Development/Plasticity/Repair

p130^{CAS} Is Required for Netrin Signaling and Commissural Axon Guidance

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Netrins are an important family of axon guidance cues. Here, we report that netrin-1 induces tyrosine phosphorylation of p130^{CAS} (Crk-associated substrate). Our biochemical studies indicate that p130^{CAS} is downstream of the Src family kinases and upstream of the small GTPase Rac1 and Cdc42. Inhibition of p130^{CAS} signaling blocks both the neurite outgrowth-promoting activity and the axon attraction activity of netrin-1. p130^{CAS} RNA interference inhibits the attraction of commissural axons in the spinal cord by netrin-1 and causes defects in commissural axon projection in the embryo. These results demonstrate that p130^{CAS} is a key component in the netrin signal transduction pathway and plays an important role in guiding commissural axons *in vivo*.

Key words: **netrin-1; p130^{CAS}; Rac1; Cdc42; signaling; commissural axons**

Introduction

Netrins are a family of secreted proteins that can both promote axon outgrowth and guide growth cone navigation in species ranging from *Caenorhabditis elegans* to mammals (Tessier-Lavigne et al., 1988; Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994; Kolodziej et al., 1996; Mitchell et al., 1996). The receptors for uncoordinated protein 6 (UNC-6)/netrin were uncovered in *C. elegans* as UNC-40 and UNC-5 (Leung-Hagesteijn et al., 1992; Chan et al., 1996). The mammalian homologs of UNC-40 are deleted in colorectal cancer (DCC) and neogenin (Keino-Masu et al., 1996; Fazeli et al., 1997). DCC contains a large extracellular domain, a single transmembrane domain, and a cytoplasmic region with three conserved domains, P1, P2, and P3 (Chan et al., 1996; Keino-Masu et al., 1996). Our studies of signal transduction mechanisms mediating netrin attraction have focused on events downstream of DCC.

We and others have shown recently that the P3 domain of DCC interacts with the Src family tyrosine kinase Fyn and the focal adhesion kinase (FAK) and that these kinases are essential for attractive signaling by netrin (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Previous studies have found that netrin activates the small GTPases Rac1 and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002). However, the small GTPases can be downstream of a number of molecules, whereas FAK can regulate a fair number of molecules, including the phospholipase C (PLC)-γ, phosphoinositol-3 (PI3)-kinase, Akt, mitogen-activated protein (MAP) kinases, paxillin, p130^{CAS}, Crk, and Graf (Parsons et al., 2000; Schaller, 2001; Hanks et al., 2003). Therefore, what functions downstream of FAK in netrin signaling is not clear.

p130^{CAS} [Crk-associated substrate (Cas)] was first identified as a highly phosphorylated protein in cells transformed by v-crk (Matsuda et al., 1990; Birge et al., 1992; Sakai et al., 1994) and v-src (Reynolds et al., 1989; Kanner et al., 1991; Sakai et al., 1994) oncogenes. It binds to multiple cellular proteins and is involved in a variety of biological processes, including cell adhesion, cell migration, growth factor stimulation, cytokine receptor engagement, bacterial infection, cell proliferation, and survival (for review, see O'Neill et al., 2000; Bouton et al., 2001). p130^{CAS} knockout mice are embryonically lethal with cardiovascular defects (Honda et al., 1998). p130^{CAS} has multiple protein–protein interaction motifs, including an Src-homology 3 (SH3) domain, a proline-rich segment, a YXXP domain (substrate-binding domain) containing 15 repeats of a four amino acid sequence (tyrosine-X-X-proline), a serine-rich region, and a C-terminal domain (for review, see O'Neill et al., 2000; Bouton et al., 2001). Tyrosine residues in p130^{CAS} are functionally important for cell migration and biochemically phosphorylated by Src (Manie et al., 1997; Ruest et al., 2001; Brabek et al., 2005).

We report here that p130^{CAS} is a key component in the netrin attraction pathway, functioning downstream of Fyn and FAK and upstream of Rac1, providing a link from the tyrosine kinases to one of the small GTPases.

Received Oct. 24, 2006; revised Dec. 12, 2006; accepted Dec. 14, 2006.

This work was supported by the National Institutes of Health (Y.R., K.G., J.W., S.T.H.). We thank Dr. R. J. Miller and Dr. Peter T. Toth for help with confocal imaging, Dr. E. T. Stoeckli for the anti-axonin-1 antibody, Dr. R. Sakai for anti-p130^{CAS} antibody, and Justin Meyer for assistance.

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DOI:10.1523/JNEUROSCI.4616-06.2007

 0270-6474/07/270957-12$15.00/0

Materials and Methods

We used the following antibodies: anti-Fyn, anti-actin, anti-p130^{Cas}, and anti-phospho-FAK (Santa Cruz Biotechnology, Santa Cruz, CA); CAS phosphotyrosines 165, 249, and 410 antibodies (Cell Signaling Technology, Beverly, MA); anti-FAK (Santa Cruz Biotechnology; Transduction Laboratories, Lexington, KY); anti-Cdc42, anti-Rac1, and anti-DCC (BD Biosciences, Franklin Lakes, NJ); anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY); anti-phospho-Src (Y418) (Cell Signaling Technology, Beverly, MA); anti-hemagglutinin (HA) (Covance, Princeton, NJ); anti-axonin-1 (gift from E. T. Stoeckli, University of Zurich, Zurich, Switzerland); and anti-p130 ^{Cas} for immunohistochemistry (gift from R. Sakai, National Cancer Research Institute, Tokyo, Japan). PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyra-zolo [3,4-d]pyrimidine] and 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3) were obtained from Calbiochem (La Jolla, CA).

Plasmids encoding DCC-ΔP1 (Δ1147–1170), ΔP2 (Δ1335–1356), ΔP3 (Δ1412–1447), 1/2 ΔP3 (Δ1426–1447); Y1420F; and 4Y/F (Y1261F, Y1272F, Y1363F, Y1420F) have been described previously (Li et al., 2004). p130^{Cas} (F15) is a mouse p130^{CAS} mutant, in which all 15 YXXP tyrosines in the substrate domain have been mutated to phenylalanines (Shin et al., 2004).

The targeted sequence of short hairpin-based RNA (shRNA) construct of p130^{CAS} is: GACATCTACCAAGTTCCTC. The human U6 promoter in pAVU6 + 27 was replaced by the mouse U6 promoter from mouse genomic DNA by PCR. The target sequence was inserted between the *Sal*I site and the *Xba*I site. The mouse U6 promoter is known to function in chickens (Bron et al., 2004; Dai et al., 2005). The targeted sequence of small interfering RNA (siRNA) is: GAUGUGCUGUGGAUGGA-CACGAAC AAA.

Netrin-1 protein was purified with anti-myc tag affinity matrix from the conditioned media of human embryonic kidney 293 (HEK293) cells stably secreting netrin-1. The control was made by sham purification from the conditioned media from HEK293 cells that had not be transfected with a cDNA expressing the myc-tagged netrin-1.

Dissociated primary neuron cultures. The dissociated culture procedure was done as described previously (Liu et al., 2004) with some modifications. Briefly, embryos were removed from freshly killed pregnant mice of the appropriate stage. The brain or the spinal cord was dissected in cold HBSS medium (Invitrogen, San Diego, CA), and meninges were removed. The cortices or spinal cords were cut into small pieces with scissors and trypsinized at 37°C for 15 min. After triturating several times, cells were resuspended in DMEM supplemented with heat-inactivated fetal calf serum (Invitrogen) and 20 U/ml of penicillin/streptomycin. Cells were grown on the poly-D-lysine (PDL; 100 μg/ml)-coated dishes at 37°C in a 5% CO_2 incubator overnight.

For immunocytochemistry of p130^{CAS} and DCC in dissociated primary neurons, cells were fixed for 10 min in 4% prewarmed paraformaldehyde solution (127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA, 10 mM PIPES) and permeabilized in 0.5% Triton X-100 for 15 min after washed three times with PBS. Cells were blocked in the blocking buffer (PBS containing 3% BSA and 0.1% Triton X-100) at room temperature for 1 h and incubated with the anti-p130^{CAS} antibody (rabbit, 1:200) and the anti-DCC antibody (mouse, 1:1000) at 37°C for 1 h. After being washed on coverslips in 1 × PBS three times, cells were incubated with the second antibodies (anti-rabbit-Cy3, 1:200; anti-mouse-Cy2, 1:200) at 37°C for 1 h. Images were taken under a confocal microscope.

For analysis of neurite outgrowth, cortical neurons were isolated from E15 mouse embryos and dissociated. Neurons (4×10^6/group) were mixed with Venus yellow fluorescent protein (YFP) (1 μg) plus control vector (4 μg) or p130^{CAS} shRNA construct (4 μg) and immediately placed in Nucleofector (Amaxa Biosystems, Gaithersburg, MD). The

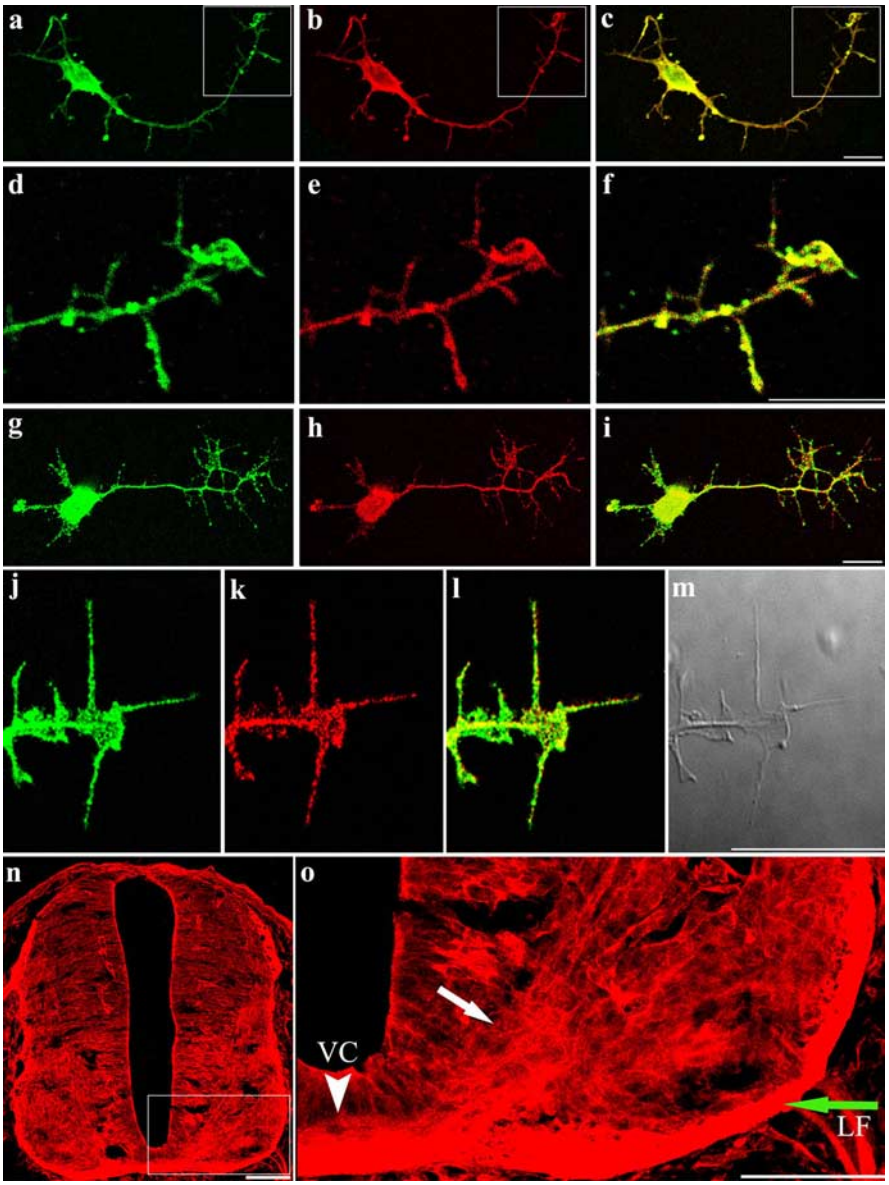

Figure 1. Expression of p130^{CAS} in primary culture neurons. ***a–f***, Expression of DCC (***a***, ***d***) and p130^{CAS} (***b***, ***e***) in primary cortical neurons from E15 mice. ***c*** is the merged image of ***a*** and ***b***. ***f*** is the merged image of ***d*** and ***e***. ***d***, ***e***, and ***f*** are higher-magnification images of the boxed regions in ***a***, ***b***, and ***c***, respectively. Neurons from E15 mice cortex were cultured overnight and immunostained with anti-DCC (G97–449) and anti-p130^{CAS} (SC-860). Scale bar, 10 μm. ***g–m***, Expression of DCC (***g***, ***j***) and p130^{CAS} (***h***, ***k***) in E13 primary spinal cord neurons. ***i*** is the superimposed images of ***g*** and ***h***. ***l*** is the superimposed image of ***j*** and ***k***. ***m*** is the phase image of ***j***, ***k***, and ***l***. ***j–m*** are higher-magnification images showing DCC and p130^{CAS} expression in the growth cone of embryonic spinal cord neurons. Scale bar, 10 μm. ***n–o***, Expression of p130^{CAS} in E13 mice spinal cord. ***o*** is the higher-magnification image of the boxed region of image ***n***. p130^{CAS} is strongly expressed in the commissural axons as they project toward the floor plate (white arrow), the ventral commissural region (VC; arrowhead), and lateral fasciculus (LF; green arrow). Scale bar, 100 μm.

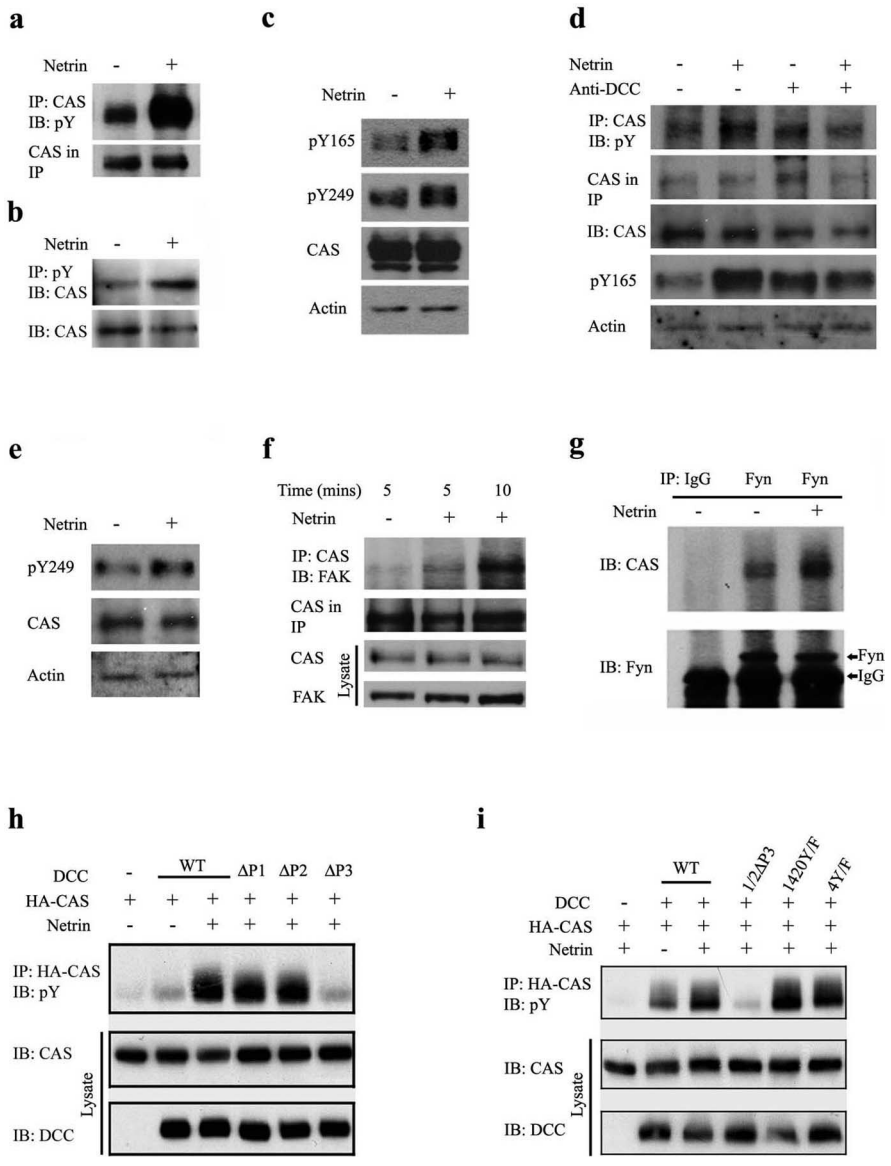

Figure 2. Increase of p130^{CAS} tyrosine phosphorylation by netrin-1. ***a–c***, Induction of p130^{CAS} tyrosine phosphorylation in dissociated cortical neurons by netrin-1. The anti-p130^{CAS} antibody (***a***) or the anti-phosphotyrosine (pY) antibody (***b***) was used to immunoprecipitate proteins from cortical neurons treated with the sham-purified control (left lane) or netrin-1 (right lane), and the blot was analyzed with the anti-phosphotyrosine antibody (***a***) or the anti-p130^{CAS} antibody (***b***). Tyrosine phosphorylation of p130^{CAS} tyrosine residues 165 and 249 was increased in dissociated cortical neurons by netrin-1 (***c***). ***d***, ***e***. Netrin-1 increased tyrosine phosphorylation of p130^{CAS} at tyrosine residues 165 and 249 in dissociated E13 dorsal spinal cord neurons (***d***, ***e***), which was blocked by the anti-DCC antibody (***d***). ***f***, Interaction of endogenous p130^{CAS} and FAK in E15 cortical neurons. Cortical neurons were treated with the 250 ng/ml purified netrin-1 for 5 or 10 min. Extracts from 2.5×10^7 cortical cells were immunoprecipitated with an anti-p130^{CAS} antibody and analyzed with an anti-FAK antibody. ***g***, Interaction of endogenous p130^{CAS} and Fyn in cortical neurons. Left lane, Immunoprecipitation (IP) with IgG; middle lane, IP with an anti-Fyn antibody; right lane, IP with an anti-Fyn antibody. ***h***, P3 domain in DCC is required for induction of p130^{CAS} tyrosine phosphorylation by netrin-1. HEK 293 cells were transfected with a DCC construct and a construct encoding p130^{CAS} tagged by HA (HA-CAS) and treated with netrin-1 or the sham-purified control for 20 min. Tyrosine phosphorylation of p130^{CAS} was determined by IP with the anti-HA antibody and analyzed by probing the blots (IB) with the anti-phosphotyrosine antibody. DCC-ΔP1, ΔP2, ΔP3, and Δ1/2P3 correspond to deletion of residues of DCC intracellular domains 1147–1171, 1335–1356, 1412–1447, and 1426–1447, respectively. ***i***, The C-terminal half of P3 domain of DCC is essential for netrin induction of tyrosine phosphorylation of p130^{CAS}. 1420Y/F and 4Y/F indicate mutations in the tyrosine(s) of the intracellular domain of DCC, Y1420, and Y1261, Y1272, Y1363 and Y1420, respectively.

cells were moved into warm media (containing 10% FCS) after electroporation using O-005 program and plated on laminin (5 μg/ml) and poly-L-lysine (50 μg/ml)-coated coverslips and cultured in DMEM, 10% FCS, and penicillin/streptomycin at 37°C with 5% CO_2 for 2 h. After settling on the coverslips, cells were cultured in DMEM with B27 (Invitrogen) and penicillin/streptomycin at 37°C with 5% CO_2 for 20 or 40 h within purified netrin-1 (250 ng/ml) or with the sham-purified control. Cells were then fixed with 4% PFA for 20 min and stained with phalloidin (Invitrogen, Eugene, OR). Nuclei were visualized with Hoechst dye.

Immunoprecipitation and Western blot analysis. Cortical cells were lysed with a modified radioimmunoprecipitation assay buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 1× protease inhibitor mixture (Roche Molecular Biochemicals)] as described previously (Liu et al., 2004). Lysates were immunoprecipitated with specific antibodies and protein A/G agarose beads for 3 h or overnight at 4°C. For the immunoblotting, the washed immunoprecipitates were boiled in 1× SDS sample buffer for 5 min.

For immunoprecipitation of HEK cell lines, HEK293 cells were transfected with the Lipofectamine (Invitrogen) method. Cells were lysed 48 h after transfection in mild lysis buffer (MLB) (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1% NP-40, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin) and followed by incubation with specific antibodies for 2 h before protein A/G-agarose beads were added at 4°C.

For Western blot analysis, protein extracts were separated with 7.5% SDS-PAGE. Western blots were visualized with the enhanced chemiluminescence kit (GE Healthcare, Arlington Heights, IL).

Rac1 and Cdc42 activity assays. Two × 10^5 HEK293 cells stably expressing DCC were transfected with the wild-type p130^{CAS} or p130^{CAS} (F15) constructs (5 μg/group) by the calcium phosphate method. Forty-eight hours after transfection, cells were stimulated with purified netrin-1 (500 ng/ml) or the sham-purified control for 5 min. After being washed with 1× PBS once, cells were lysed with MLB lysis buffer (25 mM HEPES, pH 7.5, 1% NP-40, 150 mM NaCl, 10% glycerol, 1 mM EDTA, and 10 mM MgCl2 containing protease inhibitor mixture) and centrifuged at 15,000 × *g* at 4°C for 15 min. The levels of active GTP-bound GTPase (Rac1, Cdc42) were measured as follows: 30 μg of the GST-Cdc42/Rac-interactive binding domain of p21-activated kinases (PAK) (CRIB) of PAK was coupled to glutathione-Sepharose beads (GE Healthcare) at 4°C for 30 min. These beads were used to pull down GTP-bound forms of Rac1 and Cdc42. The resulting supernatants after centrifugation were incubated with Sepharose bead-associated GST-PAK for 45 min at 4°C. The beads were washed three times with the lysis buffer, and the bound small GTPase proteins were separated by 15% SDS-PAGE. Western blot analysis was performed with anti-Rac1 and anti-Cdc42 antibodies (Wong et al., 2001).

Commissural axon turning assay. White leghorn chicken embryos were collected and staged according to Hamburger and Hamilton (1951). The electroporation procedure was essentially done as described previously (Liu et al., 2004). DNA solution was injected into the central canal of the neural tube of chick embryos *in ovo* at stages 12–15. The electroporation program was 25 V, 5 ms, 5 pulses (ECM830; BTX Instrument Division of

Genetronics, San Diego, CA). Embryos were isolated at stages 20–21 and examined for YFP fluorescence under the microscope. The half of the spinal cord showing fluorescence was isolated as an open-book preparation and cocultured with an aggregate of control or netrin-1 secreting HEK cells for 24 h. Axon turning was counted when the angle of turning toward the HEK aggregate was >5°. The percentage of turning axons was calculated from the numbers of fluorescent axons turning toward the HEK cell aggregate divided by the total numbers of fluorescent axons within 300 μm of the HEK cell aggregates. Images were collected by a confocal microscope.

Analysis of commissural axon projection in vivo. Collection of chick embryos and electroporation procedures were described above. Chick embryos were killed between stages 22 and 23 after electroporation. This stage was chosen for analysis, because it is the time when the commissural axons in the lumbosacral region cross the midline (Stoeckli et al., 1997; Bourikas et al., 2005). The lumbosacral region of the spinal cord was isolated and the fluorescence examined under a microscope. Samples expressing green fluorescent protein (GFP) were chosen for opening at the roof plate (open-book preparation). Whole-mount immunostaining of the spinal cord was performed after fixation. Briefly, samples were permeabilized in 0.5% Triton for 15 min and blocked for 30 min in the blocking buffer (PBS containing 5% goat serum and 0.1% Triton X-100) at room temperature. Tissues were incubated with the first antibody (rabbit, anti-axonin-1, 1:1000) in the blocking buffer at 37°C for 2 h. After being rinsed with 1× PBS, tissues were incubated with the second antibody (anti-rabbit-Cy3 antibody, 1:200) at 37°C for 2 h. After being washed with PBS three times, the spinal cord in the open-book preparation was mounted in Gel/Mount (Biomeda, Foster City, CA). Images were taken under the confocal microscope. The percentage of axons reaching the floor plate was quantified from the numbers of fluorescently labeled commissural axons arriving at or crossing over the floor plate divided by the total numbers of fluorescent axons within 100 μm from the floor plate.

The lumbosacral region of the spinal cord expressing GFP at stage 23 was collected. Transverse sections of 200 μm were cut and mounted in Gel/Mount. Images were also taken under the confocal microscope.

In vivo electroporation of pregnant mice was performed as described previously (Saba et al., 2003) with minor modifications. The embryonic day 10.5 (E10.5) pregnant mice were anesthetized by intraperitoneal injection of 10% Nembutal solution. The abdominal skin and wall were cut and the uterus was carefully taken out. The DNA solution was injected into the central canal of the spinal cord. The electroporation program was 22 V, 5 ms, 5 pulses (BTX, ECM830). The uteri were carefully put back into the abdominal cavity, and the abdominal wall and skin were sewn up. The mice were killed 2 d later at stage E12.5 and fixed in 4% paraformaldehyde solution. Sections of embryos spinal cord (200 μm) were transversely cut and mounted in Gel/Mount. The fluorescent images were also taken under the confocal microscope.

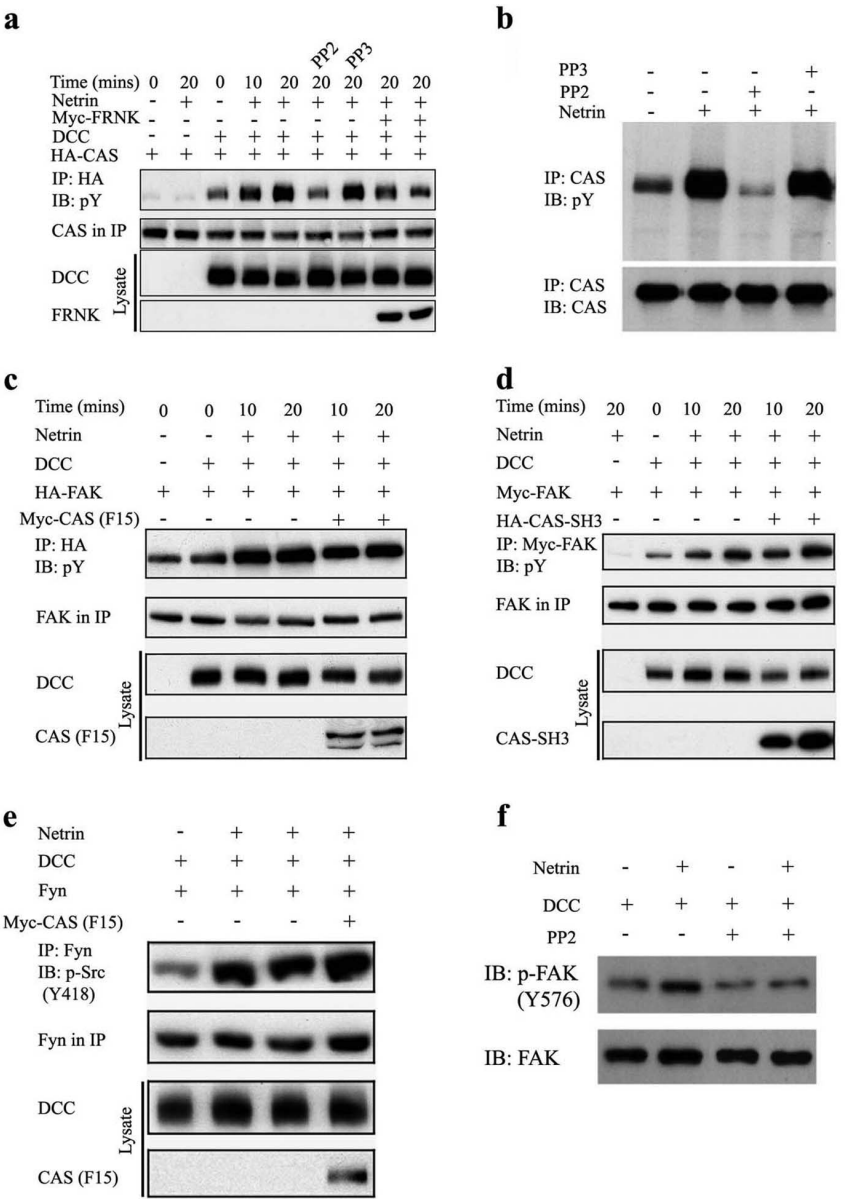

Figure 3. Induction of p130^{CAS} tyrosine phosphorylation by netrin-1 required FAK and Fyn. ***a***, The C-terminal domain of FAK (FRNK) and Src family kinase-specific inhibitor PP2 inhibited netrin-induced p130^{CAS} tyrosine phosphorylation in HEK cells. HEK293 cells were transfected with DCC, HA-CAS, and Myc-FRNK. Tyrosine phosphorylation of p130^{CAS} was determined by immunoprecipitation with an anti-HA antibody and immunoblotting with the anti-phosphotyrosine antibody. ***b***, PP2, but not PP3, blocked netrin-stimulated tyrosine phosphorylation of endogenous p130^{CAS} in primary neurons. Cortical cells (6×10^6 plate/well) were stimulated with netrin-1. The anti-p130^{CAS} antibody was used to immunoprecipitate proteins, and the blot was analyzed with the anti-phosphotyrosine antibody (top) or anti-p130^{CAS} antibody (bottom). ***c***, A p130^{CAS} mutant, myc-p130^{CAS} (F15), could not block netrin-induced FAK phosphorylation. HEK cells were transfected with DCC, HA-FAK, and myc-p130^{CAS} (F15). The anti-HA antibody was used to immunoprecipitate the extracts, and anti-phosphotyrosine was used to probe the immunoblots. The bottom panel showed Western blot analysis with the anti-FAK antibody to control for amount of input proteins. ***d***, The p130^{CAS} mutant, CAS-SH3 tagged by HA, also could not inhibit netrin-stimulated FAK phosphorylation. HEK cells were transfected with DCC, myc-FAK, and HA-CAS-SH3. FAK phosphorylation was detected by immunoprecipitation with anti-myc antibody immunoprecipitate and immunoblot with the anti-phosphotyrosine antibody. ***e***, p130^{CAS} (F15) could not block Fyn tyrosine phosphorylation. HEK293 cells were transfected with DCC, Fyn, and myc-p130^{CAS} (F15). Fyn tyrosine phosphorylation was analyzed by immunoprecipitation with an anti-Fyn antibody and immunoblotting with an anti-phospho-Src antibody. ***f***, Netrin-1 increased FAK phosphorylation at tyrosine residue 576 in the p130$^{CAS-/-}$ mouse embryonic fibroblasts, which were blocked by PP2 .

Results

Netrin-1 stimulates tyrosine phosphorylation of p130^{CAS} and its association with FAK and Fyn

We used antibodies for p130^{CAS} and DCC to examine whether p130^{CAS} is expressed in axons and growth cones containing DCC.

We found coexpression of p130^{CAS} with DCC in the soma and cytoplasmic membrane as well as the axons, axonal branches, and the growth cones of primary neurons from the neocortex of E15 mice (Fig. 1*a–f*). Confocal analysis indicates partial colocalization of p130^{CAS} with DCC (Fig. 1*c*,*f*). Similar observations were made when anti-p130^{CAS} and anti-DCC antibodies were used to examine their localization in primary neurons from the dorsal spinal cord of E13 mouse embryos (Fig. 1*g–m*). Immunohistochemistry staining showed that p130^{CAS} was strongly expressed in commissural axons of E13 embryonic spinal cord (Fig. 1*n–o*).

We observed that netrin-1 increased the phosphorylation of proteins of ~130 kDa, one of which was FAK (Liu et al., 2004). Antibody depletion experiments showed that FAK was not the only protein in that band (Liu et al., 2004). To determine whether p130^{CAS} is among them, we examined the p130^{CAS} tyrosine phosphorylation after netrin-1 stimulation of dissociated E15 primary cortical neurons. We either immunoprecipitated extracts with the anti-p130^{CAS} antibody and probed the Western blots with the anti-phosphotyrosine antibody or reversely immunoprecipitated with the anti-phosphotyrosine antibody and probed the Western blots with the anti-p130^{CAS} antibody. Results from both procedures showed that tyrosine phosphorylation of p130^{CAS} was induced by netrin-1 within 5 min (Fig. 2*a*,*b*). To directly detect the tyrosine phosphorylation of p130^{CAS}, we used antibodies recognizing phosphotyrosines at residues 165 and 249 of p130^{CAS}. Netrin-1 increased tyrosine phosphorylation at both sites of p130^{CAS} in E15 primary cortical neurons (Fig. 2*c*) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and in dissociated E13 dorsal spinal cord neurons (Fig. 2*d*,*e*). Induction of tyrosine phosphorylation in p130^{CAS} was inhibited by a function-blocking antibody against DCC (Fig. 2*d*).

p130^{CAS} is known to form a protein–protein interaction complex with the Src family kinases and FAK. Because netrin-1 activates Fyn and FAK (Li et al., 2004; Liu et al., 2004; Ren et al., 2004), we performed immunoprecipitation experiments to test whether netrin-1 regulated the interaction of p130^{CAS} with Fyn and FAK. Netrin-1 increased the interaction of p130^{CAS} with FAK (Fig. 2*f*) and Fyn (Fig. 2*g*) in E15 primary cortical neurons.

There are three conserved intracellular domains (P1, P2, and P3 in DCC), among which P3 is required for netrin-induced Fyn and FAK phosphorylation (Li et al., 2004; Ren et al., 2004). We investigated the domains of DCC involved in p130^{CAS} tyrosine phosphorylation after netrin-1 stimulation. Deletion of P1 (residues 1147–1171) or P2 (residues 1335–1356) did not inhibit the induction of p130^{CAS} phosphorylation by netrin-1 (Fig. 2*h*). However, deletion of P3 domain (residues 1412–1447) (Fig. 2*h*) or half of the P3 domain (residues 1426–1447) blocked the effect of netrin-1 on p130^{CAS} phosphorylation (Fig. 2*i*). Furthermore, mutation of one tyrosine phosphorylation site (1420Y/F) or all of sites (1261Y/F, 1272 Y/F, 1363 Y/F, Y 1420 Y/F) of DCC intracellular domains had no effect on the induction of p130^{CAS} tyrosine phosphorylation by netrin-1 (Fig. 2*i*). The domain in DCC required for p130^{CAS} phosphorylation is similar to that for Src and FAK phosphorylation (Li et al., 2004).

p130^{CAS} is downstream of FAK and Src but upstream of Rac1 and Cdc42

FAK and Src family kinases can be activated by netrin-1 (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). To determine their relationship with p130^{CAS}, we tested the effect of FAK and Src inhibition on p130^{CAS} phosphorylation, as well as the effect of inhibition on FAK and Fyn phosphorylation.

To examine the role of FAK in netrin-induced p130^{CAS} tyrosine phosphorylation, HEK293 cells were cotransfected with DCC, p130^{CAS} tagged with the hemagglutinin epitope (HA-CAS), and a myc epitope tagged form of FRNK (FAK-related nonkinase), a dominant-negative FAK mutant containing the C-terminal noncatalytic region of FAK that can bind to FAK-interacting proteins but cannot function as an active kinase. p130^{CAS} phosphorylation was assayed by immunoprecipitation with the anti-HA antibody and probed with the anti-phosphotyrosine antibody. Netrin-1 induced tyrosine phosphorylation of p130^{CAS} in a DCC-dependent manner (Fig. 3*a*). The induction of p130^{CAS} tyrosine phosphorylation by netrin-1 was inhibited by expression of FRNK (Fig. 3*a*), indicating that FAK is required for p130^{CAS} phosphorylation. Netrin-induced p130^{CAS} tyrosine phosphorylation was also inhibited by PP2, a pharmacological inhibitor of the Src family kinases, but not PP3, an inactive control for PP2 (Fig. 3*a*). Netrin induction of tyrosine phosphorylation of endogenous p130^{CAS} in E15 neurons was also inhibited by PP2 (Fig. 3*b*). These findings indicate that netrin-induced tyrosine phosphorylation of p130^{CAS} requires FAK and Src family kinases.

To determine whether netrin-induced FAK phosphorylation depends on p130^{CAS}, we used two p130^{CAS} mutants: p130^{CAS} (F15), in which all of 15 tyrosine residues in the substrate binding domain were mutated to phenylalanine; and HA-CAS-SH3, which contains only the SH3 domain of p130^{CAS}. p130^{CAS} (F15)

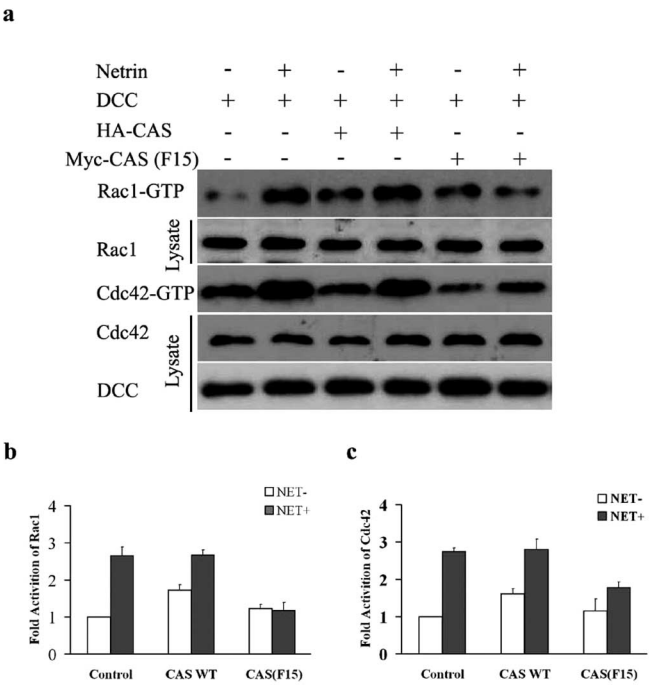

Figure 4. Involvement of p130^{CAS} in netrin-induced activation of Rac1 and Cdc42. HEK293 cells were transfected with DCC and either the full-length p130^{CAS} (HA-CAS) or the dominant-negative p130^{CAS} mutant myc-p130^{CAS} (F15). Cell lysates were incubated with GST-PAK1-CRIB fusion protein and GTP-bounded Rac1, or GTP-bounded Cdc42 was detected by Western blot analysis with the anti-Rac1 and anti-Cdc42 antibodies, respectively. ***a*** shows an example when Rac1 and Cdc42 activation by netrin-1 was completely inhibited by p130^{CAS} (F15). ***b*** and ***c*** show statistics of Rac1 and Cdc42 activation from three independent experiments. In ***b***, netrin could not induce Rac1 activation in the presence of p130^{CAS} (F15): the difference between the netrin (+) and netrin (−) groups in the presence of p130^{CAS} (F15) is not statistically significant ($p > 0.1$). In ***c***, p130^{CAS} (F15) blocked netrin-induced Cdc42 activation; the difference between the netrin (+) and netrin (−) groups in the presence of p130^{CAS} (F15) is not statistically significant ($p = 0.06$). Error bars are SEM from three independent experiments.

acts as a dominant-negative mutant, because the tyrosine residues in the substrate binding domain are required for downstream signaling (Manie et al., 1997; Shin et al., 2004; Brabek et al., 2005). HA-CAS-SH3 acts as a dominant-negative, because it can bind to proteins interacting with SH3 but lacks domains that interact with downstream signals (Shin et al., 2004; Brabek et al., 2005). These mutants were individually transfected into HEK293 cells expressing DCC and FAK. Neither could block netrin-induced tyrosine phosphorylation of FAK (Fig. 3*c*,*d*). Similarly, Fyn phosphorylation after netrin-1 stimulation could not be inhibited by p130^{CAS} (F15) (Fig. 3*e*). To rule out the possibility that the failure of the CAS dominant-negative mutants to block netrin-1 induced FAK phosphorylation could be attributable to the presence of endogenous p130^{CAS} in HEK cells, we used mouse embryonic fibroblasts lacking p130^{CAS} (CAS$^{-/-}$ MEF). After DCC was transfected into CAS$^{-/-}$ MEFs, netrin-1 increased FAK phosphorylation in these cells (Fig. 3*f*), indicating that the absence of endogenous p130^{CAS} could not block FAK phosphorylation. Netrin induction of FAK phosphorylation in CAS$^{-/-}$ MEFs could be reduced by PP2, the Src family kinase inhibitor (Fig. 3*f*). Together, these results indicate that p130^{CAS} is downstream of FAK and the Src family kinases in netrin-1 signaling.

Netrin is known to activate the small GTPase of Rho family: Rac1 and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). To study whether p130^{CAS} is involved in Rac1 and Cdc42 activation, we introduced the p130^{CAS} (F15) mutant into HEK293 cells expressing DCC and HA-CAS. As described previously (Wong et al., 2001), the GTP-bound active forms of Rac1 and Cdc42 were pull-down by GST fusion protein of the CRIB domain of PAK1 and detected by specific antibodies to Rac1 and Cdc42 (Miyamoto et al., 2006). As shown in other studies (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005), netrin-1 activated both Rac1 and Cdc42 (Fig. 4). p130^{CAS} (F15) blocked netrin activation of Rac1 and Cdc42 (Fig. 4). These results indicate that p130^{CAS} is required in the signaling pathway from netrin-DCC to Rac1 and Cdc42.

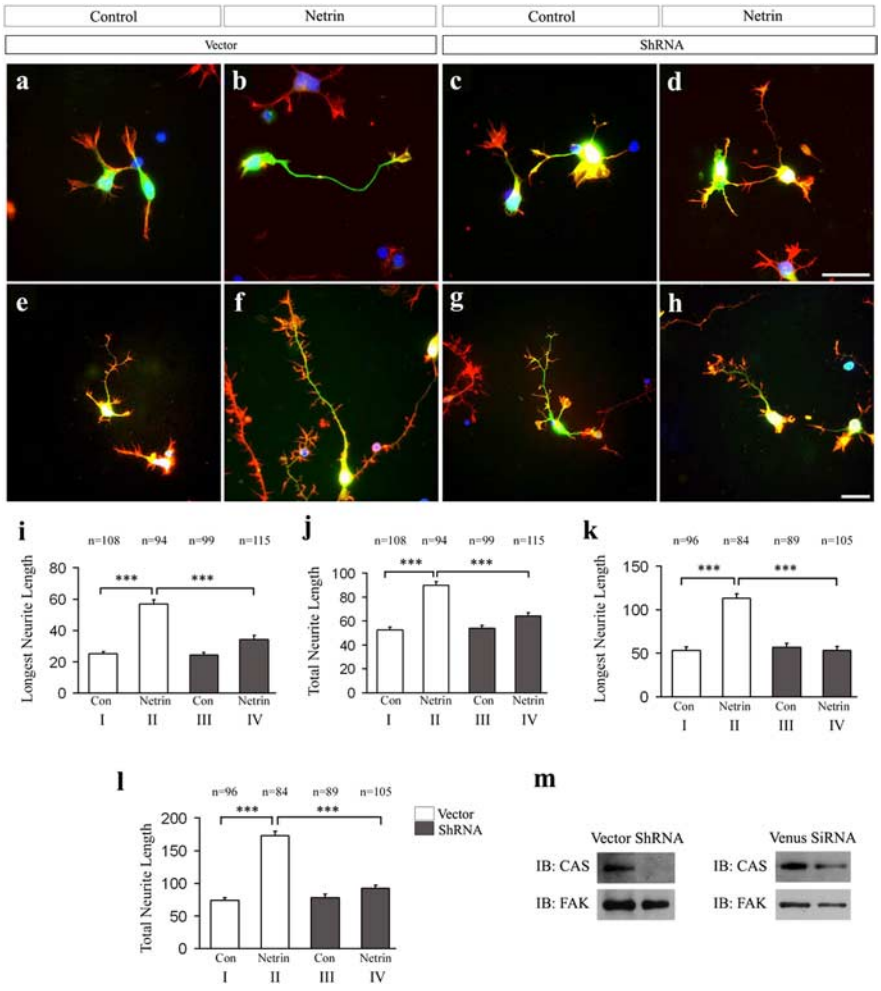

Figure 5. The induction of neurite outgrowth of dissociated cortical neurons by netrin-1 was blocked by p130^{CAS} RNAi. Cortical neurons from E15 mouse embryos were cotransfected with Venus YFP and the p130^{CAS} shRNA (***c***, ***d***, ***g***, ***h***) or the control shRNA vector (***a***, ***b***, ***e***, ***f***) and plated on coverslips coated with PDL and laminin. Neurons were stained with rhodamine-phalloidin (red) and Hoechst. Only the neurites of YFP-positive neurons not in contact with other cells were measured and used in the statistical analyses. Data are mean ± SEM from three separate experiments. Scale bar, 20 μm. ***a–d***, Neurite outgrowth from YFP-positive E15 cortical neurons transfected with control vectors (***a***, ***b***) or p130^{CAS} shRNA (***c***, ***d***) in the presence of purified netrin-1 (***b***, ***d***) or in the sham-purified control (***a***, ***c***) after culturing for 20 h. Transfected neurons were labeled by YFP, and filamentous actin was visualized by staining with rhodamine-coupled phalloidin (red). p130^{CAS} shRNA inhibited the promotion of neurite outgrowth by netrin (***b***, ***d***, ***i***, ***j***). ***e–h***, Similar to ***a–d*** except that neurons were cultured for 40 h. Scale bar, 20 μm. ***i***, ***j***, Quantification of netrin-1-induced neurite outgrowth of cortical neurons cultured for 20 h. Both the length of the longest neurite from each neuron (***i***) and the total length of all neurites from each neuron (***j***) were inhibited by p130^{CAS} shRNA. The *p* values are <0.0001 between RNAi-treated neurons and control neurons. The length on the *y*-axis is in micrometers. ***k***, Quantification of the length of the longest neurite from each cortical neuron cultured for 40 h. Group I, 53.26 ± 4.15 μm; Group II, 113.08 ± 5.44 μm; Group III, 56.67 ± 4.46 μm; Group IV, 53.31 ± 4.53 μm. The difference between Groups I and II is very significant (*p* value <0.0001); the difference between Groups II and IV is very significant ($p < 0.0001$); the difference between Groups III and IV is not significant ($p = 0.577$). These results indicate that netrin promoted neurite outgrowth (compare Groups I and II) and that p130^{CAS} shRNA significantly inhibited the effect of netrin (compare Groups II and IV). ***l***, Quantification of the total length of all neurites from each cortical neuron cultured for 40 h. Group I, 73.55 ± 4.45 μm; Group II, 173.00 ± 6.58 μm; Group III, 78.15 ± 4.66 μm; Group IV, 91.6 ± 5.53 μm. ***m***, p130^{CAS} shRNA significantly reduced p130^{CAS} protein level in E15 primary cortical neurons (left) 3 d after shRNA transfection (left). p130^{CAS} protein level is reduced in dissociated cortical neurons 3 d after siRNA transfection (right).

p130^{CAS} is essential for netrin-1-induced neurite outgrowth of cortical neurons

Netrin can promote neurite outgrowth and axon turning. To study the function of p130^{CAS}, we designed short hairpin based RNA interference (RNAi) constructs and small interfering RNA targeting a sequence common to mouse and chicken p130^{CAS}. One shRNA construct and one siRNA significantly reduced the level of endogenous p130^{CAS} protein in E15 cortical neurons (Fig. 5*m*), and these RNAi reagents were used in subsequent experiments.

Neurite outgrowth promotion activity of netrin-1 was assayed in primary cortical neurons from E15 mice. They were transfected with a construct expressing Venus YFP together with the control shRNA vector or the p130^{CAS} shRNA. These neurons were stimulated with netrin-1 and cultured for 20 h. In neurons

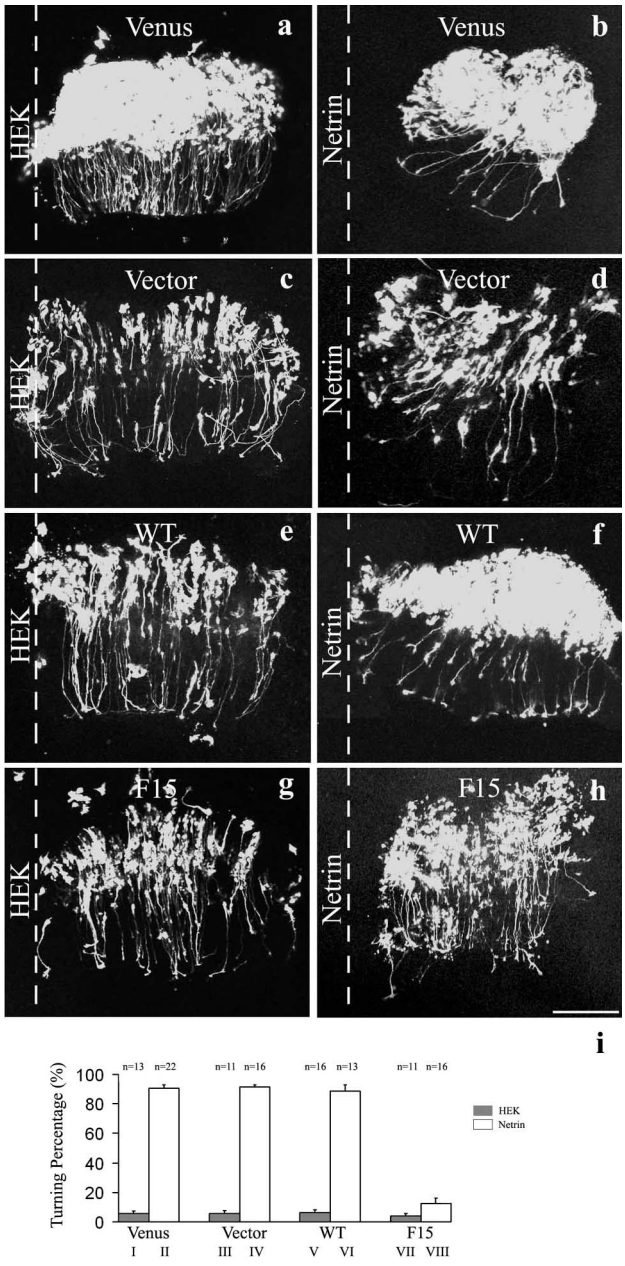

Figure 6. Inhibition of netrin-induced turning of spinal cord axons by the p130^{CAS} (F15) mutant. Electroporation of Venus GFP into the neural tube of chick embryos allowed visualization of axons. Netrin attraction was indicated by the turning of axons toward aggregates of netrin-1 secreting HEK cells (Liu et al., 2004). In ***a***, ***c***, ***e***, and ***g***, neural tube explants were cocultured with control HEK cells, and axons do not turn toward these cells. In ***b***, ***d***, ***f***, and ***h***, neural tube explants were cocultured with aggregates of HEK cells secreting netrin-1. In ***a*** and ***b***, Venus GFP alone was electroporated into the neural tube. In ***c*** and ***d***, Venus GFP was electroporated into the neural tube together with the control vector. In ***e*** and ***f***, Venus GFP was electroporated into the neural tube together with the wild-type p130^{CAS}. In ***g*** and ***h***, Venus GFP was coelectroporated with the p130^{CAS} (F15) mutant. Only the p130^{CAS} (F15) mutant inhibited axon turning toward the netrin source. Scale bar, 100 μm. ***i***, Quantification of axon turning. Turning percentage was calculated from the number of axons extended toward the netrin source divided by the total number of axons within 300 μm from the edge of the HEK cell aggregates. Only axons that had an angle of turning $>5°$ were counted. Data are mean $\pm$ SEM. Group I (example shown in ***a***), 5.7 $\pm$ 1.7%; Group II (exemplified in ***b***), 90.6 $\pm$ 2.1%; Group III (exemplified in ***c***), 5.8 $\pm$ 2.0; Group IV (as in ***d***), 91.3 $\pm$ 1.7%; Group V (as in ***e***), 6.2%$\pm$2.0%; Group VI (as in ***f***), 88.7 $\pm$ 4.1%; Group VII (as in ***g***), 4.1 $\pm$ 1.6%; Group VIII (as in ***h***), 12.4 $\pm$ 3.6%. The numbers on the top (*n*) indicate the numbers of explants tested. Net, Netrin-1-secreting HEK cell aggregates. *p* values are as follows (Student's *t* test): $<$0.0001 between Groups I and II; $<$0.0001 between Groups III and IV; $<$0.0001 between Groups V and VI; $<$0.0001 between Groups VIII and II, IV, or VI. Error bars are SEM.

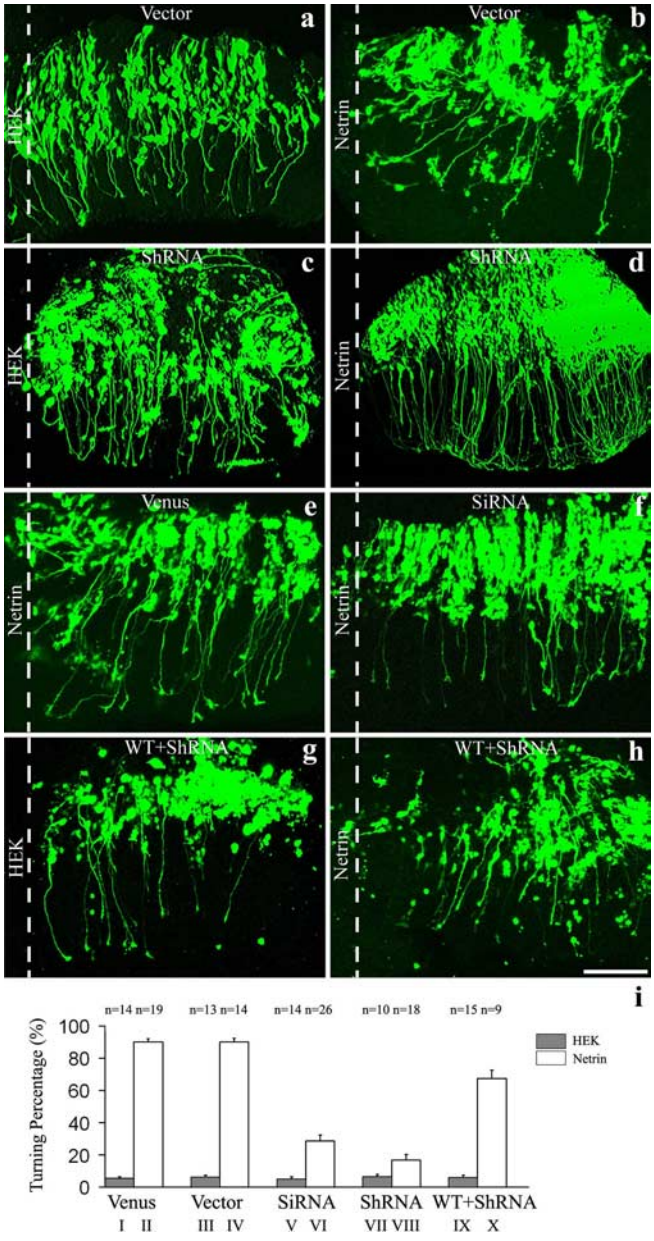

Figure 7. Requirement of p130^{CAS} for netrin-induced attractive turning of spinal cord axons. Venus YFP was electroporated into the chick neural tube with the control vector or p130^{CAS} shRNA or wild-type p130^{CAS} plus shRNA or siRNA. ***a***, ***c***, ***g***, Aggregates of control HEK cells could not attract the commissural axons. ***b***, ***e***, An aggregate of netrin-secreting HEK cells attracted the commissural axons transfected with Venus YFP only (***e***) or with Venus YFP and the control vector (***b***). ***d***, p130^{CAS} shRNA blocked the attraction of the commissural axons by netrin-1. ***f***, The attractive effect on the commissural axons by netrin-1 was also blocked by p130^{CAS} siRNA. ***h***, Cotransfected p130^{CAS} shRNA with wild-type p130^{CAS} rescued the attraction of netrin-1. Scale bar, 100 μm. ***i***. Quantification of axon turning. The turning percentages were as follows: 5.4 $\pm$ 1.0% (Group I, not shown); 90.1 $\pm$ 2.0% (Group II, as exemplified in ***e***); 6.1 $\pm$ 1.3% (Group III, example shown in ***a***); 90.3 $\pm$ 2.1% (Group IV, as exemplified in ***b***); 5.0 $\pm$ 1.4% (Group V, not shown); 28.5 $\pm$ 3.9% (Group VI, ***f***); 6.3 $\pm$ 1.6 (Group VII, as exemplified in ***c***); 16.8 $\pm$ 3.4% (Group VIII, as exemplified in ***d***); 6.0 $\pm$ 1.5% (Group IX, ***g***); 67.3 $\pm$ 5.2% (Group X, ***h***). WT, Wild type of p130^{CAS}. *p* values are as follows (Student's *t* test): $<$0.0001 between Groups I and II, Group III, and IV; $<$0.0001 between Groups II and VI, Group IV and VIII; $<$0.0001 between Group VIII and X. Error bars are SEM.

transfected with the control vector (Fig. 5*a*,*b*), neurite outgrowth was stimulated by netrin-1: without netrin treatment, the length of the longest neurite of each neuron is 25.15 $\pm$ 1.26 μm, and the total length of all neurites from each neuron is 52.62 $\pm$ 2.27 μm;

with netrin treatment, the length of the longest neurite of each neuron is 56.91 ± 2.72 μm, and the total length of all neurites from each neuron is 90.04 ± 3.03 μm (Fig. 5*i*,*j*).

p130^{CAS} shRNA inhibited netrin-induced neurite outgrowth: the length of the longest neurite per neuron was increased from 24.51 ± 1.52 μm without netrin to 34.13 ± 2.58 μm with netrin, and the total length of all neurites per neuron was increased from 54.06 ± 2.18 μm without netrin to 64.00 ± 2.98 μm with netrin (Fig. 5*c*,*d*,*i*,*j*). Comparing netrin-induced outgrowth from vector-transfected neurons (Fig. 5*i*,*j*, group II) to netrin-induced outgrowth from p130^{CAS} shRNA-transfected neurons (Fig. 5*i*,*j*, group IV), the difference is statistically significant [$p < 0.0001$ between groups II and IV (Fig. 5*i*,*j*)]. Similar effects of p130^{CAS} shRNA on netrin-induced neurite outgrowth were observed after 40 h of RNAi transfection (Fig. 5*e–h*,*k*,*l*). The neurite outgrowth promotion activity of netrin-1 was not blocked by the control shRNA (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Together, these results indicate that p130^{CAS} is required for netrin-induced neurite outgrowth from primary neurons.

p130^{CAS} is required for axon attraction by netrin-1

The commissural axons in the neural tube are a model for studying netrin attraction (Tessier-Lavigne et al., 1988; Kennedy et al., 1994; Serafini et al., 1994). To determine whether p130^{Cas} is required for axon turning toward netrin-1, we used the open-book assay with commissural axons from the neural tube of chick embryos. As described previously (Liu et al., 2004), the Venus YFP construct was electroporated into the chick neural tube at stages 12–15. An explant of the neural tube was isolated and the dorsal midline was cut open and laid out as an "open book" (for detailed diagram, see Liu et al., 2004). An aggregate of HEK cells was placed to one side of the open book. Projecting axons were visualized by Venus YFP expression. More than 90% of axons projecting from the dorsal spinal cord of chick embryos electroporated at these stages were commissural axons as confirmed by immunostaining with the anti-axonin-1 antibody (Figs. 8, 9) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

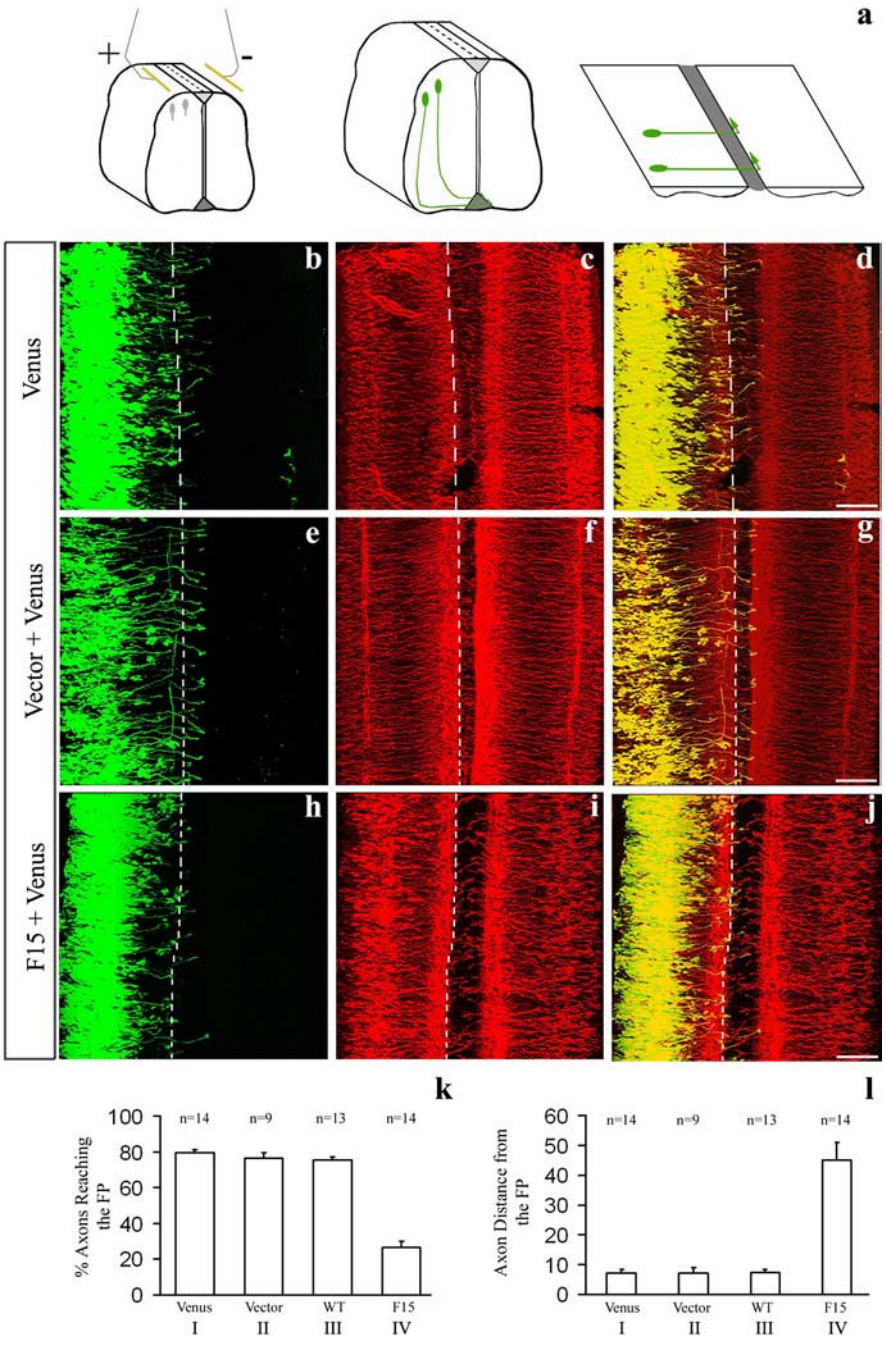

Figure 8. Inhibition of commissural axon projection *in vivo* by p130^{CAS} (F15) mutant. ***a***, Schematic diagram showing the experimental design. DNA was injected into chick neural tube *in ovo* at stage 12, and samples were isolated and fixed at stage 23. Anti-axonin-1 immunostaining shows the commissural axons (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). ***b–d*** show neurons electroporated with Venus YFP only. ***e–g*** are neurons electroporated with Venus YFP and the control vector. ***h–j*** are neurons electroporated with Venus YFP and p130^{CAS} (F15). ***b***, ***e***, and ***h*** show YFP images. ***c***, ***f***, and ***i*** show anti-axonin-1 antibody immunostaining. ***d***, ***g***, and ***j*** are merged images. Scale bar, 100 μm. ***k***, Quantification of the percentage of axons reaching the floor plate. Group I (Venus YFP alone), 79.6 ± 2.0%; Group II (YFP coelectroporated with the vector), 76.5 ± 3.0%; Group III (YFP coelectroporated the wild-type p130^{CAS}), 75.4 ± 2.0%; Group IV (YFP coelectroporated with p130^{CAS} F15 mutant), 26.8 ± 3.0%. Group IV is significantly different from any other group (*p* values <0.0001; Student's *t* test). Numbers of chick embryos examined are shown as *n* in each column. WT, Wild-type p130^{CAS}; F15, p130^{CAS} (F15). ***l***, Quantification of the average distance of axons away from the floor plate. Group I, 7.2 ± 1.1 μm; Group II, 7.1 ± 2.0 μm; Group III, 7.4 ± 1.0 μm; Group IV, 45.1 ± 5.76 μm. *p* value <0.0001 between group IV and group I, II, or III. Error bars are SEM.

Commissural axons projected straight toward the floor plate when the neural tube explants were cocultured with control HEK293 cells (Fig. 6*a*). Axons turned toward HEK cells secreting netrin-1 (Fig. 6*b*). Cotransfection of Venus YFP with either the vector or the wild-type p130^{CAS} did not affect netrin attraction (Fig. 6*d*,*f*). However, when the dominant-negative mutant p130^{CAS} (F15) was cotransfected into the commissural axons

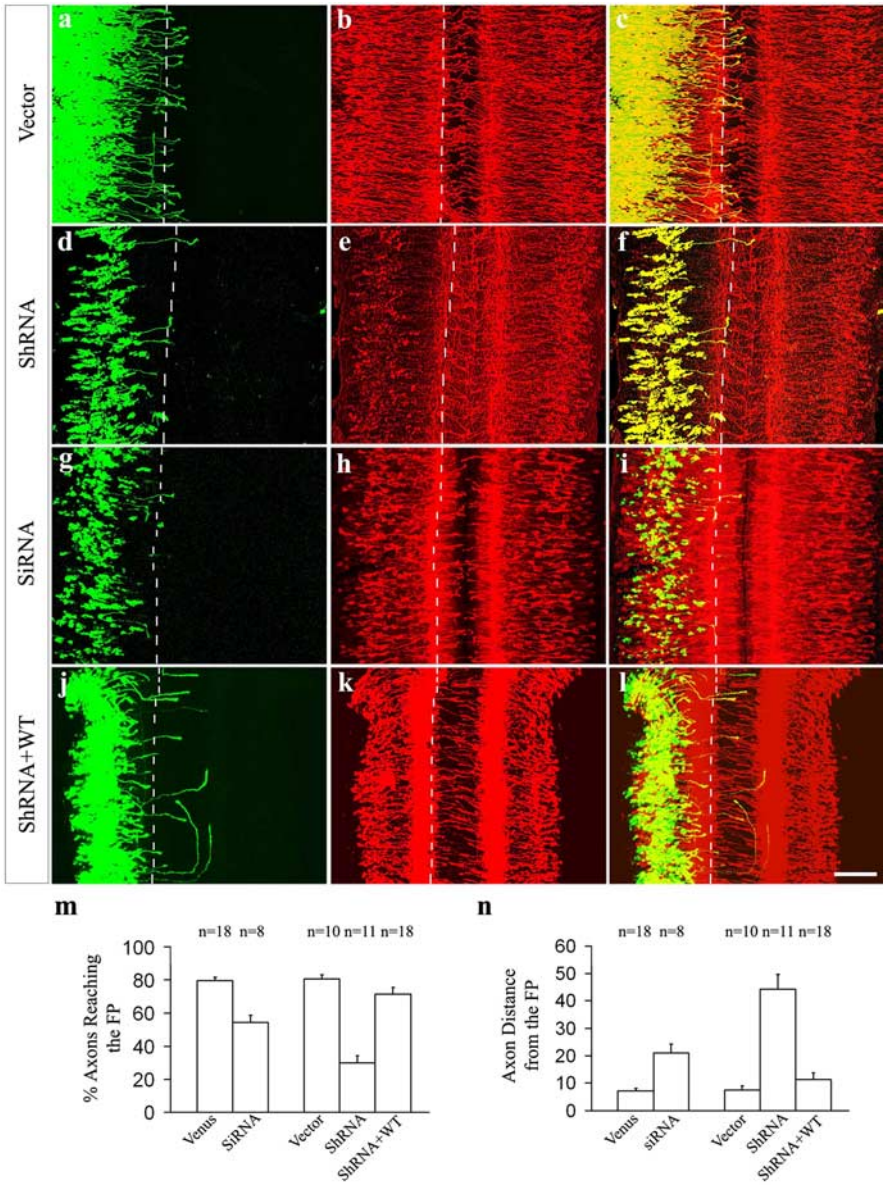

Figure 9. Inhibition of spinal cord commissural axon projection *in vivo* by p130^{CAS} RNAi. ***a–c***, Venus YFP was coelectroporated with the control shRNA vector into the chick neural tube. ***d–f***, Venus YFP was coelectroporated with p130^{CAS} shRNA. ***g–i***, p130^{CAS} siRNA was coelectroporated with Venus YFP. ***j–l***, p130^{CAS} shRNA was coelectroporated with wild-type p130^{CAS} and Venus YFP. ***a***, ***d***, ***g***, and ***j*** are YFP images; ***b***, ***e***, ***h***, and ***k*** are images of anti-axonin-1 antibody immunostaining; ***c***, ***f***, ***i***, and ***l*** are merged images. ***g***, Quantification of the percentage of axons reaching the floor plate: 79.6 ± 2.0% for commissural axons expressing Venus YFP only; 54.5 ± 4.1% for siRNA group; 80.8 ± 2.6% for commissural axons expressing Venus YFP and the control vector; 29.9 ± 4.1% for commissural axons expressing Venus YFP and p130^{CAS} shRNA; 71.6 ± 4.0% for shRNA and wild-type group ($p < 0.001$ between Venus YFP and siRNA group; $p < 0.0001$ between vector and shRNA group; $p < 0.0001$ between shRNA and shRNA plus wild-type group. Student's *t* test). ***h***, Quantification of the average distance of axons away from the floor plate: 7.2 ± 0.9 for Venus YFP; 20.9 ± 3.3 for siRNA; 7.6 ± 1.5 μm for the control vector; 44.3 ± 5.4 μm for p130^{CAS} shRNA; 11.4 ± 2.3 for shRNA plus wild type ($p < 0.01$ between Venus YFP and siRNA group; $p < 0.0001$ between vector and shRNA group; $p < 0.0001$ between shRNA and shRNA plus wild-type group). The numbers on the top (*n*) indicate the number of embryos tested. Scale bar, 100 μm. Error bars are SEM.

together with Venus YFP, attraction by netrin was significantly inhibited (Fig. 6*h*,*i*).

To confirm the role of p130^{CAS}, we introduced p130^{CAS} shRNA or the vector shRNA into the neural tube by electroporation. When the vector shRNA was electroporated into the neural tube together with Venus YFP, commissural axons turned toward netrin-1 (Fig. 7*b*,*i*), whereas p130^{CAS} shRNA cotransfected with Venus YFP significantly inhibited axon turning toward netrin (Fig. 7*d*,*i*). Axon turning toward netrin was also inhibited by p130^{CAS} siRNA (Fig. 7*e*,*f*,*i*). The effect of p130^{CAS} siRNA on commissural axon turning could be rescued by the overexpression of wild-type p130^{CAS} (Fig. 7*g*,*h*,*i*), indicating that the effect was specifically a result of p130^{CAS} knockdown. Immunohistochemistry with the anti-axonin-1 antibody recognizing commissural axons revealed that only commissural axons transfected with p130^{CAS} RNAi did not turn toward netrin, whereas untransfected axons were still attracted by netrin-1 (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

To test whether p130^{CAS} knockdown by shRNA and siRNA caused the commissural axons to lose the turning ability, rather than only losing netrin responsiveness, we used the open-book preparation to assay for axon turning by the roof plate (supplemental Fig. 4*a*, available at www.jneurosci.org as supplemental material). As reported previously (Augsburger et al., 1999), the roof plate repelled the commissural axons (supplemental Fig. 4*b*,*c*, available at www.jneurosci.org as supplemental material). The repulsive response of commissural axons was not inhibited by p130^{CAS} shRNA or siRNA (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). Thus, results from both the p130^{CAS} (F15) mutant and the p130^{CAS} RNAi demonstrate a requirement of p130^{CAS} in axon attraction by netrin-1.

p130^{CAS} is involved in commissural axon projection *in vivo*

Experiments discussed above have shown that p130^{CAS} plays an important role in netrin signaling and netrin function *in vitro*. To determine the *in vivo* functional role of p130^{CAS}, we examined the phenotype of p130^{CAS} (F15) mutant and p130^{CAS} RNAi on commissural axon projection in the chick embryos.

Venus YFP was introduced by electroporation together with a control vector, or with the p130^{CAS} (F15) mutant into the neural tube of stage 12 chick embryos. Embryos were allowed to develop until stage 23 when they were killed and the lumbosacral segments of the spinal cord were isolated. The open-book preparation was made and immunostained with the anti-axonin-1 antibody, which recognized the commissural axons (Stoeckli et al., 1997). More than 90% of axons expressing Venus YFP were marked commissural axons (Fig. 8*b–j*). By stage 23, most commissural axons expressing Venus YFP had reached the floor plate (see Fig. 8*b–d* for an example and Fig. 8*k* for statistics). Although the vector alone did not affect projection of commissural axons (Fig. 8*e–g*), p130^{CAS} (F15) significantly in-

hibited the projection of commissural axons (see Fig. 8*h*,*i*,*j*, for an example and Fig. 8*k*,*l*, for statistical analysis).

The effect of p130^{CAS} (F15) was further confirmed with the p130^{CAS} shRNA and siRNA (Fig. 9). Although the majority of the commissural axons in the spinal cord transfected with the control vector or Venus YFP reached the floor plate (Figs. 8*b–d*, 9*a–c*,*m*,*n*), only a small number of axons in p130^{CAS} shRNA or siRNA transfected group reached the floor plate (Fig. 9*d–l*,*m*,*n*). Although the open-book preparation showed obvious defects of commissural axon projection *in vivo*, it was difficult to evaluate the axon turning. To examine whether the knock-down of p130^{CAS} disrupts the commissural axon turning in addition to inhibiting axon extension *in vivo*, we cut the transverse section of chick spinal cord (stage 23) after electroporation (Fig. 10). In addition to the inhibition of axon extension, some commissural axons transfected with p130^{CAS} shRNA or siRNA were misguided (Fig. 10*c*,*c*'), compared with control groups (Fig. 10*a*,*a*',*b*,*b*'). Overexpression of wild-type p130^{CAS} rescued the effects of p130^{CAS} RNAi knock-down on commissural axon extension and turning (Figs. 9*j–n*, 10*d*,*d*'). The p130^{CAS} knock-out mice are currently not available, and instead we introduced the Venus YFP construct and p130^{CAS} shRNA into mice neural tube at E10.5 and did electroporation *in vivo*. The embryos were killed at stage 12.5, and the transverse sections of spinal cords were obtained. The projection of some commissural axons was decreased and misguided in RNAi group (Fig. 10*g*,*g*') compared with the Venus YFP group (Fig. 10*e*,*e*') and vector group (Fig. 10*f*,*f*'). These results indicate p130^{CAS} is involved in the projection and pathfinding of commissural axons *in vivo*.

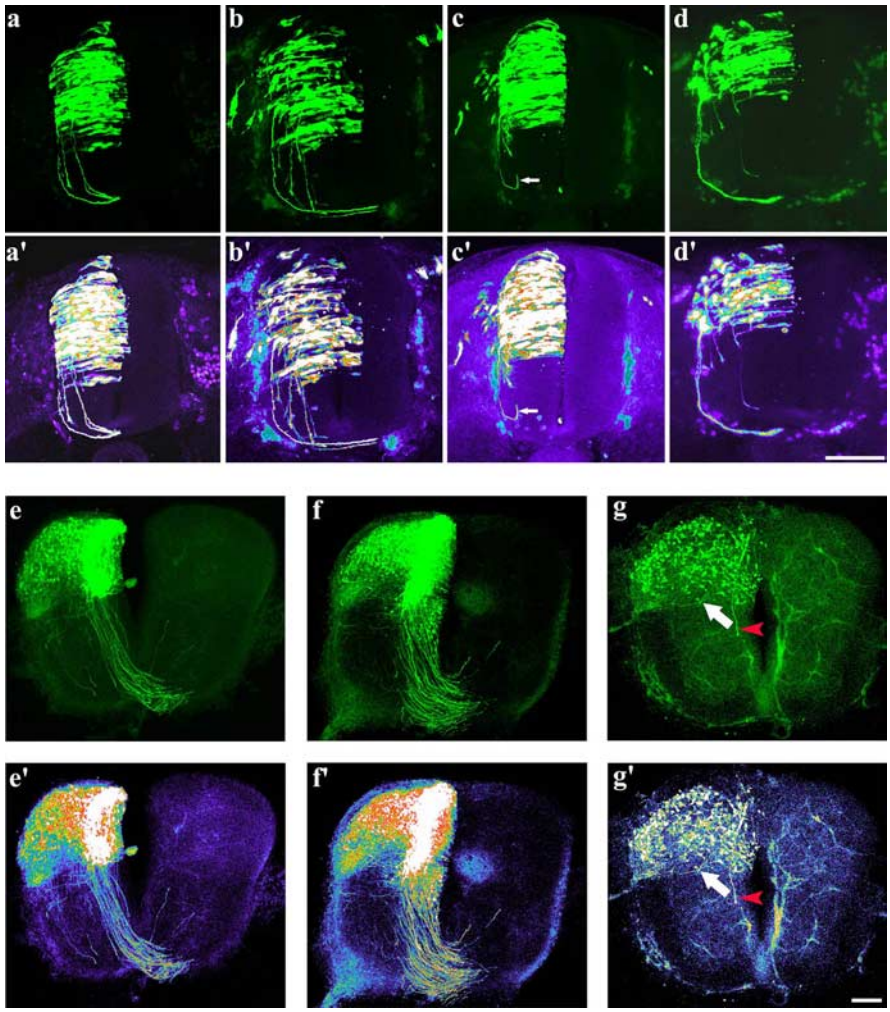

Figure 10. Wild-type p130^{CAS} rescue of commissural defects caused by p130^{CAS} RNAi. The chick neural tube was electroporated with Venus YFP only (***a***, ***a'***), Venus YFP plus shRNA vector (***b***, ***b'***), Venus YFP plus shRNA (***c***, ***c'***), or Venus YFP plus shRNA plus wild-type p130^{CAS} (***d***, ***d'***). ***a'–d'*** are the monochrome images of ***a–d***. p130^{CAS} shRNA not only inhibited the commissural axon extension but also caused aberrant pathfinding (***c***, ***c'***). Overexpression of wild-type p130^{CAS} rescued the defect of p130^{CAS} shRNA on commissural axon projection and turning (***d***, ***d'***). The effect of p130^{CAS} shRNA on the spinal cord commissural axon projection was also observed in embryonic mice. E10.5 mouse neural tube was electroporated with Venus YFP only (***e***, ***e'***), Venus YFP plus shRNA vector (***f***, ***f'***), or Venus YFP plus shRNA (***g***, ***g'***). ***e'***, ***f'***, and ***g'*** are images of ***e***, ***f***, and ***g***. The arrow shows a misguiding axon, and the arrowhead indicates the short commissural axon. Scale bar, 100 μm.

Discussion

Our results indicate p130^{CAS} is an important component in the netrin signaling pathway acting between tyrosine kinases and the small GTPase, and p130^{CAS} is essential for commissural axon guidance.

Previous studies have shown the involvement of multiple molecules in netrin signaling, but it was not clear whether they represent different pathways or whether they converge. For example, although the cytoplasmic tyrosine kinases Fyn and FAK (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004) and the small GTPases Rac1 and Cdc42 (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005) are the most extensively studied molecules in netrin signaling, their relationship was unknown. Results shown here demonstrate that p130^{CAS} is downstream of Fyn and FAK and upstream of Rac1 and Cdc42.

Our results show that p130^{CAS} is expressed in the commissural axons in the embryonic spinal cord and colocalized with DCC. We have several pieces of biochemical evidence that place p130^{CAS} in the netrin signal transduction pathway. In both primary neurons and HEK cells, netrin can stimulate tyrosine phosphorylation of p130^{CAS} (Figs. 2, 3). Netrin also stimulates the formation of a protein–protein interaction complex with Fyn and FAK (Fig. 2*f*,*d*). Netrin stimulation of p130^{CAS} phosphorylation requires DCC (Fig. 2*d*,*h*,*i*). p130^{CAS} is downstream of FAK and Src family kinases, because the inhibition of FAK and Src kinases decreases netrin-induced p130^{CAS} phosphorylation (Fig. 3*a*,*b*), which is consistent with the finding that the same domains in DCC required for FAK phosphorylation (Li et al., 2004; Ren et al., 2004) are required for netrin-induced phosphorylation of p130^{CAS} (Fig. 2*h*,*i*). Unlike the relationship between the Src kinases and FAK, which are mutually dependent on each other (Li et al., 2004; Liu et al., 2004; Ren et al., 2004), p130^{CAS} is not required for netrin-induced phosphorylation of FAK and Fyn, because dominant-negative mutants of p130^{CAS} could not inhibit the phosphorylation of FAK and Fyn (Fig. 3*c–e*), and netrin-

induced phosphorylation of FAK could not be blocked by eliminating $p130^{CAS}$ (Fig. 3*f*). Therefore, $p130^{CAS}$ is clearly downstream of FAK and Src kinases in the netrin pathway.

The Rho family of small GTPases plays important roles in growth cone motility and axon guidance. Netrin can stimulate both Rac1 and Cdc42, and netrin function requires both of them (Li et al., 2002; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Our results show that inhibition of $p130^{CAS}$ blocks netrin-1-induced activation of Rac1 and Cdc42 (Fig. 4), indicating that $p130^{CAS}$ acts upstream of Rac1 and Cdc42 in netrin-1 signaling. Because $p130^{CAS}$ cannot directly activate Rac1 and Cdc42, it will be interesting to identify the molecule that links $p130^{CAS}$ to Rac1 and Cdc42. Similarly, there are no studies linking any of the other components previously implicated in netrin signaling such as cyclic nucleotides, PLC, PI3K, MAP kinases, or calcium.

The functional roles of $p130^{CAS}$ were studied by both *in vitro* and *in vivo* experiments. Netrin-1 can stimulate the growth of neurites from cortical neurons. The $p130^{CAS}$ RNAi inhibited neurite outgrowth induced by netrin-1 (Fig. 5). Using the turning assay, we also show that both the dominant-negative $p130^{CAS}$ mutant F15 and $p130^{CAS}$ RNAi inhibited axon attraction by netrin-1 (Figs. 6, 7). These results indicate that $p130^{CAS}$ is required for netrin function. *In vivo* studies with chick and mouse spinal cords show that F15 and $p130^{CAS}$ RNAi cause failure of commissural axons to reach the floor plate (Figs. 8, 9). When either netrin or DCC is defective in mice (Serafini et al., 1996; Fazeli et al., 1997), the projection of commissural axons is also defective. However, other cues have also been implicated in commissural axon guidance. Bone morphogenetic proteins contribute as a repellent to the initial guidance of commissural axons in a ventral direction (Augsburger et al., 1999). Sonic hedgehog has been thought to be an attractant made in the floor plate for the commissural axons. Our result from the open-book turning assay suggests that $p130^{CAS}$ is not involved in mediating the repulsive response of commissural axons to the roof plate (supplemental Fig. 4, available at www.jneurosci.org as supplemental material). However, it remains to be determined whether $p130^{CAS}$ is involved in the downstream signaling pathway(s) of other guidance cues.

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