Supplemental Figures and Legends

Figure S1 (related to Fig. 1)

Protein Sequence Report

Sequence	Accession	Sf	Xcorr	deltaCn	Sp
NLHQSGFSLSGAQIDDNIPR	sp|o08553|dpyl2	0.98	4.81	0.51	2128
THNSALEYNIFEGM*ECR	sp|o08553|dpyl2	0.97	4.96	0.53	1332
GIQEEMEALVK	sp|o08553|dpyl2	0.93	3.3	0.19	1625
SAAEVIAQAR	sp|o08553|dpyl2	0.93	3.22	0.22	1368
GSPLVVISQGK	sp|o08553|dpyl2	0.92	3.48	0.3	1100
DIGAIAQVHAENGDIIAEEQQR	sp|o08553|dpyl2	0.92	4.04	0.29	1128
MVIPGGIDVHTR	sp|o08553|dpyl2	0.91	3.18	0.28	818
IVNDDQSFYADIYM*EDGLIK	sp|o08553|dpyl2	0.9	3.57	0.35	1025
KPFPDFVYK	sp|o08553|dpyl2	0.87	2.99	0.27	766
IVLEDGTLHVTEGSGR	sp|o08553|dpyl2	0.86	3.36	0.41	790
FQLTDSQIYEVLSVIR	sp|o08553|dpyl2	0.84	3.48	0.28	1062
FQM*PDQGM*TSADDFFQGTK	sp|o08553|dpyl2	0.84	3.55	0.41	584
GTVVYGEPITASLGTDGSHYWSK	sp|o08553|dpyl2	0.81	3.44	0.5	362
THNSALEYNIFEGMECR	sp|o08553|dpyl2	0.8	2.96	0.29	793
QIGENLIVPGGVK	sp|o08553|dpyl2	0.77	3.26	0.2	725
FQLTDSQIYEVLSVIR	sp|o08553|dpyl2	0.72	2.5	0.34	730
MSVIWDK	sp|o08553|dpyl2	0.7	2.45	0.08	609
M*VIPGGIDVHTR	sp|o08553|dpyl2	0.61	2.55	0.26	636
GSPLVVISQGK	sp|o08553|dpyl2	0.61	2.15	0.17	888
M*SVIWDK	sp|o08553|dpyl2	0.61	1.96	0.03	644
IVNDDQSFYADIYMEDGLIK	sp|o08553|dpyl2	0.58	2.61	0.34	514
VFNLYPR	sp|o08553|dpyl2	0.56	1.87	0.01	506
FQLTDSQIYEVLSVIR	sp|o08553|dpyl2	0.56	2.28	0.26	601
ISVGSDADLVIWDPDSVK	sp|o08553|dpyl2	0.45	2.1	0.23	415
IVNDDQSFYADIYMEDGLIK	sp|o08553|dpyl2	0.44	2.26	0.34	477
MVIPGGIDVHTR	sp|o08553|dpyl2	0.41	2.42	0.24	395
M*DENQFVAVTSTNAAK	sp|o08553|dpyl2	0.37	2.51	0.07	728
SAAEVIAQAR	sp|o08553|dpyl2	0.17	1.62	0.2	340

Sf, Sequest final score
Xcorr, Sequest cross-correlation score
DCn, Xcorr difference between the top ranked and next best sequence
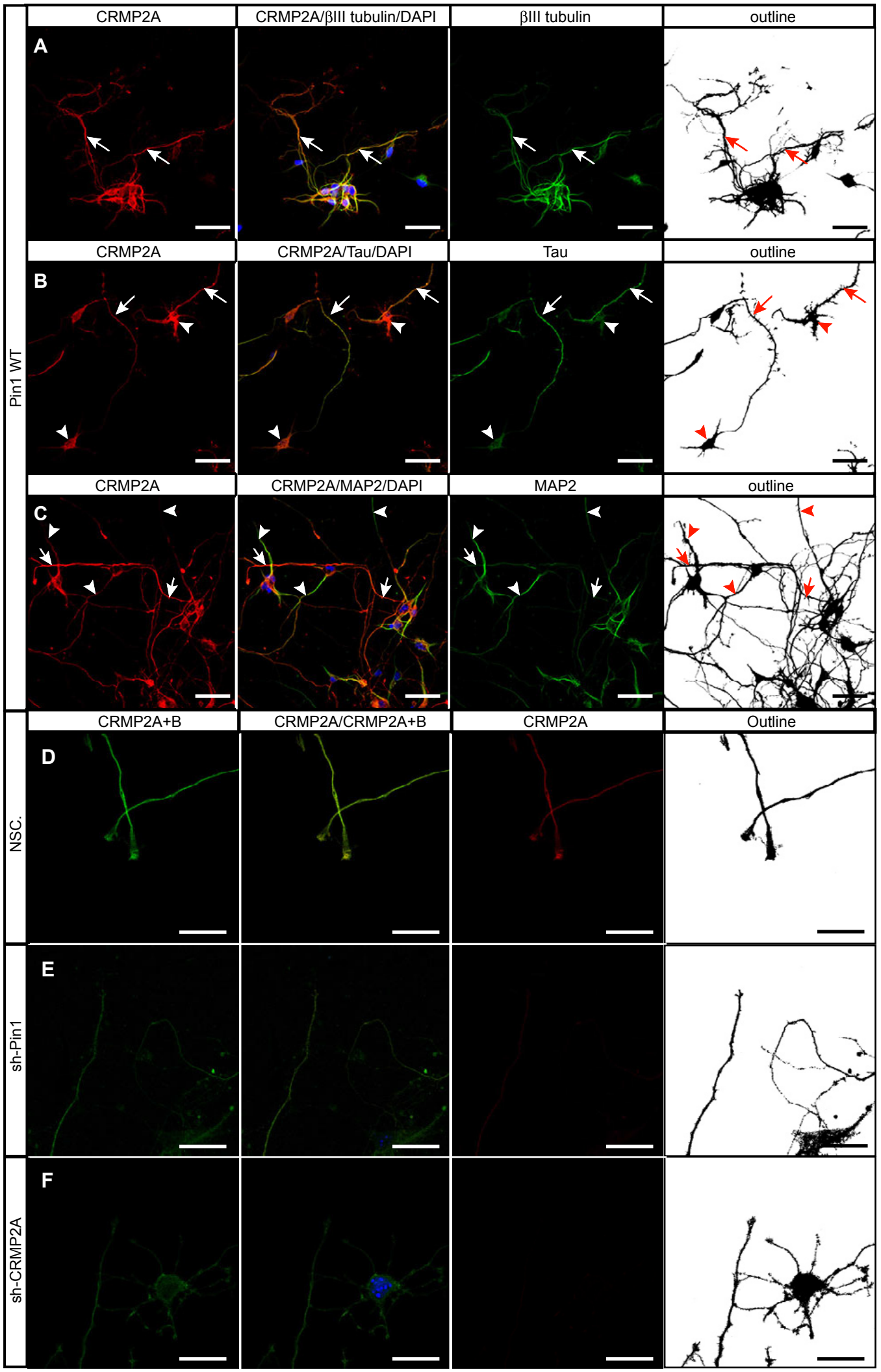**Sp**, Sequest preliminary score

Figure S2
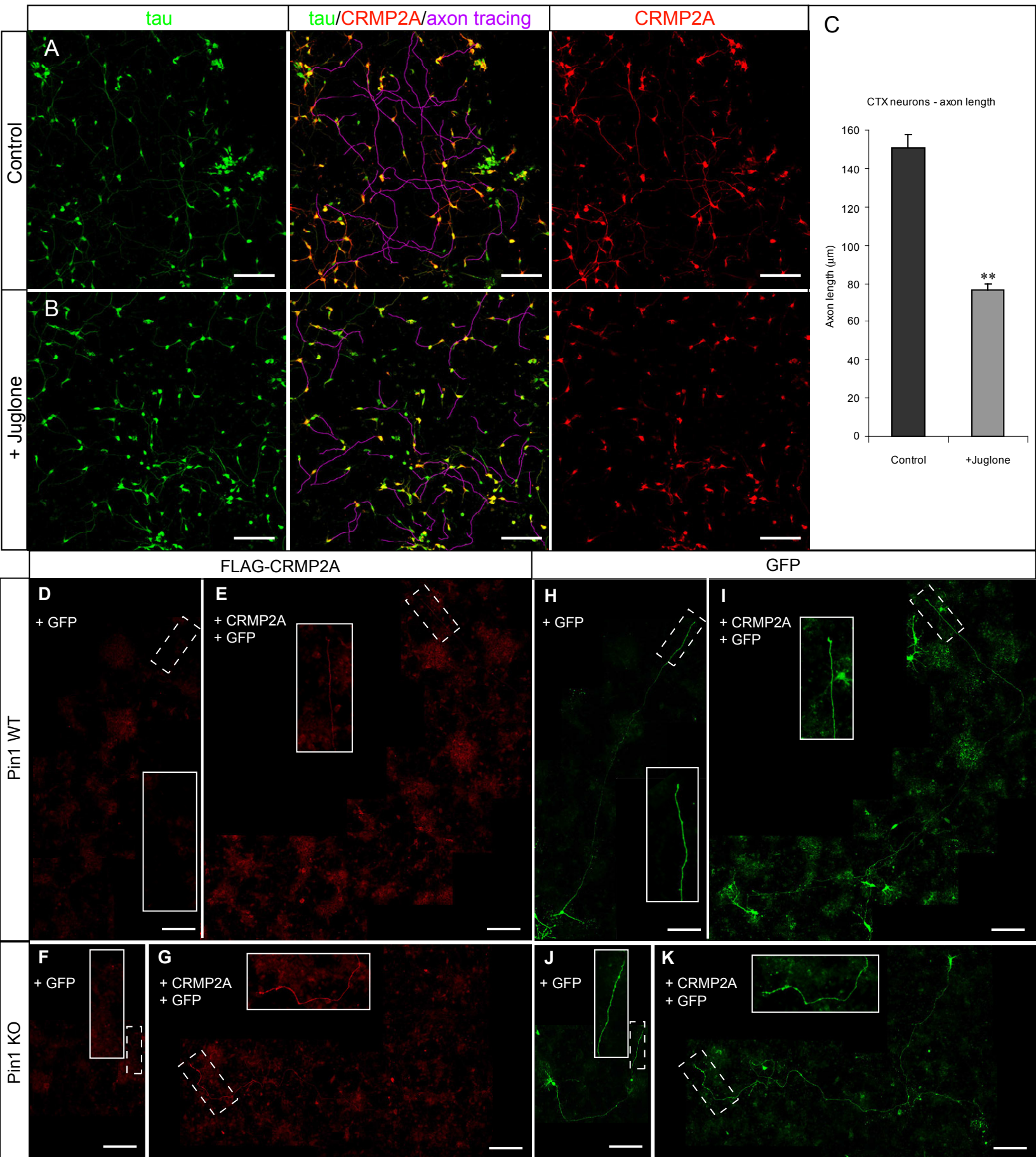
(related to Fig. 3)

Figure S3   (Related to Fig. 4)

Figure S4 (related to Fig.5)

α-tubulin/β-actin	Pin1	α-tubulin/β-actin	Pin1

Pin1 WT

A Cont.

B +LPA 1 μM

C +LPA 100 μM

Pin1 KO

D Cont.

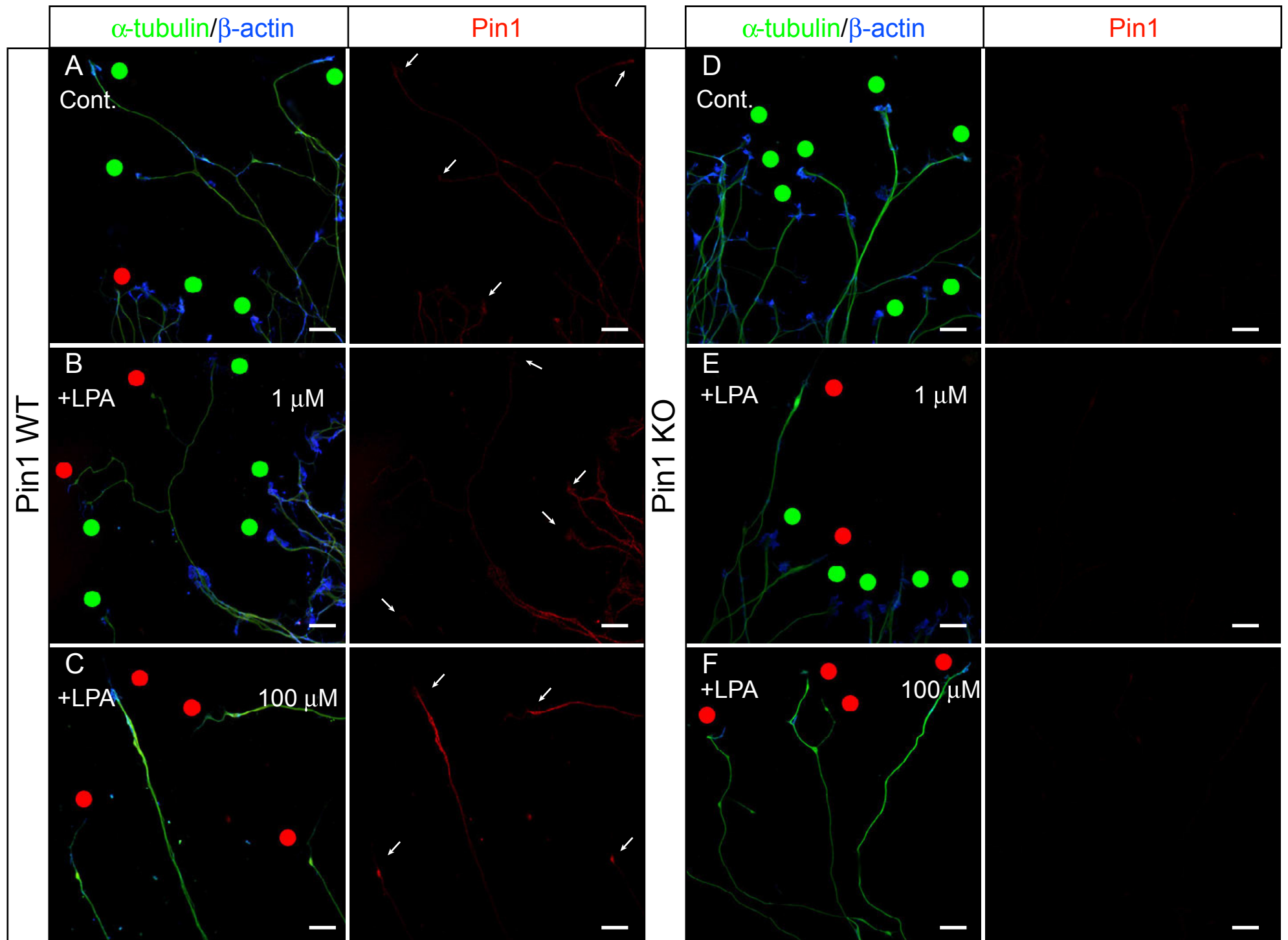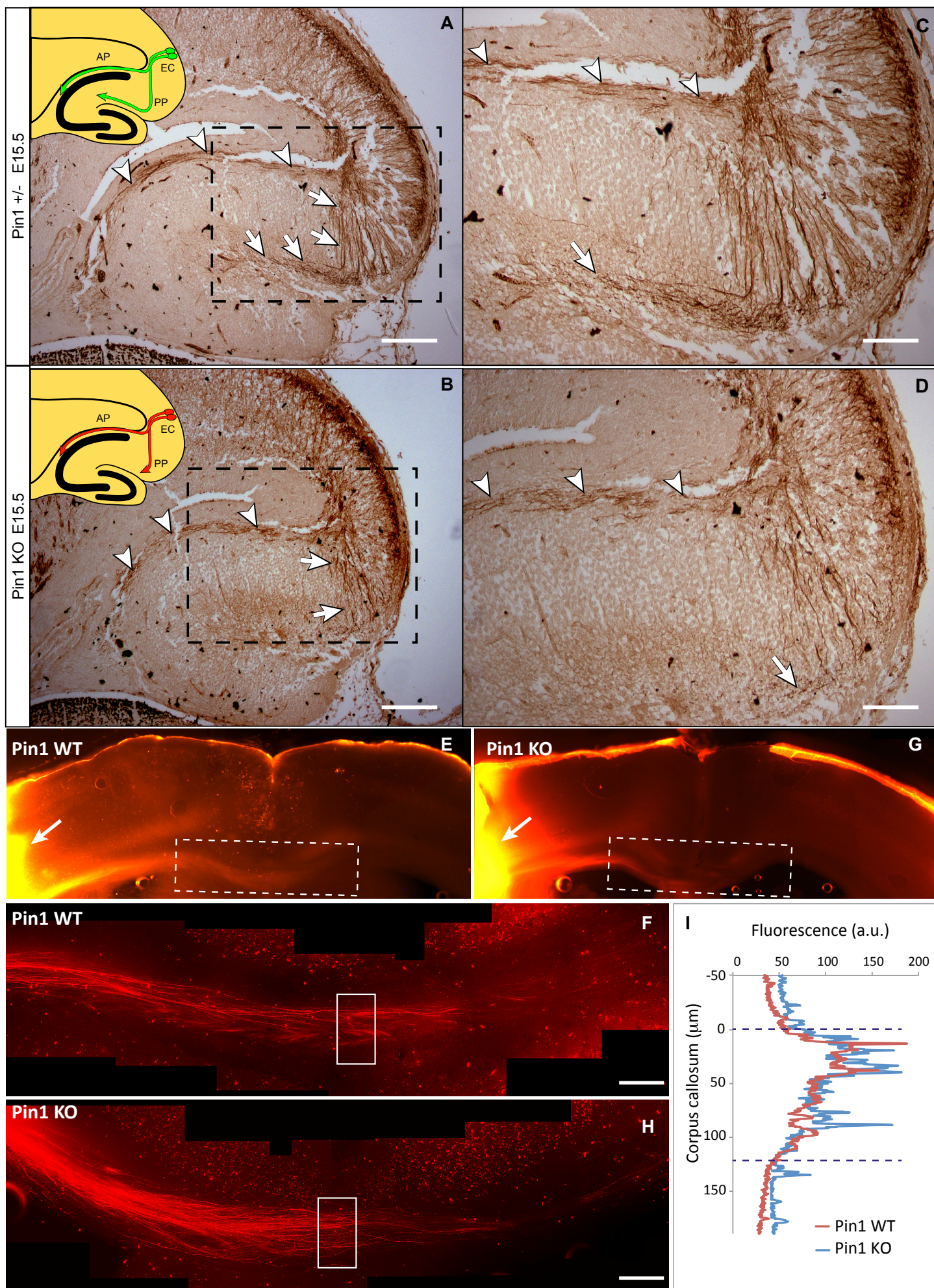E +LPA 1 μM

F +LPA 100 μM

Figure S5

Figure Legends:

Figure S1 Identification of a major Pin1-binding protein as CRMP2 by LC-MS/MS. Related to Figure 1

Sequences and their scores of 28 CRMP2 peptides identified LC-MS/MS in GST-Pin1 bound proteins isolated from postnatal brain lysates.

Figure S2 Knockdown of Pin1 or CRMP2A leads to reduction of total CRMP2 level in the vicinity of growth cones. Related to Figure 3

(A) CRMP2A is strongly expressed in primary cortical neurons, as detected by neuron marker βIII tubulin (arrows). (B) Strongest CRMP2A staining is present in distal axons (arrows), as identified by co-immunostaining with tau. CRMP2A is also present in neuronal cell bodies (arrowheads). (C) CRMP2A is present also in dendrites of primary cortical neurons (arrowheads), as identified by the dendritic marker MAP2, even though its level seems lower when compared to CRMP2A level in distal axons (arrows).

Knockdown of Pin1 (E) or CRMP2A (F) in the Pin1 WT primary cortical neurons significantly reduces level of CRMP2A as well as total CRMP2 (CRMP2A+B) in the vicinity of growth cones, when compared to control cortical neurons infected with a non-silencing lentiviral vector (D). (Scale bars A – C 50 μm, D – F 20 μm).

Figure S3 Inhibition of Pin1 isomerase activity reduces axon growth and CRMP2A axon levels in primary cortical neurons. Overexpressed FLAG-tagged CRMP2A accumulates in distal axons. Related to Figure 4

Treatment of 1 DIV cortical neurons with Juglone, an inhibitor of Pin1 isomerase activity for 3 days (B) reduces CRMP2A levels in axons and axon growth as analyzed by CRMP2A and tau double immunostaining. (A) Untreated controle. (C) Quantification of axon length. ($p <$

2e-06). (E-K) Expression of FLAG-tagged CRMP2A leads to CRMP2A accumulation in distal axons both in Pin1 WT (E) and KO (G) primary cortical neurons, as visualized by anti-FLAG antibodies. No FLAG signal was detected in vector-transfected Pin1 WT (D) or KO (F) neurons. (H-K) GFP signal used to trace the transfected neurons in (D-G). (Scale bars 100 μm).

Figure S4 Pin1 KO does not increase sensitivity to LPA treatment. Related to Figure 5

Primary Pin1 WT and KO cortical neurons were treated with 0.1, 1, 10 and 100 μM LPA, fixed and triple immunostained with anti-Pin1, β-tubulin and β-actin antibodies, followed by growth cone collapse analysis, revealing a similar percentage of collapsed growth cones in Pin1 WT (A-C) and KO (D-F) DRGs. Red dots – collapsed growth cones; green dots – intact growth cones. Similarly, intensity of Pin1 immunostaining in Pin1 WT DRG growth cones (A-C) is not significantly different compared to Pin1 KO DRGs upon low (B, E) or high (C, F) LPA stimulation and normalization to β-tubulin levels. (Scale bars: 20 μm).

Figure S5 Alvear pathway of the enthorino-hippocampal projections at E15.5 Pin1 KO embryos and fasciculation of somatosensory (S1) axons at midline of corpus callosum in adult Pin1 KO mice. Related to Figure 6

NF-L immunostaining of E15.5 brain horizontal sections shows normal development of the Pin1 KO (B, D) alvear projections (arrowheads), while growth of perforant fibers is significantly reduced (arrows) when compared to Pin1 WT littermates (A, C). The fasciculation of S1 axons in midline corpus callosum is similar in Pin1 WT (E, F) and Pin1 KO (G, H) adult mice (3 month old) (arrows indicates the place of DiI crystal insertion). (I) Fluorescence intensity of S1 axons along the D-V axis at the midline shows similar distribution and diameter of the axon bundle in Pin1 WT and Pin1 KO mice. EC–entorhinal

cortex, PP–perforant pathway, AP–alvear pathway (Scale bars: A, B 200 μm; C, D, F, H 100 μm).

Experimental procedures (Supplemental Materials)

Plasmids

Mouse *Crmp2A* and *Crmp2B* were cloned with a 5' FLAG tag into pCDNA3.1 (Invitrogen). Point mutations in Crmp2A–S27A, -S623A, -S27A+S623A were introduced using the QuikChangeTM site-directed mutagenesis kit according (Stratagene) according to the manufacturer's instructions and confirmed by sequencing. AP-Sema3A expression vector was kindly provided by Alex Kolodkin(Messersmith et al., 1995)

Animals

All experimental procedures were performed in compliance with animal protocols approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Pin1 KO mice and their WT littermates in pure C57BL/6J background were used in the experiments, as described previously(Liou et al., 2003; Pastorino et al., 2006). Zebrafish were mated, staged, and raised as described (Westfield, 2000) in accordance with IACUC guidelines.

Antibodies

The rabbit anti-CRMP2A and anti-CRMP2B antisera were generated by Proteintech Group Inc and affinity purified, as described(Lu et al., 1999a). Briefly, for rabbit immunization an affinity purified GST-tagged N-terminal fragment (a.a. 1-108) of CRMP2A, and affinity purified GST-tagged CRMP2B were used. For CRMP2A and total CRMP2 double immunostaining affinity purified sheep anti-CRMP2 polyclonal antibody was used which was described previously(Cole et al., 2006). In the immunostaining experiments, the antibodies were used in the following dilutions: anti-tau (dc25, rPeptide) 1:500, anti-βIII tubulin (Millipore) 1:500, anti-MAP2 (Abcame) 1:500, anti-NF-L (NR4; Sigma) 1:300, anti-NF-M (AB1987,Chemicon)

1:1000, anti-FLAG (F7425, Sigma) 1:1000, anti-neurofilament (2H3, developmental studies hybridoma bank) 1:100, anti-Pin1(Liou et al., 2003) 1:1000. For triple labeling of cultured DRGs anti-Pin1 (P4190, US Biological) 1:300, anti-β-tubulin-FITC (Sigma) 1:50, anti-β-actin (Sigma) 1:1000, anti-acetylated tubulin (Sigma) 1:1500 were used. As secondary antibodies either fluorescent Alexa 405-, 488-, 546- or 594-conjugated antibodies (Invitrogen, 1/300) were used, or Vectastain ABC elite kit (Vector) according to the manufacturer's protocol and detected with diaminobenzidine. For Pin1 co-immunoprecipitation experiments, rabbit monoclonal anti-Pin1 antibodies were used (Epithomics, 1:50).

GST pulldown assays and co-immunoprecipitations

GST pulldown, immunoprecipitation, and immunoblotting analyses were performed as described(Lu et al., 1999b; Yaffe et al., 1997) Briefly, SH-SY5Y or HEK293T cells were transfected with plasmid DNA overnight and then lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 10% glycerol, 1% Triton X100, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 50 µg/ml phenylmethylsulfonyl fluoride and 1 mM DTT. The cellular supernatants were incubated with either 1 µM GST or GST-Pin1 (pulldown assays) or a specific antibody (co-immunoprecipitation) for 2 hr at 4°C and then 15 µl of glutathione agarose beads (pulldown assays) or protein Agarose (co-immunoprecipitation) were added, followed by further incubation for 2 h at 4°C. The precipitated proteins were washed 4–6 times in the same buffer and subjected to immunoblotting analysis, or stained in gel using silver staining (SilverXpress, Invitrogen) or coomassie brilliant blue (Fisher) for mass spectrometry analyses.

Protein stability assay

Protein stability assay was carried out as described previously(Lim et al., 2008; Ryo et al., 2001). Briefly, cells were transfected stably or transiently with expression plasmids, as indicated. 18 h after transfection cycloheximide (100 μg/ml) was added to the media to block new protein synthesis. Cells were harvested at each time points, and total lysates were analyzed by immunoblot with anti-FLAG and anti-β-actin antibodies. For primary cortical neuron stability assay, neurons were treated at 6DIV with 50 μg/ml cycloheximide and analyzed as described above.

Tandem Mass Spectrometry

Coomassie-stained SDS-PAGE gel bands containing FLAG-CRMP2A were excised and subjected to reduction with DTT and alkylation with iodoacetamide in-gel digestion with trypsin overnight at pH 8.3. Reversed-phase microcapillary liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to identify phosphorylated and non-phosphorylated CRMP2A sequences using a EASY-nLC nanoHPLC (Proxeon Biosystems, Odense, Denmark) using a self-packed 75μm-id × 15-cm C_{18} column connected to a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) in data-dependent acquisition and positive ion mode at 300 nL/min. MS/MS spectra collected via collision-induced dissociation (CID) in the ion trap were searched against the concatenated target and decoy (reversed) Swiss-Prot protein databases using Sequest [Proteomics Browser Software (PBS), Thermo Scientific] with differential modifications for Ser/Thr/Tyr phosphorylation (+79.97) and the sample processing artifacts Met oxidation (+15.99), Cys alkylation (+57.02) and deamidation of Asn and Gln (+0.984). Phosphorylated and unphosphorylated peptide sequences were identified if they initially passed the following Sequest scoring thresholds: 1+ ions, Xcorr ≥ 2.0 Sf ≥ 0.4, p ≥ 5; 2+ ions,

Xcorr ≥ 2.0, Sf ≥ 0.4, p ≥ 5; and 3+ ions, Xcorr ≥ 2.60, Sf ≥ 0.4, p ≥ 5 against the target protein database. Passing MS/MS spectra were manually inspected to be sure that all **b-** and **y-**fragment ions aligned with the assigned sequence and modification sites. Determination of the exact sites of phosphorylation was aided using *FuzzyIons* and *GraphMod* software (PBS, Thermo Fisher Scientific).

Dorsal root ganglia cultures and collapse assays

Collapse assays were performed as described before(Kapfhammer et al., 2007). Briefly, dorsal root ganglia (DRG) were dissected from E15.5 embrya in HBSS (Invitrogen) supplemented with 20mM HEPES pH 7.3. Isolated DRGs were plated on to laminin and poly-D-lysine-coated glass coverslips and maintained in neurobasal media supplemented with factor B27 (Invitrogen) and 2 mM L-glutamine (Invitrogen) and NGF (R&D systems, 25 ng/ml). I day after plating the 1/2 of the culture media was carefully removed, supplemented with a specific Sema3A (R&D systems), LPA (Sigma) concentration and slowly returned to the DRG cultures. After 30 min incubation the DRGs were fixed with 4% PFA/PBS, and immunostained with anti-Pin1, anti-β-tubulin-FITC conjugate and anti-β-actin antibodies and number of intact and collapsed growth cones was analyzed in confocal microscope images. Criteria used to distinguish intact and collapsed growth cones were described elsewhere(Kapfhammer et al., 2007). 3D co-cultures of mouse DRG explantswere were performed as described before (Messersmith et al., 1995; Tessier-Lavigne et al., 1988). Briefly, mouse DRGs were isolated from E12.5 embryos embedded in collagen gels mixed with 50% matrigel together with SH-SY5Y cells (mock transfected or transfected with AP-Sema3A vector (kindly provided by A. Kolodkin) and cultured for 44 hours. Subsequently, cultures were fixed, immunostained with

anti NF-M antibodies and the distance of the collapsed axons from the clustered AP-Sema3A expressing cells was measured.

Compartmented chamber cultures:

Compartmented chamber cultures were prepared as described previously (Heerssen et al., 2004; Pazyra-Murphy et al., 2009). Briefly, dorsal root ganglia (DRGs) from embryonic day 15 (E15) rats were dissected and plated in the center compartment of a Teflon divider attached to collagen coated tissue culture plate (Camp10; Tyler Research) (Campenot, 1982). Cultures were maintained in DMEM with 5% horse serum, 1% penicillin–streptomycin, and 0.3 μM cytosine arabinoside (AraC) at 37.0°C, 7.5% CO2. Neurotrophins were added to the cell body compartment at 10 ng/ml BDNF (Peprotech) and 10 ng/ml NGF (Peprotech) and to the axonal compartment at 100 ng/ml BDNF and 100 ng/ml NGF for 3 days. On day 4, media were replaced and the 0.3 μM AraC was omitted. On day 6, neurotrophins were completely removed from the cell body compartment and reduced to 1 ng/ml in the axonal compartments for 3–4 d before use. Cultures were visually checked for leakage and to ensure that axons successfully grew into the side compartments before use.

Morpholino knockdown in zebrafish:

Morpholinos targeting NRP1 and Pin1 were synthesized by Gene Tools (Philomath, OR) and solubilized in water at 1 mM concentration according to the manufacturer's instructions. The sequence of NRP1 targeting morpholino was described elsewhere (Lee et al., 2002), the Pin1-silencing morpholino (5'ACACACGCCCTGAGAAGCAAATAAA3') was designed to target 5' splice junction of exon2 of zPin1. For injections, phenol red (Sigma, P0290) was added to morpholino solutions (20% final conc.). NRP1-MO and Pin1-MO morpholinos were

mixed 1:1 (for double gene silencing) or diluted (to 0.5 mM) in water (for single gene silencing). 1 nl of morpholino/phenol red solution was injected into one to two-cell stage embryos. 1-day-old zebrafish embryos were immunostained using acetylated-tubulin antibodies (Sigma) and embedded in Methocell (Sigma) for analysis using Nikon AZ100 microscope. The motor neurons were analyzed similar as described before (Feldner et al., 2005), briefly: Only the rostral 10 pairs of motor nerves were scored. The embryos were scored as abnormal when any of the following defects were observed: motor nerves were branched at or above the ventral edge of the notochord; the motor axons were truncated (they did not reach the horizontal myoseptum); multiple axons exited the spinal cord (multiple exits); tubulin-immunopositive cells were present in the ventral motor pathway. At least two, and for most three or more, experiments were performed for each treatment. Values were presented as mean + S.E.M. statistical significance was quantified using student's T-test.

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