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Regulation of apoptosis by *C. elegans* CED-9 in the absence of the C-terminal transmembrane domain

FJ Tan[1], AZ Fire[2] and RB Hill*[,1,3]

Bcl-2 proteins regulate apoptosis in organisms as diverse as mammals and nematodes. These proteins are often localized at mitochondria by a C-terminal transmembrane domain. Although the transmembrane domain and mitochondrial localization are centrally involved in specific cases of vertebrate Bcl-2 activity, the significance of this localization is not clear for all species. Studying the *Caenorhabditis elegans* Bcl-2 homolog CED-9, we found that the transmembrane domain was both necessary and sufficient for localization at mitochondrial outer membranes. Furthermore, we found that in our assays, *ced-9* transgenes lacking the transmembrane domain, although somewhat less active than equivalent transgenes derived from wild-type *ced-9*, rescued embryonic lethality of *ced-9(lf)* animals and responded properly to upstream signals in controlling the fate of Pn.aap neurons. Both of these apoptotic activities were retained in a construct where CED-9 lacking the transmembrane domain was targeted to the cytosolic surface of the endoplasmic reticulum and derived organelles, suggesting that in wild-type animals, accumulation at mitochondria is not essential for CED-9 to either inhibit or promote apoptosis in *C. elegans*. Taken together, these data are consistent with a multimodal character of CED-9 action, with an ability to regulate apoptosis through interactions in the cytosol coexisting with additional evolutionarily conserved role(s) at the membrane.
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Mammalian Bcl-2 proteins regulate apoptosis by interacting with both cytosolic proteins and intracellular membranes.[1,2] Although the precise nature of these interactions remains unclear, mutational analysis of the mammalian Bcl-2 protein Bcl-x_L revealed that both protein and membrane interactions contribute to the regulation of apoptosis.[3] These membrane interactions may be evolutionarily conserved in *Caenorhabditis elegans*, where the primary Bcl-2 homolog CED-9 localizes predominantly at mitochondria.[4] However, whether mitochondrial membrane interactions contribute to the regulation of apoptosis by CED-9 is not resolved. In an attempt to address this question, we have characterized a mitochondrial outer membrane localization signal in CED-9, and assessed its role in regulating apoptosis in *C. elegans*.

In mammals, regulation of apoptosis has been proposed to follow a rheostat model, whereby apoptosis is triggered when the activity of proapoptotic Bcl-2 proteins is greater than the activity of antiapoptotic Bcl-2 proteins.[1] Although altering the balance of these opposing activities can be achieved through multiple mechanisms,[1,2] mitochondrial localization is important for Bcl-2 proteins. This localization allows Bcl-2 proteins to modulate the permeability of mitochondrial outer membranes, which controls the efflux of apoptotic factors such as cytochrome *c*. While an exact mechanism for regulating mitochondrial outer membrane permeability is still unknown, Bcl-2 proteins may function by directly remodeling mitochondrial membranes[2] or by regulating proteins involved in the mitochondrial morphology changes that occur during cell death.[5] Regardless of the exact mechanism, mitochondrial localization of Bcl-2 proteins appears as a common feature in these models.

The *C. elegans* Bcl-2 homolog CED-9 has been proposed to function somewhat distinctly to regulate apoptosis. As the primary Bcl-2 homolog in *C. elegans*, CED-9 appears to possess both antiapoptotic and proapoptotic activities.[6,7] The antiapoptotic activity of CED-9 is thought to depend primarily on its ability to bind and sequester CED-4, a cytosolic caspase-activating factor that is homologous to mammalian Apaf-1. While mammalian Apaf-1 requires binding of cytochrome *c* to activate caspase-9, activation of the *C. elegans* caspase CED-3 *in vitro* can occur with just the addition of CED-4.[8] This ability to reconstitute the *C. elegans* apoptosis machinery without cytochrome *c* is consistent with the structure of CED-4, which differs from Apaf-1 in that it does not appear to contain an autoinhibitory domain that binds cytochrome *c*. It should be noted, however, that the proposed independence from cytochrome *c* has not been tested directly *in vivo* for *C. elegans*. In any case, this independence from cytochrome *c* does not rule out a role for CED-9 at mitochondria. In fact, two *C. elegans* mitochondrial proteins, CPS-6 and WAH-1, are released into the cytosol upon activation of the cell death pathway and enhance the efficiency of programmed cell death.[1] The proapoptotic activity of CED-9[6,9] is less known. One proposed mechanism involves regulating mitochondrial dynamics by interacting with the mitochondrial fission[10] and fusion machinery,[11] which are

[1]Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA; [2]Departments of Pathology and Genetics, Stanford University SOM, Stanford, CA 94305, USA and [3]Department of Chemistry, Johns Hopkins University, Baltimore, MD 21218, USA
*Corresponding author: RB Hill, Department of Biology, Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218, USA.
Tel: +1 410 516 6783; Fax: +1 702 441 2490; E-mail: hill@jhu.edu
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also implicated in mammalian apoptosis. However, it is unclear whether CED-9 interactions with mitochondria are required for its proapoptotic activity. Taken together, these observations raise a number of questions regarding the relationship between mitochondrial localization and apoptotic regulation by CED-9.

The membrane localization CED-9 and other of Bcl-2 proteins appears to be mediated through a C-terminal transmembrane domain (TM).[12] Although not as closely conserved as the four canonical Bcl-2 homology regions, this element is present throughout most of the Bcl-2 family.[13] Deletion analysis of the transmembrane domain in mammalian Bcl-2 family members generally reveals a shift from mitochondrial to cytosolic localization.[14,15] This change in localization may[15,16] or may not[14,17] block function, depending on the cell line and apoptotic stimuli. For CED-9, heterologous experiments in mammalian cell lines and biochemical reconstitution experiments suggest that CED-9 can interact with CED-4 to inhibit apoptosis independently of its transmembrane domain.[8,11,18] However, these findings contrast with a study suggesting that CED-9 activity may also be regulated via transmembrane domain interactions with the proapoptotic protein ceBNIP-3.[19] These studies leave open the question whether the transmembrane domain is required for CED-9 activity *in vivo*. Therefore, we have tested the requirement for the CED-9 transmembrane domain during developmental apoptosis in *C. elegans*.

Results

Assay for CED-9 regulation of apoptosis during embryogenesis. Two distinct periods of apoptosis in *C. elegans* account for the death of 131 cells during development: 113 cells die during embryogenesis, whereas 18 cells die during the L1/L2 larval stages.[20] In comparison, animals homozygous for loss-of-function *ced-9* alleles exhibit many ectopic cell deaths, as observed during embryogenesis and larval development.[7] To further examine the requirement for CED-9 in regulating apoptosis, we employed assays to probe each of these two periods of cell death (Figure 1).

The first assay tests whether or not a specific CED-9 construct can prevent ectopic cell deaths during embryogenesis in the absence of any wild-type CED-9 protein. We used the strong loss-of-function *ced-9* allele *n2812* (*ced-9(lf)*),[21] a nonsense allele that exhibits a maternal effect lethality whereby *ced-9(lf)* embryos lacking both zygotically expressed and maternally deposited CED-9 fail to complete development.[7,22] To produce a population that would die unless embryos produced functional CED-9, we mated *ced-9(lf)/+; ced-3(+)* males with *ced-9(lf); ced-3(lf)* hermaphrodites (Assay I, Figure 1). In these matings, cross-progeny express wild-type CED-3 in the zygote, resulting in embryonic lethality unless functional CED-9 is present. When we tested this assay, we observed 127 cross-progeny animals carrying one wild-type copy of *ced-9(+)* and zero cross-progeny animals homozygous for *ced-9(lf)* (Table 1), confirming that functional CED-9 is required early in development for viability.

To evaluate this assay as a transgene-based assay for testing CED-9 activity in embryonic lethality, we restored

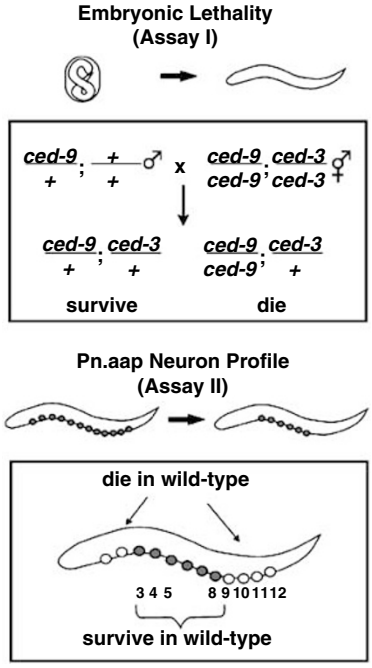


Figure 1 Schematic diagram of two assays that probe for CED-9 function at two distinct stages of apoptosis during *C. elegans* development. (Assay I) Improper regulation of apoptosis can result in embryonic lethality. To test whether various CED-9 constructs could properly regulate apoptosis, we employed an assay where *ced-9(n2812*lf*)* animals would die early in development unless functional CED-9 was produced from a transgene. In this assay, we used hermaphrodites lacking the CED-3 caspase to ensure viability without functional CED-9 protein (*ced-9(n2812*lf*) III; ced-3(n717*lf*) IV*). When these hermaphrodites were mated with males carrying a functional *ced-3* allele (*ced-9(n2812*lf*)/hT2[qIs48](I;III); nIs106[lin-11::gfp] X*), the homozygous *ced-9*(*n2812lf*) cross-progeny were dependent on functional CED-9 for survival. Three hermaphrodites were mated with four males, as described in Materials and Methods, a minimum of three times per construct. Cross-progeny were identified by GFP expression in the Pn.aap neurons and surrounding vulval tissues. Homozygous *ced-9(n2812*lf*)* animals were identified among this group by the lack of pharyngeal GFP expression from the *hT2[qIs48]* balancer chromosome;[38] *ced-9(n2812*lf*)/hT2[qIs48]* animals displaying the RoI phenotype ('*ced-9(n*2812lf*)/+* RoI') are reported as evidence that the cross was successful. Note that scoring of the RoI phenotype in rescued *ced-9(n2812*lf*)* homozygotes was not definitive, and thus the ratios between rescued *ced-9(n2812*lf*)* homozygous and *ced-9(n2812*lf*)/+* RoI heterozygous cross-progeny are of at best limited value in determining quantitative rescue frequencies. To control for any embryonic lethality that resulted from the chosen genetic backgrounds, as well as deviations from expected progeny ratios, we performed control experiments whereby *ced-9(lf)/+; ced-3(+)* males were mated with hermaphrodites wild type for the *ced-9* locus (see Materials and Methods). (Assay II) The presence or absence of Pn.aap neuron cells, as reported by an integrated *lin-11::gfp* transgene,[9,24] provides cell-specific information on the proper regulation of apoptosis. These Pn.aap neurons are so called due to their cell division lineage (anterior–anterior–posterior) from one of the 12 embryonic P blast cells, where 'n' corresponds to the position in the ventral cord along the anterior–posterior axis. In a larval animal, these 12 neurons can be found evenly spaced from anterior P1.aap to posterior P12.aap along the ventral side of the animal.[20] During the course of development, six cells ($n = 1, 2, 9–12$) undergo apoptosis, while six mid-body cells ($n = 3–8$) differentiate into ventral cord neurons. For reasons of optical reproducibility, we monitored the cell deaths of only eight cells. Pn.aap neuron profiles were collected by examining GFP expression in populations of adult hermaphrodite *ced-9(n2812*lf*); nIs106[lin-11::gfp]* animals with a dissecting epifluorescence microscope. The population of animals were collected from *ced-9(n2812*lf*)/hT2[qIs48]; nIs106[lin-11::gfp]* mothers carrying a *gfp::ced-9* transgene, unless otherwise noted. *ced-9(n2812*lf*)* homozygotes were identified by the lack of pharyngeal GFP expression from the *hT2[qIs48]* balancer chromosome, whereas the *gfp::ced-9* transgene was identified by the RoI phenotype. At least 30 animals were scored for each profile

Table 1 Viability rescue characteristics of *ced-9* transgenes

Extrachromosomal array	Progeny observed	
	***ced-9(n2812*lf*)/+* RoI**	***ced-9(n2812*lf*)/ ced-9(n2812*lf*)***
None	127	0
$P_{ced\text{-}9}$*gfp::ced-9*-A	40	80
$P_{ced\text{-}9}$*gfp::ced-9*-B	42	48
$P_{ced\text{-}9}$*gfp::ced-9*-C	14	35
(1/5th) $P_{ced\text{-}9}$*gfp::ced-9*-A	111	186
(1/5th) $P_{ced\text{-}9}$*gfp::ced-9*-B	72	108
(1/5th) $P_{ced\text{-}9}$*gfp::ced-9*-C	67	114
(1/5th) $P_{ced\text{-}9}$*gfp::ced-9*-D	34	50
(1/20th) $P_{ced\text{-}9}$*gfp::ced-9*-A	29	39
(1/20th) $P_{ced\text{-}9}$*gfp::ced-9*-B	38	54
(1/20th) $P_{ced\text{-}9}$*gfp::ced-9*-C	21	12
(1/20th) $P_{ced\text{-}9}$*gfp::ced-9*-D	35	44

Three *ced-9(n2812*lf*); ced-3(n717*lf*)* hermaphrodites were mated with four *ced-9(n2812*lf*)/hT2[qIs48]; nIs106[lin-11::gfp]* males, and cross-progeny were scored as described in Figure 1, Assay I. Note that hermaphrodite ratios between *hT2[qIs48]* and *ced-9(n2812*lf*)* are somewhat diverged from 1 : 1 as a consequence of segregation-distortion effects of the *hT2[qIs48]* balancer chromosome discussed in more detail in the Materials and Methods section.

CED-9 regulation of apoptosis in loss-of-function *ced-9* animals using an essentially complete *ced-9* transgene. We produced transgenic lines expressing CED-9 with green fluorescent protein (GFP) fused to the N terminus (*gfp::ced-9)*, and driven by a *ced-9* promoter from an extrachromosomal DNA array. Transgenic lines were produced in the *ced-9(lf); ced-3(lf)* genetic background lacking functional CED-9 to ensure that only our specified form of CED-9 was present.

In multiple *gfp::ced-9* transgenic lines that were generated independently, GFP appeared in punctate structures in a subset of cells during embryogenesis (Supplementary Figure 1a). For all three independently produced transgenic lines, *ced-9(lf)/ced-9(lf)* cross-progeny were observed ($P_{ced\text{-}9}$*gfp::ced-9*-A,B,C; Table 1), indicating that mosaic expression of *gfp::ced-9* transgenes is sufficient for viability, and may indicate that different cells require different amounts of CED-9 activity. The relative number of cross-progeny from each genotype was similar to transgenic lines expressing CED-9 without GFP fused to the N terminus (Supplementary Figure 2a), suggesting that GFP does not prevent CED-9 from regulating apoptosis. Additionally, transgene rescue requires functional CED-9, as no rescue was observed with a *ced-9* construct missing the sixth α-helix of CED-9 (*gfp::ced-9*$\Delta\alpha$*6*) that we predicted to be non-functional (Supplementary Figure 3a).

Although this construct was driven by a copy of the *ced-9* promoter, appropriate expression of CED-9 from the multi-copy extrachromosomal DNA array is a concern.[23] As bulk assessment of CED-9 expression levels by Western blot analyses was of limited value (also see Supplementary Figure 4), we addressed the CED-9 dose response by examining sets of transgenic lines that were generated independently using a range of DNA concentrations. Keeping the total DNA concentration fixed in the injection mixes, we produced independent sets of transgenic lines in which the amount of *gfp::ced-9* plasmid DNA was reduced by 5- or 20-fold. Using these injection mixes, GFP expression was still observed by microscopic analysis, and transgenic *ced-9(lf)/ced-9(lf)* animals were still viable ((1/5th) and (1/20th)$P_{ced\text{-}9}$*gfp::ced-9*; Table 1).

Assay for cell-specific CED-9 regulation of apoptosis in ventral cord neurons. While the embryonic lethality assay reflects the requirements for CED-9 to prevent widespread apoptosis during embryogenesis, we sought to assay cell-specific regulation of apoptosis. One group of cells that undergo post-embryonic apoptosis is the 12 ventral cord neurons in the Pn.aap equivalence group. Using an integrated *lin-11::gfp* reporter (*nIs106[lin-11::gfp] X*), previously used to study apoptosis,[9,24] Pn.aap cells that survive express GFP, and are readily distinguished from cells that die and disappear (Assay II, Figure 1). As previously observed, one additional benefit to assaying apoptosis in these cells is that they capture both antiapoptotic and proapoptotic activities of CED-9[9] (as described below).

As a baseline measure for the efficacy of the *lin-11::gfp* reporter, populations of otherwise wild-type *nIs106[lin-11::gfp]* animals were scored for Pn.aap cells (the Pn.aap neuron profile). Of the eight cells that could be reliably scored, four survive in *nIs106[lin-11::gfp]* animals ($n = 3$–5, 8), while four cells die ($n = 9$–12; Figure 2a). Among these animals, the Pn.aap neuron profile faithfully reflected the previously described cell lineage.[20] As expected, in *ced-3(n717*lf*); nIs106[lin-11::gfp]* animals lacking the CED-3 caspase, no cell deaths were observed (Figure 2b). To obtain a *ced-9* loss-of-function reference for the assay, we assessed the Pn.aap neuron profile among *ced-9(lf); nIs106[lin-11::gfp]* animals that survived due to maternal deposition of CED-9. In these maternally rescued populations, three cells that survive in wild-type animals (cells 3–5) did not survive as often. Additionally, we observed the opposite effect for the four cells that normally die in wild-type animals (cells 9–12), which survived at a higher frequency than wild type (Figure 2c). The lack of death in these cells is consistent with previous observations[9] and with the proposed roles for CED-9 in promoting apoptosis.[6]

To test the capacity of GFP::CED-9 to properly regulate apoptosis, we examined the Pn.aap neuron profile of *gfp::ced-9* transgenic lines. Because *ced-9(lf)* animals carrying *gfp::ced-9* transgenes are sterile, we could only examine *ced-9(lf)* animals from heterozygote *ced-9(lf)/+* mothers. These maternally rescued progeny contain both maternally deposited wild-type CED-9 and GFP::CED-9. In these *gfp::ced-9* transgenic animals, the four cells that survive in wild-type animals were present at frequencies comparable to wild type (Figure 2d). The cells that die in wild-type animals but persist in maternally rescued *ced-9(lf)* animals lacking a *ced-9* transgene were no longer present in one of the two lines examined (Figure 2d). Pn.aap neuron profiles similar to wild-type animals were also seen for *gfp::ced-9* transgenic animals produced using lower amounts of plasmid DNA (Figure 2e and f). Similar profiles were seen for transgenic animals expressing CED-9 without GFP fused to the N terminus (Supplementary Figure 2b), again supporting that GFP does not interfere with the ability of CED-9 to regulate apoptosis. These results contrast with *gfp::ced-9*$\Delta\alpha$*6* transgenic animals that displayed a Pn.aap neuron profile similar to *ced-9(lf)* animals,

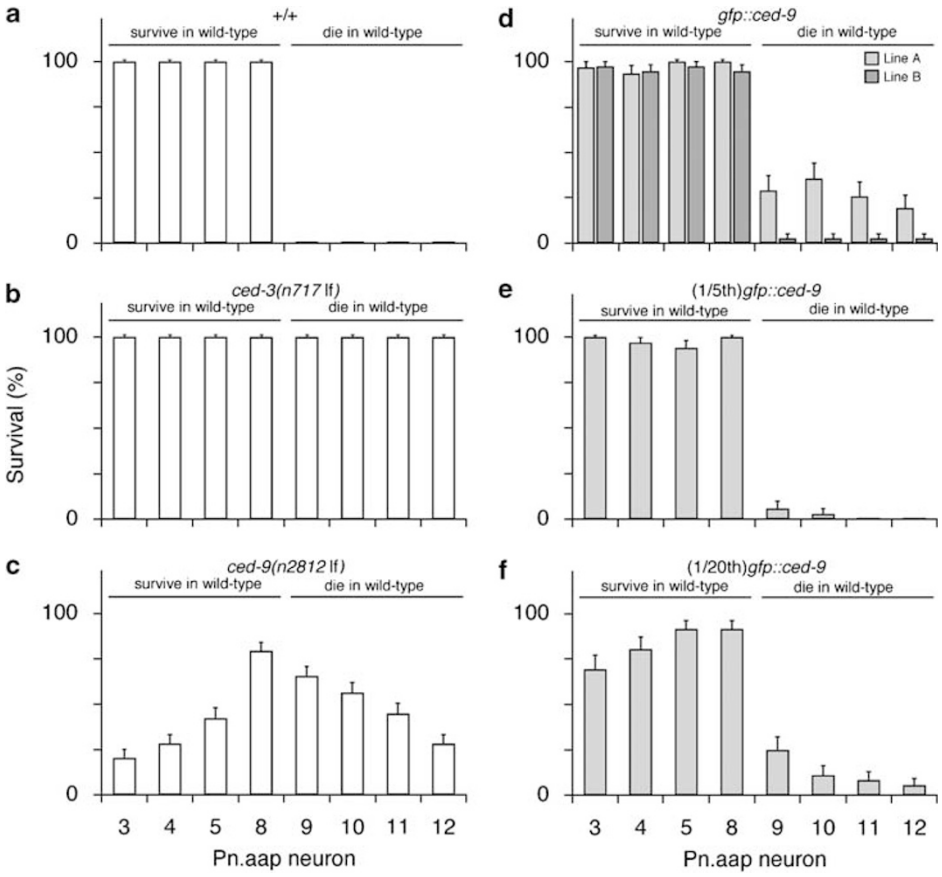


Figure 2 Lack of CED-9 protein results in an aberrant Pn.aap neuron profile. Pn.aap neuron profiles indicate the percentage of the population (*y*-value) where a Pn.aap neuron was observed at the indicated position (*x*-value) by GFP expression from a *lin-11::gfp* transgene. Shown are profiles of (**a**) otherwise wild-type animals carrying only the *nIs106[lin-11::gfp]* transgene and (**b**) *ced-3(n717*lf*); nIs106[lin-11::gfp]* animals unable to activate apoptosis in the Pn.aap neurons. (**c**) Maternally rescued *ced-9(n2812*lf*); nIs106[lin-11::gfp]* animals were examined by placing 10 heterozygous loss-of-function *ced-9* animals (*ced-9(n2812*lf*)/hT2[qIs48]; nIs106[lin-11::gfp]*) on a plate and scoring the homozygous *ced-9(n2812*lf*)* self-progeny ($n=78$). (**d**–**f**) The ability of various *gfp::ced-9* transgenes to properly pattern Pn.aap neurons was tested by examining descendents of the embryonic lethality assay, as described in Materials and Methods. (**d**) Profiles for two of the independently produced transgenic lines, *gfp::ced-9*-A ($n=31$) and *gfp::ced-9*-B ($n=38$), are displayed as the first and second set of bars, respectively. Profiles for transgenic animals produced using a lower ratio of *gfp::ced-9* to marker plasmid are shown in (**e**) and (**f**) for a five-fold reduction ((1/5th)*gfp::ced-9*-A ($n=34$)) and a 20-fold reduction ((1/20th)*gfp::ced-9*-A ($n=36$)), respectively. Error bars represent standard errors, as calculated for binomial distributions

as expected for lines expressing non-functional forms of CED-9 (Supplementary Figure 3b).

The wild-type Pn.aap neuron profiles suggest that GFP::CED-9 is not significantly overexpressed, as we do not observe excessive survival of the posterior Pn.aap neurons that normally die ($n=9$–12). Cases of excessive survival can be seen in *ced-3(lf)* animals deficient for components of the apoptotic machinery (Figure 2b) and in animals with a gain-of-function CED-9 mutant deficient in transmitting signals along the apoptosis pathway (see below; Figure 5).

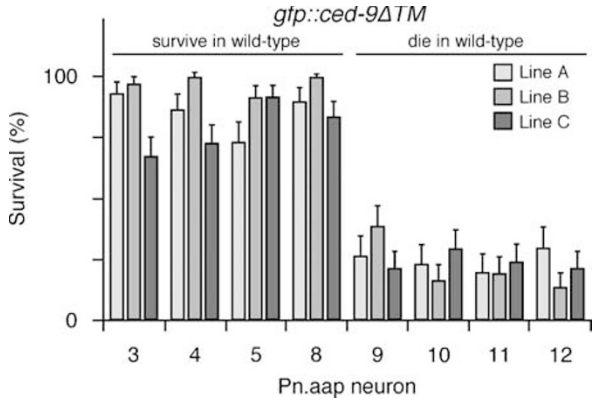
Evidence that the C-terminal 29 residues of CED-9 contribute to, but are not essential for, regulation of apoptosis. To test whether the C-terminal transmembrane domain contributes to CED-9 activity, we produced transgenic lines carrying a 'tail-less' *gfp::ced-9*Δ*TM* construct that lacks the C-terminal 29 residues of CED-9. Similar to embryos carrying *gfp::ced-9* transgenes, embryos expressing the tail-less *gfp::ced-9*Δ*TM* construct displayed a mosaic expression pattern (Supplementary Figure 1b). However, instead of punctate localization, GFP appeared diffuse throughout the cell. When tested for the ability to prevent embryonic lethality, the truncated construct appeared as effective as the full-length construct in three independent lines ($P_{ced\text{-}9}$*gfp::ced-9*Δ*TM*-A,B,C; Figure 3a). When tested for the ability to restore a proper Pn.aap neuron profile, the cells that survive in wild-type animals were present in *gfp::ced-9*Δ*TM* transgenic animals at near wild-type frequencies in three independent lines (Figure 3b). In these animals, however, the cells that normally die in wild-type animals were present at frequencies intermediate between wild-type animals and maternally rescued *ced-9(lf)* animals (Figure 3b).

To assess the dose response to the *gfp::ced-9*Δ*TM* construct, we decreased the plasmid DNA concentration of *gfp::ced-9*Δ*TM* and generated six independent lines. Only one out of these six lines was able to effectively prevent embryonic lethality ((1/5th)$P_{ced\text{-}9}$*gfp::ced-9*Δ*TM*; Figure 3a). On examining these lines, GFP expression by fluorescence microscopy was observed only in the one line with rescue activity. When

a

Extrachromosomal Array	Progeny observed	
	*ced-9(n2812*lf*)/+* Rol	*ced-9(n2812*lf*)/ced-9(n2812*lf*)*
P_{ced-9}*gfp::ced-9ΔTM*-A	34	42
P_{ced-9}*gfp::ced-9ΔTM*-B	23	28
P_{ced-9}*gfp::ced-9ΔTM*-C	14	19
(1/5th) P_{ced-9}*gfp::ced-9ΔTM*-A	35	21
(1/5th) P_{ced-9}*gfp::ced-9ΔTM*-B	20	0
(1/5th) P_{ced-9}*gfp::ced-9ΔTM*-C	41	0
(1/5th) P_{ced-9}*gfp::ced-9ΔTM*-D	95	1
(1/5th) P_{ced-9}*gfp::ced-9ΔTM*-E	128	0
(1/5th) P_{ced-9}*gfp::ced-9ΔTM*-F	67	0

b



c

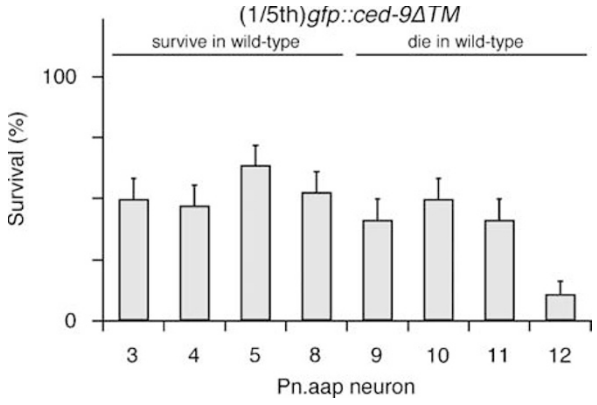


Figure 3 CED-9 regulates apoptosis without its putative transmembrane domain in two *in vivo* assays. The C terminus of CED-9 contains a putative transmembrane domain. Transgenic lines were produced with a *ced-9* construct lacking the C-terminal 29 residues (*gfp::ced-9ΔTM*) to test for the role of this domain. (**a**) The function of *gfp::ced-9ΔTM* transgenic lines was tested by mating three transgenic *ced-9(n2812*lf*); ced-3(n717*lf*)* hermaphrodites with four *ced-9(n2812*lf*)/hT2[qIs48]; nIs106[lin-11::gfp]* males, and cross-progeny were scored. Multiple independently produced transgenic lines (marked A, B, C, etc.) were tested for a standard *gfp::ced-9ΔTM* concentration and 1/5th the concentration. (**b** and **c**) The ability of various *gfp::ced-9ΔTM* transgenes to properly pattern Pn.aap neurons was tested by examining descendents of the embryonic lethality assay. (**b**) Profiles for three of the independently produced transgenic lines, *gfp::ced-9ΔTM*-A ($n=30$), *gfp::ced-9ΔTM*-B ($n=36$) and *gfp::ced-9ΔTM*-C ($n=37$), are displayed as the first, second and third set of bars, respectively. A profile for the one *gfp::ced-9ΔTM* transgenic line produced using a five-fold lower ratio of *gfp::ced-9ΔTM* to marker plasmid that effectively rescues embryonic lethality ((1/5th)*gfp::ced-9ΔTM*-A) is shown in (**c**) ($n=36$). Error bars represent standard errors, as calculated for binomial distributions

the Pn.aap neuron profile of this one functional line was examined, the pattern was similar to that seen with *gfp::ced-9Δα6* animals and maternally rescued *ced-9(lf)* animals without a *ced-9* transgene (Figure 2c). By contrast (as described above), this effect was not observed when plasmid DNA concentrations were reduced for the full-length CED-9 construct (Table 1 and Figure 2f).

The transmembrane domain of CED-9 is necessary and sufficient for localization at mitochondrial outer membranes. To better resolve the subcellular distribution of CED-9 and CED-9ΔTM, and to explore the role of the transmembrane domain in mitochondrial localization, we created transgenic lines of *gfp::ced-9* driven by a *myo-3* promoter in a *ced-9(lf); ced-3(lf)* genetic background. This

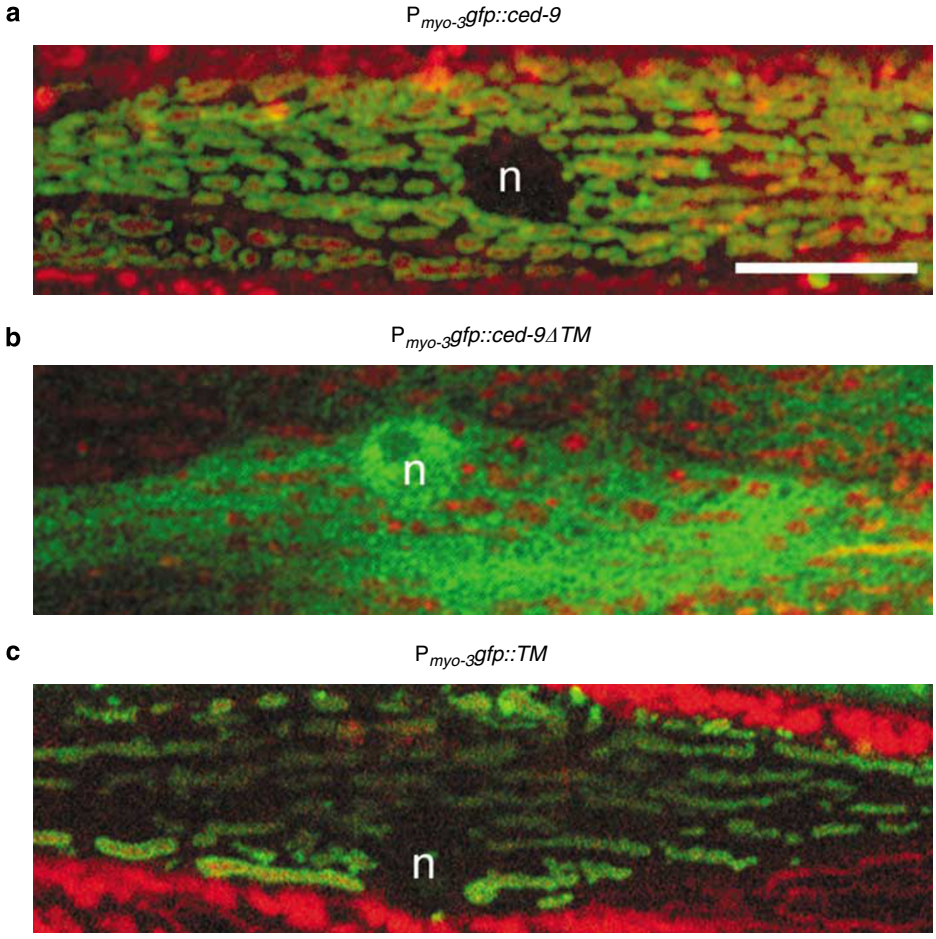


Figure 4 The transmembrane domain of CED-9 is necessary and sufficient for localization at mitochondrial outer membranes. The importance of the CED-9 transmembrane domain for localization at mitochondria was tested by examining GFP fluorescence in the body wall muscles of animals transgenic for a *gfp::ced-9* transgene. Confocal images were obtained from transgenic *ced-9(n2812*lf*); ced-3(n717*lf*)* animals stained with MitoTracker Red, as described in Materials and Methods. Animals carry an extrachromosomal array driven by a *myo-3* promoter for (**a**) *gfp::ced-9*, (**b**) *gfp::ced-9*Δ*TM* and (**c**) *gfp::TM*. The scale bar corresponds to 10 μm, and 'n' marks the nucleus

promoter drives gene expression in the body wall muscles, a group of cells with stereotypical tubular mitochondria that are well suited for epifluorescence microscopy.[25] GFP::CED-9 was localized around the periphery of vesicular bodies in multiple adult animals, consistent with the punctate localization seen in embryos where GFP::CED-9 was driven by a copy of the *ced-9* promoter (Supplementary Figure 1a). Counterstaining with MitoTracker Red, which accumulates in the mitochondrial matrix, indicated that GFP::CED-9 was localized at the outer surface of mitochondria (Figure 4a).

To test whether this mitochondrial outer membrane localization was determined by the putative C-terminal transmembrane domain, we examined transgenes lacking the C-terminal 29 amino acids of CED-9 (*gfp::ced-9*Δ*TM*). GFP was no longer restricted to mitochondria and appeared predominantly in the cytosol (Figure 4b). Additionally, the presence of some fluorescent signal in the nucleus indicates that GFP::CED-9, or a derivative thereof, is not significantly restricted to intracellular membranes and could traverse the nuclear envelope.

The transmembrane domain was sufficient for localization at mitochondrial outer membranes, as GFP fused to the C-terminal 29 residues of CED-9 (*gfp::TM*) effectively localized at mitochondria (Figure 4c). This mitochondrial outer membrane localization appears specific, as no other GFP fusion constructs in our laboratory with numerous other fusion partners have exhibited a similar pattern in *C. elegans* (data not shown).

The gain-of-function CED-9 mutation G169E does not require the transmembrane domain to regulate apoptosis. To further explore the relationship between localization and CED-9 regulation of apoptosis, we next asked whether CED-9ΔTM protein was still capable of blocking apoptosis in a manner similar to the gain-of-function *ced-9* allele *n1950*sd. This allele results in a G169E mutation that interferes with binding of the proapoptotic EGL-1 protein to CED-9, preventing activation of CED-4 and the apoptosis pathway.[6,26] If this mutation interferes primarily through modulation of cytosolic binding activities, then one would predict that the transmembrane domain would not be

a

Extrachromosomal Array	Progeny observed	
	*ced-9(n2812*lf*)*/+ Rol	*ced-9(n2812*lf*)/ced-9(n2812*lf*)*
P*ced-9gfp::ced-9(G169E)ΔTM*-A	24	33
P*ced-9gfp::ced-9(G169E)ΔTM*-B	16	27
P*ced-9gfp::ced-9(G169E)ΔTM*-C	5	3

b

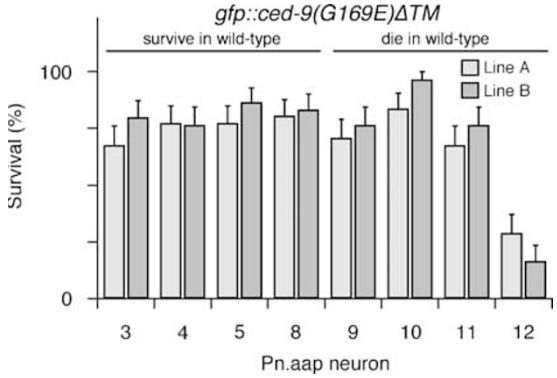


Figure 5 A gain-of-function CED-9 variant (G169E) retains activity in the absence of a transmembrane domain. The gain-of-function CED-9 mutation G169E interferes with EGL-1 binding and activation of the apoptosis pathway. Transgenic lines were produced with a *ced-9* construct containing the G169E mutation and lacking the transmembrane domain to test whether this mutation still interferes with activation of the apoptosis pathway in the absence of a transmembrane domain. (**a**) The activity of *gfp::ced-9(G169E)ΔTM* transgenic lines was tested by mating three transgenic *ced-9(n2812*lf*); ced-3(n717*lf*)* hermaphrodites with four *ced-9(n2812*lf*)/hT2[qIs48]; nIs106[lin-11::gfp]* males, and cross-progeny were scored. Multiple independently produced transgenic lines (marked A, B and C) were tested for a standard *gfp::ced-9(G169E)ΔTM* concentration. (**b**) The ability of two *gfp::ced-9(G169E)ΔTM* transgenes to pattern Pn.aap neurons was tested by examining descendents of the embryonic lethality assay. Profiles for two of the independently produced transgenic lines, *gfp::ced-9(G169E)ΔTM*-A ($n = 31$) and *gfp::ced-9(G169E)ΔTM*-B ($n = 30$), are displayed as the first and second set of bars, respectively. Error bars represent standard errors, as calculated for binomial distributions

essential. Such a *gfp::ced-9(G169E)ΔTM* construct was able to prevent embryonic lethality in three independent lines (P*ced-9gfp::ced-9(G169E)ΔTM*-A,B,C; Figure 5a). When we examined the Pn.aap neuron profiles for this construct (Figure 5b), we observed that most of the cells were present, reminiscent of *ced-3(lf)* (Figure 2b) and *ced-9(n1950*sd*)* animals (data not shown).

Evidence that targeting CED-9 to the cytosolic surface of the endoplasmic reticulum and derived organelles does not prevent apoptotic regulation. One possible interpretation from the localization and rescue experiments is that CED-9ΔTM resides solely in the cytosol, and can act in the absence of any mitochondrial localization. However, we cannot rule out a small population of protein transiently associating with mitochondrial outer membranes, as suggested by studies with human Bcl-2 proteins lacking the transmembrane domain.[3,27–29] Therefore, we attempted to sequester CED-9 away from mitochondria, yet still allow access to any necessary cytosolic components.

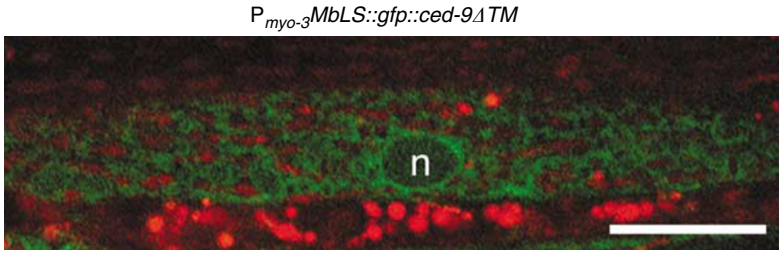
To intentionally mislocalize CED-9, we fused *gfp::ced-9ΔTM* to an N-terminal *pat-3* integrin secretion signal and transmembrane domain (*MbLS::gfp::ced-9ΔTM*).[30] This construct should result in GFP::CED-9ΔTM tethered to the cytosolic surface of the endoplasmic reticulum and derived organelles. When expressed by the *myo-3* promoter, GFP was seen localized to intracellular reticulate structures, as well as perinuclear membranes (Figures 6a). These structures did not counterstain with MitoTracker Red, suggesting specific targeting to non-mitochondrial membrane surfaces in the cytosol such as the endoplasmic reticulum. Additionally, GFP fluorescence was absent from the nucleus, suggesting that this construct was no longer free to traverse the nuclear envelope. Once again, similar to embryos carrying a P*ced-9gfp::ced-9* transgene, embryos expressing a P*ced-9 MbLS::gfp::ced-9ΔTM* transgene had GFP localized to intracellular membranes in a subset of cells (Supplementary Figure 1c).

In the embryonic lethality assay, CED-9 lacking the transmembrane domain and targeted to the endoplasmic reticulum and derived organelles rescued as effectively as the full-length and transmembrane domain deletion constructs in three independent lines (P*ced-9MbLS::gfp::ced-9ΔTM*-A,B,C; Figure 6b). This construct was also able to restore the wild-type Pn.aap neuron profile in three independent lines (Figure 6c). However, similar to the transmembrane domain deletion construct, attempts to reduce the amount of plasmid DNA used to produce the *ced-9* transgenic lines resulted in a loss of rescue ((1/5th)P*ced-9MbLS::gfp::ced-9ΔTM*-A,B,C; Figure 6b). Also similar to the transmembrane domain deletion construct, we observed no GFP fluorescence in these lines by fluorescence microscopy.

Discussion

The ability of CED-9 to regulate apoptosis is essential to the viability of *C. elegans*.[7] By exploring the localization and activity of CED-9, this work suggests that the C-terminal

a



b

Extrachromosomal Array	Progeny observed	
	*ced-9(n2812*lf*)*/+ RoI	*ced-9(n2812*lf*)/ced-9(n2812*lf*)*
P *ced-9* *MbLS::gfp::ced-9ΔTM*-A	57	23
P *ced-9* *MbLS::gfp::ced-9ΔTM*-B	54	48
P *ced-9* *MbLS::gfp::ced-9ΔTM*-C	47	74
(1/5th) P *ced-9* *MbLS::gfp::ced-9ΔTM*-A	88	0
(1/5th) P *ced-9* *MbLS::gfp::ced-9ΔTM*-B	109	0
(1/5th) P *ced-9* *MbLS::gfp::ced-9ΔTM*-C	93	0

c

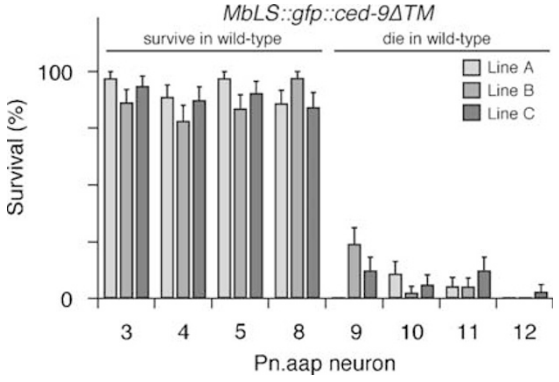


Figure 6 Activity of CED-9ΔTM targeted to non-mitochondrial cytoplasmic membranes. The N-terminal signal sequence and transmembrane domain from the *pat-3* integrin were fused to the N-terminus of *gfp::ced-9ΔTM* in an attempt to limit the access of CED-9 to mitochondrial outer membranes. This membrane localization signal (*MbLS*) should initially target protein to the cytosolic surface of the endoplasmic reticulum and derived organelles. (**a**) GFP expression in body wall muscles was examined to determine the subcellular localization of the *MbLS::gfp::ced-9ΔTM* construct driven by the *myo-3* promoter. Confocal images were obtained from transgenic *ced-9(n2812*lf*); ced-3(n717*lf*)* animals stained with MitoTracker Red. The scale bar corresponds to 10 μm, and 'n' marks the nucleus. (**b**) The activity of *MbLS::gfp::ced-9ΔTM* transgenic lines was tested by mating three transgenic *ced-9(n2812*lf*); ced-3(n717*lf*)* hermaphrodites with four *ced-9(n2812*lf*)/hT2[qIs48]; nIs106[lin-11::gfp]* males, and cross-progeny were scored. Multiple independently produced transgenic lines (marked A, B and C) were tested for a standard *MbLS::gfp::ced-9ΔTM* concentration and 1/5th the concentration. (**c**) The ability of three *MbLS::gfp::ced-9ΔTM* transgenes to properly pattern Pn.aap neurons was tested by examining descendents of the embryonic lethality assay. Profiles for three of the independently produced transgenic lines, *MbLS::gfp::ced-9ΔTM*-A ($n=36$), *MbLS::gfp::ced-9ΔTM*-B ($n=37$) and *MbLS::gfp::ced-9ΔTM*-C ($n=32$), are displayed as the first, second and third set of bars, respectively. Error bars represent standard errors, as calculated for binomial distributions

transmembrane domain of CED-9 that targets this protein to mitochondria is not essential to regulate apoptosis in *C. elegans*. CED-9 transgenes lacking the transmembrane domain were able to prevent embryonic lethality and ensure proper patterning of the Pn.aap neuron profile. This latter assay appears to require CED-9 to respond to upstream regulatory signals to prevent or enhance apoptosis.

Studying Bcl-2 protein structure–activity relationships during *C. elegans* development allows the measurement of both pro- and antiapoptotic activities in a physiologically relevant set of cell deaths. This system also avoids the difficulties of manipulating multiple, redundant Bcl-2 genes present in mammals. Our assays, however, have the challenge of potential variability in transgene expression commonly encountered in *C. elegans*.[23] Despite this concern, CED-9 expression levels and patterns are likely in a physiologically relevant range. First, we observed functional rescue (viable animals) in the expected ratio when wild-type *ced-9* constructs were used for transformation. Second, Pn.aap cell deaths in *ced-9* transgenic animals were reproducible and equivalent to those seen in wild-type animals, and not what one would expect from overexpression. Finally, these observations were repeatable with numerous independent transgenic lines at a range of DNA concentrations.

Our results extend earlier reports on the proapoptotic activity of CED-9. Although the Pn.aap cells that normally die in wild type ($n=9$–12) are partially present in transgenic *gfp::ced-9ΔTM* lines (Figures 2a and 3b), we observed a near

wild-type Pn.aap neuron profile with transgenic *MbLS::gfp::ced-9ΔTM* lines. In these lines, CED-9 lacking the transmembrane domain is targeted to the cytosolic surface of the endoplasmic reticulum and derived organelles (Figure 6c). The difference in activities between the ΔTM transgenes may result from a difference in protein expression levels (Supplementary Figure 4). Alternatively, intracellular membranes may cause a conformational change in CED-9 that is involved in its proapoptotic activity. Such membrane-induced conformational changes of mammalian Bcl-2 proteins have been reported, and are suggested to be important for activity.[28,29,31,32]

Regulation of apoptosis in *C. elegans* by CED-9 lacking the transmembrane domain is consistent with previous studies. Overexpression of murine Bcl-2ΔTM (and augmenting the endogenous pool of Bcl-2 proteins) in sympathetic neurons reduced the rate of apoptosis when cells were deprived of NGF, and also reduced the rate of apoptosis in L929 fibroblasts treated with TNF.[14] Additionally, overexpression of murine Bcl-x_LΔTM in the IL-3-dependent DA-1 cell line delayed the onset of apoptosis after IL-3 withdrawal.[33] Finally, CED-9ΔTM was able to prevent apoptosis induced by heterologous expression of CED-4/CED-3 in HeLa cells, although CED-9 could not prevent intrinsic apoptosis pathways in this system.[11] Our study furthers these findings by suggesting that CED-9 may regulate apoptosis during *C. elegans* development without the transmembrane domain and without the contribution of endogenous wild-type CED-9 molecules.

Our findings do not rule out non-essential roles for CED-9 at mitochondria. One role may be to help maintain mitochondrial morphology, an activity recently described for several mammalian Bcl-2 proteins[11,34,35] and CED-9.[11] For CED-9, this effect may be direct or through interactions with other mitochondrial proteins, such as the dynamin-related protein DRP-1.[10,11] Mitochondrial fragmentation induced by DRP-1 overexpression was shown to involve CED-9, although it is unclear whether this fragmentation requires the CED-9 transmembrane domain or CED-9 localization at mitochondria.[10] If DRP-1 activity in apoptosis requires CED-9 as proposed,[10] then our observation of a near wild-type Pn.aap profile of ER-targeted CED-9ΔTM suggests that DRP-1 activity does not require the CED-9 transmembrane domain or mitochondrial localization (Figure 6a and c). However, as mutations that inhibit DRP-1-mediated mitochondrial fragmentation only have a modest effect on apoptosis *in vivo*,[10] defects in DRP-1 activity may not be detected by our assays. Understanding the importance of CED-9 in these processes will require assays that directly detect mitochondrial morphology and not cell viability.

In conclusion, our results support a model whereby CED-9 primarily regulates apoptosis by sequestering CED-4, and further this model by suggesting that CED-9 membrane interactions are not essential for viability. Perhaps CED-9 membrane interactions serve to enhance apoptotic efficiency in some manner such as regulating mitochondrial morphology. Given that a C-terminal transmembrane domain is also found in many mammalian Bcl-2 proteins, our results imply that a biological function at mitochondrial outer membranes that is not essential for viability may be evolutionarily conserved between nematode and mammalian Bcl-2 proteins.

Materials and Methods

Genetic methods and strains. *C. elegans* were cultured and maintained as described previously[20] on MYOB agar[36] at 20°C. The following mutations were used: LGIII: *ced-9(n2812;* Q46amber*)*;[21] LGIV: *ced-3(n717;* splice acceptor mutation, exon 7*)*;[37] and LGX: *nIs106*[P_{lin-11}*gfp*].[24] The P_{lin-11}*gfp* integrated transgene expresses GFP in the Pn.aap ventral cord neurons, and was generously provided by Scott Cameron (UT Southwestern Medical Center, Dallas, TX, USA). *hT2[qIs48](I;III)* was used as a dominant balancer chromosome, which spans the *ced-9* locus. *qIs48* is an insertion of the *ccEx9747*[P_{myo-2}*gfp* P_{pes-10}*gfp* $P_{F22B7.9}$*gfp]* transgene onto *hT2* that can be followed by GFP expression in the pharynx.[38]

Germline transformations were performed as described previously.[23] We generally injected the *ced-9* constructs (approximately 6.8 kb) at 33.3 μg/ml along with the pRF4 plasmid containing the *rol-6(su1006)* gene at 83.3 μg/ml as the co-injection marker into *ced-9(n2812); ced-3(n717)* animals. Assuming a 1 Mb extrachromosomal array[39,40] this concentration would correspond to roughly 40 copies of the *ced-9* construct in each nucleus, many of which are likely inactivated in any given nucleus through the action of *C. elegans* foreign DNA silencing systems.[23,41] In experiments to reduce the amount of *ced-9* plasmid DNA, the ratio of *ced-9* plasmid DNA to the pRF4 marker plasmid DNA was altered, while keeping total DNA concentration roughly constant. Reducing the amount of *gfp::ced-9* plasmid DNA used by 5- or 20-fold, corresponding to a final concentration of 6.7 μg/ml and 1.7 μg/ml, respectively, would yield roughly eight- and two copies of *gfp::ced-9* per extrachromosomal array based on an assumed 1 Mb extrachromosomal array.[23] It is important to note, however, that the correlation between extrachromosomal gene copy number and expression levels is highly variable.[23]

Animals containing an extrachromosomal *ced-9* transgene were identified by the Rol phenotype. In some cases, spontaneous integrants of the extrachromosomal transgene were obtained. These integrants retained the same level and pattern of GFP expression, as well as the same functional activity.

Plasmid constructs. The wild-type *Nco*I/*Xho*I 3723 nt *ced-9* polycistronic region[21] is contained in the cosmids C41B4 and F11D2. The P_{myo-3}*gfp::ced-9* construct (pFT37.11) was made by PCR amplification of the 1909 nt *ced-9* coding region from cosmids provided by Alan Coulson (Sangre Center, UK), and cloned into pPD181.44 (L6890, a plasmid derived from components of the Fire Lab Vector kit (Addgene Inc.) that expresses in the body wall muscles). The P_{myo-3}*gfp::ced-9* construct contains a CPGDRWSS peptide linker between the C terminus of GFP and the N terminus of CED-9, and a *let-858 3′* region downstream of *ced-9*. The P_{myo-3}*gfp::ced-9ΔTM* construct (pFT37.27) was made in a similar fashion, but without amplification of nt 1820–1906 in the coding region. The P_{myo-3}*gfp::TM* construct (pFT46.1) was made by amplifying only nt 1820–1909, and contains the additional amino acids MH between the peptide linker and the start of the transmembrane domain.

To create rescue constructs, the *ced-9* promoter (including the first two codons, but with the ATG mutated to ATA) was PCR amplified from genomic DNA. The resulting PCR product containing 1199 nt of the *ced-9* locus was cloned into pPD118.37 (L3793, a plasmid derived from components of the Fire Lab Vector kit). Rescue constructs were created by transferring in the *gfp::ced-9* regions from the aforementioned P_{myo-3} constructs (pFT38.21 and pFT38.29, respectively). GFP was retained at the N terminus of CED-9 to verify transgene expression and subcellular localization.

The *MbLS::gfp::ced-9ΔTM* constructs (pFT44.2 and pFT45.70) were made by transferring in the *pat-3* integrin secretion signal and transmembrane domain from pPD122.36 (L4057). The *gfp::ced-9Δα6* construct (pFT47.34) was made by cutting the *Bam*HI/*Pst*I sites of *ced-9* and cloning in a bridging oligonucleotide that results in a deletion of helix 6 (amino acids 199-FVYTSLFIKTR-209).

The sequences of all coding regions amplified by PCR were determined to ensure that no point mutations were introduced by PCR. Sequences are available upon request, and plasmids will be deposited in Addgene (www.addgene.org).

MitoTracker staining. Young adults for mitochondrial colocalization experiments were collected from animals grown in the dark on MYOB agar plates containing 1.25 μM MitoTracker Red CMXRos (M-7512, Molecular Probes). Animals were anesthetized in either 0.5 mM levamisole or 0.5% 1-phenoxy-2-propanol. Images were collected with a Nikon PCM2000 laser scanning confocal

microscope coupled to an Eclipse E-600 microscope. Excitation and emission wavelengths were 488 and 515 nm for GFP, and 543 and 605 nm for MitoTracker Red. Images were integrated over time and spatially averaged.

Embryonic lethality rescue assay. Transgenic *ced-9(n2812); ced-3(n717)* L4 hermaphrodite animals carrying an extrachromosomal *gfp::ced-9* transgene were mated with *hT2[qIs48]/ced-9(n2812); nIs106* males and allowed to lay eggs for 1 day before being transferred to a fresh mating plate. This mating scheme prevented mixing of progeny generations, and was repeated one more time, for a total of 3 days worth of eggs per cross. Plates were scored between 3 and 4 days after removal of the parents for cross-progeny carrying *nIs106* by GFP expression in the Pn.aap ventral cord neurons, for the *hT2[qIs48]* balancer chromosome by GFP expression in the pharynx and for the *gfp::ced-9* transgene by the Rol phenotype. At least three separate crosses were conducted for each transgene.

Note that for crosses involving *hT2[qIs48]/ced-9(n2812lf); nIs106[lin-11::gfp] X* males, there is a reproducible distortion of progeny ratios (balancer/*ced-9* chromosome), due at least in part to meiotic pairing in the male parent between the X chromosome (which is otherwise unpaired in the *C. elegans* XO male) and the *hT2[qIs48]* balancer (some quantitative distortion could also be due to lower viability of *hT2[qIs48]* cross-progeny, although this effect seems at most marginal). Examples of distributive pairing are observed frequently with unpaired genetic elements during *C. elegans* meiosis.[42] For the crosses described in this work, the distortion is approximately two-fold: in control crosses where *hT2[qIs48]/ced-9(n2812lf); nIs106[lin-11::gfp] X* males are crossed with *unc-69(e587)III* hermaphrodites, the *hT2[qIs48]* balancer was present in 31% of hermaphrodite cross-progeny (286 of 921 animals scored) and 62% of male cross-progeny (568 of 921 animals scored). Complete rescue by a *ced-9* transgene would be expected to restore an $\sim 2:1$ ratio of non-*hT2[qIs48]*-bearing to *hT2[qIs48]*-bearing cross-progeny, while incomplete rescue (some transgene-bearing animals dying due to insufficient *ced-9* activity) would be expected to yield a lower ratio. Partial rescue for some of the transgenic lines, suggested by a <2-fold ratio, could result from either insufficient activity of the relevant *ced-9* construct or from mosaic expression. Some dose-dependence of *ced-9* rescue is observed with the wild-type *gfp::ced-9* construct (Table 1) in that lower concentrations tend to give a somewhat lower *ced-9(n2812lf)/ced-9(n2812lf)* to *ced-9(n2812lf)/+* Rol ratio. Modest differences in ratio are also observed between transgenic lines, making precise quantitative interpretation of progeny ratios somewhat challenging.

Additionally, *ced-9(lf)* cross-progeny were virtually sterile, producing no viable *ced-3(+)* self-progeny. This observation suggests that like the majority of *C. elegans* transgenes, *gfp::ced-9* transgenes were silenced in the maternal germ line, thus preventing GFP::CED-9 protein from being deposited effectively into oocytes.

Pn.aap neuron profile assay. Lines carrying a *gfp::ced-9* transgene with the genotype *hT2[qIs48]/ced-9(n2812); nIs106* were constructed from cross-progeny from the embryonic lethality assays using standard genetic techniques. Pn.aap neurons in *ced-9(n2812); nIs106* animals carrying a *gfp::ced-9* transgene were visualized and scored using a dissecting epifluorescent microscope.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)