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Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline

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SUMMARY

Development of the nematode *Caenorhabditis elegans* is highly reproducible and the fate of every somatic cell has been reported. We describe here a previously uncharacterized cell fate in *C. elegans*: we show that germ cells, which in hermaphrodites can differentiate into sperm and oocytes, also undergo apoptotic cell death. In adult hermaphrodites, over 300 germ cells die, using the same apoptotic execution machinery (*ced-3*, *ced-4* and *ced-9*) as the previously described 131 somatic cell deaths. However, this machinery is activated by a distinct pathway, as loss of *egl-1* function, which inhibits somatic cell death, does not affect germ cell apoptosis. Germ cell death requires ras/MAPK pathway activation and is used to maintain germline homeostasis. We suggest that apoptosis eliminates excess germ cells that acted as nurse cells to provide cytoplasmic components to maturing oocytes.

Key words: Germline, Programmed cell death, Apoptosis, *Caenorhabditis elegans*, Ras/MAPK pathway, Oocyte

INTRODUCTION

The nematode *Caenorhabditis elegans* is characterized by a highly reproducible development. The patterns of cell divisions, cell migrations and cell deaths that give rise to the adult animal are nearly invariant and have been completely described. Thus, the fate of every somatic cell arising during *C. elegans* development is known (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1980, 1983).

Of the 1090 cells that are generated during development of the hermaphrodite, 131 are fated to undergo programmed cell death (apoptosis). Analysis of these deaths revealed a genetic pathway that controls the programmed death and elimination of apoptotic cells in *C. elegans* (reviewed by Ellis et al., 1991b; Horvitz et al., 1994). Biochemical characterization of the genes in this pathway has led to the identification of an apoptotic machinery. Briefly, the proximal cause of apoptosis in *C. elegans* is the activation of the caspase CED-3 (CEll Death abnormal) from the inactive zymogen (proCED-3) into the mature protease (Yuan et al., 1993; Xue et al., 1996). This activation is mediated by the Apaf-1 homolog CED-4 (Yuan and Horvitz, 1992; Zou et al., 1997). In cells that should survive, CED-3 and CED-4 pro-apoptotic activity is antagonized by the Bcl-2 family member CED-9 (Hengartner et al., 1992; Hengartner and Horvitz, 1994a). CED-9 has been proposed to prevent death by sequestering CED-4 and proCED-3 in an inactive ternary complex, the apoptosome (Hengartner, 1997). In cells fated to die, CED-9 is in turn inactivated by the pro-apoptotic BH3 domain-containing protein EGL-1 (EGg-Laying defective), likely through a direct protein-protein interaction (Conradt and Horvitz, 1998). How the apoptotic machinery is controlled such that it only becomes activated in cells that should die is not yet understood.

Each of the key *C. elegans* cell death genes has one or several mammalian homologs, and all the classes of protein-protein interactions described for *C. elegans* cell death proteins have also been detected for their mammalian counterparts (reviewed by Hengartner, 1997, 1998), suggesting that the apoptotic machinery identified in *C. elegans* is evolutionarily conserved and mediates at least a subset of apoptotic deaths in mammals.

Unlike somatic cells, germ cells in *C. elegans* do not have a fixed lineage and populations of cells, rather than individual cells, are regulated, albeit through strict and well-characterized control mechanisms (reviewed by Schedl, 1997). The germline is one of the best characterized and most extensively studied cell types in *C. elegans*, as it is readily amenable to genetic and developmental analysis (Kimble and Ward, 1988; L'Hernault, 1997; Schedl, 1997).

The gonad of the *C. elegans* adult hermaphrodite consists of two symmetrical, U-shaped tubes, which are linked to a common uterus (Fig. 1A). The distal end of each gonadal tube is capped by a somatic distal tip cell (DTC). The LAG-2 growth factor, expressed by the DTC, maintains the stem cell potential of the adjacent germ cells and promotes their mitotic

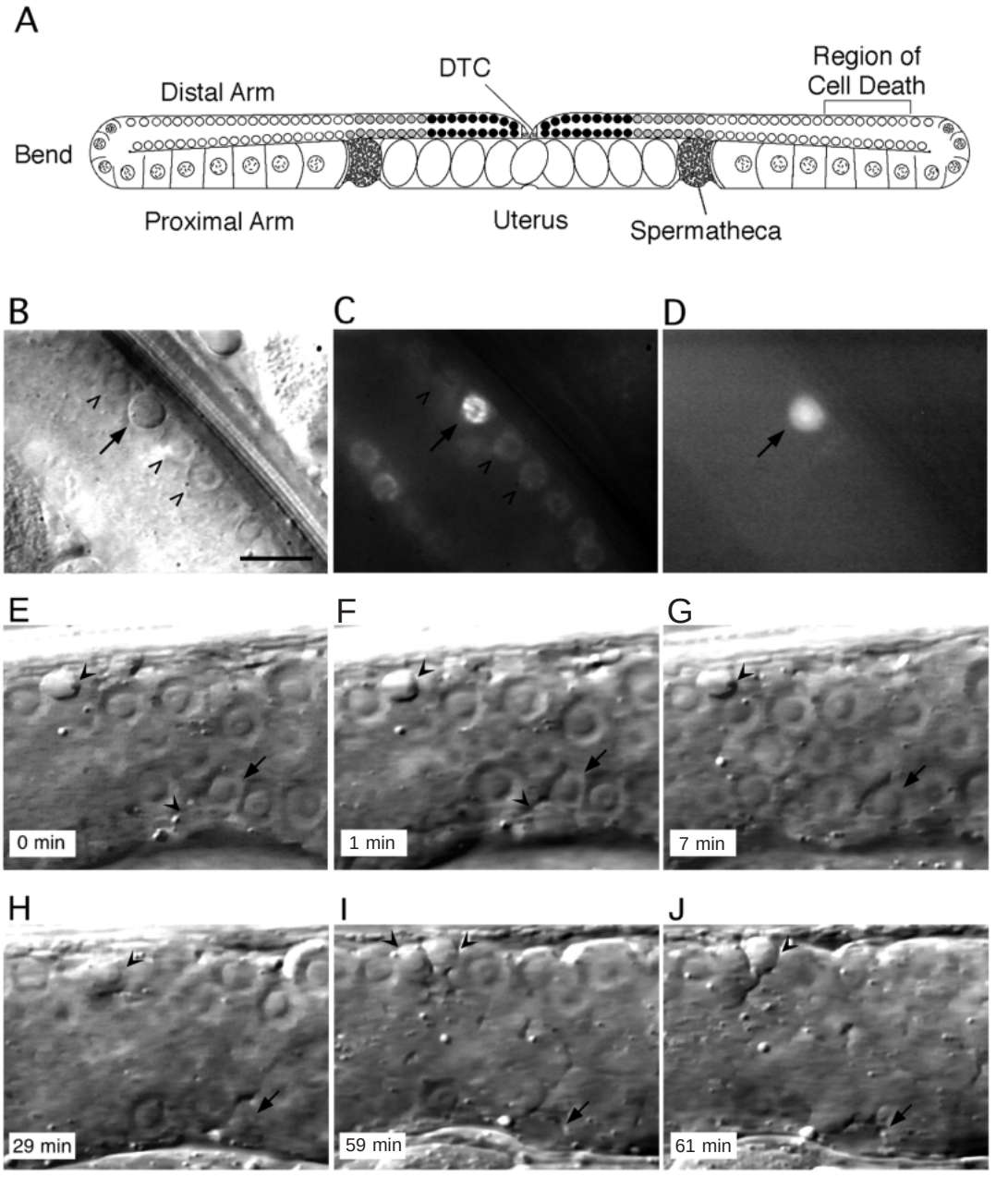

Fig. 1. Morphological features of germ cell deaths. (A) Schematic diagram of an adult hermaphrodite *C. elegans* gonad. Two gonad arms join at a common uterus. Germ nuclei are syncytial in the distal halves (furthest from the uterus) of both arms. The most distal nuclei (about twelve nuclear lengths at the distal end) are kept in mitosis (dark circles) through a proliferation signal sent by the distal tip cell (DTC). The nuclei enter a transition zone of early meiosis I (gray circles) and progress to pachytene of Meiosis I (small white circles). Diakinesis and cellularization of nuclei into oocytes occur around the bend. Sperm are located within the spermatheca. The region where programmed cell deaths normally occur is indicated above the right gonad arm. Adapted from Kirby et al., 1990. (B-D) Hoechst 33342/SYTO 12 double staining of a wild-type hermaphrodite 24 hours after the L4/adult molt. Germ cell corpse is indicated by an arrow, surrounding healthy nuclei by arrowheads. Bar, 10 μm. (B) Nomarski optics view. The dying germ cell (flat circular structure) has cellularized away from the common syncytium. (C) Hoechst 33342 staining. Note chromosome condensation and marginalization in the apoptotic nucleus as compared to the surrounding healthy nuclei. (D) SYTO 12 staining. Note that the live, neighboring cells fail to stain. (E-J) Time course of a germ cell death. Arrows indicate dying cell (or in E, nucleus about to die). Arrowheads indicate other germ cell deaths. The germ cell corpse shown here never became as refractile as others, but both appearances are normal.

proliferation throughout the reproductive life of the animal. Germ cells positioned beyond the influence of the DTC signal enter meiosis and progress into the pachytene stage of meiosis I. Near the bend of the gonadal tube, cells exit pachytene in response to activation of the ras/MAPK pathway and begin to enlarge in size. In the proximal arm, germ cells progress to diakinesis of prophase I and increase in size to form oocytes as they migrate in a single row towards the uterus.

The mitotic and early meiotic germ cell nuclei are only partially enclosed by a plasma membrane and are thus part of

a large syncytium (Figs 1A, 2A). Interestingly, nuclei within this syncytium are not synchronized, such that a given syncytium will contain at the same time nuclei at all the various stages of mitosis and early meiosis. Because syncytial germ cell nuclei appear to act as independent units, we will refer to them as 'germ cells' even though they share a common cytoplasm.

Previous work has established that germ cells have three fates available to them: they can either undergo mitosis, or they can enter meiosis and differentiate into either sperm or oocytes (Ellis and Kimble, 1994). Here we report our characterization of a fourth major germ cell fate: apoptotic cell death. We show that, in normal adult hermaphrodites, over half of all potential oocytes are eliminated during meiotic maturation. Germ cell death is mediated by the same core execution machinery, i.e. *ced-3* and *ced-4*, as are all developmental deaths. However, activation of this machinery is regulated differently in the soma and the germline: loss-of-function mutations in *egl-1*, as well as a rare gain-of-function mutation in *ced-9*, both of which completely prevent somatic cell death, have little effect on germ cell death. Our results suggest that germ cell death in the adult hermaphrodite germline serves a homeostatic purpose by eliminating excess germ cell nuclei that acted as nurse cells.

MATERIALS AND METHODS

Mutations and strains

Methods for culturing *C. elegans* have been described by Brenner (1974). All strains were grown at 20°C unless otherwise indicated. All mutants used in these studies were derived from the wild-type variety Bristol strain N2. Other wild-type species used were *Caenorhabditis briggsae*, *Caenorhabditis vulgaris*, *Caenorhabditis remanei*, *Pelodera strongyloides*, *Panagrellus redivivus* and *Rhabditella axei*. *let-60(dx16)* and *lin-45(dx19)* are recessive sterile mutations that were isolated after exposure to 310 nm UV light (E. L., unpublished). The following mutations are described by Riddle et al. (1997) or the references cited therein: LG I: *mek-2(n2678)*, *fog-1(q253*ts*)*, *unc-15(e1214*am*)*, *unc-13(e51)*, *gld-1(oz10)*, *gld-1(q485)*, *gld-1(q93oz50)*, *gld-1(q126)*, *gld-1(q266)*, *gld-1(q343)*, *ces-1(n703*gf*)*, *ced-1(e1735)*, *glp-4(bn2)*, *ces-2(n732)*; LG III: *mpk-1(oz140)*, *ced-4(n1162)*, *lon-1(e185)*, *ced-6(n1813)*, *dpy-19(e1259)*, *ced-7(n1892)*, *mog-1(q223)*, *unc-69(e587)*, *ced-9(n1950*gf*)*, *ced-9(n1653*ts*)*, *ced-9(n1950n2161)*, *ced-9(n1950n2077)*, *ced-9(n2812)*, *tra-1(e1575*sd*)*, *tra-1(e1076)* (Hodgkin and Brenner, 1977); LG IV: *ced-2(e1752)*, *ced-10(n1993)*, *unc-5(e53)*, *fem-1(e1965)*, *mor-2(e1125)*, *lin-45(dx19)*, *let-60(n1046*sd*)*, *let-60(s1124)*, *let-60(dx16)*, *unc-24(e138)*, *fem-3(e1996*sd*)*, *ced-5(n1812)*, *him-8(e1489)*, *dpy-20(e1282*ts*)*, *ced-3(n717)*; LG V: *her-1(n695*sd,ts*)*, *him-5(e1490)*, *egl-1(n986*dm*)*, *egl-1(n1084n3082)*; LG X: *ced-8(n1891)*, *nuc-1(e1392*am*)*, *sdc-1(n485)*; rearrangements and duplications: *qC1(III)*, *eDp6(III;f)*, *hT2(I;III)* and *nDp4(I;V)*.

All *ced-9* alleles except for *n1653* and *n1950* were maintained with the duplication *qC1*. *fem-1(e1965)* was maintained in *trans* to *unc-5(e53) mor-2(e1125)*. *fem-3(e1996)* was maintained heterozygous with *unc-24(e138) dpy-19(e1259)*. *gld-1* alleles *q266*, *oz10*, and *q93oz50* were maintained with *nDp4(I;V)*. *gld-1(q485)/unc-13(e51) gld-1(q126)* maintained both *q485* and *q126*. *gld-1(q343)* was maintained heterozygous with *unc-15(e1214)*. *mog-1(q223)* was maintained in trans to *dpy-19(e1259) unc-69(e587)*. *tra-1(e1076)* was maintained with *eDp6(III;f)*.

Cell corpse assays

We studied the appearance and number of cell corpses in the germline of nematodes by mounting animals in a drop of M9 salt solution containing 30 mM NaN_3 (Hodgkin, 1980) and observing the animals using Nomarski optics (Ellis et al., 1991a). Corpses are cellularized and more refractile than syncytial nuclei or oocytes and can readily be identified under high magnification. Average corpse numbers with the standard error of the mean (s.e.m.) were determined by the Statview II program (Abacus Concepts, Incorporated, Berkeley, California).

To study the kinetics of death and degradation of germ cells, adult wild-type nematodes (24 to 36 hours post L4/adult molt) were mounted in S Basal or 3 or 5 mM levamisole. Levamisole prevents animals from moving but does not affect the germline or the gonad (Sulston and Brenner, 1974). The germlines of individual animals were observed for 1 to 3 hours.

Time course assay

To study the number, timing and distribution of cell corpses in the germline, L4 stage larvae were transferred to new plates. Starting at the L4/adult molt, and every 12 hours thereafter, animals were anesthetized and observed using Nomarski optics (Ellis et al., 1991a). For each animal observed, only one arm was scored, as the other arm was usually concealed by the intestine.

SYTO 12 assay

To obtain an estimate of the relative numbers of corpses in different genetic backgrounds, 36-hour adult animals were stained with SYTO 12 (Molecular Probes, Eugene, OR), a vital dye that preferentially stains apoptotic germ cells. Animals were stained by incubating in a 33 μM aqueous solution of SYTO 12 for 4-5 hours at 23°C, then transferred to seeded plates to allow stained bacteria to be purged from the gut. After 30-60 minutes, animals were mounted on agarose pads and inspected using a Nikon Microphot-SA, equipped with standard epifluorescence filters and Nomarski optics. Only animals that stained brightly were scored. In wild-type animals, SYTO 12 has a granular cytoplasmic staining pattern in mid- to late-stage oocytes and in embryos. The identity of the stained granules was not determined. However, they might represent mitochondria and/or lysosomes. In wild-type animals, the posterior half gonad typically stained more brightly than the anterior half. SYTO 12 stains both corpses visible by Nomarski optics as well as late-stage germ cell corpses, which cannot be scored by Nomarski optics. Thus, the number of SYTO-positive germ cells is consistently higher than the number of corpses observed using Nomarski optics.

Photography

Double staining with SYTO 12 and Hoechst 33342 (Sigma) was done by incubating animals in an aqueous solution of 33 μM SYTO 12, 67 μM Hoechst 33342 for 6 hours at room temperature. In some cases, animals were anesthetized using 5 mM levamisole to facilitate photography. For image acquisition, a Sony AVC-D7 CCD camera was connected to a Scion LGIII frame grabber installed in a Quadra 700 computer and frames were taken using NIH Image software (as modified by Scion). Composite images were assembled and edited using Adobe PhotoShop 3.0 on a Power Macintosh 8100/80AV.

Electron microscopy

Adult hermaphrodites were fixed in 0.8% glutaraldehyde, 0.7% OsO_4, 0.1 M cacodylate buffer for 1 hour on ice. Subsequently, samples were cut and postfixed in 2% OsO_4, 0.1 M cacodylate buffer, mounted into an agar block, dehydrated in a series of alcohols and embedded in a mixture of Epon-Araldite. Thin sections (50 nm) were cut on an Ultracut E and pictures were taken with a JEOL 1200× electron microscope at 80 kV.

RESULTS

C. elegans germ cells undergo programmed cell death

Anecdotal observations of dead cells in the *C. elegans* germline

have previously been made (Sulston, 1988; White, 1988; J. Kimble, personal communication). To corroborate these findings, we examined gonads from wild-type animals at various stages of development. We found that the germlines of adult hermaphrodites, but not those of larvae or adult males, consistently contained a small number of condensed structures when observed using Nomarski microscopy (Fig. 1B). These structures were restricted to a region of the germline occupied by syncytial germ cells in the pachytene stage of meiosis I (Fig. 1A). The number of these structures gradually increased throughout the reproductive life of the animals. Observations of individual cells indicated that these condensed structures arose from normal-looking germ cells. Over a time frame of about 20 minutes, the cytoplasm of these cells became increasingly refractive and finally melded with the nucleus to become a highly refractive uniform structure. These condensed structures were transient in nature and typically disappeared within an hour of their appearance (Fig. 1E-J). The radical morphological changes and subsequent elimination of these germ cells suggests that they are either degenerating or undergoing programmed cell death.

The condensed intermediates seen using Nomarski optics were highly reminiscent of the somatic programmed cell deaths that occur during *C. elegans* development (Sulston and Horvitz, 1977). To test the hypothesis that the germ cell deaths that we observed might be apoptotic in nature, we characterized them further using both morphological and genetic approaches.

We first tested whether dying germ cells show other features characteristic of apoptotic deaths, such as chromatin condensation and staining with vital dyes that are taken up by apoptotic cells (Kerr et al., 1972; Wyllie et al., 1980; Abrams et al., 1993; White et al., 1994). We adapted a Hoechst 33342 DNA-staining protocol for use on live animals and found that the nuclei of refractive cells (as determined by Nomarski microscopy) showed DNA condensation characteristic of apoptosis (Fig. 1B,C). Staining with the DNA intercalator dye 4′, 6-diamidino-2-phenyline (DAPI) gave similar results (data not shown). The vital dye acridine orange (AO) has been used successfully in *Drosophila* to specifically stain apoptotic cells in live animals (Abrams et al., 1993; White et al., 1994). We found that this dye, as well as the nucleic acid stain SYTO 12, specifically stained dying germ cells identified by Nomarski optics or the Hoechst dye (Fig. 1D; T. L. G., E. L., A. Samuelson, S. Milstein, and M. O. H., unpublished).

To obtain additional evidence confirming the apoptotic nature of dying germ cells, we analyzed cross sections of young adult hermaphrodite gonads by electron microscopy. While most sections contained only healthy, syncytial germ cells, we did find that a small fraction of germ cell nuclei exhibited morphological features characteristic of early stages of apoptotic cell death (Fig. 2B-D). Our ultrastructural studies also resolved the apparent dilemma of how individual cell nuclei within a syncytium can die while the rest of the syncytium survives. We found that cells fated to die rapidly cellularize away from the common syncytium, thereby physically isolating the doomed nucleus from its neighbors. The newly generated cell, which contains only limited amounts of cytoplasm (Fig. 2C,E), is subsequently recognized and engulfed by the gonadal sheath cells that surround the germline (Fig. 2C,D). The swift phagocytosis and degradation of apoptotic germ cells by sheath cells might explain why we failed to observe any dying germ cells at advanced stages of apoptosis in normal animals. Consistent with this hypothesis, if engulfment is inhibited (by genetic means, see below), persisting dead germ cells accumulate in the gonad and undergo further nuclear and cytoplasmic condensation (Fig. 2E,F).

C. elegans cell death genes function in germ cell apoptosis

14 genes have been identified that function in programmed cell death during *C. elegans* development (reviewed by Ellis et al., 1991b; Horvitz et al., 1994). We found that many of the developmental cell death genes also function in the germline, as animals mutant in these genes showed altered patterns of germ cell death.

For example, the genes *ced-3* and *ced-4* are required for programmed cell death in the *C. elegans* soma: strong loss-of-function (lf) mutations in either gene prevent all 131 cell deaths that normally occur during hermaphrodite development. We found that both genes are also required for germ cell death, as few if any germ cell corpses were seen in *ced-3* or *ced-4* mutant animals (Fig. 3A).

The *ced-9* gene protects *C. elegans* cells from programmed cell death: loss of *ced-9* function results in the death of many cells that normally survive (Hengartner et al., 1992). In *ced-9(lf)* animals, we observed increased levels of germ cell death, indicating that *ced-9* also has a protective function in the germline (Fig. 3B and data not shown).

Efficient phagocytosis of apoptotic cells in *C. elegans* requires the function of at least six genes: *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* (Ellis et al., 1991a; Horvitz et al., 1994). We found that animals mutant for any one of these genes accumulated many more cell corpses in the germline than wild-type animals (Fig. 3C). In older animals, many unengulfed cell corpses swelled and eventually lysed, suggesting that these lingering corpses were undergoing secondary necrosis, as has been observed for mammalian corpses that escape ingestion (Wyllie et al., 1980). In addition, cellular debris accumulated in front of the spermatheca and in the uterus of these mutants. We surmise that this debris was the result of necrotic lysis or possibly the collapse of corpses forced through the spermatheca by the progression of oocytes. A seventh gene, *ced-8*, is involved in the timely phagocytosis of embryonic, but not larval, corpses (Ellis et al., 1991a; Horvitz et al., 1994). A time course study of germ cell death in *ced-8* mutant animals revealed no significant difference from that in the wild type (Fig. 3C), suggesting that *ced-8* is not required for the rapid engulfment of germ cell corpses.

The *nuc-1* (NUClease abnormal) gene is required for the degradation of DNA within engulfed corpses (Hedgecock et al., 1983). We found that, in *nuc-1* mutants, sheath cells contained DAPI-positive vacuoles with undegraded DNA. The absence of DAPI-positive vacuoles in the germline of *nuc-1* mutants suggests that dying germ cells are not reabsorbed by the germline syncytium but rather are engulfed by somatic sheath cells. DAPI-positive vacuoles mildly increased in size and number as animals aged, consistent with our observation that germ cell deaths occur continuously throughout the reproductive life of the animal (Fig. 3A and data not shown). DNA degradation also failed to occur when engulfment was

blocked (e.g., in *ced-5* mutants), indicating that, similar to somatic apoptosis (Hedgecock et al., 1983), germ cell apoptosis requires phagocytosis for *nuc-1*-mediated DNA degradation (data not shown).

Mutations in the genes *ces-1* and *ces-2* (CEll death Specification abnormal) affect a specific subset of somatic programmed cell deaths (Ellis and Horvitz, 1991). These genes could be required to specify the identity of the affected cells, or could participate in a cell-type-specific pathway that controls activation of the apoptotic machinery (reviewed by Driscoll, 1992; Hengartner and Horvitz, 1994b). We found no difference in germline corpse numbers between any of these mutants and wild type (Fig. 3E), suggesting that *ces-1* and *ces-2* play no role in germ cell death.

Somatic cells and germ cells use different mechanisms to regulate the apoptotic machinery

Despite the many similarities in the genetic regulation of somatic and germ cell deaths, we observed a few striking differences. We found that the *ced-9(n1950)* gain-of-function mutation, which completely prevents cell death during hermaphrodite development (Hengartner et al., 1992), has little if any effect on germ cell death (Fig. 3D). To confirm this result, we repeated our time-course analysis in a *ced-1(e1735)*

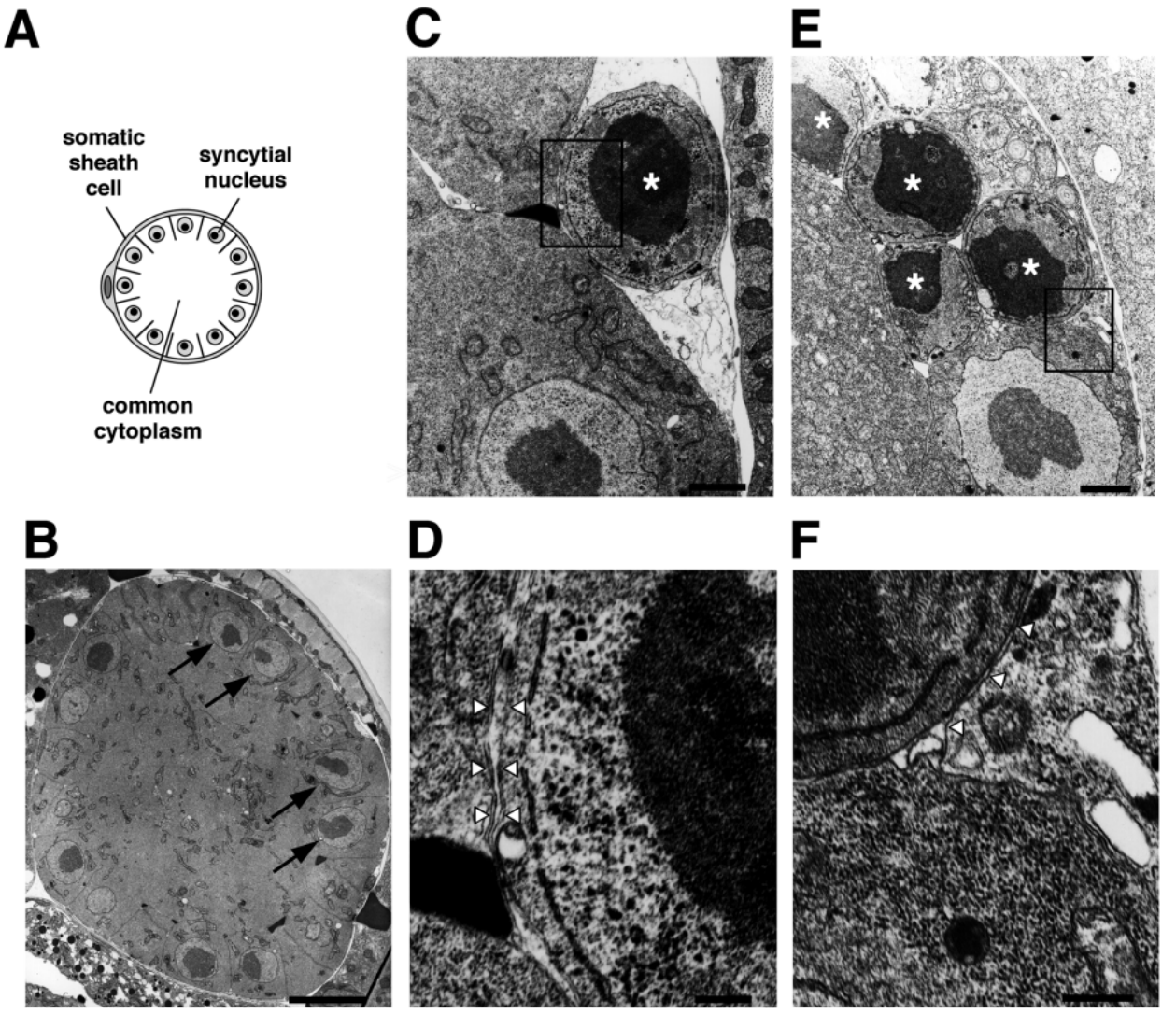

Fig. 2. Dying *C. elegans* germ cells show ultrastructural characteristics typical of apoptotic cell death. **(**A) Schematic cross section through the distal arm of the adult hermaphrodite gonad. Syncytial germ cell nuclei are located in small alcoves around the gonad periphery and are surrounded by membranes on all but one side, which is open to the common cytoplasmic core. The germline is surrounded by somatic sheath cells. (B) Electron micrograph of a wild-type adult syncytial gonad. No deaths are apparent in this cross section. Arrows indicate healthy nuclei. Bar, 5 μm. (C) Germ cell death in a wild-type adult hermaphrodite. The nucleus has cellularized and condensed, and the cell has little cytoplasm. The corpse (marked with an asterisk) is entirely engulfed by the somatic sheath cell. Box indicates region magnified in D. Bar, 1 μm. (D) Magnification of box in C. There is a thin bridge of cytoplasm from the sheath cell separating the engulfed cell corpse from the syncytium. Note the four membranes (left to right, flanked by arrowheads): one from the germline syncytium, two from the sheath cell, and one from the corpse. Bar, 0.25 μm. (E) Germ cell deaths in a *ced-5(n1812)* adult hermaphrodite. Four nuclei (marked with an asterisk) have cellularized and condensed. None of the corpses is engulfed by the sheath cell. Box indicates region magnified in F. Bar, 1 μm. (F) Magnification of box in E. Note that the sheath cell has not engulfed any of the corpses. Arrowheads indicate sheath cell membrane. Bar, 0.25 μm.

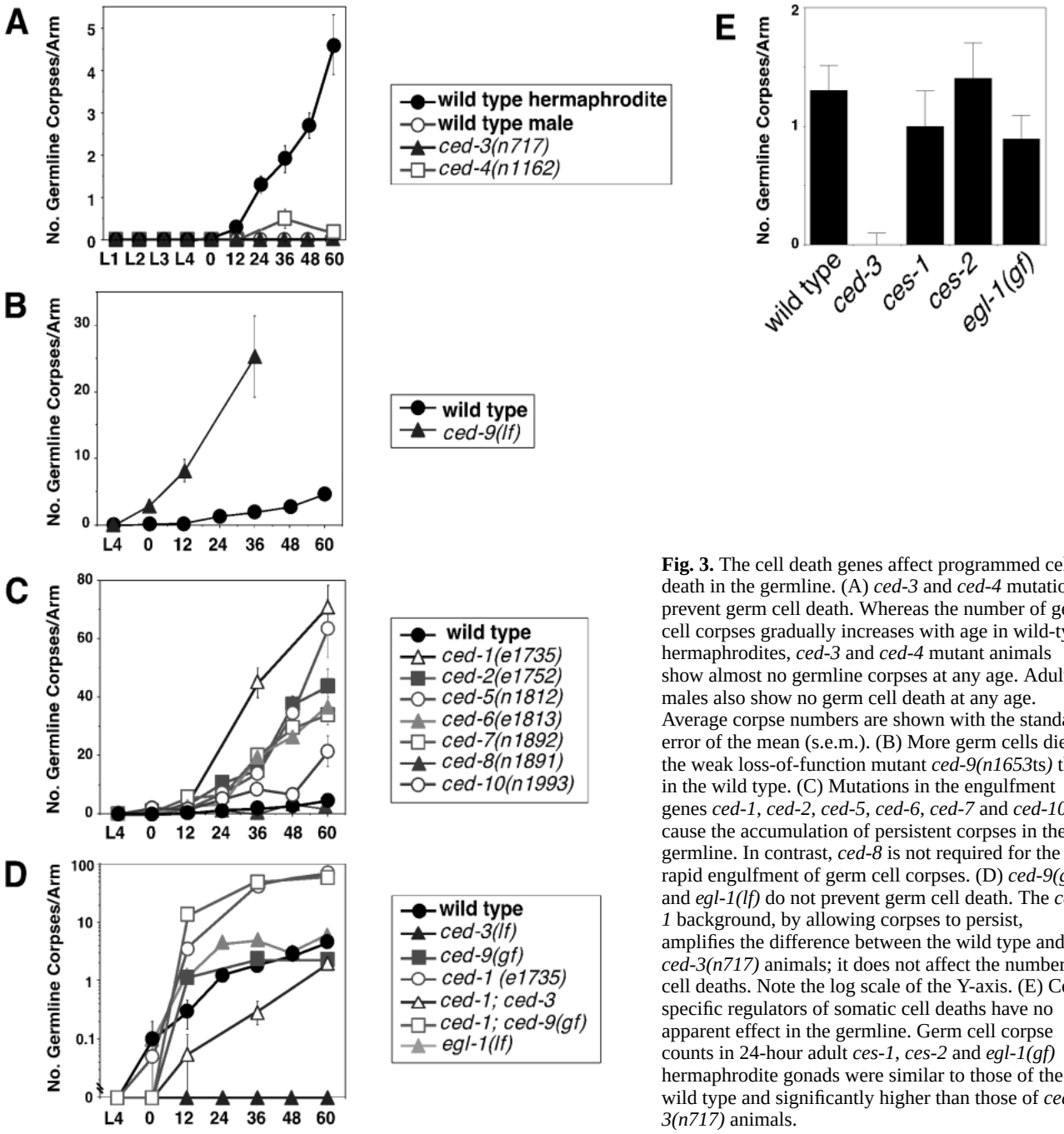

Fig. 3. The cell death genes affect programmed cell death in the germline. (A) *ced-3* and *ced-4* mutations prevent germ cell death. Whereas the number of germ cell corpses gradually increases with age in wild-type hermaphrodites, *ced-3* and *ced-4* mutant animals show almost no germline corpses at any age. Adult males also show no germ cell death at any age. Average corpse numbers are shown with the standard error of the mean (s.e.m.). (B) More germ cells die in the weak loss-of-function mutant *ced-9(n1653*ts*)* than in the wild type. (C) Mutations in the engulfment genes *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7* and *ced-10* cause the accumulation of persistent corpses in the germline. In contrast, *ced-8* is not required for the rapid engulfment of germ cell corpses. (D) *ced-9(gf)* and *egl-1(lf)* do not prevent germ cell death. The *ced-1* background, by allowing corpses to persist, amplifies the difference between the wild type and *ced-3(n717)* animals; it does not affect the number of cell deaths. Note the log scale of the Y-axis. (E) Cell-specific regulators of somatic cell deaths have no apparent effect in the germline. Germ cell corpse counts in 24-hour adult *ces-1*, *ces-2* and *egl-1(gf)* hermaphrodite gonads were similar to those of the wild type and significantly higher than those of *ced-3(n717)* animals.

background. The *ced-1(e1735)* mutation allows corpses to persist and thus provides a more sensitive assay for germ cell death, as it amplifies the difference in germ cell corpse number between wild-type and *ced-3* mutant animals. Again we found that *ced-9(n1950)* is essentially indistinguishable from the wild-type allele in the *ced-1(e1735)* background.

The gene *egl-1* is a negative regulator of *ced-9* and is required for all somatic deaths (Conradt and Horvitz, 1998). Surprisingly, *egl-1(lf)* mutants are indistinguishable from the wild type with respect to germ cell apoptosis (Fig. 3D; Conradt and Horvitz, 1998), suggesting that *egl-1* is not involved in this process. In summary, our data indicate that the molecular mechanisms that regulate the core apoptotic complex differ significantly between the soma and the germline.

Germ cell death only occurs during oogenesis

What triggers germ cell death? In wild-type nematodes, germ cell deaths occur exclusively in adult hermaphrodites; we observed no germ cell deaths in either adult males or in larvae of either sex (Fig. 3A and data not shown). The timing and location of germ cell deaths suggest that they are associated with oogenesis, which is also restricted to adult hermaphrodites. To test this hypothesis, we examined the effect on germ cell death of mutations that affect sexual identity or germ cell differentiation (Table 1).

Table 1. Germ cell death requires oogenesis

Genotype	Karyo-type	Somatic phenotype	Germline phenotype	*n*	No. of germ corpses per gonad arm
wild type	XX	female	sperm, then oocytes	100	2
wild type	XO	male	sperm	100	0
*sdc-1(n485*lf*)*	XX	intersex	sperm	16	0
*fog-1(q253*lf*)*	XO	male	oocytes	10	3
gld-1(q126)	XO	male	sperm, then oocytes	32	6
gld-1(oz10)	XX	female	sperm	11	0
*mog-1(q223*lf*)*	XX	female	sperm	30	0
*her-1(n695*gf*)*	XX	intersex	sperm, then oocytes	7	>20
*tra-1(e1076*lf*)*	XX	male	sperm, then oocytes	19	7
gld-1(q485)	XX	female	tumorous	23	0
glp-4(bn2)	XX	female	arrest at mitotic prophase	21	0

Germ cell corpses were scored 36 hours after the L4/adult molt using Nomarski optics, as described in Materials and Methods. Because the germline of sex determination mutants is often abnormal, we also crossed the sex determination and germline proliferation mutations into a *ced-3(n717)* or *ced-4(n1162)* background and/or an engulfment mutant background and re-scored the double mutants for germ cell corpses. As expected, we observed almost no corpses in the *ced-3(lf)* double mutants, nor did the introduction of an engulfment defect uncover any germ cell death in mutants that had no apparent germ cell corpses (data not shown). Thus, the apparent cell corpses that we identified in these mutants were indeed comparable to those that we saw in wild-type animals and not oddly formed oocytes, sperm or a germline abnormality.

In *C. elegans*, the fundamental difference between males and hermaphrodites is the ratio of sex chromosomes to autosomes (the X:A ratio): males carry one X chromosome, hermaphrodites two (Meyer, 1997). To assess whether the X:A ratio determines the extent of germ cell apoptosis, we analyzed the germline of masculinized XX animals, which only produce sperm instead of oocytes (*sdc-1*(*lf*), *tra-1*(*lf*)). These animals exhibited no germline corpses. In contrast, feminized XO animals (*fem-1*(*gf*), *tra-1*(*gf*)), which produce oocytes, had germ cell death. Together, these results suggest that physiological germ cell apoptosis is determined not by sex chromosome dosage but rather by the sex of the animal (Table 1).

Because the soma controls many germline events (Clifford et al., 1994), we next asked whether germ cell apoptosis is determined by the sex of the soma or the sex of the germline. We found that germ cell death still occured in XO animals with a male soma but a feminized germline (*fog-1*(*lf*) and *gld-1(q126)*) (Table 1). Conversely, mutations (*gld-1(oz10)* and *mog-1*(*lf*)), which masculinize the germline of XX hermaphrodites such that only sperm are produced, resulted in the absence of germ cell corpses. We also observed dying germ cells in animals in which the germline was incompletely masculinized and still generated both sperm and oocytes (e.g., in XX intersex or pseudomale animals; *sdc-1*(*lf*), *her-1*(*gf*), and *tra-1*(*lf*)). These results indicate that germ cell death requires a female germline and is independent of the sex of the soma.

Finally, we asked whether entry into meiosis and differentiation into oocytes are required for germ cells to die. Mutations have been identified that affect the ability of germ cells to proliferate and differentiate (Ellis and Kimble, 1994). For example, in *gld-1(q485)* hermaphrodites, meiotic differentiation is blocked, resulting in a tumorous germline filled with mitotically proliferating germ cells (Francis et al., 1995). We found that these animals did not contain germ cell corpses in the tumorous mitotic region (Table 1). However, some germ cells entered meiosis in older *gld-1(q485)* hermaphrodites; such cells were capable of undergoing cell death. Similarly, we found no germ cell corpses in *glp-4* mutants, in which germ cells were blocked in mitotic prophase and failed to proliferate and differentiate (Table 1). Together, these results suggest that mitotic germ cells are protected from apoptosis. Since we observed germ cell deaths if, and only if, oogenesis was also occurring, both in the wild type and in the various mutant backgrounds tested, we infer that germ cell death is either an integral part or a direct by-product of the oogenic program.

Germ cell apoptosis requires ras/MAPK pathway activation

In wild-type animals, germ cell death occurs near the region where cells exit the pachytene stage (Fig. 2A and data not shown). To test whether apoptotic germ cell death might be functionally linked to meiotic progression, we examined whether mutations that affect differentiation or meiotic cell cycle progression influence germ cell death. Previous studies have shown that the ras/MAPK signaling cascade is required for exit of *C. elegans* germ cells from pachytene arrest (Church et al., 1995). Known members of this signaling pathway include LET-60 Ras, LIN-45 Raf, MEK-2 MAPK/ERK kinase and MPK-1 MAP kinase (MAPK) (Kayne and Sternberg, 1995). Strong loss-of-function alleles in any of these four genes block exit from pachytene during oogenesis, resulting in sterility (Church et al., 1995, and E. L., T. Schedl, R. Francis, M.-H. Lee, and K. Kornfeld, unpublished data). We found that these mutations also block programmed germ cell death (Table 2). Thus, activation of the ras/MAPK

Table 2. Germ cell death requires ras/MAPK pathway activation

Genotype	*n*	No. of SYTO 12 positive cells
wild type	31	17
ced-9(n2812)	25	25
ced-3(n717)	41	0.6
ced-4(n1162)	34	0.0
let-60(dx16)	56	1.1
let-60(s1124)	19	0.9
ced-4(n1162); let-60(dx16)	49	0.3
*let-60(n1046*sd*)*	17	6.1
lin-45(dx19)	56	0.04
ced-9(n2812); lin-45(dx19)	26	9.4
mek-2(n2678)	43	≤0.2
mek-2(n2678); ced-9(n2812)	26	4.6
mek-2(n2678); ced-3(n717)	42	≤0.1
mpk-1(oz140)	44	≤0.3

Germ cell corpses were scored 36 hours after the L4/adult molt using the vital dye Syto12, as described in Materials and Methods. Animals with loss-of-function mutations in the ras/MAPK pathway (*let-60(ras), lin-45(raf), mek-2*(MAPK/ERK kinase) and *mpk-1*(MAP kinase)) have smaller gonads and fewer germ cells, which might account for the lower numbers of germ cell deaths observed in the *ced-9(n2812)* double mutants with *lin-45(dx19)* and *mek-2(n2678)* when compared to the wild type or *ced-9(n2812)* alone.

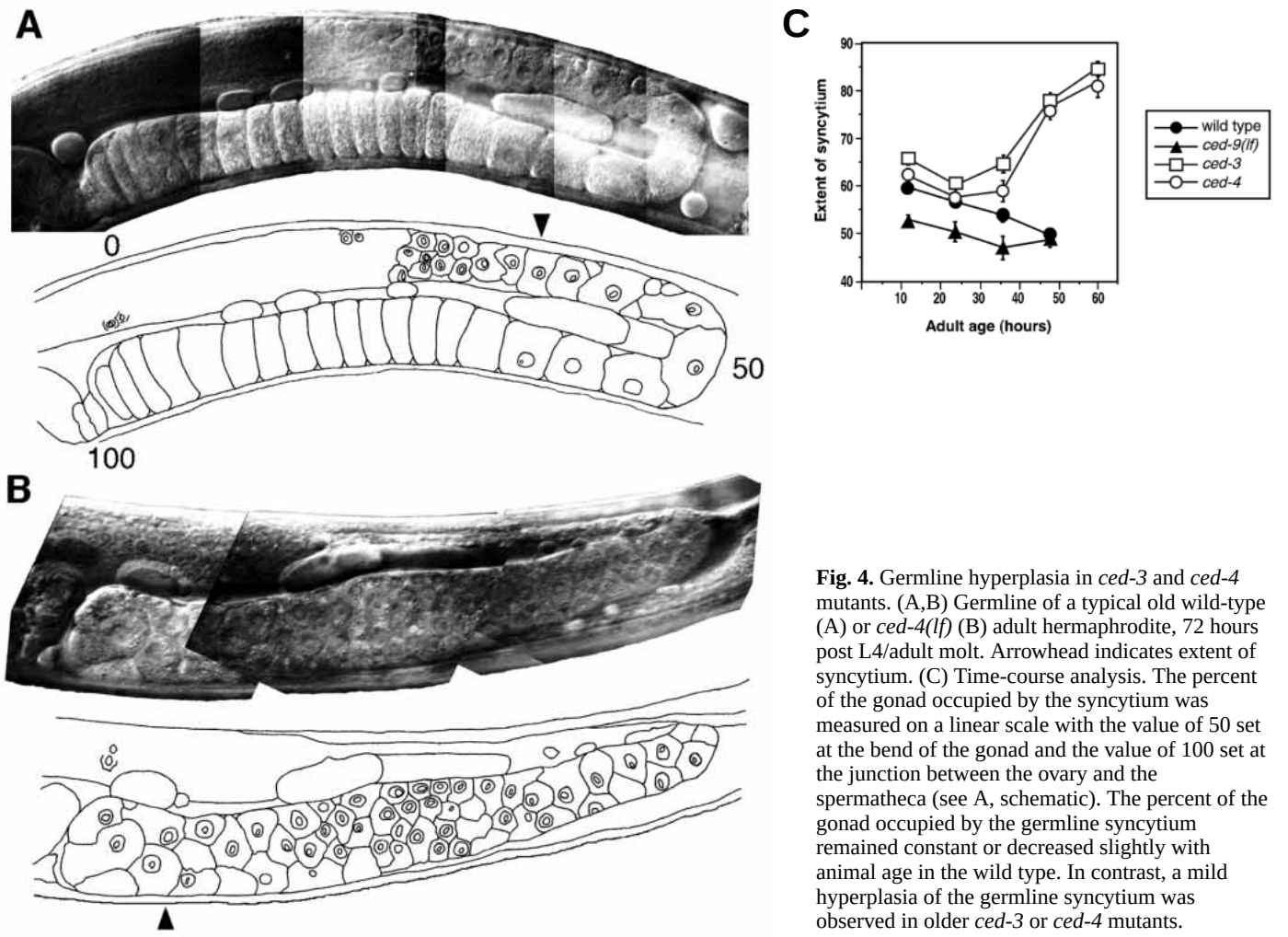

Fig. 4. Germline hyperplasia in *ced-3* and *ced-4* mutants. (A,B) Germline of a typical old wild-type (A) or *ced-4(lf)* (B) adult hermaphrodite, 72 hours post L4/adult molt. Arrowhead indicates extent of syncytium. (C) Time-course analysis. The percent of the gonad occupied by the syncytium was measured on a linear scale with the value of 50 set at the bend of the gonad and the value of 100 set at the junction between the ovary and the spermatheca (see A, schematic). The percent of the gonad occupied by the germline syncytium remained constant or decreased slightly with animal age in the wild type. In contrast, a mild hyperplasia of the germline syncytium was observed in older *ced-3* or *ced-4* mutants.

pathway, which is required for germ cells to exit pachytene, is also a prerequisite for germ cell death.

The failure of pachytene-arrested cells to undergo programmed cell death could be explained either by an absence of the death machinery in these cells or by the presence of a death-suppressing activity. To distinguish between these two possibilities, we constructed double mutants carrying strong loss-of-function mutations in a ras/MAPK pathway gene and in the cell death suppressing gene *ced-9*. If the cell death machinery were already present in pachytene-arrested cells, then the absence of *ced-9* might result in its activation. Consistent with this hypothesis, germ cell death reappeared in *ced-9; lin-45* and *mek-2; ced-9* double mutants (Table 2). These results suggest that pachytene-arrested cells have the ability to die, but are normally protected from apoptosis through a *ced-9*-dependent mechanism.

Cell death is required for the maintenance of germline homeostasis

In adult hermaphrodites, germ cell differentiation and progression through the gonad are coordinated in such a way that the position of a germ cell within the gonad is a reliable indicator of its developmental status. Cell death appears to be required to maintain this steady-state structure: in strong *ced-3* and *ced-4* loss-of-function mutants, the absence of germ cell death results in a gradual increase in the number of syncytial germ cells (Fig. 4). As the syncytium expands to accommodate the extra germ cell nuclei, the area devoted to fully grown oocytes decreases over time (Fig. 4C). In old *ced-3* or *ced-4* animals, this syncytial hyperplasia is so extensive that occasionally only one or two full-sized oocytes are present within a gonad, as opposed to greater than a dozen oocytes in wild-type animals (Fig. 4A,B).

Germ cell death is conserved among nematode species

To determine whether germ cell death also occurs in other nematodes, we looked for corpses in the germlines of other nematode species. We found that *C. briggsae*, *C. vulgaris* and *C. remanei*, three close relatives of *C. elegans*, as well as *Pelodera strongyloides*, a more distant relative (Fitch and Thomas, 1997), had programmed cell deaths in their germlines (data not shown). Another distant nematode relative, *Rhabditella axei*, is distinctive among the species that we observed because its germline is composed of germ nuclei within large compartments. We failed to observe any germ cell death in this species. *Panagrellus redivivus*, another free-living nematode, has germ cell death in both males and females (Sternberg and Horvitz, 1981). These observations indicate

germline programmed cell death is evolutionarily conserved among nematode species.

DISCUSSION

Germ cell apoptosis in *C. elegans*

Apoptosis is a common feature of metazoan germline development (Tilly, 1996). Despite its prevalence, little is known about the molecular regulation of programmed cell death in the germline. Here we show that cell death is a major outcome for germ cells in the adult *C. elegans* hermaphrodite and provide the first detailed description of the morphology, genetics and biology of germ cell death in this organism. We establish that germ cell death is extensive, apoptotic in nature and strictly dependent on oogenesis. We also provide genetic evidence that regulation of the apoptotic machinery occurs via mechanisms distinct from those used in the soma.

More than half of all female germ cells die

Cell death is a very common fate in the *C. elegans* female germline. A wild-type hermaphrodite generates about 2000 germ cells over its lifetime and produces about 300 sperm and a slightly larger number of mature oocytes (Schedl, 1997). We have observed over 70 germline corpses in gonad arms of old animals in which cell deaths are not efficiently removed because of a mutation that blocks cell-corpse engulfment (Fig. 3C), and over 100 in some animals with two such mutations (data not shown), indicating that at least 100 cells must die in each gonad arm. However, the total number of germ cell deaths is likely to be much higher, for two reasons. First, the mutations that affect engulfment are incompletely penetrant and only prevent a fraction of all corpses from being engulfed (Hedgecock et al., 1983; Ellis et al., 1991a). Second, germ cell corpses are also eliminated by passage into the uterus or by secondary necrosis (data not shown). Thus, we estimate that programmed cell death is the fate of about half of female germ cells.

This estimate is supported by our analysis of the kinetics of germ cell apoptosis. It takes approximately 1 hour for a cell to die and disappear, and about 2-3 corpses can be observed at any given time (Figs 1E-J, 3A). Integration of the number of corpses observed over time in wild-type adult hermaphrodites suggests that approximately 150 germ cells die within the first 3-4 days of adulthood in each gonad arm (Fig. 3A).

Germ cells die by apoptosis

Several lines of evidence indicate that the massive amounts of germ cell deaths observed in adult wild-type hermaphrodites are apoptotic in nature. First, the dying germ cells resemble the programmed cell deaths that occur during *C. elegans* development and exhibit many of the morphological and ultrastructural features characteristic of apoptosis, including cytoplasmic and nuclear condensation, chromatin aggregation, and rapid recognition and phagocytosis by surrounding cells (Figs 1, 2). Second, dying germ cells can be labeled with fluorescent dyes that specifically stain apoptotic cells (Fig. 1D and data not shown). Third, the genetic pathway that mediates apoptosis during development also functions in the germline: *ced-3* and *ced-4* are required for germ cells to die (Fig. 3A) and *ced-9* is required to protect germ cells from apoptotic death. Mutants homozygous for a weak loss-of-function mutation of *ced-9*, *n1653*, have many more cell deaths in the germline than wild-type animals (Fig. 3B). In strong (putative null) *ced-9* mutants, almost all potential oocytes die by programmed cell death (T. L. G. and M. O. H., unpublished), which probably accounts for the low brood size observed of *ced-9(lf)* mutants (Hengartner et al., 1992).

Germ cell death and somatic cell death are regulated by different pathways

Interestingly, although *ced-9* is vital for germ cell survival, the gain-of-function mutation *ced-9(n1950)*, which completely suppresses somatic cell death, does not prevent germ cell death

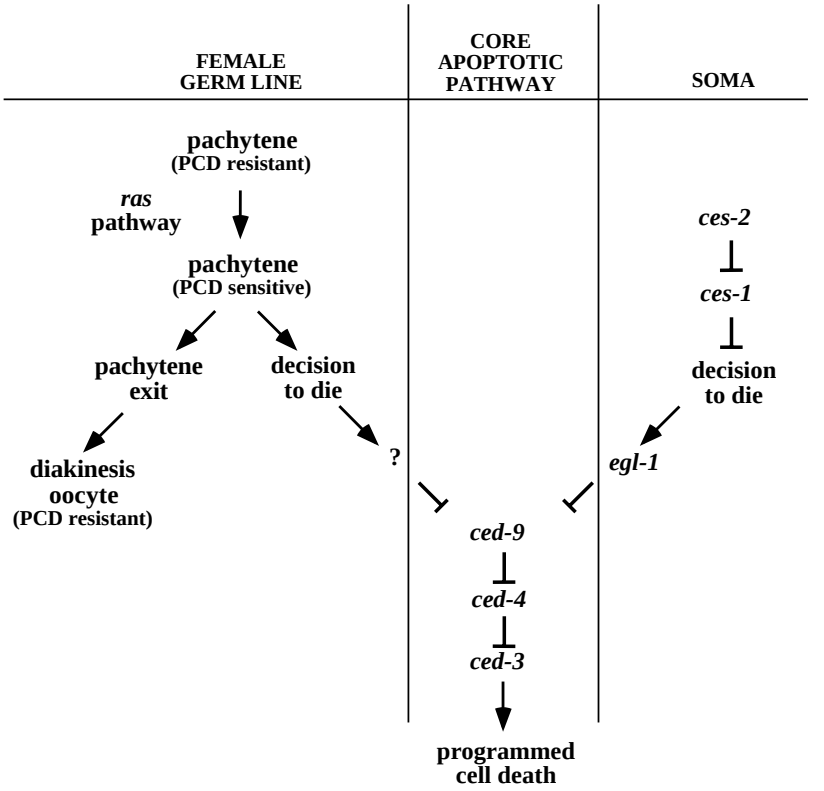

Fig. 5. Distinct signals control apoptosis in the soma and in the germline. In the soma, cell-type-specific regulators of cell death determine cell survival. For example, the *ces-2* and *ces-1* genes control the life-versus-death decision of the two NSM sister cells. Most if not all somatic deaths are mediated by the BH3 gene *egl-1*. In the germline, activation of the ras/MAPK pathway promotes progression of meiotic germ cells from an apoptosis-resistant, pachytene stage to a transient selection stage, where cells are highly sensitive to apoptosis. Via a currently unknown mechanism, some cells are rescued from death and proceed into diakinesis, at which point they are again protected from death. The remaining cells activate the apoptotic machinery and die.

(Fig. 3D). The *n1950* mutation (G169E) alters an invariant glycine residue located in the conserved BH1 domain, which is found in most CED-9/Bcl-2 family members (Hengartner and Horvitz, 1994b). The requirement of this domain for interaction between pro-survival Bcl-2 family members and the BH3 domain of pro-apoptotic Bcl-2 family members (Reed, 1994; Yin et al., 1994) suggests that *n1950* might affect the interaction of CED-9 with pro-apoptotic *C. elegans* proteins. An excellent candidate for such an interactor is the BH3 domain protein EGL-1, which physically interacts with CED-9 and is essential for all developmental cell deaths (Conradt and Horvitz, 1998). Since *ced-9(gf)* and, more importantly, *egl-1* mutants do not affect germ cell apoptosis (Fig. 3D, E), we propose that distinct CED-9 partners might be used to control germline and somatic cell death (Fig. 5).

Germ cell death occurs only during oogenesis and requires ras/MAPK pathway activation

Germ cell death is observed only during a specific stage of oogenesis, shortly before germ cells exit the pachytene stage of prophase I. Mutations that inactivate the ras/MAPK signaling cascade prevent cells from exiting pachytene arrest (Church et al., 1995) and also prevent germ cell death (Table 2). The ras/MAPK pathway determines the fate of several somatic cell lineages. For example, activation of this pathway by the EGF receptor homolog LET-23 is necessary and sufficient for the six vulval precursor cells to differentiate and form vulval tissue (reviewed by Kayne and Sternberg, 1995). In contrast, the ras/MAPK pathway appears to play a permissive (rather than instructive) role in the specification of germ cell death, as activation of the pathway is necessary, but not sufficient, for germ cells to die, as germ cell death levels are not increased in mutations that hyperactivate the pathway (Table 2).

The ras/MAPK pathway might directly regulate the cell death machinery, presumably via phosphorylation. An interaction between raf and Bcl-2 has been reported in mammalian cells (Wang et al., 1996), and phosphorylation modulates the activity of both Bcl-2 and Bad (Datta et al., 1995; Chang et al., 1997; del Peso et al., 1997; reviewed by Chao and Korsmeyer, 1998). Alternatively, ras/MAPK pathway-dependent signaling might indirectly affect germ cell apoptosis, e.g., by promoting the progression of pachytene-stage cells, which are resistant to apoptosis (Table 2), to a later differentiation stage that is more sensitive to pro-apoptotic signals (Fig. 5). Presumably, further differentiation restores death resistance to the mature oocytes (Fig. 5). Alternation between death-resistant and death-sensitive stages during differentiation is a common tactic used to selectively eliminate subpopulations of cells and has been reported to occur during germ cell maturation in chicken ovaries, as well as during B and T cell development (MacLennan, 1994, Robey and Fowlkes, 1994).

Germ cell death might be used to eliminate meiotic cells that act as nurse cells

Does the extensive apoptosis that occurs during oogenesis serve any purpose? One possibility is that an oogenesis-specific checkpoint uses apoptosis to eliminate unfit cells, e.g., germ cells with damaged DNA. This hypothesis would imply that over half of the female germ cells are so defective that they have to be removed. However, a number of observations suggest that the cells that die under normal growth conditions are healthy. First, all germ cell nuclei appear grossly similar when stained for DNA or observed using electron microscopy (except for cells already undergoing programmed cell death; Figs 1, 2). Second, *ced-3* and *ced-4* mutants (in which no germ cell deaths occur) do not show any increase in the number of defective oocytes or in embryonic lethality (Ellis and Horvitz, 1986; Hengartner et al., 1992; and data not shown). Thus, germ cells that are rescued from death in these mutants seem to be healthy and functionally equivalent to the cells that normally survive.

A second possibility consistent with our results is that apoptosis is used to cull germ cells that are created in excess to synthesize cytoplasmic components required by mature oocytes. Unlike mammals and *Drosophila*, *C. elegans* has no morphologically distinct nurse cells. Rather, this function is performed by early meiotic germ cells, which show a high level of transcriptional activity (Starck, 1977). Transcripts generated by these cells are exported to the common cytoplasmic core and subsequently incorporated into the maturing oocytes (Gibert et al., 1984). As is the case in other species, *C. elegans* presumably needs many nurse cells to provide for each oocyte. Because *C. elegans* nurse cells are also gametic precursors, there are many more candidate oocyte nuclei than can be accomodated by the available cytoplasm. We propose that *C. elegans* uses programmed cell death to solve this problem: once their function as nurse cells is fulfilled, excess nuclei might be eliminated by a stochastic selection mechanism that matches the number of oocyte precursors that are allowed to survive and differentiate to the amount of common cytoplasm. The nature of this putative selection process is at present unclear but should be amenable to genetic analysis.

The idea that germ cell death is used to eliminate meiotic cells that act as nurse cells can account for several apparently unrelated observations. First, it explains why germ cell death is only observed in adult hermaphrodites. Larvae have no germ cell death because oogenesis starts only very late in larval development. Males have no germ cell death because sperm contain little cytoplasm and therefore all germ cells can be allowed to form gametes. Second, the timing of germ cell deaths within the oogenic pathway – shortly before germ cells enlarge in size to form mature oocytes – would maximize the time that germ cells can function as nurse cells, while still allowing for the elimination of excess cells before they accumulate too much cytoplasm. Indeed, the suggestion that nuclei are expendable but cytoplasm is valuable might explain why dying germ cells pump almost all of their cytoplasm into the common cytoplasmic core before pinching off the syncytium (Fig. 2C,D).

While, in *C. elegans*, germ cell deaths are normally restricted to pachytene stage pre-oocytes, it might be possible under special circumstances or in other nematodes for other germ cell types to die. For example, in the nematode *Panagrellus redivivus*, germ cell deaths occur in both the male and the female germlines (Sternberg and Horvitz, 1981). The biological rationale for these deaths is likely to be distinct, suggesting that germ cell death in nematodes can be regulated at multiple levels.

Death in a syncytium

How does the germline syncytium prevent the apoptotic

machinery from spreading from a condemned cell to other nuclei? Dying nuclei rapidly cellularize away from the common syncytium. This process may sequester pro-apoptotic factors, such as active caspases, away from the cells that should survive and become oocytes. However, cellularization and presentation of signals for engulfment must temporally follow the initial activation of *ced-3* and *ced-4*, as these events do not occur in animals mutant for either gene. The nature of the cellularization machinery is unknown; an attractive hypothesis is that its activation is triggered by cleavage of specific substrates by the CED-3 protease.

A genetic pathway for germ cell death

Genetic analysis of programmed cell death in *C. elegans* has been used with great success to identify the molecular basis of apoptosis. These studies revealed that the central machinery that controls all programmed cell deaths has been conserved through evolution (Yang et al., 1995; Zou et al., 1997; Hengartner, 1997, 1998). We have shown here that the soma and germline use a common apoptotic execution machinery. However, these two types of tissues must use different regulatory mechanisms to control activation of this machinery, as neither the BH3-domain protein EGL-1 nor the gain-of-function mutation in *ced-9* affects germ cell death. We propose the existence of a distinct genetic pathway that specifically controls the decision between differentiation and death in the germline. Further analysis of *C. elegans* germ cell death may thus identify additional regulatory mechanisms that control apoptosis in mammals.

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