Autophagy activity contributes to programmed cell death in Caenorhabditis elegans

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Keywords: autophagy, apoptosis, genotoxic stress, C. elegans

Abbreviations: ATG, autophagy-related (gene); epg, ectopic PGL granules; DAPI, 4,6-diamino-2-phenyl indole; GFP, green fluorescent protein

The physiological relationship between autophagy and programmed cell death during C. elegans development is poorly understood. In C. elegans, 131 somatic cells and a large number of germline cells undergo programmed cell death. Autophagy genes function in the removal of somatic cell corpses during embryogenesis. Here we demonstrated that autophagy activity participates in germ-cell death induced by genotoxic stress. Upon γ ray treatment, fewer germline cells execute the death program in autophagy mutants. Autophagy also contributes to physiological germ-cell death and post-embryonic cell death in ventral cord neurons when ced-3 caspase activity is partially compromised. Our study reveals that autophagy activity contributes to programmed cell death during C. elegans development.

Introduction

Autophagy is a lysosome-mediated degradation process, involving the formation of an enclosed double-membrane structure, called the autophagosome, and its subsequent delivery to the lysosome for degradation.1,2 In response to various stresses, such as starvation or growth factor withdrawal, autophagy nonselectively degrades a portion of the cytosol to provide energy, and thus functions as a cell survival mechanism.3 Inactivation of autophagy triggers apoptosis under these conditions. Autophagy also acts as a quality control system by selectively removing misfolded proteins or damaged organelles.3 Neural-specific depletion of the autophagy genes Atg5, Atg7, or Ei24 causes accumulation of cytoplasmic protein inclusions and massive neuronal cell death.4,6 Autophagy also acts as a cell death-executing mechanism.7 Autophagy promotes cell death in the context of impaired apoptosis factors in several cell lines, including L929 mouse fibroblast cells and macrophages in the presence of the pan-caspase inhibitor zVAD, and bax and bak1 knockout fibroblasts in the presence of etoposide.8,9 Pioneering work in Drosophila has revealed that autophagy contributes to physiological cell death through different mechanisms in several tissues during development. Autophagy per se functions as a cell death mechanism in degeneration of the midgut;10 autophagy acts downstream of, or in parallel to, caspase activation in metamorphic cell death of the salivary gland;11,12 and autophagy acts upstream of caspase activation to promote cell death in the female germline.13,14 In addition to participating in the cell death program, autophagy is also essential for exposure of the “eat-me” signal phosphatidylserine by apoptotic cells.15 Through this mechanism, autophagy is involved in the clearance of apoptotic cell corpses during chick retinal development and cavitation in mouse embryoid bodies.15,16

During C. elegans development, 131 somatic cells are programmed by their cell lineage to undergo programmed cell death (PCD).17 The death program is executed through a cascade of apoptosis factors, initiated by transcriptional activation of egl-1, which encodes a BH3-only protein. EGL-1 binds to the anti-apoptotic protein CED-9 (ortholog of mammalian BCL2), and subsequently leads to activation of the proapoptotic proteins CED-4 (homolog of mammalian APAF1) and CED-3 (homolog of caspases).18 Apoptotic cell corpses are engulfed by neighboring cells, in which corpse-containing phagosomes undergo sequential maturation processes and are eventually digested by lysosomes.18 During C. elegans oogenesis, a large proportion of germline cells at the meiotic pachytene stage undergo PCD and act as nurse cells to provide cytoplasmic components for maturing oocytes.19 Physiological germ-cell apoptosis is executed via the core apoptotic machinery, composed of CED-3, CED-9, and CED-4, but independent of EGL-1.19 DNA damage or

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Submitted: 12/31/2012; Revised: 08/06/2013; Accepted: 08/08/2013
http://dx.doi.org/10.4161/auto.26095

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Various environmental stresses also trigger the death program in meiotic pachytene-stage germ cells.\textsuperscript{20} DNA damage activates the mammalian TP53 ortholog CEP-1, which induces transcription of \textit{egl-1} and another BH3 domain-only protein encoding gene, \textit{ced-13}.\textsuperscript{21-23} In autophagy mutant embryos, the number of cell corpses is increased as a result of a slower cell corpse clearance.\textsuperscript{24} Here we showed that autophagy also contributes to germ-cell death induced by genotoxic stress, and to physiological germ-cell death process. We found that the number of germ-cell corpses is much higher due to their persistence and thus provides a more sensitized background to detect differences in the cell death program during \textit{C. elegans} development.

**Results**

**Autophagy is required for germ-cell death**

The \textit{C. elegans} germline is syncytial and during oogenesis, nuclei destined to die become cellularized. The death of germ cells is restricted to late-stage meiotic pachytene cells close to the bend region of the U-shaped gonad.\textsuperscript{19} Germ-cell corpses display refractile, raised button-like morphology and thus, can be easily identified by Normarski Differential Interference Contrast microscopy (DIC). The corpses are removed by somatic sheath cells and the process is mediated by the \textit{ced-1} and \textit{ced-5} parallel pathways as in the somatic cell corpse removal pathway.\textsuperscript{19} We first examined the role of autophagy in physiological germ-cell death. In wild-type animals, there are about 1.9 and 3.3 germ-cell corpses 24 and 48 h post L4, respectively. In mutants of autophagy genes acting at distinct steps of the pathway, including \textit{atg-3}, \textit{lgl-1}, \textit{atg-5}, \textit{epg-1/atg13}, \textit{epg-4}, and \textit{arg-2}, the number of germ-cell corpses showed no significant difference compared with wild type (Fig. 1A–C). We thus checked the number of cell corpses in engulfment gene mutants, in which the number of cell corpses is much higher due to their persistence and thus provides a more sensitized background to detect differences in the cell death process. We found that the number of germ-cell corpses in double mutants of \textit{ced-1}, \textit{ced-5}, or \textit{ced-12} with autophagy gene mutations was comparable with engulfment gene single mutants (Fig. 1D–F, I, and L), indicating that autophagy is not required for physiological germ-cell death.

We next determined the occurrence of germline cell death in young adult wild-type worms (12 h post L4) treated with 120 Gy \(\gamma\) radiation, and found that the germ-cell corpse number was dramatically increased to 11.6 (Fig. 1G and M). Compared with wild-type animals, the number of germ-cell corpses after \(\gamma\) ray treatment was significantly lower in autophagy mutants including \textit{atg-3}, \textit{lgl-1}, \textit{atg-5}, \textit{epg-1/atg13}, \textit{epg-4}, and \textit{arg-2} (Fig. 1H and M). For example, there were only 3.6 cell corpses in \textit{epg-4} mutants. We also introduced cell corpse engulfment gene mutations into different autophagy mutants and determined the number of cell corpses after \(\gamma\) ray irradiation. \textit{ced-1}, \textit{ced-12}, and \textit{ced-5} mutants had much more germ-cell corpses after DNA-damage induction than untreated animals (Fig. 1J and N). Simultaneous depletion of \textit{atg-3}, \textit{epg-5}, or \textit{atg-2} dramatically reduced the germ-cell corpse number compared with \textit{ced-1}, \textit{ced-12}, or \textit{ced-5} single mutants after \(\gamma\) ray treatment (Fig. 1K and N). To determine whether autophagy mutants show a reduced number of germ-cell corpses induced by other DNA-damage agents, we treated autophagy single- and autophagy engulfment double-mutants with ethylnitrosourea (ENU), a DNA-alkylating agent causing a broad spectrum of DNA lesions. ENU treatment elevated germ-cell death in both wild-type and engulfment-gene single mutants (Fig. 2A and B). Significantly fewer germ-cell corpses were observed in autophagy single and autophagy engulfment double mutants upon ENU treatment (Fig. 2A and B). Taken together, autophagy mutants contain a reduced number of germ-cell corpses induced by genotoxic stress.

The reduced germ-cell corpse phenotype in autophagy mutants could result from fewer germ-line cells undergoing programmed cell death, or faster engulfment and degradation of dying cells than in wild-type animals. To distinguish between these two possibilities, we used time-lapse microscopy to measure the duration of germ-cell corpses in wild type and autophagy mutants 8 h after \(\gamma\) ray treatment. In wild-type animals, the duration of germ-cell corpses was about 58.1 min, while in \textit{atg-2} and \textit{atg-3} mutant worms the germ-cell corpses persisted approximately 58.8 and 56.6 min respectively (Fig. 2C). These results indicate that unlike in somatic cell death during embryogenesis, the processes involved in engulfment and degradation of germ-cell corpses are not defective in autophagy mutants. The functional translational reporters for \textit{epg-1} and \textit{epg-5}, driven by their own promoters, exhibited no evident expression in gonadal sheath cells, which are main engulfing cells for germ-cell corpses (Fig. S1A–S1D). Ectopic expression of \textit{epg-5} in sheath cells did not rescue the reduced germ-cell corpse phenotype in \textit{epg-5} mutants upon \(\gamma\) ray treatment (Fig. S1I). Immunostaining revealed that \textit{LGG-1} (ortholog of yeast \textit{Atg8}) was expressed in germ cells (Fig. S1E–S1H). Taken together, these results suggest that autophagy activity functions in the execution of the cell death program in germ cells.

Germ-cell apoptosis induced by genotoxic stress requires the core cell death pathway. Mutations in genes essential for programmed cell death, including \textit{ced-3} and \textit{ced-4}, block the cell death program. To determine whether the \(\gamma\) ray-induced germ-cell apoptosis in autophagy mutants is dependent on the core cell death pathway, we generated \textit{epg-5} double with \textit{ced-3(1f)} and \textit{ced-4(1f)} and found that strong loss-of-function mutations in \textit{ced-3} and \textit{ced-4} almost completely blocked the occurrence of germ-cell corpses in autophagy mutants with or without \(\gamma\) ray treatment (Fig. 2D). Consistent with the previous study,\textsuperscript{19} the \textit{ced-9(gf)} mutation had little effect on germ-cell death induced by genotoxic stress in \textit{epg-5} mutants.

We further determined whether autophagy acts in parallel to, downstream of, or upstream of the death pathway to promote germ-cell death. CEP-1 plays an essential role in DNA damage-induced germ-cell death. We found that loss of \textit{cep-1} activity almost completely blocked germ-cell death in autophagy mutants in response to \(\gamma\) ray irradiation (Fig. S1J). CSP-2, which shares sequence similarity to both catalytic subunits of CED-3 caspase, functions as an apoptosis inhibitor. Loss of \textit{csp-2} promotes apoptosis in germ cells. Mutations in autophagy genes showed no effect on physiological germ-cell death in \textit{cep-2} mutants.
Figure 1. Autophagy is required for genotoxic germ-cell death. (A and B, D and E) DIC images of germ-cell corpses in wild-type (A), atg-2(bp576) (B), ced-1(e1735) (D) and ced-1(e1735); atg-2(bp576) (E) worms without γ ray treatment. Germ-cell corpses are indicated by arrowheads. The left gonadal arms are shown in (A and B) and the right gonadal arms are shown in (D and E). Scale bars: 5 μm. (G, H, J, and K) DIC images of induced germ-cell corpses after 120 Gy γ ray irradiation in wild-type (G), atg-2(bp576) (H), ced-1(e1735) (J), and ced-1(e1735); atg-2(bp576) (K) worms. Germ-cell corpses are indicated by arrowheads. The left gonadal arm is shown in (J) and the right gonadal arms are shown in (G and H) and (K). Scale bars: 5 μm. (C, F, I, and L) Germ-cell corpses were scored 24 and 48 h post L4 from one gonad arm in the indicated strains. At least 20 animals were scored. Data are shown as mean ± SEM. Unpaired t-tests were performed for statistical analysis. No significant difference was observed. (M and N) Germ-cell corpses were scored 24 h after 120 Gy γ ray irradiation from one gonad arm in the indicated strains. At least 20 animals were scored. Data are shown as mean ± SEM. Unpaired t-tests were performed for statistical analysis. *P < 0.05, **P < 0.0001; all other points had P > 0.05.
**Figure 2.** Autophagy is required for physiological germ-cell death in ced-3 hypomorphic mutants. (A and B) Autophagy is required for germ-cell death upon ENU treatment. Twelve hours post L4 worms were treated with 5 mM ENU for 4 h and germ-cell corpses from one gonad arm of each animal were scored 24 h post ENU treatment. At least 15 animals were scored. Data are shown as mean ± SEM. Unpaired t-tests were performed for statistical analysis. *P < 0.05, **P < 0.0001; all other points had P > 0.05. (C) Four-dimensional microscopy analysis of germ-cell corpse duration in wild type, atg-2(bp576) and atg-3(bp412) animals 8 h after 120 Gy γ-ray irradiation. The duration of 19 cell corpses was monitored. Data are shown as mean ± SEM. (D) The ced-3(1f) and ced-4(9f) mutations prevent germ-cell death in autophagy mutant backgrounds. ced-9(gf) has little or no effect on germ-cell death after γ-ray irradiation. Germ-cell corpses were scored 24 h post L4 or 24 h post 120 Gy γ-ray irradiation from one gonad arm in the indicated strains. At least 15 animals were scored. Mutant alleles used here are ced-3(n717), ced-4(n1162) and ced-9(n1950). Data are shown as mean ± SEM. Unpaired t-tests were performed for statistical analysis. *P < 0.05, **P < 0.0001; all other points had P > 0.05. (E) Relative fold induction of egl-1 and ced-13 mRNA in indicated animals 12 h after 120 Gy γ-ray irradiation. egl-1 and ced-13 fold induction was averaged from three independent RT-PCR. (F) Relative expression levels of ced-13 and egl-1 in wide type (N2), atg-2(bp576) and atg-3(bp412) animals 12 h after γ-ray irradiation by real-time PCR. Three independent experiments were done. Data are shown as mean ± SEM. Unpaired t-test was performed for statistical analysis. *P < 0.05, **P < 0.0001; all other points had P > 0.05. (G and H) Autophagy promotes physiological germ-cell death in weak ced-3 background. Germ-cell corpses were scored 24 and 48 h post L4 from one gonad arm in the indicated strains. At least 15 animals were scored. Data are shown as mean ± SEM. Unpaired t-test was performed for statistical analysis. *P < 0.05, **P < 0.0001; all other points had P > 0.05.
(Fig. S1K). Upon γ ray treatment, the number of germ-cell corpses is increased in csp-2 mutants. Mutations in atg-2 and atg-3 suppressed the enhanced germ-cell death phenotype in csp-2 mutants (Fig. S1L). Loss of function of prmt-5 negatively regulates the activity of cep-1 in DNA-damage induced apoptosis.23 In prmt-5 mutants, the number of germline cell corpses is increased upon γ ray treatment. Mutations in atg-3 suppressed the enhanced germ-cell death phenotype in prmt-5 mutants (Fig. 1N). These results indicate that autophagy functions downstream or in parallel to prmt-5 and cep-2. In response to DNA damage-induced germ-cell death, CEP-1 activates the transcription of egl-1 and ced-13.22 We performed quantitative RT-PCR and found that levels of egl-1 and ced-13 mRNA in autophagy mutants were significantly increased after γ ray irradiation but were comparable to wild-type animals (Fig. 2E and F), suggesting that autophagy functions downstream of, or in parallel to, EGL-1 and CED-13 in genotoxic germ-cell death.

Autophagy activity contributes to physiological germ-cell death and cell death in ventral cord neurons in a weak loss-of-function ced-3 mutant

We next determined whether autophagy activity contributes to physiological germ-cell death in animals carrying the partial loss-of-function mutation ced-3(n2438). The number of germ-cell corpses in ced-12; epg-5, ced-12; atg-3, ced-1; atg-3, ced-1; atg-2, and ced-1; epg-5 double-mutants was comparable with the ced-12 or ced-1 single mutants (Fig. 1F and I). However, when caspase activity is limited, epg-5(tm3425), atg-2(bp576), and atg-3(bp412) significantly reduced the number of germ-cell corpses in ced-12 and ced-1 mutants (Fig. 2G and H), indicating that autophagy contributes to physiological germ-cell death when ced-3 caspase activity is partially compromised.

We further examined the role of autophagy in cell death in the ventral cord of larval animals in ced-3(n2438) and ced-4(n2273) mutants. The neuroblast W and 12 P (P1-P12) undergo several rounds of division during larval development to give rise to ventral cord neurons. Among them, six Pn.aap cells (Pn.aap cells are the posterior daughters of the anterior daughters of the anterior daughters of P blast cells) generated by P3-P8 precursor cells differentiate into ventral cord motor neurons.17 These neuroblasts also generate 10 cells that undergo programmed cell death during larval development to give rise to ventral cord, but its loss of function suppresses the cell death in weak ced-3 mutants, suggesting that autophagy activity contributes to execution of the cell death program and prevents survival in mutants with partially compromised ced-3 activity. Our study indicated that autophagy participates in the cell death program during C. elegans development.

Materials and Methods

C. elegans strains

Strains of C. elegans were cultured at 20 °C. The wild-type strain was N2 Bristol. Mutant alleles used in this study as follows. LGI: ced-1(e1735), ced-12(n3261), atg-5(bp546), cep-1(gk138). LGII: epg-5(bp425), lgg-1(bp500). LGIII: epg-4(bp414), epg-1(bp425), atg-2(bp576), atg-5(bp412), atg-2(bp576), cep-1(gk138), and ced-4(n2438). LG IV: epg-4(bp425), epg-5(bp414), atg-5(bp576), ced-4(n2273), and ced-4(n1162). LGV: atg-18(gk378). LGVI: atg-18(gk378). LG VII: atg-2(bp576). Strains carrying transgenic arrays used in this study include qxEx4091(Plin-11::gfp), qxEx4092(Plin-11::epg-5), bps141(Plin-11::gfp), and n196(Plin-11::gfp).
Reporters for *epg-1* and *epg-5* were constructed by a PCR fusion-based approach. In brief, the fused PCR products were derived from two overlapping PCR DNA fragments. One contained the promoter region and the entire ORF of *epg-1* and *epg-5*. The other contained *gfp* and the *unc-54* 3'UTR from pPD95.79. The sequences for constructing reporters were: *epg-1* (WRM0625aG04, nt 12,176 to 16,076), *epg-5* (F54H5: nt 1-3032, and C56C10: nt 29155-35166).

Quantification of genotoxic-induced germ-cell corpses

L4 worms were aged for 12 h then treated with 120 Gy γ rays (Gammacell 1000). Germ-cell corpses were quantified 24 h post-irradiation under Nomarski optics as described. To induce germline apoptosis by ENU (Sigma, N3385), L4 worms aged for 12 h were incubated in M9 medium (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 ml 1 M MgSO₄ in 1 L water) containing OP50 bacteria and 5 mM ENU for 4 h. Worms were then recovered on nematode growth medium plates (dissolve 3 g NaCl, 17 g agar, 2.5 g peptone in 975 ml H₂O, autoclave and then add 1 ml CaCl₂ (1 M), 1 ml MgSO₄ (1 M), 1 ml cholesterol (5 mg/ml in ethanol), 1 ml OP50.

![Figure 3.](image-url) Autophagy activity contributes to somatic cell death in ced-3 and ced-4 hypomorphic mutants. (A–E) Fluorescence microscopy images of wild type (A), ced-3(n2438) (B), lgg-1(bp500) (C), atg-3(bp412) ced-3(n2438) (D), and lgg-1(bp500); ced-4(n2273) (E) mutants. *lin-11::gfp* is present in all backgrounds. White arrowheads indicate P3.aap-P8.aap, which normally survive; yellow arrowheads indicate Pn.aap cells of P1, P2, and P9-P12 cells, which normally die. Scale bar: 100 μm. (F) Quantification of extra survival of Pn.aap cells in the indicated genotypes. The number of extra surviving motor neurons in the ventral cord was compared in animals carrying a weak ced-3 or ced-4 mutation or in combination with mutations causing defective autophagy genes. Asterisks indicate *P* < 0.05 and double asterisks indicate *P* < 0.0001 (unpaired t-test). Data are shown as mean ± SE. At least 30 L4 larvae were scored for each genotype.
25 ml potassium phosphate buffer (1 M), mix the solution and pour into petri dishes for 24 h and germ-cell corpses were scored.

Four-dimensional microscopy
Four-dimensional (4D) microscopy analysis of germ-cell corpse duration was performed using a Zeiss Axioimager M1 (National Institute of Biological Sciences, Beijing) coupled with an AxioCam monochrome digital camera (National Institute of Biological Sciences, Beijing). L4 worms aged 12 h were treated with 120 Gy γ radiation and images were captured 8 h post irradiation. Images were processed and viewed using Axiovision Rel.4.5 software.

Pn.aap cell survival
The number of surviving Pn.aap cells was scored in L4 larvae carrying the nls96(Gfp) integrated array under a Zeiss Axioimager M1 fluorescence microscope.

Immunostaining
LGG-1 staining was performed as previously described with some modifications.10 Hermaphrodites were dissected in a 40 μl drop of gonad buffer [20 mM NaCl, 30 mM KCl, 3 mM Na2HPO4, 3 mM MgCl2, 20 mM HEPES, 2 mM CaCl2, 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin (Amresco, 0558)], 10 mM glucose, 33% FCS (GIBCOL, 100099-μL) stage animals were washed off with M9 buffer then rinsed twice with M9 before total RNA was extracted. The dry total RNA was resuspended in 50 μl DEPC-treated water and 1 μg was used for cDNA synthesis according to the manufacturer's instructions (SuperScript III, Invitrogen, 18080-051).

RT–PCR analysis was performed using the SYBR Premix Ex Taq (TaKaRa, DRR081A) on an Applied Biosystems 7500 Fast Real-Time PCR System (National Institute of Biological Sciences, Beijing). The mRNA level of targeted genes was normalized to that of col-10 for N2 worms. The following primers were used to detect cd-13 and egl-1: cd-13: 5’-TTTCTCCCGC TGTTGTCAT TAGG-3’ (F); 5’-GTCAAACCTG TCGCCATATA CTG-3’ (R); egl-1: 5’-TGTTCTACTC CTGTCCTC-3’ (F); 5’-GACATCATCT GACATCG-3’ (R).

Statistical analysis
The standard error of the mean (SEM) was used as the y error bar for bar charts plotted from the mean value of the data. Data derived from different genetic backgrounds were compared by the Student two way unpaired t-test. Data were considered statistically different at P < 0.05, P < 0.0001 is indicated with double asterisks and P < 0.05 with a single asterisk.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Dr Isabel Hanson for editing the manuscript. This work was supported by the National Basic Research Program of China (2013CB910100, 2011CB910100) and also a grant from the National Natural Science Foundation of China (31225018) to HZ. The research of HZ was supported in part by an International Early Career Scientist grant from the Howard Hughes Medical Institute.

Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/autophagy/article/26152

References

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