Autophagy genes protect against *Salmonella typhimurium* infection and mediate insulin signaling-regulated pathogen resistance

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A conserved insulin-like pathway modulates both aging and pathogen resistance in *Caenorhabditis elegans*. However, the specific innate effector functions that mediate this pathogen resistance are largely unknown. Autophagy, a lysosomal degradation pathway, plays a role in controlling intracellular bacterial pathogen infections in cultured cells, but less is known about its role at the organismal level. We examined the effects of autophagy gene inactivation on *Salmonella enterica* Serovar Typhimurium (*Salmonella typhimurium*) infection in 2 model organisms, *Caenorhabditis elegans* and *Dictyostelium discoideum*. In both organisms, genetic inactivation of the autophagy pathway increases bacterial intracellular replication, decreases animal lifespan, and results in apoptotic-independent death. In *C. elegans*, genetic knockdown of autophagy genes abrogates pathogen resistance conferred by a loss-of-function mutation, *daf-2(e1370)*, in the insulin-like tyrosine kinase receptor or by overexpression of the DAF-16 FOXO transcription factor. Thus, autophagy genes play an essential role in host defense in vivo against an intracellular bacterial pathogen and mediate pathogen resistance in long-lived mutant nematodes.

Immune functions decline with age (1), but the mechanisms underlying immunosenescence are not well understood. Because a conserved insulin-like signaling pathway controls both aging and pathogen resistance in *Caenorhabditis elegans* (2, 3), the identification of cellular innate immune functions regulated by this pathway may help elucidate the basis of age-related declines in immunity. One candidate target is autophagy, a lysosomal degradation pathway that decreases with age, is implicated in the degradation of intracellular bacteria, and is required for the lifespan extension of nematodes with mutation in the *daf-2* insulin-like signaling pathway (4–7).

The autophagy pathway is mediated by evolutionarily conserved genes (called *atg* genes) and, in vitro, targets both extracellular bacteria that invade intracellularly and intracellular bacterial pathogens for lysosomal degradation (4). Autophagy limits the intracellular growth of *Listeria monocytogenes* in *Drosophila* (8), suggesting a role for autophagy in defense against intracellular bacteria in vivo. Besides promoting the direct degradation of intracellular pathogens, autophagy has other functions in immunity (4), including the delivery of microbial genetic material or peptides to endosomes or MHC Class II loading compartments, respectively, for activation of innate or adaptive immunity. Furthermore, a polymorphism in the *atg* gene, *ATG16L1*, is linked to genetic susceptibility to the inflammatory bowel disorder, Crohn's disease, which has led to the speculation that mutations in the autophagy pathway may alter the normal gut response to enteric bacterial pathogens (9). Indeed, loss of Atg16l1 in mouse models recapitulates certain aspects of the pathology of human Crohn's disease, and the Crohn's disease-associated *ATG16L1* variant may have impaired antibacterial autophagy function in a human gut epithelial cell line (10).

The unicellular organism *Dictyostelium discoideum* and the multicellular organism *C. elegans* are useful models for studying interactions between hosts and pathogens (11, 12), including the gram-negative bacterium *Salmonella typhimurium*. *Salmonella* is a pathogen that causes human food-borne illness worldwide, infects both mammalian intestinal epithelial cells and macrophages, and has an increased propensity to cause invasive, extra-intestinal disease in the elderly (1, 13). In *C. elegans*, *S. typhimurium* establishes persistent infection in the intestinal lumen but does not replicate inside intestinal epithelial cells (14, 15), suggesting that host defense mechanisms to combat *Salmonella* infection may be more successful in nematode than in mammalian cells. In *Dictyostelium*, a soil amoeba that is highly susceptible to invasion by bacterial pathogens that commonly infect human macrophages (12), *Salmonella* is efficiently taken up but fails to multiply intracellularly. Therefore, the identification of factors that govern susceptibility to *Salmonella* infection in the nematode and soil amoeba may be relevant to understanding *Salmonella* host-pathogen interactions in mammalian intestinal epithelial cells and macrophages, respectively.

Long-lived mutant nematodes display resistance to *Salmonella* infection (2, 16). In *C. elegans*, the *daf-2* insulin-like signaling pathway is a well-characterized regulator of pathogen resistance, longevity, and autophagy (2, 3, 5, 16). Nematodes with loss-of-function mutations in the insulin-like tyrosine kinase receptor *daf-2* have extended lifespan and pathogen-resistant phenotypes (3, 16), including resistance to infection with *S. typhimurium*. The major target of the DAF-2 pathway is DAF-16, a forkhead transcription factor required for both longevity and pathogen resistance in *daf-2* mutants (16). Moreoever, DAF-2 negatively regulates autophagy, and lifespan extension in *daf-2* mutants requires *atg* genes (5–7). These findings, coupled with the role of autophagy in pathogen degradation, led us to postulate that autophagy may represent a mechanism of innate immunity against *Salmonella* infection in vivo that is required for the pathogen resistance of long-lived mutant nematodes.

To evaluate the role of autophagy in pathogen resistance, we used feeding RNAi to inactivate several *C. elegans atg* genes, including *bec-1* and *lgg-1*, in *S. typhimurium*-infected wild-type, and long-lived *daf-2*-mutant and DAF-16 over-expressing nematodes. We also examined *S. typhimurium* infection in *Dictyostelium* with null mutations in three *atg* genes, *ATG1*, *ATG6*, and *ATG7*. Our results

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indicate that *atg* genes play a conserved role in antibacterial host defense in vivo and mediate pathogen resistance in long-lived mutant animals.

Results

Autophagy Mediates Host Defense Against *Salmonella* in *C. elegans*. To evaluate whether *atg* genes function in host defense against *Salmonella* infection in *C. elegans*, we used feeding RNAi to silence two previously characterized *C. elegans atg* genes, *bec-1* and *lgg-1* (5), which are orthologs of yeast *ATG6* and *ATG8* and function in autophagic vesicle nucleation and autophagic vesicle expansion, respectively. The survival of *Salmonella*-infected animals treated with either *bec-1* or *lgg-1* RNAi was significantly shortened as compared to *Salmonella*-infected control vector-treated animals (Fig. 1 *A* and *B*, Table S1)] . Although a null mutation in *bec-1* or high-dose *atg* gene RNAi injection is embryonically lethal (5, 17), feeding RNAi against *bec-1* or *lgg-1* did not shorten the lifespan of N2 (wild-type) animals exclusively fed nonpathogenic *Escherichia coli* (Fig. 1 *A* and *B*). We confirmed that feeding RNAi decreased the level of *atg* gene RNA by RT-PCR, and that *bec-1* feeding-RNAi treatment exerted previously described effects of *atg* gene inhibition [e.g., abnormal dauer development in *daf-2(e1370)* mutants and autophagy inhibition in worms that transgenically express the autophagy marker GFP::LGG-1] (data not shown). We also knocked down the expression of *atg-7* (involved in autophagic vesicle expansion) in N2 worms using RNAi. Similar to *bec-1* and *lgg-1* RNAi, the *atg-7*-RNAi animals were more susceptible to *Salmonella* infection than control animals (Fig. S1*A*, Table S2). Thus, inactivation of three different *atg* genes increased nematode susceptibility to lethal *Salmonella* infection. This is unlikely to reflect nonspecific or off-target effects of *atg* gene-RNAi treatment because no alterations in *Salmonella* susceptibility were observed in nematodes treated with feeding RNAi against two irrelevant control genes, *unc-22* (required for normal muscle function) or *him-3* (required for meiotic chromosome segregation) (Fig. S1 *B* and *C*, Table S2). Furthermore, neither *bec-1*-RNAi treatment [similar to a previous report by Hansen et al (7)] nor *Salmonella* infection altered pharyngeal pumping rates (Fig. S2), suggesting that the increased pathogen susceptibility in *atg* gene RNAi-treated animals is not because of alterations in food (bacterial) uptake.

BEC-1 interacts with CED-9 (a Bcl-2 homolog) and disruption of *bec-1* triggers apoptosis during embryo development (17). Therefore, we studied apoptosis-deficient nematodes with a loss-of-function mutation in the *C. elegans* caspase, *ced-3(n717)* to examine whether apoptosis is involved in the increased susceptibility of autophagy-deficient worms to *Salmonella* infection. *Salmonella* infection decreased the lifespan of wild-type worms more than that of *ced-3(n717)*-mutant animals (Fig. 1*C* and Table S1), indicating that the *ced-3* mutation protects against *Salmonella* infection. [The basis for the difference between this finding and those of Aballay et al. (18) is unclear, but might reflect subtle differences in genetic strains]. Importantly, *bec-1* RNAi significantly decreased the lifespan of *Salmonella*-infected *ced-3*-mutant animals (Fig. 1*D* and Table S1); these animals had similar mortality as *bec-1* RNAi-treated *Salmonella*-infected wild-type animals (Fig. 1*A*). Therefore, the mechanism of increased *Salmonella* sensitivity because of *atg* gene inactivation does not involve caspase-dependent apoptosis.

***bec-1* Restricts Bacterial Replication and Cytopathology in *C. elegans* Intestinal Epithelial Cells.** To examine the mechanism by which *atg* genes protect against *Salmonella* infection, we performed EM analyses of control vector- and *bec-1*-RNAi-treated animals (Fig. 1 *E–J*). Immediately after a 2-day *Salmonella* ingestion period, few organisms were observed in the intestinal lumen and some bacteria were observed inside the intestinal epithelial cells (Fig. 1 *E* and *F*). In the control group, intracellular bacteria were found primarily inside early intact autophagosomes or autolysosomes (Fig. 1*E* and data not shown), but rarely inside cytoplasmic *Salmonella*-containing vacuoles (SCVs). In contrast, in *bec-1*-RNAi animals,

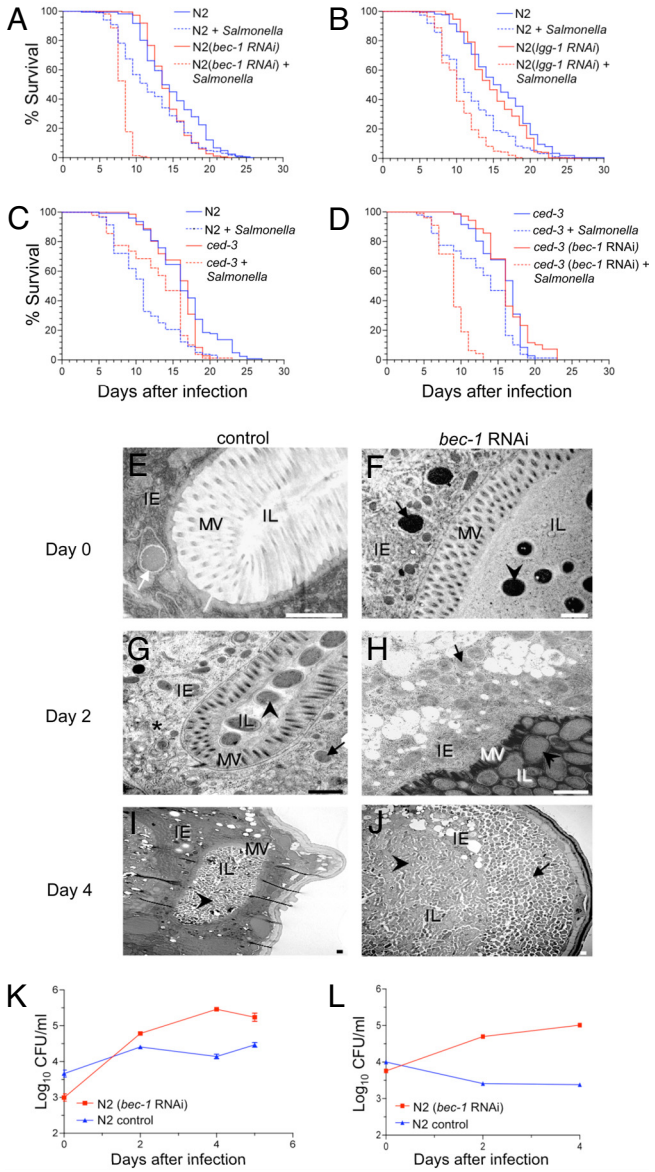

Fig. 1. *Atg* genes mediate host defense against *Salmonella* in *C. elegans*. (*A* and *B*) Survival curves of wild-type (N2) animals treated with either control vector or indicated *atg* gene RNAi-feeding plasmids following a 2-day exposure to *S. typhimurium* or normal food (i.e., nonpathogenic *Escherichia coli*) at 20 °C (see *SI Materials and Methods* for details). (*C* and *D*) Survival curves of wild-type (N2) and *ced-3(n717)*-mutant animals following a 2-day exposure to *S. typhimurium* or normal food without *atg* gene RNAi treatment (*C*) or with control vector or *bec-1* RNAi treatment (*D*) at 20 °C. For (*A*) to (*D*), see Table S1 for statistical details. (*E–J*) Representative EMs of *Salmonella*-infected control N2 animals and *bec-1*-RNAi animals at day 0 (*E* and *F*), 2 (*G* and *H*), and 4 (*I* and *J*) after a 2-day *Salmonella* ingestion period. (*F*, *G*, *I*, and *J*) Arrowheads denote intraluminal bacteria. (*E*) White arrow denotes bacterial-containing early autophagosome. (*G*) Asterisk denotes autolysosome with partially degraded bacterial debris. (*F–H* and *J*) Black arrows denote SCVs inside intestinal epithelial cells. IE, intestinal epithelial cells; IL, intestinal lumen; MV, microvilli. (Scale bars, 1 μm.) (*K* and *L*) Growth curves of *S. typhimurium* in N2 animals treated with either control vector or *bec-1*-RNAi feeding plasmids in the absence (*K*) or presence (*L*) of 100 μg/ml gentamicin. For (*K*) and (*L*), values represent mean ± SEM for triplicate samples of ≈10 animals per treatment group per sample. Similar results were observed in two independent experiments.

the cytoplasm contained intact SCVs (Fig. 1*F*). At day two after *Salmonella* ingestion, the number of organisms in the intestinal lumen increased in both groups, but more dramatically in the *bec-1*-RNAi animals (Fig. 1 *G* and *H*). Moreover, in control animals

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the intestinal epithelial cells contained rare, visible intact bacteria and numerous autolysosomes containing bacterial debris (Fig. 1*G*), whereas in *bec-1*-RNAi animals the intestinal epithelial cells contained numerous intact bacteria (which were similar in size and morphology to those found in the intestinal lumen) and very few autophagosomes or autolyososmes (Fig. 1*H*). At this stage, the epithelial cells in both groups had intact basement membranes and microvilli, although increased cytoplasmic vacuolization was observed in *bec-1*-RNAi animals. However, at day 4 after *Salmonella* ingestion, in the *bec-1*-RNAi group the intestinal epithelial cells were completely destroyed in most animals, and there were massive sheets of bacteria extending from the intestinal lumen to the body wall muscle (Fig. 1*J*). In contrast, the epithelial cells remained largely intact in the control animals (Fig. 1*I*), indicating that the *atg* gene *bec-1* successfully protects the intestinal epithelium cells from overwhelming bacterial infection and cellular destruction.

These EM analyses suggest that *Salmonella* invades the intestinal epithelial cells in both control and *bec-1*-RNAi animals; however, in the former group the bacteria are efficiently degraded by the autophagy pathway, whereas in the latter group the intracellular bacterial population expands, leading to extensive cytoplasmic destruction and premature death of the organism. To confirm that *Salmonella* replicates intracellularly in *bec-1*-RNAi animals, we compared bacterial growth curves in animals cultured in the presence or absence of gentamicin, an antibiotic that inhibits extracellular but not intracellular *Salmonella* replication. In the absence of gentamicin, bacterial colony counts increased gradually over time in both control and *bec-1*-RNAi animals, with a larger magnitude of increase in the *bec-1*-RNAi animals ($P < 0.0001$, *t*-test) (Fig. 1*K*). In the presence of gentamicin, no increase was observed in bacterial colony counts in control animals (Fig. 1*L*). In contrast, in *bec-1*-RNAi animals, the *Salmonella* growth curve paralleled that observed in the absence of gentamicin, indicating that *Salmonella* replicates intracellularly in autophagy-deficient nematodes.

***Atg* Genes Restrict Intracellular Bacterial Replication and Host Cellular Destruction in *Dictyostelium*.** To further confirm that *atg* genes restrict intracellular *Salmonella* multiplication independently of bacterial invasion (which is difficult to assess in vivo in a multicellular organism), we used a unicellular model host organism, *D. discoideum*. Unlike many bacteria that replicate intracellularly in mammalian macrophages, *Salmonella* is rapidly degraded after internalization and is nonpathogenic in *Dictyostelium* (12). We found that GFP-labeled *Salmonella* invaded wild-type *Dictyostelium* and previously described *Dictyostelium* mutants (19) lacking the *atg* genes, *ATG1* (a serine/threonine kinase involved in autophagy induction), *ATG6* (the ortholog of *bec-1*), and *ATG7* with similar kinetics and to a similar degree (Fig. 2*A*). In *Dictyostelium* that transgenically express a fluorescent autophagy marker protein, GFP-Atg8 (19), CFP-labeled *Salmonella* colocalized with GFP-Atg8 by 2-h postinfection (p.i.) (Fig. 2*B*), indicating that internalized bacteria are efficiently targeted to autophagosomes. Similarly, EM analysis revealed the degradation of *Salmonella* inside autolysosomes in wild-type *Dictyostelium* (Fig. 2*C*). In contrast, in *atg1*-, *atg6*-, and *atg7*-mutant *Dicytostelium*, no bacteria were observed inside autophagosomes or autolysosomes (Fig. 2*C*). Instead, the bacteria were observed exclusively in intact SCVs containing single bacteria or in larger vacuoles that contained multiple bacteria. Thus, the autophagic machinery is not necessary for bacterial invasion or formation of SCVs, but is required for the fusion of SCVs with lysosomal compartments. By 24-h p.i., *Salmonella* were rarely visible in wild-type *Dictyostelium* and the cytoplasm appeared normal, whereas *atg1*-, *atg6*-, and *atg7*-mutant *Dictyostelium* exhibited severe cytopathology, including extensive cytoplasmic vacuolization and disruption of plasma membrane integrity (data not shown).

These findings are consistent with autophagic degradation of intracellular *Salmonella* in wild-type *Dicytostelium* and intracellular multiplication of *Salmonella* with resulting cytopathology in

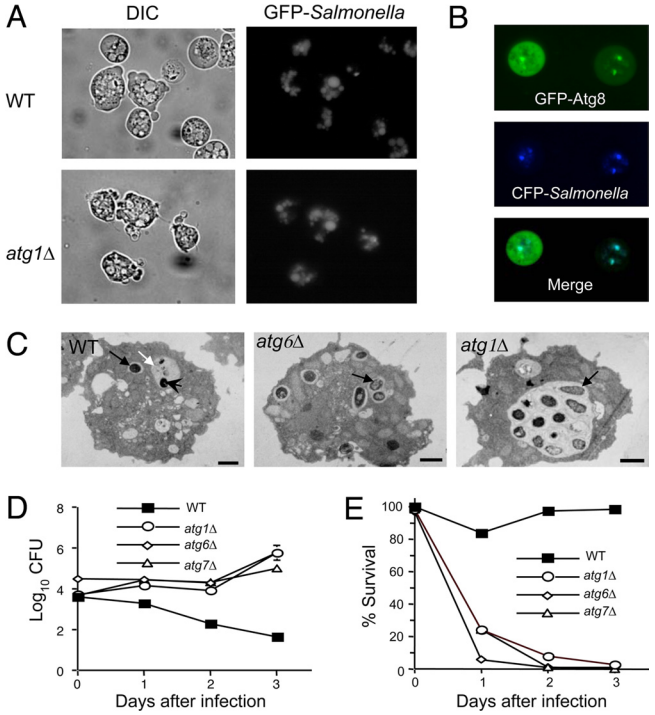

Fig. 2. *Atg* genes restrict intracellular *Salmonella* replication and pathogenicity in *D. discoideum*. (*A*) Internalization and vacuolar localization of GFP-*Salmonella* in both wild-type (WT) and *atg1* mutant *Dictyostelium* at 2-h postinfection (p.i.). Similar results were observed in *atg6*Δ and *atg7*Δ *Dictyostelium* (data not shown). (*B*) Colocalization of CFP-*Salmonella* and a transgenic autophagosomal marker, GFP-Atg8, at 2-h p.i. in wild-type *Dictyostelium*. (*C*) Representative EMs 12-h p.i. of wild-type *Dictyostelium* showing an intact SCV (*black arrow*) and an autolysosome (*white arrow*) containing a partially degraded *Salmonella* bacterieum (*arrowhead*), and of *atg1*Δ and *atg6*Δ *Dictyostelium* that lack autolysosomes but have SCVs (*black arrows*) that contain multiple organisms, indicative of active intracellular bacterial multiplication. (Scale bars, 1 μm.) (*D*) Growth of intracellular *Salmonella* in wild-type and *atg* gene-mutant *Dictyostelium* strains. (*E*) Survival of *Dictyostelium* infected in (*D*). For (*D*) and (*E*), results represent mean ± SEM for triplicate samples and similar results were observed in three independent experiments.

autophagy-deficient amoebae. To confirm this, we measured *Salmonella* replication (Fig. 2*D*) and cell survival (Fig. 2*E*) in the presence of gentamicin. In wild-type *Dictyostelium*, there was no bacterial growth (Fig. 2*D*), and the *Dictyostelium* remained healthy with a long-term survival curve that parallels that of amoebae grown in axenic media (Fig. 2*E* and data not shown). In contrast, in *atg1*-, *atg6*-, and *atg7*-mutant *Dictyostelium*, bacterial colony counts increased over time (Fig. 2*D*), indicating intracellular multiplication, and the *Dictyostelium* died within 24- to 72-h p.i. (Fig. 2*E*). Because *Dictyostelium* lack caspases or other essential components of the apoptotic machinery (20), this *Salmonella*-induced death of autophagy-deficient amoebae, like that of autophagy-deficient nematodes, does not involve caspase-dependent apoptosis. Taken together, these findings demonstrate that the autophagic machinery restricts intracellular bacterial multiplication and cytopathology in two model organisms, *C. elegans* and *Dictyostelium*.

Autophagy Deficiency Abrogates Pathogen Resistance of *daf-2*-Mutant Adults. To evaluate whether autophagy is an effector mechanism of enhanced *Salmonella* resistance in long-lived *C. elegans* mutants, we first examined whether autophagy is induced in adult nematodes with a loss-of-function mutation in *daf-2* (2). Using a previously described assay that measures autophagosomes in the seam cell, a type of specialized hypodermal cell that has detectable basal- and stimulus-induced autophagy (5), we observed a higher number of

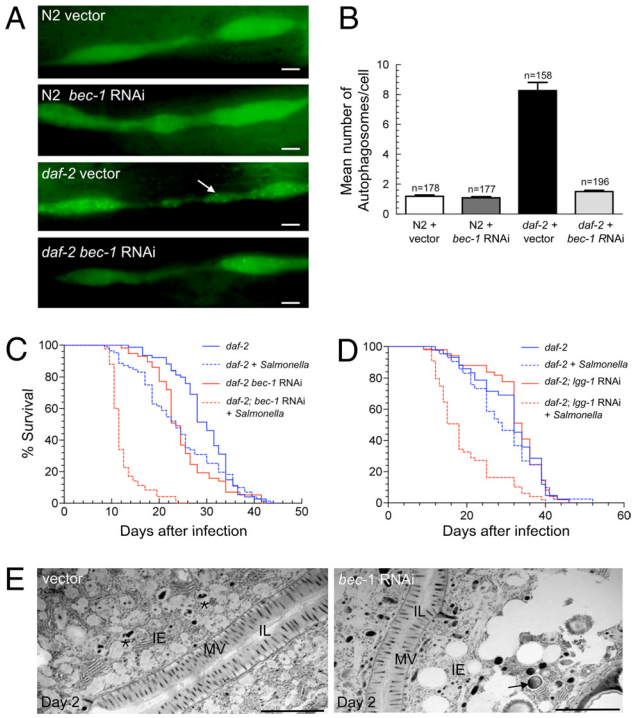

Fig. 3. *Atg* genes mediate insulin signaling-regulated resistance against *Salmonella* in *C. elegans*. (*A*) Representative images of autophagosomes (GFP::LGG-1 dots) in seam cells of N2 wild-type and *daf-2*-mutant animals treated either with RNAi control vector or *bec-1* RNAi. The arrow denotes a representative autophagosome. (Scale bars, 2 μM.) (*B*) Quantification of autophagosomes per seam cell (mean ± SEM) for each genotype. *n* = number of seam cells per group in ≈20 animals. Similar results were obtained in two independent experiments. (*C* and *D*) Survival curves of *daf-2(e1370)*-mutant animals treated with either control or indicated *atg* gene-RNAi feeding plasmids following a 2-day exposure to *S. typhimurium* or normal food at 20 °C. See Table S1 for statistical details. (*E*) Representative EMs of *Salmonella*-infected control and *bec-1*-RNAi *daf-2(e1370)* animals two days after a 2-day *Salmonella* ingestion period. The asterisk denotes a representative autolysosome containing partially degraded bacteria. The arrow denotes representative SCV. (Scale bars, 2 μM.)

autophagosomes in *daf-2(e1370)*-mutant adults than in N2 wild-type worms ($P < 0.0001$, *t*-test), which was suppressed by *bec-1*-RNAi treatment (Fig. 3 *A* and *B*). Thus, the DAF-2 signaling pathway negatively regulates autophagy in the adult worm, consistent with the report by Hansen et al. (7).

Next, we examined whether *atg* genes are required for the pathogen-resistance phenotype of *daf-2(e1370)*-mutant adults. Because *atg* genes have previously been shown to be required for the lifespan extension of *daf-2* mutants (5, 6), we sought to avoid confounding effects of autophagy on lifespan regulation that occur independently of *daf-2*-mediated pathogen resistance. To do this, we used a low dose of the RNAi inducer isopropyl-β-D-thiogalactopyranoside (IPTG) (1 nM) that was titrated to identify a concentration at which *bec-1* RNAi and *lgg-1* RNAi did not have significant effects on the lifespans of uninfected *daf-2(e1370)*-mutant animals (Fig. 3 *C* and *D* and Table S1). Similar to previous reports with other bacterial pathogens (3), the control *daf-2(e1370)*-mutant animals survive longer than wild-type animals when infected with *S. typhimurium* (Fig. 3 compared to Fig. 1 and Table S1). *Bec-1* and *lgg-1* RNAi significantly shortened the lifespan of *Salmonella*-infected *daf-2(e1370)* animals at doses that only minimally shortened the lifespan of *E. coli*-fed control *daf-2(1370)* animals (Fig. 3 *C* and *D*, Table S1). Indeed, the mean lifespan of *bec-1*-RNAi or *lgg-1*-RNAi *Salmonella*-infected *daf-2* animals was similar to that of control *Salmonella*-infected wild-type N2 animals (Fig. 1 *A* and *B*, Table S1), indicating that *atg* gene knockdown is sufficient to completely abrogate pathogen resistance conferred by a mutation in the insulin-like signaling pathway.

Similar to our observations in N2 animals, EM studies of *Salmonella*-infected *daf-2(e1370)* mutants demonstrated enhanced autophagic degradation of bacteria in control vs. *bec-1*-RNAi animals (Fig. 3*E*). Compared to N2 animals, there were fewer bacteria in the intestinal lumen of both control and *bec-1*-RNAi animals, and in control animals there was a marked increase in the number of autolysosomes containing partially degraded bacteria. Similar to *bec-1*-RNAi N2 animals, *bec-1*-RNAi *daf-2(e1370)* mutants displayed an increase in intact SCVs in intestinal epithelial cells, as well as increased intestinal epithelial cell pathologic changes, such as cytoplasmic vacuolization and destruction of the apical and basement membranes. These morphological observations suggest that increased intestinal epithelial cell autophagic activity may partially underlie the pathogen resistance of *daf-2(e1370)* mutants. Of note, pharyngeal pumping (and predicted bacterial uptake) was not altered in infected or uninfected *daf-2(e1370)* vs. N2 animals, nor in vector control vs. *bec-1*-RNAi *daf-2(e1370)* mutants (Fig. S2).

Autophagy Deficiency Blocks Pathogen Resistance Conferred by DAF-16 Over-Expression. The *daf-2* insulin-like signaling negatively regulates the activity of a forkhead transcription factor, DAF-16 (2), and *daf-16* is required for the pathogen-resistance phenotype of *daf-2* mutants (3). Thus, we postulated that DAF-16 over-expression might induce *atg* gene-dependent pathogen resistance. We introduced, by genetic crossing, the autophagy marker GFP::LGG-1 into TJ356 animals that carry additional *daf-16* gene copies (21) and found that the number of autophagosomes per seam cell was significantly increased in TJ356 animals ($P < 0.0001$, *t*-test) in a manner that is suppressed by *bec-1*-RNAi treatment (Fig. 4 *A* and *B*). We also confirmed that seam cell autophagy was increased in another DAF-16 over-expressing strain, CF1139 (Fig. S3). The increased number of GFP::LGG-1 punctuate dots (autophagosomes) in the seam cells of TJ356 and CF1139 animals is unlikely to be a result of GFP over-expression per se (these strains express a DAF-16::GFP fusion protein) because we did not observe a similar increase in punctuate dots in GFP::LGG-1 animals crossed to another strain, JR667, that over-expresses GFP in seam cells (22) (Fig. S4). Furthermore, EM analyses revealed increased numbers of autolysosomes in the seam cells of TJ356 vs. N2 animals (see representative images in Fig. S5 *A* and *B*).

Next, we examined whether autophagy induction is required for the pathogen resistance of TJ356 animals (23). The *Salmonella* infection survival assay was performed at 25 °C to reproduce published experimental conditions in which pathogen resistance was observed (23). *Salmonella*-infected TJ356 animals lived significantly longer than control N2 wild-type worms (Fig. S5*C* and Table S2), indicating that DAF-16 over-expression confers resistance to *Salmonella* infection. This resistance to *Salmonella* infection was blocked by *atg* gene knockdown; *bec-1* and *lgg-1* RNAi significantly shortened the lifespan of *Salmonella*-infected TJ356 animals but did not shorten the lifespan of *E. coli*-fed control TJ356 animals (Fig. 4 *C* and *D* and Table S1). Similar to N2 animals, EM analyses of *Salmonella*-infected TJ356 animals revealed increased luminal bacteria and decreased lysosomal bacterial degradation in intestinal epithelial cells in *bec-1*-RNAi vs. control animals (Fig. 4*E*). We also examined the pathogen-resistance phenotype of CF1139 animals. *Salmonella*-infected CF1139 animals lived significantly longer than control animals, although the resistance was not as strong as in TJ356 animals, and this pathogen resistance was blocked by both *bec-1* and *lgg-1* RNAi (Table S2). Together, these data indicate that *atg* genes are required for resistance to *Salmonella* both in worms with a loss-of-function mutation in the DAF-2 insulin-like tyrosine kinase receptor or with over-expression of the DAF-16 forkhead transcription factor.

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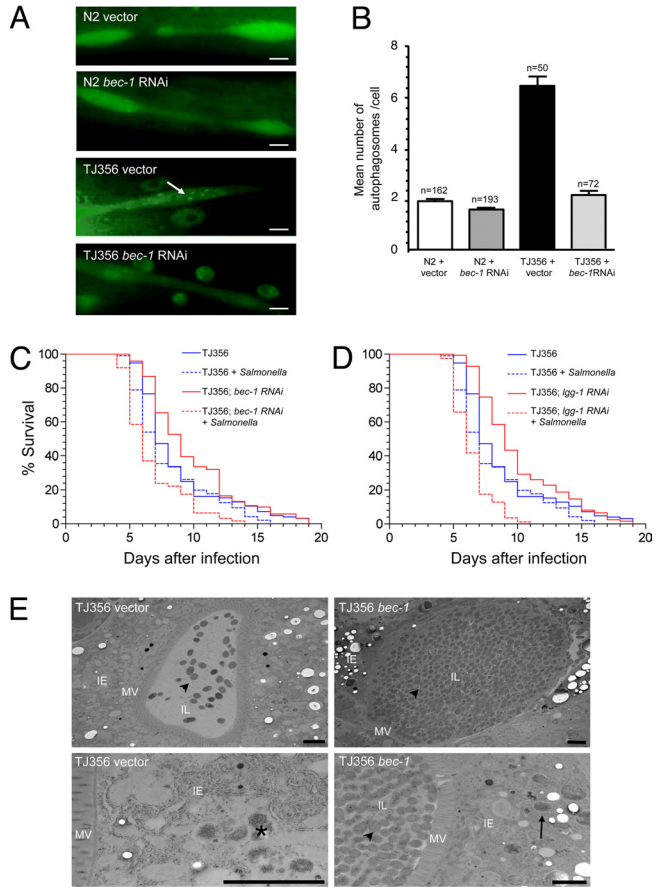

Fig. 4. *Atg* genes are required for DAF-16-mediated resistance to *Salmonella* infection. (*A*) Representative pictures of autophagosomes in seam cells of N2 wild-type and TJ356 animals treated either with RNAi control vector or *bec-1* RNAi. TJ356 is heterozygous for both *gfp*::*lgg-1* and *daf-16*::*gfp*. The oval-shaped green staining near the seam cells in TJ356 animals represent nuclei of intestinal cells that express DAF-16::GFP. The arrow denotes a representative autophagosome. (Scale bars, 2 μM.) (*B*) Quantification of autophagosomes per seam cell (mean $\pm$ SEM) for each genotype. n = number of seam cells per group in $\approx$20 animals. Similar results were obtained in two independent experiments. (*C* and *D*) Survival curves of TJ356 animals treated with either control or indicated *atg* gene RNAi-feeding plasmids following exposure to *S. typhimurium* or normal food at 25 °C. n = number of animals per treatment group. See Table S1 for statistical details. (*E*) Representative EMs of *Salmonella*-infected control and *bec-1*-RNAi TJ356 animals two days after a 2-day *Salmonella* ingestion period. The asterisk denotes a representative autolysosome containing partially degraded bacteria. Arrowheads represent intestinal luminal bacteria and the arrow denotes representative SCV-containing bacteria. (Scale bars, 2 μM.)

Discussion

Our findings demonstrate that *atg* genes play an evolutionarily conserved role in host defense against the mammalian intracellular bacterial pathogen, *S. typhimurium*. In *C. elegans* and *Dictyostelium*, the genetic knockdown or knockout of essential *atg* genes dramatically alters the fate of the invading bacterium and of the host organism. The invading bacterium, rather than being targeted for lysosomal degradation, establishes an intracellular replicative niche, which leads to cellular destruction and premature organismal death. Thus, in these two model organisms, the presence or absence of *atg* genes is sufficient to determine whether *Salmonella* is a successful intracellular pathogen.

In mammals, *Salmonella* is phagocytosed by macrophages and can invade nonphagocytic cells through the activity of the type-III secretion system (24). *Salmonella* replicates intracellularly within a specialized compartment, the SCV, and to be a successful intracellular pathogen, *Salmonella* must avoid fusion of the SCV with the lysosome (24). Our results indicate that in both *Dictyostelium* and *C. elegans*, *Salmonella* invades intracellularly, resides in SCVs, and is targeted to lysosomes for degradation in wild-type but not autophagy-deficient organisms. Therefore, we propose that the autophagic machinery is used by these organisms to deliver *Salmonella* to the lysosome. Autophagy likely plays a similar role in mammalian cells, as previous in vitro studies have shown that autophagy inactivation enhances *Salmonella* replication in macrophages (25). Perhaps a major difference in *Salmonella* infection in mammalian cells (where it replicates intracellularly) versus that in lower eukaryotic organisms (where it cannot replicate intracellularly) may relate to the ability of the bacterium to partially antagonize the host autophagic machinery. This could explain why genetic inactivation of the autophagic machinery in lower eukaryotes (such as *C. elegans* or *Dictyostelium*) mimics *Salmonella* pathogenesis in mammalian cells, in that the bacteria can replicate inside and destroy intestinal epithelial cells and phagocytic cells.

Our findings clearly indicate that *atg* genes function in a cell-autonomous manner, in intestinal epithelial cells in the nematode or in the unicellular organism, *Dictyostelium*, to limit intracellular bacterial replication and cellular destruction. In the presence of gentamicin that kills extracellular bacteria, *Salmonella* only multiplies successfully and only causes cytopathology when *atg* genes are inactivated. However, in *C. elegans* we cannot exclude multifactorial bases for increased bacterial multiplication or for increased animal mortality in autophagy-deficient organisms. We observed a defect in intestinal epithelial cell bacterial lysosomal degradation in *atg* gene RNAi-treated animals, but increased bacterial replication might also result from enhanced epithelial cell bacterial uptake or invasion or impaired intestinal epithelial cell secretion of antimicrobial peptides. Indeed, in autophagy-deficient organisms, we observed an increase in luminal bacteria, and in *daf-2*-mutant animals (which have higher levels of basal autophagy), a decrease in luminal bacteria. Recently, mice defective in intestinal expression of the *atg* genes, *Atg16L1* or *Atg5*, were found to have defects in the secretory function of the Paneth cell, a key cell type involved in gut antimicrobial peptide secretion (10). Interestingly, several genes encoding antibacterial lysozymes were induced in *daf-2* mutants, including 2 intestinally expressed genes, *lys-7* and *lys-8* (26). Furthermore, genes encoding *C. elegans* antimicrobial peptides, *abf-2* and *spp-1*, are induced during *Salmonella* infection and required to limit *Salmonella* infection in the intestinal lumen (27). Thus, it is possible that, in addition to restricting intracellular bacterial multiplication, autophagy is also involved in the production and secretion of antimicrobial peptides that limit intraluminal replication.

The mechanisms by which *atg* gene inactivation increases susceptibility to lethal *Salmonella* infection are not completely understood. Ultrastructural analyses of both *Salmonella*-infected nematodes and slime molds at later time points after infection reveal virtually complete cellular effacement by bacteria. Thus, one speculation is that the high bacterial burden or harmful products secreted by the bacteria leads to cytotoxicity. Although *Salmonella* has been reported to cause apoptotic death of intestinal epithelial cells (28), we did not observe a role for apoptosis in the increased lethality of *Salmonella*-infected autophagy-deficient nematodes. Another possibility is that, in addition to its role in decreasing the intracellular bacterial burden, autophagy, as a prosurvival mechanism, functions to "buffer" the stress imposed by intracellular bacterial infection.

Our findings not only identify an important role for *atg* genes in antibacterial host defense in vivo, they also identify *atg* genes as mediators of the pathogen-resistance phenotype of long-lived mutant nematodes. Pathogen resistance in *daf-2*-mutant animals requires at least two essential *atg* genes: *bec-1* and *lgg-1*. Thus, autophagy is likely a specific innate effector that mediates not only lifespan extension (5–7), but also pathogen resistance in nematodes with mutation in the insulin-like signaling pathway. This connection

between autophagy-mediated lifespan extension and pathogen resistance may be evolutionarily conserved. Perhaps age-related decreases in autophagy that occur in mammals may be mechanistically linked to age-related increases in susceptibility to certain infectious diseases, including those caused by intracellular bacterial pathogens.

One apparent discrepancy between our findings and a previous report (7) relates to the role of DAF-16 in autophagy regulation. In the present study, we found that two different DAF-16 over-expressing strains had increased levels of autophagy. However, Hansen et al. (7) found that a *daf-16* null mutation did not block autophagy induction in *daf-2* mutants. One potential explanation for this discrepancy is that another unidentified protein may function redundantly with DAF-16 in autophagy regulation. This might explain why a *daf-16* null mutation has no effect on autophagy, whereas DAF-16 over-expression induces autophagy. Further studies are required to more clearly delineate the role of DAF-16, and other potential transcription factors downstream of DAF-2, in mediating autophagy and pathogen resistance in *C. elegans*. However, of note, forkhead transcription factors seem to play an evolutionarily conserved role in autophagy induction; FOXO over-expression induces autophagy in *Drosophila* (29) and Foxo3 regulates autophagy in mouse muscle cells (30, 31).

In conclusion, our data demonstrate that autophagy is a critical host-defense mechanism that limits intracellular infection with the bacterial pathogen *S. typhimurium*. Based upon our findings in nematode intestinal epithelial cells and in *Dictyostelium*, we speculate that the autophagic machinery may play a conserved role in protecting mammalian epithelial cells and phagocytes from bacterial attack. Furthermore, our data demonstrate a critical role for autophagy in mediating insulin-like signaling-regulated pathogen resistance in long-lived mutant nematodes. Thus, human *atg* gene polymorphisms (e.g., the Crohn's *ATG16L* variant) or age-related changes that reduce autophagy may contribute to impaired intestinal immunity to bacterial pathogens.

Materials and Methods

***C. elegans, Dictyostelium,* and *Salmonella* Strains.** Wild-type strains were the *C. elegans* Bristol strain N2, the *D. discoideum* strain DH1, and *Salmonella enterica* Serovar Typhimurium ATCC14028s (*S. typhimurium*). All mutant strains have been previously published and are described in the *SI Materials and Methods*. Strains TJ356 and CF1139 were out-crossed with our laboratory strain of N2 before *Salmonella* infection experiments. See details in the *SI Materials and Methods*.

RNAi Methods. *C. elegans* feeding-RNAi experiments were performed as described (32), with the exception that a lower dose (1 nM instead of 1 mM) of IPTG was used to induce expression of *bec-1* RNAi in *daf-2(e1370)*-mutant animals and DAF-16 over-expression strains (TJ356 and CF1139) to avoid decreases in lifespan in uninfected animals. The construction of feeding-RNAi plasmids is described in the *SI Materials and Methods*.

***Salmonella* Infection and Survival Studies.** All *C. elegans* RNAi treatment, *Salmonella* infection, and lifespan experiments were performed at 20 °C unless otherwise indicated, as described in detail in the *SI Materials and Methods*. All survival experiments were repeated at least two times, and the results shown represent an analysis of the combined data for the total number of animals per experimental group in all experiments. The results of all individual experiments were similar to that of the combined data. The mean lifespans, total number of worms per group, total censored animals, and survival statistical analyses are listed in Tables S1 and S2. See the *SI Materials and Methods* for details of statistical methods. *Salmonella* infection and survival studies in *Dictyostelium* were performed as described in the *SI Materials and Methods*.

Measurement of Bacterial Growth. See the *SI Materials and Methods* for further details of bacterial growth measurement.

Microscopic Analyses. For transmission EM of *C. elegans*, ≈100 adult nematodes per experimental group were collected and processed as previously described (5). Transmission EM analysis of *Dictyostelium* was performed as previously described (33). Light microscopic analyses of fluorescent nematodes and amoebae were performed using a Zeiss Axioplan2 Imaging microscope.

Autophagy Induction Analysis. N2- and *daf-2(e1370)*-transgenic strains carrying the *gfp::lgg-1* autophagy marker were described previously (5) and similar methods were used to measure seam cell autophagy in young adults (i.e., within 12 h beyond the L4 stage). The generation of TJ356 and CF1139 animals carrying the *gfp::lgg-1* autophagy marker is described in the *SI Materials and Methods*.

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