

Published in final edited form as:

Autophagy. 2008 November ; 4(8): 1034–1041.

Autophagy protects from hypoxic injury in *C. elegans*

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Abstract

Macroautophagy has been implicated in a variety of pathological processes. Hypoxic/ischemic cellular injury is one such process in which autophagy has emerged as an important regulator. In general, autophagy is induced after an hypoxic/ischemic insult; however, whether the induction of autophagy promotes cell death or recovery is controversial and appears to be context dependent. We have developed *C. elegans* as a genetically tractable model for the study of hypoxic cell injury. Both necrosis and apoptosis are mechanisms of cell death following hypoxia in *C. elegans*. However, the role of autophagy in hypoxic injury in *C. elegans* has not been examined. Here, we found that RNAi knockdown of the *C. elegans* homologs of beclin 1/Atg6 (*bec-1*) and LC3/Atg8 (*lgg-1*, *lgg-2*), and mutation of Atg1 (*unc-51*) decreased animal survival after a severe hypoxic insult. Acute inhibition of autophagy by the type III phosphatidylinositol 3-kinase inhibitors, 3-methyladenine and Wortmannin, also sensitized animals to hypoxic death. Hypoxia-induced neuronal and myocyte injury as well as necrotic cellular morphology were increased by RNAi knockdown of BEC-1. Hypoxia increased the expression of a marker of autophagosomes in a *bec-1*-dependent manner. Finally, we found that the hypoxia hypersensitive phenotype of *bec-1(RNAi)* animals could be blocked by loss-of-function mutations in either the apoptosis or necrosis pathway. These results argue that inhibition of autophagy sensitizes *C. elegans* and its cells to hypoxic injury and that this sensitization is blocked or circumvented when either of the two major cell death mechanisms is inhibited.

Keywords

Autophagy; Cell death; Hypoxia; Apoptosis; Necrosis

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved mechanism to recycle proteins and organelles.^{1,2} Autophagy has been most extensively studied in yeast where it functions in adaptation to and survival during nutrient deprivation.³ In metazoans, autophagy has been implicated in a variety of physiological and pathological processes. In *C. elegans*, RNAi-knockdown of homologs of yeast autophagy genes shortens lifespan and interferes with formation of stress resistant dauer larvae.⁴ In *Drosophila*, autophagy is induced by starvation and redistributes nutrients during larval development.^{5,6} In mammals, autophagy has been functionally linked to survival during growth factor deprivation in mouse bone marrow cell lines⁷ and during the early mouse neonatal period where autophagy is upregulated in tissues with increased metabolic demands.⁸ More

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recently, autophagy has been implicated in a variety of pathological conditions including neurodegenerative diseases, myocardial and cerebral hypoxia/ischemia, tumor formation, and bacterial pathogenicity.^{2, 9} In each of these disease states, the precise role of the autophagy pathway is unclear. In particular, whether autophagy is contributing to and/or an adaptive reaction against the cellular pathology is an unresolved and fundamental question.

Hypoxic/ischemic injury is one such pathological condition, in which autophagy may play a role. Multiple studies have reported an increase in markers of autophagy after hypoxic/ischemic injury of mammalian cells.¹⁰⁻¹⁵ In particular, increases of autophagic activity have been well documented after hypoxia/ischemia of cardiac myocytes and cerebral neurons.^{16, 17} In some models, autophagy appears to promote cell death following hypoxia/ischemia whereas in others it may function to promote survival. What determines whether autophagy promotes death or survival is not yet clear. One hypothesis with some experimental support is that normally autophagy inhibits apoptotic cell death. Thus, in tissues or conditions where apoptosis is the dominant form of cell death following hypoxic injury, autophagy would be expected to promote survival.

C. elegans has made crucial contributions towards our understanding of the fundamental mechanisms of cell death. The core pathway for apoptosis was defined in *C. elegans* using elegant classical genetic techniques.¹⁸ Potent modulators of necrotic cell death have also been identified in *C. elegans* by mutagenesis screens.¹⁹⁻²⁴ Motivated by these successes, we and others have developed *C. elegans* as genetically tractable model for hypoxic adaptation and injury and have identified genes that either promote or suppress hypoxic cell death.²⁵⁻³⁵ *C. elegans* is relatively hypoxia resistant compared to most mammalian cells. Six hours of near anoxic incubation of *C. elegans* is required to produce permanent behavioral deficits and a measurable level of whole animal death.³² About half of adult animals are killed by 12 hours of hypoxia and all by 24 hours. Both neurons and myocytes die prior to and contribute to whole animal death. Morphological criteria indicate that necrosis is one mechanism of cell death. Mutations in the *C. elegans* programmed cell death pathway produce whole animal hypoxia resistance implicating apoptosis as also mediating a substantial fraction of the hypoxic cell death.^{36, 37} The role of autophagy in hypoxic injury in invertebrates has yet to be defined. Here, we use genetic and pharmacological reagents to examine the role of autophagy in hypoxic injury in *C. elegans*.

MATERIALS AND METHODS

Strains and cultivation

Except for RNAi experiments, all strains were grown at 20° on nematode growth medium NGM agar plates seeded with OP50 bacteria.³⁸ The wild-type strain used was N2 *var* Bristol and was the background for all strains and for all RNAi experiments unless otherwise indicated. PD4251 containing [*nls::pmyo-3::GFP*]³⁹ and SK4005 containing *zds4*[*pme-4::GFP;lin-15(+)*] were kindly provided by Monica Driscoll and Scott Clark.⁴⁰ QU1 containing *izEx1*[*Plgg-1::GFP::lgg-1 + rol-6(su1006)*] was kindly provided by Alicia Melendez and Beth Levine.⁴ MT1522 *ced-3(n717)*, MT3002 *ced-3(n1286)*, MT2547 *ced-4(n1162)*, MT4770 *ced-9(n1950gf)*, ZB1028 *crt-1(bz29)*, CB369 *unc-51(e369)*, and CB1189 *unc-51(e1189)* were obtained from the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

RNAi experiments

RNAi bacterial strains were obtained from the Ahringer Library⁴¹ and were grown overnight in Luria Broth with 100 µg/ml ampicillin at 37° and then diluted 1:100 in 2xYT medium with the same antibiotics and grown at 37° with shaking until reaching an OD of 0.4. 150 µl

of the RNAi bacteria plus 1mM IPTG was then added to each NGM plate containing 50µg/ml of ampicillin. After 2 days of bacterial growth, animals were added to the RNAi plates. Animals were grown for two generations from egg to adult on RNAi plates plus 1mM IPTG; the second generation was age synchronized by transferring adults to a new RNAi plate for four hours then removing the parents to produce a synchronous brood of approximately 100 animals/plate. The empty vector L4440 was used as the negative control for all RNAi experiments and L4440 was grown and induced identically to other RNAi bacteria.

Hypoxic exposure

Synchronized populations of well-fed animals were transferred from their agar plates to a 1.5 ml eppendorf tube with 1 ml M9 buffer; the buffer was exchanged three times to remove bacteria and the final wash was removed down to 100 µl; the tubes were placed into the hypoxia chamber as described previously³² and incubated for the specified period at 26° then removed from the chamber, transferred to NGM agar plates seeded with OP50 bacteria to recover for 24 four hours in normoxia at 20° before scoring. Animals without pharyngeal pumping and without spontaneous or touch-evoked movement were scored as dead. One day post L4 animals were used for scoring whole animal death assays; one day post egg animals were used for scoring cell pathology and GFP::LGG-1 density.

Autophagy inhibition with 3MA and wortmannin

A frozen 10 µl aliquot of 100 mM 3-methyladenine (3MA – Sigma #M9281) or 1mM wortmannin (Sigma #W1628) in DMSO was thawed and diluted into 90 µl of M9 buffer in minifuge tubes with about 100 adult worms to achieve the specified 3MA concentrations. The control incubations were in 10% DMSO in M9. The tubes were immediately placed into the hypoxia chamber for the hypoxic incubation for the specified time, and the worms recovered and scored as usual.

Neuronal and muscular degeneration assay

L1 and L2 larvae underwent twelve hours of hypoxic incubations as described above. All pathologies were scored on surviving animals after 5 hours of recovery by an observer blinded to condition. Nomarski optics was used for scoring necrotic cells. Markedly swollen cells with no apparent nuclei were scored as necrotic. For muscle pathology, muscle nuclei were visualized and counted using nuclear-localized GFP driven by a muscle-specific promoter - *pmyo-3::GFP* as described previously.³² Axonal beading pathology was scored in mechanosensory neurons visualized with *pme-4::GFP* as described previously.³²

Quantitative Real-time PCR analysis—A synchronous population of wild-type animals was treated for two generations with *bec-1*, *lgg-1*, or L4440 empty vector RNAi. RNA was then isolated from adult animals (one day post L4) by a Trizol freeze-cracking method. cDNA was synthesized with a RETROscript random decamer kit (Ambion, Austin, TX) with 2 µg of total RNA as template. Quantitative real-time PCR was performed with SYBR green PCR master mix (Applied Biosystems, Foster City, CA) in an Applied Biosystems 7500-fast RT PCR instrument with a Rox passive-reference dye. Primers were constructed to amplify a 100 bp fragment of the *bec-1* transcript, a 97 bp fragment of the *lgg-1* transcript, or 114 bp fragment of the *act-1* β-actin transcript, which was used as the endogenous control (housekeeping gene). Standard PCR amplification with the primer sets produced single bands migrating at the correct size. Fold-expression changes were calculated with the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ is the RNAi ΔCT [RNAi cycle threshold(CT) value – ACT-1 CT value] subtracted by the control ΔCT [L4440 cycle threshold(CT) value – ACT-1 CT value]. In all cases, raw SYBR fluorescence values were normalized to the passive-reference dye ROX. Primer sequences were as follows:

bec-1 – Forward: CCCATCTGATGCTCCAGTTT

Reverse: CAACTGCAAGAATCGACGAA

Igg-1 – Forward: TCCAACCTCGTCCAGAAGATGCTC

Reverse: TGCTGATGGTCCTGGTAGAGTTGT

act-1 – Forward: GCTGGACGTGATCTTACTGATTACC

Reverse: GTAGCAGAGCTTCTCCTTGATGTC

RESULTS

Two distinct steps in the autophagy pathway were disrupted in order to test the role of autophagy in hypoxic injury in *C. elegans*. *bec-1* encodes the *C. elegans* homolog of beclin1/Atg6.^{4, 42, 43} *unc-51* encodes an ortholog of Atg1^{4, 44}. Beclin1 and Atg1 promote initiation of autophagy, and their *C. elegans* homologs have been shown to be required for normal autophagy^{4, 44}. *Igg-1* and *Igg-2* encode *C. elegans* homologs of LC3/Atg8. LC3 is critical for autophagosome formation and maturation. To test the role of these genes in hypoxic sensitivity, we used RNAi constructs against *bec-1*, *Igg-1*, and *Igg-2* and tested *unc-51* loss-of-function mutants. *bec-1(RNAi)* significantly increased hypoxia-induced death of wild type *C. elegans* compared to the empty vector control (Fig. 1A). Similarly, RNAi knockdown of *Igg-1* and *Igg-2* increased hypoxic death (Fig. 1A). Two *unc-51(lf)* mutants also had a hypoxia hypersensitivity phenotype (Fig. 1B). Thus, the autophagy genes, *bec-1*, *Igg-1*, *Igg-2*, and *unc-51*, promote organismal survival after severe hypoxia.

Because *bec-1* is required for normal development,⁴² the hypoxic hypersensitivity of *bec-1* inactivation might not be due to an acute requirement for BEC-1 but rather to some temporally remote developmental effect. To inactivate autophagy acutely, we used the type III phosphatidylinositol-3 kinase (PI-3 kinase) inhibitor 3-methyladenine (3MA). 3MA is a widely-used inhibitor of autophagy.^{45–47} When given to adults just prior to hypoxic incubation, 3MA dose dependently increased the lethality of hypoxia with an EC₅₀ of approximately 10 mM (Fig. 1C); this concentration is similar to that required to inhibit autophagy in mammalian cells.⁴⁷ Multiple trials with 10 mM 3MA confirmed that it significantly increased hypoxic death (Fig. 1D). Wortmannin, another PI-3 kinase inhibitor that inhibits autophagy,^{46, 48} also increased hypoxic lethality in adults (Fig. 1D). These data demonstrate that autophagy is required just prior to, during, or after hypoxia for normal recovery from a hypoxic insult. Notably, both 3MA and Wortmannin produced a relatively small but nevertheless significant increase in death following normoxic incubation as well. The normoxic incubation is performed in liquid at 26°C but the tubes are placed in a normoxic incubator. Thus, both 3MA and Wortmannin sensitize to this temperature stress as well. Thus, the increased death observed after hypoxic incubation in 3MA and Wortmannin likely represents sensitization to both temperature and hypoxia.

Autophagy is required for a normal recovery from hypoxic injury in *C. elegans* but does autophagic activity respond in some way to hypoxic injury? To address this question, we used GFP-labeled LGG-1, which is concentrated on autophagosomes and has been shown in several studies to be a reliable indicator of autophagosome number in *C. elegans*.^{4, 44, 49–51} GFP::LGG-1 puncta were markedly increased by a sublethal hypoxic insult (Fig. 2). This induction of puncta was diminished but not abolished by both *bec-1(RNAi)* and 3MA consistent with the puncta being a marker of autophagosomes. To quantitate the level of knockdown of *bec-1* or *Igg-1* expression by the respective RNAi, we performed quantitative RT-PCR on adult worms that had been cultured for two generations on *bec-1(RNAi)* or *Igg-1(RNAi)*. We were not able to get reliable data with primers against *Igg-2*. Compared to growth on empty vector, the abundance of *bec-1* transcript was 57% and 49% in

bec-1(RNAi)-treated animals in two independent experiments. For *Igg-1(RNAi)*, the transcript levels were 55% and 74% relative to empty vector. Knockdown is clearly incomplete; thus, a reduced, but not absent, level of autophagy is responsible for the hypoxic hypersensitivity phenotype.

Neurons and myocytes are two cell types that are particularly prone to hypoxic injury in other organisms. In addition, the determinants of hypoxic injury of these cell types are of considerable clinical interest because they are injured in common human diseases such as stroke and ischemic heart disease. In the case of neuronal injury in *C. elegans*, previously we have observed axonal beading in animals that survive a sublethal hypoxic incubation.^{32, 37} We tested whether reduction of *bec-1* activity influences this axonal pathology by examining mechanosensory axons labeled with GFP. *bec-1(RNAi)* increased hypoxia-induced axonal beading of the type observed previously (Fig. 3A–D), but we also observed severe axonal tortuosity and large gaps in the axons (Fig. 3E,F). Pathology of any of three types was significantly more prevalent (Fig. 3G) and more severe (Fig. 3H) in animals treated with *bec-1(RNAi)*. Beading, tortuosity, and gaps are typical of traumatic and ischemic axonal pathology in mammalian models,^{52–55} but only beading or complete loss of the neuron had been previously observed in *C. elegans*. All three axonal pathological features are thought to be ultimately a consequence of loss of membrane integrity and dysfunctional axonal transport.^{56, 57}

C. elegans has approximately 90 mononucleated myocytes that comprise the striated body wall muscle. Using a nuclear-localized GFP driven by a muscle-specific promoter, we have previously observed nuclear fragmentation and disappearance following a sublethal hypoxic insult³². *bec-1(RNAi)* significantly worsened the hypoxia-induced loss of myocyte nuclei (Fig. 4). Thus, BEC-1 functions to protect both neurons and myocytes from hypoxic injury and cell death.

Mammalian cells die from both necrotic and apoptotic mechanisms after hypoxic/ischemic injury. Early after a hypoxic insult, most cells die a necrotic death whereas later apoptotic death tends to be more prevalent.⁵⁸ In *C. elegans*, hypoxia results in necrosis of a subset of *C. elegans* cells particularly those within and around the pharynx.³² Necrotic cell death of neurons in *C. elegans* has been extensively studied using gain-of-function mutants in ion channel subunits.²¹ Interestingly, inhibition of autophagy suppresses ion channel-mediated necrosis of neurons.⁴⁹ However the role of autophagy in hypoxia-induced necrotic cell death has not been examined in *C. elegans* and is relatively unexplored in mammalian models as well. After hypoxic incubation, *bec-1(RNAi)* animals had an increased number of necrotic cells compared to vector controls (Fig. 5). Given the distortion of the anatomy by the necrotic process, the type of cells dying by necrosis was not determined. Nevertheless, we can conclude that BEC-1 activity inhibits necrotic cell death after a hypoxic insult.

Apoptosis and autophagy interact in a complex manner.⁵⁹ Cellular insults can induce both apoptosis and autophagy. Apoptosis and autophagy appear in many cases to be mutually inhibitory. Inhibition of autophagy unmasks apoptosis, and likewise apoptosis inhibition may enhance autophagic cell death. However, in other models, the pro-apoptotic protein Bnip3 appears to promote autophagy. Consistent with mutual inhibition, reduction of BEC-1 activity in *C. elegans* was found to increase the number of apoptotic cell corpses in both the germline and in developing embryos.⁴² The increase in apoptosis in *bec-1* mutant animals could be blocked by a loss-of-function mutation of *ced-3*, the canonical *C. elegans* caspase gene. We have previously shown that loss-of-function mutations in the pathway leading to CED-3-mediated apoptosis reduces *C. elegans* hypoxic death.³⁷ Thus, a reasonable hypothesis for the mechanism of the hypoxia hypersensitive phenotype of *bec-1(RNAi)* animals is that knockdown of BEC-1 activity increases apoptotic cell death and thereby

sensitize animals to hypoxic injury. We tested this hypothesis by measuring hypoxic death in *bec-1(RNAi)* animals in wild type versus apoptosis-defective mutant backgrounds. As reported previously, a *ced-9(gf)* mutant, a *ced-4(lf)* mutant, and two *ced-3(lf)* mutants were hypoxia resistant relative to the wild type strain N2 (Fig. 6A). Importantly, the hypoxia resistance of all of the tested apoptosis mutants was not diminished by *bec-1(RNAi)* treatment. Thus, the hypoxic hypersensitivity of *bec-1(RNAi)* is not apparent when apoptosis is blocked. Similarly, neither *lgg-1* nor *lgg-2* RNAi increased the hypoxic sensitivity of a *ced-3(lf)* mutant (Fig. 6B).

To ask whether this epistatic relationship is unique to apoptosis mutants or might also be shared by mutations that suppress necrotic cell death, we tested the effect of *bec-1(RNAi)* in a loss-of-function mutant of *crt-1(bz29)*. *crt-1* encodes a calreticulin, and *crt-1* mutants have been shown to suppress ion-channel-mediated necrosis²⁴. *crt-1(bz29lf)* was markedly hypoxia resistant, and *bec-1(RNAi)* was not effective at suppressing its resistance (Fig. 6A).

DISCUSSION

We have shown that inhibition of autophagy is deleterious to the survival of *C. elegans* following a severe hypoxic insult. A reduction in the activity of autophagy genes not only increases animal death but also worsens the cellular pathology seen after hypoxia. Following a damaging hypoxic incubation, we observed an increase in punctated LGG-1 expression in presumptive autophagosomes, an observation consistent with the induction of autophagic activity in response to hypoxic injury. Finally, we found that the hypoxic hypersensitivity produced by knockdown of autophagy in wild type animals is not observed in apoptosis pathway mutants or in a mutant that suppresses ion-channel-mediated necrosis. For discussion, we want to place ours and other autophagy findings in *C. elegans* in the context of what is known about autophagy and hypoxia/ischemia in other organisms.

A number of recent studies have examined the relationship between autophagy and hypoxic/ischemic injury in mammalian cells. Hypoxia and/or ischemia has been shown consistently to result in an increase in the number of autophagosomes in various models. For example, Yan and coinvestigators showed that ischemia increased markers of autophagy in pig myocardium.¹¹ Similarly, autophagic activity was increased after cerebral hypoxia/ischemia in mice.⁶⁰ However, whether induction of autophagy is pro-adaptive or maladaptive was not addressed in these studies. Subsequent studies indicate that autophagy can either promote or prevent cell death following hypoxia/ischemia. Simulated ischemia and reperfusion of rat primary cardiac myocytes was shown to induce autophagosome-like vesicles, and cardiac myocytes death following simulated ischemia/reperfusion was ameliorated by both 3MA and beclin1 siRNA treatments.¹⁴ Consistent with the promotion of hypoxic/ischemic cell death by autophagy, this study also showed that beclin1 overexpression increased myocyte death. Mice challenged with carotid ligation followed by a mild hypoxic exposure were found to have increased autophagosome numbers in hippocampal neurons, and a strain with selective neuronal knockout of the *Atg7* gene had reduced hippocampal neuronal death following hypoxia/ischemia.¹⁵ Finally, in a pure hypoxia-induced cell death protocol, multiple mammalian cell lines were made hypoxia resistant by 3MA, beclin1 siRNA, or *Atg5* siRNA.⁶¹

The evidence for a protective role of autophagy in mammalian cells against hypoxic/ischemic cell death is less extensive. In a simulated hypoxia/ischemia experimental paradigm, a mouse cardiac permanent cell line was protected from apoptotic cell death by both beclin-1 and *Atg5* overexpression.^{13, 62} These same investigators also showed that transfection of an *Atg5* dominant negative construct increased apoptotic cell death.^{12, 13} Similarly, 3MA treatment resulted in an increased death of primary neonatal rat cardiac

myocytes after hypoxia and reoxygenation.⁶³ While extensive evidence supports the conclusion that basal levels of autophagy are important for the health of neurons,^{64–66} whether autophagy can protect mammalian neurons following hypoxic/ischemic insults is unclear. Thus, the available evidence in mammalian cells indicates that autophagy can serve either a destructive or protective role following a hypoxic/ischemic insult. The factors that regulate whether activation of autophagy enhances or inhibits cell death following hypoxia are unknown.

As in mammalian cell types, autophagy in *C. elegans* can either be pro-adaptive or maladaptive. A regulatory role for autophagy has been particularly well established for *C. elegans* lifespan where autophagy appears to promote long life. The long lifespan phenotype of reduction-of-function mutations in the *daf-2* gene, which encodes an insulin/IGF receptor homolog that limits lifespan, is strongly suppressed by reduction-of-function mutations and RNAi knockdown of *C. elegans* autophagy genes.⁴ Additionally, caloric restriction-induced lifespan extension requires an intact autophagy pathway.^{51, 67, 68}

For survival from severe caloric restriction, that is starvation, the role of autophagy appears to be more complex. Prolonged and severe starvation is lethal to developing *C. elegans*,^{69, 70} and the lethality of starvation is increased by inhibition of *bec-1*.⁷⁰ However, *C. elegans* mutants where muscarinic signaling in the pharynx is hyperactivated have increased death during starvation, and this hypersensitivity to starvation can be ameliorated by *bec-1* RNAi.⁷⁰ Hyperactivation of muscarinic signaling was shown to increase markers of autophagy above background levels, consistent with the hypothesis that excessive autophagy can promote death from starvation. Thus, as for hypoxia-induced death in mammals, autophagy can either prevent or promote death from starvation depending on regulatory factors, perhaps one of which is second messenger pathways such as that activated by muscarinic receptors in *C. elegans*.

Finally, we would like to consider our finding that the hypoxic hypersensitivity produced by a reduction in autophagy is dependent on the activity the apoptosis and necrosis pathways. A direct interaction between autophagy and apoptosis was initially demonstrated by the finding that beclin-1 binds to Bcl-2 in mammalian cells.⁷¹ These investigators subsequently showed that Bcl-2 acts to inhibit beclin-1-mediated autophagy.⁷² In *C. elegans*, BEC-1 was shown to interact with the Bcl-2 homolog CED-9, and inactivation of *bec-1* produced ectopic apoptotic cell death.⁴² We found that the hypoxic hypersensitivity phenotype of *bec-1(RNAi)* animals is suppressed by loss-of-function mutants in the apoptosis pathway. Thus, our results are consistent with *bec-1* inactivation enhancing apoptotic death following hypoxia, perhaps through a direct disinhibition of the canonical apoptosis pathway. However, a hypoxia-resistant necrosis-defective mutation also suppressed the hypoxic hypersensitivity of *bec-1(RNAi)*. Thus, the suppression by the apoptosis mutants can just as easily be explained by an indirect mechanism where a block of terminal cell death effectors, whether necrotic or apoptotic, negates the sensitization produced by a partial inhibition of autophagy. These complex genetic interactions suggest that the activity of apoptotic and necrotic mediators in cells injured by hypoxia will influence the effect of the autophagy pathway in cell death and survival.

Acknowledgments

Supported by NINDS (R01 NS045905), an American Heart Association Established Investigator Award, and a McKnight Endowment Fund for Neuroscience, Neuroscience of Brain Disorders Award.

Abbreviations

3MA	3-methyladenine
GFP	Green fluorescent protein
RNAi	Double-stranded RNA-mediated interference

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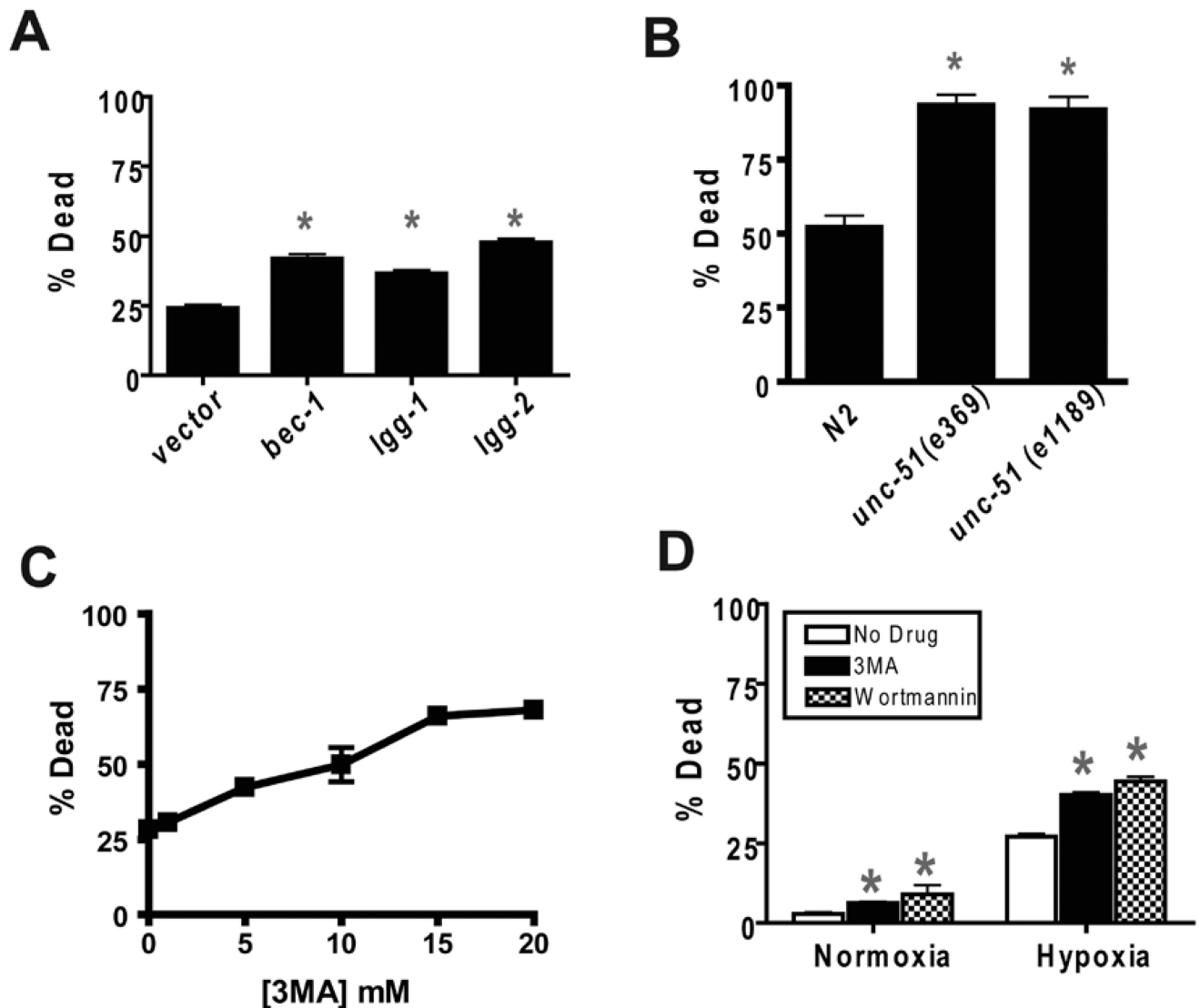


Figure 1. Inhibition of autophagy increases hypoxic sensitivity. (A) Hypoxic sensitivity of animals treated with RNAi against *bec-1*, *lgg-1*, and *lgg-2* autophagy genes compared to empty vector control. Second generation RNAi-treated adult animals were scored as alive or dead after a 24 hour recovery from a twelve hour hypoxic incubation. Error bars are mean \pm sem of 5 trials. * $p < 0.01$ vs vector control, two-tailed t-test. (B) Hypoxic sensitivity of loss-of-function mutants of *unc-51*. % dead following recovery from a twelve hour hypoxic incubation. The N2 control animals were scored contemporaneously with the *unc-51* mutants. * $p < 0.01$ vs N2, two-tailed t-test. (C) Concentration/response curve for the effect of 3-methyladenine (3MA) on hypoxic sensitivity. Adult animals were transferred into M9 buffer containing the indicated concentrations of 3-MA then immediately placed into the hypoxia chamber for twelve hours, recovered on agar plates without 3MA for 24 hours then scored as alive or dead. Error bars are mean \pm sem of 5 trials. (D) Effect of 3MA (10 mM) and wortmannin (100 μ M) on death after normoxic or hypoxic incubation. Wortmannin experiments are otherwise identical to that described for 3MA. Bars are mean \pm sem of 9 trials for 3MA and 3 trials for wortmannin. * $p < 0.01$ vs No Drug, two-tailed t-test.

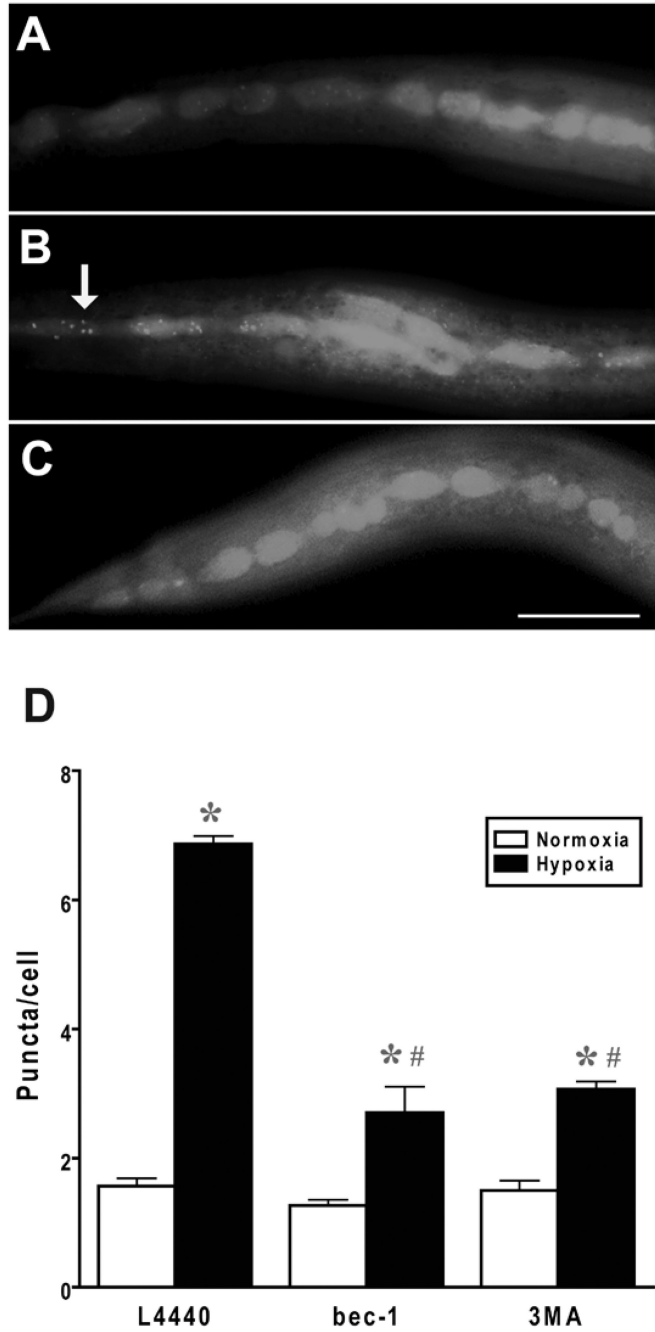


Figure 2. Effect of hypoxia on autophagosome density. Autophagosomes were labeled by LGG-1::GFP as described previously⁴ and the number of GFP-labeled puncta were scored after recovery from a twelve hour normoxic or hypoxic incubation by an observer blinded to condition. (A) Normoxic vector-treated animal with diffuse LGG-1::GFP expression in hypodermal seam cells. (B) Hypoxic vector-treated animal with multiple LGG-1::GFP puncta visible (arrow). (C) Hypoxic *bec-1(RNAi)* animal with a reduction in LGG-1::GFP puncta. (D) Quantification of puncta/seam cell. # animals: Vector normoxia – 21, Vector hypoxia – 20, *bec-1(RNAi)* normoxia – 7, *bec-1(RNAi)* hypoxia – 9. * p < 0.01 vs normoxia; # p < 0.01 vs vector. Scale bar = 20 μ m.

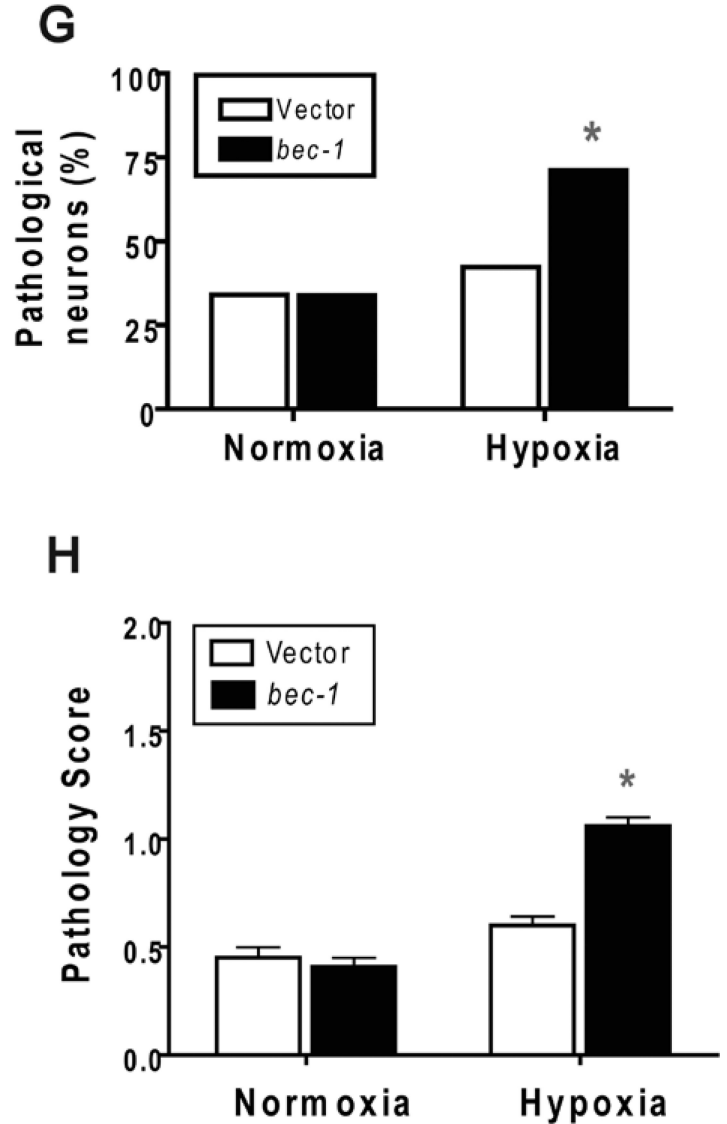
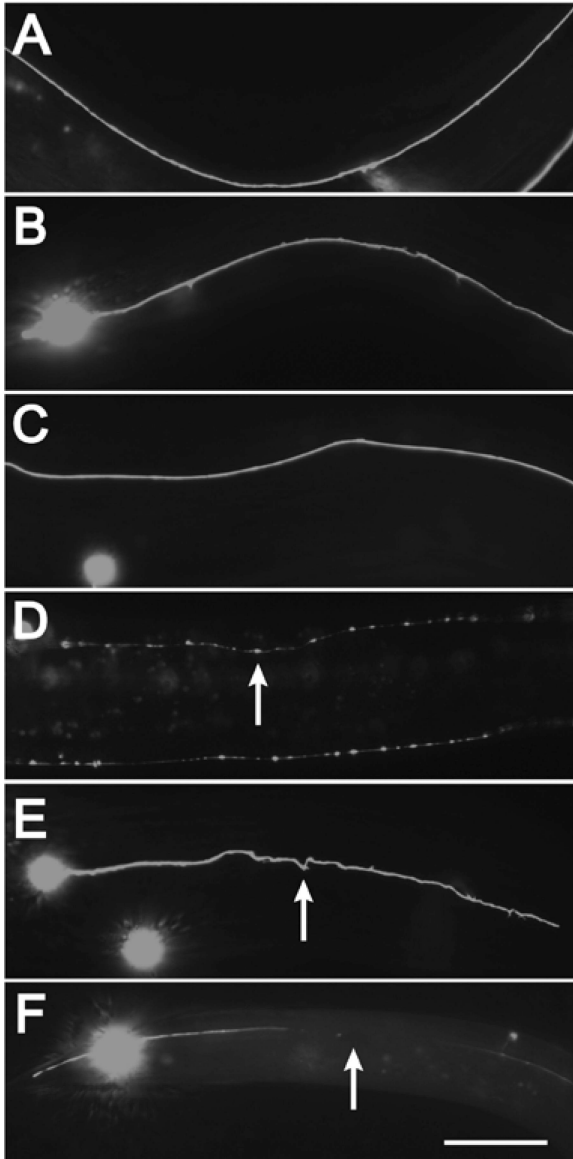


Figure 3. Effect of *bec-1* knockdown on hypoxic axonal pathology. Wild type larval animals with vector or *bec-1* RNAi treatment underwent a twelve hour hypoxic or normoxic incubation then scored after recovery for axonal pathology by an observer blinded to condition. pMEC-4::GFP was used to visualize mechanosensory axons. Axonal beading, tortuosity, and gaps were scored. (A) Normoxic vector-treated animal showing normal axonal morphology. (B) Hypoxic vector-treated animal showing little pathological changes. (C) *bec-1(RNAi)* normoxic animal with normal axonal morphology. (D) *bec-1(RNAi)* hypoxic animal with axonal beading. Arrow indicates a bead. (E) *bec-1(RNAi)* hypoxic animal with tortuous axon pathology. Arrow indicates tortuous axon. (F) *bec-1(RNAi)* hypoxic animal with large axon gap. Arrow indicates gap. (G) The prevalence of pathological neurons following hypoxia is increased by *bec-1(RNAi)*. % of neurons with any pathology was scored. * - $p < 0.01$, Fisher's exact test. (H) The severity of axonal pathology following hypoxia is increased in *bec-1(RNAi)* animals. The severity of the axonal pathology for each axon was scored on a 0 – 2 scale. Severe beading, tortuosity, and/or large gaps were scored

as a 2, milder but present pathology as a 1, and no pathology a 0. Data are mean \pm sem of at least 100 animals/condition scored by an observer blinded to condition. * - $p < 0.01$ vs vector control, two-tailed t-test. Scale bar = 20 μm .

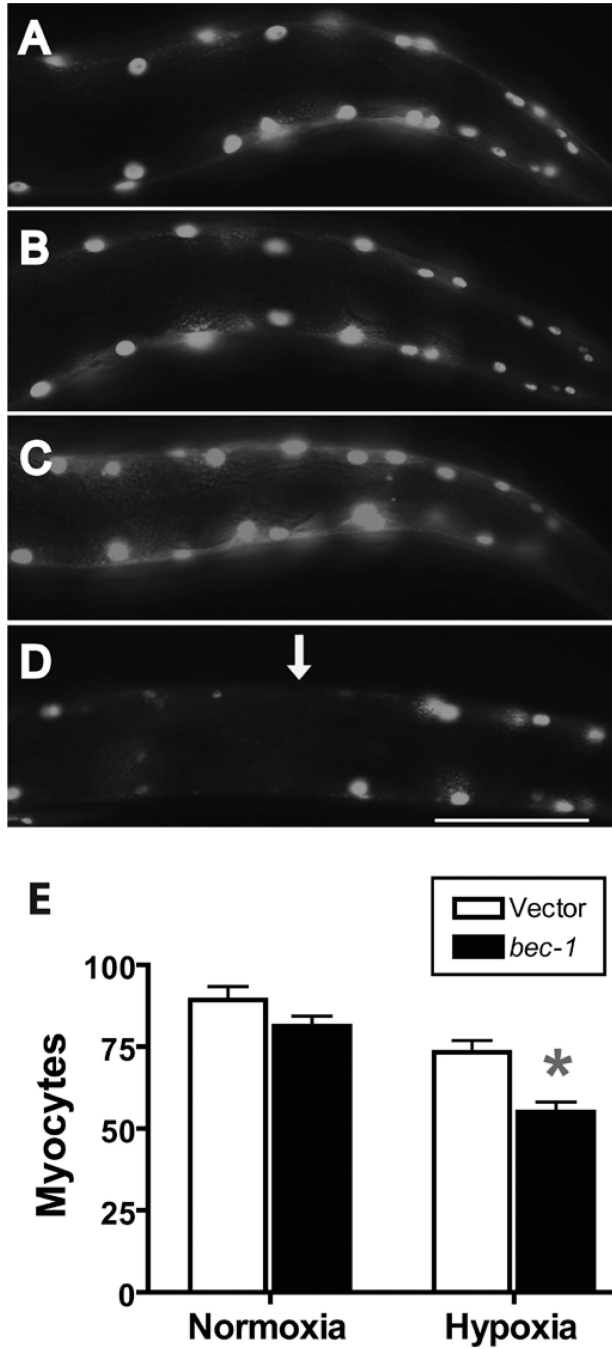


Figure 4. Effect of *bec-1* knockdown on hypoxic muscle pathology. Wild type larval animals with vector or *bec-1* RNAi treatment underwent a twelve hour hypoxic or normoxic incubation then scored after recovery for myocyte number. Nuclear-localized GFP expressed in body wall muscles was used to visualize and count myocytes. (A) Normoxic vector-treated animal showing normal myocyte nuclear morphology and number. (B) Hypoxic vector-treated animal showing normal myocyte nuclear morphology and number. (C) Normoxic *bec-1(RNAi)*-treated animal showing normal myocyte nuclear morphology and number. (D) Hypoxic *bec-1(RNAi)*-treated animal showing pale or absent nuclei (arrow). (E) Comparison of myocyte number in *bec-1(RNAi)* vs vector-treated animals after normoxic or

hypoxic incubation. Data are mean \pm sem of 35 animals/condition. * - $p < 0.01$ vs vector control. Scale bar = 20 μm .

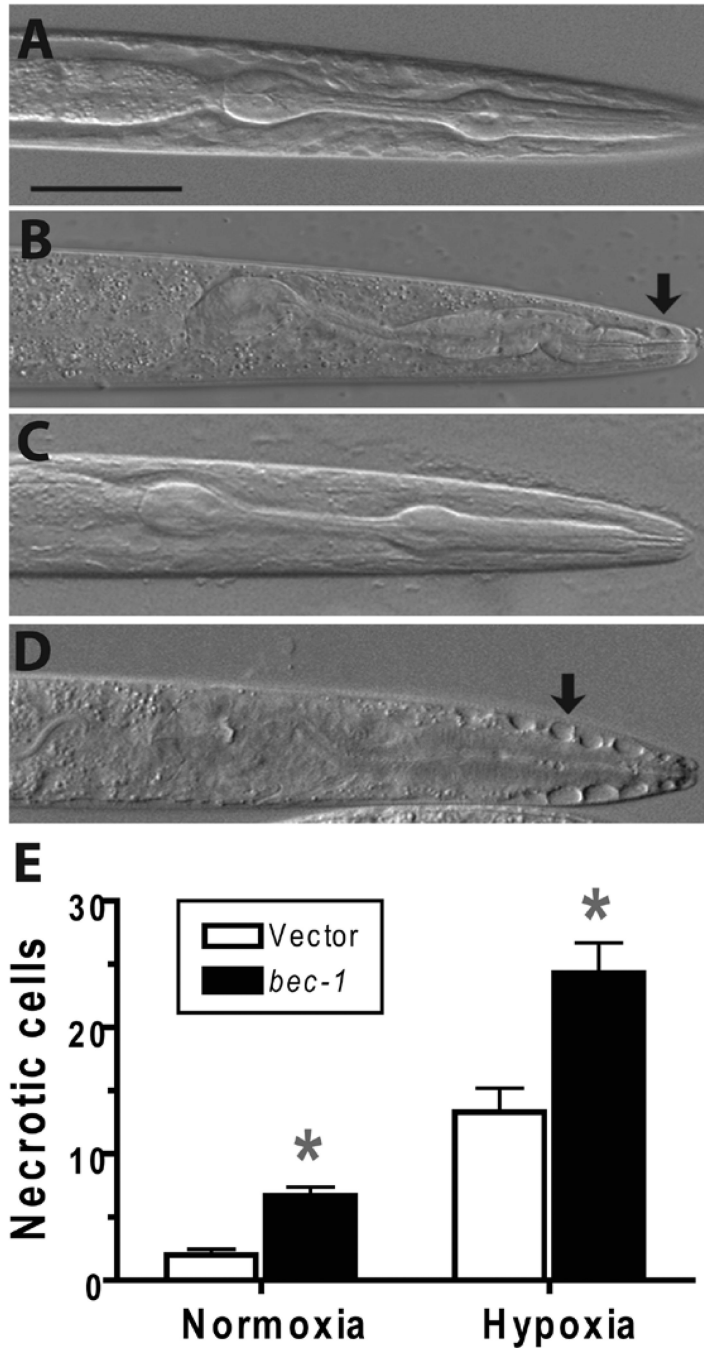


Figure 5. Effect of *bec-1* knockdown on necrotic pathology. Wild type larval animals with vector or *bec-1* RNAi treatment underwent a twelve hour hypoxic or normoxic incubation then scored after recovery for myocyte number. Necrotic cells were visualized and counted using Nomarski optics. Cells with markedly swollen morphology and lack of nuclei were scored as necrotic. (A) Normoxic vector-treated animal showing normal cellular morphology in the pharyngeal region. (B) Hypoxic vector-treated animal showing a few necrotic-appearing cells (arrow). (C) Normoxic *bec-1(RNAi)* animal showing normal cellular morphology. (D) Hypoxic *bec-1(RNAi)*-treated animal showing multiple necrotic cells adjacent to the anterior pharynx (arrow). (E) Comparison of necrotic cell number in *bec-1(RNAi)* vs vector-

treated animals after normoxic or hypoxic incubation. Data are mean \pm sem of 35 animals/condition. * - $p < 0.01$ vs vector control. Scale bar = 20 μm .

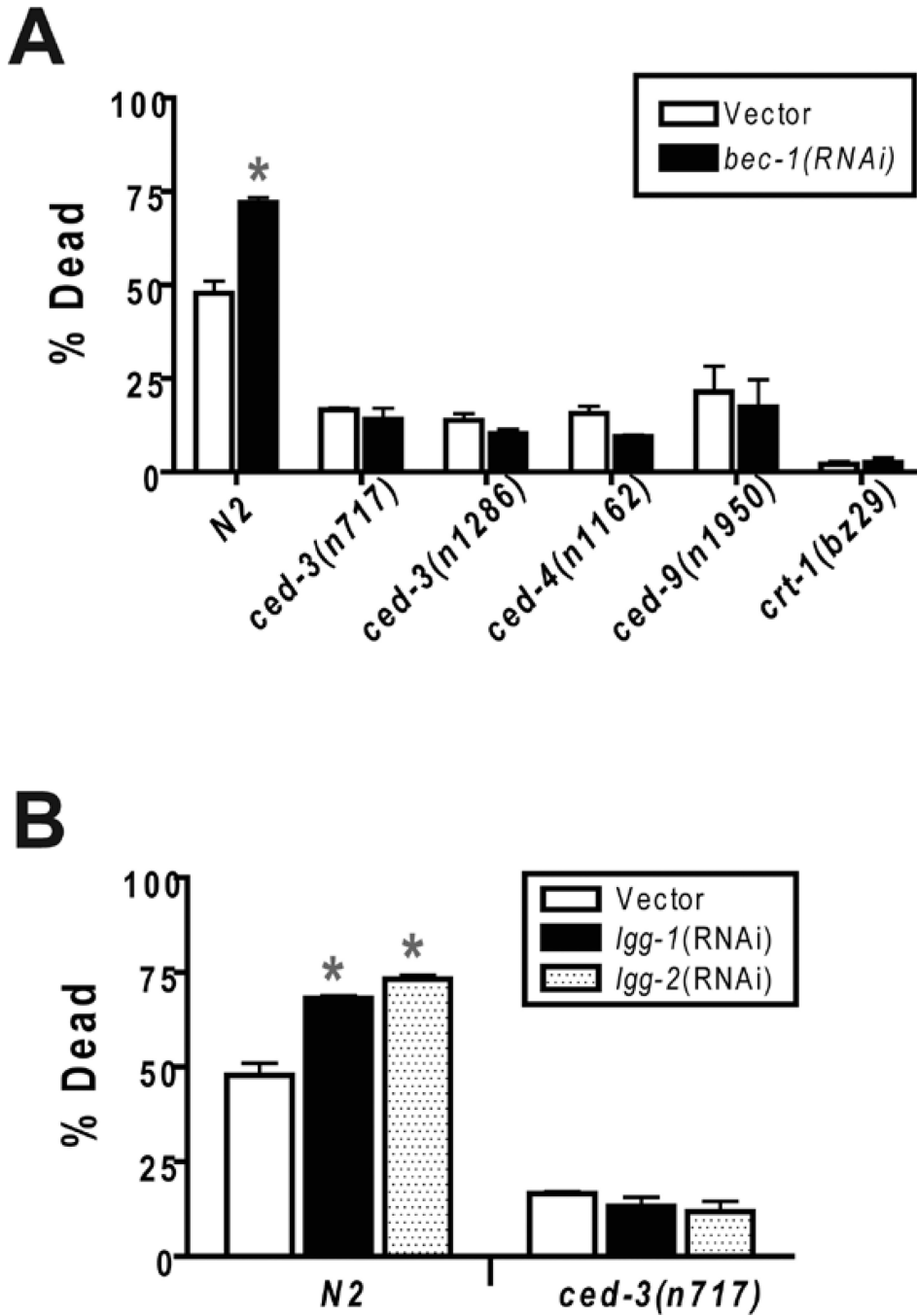


Figure 6. Epistatic relationship between autophagy pathway RNAi's and apoptosis pathway and calreticulin mutants. Second generation RNAi- or vector control-treated adult wild type or mutant animals were scored as alive or dead after recovery from a twelve hour hypoxic incubation. (A) Apoptosis pathway and calreticulin mutants are hypoxia resistant and are epistatic to *bec-1(RNAi)*. Error bars are mean \pm sem of at least 4 trials for each condition and genotype. * different from vector control, $p < 0.01$, two-tailed t-test. (B) Epistatic relationship of *lgg-1* and *lgg-2* RNAi with a *ced-3(lf)* mutant. Error bars are mean \pm sem of at least 4 trials for each condition and genotype. * different from vector control, $p < 0.01$, two-tailed t-test.