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COMMENTARY

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# How are necrotic cells recognized by their predators?

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**ABSTRACT**

Necrosis is a type of cell death often caused by cell injury and is linked to human diseases including neuron degeneration, stroke, and cancer. Cells undergoing necrosis are engulfed and degraded by engulfing cells, their predators. The mechanisms by which necrotic cells are recognized and removed remain elusive. Here we comment on our recent findings that reveal new molecular mechanisms of necrotic-cell recognition. Through studying the *C. elegans* touch neurons undergoing excitotoxic necrosis, we identified a receptor/ligand pair that enables engulfing cells to recognize necrotic neurons. The phagocytic receptor CED-1 is activated through interaction with its ligand phosphatidylserine (PS), exposed on the surface of necrotic cells. Furthermore, against the common belief that necrotic cells have ruptured plasma membrane, we found that necrotic *C. elegans* touch neurons actively present PS on their outer surfaces while maintaining plasma membrane integrity. We further identified 2 mechanisms governing the presentation of PS, one of which is shared with cells undergoing apoptosis, a "cell suicide" event, whereas the other is unique to necrotic neurons. The influx of $Ca^{2+}$, a key necrosis-triggering factor, is implicated in activating a neuronal PS-scramblase for PS exposure. We propose that the mechanisms controlling PS-exposure and necrotic-cell recognition by engulfing cells are likely conserved from worms to humans.

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## Introduction

Necrosis and apoptosis are 2 morphologically distinct types of cell death events. Whereas apoptotic cells shrink in size and condense in chromatin, necrotic cells swell to many folds of their original sizes.[1,2] Necrosis is most frequently observed during cell injury, and is closely associated with diseases such as stroke, neurodegeneration, chronic inflammation, and cancer.[3–7] Recent discoveries made in multiple organisms demonstrated that cells possess genetic pathways that specifically trigger necrosis in response to extracellular or intracellular stimuli.[8–11] Unlike apoptosis, known necrosis-triggering pathways appear to be independent of the activities of caspases, which are cysteine proteases.[10,12] Despite the different mechanisms of death, both necrotic and apoptotic cells are engulfed by phagocytes and degraded inside phagosomes.[13,14] Efficient clearance of necrotic cells from animal bodies helps to resolve the wounded area, and is further essential for reducing harmful inflammatory and auto-immune responses induced by the contents of necrotic cells.[14,15]

Pioneering researchers have established the nematode *Caenorhabditis elegans* as an effective model system for investigating the mechanisms of apoptosis and necrosis.[10,16,17] In *C. elegans*, a number of mutations in certain ion channel subunits belonging to the DEG/ENaC superfamily and referred to as degenerins, in the acetylcholine receptor, in trimeric GTPases, and in a few other proteins induce specific neurons to undergo necrotic cell death that mimics the excitotoxic necrosis, which occurs during stroke, trauma, and neurodegenerative disorders in humans.[10] In particular, dominant (*dm*) mutations in *mec-4*, which encodes a core subunit of a multimeric, mechanically gated sodium channel specifically expressed in the touch neurons, trigger the necrosis of 6 mechanosensory (touch) neurons required for sensing gentle mechanical stimuli along the body wall.[18–20] In *mec-4 (dm)* mutants, these dying neurons swell to many

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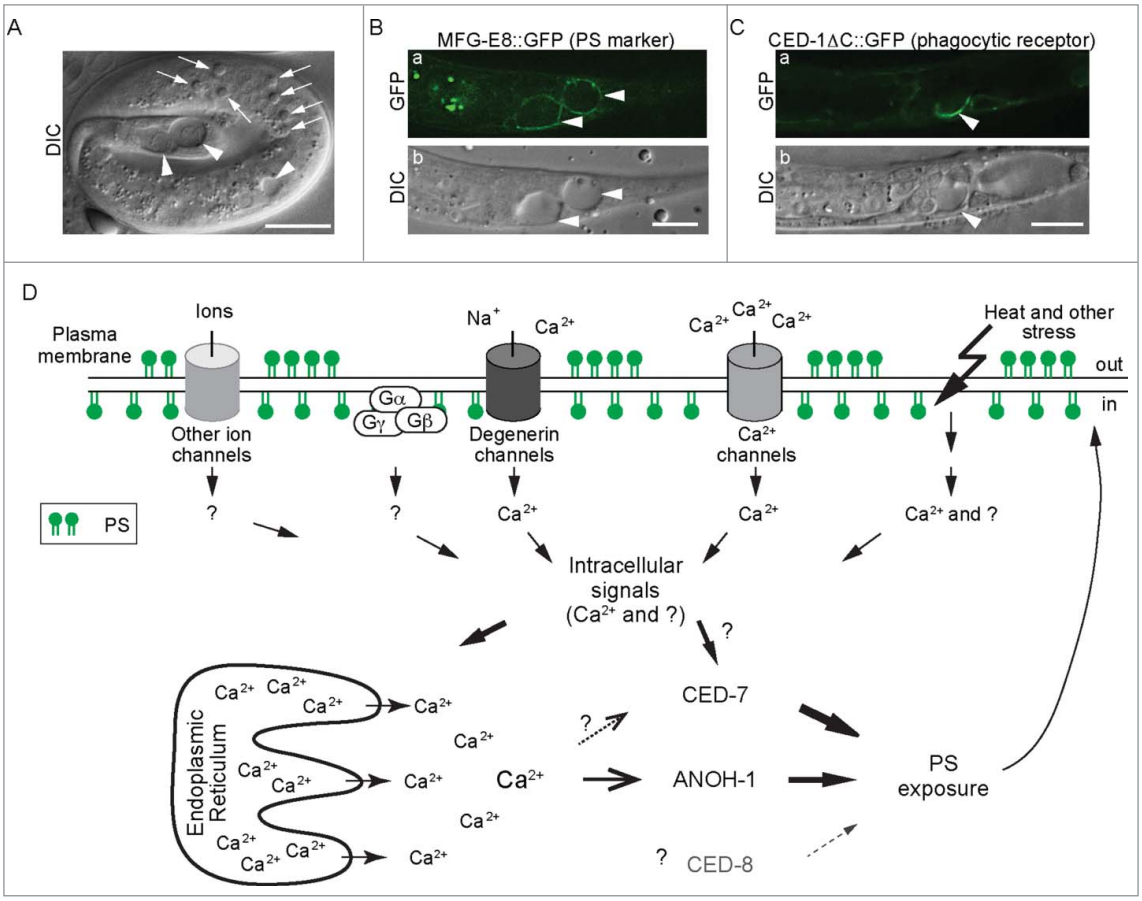


**Figure 1.** Phosphatidylserine (PS) is actively presented on the surface of necrotic neurons and attracts engulfing cells (A-C) DIC and epi-fluorescence images of *ced-1(e1735); mec-4(e1611)* mutant embryo (A) and L1 larvae (B and C). Arrows mark apoptotic cells. Arrowheads mark necrotic touch neurons PLML and PLMR. Scale bars indicate 5 $\mu$m (A) and 10 $\mu$m (B and C), respectively. (D) Diagram depicting our proposed mechanisms of PS exposure out of a necrotic neuron. See text for detailed explanation of the models. The cylinders represent various types of ion channels, which are made constitutively open by dominant mutations in certain subunits. A dominant mutation of the G$\alpha$ subunit of the trimeric G-protein also induces neuronal necrosis. Heat and other necrosis inducing stress are also indicated. $Ca^{2+}$ influx is a prominent trigger that activates the PS-exposure mechanisms through inducing $Ca^{2+}$ release from the ER and probably also through other unknown mediators. In addition, there might be other intracellular signals (short, solid arrows) triggered by the necrotic stress. The thickness of the long solid lines bearing solid arrows represents the relative contribution of each of CED-7 and ANOH-1 to the overall PS exposure activity. The solid line bearing an open arrow indicates activation of ANOH-1 by $Ca^{2+}$. The dashed line bearing open arrows indicate $Ca^{2+}$ might activate CED-7. Although CED-8 participates in necrotic cell-removal, whether it acts to facilitate the PS-exposure is unknown (represented by the dashed straight line bearing a solid arrow). All three proteins should function on the plasma membrane.

times their original sizes and are easily distinguishable from living or apoptotic cells under Differential Interference Contrast (DIC) optics by their giant sizes (Fig. 1A).[13,18] Unlike apoptosis, *mec-4(dm)*-induced cell death does not require CED-3 caspase activity.[21] Instead, the MEC-4(dm) mutations result in hyperactive channel conductivity of $Na^+$ and $Ca^{2+}$ and induce touch neurons to undergo necrosis.[19,22]

Despite dying through different mechanisms, the engulfment of both necrotic and apoptotic cells by their neighboring cells requires the functions of the same 7 *ced* (cell death defective) genes,[23] indicating the presence of certain common dying cell-removal mechanisms. On the other hand, the distinct cellular features observed during macrophage engulfment of necrotic mammalian cells[24] imply that unique pathways exist to clear necrotic and apoptotic cells.

Phosphatidylserine (PS), a membrane phospholipid, is known as an "eat me" signal presented on the surface of apoptotic cells and is recognized by phagocytic receptors such as *C. elegans* CED-1, *Drosophila* Draper, and mammalian Tim4 and BAI1, leading to the initiation of their engulfment.[25–29] In living cells, PS is almost exclusively localized to the inner leaflet of the plasma membrane, at least partially due to an ATP-dependent aminophospholipid translocase

activity that selectively returns PS from the outer to the inner leaflet.[30–32] During the early stage of apoptosis, PS is detected on the outer leaflet, suggesting a process of trans-bilayer redistribution.[30,31] Phospholipid scramblases, by catalyzing the random, bi-directional "flip-flop" of phospholipids across the membrane bilayer, could potentially counter the aminophospholipd translocase activity.[33] Indeed, recently, it was found that the mouse Xk-related protein 8, a phospholipid scamblase, and CED-8, its *C. elegans* homolog, mediate PS exposure during apoptosis.[34,35] Both Xk8 and CED-8 were found to be activated by caspase cleavage.[34,35] On the other hand, the mouse transmembrane protein 16F (TMEM16F), which was found to act as a novel $Ca^{2+}$-activated phospholipid scramblase,[36] does not seem to be involved in PS exposure on apoptotic cell surfaces.[37] These results suggest that different phospholipid scramblases might function in different cell types and respond to different stimuli. In addition to the scramblases, the mammalian ATP-binding-cassette transporter A1 (ABCA1) has been implicated in the translocation of PS from the inner to the outer leaflet,[38,39] although evidence to the contrary also exists.[40]

Previously, using a GFP-tagged, secreted PS reporter (MFG-E8::GFP), we have detected the presentation of PS specifically on the surface of apoptotic cells during *C. elegans* development.[41] We have further identified 2 alternative mechanisms that promote PS exposure in apoptotic somatic and germ cells, respectively.[41] The PS exposure on apoptotic cell surface during embryonic development, which is necessary for their engulfment, relies on the function of *C. elegans* CED-7, a homolog of mammalian ABCA1 transporters.[41] Recently, we investigated how necrotic touch neurons in *C. elegans* are recognized and engulfed.[42] Specifically, we addressed the following questions:

1. Do necrotic and apoptotic cells share common "eat me" signal molecules and are they recognized by the same phagocytic receptors?
2. Do necrotic cells actively externalize the "eat me" signal molecules to their surface? And, if the answer is yes, is (are) the PS-externalization mechanism(s) similar to that discovered in apoptotic cells?
3. Do different PS-exposure mechanisms work together to facilitate the removal of dying cells?
4. Are the PS-exposure mechanisms general or specific responding to different death triggering signals and in different cell types?

In this commentary, we will discuss our findings in the context of current understanding of necrotic cell clearance. We propose that Calcium-dependent PS exposure is a necrosis-specific mechanism employed by multiple types of cells. Moreover, multiple molecular mechanisms cooperate to regulate PS exposure in response to necrosis signals.

## *PS is an "eat me"signal exposed on the surface of necrotic touch neurons and recognized by the phagocytic receptor CED-1*

Using the secreted MFG-E8::GFP reporter, a high-affinity PS-binding protein that specifically detects PS on the outer surface of cells, we detected strong PS signal on the surface of necrotic but not live touch neurons in the *mec-4(dm)* mutant background (Fig. 1B).[42] Using a live-cell imaging protocol that we established for touch neurons, we monitored the accumulation of the PS signal on surface of necrotic touch neurons. We found that the exposure of PS was an early event detectable approximately 30–90 min after the initiation of cell morphology change was visible under the DIC optics.[42] The fact that both necrotic and apoptotic cells expose PS on their surfaces implies that PS might serve as a common "eat me" signal to attract engulfing cells to cells that die of different mechanisms. Supporting this theme, we discovered the novel function of phagocytic receptor CED-1 in recognizing necrotic cells in addition to apoptotic cells (Fig. 1C).[42] The extracellular domain of CED-1 (CED-1Ex) is capable of attaching to the surface of necrotic cells *in vivo*, suggesting an extracellular ligand-receptor interaction. Furthermore, we have detected direct and selective *in vitro* interaction between CED-1Ex and acidic phopsholipids, including PS. Most importantly, as shown by Chung et al.[23] and confirmed by us,[42] loss-of-function mutations of *ced-1* result in severe defect in the removal of necrotic touch neurons as well as apoptotic cells. As a result, these kinds of dying cells are retained in the body. Together, the above results strongly indicate that CED-1 acts as a phagocytic receptor that recognizes both apoptotic and necrotic cells.

CED-1 belongs to a family of homologous transmembrane proteins that include *Drosophila* Draper, mouse Jedi and mEGF10, and human mEGF10 and 11.[43–45] Draper, like CED-1, directly associates with PS exposed to the surface of apoptotic cells.[29] Draper acts as a phagocytic receptor for both apoptotic cells and for injured or pruned neuronal processes.[46] Likewise, mouse Jedi and mEGF10 as well as human mEGF10 also participate in the recognition of apoptotic cells.[47,44,48] CED-1 and its homologs thus play conserved roles as phagocytic receptors in different organisms. Our discovery of CED-1 as a phagocytic receptor for necrotic cells implies that the homologs of CED-1 might also recognize necrotic cells.

As an "eat me" signal, PS is also known to attract phagocytic receptors via an indirect mechanism. Secreted bridging molecules such as mouse MFG-E8 bring dying and engulfing cells together by interacting simultaneously with both PS and phagocytic receptors.[49] *C. elegans* TTR-52, a transthyretin-like secreted protein, was proposed to act as a bridging molecule that links PS on apoptotic cells to CED-1 on engulfing cell surfaces.[50] We propose that the direct and indirect interactions between PS and CED-1 provide 2 distinct and probably complementing molecular mechanisms to promote the effective recognition of dying cells by CED-1.

## PS is actively presented on the surface of necrotic touch neurons through 2 distinct mechanisms

### Necrotic touch neurons do not seem to rupture

According to a long-existing notion, injured cells undergo necrosis and lose plasma membrane integrity.[51–53] Thus comes the general assumption that the PS detected on necrotic cell surfaces is a result of the rupture of necrotic cell membranes.[53] In the recent years, accumulating evidence has demonstrated that in addition to cell injury, necrosis is induced by genetic programs.[8] In short, multiple molecular mechanisms exist that induce and execute necrosis;[8,12] thus all necrotic cells do not necessarily lose plasma membrane integrity. In addition, the observed loss of membrane integrity of necrotic cells in culture, where they are not attacked by phagocytes, does not necessarily represent what happens inside animal bodies, where engulfing cells target dying cells at early stages of their death.[13,42] Previously, those necrosis events that occur inside animal bodies were rarely examined for plasma membrane integrity. We monitored the subcellular localization of particular GFP or mRFP reporters expressed either inside necrotic cells or outside necrotic cells as secreted proteins, as well as propidium iodide, a small molecule dye that is not plasma membrane permeable, within a 36-hr observation period starting 3 hrs after necrosis was initiated. We did not observe any internalization of the secreted GFP molecules expressed from cells neighboring the necrotic touch neurons or of propidium iodide.[42] We also failed to observe any externalization of necrotic cell-expressed GFP or mRFP signals.[42] These results indicate that the touch neurons undergoing excitotoxic necrosis maintain plasma membrane integrity during a long period of time - throughout embryonic and larval development. Our observation is consistent with electron microscopic studies of rat brains and *C. elegans* touch neurons, which reported the swelling of necrotic cells and the presence of surrounding phagocytes, yet no loss of plasma membrane integrity.[54,13] These results indicate that the common notion that necrotic cells lose plasma membrane integrity is not necessarily true for all kinds of necrotic cells, in particular in the developmental context, in which the dying cells are swiftly engulfed. They further imply that in order for PS to be present on the surface of necrotic touch neurons, an active PS exposure mechanism(s) must exist. Our genetic studies revealed at least 2 separate PS-exposure activities that promote PS exposure on the surface of necrotic cells.

### A common PS-exposure mechanism employed by both apoptotic and necrotic cells

To identify the molecular mechanism(s) that drives PS exposure, we first tested the function of *C. elegans* CED-7, a member of the ABC transporter family.[55] CED-7 regulates PS exposure on the surface of apoptotic cells.[41] Mouse ABCA1 was also reported to participate in PS redistribution during apoptotic cell clearance.[38,56] We found that inactivation of *ced-7* greatly reduces the frequency as well as intensity of the PS signal detected on necrotic cell surface.[42] Furthermore, engineered tissue-specific expression of *ced-7* in touch neurons but not in engulfing cells rescues this defect, indicating that CED-7 functions cell-autonomously to promote PS exposure.[42] This discovery, together with the previous one revealing a key role of CED-7 in promoting PS exposure on the surface of apoptotic cells,[41] further demonstrate that the

presentation of the "eat me" signal relies on conserved mechanism(s) during different types of cell death. We further discovered that CED-7 has 2 distinct functions, one in necrotic and the other in engulfing cells, both of which contribute to the efficient removal of necrotic touch neurons.[42]

How CED-7 acts in necrotic cells to promote PS exposure needs to be further elucidated. CED-7 is ubiquitously expressed.[55] There thus must be dying cell-specific mechanisms that activate CED-7. Whether the CED-7 activation mechanisms are common or distinct in necrotic and apoptotic cells remains unknown. Moreover, the engulfing cell-specific function of CED-7 is a mystery and requires further investigation. Previous research suggests that engulfing cells might also externalize PS and that ABC transporters might be involved in this event.[38,57] The function of this event awaits clarification.

### A distinct, touch neuron-specific, PS-exposure mechanism

In *ced-7* null mutants, PS was still detectable, albeit at lower levels, on the surface of necrotic touch neurons, indicating the presence of an additional PS exposure-promoting activity.[42] We found that inactivating *anoh-1* (*ano*ctamin *h*omolog *1*), which encoded the *C. elegans* homolog of mammalian TMEM16F, greatly reduced the PS signal on necrotic cell surface.[42] We further found that ANOH-1 functioned in necrotic touch neurons to promote PS exposure.[42] Consistently, ANOH-1 is primarily expressed in neurons, including touch neurons.[42,58]

The vertebrates TMEM16 family of proteins, also known as anoctamins, are divided into 2 subfamilies based on 2 distinct $Ca^{2+}$-dependent biochemical activities: $Cl^-$ channels and phospholipid scramblases.[37,59] Remarkably, TMEM16F possesses both biochemical activities.[36,60–64] Mammalian TMEM16F promotes cellular PS exposure in response to $Ca^{2+}$ ionophore[60,62–64] yet not to apoptotic stimuli.[37] Similarly, we failed to observe any defect in the removal of apoptotic cells in *C. elegans* embryos and adult gonads in *anoh-1* null mutants,.[42] This observation suggests either that *anoh-1* is not involved in promoting PS exposure on the surface of apoptotic cells, or that *anoh-1* is involved but only makes a minor contribution. In the latter case, in *anoh-1* mutants, PS level on the surface of apoptotic cells might be reduced yet is still sufficiently high to promote engulfment.

On the other hand, the $Ca^{2+}$-activated phospholipid scramblase activity of TMEM16F provides an important clue toward revealing a necrosis-specific PS-exposure mechanism. As an evolutionarily conserved feature, $Ca^{2+}$ influx is known to be an effective trigger of the excitotoxic death of neurons in metazoan organisms including *C. elegans* and mammals.[65–67] Particularly in *C. elegans* touch neurons, the dominant mutation in MEC-4 leads to $Ca^{2+}$ influx, resulting in the excitotoxic necrosis.[19,22] We propose that during $Ca^{2+}$-activated necrosis of touch neurons, $Ca^{2+}$ directly activates the PS-exposure activity of ANOH-1. Xu et al discovered that necrotic cell death under a number of excitotoxic conditions requires the release of $Ca^{2+}$ from the endoplasmic reticulum (ER).[65] ER is a known calcium storage pool in cells.[68,69] Moreover, the influx of $Ca^{2+}$ from the extracellular environment is known to further induce the release of $Ca^{2+}$ from the lumen of the ER to the cytoplasm.[70,71] We propose that, like *mec-4(dm)*-induced necrosis, the *mec-4(dm)*-induced PS exposure on touch neuron surface also requires the $Ca^{2+}$-release from the ER, an event that further increases the concentration of $Ca^{2+}$ in the cytoplasm and facilitates the activation of ANOH-1 (Fig. 1D). Further development based on our findings will establish a novel $Ca^{2+}$-dependent mechanism leading to the exposure of the "eat me" signal molecules and the recognition of necrotic touch neurons.

### The Calcium-dependent PS exposure might be a necrosis-specific mechanism employed by multiple types of neurons and even non-neuronal cells

The $Ca^{2+}$-dependent PS-exposure mechanism we discovered in touch neurons in the *mec-4(dm)* mutant background might apply to the necrosis induced by genetic alterations of other degenerin family channel proteins,[72] or of other types of channel proteins (Fig. 1D). For instance, a dominant mutation in DEG-3, an acetylcholine-gated $Ca^{2+}$ channel that displays high permeability to $Ca^{2+}$, results in the necrosis of touch cells.[20,73] The constitutive $Ca^{2+}$ influx in the *deg-3* mutant background could activate ANOH-1 for PS exposure. In support of this hypothesis, we detected the exposure of PS on the surface of necrotic touch neurons in *deg-3* mutants.[42]

In addition to touch neurons, other types of neurons are also induced to undergo excitotoxtic necrosis by hyper-influx of $Ca^{2+}$. For example, certain

interneurons and sensory neurons are induced to undergo necrosis by the same dominant mutations in *deg-3*.[20,73] Dominant mutation in *deg-1* and *unc-8*, both of which encode degenerin ion channels, induce the necrosis of interneurons[74] and motor neurons,[75] respectively. Furthermore, hyperactive channels rely on trimeric G proteins to signal downstream. Expression of an active Gα subunit (*gsa-1(QL)*) results in the necrosis of motor neurons.[76] The necrosis induced by the hyperactive mutant Gα protein requires the release of $Ca^{2+}$ from ER,[65] implying increased $Ca^{2+}$ concentration in the cytoplasm. Consistently, besides touch neurons, the expression of ANOH-1 is also detected in many other neurons. Combining these lines of evidence together, we speculate that multiple types of neurons rely on ANOH-1, which is activated by $Ca^{2+}$, to promote PS exposure during necrosis (Fig. 1D).

Besides excitotoxic signal generated by hyperactive channels or downstream signaling molecules, necrosis of neurons is also inducible by other kinds of stress, such as heat stroke, hypoxia, and hypo-osmotic shock.[77] In *C. elegans*, heat treatment induces widespread necrosis in many cells, including neurons.[78] As heat treatment was detected to increase cytoplasmic $Ca^{2+}$ concentration, and as moderating $Ca^{2+}$ release from intracellular organelles protects heat-induced necrosis, Kourtis et al. proposed that heat stroke might induce necrosis through increasing cytoplasmic $Ca^{2+}$ concentration.[78] Heat-induced necrotic cells might thus also utilize a $Ca^{2+}$-activated phospholipid scramblase such as ANOH-1 to expose PS on their surface (Fig. 1D). Moreover, the study of heat-induced necrosis also suggests that the $Ca^{2+}$-induced PS exposure mechanism might not be limited to neurons.[78] Although ANOH-1 expression is primarily observed in neurons, non-neuronal cells might utilize other putative phospholipid scramblases[41] that response to necrotic signals.

### *Multiple molecular mechanisms cooperate to regulate PS exposure in response to necrosis signals*

We discovered that the *anoh-1(-) ced-7(-)* double mutants display more severe defects in PS-exposure and necrotic cell-removal than each single mutant alone.[42] Since each mutant allele analyzed is a null allele, according to the principle of epistasis grouping analysis, a further enhanced phenotype of the double mutants over the stronger single mutant phenotype indicates that the 2 genes being tested act in separate pathways. ANOH-1 and CED-7 are likely to both contribute to the overall PS-exposure activity. We propose that they act in 2 independent and partially redundant pathways (Fig. 1D). Our discoveries have further established that cells die of different mechanisms employ both common (ABC transporter-based) and unique (TMEM16F-like phospholipid scramblase-based) molecular activities to present a common "eat me" signal. Assuming that a certain level of PS is needed to efficiently attract phagocytic receptor molecules, since a necrotic *C. elegans* touch neuron possesses a surface area many times of that of an apoptotic cell, the robust and timely exposure of PS on the large surface area might rely on the cooperation of multiple molecular activities such as those represented by CED-7 and ANOH-1. Currently, it is unknown whether CED-7 or mouse ABCA1 is activated by $Ca^{2+}$. The possibility exists that in necrotic neurons, $Ca^{2+}$ influx might enhance the activity of CED-7. We further found that CED-8, a homolog of the mammalian phospholipid scramblase Xk8,[34,35] also made a modest contribution to the removal of necrotic cells: *ced-8* loss-of-function mutation results in a transient delay of necrotic touch neuron removal in the L1 but not later larval stages.[42] Comparing to the phenotypes displayed by *ced-7* or *anoh-1* mutants, this phenotype is much weaker and transient. We also found that *ced-8* and *anoh-1* acted in 2 independent pathways to promote necrotic cell removal.[42] Currently, it is unknown whether CED-8 facilitates PS-exposure to the surface of necrotic cells; moreover, the functional relationship between *ced-7* and *ced-8* in the context of necrotic cell removal remains to be elucidated. CED-8 might represent a third pathway that is in parallel to both the CED-7 and the ANOH-1 pathways (Fig. 1D).

$Ca^{2+}$ homeostasis is closely associated with neuron degeneration conditions in both *C. elegans* and mammals.[69,77] Our findings have a broader implication in the physiological role of the clearance of many kinds of degenerative neurons resulted from pathological conditions or aging.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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