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Intracellular LINGO-1 Negatively Regulates Trk Neurotrophin Receptor Signaling

James S. Meabon^{1,2}, Rian de Laat³, Katsuaki Ieguchi⁴, Dmitry Serbzhinsky⁵, Mark P. Hudson⁶, B. Russel Huber¹, Jesse C. Wiley⁷, and Mark Bothwell^{6,*}

¹Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, WA 98195

²Mental Illness Research Education and Clinical Center, VA Medical Center, Seattle, WA 98108

³Immusoft, Seattle, WA 98103

⁴Department of Pharmacology, Tokyo Women's Medical University, Tokyo, Japan

⁵Seattle Biomedical Research Institute, Seattle, WA 98109

⁶Department of Physiology & Biophysics, University of Washington, Seattle, WA 98195

⁷Department of Comparative Medicine, University of Washington, Seattle, WA 98195

Abstract

Neurotrophins, essential regulators of many aspects of neuronal differentiation and function, signal via four receptors, p75, TrkA, TrkB and TrkC. The three Trk paralogs are members of the LIG superfamily of membrane proteins, which share extracellular domains consisting of leucine rich repeat and C2 Ig domains. Another LIG protein, LINGO-1 has been reported to bind and influence signaling of p75 as well as TrkA, TrkB and TrkC. Here we examine the manner in which LINGO-1 influences the function of TrkA, TrkB and TrkC. We report that Trk activation promotes Trk association with LINGO-1, and that this association promotes Trk degradation by a lysosomal mechanism. This mechanism resembles the mechanism by which another LIG protein, LRIG1, promotes lysosomal degradation of receptor tyrosine kinases such as the EGF receptor. We present evidence indicating that the Trk/LINGO-1 interaction occurs, in part, within recycling endosomes. We show that a mutant form of LINGO-1, with much of the extracellular domain deleted, has the capacity to enhance TrkA signaling in PC12 cells, possibly by acting as an inhibitor of Trk down-regulation by full length LINGO-1. We propose that LINGO-1 functions as a negative feedback regulator of signaling by cognate receptor tyrosine kinases including TrkA, TrkB and TrkC.

*Corresponding Author: Mark Bothwell, Ph.D., University of Washington, Dept. of Physiology & Biophysics, Institute for Stem Cell and Regenerative Medicine, 850 Republican St., Seattle, WA 98109, U.S.A. Tel: +1-206-543-7924, Fax: +1-206-685-0619, mab@u.washington.edu.

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Keywords

LINGO-1; LRRN6a; TrkA; TrkB; TrkC; LINX

INTRODUCTION

LINGO-1 is a member of the LIG gene super-family whose members are type I membrane proteins possessing extracellular domains composed of Leucine rich repeat and C2 immunoglobulin-like domains. 17 LIG proteins are represented among 6 subfamilies: LINGO, LRIG, ISLR/LINX, NGL, AMIGO and TRK (Mandai et al 2009). Though LINGO-1 was originally identified as an essential component of a cell surface LINGO-1/p75^{NTR}/NgR receptor complex mediating axon growth cone collapse (Mi et al 2004) recent reports demonstrate roles for LINGO-1 in the modulation of TrkB and EGF receptor (EGFR) tyrosine kinase activity (Fu et al., 2010; Inoue et al., 2007; Lee et al., 2007; Trifunovski et al., 2004). However, the mechanism of this effect remains poorly understood. We have previously described LINX, a protein structurally related to LINGO-1, as an effector of Trk and Ret receptor tyrosine kinase signaling and a modulator of motor and sensory neuron axon guidance (Mandai et al., 2009). In the course of those studies, we performed co-immunoprecipitation experiments, which revealed that multiple LIG family members, including LINGO-1, LINX, NGL-1, AMIGO-1 and LRIG1 formed physical complexes with TrkA, TrkC and Ret. These experiments also revealed that over-expression of LINGO-1 or LRIG1 markedly down-regulated expression of TrkA, TrkC and Ret, whereas LINX, NGL-1 and AMIGO-1 did not (Mandai et al., 2009).

A number of studies have reported roles for LIG family members in modulating receptor tyrosine kinase signaling. The mechanism by which LRIG1 negatively regulates receptor tyrosine kinases, including EGF receptor and other members of the ErbB family, as well as Met and Ret, has been described (Gur et al., 2004; Laederich et al., 2004; Ledda et al., 2008; Shattuck et al., 2007). EGF receptor activity transcriptionally upregulates LRIG1 expression and upregulated LRIG1, in turn, promotes lysosomal degradation of endocytically internalized EGF receptor, representing a negative feed-back mechanism (Gur et al., 2004; Laederich et al., 2004). Genetic knockout of LRIG1 increases ErbB signaling in intestinal stem cells, which leads to abnormal cell cycle dynamics and increased cell progenitor expansion (Wong et al., 2012) LRIG1 expression correlates with better prognoses in several human cancers where dysregulated ErbB's are thought to promote progression of the disease (Krig et al., 2011; Lindström et al., 2008; Tanemura et al., 2005). These reports prompted us to ask whether LINGO-1 mediates negative regulation of Trk signaling by a similar mechanism.

Here we describe features of the regulatory interaction of LINGO-1 with Trk proteins. Our findings suggest a model in which activation of Trk receptors and accompanying endocytosis leads to formation of LINGO-1/Trk complexes in recycling endosomes. This interaction promotes delivery of Trks into lysosomes leading to Trk degradation. Evidence is presented supporting a role of LINGO-1 as a physiological negative regulator of Trk function.

MATERIALS AND METHODS

Plasmid constructs

A eukaryotic expression plasmid for full length human LINGO-1 [hL1], IMAGE: 4214343, was purchased from Thermo Scientific Open Biosystems (Pittsburgh, PA). LINGO-1 missing the intracellular domain [LINGO-1- ICD-V5] were created according to manufacturer's instructions with PCR generated DNA fragments using the forward oligonucleotide primer 5'-CACCATGCAGGTGAGCAAGAGG-3' and the reverse primers 5'-TATCATCTTCATGTTGAACTTGCG-3' and 5'-GTTGCCCTTGCCCCGGCTCCA-3', respectively, and the pcDNA3.1 Directional TOPO expression kit (Invitrogen, Grand Island, NY). The sequence for the transmembrane and intracellular domain of LINGO-1 was amplified via 5'-CACGGATCCAAGACCTCATCATCGCCACC-3' and 5'-CACTCTAGACTCATATCATCTTCATGTTGAACT-3' oligonucleotides and was spliced into and expressed from pSecTagB (BamH1-XbaI). The kinase incompetent pCMV5 rat TrkA D671A [TrkA-KD] and pCMV5 rat TrkB D693A [TrkB-KD] have been described previously (Schechter et al 2010). Empty vector control is pcDNA3.1/Zeo (-) and was purchased from Invitrogen (Grand Island, NY). A carboxy-terminal myc tagged version of mouse LINX [LINX-myc] is in the vector pcDNA3.1 myc/His(-)A (Invitrogen, Grand Island, NY) and described previously (Mandai et al., 2009). A plasmid vector driving the expression of an amino-terminal myc tagged version of human LINGO-1 with a deleted extracellular domain, a non-native secretion signal and IRES mediated DsRed expression [DsRed ECDdelta-hL1] was created in a two step process using the three plasmids: IMAGE clone 4214343 (Thermo Scientific Open Biosystems, Huntsville, AL, USA), pSecTagB (Invitrogen) and pIRES2-DsRed2 (Clontech, Mountain View, CA, USA). First, a DNA fragment encoding myc-ECDdelta-hL1 was created using standard Taq based PCR, the IMAGE clone and the oligonucleotide primers 5'-CACGGATCCATGGAGCAAAAGCTCATTCTGAAGAGGACTTGAATGAAAAGACCCTCATCATCGCCACC-3' and 5'-CACGAATTCCTCATATCATCTTCATGTTGAACTTGCGGG-3'. This fragment was digested with the endonucleases BamH1 and EcoR1 and ligated into a similarly digested pSecTagB using T4 DNA ligase. The resulting 371 base pair CDS was removed via NheI and EcoR1 and inserted into an identically opened pIRES2-DsRed2. Rat TrkA and rat TrkB constructs were gifts from Moses Chao and NgR-Flag was a gift from Z.-g. He. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa).

Lentivirus construction

We thank Dr. William Osborne for providing the lentiviral transfer vector pRRL- cPPT-CMV-X-PRE-SIN to create pRRL-cPPT-CMV-hLINGO-1-IRES-hrGFPII-PRE-SIN. Wild type LINGO-1 (IMAGE clone 4214343) was isolated using HindIII, followed by heat inactivated (65 °C for 20 minutes), blunt ended with T4 DNA polymerase followed by another round of heat inactivation (75 °C for 20 minutes) and then further digested with EcoR1. The product (2350 bp) was run on a 1% agarose gel and band purified. The resulting purified fragment was ligated into pRRL- cPPT-CMV-X-PRE-SIN linearized by EcoR1 and EcoR5. Correct insertion of LINGO-1 into the lentiviral vector was verified by direct sequencing of the final product.

Cell culture, transfection and treatments

For primary cortical neuronal cultures, C57BL/6 p0 mouse pup brains were acutely removed and dissociated as previously described (Young et al 2009). All procedures involving mice were approved by the IACUC committee of the University of Washington. Purified neural cultures were plated at 1.25×10^5 to 2.5×10^5 cells per 18 mm^2 on poly D-lysine (Sigma, St. Louis, MO) coated glass coverslips and allowed to grow 5–14 DIV in complete NBA media (Invitrogen, Grand Island, NY).

HEK293 and PC12 cells were routinely cultured and transfected as previously described (Kanning et al., 2003). For transfection experiments, cells were plated at 30–50% confluency in 6-well plates 20–24 hr prior to transient transfection and transfected using Lipofectamine 2000 (2.5 $\mu\text{l}/\mu\text{g}$ LIPO:DNA; Invitrogen, Grand Island, NY) and 1 μg (unless otherwise indicated) of plasmid DNA (per construct transfected) in DMEM+10% fetal bovine serum without antibiotics. The total amount of transfected DNA was held constant between conditions within a given experiment with empty vector control plasmid substituting for any remainder required. Cells were harvested 24 hr post transfection.

Dissociated cortical cells were transfected using Lipofectamine 2000 (0.5 $\mu\text{l}/0.8 \mu\text{g}$ plasmid) and 0.8 μg plasmid for 1.5 hr, after being switched into penicillin/streptomycin-free media. After transfection, the media containing the Lipofectamine/DNA mixture was removed and fresh NBA plus B27 supplement was supplied. Neuronal cells for immunocytochemistry and subsequent confocal microscopy were allowed to culture for 72 hr post transfection. NGF (a gift from Genentech, Inc) was used at 100 ng/ml in DMEM plus penicillin/streptomycin for 15 min after incubating cells in serum-free media for 2 hr. Neurotrophin treatment was terminated by ice cold phosphate-buffered saline (PBS) rinse (3x, 10 sec) at 4 C, followed by ice cold homogenization supplemented with sodium orthovanadate. Where indicated, cultures were subjected to the following treatments in fresh media: DMSO (Sigma, St. Louis, MO; 1:1000), bafilomycin A1 [BAF] (Calbiochem, #196000, 0.1 μM , 12 hr), or Epoxomicin [EPO] (EMD, Billerica, MA; 1 μM , 4–12hrs). Neuronal cultures were transduced using 3 MOI of virus. Transduced neurons were cultured for 5 days without changing the media. Cell death assays using trypan blue exclusion were accomplished following the manufacturer's protocol (Life Technologies, Grand Island, NY).

Generation of stable cell lines and PC12 differentiation

PC12 cell populations stably expressing DsRed-hL1, DsRed-ECD -hL1 or empty vector controls were generated by Lipofectamine-mediated transfection followed by selection in growth medium containing G418 (Invitrogen, Grand Island, NY) (200 $\mu\text{g}/\text{ml}$ –400 $\mu\text{g}/\text{ml}$; 3–4 months). Mixed populations were used for studies at the end of stabilization period. For differentiation, PC12 cells were grown in PC12 growth media supplemented with 100 ng/ml NGF for 5 days in 6-well tissue culture dishes before being imaged on Zeiss AxioSkop II inverted microscope at 20x. PC12 cells were considered to be neuronally differentiated if their longest neurite was greater than 2.5 times the cell body width. Measurements were made using AxioSkop LSM Image Browser. HEK293 cells stably expressing transfected LINGO-1 were produced by Lipofectamine-mediated transfection with plasmids containing a neomycin resistance cassette, followed by selection in growth medium containing G418.

Antibodies

The following antibodies were used as specified: rabbit anti-LINGO-1 (Upstate, Billerica, MA; 1 µg/ml), rabbit anti-Trk (Santa Cruz Biotech., Dallas, TX; 1 µg/ml), goat anti-phospho-TrkB (a gift from Dr. Moses Chao, 0.3 µg/ml), goat anti-TrkB (R&D Systems, Minneapolis, MN; 0.1 µg/ml), mouse anti-phosphotyrosine Clone 4G10 (Millipore, Billerica, MA; 1 µg/ml), goat anti-pyruvate kinase (Rockland Immunochemicals, Gilbertsville, PA, 1 µg/ml), mouse anti-V5 (Invitrogen, Grand Island, NY; 1 µg/ml).

Immunoprecipitation, gel electrophoresis and immunoblot analysis

Cell homogenization, immunoprecipitation, BCA analysis, SDS PAGE, semi-dry electrotransfer and immunoblotting was performed as described previously (Kanning et al 2003a). Immunoprecipitation from whole p0 mouse brain was conducted using the following protocol: p0 mouse brains were acutely dissected and snap frozen in liquid nitrogen. A mortar and pestle was pre-chilled with liquid nitrogen and the tissue was submerged in liquid nitrogen while being ground into a fine dust. Low salt homogenization buffer (10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 2 mM EDTA (pH 8.0), 5 mM EGTA (pH 8.0), Protease inhibitor cocktails) was added to the brain dust which was further homogenized by trituration with decreasing diameters of needles from 18 to 25 gauge. After the 25-gauge needle allowed for easy (if slow) uptake into the needle the slurry was spun at high speeds, the pellet brought up in hypotonic buffer, and the solution incubated on a nutator for one hour at 4 °C. After ultracentrifugation for 1 hour at 100,000 x g at 4 °C the supernatant was brought to a final NaCl concentration of 0.15 M. The lysate was pre-cleared by rotating for an hour at 4 °C with Protein A Sepharose beads before being aliquoted into different tubes for the various antibodies for immunoprecipitation.

Immunocytochemistry

Coverslip-grown cultures were rinsed with PBS (pH 7.4) then fixed with 4% formaldehyde (4% w/v PFA in PBS) for 30 minutes. Cover slips were then rinsed with PBS and permeabilized with 0.25% v/v Triton-X100 in PBS for 15 minutes. Permeabilization was followed by another PBS rinse. Cover slips were then blocked in 10% w/v bovine serum albumin in PBS for 15 minutes then incubated overnight at 4 °C in primary antibody mixtures. At the conclusion of incubation, primary antibodies were removed and cover slips were rinsed three times with PBS (one quick, two for 10 minutes each) and then incubated for 3 hours at room temperature in appropriate secondary reagents. Cover slips were then washed six times with cold PBS (three brief, three for 10 minutes each), incubated with Hoechst 33258 in dH₂O for 7 minutes, post-fixed with 4% PFA (10 minutes), rinsed with PBS and then washed twice for 10 minutes in dH₂O. Fixed cells were imaged using a Zeiss 510 META confocal microscope with excitations at 405nm, 488nm and 543nm. Images were routinely acquired at a depth yielding maximum fluorophor signal throughout the highest amount of neuritic arborization resolvable. Only healthy cells with normal morphology and non-pyknotic nuclei were imaged. All images were obtained in multi-track, multi-frame mode with line averaging set to 4.

Densitometry and statistics

Densitometry of subject bands (minus background) were quantified from immunoblot images using NIH ImageJ. Subsequent analysis was conducted using Microsoft Excel. Normalization of conditions was calculated as treatment conditions divided by baseline conditions (or control treatments). Statistical analysis of samples was conducted using Student's t-test. Values with $p < 0.05$ were considered significant. Graphs represent mean $\pm$ standard error of the mean.

RESULTS

LINGO-1 physically associates with Trk paralogs

We have previously demonstrated that a carboxy-terminally tagged LINGO-1 formed complexes with carboxy-terminally tagged TrkA and TrkC (Mandai et al., 2009). However, under the conditions employed no interaction of TrkB with LINGO-1 was observed. Because TrkB is highly expressed in brain and LINGO-1 modulation of TrkB function has been demonstrated in neural models (Fu et al., 2010; Mi et al., 2005) we wished to assess whether LINGO-1 binds TrkB. We also wished to verify that these interactions occurred with native non-tagged proteins. To address these questions, we transiently transfected HEK293 cells with plasmids encoding these proteins (and for comparison, TrkA) and observed that LINGO-1 was co-immunoprecipitated with either TrkA or TrkB (Figure 1A). However, more LINGO-1 co-immunoprecipitated with TrkA than with TrkB. Conversely, we were able to detect TrkA or TrkB in LINGO-1 immunoprecipitates (shown for TrkB in Figure 1B). The differential avidity between TrkA and TrkB for interaction with LINGO-1 accounts for the apparent absence of LINGO-1/TrkB complexes in our previously published study.

LRIG1-mediated degradation of EGF receptors is stimulated by activation of the receptors by EGF (Gur et al., 2004). Is the functional interaction of LINGO-1 with Trks similarly dependent on Trk activation? Addressing this question was problematic because Trks become constitutively activated when over-expressed by transfection (Schechter et al., 2010). Therefore, to address this question, we compared the association of LINGO-1 with wild type TrkA or TrkB and TrkA or TrkB bearing an inactivating mutation in the tyrosine kinase active site (TrkA-kinase dead, TrkA-KD; TrkB-kinase dead, TrkB-KD). LINGO-1 association with TrkA and TrkB in Trk immunoprecipitates was greatly diminished for the inactive Trk mutants (Figure 1A, 1B). The association of LINX with TrkA or TrkC was similarly dependent on Trk tyrosine kinase activity (Figure 1C). LINX does not associate with kinase-active TrkB (Mandai et al, 2009), so it was not feasible to try to assess whether the association is diminished with kinase-dead TrkB. Activation of Trks influences their trafficking, both in the secretory pathway (Schechter et al., 2010) and in the endocytic pathway (Bhattacharyya et al. 2002). Thus, the enhanced Trk/LINGO-1 association with kinase-active Trks may reflect increased localization of Trks to LINGO-1 expressing membrane compartments, rather than increased avidity of Trk/LINGO-1 complexes.

As LINX promotes Trk signaling (at least for TrkA) (Mandai et al., 2009) while LINGO-1 inhibits Trk signaling, it is noteworthy that LINX up-regulated Trks (Figure 1C) while

LINGO-1 down-regulated Trks (Figure 1A, 1B). While LINGO-1 down-regulated TrkB, LINX, which does not bind TrkB, had no effect on the level of TrkB expression (Figure 1C). Curiously, although association of LINX with kinase-dead TrkA and TrkC was greatly diminished, nevertheless kinase-dead TrkA and TrkC were upregulated when LINX was expressed (Figure 1C). This suggests that even weak or transient association of LINX with Trks promotes accumulation of Trks. Alternatively, we cannot rule out the possibility that effects of LINX on TrkA and TrkC expression are independent of formation of LINX/Trk complexes.

Although a functional interaction between LINGO-1 and TrkB *in vivo* in retinal tissue has been described (Fu et al., 2010), and we have described complexes between LINGO-1 and TrkA and TrkC in cultured cells over-expressing these proteins (Mandai et al., 2009), previously studies have not determined whether complexes between Trk proteins and LINGO-1 occur in brain *in vivo*. To assess whether such complexes exist in brain tissue, we employed immunoblot analysis to assess Trk immunoreactivity in LINGO-1 immunoprecipitates from detergent extracts of neonatal mouse brain cortex. For this experiment we employed an antibody that recognizes all three Trk paralogs (TrkA, TrkB, and TrkC) and an antibody that recognizes three of the four LINGO paralogs (LINGO-1, LINGO-2, LINGO-3). For this and subsequent experiments, we will refer generically to Trk and LINGO immunoreactivity whenever the specific paralog identity is unknown. For comparison, we also assessed whether Trk co-immunoprecipitated with brain proteins previously shown to bind Trk, including p75^{NTR} (Bibel et al., 1999) and LINX (Mandai et al., 2009). This experiment revealed that LINGO/Trk complexes do occur in brain tissue, and confirmed the association of Trk with p75^{NTR} and LINX with Trk (Figure 1D).

LINGO-1 regulates endogenously expressed Trks

To assess whether LINGO-1 negatively regulates expression of natively expressed Trks, we employed mouse cortical neuronal cultures. It was first necessary to confirm that individual cortical neurons express both LINGO-1 and TrkB. Cortical neurons populations are known to express both TrkB (Klein et al., 1989) and LINGO-1 (Carim-Todd et al., 2003) and BDNF-induced TrkB activity up-regulates LINGO-1 mRNA in hippocampal neurons (Trifunovski et al., 2004). However, the extent of co-expression of LINGO-1 and TrkB in cortical neurons has not been reported. Furthermore, as LINGOs interact preferentially with activated Trks, we were particularly interested to know whether LINGO is co-localized with activated TrkB at the subcellular level. To address this question we employed confocal immunofluorescence microscopy using a pan-LINGO antibody and an antibody recognizing the tyrosine-phosphorylated form of TrkB. LINGO was localized to intracellular puncta, representing intracellular membrane vesicles, as we have described previously (Meabon et al., 2015). Phospho-TrkB immunoreactivity was localized to a subpopulation of LINGO-immunopositive puncta (Figure 2A). Activated TrkB undergoes endocytosis, leading to accumulation in Rab11-positive recycling endosomes (Lazo et al., 2013), while LINGO-1 is localized to a variety of intracellular membrane compartments, including Rab11-positive vesicles (Meabon et al., 2015). Thus the punctate co-localization of LINGO and phospho-Trk in Fig. 2A may represent co-localization in intracellular vesicular membrane compartments including recycling endosomes.

Does LINGO-1 negatively regulate Trk protein in cortical neurons? We addressed this question by using a lentiviral vector to overexpress LINGO-1 in cultured cortical neurons. Application of LINGO-1 lentivirus to cortical neuronal cultures substantially elevated LINGO-1 expression above levels in uninfected controls and reduced Trk expression when compared to uninfected cultures or to cultures infected with control GFP-expressing lentiviral vector (Figure 2B).

To assess the specificity of LINGO-1 dependent down-regulation of endogenous Trk expression in dissociated primary mixed cortical cultures, we transfected cultures with wild type LINGO-1 and compared its effects to the effects of expression of LINX or of FGFR2 bearing a V5 tag and containing an inactivating mutation in the tyrosine kinase domain (FGFR2-KD). Because we lacked lentiviral vectors encoding all these proteins we employed lipofectamine-mediated plasmid transfection for this experiment. Lipofectamine-mediated transfection of primary cortical neurons is inefficient, yielding productive expression of plasmid-encoded genes in only a few percent of neural cells. In cultures transfected with LINGO-1 plasmid we observed that a small percentage of neurons expressed dramatically higher levels of LINGO-1. In such cells, Trk immunoreactivity (presumably representing mainly TrkB) was generally suppressed below the threshold for detection (Figure 2C). In contrast, overexpression of the negative controls, FGFR2-KD and LINX, did not reduce Trk expression.

Since artificially increasing LINGO-1 expression down-regulates Trks, one might expect that diminishing naturally occurring LINGO-1 expression would up-regulate Trks. To determine whether this predication is correct, we employed transfection of a plasmid encoding LINGO-1 shRNA. However, as cortical neurons transfect with poor efficiency, we employed the neuronal cell line, N2a, for this experiment. N2a cells natively express LINGO-1, TrkA and TrkB. Quantification of densitometric scans of immunoblot data demonstrated that transfection of plasmid encoding LINGO-1 shRNA down-regulated LINGO-1 by about 50% (Figure 3A). This degree of LINGO-1 down-regulation significantly up-regulated endogenous Trk expression (Figure 3B).

To confirm that natively expressed LINGO-1 controls Trk expression in another cell type, we employed HEK293 cells, which natively express LINGO-1, albeit at a low level (Meabon et al., 2015). HEK293 cells were transiently transfected with a TrkA expression plasmid in combination with plasmids expressing LINGO-1 shRNA, LINGO-1 or LINX. Immunoblot analysis revealed that increased expression of LINGO-1 diminished TrkA expression in proportion to the amount of TrkA plasmid transfected, while expression of LINX up-regulated TrkA. Exposure to LINGO-1 shRNA up-regulated TrkA, implying that natively expressed LINGO-1 in HEK293 cells suppresses TrkA expression (Figure 3C).

LINGO-1 promotes lysosomal degradation of Trk

Endogenous LINGO-1 down regulates plasmid-expressed Trk. This finding supports the conclusion that the mechanism involved is post-translational in nature and is consistent with LINGO-1's role in regulating other receptors such as EGFR (Inoue et al., 2007). LRIG1, a LIG family member akin to LINGO-1, down-regulates EGFR by promoting lysosomal degradation of EGFR (Stutz et al., 2008). Like EGFR, activated Trks are lysosomally

degraded (Geetha and Wooten, 2008) suggesting the hypothesis that LINGO-1 mediated Trk down-regulation employs the endolysosomal pathway. We tested this hypothesis by examining the effect of the lysosome inhibitor, bafilomycin A1, on LINGO-1 induced TrkA down-regulation in HEK293 cells. Lysosomal inhibition rescued TrkA from LINGO-1-mediated down-regulation (Figure 4A). In contrast, the proteasome inhibitor epoxomicin had no effect. Similar results were obtained for TrkB (data not shown).

We have shown that LINGO-1 associates more extensively with activated Trk proteins than with Trk proteins bearing inactivating mutations in their tyrosine kinase domains. Activation of Trks at the cell surface promotes internalization and continued signaling from endosomal vesicles (Grimes et al., 1996) including Rab11-positive recycling endosomes (Ascaño et al., 2009). This suggests the possibility that the preferential association of activated Trks with LINGO-1 may result from activity-dependent trafficking of Trks into intracellular membrane compartments where LINGO-1 resides. To test this hypothesis, we transfected HEK293 cells with plasmids encoding LINGO-1 and TrkA, achieving levels of TrkA expression sufficient to cause ligand-independent TrkA activation. We examined whether inhibition of endocytosis influenced association of TrkA with LINGO-1. Addition of 225 mM sucrose to cell culture medium inhibits endocytosis by a mechano-osmotic mechanism (Heuser and Anderson, 1989). Consistent with previous results, LINGO-1 was co-precipitated in TrkA immunoprecipitates from cells not exposed to high sucrose conditions. However, after exposure of cells to 225 mM extracellular sucrose, no LINGO-1 was detected in TrkA immunoprecipitates (Figure 4B), supporting the hypothesis that endocytosis of TrkA promotes association of TrkA with LINGO-1. Blockade of endocytosis by 225 mM sucrose also prevented down-regulation of TrkA by LINGO-1 (Figure 4C). These results support the hypothesis that endocytosis of TrkA promotes interaction of TrkA with LINGO-1 in endosomes, leading to subsequent lysosomal degradation of TrkA.

A major pathway for TrkA endocytosis of activated TrkA employs clathrin-coated vesicles. However, some TrkA endocytosis occurs by clathrin-independent mechanisms (Valdez et al., 2007). Does LINGO-1-mediated down-regulation of TrkA require clathrin-mediated endocytosis? To address this question we assessed the effect of chlorpromazine, a drug that inhibits formation of clathrin lattices (Wang et al., 1993). Chlorpromazine failed to inhibit LINGO-1 dependent down-regulation of TrkA (Figure 4C), suggesting that LINGO-1 promotes lysosomal degradation of TrkA by a mechanism that does not require clathrin-mediated endocytosis.

Careful examination of these immunoblot data reveals a feature that may shed light on the mechanism of action of LINGO-1. TrkA appears on immunoblots as a doublet of two closely spaced bands. The lower (smaller) TrkA species is known to represent TrkA with immature high-mannose N-glycans, reflecting protein that has not completed trans-Golgi mediated processing (Scheeterson et al., 2010). The immunoblots (for example, Figure 4C) consistently demonstrate that LINGO-1 promotes down-regulation of the immature TrkA species as well as the mature TrkA species. Thus, LINGO-1 may promote lysosomal delivery of activated TrkA in the secretory pathway as well as in the endocytic pathway. The manner in which 225 mM extracellular sucrose might affect the secretory pathway is

unknown. It is noteworthy that physiologically important neurotrophin-independent activation of Trks occurs within the secretory pathway (Rajagopal et al., 2004).

LINGO-1 negatively regulates functional TrkA signaling

As a tool to assess the functional importance of LINGO-1/Trk interactions we created a dominant-negative inhibitory form of LINGO-1. As a mutant form that might interfere with functional interactions of native LINGO-1 with Trks, we produced a form of LINGO-1 (ECD -hL1) in which much of the extracellular domain is deleted. The function of ECD -hL1 was initially assessed in HEK293 cells transiently transfected with TrkA in combination with native or mutant LINGO-1. In contrast to native LINGO-1, ECD -hL1 did not down-regulate TrkA (Figure 5A).

The failure of ECD -hL1 to down-regulate Trks might result from diminished ability to bind to Trks. To examine this possibility, we transiently transfected HEK293 cells with TrkA and ECD -hL1 or full length LINGO-1 and asked whether ECD -hL1 was present in TrkA immunoprecipitates. We observed that ECD -hL1 was pulled down in TrkA immunoprecipitates as effectively as full length LINGO-1 (Figure 5B).

If ECD -hL1 binds TrkA without down-regulating TrkA, it might be expected to competitively block down-regulation of TrkA by full-length LINGO-1. To test the functional effects of ECD -hL1 we employed the neuronal cell line PC12, because it is a widely employed system for study of functional signaling by TrkA. PC12 cells express LINGO-1 and TrkA endogenously, and undergo neuronal differentiation in response to TrkA activity. We produced stably transfected populations of PC12 cells over-expressing native LINGO-1 or ECD -hL1, using cells transfected with empty vector as a negative control.

Immunoblot analysis revealed that PC12 cells stably transfected with LINGO-1 had elevated levels of both ~90 kDa and ~15 kDa LINGO-1 proteins, while PC12 cells stably transfected with ECD -hL1 had elevated levels of ~15 kDa LINGO-1 only (Figure 5C). This production of a 15 kDa LINGO-1 fragment when LINGO-1 is over-expressed is consistent with a published report that proteolytic processing of LINGO-1 generates a fragment of this size (Rice et al., 2013). Interestingly, comparison of levels of endogenous LINGO-1 expression in PC12 populations stably transfected with ECD -h1 or a control vector indicates that ECD -hL1 diminishes expression of endogenously encoded full length LINGO-1 (Figure 5C). We assessed whether over-expression of LINGO-1 or ECD -hL1 influenced Trk signaling employing immunoblot analysis, with an antibody against tyrosine-phosphorylated TrkA to monitor TrkA activation. As TrkA does not exhibit neurotrophin-independent activation in the PC12 cell line, we exposed cells briefly (15 minutes) to 100 ng/ml NGF before lysing cells for analysis.

The level of LINGO-1 over-expression achieved in the stably transfected PC12 cell line had little effect on the total amount of TrkA expressed (Figure 5C), possibly because LINGO-1 mediated TrkA down-regulation requires TrkA activation and the period of NGF-mediated activation was too brief to allow lysosomal degradation of the TrkA pool. However, cells over-expressing LINGO-1 contained less activated tyrosine-phosphorylated TrkA.

Remarkably, PC12 cells over-expressing ECD -hL1 exhibited substantially increased levels of tyrosine-phosphorylated TrkA (Figure 5C). Interestingly, expression of ECD -hL1 caused down-regulation of endogenous full-length LINGO-1. Thus, the increased activation of TrkA in cells expressing ECD -hL1 may be due to diminished levels of native LINGO-1 as well as competitive inhibition of binding of native LINGO-1 to TrkA.

The morphologies of wild-type PC12 cells and PC12 populations over-expressing LINGO-1 and ECD -hL1, after 5-day exposure to 100 ng/ml NGF, are illustrated in Figure 5D. Although the increased TrkA activity associated with ECD -hL1 over-expression might have been predicted to enhance neuronal differentiation of PC12 cells, in fact, over-expression of ECD -hL1 inhibited morphological differentiation of PC12 cells (Figure 5E). Over-expression of LINGO-1 increased the proportion of non-viable cells, as assessed by trypan blue dye exclusion, while over-expression of ECD -hL1 had no effect on cell viability (Figure 5F).

DISCUSSION

The results of our study indicate that each of the three Trk paralogs, TrkA, TrkB and TrkC, physically associate with LINGO-1 and are directed into a lysosomal degradation pathway as a result of this interaction. Our results indicate that the LINGO-1/Trk interaction and subsequent Trk degradation is enhanced when Trk proteins are activated, either spontaneously or as a result of binding neurotrophins.

It is well known that activation of Trk proteins at the cell surface causes endocytosis of the receptor, and that the internalized receptors in early endosomes may alternatively traffic to the late endosome pathway leading to lysosomal degradation, or the recycling endosome pathway leading to return to the cell surface or retrograde axonal transport (Yu et al., 2011). Furthermore, receptors within the endocytic pathway may continue to signal. Consequently, mechanisms that influence which endocytic pathway Trks follow may control the dynamics of receptor signaling as well as the cellular levels of receptor expression. We have reported that there is little LINGO-1 at the cell surface. Instead, LINGO-1 is observed in intracellular membranes of the secretory and endocytic pathway. We propose a model in which LINGO-1 encounters Trk proteins in endosomes following activity-dependent Trk endocytosis. The association of LINGO-1 with Trk then directs Trks away from the recycling pathway and into the endolysosomal pathway for degradation. The ability of ECD -hL1, a dominant-negative inhibitor of Trk function, to promote accumulation of tyrosine-phosphorylated TrkA after NGF exposure is consistent with this model.

This simple model is unlikely to fully account for the effects of LINGO-1 because the data we have presented indicate that LINGO-1 preferentially associates with, and promotes lysosomal degradation of, Trk proteins undergoing activation within the secretory pathway as well as within the endocytic pathway. Importantly, the secretory and endocytic pathways of Trk trafficking are not entirely distinct, as endocytically internalized Trk receptors traffic back into the trans-Golgi network, via the retromer system (Butowt and von Bartheld, 2001; Klinger et al., 2015). Further study will be required to fully characterize the manner in which Trk activity controls LINGO-dependent Trk degradation.

Reports that neurotrophin/Trk signaling transcriptionally upregulates LINGO-1, coupled with the ability of LINGO-1 to suppress Trk signaling, suggests that LINGO-1 may function in a negative feedback loop to achieve homeostatic control of neurotrophin signaling. Although Trk receptors are typically activated at the cell surface by neurotrophins, neurotrophin-independent activation of Trks within the secretory pathway are functionally important in some physiological contexts. For example, EGF-dependent activation of TrkB receptors in the secretory pathway of embryonic neuronal precursors is essential for normal cortical development (Puehringer et al., 2013). Thus, it may be physiologically important that LINGO-1 can promote degradation of activated Trks in both the endocytic and secretory pathways.

The prior report that LINGO-1 inhibits TrkB signaling suggested that it did so by reducing the level of activated TrkB, without influencing the total level of TrkB protein (Fu et al., 2010). In contrast, our results indicate that LINGO-1 inhibits Trk signaling by lysosome-dependent Trk degradation and down-regulation. However, apparent differences of our results from the results reported by Fu et al. may reflect different dynamics of Trk trafficking in different cell populations. In neurons where activated Trk proteins are mainly fated to enter the lysosomal degradation pathway, interactions with LINGO-1 may speed the degradation of activated Trk proteins without significantly influencing the total pool of Trk protein. On the other hand, in neurons where Trk proteins generally are returned to the plasma membrane by the recycling pathway following activation and endocytosis, enhanced diversion of endocytic pools of Trk into the lysosomal degradation pathway would diminish the total pool of Trk protein.

Our studies in PC12 cells yielded the surprising finding that enhanced TrkA activation resulting from ECD -hL1-mediated inhibition of LINGO-1 function inhibited neuronal differentiation. This effect may reflect diminished LINGO-1 dependent degradation of other LINGO-1 targets such as EGF receptors, which inhibit neuronal differentiation of PC12 cells (Huff et al., 1981). Our finding is consistent with a prior report that LINGO-1 inhibition delays neuronal differentiation (Lööv et al., 2012).

Multiple members of the LIG superfamily of membrane proteins may function in the control of signaling by receptor tyrosine kinases. We have shown that LINX promotes signaling by the NGF receptor TrkA and the GDNF receptor subunit Ret (Mandai et al., 2009) while others have shown that LRIG1 negatively regulates the GDNF receptor subunit Ret (Ledda et al., 2008), as well as the HGF receptor MET (Shattuck et al., 2007), and multiple members of the ErbB family of receptors for EGF and neuregulin ligands (Laederich et al., 2004). Receptor tyrosine kinases negatively regulated by LINGO-1 include TrkA, TrkB and TrkC (present results and Fu et al., 2010) and EGFR/ErbB1 (Inoue et al., 2007). Receptor tyrosine kinases are not the only cellular targets of LIG protein-mediated regulation, however, as a *C. elegans* LRIG ortholog positively regulates signaling by BMP receptors (Gumienny et al 2010) and LINGO-1 enables signaling by NgR/p75^{NTR} complexes (Mi et al., 2004) and promotes lysosomal degradation of the β -amyloid precursor protein (de Laat et al., 2015).

It is interesting to note that two different members of the LIG family of proteins regulate Trk signaling oppositely. We propose that a shared characteristic of LIG family members is that they function by controlling the intracellular trafficking of the receptors they regulate. LINX promotes signaling by TrkA, while LINGO-1 inhibits signaling by all three Trk paralogs. We suggest that LINX and LINGO-1 may act by differently influencing the trafficking of activated Trk receptors. LINGO-1 promotes Trk degradation while LINX promotes Trk accumulation, at least in our artificial cultured cell system. This suggests that LINX may act by extending the lifetime of activated TrkA in the endolysosomal pathway, delaying lysosomal degradation and/or by promoting TrkA return to the plasma membrane by the recycling pathway. In some cellular contexts these effects may also increase the total amount of TrkA protein expressed as we observed experimentally. Thus LINGO-1 and LINX may regulate Trk signaling oppositely by respectively promoting or inhibiting delivery of Trk endosomes to lysosomes. Future studies should address this hypothesis more thoroughly.

LINGO-1 has been genetically linked to essential tremor, one of the most common movement disorders (Jiménez-Jiménez et al., 2012) and increased levels of LINGO-1 protein have been reported in cerebellum in essential tremor patients (Delay et al., 2014; Kuo et al., 2013). Our results suggest the possibility that increased LINGO-1 associated with essential tremor might diminish Trk-mediated neurotrophin signaling, providing a potential mechanism for etiology of this disease.

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Abbreviations

Trk	Tropomyosin-related kinase
LINGO	Leucine-rich repeat Ig-domain containing <u>N</u> ogo-Interacting Protein
EGFR	Epidermal growth factor receptor
NGF	Nerve Growth Factor
BDNF	Brain Derived Neurotrophic Factor
hL1	human LINGO-1
ECD -hL1	LINGO-1 construct missing the extracellular domain (expresses intracellular and transmembrane domains)
hL1- ICD	LINGO-1 construct missing the intracellular domain (expresses extracellular and transmembrane domains)

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Highlights

Function of LINGO-1 as a negative regulator of signaling by neurotrophin receptors TrkA, TrkB and TrkC is proposed.

LINGO-1 is shown to associate preferentially with activated (tyrosine-phosphorylated) Trk receptors.

LINGO-1 promotes lysosomal degradation of the three Trk paralogs.

Figure 1. LINGO-1 and LINX form complexes with Trks

A) HEK293 cells were transiently transfected with non-tagged wild-type versions of TrkA, TrkB, or kinase-dead TrkA and/or human LINGO-1 (hL1) and harvested 24 hr later. Detergent cell extracts (whole cell extract, WCL) or anti-Trk immunoprecipitates (IP) were subjected to SDS polyacrylamide gel electrophoresis, and immunoblots were probed with anti-Trk or anti-LINGO. Whole cell extract blots were stripped and re-probed with anti-pyruvate kinase (PK) to demonstrate consistency of gel loading. A vertical line demarcation between the TrkA-KD/hL1 sample and other samples indicates where an irrelevant lane was deleted from the image. Under conditions of similar levels of TrkA and TrkB expression, LINGO-1 showed greater association with TrkA, than with TrkB, and reduced association with kinase dead TrkA. B) Co-immunoprecipitation experiments were performed as in (A) except that immunoprecipitation with anti-LINGO was followed by immunoblotting with anti-Trk. LINGO-1 associated more extensively with kinase-active TrkB than with kinase-dead TrkB. C) HEK293 cells were transiently transfected with non-tagged native or kinase dead TrkA, TrkB or TrkC and in some cases, with myc-LINX. Detergent cell extracts or anti-myc immunoprecipitates from cell extracts were subjected to SDS polyacrylamide gel electrophoresis. Western blots were probed with anti-Trk to reveal the extent and activity-dependence of Trk/LINX associations. D) Snap frozen mouse cortex crushed via pestle in a liquid nitrogen cooled mortar was clarified at 100,000 x g (4 °C, 1hr), resuspended in lysis buffer and aliquoted. Aliquots were subjected to immunoprecipitation with antibodies against various putative Trk-associating transmembrane proteins and immunoblots were subjected to SDS polyacrylamide gel electrophoresis and examined via immunoblot. Trk

immunoreactive bands were detected in p75^{NTR}, LINX and LINGO immunoprecipitates and not with a non-specific IgG control.

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Figure 2. Co-localization and functional interaction of LINGO and Trk in cultured mouse cortical neurons

A) Neuronal cultures prepared from neonatal mouse cortex were exposed to 20 ng/ml BDNF for 15 minutes, and then fixed and processed for confocal immunofluorescence microscopy. LINGO-1 co-localization with phosphorylated TrkB was observed in discrete puncta. Scale bars equal 5 μ m B) Immunoblot analysis was performed to assess LINGO and Trk expression in mouse cortical neuronal cultures transduced with a lentiviral vector expressing LINGO-1, or control lentiviral vectors. Infection with the LINGO-1 lentivirus elevated LINGO expression and decreased Trk expression, relative to controls infected with GFP lentivirus, or non-infected controls. Immunoblots show representative results of 6 biological replicates. C) Using confocal immunofluorescence microscopy, Trk (green) expression was examined in dissociated mouse cortical neuronal cultures (10DIV) transfected with LINGO-1, kinase dead FGFR2 (FGFR2-KD-V5) or LINX (red) plasmid constructs. Only highly over-expressing transfected cells with non-pyknotic nuclei were examined to determine the effect of over-expression on Trk levels. Cells over-expressing LINGO-1 exhibited lower Trk levels compared to cells expressing kinase dead FGFR2 or LINX. Blue is DAPI DNA stain. Scale bars equal 5 μ m.

Figure 3. Down-regulation of endogenous LINGO-1 expression with LINGO-1 shRNA increases Trk expression

We produced a plasmid expressing shRNA against a sequence that is identical in mouse and human LINGO-1 transcripts. The neuronal cell line N2a natively expresses LINGO-1, TrkA and TrkC. We employed immunoblot analysis to quantify LINGO and Trk immunoreactivity in N2a cells transiently transfected with LINGO-1 shRNA. A) Transient transfection with LINGO-1 shRNA diminished LINGO immunoreactivity. Bar graphs display mean $\pm$ s.e.m. B) Diminished LINGO expression was accompanied by increased Trk expression. N=4 replicate cultures. ** $p < 0.01$ (2-tailed t-test). C) Immunoblot analysis of detergent lysates from HEK293 cells transfected with plasmids encoding TrkA and LINGO-1, LINX, or shRNA against LINGO-1. HEK293 cells express LINGO-1 endogenously. Left panel shows that over-expression of LINGO-1 down-regulates TrkA in proportion to the amount of LINGO-1 plasmid transfected. Right panel shows that transfection with a plasmid encoding LINX increases expression of TrkA while a plasmid encoding LINGO-1 shRNA increases TrkA expression in proportion to the amount of shRNA transfected.

Figure 4. LINGO-1 mediates degradation of Trk via a lysosomal pathway

A) Down-regulation of TrkA by LINGO-1 is mediated by lysosomal degradation. TrkA was expressed with or without transfected LINGO-1 in HEK293 cells and TrkA quantities were assessed by immunoblot analysis. Exposure of cells to lysosome inhibitor bafilomycin A1 (BAF; 100 nM, 12 hours) prevented LINGO-1 mediated TrkA degradation whereas the proteasome inhibitor epoxomicin (Epo; 1 μ M, 12 hours) did not rescue TrkA expression. DMSO, the vehicle for BAF and Epo, was used as a negative control. ** $p < 0.001$; Student's t-test. N=3 replicate cultures. B) Inhibition of endocytosis by exposure of cells to 225 mM extracellular sucrose diminishes association of LINGO-1 with TrkA. HEK293 cells were transfected with LINGO-1 and TrkA and association of LINGO-1 with TrkA was probed by immunoprecipitation of TrkA followed by detection of LINGO-1 on SDS gel immunoblots. Immunoblot for β actin was used to assess consistency of lane loading. Exposure of cells to growth medium supplemented with 225 mM sucrose to block endocytosis inhibited LINGO-1/TrkA association. C) Inhibition of TrkA endocytosis reduces LINGO-1 mediated down-regulation of TrkA. TrkA was expressed alone or with LINGO-1 as in A and B, and effect of inhibition of endocytosis by 225 mM sucrose was assessed by immunoblot analysis. Bar graphs plot ratio of TrkA quantity observed with and without co-expressed TrkA.

Figure 5. Confirmation of LINGO-mediated Trk down-regulation employing a dominant negative inhibitor of LINGO function

A) LINGO-1 extracellular domain is required for effective down-regulation of TrkA. HEK293 cells were transfected with combinations of TrkA and wild-type LINGO-1 or a mutated form of LINGO-1 lacking most of its extracellular domain (ECD Δ -hL1). TrkA expression was examined by immunoblot analysis as a function of the type of LINGO-1 construct expressed. TrkA expression was diminished by LINGO-1 but not by ECD Δ -hL1. B) ECD Δ -hL1 retains ability to associate with TrkA. Trk was immunoprecipitated from detergent lysates of HEK293 cells transfected with Trk and LINGO-1 constructs and the presence of LINGO-1 immunoreactivity was assessed on immunoblots. The 15 kDa ECD Δ -hL1 was co-immunoprecipitated with TrkA almost as effectively as full length LINGO-1. C) ECD Δ -hL1 increases NGF-dependent TrkA activation in a neuronal cell line, PC12. PC12 lines stably transfected with LINGO-1 or ECD Δ -hL1 were treated with 100 ng/ml NGF for 15 minutes and detergent lysates were examined by immunoblot analysis, with antibodies for active tyrosine phosphorylated Trk (pTrk), total Trk, loading control (pyruvate kinase, PK) and LINGO-1. Over-expression of ECD Δ -hL1 diminished LINGO-1 expression and enhanced TrkA activation. Transfection with LINGO-1 increased LINGO-1 expression but did not diminish levels of total or activated TrkA. D) Low density cultures of PC12 cells stably transfected with LINGO-1 constructs were exposed to 100 ng/ml NGF for 5 days. Differential interference microscopy was used to examine cell morphology. E) The percentage of cells bearing neurites were quantified and expressed as mean $\pm$ SEM for 12 replicate cultures for the LINGO-1 and ECD Δ -hL1 lines and 9 replicate cultures for the control empty plasmid line. F) The proportion of viable cells was assessed by trypan blue

exclusion. Over-expression of LINGO-1 decreased cell viability. N=12 replicate cultures for each condition. Graphs represent mean $\pm$ s.e.m. **p<0.01.

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