Death & Fate: Nobel Lessons from an Elegant Worm

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Summary

Cell fate and cell death are central to any multicellular organism's normal development. Our lab pioneered the use of the nematode Caenorhabditis elegans in studying the fundamental processes that control cell fate and death, two events that cannot be divorced from one another. We discovered several cell lineage mutants, including lin-4, let-7, lin-12 and lin-14, which affect developmental timing and control. The effects of some of these mutations on the development of the vulva are examined here, which includes programmed cell death (PCD). The molecular genetic pathway of PCD was determined over the course of more than 20 years of study. The following mutations are known to act in PCD; ces-2, ces-1, tra-1, egl-1, ced-9, ced-3, ced-4, ced-11, ced-1, ced-6, ced-7, ced-2, ced-5, ced-10, ced-12 and nuc-1. The discovery of human homologues of most of these genes has shown that the PCD pathways in worms and humans are evolutionarily conserved. This conservation has proved to have immense implications on research for cures of many human disorders like neurodegenerative diseases and cancer, which are hypothesized to be caused by malfunctions in the PCD pathway. C. elegans has proved to be a truly noble organism, providing targets in the PCD pathway for intervention aimed at developing potential therapies.

Introduction

I was delighted to be accepted as a researcher in Sydney Brenner's laboratory at the Medical Research Council Laboratory of Molecular Biology in England, in 1974. Sidney Brenner had established *C. elegans* as a model system to study genetics.[1] There, I started my studies of *C. elegans* with John Sulston, who was trained as an organic chemist. John used his chemical expertise to study the neurochemistry of the nematode. He was studying the larval ventral nervous system, by using Nomarski differential interference contrast optics to visualize individual cells within the animal.[2] By looking at single cells, he could follow each cell division and the fates of the descendent cells.

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We already knew that some cells died by "programmed cell death" (PCD) in the normal process of development.[3] In my studies of the *C. elegans* cell lineage, we observed that many cells undergo PCD at different times during development. Since the *C. elegans* cell lineages are so rigid and invariant, we hypothesized that cell death was also a fate. Our hypothesis has been supported by the data from our lab and other laboratories who have been studying PCD. Most importantly, the conservation of genes in the PCD or apoptosis pathway between nematodes and humans has had implications on the search for cures of neurodegenerative diseases and cancers, thought to be caused by abnormalities in the PCD pathway.

C. elegans has been an ideal organism to study cell lineage and PCD. It has proved to be a truly noble organism which has revealed the genetic similarities that it shares with us. I hope that future studies in *C. elegans* will help unfold many more mechanisms and pathways that are common to both nematodes and humans. In this paper, I will describe our discovery of genes affecting cell lineage, the development of the vulva, and programmed cell death.

Cell Lineage and Genetics

I was very excited to be working with John. Together, we characterized the post-embryonic cell lineages of *C. elegans*.[4] The lineages of the cells in the gonad were described two years later.[5] But the embryonic cell lineages, which were much more difficult to characterize, was done later by John along with other colleagues.[6] These studies defined the complete cell lineage of *C. elegans*.

Now that we knew the rigid and invariant cell lineages, we wanted to know the mechanisms underlying them. One method was to kill individual cells using a laser micro beam and thus study the effects of individual cells. The second was through genetics. Following up on our previous study, John and I isolated and characterized 24 mutants of the post-embryonic cell lineages of the nematode.[7, 8] Many of the cell lineage mutants we isolated controlled developmental timing rather than being homeotic (mutations in these genes cause body parts usually found in one part of the body to be found elsewhere). We named these, heterochronic mutants. There are two types of heterochronic mutants; a mutation that can cause early developmental events to occur late, and mutations that cause late development events to occur early.[9] *Lin-4* (lin, cell lineage abnormal) and *unc-86* (unc, uncoordinated movement) were found to cause repetition of cell division patterns normally associated with their descendents.[10] Similar genes have been identified in other metazoans and are necessary for the propagation of stem cells.[10] We also identified *let-7*, which coded for a 21 nucleotide RNA required to control developmental timing in the adult larva.[11] Loss of *let-7* caused reiterations whereas increased *let-7* gene dosage caused precocious development.[11]

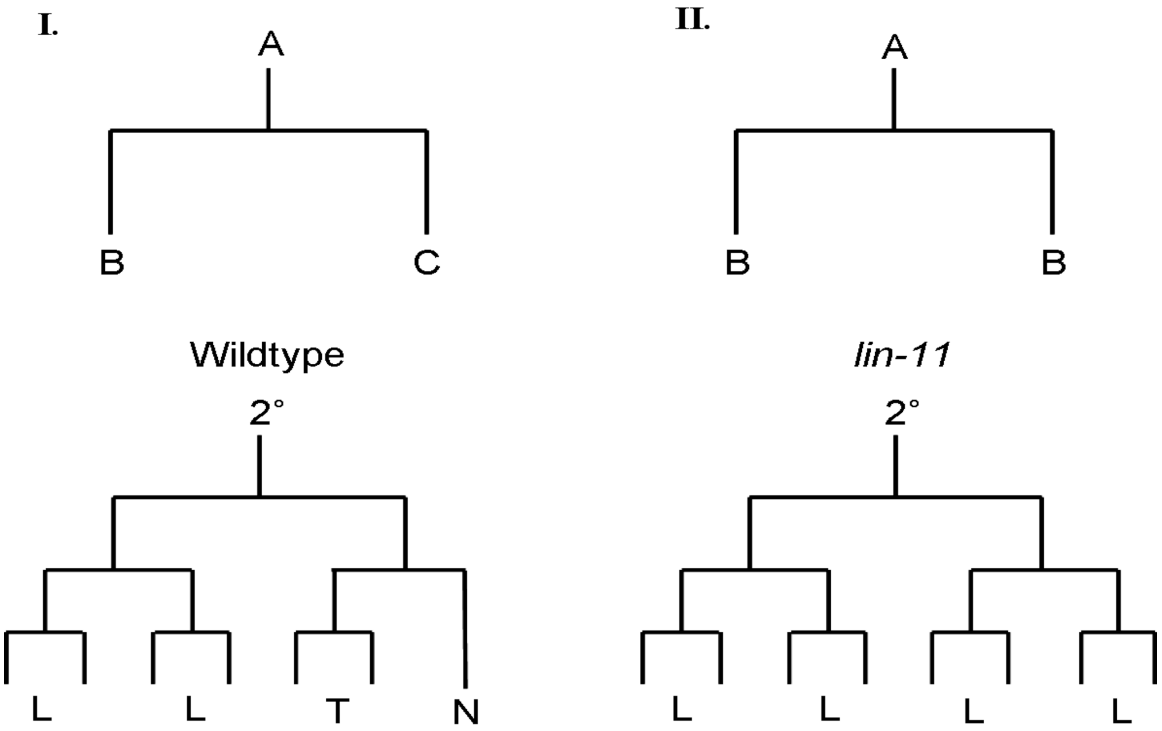

Figure 1. I. Each cell (A) has a fate, which could be to differentiate into a specific cell type or to undergo a specific differentiation pattern (B and C). In I, cell A gives rise to daughter cells B and C, which have different fates. In II, cell A, produces two cells B and B, which have the same fates. Similarly, in wildtype animals, the 2° blast cells divides to produce daughter cells with different fates, while in *lin-12* mutants, all the 2° blast cells have the same fate. N, no division; T, transverse division; L, longitudinal division (adapted from Freyd et al., 1990) [13].

All cells can be thought of as having a certain fate. The fate could be to differentiate into specific cell types or to undergo a specific pattern of cell division.[9] For example, cell A can divide to produce two cells B and C, where B and C are different from each other and from the mother cell A. We found that *Unc-86* and *lin-11* control cell diversity.[12] Mutating *lin-11* caused daughter cells to be like each other instead of differentiating (Figure 1).[13] We also found that *unc-86* and *lin-11* have counterparts in other organisms. In fact, they are both transcription factors necessary for the generation of cell diversity. [14, 13]

In order to differentiate between cells that have direct as opposed to indirect effects, we named some genes "important developmental control genes" (IDCGs). Mutations in these genes have opposite developmental effects.[15] *Lin-12*, an IDCG was first found to affect the development of the vulva.[16, 12] We found that *lin-12* acts at the time cell fate is determined, and an increase or decrease in *lin-12* activity causes alternative fates.[16] Victor Ambros, a post doctoral fellow in my laboratory isolated and characterized *lin-14*, another heterochronic mutant which was also an IDGC.[17] Increasing *lin-14* activity lead to retarded development, whereas reducing *lin-14* activity caused precocious development.[17] *Lin-14* also controls cell fates in a number of cell lineages including the hypodermis, mesoderm and intestines.[17] We later found that a reduction in *lin-14* protein levels during development causes the expression of different cell fates at successive developmental stages.[18]

Development of the Vulva

The vulva is the opening of the uterus to the environment. It is used for egg laying and mating. In the course of our cell lineage studies, we have studied the genetic pathway necessary for the development of the vulva which involves 3 rounds of cell division. In *C. elegans,* mutations in the vulva are easily identifiable and thus easier to study.[12] Vulval mutations can result in an animal with no vulva, termed "vulvaless." Since the vulva is required for egg-laying, in vulvaless hermaphrodites, the eggs are self-fertilized and hatch *in utero*, causing the death of the parent, but not the progeny. Mutations can also result in a "multivulva" phenotype, where the animal has multiple ectopic vulvae.

We found that in some vulvaless mutants (*lin-2, lin-3, and lin-7*), the first round of cell divisions are normal, however, the second round of cell divisions generally fail.[8] In the hermaphrodite multivulva mutants, (*lin-1* and the double mutant *lin-8*; *lin-9*), all cells that undergo the first round of cell divisions (P3.p-P8.p), also undergo the second round of cell divisions.[8] The additional vulvae are caused due to further cell divisions in P3.p-P6.p in the *lin-8; lin-9* mutants.[8]

In a study, we found that, of the 23 genes that affect the normal development of the vulva, most

are homeotic genes.[19] 15 of these genes are involved in intracellular signaling to the extracellular signal that induces vulval development.[19] Later studies showed that the fate of each vulval precursor cell (VPC) depended on two signaling pathways, one graded inductive signal functioning from a distance and a short range lateral signal between the VPCs. We showed that *lin-12* acts in the lateral signaling pathway and specifies 2˚, one of the three VPC cell fates. The vulvaless and multivulva genes act in the inductive signaling pathway and control 1˚ fate independent of *lin-12,* and 2˚ through *lin-12*.[20]

The genetic studies from my laboratory, along with other studies, have helped in elucidating the Ras pathway for signal transduction. We found that *let-60* is an essential ras gene and it acts as a switch in the inductive signal pathway for vulval development.[21] Thus, recessive mutations that reduce *let-60* lead to a vulvaless mutant phenotype (3˚ fate), whereas dominant mutations that increase *let-60* activity lead to development of the vulva, even in the absence of the inductive signal (1˚ and 2˚ fate). [21] Our studies of the Ras pathway have showed that this pathway is conserved in worms and mammals. We found that two genes *lin-35* and *lin-53*, code for proteins very similar to the tumor suppressor protein Rb and its binding protein RbAp48, respectively.[22] These proteins act as transcription factors that antagonize the Ras pathway thereby inhibiting vulval formation. Recently, we discovered that *lin-8* codes for nuclear protein LIN-8, which interacts with LIN-35 Rb. Thus, we have shown compelling evidence that vulval induction is controlled by transcriptional regulation of gene expression.

Programmed Cell Death (PCD)

Naturally occurring cell death or "programmed" cell death occurs in vertebrates [24] and invertebrates [25] during normal animal development. It is a fundamental part of development which was observed, but not very well studied. During my studies of the *C. elegans* cell lineage I was intrigued by the phenomena of programmed cell death. Of the 959 cells generated during the normal development of the hermaphrodite worm, an additional 131 cells are generated which undergo programmed cell death.[4] Interestingly, 105 of these 131 deaths occurred in the development of the nervous system.[6]

From our cell lineage studies, we knew that diverse cells in different developmental stages undergo programmed cell death, giving rise to the invariant cell lineages of *C. elegans*. In cells undergoing PCD, the morphological changes occurring were all very similar. This made identification of these cells by direct screening fairly simple. The first sign of a cell undergoing cell death is that there is a slight increase in its refractility. Next, the nucleus of the dying cell becomes very refractile and looks like a flat button; this lasts for about 10-30 minutes. In the last stage, the dying cell is engulfed and the nucleus decreases in refractility, becomes crumpled and finally disappears.[26] We reasoned that since PCD is a series of morphological changes, then it could be treated as a cell fate. We decided to use genetic methods to identify genes that induce the cell to die and genes that carry out the execution.

Identification of Genes in the Cell Death Pathway

The first gene in the PCD pathway was *nuc-1* (nuclease abnormal). This gene was identified by John Sulston in his studies of the cell lineages of the ventral nervous system. He used Feulgen DNA staining to observe these cells. In the *nuc-1* mutants, the DNA of dead cells was still visible and did not perish. John showed that these mutants lacked endonuclease activity required to fragment the DNA of dead cells.[2]

Cell Engulfment

In 1983, Ed Hedgecock *et al.* discovered two more cell death genes, *ced-1* and *ced-2* (cell death abnormal). In these mutants, phagocytosis or the normal process of cell engulfment does not occur, making the cell corpses persist in the animal. These corpses can be visualized, since dead cells become refractile. Hedgecock *et al.* also saw that in *ced-1* and *ced-2* mutants, the DNA of dead cells persist. By creating a double mutant with *ced-1/ced-2* and *nuc-1* knocked out, they showed that *ced-1* and *ced-2* are required for *nuc-1* activation, and thus placed *ced-1* and *ced-2* upstream of *nuc-1*.[27] This also suggested that *nuc-1* was probably expressed by the engulfing cell.

In later studies, my laboratory discovered five new genes that played a role in cell engulfment. We named these *ced-5*, *ced-6*, *ced-7*, *ced-8* and *ced-10*.[28] We observed that mutations in each of these genes prevented cell engulfment, thus these genes must be involved in cell recognition or initiation of phagocytosis. We made double mutants which suggested that these genes act in distinct and partially redundant pathways. In double mutants of *ced-2*, *ced-5* and *ced-10,* the same number of cells were unengulfed as in *ced-5* single mutants alone. However in double mutants between *ced-2*, *ced-5* or *ced-10*, and *ced-1*, *ced-6*, *ced-7 or ced-8*, there were more unengulfed cells.[28] These results suggested that *ced-2*, *ced-5* and *ced-10* act in one cell engulfment pathway while *ced-1*, *ced-6* and *ced-7* act in a second pathway.[28] We also found that *ced-12* is part of the *ced-2*, *ced-5* and *ced-10* pathway.

Later, we found that CED-1, CED-6 and CED-7 proteins are part of a signaling pathway that is essential for cell corpse recognition. We also found that CED-1 is a transmembrane receptor, similar to the human scavenger receptor SREC, which clusters around dead cells.[29] CED-6 acts downstream of CED-1 and is similar to mammalian adaptor proteins that contain phosphotyrosine binding (PTB) domains[29], while CED-7 is similar to the ATP binding cassette transporters[30] and promotes cell corpse recognition by exposing a phospholipid ligand on the surfaces of cell corpses.[29]

We also found that CED-2, CED-5 and CED-10 proteins are components of a conserved Rac GTPase signaling pathway. The Rho/Rac pathway is involved in dynamic processes such as cell motility, phagocytosis, axonal guidance and cytokinesis.[31] CED-2 is similar to the mammalian adaptor protein CrkII, which is implicated in transmembrane-receptor mediated signaling pathways.[32] CED-5 was found to be similar to human DOCK180, a Crk-interacting protein [33], while CED-10 is a *C. elegans* homolog of mammalian Rac. *Ced-12* encodes a protein which is also part of the CED-10 Rac pathway.[34] We also found that defects in

CED-2, CED-5 and CED-12 not only affect cell engulfment, but also cell migration.[32]

Ced-3

In order to identify genes abnormal in the PCD pathway, we decided to use the *ced-1* and *ced-2* mutants, since apoptotic cells, despite being visible, are engulfed and degraded quite rapidly. In *ced-1* and *ced-2* animals, cells that normally undergo PCD die, but are not engulfed and persist for a few hours. Hilary Ellis, a graduate student in my laboratory, mutagenized the *ced-1* strain and found a mutant where no cell corpses could be seen. We named this gene *ced-3*. We showed that in *ced-3* mutants almost all of the 131 cells that normally die survive.[35, 26]

We had showed that *ced-3* is required for PCD to occur. This meant that PCD was among fundamental biological processes encoded for by genes, like cell division, cell differentiation, etc. Next, we came up with a genetic pathway for cell death where *ced-3* acts upstream of *ced-2* and *ced-1* which act upstream of *nuc-1*.

In 1993, we found that *ced-3* encodes a protein similar in sequence to the mammalian interleukin-1 beta-converting enzyme (ICE).[36] ICE cleaves the pro form of the cytokine interleukin-1-beta, thereby converting it into the active form.[37] Later we showed evidence that CED-3 is a cysteine protease which cleaved substrates similar to the mammalian CPP32 protease, rather than to ICE.[38] We also showed that CED-3 protease activity is required for PCD to occur.[38] There has also been extensive evidence from other laboratories that caspases play an important role in PCD.[39, 40] Together these studies provided evidence that the cell death pathway is conserved between nematodes and humans.

Ced-4

In the course of our cell lineage studies, we discovered and characterized 145 mutants that are defective in egg laying.[41] One of these mutants lacked the hermaphrodite specific motor neurons (HSNs) required for egg laying. We named this gene *egl-1* (egg-laying abnormal). We thought that since the HSN motor neurons were missing, they probably died by PCD, because of the *egl-1* gain of function (gf) mutation, that is, a mutation that abnormally activates *egl-1.* We created a double mutant *egl-1; ced-3* and compared it to *egl-1* and saw that mutating *ced-3* leads to the survival of the HSNs showing that these cells die by PCD.[26] We looked for other mutations that block the *egl-1* phenotype and discovered *ced-4*, which acts very similar to *ced-3*. We now know that *egl-1* acts upstream of *ced-3* and *ced-4*, since an *egl-1*(gf) mutation leads to PCD, while blocking *ced-3* or *ced-4* does not.

Next, we wanted to know if *ced-3* and *ced-4* act within the dying cells or are activated in some neighboring cells. We used genetic mosaic analyses, where a single animal has some cells that are mutant (*ced-3*/*ced-4*) and some that are not, to answer our question. We discovered that *ced-3* and *ced-4* act cell autonomously, i.e. they act within the dying cells.[42] This implied that these cells committed suicide.

We did not know if *ced-3* and *ced-4* acted together, or if one gene was upstream of the other. Overexpression of *ced-4* using a transgene in *ced-3* mutants did not increase the number of cells dying by PCD, showing that *ced-3* activity was required.[43] Also, overexpression of *ced-3* in *ced-4* mutants did not require *ced-4* function, providing further evidence that *ced-4* acts upstream of *ced-3* [43].

Our laboratory found that *ced-4* encodes a novel protein that is highly expressed during programmed cell death.[44] It was later found that CED-4 was similar to apaf-1 (apoptotic protease activating factor), a human protein that is highly expressed during PCD (apoptosis).[45] This was very significant as like CED-3, it showed that the PCD pathway is conserved between nematodes and humans.

The ced-9 Gene Prevents Programmed Cell Death

In 1992, a study from our laboratory showed that the activity of the *ced-9* gene protects cells against PCD.[46] We showed that a *ced-9* (gf) mutation prevents the cell deaths that occur during normal *C. elegans* development.[46] Mutations in *ced-9* (gf) and *ced-3* and *ced-4* all prevented PCD and about the same number of extra cells were found in all three mutants.[46] We also found that a *ced-9* (lf) mutation, i.e. a mutation that inactivates *ced-9* causes the death of embryos [46]. In order to place *ced-9* in our developing PCD pathway, we created double mutants using *ced-9* (lf), and *ced-3* or *ced-4*. Mutations in either *ced-3* or *ced-4* completely suppressed PCD in *ced-9* (lf) animals. This meant that *ced-9* was upstream of *ced-3* and *ced-4* as these genes were required for the *ced-9* (lf) mutation to have its effect [46].

We asked if *ced-9* protects against PCD, by inhibiting *ced-3*, *ced-4* or both, and found out that protection conferred by *ced-9* against *ced-3* killing, is reduced when *ced-4* is mutated.[43] We also saw that overexpressing *ced-9* can protect against *ced-4* overexpression.[43] Our results suggested that *ced-9* inhibits *ced-3*, at least partly by inhibiting *ced-4*.

Our laboratory discovered that *ced-9* encodes a protein which shows 23% identity to the product of the human proto-oncogene Bcl-2.[37] We also showed that expressing human Bcl-2, can substitute for *ced-9* in *ced-9* mutants by protecting against PCD.[37] This discovery generated great interest in the worm cell-death pathway. Moreover, *C. elegans* proved to be an ideal model system for the study of the PCD pathway as so many genes were found to be conserved between nematodes and humans.

Our next question was what controls *ced-9* activity? We had already isolated the *egl-1* (gf) mutant which caused the hermaphrodite specific neurons to undergo PCD. We found that in *egl-1* (lf) mutations, not only do the HSNs survive, but cells in the anterior pharynx also survive.[47] Thus, *egl-1,* like *ced-9* (gf), *ced-3*(lf) and *ced-4* (lf), blocks all general programmed cell deaths.[47] We cloned *egl-1* and found that the EGL-1 protein contains a 9 amino acid region which is similar to the BH3 domain of the Bcl-2 like and BH3 containing proteins.47 Using an in vitro binding assay we found that EGL-1 and CED-9 proteins interact physically. These results suggested that EGL-1 may be a member of the cell death activator proteins.

Other Genes in the Programmed Cell Death Pathway

In our studies of the cell death pathway we discovered two genes that control the deaths of specific cells. We

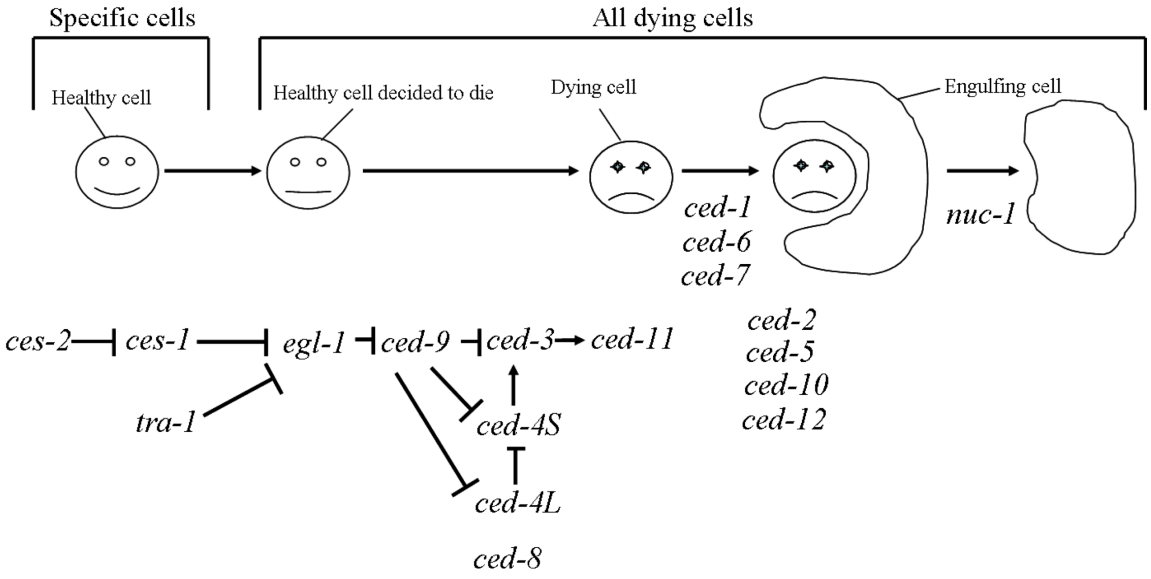

Figure 2. The Molecular Genetic Pathway of Programmed Cell Death (PCD) in *C. elegans*.
Ces-2 and *ces-1* are required by specific cells to decide to die. *Ces-1* and/or *tra-1* inhibit *egl-1*, which negatively controls the activity of *ced-9*, an inhibitor of PCD, by controlling *ced-3* and *ced-4*. *Ced-3* is activated by *ced-4S* and *ced-3* activates *ced-11*. Two parallel and partially redundant pathways regulate cell engulfment. These are the *ced-1, ced-6, ced-7* pathway and the *ced-2, ced-5, ced-10* and *ced-12* pathway. *Nuc-1* is required for DNA degradation in the dead cell. (Adapted from Worms, Life and Death, by H.R. Horvitz).[9]

named these genes ces-1 and ces-2 (ces, cell death specification).48 We discovered that a gain of function mutation in ces-1 prevented the deaths of the sisters of the serotonergic neurosecretory motor (NSM) neurons, and the sisters of the I2 neurons which die in normal development, whereas a loss of function mutation in ces-1 caused the NSM sisters to die. A reduction of ces-2 activity also lead to the survival of the NSM sisters, showing that ces-2 was needed to cause PCD.48

We created double mutants of animals with ces-1 (lf) and a mutation in either ced-3 or ced-4. We found that the NSM sisters lived just as they did in the ced-3 and ced-4 mutations. This indicated that ces-1 was upstream of ced-3 and ced-4. We also found that in a ces-1 ces-2 double mutant, the NSM sisters died, just as they do in loss of ces-1 (lf) mutants or in ces-2 mutants.48 These results along with genetic studies helped us place these two genes in the genetic pathway of PCD (Figure 2).

Our lab discovered that both ces-1 and ces-2 encode transcription factors with human homologues. Ces-1 encodes a transcription factor of the zinc finger family, whose human counterpart is SLUG, 49 while ces-2 encodes a transcription factor which is similar to the product of the human proto-oncogene HLF (hepatic leukemia factor).9 Both HLF and SLUG have been shown to regulate PCD in mammalian cells and may play a role in cancer by affecting PCD.

Tra-1 is another gene which regulates the deaths of specific cells. It regulates the sexually dimorphic programmed cell deaths of the HSN neurons (which die in males and survive in females).50 TRA-1A, coded for by tra-1, is also a zinc finger protein which acts as a transcription factor. In hermaphrodites, which have high TRA-1 activity, it binds to egl-1 and represses it. Repressing egl-1 allows ced-9 to block ced-4 and ced-3, preventing PCD (Figure 2). In egl-1 (gf) mutations, the binding site of TRA-1 is disrupted, causing egl-1 to be highly expressed and leading to the death of HSN neurons.50

Conclusion

Our studies of cell lineage and programmed cell death have enabled us to discover the basic pathway for cell death in C. elegans (Figure 2). However, in spite of knowing so much about the PCD pathway in C. elegans, there are still many unanswered questions. For instance, what makes the 131 cells in the developing hermaphrodite decide to die? What are the specific substrates of the caspases? What are the other genes in the PCD pathway? Current research in our lab is trying to answer these questions.

The discovery of human homologues of most of the genes in the PCD pathway has shown that the PCD pathways in worms and humans are evolutionarily conserved. This conservation has proved to have immense implications on research for cures of many human disorders like neurodegenerative diseases and cancer, which are hypothesized to be caused by malfunctions in the PCD pathway. Too much PCD is thought to cause Alzheimer's disease, Parkinson's disease and Huntington's disease while too little PCD may be involved in cancer. By elucidating targets for possible neurodegenerative disease therapies, C. elegans has proven to be a useful model organism in the study of the PCD pathway.

C. elegans has yet again been shown to be a superb model organism for genetic studies to reveal pathways and mechanisms that are conserved in many organisms. The recent RNA interference studies in C. elegans have shed considerable light on the regulation of gene expression in humans, animals and plants. This important discovery in C. elegans was recognized by the Nobel Assembly, which awarded the Nobel Prize in physiology or medicine for 2006, jointly to Dr. Andrew Z. Fire and Dr. Craig C. Mellow. This is the second Nobel Prize for work done in C. elegans; the real hero.

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