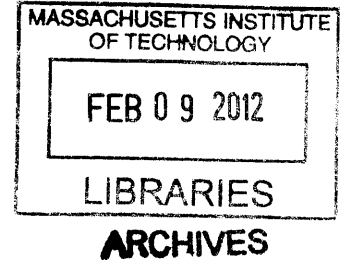# Physiological IRE-1-XBP-1 and PEK-1 Signaling in *Caenorhabditis elegans* Larval Development and Immunity

by

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Physiological IRE-1-XBP-1 and PEK-1 Signaling in *Caenorhabditis elegans* Larval Development and Immunity

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ABSTRACT

The endoplasmic reticulum (ER) is responsible for the folding and processing of approximately one third of proteins in eukaryotic cells, and homeostasis in this compartment is tightly regulated. The Unfolded Protein Response, or UPR, is activated in response to perturbations in protein folding in the ER, collectively termed ER stress. This compensatory mechanism, mediated by IRE-1, PERK-1/PEK-1 and ATF-6 in metazoans, resolves an overcrowded ER lumen in part through the increase of protein degradation and folding. Typical studies focus on activation of the UPR in response to characterized chemical agents that potently alter ER function or protein stability and folding, leaving physiological or native roles for the UPR relatively uncharacterized. Richardson et al previously demonstrated a role for the UPR in innate immunity in *C elegans*. Here, in an effort to understand this role, we demonstrate that intestinal expression of XBP-1 is sufficient to overcome PMK-1 dependent larval lethality on a lawn of pathogenic *Pseudomonas aeruginosa*. Further, we demonstrate that XBP-1 deficiency results in constitutive ER stress even in the absence of pathogenic infection. This elevated ER stress is reflected by increased activities of both IRE-1 and PEK-1 under physiological conditions. Our data suggest that negative feedback loops involving the activation of IRE-1-XBP-1 and PEK-1 pathways serve essential roles not only at the extremes of ER stress but also in the maintenance of ER homeostasis under physiological conditions.

Thesis Supervisor: Dennis Kim
Title: Associate Professor of Biology

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First, I would like to thank Dennis Kim for his unwavering support, patience and insight. I am grateful to all members of the Kim lab for their generosity, and creating a truly fun place to work and be. This work would not have been possible without Claire Richardson, and her inclusivity and scientific skills are incredibly appreciated.

Outside of the lab, I have benefited greatly by the honesty and support of faculty, friends, and family. In particular, I would like to thank Frank Solomon, David Gifford, Nick Polizzi, Angela Luu, Sean Clarke, and my sister, Jennifer Kinkel. You have each contributed to my life in immeasurably positive ways.

Introduction:

ER and UPR

The endoplasmic reticulum (ER) is the first separate compartment in the secretory pathway of cells. Here, proteins destined for the plasma membrane, lysosomes or extracellular secretion are folded and modified prior to trafficking through the Golgi apparatus. To facilitate entry into the ER, nascent amino acid chains marked with an N-terminal signal sequence of 5-10 hydrophobic amino acids are bound by the cytosolic signal recognition particle (SRP). Once SRP is bound, translation pauses while the entire complex is transferred to the ER membrane, where the peptide chain is inserted through Sec61 pores into the lumen of the ER (reviewed in Brodsky et al, 2011).

Once inside the ER lumen, the peptide chain must be properly folded. Although the native conformations of proteins are typically the most energetically favorable, proteins rarely fold properly spontaneously. The interior of the ER is specially equipped with molecular chaperones that minimize misfolded intermediates and increase the likelihood of correct folding. One ER localized chaperone, Grp78/BiP, a member of the highly conserved Hsp70 family of chaperones, binds nascent chains as they are transferred through the Sec61 complex into the ER (Figure 1A). This chaperone, with its conserved N-terminal ATPase domain, and 15KDa C-terminal substrate-binding domain, acts in concert with DnaJ co-chaperones (reviewed in Schröder, 2005). Cycles of ATP catalysis enable these proteins to repetitively bind and release hydrophobic regions of peptide. These repetitive binding events shield hydrophobic domains

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from incorrect intramolecular interactions and prevent aggregation, passively

facilitating proper folding. After several rounds of BiP/DnaJ co-chaperone

interactions, a second ER resident chaperone, GRP94, functions to preferentially

bind partially folded substrates (reviewed in Gething, 1999). It is thought that this

chaperone effectively 'holds' substrates during periods of stress.

In addition to associations with BiP and GRP94, peptides are often

glycosylated on their way to their proper folded conformation. Peptides with an

Asn-X-Ser/Thr consensus sequence are glycosylated with the core

oligosaccharide $GlcNAc_2Man_9Glu_3$ during import by oligosaccharyltransferase

(OST) (reviewed in Schröder, 2008). After immediate removal of the two terminal

glucose residues by α-glucosidase I and II, the remainder of the sugar moiety

associates with lectin chaperones calnexin and calreticulin to promote folding.

The final glucose residue is removed by α-glucosidase II, freeing the protein from

lectin chaperones. If the protein remains unfolded, hydrophobic patches and

$GlcNAc_2Man_9$ will be recognized by UDP-Glc glycoprotein glycosyltransferase

(UGT). The peptide will be reglucosylated, and reenter another cycle of

calnexin/calreticulin binding and release (Aebi et al. 2010). Once the client

protein is properly folded, it will exit the ER. If a protein repeatedly fails to fold in

this cycle, Mannosidase I, a competitor for binding with UGT, will eventually

attach to the peptide. Once bound, Mannosidase I will remove the mannose

residues, and the peptide will be destroyed through ER associated degradation

(ERAD). Through ERAD, proteins reenter the cytoplasm, where they are

degraded by the proteosome (Schröder and Kaufman, 2005).

Chaperone proteins also collectively function to monitor homeostasis in the ER, in addition to their role promote peptide folding (Figure 1B). The ER is a site for lipid synthesis and calcium storage as well as protein folding and trafficking, and perturbations in any of these metabolic functions result in the accumulation of unfolded or misfolded peptides. As unfolded peptides accumulate in the lumen of the ER, excess pools of chaperones are depleted and the Unfolded Protein Response (UPR) is activated, managing potentially lethal ER stress (Liu et al. 2002). To alleviate stress, the UPR upregulates the synthesis of chaperones such as BiP and components of ER-asssociated degradation (ERAD), promotes ER expansion, and attenuates translation (Harding, 2006a).

Aspects of the UPR are conserved from yeast to humans, stressing the importance of this system to regulate ER homeostasis. In metazoans the UPR is comprised of three branches, each mediated by a transmembrane luminal sensor (Figure 1B). IRE-1 (Inositol requiring protein-1), PERK/PEK-1 (protein kinase RNA (PKR)-like ER kinase), and ATF-6 are each situated in the ER membrane, bound to chaperones (Mori, 2009; Ron and Walter, 2007; Schröder and Kaufman, 2005).

IRE-1

The earliest branch of the UPR is activated by the integral membrane protein, IRE-1, and is present in all eukaryotes. Depletion of glucose or the use of agents that perturb the ER environment in mammalian RNA virus transformed

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cells were shown to upregulate GRP78/94, now called BiP, in the mid-1970s (Shiu et al, 1977). This finding led to the discovery of the sequestration of partially folded proteins by BiP, and a 22-bp cis acting element upstream of BiP in yeast that could confer ER stress inducibility to reporter genes. Use of this latter discovery to screen for yeast strains defective in ER stress inducible gene expression led to the identification of the ER targeted serine/threonine kinase now known as IRE-1 (Mori et al, 1992; Cox and Walter, 1993).

IRE-1 is equipped with a lumenal domain to sense the protein-folding environment in the ER and a cytoplasmic effector portion with kinase function. Normally bound to BiP, IRE-1 is freed to oligomerize when BiP is titrated away by an accumulation of substrates in need of proper folding. This release, or the potential direct binding of unfolded proteins to a region that resembles binding domains of major histocompatibility complexes, stimulates trans-autophosphorylation on the cytoplasmic face of IRE-1. Rather than a typical phosphorylation cascade, this activates the precise endonucleolytic cleavage of the only known substrate for IRE-1, an mRNA encoding a bZIP transcription factor called Hac1 in yeast, or Xbp1 in metazoans. Cut twice and religated, the spliced mRNA encodes an activator of UPR target genes. While in yeast the presence of the Hac1 intron represses translation, in metazoans both precursor and spliced forms of XBP1 are translated into proteins with distinct functions. The precursor mRNA is labile and represses UPR target genes, whereas the stable spliced mRNA is a potent activator (reviewed in Ron and Walter, 2007). \

PEK-1

Similar in structure to IRE-1, the metazoan UPR protein PERK oligomerizes in response to ER stress, and is activated by transautophosphorylation of an activation loop on its cytoplasmic domain. Further and extensive phosphorylation of this large kinase loop allows for the recruitment and phosphorylation of its single known effector, eukaryotic translation initiation factor-2 (eIF2) on Ser51. This inhibitory phosphorylation prevents eIF2β from recycling eIF2 to the GTP-bound active form, thereby globally diminishing translation. This attenuation serves to diminish the secretory load of the ER (Harding et al., 2000a). Furthermore, limiting the activity of eIF2α leads to ribosomal skipping through 5' untranslated regions (UTRs) with short upstream inhibitory open reading frames (ORFs). Consequently, in metazoan cells, PERK activation also selectively results in an increase in translation of ATF-4, a transcription factor that regulates stress responses (Harding et al., 2000b).

ATF-6

The final metazoan specific ER stress transducer is ATF-6, which differs in structure and activation from those above. Synthesized as an inactive precursor, ATF-6 is inserted into the ER membrane with a stress-sensing portion facing the ER lumen. During ER stress, ATF-6 is first transported into the Golgi apparatus where it undergoes sequential proteolysis by site 1 protease (S1P) and site 2 protease (S2P) thereby releasing the cytosolic domain. The freed DNA binding

portion of ATF-6 translocates to the nucleus where it activates UPR genes (Yoshida et al., 2000).

UPR and development

The experimental analysis of UPR signaling both in yeast and in mammalian cells has been greatly facilitated by the use of chemical agents that induce ER stress, such as the N-linked glycosylation inhibitor tunicamycin, the calcium pump inhibitor thapsigargin, and the reducing agent dithiotreitol (DTT). However, the activation of the UPR under physiological conditions is less well understood (Rutkowski and Hedge, 2010). Constitutive IRE-1 activity has been observed in diverse types of mammalian cells, particularly with high secretory activity or in the setting of increased inflammatory signaling. (Lee et al., 2008; Schröder and Kaufman, 2005; Shaffer et al., 2004; Todd et al., 2008; Zhang et al., 2006) These studies suggest critical roles for IRE-1-XBP-1 signaling in physiology and development, some of which have been proposed to be independent of its role in maintaining protein folding homeostasis in the ER.

Genetic studies suggest essential roles for UPR signaling in animal development. Genetic studies in mice, focusing on either the IRE-1-XBP-1 or the PERK pathway, have shown that each functions in the development of specialized cell types, including plasma cells, pancreatic beta cells, hepatocytes, and intestinal epithelial cells (Lee et al., 2008; Schröder and Kaufman, 2005; Shaffer et al., 2004; Todd et al., 2008; Zhang et al., 2006). In *Caenorhabditis elegans*, mutants deficient in any one of the three branches of the UPR are viable, but combining a deficiency in the IRE-1-XBP-1 pathway with loss-of-

function mutations in either the ATF-6 or PEK-1 branch has been reported to result in larval lethality (Shen et al., 2001; Shen et al., 2005). These studies suggest that the UPR is required for animal development, but the nature of this essential role has not been defined.

Immunity and UPR

Recently, Richardson et al showed that XBP-1 is required for *C. elegans* larval development on pathogenic *Pseudomonas aeruginosa*, conferring protection to the *C. elegans* host against the ER stress caused by its own secretory innate immune response to infection (Richardson et al., 2010). This study established that the innate immune response to microbial pathogens represents a physiologically relevant source of ER stress that necessitates XBP-1. Here, we sought to further understand this requirement for XBP-1 through tissue specific expression of XBP-1 in *xbp1* mutants and analysis of the requirement for expression during larval development on pathogen.

Moreover, based on the constitutive IRE-1 activity observed in other systems, we hypothesized that the requirement for XBP-1 during innate immunity might be related to its function in maintaining protein folding homeostasis even in the absence of ER stress. We sought to better understand the consequences of UPR deficiency under physiological conditions during *C. elegans* larval development. We describe our studies that suggest that even in the absence of ER stress induced by exogenously administered chemical agents, the IRE-1-XBP-1 pathway, in concert with the PEK-1 pathway, functions in a homeostatic loop that is under constitutive activation during *C. elegans* larval development.

Our data implicate an essential role for the UPR in ER homeostasis, not only in the response to toxin-induced ER stress, but also under basal physiological conditions

Methods:

Strains

C. elegans strains were constructed and propagated according to standard methods on *E. coli* OP50 at 16°C (Brenner, 1974). The *smg-2(qd101)* allele was isolated by K. Reddy and contains a C→T nonsense mutation at nucleotide 1189 of the spliced transcript. The following strains were used in the study: N2 Bristol, ZD627 *smg-2(qd101)*, ZD607 *smg-2(qd101);xbp-1(zc12)*, RB772 *atf-6(ok551)*, RB545 *pek-1(ok275)*, ZD510 *xbp-1(tm2482);pek-1(ok275)*, ZD524 *xbp-1(zc12);pek-1(tm629)*. Double mutants were made between *xbp-1* and *pek-1* by crossing strains marked with GFP: *xbp-1*(III)*;pT24B8.5::GFP(agIs220)*(X) and *pT28B8.4::GFP(agIs219)*(III)*;pek-1*(X). GFP-negative F2s were singled, propagated, and genotyped by PCR.

Cloning

The following constructs were generated *pxbp-1-xbp-1::GFP-unc-54 3'UTR* (endogenous expression), *pges-1-xbp-1-unc-54 3'UTR* (intestinal expression), and *punc-119-xbp-1-unc-54 3' UTR* (neuronal expression) using nested PCR (Hobert et al., 2002). GFP fused to the unc-54 3' UTR was amplified from pPD95.75 using the following primers: C: 5'-AGCTTGCATGCCTGCAGGTCGACT-3', D: 5'-

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AAGGGCCCGTACGGCCGACTAGTAGG-3', D*-

GGAAACAGTTATGTTTGGTATATTGGG-3'. In parallel, *xbp-1* was amplified

from cosmid R74 using the following primers: A: 5'-

TCCAAATGATGCTCATGCTCAGG-3', A*: 5'-

AAGACAGTACGATGTTAGTGGTCC-3', A**: 5'-

ACTTTGAAGGATTTGATGTCTCCG-3', B: 5'-

AGTCGACCTGCAGGCATGCAAGCTATTTTGGAAGAAATTTAGGTCGATTG-3'.

The *ges-1* promoter region was amplified from cosmid C29B10 using primers F:

5'-GAACGCACATTCATCGTGATGATGTGG-3' and R: 5'-

CGTTTTGGATAGTTGCTCATGCCTGAAACTGTAAGACGCAGACG. The *unc-119* promoter was amplified with primers F: 5'-

GCGGCTCCAATCGGAAACGCGAAC-3' and R: 5'-

CGTTTTGGATAGTTGCTCATAGGTTGCCGAGCCGGGTGCGATC-3'. Linear

DNA was microinjected as previously described (Mello et al., 1991; Mello and

Fire, 1995), and lines were selected based on the inheritance of *myo-2*-cherry.

RNA isolation and quantitative RT-PCR

For the experiment in Figure 5B, and 5C, L1 larvae were synchronized by

hypochlorite treatment, washed onto *E. coli* OP50 plates, and grown for 40 h at

16°C to the L3 stage, when they were washed in M9 to plates containing *E. coli*

OP50 or *E. coli* OP50 with 5 µg/ml tunicamycin and frozen in liquid nitrogen. For

P. aeruginosa treatment, *P. aeruginosa* strain PA14 was grown in Luria Broth

(LB), and 25 µl overnight culture was seeded onto 10 cm NGM plates. Plates

were incubated first at 37°C for 1 d, then at room temperature for 1 d. All RNA

extraction, cDNA preparation, qRT-PCR methods and specific primers to detect

xbp-1 mRNA were as described previously (Richardson et al., 2010).

Immunoblotting

For all immunoblots strains were synchronized by hypochlorite treatment and

washed onto *E.coli* OP50 plates. For the experiment in Figure 2A, strains were

grown at 20°C for 23 hours, L2/L3 worms were then washed in M9 onto

treatment plates containing 50 μg/mL tunicamycin for incubation at 25°C for 4

hours. For the experiment in Figure 2b, strains were grown at 16°C for 55 hours

until the L4 stage and harvested without treatment. All strains were collected and

rinsed 2 times in M9. Worm pellets were resuspended in an equal volume of 2x

lysis buffer containing 4% SDS, 100 mM Tris Cl, pH 6.8, and 20% Glycerol. After

boiling for 15 minutes with occasional vortexing to aid in dissolution, lysates were

clarified by centrifugation. Protein samples (50μg of total lysate loaded per lane)

were separated with a 12% SDS-PAGE and transferred to nitrocellulose (Bio-

rad). Western blots were blocked for 1H at RT in 5% milk in PBST and probed

with (1:10,000) anti-eIF2α (Nukazuka et al. 2008), (1:1,000) anti-phospho-eIF2α

(Cell Signaling Technology), or (1:10,000) anti-tubulin (E7 Developmental

Hybridoma Bank, Iowa City). All primary antibodies were diluted in 5% milk in

PBST. Following incubation with anti-rabbit or anti-mouse IgG antibodies

conjugated with horseradish peroxidase (HRP) (Cell Signaling Technology),

signals were visualized with chemiluminescent HRP substrate (Amersham).

Development assays

For development assays, strains were egg laid on 4-5 prepared plates for no more than 3 h (at least 110 eggs for each strain and treatment). Development was monitored daily for 3 d. *P. aeruginosa* PA14 plates were prepared as described (Kim et al., 2002).

Results:

Tissue specific rescue of lethality on pathogen in *C. elegans xbp-1* mutants

Previously, Richardson et al demonstrated the requirement for the IRE-1-XBP-1 branch of the UPR in *C. elegans* in protection against immune activation. Importantly, *xbp-1* loss-of-function mutants exhibited larval lethality in the presence of pathogenic bacteria in a PMK-1-dependent manner. To better understand the mechanisms behind the physiological requirements for the UPR in this context, here we investigate the cells and tissues in which the UPR components are required. We fused tissue-specific reporters to the xbp-1 gene, and injected these constructs into the *xbp-1(tm2482)* mutant (Figure 3A). Our preliminary data suggest that expression of *xbp-1* in the intestine is sufficient to rescue larval development in the presence of pathogenic *P. aeruginosa* (Figure 3B). This evidence is corroborated by the solely intestinal expression of *hsp-4::gfp*, a UPR reporter, seen by Richardson et al. upon pathogen exposure (Richardson et al., 2010). Importantly, we infer that even in the absence of complete rescue, intestinal expression of XBP-1 is sufficient to restore this involved and complicated process, as fertile gravid worms were seen in this context (Figure 3C).

Prior work from Bischof et al also show that expression of *xbp-1* exclusively in the intestine with an *app-1* promoter is sufficient to rescue *xbp-1* deficient mutants from PMK-1 dependent lethality during exposure to *Bacillus thuringiensis* toxin (Bischof et al., 2008). Importantly, although the endogenous promoter driving XBP-1 fused to GFP also shows partial rescue of pathogen dependent *xbp-1(tm2482)* larval lethality, no fluorescence was visible. Consequently, we conclude that low expression may have contributed to the inefficiency of this rescue.

To further investigate the tissue specific requirement for XBP-1 in phenotypes related to *xbp-1* deficiency, tissue specific constructs were also introduced into the *xbp-1;pek-1* double mutants, which exhibit lethality at elevated temperatures, or during immune activation (Richardson et al., 2012). Rescue of this phenotype remains to be rigorously tested, but preliminary results imply intestinal expression may be insufficient to rescue the lethality associated with *xbp-1;pek-1* mutants in these conditions.

Increased constitutive PEK-1 activation in *C. elegans xbp-1* mutants

If loss of XBP-1 resulted in an increase in basal ER stress, we might anticipate compensatory activation of the PEK-1 and/or ATF-6 pathways in the absence of XBP-1. Here, we sought to determine levels of PEK-1 activity in an *xbp-1* mutant through the detection of eIF-2α phosphorylation. Antibodies raised against mammalian eIF-2α and specifically phosphorylated eIF-2α (P-eIF-2α) cross-react with the highly homologous *C. elegans* protein (Hamanaka et al.,

2005; Nukazuka et al., 2008). We observed a single band in immunoblots using these antibodies with lysates from WT *C. elegans* (Figure 2A). We observed that eIF-2α phosphorylation was induced by a 4 h exposure to a high dose of tunicamycin in a PEK-1-dependent manner (Figure 2A). Of note, eIF-2α phosphorylation appears to be a relatively insensitive measure of ER stress, as we did not observe a significant increase in eIF-2α phosphorylation in response to standard doses of tunicamycin sufficient to induce *xbp-1* mRNA splicing.

We next determined PEK-1 activity under basal physiological conditions, specifically in the *xbp-1* mutant. Paralleling our observations of increased *xbp-1* mRNA splicing in the *xbp-1* mutant, we observed induction of PEK-1-mediated eIF-2α phosphorylation relative to WT in the absence of exogenously administered agents to induce ER stress (Figure 2B). The magnitude of the effect of XBP-1 deficiency on PEK-1 activity was comparable to the induction of PEK-1 in WT by treatment with high-dose tunicamycin.

Elevation of constitutive IRE-1 activity in *C. elegans xbp-1* mutants

The detection of IRE-1 activity provides a sensitive and responsive measure of ER stress. Most methods to measure IRE-1 activity require functional IRE-1-XBP-1 output, relying on either detection of the activated spliced form of the *xbp-1* mRNA or the transcriptional activity of the resulting XBP-1 protein. In order to follow IRE-1 activity in the absence of a functional XBP-1 protein, we utilized the *C. elegans xbp-1(zc12)* mutant, which has a C→T

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mutation that results in an early premature stop codon (Calfon et al., 2002). We reasoned that we could detect IRE-1-mediated splicing of *xbp-1(zc12)* mRNA by quantitative RT-PCR (qPCR), as we have done previously for wild type *xbp-1* mRNA, as a measure of IRE-1 activity (Richardson et al., 2010).

We anticipated, however, that the *xbp-1(zc12)* mRNA might be degraded by nonsense-mediated decay (NMD) (Rehwinkel et al., 2006), which would reduce the abundance of *xbp-1(zc12)* mRNA (Figure). Thus, we constructed a strain carrying *xbp-1(zc12)* and a null allele of *smg-2*, the *C. elegans* homolog of the NMD component Upf1 (Page et al., 1999). Indeed, we observed that the level of *xbp-1* mRNA in the *xbp-1(zc12)* mutant was markedly diminished compared with the level of *xbp-1(zc12)* mRNA in the *smg-2(qd101);xbp-1(zc12)* mutant (Fig. 1b). These data confirm that *xbp-1(zc12)* mRNA is a substrate for the NMD pathway, but that inhibition of NMD permits detection of *xbp-1(zc12)* mRNA. As expected from the predicted truncated protein product made from translation of the *xbp-1(zc12)* mRNA (Fig. 1a), loss of NMD had no effect on the null phenotype of the *xbp-1(zc12)* allele, as assessed by the effect of the *smg-2(qd101)* mutation on expression of an *xbp-1*-regulated gene, the *C. elegans* BiP homolog *hsp-4* (Fig. 1b). We next examined the level of WT *xbp-1* mRNA in the *smg-2(qd101)* mutant, and we observed that NMD inhibition increased the level of *xbp-1* mRNA 2-fold relative to WT *C. elegans* (Fig. 1c), which suggests that the NMD complex may function to decrease the level of WT *xbp-1* mRNA. This observation is consistent with prior observations that stress-induced genes may be NMD targets (Gardner, 2008) although we hypothesize that the relatively early

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termination codon present in the *xbp-1* mRNA prior to IRE-1-mediated splicing may also contribute to recognition and degradation by the NMD pathway. Consistent with this explanation, after exposing both the WT and *smg-2(qd101)* strains to tunicamycin for 4 h, the level of IRE-1-spliced *xbp-1* mRNA was similar between the two strains (Fig. 1c). Taken together, the increase in levels of IRE-1 and PEK-1 activities in the *xbp-1* mutant suggests that XBP-1 deficiency is accompanied by a marked increase in constitutive ER stress under basal physiological conditions.

Discussion:

We have shown that the IRE-1-XBP-1 and PEK-1 pathways function together to maintain ER homeostasis in *C. elegans* under physiological conditions. We found that XBP-1 deficiency results in marked activation of both IRE-1 and PEK-1, reflecting constitutive ER stress. We further showed that intestinal XBP-1 is required for protection against the stress of immune activation during larval development on *P. aeruginosa*.

The increase of IRE-1 and PEK-1 activity in the setting of XBP-1 deficiency, under standard growth conditions in the absence of exogenous agents to induce ER stress, provides strong evidence for homeostatic activity of the IRE-1-XBP-1 signaling pathway under physiological conditions (Fig. 7a), and not simply at the extremes of ER stress induced by pharmacological treatment or in specialized secretory cell types. In previously described mouse models, XBP-1 deficiency in intestinal epithelial cells (IEC) resulted in marked intestinal

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inflammation that may contribute to the observed activation of not only IRE-1 but also of PERK, as measured by expression of one of its downstream effectors, CHOP (Kaser et al., 2008). In mammals, the transcription factor CHOP promotes apoptosis of mammalian cells that experience prolonged ER stress (Zinszner et al., 1998), and indeed, the majority of Paneth and goblet cells underwent apoptosis in the $Xbp1^{-/-}$ IECs. Our observations are consistent with the idea that $Xbp1^{-/-}$ IECs may be predisposed to detrimental consequences of additional ER stress caused by intestinal inflammation because of dysregulation of basal ER homeostasis due to XBP-1 deficiency.

Our observations that PEK-1, in concert with XBP-1, function to protect against ER stress from immune activation differ from observations in mouse macrophages, in which TLR stimulation was shown to activate IRE-1, but PERK activation was reported to be suppressed rather than elevated (Martinon et al., 2010; Woo et al., 2009). This difference may be due to roles for XBP-1 in macrophages that extend beyond its function in maintaining ER homeostasis. Indeed, when stimulated by TLRs in macrophages, the IRE1-XBP1 pathway was shown to induce expression of immune effectors rather than typical UPR genes, suggestive that the IRE1-XBP1 pathway may have been co-opted in macrophages to promote macrophage-specific function independent of the UPR (Martinon et al., 2010).

Our data support the idea that UPR signaling does not function simply in response to the extremes of ER stress, as when induced by tunicamycin or by the elevated secretory load of specialized cells such as plasma cells, but instead,

as a critical pathway in the maintenance of ER homeostasis during normal growth and development in *C. elegans* (Figure 6). The diverse and dramatic consequences of XBP-1 deficiency on development and disease, taken together with our observations on the effect of XBP-1 deficiency on basal ER stress levels, underscore the critical role of ER homeostasis and homeostatic UPR signaling in both normal physiology and disease.
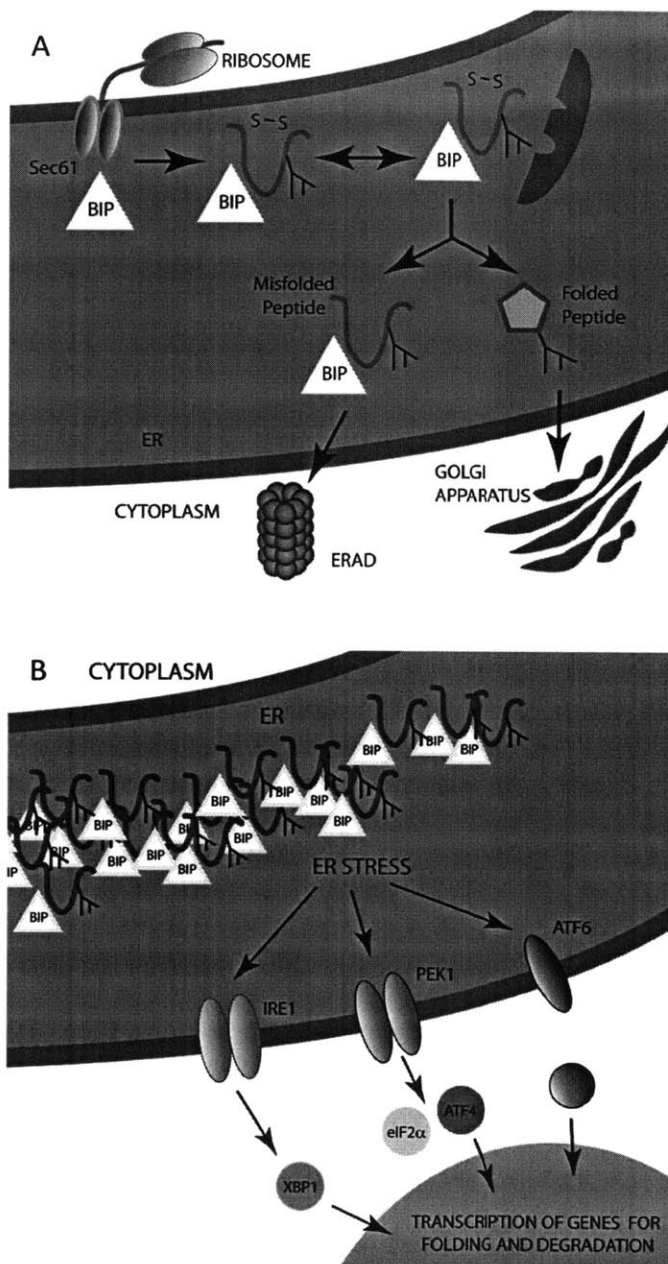
Figures:

Figure 1: Protein folding and the UPR. The role of Bip in both folding (A) and detection of elevated levels of misfolded or unfolded proteins (B). BiP reversibly binds peptides as they are translated into the ER through the Sec61 channel. Additionally, BiP is titrated away from the lumenal portions of UPR effector proteins as wrongly or incompletely folded peptides accumulate.
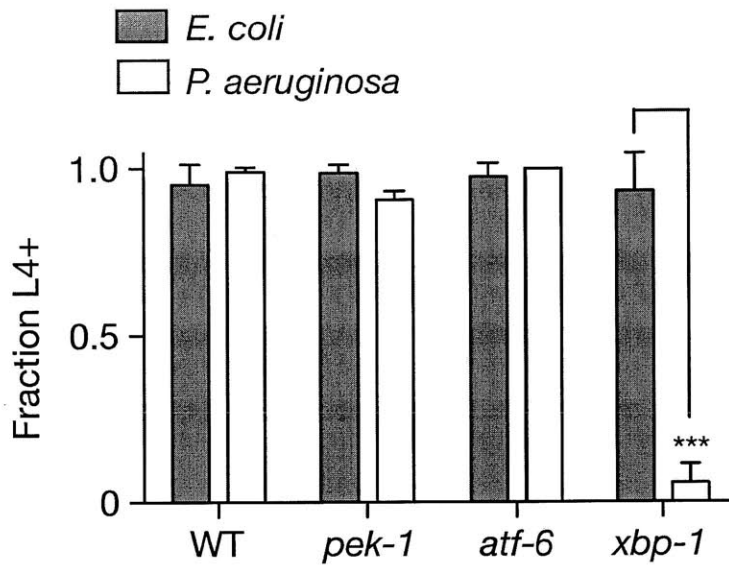
Figure 2: XBP-1 is required for *C. elegans* larval development on pathogenic *Pseudomonas aeruginosa*. Development of indicated mutants to the L4 larval stage or older after 3 days at 25°C on either *P. aeruginosa* PA14 or *E. coli* OP50. Two-way ANOVA with Bonferroni post test, n = 4 plates, *** $P < 0.001$. Results are representative of three independent experiments. (Taken from Richardson et al, 2010)
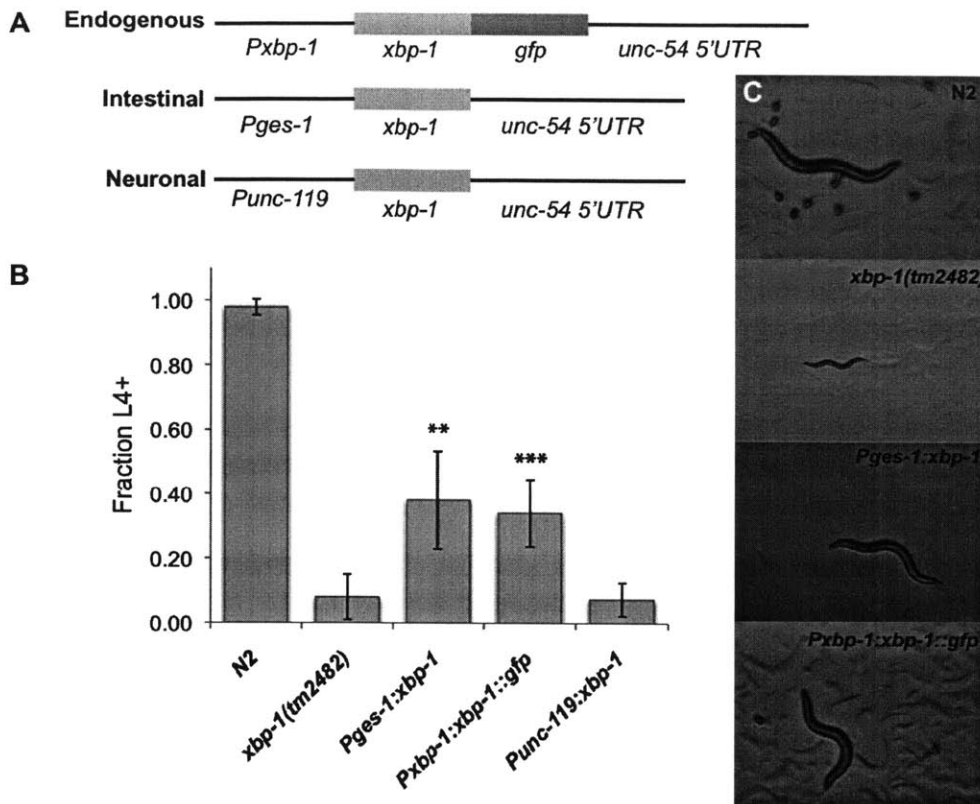
Figure 3: Intestinal Expression of XBP-1 is sufficient to restore larval development on *P aeruginosa.* Constructs with tissue specific promoters were constructed (A). Development of indicated transgenic worms to the L4 larval stage or older after 3 days at 25°C on *P. aeruginosa* PA14. Two way t-test, n>4 plates. ** P <0.0005, *** P <0.0001 (B). DIC microscopy indicating latest developmental stage reached for indicated transgenic worms at 25°C on *P. aeruginosa* PA14 (C).
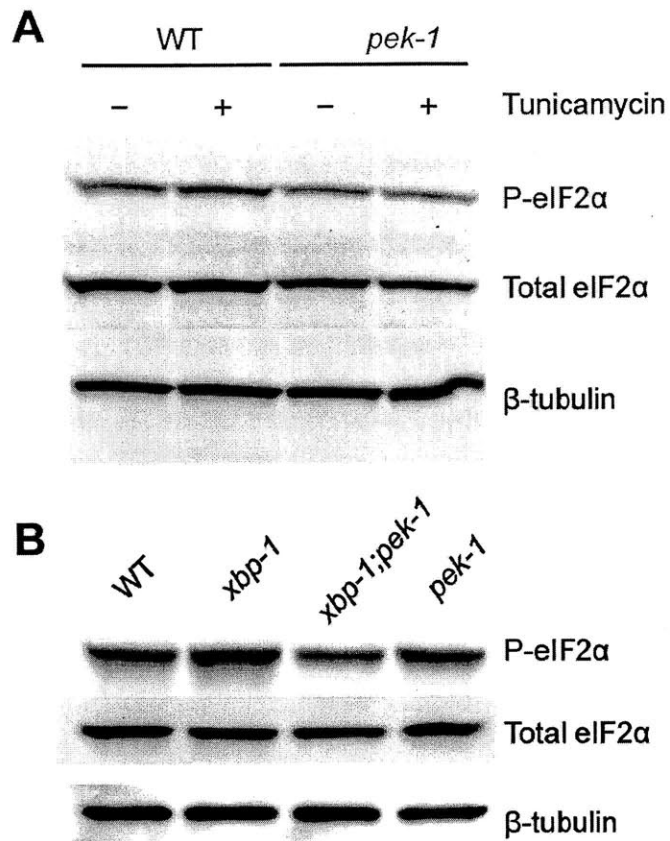
Figure 4: XBP-1 deficiency increases PEK-1 dependent phosphorylation of eIF2α. PEK-1 dependent phosphorylation of eIF2α is induced by high dose (50 µg/ml) tunicamycin. Western blot of P-eIF2α, total eIF2α and tubulin in WT and *pek-1(ok275)* strains grown to the L4 stage and then shifted to plates with or without tunicamycin (50 µg/mL) for 4 hours (A). PEK-1 dependent phosphorylation of eIF2α is induced by XBP-1 deficiency. Western blot of P-eIF2α, total eIF2α and tubulin in WT, *xbp-1(tm2482)*, *xbp-1(tm2482); pek-1(ok275)* and *pek-1(ok275)* strains grown to the L4 stage (B).
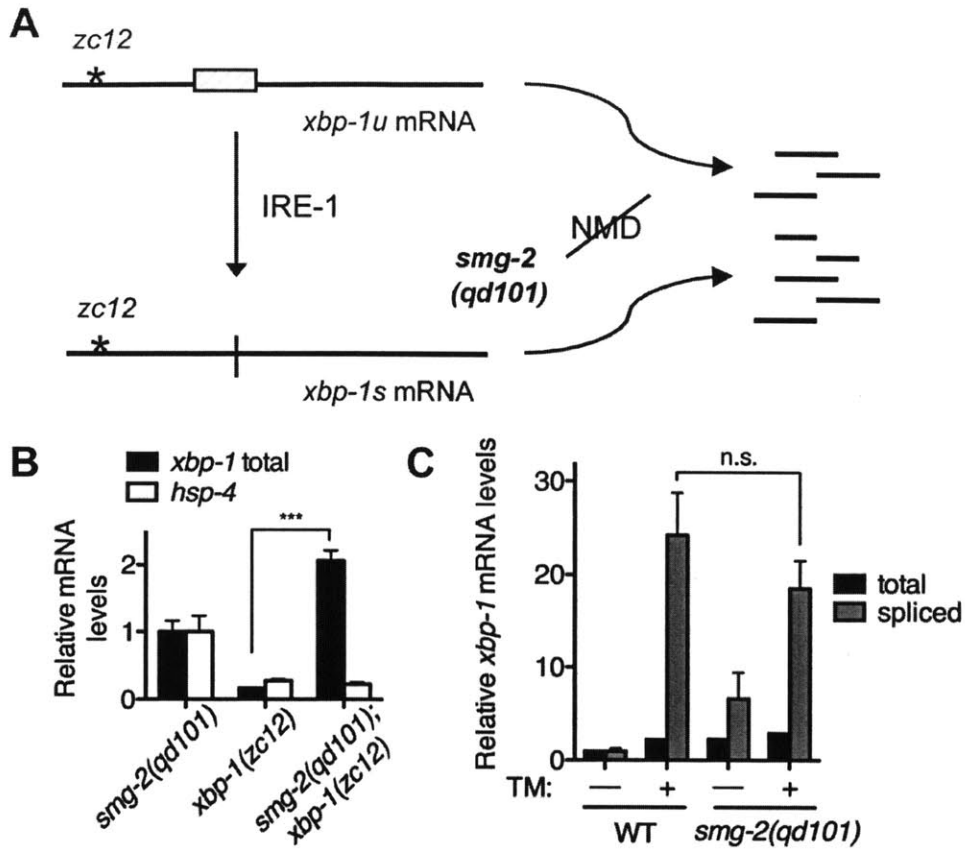
Figure 5: XBP-1 deficiency results in a dramatic increase in IRE-1 activity.
Detection of *xbp-1* mRNA splicing by IRE-1 in the *C. elegans xbp-1(zc12)*
mutant. Schematic of the unspliced and spliced *xbp-1* mRNA, noting the position
of the premature termination codon present in the *xbp-1(zc12)* allele. The *smg-
2(qd101)* mutation results in inactivation of the NMD pathway, stabilizing the *xbp-
1(zc12)* mRNA for detection (A). Quantitative real-time PCR measurements of
mRNA levels in the *xbp-1(zc12)* and *smg-2(qd101);xbp-1(zc12)* mutants relative
to the *smg-2(qd101)* mutant synchronized in the L3 stage (B). Quantitative real-
time PCR measurements of levels of total and spliced *xbp-1* mRNA in the *smg-
2(qd101)* strain relative to WT grown to the L3 stage and then shifted to plates
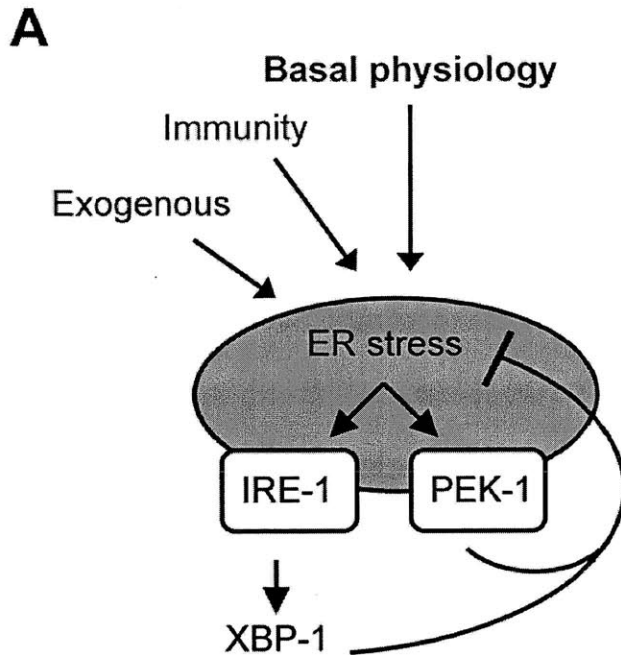with or without tunicamycin (5 µg/mL) for 4 h (C).

25

A

Figure 6: Maintenance of ER homeostasis through activation of the IRE-1 and PEK-1 pathways under basal physiological conditions during development. The increase in both IRE-1 and PEK-1 activities in XBP-1 deficiency in the absence of exogenous compounds to impose ER stress implies that UPR signaling maintains ER homeostasis not only in response to the extremes of ER stress, but also under basal physiological conditions.

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