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Review

Noncanonical cell death programs in the nematode *Caenorhabditis elegans*

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Genetic studies of the nematode *Caenorhabditis elegans* have uncovered four genes, *egl-1* (BH3 only), *ced-9* (Bcl-2 related), *ced-4* (apoptosis protease activating factor-1), and *ced-3* (caspase), which function in a linear pathway to promote developmental cell death in this organism. While this core pathway functions in many cells, recent studies suggest that additional regulators, acting on or in lieu of these core genes, can promote or inhibit the onset of cell death. Here, we discuss the evidence for these noncanonical mechanisms of *C. elegans* cell death control. We consider novel modes for regulating the core apoptosis genes, and describe a newly identified cell death pathway independent of all known *C. elegans* cell death genes. The existence of these noncanonical cell death programs suggests that organisms have evolved multiple ways to ensure appropriate cellular demise during development.
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Cell death is an important process in metazoan development, and coordinated control of cell division, differentiation, and death governs many aspects of morphogenesis. Often, waves of cell proliferation are followed by cell death, which serves to sculpt and delete structures, adjust cell numbers, and eliminate aberrantly produced cells.[1–4] Animals defective in cell death can display severe developmental abnormalities and may not survive to adulthood.[5–7]

Studies of the nematode *Caenorhabditis elegans* provided initial evidence that developmental cell death has an underlying genetic basis.[8] A transparent cuticle and nearly invariant, lineage-restricted development have allowed the construction of cell death maps describing which cells die, when they die, and where they die during *C. elegans* development. These maps, coupled with genetic tractability, have made *C. elegans* a powerful system in which to study cell death. In the developing *C. elegans* hermaphrodite, two waves of somatic cell death occur: 113 cells die during embryonic development, and an additional 18 somatic cells die in early postembryonic stages.[9,10] A third wave of cell death occurs in the adult hermaphrodite germ line, where roughly half of developing germ cells die.[11]

Unlike dying cells in many other organisms, most somatic cells fated to die in *C. elegans* are not differentiated and succumb less than 30 min after they are born. Four cells fated to die in the hermaphrodite, the cephalic companion neurons (CEM) neurons, live for nearly 3 h before dying. In addition, three other cells fated to die, MS.pppaaa, the sister cell of the Z1 germ-line precursor cell, the tail-spike cell, and the male-specific linker cell, acquire differentiated features, and survive for six, five, and 30 h, respectively, before they die.[9,10] Although the death of the MS.pppaaa cell has not been examined in detail, the CEM neurons, the tail-spike cell, and the linker cell have been shown to employ noncanonical cell death programs (see below), suggesting that elimination of longer-lived, differentiated cells may require special cell death mechanisms. Here, we review the programs leading to the demise of these, and of other *C. elegans* cells exhibiting alternative modes of cell death regulation.

The Canonical *C. elegans* Cell Death Program

Four core genes that mediate cell death during *C. elegans* development have been described. Loss-of-function (lf) mutations in *egl-1*, *ced-4*, and *ced-3* result in the survival of many cells destined to die, and overexpression of these genes promotes cell death. These results indicate that *egl-1*, *ced-4*, and *ced-3* function normally to promote cellular demise.[8,12–15] By contrast, *ced-9*(lf) mutations result in excessive cell death and organismal lethality, and a *ced-9* gain-of-function (gf) mutation blocks cell death, suggesting that *ced-9* functions normally to protect against cell death.[16,17] Epistasis studies between these genes suggest a linear pathway of action, in which *egl-1* inhibits *ced-9*,[12] which, in turn, inhibits *ced-4*, preventing activation of *ced-3*[15] (Figure 1).

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Keywords: *C. elegans*; apoptosis; linker cell; neurodegeneration; tail-spike cell
Abbreviations: BH3, Bcl-2 Homology domain 3; CEM neurons, cephalic companion neurons; Cdx2, caudal-related homeobox 2; MAPK, mitogen-activated protein kinase; GFP, green fluorescent protein; RT–PCR, reverse transcriptase-PCR; DEG/ENaC, degenerin and epithelial Na^+ channel
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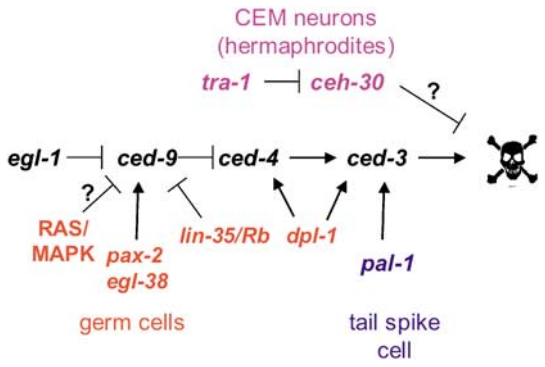

Figure 1 Noncanonical mechanisms for cell death initiation in *C. elegans*. Execution of the canonical cell death program (black letters) is thought to be initiated by activation of *egl-1*, which, in turn, inhibits *ced-9*, allowing *ced-4* to activate *ced-3*. Interaction of noncanonical regulators with this core cell death pathway has been documented in the following settings: (1) germ cell death can be prevented by *pax-2, egl-38,* and *lin-35* transcriptional regulation of *ced-9* or stimulated by *dpl-1* transcriptional regulation of *ced-4* and *ced-3*. In addition, RAS/MAPK may promote germ cell death through an unknown mechanism. (2) Tail-spike cell death is initiated by *pal-1* transcriptional upregulation of *ced-3*, and still requires *ced-4*. (3) The sexually dimorphic survival of the cephalic companion neurons (CEM) neurons is regulated by the sex determination pathway (*tra-1*) through transcriptional activation of *ceh-30*. The mechanism of *ceh-30*'s anti-apoptotic activity is unknown

Molecular characterization of the core cell death genes and their subsequent biochemical study revealed that the genetic interactions observed between these genes reflect physical-binding events that ultimately control the activation of CED-3, a cysteine protease of the caspase family.[18] In the absence of apoptotic stimuli, CED-9, an anti-apoptotic member of the B-cell lymphoma/leukemia-2 protein family, binds to and sequesters CED-4 (apoptosis protease activating factor-1) to the outer mitochondrial membrane.[19–21] In cells destined to die, EGL-1, a Bcl-2 homology domain 3 (BH3)-only protein, binds to CED-9, disrupting its interaction with CED-4.[12,22,23] CED-4 is then free to promote CED-3 activation and subsequent cellular destruction.[19,20,24,25]

The linear nature of the canonical *C. elegans* cell death pathway has led to the hypothesis that specification of which cells live and which cells die during development is accomplished by controlling the levels of EGL-1. Thus, it has been proposed that in cells destined to live, EGL-1 activity is low, and that in cells destined to die, EGL-1 activity is high. Indeed, studies of a small number of cells fated to die have shown that expression of *egl-1* is under the control of known developmental transcription factors.[26–31] However, careful examination of the genetic interactions between *C. elegans* cell death genes suggests that the story is not so simple. If the linear model is correct, in double mutant animals containing both *ced-9*(lf) and *ced-3*(weak lf) alleles, the status of EGL-1 should be irrelevant, because its target, CED-9, is absent. However, many cells still die appropriately in these animals, and few, if any, ectopic cell deaths are observed[17] (S Shaham unpublished data). These results suggest either that EGL-1 can target proteins other than CED-9, or, perhaps more likely, that other cell death initiation mechanisms independent of EGL-1 must exist. As we review here, a number of EGL-1-independent regulatory interactions have been described.

EGL-1-Independent Cell Death in the *C. elegans* Germ Line

In adult *C. elegans* hermaphrodites, roughly 50% of developing germ cells undergo programmed cell death. Characterization of the underlying death program in these cells has revealed noncanonical initiation of the process.[11]

The distal ends of the hermaphrodite gonad arms are capped by a distal tip cell (DTC) that maintains germ cells in a mitotic state.[32,33] As they move away from the DTCs, germ cells enter meiosis, and arrest at the pachytene stage of meiosis I. Activation of the RAS/mitogen-activated protein kinase (MAPK) signaling pathway is then required for the germ cells to exit pachytene.[34] It is also at this stage of development that germ cells undergo programmed cell death. As they die, they are rapidly cellularized away from the germline syncytium, and are engulfed by gonadal sheath cells.[11] Strong loss-of-function mutations in *ced-3* or *ced-4* prevent nearly all germ cell deaths, as they do in the soma. Conversely, CED-9 functions to protect germ cells against death. In animals, homozygous for a temperature-sensitive *ced-9*(lf) allele, the number of cell deaths observed in the germ line is nearly three times than that observed in wild-type animals. Surprisingly, however, neither *egl-1*(lf) mutations nor a *ced-9*(gf) mutation, reported to disrupt binding to EGL-1,[23,35] affects germ cell death. Thus, in the germ line, *egl-1* cannot serve to integrate signals that control cell death onset.

How is cell death initiation regulated in germ cells? One possibility involves the RAS/MAPK pathway, which regulates meiotic entry of germ cells (Figure 1). Gumienny[11] demonstrated that loss-of-function mutations in components of this pathway indeed block the accumulation of dying germ cells. However, gain-of-function mutations in the pathway fail to increase the number of dying cells, suggesting that the RAS/MAPK pathway establishes the competency of germ cells to die, but additional genes must also interact with the cell death machinery.

Studies of the Pax2/5/8-related proteins EGL-38 and PAX-2 suggest that these transcriptional regulators may directly control the activity of the cell death machinery in the germ line.[36] Genetically reducing the levels of either EGL-38 or PAX-2 proteins, using loss-of-function mutations in these genes, increases the number of dying germ cells, consistent with the idea that *egl-38* and *pax-2* normally function to protect against cell death. *egl-38*(lf); *pax-2*(lf) double mutants display additive accumulation of dying germ cells, suggesting that they may function in parallel. Overexpressing, either *egl-38* or *pax-2*, using a heat-shock promoter reduces the number of dying germ cells and does so only in animals in which *ced-9* is intact. These results are consistent with a model in which *egl-38* and *pax-2* function genetically upstream of *ced-9* to promote *ced-9* function. Supporting this hypothesis, overexpressing CED-9 using a heat-shock promoter in *egl-38*(lf); *pax-2*(lf) animals, suppresses the increase in germ cell death. Using real-time reverse transcriptase (RT)–PCR, Park *et al.*[36] demonstrated that *ced-9* transcript levels were decreased in *egl-38*(lf); *pax-2*(lf) animals, and increased in response to induced expression of EGL-38 and PAX-2. Furthermore, using chromatin immunoprecipitation, these authors showed

that EGL-38 and PAX-2 physically bind to the *ced-9* promoter. These results suggest that control of *ced-9* transcription may provide an avenue for the noncanonical regulation of caspase-dependent cell death in the *C. elegans* germ line (Figure 1).

In cultured mammalian cells, Pax5 can interact with the RB protein,[37,38] and a recent study reported that the RB complex controls somatic cell death in *C. elegans*,[39] suggesting that similar interactions might occur in this organism as well. Indeed, Schertel and Conradt[40] demonstrated that loss of *lin-35/*Rb also blocks cell death in the *C. elegans* germ line. Furthermore, using quantitative real-time PCR, these authors demonstrated that in *lin-35* loss-of-function mutants, *ced-9* transcript in the germ line increases fivefold. These results suggest perhaps that LIN-35 interacts with EGL-38 and/or PAX-2 to control *ced-9* mRNA levels; however, this model has not been directly tested. Additional components of the RB complex were also suggested to regulate mRNA levels of *ced-3* and *ced-4* independently of *lin-35*/RB[40]; however, in mutants of these components, only a twofold reduction in *ced-3/4* RNA was detected, suggesting that these components have a more minor role in controlling germ-line cell death.

It is important to note that correlations between the levels of *ced-9* transcript and of EGL-38, PAX-2, and LIN-35 proteins in wild-type germ cells have not been examined, leaving open the possibility that these proteins have only permissive roles in regulating cell death initiation.

Transcriptional Control Downstream of *egl-1* and *ced-9* in the CEM Neurons

The idea that transcriptional control of cell death genes other than *egl-1* could be used as a mechanism for regulating cell death has also emerged from studies of the sexually dimorphic survival of the CEM neurons.[41] Death of the CEM neurons in wild-type hermaphrodites can be blocked by loss-of-function mutations in *egl-1*, *ced-4*, or *ced-3*, or by a gain-of-function mutation in *ced-9*, indicating that the canonical apoptotic machinery mediates this specific cell death.[41,42] However, survival of the CEMs in wild-type males may not be mediated by *ced-9*, since in *ced-9*(lf); *ced-3*(weak lf) males most CEM neurons still survive,[41] unlike other cells destined to die.

Recent studies have demonstrated that the homeodomain transcription factor CEH-30 and the zinc-finger transcriptional regulator TRA-1A are important in the decision of CEM neurons to live or die. Schwartz and Horvitz[41] and Peden *et al*.[42] showed that loss-of-function mutations in *ceh-30* promote the death of male CEM neurons, suggesting that *ceh-30* normally inhibits CEM death. This idea is further supported by the demonstration that a gain-of-function mutation in *ceh-30* causes CEM neurons to inappropriately survive in hermaphrodites.[41] The *ceh-30*(gf) mutation alters an intronic consensus sequence for binding of TRA-1A, a regulator of sexual differentiation expressed only in hermaphrodites.[26,43] Thus, in *ceh-30*(gf) hermaphrodites, weakened TRA-1A binding mimics the low wild-type levels of TRA-1A in males, thereby allowing CEH-30 to exert its anti-apoptotic influence to promote CEM survival.

In the presence of CEH-30, the CEM neurons survive even in the absence of CED-9,[41] indicating that CEH-30 protects male CEM neurons independently of CED-9, suggesting that control of EGL-1 activity in this context cannot mediate cell survival (Figure 1). How CEH-30 functions, and what its targets are is still not known; however, defects in the mammalian protein most related to CEH-30, Barhl1, also promote increased sensory neuron cell death,[44,45] suggesting that the protective effect of this class of transcriptional regulators may be conserved.

EGL-1/CED-9-Independent Cell Death of the *C. elegans* Tail-Spike Cell

Studies of the death of the *C. elegans* tail-spike cell have also revealed that cell death initiation can be mediated by noncanonical transcriptional regulation. These studies also reveal that tail-spike cell death deviates even further from the canonical cell death pathway.[46]

During embryogenesis, the tail-spike cell dies 5 h after its birth, displaying differentiated features including an extended filamentous process that may serve to guide morphogenesis of neighboring hypodermal cells.[10] Tail-spike cell death is only partially dependent on *egl-1*: whereas strong loss-of-function mutations in *ced-3* or *ced-4* completely block tail-spike cell death, strong loss-of-function mutations in *egl-1* only block tail-spike cell death in 30% of animals.[46] Surprisingly, tail-spike cell death is also partially independent of *ced-9*: gain-of-function mutations in *ced-9*, which prevent most other somatic cell deaths, have virtually no effect on tail-spike cell death. Furthermore, a previously described pro-apoptotic function of *ced-9*[17] is entirely absent in the tail-spike cell. Taken together, these results suggest that a novel mechanism regulates caspase activity in the tail-spike cell to promote its death. This mechanism must display remarkable temporal accuracy, since inappropriate early activation of the cell death program would be predicted to lead to defects in hypodermal cell morphogenesis.

Although the nature of the temporal regulator is unknown, Maurer *et al*.[46] demonstrated that this regulator acts, at least in part, through transcriptional control of the *ced-3* caspase. In animals carrying *ced-3* promoter::green fluorescent protein (GFP) reporters, GFP expression was observed in the tail-spike cell only 20–30 min before death ensued. The same promoter sequence driving a *ced-3* cDNA was able to rescue the tail-spike cell death defect in *ced-3*(lf) mutants. These results indicate that expression of *ced-3* just prior to tail-spike cell death is sufficient to promote its death. Maurer *et al*.[46] identified mutants in which transcriptional induction of *ced-3* failed to occur, and which led to inappropriate survival of the tail-spike cell. Two such mutants carried lesions in the homeodomain transcription factor gene *pal-1*. The authors went on to show that PAL-1 protein can directly bind to redundant conserved elements within the *ced-3* promoter, and that these elements were required specifically for tail-spike cell death but not for other cell deaths (Figure 1). Thus, PAL-1 either directly influences the onset of cell death, or acts permissively to set a point beyond which this event can occur.

The transcription of caspases has not been extensively studied in any organism, and the results of the tail-spike cell

studies suggest that caspase transcription may be important in regulating developmental cell death. The mammalian homolog of PAL-1, caudal-related homeobox 2 (Cdx2), is an intestinal tumor suppressor, and mutations in Cdx2 promote intestinal and colon tumor formation.[47–49] Strikingly, in the intestinal epithelium, Cdx2 protein expression is highest in the outer, most differentiated, cell population, which normally undergoes apoptotic cell death.[50] It is tempting to speculate therefore that as with PAL-1 in *C. elegans*, Cdx2 might function to regulate caspase transcription in the intestine.

A Novel Morphologically Conserved Program Mediates Linker Cell Death

Recent work examining the death of the *C. elegans* male-specific linker cell has provided strong evidence for a *bona fide* non-apoptotic developmental cell death program in this animal.[51] Strikingly, all four core apoptotic genes as well as all known engulfment mediators appear irrelevant for this cell's demise.[51]

The linker cell is born in the second larval stage in the central region of the animal, and leads the migration of the male gonad behind it[52,53] (Figure 2a). The path of migration terminates at the posterior end of the animal, placing the vas deferens in close apposition to the cloaca. Upon its death during the fourth larval stage (L4)-to-adult transition, the linker cell is engulfed and removed by either the U.lp or U.rp epithelial cells (henceforth abbreviated as U.l/rp cells).[53] It is thought that linker cell death occurs in order for the gonad to fuse to the cloaca, thereby creating an exit channel for sperm. Indeed, at least some mutants that block linker cell death accumulate sperm within the male gonad, presumably leading to sterility (ES Blum and S Shaham, unpublished data).

Unlike most cells that die during *C. elegans* development, the linker cell lives for over 30 h after it is born, and becomes highly differentiated. Anecdotal reports had suggested that linker cell death may depend on engulfment by the U.l/rp cells.[53] However, when Abraham *et al*.[51] ablated the U.l/rp grandparental precursor cell, U, the linker cell still died normally, indicating that engulfment by the U.l/rp cells is not required for linker cell death. These and other results suggest that local cues from the cloacal region may only be required to fine tune efficient destruction of the linker cell.

Temporal, rather than spatial cues, however, profoundly influence the onset of linker cell death. *C. elegans* possesses a developmental timing program that communicates the developmental stage of the organism to individual cells in the animal. Thus, specific cells in mutants of this timing program will execute early or delayed cell fates, depending on the nature of the timing mutant examined.[54,55] The zinc-finger transcription factor *lin-29* is the most downstream known gene in the developmental timing pathway, and acts at the transition from the L4 to the adult.[54,56,57] Strikingly, the linker cell inappropriately survives in 53% of *lin-29*(lf) males. Mutations in the *let-7* microRNA gene, which activates *lin-29* function through the *lin-41* gene, also inhibit linker cell death.[51,58,59] *lin-29* is expressed in the linker cell beginning at the L3/L4 stage, and mosaic studies demonstrated that this gene functions cell autonomously within the linker cell to promote

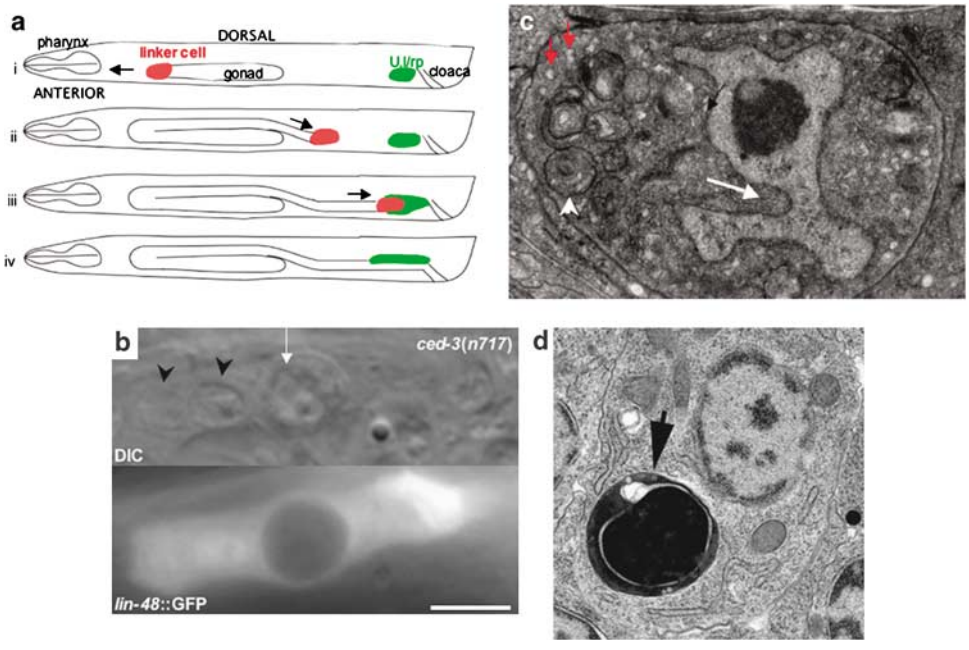

Figure 2 Linker cell death in *C. elegans*. (**a**) Linker cell migration in adult males. The linker cell is born in second larval stage (L2) animals and begins migrating anteriorly (i). By early L4, the linker cell is migrating posteriorly on the dorsal side (ii). By late L4, the linker cell has reached the cloaca, begins to die, and is engulfed by the U.l/rp cells (iii). In adult males, the linker cell has died and has been removed; the vas deferens is now connected to the cloaca (iv). (**b**) A dying and fully engulfed linker cell (arrow) in a *ced-3*(*n717*lf) male expressing a *lin-48* promoter::GFP reporter in the U.l/rp engulfing cell. Arrowheads, nuclei of engulfing cell. DIC (top) and fluorescence (bottom) images. Scale bar, 5 μm. (**c**) Electron micrograph of a dying linker cell. Note the crenellated (indented) nucleus (white arrow), large membrane-bound vesicles (white arrowhead), and white 'empty' structures (red arrows), which may represent swollen endoplasmic reticulum. (**d**) Electron micrograph of an engulfed apoptotic cell (arrow) in *C. elegans*. Note the compaction of darkened cytoplasm around the nucleus

its demise. These results, together with the cell ablation and migration studies suggest that the linker cell employs a cell-autonomous program for its death. Because *lin-29* is expressed in other cells not destined to die, as well as in the migrating linker cell, it is unlikely that *lin-29* is sufficient to promote linker cell death. Thus, *lin-29* might act together with other genes to transcriptionally control death-promoting genes.

Attempts to characterize possible targets for *lin-29* among known *C. elegans* cell death genes revealed that linker cell death is independent of all known cell death genes, including the core apoptotic genes *egl-1*, *ced-9*, *ced-4*, and *ced-3*.[51] These studies extend initial observations suggesting that linker cell death may only partially depend on *ced-3* and *ced-4*.[8] Further studies suggest that linker cell death does not require any caspase activity, because loss-of-function mutations in the other known *C. elegans* caspase-related genes, *csp-1*, *-2*, and *-3,* or mutations in both *ced-3* and *csp-1* did not affect linker cell death.[60]

Although caspase-independent death has been claimed to occur in a number of settings in vertebrate systems,[61–64] all such studies rely on examination of only selected caspase-deficient mutants, or on inhibitor studies. Because vertebrates contain roughly a dozen caspases, with differing substrate and inhibitor specificities, caspase dependence is very difficult to rule out, and in all these studies it is possible that caspases that have not been examined, or combinations of caspases, are relevant for the process at hand. In *C. elegans*, however, only four caspase-related genes exist,[18,60] and only two, *ced-3* and *csp-1*, have enzymatic function.[60] Thus, the studies of Abraham *et al*.[51] make a strong case for the argument that caspases are not essential for linker cell death.

To examine linker cell death, Abraham *et al*.[51] used a GFP reporter to follow linker cell fate, and it has been suggested that these reporters may in some way influence the death process. However, careful examination of animals carrying a GFP reporter transgene in the U.l/rp engulfing cell, but not in the linker cell, showed that in 33/36 *ced-3*(lf) mutant males and 39/39 *ced-4*(lf) mutant males, the linker cell died and was engulfed during the time window examined (0–2 h after the L4-to-adult transition; Figure 2b), alleviating any concerns regarding reporter interference (M Abraham and S Shaham, unpublished data).

Linker cell death is also independent of calpains and aspartyl proteases that have been shown to mediate necrotic cell death (see below),[65] and it is also unlikely that the linker cell undergoes autophagic cell death. The autophagosome-tethered reporter LGG-1::GFP is not expressed at higher levels in the dying linker cell, and mutations in the autophagy genes *bec-1* and *unc-51*, homologs of beclin and APG1, respectively, have no effect on linker cell death. Furthermore, the morphology of dying linker cells does not appear similar to autophagic cell death (Figure 2c).

Phagocytosis of the linker cell also appears to have unique genetic and cell-biological features, since engulfment of the cell by the U.l/rp cells is independent of all known engulfment genes in *C. elegans*.[51] Specifically, neither single nor double combinations of mutations in genes of the two known cell death engulfment pathways blocks linker cell engulfment. Consistent with these genetic results, the CED-1 protein, a transmembrane protein that accumulates on engulfing cell membranes surrounding dying cells,[66] was never observed around the linker cell. Although engulfment by the U.l/rp cells is not necessary for linker cell death, the role of engulfment in wild-type linker cell death has not been directly assessed, since no known mutations block its engulfment. While it appears that in wild-type animals, linker cell engulfment is mediated, at least in part, by an unknown mechanism, in *him-4*(lf) mutant animals, CED-1 clustering is seen around the dying linker cell. The observation that this clustering begins to occur after a linker cell corpse becomes visible suggests that the linker cell may initiate cell death independently of engulfment.

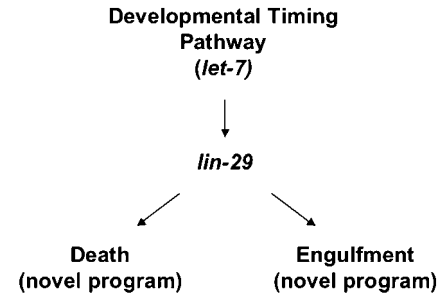

Figure 3 Model for linker cell death. Linker cell death is regulated cell autonomously by *lin-29*, a component of the *C. elegans* developmental timing pathway that controls the L4-to-adult transition. *lin-29* is required for both death and engulfment of the linker cell, mediated by novel programs

The unique genetic features of linker cell death suggest that the process is unlikely to proceed by apoptosis. Consistent with this hypothesis, electron microscopy of dying linker cells revealed non-apoptotic features, including nuclear crenellation (indentation), absence of chromatin condensation, organelle swelling, and accumulation of cytoplasmic membrane-bound structures[51] (Figures 2c and d). Remarkably similar features are seen during the normal developmental death of chick ciliary ganglion cells[67] and chick spinal cord motor neurons,[68] a type of cell death that has been coined 'type III' cell death.[69,70] These observations suggest that linker cell death is morphologically conserved. Identification of genes required for linker cell death and examination of their mammalian counterparts for roles in type III cell death may help to determine whether the cell death program required to kill the linker cell is evolutionarily conserved (Figure 3).

C. elegans Mutants Displaying Noncanonical Cell Death Programs

A number of *C. elegans* mutant strains carrying defined genetic lesions display inappropriate cell death patterns in which cells normally destined to live instead die. In several of these mutants, cell death is mediated either by a modified version of the canonical developmental cell death pathway, or by a completely different mechanism. Although these alternate cell death programs have no known parallels during normal *C. elegans* development, their existence suggests that they might reflect underlying endogenous programs. We briefly describe these programs below.

Knockdown by RNA interference of the inhibitor of cell death (*icd-1*) gene promotes the death of many cells in the *C. elegans* embryo in a *ced-4*-dependent, yet *ced-3*-independent manner, and overexpression of ICD-1 can block some normally occurring embryonic cell deaths.[71] ICD-1 localizes to mitochondria and is homologous to the β-subunit of the nascent-polypeptide-associated complex, which is thought to regulate protein localization during translation.[71] Electron microscopy of dying cells in an *icd-1*(RNAi); *ced-3*(lf) double mutant revealed features of apoptosis, including condensed chromatin, suggesting that such features can be generated in the absence of caspases, or that a different *C. elegans* caspase[60] substitutes for *ced-3* in these dying cells.

Loss-of-function mutations in the *pvl-5* gene cause incompletely penetrant embryonic lethality, and loss of sensory structures in the male tail.[72] Furthermore, in animals carrying the *pvl-5*(*ga87*) mutation, some P*n*.p cells, a group of precursor cells that generate hypodermal and vulval cells,[9] undergo inappropriate cell death.[72] Mutant animals also exhibit semi-penetrant developmental defects including embryonic lethality and gonadal migration defects. The P*n*.p cell deaths can be suppressed by *ced-3*(lf) and *ced-9*(gf) mutations but not by *ced-4*(lf) or *egl-1*(lf) mutations. Similarly, a small number of embryonic deaths in *pvl-5*(*ga87*) mutants are suppressed by *ced-3*(lf) but not *ced-4*(lf) mutations. Interestingly, mutations in both known cell-corpse engulfment pathways[73] also suppress P*n*.p cell death.[72] These results suggest that *pvl-5* may normally be required to prevent *ced-3*-dependent cell death in a select group of cells. The molecular identity of the *pvl-5* gene is not yet known.

The semi-dominant alleles of the *lin-24* and *lin-33* genes have also been shown to affect the survival of the P*n*.p cells.[74] These alleles promote *ced-3*- and *ced-4*-independent cell death, and dying cells display an abnormal morphology when viewed using Nomarski optics. Mutations in some *C. elegans* engulfment genes protect against *lin-24* or *lin-33*-induced death.[75]

Perhaps the best characterized mutation-induced non-apoptotic cell death type in *C. elegans* is a necrotic-like neuronal death induced by dominant (*d*) gain-of-function mutations that hyperactivate the ion-channel subunits *mec-4*[76,77] or *deg-1*[78] (degeneration inducing) of the degenerin and epithelial Na^+ channel (DEG/EnaC) family.

In this necrotic death type, the cell bodies of dying neurons swell and can be readily viewed in living animals (Figure 4a). At the ultrastructural level (Figure 4b), *mec-4(d)*-induced neurodegeneration involves initial formation of electron-dense multilamellar figures near the plasma membrane that appear to coalesce, later intracellular vacuolation, nuclear distortion and chromatin clumping, and final degradation of intracellular contents.[79] Necrotic neurons may lyse, but more often appear to be removed by phagocytosis before membrane disruption.[79,80] Interestingly, the *C. elegans* genes involved in phagocytotic removal of apoptotic corpses are also needed for efficient elimination of necrotic corpses, suggesting common recognition and removal mechanisms of apoptotic and necrotic cells.[80]

Toxic *mec-4(d)* mutations encode amino-acid substitutions that increase channel conductance.[77,81–83] Genetic perturbations of other *C. elegans* channels, including those

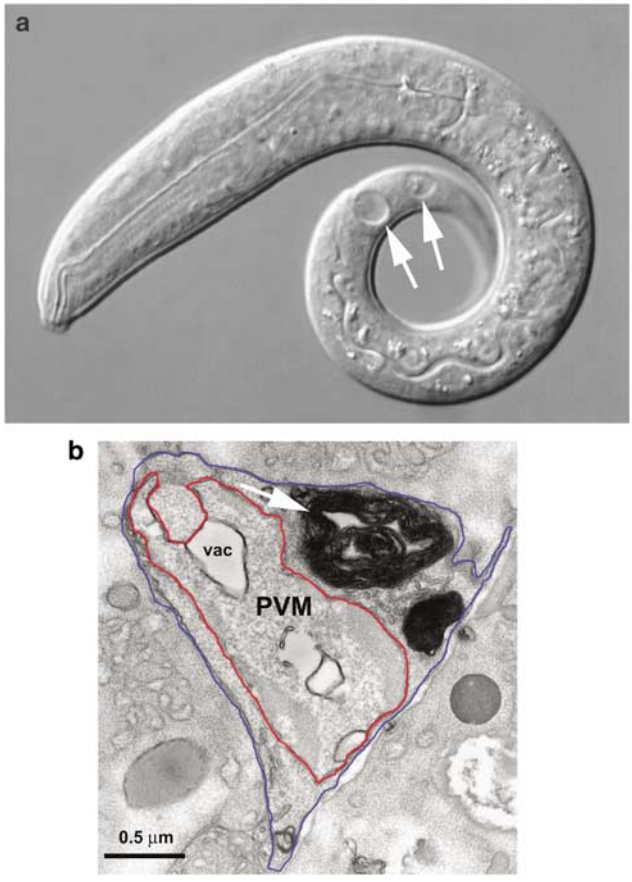

Figure 4 Morphological features of necrotic-like cell death in *C. elegans*. (**a**) Necrotic touch receptor neurons become dramatically swollen as neurons die. Indicated by white arrows are the two posterior PLM neurons that have become necrotic in a first larval (L1) stage animal. Note that necrosis is readily apparent in transparent living animals using low power magnification. Photo courtesy of Yury Nunez. (**b**) Electron micrograph showing ultrastructural features of a necrotic PVM neuron in a *mec-4(d)* mutant. Blue trace parallels the plasma membrane of the PLM. White arrow indicates electron-dense whorls that are prominent in dying neurons. The nucleus is outlined in red and features vacuoles and clumped chromatin. Reprinted with permission from Hall *et al*.[79]

that reduce desensitization of the DEG-3 acetylcholine receptor[84,85] and expression of constitutively active Gαs[86,87] (thought to induce glutamate excitotoxicity), can also initiate necrosis. Mutant ion channels that are all neurotoxic can conduct calcium,[83] and thus elevated calcium influx through plasma membrane ion channels appears to be a common necrosis-triggering event. In mammals, analogous glutamate excitotoxicity[88] and DEG/ENaC channel hyperactivation[89] are important in neuronal death consequent to stroke and ischemia.

Genetic studies suggest a general model for ion-channel-initiated necrosis in which catastrophic ER calcium release figures prominently. Null alleles of the ER Ca^{2+}-binding chaperone calreticulin, which are important in maintenance of intracellular Ca^{2+} stores,[90] strongly suppress both *mec-4(d)*-induced necrosis[91] and glutamate-dependent excitotoxicity (Driscoll and Mano, unpublished). Mutations in ER Ca^{2+}-release channels IP3-receptor ITR-1 and ryanodine receptor UNC-68 also significantly suppress death, as can

dantrolene treatment, which blocks ER Ca^{2+} release. These studies suggest that the intracellular Ca^{2+} concentration must rise to critical levels for progression through necrosis and that the ER is an important source of the extreme Ca^{2+} elevations required for necrotic neuronal death.[92] How excess ion influx through plasma membrane ion channels provokes the deleterious release of ER calcium stores remains to be elaborated.

C. elegans calcium-activated proteases (calpains TRA-3 and CLP-1) are required for efficient progression through necrosis[65] and may be activated by ER calcium release. Roles for the lysosome in necrosis are underscored by the requirement for vacuolar-ATPase-mediated lysosomal acidification[93] and by the existence of the intracellular anti-necrosis serine protease inhibitor serpin SRP-6, which defends against lysosomal protease release during general tissue necrosis.[94] Finally, disruption of autophagy genes that impact lysosomal function can also influence the efficacy of channel-induced necrosis.[95,96]

Concluding Remarks

Studies of normally occurring developmental cell death in *C. elegans* have been instrumental in uncovering a molecular pathway driving this process. The studies reviewed here suggest that not all components of this pathway are required to execute cell death in all cells, and that regulation of developmental cell death can occur not only by regulating the upstream BH3-only-encoding gene *egl-1*, but also by impinging on other, and perhaps all, components of the core apoptotic machinery. Studies of the *C. elegans* linker cell demonstrate *in vivo* roles for a non-apoptotic cell death program that can function independently of all known cell death genes. Given the conservation of the apoptotic cell death machinery across metazoans, studies of noncanonical regulation of this machinery, as well as the novel cell death pathways leading to linker cell death and necrotic death, may provide insight into the regulation of cell death in some, and perhaps all other animals.

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