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Inactivation of the Autophagy Gene *bec-1* Triggers Apoptotic Cell Death in *C. elegans*

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Summary

Programmed cell death (PCD) is an essential and highly orchestrated process that plays a major role in morphogenesis and tissue homeostasis during development. In humans, defects in regulation or execution of cell death lead to diabetes, neurodegenerative disorders, and cancer [1]. Two major types of PCD have been distinguished: the caspase-mediated process of apoptosis and the caspase-independent process involving autophagy [1, 2]. Although apoptosis and autophagy are often activated together in response to stress [3], the molecular mechanisms underlying their interplay remain unclear. Here we show that BEC-1, the *C. elegans* ortholog of the yeast and mammalian autophagy proteins Atg6/Vps30 and Beclin 1, is essential for development. We demonstrate that BEC-1 is necessary for the function of the class III PI3 kinase LET-512/Vps34, an essential protein required for autophagy, membrane trafficking, and endocytosis. Furthermore, BEC-1 forms a complex with the antiapoptotic protein CED-9/Bcl-2, and its depletion triggers CED-3/Caspase-dependent PCD. Based on our results, we propose that *bec-1* represents a link between autophagy and apoptosis, thus supporting the view that the two processes act in concerted manner in the cell death machinery.

Results and Discussion

Autophagy is a highly regulated intracellular degradation process that involves the formation of subcellular membranes to sequester cargo for degradation [2]. It permits cells to survive during starvation, to undergo structural remodeling during differentiation, and may be important, together with apoptosis, for tumor suppression [2–4]. Genes that constitute the basic machinery of autophagy are well characterized in yeast, and their metazoan orthologs have also been identified. For example, the gene *beclin 1* is the mammalian counterpart of the yeast autophagy gene *ATG6/VPS30* [5]. It acts as a haploinsufficient tumor suppressor gene and is essential for early embryonic development in the mouse [6, 7]. Beclin 1 was found to bind Bcl-2 [8], an important negative regulator of apoptosis, and to protect neuronal cells against virus-induced PCD, both suggesting an implication in apoptosis. Furthermore, very recently it was shown that plants silenced for BECLIN1 show increased programmed cell death during the innate immune response [9]. To investigate whether Beclin 1-type autophagy proteins may have a general role in apoptosis, we focused on *bec-1*, the single *beclin 1* ortholog in *C. elegans*, where apoptosis was extensively studied. Recently, it has been shown that *bec-1* is required for autophagy in hypodermal seam cells during dauer development [10], but a function in apoptosis has not yet been described.

bec-1 Is Ubiquitously Expressed throughout Development

To study the expression pattern of *bec-1*, we performed a Northern blot analysis of poly(A)$^+$ RNA isolated from wild-type animals at different developmental stages (Figure 1A). *bec-1* mRNA was present in all developmental stages; however, the strongest expression was observed during embryogenesis. The increased level of *bec-1* mRNA in adults presumably results from the gonad and/or embryos, since *glp-1* mutants, which do not contain germ cells, showed low steady-state levels of *bec-1* transcript. We also generated a rescuing *bec-1::gfp* translational fusion construct, containing 5.6 kb of promoter region and the full-length *bec-1* coding sequence fused with the GFP reporter gene. Consistent with the Northern analyses, BEC-1::GFP was ubiquitously expressed throughout development. Its expression level appeared to be most intense in differentiating organs, such as the presumptive ventral nerve cord, the intestine, the developing vulva, and the somatic gonad. The distribution of BEC-1::GFP corresponded to the expression pattern of a transcriptional *bec-1::gfp* fusion construct reported earlier [10]. The BEC-1::GFP fusion protein was located in the cytoplasm of many cell types and often accumulated in vesicle-like structures (Figure 1B). Some expression was also observed in the nucleus.

bec-1 Is Essential for Development

To assess the function of *bec-1*, we analyzed the two mutant alleles *ok691* and *ok700*. Both alleles are deletion derivatives (Figure 1C) and probably represent strong loss-of-function or null alleles. *bec-1(ok691)* and *bec-1(ok700)* animals displayed a highly penetrant lethal phenotype (Table 1). Mutant embryos had various morphogenetic defects and died at different stages during embryogenesis. The cells of the arrested early embryos often contained abnormal granules and sometimes dis-

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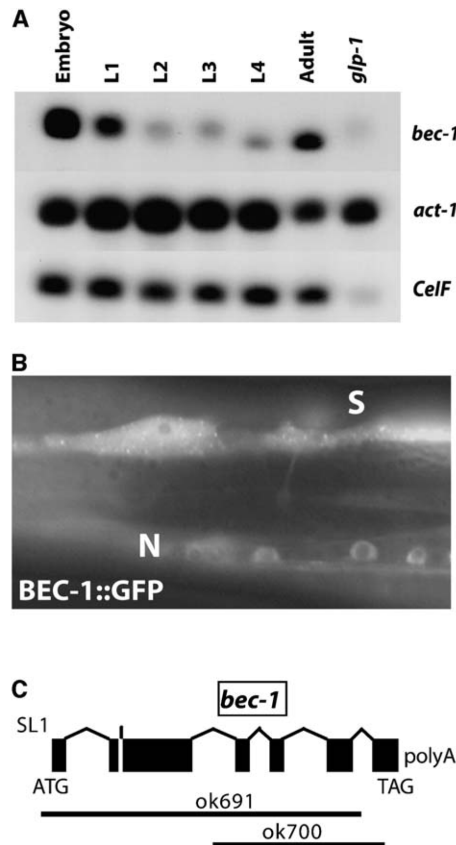

Figure 1. Expression Pattern of *bec-1*

(A) Developmental Northern blot analysis of *bec-1* mRNA. Poly(A)+ RNA was purified from staged worm cultures as indicated. *glp-1 (q224)* ^{ts} mutant adults raised at restrictive temperature have virtually no germ line [17]. As loading controls, we used CelF RNA [18] and *act-1* RNA [19] that is equally expressed in wild-type and *glp-1* adults. Northern blot analysis was performed as described [20].

(B) Distribution of BEC-1::GFP. Fluorescent microscopy shows accumulation of BEC-1::GFP in the cytoplasm of seam hypodermal (S) and neuronal (N) cells of an early transgenic L3 larva. The *bec-1::gfp* fusion construct corresponded to the *gfp* reporter gene fused to a 9 kb genomic fragment containing the full-length *bec-1* coding region and 5.6 kb of upstream sequences.

(C) Structure of the *bec-1* gene. Sequencing of the *bec-1* full-length cDNA clone yk391b8 confirmed the exon-intron structure predicted by GENEFINDER. The start and stop codons and the spliced leader (SL1) are shown. The bars indicate the deletions associated with alleles *ok691* and *ok700* (provided by the *C. elegans* Knockout Consortium). Relative to the ATG start codon (position 1–3), *ok691* removes the genomic sequences between positions −291 (A) and +2391 (T), whereas *ok700* deletes the sequences between nucleotide positions +1274 (G) and +2655 (T).

played incomplete cytokinesis, resulting in the formation of binucleated cells. Animals that passed embryonic development arrested during the L2/L3, the L3/L4, or the L4/adult molt, and only a few animals reached adulthood (Table 1). The molting-defective worms failed to shed their old cuticles (data not shown). *bec-1* RNAi-depleted worms showed the same phenotype as *bec-1* mutant animals, although at lower penetrance (Table 1). This was probably due to an incomplete inactivation of *bec-1*. In fact, *bec-1(RNAi)* strongly but not completely

Table 1. *bec-1* Mutant Analysis

		Embryonic Arrest	Larval Arrest	Adults
bec-1(ok691)	n = 2486	92.7%	7%[a]	0.3%[b]
bec-1(ok700)	n = 2326	85.1%	14.7%[a]	0.2%[b]
Wild-type[c]	n = 20	10.6%	0	89.4%
bec-1(RNAi)	n = 45	16.2% (F1) 95.1% (F2)	0.8% (F1)[d]	83%

For the *bec-1(RNAi)* analysis, wild-type L4 larvae (P0) were put on HT115 bacteria expressing ds *bec-1* RNA (see Supplemental Experimental Procedures). Analysis was done at 25°C.
[a] Arrest mainly at the L3/L4 molt, sometimes also at the L2/L3 or the L4/adult molts.
[b] Sterile adults with a few deformed eggs.
[c] Wild-type worms grown on HT115 bacteria not expressing ds *bec-1* RNA as control.
[d] All *bec-1(RNAi)* animals arrested at the L4/adult molt.

reduced the GFP fluorescence in *bec-1::gfp* worms (data not shown). Many of the *bec-1(RNAi)* F1 adults displayed various defects, including large vacuoles in the intestine, vulval protrusions, or an extra vulva and an extended outer nuclear membrane in the germ cells. Fertility was highly reduced, and occasionally individuals were sterile. The majority of the fertilized oocytes developed into severely malformed, nonviable F2 embryos (Table 1). Altogether, the phenotypic analysis of *bec-1* mutants and *bec-1(RNAi)* animals showed that *bec-1* is essential for development.

Disruption of *bec-1* Triggers Apoptosis

The mammalian Beclin 1 was shown to bind to Bcl-2 [8]. To see whether the molecular relationship between Beclin 1 and Bcl-2 is evolutionarily conserved, we have tested a possible interaction between BEC-1 and CED-9, the *C. elegans* ortholog of Bcl-2 [11, 12]. Both in vitro pull-down assays and coimmunoprecipitation experiments from worm extracts revealed that the two proteins form a complex (Figure 2). Because no anti-BEC-1 antibody was available, the in vivo interaction between BEC-1 and CED-9 was tested using anti-GFP antibodies to precipitate a rescuing extrachromosomal array-driven BEC-1::GFP fusion construct expressed in *bec-1* mutant worms. To assess a possible role of *bec-1* in apoptosis, we have determined the number of apoptotic cell corpses in *bec-1* mutant and *bec-1(RNAi)* animals. By recording developing *bec-1(RNAi)* embryos with four-dimensional microscopy, we counted a significantly higher number of cell corpses in *bec-1* mutant animals than in wild-type worms (Figure 3A). Furthermore, the germline of *bec-1(RNAi)* hermaphrodites also contained as many as nine cell corpses in average per gonad arm, while wild-type hermaphrodites had only two (Figure 3B). As an additional marker for PCD, we performed the TUNEL assay in which nicked DNA ends generated during apoptosis are labeled [13]. Consistent with the previous results, we found a significantly increased number of TUNEL-stained nuclei in both *bec-1* mutant and *bec-1(RNAi)* embryos as compared to wild-type embryos (Figures 3C–3E). The number of TUNEL-positive cells in *bec-1* mutants was higher than in *bec-1(RNAi)* animals (Figure 3E), again suggesting that

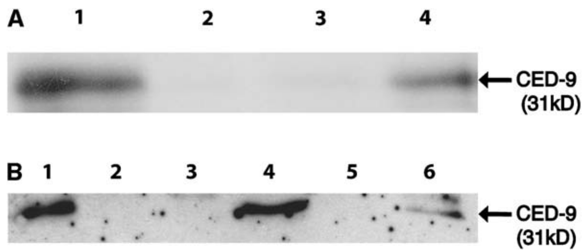

Figure 2. BEC-1 Interacts with CED-9

(A) In vitro pull-down experiment showing that BEC-1 binds to CED-9. Lane 1: input (10% of the amount of ^{35}S-methionine-labeled CED-9 protein used for lanes 2–4); lane 2: labeled CED-9 protein and GST alone as negative control; lane 3: labeled CED-9 protein and FBF::GST as negative control; lane 4: labeled CED-9 protein and BEC-1::GST. For experimental details, see Supplemental Data.

(B) Coimmunoprecipitation of BEC-1::GFP with CED-9. The Western blot was probed with anti-CED-9 antibodies. Lane 1: wild-type extract; lanes 2 and 3: wild-type extract precipitated with mouse IgG (lane 2) and anti-GFP antibody (lane 3); lane 4: extract from *bec-1(ok700);*[*bec-1::gfp+rol-6(su1006)*] animals; lanes 5 and 6: extract from *bec-1(ok700);*[*bec-1::gfp+rol-6(su1006)*] animals precipitated with mouse IgG (lane 5) and anti-GFP antibody (lane 6). For experimental details, see Supplemental Data.

bec-1 inactivation by RNAi is incomplete. The excess of apoptotic cell corpses observed in *bec-1(RNAi)* animals was blocked by *ced-3(lf)* or *ced-9(gf)* mutations (see Figures 3B and 3F), thus providing convincing evidence that the ectopic cell death occurring upon inactivation of *bec-1* involves the canonical *ced-3*/caspase-mediated cell death pathway.

The mammalian Beclin 1 was shown to bind to Bcl-2 [8], but its participation in apoptosis has not yet been directly demonstrated. No changes in apoptosis were observed in mammary epithelial cells of heterozygous beclin 1 mice [7]. Although a large number of acridine-orange-positive cells were found in homozygous beclin 1 mutant mice, they were not considered to be indicative for increased apoptosis [6]. In contrast, our results clearly show that in *C. elegans* homozygous *bec-1* mutants and *bec-1(RNAi)* animals displayed an elevated number of apoptotic cell corpses, thereby revealing a role for BEC-1 in apoptosis.

BEC-1 Is Necessary for the Function of the Class III PI3 Kinase LET-512/Vps34

The Bec-1 phenotype shared striking similarities with that of *let-512*/*VPS34* mutant worms, including lethality and molting defects, as well as alterations in the outer nuclear membrane and in the ER ([14] and results not shown). LET-512, the *C. elegans* ortholog of the yeast and mammalian phophatidylinositol (PtdIns) 3-kinase Vps34, is a key regulator of membrane trafficking. In yeast and mammals, Atg6/Vps30/Beclin 1 and Vps34 form a complex. The interaction between the two proteins is required for autophagy [15, 16], but the underlying mechanisms are not clear. To gain further insight, we tested a possible interaction between LET-512/Vps34 and BEC-1. Coimmunoprecipitation experiments revealed that the two proteins associated with each other in vivo (Figure 4A). To further assess their functional relationship, we examined the cellular distribution of the lipid product PtdIns 3-phosphate of LET-512/Vps34 in *bec-1(RNAi)* animals. We found that, like in *let-512(lf)* mutants [14], PtdIns 3-phosphate was absent from all microscopically detectable cytoplasmic membranes and vesicles in *bec-1(RNAi)* larvae (Figures 4B

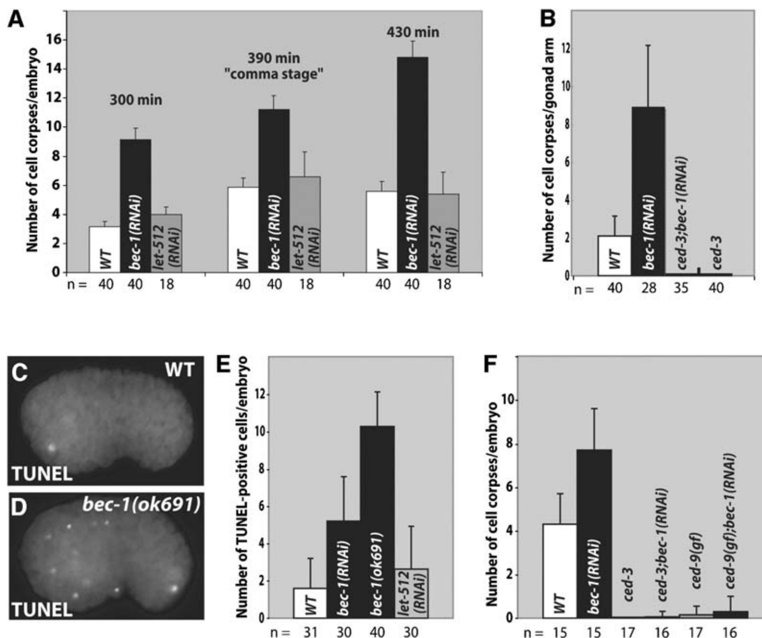

Figure 3. Disruption of *bec-1* Triggers Apoptosis

(A) Quantification of cell corpses in wild-type, *bec-1(RNAi)*, and *let-512(RNAi)* embryos. The developmental stage was normalized to wild-type embryos grown at 22°C for 300, 390, and 430 min. Embryos were recorded under Nomarski optics with a "four-dimensional" video recording system. The numbers of analyzed individuals are indicated. Error bars are SEM.

(B) Quantification of cell corpses by Nomarski optics in the germline of wild-type, *bec-1(RNAi)*, *ced-3(n2433);bec-1(RNAi)*, and *ced-3 (n2433)* hermaphrodites. The number of analyzed individuals is indicated. The data shown are means ± SEM.

(C–E) TUNEL staining. TUNEL-stained wild-type (C) and *bec-1(ok691)* mutant comma stage embryos (D). (E) Quantification of TUNEL-positive nuclei in wild-type, *bec-1 (RNAi)*, *bec-1(ok691)*, and *let-512(RNAi)* comma stage embryos. The number of embryos analyzed is indicated. The data shown are means ± SEM. TUNEL staining was performed as described [13].

(F) The production of extra apoptotic cells corpses in *bec-1(RNAi)* embryos is blocked by *ced-3(lf)* or *ced-9(gf)* mutations. Quantification of cell corpses by Nomarski optics in wild-type, *bec-1(RNAi)*, *ced-3(n2433)*, *ced-3(n2433);bec-1(RNAi)*, *ced-9(n1950)*, and *ced-9(n1950);bec-1(RNAi)* comma stage embryos. The wild-type embryos came from worms that were grown on HT115 bacteria not expressing ds *bec-1* RNA. The number of analyzed individuals is indicated. The data shown are means ± SEM.

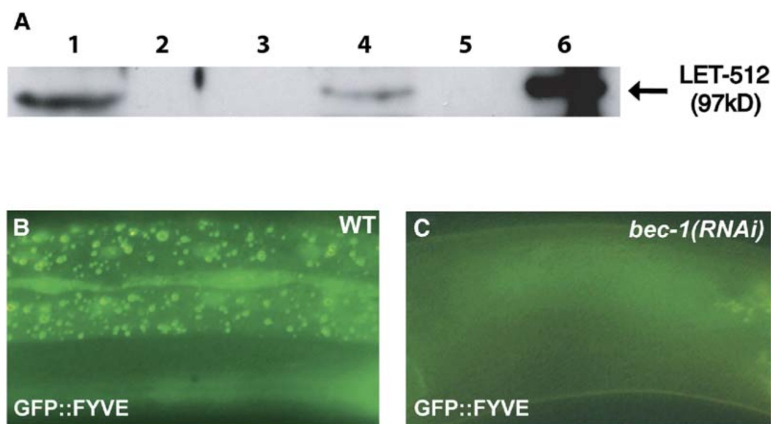

Figure 4. BEC-1 Interacts with LET-512/Vps34 and Is Required for Its Function

(A) Coimmunoprecipitation of BEC-1 with LET-512/Vps34. Western blot probed with anti-LET-512/Vps34 antibodies. Lane 1: wild-type extract; lanes 2 and 3: wild-type extract precipitated with mouse IgG (lane 2) and anti-GFP antibody (lane 3); lane 4: extract from *bec-1(ok700);[bec-1::gfp+rol-6(su1006)]* animals; lanes 5 and 6: extract from *bec-1(ok700);[bec-1::gfp+rol-6(su1006)]* animals precipitated with mouse IgG (lane 5) and anti-GFP antibody (lane 6). For experimental details, see Supplemental Data.

(B and C) Distribution of the GFP::FYVE fusion protein [GFP::2xT10G3.5(FYVE)] that binds to the PtdIns 3-phosphatase product of LET-512/Vps34 [16], in wild-type (B) and *bec-1(RNAi)* (C) larvae.

and 4C). Taken together, these data demonstrate that BEC-1 is required for the function of LET-512/Vps34.

Two Different Functions, Two Different Complexes?

Despite the fact that BEC-1 interacts in vivo with LET-512, we observed no significant increase in the number of cell corpses in *let-512(RNAi)* animals (Figures 3A and 3E). This suggested that the role of BEC-1 in apoptosis is independent of LET-512/Vps34. Since BEC-1 interacts with both LET-512/Vps34 and CED-9/Bcl-2, we tested whether the three proteins coexist in a trimeric complex. However, we found no interaction between CED-9/Bcl-2 and LET-512/Vps34 by coimmunoprecipitation (data not shown). Together with the genetic data above, this result provides some preliminary evidence that BEC-1 may form two separate and functionally distinct complexes with LET-512/Vps34 and CED-9/Bcl-2. Its interaction with LET-512/Vps34 is required for the function of the PtdIns 3-kinase that plays an essential role in membrane trafficking and endocytosis [14]. *bec-1* mutants were defective in both processes (see Supplemental Results available with this article online), suggesting that BEC-1 might participate in these mechanisms as an essential component of the PtdIns 3-kinase complex. Since the interaction between BEC-1 and LET-512/Vps34 is evolutionarily conserved in yeast and mammals, we expect a similar function for Atg6/Vps30/beclin 1 in these species. Moreover, BEC-1 forms a complex with CED-9/Bcl-2, but the functional significance of this interaction is not clear. Our results show that BEC-1 is necessary for the control of apoptosis in the germline and in somatic cells of *C. elegans*; however, its exact role in the canonical apoptotic cell death pathway remains to be established. Since BEC-1 promotes autophagy [10] and prevents ectopic apoptosis (this study), the two processes seem to be inversely related. The multifunctional protein BEC-1 might, therefore, have an essential role in integrating and fine-tuning the two basic mechanisms of cell death involved in developmental and pathological processes as well as in cellular stress responses.

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, and a figure and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/16/1513/DC1/.

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