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Pax2/5/8 proteins promote cell survival in *C. elegans*

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Programmed cell death, or apoptosis, plays an important role during normal development, and is disrupted in a range of disease states. Although the key molecular events that occur during apoptosis are well characterized, less is known about the regulatory inputs that influence whether a cell will live or die. Work in mouse and human cells has shown that Pax transcription factors can influence cell death and promote cell survival, but the mechanism for their activity is not clear. Here, we show that two *Pax2/5/8*-related genes (*egl-38* and *pax-2*) influence both somatic and germline cell death in *C. elegans*. Using genetic and molecular experiments, we show that the Pax proteins act as transcriptional regulators of *ced-9*, the *C. elegans bcl-2* gene. These results identify a mechanism for Pax2/5/8-mediated regulation of cell death, and underscore the importance of transcriptional regulation of core apoptotic pathway genes in influencing cell survival.

KEY WORDS: Apoptosis, *ced-9*, *Caenorhabditis elegans*, Transcriptional regulation, *egl-38*, *pax-2*

INTRODUCTION

Programmed cell death (apoptosis) is a process of regulated cell death that is important for normal development and physiological homeostasis in all animals (reviewed by Lockshin and Zakeri, 2004). It provides a regulated process to refine organ structure or remove cells that are damaged or no longer required. Apoptosis is mediated by a core biochemical pathway that regulates the activation of caspases (reviewed by Danial and Korsmeyer, 2004; Yan and Shi, 2005). These enzymes act to digest cellular proteins systematically, and thus their activity can result in irreversible damage to cells. Caspase activity is regulated by the proteolytic processing of a pro-caspase to its active form, as well as by inhibitor of apoptosis proteins (IAPs). The importance of tight regulation of caspase activity and the cell-death pathway is evident from the fact that an inappropriate increase or decrease in apoptosis levels is a hallmark of many human diseases, including cancer, neurodegenerative disorders and autoimmune disease. Biochemical and genetic studies have been central to identifying the components and mechanistic details of the core apoptotic pathway. However, less is understood about the potential regulatory inputs that promote survival or trigger apoptosis, especially during normal development.

The nematode *C. elegans* has served as a pioneering model for gene discovery and functional analysis of apoptosis in vivo (reviewed by Lettre and Hengartner, 2006). Genetic studies in *C. elegans* have identified a core apoptotic pathway that shares molecular components with other organisms. Both somatic cells that die during the normal process of embryonic and larval development, as well as germ cells that die in the adult as part of gamete formation and germline homeostasis, use this core pathway. However, the regulatory inputs that influence somatic and germline cell death differ. Cell lineage and developmental cell fate specification mechanisms play an important role in regulating which somatic cells will die, and the pattern of cell death is highly reproducible from one animal to the next (Sulston and Horvitz, 1977; Sulston et al., 1983). By contrast, there is a variable pattern of cell death in the germline, and the regulatory processes include DNA damage-induced checkpoint controls, the activity of signal transduction pathways and bacterial infection (Aballay and Ausubel, 2001; Gartner et al., 2000; Gumienny et al., 1999). Although many genes that are important for regulating either somatic or germline cell death have been identified, it is clear that new genes remain to be discovered (e.g. Lettre et al., 2004). Importantly, aside from core apoptotic genes (the caspase gene *ced-3*, the Apaf-1 gene *ced-4*, the Bcl-2 gene *ced-9* and the BH3-only gene *egl-1*; see Fig. 4A), regulatory genes that impact apoptosis in both somatic and germline cells have not been identified.

This work focuses on the role of Pax2/5/8 proteins in regulating apoptosis. Pax proteins are transcription factors identified by the presence of the DNA-binding paired domain (reviewed by Chi and Epstein, 2002). Pax proteins fall into four subgroups based on their DNA-binding specificity, sequence similarity within the paired domain and the presence or absence of other sequence motifs. The Pax2/5/8 subgroup is defined by the mammalian *Pax2*, *Pax5* and *Pax8* gene products. Genetic studies of *Pax2/5/8* genes in a range of animals indicate that they are important for the normal development of subsets of cells and tissues, and that they can be key regulators in the development of organs. At the cellular level, Pax2/5/8 proteins can promote proliferation and cell survival, and influence differentiation. This effect is observed in both gain- and loss-of-function conditions. For example, increased expression of *Pax2* in mouse kidney results in renal hyperplasia (Dressler et al., 1993), and inappropriate expression of Pax genes is associated with primary cancer cells and cancer cell lines (Muratovska et al., 2003; Wu et al., 2005). By contrast, *Pax2* mutant mice and human individuals with *PAX2* mutations exhibit renal hypoplasia (Sanyanusin et al., 1995; Torres et al., 1995). Importantly, interfering with Pax gene expression in cancer cells promotes apoptosis, showing that Pax gene expression protects cells from cell death (Bernasconi et al., 1996; Buttiglieri et al., 2004; Muratovska et al., 2003). Similarly, the hypoplastic defect in *Pax2/+* mutant mice can be suppressed by caspase inhibitors, demonstrating that the reduced kidney development results, at least in part, from increased apoptosis (Clark et al., 2004; Dziarmaga et al., 2003). Although the evidence that at least some Pax genes act to protect cells from apoptosis is strong, the molecular mechanism for this function is not well understood.

In order to better understand how *Pax2/5/8* genes influence cell death, we have studied their function in *C. elegans*. This organism has two genes of the *Pax2/5/8* class: *egl-38* and *pax-2*. Although

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these genes result from a recent gene duplication, both have some non-redundant features (Wang et al., 2004). Here, we show that the *C. elegans Pax2/5/8* genes act similarly to the mammalian genes to promote cell survival. The two genes influence both somatic and germline cell death, demonstrating that they have a global role in modulating apoptosis. We have used molecular and genetic experiments to demonstrate that these Pax proteins affect the core apoptotic pathway by directly modulating the transcription of *ced-9*, the only *C. elegans* pro-survival *bcl-2* gene. This work defines a mechanism for Pax2/5/8 factors in influencing cell death, and identifies transcriptional regulation of *bcl-2/ced-9* as a potential regulatory point at which to modulate cell death during normal development.

MATERIALS AND METHODS

C. elegans strains and strain construction

C. elegans strains were cultured according to standard techniques (Sulston and Hodgkin, 1988). Mutations are described in wormbase (http://www.wormbase.org/). Mutations used are as follows.

Linkage group I (LG I): *cep-1(gk138)*.

LG III: *mpk-1(oz140)*, *ced-4(n1162)*, *ced-6(n1813)*, *ced-9(n1950 n2161)*, *unc-119(e2498)*.

LG IV*: pax-2(ok935)*, *egl-38(n578)*, *egl-38(sy294)*, *egl-38(sy287)*, *egl-38(gu22)*, *let-60(n1046)*, *dpy-20(e1282)*, *ced-3(n717)*.

LG X: *lin-2(e1309)*.

Integrated transgenes were *saIs21* (*hsp::egl-38*) (Zhang et al., 2005), *wdIs5* (*unc-4::gfp*) (Lickteig et al., 2001; Pflugrad et al., 1997). The alleles chosen for epistasis analysis with *pax-2* and *egl-38* were selected because they have been used for cell death assays in similar experiments (Gumienny et al., 1999; Lettre et al., 2004), and, except for *let-60(n1046)*, are considered to be genetically null or strong reduction-of-function alleles. The *pax-2(ok935)* allele is a deletion that includes exons coding for the DNA-binding domain, and is considered a genetic null. The *egl-38(n578)* allele is a mis-sense mutation that alters the sequence of the EGL-38 DNA-binding domain (Chamberlin et al., 1997). This allele preferentially disrupts a subset of *egl-38* functions, and is considered to be a non-null hypomorph. This allele was selected for our cell death assays because it exhibits the strongest defect in germline apoptosis, yet is homozygous viable.

RNA-mediated gene interference

cDNA fragments corresponding to *ced-10*, *egl-1*, *ced-3* and *ced-4* were amplified using RT-PCR and cloned into pBluescript (all primer sequences not listed are available on request). The cDNA inserts were transferred to pPD129.36 (pPD vectors were a gift from A. Fire), and the resultant clones were transformed into HT115. Bacteria were cultured on NGM plates containing 25 μg/ml carbenicillin, 10 μg/ml tetracyclin and 1 mM IPTG, according to the method of Kamath et al. (Kameth et al., 2001). Three to four L4 hermaphrodites were incubated on plates to produce offspring, and then removed after 24 hours. Their offspring were maintained on the RNAi feeding plates until being analyzed for cell death, described below. For germline cell death, L4 animals from treated mothers were transferred to fresh RNAi-feeding plates, and germline cell death was examined after 36 hours.

Apoptosis assays

Germline apoptosis was assessed in staged adult animals (generally 24 hours post L4/adult molt) and stained with SYTO12 according to the protocols of Gumienny et al. (Gumienny et al., 1999). Each genetic or experimental condition included at least 20 animals. For induced expression of PAX-2, EGL-38, CED-9 or MEV-1, animals bearing *hsp::pax-2*, *hsp::egl-38*, *hsp::ced-9* or *hsp::mev-1* transgenes were selected as L4 larvae, as above, and allowed to mature for 24 hours. Protein expression was induced for 30 minutes at 35ºC, and the animals were recovered to room temperature for 30 minutes and stained with SYTO12 for 4 hours. For experiments in engulfment-defective strains, animals were selected as above. However, the number of germ cell corpses was scored using the characteristic cell morphology (described below) under Nomarski optics. Data are reported as mean number of corpses or of SYTO12-positive cells ±the standard error of the mean (s.e.m.).

Somatic cell apoptosis was assessed in comma-stage embryos using Nomarski optics. In general, dying cells appear as refractile discs that are clearly distinct from other cell types (Sulston and Horvitz, 1977). Each genetic condition included at least 45 animals. The number of VC motoneurons was scored as number of ventral cord *unc-4::gfp*-positive cells in L2 larvae. Each genetic condition included at least 35 animals.

Genetic mosaic analysis

egl-38(n578) animals were injected with a mixture of 10 ng/μl of C04G2 cosmid (*egl-38* rescuing cosmid) (Chamberlin et al., 1997), 15 ng/μl of a cytoplasmically localized transformation marker (*myo-2::gfp*) (Okkema et al., 1993) and 100 ng/μl of a ubiquitously expressed nuclear marker (*sur-5::gfp*) (Gu et al., 1998; Yochem et al., 1998). Mosaic analysis for *egl-38* was conducted by selecting mid-L4 stage animals and scoring a panel of cells derived from different lineage precursors for the presence or absence of *sur-5::gfp* (see Fig. 5). These animals were recovered, allowed to mature for 24 hours, and were then stained with SYTO12 and analyzed for apoptosis, as described above. The offspring of each mosaic animal were subsequently assayed for GFP to determine whether the transgene was present in the germline.

Construction of *hsp::pax-2*, *hsp::ced-9*, *hsp::mev-1* and *ced-9::gfp* reporter transgenes

The heat-inducible clones (*hsp::pax-2*, *hsp::ced-9* and *hsp::mev-1*) were constructed in the same manner as *hsp::egl-38* (Zhang et al., 2005). cDNA coding for each gene was amplified by RT-PCR of wild-type RNA and cloned downstream of the *hsp16-41* promoter in pPD49.83. The *pax-2* cDNA was incorporated into a modified pPD49.83 vector that includes 3′ sequences coding for the FLAG epitope. All of the clones were sequenced to confirm that no mutations were introduced by PCR. Transgenes were produced by the microinjection of 50-100 ng/μl *hsp* plasmid DNA together with either 15 ng/μl pDP#MM016 [*unc-119(+)* plasmid] (Maduro and Pilgrim, 1995) into the mitotic germline of *unc-119(e2498)* animals, or 9 ng/μl *myo-2::gfp* (Okkema et al., 1993) into the mitotic germline of wild type (N2) or *pax-2(ok935) egl-38(n578)* mutants, according to the method of Mello et al. (Mello et al., 1991). The function associated with the *hsp::pax-2* transgene used in epistasis with *ced-9* (Fig. 4B,C) was confirmed by assaying its effects in non-Ced-9 siblings. To detect induced protein expression from clones with the FLAG tag (Fig. 2B), transgenic animals were heat-shocked, as above, and total protein was isolated as described (Zhang et al., 2005). Total protein (40 μg) was used for western blot analysis. The blot was probed with AP-conjugated monoclonal anti-FLAG antibody (diluted 1:700; Sigma M2) and detected with BCIP/NBT.

Genomic clones (Fig. 7) of the *ced-9* gene were constructed using PCR, with the T07C4 cosmid DNA as template (construction details are available upon request). The full-length clone was confirmed to rescue the defects associated with *ced-9(n1950 n2161)*. Sequences corresponding to the *gfp* gene were amplified using PCR from the vector pPD95.69, and cloned into a unique *Pst*I site of exon 3 of the *ced-9* gene, producing a translational fusion clone, *ced-9::gfp*. The insert was sequenced to verify it, and *ced-9::gfp* was introduced into animals as transgenes, as described above, using 40 ng/μl *ced-9::gfp* and 9 ng/μl *myo-2::gfp* as a transgene marker. The deletion clones were derivatives of the full-length clone. The mutant clone was generated by following the QuickChange site-directed mutagenesis protocol (Stratagene) with primers LP (5′-ACAAACCCCGACTCTAGATCTCTGCTCCGAAACAGATTTTC-3′) and RP (5′-GAAAATCTGTTTCGGAGCAGAGATCTAGAGTCGGGGTTTGT-3′). The introduced *Bgl*II site was confirmed by restriction digestion, and the clone was confirmed by sequencing. At least 150 of various stages of embryos from each line were assessed for the pattern and strength of GFP expression.

Quantitative RT-PCR

Worms were harvested from NGM plates, with or without heat shock treatment (described above), and lysed by sonication. Total RNA was extracted with Trizol (Invitrogen) and cleaned according to the RNeasy protocol (Qiagen). Total RNA (2 μg) from each sample was used for reverse

transcription using random hexamer priming and SuperScript II reverse transcriptase (Invitrogen). The resulting first-strand cDNA was used for PCR analysis (specific primers are available upon request). Quantitative PCR was performed using iCycler iQ Real-Time PCR (BioRad) with BioRad iQ SYBR Green supermix reagents. The PCR was performed for 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds. The abundance of *ced-9*, *mev-1*, *ced-3*, *ced-4* and *egl-1* was normalized to the levels of *act-2*, and the fold induction was calculated by dividing the relative abundance of each genotype by that of wild-type and non-heat shocked animals. Each experimental condition was replicated three times, from two independent batches of RNA (six replicates total).

Chromatin immunoprecipitation

The chromatin immunoprecipitation protocol was based on those of Upstate and Chu et al. (Chu et al., 2002), with the following modifications. Transgenic lines harboring the *hsp::egl-38::FLAG* or *hsp::pax-2::FLAG* transgene were heat shocked at 35°C for 45 minutes and harvested 1 hour after heat shock treatment. Worms were subjected to a 1% formaldehyde solution for 30 minutes at room temperature to promote the cross-linking of DNA and proteins. After chelating the formaldehyde by adding 500 μl of 2.5 M glycine, worms were harvested in 1 ml of ChIP buffer [50 mM HEPES (pH 7.6), 1 mM EDTA, 140 mM NaCl, 0.5% NP-40, 10% glycerol, 5 mM DTT and protease inhibitor cocktail (Calbiochem)] and sonicated five times for 30 seconds. The cross-linked chromosomal DNA was sheared by sonication (30 seconds for 10 cycles) and the resulting lysates were used for immunoprecipitation. One quarter of the solution was reserved to assay input DNA. Immunoprecipitation was performed by incubating the lysate with either anti-FLAG (Sigma, 1:100) or anti-Histone (Upstate, 1:100) antibody for 1 hour at 4°C followed by binding of antibody with protein A/G-agarose beads (Calbiochem). Beads without antibody were used as a negative control. The immune complexes were washed once with low-salt ChIP buffer (above), once with high-salt ChIP buffer (as above, but 1 M NaCl), once with LiCl buffer [10 mM Tris-Cl (pH 8.0), 0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA] and twice with TE buffer. The immunoprecipitates were eluted twice with 250 μl of elution buffer [100 mM $NaHCO_3$, 2%(w/v) SDS]. At this point, 10 μl of 10 mg/ml of RNAse A was added in order to degrade RNA. The solution was incubated at 65°C overnight to reverse cross-links, and treated with 5 μl of Proteinase K (10 mg/ml) to degrade any remaining proteins. After precipitating DNA with ethanol for 30 minutes at –80°C, the precipitate was dissolved in 100 μl of TE buffer and purified using a PCR-DNA purification column (Qiagen). To detect precipitated DNA, PCR was performed using 36 cycles of 95°C for 45 seconds, 50°C for 30 seconds and 72°C for 45 seconds with primer sets for *ced-9* upstream region (LP, 5′-ATAGTTGATATACCAAATTGTG-GGC-3′; RP, 5′-GGACAAAACTTCATTCCGACGT-3′) and *ced-9* coding region (LP, 5′-CGGACAACTCGCTGACGAAT-3′; RP, 5′-GGCTCT-TCCCAATCATTGAT-3′).

RESULTS

C. elegans Pax2/5/8 genes protect germline cells from apoptosis

Molecular and genetic data suggest that a single *Pax2/5/8* gene underwent duplication in the evolution of *C. elegans*, but that the two gene copies have distinct functional and structural features (Wang et al., 2004). Animals homozygous for a null mutation in one of the genes (*pax-2*) are viable and fertile, whereas animals homozygous for a null mutation in the other (*egl-38*) die as young larvae. However, several reduction-of-function but non-null alleles of *egl-38* have been identified, allowing its function to be assessed in a range of larval and adult tissues (Chamberlin et al., 1999; Chamberlin et al., 1997; Trent et al., 1983; Zhang et al., 2005). To investigate whether *C. elegans Pax2/5/8* genes affect apoptosis, we assessed germline apoptosis in *pax-2* and *egl-38* mutants, and found that animals mutant for either gene exhibit an increase in cell death (Fig. 1). In addition, double mutants between *pax-2(ok935)* and *egl-38(n578)*, the *egl-38* allele with the strongest apoptosis

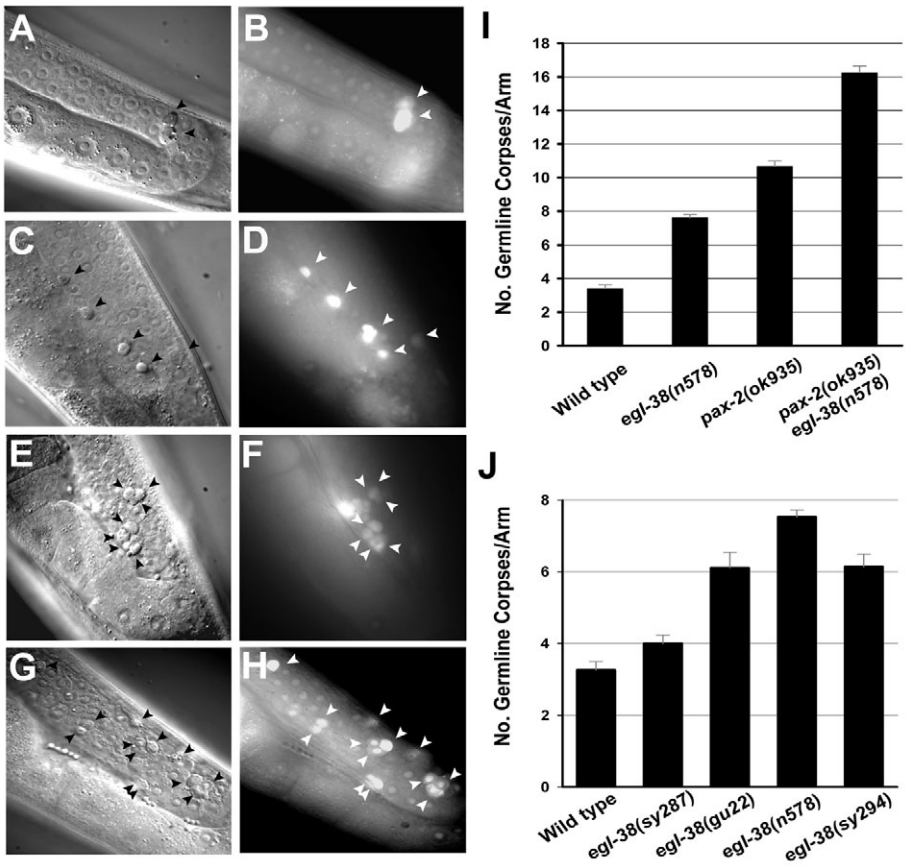


Fig. 1. *C. elegans Pax2/5/8* mutants have increased germline cell apoptosis. (A-H) Nomarski DIC (A,C,E,G) and epifluorescence (B,D,F,H) images of the gonad of adult hermaphrodite animals, stained with SYTO12, which preferentially labels dying germ cell nuclei (Gumienny et al., 1999). Arrowheads indicate dying cells or clusters of dying cells. (**A**,**B**) Wild type. (**C**,**D**) *egl-38(n578)*. (**E**,**F**) *pax-2(ok935)*. (**G**,**H**) *pax-2(ok935) egl-38(n578)*. (**I**) Average number of cell corpses per gonad arm for wild type and *Pax2/5/8* mutants. Error bars represent s.e.m. for at least 20 animals in each category. (**J**) Average number of cell corpses per gonad arm for different *egl-38* mutants. Data presented as in I.

defect, display an increase in germline apoptosis that is approximately additive (Fig. 1G-I). Thus, increased germline apoptosis is a defect associated with mutants of both of the *C. elegans Pax2/5/8* genes.

To test whether the *Pax2/5/8* genes can act as a switch to affect the cell-death decision, we induced the expression of each gene throughout otherwise wild-type animals by expressing each under the control of a heat-shock promoter (Fig. 2). Induced expression of either PAX-2 or EGL-38 results in a significant decrease in the number of apoptotic cells in the germline, indicating that the proteins can protect cells from apoptosis that would otherwise die. Taken together, our results show that the *Pax2/5/8* genes *egl-38* and *pax-2* influence whether germline cells undergo apoptosis. Increased gene activity can protect cells that would normally die, whereas reduced gene activity results in the death of cells that would normally live.

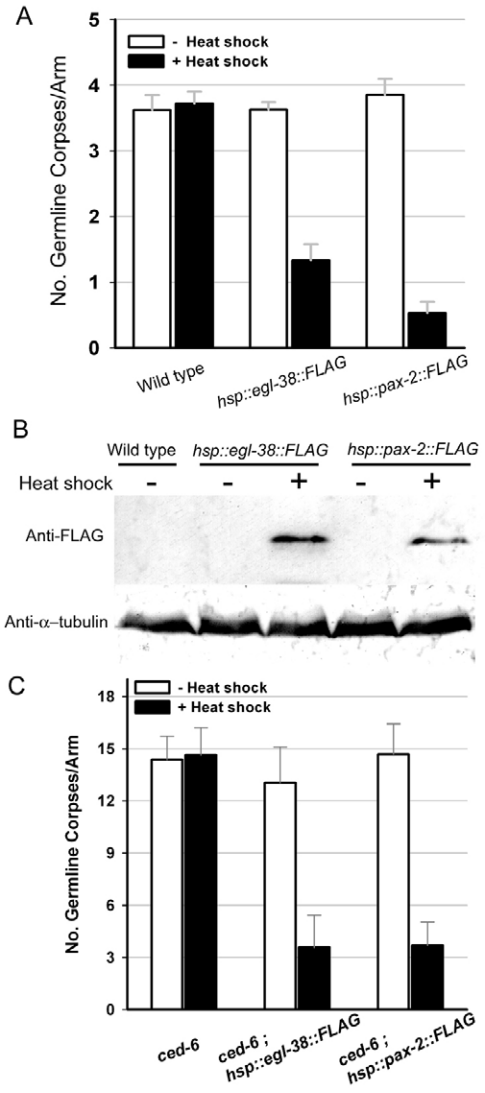


Fig. 2. Induced expression of *C. elegans Pax2/5/8* genes protects germline cells from apoptosis. (**A**) Induced expression of either *egl-38* or *pax-2* reduces germline apoptosis. Data presented as in Fig. 1. (**B**) Western blot for heat-treated lines in A, showing expression of the FLAG-tagged proteins. (**C**) Germline apoptosis assay in an engulfment-defective mutant background (*ced-6*) to increase the baseline number of cell corpses. Data presented as in Fig. 1.

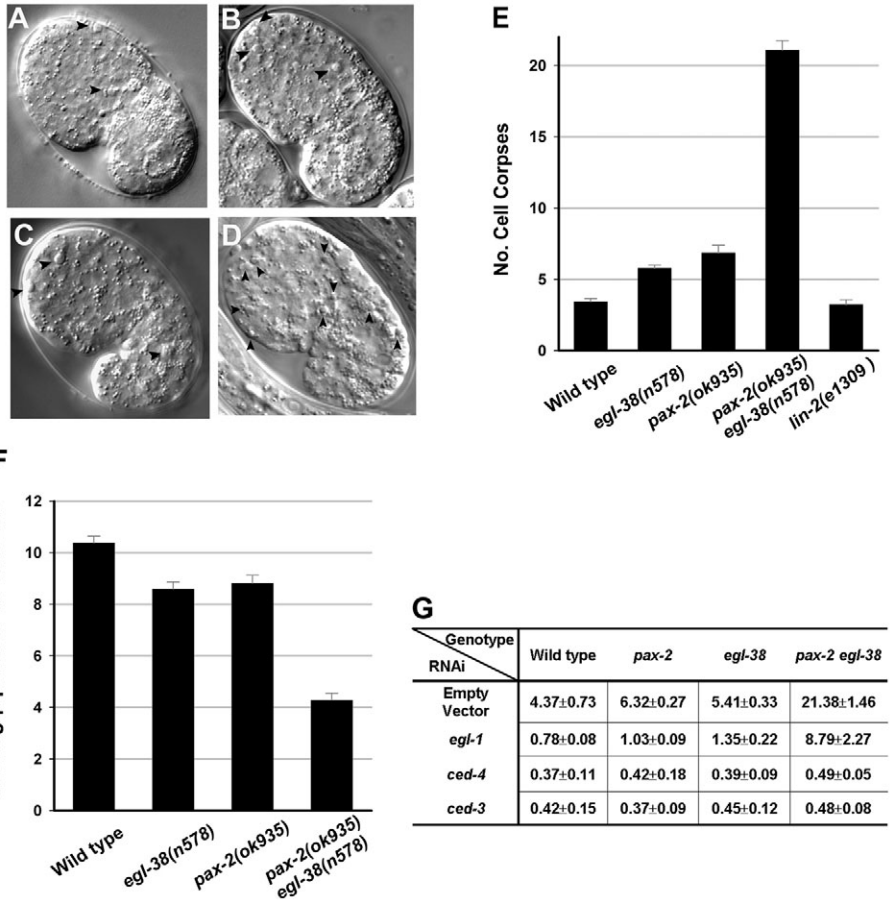
C. elegans Pax2/5/8 mutants exhibit inappropriate apoptosis of somatic cells

In order to determine whether *pax-2* and *egl-38* affect somatic, as well as germline, cell survival, we observed double-mutant embryos. We found that they exhibit a striking increase in the number of cell corpses that are characteristic of apoptotic cell death (Fig. 3A-E). Animals homozygous for mutations in only one of the two genes also exhibit a small but significant increase in cell corpses. As an additional assay for loss of somatic cells, we used an *unc-4::gfp* reporter transgene that is expressed in the VA motoneurons (Lickteig et al., 2001; Miller and Niemeyer, 1995). The VA motoneurons derive from the postembryonic division of ventral cord P cells, a lineage that exhibits increased apoptosis in other mutants, such as *ced-9* (Hengartner et al., 1992). We find that *pax-2 egl-38* double mutants have fewer *unc-4::gfp*-positive cells than do the wild type (Fig. 3F). Consistent with an increase in apoptosis, the *pax-2(ok935) egl-38(n578)* double mutants are uncoordinated, and exhibit a level of embryonic and larval lethality (data not shown). We conclude that the *C. elegans Pax2/5/8* genes protect somatic, as well as germline, cells from inappropriate apoptosis. Although single mutants exhibit some somatic cell defects, the effect is significantly enhanced when both *pax-2* and *egl-38* are mutant, indicating that there is partial redundancy between the two genes for this function.

egl-38 and *pax-2* act upstream of *ced-9* in the core apoptotic pathway

To determine genetically where *egl-38* and *pax-2* act to influence cell death, we constructed double mutants and assayed germline apoptosis (Fig. 4B). We find that apoptosis in *egl-38* and *pax-2* mutants is suppressed by mutations in either the caspase gene *ced-3* or the Apaf-1-related gene *ced-4*, indicating that the cell deaths in *Pax2/5/8* mutants use the caspase-mediated apoptotic pathway. *ced-3* and *ced-4* are also epistatic to *egl-38* and *pax-2* in somatic cells (Fig. 3G). As *Pax2/5/8* and *ced-9* loss-of-function mutants each exhibit the same defect of increased apoptosis, we tested epistasis between these genes by comparing the effect of induced *hsp::pax-2* expression in wild type with that in *ced-9* mutants. We find that the germline cell survival effects resulting from induced expression of PAX-2 require functional *ced-9*, showing that the protection from apoptosis is mediated through the activity of *ced-9*. Consistent with this gene order, induced expression of *ced-9* is also sufficient to bypass the cell-death defect in *pax-2 egl-38* double mutants (see below).

Two main regulatory pathways influence germline apoptosis (Fig. 4A). Normal physiological germ-cell death requires the activity of a ras/MAPK pathway (Gumienny et al., 1999). Genotoxic treatments, such as ionizing radiation, also promote germline cell death, and response to such treatments is mediated through checkpoint controls and p53 (Derry et al., 2001; Gartner et al., 2000; Schumacher et al., 2001). We constructed double mutants between key downstream genes in each of these pathways, and in the *Pax2/5/8* genes (Fig. 4B,C). In general, we found that the double mutants between *pax-2* or *egl-38* and the MAP kinase gene *mpk-1* or the p53 gene *cep-1* exhibit germline cell death patterns intermediate to either single mutant. Although this result does not provide a strict epistatic relationship, we can conclude that blocking either of these two regulatory pathways does not block the production of dying cells in *Pax2/5/8* mutants, arguing that the *Pax2/5/8* genes do not act upstream in either of these defined regulatory pathways. Double mutant analysis between *ced-9* and regulatory genes such as *mpk-1* and *cep-1* has yielded similar intermediate phenotypes, and it is possible that the intermediate pattern of apoptosis reflects other germline functions for the genes under study (Gartner et al., 2000;



G

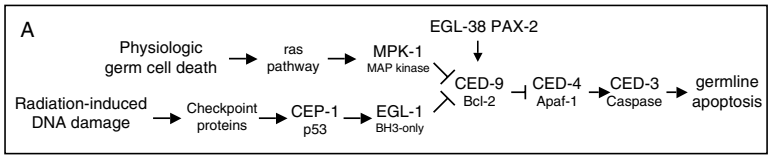
Genotype / RNAi	Wild type	*pax-2*	*egl-38*	*pax-2 egl-38*
Empty Vector	4.37±0.73	6.32±0.27	5.41±0.33	21.38±1.46
egl-1	0.78±0.08	1.03±0.09	1.35±0.22	8.79±2.27
ced-4	0.37±0.11	0.42±0.18	0.39±0.09	0.49±0.05
ced-3	0.42±0.15	0.37±0.09	0.45±0.12	0.48±0.08

Fig. 3. *C. elegans Pax2/5/8* mutants have increased somatic cell apoptosis. (A-D) Representative comma-stage embryos from different genotypes. Cell corpses visible in the plane of focus are indicated with an arrowhead. (**A**) Wild type. (**B**) *egl-38(n578)*. (**C**) *pax-2(ok935)*. (**D**) *pax-2(ok935) egl-38(n578)*. (**E**) Average number of visible cell corpses in comma to 1.5-fold stage embryos for wild type and *Pax2/5/8* mutants. *lin-2(e1309)* is included as a control for eggs retained in an egg-laying defective mother. Error bars represent s.e.m. for at least 45 animals in each category. (**F**) *unc-4::gfp*-positive VC neurons in wild type and *Pax2/5/8* L2 stage mutant animals. (**G**) Genetic epistasis experiments between *C. elegans Pax2/5/8* genes and other genes that affect somatic cell apoptosis. Animals of different genotypes were treated with RNAi induced by bacterial feeding (see Materials and methods) and assessed as in E.

Gumienny et al., 1999; Lettre et al., 2004). Collectively, we interpret that the *Pax2/5/8* genes act upstream of *ced-9* in the core apoptotic pathway, but downstream or in parallel to other regulatory pathways known to influence germline apoptosis.

EGL-38 can act within the germline lineage to influence germline cell survival

To understand better how the *Pax2/5/8* genes influence germline cell survival, we performed mosaic analysis for *egl-38* to determine which cells require its function. We introduced an extra-chromosomal transgene with *egl-38(+)*-rescuing DNA and *sur-5::gfp*, which marks transgene-bearing cells with GFP (see Materials and methods; Yochem et al., 1998), into *egl-38(n578)* animals. This transgene can be lost at cell division, resulting in genetically mosaic animals. We selected mid-L4 animals from this strain, and observed a panel of somatic cells derived from different embryonic precursors to infer where the transgene was lost during development (Fig. 5). These animals were recovered and examined 24 hours later for germline apoptosis. As *sur-5::gfp* can be detected in most somatic cells, but not germline cells, we



B

Genotype	wild type	*egl-38(n578)*	*pax-2(ok935)*	*hsp::egl-38*	*hsp::pax-2*
wild type	3.06 ± 0.14	6.17 ± 0.27	11.8 ± 0.25	1.13 ± 0.22	0.53 ± 0.17
ced-3(n717)	0.06 ± 0.03	0.06 ± 0.03	0.05 ± 0.02	N/D	N/D
ced-4(n1162)	0.43 ± 0.15	0.6 ± 0.16	0.46 ± 0.16	N/D	N/D
ced-9(n1950 n2161)	16.3 ± 0.86	N/D	N/D	N/D	16.92 ± 0.86
mpk-1(oz140)	0.11 ± 0.07	3.29 ± 0.24	6.05 ± 0.30	N/D	N/D
cep-1(gk138)	1.35 ± 0.12	3.47 ± 0.15	6.70 ± 0.26	N/D	N/D

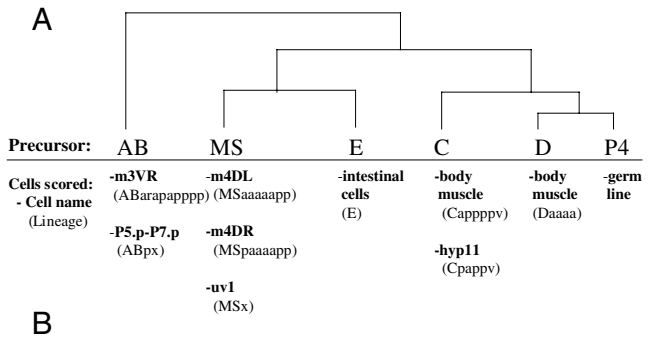
C

Genotype	wild type	*egl-38(n578)*	*pax-2(ok935)*	*hsp::egl-38*	*hsp::pax-2*
wild type	9.46 ± 2.43	25.7 ± 1.54	35.8 ± 4.9	4.28± 1.21	4.01 ± 0.45
ced-3(n717)	1.03 ± 0.22	1.12 ± 0.31	1.08 ± 0.75	N/D	N/D
ced-4(n1162)	1.11 ± 0.35	1.08 ± 0.12	1.05 ± 0.46	N/D	N/D
ced-9(n1950 n2161)	49.77±2.58	N/D	N/D	N/D	46.84±3.81
mpk-1(oz140)	1.89 ± 0.68	20.98 ± 2.33	28.38 ± 3.69	N/D	N/D
cep-1(gk138)	3.26 ± 0.95	17.54 ± 2.77	26.88 ± 4.53	N/D	N/D

Fig. 4. Regulatory pathways that influence germline cell death. (**A**) The pathways that influence *C. elegans* germline cell apoptosis. Proteins discussed in the text are included. A physiological germ cell death is mediated through the ras pathway, with MPK-1 (MAP kinase) representing the most downstream component. Genotoxic treatments such as radiation induce checkpoint genes and p53-mediated apoptosis. Both pathways act to regulate a core pathway required for all normal cell deaths in *C. elegans*. EGL-38 and PAX-2 are drawn to act in parallel to the other two pathways, but could alternatively act downstream of MPK-1 (or CEP-1). (**B**) Genetic epistasis experiments between *C. elegans* Pax2/5/8 genes and other genes that affect germline apoptosis, carried out in an otherwise wild-type genetic background. (**C**) Genetic epistasis experiments between *C. elegans* Pax2/5/8 genes and other genes that affect germline apoptosis, carried out in animals RNAi-depleted for *ced-10*, a gene required for corpse engulfment. Germ-cell corpses were quantified as described in Materials and methods. Data are mean number of cell corpses per gonad arm±s.e.m. Wild type is N2.

determined whether the transgene was present or absent in the germline precursor (P4) by observing whether GFP was expressed in the offspring. Animals with the transgene present in all somatic, as well as in germline, cells exhibited germline apoptosis levels similar to wild type, whereas animals with the transgene absent from all cells exhibited germline apoptosis levels similar to *egl-38(n578)* mutants (an average of 3.04±0.18 and 6.93±0.21 corpses per gonad arm, respectively). Mosaic animals (lacking the transgene in one or more embryonic precursor) that retained the transgene in the germline were similar to wild type. In particular, we focused on cells of the MS lineage (precursor to the somatic gonad) and the E lineage (precursor to the intestine), as these cells surround the germline cells and represent potential sources of survival signal(s). However, we found that all 16 animals that retained the transgene in the germline but lost it in either the MS lineage, or the E lineage, or both, exhibited normal patterns of apoptosis (2.75±0.43 corpses per gonad arm). Thus, *egl-38* is not required in these somatic tissues to promote germline cell survival.

A



B

Mosaic Cat.	AB	MS	E	P4	# animals	≤5 deaths # wild type	>5 deaths # mutant	% wild type	% wild type
1	+	+	+	+	71	64	7	90	90
2	—	+	+	+	10	9	1	90	
3	+	—	+	+	6	5	1	83	
4	—	—	+	+	0				
5	+	+	—	+	6	6		100	88
6	—	+	—	+	3	2	1	67	
7	+	—	—	+	1	1		100	
8	—	—	—	+	0				
9	+	+	+	—	15	11	4	73	
10	—	+	+	—	3	3		100	
11	+	—	+	—	2	2		100	
12	—	—	+	—	0				72
13	+	+	—	—	5	3	2	60	
14	—	+	—	—	2	1	1	50	
15	+	—	—	—	2	1	1	50	
16	—	—	—	—	102	17	85	17	17

Fig. 5. Mosaic analysis for *egl-38* function. (**A**) Cell lineage chart of the early cell divisions in *C. elegans*, indicating the embryonic source of the cells listed below, which were assayed in mosaic animals (see Sulston et al., 1983). An 'x' indicates that the cells derive from more than one cell in the lineage. (**B**) Summary of the observations from mosaic animals, grouped according to category. For clarity, the mosaic animals were classified as wild type if they exhibited five or fewer corpses per gonad arm, and classified as mutant if they exhibited six or more corpses per gonad arm (beyond two standard deviations from the average of animals with the transgene present in all lineages). See text for additional analysis. All animals lacking the transgene in their offspring were classified as P4-negative. A more stringent analysis of germline loss (including in the analysis only animals that lack the transgene in D as well as P4 but retain the transgene in some other somatic lineages) yields a slightly lower, but similar proportion of animals classified as wild type (11 of 18, or 61%).

Animals that had lost the transgene in the germline but retained it in somatic cells exhibited increased apoptosis (4.03±0.28 corpses per gonad arm), although apoptosis levels were less than those observed in animals lacking the transgene in all cells. This result suggests that *egl-38* may be able to influence germline survival by functioning in somatic cell(s), in addition to its role in the germline. Altogether, however, the mosaics support the idea that this *Pax2/5/8* gene has a role within germline tissue to promote cell survival.

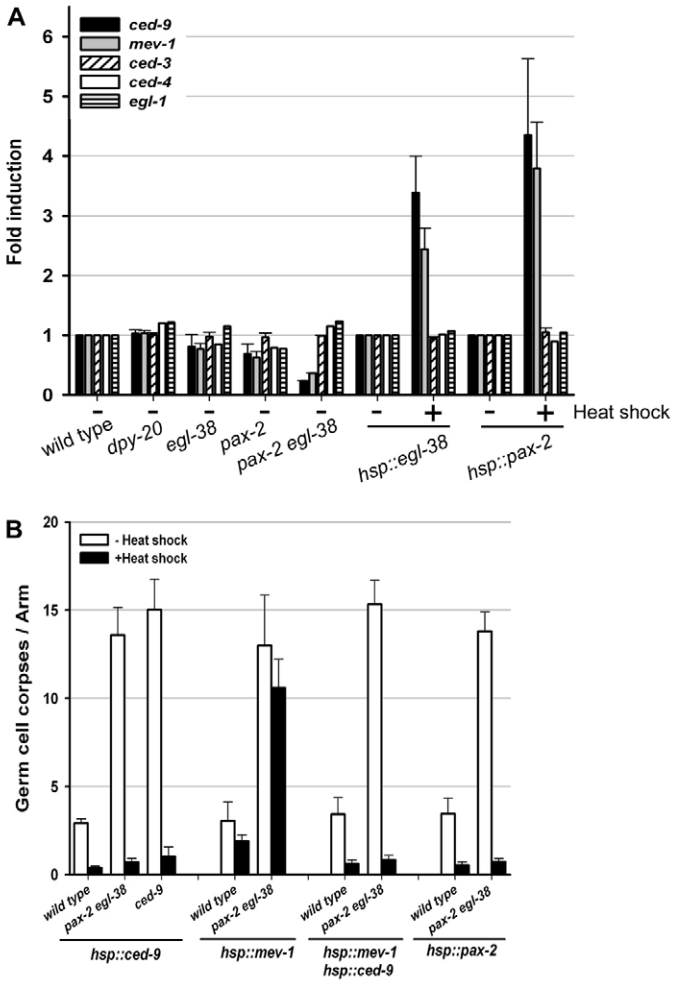


Fig. 6. *ced-9* acts downstream of the *Pax2/5/8* genes. (**A**) *ced-9* and *mev-1* transcript abundance is altered in response to *Pax2/5/8* gene activity. Quantitative RT-PCR results for *ced-9*, *mev-1*, *ced-3*, *ced-4* and *egl-1* genes in different *Pax2/5/8* genetic backgrounds, normalized to the *act-2* gene. Wild type (N2) and *dpy-20* [the genetic background for *egl-38(n578)*] serve as controls. *ced-9* and *mev-1* transcript abundance is decreased in *egl-38 pax-2* double mutants, and increased in response to induced expression of *egl-38* or *pax-2* (*hsp::egl-38* and *hsp::pax-2*). The transcript abundance of the other cell death pathway genes is unaltered. Separate experiments indicate that *ced-9* and *mev-1* abundance is not altered in response to heat-shock in wild-type animals (data not shown). Error bars indicate standard deviation. (**B**) Induced expression of *ced-9* can bypass the cell death defect of *pax-2 egl-38* double mutants. By contrast, induced expression of *mev-1* alone, or in combination with *ced-9*, does not significantly impact the cell death effect. Induced expression of *pax-2* in wild type and *pax-2 egl-38* mutants is included as a control. Data presented as in Fig. 1.

EGL-38 and PAX-2 regulate the transcription of *ced-9*, the *C. elegans bcl-2* gene

The genetic epistasis results focused our research on understanding how the Pax2/5/8 proteins might modulate the activity of *ced-9*. As Pax2/5/8 proteins act by influencing the transcription of other genes, we tested whether these proteins alter *ced-9* transcription. Using real-time RT-PCR, we found that *ced-9* transcript abundance is indeed altered in response to *Pax2/5/8* gene activity. The transcript is diminished in *pax-2 egl-38* double mutants, and increased in response to induced expression of EGL-38 or PAX-2 (Fig. 6A). Changes in mRNA abundance are not seen in other genes important for cell death, including *ced-3*, *ced-4* and *egl-1*, arguing that there is not a widespread alteration of cell death gene transcription in response to *pax-2* and *egl-38*, or in animals with altered cell death patterns. In the *C. elegans* genome, the *ced-9* gene is part of an operon with another gene, *mev-1* (Hengartner and Horvitz, 1994) (*mev-1* is also known as *cyt-1*; Fig. 7A). In operons of this type, the genes are initially transcribed as a single RNA, which is then processed through trans-splicing into separate mRNAs (reviewed by Blumenthal and Gleason, 2003). Thus, genes within an operon are subject to the same transcriptional regulatory inputs. We characterized the *mev-1* transcript in the different genetic backgrounds, and found that the abundance of this gene is altered in response to *Pax2/5/8* activity in a manner similar to *ced-9*. These results are consistent with a model in which the Pax2/5/8 proteins alter the transcription of the *mev-1 ced-9* operon.

We reasoned that, if altered transcription of the *mev-1 ced-9* operon is important for the apoptosis effects of the *Pax2/5/8* genes, then expressing *mev-1* or *ced-9* in a *pax-2 egl-38*-independent manner should bypass the cell death defect. To test this idea, we developed transgenes that expressed *mev-1* or *ced-9* under the control of a heat-inducible promoter, and introduced them into *pax-2 egl-38* mutants (Fig. 6B). We found that *hsp::ced-9*, but not *hsp::mev-1*, can promote the survival of germ cells in *pax-2 egl-38* mutants. This result supports the idea that *ced-9* acts downstream of the *Pax2/5/8* genes, and is consistent with the idea that altered transcription of the *mev-1 ced-9* operon is important for the cell survival effects mediated by the *Pax2/5/8* genes.

As *mev-1* and *ced-9* transcripts are altered in response to *Pax2/5/8* gene activity, we tested whether the operon could be a direct target for the Pax2/5/8 proteins. Starting with a clone of genomic DNA that includes both *mev-1* and *ced-9*, we created a reporter gene by introducing the coding sequences for GFP into the third exon of *ced-9* (Fig. 7). We found that when this clone is introduced into animals as part of a transgene, the GFP-tagged CED-9 is expressed broadly in pre-elongation embryos in a cytoplasmic lattice-like pattern, as has been reported for the CED-9 protein (Chen et al., 2000) (Fig. 7B-

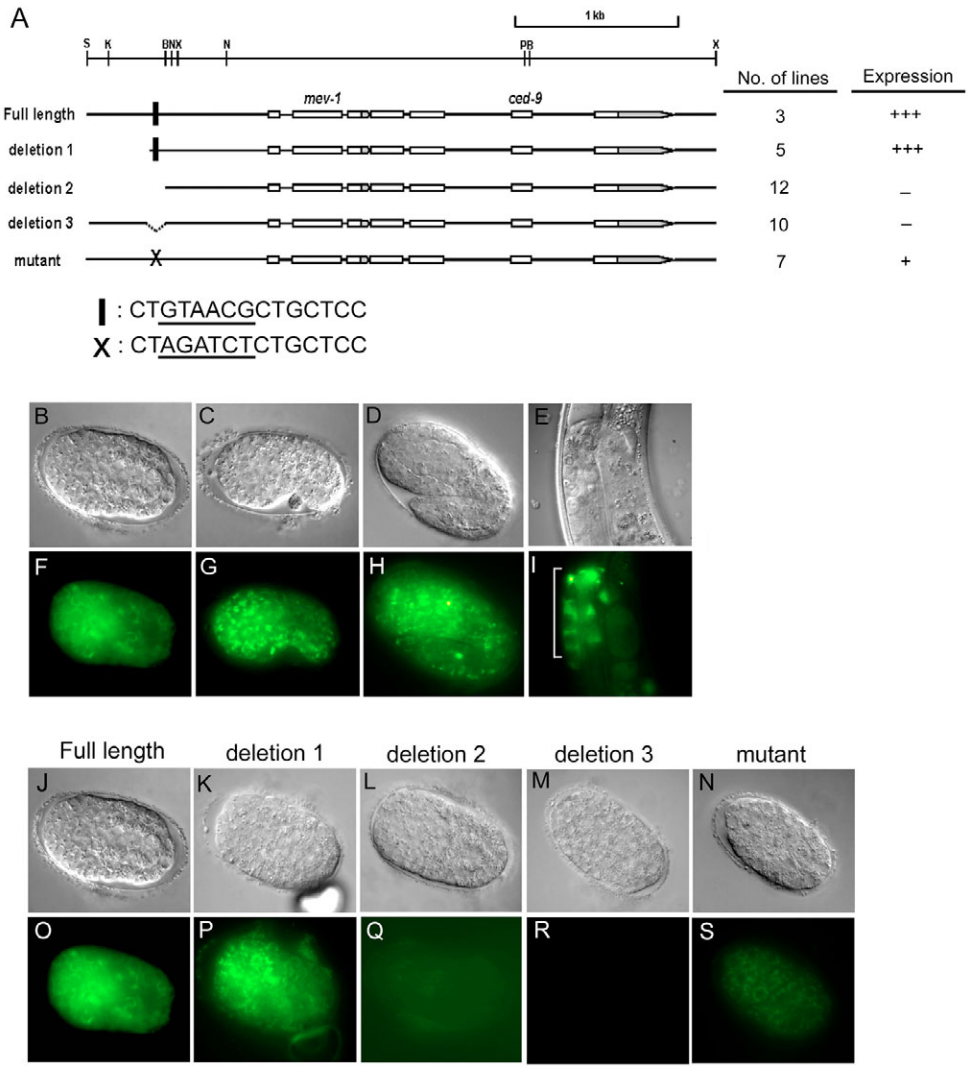


Fig. 7. A deletion analysis identifies an upstream regulatory element important for *ced-9* transcription. (**A**) Clones tested for *ced-9::gfp* expression and *ced-9* rescue. The sequences coding for GFP are inserted into a unique *Pst*I site in the third exon of *ced-9*. Deletions 1-3 correspond to three deleted clones that center on a potential Pax response element, indicated with a black bar on the clones. In the mutant clone, this element is altered, indicated by 'X'. The sequence for both the wild-type and the mutant element is shown below the diagram. (**B-I**) Expression of the full length *ced-9::gfp* clone in embryos [(B,F) prior to elongation, (C,G) at comma stage, (D,H) at 1.5-fold stage] and (E,I) in adult gonad. (B-E) DIC images. (F-I) The same animals under epifluorescence to visualize GFP. In I, the bend of the gonad arm is shown and the *ced-9* expression is marked with a bracket. (**J-S**) Representative embryos bearing each of the transgenes summarized in A. (J-N) DIC images. (O-S) The same embryos under epifluorescence to visualize GFP. All embryos are at a stage similar to that shown in B and F.

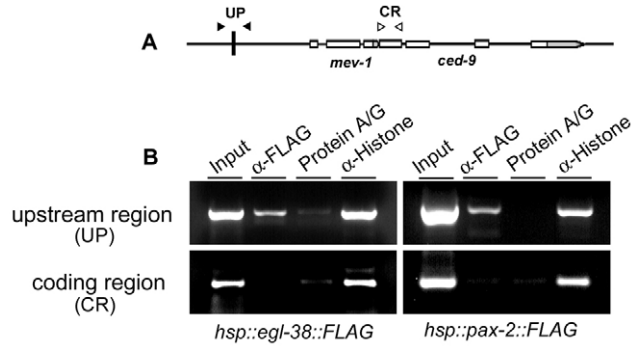


Fig. 8. EGL-38 and PAX-2 directly regulate *ced-9* transcription in vivo. (**A**) Diagram of the *mev-1 ced-9* genomic region, showing the position of primers used for chromatin immunoprecipitation. Black arrowheads correspond to primers for the upstream region (UP); white arrowheads correspond to primers for the coding region (CR). (**B**) PCR of DNA recovered from chromatin immunoprecipitation. Input lane corresponds to DNA recovered without immunoprecipitation. Two representative experiments are shown, one each for a strain expressing EGL-38 (*hsp::egl-38::FLAG*) and PAX-2 (*hsp::pax-2::FLAG*). The experimental proteins (EGL-38 and PAX-2) are tagged at the C-terminus with a FLAG peptide (Fig. 2B), and can be immunoprecipitated with an α-FLAG antibody. PCR of DNA recovered from immunoprecipitation using α-FLAG antibody, protein A/G beads (negative control) or α-Histone (positive control) are shown for representative experiments using EGL-38 (left) and PAX-2 (right). In both cases, the Pax2/5/8 protein preferentially immunoprecipitates DNA in the upstream region.

I). In addition, we found that CED-9::GFP is enriched in the gonadal region where germ cell apoptosis is observed (Fig. 6E,I). A shorter reporter clone that lacks ~500 bp upstream of *mev-1* fails to show robust CED-9::GFP expression (Fig. 6L,Q). This result identifies that there are regulatory sequences upstream of *mev-1* that are important for the normal expression of *ced-9*, and is consistent with mutant rescue experiments that showed sequences upstream of *mev-1* were required for optimal rescue of *ced-9* mutant defects (Hengartner and Horvitz, 1994). Within this functionally defined upstream regulatory region, we identified one sequence with similarity to sequences bound by Pax2/5/8 proteins. Mutant clones that delete or disrupt this site fail to express abundant CED-9::GFP (Fig. 6L-N, Q-S), showing that the site is necessary for the activity associated with the full-length clone. We conclude that this site is important for the normal expression of *ced-9*. The simplest interpretation based on gene organization and the location of the functional sequence, is that the site affects the transcription of the *mev-1 ced-9* operon.

The results of these experiments suggested a model in which the Pax2/5/8 proteins PAX-2 and EGL-38 act as direct positive regulators of the transcription of *ced-9* by binding to regulatory sequences upstream of *mev-1*. To test this model in vivo, we used chromatin immunoprecipitation. The *hsp::pax-2* and *hsp::egl-38* transgenes that can promote germline cell survival include sequences for a FLAG epitope tag at the 3′ end of each cDNA (Fig. 2B). We used these transgenes to induce the expression of each Pax2/5/8 protein in animals, and immunoprecipitated the tagged proteins and associated DNA. We found that the induced PAX-2 or EGL-38 proteins preferentially precipitate DNA sequences that include the defined response element, whereas other sequences, such as ones corresponding to the coding region of *ced-9*, are not enriched in the precipitated DNA (Fig. 8A,B). We conclude that the *C. elegans* Pax2/5/8 proteins influence apoptosis by directly binding to sequences upstream of *mev-1*, promoting transcription of the *mev-1 ced-9* operon.

DISCUSSION

A conserved role for Pax2/5/8 transcription factors in apoptosis

Our results demonstrate that the *C. elegans Pax2/5/8* genes are important for cell survival. As mammalian *Pax2/5/8* genes also promote cell survival, influencing apoptosis is a conserved function for this gene class. We propose that the mechanism of action for *C. elegans* Pax2/5/8 proteins is direct transcriptional regulation of a key apoptosis gene, *ced-9/bcl-2*. In this model, the Pax2/5/8 proteins play a broad role in the normal transcriptional regulation of *ced-9*, as mutants exhibit increased, inappropriate, apoptosis in both germline and somatic cells. In *C. elegans* and other animals, Pax genes have important functions in the normal development of organs, cells and tissues, in addition to their role in promoting cell survival. An emerging idea is that coordinating the distinct but important developmental features of cell survival and cell fate specification is a core feature of Pax genes, and underlies their role in organ and tissue development in all animals.

Transcriptional regulation of the *mev-1 ced-9* operon by Pax2/5/8 factors

ced-9 is the downstream gene in a two-gene operon that includes the succinate dehydrogenase cytochrome b subunit gene *mev-1* (Hengartner and Horvitz, 1994). Our work suggests that altered *ced-9* transcription is sufficient to account for the cell death phenotype associated with the *Pax2/5/8* mutants, although the Pax2/5/8 proteins may also influence the expression of other genes that affect cell survival. Mutations in *mev-1* confer reduced stress tolerance, and can promote inappropriate somatic apoptosis, which is dependent on the caspase CED-3 (Senoo-Matsuda et al., 2003; Senoo-Matsuda et al., 2001). Although the specific role for *mev-1* in apoptosis has not been determined, mitochondria play a role in regulating cell death in *C. elegans,* as they do in other organisms (Jagasia et al., 2005), and, thus, *mev-1* may influence aspects of mitochondrial function important for normal cell survival. Co-transcriptional regulation of the operon by the Pax2/5/8 proteins provides a mechanism to coordinate the expression of genes that have a protective role for the cell, or that contribute to mitochondrial biogenesis.

Dynamic spatial and temporal regulation of *ced-9* expression

We and others have found that *ced-9* is not expressed uniformly in all cells throughout the development of *C. elegans* (Chen et al., 2000; Hill et al., 2000). Indeed, in contrast to what might be predicted with respect to its anti-apoptotic function, *ced-9* is enriched at times when the majority of normal apoptosis takes place. Endogenous CED-9 protein, as well as our CED-9::GFP, is most abundant in embryos as cells begin to leave the cell cycle and differentiate or initiate apoptosis. We also observe enriched CED-9::GFP in the region of the gonad where germline apoptosis takes place. Germline expression has not been reported for the CED-9 protein (Chen et al., 2000), but is observed using *ced-9* RNA in situ analysis (the Nematode Expression Pattern DataBase, http://nematode.lab.nig.ac.jp/), suggesting that the difference between CED-9::GFP and CED-9 detected by antibody may reflect a difference of stability or post-transcriptional regulation between the endogenous and the GFP-tagged proteins. To explain why *ced-9* transcription is upregulated at times and locations where cell death

is most prevalent, we envisage that there are developmental conditions or signals that promote apoptosis, and that CED-9 expression is raised at these times to limit which cells ultimately die.

Transcription as a mechanism to influence cell death

The decision of a cell to undergo apoptosis is mediated by a regulatory cascade that modulates the activity of proteins. However, the relative abundance of these proteins is crucial to the activity of the cascade, providing important parallel mechanisms to regulate the output of the pathway. Increased transcriptional regulation of *bcl-2* and related pro-survival genes protect cells from apoptosis in mammals and *C. elegans* (McDonnell et al., 1989; Vaux et al., 1988; Vaux et al., 1992), and the *bcl-2* gene was originally identified as a constitutively overexpressed oncogene that contributes to follicular B-cell lymphoma (Tsujimoto et al., 1984). Our results demonstrate that transcriptional regulation of *ced-9/bcl-2* by Pax2/5/8 proteins contributes to normal cell survival, and that cells are sensitive to the level of *pax-2* and *egl-38* activity. This sensitivity identifies Pax2/5/8 protein activity as a potential regulatory point to influence cell death, although future experiments are required to test whether these factors normally respond to regulatory input in vivo, or rather contribute more towards maintaining basal levels of *ced-9* transcription. We hypothesize that PAX-2 and EGL-38 transcription of *ced-9* provides a mechanism to influence cell death decisions, as this mechanism is also observed for other cell death pathway components. For example, in *C. elegans*, transcriptional regulation of the pro-apoptotic BH3 domain-only gene *egl-1* in both germline and somatic cells influences whether a cell will die (Hofmann et al., 2002; Thellmann et al., 2003). As EGL-1 inhibits CED-9 activity, the balance of expression between these two genes can determine the cell death decision. We anticipate that, during normal development, altering the transcription of core apoptosis pathway genes will be a widely employed mechanism to reproducibly link cell fate and apoptosis.

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