INTERACTION OF MINT2 WITH TRKA IS INVOLVED IN REGULATION OF NERVE GROWTH FACTOR-INDUCED NEURITE OUTGROWTH

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TrkA receptor signaling is essential for nerve growth factor (NGF)-induced survival and differentiation of sensory neurons. To identify possible effectors or regulators of TrkA signaling, yeast two-hybrid screening was performed using the intracellular domain of TrkA as bait. We identified muc18-1 interacting protein 2 (Mint2) as a novel TrkA-binding protein and found that the PTB domain of Mint2 interacted with TrkA in a phosphorylation- and ligand-independent fashion. Co-immunoprecipitation assays showed that endogenous TrkA interacted with Mint2 in rat tissue homogenates, and immunohistochemical evidence revealed that Mint2 and TrkA co-localized in rat DRG neurons. Furthermore, Mint2 overexpression inhibited NGF-induced neurite outgrowth in both PC12 and cultured DRG neurons, while inhibition of Mint2 expression by RNA interference facilitated NGF-induced neurite outgrowth. Moreover, Mint2 was found to promote the retention of TrkA in the Golgi apparatus and inhibit its surface sorting. Taken together, our data provide evidence that Mint2 is a novel TrkA regulating protein that affects NGF-induced neurite outgrowth, possibly through a mechanism involving retention of TrkA in the Golgi apparatus.

The neurotrophin family member nerve growth factor (NGF) is essential for proper development, patterning, and maintenance of nervous systems. (1,2). NGF has two known receptors; TrkA, a single-pass transmembrane receptor tyrosine kinase that binds selectively to NGF, and p75, a transmembrane glycoprotein that binds all members of the neurotrophin family (3,4). NGF binding activates the kinase domain of TrkA leading to autophosphorylation (5). The resulting phosphotyrosines become docking sites for adaptor proteins involved in signal transduction pathways that lead to the activation of Ras, Rac, phosphatidylinositol 3-kinase (PI3-K), phospholipase C-γ (PLC-γ) and other effectors (2,6). Many of these TrkA interacting adaptor proteins have been identified and include, Grb2, APS, SH2B, fibroblast growth factor receptor substrate 2 (FRS-2), Shc and human tumorous imaginal disc 1 (TID1) (7-10). The identification of these binding partners has contributed greatly to our understanding of the mechanisms underlying the functional diversity of NGF-TrkA signaling.

Studies have indicated that the transmission of NGF signaling in neurons involves retrograde transport of NGF-TrkA complexes from the neurite tip to the cell body (11-14). TrkA associates with components of cytoplasmic dynein, and it is thought that vesicular trafficking of neurotrophins occurs via direct interaction of Trk receptors with the dynein motor machinery (14). Furthermore, the atypical protein kinase C interacting protein, p62, associates with TrkA and plays a novel role in connecting receptor signals with the endosomal
signaling network required for mediating TrkA-induced differentiation (15). Recently, the membrane trafficking protein Pincher has been found to mediate macroendocytosis underlying retrograde signaling by TrkA (16). Despite the progress made to date in understanding Trk complex internalization and trafficking, the mechanisms remain poorly understood.

Mint2 (muc18-1 interacting protein 2) belongs to the Mint protein family, which consists of three members, Mint1, Mint2 and Mint3. Mints proteins were first identified as interacting proteins of the synaptic vesicle docking protein Munc18-1 (17,18). Mint1 is also sometimes referred to as mLIN-10, as it is the mammalian orthologue of the Caenorhabditis elegans LIN-10 (19). Additionally, Mint1, Mint2 and Mint3 are also referred to as X11α or X11, X11β or X11L (X11-like), and X11γ or X11L2 (X11-like 2), respectively (20). All Mints contain a conserved central phosphotyrosine binding (PTB) domain and two contiguous C-terminal PDZ domains (repeated sequences in the brain-specific protein PSD-95, the Drosophila septate junction protein Discs large, and the epithelial tight junction protein ZO-1) (17,18,21). Mint1 and Mint2 are expressed only in neuronal tissue (17), whereas Mint3 is ubiquitously expressed (18). Although the function of Mints proteins is not fully clear, their interactions with the docking and exocytosis factors Mun18-1 and CASK, ADP-ribosylation factor (Arf) GTPases involved in vesicle budding (22), and other synaptic adaptor proteins, such as neurabin-II/spinophilin (23), tamalin (24) and kalirin-7 (25), all suggest possible roles for Mints in synaptic vesicle docking and exocytosis. Mint proteins have also been implicated in the trafficking and/or processing of β-APP (β-amyloid precursor protein). Through their PTB domains, all three Mints bind to a motif within the cytoplasmic domain of β-APP (21,26-29), and Mint1 and Mint2 can stabilize β-APP, affect β-APP processing, and inhibit the production and secretion of Aβ (28,30-32). Although the mechanisms by which Mints inhibit β-APP processing are not yet well known, Mints and their binding partners have emerged as potential therapeutic targets for the treatment of Alzheimer's disease.

To uncover new TrkA interacting factors and gain insight into the mechanisms that guide TrkA intracellular trafficking and other aspects of TrkA signaling, we conducted a yeast two-hybrid screen of a brain cDNA library using the intracellular domain of TrkA as bait. The screen identified several candidate TrkA interacting proteins, one of which was Mint2. Follow-up binding assays showed that the PTB domain of Mint2 alone was necessary and sufficient for mediating the interaction with TrkA. Endogenous Mint2 was also co-immunoprecipitated and co-localized with TrkA in rat DRG tissue. Overexpression and knockdown studies showed that Mint2 could significantly inhibit NGF-induced neurite outgrowth in both TrkA-expressing PC12 cells and DRG neurons. Moreover, Mint2 was found to induce the retention of TrkA in the Golgi apparatus and inhibit its surface sorting. Our results suggest that Mint2 is a novel regulator of TrkA receptor signaling.

**MATERIALS AND METHODS**

*Plasmid constructs* Full-length TrkA constructs were generous gifts from Dr. David D. Ginty and full-length Mint2 constructs were provided by Dr. Thomas C. Südhof. The intracellular domain of human TrkA was generated by PCR and cloned in-frame into the LexA fusion vector pGilda (Clontech, Palo Alto, CA) as bait pGilda-TrkA<sup>IC</sup>. Full-length Mint2 and the PTB, PDZ1, PDZ2 and N-terminal domains of Mint2 were amplified by PCR and subcloned into pB42AD vectors. To construct the myc-tagged Mint2 (myc-Mint2) and GFP-tagged Mint2 (GFP-Mint2), we sub-cloned full-length Mint2 into pcDNA3 (Invitrogen, Carlsbad, CA) and pEGFP-N2 vector (Clontech, Palo Alto, CA) vectors, respectively. The K547N TrkA mutant was generated by applying the QuickChange site-directed mutagenesis method.
from Stratagene (La Jolla, CA). All constructs were fully sequenced before being used for transformation or transfection.

For inhibition of Mint2 expression by siRNA, three unique 21-bp nucleotide sequences were designed and synthesized by GeneChem (Shanghai, China). They were: No.1 (bp 948-969), 5'-GCCUGAGCAUGACCAGUAUtt-3'; No.2 (bp 1192-1213), 5'-GGUUUGCAAUGCUCUUUAttt-3', and No.3 (bp 1468-1489), 5'-GCGUUGCUAAGUCACAAAtt-3'. To determine the knock-down efficacy of the siRNAs, HEK 293 cells in a 12-well culture plates were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with myc-Mint2 plasmid and nonsense sequence or myc-Mint2 plasmid and siRNA. Cells were lysed 48 hours after transfection and lysates were subjected to immunoblotting analysis. The most efficient siRNA sequence was inserted into pSuper vector.

Yeast two-hybrid screening
Yeast two-hybrid screening was performed according to the manufacturer’s protocols (Clontech, Palo Alto, CA). The Saccharomyces cerevisiae yeast strain EGY48 (MATa, his3, trpl, ura3, LEU2::plexAop6-LEU2), the LexA yeast two-hybrid system and MatchMaker human brain cDNA library were all purchased from Clontech. The pGilda-TrkA IC construct and human brain cDNA library fused to pB42AD were co-transformed into the EGY48 strain with PEG/LiAc solution. The co-transformants were plated on SD-Gal Ura-His-Trp-Leu drop-out galactose induction medium for 3-4 days at 30°C to induce the expression of reporter proteins fused with the activation domain. Filter-lift color assays and Leucine-deficient (Leu -) culture assays were performed as described previously (33). Equal amounts of immobilized GST fusion proteins were mixed and incubated for 3 h at 4°C with PC12 cell extract in GST binding buffer containing 40 mM HEPES, 50 mM Na acetate, 200 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 0.5% NP-40, and protease inhibitors. Glutathione beads were washed three times in binding buffer and boiled in SDS sample buffer to elute bound proteins, which were then resolved by SDS-PAGE and followed by Western blot analysis and visualized with enhanced chemiluminescence (ECL, Pierce, Rockford, IL).

Preparation of tissue homogenates and immunoprecipitation
DRG, cerebellum and basal forebrain tissue were dissected from adult male Sprague Dawley rats and dissociated. Samples were homogenized in a pestle tissue grinder in solubilization buffer (25mM HEPES-NaOH, pH 7.4, 125 mM K-acetate, 5 mM MgCl2, 0.32 M sucrose, and 1% Triton X-100). Proteins solubilized from each homogenate were quantified using the BCA method. Equal amounts of protein were incubated with primary antibodies for 3 hours at 4°C under mild agitation. Protein G-agarose beads (Roche, Indianapolis, IN) were added for 3 hours, and immunoprecipitated samples were then washed three times with solubilization buffer, boiled 3-5 minutes in sample-loading buffer and...
subjected to western blotting analysis. Antibodies used in western blot and immunoprecipitation experiments included: rabbit anti-TrkA, phospho (Tyr490; Abcam, Cambridge, UK); rabbit anti-TrkA(763) (Santa Cruz, CA, USA); rabbit anti-TrkA (Chemicon, Temecula, CA); mouse anti-Mint2 (BD biosciences, San Jose, CA); rabbit anti-myc (Sigma, St Louis, MO); mouse anti-GFP (Roche, Indianapolis, IN); polyclonal GPP130 (1:400, Covance, Princeton, New Jersey); rabbit anti-calnexin (Sigma, St Louis, MO); rabbit anti-actin (Chemicon, Temecula, CA); rabbit anti-p75NTR (Chemicon, Temecula, CA); monoclonal anti-actin (Sigma, St Louis, MO); mouse IgG (Santa Cruz, CA, USA); rabbit IgG (Santa Cruz, CA, USA).

Immunohistochemistry and immunocytochemistry
Adult male Sprague Dawley rats, weighing 200-250g, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate buffer (PB) pH 7.4, followed by perfusion with 4% paraformaldehyde with 0.2% saturated picric acid in 0.1 M PB. DRG tissues were removed, kept for 1.5 hours in the same fixative at 4°C, and then cryoprotected overnight at 4°C in 0.01 M PBS pH 7.4, containing 20% sucrose. Tissues were sectioned into 14 µm slices with a Leica 1900 cryostat and mounted on glass slides. Sections were washed in PBS, incubated with primary antibodies (rabbit anti-TrkA 1:200, Chemicon, Temecula, CA; mouse anti-Mint2 1:500, BD biosciences, San Jose, CA) in PBS containing 3% BSA and 0.3% Triton X-100 at 4°C overnight. After three washes in PBS, sections were incubated with FITC-conjugated donkey anti-rabbit (1:100) and rhodamine-conjugated donkey anti-mouse (1:100) antibodies (both from Jackson ImmunoResearch, West Grove, PA). Sections were washed, placed on coverslips and examined with a Leica SP2 confocal microscope.

PC12 cells were fixed in PBS containing 4% formaldehyde for 15 min at room temperature. After fixation, cells were rinsed with PBS, blocked and permeabilized in PBS (containing 5% BSA, 0.1% Triton) and stained with monoclonal anti-myc (1:500, BD biosciences, San Jose, CA) with wheat germ agglutinin (WGA) -conjugated Alexa Fluor 633 (1:1500, Invitrogen, Carlsbad, CA) and polyclonal anti-rabbit myc (1:500, Sigma, St Louis, MO) in the same buffer at 4°C overnight. A collection of fluorophore-conjugated secondary antibodies were used including donkey anti-mouse-TRITC, donkey anti-rabbit-Cy5 or donkey anti-mouse-Cy5 (Jackson ImmunoResearch, West Grove, PA).

Neurite outgrowth assay PC12 cells were transfected with pEGFP-N2 alone or pEGFP-N2 and Myc-Mint2 and seeded on poly-D-lysine coated cover slips at a density of 3.5×10⁵ cells/ml. NGF (100 ng/ml, Sigma, St Louis, MO) was added directly to the medium to initiate cell differentiation. Cells were then incubated for 3d in the presence of NGF before being fixed and permeabilized for immunocytochemistry. The PC12 cell differentiation assay was performed as described before (34), and cells possessing one or more neurites of a length more than twice the diameter of the cell body were scored as positive. 100 cells per group were randomly counted for each experiment.

Primary DRG neuron cultures were generated as described before (35). Briefly, DRGs were removed from E17 Sprague Dawley rat embryos and pooled in ice-cold Hanks buffed salt solution. Tissue sections were dissociated by consecutive treatment with 0.25% trypsin, followed by careful trituration at 37°C for 45 minutes. Following centrifugation, the pellet was dispersed in 10% FBS/DMEM. Cells were then electroporated (Amaxa Biosystems, Koln, Germany) and plated directly on acid-washed coverslips precoated with 10 µg/ml mouse laminin (Invitrogen, Carlsbad, CA) in 10% FBS/DMEM (35). The media was then changed to Neurobasal media with B27 supplement (Invitrogen, Carlsbad, CA) with 100ng/ml NGF (Sigma, St Louis, MO). Cells were maintained at 37°C in 5% CO₂ for 3 days, then
fixed and immunostained with anti-MAP2 antibodies (Chemicon, Temecula, CA), and donkey anti-mouse-TRITC (Jackson ImmunoResearch, West Grove, PA). Neurite outgrowth was quantification as reported in our previous study (34). The longest neurite per neuron was measured by Image-pro Plus software (Olympus, Japan), and total neurite length was measured by Neurulucida software (MicroBrightField Inc, USA). For each experiment, 150 neurons were scored.

**Surface biotinylation assay** Transfected PC12 cells were starved overnight before being treated with 100 ng/ml NGF (Sigma, St Louis, MO) at 37°C for 30 min. Surface biotinylation was performed according to a previous published protocol (36). Briefly, cells were washed in PBS (Ca²⁺/Mg²⁺) containing Sulfo-NHS-LC-Biotin (0.5 mg/ml, Pierce, Rockford, IL) for 45 min at 4°C. After extensive washing with ice-cold PBS containing 50 mM glycine, cells were lysed. Biotinylated proteins were precipitated with ImmunoPure Immobilized Streptavidin (Pierce, Rockford, IL) overnight at 4°C. Samples were loaded on SDS-PAGE, transferred, probed with antibody against GFP (1:2000, Roche, Indianapolis, IN), and visualized with enhanced chemiluminescence (ECL, Pierce, Rockford, IL).

Internalization of GFP-TrkA was also analyzed using a cell-surface biotinylation assay (37). Briefly, cells were subjected to biotinylation on ice with the reversible membrane impermeable derivative of biotin (1.5 mg/mL sulfo-NHS-S-S-biotin in PBS, Pierce, Rockford, IL). Internalization was allowed to occur by incubation at 37°C with media containing NGF (100 ng/ml) for 30 min. The remaining cell-surface biotin was cleaved by reducing its disulfide linkage with glutathione cleavage buffer and cells were lysed with RIPA buffer. Biotinylated proteins were precipitated using UltraLink immobilized Neutravidin beads (Pierce, Rockford, IL), eluted from the beads with boiling Laemmli buffer, resolved by SDS-PAGE, and immunoblotted with an antibody directed against GFP (1:2000, Roche, Indianapolis, IN).

**Gradient Fractionation Assay** PC12 cells from eight 10-cm plates were collected by shake-off and centrifugation for 5 min (2,000 rpm in a SA-600 rotor, Sorvall, Newtown, CT), washed once in PBS (containing 1 mM EDTA), washed once in sucrose buffer (250 mM sucrose, 10 mM triethylamine pH 7.4, 1 mM EDTA, with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, and 10 mg/ml pepstatin A), homogenized by 20 passages through a 25-gauge needle in 0.5 ml buffer (50 mM NaCl, 10 mM triethylamine, pH 7.4, 1 mM EDTA, with protease inhibitors), and centrifuged at 1,000 × g for 2 min. The postnuclear supernatant was then fractionated on either velocity gradients or flotation gradients. In the standard velocity gradient procedure the postnuclear supernatant (0.4 ml) was layered on linear 5–25% (vol/vol) glycerol gradients (5 ml in 10 mM triethylamine, pH 7.4, and 1 mM EDTA on a 0.5 ml 80% sucrose cushion) and centrifuged at 150,000 × g for 30 min in a SW50.1 rotor (Beckman, Fullerton, CA), and 0.4 ml fractions were collected from the top. The sedimentation coefficient was calculated using a proportionality constant value of 3.38 cm, as described previously (Fritsch, 1973). For flotation gradient analysis, the postnuclear supernatant was adjusted to 1.6 M sucrose (1.1 ml), layered on 2 M sucrose (0.5 ml), covered with three sucrose layers (1.1 ml each: 1.4 M, 1.2 M, 0.8 M), and centrifuged at 105,000 × g for 105 min in a SW50.1 rotor (Beckman), and 0.4-ml fractions were collected from the top (38). All steps were carried out at 0–4°C. Immunoblotting was used to assay each fraction for the presence of TrkA-GFP (anti-GFP, Roche, Indianapolis, IN), Myc-Mint2 (anti-Myc, Sigma, St Louis, MO) GPP130 (anti-GPP130, Covance, Princeton, New Jersey) (39), and Calnexin (anti-calnexin Sigma, St Louis, MO) (40).

**RESULTS**

**Identification of Mint2 as a potential binding**
partner of TrkA by yeast two-hybrid screening. In an attempt to identify proteins that associate with TrkA, we conducted a yeast two-hybrid screen of a human brain cDNA library using the intracellular domain of TrkA (TrkAIC) as bait (Fig. 1A). To construct the bait for the two-hybrid screen, the TrkAIC (amino acids 443-799) coding sequence was fused in frame to the DNA-binding domain of LexA using the pGilda vector. The brain cDNAs were sub-cloned into the B42 activation domain vector pB42AD and were co-transformed with the bait plasmid into yeast strain EGY48. About $3 \times 10^6$ co-transformants were plated on leucine-deficient medium, and activation of the LEU reporter gene was used to select for protein-protein interactions. Approximately 269 Leu-reporter gene positive clones were isolated in the growth assay screen, and of these, 78 clones scored positive for the β-galactosidase filter-lift assay; false positives were further eliminated by testing against LexA-lamin. The cDNA inserts of the remaining 11 clones were then sequenced. One of the cDNAs was identified to be a 672 bp fragment corresponding to residues 286-539 of the Mint2 gene, a region encompassing the entire PTB domain and flanking sequence, as shown in Figure 1B.

The PTB domain of Mint2 mediates the interaction with TrkA. To further confirm the presumptive interaction between Mint2 and TrkA, we tested the ability of full length Mint2 to associate with TrkA. Both β-galactosidase activity and restricted growth assays showed that TrkA could interact with full length Mint2 in yeast, whereas the EGF receptor could not (Fig. 2A). Since Mint2 contains multiple domains including an N-terminal region, a central PTB domain and two C-terminal PDZ domains arranged in tandem (Fig. 1B), we subcloned fragments of rat Mint2 corresponding to these regions (N-terminal region, 1-368 aa; PTB domain, 369-532 aa; PDZ1 domain, 566-650 aa; PDZ2 domain, 662-735 aa) into prey vectors, and tested which region was critical for TrkA interaction. Our results showed that only the PTB domain was capable of binding TrkA; no interaction between TrkA and other regions of Mint2 occurred (Fig. 2A). To determine whether this interaction was dependent on the phosphorylation state of TrkA, we used a yeast two-hybrid assay to test whether Mint2 could interact with a catalytically inactive TrkAIC construct in which Lysine 547, the essential ATP binding site, was substituted for Glutamine (K547N; 41-44). We found that Mint2 was capable of binding to this construct as well, suggesting that the interaction is phosphorylation-independent (Fig. 2A).

To confirm our yeast two-hybrid assay results, we used a GST pull-down assay to examine the interaction between GST-tagged Mint2 and endogenous TrkA in PC12 cells (45). PC12 cells with or without NGF treatment were lysed and subjected to GST pull-down followed by Western blotting with anti-TrkA antibody. As shown in Figure 2B, Mint2 could bind to TrkA in both NGF-treated and untreated PC12 cells. As shown in Figure 2C, expression of a GST-tagged PTB domain construct was both necessary and sufficient to pull-down TrkA in PC12 extract. Thus, the results from our yeast two-hybrid and GST pull-down assays indicate that TrkA can interact with Mint2, and this interaction is mediated by the PTB domain of Mint2.

Endogenous Mint2 interacts with TrkA in rat tissue homogenates. To examine whether endogenous TrkA and Mint2 can complex together in mammalian tissue in vivo, we performed immunoprecipitation assays with homogenates from different tissues of the adult rat, including DRG, cerebellum and basal forebrain. Homogenates were immunoprecipitated with anti-TrkA or anti-Mint2, and immunoblotted with anti-Mint2 or anti-TrkA. As shown in Figure 3, TrkA could co-immunoprecipitate Mint2, and vice versa, in DRG and the basal forebrain, but not the cerebellum. Neither Mint2 nor TrkA was immunoprecipitated by control IgG in DRG tissue (data not shown), further confirming the specificity
of the interaction.

**TrkA co-localizes with Mint2 in rat DRG neurons.** We next examined whether TrkA and Mint2 co-localize in neurons. Since TrkA is expressed in DRG neurons (46) and Mint2 is distributed ubiquitously in the nervous system (47), immunohistochemical analysis was carried out on adult rat DRG slices to determine the subcellular distribution of TrkA and Mint2 in DRG neurons. As shown in Fig. 4, fluorescent confocal imaging revealed the expression of TrkA (green) and Mint2 (red) in the DRG neurons. The merged images showed that TrkA and Mint2 could co-localize in the same neurons of DRG and their distributions were highly overlapped. As a control, no staining was observed when the primary antibody was removed from our staining procedure, showing that the staining was specific (data not shown).

Mint2 negatively regulates NGF-induced neurite outgrowth in PC12 cells and DRG neurons. NGF signaling through TrkA induces PC12 cell differentiation, a process that includes the growth of neurites (45). Since Mint2 was not expressed in PC12 cells (Fig. 5A), we co-transfected PC12 cells with pEGFP and either myc-tagged Mint2 or a control construct (pcDNA3) at a ratio of 1:10 and compared neurite outgrowth after NGF stimulation to identify a physiological role of Mint2 in TrkA signaling. Statistical analysis revealed that compared to the control group, ectopic expression of myc-Mint2 markedly reduced the ratio of neurite-bearing cells after NGF treatment, suggesting that Mint2 negatively regulates TrkA signaling in PC12 cells (Fig. 5B and C). To determine how Mint2 might affect NGF signaling, we first examined the expression of NGF receptors in Mint2 expressing PC12 cells, both with and without NGF stimulation, but did not observe any difference in the levels of TrkA or p75 in Mint2-expressing cells compared to control cells (Fig. 5D). Interestingly, the level of p-TrkA was lower in the Mint2 over-expressing group relative to the control group after exposure to NGF, suggesting that Mint2 prevents robust NGF-induced TrkA activation and thereby inhibits neurite outgrowth. We next investigated the interaction between TrkA and Mint2 following NGF treatment by co-immunoprecipitation. As shown in Figure 5E, the interaction was not affected by NGF treatment, consistent with the results of our GST-Mint2 pull-down assays (Fig. 2B). Neither Mint2 nor TrkA was immunoprecipitated by control IgG in Mint2-transfected PC12 cells either with or without NGF stimulation (data not shown), further confirming the specificity of the interaction.

Since both Mint2 and TrkA are endogenously expressed in DRG neurons (Fig.4), we next performed a neurite outgrowth assay on cultured DRG neurons extracted from E16 rats. We first assessed the effect of RNAi-mediated silencing of Mint2 in HEK293 cells by measuring protein levels of exogenous Mint2. Three independent Mint2-siRNA sequences were tested, among which one (#1) afforded the most robust knock-down efficacy (Fig. 6A). When the selected GFP-tagged Mint2-siRNA was transfected into DRG neuron, the endogenous Mint2, which are mainly accumulated near the nucleus(20) was successfully knocked down (Fig. 6B), further indicating the high efficiency of the Mint2 RNAi. We then measured neurite outgrowth in cultured DRG neurons transfected with GFP-tagged Mint2-siRNA, GFP-tagged nonsense-siRNA or Mint2/GFP after 3 days of exposure to NGF. MAP2 staining was performed to distinguish neurons from glial cells. As shown in Figure 6C, neurite outgrowth was dramatically enhanced in DRG neurons transfected with Mint2-siRNA and attenuated in neurons transfected with Mint2. Statistical analysis revealed that NGF-induced neurite outgrowth, measured as both the length of the longest neurite and total neurite length, was significantly increased in the Mint2 RNAi group and decreased in Mint2 overexpression group compared to the control (Fig. 6D and E). These results show that endogenous Mint2 acts as a negative regulator of NGF-induced neurite
outgrowth, possibly by suppressing TrkA activation.

Mint2 reduces surface localization of TrkA. To uncover the mechanism by which Mint2 inhibits NGF-induced neurite outgrowth, GFP-TrkA was co-transfected with either myc-Mint2 or myc into PC12 cells at a ratio of 1:10. 24 hours after transfection, cells were treated with or without NGF for 30 min. After fixation, cells were stained with anti-myc monoclonal antibody and the plasma membrane and Golgi apparatus marker Wheat Germ Agglutinin (WGA)-conjugated Alexa Fluor 633 (48,49). As shown in Figure 7A, GFP-TrkA was mainly localized at the cell surface without NGF treatment and internalized after NGF stimulation, consistent with a previous report (50). In contrast, the majority of myc-Mint2 co-localized with WGA, suggesting that myc-Mint2 was mainly localized in the Golgi apparatus, as has been suggested previously (47). Moreover, GFP-TrkA was found to co-localize with myc-Mint2 and WGA.

To quantitatively analyze the effect of Mint2 on TrkA trafficking, surface protein biotinylation assay was performed on PC12 cells that expressed GFP-TrkA together with myc-Mint2 or myc. We first examined the surface GFP-TrkA. As shown in Figure 7B and C, NGF stimulation significantly reduced surface GFP-TrkA in PC12 cells either transfected with myc-Mint2 or myc, suggesting efficient internalization induced by NGF. Interestingly, in cells transfected with myc-Mint2, the level of GFP-TrkA at the surface was significantly reduced both at rest and after NGF stimulation compared to myc-transfected controls (Fig. 7B and C). Further experiments by measuring internalized GFP-TrkA revealed that NGF-induced internalized GFP-TrkA was significantly decreased when Mint2 was overexpressed (Fig. 7D and E). Previous studies have shown that the internalization of TrkA is dependent on dynamin (51,52). We therefore used a dominant-negative form of dynamin (dynamin-K44A, DN-dynamin) to test whether we could suppress GFP-TrkA internalization. Indeed, when DN-dynamin was co-transfected with GFP-TrkA, NGF treatment did not lead to a reduction of surface GFP-TrkA (Fig. 7F and G), suggesting efficient blockage of internalization. However, decreased levels of surface GFP-TrkA were still observed when Mint2 was overexpressed along with DN-dynamin and GFP-TrkA (Fig. 7F and G). Taken together, these results suggest that Mint2 inhibited the surface sorting of GFP-TrkA.

To further clarify the effect of Mint2 on TrkA trafficking, a gradient fractionation assay was carried out on transfected PC12 cells. Using the Golgi protein GPP130 and calnexin to identify Golgi and ER-enriched fractions, respectively (53), we found that the level of TrkA-GFP in the Golgi fractions was greatly increased in myc-Mint2 expressing cells relative to control cells (Fig. 8A and B). Golgi retention was reversed in cells co-expressing Mint2-siRNA and Myc-Mint2 (Fig. 8C). Moreover, co-immunoprecipitation revealed that Mint2 and GFP-TrkA interacted with each other in both Golgi and ER fractions (Fig. 8D and E). Thus, these results suggest that Mint2 interacts with TrkA to promote TrkA retention in the Golgi apparatus.

**DISCUSSION**

Despite the crucial importance of TrkA-mediated signaling in the nervous system, the mechanisms that regulate its functions and trafficking remain poorly understood. In this study, we wished to identify novel TrkA-binding proteins that may represent important effectors or regulators, in the hopes of obtaining a better understanding of the varied mechanisms underlying TrkA signaling. Using the intracellular domain of TrkA as bait, we performed a yeast two-hybrid screen and identified Mint2 as a candidate TrkA-binding partner.

In our yeast two-hybrid screen, a fragment that included the entire PTB domain of Mint2 was isolated as a candidate binding partner of TrkA. Although structural studies have suggested that the
interaction between the C-terminal tail and the PDZ domains of Mint1 has an important regulatory role in the function of Mint1 (54), it was demonstrated here by both yeast two-hybrid and GST pull-down assay that the PTB domain alone was necessary and sufficient for mediating the interaction of Mint2 with the intracellular domain of TrkA. The PTB domain is well-known for phosphotyrosine recognition and preferentially binds to phosphorylated proteins containing an NPXpY motif (where pY is phosphotyrosine and X is any amino acid) (55). However, many studies have shown that PTB domain-like modules can also bind to proteins independent of tyrosine phosphorylation or even the canonical NPXY motif. For example, the PTB domains of Mint1 and Fe65 both bind with high affinity to a small peptide containing an NPXY sequence derived from a region of β-amyloid precursor protein, and this stable complex is not affected by tyrosine phosphorylation (21,29,56,57). Similarly, in the present study, Mint2 was capable of binding a kinase-dead form of TrkA in a yeast two-hybrid assay. Moreover, our results showed that the Mint2 PTB domain could bind directly to endogenous TrkA in PC12 cells in the absence of NGF by using GST-pull down assay, and interactions between TrkA and Mint2 were not affected by NGF treatment by immunoprecipitation analysis. Thus, our results provide another example showing that PTB domains can bind to receptor tyrosine kinase in a ligand-independent and phosphorylation-independent manner.

Upon NGF binding to its receptor, the tyrosine kinase domain of TrkA is activated, and those phosphotyrosines become docking sites for adaptor proteins and effectors (2,6). Thus far to our knowledge, few other studies have demonstrated phosphorylation-independent interaction of TrkA (14). Our data provided evidence that Mint2 interacted with the TrkA receptor tyrosine kinases in an activity-independent manner, suggesting that Mint2 is more likely serving as a regulator protein for TrkA, as opposed to a downstream signaling molecule.

The Mint family are well-characterized for their roles in the formation of multi-protein complexes and their ability to regulate the signaling and trafficking of membrane proteins (20). There is evidence that Mint1 and Mint2 are enriched in the Golgi apparatus but are also distributed throughout axons and dendrites (28,47). Mints bind to munc-18, a protein essential for synaptic vesicle exocytosis, and to CASK, which is involved in targeting and localization of synaptic membrane proteins (58). The Mint family ortholog in C. elegans, Lin-10, which is required for postsynaptic localization of the glutamate receptor GLR1 in nematodes (59), is present in dendrites and spines, and also enriched in the trans-Golgi network; targeting to the Golgi is dependent on its PDZ domain (60). Mints also bind to ADP-ribosylation factor (Arf), and co-localize with Arf and β-APP in regions of the Golgi/trans-Golgi network. Mints bind Arfs through a region of the PTB domain and the PDZ2 domain, and Arf-Mint interaction is necessary for increased cellular accumulation of β-APP produced by Mint overexpression (22). Recently Mint3 was reported to regulate trafficking of Furin and to play an obligate role in the traffic of APP from the trans-Golgi network to the plasma membrane (61,62). These studies indicate that Mint2 may regulate receptor sorting and protein trafficking, and that its PDZ domain may serve as a Golgi retention signal. In the present study, we found that Mint2 overexpression induced the retention of TrkA in the Golgi apparatus and inhibited TrkA surface sorting.

Expression of TrkA at the cell surface is critical for proper NGF signaling, however little is known of the mechanisms that regulate TrkA sorting to the plasma membrane. Here, we found that Mint2 inhibits TrkA sorting to the cell membrane, most likely by inducing its retention in Golgi apparatus. We also found that overexpression of Mint2 resulted in a significant decrease in NGF-induced neurite outgrowth, in both PC12 cells and DRG.
neurons, whereas knocking down Mint2 facilitated NGF-induced neurite outgrowth. Thus, Mint2-mediated retention of TrkA in the Golgi may provide a mechanism by which functional regulation of TrkA is achieved during sensory neuron development.

REFERENCES

1. Huang, E. J., and Reichardt, L. F. (2001) Annu Rev Neurosci 24, 677-736
2. Huang, E. J., and Reichardt, L. F. (2003) Annu Rev Biochem 72, 609-642
3. Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986) Cell 47(4), 545-554
4. Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991) Science 252(5005), 554-558
5. Gryz, E. A., and Meakin, S. O. (2000) Oncogene 19(3), 417-430
6. Kaplan, D. R., and Miller, F. D. (2000) Curr Opin Neurobiol 10(3), 381-391
7. Liu, H. Y., MacDonald, J. I., Hryciw, T., Li, C., and Meakin, S. O. (2005) J Biol Chem 280(20), 19461-19471
8. MacDonald, J. I., Gryz, E. A., Kubu, C. J., Verdi, J. M., and Meakin, S. O. (2000) J Biol Chem 275(24), 18225-18233
9. Meakin, S. O., MacDonald, J. I., Gryz, E. A., Kubu, C. J., and Verdi, J. M. (1999) J Biol Chem 274(14), 9861-9870
10. Qian, X., Riccio, A., Zhang, Y., and Ginty, D. D. (1998) Neuron 21(5), 1017-1029
11. Grimes, M. L., Zhou, J., Beattie, E. C., Yuen, E. C., Hall, D. E., Valletta, J. S., Topp, K. S., LaVail, J. H., Bunnett, N. W., and Mobley, W. C. (1996) J Neurosci 16(24), 7950-7964
12. Kuruvilla, R., Ye, H., and Ginty, D. D. (2000) Neuron 27(3), 499-512
13. Riccio, A., Pierchala, B. A., Ciarallo, C. L., and Ginty, D. D. (1997) Science 277(5329), 1097-1100
14. Yano, H., Lee, F. S., Kong, H., Chuang, J., Arevalo, J., Perez, P., Sung, C., and Chao, M. V. (2001) J Neurosci 21(3), RC125
15. Geetha, T., and Wooten, M. W. (2003) J Biol Chem 278(7), 4730-4739
16. Valdez, G., Akmentin, W., Philippidou, P., Kuruvilla, R., Ginty, D. D., and Halegoua, S. (2005) J Neurosci 25(21), 5236-5247
17. Okamoto, M., and Sudhof, T. C. (1997) J Biol Chem 272(50), 31459-31464
18. Okamoto, M., and Sudhof, T. C. (1998) Eur J Cell Biol 77(3), 161-165
19. Whitfield, C. W., Benard, C., Barnes, T., Hekimi, S., and Kim, S. K. (1999) Mol Biol Cell 10(6), 2087-2100
20. Rogelj, B., Mitchell, J. C., Miller, C. C., and McLoughlin, D. M. (2006) Brain Res Rev 52(2), 305-315
21. Borg, J. P., Ooi, J., Levy, E., and Margolis, B. (1996) Mol Cell Biol 16(11), 6229-6241
22. Hill, K., Li, Y., Bennett, M., McKay, M., Zhu, X., Shern, J., Torre, E., Lah, J. J., Levey, A. I., and Kahn, R. A. (2003) J Biol Chem 278(38), 36032-36040
23. Ide, N., Hata, Y., Hirao, K., Irie, M., Deguchi, M., Yao, I., Satoh, A., Wada, M., Takahashi, K., Nakanishi, H., and Takai, Y. (1998) Biochem Biophys Res Commun 244(1), 258-262
24. Kitano, J., Yamazaki, Y., Kimura, K., Masukado, T., Nakajima, Y., and Nakanishi, S. (2003) J Biol Chem 278(17), 14762-14768
25. Penzes, P., Johnson, R. C., Sattler, R., Zhang, X., Huganir, R. L., Kambampati, V., Mains, R. E., and Eipper, B. A. (2001) Neuron 29(1), 229-242
26. McLoughlin, D. M., and Miller, C. C. (1996) FEBS Lett 397(2-3), 197-200
27. Tanahashi, H., and Tabira, T. (1999) Neuroreport 10(12), 2575-2578
28. Tomita, S., Ozaki, T., Taru, H., Oguchi, S., Takeda, S., Yagi, Y., Sakiyama, S., Kirino, Y., and Suzuki, T. (1999) J Biol Chem 274(4), 2243-2254
29. Zhang, Z., Lee, C. H., Mandiyan, V., Borg, J. P., Margolis, B., Schlessinger, J., and Kuriyan, J. (1997) EMBO J 16(20), 6141-6150
30. Borg, J. P., Yang, Y., De Taddeo-Borg, M., Margolis, B., and Turner, R. S. (1998) J Biol Chem 273(24), 14761-14766
31. McLoughlin, D. M., Irving, N. G., Brownlees, J., Brion, J. P., Leroy, K., and Miller, C. C. (1999) Eur J Neurosci 11(6), 1988-1994
32. Sastre, M., Turner, R. S., and Levy, E. (1998) J Biol Chem 273(35), 22351-22357
33. Zhang, Y., Yan, Z., Farooq, A., Liu, X., Lu, C., Zhou, M. M., and He, C. (2004) J Mol Biol 343(4), 1147-1155
34. Zhang, Y., Zhu, W., Wang, Y. G., Liu, X. J., Jiao, L., Liu, X., Zhang, Z. H., Lu, C. L., and He, C. (2006) J Cell Sci 119(8), 1666-1676
35. Brown, M., Jacobs, T., Eickholt, B., Ferrari, G., Teo, M., Monfries, C., Qi, R. Z., Leung, T., Lim, L., and Hall, C. (2004) J Neurosci 24(41), 8994-9004
36. Bao, L., Jin, S. X., Zhang, C., Wang, L. H., Xu, Z. Z., Zhang, F. X., Wang, L. C., Ning, F. S., Cai, H. J., Guan, J. S., Xiao, H. S., Xu, Z. Q., He, C., Hokfelt, T., Zhou, Z., and Zhang, X. (2003) Neuron 37(1), 121-133
37. Kuruvilla, R., Zweifel, L. S., Glebova, N. O., Lonze, B. E., Valdez, G., Ye, H., and Ginty, D. D. (2004) Cell 118(2), 243-255
38. Nagata-Kuno, K., Hino, Y., Nanri, H., Shibata, Y., and Minakami, S. (1990) Exp Cell Res 191(2), 273-277
39. Linstedt, A. D., Mehta, A., Suharn, J., Reggio, H., and Hauri, H. P. (1997) Mol Biol Cell 8(6), 1073-1087
40. Linstedt, A. D., and Hauri, H. P. (1993) Mol Biol Cell 4(7), 679-693
41. Eggert, A., Ikegaki, N., Liu, X., Chou, T. T., Lee, V. M., Trojanowski, J. Q., and Brodeur, G. M. (2000) Oncogene 19(16), 2043-2051
42. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241(4861), 42-52
43. Tarr, P. E., Roncarati, R., Pelicci, G., Pelicci, P. G., and D'Adamio, L. (2002) J Biol Chem 277(19), 16798-16804
44. Wolf, D. E., McKinnon-Thompson, C., Daou, M. C., Stephens, R. M., Kaplan, D. R., and Ross, A. H. (1998) Biochemistry 37(9), 3178-3186
45. Chao, M. V., and Hempstead, B. L. (1995) Trends Neurosci 18(7), 321-326
46. Carroll, S. L., Silos-Santiago, I., Frese, S. E., Ruit, K. G., Milbrandt, J., and Snider, W. D. (1992) Neuron 9(4), 779-788
47. Nakajima, Y., Okamoto, M., Nishimura, H., Obata, K., Kitano, H., Sugita, M., and Matsuyama, T. (2001) Brain Res Mol Brain Res 92(1-2), 27-42
48. Gennuso, F., Fernetti, C., Tirotto, C., Testa, N., L’Episcopo, F., Caniglia, S., Morale, M. C., Ostrow, J. D., Pascolo, L., Tiritelli, C., and Marchetti, B. (2004) Proc Natl Acad Sci U S A 101(8), 2470-2475
49. Ramoino, P., Fronte, P., Fato, M., Beltrame, F., Robello, M., and Diaspro, A. (2001) Eur Biophys J 30(5), 305-312
50. Jullien, J., Guii, V., Derrington, E. A., Darlix, J. L., Reichardt, L. F., and Rudkin, B. B. (2003) J Biol
FOOTNOTES

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FIGURE LEGENDS

Fig. 1. Schematic representation of the bait and prey used in the yeast two-hybrid screen. (A) Schematic representation of full length TrkA and the pGilda-TrkAIC bait. TrkA has a single transmembrane domain and a single cytoplasmic tyrosine kinase domain. C, cysteine clusters; LRR, leucine-rich repeats; Ig, immunoglobulin-like domain; TM, transmembrane domain; TK, tyrosine kinase domain. (B) The two-hybrid screen identified the region of Mint2 containing the PTB domain (amino acids 286-539) as a binding partner for TrkAIC. N, N-terminal region; PTB, the phosphotyrosine binding domain; PDZ, the PDZ domain.

Fig. 2. Mint2 interacts with TrkA in yeast two-hybrid and GST pull-down assays. (A) The interaction of Mint2 with TrkAIC was analyzed by filter assay for LacZ reporter gene β-galactosidase activity and Leucine-deficient growth assay for LEU reporter gene expression. The indicated Mint2 domains fused in frame into pB42AD vector were co-transformed with TrkAIC (pGilda-TrkAIC), a kinase-dead TrkAIC
(pGilda-TrkAIC (K547N)), the intracellular domain of the EGF receptor (pGilda-EGFRIC), or the vector alone (pGilda) into the yeast reporter strain EGY48. Positive and negative controls were described in methods. (B) PC12 cells expressing the indicated constructs, either treated with NGF or left untreated, were lysed and subjected to GST pull-down, followed by Western blotting with anti-TrkA antibody (Chemicon). The whole cell lysate is shown as an input control. (C) Various GST fusion proteins were incubated with PC12 cell extracts containing endogenous TrkA. After GST pull-down, GST complexes were immunoblotted with anti-TrkA (Santa Cruz) for Western blot analysis. 1: GST-Mint2-N (68kD); 2: GST-PTB (45kD); 3: GST-PDZ1 (34kD); 4: GST-PDZ2 (34kD); 5: GST (negative control; 27kD); 6: PC12 cell lysate (positive control).

Fig. 3. Endogenous Mint2 associates with TrkA in rat tissue homogenates. Tissue homogenates from adult rat DRG, forebrain and cerebellum were prepared as described in the Methods, and immunoprecipitated with anti-TrkA or anti-Mint2 antibodies, followed by immunoblotting with anti-Mint2 or anti-TrkA antibodies. Protein expression levels in tissue homogenates were detected by immunoblotting with anti-Mint2 or anti-TrkA antibodies.

Fig. 4. TrkA and Mint2 co-localize in rat DRG neurons. Sections of rat DRG were co-immunostained with anti-TrkA antibodies (green) and anti-Mint2 antibodies (red). Merged images show TrkA and Mint2 co-localization in rat DRG neurons (yellow). Scale bars, 10 µm.

Fig. 5. Mint2 overexpression attenuates NGF-induced PC12 cell differentiation. (A) Western blot showing Mint2 expression in DRG neurons, but not in PC12 cells. (B) PC12 cells were co-transfected with pEGFP-N2, together with pcDNA3 as control or with pcDNA3-Mint2. After three days with or without NGF, neurite outgrowth was assessed as an indicator of neuronal differentiation. Scale bar, 10µm. (C) Quantification of neuronal differentiation of PC12 cells. Values represent mean ± s.d., ** indicates significant difference (p<0.01, Student’s t-test). (D) Western blotting was carried out to detect the levels of TrkA, p-TrkA and p75 proteins in various groups. After NGF treatment, decreased levels of p-TrkA were detected in Mint2 overexpressing PC12 cells compared to those seen in wild type PC12 cells. (E) Co-immunoprecipitation analysis showing that the interaction between TrkA and Mint2 was not affected by NGF stimulation.

Fig. 6. Mint2 overexpression inhibits NGF-induced neurite outgrowth in cultured DRG neurons. (A) Mint2 RNAi efficiency was assessed by Western blot analysis. HEK 293 cells were transfected with myc-Mint2 (lane 1), myc-Mint2 and nonsense-RNAi (lane 2), myc-Mint2 and the Mint2-siRNA constructs at 0.5 µg (lane3, No.1; lane 5, No.2; lane 7, No.3) or 1µg (lane 4, No.1; lane 6, No.2; lane 8, No.3). Mint2 was detected by blotting with a monoclonal anti-myc antibody. Actin IB was used as a loading control. (B) GFP-tagged Mint2-siRNA or GFP-tagged nonsense-siRNA plasmids were transfected into primary rat DRG neuron cultures (left panels). 24h post-transfection, cultures were immunostained with anti-Mint2 antibody to examine the expression of Mint2 (right panels). Scale bar, 10 µm. (C) Primary rat DRG neuron cultures were transfected with GFP tagged nonsense-siRNA, Mint2/GFP, or Mint2-siRNA plasmids, and subsequently incubated with NGF for 3 days. The transfected neurons were visualized as both GFP (green) positive and MAP2 staining (red) positive. Scale bar, 20 µm. (D, E) Quantification of neurite outgrowth in cultured DRG neurons. Image-pro Plus analysis software was used to quantify the length of the longest neurite (D), and Neuroulucida software analysis software was used to quantify total neurite length (E). Data are presented as means ± s.d.. ** indicates significant difference (p<0.01, Student’s t-test) compared with
control.

**Fig. 7.** Mint2 reduces surface localization of TrkA. (A) PC12 cells co-transfected with GFP-TrkA (green) together with myc or myc-Mint2, were incubated with or without NGF for 30 min, and then double-stained with anti-myc monoclonal antibody (red) and WGA (blue). GFP-TrkA was found to co-localize with myc-mint2 and WGA. (B) PC12 cells co-transfected with GFP-TrkA together with myc or myc-Mint2 were starved overnight before being treated with NGF, and then subjected to cell surface biotinylation. Biotinylated proteins were recovered by incubating cell lysates with avidin-agarose. The presence of TrkA in surface fractions was analyzed by Western blotting with anti-GFP antibody, and quantified as shown in (C). (D) Prior to NGF treatment, cell surface biotinylation was performed on transfected PC12 cells, and cell-surface biotin was then cleaved off using glutathione cleavage buffer. The biotinylated proteins were recovered by incubating cell lysates with avidin-agarose. The internalized fraction of GFP-TrkA was analyzed by Western blotting with anti-GFP antibody, and quantified as shown in (E). (F) DN-dynamin was co-transfected to block the internalization of TrkA. The surface fraction of GFP-TrkA was detected as described in (B), and quantified as shown in (G). Data represent mean ± s.d. of three independent experiments. **p<0.01 and *p<0.05 indicate significant difference (Student’s t-test) compared with the indicated group.

**Fig. 8.** Mint2 induces retention of TrkA in the Golgi apparatus. (A-C) The postnuclear supernatants of transfected PC12 cells were fractionated and immunoblotted using antibodies against GPP130 (Golgi marker) and calnexin (ER marker). Anti-GFP was used to blot for GFP-TrkA, and anti-Myc was used to blot for Myc-Mint2. PC12 cells were transfected with GFP-TrkA together with a pcDNA3 control vector (A), with Myc-Mint2 (B) or with Mint2-siRNA/Myc-Mint2 (C). (D, E) The interaction between myc-Mint2 and GFP-TrkA was detected by co-immunoprecipitation using the Golgi fractions (D) or ER fractions (E) from transfected PC12 cells as indicated.
Mint2 (amino acids 286-539) region isolated from the yeast two-hybrid

LPEAKHPGDPQGRFHKPTRTPEERLKWPHEQVCNGLLEQPRLKQRSDLNPGVDDNINPETKKVASFPSFVAVPGPECEDLIDGIIFAANYLGSTQSSERTPSKNIRMMQAQEAVSRVKRMQKAAKIKKKANSEGDAQTLTTEVFIDISTQRIKVLNADTTQETMMDHALRTRSYIADIGNIVLMLARRRMPRSSASQDCETTPGQAEGKQKYKMI
CHVFSEDAQLIAQSIGQAFSVAYQEFLRANGINPEDLSQ
**Fig. 2**

### A

| Bait                  | Prey              | Filter Assay | Growth Assay |
|-----------------------|-------------------|--------------|--------------|
| pGilda-TrkAIC         | pB42AD-Mint2      | +            | +            |
| pGilda-TrkAIC         | pB42AD-Mint2-N    | -            | -            |
| pGilda-TrkAIC         | pB42AD-Mint2-PTB | +            | +            |
| pGilda-TrkAIC         | pB42AD-Mint2-PDZ1| -            | -            |
| pGilda-TrkAIC         | pB42AD-Mint2-PDZ2|              |              |
| pGilda-TrkAIC (K547N)| pB42AD-Mint2      | +            | +            |
| pGilda-EGFRIC         | pB42AD-Mint2      | -            | -            |
| pGilda-53             | pB42AD-T          | +            | +            |
| pGilda                | pB42AD            | -            | -            |

### B

- NGF: - - + +
- TrkA: + + + +
- GST: + - + -
- GST-Mint2: - + - +

### C

**Precipitation**

- IB: TrkA

**Coomassie blue staining**

- Input IB: TrkA

**Precipitation**

- IB: TrkA

**Coomassie blue staining**

- Input IB: TrkA

Buffer concentrations:

- 140 kD
- 110 kD
- 110 kD
- 27 kD
- 140 kD
- 110 kD
- 140 kD
- 68 kD
- 45 kD
- 34 kD
- 27 kD
- 140 kD
- 68 kD
Fig. 3

| DRG | forebrain cerebellum |
|-----|----------------------|
| IP: anti-TrkA | IB: anti-Mint2 |
| IB: anti-TrkA | 120kD |
| IP: anti-Mint2 | IB: anti-TrkA |
| IB: anti-TrkA | 140kD |
| IP: anti-Mint2 | IB: anti-Mint2 |
| IB: anti-Mint2 | 120kD |
| IB: anti-Mint2 | IB: anti-TrkA |
| IB: anti-TrkA | 140kD |
Fig. 4
Fig. 5

A

![Image showing DRG PC12, Mint2, and actin bands with Molecular weights: Mint2 120kD, actin 42kD.]

B

![Image showing NGF- and NGF+ conditions with pcDNA3/GFP and Mint2/GFP labeled regions. Scale bar: 10µm.]

C

![Bar graph showing PC12 cell differentiation rate (%). Bars represent NGF- and NGF+. Error bars indicate standard error. Significant difference indicated by **.]

D

![Table showing NGF conditions with Myc-Mint2, TrkA, p-TrkA, p75, Mint2, and actin bands with Molecular weights: TrkA 140kD, p-TrkA 140kD, p75 75kD, Mint2 120kD, actin 42kD.]

E

![Western blot showing IP: TrkA, IB: Mint2, and IP: Mint2, IB: TrkA with Molecular weights: NGF- 120kD, NGF+ 140kD.]

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Fig. 8

A

| Lanes | GPP130 | Calnexin | GFP-TrkA |
|-------|--------|----------|----------|
| 1-13  |        |          |          |

B

| Lanes | GPP130 | Calnexin | GFP-TrkA | Myc-Mint2 |
|-------|--------|----------|----------|-----------|
| 1-13  |        |          |          |           |

C

| Lanes | GPP130 | Calnexin | GFP-TrkA | Myc-Mint2 |
|-------|--------|----------|----------|-----------|
| 1-13  |        |          |          |           |

D

- Myc-Mint2: - + +
- GFP-TrkA: + + +
- Mint2 siRNA: - - +

E

- Myc-Mint2: - + +
- GFP-TrkA: + + +
- Mint2 siRNA: - - +
Interaction of mint2 with TRKA is involved in regulation of nerve growth factor-induced neurite outgrowth

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