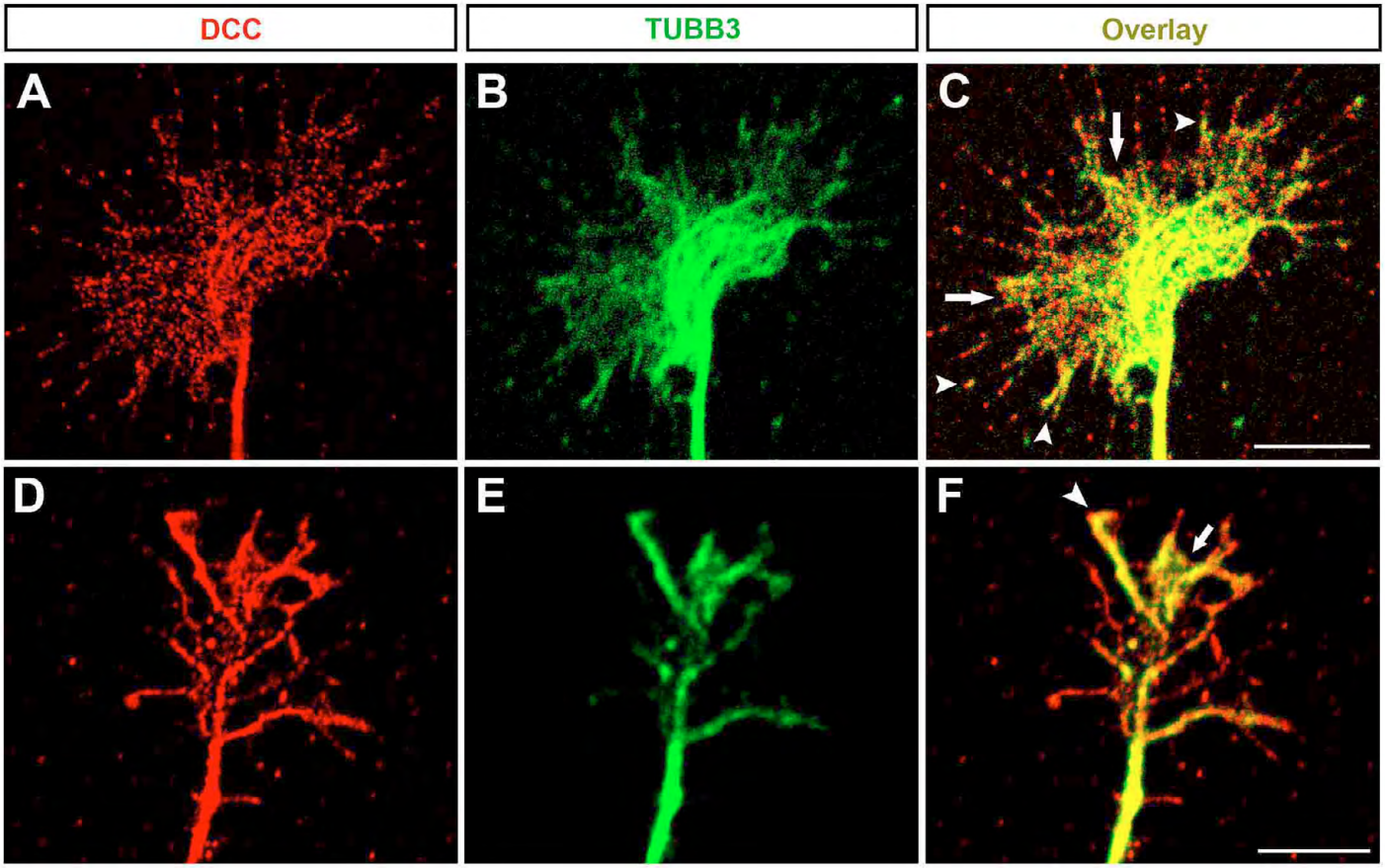

Fig. S1. Subcellular colocalization of DCC with TUBB3 in the growth cone of primary neurons. (**A–C**) Localization of DCC (A) and TUBB3 (B) in dissociated commissural neurons from E11 mouse spinal cords cultured for 4 days. Panel C is merged images of A and B. Quantitative analysis of fluorescence colocalization was performed on original images (Volocity, Version 4.0, Improvision, Waltham, MD, USA). Background was corrected and a free-hand ROI was drawn around the outer edge of the growth cone including lamellipodia and filopodia, but excluding the central core area where pixel intensities tended to be saturated. To evaluate the degree of colocalization of green and red fluorescence within the specified ROI, Pearson's Correlation Coefficient, r, was calculated using the colocalization module in Volocity (means ± S.D.). The value of Pearson's correlation coefficient in A–C was 0.71 ± 0.08. (**D–F**) DCC colocalized with TUBB3 in the growth cones of dissociated cortical neurons from E15 mouse cortexes cultured for 4 days. Panel F is merged images of D and E. The value of Pearson's correlation coefficient of DCC and TUBB3 in the P region of growth cones in D–F was 0.74 ± 0.07. White arrowheads, examples of colocalization sites of DCC and TUBB3 in filopodia; White arrows, examples of overlap in lamellipoda. Scale bars: 10 μm.

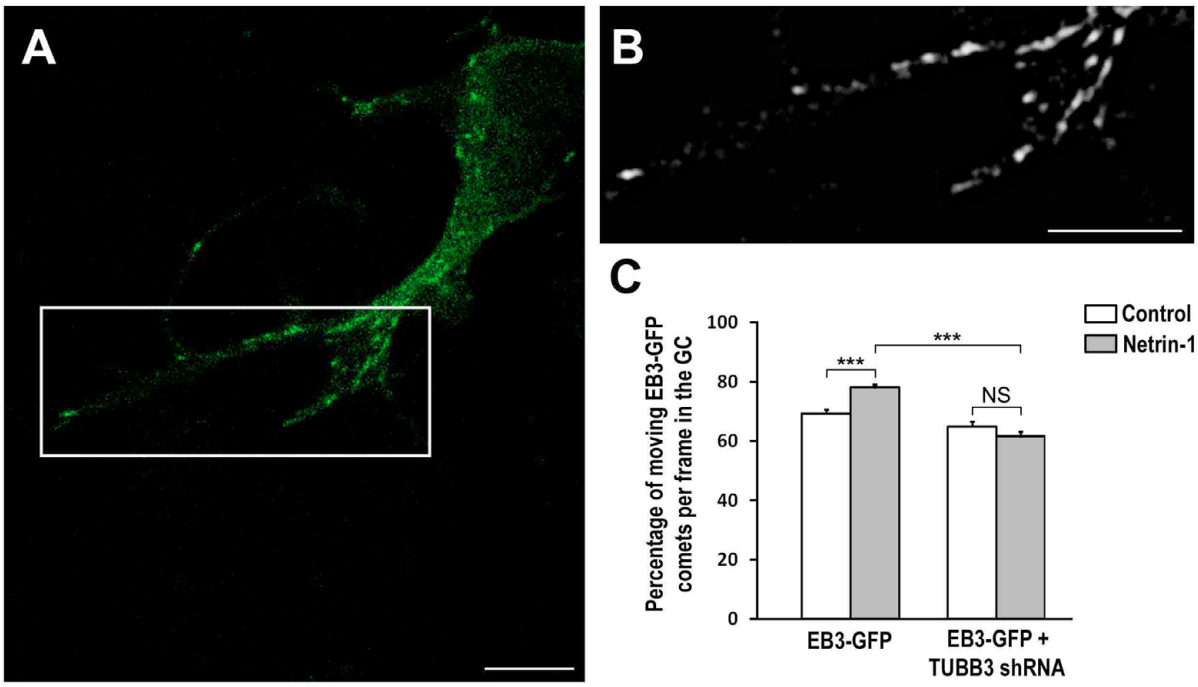

Fig. S2. Time-lapse recording of EB3-GFP in dissociated E13 dorsal spinal cord neurons. (**A**) EB3-GFP comets in transfected spinal cord neurons. E13 dorsal spinal cord neurons were dissociated and nucleofected with EB3-GFP. Primary neurons were cultured for 1–3 days before live imaging in a 37°C chamber using a Leica TCS SP8 confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL). Images were acquired every 1–4 s over 60 frames per growth cone and were corrected for photobleaching and background subtraction. The box in A represents the growth cone in the EB3-GFP-transfected neuron and is magnified in B. (**B**) Images were passed through a bandpass filter before particle detection and trajectories of EB3-GFP comets in the growth cone (GC) using the Particle Tracker plugin for ImageJ (Sbalzarini and Koumoutsakos, 2005). (**C**) Quantification of the percentage of moving EB3-GFP comets to total EB3-GFP per frame in the growth cone of E13 dorsal spinal cord neurons (see supplementary material Movies 1–4). Primary neurons transfected with either EB3-GFP only or EB3-GFP and TUBB3 shRNA together were imaged with or without netrin-1 stimulation (200 ng/ml). Only comets that could be tracked for more than 3 frames were considered moving comets. Data are reported as mean ± s.e.m (2 growth cones in each group). NS, not significant; ***, $P<0.001$ (One-way ANOVA with Tamhane's T2 for post-hoc comparisons). GC, growth cone. Scale bars: 5 μm.

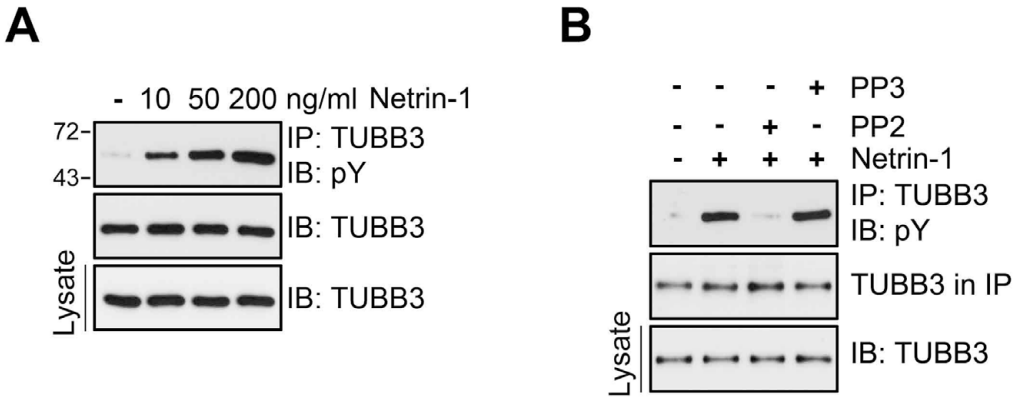

Fig. S3. Src family kinase activity is required for netin-1-induced TUBB3 tyrosine phosphorylation, related to Fig. 3B,D. (**A**) Netrin-1 induces tyrosine phosphorylation of endogenous TUBB3 in dissociated E15 cortical neurons (the reciprocal IP for Fig. 3B). TUBB3 was immunoprecipitated using anti-TUBB3 and the membrane was blotted for tyrosine phosphorylation using anti-pY. Primary neurons were stimulated with purified netrin-1 (200 ng/ml). (**B**) The induction of TUBB3 tyrosine phosphorylation by netrin-1 was inhibited by PP2, not PP3 (the reciprocal IP for Fig. 3D). Primary E15 cortical neurons were treated with purified netrin-1 (200 ng/ml) in the presence of PP2 or PP3.

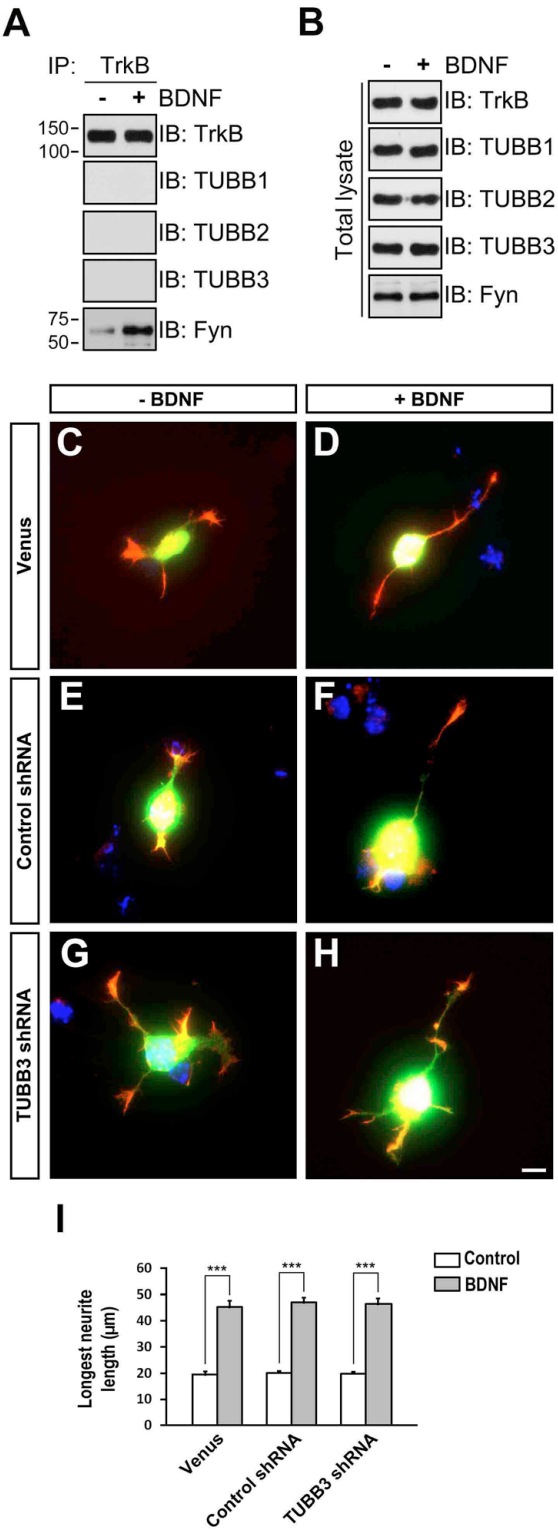

Fig. S4. TUBB3 could not bind to endogenous TrkB, a BDNF receptor, and was not involved in BDNF-promoted neurite outgrowth. (**A**,**B**) TrkB did not form protein–protein interaction complexes with TUBB1, TUBB2 and TUBB3 in primary cortical neurons with or without purified BDNF treatment. In contrast, the interaction of endogenous TrkB with Fyn was increased by BDNF stimulation. The anti-TrkB antibody was used to immunoprecipitate proteins and the blot was analyzed with anti-TUBB1, anti-TUBB2, anti-TUBB3 or anti-Fyn. Equivalent amounts of input TrkB, TUBB1, TUBB2, TUBB3 and Fyn in cortical lysates (B). (**C–I**) Cortical neurons from E15 mouse embryos were transfected with either Venus YFP only (C,D), Venus YFP plus TUBB3 control siRNA (E,F) or TUBB3 shRNA (G,H), respectively, and then cultured on coverslips coated with PLL. Neurons were cultured after 40 h and stained with the Alexa Fluor® 555 phalloidin and DAPI. Purified BDNF (50 ng/ml) induced the neurite outgrowth from YFP-positive neurons transfected with Venus YFP only (C,D) and Venus YFP plus control siRNA (E,F). Expression of TUBB3 shRNA did not affect BDNF-induced neurite outgrowth (G,H). (I) Quantification of the length of the longest neurite from individual neuron. The neurites of YFP-positive neurons not in contact with other neurons were quantified and used in the statistical analyses. Data are reported as mean ± s.e.m. ***, $P<0.001$ (One-way ANOVA with Fischer LSD for post-hoc comparisons). Scale bar: 10 μm.

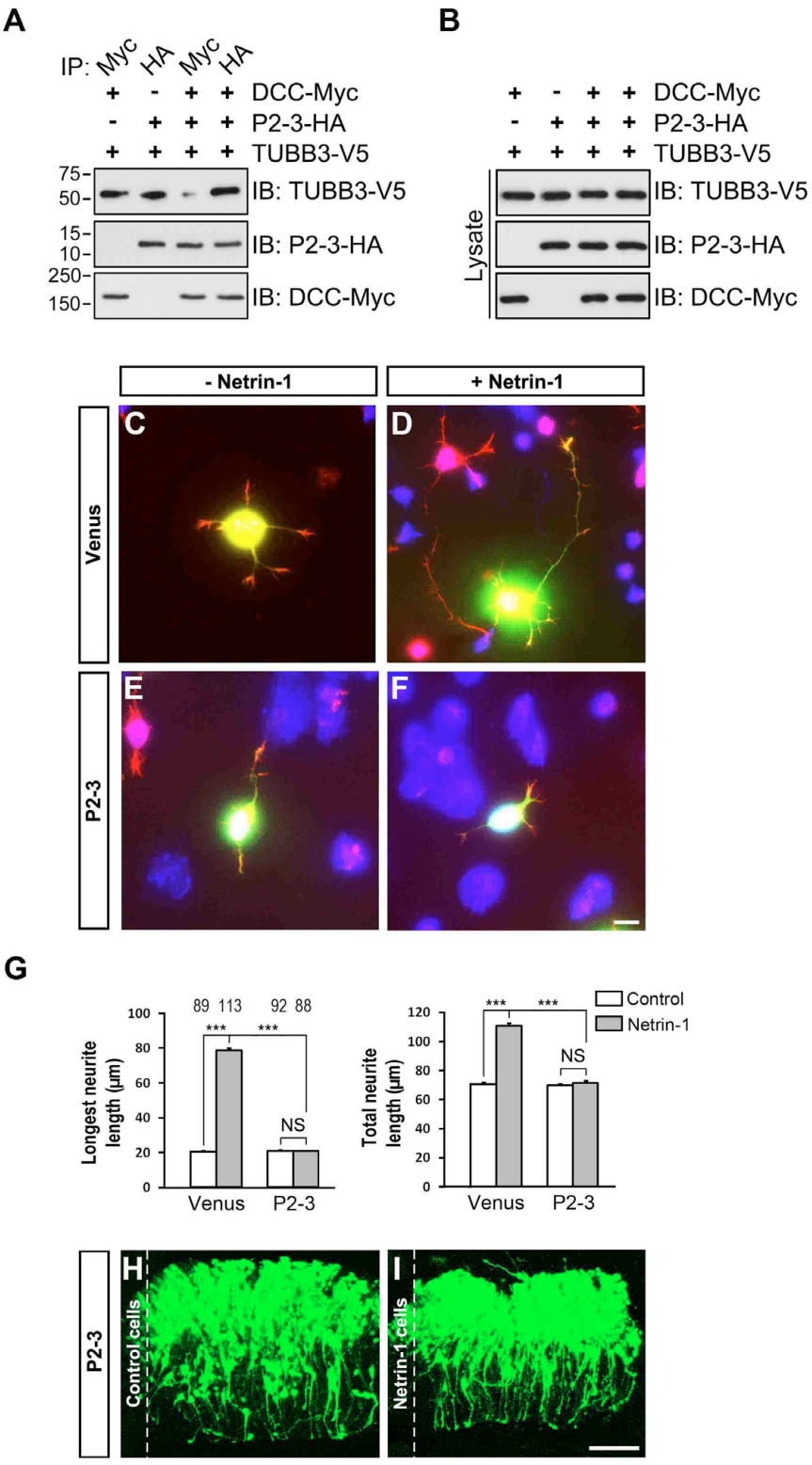

Fig. S5. The DCC intracellular P2-3 domain inhibited the interaction of TUBB3 with full-length wild-type DCC as well as netrin-1-induced neurite outgrowth and commissural axon attraction. (**A**,**B**) TUBB3-V5 were co-transfected with either wild-type full-length DCC-Myc, DCC P2-3-HA or full-length DCC-Myc and DCC P2-3-HA together into HEK293 cells. Cell lysates were immunoprecipitated using either anti-Myc or anti-HA and the membrane was blotted for TUBB3 using anti-V5. (B) Equivalent amounts of input TUBB3-V5, DCC P2-3-HA and DCC-Myc. (**C–G**) Expression of DCC P2-3 domain inhibited netrin-1-induced neurite outgrowth of cortical neurons. E15 mouse cortical neurons were transfected with either Venus YFP only (C,D) or Venus YFP plus DCC P2-3 (E,F). Primary neurons were treated with either purified netrin-1 (D,F) or the sham-purified control (C,E). (G) Quantification of netrin-1-induced neurite outgrowth. The numbers on the top of each bar indicate the numbers of neurons tested in the corresponding groups. Data are mean ± s.e.m. NS, not significant; ***, $P<0.001$ (One-way ANOVA with Fischer LSD for post-hoc comparisons). (**H**,**I**) Expression of DCC intracellular P2–3 domain blocked netrin-1 attraction of chick spinal commissural axons. Axons expressing Venus YFP and DCC P2-3 domain were not attracted by the aggregates of HEK293 cells secreting netrin-1 (quantification in Fig. 6L, group I). Scale bar: 10 µm (F); 100 µm (I).

Movie 1. Live imaging of EB3-GFP comets in an E13 dorsal spinal cord neuron.

Movie 2. Live imaging of EB3-GFP comets in an E13 dorsal spinal cord neuron after netrin-1 stimulation.

Movie 3. Live imaging of EB3-GFP comets in an E13 dorsal spinal cord neuron transfected with TUBB3 shRNA in the absence of netrin-1.

Movie 4. Live imaging of EB3-GFP comets in an E13 dorsal spinal cord neuron transfected with TUBB3 shRNA in the presence of netrin-1.