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C. elegans orthologs of components of the RB tumor suppressor complex have distinct pro-apoptotic functions

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To obtain insight into the role of the retinoblastoma susceptibility gene (*Rb*; also known as *Rb1*) in apoptosis, we analyzed *Caenorhabditis elegans* mutants lacking a functional *lin-35* RB gene. We found that the loss of *lin-35* function results in a decrease in constitutive germ cell apoptosis. We present evidence that *lin-35* promotes germ cell apoptosis by repressing the expression of *ced-9*, an anti-apoptotic *C. elegans* gene that is orthologous to the human proto-oncogene *BCL2*. Furthermore, we show that the genes *dpl-1* DP, *efl-1* E2F and *efl-2* E2F also promote constitutive germ cell apoptosis. However, in contrast to *lin-35*, *dpl-1* (and probably also *efl-1* and *efl-2*) promotes germ cell apoptosis by inducing the expression of the pro-apoptotic genes *ced-4* and *ced-3*, which encode an APAF1-like adaptor protein and a pro-caspase, respectively. Based on these results, we propose that *C. elegans* orthologs of components of the RB tumor suppressor complex have distinct pro-apoptotic functions in the germ line and that the transcriptional regulation of components of the central apoptosis machinery is a critical determinant of constitutive germ cell apoptosis in *C. elegans*. Finally, we demonstrate that *lin-35*, *dpl-1* and *efl-2*, but not *efl-1*, function either downstream of or in parallel to *cep-1* p53 (also known as TP53) and *egl-1* BH3-only to cause DNA damage-induced germ cell apoptosis. Our results have implications for the general mechanisms through which RB-like proteins control gene expression, the role of RB-, DP- and E2F-like proteins in apoptosis, and the regulation of apoptosis.

KEY WORDS: *C. elegans*, Apoptosis, Germ line, lin-35 RB

INTRODUCTION

The retinoblastoma susceptibility gene (*Rb*; also known as *Rb1*) is functionally inactivated in most human solid tumors (reviewed by Lipinski et al., 2001; Liu et al., 2004; Stevaux and Dyson, 2002). The tumor-suppressing activity of *Rb* has been attributed to its ability to block cell proliferation. Specifically, the RB protein can bind to heterodimers composed of a member of the DP protein family and an 'activating' member of the E2F family of sequence-specific DNA-binding proteins (E2F1, E2F2, E2F3) (E2F-DP); this blocks the ability of E2F-DP to activate the transcription of genes, the products of which are required for G1–S phase transition (reviewed by Attwooll et al., 2004; Dimova and Dyson, 2005). Independently of its role in cell proliferation, *Rb* also plays a role in other processes such as differentiation and apoptosis (reviewed by Chau and Wang, 2003; Lipinski and Jacks, 1999; Liu et al., 2004; Stevaux and Dyson, 2002).

The inactivation of the apoptotic pathway contributes to tumorigenesis in many types of cancers (reviewed by Cory and Adams, 2002; Danial and Korsmeyer, 2004). In mice lacking a functional *Rb* gene ($Rb^{-/-}$), ectopic apoptosis is observed in the peripheral and central nervous system as well as the ocular lens, indicating that *Rb* can block apoptosis in these tissues. The fact that *Rb* has anti-apoptotic activity seems counterintuitive for a tumor suppressor, as it suggests that the loss of *Rb* function not only results in increased proliferation, but also increased apoptosis. Indeed, in many *Rb*-deficient tumors, the apoptotic pathway is found to be inactivated by mutation as well, thus suggesting that the loss of *Rb* function induces tumorigenesis through a mechanism that is dependent on the inactivation of the apoptotic pathway (Chau and Wang, 2003). However, in certain tissues, the loss of *Rb* function appears to be sufficient for tumorigenesis, which indicates that *Rb* might not have anti-apoptotic activity in all tissues and that its role in apoptosis might therefore be tissue-specific (reviewed by Sherr and McCormick, 2002).

The genetic analysis of the role of mammalian *Rb* in apoptosis has been hampered by the fact that apart from *Rb*, two *Rb*-like genes [*p107* (*Rbl1*) and *p130* (*Rbl2*)] exist. Furthermore, mammals have at least three *Dp*-like genes and eight *E2f*-like genes (Attwooll et al., 2004; Christensen et al., 2005; Dimova and Dyson, 2005; Logan et al., 2005; Maiti et al., 2005; Milton et al., 2006). By contrast, the *C. elegans* genome encodes one ortholog of RB, LIN-35, one ortholog of DP, DPL-1, and two E2F-like proteins, EFL-1 and EFL-2 (Ceol and Horvitz, 2001; Lu and Horvitz, 1998). *lin-35*, *dpl-1* and *efl-1* are members of the class B synthetic multivulval (synMuv) genes, which were originally identified because of their role in vulval development: synMuv B genes act redundantly with class A and class C synMuv genes to antagonize *let-60* RAS, *mpk-1* MAPK signaling in the vulval precursor cells (VPCs), thereby blocking vulval differentiation (Ceol and Horvitz, 2004; Ferguson and Horvitz, 1989). The fact that the loss of *lin-35*, *dpl-1* or *efl-1* function causes similar rather than opposite phenotypes suggests that, at least during vulval differentiation, *lin-35*, *dpl-1* and *efl-1* function together to repress gene transcription.

Animals lacking *lin-35* function are viable and overall have a wild-type appearance, although they are less fertile and show enhanced sensitivity to the inactivation of gene function by RNA interference (Boxem and van den Heuvel, 2001; Fay et al., 2002; Lu and Horvitz, 1998; Thomas and Horvitz, 1999; Wang et al., 2005). Furthermore, *lin-35* functions redundantly in a number of processes other than vulval differentiation, such as cell cycle progression, larval development, somatic gonad development, pharynx differentiation and transgene expression (Bender et al., 2004; Boxem and van den Heuvel, 2001; Cardoso et al., 2005; Chesney et al., 2006; Cui et al., 2004; Fay et al., 2002; Fay et al., 2003; Fay et al.,

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DEVELOPMENT

2004; Hsieh et al., 1999; Wang et al., 2005). Therefore, like mammalian *Rb*, *C. elegans lin-35* plays a role in cell proliferation and differentiation. Whether *lin-35* also plays a role in apoptosis has not previously been investigated.

During *C. elegans* development, 131 of the 1090 somatic cells that are formed undergo apoptosis, a process referred to as 'developmental apoptosis' (Sulston and Horvitz, 1977; Sulston et al., 1983). Developmental apoptosis is determined by the essentially invariant somatic cell lineage of *C. elegans* and is executed through a conserved apoptotic pathway (reviewed by Horvitz, 2003; Lettre and Hengartner, 2006). Specifically, developmental apoptosis is dependent on the pro-apoptotic genes *ced-4* and *ced-3*, which encode an APAF1-like adaptor protein and a pro-caspase, respectively. In cells destined to live, the ability of the CED-4 protein to induce pro-CED-3 activation and, hence, the execution of apoptosis, is blocked by the anti-apoptotic protein CED-9, the *C. elegans* ortholog of the human proto-oncoprotein BCL2. In cells destined to die, the pro-apoptotic gene *egl-1*, which encodes a BH3-only (BH3, BCL2 homology domain 3) protein, is upregulated at the transcriptional level. EGL-1 protein can interact with and block CED-9, thereby allowing CED-4 activation.

Apoptosis also occurs in the germ line of adult *C. elegans* hermaphrodites (Sulston, 1988), where more than 50% of all germ cells in the pachytene stage of prophase of meiosis I undergo apoptosis, a process referred to from hereon as 'constitutive germ cell apoptosis' (Gumienny et al., 1999). Like developmental apoptosis, constitutive germ cell apoptosis is dependent on the genes *ced-4* and *ced-3* and is blocked by *ced-9*. However, unlike developmental apoptosis, constitutive germ cell apoptosis is not dependent on *egl-1* (Gumienny et al., 1999). Finally, the exposure of adult *C. elegans* hermaphrodites to genotoxic agents, such as ionizing radiation (IR), causes large numbers of germ cells in the pachytene stage to undergo apoptosis, a process referred to as 'DNA damage-induced germ cell apoptosis' (Gartner et al., 2000). DNA damage-induced germ cell apoptosis is at least partially dependent on *egl-1*, which is transcriptionally upregulated in response to DNA damage in a manner that is dependent on the *C. elegans* ortholog of the mammalian *p53* gene (also known as *Tp53*), *cep-1* (Derry et al., 2001; Hofmann et al., 2002; Schumacher et al., 2001).

In this article, we report that *lin-35* RB as well as *dpl-1* DP, *efl-1* E2F and *efl-2* E2F, are required for germ cell apoptosis in *C. elegans*. Surprisingly, however, the pro-apoptotic role of *lin-35* appears to be distinct from the pro-apoptotic roles of *dpl-1*, *efl-1* and *efl-2*.

MATERIALS AND METHODS

General methods and strains

C. elegans strains were cultured at 20°C as described (Brenner, 1974). The wild-type strain used was *C. elegans* var. Bristol (N2). Alleles used are listed below and are described (Riddle, 1997), except where noted otherwise. LGI: *lin-35*(*n745*, *n2996*) (Lu and Horvitz, 1998), *cep-1*(*lg12501*) (Schumacher et al., 2001), *hus-1*(*op241*) (Hofmann et al., 2002), *ppw-1*(*pk1425*) (Tijsterman et al., 2002), *rrf-1*(*pk1417*) (Simmer et al., 2003). LGII: *dpl-1*(*n3643, n3316*) (Ceol and Horvitz, 2001), *unc-4*(*e120*), *unc-52*(*e444*), *dpy-10*(*e128*), *rrf-3*(*pk1426*) (Simmer et al., 2002), *efl-2*(*tm2359*) (National BioResource Project, Tokyo, Japan) (*tm2359* is a 332 bp deletion with a 47 bp insertion that removes sequences 5′ to the predicted transcriptional start site of the *efl-2* gene and most of exon 1. Exon 1 encodes the putative dimerization domain and DNA-binding domain of the EFL-2 protein. For this reason, *tm2359* is predicted to be a strong loss-of-function or null allele of the *efl-2* gene). LGIII: *ced-9*(*n1653*ts, *n2812*) (Hengartner and Horvitz, 1994), *ced-6*(*n2095*), *dpy-19*(*e1259*), *glp-1*(*q339*) (Edgley et al., 1995), *ceh-13*(*sw1*) (Brunschwig et al., 1999), *dpy-17*(*e164*), *nDf20*, *nDp2[unc-86*(*e1416*)*]* (*III;f*) (Finney et al., 1988). LGIV: *dpy-20*(*e1282*). LGV: *bcIs39* (P_{lim-7}*ced-1::gfp*) (Schumacher et al., 2005; Zhou et al., 2001), *dpy-21*(*e428*), *unc-76*(*e911*), *efl-1*(*n3318*) (Ceol and Horvitz, 2001), *rde-1*(*ne300*) (Tabara et al., 1999).

Quantification of germ cell apoptosis

For constitutive germ cell apoptosis, hermaphrodites were synchronized at the fourth larval (L4) stage and analyzed 12, 24, 36 and 48 hours post the L4 stage. Animals were anesthetized using 80 mM sodium azide and mounted on slides using 2% agarose pads. Apoptotic germ cells were detected using DIC and epifluorescence. For *ced-9*(*n1653*ts)-induced germ cell apoptosis, animals were synchronized at the L4 stage, cultivated at 20°C for 12 hours and shifted to 25°C for 24 hours before being analyzed. For *ced-9*(*n2812*)/+-induced germ cell apoptosis, animals were synchronized at the L4 stage and analyzed by DIC 40 hours post the L4 stage. For DNA damage-induced germ cell apoptosis, hermaphrodites were synchronized at the L4 stage and exposed to 120 Gy using a ^{137}Cs source (J. L. Shepherd, San Fernando, CA) with a dose rate of 10 Gy/minute. 24 hours post-irradiation, apoptotic germ cells were scored using DIC. *efl-1*(*n3318*) and *efl-2*(*tm2359*) animals were irradiated 12 hours post the L4 stage and analyzed 24 hours post-irradiation.

Transgenic animals

The P_{lim-7}*lin-35* plasmid was constructed by fusing the *lin-35* cDNA to the *lim-7* promoter (2.23 kb upstream of transcriptional start site) (Hall et al., 1999). This fusion was blunt-cloned into the *Eco*RI site of vector pPD95.67 (gift from Dr A. Fire, Stanford University School of Medicine, CA). The resulting plasmid was injected at a concentration of 1 ng/μl using the plasmid pRF4 [*rol-6*(dm)] as co-injection marker at a concentration of 100 ng/μl (Kramer et al., 1990). The cosmid *C32F10* [*lin-35*(+)] was injected at a concentration of 10 ng/μl using the plasmid pPD93.97 (P_{myo-3}*gfp*) (gift from Dr A. Fire) as co-injection marker at a concentration of 50 ng/μl.

RNAi experiments

RNAi experiments were performed by feeding as described (Timmons et al., 2001). Briefly, animals were exposed to RNAi plates containing 6 mM IPTG. Animals of the F1 generation were synchronized at the L4 larval stage, transferred to fresh RNAi plates and analyzed after the indicated time. An unrelated gene (*F53B3.2*) was used as control RNAi.

Semi-quantitative and quantitative expression analyses

For semi-quantitative RT-PCR, hermaphrodites were synchronized at the L4 stage and irradiated with 100 Gy 24 hours post the L4 stage. Total RNA was isolated from 200 animals 3 hours post-irradiation using Trizol (Invitrogen) and purified by chloroform extraction and isopropanol precipitation. 1 μg of total RNA was treated with DNase and reverse transcribed into cDNA using the SuperScript III First Strand Kit (Invitrogen) and oligo-dT primers. Equal amounts of cDNA were used as template for gene-specific PCR with appropriate primers. A plasmid containing the *egl-1* cDNA was used as positive control. Water was used as negative control. *tbg-1* (encoding γ-tubulin) RT-PCR was performed as a control. For quantitative real-time RT-PCR, total RNA was isolated from about 40 gonads dissected from hermaphrodites 27 hours post the L4 stage as described for semi-quantitative RT-PCR. 400 ng of total RNA was reverse transcribed into cDNA. Gene-specific PCR reactions using equal amounts of cDNA, appropriate primers and TaqMan probes were performed with an Opticon DNA Engine (MJ Research). *tbg-1* was used as external control. Test gene C_T values were normalized to *tbg-1* C_T values and relative expression levels were derived with the comparative C_T method (Livak and Schmittgen, 2001).

Western analysis

About 100 (wild-type) or 120 [*lin-35*(*n745*) and *dpl-1*(*n3643*)] gonads were dissected in PBS from synchronized hermaphrodites 27 hours post the L4 stage, transferred into 2× sample buffer and boiled for 5 minutes. Proteins were separated on 10% SDS-PAGE gels. Western analysis was performed using CED-4- and CED-9-specific antibodies as described (Chen et al., 2000) and HRP-coupled secondary antibodies. Loading controls were detected with antibodies specific for β-actin (AC15, Sigma) and β-tubulin (N357, Amersham). NIH image software was used for quantification.

RESULTS

The loss of *lin-35* RB function results in a decrease in constitutive germ cell apoptosis

To determine whether the *C. elegans* LIN-35 RB protein plays a role in developmental apoptosis, we analyzed animals homozygous for the *lin-35* loss-of-function (lf) mutation *n745*. *n745* is a nonsense mutation predicted to truncate the 961 amino acid LIN-35 protein after amino acid 150. For this reason, it has been proposed that *n745* might completely eliminate the function of the *lin-35* gene (Lu and Horvitz, 1998). We found no defect in developmental apoptosis in *lin-35*(*n745*) animals, indicating that *lin-35* RB is not required for developmental apoptosis at least in a wild-type background (see Table S1 in the supplementary material; data not shown). However, it has recently been demonstrated that in a sensitized genetic background, *lin-35* promotes developmental apoptosis (Reddien et al., 2007).

Next we analyzed constitutive germ cell apoptosis in *lin-35*(*n745*) animals. Germ cells undergoing apoptosis become refractile and are engulfed by the sheath cells, which are part of the somatic gonad and which express the scavenger receptor-like protein CED-1 on their cell surface (Zhou et al., 2001). Therefore, constitutive germ cell apoptosis was quantified using a combination of DIC and CED-1::GFP ($P_{lim\text{-}7}$*ced-1::gfp*, *bcIs39*) fluorescence microscopy. As a result of the efficient engulfment of apoptotic germ cells by the sheath cells, the number of apoptotic germ cells per gonad arm that can be detected at any given time is very low [4.0 apoptotic germ cells on average in hermaphrodites 48 hours post the fourth larval (L4) stage] (Gumienny et al., 1999). For this reason, we analyzed constitutive germ cell apoptosis in the background of *ced-6*(*n2095*). *n2095* is a loss-of-function mutation in *ced-6*, a gene which is required for the efficient engulfment of apoptotic germ cells (Ellis et al., 1991). In the *ced-6* mutant background, apoptotic germ cells accumulate, resulting in a greatly increased number of apoptotic germ cells that can be detected at any given time (55.3±5.4 apoptotic germ cells on average in hermaphrodites 48 hours post the L4 stage) (Fig. 1A, +/+). We found that *lin-35*(*n745*) reduced the number of apoptotic germ cells in *ced-6*(*n2095*) animals by more than 50% [Fig. 1A, *lin-35*(*n745*)]. Similarly, the *lin-35* loss-of-function mutation *n2996*, a nonsense mutation predicted to truncate the LIN-35 protein after amino acid 304, reduced the number of apoptotic germ cells in *ced-6*(*n2095*) animals by more than 50%. For comparison, in *ced-6*(*n2095*) animals homozygous for a loss-of-function mutation in the *ced-3* gene, almost no apoptotic germ cells were detected [Fig. 1A, *ced-3*(*n717*)].

When compared with wild-type hermaphrodites, *lin-35*(*n754*) hermaphrodites are less fertile (Boxem and van den Heuvel, 2001; Fay et al., 2002; Lu and Horvitz, 1998; Thomas and Horvitz, 1999). Therefore, the decrease in the number of apoptotic germ cells in *lin-35*(*n745*) animals might be the result of a decrease in constitutive germ cell apoptosis or, alternatively, a decrease in germ cell proliferation. To distinguish between these two possibilities, we assessed constitutive germ cell apoptosis in animals homozygous for *e1282*, a loss-of-function mutation in the *dpy-20* gene, which encodes a novel, BED zinc-finger protein required for normal body morphology (Clark et al., 1995). Germ cell proliferation in *dpy-20*(*e1282*) animals is compromised to a degree similar to that in *lin-35*(*n745*) animals (see Table S2 in the supplementary material); however, the *dpy-20*(*e1282*) mutation had no effect on the number of apoptotic germ cells [Fig. 1A, *dpy-20*(*e1282*)]. This finding indicates that the decrease in the number of apoptotic germ cells detected in *lin-35*(*n745*); *ced-6*(*n2095*) animals is a result of a decrease in constitutive germ cell apoptosis rather than a decrease in germ cell proliferation. This conclusion is supported by the following two observations: first, a partial loss-of-function mutation in the gene *dpl-1*, *n3643*, does not compromise germ cell proliferation but, like *lin-35*(*n745*), decreases the number of apoptotic germ cells in *ced-6*(*n2095*) animals by more than 50% (see below); second, the loss of *lin-35* function completely blocks DNA damage-induced germ

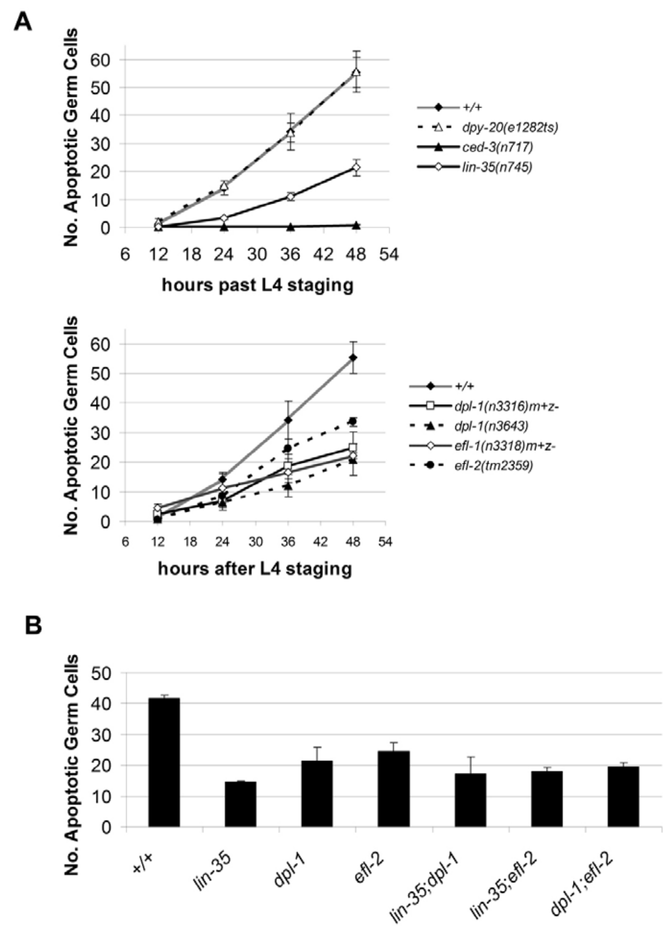

Fig. 1. The loss of *lin-35*, *dpl-1*, *efl-1* or *efl-2* function results in a reduced level of constitutive germ cell apoptosis. (**A**) Time-course analysis of constitutive germ cell apoptosis, which was analyzed in *C. elegans* hermaphrodites of the indicated genotypes. Average numbers of apoptotic germ cells per gonad arm of four independent experiments are shown. Error bars represent standard deviations. For each experiment, a minimum of ten animals per genotype and time point was scored double blind. All strains analyzed were homozygous for the mutation *ced-6*(*n2095*). Except for the strain *efl-1*(*n3318*), all strains were also homozygous for the $P_{lim\text{-}7}$*ced-1::gfp* integration *bcIs39*. m^+z^- indicates that animals analyzed were homozygous mutant progeny of heterozygous animals. The complete genotypes of *dpl-1*(*n3316*) and *efl-1*(*n3318*) animals were *dpl-1*(*n3316*) *unc-4*(*e120*); *ced-6*(*n2095*); *bcIs39* and *ced-6*(*n2095*); *efl-1*(*n3318*), respectively. (**B**) Analysis of constitutive germ cell apoptosis 40 hours post the L4 stage. Average numbers of apoptotic germ cells per gonad arm of two independent experiments are shown. Error bars represent standard deviations. For each experiment, a minimum of ten animals per genotype was scored blind. All strains were homozygous for *ced-6*(*n2095*) and *bcIs39*. Mutations of *lin-35*, *dpl-1* and *efl-2* used were: *lin-35*(*n745*), *dpl-1*(*n3643*) and *efl-2*(*tm2359*).

DEVELOPMENT

Table 1. *lin-35* is required in the germ line and the somatic gonad to promote constitutive germ cell apoptosis

A

Genotype	No. apoptotic germ cells (*n*)	% animals with high level *ced-1::gfp* expression in all sheath cells (*n*)
+/+	41.3±6.7 (13)	100 (37)
lin-35(*n745*)	17.5±4.4 (13)	79 (33)
lin-35(*n745*); *lin-35*(+) line 1	36.5±4.4 (12)	ND
lin-35(*n745*); *lin-35*(+) line 2	17.7±2.0 (6)	ND
lin-35(*n745*); P_{lim-7}*lin-35* line 1	21.7±4.2 (11)	72 (11)
lin-35(*n745*); P_{lim-7}*lin-35* line 2	18.9±5.3 (13)	100 (13)*
lin-35(*n745*); P_{lim-7}*lin-35* line 3	20.7±3.7 (14)	93 (14)
lin-35(*n745*); P_{lim-7}*lin-35* line 4	19.8±3.9 (13)	85 (13)

B

Genotype	No. apoptotic germ cells (*n*)	
	+	*rrf-1*(*pk1417*)
Control(*RNAi*)	34.5±2.3 (10)	27.9±2.8 (13)
lin-35(*RNAi*)	15.3±2.5 (12)	10.5±2.3 (12)

C

Genotype	No. apoptotic germ cells (*n*)	
	+	*ppw-1*(*pk1425*)
Control(*RNAi*)	32.5±2.2 (12)	34.1±2.5 (12)
lin-35(*RNAi*)	16.2±3.4 (12)	16.7±4.3 (12)

(A) *lin-35* expression in the somatic gonad is insufficient to rescue the germ cell apoptosis phenotype of *lin-35*(*n745*) animals. Transgenic animals were generated and the presence of apoptotic germ cells determined 40 hours after the L4 stage. All strains were homozygous for the mutation *ced-6*(*n2095*) and the stable P_{lim-7}*ced-1::gfp* integration *bcIs39*. Average numbers of apoptotic germ cells per gonad arm±standard deviation (left column) and percentage of animals with high level of *ced-1::gfp* expression in all sheath cells (right column) are shown. Not all sheath cell pairs express high levels of *ced-1::gfp* in *lin-35*(*n745*) mutants. We used this defect as a measure of the rescuing ability of the P_{lim-7}*lin-35* transgene. (B,C) Reducing *lin-35* activity in either the somatic gonad (B) or the germ line (C) reduces constitutive germ cell apoptosis. The presence of apoptotic germ cells was determined 40 hours after the L4 stage. All strains were homozygous for the mutation *ced-1*(*e1735*) (B) or the mutation *ced-6*(*n2095*) and the stable P_{lim-7}*ced-1::gfp* integration *bcIs39* (C). Average numbers of apoptotic germ cells per gonad arm±standard deviation are shown. *F53B3.2*(*RNAi*) was used as control(*RNAi*).
ND, not determined.
*The frequency of *ced-1::gfp*(+) sheath cells is significantly greater in *lin-35*(*n745*); P_{lim-7}*lin-35* line 2 than in *lin-35*(*n745*) (P<0.05; z-test).

cell apoptosis (see below). Based on these observations, we conclude that the *lin-35*/*Rb* gene promotes constitutive germ cell apoptosis.

To determine where *lin-35* function is required to promote constitutive germ cell apoptosis, we generated transgenic *lin-35*(*n745*) animals carrying extrachromosomal arrays composed of cosmid *C32F10*, which contains the wild-type *lin-35* locus [*lin-35*(+)], or a transgene expressing the wild-type *lin-35* cDNA under the control of the *lim-7* promoter (P_{lim-7}*lin-35*). We found that *lin-35*(+), which has been shown to rescue *lin-35* function in the somatic gonad as well as the germ line (Fay et al., 2002), but not P_{lim-7}*lin-35*, which presumably rescues *lin-35* function specifically in the somatic gonad (Hall et al., 1999), was able to rescue the germ cell apoptosis phenotype of *lin-35*(*n745*) animals (Table 1A). Furthermore, reducing *lin-35* function by RNAi in a *rrf-1* mutant background, which is defective for RNAi in somatic tissues, or a *ppw-1* mutant background, which is defective for RNAi in the germ line, reduced constitutive germ cell apoptosis by about 50% (Table 1B and C). These results demonstrate that *lin-35* expression in either somatic gonad or germ line is insufficient to promote constitutive germ cell apoptosis. Therefore, we propose that *lin-35* function is required in both the somatic gonad and the germ line to promote constitutive germ cell apoptosis.

Table 2. Suppression of germ cell apoptosis in *ced-9*(*n2812*); *ced-3*(*n2427*) animals

Genotype	No. apoptotic germ cells	*n*
+/+	3.0±0.4	44
ced-9(*n2812*); *ced-3*(*n2427*)	13.9±3.2	61
lin-35(*n745*); *ced-9*(*n2812*); *ced-3*(*n2427*)	13.4±2.9	55
dpl-1(*n3643*); *ced-9*(*n2812*); *ced-3*(*n2427*)	6.1±0.2	48
efl-2(*tm2359*); *ced-9*(*n2812*); *ced-3*(*n2427*)	5.5±1.2	46

The presence of apoptotic germ cells was determined by DIC 40 hours post the L4 stage. Average numbers of apoptotic germ cells per gonad arm±standard deviations are shown.

lin-35 RB promotes constitutive germ cell apoptosis by blocking the function of *ced-9* BCL2

In an otherwise wild-type background, the temperature-sensitive, partial *ced-9* loss-of-function mutation *n1653* causes massive germ cell apoptosis at the non-permissive temperature (25°C), often resulting in gonad arms with severely damaged germ lines (Gumienny et al., 1999; Hengartner et al., 1992). We found that *lin-35*(*n745*) reduced the number of gonad arms with severely damaged germ lines in *ced-9*(*n1653*ts) animals from 83% [n=30; *ced-9*(*n1653*ts)] to 20% [n=40; *lin-35*(*n745*); *ced-9*(*n1653*ts)] (Fig. 2A). In addition, in gonad arms with undamaged germ lines, *lin-35*(*n745*) reduced the number of apoptotic germ cells detectable from 26.0±7.2 [*ced-9*(*n1653*ts); n=14] to 4.1±2.8 [*lin-35*(*n745*); *ced-9*(*n1653*ts); n=47]. Furthermore, we found that *lin-35*(*n745*) completely suppresses germ cell apoptosis induced by the loss of one copy of the *ced-9* gene (see below). The potential *ced-9*-null allele, *n2812*, is a nonsense mutation predicted to truncate the 280 amino acid CED-9 protein after amino acid 45 (Hengartner and Horvitz, 1994). *ced-9*(*n2812*) causes massive germ cell apoptosis resulting in 100% (n>40) gonad arms with severely damaged germ lines at all growth temperatures (Gumienny et al., 1999; Hengartner et al., 1992). *lin-35*(*n745*) failed to suppress the massive germ cell apoptosis in *ced-9*(*n2812*) hermaphrodites and did not affect the percentage of gonad arms with severely damaged germ lines (100%; n>40).

The finding that *lin-35*(*n745*) suppresses the partial but not complete loss of *ced-9* function is consistent with the model that *lin-35* acts upstream of *ced-9* to block *ced-9* function. Alternatively, *lin-35* might act downstream of *ced-9* and the failure of *lin-35*(*n745*) to suppress the complete loss of *ced-9* function might be due to irreversible germ line damage. To distinguish between these two possibilities, we analyzed germ cell apoptosis in *ced-9*(*n2812*) animals homozygous for *n2427*, a weak loss-of-function mutation of the *ced-3* gene, which acts downstream of *ced-9*. *ced-3*(*n2427*) partially suppressed the germ line phenotype caused by *ced-9*(*n2812*): in *ced-3*(*n2427*); *ced-9*(*n2812*) animals, 0% of the gonad arms were severely damaged (n>40), but when compared with wild-type animals (+/+), an elevated level of germ cell apoptosis could be detected [Table 2, *ced-3*(*n2427*); *ced-9*(*n2812*)]. We found that *lin-35*(*n745*) failed to suppress the elevated level of germ cell apoptosis in *ced-3*(*n2427*); *ced-9*(*n2812*) animals [Table 2, *lin-35*(*n745*); *ced-3*(*n2427*); *ced-9*(*n2812*)]. Therefore, the ability of *lin-35*(*n745*) to reduce constitutive germ cell apoptosis is dependent on the presence of a *ced-9* gene that is at least partially functional. Based on these results, we conclude that the *lin-35* RB gene acts upstream of *ced-9* BCL2 to inhibit its function.

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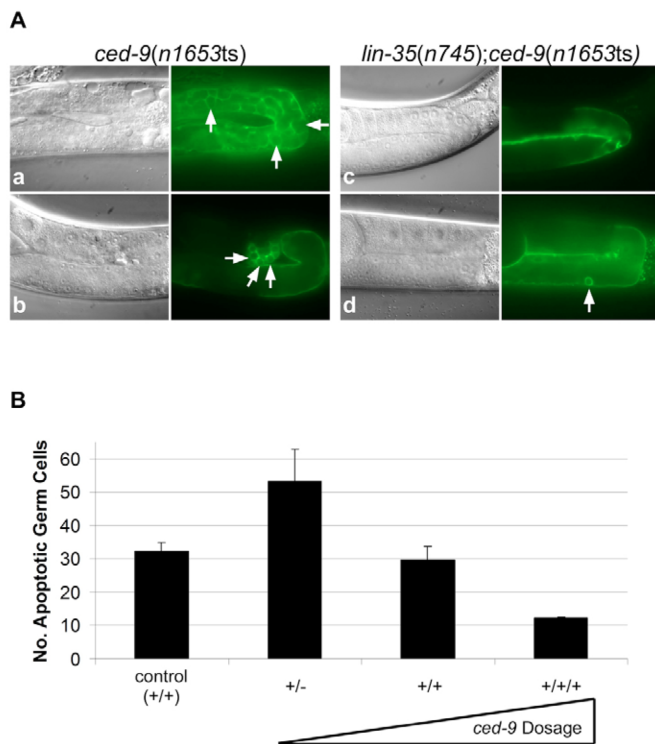

Fig. 2. Role of *ced-9* in *lin-35*-dependent germ cell apoptosis. (**A**) The loss of *lin-35* function suppresses germ cell apoptosis caused by the partial loss of *ced-9* function. DIC and fluorescence images of gonad arms of *ced-9*(*n1653*ts) and *lin-35*(*n745*)*; ced-9*(*n1653*ts) *C. elegans* hermaphrodites. Both strains were homozygous for the P_{lim-7}*ced-1::gfp* integration *bcIs39.* White arrows point to apoptotic germ cells. (**Aa**) Gonad of a *ced-9*(*n1653*ts) hermaphrodite that is severely damaged. (**Ab**) Gonad of a *ced-9*(*n1653*ts) hermaphrodite that is not severely damaged but contains a large number of apoptotic germ cells. (**Ac**,**Ad**) Gonads of *lin-35*(*n745*)*; ced-9*(*n1653*ts) hermaphrodites that are not severely damaged and contain low numbers of apoptotic germ cells. (**B**) The dosage of *ced-9* determines the level of constitutive germ cell apoptosis. Constitutive germ cell apoptosis was analyzed 40 hours post the L4 stage. Average numbers of apoptotic germ cells per gonad arm are shown. Error bars represent standard deviations of three independent experiments. For each genotype, a minimum of 20 animals was scored blind (*n*=20-51). The complete genotype of the animals was: *ced-1*(*e1735*) [control (+/+)], *ced-1*(*e1735*)*; nDf20; nDp2[unc-86*(*e1416*)*]* (+/–), *ced-1*(*e1735*)*; nDf20/dpy-17*(*e164*)*; nDp2[unc-86*(*e1416*)*]* (+/+) and *ced-1*(*e1735*)*; dpy-17*(*e164*)*; nDp2[unc-86*(*e1416*)*]* (+/+/+).

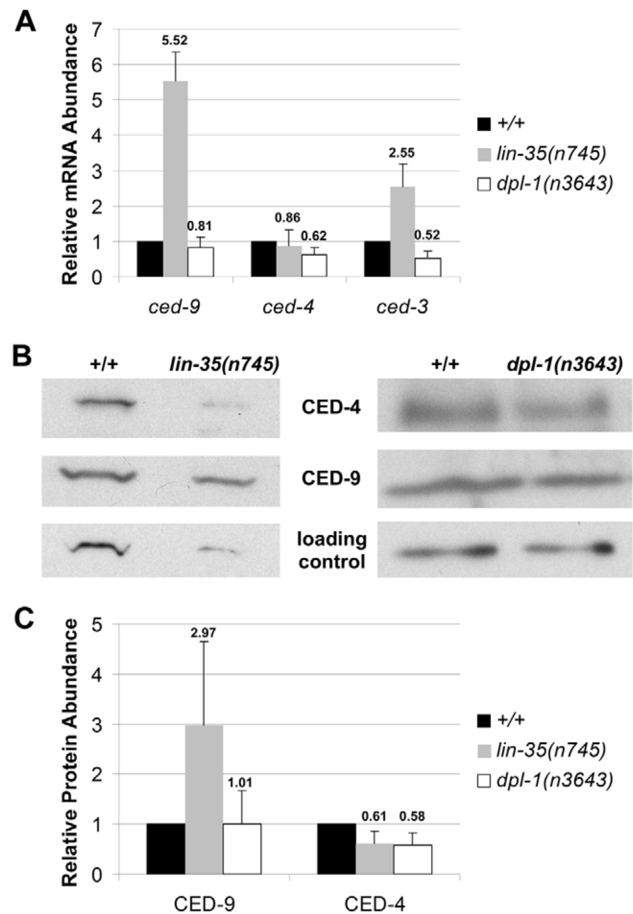

Fig. 3. Analysis of *ced-9*, *ced-4*, and *ced-3* expression in *lin-35* and *dpl-1* mutant gonads. (**A**) Quantitative real-time RT-PCR experiments using cDNAs isolated from gonads dissected from wild-type (+/+), *lin-35*(*n745*) or *dpl-1*(*n3643*) *C. elegans*. Average relative abundances of *ced-9*, *ced-4* and *ced-3* mRNAs of three independent experiments, each performed in triplicate, are shown. Error bars represent standard deviations. (**B**) Western analyses using proteins extracted from gonads dissected from wild-type (+/+), *lin-35*(*n745*) and *dpl-1*(*n3643*) hermaphrodites. The protein β-actin [*lin-35*(*n745*)] or β-tubulin [*dpl-1*(*n3643*)] was used as loading control. One representative experiment is shown for each. (**C**) Quantification of the abundance of CED-9 and CED-4 proteins in the gonad of wild-type (+/+), *lin-35*(*n745*) and *dpl-1*(*n3643*) animals. Quantification was performed using NIH image software. Average relative abundances of CED-9 and CED-4 protein of three [*lin-35*(*n745*) and two *dpl-1*(*n3643*)] independent experiments are shown. Error bars represent standard deviation.

The loss of *lin-35* RB function results in increased levels of *ced-9* mRNA and CED-9 protein in the germ line

C. elegans LIN-35 RB most probably functions by regulating gene transcription. Therefore, using quantitative real-time reverse transcriptase (RT)-PCR, we determined whether the loss of *lin-35* function has an effect on the level of *ced-9* mRNA in the germ line. We found that *ced-9* mRNA was on average 5.5-fold more abundant in the germ line of *lin-35*(*n745*) animals than in the germ line of wild-type (+/+) animals (Fig. 3A). In addition, *ced-3* mRNA was on average 2.5-fold more abundant in the germ line of *lin-35*(*n745*) animals than in the germ line of wild-type animals. By contrast, the abundance of *ced-4* mRNA was not affected by *lin-35*(*n745*).

We also determined the level of CED-9 protein in the germ line of *lin-35*(*n745*) animals. We found that the level of CED-9 protein was on average 3.0-fold higher in the germ line of *lin-35*(*n745*) animals than in the germ line of wild-type (+/+) animals (Fig. 3B,C and see Fig. S1 in the supplementary material). In addition, we found that in the germ line of *lin-35*(*n745*) animals, the level of CED-4 protein was slightly reduced. [Owing to the lack of a CED-3-specific antibody, we were unable to determine the level of endogenous CED-3 protein in the germ line of *lin-35*(*n745*) and wild-type animals.] We conclude that the *lin-35* RB gene is required to directly or indirectly repress the transcription of *ced-9* BCL2 in the germ line. Furthermore, *lin-35* RB might also play a role in controlling the expression of *ced-4* APAF1 and *ced-3* caspase in the germ line.

Table 3. The gene dosage of *ced-9*, *ced-4* and *ced-3* is important for germ cell apoptosis

Genotype	No. apoptotic germ cells	*n*
+/+	3.0±0.4	44
+/*qC1*	3.1±0.7	43
ced-9(*n2812*)/*qC1*	10.7±1.6	79
lin-35(*n745*); *ced-9*(*n2812*)/*qC1*	2.6±0.6	49
+/+*	34.5±4.6	34
ced-3(*n717*)*	0.3±0.1	51
ced-4(*n1162*)*	0.3±0.1	47
ced-3(*n717*)/+*	31.9±0.8	35
ced-4(*n1162*)/+*	34.5±3.6	34
ced-4(*n1162*)/+; *ced-3*(*n717*)/+*	8.4±2.7	56

The presence of apoptotic germ cells was determined 40 hours post the L4 stage by DIC. Average numbers of apoptotic germ cells per gonad arm±standard deviations are shown.
*These strains also were homozygous for the mutation *ced-1*(*e1735*), which blocks the efficient engulfment of apoptotic germ cells.

The dosage of *ced-9* determines the level of germ cell apoptosis

Hermaphrodites trans-heterozygous for the potential *ced-9*-null mutation *n2812* and the balancer chromosome *qC1* [*ced-9*(*n2812*)/*qC1*] overall are wild type. However, we found that, when compared with wild-type animals (+/+) or animals heterozygous for *qC1* (+/*qC1*), *ced-9*(*n2812*)/*qC1* animals had an increased number of apoptotic germ cells (Table 3). This observation indicates that the *ced-9* gene is haploinsufficient for the repression of constitutive germ cell apoptosis. To further characterize the effect of *ced-9* dosage on germ cell apoptosis, we analyzed germ cell apoptosis in engulfment-defective animals with one (+), two (+/+) or three (+/+/+) copies of the *ced-9* gene. With increasing *ced-9* dosage, we detected decreasing numbers of apoptotic germ cells (Fig. 2B). Thus, changes in the dosage of *ced-9* BCL2 and, hence, most likely in the expression of *ced-9* BCL2, strongly affect the level of germ cell apoptosis. Furthermore, the increase in apoptotic germ cells observed in animals of the genotype *ced-9*(*n2812*)/*qC1* was completely suppressed by *lin-35*(*n745*) (*lin-35*(*n745*); *ced-9*(*n2812*)/*qC1* (Table 3). Therefore, we conclude that the decreased level of constitutive germ cell apoptosis detected in animals lacking *lin-35* RB function is a consequence of increased *ced-9* expression in the germ line.

dpl-1 DP, *efl-1* E2F and *efl-2* E2F promote constitutive germ cell apoptosis

C. elegans LIN-35 RB protein has been shown to physically interact with the DP ortholog DPL-1 and the E2F-like protein EFL-1 (Ceol and Horvitz, 2001). Therefore, we determined the effect of decreasing *dpl-1*, *efl-1* or *efl-2* function on constitutive germ cell apoptosis. [As observed for the loss of *lin-35* function, decreasing *dpl-1*, *efl-1* or *efl-2* function did not affect developmental apoptosis (see Table S1 in the supplementary material).] We found that a partial (*n3643*) or strong (*n3316*) loss-of-function mutation in the *dpl-1* gene reduced constitutive germ cell apoptosis in a *ced-6*(*n2095*) background by more than 50% [Fig. 1A, *dpl-1*(*n3643*), *dpl-1*(*n3316*)]. Furthermore, strong loss-of-function mutations in *efl-1*, *n3318*, or *efl-2*, *tm2359*, decreased constitutive germ cell apoptosis in a *ced-6*(*n2095*) background by about 40-50% [Fig. 1A, *efl-1*(*n3318*), *efl-2*(*tm2359*)]. To determine whether *efl-1* and *efl-2* have partially redundant functions in constitutive germ cell apoptosis, we attempted to construct a *C. elegans* strain lacking both *efl-1* and *efl-2* function. However, we were unable to obtain such a strain (our unpublished observations). For this reason, we consider it likely that *efl-1* and *efl-2* have redundant functions in an essential process. *dpl-1*(*n3316*) and *efl-1*(*n3318*) animals are sterile; however, *dpl-1*(*n3643*) and *efl-2*(*tm2359*) animals are fertile and their fertilities are comparable to the fertility of wild-type animals (Ceol and Horvitz, 2001) (see Table S2 in the supplementary material; data not shown). Therefore, we propose that, like *lin-35* RB, the genes *dpl-1* DP, *efl-2* E2F and most likely also *efl-1* E2F promote constitutive germ cell apoptosis.

Reducing the function of *dpl-1* in *lin-35*(*n745*); *ced-6*(*n2095*) animals (*lin-35; dpl-1*) or *efl-2*(*tm2359*); *ced-6*(*n2095*) animals (*dpl-1; efl-2*) or reducing the function of *lin-35* in *efl-2*(*tm2359*); *ced-6*(*n2095*) animals (*lin-35; efl-2*) did not result in a further decrease in the number of apoptotic germ cells (Fig. 1B). These observations suggest that the inactivation of *C. elegans* orthologs of components of the RB complex reduces constitutive germ cell apoptosis by about 50%.

The roles of *dpl-1* DP and *efl-2* E2F in constitutive germ cell apoptosis are distinct from the role of *lin-35* RB

In contrast to *lin-35*(*n745*), reducing the function of *dpl-1* or *efl-2* decreased the number of apoptotic germ cells detected in *ced-9*(*n2812*); *ced-3*(*n2427*) animals by 56% and 60%, respectively (Table 2). [We have been unable to construct a strain of the genotype *efl-1*(*n3318*); *ced-9*(*n2812*); *ced-3*(*n2427*), and consider it likely that animals of this genotype are not viable (our unpublished observations).] Furthermore, we found that the levels of *ced-9* mRNA and CED-9 protein in the germ line of *dpl-1*(*n3643*) animals were not greater than in the germ line of wild-type (+/+) animals (Fig. 3). These results demonstrate that *dpl-1* and *efl-2* do not promote constitutive germ cell apoptosis by inhibiting *ced-9* expression. Therefore, the pro-apoptotic roles of *dpl-1* DP and *efl-2* E2F (and possibly *efl-1* E2F) are distinct from that of *lin-35* RB.

By contrast, we found that *dpl-1*(*n3643*) reduced the levels of *ced-3* and *ced-4* mRNAs in the germ line by about 40% and 50% (Fig. 3A), respectively, and the level of CED-4 protein by 40% (Fig. 3B,C). To determine whether a reduction of *ced-3* or *ced-4* expression by about 50% has an effect on constitutive germ cell apoptosis, we analyzed engulfment-defective animals heterozygous for strong loss-of-function mutations in *ced-3* [*ced-3*(*n717*)/+], *ced-4* [*ced-4*(*n1162*)/+] or *ced-3* and *ced-4* [*ced-4*(*n1162*)/+; *ced-3*(*n717*)/+]. We found that the lack of one functional copy of either *ced-3* or *ced-4* was not sufficient to reduce constitutive germ cell apoptosis, but the lack of one functional copy of both *ced-3* and *ced-4* reduced constitutive germ cell apoptosis by 75% (Table 2). Therefore, the simultaneous reduction by 50% of the dosage of *ced-3* and *ced-4* and, hence, most likely of the expression of *ced-3* and *ced-4*, strongly reduces constitutive germ cell apoptosis. Based on these results, we conclude that the reduction in constitutive germ cell apoptosis observed in animals lacking *dpl-1* function is a result of decreased levels of *ced-3* and *ced-4* mRNA. Therefore, *dpl-1* DP, and most likely also *efl-1* E2F and *efl-2* E2F, promotes constitutive germ cell apoptosis by inducing the expression of the genes *ced-4* APAF1 and *ced-3* caspase in the germ line.

lin-35 RB, *dpl-1* DP and *efl-2* E2F are required for DNA damage-induced germ cell apoptosis

Next, we determined whether *lin-35*, *dpl-1*, *efl-1* and *efl-2* also promote DNA damage-induced germ cell apoptosis (Gartner et al., 2000). Since the exposure to genotoxic agents induces massive germ cell apoptosis, DNA damage-induced germ cell apoptosis was analyzed in a wild-type background rather than the *ced-6*(*n2095*) background. The gene *cep-1* is required for DNA

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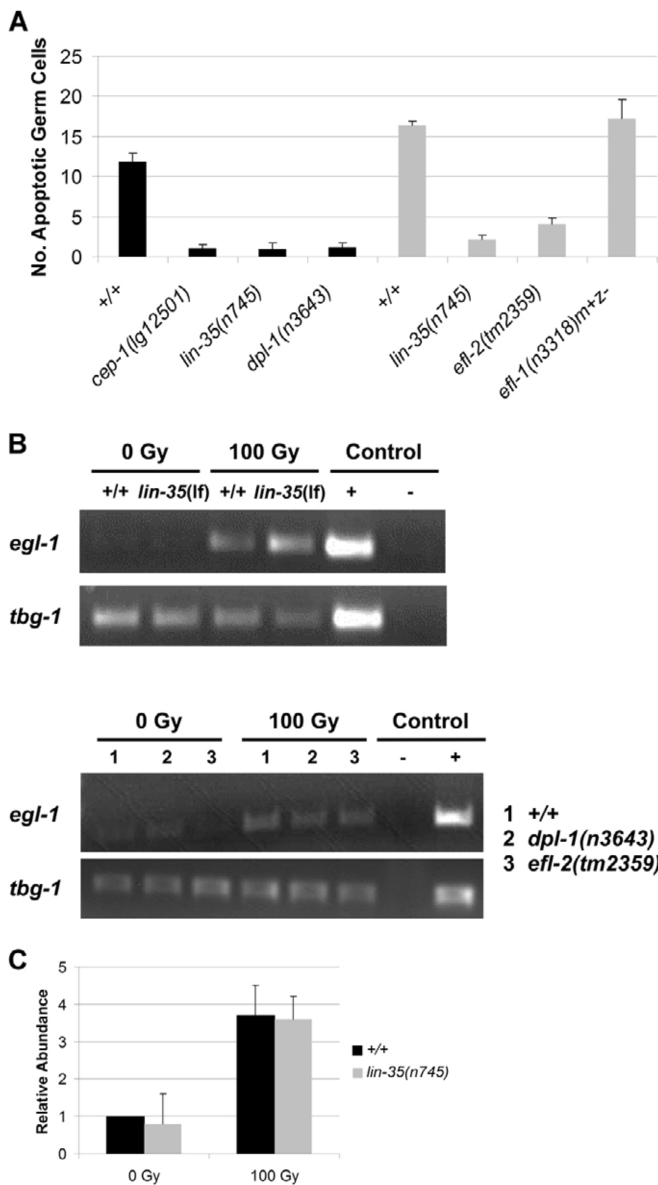

Fig. 4. Response to DNA damage in *lin-35*, *dpl-1*, *efl-1* and *efl-2* mutants. (**A**) Analysis of DNA-damage-induced germ cell apoptosis. For the data represented by the black bars, hermaphrodites were synchronized and irradiated at the L4 stage and germ cell apoptosis analyzed 24 hours post-irradiation. For the data represented by the gray bars, hermaphrodites were synchronized at the L4 stage, irradiated 12 hours post the L4 stage, and germ cell apoptosis analyzed 24 hours post-irradiation. Average numbers of apoptotic germ cells per gonad arm of four (black bars; *n*=61-79) or three (gray bars; *n*=38-54) independent experiments are shown. Error bars represent standard deviations. Strains were scored blind. m^+z^- indicates that animals analyzed were homozygous mutant progeny of heterozygous animals. (**B**) Semi-quantitative *egl-1* RT-PCR experiments using cDNAs isolated from unirradiated (0 Gy) or irradiated (100 Gy) wild-type (+/+), *lin-35*(*n745*) [*lin-35*(lf)], *dpl-1*(*n3643*) or *efl-2*(*tm2359*) animals. Purified cDNA was used as positive control (+), water as negative control (–). *tbg-1* RT-PCR was performed as the control. Representative experiments of three [*lin-35*(*n745*)] and two [*dpl-1*(*n3643*), *efl-2*(*tm2359*)] independent experiments are shown. (**C**) Quantitative *egl-1* RT-PCR experiments using cDNAs isolated from unirradiated (0 Gy) or irradiated (100 Gy) wild-type (+/+) or *lin-35*(*n745*) gonads. Average relative mRNA abundances of two or three independent experiments, each performed in triplicates, are shown. Error bars represent standard deviations.

damage-induced germ cell apoptosis and encodes the *C. elegans* ortholog of p53 (Derry et al., 2001; Schumacher et al., 2001). We found that, like the loss of *cep-1* function, reducing *lin-35*, *dpl-1* or *efl-2* function completely blocked DNA damage-induced germ cell apoptosis [Fig. 4A, *lin-35*(*n745*), *dpl-1*(*n3643*), *efl-2*(*tm2359*)]. By contrast, reducing *efl-1* function had no effect on this process [*efl-1*(*n3318*)]. Furthermore, the loss of *lin-35*, *dpl-1* or *efl-2* function did not result in a defect in DNA damage-induced cell-cycle arrest, indicating that *lin-35*, *dpl-1* and *efl-2* are not required for the DNA-damage checkpoint (see Fig. S2 and Table S3 in the supplementary material; data not shown). Based on these results, we conclude that *lin-35* RB, *dpl-1* DP and *efl-2* E2F, but not *efl-1* E2F, are specifically required for DNA damage-induced germ cell apoptosis.

The loss of *lin-35* RB, *dpl-1* DP or *efl-2* E2F function does not affect the *cep-1* p53-dependent expression of *egl-1* BH3-only in response to DNA damage

DNA damage-induced germ cell apoptosis is in part mediated by the *cep-1*-dependent upregulation at the transcriptional level of the BH3-only gene *egl-1* (Hofmann et al., 2002). To determine whether the block in DNA damage-induced germ cell apoptosis caused by the loss of *lin-35*, *dpl-1* or *efl-2* function is a result of a defect in the upregulation of *egl-1*, we analyzed the level of *egl-1* mRNA in the germ line of *lin-35*(*n745*), *dpl-1*(*n3643*) and *efl-2*(*tm2359*) animals. Using semi-quantitative and quantitative real-time RT-PCR, we found that in response to DNA damage the levels of *egl-1* mRNA increased to similar levels in the germ line of wild-type (+/+) and *lin-35*(*n745*) animals (Fig. 4B,C). Similarly, using semi-quantitative real-time RT-PCR, we found that the levels of *egl-1* mRNA increased to similar levels in the germ line of wild-type (+/+), *dpl-1*(*n3643*) and *efl-2*(*tm2359*) animals (Fig. 4B). Therefore, the loss of *lin-35*, *dpl-1* or *efl-2* function does not affect the *cep-1*-dependent upregulation of *egl-1* transcription. These results are consistent with the model that *lin-35* RB, *dpl-1* DP and *efl-2* E2F act downstream of or in parallel to *cep-1* p53 and *egl-1* BH3-only to promote DNA damage-induced germ cell apoptosis.

DISCUSSION

C. elegans lin-35 RB promotes constitutive germ cell apoptosis by repressing the transcription of *ced-9* BCL2

C. elegans lin-35 has pro-apoptotic activity in the hermaphrodite germ line. This pro-apoptotic activity is mediated by the transcriptional repression in the germ line of the anti-apoptotic *ced-9* BCL2 gene (Fig. 5A). In support of this conclusion, microarray-based expression profiling of germ lines identified the *ced-9* gene as a transcriptional target of LIN-35 that is repressed by *lin-35* activity (Chi and Reinke, 2006). Furthermore, our results indicate that the pro-apoptotic activity of *lin-35* in the germ line is dependent on the expression of *lin-35* not only in the germ line but also the somatic gonad. Hence, *lin-35* acts in both a cell-autonomous and cell non-autonomous manner to promote constitutive germ cell apoptosis. *lin-35* appears to be expressed in the germ line (Reinke et al., 2004) and it has previously been shown to play a role in the somatic gonad (Bender et al., 2004). Furthermore, *lin-35* has been shown to act in a cell non-autonomous manner to antagonize vulval differentiation in the VPCs (Cui et al., 2006; Myers and Greenwald, 2005). Therefore, the LIN-35 protein might function in the germ line to directly or indirectly cause the repression of *ced-9* transcription in the germ line and it might also affect an activity of the somatic gonad that non-autonomously promotes the repression of *ced-9* transcription in the germ line.

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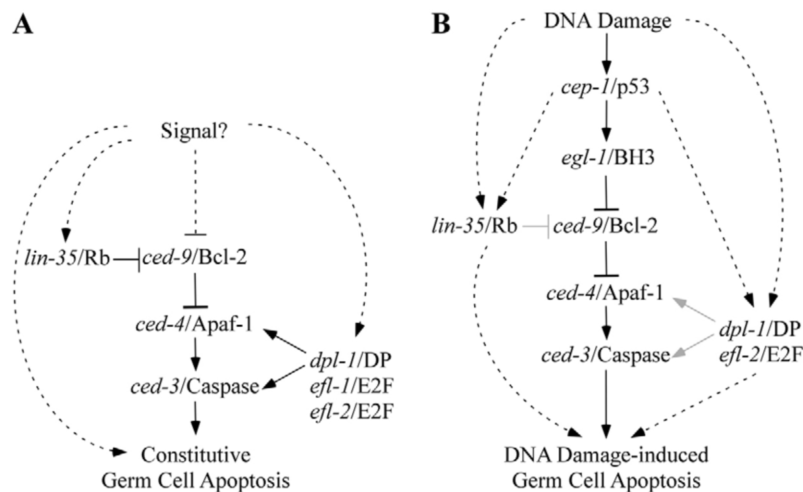

Fig. 5. Genetic pathways for germ cell apoptosis in *C. elegans*. (**A**) The genetic pathway for constitutive germ cell apoptosis. The genes *lin-35*, *dpl-1*, *efl-2* and, most likely, *efl-1*, promote constitutive germ cell apoptosis by blocking the function of the anti-apoptotic gene *ced-9* or by enhancing the function of the pro-apoptotic genes *ced-4* and *ced-3*, respectively. Furthermore, a *lin-35*, *dpl-1*, *efl-1* and *efl-2*-independent pathway most likely acts in parallel to *lin-35*, *dpl-1*, *efl-1* and *efl-2* to promote constitutive germ cell apoptosis. Solid lines represent confirmed genetic interactions. (**B**) The genetic pathway for DNA damage-induced germ cell apoptosis. *lin-35*, *dpl-1* and *efl-2* function downstream of or in parallel to *cep-1* to cause DNA damage-induced germ cell apoptosis by controlling the expression of unknown target genes. See text for details.

The loss of *lin-35* function also consistently resulted in an increase in the level of *ced-3* mRNA and a decrease in the level of CED-4 protein. We speculate that rather than being a specific effect of the loss of *lin-35* function, the changes in *ced-3* and *ced-4* expression observed might be the result of *lin-35*-dependent changes in the somatic gonad. Furthermore, the increase in *ced-3* expression observed in *lin-35*(*n745*) animals might explain why the loss of *lin-35* function only blocks 50% of constitutive germ cell apoptosis and why the defect in constitutive germ cell apoptosis observed in *lin-35*(*n745*); *dpl-1*(*n3643*) animals is not stronger than in *lin-35*(*n745*) animals.

C. elegans dpl-1 DP, *efl-1* E2F and *efl-2* E2F promote constitutive germ cell apoptosis

Like *lin-35*, *C. elegans dpl-1*, *efl-1* and *efl-2* have pro-apoptotic activities, at least in the hermaphrodite germ line. However, in contrast to the pro-apoptotic activity of *lin-35*, the pro-apoptotic activity of *dpl-1*, and most likely of *efl-1* and *efl-2*, is mediated through the transcriptional enhancement of the pro-apoptotic genes *ced-4* APAF1 and *ced-3* caspase (Fig. 5A). Microarray-based expression profiling of germ lines failed to identify *ced-4* and *ced-3* as transcriptional targets of EFL-1 and DPL-1 (Chi and Reinke, 2006). However, we believe that this failure can be explained by the relatively small decrease in the levels of *ced-4* and *ced-3* mRNAs caused by the loss of *dpl-1* or *efl-1* function and the threshold level used in this study. *efl-1* and *dpl-1* both are expressed in the germ line (Ceol and Horvitz, 2001; Page et al., 2001). Therefore, we propose that the DPL-1, EFL-1 and EFL-2 proteins act in a cell-autonomous manner to either directly or indirectly enhance the transcription in the germ line of *ced-4* and *ced-3*.

The overexpression of the mammalian *E2f1* gene in tissue culture cells can induce apoptosis. Furthermore, *E2f1* overexpression results in the transcriptional activation of a number of pro-apoptotic genes, such as the genes encoding the BH3-only proteins NOXA and PUMA, APAF1 and Caspase 3, 7, 8 and 9 (Attwooll et al., 2004; Dimova and Dyson, 2005). Furthermore, studies on the role of the *Drosophila* E2F-like protein dE2F1 in apoptosis indicate that it has pro-apoptotic activity, at least in cells of the intervein region of wing discs, where dE2F1 promotes apoptosis in response to DNA damage, which probably results from the transcriptional upregulation of a pro-apoptotic gene(s) (Moon et al., 2005). Therefore, the activation of apoptosis by the E2F-DP-dependent transcriptional activation of pro-apoptotic genes is a mechanism conserved among *C. elegans*, *Drosophila* and mammals.

The pro-apoptotic function of *lin-35* RB in constitutive germ cell apoptosis is independent of *dpl-1* DP and *efl-2* E2F

The function of *lin-35* RB in constitutive germ cell apoptosis is independent of the functions of *dpl-1* DP, *efl-2* E2F and probably also of *efl-1* E2F. This notion is supported by microarray-based expression profiling of germ lines, which revealed that there is extensive overlap between the target genes of DPL-1 and EFL-1, but not between the target genes of LIN-35 and DPL-1 or EFL-1 (Chi and Reinke, 2006). Mammalian RB protein is thought to control gene expression almost exclusively through binding to E2F-DP complexes. However, mutant RB proteins that are unable to bind to E2F-DP complexes retain certain aspects of RB function (Sellers et al., 1998). This observation suggests that RB could have functions that are independent of E2F-DP activity. Indeed, RB can interact with a variety of transcription factors other than E2F-DP, such as MyoD, AP-2, C/EBPs and Pax5 (Eberhard and Busslinger, 1999; Macaluso et al., 2006; Macleod, 1999; Sato et al., 2001). However, the significance of these interactions is unclear. Interestingly, *egl-38* and *pax-2*, two *C. elegans* genes related to mammalian *Pax2/5/8*, promote *ced-9* expression in somatic tissues and in the germ line and possibly encode direct activators of *ced-9* transcription (Park et al., 2006). Thus, we speculate that the LIN-35 RB protein might interact with the EGL-38 and PAX-2 proteins and antagonize their ability to promote *ced-9* BCL2 transcription.

The levels of CED-9 BCL2, CED-4 APAF1 and CED-3 caspase control constitutive germ cell apoptosis

In contrast to developmental apoptosis, which is determined by the essentially invariant somatic cell lineage and is dependent on the pro-apoptotic gene *egl-1* BH3-only, constitutive germ cell apoptosis is a stochastic event that is coupled to meiotic cell-cycle progression and which occurs in an *egl-1* BH3-only-independent manner (Conradt and Horvitz, 1998; Gumienny et al., 1999). We found that the levels of CED-9, CED-4 and CED-3 proteins are critical for constitutive germ cell apoptosis: lowering the dosage of the *ced-9* gene increases constitutive germ cell apoptosis, whereas increasing the dosage of *ced-9* or lowering the dosage of *ced-4* and *ced-3* decreases constitutive germ cell apoptosis. Therefore, the amount of CED-9 protein is a limiting factor in a germ cell's quest for survival and, conversely, the amounts of CED-4 and CED-3 proteins are limiting factors in a germ cell's quest for demise. We propose that the combination of *lin-35*-dependent repression of *ced-9* transcription and *dpl-1*-dependent

enhancement of *ced-4* and *ced-3* transcription ensure that the relative levels of CED-9, CED-4 and CED-3 proteins are such that more than 50% of the germ cells undergo apoptosis. How the pro-apoptotic activities of *lin-35* and *dpl-1* are regulated remains to be determined. Furthermore, it remains to be determined whether *lin-35* and *dpl-1* are targets of apoptotic signaling pathways that determine the extent of germ cell apoptosis or whether they are components of a general machinery that sets the level of *ced-9*, *ced-3* and *ced-4* mRNAs in the germ line. Finally, because the loss of *lin-35* and *dpl-1*, *efl-1* or *efl-2* function causes a 50% decrease in constitutive germ cell apoptosis, the activities of additional, as yet uncharacterized factors must contribute to the life-versus-death decision within germ cells (Fig. 5A).

The regulation at the transcriptional level of members of the family of pro- and anti-apoptotic BCL2-like protein is a well-established mechanism to control apoptosis (Cory and Adams, 2002). However, at least to our knowledge, the regulation at the transcriptional level of APAF1-like proteins and caspases has so far not been demonstrated to be a physiologically important mechanism for apoptosis regulation (Adams, 2003; Danial and Korsmeyer, 2004). It will be of interest to determine whether the transcriptional regulation of *Apaf1*-like or caspase genes controls apoptosis in species other than *C. elegans*.

lin-35 RB, *dpl-1* DP and *efl-2* E2F are required for DNA damage-induced germ cell apoptosis

lin-35, *dpl-1* and *efl-2*, but not *efl-1*, are required for DNA damage-induced germ cell apoptosis. Interestingly, in response to DNA damage, the level of *ced-9* mRNA increases about 2-fold and the levels of *ced-4* and *ced-3* mRNAs decrease by about 50% in the germ line of wild-type hermaphrodites (our unpublished observations). Therefore, the transcriptional regulation of *ced-9*, *ced-4* and *ced-3* does not appear to be a determinant of DNA damage-induced germ cell apoptosis. Instead, we propose that in response to DNA damage, *lin-35*, *dpl-1* and *efl-2* control the expression of different target genes that encode critical determinants of DNA damage-induced germ cell apoptosis (Fig. 5B). Candidate genes are *cep-1* p53 and *egl-1* BH3-only, which are required for DNA damage-induced germ cell apoptosis. However, we found that *lin-35*, *dpl-1* and *efl-2* act downstream of or in parallel to *cep-1* and *egl-1* to cause DNA damage-induced germ cell apoptosis (Fig. 5B). Finally, we have evidence that apart from *lin-35* and *dpl-1*, additional synMuvB genes are required for DNA damage-induced germ cell apoptosis (our unpublished observations). Orthologs of synMuvB proteins in other species have been implicated in chromatin remodeling and transcriptional repression (reviewed by Korenjak and Brehm, 2005; Lipsick, 2004). Therefore, we speculate that in response to DNA damage, LIN-35, DPL-1, EFL-2 and additional synMuvB proteins might assemble to form a transcriptional repressor complex, which represses the transcription of a gene or genes, the product(s) of which can block DNA damage-induced germ cell apoptosis (Fig. 5B).

Implications for the role in apoptosis of mammalian *Rb*

Currently, the prevailing model is that mammalian *Rb* has anti-apoptotic activity. This model is based on the observation that mice lacking *Rb* function exhibit ectopic apoptosis. However, the fact that ectopic apoptosis in $Rb^{-/-}$ mice is observed only in a limited number of tissues suggests that the role of *Rb* in apoptosis might be tissue-specific. This notion is supported by observations indicating that mutations in the *Rb* gene are sufficient to cause pituitary and thyroid tumors in mice and small-cell lung cancer in humans, and that tumorigenesis in these types of tumors does not appear to depend on the concomitant inactivation by mutation of the apoptotic pathway (Chau and Wang, 2003; Hitchens and Robbins, 2003; Hu et al., 1994; Lipinski and Jacks, 1999; Sherr and McCormick, 2002; Williams et al., 1994). Based on these facts and on our finding that *C. elegans lin-35* RB has pro-apoptotic activity in the germ line, we hypothesize that mammalian *Rb* functions to promote apoptosis in certain tissues, such as tissues giving rise to pituitary and thyroid tumors, and small-cell lung cancer. Furthermore, we hypothesize that in these tissues, the pro-apoptotic activity of *Rb* contributes to the tumor-suppressing activity of *Rb*, which so far has mainly been attributed to its anti-proliferative activity. We also speculate that, in analogy to LIN-35 RB, the putative pro-apoptotic activity of the RB protein could be mediated by the transcriptional repression of anti-apoptotic *Bcl2*-like genes (Cory and Adams, 2002). A comprehensive understanding of the role in apoptosis of mammalian *Rb* in different tissues and contexts will almost certainly improve our knowledge of the tumor-suppressing activity of *Rb* and, hence, tumorigenesis in *Rb*-deficient tumors.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/20/3691/DC1

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DEVELOPMENT

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