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MULTI-AUTHOR REVIEW

Programmed cell death and clearance of cell corpses in *Caenorhabditis elegans*

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Abstract Programmed cell death is critical to the development of diverse animal species from *C. elegans* to humans. In *C. elegans*, the cell death program has three genetically distinguishable phases. During the cell suicide phase, the core cell death machinery is activated through a protein interaction cascade. This activates the caspase CED-3, which promotes numerous pro-apoptotic activities including DNA degradation and exposure of the phosphatidylserine "eat me" signal on the cell corpse surface. Specification of the cell death fate involves transcriptional activation of the cell death initiator EGL-1 or the caspase CED-3 by coordinated actions of specific transcription factors in distinct cell types. In the cell corpse clearance stage, recognition of cell corpses by phagocytes triggers several signaling pathways to induce phagocytosis of apoptotic cell corpses. Cell corpse-enclosing phagosomes ultimately fuse with lysosomes for digestion of phagosomal contents. This article summarizes our current knowledge about programmed cell death and clearance of cell corpses in *C. elegans*.

Keywords Programmed cell death · Apoptotic cell · Cell corpse clearance · *C. elegans*

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Introduction

Programmed cell death (herein also referred to as apoptosis) is an evolutionarily conserved cellular process that is of fundamental importance to living beings. The developmental cell death in the nematode *C. elegans* provides a unique and outstanding system for deciphering the cell death program at the single-cell level using genetic and cell biological approaches. In the lifetime of a *C. elegans* hermaphrodite, three waves of programmed cell death occur in two types of tissues: the soma and the gonad [1]. In hermaphrodites, 113 out of 628 somatic cells die during embryogenesis, and a further 18 cells die in the larval stage 2 (L2 stage). Thus, out of a total of 1090 somatic cells generated during the development of an adult hermaphrodite, 131 undergo programmed cell death [2, 3]. These deaths occur in an invariant pattern by following a cell-lineage-specific manner and cannot be induced by environmental stress. In adult hermaphrodite gonads, up to half of the germ cells undergo apoptosis during oogenesis. In addition, germ cell death can be induced by genotoxic or non-genotoxic stress [4, 5]. In general, developmental cell death involves three distinct phases: (1) death fate specification, (2) activation of the core cell death machinery for cell killing, and (3) programmed clearance of cell corpses [1, 6]. In the following sections, we provide a brief review of our current understanding of programmed cell death and cell corpse clearance in the model animal *C. elegans*.

Mechanisms of cell killing

The core cell death machinery

Both somatic and germ cell deaths are essentially controlled by a core cell death machinery that activates the cell suicide process. By genetic screening and gene cloning, four genes encoding members of the core cell death machinery have been identified, namely *ced-3*, *ced-4*, *ced-9*, and *egl-1* [7, 8]. Strong loss-of-function (lf) mutations of any of *ced-3*, *ced-4*, and *egl-1*, or gain-of-function (gf) mutation of *ced-9*, essentially block most, if not all, of the somatic cell death [9–11]. Strong *ced-9*(*lf*) mutants, on the contrary, exhibit a large number of ectopic cell deaths and cannot survive [11]. Epistasis analysis revealed that the ectopic cell death resulting from *ced-9*(*lf*) is suppressed by loss of *ced-3* or *ced-4*, indicating that *ced-3* and *ced-4* act downstream of *ced-9* [11]. While *ced-4* overexpression fails to induce cell killing in *ced-3*(*lf*) mutants, *ced-3* overexpression kills cells in *ced-4*(*lf*) mutants, suggesting that *ced-3* acts downstream of *ced-4* in the cell death pathway [12]. In addition, *egl-1*(*gf*) mutation causes ectopic death of hermaphrodite-specific neurons (HSNs), which is however suppressed by *ced-9*(*gf*) mutations [9]. These facts suggest that these genes act in a linear genetic pathway, in which *ced-3* functions furthest downstream and *egl-1* acts furthest upstream, to control the cell killing process [7, 8] (Fig. 1).

ced-3 encodes the founding member of the caspase family of proteases that need to be processed for activation [13]. CED-3 is the major caspase that provides protease activity for apoptosis in *C. elegans* [13, 14]. *ced-4* encodes a *C. elegans* homolog of mammalian Apaf1, with both containing a caspase-recruitment domain (CARD) and nucleotide-binding motifs [15, 16]. The CED-9 protein is similar to human anti-apoptotic proteins Bcl2/Bcl-xL [17]. EGL-1 is a BH3-only protein, and is similar to several BH3-only pro-apoptotic members of the Bcl2 family of apoptosis regulators [9]. The similarities shared by major cell death regulators in *C. elegans* and mammals suggest that the regulatory mechanisms underlying programmed cell death are evolutionarily conserved [7, 8]. In vitro biochemical studies have revealed that CED-4 and CED-9 physically interact with one another [18–20]. EGL-1 binds to CED-9 with higher affinity, causing a major conformational change of CED-9 and consequently disruption of CED-9:CED-4 interaction [21, 22]. In *C. elegans* embryos, CED-9 and CED-4 colocalize to mitochondria and the mitochondrial localization of the latter is dependent on the former. Overexpression of EGL-1 leads to translocation of

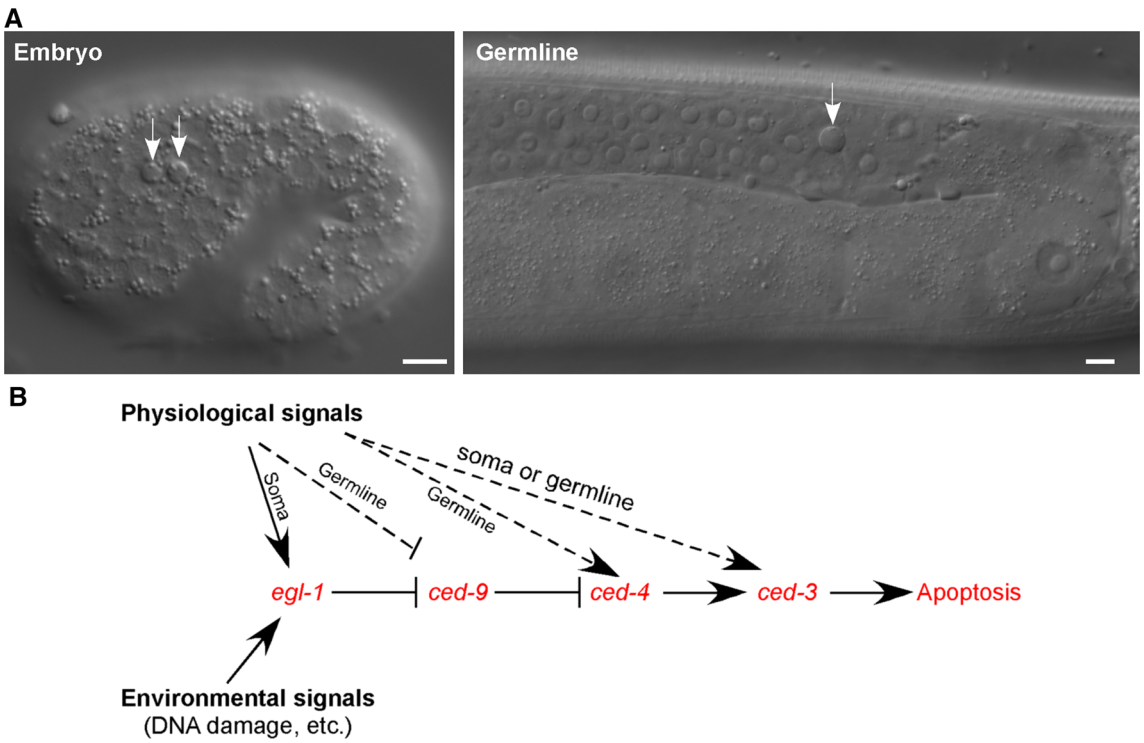

Fig. 1 Activation of programmed cell death in *C. elegans*. **a** Representative images of refractile "button-like" apoptotic cells (indicated by *arrows*) in an embryo (*left*) and a gonad arm (*right*) of a hermaphrodite. *Bars* 5 μm. **b** Genetic pathway for cell death activation in *C. elegans*. Death signals may be transduced to *egl-1*, or *ced-9*, or *ced-3* to activate apoptosis (see the text). Activating and inhibitory effects are designated by *down arrow* and *perpendicular symbol*, respectively; *dashed lines* represent proposed interactions

CED-4 to the perinuclear region and ectopic cell death [23]. These in vitro and in vivo observations suggest a mechanistic model for cell death activation in *C. elegans*: CED-4 is normally sequestered on mitochondria by interacting with mitochondrion-localizing CED-9 in living cells. In cells destined to die, EGL-1 is transcriptionally activated and interacts with CED-9, resulting in translocation of CED-4 from the mitochondrial surface to the perinucleus, which in turn promotes the activation of the CED-3 caspase and hence cell death [7, 8]. Indeed, crystal structural studies revealed that CED-4 binds to CED-9 in a 2:1 ratio. After being freed from the CED-4/CED-9 complex by EGL-1, the CED-4 dimer further oligomerizes, facilitating auto-cleavage of the CED-3 zymogen into p17 and p15 subunits required for formation of active caspase [14, 24, 25]. Further crystal structural analysis suggests that eight CED-4 molecules form an apoptosome consisting of four asymmetric dimers. The octameric CED-4 apoptosome adopts a funnel-shaped structure and accommodates within its conical internal space two molecules of CED-3 zymogen via their L2′ loops, thus facilitating CED-3 activation by autocleavage. The activated CED-3 molecules remain associated with the CED-4 apoptosome and thus the protease activity is stimulated [26, 27].

Other cell death regulators

Several other factors have been shown to regulate apoptosis through the core cell death machinery in *C. elegans.* A mitochondrial ATP translocase, ANT-1.1/WAN-1, was found to interact with CED-4 to promote apoptosis. Loss of function of ANT-1.1/WAN-1 strongly decreases cell death in both soma and germline [28]. CED-4L, a CED-4 variant resulting from alternative splicing, plays an anti-apoptotic role in contrast to the predominant pro-apoptotic function of CED-4. More recently, SPK-1, a serine-arginine-rich (SR) protein kinase involved in splicing, was found to play an anti-apoptotic role, probably by affecting the alternative splicing of *ced-4* mRNA [29, 30]. In certain cell types, such as the tail-spike cell, EGL-1 seems to play a minor role; instead, the F-box protein DRE-1/FBXO10 acts in parallel with EGL-1 to inactivate CED-9 and promote apoptosis of tail-spike cells [31]. In addition, appropriate translation initiation may also play a critical role in apoptosis. For example, the translation regulators GCN-1 and ABCF-3, yeast homologs of which control eIF2a phosphorylation, promote apoptosis of most somatic cells and DNA damage-induced germ cell death in a CED-3-dependent, but CED-9- or EGL-1-independent manner [32]. Similarly, the translation initiation factor subunit K EIF-3.K was found to promote apoptosis through CED-3, but not CED-4 [33]. These findings suggest that apoptosis activation is regulated at multiple levels.

Cleavage of protein targets by CED-3 in apoptosis

The CED-3 caspase, once activated, is believed to kill cells by proteolytic cleavage of a broad spectrum of protein targets. However, only a few CED-3 substrates have been characterized to date. The cleavage of CED-9 by CED-3 was found to be important for apoptosis [34]. In recent years, it has been shown that the Dicer/DCR-1 ribonuclease is a substrate of CED-3. During apoptosis, cleavage of DCR-1 by CED-3 converts it into a deoxyribonuclease that initiates chromosome fragmentation [35, 36] (see below). In addition, the mitochondrial fission protein DRP-1/Drp1 and the multipass transmembrane protein CED-8/Xk1 are also subjected to CED-3 cleavage, generating truncated DRP-1 and CED-8 that promote elimination of mitochondria and exposure of phosphatidylserine (PtdSer) as an "eat me" signal on the apoptotic cell surface, respectively [37, 38]. More recently, it was found that CPS-2/CNT-1, an Arf GTPase activating protein (Arf GAP), undergoes CED-3-dependent processing during apoptosis. The resulting tCNT-1 is capable of binding to phosphatidylinositol (3,4,5)-triphosphate (PIP3) and translocates from the cytoplasm to the plasma membrane, where it antagonizes binding of AKT to PIP3 and consequently disables AKT activation and its pro-survival activity [39].

Role of CED-3-like caspases in programmed cell death

In addition to *ced-3*, the *C. elegans* genome contains three other caspase genes, *csp-1*, *csp-2*, and *csp-3* [40]. CSP-1 plays a pro-apoptotic role in a subset of cells during embryogenesis. Unlike CED-3, however, CSP-1 is not regulated by either CED-4 or CED-9, suggesting that the CSP-1 caspase acts independently of the canonical genetic pathway of apoptosis to promote cell death [41]. Interestingly, CSP-3 and CSP-2 do not play a pro-apoptotic role; instead, they act as anti-apoptotic factors by inhibiting CED-3 autoactivation [42, 43]. CSP-3 is similar to the small subunit of CED-3 and associates with its large subunits, thereby inhibiting autoactivation of the CED-3 zymogen in somatic cells. CSP-2 shares sequence homology with both large and small subunits of CED-3. Like CSP-3, CSP-2 interacts with the CED-3 zymogen and inhibits CED-3 autoactivation in germline cells. Nevertheless, CSP-3 and CSP-2 inhibit neither CED-4-mediated CED-3 activation nor the activity of activated CED-3 [42, 43]. In many other species, members of the IAP (inhibitor of apoptosis protein) family play important roles in inhibiting caspase activity. No apparent IAP homologs exist in *C. elegans*, but nevertheless the activity of CED-3 is tightly regulated by different mechanisms to ensure the precise control of cell death during development.

Caspase-activated apoptotic DNA degradation

In mammals, the activation of effector caspases, e.g., caspase 3 or 7, leads to cleavage of DFF45/ICAD, which normally interacts with the DFF40/CAD deoxyribonuclease to keep its activity in check. Cleavage of DFF45/ICAD activates DFF40/CAD, which induces chromosome fragmentation by cleaving the internucleosomal DNA [44–47]. In *C. elegans*, there are no obvious homologs of DFF45/ICAD and DFF40/CAD. Surprisingly, the Dicer/DCR-1 ribonuclease, which is responsible for generating small interference RNAs (siRNAs), microRNAs (miRNAs), and other small RNAs, plays a critical role in chromosome fragmentation during apoptosis in *C. elegans* [35]. Inactivation of *dcr-1* strongly suppresses the generation of DNA breaks and reduces embryonic cell death. Mechanistically, DCR-1 is cleaved by the CED-3 caspase to produce a C-terminal fragment with deoxyribonuclease activity. Thus, the conversion of the DCR-1 ribonuclease into a deoxyribonuclease by CED-3 induces chromosome fragmentation and promotes apoptosis [35]. The fragmented DNA is further degraded by synergistic action of a group of nucleases and their regulators, including CPS-6/EndoG, NUC-1, WAH-1/AIF, CRN-1 to -6, and CYP-13 [48–52]. Loss or reduction of function of these genes leads to accumulation of DNA breaks that can be visualized with TUNEL (terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate (dUTP) nick end labeling) assays. Furthermore, inactivation of most of these genes, except *nuc-1* and *crn-6*, causes a significant delay in appearance of apoptotic cell corpses during embryonic development, suggesting that timely degradation of apoptotic DNA is important for progression of programmed cell death [49, 50, 52]. Interestingly, both WAH-1/AIF and CPS-6/EndoG are mitochondrial proteins. WAH-1/AIF physically interacts with CPS-6/EndoG and enhances its nuclease activity. Ectopic expression of EGL-1 triggers the translocation of WAH-1 from mitochondria to nuclei in a CED-3-dependent manner, suggesting an important role of mitochondria in *C. elegans* programmed cell death as in mammals [48, 51].

Specification of cell death fates

Death fate specification in somatic cells

The fact that 131 cells out of the total 1090 cells in a hermaphrodite undergo apoptosis suggests that the death fates of these cells are specifically determined. Studies in several cell types indicate that the cell death initiator EGL-1 is transcriptionally activated in individual cells destined to die [53, 54]. This is achieved by binding of specific transcription regulators to *cis*-elements in the *egl-1* gene. For example, LIN-3/EGF acts through the ETS domain-containing transcription factor LIN-1 to activate *egl-1* expression to induce apoptosis of a subset of cells [55]. In the NSM (neurosecretory motor neuron) sister cell that is doomed to die, *egl-1* transcription is upregulated by a heterodimer composed of two helix-loop-helix proteins HLH-2 and HLH-3. In contrast, *egl-1* expression is inhibited by the transcription repressor CES-1/SLUG in the surviving NSMs. While CES-1 expression is seen in NSMs, it is suppressed by the basic leucine-zipper transcription factor CES-2 and the DNaJ domain-containing protein DNJ-11 in the NSM sister cell, allowing HLH-2/-3-dependent upregulation of *egl-1* and consequently cell death. In addition, *ces-1, ces-2* and *dnj-11* are required for proper control of asymmetric cell division of NSM neuroblasts, suggesting that asymmetric cell division is critical for specification of cell death [56–59]. This is supported by observations that disruption of asymmetric cell division by loss of function of *pig-1*, an AMP-activated protein kinase gene, leads to survival of extra cells instead of death in several lineages, including the HSN/PHB, I2, M4, and PLM/ALN precursors [60, 61]. In addition, the Arf GAP CNT-2, the cytohesin GRP-1, and the transcription factor TOE-2 were also found to regulate asymmetric division of Q.p precursors, affecting death of daughter cells [62–64].

The sex-specific deaths of hermaphrodite-specific neurons (HSNs) in males and of male-specific sensory CEM (cephalic male) neurons in hermaphrodites are also controlled by transcriptional activation of *egl-1*. In hermaphrodite HSNs, the zinc-finger protein TRA-1A binds to a DNA region 6.4 kb downstream of the *egl-1* start codon, repressing the transcription of *egl-1* [65]. In males, the TRA-1A protein is inactivated through proteasomal degradation triggered by high-level expression of a secreted protein, HER-1. HER-1 binds to and inactivates a transmembrane protein, TRA-2, leading to assembly of an E3 ubiquitin ligase complex composed of six proteins (FEM-1, -2, -3, ELC-1, CUL-2, and RBX-1) and consequently degradation of TRA-1A. As a result, *egl-1* is upregulated to induce the death of HSNs [66–68]. In contrast, *egl-1* transcription is repressed by the BarH domain-containing protein CEH-30 in male-specific CEMs, allowing CEM survival. In hermaphrodites, however, TRA-1A protein is present at high levels and directly represses CEH-30 transcription, thereby activating *egl-1* transcription and resulting in death of CEMs [69, 70].

For the P11.aaap cell in the ventral cord, the sister cell of the RID neuron in the anterior ganglion, or the sister cell of the M4 motor neuron in the pharynx, the death fates are also specified by activation of *egl-1* by specific transcription factors. The *Antennapedia* homolog MAB-5 forms a complex with the Pbx homolog CEH-20 and binds to a

Pbx/Hox-binding site downstream of the *egl-1* open reading frame to activate its expression, thus triggering the death of the P11.aaap cell [71]. In the RID sister cell, the COE (Collier/Olf/EBF) transcription factor UNC-3 acts together with CBP-1/p300 to activate *egl-1* expression and induce cell death [72]. In the M4 sister cell, *egl-1* expression is activated by the Six family Hox protein CEH-34 and the EYA domain-containing protein EYA-1, which form a complex and bind to a 5′ *cis*-element in the *egl-1* gene [73]. Additionally, *egl-1* expression in the M4 sister cell and the sister cell of the AQR sensory neuron is controlled by SPTF3, an Sp1 transcription factor [60]. Interestingly, SPTF-3 also drives the expression of *pig-1*, which acts through a CED-3-independent pathway to promote apoptosis of these two cell types, suggesting that a single transcription factor controls two parallel programs to specify cell death fates [60].

An alternative mechanism to specify the death fate is transcriptional activation of *ced-3* instead of *egl-1*. Tail-spike cells are highly differentiated and long-lived prior to death. Interestingly, the majority of spike cell deaths are independent of *egl-1*, but require *ced-4* and *ced-3*. Studies revealed that PAL-1, a *Caudal* homeodomain protein, promotes *ced-3* expression by binding to a *cis*-element in the *ced-3* promoter, thus inducing the death of tail-spike cells [74]. It remains to be understood how elevated *ced-3* levels efficiently trigger death of tail-spike cells, but not other cells.

Signal transduction leading to apoptosis of germ cells

In *C. elegans*, germ cell apoptosis appears to be specific to hermaphrodites. It is estimated that up to a half of germ cells undergo apoptosis during oogenesis in an adult hermaphrodite, while no cell death takes place in male gonads [4]. Intriguingly, physiological germ cell death requires *ced-4* and *ced-3*, but not *egl-1*. In addition, unlike in somatic tissues, gain-of-function mutation of the cell death inhibitor CED-9 does not prevent physiological germ cell death even though its loss of function still causes excessive apoptosis [4]. Thus, the cell death initiator that activates the core cell death machinery for germ cell apoptosis remains to be understood. Nevertheless, it was found that the Ras/Mitogen-activated kinase (MAPK) signaling pathway plays an essential role in germ cell death under physiological conditions. Genes involved in MAPK signaling, such as LIP-1, a phosphatase, and GLA-3, an RNA-binding protein, may antagonize germ cell apoptosis by inhibiting MAPK signals [75, 76]. It is suggested that MAPK signaling probably regulates *ced-9* expression in a negative manner to trigger germ cell killing via CED-4 and CED-3, as evidenced by the observation that reduction in CED-9 levels can cause germ cell apoptosis in the absence of MAPK signaling [4]. Consistent with this, LIN-35/RB, PAX-2 and EGL-38 affect physiological germ cell death by regulating *ced-9* expression positively or negatively. In addition, transcriptional regulation of *ced-3* and *ced-4* expression levels is also important for physiological germ cell death [77, 78].

Unlike somatic cells, germ cells can be induced to die via apoptosis as a result of environmental stress, particularly genotoxic stress. DNA damage-induced germ cell apoptosis requires the cell death initiator gene *egl-1* to trigger the core cell death machinery [5]. *egl-1* and the other BH3-only protein-encoding gene, *ced-13*, are transcriptionally upregulated by CEP-1/p53 in response to DNA damage [79–81]. This resembles the p53-mediated upregulation of the BH3-only cell death initiators PUMA and Noxa in mammalian cells [82, 83]. As in mammals, DNA damage in *C. elegans* germ cells is sensed and transduced by checkpoint signaling pathway orchestrated by ATM-1/ATM, ATL-1/ATR, and the 9-1-1 complex. Loss of MRT-2/Rad1, HUS-1/hus-1, and CLK-2/Rad5 essentially blocked germ cell apoptosis induced by ionizing irradiation [5]. In addition, CEP-1/p53 activity can be regulated through different mechanisms [84]. For example, the GLD-1 translational repressor negatively regulates CEP-1 by binding to the 3′ UTR of *cep-1* mRNA to inhibit its translation [85]. The protein arginine methyl transferase PRMT-5, on the other hand, forms a complex with CEP-1 and CBP-1/p300 and methylates the latter, thus affecting transcriptional activation of *egl-1* by CEP-1 in response to ionizing irradiation [86]. Intriguingly, the protein deacetylase SIR-2.1 acts in parallel with CEP-1 and plays an essential role in DNA damage-induced apoptosis [87]. As well as exogenous genotoxic stress, failure in chromosome synapsis or defects in repair of DNA double-strand breaks (DSBs) during meiosis also lead to elevated levels of germ cell apoptosis. However, synaptic failure and defective DSB repair appear to trigger apoptosis by distinct mechanisms. If not repaired properly, DNA breaks generated by SPO-11, a topoisomerase-like nuclease, trigger *egl-1*-dependent apoptosis through MRT-2/HUS-1-CEP-1 signaling, the same pathway activated by exogenous DNA damaging insults [84, 88]. In contrast, synaptic failure resulting from defects in the pairing center or mutations in the synaptonemal complex (SC) trigger apoptosis through the synapsis checkpoint PCH-2, a *C. elegans* homolog of the yeast AAA-adenosine triphosphatase (AAA-ATPase) Pch2 [89], though it remains unknown how PCH-2 mediates death signals to the EGL-1 cell death initiator. Thus, mutations of regulators important for genomic stability, such as the chromodomain protein MRG-1/Mrg15, may trigger germ cell apoptosis through either pathway, or both [88, 90, 91].

Nonapoptotic cell death in *C. elegans*

When the canonical apoptosis pathway is inhibited, a number of embryonic cells still undergo programmed cell death by shedding in a caspase-independent manner [92]. Cell shedding requires the *pig-1* gene, which encodes an AMPK-related serine-threonine kinase. It also involves a kinase complex composed of PAR-4, STRD-1, and MOP-25.1/-25.2, the *C. elegans* homologs of mammalian tumor suppressor LKB1 and its interacting proteins STADα and MO25α. The PAR-4/LKB1 kinase complex probably phosphorylates PIG-1, which promotes shed-cell detachment by inhibiting expression of cell-adhesion molecules on the surface. Thus, cell shedding can compensate for the canonical death pathway in certain cells that are destined to die during animal development [92, 93].

The linker cell is essential for the extension and development of the gonad in *C. elegans* males. It is born at the L2 stage, but dies after the L4/adult transition to facilitate the fusion between the vas deferens and cloaca. The death of the linker cell is independent of the CED-3 caspase and other apoptotic machinery components. Instead, it requires the microRNA *let-7* and Zn-finger protein LIN-29 [94]. In addition, the death of the linker cell requires PQN-41, a polyglutamine-repeat protein of which expression is controlled by the mitogen-activated protein kinase kinase SEK-1. *pqn-41* acts in parallel to *lin-29* to promote linker cell death in a nonapoptotic manner, which is morphologically similar to polyglutamine-induced neuron degeneration in vertebrates [95].

In addition to developmental nonapoptotic cell deaths as described above, *C. elegans* cell death can also be induced in a nonapoptotic way by a broad range of environmental or physiological stimuli, or by heterologous expression of human disease-related genes. For more detailed review, please refer to Vlachos and Tavernarakis [96].

Clearance of apoptotic cell corpses

Apoptotic cells generated by programmed cell death are quickly removed to prevent inflammatory and autoimmune responses and maintain tissue homeostasis. During this process, apoptotic cells expose "eat me" signals, which are recognized by phagocytes to trigger signaling cascades, leading to internalization and degradation of cell corpses. Mutations in genes involved in cell corpse engulfment causes survival of some cells that are programmed to die, indicating that cell corpse engulfment contributes to cell killing [97, 98]. As in the case of the cell killing machinery, evolutionarily conserved genes have been identified, which control cell corpse recognition, engulfment, and degradation through mechanisms that are utilized in both worms and humans.

Surface-exposed phosphatidylserine serves as an "eat me" signal for cell corpse removal

Phosphatidylserine (PtdSer), which is restricted to the inner leaflet of plasma membranes in living cells, is exposed on the surface of dying cells and serves as an "eat me" signal to trigger phagocytosis in mammals [99]. By employing an ex vivo Annexin V-based staining protocol, selective exposure of PtdSer on apoptotic cells was first observed in the *C. elegans* germline and was later confirmed in soma by expressing secreted PtdSer-binding reporters; these findings indicated that externalization of PtdSer is an evolutionarily conserved apoptotic event [100–103]. The P4-ATPase TAT-1 maintains membrane PtdSer asymmetry by promoting PtdSer movement to the cytosolic leaflet. Loss of TAT-1 causes ectopic PtdSer exposure on the surface of normal living cells, leading to random cell loss due to indiscriminate removal by neighboring phagocytes [101]. On the other hand, CED-8 and human Xkr8, the plasma membrane-localized XK membrane transporter, are activated by caspase cleavage to promote PtdSer externalization in apoptotic cells [37, 104]. WAH-1, an apoptogenic factor released from mitochondria during apoptosis, activates phospholipid scramblase SCRM-1 to promote PtdSer exposure on the apoptotic cell surface [100] (Fig. 2). Loss of CED-8, WAH-1, or SCRM-1 reduces PtdSer externalization in apoptotic cells and compromises cell corpse engulfment, indicating that surface-exposed PtdSer serves as an evolutionarily conserved "eat me" signal for cell corpse recognition. PtdSer

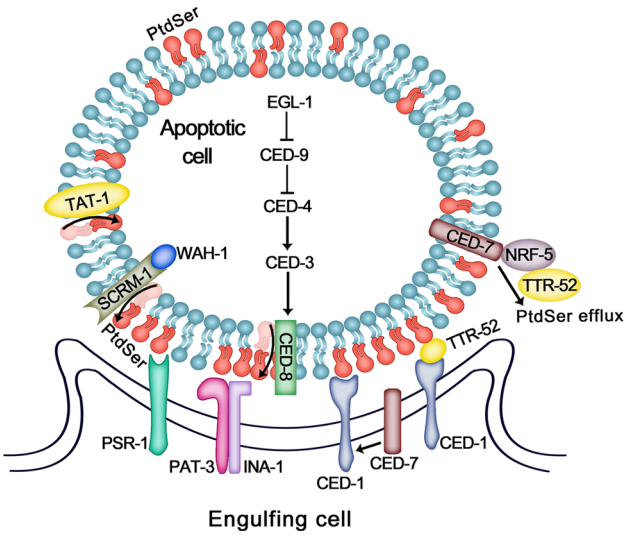

Fig. 2 Externalization and recognition of the PtdSer "eat me" signal in *C. elegans*. Known regulators of PtdSer externalization and PtdSer-recognizing receptors are shown. Activating and inhibitory effects are designated by *down arrow* and *perpendicular symbol*, respectively. CED-7, NRF-5, and TTR-52 mediate PtdSer efflux from the apoptotic cell [113, 114]

externalization is reduced, but not eliminated in the three mutants (*ced-8, wah-1, scrm-1*), suggesting that additional genes or mechanisms are involved in this process. In addition to apoptotic cells, PtdSer is also observed on the surface of necrotic cells and may serve as the signal for clearance [105].

Recognition of cell corpses

Apoptotic cells can be recognized either directly by phagocytic receptors or indirectly through secreted PtdSer-binding "bridging molecules". TTR-52, a transthyretin-like extracellular protein secreted from non-apoptotic intestine cells, mediates recognition of apoptotic cells by the phagocytic receptor CED-1 (MEGF10) [106, 107]. TTR-52 forms a dimer, binds surface-exposed PtdSer, and interacts with the extracellular ERM domain of CED-1, thus acting as a bridging molecule to cross-link the PtdSer "eat me" signal with the CED-1 receptor [108]. CED-7, an ABC transporter that acts in the same genetic pathway with CED-1 and TTR-52, is required for enrichment of CED-1 around cell corpses [106, 109]. How CED-7 mediates cell corpse recognition by CED-1 remains unclear. The extracellular domain of CED-1 associates with PtdSer in vitro, suggesting that CED-1 may recognize cell corpses by binding directly to PtdSer [105]. Thus, multiple mechanisms may be engaged to mediate recognition of cell corpses by the CED-1 receptor (Fig. 2). In addition to mediating recognition of the PtdSer "eat me" signal on apoptotic cells, CED-7 and TTR-52 are involved in regulating PtdSer appearance on engulfing cells, a phenomenon observed previously in macrophages and differentiating monocytes [110–114]. NRF-5, an extracellular lipid binding and transfer protein, is recruited to apoptotic cells by CED-7 and cooperates with CED-7 and TTR-52 to mediate PtdSer transfer from apoptotic cells to the adjacent phagocytes and thus facilitate recognition and engulfment by the PtdSer-positive phagocytes [113]. The efflux of PtdSer from apoptotic cells may involve generation of extracellular PtdSer-containing vesicles and CED-1 may provide a docking site to unload PtdSer or to promote fusion of PtdSer vesicles with the phagocyte [114].

In the genetic pathway parallel to CED-1/TTR-52/CED-7, multiple phagocytic receptors have been identified (Figs. 2, 3). The *C. elegans* phosphatidylserine receptor PSR-1 serves as an evolutionarily conserved PtdSer receptor to mediate cell corpse recognition. PSR-1 enriches on and clusters around apoptotic cells in vivo, binds PtdSer in vitro through a conserved lysine-rich motif, and may undergo PtdSer-induced oligomerization [115]. It acts through the CED-2, CED-,5 and CED-12 signaling pathway to promote clearance of apoptotic and necrotic cells and is also responsible for removing PtdSer-positive living cells caused by loss of TAT-1 [101, 116] (Fig. 3). PSR-1 also plays a role in axonal fusion during regeneration, a non-apoptotic process that requires both the PtdSer-binding and Fe(II)-dependent Jmjc activity of PSR-1 [117]. Thus, PSR-1 may regulate multiple processes through a PtdSer-mediated mechanism. Like PSR-1, the *C. elegans* integrin INA-1 (integrin α)/PAT-3 (integrin β) may recognize the PtdSer signal and promote cell corpse engulfment through the CED-2/5/12 pathway [118] (Fig. 2). SRC-1, a non-receptor tyrosine kinase, binds to both INA-1 and CED-2 to relay the engulfment signal from INA-1 to the CED-2/5/12 signaling complex [118] (Fig. 3). PAT-2, another integrin α subunit, functions specifically in muscle cells to mediate cell corpse recognition and engulfment through the small GTPase CDC-42, and the Frizzled homolog MOM-5 may act in early embryos to mediate recognition of apoptotic cells through the CED-2/5/12 pathway [119, 120] (Fig. 3). It is unclear whether PAT-2 or MOM-5 recognizes PtdSer on the apoptotic cell surface.

Engulfment of cell corpses

Upon recognition of the apoptotic cell by receptors, signaling cascades are activated in phagocytes to initiate the engulfment process. CED-12 (ELMO) and CED-5 (DOCK180) act as a bipartite GEF to activate Rac GTPase CED-10, which in turn leads to rearrangement of the actin cytoskeleton for cell corpse engulfment [121–125]. CED-2 (CrkII), an SH2/SH3 domain-containing adaptor protein, or the UNC-73 (Trio)-MIG-2 (RhoG) module activates the CED-12/CED-5 complex, thus linking upstream engulfment signals to the activation of CED-10 (Rac1) [125, 126] (Fig. 3). Loss-of-function mutations of *unc-73* or *mig-2* enhance the phenotype of other engulfment-defective mutants, but cause no defects on their own, suggesting that they provide a minor input into the CED-12/CED-5 complex [126]. The CED-2/5/12 pathway controls both cell corpse engulfment and migration of distal tip cells (DTCs), suggesting common regulatory mechanisms in both processes. In the CED-1 pathway, the phosphotyrosine-binding domain (PTB)-containing adaptor CED-6 (GULP) interacts with the cytoplasmic tail of the CED-1 receptor to transduce engulfment signals to downstream effectors including large GTPase DYN-1 (dynamin), which is important for engulfment and degradation of cell corpses [127–129] (Fig. 3). Interestingly, two recent studies report that clathrin and its adaptor proteins AP2 and epsin, which are key regulators of receptor-mediated endocytosis, act downstream of CED-6 to promote actin reorganization [130, 131] (Fig. 3). In response to CED-1 signaling, AP2 and CHC-1 form a complex with CED-1 and CED-6 and induce cytoskeleton remodeling, leading to internalization of apoptotic cells [130]. The Rac GTPase CED-10 may

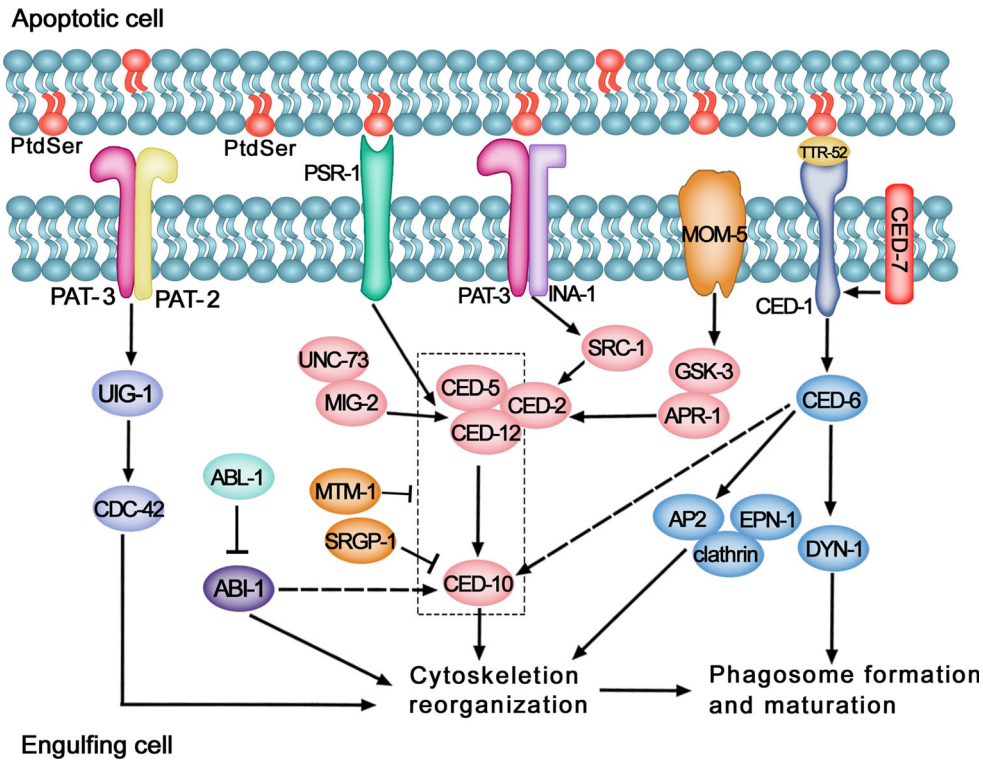

Fig. 3 Multiple signaling pathways mediate cell corpse engulfment in *C. elegans*. Activating and inhibitory effects are designated by *down arrow* and *perpendicular symbol*, respectively. *Dashed arrows* indicate the proposed activation

also act downstream of the CED-1 pathway and thus functionally link the two engulfment pathways [132]. In response to PAT-2/PAT-3 integrin signaling, the small GTPase CDC-42 (Cdc42) is activated by its GEF UIG-1 (Clg), leading to cytoskeleton reorganization and cell corpse internalization [119, 133] (Fig. 3).

Cell corpse engulfment is also controlled by negative regulators. Myotubularin MTM-1 is a plasma membrane-localizing lipid phosphatase. It negatively regulates cell corpse engulfment through the CED-5/12/10 module, probably by controlling PtdIns3P or PtdIns(3,5)P_2 levels on the cell membrane [134, 135]. SRGP-1 functions as a GTPase activating protein (GAP) to negatively regulate CED-10 activity and thus inhibit engulfment [136]. The tyrosine kinase ABL-1 (abl) acts through its interacting protein ABI-1 (abi) to inhibit cell corpse engulfment by acting on CED-10 or an independent pathway [137] (Fig. 3).

Phagosome formation and maturation

To internalize apoptotic cells, phagocytes extend pseudopods that fuse to form membrane-bound vesicles (phagosomes) enclosing cell corpses. Phagosome scission separates the vesicle from the plasma membrane, which completes engulfment and initiates a maturation process. Maturation of apoptotic cell containing phagosomes, which in many ways parallels endosome progression, involves sequential interactions with early endosomes, late endosomes and lysosomes to yield phagolysosomes where apoptotic cells are degraded. In the past years, a large number of genes have been identified that function in a stepwise manner to regulate a variety of membrane remodeling events, leading to formation and maturation of phagosomes.

Phagosome sealing

During mammalian phagocytosis of invading microorganisms, dynamic changes in phospholipid composition have been observed, which define stages of phagosome formation and maturation [138, 139]. In *C. elegans* apoptotic cell clearance, however, phagosome formation has not been clearly distinguished from the maturation stage, largely due to lack of in vivo assays to determine phagosomal sealing. In a recent study, we followed dynamic changes of various phosphoinositide reporters and examined sealing of apoptotic cell-containing phagosomes. We found that PtdIns(4,5)P_2 and PtdIns3P transiently accumulate on unsealed and fully sealed phagosomes, respectively, and are both involved in phagosome closure [140]. We identified a coincident detection mechanism that regulates phagosome sealing through LST-4/DYN-1 and couples sealing with the switch of membrane identity from PtdIns(4,5)P_2-enriched unsealed phagosomes to PtdIns3P-enriched fully sealed phagosomes [140]. In this process, the

myotubularin phosphatase MTM-1 acts as an effector of PtdIns(4,5)P_2 that coordinates with the class II PI3 kinase PIKI-1 to control PtdIns3P levels on unsealed phagosomes (Fig. 4a). Thus, MTM-1 acts at both early and late steps of cell corpse engulfment. Loss of *mtm-1* accelerates initiation of engulfment, but impairs phagosomal sealing, suggesting that PtdIns3P turnover may have distinct effects on apoptotic cell clearance at different stages. We found that the SNX9 family protein LST-4 and the *C. elegans* dynamin DYN-1, two key regulators of apoptotic cell removal [130, 141, 142], are both required for phagosomal sealing. LST-4 is recruited by a coincident detection code, consisting of PtdIns(4,5)P_2, PtdIns3P and MTM-1, to unsealed phagosomes where it recruits and acts through DYN-1 to regulate sealing, probably through a similar mechanism as in scission of endocytic vesicles (Fig. 4a) [130, 140, 142, 143]. CHC-1 and AP2 may also regulate phagosomal association of LST-4 either directly by forming a complex with the latter or indirectly by affecting the coincident detection code that recruits LST-4 [130]. Like in mammalian phagocytosis, sealing of apoptotic cell-containing phagosomes coincides with and requires timely depletion of PtdIns(4,5)P_2, a process that involves the inositol-5-phosphatase OCRL-1 [140, 144]. Elimination of PtdIns(4,5)P_2 by OCRL-1 may release MTM-1, LST-4 and DYN-1, which completes the scission process and allows subsequent accumulation of PtdIns3P on fully sealed phagosomes (Fig. 4a). It remains unclear how OCRL-1 is recruited to phagosomes. It is possible that LST-4/DYN-1 plays a role in OCRL-1 recruitment or RAB-5 may be involved in this process as in mammalian phagocytosis [144].

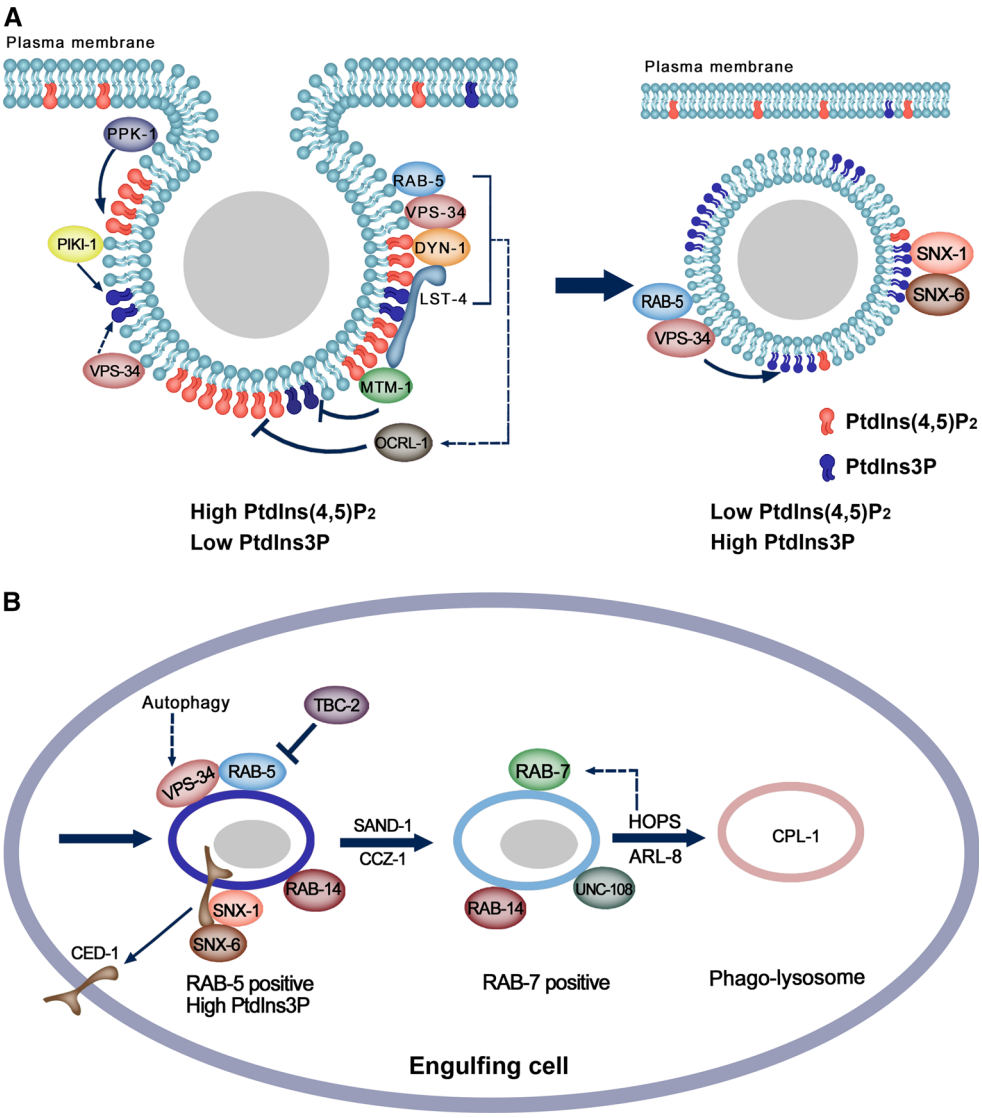

Fig. 4 Formation and maturation of apoptotic cell-containing phagosomes in *C. elegans*. Stepwise regulation of phagosome formation (**a**) and maturation (**b**) is shown. Activating and inhibitory effects are designated by *down arrow* and *perpendicular symbol*, respectively. *Dashed arrows* indicate the proposed activation or recruitment that requires further investigation

PtdIns3P and its effectors

PtdIns3P is a key marker and maturation determinant of early phagosomes. The class III and II PI3 kinases VPS-34 and PIKI-1 coordinate to regulate PtdIns3P generation and accumulation on phagosomes [145, 146]. PIKI-1 associates with extending pseudopods and nascent phagosomes where it acts with MTM-1 to control PtdIns3P levels for phagosomal sealing [140] (Fig. 4a). VPS-34 contributes to PtdIns3P generation at the sealing stage, but probably plays a major role in producing PtdIs3P on sealed phagosomes as both MTM-1 and PIKI-1 are released at this stage [140] (Fig. 4). Autophagy may mediate in part the regulation of phagosomal PtdIns3P by VPS-34, thus contributing to the clearance of apoptotic cells [146, 147]. PIKI-1 and VPS-34 are reported to regulate phagosome maturation by acting sequentially to control PtdIns3P oscillation on phagosomes, a process antagonized by MTM-1 [145]. PtdIns3P waves, however, appear to associate with clearance of specific corpses (C1, C2 and C3) and do not involve functions of MTM-1 [140]. Deciphering how PtdIns3P oscillation contributes to cell corpse clearance requires further investigation.

As a mediator of membrane trafficking, PtdIns3P regulates phagosome maturation through its effectors. LST-4 is reported as a PtdIns3P effector that promotes phagosome maturation [142]. However, we found that LST-4 associates with PtdIns(4,5)P_2-enriched unsealed phagosomes and is released before PtdIns3P accumulates. LST-4 binds to PtdIns(4,5)P_2-liposomes more efficiently than PtdIns3P-liposomes and loss of LST-4 blocks phagosomal sealing and PtdIns3P accumulation [140]. Thus, LST-4 plays a key role at the scission stage before phagosome maturation starts. SNX-1 and SNX-6, two essential subunits of the retromer sorting nexin dimer, promote cell corpse clearance by mediating recycling of the CED-1 receptor [148]. Loss of retromer inhibits recycling of CED-1 from the phagosome to the cell membrane, causing lysosomal degradation of the receptor [148]. SNX-1 associates with phagosomes after MTM-1 is released, suggesting that SNX-1 acts after phagosome sealing (unpublished data). As PX-BAR domain-containing sorting nexins, SNX-1, and SNX-6 may act as PtdIns3P-binding effectors to mediate CED-1 recycling and regulate phagosome maturation [142, 148] (Fig. 4).

Rab GTPases

Maturation of apoptotic cell-containing phagosomes involves sequential actions of at least four Rab GTPases (Fig. 4b). RAB-5 associates with early phagosomes to promote PtdIns3P generation, probably by activating VPS-34 [149] (Fig. 4b). RAB-5 recruitment might be mediated through direct interactions with VPS-34 and DYN-1 [149], but more work is needed to reveal how this occurs on phagosomes and whether other recruitment mechanisms are involved. How RAB-5 is activated on phagosomes remains unclear. The GTPase activating protein TBC-2 inactivates RAB-5 to release it from phagosomal membranes, thereby promoting progression of phagosome maturation through the RAB-5-positive stage [150] (Fig. 4b). SAND-1 (Mon1) and CCZ-1 (Ccz1) function downstream of RAB-5 to regulate phagosome maturation, probably by promoting phagosome progression from the RAB-5-positive stage to the RAB-7-positive stage [151, 152] (Fig. 4b). Mammalian Mon1 interacts with GTP-bound Rab5, while the Mon1–Ccz1 complex binds Rab7 and may influence Rab7 activation, suggesting that Mon1–Ccz1 may function to promote the Rab5 to Rab7 transition [151]. Whether SAND-1-CCZ-1 utilizes a similar mechanism to regulate phagosome progression remains to be determined. RAB-7 functions at late maturation stages to promote phagolysosome formation [149, 153]. HOPS complex components may mediate fusion between phagosomes and lysosomes by acting downstream of RAB-7, but whether any of them act directly as RAB-7 effectors remains unclear [149, 154]. ARL-8, an Arf-like small GTPase, also contributes to phagolysosome formation and its recruitment to phagosomes requires RAB-7 and HOPS complex components [155] (Fig. 4b). UNC-108 (Rab2) and RAB-14 (Rab14) function downstream of RAB-5 activation to regulate phagosome acidification and phagolysosome formation in a redundant manner [156–158] (Fig. 4b). RAB-14, UNC-108 and RAB-7 function in sequential steps to regulate phagolysosome formation: RAB-14 and UNC-108 recruit lysosomes, whereas RAB-7 mediates docking and fusion of lysosomes with phagosomes [158]. As effectors of UNC-108 and RAB-14 have not been identified to date, it is unclear how they act to regulate phagosomal maturation. By fusing with lysosomes, phagosomes acquire a variety of lysosomal hydrolases for destruction of apoptotic cells. Interestingly, loss of CPL-1, the *C. elegans* cathepsin L, causes defective degradation of cell corpses in phagolysosomes, whereas inactivation of other lysosomal proteases individually does not obviously impair cell corpse clearance [159]. Thus, lysosomal proteases do not contribute equally to cell corpse degradation. CPL-1 may play a leading role in degrading or initiating the degradation of apoptotic cells, whereas other proteases may act coordinately to digest cell corpse debris.

Removal of non-apoptotic targets by the cell corpse clearance machinery

In addition to cell corpses, non-apoptotic targets are found to be removed by the apoptotic cell clearance machinery

including the residual body generated during spermatogenesis and the midbody formed during cell division. Both structures may expose PtdSer on their surfaces and are recognized and engulfed by the same molecular machinery that removes apoptotic cells [160, 161]. Residual body-containing phagosomes undergo a similar maturation process as in cell corpse degradation, resulting in the final destruction of residual bodies in phagolysosomes. Efficient removal of residual bodies is essential for sperm quantity and transfer, indicating the functional importance of the cell corpse clearance pathway in this process [160].

Perspective

Our understanding of the molecular machinery that governs cell death specification, execution, and cell corpse clearance in *C. elegans* has grown rapidly in the past decades. Nonetheless, many important questions remain to be answered. For example, what are the death signals and the transcription regulators that activate the cell death initiator to trigger apoptosis of distinct cell types during animal development? In cell corpse clearance, are there other "eat me" signals in addition to PtdSer? Are there "do not eat me" or "find me" signals that help to distinguish between live and dying cells in worms? How is the metabolism of PtdIns3P, which transiently accumulates on fully sealed phagosomes, controlled during phagosome maturation? What are the effectors of PtdIns3P and Rab GTPases that drive phagosome maturation? Future investigations are also needed to understand how lysosomal enzymes (proteases, lipases, nucleases, glycosidases, phosphatases) coordinate to fully destroy cell corpses and how lysosomes are reformed from phagolysosomes. Notably, new types of developmental cell death that are non-canonical, yet evolutionarily conserved have been recently uncovered in *C. elegans* [92, 95] and non-apoptotic targets removed by the cell corpse clearance pathway have been identified. Thus, broader roles of cell death and cell corpse clearance in animal development and defense will be revealed. Future studies on *C. elegans* will certainly deepen our understanding of this key process as always.

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