

# Autophagy genes function in apoptotic cell corpse clearance during *C. elegans* embryonic development

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**Abbreviations:** *bec-1*, *C. elegans* ortholog of yeast *VPS30*/mammalian *BECN1*; BH3, BCL2 homology 3; *C. elegans*, *Caenorhabditis elegans*; DIC, differential interference contrast; *lgg-1*, *C. elegans* ortholog of yeast *ATG8*/mammalian *MAP1LC3A*; mRFP, monomeric red fluorescent protein; PS, phosphatidylserine; PtdIns3-kinases, phosphatidylinositol 3-kinases

Efficient apoptotic corpse clearance is essential for metazoan development and adult tissue homeostasis. Several autophagy proteins have been previously shown to function in apoptotic cell clearance; however, it remains unknown whether autophagy genes are essential for efficient apoptotic corpse clearance in the developing embryo. Here we show that, in *Caenorhabditis elegans* embryos, loss-of-function mutations in several autophagy genes that act at distinct steps in the autophagy pathway resulted in increased numbers of cell corpses and delayed cell corpse clearance. Further analysis of embryos with a null mutation in *bec-1*, the *C. elegans* ortholog of yeast *VPS30*/*ATG6*/mammalian *beclin 1* (*BECN1*), revealed normal phosphatidylserine exposure on dying cells. Moreover, the corpse clearance defects of *bec-1(ok691)* embryos were rescued by *BEC-1* expression in engulfing cells, and *bec-1(ok691)* enhanced corpse clearance defects in nematodes with simultaneous mutations in the engulfment genes, *ced-1*, *ced-6* or *ced-12*. Together, these data demonstrate that autophagy proteins play an important role in cell corpse clearance during nematode embryonic development, and likely function in parallel to known pathways involved in corpse removal.

## Introduction

Apoptosis is a conserved process that removes unwanted cells, and its proper regulation is essential for normal development and tissue homeostasis. Not only is the execution of cell killing tightly controlled, the removal of apoptotic corpses is also highly regulated, and defects in apoptotic cell corpse clearance contribute to inflammation and autoimmunity.<sup>1,2</sup> Upon cell death execution, apoptotic cells display markers, so-called “eat-me” signals, that signal neighboring cells or specialized phagocytes to internalize and degrade the dead cell corpses. The best-studied “eat-me” signal is the exposure of phosphatidylserine (PS) on the outer leaflet of the apoptotic cell membrane,<sup>3</sup> although other cell death markers such as altered sugar moieties and other phospholipids are also important in labeling corpses for engulfment.<sup>4</sup>

The engulfment of apoptotic cell corpses is controlled by two evolutionarily conserved, partially redundant pathways that were discovered primarily through genetic studies in the nematode

worm *Caenorhabditis elegans*.<sup>5,6</sup> The first pathway, composed of CED-1, CED-6, CED-7 and DYN-1 proteins, is important for promoting membrane expansion of pseudopods during engulfment and for the degradation of apoptotic cells inside phagosomes.<sup>7,8</sup> The second pathway includes the CED-2, CED-5 and CED-12 proteins that are important for cytoskeletal reorganization during apoptotic corpse removal, and that function upstream of the Rho family GTPase CED-10 (*RAC1*).<sup>5,9</sup> CED-10 may also mediate certain activities of the CED-1 pathway.<sup>7,9</sup> Phagosomes containing engulfed cell corpses mature by sequentially recruiting proteins including PtdIns3-kinases, Rab GTPases, and the homotypic fusion and vacuole protein sorting (HOPS) complex, leading to the formation of phagolysosomes wherein the engulfed cell corpses are degraded.<sup>10-12</sup>

Macroautophagy (hereafter referred to as autophagy) is a lysosome-mediated “self-eating” process which is highly conserved from yeast to human (for a review, see refs. 13 and 14). More than 30 autophagy genes regulate the multistep autophagy

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process, and the deregulation of autophagy has been linked to a variety of diseases including cancer and neurodegenerative diseases.<sup>13</sup> The initiation of autophagy involves the formation of autophagosomes, double-membraned vacuoles surrounding the intracellular materials targeted for degradation, including damaged organelles, protein aggregates and intracellular pathogens. Autophagosomes eventually fuse with lysosomes, resulting in the acidification and degradation of their contents. The process of autophagy produces ATP and metabolic precursors such as amino acids and fatty acids, which allows cells to maintain nutrient and energy homeostasis, and thereby enhances survival during stress. Besides autophagy, components of the autophagy machinery may participate in other membrane-trafficking events, including endocytosis and endocytic trafficking, phagocytosis, secretion and the recruitment of GTPases and other signaling molecules to intracellular membranes.<sup>15-23</sup>

There are several lines of evidence indicating that the autophagy pathway, or at least certain autophagy proteins, contribute to efficient apoptotic corpse clearance, either by generating “eat-me” signals in dying cells or through a role in the phagocytosis of apoptotic corpses. Qu et al.<sup>24</sup> demonstrated defective PS exposure on dying cells and defective apoptotic corpse clearance in mammalian embryoid bodies lacking either *Becn1* or *Atg5*. Mellen et al.<sup>25,26</sup> demonstrated defective PS exposure on dying cells and defective apoptotic corpse clearance in chick embryo retinas treated with the autophagy inhibitor, 3-methyladenine. Martinez et al.<sup>27</sup> showed that the autophagy genes, *BECN1*, *ATG5* and *ATG7*, but not *ULK1*, are required for the phagocytosis of apoptotic cells by macrophages, whereas Konishi et al.<sup>28</sup> found that only *BECN1*, and neither *ATG5* nor *ULK1*, are required for macrophage engulfment of apoptotic cells. In *C. elegans*, Ruck et al.<sup>29</sup> demonstrated a role for the *C. elegans* orthologs of *VPS30/ATG6/BEEN1*, *ATG18* and *ULK1* (*bec-1*, *atg-18*, *unc-51*, respectively) in germ cell corpse clearance in the adult gonad, and Li et al.<sup>30</sup> demonstrated a role for the *C. elegans* autophagy genes, *lgg-1* (ortholog of *ATG8/MAP1LC3A/LC3*), *atg-18* and *epg-5*, but not *unc-51*, in Q cell neuroblast corpse clearance in the L1 larval stage.

These studies suggest that autophagy proteins may be crucial for apoptotic corpse clearance during programmed cell death. However, no previous studies have directly examined whether autophagy proteins are essential for apoptotic corpse clearance during embryonic development. Of note, Takacs-Vellai et al.<sup>31</sup> reported that a null mutation in the autophagy gene, *bec-1*, increased the number of apoptotic cell corpses in *C. elegans* embryos. These results were hypothesized to reflect increased caspase-dependent apoptosis in the setting of BEC-1 deficiency. An open question is whether defects in apoptotic corpse engulfment may also contribute to increased apoptotic corpse numbers in *bec-1*-null *C. elegans* embryos. In addition, it is unknown whether the phenotype of increased corpses in *C. elegans* is specific to mutation of *bec-1*, a gene that has multiple functions in membrane trafficking<sup>16,17</sup> and that encodes a protein which interacts with the antiapoptotic protein, CED-9/BCL2,<sup>32</sup> or whether this phenotype is also observed in other autophagy mutant nematodes.

To address these questions, we analyzed cell corpse numbers and the kinetics of cell corpse clearance in *C. elegans* embryos

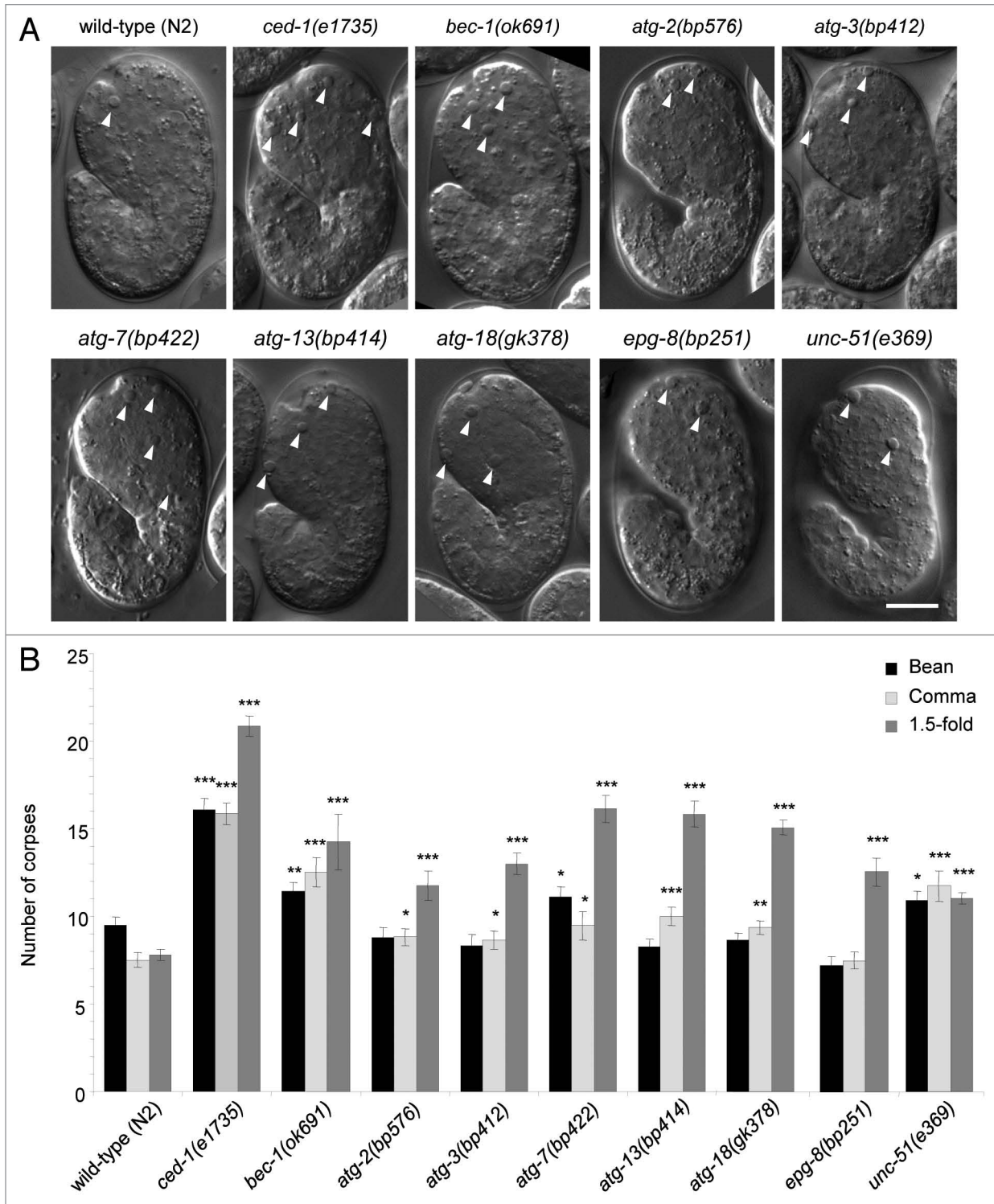
with mutations in autophagy genes that act at different stages in the autophagy pathway. Together, our results suggest that autophagy genes are important for efficient apoptotic corpse clearance during *C. elegans* embryonic development by a mechanism that is genetically distinct from the *ced-1*, *ced-6*, *ced-7*, *dyn-1* and the *ced-2*, *ced-5*, *ced-12* engulfment pathways.

## Results

**Increased numbers of apoptotic cell corpses are detected in *C. elegans* embryos with mutations in autophagy genes.** In *C. elegans*, 113 apoptotic cell corpses are generated during embryogenesis.<sup>33</sup> These cell corpses show a distinct highly refractile, “button-like” appearance under Nomarski differential interference contrast (DIC) microscopy. To investigate whether other autophagy genes play roles in apoptosis similar to that previously described for *bec-1*,<sup>31</sup> we used DIC microscopy to determine the numbers of apoptotic cell corpses detected during *C. elegans* embryogenesis in eight strains with loss-of-function mutations in autophagy. We focused on three morphologically distinct embryonic developmental stages including bean, comma and 1.5-fold stages, that correspond to ~320 min, ~380 min and ~420 min after the first cleavage, respectively.<sup>34</sup> We included analyses of the wild-type (N2) strain, and a well-studied engulfment mutant, *ced-1(e1735)*,<sup>5,7,35</sup> as controls. The eight autophagy-deficient strains included nematodes with mutations in two genes, *atg-13* and *unc-51*, that are part of the serine/threonine kinase induction complex;<sup>36</sup> two genes, *bec-1* and *epg-8* (which shares low similarity with mammalian *ATG14*),<sup>37</sup> that are part of the class III PtdIns3-kinase complex involved in vesicle nucleation;<sup>36</sup> two genes, *atg-3* and *atg-7*, that are part of the protein conjugation system involved in ATG8/LC3 lipidation and vesicle expansion and completion;<sup>36</sup> and two genes, *atg-2* and *atg-18*, that are important for the recycling of autophagy machinery from mature autophagosomes.<sup>36</sup>

We found that, compared with wild-type embryos, all autophagy mutant strains examined had significantly more cell corpses at the 1.5-fold stage (Fig. 1A and B). Some strains also had increased numbers of cell corpses detected at the bean [*bec-1(ok691)*, *atg-7(bp412)*, *unc-51(e369)*] or comma [*bec-1(ok691)*, *atg-2(bp576)*, *atg-3(bp412)*, *atg-7(bp422)*, *atg-13(bp414)*, *atg-18(gk378)*, *unc-51(e369)*] stages. As these genes function in different steps of the autophagy pathway, these results suggest that the autophagy pathway, rather than potential autophagy-independent functions of individual autophagy genes, regulates apoptotic cell corpse number during *C. elegans* embryogenesis.

Among the autophagy mutant alleles tested, only *bec-1(ok691)* animals are maintained as heterozygotes due to developmental and fertility defects in homozygous animals. Therefore, *bec-1(ok691)* homozygous embryos from heterozygous adults are expected to carry a maternal supply of the *bec-1* gene product. To further deplete the maternal *bec-1* gene product, a strain was constructed in which the homozygous *bec-1(ok691)* animals were kept alive and fertile by an extrachromosomal array expressing a functional BEC-1::mRFP transgene.<sup>38</sup> Since the expression of the repetitive extrachromosomal array is repressed in the *C. elegans*



**Figure 1.** Detection of increased numbers of cell corpses in autophagy-mutant strains during *C. elegans* embryogenesis. (A) Representative DIC images and (B) quantification of numbers of apoptotic corpses in indicated *C. elegans* genotype, as detected by DIC microscopic analysis of the head region during bean, comma and 1.5-fold stages of embryogenesis. Wild-type (N2) and the engulfment mutant *ced-1(e1735)* strains were included for comparison. Arrowheads denote representative apoptotic corpses. Scale bar: 10  $\mu$ m. Bar graph shows mean  $\pm$  s.e.m. from at least 20 embryos for each genotype at each stage. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. wild-type embryos at the same stage of development; t-test.

germline,<sup>39,40</sup> and the arrays are randomly lost during meiosis,<sup>41</sup> the mRFP(-) embryos therefore lack both maternal and zygotic *bec-1*. We found that *bec-1(ok691)* (*m-z-*) (*m*, maternal gene

product; *z*, zygotic gene product) embryos had a more striking increase in the number of cell corpses detected than *bec-1(ok691)* (*m+z-*) embryos as compared with wild-type animals [Fig. S1A;

$p < 0.001$ ; 2-way ANOVA analysis for comparison of magnitude of increase in *bec-1(ok691)(m+z-)* embryos vs. in *bec-1(ok691)(m+z-)* embryos for both bean and comma stages]. Thus, the analysis of *bec-1(ok691)(m+z-)* animals may underestimate the physiological consequences of complete loss of *bec-1* gene function. However, *bec-1(ok691)(m+z-)* animals displayed a more severe developmental defect than those of *bec-1(ok691)(m+z-)* animals; many *bec-1(ok691)(m+z-)* embryos were developmentally arrested prior to the bean stage, making it difficult to perform more detailed analyses in this strain. Therefore, subsequent experiments using *bec-1(ok691)* were performed with *bec-1(ok691)(m+z-)* embryos.

**Delayed clearance of apoptotic cell corpses in autophagy mutant embryos.** The observation of increased numbers of cell corpses during development may be due to increased cell death events, delayed cell corpse removal or both. To investigate whether the removal of cell corpses was delayed in autophagy mutant animals, we performed time-lapse DIC microscopy to analyze the duration of apoptotic cell corpse persistence. We analyzed at least one representative mutant strain for each group of autophagy genes (i.e., induction, vesicle nucleation, vesicle expansion/completion and recycling). In wild-type embryos, apoptotic cell corpses persisted an average of  $21.3 \pm 1.4$  min, with less than 6% of the cell corpses persisting more than 40 min and no corpses persisting for more than 50 min (Fig. 2). The autophagy mutant embryos, *bec-1(ok691)*, *atg-2(bp576)*, *atg-7(bp422)*, *atg-18(gk378)* and *unc-51(e369)* all showed notable delays in apoptotic cell corpse clearance, with corpses that persist for more than 60 min (Fig. 2). The magnitude of delay varied in different mutants; the most severe defect was observed in *bec-1(ok691)* embryos, with nearly 75% of corpses persisting more than 60 min. These kinetic analyses demonstrate that several different autophagy genes are required for efficient apoptotic corpse clearance during embryonic development.

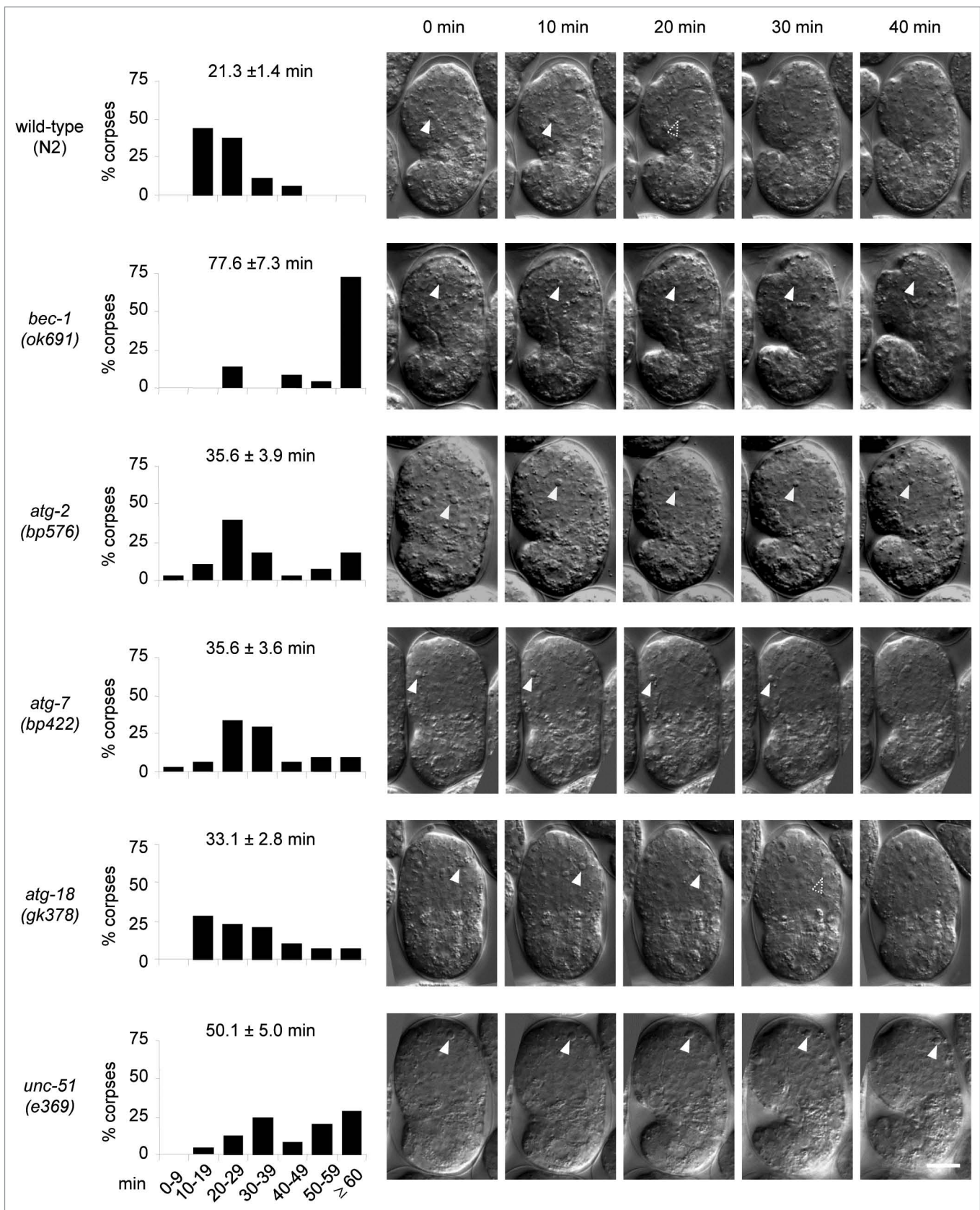
To determine whether the delay in cell corpse clearance is due to a defect in engulfment or in the degradation of engulfed cell corpses, we monitored the localization of the reporter  $P_{ced-1}::gfp$ . CED-1 is a key cell surface receptor important for engulfment, and cell corpse engulfment can be detected by observing CED-1::GFP surrounding cell corpses in engulfing cells that express CED-1::GFP.<sup>35</sup> Similar percentages of cell corpses were engulfed in *bec-1(ok691)* embryos as compared with wild-type controls during the bean, comma and 1.5-fold stages (Fig. 3). Thus, in *bec-1(ok691)(m+z-)* animals, delayed corpse clearance appears to be due to defects in the degradation of engulfed cell corpses, rather than defects in phagocytosis. However, *bec-1(ok691)(m+z-)* embryos showed a significant defect in corpse engulfment (Fig. S1B). Therefore, *bec-1* may play roles in both cell corpse engulfment and in the degradation of engulfed cell corpses.

**The autophagy gene *bec-1* is not required for the exposure of PS in *C. elegans* embryos.** In previous reports, autophagy gene mutation or pharmacological inhibition of autophagy resulted in PS exposure defects and persistent apoptotic cell corpses in developing mouse embryoid bodies<sup>24</sup> and chick retina.<sup>25</sup> However, in

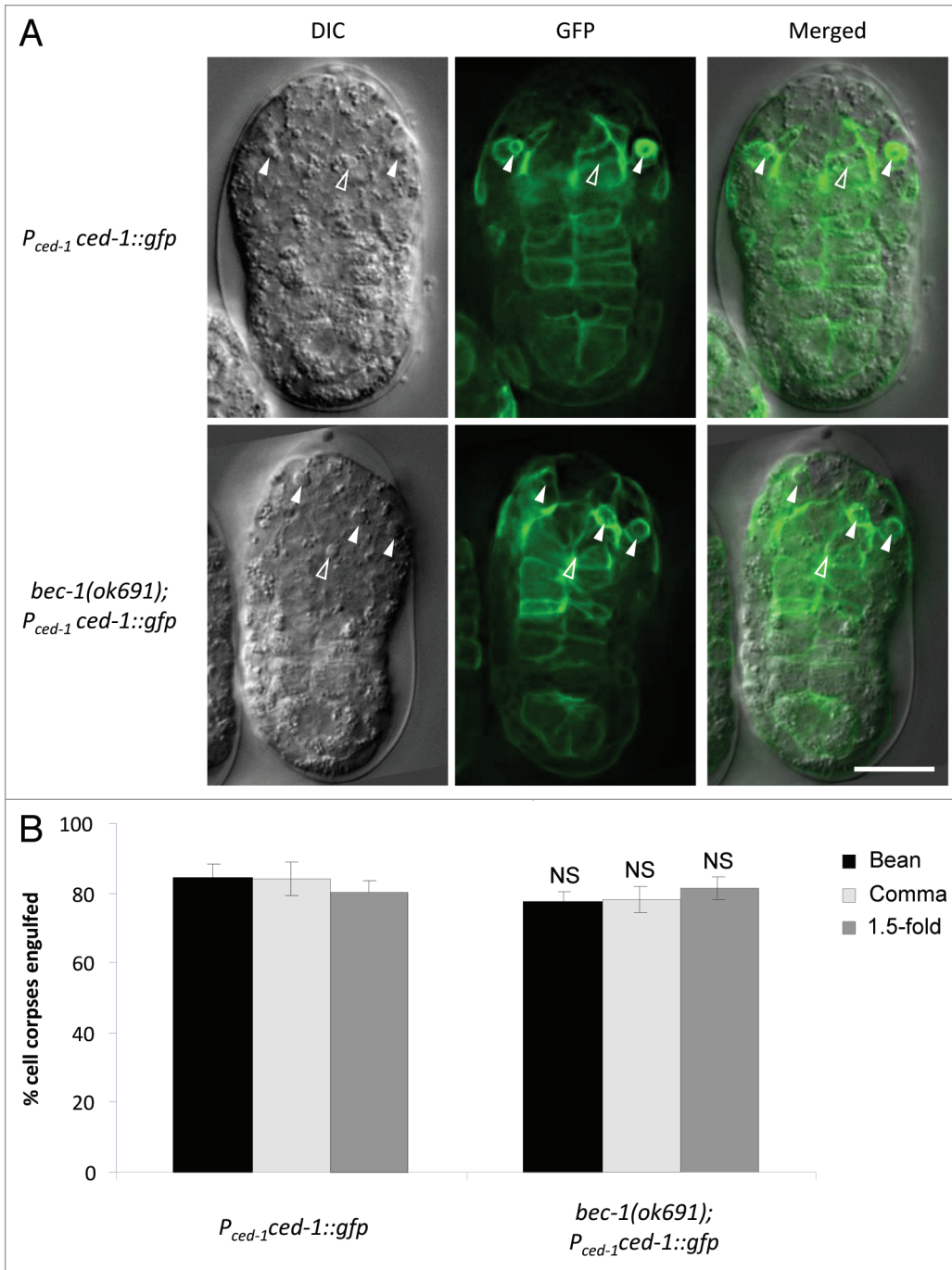
another study, autophagy inhibition did not block PS exposure in dying cells in the chick optic nerve or in later stages of retinal development.<sup>26</sup> To determine whether autophagy is required for PS exposure on apoptotic corpses in live *C. elegans* embryos, we expressed GFP-labeled human Annexin V under the control of the *dym-1* promoter, which drives ubiquitous expression in embryos.<sup>7</sup> Annexin V is a protein which binds to PS and is commonly used to detect PS exposure on the surface of apoptotic cells. The SEL-1 signal peptide domain was added to the N-terminus of GFP::Annexin V to promote the secretion of the fusion protein.<sup>42</sup> Since *bec-1(ok691)* mutants showed the most severe cell corpse clearance defect (Figs. 1 and 2), we compared GFP::Annexin V fluorescence in the embryos of wild-type N2, *ced-1(e1735)*, *bec-1(ok691)* animals and *ced-1(e1735); bec-1(ok691)* animals (Fig. 4). In the wild-type and the engulfment mutant *ced-1(e1735)* animals, GFP::Annexin V was highly enriched around apoptotic cell corpses with the GFP::Annexin V signal forming a ring-like structure decorated with aggregated puncta (Fig. 4E and F). The localization of GFP::Annexin V in vivo was better visualized after deconvolution of the epifluorescence images removing the optic distortion using a point spread function (Fig. 4I and J). Similar GFP::Annexin V rings were also detected around apoptotic cell corpses in *bec-1(ok691)* and *ced-1(e1735); bec-1(ok691)* embryos (Fig. 4G, H, K and L). Thus, these results demonstrate that *bec-1* is not required for PS exposure in dying cells during *C. elegans* development.

**Partial rescue of the persistent cell corpse phenotype in *bec-1(ok691)* embryos by expression of *bec-1* under the control of the *ced-1* promoter but not the *egl-1* promoter.** The lack of a defect in PS exposure in apoptotic corpses in *bec-1(ok691)* embryos suggested that autophagy in the dying cell may not be essential to generate engulfment signals, and rather, that the requirement for autophagy genes in the timely degradation of apoptotic corpses in *C. elegans* embryogenesis may instead reflect the recently described function of components of the autophagy machinery in the removal of apoptotic corpses by phagocytic cells. To evaluate this hypothesis, we determined whether the corpse clearance defect of *bec-1(ok691)* animals could be rescued by expressing *bec-1* full-length cDNA under the control of the *egl-1* promoter, which is active in apoptotic corpses,<sup>35,43</sup> and/or by expressing *bec-1* full-length cDNA under the control of the *ced-1* promoter, which is active in healthy cells capable of engulfment.<sup>35</sup> In both cases, we monitored the expression constructs by fusing an mCherry moiety to the N-terminus of BEC-1 (Fig. 5A and B).

We confirmed that  $P_{egl-1}::mcherry::bec-1$  led to the expression of mCherry::BEC-1 exclusively in dying cells (Fig. 5A). This is predicted as EGL-1 is a BH3 domain protein that functions as an upstream activator of apoptosis, and its expression is detected only in cells undergoing apoptosis.<sup>35,43</sup> Consistent with the lack of a defect in PS exposure in *bec-1(ok691)* embryos, we found that the expression of *bec-1* driven by the *egl-1* promoter in apoptotic cells failed to reduce the number of cell corpses in *bec-1(ok691)* embryos (Fig. 5C). Together, these results suggest that *bec-1* expression does not play a significant role in the dying cell to mediate apoptotic corpse clearance during *C. elegans* embryonic development.



**Figure 2.** For figure legend, see page 129.

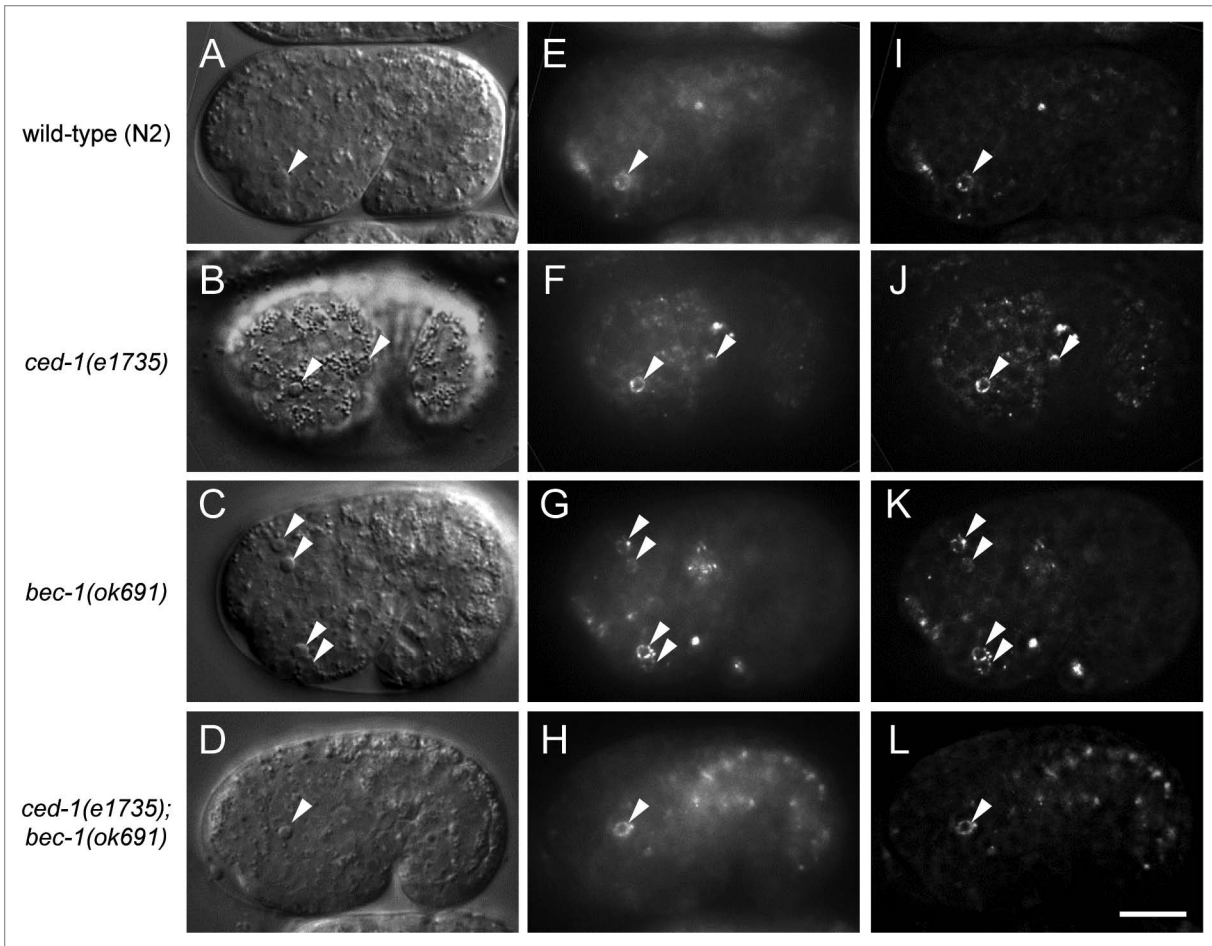


**Figure 3.** Engulfment of cell corpses in *bec-1(ok691)* mutant embryos. **(A)** Representative micrographs showing the expression of CED-1::GFP epi-fluorescence (middle) with the corresponding DIC (left) and merged (right) images in wild-type (upper panel) and *bec-1(ok691)* (lower panel) embryos at the bean stage. *P<sub>ced-1</sub>* was used to drive the expression of CED-1::GFP. Solid arrowheads denote engulfed cell corpses detected by the presence of CED-1::GFP surrounding the corpse and CED-1::GFP-expression in the engulfing cell. Open arrowheads denote cell corpses that are not surrounded by CED-1::GFP. **(B)** Quantification of apoptotic cell corpses engulfed detected using the CED-1::GFP marker during bean and comma stages of embryogenesis. Bar graph shows mean  $\pm$  s.e.m. for at least 10 embryos for each genotype at each stage. NS, not significant; t-test. Scale bars: 10  $\mu$ m.

In contrast, the expression of mCherry::BEC-1 under the control of the *ced-1* promoter (which is widely used to drive transgene expression in engulfing cells<sup>35</sup>) resulted in nearly complete rescue of the corpse clearance defect in *bec-1(ok691)* embryos (Fig. 4C). The incomplete rescue may be due to different strength of expression from the transgene compared with the endogenous promoter and/or to the mosaic inheritance of the transgene. Although we

occasionally detected the expression of *P<sub>ced-1</sub>mcherry::bec-1* in apoptotic cell corpses, the number of mCherry-positive corpses was too small to account for the magnitude of the rescue by the transgene. Taken together with our findings of a lack of corpse clearance defect rescue in embryos with *egl-1* promoter-driven expression of mCherry::BEC-1, these data indicate that BEC-1 expression in engulfing cells is essential for efficient apoptotic corpse clearance.

**Figure 2 (See opposite page).** Defective clearance of cell corpses in *C. elegans* autophagy-mutant strains. Histogram distributions of the duration of cell corpse persistence as observed by time-lapse DIC microscopy. The y-axis represents the percentages of cell corpses that persist within the specified duration range indicated on the x-axis. The numbers on top of each histogram indicate the mean duration ( $\pm$  s.e.m.). More than 22 corpses from more than five embryos were randomly chosen and followed for each genotype. DIC micrographs show apoptotic corpses (indicated by the arrowheads) in 10 min intervals from the appearance of a corpse (0 min) to 40 min. Scale bar: 10  $\mu$ m.



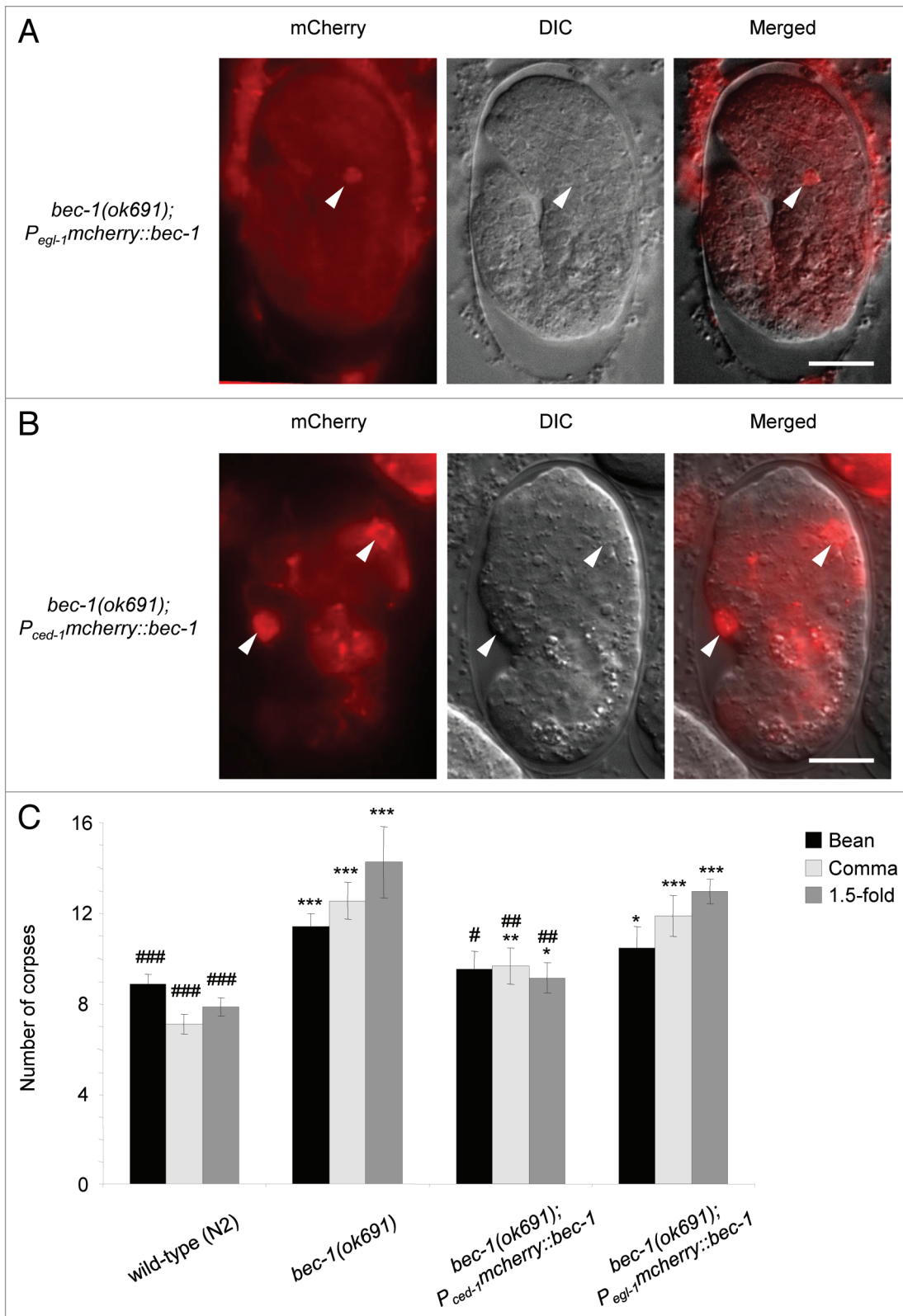
**Figure 4.** Lack of requirement for *bec-1* in the exposure of phosphatidylserine in *C. elegans* corpses. Representative DIC images (A–D), the corresponding epifluorescence images (E–H), and the corresponding deconvolution images (I–L) showing cell corpses labeled with GFP::Annexin V in vivo in the indicated *C. elegans* genotype. GFP::Annexin V expression was driven by the *dyn-1* promoter as an integrated transgene. Arrows denote corpses labeled with GFP::Annexin V rings. Scale bar: 10  $\mu$ m.

Enhancement of corpse clearance defects in engulfment mutants, *ced-1(e1735)*, *ced-6(n1813)* and *ced-12(n3261)* by *bec-1(ok691)*. Our results above indicate that *bec-1* functions in apoptotic corpse clearance during *C. elegans* embryogenesis. Therefore, we investigated whether *bec-1* functions with or in parallel to two pathways that have been previously shown to be required for apoptotic corpse removal in *C. elegans*, the *ced-1*, *ced-6*, *ced-7*, *dyn-1* pathway and the *ced-2*, *ced-5*, *ced-12* pathway. Because the *bec-1* gene is located on chromosome IV, we performed genetic epistasis analysis by generating double mutants of *bec-1(ok691)* with strong loss-of-function alleles of known

engulfment genes that are not on the same chromosome, including *ced-1*, *ced-6* and *ced-12*.<sup>35,44,45</sup>

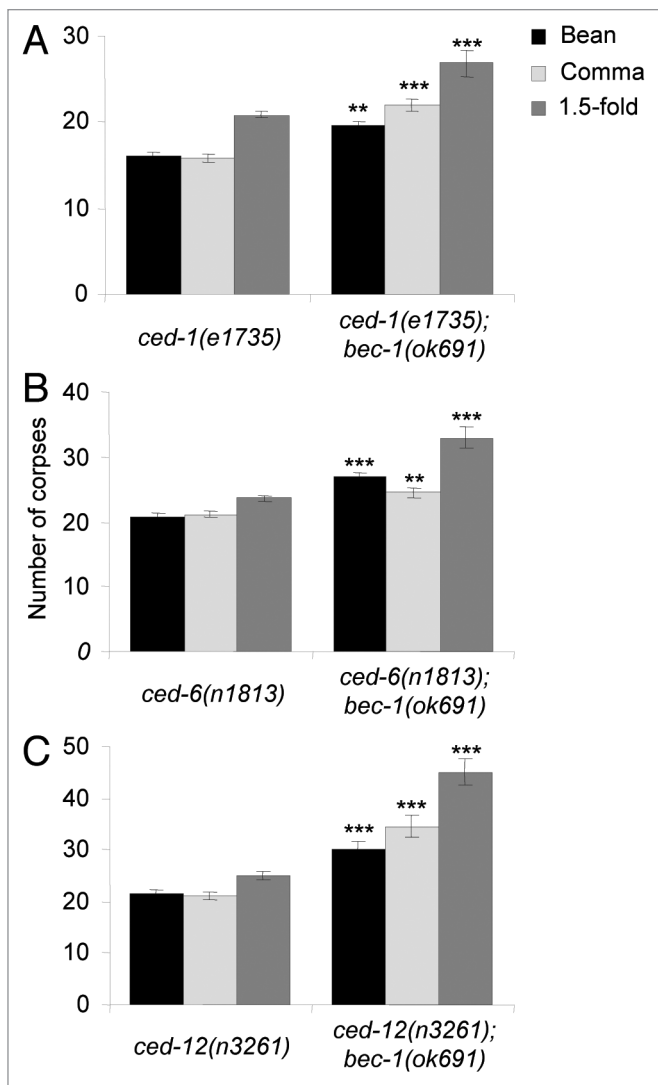
We found that the number of cell corpses detected in *ced-1(e1735)*, *ced-6(n1813)* or *ced-12(n3261)* single mutants (Fig. 6A–C) was greater than that observed in *bec-1* single mutants (see Fig. 1). Of note, double mutants of *bec-1(ok691)* with *ced-1(e1735)*, *ced-6(n1813)* or *ced-12(n3261)*, all showed further increases in the numbers of cell corpses detected at bean, comma, and 1.5-fold stages compared with the single *ced-1(e1735)*, *ced-6(n1813)* or *ced-12(n3261)* mutant (Fig. 6A–C). These results suggest that *bec-1* does not function through the

**Figure 5 (See opposite page).** Partial rescue of corpse clearance defect of *bec-1(ok691)* mutant animals by *ced-1* promoter-driven expression of *bec-1*, but not *egl-1* promoter-driven expression of *bec-1*. (A) Representative immunofluorescence micrographs showing the expression of  $P_{egl-1}$  *mcherry::bec-1* (left) with the corresponding DIC (middle) and merged (right) images in a *bec-1(ok691)*. The embryo was stained with anti-dsRed antibodies. (B) Representative micrographs of  $P_{ced-1}$  *mcherry::bec-1* expression in a *bec-1(ok691)* embryo. mCherry fluorescence is shown in the left panel, with the corresponding DIC image in the middle, and merged image in the right panel. For (A and B), arrowheads denote representative cells with MCHERRY::BEC-1 expression. (C) Quantification of apoptotic corpse numbers detected by DIC microscopy in the head region during bean, comma and 1.5-fold stages of embryogenesis. Bar graph shows mean  $\pm$  s.e.m. for at least 20 embryos for each genotype at each stage.  $P_{ced-1}$  was used to drive the expression of N' mCherry-fused *bec-1* full-length cDNA in cells capable of engulfment.  $P_{egl-1}$  was used to drive the expression of *mcherry::bec-1* in cells undergoing apoptosis. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  represent statistical comparison vs. wild-type embryos and # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$  represent statistical comparison vs. *bec-1(ok691)* embryos; t-test. Scale bars: 10  $\mu$ m.



**Figure 5.** For figure legend, see page 130.





**Figure 6.** Enhancement of corpse clearance defects by *bec-1(ok691)* in engulfment mutants, *ced-1(e1735)* (A), *ced-6(n1813)* (B), and *ced-12(n3261)* (C). Bar graphs show mean  $\pm$  s.e.m. at bean, comma, and 1.5-fold stages during embryogenesis. At least 20 embryos for each genotype were analyzed at each stage. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  for comparison of double mutant on right vs. single mutant on left; t-test.

*ced-1*, *ced-6* and *ced-7* pathway nor through the *ced-2*, *ced-5* and *ced-12* pathway for corpse removal.

## Discussion

Our results demonstrate that several different autophagy gene mutant nematodes have increased embryonic corpses and delayed apoptotic corpse clearance. To our knowledge, these findings represent the first demonstration of a role for autophagy genes in apoptotic corpse clearance during embryonic development in an intact organism. These findings extend previous studies indicating a role for autophagy genes in corpse clearance in the *C. elegans* adult gonad<sup>29</sup> and L1 larval stage Q cells.<sup>30</sup> In contrast to results in mammalian embryoid bodies,<sup>24</sup> an in

vitro model for mammalian embryonic development, we did not find that autophagy genes were necessary for PS exposure in dying cells. Rather, our results are consistent with the emerging evidence that the autophagy machinery has a crucial function in mediating the efficient clearance of apoptotic corpses by phagocytes.<sup>27-30</sup>

The precise mechanism(s) by which autophagy genes mediate efficient apoptotic corpse clearance remain unclear. Other studies in autophagy-deficient mammalian macrophages<sup>27</sup> and post-embryonic corpse clearance in *C. elegans*<sup>30</sup> point to a defect in apoptotic corpse degradation, rather than uptake. For example, in *C. elegans* Q cell corpse clearance, the recruitment of phagosome/lysosome markers RAB5A, RAB7A and CTNS are delayed in two autophagy mutants, *atg-18(gk378)* and *epg-5(tm3425)*,<sup>30</sup> suggesting that autophagy plays a role in the degradation of engulfed cell corpses by regulating the process of phagosome maturation. This is consistent with studies that have demonstrated a role for certain autophagy proteins in phagolysosomal maturation in other biological contexts, including macrophage or dendritic cell engulfment of toll-like receptor ligands.<sup>15,27,46</sup> Our study further supports the concept that the autophagy gene, *bec-1*, may function in the degradation of engulfed apoptotic corpses. Moreover, complete loss of both maternal and zygotic *bec-1* also results in a defect in apoptotic corpse engulfment. Thus, autophagy genes may play roles in both apoptotic corpse engulfment and apoptotic corpse degradation during *C. elegans* embryogenesis.

The PIK3C3/VPS34-BECN1-containing class III PtdIns3P kinase complex plays a conserved role in apoptotic corpse removal in both mammalian cells<sup>27</sup> and in *C. elegans*.<sup>29</sup> This complex has autophagy-independent membrane trafficking functions, including a role in endocytosis,<sup>16,17</sup> endocytic retrograde transport<sup>29</sup> and LC3 recruitment to phagosomes;<sup>15</sup> such functions may explain the more severe corpse clearance defect observed in our study in *bec-1* vs. other autophagy gene mutant animals. In addition, BECN1 has been shown to interact with the mammalian ortholog of CED-10, the Rho GTPase, RAC1, and proposed to work in concert with RAC1 to coordinate actin dynamics and promote efficient apoptotic cell engulfment.<sup>28</sup> It will be interesting to examine whether *C. elegans* BECN1 interacts with CED-10 and is required for its function in *C. elegans* corpse removal; this possibility may also explain the more severe corpse clearance defect in *bec-1* vs. other autophagy gene mutant nematodes.

Although the *bec-1(ok691)* corpse clearance delay phenotype was the most severe, we also observed corpse clearance defects in nematodes with mutations in other autophagy genes that are not part of the class III PtdIns 3-kinase complex, including *atg-2*, *atg-7*, *atg-18* and *unc-51*. The differences in severity of corpse clearance defects may reflect differences in the underlying molecular basis of each mutant allele used (*bec-1(ok691)*,<sup>29</sup> *atg-2(bp576)* (Dr. Hong Zhang, personal communication), and *atg-18(gk378)*<sup>47</sup> are predicted null alleles; *unc-51(e369)* is a strong loss-of-function allele;<sup>48</sup> whereas *atg-7(bp422)* has a single amino acid change in the encoded protein that is predicted to affect protein activity);<sup>49</sup> partial gene redundancy with homologs that are not detected by primary sequence similarities; and/or autophagy

pathway-independent functions of the encoded autophagy protein. Nonetheless, the overlapping phenotypes in *bec-1*, *atg-2*, *atg-7*, *atg-18* and *unc-51* mutant animals provide strong genetic support that the autophagy pathway, rather than additional functions of autophagy genes, may be required for the proper removal of apoptotic cell corpses.

The role of *unc-51*, the *C. elegans* ortholog of Atg1/ULK1, in embryonic corpse clearance is noteworthy, as *unc-51* and *ULK1* were found to be dispensable for *C. elegans* Q cell corpse clearance and mammalian macrophage engulfment of apoptotic cells.<sup>28,30</sup> However, our results are consistent with the previous report in *C. elegans* which found a role for *unc-51* in germ cell corpse clearance in the adult gonad.<sup>29</sup> It is not yet known whether *unc-51/ULK1* truly functions in a cell-type or developmental stage-specific manner in apoptotic corpse clearance, or whether other factors may explain the contrasting findings in different studies. As UNC-51 and ULK1 are reportedly not involved in phagosomal maturation,<sup>29,30</sup> the requirement for UNC-51 in efficient embryonic corpse clearance (the present study) and germ cell clearance in the adult gonad<sup>29</sup> may be an important clue that the autophagy pathway contributes via other mechanisms to apoptotic corpse clearance in certain developmental contexts.

Our epistasis analyses indicate that *bec-1* likely functions in parallel with the two cell corpse clearance pathways, the *ced-1*, *ced-6*, *ced-7* and *dyn-1* pathway, and *ced-2*, *ced-5* and *ced-12* pathway. It is possible that *bec-1* is part of a third pathway that converges on *ced-10*, especially in view of the recently described interaction between the mammalian orthologs of BEC-1 and CED-10.<sup>28</sup> Alternatively, *bec-1* and other autophagy genes may function in parallel to *ced-10* in promoting efficient apoptotic corpse clearance. Further genetic studies will be required to determine the precise relationships between *bec-1* (and other autophagy genes) and the major known *C. elegans* engulfment pathways. Such studies should facilitate a deeper understanding of the molecular and cellular events that likely play a conserved role in apoptotic corpse clearance.

## Materials and Methods

**Strains and genetics.** *C. elegans* strains were maintained at 23°C as described.<sup>30</sup> All strains were grown on nematode growth media (NGM) plates and fed *E. coli* bacteria, strain DA837. *C. elegans* var Bristol strain N2 obtained from the *Caenorhabditis* Genetics Center was used as the wild-type reference. The following mutants were used: LGI (linkage group I), *ced-1(e1735)*, *ced-12(n3261)*, *epg-8(bp251)*; LGIII (linkage group III), *atg-13(bp414)*, *ced-6(n2095)*; LGIV (linkage group IV), *atg-3(bp412)*, *atg-7(bp422)*, *bec-1(ok691)*; LGV (linkage group V), *atg-18(gk378)*, *unc-51(e369)*, *enIs35[P<sub>ced-1</sub>ced-1::gfp*, *P<sub>ced-1</sub>2xFYVE::mRFP*, *unc-76(+)*]; and LGX (linkage group X), *atg-2(bp576)*. The balancer for LGIV; LGV, *nTI[qIs51]*, was used to balance *bec-1(ok691)* mutants. The mutants *atg-2(bp576)*, *atg-3(bp412)*, *atg-7(bp422)*, *atg-13(bp414)* and *epg-8(bp251)* were kindly provided by Dr. Hong Zhang (National Institute of Biological Sciences, Beijing, China). The extrachromosomal

transgene *enEx919[P<sub>bec-1</sub>bec-1::mRFP]* was introduced to rescued *bec-1(ok691)* to study the maternal effect of *bec-1* in *bec-1(ok691)*.

**Microscopy and quantification.** Live embryos were collected onto 3% agarose pads for imaging on a Zeiss Imager M2 microscope equipped with Nomarski differential interference contrast (DIC) and epifluorescence optics. Stack images with 0.8 μm between each slice were acquired with a CoolSnap HQ<sup>2</sup> CCD camera using Axiovision software (Zeiss). Apoptotic cell corpses were identified by their highly refractile appearance under DIC, and the corpses in the head region of embryos were scored at different developmental stages defined on the basis of the morphology of the embryos. Embryos that were developmentally arrested were excluded from analysis. For time-lapse imaging, embryos were mounted onto 3% agarose pads sealed with petroleum jelly. Stack DIC images with 0.8 μm between each slice were recorded at 2 min intervals for over 2 h to score the duration of apoptotic cell corpses. Less than five corpses were randomly chosen per embryo and > 22 corpses were analyzed for each genotype. p-values for pair-wise comparison were calculated using a t-test. For GFP::Annexin V and CED-1::GFP detection, deconvolution of Z-stack epifluorescence images was performed with AutoQuant software (Bitplane) using a blind deconvolution algorithm (20 iterations, medium noise). Engulfment of cell corpses in *bec-1(ok691)(m+z-)* was analyzed by the detection of CED-1::GFP surrounding the cell corpses inside engulfing cells that express CED-1::GFP. Engulfment of cell corpses in *bec-1(ok691)(m-z-)* was analyzed using the *P<sub>ced-1</sub>ced-1c::gfp* reporter, which expresses the cytoplasmic fragment of CED-1 fused to GFP. Engulfed cell corpses were recognized as dark spheres surrounded by cytoplasmic CED-1c::GFP.

**Plasmid construction and transgenic strains.** For *Pced-1mcherry::bec-1*, a full-length *bec-1* cDNA fragment with N-terminal Sall-BamHI sites and a C-terminal KpnI site was cloned into the Sall and KpnI sites of pZZ954, which contains a 5.1 kb *ced-1* promoter and the *unc-54* 3'UTR. To visualize expression of the construct, a Sall-mcherry-BamHI fragment was cloned into the resulting plasmid to create *Pced-1mcherry::bec-1*. *Pced-1mcherry::bec-1* was maintained as extrachromosomal arrays generated by microinjection into *bec-1(ok691)/nTI[qIs51]* together with the dominant roller marker pRF4 [*rol-6(su1006)*]. Embryos negative for *nTI[qIs51]* GFP and positive for mCherry were used to quantify the number of apoptotic cell corpses.

For *Pegl-1mcherry::bec-1*, a full-length *bec-1* cDNA fragment was inserted between the BamHI-AgeI sites of pZZ609, which contains a 1 kb *egl-1* promoter and a 5.6 kb *egl-1* 3'UTR. mCherry was cloned into the BamHI site upstream of *bec-1* to create *Pegl-1mcherry::bec-1*. The plasmid containing *Pegl-1mcherry::bec-1* was injected into *bec-1(ok691)/nTI[qIs51]* together with the dominant roller injection marker pRF4 [*rol-6(su1006)*] to generate extrachromosomal transgenic lines. The extrachromosomal arrays were integrated by irradiation followed by five outcrosses. Expression of the transgene was confirmed by RT-PCR and immunostaining. For immunofluorescence staining, the embryos were fixed with 4% paraformaldehyde, freeze-cracked and treated with dimethylformamide (DMF, Sigma-Aldrich Corp., D4551). The embryos were stained with

anti-dsRed antibodies (rabbit, 1:100 in PBS, Clontech 632496) and labeled with goat-anti-rabbit AlexaFluor594 (donkey, 1:250 in PBS, Invitrogen A21207). After five outcrosses, live embryos negative for *nT1[qIs51]* GFP were used to quantify the number of apoptotic cell corpses.

For *Pdyn-Isel-Isp::gfp::annexinV*, a 3.2 kb *dyn-1* promoter was cloned to drive expression of *gfp::annexinV* in the L754 vector. An 85 amino acid *sel-1* signal peptide was amplified from pSZ8 (gift of Dr. Michael O. Hengartner, University of Zurich, Switzerland) and inserted into the N-terminus of *gfp::annexinV* to facilitate secretion of the protein. The plasmid containing *Pdyn-Isel-Isp::gfp::annexinV* was injected into *ced-1(e1735)* together with the dominant roller injection marker pRF4 [*rol-6(su1006)*] to generate extrachromosomal transgenic lines. The extrachromosomal arrays were integrated by irradiation followed by five outcrosses.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Material

Supplemental material may be downloaded here: [www.landesbioscience.com/journals/auto/article/22352/](http://www.landesbioscience.com/journals/auto/article/22352/)

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