Supplemental Information

Slitrk5 mediates BDNF-dependent TrkB receptor trafficking and signaling

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Inventory of Supplemental Information

Supplemental Experimental Procedures

Supplemental Data

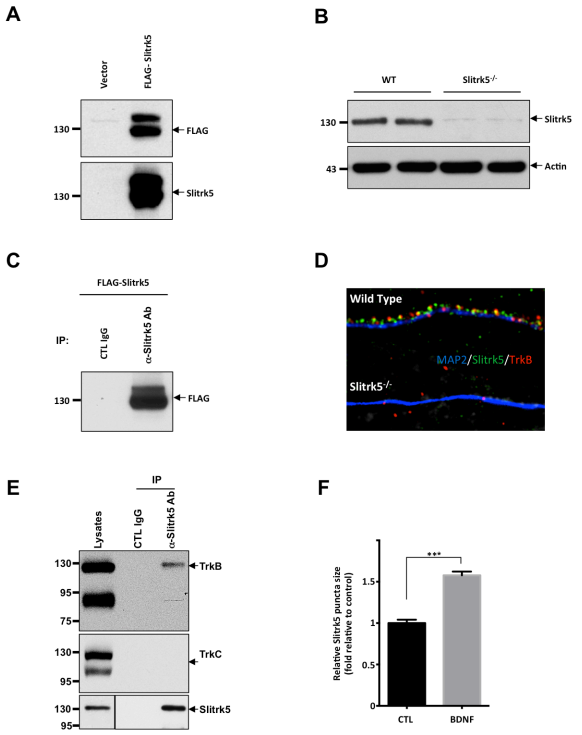


Figure S1. Slitrk5 specific antibody was generated, related to Figure 1.

(a) Anti-Slitrk5 antibody was tested for Western blot analysis by using HEK293T ovrexpressing FLAG-tagged Slitrk5 and empty vector. (b) Specificity of anti-Slitrk5 antibody was further tested with WT and *Slitrk5*$^{-/-}$ brain lysates. (c) Anti-Slitrk5 antibody was tested for immunoprecipitation. HEK293T cells were transfected with FLAG-tagged Slitrk5. Cell lysates were immunoprecipitated with control IgG or anti-Slitrk5 antibodies, and resulting precipitates were subjected to immunoblot analysis with anti-FLAG antibodies. (d) Anti-Slitrk5 antibody was tested for immunostaining. DIV14 WT and *Slitrk5*$^{-/-}$ hippocampal neurons were fixed with Methanol and incubated with rabbit anti-Slitrk5 (green), goat anti-TrkB (red), and chicken anti-MAP2 (blue) antibodies. (e) Endogenous association of Slitrk5 and TrkB. Mouse whole-brain lysates (2 months old) were subjected to immunoprecipitation with anti-Slitrk5 antibody (Millipore) or control

IgG. The immune protein complex was then eluted, and TrkB, TrkC, and Slitrk5 were detected by immunoblotting. (f) Quantification of Slitrk5 punta size in Fig. 1I. BDNF treatment enlarges the size of Slitrk5 puncta. Results are means ± SEM from 3 independent experiments. 10-20 neurons were analyzed per condition per experiment. ***P<0.001 significantly different from control condition (Student's t test).

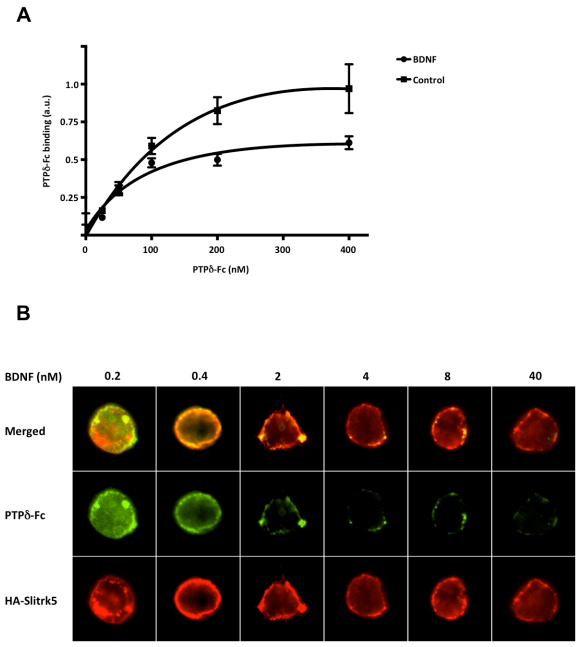


Figure S2. PTPδ and TrkB compete for binding to Slitrk5, related to Figure 2.

(a) PTPδ-Fc binding to Slitrk5 proteins is affected by BDNF. HA-Slitrk5-expressing HEK293-TrkB cells were pretreated with DMSO or 80 nM Dynasore for 15 min to exclude the effect of TrkB-Slitrk5 endocytosis on PTPδ-Fc binding. Cells were incubated with increasing amount of PTPδ-Fc in the presence or absence of BDNF (25ng/ml). After washing and fixation, fluorescence intensity of bound PTPδ-Fc was measured. (b) Representative image showing BDNF-induced dissociation of pre-bound PTPδ-Fc from HA-Slitrk5-expressing HEK293-TrkB cells. HA-Slitrk5-expressing HEK293-TrkB cells were pre-incubated with saturating condtion of PTPδ-Fc (400 nM) for 1hr. After washing, cells were incubated with indicated dose of BDNF for 30 min. Remaining PTPδ-Fc binding was analyzed by immunofluorescence microscopy.

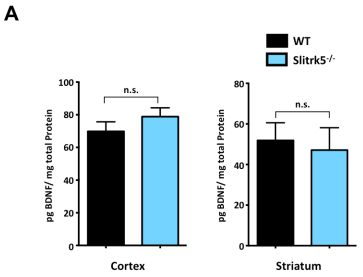


Figure S3. BDNF protein levels were not altered in the brain of *Slitrk5*$^{-/-}$ mice, related to Figure 3.

(a) BDNF levels determined by ELISA were not changed in the cortex and striatum of 3 months old *Slitrk5*$^{-/-}$ mice compared with WT animals (n=5, for each genotype).

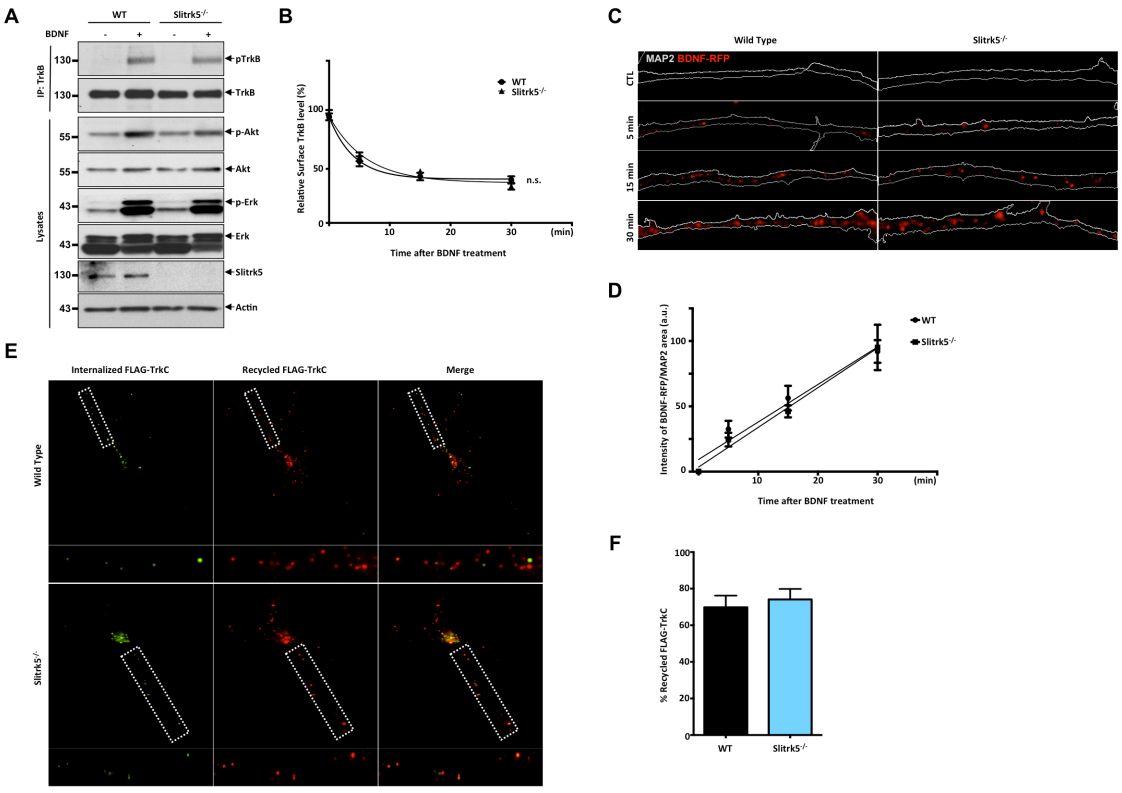


Figure S4. Acute TrkB receptor signaling, TrkB internalization, and TrkC receptor recycling were not affected in Slitrk5$^{-/-}$ striatal neurons, related to Figure 4.

(a) TrkB receptor responsiveness in striatal neurons to low BDNF concentrations in the presence and absence of Slitrk5. DIV6 striatal neurons from WT and *Slitrk5*$^{-/-}$ mice were treated with or without BDNF (1 ng/ml) for 30 min. Phosphorylation of TrkB, Akt and Erk were examined by Western blot analysis. (b) TrkB receptor internalization was not affected in *Slitrk5*$^{-/-}$ striatal neurons. Cultured striatal neurons from WT and *Slitrk5*$^{-/-}$ mice were transduced with FLAG-tagged TrkB lentivirus at DIV2. Neurons were treated with or without BDNF after labeling surface FLAG-TrkB with anti-FLAG M2 antibody at DIV6. Surface remaining FLAG-TrkB was labeled with Alexa-488 dye-conjugated anti-mouse secondary antibody, while internalized FLAG-TrkB was labeled with Cy3-conjugated anti-mouse secondary antibody after permeablization with 0.2% Triton X-100-containing PBS. Internalization of FLAG-TrkB was examined by fluorescent ratiometric analysis.

Results are means ± SEM from 3 independent experiments determined from analysis of 30 neurons per condition per experiment. (n.s. not significant, Student's t test) (c) Representative images of BDNF-RFP internalization. DIV6 striatal neurons from WT and *Slitrk5*$^{-/-}$ mice were incubated with RFP-tagged BDNF for indicated times. Neurons were fixed, permeabilized, and immunostained with anti-MAP2 antibody to visualize dendirtes. Internalization of RFP-tagged BDNF in MAP2 area was examined by immunofluorescence microscopy. Results are means ± SEM from 3 independent experiments determined from analysis of 30 neurons per condition per experiment. (d) Quantification of (c). (e) Representative images showing intact recycling of TrkC in *Slitrk5*$^{-/-}$ striatal neuron. WT and *Slitrk5*$^{-/-}$ striatal neurons were transfected at DIV4 with FLAG-tagged TrkC. NT3-induced TrkC recycling was measured with live-cell fluorescence ratiometric recycling assay at DIV6. (f) Quantification of (e). Results are means ± SEM from 3 independent experiments determined from analysis of 30 neurons per condition per experiment.

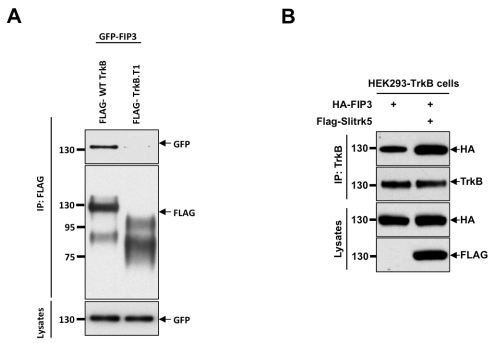


Figure S5. Slitrk5 facilitates Rab11-FIP3 binding to full-length TrkB, related to Figure 6.

(a) Rab11-FIP3 specifically binds to full-length TrkB, but not to truncated TrkB.T1. HEK293T cells were co-transfected with cDNAs encoding GFP-tagged Rab11-FIP3, and FLAG-tagged WT TrkB or FLAG-tagged TrkB.T1. Cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-GFP antibodies. (b) Slitrk5 facilitates Rab11-FIP3 binding to TrkB. HEK293-TrkB cells were co-transfected with cDNAs encoding HA-tagged Rab11-FIP3, and control vector or FLAG-tagged Slitrk5. Cell lysates were immunoprecipitated with anti-TrkB antibodies and immunoblotted with anti-HA antibodies.

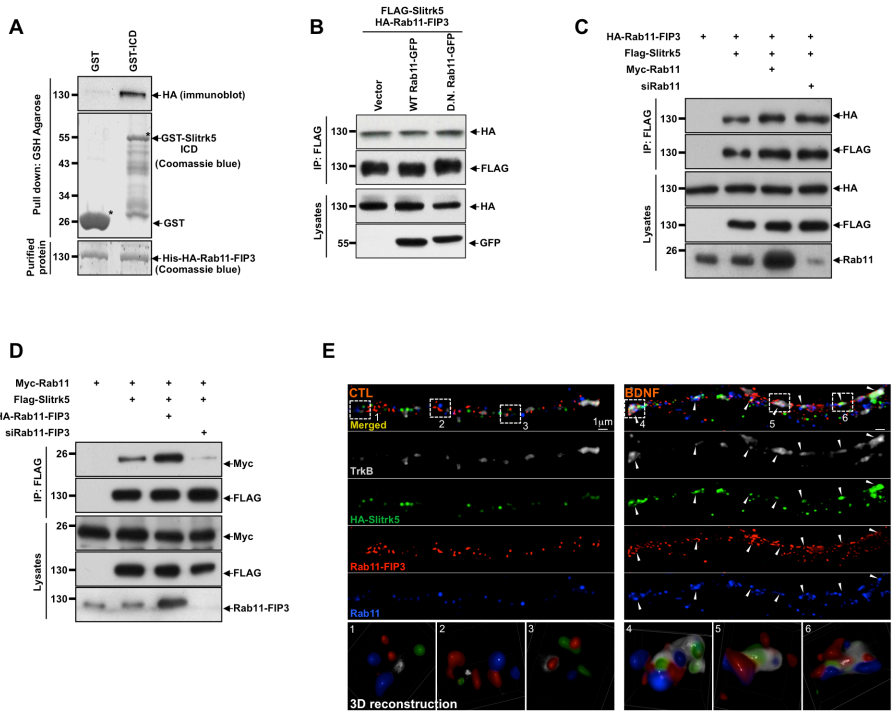
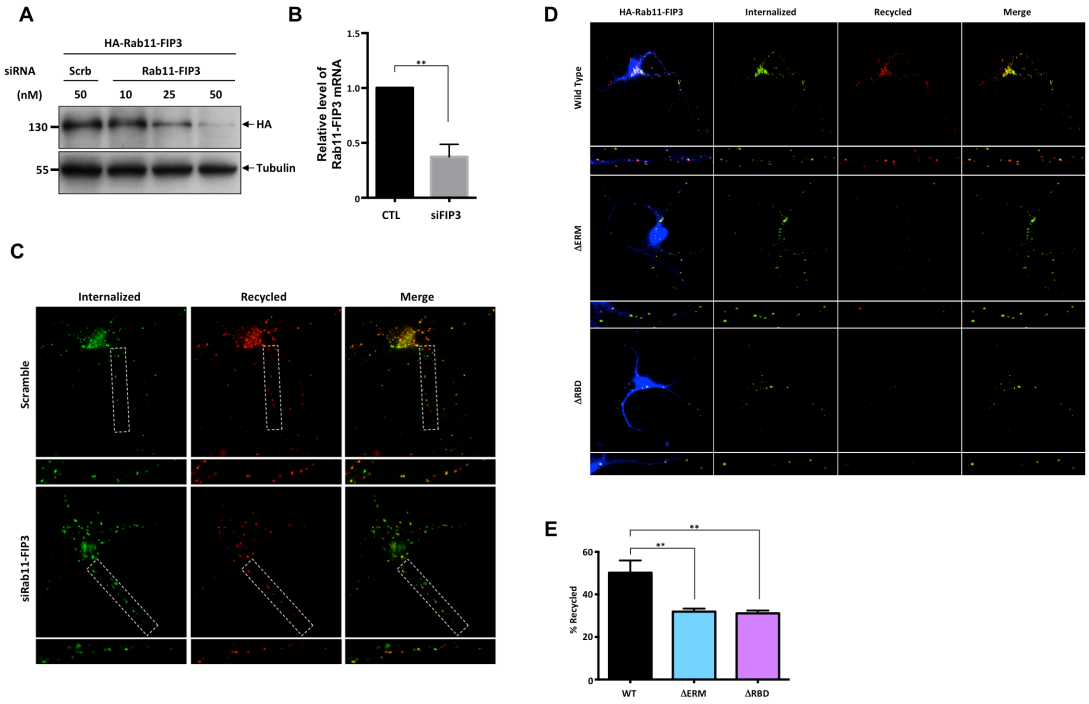


Figure S6. Rab11-FIP3 binds to Slitrk5, related to Figure 6.

(a) Slitrk5 directly binds to Rab11-FIP3. GST-tagged intracellular domain (ICD) of Slitrk5 and His-HA-tagged Rab11-FIP3 were expressed in E. coli (BL21) and purified. The purified His-HA-Rab11-FIP3 proteins were incubated with GST or GST-Slitrk5-ICD bound beads at 4°C for 2 hr. After extensive wash with PBS buffer, the bound proteins were analyzed by SDS-PAGE followed by staining with Coomassie blue (Sigma) or immunoblotting with anti-HA antibodies. (b), (c), (d) Representative blots showing Rab11-independent binding of Slitrk5 and Rab11-FIP3. (b) HEK293T cells were co-transfected with cDNAs encoding FLAG-tagged Slitrk5, HA-tagged Rab11-FIP3, and WT Rab11-GFP or dominant negative form of Rab11-GFP. Cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-HA antibodies. (c) HEK293T cells were co-transfected with cDNAs encoding FLAG-tagged Slitrk5, HA-tagged Rab11-FIP3, and Myc-tagged WT Rab11 or siRNA for Rab11. Cell

lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-HA antibodies. (d) HEK293T cells were co-transfected with cDNAs encoding FLAG-tagged Slitrk5, Myc-tagged WT Rab11, and HA-tagged Rab11-FIP3, or siRNA for Rab11-FIP3. Cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-Myc antibodies. (e) Representative image showing presence of Rab11 in TrkB/Slitrk5/Rab11-FIP3 complex. WT striatal neurons were transfected at DIV4 with HA-tagged Slitrk5. At DIV6, striatal neurons were treated with or without BDNF (25ng/ml) for 30 min after incubating with anti-TrkB antibody to specifically label surface TrkB. HA-tagged Slitrk5, Rab11-FIP3, and Rab11 were visualized with specific antibodies after fixation and permeabilization. Super resolution images were acquired using a Nikon N-SIM structured illumination microscope. The images were 3D reconstructed (IMARIS) to better illustrate the distributions of four molecules.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HA-Rab11-FIP3
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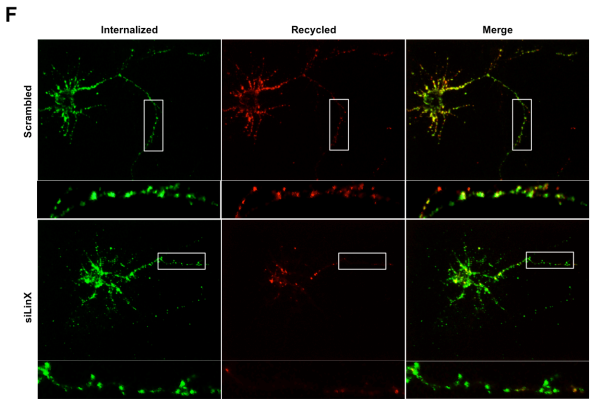
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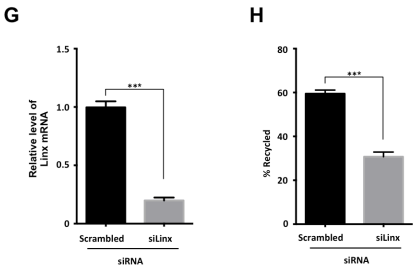
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Scrambled
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Figure S7. Rab11-FIP3 is required for TrkB recycling, related to Figure 7.

(a) HEK293T cells were co-transfected with cDNAs encoding HA-tagged Rab11-FIP3, and siRNA targeting Rab11-FIP3 or control siRNA. Cell lysates were immunoblotted with anti-HA antibodies. (b) Specific downregulation of endogenous Rab11FIP3 mRNA by Rab11FIP3 siRNA. DIV5 cortical neurons were transfected with 100nM siRNA as indicated. After 72h, relative levels of Rab11FIP3 mRNA were quantified by real-time PCR analysis. The data were normalized by using β-actin as an internal standard. (**P<0.01, Student's t-test) (c) Impaired recycling of TrkB by Rab11-FIP3 knockdown. Striatal neurons were co-transfected at DIV4 with FLAG-tagged TrkB, and either siRNA targeting Rab11-FIP3 or control siRNA. BDNF-induced TrkB recycling was measured with live-cell fluorescence ratiometric recycling assay at DIV6, as described in Figure 6F. Representative images are shown here. (d) Representative images showing defective recycling of TrkB in Rab11-FIP3ΔERM and Rab11-FIP3ΔRBD-expressing striatal neurons. WT striatal neurons were co-transfected at DIV4 with FLAG-tagged TrkB and WT or deletion mutants of HA-tagged Rab11-FIP3. BDNF-induced TrkB recycling was measured with live-cell fluorescence ratiometric recycling assay at DIV6. (e) Quantification of (d). Results are means ± SEM from 3 independent experiments determined from analysis of 30 neurons per condition per experiment. (f) Representative images showing defective recycling of TrkA in Linx siRNA transfected cortical neuron. WT cortical neurons were co-transfected at DIV4 with FLAG-tagged TrkA and either siRNA targeting Linx or control siRNA. NGF-induced TrkA recycling was measured with live-cell fluorescence ratiometric recycling assay at DIV6. (g) Specific downregulation of endogenous Linx mRNA by Linx siRNA. DIV5 cortical neurons were transfected with 100nM siRNA as indicated. After 72h, relative levels of Rab11FIP3 mRNA were quantified by real-time PCR analysis. The data were normalized by using β-actin as an

internal standard (***P<0.001, Student's t-test). (h) Quantification of (f). Results are means ± SEM from 3 independent experiments determined from analysis of 30 neurons per condition per experiment (***P<0.001, Student's t-test).

Supplemental Experimental Procedures

Mice

All animal procedures were approved by the Institutional Animal Care and Use Committees of Weill Cornell Medical College and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Generation of the *Slitrk5*$^{-/-}$ mice and genotyping method were described previously (Shmelkov et al., 2010). Mice were generated from heterozygous mouse intermatings, were weaned and sex segregated at postnatal Day 21, and in nearly all cases wild type (WT), Slitrk5$^{-/+}$, and *Slitrk5*$^{-/-}$ were mixed housed in a single cage.

Reagents and Antibodies

Human recombinant BDNF and NGF were purchased from PeproTech (RockyHill, NJ, USA). Rabbit polyclonal anti-Slitrk5 antibody was generated against synthetic peptides (EPREDLLSPVQDADRFYR). Affinity purified anti-Slitrk5 antibody was tested for Western blot and immunoprecipitation. Anti-Slitrk5 antibody recognized ectopically expressed FLAG-tagged Slitrk5 in HEK293T cell lysates (Figure S1A) and specifically detected protein of M.W. ~130 kDa in brain lysates of WT mouse with Western blot analysis but not in those of *Slitrk5*$^{-/-}$ mouse (Figure S1B). Anti-Slitrk5 antibody was able to precipitated ectopically expressed FLAG-tagged Slitrk5 (Figure S1C). Rabbit anti-MAPK1/2, goat anti-actin, goat anti-GAD65/67, and mouse anti-phospho-Tyr (pY99) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-Akt, rabbit anti-phospho-Akt^{S473} and rabbit anti-phospho-MAPK1/2 antibodies were obtained from Cell Signaling (Beverly, MA, USA). Chicken anti-MAP2 and rabbit anti-hemagglutinin (HA) were obtained from Abcam (Cambridge, MA, USA). Rabbit anti-Rab11, mouse anti-Transferrin, and Alexa Fluor dye-conjugated

secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Mouse anti-Flag antibodies (M1, M2 and rabbit polyclonal Antibodies) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or rabbit IgG, and HRP-conjugated rabbit anti-goat IgG were purchased from Calbiochem (La Jolla, CA, USA). The other antibodies were purchased as follows: mouse anti-HA antibody (HA.11) from Covance (Emeryville, CA, USA), rabbit anti-TrkB from Millipore (Temecula, CA, USA), rabbit anti c-Myc antibodies from Bethyl Laboratories (Montgomery, TX), mouse anti-GFP from Abgent (San Diego, CA, USA), mouse anti-TrkB from BD transduction laboratories (San Jose, CA, USA), goat anti-TrkB from R&D systems (Minneapolis, MN, USA). Generation of rabbit anti-Rab11-FIP3 antibody was described previously (Wilson et al., 2005). All cell culture reagents were from Invitrogen. The other reagents were from Sigma-Aldrich.

Plasmid Constructs and siRNA

Human Slitrk5 cDNA (OpenBiosystems, MGC Human SLITRK5 Sequence-Verified cDNA, Accession: BC098106, Clone ID: 40012375) was subcloned into pCCL-PGK vector by using BamHI and XhoI sites. The amino terminal FLAG or HA epitope tag was added to the 5′ end of Slitrk5 by PCR. Slitrk5-GFP construct was generated by adding GFP epitope tag to the 3’ end of Slitrk5 by PCR. Human Slitrk1 (OpenBiosystems, MGC Human SLITRK1 cDNA, Accession: BC051738 Clone ID: 4816570), Slitrk2 (OpenBiosystems, MGC Human SLITRK2 Sequence-Verified cDNA, Accession: BC113012, Clone ID: 40028693), and Slitrk5 cDNA were subcloned into pcDNA3.1 neo expression vector by using BamHI and XbaI. Human Slitrk3 (OpenBiosystems, MGC Human SLITRK3 Sequence-Verified cDNA, Accession: BC114621, Clone ID: 40036558) was subcloned into pcDNA3.1 neo expression vector by using BamHI and NotI. The amino terminal FLAG epitope or HA epitope tag was

added to the 5′ end of Slitrk isotypes by PCR. Rat TrkB and TrkC cDNAs were subcloned into pcDNA3.1Neo expression vector (Invitrogen) by using EcoRI and EcoRV sites. FLAG-LRR1(S1) Slitrk5 and FLAG-LRR2(S1) Slitrk5 constructs were generated by replacing LRR1 or LRR2 domain of Slitrk5 with that of Slitrk1. FLAG-LRR(C) TrkB and FLAG-Ig1(C) TrkB constructs were generated by replacing LRR or IG-C1 domain of TrkB with respective domains of TrkC by means of two-step PCR. HA-ΔECD Slitrk5, HA-ΔLRR1 Slitrk5, HA-ΔLRR2 Slitrk5, HA-Rab11FIP3, Rab11FIP3-GFP, FIP1C-GFP, FIP5-GFP, HA-FIP3delRBD, HA-FIP3delERM and serial C-terminal deletion mutants or Rab11-FIP3 binding-deficient mutant of Slitrk5 were generated by a PCR based methods. All of the constructs were confirmed by DNA sequence to exclude potential PCR-introduced mutations. Human PTPδ (OpenBiosystems, MGC Human PTPRD Sequence-Verified cDNA, Accession: BC106714, Clone ID: 40027582) was subcloned into pCCL-PGK vector by using SpeI and XhoI sites, and extracellular domain of PTPδ was subcloned into pSecTag2 vector by using HindII and XhoI sites to generate Fc-tagged version of PTPδ ECD.

The siRNAs targeting Rab11-FIP3 (5'-AGCTCCTGTGCAAGATGGA-3') and scramble siRNA are synthesized by RIBOBIO Company (Guangzhou, China) and were transfected with Lipofectamine® 2000 reagents following the manufacturer's instructions (Invitrogen).

The pSuper-RFP or pSuper-EGFP vectors (OligoEngine, Seattle, WA) were used to transcribe functional small interfering RNA (siRNA) of rat Linx. In the vectors, oligonucleotides targeting different gene were inserted into the downstream of H1 promotor, with their veracity confirmed by double digestion and sequencing. The target sequences for rLinx were as follows: GCATTCAACCAGAGCTCAGAT. The expression levels of Linx in PC12 cells transfected with the resulting siRNA or the scramble siRNA

were analyzed by qRT-PCR. Knock-down of Rab11 expression of was performed by RNA interference using siRNA oligonucleotides as follows: 5'-GAA ACG AGU UCA AXX UAG A-3'.

Real-time PCR analysis

Total RNA was isolated from neurons using TRNzol-A+ RNA isolation reagent (TIANGEN) according to the manufacturer's instructions. Reverse transcription was conducted using the RevertAid First Strand cDNA Synthesis Kit (Fermentas), and relative transcript expression levels were measured by quantitative real-time PCR using a SYBR Green-based method. β-actin was applied as an internal control. The following primers were used: Rab11FIP3: 5′-TGTAGCCAGTGCCACAAAC-3′ forward and 5′-TCCAGGGCCTCCATAGTC-3′ reverse; rLinX: 5′-CCA AAG CCA ACC AAG AAG AGT T-3′ forward and 5′- GTC TGC CTG TAG TTG CCG TTT AT-3′ reverse; β-actin: 5′-TCCATCATGAAGTGTGACGT-3′ forward and 5′-GAGCAATGATCTTGATCTTCAT-3′ reverse. The threshold cycle for each sample was chosen from the linear range and converted to a starting quantity by interpolation from a standard curve run on the same plate for each set of primers. The Rab11FIP3 and rLinx RNA levels were normalized for each well to the β-actin mRNA levels using the $2^{-\Delta\Delta CT}$ methods. Each experiment was repeated three times.

BDNF ELISA

To measure endogenous BDNF levels, a BDNF enzyme-linked immunosorbent assay (ELISA) was used (BDNF Emax Immunoassay System, Promega, Madison, WI) with recombinant BDNF as a standard. This methodology demonstrates low cross-reactivity (<3%) with other neurotrophic factors and is capable of detecting a minimum of 15.6 pg/ml of BDNF. Briefly, WT and *Slitrk5*$^{-/-}$ mice were sacrificed by cervical dislocation

and then decapitation. Brains were collected on ice, and then cortical and striatal regions were dissected, lysed in lysis buffer (0.1 M Tris HCl, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 1 % Triton X-100, 1 mM PMSF, and protease inhibitor cocktail (Sigma)). Lysates were centrifuged for 10 min at 4°C and the clarified supernatant was collected. Using the Bradford method, total levels of protein were quantified. Tissue and assay were prepared and run in accordance with the manufacturers suggested protocol. BDNF levels were then corrected based on the total amount of protein loaded.

Neuronal culture

All reagents used to prepare primary neuronal cultures were purchased from Invitrogen, except glucose that was from Sigma. The striatum of E16 mouse embryos were dissected in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution supplemented with 0.37% glucose and digested in the same medium supplemented with 0.05% trypsin. Striatal cells were mechanically dissociated with fire-polished Pasteur pipettes and plated in plating medium (MEM containing 2 mM glutamine, supplemented with 10% FBS, 1 mM pyruvate, 0.37% glucose, and 25 U/ml penicillin/streptomycin). Striatal neurons were grown on poly-D lysine/laminin-coated glass coverslips at a cell density of $1.5x10^4$ cells/cm^2 for immunocytochemistry or on poly-D lysine/laminin-coated culture dishes at a density of $1x10^5$ cells/cm^2 for biochemical experiments. Plated neurons were kept in a humidified incubator at 37°C and 5% CO2. Cells were maintained in serum-free Neurobasal medium with B-27 supplement, 0.5 mM glutamine, 25 μM glutamate, and 25 U/ml penicillin/streptomycin, and AraC (Cytosine-1-β-D-arabinofuranoside) was added at DIV2. Cultures were grown for 6 days before being used for experiments, and media were changed every 3 days. Neurons were transfected with Lipofectamine 2000 transfection reagent following the manufacturer's instructions (Invitrogen) or with

lentiviral vectors. Forty-eight hours after Lipofectamine 2000 transfection or 4 days after lentiviral transduction, experiments were performed.

Production of lentivirus

Lentivirus was produced by co-transfecting HEK293T cells with the transfer plasmids, envelope plasmids and packaging plasmids using Lipofectamine 2000 transfection reagent following the manufacturer's instructions. Briefly, a total of 5×10^6 HEK293T cells were seeded in 10 cm dishes 24 hours prior to transfection in 10 ml complete DMEM medium and incubated at 37°C in a 5% CO2 incubator. A total of 20 μg of plasmid DNA was used for the transfection of one dish: 3.5 μg of the envelope plasmid pMD.G, 6.5 μg of packaging plasmid, and 10 μg of transfer vector plasmid. The medium was replaced next morning and the virus-containing media were harvested at 48 hour and 72 hour after transfection, pooled and centrifuged at 1000 rpm for 10 min, and then filtered through 0.45μm filter (Millipore, MA). The supernatants were aliquoted and stored at -80°C. For in vitro experiments, neurons were transduced with respective lentivirus vectors in the presence of Polybrene (8 μg/ml). Viral p24 antigen concentration was determined by immunocapture (Alliance; DuPont-NEN) for the titration of lentivirus.

Preparation of glutathione S-transferase fusion proteins and in vitro binding assay.

The glutathione S-transferase (GST)–SliTrk5-ICD constructs include the V^{686}-F^{958}. These cDNA fragments were generated by PCR and subcloned into the pGEX-4T-1 vector (Amersham Biosciences, Arlington Heights, IL) respectively using the BamHI and XhoI sites. The fusion protein was expressed in BL21 Escherichia coli (DE3 strain; Novagen, Madison, WI) and immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences). HA-FIP3 was subcloned into pET28a (Novagen, Madison, WI)

to yield His-FIP3. His-tagged FIP3 were purified using Ni^{2+} affinity purification (Qiagen). The purified His-HAFIP3 proteins were incubated with GST-fusion proteins bound beads in pull down buffer (20mM Tris, 1% BSA, 0.5% TritonX-100) at 4°C for 2 hr. After extensive wash with PBS buffer, the bound proteins were analyzed by SDS-PAGE followed by staining with Coomassie blue (Sigma) or immunoblotting with anti-HA antibodies.

Western blot analysis

Brain tissue, neuronal and cell line cultures were lysed in RIPA buffer (150 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) containing protease and phosphatase inhibitors (2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Lysates were clarified by centrifugation at 12,000 g for 15 min and protein concentrations were determined by Lowry assays (DC protein assay kit, Bio-Rad). The protein samples were boiled in an LDS sample buffer (NuPAGE® Novex, Invitrogen, Carlsbad, CA) for 5 min, and then were separated on a 10% Nupage Bis-Tris Gel (NuPAGE® Novex, Invitrogen, Carlsbad, CA) and transferred to PVDF membranes. These were then blocked for 1 h in TBS with 0.1% Tween 20 (TBS-T) and 5% low-fat milk. The incubation with the primary antibodies was performed overnight at 4ºC in TBS-T with 3% BSA, followed by washes in TBS-T and incubation with HRP secondary antibodies at room temperature for 1 h. Immunoreactive proteins were visualized by ECL detection and film autoradiography. Striping was performed by washing the membranes in 0.1M Glycine pH 2.5 for 15 min, followed by another wash in 1% SDS for 15 min. Each experiment was repeated at least three times.

Immunoprecipitation

Lysates from cultured cells or brain tissue were prepared as described above. After centrifugation at 12,000 x g for 15 min, the supernatant was incubated with antibody-coupled agarose beads for overnight at 4°C, with constant rotation. After washing three times with the lysis buffer, the bound proteins were eluted by boiling in LDS sample buffer for 5 min. Eluted proteins were resolved by SDS-PAGE.

Endocytosis assay

To quantitatively analysis TrkB endocytosis, DIV2 striatal neurons were transfected with FLAG-tagged TrkB lentivirus. After starvation as described above, live neurons were incubated with anti-FLAG antibodies (M2) to specifically label cell surface FLAG-tagged TrkB receptors and TrkB internalization was induced by BDNF treatment on DIV6. After receptor endocytosis had driven, neurons were fixed and remaining surface receptor populations were labeled with Alexa-488 dye-conjugated anti-mouse antibodies (dilution 1/300) for 20 min at RT. Neurons were permeabilized after washing three times with PBS, internalized receptors were visualized with Alexa-568 dye-conjugated anti-mouse antibodies (dilution 1/300) at RT for 20 min. Neurons were examined by fluorescence microscopy and staining intensities of each fluor in individual neurons were quantified using MetaMorph software (Molecular Devices, Sunnyvale, CA). Values corresponding to the internalized receptors (green) were divided by the total fluorescence values (red + green) and normalized to untreated controls. Each experiment was repeated at least three times.

Surface biotinylation and degradation assay

Surface biotinylation was performed to specifically detect TrkB receptors present in the plasma membrane and to measure their proteolysis, as described previously

(Rajagopal et al., 2004). Briefly, dissociated striatal neuron cultures were washed twice with ice-cold PBS supplemented with Ca^{2+} and Mg^{2+} (PBS^{++}) and incubated with 500 μg/ml of sulfo-NHS-S-biotin (Pierce Chemical, Rockford, IL) for 20 min on ice with gentle agitation. Unreacted biotin was quenched and removed with 50mM Glycine in PBS^{++}. Biotinylated neurons were then transferred to pre-warmed medium containing BDNF for 90 min, and then neurons were immediately chilled on ice and lysates were prepared as described above. Biotinylated proteins were isolated from cell extracts by immobilization on high capacity streptavidin-conjugated Sepharose beads (Pierce Chemical, Rockford, IL). Washed beads were eluted with LDS sample buffer, and eluted proteins were resolved by SDS-PAGE. Each experiment was repeated at least three times.