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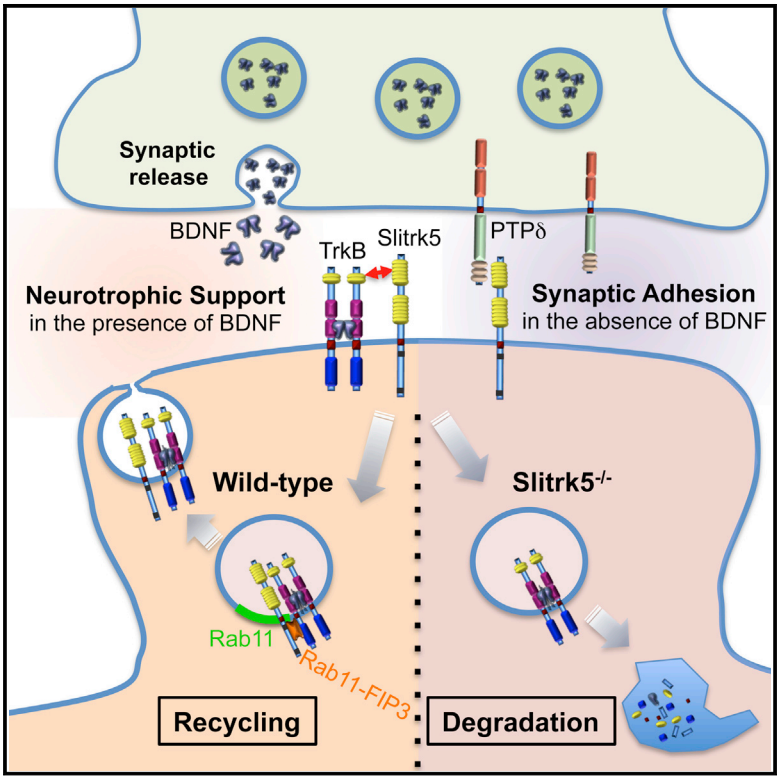
Developmental Cell

Slitrk5 Mediates BDNF-Dependent TrkB Receptor Trafficking and Signaling

Graphical Abstract



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In Brief

Slitrk family proteins are emerging as candidate genes involved in neuropsychiatric disorders. Song et al. show that Slitrk5 modulates BDNF-dependent biological responses by directly regulating TrkB receptor recycling via recruitment of Rab11-FIP3.

Highlights

- Slitrk5, a cell-surface LRR protein, is a co-receptor facilitating TrkB signaling
- TrkB and Slitrk5 form a physical complex through their LRR domains
- TrkB and PTPδ compete for the first LRR domain of Slitrk5
- Slitrk5 regulates TrkB receptor recycling by facilitating recruitment of Rab11-FIP3

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Slitrk5 Mediates BDNF-Dependent TrkB Receptor Trafficking and Signaling

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SUMMARY

Recent studies in humans and in genetic mouse models have identified Slit- and NTRK-like family (Slitrks) as candidate genes for neuropsychiatric disorders. All Slitrk isotypes are highly expressed in the CNS, where they mediate neurite outgrowth, synaptogenesis, and neuronal survival. However, the molecular mechanisms underlying these functions are not known. Here, we report that Slitrk5 modulates brain-derived neurotrophic factor (BDNF)-dependent biological responses through direct interaction with TrkB receptors. Under basal conditions, Slitrk5 interacts primarily with a transsynaptic binding partner, protein tyrosine phosphatase δ (PTPδ); however, upon BDNF stimulation, Slitrk5 shifts to *cis*-interactions with TrkB. In the absence of Slitrk5, TrkB has a reduced rate of ligand-dependent recycling and altered responsiveness to BDNF treatment. Structured illumination microscopy revealed that Slitrk5 mediates optimal targeting of TrkB receptors to Rab11-positive recycling endosomes through recruitment of a Rab11 effector protein, Rab11-FIP3. Thus, Slitrk5 acts as a TrkB co-receptor that mediates its BDNF-dependent trafficking and signaling.

INTRODUCTION

Members of the Slit- and NTRK-like family (Slitrks) are type 1 transmembrane proteins that localize to and function at CNS synapses where they mediate synapse formation through transsynaptic interactions of their ectodomains with presynaptic binding partners (Linhoff et al., 2009; Takahashi et al., 2012; Yim et al., 2013). Their cytoplasmic tails, larger than those of other well-studied synaptic adhesion molecules (SAMs), e.g., neurexins, neuroligins, leucine-rich repeat transmembranes (LRRTMs), synaptic cell adhesion molecules (SynCAMs), N-cadherins, and L1 adhesion molecules (Aruga and Mikoshiba, 2003; Proenca et al., 2011), suggest functional interactions with other molecules. Slitrks have structural and functional similarity to tropomyosin receptor kinase (Trk) neurotrophin receptors. They contain N-terminal leucine-rich repeat (LRR) domains and intracellular tyrosine-based motifs similar to those found in TrkA, TrkB, and TrkC. Furthermore, Slitrks mediate biological functions similar to Trk receptors, including neurite outgrowth and dendritic elaboration, synapse formation, and neuronal survival (Aruga and Mikoshiba, 2003; Ko, 2012; Proenca et al., 2011; Yim et al., 2013). It is well established that Trk receptors mediate this broad range of functions through their selective binding to three major ligands: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) (Chao, 2003).

A key question is how such a limited repertoire of neurotrophins and Trk receptors mediates such a diverse array of

biological actions. In the peripheral nervous system (PNS), the basic strategy to generate signaling diversity has been elucidated. In sensory and sympathetic neurons, Trk receptors are expressed at high levels and form combinatorial complexes with their co-receptor $p75^{NTR}$ at the plasma membrane. These interactions increase their affinity and selectivity for specific neurotrophins (Benedetti et al., 1993; Hempstead et al., 1991; Kuruvilla et al., 2004; Lee et al., 1994). In contrast, in the adult CNS, the strategy underlying signaling diversity remains essentially unknown. Whereas TrkB and TrkC receptors are expressed at high levels in the CNS, $p75^{NTR}$ has a limited distribution, restricted primarily to the basal forebrain (Huang and Reichardt, 2003; Lee et al., 2001). Hence, it is likely that regulation of Trk function in the CNS involves alternative molecules.

Slitrks are excellent candidates to perform such regulatory functions. Of the six Slitrk family members expressed in the CNS (Aruga and Mikoshiba, 2003; Shmelkov et al., 2010), Slitrk5 provides the more compelling links with the neurotrophin system. *Slitrk5*$^{-/-}$ mice display altered anatomical and neuronal morphological phenotypes in the striatum similar to those observed in the striatum of mice with genetic deficiencies in BDNF or TrkB (*Emx-BDNF*, *Tau-BDNF*, *Dlx5/6-TrkB*, and *Bf1-BDNF* knockout [KO] mice) (Baquet et al., 2004; Baydyuk et al., 2011; Li et al., 2012; Rauskolb et al., 2010; Shmelkov et al., 2010). This raises the intriguing question of whether in the CNS Slitrk5 functions through direct interaction and modulation of the neurotrophin system. Here, we present mechanistic evidence demonstrating that Slitrk5 acts as a co-receptor of TrkB that modulates its postendocytic recycling to facilitate BDNF-dependent signaling responses. Our study identifies a key regulator of the diverse array of BDNF functions in the CNS.

RESULTS

Slitrk5 Interacts with TrkB Receptors

To determine whether Slitrk5 and TrkB receptors are physically associated, we carried out co-immunoprecipitation studies in cell lines and primary neurons. Studies using HEK293T cells transfected with FLAG-tagged TrkB and wild-type (WT) Slitrk5 plasmids (Figure 1A) or HEK293 cells stably expressing TrkB (HEK293-TrkB) transfected with GFP-tagged Slitrk5 (Figure 1B) clearly demonstrated that the two proteins interact. Furthermore, co-immunoprecipitation studies in brain lysates using anti-TrkB antibodies or anti-Slitrk5 antibodies demonstrated that endogenous Slitrk5 and TrkB interact in neurons (Figures 1C and S1E). This interaction is specific, as it was not observed for other Slitrk members (Slitrk1–3) (Figure 1D), or for the other major CNS neurotrophin receptor, TrkC, in primary cultured neurons (Figures 1E and S1E). To map the Slitrk5 and TrkB domains that mediate their interaction, we utilized a chimera-based approach. The extracellular domain of Slitrk5 encodes two LRR domains (Figure 1F). LRR domains often mediate protein-protein interactions (Gay et al., 1991; Mandai et al., 2009). We swapped the extracellular domains of Slitrk5 with the corresponding domains of another Slitrk (Slitrk1) (Figure 1F). After transfection of HEK293-TrkB cells with the chimeric Slitrk5 constructs, their interaction with TrkB was assessed by co-immunoprecipitation and immunoblot analyses. These studies demonstrated that the interaction of Slitrk5 with TrkB was mediated by its first LRR (LRR1) domain (Figure 1F). Complementary studies with chimeras between TrkB and TrkC demonstrated that binding of TrkB with Slitrk5 is mediated by its single LRR domain (Figure 1G). Taken together, these studies demonstrate that Slitrk5 and TrkB interact specifically via their extracellular LRR domains.

Next, we carried out co-immunoprecipitation experiments to examine whether the interaction between Slitrk5 and TrkB is modulated by BDNF-dependent TrkB activation. These experiments showed that, compared to the control condition (10% fetal bovine serum [FBS]; Figures 1A and 1B), serum starvation (0% FBS) significantly reduced the basal interaction between FLAG-tagged Slitrk5 and TrkB (Figure 1H). In contrast, upon BDNF stimulation, the interaction between Slitrk5 and TrkB was significantly increased, and was blocked by pretreatment with K252a, an inhibitor of Trk kinases (Figure 1H). These studies suggest that Slitrk5 optimally interacts with a TrkB receptor complex that is activated by BDNF.

We next considered whether Slitrk5 and TrkB receptors co-localized within neurons. To test this hypothesis, we examined the subcellular localization of endogenous Slitrk5 and TrkB receptors in cultured striatal neurons using structured illumination microscopy (SIM), a form of high-resolution microscopy that uses a high-frequency striped pattern of light to illuminate the sample in multiple angles to enhance image resolution up to 85 nm (Gustafsson, 2000). In untreated striatal neurons, TrkB and Slitrk5 localized separately in dendrites, visualized with microtubule-associated protein 2 (MAP2) staining (Figure 1I, left panels; quantified in Figure 1J). In contrast, after BDNF treatment, TrkB receptors significantly co-localized with Slitrk5 in enlarged punctate structures, presumably reflecting its presence in endosomes (Figure 1I, right panels; quantified in Figures 1J and S1F). The co-localization of TrkB and Slitrk5 was visualized with co-localization highlighter (ImageJ, NIH), as well as with 3D reconstruction (IMARIS, Bitplane). Together, these interaction and immunocytochemical studies suggest that Slitrk5 and TrkB receptors interact in a BDNF-dependent manner.

BDNF Shifts Slitrk5 Binding from PTPδ to TrkB Receptors

Recently, one of the Slitrk isotypes, Slitrk3, was reported to mediate inhibitory synapse formation through transsynaptic interactions with presynaptic receptor-type protein tyrosine phosphatase δ (PTPδ) (Takahashi et al., 2012). Takahashi et al. (2012) also showed that Slitrk5 was capable of interacting with PTPδ. Therefore, we investigated whether the BDNF-induced cis-interaction of Slitrk5 with TrkB disrupts the trans-interaction of Slitrk5 with PTPδ (Figure 2). First, utilizing a binding assay in which we exposed HEK293T cells expressing full-length or mutant Slitrk5 to soluble purified PTPδ ectodomain fused to the Fc region of human immunoglobulin (PTPδ-Fc), we showed that Slitrk5 binds PTPδ through its LRR1 domain (Figures 2A and 2B). Next, using a heterophilic cell adhesion assay (Fogel et al., 2007), we tested whether the presence of TrkB receptors in cells expressing Slitrk5 affected the trans-interaction of Slitrk5 with PTPδ. Briefly, to mimic cis- and trans-interactions of Slitrk5 with TrkB and PTPδ, respectively, in the neuronal synapse, we co-cultured HEK293 cells co-expressing FLAG-Slitrk5 and TrkB with HEK293T cells expressing hemagglutinin (HA)-PTPδ. Surface proteins were labeled with respective antibodies at the

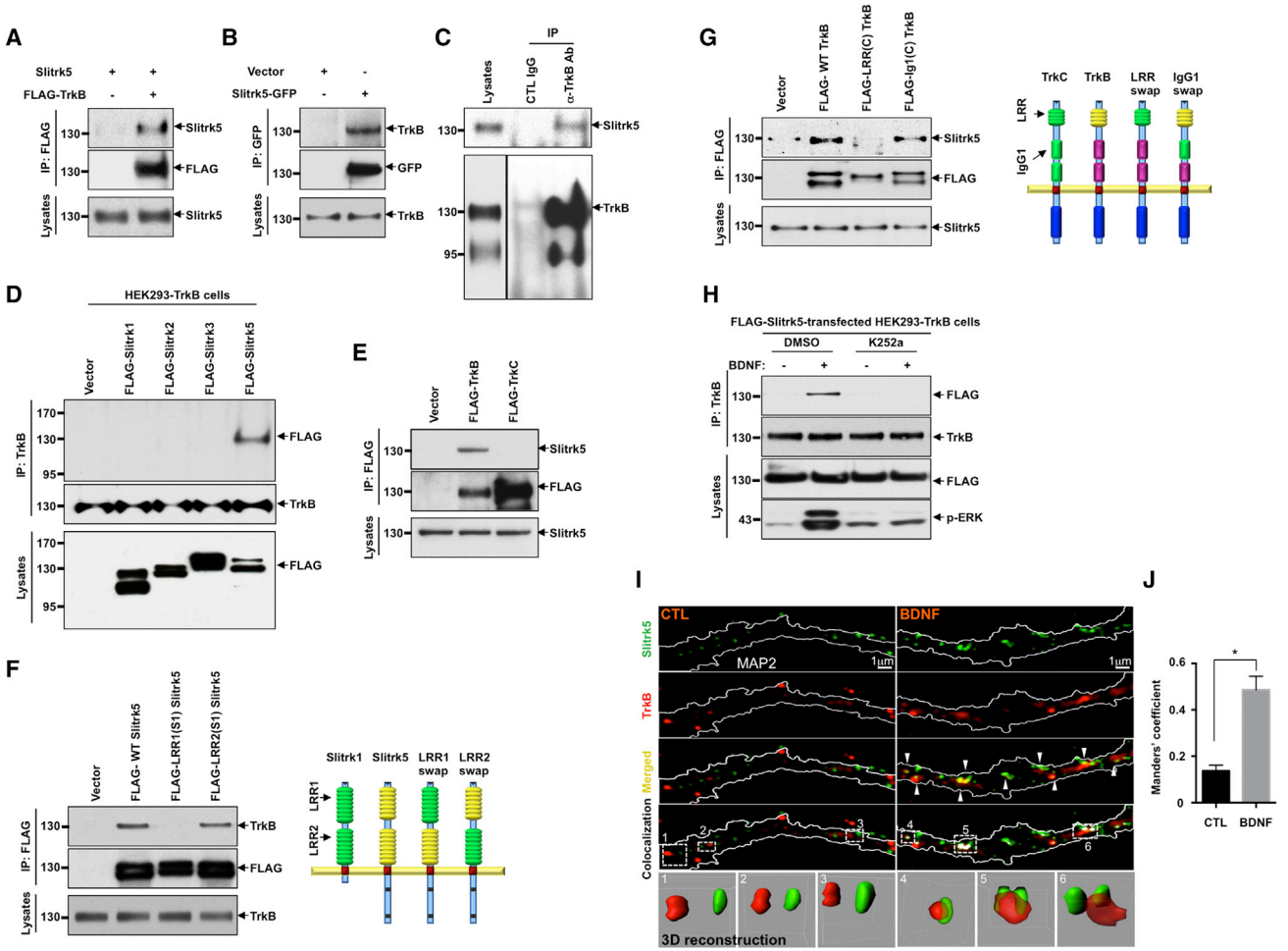

Figure 1. TrkB Receptors Interact and Co-localize with Slitrk5

(A) Interaction between TrkB receptors and Slitrk5 was assessed in HEK293T cells overexpressing cDNAs encoding FLAG-TrkB, Slitrk5, and empty vector. Cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-Slitrk5 antibodies.

(B) Interaction between TrkB receptors and Slitrk5 was assessed in HEK293-TrkB cells. Lysates from Slitrk5-GFP or empty vector-transfected HEK293-TrkB cells were immunoprecipitated with anti-GFP antibodies and immunoblotted with anti-TrkB antibody.

(C) Endogenous association of Slitrk5 and TrkB. Mouse whole-brain lysates (2 months old) were subjected to immunoprecipitation with anti-TrkB antibody (Millipore) or control IgG. The immune protein complex was then eluted, and TrkB and Slitrk5 were detected by immunoblotting.

(D and E) Interaction between TrkB and Slitrk5 is specific. (D) Interaction between TrkB and Slitrk family members. FLAG-tagged Slitrk family isotypes were expressed in HEK293-TrkB cells. Cell lysates were immunoprecipitated with anti-TrkB antibodies (Millipore) and immunoblotted with anti-FLAG (M2) antibodies. (E) Dissociated embryonic day 16 (E16) mouse cortical neurons were electroporated (Amaxa) with FLAG-TrkB or FLAG-TrkC. At DIV6, cell lysates were precipitated with anti-FLAG (M2) antibodies and immunoblotted with anti-Slitrk5 antibodies.

(F) First LRR domain of Slitrk5 mediates TrkB binding. HEK293-TrkB cells were transfected with FLAG-tagged WT Slitrk5, chimeric FLAG-LRR1-domain-swapped Slitrk5 (FLAG-LRR1(S1) Slitrk5), and LRR2-domain-swapped Slitrk5 [FLAG-LRR2(S1) Slitrk5] or empty vector. Cell lysates were precipitated with anti-FLAG (M2) antibodies and immunoblotted with anti-TrkB antibodies (Millipore). Schematic representation of chimeric Slitrk5 mutants shown on the right.

(G) LRR domain of TrkB mediates Slitrk5 binding. Dissociated E16 mouse cortical neurons were transfected with FLAG-tagged WT TrkB, LRR domain-swapped TrkB (FLAG-LRR(C) TrkB), and IgG1 domain-swapped TrkB [FLAG-IgG1(C) TrkB] or empty vector. At DIV6, cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-Slitrk5. Schematic representation of chimeric TrkB mutants shown on the right.

(H) BDNF-dependent and TrkB kinase activity-dependent binding of TrkB and Slitrk5. After transfection of FLAG-tagged Slitrk5 into HEK293-TrkB cells, cells were pretreated with DMSO or K252a for 30 min to inhibit kinase activity of TrkB after overnight serum starvation. Cells were treated with or without BDNF, and their binding was examined by immunoprecipitation with anti-TrkB antibodies followed by immunoblotting with anti-FLAG antibodies.

(I) TrkB receptors co-localize with Slitrk5 in a BDNF-dependent manner. DIV6 striatal neurons were treated with or without BDNF (25 ng/ml) for 30 min after incubating with anti-TrkB antibody to specifically label surface TrkB. Endogenous Slitrk5 and MAP2 were visualized with specific antibodies after fixation and permeabilization. Super-resolution images were acquired using a Nikon N-SIM structured illumination microscope. The co-localization of TrkB and Slitrk5 was presented using co-localization highlighter (ImageJ) and 3D reconstruction (IMARIS) to show more convincing co-localization signals.

(J) Histogram showing mean Manders' coefficients for co-localization of TrkB and Slitrk5 (n = 20 for each condition). High Manders' coefficients indicate better co-localization of TrkB with Slitrk5. Results are means ± SEM from three independent experiments. 20–30 neurons were analyzed per condition per experiment. *$p < 0.05$ significantly different from control condition (Student's t test).

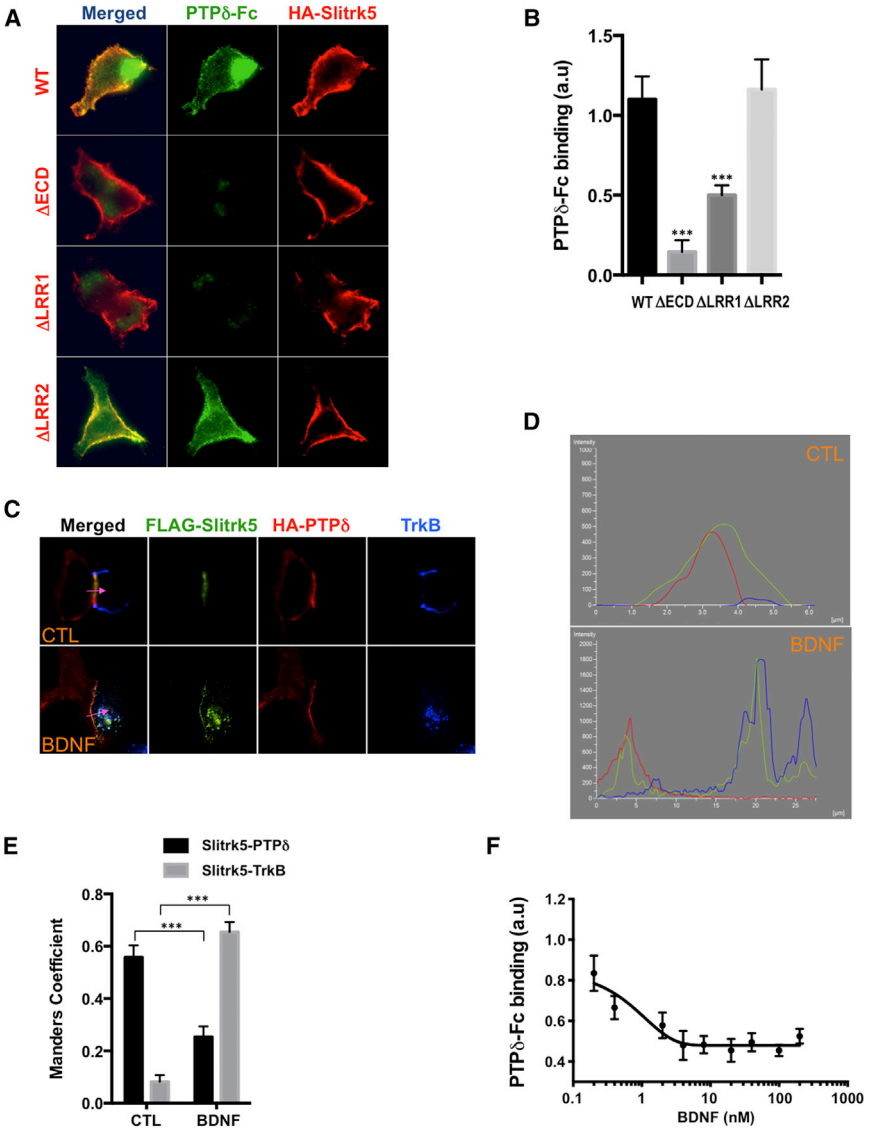

Figure 2. PTPδ and TrkB Compete for Binding to Slitrk5

(A) Slitrk5 binds PTPδ through LRR1. Soluble purified PTPδ-Fc chimeras were added to HEK293T cells expressing WT and indicated deletion mutants of Slitrk5, and the binding was analyzed by immunofluorescence microscopy. Note that the observed binding to WT Slitrk5 is abolished by deletion of Slitrk5's extracellular domain (ECD) or LRR1, but not LRR2.

(B) Quantitative analysis of these results. Results are means ± SEM from three independent experiments. 20–30 cells were analyzed per condition per experiment. ***$p < 0.0001$ significantly different from WT condition (one-way ANOVA and Dunnett's multiple comparisons test).

(C) BDNF displaces Slitrk5 binding from PTPδ to TrkB. Heterophilic cell adhesion assay in which HEK293-TrkB cells expressing FLAG-Slitrk5 are co-cultured with HEK293T cells expressing HA-PTPδ. Surface proteins were visualized by fluorescence microscopy in the presence or absence of BDNF treatment (as described in Supplemental Experimental Procedures).

(D) Fluorescence intensity trace of FLAG-Slitrk5 (green), HA-PTPδ (red), and TrkB (blue) scanning across corresponding purple arrow as indicated in (C).

(E) Quantitative colocalization analysis of FLAG-Slitrk5-HA-PTPδ and FLAG-Slitrk5-TrkB in the presence or absence of BDNF treatment in (C). Results are means ± SEM from three independent experiments. 10–15 cells showing heterophilic adhesion were analyzed per condition per experiment. ***$p < 0.0001$ significantly different from control condition (two-way ANOVA and Sidak's multiple comparisons test).

(F) 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 condition of PTPδ-Fc (400 nM) for 1 hr. After washing, cells were incubated with indicated dose of BDNF for 30 min. Remaining PTPδ-Fc binding was analyzed by immunofluorescence microscopy. Results are means ± SEM from three independent experiments. 20–30 cells were analyzed per condition per experiment.

beginning of the experiments, and their localization was analyzed by fluorescence microscopy for cis- and trans-interactions in the presence or absence of BDNF treatment. In the absence of BDNF, all FLAG-Slitrk5 was recruited to regions of heterophilic interaction with HA-PTPδ, where the interacting proteins formed stretched zipper-like structures, with minimal co-localization with TrkB (Figures 2C–2E). In contrast, upon addition of BDNF, FLAG-Slitrk5 co-localized with TrkB in punctate endosomal structures. Quantification of their co-localization showed that the binding preference of FLAG-Slitrk5 shifted from PTPδ to TrkB upon BDNF stimulation (Figures 2D and 2E). To further support this notion of a ligand-dependent shift in interactions, we employed additional quantitative approaches. We examined PTPδ-Fc binding to Slitrk5 with increasing amounts of PTPδ-Fc in the presence or absence of BDNF (25 ng/ml). Results indicate that the maximal binding capacity (B_{max}) of PTPδ-Fc, but not dissociation constant (K_D), was decreased in the presence of BDNF (Figure S2A). This result indicates that available PTPδ-Fc binding sites were reduced by BDNF treatment without changing affinity of PTPδ-Fc to Slitrk5. The apparent K_D value of PTPδ-Fc binding to Slitrk5 was ~100 nM regardless of BDNF treatment. Next, we measured dissociation of pre-bound PTPδ-Fc to surface-expressed HA-Slitrk5 with increasing BDNF concentrations. In accordance with previous competition data (Figures 2C–2E), BDNF potently induced dissociation of pre-bound PTPδ-Fc from HA-Slitrk5-expressing HEK293-TrkB cells (Figures 2F and S2B). Based on the PTPδ-Fc dissociation curve, the K_D value of BDNF to HA-Slitrk5 was ~0.9 nM. These results suggest that both PTPδ and TrkB compete for binding to Slitrk5 and that, whereas under basal conditions the interaction with PTPδ predominates, BDNF stimulation directs Slitrk5 to cis-interactions with TrkB receptors.

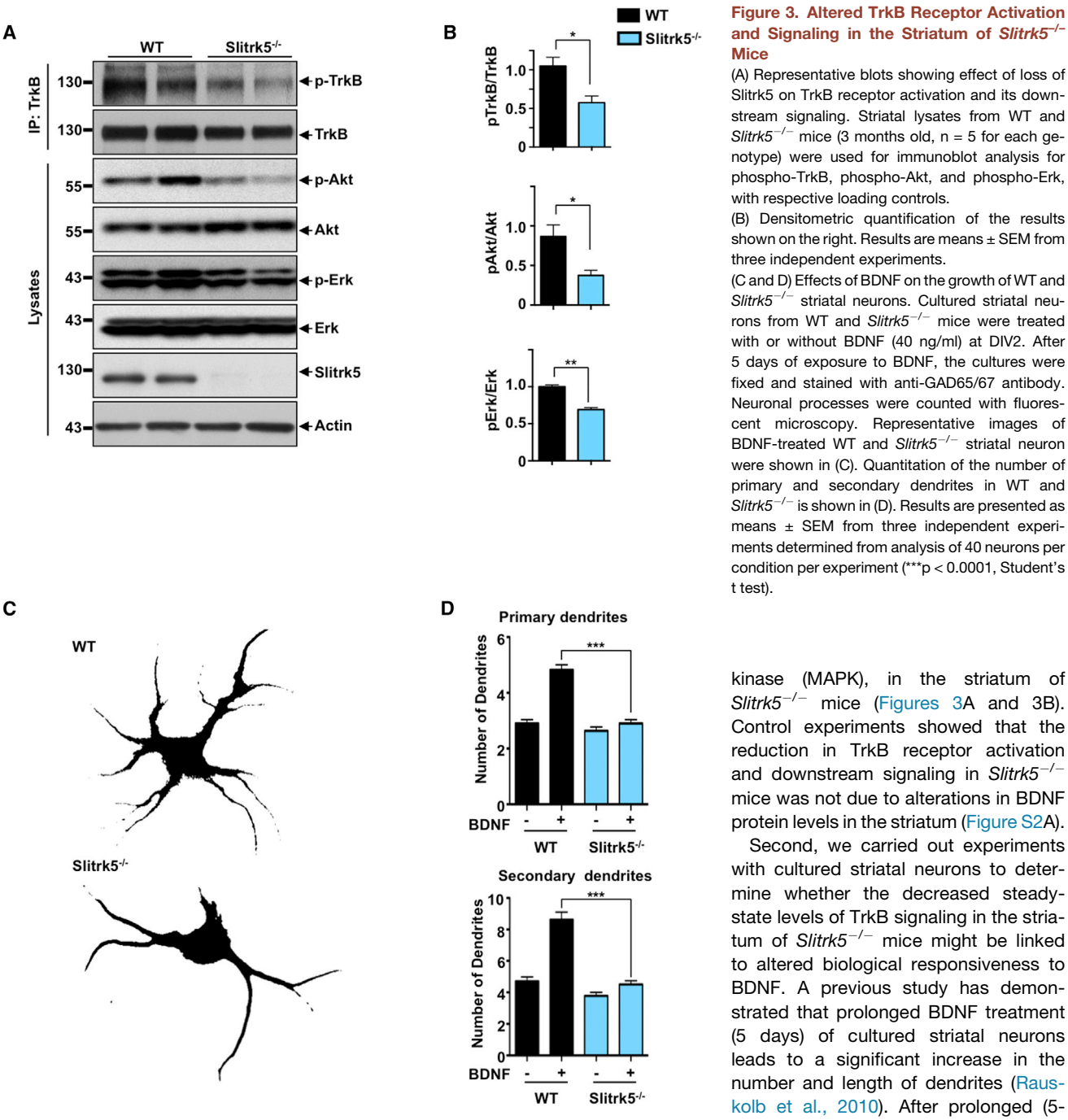

Figure 3. Altered TrkB Receptor Activation and Signaling in the Striatum of *Slitrk5*$^{-/-}$ Mice

(A) Representative blots showing effect of loss of Slitrk5 on TrkB receptor activation and its downstream signaling. Striatal lysates from WT and *Slitrk5*$^{-/-}$ mice (3 months old, n = 5 for each genotype) were used for immunoblot analysis for phospho-TrkB, phospho-Akt, and phospho-Erk, with respective loading controls.

(B) Densitometric quantification of the results shown on the right. Results are means ± SEM from three independent experiments.

(C and D) Effects of BDNF on the growth of WT and *Slitrk5*$^{-/-}$ striatal neurons. Cultured striatal neurons from WT and *Slitrk5*$^{-/-}$ mice were treated with or without BDNF (40 ng/ml) at DIV2. After 5 days of exposure to BDNF, the cultures were fixed and stained with anti-GAD65/67 antibody. Neuronal processes were counted with fluorescent microscopy. Representative images of BDNF-treated WT and *Slitrk5*$^{-/-}$ striatal neuron were shown in (C). Quantitation of the number of primary and secondary dendrites in WT and *Slitrk5*$^{-/-}$ is shown in (D). Results are presented as means ± SEM from three independent experiments determined from analysis of 40 neurons per condition per experiment (***$p < 0.0001$, Student's t test).

TrkB Receptor Signaling Is Impaired in the Striatum of *Slitrk5*$^{-/-}$ Mice

Next, we investigated whether Slitrk5 affects BDNF-mediated TrkB signaling. First, we examined the impact of Slitrk5 on steady-state BDNF-TrkB signaling in vivo. Western blot analyses of striatal lysates obtained from adult WT and *Slitrk5*$^{-/-}$ mice showed that TrkB receptor activation was significantly reduced in *Slitrk5*$^{-/-}$ mice relative to WT mice (Figures 3A and 3B). This effect on TrkB receptor activation was also reflected in a reduction in the activation of its downstream targets, Akt and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK), in the striatum of *Slitrk5*$^{-/-}$ mice (Figures 3A and 3B). Control experiments showed that the reduction in TrkB receptor activation and downstream signaling in *Slitrk5*$^{-/-}$ mice was not due to alterations in BDNF protein levels in the striatum (Figure S2A).

Second, we carried out experiments with cultured striatal neurons to determine whether the decreased steady-state levels of TrkB signaling in the striatum of *Slitrk5*$^{-/-}$ mice might be linked to altered biological responsiveness to BDNF. A previous study has demonstrated that prolonged BDNF treatment (5 days) of cultured striatal neurons leads to a significant increase in the number and length of dendrites (Rauskolb et al., 2010). After prolonged (5-day) exposure to BDNF (40 ng/ml), WT striatal neurons displayed 4.82 ± 0.19 primary dendrites and 8.62 ± 0.48 secondary dendrites, a 2-fold increase compared with untreated neurons. In contrast, after 5 days of BDNF exposure, *Slitrk5*$^{-/-}$ neurons developed only 2.91 ± 0.14 primary dendrites and 4.5 ± 0.24 secondary dendrites on average, which was not significantly different from untreated neurons (Figures 3C and 3D). These in vitro findings indicate that the absence of Slitrk5 causes no impairment in BDNF-independent morphogenesis of striatal neurons, but nearly abrogates their response to chronic BDNF. Taken together, these in vivo and in vitro results demonstrate that Slitrk5 is necessary

for optimal long-term BDNF-dependent TrkB signaling in striatal neurons.

Slitrk5 Plays a Pivotal Role in the Endocytic Recycling of TrkB Receptors

To obtain additional insight into the mechanisms underlying the role of Slitrk5 in BDNF-mediated TrkB signaling, we conducted a series of biochemical and fluorescence-based immunocytochemical assays. Initially, we investigated whether Slitrk5 acts, like $p75^{NTR}$, by enhancing Trk receptor responsiveness to its ligand. To this end, we tested in WT versus *Slitrk5*$^{-/-}$ striatal neurons whether a low dose of BDNF (1.0 ng/ml; 15 min) can acutely activate TrkB receptors more potently in the presence of Slitrk5. We chose an early time point at which stage minimal TrkB receptor recycling or degradation occurs (Chen et al., 2005). Our studies demonstrated no change in short-term TrkB activation or its downstream signaling pathways (Akt, ERK/MAPK) in response to low-dose BDNF (Figure S4A). Next, we hypothesized that the decreased steady-state TrkB signaling observed in *Slitrk5*$^{-/-}$ striatum (Figure 3A) may be due to alterations in endocytic TrkB receptor trafficking at a particular stage after ligand binding. It has been established that upon binding to neurotrophins, Trk receptors are rapidly internalized in a clathrin-dependent manner that engages endocytic adaptors (Grimes et al., 1997; Zheng et al., 2008; Zhou et al., 2011). Trk receptor endocytosis is also required for certain ligand-mediated downstream signaling (Riccio et al., 1997; Zheng et al., 2008; Zhou et al., 2011). To determine whether Slitrk5 functions to regulate endocytosis, we utilized a fluorescent ratiometric internalization assay we developed to study Trk receptors (Chen et al., 2005), based on similar assays to study other signaling receptors such as epidermal growth factor (EGF) receptors and G protein-coupled receptors (GPCRs) (Gage et al., 2001; Tanowitz and von Zastrow, 2003; Vargas and Von Zastrow, 2004). Quantification of these results confirmed that there was no significant difference in TrkB endocytosis between WT and *Slitrk5*$^{-/-}$ striatal neurons after BDNF treatment (Figure S4B), in agreement with our observation of similar levels of BDNF-induced TrkB activation at this time point. An alternative internalization assay using red fluorescent protein (RFP)-tagged BDNF confirmed that there was no alteration in BDNF-dependent TrkB internalization in the absence of Slitrk5 (Figures S4C and S4D).

The fate of Trk receptors after ligand binding and receptor internalization has significant impact on the physiological responses to neurotrophins, as it determines the strength and duration of signaling cascades initiated by activated Trk receptors. One of the established postendocytic pathways of Trk receptors is their retrograde trafficking from axons to cell bodies, which is reportedly required for trophic responses in the PNS, but not in the CNS (Ginty and Segal, 2002; Riccio et al., 1997). Alternatively, Trk receptors can undergo (1) trafficking to lysosomes, which is reflected in decreased number of surface Trk receptors and decreased responsiveness to ligand (Sommerfeld et al., 2000); or (2) recycling to the plasma membrane, which can lead to functional resensitization of cell-surface-specific signaling events (Chen et al., 2005; Huang et al., 2009). The mechanisms regulating the sorting of endocytosed Trk receptors into these diverse pathways, a complex and highly regulated process, remain unclear.

To investigate whether interactions with Slitrk5 control the postendocytic fates of TrkB, we examined the fate of TrkB receptors after BDNF treatment in WT and *Slitrk5*$^{-/-}$ striatal neurons, using standard biotinylation experiments in which cell-surface TrkB receptors are labeled and their degradation measured after ligand treatment by immunoblot analysis (Arévalo et al., 2006; Chen et al., 2005). In the WT striatal neurons, ~30% of TrkB was degraded within 90 min after BDNF treatment (25 ng/ml). Interestingly, we observed increased TrkB degradation (~70% in 90 min) in *Slitrk5*$^{-/-}$ striatal neurons (Figures 4A and 4B). The rate of TrkB degradation was reduced to control levels after lentiviral transduction of WT Slitrk5 into *Slitrk5*$^{-/-}$ neurons, but not after transduction of a chimeric Slitrk5 lacking its endogenous LRR1 domain [LRR1(S1)-Slitrk5] that has been shown not to interact with TrkB receptors.

How does the absence of Slitrk5 in cultured striatal neurons promote accelerated ligand-dependent degradation of TrkB? We hypothesized that Slitrk5 might promote TrkB recycling and its absence might allow targeting of endocytosed TrkB receptors to the degradative pathway. To test this hypothesis, we used a live-cell assay that was previously used to study recycling to the plasma membrane for TrkB and TrkA receptors (Chen et al., 2005; Huang et al., 2009, 2013). This assay is based on the FLAG-epitope system that allows rapid removal of fluoresceinated FLAG antibodies that bind to extracellular FLAG epitopes on signaling receptors in a calcium-dependent manner (Guan et al., 1992; Tanowitz and von Zastrow, 2003; Vargas and Von Zastrow, 2004). In WT striatal neurons, the level of TrkB recycling was 51% ± 1.4% (Figures 4C and 4D). In contrast, in the *Slitrk5*$^{-/-}$ striatal neurons, the level of ligand-dependent TrkB recycling was significantly reduced (33% ± 0.5%). This reduction in TrkB recycling was specifically rescued after lentiviral transduction of WT Slitrk5 (56% ± 1.8%), but not after transduction of TrkB binding-deficient Slitrk5 (39% ± 1.8%) (Figures 4C and 4D). In accordance with the binding experiments (Figures 1E and S1E), TrkC recycling was not affected in the absence of Slitrk5 (Figures S4E and S4F). Together, these results suggest that Slitrk5 is required for efficient TrkB receptor recycling after ligand treatment in striatal neurons.

Slitrk5 Facilitates TrkB Receptor Recruitment into Rab11 Endosomes

The experiments described above indicate that the persistent downregulation of TrkB receptors in *Slitrk5*$^{-/-}$ striatal neurons may be due to its reduced recycling rate, possibly explaining our findings of decreased steady-state BDNF-TrkB signaling in the striatum of *Slitrk5*$^{-/-}$ mice (Figure 3A), and altered striatal neuronal morphology (Shmelkov et al., 2010). To elucidate how Slitrk5 regulates TrkB receptor recycling, we hypothesized that Slitrk5 might act as a specialized sorting protein that enhances recycling of endocytosed TrkB receptors and prevents their incorporation into a degradative route. Recent studies have emphasized the importance of targeting TrkB receptors to a Rab11-positive recycling endosomes as an essential step for the physiological function of TrkB signaling (Huang et al., 2013; Lazo et al., 2013). Furthermore, it has been shown that TrkB can form a complex with Rab11 that may modulate synaptic plasticity (Huang et al., 2013) as well as BDNF-dependent dendritic branching (Lazo et al., 2013). To test our hypothesis, we

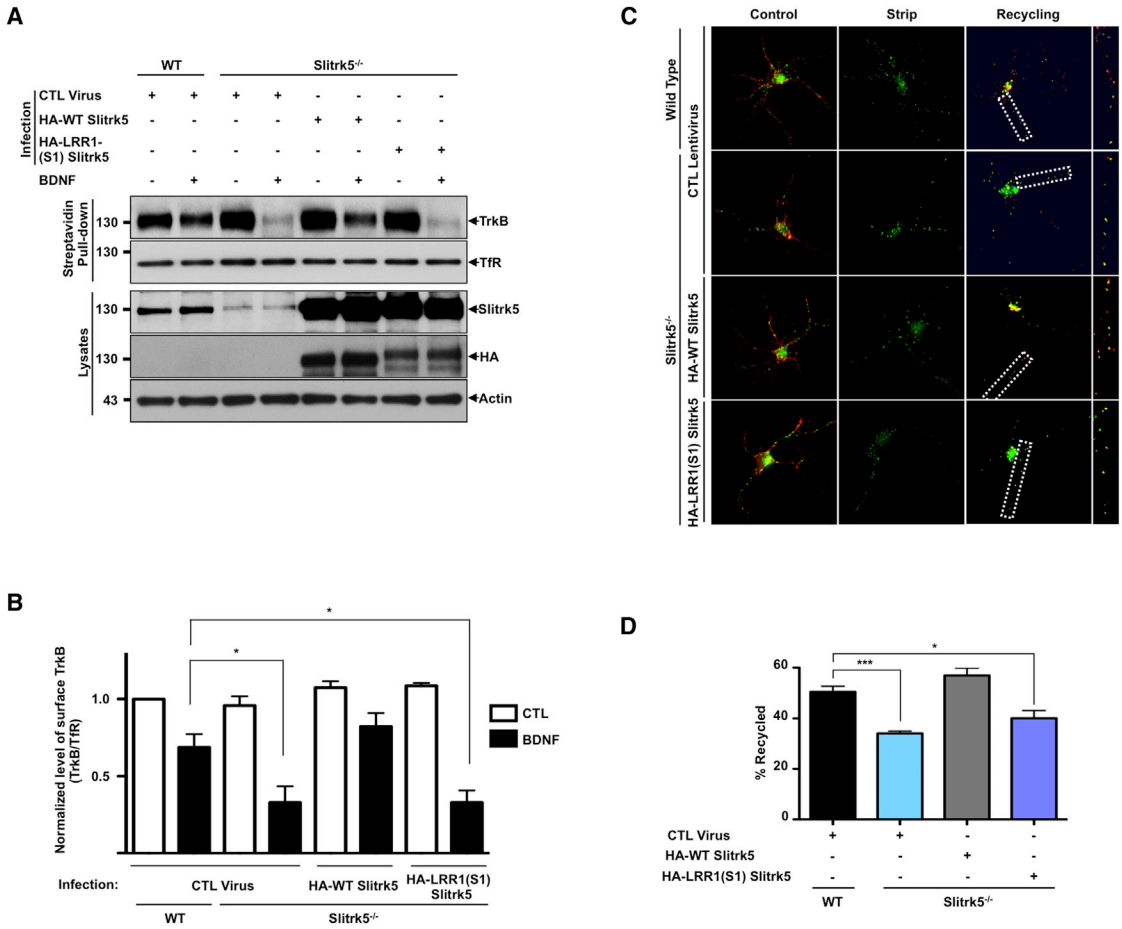

Figure 4. Slitrk5 Plays a Pivotal Role in Ligand-Dependent TrkB Receptor Recycling

(A) Representative blot showing enhanced BDNF-induced TrkB degradation in *Slitrk5*$^{-/-}$ striatal neurons. WT and *Slitrk5*$^{-/-}$ striatal neurons were transduced with HA-tagged WT Slitrk5, or chimeric HA-tagged-LRR1-domain-swapped (HA-LRR1(S1) Slitrk5)-expressing, or empty vector lentivirus at DIV2. Neurons were surface biotinylated and incubated at 37°C for 90 min in the absence or presence of 25 ng/ml BDNF at DIV6. Surface-labeled receptors were detected by streptavidin pull-down followed by anti-TrkB immunoblotting.

(B) Densitometric quantification of the results from three independent experiments shown in (A) (*$p < 0.05$, Student's t test).

(C and D) TrkB recycling was impaired in *Slitrk5*$^{-/-}$ striatal neurons. TrkB recycling was examined in WT and *Slitrk5*$^{-/-}$ neurons with live-cell fluorescence ratiometric recycling assay. WT and *Slitrk5*$^{-/-}$ striatal neurons were co-transfected with FLAG-tagged TrkB lentivirus, HA-tagged WT Slitrk5, or chimeric HA-tagged-LRR1-domain-swapped (HA-LRR1(S1) Slitrk5)-expressing, or empty vector lentivirus at DIV2. Internalization of TrkB receptor was induced by BDNF treatment for 30 min after labeling surface FLAG-tagged TrkB with Alexa 488 dye-conjugated anti-FLAG (M1) antibody. Remaining surface anti-FLAG (M1) antibodies were removed with EDTA-containing Ca^{2+}/Mg^{2+}-free PBS, and then recycling of FLAG-tagged TrkB was monitored in the presence of Cy3-conjugated anti-mouse secondary antibody in culture medium. (C) Representative images from FLAG-tagged TrkB recycling experiment in striatal neurons. Control refers to the 100% surface TrkB receptor control, and Strip refers to the 0% recycled control. The right panels of each images showed enlarged images of framed regions. (D) Receptor recycling was quantitated as described in Experimental Procedures. Results are presented as means ± SEM from three independent experiments determined from analysis of 30 neurons per condition per experiment (***$p < 0.001$, *$p < 0.05$, Student's t test).

initially examined whether Slitrk5 mediates recycling of TrkB into Rab11-positive endosomes in cultured striatal neurons. After transduction with lentivirus encoding HA-tagged Slitrk5 construct, days in vitro 6 (DIV6) WT striatal neurons were labeled using a "live-feeding" method to specifically visualize cell-surface HA-tagged Slitrk5 and endogenous TrkB receptors with their respective antibodies (Chen et al., 2005; Huang et al., 2009, 2013). Neurons were stained for Rab11 after fixation and permeabilization. In the absence of BDNF treatment, only minimal co-localization of TrkB, HA-tagged Slitrk5, and Rab11 was detected in WT striatal neurons. In contrast, after 30 min of BDNF treatment, there was a significant overlap of Slitrk5 and TrkB in Rab11-positive compartments (Figure 5A). This result suggests that Slitrk5 and TrkB receptors are sorted to Rab11-positive compartments after ligand treatment. We next investigated whether Slitrk5 is required for TrkB localization to the Rab11-positive compartments. In WT striatal neurons, there was 3-fold increase in TrkB co-localization with Rab11 at 30 min after BDNF treatment (Figures 5B and 5C). This colocalization was significantly reduced after similar treatment of BDNF in *Slitrk5*$^{-/-}$ striatal neurons (Figures 5B and 5C). Together, these results suggest that Slitrk5 plays an important role in TrkB recycling by facilitating TrkB sorting into Rab11-positive compartments after BDNF treatment.

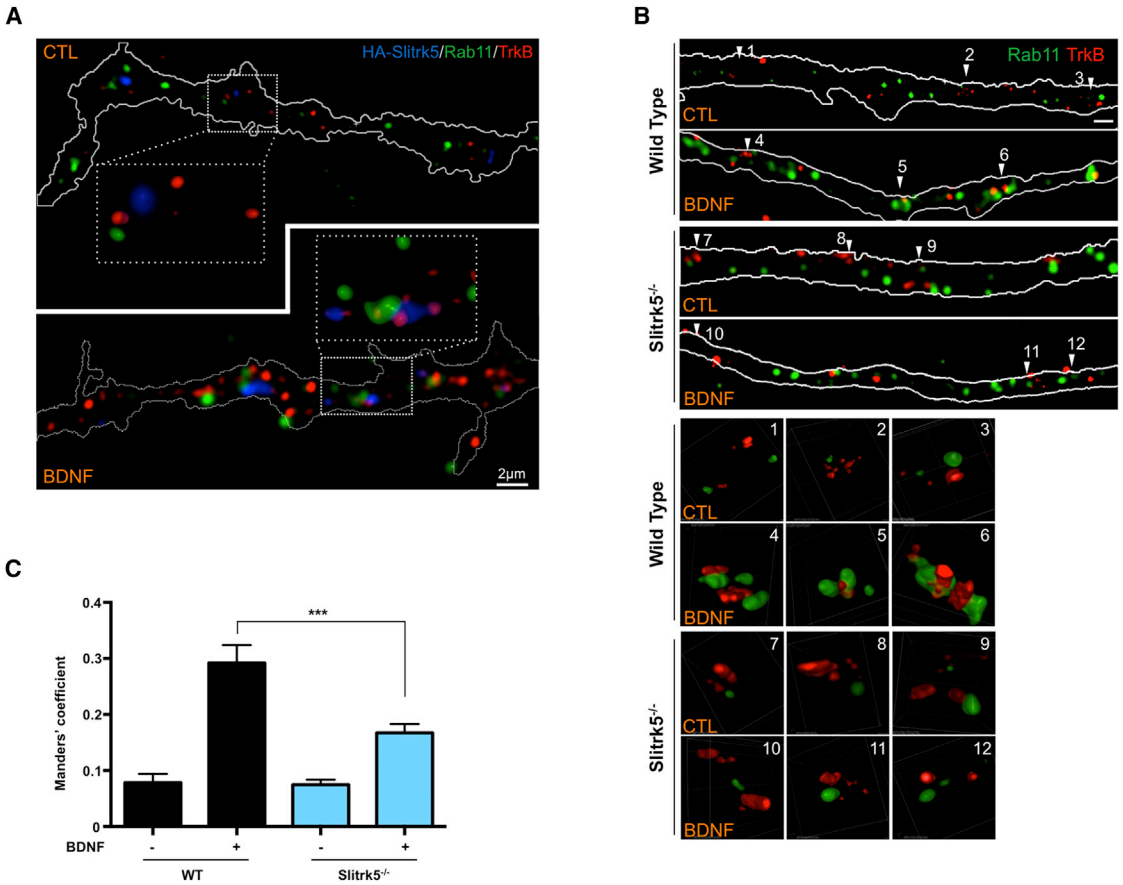

Figure 5. Slitrk5 Facilitates TrkB Receptor Recruitment into Rab11-Positive Compartments

(A) Representative images showing co-localization of TrkB receptors and Slitrk5 in Rab11-positive compartments. WT striatal neurons were transduced with HA-tagged Slitrk5 lentivirus at DIV2. Live neurons were incubated with anti-TrkB and anti-HA antibody to specifically label cell-surface proteins and then stimulated with or without BDNF for 30 min at DIV6. Rab11 was stained after fixation and permeabilization. Super-resolution images were acquired using a Nikon N-SIM structured illumination microscope.

(B) Representative images showing requirement of Slitrk5 for TrkB localization into Rab11-positive compartments after BDNF treatment. Co-localization of TrkB receptors and Rab11 was examined with WT and *Slitrk5*$^{-/-}$ striatal neurons in the presence or absence of BDNF. Lower panels show enlarged images of numbered regions in upper panels.

(C) Co-localization of TrkB and Rab11 was quantitated as described in Experimental Procedures. Results are presented as means ± SEM from three independent experiments determined from analysis of n ≥ 30 neurons per condition per experiment (***p < 0.001, Student's t test).

Slitrk5 Facilitates Rab11-FIP3 Recruitment of TrkB Receptors to Rab11 Compartments

To obtain additional mechanistic insight on how Slitrk5 mediates the sorting of endocytosed TrkB to the Rab11-positive compartments, we screened for TrkB interacting proteins using a yeast two-hybrid assay. An 85-amino-acid (aa) intracellular juxtamembrane region of the TrkB receptor was selected as bait, since the region was shown to be important for endocytic TrkB recycling (Chen et al., 2005; Huang et al., 2009). Among the positive clones was Rab11-FIP3, a protein that has been also established to interact with ADP-ribosylation factors (ARF5, ARF6) as well as with motor proteins (kinesin I, dynein light intermediate chain) (Horgan et al., 2010; Prekeris, 2003; Simon and Prekeris, 2008), and was previously shown to modulate recycling of various cargoes (Horgan and McCaffrey, 2009; Prekeris, 2003). We confirmed with co-immunoprecipitation studies that Rab11-FIP3 and TrkB receptors interacted (Figure S5A). To test whether there was a physical interaction between Slitrk5 and Rab11-FIP3, we transfected FLAG-tagged Slitrk5 and HA-tagged Rab11-FIP3 plasmids into HEK293T cells and confirmed their binding with co-immunoprecipitation study (Figure 6A). Additional experiments with a panel of Rab11-FIPs demonstrated that Slitrk5 bound selectively and directly to Rab11-FIP3, but not to other Rab11-FIPs (Figures 6B and S6A), and facilitated the interaction between TrkB and Rab11-FIP3 (Figure S5B). Rab11 was not required for Rab11-FIP3 binding to Slitrk5; however, Rab11-FIP3 was essential for the recruitment of Rab11 to Slitrk5 (Figures S6B–S6D). In parallel with this biochemical study, we performed fluorescent microscopy studies to assess co-localization of Slitrk5, TrkB, and Rab11-FIP3 in cultured striatal neurons. Endogenous TrkB and transfected HA-tagged Slitrk5 were visualized using the live-feeding method with respective antibodies to specifically label cell-surface TrkB and HA-tagged Slitrk5, and then endogenous

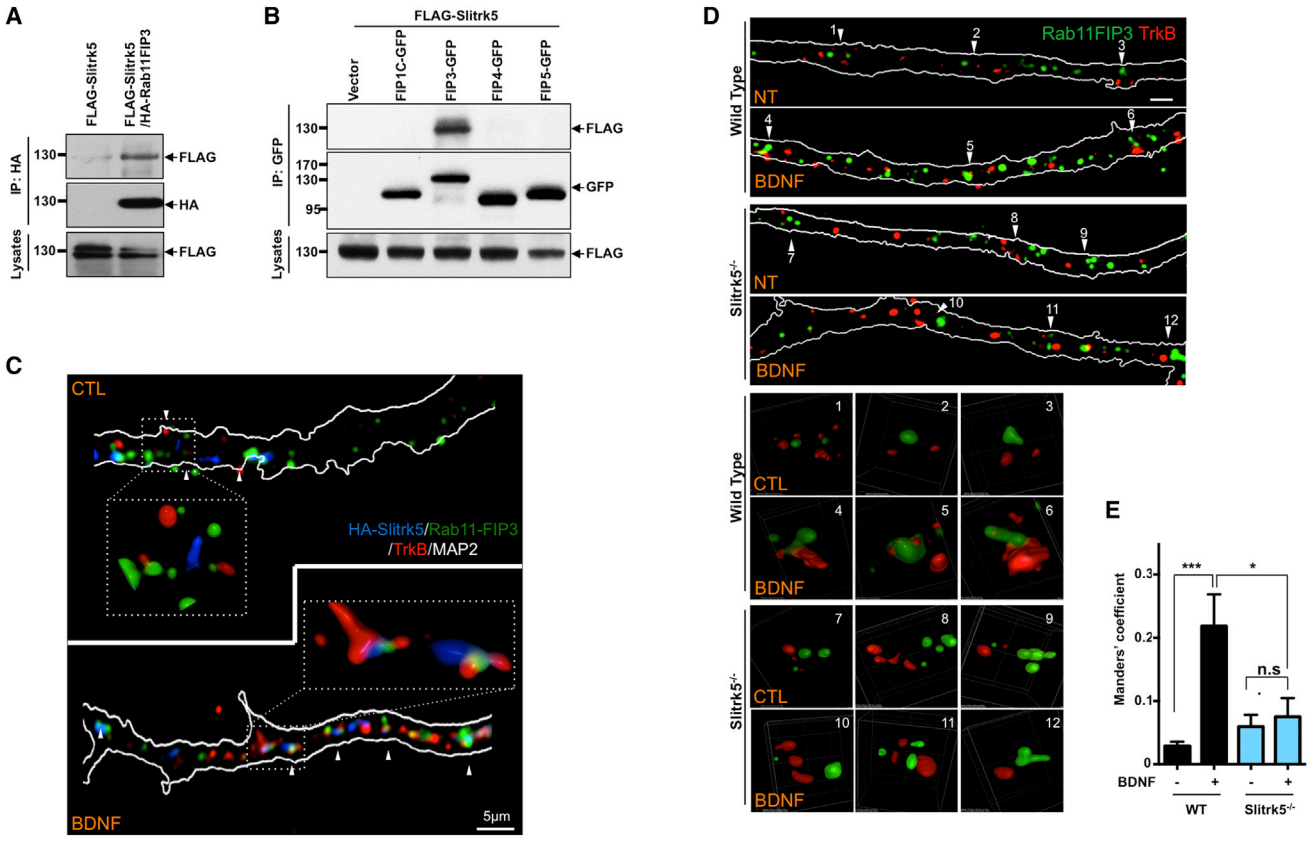

Figure 6. Slitrk5 Interacts with Rab11-FIP3 to Facilitate TrkB Receptor Trafficking to Rab11-Positive Recycling Endosomes

(A and B) Representative blots showing specific interaction between Slitrk5 and Rab11-FIP3. (A) HEK293T cells were transfected with cDNAs encoding FLAG-Slitrk5, and HA-Rab11-FIP3. Cell lysates were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-FLAG antibodies. (B) HEK293T cells were transfected with cDNAs encoding FLAG-Slitrk5, and either empty vector, Rab11-FIP3-GFP, FIP1C-GFP, FIP4-GFP, or FIP5-GFP. Cell lysates were immunoprecipitated with anti-GFP antibodies and blotted with anti-FLAG antibodies.

(C) Representative image showing the co-localization of TrkB and Slitrk5 with Rab11-FIP3 with BDNF-dependent manner. WT striatal neurons expressing HA-Slitrk5 were stimulated with or without BDNF after incubating with anti-TrkB and anti-HA antibody for surface protein labeling. Neurons were stained with anti-Rab11-FIP3 antibody after fixation and permeabilization. Super-resolution images were acquired using a Nikon N-SIM structured illumination microscope.

(D) Representative images showing requirement of Slitrk5 for TrkB receptor localization in Rab11-FIP3 compartments. Co-localization of TrkB and Rab11-FIP3 was examined with WT and *Slitrk5*$^{-/-}$ striatal neurons in the presence or absence of BDNF. Lower panels show enlarged images of numbered regions in upper panels.

(E) Co-localization of TrkB and Rab11-FIP3 was quantitated as described in Experimental Procedures. Results are presented as means ± SEM from three independent experiments determined from analysis of n ≥ 20 neurons per condition per experiment (*p < 0.05, ***p < 0.001, Student's t test).

Rab11-FIP3 was imaged after fixation and permeabilization. The results showed that, indeed, TrkB and Slitrk5 were co-localized with Rab11-FIP3 after BDNF treatment (Figure 6C). We were also able to see co-localization of TrkB, Slitrk5, Rab11-FIP3, and Rab11 after BDNF treatment (Figure S6E). These findings support a scenario in which Slitrk5 recruits Rab11-FIP3 to TrkB receptors during postendocytic trafficking to properly target TrkB receptors to the recycling pathway.

We next investigated whether Slitrk5 is required for TrkB localization to the Rab11-FIP3-positive compartments. We used a live-feeding method to specifically visualize cell-surface TrkB receptors with anti-TrkB antibodies (Chen et al., 2005; Huang et al., 2009, 2013) and stained for Rab11-FIP3 after fixation and permeabilization. Results showed impaired TrkB localization in Rab11-FIP3-positive compartments in *Slitrk5*$^{-/-}$ striatal neurons after BDNF treatment (Figures 6D and 6E). However, there was a significant overlap of TrkB in compartments that were positive for Rab11-FIP3 in WT striatal neurons after BDNF treatment (Figures 6D and 6E).

Slitrk5-Mediated Rab11-FIP3 Recruitment Is Required for TrkB Recycling

To further examine whether Rab11-FIP3 is directly involved in TrkB recycling, we performed live-cell fluorescent ratiometric recycling assays with control small interfering RNA (siRNA) or Rab11-FIP3 siRNA-transfected striatal neurons. Knockdown efficiency of siRNA targeting Rab11-FIP3 was validated with western blot and qPCR analyses (Figures S7A and S7B). Quantification of these results by ratiometric analysis confirmed that TrkB recycling was impaired in striatal neurons upon Rab11-FIP3 knockdown (36.95% ± 1.75%) compared to control siRNA transfected (48.12% ± 2.48%) (Figures 7A and S7C). We also

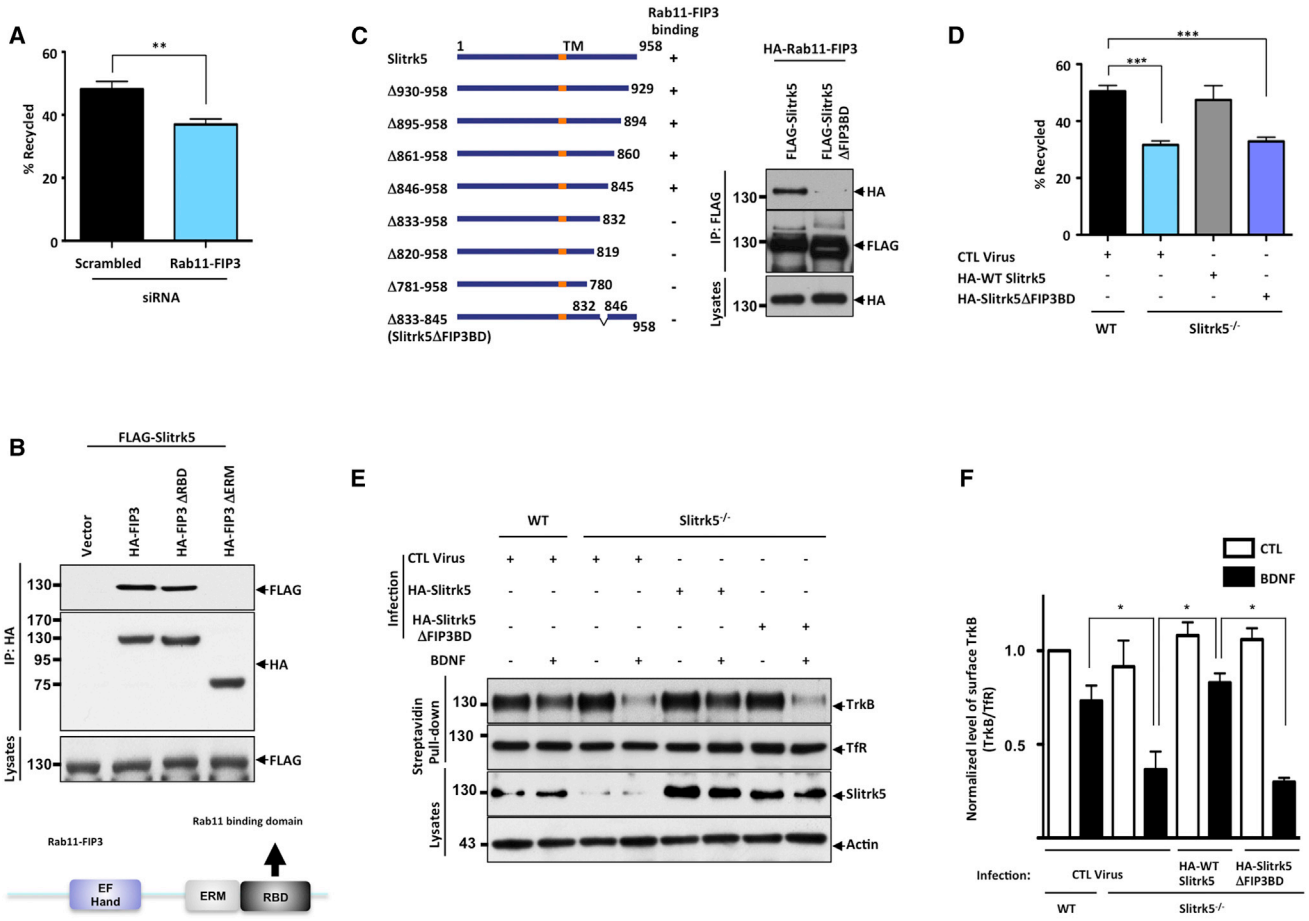

Figure 7. Slitrk5-Mediated Rab11-FIP3 Recruitment Is Required for TrkB Recycling

(A) TrkB recycling was quantified with live-cell fluorescence ratiometric recycling assay in the control siRNA (Scrb) or Rab11-FIP3 siRNA-transfected striatal neuron. Knockdown efficiency of siRNA targeting Rab11-FIP3 and representative images of live-cell fluorescence ratiometric recycling assay are shown in Figures S4A–S4C. The error bars represent the SEM of three independent experiments ($n \geq 30$ cells for each condition per experiment (**$p < 0.001$, Student's t test).

(B) ERM domain of Rab11-FIP3 mediates Slitrk5 binding. HEK293T cells were co-transfected with cDNAs encoding FLAG-Slitrk5, and either empty vector, HA-Rab11-FIP3, HA-Rab11-FIP3ΔRBD, or HA-Rab11-FIP3ΔERM. Cell lysates were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-FLAG antibodies. Schematic of Rab-FIP3 denoting established domains (EF Hand, ERM domain, and RBD [Rab11 binding domain]).

(C) Mapping domain in Slitrk5 that mediates association with Rab11-FIP3. (Left) Schematic representation shows a series of Slitrk5 deletion mutants that were tested for the capacity to interact with Rab11-FIP3. HEK293T cells were co-transfected with cDNAs encoding HA-Rab11-FIP3 and either FLAG-Slitrk5 or FLAG-Slitrk5ΔFIP3BD. (Right) Cell lysates were immunoprecipitated with anti-FLAG antibodies and immunoblotted with anti-HA antibodies.

(D) Rab11-FIP3 binding is required for Slitrk5 to rescue reduced recycling of TrkB in *Slitrk5*$^{-/-}$ striatal neurons. WT and *Slitrk5*$^{-/-}$ striatal neurons were co-transfected at DIV2 with FLAG-tagged TrkB lentivirus, and either empty vector, HA-Slitrk5, or HA-tagged Rab11-FIP3 binding-deficient Slitrk5 (HA-Slitrk5ΔFIP3BD). BDNF-induced TrkB recycling was measured with live-cell fluorescence ratiometric recycling assay at DIV6, as described in Experimental Procedures. The error bars represent the SEM of three independent experiments ($n \geq 30$ cells for each condition per experiment (***$p < 0.0001$, Student's t test).

(E) A cell-surface biotinylation assay shows that RAB11-FIP3 binding-deficient Slitrk5 is not able to rescue enhanced degradation of TrkB. WT and *Slitrk5*$^{-/-}$ striatal neurons were transduced with empty vector, HA-tagged WT Slitrk5, or HA-Slitrk5ΔFIP3BD-expressing lentivirus at DIV2. At DIV6 neurons were surface biotinylated and incubated in the presence or absence of BDNF (25 ng/ml; 90 min). Cell lysates were subjected to avidin pull-down, and TrkB levels were assessed by immunoblotting with anti-TrkB antibodies.

(F) Densitometric quantification of the results was shown. The error bars represent the SEM of three independent experiments (*$p < 0.05$, Student's t test).

confirmed that TrkB recycling was impaired in striatal neurons transfected with Rab11-FIP3ΔRBD or ΔERM mutant, presumably due to the impaired Rab11 binding or Slitrk5 binding, respectively (Figures S7D and S7E).

Rab11-FIP3 contains several conserved domains that may be involved in protein-protein interactions. The central ezrin/radixin/moesin (ERM) domain is known to interact with actin cytoskeleton while Rab11 binding domain is localized in the C terminus. To determine the Rab11-FIP3 domain responsible for binding to Slitrk5, we constructed a series of deletion mutants and tested their ability to interact with Slitrk5. Co-immunoprecipitation experiments showed that the ERM domain of Rab11-FIP3 is essential for the Slitrk5 binding (Figure 7B).

We next mapped the Rab11-FIP3 binding region on Slitrk5 using a series of C-terminal deletion mutants of Slitrk5. We found that deletion of 13 amino acids in the intracellular region of Slitrk5

abolished Rab11-FIP3 binding (Figure 7C). Next, we took advantage of this Rab11-FIP3 binding-deficient mutant (Slitrk5Δ FIP3BD) to determine whether Rab11-FIP3 is necessary for ligand-dependent TrkB recycling. Using fluorescent ratiometric recycling assays, we demonstrated that Slitrk5ΔFIP3BD was not able to rescue the decreased recycling of TrkB in *Slitrk5*$^{-/-}$ striatal neurons (Figure 7D) in contrast to WT Slitrk5. In accordance with this result, a TrkB degradation experiment showed that Slitrk5ΔFIP3BD was not able to reverse the enhanced rate of TrkB degradation in *Slitrk5*$^{-/-}$ striatal neurons (Figures 7E and 7F). These complementary experiments highlight the importance of Rab11-FIP3 binding to Slitrk5 in mediating efficient TrkB recycling in striatal neurons and suggest that Slitrk5 plays a pivotal role in the Rab11-mediated TrkB recycling by facilitating the recruitment of a Rab11 interacting protein, Rab11-FIP3, into the TrkB receptor complex for efficient targeting to the recycling endosomes.

DISCUSSION

LRR domain-containing proteins play pivotal roles in the regulation of various neuronal functions, such as neurite outgrowth, synapse formation, and dendritic morphogenesis. Two basic mechanisms are employed by LRR proteins to execute such functions. First, LRR proteins function in *trans* as cell-cell adhesion molecules that mediate axon-dendrite adhesion (Gur et al., 2004; Laederich et al., 2004; Lin et al., 2003; Shattuck et al., 2007; Zhao et al., 2008). Second, LRR proteins act in *cis* to regulate cell-surface receptor function (Gur et al., 2004; Laederich et al., 2004; Shattuck et al., 2007; Zhao et al., 2008). Here, we have shown that Slitrk5, a postsynaptic plasma membrane protein containing extracellular LRR domains, interacts under basal conditions with the presynaptic adhesion molecule PTPδ in *trans*, but in the presence of BDNF, shifts to a cis-interaction with TrkB receptor that mediates its postendocytic recycling, leading to functional resensitization of neurotrophic signaling. Of note, the Slitrk5 interaction with TrkB receptors represents the first reported interaction among LRR proteins and receptor tyrosine kinases (RTKs) that is mediated by the respective LRR domains. This likely contributes to the high specificity of this interaction and to the exclusion of other Slitrk and Trk family members (Figure 1). In contrast to other LRR-containing proteins, such as Lrigs, which negatively regulate epidermal growth factor receptor (EGFR) and Met, Slitrk5 positively regulates TrkB receptor activity. In addition, a recent study shows that another LRR-containing protein, Linx, is required for NGF-TrkA and glial cell line-derived neurotrophic factor (GDNF)-GDNF α1 (GFRα1)/Ret-mediated sensory and motor axonal projections. Linx forms physical complex with Trk receptors (TrkA and TrkC, but not with TrkB) and Ret receptors; however, it was not determined how Linx modulates RTK complexes in that study (Mandai et al., 2009). Linx has a relatively short cytoplasmic domain (134 aa) compared to Slitrk5 (271 aa), and does not have features required for downstream signaling in the cytoplasmic domain, e.g., a tyrosine phosphorylation site or a Src homology 2 (SH2) domain binding site, both observed in the intracellular domain of Slitrk5. Interestingly, we found that Linx is required for the recycling of TrkA (Figures S7F–S7H). Together with the suggested role of Lrigs on EGFR ubiquitination and downregulation (Gur et al., 2004; Laederich et al., 2004), these results suggest that LRR protein-mediated regulation of trafficking fate could be a universally employed mechanism for modulating RTK signaling.

In addition to the originally established function of Slitrk family members in regulating neuronal processes outgrowth (Abelson et al., 2005; Aruga and Mikoshiba, 2003), recent studies have focused on Slitrks' involvement in synapse formation. All Slitrk family members have been shown to induce presynaptic neuronal differentiation in a cellular co-culture system (Takahashi et al., 2012). Slitrks have been shown to interact with presynaptic receptor-type PTPδ. Thus, Slitrks were only considered to mediate a trans-interaction with a presynaptic receptor-type protein tyrosine phosphatase. Our current studies elucidate a new aspect of Slitrk5 function in which Slitrk5 has a cis-interaction with activated TrkB receptors on the surface of postsynaptic sites via extracellular interactions. Intriguingly, the cis-interactions of Slitrk5 with TrkB receptors compete with trans-interactions with presynaptic partners PTPδ, and the competition was modulated by BDNF stimulation (Figure 2). It will be important to study further the significance of the interplay between these three synaptic molecules during steady-state and activity-dependent synaptic remodeling. It is interesting to note that significant co-localization of Slitrk5 and TrkB receptors occurs only after BDNF treatment, suggesting that the interaction is enhanced after TrkB dimerization, involving the two LRR domains in the TrkB dimer, leading to optimal interaction with the LRR1 domain of Slitrk5. The interaction between the extracellular domains of Slitrk5 and TrkB allows their intracellular interaction with Rab11-FIP3, which mediates the recycling of TrkB receptors to the cell surface via Rab11 recycling endosomes, while not trafficking the other TrkB isoform, truncated TrkB receptor, to the same compartments. Truncated TrkB receptors have been shown to act in a dominant-negative manner, sequestering BDNF from full-length TrkB (Eide et al., 1996). In striatal neurons, with limiting BDNF supplies, Slitrk5 could thus act to efficiently recycle activated TrkB receptors, facilitating BDNF-dependent signaling pathways.

In summary, the present study identifies an unanticipated role of a cell-surface transmembrane protein in regulating TrkB receptor endocytic trafficking to recycling endosomes, leading to facilitation of neurotrophic signaling in neurons. Engagement of TrkB receptors with Slitrk5, as a co-receptor, represents a new mechanism of how neurons within a particular brain region with limiting BDNF levels can expand the strength and duration of neurotrophic factor signaling. The striatum is the largest component of the basal ganglia, and loss of striatal function has been implicated in neurodegenerative disorders such as Huntington's disease and Parkinson's disease. While neurotrophins and neurotrophin receptors have been thought of as potential therapeutic targets for these disorders, there has been limited success in these lines of investigation due to pharmacokinetic and delivery issues. Slitrk5 represents a new potential target for therapeutics for these neurodegenerative disorders in which selective facilitation of BDNF-dependent signaling could be achieved in a region-specific manner by enhancing interaction of TrkB receptors with Slitrk5 in the absence of exogenous neurotrophic factors.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

The reagents, antibodies, plasmid construct, siRNA, and primers used in this study are available in Supplemental Experimental Procedures.

Production of Soluble PTPδ-Fc Protein and Binding Assays

Based on previously described methods (Takahashi et al., 2012), PTPδ-Fc was generated using HEK293 cells transfected with the expression vectors, and purified from culture media. For testing binding of soluble PTPδ-Fc, HEK293T or HEK293-TrkB cells on coverslips were transfected with the expression vectors for WT and deletion mutants of HA-tagged Slitrk5 and grown for 24 hr. The transfected cells were washed with extracellular solution (ECS; 168 NaCl mM, 2.4 KCl mM, 20 mM HEPES [pH 7.4], 10 mM D-glucose, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$) that contained 100 μg/ml BSA (ECS/BSA) and then incubated with ECS/BSA that contained 100 nM purified PTPδ Fc-fusion protein for 1 hr at room temperature in the presence or absence of BDNF. The cells were washed in ECS, fixed with 4% paraformaldehyde, and incubated first with blocking solution and then with mouse anti-HA antibody. Cells were incubated with subtype-specific fluorescenated secondary antibodies and analyzed by fluorescence microscopy with Alexa 488 dye-conjugated anti-human immunoglobulin G (IgG) (H^+L) antibodies (donkey IgG, 1:400, Jackson ImmunoResearch Laboratories) for labeling of bound Fc proteins. For the PTPδ-Fc dissociation experiment, Slitrk5-expressing HEK293-TrkB cells were incubated with purified PTPδ Fc-fusion protein for 1 hr at room temperature. After washing with ECS, PTPδ Fc-bound cells were incubated with indicated dose of BDNF for 30 min at room temperature. For quantification, we measured the average intensity of bound Fc protein per COS cell area, subtracted for off-cell background.

Super-Resolution Microscopy

For super-resolution analysis, imaging was performed on Nikon's structured illumination microscope (N-SIM) Nikon Eclipse Ti that can bring the resolution to 100–85 nm, equipped with an ANDOR camera. Images of Alexa fluors 488, 568, and 647 were acquired in 3D-SIM mode using 100× Apo total internal reflection fluorescence (TIRF) lens with 1.49 numerical aperture (NA). For every z stack, 15 images were generated, resulting from three directions and five phases, which were subsequently reconstructed using NIS-Elements software with SIM plugin (Nikon) to generate super-resolution data. In order to outline dendritic morphology, MAP2 images were acquired using TIRF 405 laser and combined with SIM images. The background was subtracted and maximum intensity projection-collapsed images are portrayed along with cropped segments represented in volume view. Each experiment was repeated at least three times. See the Supplemental Experimental Procedures for details on neuronal culture and imaging sample preparation.

Analysis of Trk Receptor Recycling by using Fluorescence Ratio Microscopy

To quantify the extent of TrkB recycling in individual neurons, an adapted version of previous receptor recycling methods (Chen et al., 2005; Tanowitz and von Zastrow, 2003; Vargas and Von Zastrow, 2004) was employed. Details are available in Supplemental Experimental Procedures.

Statistical Analyses

Statistical analyses were performed using Prism v.5.0 software (GraphPad). Statistical significance was considered at $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ between the means of a minimum of three groups and was determined using Student's t test or one-way or two-way ANOVA test as indicated in the figure legends. Results are expressed as the mean ± SD. All experiments were done with at least three independent biological replicates.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.04.009.

AUTHOR CONTRIBUTIONS

M.S., C.C.P., Z.-Y.C., E.R.-B., and F.S.L. developed the research. M.S. designed the experiments and conducted biochemical and functional characterization for the research. I.D., D.J., and M.E. conducted biochemical analysis. J.G., J.K., and J.B.D. optimized and performed super-resolution imaging studies. M.S. analyzed, quantified, and interpreted the acquired images. S.-H.H. and Z.-Y.C. conducted Rab11-FIP3 knockdown studies and generated constructs. E.C. generated various TrkB constructs. R.S. and E.R.-B. supported Rab11 imaging studies. S.V.S. and S.R. generated and analyzed the Slitrk5 KO mice. R.P. generated Rab11-FIP-related reagents. M.V.C. and B.L.H. supported yeast two-hybrid experiments. M.S. and F.S.L. wrote the manuscript with assistance from B.L.H., Z.-Y.C., and E.R.-B.

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REFERENCES

Abelson, J.F., Kwan, K.Y., O'Roak, B.J., Baek, D.Y., Stillman, A.A., Morgan, T.M., Mathews, C.A., Pauls, D.L., Rasin, M.R., Gunel, M., et al. (2005). Sequence variants in SLITRK1 are associated with Tourette's syndrome. Science *310*, 317–320.

Arévalo, J.C., Waite, J., Rajagopal, R., Beyna, M., Chen, Z.Y., Lee, F.S., and Chao, M.V. (2006). Cell survival through Trk neurotrophin receptors is differentially regulated by ubiquitination. Neuron *50*, 549–559.

Aruga, J., and Mikoshiba, K. (2003). Identification and characterization of Slitrk, a novel neuronal transmembrane protein family controlling neurite outgrowth. Mol. Cell. Neurosci. *24*, 117–129.

Baquet, Z.C., Gorski, J.A., and Jones, K.R. (2004). Early striatal dendrite deficits followed by neuron loss with advanced age in the absence of anterograde cortical brain-derived neurotrophic factor. J. Neurosci. *24*, 4250–4258.

Baydyuk, M., Russell, T., Liao, G.Y., Zang, K., An, J.J., Reichardt, L.F., and Xu, B. (2011). TrkB receptor controls striatal formation by regulating the number of newborn striatal neurons. Proc. Natl. Acad. Sci. USA *108*, 1669–1674.

Benedetti, M., Levi, A., and Chao, M.V. (1993). Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc. Natl. Acad. Sci. USA *90*, 7859–7863.

Chao, M.V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat. Rev. Neurosci. *4*, 299–309.

Chen, Z.Y., Ieraci, A., Tanowitz, M., and Lee, F.S. (2005). A novel endocytic recycling signal distinguishes biological responses of Trk neurotrophin receptors. Mol. Biol. Cell *16*, 5761–5772.

Eide, F.F., Vining, E.R., Eide, B.L., Zang, K., Wang, X.Y., and Reichardt, L.F. (1996). Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J. Neurosci. *16*, 3123–3129.

Fogel, A.I., Akins, M.R., Krupp, A.J., Stagi, M., Stein, V., and Biederer, T. (2007). SynCAMs organize synapses through heterophilic adhesion. J. Neurosci. *27*, 12516–12530.

Gage, R.M., Kim, K.A., Cao, T.T., and von Zastrow, M. (2001). A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. J. Biol. Chem. *276*, 44712–44720.

Gay, N.J., Packman, L.C., Weldon, M.A., and Barna, J.C. (1991). A leucine-rich repeat peptide derived from the Drosophila Toll receptor forms extended filaments with a beta-sheet structure. FEBS Lett. *291*, 87–91.

Ginty, D.D., and Segal, R.A. (2002). Retrograde neurotrophin signaling: Trk-ing along the axon. Curr. Opin. Neurobiol. *12*, 268–274.

Grimes, M.L., Beattie, E., and Mobley, W.C. (1997). A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. Proc. Natl. Acad. Sci. USA *94*, 9909–9914.

Guan, X.M., Kobilka, T.S., and Kobilka, B.K. (1992). Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide. J. Biol. Chem. *267*, 21995–21998.

Gur, G., Rubin, C., Katz, M., Amit, I., Citri, A., Nilsson, J., Amariglio, N., Henriksson, R., Rechavi, G., Hedman, H., et al. (2004). LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. EMBO J. *23*, 3270–3281.

Gustafsson, M.G.L. (2000). Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. J. Microsc. *198*, 82–87.

Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Chao, M.V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature *350*, 678–683.

Horgan, C.P., and McCaffrey, M.W. (2009). The dynamic Rab11-FIPs. Biochem. Soc. Trans. *37*, 1032–1036.

Horgan, C.P., Hanscom, S.R., Jolly, R.S., Futter, C.E., and McCaffrey, M.W. (2010). Rab11-FIP3 links the Rab11 GTPase and cytoplasmic dynein to mediate transport to the endosomal-recycling compartment. J. Cell Sci. *123*, 181–191.

Huang, E.J., and Reichardt, L.F. (2003). Trk receptors: roles in neuronal signal transduction. Annu. Rev. Biochem. *72*, 609–642.

Huang, S.H., Zhao, L., Sun, Z.P., Li, X.Z., Geng, Z., Zhang, K.D., Chao, M.V., and Chen, Z.Y. (2009). Essential role of Hrs in endocytic recycling of full-length TrkB receptor but not its isoform TrkB.T1. J. Biol. Chem. *284*, 15126–15136.

Huang, S.H., Wang, J., Sui, W.H., Chen, B., Zhang, X.Y., Yan, J., Geng, Z., and Chen, Z.Y. (2013). BDNF-dependent recycling facilitates TrkB translocation to postsynaptic density during LTP via a Rab11-dependent pathway. J. Neurosci. *33*, 9214–9230.

Ko, J. (2012). The leucine-rich repeat superfamily of synaptic adhesion molecules: LRRTMs and Slitrks. Mol. Cells *34*, 335–340.

Kuruvilla, R., Zweifel, L.S., Glebova, N.O., Lonze, B.E., Valdez, G., Ye, H., and Ginty, D.D. (2004). A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell *118*, 243–255.

Laederich, M.B., Funes-Duran, M., Yen, L., Ingalla, E., Wu, X., Carraway, K.L., 3rd, and Sweeney, C. (2004). The leucine-rich repeat protein LRIG1 is a negative regulator of ErbB family receptor tyrosine kinases. J. Biol. Chem. *279*, 47050–47056.

Lazo, O.M., Gonzalez, A., Ascano, M., Kuruvilla, R., Couve, A., and Bronfman, F.C. (2013). BDNF regulates Rab11-mediated recycling endosome dynamics to induce dendritic branching. J. Neurosci. *33*, 6112–6122.

Lee, F.S., Kim, A.H., Khursigara, G., and Chao, M.V. (2001). The uniqueness of being a neurotrophin receptor. Curr. Opin. Neurobiol. *11*, 281–286.

Lee, K.F., Davies, A.M., and Jaenisch, R. (1994). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. Development *120*, 1027–1033.

Li, Y., Yui, D., Luikart, B.W., McKay, R.M., Li, Y., Rubenstein, J.L., and Parada, L.F. (2012). Conditional ablation of brain-derived neurotrophic factor-TrkB signaling impairs striatal neuron development. Proc. Natl. Acad. Sci. USA *109*, 15491–15496.

Lin, J.C., Ho, W.H., Gurney, A., and Rosenthal, A. (2003). The netrin-G1 ligand NGL-1 promotes the outgrowth of thalamocortical axons. Nat. Neurosci. *6*, 1270–1276.

Linhoff, M.W., Laurén, J., Cassidy, R.M., Dobie, F.A., Takahashi, H., Nygaard, H.B., Airaksinen, M.S., Strittmatter, S.M., and Craig, A.M. (2009). An unbiased expression screen for synaptogenic proteins identifies the LRRTM protein family as synaptic organizers. Neuron *61*, 734–749.

Mandai, K., Guo, T., St Hillaire, C., Meabon, J.S., Kanning, K.C., Bothwell, M., and Ginty, D.D. (2009). LIG family receptor tyrosine kinase-associated proteins modulate growth factor signals during neural development. Neuron *63*, 614–627.

Prekeris, R. (2003). Rabs, Rips, FIPs, and endocytic membrane traffic. ScientificWorldJournal *3*, 870–880.

Proenca, C.C., Gao, K.P., Shmelkov, S.V., Rafii, S., and Lee, F.S. (2011). Slitrks as emerging candidate genes involved in neuropsychiatric disorders. Trends Neurosci. *34*, 143–153.

Rauskolb, S., Zagrebelsky, M., Dreznjak, A., Deogracias, R., Matsumoto, T., Wiese, S., Erne, B., Sendtner, M., Schaeren-Wiemers, N., Korte, M., et al. (2010). Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth. J. Neurosci. *30*, 1739–1749.

Riccio, A., Pierchala, B.A., Ciarallo, C.L., and Ginty, D.D. (1997). An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. Science *277*, 1097–1100.

Shattuck, D.L., Miller, J.K., Laederich, M., Funes, M., Petersen, H., Carraway, K.L., 3rd, and Sweeney, C. (2007). LRIG1 is a novel negative regulator of the Met receptor and opposes Met and Her2 synergy. Mol. Cell. Biol. *27*, 1934–1946.

Shmelkov, S.V., Hormigo, A., Jing, D., Proenca, C.C., Bath, K.G., Milde, T., Shmelkov, E., Kushner, J.S., Baljevic, M., Dincheva, I., et al. (2010). Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive-like behaviors in mice. Nat. Med. *16*, 598–602.

Simon, G.C., and Prekeris, R. (2008). Mechanisms regulating targeting of recycling endosomes to the cleavage furrow during cytokinesis. Biochem. Soc. Trans. *36*, 391–394.

Sommerfeld, M.T., Schweigreiter, R., Barde, Y.A., and Hoppe, E. (2000). Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. J. Biol. Chem. *275*, 8982–8990.

Takahashi, H., Katayama, K., Sohya, K., Miyamoto, H., Prasad, T., Matsumoto, Y., Ota, M., Yasuda, H., Tsumoto, T., Aruga, J., et al. (2012). Selective control of inhibitory synapse development by Slitrk3-PTPdelta trans-synaptic interaction. Nat. Neurosci. *15*, 389–398.

Tanowitz, M., and von Zastrow, M. (2003). A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. J. Biol. Chem. *278*, 45978–45986.

Vargas, G.A., and Von Zastrow, M. (2004). Identification of a novel endocytic recycling signal in the D1 dopamine receptor. J. Biol. Chem. *279*, 37461–37469.

Yim, Y.S., Kwon, Y., Nam, J., Yoon, H.I., Lee, K., Kim, D.G., Kim, E., Kim, C.H., and Ko, J. (2013). Slitrks control excitatory and inhibitory synapse formation with LAR receptor protein tyrosine phosphatases. Proc. Natl. Acad. Sci. USA *110*, 4057–4062.

Zhao, H., Tanegashima, K., Ro, H., and Dawid, I.B. (2008). Lrig3 regulates neural crest formation in Xenopus by modulating Fgf and Wnt signaling pathways. Development *135*, 1283–1293.

Zheng, J., Shen, W.H., Lu, T.J., Zhou, Y., Chen, Q., Wang, Z., Xiang, T., Zhu, Y.C., Zhang, C., Duan, S., and Xiong, Z.Q. (2008). Clathrin-dependent endocytosis is required for TrkB-dependent Akt-mediated neuronal protection and dendritic growth. J. Biol. Chem. *283*, 13280–13288.

Zhou, P., Alfaro, J., Chang, E.H., Zhao, X., Porcionatto, M., and Segal, R.A. (2011). Numb links extracellular cues to intracellular polarity machinery to promote chemotaxis. Dev. Cell *20*, 610–622.