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# An Essential Role for XBP-1 in Host Protection against Immune Activation in *C. elegans*

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## Abstract

The detection and compensatory response to the accumulation of unfolded proteins in the endoplasmic reticulum (ER), termed the Unfolded Protein Response (UPR), represents a conserved cellular homeostatic mechanism with important roles in normal development and in the pathogenesis of disease[1]. The IRE1-XBP1/Hac1p pathway is a major branch of the UPR that has been conserved from yeast to human[2],[3],[4],[5],[6]. XBP-1 is required for the differentiation of the highly secretory plasma cells of the mammalian adaptive immune system[7],[8], but recent work also points to reciprocal interactions between the UPR and other aspects of immunity and inflammation[9],[10],[11]. We have been studying innate immunity in the nematode *Caenorhabditis elegans*, having established a key role for a conserved PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway in mediating resistance to microbial pathogens[12]. Here, we show that during *C. elegans* development, XBP-1 has an essential role in protecting the host during activation of innate immunity. Activation of the PMK-1-mediated response to infection with *Pseudomonas aeruginosa* induces the XBP-1-dependent UPR. Whereas a loss-of-function *xbp-1* mutant develops normally in the presence of relatively non-pathogenic bacteria, infection of the *xbp-1* mutant with *P. aeruginosa* leads to disruption of ER morphology and larval lethality. Unexpectedly, the larval lethality phenotype on pathogenic *P. aeruginosa* is suppressed by loss of PMK-1-mediated immunity. Furthermore, hyperactivation of PMK-1 causes larval lethality in the *xbp-1* mutant even in the absence of pathogenic bacteria. Our data establish innate immunity as a physiologically relevant inducer of ER stress during *C. elegans* development and suggest that an ancient, conserved role for XBP-1 may be to protect the host organism from the detrimental effects of mounting an innate immune response to microbes.

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We began our analysis of the role of the UPR in *C. elegans* immunity by asking if the UPR is activated upon exposure of *C. elegans* larvae to *P. aeruginosa* strain PA14, a human opportunistic pathogen that can also infect and kill *C. elegans*[13]. First, we assessed expression of the transgenic transcriptional reporter *Phsp-4:: GFP(zcIs4)* (the *C. elegans*

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**Author Contributions** C.E.R. and D.H.K. conceived and planned experiments. C.E.R. and T.K. performed experiments. C.E.R. and D.H.K. analyzed the data and wrote the paper.

**Supplementary Information** is linked to the online version of this paper atwww.nature.com/nature.

*hsp-4* gene encodes a homolog of mammalian BiP/GRP78), which reflects activation of the IRE-1-XBP-1 branch of the UPR[5]. After exposure of *C. elegans* larvae to *P. aeruginosa*, GFP expression was induced in the intestine, the site of infection (Fig. 1a). We confirmed the pathogen-induced activation of the endogenous *hsp-4* gene by quantitative RT-PCR (qRT-PCR) (Fig. 1b).

The mechanism by which IRE-1 activates XBP-1 in response to ER stress is conserved from yeast to mammals[2],[4],[5],[6]. Upon accumulation of unfolded proteins in the ER, the integral ER-membrane protein IRE-1 activates XBP-1 by alternative splicing of the *xbp-1* mRNA, thereby changing the reading frame. The activated "spliced form" of XBP-1 regulates expression of genes involved in ER homeostasis, such as those encoding chaperones. Using qRT-PCR to quantify the IRE-1-mediated splicing of *xbp-1* mRNA after exposure to *P. aeruginosa*, we found there was a marked induction of IRE-1-spliced *xbp-1* transcript within 4 h of exposure to pathogen (Fig. 1c).

We hypothesized that the rapid activation of the IRE-1-XBP-1 pathway after *P. aeruginosa* exposure might in fact be caused by induction of the host immune response. A conserved p38 MAPK pathway mediates innate immunity in organisms ranging from *C. elegans* to humans[12],[14]. We have previously shown that loss-of-function of the p38 MAPK ortholog PMK-1 results in markedly enhanced susceptibility to killing of *C. elegans* by *P. aeruginosa*. In the *pmk-1(km25)* mutant, we found no increase in IRE-1-spliced XBP-1 mRNA and no induction of the *Phsp-4::GFP* reporter in response to *P. aeruginosa* (Fig. 1). RNAi of *sek-1*, encoding a MAPKK required for PMK-1 activation[12], also abrogated induction of *Phsp-4::GFP* expression in response to *P. aeruginosa* infection (Supplementary Fig. 1a).

Consistent with the induction of XBP-1 splicing arising as a consquence of a transcriptional response to infection, we found that a mutant, *atf-7(qd22 qd130)*, carrying a mutation in a conserved transcription factor that abrogates the pathogen-induced expression of genes regulated by the PMK-1 pathway[15], also blocked induction of the *Phsp-4::GFP* reporter in response to *P. aeruginosa* PA14 (Supplementary Fig. 1b). Activation of the IRE-1-XBP-1 pathway in response to tunicamycin (an N-glycosylation inhibitor), however, occurred in the *pmk-1* mutant as in the wild-type strain N2 (WT), as reported by Bischof et al. in their study of PMK-1-dependent activation of the IRE-1-XBP-1 pathway in the *C. elegans* response to a pore-forming toxin from *Bacillus thurigiensis*[16]. Whereas our data show that activation of the IRE-1-XBP-1 pathway by *P. aeruginosa* infection is dependent on the PMK-1 pathway, we did not observe a reciprocal dependence of activation of the PMK-1 pathway on XBP-1 (Supplementary Fig. 2).

We next asked if XBP-1 is required for resistance to *P. aeruginosa*, focusing on its role during larval development. Synchronized eggs of WT and mutants deficient in each of the three branches of the UPR were propagated on plates with *P. aeruginosa* PA14 as the only food source and development was monitored over time. Nearly all the eggs of the WT strain developed to at least the fourth larval stage (L4) in this assay, comparable to their growth and development on the relatively nonpathogenic bacterial strain and standard laboratory food source *Escherichia coli* OP50 (Fig. 2a). In contrast, the *xbp-1(zc12)* mutant on *P.*

*aeruginosa* exhibited severely attenuated larval development and growth, as measured by the rate of progression between molts, size of larvae, and ability of larvae to reach the L4 stage by 72 h (Fig. 2a), eventually resulting in larval lethality. Loss-of-function mutations in the each of the other two major branches of the UPR, *atf-6(ok551)* and *pek-1(ok275)*[4,17], did not affect larval development in the presence of *P. aeruginosa* (Fig. 2a). These data implicate a specific and essential role for the IRE-1-XBP-1 branch of the UPR in *C. elegans* development in the presence of *P. aeruginosa*.

The *xbp-1* developmental phenotype was alleviated in the presence of the *gacA* mutant of *P. aeruginosa* PA14 (Supplementary Fig. 3a), which has diminished pathogenicity in *C. elegans* and mammals[13,18]. On a lawn of wild-type *P. aeruginosa* PA14 mixed with relatively non-pathogenic *E. coli*, the *xbp-1* mutant again exhibited the severely attenuated developmental phenotype that was observed in the presence of *P. aeruginosa* alone, suggestive that the pathogenicity of *P. aeruginosa*, not a nutritional deficiency, caused the *xbp-1* developmental phenotype (Fig. 2b and Supplementary Fig. 3b). Behavioural avoidance of pathogenic bacteria[19,20] can influence survival[21,22,23], but we found that the development of WT was unaffected by experimental conditions in which the *P. aeruginosa* could not be avoided, demonstrating that the inability of the *xbp-1* mutant to develop on *P. aeruginosa* was not due to a defect in behavioural avoidance (C. E. R. and D. H. K., unpublished data). In addition, the attenuated development and death of the *xbp-1* mutant on *P. aeruginosa* was also unaffected by the blocking of cell necrosis (Supplementary Table 1).

WT and *xbp-1* mutant larvae exhibit characteristic ribosome-studded tubular structures of the ER by transmission electron microscopy when propagated on relatively non-pathogenic *E. coli* OP50 (Fig. 2c). The ER architecture of WT worms was not markedly perturbed upon exposure to *P. aeruginosa* (Fig. 2c). In contrast, *xbp-1(zc12)* larvae propagated on *P. aeruginosa* PA14 revealed disruption in ER morphology of *xbp-1* mutant worms upon pathogen exposure (Fig. 2c), with localized regions of dilated ER lumen, comparable to that induced by tunicamycin treatment of *xbp-1(zc12)* worms (Supplementary Fig. 4). Such changes in ER morphology are consistent with ER stress and perturbation[24], and strongly suggest that a disruption in ER homeostasis is induced by PA14 infection in the *xbp-1* mutant.

We considered whether the developmental lethality of the *xbp-1* mutant might be due to the toxicity of PA14 arising from accelerated infection due to diminished resistance to *P. aeruginosa* in the *xbp-1* mutant. Because activation of the PMK-1 pathway results in the splicing of XBP-1 upon *P. aeruginosa* exposure, we first asked whether XBP-1 confers a survival benefit against *P. aeruginosa* by facilitating resistance to intestinal infection. Diminished activation of PMK-1-dependent innate immunity is accompanied by an increased rate of *P. aeruginosa* accumulation in the intestine[12](Fig. 2d). If XBP-1 functioned downstream of PMK-1 to promote the immune response to *P. aeruginosa*, we would anticipate that *xbp-1* deficiency would lead to an increased rate of *P. aeruginosa* accumulation relative to WT. Interestingly, in spite of the severely attenuated development and survival of the *xbp-1* mutant larvae on *P. aeruginosa*, the kinetics of accumulation of a PA14-derived strain of *P. aeruginosa* expressing GFP in the *xbp-1(zc12)* mutant were comparable to that observed for WT (Fig. 2d). Although the *pmk-1(km25)* mutant exhibited

an increased rate of accumulation of *P. aeruginosa* (Fig. 2d), larval development and survival was markedly greater than that observed for the *xbp-1* mutant (Figs. 3a and 3b, and Supplementary Fig. 5). These data decouple enhanced susceptibility to *P. aeruginosa* infection from the attenuated larval development phenotype of the *xbp-1* mutant and suggest that while activation of XBP-1 by *P. aeruginosa* infection is PMK-1-dependent, the primary protective role of XBP-1 is not to facilitate the killing of *P. aeruginosa*.

In view of these data, we considered whether the XBP-1-dependent UPR functions in this setting to protect against the ER stress induced by the immune response itself. If this were the case, then diminishing the immune response might actually improve the survival of the *xbp-1* mutant on *P. aeruginosa*. To test this hypothesis, we examined the larval development of a *xbp-1(zc12);pmk-1(km25)* double mutant. Strikingly, the *xbp-1;pmk-1* double mutant showed markedly increased development and survival relative to the *xbp-1* mutant, to a degree that was comparable to the development and survival of the *pmk-1* mutant (Figs. 3a and 3b). RNAi of *sek-1*, and RNAi of *atf-7* in the *xbp-1(zc12)* mutant each alleviated the attenuated development phenotype in the presence of *P. aeruginosa* (Supplementary Figs. 6a and b). These data indicate that activation of the PMK-1-dependent transcriptional innate immune response to pathogen is indeed detrimental to survival during development in the absence of the IRE-1-XBP-1 pathway, and that the principal mechanism by which XBP-1 promotes development and survival during infection with *P. aeruginosa* is by protecting against the innate immune response.

To separate further the effect of toxicity caused by pathogenic *P. aeruginosa* from that caused specifically by immune activation on the *xbp-1* developmental phenotype, we next asked if the *xbp-1* mutant would be compromised in the setting of hyperactivation of the PMK-1-mediated immune response in the absence of *P. aeruginosa*. RNAi against *vhp-1*, which encodes a MAPK phosphatase that negatively regulates PMK-1, results in hyperactivation of PMK-1[25]. We observed that RNAi of *vhp-1* resulted in sustained induction of the *Phsp-4::GFP(zcIs4)* reporter with the relatively non-pathogenic, standard laboratory food source *E. coli* OP50 as the only bacteria present in the assay (Fig. 4a). RNAi-mediated knockdown of *vhp-1* had little effect on development of WT (Fig. 4b). In contrast, RNAi of *vhp-1* severely impaired development of the *xbp-1(zc12)* mutant in the absence of pathogenic bacteria (Fig. 4b) in a manner similar to that observed for the *xbp-1* mutant when propagated on *P. aeruginosa* (Fig. 2a). Both the induction of *hsp-4* and the larval developmental phenotype of the *xbp-1* mutant resulting from RNAi of *vhp-1* were suppressed by *pmk-1* (Figs. 4a and 4b), demonstrating that the observed effects of RNAi of *vhp-1* were mediated by the PMK-1-dependent immune response.

Our data suggest that the XBP-1-mediated UPR plays an essential role during *C. elegans* larval development by protecting against the activation of innate immunity. Transcriptional profiling of the *C. elegans* response to infection with *P. aeruginosa*[26],[27] has revealed the induction of at least 300 genes[26], nearly half of which are predicted to be trafficked through the ER. Although this innate immune response promotes pathogen resistance, retarding the intestinal accumulation of *P. aeruginosa* and substantially increasing the fraction of the population that survives through larval development (as illustrated by the survival of WT relative to the *pmk-1(km25)* mutant (Fig. 3 and Supplementary Fig. 5)), our data suggest that

innate immunity also constitutes a physiologically relevant source of ER stress that necessitates the compensatory activity of the UPR in *C. elegans* larval growth and development. An ancient role for the UPR may be to facilitate the development and survival of metazoan cell types that are exposed to the environment and mount a defensive, secretory response to microbial pathogens and abiotic toxins[4,28]. Our data not only support this hypothesis, but more specifically suggest that in fact the essential role of XBP-1 lies not in the neutralization of these environmental insults, but instead in conferring protection against the potentially lethal ER stress that is induced by this response. Our findings support the idea that the microbiota of multicellular organisms may represent the most commonly encountered and physiologically relevant inducer of such ER stress. Consistent with this hypothesis is the Paneth cell death observed in *XBP1* intestinal knockout mice[10]. Evolutionary conservation of the function of XBP-1 that we have defined in *C. elegans* would implicate the UPR in protection against ER toxicity caused by activation of immune and inflammatory pathways in mammals, where the UPR may have a critical role in the pathogenesis of infectious and inflammatory autoimmune diseases.

## Methods Summary

*C. elegans* strains were maintained as described[29]. Strains used in this study were N2 Bristol WT strain, SJ4005 *Phsp-4::GFP(zcIs4)*, ZD305 *pmk-1(km25);Phsp-4::GFP(zcIs4)*, ZD441 *atf-7(qd22 qd130); Phsp-4::GFP(zcIs4)*, ZD361 *xbp-1(zc12)*, RB545 *pek-1(ok275)*, RB772 *atf-6(ok551)*, ZD363 *xbp-1(zc12);pmk-1(km25)*, ZD416 *xbp-1(tm2457)*, ZD417 *xbp-1(tm2457);pmk-1(km25)*, ZD418 *xbp-1(tm2482)* and ZD419 *xbp-1(tm2482);pmk-1(km25)*. Strain construction is detailed in Supplementary Methods. All *P. aeruginosa* (strain PA14) plates were prepared as described[13], exceptions are detailed in Supplementary Methods. For tunicamycin treatments, tunicamycin was added to *E. coli* plates at 5 μg/ml final concentration. Images were acquired with an Axioimager Z1 microscope using worms anesthetized in 0.1% sodium azide. Transmission electron microscopy was performed as described in Supplementary Methods. For RNA analysis, L1 larvae were synchronized by hypochlorite treatment, washed onto OP50 plates and grown at 20 C for 23 h on OP50, then washed in M9 onto treatment plates. After 4 h, worms were washed off plates and frozen in liquid nitrogen. RNA and qRT-PCR methods are detailed in Supplementary Methods. For development assays, *E. coli* OP50 plates were prepared in parallel with PA14 plates. Strains were egg laid in parallel onto 4 plates each of PA14 and OP50 (at least 80 eggs for each strain and treatment), and fraction of worms growing to at least the L4 larval stage ("L4+") between the plates was averaged. The L4 stage of development was chosen due to ease of scoring vulval development. RNAi by feeding of bacteria[30] was performed as described by propagating stains on *E. coli* HT115 carrying the RNAi clones as indicated at 20° C, and adult progeny were used to lay eggs in experiments, which were performed at 25° C. Details regarding RNAi clones are in Supplementary Methods. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc.).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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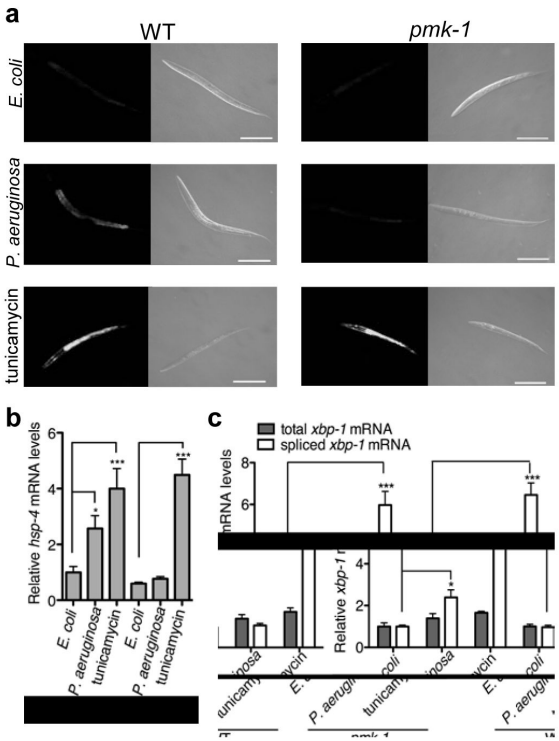


**Figure 1. PMK-1 p38 MAPK-dependent activation of the IRE-1-XBP-1-dependent UPR by *P. aeruginosa* infection**

**(a)** Fluorescence and DIC microscopy of WT and mutant larvae carrying the *Phsp-4:GFP(zcIs4)* transgene 24 h after egg lay on indicated treatments (Scale bar = 100μm). **(b)** qRT-PCR of endogenous *hsp-4* mRNA levels. **(c)** qRT-PCR analysis of total and spliced *xbp-1* mRNA levels. Values represent fold change relative to WT on OP50 ± s.e.m. (n = 4 independent experiments, *P < 0.05 and ***P < 0.001, two-way ANOVA with Bonferroni post test).

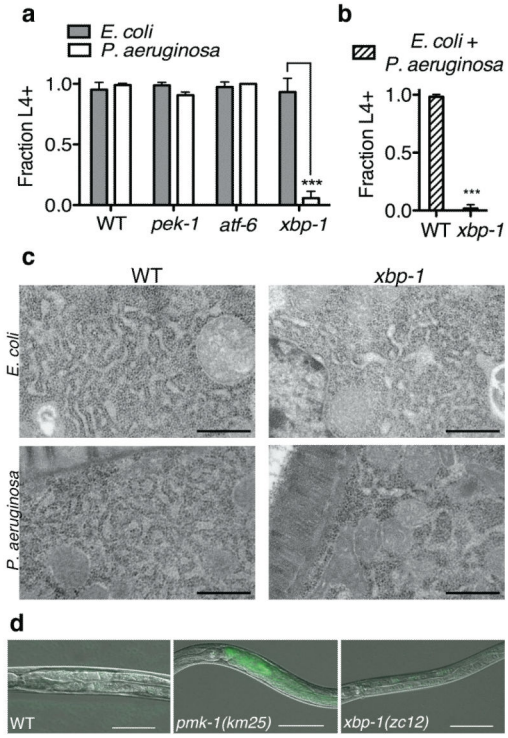


**Figure 2. XBP-1 is required for *C. elegans* development and survival on *P. aeruginosa***

**(a)** Development of indicated mutants to the L4 larval stage or older after 3 d at 25° C on either *P. aeruginosa* PA14 or *E. coli* OP50, or (b) mixture of PA14 and *E. coli* HB101. Plotted is mean ± s.d. (n = 3-4 plates, ***P<0.001, two-way ANOVA with Bonferroni post test in (a), and n = 4 plates, *** p<0.001 two tailed t-test in (b)). Results in (a) and (b) are representative of 3 independent experiments. **(c)** Transmission electron microscopy of L3 larvae at 60,000x magnification (Scale bar = 500 nm). **(d)** Fluorescence and DIC microscopy overlay of larvae 40 h after egg-lay on *P. aeruginosa* PA14 expressing GFP.

Strain Construction

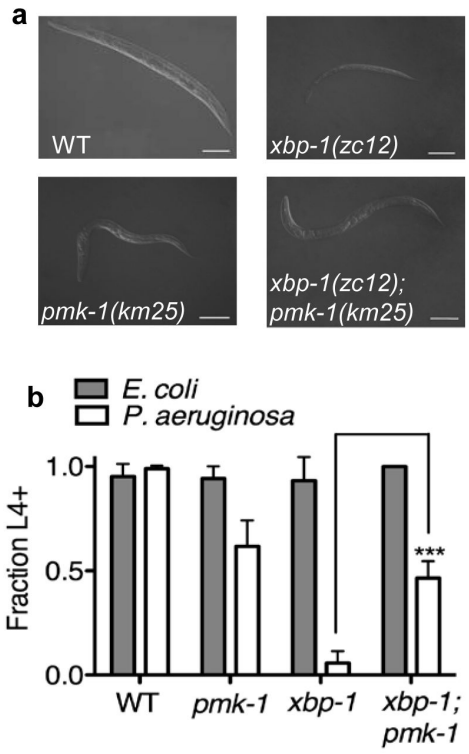


**Figure 3. Suppression of the *P. aeruginosa*-induced larval lethality phenotype of *xbp-1(zc12)* by *pmk-1(km25)***

(**a**) DIC microscopy of representative worms of each genotype 3 d after egg-lay on *P. aeruginosa* PA14 (Scale bar = 100 μm). (**b**) Development of indicated mutants to the L4 larval stage or older after 3 d at 25° C on either *P. aeruginosa* PA14 or *E. coli* OP50. Plotted is the mean ± s.d. (n = 3-4 plates, ***P < 0.001, two-way ANOVA with Bonferroni post test). Representative results of 3 independent experiments.

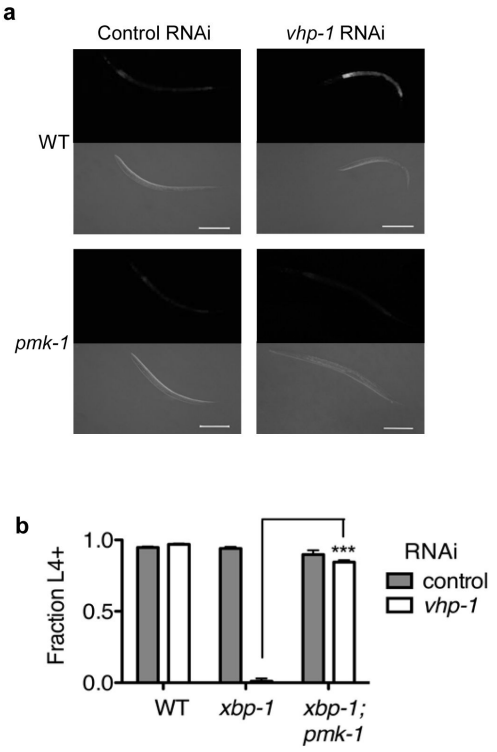


**Figure 4. XBP-1 is required for development and survival during pathogen-independent constitutive activation of PMK-1**

**(a)** Fluorescence and DIC microscopy of WT and xbp-1 larvae carrying the *Phsp-4:GFP(zcIs4)* transgene, which have been treated with control (empty vector) or *vhp-1* RNAi, 24 h after egg lay. (b) Development to the L4 larval stage or older after 3 d at 25° C of WT, *xbp-1*, and *xbp-1;pmk-1* mutants that have been subjected to RNAi of *vhp-1* and propagated on *E. coli* OP50.

Plotted is mean ± s.e.m (n = 3 independent experiments, ***P < 0.001, two-way ANOVA with Bonferroni post test).