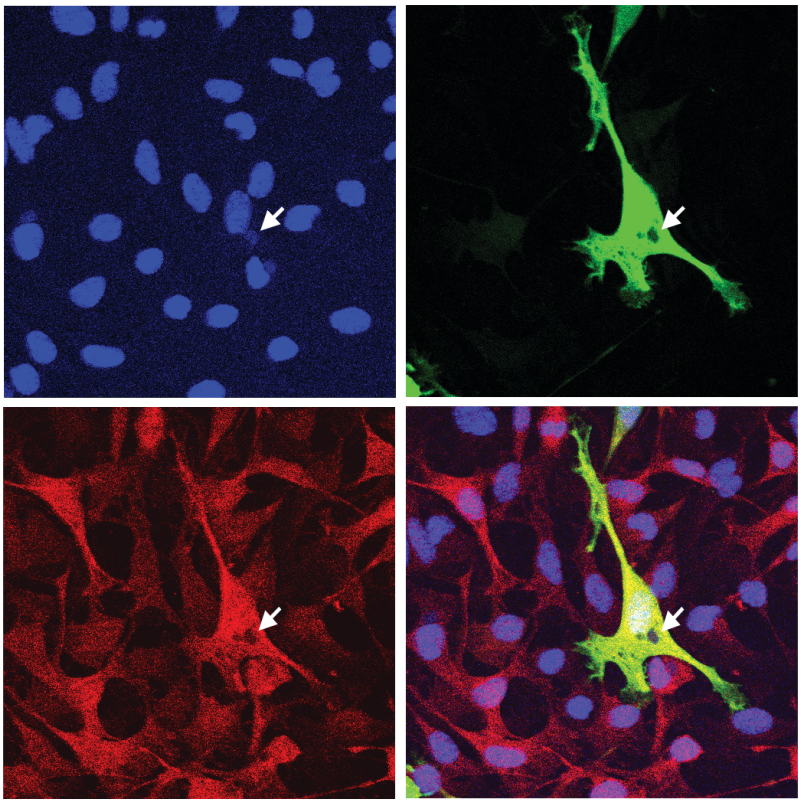
Glial Precursors Clear Sensory Neuron Corpses during Development via Jedi-1, an Engulfment Receptor

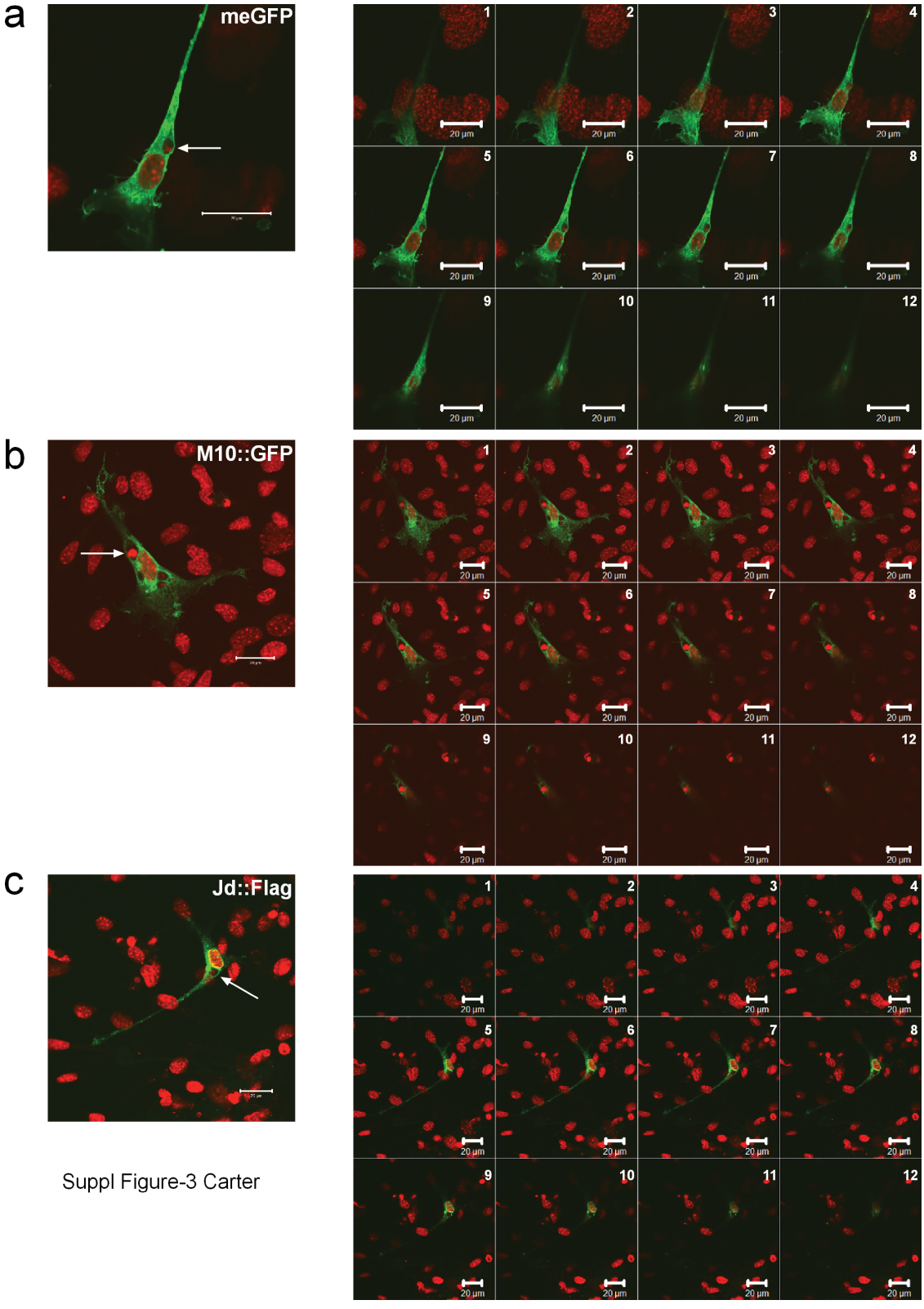
Hsiao-Huei Wu, Elena Bellmunt, Jami L. Scheib, Victor Venegas, Cornelia Burkert, Louis F. Reichardt, Zheng Zhou, Isabel Farinas, and Bruce D. Carter



Supplementary Figure 1. Glial precursors engulf apoptotic neurons in culture. Primary mixed cultures of sensory neurons and SGCs were derived from dissociated E15 rat DRG and cultured with NGF for 2 days. The cells were then transfected with meGFP, and NGF removed to induce neuronal apoptosis. The cultures were fixed 2 days later and immunostained for BFABP. Images were compiled from orthogonal optical sections showing apoptotic nuclear remnants [TO-PRO3, blue] inside the cytoplasm of meGFP+ [green] glial cells [α-BFABP, red].

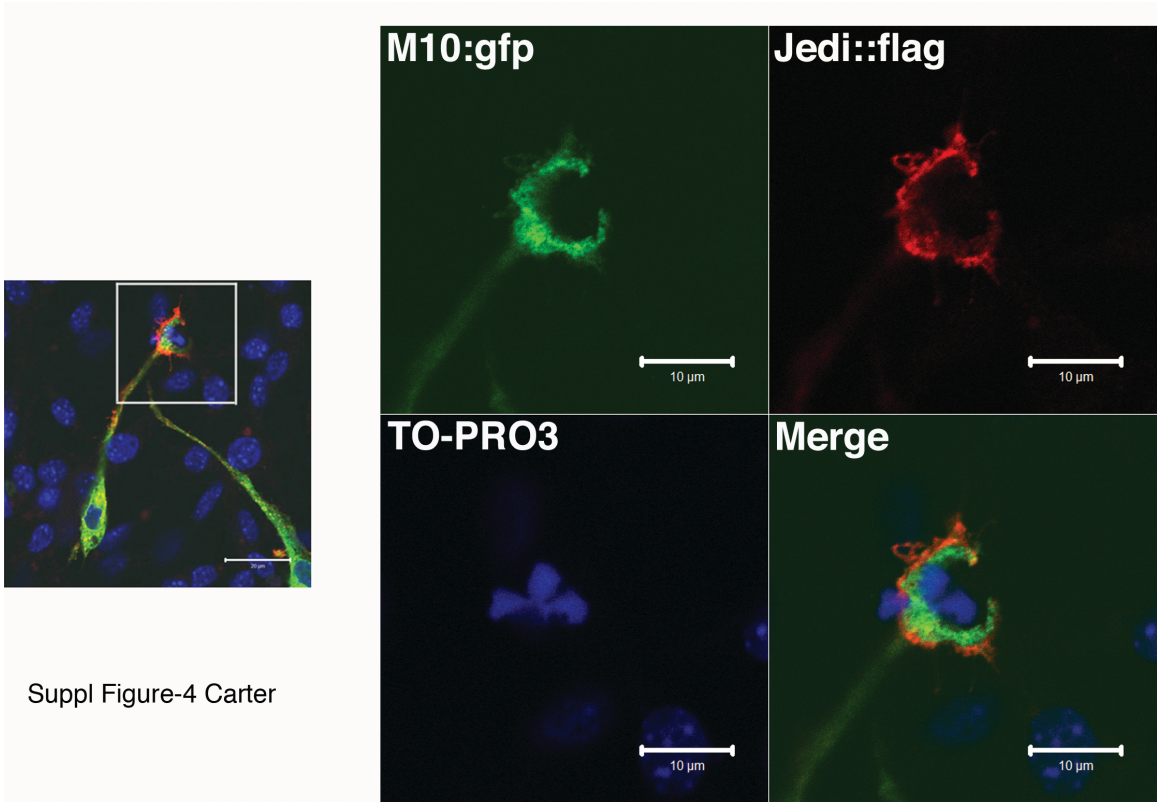
Suppl Figure-2 Carter

Supplementary Figure 2. Sequence alignments of predicted intracellular domains of CED-1, Draper, and their putative mammalian homologs. This alignment was constructed manually and was partially based on the result obtained from CLUSTALW (http://align.genome.jp/). NPXY motifs are shown in bold and underlined. YXXL motifs are shown in bold and italic characters. Other conserved amino acids between Draper, CED-1 and most of the mammalian homologs are indicated in boxes.

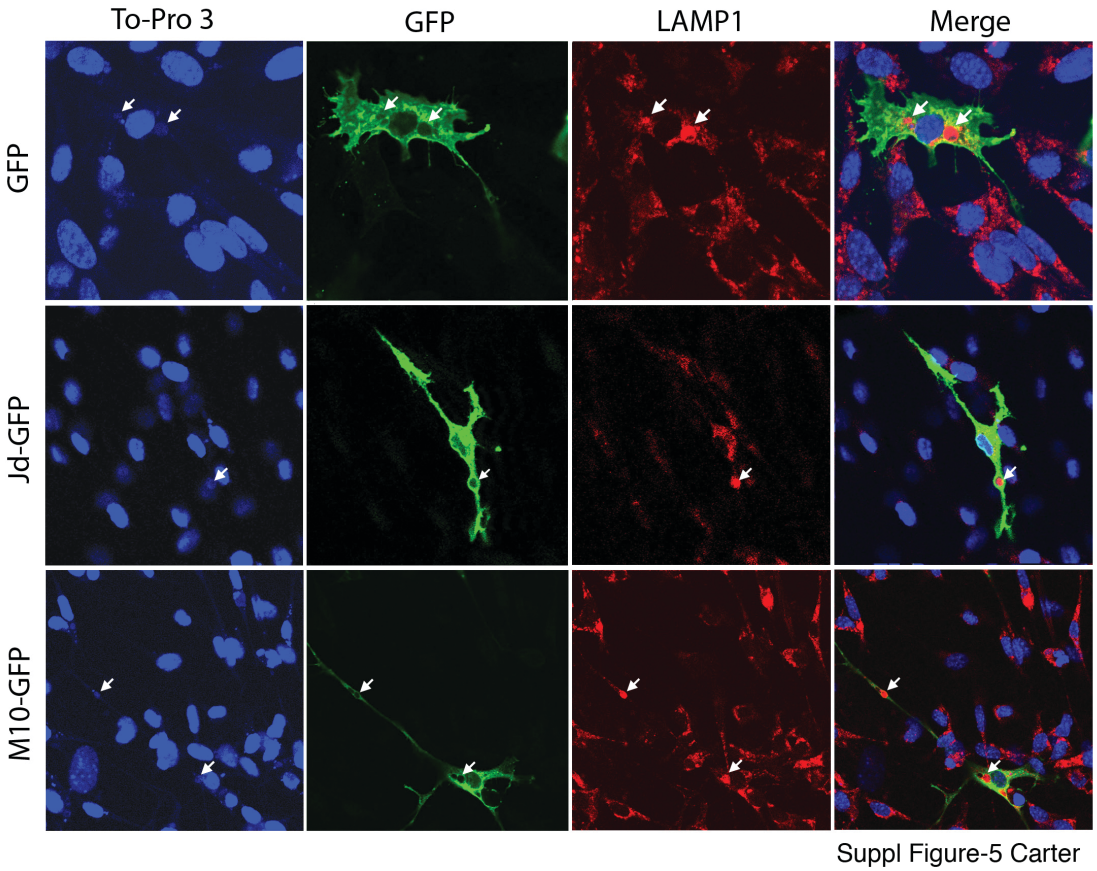


Suppl Figure-3 Carter

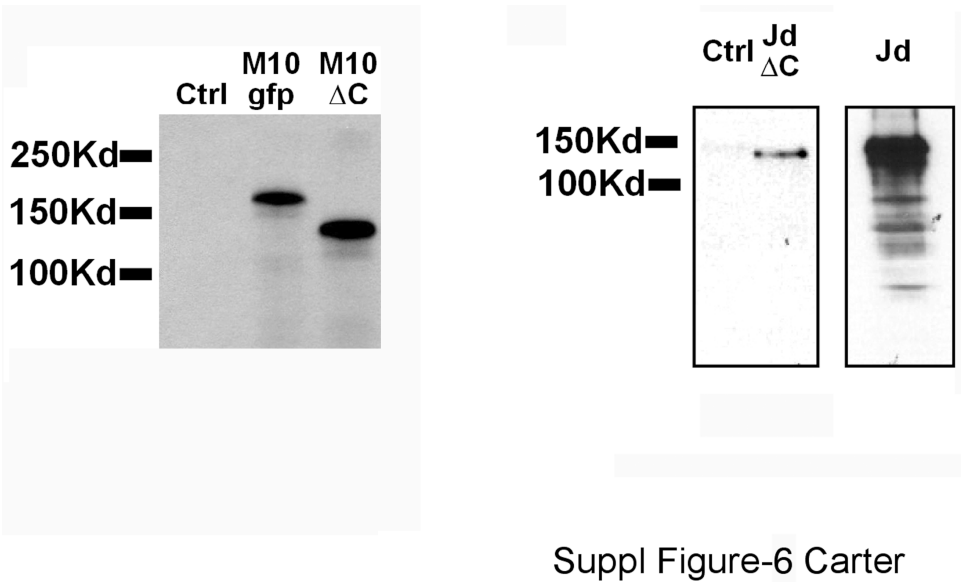
Supplementary Figure 3. Over expression of Jedi-1 or MEGF10 increases engulfment of apoptotic nuclei by glial cell precursors. meGFP (a), MEGF10::GFP (b), or Jedi-1::Flag (c) were transfected into glial cells in DRG mixed cultures and NGF was withdrawn from the growth medium to induce neuronal death. Cultures were fixed 2 days after NGF withdrawal and immunostained with anti-GFP antibody or anti-Flag followed by fluorescently labeled secondary antibodies and TO-PRO3 to stain the nuclei. (Immunostained cells are depicted in green and nuclei in red.) The cultures were then visualized by confocal microscopy. Frame numbers for each Z-axis section are shown on the top right corner of each panel. Arrows point to an engulfed nucleus. Note that while meGFP appeared evenly distributed in the cells, some MEGF10::GFP and Jedi-1::Flag appeared more localized to the area around apoptotic nuclei containing vacuoles.



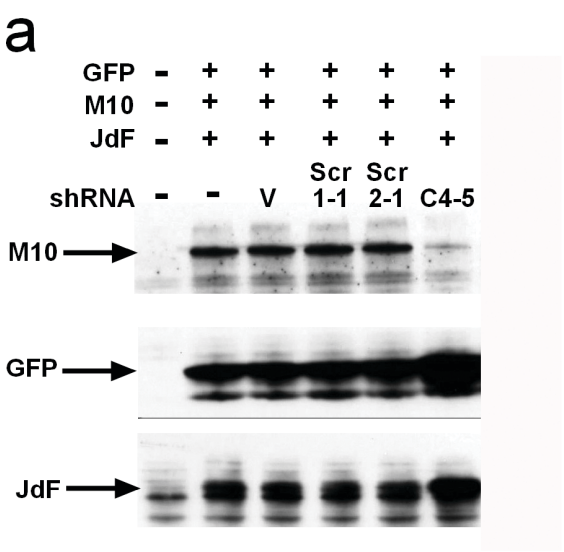
Supplementary Figure 4. Localization of Jedi-1 and MEGF10 to a phagocytic cup. MEGF10::GFP (green) and Jedi-1::Flag (red) were co-transfected into DRG mixed cultures and NGF was withdrawn from the growth medium to induce neuronal death. Cultures were fixed 2 days after NGF withdrawal and immunostained with anti-GFP antibody and anti-Flag followed by secondary fluorescent antibodies and TO-PRO3 to stain the nuclei (blue). Depicted is an example of a glial cell engulfing an apoptotic body with Jedi and MEGF10 clustered at what appears to be the phagocytic cup. (The boxed area is shown enlarged on the right with each fluorophore separated.)

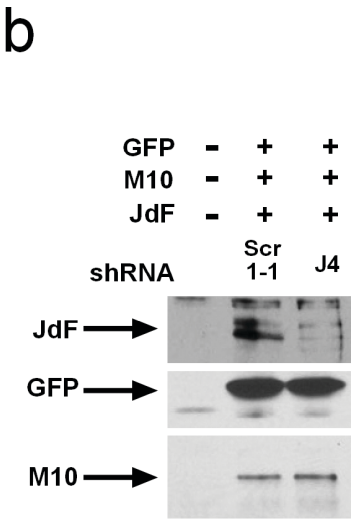


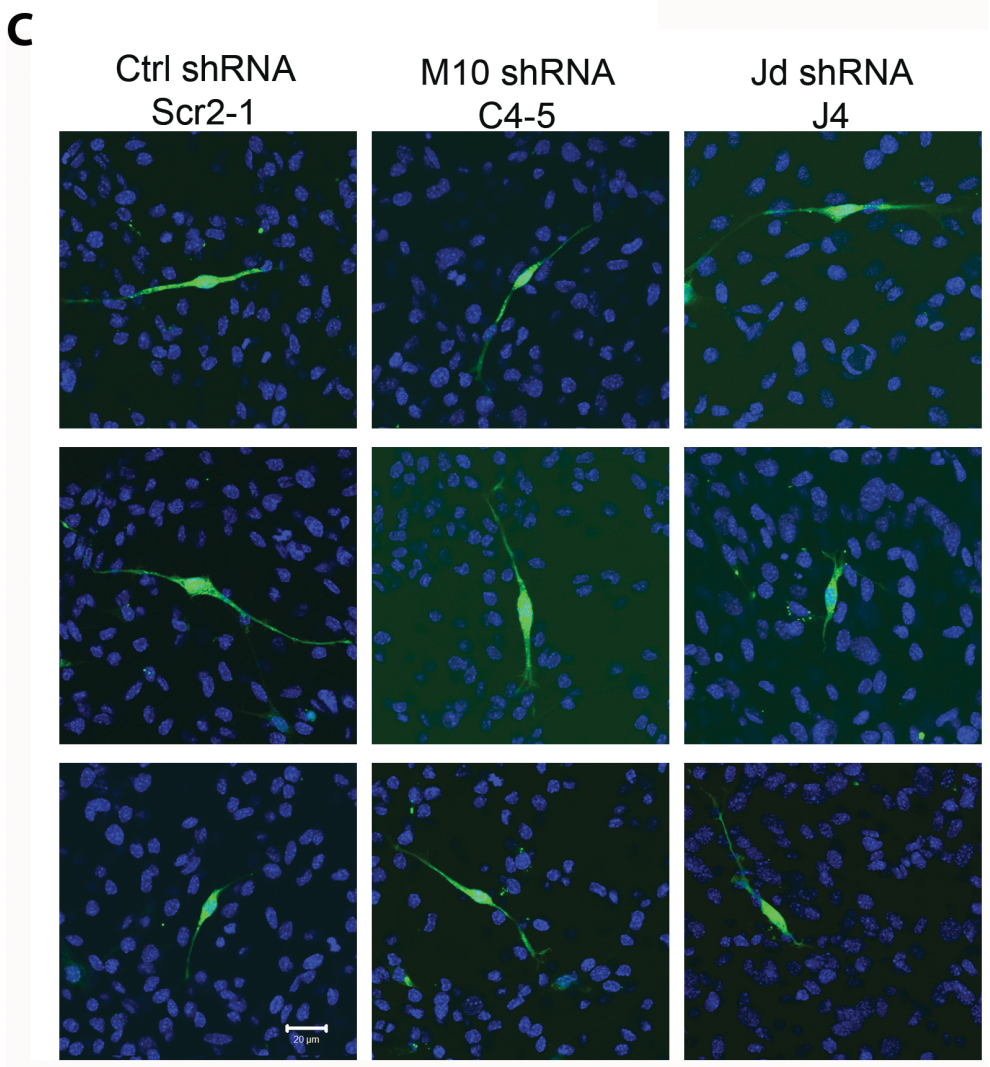
Supplementary Figure 5. Phagocytosed apoptotic nuclei end up in lysosomes in glial cells over expressing GFP, Jedi-1 or MEGF10. meGFP (top row), Jedi-1::GFP (middle row) or MEGF10::GFP (bottom row), were transfected into primary DRG mixed cultures and NGF was withdrawn from the growth medium to induce neuronal death. Cultures were fixed 2 days after NGF withdrawal and immunostained with anti-LAMP1 (to label lysosomes) and anti-GFP antibody followed by fluorescently labeled secondary antibodies and TO-PRO3 to stain the nuclei. (Immunostained cells are depicted in green, LAMP1 in red and nuclei in blue.) The cultures were then visualized by confocal microscopy.



Supplementary Figure 6. Expression of full-length and truncated fusion constructs in HEK293 cells. HEK 293 cells were transfected with vector (Ctrl), full length MEGF10::GFP (M10gfp) or Jedi-1::Flag (Jd), or a C-terminal deletion mutant of MEGF10 (M10ΔC) or Jedi (JdΔC) and the proteins detected by Western blot using anti-GFP for MEGF10 constructs and anti-Flag for Jedi constructs.







Suppl Figure-7 Carter

Supplementary Figure 7. Knockdown of Jedi-1 or MEGF10 does not alter satellite precursor cell morphology. (a) Western blot of cell lysates after shRNA-mediated gene silencing shows specific knockdown of MEGF10 but no effect on GFP and Jedi-1 expression. Plasmids transfected into HEK 293 cells are indicated on the top of the blot. Anti-GFP was used to detect MEGF10::GFP (M10) and GFP; anti-Flag was used to detect Jedi-1::Flag (JdF). C4-5, shRNA specific for MEGF10; Scr1-1 and Scr2-1, nontargeting shRNA controls; V, pSIREN-RetroQ-ZsGreen vector. (b) Similar to (a); except, shRNA specific to Jedi-1 (J4) was used to show specific knockdown of Jedi-1 but no effect on GFP and MEGF10. (c) The shRNA vectors targeting MEGF10 (C4-5), Jedi (J4) or a scrambled control (Scr1-1) were transfected into glial cells in primary DRG mixed cultures and NGF was withdrawn from the growth medium to induce neuronal death. Cultures were fixed 2 days after NGF withdrawal. Three representative transfected cells are depicted for each targeting vector (shown in green). Note that there is no significant difference in morphology.

Supplementary Methods

MEGF10::GFP and related constructs:

Mouse MEGF10 cDNA (Accession # BC07564; clone ID:30620548; Open Biosystems) was subcloned into a modified pEGFP-N3 vector (Clonetech) in which the EGFP protein was mutated at amino acid position 206 from an alanine to a lysine resulting in an EGFP protein with limited ability to form homodimer (kind gift of Dr. Graham Carpenter at Vanderbilt University Medical School). A BamHI site was created at the end of the coding sequence of MEGF10 in order to create a fusion protein with the GFP at its C-terminus (MEGF10::GFP). To generate a GFP fusion protein with the intracellular domain of MEGF10 truncated (MEGF10ΔC::GFP), a BamHI site was created at amino acid position 887 and 888 (10 amino acids after the putative transmembrane domain; Ensemble mouse genomic server ENSMUSP00000075174) in the MEGF10::GFP construct. MEGF10ΔC::GFP was generated by excision of the intracellular domain by BamHI digestion and relegation.

Jedi-1::Flag and related constructs:

Jedi-1 cDNA in pCMV-SPORT6 vector (accession # BC042490; protein ID: AAH42490) was purchased from Invitrogen. After comparison with other cDNA sequences in the data base (for example, GenBank accession # AF440279, Protein ID: AAL33583), we found that this cDNA is missing sequences encoding amino acid 375-405. Using RT-PCR, we determined that the major form of cDNA in E15 mouse embryonic tissues contained sequences encoding these missing amino acids (data not shown). We then used mRNA obtained from E15 mouse spinal cord and DRG to obtain portions of Jedi-1 cDNA and generated a full-length mouse Jedi-1 cDNA using BC042490 as the base. A Flag-tagged full length Jedi-1 cDNA in pCMV SPORT6 vector was then generated by inserting the Flag tag sequences (DYKDDDDK) right after Jedi-1 coding sequences followed by a stop codon. To generate a GFP fusion protein with the intracellular domain of Jedi-1 truncated, an Apa I site was created 7-8 amino acids after the predicted transmembrane domain (Ensemble mouse genomic server ENSMUSP00000029714) and a Sal I site was created about 30 nucleotides upstream of the start site. The sequences encoding the extracellular domain, transmembrane domain, and 8 residues after transmembrane domain were subcloned into the modified pEGFP-N3 GFP vector described above.

shRNA:

Potential shRNA target sites of MEGF10 or Jedi-1 were selected using RNAi Target Sequence Selector from Clontech. shRNA oligos were designed using the target sequences shown below with a hairpin loop sequence (5'-TTCAAGAGA). Oligonucleotides were synthesized by OPERON and were cloned into pSIREN-RetroQ-ZsGreen vector (Clontech). for MEGF10: C4-5, 5'-GCTGTAACCTAACGTGTCA-3'; C6-2, 5'-GGTCATCAATGCAGACTAT-3' for Jedi-1:

J4, 5'-CACTTACGGGATCAACTGT-3' control Scr1-1, 5'-GCGTTGAATCGCCTACAAT-3' (created using the web based program: http://bioinfo.wistar.upenn.edu/siRNA/searchScramRNA.cgi , developed by University of Pennsylvania, using C4-5 sequences as a starting point; when blasted against the NCBI mouse database we found no significant homology to *megf10*, *Jedi-1,* or other sequences.)

Western Blot Analysis:

HEK293 cells were transfected with indicated plasmids containing cDNAs or shRNA using Effectene (Qiagen) as recommended by the manufacturer. Cells were harvested in PBS and lysed in buffer consisting of 20 mM Tris-HCl, pH7.6, 2% (v/v) Triton X-100, 4 mM EDTA, 150 mM NaCl, 20% (v/v) glycerol, a mixed protease inhibitor (Roche) and 2 mM PMSF. Protein concentration was determined by *DC* Protein Assay (BioRad). Lysates were fractionated with 10% or 7.5% SDS PAGE and subjected to Western blotting with the indicated antibodies: mouse anti-Flag 1:1000 (Sigma); mouse anti-GFP 1:500 (Roche).