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Noncanonical cell death in the nematode *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis. elegans* has served as a fruitful setting for cell death research for over three decades. A conserved pathway of four genes, *egl-1*/BH3-only, *ced-9*/Bcl-2, *ced-4*/Apaf-1, and *ced-3*/caspase, coordinates most developmental cell deaths in *C. elegans*. However, other cell death forms, programmed and pathological, have also been described in this animal. Some of these share morphological and/or molecular similarities with the canonical apoptotic pathway, while others do not. Indeed, recent studies suggest the existence of an entirely novel mode of programmed developmental cell destruction that may also be conserved beyond nematodes. Here we review evidence for these noncanonical pathways. We propose that different cell death modalities can function as backup mechanisms for apoptosis, or as tailor-made programs that allow specific dying cells to be efficiently cleared from the animal.

Keywords

cell death; apoptosis; necrosis; linker; elegans; morphology

1. INTRODUCTION

Cell death, programmed or otherwise, is a ubiquitous biological phenomenon. Programmed cell death is required for development, homeostasis, and the response to pathological insults in virtually all animals, from sponges to humans[1]. In humans, disease processes are often accompanied by either causal or incidental cell death[2]. Thus, a broad understanding of cell death programs may yield insights into, and possibly treatments for, human pathologies.

The nematode *Caenorhabditis elegans* has proven to be an invaluable tool for dissecting programmed cell death mechanisms. Several aspects of this organism make it well suited for cell death research. Like other nematodes, *C. elegans* has an essentially invariant cell lineage[3], where death features prominently as a common fate. Dying cells are easy to observe in intact, developing animals, which are small and possess a transparent cuticle. Simple genetics and animal husbandry[4], efficient RNA interference[5], and a fully sequenced and heavily annotated genome[6] have enabled investigators to identify genes

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involved in the control and execution of developmental programmed cell death and to uncover mutations and conditions leading to pathological cellular demise.

A molecular description of apoptotic cell death emerged from studies of *C. elegans* in the 1980s and 90s. Horvitz and colleagues identified mutants that define four core apoptotic genes [7]: the BH3-only-like gene *egl-1*, the Bcl-2-like *ced-9*, the Apaf-1-like *ced-4*, and the caspase *ced-3*[8]. Mutations in these genes abolish the death of almost all cells fated to die, and their roles in cell death are largely conserved in all metazoans examined. Most somatic *C. elegans* cells destined to die specifically induce *egl-1* transcription[9]. EGL-1 protein then binds to CED-9[10], disrupting its interaction with CED-4[11,12], thereby freeing CED-4 to activate CED-3, promoting cell death[7,13].

Despite the great success of these early genetic studies, which relied on tracking the survival of groups of cells, they did not initially identify programs unique to individual cells. Partially redundant pathways would have also been more difficult to detect, as mutations in individual components would likely yield only weak defects. Later genetic screens in many labs, seeking mutations affecting the deaths of individual or small groups of cells, uncovered new forms of cell death that deviate partially or entirely from the canonical molecular pathway for apoptosis. Here we discuss these recent studies.

2. PATHOLOGICAL CELL DEATH INDUCED BY GENOME LESIONS AND ENVIRONMENTAL STRESS

2.1 ION CHANNEL MUTATIONS

Genetic studies in *C. elegans* identified three proteins, MEC-4[14], DEG-1[15], and UNC-8[16], whose activation by gain-of-function mutations inappropriately promotes neuronal death. Electron microscope reconstructions demonstrate that dying neurons accumulate progressively larger vacuoles and electron-dense membranous whorls, as well as what appear to be nuclear chromatin clumps. Changes in nuclear shape are also evident (Fig. 1.A) [17]. Late in the process, organelle swelling and lysis can be seen.

The three affected proteins are ENaC-type cation channels, the so-called degenerins, that conduct predominantly sodium[18], but also calcium[19], and cell death inducing mutations increase their open channel probability [20]. Thus, abnormal ion homeostasis is likely the initiating insult that leads to cell swelling and death. Gain-of-function mutations in the nicotinic acetylcholine receptor DEG-3[21], another cation channel, also have similar effects.

While the mechanistic details of this pathological cell death process are still not entirely worked out, a prominent role for intracellular calcium release has been suggested. Mutants in the *C. elegans* homolog of the endoplasmic reticulum (ER) calcium-binding chaperone, calreticulin, attenuate MEC-4(gf)-mediated neuronal cell death[22]. Similarly, mutations in calnexin, another ER calcium-binding protein, in ITR-1, the *C. elegans* ER IP_3 receptor, and in the ryanodine receptor ER release channel, UNC-68, also attenuate cell death (Fig. 2), as does the calcium chelator EGTA. Cell death can be restored in these suppressed animals by thapsigargin, which blocks the ER calcium influx pump and causes calcium release from the

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ER. Thapsigargin treatment also results in occasional cell death in wild-type animals, suggesting that cytosolic calcium elevation may be sufficient to promote cell death. Consistent with this idea, the *deg-3(*gf*)* mutations, which likely cause cytosolic calcium increase without the need for additional ER calcium, cannot be suppressed by mutations that block ER calcium release[22]. Additionally, heat shock is also able to induce calcium-dependent necrosis, perhaps by denaturing crucial regulators of calcium homeostasis[23].

While calcium has many functions in the cell, its requirement for the activation of cytosolic calpain and cathepsin proteases may play at least some role in cell degeneration[24]. Overexpression of these proteases is sufficient to cause death with similar morphology, and RNAi-mediated knockdown of the calpains CLP-1 and TRA-3, or the cathepsins ASP-3 and ASP-4 inhibits cell death progression in *mec-4*(gf) mutants. While double calpain or double cathepsin knockdowns enhances cell survival in this background, reducing expression of one of each does not, suggesting that calpains and cathepsins might function in a linear pathway in which elevated cytosolic calcium activates calpains, which, in turn, promote cathepsin activation and cell demise (Fig. 2). However, this model has not been rigorously tested.

Several other cytoplasmic cathepsins exist in *C. elegans* that do not seem to affect activated-channel induced neuronal death. Whether this requirement for select proteases reflects cell-type-specific expression of these proteins or substrate specificity is not clear.

Calcium may not be the only ion involved in degenerin-induced cell death. Mutations in subunits of the vacuolar-H+-ATPase (V-ATPase) ameliorate both degenerin-mediated and thapsigargin-induced death[25], suggesting that cytosol acidification could function downstream of calcium elevation to promote cell death (Fig. 2). Treating *C. elegans* with weak lysotropic bases or impairing lysosomal biogenesis can also attenuate calcium-dependent cell death, suggesting a possible role for this organelle in cytosol acidification [26]. How protons may affect cytosolic protease activation, if at all, is not known, but lysosomes might also contribute to cellular demise by leaking their normally sequestered acid hydrolases into the cytoplasm.

Neuronal cell death accompanied by cell swelling can also be induced in *C. elegans* by constitutive activation of the $G_{\alpha s}$ protein [27,28], which functions through the adenylyl cyclase ACY-1 to transmits signals from metabotropic neurotransmitter receptors. This death is weakly dependent on the voltage-gated calcium channel subunit UNC-36 and on the vesicular glutamate transporter EAT-4[27], suggesting that neuronal activity may modulate sensitivity to pathological cell death. Indeed, deletion of the *C. elegans* glutamate transporter *glt-3*, which presumably leads to higher extracellular glutamate levels, cooperates with $G_{\alpha s}$ overexpression to enhance neuronal cell death[29].

The studies of degenerative cell death in *C. elegans* neurons raise the possibility that similar processes contribute to human nervous system pathologies. For example, as in *C. elegans*, neuronal cell death induced in a mouse stroke model depends on both calcium and low pH. However, in this system, acid seems to function upstream of calcium release[30]. Glutamate-induced toxicity, thought to be an important facet of cell death induction in stroke, may also promote cell death through neuronal second messengers[31].

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2.2 NAD METABOLISM DEFECTS

While neuronal cell death has featured prominently in studies of degenerative cell death in *C. elegans*, the degeneration of non-neuronal cells in response to specific gene mutations has also been described. In *pnc-1* mutant larvae, the uterine uv1 cells die with a vacuolated morphology through a process requiring calpains and aspartyl proteases[32]. Cell death seems to be a response to overabundance of nicotinamide (NAM), which PNC-1, a nicotinamidase homolog, converts to nicotinic acid. Indeed, feeding animals NAM also promotes uv1 vacuolation and death[33]. Why uv1 cells are sensitive to NAM accumulation is not understood. One possibility is that NAM levels alter the generation and/or function of nicotinamide adenine dinucleotide (NAD), a key respiration intermediate. However, muscle cells whose energy requirements are likely much higher, remain intact in *pnc-1* mutants[33]. Boosting EGF signaling, which promotes uv1 specification, suppresses cell death, suggesting that an EGF-repressible NAD consumer, or its product, may be involved. However, if and how such an NAD consumer causes calpain and/or aspartyl protease activation is unclear.

How vacuoles accumulate within neuronal or non-neuronal *C. elegans* cells undergoing degenerative cell death is not well understood. Notably, expressing human caspase-3 in *C. elegans* body wall muscle can promote vacuole formation in these cells as well, rather than the more classical, refractile appearance induced in other cells[34]. Thus, it is possible that both apoptotic and degenerative cell death regulators in *C. elegans* engage common targets. Identification of such targets would be required to confirm this idea.

2.3 CELL DIFFERENTIATION MUTATIONS

In vertebrates and in *Drosophila*, cell death is often induced in response to a failure in cell fate specification or differentiation[35], perhaps as a result of disturbances in proteostasis[36,37]. This may also be the case in *C. elegans*. For example, LIN-26, a Zn-finger transcription factor, normally promotes hypodermal and glial cell fate. A reduction-of-function mutation in this gene results not only in excess neuron production, but also in vacuolation and death of hypodermal and glial cells[38]. While the mechanism promoting cell demise in this case is not known, it is independent of the *ced-3* caspase (M. Labouesse, personal communication).

Developmental failure also seems to lead to cell death in animals carrying mutations in the *unc-83* and *unc-84* genes, which encode KASH and SUN domain proteins, respectively, that anchor the nucleus to the cytoskeleton[39]. In these mutants, nuclei of the P epithelial blast cells fail to migrate ventrally along with the rest of the cell body, resulting in elongated cells that eventually die in a *ced-3*-independent manner[40,41]. Unlike hypodermal cell death in *lin-26* mutants, dying cells in *unc-83/84* mutants are not vacuolated, instead adopting a refractile appearance common to naturally dying cells in *C. elegans*. Double mutants of *unc-84* and genes that block P cell migration do not exhibit P cell death[40]. Furthermore, failure of nuclear migration is not generally lethal to cells, since, in *unc-83* mutants, other cells exhibit nuclear migration failure without death[41]. These observations suggest that the disconnect between cell migration and nuclear migration must trigger a cell-specific response that leads to death. Genetic screens for cell death suppressors could reveal the key

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players in this pathological process, and should reveal whether it is possible to interrupt cell death without restoring nuclear migration.

While cell death in response to failed differentiation in *C. elegans* is *ced-3*-independent, and likely caspase independent, this is not the case in other animals[42-45]. The source of this difference is not clear, but suggests that at least in somatic cells in *C. elegans*, caspases and other apoptotic genes respond mainly to programmed stimuli. *C. elegans* germ cells can engage caspases in response to irradiation and other DNA lesions[46], suggesting that damage responses in this tissue may be more akin to generalized responses in vertebrates.

2.4 *lin-24/lin-33* MUTANTS

Dominant mutations in two genes, *lin-24* and *lin-33*, promote the inappropriate deaths of P*n*.p cells, daughter cells of the P cells affected by *unc-83* and *unc-84* mutations[47]. Dying cells assume a refractile, non-vacuolated appearance under Differential Interference Contrast (DIC) optics. Electron microscopy revealed that dying cells exhibit electron-dense nuclear puncta, but otherwise normal nucleoplasm, dilation of the nuclear envelope, dense membranous cytoplasmic whorls, and disrupted mitochondria (Fig 1.B). Some dying cells can recover and reacquire normal morphology, while others can recover but possess a small nucleus. P*n*.p cell fate is also affected in these mutants, but whether fate changes result from developmental cues missed because of injury or are independent defects is not clear.

Programmed cell death in *C. elegans* is usually an all-or-none process, however, recovery from death is also seen in animals doubly mutant for weak mutations in *ced-3* and genes promoting apoptotic cell corpse engulfment. In these animals, progeny of P*n*.p cells begin to undergo normal developmental cell death, acquire a refractile appearance, only to recover and inappropriately survive[48]. This phenomenon is only seen in engulfment mutant backgrounds, demonstrating that engulfment can modulate cell susceptibility to death. Indeed, one *C. elegans* cell, B.al/rapaav, always survives in engulfment-defective mutants, despite additional dependence on *ced-3* caspase activity for death[48], and, rarely, cells in animals lacking all four *C. elegans* caspase-related genes die and are engulfed[49], hinting perhaps at a role for engulfment in cell death. Remarkably, cell death in dominant *lin-24/33* mutants can also be attenuated by mutations in engulfment genes[47]. While *ced-3* caspase does not seem to play a role in *lin-24/33* mediated cell death, mutations in *egl-1*/BH3-only and *ced-4*/Apaf-1 weakly interfere with the process. The sites of action of *lin-24/33* or any of the modifying genes in the context of P*n*.p cell death are not known.

Deleting *lin-24, lin-33*, or both has no obvious effects on *C. elegans* development, or cell survival. However, loss of function of either gene prevents P*n*.p cell death by dominant mutations in the other, suggesting that the encoded proteins may function in a complex. LIN-24 protein contains a domain similar to bacterial toxins, and both loss- and gain-of-function alleles alter this conserved domain[47]. The predicted LIN-33 protein does not resemble other known genes. Bacterial toxins homologous to LIN-24 kill eukaryotic cells by forming oligomeric pores in the plasma membrane, a mechanism shared with the membrane attack complex of the vertebrate blood complement system[50] as well as with the perforins of cytotoxic T lymphocytes and NK cells[51]. One possibility, therefore, might be that LIN-24/33 mutant proteins are inappropriately released from neighboring cells to promote

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P*n*.p cell death by poking holes in their membranes. Engulfment mutants might suppress death by preventing contact between P*n*.p cells and their killer neighbors. It is equally plausible that LIN-24/33 function in the dying cell to introduce membrane pores, and that engulfing cells, sensing membrane perturbations, finish off the weakened P*n*.p cells.

2.5 A LATENT APOPTOTIC PATHWAY IN P*n*.p CELLS?

That apoptotic genes modulate *lin-24/33* dependent P*n*.p cell death suggests that this death process may be mechanistically related to apoptosis. Support for this notion comes from studies of loss-of-function mutations in the gene *pvl-5*. Animals carrying such mutations exhibit inappropriate P*n*.p cell death, which occurs at the same developmental time as *lin-24/33*-mediated deaths. Dying cells in *pvl-5*(lf) animals can recover, and recovered cells frequently exhibit an ovoid morphology and shrunken nucleus as in *lin-24/33* mutants[52]. Intriguingly, *pvl-5*-mediated cell death requires *ced-3* caspase and can be suppressed by *ced-9*(gf) mutations, suggesting inappropriate initiation of an apoptosis-related pathway in P*n*.p cells.

Nonetheless, *pvl-5* and *lin-24/33* mediated Pn.p cell deaths are not identical. *pvl-5* mutations can cause P*n*.p cell vacuolation not reported in *lin-24/lin-33* mutants. Moreover, the cell fate defects resulting from the two lesions are likely to be different: *lin-24/33* mutants lack the hermaphrodite vulva, which is normally generated by P*n*.p cell descendents, whereas *pvl-5* mutants exhibit a protruding vulva defect. Furthermore, while *lin-24/lin-33*-mediated death is weakly suppressed by mutations in all core cell death genes except for *ced-3, pvl-5(*lf*)*-mediated P*n*.p cell death is suppressed by *ced-9(*gf*),* but not by *egl-1* or *ced-4* loss-of-function mutations. Finally, *pvl-5* mutants suffer a small number of *ced-3*-dependent ectopic cell deaths in cells other than the P*n*.p cells, a defect not reported for *lin-24/33* mutants. The molecular identity of the *pvl-5* gene is not known, but it maps to a different chromosome from *lin-24* and *lin-33*. How *pvl-5* regulates *ced-3* function is also not understood.

In *Pristionchus pacificus* and other nematodes more distantly related to *C. elegans*, some or all P*n*.p cells that are not destined to contribute to vulva formation die by *ced-3*-dependent apoptosis[53,54]. All P*n*.p cells have the capacity to die in *Pristionchus*, as mutants in the Hox gene *Ppa-lin-39* are vulvaless because all P*n*.p cells die. Cell death is blocked, and vulva formation is restored in animals also carrying *Ppa-ced-3* mutations. A genetic screen for *Ppa-lin-39* suppressors recovered 22 alleles of *Ppa-ced-3*, but only two alleles of other genes. This may suggest that in *Pristionchus* P*n*.p cells, *ced-3* caspase is the main cell death effector, a profile resembling that of *pvl-5*-induced cell death, although differences in gene mutability could also account for this mutational profile.

Taken together, these studies suggest that *C. elegans lin-24/33* and *pvl-5* mutations may uncover a silenced apoptotic program that is still functional in other nematodes (Fig. 3). However, the differential requirement for *ced-3* and *ced-4* in these death processes remains puzzling. RNAi against *icd-1*, the beta subunit of the nascent polypeptide associated complex (β-NAC), causes widespread ectopic cell death during *C. elegans* development[55]. As in *lin-24/33* mutants, loss of *ced-4*, but not of *ced-3*, suppresses *icd-1(RNAi)*-mediated death, and other *C. elegans* caspases play only minor roles in this process[49]. One must,

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therefore, entertain the possibility that *ced-4* can promote cell death in the absence of caspases.

2.6. CELL SHEDDING IN CASPASE MUTANTS

In embryos lacking all four *C. elegans* caspases, six cells are shed from the anterior sensory depression and the ventral pocket[56]. These cells express *egl-1*, but are still shed in *egl-1(*lf*), ced-9(*gf*),* and *ced-4(*lf*)* mutants as well. In wild-type embryos, shed cells die normally and are engulfed by neighboring cells. Mutations in the MELK kinase PIG-1 and in its activating kinase PAR-4/LKB1 prevent shed cell accumulation. These observations have led to the hypothesis that cell shedding is a cell death program that functions in parallel to CED-3 caspase, perhaps as a backup program, and that PIG-1 is a key activator of this program. Perhaps the strongest evidence in favor of this model is that shed corpses exhibit some features reminiscent of apoptosis (Fig. 1.C).

Cell shedding does not appear to take place in wild-type animals and *pig-1* expression is not sufficient to promote cell death. These and other results have raised the possibility that cell shedding may not be a cell death program *per se*, but a passive result of *ced-3* caspase loss[57]. The transcriptome of dying cells may be under reduced selective pressure, and, if so, dying cells may be poorly differentiated. Indeed, while inappropriately surviving cells in *ced-3* mutants can acquire fates of their sister cells or their progeny, differentiation reporters are weakly expressed in many of these surviving cells[7], and fate acquisition is often incomplete[58]. It is possible, therefore, that in *ced-3* mutants, these six inappropriately surviving cells have poor expression of cell adhesion proteins, and are passively extruded from the animal in response to movements of adjacent cells. *pig-1* mutations could, in this model, enhance the differentiation of the “undead” cells towards their adhesive sister cell fate, thereby preventing shedding. PIG-1 has been implicated in the control of asymmetric cell division[59,60] and plays important roles in cell fate specification throughout the embryo[61]. In the context of cell shedding, *pig-1* mutants inappropriately express the α-catenin HMP-1 on the surface of would-be shed cells, and one of these cells expresses reporters specific for its sister cell progeny, the excretory cell[56].

If *pig-1* does regulate a novel cell death process, the expectation would be that its role and its targets in all dying cells be the same. Whether this is the case is unclear, however, *pig-1* mutations also affect the death and specification of the sister cell of the M4 pharyngeal neuron[62], and this cell is not shed and remains adhesive in *ced-3* mutants[58].

3. DEVELOPMENTAL CELL DEATHS THAT DO NOT FOLLOW THE CANONICAL APOPTOTIC PATHWAY

3.1 GERMLINE CELL DEATH

In adult *C. elegans* hermaphrodites, about half of female germ cells die by apoptosis before developing into mature oocytes[63]. Unlike dying somatic cells, whose identities are invariant, germ cells, which occupy a syncytium and appear identical, seem to die stochastically. Competence to die is imparted by ephrin and Ras/MAPK signaling, probably

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originating from surrounding sheath cells, resulting in germ cell exit from meiotic pachytene[64,65].

While germline cell death requires *ced-3, ced-4*, and *ced-9*, it is independent of *egl-1* and is not blocked by a gain-of-function mutation in *ced-9* that prevents somatic cell death[63]. Mutations in the Pax2-related genes *egl-38* and *pax-2* promote excess germ cell death. Genetically, *egl-38* and *pax-2* seem to function upstream of *ced-9*, a model supported by the observation that EGL-38 and PAX-2 proteins bind to regulatory sequences near the *ced-9* gene. It is therefore possible that in the germline, Pax2 proteins substitute for EGL-1. Nonetheless, in the soma, *egl-1* transcription is induced in dying cells. This does not seem to be the case for *egl-38* and *pax-2*[66], suggesting that these genes may act permissively to set *ced-9* levels in germ cells. Thus, other inputs into the apoptotic pathway may control the decision to promote germ cell death. The involvement of genes acting in gonadal sheath cells in germ cell death competence[67,68] raises the possibility that regulation of germ cell death could have cell-autonomous and non-autonomous components, which would allow the animal to make decisions about germ cell death based on both the overall state of the animal[69-73] as well as the integrity of individual germ cell genomes[74].

3.2 TAIL-SPIKE CELL DEATH

The genetic requirements for germ cell death are mirrored, in part, in the *C. elegans* tail-spike cell. This binucleate cell, which arises by cell fusion, sends a slender posterior process that seems to serve as a scaffold for molding the *C. elegans* tail. *ced-*3 and *ced-4* are absolutely required for tail-spike cell death, but *egl-1* plays only a minor role, and a gain-of-function mutation in *ced-9* has no effect[75]. Studies of *ced-3* transcription revealed that its expression is induced in the tail-spike cell about 25 minutes before morphological signs of cell death are apparent. The homeodomain transcription factor PAL-1 promotes *ced-3* expression in the tail-spike cell by binding to three redundant sites upstream of the *ced-3* gene. These results suggest that transcriptional induction of *ced-3*, and not of *egl-1*, may be the key regulatory event promoting tail-spike cell death.

Additional layers of control also exist. A recent study demonstrated that tail-spike cell death requires the F-box protein DRE-1. Genetic and molecular evidence supports the idea that DRE-1 functions in a Skp/Cullin/F-box (SCF) complex in parallel to EGL-1 and likely upstream of CED-9. An attractive model is that DRE-1 substitutes for EGL-1 by inactivating CED-9 through ubiquitination and degradation, thereby creating a permissive environment for newly translated CED-3. Support for this model comes from studies of human FBX010 and BCL2, proteins similar to DRE-1 and CED-9, respectively. In a subset of B-cell lymphomas, FBX010 expression can promote BCL2 degradation. Furthermore, in these same lines, FBX010 expression promotes cell death[76].

Mutations in FBX010 are found in some patients with B-cell lymphomas, and expression of the gene is reduced in many others. Furthermore, RNAi against FBX010 in tumor cells promotes their survival[76]. These results suggest that FBX010 may function as a tumor suppressor gene. Mutations in Cdx2, the human homolog of *C. elegans pal-1* promote intestinal tumors[77], suggesting that this gene is a tumor suppressor as well. These observations raise the intriguing possibility that while tail-spike cell death control exhibits

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non-canonical features in *C. elegans*, similar regulatory mechanisms may play integral roles in controlling tumorigenesis in humans.

3.3 SEX-SPECIFIC DEATH OF CEM NEURONS

The sexually dimorphic CEM cells survive in males, differentiating into neurons that help orchestrate the male's complex mating behavior[78]. In hermaphrodites, which do not exhibit this behavior, the neurons die[79]. CEM cell death requires all four core cell death genes. Yet, as in the germline and tail-spike cells, CEM cell death regulation appears to require transcriptional activation of the *ced-3* caspase gene. Although *egl-1* expression is still induced in CEM neurons, this induction is not always sufficient to promote CEM death. In males carrying mutations in *unc-86*, a gene encoding a POU homeodomain transcription factor, *egl-1* expression is unaltered, but CEMs fail to die. Genetics and expression studies revealed that UNC-86 protein, LRS-1, a tRNA synthetase, and UNC-132, a novel protein, control CEM demise by promoting *ced-3* transcription [80,81]. Nonetheless, whether *ced-3* or *egl-1* transcription is the rate-determining step in CEM cell death remains unclear.

ced-3 transcription in CEMs seems to be counteracted by CEH-30, a BarH1-related transcription factor. CEH-30 functions genetically downstream of *egl-1* and *ced-9*[80,82]. A *ceh-30* gain-of-function allele alters an intronic consensus sequence for binding by TRA-1A, a Gli-related protein that is an effector of the sex determination machinery promoting hermaphrodite identity[83,84]. This observation suggests that TRA-1A normally represses *ceh-30* in hermaphrodites.

CEM neurons and the tail-spike cell survive for an extended duration after they are generated and before succumbing to cell death. Likewise, both cell types actively control transcription of *ced-3*. This correlation raises the possibility that in these long-lived cells destined to die, there is a need to replenish CED-3 protein to promote cell death. Indeed, *ced-3* transcriptional reporter studies suggest that while the gene is widely expressed, its transcription is mainly confined to early embryogenesis[85], before most cell death takes place. Thus, cells that are longer lived may need to re-express the gene to promote their demise.

3.4 THE USE OF ALTERNATE CASPASES IN DYING CELLS

The *C. elegans* genome contains three caspase-encoding genes in addition to *ced-3*: *csp-1, csp-2*, and *csp-3*[86]. While CSP-1 protein has caspase activity *in vitro*[86], and its overexpression can promote cell death in *C. elegans*[49], neither *csp-2* nor *csp-3* seem to encode catalytically active enzymes. CSP-2 has a catalytic cysteine, but lacks conserved residues surrounding the active site, and CSP-3 lacks the large caspase subunit and its active site.

csp-1 may play a minor role in somatic cell death. While mutants in the gene have no obvious cell death defects, enhanced cell survival is observed in conjunction with weak mutations in *ced-3* caspase[49]. Enhancement is cell-specific, as only some cells destined to die, such as the sister of the pharyngeal M4 neuron, are affected. The activity of CSP-1 does not appear to be regulated by CED-4, as *ced-4* lesions do not inhibit ectopic cell death

mediated by CSP-1. It seems, therefore, that if CSP-1 has a role in cell death, it may respond to different cues than CED-3.

Loss-of-function mutations in the *csp-2* and *csp-3* genes have been reported to enhance cell death in the germline[87] and soma[88] respectively, although this observation has been challenged[49]. A suggested mechanism for these effects is that these caspase-related proteins bind CED-3 or CSP-1 to inhibit their activities[87,88]. However, given the weak cell death effects of mutants in these genes, testing models regarding their activities remains challenging.

5. NON-APOPTOTIC, CASPASE-INDEPENDENT LINKER CELL DEATH

The male-specific linker cell leads the developing male gonad on a stereotyped elongation path and, upon its death, permits the lumen of the vas deferens to fuse with the cloaca to allow sperm exit[89]. Linker cell death is independent of all *C. elegans* caspases and other apoptotic cell death genes[7,49,90], and also seems to proceed independently of genes controlling apoptotic cell engulfment and proteases involved in other cell death forms in *C. elegans*[90]. While linker cell death was initially thought to proceed non-autonomously, through engulfment by the U.l/rp cell[79,89], recent studies demonstrate important cell autonomous components involved in the process[90,91].

Consistent with the unique genetic requirements, dying linker cells are morphologically distinct from apoptotic cells in which chromatin condensation and cytoplasmic shrinkage are generally evident. Dying linker cells maintain open chromatin, and display progressive nuclear envelope crenellation leading to formation of a flower-shaped nucleus never observed outside this setting in *C. elegans*. Mitochondrial and endoplasmic reticulum (ER) swelling is also observed (Fig 1.D)[90].

The death of the linker cell requires both temporal and spatial cues. Mutations in the microRNA gene *let-7* and the Zn-finger transcription factor gene *lin-29* block linker cell death. These genes are components of a developmental timing program, the heterochronic pathway, that communicates the developmental stage of animals to individual cells within the animal. LIN-29 functions together with the MAB-10 transcriptional cofactor[92], and both proteins are present in the nucleus of the linker cell during its migration and death (Fig. 4). Mutation in the *him-4* gene, encoding a secreted immunoglobulin family member, reroute the linker cell migration path, so that cells often end up in the head, instead of the tail. While most linker cells die on time even in the head, about 13% of *him-4* mutants exhibit linker cell survival, suggesting that local spatial cues may be important for linker cell death. Our recent studies suggest a role for Wnt signaling in transducing such a spatial cue, (M. Kinet and S. Shaham, unpublished observations).

Dying linker cells morphologically resemble cells that die during normal vertebrate development. For example, approximately half of the cells initially present in the developing chick ciliary ganglion die during development. Electron microscopy fails to reveal apoptotic features in dying cells., but does uncover cells with swollen mitochondria and ER[93]. Nuclear crenellation can also be observed in these cells, becoming more pronounced when ganglion neurons are deprived of their target organ[93]. Developmental death of spinal

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motor neurons proceeds, slowed but unabated, in the absence of caspase-3 or caspase-9, and dying cells exhibit open chromatin, swollen ER and mitochondria, and cytoplasmic vacuolation[94]. Crenellated nuclei and swollen ER and mitochondria are also observed in dying neurons of patients with poly-glutamine expansion diseases, such as Huntington's disease and some spinocerebellar ataxias, as well as in mouse models for those disease[91]. Strikingly, the gene *pqn-41*, which encodes a protein containing a 427 amino-acid C-terminal domain rich in glutamines, is required for linker cell death. *pqn-41* seems to function in the same pathway as the conserved MAPKK SEK-1 and its adapter protein TIR-1 to promote linker cell death (Fig. 4) [91]. *pqn-41* is not required for other cell deaths in *C. elegans*, and ectopic expression of the rescuing PQN-41C isoform does not precociously kill the linker cell or other cells, suggesting that it must function with other components to promote linker cell death.

Recently, the TIR-1 homologs dSarm and Sarm have been implicated in distal neurite degeneration following axotomy in *Drosophila* and mice, respectively[95]. That the same protein promotes degenerative processes in all three species is intriguing, and bolsters the possibility of a connection between linker cell death and cell death processes in humans. Further, excitotoxic injury to mouse retinal ganglion cells induced by kainate treatment also requires Sarm[96], suggesting mechanistic commonalities between excitotoxic necrotic death and other degenerative deaths that may explain some of the observed morphological parallels . Nonetheless, it is still too early to tell whether the morphological and molecular similarities between linker cell death, normal vertebrate cell death, polyglutamine-mediated cell death, and axon degeneration represent true conservation or happenstance.

Why does the linker cell not die by apoptosis? The cell is larger than other cells that succumb to apoptosis and likely harbors extensive functional machinery required for its long migration and the concomitant morphological stages through which it must progress[97]. For these reasons, the linker cell might require an alternate program to deal with its degradation. A similar idea has been invoked for the degeneration of *Drosophila* salivary glands, although in this case cell death remains caspase-dependent[98]. Alternatively, the linker cell death program may ensure that the cell will be engulfed by a specific phagocyte. Supporting this idea, the engulfment of dying linker cells is independent of genes required for apoptotic corpse engulfment[90]. Furthermore, the engulfing U.l/rp cell does not cluster CED-1: :GFP at membranes making contact with the linker cell, as is the case for apoptotic cells. CED-1: :GFP does surround mistargeted dying linker cells in *him-4* mutants, suggesting that the cell can be engulfed by this mechanism, and that other cells may not express the physiological engulfment program utilized by the U.l/rp cells. Mistargeted *him-4* cell corpses often persist much longer than wild-type corpses[90], suggesting that the *ced-1*-mediated engulfment process used at these locations is not as efficient as the physiological program engulfing the dying linker cell. Supporting this notion, CED-1: :GFP surrounding mistargeted linker cell corpses can be incomplete[90], a phenomenon never seen in apoptotic corpse engulfment but reminiscent of the incomplete engulfment seen in *ced-1* mutants[99]. Corpses of cells in animals with ablated U.l/rp cells also persist in the terminal vas deferens (M. Abraham and S. Shaham, unpublished observations), appearing to block

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sperm exit, suggesting that terminal vas deferens cells are not competent for engulfment using either canonical or linker cell processes.

The cell-specific competence for expressing the physiological engulfment program combined with the selective efficiency between specific death and engulfment programs suggests a strategy for targeting specific cells to specific phagocytes. Such a strategy may have specific anatomical imperatives in the worm, but, in animals with cellular immune systems, it is the rule rather than the exception.

6. CONCLUSION

C. elegans has been appropriately lauded as a system for studying programmed cell death, as studies in this organism laid the foundations for understanding the conserved process of apoptosis. *C. elegans* has also been used to study cell death induced by environmental toxicants[100,101], excellent studies in their own right with clear relevance to humans but outside of the scope of our present discussion. Here we have reviewed experiments suggesting that this animal still has much to offer in the context of programmed cell death research. From the identification of a novel morphologically conserved developmental cell death program, to the characterization of different degenerative processes, *C. elegans* continues to be an exciting venue for uncovering basic mechanisms that control cell viability normally and in pathological states. Several of the seemingly disparate cell death phenomena described in this review share common threads, either morphological (Fig. 1) or molecular. As our understanding of cell death processes expands, additional interconnections may emerge, providing insight into what key cellular aspects must be dismantled for cells to give up the ghost.

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Highlights

- The core apoptotic pathway can be regulated at any step.
- Death processes share morphological features and possibly downstream effectors.
- Linker cell death is a novel death program possibly relevant to human biology.

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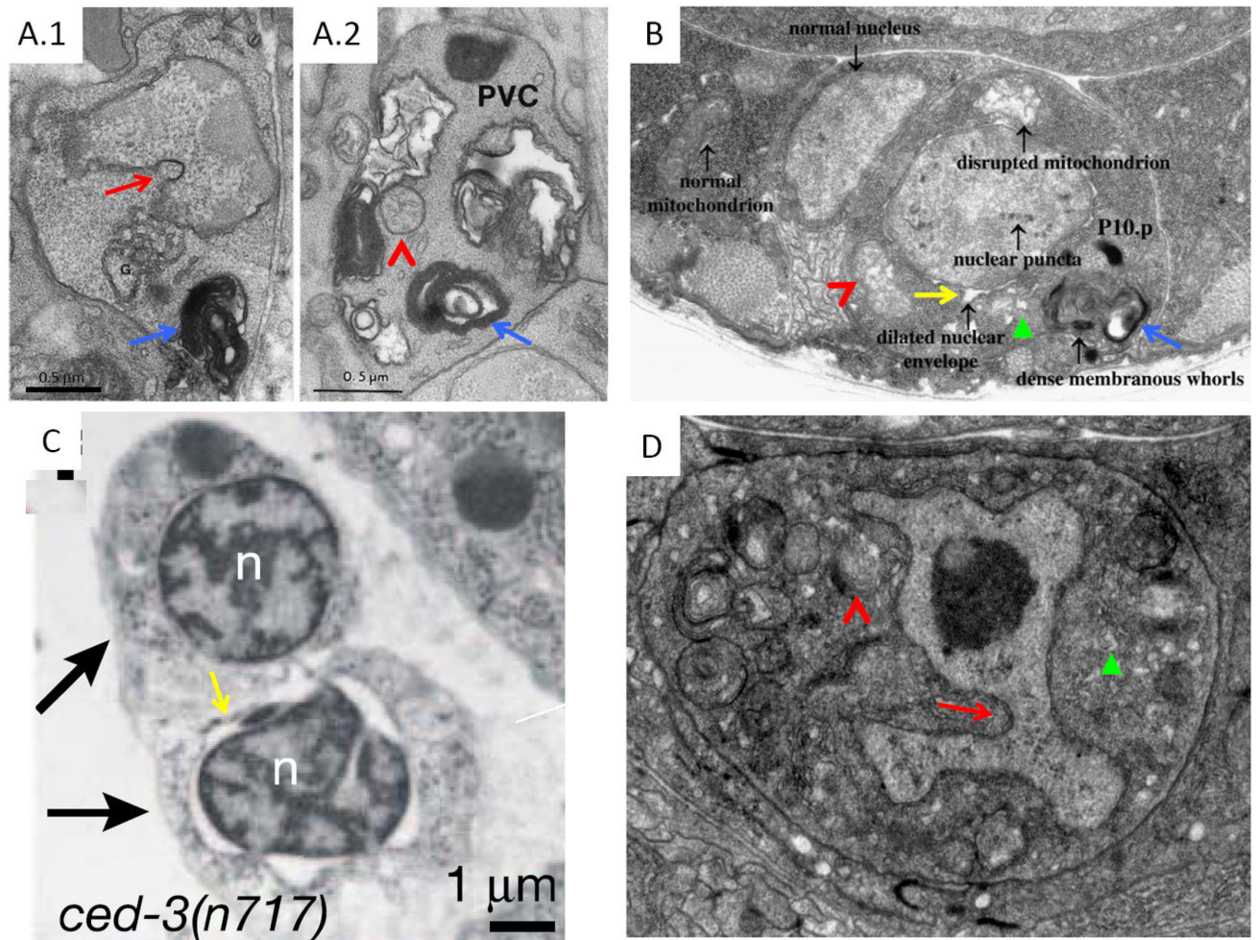

Figure 1.

Different cell death pathways share morphological features. A. PVM neuron (A.1) of a *mec-4(*gf*)* mutant and PVC neuron (A.2) of a *deg-1(*gf*)* mutant, Reproduced with permission from [17]. B. P10.p cell in a *lin-33(gf)* animal. Reproduced with permission from [47]. C. Shed cells (arrows) in a *ced-3(n717)* embryo. Reproduced with permission from [56]. D. Dying linker cell. Reproduced with permission from [90]. Nuclear indentations, red arrows. Membranous whorls, blue arrows. Dilated ER, green arrowheads. Dilated nuclear envelope, yellow arrows. Dilated mitochondria, red carrats. Dark intranuclear structure in D is the linker cell nucleolus.

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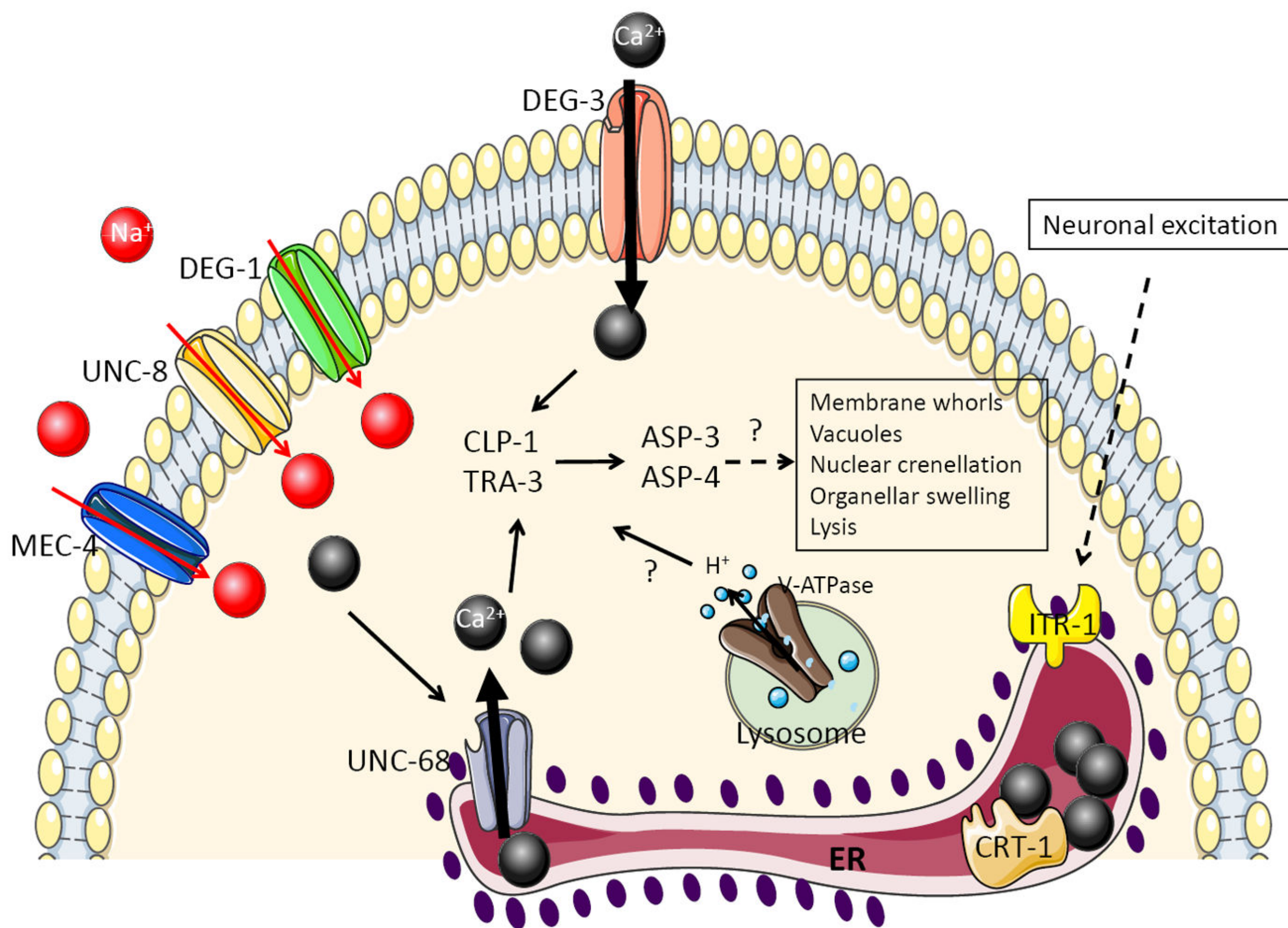

Figure 2.

Mechanisms of ion channel mutation induced death in *C. elegans.*

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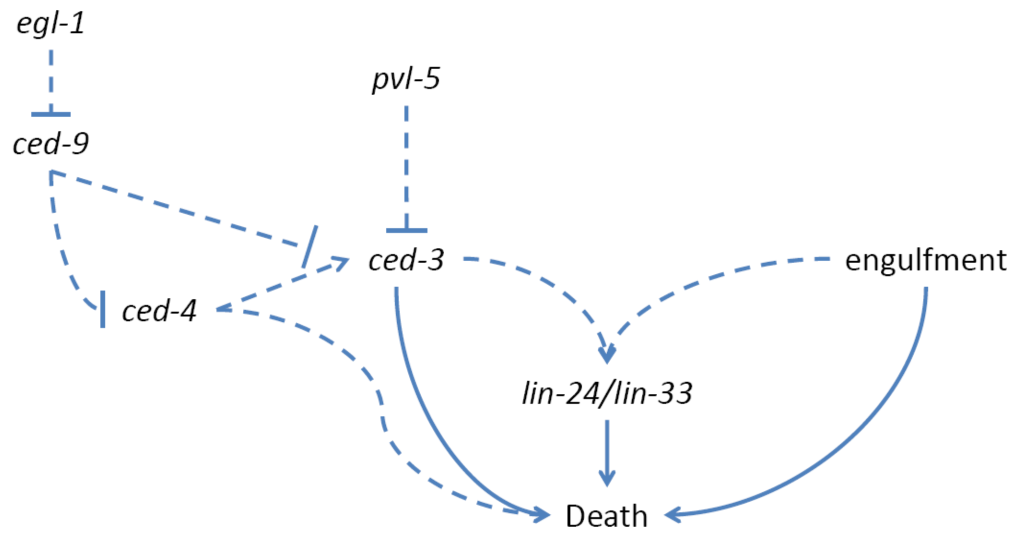

Figure 3.
Possible P*n*.p cell death pathways in P*n*.p cells. Dashed arrows indicate tentative relevant genetic interactions.

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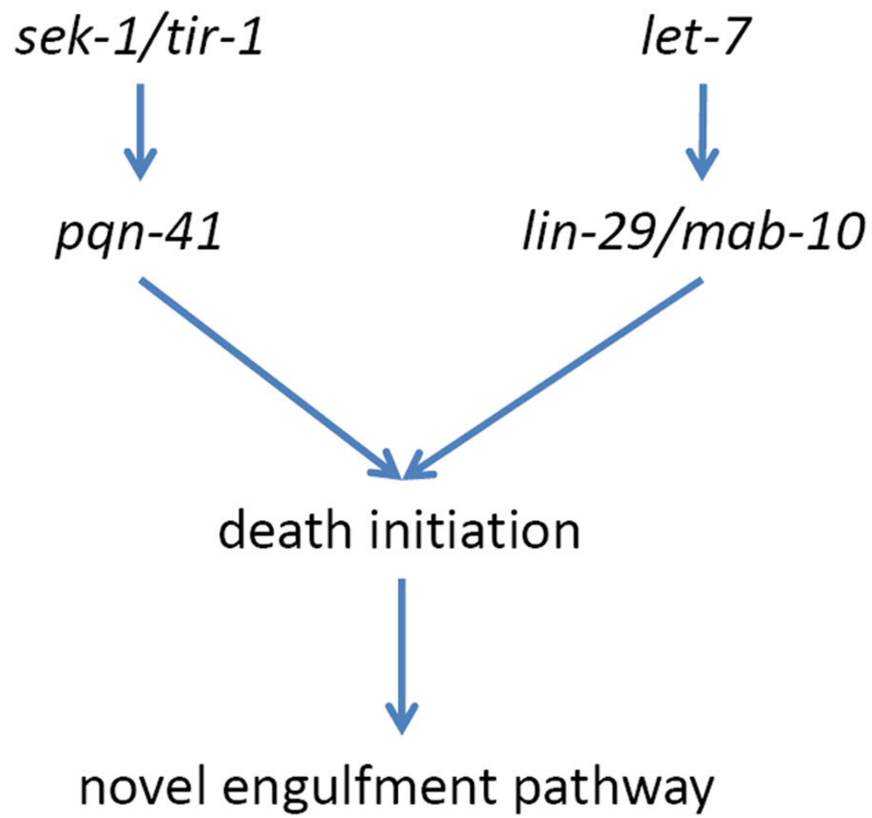

Figure 4.
Genetics of linker cell death. Arrows, plausible genetic interactions.