

# Complementary Signaling Pathways Regulate the Unfolded Protein Response and Are Required for *C. elegans* Development

Xiaohua Shen,<sup>1</sup> Ronald E. Ellis,<sup>4</sup> Kyungho Lee,<sup>2</sup> Chuan-Yin Liu,<sup>1</sup> Kun Yang,<sup>3</sup> Aaron Solomon,<sup>5</sup> Hiderou Yoshida,<sup>6</sup> Rick Morimoto,<sup>5</sup> David M. Kurnit,<sup>3</sup> Kazutoshi Mori,<sup>6</sup> and Randal J. Kaufman<sup>1,2,7</sup>

<sup>1</sup>Department of Biological Chemistry

<sup>2</sup>Howard Hughes Medical Institute

<sup>3</sup>Pediatrics and Human Genetics  
University of Michigan Medical School  
Ann Arbor, Michigan 48109

<sup>4</sup>Department of Molecular, Cellular  
and Developmental Biology  
University of Michigan  
Ann Arbor, Michigan 48109

<sup>5</sup>Department of Biochemistry, Molecular Biology  
and Cell Biology

Rice Institute for Biomedical Research  
Northwestern University  
Evanston, Illinois 60208

<sup>6</sup>Graduate School of Biostudies  
Kyoto University  
Sakyo-ku, Kyoto 606-8304  
Japan

## Summary

The unfolded protein response (UPR) is a transcriptional and translational intracellular signaling pathway activated by the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER). We have used *C. elegans* as a genetic model system to dissect UPR signaling in a multicellular organism. *C. elegans* requires *ire-1*-mediated splicing of *xbp-1* mRNA for UPR gene transcription and survival upon ER stress. In addition, *ire-1/xbp-1* acts with *pek-1*, a protein kinase that mediates translation attenuation, in complementary pathways that are essential for worm development and survival. We propose that UPR transcriptional activation by *ire-1* as well as translational attenuation by *pek-1* maintain ER homeostasis. The results demonstrate that the UPR and ER homeostasis are essential for metazoan development.

## Introduction

Protein conformational diseases, such as  $\alpha$ 1-antitrypsin deficiency and cystic fibrosis, are associated with the accumulation of unfolded proteins in the ER (Aridor and Balch, 1999; Kaufman, 1999; Kopito and Ron, 2000). Expression of mutant or even some wild-type proteins, viral infection, energy or nutrient depletion, extreme environmental conditions, or stimuli that elicit excessive calcium release from the ER lumen compromise protein-folding reactions in the ER, causing unfolded proteins to accumulate, and initiate signals that are transmitted

to the cytoplasm and nucleus. This adaptive response includes: (1) the transcriptional activation of genes encoding ER-resident chaperones and folding catalysts and protein degrading complexes that augment ER folding capacity, and (2) translational attenuation to limit further accumulation of unfolded proteins in the ER (Kaufman, 1999; Mori, 2000). In mammals, this signal transduction cascade, termed the unfolded protein response (UPR), is mediated by three types of ER transmembrane proteins: the protein-kinase and site-specific endoribonuclease IRE1 (Tirasophon et al., 1998; Wang et al., 1998); the eukaryotic translation initiation factor 2 kinase, PERK/PEK (Shi et al., 1998; Harding et al., 1999); and the transcriptional activator ATF6 (Yoshida et al., 1998, 2001a). If adaptation is not sufficient, an apoptotic response is initiated leading to activation of JNK protein kinase and caspases 7, 12, and 3 (Urano et al., 2000a; Nakagawa et al., 2000; Yoneda et al., 2001). Two perplexing questions in biology and medicine are: (1) what is the physiological role of the UPR in the absence of ER stress, and (2) how do cells decide between adaptation and cell death responses upon activation of the UPR?

In *Saccharomyces cerevisiae*, the UPR is controlled by the ER transmembrane protein kinase/endoribonuclease Ire1p (Nikawa and Yamashita, 1992; Cox et al., 1993; Mori et al., 1993). Following ER stress, Ire1p is essential for survival by initiating splicing of the mRNA encoding the basic leucine zipper (bZIP) transcription factor Hac1p (Cox and Walter, 1996; Kawahara et al., 1997; Mori et al., 2000). Whereas unspliced *HAC1* mRNA is poorly translated, spliced *HAC1* mRNA is efficiently translated to yield a protein that acts as a more potent transcriptional activator (Chapman and Walter, 1997; Kawahara et al., 1997; Mori et al., 2000). As the cellular level of Hac1p increases, the transcription of genes harboring UPR elements (UPREs) in their promoters is activated.

In the mammalian genome, there are two homologs of yeast IRE1, *Ire1 $\alpha$*  and *Ire1 $\beta$* . Whereas IRE1 $\alpha$  is expressed in all cells and tissues, IRE1 $\beta$  expression is primarily restricted to intestinal epithelial cells (Bertolotti et al., 2000). Upon overexpression, the endoribonuclease of either IRE1 $\alpha$  or IRE1 $\beta$  is sufficient to activate the UPR transcriptional response (Tirasophon et al., 1998, 2000; Wang et al., 1998). Therefore, IRE1-mediated splicing of an RNA target is likely one mechanism that activates the UPR. However, previously, a *HAC1* homolog had not been identified in the sequenced genomes of *C. elegans* or *D. melanogaster*, or in the sequences available from the human or murine genomes. Interestingly, deletion of either or both the *Ire1 $\alpha$*  and *Ire1 $\beta$*  genes did not interfere with transcriptional activation of several UPR genes or survival following ER stress in cultured mouse cells (Urano et al., 2000a, 2000b; Lee et al., 2002). Therefore, at least one additional inductive and adaptive mechanism exists. Finally, overexpression of either *Ire1 $\alpha$*  or *Ire1 $\beta$*  was also linked to apoptosis, leading to the question as to whether these pathways are adaptive or apoptotic responses to ER stress (Iwawaki et al., 2001).

<sup>7</sup>Correspondence: kaufmanr@umich.edu

To date, there is no evidence that directly supports the notion that metazoan cells require IRE1 to induce the UPR.

PERK and IRE1 display homology in their luminal domains and sense the same ER stresses through a similar mechanism (Kaufman, 1999; Bertolotti et al., 2000; Liu et al., 2000). PERK/PEK phosphorylates the  $\alpha$  subunit of eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) at Ser<sup>51</sup> to attenuate protein synthesis, thereby reducing protein folding load (Shi et al., 1998; Harding et al., 1999; Scheuner et al., 2001). Transcriptional induction of BiP (GRP78), a marker for the UPR, was significantly reduced both in *Perk* null cells and in cells bearing a homozygous Ser<sup>51</sup>→Ala mutation (S51A) in the eIF2 $\alpha$  gene to prevent phosphorylation (Harding et al., 2000; Scheuner et al., 2001). In addition, *Perk*<sup>-/-</sup> cells and S51A eIF2 $\alpha$  mutant cells were exceptionally sensitive to apoptosis induced by ER stress, suggesting that the translational component of the UPR is required for cultured cells to survive this stress (Harding et al., 2000; Scheuner et al., 2001).

Surprisingly, although the role of the UPR in cultured cells is well characterized, the role of the UPR in development is not known. The UPR appears intact in *Ire1*-deleted cells in culture, however, *Ire1* $\alpha$ <sup>-/-</sup> mouse embryos die around day 10.5 of gestation (Urano et al., 2000a,b; Lee et al., 2002). *Ire1* $\beta$ <sup>-/-</sup> mice survive without a significant phenotype, although they display increased sensitivity to experimental-induced colitis, consistent with its expression restricted to this tissue (Bertolotti et al., 2001). Mice harboring a deletion in *Perk* or a mutation at the PERK phosphorylation site in eIF2 $\alpha$  survive to birth, at which point they develop disturbances in glucose metabolism, pancreatic  $\beta$ -cell insufficiency, and hypoinsulinemia (Harding et al., 2001; Scheuner et al., 2001). In addition, homozygous *Perk* deletion in humans causes infancy onset type-I diabetes (Delepine et al., 2000). These studies demonstrate that mutations in these genes cause human disease and that the UPR plays an important role in development. To develop a simple genetic model for dissecting the UPR in higher eukaryotes, we studied the requirement for homologs of mammalian *Ire1* and *Perk* in *C. elegans*. We show that in *C. elegans*, *ire-1* controls ER stress-induced novel splicing of mRNA that encodes the transcription factor XBP-1, the *C. elegans* homolog of yeast Hac1p. Furthermore, our data suggest that splicing of *xbp-1* mRNA is required for activation of the UPR and this pathway is partially redundant with *pek-1* signaling as a requirement for larval stage 2 development in *C. elegans*.

## Results

### Transcription of *hsp-3* and *hsp-4* Is Induced upon ER Stress in *C. elegans*

The most well characterized transcriptional target of the UPR is the gene encoding BiP (*grp78*) (Kaufman, 1999). *C. elegans* has two homologs of mammalian BiP, HSP-3, with a KDEL ER-retention motif, and HSP-4, with a HDEL ER-retention motif. By contrast, yeast and mammals have either BiP-HDEL or BiP-KDEL, respectively (Kaufman, 1999). In order to determine whether *C. elegans* has a UPR, the expression of *hsp-3* and *hsp-4* were analyzed upon ER stress induced by dithiothreitol (DTT),

a reducing reagent that disrupts disulfide bond formation in the ER. Northern blot and quantitative *Taqman* RT-PCR analysis showed that in mixed stage worms grown in liquid culture, expression of both *hsp-3* and *hsp-4* increased with time, and reached a plateau at 4–6 hr (Figures 1A and 1B). At the plateau, *hsp-3* was induced about 2-fold, and *hsp-4* was induced about 9-fold in mixed stage animals. Furthermore, the basal expression of *hsp-3* was about 5-fold higher than *hsp-4*. Thus, *C. elegans* has a UPR and *Taqman* RT-PCR allows us to analyze the UPR in single worms.

### RNA Interference Shows that Either *ire-1* or *pek-1* Is Required for Larval Development in *C. elegans*

We designated the *C. elegans* homologs for mammalian *Ire1* and *Perk* as *ire-1* and *pek-1*, respectively. Using RT-PCR, we cloned and sequenced both genes (Figure 2A; Genbank accession number: AF435952 for *ire-1* and AF435953 for *pek-1*). To study the requirements for *ire-1* and *pek-1* in the UPR and development, RNA interference (RNAi) was used to inactivate each gene. The *ire-1(RNAi)* and *pek-1(RNAi)* animals displayed a linear growth identical to the mock control progeny from adults injected with buffer alone. They became late L2 larvae at 1.5 days after eggs were laid and matured to adulthood at 3 days. The *ire-1(RNAi); pek-1(RNAi)* animals also became early L2 larvae by 1.5 days, and at that time were indistinguishable from controls or the single mutants. However, after the *ire-1(RNAi); pek-1(RNAi)* animals reached L2, they became very sluggish and sick. Six days after eggs were laid, 90% (n = 120) remained as L2 larvae, identified by germline morphology.

Close examination of *ire-1(RNAi); pek-1(RNAi)* animals revealed small vacuoles in the intestinal cells at 1.5 days after the eggs were laid. These vacuoles increased in number and size by 2.5 days (Figure 2E). By 4 days, the connection between the intestine and pharynx narrowed, so bacteria could not pass through to the intestine. Furthermore, the intestine fragmented, and large empty spaces appeared in the worm. By 5 or 6 days, most intestinal tissues degraded and the cytoplasm of the intestinal cells disappeared, with only nuclei remaining distinct (Figure 2E). This phenotype is characteristic of necrosis (Wyllie, 1981; Hall et al., 1997). These RNAi results show that *ire-1* and *pek-1* are redundant genes that control a pathway essential for larval development.

### *ire-1* and *pek-1* Deletion Mutants Are Viable

To confirm the RNAi results, we identified deletion mutants of *ire-1* and *pek-1*. The *ire-1(v33)* null mutation was isolated from an EMS-mutagenized worm library by screening short alleles by nested PCR. We found an 878 bp deletion extending from -199 bp upstream of the ATG start codon to bp 679 of the *ire-1* gene (Figure 2A). The *ire-1(v33)* mutants were viable, but their growth was somewhat slower than observed for wild-type animals.

We found that the *pek-1(ok275)* mutant (isolated by the *C. elegans* Gene Knockout Consortium, Oklahoma) had a 2013 bp deletion, extending from 495 bp to 2507 bp in the *pek-1* gene. Sequencing analysis showed that

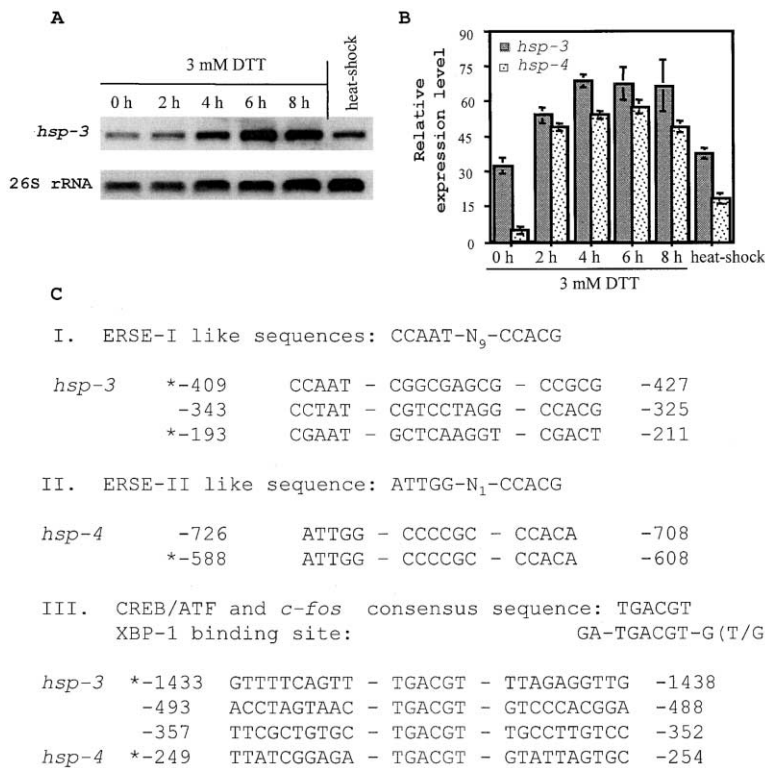


Figure 1. *C. elegans* Has an Unfolded Protein Response

(A) Northern blot analysis of *hsp-3* expression in response to DTT. Mixed stage worms grown in liquid medium were treated with DTT or heat shock at 30°C for 1 hr.

(B) Quantitative *Taqman* RT-PCR analysis of *hsp-3* and *hsp-4* expression. About 20 ng of total RNA prepared as above was used for each *Taqman* reaction. Expression of *hsp-3* and *hsp-4* was normalized to *act-1/act-3*. The error bars represent standard deviation calculated from three reactions.

(C) Potential UPR regulatory elements in the promoters of *hsp-3* and *hsp-4*. Numbers are relative to the ATG start codon. The asterisks refer to complementary sequences that are in the promoter regions of the respective genes.

the transcript was missing a 1535 bp (from 280 bp to 1815 bp) fragment that included exons 3 to part of exon 8 (Figure 2A). Although the deletion was in frame, loss of the transmembrane domain predicts the mutant PEK-1 is mislocalized to the ER lumen, causing a loss of function. These *pek-1(ok275)* mutants were indistinguishable from the wild-type under normal growth conditions.

#### *ire-1(v33); pek-1(ok275)* Double Mutants Arrest as L2 Larvae with Intestinal Degeneration

Based on our RNAi results, we expected that *ire-1(v33); pek-1(ok275)* homozygous mutants would be dead. To ensure a stable supply of these animals, we constructed the strain *ire-1(v33)/mnC1; pek-1(ok275)*, in which the *ire-1(v33)* mutation is balanced by the marker chromosome *mnC1*. According to Mendelian genetics, one quarter of the progeny should be *ire-1(v33); pek-1(ok275)* homozygotes. We studied a total of 1287 eggs laid by *ire-1(v33)/mnC1; pek-1(ok275)* animals. Three days after eggs were laid, we found that 27% of *ire-1(v33)/mnC1; pek-1(ok275)* progeny failed to mature into wild-type (the phenotype of the heterozygous parents) or Dyp Unc adults (the phenotype of *mnC1*) (Figure 2B). Instead, we observed many L2-arrested animals having *ire-1(v33); pek-1(ok275)* genotypes (Figure 2C). These *ire-1(v33); pek-1(ok275)* double mutants showed intestinal degeneration like that observed in the RNAi studies (Figure 2D).

#### *xbp-1* mRNA Is an IRE-1 Substrate Required for IRE-1 Signaling

To elucidate the mechanism for *hsp-3* and *hsp-4* induction, their promoter regions were analyzed. The *hsp-3*

promoter has three ERSE-I-like sequences (*ER* stress element, Figure 1C) (Yoshida et al., 1998). In contrast, *hsp-4* lacks ERSE-I sites but has two identical sequences similar to ERSE-II (Kokame et al., 2000). In addition, the *hsp-4* promoter contains a mammalian XBP1 (*X* box DNA binding protein) binding site (Clauss et al., 1996), while the *hsp-3* promoter contains three ATF/CREB recognition sites (Kataoka et al., 1994; Koldin et al., 1995). The mammalian XBP1 recognition site in the *hsp-4* promoter suggested the potential importance of *C. elegans* XBP-1 in regulation of *hsp-4* expression upon ER stress.

In the course of these studies, we identified a putative mammalian homolog for yeast *HAC1*—the bZIP transcription factor *Xbp1* (Yoshida et al., 2001b). The protein sequence of human XBP1 was used to search the *C. elegans* protein database, and we identified a hypothetical protein encoded by the gene R74.3, which we designated *xbp-1*. *C. elegans* XBP-1 has a conserved bZIP domain and shares no amino acid homology with human XBP1 or yeast *HAC1* outside of the bZIP region. The *xbp-1* gene contains an additional open reading frame that is in +1 register with the *xbp-1* initiation AUG codon (Figure 3A). Quantitative *Taqman* RT-PCR showed that total *xbp-1* transcription increased 2–3 fold upon ER stress induced by DTT or by inhibition of N-linked glycosylation by tunicamycin treatment (data not shown). Splicing of *xbp-1* mRNA to remove 23 bases was induced between 30 min–1 hr after tunicamycin treatment in wild-type L2 larvae (Figures 3B and 3C). Significantly, this novel mRNA species was not detected in *ire-1(v33)* mutants (Figure 3C). Therefore, excision of the 23 base sequence requires IRE-1 and would generate a +1 translation shift into the second reading frame.

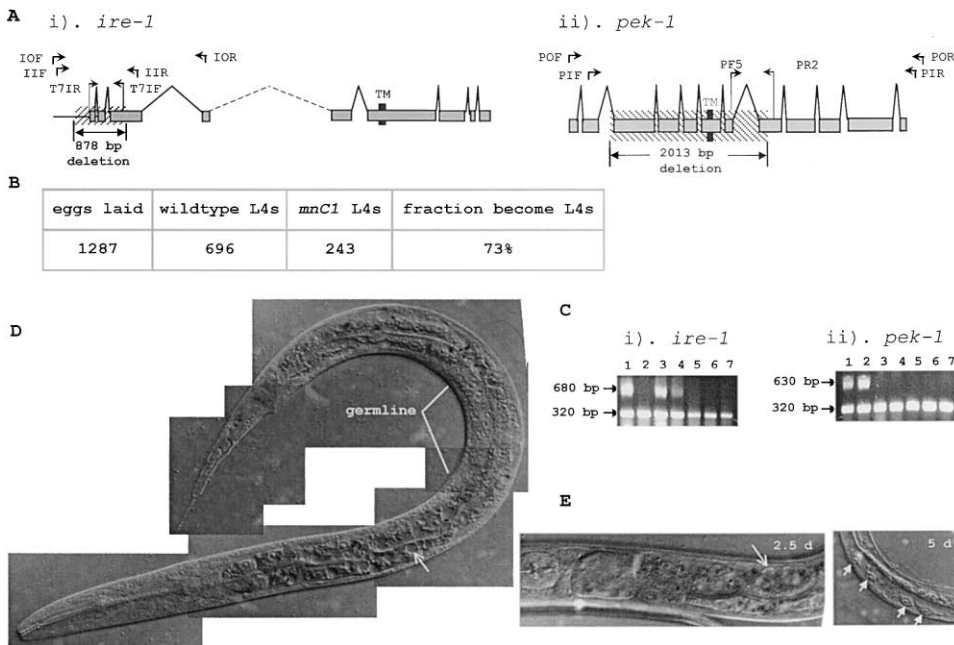


Figure 2. *ire-1* and *pek-1* Signal Redundant Pathways Required for Larval Development

(A) Isolation of *ire-1(v33)* and *pek-1(ok275)* deletion alleles. The *ire-1* gene (C41C4.4) maps to chromosome II, and features one unusually large intron (7.7 kb, indicated by — —) that contains a second gene: *unc-105*. The *pek-1* gene (F46C3.1) maps to chromosome X. Positions of primers used for PCR reactions are depicted. Regions deleted are indicated. TM, transmembrane domain.

(B) Isolation of *ire-1(v33); pek-1(ok275)* homozygotes. *ire-1(v33)/mnC1; pek-1(ok275)* animals are wild-type and *mnC1; pek-1(ok275)* are Dpy Uncs. In addition, we observed animals arrested as L2 larvae.

(C) PCR analysis confirmed that L2-arrested animals were *ire-1(v33); pek-1(ok275)* homozygotes. Lane 1, N2; Lane 2, *ire-1(v33)*; Lane 3, *pek-1(ok275)*; Lane 4, *ire-1(v33)/mnC1; pek-1(ok275)*; Lanes 5–7 are L2-arrested larvae. The internal control reaction using primers PF2/PR5 yielded a 320 bp band. (i) To analyze the *ire-1* gene, primers from inside the *ire-1* deletion (T7IF/T7IR) were used. *ire-1(v33)* (lane 2) and L2-arrested larvae (lanes 5–7) were missing a 680 bp band. (ii) To analyze the *pek-1* gene, the primers PF5 from inside the *pek-1* deletion and PR2 were used. All of *pek-1(ok275)* homozygotes and L2-arrested larvae were missing a 630 bp band (lanes 3–7).

(D) and (E) Nomarski micrograph of a 3-day-old *ire-1(v33); pek-1(ok275)* mutant (D) and *ire-1(RNAi); pek-1(RNAi)* animal (E). Open arrows indicate vacuoles in intestinal cells. Closed arrows indicate the nuclei of necrotic intestinal cells. The germ line *ire-1(v33); pek-1(ok275)* animals did not develop past the L2 stage.

The 23 base intron is predicted to form an RNA secondary structure containing two stem-loop signatures with seven-membered rings, similar to that found in yeast *HAC1* mRNA (Figure 3D). To test whether *xbp-1* mRNA can be cleaved by IRE-1, an in vitro cleavage assay was performed using human IRE1 $\alpha$  expressed in COS-1 monkey cells. Western blot analysis confirmed that both the wild-type and endoribonuclease mutant (K907A) IRE1 $\alpha$  were expressed (Figure 3E). Human IRE1 $\alpha$  cleaved the *C. elegans xbp-1* RNA substrate (399 nt fragment) at the expected 5' and 3' cleavage sites, releasing the 23 nt intron and yielding two fragments (266 nt and 110 nt) that were detected on a polyacrylamide gel (Figure 3F, lane 3). Although the IRE1 $\alpha$  endoribonuclease mutant (K907A) was expressed at a much higher level as previously described (Tirasophon et al., 1998, 2000), it cleaved *xbp-1* RNA to a much lesser extent (Figure 3F, lane 2). These results demonstrate that the RNase activity of IRE1 $\alpha$  is required for *xbp-1* cleavage. Mutation of the conserved sites (–3, –1, and +3) in both the 5' and 3' loops interfered with the cleavage reaction (Figure 3F, lanes 5, 9, 11, 14, 16, and 17). By contrast, mutation of the nonconserved base (–2) in either the 5' or 3' loop did not prevent cleavage (Figure 3F, lanes 7 and 15). Moreover, double mutations at either

–1 or –3 sites within both the 5' and 3' loops abolished or significantly reduced cleavage, respectively (Figure 3F, lanes 12 and 13).

We also tested the genetic interaction between *xbp-1* and *pek-1*. Though *pek-1(ok275); xbp-1(RNAi)* eggs hatched normally, they arrested at or prior to the L2 larval stage (Figure 4A). In addition, the *pek-1(ok275); xbp-1(RNAi)* animals showed an intestinal defect resembling that of *ire-1(v33); pek-1(ok275)* double mutants (Figure 4B). By contrast, inactivating *xbp-1* in either *ire-1* or in wild-type worms did not interfere with development. Therefore, RNAi experiments demonstrated that *xbp-1* and *pek-1* mediate redundant pathways that are essential for worm development, and our results are consistent with *xbp-1* acting downstream of *ire-1* in the same pathway.

#### *ire-1*, *xbp-1*, and *pek-1* Are Required for the UPR in *C. elegans*

To determine if silencing *ire-1*, *xbp-1*, and *pek-1* expression would affect the UPR, quantitative *Taqman* RT-PCR was used to analyze *hsp-3* and *hsp-4* expression in affected animals. Since *ire-1(v33); pek-1(ok275)* and *pek-1(ok275); xbp-1(RNAi)* mutants did not grow to adulthood, we reasoned that the pathway mediated by

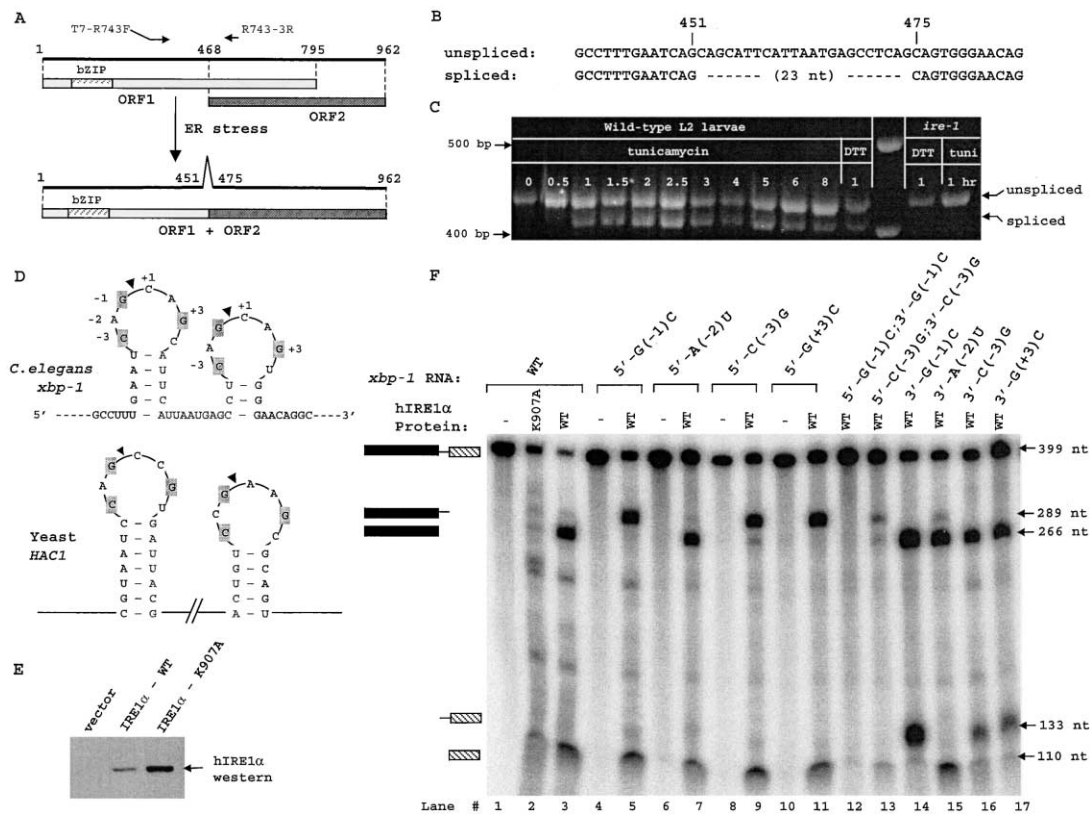


Figure 3. *C. elegans xbp-1* mRNA Is the Substrate for the Endoribonuclease Activity of IRE-1

(A) Schematic representation of the two large open reading frames encoded by *xbp-1* transcripts before and after stress-induced splicing. Numbers above the mRNA denote nucleotide positions with the translation start site set at 1. Following ER stress, splicing of an unconventional intron between nucleotides 451 and 475 results in a combined, longer ORF. Primers T7-R743F/R743-3R (arrows) were used to detect *xbp-1* spliced products and to prepare templates for in vitro cleavage.

(B) Nucleotide sequences from cDNAs corresponding to unspliced and spliced forms of *xbp-1* mRNA.

(C) Time course of *xbp-1* mRNA splicing. RNA prepared from drug-treated wild-type L2 larvae and mixed-staged *ire-1(v33)* mutants was analyzed by RT-PCR and agarose gel electrophoresis.

(D) Secondary structures of the splice sites of *C. elegans xbp-1* and *S. cerevisiae HAC1* mRNAs. The six nucleotides that are indispensable for the yeast *HAC1* mRNA cleavage reaction are conserved in *xbp-1* mRNA and highlighted in the diagram. Based on the *HAC1* mRNA cleavage reaction, the potential cleavage sites in *xbp-1* mRNA were identified (arrowheads). Cleavage at these two sites and subsequent ligation of the 5' and 3' fragments would yield the spliced form of *xbp-1* mRNA.

(E) Western blot analysis of human IRE1 $\alpha$  wild-type and endoribonuclease mutant (K907A) expressed in COS-1 cells.

(F) In vitro cleavage of *C. elegans xbp-1* RNA by human IRE1 $\alpha$ . Human IRE1 $\alpha$  protein was immunoprecipitated and incubated with the *xbp-1* RNA substrates (399 nt). Wild-type *xbp-1* RNA yields two cleavage fragments (266 nt and 110 nt) detected by polyacrylamide gel electrophoresis (lane 3). Each mutant prepared was a transition mutation. Cleavage in the 5' and 3' loops was detected by the appearance of 289 nt (lanes 5, 9, and 11) and 133 nt (lanes 14, 16, and 17) fragments, respectively. Control reactions were performed without adding immunoprecipitated hIRE1 $\alpha$  proteins (lanes 1, 4, 6, 8, and 10).

*ire-1/xbp-1* and *pek-1* might be required for L2 development. Therefore, individual 1.5-day-old L2 larvae were studied. Expression of the two *hsp* genes was normalized to that of *act-1* and *act-3*.

In wild-type L2 larvae, the basal expression of *hsp-3* was about 19-fold higher than that of *hsp-4* (Figures 5A and 5B). In contrast to 2- and 10-fold induction of *hsp-3* and *hsp-4*, respectively, in mixed staged animals (Figure 1B), in L2-stage larvae, expression of *hsp-3* and *hsp-4* was induced 9.3-fold and 61-fold, respectively, upon DTT treatment. Furthermore, expression of *hsp-3* and *hsp-4* was induced 3.9- and 29-fold, respectively, upon tunicamycin treatment. In *ire-1(v33)* mutants, the basal expression of the two *hsp* genes was similar to that of N2 animals. However, the induction of the *hsp-3* gene by DTT or tunicamycin was greatly reduced, and that

of *hsp-4* was almost abolished. Therefore, *ire-1* is required to activate the UPR in *C. elegans*.

As with *ire-1(v33)* mutants, induction of both *hsp* genes was abolished in *xbp-1(RNAi)* animals (Figures 5A and 5B). Furthermore, *pek-1(ok275); xbp-1(RNAi)* animals were defective in inducing both *hsp* genes. By contrast, *pek-1(ok275)* mutants were able to activate transcription of both *hsp* genes to a similar extent as the wild-type. However, the basal expression of both *hsp* genes was increased in the *pek-1(ok275)* mutant. It is possible that *pek-1(ok275)* mutants experience endogenous ER stress during development, consistent with a model where PEK-1 limits ER stress by attenuating protein synthesis. Overall, these results suggest that *ire-1/xbp-1* and *pek-1* play partially complementary roles in eliminating ER stress, where *ire-1/xbp-1* signals

A

day	day 0		day 1				day 2			day 3			day 5	
developmental stage	egg	egg	L1	L2	L3 or older	dead	L2	L3 or older	dead	adult	dead			
number	725	2	723	348	2	373	80	11	632	11	712			
ratio	100%	0%	100%	48%	0%	51%	11%	2%	87%	2%	98%			

B

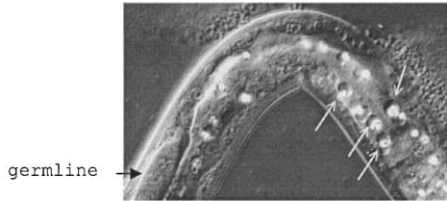


Figure 4. RNAi Shows that *C. elegans* *xbp-1* and *pek-1* Are Redundant Genes Required for Larval Development

(A) Growth of *pek-1(ok275); xbp-1(RNAi)*. Though *pek-1(ok275); xbp-1(RNAi)* eggs hatched normally, significant death (51% of 723 hatched larvae) was observed at day 2 after eggs were laid. By 5 days, nearly 98% of *pek-1(ok275); xbp-1(RNAi)* larvae were dead. (B) Nomarski micrograph of a 2.5-day-old *pek-1(ok275); xbp-1(RNAi)* L2 larvae. The worm is oriented with anterior to the right and ventral up. Open arrows indicate vacuoles present in the intestinal cells. Many distinct granules are also observed.

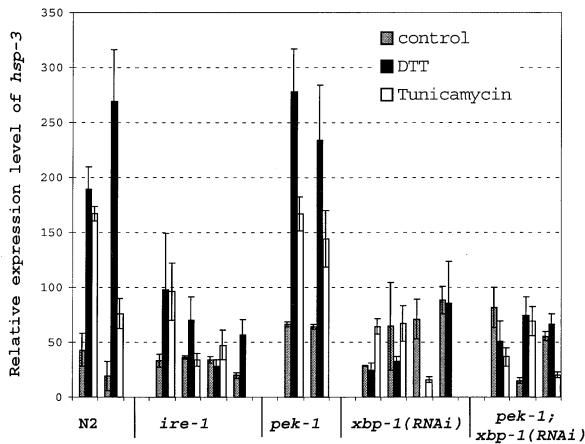
to activate UPR transcription and *pek-1* signals to attenuate protein synthesis.

#### Mutant Animals Are Sensitive to Tunicamycin

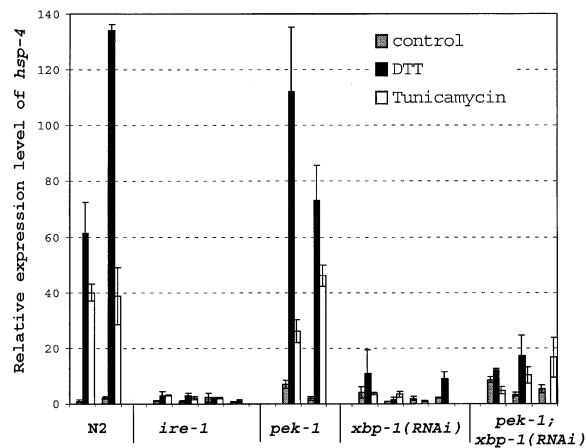
We hypothesize that *ire-1* and *pek-1* protect animals from conditions that promote the accumulation of unfolded proteins in the ER lumen. To test this hypothesis, we studied the survival of wild-type (N2) and mutants upon induction of ER stress by tunicamycin. The growth

of N2 animals was not affected until the tunicamycin concentration reached 5  $\mu\text{g/ml}$  (Figure 6A). At 5  $\mu\text{g/ml}$ , only 8% of N2 matured to the L4 stage or older after 3 days, 29% were arrested at or prior to the L3 stage, and 63% were dead. The arrested N2 animals had many vacuoles in their intestinal cells (data not shown). These vacuoles were indicative of a necrotic cell death, much like that observed in *ire-1(v33); pek-1(ok275)* mutants. In the absence of tunicamycin, 72% of *ire-1(v33)* mutants

A



B



<i>hsp-3</i>	basal (SD)	fold induction DTT (SD)	fold induction tunicamycin (SD)	<i>hsp-4</i>	basal (SD)	fold induction DTT (SD)	fold induction tunicamycin (SD)
N2	31 (17)	9.3 (6.9)	3.9 (0.1)	N2	1.6 (0.9)	61 (0.2)	29 (15.8)
<i>ire-1(v33)</i>	31 (7.5)	2.2 (1.0)	1.8 (1.0)	<i>ire-1(v33)</i>	1.3 (0.7)	2.2 (1.0)	1.9 (1.0)
<i>pek-1(ok275)</i>	65 (1.4)	3.9 (0.4)	2.4 (0.2)	<i>pek-1(ok275)</i>	4.5 (3.5)	26 (14.5)	13.4 (13.6)
<i>xbp-1(RNAi)</i>	63.0 (25.2)	0.8 (0.2)	1.2 (1.0)	<i>xbp-1(RNAi)</i>	2.3 (1.3)	2.9 (1.2)	1.7 (1.7)
<i>pek-1(ok275); xbp-1(RNAi)</i>	50.7 (33.7)	2.3 (2.4)	1.8 (2.4)	<i>pek-1(ok275); xbp-1(RNAi)</i>	5.7 (2.6)	3.3 (2.7)	2.3 (1.5)

Figure 5. *ire-1* and *xbp-1* Are Required for Transcriptional Regulation of the UPR in *C. elegans*

Individual 1.5-day-old L2 larvae were treated with M9 buffer (control), DTT (2.5 mM), or tunicamycin (28  $\mu\text{g/ml}$ ) for 4 hr. Each column in the figure represents one independent treatment and RNA isolation. Expression of *hsp-3* and *hsp-4* was normalized to that of *act-1/act-3*. The error bars show standard deviation based on the normalized duplicate or triplicate reactions. A numerical summary of basal expression and fold induction is shown below. Standard deviations (SD) were calculated from independent experiments.

(A) Relative expression of *hsp-3*.

(B) Relative expression of *hsp-4*.

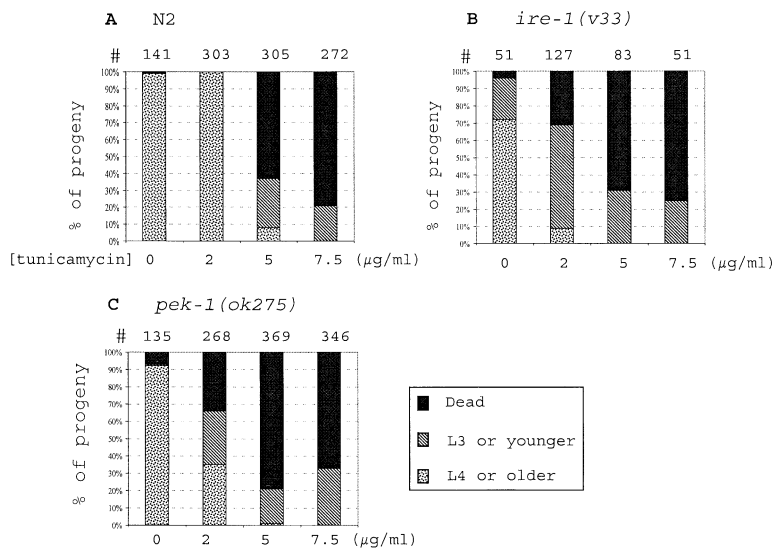


Figure 6. Mutant Animals Are More Sensitive to Tunicamycin

Eggs from each strain were laid on plates containing different concentrations of tunicamycin, counted, and studied after 3 days. The number of eggs studied is listed above each column. The X axis represents tunicamycin concentration. The total worm population was grouped into three fractions: animals that matured to L4 or older, animals that arrested at or prior to the L3 stage, and dead animals. Each fraction was plotted as the percentage of total eggs laid (Y axis).

(A) N2.

(B) *ire-1(v33)*.

(C) *pek-1(ok275)*.

matured to the L4 stage or older within 3 days (Figure 6B). On plates with 2  $\mu\text{g/ml}$  of tunicamycin, only 9% of *ire-1* animals matured to the L4 stage or older, 60% arrested at or prior to the L3 stage, and 31% were dead. As for *pek-1(ok275)* mutants on plates with 2  $\mu\text{g/ml}$  of tunicamycin, only 35% matured to the L4 stage or older, 31% arrested at or prior to the L3 stage, and 34% were dead (Figure 6C). Thus, both *ire-1(v33)* and *pek-1(ok275)* mutants were sensitive to tunicamycin treatment at 2  $\mu\text{g/ml}$ , whereas N2 animals were resistant to this concentration. Furthermore, *ire-1(v33)* mutants appeared more sensitive to tunicamycin than did *pek-1(ok275)*. The double mutant was exquisitely sensitive to tunicamycin (data not shown). These results demonstrate that *ire-1* and *pek-1* provide adaptive functions upon ER stress.

## Discussion

### *C. elegans* Has a UPR that Resembles that of Mammals

Due to the complexity of the mammalian UPR and the difficulty of using a genetic analysis, we initiated study of *C. elegans* as a genetic model to dissect UPR signaling, and study the physiological function of this pathway in an animal. First, we demonstrated that *C. elegans* responds to agents that specifically disrupt protein folding in the ER by transcriptional induction of *hsp-3* and *hsp-4*, two homologs of mammalian BiP—a marker for the UPR in yeast and mammals (Kaufman, 1999). Neither of these two *hsp* genes have transcriptional elements (UPREs) found in yeast UPR-responsive genes, but rather have ERSE-I (Yoshida et al., 1998), ERSE-II (Kokame et al., 2000), and XBP-1 (Clauss et al., 1996) binding sites, implying that the *C. elegans* UPR more closely resembles that of mammals. We then characterized the *C. elegans* homologs of human *Ire1* and *Perk/Pek* to elucidate their roles in signaling the UPR. We isolated a viable *ire-1(v33)* null allele that exhibited slow growth and a small brood size, consistent with the ability of *IRE1* mutant yeast to grow in the absence of ER stress

(Nikawa and Yamashita, 1992; Cox et al., 1993; Mori et al., 1993). Surprisingly, *pek-1(ok275)* mutant worms did not exhibit a discernable phenotype. Although the yeast genome does not encode a *pek-1* homolog, *Perk*<sup>-/-</sup> mice and humans experience progressive diabetes mellitus and exocrine pancreatic insufficiency due to death of pancreatic cells (Delepine et al., 2000; Harding et al., 2001). Thus, in mammals, PERK signaling has acquired a role in maintaining pancreas function and/or survival, perhaps by protecting secretory cells from ER stress.

### *xbp-1* RNA Is a Substrate for IRE-1

The existence of an XBP1 binding motif in the *hsp-3* and *hsp-4* promoters suggested a potential role for *C. elegans* XBP-1 in induction of both *hsp* genes. XBP1 was previously isolated through a yeast one-hybrid screen for proteins that bind ERSE-1 (Yoshida et al., 1998). In addition, XBP1 mRNA is induced upon activation of the UPR (Yoshida et al., 2000; Lee et al., 2002). Here, we showed that *C. elegans xbp-1*, a basic leucine zipper transcription factor, is required for transcriptional activation of *hsp-3* and *hsp-4*. In addition, human IRE1 $\alpha$  directly cleaves *xbp-1* mRNA in vitro at two sites that have stem-loop signatures with seven-membered rings, similar to the Ire1p cleavage sites in yeast *HAC1* mRNA. Residues required for Ire1p cleavage of yeast *HAC1* mRNA are conserved and required for cleavage in *C. elegans xbp-1* mRNA. The out-of-frame splicing of *xbp-1* mRNA mediated by IRE-1 is predicted to generate a novel protein that replaces 114 carboxyl-terminal amino acids with 162 amino acids and that are rich in Gln and Ser residues. In yeast, the *HAC1* mRNA splicing reaction activates the UPR through increased translational efficiency of spliced *HAC1*<sup>i</sup> mRNA and increased activation potential of Hac1p<sup>i</sup> produced from *HAC1*<sup>i</sup> mRNA (Chapman and Walter, 1997; Kawahara et al., 1997; Mori et al., 2000). Translation of unspliced *HAC1* mRNA is inhibited by base-pairing between the *HAC1* intron and the 5' untranslated region of *HAC1* mRNA (Rueggsegger et al., 2001). This type of base-pairing does not occur in *C. elegans*, since no homology exists between the 23

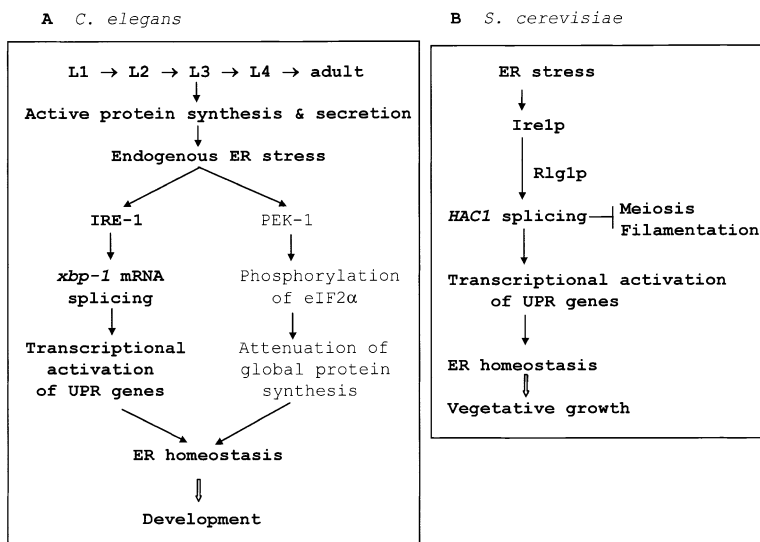


Figure 7. Pathways that signal the UPR during Development in *C. elegans* and *S. cerevisiae*

Pathways that are supported by direct evidence are printed in bold.

(A) The UPR in *C. elegans*. During development, active protein synthesis and secretion might generate endogenous ER stress, which would activate IRE-1 and PEK-1. Activated IRE-1 splices *xbp-1* mRNA, resulting in translation of an active bZIP transcriptional factor. The transcriptional activation of the UPR controlled by IRE-1 and XBP-1, and attenuation of global protein synthesis by PEK-1, should increase the folding capacity of the cell and decrease the protein-folding load, so that ER homeostasis is maintained, allowing for proper development.

(B) The UPR in *S. cerevisiae*. In *S. cerevisiae*, the UPR is a simple, linear pathway requiring only Ire1p, the bZIP transcription factor Hac1p, and tRNA ligase Rlg1p (Sidrauski et al., 1996). ER stress-induced *HAC1* mRNA splicing mediated by Ire1p suppresses yeast differentiation and allows vegetative growth (Schroder et al., 2000).

base intron and other sequences within *xbp-1* mRNA. At present, we do not know whether the IRE-1-dependent splicing of *C. elegans xbp-1* mRNA acts to increase the translational efficiency of *xbp-1* mRNA and/or the activation potential of XBP-1 protein. In mammals, the spliced form of *Xbp1* mRNA produces a protein that is a better transcriptional activator (Yoshida et al., 2001b; Lee et al., 2002).

***ire-1/xbp-1*, and Not *pek-1*, Is Required for Transcriptional Regulation of the UPR in *C. elegans***

The induction of *hsp-3* and *hsp-4* was abolished in the *ire-1(v33)* mutant and by inactivating *xbp-1* with RNAi. The induced expression levels of the two *hsp* genes in *pek-1(ok275)* mutant animals were comparable to those in N2 animals, suggesting that *pek-1* signaling is not required for transcriptional activation of the UPR (Figure 7). By contrast, in the mammalian UPR, PERK signaling is required for maximal transcriptional activation (Harding et al., 2000; Scheuner et al., 2001). In addition, ER stress-induced proteolytic cleavage of the ER-bound ATF6 transcription factor is required for transcriptional induction of *Xbp1* and to activate the UPR in mammals (Ye et al., 2000; Yoshida et al., 2001b; Lee et al., 2002). However, inactivation of *C. elegans atf-6* by RNAi did not produce a significant phenotype in either the wild-type or *pek-1(ok275)* mutant strains (data not shown). Although induction of *hsp-3* and *hsp-4* genes was not affected in *atf-6(RNAi)* animals (data not shown), at this point we cannot rule out that the silencing was incomplete.

**The UPR Protects Animals from ER Stress and Provides an Essential Role in Development**

Upon growth in the presence of tunicamycin to induce ER stress, wild-type animals arrested at or prior to the L3 stage and displayed intestinal cell necrosis similar to that observed in *ire-1(v33); pek-1(ok275)* double mu-

nants growing in the absence of any exogenous ER stress inducer. It seems possible that one important function for the UPR during development is to respond to an increased protein folding demand that occurs as cells differentiate (Figure 7A). The intestine was the organ most visibly defective in worms with a compromised UPR, suggesting that intestinal cells experience ER stress during the L2 developmental stage. Because of increased synthesis of secretory proteins, such as digestive enzymes and molting hormones, that occurs in the intestinal cells, tunicamycin might selectively exacerbate protein-folding problems in these cells to overwhelm IRE-1 and PEK-1 in wild-type animals, causing a phenotype like that of *ire-1(v33); pek-1(ok275)* double mutants. Indeed, *pek-1*-promoter-GFP fusion constructs showed that *pek-1* was strongly expressed in intestinal cells, consistent with an important role in that tissue (data not shown). Furthermore, loss of function of either *ire-1* or *pek-1* increased sensitivity to tunicamycin, consistent with the hypothesis that *ire-1* and *pek-1* alleviate ER stress. Finally, *ire-1(v33)* mutants were more sensitive to tunicamycin than *pek-1(ok275)* mutants, consistent with our observation that *ire-1* plays the major role in transcriptional activation of the UPR upon ER stress.

However, the UPR may provide additional roles in development. For example, the UPR may provide an essential anti-inflammatory response in intestinal cells, thus preventing necrotic death. The *C. elegans* intestine is the first organ to encounter many environmental toxins and infectious agents. Alternatively, a defective UPR might disrupt the secretion of proteins required for development, thus resulting in an L2 arrest. Finally, it is possible that *ire-1* and *pek-1* act as nutritional sensors in the gut to produce hormones that regulate metabolism, possibly similar to the proposed requirement for PERK in pancreatic  $\beta$ -cell function in mice and humans (Delepine et al., 2000; Scheuner et al., 2001; Harding et al., 2001).

It was recently shown that mammalian XBP1 is re-

quired for liver development, cardiomyocyte survival, and terminal differentiation of B lymphocytes to immunoglobulin high-secreting plasma cells (Masaki et al., 1999; Reimold et al., 2000, 2001). Based on our findings in *C. elegans* presented here, we propose that both plasma cell and hepatocyte functions require an expanded ER compartment to accommodate the large amount of secretory protein biosynthesis and secretion. This function is mediated by activation of IRE1-mediated cleavage of *Xbp1* mRNA in response to the increased secretory protein load that occurs as these cells differentiate. Therefore, we hypothesize that the UPR is essential for the function of cells and organs engaged in high level secretory protein synthesis.

In mammals, excessive ER stress results in apoptosis, possibly mediated by caspase 7 and/or caspase 12 (Nakagawa et al., 2000; Yoneda et al., 2001). Since intestinal cell death in *ire-1(v33); pek-1(ok275)* double mutants is necrotic, it seems unlikely to involve programmed cell death signaling pathways. Necrotic-like neuronal death in *C. elegans* is induced by gain-of-function mutations in two genes, *mec-4* and *deg-1*, that encode proteins similar to subunits of the vertebrate amiloride-sensitive epithelial Na<sup>+</sup> channel (Hall et al., 1997). Release of ER Ca<sup>2+</sup> stores appears to be a critical step in necrotic cell death in *C. elegans* (Xu et al., 2001). Mutations in the major Ca<sup>2+</sup> binding chaperone in the ER, calreticulin, or in the ER Ca<sup>2+</sup> release channels *itr-1* (IP3 receptor), and *unc-68* (ryanodine receptor) significantly suppress *mec-4(d)*-induced degeneration (Xu et al., 2001). In addition, specific aspartyl proteases of the cathepsin D family are required for the efficient execution of necrotic-like cell death and mutations that reduce cathepsin D activity suppress *mec-4(d)*-induced degeneration (N. Tavernarakis and M. Driscoll, personal communication). Therefore, it is possible that ER stress might activate *itr-1* and/or *unc-68* to release calcium from the ER and activate aspartyl proteases that cause necrosis.

### ***C. elegans* Provides a Genetic Model to Study the UPR**

One of the outstanding questions regarding the requirement of IRE1 in animals is whether *Xbp1* is the only downstream effector of IRE1. Although the UPR and developmental defects in *ire-1(v33); pek-1(ok275)* and *pek-1(ok275); xbp-1(RNAi)* mutant animals are very similar, there are subtle differences. For example, distinct granules appear in the intestine of the latter that are not observed in the *ire-1(v33); pek-1(ok275)* mutants. In future studies, we should be able to determine whether expression of spliced *xbp-1* corrects all the defects observed in *ire-1(v33)* mutants. Elucidating the role of *ire-1* in worms might provide insight into the basis of intestinal disease in humans. Furthermore, the simplified genetic system described here should provide insight into the roles and interactions of *ire-1/xbp-1* and *pek-1* pathways. Nematodes should be ideal for elucidating the UPR as well, because these pathways are similar but simpler than those in mammals, and worms with mutations in these pathways are viable and convenient for analysis. Analysis of downstream targets should provide insight into how these two signaling pathways act in disease and in health. Finally, the growth defect in *C.*

*elegans* harboring defects in *ire-1/xbp-1* and/or *pek-1* signaling pathways may provide an ideal model to identify and evaluate molecules that influence their signaling for drug development.

### **Experimental Procedures**

#### **Strains and General Methods**

The strain N2 (Bristol) was used as the wild-type strain. The strain JJ529 *rol-1(e91) mex-1(zu121)/mnC1 [dpy-10(e128) unc-52(e444)]* was used to construct *ire-1(v33)/mnC1; pek-1(ok275)* (Sigurdson et al., 1984). The *pek-1(ok275)* mutant was obtained from the *C. elegans* Gene Knockout Consortium, Oklahoma. *C. elegans* strains were cultivated at 20°C unless otherwise indicated (Brenner, 1974). Primers used in this study are posted as an online supplement (available online at [http://www.cell.com/cgi/content/full/\[107\]/7/893/DC1](http://www.cell.com/cgi/content/full/[107]/7/893/DC1)).

#### **Drug Treatments**

For Northern analysis, mixed stage nematodes grown in liquid culture were treated with 3 mM of DTT (Calbiochem) for up to 8 hr. Heat shock treatment was performed at 30°C for 1 hr. For single worm analysis, individual L2 larvae grown on plates were treated with 2.5 mM of DTT or 28 µg/ml of tunicamycin (Calbiochem) for 4 hr. To study survival to tunicamycin, gravid adults were allowed to lay eggs on plates containing tunicamycin (0 to 7.5 µg/ml) for 4 hr and then removed from the plates. Eggs were counted and studied 3 days later.

#### **Northern Blot Analysis**

Total RNA preparation and Northern blot analysis was performed as described (Chen et al., 2000). The blot was hybridized sequentially with digoxigenin (DIG)-labeled DNA probes for *hsp-3* and for 26S rRNA prepared with DIG-high prime labeling Kit (Roche).

#### **RT-PCR and DNA Sequence Analysis of *xbp-1* Transcripts**

RNA was isolated from wild-type L2 larvae and mixed-stage *ire-1* mutants treated with DTT or tunicamycin (MRC, Inc). First-strand cDNA was synthesized using oligo-dT primer (Promega) and amplified using primers T7-R743F and R743-2R. PCR fragments were sequenced. PCR-amplified first-strand cDNA with the primers T7-R743F and R743-3R, producing a 426 bp (unspliced) and a 403 bp fragment (spliced) that were separated on a 2.2% agarose gel.

#### **In Vitro RNA Cleavage**

In vitro cleavage of *xbp-1* mRNA was performed as described by Sidrauski and Walter (1997) and Tirasophon et al. (1998). A 399 bp wild-type *xbp-1* DNA fragment fused to the T7 promoter was amplified from *C. elegans* first-strand cDNA using the primers (T7-R743F and R743-3R). T7 promoter-fused mutant *xbp-1* DNA fragments were generated by overlapping PCR using primer pairs: CE-5'G(-1)C/-AS, CE-5'A(-2)T/-AS, CE-5'C(-3)G/-AS, CE-5'G(+3)C/-AS, CE-3'G(-1)C/-AS, CE-3'A(-2)T/-AS, CE-3'C(-3)G/-AS, and CE-3'G(+3)C/-AS. The <sup>32</sup>P-labeled *xbp-1* RNA was produced by in vitro transcription (Boehringer Mannheim). Wild-type and endoribonuclease mutant (K907A) hIRE1 $\alpha$  were prepared as described (Tirasophon et al., 1998). Purified *xbp-1* RNA fragments were incubated with wild-type or mutant hIRE1 $\alpha$  proteins at 30°C for 1 hr. The reaction mixes were separated on 5% denaturing polyacrylamide gels, and analyzed by autoradiography.

#### **Quantitative Taqman RT-PCR Analysis**

*Taqman* RT-PCR was performed as described by Heid et al. (1996). The lack of DNA contamination in RNA preparations was confirmed by a 1000-fold decrease in quantitative PCR yield when reverse transcriptase was omitted. A unique sequence in the 3' UTR of *act-1* and *act-3* was amplified using *Taqman* PCR (primers act-F/act-R and act-probe). The primers (*hsp-3-F/hsp-3-R*) and the *hsp-3*-probe were used for detecting *hsp-3* transcripts. The primers (*hsp-4-F* and *hsp-4-R*) and the *hsp-4*-probe were used for detecting *hsp-4* transcripts. The relative expression of *hsp-3* and *hsp-4* was normalized to the average signals of *act-1/act-3*.

### RNA Interference

PCR was used to amplify fragments flanked by the T7 promoter at both the 5' and 3' ends. The primer pair T7IF and T7IR amplified a 521 bp region from the ATG start codon on the *ire-1* gene. T7PF and T7PR amplified a 526 bp region from the ATG start codon on the *pek-1* gene. T7-R743F and T7-R743R amplified a 480 bp of exon II of the *xbp-1* gene. Amplified templates were transcribed in vitro to yield dsRNA (Chen et al., 2000) for injection as described (Fire et al., 1998). Only progeny hatched from eggs laid between 12 to 24 hr post injection were studied.

### Isolation of *ire-1(v33)* and *pek-1(ok275)* Deletion Mutants

Nested PCR (primers IOF/IOB and IIF/IIR) was used to screen for shorter alleles of *ire-1* in an EMS mutagenized worm library that was composed of  $1.2 \times 10^6$  mutagenized genomes. The wild-type *ire-1* allele amplified a 2441 bp fragment compared to a 1564 bp fragment from the *ire-1(v33)* deletion allele. A homozygous mutant *ire-1(v33)* was identified by PCR using primers T7IF/T7IR from inside the deleted region. The *ire-1(v33)* mutant strain was backcrossed five times to animals of N2 background. Nested PCR (POF/POR and PIF/PIR) was used to characterize the *pek-1(ok275)* deletion mutant, which generated a 952 bp fragment compared to a 2965 bp fragment from the wild-type allele. A PCR reaction with the primers PF5 (from inside the deletion region) and PR2 identified homozygous *pek-1* mutants.

### Construction of the Strain *ire-1(v33)/mnC1; pek-1(ok275)*

First, *pek-1(ok275)* males were mated to the *rol-1(e91) mex-1(zu121)/mnC1 [dpy-10(e128) unc-52(e444)]* hermaphrodites. The *mnC1/+; pek-1(ok275) /+* hermaphrodite progeny were mated to *ire-1(v33)/+* males. Then, *ire-1(v33)/mnC1; pek-1(ok275) /+* animals were selected by PCR, and self fertilized to generate the *ire-1(v33)/mnC1; pek-1(ok275)* strain.

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