

Glial precursors clear sensory neuron corpses during development via Jedi-1, an engulfment receptor

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During the development of peripheral ganglia, 50% of the neurons that are generated undergo apoptosis. How the massive numbers of corpses are removed is unknown. We found that satellite glial cell precursors are the primary phagocytic cells for apoptotic corpse removal in developing mouse dorsal root ganglia (DRG). Confocal and electron microscopic analysis revealed that glial precursors, rather than macrophages, were responsible for clearing most of the dead DRG neurons. Moreover, we identified Jedi-1, an engulfment receptor, and MEGF10, a purported engulfment receptor, as homologs of the invertebrate engulfment receptors Draper and CED-1 expressed in the glial precursor cells. Expression of Jedi-1 or MEGF10 in fibroblasts facilitated binding to dead neurons, and knocking down either protein in glial cells or overexpressing truncated forms lacking the intracellular domain inhibited engulfment of apoptotic neurons. Together, these results suggest a cellular and molecular mechanism by which neuronal corpses are culled during DRG development.

The extensive neuronal cell death that occurs during the ontogenesis of the peripheral ganglia was first described in the developing chick embryo, leading to the discovery of nerve growth factor (NGF)^{1,2}. An important part of this 'tissue-sculpting' process is to properly dispose of degenerated cellular components, thereby avoiding any inflammatory response³. Although much progress has been made in understanding the regulation of neuronal cell death⁴, little is known about how the vast pool of neuronal corpses is eliminated.

In the developing mammalian CNS, glial cells and microglia have been implicated in the clearance of apoptotic neurons. Infiltration of F4/80-positive macrophages from the developing mouse vasculature into the retina and brain is associated with neuronal death. These invading macrophages further differentiate into microglia and engulf and degrade the apoptotic debris^{5,6}. Early electron microscopy studies in the developing chick peripheral nervous system (PNS) suggested that macrophages, as well as satellite glial cells and their precursors, may be involved in clearing neuronal corpses^{7,8}; nonetheless, the potential function of these glial cells in engulfment and the molecular mechanism involved have not been explored.

The engulfment process used by professional phagocytic cells, including macrophages and dendritic cells, is known to involve an array of receptors on the phagocytes that are able to sense 'find me' and 'eat me' cues exposed by dying cells and 'don't eat me' signals from healthy cells^{9–12}. Whether any of these receptors or cues is involved in clearing dead neurons during PNS development is not known. Recently, a *Drosophila* engulfment receptor, Draper, was identified that is structurally and functionally similar to CED-1, a

phagocytic receptor found in *Caenorhabditis elegans*^{13,14}. Draper is expressed exclusively in macrophages and glia, and Draper-deficient embryos had defects in the clearance of neuronal corpses and degenerating axons^{13,15–17}.

Clearance of apoptotic cells is not just for waste disposal. Non-ingested apoptotic cells typically undergo secondary necrosis, which not only activates immature dendritic cells to become immunogenic, but also exposes normally sequestered self-antigens¹⁸, resulting in an increased risk for autoimmune disease later in life³. To gain insight into how apoptotic neurons are eliminated during DRG development, we investigated the cellular and the molecular mechanisms underlying this clearance process. We found that satellite glial cell (SGC) precursors were the primary cell type responsible for dead neuron clearance. We also identified two receptors that are homologous to CED-1 and Draper as mediators of this engulfment process: MEGF10, recently reported to be a CED-1 homolog¹⁹, and Jedi-1, an engulfment receptor (also known as PEAR1 or MEGF12).

RESULTS

Apoptotic DRG neurons are engulfed by SGC precursors

In the mouse embryonic DRG, approximately 50% of the sensory neurons undergo apoptosis, starting around embryonic day 11 (E11), peaking at E13, then tapering off about E15 (refs. 20,21). To determine how these neuron corpses are cleared, we first considered the possibility that macrophages could be responsible. Notably, we found only a few sporadic macrophages, detected by the macrophage-specific antigen F4/80 (ref. 6), in the mouse DRG during the period of

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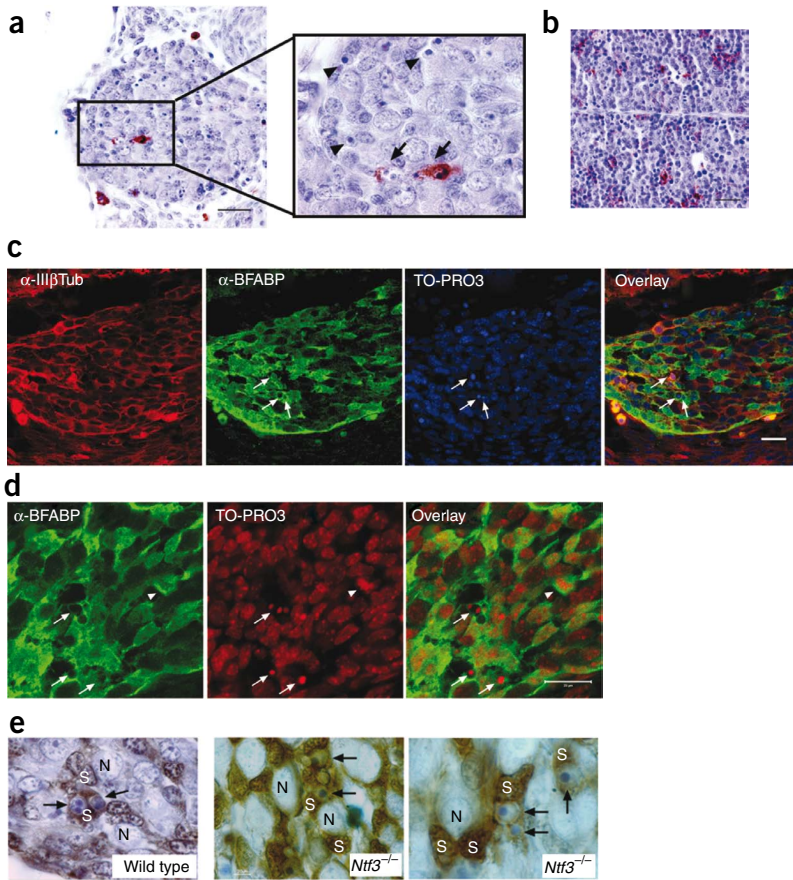
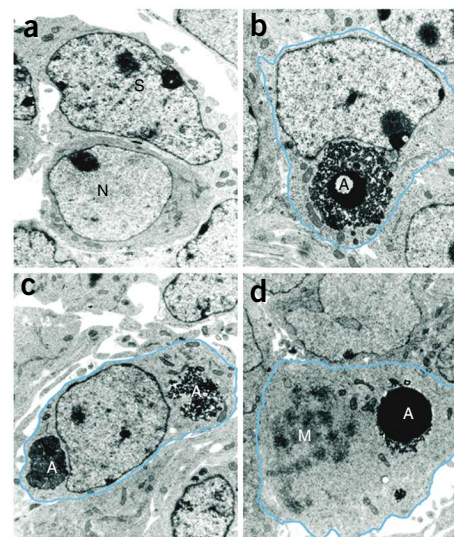


Figure 1 Neuron corpses in developing DRG are engulfed by BFABP-positive SGC precursors. (a,b) Immunostaining with F4/80 antibody was used to detect the presence of macrophages in paraffin sections from E12.5 mouse DRG (a) or liver (b). Arrows point to cell corpses engulfed by macrophages, which were rare. Arrowheads point to several cell corpses that are not engulfed by macrophages. (c,d) Cryosections from E12.5 mouse DRG were immunostained with antibody to BFABP to label satellite glial cells and antibody to type III β -tubulin (α -III β Tub) to label neurons. Cell nuclei were counterstained with TO-PRO3 iodide (642 of 661), a monomeric cyanine nucleic acid stain. Arrows indicate apoptotic cells engulfed by BFABP-positive cells (c). Dorsal is to the right. An enlarged view of nuclear fragments engulfed by BFABP-positive cells, indicated by the arrows, is shown in d. The arrowhead indicates a BFABP-positive SGC precursor undergoing mitosis. Scale bars represent 20 μ m in a–d. (e) Immunohistochemical staining of E12.5 wild-type and *Ntf3*^{-/-} DRGs with antibody to BFABP (brown) on 5- μ m-thick sections counterstained with toluidine blue. S, SGC precursors; N, neurons. Arrows point to several apoptotic neurons engulfed/enveloped by BFABP-positive SGC precursors.

cytoplasm (Fig. 1d and data not shown). To be sure that macrophages were not expressing the glial marker, we performed double labeling with antibodies to BFABP and F4/80 and confirmed

that these were separate cell populations (data not shown).

To further demonstrate that SGCs were the primary cell type engulfing apoptotic bodies, we examined ganglia of E11–13 embryos at the electron-microscopy level. Healthy neurons, morphologically characterized by a large nucleus with finely dispersed chromatin and abundant cytoplasm, were typically positioned near elongated cells with a polymorphic nucleus exhibiting a perinuclear chromatin ring and chromatin clumps, typical of satellite glia (Fig. 2a). In 100% of the cases in which a cell was surrounding an apoptotic body (53 apoptotic bodies total, in DRG sections from E11, $n = 15$; E12, $n = 22$; E13, $n = 16$),

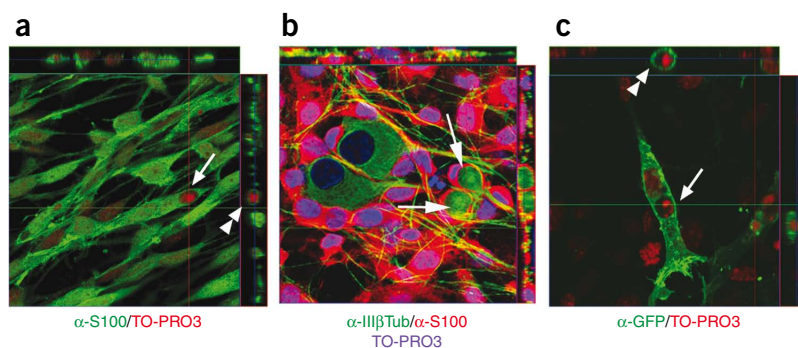


naturally occurring cell death (Fig. 1a). Specifically, just $0.65 \pm 0.58\%$ ($n = 3$) of the total number of cells in the ganglia were F4/80 positive at E11 and $0.65 \pm 0.65\%$ at E13 ($n = 3$). Moreover, even though F4/80-positive cells inside mouse DRG appeared to be encircling dead neurons (Fig. 1a), most of the apoptotic cells were not associated with macrophages (Fig. 1a). This was not a result of a lack of macrophages during this stage or a limitation of detection, as many F4/80-positive macrophages were present in the liver in the same section (Fig. 1b).

The primary cell types in the embryonic DRG are neurons and SGC precursors^{22–25} (Fig. 1c). SGC precursors arise after E10.5 in mice^{24,26–28}, corresponding to the time of cell death of DRG neurons, and eventually surround the mature neurons, thus positioning themselves for engulfment of neuronal corpses. Using an antibody to brain fatty acid-binding protein (BFABP), a marker of SGC precursors^{22–25}, we found that most of the apoptotic nuclei appeared to be engulfed by BFABP-positive cells (Fig. 1c–e). We quantified the number of glial precursors surrounding dead cells during the period of normal neuronal apoptosis in the DRG and found that $86.2 \pm 5.4\%$ of the apoptotic bodies were associated with BFABP-positive cells at E11, $73.4 \pm 2.8\%$ at E12 and $77.1 \pm 3.6\%$ at E13 ($n = 3$). Confocal images with serial optical sections acquired at a higher magnification revealed that several apoptotic nuclear remnants were present inside the BFABP-positive

Figure 2 Electron micrographs of apoptotic bodies engulfed and ingested by SGC precursors in embryonic E12.5 DRGs. (a) A healthy neuron (N) characterized by finely dispersed chromatin and abundant cytoplasm adjacent to a SGC precursor (S) with a characteristic pleomorphic nucleus with chromatin clumps. (b) An apoptotic cell (A) engulfed by a SGC precursor. (c) Debris of two ingested cells inside a SGC precursor. (d) An ingested apoptotic cell present in a cell that is undergoing mitosis (M, mitotic figure, condensing chromosomes).

Figure 3 Glial cells engulf dead neurons induced by NGF withdrawal *in vitro*. Primary mixed cultures of sensory neurons and SGCs were derived from dissociated E15 rat DRG and cultured with NGF for 2 d. The NGF was then removed to induce neuronal apoptosis and the cultures were fixed and immunostained 2 d later. **(a,b)** Images were compiled from orthogonal optical sections showing apoptotic nuclear remnants (TO-PRO3, red) inside the cytoplasm of glial cells (α -S100, green) **(a)** and neuron corpses (α -III β Tub, green) enveloped by glial cells (α -S100, red) **(b)**. Nuclei were stained with TO-PRO3 (blue). **(c)** Apoptotic nuclear remnants (TO-PRO3, red) present in glial cells expressing membrane bound GFP (green). The window on the top of each panel shows the z axis stacks at the specific y axis location (indicated by a green line crossing the panel) and that on the right shows the z axis stacks at the specific x axis location (red line crossing the panel). Arrows and arrowheads indicated engulfed apoptotic bodies.



the ultrastructure of the engulfing cell resembled that of satellite cells (**Fig. 2b,c**). We did not find any macrophage-like cells engulfing cell corpses in the electron micrographs. Moreover, we occasionally observed engulfing cells undergoing mitosis (**Fig. 2d**). Mitotic figures were also observed in BFABP-positive cells in the ganglia (**Fig. 1d**) and $29.3 \pm 1.8\%$ of the BFABP-positive cells at E12 colabeled with BrdU 1 h following injection. Because nearly all detectable apoptotic cells in the DRG at this time are neurons²¹ and this is the period during which SGC precursors are proliferating, these observations indicate that SGC precursors are responsible for engulfing the dying neurons.

To further pursue this possibility, we asked whether elevated apoptosis in the developing DRG could be handled by SGC precursors or would lead to macrophage invasion. Using neurotrophin 3 null (*Ntf3*^{-/-}) mice, which lose 70% of their sensory neurons between E11 and E13 (refs. 20,29,30), we examined the clearing of dead neurons in the E12 DRG. As in the wild-type ganglia, the vast majority of apoptotic cells were surrounded by BFABP-positive cells ($74.6 \pm 6.0\%$, $n = 3$ embryos; **Fig. 1e**), whereas only $3.1 \pm 2.5\%$ were associated with F4/80-positive cells ($n = 3$ embryos). This result indicates that SGC precursors are the primary engulfing cell type during DRG development and are sufficient for the job even when the pool of dead neurons is markedly increased.

The ability of glial precursors to ingest apoptotic neurons could also be demonstrated *in vitro*. We established an *in vitro* engulfment assay using dissociated ganglia from E15 rat or E13 mouse embryos. The cells

were first grown in the presence of NGF for 2–3 d to keep the sensory neurons alive, followed by removal of NGF to induce apoptosis. The glial cells were detected with an antibody to BFABP or S100, which labels both SGC and Schwann cells, which most of the glia eventually convert to in culture³¹. Virtually all non-neuronal cells in the cultures were BFABP and S100 positive; we did not detect any fibroblasts by Thy1.1 staining and we did not find any F4/80-positive cells ($n = 3$). Following 2 d of NGF withdrawal, 82% of the neurons were apoptotic and the engulfed and ingested dead neurons were exclusively detected inside the glial cells on the basis of S100 (**Fig. 3a,b**) and BFABP (**Supplementary Fig. 1**) immunostaining and confocal microscopy. We also expressed a membrane-bound form of GFP³² (mGFP) in the glial cells to more clearly visualize the internalized corpses. Under these conditions, engulfed nuclear remnants inside phagosomes were clearly observed (**Fig. 3c**). Taken together with our

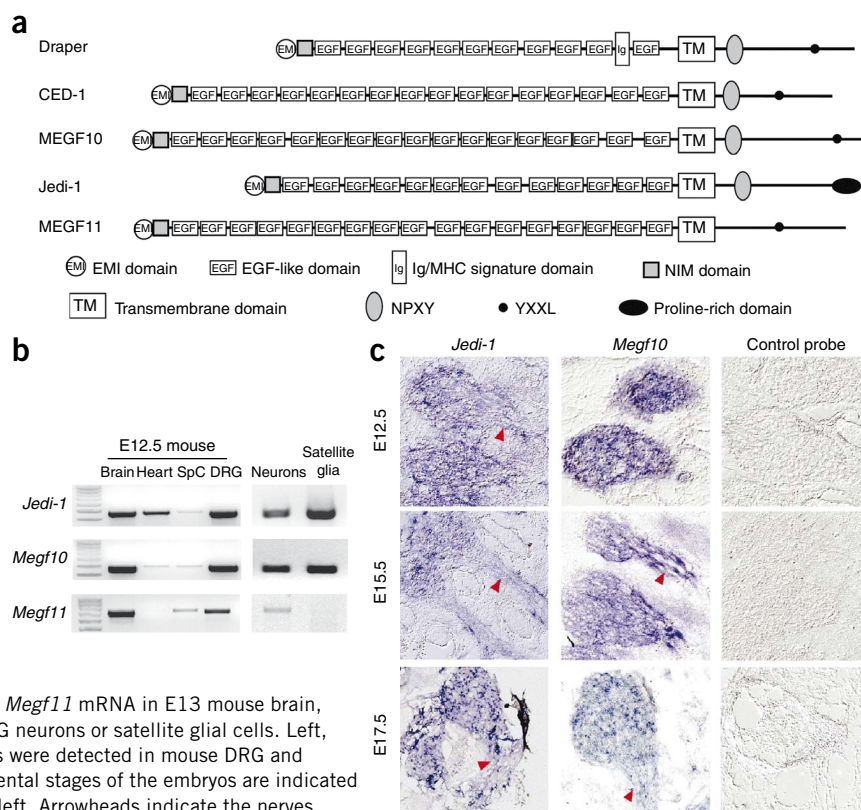
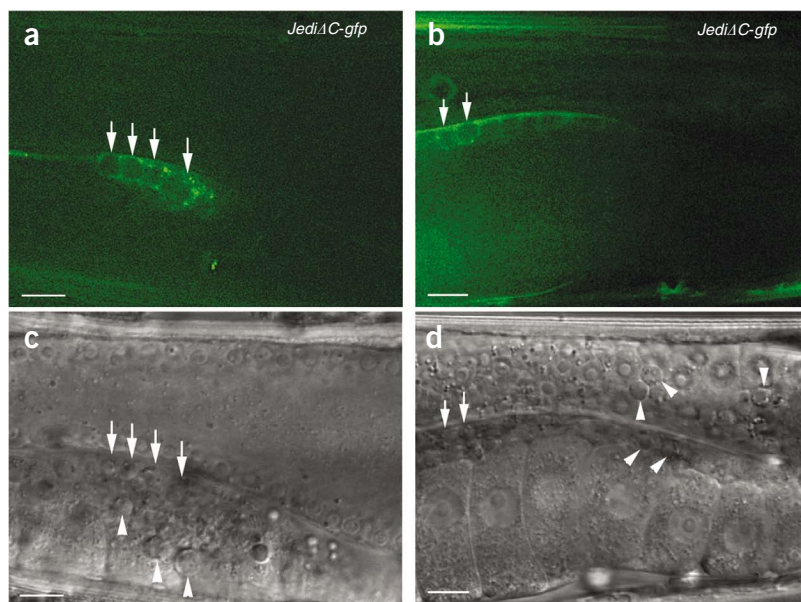


Figure 4 Putative Draper and CED-1 homologs, Jedi-1 and Megf10, are expressed in developing peripheral glial cells. **(a)** Schematic representation of the modular architecture of Draper, CED-1 and possible mammalian homologs. A key for the predicted domains and motifs is shown on the bottom (see **Supplementary Fig. 1** for the sequence alignments of their predicted intracellular domains). **(b)** RT-PCR detection of *Jedi-1*, *Megf10* or *Megf11* mRNA in E13 mouse brain, heart, spinal cord (SpC), whole DRG and purified DRG neurons or satellite glial cells. Left, 1-kb DNA markers. **(c)** *Jedi-1* and *Megf10* transcripts were detected in mouse DRG and developing glial cells alongside axons. The developmental stages of the embryos are indicated on the left. For the sagittal sections, dorsal is on the left. Arrowheads indicate the nerves.



The number of germ cell corpses in wild-type *C. elegans* hermaphrodites expressing *JediΔC-gfp*

Genotype	Transgene	Transgenic line	Number of germ cell corpses	n	P value
Wild type	None	NA	2.9 ± 2.6	39	NA
Wild type	<i>JediΔC-gfp</i>	1	9.5 ± 4.0	22	<0.005
Wild type	<i>JediΔC-gfp</i>	2	8.2 ± 5.1	18	<0.005

Figure 5 The extracellular domain of Jedi-1 recognizes cell corpses when expressed in *C. elegans* engulfing cells. Transgenic worms expressed JediΔC-GFP in engulfing cells under the control of the *ced-1* promoter. All of the worms that we analyzed were adult hermaphrodites aged 48 h post-mid L4 larval stage. (a–d) GFP (a,b) and corresponding differential interference contrast microscopy (c,d) images of part of adult *ced-1(e1735)* hermaphrodite gonads to indicate the clustering of JediΔC-GFP around germ cell corpses. Arrows indicate a few germ cell corpses labeled with JediΔC-GFP on their surfaces. Arrowheads indicate examples of germ cell corpses not labeled by JediΔC-GFP. Dorsal is to the top. Midbody is to the left. Scale bars represent 10 μm. The table indicates the number of germ cell corpses in wild-type *C. elegans* hermaphrodites expressing JediΔC-GFP scored under DIC optics in one gonadal arm of each adult hermaphrodite staged 48 h post L4 larval stage. Data are presented as mean ± s.d. n indicates the number of worms scored. The data obtained from transgenic and the wild-type control worms were compared and P values were obtained from two-tailed Student's *t* tests.

in situ hybridization (ISH; Fig. 4c). At all of the ages that we examined (E12.5, E15.5 and E17.5), both *Jedi-1* and *Megf10* were observed in the ganglia and in the cells along the nerves, consistent with the location of SGC precursors and immature Schwann cells.

in vivo findings, these results suggest that SGC precursors are the primary cell type responsible for clearing neuronal corpses during the period of naturally occurring cell death in the developing DRG.

Jedi-1 and MEGF10 are expressed in SGC precursors

The molecular mechanisms underlying apoptotic cell clearance in the mammalian PNS during development are not known. Draper, a *Drosophila* protein that is homologous to the *C. elegans* CED-1 receptor, was identified as an engulfment receptor that is expressed on glial cells and that is required for clearing degenerating neurons and axons^{13–17,33}; therefore, we speculated that a Draper/CED-1-like engulfment receptor might exist in SGC precursors to mediate phagocytosis of dead neurons. Three mammalian proteins, MEGF10, MEGF11 and Jedi-1, were identified as being highly homologous to Draper and CED-1 using BLASTP (National Center for Biotechnology Information). Two regions in the intracellular domain of CED-1 are required for its engulfment function: an NPXY motif that may serve as a phosphotyrosine binding site and an YXXL motif, a Src Homology 2 (SH2) domain binding site¹⁴. Draper and MEGF10 have both NPXY and YXXL motifs, whereas Jedi-1 has an NPXY sequence and MEGF11 has an YXXL in their putative intracellular regions (Fig. 4a and Supplementary Fig. 2).

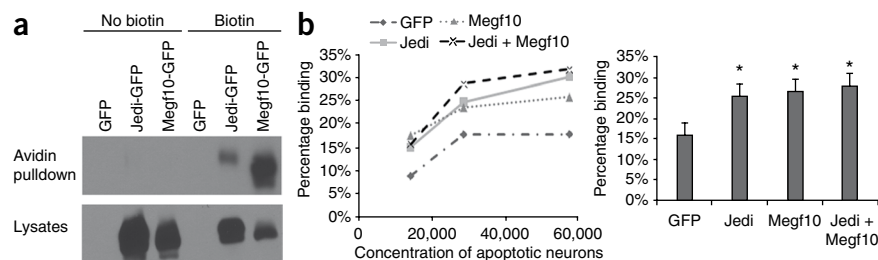
To determine whether Jedi-1, MEGF10 or MEGF11 could mediate engulfment by SGC precursors, we examined their expression in these cells by reverse-transcription PCR (RT-PCR). The mRNAs for all of these proteins were present in E12.5 mouse brain and whole DRG (Fig. 4b); however, only MEGF10 and Jedi-1 were expressed in isolated SGC precursors, indicating that MEGF11 is unlikely to function as an engulfment receptor in DRG development. Curiously, the mRNA for all three proteins was detected in neurons, although their function there is not known. We then analyzed the expression pattern of *Jedi-1* (also known as *Pear1*) and *Megf10* in the developing mouse DRG at different developmental stages using

Jedi-1 and MEGF10 facilitate binding to apoptotic cells

Recently, MEGF10 was proposed as a putative CED-1 homolog because it could promote dead thymocyte engulfment when ectopically expressed in HeLa cells^{19,34} and expression of an MEGF10-GFP fusion protein under the control of the *ced-1* promoter in *ced-1(e1735)* mutant *C. elegans* partially rescued the engulfment defect of *ced-1(e1735)*¹⁹. Whether Jedi-1 exerts a function similar to CED-1 has never been examined. CED-1 and its truncated form, which lacks the intracellular domain (ICD) (CED-1ΔC), similarly cluster around neighboring cell corpses, owing to their ability to recognize an apoptotic cell-surface signal(s)¹⁴. We expressed a Jedi-1 protein with a truncated intracellular domain tagged with GFP at its C terminus (JediΔC-GFP) in engulfing cells under the control of the *ced-1* promoter in *C. elegans* and observed cell-surface presentation of a fraction of JediΔC-GFP molecules, although much of it remained inside the cells. Notably, the portion of JediΔC-GFP present on the surface of gonadal sheath cells, which are engulfing cells for apoptotic germ cells, was clustered around some of the germ cell corpses (Fig. 5). This localized enrichment of JediΔC-GFP around cell corpses suggests that the extracellular domain of Jedi-1 is capable of recognizing a signal on the surface of the dying cell, similar to CED-1. Furthermore, expression of JediΔC-GFP in wild-type worms resulted in the presence of excessive germ cell corpses (Fig. 5). This result suggests that, among other possibilities, JediΔC-GFP might interfere with the normal engulfment of dead cells by associating with the 'eat me' cue on the surface of cell corpses, thereby preventing endogenous CED-1 or other unknown engulfment receptors from binding these cues and transducing the normal engulfment signal. The expression of CED-1ΔC similarly results in the inhibition of cell-corpse engulfment¹⁴. We also tested whether ectopic expression of full-length

Figure 6 Jedi-1 and MEGF10 expressed in HEK293 cells enable binding to dead neurons.

(a) HEK293 cells were transfected with GFP-tagged Jedi-1 or MEGF10 and their expression on the cell surface analyzed by biotinylation of the surface proteins followed by precipitation with avidin beads and western blotting with antibody to GFP. The biotin reagent was not added to some cells (no biotin) to confirm the specificity of the pull down. The expression of Jedi-GFP and MEGF10-GFP in total cell lysates is shown in the lower panel. (b) Jedi-1- and/or MEGF10-transfected cells were incubated at 4 °C with the indicated number of neuronal corpses, induced to undergo apoptosis by NGF withdrawal for 24 h and labeled with propidium iodide. After washing off the unbound dead cells, the cultures were fixed with 10% formalin. The percentages of GFP-positive cells with at least one propidium iodide-positive corpse bound are shown (one representative experiment of three is depicted). The lower panel shows the mean \pm s.e.m. percentage binding at the highest concentration of neurons (* $P < 0.01$ relative to GFP-transfected cells, $n = 4$).

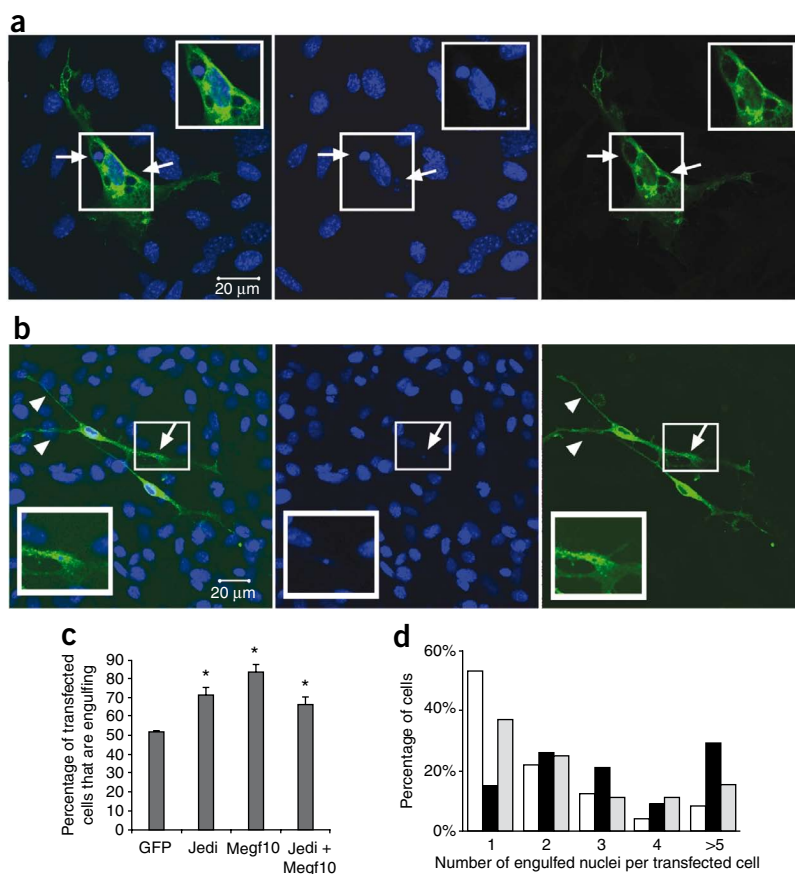


Jedi-1 (Jedi-GFP) in *C. elegans* engulfing cells could also rescue the cell-corpse removal defects in *ced-1* (*e1735*) mutants. Unfortunately, ectopically expressed Jedi-GFP was retained inside cells, forming protein aggregates and did not result in any rescue of the *ced-1* mutant (data not shown).

Although CED-1, Draper and MEGF10 are thought to be receptors for apoptotic cells, none of the ligands have been identified and it has not been directly demonstrated that these proteins specifically mediate binding, as opposed to just facilitating the engulfment process. To determine whether Jedi-1 or MEGF10 can act as receptors for dead neurons, we transiently expressed them in HEK293 cells and added various concentrations of apoptotic neurons, labeled with propidium iodide. We confirmed that Jedi-1 and MEGF10 were expressed and trafficked to the cell surface (Fig. 6a) and then incubated the cells expressing these proteins with neuronal corpses at 4 °C to prevent engulfment, as we wanted to specifically assess binding

and not internalization. After rinsing, the cultures were fixed and the number of dead neurons attached to the transfected cells was scored. Some binding occurred to the control cells, most likely as a result of other endogenous proteins that can facilitate binding to dead cells, such as integrins or PSR¹²; however, expression of either Jedi-1 or MEGF10 significantly increased binding to neuronal corpses ($P < 0.01$ relative to GFP-transfected cells, $n = 4$; Fig. 6b). This result indicates that these proteins can function as receptors, enhancing binding in the absence of any internalization. Expression of both proteins did not further increase the binding (Fig. 6b), which may indicate that they are part of a single binding complex, but the affinity and any cooperativity could not be accurately determined, as the ligand source is an entire dead neuron and not a small, freely diffusible molecule.

Figure 7 Ectopic expression of Jedi-1 or MEGF10 in glial cells promotes neuronal corpse engulfment. DRG from E13.5 mice were dissociated and grown in the presence of NGF for 2 d. The glial cells were then transfected with plasmids expressing MEGF10-GFP, Jedi-1-Flag or meGFP and NGF was removed from the cultures. The cells were fixed after 2 d without NGF and immunolabeled with antibodies to GFP or Flag. (a–d) z axis optical stacks of confocal images of glial cells expressing MEGF10-GFP (MEGF10, a, c, d), Jedi-1-Flag (Jedi, b–d) or meGFP (c, d) were acquired and the numbers of transfected cells containing at least one ingested nuclear remnant (condensed TO-PRO3 staining) was quantified. Arrows indicate the location of engulfed apoptotic nuclei in these cells. Arrowheads indicate the long processes in Jedi-1-Flag-expressing cells. In c, the error bars indicate mean \pm s.d. ($P = 0.0002$, one-way ANOVA). The number of engulfed nuclei per engulfing cell was also determined and expressed as the percentage of transfected cells containing the indicated number of apoptotic nuclei (open bar, meGFP; black bar, MEGF10-GFP; gray bar, Jedi-Flag; d). Based on a χ^2 analysis, there was a significant difference between all three groups of cells (expressing Jedi-1, MEGF10 and meGFP, $P < 0.0001$). Scale bars represent 20 μ m.



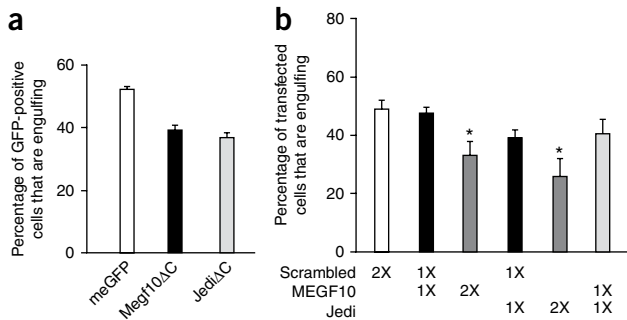


Figure 8 Neuronal corpse engulfment by glial cells requires endogenous Jedi-1 and MEGF10. The neuronal engulfment assay described in **Figure 7a** was used to determine whether endogenous MEGF10 and Jedi-1 are required for engulfment. **(a)** C-terminal truncated forms of MEGF10 or Jedi-1 (Megf10ΔC or JediΔC, respectively), as well as meGFP, were transfected into glial cells, and the number of GFP-positive cells with engulfed apoptotic nuclei were counted 2 d after NGF withdrawal. Error bars represent mean \pm s.d. ($P = 0.0005$, one-way ANOVA). **(b)** Plasmids bi-cistronically expressing ZsGreen and shRNAs targeting *Megf10*, *Jedi-1* or non-targeting shRNA (scrambled) were transfected into the glial cells. A total of 8 μ g of DNA was transfected, either in combinations or as a single plasmid, and the number of ZsGreen-positive cells containing engulfed corpses were counted after 2 d. Error bars represent mean \pm s.d. ($P = 0.0008$, one-way ANOVA).

Modifying Jedi-1 or MEGF10 expression alters engulfment

To investigate the involvement of Jedi-1 and/or MEGF10 in neuronal corpse engulfment by SGC precursors, we used the engulfment assay described above (**Fig. 3**). DRGs from E13.5 mouse embryos were dissociated and grown in the presence of NGF for 2 d. NGF was then removed from the culture and, at the same time, the glial cells were transfected with either a control (meGFP) or other transgene. Images of cells expressing these transgenes were acquired with full z axis optical sections and the numbers of transfected cells containing at least one fully internalized apoptotic nucleus were determined (**Fig. 7** and **Supplementary Fig. 3**). Under these conditions, engulfed apoptotic nuclei were observed in about 50% of meGFP-expressing glial cells (**Fig. 7c**). In contrast, overexpression of MEGF10 or Jedi-1 enhanced engulfment of dead neurons by the glial cells; the number of Flag- or GFP-positive cells that had engulfed an apoptotic nucleus increased by approximately 50 and 80% when overexpressing either Jedi-1-Flag or MEGF10-GFP, respectively (**Fig. 7** and **Supplementary Fig. 3**). Transfection of both receptors into the cells did not further enhance phagocytosis (**Fig. 7c**), suggesting that there is an endogenous component in the pathway that is limiting or that the two proteins converge on a common pathway.

MEGF10-GFP and Jedi-1-Flag accumulated around vacuoles containing apoptotic nuclei (**Fig. 7a,b** and **Supplementary Fig. 3**) and in what appeared to be phagocytic cups (**Supplementary Fig. 4**). The vacuoles were identified as lysosomes or late endosomes by LAMP-1 labeling (**Supplementary Fig. 5**). Notably, even though the overall number of cells that were engulfing increased when transfected with Jedi-1 or MEGF10, these cells exhibited some differences; cells expressing MEGF10-GFP contained more vacuoles with apoptotic nuclei than those expressing Jedi-1-Flag, which typically had more long processes and lamellipodia (**Fig. 7b**). To quantify this difference in engulfment, we scored the number of vesicles with nuclear fragments in each transfected cell. Three or more apoptotic corpses were found in 70% of MEGF10-transfected cells, but only in ~30% of Jedi-transfected cells, which more often contained one or two engulfed nuclei (**Fig. 7d**). These observations suggest that overexpression of either Jedi-1 or MEGF10 can ultimately increase the engulfment of dead cells, although there appear to be some differences in their mechanisms of action.

The effect of expressing a mutant Jedi-1 that lacked the ICD in *C. elegans* suggested that this construct could act as an inhibitor of engulfment, preventing the endogenous phagocytic receptor(s) from binding and/or internalizing apoptotic cells (**Fig. 5**). Indeed, we found that transfection of glial cells with either JediΔC-GFP or MEGF10ΔC-GFP in the engulfment assay led to a ~30% decrease in the number of cells that were engulfing dead neurons when compared with those transfected with meGFP (**Fig. 8a** and **Supplementary Fig. 6**). The

ability of the truncated MEGF10 and Jedi-1 to reduce engulfment suggests that the mutants interfere with the action of the endogenous protein. Therefore, to directly determine whether endogenous Jedi-1 or MEGF10 is required for neuronal corpse clearance, we used short hairpin RNA (shRNA) to target *Jedi-1* and *Megf10* (**Supplementary Fig. 7**). Knocking down *Jedi-1* or *Megf10* in the glial cells did not alter their morphology (**Supplementary Fig. 7**); however, it did result in a 40–50% decrease in the number of cells with internalized apoptotic neurons relative to those expressing a scrambled shRNA (**Fig. 8b**). There was no difference between the number of engulfing glial cells transfected with the control shRNA (Scr1-1) and those transfected with meGFP (54.3 ± 1.0 versus 53.4 ± 4.3). Knocking down both Jedi-1 and MEGF10 did not further reduce the ability of the transfected cells to engulf apoptotic bodies, further suggesting that the two proteins may function in a common pathway (**Fig. 8b**). These results indicate that endogenous Jedi-1 and MEGF10 are necessary for neuronal corpse clearance in the embryonic DRG.

DISCUSSION

Although it has long been recognized that there is extensive cell death in the developing PNS, the mechanisms responsible for disposing of the cellular ‘waste’ have remained an open question. Our findings indicate that SGC precursors in the DRG are the primary cell type responsible for clearing neuronal corpses generated during the period of naturally occurring cell death. In addition, we identified Jedi-1, a previously unknown engulfment receptor, and MEGF10 as two CED-1 homologs that were expressed in the glial cells and were involved in phagocytosing neuronal corpses. Thus, these results reveal the cellular and molecular basis for clearing the neuronal waste generated during the development of sensory ganglia.

Macrophages carry out the removal of cellular debris in many tissues during development or after injury and these cells are known to increase in the DRG after injury to the sciatic nerve³⁵. However, we found few macrophages in the developing DRG, even in animals with unusually high numbers of apoptotic neurons (*Ntf3*^{-/-} mice). The phagocytic ability of glial cells in the developing nervous system has been known for some time, although the importance has largely been unrecognized. Axonal fragment ingestion by Schwann cells during Wallerian degeneration after injury was first described some 40 years ago⁸. However, macrophages subsequently invaded the nerve and clear most of the debris, thereby overshadowing the contribution of the Schwann cells³⁶. A more recent study demonstrated that Schwann cells have an important phagocytic role in synapse elimination during the development of the neuromuscular junction³⁷. Early electron microscopy studies of the developing chick embryo suggested the presence of degenerated axons and apoptotic neurons in astrocytes, satellite glial cells and Schwann cells^{7,8,38}; however, the identity of the engulfing

cells was not confirmed because of the absence of immunological markers. Furthermore, in some cases, contradictory observations were reported, suggesting that macrophages were clearing the debris⁸. Our findings demonstrate that SGCs, rather than macrophages, are the primary phagocytes responsible for clearing the dead neurons generated during the normal development of the DRG.

The physiological roles of SGCs are not well understood. They are found in sensory, sympathetic and parasympathetic ganglia, where they cluster around each neuron and regulate the extracellular environment, taking up neurotransmitters similar to astrocytes³⁹. SGCs also produce numerous neuroactive agents, such as neurotrophins and bradykinin, although the functions of these in the ganglia are not clear³⁹. Following injury, the SGCs undergo a morphological change, begin to proliferate and release many of these factors, leading some to suggest that they are involved in neuropathic pain^{39,40}. The phagocytic ability characterized here provides a glimpse into an important function of SGCs. Whether the glial cells remain the primary cell type responsible for phagocytosing dead neurons in more mature animals, such as after axotomy or other inducers of neurodegeneration, remains to be determined.

The molecular mechanisms involved in apoptotic neuron clearance in the developing mammalian PNS were previously unknown. In *C. elegans*, CED-1 was identified as a receptor required for engulfment of apoptotic cells¹⁴. Draper, the *Drosophila* homolog of CED-1, was shown to mediate dead neuron removal during development¹³ and to eliminate degenerating axons during metamorphosis and after injury^{15,16,33}. We identified MEGF10 and Jedi-1 as possible homologs on the basis of their predicted structural organization and their expression pattern. MEGF10 was previously suggested to be an engulfment receptor¹⁹, but little is known about Jedi-1. It was shown to be phosphorylated on platelet activation, although its role in this process was not determined, and its overexpression in hematopoietic progenitors reduced the number of cells that committed to a myeloid lineage, suggesting that it is involved in the differentiation of these cells. We found that Jedi-1 functions as a phagocytic receptor and is involved in clearing dead sensory neurons.

Notably, both Jedi-1 and MEGF10 promoted the engulfment of neuronal corpses by SGCs (Figs. 7 and 8); however, overexpression or knockdown of both proteins was no more effective than altering the expression of either alone. We therefore suggest that these receptors may converge on a common pathway or even form a complex. However, they also exerted somewhat different actions when overexpressed in glial cells, suggesting that they have some non-overlapping functions. Almost all of the cells transfected with MEGF10 had engulfed more than one apoptotic nuclei ($90.6\% \pm 1.3$) and most had numerous apoptotic nuclear remnants in lysosomes. This could be a result of an increase in the number of dead neurons taken up by a single cell or an increase in the number of lysosomes digesting the engulfed material. Increased vacuole formation has been described in HEK293 cells overexpressing MEGF10, even when no apoptotic cells were added³⁴. On the other hand, most of the Jedi-1-overexpressing glial cells appeared to be slimmer with longer processes (Fig. 7b) and contained only one or two visible vacuoles with ingested nuclei, unlike those overexpressing MEGF10 (Fig. 7d). It is possible that Jedi-1 has the ability to promote both engulfment and degradation of apoptotic cells, similar to CED-1 (ref. 41), whereas MEGF10 only carries out the engulfment activity. Of course, we cannot rule out the possibility that overexpression of either protein indirectly increases engulfment, for example, by increasing process formation. In any case, these differences suggest that, on activation by the 'eat me' signals, MEGF10 and Jedi-1 trigger, at least partially, non-overlapping molecular pathways for engulfment.

Such a need for multiple receptors is typical of the phagocytic process. Multiple ligands and receptors are implicated in the recognition and uptake of apoptotic cells by 'professional' phagocytes such as macrophages^{12,42}. For phosphatidylserine alone, the most well-studied 'eat me' cue, there are at least four transmembrane receptors, PSR, Tim4, BAI1 and Stabilin-2, that have been shown to bind this phospholipid and transduce an engulfment signal⁹. Even in *C. elegans*, there are two partially redundant pathways that mediate cell corpse removal, with the *ced-1*, *ced-6* and *ced-7* genes functioning in one pathway and the *ced-2*, *ced-5*, *ced-10* and *ced-12* genes acting in the other⁴³. Recently, a second *Drosophila* receptor, SIMU, was identified and was proposed to function as the recognition receptor⁴⁴, with Draper acting primarily in the engulfment and degradation process through recruitment of the src family kinases⁴⁵.

Programmed cell death and process elimination are essential for the development and maintenance of functional nervous systems. Consequently, considerable effort has been made to understand the molecular mechanisms involved in these events; however, less attention has been given to the resulting byproducts. During such regressive processes, large amounts of degenerated and excess cellular debris are generated that need to be efficiently eliminated. Several reports have provided convincing evidence for a link between defective clearance of apoptotic cells and the development of autoimmunity^{3,46,47}. Thus, it is likely that defects in neural waste clearance during development predisposes an organism to autoimmune attack on the nervous system later in life, although this has yet to be demonstrated.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

H.-H.W. and B.D.C. initiated and developed the overall concept and design of the project. H.-H.W. also performed, analyzed and interpreted most of the experiments and prepared the initial version of the manuscript. E.B. performed the quantitative histological analysis of neuronal corpse engulfment in *Ntf3*^{+/+} and *Ntf3*^{-/-} mice. J.L.S. generated some of the Jedi-1 and MEGF10 constructs, performed the binding experiment and some of the immunostaining analyses. V.V. performed all of the experiments with *C. elegans*. C.B. assisted with the immunostaining on sections and generated the shRNA construct for MEGF10. L.F.R. provided technical expertise for electron microscopy analysis and critical intellectual input for this study. I.F. performed the electron microscopy analysis, supervised the quantitative histological analysis and provided intellectual input. Z.Z. designed and supervised the *C. elegans* study and provided intellectual input. B.D.C. directed the overall project and prepared the final version of the manuscript.

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ONLINE METHODS

Animals. CD-1 mice, Sprague-Dawley rats or *Ntf3*^{+/+} and *Ntf3*^{-/-} mice²⁰ were used for these studies. All experimental procedures using animals were approved by the Institutional Animal Care and Use Committee at Vanderbilt University, the committee on Animal Research at the University of Valencia and conformed to US National Institutes of Health and EU guidelines.

ISH. Embryos were dissected in cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (wt/vol) overnight at 4 °C and transferred to 30% sucrose/PBS (wt/vol) before being cryosectioned at 20 μm. ISH on sections were performed as described previously⁴⁸. We used digoxigenin-labeled cRNA probes to *Jedi-1* (probes 1 and 2, 467–931 bp and 871–1,347 bp of GenBank #AF444274), *megf10* (probes 1 and 2, 1,326–1,828 bp and 2,369–2,857 of GenBank #NM_001001979).

Electron microscopy. Isolated E11.5–13.5 mouse embryos were fixed by immersion in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h and thoroughly washed with phosphate buffer. Body fragments containing lumbar DRGs were postfixed with 1% osmium tetroxide (wt/vol) in phosphate buffer for 90 min at 20–25 °C, dehydrated through a graded ethanol series followed by propylene oxide and embedded in araldite (Durcupan, Fluka). Samples were sectioned at 1 μm and sections were stained with 1% toluidine blue (wt/vol) for ganglia identification. Ultrathin 60-nm-thick sections from lumbar ganglia were collected on formvar-coated slot grids, stained with uranyl acetate and lead citrate, and examined with a Jeol JEM-1010 electron microscope.

RT-PCR. Total RNA from E12.5 CD-1 mouse brain, heart, spinal cord, DRG and cultured embryonic satellite cells¹⁹ or neurons was extracted with TRIzol reagent (Invitrogen) per manufacturer's recommendation and reverse-transcribed using random primers and Superscript II reverse transcriptase (Invitrogen) following treatment with DNase I (DNA-free kit, Ambion). Resulting cDNAs were analyzed by PCR. We used primers to *Jedi-1* (forward, 5'- CCT GCA GCT GCC CAC CGG GCT GGA -3'; reverse, 5'- CCT GGC AGC CCG GGC CAT GCG TGT -3'), *Megf10* (forward, 5'-GGC GCG CCT GTG TCC CGA GGG GCT TT-3'; reverse, 5'-CGG GCG CAC AGG TGC AGG TCC CAT CC-3') and *Megf11* (forward, 5'-GCG CGC CAC GGA AGC AGG CCC CGA TG-3'; reverse, 5'-CCA GCC TCG GAA GCC CGG GGC GCA CA-3').

Plasmids and ISH probes. For information regarding cDNA constructs, truncated fusion constructs, ISH probes, and shRNA containing plasmids please see the **Supplementary Methods**.

Neuronal engulfment assay. DRG from E13.5 mouse CD1 embryos or E15 rat embryos were dissociated with collagenase A (1 mg ml⁻¹, Roche) plus 0.05% trypsin (1:250, Gibco) and triturated. We then plated 50,000 cells onto a 25-mm round coverslip (#1.5, 0.17 mm, Warner Instruments) coated with collagen. For mouse cultures, cells were grown in mouse-engulfing medium (MEGM, 1:1 UltraCULTURE serum-free medium (BioWhittaker): Neural Basal medium (Invitrogen) supplemented with 3% fetal bovine serum (FBS, vol/vol, Hyclone), N2 and B27 (Invitrogen)). For rat cultures, cells were grown in Basal Medium Eagle (Invitrogen) supplemented with 0.4% glucose, (wt/vol) 3% FBS and N2. Cultures were first grown in the presence of 50 ng ml⁻¹ NGF (Harlan) for 2 d, then the NGF was removed by washing the cultures twice and refeeding with a 1:10,000 dilution of monoclonal antibody to mouse NGF (Chemicon) to remove any remaining NGF. For mouse cultures, 50 ng ml⁻¹ of glial growth factor (R&D Systems) were added to ensure the survival of immature peripheral glial cells. Cells were then transfected with the plasmids indicated using Effectene (Qiagen) according to the manufacturer's recommendation.

DRG SGCs isolation. DRG SGCs were isolated as previously described²⁵ with the following modifications. E13.5 mouse DRGs were cultured as explants on 35-mm dishes coated with poly-D-lysine and laminin (Invitrogen) in MEGM with 25 ng ml⁻¹ NGF and 50 ng ml⁻¹ glial growth factor or 1 μM insulin (Sigma) for 3 d to allow cell migration. Neuron soma and glia still associated with the explants were pinched out using fine forceps and dissecting scissors. The remaining satellite glial cells were dissociated by trypsinization and replated to a 10-cm dish coated with poly-D-lysine/laminin in MEGM with 1 μM insulin without NGF for 2 d.

Immunostaining and quantification. Embryos, processed as for *in situ* analysis, were cryosectioned at 12 μm, permeabilized and blocked with 0.5% Triton X-100 (wt/vol) in 10% bovine calf serum (wt/vol) and incubated with primary antibodies. For primary antibodies, we used rabbit antibody to BFABP (1:1,000, kind gift from T. Muller²², Max Delbruck Center, F4/80 (1:100, Serotec), rabbit antibody to S100β (1:4, ImmunoStar) and mouse antibody to neuronal class III β-tubulin (Tuj1, 1:1,000, Constance). TO-PRO3 (Invitrogen) was used according to the manufacturer's recommendation. For immunohistochemistry, *Ntf3*^{+/+} and *Ntf3*^{-/-} embryos were processed as described previously²⁰. To quantify neuronal engulfment *in vivo*, we counted the proportion of apoptotic bodies that appeared to be surrounded by BFABP- or F4/80-positive cells in sections from at least six DRGs from at least three embryos at each age.

For immunofluorescence staining of cultured cells, cultures were fixed with 4% paraformaldehyde or 3.5% formaldehyde (vol/vol) and incubated with primary antibodies to S100β (1:4), BFABP (1:1,000), Flag M2 (1:500, Sigma), TUJ1 (1:1,000), F4/80 (1:100), Thy1.1 (1:25, Serotec), LAMP1 (1D4B; 3.9 mg ml⁻¹; the LAMP1 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa) and chicken antibody to GFP (1:500, Abcam). Cultures were then incubated with 200 μg ml⁻¹ of DNase-free RNase A in PBS for 30 min at 20–25 °C, rinsed, and nuclei were visualized with 1 μM TO-PRO3 (Invitrogen). For secondary antibodies, we used Alexa488-conjugated goat antibody to rabbit (1:1,000) and Alexa488-conjugated goat antibody to mouse (1:200) from Invitrogen, and Rhodamine X-conjugated donkey antibody to mouse (1:200–400) and Cy2-conjugated donkey antibody to chicken (1:200) from Jackson ImmunoResearch Laboratories. Photomicrographs of z axis series were taken using a Zeiss LSM 510 inverted confocal microscope (Cell Imaging Shared Resource at Vanderbilt University Medical Center) and analyzed with LSM image Browser from Zeiss. For each experiment, at least 50 cells from each condition (done as duplicates or triplicates for each experiment) were counted. The results were obtained from 2–3 independent experiments.

Quantitation of cell proliferation. Pregnant females were injected with 50 μg per kg of body weight bromodeoxyuridine 1 h before being killed. Embryos were fixed in Carnoy's solution and embedded in paraffin. Sections were treated with 2 N HCl for 30 min at 37 °C, neutralized in 0.1 M sodium borate (pH 8.5) for 5–10 min and immunostained using mouse monoclonal antibodies to BrdU (1:300; Dako) and BFABP (1:1,000).

Apoptotic neuron binding assay. DRG neurons from E13.5 CD1 mouse embryos were isolated as described above, plated on collagen-coated coverslips and grown in UltraCULTURE media with 50 ng ml⁻¹ NGF (Harlan). Non-neuronal cells were eliminated by two 48-h treatments with uridine (10 μM) and fluorodeoxyuridine (10 μM). NGF was removed to induce apoptosis by rinsing and addition of antibody to NGF (1:10,000). After 24 h, the neurons were harvested by scraping, stained with propidium iodide for 20 min, rinsed and added to transfected HEK293 cells.

HEK293 cells were plated onto collagen-coated eight-well glass chamber slides at a density of 20,000 cells per well in DMEM with 10% FBS (Sigma). After 24 h, the cells were transfected using Effectene (Qiagen) and the indicated number of propidium iodide-stained apoptotic neurons were added 48 h later for 1 h at 4 °C. Unbound neurons were washed away with three rinses of PBS and fixed with 10% formalin. To quantify, we counted at least 100 GFP-positive HEK293 cells per condition. The percentage of GFP-positive cells with at least one propidium iodide-positive corpse bound was calculated.

Surface biotinylation. HEK293 cells were transfected with GFP-tagged *Jedi* or *MEGF10* and the surface expression analyzed 48 h later by biotinylation of the receptor at 4 °C using EZ-Link Sulfo-NHS-Biotin (Pierce) according to the manufacturer's instructions. The biotinylated proteins were precipitated using avidin-agarose (Pierce) and subjected to SDS-PAGE and western blotting using antiserum to GFP (1:500, Roche).

Analysis of the ectopic expression of *Jedi*ΔC-GFP in *C. elegans*. The *ced-1–Jedi*ΔC-*gfp* and *ced-1–Jedi-gfp* fusion constructs were generated from full-length *Jedi-1*–Flag or *Jedi*ΔC-GFP and the *ced-1* promoter¹⁴ and were introduced as

extrachromosomal arrays into wild-type or *ced-1(e1735)* backgrounds. Transgenic lines were obtained using standard microinjection techniques⁴⁹ and the transgenic worms were identified by GFP expression using a fluorescence microscope. Germ cell corpses in hermaphrodite gonads were analyzed using Axioplan 2 compound microscope (Carl Zeiss) with Nomarski DIC accessories, Axiocam camera and the AxioVision v4.3 imaging software. Germ cell corpses labeled with Jedi Δ C-GFP were identified by fluorescence microscopy under the DeltaVision Deconvolution Microscope (Applied Precision). Fluorescence images were deconvolved using the SoftWoRx software (Applied Precision).

Statistical analysis. The cell corpse data obtained from transgenic and the wild-type control *C. elegans* were compared and *P* values were obtained from unpaired, two-tailed Student's *t* tests. The amount of dead neurons binding to

transfected HEK293 cells was compared using *P* values obtained from paired, two-tailed Student's *t* tests. The data from over expressing Jedi-1 and/or MEGF10 in glial cells shown in **Figure 7c** was analyzed using a one-way ANOVA with a Dunnett's post-test and the data in **Figure 7d** were evaluated using a Mantel-Haenszel χ^2 analysis. To evaluate the effects of overexpressing the truncated mutants of Jedi-1 and MEGF10 and knocking them down in the engulfment assay shown in **Figure 8**, we subjected the data to a one-way ANOVA with a Dunnett's post-test.

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