amplified by RT-PCR (One step, Life Technologies) from E10.5 *opb*[1], *opb*[2] and C57BL6/J embryos using forward and reverse primers (5′-TGGTTT GAGGGAGACAGTGTGG-3′ and 5′-CGCCATCTACATTCTGAGGAGCAC-3′, respectively). cDNAs from three embryos per genotype were separately amplified, subcloned and sequenced, yielding consistent results. An A to T change at position +115 and a C to T change at position +238 of the open reading frame (both causing nonsense mutations) were observed in *opb*[1] and *opb*[2] mutants, respectively. The change in *opb*[2] created a Taq1 restriction-fragment length polymorphism, which was assayed in genomic DNA using the marker *Rab23C238T* (forward: 5′-ATGCCCTCTGTGATGGAGACTG-3′; reverse: 5′-AACACCCCCGTCATT CAAAAC-3′) to confirm the linkage of *opb*[2] with the change at position +238. Genotyping with *Rab23C238T* confirmed the change in the genomic DNA of eight additional *opb*[2] homozygous embryos.

Received 21 January; accepted 1 June 2001.

1. Günther, T., Struwe, M., Aguzzi, A. & Schughart, K. *Open brain*, a new mouse mutant with severe neural tube defects, shows altered gene expression patterns in the developing spinal cord. *Development* **120,** 3119–3130 (1994).
2. Eggenschwiler, J. T. & Anderson, K. V. Dorsal and lateral fates in the mouse neural tube require the cell-autonomous activity of the *open brain* gene. *Dev. Biol.* **227,** 648–660 (2000).
3. Chiang, C. *et al.* Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383,** 407–413 (1996).
4. Goodrich, L. V. & Scott, M. P. Hedgehog and patched in neural development and disease. *Neuron* **21,** 1243–1257 (1998).
5. Matise, M. P. & Joyner A. L. *Gli* genes in development and cancer. *Oncogene* **18,** 7852–7859 (1999).
6. Ingham, P. W. Transducing Hedgehog: the story so far. *EMBO J.* **17,** 3505–3511 (1998).
7. Methot, N. & Basler, K. An absolute requirement for Cubitus interruptus in Hedgehog signaling. *Development* **128,** 733–742 (2001).
8. Kasarskis, A., Manova, K. & Anderson, K. V. A phenotype-based screen for embryonic lethal mutations in the mouse. *Proc. Natl Acad. Sci. USA* **95,** 7485–7490 (1998).
9. Milenkovic, L., Goodrich, L. V., Higgins, K. M. & Scott, M. P. Mouse *patched1* controls body size determination and limb patterning. *Development* **126,** 4431–4440 (1999).
10. Forbes, A. J., Nakano, Y., Taylor, A. M. & Ingham, P. W. Genetic analysis of hedgehog signalling in the *Drosophila* embryo. *Development* (Suppl.) **119,** 115–124 (1993).
11. Goodrich, L. V., Johnson, R. L., Milenkovic, L., McMahon, J. A. & Scott, M. P. Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse *patched* gene by Hedgehog. *Genes Dev.* **10,** 301–312 (1996).
12. Goodrich, L. V., Milenkovic, L., Higgins, K. M. & Scott, M. P. Altered neural cell fates and medulloblastoma in mouse *patched* mutants. *Science* **277,** 1109–1113 (1997).
13. Litingtung, Y. & Chiang, C. Specification of ventral neuron types is mediated by an antagonistic interaction between shh and gli3. *Nature Neurosci.* **10,** 979–985 (2000).
14. Echelard, Y. *et al.* Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430 (1993).
15. Mo, R. *et al.* Specific and redundant functions of *Gli2* and *Gli3* zinc finger genes in skeletal patterning and development. *Development* **124,** 113–23 (1997).
16. Lee, K. J., Dietrich, P. & Jessell, T. M. Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. *Nature* **403,** 734–740 (2000).
17. McMahon, J. A. *et al.* Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12,** 1438–1452 (1998).
18. Liem, K. F., Jessell, T. M. & Briscoe, J. Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* **127,** 4855–4866 (2000).
19. Olkkonen, V. M. *et al.* Isolation of a mouse cDNA encoding Rab23, a small novel GTPase expressed predominantly in the brain. *Gene* **138,** 207–211 (1994).
20. Pereira-Leal, J. B. & Seabra, M. C. The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functionalspecificity in the Ras superfamily. *J. Mol. Biol.* **301,** 1077–1087 (2000).
21. Ostermeier, C. & Brunger, A. T. Structural basis of Rab effector specificity: crystal structure of the small G protein Rab3A complexed with the effector domain of rabphilin-3A. *Cell* **96,** 363–374 (1999).
22. Bock, J. B., Matern, H. T., Peden, A. A. & Scheller, R. H. A genomic perspective on membrane compartment organization. *Nature* **409,** 839–841 (2001).
23. Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J. & Zerial, M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell. Biol.* **149,** 901–914 (2000).
24. Wilson, S. M. *et al.* A mutation in Rab27a causes the vesicle transport defects observed in ashen mice. *Proc. Natl Acad. Sci. USA* **97,** 7933–7938 (2000).
25. Hui, C. C. & Joyner, A. L. A mouse model of Greig cephalopolysyndactyly syndrome: the *Extra-toes*[J] mutation contains an intragenic deletion of the *Gli3* gene. *Nature Genet.* **3,** 241–246 (1993).
26. Capdevila, J., Pariente, F., Sampedro, J., Alonso, J. L. & Guerrero, I. Subcellular localization of the segment polarity protein patched suggests an interaction with the wingless reception complex in *Drosophila* embryos. *Development* **4,** 987–998 (1994).
27. Incardona, J. P. *et al.* Receptor-mediated endocytosis of soluble and membrane-tethered Sonic hedgehog by Patched-1. *Proc. Natl Acad. Sci. USA* **97,** 12044–12049 (2000).
28. Denef, N., Neubuser, D., Perez, L. & Cohen, S. M. Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **120,** 521–531 (2000).
29. Carstea, E. D. *Niemann-Pick C1* disease gene: homology to mediators of cholesterol homeostasis. *Science* **277,** 228–231 (1997).
30. Holtta-Vuori, M., Maatta, J., Ullrich, O., Kuismanen, E. & Ikonen, E. Mobilization of late-endosomal cholesterol is inhibited by Rab guanine nucleotide dissociation inhibitor. *Curr. Biol.* **10,** 95–98 (2000).

Acknowledgements

Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. We thank P. Beachy and M. Scott for discussions and for gifts of the *Shh* mice and *Patched–lacZ* mice; M. Rosen for discussions about Rab structure; and M. J. García-García, K. Brennan, J. Timmer, L. Niswander and T. Bestor for helpful comments on the manuscript. This work was supported by an NIH grant (K.V.A.), the Lita Annenberg Hazen Foundation and an ACS postdoctoral fellowship to J.T.E.

Correspondence and requests for materials should be addressed to K.V.A. (e-mail: k-anderson@ski.mskcc.org).

Phagocytosis promotes programmed cell death in *C. elegans*

Peter W. Reddien*, Scott Cameron*† & H. Robert Horvitz*

* *Howard Hughes Medical Institute, Department of Biology, 68-425, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA*

† *Division of Pediatric Hematology Oncology, Children's Hospital/Dana Farber Cancer Institute, Boston, Massachusetts 02115, USA*

In the nematode *Caenorhabditis elegans* programmed cell death requires the killer genes *egl-1*, *ced-4* and *ced-3* (refs 1 and 2), and the engulfment of dying cells requires the genes *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-10* and *ced-12* (refs 3–5). Here we show that engulfment promotes programmed cell death. Mutations that cause partial loss of function of killer genes allow the survival of some cells that are programmed to die, and mutations in engulfment genes enhance the frequency of this cell survival. Furthermore, mutations in engulfment genes alone allow the survival and differentiation of some cells that would normally die. Engulfment genes probably act in engulfing cells to promote death, as the expression in engulfing cells of *ced-1*, which encodes a receptor that recognizes cell corpses[6], rescues the cell-killing defects of *ced-1* mutants. We propose that engulfing cells act to ensure that cells triggered to undergo programmed cell death by the CED-3 caspase[7] die rather than recover after the initial stages of death.

During metazoan development, programmed cell death acts in the shaping of tissues, the refinement of neuronal connections, and the removal of unnecessary or damaged cells[8]. Cells dying by programmed cell death are recognized and rapidly engulfed by phagocytic cells[9]. In *C. elegans*, engulfment can begin even before the completion of the cell division that generates a cell programmed to die[10]. *C. elegans* genes that control the killing and engulfment processes of programmed cell death have been identified, and the molecular mechanisms underlying these processes in *C. elegans* have proven to be evolutionarily conserved[11,12]. On the basis of their genetic interactions, the engulfment genes fall into two partially redundant sets, *ced-1*, *ced-6*, *ced-7* and *ced-2*, *ced-5*, *ced-10* and *ced-12*, which have been proposed to define two parallel and partially redundant pathways (ref. 4; and Z. Zhou and H.R.H., unpublished results). The CED-7 ABC transporter[13] functions with the CED-1 SREC receptor in corpse recognition[6]. CED-6 is an adaptor-like protein[14] and may transduce signals from CED-1. The CED-2 CrkII, CED-5 DOCK180, and CED-10 Rac proteins have been proposed to act in a signalling pathway that controls cytoskeletal extensions of cell surfaces around dying cells during engulfment[15,16]. That cell corpses are formed in animals which have defective cell-corpse engulfment indicates that engulfment is not essential for cells to die by programmed cell death.

We sought new killer genes by screening for mutations (P.W.R. and H.R.H., unpublished results) that enhanced the defect in programmed cell death of the *ced-3* partial-loss-of-function mutation *n2427* (ref. 17). Unexpectedly, we isolated mutations in engulfment genes. We assayed defects in programmed cell death by counting the number of extra cell nuclei ('undead cells') in the anterior pharynges of *C. elegans*, in which 16 cell deaths normally

occur during embryogenesis[17]. Animals carrying *ced-3(n2427)* had an average of 1.5 extra cells in their anterior pharynges, whereas double mutants carrying *ced-3(n2427)* and mutations in engulfment genes had a greater number of extra cells in this region: 4.5–6.2 (Table 1, $P < 0.0001$, unpaired *t*-test). Mutations in the cell-corpse engulfment genes alone did not result in extra cells in the anterior pharynx (Table 1). The effects of engulfment mutations on cell killing were not limited to the pharynx, because double mutant animals carrying both *ced-3(n2427)* and an engulfment gene mutation displayed a substantially increased frequency of survival of V5R.paapp, a cell that dies postembryonically in the lateral ectoderm[9], compared with that of the single mutants (Table 1). For example, V5R.paapp never survived in *ced-3(n2427)* or *ced-6(n1813)* animals but survived in 40% of *ced-6(n1813); ced-3(n2427)* animals.

The engulfment mutation *ced-1(e1735)* enhanced multiple *ced-3* alleles, *ced-4* alleles (for example, *ced-4(n3312)* for V5R.paapp, $P < 0.0001$, unpaired *t*-test), and an *egl-1* allele ($P < 0.0001$, unpaired *t*-test) (Table 1). Mutations in all known engulfment genes enhanced *ced-3(n2427)* (Table 1). The engulfment genes *ced-1*, *ced-2*, *ced-5*, *ced-6* and *ced-10* appear to act in engulfing cells for cell-corpse phagocytosis[6,14–16], and *ced-7* appears to act in both engulfing and dying cells[13]. That mutations in all engulfment genes, including those of both pathways, enhanced the cell-death defects caused by *ced-3(n2427)* suggests that some aspect of the engulfment process itself is involved in the killing of cells.

We also examined the effects of engulfment gene mutations on cell death in the ventral nerve cord. Postembryonic development in

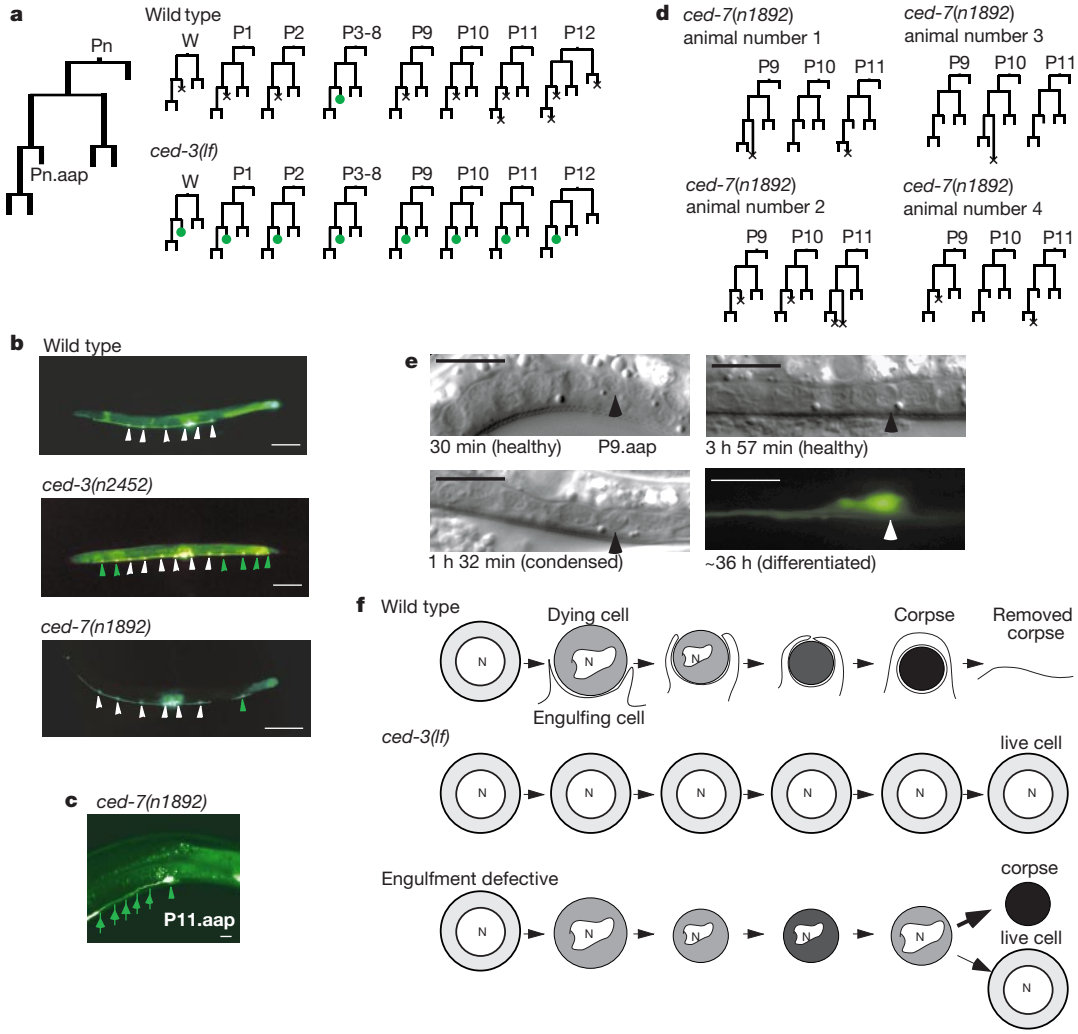

Figure 1 Engulfment mutations lead to the survival of cells programmed to die in the ventral cord. **a**, 13 blast cells (W and P1–P2) divide post-embryonically to generate motor neurons of the ventral cord[9]. P3.aap–8.aap (the posterior daughter of the anterior daughter of the anterior daughter of the P3–P8 blast cells) differentiate into VC motor neurons and express GFP from the *lin-11* promoter. W.ap, P1.aap, P2.aap and P9.aap–P12.aap undergo programmed cell death. In a *ced-3* mutant, W.ap, P1.aap, P2.aap and P9–P12.aap survive and differentiate into VC-like neurons and express GFP from the *lin-11* promoter (see **b**). **b**, A wild-type adult displayed fluorescence from P3.aap–P6.aap, vulval tissue, and P7.aap–P8.aap. A *ced-3(n2452)* adult additionally displayed fluorescence from the undead P1.aap, P2.aap, and P9.aap–P12.aap cells. Expression from undead W.ap and P1.aap is variable. A *ced-7(n1892)* adult displayed fluorescence in P11.aap. White arrowheads, P3.aap–P8.aap; green arrowheads, Pn.aap cells that failed to die. Anterior, left. Ventral, down. Scale bar, 100 μm. **c**, P11.aap in a *ced-7(n1892)* animal survived, expressed $P_{lin\text{-}11}gfp$, and extended an axon. Arrowhead, cell body. Green arrows follow the P11.aap axon trajectory. Anterior, left. Ventral, down. Scale bar, 10 μm. **d**, Cell lineages of *ced-7(n1892)* animals had normal division patterns but abnormalities in cell death. The deaths of P9.aap in animal number 1, P11.aap in animal number 2, and P10.aap in animal number 3 were delayed. In cases in which a cell lineally fated to die failed to do so, animals were observed for at least three hours after the last cell division occurred. **e**, A P9.aap cell in a *ced-7(n1892)* animal showed morphological characteristics of cell death 1 h 32 min after the cell was born. This cell recovered a normal nuclear morphology by 4 h and 36 h later, expressed GFP from the *lin-11* promoter. Black arrowhead, P9.aap. Anterior, left. Ventral, down. Scale bar, 10 μm. **f**, Model for the effects of engulfment defects on cell killing. In *ced-3(lf)* animals cell death is not initiated. Engulfment is necessary for the efficient execution of death and in engulfment-defective animals cell death is initiated but cells typically attempt to recover and occasionally survive. N, nucleus.

the ventral cord involves 13 neuroblasts, termed W, and P1–P12, which together generate 10 cells that undergo programmed cell death[9]. Six of the ventral-cord cells that die are Pn.aap cells (posterior daughters of the anterior daughters of the anterior daughters of P blast cells), specifically, those generated by the P1, P2 and P9–P12 lineages; by contrast, the Pn.aap cells generated by the P3–P8 lineages survive and differentiate into class VC motor neurons[9] (Fig. 1a). We found that the gene *lin-11*, which encodes a LIM-domain transcription factor[18] and is expressed in VC motor neurons (G. Acton, S.C. and H.R.H. unpublished observations), was also expressed in the undead VC-equivalent cells (W.ap, P1.aap, P2.aap and P9–12.aap) of a *ced-3* mutant (Fig. 1a, b), suggesting that these undead cells adopted a VC-like fate. We used the P_{lin-11}*gfp* reporter *nIs96* (see Methods) to assay the effects of engulfment mutations on Pn.aap cell deaths.

Mutations in each of the engulfment genes resulted in the low-penetrance survival of Pn.aap cells that normally die (Table 2). (That the *lin-11* promoter does not express until the L4 larval stage (our unpublished observations) and the deaths of the Pn.aap cells occur approximately 24 h earlier in the late L1/early L2 larval stage indicated that the ventral-cord fluorescence we observed was the result of transcription and translation in an undead VC-like cell rather than the persistence of green fluorescent protein (GFP) in a cell corpse.) Pn.aap cells that failed to die in engulfment mutants showed characteristics of differentiated VC neurons: neuronal nuclear morphology, elongated axons and expression of *lin-11* (Fig. 1c). We confirmed that the fluorescent cells we observed were undead VC-like neurons by examining the patterns of ventral-cord corpses in *ced-1(e1735)* L2 larvae using Nomarski microscopy, allowing these animals to develop into adults, and then determining the subsequent patterns of P_{lin-11}*gfp*-expressing cells (data not shown).

To determine if the undead VC-like cells in engulfment mutants expressed any morphological signs of initiating programmed cell death (and also to confirm directly that the ectopic fluorescent cells were undead Pn.aap cells), we directly observed the P cell lineages of *ced-7(n1892)* mutants. Although the P cell division patterns were normal, there were abnormalities in programmed cell deaths (Fig. 1d). In many cases the cells that failed to die initially displayed some of the morphological characteristics of dying cells, that is, cytoplasmic and nuclear condensation and loss of nuclear refractility. These morphological features were episodic and fluctuated between condensed and non-condensed states, eventually resulting in either a delay in the formation of a normal cell corpse or in the ultimate appearance of a normal-looking VC neuron (Fig. 1e). We observed similar episodic morphological changes in *ced-7(n1996)* and *ced-6(n1813)* mutants (data not shown). As cells that failed to die initially displayed some characteristics of dying cells, we suggest that engulfment is not necessary for the initiation of cell death but rather for the efficient execution of death once it has been initiated. We propose that engulfment actively promotes the killing process rather than passively eliminates the opportunity for recovery. In the latter case, we might expect that in the absence of engulfment the cell-death process would proceed normally up to a certain stage, but that the cells occasionally would subsequently recover. By contrast, we observed that in the absence of engulfment, cells that normally undergo programmed cell death instead often underwent episodic changes in morphology and sometimes appeared to fail to express any sign whatsoever of normal cell-corpse morphology, indicating that the cell-death process itself was abnormal in these cells. For example, two cells indicated in Fig. 1d (P11.aaap in *ced-7(n1892)* animal number 3 and P10.aap in animal number 4) showed no or minor characteristics of dying cells. CED-3 caspase activity is necessary for the initiation of the morphological changes we observed, because these changes, which appear to reflect attempts at cell death, did not occur in *ced-3(n2452)* ventral-cord lineages (data not shown). (*n2452* is a *ced-3* allele deleted for the protease-

Table 1 Mutations in engulfment genes enhance cell survival

Genotype	Percentage of animals with undead V5R.paapp cells*	Number of extra cells (anterior pharynx)†
N2	0	0.1 ± 0.05
ced-3(n2452)	100	10.8 ± 0.32
ced-1(e1735)	4	0.1 ± 0.06
ced-6(n1813)	0	0.3 ± 0.09
ced-7(n1892)	8	0.1 ± 0.05
ced-2(e1752)‡	0	0.0 ± 0.03
ced-5(n1812)	0	0.0 ± 0.00
ced-10(n1993)§	4	ND
ced-12(n3261)	0	0.0 ± 0.0 ($n = 25$)
ced-7(n1892); ced-5(n1812)	12	ND
ced-3(n2427)‖	0	1.5 ± 0.14 ($n = 47$)
ced-1(e1735); ced-3(n2427)‖	48	5.9 ± 0.36
ced-6(n1813); ced-3(n2427)‖	40	5.9 ± 0.25
ced-7(n1892); ced-3(n2427)‖	56	6.2 ± 0.34
ced-2(e1752); ced-3(n2427)‖	32	4.5 ± 0.21
ced-5(n1812); ced-3(n2427)‖	36	5.0 ± 0.36
ced-10(n1993); ced-3(n2427)§‖	44	ND
ced-12(n3261); ced-3(n2427)	40	5.6 ± 0.24 ($n = 25$)
ced-7(n1892); ced-5(n1812); ced-3(n2427)‖	84	ND
ced-3(n2438)‖	0	2.0 ± 0.20
ced-1(e1735); ced-3(n2438)‖	64	6.5 ± 0.31
ced-3(n2447)	15 ($n = 27$)	1.6 ± 0.14 ($n = 70$)
ced-1(e1735); ced-3(n2447)	57 ($n = 30$)	3.0 ± 0.27 ($n = 36$)
ced-7(n1892); ced-3(n2447)	80	4.4 ± 0.25 ($n = 37$)
ced-3(n2443)	8	1.9 ± 0.22
ced-1(e1735); ced-3(n2443)	20	3.5 ± 0.27
ced-4(n3312)	40 ($n = 45$)	2.1 ± 0.22
ced-1(e1735); ced-4(n3312)	80 ($n = 46$)	2.3 ± 0.22
ced-4(n3195)¶	23 ($n = 30$)	1.6 ± 0.24 ($n = 25$)
ced-1(e1735); ced-4(n3195)‖	80 ($n = 30$)	1.8 ± 0.36 ($n = 25$)
ced-4(n3158)	93 ($n = 15$)	3.7 ± 0.36 ($n = 20$)
ced-1(e1735); ced-4(n3158)	100 ($n = 15$)	4.6 ± 0.39 ($n = 20$)
egl-1(n3331)#	3 ($n = 30$)	3.7 ± 0.27 ($n = 35$)
ced-1(e1735); egl-1(n3331)#	47 ($n = 30$)	5.4 ± 0.33 ($n = 35$)

* The number of extra cells in the right postdeirid of L4 larvae was determined using Nomarski optics. In the wild type four cells are present, and one cell undergoes programmed cell death. Data are percentages. Sample size $n = 25$ unless otherwise shown.
† The number of extra cells in the anterior pharynges of L3 larvae was determined using Nomarski optics. In the wild type, 16 cells undergo programmed cell death in this region. Data are means ± standard error of the means. Sample size $n = 30$ unless otherwise shown.
Strains contained the following additional mutations: ‡*him-5(e1490)*; §*him-8(e1489)*; ‖*unc-30(e191)*; ¶*unc-69(e587)*; and #*nIs96*.
ND, not determined. Pharyngeal cell numbers could not be determined in these animals because of abnormal cell positions.

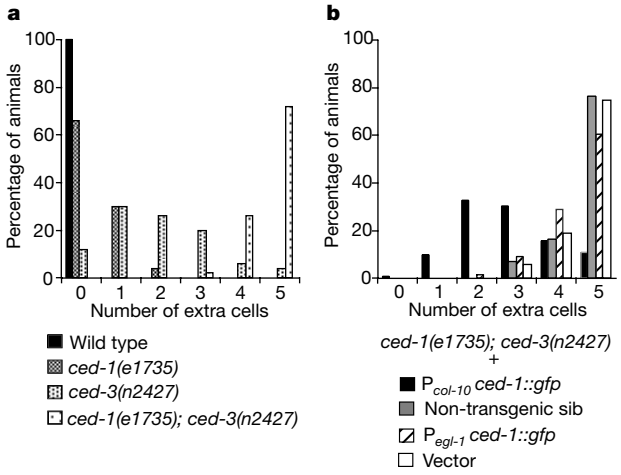

Figure 2 *ced-1* acts in engulfing cells to control cell killing. Distributions of the percentages of animals with specific numbers of extra cells in the ventral cord, as determined using P_{lin-11}*gfp* and observing fluorescent cell numbers in the regions where P2.aap and P9.aap–P12.aap reside. **a**, *ced-1(e1735)* caused some survival and enhanced the frequency of cell survival conferred by *ced-3(n2427)* in the ventral cord. 50 animals of each genotype were scored. **b**, P_{col-10}*ced-1::gfp* (engulfing cell expression) rescued the enhancement of *ced-3(n2427)* by *ced-1(e1735)* ($n = 122$). Non-transgenic siblings of transgenic P_{col-10}*ced-1::gfp* animals ($n = 127$), P_{egl-1}*ced-1::gfp* (dying cell expression) animals ($n = 66$), and P_{col-10}*gfp* (vector) animals ($n = 79$) showed distributions similar to that of *ced-1(e1735); ced-3(n2427)* animals in **a**.

encoding region of the *ced-3* gene[17].) Perhaps if a dying cell fails to reach some critical point, the cellular alterations that result from CED-3 caspase activity can be reversed and cells can survive and differentiate (Fig. 1f).

Do engulfment genes promote death by acting in engulfing or in dying cells? Dying cells in the ventral cord are engulfed by hypodermal cells[10], and expression of *ced-1* in the hypodermis can rescue *ced-1(lf)* engulfment defects[6]. *ced-1(e1735)* enhanced cell survival in the ventral cords of *ced-3(n2427)* animals (Fig. 2a), so we expressed *ced-1* in the hypodermis using the promoter of the *col-10* gene (V. Ambros, personal communication) ($P_{col\text{-}10}$*ced-1*; ref. 6) and in dying cells using the promoter of the *egl-1* gene[19] ($P_{egl\text{-}1}$*ced-1*; ref. 6) in *ced-1(e1735); ced-3(2427)* animals and determined the number of VC-like cells using the $P_{lin\text{-}11}$*gfp* reporter. Expression of *ced-1* in the hypodermis, but not in dying cells, rescued the enhancement of *ced-3(n2427)* conferred by *ced-1(e1735)* (Fig. 2b). We suggest that *ced-1*, and presumably other engulfment genes as well, acts within engulfing rather than within dying cells to promote death.

Does engulfment contribute to cell killing by inhibiting the protective function of the anti-apoptotic gene *ced-9*, which encodes a protein similar to the human anti-apoptotic protein Bcl-2 (ref. 20)? We tested whether *ced-1* mutations could enhance *ced-3(n2427)* in the absence of *ced-9* function. Defects in engulfment can significantly decrease killing even in the absence of *ced-9* function: *ced-9(n2812); ced-3(n2427)* animals had 6.3 extra cells in their pharynges, whereas *ced-1(e1735); ced-9(n2812); ced-3(n2427)* animals had 9.8 extra cells (Table 3) ($P < 0.0001$, unpaired *t*-test). Both *ced-9(n2812)* and *ced-1(e1735)* are probably null alleles[6,20]. This observation suggests that engulfment does not kill cells by inhibiting the protective function of *ced-9* but rather acts downstream of or parallel to *ced-9* protection. In addition to its protective function *ced-9* also has a killing function: whereas *ced-9* loss-of-function mutations result in the deaths of cells that should live, these mutations also enhance the cell-killing defects conferred by partial *ced-3* loss-of-function mutations[21]. The promotion of programmed cell death by engulfment appears to occur by a mechanism that is at least in part independent of the killing function of *ced-9*, as *ced-9(lf); ced-3(lf)* engulfment-defective mutants were more defective in killing than were *ced-9(lf); ced-3(lf)* mutants (Table 3).

Engulfment-promoted killing also acts at least in part independently of the killing activity of *ced-8*, which controls the timing of cell death and, like engulfment, contributes to cell killing downstream of or parallel to *ced-9* protection[22]. Specifically, *ced-1(e1735)* enhanced the killing defect of *ced-3(n2427)* in the absence of *ced-8* (Table 3) ($P < 0.0001$, unpaired *t*-test). We suggest that engulfment, like *ced-8*, defines one of multiple downstream and partially redundant cell-killing activities.

Table 3 Engulfment killing acts downstream of *ced-9* and parallel to *ced-8*

Genotype	Percentage of animals with undead V5R.paapp cells*	Number of extra cells (anterior pharynx)†
ced-8(n1891)	0	1.1 ± 0.17
ced-1(e1735); ced-8(n1891)	4	1.7 ± 0.22 ($n = 40$)
ced-3(n2427)‡	0	1.5 ± 0.14 ($n = 47$)
ced-1(e1735); ced-3(n2427)‡	48	5.9 ± 0.36
ced-3(n2427); ced-8(n1891)‡	0	5.7 ± 0.29 ($n = 45$)
ced-1(e1735); ced-3(n2427); ced-8(n1891)‡§	92	8.4 ± 0.31 ($n = 45$)
ced-9(n2812); ced-3(n2427)	0	6.3 ± 0.46
ced-9(n2812); ced-3(n2427); ced-8(n1891)	4	9.3 ± 0.40
ced-1(e1735); ced-9(n2812); ced-3(n2427)	80	9.8 ± 0.36

* The number of cells in the right postdeirid of L4 larvae was determined using Nomarski optics. In the wild type four cells are present, and one cell undergoes programmed cell death. Data are percentages. Sample size $n = 25$.
† The number of extra cells in the anterior pharynges of L3 larvae was determined using Nomarski optics. In the wild-type, 16 cells undergo programmed cell death in this region. Data are means ± standard error of the means. Sample size $n = 30$ unless otherwise shown.
Strains also contained the following mutations: ‡*unc-30(e191)*; §*sem-4(n1378)*.

The death of at least one cell type that normally dies during *C. elegans* development may be completely dependent on engulfment. The male-specific left–right homologues B.alapaav and B.arapaav can survive in engulfment-defective mutant animals[3] and can also survive if their engulfing cell, P12.pa, is ablated using a laser microbeam[23]. We confirmed that B.alapaav/B.arapaav dies in an engulfment-dependent manner and found that this death requires the function of the CED-3 caspase (data not shown). That engulfment can lead to a caspase-dependent death suggests that engulfing cells can trigger a signal transduction pathway that contributes to caspase activation and perhaps other aspects of programmed cell death. Consistent with the hypothesis that engulfing cells can signal changes within dying cells, the activities of two engulfment genes necessary for cell-corpse recognition, *ced-1* and *ced-7* (ref. 6), are required for the generation of free DNA 3′-hydroxyl ends within dying cells, a process that can occur without cell-corpse engulfment[24] and that is a hallmark of apoptotic cells[25].

We propose that engulfing cells promote the suicides of many and possibly all cells triggered to initiate programmed cell death. As described above, in the case of B.alapaav/B.arapaav an engulfing cell is necessary for death. As in *C. elegans*, in mammals dying cells are rapidly recognized by other cells and engulfed. The removal or

Table 2 Engulfment mutations result in the survival of cells that normally die in the ventral cord

Genotype	Number of corpses (range)*	Average number of extra VC-like cells†	Percentage of animals with surviving Pn.aap cells‡
$P_{lin\text{-}11}$*gfp*	0.0 ± 0.0	0.0 ($n = 50$)	0
ced-3(n2452); $P_{lin\text{-}11}$*gfp*	0.0 ± 0.0	5.0 ($n = 50$)	100
ced-1(n1995); $P_{lin\text{-}11}$*gfp*	6.3 ± 3.9	0.08 ($n = 123$)	7
ced-1(n1951); $P_{lin\text{-}11}$*gfp*	20.8 ± 4.1	0.21 ($n = 100$)	21
ced-1(e1735); $P_{lin\text{-}11}$*gfp*	24.8 ± 3.0	0.36 ($n = 150$)	31
ced-2(n1994); $P_{lin\text{-}11}$*gfp*	19.9 ± 4.2	0.35 ($n = 100$)	29
ced-5(n2098); $P_{lin\text{-}11}$*gfp*	24.2 ± 3.8	0.24 ($n = 243$)	21
ced-5(n1812); $P_{lin\text{-}11}$*gfp*	31.2 ± 3.1	0.44 ($n = 100$)	35
ced-6(n1813); $P_{lin\text{-}11}$*gfp*	20.2 ± 3.1	0.57 ($n = 150$)	45
ced-7(n1996); $P_{lin\text{-}11}$*gfp*	35.0 ± 5.7§	0.95 ($n = 253$)	67
ced-7(n2001); $P_{lin\text{-}11}$*gfp*	32.6 ± 3.6§	1.47 ($n = 100$)	79
ced-7(n1892); $P_{lin\text{-}11}$*gfp*	42.9 ± 3.8§	1.42 ($n = 150$)	80
ced-10(n1993); $P_{lin\text{-}11}$*gfp*	15.5 ± 4.0	0.24 ($n = 150$)	24
ced-10(n3246); $P_{lin\text{-}11}$*gfp*	29.6 ± 4.7	0.47 ($n = 135$)	31
ced-12(n3261); $P_{lin\text{-}11}$*gfp*	24.5 ± 3.7	0.31 ($n = 100$)	31
ced-1(e1735); ced-2(n1994); $P_{lin\text{-}11}$*gfp*	37.7 ± 4.7‖	0.48 ($n = 127$)	37

* Average numbers of persistent cell corpses were determined in the heads of at least 25 L1 larvae at the four-cell gonad stage using Nomarski microscopy. Error indicates standard deviation.
† Average numbers of fluorescent cells caused by expression from $P_{lin\text{-}11}$*gfp*, *nls96*, in P2, 9, 10, 11, and 12-derived regions were determined using a stereomicroscope equipped with an ultraviolet light source (Kramer Scientific).
‡ The percentages of animals that had at least one fluorescent cell in the P2, 9, 10, 11, and 12-derived regions were determined.
§ Cell corpses in the heads of *ced-7* mutant animals were counted in four-fold embryos, because corpse number in *ced-7* mutant larvae decreases sharply with time[4].
‖ *ced-1(e1735); ced-2(n1994)* double mutants had similar defects in cell-corpse engulfment in the ventral cords of *ced-1(e1735)* mutants (6.2 ± 0.2 ($n = 30$) and 6.1 ± 0.1 ($n = 60$) out of seven possible corpses in the posterior ventral cords, respectively).

inhibition of macrophages can disrupt the remodelling of tissues in the mouse eye or tadpole tail regression, processes that involve apoptosis[26,27]. Furthermore, the elimination of macrophages in the anterior chamber of the rat eye results in the survival of vascular endothelial cells that normally undergo apoptosis[28]. These findings suggest a potential role for macrophages in promoting the deaths of cells during tissue regression and suggest that engulfment may be involved in facilitating programmed cell death in some tissues in vertebrates. If so, the pharmacologic inhibition of engulfment in humans may be of therapeutic benefit in situations in which cells are poised between survival and death, such as in ischemic regions surrounding areas of infarction or in slow neurodegenerative diseases[8]. □

Methods

Strains and genetics

All strains were grown at 20 °C on NGM (nematode growth medium) agar with *Escherichia coli* OP50 bacteria. The wild-type strain was N2. The following alleles were used (the molecular nature of the alleles of cell-death genes is also listed): LGI: *sem-4(n1378); ced-12(n3261)*, loss of function (Z. Zhou and H.R.H., unpublished results), *ced-1(e1735)*, Q375opal[6]; *ced-1(n1951)*, C501Y[6]; *ced-1(n1995)* P124L[6]. LGIII: *ced-4(n3158)*, S163F (B. Hersh, S.C. and H.R.H., unpublished); *ced-4(n3195)*, R63H (S.C. and H.R.H., unpublished); *ced-4(n3312)*, A394T (B. Hersh, P.W.R. and H.R.H., unpublished); *ced-6(n1813)*, deletion[14]; *ced-7(n1892)*, loss of function[13]; *ced-7(n1996)*, R5ochre[13]; *ced-7(n2001)*, W1540ochre[13]; *unc-69(e587); ced-9(n2812)*, Q46amber[20]. LGIV: *ced-2(e1752)*, W153opal G192E[16]; *ced-2(n1994)*, R102opal[16]; *ced-10(n1993)*, V190G[16]; *ced-10(n3246)*, G60R[16]; *him-8(e1489); ced-5(n1812)*, E28opal[15]; *ced-5(n2098)*, splice mutation[15]; *unc-30(e191); ced-3(n2427)*, G474R[17]; *ced-3(n2438)*, G474R[17]; *ced-3(n2443)*, P400S[17]; *ced-3(n2447)*, S446L[17]; *ced-3(n2452)*, deletion[17]. LGV: *him-5(e1490), egl-l(n3331)*, A59P (S.C. and H.R.H., unpublished results), *nIs96*. LGX: *ced-8(n1891)*, W219amber[22]; *lin-15(n765ts), nIs106*. Extra cells and cell corpses were visualized using Nomarski optics. VC neurons expressing GFP were visualized using a dissecting light microscope equipped with an ultraviolet light source (Kramer Scientific). Cell lineage analysis was performed as previously described[9].

Generation of the $P_{lin\text{-}11}gfp$ reporter

To construct the $P_{lin\text{-}11}gfp$ reporter we fused the *gfp* gene to an *Nsi*I fragment of genomic DNA containing 5.2 kb of 5′ sequence and the ATG of the *lin-11* gene[18]. Reporter DNA was injected at 50 ng μl^{-1} into *lin-15(n765)* mutants using the *lin-15*(+) plasmid pL15EK[29] as a rescuing co-injection marker. Non-Muv (that is, non-Lin-15) animals with bright expression of GFP in the VC neurons were irradiated with 4,000 rad from a cobalt source, and chromosomal integrants were identified as animals that stably transmitted the transgene to all progeny (that is, all progeny were non-Muv). The *nIs96* and *nIs106* integrants were backcrossed to N2 six times, and the integration sites were mapped to LGV and LGX, respectively.

Cell autonomous rescue

$P_{col\text{-}10}$*ced-1::gfp*, $P_{egl\text{-}1}$*ced-1::gfp*, and $P_{col\text{-}10}gfp$ were previously described[6]. We injected these constructs as 75 ng μl^{-1} with *ttx-3::gfp* (ref. 30) at 50 ng μl^{-1} as the co-injection marker into *ced-1(e1735); ced-3(n2427); nIs96* animals. Transgenic animals were identified by GFP fluorescence in the AIY.

Received 19 February; accepted 8 May 2001.

1. Ellis, H. M. & Horvitz, H. R. Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* **44,** 817–829 (1986).
2. Conradt, B. & Horvitz, H. R. The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell* **93,** 519–529 (1998).
3. Hedgecock, E. M., Sulston, J. E. & Thomson, J. N. Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science* **220,** 1277–1279 (1983).
4. Ellis, R. E., Jacobson, D. M. & Horvitz, H. R. Genes required for the engulfment of cell corpses during programmed cell death in *Caenorhabditis elegans*. *Genetics* **129,** 79–94 (1991).
5. Chung, S., Gumienny, T. L., Hengartner, M. O. & Driscoll, M. A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in *C. elegans*. *Nature Cell Biol.* **2,** 931–937 (2000).
6. Zhou, Z., Hartwieg, E. & Horvitz, H. R. CED-1 is a transmembrane receptor that mediates cell corpse engulfment in *C. elegans*. *Cell* **104,** 43–56 (2001).
7. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. & Horvitz, H. R. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* **75,** 641–652 (1993).
8. Vaux, D. L. & Korsmeyer, S. J. Cell death in development. *Cell* **96,** 245–254 (1999).
9. Sulston, J. E. & Horvitz, H. R. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol.* **56,** 110–156 (1977).
10. Robertson, A. M. G. & Thomson, J. N. Morphology of programmed cell death in the ventral nerve cord of *C. elegans* larvae. *J. Embryol. Exp. Morphol.* **67,** 89–100 (1982).
11. Metzstein, M. M., Stanfield, G. M. & Horvitz, H. R. Genetics of programmed cell death in *C. elegans*: Past, present and future. *Trends Genet.* **14,** 410–416 (1998).
12. Albert, M. L., Kim, J. I. & Birge, R. B. alpha Vβ5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. *Nature Cell Biol.* **2,** 899–905 (2000).
13. Wu, Y. C. & Horvitz, H. R. The *C. elegans* cell corpse engulfment gene *ced-7* encodes a protein similar to ABC transporters. *Cell* **93,** 951–960 (1998).
14. Liu, Q. A. & Hengartner, M. O. Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in *C. elegans*. *Cell* **93,** 961–972 (1998).
15. Wu, Y. C. & Horvitz, H. R. *C. elegans* phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. *Nature* **392,** 501–504 (1998).
16. Reddien, P. W. & Horvitz, H. R. CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*. *Nature Cell Biol.* **2,** 131–136 (2000).
17. Shaham, S., Reddien, P. W., Davies, B. & Horvitz, H. R. Mutational analysis of the *Caenorhabditis elegans* cell-death gene *ced-3*. *Genetics* **153,** 1655–1671 (1999).
18. Freyd, G., Kim, S. K. & Horvitz, H. R. Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. *Nature* **344,** 876–879 (1990).
19. Conradt, B. & Horvitz, H. R. The TRA-1A sex determination protein of *C. elegans* regulates sexually dimorphic cell deaths by repressing the *egl-1* cell death activator gene. *Cell* **98,** 317–327 (1999).
20. Hengartner, M. O. & Horvitz, H. R. *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* **76,** 665–676 (1994).
21. Hengartner, M. O. & Horvitz, H. R. Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. *Nature* **369,** 318–320 (1994).
22. Stanfield, G. M. & Horvitz, H. R. The *ced-8* gene controls the timing of programmed cell deaths in *C. elegans*. *Mol. Cell* **5,** 423–433 (2000).
23. Sulston, J. E., Albertson, D. G. & Thomson, J. N. The *Caenorhabditis elegans* male: postembryonic development of nongonadal structures. *Dev. Biol.* **78,** 542–576 (1980).
24. Wu, Y. C., Stanfield, G. M. & Horvitz, H. R. NUC-1, a *Caenorhabditis elegans* DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis. *Genes Dev.* **14,** 536–548 (2000).
25. Wyllie, A. H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555–556 (1980).
26. Lang, R. A. & Bishop, J. M. Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell* **74,** 453–462 (1993).
27. Little, G. H. & Flores, A. Inhibition of programmed cell death by catalase and phenylalanine methyl ester. *Comp. Biochem. Physiol. Physiol.* **105,** 79–83 (1993).
28. Diez-Roux, G. & Lang, R. A. Macrophages induce apoptosis in normal cells in vivo. *Development* **124,** 3633–3638 (1997).
29. Clark, S. G., Lu, X. & Horvitz, H. R. The *Caenorhabditis elegans* locus *lin-15*, a negative regulator of a tyrosine kinase signaling pathway, encodes two different proteins. *Genetics* **137,** 987–997 (1994).
30. Hobert, O. *et al.* Regulation of interneuron function in the *C. elegans* thermoregulatory pathway by the *ttx-3* LIM homeobox gene. *Neuron* **19,** 345–357 (1997).

Acknowledgements

We thank B. Galvin, B. Hersh and Z. Zhou for comments. P.W.R. was supported by a National Science Foundation Fellowship. S.C. was supported by a Howard Hughes Medical Institute (HHMI) postdoctoral fellowship, a Merck/MIT collaborative fellowship, and an NIH training grant. H.R.H. is an Investigator of the HHMI.

Correspondence and requests for materials should be addressed to H.R.H. (e-mail: horvitz@mit.edu).

Engulfment genes cooperate with *ced-3* to promote cell death in *Caenorhabditis elegans*

Daniel J. Hoeppner*†‡, Michael O. Hengartner* & Ralf Schnabel§

** Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, New York 11724, USA*
† Graduate Program in Genetics, Department of Molecular Genetics and Microbiology, SUNY at Stony Brook, Stony Brook, New York 11794, USA
§ Institut fuer Genetik, TU Braunschweig, Spielmannstrasse 7, D-38106 Braunschweig, Germany

Genetic studies have identified over a dozen genes that function in programmed cell death (apoptosis) in the nematode *Caenorhabditis elegans*[1–3]. Although the ultimate effects on cell survival or engulfment of mutations in each cell death gene have been extensively described, much less is known about how these mutations affect the kinetics of death and engulfment, or the interactions between these two processes. We have used four-dimensional-Nomarski time-lapse video microscopy to follow in detail how cell death genes regulate the extent and kinetics of

‡ Present address: NINDS-LMB, Building 36, National Institutes of Health, Bethesda, Maryland 20892-4092, USA.