

DPL-1 DP, LIN-35 Rb and EFL-1 E2F Act With the MCD-1 Zinc-Finger Protein to Promote Programmed Cell Death in *Caenorhabditis elegans*

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ABSTRACT

The genes *egl-1*, *ced-9*, *ced-4*, and *ced-3* play major roles in programmed cell death in *Caenorhabditis 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.

PROGRAMMED cell death is important for many aspects of animal development, including morphogenesis, homeostasis, and neuronal refinement (GLÜCKSMANN 1951; SAUNDERS 1966; JACOBSON *et al.* 1997). 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 procaspase (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

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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° on NGM agar with *Escherichia 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:

- LG1: *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 [qIs48]* (MATHIES *et al.* 2003), *nIs128* (H. SCHWARTZ and H. R. HORVITZ, unpublished observations).
- LGII: *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)*, *mIn1 [dpy-10(e128) mIs14]* (EDGLEY and RIDDLE 2001), *mnC1 [dpy-10 unc-52]*.
- LGIII: *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; and this work), *ced-4(n3379, n3392)* (this work), *hT2 [qIs48]* (MATHIES *et al.* 2003).
- LGIV: *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; and this work), *dpy-4(e1166)*, *nT1 [qIs51]* (MATHIES *et al.* 2003).
- LGv: *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)* (DUFOURCQ *et al.* 2002), *unc-76(e911)*, *nIs96* (REDDIEN *et al.* 2001).
- LGx: *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*. Zero of 15 Rol-1 non-Dpy-10 animals from *dpy-10 rol-1+/+n3376* carried *n3376*. Twenty of 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. Zero of 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 ~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'-AAGCC CCCTTGAAAAATGAGC-3' is ~1.1 kb; and 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 ~1.3 kb; and 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'-CCATCATTTGTCAATTGGAGCG-3' and PWR.G22 5'-AGGGTAGGGGCACGGTAGATAAAG-3' is ~1.2 kb; and 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'-CATTGGTAGTTGTC GGCTTCCTG-3' and PWR.G32 5'-CCTTTTCATTTTTCGGG TGTCC-3' is ~1.5 kb; and 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 to the left of *n3376*, and one recombinant was found to the left of *nP91* and to 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, Grand Island, NY). DNA sequences were determined using an automated ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA). RNAi was performed by microinjection or feeding, as previously described (FIRE *et al.* 1998; TIMMONS *et al.* 2001). RNAi of *egl-1*, *egl-2*, and *lin-35* was performed using *egl-1*, *egl-2*, and *lin-35* cDNAs as previously described (LU and HORVITZ 1998; CEOL and HORVITZ 2001). 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 1H), using primers carrying the T7 promoter, I.9, 5'-GATCGATAATACGACTCACTATAG GGCGGAAAAATCCGCCAAAAAAAATCGG-3', and I.10, 5'-GATCCATAATACGACTCACTATAGGGGATCACAGAGTCGATCGATTACAGG-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_{lin-11} *gfp* reporter allows the deaths of specific cells in the ventral cord to be assessed (REDDIEN *et al.* 2001). The P_{lin-11} *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_{lin-11} *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.

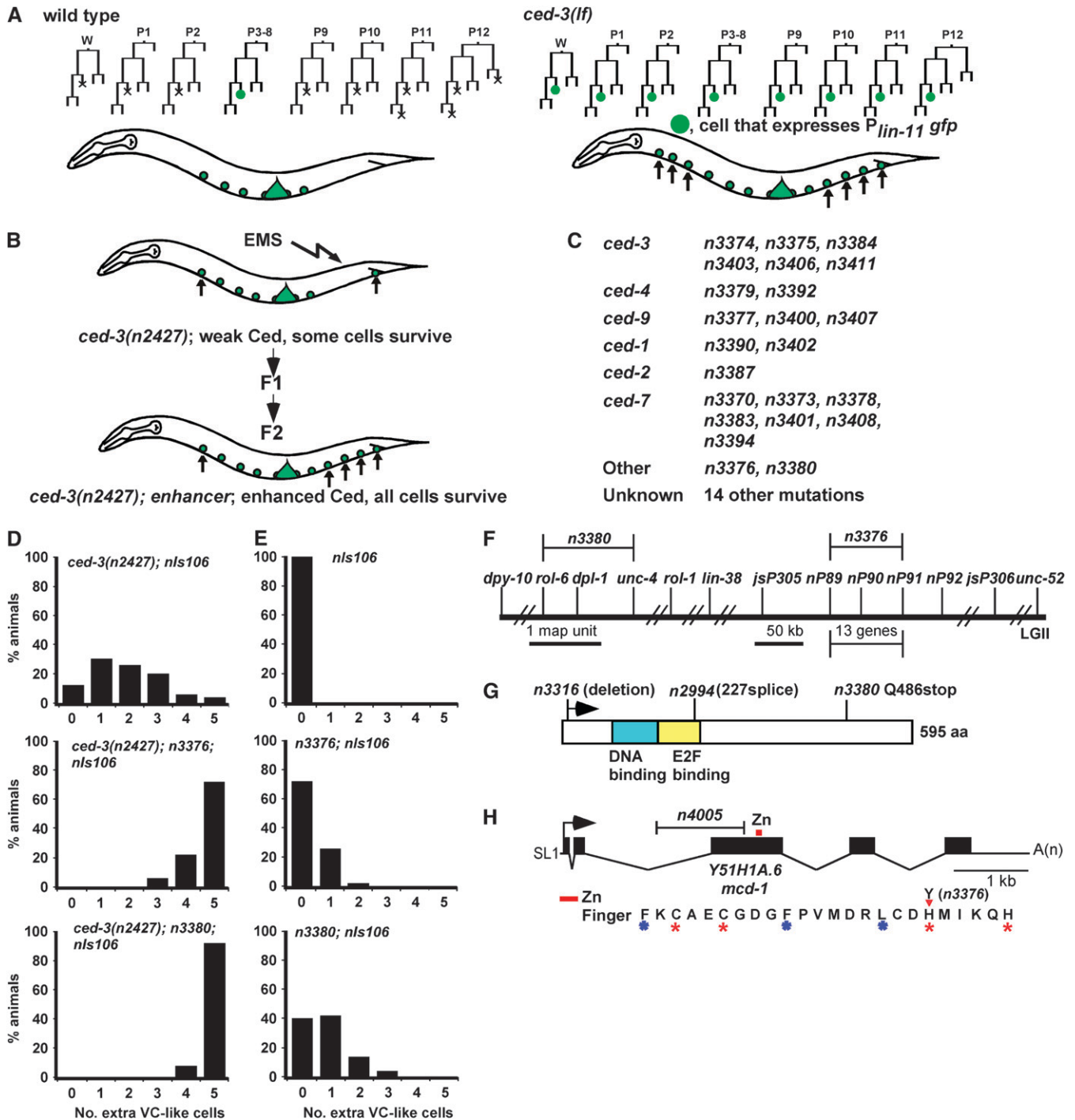


FIGURE 1.—The *mcd-1* Zn finger and *dpl-1* DP genes were identified as cell death-promoting genes from a genetic screen. (A) Ventral cord cell lineage diagrams. W, W blast cell. P, P blast cell. Arrows, cells that fail to die and that express $P_{lin-11} gfp$. In the wild type, P3–8.aap (a, anterior daughter; p, posterior daughter) survive and express $P_{lin-11} gfp$. W.ap, P1–2.aap, and P9–12.aap normally die (SULSTON and HORVITZ 1977). In *ced-3(lf)* animals, no cell death occurs and W.ap, P1–2.aap, and P9–12.aap survive and express $P_{lin-11} gfp$ (REDDIEN *et al.* 2001). The large green midbody region reflects GFP expression in the vulva from the *lin-11* promoter. (B) Schematic of the *ced-3(n2427)* enhancer screen (see text for details). (C) Results from the *ced-3* enhancer screen. From ~13,000 mutagenized haploid genomes, 37 mutations were isolated. (D and E) Distribution of percentages of animals with zero to five extra cells in the ventral cord. The five cells P2.aap and P9–12.aap were scored using the assay described. At least 50 young adult animals of each genotype were scored. *nls106*, $P_{lin-11} gfp$ 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 ~75 kb. 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 is at Q486. (H) The gene *Y51H1A.6* is *mcd-1*

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 ~12–13 extra cells in this region, whereas animals carrying weak alleles of *ced-3* (for example, *n2427*) have ~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 (supplemental Table S1 at <http://www.genetics.org/supplemental/>), suggesting that mutations that caused alterations in cell number in *ced-3(n2427)* animals would reflect specific effects on cell death rather than nonspecific effects on animal health.

A genetic screen for enhancers of *ced-3(n2427)*: Using the $P_{lin-11} gfp$ 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 $P_{lin-11} gfp$ (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 6 strains that likely carry mutations in *ced-3*, 2 in *ced-4*, and 3 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 2 of these strains carry a mutation in *ced-1*, 7 carry one in *ced-7*, and 1 carries 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 1, D and 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-like cells in their ventral cords, whereas only 4% of *ced-3(n2427)* single-mutant animals had all five extra VC-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 (supplemental Figure S2A at <http://www.genetics.org/supplemental/>). 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 that *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

(see text for details). The 5' end carries an SL1 *trans*-spliced leader sequence (see text), and the 3'-UTR is ~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. *n3376* is a C-to-T mutation resulting in an H277Y substitution in the MCD-1 protein and is indicated by a red arrowhead.

TABLE 1

n3376 and *n3380* affect many programmed cell deaths and are mutations in *Y51H1A.6* and *dpl-1*, respectively

Genotype	No. of extra cells (anterior pharynx) ^a
Wild type	0.1 ± 0.1 (<i>n</i> = 20)
<i>ced-3(n2452)</i>	11.0 ± 0.4 (<i>n</i> = 20)
<i>ced-3(n2427)^b</i>	1.8 ± 0.2 (<i>n</i> = 40)
<i>dpl-1(n3380); ced-3(n2427)^b</i>	6.1 ± 0.4 (<i>n</i> = 20)
<i>mcd-1(n3376); ced-3(n2427)^b</i>	5.9 ± 0.4 (<i>n</i> = 20)
<i>dpl-1(n3380)^b</i>	0.2 ± 0.1 (<i>n</i> = 20)
<i>mcd-1(n3376)^b</i>	0.6 ± 0.2 (<i>n</i> = 30)
<i>dpl-1(n2994); ced-3(n2427)^b</i>	6.1 ± 0.4 (<i>n</i> = 20)
<i>dpl-1(n3316 (M+)); ced-3(n2427)^{b,c,d}</i>	3.7 ± 0.7 (<i>n</i> = 9)
<i>dpl-1(n3316 (Mn3316/n3380)); ced-3(n2427)^{b,c,e}</i>	7.3 ± 0.7 (<i>n</i> = 10)
<i>Y51H1A.6(RNAi); ced-3(n2427)^b</i>	6.3 ± 0.5 (<i>n</i> = 15)
<i>mcd-1(n4005)^b</i>	1.0 ± 0.2 (<i>n</i> = 20)
<i>mcd-1(n4005); ced-3(n2427)</i>	5.9 ± 0.3 (<i>n</i> = 40)
<i>mcd-1(n3376/n4005); ced-3(n2427)^b</i>	6.5 ± 0.7 (<i>n</i> = 6)

^a The number of extra cells in the anterior pharynx of L3 larvae was 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 that the parental genotype of these *n3316/n3380* animals was *n3316/n3380*.

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 nonvulval cells adopting a vulval fate; the resulting phenotype is termed multivulva (*Muv*) (HORVITZ and SULSTON 1980; FERGUSON and HORVITZ 1989; CEOL and HORVITZ 2004). 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 ~75 kbp containing 13 predicted genes (Figure 1F, see MATERIALS AND METHODS). We inhibited the function of candidate genes in this region by RNA-mediated genetic interference (RNAi) (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 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 (Figure 1H, supplemental Figure S1 at <http://www.genetics.org/supplemental/>). This C2H2 zinc finger (FKCAECCGDGFPVMDRLCDHMIKQH) 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 that 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* (LU and HORVITZ 1998; CEOL and HORVITZ 2001; 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 (HELIN 1998; BRACKEN *et al.* 2004) 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; supplemental Figure S2, B and C, at <http://www.genetics.org/supplemental/>). 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

TABLE 2
***efl-1* E2F and *lin-35* Rb promote cell death**

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

^a The number of extra cells was determined as described in Table 1.

^b This strain was also homozygous for *nIs106*.

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

^d This strain was also homozygous for *unc-32(e189)*.

^e This strain was also homozygous for *lin-61(n3446)*.

^f This strain was also homozygous for *unc-52(e444)*.

^g This strain was also homozygous for *dpy-5(e61)*.

^h This strain was also heterozygous for *nIs106*.

ⁱ (M+) is described in Table 1.

^j This strain was also homozygous for *unc-46(e177)*.

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

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 (supplemental Figure S2C and data not shown). Our data suggest that *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, supplemental 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, supplemental Figure S2D). *lin-37* encodes a protein similar to the Myb-interacting Mip40 protein (KORENIAK

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.* 2000), *chd-3* (VON ZELEWSKY *et al.* 2000), *lin-40* (*egr-1*) (SOLARI *et al.* 1999; CHEN and HAN 2001b), 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 that 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 (FERGUSON *et al.* 1987; BEITEL *et al.* 1990; 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 (supplemental Figure S2E at <http://www.genetics.org/supplemental/>). 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 pharynxes of appropriate double- and

TABLE 3

dpl-1 DP, *mcd-1*, *egl-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*

Genotype	No. of extra cells (anterior pharynx) ^a
A. Mutations that enhance cell death defects caused by <i>ced-3(n2427)</i>	
<i>ced-3(n2427)^b</i>	1.8 ± 0.2 (n = 40)
<i>dpl-1(n3380); ced-3(n2427)^b</i>	6.1 ± 0.4 (n = 20)
<i>mcd-1(n3376); ced-3(n2427)^b</i>	5.9 ± 0.4 (n = 20)
<i>mcd-1(n4005); ced-3(n2427)^b</i>	5.9 ± 0.3 (n = 40)
<i>egl-1(RNAi); ced-3(n2427)^{b,c}</i>	4.9 ± 0.5 (n = 20)
<i>lin-35(n745); ced-3(n2427)^{b,d}</i>	5.1 ± 0.4 (n = 20)
<i>ced-1(e1735); ced-3(n2427)^b</i>	5.9 ± 0.4 (n = 30)
<i>ced-3(n2427); ced-8(n1891)</i>	5.7 ± 0.3 (n = 45)
<i>ced-9(n2812); ced-3(n2427)^e</i>	6.3 ± 0.5 (n = 30)
B. Combinations of mutations that do not cause additive defects	
<i>dpl-1(n3380) mcd-1(n3376); ced-3(n2427)^{b,f,g}</i>	5.9 ± 0.2 (n = 67)
<i>dpl-1(n3380) mcd-1(n4005); ced-3(n2427)^{b,f}</i>	5.4 ± 0.3 (n = 25)
<i>dpl-1(n3380); egl-1(RNAi); ced-3(n2427)^{b,c}</i>	6.6 ± 0.4 (n = 20)
<i>mcd-1(n3376); egl-1(RNAi); ced-3(n2427)^{b,c}</i>	6.2 ± 0.4 (n = 20)
<i>lin-35(n745); dpl-1(n3380); ced-3(n2427)^{b,d}</i>	6.5 ± 0.4 (n = 20)
<i>lin-35(n745); mcd-1(n3376); ced-3(n2427)^b</i>	6.4 ± 0.4 (n = 20)
C. Combinations of mutations that cause additive defects	
<i>ced-1(e1735); dpl-1(n3380); ced-3(n2427)^{b,f}</i>	9.6 ± 0.3 (n = 20)
<i>ced-1(e1735); mcd-1(n3376); ced-3(n2427)^b</i>	8.8 ± 0.3 (n = 40)
<i>dpl-1(n3380); ced-3(n2427); ced-8(n1891)</i>	8.5 ± 0.4 (n = 25)
<i>mcd-1(n3376); ced-3(n2427); ced-8(n1891)</i>	9.0 ± 0.5 (n = 20)
<i>dpl-1(n3380); ced-9(n2812); ced-3(n2427)^b</i>	10.6 ± 0.3 (n = 45)
<i>mcd-1(n3376); ced-9(n2812); ced-3(n2427)^{b,e}</i>	9.4 ± 0.4 (n = 45)

^aThe number of extra cells was determined as described in Table 1.

^bThis strain was also homozygous for *nIs106*.

^cThe gene *egl-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 *egl-1* and *egl-2* act partially redundantly for some processes; we therefore inhibited both genes.

^dThis strain was also homozygous for *unc-13(e1091)*.

^eThis strain was also homozygous for *unc-30(e191)*.

^fThis strain was also homozygous for *unc-4(e120)*.

^gThis strain was also homozygous for *rol-1(e91)*.

triple-mutant animals. *dpl-1*, *mcd-1*, *lin-35*, and *egl-1* did not appear to act additively (Table 3B, supplemental Table S2B at www.genetics.org/supplemental/). *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 (supplemental Figure S2F at www.genetics.org/supplemental/) but not in their anterior pharynges (supplemental 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); ced-3(n2427)* or *mcd-1(n4005); ced-3(n2427)* animals (Table 3B). That *dpl-1(n3380)* and *mcd-1(n3376)* or *mcd-1(n4005)* can con-

fer additive defects with other mutations that caused similar levels of cell-death defects (*ced-1*, *ced-8*, and *ced-9*, see below, Table 3C) indicates that changes in cell-killing efficiency are within the detectable range of this assay. Thus, *dpl-1*, *mcd-1*, *lin-35*, and *egl-1* might act together to promote cell death.

***dpl-1* and *mcd-1* promote cell death in a manner independent of *ced-1* and *ced-8*:** We asked whether *dpl-1* and *mcd-1* act together with other subtle cell-death regulators. Mutations in engulfment genes (e.g., *ced-1*) can decrease cell killing (REDDIEN *et al.* 2001). Mutations in *ced-8* cause a delay in cell death, enhance weak alleles of *ced-3*, and cause very weak defects in cell killing on their own (STANFIELD and HORVITZ 2000). We observed that mutations in *mcd-1* and *dpl-1* enhanced cell-killing defects conferred by *ced-1(e1735)* or *ced-8(n1891)* (Table 3C). Because *ced-1(e1735)* and *ced-8(n1891)* are both null or strong loss-of-function alleles (STANFIELD and HORVITZ 2000; ZHOU *et al.* 2001), our data indicate that *mcd-1* and *dpl-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 (supplemental Table S2C and supplemental Figure S2F at www.genetics.org/supplemental/). 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 upregulated to promote programmed cell death (CONRADT and HORVITZ 1998, 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

TABLE 4

mcd-1(n3376) and *dpl-1(n3380)* suppress *ced-4(n2273)* *ced-9(n1653)* and *ced-9(n1950 n2161)* maternal-effect lethality

Maternal genotype	Genotype ^a	No. of viable progeny (n) ^b
<i>ced-4(n2273) ced-9(n1653)/qC1</i>	<i>ced-4(n2273) ced-9(n1653)</i>	0 (many)
<i>mcd-1(n3376); ced-4(n2273) ced-9(n1653)/qC1</i>	<i>mcd-1(n3376); ced-4(n2273) ced-9(n1653)</i>	4.9 ± 3.1 (n = 20)
<i>dpl-1(n3380); ced-4(n2273) ced-9(n1653)/qC1</i>	<i>dpl-1(n3380); ced-4(n2273) ced-9(n1653)</i>	18.5 ± 12.1 (n = 15)
Maternal genotype	Genotype ^a	No. of hatched progeny (n) ^c
<i>unc-69(e587) ced-9(n1950 n2161)/qC1</i>	<i>unc-69(e587) ced-9(n1950 n2161)</i>	0.0 ± 0.0 (n = 6)
<i>dpl-1(3380); unc-69(e587) ced-9(n1950 n2161)/qC1</i>	<i>dpl-1(3380); unc-69(e587) ced-9(n1950 n2161)</i>	29.4 ± 14.0 (n = 12)
<i>mcd-1(n4005); unc-69(e587) ced-9(n1950 n2161)/qC1</i>	<i>mcd-1(n4005); unc-69(e587) ced-9(n1950 n2161)</i>	18.1 ± 11.0 (n = 12)

All animals were homozygous for *nIs106*.

^a *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)*.

^b The number of progeny was determined by counting larval stage 3 (L3) or older animals on plates 4 days after young adults were placed onto petri plates. Data are means ± standard deviations.

^c *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.

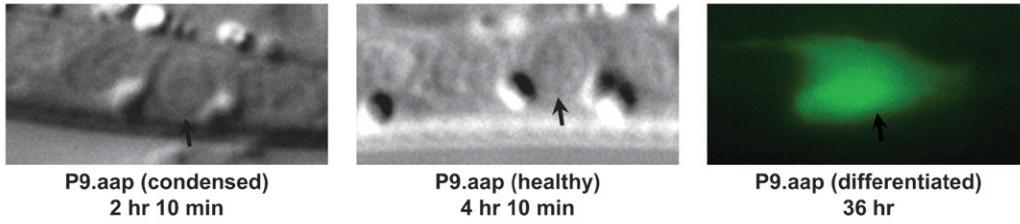
indicate that *dpl-1* and *mcd-1* have cell-death-promoting activity downstream of or independent of the cell-death protective activity of *ced-9*. In addition, the data indicate that *dpl-1* and *mcd-1* act independently from the cell-death-promoting activity of *ced-9*. Because *egl-1* acts to inhibit *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. HORVITZ, 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

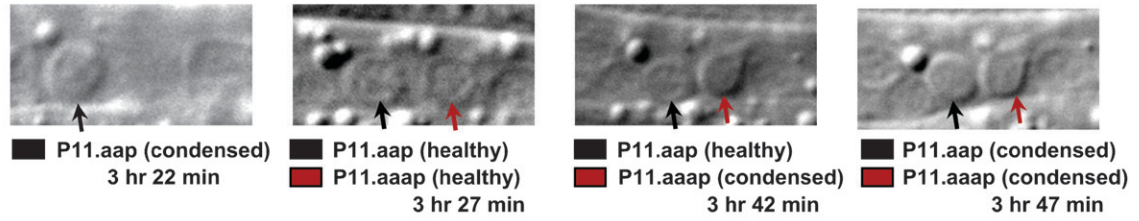
loss-of-function mutant *ced-9(n1950 n2161)* (Table 4). Therefore, *dpl-1* and *mcd-1* likely are active in cells that normally live and can promote the ability of such cells to die when cell-death inhibitory activity is reduced. Cell deaths in *C. elegans* are normally invariant among individuals (SULSTON 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 2, A and B). Some cells ultimately survived following these episodic morphological changes. Specifically, 4 of 24 Pn.aap cells failed to die in the time period observed in *dpl-1(n3380); P_{lin-11} gfp* animals, and all 4 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 3 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).

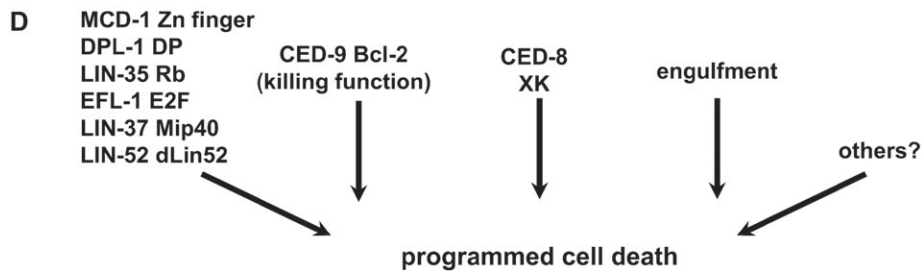
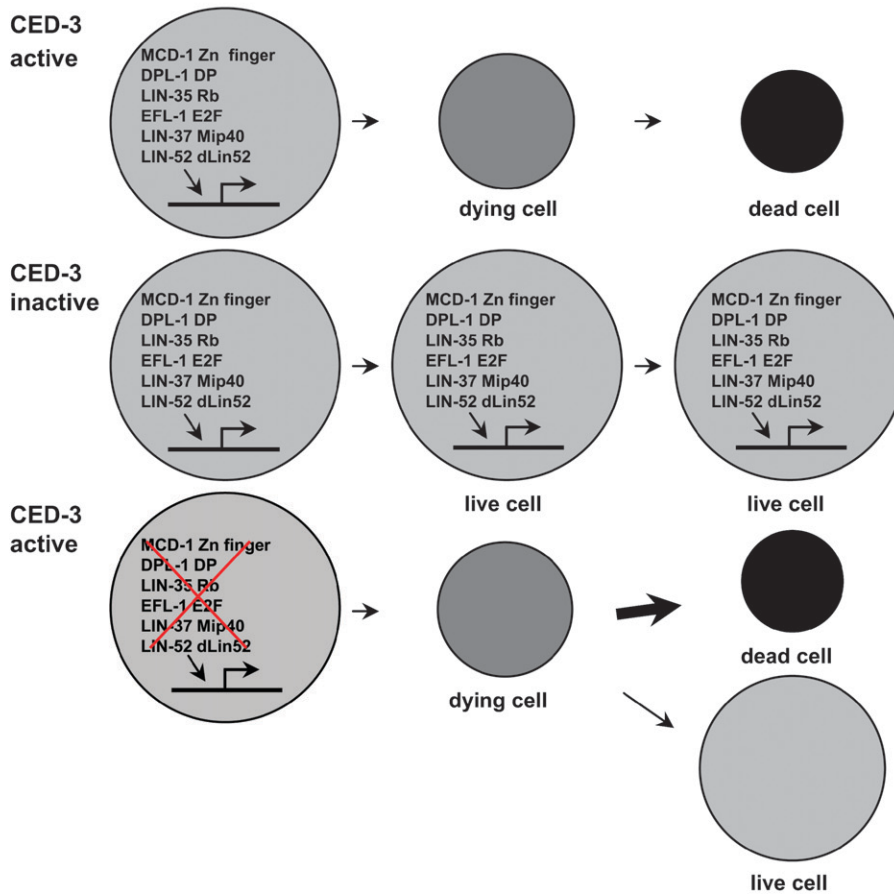
A *dpl-1(n3380); nls106*



B *mcd-1(n4005); nls106*



C Cell specified to die



***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 notable 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 nonnull 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-1* 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 (HARBOR 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

FIGURE 2.—Abnormalities in the cell-death process in *dpl-1(n3380)* and *mcd-1(n4005)* animals. (A) P9.aap in a *dpl-1(n3380); nIs106* animal was condensed 2 hr 10 min after its generation. By 4 hr 10 min after its generation, P9.aap had recovered and appeared morphologically normal; 36 hr later, P9.aap expressed P_{*lin-11*}*gfp*. P9.aap is indicated by an arrow. Anterior, left. Posterior, right. Dorsal, top. Ventral, bottom. In the 4-hr 10-min image anterior is right and posterior is left. (B) P11.aap in an *mcd-1(n4005); nIs106* animal condensed 3 hr 22 min after its generation. This cell had first displayed attributes of a dying cell 1 hr 35 min after its generation. Three hours 27 min after generation P11.aap recovered, and P11.aap had normal nuclear morphology. Three hours 42 min after generation, P11.aap condensed, and P11.aap was condensed as well. Three hours 47 min after generation, P11.aap recovered. P11.aap was observed until 5 hr 25 min after generation without any further obvious attempts at death. Thirty-six hours later, no GFP fluorescence was detected in the P11 region, indicating that this cell either failed to express *lin-11* or ultimately died. P11.aap is indicated by a black arrow and P11.aap is indicated by a red arrow. Anterior, left. Posterior, right. Dorsal, bottom. Ventral, top. (C) Model for the effects of *dpl-1* and *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. Bottom, in the absence of *mcd-1* or *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 5

***mcd-1* loss of function causes synthetic lethality with some class B synMuv mutations**

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

^a The growth phenotype was assayed at 20° and 25° by picking L4 larvae and quantifying the time until >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 *dpy-5*(e61).

^d The development of this strain was delayed 2 days as compared to the *mcd-1* single-mutant strain or the respective class B synMuv mutant strain at 20°.

^e Strain also contains *unc-4*(e120).

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

^g One hundred percent of the animals arrested as larvae in size similar to that of L1 larvae, whereas the *mcd-1* single-mutant and the respective class B synMuv mutant strains do not.

^h At 20° animals were small, infertile Muv adults, sick slow-growing larvae, or arrested larvae. At 22.5° and 25°, 100% of the animals arrested as larvae in size similar to that of L1 larvae.

ⁱ Strain also contained *unc-46*(e177).

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 (MORGENBESSER *et al.* 1994; MACLEOD *et al.* 1996). It is possible that Rb-like genes promote cell death in mammals but that this defect is obscured by cell-cycle abnormalities; cell-cycle abnor-

malities 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); *nIs106*, *n* = 8; *mcd-1*(n4005); *nIs106*, *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, 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-37*, and *lin-52* act together to regulate cell death via transcriptional regulation of specific targets that affect the cell-death process rather than act nonspecifically 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 (LANDSBERG *et al.* 2003; FROLOV and DYSON 2004).

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 (METZSTEIN *et al.* 1996; METZSTEIN and HORVITZ 1998; CONRADT and HORVITZ 1999; 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 adds to an increasing number of functional categorizations of synMuv genes. Subsets of synMuv genes control at least 14 diverse biological processes in *C. elegans* (FERGUSON and HORVITZ 1989; BOXEM and VAN DEN HEUVEL 2001, 2002;

CHEN and HAN 2001a; PAGE *et al.* 2001; DUFOURCQ *et al.* 2002; FAY *et al.* 2002, 2003, 2004; UNHAVAITHAYA *et al.* 2002; BENDER *et al.* 2004; CEOL and HORVITZ 2004; CUI *et al.* 2004; GARBE *et al.* 2004; REDDY and VILLENEUVE 2004; 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 coregulators 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 (XUE *et al.* 1998; KORENJAK *et al.* 2004; LEWIS *et al.* 2004). 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*, *eft-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 article. 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 article 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.

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