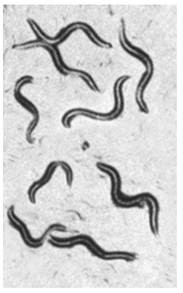

The genome sequence of *Caenorhabditis elegans* is due to be completed around the end of this year. *C. elegans* will therefore be the first animal to have its genome completely sequenced. To mark this outstanding achievement, we are publishing a series of articles celebrating worm genetics. Articles will review the contributions that worm genetics has made to fundamental aspects of biology, such as cell death, signal transduction, sex determination and neurobiology.

Genetics of programmed cell death in *C. elegans*: past, present and future

MARK M. METZSTEIN (mmmetz@mit.edu)
GILLIAN M. STANFIELD (gillian@mit.edu)
H. ROBERT HORVITZ (horvitz@mit.edu)

Genetic studies of the nematode Caenorhabditis elegans have defined a variety of single-gene mutations that have specific effects on programmed cell death. Analyses of the genes defined by these mutations have revealed that cell death is an active process that requires gene function in cells that die. Specific genes are required not only to cause cell death but also to protect cells from dying. Gene interaction studies have defined a genetic pathway for the execution phase of programmed cell death in C. elegans. Molecular and biochemical findings are consistent with the pathway proposed from these genetic studies and have also revealed that the protein products of certain cell-death genes interact directly. This pathway appears to be conserved among organisms as diverse as nematodes and humans. Important questions remain to be answered about programmed cell death in C. elegans. For example, how does a cell decide to die? How is cell death initiated? What are the mechanisms of action of the cell-death protector and killer genes? What genes lie downstream of the cell-death execution pathway? The conservation of the central cell-death pathway suggests that additional genetic analyses of programmed cell death in C. elegans will help answer these questions, not only for this nematode but also for other organisms, including ourselves.

The elimination of unwanted cells by programmed cell death is an important developmental and homeostatic process in multicellular organisms, including the nematode *Caenorhabditis elegans*[1]. During the development of the *C. elegans* hermaphrodite, 1090 cells are generated, of which 131 undergo programmed cell death[2–4] (Fig. 1). Genetic analyses of *C. elegans* have identified genes that function in programmed cell death, ordered these genes in a genetic pathway and led to the finding that similar genes control programmed cell death in other organisms, including mammals. In this review, we concentrate on the genetic and developmental studies of *C. elegans* that have resulted in these discoveries. We mention only briefly molecular biological and biochemical studies of programmed cell death, which have been described in a number of recent reviews[5–8]. We focus primarily on mechanisms of cell killing and, for this reason, do not discuss in detail the engulfment or degradation of cell corpses.

egl-1, *ced-4*, *ced-3* and *ced-9* are global regulators of programmed cell death in *C. elegans*

Three *C. elegans* genes, *egl-1*, *ced-4* and *ced-3*, seem to be required for all somatic programmed cell death to occur. Loss-of-function (lf) mutations in *egl-1*, *ced-4* or *ced-3* lead to the survival of essentially all cells that undergo programmed cell death during wild-type development[9,10]. The *egl-1* (*egl*, egg laying defective) gene was defined originally by gain-of-function (gf) mutations that cause a dominant egg-laying defect attributable to the loss of functional HSN neurons in hermaphrodites[11], a defect found to be caused by the ectopic programmed deaths of these neurons[9]. The isolation of a *cis*-dominant suppressor of the *egl-1*(gf) egg-laying defect revealed that the loss of *egl-1* function causes an absence of programmed cell death[10]. The first allele of *ced-4* (*ced*, cell death abnormal) was identified as an extragenic suppressor of the egg-laying defect of *egl-1*(gf) mutants[9]. Mutations in *ced-3* were identified as suppressing the presence of persistent cell corpses seen in mutant animals defective in cell-corpse engulfment[9]. Subsequently, many more alleles of *ced-4* and *ced-3* have been obtained by screening for either the absence of cell corpses (e.g. Ref. 12), or the presence of extra living cells that would have died in wild-type animals (undead cells; e.g. Ref. 13), or by suppressing the lethality caused by loss-of-function mutations in the cell-death protection gene *ced-9* (see below; e.g. Ref. 14).

The activity of the gene *ced-9* protects most, if not all, cells from undergoing programmed cell death during *C. elegans* development. Loss-of-function mutations in *ced-9* lead to sterility and maternal-effect lethality, as a consequence of the ectopic activation of programmed cell death in cells that normally live[15]. In *ced-9*(lf) animals many cell types, including neurons, ectodermal

 0168-9525/98/$19.00
PII: S0168-9525(98)01573-X

cells and even undifferentiated blast cells, can activate the death program[15]. All of these deaths are suppressed by mutations in *ced-4* and *ced-3* (Ref. 15). *ced-9* was defined originally by a gain-of-function allele that blocks programmed cell death (see below). Loss-of-function alleles were obtained by identifying *cis*-dominant suppressors.

The molecular characterization of *C. elegans* cell-death genes has been key in revealing some of the biochemical mechanisms of programmed cell death in all animals. The discovery that *ced-3* encodes a member of the caspase (cysteine aspartate-specific protease) family of proteases[16,17] was instrumental in identifying a role for mammalian caspases in the regulation of programmed cell death[5]. A protein related to the CED-4 protein, APAF-1, appears to be involved in effecting programmed cell death in mammalian cells[18], and both of these proteins might require nucleotide binding to function[19–21]. The cloning of the *ced-9* gene revealed that its protein product is similar to the human Bcl-2 protein[22], which had been implicated in programmed cell death in mammalian cell culture experiments[23]. This discovery, along with the findings that human Bcl-2 could block programmed cell death in *C. elegans* and could substitute for the *C. elegans ced-9* gene in *ced-9*-deficient mutants[22,24], strongly suggests that the molecular mechanism of programmed cell death is evolutionarily conserved. Finally, EGL-1 has functional and molecular similarity to the 'BH3-only' (BH, Bcl-2 homology domain) subfamily of Bcl-2-like proteins: BH3-only proteins are capable of inducing programmed cell death when overexpressed in mammalian cell systems (Ref. 10 and references therein). The *egl-1* gene is the first member of the BH3-only family for which a loss-of-function phenotype has been described, and this phenotype indicates not only that a gene of this class can cause programmed cell death when overexpressed, but also that such a gene normally functions in this way.

Genetic evidence suggests that *egl-1*, *ced-4* and *ced-3* function exclusively in programmed cell death. Programmed cell death is not essential for the viability of *C. elegans*, and animals homozygous for loss-of-function mutations in *egl-1*, *ced-4* or *ced-3* appear superficially normal in size and movement[9]. Undead cells in *C. elegans* can differentiate[9,13,25] and can even function[26]. Loss-of-function alleles of *ced-4* include some that are null by genetic and molecular criteria[12]. More than 90 alleles of *ced-3* have been obtained, of which more than 60 have been analyzed genetically and molecularly (Ref. 16; H.R. Horvitz *et al.*, unpublished), and some are altered in residues essential for CED-3 enzymatic activity (Refs 16, 27; S. Shaham, B. Davies, P. Reddien and H.R. Horvitz, unpublished). However, no allele is known that is unambiguously predicted to eliminate expression of the entire CED-3 protein. Based on this consideration, it is conceivable that *ced-3* has a role during the development of *C. elegans* that is unrelated to its enzymatic activity and that null alleles of *ced-3* lead to additional (possibly lethal) defects. There is only one known loss-of-function allele of *egl-1*. This allele behaves as a null by genetic criteria, but molecular analysis indicates that at least a portion of the EGL-1 protein could still be produced, so it is not clear whether this mutation completely eliminates *egl-1* activity[10].

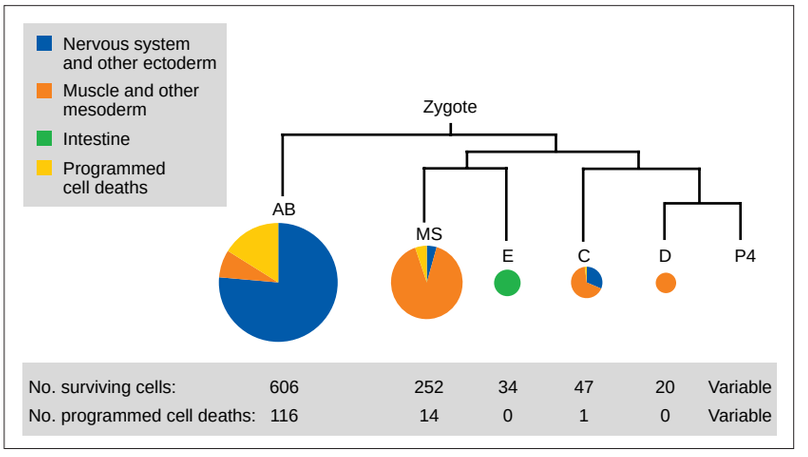

FIGURE 1. Distribution of programmed cell deaths within the cell lineage of the *Caenorhabditis elegans* hermaphrodite. Horizontal lines indicate early embryonic cell divisions, colors represent cell fates and areas are proportional to the total numbers of embryonic and postembryonic descendants from the primary blastomeres AB, MS, E, C and D. The germline, generated from blast cell P4, unlike other tissues in *C. elegans*, continuously proliferates and gives rise to variable numbers of cells and cell deaths[3,64]. Programmed cell deaths occur in the AB, MS and C cell lineages, but not in the E cell lineage, which produces only intestine, or the D cell lineage, which produces only muscle[2,4,44]. Cell death is a particularly common fate in the AB cell lineage, which produces most of the nervous system and in which 116 of the 722 cells generated proceed to die. Adapted from Ref. 4.

Because *ced-9* (lf) causes early lethality it is difficult to know whether *ced-9* is required for any process other than the regulation of programmed cell death. However, double mutants between a null allele of *ced-9* and either *ced-3* or *ced-4* do not appear to have any defects beyond those seen in *ced-3* or *ced-4* single mutants[14], arguing that *ced-9* function is as cell-death specific as are the functions of *ced-3* and *ced-4*.

Pathway to death

Genetic experiments have ordered the functions of *egl-1*, *ced-9*, *ced-4* and *ced-3*. First, as described above, loss-of-function mutations in *ced-9* lead to lethality by causing the ectopic activation of programmed cell death. These deaths are suppressed by loss-of-function mutations in *ced-4* or *ced-3*, indicating that *ced-9* normally functions to negatively regulate *ced-4* and *ced-3* (Ref. 15). A simple pathway consistent with these observations places *ced-9* genetically upstream of *ced-4* and *ced-3* (Fig. 2a). However, these observations do not exclude the possibility that *ced-9* might function biochemically to inhibit the activities rather than the expression or activation of *ced-4* and *ced-3* (e.g. Ref. 28). By contrast, loss of *egl-1* function, while preventing somatic cell death just as does loss of *ced-4* or *ced-3* function, does not suppress the lethality resulting from a loss of *ced-9* function[10]. This finding suggests

(a)

Genotype	Phenotype	
	Viable?	Programmed cell death
Wild-type	Yes	Normal
ced-9(lf)	No	Excess
ced-4(lf)	Yes	Reduced
ced-3(lf)	Yes	Reduced
egl-1(lf)	Yes	Reduced
ced-4(lf);*ced-9*(lf)	Yes	Reduced
ced-9(lf);*ced-3*(lf)	Yes	Reduced
ced-9(lf);*egl-1*(lf)	No	Excess

(b)

Overexpressed gene	Genotype	Cell killing
ced-3	*ced-4*(+)	+++
ced-4	*ced-3*(+)	+++
ced-3	*ced-4*(lf)	++
ced-4	*ced-3*(lf)	+/–

(c)

Genotype			
ced-4	*ced-9*	*ced-3*-induced killing	
+	lf	++++	*ced-9* protection
+	+	+++	
lf	lf	++	No *ced-9* protection
lf	+	++	

(d)

Genotype	*egl-1*-induced killing
Wild-type	+++
ced-3(lf)	–
ced-4(lf)	–
ced-9(gf)	–

FIGURE 2. Interactions among *egl-1*, *ced-9*, *ced-4* and *ced-3*. Genetic epistasis experiments and studies in which *egl-1*, *ced-9*, *ced-4* and *ced-3* have been overexpressed to kill cells in different genetic backgrounds have suggested an order of action of these genes[10,15,29]. The relative efficiency of cell killing is represented by the number of plus (+) signs, as interpreted from data in Refs 10, 29. (a) Loss-of-function mutations in *ced-4* or *ced-3* suppress the lethality caused by loss of *ced-9* function, suggesting that *ced-9* functions upstream of *ced-4* and *ced-3*. By contrast, a loss of function mutation in *egl-1* does not suppress *ced-9* (lf) lethality, suggesting that *egl-1* functions upstream of *ced-9*. (b) The overexpression of *ced-3* or *ced-4* can kill cells in wild-type *Caenorhabditis elegans*. In the absence of *ced-4* activity, overexpressed *ced-3* can still kill, albeit at a slightly reduced efficiency. By contrast, in the absence of *ced-3* activity, *ced-4* is almost incapable of killing cells. These results suggest *ced-4* acts upstream of *ced-3* to activate programmed cell death. (c) Endogenous *ced-9* activity protects cells from programmed cell death induced by overexpression of *ced-3*, because killing is more efficient in a *ced-9*(lf) background than in a *ced-9*(+) background. While elimination of *ced-4* function reduces the killing activity of *ced-3*, this residual *ced-3* activity is no longer inhibited by *ced-9*, suggesting that *ced-9* functions to protect against programmed cell death, at least in part, by negatively regulating *ced-4*. (d) The overexpression of *egl-1* can also kill cells. This killing is blocked by loss-of-function mutations in *ced-4* or *ced-3* or by a gain-of-function mutation in *ced-9*, suggesting that *egl-1* acts upstream of these genes.

that *egl-1* acts genetically upstream of and functions as a negative regulator of *ced-9* (Fig. 2a).

Because in a *ced-9* loss-of-function mutant background the allelic states of *ced-4* and *ced-3* affect the fates of cells that would normally live, either the *ced-4* and *ced-3* gene products are normally present in most cells before the time at which cells differentiate or the loss of *ced-9* activity leads to the ectopic expression of *ced-4* and *ced-3*. Although the expression patterns of *ced-4* and *ced-3* at the RNA and protein levels have not been described, other molecular genetic evidence suggests that the former model is more likely to be correct. Specifically, transcriptional overexpression of *ced-4* and/or *ced-3* from transgenes, at least in certain types of developing *C. elegans* neurons, causes these cells to undergo programmed cell death[29]. The efficiency of this killing is dependent on the genotype of the animal carrying the transgene[29]. For instance, killing by *ced-3* overexpression is more efficient when the transgenic animal is wild-type for *ced-4* and/or mutant for loss-of-function of *ced-9* (as described in more detail below). Because changes in the *ced-4* or *ced-9* genotype influence killing, *ced-4* and *ced-9* products are likely to be normally present in these cells[29]. Similar arguments suggest that *ced-3* is also likely to be present.

Transcriptional overexpression has also been used to order the functions of cell-death genes. Specifically, in the absence of *ced-4* activity, *ced-3* overexpression is able to kill cells, although at a reduced level in comparison with killing in a wild-type *ced-4* background (Fig. 2b). By contrast, when *ced-3* activity is reduced, *ced-4* overexpression kills very inefficiently (Fig. 2b). These data suggest that *ced-3* is needed for *ced-4*-induced killing but that *ced-4* is not needed for *ced-3*-induced killing. One simple model is that *ced-4* potentiates *ced-3* activity and that a sufficiently high level of *ced-3* can bypass the normal requirement for *ced-4* activity. Similar results have been obtained by the overexpression of *ced-4* and *ced-3* in insect cells[30].

Why, then, is there any killing by overexpressed *ced-4* in a *ced-3*(lf) background? One possibility is that killing is a consequence of *ced-4*-induced activation of the residual activity of the *ced-3* alleles tested (as described above, none of the known *ced-3* alleles is definitively a null mutation). Another possibility is that the overexpression of *ced-4* leads to the apparent absence of the cell not because the cell has died but, rather, because it is misplaced or not generated. We favor a third possibility: perhaps overexpressed *ced-4* can trigger programmed cell death independently of

ced-3, either through another caspase or independently of caspase activity. At least two additional genes with similarity to *ced-3* are present in *C. elegans* genomic sequences (P. Reddien and H.R. Horvitz, unpublished; M. Hengartner, pers. commun.).

Biochemical data have supported the hypothesis that *ced-4* is an upstream activator of *ced-3* and have provided a possible physical basis for their interaction. The CED-4 protein might bind directly to the proenzyme form of the CED-3 protein to facilitate its proteolytic conversion to the active enzyme[20,31,32]. In mammals APAF-1 (a CED-4-related protein) binds to and stimulates the processing of caspase-9 (a CED-3-like protein)[18,21]. High levels of CED-3 might relieve the need for CED-4 to facilitate conversion from the proenzyme form, explaining why *ced-3* overexpression can kill in the absence of *ced-4* activity.

Similar overexpression experiments suggest that *ced-9* acts by inhibiting *ced-4* function[29] (Fig. 2c). The presence of an endogenous wild-type *ced-9* allele partially inhibits the ability of overexpressed *ced-3* to kill cells that normally live: killing by ectopically expressed *ced-3* is less efficient in a *ced-9* wild-type animal than in a *ced-9* loss-of-function animal. However, in a *ced-4* mutant background, killing by *ced-3* is no less efficient in animals carrying a wild-type *ced-9* allele than in animals carrying a *ced-9*(lf) allele. These results indicate that, in the absence of *ced-4* activity, *ced-9* fails to protect against killing by overexpressed *ced-3*. Thus, *ced-9* protection against *ced-3* killing is mediated at least in part by *ced-4* (Fig. 2c). Results consistent with this hypothesis were also obtained by examining the effects of expression of combinations of *ced-9*, *ced-4* and *ced-3* in insect and mammalian cells[30,31].

A physical basis for the results of these genetic studies is suggested by the observation that CED-9 and CED-4 can interact directly (reviewed in Ref. 33). This interaction has been proposed to block the activity of CED-4, although the mechanism of such an inhibition is unknown. An interaction between CED-9 and CED-4 has been demonstrated *in vitro* and in yeast, insect and mammalian cells but not yet directly in *C. elegans*, although some *ced-9* and *ced-4* allele-specific interactions have been observed[34]. The human CED-4-like protein APAF-1 and a Bcl-2 family member, Bcl-x_L, might also interact directly[35,36].

Overexpression of *egl-1* can kill cells that normally live[10]. This killing is dependent on *ced-4* and *ced-3*, placing *egl-1* genetically upstream of these genes (Fig. 2d). As described above, *egl-1* also seems to function upstream of and act as a negative regulator of *ced-9*. The EGL-1 protein binds to the CED-9 protein, and it has been proposed that EGL-1 acts by releasing CED-4 from CED-9 (Ref. 10). Similarly, in mammals BH3-only proteins bind Bcl-2 family proteins (various references cited in Ref. 10).

Thus, current genetic and molecular data suggest the following model for the activation of programmed cell death (Fig. 3). After activation by upstream signals, the EGL-1 protein interacts with CED-9 and releases CED-4 protein from membrane-associated CED-9 protein (CED-9, like other Bcl-2 family members, has a hydrophobic C-terminus that probably causes it to be membrane-associated)[37]. Free CED-4 then interacts with

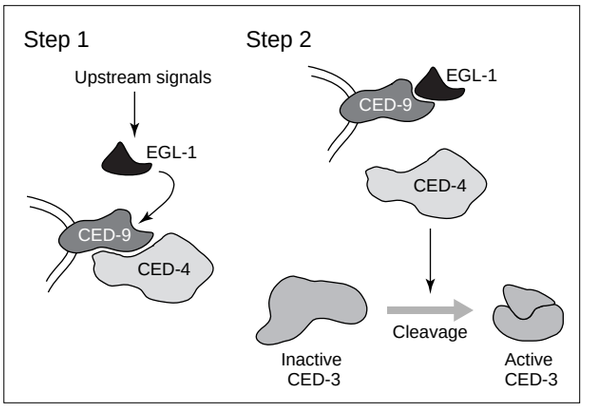

FIGURE 3. A model for the activation of programmed cell death in *Caenorhabditis elegans*. (a) Step 1: upstream signals lead to the production or activation of the EGL-1 protein. EGL-1 binds to CED-9, shown localized to cell membranes, leading to the release of CED-4 from CED-9. Although shown as a monomer, CED-9 likely functions as a dimer, as do other Bcl-2 family proteins[7]. (b) Step 2: free CED-4 then promotes the proteolytic cleavage of the proenzyme form of CED-3; p17 and p13 subunits derived from this processing assemble into the active form[27] (active caspases are thought to be tetramers, consisting of dimers of such a heterodimer; reviewed in Ref. 65). It is possible that CED-3 is complexed with CED-4 before EGL-1 mediates the release of CED-4 from CED-9.

and facilitates the processing of inactive pro-CED-3 to the active enzyme. The active caspase acts as the mediator of downstream events in cell death, eventually leading to the destruction of the cell by cleaving and, thereby, activating additional killing proteins and/or inactivating additional protecting proteins or proteins needed for cellular homeostasis.

ced-9 and *ced-4* have protecting and killing activities

Genetic evidence suggests that, in addition to its protective role, *ced-9* might also activate programmed cell death, at least in cells that normally die. Specifically, the survival of cells in weak *ced-3* mutants is enhanced by loss-of-function alleles of *ced-9* (Ref. 14). Two genes related to *ced-9*, *bcl-x* and *bcl-2*, each encode opposing cell-death activities, but there is no evidence that the opposing activities of *ced-9* are generated either by alternative splicing, as for *bcl-x* (Ref. 38), or by proteolytic processing, as for *bcl-2* (Ref. 39). A gain-of-function allele of *ced-9*, *n1950*, prevents programmed cell death, just as do *egl-1*, *ced-4* and *ced-3* loss-of-function mutations[15]. Overexpression of wild-type *ced-9* can also block programmed cell death[14,22,28], so it is possible that the *ced-9* mutation, *n1950*, acts to increase an essentially wild-type *ced-9* activity. Alternatively, it is possible that the *n1950* mutation inactivates the killing function of *ced-9* while leaving its protective function intact. Because the *n1950* mutation alters the BH1 domain of the CED-9 protein, a domain that is known to be involved in protein–protein interactions in other CED-9/Bcl-2-family proteins[40,41], one possibility is that the *n1950* mutation alters the specificity of protein–protein interactions. It should be noted, however, that the equivalent change in the CED-9 homolog Bcl-2 behaves as a loss-of-function mutation and fails to protect against cell death in mammalian cells[41] and in *C. elegans*[14].

Thus, one should be cautious about drawing conclusions concerning the function of the CED-9 BH1 domain based upon other Bcl-2 family members and, more generally, concerning the function of the BH1 domain of any Bcl-2 family protein based upon any other family member.

ced-4 can also function as a cell-death activator and as a cell-death inhibitor. These opposing activities appear to result from two protein isoforms of CED-4 encoded by alternatively spliced transcripts[34]. The discovery of an alternatively spliced *ced-4* transcript was based upon comparative genomic studies. Introns in *C. elegans* and the related nematode species *Caenorhabditis briggsae* or *Caenorhabditis remanei* (formerly *C. vulgaris*[42]) tend to be poorly conserved in sequence (although not in position)[42]. The *ced-4* gene appeared to be an exception, because within the third intron there is a 72-nucleotide region of very high (>90%) identity among all three species. This region proved to correspond to an alternatively spliced exon that is present in a second, less abundant, *ced-4* transcript and that is predicted to produce a protein, CED-4L (L, long), with 24 additional amino acids inserted between residues 212 and 213 of the 549-amino acid killing form, CED-4S (S, short). When overexpressed from a transgene CED-4S causes programmed cell death, while when overexpressed from a transgene CED-4L can protect against programmed cell death. Overexpression of *ced-4L* can also protect against ectopic programmed cell deaths caused by *ced-9* loss of function, indicating that *ced-9* functions genetically upstream of *ced-4L*, just as it functions genetically upstream of *ced-4S*. It is not known how the splicing choice is controlled or whether this control is differentially regulated in different cells (e.g. those that normally live and those that normally die) to alter their susceptibility to death-inducing signals or perhaps to initiate the cell death program. Furthermore, how the CED-4L protein inhibits programmed cell death is unknown. The simplest model is that CED-4L functions as an interfering dominant-negative form of the CED-4 protein: for example, CED-4L could interact with the same targets as does CED-4S but not activate these targets, while blocking CED-4S from doing so. Consistent with this hypothesis, recent biochemical evidence suggests that CED-4L can bind to, but not promote the processing of, the pro-enzyme form of CED-3 (Ref. 43).

It is possible that the killing function of *ced-9* and the protective function of *ced-4* are related. CED-9 might kill by binding to and inhibiting the activity of CED-4L, much in the same way that CED-9 protects by binding to and inhibiting the activity of CED-4S (see Ref. 34).

Developmental regulation of programmed cell death

How does a cell decide to undergo programmed cell death? In mammals, cell interactions act to trigger at least some programmed cell deaths. In *C. elegans,* a few cell deaths depend upon interacting cells[44], but many are probably cell-autonomous[45]. How might such cell-autonomous deaths be initiated? We suggest that there are two ways to think about the existing observations. First, programmed cell deaths might be triggered by an underlying cellular defect, such as a defect in differentiation. Second, programmed cell death might be a differentiated fate expressed as a consequence of the normal process of selecting among a set of differentiation choices. Undergoing programmed cell death is similar in a number of ways to adopting any differentiated fate, such as becoming a neuron or muscle cell. Like other cell fates in *C. elegans*, cell deaths occur throughout the cell lineage (Fig. 1), and the majority of cell deaths occur during the developmental period when most other cells terminally differentiate[2,4]. Also, like other cell fates in *C. elegans*, programmed cell death is observed in an essentially invariant pattern with the same lineally equivalent cells undergoing programmed cell death from animal to animal. This reproducibility in the cells that die suggests that programmed cell death in *C. elegans* is not a result of stochastically occurring defects in cellular physiology. Furthermore, patterns of programmed cell deaths can be altered by mutations in genes known to be involved in the control of other cell fates (e.g. Refs 46–48), further indicating that programmed cell death is no different from developmental cell fates in general.

How do developmental signals control the activities of the killing machinery, which includes at least *egl-1*, *ced-9*, *ced-4* and *ced-3*, to initiate the death process in particular cells? Although some understanding has been reached of how certain cell–cell interactions in the mammalian immune system lead to the activation of the cell-death machinery (reviewed in Ref. 49), much less is known about how the machinery is activated during development in *C. elegans* or in any other organism. One possibility is that the activity of one or more of these genes is under transcriptional control. Although many living cells in *C. elegans* probably express *ced-4* and *ced-3*, transcriptional overexpression of either of these genes, or of *egl-1*, is sufficient to cause the deaths of at least some cell types[10,29]. Thus, elevation of *egl-1*, *ced-4* and/or *ced-3* transcription could be a mechanism used to initiate programmed cell death. The direct transcriptional regulation of the *ced-9* gene does not seem as likely to be responsible for initiating cell death (at least during embryogenesis), because this gene shows a strong maternal component and no zygotic *ced-9* activity is required for the normal regulation of programmed cell death during embryogenesis[15].

Genetic analysis has identified two genes, *ces-1* and *ces-2* (*ces,* cell death specification) that control a subset of programmed cell deaths in *C. elegans*. While mutations in *ces-1* or *ces-2* block the deaths of certain neural cells[13], no other discernible cell-death, cell-lineage or cell-fate defects have been observed in *ces-1* or *ces-2* mutants, suggesting that *ces-1* and *ces-2* are specifically involved in regulating the programmed cell deaths of these cells. *ces-2* encodes a member of the basic-leucine zipper (bZIP) family of transcription factors[50], consistent with the hypothesis that programmed cell death is controlled at the level of differential gene expression. Mammalian members of the bZIP family, which is most similar to CES-2 (the proline and acid rich or PAR family), might also have a role in the cell-specific regulation of programmed cell death[50,51]. It is conceivable that CES-2 is a direct transcriptional regulator of *egl-1*, *ced-4* or *ced-3*. It is important to note, however, that the Ces phenotype

of *ces-1* mutant animals is caused by gain-of-function alleles and that of *ces-2* animals is caused by a partial loss-of-function allele[13]. Because these alleles do not result in a complete loss of gene activity, it remains possible that the cell-specific cell-death phenotypes of *ces-1* and *ces-2* animals represent an aspect of a more general requirement for these genes in programmed cell death or in cell-fate determination.

Finally, the regulation of *ced-9* activity is unlikely to be the only mechanism controlling the life or death decision. Animals that are doubly mutant for a null allele of *ced-9* and weak alleles of *ced-3* are viable, and do not have a large number of ectopic cell deaths[14]. However, in such animals the majority of cells that normally die during wild-type development still do so[14], indicating that, in this genetic background, cells that normally die are more susceptible to programmed cell death than are cells that normally live. Because *ced-9* is inactive in these mutants, this difference in susceptibility cannot arise from differential regulation of *ced-9* activity. Hence, some death-promoting signals must feed into components of the cell-death pathway independently of *ced-9*.

In the throes of death

As cells undergo programmed cell death, their corpses are rapidly engulfed by, and then degraded within, engulfing cells. In *C. elegans* most corpses are engulfed by their closest neighbors (Ref. 52 and J. Sulston and J. White, pers. commun.). Six genes, *ced-1*, *-2*, *-5*, *-6*, *-7* and *-10*, important for cell-corpse engulfment in *C. elegans*, have been discovered in genetic screens by visually identifying mutants that contain unengulfed cell corpses[53,54]. (The gene *ced-8*, which was originally classified as an engulfment *ced* gene, is instead involved in a different aspect of the cell-death process; G.M. Stanfield, M. Hengartner and H.R. Horvitz, unpublished.) Recent papers describe molecular analyses of three of the six engulfment genes[55–57]. At least one gene, *nuc-1* (*nuc*, nuclease), is involved in the degradation of the DNA of cell corpses[58]. In *nuc-1* animals, the condensed DNA of cell corpses persists indefinitely within engulfing cells. *nuc-1* appears to encode or control the activity of a nuclease that acts not only in cell death but also in other processes, because *nuc-1* mutants are unable to digest bacterial DNA in their guts and have a large reduction in at least one biochemically-defined endonuclease activity present in wild-type animals[53,59].

Mutations in *nuc-1* or in any of the engulfment genes, alone or in combination, do not prevent the execution of cell deaths. The wild-type pattern of cell deaths occurs, and the timing of the appearance of cell corpses is not altered[53]. Thus, the activities of these genes are not required for cell killing. One model consistent with these data is that multiple, independent activities are required to effect different aspects of the post-execution death program, such as the morphological changes in the dying cell, engulfment and cellular degradation. Loss of any one of these functions is not sufficient to block programmed cell death in *C. elegans*. It is also possible that mutations in *nuc-1* and/or in the engulfment *ced* genes affect programmed cell death nonspecifically; that is, these genes are involved in general cellular functions in living and dying cells, and dying cells are more sensitive than living cells to defects in those functions.

Beyond the valley of the shadow of death

Genetic analysis has led to the identification of key central regulators of programmed cell death in *C. elegans*, and molecular and biochemical studies are providing clues about the mechanisms of action of these genes and their protein products. Nonetheless, many aspects of the regulation and function of the *egl-1*, *ced-9*, *ced-4* and *ced-3* genes are still unknown. For instance, *ces* genes have been identified for only a small subset of cell types. These cell types might have unique cell-death controls, but it seems more likely that many *ces* mutants have been overlooked because their phenotypes are subtle. How is *egl-1*, the most upstream component of the general cell-death machinery, regulated? How does CED-4 trigger the conversion of inactive CED-3 to the active form? How are relative levels of CED-4L and CED-4S controlled? What is the basis for the killing activity of *ced-9* and the protective activity of *ced-4L*? What are the steps downstream of CED-3 activation that mediate cell-corpse formation, engulfment and degradation? Further genetic analysis using *C. elegans* should help reveal the answers to these questions.

While there have been extensive screens to identify mutations that can suppress the lethality conferred by *ced-9* (lf) mutations (H.R. Horvitz *et al*., unpublished), there has been only limited screening for genes that, like *egl-1*, function to promote cell death upstream of *ced-9*. Furthermore, most screens for cell-death mutants have required that animals be viable, so other protector genes like *ced-9* might not have been identified. Genes with redundant functions in cell killing would also probably have been missed in previous screens. Searching for enhancers or suppressors of cell killing in genetically sensitized backgrounds (e.g. Ref. 60) or examining cell death in animals carrying chromosomal deletions (e.g. Refs 61, 62) offer two approaches to the identification of such genes.

What are the targets of CED-3 proteolysis? There might be a small number of critical targets that are activated to effect the downstream events of cell death. Alternatively, there might be multiple targets with important cellular functions that are inactivated by CED-3 proteolysis. This latter model assumes that although cell death initiates as a regulated process, it proceeds by eliminating functions that are required for normal cellular homeostasis. Another possibility is that *ced-3* functions to inactivate a cell-death protector. Loss of function of such a gene would presumably confer a lethal phenotype and would not be suppressed by any known cell-death mutant. The identification and characterization of genes involved in the generation of the conserved morphological changes in cell corpses, in cell-corpse degradation and in the generation of engulfment signals might reveal which, if any, of these models is true. Few genetic screens have sought genes involved in corpse formation and breakdown. Perhaps targets of the execution genes could be sought by screening for mutations that synthetically alter cell death patterns, for example by blocking cell death when in combination with mutations that cause other downstream defects.

While numerous proteolytic targets of mammalian caspases have been identified[63], the relevance of these targets *in vivo* to programmed cell death has been mostly untested. The genetic analysis of such genes in *C. elegans* should determine their functions.

Conclusions

Enormous progress has been made towards understanding the basic molecular mechanisms used by cells to kill themselves. Nonetheless, many questions remain. The remarkable degree of conservation of the cell-death pathway from nematodes to mammals suggests that genetic analysis of programmed cell death in *C. elegans* will continue to play a major role in revealing the mechanisms responsible for this crucial and fascinating process.

Acknowledgements

We wish to thank T. Herman, J. Agapite and myriad members of the Horvitz laboratory for helpful comments concerning the manuscript. H.R.H. is an Investigator of the Howard Hughes Medical Institute.

References

1 Ellis, R.E., Yuan, J. and Horvitz, H.R. (1991) *Annu. Rev. Cell Biol.* 7, 663–698
2 Sulston, J.E. and Horvitz, H.R. (1977) *Dev. Biol.* 56, 110–156
3 Kimble, J. and Hirsh, D. (1979) *Dev. Biol.* 70, 396–417
4 Sulston, J.E., Schierenberg, E., White, J.G. and Thomson, J.N. (1983) *Dev. Biol.* 100, 64–119
5 Thornberry, N.A. (1997) *Br. Med. Bull.* 53, 478–490
6 Kidd, V.J. (1998) *Annu. Rev. Physiol.* 60, 533–573
7 Newton, K. and Strasser, A. (1998) *Curr. Opin. Genet. Dev.* 8, 68–75
8 Bergeron, L. and Yuan, J. (1998) *Curr. Opin. Neurobiol.* 8, 55–63
9 Ellis, H.M. and Horvitz, H.R. (1986) *Cell* 44, 817–829
10 Conradt, B. and Horvitz, H.R. (1998) *Cell* 93, 519–529
11 Trent, C., Tsung, N. and Horvitz, H.R. (1983) *Genetics* 104, 619–647
12 Yuan, J. and Horvitz, H.R. (1992) *Development* 116, 309–320
13 Ellis, R.E. and Horvitz, H.R. (1991) *Development* 112, 591–603
14 Hengartner, M.O. and Horvitz, H.R. (1994) *Nature* 369, 318–320
15 Hengartner, M.O., Ellis, R.E. and Horvitz, H.R. (1992) *Nature* 356, 494–499
16 Yuan, J. *et al.* (1993) *Cell* 75, 641–652
17 Alnemri, E. *et al.* (1996) *Cell* 87, 171
18 Zhou, H. *et al.* (1997) *Cell* 90, 405–413
19 James, C., Gschmeissner, S., Fraser, A. and Evan, G.I. (1997) *Curr. Biol.* 7, 246–252
20 Chinnaiyan, A.M. *et al.* (1997) *Nature* 388, 728–729
21 Li, P. *et al.* (1997) *Cell* 91, 479–489
22 Hengartner, M.O. and Horvitz, H.R. (1994) *Cell* 76, 665–676
23 Vaux, D.L., Cory, S. and Adams, J.M. (1988) *Nature* 335, 440–442
24 Vaux, D.L., Weissman, I.L. and Kim, S.K. (1992) *Science* 258, 1955–1957
25 White, J.G., Southgate, E. and Thomson, J.N. (1991) *Philos. Trans. R. Soc. London Ser. B* 331, 263–271
26 Avery, L. and Horvitz, H.R. (1987) *Cell* 51, 1071–1078
27 Xue, D., Shaham, S. and Horvitz, H.R. (1996) *Genes Dev.* 10, 1073–1083
28 Xue, D. and Horvitz, H. (1997) *Nature* 390, 305–308
29 Shaham, S. and Horvitz, H.R. (1996) *Genes Dev.* 10, 578–591
30 Seshagiri, S. and Miller, L.K. (1997) *Curr. Biol.* 7, 455–460
31 Wu, D., Wallen, H.D., Inohara, N. and Nunez, G. (1997) *J. Biol. Chem.* 272, 21449–21454
32 Chinnaiyan, A.M., O'Rourke, K., Lane, B.R. and Dixit, V.M. (1997) *Science* 275, 1122–1126
33 Vaux, D.L. (1997) *Cell* 90, 389–390
34 Shaham, S. and Horvitz, H.R. (1996) *Cell* 86, 201–208
35 Pan, G., O'Rourke, K. and Dixit, V.M. (1998) *J. Biol. Chem.* 273, 5841–5845
36 Hu, Y. *et al.* (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 4386–4391
37 Nguyen, M. *et al.* (1993) *J. Biol. Chem.* 268, 25265–25268
38 Boise, L.H. *et al.* (1993) *Cell* 74, 597–608
39 Cheng, E.H. *et al.* (1997) *Science* 278, 1966–1968
40 Muchmore, S.W. *et al.* (1996) *Nature* 381, 335–341
41 Yin, X.M., Oltvai, Z.N. and Korsmeyer, S.J. (1994) *Nature* 369, 321–323
42 Fitch, D.H.A. and Thomas, W.K. (1997) in *C. elegans* (Vol. 2) (Riddle, R.L., Blumenthal, T., Meyer, B.J. and Priess, J.R., eds), pp. 815–850, Cold Spring Harbor Laboratory Press
43 Chaudhary, D., O'Rourke, K., Chinnaiyan, A.M. and Dixit, V.M. (1998) *J. Biol. Chem.* 273, 17708–17712
44 Sulston, J.E., Albertson, D.G. and Thomson, J.N. (1980) *Dev. Biol.* 78, 542–576
45 Sulston, J.E. and White, J.G. (1980) *Dev. Biol.* 78, 577–597
46 Wang, B.B. *et al.* (1993) *Cell* 74, 29–42
47 Clark, S.G., Chisholm, A.D. and Horvitz, H.R. (1993) *Cell* 74, 43–55
48 Guenther, C. and Garriga, G. (1996) *Development* 122, 3509–3518
49 Cohen, G.M. (1997) *Biochem. J.* 326, 1–16
50 Metzstein, M.M. *et al.* (1996) *Nature* 382, 545–547
51 Inaba, T. *et al.* (1996) *Nature* 382, 541–544
52 Robertson, A.M.G. and Thomson, J.N. (1982) *J. Embryol. Exp. Morphol.* 67, 89–100
53 Hedgecock, E., Sulston, J. and Thomson, J.N. (1983) *Science* 220, 1277–1279
54 Ellis, R.E., Jacobson, D.M. and Horvitz, H.R. (1991) *Genetics* 129, 79–94
55 Wu, Y.C. and Horvitz, H.R. (1998) *Nature* 392, 501–504
56 Liu, Q.A. and Hengartner, M.O. (1998) *Cell* 93, 961–972
57 Wu, Y.C. and Horvitz, H.R. (1998) *Cell* 93, 951–960
58 Sulston, J.E. (1976) *Philos. Trans. R. Soc. London Ser. B* 275, 287–298
59 Hevelone, H. and Hartman, P.S. (1988) *Biochem. Genet.* 26, 447–461
60 Hay, B.A., Wassarman, D.A. and Rubin, G.M. (1995) *Cell* 83, 1253–1262
61 White, K. *et al.* (1994) *Science* 264, 677–683
62 Terns, R.M. *et al.* (1997) *Genetics* 146, 185–206
63 Rosen, A. and Casciola-Rosen, L. (1997) *J. Cell. Biochem.* 64, 50–54
64 Sulston, J.E. (1988) in *The Nematode Caenorhabditis elegans* (Wood, W.B. and the Community of *C. elegans* Researchers, eds), pp. 123–155, Cold Spring Harbor Laboratory Press
65 Nicholson, D.W. and Thornberry, N.A. (1997) *Trends Biochem. Sci.* 22, 299–306

M.M. Metzstein, ***G.M. Stanfield*** *and* ***H.R. Horvitz*** *are in the Howard Hughes Medical Institute, Department of Biology, Room 68-425, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.*