Supporting Information

Jia et al. 10.1073/pnas.0813319106

SI Materials and Methods

***C. elegans* and *D. discoideum* Mutant Strains.** Strains obtained from *Caenorhabditis* Genetics Center include JR667 [*unc-119(e2498*::*Tcl)* III; *wls51[SCM*::*gfp,unc-119(+)]*], CF1139 [*daf-16(mu86)* I; muIs61], and CB1370 [*daf-2(e1370)*]. TJ356 (1) was provided by A. Aballay (Duke University Medical Center) and MT1522 [*ced-3(n717)*] (2) was provided by S. Cameron (University of Texas Southwestern Medical Center). The *D. discoideum* strains (provided by R. Kessin, Columbia University College of Physicians and Surgeons, New York), DH1 (wild-type), HR79 (*atg7* mutant), HR131 (*atg1* mutant), HR174 (*atg6* mutant), and GFP-*Atg8* transgenics have been previously described (3, 4). TJ356 animals were out-crossed four times to our laboratory's N2 strain. N2 males were crossed to TJ356 hermaphrodites. The roller male progeny were picked up, crossed to N2 hermaphrodites, and the roller hermaphrodites from these crossed progeny were picked up. This crossing process was repeated 1 time with new TJ356 animals that were out-crossed two times to obtain TJ456 animals that were back-crossed to N2 animals for four generations. A similar strategy was used to out-cross CF1139 animals, with the exception that CF1139 male segregants instead of N2 males were used for the first out-cross. Therefore, the final CF1139 train was out-crossed for three generations to our laboratory's N2 strain.

***C. elegans* RNAi Methods.** The cDNA fragments corresponding to sequences of *bec-1*, *lgg-1*, and *atg-7* were cloned into the RNAi vector L4440, and the resulting plasmids were transformed into HT115 *E. coli* bacteria. The primer sequences used to amplify *bec-1*, *lgg-1*, and *atg7* are: *bec-1* forward, 5′ATGACGACCCAACGAAGCCATA-3′; *bec-1* reverse, 5′-CTAAATAGGCGATCTGAGAGCAT-3′; *lgg-1* forward, 5′-ATGGCCACG-TTTGTTCCCTTTG-3′; *lgg-1* reverse, 5′-GCCAGCGAGCTTCCCTTGAAT-3′; *atg7* forward, 5′-ATGGCCACG-TTTGTTCCCTTTG-3′; *atg7* reverse, 5′-GCCAGCGAGC-TTCCCTTGAAT-3′. *unc-22* and *him-3* RNAi plasmids were provided by S. Cameron (University of Texas Southwestern Medical Center). To perform RNAi at 20 °C, L4 hermaphrodites were put on RNAi and control plates for 36 h. The worms were then transferred to fresh RNAi and control plates, respectively. After 24 h, the parental worms were removed and the progeny were allowed to develop. The L4 hermaphrodites of RNAi-treated progeny were collected for experiments. These animals were on RNAi during development. The procedures for RNAi at 15 °C were similar except that the incubation time was extended at each step as described by Kamath et al. (5).

***Salmonella* Infection and Survival Studies.** For experiments involving N2, *daf-2(e1370)*, and CF1139 nematodes, RNAi-treated L4 stage nematodes were transferred to NGM agar plates containing 100 μl of *S. typhimurium* that had been cultured overnight in LB broth and incubated on plates for 6 h at room temperature. After 48 h of infection, animals were transferred to fresh feeding RNAi or vector-control plates, and survival was scored daily. Infection of TJ356 animals was performed following the description by Singh and Aballay (6). RNAi treatment was conducted at 15 °C. RNAi-treated and control L4 worms were collected and maintained at 15 °C for 24 h, and then shifted to 25 °C and placed on *Salmonella* plates prepared as described above for the remainder of the experiment. The RNAi treatment of *daf-2(e1370)* animals was performed at 15 °C. For survival assays, animals were transferred to fresh plates every day during the reproductive period, and every other day thereafter. Animals were scored as dead when they failed to respond to touch. Animals that died from internal hatching or vulval rupture were excluded from analysis.

For *Dictyostelium Salmonella* infection and survival studies, wild-type or autophagy gene-mutant *D. discoideum* cells were infected with *S. typhimurium* at a multiplicity of infection of 4 CFUs per cell for 3 h. At the end of 3 h, *Dictyostelium* were washed, incubated for 30 min in media containing 50-μg/ml gentamicin, and then maintained in media containing 5-μg/ml gentamicin to kill extracellular bacteria. At serial time points after infection, cell viability was determined by trypan blue exclusion, using at least 100 cells per genotype per time point. *S. typhimurium* harboring eGFP or eCFP were visualized by differential interference contrast and epifluorescence microscopy.

Measurement of Bacterial Growth. At serial time points after infection, ≈10 nematodes per experimental group were washed with M9 buffer, mechanically disrupted, and the disruption solution was plated on *Salmonella*-selective XLD agar plates (Xylose Lysine Desoxycholate, EMD Chemical Inc.). For gentamicin treatment, infected animals were washed three times with 1-ml M9 buffer. The washed worms were then transferred to 500-μl M9 buffer containing 100-μg/ml gentamicin and incubated at room temperature for 2 h. After gentamicin treatment, the animals were transferred to fresh feeding RNAi or vector-control plates and bacterial growth were measured as described above. For measurement of bacterial growth in *Dictyostelium*, *Dictyostelium* were infected as described in the above section on "*Salmonella* Infection and Survival Studies," and at serial time points after infection, intracellular bacteria titers were determined by plating cell lysates on LB agar.

Autophagy Induction and Strain Construction. N2 roller males carrying the *gfp*::*lgg-1* autophagy marker were crossed with TJ356 L4 hermaphrodites. When a large percentage of male progeny was observed, L4 hermaphrodites of cross progeny were collected, mounted on an agar pad, and examined under a stereomicroscope equipped for epifluorescence. The animals showing a GFP expression pattern of both *gfp*::*lgg-1* and *daf-16*::*gfp* were recovered and put on RNAi and control plates. The RNAi-treated L4 stage progeny were then examined for the expression of both *gfp*::*lgg-1* and *daf-16*::*gfp*, and the GFP punctate dots corresponding to autophagosomes in the seam cells were counted at 1,000× magnification. Five to 10 seam cells were scored for each animal and approximately 20 animals were examined for each strain in each experiment. The animals used for counting autophagosomes were heterozygous for both *gfp*::*lgg-1* and *daf-16*::*gfp*. The same strategy was used to count autophagosomes in CF1139 and JR667 animals.

Pharyngeal Pumping Assay. Forty *bec-1*-RNAi and control RNAi-treated L4 animals were transferred to fresh RNAi plates and control plates. The pumping of 20 randomly chosen animals was counted under a dissecting microscope at 100× magnification. For day-1- and day-2-old animals, 40 *bec-1*-RNAi and control RNAi-treated L4 animals were kept on either *S. typhimurium* plates or RNAi or control-RNAi plates for two days. On each day, 20 animals were randomly chosen and their pumping was scored under a dissecting microscope at 100× magnification. The same method was used to examine the effect of *bec-1* RNAi on pharyngeal pumping of *daf-2(e1370)*-mutant animals.

Statistical Analyses of Survival Data. The Kaplan-Meier method was used to estimate survival curves. The log-rank test was used to determine statistical significance of differences in survival between two groups and Cox proportional-hazards models were used to compare survival times and estimate hazard (risk) ratios among several groups. Analyses were performed with GraphPad Prism 5 (GraphPad Software, Inc.) and SAS 9.1.3 (SAS Institute).

1. Henderson ST, Johnson TE (2001) *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans. Curr Biol* 11(24):1975–1980.
2. Ellis HM, Horvitz HR (1986) Genetic control of programmed cell death in the nematode *C. elegans*. *Cell* 44(6):817–829.
3. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH (2003) Macroautophagy is required for multicellular development of the social amoeba *Dictyostelium discoideum. J Biol Chem* 278(20):17636–17645.
4. Otto GP, Wu MY, Kazgan N, Anderson OR, Kessin RH (2004) *Dictyostelium* macroautophagy mutants vary in the severity of their developmental defects. *J Biol Chem* 279:15621–15629.
5. Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans. Genome Biol*, 10.1186/gb-2000-2-1-research0002.
6. Singh V, Aballay A (2006) Heat-shock transcription factor (HSF)-1 pathway required for *Caenorhabditis elegans* immunity. *Proc Natl Acad Sci USA* 103(35):13092–13097.

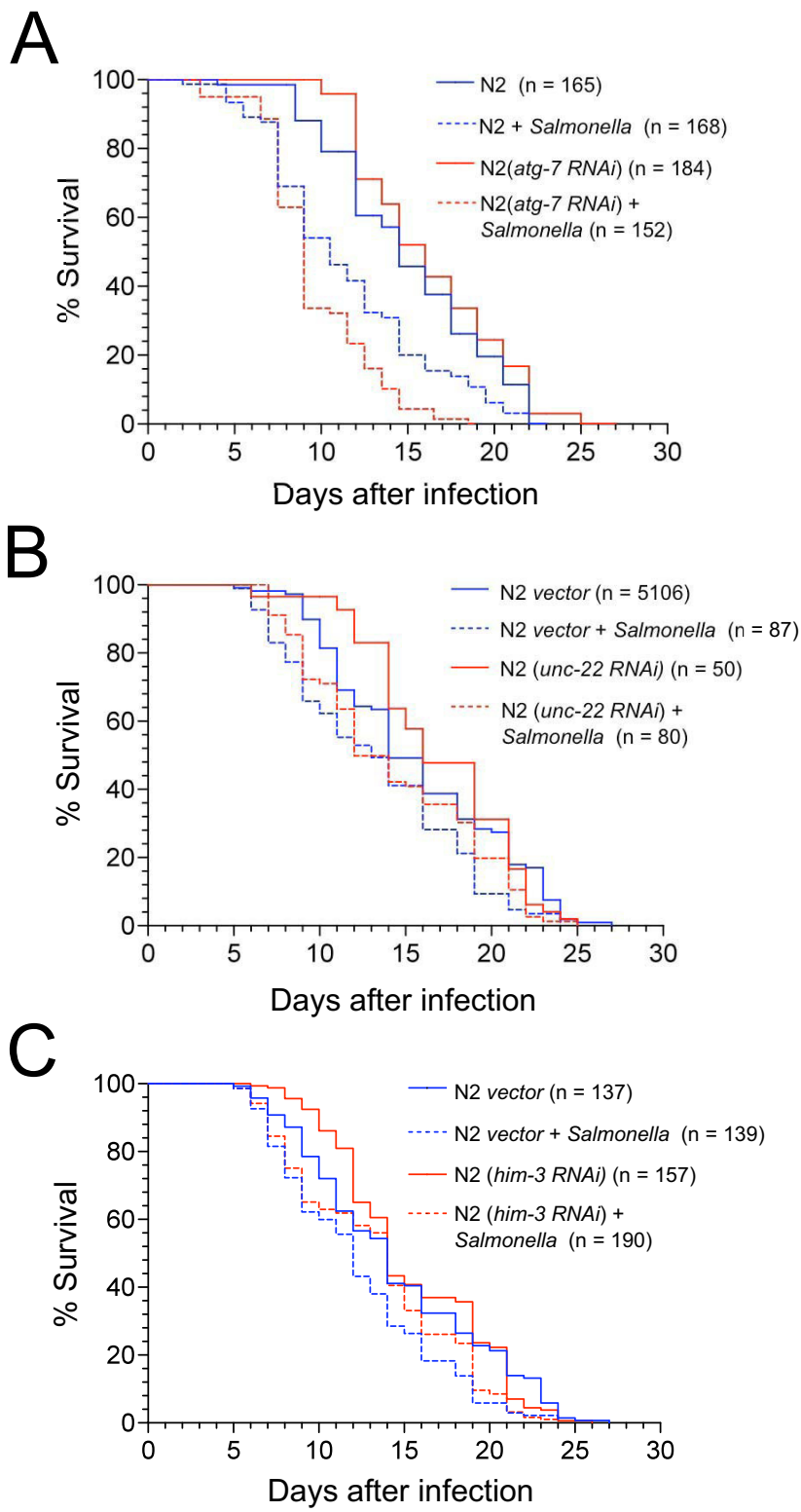

Fig. S1. Effect of different RNAi treatments on *C. elegans* susceptibility to lethal *Salmonella* infection. Survival curves of wild-type (N2) animals treated with either control vector or *atg-7* RNAi-feeding plasmids (*A*), control vector or *unc-22* RNAi-feeding plasmids (*B*), or control vector or *him-3* RNAi-feeding plasmids (*C*) following exposure to *S. typhimurium* or normal food. *n* = number of animals per treatment group. See Table S2 for statistical details.

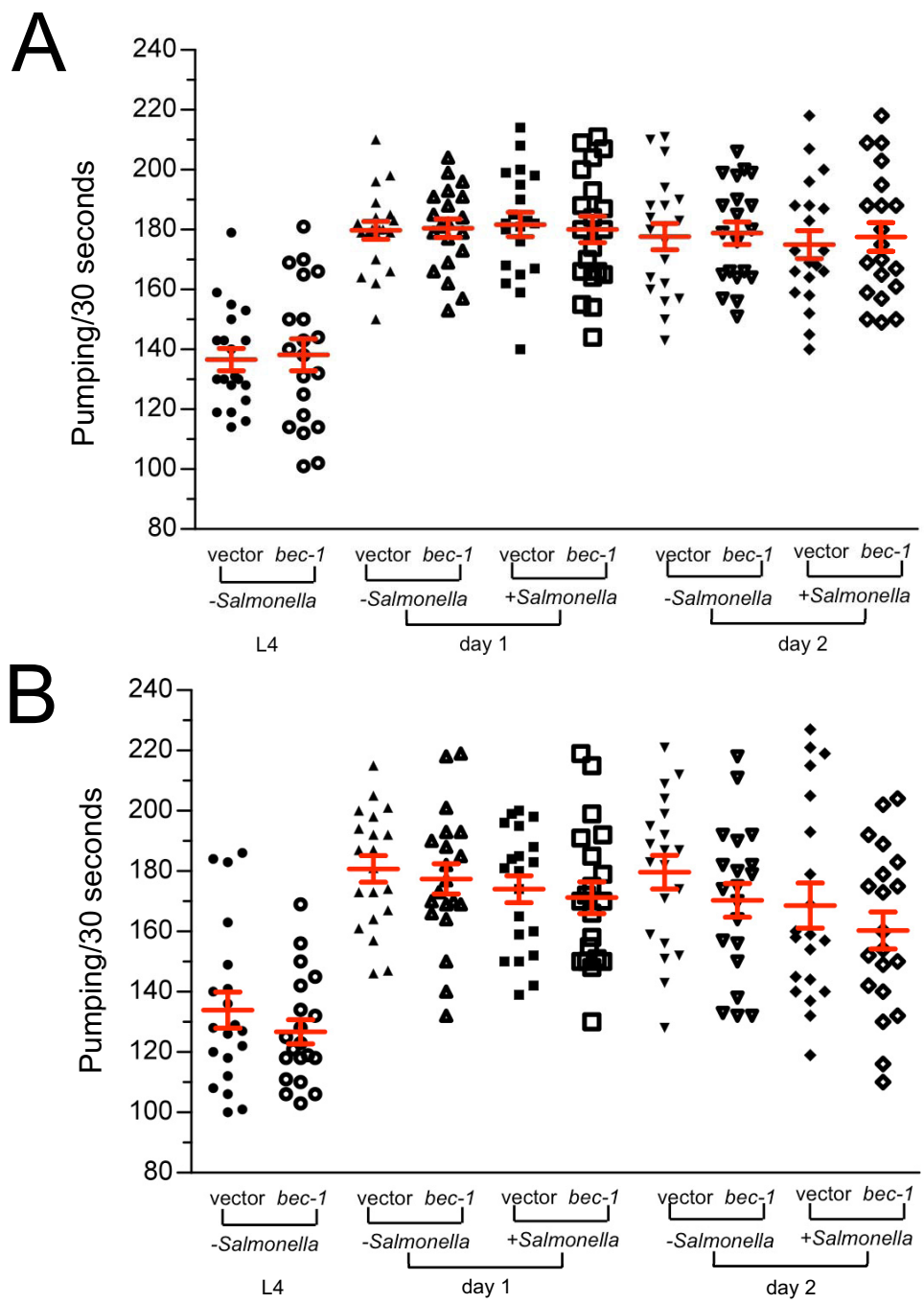

Fig. S2. Lack of effect of *Salmonella* infection or *bec-1* RNAi treatment on pharyngeal pumping of *C. elegans* wild-type animals (N2) and *daf-2(e1370)* animals. The pumping rate of 20 animals in each group was counted, and a *t*-test was performed to determine statistical significance. (*A*) Pharyngeal pumping of N2 animals. For the non-*Salmonella* infection group, for *bec-1* RNAi vs. vector, $P = 0.81$ at the L4 stage, $P = 0.78$ at Day 1, and $P = 0.84$ at Day 2. For the *Salmonella*-infected group, for *bec-1* RNAi vs. vector, $P = 0.78$ at Day 1, and $P = 0.71$ at Day 2. (*B*) Pharyngeal pumping of *daf-2* animals. For the non-*Salmonella* infection group, for *bec-1* RNAi vs. vector, $P = 0.32$ at the L4 stage, $P = 0.63$ at Day 1, and $P = 0.24$ at Day 2. For the *Salmonella*-infected group, for *bec-1* RNAi vs. vector, $P = 0.69$ at Day 1, *and* $P = 0.39$ at Day 2. There is no statistical difference between the pharyngeal pumping rates of vector-control treated N2 and *daf-2* animals. For the non-*Salmonella* infection group, for *daf-2* vs. N2, $P = 0.70$ at the L4 stage, $P = 0.86$ at Day 1, and $P = 0.77$ at Day 2. For the *Salmonella*-infected group, for *daf-2* vs. N2, $P = 0.21$ at Day 1, and $P = 0.47$ at Day 2.

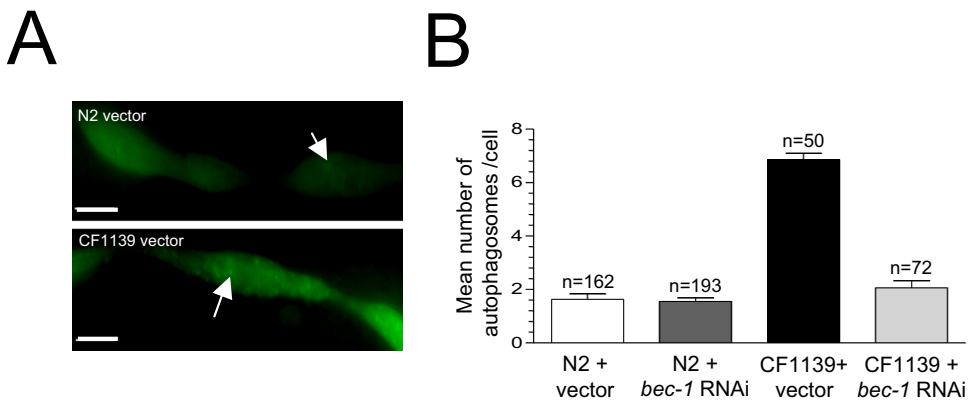

Fig. S3. Autophagy is induced in CF1139 animals that over-express DAF-16. (*A*) Representative pictures of autophagosomes in seam cells of animals that are heterozygous for both *gfp*::*lgg-1* and *daf-16*::*gfp*. Arrows denote representative autophagosomes. (Scale bars, 2 μM.) (*B*) *bec-1* RNAi suppresses autophagy induction induced by DAF-16 over-expression. 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.

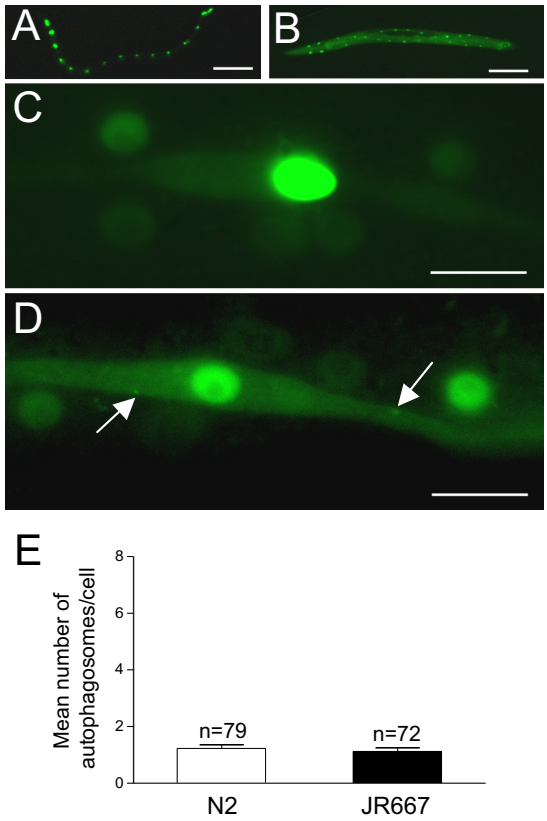

Fig. S4. Lack of effect of seam cell over-expression of GFP on autophagosome accumulation. (*A*–*D*) Representative images showing JR667 animals expressing seam cell GFP protein (*A* and *C*) and seam cell GFP protein plus GFP::LGG-1 (*B* and *D*). Arrows denote representative autophagosomes. (Scale bars for *A* and *B*, 100 μm; for *C* and *D*, 2 μM.) (*E*) 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 observed in two independent experiments.

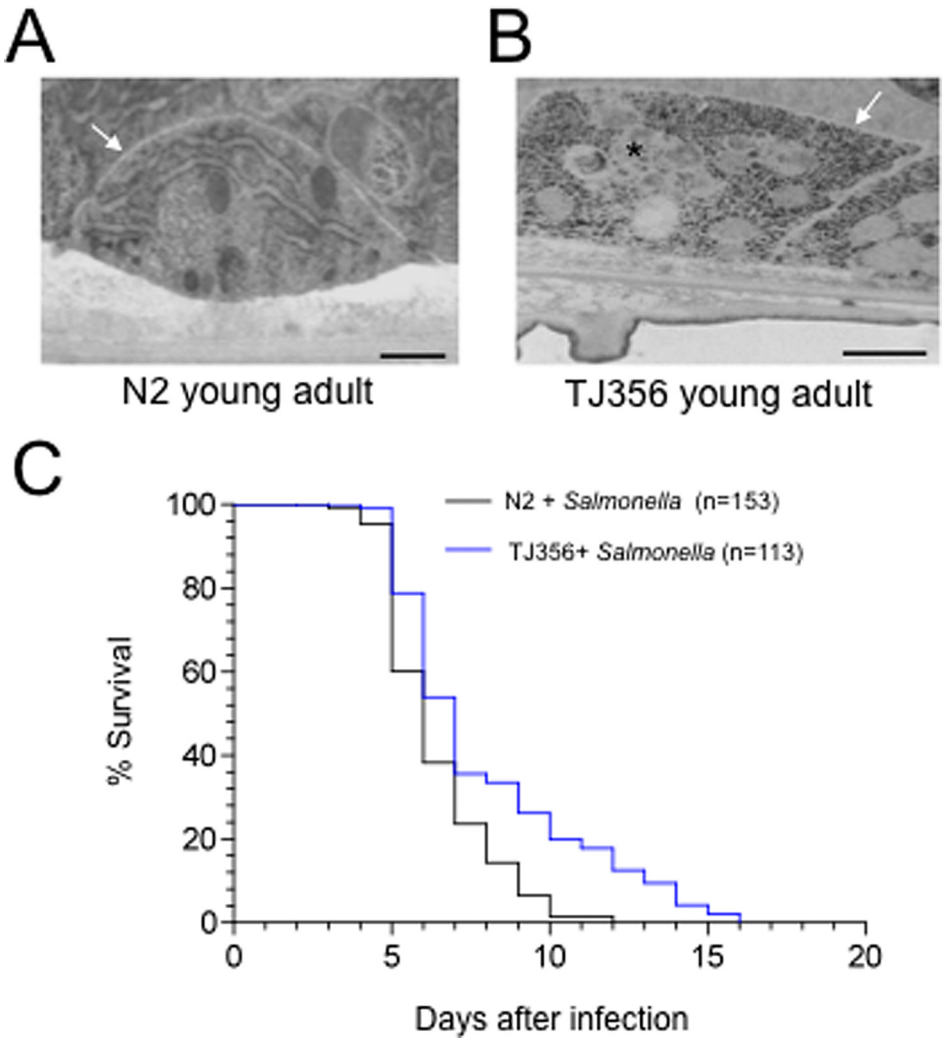

Fig. S5. Over-expression of DAF-16 induces autophagy and confers pathogen resistance. (*A* and *B*) Representative electron micrographs of seam cells in young-adult stage N2 and TJ356 animals. The asterisk denotes representative autolysosome. Arrows denote seam cells. (Scale bars, 2 μM.) (*C*) Survival curves of N2 and TJ356 animals infected with *S. typhimurium* at 25 °C. *n* = number of animals per genotype. See Table S2 for more detailed analyses of lifespan experiments.

Table S1. Statistical analysis of lifespan data in Figs. 1–5

Strain	Mean lifespan[a]	Number/censored[b]	*P* value[c]
N2 vector	15.4	165/7	
N2 *bec-1*	14.7	184/8	<0.01
N2 vector + *Salmonella*	12.6	168/27	
N2 *bec-1* + *Salmonella*	8.6	152/21	<0.0001
N2 vector	15.8	200/15	
N2 *lgg-1*	15.7	219/13	0.96
N2 vector + *Salmonella*	11.9	200/15	
N2 *lgg-1* + *Salmonella*	10.3	190/49	<0.0001
N2 vector	16.5	126/10	
ced-3 vector	15.6	71/9	0.01
ced-3 bec-1	16.3	69/3	0.20
N2 vector + *Salmonella*	11.1	158/18	
ced-3 vector + *Salmonella*	12.9	81/13	0.01
ced-3 bec-1 + *Salmonella*	8.9	97/8	<0.0001
daf-2 vector[d]	30.1	74/18	
daf-2 bec-1[d]	25.6	57/79	<0.01
daf-2 vector + *Salmonella*[d]	24.8	73/32	
daf-2 bec-1 + *Salmonella*[d]	13.0	40/275	<0.0001
daf-2 vector[d]	31.3	42/29	
daf-2 lgg-1[d]	32.5	49/11	0.71
daf-2 vector + *Salmonella*[d]	29.2	42/29	
daf-2 lgg-1 + *Salmonella*[d]	19.3	57/15	<0.0001
TJ356 vector[e]	8.7	134/39	
TJ356 *bec-1*[e]	9.7	126/25	0.02
TJ356 vector + *Salmonella*[e]	7.9	113/126	
TJ356 *bec-1* + *Salmonella*[e]	6.7	114/91	<0.0001
TJ356 vector[e]	8.7	134/39	
TJ356 *lgg-1*[e]	9.9	127/32	<0.01
TJ356 vector + *Salmonella*[e]	7.9	113/126	
TJ356 *lgg-1* + *Salmonella*[e]	6.4	117/104	<0.0001

[a]Mean adult lifespan in days. Two independent trials were pooled.

[b]Number denotes population size. Censored animals were not included in the statistical analysis. Most of these animals died of internal hatching. Others were either lost during experiment or died of vulva rupture.

[c]*P* values (log-rank test) for mean life span of the entire population compared to corresponding control.

[d]Cox proportional-model hazard ratios were 1.74 (95% CI: 1.21, 2.50) for *bec-1* RNAi- vs. control vector-treated uninfected *daf-2(e1370)* animals ($P = 0.003$), 6.87 (95% CI: 4.18, 11.29) for *bec-1* RNAi- vs. control vector- treated *Salmonella*-infected *daf-2(e1370)* animals ($P < 0.0001$), 0.93 (95% CI: 0.61, 1.41) for *lgg-1* RNAi- vs. control vector-treated uninfected *daf-2(e1370)* animals (P = NS), and 2.82 (95% CI: 1.82, 4.37) for *lgg-1* RNAi- vs. control vector-treated *Salmonella*-infected *daf-2(e1370)* animals ($P < 0.0001$).

[e]The *Salmonella* infection survival assay was performed at 25°C, as previously described (6), in order to mimic published experimental conditions in which pathogen resistance was observed.

Table S2. Statistical analysis of lifespan data in the SI figures

Strain	Mean lifespan[a]	Number/censored[b]	*P* value[c]
N2 vector	15.9	63/6	
N2 *atg-7*	17.1	67/7	0.13
N2 vector + *Salmonella*	12.1	67/10	
N2 *atg-7* + *Salmonella*	10.4	70/10	<0.01
N2 vector	15.7	106/8	
N2 *unc-22*	16.9	50/8	0.55
N2 vector + *Salmonella*	13.3	87/12	
N2 *unc-22* + *Salmonella*	14.2	80/11	0.18
N2 vector	14.5	137/13	
N2 *him-3*	15.4	157/9	0.20
N2 vector + *Salmonella*	12.3	139/12	
N2 *him-3* + *Salmonella*	13.3	190/22	0.05
N2 vector + *Salmonella*[d]	6.4	153/49	
TJ356 vector + *Salmonella*[d]	7.9	113/126	<0.0001
N2 vector + *Salmonella*[d]	8.3	187/34	
CF1139 vector + *Salmonella*[d]	8.9	193/45	0.04
CF1139 vector[d]	10.7	218/19	
CF1139 *bec-1*[d]	11.8	186/21	<0.01
CF1139 vector + *Salmonella*[d]	8.9	193/45	
CF1139 *bec-1* + *Salmonella*[d]	7.03	199/20	<0.0001
CF1139 vector[d]	10.7	218/19	
CF1139 *lgg-1*[d]	10.4	185/16	0.39
CF1139 vector + *Salmonella*[d]	8.9	193/45	
CF1139 *lgg-1* + *Salmonella*[d]	6.8	192/34	<0.0001

[a]Mean adult lifespan in days. Two independent trials were pooled.
[b]Number denotes population size followed by number of animals censored from analysis. Most of the censored animals died of internal hatching. Others were either lost during the experiment or died of vulva rupture.
[c]*P* values (log-rank test) for mean life span of the entire population compared to corresponding control.
[d]The *Salmonella* infection survival assay was performed at 25°C, as previously described (6), in order to mimic published experimental conditions in which pathogen resistance was observed.