Supplementary Materials and Methods

Sympathetic neuron treatments. Stimulation of SCG neurons with Sema3A was performed using tdT-Sema3A or AP-Sema3A (5nM) along with NGF (25ng/ml) for 6 hrs or overnight (37°C, 8% CO_2). To inhibit caspases, the pan-caspase inhibitor Boc-Asp(OMe)-fluoromethyl ketone (BAF, 50 μM, Sigma) or the caspase-8 specific inhibitor (Caspase-8 Inhibitor II, 5 μM, Calbiochem) were applied concomitant with Sema3A. In compartmentalized cultures, only the distal axons were stimulated, and controls consisted of either tdT alone or AP-Fc (5nM). For retrograde labeling, FluoSphere carboxylate beads (Yellow-Green, Invitrogen) were applied to the distal axons at a 1:2000 dilution of the stock, and cell bodies imaged 24 hrs later. In compartmentalized cultures, microtubule polymerization was arrested in axons in the distal axon compartment by pretreating axons with nocodazole (20μg/ml; Sigma) for 1 hr prior Sema3A treatment and then with 10μg/ml nocodazole along with Sema3A treatment for 6 hours.

siRNA silencing assays. siRNAs targeting Npn-1 (80 nM), PlexinA3 (20 nM) and PlexinA4 (20 nM) were used for RNA interference experiments with the following target sequences:5´-CGGGCGCTTTCCGCAGCGATA-3´, 5´ACCGTGTACAAGGGTATTC CA-3´ and 5´-TACAGCAACTTTGA TGATAAA-3´, respectively (Qiagen). A scrambled siRNA was used as control (20 nM, Ambion). Primary sympathetic neurons at 15 DIV were transfected with these siRNAs using the i-fect siRNA transfection reagent (Neuromics) according to the manufacturer's protocol. After 48 hrs, the transfected neurons were subjected to either immunofluorescence labeling or cell lysis for immunoblotting.

Microscopy and quantification of apoptosis. Immunofluorescence and TUNEL imaging was performed using fluorescence inverted microscopes (Carl Zeiss, AxioVert 200M and Olympus, IX71, Germany). The tdT-Sema3A and FluoSphere retrograde transport in SCG compartmentalized

cultures were visualized using a confocal microscope (Leica microsystems, TCS SP5II, Germany). Images were analyzed by using the image acquisition Zeiss Axiovision or Leica LAS AF software (Carl Zeiss and Leica microsystems, respectively). For activated caspase-3 immunolabeling and TUNEL assays, every third 10µm section throughout the entire ganglion was analyzed for activated caspase-3-positive or TUNEL-positive cells (predicted soma size of an SCG neuron at this age is roughly 30 µm) to avoid double counting of cells. Total neuron counts in the SCG were obtained by counting every TH+ cell in every third 10 µm section throughout the entire ganglion as well. Apoptosis was quantified for activated caspase-3 or P-c-Jun immunofluorescence in cultured SCG neurons as a percentage of activated caspase-3-positive or P-c-Jun-positive cells divided by the total number of DAPI-positive SCG neurons. Quantifications were typically performed by an observer that was naïve to the conditions and data were graphed as the mean ± standard error (SEM).

Construction and analysis of Sema3A fusion proteins. The tandem dimerized tomato-Semaphorin3A fusion protein (tdT-Sema3A) was constructed by standard PCR cloning using the *Tth* DNA polymerase (Applied Biosystems) and sequences from tdT (a generous gift from Samuel L. Pfaff) and rat Sema3A. As a negative control, the tdT-Sema3A expression plasmid was modified such that the tdT stop codon was included. Thus, this protein (referred to as tdT) consists of an expressed tdT and non-expressed Sema3A. In some of these experiments, an alkaline-phosphatase (AP) tagged Sema3A was used to assess the apoptotic effects of Sema3A on sympathetic neurons (Kolodkin et al., 1997). AP-Sema3A, which has been extensively characterized previously, has high affinity for Npn-1 (1.5 nM K_D) and is highly bioactive, as assessed by growth cone collapse assays in DRG explants (Kolodkin et al., 1997). Conditioned cell culture supernatants from transfected HEK293 cells were collected and concentrated using centrifugal filter units (MWCO 30kDa, Millipore).

Bioactivity of Sema3A fusion proteins. To confirm the functionality of the tdT-Sema3A fusion protein, HEK293T cells were transiently transfected with either pIRES-EGFP (GFP) or full-length rat Npn-1

in pIRES-EGFP (GFP-Npn-1). After 24 hours, the cells were rinsed with PBS and incubated with 5nM of either tdT or tdT-Sema3A for 90 minutes at RT. Following three additional washes with PBS, the cells were fixed with 4% paraformaldehyde (PFA) for 40 min, rinsed 3 times with PBS, and analyzed by fluorescence microscopy. To assess the bioactivity of tdT-Sema3A, a neurite repulsion assay on E18 dorsal root ganglion (DRG) explants was performed as described previously (Kolodkin et al., 1997). Briefly, HEK293T cells were transiently transfected with either tdT-Sema3A or tdT. After 24 hours, the cells were trypsinized and collected in "hanging droplets" and the droplets were incubated for 5 hrs at 37°C to form cell aggregates. In a collagen matrix, a DRG explant was placed in close proximity to aggregated HEK293T cells. After 30 min at RT, neuronal growth medium (Neurobasal medium with B-27 supplement, glutamine, penicillin/streptomycin, and 15ng/ml NGF) was added and the co-cultures were incubated at 37°C for 48 hrs. The co-cultures were then fixed with 4% PFA for 1 hour, washed 3 times with PBS, and immunolabeled with the 2H3 anti-neurofilament antibody (1:500 dilution, Developmental Studies Hybridoma Bank, University of Iowa). For quantification, the length of axonal projections on the proximal (P) and distal (D) sides of the DRG explant was measured and used to calculate the P/D ratio. 16 explants were analyzed for each condition (from 3 independent experiments). Results are presented as mean ± SEM ($P < 0.001$, unpaired t-test).

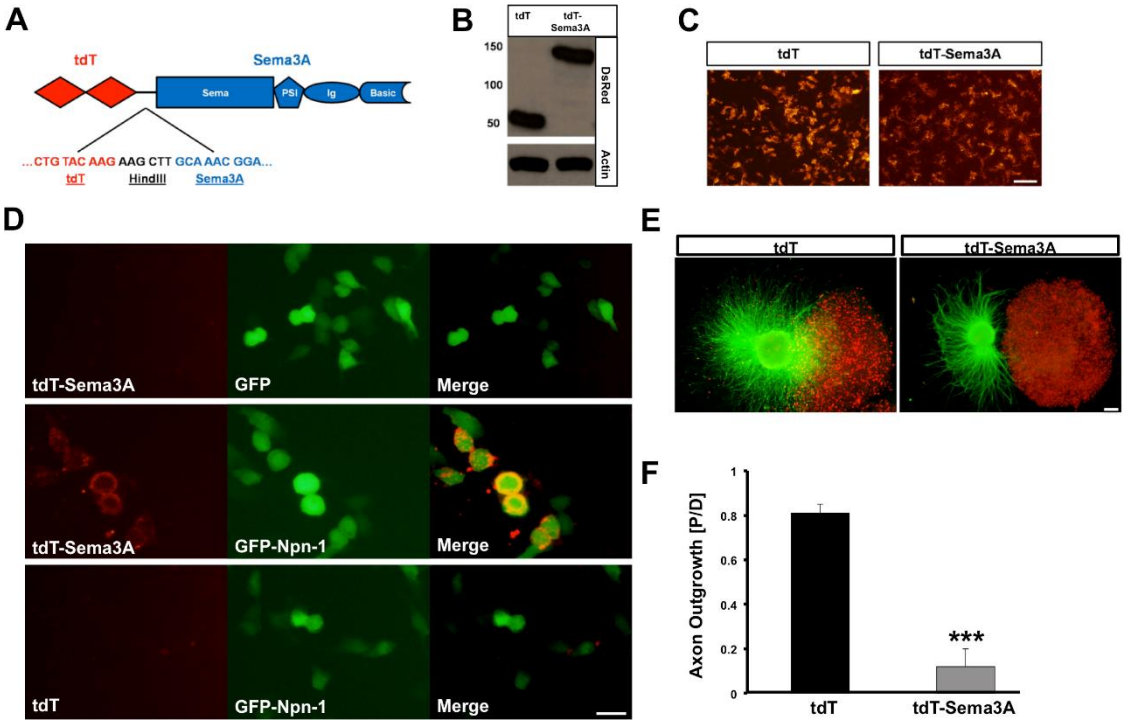

Fig. S1. Confirmation of tdT-Sema3A functionality. (A) Schematic diagram of the tdTomato-Semaphorin3A fusion protein (tdT-Sema3A). Tandem dimerized tomato was fused in frame to the amino terminus rat Sema3A (23 amino acids after the start codon - blue). Sema is the amino-terminal semaphorin domain; PSI is the plexin-semaphorin-integrin domain; Ig is the immunoglobulin-like domain; Basic is the basic C-terminal domain. (B) Immunoblotting using conditioned media from HEK293T cells transfected with either tdT or tdT-Sema3A revealed the expected protein sizes when probed with an anti-DsRed antibody. Actin immunoblotting confirmed analysis of equal amounts of protein. (C) HEK293T cells that were transfected with either tdT or tdT-Sema3A display robust tdT fluorescence. (D) HEK293T cells were transfected with either GFP or GFP-Npn-1, and binding of tdT or tdT-Sema3A was assessed 24 hours later. Binding is only observed between the tdT-Sema3A fusion protein and GFP-Npn1-transfected cells. (E) E18 rat dorsal root ganglion (DRG) explants were co-cultured in a collagen matrix with HEK293T cells expressing either tdT or tdT-Sema3A and grown for 48 hours. DRG axonal projections were labeled with 2H3 anti-neurofilament antibody. DRG

axons are repelled by the tdT-Sema3A-expressing HEK293Ts, but not by the tdT-expressing cells. (F) Quantification of the repulsion assay shown in (E). The length of axonal projections on the proximal (P) and distal (D) sides of the DRG explants was measured and used to calculate the P/D value. 16 explants from 3 independent cultures were analyzed. Gray bar (tdT); black bar (tdT-Sema3A). Results are presented as mean ± SEM. (***) is $P< 0.001$ using student's t-test. Scale bar, (A) 30 μm, (B) 200 μm.

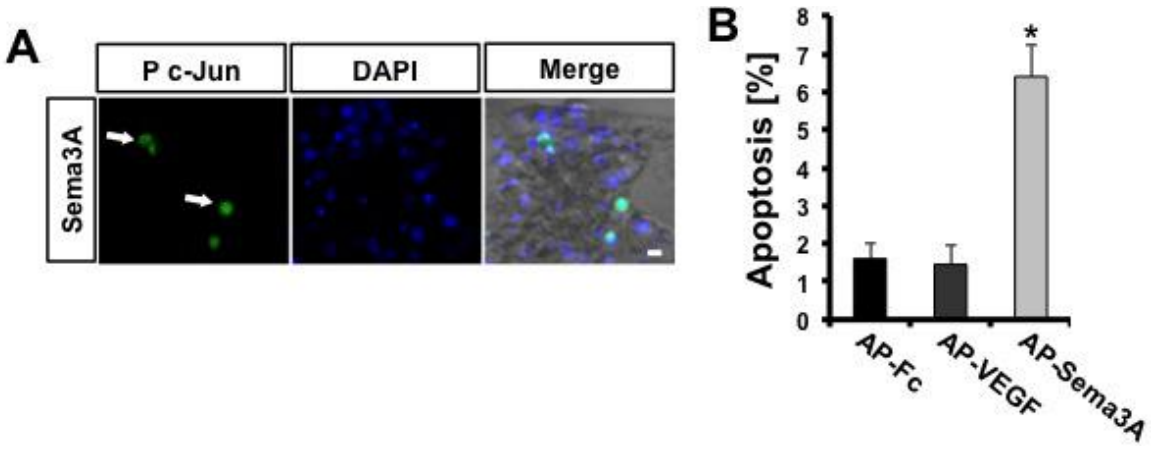

Fig. S2. Sema3A induces c-Jun phosphorylation in SCG neurons. (A) Phospho-c-Jun immunolabeling was detected in Sema3A-stimulated sympathetic neurons (arrows). (B) Quantification of the number of phospho-c-Jun positive neurons, relative to the total number of neurons, when stimulated with AP-Sema3A, AP-VEGF or AP-Fc are presented as mean ± SEM from four independent experiments. (*) $P < 0.05$, using student's t-test. Scale bar, 10μm.