Transcriptional upregulation of both *egl-1* BH3-only and *ced-3* caspase is required for the death of the male-specific CEM neurons

R Nehme¹, P Grote^{1,2}, T Tomasi^{1,4}, S Löser^{2,4}, H Holzkamp², R Schnabel³ and B Conradt^{*,1}

Most of the 131 cells that die during the development of a *Caenorhabditis elegans* hermaphrodite do so ~30 min after being generated. Furthermore, in these cells, the pro-caspase proCED-3 is inherited from progenitors and the transcriptional upregulation of the BH3-only gene *egl-1* is thought to be sufficient for apoptosis induction. In contrast, the four CEM neurons, which die in hermaphrodites, but not males, die ~150 min after being generated. We found that in the CEMs, the transcriptional activation of both the *egl-1* and *ced-3* gene is necessary for apoptosis induction. In addition, we show that the Bar homeodomain transcription factor CEH-30 represses *egl-1* and *ced-3* transcription in the CEMs, thereby permitting their survival. Furthermore, we identified three genes, *unc-86*, *lrs-1*, and *unc-132*, which encode a POU homeodomain transcription factor, a leucyl-tRNA synthetase, and a novel protein with limited sequence similarity to the mammalian proto-oncoprotein and kinase PIM-1, respectively, that promote the expression of the *ceh-30* gene in the CEMs. On the basis of these results, we propose that *egl-1* and *ced-3* transcription are coregulated in the CEMs to compensate for limiting proCED-3 levels, which most probably are a result of proCED-3 turn over. Similar coregulatory mechanisms for BH3-only proteins and pro-caspases may function in higher organisms to allow efficient apoptosis induction. Finally, we present evidence that the timing of the death of the CEMs is controlled by TRA-1 Gli, the terminal global regulator of somatic sexual fate in *C. elegans*.

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Owing to its invariant pattern of apoptotic cell death during development, *Caenorhabditis elegans* has become an important model for apoptosis studies. The central cell death machinery in *C. elegans* is composed of the anti-apoptotic BCL-2-like protein CED-9, the APAF-1-like caspase activator CED-4 and the pro-caspase proCED-3.^{1,2} At least during mid-embryogenesis when the majority of cell deaths occur, most if not all cells in developing embryos contain CED-4 as well as proCED-3, which once matured is sufficient for apoptosis induction.^{3–5} However, these cells also contain the CED-9 protein, which through its direct interaction with CED-4, blocks the ability of CED-4 to mediate proCED-3 maturation.^{3,4} As a result, apoptosis induction is prevented.

During the development of a *C. elegans* hermaphrodite, exactly 131 cells are programmed to die.⁶ The majority of these cells die ~30 min after being generated. Furthermore, in these cells, the transcriptional activation of the BH3-only gene *egl-1* is thought to be sufficient for the activation of the central cell death machinery and, hence, apoptosis induction.⁷ Specifically, the BH3-only protein EGL-1 can bind to CED-9 thereby causing CED-4 release from CED-9 and CED-4-dependent proCED-3 maturation. Interestingly, recent data suggest that the *ced-3* gene is not transcriptionally active in most of the 131 cells that are programmed to die during

development.⁴ This observation suggests that proCED-3 protein present in these cells is derived from their progenitors. It also suggests that once activated, the amount of proCED-3 protein inherited from progenitors is, in general, sufficient for apoptosis induction.

The four male-specific cephalic companion neurons (CEMs) are generated ~320 min after the first cell division. In males, the CEMs survive and differentiate into ciliated sensory neurons implicated in mating behavior.^{6,8,9} In hermaphrodites, however, the CEMs undergo apoptosis ~150 min after being generated (~470 min after the first cell division). As is the case for most of the cells that are programmed to die during development, the death of the CEMs is dependent on the genes *egl-1*, *ced-4*, and *ced-3*.^{10,11} The death of the CEMs is also dependent on the gene *tra-1*, which encodes the terminal, global regulator of somatic sexual fate, and which acts to promote female development.¹² Specifically, the TRA-1 protein, a GLI-like transcription factor, causes the CEMs in hermaphrodites to undergo apoptosis by directly repressing the transcription of the gene *ceh-30*, which encodes a Bar homeodomain transcription factor that acts to block the death of the CEMs in males.^{13,14}

How the CEH-30 protein acts on the central cell death machinery to block the death of the CEMs has so far been

¹Department of Genetics, Dartmouth Medical School, Norris Cotton Cancer Center, Hanover, NH, USA; ²Max-Planck-Institute of Neurobiology, Am Klopferspitz, Martinsried, Germany and ³Institut für Genetik, Technische Universität Braunschweig *Carolo Wilhelmina*, Braunschweig, Germany

*Corresponding author: B Conradt, Department of Genetics, Dartmouth Medical School, 7400 Remsen Building, Hanover, NH 3755, USA. Tel: 603 650 1210; Fax: 603 650 1188; E-mail: barbara.conradt@dartmouth.edu

⁴These authors contributed equally to this work.

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Abbreviations: CED, cell-death abnormal; EGL, egg-laying abnormal; CEM, cephalic companion neurons; UNC, uncoordinated

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unclear. We now present evidence that CEH-30 blocks the death of the CEMs by repressing the transcription of both the *egl-1* and *ced-3* gene. Furthermore, we identified three genes, *unc-86*, *lrs-1*, and *unc-132*, which encode a POU homeodomain transcription factor, a leucyl-tRNA synthetase, and a novel protein with limited sequence similarity to the mammalian proto-oncoprotein and kinase PIM-1, respectively, that promote *ceh-30* expression in the CEMs. Our data show that in contrast to most cells that are programmed to die during development, in the CEMs, the transcriptional upregulation of both the BH3-only gene *egl-1* and the caspase gene *ced-3* is required for apoptosis induction. We propose that coupling the transcriptional upregulation of a key activator of apoptosis induction (*egl-1*) and a component of the central, enzymatic cell death machinery (*ced-3*) ensures efficient apoptosis induction in the CEMs.

Results

***bc151*, *bc155*, and *bc159* cause differentiated CEMs to be absent from masculinized hermaphrodites or males.** An altered-function mutation of the *C. elegans* gene *sel-10*, *n1077*, which encodes an F-box protein that promotes female development, causes weak masculinization of the hermaphrodite soma.^{15–17} For example, in wild-type hermaphrodites, the four CEMs undergo apoptosis.^{6,8} In contrast, in *sel-10(n1077)* hermaphrodites, the CEMs survive like in wild-type males. To identify genes required for the survival of the CEMs, we sought mutations that cause the CEMs to be absent from *sel-10(n1077)* hermaphrodites based on the expression of *pkd-2*, a marker for differentiated CEMs

(see Materials and Methods). We identified three recessive mutations, *bc151*, *bc155*, and *bc159*, that cause the majority of CEMs to be absent from masculinized hermaphrodites as well as males (Table 1a; data not shown). The CEMs are also absent in *bc151*, *bc155*, or *bc159* masculinized hermaphrodites based on their characteristic morphology in L4 larvae (Table 1b) and in *bc151*, *bc155*, or *bc159* males based on *lov-1* expression, another marker for differentiated CEMs (Supplementary Table S1). In addition, all three mutations cause a reduced brood size and an uncoordinated or 'Unc' phenotype (Supplementary Table S2; data not shown). On the basis of these observations, we conclude that *bc151*, *bc155*, and *bc159* cause differentiated CEMs to be absent in masculinized hermaphrodites and males.

***bc151* is a loss-of-function mutation of *unc-86* POU.** *unc-86*, which encodes a POU transcription factor, has previously been shown to be required for the presence of differentiated CEMs in males.^{13,18,19} We found that *bc151* animals carry two mutations in the *unc-86* gene: first, a missense mutation leading to an aspartic acid (GAU)-to-asparagine (AAU) substitution at position 203 of the protein sequence of the UNC-86.b protein (generated by transcript *C30A5.7b*); second, a nonsense mutation, changing codon 220 of the *C30A5.7b* coding sequence (CAA) to a STOP codon (UAA) (Figure 1a). Both *unc-86* transcripts (*C30A5.7a*, *C30A5.7b*) are predicted to be affected by the two mutations. As loss-of-function mutations of *unc-86* have previously been shown to cause differentiated CEMs to be absent in males, we conclude that *bc151* is a new loss-of-function mutation of the *unc-86* gene.

Table 1 The CEMs are absent in masculinized hermaphrodites or males homozygous for *unc-86(bc151)*, *lrs-1(bc155)* or *unc-132(bc159)*.

Genotype	% GFP ⁺ CEMs (n)			
	+/+		<i>sel-10(n1077)</i>	
	XX	XO ^a	+/+ XX	<i>ced-3(n717)</i> XX
(a) Presence of differentiated CEMs in adults				
+/+	0 (Many)	100 (Many)	100 (Many)	100 (Many)
<i>egl-1(n1084 n3082)</i>	68 (356)	ND	ND	ND
<i>ced-3(n717)</i>	78 (232)	ND	ND	ND
<i>unc-86(bc151)^b</i>	ND	25 (80)	12 (178)	8 (92)
<i>lrs-1(bc155)^b</i>	ND	18 (76)	10 (224)	7 (80)
<i>unc-132(bc159)</i>	ND	0 (68)	0 (232)	0 (96)
Genotype	% CEMs (n)			
	+/+ XX	<i>sel-10(n1077)</i> XX	<i>ced-3(n717); sel-10(n1077)</i> XX	
(b) Presence of CEMs in L4 larvae				
+/+	0 (36)	100 (40)	100(36)	
<i>egl-1(n1084 n3082)</i>	96 (80)	ND	ND	
<i>ced-3(n717)</i>	98 (128)	ND	ND	
<i>unc-86(bc151)^b</i>	ND	6 (32)	4 (24)	
<i>lrs-1(bc155)^b</i>	ND	7 (48)	7 (48)	
<i>unc-132(bc159)</i>	ND	0 (20)	2 (44)	

The presence of differentiated CEMs was analyzed in adults using the *P_{pkd-2} gfp* reporter as described in Materials and Methods (part (a)). The presence of CEMs was scored at the L4 stage using DIC as described in Materials and Methods (part (b)). All strains were homozygous for the *P_{pkd-2} gfp* integration *bcl/s9*. ^aIndicates that the strains were homozygous for *him-5(e1490)* V. ^bIndicates that the strains were homozygous for *dpy-17(e164)* III.

Figure 1 Cloning of *unc-86(bc151)*, *lrs-1(bc155)*, and *unc-132(bc159)*. (a) The gene *dpy-17* used for mapping *bc151* is indicated on the genetic map (LG III). The two mutations identified in the *C30A5.7/unc-86* gene in *bc151* animals are indicated in red. (b) Genes and SNPs used for mapping *bc155* on LG III are indicated on the genetic map. Cosmids and fosmids tested for *bc155* rescue are shown below the genetic map. The *bc155* mutation in *R74.1/lrs-1* is indicated in red. (c) *dpy-11* and H10D18:28865 used for mapping *bc159* on LG V are indicated on the genetic map. Cosmids tested for *bc159* rescue are shown below. pBC430 and pBC431 represent rescuing subclones of cosmid *W08A12*. The *bc159* mutation in *W08A12.1/unc-132* is indicated in red. (d) Alignment of the protein sequences of UNC-132 (isoform a), *D. melanogaster* CG30118 and human PIM-1. The alignment was done using the EMBOSS algorithm. Identical amino acids have a black background. Amino acids with similar biochemical properties have a gray background. The blue boxes indicate the P-loop domains and the orange box the kinase domain. The amino acid that is changed as a result *bc159* (M200I) is indicated with a red asterisk. Over its entire length, the UNC-132 protein is 38.2% identical and 53.5% similar to the CG30118 protein. The similarities between UNC-132 and CG30118 are mainly found in the central (57.5% identity, 76.4% similarity) and C-terminal (42.3% identity, 59.6% similarity) regions of the two proteins. Over its entire length, the UNC-132 protein is 12.7% identical and 22.0% similar to the human kinase PIM-1. The similarity between the two proteins is mainly restricted to their N-terminal (29.9% identical, 46.8% similar) regions

***bc155* is a loss-of-function mutation of the gene *lrs-1* leucyl-tRNA synthetase.** We found that *bc155* is a mutation in the gene *lrs-1*, which encodes a leucyl-tRNA synthetase, a Class I tRNA synthetase (Figure 1b; data not shown).²⁰ *bc155* is a G-to-A transition that changes the 3' splice acceptor site of intron 3 from AG to AA, which results in reduced levels of fully spliced *lrs-1* mRNA (Figure 1b; data not shown). Both *lrs-1* transcripts (*R74.1.1*, *R74.1.2*) are predicted to be affected by the *bc155* mutation. Reducing *lrs-1* function by RNAi results in the absence of differentiated CEMs in masculinized hermaphrodites (Table 2a). On the basis of these findings, we conclude that *bc155* is a loss-of-function mutation of the gene *lrs-1*. Furthermore, we found that reducing the function by RNAi of genes encoding other Class I or Class II tRNA synthetases can result in the absence of at least 20% of differentiated CEMs in masculinized hermaphrodites (Table 2a).

***bc159* is a loss-of-function mutation of the PIM-1-like gene *unc-132*.** *bc159* is a mutation in a previously

uncharacterized gene, *W08A12.1*, which generates five transcripts. Transcript *W08A12.1.a* encodes a 501 amino-acid protein of unknown function with a P-loop domain (Figure 1c).^{21,22} As *bc159* also causes an Unc phenotype, we named *W08A12.1.unc-132*. The *bc159* mutation affects the second exon that is common to all five *unc-132* transcripts. Specifically, *bc159* is a missense mutation that leads to a methionine (AUG)-to-isoleucine (AUA) substitution at position 200 of the protein sequence of the UNC-132.a protein (generated by transcript *W08A12.1.a*). The reduction of *unc-132* function by RNAi causes differentiated CEMs to be absent in a *sel-10(n1077)* background (Table 2b), confirming that *bc159* is a loss-of-function mutation of the *unc-132* gene. *unc-132* is orthologous to a gene of unknown function in *Drosophila melanogaster*, CG30118.²¹ Among mammalian proteins, UNC-132 is most similar to the human kinase PIM-1; however, the two proteins are not orthologous.²² As is the case for UNC-132 and CG30118, PIM-1 has a P-loop domain. However, in contrast to UNC-132 and CG30118, PIM-1 also contains a kinase domain.

Table 2 The inactivation by RNAi of *lrs-1* and other genes encoding tRNA synthetases as well as *unc-132* by RNAi results in the absence of differentiated CEMs in masculinized hermaphrodites.

Genotype	tRNA synthetase affected	%GFP ⁺ CEMs (n) <i>sel-10(n1077)</i> XX	Other RNAi phenotypes
(a) Inactivation by RNAi of <i>lrs-1</i> and other genes encoding tRNA synthetases			
+/+	NA	100 (Many)	NA
Class I tRNA synthetases			
<i>lrs-1(RNAi)</i>	Leu	62 (60)	Larval arrest, lethality
<i>irs-2(RNAi)</i>	Ile	63 (248)	None detected
<i>irs-1(RNAi)</i>	Ile	83 (12)	Larval arrest, lethality
<i>vrs-2(RNAi)</i>	Val	60 (32)	Larval arrest, lethality
<i>vrs-1(RNAi)</i>	Val	90 (124)	None detected
<i>mrs-1(RNAi)</i>	Met	89 (132)	None detected
Class II tRNA synthetases			
<i>hrs-1(RNAi)</i>	His	71 (24)	Larval arrest, lethality
<i>srs-1(RNAi)</i>	Ser	95 (112)	None detected
<i>yrs-1(RNAi)</i>	Tyr	88 (68)	None detected
<i>ers-1(RNAi)</i>	Glu	85 (100)	None detected
<i>wrs-2(RNAi)</i>	Trp	75 (124)	None detected
<i>drs-1(RNAi)</i>	Asp	92 (24)	Larval arrest, lethality
<i>prs-1(RNAi)</i>	Pro	97 (116)	None detected
<i>ars-1(RNAi)</i>	Ala	88 (124)	None detected
<i>trs-1(RNAi)</i>	Thr	74 (72)	None detected
<i>nrs-1(RNAi)</i>	Asn	86 (28)	None detected
<i>frs-1(RNAi)</i>	Phe	100 (36)	Larval arrest, lethality
<i>crs-1(RNAi)</i>	Cys	91 (108)	Larval arrest, lethality
<i>krs-1(RNAi)</i>	Lys	100 (32)	Larval arrest, lethality

Genotype	% GFP ⁺ CEMs (n) <i>sel-10(n1077)</i>
(b) Inactivation of <i>unc-132</i> by RNAi	
+/+	99 (200)
<i>unc-132(RNAi ; W08A12.1b cDNA)</i>	49 (216)
<i>unc-132(RNAi ; W08A12.1c cDNA)</i>	64 (172)

The presence of differentiated CEMs was analyzed in adults using the $P_{pkd-2gfp}$ reporter as described in Materials and Methods. All strains were homozygous for the $P_{pkd-2gfp}$ integration *bcl9*. RNAi was performed by feeding as described in Materials and Methods.

Table 3 The fate of the AMso and URAs in *unc-86(bc151)*, *lrs-1(bc155)* and *unc-132(bc159)*

Genotype	% GFP ⁺ AMso (n) $P_{unc-53gfp}$	% GFP ⁺ URAs (n) $P_{glt-4glt-4::gfp}$	% GFP ⁺ URAs (n) $P_{cfl-1gfp}$
+/+	100 (120)	95 (92)	91 (124)
<i>unc-86(bc151)^a</i>	100 (100)	8 (40)	0 (100)
<i>lrs-1(bc155)^a</i>	100 (140)	93 (84)	89 (60)
<i>unc-132(bc159)</i>	100 (140)	77 (88)	93 (60)

The presence of the AMso and URAs was determined in hermaphrodites using $P_{unc-53gfp}$ (*bgEx21*) (AMso) or $P_{glt-4glt-4::gfp}$ (*akEx32*) and $P_{cfl-1gfp}$ (*nsEx37*) (URAs) as described in Materials and Methods. Strains carrying the array *akEx32* were homozygous for *lin-15(n765ts)* X. Strains carrying the array *nsEx37* were homozygous for *him-5(e1490)* V and *lin-15(n765ts)* X. ^aIndicates that the strains were homozygous for *dpy-17(e164)* III.

The loss of *unc-86*, *lrs-1*, or *unc-132* function causes a defect in CEM specification. Next, we determined the fate of the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function. Briefly, a mutation that blocks apoptotic cell death in general (*ced-3(n717)*) failed to suppress the CEM phenotype observed in these mutant backgrounds, which indicates that the CEMs do not inappropriately undergo apoptosis (Tables 1a and b). In addition, we found that the loss of *lrs-1* or *unc-132* function does not affect the presence of differentiated dorsal URAs or amphid socket cells, the sisters of the dorsal CEMs and ventral CEMs, respectively,⁶ which indicates that the CEMs are generated (Table 3). Finally, as shown below, we can detect CEMs in *unc-86* mutant embryos using a transcriptional *egl-1* reporter.

Therefore, we conclude that the CEMs are also generated in animals lacking *unc-86* function. On the basis of these observations, we propose that masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function lack differentiated CEMs as a result of a defect in the specification (rather than generation or survival) of the CEMs.

***unc-86*, *lrs-1*, and *unc-132* are required for *ceh-30* expression in the CEMs in masculinized hermaphrodites.** The Bar homeodomain transcription factor CEH-30, whose expression is under the direct control of the terminal, global regulator of somatic sexual fate, TRA-1, acts to block the death of the CEMs.^{13,14} Specifically, the loss of *ceh-30* function causes the CEMs to inappropriately die in

Figure 2 *ceh-30*, *egl-1*, and *ced-3* expression in embryonic CEMs by 4D microscopy and lineaging analysis. Schematic representation of time course analyses of the expression of the reporters $P_{ceh-30}ceh-30::gfp$, $P_{egl-1}his-24::gfp$, and $P_{ced-3}gfp$ in the ventral left embryonic CEM (CEMVL) in wild-type or *sel-10(n1077)* hermaphrodites. Time (min) is indicated on the left. Three embryos were analyzed for each reporter and genotype. White dots represent the CEM mother cells, green dots indicate CEMs expressing a particular *gfp* reporter. Green dots surrounded by purple circles indicate CEMs at the time when expression of the *gfp* reporter was first detected. (See Supplementary Figure S1 for corresponding DIC and fluorescent images.) Gray dots indicate CEMs lacking reporter expression. Embryos were prepared for 4D microscopy and lineaged starting at the 2- or 4-cell stage as described in Materials and Methods. Fluorescent stacks were taken every ~20 min after the CEM mother cell was born. No expression was detected for any of the reporters in the CEM mother cell

males as well as in masculinized hermaphrodites, indicating that *ceh-30* is required for CEM survival^{13,14} (P Grote and B Conrads, unpublished data). Using a translational *ceh-30* reporter ($P_{ceh-30}ceh-30::gfp$) and 4D lineaging analysis,^{14,23,24} we determined the expression pattern of the *ceh-30* gene in the CEMs in embryos starting at ~320 min, the time at which they are generated, until ~470 min, the time at which the CEMs normally die in hermaphrodites.⁶ We found that in wild-type hermaphrodites, in which the CEMs die, *ceh-30* expression in the CEMs can only be detected right after the cells are generated (Figure 2, +/+; Supplementary Figure S1). *ceh-30* expression cannot be detected in the mother cell and can no longer be detected in the CEMs at ~360 min or later time points (Figure 2). In masculinized hermaphrodites, however, in which the CEMs survive, *ceh-30* expression in the CEMs can be detected starting at ~400 min (Figure 2, *sel-10(n1077)*).

Next, we analyzed the expression of the *ceh-30* reporter in the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function in which, as described above, the CEMs are generated and survive, but are misspecified. We found that in these animals, at ~470 min, *ceh-30* is not expressed in most CEMs (Figures 3a and b). This result was surprising as the loss of *ceh-30* function causes the CEMs to inappropriately die in masculinized hermaphrodites. On the basis of these observations, we conclude that *unc-86*, *lrs-1*, and *unc-132* are required for the expression of *ceh-30* in the

CEMs in masculinized hermaphrodites (Figure 4). We also conclude that apart from promoting *ceh-30* expression, *unc-86*, *lrs-1*, and *unc-132* have an additional function, which is to promote CEM death in a *ceh-30*-independent manner (Figure 4).

The loss of *unc-86*, *lrs-1*, or *unc-132* function causes de-repression of *egl-1* transcription in the CEMs in masculinized hermaphrodites. Using a transcriptional reporter ($P_{egl-1}his-24::gfp$) and 4D lineaging analysis, we analyzed the expression of the *egl-1* gene in the CEMs.²⁵ We found that in wild-type hermaphrodites, in which the CEMs die, *egl-1* transcription can be detected in the CEMs starting at ~400 min (Figure 2, +/+; Supplementary Figure S1). In contrast, in masculinized hermaphrodites, in which the CEMs survive, *egl-1* transcription cannot be detected in the CEMs (Figure 2, *sel-10(n1077)*). As mentioned above, the loss of *ceh-30* function causes the CEMs to inappropriately die in masculinized hermaphrodites. We found that the loss of *ceh-30* function causes *egl-1* to be inappropriately transcribed in the CEMs in masculinized hermaphrodites at ~470 min (Figures 5a and b). These results show that *ceh-30* functions to repress *egl-1* transcription in the CEMs in masculinized hermaphrodites (Figure 4).

To determine why the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function, but not *ceh-30*

Figure 3 *unc-86*, *lrs-1*, and *unc-132* are required for *ceh-30* expression in the CEMs in masculinized hermaphrodites. (a) DIC and fluorescence images ($P_{ceh-30::gfp}$) of CEM corpses (white arrow heads) or CEMs (white arrows) in wild-type hermaphrodites (+/+), males (XO +/+), *sel-10(n1077)* hermaphrodites or *lrs-1(bc155); sel-10(n1077)* hermaphrodites. Males were identified by recovering embryos after analysis and allowing them to develop into larvae. (b) Summary of data obtained on $P_{ceh-30::gfp}$ expression. Green bars indicate the percentage of CEMs that were GFP-positive and green numbers above indicate the fraction of CEMs analyzed that were GFP-positive. Blue bars indicate the percentage of CEMs that acquired a corpse-like morphology and blue numbers above indicate the fraction of CEMs analyzed that had a corpse-like morphology. All strains analyzed were homozygous for *unc-76(e911)* and carried the extrachromosomal array *nEx1171* ($P_{ceh-30::gfp}$). The wild-type hermaphrodites and males analyzed were homozygous for *him-5(e1467ts)*. The strains *unc-86(bc151); sel-10(n1077)* and *lrs-1(bc155); sel-10(n1077)* were also homozygous for *dpy-17(e164)*

Figure 4 Genetic pathway of the CEM death. (Upper panel) In wild-type hermaphrodites (XX), *tra-1* is active in the CEMs, thereby blocking *ceh-30*. Thus, the pro-apoptotic genes *egl-1* and *ced-3* are active, which promotes CEM death. See text for details. (Lower panel) In males (XO) or masculinized hermaphrodites (*sel-10(n1077)*), *tra-1* is not active in the CEMs, which results in the activation of *ceh-30*, and, consequently, the repression of *egl-1* and *ced-3*. Thus, CEM death is inhibited. See text for details

Figure 5 *egl-1* is transcribed in surviving CEMs in masculinized *unc-86(bc151)*, *lrs-1(bc155)*, or *unc-132(bc159)* hermaphrodites. (a) DIC and fluorescence images (P_{egl-1his-24::gfp}) of CEMs (white arrows) or CEM corpses (white arrow heads) in wild-type hermaphrodites (+/+), *sel-10(n1077)*, *sel-10(n1077); ceh-30(bc272)*, or *lrs-1(bc155); sel-10(n1077)* hermaphrodites. (b) Summary of data obtained on P_{egl-1his-24::gfp} expression. Green bars indicate the percentage of CEMs that were GFP-positive and blue bars indicate the percentage of CEMs that acquired a corpse-like morphology. All strains analyzed were homozygous for the integrated P_{egl-1his-24::gfp} array *bcls37*. The strain *ceh-30(bc272); sel-10(n1077)* was also homozygous for the integrated array *bcls9*. The strains *unc-86(bc151); sel-10(n1077)* and *lrs-1(bc155); sel-10(n1077)* were homozygous for *dpy-17(e164)*

function, fail to inappropriately die, we analyzed *egl-1* transcription in the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* at ~470 min. To our surprise, we found that the CEMs in these animals transcribe *egl-1* at this time point (Figures 5a and b). This observation indicates that, unlike in masculinized hermaphrodites lacking *ceh-30* function, in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function, the transcriptional upregulation of *egl-1* in the CEMs is not sufficient for their apoptotic death.

***ced-3* is transcribed in the CEMs in hermaphrodites and its transcription in the CEMs in masculinized hermaphrodites is repressed by *ceh-30*.** Using a reporter for the caspase gene *ced-3*, it has been shown that *ced-3* is not transcribed in most of the 131 cells that are programmed to die.⁴ However, *ced-3* is actively transcribed in at least one cell that is programmed to die, the tail spike cell.⁴ Like the CEMs, the tail spike cell survives much longer than most cells programmed to die (~300 min) before it dies. For this reason, we explored whether the *ced-3* gene is transcribed in the CEMs before their deaths in hermaphrodites.

Using a transcriptional reporter (P_{ced-3gfp}) and 4D lineaging analysis, we found that in wild-type hermaphrodites, in which the CEMs die, *ced-3* transcription can be detected in the CEMs starting at ~460 min (Figure 2, +/+; Supplementary Figure S1). Therefore, the *ced-3* gene is transcriptionally active in the CEMs. Furthermore, we found that in masculinized hermaphrodites, in which the CEMs survive, *ced-3*

transcription cannot be detected (Figure 2, *sel-10(n1077)*). Next, we determined the role of *ceh-30* in *ced-3* transcription in the CEMs. We found that in masculinized hermaphrodites lacking *ceh-30* function, in which the CEMs inappropriately die, *ced-3* is transcribed in the CEMs at ~470 min (Figures 6a and b). Therefore, we conclude that *ceh-30* functions to repress *ced-3* transcription in the CEMs in masculinized hermaphrodites.

The loss of *unc-86*, *lrs-1*, or *unc-132* function does not cause activation of *ced-3* transcription in the CEMs in masculinized hermaphrodites. To determine the role of *unc-86*, *lrs-1*, and *unc-132* in *ced-3* transcriptional control in the CEMs, we analyzed the expression of the P_{ced-3gfp} reporter in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function at ~470 min. We found that, like in masculinized hermaphrodites, the *ced-3* gene is not transcribed in most CEMs at this time point (Figures 6a and b). Therefore, while the loss of *unc-86*, *lrs-1*, or *unc-132* function results in the de-repression of *egl-1* transcription in the CEMs in masculinized hermaphrodites, it does not result in the de-repression of *ced-3* transcription. On the basis of this observation we conclude that *unc-86*, *lrs-1*, and *unc-132* not only repress *ced-3* transcription in a *ceh-30*-dependent manner but also promote *ced-3* transcription in a *ceh-30*-independent manner (Figure 4). Furthermore, we propose that the CEMs in masculinized hermaphrodites lacking *unc-86*, *lrs-1*, or *unc-132* function fail to die because *ced-3* transcription in the CEMs is not activated.

Figure 6 Analysis of *ced-3* transcription in the CEMs. (a) DIC and fluorescence images ($P_{ced-3gfp}$) of CEM corpses (white arrow heads) or CEMs (white arrows) in wild-type (+/+), *sel-10(n1077)*, *sel-10(n1077); ceh-30(bc272)*, or *lrs-1(bc155); sel-10(n1077)* hermaphrodites. (b) Summary of data obtained on $P_{ced-3gfp}$ expression. Green bars indicate the percentage of CEMs that were GFP-positive, and blue bars indicate the percentage of CEMs that acquired a corpse-like morphology. All strains analyzed were homozygous for the integrated $P_{ced-3gfp}$ array *bcl9* and carried extrachromosomal arrays of $P_{ced-3gfp}$ (+/+ [*bcEx834*, *bcEx836*], *sel-10(n1077)* [*bcEx839*, *bcEx840*], *ceh-30(bc272); sel-10(n1077)* [*bcEx876*], *unc-86(bc151); sel-10(n1077)* [*bcEx870*, *bcEx871*], *lrs-1(bc155); sel-10(n1077)* [*bcEx872*, *bcEx873*], *unc-132(bc159); sel-10(n1077)* [*bcEx874*, *bcEx875*])

Discussion

Transcriptional upregulation of both *egl-1* and *ced-3* is required for the apoptotic death of the CEMs in hermaphrodites.

The transcriptional upregulation of *egl-1* is thought to be necessary and sufficient for many of the cell death events that take place during *C. elegans* development.⁷ We found that *egl-1* is transcriptionally active in the CEMs in hermaphrodites in which the CEMs die but not in masculinized hermaphrodites (and most probably males) in which the CEMs survive. However, our results show that the transcriptional upregulation of *egl-1* in the CEMs in hermaphrodites is necessary, but not sufficient for their death.

The *ced-3* gene does not appear to be transcriptionally active in many of the cells that are programmed to die during development.⁴ However, we found that *ced-3* is transcriptionally active in the CEMs in hermaphrodites in which the CEMs die but not in masculinized hermaphrodites in which the CEMs survive. Furthermore, we present data that indicate that transcriptional activation of *ced-3* in the CEMs is necessary for their death in hermaphrodites. On the basis of these findings, we propose that in contrast to most cells that are programmed to die during development, in the CEMs, the transcriptional upregulation of *egl-1* and *ced-3* is necessary for their death in hermaphrodites. This is the first demonstration that the transcriptional upregulation of both a BH3-only gene as well as a caspase gene is required for apoptosis induction.

What distinguishes the CEMs from the majority of cells that die during development is that rather than surviving for only

~30 min after being generated, they survive for ~150 min.⁶ In the majority of cells that are programmed to die proCED-3 protein is inherited from progenitors.⁴ On the basis of this observation, we propose that at the time apoptosis induction takes place in the CEMs, as a result of proCED-3 turn over, the level of progenitor-derived proCED-3 has reached a level in the CEMs that is no longer sufficient for apoptosis induction (Figure 7). For this reason, the transcriptional upregulation of not only the *egl-1* gene but also the *ced-3* gene is necessary for apoptosis induction in the CEMs.

CEH-30 represses *egl-1* and *ced-3* transcription thereby promoting CEM survival.

The inappropriate death of CEMs in males lacking *ceh-30* function is suppressed by the loss of *egl-1* function.¹⁴ This suggests that the *egl-1* gene acts downstream of the *ceh-30* gene and is a potential target of the CEH-30 transcription factor. However, analyses performed with a gain-of-function mutation of *ceh-30* suggest that the *ceh-30* gene blocks the death of the CEMs by acting in parallel to or downstream of *egl-1*.¹⁴ We present data that indicate that the inappropriate death of CEMs in masculinized hermaphrodites lacking *ceh-30* function is the result of the transcriptional de-repression of the pro-apoptotic genes *egl-1* and *ced-3*. In other words, we show that the *ceh-30* gene acts both upstream of as well as in parallel to *egl-1* to prevent the activation of the central cell death machinery. We propose that the CEH-30 protein is required to repress *egl-1* and *ced-3* transcription in the CEMs of masculinized hermaphrodites, thereby causing their survival

Figure 7 Model of the life-versus-death decision in the CEMs. (Top panel) Most cells programmed to die do so ~30 min after being generated. In these cells, proCED-3 protein inherited from the progenitor is sufficient for apoptosis induction in response to *egl-1* transcriptional activation. (Bottom panel) The CEMs in hermaphrodites die ~150 min after being generated (~470 min after the first cell division). Increasing TRA-1 activity results in decreasing CEH-30 activity, which is required for the de-repression of *egl-1* and *ced-3* transcription. The activation at the transcriptional level of the *ced-3* gene compensates for decreased levels of proCED-3. See text for details

(Figure 4). Whether CEH-30 represses *egl-1* and *ced-3* transcription directly or indirectly remains to be determined.

***ceh-30* transcription in the CEMs is dependent on *unc-86*, *lrs-1*, and *unc-132* function.** The *ceh-30* gene is transcriptionally active in the CEMs in males and masculinized hermaphrodites, but not in hermaphrodites. In hermaphrodites, *ceh-30* transcription in the CEMs is directly repressed by the GLI-like transcription factor TRA-1, which is the terminal, global regulator of somatic sexual fate^{12–14} (Figure 4). Our results indicate that the genes *unc-86*, *lrs-1*, and *unc-132*, which are required for correct CEM specification, are necessary for *ceh-30* transcriptional activation in the CEMs in masculinized hermaphrodites (Figure 4).

unc-86 encodes a POU homeodomain transcription factor that has previously been implicated in CEM specification and differentiation.^{18,19} Our results show that the UNC-86 protein is required for *ceh-30* transcription in the CEMs, which is consistent with previous studies that suggest that UNC-86 acts as a direct activator of *ceh-30* transcription.¹³ Surprisingly, *lrs-1* encodes a leucyl-tRNA synthetase. On the basis of our analyses, compromising translation by decreasing the level of at least certain aminoacyl-tRNA synthetases abrogates *ceh-30* transcription in the CEMs. Therefore, we propose that *ceh-30* transcription is particularly sensitive to perturbations of the translational machinery. Finally, *unc-132* encodes a novel protein of unknown function. The *D. melanogaster* orthologue of *unc-132*, *CG30118*, was identified in a screen for genes involved in *Drosophila* hematopoiesis.²¹ However, the mechanism through which *CG30118* acts has so far not been determined. Furthermore, we found that the N-terminal region of the UNC-132 protein shares sequence similarity with the N-terminal region of the human proto-oncoprotein PIM-1.²² PIM-1 is a serine/threonine kinase,

which is involved in various cellular processes.²⁶ However, the roles of PIM-1 in these processes are thought to be dependent on its kinase activity. As UNC-132 does not contain a classical kinase domain, it is unclear how extensive the functional similarities between PIM-1 and UNC-132 are. In summary, although UNC-86 may be a direct activator of *ceh-30* transcription, the mechanisms through which LRS-1 and UNC-132 promote *ceh-30* transcription in the CEMs remain to be elucidated.

***unc-86*, *lrs-1*, and *unc-132* promote *ced-3* transcription in the CEMs in a *ceh-30*-independent manner thereby promoting their death.** *unc-86*, *lrs-1*, and *unc-132* are required for *ceh-30* transcriptional activation in the CEMs in masculinized hermaphrodites. However, although we found that the loss of *ceh-30* function results in the de-repression of both *egl-1* and *ced-3* transcription, the loss of *unc-86*, *lrs-1*, or *unc-132* function only results in the activation of *egl-1* transcription. For this reason, we propose that apart from blocking *egl-1* and *ced-3* transcription in a *ceh-30*-dependent manner, *unc-86*, *lrs-1*, and *unc-132* also promote *ced-3* transcription in a *ceh-30*-independent manner (Figure 4). We hypothesize that the ability of *unc-86*, *lrs-1*, and *unc-132* to promote *ced-3* transcription in a *ceh-30*-independent manner is relevant in hermaphrodites in which the *ced-3* gene is relieved of *ceh-30*-dependent transcriptional repression (Figure 4, XX). Conversely, their ability to block *ced-3* transcription in a *ceh-30*-dependent manner may be relevant in males and masculinized hermaphrodites in which the *ceh-30* gene is relieved of TRA-1-dependent transcriptional repression (Figure 4, XO, *sel-10(n1077)*). How *unc-86*, *lrs-1*, and *unc-132* promote *ced-3* transcription in the CEMs in hermaphrodites remains to be determined.

Why do the CEMs survive for 150 min? The CEMs are born ~320 min after the first cell division and die in hermaphrodites at ~470 min.⁶ The TRA-1 protein has a pivotal function in the life-versus-death decision of the CEMs. In the CEMs, TRA-1 represses *ceh-30* transcription thereby causing *egl-1* and *ced-3* de-repression and apoptosis induction. The activity of TRA-1 is regulated at the post-translational level by sex-specific cleavage and proteolysis.¹² Currently, it is unclear when during embryonic development active TRA-1 protein is first generated in hermaphrodites. However, experiments using a temperature-sensitive loss-of-function mutation of the gene *tra-2*, which acts upstream of *tra-1* in somatic sex determination, suggest that active TRA-1 might be generated in hermaphrodites starting at ~320 min.²⁷ This notion is supported by the finding that the *ceh-30* gene, whose expression is directly repressed by TRA-1, is no longer expressed at ~360 min (Figures 2 and 7). On the basis of these observations, we propose that the CEMs die only at ~470 min, because a level of active TRA-1 first has to be generated in the CEMs in hermaphrodites that is sufficient for the transcriptional repression of *ceh-30*, which is a prerequisite for the transcriptional de-repression of *egl-1* and *ced-3* at ~390 min and 460 min, respectively (Figure 7). Hence, we propose that the timing of the death of the CEMs is controlled by the sex determination pathway and, in particular, its terminal, global regulator, the Gli-like transcription factor TRA-1.

Potential role for the coexpression of BH3-only and caspase genes in the developmental control of apoptosis. At least during animal development, pro-caspases are thought to be present in most if not all cells.^{1,2} Furthermore, pro-caspases are thought to be present at levels that, once they become matured and activated, are sufficient for apoptosis induction. As the maturation and activation of pro-caspases generally represents the commitment of a cell to the apoptotic fate, it is a highly regulated process.²⁸ In contrast, very little is known thus far about the contribution of transcriptional control in the regulation of pro-caspases. The most comprehensive studies available to date were performed in *D. melanogaster*. These studies suggest that transcriptional regulation of caspase genes has an important function in apoptosis induction at least during *D. melanogaster* development.^{1,29} The data presented here show that the transcriptional upregulation of the *C. elegans* caspase gene *ced-3* is required for the sexually dimorphic, apoptotic death of the CEM neurons. On the basis of these findings, we propose that the transcriptional upregulation of caspase genes is a conserved aspect of apoptosis induction during animal development. Furthermore, our data show that in *C. elegans*, genes encoding BH3-only proteins and pro-caspases can be under the same transcriptional control. We hypothesize that such coregulatory mechanisms have evolved to ensure efficient apoptosis induction during development.

Materials and Methods

Strains and general methods. *C. elegans* strains were cultured as described.³⁰ Bristol N2 was used as the wild-type strain. N2 and the CB4856 (Hawaii) strain were used for SNP mapping. Mutations and integrated transgenes

used in this study are listed below and are described³¹ except where noted otherwise: LG II: *rff-3(pk1426)*,³² LG III: *ced-4(n1162)*, *unc-86(bc151)* (this study), *lrs-1(bc155)* (this study), *dpy-17(e164)*, *unc-93(e1500sd)*. LG IV: *ced-3(n717)* LG V: *bcls37* (*P_{egl-1;his-24::gfp}*),²⁵ *egl-1(n1084 n3082)*,¹⁰ *sel-10(n1077)*, *unc-132(bc159)* (this study), *unc-76(e911)*, *him-5(e1490)*, *him-5(e1467ts)*, *dpy-11(e224)*. LG X: *ceh-30(bc272)* (P. Grote and B. Conradt; unpublished data), *lin-15(n765ts)*, *bcls9* (*P_{pkd-2gfp}*).³³ The following extrachromosomal arrays were used: *nEx1171* (*P_{ceh-30ceh-30::gfp}*),¹⁴ *bgEx21* (*P_{unc-53gfp}*),³⁴ *syEx301* (*P_{lov-1gfp}*),³⁵ *nsEx37* (*P_{ceh-1gfp}*),³⁶ *akEx32* (*P_{glt-4glt-4::gfp}*).³⁷

RNAi by feeding was performed as described using 6 mM IPTG.³⁸ The plasmids pBC966 (*W08A12.1b(RNAi)*) and pBC967 (*W08A12.1c(RNAi)*) contained full-length cDNA clones cloned as NcoI-NheI fragments into vector L4440. *sel-10(n1077)*; *bcls9* animals were mutagenized with EMS (ethyl-methanesulfonate) as described.³⁰ Germine transformations were performed as described.³⁹ Cosmids and fosmids were injected at a concentration of 10 ng/μl using pRF4 (*rol-6(su1006)*) at 50 ng/μl as coinjection marker. The plasmid *P_{ced-3gfp}* was injected at 40 ng/μl using pRF4 (*rol-6(su1006)*) (50 ng/μl) as coinjection marker. In the case of *bcls9* (*P_{pkd-2gfp}*), *syEx301* (*P_{lov-1gfp}*), *bgEx21* (*P_{unc-53gfp}*), *nsEx37* (*P_{ceh-1gfp}*), *akEx32* (*P_{glt-4glt-4::gfp}*), *bcls37* (*P_{egl-1;his-24::gfp}*), and *nEx1171* (*P_{ceh-30ceh-30::gfp}*), transgenic animals were crossed with *unc-86(bc151)*, *lrs-1(bc155)* or *unc-132(bc159)* mutants to generate *unc-86(bc151)*, *lrs-1(bc155)* and *unc-132(bc159)* strains carrying these arrays. pBC957 (*P_{ced-3gfp}*) was constructed by removing an internal, 4.6 kb EcoRV fragment containing most of the *ced-3* coding sequence from a *ced-3* reporter.⁴

***sel-10(n1077)* screen and cloning of *unc-86(bc151)*, *lrs-1(bc155)*, and *unc-132(bc159)*.** 'Undead' CEMs in *sel-10(n1077)* hermaphrodites have the characteristic CEM morphology when observed by differential interference contrast microscopy (DIC) in L4 larvae, and express CEM-specific markers in adults, such as the genes *pkd-2* (*P_{pkd-2gfp}*) and *lov-1* (*P_{lov-1gfp}*).³⁵ These observations indicate that the CEMs in *sel-10(n1077)* hermaphrodites are correctly specified and fully differentiated. We mutagenized *sel-10(n1077)* hermaphrodites carrying an integrated *P_{pkd-2gfp}* transgene (*bcls9*), screened 8500 mutagenized, haploid genomes for mutants in which differentiated CEMs were absent based on *pkd-2* expression and identified 49 mutants. Among these mutants, 21 still had CEMs based on DIC and therefore most likely harbor mutations that affect *pkd-2* expression. In addition, in 20 mutants, all aspects of the weak masculinization caused by *sel-10(n1077)* were suppressed, indicating that they most likely carry feminizing mutations or loss-of-function mutations in the *sel-10* gene. Of the remaining eight mutations, one behaved non-Mendelian, one could not be outcrossed, and three caused the CEMs to be absent in masculinized *sel-10(n1077)* hermaphrodites, but not males. The final three mutations, *bc151*, *bc155*, and *bc159*, were characterized further.

Standard genetic techniques were used to map *bc151* to the right of *dpy-17* on LG III, *bc155* between *unc-93* and *dpy-17* on LG III, and *bc159* to the left of *dpy-11* on LG V.

bc151:bc151 failed to complement *unc-86(n846)*, a *lf* allele of the gene *unc-86*, indicating that *bc151* is a loss-of-function mutation in the *unc-86* gene.

bc155:SNP mapping was used to locate *bc155* between the SNPs C30D11:9408 and R10E4:10469. The fosmid WRM0638-J8 rescued the phenotype observed in *bc155*; *sel-10(n1077)*; *bcls9* animals.

bc159:SNP mapping was used to map *bc159* to the left of SNP H10D18:26865. The cosmid W08A12 rescued the phenotype observed in *bc159*; *sel-10(n1077)*; *bcls9* animals. The *bc159* phenotype was also rescued by a 31.3-kb XcmI subclone of W08A12 (pBC430, containing the genes W08A12.1, W08A12.2, and *egr-1*) and was partially rescued by a 23.9-kb Apal subclone of W08A12 (pBC431, spanning the genes W08A12.4, *egr-1*, and W08A12.1).

Phenotypic analysis. The presence of CEMs in adults was analyzed using *bcls9* (*P_{pkd-2gfp}*) and a Zeiss Axioskop2 equipped with epifluorescence as described.³³ The % GFP-positive CEMs was calculated by dividing the number of CEMs observed by the maximum number of possible CEMs (four CEMs per animal). The presence of CEMs in L4 larvae was analyzed using DIC as described.⁸ The presence of CEMs in embryos was analyzed as follows. CEMs were identified by DIC in 1½-fold stage embryos (~450 min) based on their positions and observed for at least ~30 min. (As the positions of the two dorsal CEMs are less characteristic, these cells could not always be examined.) CEM corpses appear in two-fold embryos (~465 min). Microscopy of living embryos was performed by mounting embryos on 2% agar pads in M9 buffer, using a Zeiss Axioskop2

equipped with epifluorescence, a Micromax CCD camera (Princeton Instruments, Trenton, NJ, USA), and Metamorph software. DIC and epifluorescence images were taken every 5 min between the 1½- and 2-fold stage to determine the fate of the CEMs and the expression of the GFP reporters in embryonic CEMs.

The presence of the AMso (amphid socket) cells was scored in adults using *P_{unc-53}gfp* as described.³⁷ The presence of the URA cells was determined in adults using two different reporters, *P_{glr-4}gfp* and *P_{cfl-1}gfp*, as described.³⁶

4D microscopy and lineage analysis. 4D microscopy and lineaging analysis (as shown in Figure 2) was performed on *C. elegans* embryos as described earlier.^{23,24} Embryos were recorded at 20 °C, and GFP expression of the reporters *P_{ceh-30}ceh-30::gfp*, *P_{egl-1}his-24::gfp* and *P_{ced-3}gfp* was recorded after every 30 DIC stacks. The 4D recordings were analyzed using the SIMI BioCell software as described earlier.^{23,40} The ventral left CEM (CEMVL, ABplpaapap) was lineaged in all embryos. Starting at the 2- to 4-cell stage, cells were tracked and their 3D coordinates were saved approximately every 2 min, until at least ~465 min after the first cell division. In addition, the 3D coordinates of the CEMVL progenitor (ABplpaapap) or CEMVL were saved at the time points when GFP expression of the reporters *P_{ceh-30}ceh-30::gfp*, *P_{egl-1}his-24::gfp*, or *P_{ced-3}gfp* was recorded. Using this approach, the status of expression of these reporters in the CEM could be determined. The cell death fate of the CEMVL was determined at ~465 min by DIC.

To determine the expression of the reporters *P_{ceh-30}ceh-30::gfp*, *P_{egl-1}his-24::gfp*, or *P_{ced-3}gfp* at ~470 min after the first cell division (as shown in Figures 3, 5, and 6), we identified the CEMs in embryos ~450 min after the first cell division based on their positions using DIC and observed them for ~30 min using fluorescence microscopy. At the end of the recording, we determined the cell death fate of the CEMs using DIC (Apoptotic cells adopt a button-like, refractile appearance, which can be detected by DIC).

Conflict of interest

The authors declare no conflict of interest.

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