Supplementary Material for

Operational redundancy in axon guidance through the multifunctional receptor Robo3 and its ligand NELL2

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This PDF file includes:

Materials and Methods
Figs. S1 to S4
Table S1
Full Reference List
Materials and Methods

Animals. Mice carrying the Robo3 null allele have been described before, and genotyping was performed as originally reported (12). Mice carrying the NELL1<sup>6R</sup> point mutation have been described previously (21) and were genotyped by PCR-amplifying and sequencing part of the NELL1 genomic locus (primer sequences: TTGCCTCAACCTCAATATCC and ATAGACCAGGGGCGAGAAACC). For generation of NELL2 mutant mice (Fig. S4A), a targeting vector with loxP sites flanking NELL2 exons 2 and 3, which contain the start codon and sequences encoding the signal peptide, was generated by recombineering and introduced into mouse ES cells by standard procedures. Correctly targeted ES cell clones were identified by PCR screening of genomic DNA, the frt-flanked neomycin cassette was removed by transient expression of FlpE, and mice carrying the NELL2<sup>flox</sup> allele were generated by standard procedures. The NELL2<sup>+</sup> allele was generated by crossing NELL2<sup>flox/+</sup> mice with EIIA-Cre mice that express Cre recombinase in the germ line (22). For genotyping, the NELL2<sup>+</sup>, NELL2<sup>flox</sup>, and NELL2<sup>-</sup> alleles were detected by PCR (primer sequences: CTGGCATTGAGATCCTAGGATC, GGCAGATGGGTGATTTGGG, and GGAGCGCTCCATGGTAACC) from genomic DNA. Mice were maintained on a mixed background (NELL1 and NELL2 mutant mice) or a CD-1 background (Robo3 mutants and wild type mice).

Expression constructs and recombinant proteins. Information about plasmids for protein expression is listed in Table S1. The mouse Robo3.1 and Robo3.2 expression constructs have been described before (14). Untagged and AP-tagged proteins for AP fusion protein binding assays and repulsion experiments were expressed in situ as described for the respective assays below. For flow cytometry experiments, we used a previously described expression system (23) to tether a NELL2-EGFP fusion to the cell
surface via a GPI membrane anchor. Recombinant proteins fused to the Fc portion of human IgG or to FLAG were produced in Chinese hamster ovary (CHO) cells and purified as previously described (24). Briefly, Fc fusion proteins were extracted from transient transfection media using protein-A sepharose (Amersham Biosciences) columns. FLAG fusion proteins were purified by batch adsorption onto anti-FLAG resin followed by packing into a column, low pH elution, and neutralization. A secondary gel filtration or ion exchange column was run as a final polishing step. Protein purity was assessed by SDS-PAGE and Coomassie Blue staining, and all proteins were >90% pure.

**Protein microarray screen.** Construction of protein microarrays on epoxysilane-coated slides (Schott) and the details of the screening methodology have previously been described (15). Two protein libraries containing secreted or extracellular domains of single-transmembrane proteins were printed across two slides and used for the Robo3 ligand screen. Library 1 and Library 2 consisted of 1,334 and 624 purified protein samples, respectively, representing 686 and 562 genes (92 genes were present in both libraries) (16). A 1:1 mix of the extracellular domain of Robo3 fused to Fc and Cy5-labeled-hIgG was allowed to complex with multivalent protein-A microbeads (Miltenyi Biotech). Protein microarray slides were processed using an automated aHyb hybridization station (Miltenyi Biotech) for blocking, incubation with the Robo3-Cy5-hIgG multivalent bead complex, and wash steps. Slides were rinsed in a final PBS wash, dried, and scanned for Cy5 fluorescence using a GenePix 4000B scanner (Molecular Devices). Fluorescent intensities were analyzed using GenePix Pro 6.0 (Molecular Devices). Data analysis was carried out as described before (15).

**SPR.** Analysis of protein interactions by SPR was performed on a Biacore 3000 (GE Healthcare) at 25 °C using CM5 sensor chips in 10 mM HEPES (pH 7.4), 0.15 M NaCl,
0.005% Surfactant P20 running buffer (HBS-P) at a flow rate of 30 µl/min. The extracellular region or Ig domains of Robo3 fused to an Fc tag (Robo3-ECD-Fc and Robo3-Ig5-Fc) or the FNIII domains of Robo3 fused to a FLAG tag (Robo3-FNIII-FLAG) were immobilized to CM5 sensor chips by amine coupling and binding analysis was carried out with NELL2-Fc or negative controls injected over each surface for 3 min at concentrations of 5, 20, 25, or 50 µg/ml. Dissociation in HBS-P was allowed for 3 min. The surface of the chip was regenerated after each injection of sample with 10 mM Glycine-HCl (pH 3.0). Immobilization levels were 10100, 15200, and 1600 RU for Robo3-ECD-Fc, Robo3-Ig5-Fc, and Robo3-FNIII-FLAG, respectively. Additional binding experiments were carried out by SPR measurements on a ProteOn XPR36 (Bio-Rad Laboratories) instrument at 25 °C. Mouse Slit2-His (R&D Systems) was immobilized at a surface density of 2000 RU on an activated ProteOn GLC sensor chip using standard amine coupling procedures as described by the manufacturer. Analytes (200 nM) were injected in PBS with 0.005% Tween-20 and 0.3 M NaCl at a flow rate of 80 µl/min, and sensograms for association and disassociation phases were recorded. Analytes were injected for 300 s and allowed to disassociate for 600 s. Data was referenced with interspots and processed with the ProteOn Manager software (Bio-Rad).

**Flow cytometric analysis of protein interactions.** NELL2-EGFP-GPI was expressed in HEK293T cells. Cell surface expression was detected with anti-GFP-APC (R&D Systems). Biotin labeling of Robo3-ECD-Fc was carried out at a concentration of 2 mg/ml with 20-fold molar excess of sulfo-NHS-LC-biotin (Thermo Scientific) in PBS for 30 min. Excess non-reacted biotin reagent was removed with Zeba spin desalting columns (Thermo Scientific). To determine the EC50 for the Robo3 and NELL2 interaction, biotin-labeled Robo3-ECD-Fc was incubated with transfected cells at 4 °C for 1 h at concentrations ranging from 1 nM to 1000 nM. Binding was detected with streptavidin-
APC (BD Biosciences) followed by flow cytometric analysis. The mean APC fluorescence was calculated after gating for GFP positive cells and then plotted against their respective Robo3 concentrations on a log-scale. The EC$_{50}$ is defined as the biotin-labeled Robo3-ECD concentration required to reach 50% maximal binding to HEK293T cells expressing GPI-anchored NELL2 on the surface and was calculated from the four-parameter curve fitting of the dose-response curve using Prism 5. Presented data is representative of three independent experiments, each containing triplicate wells at 3-fold serial dilutions of Robo3-ECD-Fc starting at a 1000 nM dose. Competition reactions were set up with a fixed concentration of biotin-labeled Robo3-ECD-Fc (30 nM), and transfected cells were pre-incubated with unlabeled Robo3-ECD-Fc competitor at 1000 nM and 3000 nM at 4 °C for 1 h. Competition was measured by changes in streptavidin-APC staining for GFP-positive cells. For domain mapping experiments, transfected cells were incubated with recombinant Robo3-ECD-Fc, Robo3-FNIII-Fc, or negative control, FNDC5-Fc at 4 °C for 1 h. Protein binding was detected with APC-labeled anti-human IgG, (Jackson ImmunoResearch) and analyzed by flow cytometry.

**Cell culture and transfection.** COS-7 and HEK239 cells were grown and transfected as previously described (25). For COS-7 transfections, a DsRed2 reporter plasmid (14) was used at a 1/10 (w/w) ratio of reporter to expression plasmid.

**Neuronal explant culture.** Dorsal spinal cord explants and post-crossing explants from E11.5 mouse embryos were dissected and cultured in collagen gel as previously described (5, 20). Aggregates of COS-7 cells were prepared the day after transfection and placed 200-500 µm away from tissue explants. Explant/COS-7 co-cultures were grown for 20-22 h in DSC medium (50% OptiMEM, 45% Ham’s F-12, 1 x penicillin/streptomycin/glutamine (P/S/G) (all Gibco), 5% horse serum, 0.75% glucose),
and Netrin-1 (R&D Systems, 500 ng/ml) was included in the medium for dorsal spinal cord cultures. For growth of dorsal spinal cord explants followed by AP fusion protein binding or Robo3.1 immunohistochemistry, explants were cultured on poly-D-lysine- and N-Cadherin-coated 8-chamber glass slides with Neurobasal-A, 2% B-27 (both Gibco), 1 x P/S/G, and 500 ng/ml Netrin-1 as growth medium.

**AP fusion protein binding assays.** AP fusion proteins were expressed in HEK293 cells, and conditioned Opti-MEM was harvested after 48 h. The concentrations of AP fusion proteins were determined by measuring AP activity, and comparable concentrations were used for binding. For binding assays on COS-7 cells, cultures were processed 40-45 h after transfection. For ligand-AP binding to receptor-expressing cells, cultures were rinsed twice in AP binding buffer (HBSS (Gibco), 20 mM HEPES (pH 7.0), 0.2% BSA, 5mM CaCl$_2$, 1 mM MgCl$_2$, 2 µg/ml Heparin) and incubated with diluted AP fusion proteins in binding buffer for 1.5 h at 4 °C. After 3 rinses with binding buffer, cells were fixed for 15 min with 4% paraformaldehyde (PFA) in PBS and rinsed 3 times with 20 mM HEPES (pH 7.0), 150 mM NaCl. Endogenous AP activity was quenched by incubation at 65 °C for 3 h, cells were rinsed twice with AP buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM MgCl$_2$), and AP activity was visualized by incubating cells overnight with NBT/BCIP (Roche; 1:50) in AP buffer. The AP reaction was stopped by 3 rinses in 1 mM EDTA, 0.1% Triton X-100 in PBS, cells were fixed 30 min with 4% PFA in PBS, rinsed 3 times with PBS, and mounted using 80% glycerol in PBS. For receptor-AP binding to ligand-expressing cells, cultures were fixed using 4% PFA in PBS (30 min at 4 °C). After 3 washes in PBS (10 min each), cells were blocked 30 min with 2.5% goat serum, 0.1% Triton X-100 in PBS, and incubated overnight at 4 °C with AP fusion protein diluted in blocking solution. Cells were rinsed 3 times 5 min with PBS and fixed for 15 min with 4% PFA in PBS, at which point the staining was continued by washing
and AP quenching as described above. For ligand-AP binding to dorsal spinal cord explants, the tissue was fixed after 24 h in culture by incubating in 4% PFA in PBS for 30 min at 4 °C. The staining was continued by washing and blocking as described for receptor-AP binding to fixed cells.

**Quantification of NELL2-AP binding to commissural axons.** 4 explants and a total of at least 20 axons per animal were used for quantification. Axonal staining was measured in grayscale images of AP stainings using NIH ImageJ, and background was subtracted from axonal grayscale levels. The average axonal staining intensity was determined for each embryo, and the means from multiple embryos of the same genotype were analyzed for statistically significant differences in an unpaired two-tailed t test (n and p are indicated in figure legend).

**In situ hybridization.** Radioactive in situ hybridization on cervical and brachial mouse spinal cord sections was carried out as described before (12). For colorimetric in situ hybridization, DIG-labeled riboprobes were synthesized with DIG RNA labeling mix (Roche). Slides with cervical and brachial mouse spinal cord sections were rinsed 3 times in PBS and acetylated for 3 min in 100 mM triethanolamine, 10 mM HCl and 10 min in 100 mM triethanolamine, 25 mM acetic anhydride, 10 mM HCl. Slides were rinsed 3 times in PBS and pre-hybridized for 1 h at 65 °C in hybridization solution (50% formamide, 5 x Denhardt’s solution, 0.1% Tween-20, 0.25% CHAPS, 2.5 mM EDTA, 250 µg/ml yeast tRNA, 500 µg/ml herring sperm DNA, 50 µg/mL Heparin in 5 x SSC). Slides were then hybridized with riboprobe in hybridization solution for 30-36 h at 55 °C and washed at 55 °C in 4 x and 2 x SSC with 100 mM DTT for 20 min each. After a 10 min pre-incubation at 37 °C in RNase buffer (100 mM Tris (pH 8.0), 25 mM MgCl₂, 5 mM CaCl₂), slides were incubated for 10 min at 37 °C with 25 µg/ml RNase A (Ambion)
in RNase buffer to remove unbound riboprobe. Slides were then washed at 55 °C in 2 x SSC with 100 mM DTT and in 0.1x SSC for 20 min each. Slides were rinsed in 0.1% Tween-20 in PBS and blocked for 1 h with 10% goat serum and 0.1% Tween-20 in PBS. AP-conjugated anti-DIG antibody (Roche) was pre-absorbed with mouse embryo powder, and slides were incubated overnight with 1:1000 AP-anti-DIG in 10% goat serum and 0.1% Tween-20 in PBS. Slides were then rinsed 3 times in 0.1% Tween-20 in PBS, followed by a 10 min incubation in AP buffer (100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM MgCl2, 0.1% Tween-20, 240 µg/ml levamisole). Slides were developed at 37 °C in AP buffer with 1:50 NBT/BCIP until signal was apparent. Slides were rinsed 2 times for 10 min in PBS, post-fixed for 10 min in PBS containing 4% PFA, rinsed 3 times for 10 min in PBS, and mounted with 80% glycerol in PBS. The used riboprobes span nucleotides 535 through 1,151 of the NELL1 coding sequence (cds) (GenBank accession NM_001037906) and nucleotides 379 through 936 of the NELL2 cds (GenBank accession NM_001289653). Labeling with sense probes resulted in weak, uniform staining for each gene (data not shown). NELL2 in situ hybridization on spinal cord sections from E11.5 NELL2 mutant embryos, did not produce detectable signal (Fig. S4C,D).

**qRT-PCR.** E11.5 embryos were homogenized at 4 °C in RNA STAT-60 (Tel-Test), and RNA was isolated according to the manufacturer’s recommendations. After treatment with DNAsse I, RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). cDNA was used for quantitative PCR with iQ SYBR Green Supermix (Bio-Rad) on a StepOne Plus thermocycler (Applied Biosystems). The primers used were CATGGCCTTCCGTGTCC and CAGTGGGCCCTCAGATGC for GAPDH, CATGGAATCCCGGGTGTTACT and CTTGGCGCAGACTCCATCTGTAG for NELL2 exons 2 and 3, and CCTTAGCCTTCAGTGCTCCTCA and
TGTGCATTATTCTCTGTCCAAA for NELL2 exons 5 and 6. Linearity of the PCR reactions was confirmed by analyzing serial dilutions of samples, and each reaction was performed in duplicate. Relative NELL2 expression levels were normalized to GAPDH expression, and the means from multiple embryos of each genotype were determined and compared in an unpaired two-tailed t test (n and p are indicated in figure legend).

**Immunohistochemistry.** Unless indicated otherwise, all incubations were performed at room temperature. For immunofluorescence on cervical and brachial spinal cord sections, embryos were processed and stained as previously described (8). For immunohistochemistry on COS-7 cells, cultures were processed 40-45 h after transfection by rinsing with PBS and fixing with 2% PFA in PBS for 30 min at 4 °C. Cells were rinsed twice in PBS, blocked 10 min with 2.5% BSA and 0.1% Triton X-100 in PBS, and incubated overnight at 4 °C with primary antibodies in blocking solution. After 3 10 min washes with blocking solution, cells were incubated with secondary antibodies in blocking solution for 2 h. Cells were then washed 3 times 10 min with blocking solution, rinsed with PBS, fixed 10 min with 2% PFA in PBS, rinsed 3 more times with PBS, and mounted using Fluoromount G (Electron Microscopy Sciences). The same staining protocol was used for dorsal spinal cord explants grown on glass slides. Collagen-embedded explants were fixed in PBS containing 4% PFA for 2 h, washed three times 10 min in PBS, blocked in 2.5% goat serum and 0.1% Triton X-100 in PBS for 2 h, and incubated with primary antibody in blocking solution at 4°C overnight. After six 1 h washes in 0.1% Triton X-100 in PBS at 4°C, explants were incubated with secondary antibody in blocking solution at 4°C overnight. Explants were washed six times 1 h in 0.1% Triton X-100 in PBS at 4°C and mounted on hanging drop slides using Fluoromount G. The primary antibodies used were mouse monoclonal antibodies against TAG-1 (clone 4D7, Developmental Studies Hybridoma Bank; 1:200) and NELL1 (clone
M01, Novus; 1:100), a rat monoclonal antibody against L1 (Chemicon; 1:200), and rabbit polyclonal antibodies against TuJ1 (Covance; 1:1000), NELL2 (Sigma; 1:100), Robo3 ECD (described previously (26); 1:200), Robo3.1 (described previously (14); 1:200), and HB9 (described previously (27); 1:10000). Secondary antibodies (all from Invitrogen; 1:200) were Alexa488-conjugated goat anti-mouse, Alexa488-conjugated goat anti-rat, Alexa488-conjugated goat anti-rabbit, Alexa594-conjugated goat anti-mouse IgM, Alexa594-conjugated goat anti-rat, and Alexa594-conjugated goat anti-rabbit. Hoechst 33342 (Invitrogen; 1:1000) was added with secondary antibodies. All images were acquired on a Zeiss Axioplan 2 microscope or a Nikon Eclipse 90i microscope.

Quantification of axon growth and repulsion in vitro. Between 7 and 31 TuJ1-stained explants per experimental condition were used for quantification. Axonal bundles were traced using the NeuronJ plugin for NIH ImageJ. For post-crossing explants, the average summed length of axons per explant for each experimental condition was determined, and means from four independent experiments were compared in a paired two-tailed t test. For dorsal spinal cord explants, the total summed lengths of axons in the proximal, intermediate, and distal quadrants of all explants relative to the COS-7 aggregates were determined and divided by the total summed length from all quadrants of all explants in the experimental condition. The means for relative proximal and distal growth from several independent experiments were compared in a paired two-tailed t test (n and p are indicated in figure legends). Total summed axon length was also compared in a paired two-tailed t test (n and p are indicated in main text). For comparison of Robo3 mutant explants and controls, tissue from several Robo3-/- embryos was pooled and treated as a group and explants from wild type and Robo3 heterozygous embryos were pooled as the control group. Means for relative proximal and distal growth from three independent experiments were compared in a paired two-tailed t test (n and p are indicated in figure
legend). For comparison of wild type and Robo1<sup>−/−</sup>; Robo2<sup>−/−</sup> explants, each embryo was considered an independent experiment, and means for relative proximal and distal growth from several wild type and Robo1/2 double mutant embryos were compared in an unpaired two-tailed \( t \) test (n and \( p \) are indicated in figure legend). To quantify axon turning, axon bundles in the intermediate quadrants of dorsal spinal cord explants (see above) were scored as turning towards or away from COS-7 aggregates with a cutoff value of 10 degrees deviation from a trajectory that is perpendicular to the explant surface. The ratio of the number of axons turning towards cell aggregates to the number of axons turning away was determined for each experimental replicate, and the means from several independent experiments were compared in a paired two-tailed \( t \) test (n and \( p \) are indicated in main text).

**Quantification of commissural axon invasion of spinal cord ventral horn.** Between 4 and 9 sections per embryo were used for quantification. TAG-1-positive axons within the HB9-positive area of the spinal cord were traced using the NeuronJ plugin for NIH ImageJ. The outline of the HB9-positive area was drawn blinded to embryo genotype and excluded medially located migrating motor neurons (Fig. 4A-J). There were no significant differences in the size of the HB9-positive area between different genotypes (data not shown). The average summed axonal length per section was determined for each embryo, and the means from multiple embryos of the same genotype were analyzed for statistically significant differences by one-way ANOVA. Post-hoc comparison of all genotypes was performed using Holm’s test with \( \alpha = 0.05 \) (n and \( p \) are indicated in figure legends).
Fig. S1: Interaction between Robo3 and NELL family members. (A) An interaction screen using Robo3-ECD-Fc was performed in duplicate (Arrays 1, 2) against two extracellular protein microarray libraries, revealing that Robo3-ECD-Fc binds NELL2-Fc. Black dots represent individual protein samples. Box represents hit cutoff value of 5 after quantile normalization. (B) The Robo3-NELL2 interaction was examined in a cell-based binding assay. Netrin-1-AP specifically binds to DCC-expressing cells, Slit2-N-AP specifically binds to Robo1- and Robo2-expressing cells, and NELL2-AP binds to cells expressing Robo3.1 or Robo3.2 (inset), but not to cells expressing DCC, Robo1, or Robo2. Weak background binding is observed with Netrin-1-AP and Slit2-N-AP on untransfected cells. (C) Robo1 and Robo3 binding to Slit2 was tested by SPR. SPR sensograms show binding of Robo1-ECD-Fc but not Robo3-ECD-Fc or negative control (WIF1-Fc) at indicated concentrations to immobilized mouse Slit2-His. Observed negative binding curves for WIF1-Fc are attributed to nonspecific binding to the reference sensor surface relative to sensors with immobilized Slit2-His. (D,E) The Robo3 domains mediating NELL2 binding were mapped by SPR. SPR sensograms show
binding of NELL2 (but not negative controls) at indicated concentrations to immobilized Robo3-FNIII-FLAG (E), but not Robo3-Ig-Fc (D). (F-J) Robo3 binding to NELL2 on cells was tested by flow cytometry (FACS). Cell surface expression of NELL2 as GPI-anchored EGFP fusion was confirmed by FACS using anti-GFP-APC and total endogenous GFP in untransfected (F) and transfected (G) HEK293T cells. No binding was observed to negative control FNDC5-Fc (H) in cells expressing NELL2-EGFP-GPI using fluorescently labeled anti-hIgG antibody. Binding to Robo3-ECD-Fc (I) was observed using the same FACS assay. Binding curves on NELL2-EGFP-GPI-positive cells are shown for a fixed amount (30 nM) of biotinylated Robo3-ECD-Fc competed with unlabeled Robo3-ECD-Fc at the indicated concentrations, as determined by Streptavidin-APC detection (J). Baseline represents Streptavidin-APC binding without Biotin-Robo3-ECD-Fc. (K) The Robo3 FNIII domains are sufficient to mediate NELL2 binding, as determined by FACS using Robo3-FNIII-Fc on NELL2-EGFP-GPI-expressing cells. (L,M) Robo3 binding to NELL1 was tested in a cell-based assay. COS-7 cells were transfected with Robo3.1 and incubated with NELL1-AP. NELL1-AP binds Robo3.1-transfected, but not untransfected cells (L). In a reverse cell-based binding assay, Robo3-FNIII-AP specifically binds COS-7 cells transfected with either NELL1 or NELL2 (M). Scale bar: 200 µm in B,L,M.
Fig. S2: Failure to detect *NELL1* expression in the developing spinal cord. (A-D) Transverse spinal cord sections from E9.5 (A), E10.5 (B), E11.5 (C), and E12.5 (D) mouse embryos were used for radioactive *in situ* hybridization to detect *NELL1* mRNA. *NELL1* is not expressed in spinal cord at any of developmental time points examined. Scale bar: 200 µm in A-D.
Fig. S3: Lack of pre-crossing commissural axon response to NELL1 and post-crossing response to NELL2. (A) A schematic of commissural axon projections and the assay for commissural axon responses to NELL1 and NELL2. (B-E) COS-7 cells were transfected with the indicated expression vectors and stained with antibodies against NELL1 and NELL2 to confirm expression. A NELL1 antibody detects NELL1 protein expression in NELL1-transfected cells (C), but not control cells (B). A NELL2 antibody stains NELL2-transfected cells (E), but not control cells (D). (F) Commissural axon growth in proximal (p), intermediate (i), and distal (d) quadrants relative to COS-7 aggregates was quantified. Axon growth in the quadrant proximal to RFP-expressing cells is similar to distal growth ($p = 0.1299$, $n = 5$ independent experiments). With NELL2-expressing cells, proximal growth is significantly lower than distal growth ($p < 0.0001$, $n = 5$). Proximal growth is significantly lower with NELL2-expressing cells than with control cells ($p = 0.0009$). (G-I) Dorsal spinal cord explants from E11.5 mice were co-cultured with COS-7 cells expressing RFP (G) or RFP and NELL1 (H), stained for TuJ1, and commissural axon growth relative to cell aggregates was quantified (I). Axon growth proximal to RFP-expressing (control) cells is similar to distal growth ($p = 0.2268$, $n = 3$ independent experiments).
When confronted with NELL1-expressing cells, proximal axon growth is significantly lower than distal growth \( (p = 0.0285, n = 3) \), but proximal growth is not significantly different between control and NELL1-expressing cells \( (p = 0.4815) \). (J) Proximal growth relative to control and NELL2-expressing cell aggregates from \( Robo1^{-/-}; Robo2^{-/-} \) (n = 3 embryos) and wild type dorsal spinal cord explants (n = 6) was quantified. Proximal growth relative to NELL2-expressing cells is indistinguishable between the two genotypes \( (p = 0.1332) \). (K) A schematic of the \textit{in vitro} assay for post-crossing commissural axon responses to NELL2. (L,M) Half spinal cord explants from E11.5 mice were cultured either with RFP-expressing cells (L) or with cells expressing RFP and NELL2 (M) and labeled with TuJ1 antibody. Commissural axons leave spinal cord explants through the floor plate under both conditions. (N) Post-crossing axon growth was quantified and is not statistically different between the two experimental conditions \( (p = 0.4488, n = 4) \). Scale bar: 100 \( \mu \)m in B-E; 200 \( \mu \)m in G,H,L,M. Error bars indicate SEM.
Fig. S4: Analysis of NELL2 function in vivo. (A) A schematic of the targeting strategy to inactivate NELL2. In the NELL2 conditional allele, exons 2 and 3 are floxed. In the null allele, these exons are deleted. (B) NELL2 mRNA abundance in E11.5 NELL2−/− embryos and wild type littermates was determined by qRT-PCR for exons 2-3 and exons 5-6. NELL2 mRNA levels in the NELL2 mutant (n = 3 embryos) are significantly reduced (p = 0.0021 for exons 2-3; p = 0.0187 for exons 5-6) compared to wild type (n = 5). (C,D) Transverse spinal cord sections from E11.5 NELL2−/− embryos and wild type littermates were used for colorimetric in situ hybridization to detect NELL2 mRNA. NELL2 mRNA is detectable in the ventral horn and cells surrounding the central canal in wild type (C) but not NELL2−/− embryos (D). (E-J) Transverse sections of E11.5 spinal cords were labeled with antibodies against TAG-1 and HB9. Shown are double stainings (E-I) and an inverted grayscale image of TAG-1 labeling alone (J). Commissural axons project to the ventral midline and avoid the area occupied by motor neurons in the ventral horn (dotted outline) in Robo3+/− (E), NELL2−/− (G), and NELL1+/−; NELL2−/− (I,J) embryos, but invade this region in Robo3−/− (F) and NELL2−/−; Robo3+/− embryos (H). (K) TAG-1-
positive axon growth in the HB9-positive ventral horn was quantified. There is no significant difference between $NELL1^{-/-}; NELL2^{-/-}$ ($p = 0.4706; n = 3$) and wild type embryos. (L,M) Transverse sections of E11.5 spinal cords from wild type and $NELL2^{-/-}; Robo3^{+/+}$ mice were stained for Robo3. Shown are inverted grayscale images of the left ventral horn. Numerous C-axons (arrowheads) invade the ventral horn in $NELL2^{-/-}; Robo3^{+/+}$ (M), but not wild type embryos (L). Scale bar: 100 µm in C-J, 60 µm in L,M. Error bars indicate SEM.
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<tr>
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<td>C-term. AP</td>
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<td>Rat</td>
<td>I28-K1051</td>
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**Table S1: Protein expression constructs.** Expression was driven with a Cytomegalovirus (CMV) promoter, a combined CMV/β-actin (CAG) enhancer/promoter, a Simian virus 40 (SV40) promoter, or a Major Adeno Late (MAL) promoter. The species of origin and amino acid range for the coding sequence (cds) of interest and the position of ectopic sequences fused to the cds are indicated.
References and Notes


24. A. A. Lobito, S. R. Ramani, I. Tom, J. F. Bazan, E. Luis, W. J. Fairbrother, W. Ouyang, L. C. Gonzalez, Murine insulin growth factor-like (IGFL) and human IGFL1 proteins are induced in inflammatory skin conditions and bind to a novel tumor necrosis factor

