Cell-nonautonomous inhibition of radiation-induced apoptosis by dynein light chain 1 in Caenorhabditis elegans

TH Morthorst1 and A Olsen*1

The evolutionarily conserved process of programmed cell death, apoptosis, is essential for development of multicellular organisms and is also a protective mechanism against cellular damage. We have identified dynein light chain 1 (DLC-1) as a new regulator of germ cell apoptosis in Caenorhabditis elegans. The DLC-1 protein is highly conserved across species and is a part of the dynein motor complex. There is, however, increasing evidence for dynein-independent functions of DLC-1, and our data describe a novel dynein-independent role. In mammalian cells, DLC-1 is important for cellular transport, cell division and regulation of protein activity, and it has been implicated in cancer. In C. elegans, we find that knockdown of dlc-1 by RNA interference (RNAi) induces excessive apoptosis in the germline but not in somatic cells during development. We show that DLC-1 mediates apoptosis through the genes lin-35, egl-1 and ced-13, which are all involved in the response to ionising radiation (IR)-induced apoptosis. In accordance with this, we show that IR cannot further induce apoptosis in dlc-1(RNAi) animals. Furthermore, we find that DLC-1 is functioning cell nonautonomously through the same pathway as kri-1 in response to IR-induced apoptosis and that DLC-1 regulates the levels of KRI-1. Our results strengthen the notion of a highly dynamic communication between somatic cells and germ cells in regulating the apoptotic process.

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Apoptosis is the process in which external or internal cues induce self-elimination of the cell. Apoptosis is an evolutionarily conserved key process in the development of multicellular organisms and is also implicated in the aetiology of several diseases such as cancer and neurodegeneration.1,2 In C. elegans, invariably, 131 somatic cells undergo apoptosis during development (developmental apoptosis) of the hermaphrodite,3,4 and in the adult half of the germ cells are eliminated by apoptosis (physiological germ cell apoptosis).5 These types of apoptosis in C. elegans depend on the core apoptotic machinery comprising the caspase ced-3,6 the adaptor protein Apaf-1 homologue ced-47 and the anti-apoptotic Bcl-2 homologue ced-9.8 These types of apoptosis in C. elegans are functioning inside the dying cells to regulate the killing process. However, the genes vab-1 (ephrin receptor) and kri-1 (ankyrin-repeat protein orthologous to the human KRIT1/CCM1) have been shown to cell nonautonomously regulate physiological and IR-induced germ cell apoptosis, respectively.9,10 Apoptotic cells are removed by engulfment, and in the germline engulfment is carried out by the surrounding sheath cells.11 Two partially redundant pathways regulate engulfment.12 One pathway consists of the transmembrane receptor ced-1, the adaptor protein ced-6 (GULP) and ced-7 (ABC1).13–16 The other pathway comprises the adaptor protein ced-2 (Crlk), the guanine nucleotide-exchange factors ced-5 (DOCK180) and ced-12 (ELMO) and ced-10 (RAC1).17–19 Mutations in several engulfment genes impair the removal of dead cells, which consequently persist longer.12 Cytoplasmic dyneins are multisubunit motor protein complexes associated with microtubules. Large heavy chains comprise the bulk of the dynein complexes and confer motor activity, whereas the intermediate and light chains are accessory subunits that bind cargo.20–22 The mammalian dynein light chain DYNLL1 is highly conserved with 95% homology to the C. elegans homologue DLC-1. DYNLL1 is implicated in dynein-regulating processes such as vesicular

1Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, Aarhus C DK-8000, Denmark
*Corresponding author: A Olsen, Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, Aarhus C DK-8000, Denmark.
Tel: +45 3069 8155; Fax: +45 8612 3178; E-mail: ano@mb.au.dk

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Abbreviations: C. elegans, Caenorhabditis elegans; DLC-1, dynein light chain 1; IR, ionising radiation; RNAi, RNA interference; GFP, green-fluorescent protein; DIC, differential interference contrast; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; gaf, gain-of-function; MAPK, mitogen-activated protein kinase; Gy, grey; Unc, uncoordinated; ORF, open reading frame; NGM, nematode growth medium; DTT, dithiothreitol; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; cDNA, complementary DNA; DMEM, Dulbecco’s Modified Eagle Medium

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and protein transport, cell division, mitotic spindle formation and nuclear migration.\textsuperscript{29,30} \textit{DYNLL1} binds to a variety of proteins besides dynein including the following: the pro-apoptotic protein BimL.\textsuperscript{31} p53-binding protein 1\textsuperscript{32,33} and the cell cycle regulators Cdk2 and Ciz1.\textsuperscript{34} There is increasing evidence that \textit{DYNLL1} can act independent of its association with microtubules.\textsuperscript{35,36} Several interaction partners of \textit{DYNLL1} regulate cell viability\textsuperscript{31,33,34} and \textit{DYNLL1} is over-expressed in breast tumours.\textsuperscript{37} In accordance with a high evolutionary conservation, the \textit{C. elegans} homologue \textit{DLC-1} affects germ cell proliferation, and inactivation of \textit{dic-1} by RNA interference (RNAi) in a tumour-promoting background results in hyperproliferating and polyploid germ cells.\textsuperscript{38}

We previously conducted a whole-genome RNAi screen with a view of identifying genes conferring resistance to the chemotherapeutic drug hydroxyurea (HU) (unpublished). The genes identified in the screen were analysed for germ cell apoptosis, and RNAi against \textit{dynein light chain 1 (dic-1)} caused a significant increase in the number of apoptotic germ cells. In this study, we describe a novel role of \textit{dlc-1} in regulating IR-induced germ cell apoptosis by a cell-nonautonomous function via \textit{kri-1}. We show that \textit{DLC-1} is functioning via \textit{egl-1} and \textit{ced-13} independently of \textit{cep-1}.

\textbf{Results}

\textbf{Lack of \textit{dlc-1} induces germ cells to undergo apoptosis.}

To investigate the effect of \textit{dlc-1} inactivation on germ cell apoptosis, we treated worms with RNAi against \textit{dlc-1} and quantified apoptosis using differential interference contrast (DIC) microscopy and the \textit{CED-1::GFP} (green-fluorescent protein) reporter. During the engulfment process, the transmembrane receptor \textit{CED-1} expressed in sheath cells clusters around apoptotic cells.\textsuperscript{19} \textit{CED-1::GFP (Plim-7::\textit{ced-1::gfp, bcIs39})} can therefore be used to quantify apoptotic germ cells undergoing engulfment. The \textit{dlc-1(RNAi)} animals had significantly more GFP-positive cells than the controls (Figures 1a and b). A significant increase in the number of apoptotic cells following RNAi against \textit{dlc-1} was also seen using DIC microscopy (Figures 1a and b). The RNAi treatment reduced the expression of \textit{dlc-1} with 80–90% compared with controls (Supplementary Figure S1).

We reasoned that the increased number of apoptotic cells in the \textit{dlc-1(RNAi)} animals could be because of excessive germ cells undergoing apoptosis or an engulfment defect preventing their removal. The \textit{CED-1::GFP} marker can only be used if proper internalisation of the dead cells is taking place during the engulfment process; consequently, the \textit{CED-1::GFP} marker does not detect apoptotic cells in the engulfment-defective mutants \textit{ced-5} and \textit{ced-6}.\textsuperscript{39} We confirmed that persistent cell corpses observable with DIC in \textit{ced-5(tm1950)} and \textit{ced-6(tm1826)} mutants could not be seen with the \textit{CED-1::GFP marker (Figure 1c)}. Therefore, as the apoptotic cells in \textit{dlc-1(RNAi)} animals could be observed equally well with DIC and \textit{CED-1::GFP}, RNAi against \textit{dlc-1} did not result in an engulfment defect. Thus, \textit{dlc-1(RNAi)} animals do not phenocopy engulfment-defective mutants.

To investigate whether \textit{dlc-1} is also involved in somatic apoptosis during development, we analysed L1 larvae for the presence of persistent cell corpses in the head with DIC microscopy. Wild-type N2 larvae have no visible cell corpses, whereas engulfment mutants, such as \textit{ced-5(tm1950)} and \textit{ced-6(tm1826)}, have many persistent cell corpses\textsuperscript{18} (and Figure 1d). Inactivation of \textit{dic-1} in wild-type N2 worms did not result in persistent cell corpses, indicating that \textit{dic-1} does not affect engulfment during development. Increased apoptosis during development can be seen in engulfment-defective mutant backgrounds, as the extra dead cells are not as efficiently removed as in wild-type worms. However, RNAi against \textit{dlc-1} had no effect on the number of corpses observed in \textit{ced-5} or \textit{ced-6} mutants (Figure 1d). This suggests that RNAi against \textit{dlc-1} does not affect somatic cell death or engulfment but specifically increases germ cell apoptosis.

\textbf{DLC-1 affects apoptosis independently of the dynein motor complex.}

In addition to \textit{dic-1}, the \textit{C. elegans} genome encodes five other light chains (\textit{dlc-2–6}). We speculated whether the observed apoptotic phenotype was specific for the inactivation of \textit{dic-1} or whether it was a general phenotype for all the dynein light chains. Following their inactivation, none of the other five light chains resulted in an increased number of apoptotic germ cells (Figure 1e). Furthermore, we also investigated the effect on apoptosis after knockdown of the main dynein heavy chain, \textit{dhc-1}. As RNAi against \textit{dhc-1} causes developmental arrest, we treated N2 wild-type worms with RNAi against \textit{dhc-1} from the L4 stage and scored apoptosis 48 h later using DIC, but no effect on germ cell apoptosis was observed (Figure 1f). This indicates that the increase in apoptotic cells is specific for \textit{dic-1} and is not a general phenotype from a disrupted dynein complex.

\textbf{Lack of \textit{dlc-1} induces apoptosis through \textit{CED-3}, \textit{CED-9} and \textit{LIN-35}.}

Strong loss-of-function mutations in \textit{ced-3} or \textit{ced-4} completely inhibit both somatic and germ cell apoptosis. We inactivated \textit{dlc-1} in a mutant harbouring the strong loss-of-function allele \textit{ced-3(n717)} and expressing the \textit{CED-1::GFP} marker. The \textit{ced-3(n717)} mutation completely suppressed apoptosis because of RNAi against \textit{dic-1} (Figure 2a). \textit{ced-3-independent} apoptosis has been reported following RNAi against the inhibitor of cell death-1 (\textit{icd-1}) gene, which encodes the beta-subunit of the nascent polypeptide-associated complex (bNAC).\textsuperscript{40} Therefore, as an additional control we treated \textit{ced-3(n717)} mutants with \textit{icd-1} RNAi. In agreement with the previous report, we observed that \textit{ced-3} could not completely suppress apoptosis induced by RNAi against \textit{icd-1} (Supplementary Figure S2). Thus, inactivation of \textit{dlc-1} increases the number of apoptotic germ cells exclusively through the \textit{ced-3} pathway. As almost all types of apoptosis in \textit{C. elegans} require the core apoptotic machinery, we next decided to use an epistasis analysis approach to determine the subtype of apoptosis influenced by RNAi against \textit{dic-1}.

Physiological germ cell death is independent of the core apoptotic machinery gene \textit{ced-9}.\textsuperscript{5} We tested whether \textit{dlc-1(RNAi)}-induced apoptosis was independent of a \textit{ced-9} gf mutation (\textit{n1950}). However, \textit{dlc-1(RNAi)}-induced apoptosis was completely abolished in the \textit{ced-9(gf)} mutant, indicating that \textit{ced-9} was required for \textit{dlc-1(RNAi)} to induce apoptosis (Figure 2b). Thus, \textit{dlc-1} is not involved in physiological germ cell death.
Inactivation of dlc-1 results in a tumorous germline and unpaired chromosomes in the oocytes. Therefore, we hypothesised that these phenotypes might lead to DNA damage-induced apoptosis through CED-9. In C. elegans, DNA damage-induced apoptosis is dependent on the p53 homologue cep-1. Hence, we investigated whether dlc-1(RNAi) could induce apoptosis in cep-1(gk138) mutants. RNAi against dlc-1 induced apoptosis in the cep-1 mutants to the same extent as in wild-type N2 animals (Figure 2c). Thus, dlc-1(RNAi) does not result in apoptosis because of an increased level of DNA damage. Unpaired chromosomes can trigger a synapsis checkpoint independent of the DNA-damage checkpoint, which requires the activity of PCH-2, the C. elegans homologue of PCH2, a budding yeast pachytene checkpoint gene. We found that inactivation of dlc-1 induced apoptosis in pch-2 mutants to a similar extent as seen in wild-type N2 animals (Figure 2d). Thus, dlc-1(RNAi) does not induce apoptosis because of unpaired chromosomes.

Several pathways have been shown to feed into the core apoptotic machinery through CED-9, such as the mitogen-activated protein kinase (MAPK) and retinoblastoma (RB) tumour-suppressor pathways. Overactivation of MAPK – for example, by loss of the inhibitor GLA-3 – causes excessive germ cell apoptosis.
**Figure 2.** *dil-1(RNAi) increases apoptosis through CED-3, CED-9 and LIN-35 (a). Mean number of apoptotic cells per gonad arm after RNAi against *dil-1* in a *ced-3(n717)* mutant, scored with CE-1::GFP. (b) Mean number of apoptotic cells per gonad arm after RNAi against *dil-1* in a *ced-9(n1950)* mutant, scored with DIC. (c) Mean number of apoptotic cells per gonad arm after RNAi against *dil-1* in *cep-1(gk138)* mutants, scored with CED-1::GFP. (d) Mean number of apoptotic cells per gonad arm after RNAi against *dil-1* in *pch-2(tm1458)* mutants, scored with CE-1::GFP. (e) Mean number of apoptotic cells per gonad arm after RNAi against *dil-1* in *gla-3(ok2684)* mutants, scored with CED-1::GFP. (f) Mean number of apoptotic cells per gonad arm after RNAi against *dil-1* in *lin-35(n745)* mutants, scored with DIC. All graphs are mean (± S.D.) of at least three independent experiments. *N* = 20–40 gonad arms for each experiment. *P < 0.05*
that *dlc-1* does not affect *lin-35* and *ced-9* activity at the transcriptional level.

**DLC-1 is a part of the response to IR-induced apoptosis.** As *dlc-1*(RNAi)-induced apoptosis was independent of *cep-1* but dependent on *lin-35*, both of which are involved in response to DNA damage, we evaluated how *dlc-1*(RNAi) animals responded to DNA damage induced by IR. Interestingly, IR did not increase the number of apoptotic cells in *dlc-1*(RNAi) animals (Figure 3a), indicating that IR and *dlc-1* function in a common pathway.

In human cells, DLC-1 facilitates the transport of p53 to the nucleus in response to DNA damage.\(^{32,33}\) The lack of response to IR in *dlc-1*(RNAi) animals could arise from the mislocalisation or malfunction of CEP-1. Using western
DLC-1 regulates germ cell apoptosis
TH Morthorst and A Olsen

DLC-1 is functioning cell nonautonomously through kri-1. To study the expression and localisation of DLC-1, we generated two independent transgenic lines (P{dlc-1::dlc-1::gfp} expressing GFP-tagged DLC-1 under the regulation of the dlc-1 promotor (Figure 4). We found DLC-1 to be expressed in numerous tissues including those of the intestine (Figure 4a), body wall muscles (Figure 4b), germ cells and oocytes (Figure 4c), the rectal valve cell (Figure 4d) and unidentified cells in the head (Figure 4e). DLC-1 was expressed at all developmental stages from the one-cell embryo. To validate that the transgenic strain was expressing wild-type DLC-1, we treated it with RNAi against dlc-1 and observed that the GFP level was significantly reduced (Supplementary Figure S5), and we used western blotting to confirm that the full-length fusion protein was being expressed (data not shown).

As DLC-1 was expressed both in the germ cells and in somatic tissues, we investigated in which of these tissues inactivation of dlc-1 resulted in increased germ cell apoptosis. The ppw-1 gene encodes a member of the argonaute family of proteins and is required for efficient RNAi in germ cells.48 The rrf-1 gene encodes an RNA-directed RNA polymerase required for activation of the RNAi pathway in somatic cells.49 We treated two different alleles of rrf-1 (pk1417 and ok589) and ppw-1 (pk2505 and pk1425) with RNAi against dlc-1 and quantified the number of apoptotic cells using DIC. We observed that the increase in apoptotic cells caused by dlc-1 RNAi was completely abolished in the rrf-1 mutants but not affected in the ppw-1 mutants (Figure 5a). Therefore, inactivation of dlc-1 only in somatic cells gives rise to the increase in apoptotic cells. To verify the specificity of the rrf-1 and ppw-1 mutants, we treated them with ced-1 RNAi, which induces an engulfment defect. CED-1 functions in the somatic sheath cells but not in the germ cells. As expected, ced-1(RNAi) only induced an engulfment defect in ppw-1 mutants but not in rrf-1 mutants (Supplementary Figure S6).

As dlc-1 functions in the response to IR, we investigated whether IR regulates dlc-1 at the transcriptional or translational level. However, using qPCR no changes in dlc-1 transcription in response to IR was observed (data not shown). Likewise, we did not observe any changes in the levels of DLC-1::GFP after IR in whole animals (Figure 4f). We also examined the localisation of DLC-1::GFP after IR, but changes were observed neither in whole animals (Figure 4g) nor in sheath cells (Supplementary Figure S7). We also hypothesised that overexpression of DLC-1 might protect against IR-induced apoptosis. However, we observed no difference in the number of apoptotic cells between wild-type N2 and DLC-1::GFP animals when treated with 120 or 60 Gy (Figure 5b and Supplementary Figure S8).

The clear involvement of DLC-1 in somatic tissues in regulating germ cell apoptosis led us to investigate a possible involvement of kri-1. The KRI-1 ankyrin-repeat protein is orthologous to the human KRIT1/CCM1, and it is required for IR-induced apoptosis in a novel pathway acting in somatic cells.16 We examined the epistatic relationship between dlc-1 and kri-1 by treating kri-1(ok1251) mutants with RNAi against dlc-1. kri-1 mutants fed dlc-1 RNAi had significantly fewer apoptotic germ cells than wild-type N2 animals treated with RNAi against dlc-1 (Figure 5c). This indicates that kri-1 is necessary for dlc-1(RNAi)-induced apoptosis. Next, we used a strain expressing the functional KRI-1::GFP protein to examine whether dlc-1 could regulate the expression or localisation of KRI-1. We treated KRI-1::GFP animals with dlc-1 RNAi for 3 days and scored the expression of GFP compared with animals grown on control RNAi. Our results show that dlc-1 RNAi increases the expression of KRI-1::GFP on average with 57% (Figure 5d), demonstrating that dlc-1 can regulate KRI-1. We did not observe any change in localisation of KRI-1::GFP because of RNAi against dlc-1 (Supplementary Figure S9). To test the possibility that DLC-1 and KRI-1 might interact directly, we expressed C. elegans DLC-1 and KRI-1 in a mammalian two-hybrid system using luciferase as readout. As DLC-1 is known to bind to itself in mammalian cells, we used this interaction as a positive control (Figure 5e). However, we did not observe any interaction between DLC-1 and KRI-1 (Figure 5f). Based upon our results we propose that DLC-1 is regulating KRI-1 in response to IR-induced apoptosis to mediate a pro-apoptotic signal from somatic cells to germ cells (Figure 6).
Discussion

We have identified DLC-1 as a new gene required for proper apoptotic signalling between somatic cells and germ cells in response to IR acting through KRI-1. Our study is thus adding to the knowledge of the recently described KRI-1-mediated response to IR. Firstly, we find that KRI-1 is required for CED-3-dependent apoptosis induced by RNAi against dlc-1. Secondly, our data show that DLC-1 regulates the level of KRI-1. Thirdly, we show that the DLC-1/KRI-1 signalling pathway operates via EGL-1 and CED-13. Based upon these results we propose a model in which DLC-1 is part of the response to IR-induced apoptosis through a cell-nonautonomous pathway depending on kri-1 and independent of cep-1 (Figure 6). Consistent with this model we found that IR did not induce additional apoptosis in dlc-1(RNAi) animals. Our results strongly support that intercellular communication between somatic cells and germ cells is required for proper regulation of the cell-killing process.

Figure 4  Transgenic worm expressing Pdlc-1,dlc-1::gfp. (a) Expression of DLC-1::GFP under control of the dlc-1 promoter. (b) Expression of DLC-1::GFP in body wall muscles. (c) DLC-1::GFP is expressed in germ cells and oocytes. (d) DLC-1::GFP is expressed in the rectal valve cells. (e) DLC-1::GFP expression in unidentified cells in the head. (f) Quantification (by ImageJ) of DLC-1::GFP expression after exposure to 0 and 60 Gy. (g) Expression of DLC-1::GFP 24 h after exposure to 60 Gy.
Our model raised the possibility that dlc-1 was directly regulated by IR at the transcriptional or translational level. However, we did not observe any changes in expression or localisation of DLC-1::GFP in response to IR. Instead, IR may induce post-translational modifications to DLC-1. In mammalian cells, DLC-1 can be phosphorylated on Ser88 that inactivates DLC-1 by disrupting the homodimer. This phosphorylation can be carried out by the p21-activated kinase, Pak-1.

We have demonstrated that dlc-1(RNAi)-induced apoptosis is dependent on EGL-1 and CED-13; however, DLC-1 is not involved in transcriptional regulation of egl-1 and ced-13. Rather our model predicts a second layer of regulation of the proteins involved in IR-induced apoptosis; CEP-1 regulates the transcription of egl-1 and ced-13, whereas DLC-1 indirectly regulates their activity at the protein level through a cell-nonautonomous pathway, giving a tighter and more fine-tuned regulation of IR-induced apoptosis.

At the molecular level, several mechanisms may explain how DLC-1 regulates apoptosis. One possible underlying molecular mechanism could be that DLC-1 normally binds a pro-apoptotic signal/protein. dlc-1 inactivation or exposure to

Figure 5  DLC-1 is functioning cell nonautonomously through kri-1. (a) Mean number of apoptotic cells per gonad arm after RNAi against dlc-1 in N2, ppw-1(pk2505) and rr-1(pk1417) mutants, scored with DIC. (b) Mean number of apoptotic cells per gonad arm in P_dlc-1::gfp 24 h after treatment with 120 Gy, scored with CED-1::GFP. (c) Mean number of apoptotic cells per gonad arm after RNAi against dlc-1 in N2 and kri-1(ok1251) mutants. All graphs for apoptosis are mean (± S.D.) of at least three independent experiments. For the apoptosis assays, N = 20–40 gonad arms for each experiment. *P < 0.05. (d) Expression of KRI-1::GFP after treatment with dlc-1 RNAi for 3 days. Quantified by ImageJ. Representative of three independent experiments. (e) DLC-1–DLC-1 interaction by the use of mammalian two-hybrid system. Representative of three independent experiments (f). DLC-1–KRI-1 interaction by use of mammalian two-hybrid system.
proliferation and differentiation of the germline.\textsuperscript{53} Decoding pathways for intertissue communication is also of crucial importance in higher organism such as humans to better understand the interplay and regulation of individual organs required to maintain an intact organism.

Materials and Methods

Strains and culture conditions. All strains were maintained at 20°C on standard nematode growth medium (NGM) spotted with Escherichia coli strain OP50. The following strains were used: Wild-type N2, MD701 (bcIs23[psi-lm-7] oed-1::GFP + lin-15[+];), MT1522 ced-5(n717), ced-6(tm1826), MT4770 ced-9(n1590), FX536 ced-13(tm536), NL2098 rrf-1(pk1417), RB798 rrf-1(ok589), NL2550 pww-1(pk5205), NL3511 pww-1(pk4129), TJ1 cep-1(gk138), CA388 pch-2(tm1458), MT10430 lin-35(n745), RB2026 gla-3(ok2684), MT8375 egl-1(n1084n3082), ced-5(tm1590), egl-1(n1084n3082);ced-13(tm536), EG6699 (lt71605 II, unc-119 (ed3) III, oxEx1578, pmk-3(ok169), pmk-1(km25), sir-2.1(kc343), CF2052 kri-1(ok1251), muEx353[Pkri-1::kri-1::gfp; odr-1::rfp]; OLS400 aasII[Pdce-1::DLC-1::GFP cb-unc-119(+);] II and OLS401 aasII[Pdce-1::DLC-1::GFP cb-unc-119(+);] II.

Double mutants were made by crossing MD701 males with hermaphrodites of the desired phenotype. For cep-1::ced-1::GFP, pch-2::ced-1::GFP and gla-3::ced-1::GFP, the homozgyous F2s were selected using standard PCR (see Supplementary Table S1 for primers) and GFP expression. cep-1::ced-1::GFP was made by using the marker unc-26(e1196). The marker was first crossed to MD701, and F2s were selected by their Unc phenotype and GFP expression. This strain was then crossed to ced-1, and F2s were selected for their GFP expression and non-Unc phenotypes. To validate the cep-1 mutant, we used IR to introduce DNA damage-induced apoptosis. As expected, the cep-1 mutant had no apoptotic cells in the germline after IR (Supplementary Figure S10).

Generation of transgenic strains by MosSCI. The plasmid containing P	extsubscript{dce-1}:dlc-1::gfp was generated by using standard Gateway techniques (Invitrogen, Naerum, Denmark). pENTRY clones for the dce-1 promoter and open reading frame (ORF) were generated by using PCR products amplified with Gateway and gene-specific primers (Supplementary Table S1). The vector pJA256 (Addgene, Cambridge, MA, USA) was used as pENTRY clone for GFP. The Gateway destination vector was pCFJ150 (Addgene) containing an unc-119 rescue fragment.

MosSCI was performed as described\textsuperscript{49} using direct insertion based on injection of unc-119-animals (EG6699 strain) with an injection mix comprised of the plasmids (all from Addgene): pCFJ50 containing P	extsubscript{dce-1}:dlc-1::gfp (final conc. 15 ng/l), pCFJ801 (final conc. 50 ng/l), pMA122 (final conc. 10 ng/l), pGH8 (final conc. 2.5 ng/l) and pCFJ104 (final conc. 5 ng/l). Selection was based on pell-1 toxin, wild-type movement, absence of mCherry markers and presence of GFP.

Two independent lines OLS400 and OLS401 were created, and expression of full-length DLC-1 was confirmed by using western blotting with an antibody against GFP. The lines were subsequently back-crossed to wild-type N2 four times to eliminate background mutations.

RNAi. The RNAi clone against dlc-1(T2669.5) was from the OpenBiosystems RNAi Library (Thermo Fisher Scientific, Waltham, MA, USA). RNAi clones against dlc-2, dlc-3, dlc-4, dlc-5 and dlc-6 were generated by standard cloning techniques and inserted into pL4440 and expressed by HT115 cells. All RNAi clones were verified by sequencing (See Supplementary Table S1 for primers). RNAi was performed by feeding on NGM plates containing 1 mM isopropyl thiogalactoside and mCherry (100 ng/ml),\textsuperscript{52} Worms feed HT115 bacteria containing an empty pL4440 vector (ctrl RNAi) were used as controls.

In all germline apoptosis assays following RNAi against dlc-1, apoptosis was scored in the first generation on RNAi. For assays with RNAi against dlc-2-6, apoptosis was scored both in first and second generation on RNAi to eliminate maternal rescue. For dlc-1 RNAi, animals were treated with RNAi from L4 and apoptosis was scored 48 h later.

For developmental apoptosis in the head of L1 larvae on dlc-1 RNAi, eggs were picked onto dlc-1 RNAi plates and their progeny was scored for apoptosis.

Quantification of apoptosis. Cells undergoing apoptosis were distinguished from normal cells as refractile, button-like discs by using DIC microscopy.
Quantitative real-time PCR. Primers). Test gene technical triplicates. Water was used as negative control. All primers were PCR Master Mix (Applied Biosystems, Naerum, Denmark), was performed on a Gene-specific qRT-PCR using cDNA, appropriate primers and Power SYBR Green measured using a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). One micromolar of total RNA was reverse-transcribed into complementary DNA (cDNA) using iScript cDNA Synthesis Kit (BioRad) following the manufacturer’s protocol. Gene-specific qRT-PCR using cDNA, appropriate primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Naerum, Denmark), was performed on a Stratagene MX3000P machine (La Jolla, CA, USA). All samples were run in technical triplicates. Water was used as negative control. All primers were designed to span exon-exon boundaries (See Supplementary Table S1 for primers). Test gene Ct values were normalised to either act-1 (dcl-1, egl-17 and ced-13) or pmp-2 (lin-35 and ced-9) Ct values. Relative expression levels were calculated by using the Pfaffl method.

Mammalian two-hybrid system. Experiments were performed based on the Checkmates Mammalian Two-Hybrid system (Promega, Madison, WI, USA). C. elegans KRI-1 (isofrom a) and DLC-1 were cloned from whole-worm cDNA using standard cloning techniques (See Supplementary Table S1 for primers). KRI-1 was inserted into the pACT vector, and DLC-1 was inserted into both the pACT and pBIND vectors. HEK293T cells were seeded in 24-well plates with a density of 1.4 × 10^6 cells per well. Next day the cells were transfected at 70–90% confluence with 50 ng GFP vector, 150 ng pGL5uc, 150 ng pACT (with appropriate insert), 150 ng pBIND (with appropriate insert) and 1 μl lipofectamine 2000 (Life Technologies, Naerum, Denmark) in 50μl DMEM serum-free medium (Life Technologies) per well. After 24 h, the medium was refreshed. Forty-eight hours after transfection, the cells were lysed, and 50μl of lysate were transferred to 96-well plates. GFP fluorescence was measured using a Perkin Elmer Universal Multiplate Analyzer Fusion-Alpha FPHT. Forty microtitre luciferase (Promega) was added to each well, and fluorescence was measured in an Enspire 2300 Multi label Reader. Background levels from empty cells were subtracted and luciferase/ GFP was plotted as binding efficiency. All measurements were run in technical triplicates. Non-transfected cells, cells transfected with either pACT or pBIND without insert or both were used as negative controls.

Conflict of Interest

The authors declare no conflict of interest.

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