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## Caspase-mediated activation of *Caenorhabditis elegans* CED-8 promotes apoptosis and phosphatidylserine externalisation

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### Abstract

During apoptosis, phosphatidylserine (PS), normally restricted to the inner leaflet of the plasma membrane, is exposed on the surface of apoptotic cells and serves as an “eat-me” signal to trigger phagocytosis. It is poorly understood how PS exposure is activated in apoptotic cells. Here we report that CED-8, a *C. elegans* protein implicated in controlling the kinetics of apoptosis and a homolog of the XK family proteins, is a substrate of the CED-3 caspase. Cleavage of CED-8 by CED-3 activates its proapoptotic function and generates a carboxyl terminal cleavage product, acCED-8, that promotes PS externalization in apoptotic cells and can induce ectopic PS exposure in living cells. Consistent with its role in promoting PS externalization in apoptotic cells, *ced-8* is important for cell corpse engulfment in *C. elegans*. Our finding identifies a crucial link between caspase activation and PS externalization, which triggers phagocytosis of apoptotic cells.

### Introduction

Phospholipids are major components of the plasma membrane and are asymmetrically distributed in the inner and outer leaflets of plasma membrane, with phosphatidylcholine (PC) and sphingomyelin (SM) predominantly in the outer leaflet and phosphatidylserine (PS) and phosphatidylethanolamine (PE) in the inner leaflet of plasma membrane<sup>1</sup>. During apoptosis, the asymmetrical distribution of phospholipids is disrupted, leading to the flipping of PS to the outer leaflet of plasma membrane<sup>2,3</sup>. The externalized PS then serves as an “eat-me” signal to trigger removal of apoptotic cells<sup>4</sup>

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#### Author Contributions

Y.Z.C., J.M., E.S.L. and R.R.S. performed the experiments; Y.Z.C., J.M. and D.X. conceived and designed the experiments, analyzed and interpreted data, and wrote the manuscript.

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Caspases are a family of cysteine proteases that are activated during apoptosis to initiate multiple cell suicidal or disassembly events, including nuclear membrane breakdown, chromatin fragmentation, and mitochondria elimination<sup>5</sup>. They promote cell death execution by cleaving and activating proapoptotic caspase substrates or inactivating anti-apoptotic caspase substrates<sup>6</sup>. How PS is externalized during apoptosis and whether caspases play a direct role in activating PS externalization are poorly understood and have been a longstanding issue in the field of apoptosis<sup>4,7</sup>. In the nematode *C. elegans*, the caspase-encoding gene *ced-3* is required for the death of almost all cells programmed to die<sup>8</sup>, but how the CED-3 caspase kills the cell through cleavage of its downstream protein substrates remains largely unknown. DRP-1, a dynamin-related GTPase, and DCR-1, a ribonuclease important for small RNA biogenesis, have been identified as two important CED-3 substrates that are activated by CED-3 cleavage to promote mitochondria elimination and to initiate chromosome fragmentation during apoptosis, respectively<sup>9,10</sup>. As in mammals, PS externalization occurs during *C. elegans* apoptosis<sup>11–13</sup> and is one of the earliest cell death events observed in worms<sup>14</sup>. In animals deficient in *ced-3*, few cells undergo apoptosis and PS is not externalized in cells that are programmed to die<sup>11,14</sup>. These observations suggest that CED-3 is required to activate the PS externalization process in apoptotic cells.

The *ced-8* gene was first identified as a gene important for controlling the kinetics of apoptosis in *C. elegans*<sup>15</sup>. In *ced-8* loss-of-function (*lf*) mutants, the numbers of cell corpses in early embryonic stages are markedly reduced and more apoptotic cells appear in late embryonic stages and in the L1 larval stage, suggesting a delay of early embryonic cell deaths to later embryonic stages<sup>15</sup>. *ced-8* encodes a protein with sequence similarity to the human XK membrane transporter<sup>15</sup>. Loss of human XK has been associated with the McLeod syndrome, a hereditary disease characterized by abnormalities in the neuromuscular and hematopoietic systems, including acanthocytic red blood cells, loss of erythrocyte Kx antigen, and reduction of Kell antigens<sup>16–18</sup>. The mechanism by which *ced-8* affects apoptosis is unknown, but genetic analysis suggests that it functions downstream of or in parallel to the *C. elegans* cell death inhibitor *ced-9*<sup>15</sup>.

In this study, we present evidence that CED-8 is a substrate of the CED-3 caspase and acts downstream of CED-3 to promote cell killing. Cleavage of CED-8 by CED-3 generates an activated cleavage product, acCED-8, that is necessary and sufficient to mediate the proapoptotic functions of CED-8. Moreover, we find that CED-8 is important for PS externalization in apoptotic cells and phagocytosis and that acCED-8 can induce ectopic PS exposure in living cells. Our study thus identifies an important caspase mediator that activates PS externalization in response to caspase activation.

## Results

### CED-8 is a substrate of the CED-3 caspase

In wild-type *C. elegans* embryos, most cell deaths occur during early embryogenesis (comma, 1.5-fold, and 2-fold embryonic stages) and less cell deaths are seen during late embryogenesis (3-fold and 4-fold embryonic stages; Fig. 1a). In a strong *lf ced-8(n1891)* mutant, the numbers of cell corpses in early embryonic stages are greatly reduced and significantly more apoptotic cells are found in late embryonic stages and in the L1 larval

stage<sup>15</sup>, suggesting a delay of embryonic cell deaths (Fig. 1a). To understand how *ced-8* acts to effect apoptosis, we first determined if *ced-8* acts downstream or upstream of the CED-3 caspase. We performed epistatic analysis of *ced-8* and *ced-3* using an integrated transgene (*smls111*) that expresses an activated form of CED-3 (acCED-3) under the control of the promoter of the *C. elegans* cell death initiator *egl-1*<sup>19</sup>. When *smls111* was placed in the *ced-1(e1735); ced-3(n2433)* mutant background, in which almost all naturally occurring cell deaths in *C. elegans* are blocked by a strong *lf ced-3(n2433)* mutation [*ced-1(e1735)* blocks cell corpse engulfment and sensitizes quantification of apoptotic cells<sup>20</sup>], acCED-3 still induced ectopic cell death and an average of 8 and 11 persistent cell corpses in twofold and four-fold stage embryos, respectively (Fig. 1b). The *ced-8(n1891)* mutation significantly reduced the number of ectopic cell deaths induced by acCED-3 in *ced-1(e1735) smls111; ced-3(n2433)* embryos (Fig. 1b), indicating that *ced-8* likely acts downstream of *ced-3* to promote apoptosis.

Because effectors of apoptosis that act downstream of caspases are often substrates of caspases<sup>6,9,10,21</sup>, we tested whether CED-8 is a substrate of the CED-3 protease. A glutathione S transferase CED-8 fusion (GST-CED-8) was synthesized and labeled with <sup>35</sup>S-Methionine in rabbit reticulocyte lysate and incubated with purified CED-3 protease<sup>22</sup>. <sup>35</sup>S-Met-labeled GST-CED-8 was cleaved by CED-3, yielding multiple cleavage products of approximately 51 kD, 45 kD, 34 kD, and 28 kD (Fig. 1c, lanes 1 and 2). Based on the sizes of the cleavage products and the known consensus CED-3 cleavage sites, D/EXXD, in which X can be any amino acid<sup>22</sup>, we inferred the locations of two principle CED-3 cleavage sites: between Asp 21 and Ala 22 and between Asp 163 and Gly 164. The amino acid sequences N-terminal to these two potential cleavage sites (P<sub>4</sub> to P<sub>1</sub>; EQED and ENTD) are consistent with the consensus CED-3 cleavage sites. When Asp 21 of CED-8 was replaced by Ala and the resulting mutant protein, GST-CED-8(D21A), was examined in the CED-3 cleavage assay, the 51 kD and 28 kD cleavage products expected from CED-3 cleavage at CED-8 Asp 21 were lost (Fig. 1c, lanes 3 and 4), confirming that CED-3 cleaves CED-8 at Asp 21. Similarly, substitution of CED-8 Asp 163 with Ala blocked the production of the 45 kD and 34 kD cleavage products expected from CED-3 cleavage at CED-8 Asp 163 (Fig. 1c, lanes 5 and 6). Moreover, substitutions of both Asp 21 and Asp 163 with Ala prevented cleavage of CED-8 by CED-3 (Fig. 1c, lanes 7 and 8), indicating that these are two major *in vitro* CED-3 cleavage sites. Analysis of the CED-8 protein sequence using the TOPCONS program (Consensus prediction of membrane protein topology) yields uniformed predictions that residues 1–34 are inside of the cell, whereas residues 145–218 are extracellular and correspond to the site of the extracellular disulfide linkage of XK to Kell<sup>16,23</sup>. Therefore, Asp 163 is unlikely accessible to CED-3 *in vivo*.

We examined whether CED-8 is cleaved by CED-3 in cells undergoing apoptosis using a yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) dual reporter assay. We generated a CFP fusion to the N-terminus of wild-type CED-8 (CFP::CED-8) and a YFP fusion to the N-terminus of CED-8(D21E) [YFP::CED-8(D21E)], which is resistant to CED-3 cleavage, because D21E blocks CED-3 cleavage of CED-8 as well as D21A (Supplementary Fig. S1). Both fusion proteins were expressed in *C. elegans* under the control of the native *ced-8* promoter. In living cells, both CFP::CED-8 and

YFP::CED-8(D21E) displayed identical plasma-membrane localization indistinguishable from that of CED-8::GFP reported previously (Fig. 1d and Supplementary Fig. S2a, b)<sup>15</sup>. In cells undergoing apoptosis (arrowheads, Fig. 1d), CFP was observed instead in the cytoplasm, suggesting that following CED-3 cleavage CFP disassociated from CED-8 localized on the plasma membrane and diffused into the cytoplasm. In comparison, YFP remained associated with CED-8(D21E) and localized to the plasma membrane in these apoptotic cells (Fig. 1d), indicating that CED-3 cleaves CED-8 at Asp 21 *in vivo*.

### CED-8 cleavage by CED-3 is critical for apoptosis

We next examined whether the two *in vitro* CED-3 cleavage sites in CED-8 are important for its proapoptotic function *in vivo*. We tested whether transgenes expressing wild-type or mutant forms of CED-8::GFP fusions under the control of the *ced-8* gene promoter can rescue the delay-of-cell-death defect of the *ced-8(n1891)* mutant at two signature embryonic stages, the early comma embryonic stage and the late 4-fold embryonic stage (Fig. 1a). Compared with wild-type embryos, which had an average of 9 cell corpses in the comma stage and few cell corpse in the 4-fold stage, *ced-8(n1891)* embryos had 1–2 cell corpses in the comma stage and an average of 15 cell corpses in the 4-fold embryonic stage (Fig. 1a)<sup>15</sup>. Transgenes expressing wild-type CED-8 [ $P_{ced-8}$ CED-8::GFP] or mutant CED-8(D163A) [ $P_{ced-8}$ CED-8(D163A)::GFP] fully rescued the delay-of-cell-death defect of the *ced-8(n1891)* mutant and restored wild-type cell corpse patterns to the *ced-8(n1891)* embryos (Fig. 1e), indicating that Asp 163 is not important for CED-8's proapoptotic function. By contrast,  $P_{ced-8}$ CED-8(D21A)::GFP did not rescue the delay-of-cell-death defect of the *ced-8(n1891)* mutant (Fig. 1f), even though the GFP intensity on the plasma membrane of  $P_{ced-8}$ CED-8(D21A)::GFP transgenic embryos was similar to that of  $P_{ced-8}$ CED-8::GFP or  $P_{ced-8}$ CED-8(D163A)::GFP embryos (Supplementary Fig. S2). This result indicates that CED-3 cleavage at the Asp 21 site is critical for CED-8's proapoptotic function *in vivo*. Finally, expression of the C-terminal cleavage fragment of CED-8 by CED-3 [ $P_{ced-8}$ CED-8(22-458)::GFP] also fully rescued the *ced-8(n1891)* mutant (Fig. 1f), indicating that CED-8(22-458), which we named acCED-8, is sufficient to mediate the proapoptotic function of CED-8 and represents the CED-3-activated form of CED-8.

### CED-8 is crucial for PS externalization in apoptotic cells

To probe how *ced-8* regulates apoptosis, we examined whether *ced-8* affects the PS externalization process, a hallmark of apoptosis<sup>1,2,7</sup>, and one of the earliest detectable cell death events in *C. elegans*<sup>14</sup>. We used a genetically encoded PS sensor, secreted Annexin V::GFP fusion (ANV::GFP)<sup>14</sup>, to monitor the PS externalization process. In early comma stage wild-type *C. elegans* embryos carrying an integrated transgene *smls76*, which expresses ANV::GFP under the control of the *C. elegans* heat-shock promoters<sup>14</sup>, an average of 9 cell corpses were observed (Fig. 1a) and almost all of them were strongly labeled by ANV::GFP (Fig. 2a, d). In comma stage *smls76; ced-8(n1891)* embryos, only a few cell corpses were observed and approximately 60% of them were labeled by ANV::GFP (Fig. 1a and Fig. 2b–d). However, in those apoptotic cells that were labeled, ANV::GFP labeling often was weak or barely detectable (Fig. 2c, e), with an average fluorescent intensity approximately 11% of that seen in apoptotic cells in wild-type embryos (Fig. 2e). These results indicate that loss of *ced-8* blocks or greatly reduces PS externalization in cells

that already died at this stage. Because the death of most cells programmed to die at the comma stage was deferred to later embryonic stages, we examined whether apoptotic cells observed in late stage *ced-8(n1891)* embryos were labeled by ANV::GFP. In 4-fold *ced-8(n1891)* embryos, there are an average of 15 cell corpses, compared with an average of 0.5 cell corpse in wild-type 4-fold embryos (Fig. 1a). However, only 5% of these apoptotic cells in *smIs76; ced-8(n1891)* 4-fold embryos were labeled by ANV::GFP (Fig. 2d), indicating that PS was not externalized in cells that died in late stage *ced-8(n1891)* embryos either. We obtained similar results using another genetically encoded PS sensor, secreted GFP::Lactadherin fusion (GFP::Lact)<sup>14</sup>. In *smIs434* animals, which carry an integrated transgene expressing GFP::Lact under the control of the heat-shock promoters<sup>14</sup>, *ced-8(n1891)* not only reduced the percentage of apoptotic cells labeled by GFP::Lact (Supplementary Fig. S3a), but also markedly decreased the intensity of GFP::Lact labeling on apoptotic cells (Supplementary Fig. S3b), providing further evidence that loss of *ced-8* greatly reduces PS externalization in apoptotic cells.

We then performed time-lapse microscopy to follow the PS externalization process in cells undergoing apoptosis. As reported previously<sup>14</sup>, in wild-type *C. elegans* embryos, ANV::GFP was detected on the surface of the dying cell before it adopted the typical button-like cell corpse morphology (Supplementary Fig. S4a, b). The intensity of ANV::GFP increased as the dying cell shrank and displayed the dense button-like morphology (Supplementary Fig. S4c), and reached its maximum around the time when it was engulfed by one of its neighbors (Supplementary Fig. S4d), which were also weakly labeled by ANV::GFP<sup>14</sup>. In the *ced-8(n1891)* embryo, ANV::GFP labeling of the apoptotic cell was much weaker (Supplementary Fig. S4h–n) and was first detected after the dying cell already adopted the button-like morphology (Supplementary Fig. S4h, i), the appearance of which was delayed compared with that in wild-type embryos (20 min versus 10 min in the N2 embryo; Supplementary Fig. S4c, i). The intensity of ANV::GFP on the apoptotic cell did not increase subsequently and the apoptotic cell persisted for a significantly longer time period (120 min versus 30 min in the N2 embryo; Supplementary Fig. S4c–g, i–n), which suggests a potential defect in cell corpse engulfment (see below). The observations from the time-lapse microscopy provide further support that *ced-8* plays a critical role in PS externalization in apoptotic cells.

It is possible that in *ced-8(n1891)* animals the cells programmed to die in early embryonic stages already initiated the suicide program and exposed PS, but had not yet adopted the button-like morphology. We thus quantified the total number of ANV::GFP rings in comma stage embryos regardless of their morphology and found that wild-type embryos had an average of 12 ANV::GFP rings, most of which displayed apoptotic cell morphology (Fig. 2f). By contrast, *ced-8(n1891)* comma stage embryos had an average of 1.5 ANV::GFP rings (Fig. 2f), confirming that most cells programmed to die at the comma stage did not expose PS at this stage. We further sensitized quantification of PS exposure in apoptotic cells by inactivating the *ced-7* gene, which encodes an ATP-binding cassette (ABC) transporter important for cell corpse engulfment<sup>24</sup> and promotes time-dependent efflux of exposed PS from the surface of apoptotic cells to the extracellular space through PS-containing small vesicles<sup>14,25</sup>. Loss of *ced-7* leads to retention of exposed PS on the surface



of aged or unengulfed apoptotic cells and thus sensitizes scoring of apoptotic cells with surface exposed PS. In comma stage *ced-7(n2094)* embryos, almost all apoptotic cells (97%) were labeled by ANV::GFP, while in 4-fold *ced-7(n2094)* embryos, 52% of unengulfed cell corpses still had surface exposed PS (Fig. 2d). In comparison, only 6% of unengulfed cell corpses in *ced-1(e1735)* 4-fold embryos have surface exposed PS, due to efflux and loss of exposed PS from apoptotic cells<sup>14</sup>. In *ced-7(n2094); ced-8(n1891)* embryos, 63% of cell corpses in the comma stage were labeled by ANV::GFP and only 11% of unengulfed cell corpses were labeled by ANV::GFP in late 4-fold embryos (Fig. 2d), which had the same number of unengulfed apoptotic cells as that in *ced-7(n2094)* 4-fold embryos (Fig. 4c). These observations confirm that loss of *ced-8* also significantly reduces PS exposure in unengulfed apoptotic cells. Taken together, these results indicate that *ced-8* is crucial for PS externalization in apoptotic cells.

### CED-8 is activated by CED-3 to promote PS externalization

We next examined whether CED-3 cleavage of CED-8 at the Asp 21 site is critical for CED-8 to promote PS externalization in apoptotic cells. Transgenes expressing wild-type or altered forms of CED-8 under the control of the *ced-8* gene promoter were introduced into *smls76; ced-8(n1891)* animals. The percentage of apoptotic cells labeled by ANV::GFP and the intensity of ANV::GFP labeling in comma-stage transgenic embryos were examined (Fig. 2g). Expression of wild-type CED-8 or acCED-8 in *smls76; ced-8(n1891)* embryos restored ANV::GFP labeling of apoptotic cells to the levels seen in *smls76* embryos (Fig. 2g), indicating full rescue of the PS externalization defect. By contrast, ANV::GFP labeling of apoptotic cells in *smls76; ced-8(n1891)* embryos expressing CED-8(D21E) was similar to that seen in *smls76; ced-8(n1891)* embryos (Fig. 2g), indicating no rescue of the PS externalization defect. These results indicate that CED-3 cleavage at the Asp 21 site is also critical for activating CED-8's function in promoting PS externalization in apoptotic cells.

Given that acCED-8 is necessary and sufficient to mediate the PS externalization activity of CED-8 in apoptotic cells, we examined whether acCED-8 can induce PS exposure in non-apoptotic cells. We induced global expression of acCED-8 under the control of the *C. elegans* heat-shock promoters ( $P_{hsp}$ acCED-8) and then examined PS exposure using GFP::Lact, which is more sensitive in detecting PS exposure in living cells than ANV::GFP (Supplementary Fig. S5)<sup>14,26</sup>. In *smls434* animals expressing GFP::Lact, GFP::Lact only labeled apoptotic cells (arrowheads, Fig. 3a). Expression of acCED-8 in *smls434* embryos resulted in GFP::Lact labeling of all cells, including both living cells and apoptotic cells (Fig. 3c), suggesting that acCED-8 can induce ectopic PS exposure on the surface of all cells. In comparison, expression of full-length CED-8 ( $P_{hsp}$ CED-8) in *smls434* embryos did not alter the staining pattern of GFP::Lact or cause widespread, ectopic PS exposure on the surface of all cells (Fig. 3b). In *ced-3(n2433); smls434* animals, where almost all apoptosis is blocked, expression of acCED-8, but not full-length CED-8, still caused all cells to externalize PS (Fig. 3d, e), indicating that acCED-8 is capable of activating PS externalization independent of the CED-3 caspase and apoptosis. These results suggest that CED-8, once activated by CED-3, promotes PS externalization in cells through the generation of acCED-8.

## CED-8 is important for cell corpse engulfment

Because surface-exposed PS is an important “eat-me” signal for phagocytosis<sup>2,4,27</sup> and because cell corpses in *ced-8(n1891)* embryos appear to persist significantly longer than those in wild-type embryos (Supplementary Fig. S4), we investigated whether *ced-8* affects cell corpse engulfment in *C. elegans*, in addition to its previously reported role in regulating the kinetics of cell death<sup>15</sup>. We first used four-dimensional microscopy to measure the duration of embryonic cell corpses in *ced-8(n1891)* animals<sup>28</sup>. In wild-type embryos, on average cell corpses persisted for 34 minutes. In *ced-8(n1891)* embryos, cell corpses persisted for 96 minutes, which is almost three times longer than that of wild-type animals (Fig. 4a). These results indicate that the cell corpse engulfment process is significantly compromised in the *ced-8(n1891)* mutant.

In *C. elegans*, two partially redundant pathways mediate cell corpse removal, with *ced-1*, *ced-6*, *ced-7*, and *ttr-52* genes functioning in one pathway and *ced-2*, *ced-5*, and *ced-12* genes acting in the other<sup>29–31</sup>. To interrogate the functioning pathway of *ced-8*, we constructed and analyzed double mutants between the *ced-8(n1891)* mutation and strong *lf* alleles of the above engulfment genes. *ced-8(n1891)* markedly enhanced the cell corpse engulfment defect of the *ced-2*, *ced-5*, or *ced-12* mutants at the late 4-fold embryonic stage and L1 larval stage (Fig. 4b), but only mildly enhanced the corpse engulfment defect of the *ced-1*, *ced-6*, *ced-7*, or *ttr-52* mutants at the L1 larval stage (Fig. 4c). These results are consistent with *ced-8* acting to promote PS externalization, which serves as an “eat-me” signal for both cell corpse engulfment pathways to promote phagocytosis of apoptotic cells (Fig. 5)<sup>26,28,31</sup>.

## Discussion

As a multipass plasma membrane protein, how CED-8 affects programmed cell death has been a major unresolved issue in *C. elegans* apoptosis. In this study, we demonstrate that CED-8 acts downstream of the CED-3 caspase to promote apoptosis and is a substrate of CED-3. Cleavage of CED-8 by CED-3 is required to activate CED-8’s proapoptotic functions and generates a C-terminal cleavage product, acCED-8, that is sufficient to mediate the proapoptotic functions of CED-8. Importantly, *ced-8* is also critical for PS externalization in apoptotic cells, which is an unexpected new role for CED-8. Cleavage of CED-8 by CED-3 is also required to activate its PS externalization activity. Moreover, ectopic expression of acCED-8, but not full-length CED-8, can induce PS externalization in all living cells in the absence of the CED-3 caspase or apoptosis. Our study thus identifies a crucial link between caspase activation and PS externalization<sup>4,27</sup>.

Consistent with its role in promoting PS externalization in apoptotic cells, we find that *ced-8* is important for cell corpse engulfment. Loss of *ced-8* causes persistence of embryonic cell corpses, mildly enhances the cell corpse engulfment defects of *ced-1*, *ced-6*, *ced-7*, and *ttr-52* mutants, and strongly enhances the cell corpse engulfment defects of *ced-2*, *ced-5* and *ced-12* mutants. Because PS could be an “eat-me” signal for both cell corpse engulfment pathways in *C. elegans*<sup>26,28,31</sup>, our observations are consistent with *ced-8* acting in both engulfment pathways to promote clearance of apoptotic cells (Fig. 5). However, given the significantly stronger effect of *ced-8(n1891)* in enhancing the engulfment defect of the *ced-2*

pathway and marginal effect in enhancing the engulfment defect of the *ced-1* pathway, *ced-8* may mainly act through the *ced-1*, *ced-6*, *ced-7*, and *ttr-52* engulfment pathway to promote phagocytosis. Since *ced-1* encodes a phagocyte receptor that recognizes and promotes engulfment of apoptotic cells with surface-exposed PS through its binding partner TTR-52, a PS-binding extracellular bridging protein<sup>31,32</sup>, our finding delineates a new apoptotic signaling cascade, starting from CED-3 caspase activation during apoptosis, followed by CED-3-mediated activation of CED-8, acCED-8-mediated activation of PS exposure on the surface of apoptotic cells, and subsequent recognition and removal of PS-exposed apoptotic cells by the TTR-52 and CED-1 phagocytosis pathway, which is likely aided by the *ced-2*, *ced-5*, and *ced-12* phagocytosis pathway, also capable of recognizing and engulfing apoptotic cells with surface-exposed PS (Fig. 5).

A new study reports a similar finding that a mammalian XK family protein, Xkr8, and its *C. elegans* homolog CED-8, play a conserved role in promoting PS exposure in apoptotic cells<sup>33</sup>. The PS externalizing activity of Xkr8 also appears to be activated by caspase cleavage. Interestingly, CED-8 is cleaved and activated by the CED-3 caspase at the N-terminus, whereas Xkr8 is cleaved and potentially activated by mammalian caspases at its C-terminus (Supplementary Fig. S6). Although the activated form of Xkr8 has not been identified<sup>33</sup>, the N-terminal cleavage product of Xkr8 shares an overall sequence similarity to acCED-8, including all putative transmembrane domains. Moreover, the 21-residue CED-8 N-terminal sequence removed by CED-3 cleavage appears to be unique to *C. elegans* and is not found in Xkr8 or Xkr8 homologs in other organisms. It remains to be determined if CED-8 and Xkr8 are regulated by similar factors and activated through a conserved mechanism and whether activated CED-8 and activated Xkr8 promote PS externalization in apoptotic cells through a similar mechanism.

CED-8 shares sequence similarity to the XK transporters. However, the substrates that XK family proteins might transport remain unknown<sup>18,33</sup>. Given our new findings, it is conceivable that acCED-8 may directly transport PS across the plasma membrane to effect PS exposure. However, because CED-8 also affects the kinetics of apoptosis, it is more likely that acCED-8 is a general caspase mediator that interacts with several downstream effectors to promote cell death execution (Fig. 5). In this scenario, acCED-8 may regulate the activity of a lipid-transporting enzyme, leading to PS externalization in apoptotic cells. Moreover, because loss of *ced-8* does not completely abolish PS externalization in *C. elegans* apoptotic cells, additional mechanisms or regulators must be responsible for the residual PS externalization activity in the *ced-8* mutant during apoptosis.

## Methods

### Strains and culture conditions

*C. elegans* strains were maintained using standard procedures<sup>34</sup>. The following alleles were used in the genetic analyses: LGI, *ced-1(e1735)*, *ced-12(n3261)*, *smIs111*; LGIII, *ced-6(n2095)*, *ced-7(n2094)*, *ttr-52(tm2078)*; LGIV, *ced-2(n1994)*, *ced-3(n2433)*, *ced-5(n1812)*; LGV, *smIs76*; LGX, *ced-8(n1891)*, *smIs434*. *smIs76* and *smIs434* are integrated transgenes generated by UV irradiation of animals carrying extrachromosomal



arrays containing  $P_{hsp}ANV::GFP$  and  $P_{hsp}GFP::Lact$ , respectively<sup>14</sup>. They were backcrossed four times with wild-type animals before being used for genetic analyses.

### Transgenic animals

Transgenic animals were generated using standard protocols<sup>35</sup>.  $P_{ced-8}CED-8::GFP$ ,  $P_{ced-8}CED-8(D21A)::GFP$ ,  $P_{ced-8}CED-8(D163A)::GFP$ , or  $P_{ced-8}acCED-8::GFP$  was injected into *unc-76(e911)*; *ced-8(n1891)* animals at 5 ng/μl along with an *unc-76* rescuing plasmid (at 50 ng/μl) as a co-injection marker.  $P_{ced-8}CFP::CED-8$  and  $P_{ced-8}YFP::CED-8(D21E)$  were co-injected into *ced-1(e1375)*; *unc-76(e911)* animals at 25 ng/μl each along with the *unc-76* rescuing plasmid (at 50 ng/μl).  $P_{hsp}CED-8$  or  $P_{hsp}acCED-8$  was injected into *smls434* or *ced-3(n2433)*; *smls434* animals at 25 ng/μl along with the  $P_{sur-5}mCherry$  plasmid (25 ng/μl) as a co-injection marker, which directs mCherry expression in all somatic cells in most developmental stages<sup>36</sup>.

### Quantification of cell corpses

The number of cell corpses in living embryos or in the head region of L1 larvae was scored using Nomarski optics<sup>37</sup>.

### Quantification of ANV::GFP or GFP::Lact intensity on corpses

To score the number of cell corpses labeled by ANV::GFP or GFP::Lact, apoptotic cells were first identified using DIC optics and then examined for the presence of ANV::GFP or GFP::Lact on their surface. To measure the ANV::GFP or GFP::Lact fluorescence intensity on cell corpses, fluorescence micrographs were captured using a Zeiss Axioplan 2 microscope equipped with a CCD camera and the Slidebook 5.0 software (Intelligent Imaging Innovations). GFP intensity was determined by measuring the fluorescence signals from three random areas on the plasma membrane of each apoptotic cell (Ia) and three random areas on normal cells (Ib). The average value of Ia minus the average value of Ib was then used as the value for relative ANV::GFP or GFP::Lact intensity.

### Quantification of ANV::GFP rings

*C. elegans* embryos were mounted on slides using the egg salt buffer (118 mM NaCl, 48 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM HEPES pH 7.5) and the number of ANV::GFP rings was then scored regardless of the cell morphology.

### Heat-shock treatment

For time-lapse microscopy, *smls76* or *smls76*; *ced-8(n1891)* embryos were placed at 33°C for 30 minutes, followed by recovery at room temperature for 1 hour. For analysis of ANV::GFP or GFP::Lact labeling of comma stage embryos, embryos were placed at 33°C for 45 minutes, followed by recovery at room temperature 2 hours. For analysis of ANV::GFP or GFP::Lact labeling of 4-fold stage embryos, embryos were placed at 33°C for 45 minutes, followed by recovery at room temperature for 3 hours.

## Microscopy

For fluorescent microscopy, *C. elegans* embryos were mounted on agar pads and observed using 100X optics on Axioplan 2 microscopes (Zeiss). Fluorescence and DIC images were captured using a CCD camera (PCO SensiCam) coupled with the Slidebook 5.0 software.

To perform time-lapse microscopy, *C. elegans* embryos were mounted on slides with agar pad in egg salt buffer and cover slips were sealed with Beeswax and Vaseline (1:1). Images in a 45-micron z series (3 micron/section) were captured every 2 or 5 minutes for 180–360 minutes. Neutral density filter was used to allow only 12% light transmission. Four-dimensional microscopy analysis of cell corpse durations in the N2 and *ced-8(n1891)* embryos were performed as described previously<sup>28</sup>.

## Molecular biology

*ced-8* coding region was amplified using polymerase chain reaction (PCR) from the pBluescript KS(+)-*ced-8* vector<sup>15</sup> and subcloned into the pET-41b vector (Novagen) through its SpeI and EcoRV sites. Deletions of CED-8 or single amino acid substitutions in CED-8 were generated by site-directed mutagenesis of the resulting pET-41b-CED-8 vector. The  $P_{ced-8}$ CED-8::GFP vector was made by fusing a 1.2 kb (Sma I/Nhe I) *ced-8* promoter to a *ced-8* cDNA (Nhe I/Age I) in frame with GFP from pPD95.77. The  $P_{ced-8}$ CFP::CED-8 and  $P_{ced-8}$ YFP::CED-8(D21E) vectors were made by fusing the 1.2 kb *ced-8* promoter to CFP::CED-8 or YFP::CED-8(D21E), respectively.  $P_{hsp-7ac}$ CED-8 was made by PCR amplification of *ced-8* cDNA encoding amino acids 22-458 and subcloning the PCR fragment into the pPD48.78 and pPD48.83 vectors through Nhe I and EcoR V sites.

## CED-3 protease assays

CED-3 protease assays were carried out using the standard protocol<sup>22</sup>. Briefly, GST-CED-8, GST-CED-8(D21A), GST-CED-8(D21E), GST-CED-8(D163A), and GST-CED-8(D21A, D163A) were synthesized and labeled with <sup>35</sup>S-Methionine using TNT Transcription/Translation coupled system (Promega) and incubated with 5 ng of purified CED-3 at 30°C for 2 hours. The reactions were then resolved by 15% SDS-PAGE.

## Statistical analysis

*P* values represent analysis from two-tailed Student's *t*-tests. \**P* < 0.05; \*\**P* < 0.001.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

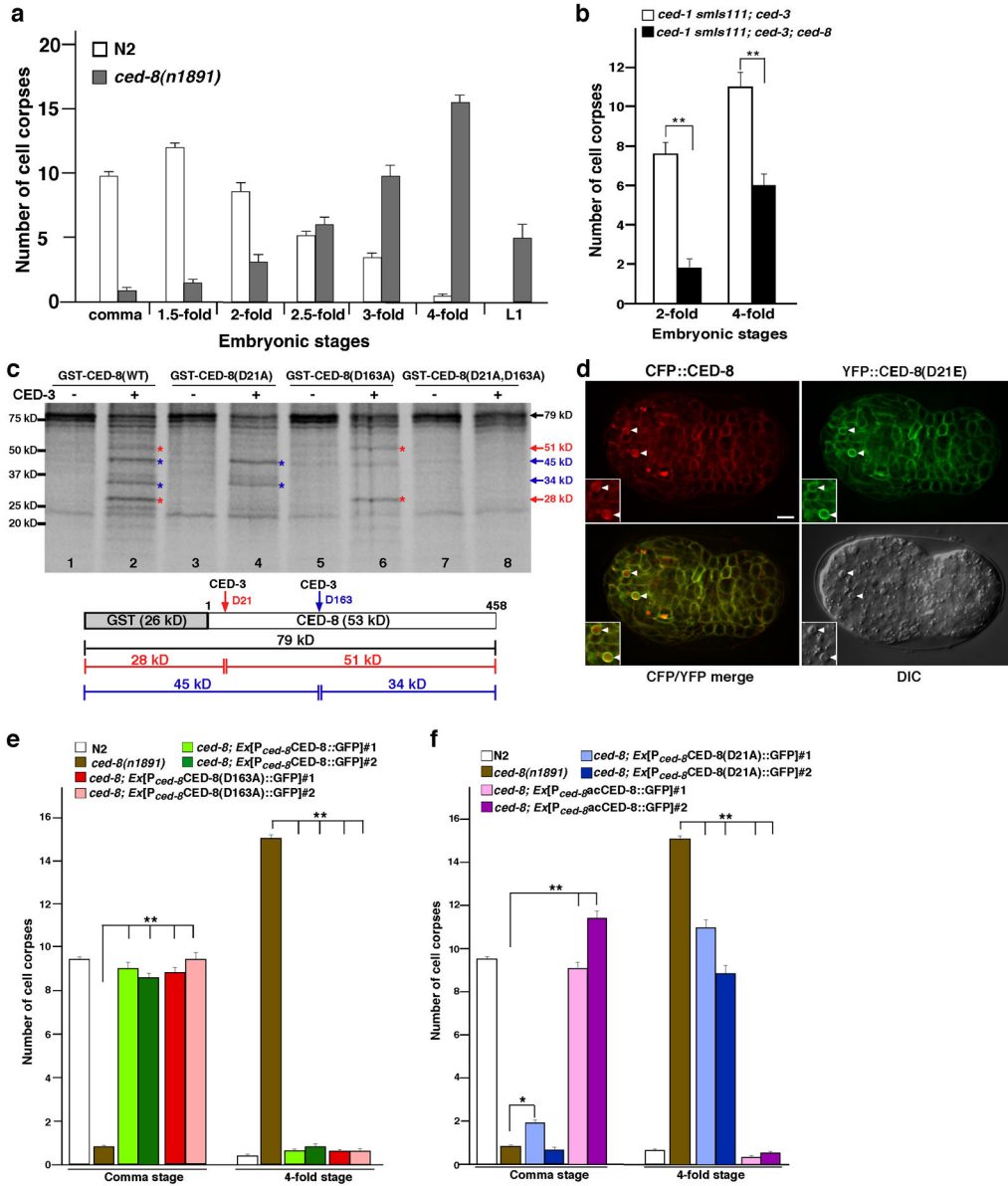
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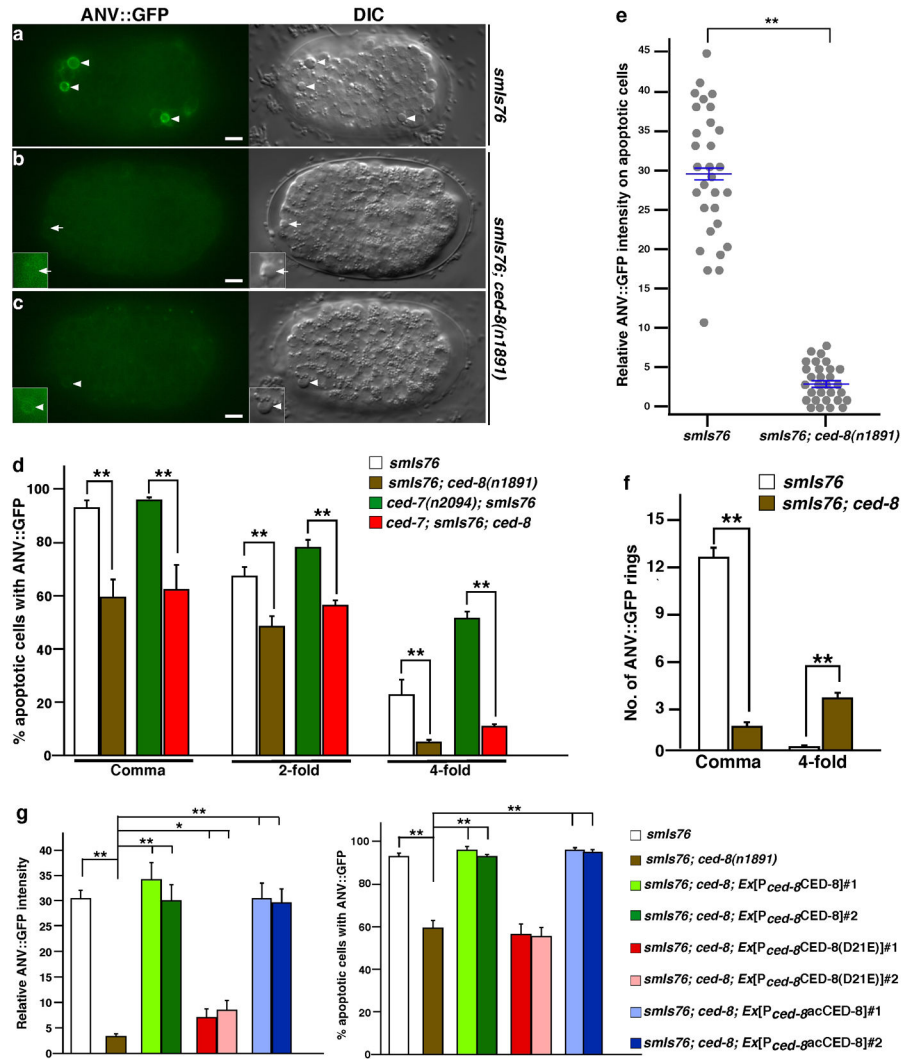


**Figure 1. *ced-8* acts downstream of *ced-3* and encodes a substrate of CED-3**

(a) Appearance of apoptotic cell corpses in different stages of wild-type (N2) and *ced-8(n1891)* embryos. (b) *ced-8* acts downstream of *ced-3*. Cell corpses were scored in 2-fold and 4-fold *ced-1(e1735) smls111; ced-3(n2433)* and *ced-1(e1735) smls111; ced-3(n2433); ced-8(n1891)* embryos. (c) Cleavage of CED-8 by CED-3 *in vitro*. Top: <sup>35</sup>S-Met-labeled GST-CED-8 proteins (wild-type or mutants) were incubated with or without 5 ng purified CED-3 protease at 30°C for 2 hours and then resolved by 15% SDS polyacrylamide gel electrophoresis (PAGE). Bottom: a schematic diagram of GST-CED-8 and cleavage products generated by CED-3 (indicated by arrows). (d) Demonstration of *in vivo* cleavage of CED-8 by CED-3. CFP, YFP, CFP/YFP merged, and differential contrast interference (DIC) images of a comma stage *ced-1(e1735)* embryo expressing CFP::CED-8 and YFP::CED-8(D21E) are shown. CFP (pseudo-color in red) is observed in the cytoplasm



of apoptotic cells (arrowheads). YFP (pseudo-color in green) remains localized to the plasma membrane. Scale bar indicates 5  $\mu\text{m}$ . **(e, f)** Rescue of the delay-of-cell-death defect of the *ced-8(n1891)* mutant by various *ced-8* transgenes. Cell corpses were scored in the following animals: **(e)** N2, *ced-8(n1891)*, *ced-8(n1891); ex[P<sub>ced-8</sub>CED-8::GFP]* arrays #1-2, and *ced-8(n1891); ex[P<sub>ced-8</sub>CED-8(D163A)::GFP]* arrays #1-2. **(f)** N2, *ced-8(n1891)*, *ced-8(n1891); ex[P<sub>ced-8</sub>CED-8(D21A)::GFP]* arrays #1-2, and *ced-8(n1891); ex[P<sub>ced-8ac</sub>CED-8::GFP]* arrays #1-2. Comma and 4-fold stage transgenic embryos were examined. **(a, b, e, f)** The y axis represents average number of cell corpses scored and error bars represent s.e.m. ( $n=15$ ). The significance of differences between results was determined by two-tailed Student's *t*-tests,  $**P < 0.001$ ;  $*P < 0.05$ .



**Figure 2. PS externalization in apoptotic cells is blocked or greatly reduced in the *ced-8* mutant** (a–c) ANV::GFP and DIC images of comma stage embryos from *smIs76* and *smIs76; ced-8(n1891)* animals are shown. Arrowheads indicate apoptotic cells labeled by ANV::GFP. In *smIs76; ced-8(n1891)* embryos, apoptotic cells either were not labeled (indicated by an arrow, b) or were very weakly labeled (c) by ANV::GFP. Insets show enhanced GFP images of apoptotic cells. Scale bars indicate 5  $\mu$ m. (d) Percentages of apoptotic cells labeled by ANV::GFP in the indicated strains and stages are shown. (e) Relative intensity of ANV::GFP that labeled apoptotic cells in comma stage embryos was quantified in the indicated strains (see Methods). Results are shown using the histogram ( $n=30$ ), with mean  $\pm$  s.e.m. indicated. Apoptotic cells that were not labeled by ANV::GFP are presented with zero intensity. (f) The number of ANV::GFP rings in the indicated strains was scored in the comma and 4-fold stage embryos regardless of their cell morphology. (g) Rescue of the PS externalization defect of the *ced-8(n1891)* mutant by various *ced-8* transgenes. Quantification of the percentage of apoptotic cells labeled by ANV::GFP and the relative intensity of ANV::GFP in transgenic embryos was performed as in (d, e). (d, f, g)

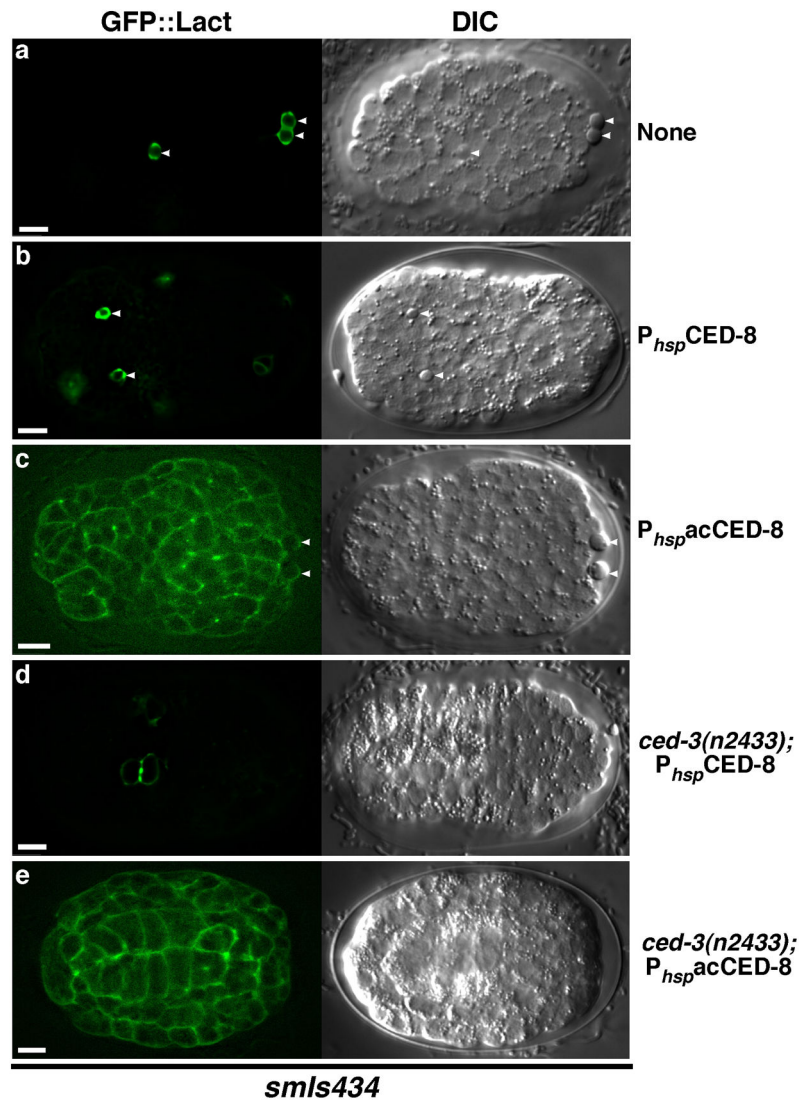
Results are presented as mean  $\pm$  s.e.m. ( $n=15$  each). The significance of differences between results was determined by two-tailed Student's *t*-tests,  $**P < 0.001$ ;  $*P < 0.05$ . Heat-shock treatment was performed at 33°C for 30 min and embryos were examined after 2 hour recovery at 20°C. Exposure time for each fluorescent image was identical (12.5 ms).

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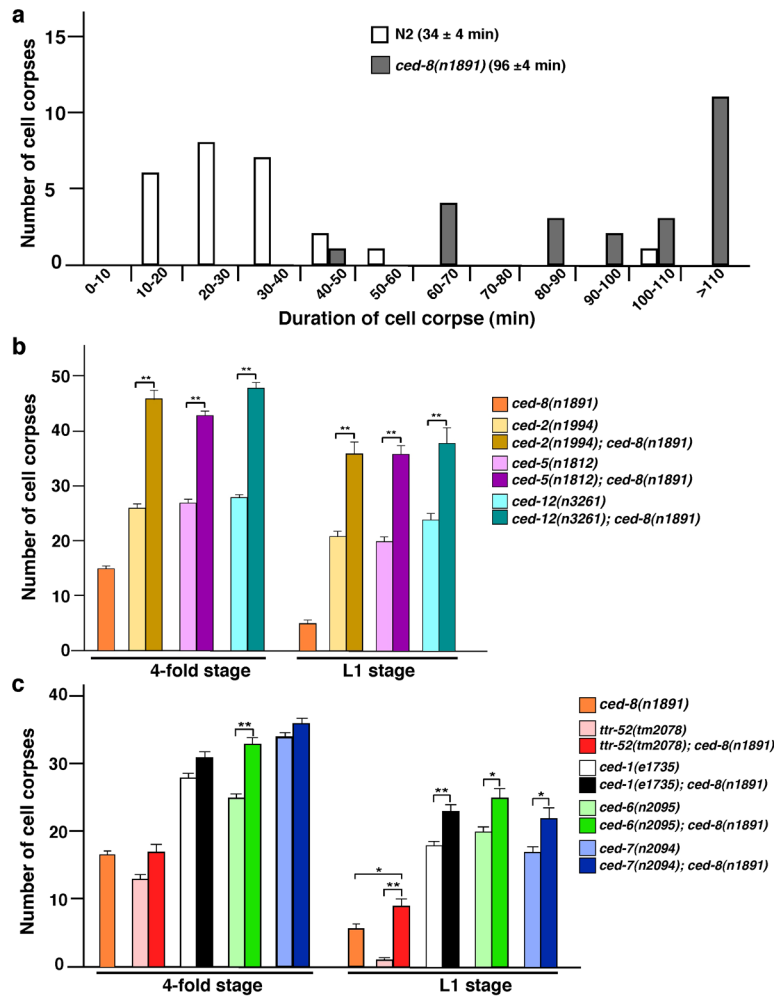
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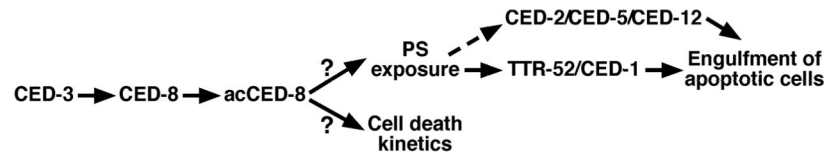
**Figure 3. Expression of acCED-8 induces ectopic PS exposure in all cells**  
**(a–e)** GFP::Lact and DIC images of comma stage embryos from the follow strains are shown: *smls434* **(a)**, *smls434; ex[P<sub>hsp</sub>CED-8]* **(b)**, *smls434; ex[P<sub>hsp</sub>acCED-8]* **(c)**, *ced-3(n2433); smls434; ex[P<sub>hsp</sub>CED-8]* **(d)**, and *ced-3(n2433); smls434; ex[P<sub>hsp</sub>acCED-8]* **(e)**. Arrowheads indicate apoptotic cells. Heat-shock treatment was performed at 33°C for 45 min and embryos were examined after 2 hour recovery at 20°C. Exposure time for each fluorescent image was identical (100 ms). Scale bars indicate 5 μm.



**Figure 4. *ced-8* is important for cell corpse engulfment in *C. elegans***

(a) Time lapse microscopy analysis of the durations of cell corpses. The persistence of 25 cell corpses from N2 embryos ( $n=3$ ) and 24 cell corpses from *ced-8(n1891)* embryos ( $n=8$ ) was monitored. The numbers in parentheses indicate the average persistence for cell corpses  $\pm$  s.e.m. The y axis indicates the number of cell corpses with a specific duration range (shown by the x axis). The durations of four cell divisions in the MS cell lineage from the MS cell to the MS.aaaa cell<sup>38</sup> were also followed to ensure that the embryos assayed had similar rates of development. The average duration of four cell divisions for N2 embryos is  $89 \pm 6$  minutes and  $91 \pm 3$  minutes for *ced-8(n1891)* embryos. (b, c) The *ced-8(n1891)* mutation enhances the corpse clearance defect of engulfment mutants. Cell corpses were scored in 4-fold embryos and in L1 larvae from the indicated strains. The y axis represents the mean of cell corpses scored in the whole embryos or in the head region of L1 larvae ( $n=15$ ). Error bar represents s.e.m. The significance of differences between results was determined by two-tailed Student's *t*-tests, \* $P < 0.05$ ; \*\* $P < 0.001$ .





**Figure 5. CED-8 working model**

CED-8 is cleaved and activated by CED-3 during apoptosis to generate acCED-8, which promotes PS externalization and normal progression of apoptosis through undefined mechanisms. Surface-exposed PS then promotes phagocytosis of apoptotic cells through TTR-52, a PS-binding extracellular bridging molecule, and its binding partner, the CED-1 phagocyte receptor. Surface-exposed PS may also promote phagocytosis of apoptotic cells through the CED-2/CED-5/CED-12 engulfment pathway.