DPL-1 DP, LIN-35 Rb, and EFL-1 E2F act with the MCD-1 Zinc-finger protein to promote programmed cell death in *C. elegans*

Peter W. Reddien*, Erik C. Andersen✝, Michael C. Huang✝✝, and H. Robert Horvitz†

*Present address:
Department of Biology
Massachusetts Institute of Technology
Whitehead Institute
9 Cambridge Center
Cambridge, MA 02142, USA

✝Howard Hughes Medical Institute
Department of Biology, 68-425
Massachusetts Institute of Technology
77 Massachusetts Avenue
Cambridge, MA 02139, USA

✝✝Present address:
Department of Neurosurgery
Pasquerilla Healthcare Center, Seventh Floor
Georgetown University Hospital
3800 Reservoir Road, NW
Washington, DC 20007
SUMMARY

The genes *egl-1*, *ced-9*, *ced-4*, and *ced-3* play major roles in programmed cell death in *C. elegans*. To identify genes that have more subtle activities, we sought mutations that confer strong cell-death defects in a genetically sensitized mutant background. Specifically, we screened for mutations that enhance the cell-death defects caused by a partial loss-of-function allele of the *ced-3* caspase gene. We identified mutations in two genes not previously known to affect cell death, *dpl-1* and *mcd-1* (modifier of cell death). *dpl-1* encodes the *C. elegans* homolog of DP, the human E2F-heterodimerization partner. By testing genes known to interact with *dpl-1*, we identified roles in cell death for four additional genes: *efl-1* E2F, *lin-35* Rb, *lin-37* Mip40, and *lin-52* dLin52. *mcd-1* encodes a novel protein that contains one zinc finger and that is synthetically required with *lin-35* Rb for animal viability. *dpl-1* and *mcd-1* act with *efl-1* E2F and *lin-35* Rb to promote programmed cell death and do so by regulating the killing process rather than by affecting the decision between survival and death. We propose that the DPL-1 DP, MCD-1 Zinc finger, EFL-1 E2F, LIN-35 Rb, LIN-37 Mip40, and LIN-52 dLin52 proteins act together in transcriptional regulation to promote programmed cell death.
INTRODUCTION

Programmed cell death is important for many aspects of animal development, including morphogenesis, homeostasis, and neuronal refinement (GLÜCKSMANN 1951; JACOBSON et al. 1997; SAUNDERS 1966). Studies of the mechanisms of programmed cell death in the nematode Caenorhabditis elegans have identified a pathway that is largely conserved in other organisms, including humans (METZSTEIN et al. 1998). Four genes -- egl-1, ced-9, ced-4, and ced-3 -- regulate essentially all somatic programmed cell death and define the core metazoan cell-death execution machinery. EGL-1, which promotes cell death, is a BH3-only protein that binds to and inhibits the CED-9 protein (CONRADT and HORVITZ 1998). CED-9, which inhibits cell death (HENGARTNER et al. 1992), is similar to the human proto-oncoprotein BCL-2 (HENGARTNER and HORVITZ 1994b; YAN et al. 2005) and localizes to mitochondria (CHEN et al. 2000). CED-9 binds CED-4 (SPECTOR et al. 1997), which localizes to mitochondria in a CED-9-dependent manner (CHEN et al. 2000), promotes cell death (ELLIS and HORVITZ 1986), and is similar to the human pro-apoptotic protein APAF-1 (YUAN and HORVITZ 1992; ZOU et al. 1997). The expression of egl-1 or the loss of ced-9 function can trigger cell death and result in a change in the localization of CED-4 from mitochondria to the perinuclear region (CHEN et al. 2000). CED-4 activates CED-3 (SHAHAM and HORVITZ 1996b), which promotes cell death (ELLIS and HORVITZ 1986) and is a defining member of a family of cysteine proteases termed caspases (YUAN et al. 1993). CED-4 can interact directly with the CED-3 pro-caspase (WU et al. 1997) and facilitate the processing of proCED-3 into active CED-3 (CHINNAIYAN et al. 1997; YANG et al. 1998).

Other C. elegans genes appear to promote cell death more subtly. For example, the gene ced-8 XK affects the timing of programmed cell deaths in C. elegans and has a minor role in cell killing (STANFIELD and HORVITZ 2000). The gene ced-9, which inhibits programmed cell death, also can promote cell death (HENGARTNER and HORVITZ 1994a). The gene cps-6 encodes a mitochondrial endonuclease G protein that likely promotes cell death (PARRISH et al. 2001). The process of the engulfment of dying cells also promotes cell death (HOEPPNER et al. 2001; REDDIEN et al. 2001). Additional genes that affect programmed cell death in C. elegans, such as those encoding proteins that mediate the ability of engulfment to promote cell death, that mediate the cell-killing activities of ced-8
or *ced-9*, or that act downstream of CED-3 remain to be identified. We reasoned that genes with subtle contributing roles in programmed cell death might be identified by screening for mutations that further decrease cell death in a genetic background in which cell death is partially impaired. Here we report the identification and characterization of genes we identified using this approach and present evidence that these genes control activities that promote programmed cell death.
MATERIALS AND METHODS

Strains: C. elegans was cultured at 20°C on NGM agar with E. coli OP50 as described (BRENNER 1974). In general, the wild-type strain was N2. For mapping with polymorphisms, the wild-type strain RC301 was used. All mutations used have been described (RIDDLE et al. 1997), unless from this work or otherwise noted. The following mutations were used:

LG I; lin-61(n3446), dpy-5(e61), lin-35(n745), lin-35(n2239, n2242) (LU and HORVITZ 1998), unc-13(e1091), unc-29(e1072), lin-53(n833) (LU and HORVITZ 1998), unc-75(e950), ced-1(e1735, n3390, n3402) (HEDGECOCK et al. 1983), hT2 [qls48] (MATHIES et al. 2003), nIs128 (H. Schwartz and H.R.H., unpublished observations)

LG II; lin-8(n111), lin-8(n2731) (THOMAS et al. 2003), eT1, dpy-10(e128), egl-27(n170), lin-56(n2728) (THOMAS et al. 2003), rol-6(e187), dpl-1(n2994, n3316, n3380) (CEOL and HORVITZ 2001), unc-4(e120), rol-1(e91), lin-38(n751), mcd-1(n3376, n4005) (this work), unc-52(e444), mln1 [dpy-10(e128) mIs14] (EDGLEY and RIDDLE 2001), mnC1 [dpy-10 unc-52]

LG III; qC1, unc-79(e1068), ced-4(n1162, n2273), dpy-17(e164), lin-37(n758), lin-36(n766), lin-9(n112), lin-13(n770), unc-32(e189), unc-16(e109), lin-52(n771), lin-52(n3718) (THOMAS et al. 2003), ced-7(n3370, n3373, n3378, n3383, n3401, n3408, n3394) (this work), unc-69(e587), unc-50(e306), ced-9(n2812, n1950 n2161, n1653, n3377, n3400, n3407) (DESAI et al. 1988; HENGARTNER et al. 1992; HENGARTNER and HORVITZ 1994b), ced-4(n3379, n3392) (this work), hT2 [qls48] (MATHIES et al. 2003)

LG IV; ced-2(n3387) (this work), dpy-20(e1282), unc-30(e191), let-60(n1876), ced-3(n2427, n2447, n2452, n3374, n3375, n3384, n3403, n3406, n3411) (SHAHAM et al. 1999), dpy-4(e1166), nT1 [qls51] (MATHIES et al. 2003)

LG V; eT1, lin-40(s1593) (SOLARI et al. 1999), tam-1(cc567) (HSIEH et al. 1999), unc-46(e177), let-418(s1617), dpy-11(e224), mys-1(n3681) (CEOL and HORVITZ 2004), egl-1(n1084 n3082) (CONRADT and HORVITZ 1998), hda-1(e1795) (DUFORCQ et al. 2002), unc-76(e911), nIs96 (REDDIEN et al. 2001)

LG X; unc-20(e112), ced-8(n1891), lin-15(n433, n744, n767), chd-3(eh4) (VON ZELEWSKY et al. 2000), nIs106 (REDDIEN et al. 2001)
**Isolation of n4005:** *mcd-1(n4005)* was isolated by screening a library of UV-trimethylpsoralen-induced deletions using PCR as described previously (JANSEN et al. 1997). The *mcd-1(n4005)* deletion begins at base 1228 after the A of ATG within the second intron and extends through base 2325 within the third exon. If the *mcd-1* mRNA in *n4005* animals is spliced from the second to the fourth exon, the product is predicted to be out-of-frame and could generate a protein of 92 amino acids.

**Genetic mapping:** Four of 52 *n3376/n3376* progeny from *n3376+/+unc-52* heterozygous animals were heterozygous for *unc-52*. 0/15 Rol-1 non-Dpy-10 animals from *dpy-10 rol-1+/+n3376* carried *n3376*. 20/21 Rol-1 non-Unc-52 recombinants between *rol-1* and *unc-52* from *rol-1+/unc-52/+n3376+* animals carried *n3376*, and 1/21 did not. 0/93 F2 animals from *n3380+/+rol-6* heterozygous animals recombined between *n3380* and *rol-6.* From 18 recombinants selected between *dpy-10+rol-1+/+n3380+* animals, 5/18 were between *dpy-10* and *n3380* and 13/18 were between *n3380* and *rol-1*. From 22 recombinants selected between *dpy-10+unc-4+/+n3380+* animals, 9/22 were between *dpy-10* and *n3380* and 13/22 were between *n3380* and *unc-4*.

**Polymorphism identification and mapping:** PCR-size polymorphisms on LGIIR between RC301 and N2 were identified by PCR-amplifying approximately 1 kb fragments from intergenic regions and analyzing fragment sizes by electrophoresis. *nP89* is located in the region of the genome represented in cosmid F08G2; the RC301 PCR product amplified with primers PWR.G1 5’- GCCGAAGAAGCGATACTGAATG -3’ and PWR.G2 5’- AAGCCCCCTTGAAAAATGAGC -3’ is approximately 1.1 kb; N2, 1.0 kb. *nP90* is located in the region of the genome represented in YAC Y51H1A; the RC301 PCR product amplified with primers PWR.G11 5’- GTCATTGTGCGTTGATGGGAG -3’ and PWR.G12 5’- TTACCGAGTGCGTTCTGTGAATC -3’ is approximately 1.3 kb; N2, 1.2 kb. *nP91* is located in the region of the genome represented in cosmid W02B8; the RC301 PCR product amplified with primers PWR.G21 5’- CCATCATTTGTCATTGGAGCG -3’ and PWR.G22 5’- AGGCTAGGGGCACCGGTAGATAAAC -3’ is approximately 1.2 kb; N2, 1.1 kb. *nP92* is located in the region of the genome represented in cosmid W07G1; the RC301 PCR product amplified with primers PWR.G31 5’-
CATTTGAGTTGTCGGCTTCCTG -3' and PWR.G32 5'-
CCTTTTCATTTTTGCGGTGTCC -3' is approximately 1.5 kb; N2, 1.2 kb. Recombinants were generated from animals heterozygous for LGII between RC301 and N2: *rol-1 n3376 unc-52 (N2)/+++ (RC301); ced-3(n2427); nIs106* animals. We isolated 20 Rol-1 non-Unc-52 recombinants and generated homozygous recombinant strains. Two recombinants were found to the right of *nP89* and the left of *n3376*, and one recombinant was found to the left of *nP91* and the right of *n3376*.

**Molecular Biology:** The *mcd-1* cDNA was excised from the phage clone yk464e11 (Y. Kohara, personal communication). To determine the 5' end of *mcd-1* messages we used a 5'-rapid amplification of cDNA ends (RACE) system (Gibco). DNA sequences were determined using an automated ABI 373A DNA sequencer (Applied Biosystems). RNAi was performed by microinjection or feeding, as previously described (FIRE et al. 1998; TIMMONS et al. 2001). RNAi of *efl-1*, *efl-2*, and *lin-35* was performed using *efl-1*, *efl-2*, and *lin-35* cDNAs previously described (CEOL and HORVITZ 2001; LU and HORVITZ 1998). RNAi of *mcd-1* was performed from a template isolated by PCR amplification of a region of the third exon of *mcd-1* (see Figure 3) using primers carrying the T7 promoter, I.9, 5'-

GATCGATAATACGACTCACTATAGGGCGGAAAATCCGCCCAAAAAAATCGG -3', and I.10, 5'-

GATCGATAATACGACTCACTATAGGGGATCACAGAGTCGATCCATTACAGG -3'.

Similar methods were used to amplify and generate RNA from *F08G2.7, Y51H1A.1, Y51H1A.2, Y51H1A.4*, and *Y51H1A.5.*
RESULTS

Defects in programmed cell death in *C. elegans* have been efficiently quantified using two assays. First, a P<sub>lin-11</sub> gfp reporter allows the deaths of specific cells in the ventral cord to be assessed (REDDIEN et al. 2001). The P<sub>lin-11</sub> gfp reporter is expressed in the six VC motor neurons, P3-8.aap (P, P blast cell; a, anterior daughter; p, posterior daughter), of the ventral cord (Figure 1A). The cells W.ap (W, W blast cell), P1-2.aap, and P9-12.aap, which die in the wild type, survive in animals with defects in programmed cell death and express P<sub>lin-11</sub> gfp (REDDIEN et al. 2001). Five of these cells, P2.aap and P9-12.aap can be reliably and easily scored for cell survival using a fluorescence stereomicroscope.

A second assay for programmed cell death uses Nomarski optics to determine the number of cell nuclei in another specific region of the animal, the anterior pharynx (HENGARTNER et al. 1992; SHAHAM et al. 1999). Animals carrying strong loss-of-function alleles of the killer gene *ced-3* have about 12-13 extra cells in this region, whereas animals carrying weak alleles of *ced-3* (for example, *n2427*) have about 1-2 extra cells in this region (SHAHAM et al. 1999). We observed that the degree of the cell-death defect conferred by the weak loss-of-function allele *ced-3(n2427)* as determined by counting the number of extra cells in anterior pharynges did not substantially vary with genetic background (Table S1), suggesting that mutations that caused alterations in cell number in *ced-3(n2427)* animals would reflect specific effects on cell death rather than non-specific effects on animal health.

**A genetic screen for enhancers of ced-3(n2427):** Using the P<sub>lin-11</sub> gfp assay described above, we observed that *ced-3(n2427)* results in 4% of animals having all five VC-like cells (P2.aap and P9-12.aap, n=50). We previously showed that mutations in engulfment genes enhance the cell-death defects conferred by *ced-3(n2427)* as assayed with P<sub>lin-11</sub> gfp (REDDIEN et al. 2001). For example, 88% of *ced-1(e1735); ced-3(n2427)* animals had all five extra VC-like cells present (n=50), whereas 0% of *ced-1(e1735)* animals (n=50) had all five extra VC-like cells present (REDDIEN et al. 2001). *ced-1* is necessary for engulfment and encodes a cell-corpse receptor (HEDGECOCK et al. 1983;
ZHOU et al. 2001). We therefore concluded that the $P_{lin-11}$ gfp assay could be used for isolating mutations that enhance $ced-3(n2427)$.

We mutagenized $ced-3(n2427); P_{lin-11}$ gfp animals, selected individual F2 progeny with five extra VC-like cells, and determined if these animals generated progeny with enhanced cell-death defects (Figure 1B). We expected to obtain alleles in the cell-death execution genes $ced-3$, $ced-4$, and $egl-1$. Because loss-of-function mutations in the $ced-9$ gene enhance the cell-death defect caused by weak alleles of $ced-3$ (HENGARTNER and HORVITZ 1994a), we anticipated that mutations in $ced-9$ would also be isolated. We isolated six strains that likely carry mutations in $ced-3$, two in $ced-4$, and three in $ced-9$ (Figure 1C). Because mutations in engulfment genes can enhance the cell-death defect caused by $ced-3(n2427)$, we also expected to isolate alleles of engulfment genes. At least 10 of the strains we isolated have defects in the engulfment of dying cells. We determined, using complementation tests, that two of these strains carry a mutation in $ced-1$, seven in $ced-7$, and one in $ced-2$ (Figure 1C). Many alleles of $ced-7$ might have been isolated because loss of $ced-7$ function strongly enhances weak cell-death defects (Reddien et al. 2001). Two mutations, n3376 and n3380, define new cell-death genes, as described below.

**The mutations n3376 and n3380 define two new cell-death genes:** Animals carrying the mutations n3376 and n3380 had defects in cell death (Figure 1D, E). Genetic mapping established that n3376 and n3380 reside at different locations on LGII (Figure 1F). Because no previously characterized *C. elegans* cell-death gene is located on LGII, these mutations define new cell-death genes. The gene defined by n3380 is a previously known gene, dpl-1 (Figure 1G, see below for details). We named the gene defined by n3376, mcd-1 (mcd, modifier of cell death). More than 60% of double mutant animals carrying mcd-1(n3376) or dpl-1(n3380) with $ced-3(n2427)$ had all five extra VC-1-like cells in their ventral cords, whereas only 4% of $ced-3(n2427)$ single mutant animals had all five extra VC-1-like cells present (Figure 1D). mcd-1(n3376) and dpl-1(n3380) also enhanced cell-death defects conferred by $ced-3(n2427)$ in the anterior pharynx (Table 1), indicating that these mutations affect programmed cell death broadly rather than specifically in the ventral cord. Furthermore, mcd-1(n3376) and dpl-1(n3380) enhanced the cell-death defect associated not only with the CED-3 G474R substitution caused by the
n2427 allele but also with the S446L substitution caused by the n2447 allele (Figure S2A). Therefore, mcd-1(n3376) and dpl-1(n3380) are not allele-specific enhancers of ced-3 and likely generally affect the ability of cells to die if programmed cell death is slightly impaired. We generated animals homozygous for mcd-1(n3376) and dpl-1(n3380) in a wild-type ced-3 background. These animals were viable with no obvious morphological abnormalities. mcd-1(n3376) and dpl-1(n3380) each conferred a weak block in programmed cell death in the presence of an intact core cell-death execution pathway, indicating mcd-1 and dpl-1 have normal roles in promoting cell death (Figure 1E).

n3380 is an allele of dpl-1 DP: We mapped n3380 between unc-4 and dpy-10 on LGII (Figure 1F, Materials and methods). We found that existing mutations in a gene in this region, dpl-1, enhanced the cell-death defect conferred by ced-3(n2427) (Table 1). dpl-1 encodes the C. elegans ortholog of the mammalian E2F-heterodimerization partner DP (CEOL and HORVITZ 2001). We performed complementation tests and determined that the dpl-1(n3316) mutation (CEOL and HORVITZ 2001) failed to complement n3380 for the enhancement of ced-3(n2427) (Table 1 and data not shown). dpl-1 is a class B synthetic multivulva (synMuv) gene (CEOL and HORVITZ 2001). The synMuv genes fall into three classes, A, B, and C. Animals carrying mutations in any two genes from different classes undergo ectopic vulval development, the result of normally non-vulval cells adopting a vulval fate; the resulting phenotype is termed multivulva (Muv) (CEOL and HORVITZ 2004; FERGUSON and HORVITZ 1989; HORVITZ and SULSTON 1980). We found that dpl-1(n3380); lin-15A(n767) animals were Muv (n767 is a class A synMuv mutation; (FERGUSON and HORVITZ 1989), indicating that dpl-1(n3380) acts as a class B synMuv mutation, like other mutations in dpl-1. We determined the sequence of the dpl-1 open reading frame in dpl-1(n3380) animals and found a C-to-T mutation predicted to result in a Q486stop (TAA) alteration in the 595-amino acid DPL-1 protein (Figure 1G). Together these results indicate that n3380 is an allele of the gene dpl-1. Because dpl-1 null alleles confer a sterile phenotype (CEOL and HORVITZ 2001), dpl-1(n3380) is not a null allele of dpl-1. dpl-1(n3380) conferred cell-death defects similar to those of the dpl-1 allele n2994 and the dpl-1 null allele n3316 (Table 1).
**n3376** is an allele of a gene predicted to encode a novel zinc-finger protein: We mapped the mutation n3376 to a region of approximately 75 kilobase pairs containing 13 predicted genes (Figure 1F, see Materials and methods). We inhibited the function of candidate genes in this region by RNAi (RNA-mediated genetic interference; (FIRE et al. 1998) and found that inhibition of Y51H1A.6, but not of F08G2.7, Y51H1A.1, Y51H1A.2, Y51H1A.4, or Y51H1A.5, caused enhancement of the cell-death defect of ced-3(n2427) animals (Table 1). We defined the structure of the Y51H1A.6 genomic locus by determining the sequence of a cDNA clone, yk464e11 (kindly provided by Y. Kohara) (Figure 1H). We determined the 5' end of the Y51H1A.6 transcript using 5' RACE (Rapid Amplification of cDNA Ends) and found Y51H1A.6 transcripts to carry the SL1 trans-spliced leader sequence, which is commonly found on C. elegans mRNAs (KRAUSE and HIRSH 1987). We identified a C-to-T mutation predicted to cause an H277Y substitution in the Y51H1A.6 protein in mcd-1(n3376) animals (Figure 1H). We isolated a deletion allele of Y51H1A.6, n4005 (Figure 1H, Materials and methods) and found that n4005 resulted in a phenotype similar to that of mcd-1(n3376) and failed to complement mcd-1(n3376) for the enhancement of ced-3 (Table 1). n4005 appears to be a strong loss-of-function allele of mcd-1. Together these findings indicate that Y51H1A.6 is the gene defined by n3376.

The mcd-1 gene is predicted to encode a highly acidic novel protein containing one candidate C2H2 zinc finger and has no other significant homology to known genes (Figures 1H, S1). This C2H2 zinc finger (FKCAECDGFVMDRLCDHMIKQH) is an exact match to canonical zinc-finger domains (Y/F-X-C-X2-4-C-X3-F-X5-L-X2-H-X3-5-H, (WOLFE et al. 2000). The mcd-1(n3376) missense mutation changes the first C2H2 histidine to a tyrosine (Figure 1H), indicating the MCD-1 zinc finger is important for the MCD-1 cell-death function. Zinc-finger domains can interact with DNA (WOLFE et al. 2000) and mediate protein-protein interactions (MACKAY and CROSSLEY 1998).

**efl-1** E2F and lin-35 Rb promote cell death: Given that dpl-1 acts in C. elegans development with the E2F-like gene efl-1 and the Rb-like gene lin-35 (CEOL and HORVITZ 2001; LU and HORVITZ 1998; PAGE et al. 2001), we asked whether these and other dpl-1-interacting genes also affect programmed cell death. DP-1 from mammals can bind to and promote DNA-binding by E2F-1 (GIRLING et al. 1993; HELIN et al. 1993). E2F proteins
can affect DNA replication, cell-cycle progression, and development in mammals (BRACKEN et al. 2004)(HELIN 1998) and can interact with the tumor-suppressor protein pRb (DYSON 1998). E2F proteins can promote programmed cell death (HARBOUR and DEAN 2000b). We found that, like dpl-1 DP, efl-1 E2F and lin-35 Rb promoted programmed cell death (Table 2A, Figure S2B, C). Because mutations in efl-1 confer sterility, we studied the role of efl-1 E2F in programmed cell death using RNAi. RNAi of efl-1 enhanced the weak cell-death defects conferred by ced-3(n2427). Perturbation of a second C. elegans E2F-like gene, efl-2, caused no observed cell-death defects (data not shown). We studied the role of lin-35 Rb in programmed cell death using the putative null allele n745. Enhancement of a weak cell-death defect was caused by lin-35(n745) (Table 2A), as well as by RNAi of lin-35 and by other putative lin-35 null alleles (Figure S2C and data not shown). Our data suggest dpl-1, lin-35, and efl-1 act together in programmed cell death as they do in vulval development. The cell-death defects of dpl-1 mutants were stronger than those of efl-1(RNAi) animals or of animals carrying a putative lin-35 null allele, n745 (LU and HORVITZ 1998), for unknown reasons. Disruption of the class B synMuv genes lin-37 and lin-52 also enhanced ced-3(n2427) (Table 2A, Figure S2D). lin-52(n771) more strongly enhanced the cell-death defect conferred by ced-3(n2427) in the anterior pharynx than in the ventral cord (Table 2A, Figure S2D). lin-37 encodes a protein similar to the Myb-interacting Mip40 protein (KORENJAK et al. 2004) and can physically interact with LIN-53 RbAP48, an Rb-interacting protein (WALHOUT et al. 2000). lin-52 encodes a novel protein with similarity to proteins of unknown function in humans and Drosophila (THOMAS et al. 2003).

Disruption of class B synMuv genes - lin-9, lin-15B, lin-36, or lin-53 -or any of the class A synMuv genes lin-8, lin-15A, lin-38, or lin-56 did not enhance ced-3(n2427) (Table 2B). Therefore, the synMuv genes likely do not control a single process that affects both vulval cell fate and cell death. Rb-associated proteins have been found in multiple complexes, including a nucleosome-remodeling and histone-deacetylase complex (NuRD; (XUE et al. 1998) and two complexes from Drosophila containing the transcription factor Myb and multiple class B synMuv-like proteins (Myb-MuvB and dREAM; (LEWIS et al. 2004). We failed to detect a cell-death role for candidate C. elegans components of a NuRD complex (Table 2B): hda-1 (LU and HORVITZ 1998), let-418 (VON ZELEWSKY et al.
chd-3 (VON ZELEWSKY et al. 2000), lin-40 (egr-1) (CHEN and HAN 2001b; SOLARI et al. 1999), and egl-27 (SOLARI et al. 1999). We also failed to detect a cell-death role for lin-9, which encodes a protein similar to a component of the Myb-MuvB and dREAM complexes (Table 2B). Together, our results suggest DPL-1 DP, EFL-1 E2F, LIN-35 Rb, LIN-52 and LIN-37 Mip40 define a novel association of class B synMuv proteins that act to promote programmed cell death. Because of lethality associated with strong loss-of-function alleles of multiple C. elegans genes encoding candidate NuRD, Myb-MuvB, and dREAM complex components, we examined animals with incomplete loss of function for some genes (Table 2B). Roles for essential candidate NuRD, Myb-MuvB, and dREAM complex components in programmed cell death therefore remain possible.

Because the synMuv genes act antagonistically to let-60 Ras in vulval development (BEITEL et al. 1990; FERGUSON et al. 1987; HAN and STERNBERG 1990; STERNBERG and HAN 1998), we asked whether the dpl-1 gene acts antagonistically to let-60 Ras in cell death. Null alleles of let-60 confer maternally rescued larval lethality and zygotically suppress the synMuv phenotype conferred by dpl-1 mutants (CEOL and HORVITZ 2001). We examined homozygous let-60(n1876) animals from heterozygous mothers. let-60(n1876) did not significantly affect the cell-death phenotype of ced-3(n2427) animals and did not suppress the enhanced cell-death defect of dpl-1(n3380); ced-3(n2427) animals (Figure S2E). These observations indicate that dpl-1 does not promote cell death by antagonizing Ras signaling.

dpl-1 and mcd-1 might act together and with lin-35 Rb and efl-1 E2F to promote cell death: To ask whether dpl-1 and mcd-1 act together or separately with one another and with lin-35 and efl-1 to promote cell death, we assayed the number of extra cells conferred by ced-3(n2427) in the anterior pharynges of appropriate double and triple mutant animals. dpl-1, mcd-1, lin-35, and efl-1 did not appear to act additively (Tables 3B, S2B). dpl-1(n3380) mcd-1(n3376) animals had slightly more extra cells in their ventral cords than did dpl-1(n3380) or mcd-1(n3376) single mutant animals (Figure S2F) but not in their anterior pharynges (Table S2B). Furthermore, dpl-1(n3380) mcd-1(n3376); ced-3(n2427) and dpl-1(n3380) mcd-1(n4005); ced-3(n2427) animals did not have more extra cells in their anterior pharynges than did dpl-1(n3380); ced-3(n2427) and mcd-1(n3376);
The dpl-1(n3380) and mcd-1(n3376) animals had defects in cell killing greater than those seen in the single mutants (Table S2C, Figure S2F). In addition, n3376 and n3380 did not cause defects in engulfment. These results indicate that dpl-1 and mcd-1 act independently from ced-8 and ced-1. Furthermore, we examined doubly mutant strains carrying a wild-type ced-3 locus and observed enhanced cell-death defects: ced-1(e1735); dpl-1(n3380) animals, ced-1(e1735); mcd-1(n3376) animals, dpl-1(n3380); ced-8(n1891) animals, and mcd-1(n3376); ced-8(n1891) animals had defects in cell killing greater than those seen in the single mutants (Table S2C, Figure S2F). In addition, n3376 and n3380 did not cause defects in engulfment. These results indicate that dpl-1 and mcd-1 control one of multiple parallel processes that redundantly and significantly contribute to cell death.

**dpl-1 and mcd-1 do not promote cell death through regulation of ced-9:** The egl-1 gene is required for essentially all programmed cell death in *C. elegans* and can be transcriptionally up-regulated to promote programmed cell death (Conradt and Horvitz 1998; Conradt and Horvitz 1999). Because DP, Rb, and E2F can act in transcriptional regulation and mcd-1 has a zinc-finger domain, we sought to determine whether these genes might affect egl-1 expression. egl-1 acts upstream of ced-9 (Conradt and Horvitz 1998). To determine whether dpl-1 and mcd-1 act downstream or upstream of the death-inhibiting role of ced-9, we asked whether mutations in these genes could enhance ced-
3(n2427) in the absence of ced-9 function using the ced-9 null allele n2812 (Table 3C). ced-9 has both a death-inhibiting and a death-promoting role (HENGARTNER and HORVITZ 1994a). We found that ced-3(n2427) animals had on average 1.8 extra cells in their anterior pharynges, while ced-9(n2812); ced-3(n2427) animals had 6.3 extra cells (Table 3A). Triple mutant animals with ced-9(n2812); ced-3(n2427) in addition to mcd-1(n3376) or dpl-1(n3380) had an enhanced number of extra cells (9.4 and 10.6, respectively, Table 3C). By contrast, null alleles of egl-1 do not enhance the cell-killing defects of ced-9(n2812); ced-3(n2427) double mutants (CONRADT and HORVITZ 1998). These results indicate that dpl-1 and mcd-1 have cell-death promoting activity downstream of or independent of the cell-death protective activity of ced-9. In addition, the data indicate dpl-1 and mcd-1 act independently from the cell-death promoting activity of ced-9. Because egl-1 acts to inhibit ced-9, dpl-1 and mcd-1 likely do not promote death by regulating egl-1 transcription.

**dpl-1 and mcd-1 can affect cells fated to live:** Embryos lacking maternal and zygotic ced-9 undergo excessive programmed cell death and die, i.e., in such embryos cells that are normally fated to live instead die (HENGARTNER et al. 1992). This lethality can be suppressed by mutations in the killer genes ced-3 and ced-4 (HENGARTNER et al. 1992). To determine if perturbation of dpl-1 and mcd-1 can affect the ectopic programmed cell death of cells that normally live, we utilized the observation that animals carrying both the ced-9(n1653) temperature-sensitive allele and the ced-4(n2273) mutation confer a synthetic maternal-effect lethality caused by excessive programmed cell death (SHAHAM and HORVITZ 1996a). The ced-4(n2273) mutation affects ced-4 splicing and affects production of the minor cell-death inhibitory CED-4L product; therefore, ced-9(n1653) ced-4(n2273) animals might confer synthetic lethality as a result of a reduction of both ced-9 death-inhibitory function and ced-4L death-inhibitory function (SHAHAM and HORVITZ 1996a). ced-9(n1653) ced-4(n2273) synthetic maternal-effect lethality can be suppressed by mutations that otherwise cause a very weak cell-death defect (E. Speliotes and H. R. H., unpublished results), suggesting that cells that normally live are poised between life and death in these animals. We found that either dpl-1(n3380) or mcd-1(n3376) partially suppressed the synthetic lethality conferred by ced-9(n1653) ced-4(n2273) (Table 4). dpl-1(n3380) or mcd-1(4005) also partially suppressed the maternal-
effect lethality conferred by the ced-9 partial loss-of-function mutant ced-9(n1950 n2161) (Table 4). Therefore, dpl-1 and mcd-1 likely are active in cells that normally live and can promote the ability of such cells to die when cell-death inhibitory activity is reduced. Cell deaths in C. elegans are normally invariant among individuals (SULSTON and HORVITZ 1977). Our observations indicate that dpl-1 and mcd-1 likely do not act to mediate the effects of lineage-regulated factors that specify cell death, because these genes can affect the ability of any cell to die. This hypothesis is supported by our direct observation of the cell-death process in mcd-1 and dpl-1 mutants using Nomarski optics (see below).

**Cells can initiate the death process and recover in dpl-1(n3380) and mcd-1(n4005) animals:** We directly observed cell death within the P9, P10, and P11 neuroblast lineages in dpl-1(n3380) and mcd-1(n4005) animals. Animals also carried the P_{lin-11} gfp reporter, allowing us to determine whether any Pn.aap cell ultimately survived and differentiated. We observed no defect in cell division patterns (dpl-1(n3380), n=8 and mcd-1(n4005), n=6). We observed that the cell-death process could initiate and be followed by episodic changes in the morphology of the dying cells (Figure 2A, B). Some cells ultimately survived following these episodic morphological changes. Specifically, four of 24 Pn.aap cells failed to die in the time period observed in dpl-1(n3380); P_{lin-11} gfp animals, and all four of these cells expressed GFP. Four of 18 Pn.aap cells failed to die in the time period observed in mcd-1(n4005); P_{lin-11} gfp animals, and three of these cells expressed GFP. The initiation of cell condensation during cell death requires the CED-3 caspase (REDDIEN et al. 2001). Therefore, we suggest that cells lacking normal dpl-1 or mcd-1 function can occasionally recover following the activation of CED-3 and the initiation of morphological changes associated with the cell-death process (Figure 2C).

**dpl-1 and mcd-1 do not affect CED-3 caspase-independent death:** Strong loss-of-function alleles of ced-3, including a deletion allele that completely lacks the CED-3 protease-encoding region, ced-3(n2452), do not completely block programmed cell death (SHAHAM et al. 1999). For example, ced-1(e1735); ced-3(n2452) animals generated a few apparent cell corpses: 7 of 50 animals had two or more corpses in the heads of L1 larvae, suggesting that a low level of cell death can occur independently of the ced-3 caspase.
*mcd-1* and *dpl-1* had no effect on these *ced-3*-independent deaths: 8 of 50 L1 larvae had two or more corpses, and 6 of 50 animals had two or more corpses in *ced-1(e1735); mcd-1(n3376); ced-3(n2452) and *ced-1(e1735); dpl-1(n3380); ced-3(n2452) animals, respectively). We therefore suggest that *dpl-1* and *mcd-1* promote cell death by affecting a process controlled by the CED-3 caspase rather than by acting independently from and parallel to CED-3 activity.

**Loss of *mcd-1* function confers synthetic lethality with mutations in *lin-35* Rb and other class B synMuv genes:** Reduction of *mcd-1* function by deletion or RNAi did not cause noticeable growth defects as compared to the wild type. However, perturbation of *mcd-1* function in combination with mutations in some class B synMuv genes conferred synthetic lethality (larval arrest) or slow growth (Table 5A). Specifically, RNAi of *mcd-1* and *mcd-1(n4005)* synthetically caused 100% arrest during the first larval stage of *lin-9(n112), lin-15B(n744), lin-35(n745), lin-37(n758),* and *lin-54(n2231) animals, slow larval growth of *lin-53(n833) animals, and slow growth and some larval arrest of animals carrying mutations in *dpl-1* and *lin-13. mcd-1(n4005)* also synthetically caused slow larval growth in animals carrying a mutation in the class C synMuv gene *mys-1* (Table 5A). Additionally, an L1-arrest phenotype was observed in *mcd-1(n4005) animals when the class B synMuv genes *lin-54, dpl-1, lin-9, or lin-37* were inactivated by RNAi. *mcd-1(n4005)* and RNAi of *mcd-1* did not cause synthetic lethality with the class B synMuv mutations *tam-1(cc567), lin-36(n766), or lin-52(n771)* (Table 5B) or with the class A synMuv mutations *lin-8(n2731), lin-15A(n767), lin-38(n751),* or *lin-56(n2728)* (Table 5C). *lin-36(n766) and lin-52(n771)* are non-null alleles (THOMAS et al. 2003). RNAi of *mcd-1* did not cause a synMuv phenotype in combination with the class B mutations *lin-36(n766) and lin-52(n771)*. These results indicate that *mcd-1* acts redundantly with most but not all class B synMuv genes for growth and viability.

**DISCUSSION**

From a screen for mutations that enhanced the cell-death defect caused by a weak *ced-3* allele we identified two new positive regulators of programmed cell death in *C. elegans,* *mcd-1* and *dpl-1. mcd-1* encodes a novel zinc-finger protein that acts together with the *dpl-
I DP gene. Because DP is the dimerization partner for the E2F transcription factor in mammals (GIRLING et al. 1993; HELIN et al. 1993), these genes likely affect C. elegans cell death via transcription. DP and E2F can act together with Rb and a number of chromatin regulators in transcriptional repression (HARBOUR and DEAN 2000a). A large number of genes encoding proteins implicated in chromatin remodeling have been identified in C. elegans that act together with dpl-1 DP in regulating vulval development. These genes, called “synMuv” for their synthetic multivulva loss-of-function phenotype (FERGUSON and HORVITZ 1989), number at least 27. We found that a few of these genes promote cell death; these genes encode an E2F-like protein (EFL-1), an Rb-like protein (LIN-35), a Mip40-like protein (LIN-37), and one novel protein with similarity to proteins in humans and Drosophila (LIN-52). Therefore, a DP/E2F-like heterodimeric transcription factor probably acts together with an Rb-like protein, the MCD-1 zinc-finger protein, a Mip40-like protein, and the LIN-52 protein to promote cell death in C. elegans via transcriptional regulation.

E2F and Rb proteins have roles in cancer and effects on cell death in mammals (HARBOUR and DEAN 2000b; SHERR and McCORMICK 2002). Similar to the case in C. elegans, loss of function of E2F-1 in mice leads to reduced cell death (FIELD et al. 1996; YAMASAKI et al. 1996). However, in contrast to C. elegans, in the mouse loss of Rb function leads to increased cell death (MACLEOD et al. 1996; MORGENBESSER et al. 1994). It is possible that Rb-like genes promote cell death in mammals but that this defect is obscured by cell-cycle abnormalities; cell-cycle abnormalities are known to trigger cell death in mammals (EVAN and LITTLEWOOD 1998). In C. elegans, an E2F-like and an Rb-like protein act together, rather than antagonistically, to regulate vulval development (CEOL and HORVITZ 2001), the cell cycle (BOXEM and VAN DEN HEUVEL 2002), and cell death (this work). Because E2F-4 and E2F-5 are known to act together with Rb to mediate transcriptional repression in mammals (FROLOV and DYSON 2004), some of the mammalian Rb and E2F genes might act together to promote cell death, as observed in C. elegans.

How might dpl-1, mcd-1, lin-35, efl-1, lin-37, and lin-52 affect C. elegans cell death? Rb and E2F/DP are cell-cycle regulators, and a misregulated cell cycle can trigger
cell death in mammals (EVAN and LITTLEWOOD 1998). However, our data are inconsistent with the hypothesis that cell-cycle abnormalities account for the enhanced cell-death defects we observed. For example, the P9-11 neuroblast lineages in *dpl-1* and *mcd-1* mutants were grossly normal (i.e., no cell cycle arrest or aberrant division patterns were observed, *dpl-1(n3380); nls106, n=8; mcd-1(n4005); nls106, n=6*). Furthermore, cell-cycle roles have been identified in *C. elegans* for a subset of *synMuv* genes, including *lin-35 Rb, dpl-1 DP, efl-1 E2F, lin-9 ALY, lin-15B, and lin-36* (BOXEM and VAN DEN HEUVEL 2001; BOXEM and VAN DEN HEUVEL 2002), but this subset does not match the subset of *synMuv* genes that affects cell death. Specifically, not all *synMuv* genes that affect the cell cycle affect cell death (*lin-9, lin-15, lin-36*), and a *synMuv* gene that does not affect the cell cycle can affect cell death (*lin-37*). Many class B *synMuv* genes probably have multiple functions in development, such as regulation of the cell cycle and regulation of cell fate. For example, many *synMuv* genes have no observed effect on the cell cycle and act together with *synMuv* genes like *dpl-1, lin-35*, and *efl-1* to regulate vulval cell fates. We propose that *dpl-1, mcd-1, efl-1, lin-35, lin-36, and lin-52* act together to regulate cell death via transcriptional regulation of specific targets that affect the cell-death process rather than act non-specifically to affect cell death as a consequence of effects on the cell cycle. Mammalian Rb and E2F-like proteins probably also have non-cell cycle roles (FROLOV and DYSON 2004; LANDSBERG *et al.* 2003).

There are multiple mammalian candidate E2F/DP transcriptional targets that could interface with the cell-death pathway. For example, E2F can trigger cell death via transcriptional activation of the p53 tumor-suppressor gene (HARBOUR and DEAN 2000b). However, we observed that a putative null allele of the *C. elegans* P53-like gene *cep-1* (DERRY *et al.* 2001; SCHUMACHER *et al.* 2001) failed to cause enhancement of the cell-death defects in *ced-3(n2427)* animals (data not shown). E2F genes can also regulate transcription of caspase (MULLER *et al.* 2001) and the *Apaf-1* (MORONI *et al.* 2001) genes. No clear candidate EFL-1 binding sites are readily apparent in *ced-3* or *ced-4* regulatory regions that are evolutionarily conserved with the related species *C. briggsae*. The initiation of cell death in *C. elegans* is known to involve transcription (CONRADT and HORVITZ 1999; METZSTEIN *et al.* 1996; METZSTEIN and HORVITZ 1998; THELLMANN *et al.* 2003), raising the possibility that *dpl-1, mcd-1*, and interacting genes regulate the
transcription of egl-1 to promote cell-death initiation. However, multiple lines of evidence are inconsistent with this hypothesis. First, egl-1 acts upstream of the cell-death inhibitory ced-9 gene (CONRADT and HORVITZ 1998), and dpl-1 and mcd-1 do not. Second, dpl-1 and mcd-1 do not act only in cells fated to die. Third, cells that fail to die in dpl-1 and mcd-1 mutants display morphological changes reflecting attempts at cell death rather than a complete failure to initiate cell death. dpl-1 and mcd-1 therefore likely control the death process rather than the life vs. death decision-making process. We do not know whether dpl-1 and mcd-1 act to control the activity of the core cell death-execution pathway (CED-9, CED-4, CED-3) or act independently. Our data indicate dpl-1 and mcd-1 do not mediate the effects of engulfment on cell death and do not act together with ced-8; engulfment and ced-8 are other known cell-death contributing factors. Because loss-of-function of mcd-1 and dpl-1 did not perturb the low level of cell death that occurs independently of the CED-3 caspase, these genes likely function in a pathway with CED-3.

**MCD-1 is a novel regulator of cell death and is synthetically required for viability with the LIN-35 Rb tumor suppressor:** MCD-1 has a single C2H2 zinc finger and no obvious homology to other domains or proteins in existing databases. Because caspases execute the death process, modulators of cell death are candidate caspase targets for proteolysis. However, MCD-1 does not contain candidate CED-3 cleavage sites conserved in the related species *C. briggsae*. Many uncharacterized zinc-finger proteins exist in mammals, one or more of which could share functional similarities with MCD-1. Given known functions for C2H2 zinc fingers, MCD-1 could be a DNA-binding protein or be involved in protein-protein interactions. *mcd-1* acts with a number of synMuv genes to regulate cell death. Because *mcd-1* is synthetically required for viability with *lin-35 Rb* and many other class B synMuv genes, *mcd-1* must act redundantly with these genes in some developmental processes. This might be because different assemblages of transcriptional regulatory factors control distinct developmental events (see below). Because Rb is a known tumor-suppressor gene, *mcd-1* and potential interacting genes could define an uncharacterized complex that acts redundantly with Rb as a tumor suppressor in mammals.
Subsets of synMuv genes control diverse aspects of biology: Our finding that a subset of synMuv genes affects cell death and that a different subset of synMuv genes is synthetically required for animal viability add to an increasing number of functional categorizations of synMuv genes. Subsets of synMuv genes control at least 14 diverse biological processes in C. elegans (BENDER et al. 2004; BOXEM and VAN DEN HEUVEL 2001; BOXEM and VAN DEN HEUVEL 2002; CEOL and HORVITZ 2004; CHEN and HAN 2001a; CUI et al. 2004; DUFOURCQ et al. 2002; FAY et al. 2002; FAY et al. 2003; FAY et al. 2004; FERGUSON and HORVITZ 1989; GARBE et al. 2004; PAGE et al. 2001; REDDY and VILLENEUVE 2004; UNHAVAITHAYA et al. 2002; WANG et al. 2005). The set of synMuv genes that affects one process is often distinct from the set affecting others. Therefore, the diverse functions of LIN-35 Rb and associated synMuv proteins could be mediated by different assemblages of co-regulators of transcription. Many of the class B synMuv genes encode proteins similar to those found in several chromatin-remodeling complexes in other organisms, including the NuRD, Myb-MuvB, and dREAM transcriptional repression complexes (KORENJAK et al. 2004; LEWIS et al. 2004; XUE et al. 1998). Our studies define a new candidate assemblage of synMuv proteins, containing DPL-1, EFL-1, LIN-35, MCD-1, LIN-37, and LIN-52 and regulating programmed cell death.

Our results with mcd-1 highlight the complex manner in which synMuv genes are utilized. For example, mutations in some of the class B synMuv genes that cause synthetic lethality with loss of mcd-1 cause no detectable defect in cell death (e. g., lin-9 and lin-15B), whereas others do cause defects in cell death (e. g., lin-35). By contrast, one class B synMuv mutation that causes a defect in cell death, lin-52(n771), is homozygous viable with mcd-1(n4005). The process that is affected by mcd-1 and synthetically required for viability with some class B synMuv genes is likely distinct from the process affected by mcd-1 during cell death, because different gene sets associate with these aspects of the mcd-1 loss-of-function phenotype. The hypothesis that mcd-1 acts in at least two processes might explain the observation that mcd-1 appears to act redundantly with lin-35 Rb, dpl-1 DP, and other class B synMuv genes for growth and viability but not for cell death.

Continued genetic studies in C. elegans should allow for the systematic dissection of the functional associations among the synMuv genes. Given that some synMuv genes are similar to human tumor-suppressor proteins (e.g., lin-35 Rb), an understanding of these
associations could have important implications for our understanding of genes regulating human cancer.

**A candidate transcriptional regulatory complex controls one of multiple redundant activities that promote programmed cell death in *C. elegans***: We have identified six new *C. elegans* cell-death genes: *mcd-1, dpl-1, efl-1, lin-35, lin-37,* and *lin-52*. These genes are not needed for most cell death; rather, they have contributing roles. The primary cell-death pathway components remain *egl-1, ced-9, ced-4,* and *ced-3*. When animals contain mutations in multiple genes that control independent, cell-death contributing activities, robust cell-death defects are observed. For example, every animal we observed with mutations in *dpl-1* and the cell-corpse engulfment gene *ced-1* had abnormal cell death in the ventral cord. Therefore, together, cell-death modifiers have very significant roles in the cell-death process. Other independent cell death-promoting activities might exist and be identified using the strategy presented in this manuscript. An understanding of cell death, a process important for disease and development, must account for the many partially redundant and largely unexplained activities that have been identified (Figure 2D). The genes described in this manuscript are candidates to define components of a transcriptional regulatory complex that contains DP, E2F, Rb, MCD-1, LIN-52, and LIN-37 proteins and that promotes programmed cell death. Given the involvement of misregulated programmed cell death in cancer and neurodegenerative disorders, we have identified a new category of genes as potential therapeutic targets for the manipulation of cell death to treat human disease.
ACKNOWLEDGMENTS

We thank Yamini Jagganath for assistance with screening. We thank Rajesh Ranganthan for co-coordinating the design and construction of the deletion library, members of the Horvitz laboratory for efforts in deletion library construction, and Beth Castor for deletion library screening. We thank Na An for maintaining the Horvitz laboratory strain collection. We thank Brendan Galvin and Hillel Schwartz for comments concerning this manuscript. P. W. R. was supported by a National Science Foundation predoctoral fellowship. E. C. A. is an Anna Fuller Graduate Fellow. H. R. H. is the David H. Koch Professor of Biology at M.I.T. and an Investigator of the Howard Hughes Medical Institute. This work was supported by National Institutes of Health grant GM24663 to H. R. H. and by the Howard Hughes Medical Institute.
LITERATURE CITED


SOLARI, F., A. BATEMAN and J. AHRINGER, 1999 The Caenorhabditis elegans genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning. Development 126: 2483-2494.


ZHOU, Z., E. HARTWIEG and H. R. HORVITZ, 2001 CED-1 Is a transmembrane receptor that mediates cell corpse engulfment in *C. elegans*. Cell **104**: 43-56.

Figure 1 The mcd-1 Zn finger and dpl-1 DP genes were identified as cell death-promoting genes from a genetic screen


(B) Schematic of the ced-3(n2427) enhancer screen (see Materials and methods for details).

(C) Results from the ced-3 enhancer screen. From approximately 13,000 mutagenized haploid genomes, 37 mutations were isolated.

(D, E) Distribution of percentages of animals with 0-5 extra cells in the ventral cord. The five cells P2.aap and P9-12.aap were scored using the assay described in Figure 1. At least 50 young adult animals of each genotype were scored. nIs106, P$_{lin-11}$ gfp reporter (REDDIEN et al. 2001).

(F) n3380 is located in the region on LGII between the genes rol-6 and unc-4. n3376 is located between two polymorphisms, nP89 and nP91, on LGII, a region of approximately 75 kilobases. See Materials and methods for details.

(G) Protein structure of DPL-1 DP. The blue box indicates the putative DNA binding region of DPL-1 and the yellow box the putative E2F binding region, as previously described (CEOL and HORVITZ 2001). The n3316 mutation is a deletion following the third codon, and n2994 is a splice-site mutation predicted to alter protein structure following amino acid 227 (CEOL and HORVITZ 2001). n3380 is a C-to-T nonsense mutation that at Q486.

(H) The gene Y51H1A.6 is mcd-1 (see text for details). The 5' end carries a SL1 trans-spliced leader sequence (see text), and the 3'UTR is approximately 750 bp. We isolated a deletion allele, n4005, that removes part of intron two and part of exon three of Y51H1A.6 (see Materials and methods). The red line labeled "Zn" depicts the zinc finger-encoding region of mcd-1. The MCD-1 zinc-finger region amino acid sequence is shown below the gene structure diagram. Red stars indicate the residues that define the C2H2 domain, and
blue stars indicate other residues conserved with the canonical C2H2 zinc finger. \textit{n3376} is a C-to-T mutation resulting in an H277Y substitution in the MCD-1 protein and is indicated by a red arrowhead.

**Figure 2 Abnormalities in the cell-death process in \textit{dpl-1(n3380)} and \textit{mcd-1(n4005)} animals**

(A) P9.aap in a \textit{dpl-1(n3380)}; \textit{nIs106} animal was condensed 2 hours 10 minutes after its generation. By 4 hours 10 minutes after its generation, P9.aap had recovered and appeared morphologically normal. 36 hours later, P9.aap expressed \textit{P\_lin-11\_gfp}. P9.aap is indicated by a yellow arrow. Anterior, left. Posterior, right. Dorsal, top. Ventral, down. In the 4 hour 10 minute image anterior is right and posterior is left.

(B) P11.aap in an \textit{mcd-1(n4005)}; \textit{nIs106} animal condensed 3 hours 22 minutes after its generation. This cell had first displayed attributes of a dying cell one hour 35 minutes after its generation. 3 hours 27 minutes after generation P11.aap recovered, and P11.aaap had normal nuclear morphology. 3 hours 42 minutes after generation, P11.aap condensed, and P11.aaap was condensed as well. 3 hours 47 minutes after generation, P11.aap recovered. P11.aap was observed until 5 hours 25 minutes after generation without any further obvious attempts at death. 36 hours later, no GFP fluorescence was detected in the P11 region, indicating this cell either failed to express \textit{lin-11} or ultimately died. P11.aap is indicated by a yellow arrow and P11.aaap is indicated by a red arrow. Anterior, left. Posterior, right. Dorsal, down. Ventral, top.

(C) Model for the effects of \textit{dpl-1} and \textit{mcd-1} on cell killing. Top, in a cell specified to die in wild-type animals, the CED-3 caspase is activated and the MCD-1 Zn finger, DPL-1 DP, LIN-35 Rb, EFL-1 E2F, LIN-37 Mip40, and LIN-52 dLin52 proteins mediate transcriptional regulation of unknown targets to allow cell death to occur. Middle, in the absence of the CED-3 caspase, cells fail to display the morphological alterations characteristic of cell death. In the absence of \textit{mcd-1} or \textit{dpl-1}, CED-3 is still activated and initiates the cell-death process. The execution of cell death occasionally fails, and cells can survive and differentiate.

(D) Multiple activities function independently and additively to promote cell death.
The MCD-1 Zn finger, DPL-1 DP, LIN-35 Rb, EFL-1 E2F, LIN-37 Mip40, and LIN-52 dLin52 proteins define a transcriptional regulatory activity that promotes cell death. This activity functions in an additive manner with the cell-killing activity of the CED-9 Bcl-2 and CED-8 XK proteins, as well as with the genes that control the process of engulfment to promote cell death. Multiple other activities could exist and act in a similar additive manner to control cell-death execution.
Table 1. \(n^{3376}\) and \(n^{3380}\) affect many programmed cell deaths and are mutations in \(Y51H1A.6\) and \(dpl-1\), respectively

<table>
<thead>
<tr>
<th>genotype</th>
<th>Number extra cells (anterior pharynx)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.1 ± 0.1 (n=20)</td>
</tr>
<tr>
<td>(ced-3(n^{2452}))</td>
<td>11.0 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>(ced-3(n^{2427})) (^b)</td>
<td>1.8 ± 0.2 (n=40)</td>
</tr>
<tr>
<td>(dpl-1(n^{3380})); (ced-3(n^{2427})) (^b)</td>
<td>6.1 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>(mcd-1(n^{3376})); (ced-3(n^{2427})) (^b)</td>
<td>5.9 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>(dpl-1(n^{3380})) (^b)</td>
<td>0.2 ± 0.1 (n=20)</td>
</tr>
<tr>
<td>(mcd-1(n^{3376})) (^b)</td>
<td>0.6 ± 0.2 (n=30)</td>
</tr>
<tr>
<td>(dpl-1(n^{2994})); (ced-3(n^{2427})) (^b)</td>
<td>6.1 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>(dpl-1(n^{3316} (M+))); (ced-3(n^{2427})) (^b, c, d)</td>
<td>3.7 ± 0.7 (n=9)</td>
</tr>
<tr>
<td>(dpl-1(n^{3316} (Mn^{3316}/n^{3380}))); (ced-3(n^{2427})) (^b, c, e)</td>
<td>7.3 ± 0.7 (n=10)</td>
</tr>
<tr>
<td>(Y51H1A.6(\text{RNAi})); (ced-3(n^{2427})) (^b)</td>
<td>6.3 ± 0.5 (n=15)</td>
</tr>
<tr>
<td>(mcd-1(n^{4005})) (^b)</td>
<td>1.0 ± 0.2 (n=20)</td>
</tr>
<tr>
<td>(mcd-1(n^{4005})); (ced-3(n^{2427}))</td>
<td>5.9 ± 0.3 (n=40)</td>
</tr>
<tr>
<td>(mcd-1(n^{3376}/n^{4005}); (ced-3(n^{2427})) (^b)</td>
<td>6.5 ± 0.7 (n=6)</td>
</tr>
</tbody>
</table>

\(^a\)the number of extra cells in the anterior pharynx of L3 larvae were determined using Nomarski optics (Hengartner et al. 1992; Shaham et al. 1999). In the wild type, 16 cells undergo programmed cell death in this region. Data are means ± standard errors of the means.
b this strain was also homozygous for *nIs106*.

c this strain was also homozygous for *dpy-10(e128)*.

d (M+) indicates that the parental genotype of these animals was heterozygous for the mutation.

e (Mn3316/n3380) indicates the parental genotype of these *n3316/n3316* animals was *n3316/n3380*. 
# Table 2. efl-1 E2F and lin-35 Rb promote cell death

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Extra Cells (anterior pharynx)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Loss of function of efl-1, lin-35, lin-37, and lin-52 decreases cell death</strong></td>
<td></td>
</tr>
<tr>
<td>ced-3(n2427)</td>
<td>1.8 ± 0.2 (n=40)</td>
</tr>
<tr>
<td>ced-3(n2427); efl-1(RNAi)</td>
<td>4.8 ± 0.3 (n=30)</td>
</tr>
<tr>
<td>lin-35(n745); ced-3(n2427)</td>
<td>5.1 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>lin-37(n758); ced-3(n2427)</td>
<td>5.5 ± 0.5 (n=15)</td>
</tr>
<tr>
<td>lin-52(n771); ced-3(n2427)</td>
<td>4.5 ± 0.3 (n=35)</td>
</tr>
<tr>
<td><strong>B. Loss of function of many synMuv genes and putative NuRD complex-encoding genes does not perturb cell death</strong></td>
<td></td>
</tr>
<tr>
<td>ced-3(n2427)</td>
<td>1.8 ± 0.2 (n=40)</td>
</tr>
<tr>
<td>lin-8(n111); lin-9(n112); ced-3(n2427)</td>
<td>1.3 ± 0.3 (n=20)</td>
</tr>
<tr>
<td>ced-3(n2427); lin-15(n767)</td>
<td>1.6 ± 0.3 (n=20)</td>
</tr>
<tr>
<td>lin-38(n751); lin-9(n112); ced-3(n2427)</td>
<td>2.3 ± 0.3 (n=14)</td>
</tr>
<tr>
<td>lin-56(2728); ced-3(n2427); lin-15(n744)</td>
<td>1.8 ± 0.2 (n=20)</td>
</tr>
<tr>
<td>lin-8(n111); lin-36(n766); ced-3(n2427)</td>
<td>1.1 ± 0.3 (n=20)</td>
</tr>
<tr>
<td>lin-53(n833); ced-3(n2427)</td>
<td>2.7 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>hda-1(e1795 M+); ced-3(n2427)</td>
<td>2.0 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>ced-3(n2427); let-418(s1617 M+)</td>
<td>1.5 ± 0.3 (n=29)</td>
</tr>
<tr>
<td>ced-3(n2427); chd-3(eh4)</td>
<td>2.3 ± 0.4 (n=20)</td>
</tr>
<tr>
<td>ced-3(n2427); lin-40(s1593 M+)</td>
<td>0.7 ± 0.2 (n=15)</td>
</tr>
</tbody>
</table>
egl-27(n170); ced-3(n2427)$^{\text{b,k}}$ 1.0 ± 0.2 (n=20)

The number of extra cells were determined as described in Table 1.

This strain was also homozygous for $nIs106$.

This strain was also homozygous for $unc-13(e1091)$.

This strain was also homozygous for $unc-32(e189)$.

This strain was also homozygous for $lin-61(n3446)$.

This strain was also homozygous for $unc-52(e444)$.

This strain was also homozygous for $dpy-5(e61)$.

This strain was also heterozygous for $nIs106$.

(M+) is described in Table 1.

This strain was also homozygous for $unc-46(e177)$.

This strain was also homozygous for $unc-4(e120)$. 
Table 3. *dpl-1* DP, *mcd-1*, *efl-1* E2F, and *lin-35* Rb define a new class of cell-death promoting genes that act together and that do not inhibit *ced-9* or act together with *ced-1* or *ced-8*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number extra cells (anterior pharynx)a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Mutations that enhance cell death defects caused by <em>ced-3(n2427)</em></strong></td>
<td></td>
</tr>
<tr>
<td><em>ced-3(n2427)</em></td>
<td>1.8 ± 0.2 (n=40)</td>
</tr>
<tr>
<td><em>dpl-1(n3380); ced-3(n2427)</em></td>
<td>6.1 ± 0.4 (n=20)</td>
</tr>
<tr>
<td><em>mcd-1(n3376); ced-3(n2427)</em></td>
<td>5.9 ± 0.4 (n=20)</td>
</tr>
<tr>
<td><em>mcd-1(n4005); ced-3(n2427)</em></td>
<td>5.9 ± 0.3 (n=40)</td>
</tr>
<tr>
<td><em>efl-1(RNAi); ced-3(n2427)</em></td>
<td>4.9 ± 0.5 (n=20)</td>
</tr>
<tr>
<td><em>lin-35(n745); ced-3(n2427)</em></td>
<td>5.1 ± 0.4 (n=20)</td>
</tr>
<tr>
<td><em>ced-1(e1735); ced-3(n2427)</em></td>
<td>5.9 ± 0.4 (n=30)</td>
</tr>
<tr>
<td><em>ced-3(n2427); ced-8(n1891)</em></td>
<td>5.7 ± 0.3 (n=45)</td>
</tr>
<tr>
<td><em>ced-9(n2812); ced-3(n2427)</em></td>
<td>6.3 ± 0.5 (n=30)</td>
</tr>
</tbody>
</table>

| **B. Combinations of mutations that do not cause additive defects**                                    |
| *dpl-1(n3380) mcd-1(n3376); ced-3(n2427)*            | 5.9 ± 0.2 (n=67)                        |
| *dpl-1(n3380) mcd-1(n4005); ced-3(n2427)*            | 5.4 ± 0.3 (n=25)                        |
| *dpl-1(n3380); efl-1(RNAi); ced-3(n2427)*            | 6.6 ± 0.4 (n=20)                        |
| *mcd-1(n3376); efl-1(RNAi); ced-3(n2427)*            | 6.2 ± 0.4 (n=20)                        |
| *lin-35(n745); dpl-1(n3380); ced-3(n2427)*           | 6.5 ± 0.4 (n=20)                        |
| *lin-35(n745); mcd-1(n3376); ced-3(n2427)*           | 6.4 ± 0.4 (n=20)                        |

<p>| <strong>C. Combinations of mutations that cause additive defects</strong>                                           |
| <em>dpl-1(n3380) mcd-1(n3376) ced-3(n2427)</em> | 6.5 ± 0.4 (n=20) |</p>
<table>
<thead>
<tr>
<th>Strain Combination</th>
<th>Number of Extra Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>ced-1(e1735); dpl-1(n3380); ced-3(n2427)</td>
<td>9.6 ± 0.3 (n=20)</td>
</tr>
<tr>
<td>ced-1(e1735); mcd-1(n3376); ced-3(n2427)</td>
<td>8.8 ± 0.3 (n=40)</td>
</tr>
<tr>
<td>dpl-1(n3380); ced-3(n2427); ced-8(n1891)</td>
<td>8.5 ± 0.4 (n=25)</td>
</tr>
<tr>
<td>mcd-1(n3376); ced-3(n2427); ced-8(n1891)</td>
<td>9.0 ± 0.5 (n=20)</td>
</tr>
<tr>
<td>dpl-1(n3380); ced-9(n2812); ced-3(n2427)</td>
<td>10.6 ± 0.3 (n=45)</td>
</tr>
<tr>
<td>mcd-1(n3376); ced-9(n2812); ced-3(n2427)</td>
<td>9.4 ± 0.4 (n=45)</td>
</tr>
</tbody>
</table>

*a* The number of extra cells were determined as described in Table 1.

*b* This strain was also homozygous for nIs106.

*c* The gene *efl-2* was also inhibited by RNAi in this strain. Because two E2F-like genes have been identified in *C. elegans* (Ceol and Horvitz 2001) it is possible that *efl-1* and *efl-2* act partially redundantly for some processes; we therefore inhibited both genes.

*d* This strain was also homozygous for unc-13(e1091).

*e* This strain was also homozygous for unc-30(e191).

*f* This strain was also homozygous for unc-4(e120).

*g* This strain was also homozygous for rol-1(e91).
Table 4. *mcd-1(n3376)* and *dpl-1(n3380)* suppress *ced-4(n2273) ced-9(n1653) and ced-9(n1950 n2161)* maternal-effect lethality

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of viable progeny (n)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ced-4(n2273) ced-9(n1653)/qC1</em></td>
<td><em>ced-4(n2273) ced-9(n1653)</em></td>
<td>0 (many)</td>
</tr>
<tr>
<td><em>mcd-1(n3376); ced-4(n2273) ced-9(n1653)/qC1</em></td>
<td><em>mcd-1(n3376); ced-4(n2273) ced-9(n1653)</em></td>
<td>4.9 ± 3.1 (n=20)</td>
</tr>
<tr>
<td><em>dpl-1(n3380); ced-4(n2273) ced-9(n1653)/qC1</em></td>
<td><em>dpl-1(n3380); ced-4(n2273) ced-9(n1653)</em></td>
<td>18.5 ± 12.1 (n=15)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of hatched progeny (n)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>unc-69(e587) ced-9(n1950 n2161)/qC1</em></td>
<td><em>unc-69(e587) ced-9(n1950 n2161)</em></td>
<td>0.0 ± 0.0 (n=6)</td>
</tr>
<tr>
<td><em>dpl-1(3380); unc-69(e587) ced-9(n1950 n2161)/qC1</em></td>
<td><em>dpl-1(3380); unc-69(e587) ced-9(n1950 n2161)</em></td>
<td>29.4 ± 14.0 (n=12)</td>
</tr>
<tr>
<td><em>mcd-1(n4005); unc-69(e587) ced-9(n1950 n2161)/qC1</em></td>
<td><em>mcd-1(n4005); unc-69(e587) ced-9(n1950 n2161)</em></td>
<td>18.1 ± 11.0 (n=12)</td>
</tr>
</tbody>
</table>

All animals were homozygous for *nIs106*.

<sup>a</sup> *ced-4(n2273) ced-9(n1653)/ced-4(n2273) ced-9(n1653)* animals were recognized by the fact that they had extra VC-like cells in the ventral cord (data not shown). Animals with four or five extra VC-like cells were picked as *ced-4(n2273) ced-9(n1653)/ced-4(n2273) ced-9(n1653)* animals. *unc-69(e587) ced-9(n1950 n2161)* homozygous animals were recognized by their Unc-69 phenotype. The *dpl-1(n3380); unc-69(e587) ced-9(n1950 n2161)/qC1* strain was also homozygous for *unc-30(e191).*
The number of progeny were determined by counting larval stage 3 (L3) or older animals on plates four days after young adults were placed onto petri plates. Data are means ± standard deviation.

dpl-1(3380); unc-69(e587) ced-9(n1950 n2161) and mcd-1(n4005); unc-69(e587) ced-9(n1950 n2161) animals arrested as larvae approximately the size of L1 larvae. We therefore quantified the number of larvae present. Data are means ± standard deviations.
Table 5. *mcd-1* loss-of-function causes synthetic lethality with some class B synMuv mutations

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Growth phenotype$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Some class B and C synMuv mutations cause synthetic growth and viability defects with <em>mcd-1(n4005)</em></strong></td>
<td></td>
</tr>
<tr>
<td><em>mcd-1(n4005)</em></td>
<td>viable$^b$</td>
</tr>
<tr>
<td><em>lin-53(n833); mcd-1(n4005)$^d$</em></td>
<td>slow growth$^c$</td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-13(n770)</em></td>
<td>slow growth$^c$</td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-54(n2231)</em></td>
<td>slow growth$^c$</td>
</tr>
<tr>
<td><em>dpl-1(n3380) mcd-1(n4005)$^f$</em></td>
<td>slow growth$^{c,g}$</td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-9(n112)</em></td>
<td>L1 arrest$^h$</td>
</tr>
<tr>
<td><em>lin-35(n745); mcd-1(n4005)</em></td>
<td>L1 arrest$^h$</td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-37(n758)</em></td>
<td>L1 arrest$^i$</td>
</tr>
<tr>
<td><em>mcd-1(n4005); mys-1(n3681)</em></td>
<td>slow growth$^c$</td>
</tr>
<tr>
<td><strong>B. Some class B synMuv mutations do not cause synthetic lethality with <em>mcd-1(n4005)</em></strong></td>
<td></td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-36(n766)</em></td>
<td>viable</td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-52(n771)</em></td>
<td>viable</td>
</tr>
<tr>
<td><em>mcd-1(n4005); tam-1(cc567)$^d$</em></td>
<td>viable</td>
</tr>
<tr>
<td><strong>C. Class A synMuv mutations do not cause synthetic lethality with <em>mcd-1(n4005)</em></strong></td>
<td></td>
</tr>
<tr>
<td><em>lin-8(n2731) mcd-1(n4005)</em></td>
<td>viable</td>
</tr>
<tr>
<td><em>mcd-1(n4005); lin-15A(n767)</em></td>
<td>viable</td>
</tr>
</tbody>
</table>
lin-38(n751) mcd-1(n4005) viable
lin-56(n2728) mcd-1(n4005) viable

a The growth phenotype was assayed at 20°C and 25°C by picking L4 larvae and quantifying the time until greater than 50% of the next generation reached the L4 larval stage.

b Viable strains can be maintained and have a growth phenotype of between 3 and 3.5 days.

c strain also contained unc-46(e177)

d strain also contained dpy-5(e61)

e The development of this strain was delayed two days as compared to the mcd-1 single mutant strain or the respective class B synMuv mutant strain at 20°C.

f strain also contains unc-4(e120)

g dpl-1(n3380) unc-4 mcd-1(n4005) animals were homozygous viable and slow-growing at 20°C. At 25°C the strain could not be maintained after 24 days with infertile adults, sick slow-growing larvae and some arrested larvae.

h 100% of the animals arrested as larvae in size similar to L1 larvae, whereas the mcd-1 single mutant and the respective class B synMuv mutant strains do not.

i At 20°C animals were either small, infertile Muv adults, sick slow-growing larvae, or arrested larvae. At 22.5°C and 25°C, 100% of the animals arrested as larvae in size similar to L1 larvae.
Figure 1

A. 

wild type

B. 

EMS

ced-3(n2427); weak Ced, some cells survive

F1

ced-3(n2427); enhancer; enhanced Ced, all cells survive

F2

ced-3(n2427); enhancer; enhanced Ced, all cells survive

C. 

ced-3  n3374, n3375, n3384
     n3403, n3406, n3411

ced-4  n3379, n3392

ced-9  n3377, n3400, n3407

ced-1  n3390, n3402

ced-2  n3387

ced-7  n3370, n3373, n3378, n3383, n3401, n3408, n3394

Other  n3376, n3380

Unknown 14 other mutations

D. 

ced-3(n2427); nls106

E. 

nls106

F. 

dpy-10 rol-6 dpl-1 unc-4 rol-1 lin-38 jsP305 nP89 nP90 nP91 nP92 jsP306unc-52

1 map unit 50 kb 13 genes LGII

G. 

n3316 (deletion) n2994(227splice) n3380 Q486stop

DNA binding E2F binding

595 aa

H. 

n4005

Zn

Y51H1A.6 mcd-1

n3376

SL1

Finger

A(n)

1 kb
Figure 2

A  *dpl-1(n3380); nls106*

- P9.aap (condensed) 2 hr 10 min
- P9.aap (healthy) 4 hr 10 min
- P9.aap (differentiated) 36 hr

B  *mcd-1(n4005); nls106*

- P11.aap (condensed) 3 hr 22 min
- P11.aap (healthy) 3 hr 27 min
- P11.aap (condensed) 3 hr 42 min
- P11.aap (healthy) 3 hr 47 min

C  

- **CED-3 active**
  - CED-1 Zn finger
  - DPL-1 DP
  - LIN-35 Rb
  - EFL-1 E2F
  - LIN-37 Mip40
  - LIN-52 dLin52

  > dying cell
  > dead cell

- **CED-3 inactive**

  > live cell
  > live cell

- **CED-3 active**

  > dying cell
  > dead cell

D  

- MCD-1 Zn finger
- DPL-1 DP
- EFL-1 E2F (killing function)
- CED-9 Bcl-2
- CED-8 Xk engulfment
- LIN-35 Rb
- LIN-37 Mip40
- LIN-52 dLin52

  > programmed cell death

- others?