Both the Caspase CSP-1 and a Caspase-Independent Pathway Promote Programmed Cell Death in Parallel to the Canonical Pathway for Apoptosis in *Caenorhabditis elegans*

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Abstract

Caspases are cysteine proteases that can drive apoptosis in metazoans and have critical functions in the elimination of cells during development, the maintenance of tissue homeostasis, and responses to cellular damage. Although a growing body of research suggests that programmed cell death can occur in the absence of caspases, mammalian studies of caspase-independent apoptosis are confounded by the existence of at least seven caspase homologs that can function redundantly to promote cell death. Caspase-independent programmed cell death is also thought to occur in the invertebrate nematode *Caenorhabditis elegans*. The *C. elegans* genome contains four caspase genes (ced-3, csp-1, csp-2, and csp-3), of which only ced-3 has been demonstrated to promote apoptosis. Here, we show that CSP-1 is a pro-apoptotic caspase that promotes programmed cell death in a subset of cells fated to die during *C. elegans* embryogenesis. csp-1 is expressed robustly in late pachytene nuclei of the germline and is required maternally for its role in embryonic programmed cell deaths. Unlike CED-3, CSP-1 is not regulated by the APAF-1 homolog CED-4 or the BCL-2 homolog CED-9, revealing that csp-1 functions independently of the canonical genetic pathway for apoptosis. Previously we demonstrated that embryos lacking all four caspases can eliminate cells through an extrusion mechanism and that these cells are apoptotic. Extruded cells differ from cells that normally undergo programmed cell death not only by being extruded but also by not being engulfed by neighboring cells. In this study, we identify in csp-3; csp-1; csp-2 ced-3 quadruple mutants apoptotic cell corpses that fully resemble wild-type cell corpses: these caspase-deficient cell corpses are morphologically apoptotic, are not extruded, and are internalized by engulfing cells. We conclude that both caspase-dependent and caspase-independent pathways promote apoptotic programmed cell death and the phagocytosis of cell corpses in parallel to the canonical apoptosis pathway involving CED-3 activation.

Introduction

The elimination of unnecessary or dangerous cells is fundamental to development, tissue homeostasis and disease mitigation in multicellular organisms. The primary mechanism of cell elimination is apoptosis, a form of cell suicide that was originally defined by evolutionarily conserved morphological characteristics that include chromatin condensation, shrinkage of the cytoplasmic volume and membrane blebbing [1] and by biochemical features like phosphatidylserine exposure and DNA fragmentation [2,3]. Apoptosis serves as a highly controlled mechanism for the removal and degradation of damaged or unnecessary cells, and blocking apoptosis can lead to catastrophic forms of cell death, such as necrosis, which can cause dangerous inflammatory responses [4]. The discovery of the CED-3 caspase as a cell-autonomous executor of programmed cell death in the nematode *Caenorhabditis elegans* led to the paradigm that the caspase family of cysteine proteases drives apoptosis through the cleavage of substrate proteins at specific peptide sequences [5,6]. Indeed, caspases have evolutionarily conserved roles in apoptosis throughout metazoa [7].

Despite the compelling causal link between caspases and apoptosis, a growing body of evidence indicates that apoptosis can occur in the absence of caspases [4]. For example, mouse cells lacking Apaf-1, an activator of the apical caspase Caspase-9, which in turn activates effector caspases, can undergo apoptosis in response to pro-apoptotic stimuli [8]. In the presence of caspase inhibitors, TNF (tumor necrosis factor) can induce a form of cell death termed necroptosis, which exhibits characteristics of both necrosis and apoptosis [4,9]. The mitochondrial flavoprotein AIF (apoptosis-inducing factor) is thought to promote apoptotic cell death in mammals even in the presence of caspase inhibitors [10]. Furthermore, cell death with aspects of apoptotic morphology occurs in non-metazoans, including unicellular eukaryotes and...
Author Summary

Caspases are cysteine proteases that in many cases drive apoptosis, an evolutionarily conserved and highly stereotyped form of cellular suicide with functions in animal development and tissue maintenance. The dysregulation of apoptosis can contribute to diseases as diverse as cancer, autoimmunity, and neurodegeneration. Caspases are often thought to be required for, or even to define, apoptosis. Although there is evidence that apoptosis can occur in the absence of caspase activity, caspase-independence can be difficult to prove, as most animals have multiple caspases. The nematode Caenorhabditis elegans has four caspases, CED-3, CSP-1, CSP-2, and CSP-3. CED-3 has a well-established role in apoptosis, but less is known about the functions of the CSP caspases. In this study, we show that CSP-1 promotes apoptosis in the developing C. elegans embryo and that CSP-1 is regulated differently than its homolog CED-3. Furthermore, we show that apoptosis and the engulfment of dying cells can occur in mutants lacking all four caspases, proving that neither apoptosis nor cell-corpse engulfment require caspase function and that caspase-independent activities can contribute to apoptosis of some cells during animal development.

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Results

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Figure 1. The B and/or C isoforms of csp-1 promote programmed cell death. (A) Representations of the intron-exon organization of the three known csp-1 mRNA isoforms (A, B, and C). Red bars indicate the csp-1 deletion alleles used in this study; arrowheads indicate the SACRG sequence that encodes the caspase active-site. The graphic was generated using the Intron-Exon Graphic Maker (N. Bhatia; www.wormweb.org). (B) Extrachromosomal arrays carrying a wild-type genomic fragment of the csp-1 locus or a mutant version that expresses only the B or C isoforms can rescue the csp-1(n4967) mutant phenotype. The csp-1 PD-only transgene contains two nonsense mutations that encode early stop codons affecting the B and C mRNA isoforms; the csp-1A-only transgene contains a mutation that changes the B/C start codon to an alanine codon; and the csp-1B/C-only transgene contains two nonsense mutations that encode early stop codons affecting the A isoform. The csp-1 transgenes were injected into csp-1(n4967); ced-3(n2436) animals, and the resulting independent lines were assayed for csp-1 rescuing activity by counting the number of extra undead cells in the anterior pharynx. The transgenes and their constructions are described in detail in Materials and Methods, and the complete data for each transgenic line are provided in Table S2. (C) RNAi knockdown of csp-1 phenocopies the csp-1 mutations. dsRNAs targeting the csp-1 pro-domain or the csp-1B isoform were in vitro transcribed and injected into the gonads of RNAi-sensitive rrf-3(pk1426); ced-3(n2436) adult hermaphrodites. Progeny of the injected adults were assayed for extra undead cells in the anterior pharynx. PD, prodomain.

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Pvec: csp-1 PD gene that expresses GFP in the six touch neurons (AVM, two ALM, PVM and two PLM neurons) in addition to the FLP and PVD neurons, we examined csp-1 mutants for missing cells that normally survive. We observed that csp-1(n4967) mutants contained a full complement of touch neurons and pharyngeal cells (Table S1). We also noted that csp-1(n4967) failed to cause ectopic cell deaths in sensitized animals carrying the loss-of-function mutation n2812 in the anti-apoptotic gene ced-9, a homolog of human BCL2 (Table S1; data not shown). These results indicate that csp-1 does not have an obvious anti-apoptotic function in the soma. Consistent with a previous report that csp-2 does not affect somatic cells [23], a mutation in csp-2 did not cause ectopic cell deaths in the somatic cells we examined (Table S1). However, we failed to observe the ectopic cell deaths in csp-3 mutants previously reported [22]. Ectopic somatic cell deaths have also been noted in animals with loss-of-function mutations in ced-9 [25] or tat-1 [26,27], which encodes an aminophospholipid translocase required for the asymmetric distribution of phosphatidylserine on the inner leaflet of the plasma membrane. As expected, we found that ced-9 mutant larvae were missing pharyngeal cells and many touch neurons; more than 80% of PLM neurons were not present in ced-9(n2812) larvae (Table S1). However, we failed to detect the previously reported ectopic cell-death defect of tat-1 mutants (ref. [26,27]; Table S1); we used the same deletion alleles for csp-3 and tat-1 and assayed the same cells that had been characterized in the previous studies.

To determine whether the C. elegans caspase homologs csp-1, csp-2 or csp-3 promote programmed cell death in the soma, we examined animals carrying csp deletion mutations for extra cells that failed to undergo programmed cell death in the anterior pharynx. As many as 16 extra cells can be counted in the anterior pharynges of mutants with strong defects in programmed cell death, e.g., ced-3(n2692) (ref. [20]; Table 1). Single mutations in csp-1, csp-2 or csp-3 failed to cause detectable defects in programmed cell death (Table 1; data not shown). However, we observed that mutations in csp-1 (but not csp-2 or csp-3) caused the survival of pharyngeal cells in sensitized strains carrying a weak mutation in the caspase gene ced-3 (Table 1). The partial loss-of-function ced-3 mutations n2427 and n2436 cause slight and intermediate defects in apoptosis, respectively (ref [17]; Table 1; data not shown). The n4967 and n5133 mutations, both of which delete the putative active site of CSP-1 (Figure 1A), enhanced the cell-death defects of ced-3(n2427) and ced-3(n2436) mutants, increasing the number of extra cells in their anterior pharynges on average by 1.4 and 2.4 cells, respectively (Table 1). These results are consistent with our RNAi experiments in which csp-1 PD dsRNA (which likely inactivated all csp-1 transcripts) was injected into the gonads of rrf-3(pk1426); ced-3(n2436) animals and caused an enhanced cell-death defect in their progeny (Figure 1C); we used the nfd-3 mutation to increase sensitivity to RNAi [29]. The cell-death defect conferred by the csp-1(n4967) mutation was rescued by extrachromosomal arrays carrying a 9 kb genomic csp-1 fragment that included the entire csp-1 coding region, 1.5 kb of genomic sequence 3’ of the csp-1A translational start codon and 3.5 kb of genomic sequence 5’ of the csp-1A/B translational stop codon (Figure 1B; Table S2). These results demonstrate that csp-1 encodes a detectable cell-killing activity that contributes to programmed cell death in C. elegans. Mutation of csp-2 and/or csp-3 neither enhanced nor suppressed the cell-death defects of strains mutant for csp-1 and/or ced-3 (Table 1; Table S3), suggesting that csp-1 and ced-3 are the only C. elegans caspase genes with functions in somatic programmed cell deaths.

The development of the anterior part of the C. elegans pharynx involves 16 programmed cell deaths, all of which appear to be
sensitive to \textit{ced-3} [17,28,30]. To test whether specific pharyngeal programmed cell deaths required \textit{csp-1}, we used GFP reporters to visualize the survival of cells fated to die, specifically the sister cells of the M4 and NSM neurons. \textit{csp-1} was partially required in \textit{csp-3(n2436)}, and double mutants with each \textit{csp} allele, \( p \) values were considered significant if less than 0.01 to correct for multiple comparisons.

\begin{table}
\centering
\caption{The caspase homolog \textit{csp-1} promotes programmed cell death in the \textit{C. elegans} anterior pharynx.}
\begin{tabular}
{|l|l|l|l|}
\hline
\textbf{genotype} & \textbf{extra cells per anterior pharynx \( \pm \) SD} & \textbf{n} & \textbf{p value} \\
\hline
The deletion of \textit{csp-1}, \textit{csp-2} or \textit{csp-3} alone does not cause a defect in programmed cell death. & & & \\
\hline
\textit{wild-type} & 0.1 \pm 0.3 & 14 & - \\
\hline
\textit{ced-3(n3692)}\textsuperscript{1} & 11.3 \pm 1.1 & 14 & <0.00001 \\
\hline
\textit{csp-1(n4967)} & 0.3 \pm 0.4 & 16 & n.s. \\
\hline
\textit{csp-1(n5133)} & 0.1 \pm 0.2 & 19 & n.s. \\
\hline
\textit{csp-1(tm917)} & 0.1 \pm 0.3 & 16 & n.s. \\
\hline
\textit{csp-2(n4871)}\textsuperscript{1} & 0.2 \pm 0.4 & 12 & n.s. \\
\hline
\textit{csp-3(n4872)}\textsuperscript{1} & 0.3 \pm 0.6 & 21 & n.s. \\
\hline
\hline
\textbf{Deletion of \textit{csp-1}, but not \textit{csp-2} or \textit{csp-3}, enhances the defects in programmed cell death caused by partial loss-of-function alleles of \textit{ced-3} and \textit{ced-4}.} & & & \\
\hline
\textit{ced-3(n2427)}\textsuperscript{1} & 1.7 \pm 1.2 & 22 & - \\
\hline
\textit{csp-1(n4967); ced-3(n2427)}\textsuperscript{2} & 3.0 \pm 1.3 & 38 & 0.0001 \\
\hline
\textit{csp-1(n5133); ced-3(n2427)} & 3.2 \pm 1.5 & 21 & 0.0008 \\
\hline
\textit{csp-1(tm917); ced-3(n2427)}\textsuperscript{2} & 2.6 \pm 1.3 & 46 & 0.004 \\
\hline
\textit{csp-2(n4871); ced-3(n2427)}\textsuperscript{1} & 1.0 \pm 0.8 & 20 & n.s. \\
\hline
\textit{csp-3(n4872); ced-3(n2427)}\textsuperscript{1} & 1.5 \pm 1.2 & 16 & n.s. \\
\hline
\textit{csp-3; csp-2; ced-3(n2427)}\textsuperscript{1} & 1.9 \pm 1.5 & 18 & - \\
\hline
\textit{csp-3; csp-1(n4967); csp-2; ced-3(n2427)} & 3.2 \pm 1.2 & 17 & 0.008 \\
\hline
\textit{ced-3(n2436)}\textsuperscript{1} & 6.2 \pm 1.4 & 37 & - \\
\hline
\textit{csp-1(n4967); ced-3(n2436)} & 8.6 \pm 1.6 & 29 & <0.00001 \\
\hline
\textit{csp-1(n5133); ced-3(n2436)} & 8.7 \pm 1.5 & 19 & <0.00001 \\
\hline
\textit{csp-1(tm917); ced-3(n2436)}\textsuperscript{2} & 7.4 \pm 1.6 & 52 & <0.00001 \\
\hline
\textit{csp-2(n4871); ced-3(n2436)}\textsuperscript{2} & 5.4 \pm 1.0 & 16 & n.s. \\
\hline
\textit{csp-3(n4872); ced-3(n2436)}\textsuperscript{1} & 5.6 \pm 1.5 & 15 & n.s. \\
\hline
\textit{csp-3; csp-2; ced-3(n2436)}\textsuperscript{1} & 4.9 \pm 1.7 & 16 & - \\
\hline
\textit{csp-3; csp-1(n4967); csp-2; ced-3(n2436)} & 8.2 \pm 1.4 & 16 & <0.00001 \\
\hline
\textit{ced-4(n3158)}\textsuperscript{1} & 5.0 \pm 2.6 & 29 & - \\
\hline
\textit{csp-1(n4967); ced-4(n3158)}\textsuperscript{1} & 6.5 \pm 2.7 & 32 & 0.03 \\
\hline
\end{tabular}
\end{table}

\textsuperscript{1}Homozygous for the integrated transgene \textit{nl106[\textit{Pm-1::gfp}].}
\textsuperscript{2}Includes animals that were either homozygous for \textit{unc-75(+)} or \textit{unc-75(e950)).
\textsuperscript{3}Homozygous for \textit{dpy-20(e1282ts).}
The \textit{csp-1} locus generates three known mRNA isoforms [15], all of which include the sequence that encodes the presumptive caspase active site (Figure 1A). The \textit{csp-1A} transcript contains a long prodomain not present in the other transcripts, and it uses an alternative start site that is 3 kb 5' to the start site of the \textit{csp-1B} and \textit{csp-1C} isoforms. To determine which isoforms are required for the cell-killing activity of \textit{csp-1}, we performed experiments in which the \textit{csp-1} rescuing transgene was mutated to express: (1) the \textit{A} isoform only, (2) the \textit{B} and \textit{C} isoforms only, or (3) a truncated version of \textit{csp-1A} including only the prodomain (PD). Extrachromosomal arrays engineered to express only \textit{csp-1-PD} or the \textit{csp-1A} isoform failed to rescue the cell death defect of \textit{csp-1(n4967)} mutants (Figure 1B; Table S2). By contrast, a \textit{csp-1} transgene lacking the \textit{csp-1A} translation start codon and predicted to express only the \textit{PD} isoform rescued the \textit{csp-1(n4967)} mutants (Figure 1B; Table S2). Consistent with these results, transgenes expressing the \textit{csp-1B} cDNA, but not the \textit{csp-1A} cDNA, under the control of the \textit{mec-7} promoter efficiently killed touch neurons (Figure 2A–2B; Table 2; data not shown); we also expressed the \textit{csp-1C} cDNA under the control of the \textit{mec-7} promoter and failed to observe killing of the touch neurons (data not shown).
not shown). Ectopic expression of csp-1B from the ser-2d and flp-15 promoters killed the OLL and I2 neurons, respectively (ref. [31]; N. Bhatla and H.R. Horvitz, unpublished results). However, we noted that tm917, a csp-1 allele that deletes coding regions of only the csp-1A transcript, enhanced significantly (albeit weakly) the cell-death defects of ced-3(n2427) and ced-3(n2436) mutants, increasing the number of extra cells in their anterior pharynges by 0.9 and 1.2 cells, respectively (Table 1). dsRNA targetting the csp-1A prodomain (csp-1-PD) caused a similar slight enhancement of the cell-death defect of ced-3(n2436) mutants (Figure 1C), suggesting that, in addition to the more robust cell-killing activity of the csp-1B transcript, csp-1A might have a weak cell-killing function.

csp-1B encodes a pro-apoptotic caspase

The proteolytic activity of caspases requires an active-site cysteine. Previously, it was shown that the CSP-1B protein can proteolytically process CED-3 in vitro and that this enzymatic activity required the active-site (SACRG) cysteine of CSP-1B, C138 [15]. We tested in vivo whether C138 was necessary by assaying the touch neuron-killing activity of mutant Pmec-7::csp-1B transgenes in which C138 was changed to a serine. We observed that the ectopic cell deaths were entirely dependent on the caspase active site (Table 2). Thus, csp-1B promotes cell death via caspase activity. The cell deaths induced by a Pmec-7::csp-1B transgene resulted in cell corpses with apoptotic characteristics (Figure 2C–D). When observed with Nomarski optics, the csp-1B-induced cell deaths exhibited a refractile button-like appearance (Figure 2C) similar to that of developmental programmed cell deaths. Transmission electron micrographs of the cell corpses showed some contraction of the cytoplasmic volume and considerable condensation of the nuclear chromatin (Figure 2D), which are general characteristics of apoptotic cells, including those generated by ced-3 cell-killing transgenes (ref. [32]; data not shown). We conclude that csp-1B encodes a functional caspase that promotes programmed cell deaths with apoptotic morphology.

csp-1B cell-killing activity is independent of ced-9 and ced-4

CED-3, like most caspases, is expressed as an inactive zymogen with an inhibitory N-terminal prodomain. Trans-auto-proteolysis

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**Table 2.** Ectopic expression of csp-1B from the mec-7 promoter can kill touch neurons, and this killing activity requires the conserved cysteine in the putative caspase active site.

<table>
<thead>
<tr>
<th>genotype</th>
<th>% survival</th>
<th>n</th>
<th>AVM</th>
<th>ALML/R</th>
<th>PVM</th>
<th>PLML/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type [nls290]</td>
<td>59</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Pmec-7::csp-1B Line #1 [nls307]</td>
<td>52</td>
<td>71</td>
<td>61</td>
<td>40</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Pmec-7::csp-1B Line #2 [nls308]</td>
<td>41</td>
<td>49</td>
<td>32</td>
<td>27</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Pmec-7::csp-1B Line #3 [nls309]</td>
<td>60</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pmec-7::csp-1B(C138S) Line #1 [nls368]</td>
<td>23</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Pmec-7::csp-1B(C138S) Line #2 [nls369]</td>
<td>23</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Pmec-7::csp-1B(C138S) Line #3 [nls370]</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
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n, number of animals assayed; for each animal, six touch neurons (AVM, ALML, ALMR, PVM, PLML and PLMR) were scored for survival using the Pmec-3::gfp reporter transgene.

*Each strain contained the transgene Pmec-7::csp-1B, which expressed GFP in the FLP, AVM, ALM, PVM, PVD and PLM neurons.

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of the CED-3 pro-protein at two aspartate residues removes the pro-domain and yields two subunits that form the active caspase [33]. CED-3 auto-activation is dependent on its prodomain and is facilitated by the association of two CED-3 pro-proteins within an octameric complex formed with the Apa-1 homolog CED-4 [34–36]. Under normal cellular conditions, CED-4 is sequestered by CED-9 at mitochondria through a direct protein-protein interaction [37–39]. In response to upstream pro-apoptotic signals and the consequent expression of the BH3-domain-only protein EGL-1, which binds to and inhibits CED-9 [40], CED-4 is released from CED-9 and translocates to the nuclear periphery [37,41], where it facilitates CED-3 activation [38]. Thus, the activation of CED-3 is controlled by an apoptosis pathway involving a BH3-domain-only protein, a member of the Bel-2 family of apoptosis regulators, and a homolog of the apoptosome complex protein Apa-1. The basic elements of this apoptosis pathway are evolutionarily conserved in mammals and are responsible for the activation of caspases in response to cell-intrinsic apoptotic stimuli [7].

Consistent with the role of ced-9 in negatively regulating ced-3 activation, it was previously shown that null mutations of ced-9 enhance the touch neuron-killing activities of P<sub>mas</sub>:p-csp-1A transgenes [32]. (These experiments were performed using a ced-3(null) background to suppress the ced-3-dependent inviability of ced-9(null) animals.) Furthermore, this enhancement is dependent on ced-4 [32], indicating that the absence of CED-9 activates endogenous CED-4 within the touch neurons and that CED-4 activation elevates CED-3 activity. Unlike the CED-3 zymogen, CSP-1B lacks a long prodomain, suggesting that it might be activated via an alternative mechanism (i.e., independently of CED-4 and CED-9). To determine whether these canonical apoptosis regulators control CSP-1B activation, we introduced the ced-9(n2812) mutation into ced-3(n3692) strains carrying P<sub>mas</sub>:p-csp-1B transgenes and assessed the effect of this ced-9 null mutation on PLM survival. In contrast to its effects on P<sub>mas</sub>:p-ced-3 mediated PLM killing, ced-9(n2812) failed to enhance PLM killing in P<sub>mas</sub>:p-csp-1B strains with a ced-3(n3692) mutant background (Figure 3A). Instead, ced-9(n2812) partially suppressed csp-1B-mediated PLM death (Figure 3A). CED-9 has a poorly understood pro-apoptotic activity in addition to its anti-apoptotic role in CED-4 inhibition [42], and it is possible that this ced-9 pro-apoptotic activity contributed to the deaths of cells expressing ectopic CSP-1B. Nevertheless, our results indicate that csp-1B-mediated cell killing, unlike ced-3-mediated cell killing, is negatively regulated by ced-9 and suggest that CSP-1B is activated independently of CED-9.

We also observed that the expression of a P<sub>mas</sub>:p-csp-1A transgene in ced-3(null) mutant strains failed to cause PLM cell death, even in a ced-9(null) background (Figure S1). These results suggest that the CSA-1 isoform (which contains a long prodomain similar to that of CED-3) does not promote programmed cell death, even in the absence of the anti-apoptotic protein CED-9. A role for csp-1C in cell death cannot be excluded entirely, as it is possible that endogenous CSA-1A requires a co-factor not present in the touch neurons to mediate cell killing.

Since CSP-1B can proteolytically cleave CED-3 <em>in vitro</em> [15], we tested whether the csp-1B cell-killing activity requires the endogenous ced-3 and ced-4 genes. The ced-3(n3692) and ced-4(n1162) mutations weakly suppressed csp-1B-mediated PLM death (Figure S2A), and it is possible that the endogenous ced-3 and ced-4 genes can in part promote programmed cell death through ced-3. Nonetheless, most csp-1B cell-killing activity was independent of ced-4 and ced-3 (Figure S2B). Loss of endogenous csp-1 failed to suppress PLM death in strains carrying P<sub>mas</sub>:p-ced-3 or P<sub>mas</sub>:p-ced-4 transgenes (Figure S3C–S3D). Together, our results are consistent with a model in which csp-1B promotes programmed cell death at least mostly independently of and in parallel to the canonical apoptosis pathway (Figure 3E).

csp-1 expression in the maternal germline contributes to embryonic programmed cell death

To determine which <i>C. elegans</i> cells express csp-1, we directly visualized endogenous csp-1 transcripts via fluorescence <em>in situ</em> hybridization (FISH) experiments using Cy5- and ALEXA-labelled probes complementary to the csp-1B transcript (i.e., targeted to all csp-1 transscripts) or to the csp-1A prodomain (specific to the csp-1A transcript). To our surprise, csp-1 mRNA was not detectable in the somatic cells of wild-type or egf-1(n1984 n3082) mutant embryos, larvae or adult hermaphrodites (data not shown). By contrast, csp-1 transcripts were present in the germines of L4-stage larval and adult hermaphrodites (Figure 4A–4B). This expression was restricted to the late pachytene stage of meiosis I in both L4 larval gonads (in pachytenic nuclei adjacent to differentiating sperm) and adult gonads (in pachytenic nuclei adjacent to the bend of the gonad arm) (Figure 4A–4B). Both csp-1A and csp-1B/C transcripts were expressed in the adult pachytene germ cells, as indicated by the presence of FISH foci recognized by the csp-1A prodomain probes and foci recognized primarily by the csp-1B probes and only weakly by the csp-1A probes (Figure 4C). Stochastic and ionizing radiation (IR)-induced germine cell deaths occur during the late pachytene stage of oocyte development in adult gonads [43,44]. However, csp-1 (unless ced-3 was not required for either stochastic or IR-induced germine apoptosis, even in ced-3(n2436) strains sensitized for defects in germ-cell death (Figure 4D). In these experiments, apoptotic germ cells were identified using a transgene that expresses a functional GFP::CED-1 fusion protein that envelopes dying cells engulfed by the gonadal sheath [45,46]. We also failed to detect differences in either stochastic or IR-induced germine cell death between csp-1 mutants and wild-type animals in experiments in which apoptotic germ cells were quantified by acridine orange staining or by direct observation of their refractile morphology using Nomarski optics (data not shown). We also noted that the level of csp-1 transcript expression in the germline (as determined by FISH) was not affected by either ionizing radiation or by mutation of egf-1 or ced-3 (data not shown).

Since we detected csp-1 expression in the adult germine but not in somatic cells of the embryo, we tested whether maternally supplied csp-1 transcript was necessary for the zygotically functioning csp-1 in programmed cell death. Indeed, in sensitized genetic backgrounds (ced-3(n2427) and ced-3(n2436), csp-1(+) progeny of csp-1(n4967) hermaphrodites (M+Z+) animals had more undeformed pharyngeal cells than the csp-1(−) progeny of csp-1(+) hermaphrodites (M+Z+ animals) or the csp-1(n4967) progeny of csp-1(−) hermaphrodites (M+Z− animals) (Table 5). Thus, csp-1 expressed in the maternal germine is necessary for the csp-1 pro-apoptotic activity in embryonic programmed cell deaths. Given that we could not detect csp-1 expression in either embryos or larvae, it is therefore not surprising that the postembryonic programmed cell deaths of the ventral cord and postdeirid sensilla were unaffected by mutation of csp-1 (Table S4).

Programmed cell deaths occur in animals completely lacking all caspase genes

Most programmed cell deaths in <i>C. elegans</i> require ced-3 [20]. However, some cells die in mutants completely lacking ced-3. We previously reported that a subset of cells fated to die can be eliminated from ced-3 mutant embryos via a cell-shedding
mechanism [19]. In that study, we noted that cell shedding from ced-3 mutants occurs independently of csp-1, csp-2 and csp-3; quadruple mutants lacking all four caspases also generate shed cells, indicating that cell elimination by this mechanism is completely caspase-independent [19]. Like most programmed cell deaths, the cells generated by caspase-independent extrusion are apoptotic in appearance. However, unlike caspase-dependent cell corpses, shed cells do not undergo phagocytosis by engulfing cells. The death of the male linker cell, which also occurs independently of ced-3, is non-apoptotic and requires the heterochronic protein LIN-29, its binding partner MAB-10 [47], and the polyglutamine repeat protein PQN-41 (ref. [18,24]; Table S5). Previously it was shown that this cell death occurs in double-mutant males in which ced-3 and an additional csp gene (csp-1, csp-2 or csp-3) were inactivated [18]. We have now examined males lacking all four caspases and observed that the linker cell died in 100% of csp-3;
The *csp-3; csp-1; csp-2 ced-3* quadruple mutants were viable and fertile. Thus, both zygotic and maternal caspase contributions were eliminated. Our results therefore confirm that this cell death is indeed completely caspase-independent.

In addition, cell corpses are visible in the heads of larvae carrying null alleles of *ced-3* (ref. [17]; Table 4). All programmed cell deaths in the developing heads of wild-type animals occur embryonically and are engulfed and degraded prior to hatching (ref. [30,48]; Table 4). To detect *ced-3*-independent programmed cell deaths in larval heads, we used mutations (e.g., *ced-1(e1735)*, *ced-6(n2095)* or *ced-7(n1996)*) that cause defects in cell-corps engulfment and result in the persistence of many embryonic cell corpses into larval stages (ref. [49,50]; Table 4). Like most wild-type cell corpses, the *ced-3*-independent cell corpses were refractile in appearance as observed with Nomarski optics and were not extruded from the animal (data not shown). We also observed that larvae mutant for *ced-4* or *egl-1* contained similar cell corpses, demonstrating that their generation does not require the canonical pro-apoptotic pathway that mediates most programmed cell deaths (Table 4).

We tested whether the small number of cell corpses visible in *ced-3* larval heads are generated by the other *C. elegans* caspase genes and found that all double, triple and quadruple caspase mutants that we examined contained a small number of refractile corpses (Table 4). For example, 39% of *csp-3; csp-1* mutant animals contained at least one refractile cell corpse (Table 4), indicating that...
these programmed cell deaths occur in animals lacking all C. elegans caspases. We observed caspase-independent cell corpses in different regions of the larval head, including positions internal and external to the pharynx, which suggests that multiple cell lineages – at low frequencies – generated caspase-independent cell corpses. Surprisingly, we discovered that engulfment-competent ced-3 and ced-3; csp-1; csp-2 ced-3 mutants also contained refractile cell corpses (Table 4). The number of cell corpses per ced-3 or ced-3; csp-1; csp-2 ced-3 larva increased until 12 to 24 hours post hatching (see below; data not shown), indicating that at least some of the cell deaths occurred after embryogenesis. Given that all programmed cell deaths in the head normally occur embryonically and that cell corpses are never observed in the heads of wild-type larvae, we concluded that timing of cell deaths in these ced-3 mutants was delayed. Thus, caspase-independent cell corpses can undergo an inefficient programmed cell death with slow kinetics in the absence of CED-3 activity, indicating that these cells likely die via CED-3-mediated apoptosis in wild-type animals.

Caspase-independent cell corpses exhibit apoptotic morphology

Despite the strong causal link between caspase activation and apoptosis, recent studies have demonstrated that many morphological and biochemical changes associated with apoptosis can occur in the absence of caspases [4,19,21]. For example, in C. elegans the shed cells of csp-3; csp-1; csp-2 ced-3 quadruple mutants exhibit phosphatidylserine exposure, expression of the pro-apoptotic Bcl-2 homologue ced-3, and DNA cleavage [22,23].

---

**Table 3.** csp-1 is maternally required for programmed cell deaths that occur embryonically in the presumptive anterior pharynx.

<table>
<thead>
<tr>
<th>zygotic genotype</th>
<th>maternal genotype</th>
<th>extra cells per ant. pharynx ± SD</th>
<th>n</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ced-3(n2427)</td>
<td>ced-3(n2427)</td>
<td>1.0 ± 0.9</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>csp-1(n4967); ced-3(n2427)</td>
<td>csp-1(n4967); ced-3(n2427)</td>
<td>3.2 ± 1.2</td>
<td>20</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>csp-1(n4967); ced-3(n2427)</td>
<td>ced-3(n2427)</td>
<td>1.7 ± 1.0</td>
<td>20</td>
<td>0.018</td>
</tr>
<tr>
<td>ced-3(n2436)</td>
<td>ced-3(n2436)</td>
<td>6.2 ± 1.2</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>csp-1(n4967); ced-3(n2436)</td>
<td>csp-1(n4967); ced-3(n2436)</td>
<td>8.0 ± 1.8</td>
<td>18</td>
<td>0.002</td>
</tr>
<tr>
<td>csp-1(n4967); ced-3(n2436)</td>
<td>ced-3(n2436)</td>
<td>6.3 ± 1.3</td>
<td>18</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

*aHeterozygous for unc-30(e191)/+.
*bHeterozygous for unc-75(e950)/+.

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**Table 4.** Cell deaths occur in the absence of all C. elegans caspase genes.

<table>
<thead>
<tr>
<th>genotype</th>
<th>n</th>
<th>% with ≥1 corpse</th>
<th>corpses per head ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ced-1(e1735)</td>
<td>23</td>
<td>100</td>
<td>21.3 ± 5.3</td>
</tr>
<tr>
<td>ced-1; ced-3(n3692)</td>
<td>49</td>
<td>27</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>ced-1; csp-1; ced-3(n3692)</td>
<td>29</td>
<td>100</td>
<td>3.4 ± 4.7</td>
</tr>
<tr>
<td>ced-1; egl-1(n1084 n3082)</td>
<td>26</td>
<td>50</td>
<td>0.6 ± 0.7</td>
</tr>
<tr>
<td>ced-1; csp-1(n4967)</td>
<td>24</td>
<td>100</td>
<td>20.7 ± 5.6</td>
</tr>
<tr>
<td>ced-1; csp-2(n4871)</td>
<td>30</td>
<td>27</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>ced-1; csp-3(n4872); ced-1(n3692)</td>
<td>24</td>
<td>21</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>ced-1(n4967); csp-2(n4871)</td>
<td>21</td>
<td>100</td>
<td>30.1 ± 4.4</td>
</tr>
<tr>
<td>ced-7; ced-3(n3692)</td>
<td>27</td>
<td>23</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td>csp-3(n4872); ced-7</td>
<td>19</td>
<td>100</td>
<td>30.3 ± 4.3</td>
</tr>
<tr>
<td>csp-3(n4967); ced-7; ced-3(n3692)</td>
<td>27</td>
<td>37</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td>csp-3(n4872); ced-7; ced-3(n3692)</td>
<td>32</td>
<td>34</td>
<td>0.4 ± 0.6</td>
</tr>
<tr>
<td>ced-6(n2095)</td>
<td>24</td>
<td>100</td>
<td>19.8 ± 4.3</td>
</tr>
<tr>
<td>ced-3(n4967); ced-6; csp-2(n4871)</td>
<td>36</td>
<td>39</td>
<td>0.4 ± 0.6</td>
</tr>
<tr>
<td>wild-type</td>
<td>28</td>
<td>0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>ced-3(n3692)</td>
<td>43</td>
<td>14</td>
<td>0.1 ± 0.4</td>
</tr>
<tr>
<td>ced-3(n2452)</td>
<td>27</td>
<td>41</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td>csp-3(n4872); ced-7; ced-3(n3692)</td>
<td>25</td>
<td>16</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>csp-3(n4967); ced-7; ced-3(n2452)</td>
<td>34</td>
<td>26</td>
<td>0.3 ± 0.4</td>
</tr>
</tbody>
</table>

The number of refractile cell corpses per head was counted in L1 larvae within one hour of hatching.

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apoptotic BH3-only gene egl-1, and chromatin condensation [19]. To determine whether these apoptotic attributes are evident in caspase-independent programmed cell deaths that do not involve extrusion of the dying cell from the embryo, we characterized the cell corpses visible in caspase-deleted larvae (Figure 5 and Figure 6). In most of these experiments, we used strains with the wild-type csp-3 allele, because (1) csp-3 lacks a caspase active-site [15]; (2) although previous studies reported that csp-3 has an anti-apoptotic function in somatic cells [22], we were unable to replicate those findings (Table S1); and, (3) the presence or absence of a csp-3 mutation had no effect on the frequency or appearance of caspase-independent corpses (Table 4; Figure 6B; data not shown). Like ced-3-mediated programmed cell deaths in wild-type animals, the caspase-independent corpses expressed egl-1, the upstream activator of the canonical apoptosis pathway (Figure 5A). Also, these cell corpses displayed phosphatidylserine on their cell surfaces, as indicated by the phosphatidylserine-binding reporter MFG-e8::Venus (Figure 5B), and exhibited many of the morphological hallmarks of apoptosis, including contraction of cytoplasmic volume and, in some but not all cases, condensation of nuclear chromatin (Figure 5C).

Figure 5. The cell corpses of caspase-deleted mutants are cytologically and morphologically apoptotic. (A) Nomarski DIC and fluorescence images of a cell corpse within the head of a ced-1(e1735); csp-1(n4967); csp-2(n4871) ced-3(n3692) L1 larva carrying the integrated transgene nls342[P\text{egl-1::gfp}], a transcriptional reporter that expresses GFP under the control of the BH3 domain-only encoding gene egl-1. (B) Nomarski DIC and fluorescence images of a cell corpse within the head of a ced-1(e1735); csp-1(n4967); csp-2(n4871) ced-3(n3692) L1 larva carrying the extrachromosomal array nEx1646[P\text{dyn-1::mfg-e8::Venus}], a fusion protein that binds to cell-surface exposed phosphatidylethanolamine. (C) Representative transmission electron micrographs of cell corpses from ced-1(e1735); csp-1(n4967); csp-2(n4871) ced-3(n3692) larvae 24 hrs post hatching. "n", nuclei of the cell corpses; scale bars, 0.5 microns. Note the difference in chromatin condensation between the two cell corpses.

doi:10.1371/journal.pgen.1003341.g005

Figure 6. Caspase-independent cell corpses are engulfed and degraded. (A) Nomarski DIC and fluorescence images of a cell corpse from a ced-1(e1735); csp-1(n4967); csp-2(n4871) ced-3(n3692) L1 larva carrying the integrated transgene nIs400[P\text{ced-1::ced-1\_DC::gfp}], which expresses a non-rescuing CED-1\_DC::GFP fusion protein. CED-1 is a transmembrane receptor that is expressed on engulfing cells, binds to apoptotic cell corpses, and is required for phagocytosis [46]. (B) Nomarski DIC and fluorescence images of a cell corpse from a csp-3(n4872); csp-1(n4967); csp-2(n4871) ced-3(n3692) L1 larva stained with acridine orange (AO), which fluoresces in engulfed cell corpses undergoing degradation in endosomal compartments. (C) The fraction of csp-1(n4967); csp-2(n4871) ced-3(n3692) and ced-1(e1735); csp-1(n4967); csp-2(n4871) ced-3(n3692) with 0, 1, 2 or >2 cell corpses at different time points post hatching. Asterisks indicate p<0.05 in a Mann-Whitney test comparing the two genotypes at a given time point.

doi:10.1371/journal.pgen.1003341.g006
Additionally, we noted that the caspase–independent cell corpses frequently stained with acridine orange (Figure 6A), suggesting that these corpses are engulfed, internalized and degraded via endosomal pathways, as are canonical programmed cell deaths [30,49,49,51]. Indeed, we found that the caspase–independent corpses were recognized by CED-1 (Figure 6B), a receptor expressed on engulfing cells required for the efficient phagocytosis of cell corpses [46,49,50]. The recognition of caspase–independent cell corpses by CED-1 appeared to be functionally important, as ced-1; csp-1; csp-2 ced-3 larvae contained more corpses than csp-1; csp-2 ced-3 larvae (Figure 6C). Given that ced-1 and other genes that function in cell–corpses engulfment promote programmed cell death [52,53], it is unlikely that the ced-1(e1735) loss-of-function mutation caused additional cell deaths in the caspase–deleted mutants. Instead, the extra corpses in ced-1 mutant larvae likely reflected an engulfment defect, consistent with the comparatively rapid degradation and disappearance of most caspase–independent corpses in ced-1(+ ) larvae within the 36-hour period after hatching (Figure 6C). We conclude that caspases are not required for programmed cell deaths to be recognized by the engulfment machinery, internalized and degraded. In short, many aspects of apoptosis, including phagocytosis – the ultimate fate of apoptotic cells – can occur without caspases. We conclude that a parallel, caspase–independent pathway contributes to programmed cell death in *C. elegans* and can execute most cellular changes associated with apoptosis.

**Discussion**

Our experiments revealed unexpected complexities in the execution of apoptosis in *C. elegans*. While the CED-3 caspase is clearly the primary effector of programmed cell death, we demonstrated the existence of additional caspase–dependent and caspase–independent contributions to developmental apoptosis. Specifically, we observed that maternally-expressed caspase gene csp-1 (but not csp-2 or csp-3) promotes the deaths of a subset of cells programmed to die during *C. elegans* embryogenesis (Figure 1 and Figure 4; Table 1 and Table 3). Furthermore, ectopic expression of the csp-1B isoform of csp-1 is sufficient to cell–autonomously kill cells that normally survive. These ectopic apoptotic cell deaths require the active site cysteine (C138) of CSP-1B, indicating that a caspase–like proteolytic function is responsible for its cell–killing activity (Table 2). The *C. elegans* genome therefore expresses at least two pro–apoptotic caspases, CED-3 and CSP-1B, to mediate programmed cell deaths. Nevertheless, the additional caspase activity conferred by csp-1 cannot account for ced-3–independent programmed cell deaths that have been observed in *C. elegans*. For example, the non–apoptotic death of the male linker cell and the extrusion of shed cells were already known to be caspase–independent [18,19]. Here we demonstrate that cells in caspase–deleted animals can undergo an apoptosis–like programmed cell death followed by engulfment, indicating that the complete apoptotic program can occur in the absence of caspases. Thus, in addition to CED-3 and CSP-1B, there are caspase–independent cell–killing activities that contribute to programmed cell deaths. CSP-1B is regulated by a mechanism distinct from that of CED-3

The caspases CED-3 and CSP-1B appear to be regulated differently. The auto-activation of CED-3 is facilitated by the Apaf-1 homolog CED-4 in a protein–protein interaction that requires the CED-3 prodomain [34–36]. In the absence of a pro–apoptotic signal, CED-9 sequesters CED-4 [37], thereby preventing its association with the inactive CED-3 proprotein. The CDP-1B proprotein lacks a long prodomain, suggesting that it is not activated through an association with the CED-4 octamer in cells undergoing apoptosis. Consistent with this expectation, we observed that the cell–killing activity of csp-1B transgenes, unlike that of ced-3 transgenes, was not negatively regulated by ced-9 (Figure 5). Furthermore, based on our genetic experiments (Figure 3) and the in vitro studies of Shaham [15], it does not appear that CSP-1B is activated by CED-3. We therefore propose that CSP-1B is regulated by a mechanism different from the canonical programmed cell death pathway that activates CED-3 and that CSP-1B likely promotes cell killing in parallel to CED-3 (Figure 3E).

There are no known or candidate regulators of csp-1. It is possible that csp-1 is controlled entirely at the transcriptional level and that csp-1 contributes a minor, sub-lethal pro–apoptotic activity to all cells within the *C. elegans* embryo. Indeed, only using sensitized backgrounds with partial defects in programmed cell death did we detect the pro–apoptotic function of csp-1. Nevertheless, we expect that it will be possible to identify regulators and effectors of csp-1 through genetic screens for mutants that modify the cell–killing activity of csp-1B transgenes.

**Do the csp genes have non–apoptotic functions?**

Given the minor contribution of csp-1 to programmed cell death and the lack of a detectable role of csp-2 or csp-3 in apoptosis (Table 1; Table S1; data not shown), it is tempting to speculate that the csp genes have non–apoptotic functions in *C. elegans*. In *C. elegans*, ced-3 functions in axon regeneration following laser ablation [54]. In mammalian and *Drosophila* neurons, caspases have functions in dendritic pruning, axon guidance and the synaptic changes underlying long-term depression [14]. Caspase function is also required for the maturation of *Drosophila* sperm [55]. Interestingly, we observed robust expression of csp-1 in the germ cells of *L.4* and adult hermaphrodites, specifically in the late pachytene nuclei (Figure 4). We also observed temporally and spatially restricted csp-2 and csp-3 mRNA expression in the late pachytene nuclei of the *L.4* larval germ line (data not shown), suggesting that the csp genes might have functions in germ cell development. However, mutant hermaphrodites and males carrying all tested combinations of csp-1, csp-2 and csp-3, including the triple csp mutant, were viable, fertile and failed to exhibit obvious brood–size defects that would suggest abnormalities in sperm or oocyte differentiation (data not shown).

**csp-1B as a tool for the genetic ablution of cells**

Genetically encoded cell–killing activities provide an efficient and convenient method for determining cellular function through cell ablation. Killer genes such as ced-3 have been used under the control of various promoters to ablature specific cells [32,45,56,57]. However, the potent cell–killing activity of ced-3 transgenes can cause organismic inviability, particularly if the promoter expression is not exclusive to a small number of cells (see below), csp-1B overexpression using the mec-7 and flp-15 promoters efficiently killed the touch and I2 neurons, respectively (Figure 2; Table 2; N. Bhatla and H.R. Horvitz, personal communication). The mec-7 and flp-15 promoters are relatively strong, as they also robustly induced gfp expression in these cells, such that the neural processes were visible with a dissecting microscope equipped with fluorescence optics. By contrast, the odr-1 promoter did not produce detectable GFP expression in the neurites of the AWB, AWG and I1 neurons, and csp-1B under the control of the odr-1 promoter failed to kill these cells even when injected at plasmid concentrations as high as 100 ng/µl (N. Bhatla and H.R. Horvitz, unpublished results). Thus, high levels of csp-1B expression might
be required to kill most cells, making the use of csp-1B as a cell-ablation tool appropriate in situations in which the promoter sequence strongly drives expression in targeted cells and/or weakly promotes expression in additional cells not intended to be targets. For example, the \( \text{P}_{\text{ mec-7::csp-1B}} \) constructs, which were injected at a concentration of 15 ng/\( \mu \)l, produced csp-1B expression outside of the touch neurons that was detectable by fluorescence in situ hybridization. However, this level of csp-1B expression was sub-lethal and did not induce cell death or other cellular defects outside of the touch neurons (data not shown). By contrast, P\text{lethal} and did not induce cell death or other cellular defects in the touch neurons that was detectable by fluorescence in situ hybridization. This result is consistent with previous reports that the concentration of 15 ng/\( \mu \)l produced expression outside of the touch neurons.

For example, the P\text{lethal} promotes expression in additional cells not intended to be targets. An ablation tool appropriate in situations in which the promoter would be required to kill most cells, making the use of csp-1B as a cell-ablation tool appropriate in situations in which the promoter sequence strongly drives expression in targeted cells and/or weakly promotes expression in additional cells not intended to be targets. For example, the \( \text{P}_{\text{ mec-7::csp-1B}} \) constructs, which were injected at a concentration of 15 ng/\( \mu \)l, produced csp-1B expression outside of the touch neurons that was detectable by fluorescence in situ hybridization. However, this level of csp-1B expression was sub-lethal and did not induce cell death or other cellular defects outside of the touch neurons (data not shown). By contrast, P\text{lethal} and did not induce cell death or other cellular defects in the touch neurons that was detectable by fluorescence in situ hybridization. This result is consistent with previous reports that the concentration of 15 ng/\( \mu \)l produced expression outside of the touch neurons.

What is the role of ced-3-independent cell-killing activities that have minor contributions to programmed cell death?

Although the csp-1 gene contributes a cell-killing activity to normal programmed cell deaths (Table 1), csp-1 and the other csp genes are not responsible for the ced-3-independent programmed cell deaths present in the heads of ced-3 larvae (Table 4). These deaths, like those of the male linker cell (ref. [10], Table S5) and the embryonic shell cells [19], are caspase-independent – a surprising result in light of our observations that these cell corpses are morphologically apoptotic (Figure 5) and are engulfed (albeit with slower kinetics) like normal programmed cell deaths (Figure 6). Thus, the complete apoptotic program including cell-corpus internalization can occur in the absence of caspases in C. elegans, suggesting that the cellular changes accompanying apoptosis do not require proteolysis by the caspase family of proteases. Moreover, it is clear that apoptotic programmed cell deaths are achieved through the integration of independent cell-killing activities from CED-3, CSP-1B and an unknown caspase-independent source.

Given the minor cell-killing effects of the CSP-1B and the caspase-independent pathways, why might cell-killer activities in addition to that of CED-3 have evolved? It is possible that different cells, even within the set of C. elegans cells that died to fate, are differentially sensitive to pro-apoptotic signals and that additional caspase and caspase-independent pathways ensure efficient and complete cell death under diverse environmental and developmental conditions. Interestingly, the postembryonic programmed cell deaths of the ventral cord are more sensitive to weak ced-3 mutations than are the embryonic programmed cell deaths in the presumptive anterior pharynx: ced-3 mutations that have weak effects in the anterior pharynx typically have stronger effects in the ventral cord (ref. [17]; data not shown). We observed a complementary function for csp-1, which promotes apoptosis in the anterior pharynx (Table 1) but not in the ventral cord (Table S4).

In summary, multiple pro-apoptotic caspase functions in programmed cell death in C. elegans, Drosophila and vertebrates. Furthermore, as we and others have shown, there are additional caspase-independent contributions to programmed cell deaths in C. elegans. We identified C. elegans caspase-independent cell deaths that are essentially identical to wild-type programmed cell deaths based on their apoptotic appearance and their recognition and internalization by engulfing cells. We expect that caspase-independent pro-apoptotic activities are present in other metazoa and that their identification will be of major importance to our understanding of cell death in the contexts of development and disease.

Materials and Methods

Strains

All C. elegans strains were cultured as described previously [58] and maintained at 20°C. We used Bristol N2 as the wild-type strain, and the mutations used in our experiments are listed below:

- LG I, unc-75(e950), ced-1(e1735), csp-5(n4872, tm2260, tm2286), nIs177[P\text{ mec-7::gfp}] [59]
- LG II, csp-2(n4967, n5133, tm617), mab-10(n5117), lin-29(n626)
- LG III, ced-4(n1162, n5158), ced-6(n2093), ced-7(n1996), ced-9(n1653, n2812), tat-1(n1034), nIs308[P\text{ mec-7::cep-1B}, P\text{ mec-7::gfp}, P\text{ mec-7::ced-1::gfp}] [19]
- LG IV, csp-2(n4871), ced-5(n1812), dpy-20(6w1282), unc-30(e191), ced-3(n2427, n2436, n2452, n3692), nls309[P\text{ mec-7::csp-1B}, P\text{ mec-7::gfp}]
- LG V, gfp-1(n1084 n3082), heIs39[cm\text{ mec-7::gfp}] [45], lsIs42[P\text{ csp-1::4xNLS::gfp}] [59], qIs56[P\text{ csp-1::gfp}]
- LG X, ced-8(n1891), bIs18[P\text{ mec-3::gfp}] [22], nIs106[P\text{ mec-11::gfp}] [52]

Unknown linkage. nIs290(P\text{ mec-7::gfp}); nIs307(P\text{ mec-7::csp-1B}, P\text{ mec-7::gfp}, nIs368-370[P\text{ mec-7::csp-1B}(C1958), P\text{ mec-7::gfp}]; nIs398[P\text{ mec-7::mrg-4::e2::Venus}] [19,60]

Extrachromosomal arrays. nEx1646[pg\text{ mec-3::gfp}]; nEx1645-611[ced-1(+) (pDD071)], nEx1604-9[csp-1B/C only (pDD030)], nEx1614-16[csp-1A only (pDD029)], nEx1617-19[csp-1-PD (pDD029)]

Plasmids

The \( \text{P}_{\text{ mec-7::csp-1B}} \) and \( \text{P}_{\text{ mec-7::csp-1A}} \) plasmids were toxic to the animals when injected at concentrations above 1 ng/\( \mu \)l, produced expression outside of the touch neurons (data not shown), suggesting that cells are very sensitive to ectopic ced-3 and that using ced-3 as a cell ablation tool is potentially problematic when promoter expression is not restricted to a small number of targeted cells.
amplicon was digested with BglII and XhoI and then ligated into pL4440. The RNAi plasmid pL4440::csp-1B (pDD061) was constructed using PCR to amplify the csp-1B cDNA with the primers 5'-ggagagatcctcgaggaagagc-3' and 5'-gctcgagtagctagcatcgc-3', which incorporate the restriction sites BglII and XhoI, respectively. The resulting csp-1B amplicon was digested with BglII and XhoI and then ligated into pL4440.

RNAi experiments
The in vitro transcription, purification, preparation and microinjection of csp-1-PD (pDD060) and csp-1B (pDD061) dsRNA were performed as described previously [61].

Fluorescence in situ hybridization
The fixation of embryos and larval and adult animals, the conjugation of Cy5 or ALEXA594 fluorescent probes to in situ oligo probes, and the hybridization of oligos to fixed samples were performed as described previously [62]. All images were acquired using an inverted Nikon TE-2000 compound microscope equipped for fluorescence microscopy (Prior Scientific). Images were acquired with a PIXIS camera (Princeton Instruments) controlled by MetaMorph software (Molecular Devices) and modified for publication with ImageJ software (NIH). The “total csp-1” set of probes included 32 distinct 20-nucleotide sequences complementary to csp-1B (Biosearch Technologies, Inc). This set of oligos was conjugated to the fluorophore Cy5 (GE Healthcare) and hybridized to all three csp-1 mRNA isoforms (csp-1A, csp-1B and csp-1C). The “csp-1A” set of probes included 32 distinct 20-nucleotide sequences complementary to the region of csp-1A that encodes the prodomain. This set of oligos was conjugated to the fluorophore ALEXA594 (Invitrogen) and hybridized specifically to the csp-1A mRNA isoform. Probe sequences are listed in Table S6.

Cell-death assays and microscopy
The numbers of undead cells that failed to undergo programmed cell death in the anterior pharynges and postdeirid sensilla of L3 larvae were determined by direct observation using Nomarski optics as described previously [28]. Persistent cell corpses in larval heads also were quantified by direct observation using Nomarski optics; for this assay, larvae were staged by the time of hatching. For other cell-death assays, the ventral cord cells of young adults, the M4 neuron and its undec sister cell of L3 larvae, the touch neurons of L4 larvae, and the germ cell corpses of adult hermaphrodite gonads were identified using previously described GFP reporter transgenes [45,52,59]. For experiments involving ionizing radiation, L4 larvae were exposed to gamma irradiation from a Co-60 source. All strains were analyzed using a Zeiss Axioskop II compound microscope equipped for fluorescence microscopy. Images were acquired with an ORCA camera (Hamamatsu) controlled by OpenLab software (Perkin Elmer) and modified for publication using ImageJ (NIH).

Transmission electron microscopy
L1-stage larvae were fixed, stained and sectioned for transmission electron microscopy as described previously [43]. Stained sections were imaged with a JEM-1200EX II microscope (JEOL) using an AMT XR41 CCD camera.

Supporting Information
Figure S1 Transgenes that ectopically express csp-1A in the touch neurons lack cell-killing activity in both the presence and absence of the apoptosis regulator CED-9. The percentages of PLM cells that survive in strains carrying P_mec-1::csp-1A transgenes. All strains contained the ced-3(n3692) mutation, which suppresses ced-9(n2812) inviability, n.s., p>0.05 in a Fisher’s exact test. (PDF)

Table S1 The deletion of csp-1, csp-2 or csp-3 does not cause the deaths of cells that normally survive. (A). The touch neurons survive in csp mutants. The survival of AVM, ALML/R, PVM and PLML/R was scored using the transcriptional reporters P_mec-4::gfp (nIs290) or P_mec-3::gfp (bzIs100). n, animals scored. (B). Mutants carrying csp deletions have the same number of pharyngeal cells as wild-type animals. The following pharyngeal cells were scored: the neurons I1, I2, I3, MC, MI, M3, M4 and NMS; the epithelial cells e1, e2, and e3; and, the muscle cells m1 and m2. In total, 34 cells were scored per pharynx. n, animals scored; SD, standard deviation. (DOC)

Table S2 The defect in programmed cell death of csp-1(n4967) animals is rescued by transgenes that contain the endogenous csp-1 promoter and coding regions. Mutations that alter the start of the B and C splicing isoforms of csp-1 disrupt the rescuing activity of the csp-1 transgene. The transgenes are described in detail in the legend of Figure 1 and in Materials and Methods. A Student’s t-test was used to compare the survival of csp-1(n4967); ced-3(n2436) strains with csp-1 transgenes to the csp-1(n4967); ced-3(n2436) parental strain. p values were considered significant if less than 0.01 to correct for multiple comparisons. (DOC)

Table S3 The deletion of csp-2 or csp-3 does not modify the defects in programmed cell death of csp-1 and ced-3 mutants. The average number of extra, undead cells in the pharynx was determined for each genotype. n, number of animals scored; SD, standard deviation. For the statistical comparisons between ced-3(n2427) and ced-3(n2436) and double mutants with each csp allele, p values were considered significant if less than 0.02 to correct for multiple comparisons. (DOC)

Table S4 csp-1 promotes the programmed cell death of (A) the M4 sister cell but not those of (B) the VC-like cells in the ventral cord and of (C) the V5.praap cell in the postdeirid sensillum. The survival of the M4 sister cell was scored using the integrated transgene nIs177[P_ceh-28::gfp]. The number of extra VC-like cells was determined using the integrated transgene nIs106[P_mec-4::gfp]. The survival of V5.praapp was determined via direct observation using Nomarski optics. (DOC)

Table S5 The male linker cell dies in animals lacking all four caspases. (DOC)

Table S6 Sequences of DNA probes used for fluorescence in situ hybridization (FISH) experiments. The csp-1A oligos hybridize to the region of csp-1A that encodes the prodomain and are therefore specific to the csp-1A isoform. The “total” csp-1A oligos hybridize to a region present in all known csp-1 mRNA isoforms. (DOCX)

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Author Contributions
Conceived and designed the experiments: DPD VH HRH. Performed the experiments: DPD VH. Analyzed the data: DPD VH HRH. Contributed

References