam2 was not effective (Figs. 3E and 4, D–F, and Figs. S3 and S5).

Because Rufy3-KO mice immediately died after birth with severe cyanosis but did not show marked developmental defects of the nervous system, we suspect that this death is probably due to a circulation problem. RPIP8, another binding partner of Rap2 enriched in brain that also has a RUN domain, may partially and functionally rescue the Rufy3 deficiency (21). Rufy3 was previously characterized as a regulator of neuronal cell polarity and to act in a cell-autonomous manner (14) as we confirmed using Rufy3−/− neurons (Fig. 1, C and D). However, polarity determination was delayed in Rufy3−/− neurons more markedly than in LN-dependent neurons (Fig. 1, D–F), consistent with our recent results showing that GPM6a, Rap2, and}

**Figure 1. Generation of Rufy3-KO mice and abnormalities in the polarity of their neurons.** A, strategy for conditional inactivation of the mouse Rufy3 gene. B, BamHI site; Neo, neomycin resistance gene; DT, diphtheria toxin gene. B, Southern blot analysis of genomic DNA isolated from WT ES cells and chimeric clones. The DNA was digested with BamHI and hybridized with 5’ and 3’ probes. Each band was detected at the expected size. C, schematic models of the time course in neuronal polarity determination of the cultured neurons plated on LN. The schematics show the representative neuronal shapes at each time point. Developmental stages of the neurons were morphologically defined according to the definition of Dotti (19); i.e., stage 1, cells with no neurite (gray); stage 2, multipolar cells (green); and stage 3, polarized cells (pink). In the WT, the neurons dramatically changed from stage 1 to 3 within 1–4 h after plating on LN. In contrast, the gpm6a−/− neurons still remained at stages 1 to 2 at the same time course (see Ref. 9). D–G, cortical neurons derived from WT or Rufy3−/− mice were cultured for 30 min or 48 h on LN. D, the plasma membrane (green) and F-actin (red) were stained using Dil and phalloidin, respectively. Scale bars, 50 (upper) and 20 μm (lower). E and F, percentage of neurons at stage 1, 2, or 3 from WT or Rufy3−/− mice plated for 30 min (E) or 48 h (F) on LN (see also Ref. 9) (one-way analysis of variance with Tukey’s multiple comparisons test; n 500 cells (E) and n 300 cells (F) for each group; error bars represent S.D. of the percentage of the stage 3 neurons in each group). G, the numbers of neurites of WT or Rufy3−/− mice after 48-h culture (two-tailed t test (means ± S.D.); WT (1.203 ± 0.661) versus Rufy3−/− (1.30 ± 1.944); ****, p < 0.0001; n = 300 from five distinct preparations; error bars represent S.D.). H, in LN-dependent cultures, growth cones (arrowheads) protruded from the GPM6a-enriched membrane area (asterisk) in the cortical neurons of WT but not from Rufy3−/− mice. Scale bar, 20 μm. I, time-lapse imaging of EGF-GPM6a overexpressed in the cortical neurons of WT and Rufy3−/− mice for 80 min after plating on LN. The fluorescence intensities of EGF-GPM6a are shown as pseudocolor images. Neurites (arrowheads) protruded from the GPM6a-enriched membrane area (asterisk) in WT but not in Rufy3−/−. Scale bar, 20 μm. P-LILys. AASBMB J. Biol. Chem. (2017) 292(51) 20936 –20946 20939
Tiam2/STEF are highly concentrated in lipid rafts and that the presence of Rap2 and Tiam2/STEF are GPM6a-dependent (9).

Rufy3 was previously reported to interact with fascin and control axon growth (14); however, our results did not show its colocalization with F-actin (Fig. S3). Because fascin is specifically localized in the F-actin bundles of the P-domain (Fig. S3; also see Ref. 20) and Rufy3 was mainly localized in the membrane area (Fig. S3), we could not confirm colocalization of these two proteins as reported previously (14) using our antibody (Fig. S3).

**Abnormal polarity in Rufy3-KO neurons**

![Image](image-url)
Previous in vitro studies showed that Rufy3 interacts with other small G proteins, including Rab family members such as Rab33 (22) reported to be involved in axon growth (23). However, we observed that Rufy3 was mainly distributed to the plasma membrane or the cytosol (Fig. 2A and Fig. S3A) and did not show the vesicular distribution along the cytoskeleton. These results indicate that Rufy3 is physiologically bound to Rap2 in the developmental stages of neurons unlike Rab33.

In conclusion, Rufy3 is believed to be an adapter of Rap2 as an upstream protein for the determination of neuronal polarity. We recently visualized the recycling of lipid raft domains in the growth cone using super-resolution microscopy (24) and will apply this new methodology to study the dynamics of Rufy3 using Rufy3-KO mouse neurons.

**Experimental procedures**

**Animals**

We obtained C57BL/6J BAC genomic clone RP24-132B11 mice, which contain the Rufy3 gene, from the BACPAC Resources Center. To construct the Rufy3-targeting vector, a 0.86-kb drug fragment carrying exon 3 of Rufy3 was amplified by PCR and inserted into the KpnI-Sacl sites of a middle entry clone plasmid (pDM-1). In this plasmid, a DNA fragment of phosphoglycerate kinase promoter–driven neomycin resistance gene-poly(A) (pgk-Neo) is flanked by two fRT sites and a loxp sequence located 249 bp upstream of exon 3, and a second loxP sequence is placed 269 bp downstream of exon 3. The 3.02-kb upstream and 5.45-kb downstream homologous genomic DNA fragments were subcloned into the 5’ entry clone (pD5UE-2) and 3’ entry clone (pD5DE-2), respectively, using a Quick and Easy BAC modification kit (Gene Bridges, Dresden, Germany). To target vector assembly, the three entry clones were recombined to a destination vector plasmid (pDEST-DT; containing a CAG promoter–driven diphtheria toxin gene) using a MultiSite Gateway Three-fragment Vector construction kit (Invitrogen). The Rufy3-flox mouse line was established by introducing the linearized targeting vector into the C57BL/6N–derived embryonic stem cell line RENKA, and then recombinant clones were selected using medium containing 175 μg/ml G418. ES cells were cultured as described previously (23). The targeted clones were confirmed by Southern blot analysis using 5’, 3’, and neomycin resistance gene probes. Chimeric mice were generated as described previously (25).

Briefly, targeted clones were microinjected into eight–cell-stage embryos of the CD-1 mouse strain. The resulting chimeric embryos were developed to the blastocyst stage by incubation for more than 24 h and then transferred to pseudopregnant CD-1 mouse uteruses. Germ line chimeras were crossed with C57BL/6N female mice, and the heterozygous offspring were crossed with a Tlcn-Cre deleter mouse line (26) to establish the Rufy3 knock-out mouse line.

**Neuronal cell culture**

Dissociated neurons were cultured as described previously (9). The cortices of E14.5 embryonic mice were dissected in cold DMEM (Wako) and incubated in AccuMax (Innovative Cell Technologies) in Hanks’ balanced salt solution (Wako) for 10 min at 37 °C. Neurons were suspended in 10% FBS (Gibco) in DMEM, filtered through a 40-μm Cell Strainer (BD Falcon), then counted, and plated on coverslips coated with LN (2 μg/ml; Invitrogen) using Neurobasal medium (Gibco) supplemented with B27, GlutaMAX (×1 final concentration; Gibco), and penicillin/streptomycin. For transfection, dissociated neurons (1 × 10^6) or COS-7 cells (1 × 10^6) were placed in 2-mm gap cuvettes (BEX Co., Ltd., Tokyo, Japan) containing 100 μl of Opti-MEM (Gibco) with 2–5 μg of plasmid DNA and then transfected using 10 direct-current 20-V electrical pulses at intervals of 50 ms using an electroporator (CUVY21 Vitro-EX, BEX Co., Ltd.).

**Plasmid construction**

cDNA fragments of GPM6a and Rufy3 were amplified from a mouse brain cDNA library by PCR and subcloned into pmCherry-C1 (Clontech), pEGFP-C1 (Clontech), or pEF1-Myc plasmid vector (Clontech). DNA fragments encoding the RUN domain (aa 1–255), two coiled-coil domains (CC1 and CC2 domains; aa 255–447), and RUN-CC1 (aa 83–396) of Rufy3 were coexpressed with FLAG-GPM6a in COS-7 cells. EGFP-Rufy3 fragments and coimmunoprecipitation (IP) of GPM6a were detected with both anti-GFP and anti-FLAG antibodies using Western blotting (WB), respectively. C. EGFP-Rufy3 (green) changes its localization upon coexpression with mCherry-GPM6a (magenta) in COS-7 cells. The translocated EGFP-GPM6a was colocalized with mCherry-GPM6a at the tips of the protrusions (arrow). A high magnification image of the protrusions in the inset is shown in the left panel. Scale bar, 10 μm. D and E, translocation of EGFP-Rufy3 to DRM fractions, induced by coexpression of FLAG-GPM6a in COS-7 cells, with or without FLAG-GPM6a. D, translocation of EGFP-Rufy3, induced by coexpression of FLAG-GPM6a in COS-7 cells, with or without FLAG-GPM6a. The cells were lysed using 1% Bri198 and the whole-cell extract (Ex) was fractionated into DRM and non-DRM fractions. The cells were lysed using 1% Bri198 and fractionated into DRM and non-DRM fractions. The distribution of EGFP-Rufy3 was analyzed using EGFP antibody. Endogenous flotillin 1 was used as a lipid raft marker. EGFP-Rufy3 was partially translocated from the non-DRM to the DRM fractions. E, ratio of immunofluorescence of EGFP-Rufy3 in the DRM fractions to that in total EGFP-Rufy3 (two-tailed t test (means ± S.E.); Rufy3 (2.41 ± 0.25) versus Rufy3 + GPM6a (1.122 ± 0.74); ****, p < 0.0001; n = 5; error bars represent S.E.). F, differential localization of endogenous Rufy3 between WT and GPM6a-KO brains. Homogenized brains (E14.5 WT or gpm6a/−/− embryos) were treated with 1% Bri98, DRMs were fractionated, and the distribution of Rufy3 was analyzed as described above. In WT but not in gpm6a/−/−, Rufy3 was partially distributed in the DRM fractions. G, ratio of Rufy3 in the DRM fractions to its total amount (two-tailed t test (means ± S.E.); WT (5.45 ± 0.2452) versus gpm6a/−/− (2.085 ± 0.3087); ****, p < 0.0001; n = 5; error bars represent S.E.).
Transfection of neurons

Dissociated neurons (1 × 10^6) or COS-7 cells (1 × 10^6) were placed in 2-mm gap cuvettes containing 100 μl of Opti-MEM with 2–5 μg of plasmid DNA and then transfected as described under “Neuronal cell culture.”

Immunofluorescence labeling

Indirect immunofluorescence labeling was performed as described previously (9) using the antibodies shown in Table S1. The cells were fixed for 30 min with 4% paraformaldehyde (PFA) in PBS (pH 7.4), and then the cells were permeabilized for
5 min with 0.1% Triton X-100 in PBS and incubated in 2% BSA in PBS for 1 h at room temperature, in 2% BSA in PBS for 1 h, and with 2% BSA in PBS containing primary antibodies overnight at 4 °C. Subsequently, the cells were rinsed and incubated with secondary antibody for 3 h three times with PBS and then mounted in fluorescent mounting medium (Dako).

Coronal slices (50 μm thick) were prepared from fixed brains using a Leica VT1000S Vibratome. The sections were permeabilized with 1% Triton X-100 in PBS for 1 h at room temperature and then blocked with 5% BSA in PBS for 1 h. Slices were incubated with 5% BSA in PBS containing primary antibodies overnight at 4 °C, rinsed with PBS, incubated with secondary
antibody for 3 h, then washed with PBS, and mounted in fluorescent mounting medium. Preparations were examined under Olympus BX63 optical, Olympus FV2000 confocal, and Zeiss LSM510 confocal microscopes.

E16.5 mice were fixed by cardiac perfusion with 4% PFA in PBS (pH 7.4). The brains were dissected and soaked in 4% PFA in PBS (pH 7.4) at 4 °C overnight, and then the fixed brains were stored in 30% sucrose in PBS at 4 °C. The brains were frozen in powdered dry ice and embedded in OCT compound (Tissue-Tek, Sakura Finetek USA Inc.), and 30-μm-thick coronal sections were sliced using a sliding cryotome (Leica CM1850). Coronal sections were rinsed with PBS and incubated in 0.1% Triton X-100 and 3% H2O2 in PBS for 15 min at room temperature. After rinsing with PBS, the sections were incubated overnight at 4 °C with anti-Rufy3 antibody (1:1000) in 5% BSA in PBS, then rinsed with PBS, and incubated with biotinylated anti-rabbit IgG antibody (1:200) for 30 min at 37 °C. After rinsing with PBS, the sections were incubated in avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 30 min at 37 °C, rinsed with PBS, and then incubated in 50 mM

Figure 5. Loss of Rufy3 disturbs the accumulation of neuronal polarity determinants in the growth cone. A, immunofluorescence staining of GPM6a (red) and Par3 (green) in WT and rufy3−/− cortical neurons (48 h after plating on LN). Arrows, growth cones. Scale bar, 50 μm. B, immunofluorescence intensity plot profiles of anti-GPM6a (red) and anti-Rufy3 (green) from the axon shaft to the growth cone (tip) are shown as dotted lines in A. C, ratio of the immunofluorescence intensity of Par3 in the growth cone versus that of the axon (two-tailed t test (means ± S.E.); WT (2.87 ± 0.23) versus rufy3−/− (1.06 ± 0.07); ****, p < 0.0001; n = 25; error bars represent S.E.). D, immunofluorescence staining of phalloidin (red) and anti-STEF antibody (green) in WT and rufy3−/− cortical neurons (48 h after plating on LN). High magnification images of insets are shown in the left panels. Arrows, growth cones. Scale bars, 100 (left) and 20 μm (right). E, detailed fluorescence plot profiles of each fluorescence image along the a–b lines in D are shown.
Tris-HCl (pH 7.4) containing 0.01% H₂O₂ and 0.01% diaminobenzidine tetrahydrochloride at 37 °C for 10 min. The stained sections were mounted on MAS-coated glass slides (Matsunami Glass, Osaka, Japan) using fluorescent mounting medium. Bright-field images were taken with an Olympus BX63 microscope and DP-72 charge-coupled device camera.

**Time-lapse experiment**

Time-lapse recording of EGFP-GPM6a or EGFP-Rap2 was analyzed as described previously (9). pEGFP-C1-GPM6a or pEGFP-C1-Rap2 plasmid was electroporated into dissociated E14.5 mouse cortical neurons, and the transfected cells were precultured in L-15 medium (Gibco) supplemented with 10% FBS, B27, GlutaMAX, 0.45% glucose, and penicillin/streptomycin in a Lipidure-coated 96-well plate (NOF Corp., Japan) at 37 °C without CO₂ for 24 h. The cells were dissociated with AccuMax and plated on a glass-bottom dish coated with LN. EGFP-positive neurons were observed in a temperature-controlled incubation chamber (at 37 °C; Tokai Hit) fitted onto a confocal microscope (Olympus FV2000). Fluorescence images were obtained with a cooled charge-coupled device camera and processed using image analysis software (Metamorph, Molecular Devices, CA).

**Polarity assay**

Neuronal polarity was analyzed as described previously (9). Briefly, at 30 min after plating, neurons with a single short filopodium or lamellipodium protruding within a 90° angle from the center were considered as monopolar, i.e. stage 1 neurons. Neurons with two or more filopodia or lamellipodia protruding within a 90° angle were considered as bi- or multipolar, i.e. stage 2 neurons. Neurons with a lamellipodium that formed at an angle over 180° or those with no filopodia or lamellipodia were defined as neutral or intact, i.e. stage 3 neurons.

At 18–72 h after plating, neurons projecting single major neurites that were at least twice as long as other protrusions were counted as polarized, i.e. stage 3 neurons. Neurons with minor and no major neurites were counted as unipolarized, i.e. stage 2 neurons. Neurite tracing and measurement of neurite length were conducted using ImageJ with the NeuronJ plugin module.

**Pulldown assay and immunoprecipitation assay**

COS-7 cells transfected by electroporation were washed with cold PBS and lysed with lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA). The homogenate was sonicated and centrifuged at 10,000 g at 4 °C. The supernatants were gently mixed with an equal volume of 80% sucrose lysis buffer. The samples were overlaid with a discontinuous gradient of 35% sucrose lysis buffer (4 ml) to 5% sucrose lysis buffer (4 ml). The gradients were subjected to ultracentrifugation at 33,600 rpm (20,000 × g) for 18 h in a Hitachi ST40 Ti rotor. Twelve fractions (1 ml/fraction) were collected from the top of a centrifugation tube by a Hitachi gradient generator. The DRM fraction was positioned at the interface between 35% and 5% sucrose (fractions 3–5).

**Colocalization assay**

We used the ImageJ plugin Colocalization Colormap to determine protein colocalization to automatically quantify the correlation between a pair of pixels. Distributions of the normalized mean deviation product value shown with a color scale and average of normalized mean deviation products were compared.

**In utero electroporation**

In utero electroporation was performed on E14.5 embryos using pregnant ICR (Japan SLC, Inc.) mice as described previously (9, 28) with some modifications. In brief, pregnant mice were deeply anesthetized by intraperitoneal administration of pentobarbital (48.6 mg/kg of body weight), and their uterine horns were exposed. Plasmid DNA purified using a Qiagen Plasmid Maxi kit was dissolved in HBS buffer solution (10 mM Hepes (pH 7.4), 150 mM NaCl) with 0.01% Fast Green solution. Approximately 1 µl of plasmid solution was injected into the lateral ventricle of each embryo using a processed micropipette made from a glass tube (number 3-000-105-G, Drummond) and the Animal Resource Department of Niigata University for the generation and maintenance of Rufy3-KO mice.
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