Cell, Vol. 93, 951–960, June 12, 1998, Copyright ©1998 by Cell Press

The *C. elegans* Cell Corpse Engulfment Gene *ced-7* Encodes a Protein Similar to ABC Transporters

Yi-Chun Wu and H. Robert Horvitz*
Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

The *C. elegans* gene *ced-7* functions in the engulfment of cell corpses during programmed cell death. We report that the CED-7 protein has sequence similarity to ABC transporters, is broadly expressed during embryogenesis, and is localized to the plasma membrane. Mosaic analysis revealed that *ced-7* functions in both dying cells and engulfing cells during the engulfment process. We propose that CED-7 functions to translocate molecules that mediate homotypic adhesion between the cell surfaces of the dying and engulfing cells. Like CED-7, the mammalian ABC transporter ABC1 has been implicated in the engulfment of cell corpses, suggesting that CED-7 and ABC1 may be functionally similar and that the molecular mechanism underlying cell corpse engulfment during programmed cell death may be conserved from nematodes to mammals.

Introduction

Programmed cell death is an important cellular process in development and homeostasis (reviewed by Ellis et al., 1991b; Steller, 1995; Jacobson et al., 1997). Once cells undergo programmed cell death, their corpses are swiftly engulfed by other cells and degraded (reviewed by Ellis et al., 1991b; Savill et al., 1993). The engulfment process, which removes dying cells before they can lyse and release potentially harmful cytoplasmic contents, is important for tissue remodeling and for the resolution of the inflammatory response (e.g., for the removal of senescent or apoptotic lymphocytes) (Savill et al., 1993; Hart et al., 1996; Savill, 1997). Cell corpse engulfment is a multistep process and involves the recognition of a dying cell followed by the extension of pseudopodia and the envelopment of the dying cell by an engulfing cell. Studies of vertebrates have identified a number of molecules that may participate in the recognition step of engulfment. The exposure of phosphatidylserine on the surfaces of dying cells may act as a marker for their recognition by macrophages (Fadok et al., 1992a, 1992b). Lectin-like proteins (Duvall et al., 1985) and adhesion molecules, such as vitronectin (Savill et al., 1990; Fadok et al., 1992b) and CD36 (Savill et al., 1991, 1992), on the surfaces of macrophages have been implicated in the recognition of specific carbohydrates and charge-sensitive moieties on the surfaces of dying cells, respectively. However, the mechanism of cell corpse engulfment remains largely unknown.

*To whom correspondence should be addressed.

In the nematode *Caenorhabditis elegans*, 131 of 1090 somatic cells generated during hermaphrodite development undergo programmed cell death (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). Genetic studies have identified six genes, *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* (*ced*, *c*ell *d*eath abnormal), that control the engulfment of cell corpses (Hedgecock et al., 1983; Ellis et al., 1991a). Mutations in any of these genes block the engulfment of many cell corpses (Ellis et al., 1991a), including cell corpses in the germline (Hengartner, 1997; M. Hengartner and H. R. H., unpublished data). The unengulfed cell corpses persist and are readily distinguishable using Nomarski optics by their refractile appearance. This mutant phenotype can be quantified by counting the number of persistent cell corpses (Ellis et al., 1991a). Genetic analysis suggests that the six engulfment genes fall into two groups: *ced-1*, *ced-6*, and *ced-7* are in one group, and *ced-2*, *ced-5*, and *ced-10* are in the other (Ellis et al., 1991a). Single mutants or double mutants within the same group show relatively weak engulfment defects, whereas double mutants between the two groups show strong engulfment defects. One model consistent with these observations is that the two groups of genes are involved in two distinct but partially redundant pathways in the engulfment process (Ellis et al., 1991a). Of the six engulfment genes, *ced-5* has been cloned. The sequence of the CED-5 protein is similar to that of human DOCK180 and *Drosophila* Myoblast City (Wu and Horvitz, 1998). CED-5 is likely to function in engulfing cells by effecting a reorganization of the cytoskeleton as engulfing cells extend their surfaces to envelop cell corpses (Wu and Horvitz, 1998).

Of the somatic programmed cell deaths, 90% occur during embryogenesis, and the remainder occur during early larval development (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). Unlike *ced-1*, *ced-2*, *ced-5*, *ced-6*, and *ced-10*, the *ced-7* gene appears to be specific for embryonic cell death as opposed to larval cell death, since *ced-7* mutations significantly perturb the engulfment of cell corpses in embryos but not in larvae (Ellis et al., 1991a). We have cloned and characterized the *ced-7* gene.

Results

Positional Cloning of *ced-7*

The *ced-7* gene was previously localized to the region between *glp-1* and *unc-50* on chromosome III (Ellis et al., 1991a) (Figure 1A). This interval corresponds to approximately 1.2 Mb on the *C. elegans* physical map (Coulson et al., 1986). To define better the region containing *ced-7*, we mapped *ced-7* with respect to the cloned gene *emb-9* (Guo et al., 1991) and the restriction fragment length polymorphisms (RFLPs) *eP7* (Greenwald et al., 1987) and *stP127* (Williams et al., 1992), which were previously mapped to this region. Our results (see Experimental Procedures) localized *ced-7* to an approximately 300 kb region between *emb-9* and *eP7*

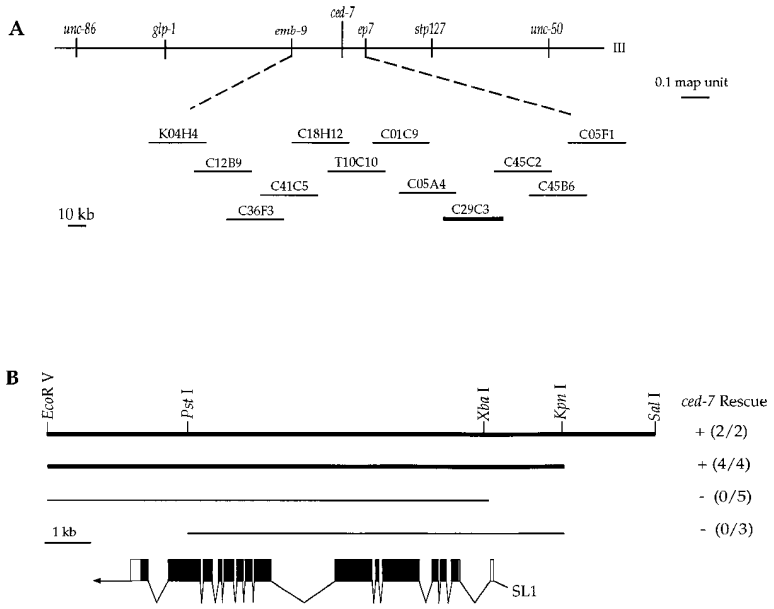


Figure 1. Molecular Cloning of the *ced-7* Gene

(A) The genetic map near the *ced-7* locus on chromosome III is shown above. The cosmid clones shown below were tested for their abilities to rescue the *ced-7* engulfment defect. The cosmid C29C3 (shown in bold) rescued the defect.

(B) A partial restriction map of one subclone of the C29C3 cosmid with *ced-7* rescuing activity is shown. The fragments derived from this subclone were used to define the minimal region containing the *ced-7* rescuing activity based upon germline transformation experiments. Plus, rescue; minus, no rescue. Numbers in parentheses indicate the number of rescued lines and the total number of transgenic lines. The structure of the *ced-5* gene was deduced by comparing the sequences of genomic DNA and cDNAs. A 5′ SL1 *trans*-spliced leader is indicated. Boxes represent exons. Closed boxes indicate the *ced-5* open reading frame; open boxes indicates the untranslated region. The transcription direction is from right to left.

(Figure 1A). We tested 12 overlapping cosmids from this region for their abilities to rescue the persistent cell corpse phenotype of *ced-7(n1892)* mutants. The cosmid C29C3 rescued the Ced-7 mutant phenotype. By testing subclones from this cosmid, we localized the *ced-7* rescuing activity to an 11 kb fragment (Figure 1B). Further deletions into this fragment from either the right or the left ends abolished its rescuing activity.

ced-7 Sequence and Mutant Alleles

We used the 11 kb genomic rescuing fragment to isolate *ced-7* cDNA clones and defined the 5′ end of the *ced-7* message using the RACE (*r*apid *a*mplification of *c*DNA *e*nds) method (Frohman et al, 1988). The sequences of these cDNAs revealed an open reading frame of 1704 amino acids (Figure 2), a 5′ SL1 *trans*-spliced leader found at the 5′ end of many *C. elegans* transcripts (Krause and Hirsh, 1987), and a 3′ poly(A) tract, confirming that we had identified the complete *ced-7* transcription unit. The *ced-7* genomic sequence has been determined by *C. elegans* genomic sequencing project, and its exons have been predicted by the Genefinder program (Wilson et al., 1994). Ten of fourteen exons predicted are consistent with those of the *ced-7* cDNA. Northern analysis using the *ced-7* cDNA as a probe revealed a single band of 5.8 kb, consistent with the size of the full-length *ced-7* cDNA (data not shown). The expression of the *ced-7* cDNA under the control of *C. elegans* heat shock promoters (Stringham et al., 1992) rescued the engulfment defect of *ced-7* mutant animals (Table 1), indicating that the *ced-7* cDNA encodes a functional CED-7 protein.

We identified molecular lesions in eight *ced-7* alleles (Table 2), confirming that we have correctly identified the *ced-7* gene. The alleles *n1996* and *n2094* are early nonsense mutations, presumably resulting in the absence of more than 90% of the CED-7 protein. These alleles result in no detectable CED-7 protein expression as assayed by Western blot analysis (Figure 5A) or antibody staining of whole-mount embryos and larvae (see Figure 5C), consistent with the two alleles being null. One *ced-7* allele, *n1892*, appeared to contain no mutations in the *ced-7* coding sequence and splice junction sites. This allele may contain an alteration in a regulatory region of the gene, since the *ced-7(n1892)* mutant did not express detectable CED-7 protein as assayed by Western blot analysis and antibody staining (data not shown).

ced-7 Encodes a Protein Similar to ABC Transporters

A search of protein databases with the predicted CED-7 protein sequence revealed that CED-7 is similar to ABC (*A*TP-*b*inding *c*assette) transporters (reviewed by Higgins, 1992; Fath and Kolter, 1993). Like other members of the ABC transporter superfamily, CED-7 consists of two similar halves. Each half contains a hydrophobic region with six putative transmembrane domains, a predicted ATP *n*ucleotide-*b*inding *d*omain (NBD), which includes the Walker A motif $GX_4GK(S/T)$ (Walker et al., 1982) and the Walker B motif $(R/K)X_{6-8}hyd_4D$ (hyd, hydrophobic residues; Walker et al., 1982), and the ABC signature sequence (L/Y)SGG(Q/M), which is diagnostic for ABC transporters (Higgins, 1992) (Figures 2 and 3).

ABC transporters have been identified that mediate the transport of a diversity of substrates, including ions, sugars, vitamins, phospholipids, peptides, and proteins (Higgins, 1992; Ruetz and Gros, 1994). The mechanism by which each ABC transporter achieves its substrate specificity is poorly understood.

CED-7 is most similar to the ABC1 subfamily, which includes the mouse ABC1 (Luciani et al., 1994), the mouse ABC2 (Luciani et al., 1994), the human ABC-C (ABC3) (Klugbauer and Hofmann, 1996; Connors et al., 1997), the human ABCR (Allikmets et al., 1997), and the bovine rim (Illing et al., 1997) proteins. CED-7 is most

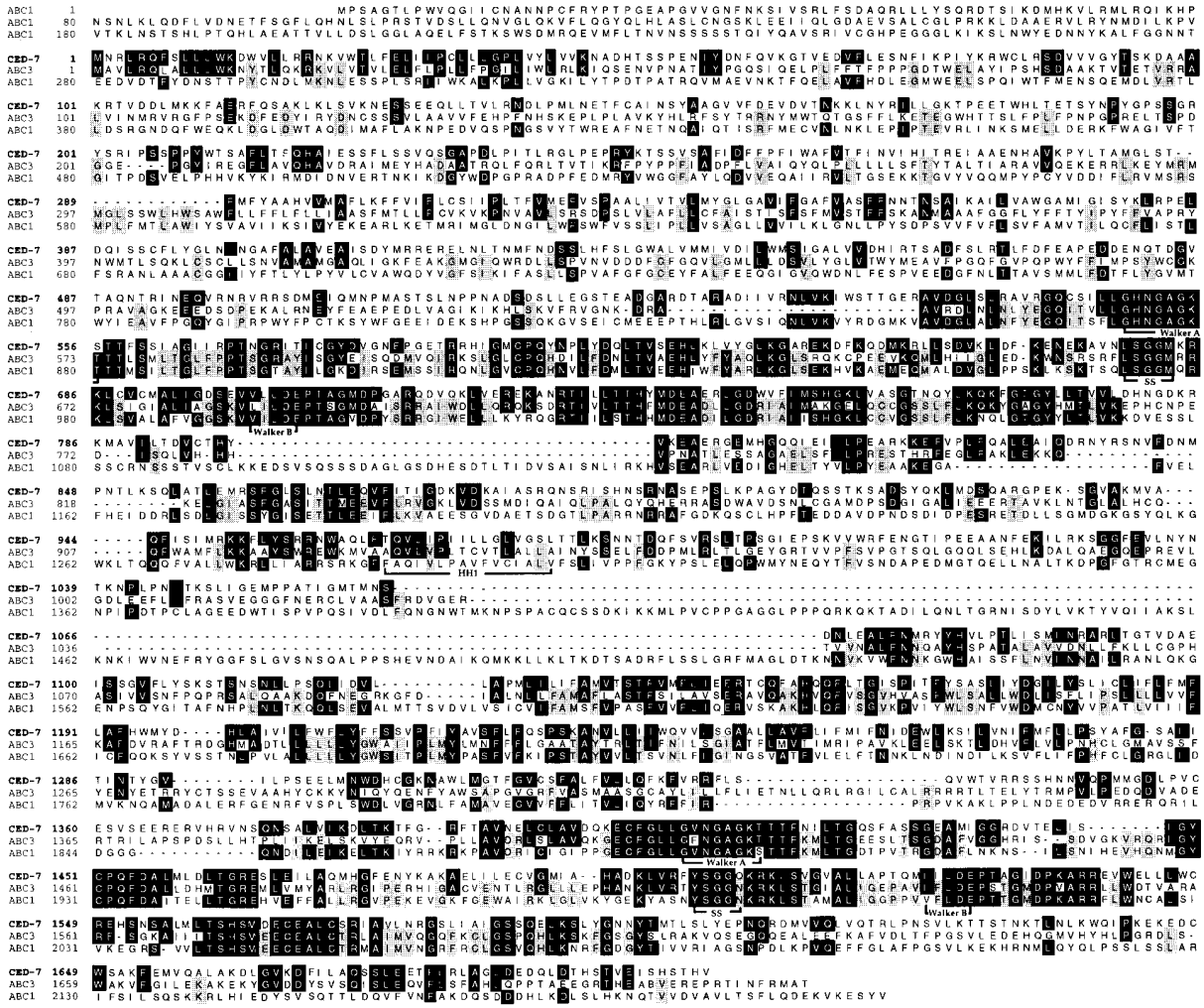


Figure 2. CED-7 Protein Sequence and Motifs

The CED-7 protein sequence is aligned with the human ABC-3 (Connors et al., 1997) and the mouse ABC1 (Luciani et al., 1994) protein sequences. Black boxes indicate amino acids identical between CED-7 and ABC-3 or ABC1. Gray boxes indicate amino acids identical only between ABC-3 and ABC1. The Walker A and Walker B motifs of the nucleotide-binding domains, the ABC signature sequence (SS), and the *h*ighly *h*ydrophobic domain (HH1) are indicated.

similar to the ABC-C protein and is 25% and 20% identical to the ABC-C and ABC1 proteins, respectively, throughout their entire lengths (Figure 2). The similarity of CED-7 to these two proteins is most striking in the regions of and near the Walker A and Walker B motifs (see Figure 2). In addition, CED-7 as well as members of the ABC1 subfamily all have a unique *h*ighly *h*ydrophobic domain (HH1) (Luciani et al., 1994) localized between the two halves of the protein (Figures 2 and 3). The functional or structural significance of this domain remains to be explored.

While the ABC1 protein is able to transport anions across the cell membrane of *Xenopus laevis* oocytes (Becq et al., 1997), no physiological substrates of the ABC1 subfamily of ABC transporters have been identified. ABC1 is expressed in macrophages, and the ability of macrophages to engulf apoptotic thymocytes, but not yeast cells, is severely impaired when macrophages are loaded with anti-ABC1 antibodies (Luciani and Chimini, 1996). These results suggest that ABC1 may be involved in the engulfment of cell corpses, and if so, that ABC1 and CED-7 transport similar substrates required for the engulfment process.

The First Nucleotide-Binding Site of CED-7 Is More Important than the Second for In Vivo Function

Both NBDs of some ABC transporters have been shown to be important for substrate transport (Azzaria et al., 1989; Berkower and Michaelis, 1991). Crystallographic and NMR studies of adenylate kinase have suggested that the lysine residue of the Walker A motif $GX_4GK(S/T)$ interacts with the phosphate group of the bound ATP (Pai et al., 1977; Fry et al., 1988; Saraste et al., 1990) and is important for ATP hydrolysis (Saraste et al., 1990). Mutations that change the conserved lysine residues to arginine in one or both NBDs of the ABC transporter MDR1 disrupt its drug transport activity but not its ability to bind the ATP analog 8-azido ATP, suggesting that

Table 1. Structure Function Analysis of the CED-7 Nucleotide-Binding Domains

$P_{ced\text{-}7}$ Construct[a]	Array[a]	No. Persistent Corpses (n = 15)[b]	Range of Persistent Corpses[b]
None	—	34 ± 4	29–40
CED-7	1	0 ± 0	0–2
	2	0 ± 0	0–1
	3	0 ± 0	0
CED-7(K586R)	1	37 ± 3	33–43
	2	35 ± 3	30–40
	3	35 ± 4	25–43
CED-7(K1417R)	1	11 ± 6	1–18
	2	21 ± 7	8–36
	3	13 ± 7	1–22
CED-7(K586R, K1417R)	1	35 ± 5	27–45
	2	36 ± 3	32–41
	3	34 ± 3	29–40

[a] The $P_{ced\text{-}7}$ constructs were injected into *ced-7(n1996)* mutant animals (see Experimental Procedures). Each array represents an extrachromosomal transgene carried by a different transgenic line.
[b] Cell corpses were scored in the head of 4-fold stage embryos. Mean ± SEM.

such mutations do not cause an overt alteration in protein conformation and that their effects on transport activity may be attributed to impaired ATP hydrolysis (Azzaria et al., 1989).

To assess the functional importance of the two NBDs for *ced-7* activity and to examine if the two function equivalently, we mutated the conserved lysine residues to arginine in the first (K586R) or the second (K1417R) or both (K586R, K1417R) NBDs and generated *ced-7(n1996)* transgenic animals expressing either the wild-type or mutant CED-7 proteins under the control of the endogenous *ced-7* promoter, $P_{ced\text{-}7}$. We then determined the extent of rescue of the *ced-7* engulfment defect by these transgenes (see Experimental Procedures). We found that CED-7(K1417R) retained partial rescuing activity, whereas CED-7(K586R) and CED-7 (K586R, K1417R) failed to rescue the *ced-7* engulfment defect (Table 1). We confirmed the normal expression and localization of the CED-7 proteins by these transgenic animals by anti-CED-7 antibody staining (data not shown). These results suggest that the first NBD may play a more important functional role for *ced-7* activity than does the second NBD.

Table 2. Characterization of *ced-7* Alleles

Allele	Nucleic Acid and Amino Acid Changes	Codon Position	No. Persistent Corpses (n = 20)[b]
n1996	**C**GA (R)→**T**GA (stop)	5	34 ± 4
n2094	**C**AA (Q)→**T**AA (stop)	116	36 ± 4
n3072	G**A**A (E)→G**G**A (G)	639	33 ± 6
n1997	**C**GA (R)→**T**GA (stop)	1074	34 ± 3
n2690	a**g**|AGT→a**a**|AGT	1200	16 ± 5
n1998	TG**G** (W)→TG**A** (stop)	1300	30 ± 3
n3073	**A**GA (R)→**T**AG (stop)	1332	33 ± 5
n2001	TG**G** (W)→TG**A** (stop)	1540	32 ± 4
n1892	ND[a]	ND[a]	36 ± 4

[a] We did not identify any mutations in the *ced-7* coding sequence or splice junction sites in the *n1892* allele. ND, not determined.
[b] Cell corpses in the head of L1 larvae within 1.5 hr of hatching were counted. Mean ± SEM.

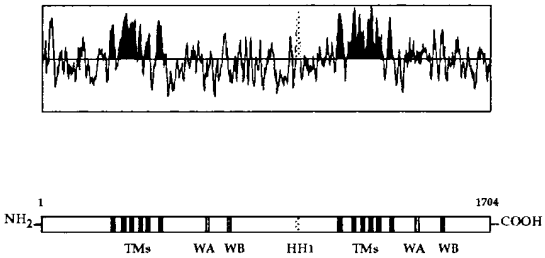


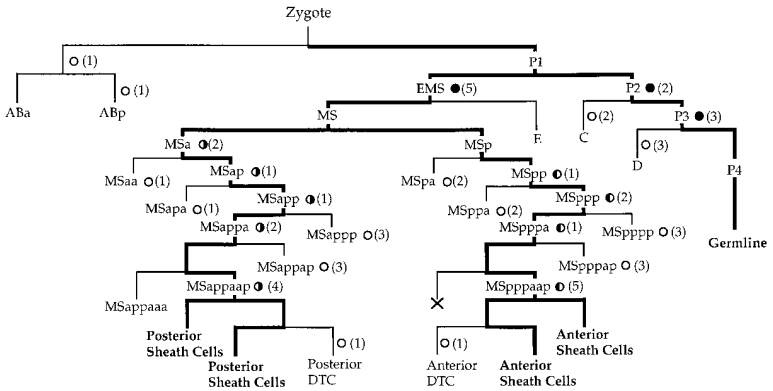
Figure 3. CED-7 Motifs and Hydropathy Profile

The hydropathy plot was generated using the algorithm and hydrophobicity values of Kyte and Doolittle (1982) for a window size of 11 residues. Hydrophobic and hydrophilic regions are above and below the central line, respectively. The 12 potential transmembrane domains are marked in black, and the HH1 domain is marked with dots. Shown below is a schematic drawing of CED-7, indicating the relative positions of the transmembrane domains (TMs), the HH1 domain, the Walker A motifs (WA, dotted boxes), and Walker B motifs (WB, hatched boxes).

CED-7 Activity Is Required in Both Dying Cells and Engulfing Cells during Cell Corpse Engulfment

To determine if *ced-7* function is required in the dying cell or the engulfing cell, we analyzed *ced-7* genetic mosaics (see Experimental Procedures). In brief, we used a strain mutant for *ced-7* and the cell-autonomous marker *ncl-1* (Hedgecock and Herman, 1995) and carrying wild-type copies of *ced-7* and *ncl-1* on a small extrachromosomal duplication of chromosome III, *qDp3* (Austin and Kimble, 1987; Seydoux and Greenwald, 1989; Clark et al., 1993). Since mutations in the *ced-7* and *ncl-1* genes cause recessive phenotypes, animals carrying *qDp3* are generally wild type. However, *qDp3* is mitotically unstable and is occasionally lost during embryonic cell divisions. Such mitotic loss generates a clone of genetically mutant *ced-7*(−) *ncl-1*(−) cells recognizable by their enlarged nucleoli (the Ncl phenotype; *nucl*eoli abnormal) by Nomarski optics in an otherwise genetically *ced-7*(+) *ncl-1*(+) background.

We used such genetic mosaics to analyze the role of *ced-7* in germline cell death. The *C. elegans* germline is syncytial. However, during programmed cell death, germline nuclei are cellularized, and the germline cell corpses are engulfed by gonadal sheath cells, which contact the germline (Hengartner, 1997; M. Hengartner, E. Hartwieg, and H. R. H., unpublished data). The germline in both the anterior and posterior arms of the gonad of adult hermaphrodites is derived from the P2 lineage, while the sheath cells of the anterior and posterior arms are derived from the blastomeres MSp and MSa, respectively (Kimble and Hirsh, 1979; Sulston et al., 1983) (Figure 4). We identified candidate mosaics in which *qDp3* had been lost in either the germline or gonadal sheath cells by screening for animals with Ncl cells specifically in the P2, MSp, or MSa lineages (Figure 4; see Experimental Procedures). If *ced-7* function is required in the dying cell, the loss of *qDp3* in the germline but not in other cells would result in persistent cell corpses in the



Phenotypes of mosaic animals (anterior, posterior germ cells):
● Ced-7, Ced-7
○ wild-type, wild-type
◐ Ced-7, wild-type
◑ wild-type, Ced-7

Figure 4. *ced-7* Mosaic Analysis

A partial representation of the *C. elegans* cell lineage (adapted from Kimble and Hirsh, 1979, and Sulston et al., 1983). The origins of the germline and of the gonadal sheath cells of the anterior or posterior gonadal arms are indicated by thick vertical and horizontal lines. The phenotypic symbols indicate the mitotic cell divisions at which the duplication was lost, and the numbers in parentheses indicate the number of animals for which that point of duplication loss was observed. The Ncl phenotype cannot be scored reliably in the P4 and E lineages.

germline. On the other hand, if *ced-7* function is required in the engulfing cell, the loss of *qDp3* in the gonadal sheath cells but not in other cells would result in persistent cell corpses in the germline. If *ced-7* function is required in both dying cells and engulfing cells, the loss of *qDp3* in either germline or gonadal sheath cells would result in persistent cell corpses. If *ced-7* function is required in either dying cells or engulfing cells, only the loss of *qDp3* in both germline and gonadal sheath cells would result in persistent cell corpses.

We identified 56 mosaic animals. Nine animals lost *qDp3* in the sheath cells of anterior gonadal arms and showed the Ced-7 phenotype in these gonadal arms with an average 26 cell corpses; these animals were phenotypically wild type in their posterior gonadal arms, with fewer than four cell corpses. Similarly, ten animals lost *qDp3* in the sheath cells of posterior gonadal arms and showed the Ced-7 phenotype in these gonadal arms, with an average 28 cell corpses, but were wild type in their anterior arms with fewer than 4 cell corpses. Five animals lost *qDp3* in the sheath cells of both anterior and posterior gonadal arms and showed the Ced-7 phenotype in both gonadal arms, with an average 25 and 29 cell corpses, respectively. *ced-7(n1892)* mutants (n = 15) showed on average 25 cell corpses in each gonadal arm, whereas wild-type animals (n = 15) had fewer than 4 cell corpses observed in each gonadal arm.)

These results indicate that *ced-7* function is required in the engulfing gonadal sheath cells. In addition, five mosaic animals in which *qDp3* was lost in the germline but not in the sheath cells also showed the Ced-7 phenotype in both gonadal arms, with an average 15 cell corpses. Therefore, *ced-7* function is important not only in engulfing cells but also in dying cells for the engulfment of cell corpses. In 27 mosaic animals in which *qDp3* was lost in the cells other than germline or gonadal sheath cells, fewer than four cell corpses were observed in each gonadal arm. This finding is consistent with the interpretation that *ced-7* function is dispensable in cells other than dying and engulfing cells during cell corpse engulfment.

CED-7 Is Widely Expressed and Is Localized to the Plasma Membrane during Embryogenesis

We raised polyclonal antibodies against recombinant CED-7 protein (see Experimental Procedures). Using affinity-purified CED-7 antibodies, we detected by Western blot analysis a protein band of apparent molecular mass 170 kDa from wild-type embryonic extracts; this size is slightly smaller than that predicted from the CED-7 amino acid sequence (191 kDa) (Figure 5A). This protein was absent in extracts of the *ced-7(n1996)* and *ced-7(n2094)* mutants (Figure 5A), confirming that this protein is the product of the *ced-7* gene.

We used the purified CED-7 antibodies to stain whole-mount animals. We found that CED-7 was widely expressed in embryos and was localized to the plasma membrane (Figure 5B). In larvae and adults, CED-7 expression appeared restricted to specific cells. Specifically, CED-7 was detected in the amphid sheath cells, the pharyngeal-intestinal valve (Figure 5D), and the phasmid sheath cells (Figure 5E). CED-7 expression was also detected in both germline precursors and germline, except sperm in larvae and adults, respectively (Figure 5F). This observation is consistent with the requirement of *ced-7* function in the germline during germline cell death. *ced-7(n1996)* and *ced-7(n2094)* mutant animals consistently lacked somatic staining, but we occasionally observed staining in oocytes in *ced-7(n1996)* and *ced-7(n2094)* mutant animals. For this reason, it is possible that the oocyte staining by anti-CED-7 antibodies is not specific.

The analysis of *ced-7* genetic mosaics showed that *ced-7* function is required in the gonadal sheath cells. However, we could not detect CED-7 expression in these cells. It is possible that in these cells CED-7 protein is expressed at levels below which it can be detected by the anti-CED-7 antibodies.

We also examined the CED-7 expression pattern in other mutants defective in the engulfment of cell corpses, *ced-1*, *ced-2*, *ced-5*, *ced-6*, and *ced-10*. The CED-7 expression pattern was not altered in these mutants. These five engulfment genes therefore do not regulate the expression or localization of the CED-7 protein.

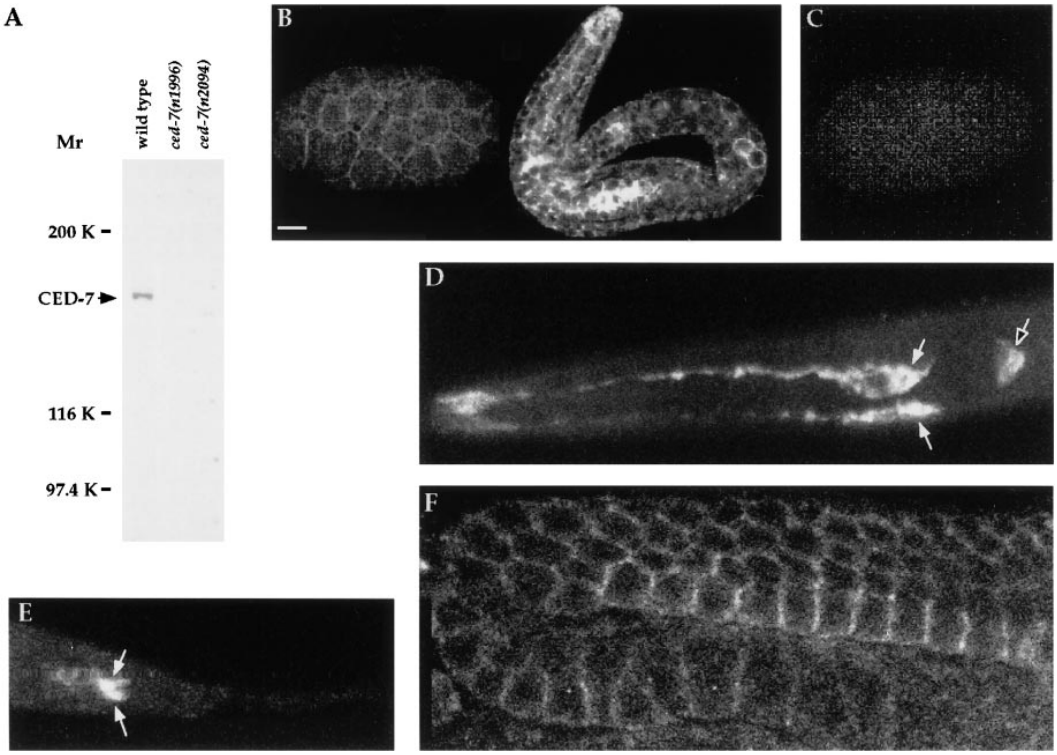


Figure 5. CED-7 Protein Expression

(A) Western blot analysis of CED-7 protein. We used affinity-purified anti-CED-7 antibodies to probe a blot of embryonic extracts from wild-type, *ced-7(n1996)*, and *ced-7(n2094)* animals. Protein extracts (200 μg) were loaded in each lane. That the equal amount of protein was loaded in each lane was confirmed by Ponceau S staining (data not shown). The sizes of molecular weight markers (High range, Bio-Rad) and the position of the CED-7 protein are indicated.

(B) Anti-CED-7 antibody staining of a wild-type embryo at approximately the 50-cell stage (left) and a wild-type embryo at ~558-cell stage (right). The two brightly stained cells on the ventral midbody of the right embryo are Z2 and Z3, the germline precursor cells. The bar represents 10 μm; the same scale applies to (C)–(F).

(C) Anti-CED-7 antibody staining of a *ced-7(n1996)* mutant embryo at approximately the 50-cell stage, prepared in parallel to (B) and lacking any specific staining.

(D) Anti-CED-7 antibody staining of the head of a wild-type L1 (first larval stage) hermaphrodite. The pharyngeal-intestinal valve and the cell bodies of the two amphid sheath cells are indicated by open and closed arrows, respectively. Anterior is to the left. This picture is a projection of four serial confocal images.

(E) Anti-CED-7 antibody staining of the tail of a wild-type L1 hermaphrodite. The two phasmid sheath cells are indicated by arrows. Anterior is to the left.

(F) Anti-CED-7 antibody staining of the germline in part of the anterior gonadal arm of a wild-type adult hermaphrodite.

None of the somatic cells in which we detected CED-7 expression in larvae or adults appear to be involved in cell corpse engulfment. We thus asked if *ced-7* has another role in these cells. Among these cells, the function of the amphid sheath cells is best understood. These cells ensheath chemosensory neurons in a pair of head sensory organs called the amphids. The amphids open to the outside, so that the chemosensory neurons are exposed to the environment. One behavior mediated by the amphids is osmotic avoidance: animals avoid high concentrations of a number of sugars and salts (Culotti and Russell, 1978). Animals in which the amphid sheath cells were killed by laser microsurgery are defective in osmotic avoidance (J. Thomas and H. R. H., unpublished data). We found that *ced-7(n1996)* and *ced-7(n2094)* mutant animals behave indistinguishably from wild-type animals in osmotic avoidance assays (data not shown), indicating that the amphid sheath cells of these *ced-7* mutant animals are at least partially functional. In addition, we also found no obvious ultrastructural defects in the amphid sheath cells and the pharyngeal-intestinal valves of *ced-7(n1996)* and *ced-7(n2094)* mutants in electron micrographs (data not shown). Therefore, CED-7 expression in the amphid sheath cells and the pharyngeal-intestinal valves may not be essential for the functions or gross morphologies of these cells.

Discussion

One prominent feature of programmed cell death is the engulfment of dying cells. We showed that the *C. elegans ced-7* gene, which functions in the engulfment process, encodes a protein with sequence similarity to ABC transporters. Like other members of the ABC transporter superfamily, the CED-7 protein has two NBDs.

The first NBD appears to be functionally more important than the second one, as the mutation of the conserved Walker lysine residue in the first NBD had a more severe effect on *ced-7* activity than did mutation of the corresponding lysine in the second NBD. Similar results were obtained in the case of CFTR (*c*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator), another member of the ABC transporter superfamily. The analysis of mutant CFTR proteins revealed that the two NBDs have distinct roles in controlling CFTR channel activity (Carson et al., 1995). The first NBD seems to control channel opening, whereas the second NBD seems to control channel closing (Carson et al., 1995). The differential importance of the two NBDs of the CED-7 protein suggests that these two NBDs are functionally distinct.

One characteristic feature of ABC transporters is the unidirectionality of substrate transport (Higgins and Gottesman, 1992; Ruetz and Gros, 1994). The transport process appears to be export rather than import in almost all ABC transporters with identified substrates, except in the case of CFTR, which acts as a channel (Higgins, 1992). Recent studies of mouse Mdr2 (Smit et al., 1993; Ruetz and Gros, 1994) and human MDR1 and MDR3 (van Helvoort et al., 1996) have shown that these ABC transporters can function as membrane flippases to translocate lipids from one monolayer of the lipid bilayer to another. Such translocation may result in the reorganization of membrane lipid composition and the redistribution or the modulation of certain cell-surface membrane proteins (Conforti et al., 1990). By analogy, CED-7 could function either as a transporter, channel, or flippase in the process of engulfment.

We found that the CED-7 protein is expressed in embryos, consistent with its role in the engulfment of embryonic cell corpses. This broad expression pattern suggests that CED-7 activity may be regulated so that CED-7-mediated engulfment specifically targets dying but not viable cells. Since CED-7 may act as a transporter, the regulation of CED-7-mediated engulfment may be achieved by controlling either the transporter activity of CED-7 or the accessibility of the substrates that CED-7 transports. From a Prosite search with the CED-7 amino acid sequence, we identified several potential phosphorylation sites (data not shown), suggesting that CED-7 activity might be regulated by phosphorylation, just as the activity of CFTR is controlled through phosphorylation by cAMP-dependent protein kinase (PKA) (Cheng et al., 1991).

The CED-7 protein is localized to the plasma membrane, consistent with its sequence as an ABC transporter. This finding suggests that CED-7 activity may be important for the interaction between the cell surfaces of the dying and engulfing cells. Such interaction between the dying and engulfing cells is required for two aspects of engulfment: the recognition process, which triggers phagocytosis, and the adhesion process, as the engulfing cell extends pseudopodia around the dying cell during phagocytosis.

ced-7 acts in both dying and engulfing cells during cell corpse engulfment. If CED-7 exports the same substrate across the membrane of dying and engulfing cells, this substrate is probably not marking dying cells for recognition by or the chemotactic attraction of neighboring engulfing cells. More likely, the function of CED-7 may be permissive rather than instructive for the engulfment of cell corpses. For example, the same substrates translocated by both dying and engulfing cells may modulate the membrane properties of both cells to facilitate their homotypic adhesion either directly or indirectly (e.g., through molecules in the extracellular matrix). Alternatively, CED-7 may translocate different molecules in dying and engulfing cells. Some ABC transporters can transport different hydrophobic molecules (Higgins, 1992).

The mammalian ABC transporter ABC1 has been suggested to act in macrophages during the phagocytosis of apoptotic cells (Luciani and Chimini, 1996). The possibility that ABC1 might also function in dying cells remains to be explored. The sequence and potential functional similarity of CED-7 and ABC1 suggests that these two proteins might be homologs. If so, the process of cell corpse engulfment in which *ced-7* acts may have been conserved through evolution, and there may well be a common molecular mechanism responsible for the engulfment of cell corpses in all metazoans.

Experimental Procedures

Strains

All strains were grown at 20°C, except where otherwise noted. All mutations were generated in a strain Bristol N2 background, the standard wild-type strain (Brenner, 1974). The following mutations were used: LGIII, *ced-4(n1162)*, *unc-86(n946)*, *emb-9(hc70)*, *ced-7(n1892*, *n1996*, *n1997*, *n1998*, *n2001*, *n2094*, *n2690*, *n3072*, and *n3073)*, *ced-9(n1950)*, and *unc-50(e306)*; LGIV, *ced-3(n717)*; and LGV, *unc-76(e911)* and the wild-type polymorphic strain RW7000, which displays multiple RFLPs when compared to N2. *emb-9(hc70)* (Guo et al., 1991) was obtained from the Caenorhabditis Genetics Center.

Mapping *ced-7*

We showed that *ced-7* lies between *emb-9* and *unc-50* by four-factor mapping: 22/56 Unc-86 non-Ced non-Unc-50, 28/56 non-Unc-86 non-Ced Unc-50, 0/56 Unc-86 Ced non-Unc-50, and 6/56 non-Unc-86 Ced-7 Unc-50 recombinants from *unc-86 ced-7(n1892) unc-50*/*emb-9* heterozygotes segregated *emb-9*.

We determined the position of *ced-7* with respect to the RFLPs *eP7* and *stP127* as described by Ruvkun et al. (1989). In brief, we obtained N2-RW7000 recombinants in the *ced-7* region by mating *unc-86 ced-7(n1892) unc-50*/+++ males with RW7000 hermaphrodites to generate *unc-86 ced-7 unc-50*(N2)/+++(RW7000) heterozygotes. From these animals, Unc-86 non-Unc-50 and Unc-50 non-Unc-86 recombinants were picked. Progeny homozygous for each recombinant chromosome were maintained, and their genotypes at the *eP7* and *stP127* loci were determined by genomic Southern blots. The relative distances, indicated as the number of recombinant chromosomes in each interval as a fraction of the total number of chromosomes examined, were *unc-86*(9/18)*ced-7*(1/18)*eP7*(5/18)*stP127*(3/18)*unc-50*.

Transgenic Animals

For the genomic rescue experiments, we injected DNAs into *ced-7(n1892)* animals at concentrations of 25–50 μg/ml with the dominant roller marker pRF4 (50 μg/ml), as previously described (Mello et al., 1992). To determine the extent of rescue, we counted the cell corpses in the head of 4-fold-stage embryos from the stably transmitting lines, using Nomarski optics, as previously described (Ellis et al., 1991a). Nonrescued embryos have about 34 corpses. Embryos with 0–5 corpses were scored as rescued for the *ced-7* engulfment defect.

For the structure function analysis of the CED-7 protein NBDs, we coinjected DNAs at concentrations of 50 μg/ml into *ced-7(n1996); unc-76(e911)* animals with two transformation markers (50 μg/ml each), the *unc-76* rescuing plasmid p76-16B (Bloom and Horvitz, 1997) to establish transgenic lines and the *egl-5*::GFP plasmid pSC212 (A. Chisholm and H.R.H., unpublished data) to identify transgenic embryos.

Plasmid Construction

To make the mutant *ced-7*(K586R) cDNA construct, we first introduced a K586R change in the first NBD by the polymerase chain reaction (PCR) using the plasmid p83.c7, which contains a full-length *ced-7* cDNA, as a template and the oligonucleotides c7r1atp, CTCGAGAAGGTTGTACT*ACG*ACCAGCTCC, and c71tm2, AGAATTCCATCGAGCCCTCC, as primers. To make the mutant *ced-7*(K1417R) cDNA, we introduced a K1417R change in the second NBD by PCR using the primers c7r2atp, ACCGGTTAAAATATT GAATGTTGTAGT*ACG*TCCAGCTCC, and c72tmp2, TGCCACCAG CCACAATTGGA. The resulting PCR products were cloned into the pBluescript SK+ vector (Stratagene) at its EcoRV site.

The sequence-confirmed constructs were cut with BglII and XhoI for the *ced-7*(K586R) and with SnaBI for the *ced-7*(K1417R) PCR products, and the DNA fragments were cloned into the p83.c7 plasmid previously cut with appropriate enzymes to generate the constructs p83.c7(K586R) and p83.c7(K1417R), respectively. To generate the p83.c7(K586R, K1417R) construct, we excised the SnaBI fragment from p83.c7(K1417R) and inserted the fragment into P83.c7(K586R) via the SnaBI sites. To construct P_{ced-7} *ced-7* and its mutant variants, we excised p83.c7 and its mutant derivatives with BglII-HpaI and cloned the fragments into the *ced-7*-rescuing plasmid pC7KEN, which contains the 11 kb *ced-7* genomic fragment, previously cut with BglII and HpaI.

Antibodies and Immunostaining

We PCR-amplified a region of *ced-7* coding sequence from codon 1339 to codon 1704 with the plasmid p83.c7 as a template and the oligonucleotides GGAGATCTTGGACTGTGCGTCGATCT and CCAG ATCTTCAGACATGTGGAATGG as primers. The resulting 1.1 kb product was cut with BglII and cloned into the pGEX-2T (Pharmacia) and pATH11 *E. coli* expression vectors (Rimm and Pollard, 1989) via their BamHI sites. Both GST-CED-7 and TrpE-CED-7 fusion proteins were present in the inclusion bodies and were purified using standard methods (Harlow and Lane, 1988). The GST-CED-7 fusion protein was further purified using 7% SDS-PAGE. The correct band was excised from the gel following visualization by soaking gels in 0.3 M $CuCl_2$ and electroelution. Gel-purified CED-7 protein was mixed with RAS adjuvant (RIBI ImmunoChem Research) in PBS and injected into two rabbits at a dose of 0.4–1.0 mg of protein per injection. The anti-CED-7 antibodies were purified by binding to a nitrocellulose filter strip carrying TrpE-CED-7 fusion protein and eluting the specifically bound antibodies with 100 mM glycine-HCl (pH 2.5).

For Western blot analysis, embryos and worms were washed off plates with dH_2O and treated with hypochlorite to obtain embryos. The embryos were then sonicated in 1× SDS sample buffer, and the extracts were fractionated using 5% SDS-PAGE and transferred to nitrocellulose membranes. The CED-7 protein was visualized using horseradish peroxidase–conjugated secondary antibodies (Bio-Rad) and chemiluminescent detection reagents (Pierce).

For immunofluorescence detection of CED-7, embryos were fixed in 2% paraformaldehyde for 20 min at room temperature and permeablized as described by Guenther and Garriga (1996), and larvae and adults were fixed in 2% paraformaldehyde for 20 min at room temperature and permeablized according to the procedure described by Finney and Ruvkun (1990). The fixed animals were stained with a 1:50 dilution of purified CED-7 antibodies at 4°C overnight, washed three times with PBST-B (1× PBS, 0.1% BSA, 0.5% Triton-X-100, 1 mM EDTA), incubated with a 1:50 dilution of FITC-conjugated goat anti-rabbit IgG (Cappel) at 37°C for 2 hr, and washed as before. Stained worms were mounted in 1% DABCO (1,4-diazabicyclo[2.2.2]octane) and 70% glycerol and visualized using a Bio-Rad MRC-500 confocal microscope.

Analysis of *ced-7* Genetic Mosaics

We analyzed *ced-7* genetic mosaics generated by the strain MT9149, *ncl-1(e1865) unc-36(e251) ced-7(n1892); qDp3(ncl-1*(+) *unc-36*(+) *ced-7*(+)). The Unc-36 phenotype is produced if *qDp3* is lost in P0, AB, or ABp (Kenyon, 1986).

We raised animals at 25°C, since this temperature was reported to increase slightly the frequency of *qDp3* mitotic loss (Clark et al., 1993). We used Nomarski optics to screen L4 non-Unc animals from the strain MT9149 for mosaic animals in which some, but not all, cells had lost the duplication *qDp3* and were Ncl. We scored some or all of the following cells to determine the points of duplication loss. Points of loss and cells scored (in parentheses) were: MSaa (m3DL, m4DL, I3, and I4), MSapa (muscle and midbody ceolomocytes), MSappp (head muscle), MSappap (head muscle), MSpa (m3DR, m4DR, and M4), MSppa (head muscle and anterior coelomocytes), MSpppp (head muscle), MSpppap (head muscle), C (midbody muscle), D (head and anterior body muscle), and AB (m3L, m3VL, m3R, m4R, and excretory canal). We identified 50 mosaic animals from 3050 non-Unc animals screened. Since we isolated only one mosaic animal in which *qDp3* was lost in the germline lineage, we screened an additional 9935 L4 non-Unc progeny and focused on identifying such mosaic animals. Since the Ncl phenotype cannot be reliably scored in the germline, we isolated mosaic animals that had lost *qDp3* in the D lineage and generated only Unc progeny to ensure that *qDp3* was lost in the germline. We isolated four mosaic animals in this way. To investigate if *ced-7* function is dispensable in the AB lineage, we also scored L4 Unc progeny with non-Ncl cells in the P1 lineage. Such animals would have lost *qDp3* in the AB or ABp lineages. From about 6000 animals scored, we isolated two mosaic animals.

All L4 mosaic animals identified were isolated from slides and transferred to Petri dishes at 20°C for recovery. After allowing the animals to recover for 48 hr, we scored the Ncl phenotype of the gonadal sheath cells and the number of cell corpses in each gonadal arm of the animals using Nomarski optics. The presence of *qDp3* in P4 (the germline) was assessed by scoring the presence of phenotypically wild-type progeny. In almost all cases, the mosaicism observed can be explained by duplication loss at a single mitosis cell division.

Acknowledgments

We thank E. James for DNA sequencing; E. Hartwieg for assistance with EM ultrastructural studies; B. Conradt, T. Herman, B. Hersh, Z. Zhou, and D. Xue for comments on this manuscript; and G. Campbell for the *ced-7* allele *n3072* and E. Speliotes for *n3073*. H. R. H. is an Investigator of the Howard Hughes Medical Institute.

Received February 27, 1998; revised May 1, 1998.

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GenBank Accession Number

The GenBank accession number for the *ced-7* sequence is AF049142.

Note Added in Proof

In the accompanying paper, Liu and Hengartner (1998) describe molecular genetic studies of another *C. elegans* cell corpse engulfment gene, *ced-6*: Liu, Q.A., and Hengartner, M.O. (1998). Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in *C. elegans*. Cell, this issue, 961–972.