Developmental Cell
Article

Unfolded Protein Response Genes Regulated by CED-1 Are Required for *Caenorhabditis elegans* Innate Immunity

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DOI 10.1016/j.devcel.2008.05.006

SUMMARY

The endoplasmic reticulum stress response, also known as the unfolded protein response (UPR), has been implicated in the normal physiology of immune defense and in several disorders, including diabetes, cancer, and neurodegenerative disease. Here, we show that the apoptotic receptor CED-1 and a network of PQN/ABU proteins involved in a noncanonical UPR response are required for proper defense to pathogen infection in *Caenorhabditis elegans*. A full-genome microarray analysis indicates that CED-1 functions to activate the expression of *pqn/abu* genes. We also show that *ced-1* and *pqn/abu* genes are required for the survival of *C. elegans* exposed to live *Salmonella enterica*, and that overexpression of *pqn/abu* genes confers protection against pathogen-mediated killing. The results indicate that unfolded protein response genes, regulated in a CED-1-dependent manner, are involved in the *C. elegans* immune response to live bacteria.

INTRODUCTION

The first line of defense against pathogens is the phylogenetically ancient innate immune system. Activation of the innate immune system upon pathogen recognition triggers intracellular signals that result in a rapid and definitive microbicidal response to invading microorganisms (Akira et al., 2006). Another key aspect of the metazoan response to pathogen infection is the activation of a primitive apoptotic genetic program (Yuan, 2006). Interestingly, the microbicidal and apoptotic processes may be highly related since the acute endoplasmic reticulum (ER) stress induced by the immune response leads to an unfolded protein response (UPR) or apoptosis when ER function cannot be restored (Hoyer-Hansen and Jaattela, 2007).

The nematode *Caenorhabditis elegans*, which lives in the soil, where it is in contact with soil-borne microbes, has evolved mechanisms to recognize different pathogens and to respond accordingly. *C. elegans*' defense against pathogen infections requires interacting pathways that control stress response, aging, and immunity (Garsin et al., 2003; Kim et al., 2002; Singh and Aballay, 2006b). These pathways regulate the expression of a wide variety of genes, including those encoding conserved immune effectors such as antimicrobial peptides, lectins, and lysozymes (Kerry et al., 2006; Mallo et al., 2002; O'Rourke et al., 2006; Shapira et al., 2006; Troemel et al., 2006). The *C. elegans* response to pathogen infection also involves an apoptotic pathway (Aballay and Ausubel, 2001; Aballay et al., 2003). Using a set of *C. elegans* mutants in which apoptosis is blocked, it was shown that infection by the human pathogen *Salmonella enterica* results in the activation of germline cell death, which is dependent on the well-characterized CED-9/CED-4/CED-3 pathway, homologous to the BCL2/APAF-1/CASPASE pathway in mammals. Moreover, *ced-3(lf)* and *ced-4(lf)* mutants were found to be hypersensitive to *S. enterica*-mediated killing, suggesting that the apoptotic pathway may be involved in a *C. elegans* defense response to pathogen attack (Aballay and Ausubel, 2001). In addition, taking advantage of both host and pathogen mutants, it was shown that *S. enterica* lipopolysaccharide acts as a pathogen-associated molecular pattern that triggers programmed cell death in *C. elegans* (Aballay et al., 2003). Similar to the homologous pathway in mammals, the pathogen-induced CED-3 pathway in *C. elegans* appears to lie downstream of a PMK-1/P38 MAPK signaling pathway (Aballay et al., 2003).

In addition to the CED pathway that controls apoptosis, two other converging CED pathways (Chung et al., 2000; Ellis et al., 1991; Gumienny et al., 2001; Henson, 2005; Kinchen et al., 2005) are known to control the process of the engulfment of dying cells and to promote apoptosis (Hoeppner et al., 2001; Reddien et al., 2001). With the exception of CED-1 and CED-7, the remaining components of the engulfment pathways appear to act intracellularly (Gumienny et al., 2001; Liu and Hengartner, 1998; Reddien and Horvitz, 2000; Wu and Horvitz, 1998; Wu et al., 2001; Yu et al., 2006; Zhou et al., 2001a). CED-7, an ABC transporter and homolog of ABCA1, is essential for the recognition of cell corpses by CED-1 (Zhou et al., 2001b). CED-1, a phagocytic receptor that recognizes cell corpses and initiates their engulfment (Zhou et al., 2001b), is a single-pass transmembrane receptor that contains various extracellular EGF repeats and an intracellular candidate signaling domain.

Here, we examine the potential role of CED-1 in *C. elegans* immunity. We found that loss-of-function *ced-1* mutants are immunocompromised animals and are rapidly killed by live bacteria. Full-genome expression analyses demonstrated that CED-1 upregulates a family of genes encoding proteins with prion-like glutamine/asparagine (Q/N)-rich domains, which are known to be activated by ER stress and are thought to aid in the UPR

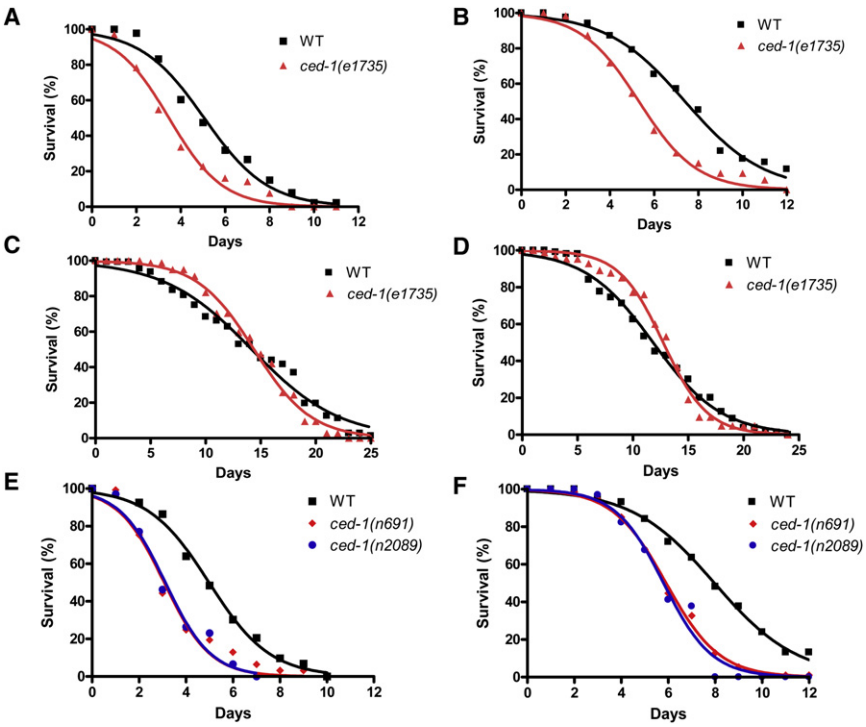

Figure 1. *ced-1* Loss-of-Function Mutants Are Immunocompromised Animals Killed by Live Bacteria

(A) Wild-type and *ced-1(e1735)* animals were exposed to live *S. enterica*: *ced-1(e1735)*, $p < 0.0001$.

(B) Wild-type and *ced-1(e1735)* animals were exposed to live *E. coli*: *ced-1(e1735)*, $p < 0.0001$.

(C) Wild-type and *ced-1(e1735)* animals were exposed to heat-killed *S. enterica*: *ced-1(e1735)*, $p > 0.1$.

(D) Wild-type and *ced-1(e1735)* animals were exposed to heat-killed *E.* coli: *ced-1(e1735)*, $p > 0.1$.

(E) Wild-type, *ced-1(n691)*, and *ced-1(n2089)* animals were exposed to live *S. enterica*: *ced-1(n691)*, $p < 0.0001$; *ced-1(n2089)*, $p < 0.0001$.

(F) Wild-type, *ced-1(n691)*, and *ced-1(n2089)* animals were exposed to live *E. coli*: *ced-1(n691)*, $p < 0.0001$; *ced-1(n2089)*, $p < 0.0001$.

For each condition, 110–140 animals were used, with the exception of *ced-1(n2089)*, for which 35 animals were used. p values are relative to wild-type animals.

(Urano et al., 2002). When expression of these genes was abrogated, the animals exhibited a wild-type life span when exposed to dead bacteria, but showed a reduced life span when exposed to live bacteria. These studies indicate that CED-1 is required for the transcriptional activation of an UPR pathway required for proper response to bacterial infections.

RESULTS

ced-1 Loss-of-Function Mutants Are Immunocompromised Animals and Are Rapidly Killed by Live Bacteria

To study the role of CED-1 in the *C. elegans* defense response, we first examined whether *ced-1(e1735)* mutants were susceptible to *S. enterica*-mediated killing. As shown in Figure 1A, *ced-1(e1735)* loss-of-function mutants (Hedgecock et al., 1983; Zhou et al., 2001b) died more quickly than wild-type animals when feeding on *S. enterica* strain 1344. The time for 50% of the nematodes to die (TD50) when fed at 25°C on live *S. enterica* was 5.09 ± 0.17 days for wild-type animals compared to 3.47 ± 0.31 days for *ced-1(e1735)* mutants, which represents a reduction of 32%. *ced-1(e1735)* animals also exhibited a 27% reduced life span when grown on live *E. coli* strain OP50 (Figure 1B). The short life span exhibited by *ced-1(e1735)* animals fed live *E. coli* is consistent with the observations that proliferating *E. coli* is a cause of death in *C. elegans* (Garigan et al., 2002), that *E. coli* grown on rich media kills *C. elegans* (Garsin et al., 2001), and that immunocompromised animals are killed and persistently colonized by *E. coli* (Kerry et al., 2006; Singh and Aballay, 2006b; Tenor and Aballay, 2008). To ensure that the difference in mortality between *ced-1(e1735)* and wild-type animals was not caused by a reduction in the overall health of the mutant, we exposed *ced-1(e1735)* and wild-type animals to both heat-killed *S. enterica* and heat-killed *E. coli*. When *ced-1(e1735)* mutants were grown on dead *S. enterica* or dead *E. coli*, they exhibited a life span comparable to that of wild-type animals (Figures 1C and 1D). Thus, *ced-1(e1735)* mutants are killed by live, but not dead, bacteria, indicating that they are immunocompromised.

We confirmed that CED-1 is required for *C. elegans*' survival on live bacteria by exposing two additional *ced-1* mutants to *S. enterica* and *E. coli* and comparing their life spans to that of wild-type animals (Figures 1E and 1F). The enhanced susceptibility to live *S. enterica* and live *E. coli* of *ced-1(n691)* and *ced-1(n2089)* mutants, which carry frameshift and missense mutations, respectively (Zhou et al., 2001b), confirms that CED-1 is required for the defense response to live bacteria. Furthermore, these results make it unlikely that the enhanced susceptibility of *ced-1* animals is caused by secondary mutations or the effect of a particular allele on a process unrelated to CED-1 function.

CED-1 Regulates *pqn/abu* Unfolded Protein Response Genes

To determine the mechanism underlying CED-1-mediated defense to live bacteria, we utilized Affymetrix GeneChip *C. elegans* Genome Arrays and hierarchical clustering to find clusters of genes commonly upregulated or downregulated in *ced-1(e1735)* mutants relative to wild-type animals grown on live *E. coli*. Hierarchical clustering identified a family of 10 *pqn/abu* genes in a 17-gene cluster of transcripts that were similarly downregulated in *ced-1(e1735)* mutants (Table 1).

The *pqn* (prion-like glutamine[Q]/asparagine[N]-rich domain-bearing protein) genes constitute a 79-member family characterized by prion-like Q/N-rich amino acid sequences. Eleven genes in the *pqn* family have been further classified as *abu* (activated in blocked unfolded protein response) (Urano et al., 2002). The *abu*

Table 1. Cluster of *C. elegans* CED-1-Regulated Genes

ORF Name	Gene Name	Expression in Wild-Type (WT)	Expression in *ced-1 (lf)* Mutant (C)	Fold Difference WT/C
D2096.6	D2096.6	1492.22 ± 149.65	81.67 ± 35.23	18.27
ZK1067.7	*pqn-95*	4087.87 ± 316.15	172.50 ± 62.85	23.70
F35A5.3	*abu-10*	1248.88 ± 371.91	62.96 ± 3.86	21.44
AC3.3/AC3.4	*abu-1/pqn-2*	1655.47 ± 331.13	60.20 ± 16.15	27.49
W02A2.3	*pqn-74*	2374.52 ± 483.42	89.93 ± 17.52	26.40
C03A7.8/C03A7.14	*abu-7/abu-8*	4137.28 ± 1144.27	101.25 ± 41.58	40.86
C03A7.7	*abu-6*	3501.07 ± 974.25	87.78 ± 44.75	39.89
F41E6.11	F41E6.11	1118.84 ± 168.85	71.95 ± 1.48	15.55
W08E12.4/W08E12.5	W08E12.4/W08E12.5	2934.21 ± 313.82	238.26 ± 245.54	12.32
Y47D3B.6	Y47D3B.6	2602.44 ± 411.67	107.46 ± 18.78	24.22
R09B5.5	*pqn-54*	1145.01 ± 316.23	41.05 ± 1.96	27.89
T01D1.6	*abu-11*	1372.46 ± 273.74	65.21 ± 9.69	21.05
T05B4.3	*phat-4*	1654.16 ± 140.24	68.66 ± 0.14	24.10
F20B10.3	F20B10.3	632.81 ± 132.49	46.21 ± 11.39	13.69
ZK662.2	ZK662.2	2173.22 ± 170.18	123.65 ± 46.22	17.58
C03A7.14	*abu-8*	4242.83 ± 1060.59	89.41 ± 36.81	47.45
C03A7.4	*pqn-5*	4765.04 ± 906.42	85.26 ± 43.12	55.89

Shown are the mean ± error of expression levels in wild-type (n = 3) and *ced-1(e1735)* (n = 2) animals. Eggs were placed in S basal to hatch overnight, causing growth arrest in L1. Synchronized L1 wild-type and *ced-1(e1735)* animals were grown on *E. coli* at 25°C for 26 hr, and RNA was isolated. Expression data were normalized, and cluster of CED-1-regulated genes was performed as described in the Experimental Procedures.

genes were found to be upregulated upon ER stress in an *xbp-1* mutant, which is defective in the canonical UPR (Urano et al., 2002). In addition, the *abu* genes are believed to encode UPR proteins, either functioning in a pathway parallel to the canonical UPR or in the ER-associated degradation of misfolded proteins (Urano et al., 2002).

Further studies revealed that 9 of the *pqn/abu* genes residing in the CED-1-regulated cluster are also grouped together in mountain 29 of the *C. elegans* three-dimensional topographical expression map (Kim et al., 2001). Since this gene expression map correlates gene regulation among different growth conditions, developmental stages, and mutant backgrounds, *pqn/abu* genes in mountain 29 have a high probability of being regulated by CED-1. In addition, we confirmed the microarray results showing that *pqn/abu* genes are downregulated in *ced-1(e1735)* mutants by performing reverse transcription-polymerase chain reaction (RT-PCR) analysis on *abu-1*, *abu-6*, *abu-7*, *abu-8*, *abu-11*, *pqn-5*, and *pqn-54*, all of which are likely to be coregulated, as they are part of mountain 29 (Figures 2A and 2B). We also performed quantitative RT-PCR (qRT-PCR) to provide another independent confirmation that *pqn/abu* genes are downregulated in *ced-1(e1735)* animals. Figure 2C shows that *abu-1* and *pqn-54* are downregulated 3.3- and 2.6-fold, respectively, in *ced-1(e1735)* mutants. Even though the degree of misregulation of *pqn/abu* genes observed by RT-PCR and qRT-PCR (Figures 2A–2C) is lower than that seen in the microarrays, the results confirm the CED-1 requirement for proper *pqn/abu* gene expression. The requirement of CED-1 for proper *abu* gene expression was further confirmed by comparing the GFP intensities of *abu-1::gfp(ZcEx8)* and *ced-1(e1735);abu-1::gfp(ZcEx8)* animals (Figure 2D). The results shown in Figure 2 confirm that CED-1 is required for the proper expression of *pqn/abu* genes and suggest that these UPR genes may be required for *C. elegans*' survival on live bacteria. The transcriptional profiling of wild-type and *ced-1(e1735)* animals grown on live *S. enterica* also indicated that *pqn/abu* genes are upregulated by CED-1 (Table S1 available online), suggesting that *pqn/abu* genes may be involved in CED-1-mediated protection against potentially pathogenic bacteria.

pqn/abu Genes Expressed in a CED-1-Dependent Manner Are Required for *C. elegans* Immunity to Live Bacteria

To test the hypothesis that *pqn/abu* genes function in *C. elegans*' immunity to pathogenic bacteria, we first compared *S. enterica* killing of wild-type nematodes to that of nematodes in which *abu-1/7/8/11* and *pqn-5/54* gene expression was abrogated by RNAi. As shown in Figures 3A and 3B, *pqn/abu* RNAi increased nematode susceptibility to *S. enterica*-mediated killing. Importantly, RNAi reduction of *pqn/abu* gene expression did not affect the nematode life span on killed *S. enterica* (Figures 3C and 3D); similar results were obtained by using live and killed *E. coli* (Figure S1). In mutant animals in *ced-1* or in any of the genes involved in apoptotic corpse clearance, several dying cells are not engulfed and remain as cell corpses in the gonads. As shown in Figure S2, no significant differences were found in the number of apoptotic corpses among control, *abu-1* RNAi, and *abu-11* RNAi animals after 24 hr of exposure to either *E. coli* or *S. enterica*. Taken together, these results indicate that these *pqn/abu* genes are required for proper *C. elegans* immunity to pathogenic bacteria without affecting *ced-1* functions related to apoptotic corpse removal.

Given the high sequence similarity among *pqn/abu* genes, the occurrence of cross-RNAi was likely. To identify potential

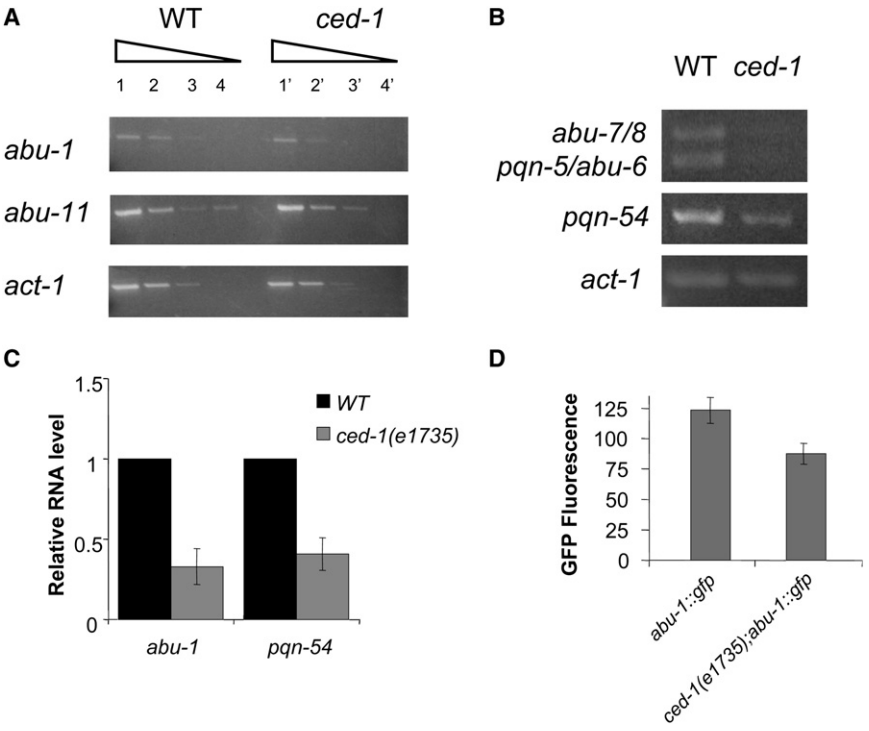

Figure 2. CED-1 Regulates *pqn/abu* Unfolded Protein Response Genes

(A) Wild-type (lanes 1–4) and *ced-1(e1735)* (lanes 1′–4′) cDNAs were stepwise 10-fold serially diluted. PCR was performed by using gene-specific primers, and expression levels of *act-1*, a housekeeping gene, was used to confirm cDNA equalization.

(B) Wild-type and *ced-1(e1735)* cDNAs were 10-fold serially diluted, and the 1:1,000 dilutions were used. PCR was performed by using gene-specific primers, and expression levels of *act-1*, a housekeeping gene, was used to confirm cDNA equalization. *abu-7* and *abu-8* mRNA are 93.4% identical, and *abu-6* and *abu-7* mRNA are 98.3% identical; although one primer set was used to amplify all four transcripts, the two groups could be differentiated by size. (A and B) L1-stage animals fed *E. coli* were grown to L4 stage. RNA was then isolated, RT-PCR was performed, and PCR products were run on a gel and stained with ethidium bromide. RT-PCR was performed in duplicate from independent RNA isolations, and similar results were achieved.

(C) qRT-PCR analysis of *abu-1* and *pqn-54* expression in *ced-1(e1735)* relative to wild-type nematodes grown on *E. coli* to L4 stage. Data were analyzed by relative quantitation by using the comparative cycle threshold method and were normalization to actin. One sample Student's exact t test indicates that differences between wild-type and *ced-1(e1735)* are significantly different ($p < 0.05$); n = 3; bars correspond to mean ± SD.

(D) GFP expression in a standard defined area encompassing the entire pharynx of L4-stage *abu-1::gfp(zcEx8)* animals and *ced-1(e1735);abu-1::gfp(zcEx8)* animals was analyzed by using max green channel intensity calculated by ImageJ 1.37v freeware. A Student's exact t test indicates that differences between *abu-1::gfp(zcEx8)* and *ced-1(e1735);abu-1::gfp(zcEx8)* are significantly different ($p < 0.05$); n = 15; bars correspond to mean ± SD.

off-target crossreactions, the BLAT algorithm was used (Kent, 2002). In *C. elegans*, cross-RNAi is known to occur when a target mRNA shares at least 95% identity, over a span of 40 or more nucleotides, to the dsRNA encoded by the RNAi construct (Rual et al., 2007). Indeed, the *abu-1*, *abu-7*, *abu-8*, *pqn-5*, and *pqn-54* RNAi constructs appear to simultaneously target as many as 17 other *pqn/abu* genes (Table S2). Our analysis revealed no potential off-target crossreactions for the *abu-11* RNAi construct (Table S2). However, based on the sequence similarity among *pqn/abu* genes, we cannot rule out the possibility that *abu-11* RNAi caused cross-RNAi effects that our BLAT analysis was not capable of predicting. On the other hand, since several

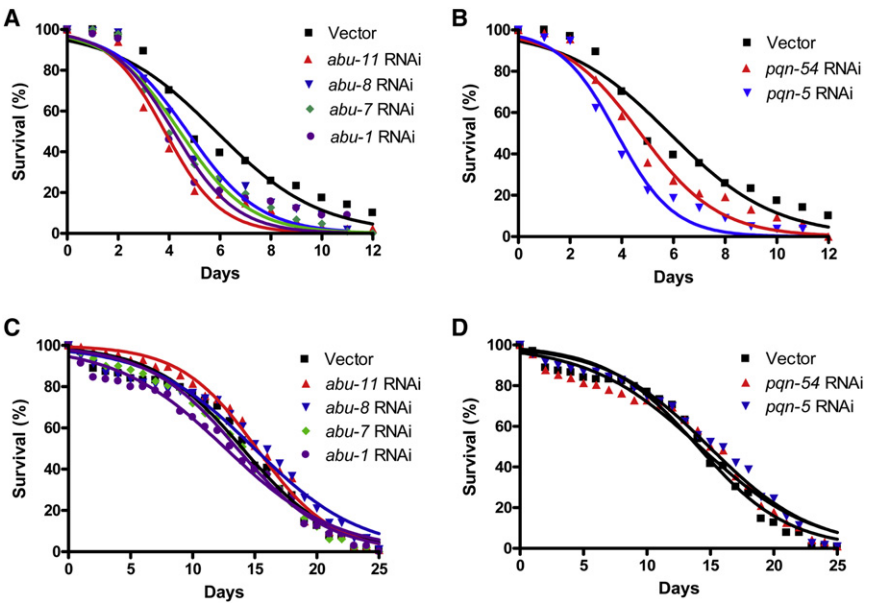

Figure 3. *pqn/abu* Genes Expressed in a CED-1-Dependent Manner Are Required for *C. elegans* Immunity

(A) Wild-type animals grown on bacteria carrying a vector control or expressing dsRNA targeting *abu* genes were exposed to live *S. enterica*: *abu-11* RNAi, $p < 0.0001$; *abu-8* RNAi, $p = 0.0032$; *abu-7* RNAi, $p < 0.0001$; *abu-1* RNAi, $p = 0.0459$.

(B) Wild-type animals grown on bacteria carrying a vector control or expressing dsRNA targeting *pqn* genes were exposed to live *S. enterica*: *pqn-54* RNAi, $p = 0.0056$; *pqn-5* RNAi, $p < 0.0001$.

(C) Wild-type animals grown on bacteria carrying a vector control or expressing dsRNA targeting *abu* genes were exposed to heat-killed *S. enterica*: *abu-11* RNAi, $p > 0.1$; *abu-8* RNAi, $p > 0.1$; *abu-7* RNAi, $p > 0.1$; *abu-1* RNAi, $p > 0.1$.

(D) Wild-type animals grown on bacteria carrying a vector control or expressing dsRNA targeting *pqn* candidate genes were exposed to heat-killed *S. enterica*: *pqn-54* RNAi, $p > 0.1$; *pqn-5* RNAi, $p > 0.1$. For each condition, 90–140 animals were used. *P* values are relative to wild-type animals fed dsRNA for vector control.

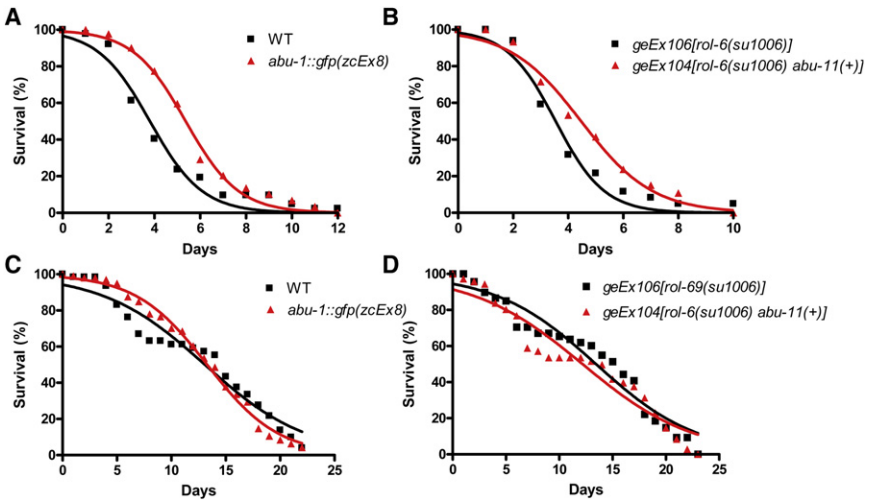

Figure 4. Overexpression of *abu-1* or *abu-11* Extends *C. elegans* Life Span on Live Bacteria

(A) Wild-type and *abu-1::gfp(zcEx8)* animals were exposed to live *S. enterica*: *abu-1::gfp(zcEx8)* $p = 0.0005$.

(B) *geEx106[rol-6(su1006)]* and *geEx104[rol-6(su1006) abu-11(+)]* animals were exposed to live *S. enterica*: *geEx104[rol-6(su1006) abu-11(+)]* $p = 0.0373$.

(C) Wild-type and *abu-1::gfp(zcEx8)* animals were exposed to heat-killed *S. enterica*: *abu-1::gfp(zcEx8)* $p > 0.1$.

(D) *geEx106[rol-6(su1006)]* and *geEx104[rol-6(su1006) abu-11(+)]* animals were exposed to heat-killed *S. enterica*: *geEx104[rol-6(su1006) abu-11(+)]* $p > 0.1$.

For each condition, 60–105 animals were used. p values are relative to control animals.

PQN and ABU proteins may interact (Li et al., 2004), it is possible that they function in a cooperative network that requires each member to be properly expressed, such that perturbing expression of a single member causes the function of the cooperative network to be impaired.

If a network of PQN/ABU proteins, involved in the UPR, is required for proper immunity to live bacteria and downregulation of a single member of the network can impair function, then the overexpression of a single member may enhance the function of the network. To test this, we next investigated whether the overexpression of ABU proteins confers resistance to pathogenic bacteria. Specifically, we compared *S. enterica*-mediated killing of control animals and animals overexpressing ABU-1 or ABU-11. Consistent with the idea that UPR proteins protect *C. elegans* from bacterial infection, animals overexpressing ABU-1 or ABU-11 were significantly more resistant to live *S. enterica* than control animals (Figures 4A and 4B). However, when fed heat-killed *S. enterica*, all transgenic animals overexpressing ABU proteins exhibited a life span comparable to that of control animals (Figures 4C and 4D). Taken together, these results indicate that the PQN/ABU proteins are required for *C. elegans*' immunity to live bacteria, and that they may be important components of the CED-1-mediated immune response.

CED-1 and a Network of UPR Proteins Are Part of a Pathway that Prevents *S. enterica* Invasion of Pharyngeal Tissue

The results described above implicate CED-1 in the regulation of the expression of genes encoding PQN/ABU proteins required for proper immune response to live bacteria. Interestingly, various *pqn/abu* genes have a reported strong expression in the pharynx (Urano et al., 2002), which constitutes one of the first physiological barriers against pathogens in *C. elegans*. In addition, it has recently been reported that the pharyngeal tissue can be invaded by *S. enterica* (Tenor and Aballay, 2008). More specifically, animals lacking TOL-1-mediated immunity exhibited a significant pharyngeal invasion, which is not observed in other immunocompromised animals such as *pmk-1(km25)* and *dbl-1(nk3)* mutants (Tenor and Aballay, 2008). Thus, we sought to determine whether CED-1 and the network of PQN/ABU proteins described here also play a role in preventing *S. enterica*'s invasion of the pharynx.

We examined the profile of bacterial accumulation in the pharyngeal tissue by feeding nematodes *S. enterica* expressing green fluorescent protein (GFP) and following the accumulation of bacteria by direct observation under the fluorescence microscope as described (Aballay et al., 2000; Tenor and Aballay, 2008). As shown in Figure 5, *ced-1(e1735)* nematodes exhibited significantly more pharyngeal invasion than that observed in wild-type nematodes (Figure 5G and comparison of Figures 5B and 5D), and intact *S. enterica* cells are observed in the pharyngeal tissue, where *abu-1* is known to be expressed (Urano et al., 2002; Figures 5E and 5F; Figure S3). By 48 hr, over 50% of the *ced-1(e1735)* nematodes exhibited infected pharynxes (Figure 5G). The *S. enterica* invasion does not appear to be a consequence of pharyngeal defects in *ced-1(e1735)* animals, since the pumping rates of *ced-1(e1735)* and wild-type animals are not perceptibly different and there is no visible *S. enterica* invasion during early infection (data not shown). Consistent with the idea that a lack of CED-1 does not affect the general physiology of the pharynx, the expression levels of pharyngeal genes in *ced-1(e1735)* animals are not significantly different from expression levels observed in wild-type animals (Table S3). In addition, the pharyngeal invasion of *ced-1(e1735)* animals is comparable to that of *abu-1* RNAi and *abu-11* RNAi animals, which greatly contrasts with the limited pharyngeal invasion observed in wild-type nematodes grown on control RNAi plates (Figure 5G). The increased pharyngeal invasion of *S. enterica* observed in *ced-1(e1735)* and *abu* RNAi nematodes is distinct since it is observed in *tol-1(nr2033)* mutants, but not in other immunocompromised animals such as *pmk-1(km25)* and *dbl-1(nk3)* mutants (Tenor and Aballay, 2008). In addition, no other *ced* engulfment mutant displays increased *S. enterica* pharyngeal invasion compared to wild-type (Figure S4), suggesting that the engulfment function of CED-1 is independent of the immune function. Consistent with this idea, we have not observed CED-1-mediated phagocytosis of *S. enterica* (A.A., unpublished data).

Our results suggest that decreased expression of UPR proteins in *ced-1(e1735)* nematodes facilitates *S. enterica*

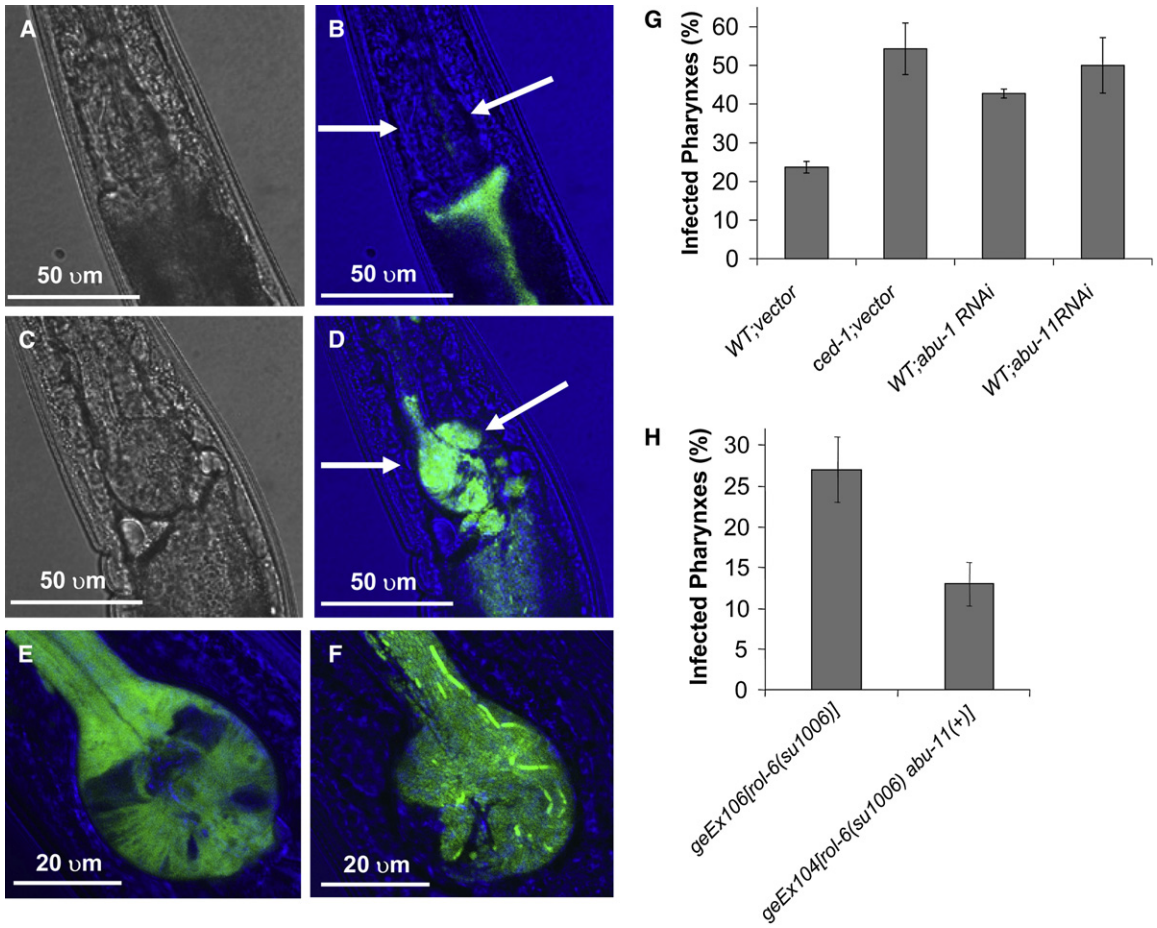

Figure 5. *pqn/abu* Genes Expressed in a CED-1-Dependent Manner Are Required for *C. elegans* Defense to Pharyngeal Invasion by *S. enterica*

(A–D) Confocal images show the pharynx of (A and B) wild-type and (C and D) *ced-1(e1735)* animals infected for 48 hr with *S. enterica* expressing GFP. (B and D) In the merged images, the terminal bulb of the pharynx is indicated with arrows.

(E) Confocal image of the terminal bulb of an *abu-1::gfp(zcEx8)* animal showing pharyngeal expression of ABU-1::GFP.

(F) Confocal image of the infected terminal bulb of a *ced-1(e1735)* animal fed *S. enterica* expressing GFP for 48 hr.

(G) The percentage of nematodes with infected pharynxes when fed *S. enterica* expressing GFP for 48 hr was determined for wild-type and *ced-1(e1735)* animals grown on bacteria carrying a vector control or expressing dsRNA targeting *abu-1* and *abu-11*.

(H) The percentage of nematodes with infected pharynxes when fed *S. enterica* expressing GFP for 48 hr was determined for *geEx106[rol-6(su1006)]* and *geEx104[rol-6(su1006) abu-11(+)]* animals.

Bars correspond to mean ± SD.

invasion of the pharyngeal tissue. To further investigate the potential role of UPR proteins in mediating protection against *S. enterica* invasion, we studied whether ABU-1 and ABU-11 overexpression can provide protection against *S. enterica* invasion. We found that, relative to control nematodes, pharyngeal invasion is lower in nematodes overexpressing ABU-11 (Figure 5H). These results suggest that UPR proteins are crucial for protection against *S. enterica* invasion of the pharyngeal tissue.

The increased susceptibility of *pqn/abu* RNAi animals, together with the microarray and RT-PCR data, indicate that PQN/ABU proteins are required for the CED-1-mediated protection against bacteria. To confirm that PQN/ABU proteins are part of a CED-1-dependent immunity, we performed *S. enterica* killing assays by using RNAi to abrogate the expression of *pqn/abu* genes in a *ced-1(e1735)* background. As would be expected if PQN/ABU proteins and CED-1 are part of the same pathway, we observed no additive effect of *pqn/abu* RNAi ablation on *ced-1(e1735)* mutants (Figures 6A and 6B). Furthermore, we did not observe any increased pharyngeal invasion when *abu-1* and *abu-11* expression was abrogated by RNAi in a *ced-1(e1735)* background (Figure 6C); *abu-1* overexpression rescues the enhanced susceptibility to *S. enterica* of *ced-1(e1735)* mutants (Figure 6D). Taken together, these results support the hypothesis that UPR proteins are part of a CED-1-dependent immunity to live bacteria.

DISCUSSION

In this study, we have shown that CED-1 is required for *C. elegans*' survival in the presence of live bacteria. Animals lacking CED-1-mediated responses were rapidly killed by the human

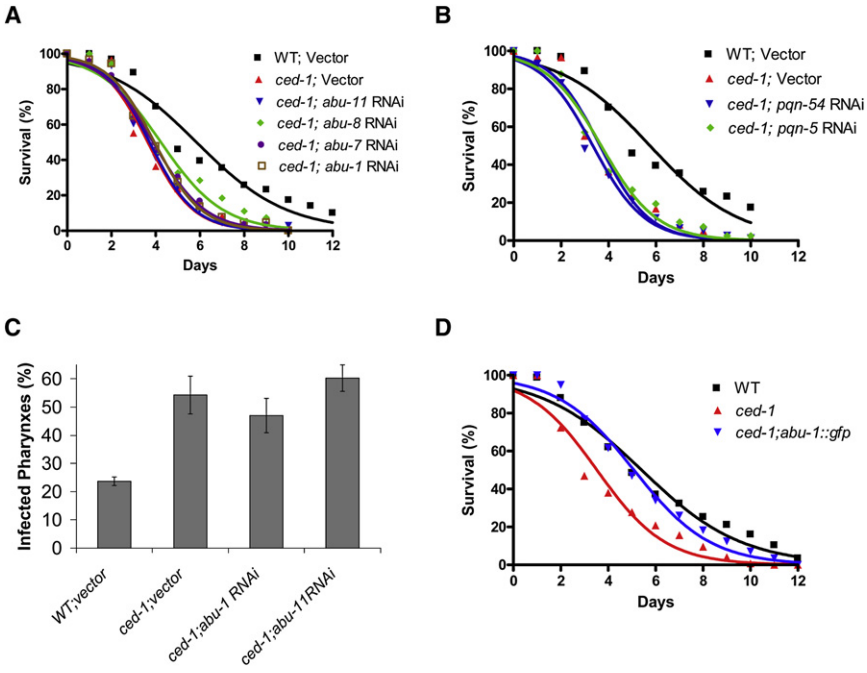

Figure 6. CED-1 and PQN/ABU Unfolded Protein Response Proteins Are Part of a Pathway Required for Innate Immunity to *S. enterica*

(A) Wild-type and *ced-1(e1735)* animals grown on bacteria carrying a vector control or expressing dsRNA targeting *abu* genes were exposed to live *S. enterica*: wild-type; vector, $p < 0.0001$; *ced-1;abu-11* RNAi, $p > 0.1$; *ced-1;abu-8* RNAi, $p > 0.1$; *ced-1;abu-7* RNAi, $p > 0.1$; *ced-1;abu-1* RNAi, $p > 0.1$. A total of 74–140 animals were used. p values are relative to *ced-1(e1735)* animals fed dsRNA for vector control.

(B) Wild-type and *ced-1(e1735)* animals grown on bacteria carrying a vector control or expressing dsRNA targeting *pqn* genes were exposed to live *S. enterica*: wild-type; vector, $p < 0.0001$; *ced-1;pqn-54* RNAi, $p > 0.1$; *ced-1;pqn-5* RNAi, $p > 0.1$. A total of 74–140 animals were used. p values are relative to *ced-1(e1735)* animals fed dsRNA for vector control.

(C) The percentage of nematodes with infected pharynxes when fed *S. enterica* expressing GFP for 48 hr was determined for wild-type and *ced-1(e1735)* animals grown on bacteria carrying a vector control or expressing dsRNA targeting *abu-1* and *abu-11* genes. Bars correspond to mean ± SD.

(D) Wild-type, *ced-1(e1735)*, and *ced-1(e1735);abu-1::gfp(zcEx8)* animals were exposed to live *S. enterica*: wild-type, $p < 0.0001$; *ced-1(e1735);abu-1::gfp(zcEx8)*, $p > 0.1$. A total of 174–179 animals were used. p values are relative to *ced-1(e1735)* animals.

pathogen *S. enterica* and by *E. coli*, which, even though it is the food source of nematodes in the laboratory, has been shown to kill immunocompromised animals (Kerry et al., 2006; Singh and Aballay, 2006b; Tenor and Aballay, 2008). However, the survival of *ced-1(lf)* animals grown on dead *S. enterica* and dead *E. coli* was comparable to that of wild-type animals, indicating that a CED-1-mediated mechanism is required for immune response to live, but not dead, bacteria. Importantly, whole-genome microarray analyses demonstrated that CED-1 regulates the transcription of *pqn/abu* genes, which are part of a noncanonical UPR response in *C. elegans* (Urano et al., 2002) and are also required for the resveratrol-mediated extension of life span on live bacteria (Viswanathan et al., 2005). Furthermore, we found that *pqn/abu* genes are required for the survival of *C. elegans* exposed to live *S. enterica*, and that their overexpression confers protection against *S. enterica*-mediated killing and invasion of the pharyngeal tissue. The results indicate that the UPR, regulated in a CED-1-dependent manner, is critical for a successful immune response to bacteria in *C. elegans*.

As in mammals, peristalsis, low pH, and antimicrobial substances prevent microbial colonization of the *C. elegans* intestine. Typically, *C. elegans* animals are propagated in the laboratory by feeding them *E. coli*. *E. coli* is effectively disrupted by the *C. elegans* pharyngeal grinder, and almost no intact bacterial cells can be found in the intestinal lumen. Once in the gut, however, pathogenic bacteria can overcome innate immune responses to proliferate and kill *C. elegans*. In the case of animals deficient in immune responses, even ordinarily benign *E. coli* can proliferate in the intestine and eventually kill the animals (Kerry et al., 2006; Singh and Aballay, 2006b; Tenor and Aballay, 2008). In fully immunocompetent animals, bacterial infections are controlled by a range of immune effectors that are upregulated upon pathogen exposure (Alper et al., 2007; Kerry et al., 2006; Mallo et al., 2002; Shapira et al., 2006; Troemel et al., 2006; Wong et al., 2007). Presumably, this upregulation of immune-related proteins requires a system of chaperones that help maintain protein homeostasis during bacterial infections (Singh and Aballay, 2006a, 2006b). Here, we show that, in addition to the chaperone system, the increased demand on protein folding in the ER during bacterial infections must be successfully alleviated by the UPR for a complete defense response to be mounted.

In all eukaryotic cells, UPR signaling confers protection against ER stress by expanding the amount of ER in the cell, enhancing the degradation of misfolded proteins, and reducing the synthesis of new proteins (Kaufman, 2002; Lin et al., 2007; Mori, 2000; Ron, 2002). However, when the UPR cannot maintain protein homeostasis due to excessive or long-term ER stress, cells typically die by apoptosis (Nakagawa et al., 2000; Nishitoh et al., 2002). Interestingly, the somatic cells of *C. elegans* have a fixed lineage or population of cells that do not undergo apoptosis after development. Therefore, the animals must use a nonapoptotic mechanism to deal with the stresses that pathogen infection causes to the somatic cells. Indeed, *S. enterica* infection has been shown to elicit apoptosis only in the cells of the *C. elegans* germline (Aballay and Ausubel, 2001; Aballay et al., 2003). This increased germline apoptosis is regulated through the CED-9/CED-4/CED-3 pathway, and mutants in this pathway that are

deficient in apoptosis exhibit increased susceptibility to *S. enterica*-mediated killing (Aballay and Ausubel, 2001). Thus, it is intriguing that an apoptotic receptor such as CED-1 upregulates UPR proteins that prevent apoptosis during ER stress. However, several lines of evidence indicate that the regulation of *pqn/abu* expression by CED-1 is part of a nonapoptotic function of this receptor. A few years ago, two seminal papers demonstrated that, in addition to its critical role in the engulfment of dying cells, CED-1 functions in the engulfing cells to ensure the apoptotic death of cells undergoing CED-3-mediated apoptosis (Hoeppner et al., 2001; Reddien et al., 2001). These studies highlight a proapoptotic function of CED-1 that is only seen in *ced-3* backgrounds and therefore should not play any role in *S. enterica*-elicited apoptosis in the germline of wild-type animals. On the other hand, the increased number of corpses in the germline of *ced-1(lf)* animals (Gumienny et al., 1999) cannot account for the increased susceptibility to *S. enterica*, as it has been demonstrated that extra corpses does not affect *C. elegans*' survival (Aballay and Ausubel, 2001). In addition, unlike the *ced-9/ced-4/ced-3* mutants deficient in apoptosis, *ced-1(lf)* mutants are hypersusceptible to not just live *S. enterica*, but also to live *E. coli*, which does not elicit apoptosis in the germline. Thus, the observation that *ced-1(lf)* animals are susceptible to live *E. coli* indicates that the CED-1-mediated defense is separate from the effects of the CED-9/CED-4/CED-3 pathway in the germline. Our results, however, suggest the possibility of crosstalk between UPR signaling and CED signaling that will require further investigation to be elucidated.

Not much is known about the regulation of *pqn/abu* genes. Interestingly, one of the known coregulators of *pqn/abu* genes is the NAD^+-dependent histone deacetylase SIR-2.1, which represses transcription of various members of this gene family (Viswanathan et al., 2005). Since ABU-1 or ABU-11 overexpression confers protection against *S. enterica*-mediated killing (Figures 4A and 4B) and ABU-1 overexpression reduces *S. enterica* invasion of the pharynx (Figure 5H), *sir-2.1(lf)* mutants would be expected to be more resistant to *S. enterica*-mediated killing. However, *sir-2.1(lf)* animals do not exhibit increased resistance to live *S. enterica,* and there is a trend toward increased susceptibility to *S. enterica* in *sir-2.1(lf)* animals ($p = 0.09$) (Figure S5). This result is consistent with the observation that *sir-2.1(lf)* animals do not exhibit an extended life span when grown in the presence of live *E. coli* (Viswanathan et al., 2005) or with the fact that *sir-2.1* promotes *daf-16* activity, which is required for proper innate immunity to *S. enterica* (Singh and Aballay, 2006b). Thus, the potential benefits of *pqn/abu* overexpression in *sir-2.1(lf)* animals appear to be compensated by a reduced *daf-16* activity.

The UPR mediated by *pqn/abu* gene products is independent of the canonical UPR mediated by the conserved HAC1-like transcription factor XBP-1 (Urano et al., 2002), making it unlikely that XBP-1 is involved in the CED-1-mediated upregulation of *pqn/abu* genes. Consistent with the idea that CED-1 specifically upregulates the expression of *pqn/abu* genes without affecting other genes involved in the UPR, we did not observe overlap between the genes regulated by XBP-1 or IRE-1 (Shen et al., 2005) and the CED-1-regulated genes (data not shown). Given the importance of the CED-1-dependent UPR in immunity to live bacteria, it would be interesting to study the role of the canonical UPR pathway in immunity.

In summary, our results provide evidence that the stress caused by bacterial infections is likely met by the CED-1-mediated upregulation of *pqn/abu* genes involved in the UPR. Our findings indicating that overexpression of *pqn/abu* genes enhances *C. elegans* survival on live bacteria and protects the animals from *S. enterica*-mediated killing and invasion of the pharyngeal tissue suggest a mechanism that can potentially be exploited to alleviate bacterial infections.

EXPERIMENTAL PROCEDURES

Bacterial and Nematode Strains

The *Escherichia coli* OP50 (Brenner, 1974) and *Salmonella enterica* serovar *Typhimurium* 1344 (Wray and Sojka, 1978) strains were used. *C. elegans* N2 Bristol, *ced-1(e1735)*, *ced-1(n691)*, and *ced-1(n2089)* strains were obtained from the *Caenorhabditis elegans* Genetics Center. The *abu-1::gfp(ZcEx8)* strain (Urano et al., 2002) was generously provided by the Ron laboratory. The *abu-11* extrachromosomal array line *geEx104[(rol-6su1006)abu-11(+)]* and strain *geEx106[(rol-6su1006)]* (Viswanathan et al., 2005) were generously provided by the Guarente laboratory. The *abu-1::gfp(ZcEx8)* transgenics were outcrossed four times to our N2 strain, which was used as the control. *geEx-106[(rol-6su1006)]* was used as a control for *geEx104[(rol-6su1006)abu-11(+)]*.

Growth Conditions

Nematodes were maintained on nematode growth medium (NGM, minimal medium containing NaCl, agar, peptone, cholesterol, $CaCl_2$, $MgSO_4$, and potassium phosphate [Brenner, 1974]) containing a lawn of *Escherichia coli* OP50 at 20°C. Synchronous populations were acquired by placing gravid adults on NGM plates containing *Escherichia coli* OP50 for 5 hr at 20°C. The gravid adults were removed, leaving the eggs to hatch and develop into 1-day-old hermaphroditic adults at 20°C for use in the different assays.

C. elegans Killing Assay

For all bacterial strains, individual bacterial colonies were inoculated into LB and grown overnight on a rotary wheel at 37°C. A total of 20 μl of culture was plated onto a 3.5 cm plate containing modified NGM (3.5% peptone instead of 2.5%). One-day-old adult hermaphroditic nematodes were transferred to lawns of the various bacteria and transferred daily to a fresh lawn until progeny were no longer produced. All experiments were performed at 25°C. Animals were considered dead upon failure to respond to touch, and animals missing from the agar plate were censored on the day of loss.

C. elegans Life Span Assay on Killed Bacteria

Synchronized young adult animals were collected by using M9 solution and were washed in antibiotic for 3 hr. Animals grown on *E. coli* OP50 were washed in M9 with 100 μg/ml ampicillin, and animals grown on *E. coli* HT115(DE3) were washed in M9 with 50 μg/ml kanamycin.

Bacteria were grown as previously described, concentrated 1:10, and then heat killed at 100°C for 1 hr. Bacterial death was confirmed by failure to grow on LB plates at 37°C overnight. A total of 100 μl of the concentrated, killed bacteria was plated onto a 3.5 cm plate containing modified NGM with the appropriate antibiotic. Animals grown on *E. coli* OP50 were put on modified NGM with 100 μg/ml ampicillin, and animals grown on *E. coli* HT115(DE3) were put on modified NGM with 50 μg/ml kanamycin. Killing assays were performed as described above.

Pharyngeal Invasion Assay

For pharyngeal invasion microscopy, 20 μl *S. enterica* expressing GFP grown in 3 ml LB with 50 μg/ml kanamycin were plated onto a 3.5 cm plate of modified NGM containing 50 μg/ml kanamycin. The plates were incubated at 37°C overnight. One-day-old adult nematodes were transferred to *S. enterica* expressing GFP strain *smo22* (Vazquez-Torres et al., 1999) and were incubated for 2 days with daily transfers to a new lawn of pathogen. After 48 hr, the nematodes were moved to a lawn of *E. coli* OP50. This enables the identification of nematodes with infected pharynxes and excludes the fluorescence from the background lawns. Nematodes were monitored for infected pharynxes by using

a Leica MZ FLIII fluorescence stereomicroscope. An infected pharynx was defined as the presence of GFP in the terminal bulb visible at 10× magnification. All experiments were performed at 25°C.

Confocal Microscopy

Nematodes were anesthetized in 1% sodium aside on an agar pad (2% agarose) and were examined with a Leica TCS SL confocal microscope with Leica Confocal software version 2.61 Build 1537 (Leica Microsystems, Heidelberg GMbH). Confocal images were imported into Adobe Photoshop for processing, including size adjustments, layering, and brightness contrast.

RNAi

We used the RNA interference technique to generate loss-of-function RNAi phenotypes by feeding nematodes with *E. coli* expressing double-stranded RNA that is homologous to a target gene (Fraser et al., 2000; Timmons and Fire, 1998). The *E. coli* strain HT115(DE3) harboring the appropriate vectors was grown in LB broth containing 100 μg/ml ampicillin and 10 μg/ml tetracycline at 37°C overnight. Bacteria were plated onto NGM plates containing 100 μg/ml ampicillin and 10 mM isopropyl β-D-thiogalactoside and were allowed to grow overnight at 37°C.

L4 nematodes were placed on RNAi-expressing lawns of bacteria and were allowed to grow to gravid adults. These gravid adults laying eggs were then transferred to new RNAi-expressing lawns of bacteria for 5 hr and were then removed. The eggs were allowed to develop into young adults at 20°C on plates containing *E. coli* strain HT115 harboring a vector control or the appropriate RNAi vectors. Animals were then transferred to plates containing *E. coli* OP50 or *S. enterica* 1344, according to the experimental conditions described for each specific assay. Bacteria strains expressing double-stranded RNA to inactivate the *C. elegans* genes were obtained from Wellcome/Cancer Research (Cambridge, UK) and Open Biosystems (Huntsville, AL). The identity of clones was confirmed by sequencing.

RNA Isolation, RT-PCR, and qRT-PCR

N2 and *ced-1(e1735)* strains were grown to gravid adults and were treated with alkaline hypochlorite (Emmons et al., 1979) to isolate eggs. Eggs were placed in S basal to hatch overnight, causing growth arrest in L1. Synchronized nematodes were then grown on NGM plates with *E. coli* OP50 or *S. enterica* SL1344 at 25°C for 26 hr. Nematodes were harvested and freeze thawed three times in liquid nitrogen before total RNA was extracted with TRIzol reagent (Invitrogen).

For RT-PCR, poly(A)$^+$ RNA was isolated by using the oligotex mRNA kit (QIAGEN). We used the oligo-dT method from the SuperScript First-Strand Synthesis System (Invitrogen) with 50 ng poly(A)$^+$ RNA isolated as described above to generate a cDNA template. PCR was performed by amplifying from the cDNA by using primers listed in Table S4. cDNA samples were 10-fold serially diluted four times. A total o f30 cycles were used for PCR of *act-1* control primers. A total of 40 cycles were used for PCR of candidate genes.

qRT-PCR was conducted by using the Applied Biosystems Taqman One-Step Real-time PCR protocol with SYBR Green fluorescence (Applied Biosystems) on an Applied Biosystems StepOnePlus Real Time-PCR System. Independent RNA preparations were measured at least twice and were normalized to the housekeeping genes *act-1*, *act-3*, and *act-4* (pan-actin). Gene expression in *ced-1(e1735)* was compared to wild-type by using the comparative Ct method, and normalization to actin was used.

Microarray Analyses

Nematodes were grown and infected essentially as described above. Briefly, N2 and *ced-1(e1735)* strains were grown to gravid adults, and eggs were isolated and placed in S basal to hatch overnight. Synchronized nematodes were then grown on NGM plates with *E. coli* OP50 or *S. enterica* SL1344 at 25°C for 26 hr. Total RNA was obtained as described above. Affymetrix DNA microarray processing was prepared according to the manufacturer's instructions (http://www.affymetrix.com/support/technical/manual/expression_manual.affx), and targets were hybridized to the *C. elegans* GeneChip (Affymetrix, Santa Clara, CA). The microarray data were subjected to the Robust Multichip Averaging (RMA) Algorithm by using Partek Software (Partek, Inc., St. Charles, Missouri). GeneSpring Software 9.0 (Agilent Technologies) was used to perform hierarchical cluster analysis.

Cell Corpse Assay

To quantify the number of apoptotic germ cells, animals were stained with SYTO 12 (Molecular Probes) as previously described (Gumienny et al., 1999). In brief, worms were incubated in 50 μM SYTO 12 for 3–4 hr at room temperature and were then seeded on bacterial lawns to reduce the amount of stained bacteria in the gut. After 20–30 min, more than 20 animals were mounted in a drop of M9 salt solution containing 30 mM NaN_3 and were observed by using a Leica MZ FLIII fluorescence stereomicroscope. Only animals that were brightly and equally stained were scored.

GFP Fluorescence Analysis

For GFP fluorescence microscopy, 20 μl *E. coli* OP50 was plated on modified NGM plates and grown at 37°C overnight. Eggs were isolated from N2 and *ced-1(e1735)* strains by alkaline hypochlorite treatment (Emmons et al., 1979) and were grown on the OP50 plates at 20°C until the animals reached the L4 stage (~36 hr).

Animals were anesthetized by using a M9 salt solution containing 30 mM NaN_3 and were visualized with a Leica MZ FLIII fluorescence stereomicroscope. Images were taken of more than 15 worms per condition, and max green channel fluorescence was analyzed with ImageJ 1.37v freeware. Intensities were averaged, and a t test was performed by using GraphPad Prism 4.

SUPPLEMENTAL DATA

Supplemental Data include five figures and four tables and are available at http://www.developmentalcell.com/cgi/content/full/15/1/87/DC1/.

ACKNOWLEDGMENTS

We thank the *Caenorhabditis* Genetics Center (University of Minnesota) for providing all mutant strains used in this study. We also thank the Ron and Guarente laboratories for generously providing the *abu-1* and *abu-11* transgenic strains, respectively. Jennifer Tenor provided technical advice and performed the initial pharyngeal invasion assays. Holly K. Dressman provided excellent technical advice on microarray data analysis. A.A. is funded by The Whitehead Scholars Program, National Institutes of Health (NIH) Southeast Regional Center of Excellence for Emerging Infections and Biodefense (U54 AI057157), and NIH GM070977.

Received: November 15, 2007
Revised: April 10, 2008
Accepted: May 15, 2008
Published: July 7, 2008

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