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Apoptotic and Nonapoptotic Cell Death in *Caenorhabditis elegans* Development

Lauren Bayer Horowitz and Shai Shaham

Laboratory of Developmental Genetics, The Rockefeller University, New York, NY, USA; email: lbhorowitz@rockefeller.edu, shaham@rockefeller.edu

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Abstract

Programmed cell death (PCD) is an essential component of animal development, and aberrant cell death underlies many disorders. Understanding mechanisms that govern PCD during development can provide insight into cell death programs that are disrupted in disease. Key steps mediating apoptosis, a highly conserved cell death program employing caspase proteases, were first uncovered in the nematode *Caenorhabditis elegans*, a powerful model system for PCD research. Recent studies in *C. elegans* also unearthed conserved nonapoptotic caspase-independent cell death programs that function during development. Here, we discuss recent advances in understanding cell death during *C. elegans* development. We review insights expanding the molecular palette behind the execution of apoptotic and nonapoptotic cell death, as well as new discoveries revealing the mechanistic underpinnings of dying cell engulfment and clearance. A number of open questions are also discussed that will continue to propel the field over the coming years.

INTRODUCTION

Programmed cell death (PCD): cell death that is genetically programmed into the organism and functions in development and homeostasis

Apoptosis: a cell death form prevalent in animal development exhibiting cytoplasm shrinking, nuclear condensation, and plasma membrane blebbing

Phagocyte: a specialized cell that engulfs and degrades cellular debris

Caspase: a class of cysteine protease enzymes that play crucial roles in apoptosis

Cell death is a fundamental biological process required for animal development and homeostasis and also a hallmark of many pathologies. Indeed, aberrant cell death is associated with many disorders, including neurodegeneration, organ infarctions, and cancer (29). Although both developmental and pathological cell death are often described as programmed cell death (PCD), the term was originally coined in reference to the reproducibility of cell death during animal development, suggesting an underlying genetic program (74). Here, we use the term PCD in this original sense (74). PCD accompanies many developmental events and is involved in homeostatic control during morphogenesis, tissue remodeling, cell number regulation, and elimination of damaged cells, such as autoreactive immune cells (29, 33) (see the sidebar titled Roles of Programmed Cell Death in Development).

The nematode *Caenorhabditis elegans* has been indispensable in characterizing conserved PCD mechanisms. PCD is not required for *C. elegans* viability (25), and dying cells in living animals are easily observable using differential interference contrast (DIC) microscopy (98) (**Figure 1*a***). *C. elegans* also has a well-defined and invariant somatic cell lineage; in the hermaphrodite, precisely 131 of the 1,090 cells generated die at reproducible time points during development (109, 110). Furthermore, powerful genetics and genomics make *C. elegans* a prime setting for PCD gene discovery.

The term apoptosis, referring to a common cell death form characterized by cytoplasm shrinking, nuclear condensation, and plasma membrane blebbing followed by rapid engulfment by neighboring cells or dedicated phagocytes, was coined in 1972 (60) (**Figure 1*b***). The core molecular pathway mediating apoptosis was largely deciphered in *C. elegans* and demonstrated to employ conserved caspase cysteine proteases (47, 114). While apoptosis is considered an important cell death mechanism in vertebrate development, loss of key apoptotic regulators causes only minor developmental defects (30). Furthermore, while changes in apoptosis have been observed in cancers and some neurodegenerative diseases, therapeutics that target apoptotic components in these diseases, with the notable exception of Bcl-2 inhibitors used to treat hematological cancers (131), have been largely ineffective (21, 71). Recently described roles for nonapoptotic cell death programs in development and disease, however, may provide more promising results. For example, studies of linker cell–type death (LCD), a PCD process first described in *C. elegans*, uncovered a cell

ROLES OF PROGRAMMED CELL DEATH IN DEVELOPMENT

Programmed cell death (PCD) plays many important roles in development. Morphogenesis and tissue sculpting require cell death during removal of the interdigital webbing (73) or hollowing of the neural tube (35). Transient structures are also removed through PCD. For example, the mammalian Mullerian duct, which gives rise to female reproductive organs, and the Wolffian duct, which develops into the male sex organ, are degraded sex-specifically during embryogenesis (52). PCD also regulates cell number and removes defective cells in developing tissues. In the developing mammalian brain, more than 50% of neurons that are generated undergo cell death (88), and, in the immune system, over 95% of newly generated autoreactive T and B cells are culled (87). Multiple *Caenorhabditis elegans* studies provide evidence that PCD is also important for the formation of neural circuits that drive behavior (2, 25, 26, 125). In caspase mutants, undead neuronal sister cells, such as the M4 sister cell (2), differentiate into functional neurons, and a recent study showed that undead RIM sister cells even form synapses and alter behavior (66). Too much or too little PCD is associated with multiple disorders, such as neurodegeneration and cancer, respectively (29). Therefore, regulation of PCD is critical for proper development and homeostasis.

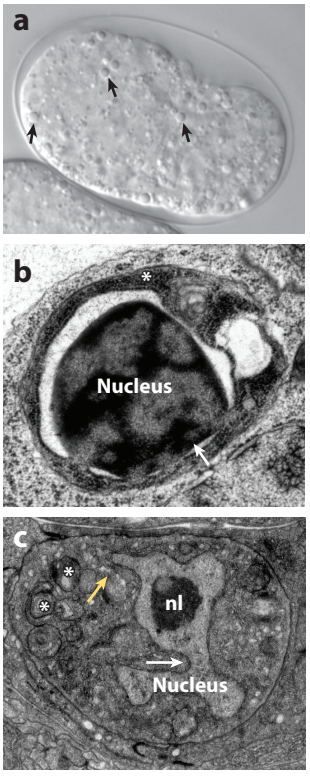

Figure 1

Morphology of dying cells. (*a*) DIC image of a *Caenorhabditis elegans* bean-stage embryo in which several cells undergo PCD. Dying cells have a refractile and raised button-like appearance (*arrows*). (*b*) Electron micrograph of a *C. elegans* cell undergoing apoptosis. Apoptotic cells display condensed chromatin (*arrow*) and a compact cytoplasm (*asterisk*). Panel adapted with permission from Reference 69. (*c*) Electron micrograph of a dying *C. elegans* linker cell, which displays nuclear crenellations (*white arrow*) and swollen organelles (the endoplasmic reticulum is indicated by the yellow arrow and the mitochondria by asterisks). Panel adapted from Reference 4 with permission from AAAS. Abbreviations: DIC, differential interference contrast; nl, nucleolus; PCD, programmed cell death.

death program with morphological and genetic distinctions from apoptosis (1). Dying linker cells display nuclear crenellations and organelle swelling and lack condensed chromatin (**Figure 1*c***). Intriguingly, a similar ultrastructure accompanies cell death in many tissues during vertebrate development and in disease, suggesting that LCD is not only conserved but also inappropriately co-opted in disease (69).

Here, we review studies over the last decade exploring *C. elegans* apoptotic and nonapoptotic PCD programs. Apoptosis, LCD, and additional noncanonical *C. elegans* PCD programs, such as cell extrusion and LC3-assocated phagocytosis (LAP), are discussed. We also review our understanding of how dying cells are cleared through phagocytosis. A summary of all *C. elegans* cell death mechanisms (developmental and otherwise) is presented in **Table 1**. Cell death that occurs in response to injury or pathology and cell death in the *C. elegans* hermaphrodite gonad, which occurs stochastically (37), are not discussed but have been reviewed elsewhere (3, 65, 138). Finally, we highlight outstanding questions and areas of inquiry that may impact our understanding of PCD in mammalian development and disease.

Linker cell–type death (LCD): a nonapoptotic and caspase-independent cell death program exhibiting nuclear crenellations, organelle swelling, and uncondensed chromatin

Cell extrusion: a process by which cells are actively expelled from a tissue or epithelial layer, typically as a response to cell death or damage

Table 1 Developmental and pathological cell death events in *Caenorhabditis elegans*

Mechanism	Cell types	Function	Morphological hallmarks	EGL-1/BH3-only dependent	CED-3/caspase dependent	References
Apoptosis	Embryonic and postembryonic somatic cells	Development	Refractile, compact cytoplasm, condensed chromatin, membrane blebbing	Yes	Yes	60, 109, 110
CCE	TSCs, CEM neurons	Pruning of morphologically complex cells	Soma is apoptotic; processes degraded and cleared separately	No	Yes	12, 32, 83
Cell extrusion	Early embryonic somatic cells	Elimination of apoptotic cells in *ced-3* mutants	Corpses shed into extraembryonic space	No	No	19, 24
Linker cell–type death	Male-specific linker cells	Formation of sperm exit channel	Nuclear crenellations, organelle swelling, uncondensed chromatin	No	No	1, 4
LAP	Second polar bodies	Degradation of polar body	Membrane integrity loss, exposure of phosphatidylserine	Yes	No	28, 129
Germ cell death	Germ cells	Homeostasis, response to genotoxic stimuli	Apoptotic morphology	No	Yes	37, reviewed in 3
Autophagy	Apoptotic cells, germ cells, ventral cord neurons	Aiding removal of somatic and germ apoptotic cells, neurodegeneration	Double-membrane vesicles, autophagosomes	Yes	No	50, 94, 117, reviewed in 138
Ferroptosis	Intestinal cells, germ cells	Resulting from the accumulation of iron-dependent lipid peroxides	Shrunken mitochondria with increased membrane density	No	No	22, 53, 95
Necrosis-like	Touch neurons, uterine uv1 cells	Caused by increased activity of cation channels, G-α protein signaling, or nicotinamide	Large vacuoles, electron-dense membranous whorls, nuclear membrane loss	No	No	40, 97, reviewed in 65

Abbreviations: CCE, compartmentalized cell elimination; LAP, LC3-associated phagocytosis; TSC, tail-spike cell.

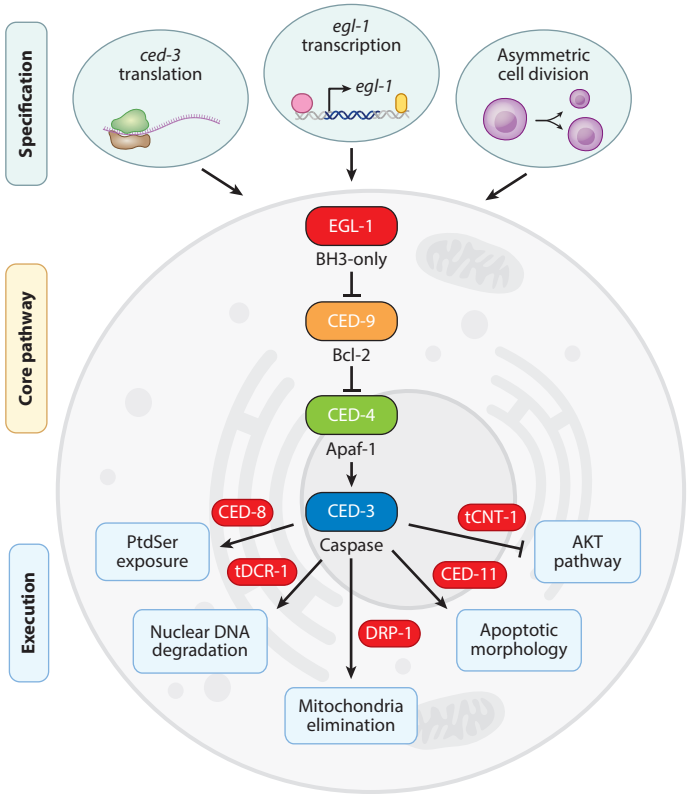

Figure 2

Apoptotic cell death in *Caenorhabditis elegans* development. Apoptosis is activated by EGL-1/BH3-only, which inhibits CED-9/Bcl-2 to cause the release of CED-4/Apaf-1 and subsequent activation of CED-3/caspase. Several factors specify certain cells to undergo apoptosis, including the regulation of *egl-1* expression by lineage-specific transcription factors, asymmetric cell division, and factors that regulate other components of the apoptotic pathway, such as *ced-3* mRNA translation. To execute apoptotic cell death, activated CED-3 cleaves many target proteins, including CED-8, which causes externalization of PtdSer; tDCR-1, which initiates nuclear DNA degradation; DRP-1, which causes mitochondrial elimination; and CED-11, which affects the apoptotic cellular morphology. CED-3 also cleaves CNT-1, generating tCNT-1, which represses the prosurvival AKT signaling pathway. Abbreviations: PtdSer, phosphatidylserine; tCNT-1, truncated CNT-1; tDCR-1, truncated DCR-1. Figure adapted from images created with BioRender.com.

APOPTOSIS

The Core Apoptotic Machinery

Apoptotic cell death is defined by its unique ultrastructure, characterized by cytoplasm compaction, chromatin condensation, and plasma membrane blebbing (60). Pioneering genetic studies in *C. elegans* demonstrated that apoptosis is controlled by a conserved cell-autonomous core pathway (14, 47, 103) (**Figure 2**). The CED-3 caspase proenzyme localizes near nuclei in many cells and is activated in cells destined to die by CED-4/Apaf-1, driving cell death (133, 141). Many cells also express CED-9/Bcl-2, which inhibits cell death by binding to CED-4, sequestering it to mitochondria (9, 43). CED-9 inhibition can be overridden by various signals that induce the transcription of *egl-1*/BH3-only. EGL-1 binds CED-9, promoting CED-4 release from mitochondria and consequent cell death (9, 14, 135).

Phagocytosis: a cellular process for internalizing and degrading cellular debris, corpses, or pathogens

CED-9–EGL-1 and CED-9–CED-4 binding have been extensively characterized (134–136). However, a mechanism for CED-3 activation by CED-4 was only recently proposed. Upon nuclear translocation, CED-4 forms an octameric apoptosome that binds inactive CED-3 on the nuclear membrane (96). X-ray crystallography and cryo-electron microscopy structures reveal that the apoptosome adopts a funnel-shaped structure that allows binding of two CED-3 zymogens inside the apoptosome hutch (51, 72). This likely allows cross-cleavage and activation of CED-3 moieties. Active CED-3 bound to the apoptosome has higher protease activity compared to free active CED-3, suggesting that the CED-3–apoptosome complex may function as a holoenzyme (72).

How CED-4 translocates from mitochondria to the nucleus is poorly understood. However, recent studies on germ cell death suggest that dynein, a motor protein that enables cargo transport on microtubules, promotes CED-4 nuclear accumulation (41, 143). CED-3 also appears to be anchored to nuclear membranes through the nuclear pore protein NPP-14, which also blocks its activation in the germline (10). Whether dynein and NPP-14 function in somatic apoptosis remains to be tested.

Unlike *Drosophila* and mice, in which apoptosis progresses through proteolytic cascades of initiator and executioner caspases, CED-3 is the only *C. elegans* caspase necessary for apoptosis (29). Nonetheless, a recent study demonstrated that a related cysteine protease, the separase SEP-1, can functionally substitute for CED-3 (54). Partial loss of *sep-1* enhances the cell death defects of weak *ced-3* mutants and can suppress the lethality of *ced-9* mutants. Because separases also function in cell division (106), the complete loss of SEP-1 function cannot be readily assessed. Thus, it is possible that SEP-1 contributes even more significantly to apoptosis execution. Whether this function is conserved is still unclear.

Specification of Apoptosis

The discovery of the core apoptotic pathway highlighted how PCD is governed by a latent intrinsic genetic program. Therefore, cell death can be considered a type of cell fate acquired during development. Below, we summarize the intrinsic and extrinsic mechanisms that specify which cells undergo apoptosis (**Figure 2**). Most mechanisms regulate EGL-1, the activator of the core apoptotic pathway. However, recent studies demonstrate that regulators of other components of the apoptotic pathway, as well as asymmetric cell division and cell size, are also important in the specification of apoptosis.

EGL-1-dependent mechanisms. Apoptosis in *C. elegans* is often controlled by lineage-specific transcription factors that regulate EGL-1/BH3-only expression (reviewed in 16, 76). For example, in hermaphrodites, the TRA-1/Gli sex determination transcription factor represses *egl-1* expression by binding to *egl-1* regulatory elements to promote the survival of hermaphrodite-specific neurons (HSNs) (15). In males, however, TRA-1 promotes *egl-1* expression and HSN cell death by inhibiting the expression of the transcription factor CEH-30, another EGL-1 repressor (83, 93, 100). Many other transcriptional regulators of *egl-1* expression have been described in other cell lineages that function similarly (reviewed in 16, 76).

TRA-1 and CEH-30 are not dedicated cell death genes, as they also affect cell differentiation and fate. Likewise, while the Collier/Olf/EBF (COE) transcription factor UNC-3 promotes the cell death of the RID neuron's sister cell, it does not promote the death of other neuronal sister cells where it is also expressed (118). Even in RID, UNC-3 is also required for neuronal differentiation (118). Therefore, it is likely that a combination of controls specifies *egl-1* expression and cell death versus cell survival. Indeed, recent work reveals that microRNAs (miRNAs) also regulate apoptosis (105). The miRNAs miR-35 and miR-58 bind to the 3′ untranslated region of *egl-1* to cooperatively repress expression, preventing the precocious death of precursor cells whose

progeny are destined to die (105). Despite their broad expression, the cell death effects of these miRNAs are lineage specific, supporting the notion of combinatorial control of EGL-1 expression.

Extrinsic signals can also influence EGL-1 activity. The epidermal growth factor (EGF)-like ligand LIN-3 functions cell nonautonomously to transcriptionally activate *egl-1* in some cells slated to die (56). Secreted LIN-3 signaling transduced by the EGF receptor LET-23 activates the ETS domain–containing transcription factor LIN-1 through a mitogen-activated protein kinase (MAPK) signaling pathway, which then promotes *egl-1* expression (56). Interestingly, LIN-3/EGF can function at a distance, as LIN-3 production in intestinal cells can promote cell death throughout the embryo (56). Whether EGF signaling directly activates *egl-1* expression or promotes a permissive cellular state for *egl-1* induction is not clear. Additionally, how EGF and other nonautonomous signals cooperate with intrinsic factors to promote *egl-1* expression and cell death is not known.

Asymmetric cell division: process in which a parent cell divides into two daughter cells with distinct sizes and properties, leading to the adoption of different cell fates

Non-EGL-1-dependent mechanisms. *C. elegans* apoptosis can also be specified through the control of apoptotic pathway components other than EGL-1 (**Figure 2**). The conserved PUF-8 RNA-binding protein promotes and inhibits apoptosis independently of *egl-1* (132). In *puf-8* mutants, some cells that normally survive die inappropriately, while in other lineages, cells that usually die instead survive. PUF-8, as well as three other RNA-binding proteins, repress *ced-3* caspase expression by binding to 3′ noncoding sites within the *ced-3* mRNA, preventing apoptosis (107, 132). *ced-9*/Bcl-2 mRNA also contains PUF protein–binding sites in its 3′ untranslated regions (132). Thus, PUF-8-dependent inhibition of *ced-3* or *ced-9* expression may block or promote apoptosis, respectively.

The eukaryotic initiation factor 3 subunit K (EIF-3.K) also promotes cell death through *ced-3* (49), and the translational regulators GCN-1 and ABCF-3 may do the same (45). These two proteins function downstream of, or in parallel to, *ced-9*; physically interact; and maintain phosphorylation of eukaryotic initiation factor 2α (eIF2α) to promote translation initiation (45).

Asymmetric cell division. In vivo apoptotic cells are often smaller than their neighbors (60), and smaller cells are more likely to undergo apoptosis than larger ones (78). In *C. elegans*, many dying cells are the smaller daughter cell of a blast cell that divides asymmetrically (109, 110), and mechanisms that function in asymmetric cell division are required for apoptosis (reviewed in 16). For example, PIG-1/MELK, a kinase required for asymmetric cell division, is important for apoptotic cell specification (17). In *pig-1* mutants, neuroblasts that normally produce a small cell slated to die and a surviving neuronal sister cell instead generate daughter cells of equal size that both survive (17). In the NSM neuroblast (NSMnb) lineage, PIG-1, working through nonmuscle myosin II (NMY-2), promotes unequal partitioning to daughter cells of the Snail-like transcription factor CES-1, which blocks *egl-1* expression (113, 124). NMY-2 is enriched on NSMnb cortical regions that give rise to NSM (124). Thus, while the larger NSM neuron receives ample CES-1 protein and survives, the smaller NSM sister cell lacks CES-1 and dies (124).

Small cell size itself may also promote apoptosis. In the NSM and Q neuroblast lineages, loss-of-function mutations in *egl-1*, *ced-4*, or *ced-3* or a *ced-9* gain-of-function mutation inappropriately produce daughter cells of similar sizes (7, 79), suggesting roles for apoptotic genes in asymmetric cell division. CED-3 protein forms a gradient across the dividing neuroblast, which results in enhanced segregation of CED-3 into the smaller apoptotic daughter cell (79). In the neuroblast, the Rho guanine nucleotide exchange factor (RhoGEF) ECT-2 promotes asymmetric cell division and apoptosis of the smaller daughter cell (101). ECT-2 is also asymmetrically localized, which unexpectedly is dependent on CED-3 (101). ECT-2 was previously shown to promote the polarization of the cortical actomyosin network during cell division (80, 99). Remarkably, ECT-2 hyperactivation results in even smaller daughter cells, and this manipulation alone is sufficient to

restore cell death to weak *ced-3* mutants (101), suggesting that cell size can indeed determine life and death.

Other proteins that regulate cell size are also linked to apoptosis control. The transcriptional regulators SPTF-3/Sp1, CES-2/HLF, and DNJ-11/MIDA1 control asymmetric neuroblast divisions that result in apoptosis (42, 44). Mutations in the Arf-GEF GRP-1 and the Arf GTPase-activating protein (GAP) CNT-2, as well as in the DEP domain–containing protein TOE-2, that control membrane dynamics during cell division, also promote cell survival in neuroblast lineages (38, 112). Interestingly, changes in tissue hydraulics that cause some germ cells to decrease in size also promote their apoptotic death (8). Precisely how small cell size promotes cell death, however, is unknown.

Execution of Apoptosis

Following apoptosis initiation, CED-3 caspase is believed to cleave and activate or inactivate proapoptotic or prosurvival targets, respectively (**Figure 2**). One CED-3 target is the DRP-1 mitochondrial fission protein, which promotes the elimination of mitochondria from apoptotic cells following cleavage (5). CED-3 also cleaves the multipass transmembrane protein and human XK-related 6 (XKR6) protein homolog CED-8 (11, 111), whose C-terminal cleavage product promotes the externalization of phosphatidylserine on apoptotic cells (11, 111). Identities of other CED-3 substrates, however, remain long-standing mysteries. Here, we summarize some previous findings (reviewed in 16) and discuss recent studies providing insight into apoptosis execution.

Nuclear DNA degradation. During apoptosis, chromosomal DNA is fragmented at internucleosomal regions by multiple deoxyribonucleases (DNases) in a stepwise fashion (92). In mammals, caspases first activate caspase-associated deoxyribonuclease (CAD), which mediates the cleavage of chromosomal DNA to generate 3′ hydroxyl DNA breaks detected by the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) assay (126). Chromosomal DNA is then further fragmented by endonucleases and DNase II enzymes (126). Although *C. elegans* has endonucleases and DNase II enzymes that function in apoptotic DNA degradation, no CAD homolog exists (92). Nonetheless, one report suggests that the Dicer ribonuclease DCR-1 may have CAD-like activity (81). DCR-1 is well known as a ribonuclease (RNase) that processes double-stranded RNA to generate small RNAs. During apoptosis, however, CED-3 cleaves DCR-1 in the middle of its N-terminal RNase domains, generating a C-terminal cleavage product, tDCR-1, with DNase activity similar to CAD (31, 81). Whether this function of Dicer is conserved in evolution is unknown.

It has been suggested that downstream of Dicer the CPS-6 mitochondrial endonuclease (90) interacts with the worm apoptosis-inducing factor homolog 1 (WAH-1) (123) and other cell death–related nucleases (CRNs), such as CRN-1 (91), to catalyze the degradation of the DNA nicks generated by tDCR-1 into single-stranded DNA gaps and double-stranded DNA breaks (92). Finally, the NUC-1 DNase II homolog, and to a lesser extent CRN-6, mediates further DNA degradation (70, 129). In the absence of cell corpse engulfment, NUC-1-dependent DNA degradation still proceeds (139). This surprising result reveals that NUC-1 functions cell-autonomously and that DNA degradation is not a consequence of engulfment but an important part of the apoptotic cell suicide program (139).

Apoptotic morphology. As apoptotic cells lose adhesion to neighboring cells, they acquire a characteristic rounded morphology that is refractile under DIC optics (**Figure 1*a***). Although these morphological changes are caspase dependent (25, 109), their mechanistic basis and functional significance were only recently probed. The CED-11 protein, a presumptive transient receptor potential (TRP) cation channel, was shown to promote shrinkage in volume as well as refractility of

apoptotic cells (23). CED-11 also mediates an increase in electron density as well as wrinkling and separation of the nuclear membrane (23). *ced-11* loss delays degradation of apoptotic phagosomes and enhances weak cell survival defects of engulfment mutants, suggesting that the morphological changes caused by loss of *ced-11* contribute to apoptotic cell death and to the efficient degradation of these cells (23). *ced-11* is expressed in dying cells, but *ced-11* loss does not enhance the cell death defects of weak *ced-3* mutants (23). Since cells still die in *ced-11* mutants, CED-11 likely functions downstream of CED-3 caspase. Because CED-11 encodes a presumptive cation channel, it may promote the shrinkage of apoptotic cells by modulating ion flow across the cell membrane (23). Other morphological changes dependent on CED-11 may therefore be consequences of the osmotic imbalance that ensues.

Cell survival pathways. The phosphoinositide 3-kinase (PI3K)-AKT signaling pathway promotes cell growth and survival in many animals. During apoptosis, PI3K-AKT signaling may be inactivated in *C. elegans* by the CED-3 caspase substrate CNT-1, mutations in which weakly inhibit or delay apoptosis (82). CED-3 caspase cleaves CNT-1 to generate truncated CNT-1 (tCNT-1), an N-terminal cleavage product (82). Unlike CNT-1, tCNT-1 binds strongly to phosphoinositide (PI) phosphatidylinositol 3-phosphate (PIP3) on the plasma membrane (82). It has been hypothesized that competition for PIP3 binding between tCNT-1 and AKT kinases inhibits PI3K-AKT signaling to promote cell death (82). However, this seems unlikely, as PIP3 moieties presumably far exceed tCNT-1 protein levels. Therefore, how PI3K-AKT signaling and other cell survival pathways are inactivated during apoptosis remains poorly understood.

Phagocytosis of Apoptotic Cells

Clearance of cell corpses by phagocytosis is an important step of PCD. Because there are no specialized phagocytes in *C. elegans*, dying cells are engulfed by neighboring cells (110). Mechanisms of phagocytosis were recently reviewed (34, 122). Briefly, phagocytosis involves three key steps: formation of the phagocytic cup surrounding the dying cell, phagosome maturation, and phagolysosome resolution (34, 122) (**Figure 3*a***). The phospholipid phosphatidylserine, normally found on the inner leaflet of the plasma membrane, translocates to the outer leaflet of dying cells and serves as an eat-me signal that triggers engulfment (116, 120). Receptors on phagocytes recognize externalized phosphatidylserine, promoting phagocytic cup formation. Once engulfment is complete, phagocytic membranes fuse, forming a sealed phagosome. The phagosome then associates and disassociates with various Rab GTPases that change the lipid composition of the phagosome membrane, acidify the phagosomal lumen, and promote lysosomal fusion (34, 63). Apoptotic cargo inside the phagolysosome is degraded using lysosomal acid hydrolases (34).

Phagocytic cup formation and subsequent engulfment are governed by CED-1-dependent and CED-5-dependent signaling pathways (**Figure 3*b***). In the CED-1-dependent pathway, dying cells are recognized by the CED-1/MEGF10 receptor on the engulfing cell, which functions with the secreted bridging molecule TTR-52 and the ABC transporter CED-7 to recognize phosphatidylserine (119, 128, 145). CED-1 then binds CED-6/GULP to regulate proteins that promote engulfment and phagosome maturation, including the dynamin GTPase DYN-1, the clathrin AP2, and EPN-1/Epsin (104, 140). A recent study revealed that CED-1/MEGF10 levels are actively maintained during engulfment to ensure proper apoptotic cell clearance (142). The TRIM-21 E3 ubiquitin ligase functions with the E2 ubiquitin enzyme UBC-21 to promote polyubiquitination and degradation of CED-1 during phagocytosis (142). In *trim-21* mutants, excessive CED-1 levels accumulate, aberrantly altering phagosome acidification by the vacuolar-type H^+-ATPase subunit VHA-10, leading to dying cell degradation defects (142).

In the CED-5-dependent engulfment pathway, the PSR-1 phosphatidylserine receptor, the MOM-5 Frizzled Wnt receptor, and the INA-1/PAT-3 integrin heterodimer recognize the dying

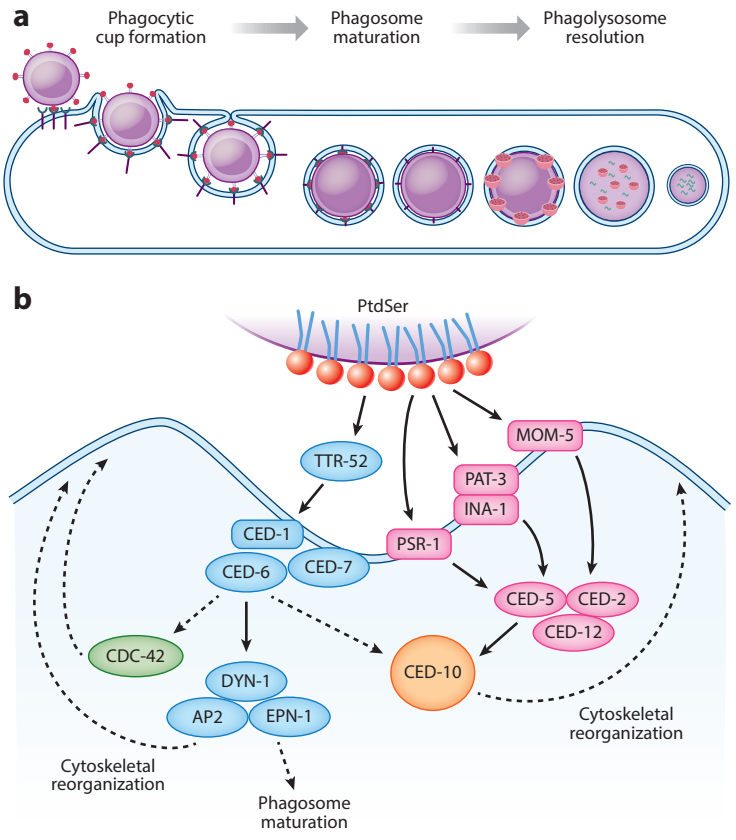

Figure 3

Phagocytosis following apoptotic cell death. (*a*) Apoptotic cells expose PtdSer eat-me signals that are recognized by phagocytic receptors and cause phagocytic cup formation around the dying cell. The ends of the phagocytic cup are sealed after engulfment, forming the phagosome, which then undergoes a maturation process that results in the recruitment and fusion of lysosomes to form the phagolysosome. During phagolysosome resolution, corpses are degraded using lysosomal hydrolases. (*b*) Formation of the phagocytic cup is controlled by the CED-1-dependent (*blue*) and CED-5-dependent (*pink*) signaling pathways. In the CED-1-dependent pathway, the CED-1 receptor functions with TTR-52 and CED-7 to recognize PtdSer on dying cells. CED-1, CED-7, and TTR-52 signal through CED-6 to regulate proteins that promote engulfment and phagosome maturation, including DYN-1, AP2, and EPN-1. In the CED-5-dependent pathway, the receptors PSR-1, MOM-5, and INA-1/PAT-3 recognize the dying cell and activate CED-5, CED-12, and CED-2. Both pathways activate CED-10, which drives the reorganization of the cytoskeleton and formation of the phagocytic cup. CDC-42 also promotes cytoskeletal rearrangements in the phagocyte, downstream of and/or in parallel to the CED-1-dependent pathway. Abbreviation: PtdSer, phosphatidylserine. Figure adapted from Reference 34 with permission from Elsevier and modified from images created with BioRender.com.

cell (6, 48, 121, 137). This is followed by CED-5/DOCK180 GEF activation, which binds CED-12/ELMO and CED-2/CRK (36, 130, 144).

Both engulfment pathways ultimately activate CED-10/Rac1 GTPase (62, 127). Activated CED-10 drives reorganization of the cytoskeleton and formation of the phagocytic cup by recruiting the actin-related protein 2/3 complex (Arp2/3) and WASP, an Arp2/4 nucleation factor, to apoptotic corpses (127). The small GTPase CDC-42 also promotes cytoskeletal rearrangements downstream of and/or parallel to the CED-1-dependent pathway (84).

NONCANONICAL FORMS OF APOPTOSIS

Most *C. elegans* cells that undergo apoptosis during development are undifferentiated and die within 30 minutes of being generated (109, 110). A few specialized cells, however, take much longer to die, and while they all require caspase genes, their cell death mechanisms exhibit significant deviations from the canonical apoptotic pathway. In this section, we describe these noncanonical forms of apoptosis that take place during normal *C. elegans* development.

Compartmentalized Cell Elimination

Compartmentalized cell elimination (CCE) is a recently described program that governs the death and clearance of morphologically complex cells, such as neurons, which have one or more cellular extensions (33). Two cell types are known to undergo this event in *C. elegans*: the tail-spike cell (TSC), a binucleate cell with a posterior process required for hermaphrodite tail morphogenesis, and the sexually dimorphic CEM neurons, which direct mating behavior in males but die in hermaphrodites (32, 77). The TSC and CEM neuron somata are cleared by neighboring cells and resemble other dying cells when viewed by DIC microscopy (32). However, the process and dendrites of the TSC and CEM neurons, respectively, are degraded differently. The proximal process/dendrite develops beaded constrictions, as viewed by fluorescence and electron microscopy, and then fragments (32). The distal segment undergoes bidirectional retraction into a distal node (32). While the TSC soma is engulfed through CED-1- and CED-5-dependent signaling, the distal TSC process is not and instead requires the fusogen EFF-1 to seal its phagosome (32). The molecular basis of CCE in CEM neurons is currently unknown.

Compartmentalized cell elimination of the tail-spike cell appears to proceed independently of EGL-1 (77). Control of this death is mediated in part by transcriptional control of *ced-3* caspase, whose expression is induced shortly before the cell dies (77). The PAL-1/Cdx2 transcription factor, required for TSC death, binds multiple regulatory sequences upstream of the *ced-3* gene to activate its expression in the TSC (77). To permit cell death, CED-9 is inhibited by the F-box protein DRE-1, which ubiquitinates and likely degrades CED-9 (12). The zinc-finger transcription factor BLMP-1 also inhibits CED-9 by repressing its transcription (55). Together PAL-1, DRE-1, and BLMP-1 are all required for activating TSC death. How they control cell process degradation, however, is unclear.

The death of CEM neurons in hermaphrodites also requires the transcriptional activation of *ced-3* (83). In males, where the CEM neurons survive, the CEH-30 transcription factor represses *egl-1* and *ced-3* expression (83, 100). In hermaphrodites, TRA-1 inhibits CEH-30, which induces *egl-1* and *ced-3* expression to promote cell death (83, 100).

Why is *ced-3* transcription a key regulatory step in the induction of cell death in the TSC and CEM neurons? Like the TSC, CEM neurons die much longer after they are born compared to other apoptotic cells (~150 min versus 30 min) (108–110). Furthermore, gene expression studies suggest that *ced-3* is transcribed in many cells in the early embryo (most of which do not die) and that CED-3 protein is then segregated to progeny cells (77, 79). Therefore, in long-lived cells destined to die, perduring CED-3 protein may eventually be degraded, necessitating reexpression of the gene and presenting an opportunity for temporal control of cell death initiation (77).

Assisted Suicide

In fourth larval–stage males, one of the two left–right homologous cells, B.alapaav or B.arapaav, undergoes PCD and is engulfed by the neighboring P12.pa cell (109). Although apoptotic genes are required for this death, engulfment genes acting in the engulfing cell also play an important role (57). In *ced-1* and *ced-10* mutants, while B.al/rapaav cells acquire some refractility under

DIC optics, chromatin condensation and/or membrane blebbing, hallmarks of apoptotic cells, are not evident and the cells persist (57). Furthermore, when the P12.pa engulfing cell is absent, other nonphagocytic cells engulf the cell and promote its death (57). Thus, unlike the strictly cell-autonomous nature of apoptotic cell death, B.al/rapaav death has been termed assisted cell suicide (57). Like the TSC and CEM neurons, B.al/rapaav cells are morphologically complex, perhaps necessitating modification to the canonical apoptotic program. How the choice is made between whether B.alapaav or B.arapaav is assigned to die is not clear. One testable possibility is that stochastic proximity to the engulfing cell determines their fates.

Alternative Caspase-Dependent Pathways

Aside from *ced-3*, the *C. elegans* genome encodes three additional caspases: *csp-1*, *csp-2*, and *csp-3* (102). Overexpression of *csp-1* induces ectopic apoptotic cell death with morphological characteristics of apoptosis, and *csp-1* deletion, but not *csp-2* or *csp-3* disruption, enhances PCD defects of weak *ced-3* mutants (20). Thus, *csp-1* may promote PCD. Genetic studies suggest that maternal *csp-1* promotes PCD in embryogenesis independently of CED-9 and CED-4 (20). Thus, it is possible that caspase-dependent pathways exist that function in parallel to the core apoptotic pathway yet converge on the same targets to effect cell demise.

NONAPOPTOTIC CELL DEATH IN *C. ELEGANS* DEVELOPMENT

Several studies reveal that apoptosis alone cannot account for all developmental cell death events. For example, mice lacking crucial apoptotic regulators, BAX, BAK, and BOK or Apaf-1, can survive to adulthood without major defects (46, 58). Therefore nonapoptotic cell death programs likely function during development, but their molecular basis is poorly understood. Studies in *C. elegans* uncovered the first example of a nonapoptotic caspase-independent cell death program required in development: LCD (1). Subsequent studies uncovered other instances of caspase-independent cell death in *C. elegans* development, including cell extrusion and LC3-associated phagocytosis. These PCD forms utilize conserved genes and may therefore also function in vertebrate development and homeostasis.

Linker Cell–Type Death

The linker cell is a male-specific leader cell that guides the elongation of the gonad during larval development. It dies during the larva-to-adult transition to facilitate formation of the sperm exit channel (61) (**Figure 4*a***). Unlike apoptotic cells, dying linker cells display nuclear crenellations and organelle swelling and lack condensed chromatin (1) (**Figure 1*c***). LCD proceeds in the absence of apoptotic genes, including *ced-3* and all other caspases, *egl-1*, *ced-4*, and *ced-9* gain-of-function mutants (1). Therefore, LCD is morphologically and genetically nonapoptotic.

Linker cell–type death molecular pathway. LCD is activated by several parallel pathways (**Figure 4*b***). The LIN-29/zinc-finger transcription factor in the developmental timing pathway specifies LCD onset (1), and two opposing Wnt signaling pathways specify where LCD should take place (64). A MAPK kinase (MAPKK) pathway is also involved, although its trigger is unknown (4). The glutamine-rich protein PQN-41, which shares features with neurodegenerative polyglutamine-repeat proteins, activates LCD in parallel or downstream of the MAPKK pathway (4). These pathways converge to activate the heat-shock transcription factor HSF-1 (64). HSF-1 activates the ubiquitin proteasome system (UPS) by stimulating transcription of the E2 ubiquitin enzyme *let-70*/Ube2D2 (64). The SET-16/mixed-lineage leukemia 3/4 (MLL3/4) chromatin regulation complex also functions with HSF-1 to promote *let-70* expression (75). The E1 enzyme UBA-1 and E3 ubiquitin ligases composed of the cullin CUL-3, the ring box domain–containing

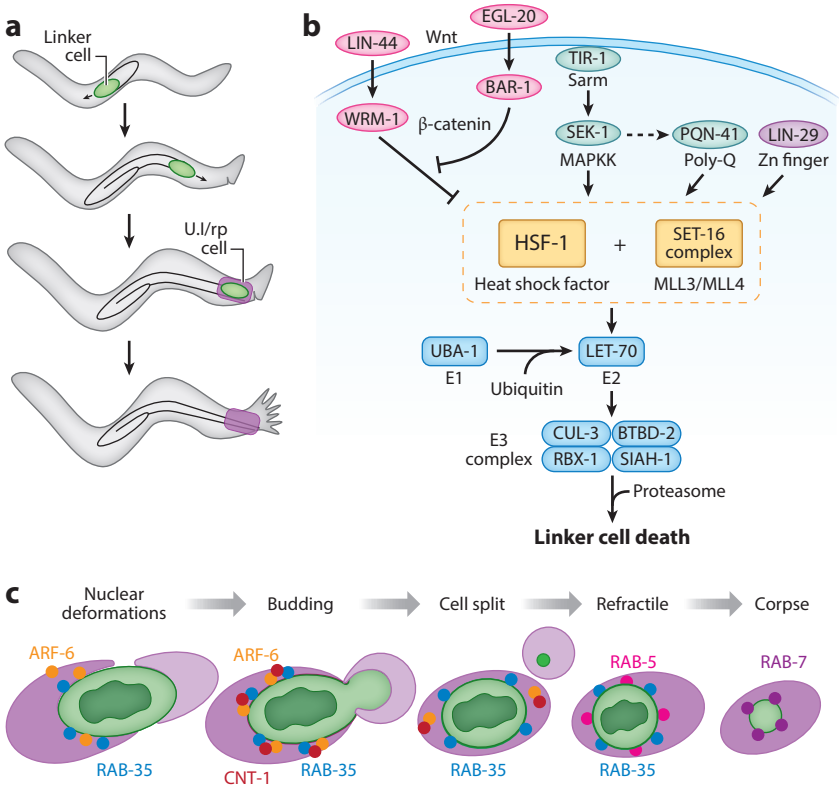

Figure 4

Linker cell–type death. (*a*) The male-specific linker cell (*green*) guides the elongation of the gonad during larval development. Once it finishes migrating at the end of the fourth larval stage, the linker cell dies and is engulfed by the U.I/rp cells (*purple*), allowing the formation of the sperm exit channel. Panel adapted with permission from Reference 69. (*b*) Three parallel pathways specify LCD: two opposing Wnt signaling pathways, a MAPKK pathway that functions with PQN-41, and the LIN-29/Zn-finger transcription factor pathway. These pathways converge on HSF-1, which acts in parallel with the SET-16 histone methyltransferase complex to stimulate the UPS by transcriptionally activating the E2 LET-70. Ubiquitin is transferred from the E1 UBA-1 through LET-70 to substrates recognized by the E3 complex, leading to their degradation by the proteasome and LCD. Panel adapted with permission from Reference 69 and modified from images created with BioRender.com. (*c*) The linker cell undergoes stereotypical morphological changes while being competitively phagocytosed and cleared by U.I/rp cells. Nuclear deformations mark the start of LCD and occur as the linker cell contacts U.I/rp cells. U.I/rp cells compete to engulf the linker cell, resulting in the cell budding and splitting into a larger nucleus-containing fragment that becomes refractile and a small fragment; both are degraded separately. As competitive phagocytosis begins, RAB-35 and ARF-6 GTPases surround the nascent phagosome. The GAP CNT-1 interacts with RAB-35 and drives the removal of ARF-6 from phagosomal membranes. RAB-35 remains on the phagosome and recruits RAB-5 to promote phagosome maturation. Afterwards, RAB-35 and RAB-5 are removed, and RAB-7 is recruited to effect formation of the phagolysosome and degradation of the linker cell. Abbreviations: GAP, GTPase-activating protein; LCD, linker cell–type death; MAPKK, mitogen-activated protein kinase kinase; MLL, mixed-lineage leukemia; Poly-Q, polyglutamine; UPS, ubiquitin proteasome system; U.I/rp, U.Ip and U.rp cells.

protein RBX-1, the BTB domain–containing protein BTBD-2, and other proteins function with LET-70 as part of the UPS to effect LCD (64). Thus, proteasomal degradation of proteins may be important for executing LCD. Supporting this notion, mutations in 19S proteasome subunit components also block LCD (64). The proteolytic targets that effect LCD, however, are unknown.

Linker cell degradation and clearance. Dying linker cells are also cleared and degraded differently from apoptotic cells (**Figure 4*c***). As the cell begins to die, two phagocytes, U.lp and U.rp (U.l/rp), simultaneously attempt to engulf the linker cell (68). At the end of this process, termed competitive phagocytosis, the linker cell splits into a large fragment containing the nucleus and a much smaller fragment, which are degraded separately with similar kinetics (68). Unlike the rapid kinetics of apoptosis, death and clearance of the linker cell can take up to 8 h (59, 68).

The dying linker cell does not expose phosphatidylserine on its cell membrane, and its engulfment does not require CED-1, CED-5, or other apoptotic engulfment genes (1, 68). Instead, the small GTPase RAB-35 mediates the onset of competitive phagocytosis and linker cell degradation (68) (**Figure 4*c***). RAB-35 localizes to extending phagocyte pseudopods and prevents the premature onset of phagocytosis. After engulfment, RAB-35 drives the removal of phosphatidylinositol 4,5-biphosphate [PI(4,5)P_2] from the phagosomal membrane, allowing recruitment of RAB-5 and RAB-7 GTPases, which mediate phagosome maturation. During both steps, RAB-35 functions by inactivating another small GTPase, ARF-6, an inhibitor of cell clearance, by binding the ARF-6 GAP CNT-1. While RAB-35 localizes to the engulfing cell and phagosome membranes during linker cell clearance, ARF-6 is only localized on these membranes transiently. ARF-6 removal coincides with PI(4,5)P_2 removal from the phagosome membrane. Thus, RAB-35 removal of ARF-6 likely drives the removal of PI(4,5)P_2 from the phagosome membranes and subsequent phagosome maturation (68).

A recent study found that RAB-35 weakly promotes phagosome maturation during apoptotic cell clearance, utilizing the GAP TBC-10 instead of CNT-1 (39), suggesting some overlap in engulfment programs between apoptosis and LCD. Indeed, if linker cell migration is blocked, the cell dies far from its normal site of demise and is then engulfed and degraded by a neighboring cell using the CED-1 engulfment receptor (1). Why the linker cell needs a dedicated engulfment program is not clear. Furthermore, the molecular basis of linker cell recognition by the U.l/rp cells remains a mystery and may divulge a new class of engulfment receptors.

Linker cell–type death conservation. The morphological hallmarks of LCD are conserved in a number of developing tissues in vertebrates, including the nervous system and reproductive tracts (69). Dying spinal motorneurons in developing chicks, for example, display crenellated nuclei, dilated organelles, and open chromatin reminiscent of dying linker cells (13, 85). Cells with LCD morphology are also found in brain samples and in mouse models of neurodegenerative states, such as Huntington's disease (18, 115). Genes that promote LCD in *C. elegans* are highly conserved, and many have been linked to cell-degenerative processes in mammals (69). In addition to the polyglutamine-repeat protein PQN-41, Sarm1, homologous to the *C. elegans* MAPKK activator TIR-1, promotes neurodegeneration following injury in mice and *Drosophila* (89). Thus, further studies of LCD may provide insight into mammalian development and disease.

Cell Extrusion

When apoptosis is blocked, such as in *egl-1*, *ced-3*, and *ced-4* loss-of-function mutants and in *ced-9* gain-of-function mutants, cells slated to die can still be shed from the developing embryo into the extraembryonic space (19). This nonapoptotic mechanism of cell elimination is termed cell extrusion (19, 86) and requires PAR-4/LKB1 and PIG-1/AMPK (19). Intriguingly, extruded cells display the hallmarks of apoptosis, including *egl-1* expression, phosphatidylserine externalization, and condensed chromatin (19), suggesting that these features are not unique to apoptotic cell death.

Cellular replication stress is an important driver of cell extrusion, as cell cycle genes with S phase–specific functions promote cell extrusion (24). Indeed, extruded cells are arrested in S phase before elimination, and shedding requires the replication stress signaling pathway, which

includes ATL-1/ATR and CHK-1/CHK1 (24). Dwivedi et al. (24) propose that in the absence of caspases, the smaller daughter cell generated by asymmetric cell division fails to inherit cellular resources to complete mitosis, is arrested in S phase, activates the replication stress response pathway, and therefore is extruded. Supporting their model, disrupting genes for nucleotide synthesis or balance causes ectopic cell extrusion (24). Of note, cell extrusion has been observed in the mammalian intestinal epithelium (67, 86), and activating the replication stress response pathway in cultured mammalian epithelial cells can induce cell extrusion (24), suggesting molecular conservation of the process. Dysregulation of cell extrusion has also been linked to epithelial diseases, including cancer (86). Therefore, further studies of cell extrusion mechanisms can provide insight into the etiology of these disorders.

Other Caspase-Independent Cell Death Events

In *ced-3* mutants, and in animals lacking all four *C. elegans* caspases, a few cells still die independently of cell extrusion (20). These dying cells display *egl-1* expression, externalize phosphatidylserine, and exhibit compacted cytoplasm and condensed chromatin (20). Like apoptotic cells, these dying cells are also engulfed and degraded by neighboring cells that express the CED-1 engulfment receptor (20). The molecular mechanism of these cell death events is not known, and it is difficult to study because it happens infrequently. Nonetheless, deciphering this mechanism may have important ramifications for understanding nonapoptotic cell death.

Clearance of Polar Bodies by LC3-Associated Phagocytosis

C. elegans polar bodies are meiotic by-products of oogenesis and are cleared in the embryo following fertilization. While the first polar body is cleared by eggshell secretion, the second polar body is internalized and degraded by early embryonic blast cells (28). In animals carrying loss-of-function mutations in *ced-3* or *ced-4*, or in *ced-9* gain-of-function mutants, polar bodies are still engulfed and continue to exhibit TUNEL reactivity (129), suggesting that second polar body elimination is nonapoptotic and caspase independent.

The second polar body shows phosphatidylserine reactivity on its outer membrane (27, 28). How this occurs is unclear, as the apoptotic scramblase CED-8 is not required (27, 28). The apoptotic engulfment receptor CED-1, recruited to the plasma membrane of embryonic blastomeres using VPS-35/PI3 kinase, RAB-5 GTPase, and SNX-6 sorting nexin (28), mediates CED-10-dependent polar body phagocytosis (28). Polar body membrane degradation in the phagolysosome is then mediated by LGG-1/LC3 (28). Following membrane breakdown, the phagolysosome tubulates to form small vesicles (28). This occurs only after amino acid release from phagolysosomes by the SLC-36.1 transporter and through activation of TORC1, which releases the BLOC-1-related complex (BORC), a multisubunit protein complex localized on lysosomal membranes (27). BORC activates the small ARF-like GTPase ARL-8 and the motor protein kinesin-1 (27). Finally, the HOPS tethering complex promotes cargo degradation in the small phagolysosomal vesicles (27).

All molecular components of second polar body elimination are conserved, and BORC has been shown to promote phagolysosome degradation in mammals (27). Whether this phagolysosome degradation pathway also promotes the final stages of apoptotic corpse degradation remains to be tested.

CONCLUSIONS

C. elegans PCD research has provided insight into the plethora of mechanisms responsible for specifying and effecting cell death during animal development. Many of these caspase-dependent

and -independent cell death programs have highly conserved components that function similarly in vertebrates. Despite the tremendous progress in *C. elegans* PCD research, many outstanding questions remain. For example, how the same apoptotic cell fate is conferred upon cells with vastly different lineages is unclear, and a complete understanding of *egl-1* expression control is also not yet available. Relevant caspase substrates that cause chromatin condensation and other aspects of the apoptotic morphology in *C. elegans* are unknown. We are also only just beginning to explore how apoptotic-like cell death is activated in the absence of caspases, as occurs during cell extrusion and polar body clearance. Nonapoptotic PCD programs in general, such as LCD, are ripe for discovery and could reveal important principles of cell death execution across phylogeny. What substrates act downstream of the UPS during LCD remain to be determined, and molecular conservation of this process in mammals will need to be explored. By addressing these and other outstanding questions, studies of PCD in *C. elegans* will continue to contribute toward our understanding of developmental cell death across species. These studies have and will continue to uncover candidate therapeutic targets for disorders associated with aberrant cell death, such as neurodegeneration and cancer.

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