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Autophagy activity contributes to programmed cell death in *Caenorhabditis elegans*

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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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various environmental stresses also trigger the death program in meiotic pachytene-stage germ cells.[20] DNA damage activates the mammalian TP53 ortholog CEP-1, which induces transcription of *egl-1* and another BH3 domain-only protein encoding gene, *ced-13*.[21-23] In autophagy mutant embryos, the number of cell corpses is increased as a result of a slower cell corpse clearance.[24] Here we showed that autophagy also contributes to germ-cell death induced by genotoxic stress, and to physiological germ-cell death and post-embryonic cell death in a mutant with partially compromised *ced-3* function. Thus, autophagy also participates in the caspase-mediated death program during *C. elegans* development.

Results

Autophagy is required for germ-cell death

The *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 arm.[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 *ced-1* and *ced-5* parallel pathways as in the somatic cell corpse removal pathway.[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 *atg-3, lgg-1*, *atg-5, epg-1/atg13, epg-4*, and *atg-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 *ced-1*, *ced-5*, or *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 γ 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 γ ray treatment was significantly lower in autophagy mutants including *atg-3*, *lgg-1, atg-5, epg-1/atg13, atg-18, atg-2*, *epg-4*, and *epg-5* (**Fig. 1H and M**). For example, there were only 3.6 cell corpses in *epg-4* mutants. We also introduced cell corpse engulfment gene mutations into different autophagy mutants and determined the number of cell corpses after γ ray irradiation. *ced-1, ced-12*, and *ced-5* mutants had much more germ-cell corpses after DNA-damage induction than untreated animals (**Fig. 1J and N**). Simultaneous depletion of *atg-3, epg-5*, or *atg-2* dramatically reduced the germ-cell corpse number compared with *ced-1, ced-12*, or *ced-5* single mutants after γ 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 germline 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 γ ray treatment. In wild-type animals, the duration of germ-cell corpses was about 58.1 min, while in *atg-2* and *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 *epg-1* and *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 *epg-5* in sheath cells did not rescue the reduced germ-cell corpse phenotype in *epg-5* mutants upon γ ray treatment (**Fig. S1I**). Immunostaining revealed that LGG-1 (ortholog of yeast 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 *ced-3* and *ced-4*, block the cell death program. To determine whether the γ ray-induced germ-cell apoptosis in autophagy mutants is dependent on the core cell death pathway, we generated *epg-5* double with *ced-3(lf)* and *ced-4(lf)* and found that strong loss-of-function mutations in *ced-3* and *ced-4* almost completely blocked the occurrence of germ-cell corpses in autophagy mutants with or without γ ray treatment (**Fig. 2D**). Consistent with the previous study,[19] the *ced-9(gf)* mutation had little effect on germ-cell death induced by genotoxic stress in *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 *cep-1* activity almost completely blocked germ-cell death in autophagy mutants in response to γ 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 *csp-2* promotes apoptosis in germ cells. Mutations in autophagy genes showed no effect on physiological germ-cell death in *csp-2* mutants

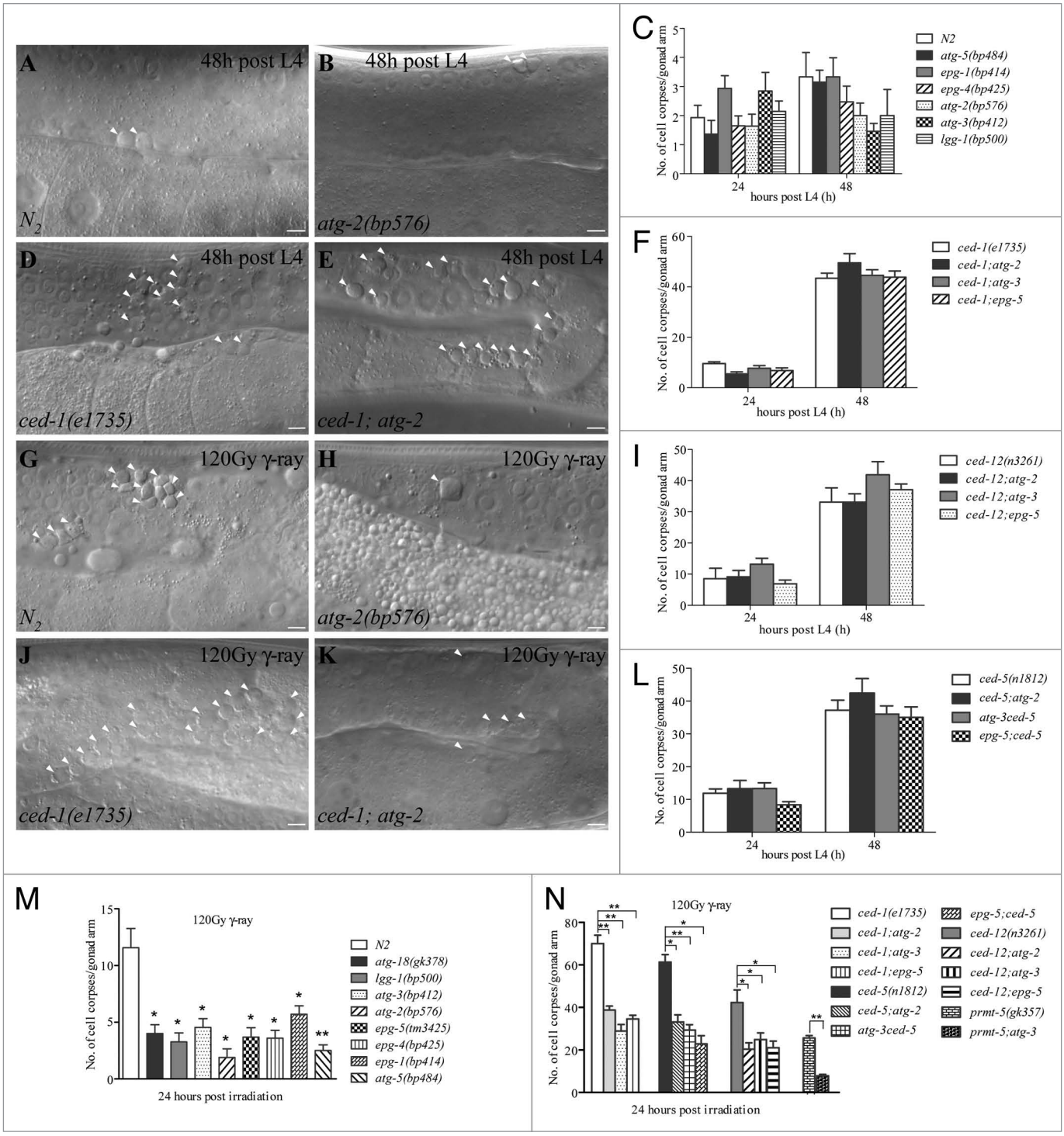

Figure 1. Autophagy is required for genotoxic germ-cell death. (**A and B, D and E**) DIC images of germ-cell corpses in wild-type (**A**), *atg-2(bp576)* (**B**), *ced-1(e1735)* (**D**) and *ced-1(e1735); atg-2(bp576)* (**E**) worms without γ ray treatment. Germ-cell corpses are indicated by arrowheads. The left gonadal arms are shown in (**A and B**) and the right gonad 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 gonad 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 $P > 0.05$.

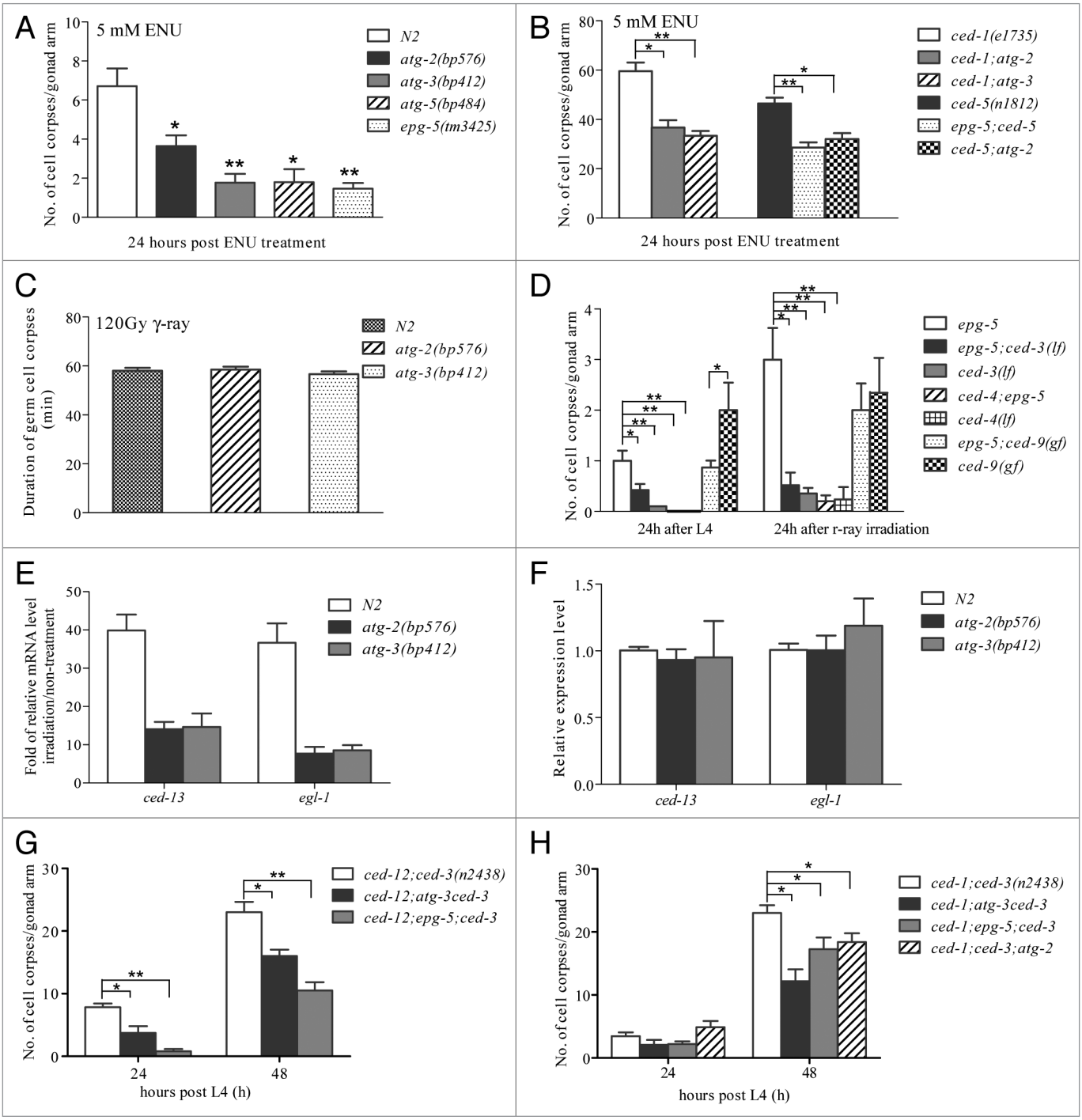

Figure 2. Autophagy is required for physiological germ-cell death in *ced-3* hypomorphic mutants. (**A and B**) Autophagy is required for germ-cell death upon ENU treatment. Twelve hours post L4 worms were treated with 5 mM ENU for 4 h and germ-cell corpses from one gonad arm of each animal were scored 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(bp414)* 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(lf)* and *ced-4(lf)* 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-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 (**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.[25] 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 *csp-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 neuroblasts 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, including P1.aap, P2.aap, and P9.aap-P12.aap. The *lin-11* gene, which encodes a LIM-domain transcription factor, is specifically expressed in ventral cord motor neurons and thus the $P_{lin\text{-}11::}gfp$ reporter specifically labels the ventral cord cells, P3.aap–P8.aap (**Fig. 3A**).[26] In strong *ced-3* or *ced-4* loss-of-function mutants, the other six Pn.aap cells (from P1, P2, and P9-P12), which normally die, also survive and differentiate into ventral cord equivalent cells expressing $P_{lin\text{-}11::}gfp$.[26] Partial loss of function of *ced-3* and *ced-4* activity allows the survival of some cells that are programmed to die (**Fig. 3B and F**).[26] We did not observe extra "undead" Pn.aap cells in autophagy mutants (**Fig. 3C and F**). However, double mutants of *atg-3(bp412)*, *lgg-1(bp500)*, *atg-2(bp576)* or *epg-5(tm3425)* with *ced-3(n2438)* had 3.8, 4.1, 2.8, and 2.8 extra ventral cord cells, respectively (**Fig. 3D and F**), compared with 2.2 extra cells in *ced-3(n2438)* single mutants. Loss of autophagy activity also promoted the survival of Pn.aap cells in *ced-4(n2273)* mutants.

We next determined whether elevated autophagy activity promotes apoptosis of ventral cord neurons in *ced-3(n2438)* mutants. We introduced a transgene overexpressing *epg-4*, $P_{hs::}epg\text{-}4$, into *ced-3(n2438)* mutants and found that ventral cord cell number labeled by $P_{lin\text{-}11::}gfp$ was decreased compared with *ced-3(n2438)* single mutants (**Fig. 3F**), indicating that extra ventral cord cells resulted from partial loss-of-function of caspase activity undergo programmed cell death when autophagy activity is elevated. Thus, loss of autophagy activity enhances the survival of Pn.aap cells in the presence of limiting caspase activity, implying that autophagy activity promotes programmed cell death in ventral cord neurons.

Discussion

The relationship between autophagy and caspase activation in cell death appears to be context-specific in *Drosophila*; autophagy and caspases act synergistically in cell death during oogenesis, they function in parallel and cooperatively in the death of salivary gland cells, and autophagy per se initiates the death program in the midgut.[27,28] During embryonic somatic cell death in *C. elegans*, autophagy activity participates in cell corpse removal.[24] Here we showed that autophagy activity acts cooperatively with caspases to promote germ-cell death following genotoxic stress and also the physiological germ-cell death and the death of ventral cord neurons in mutants with partially compromised *ced-3* and *ced-4* function. Thus, autophagy functions both in corpse removal and in the cell death program depending on developmental context. Cells with partially reduced caspase activity have the ability to reverse the course of events and escape death, even after activation of the apoptotic program. Preventing engulfment enhances the reversion from 'early death' and hence increases cell survival.[26,29] We showed here that autophagy itself does not trigger cell death in the germline and in the 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 caspase 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(tm3425), lgg-1(bp500)*. LGIII: *epg-4(bp425), epg-1(bp414), epg-4(bp425), prmt-5(gk378), ced-4(n2273), ced-4(n1162), ced-9(n1950)*. LGIV: *atg-3(bp412), ced-5(n1812), ced-3(n2438), ced-3(n717), csp-2(tm3077)*. LGV: *atg-18(gk378)*. LGX: *atg-2(bp576)*. Strains carrying transgenic arrays used in this study include *qxEx4091*($P_{ced\text{-}1::}epg\text{-}5$), *qxEx4092*($P_{ced\text{-}1::}epg\text{-}5$), *bpIs141*($P_{hs::}epg\text{-}4$), and *nIs96* ($P_{lin\text{-}11::}gfp$).

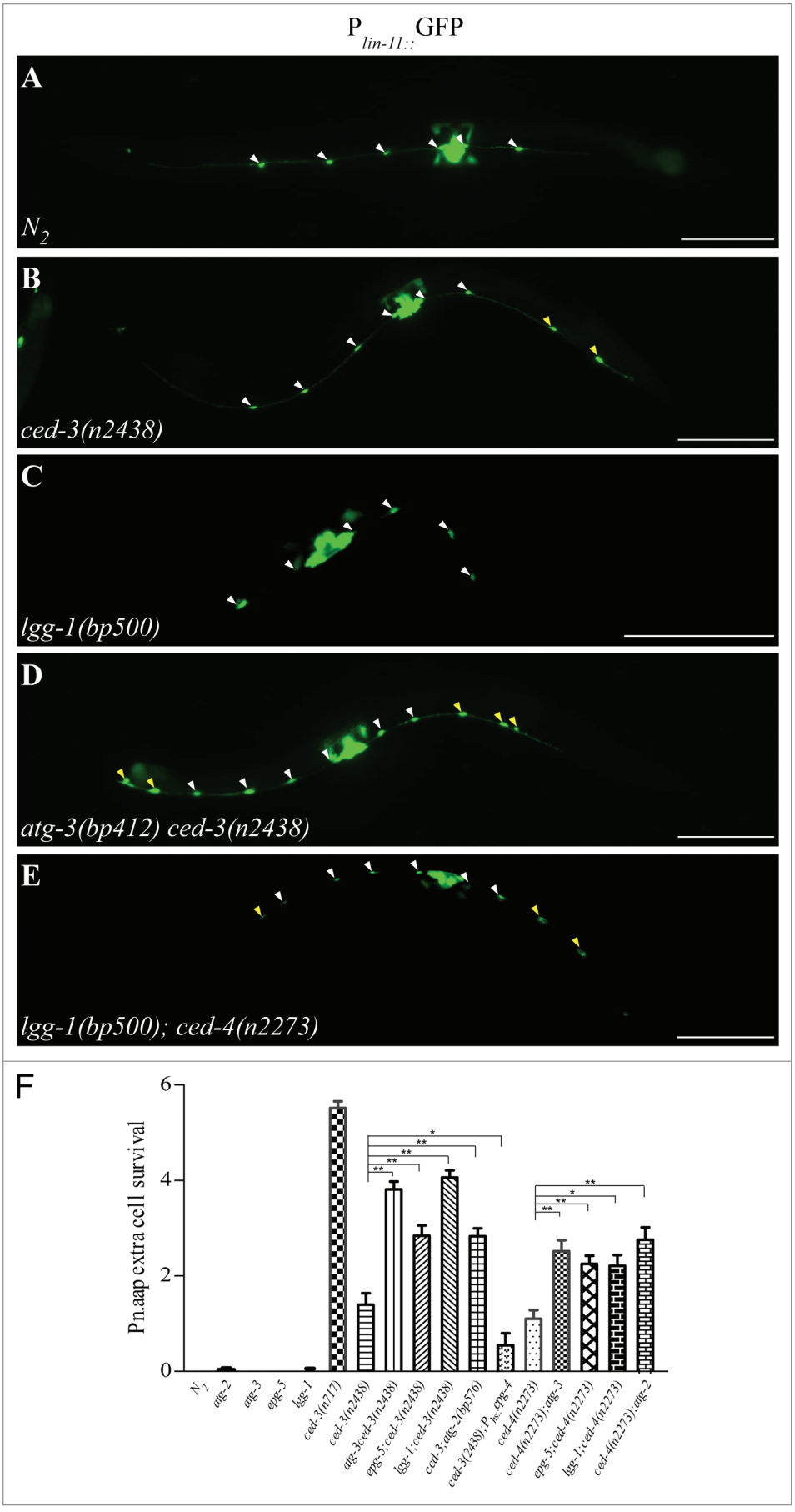

Figure 3. Autophagy activity contributes to somatic cell death in *ced-3* and *ced-4* hypomorphic mutants. (**A–E**) Fluorescence microscopy images of wild type (**A**), *ced-3(n2438)* (**B**), *lgg-1(bp500)* (**C**), *atg-3(bp412) ced-3(n2438)* (**D**), and *lgg-1(bp500); ced-4(n2273)* (**E**) mutants. *nIs96*($P_{lin-11::}gfp$) 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 ± SEM. At least 30 L4 larvae were scored for each genotype.

Reporters for *epg-1* and *epg-5* were constructed by a PCR fusion-based approach.[30,31] 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.[19]

To induce germline apoptosis by ENU (Sigma, N3385), L4 worms aged for 12 h were incubated in M9 medium (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$ 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 H_2O, autoclave and then add 1 ml $CaCl_2$ (1 M), 1 ml $MgSO_4$ (1 M), 1 ml cholesterol (5 mg/ml in ethanol),

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 for 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 *nIs96*($P_{lin\text{-}11}$::*gfp*) integrated array under a Zeiss Axioimager M1 fluorescence microscope.

Immunostaining

LGG-1 staining was performed as previously described with some modifications.[31] Hermaphrodites were dissected in a 40 μl drop of gonad buffer [20 mM NaCl, 30 mM KCl, 3 mM Na_2HPO_4, 3 mM $MgCl_2$, 20 mM HEPES, 2 mM $CaCl_2$, 50 μg/mL penicilin, 50 μg/mL streptomycin, 100 μg/mL neomycin (Amresco, 0558), 10 mM glucose, 33% FCS (GIBCOL, 100099-141)] on a slide freshly coated with poly-lysine (Sigma, P1524) to release the gonad system. Permeabilization of dissected gonad was performed by freeze-cracking methods.[32] Freeze-cracked slides were fixed, blocked, and incubated with diluted antibody at 4 °C overnight. The worms were then washed three times and incubated with Rhodamine-conjugated secondary antibody (Jackson, 112-515-003). Slides were viewed using an epifluorescence microscope or a confocal microscope (Zeiss LSM 510 Meta plus Zeiss Axiovert zoom (National Institute of Biological Sciences, Beijing).

RNA isolation, reverse transcription, and RT-PCR

Twelve hours after irradiation with 120 Gy γ rays, mixed-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 *ced-13* and *egl-1*: *ced-13*: 5′-TTTCTCCCGC TGTTGTCTAT TAGG-3′ (F); 5′-GTCAAACTCG TCGCACATAA CTG-3′ (R); *egl-1*: 5′-TGTTCTACTC CTCGTCTC-3′ (F); 5′-GACATCATCT GAGCATCG-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.

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Supplemental Materials

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

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