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A survival pathway for *Caenorhabditis elegans* with a blocked unfolded protein response

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The unfolded protein response (UPR) counteracts stress caused by unprocessed ER client proteins. A genome-wide survey showed impaired induction of many UPR target genes in *xbp-1* mutant *Caenorhabditis elegans* that are unable to signal in the highly conserved IRE1-dependent UPR pathway. However a family of genes, *abu* (activated in blocked UPR), was induced to higher levels in ER-stressed *xbp-1* mutant animals than in ER-stressed wild-type animals. RNA-mediated interference (RNAi) inactivation of a representative *abu* family member, *abu-1* (*AC3.3*), activated the ER stress marker *hsp-4::gfp* in otherwise normal animals and killed 50% of ER-stressed *ire-1* and *xbp-1* mutant animals. *Abu-1(RNAi)* also enhanced the effect of inactivation of *sel-1*, an ER-associated protein degradation gene. The nine *abu* genes encode highly related type I transmembrane proteins whose lumenal domains have sequence similarity to a mammalian cell surface scavenger receptor of endothelial cells that binds chemically modified extracellular proteins and directs their lysosomal degradation. Our findings that ABU-1 is an intracellular protein located within the endomembrane system that is induced by ER stress in *xbp-1* mutant animals suggest that ABU proteins may interact with abnormal ER client proteins and this function may be particularly important in animals with an impaired UPR.

Introduction

The ER participates in folding and posttranslational processing of proteins destined for secretion or membrane insertion. Several signaling pathways, collectively referred to as the unfolded protein response (UPR),* maintain an equilibrium between the capacity of the ER to process its client proteins and the physiological demand on the organelle (Kaufman, 1999; Mori, 2000; Patil and Walter, 2001). Inactivation of UPR signaling has deleterious consequences on cell survival and organ function (Bertolotti et al., 2001; Harding et al., 2000, 2001; Scheuner et al., 2001; Shen et al., 2001), and accumulation of malfolded proteins in the ER may play an important role in human diseases (Carrell and Lomas, 1997; Aridor and Balch, 1999).

In yeast, ER stress activates the ER resident transmembrane protein kinase and endonuclease Ire1p, leading to the posttranscriptional processing of a downstream mRNA encoding the transcription factor Hac1p. Hac1p directly induces target genes of the UPR. These target genes encode proteins involved in processing, trafficking, and degradation of ER client proteins (for reviews see Mori, 2000; Patil and Walter, 2001). The UPR has diversified considerably in metazoans. In addition to a conserved signaling pathway of IRE1 homologues and a downstream HAC1-like transcription factor, XBP-1 (Yoshida et al., 2001; Calfon et al., 2002), two new pathways, not present in yeast, have evolved. Pancreatic-enriched ER kinase (PERK) phosphorylates eukaryotic translation initiation factor 2 to attenuate protein synthesis and activate specific gene expression during ER stress (for review see Ron and Harding, 2000), and the ER stress–activated transcription factor ATF6 directly activates UPR target genes (Haze et al., 1999; Ye et al., 2000; Yoshida et al., 2001).

The coordination of these three pathways and their specific contribution to the metazoan ER stress response are unclear, though in *Caenorhabditis elegans*, the IRE1 and PERK pathways provide redundant protection against ER stress (Shen et al., 2001). We used cDNA microarrays to characterize the transcriptional response to ER stress in *C. elegans*, a simple metazoan that has counterparts of all three known components of the mammalian UPR (Shen et al., 2001; Calfon et al., 2002). We find that in *C. elegans*, the *ire-1* and *xbp-1*

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*Abbreviations used in this paper: ERAD, ER-associated degradation; PERK, pancreatic-enriched ER kinase; RNAi, RNA-mediated interference; UPR, unfolded protein response.

Key words: chaperone; protein folding; protein degradation; gene expression; functional genomics

Table I. **Genes whose activation by tunicamycin treatment is attenuated in *xbp-1(zc12)III* mutant animals**

Gene	Induction in N2	Induction in *xbp-1*	Protein
F43E2.8	1.92 ± 0.14	0.44 ± 0.25	HSP-4
C15H9.6	1.32 ± 0.06	0.68 ± 0.05	HSP-3
T27E4.8	2.85 ± 0.30	0.18 ± 0.37	HSP-16
Y46H3A.E	2.67 ± 0.34	0.49 ± 0.40	HSP16-41
Y46H3A.D	2.55 ± 0.48	−0.20 ± 0.17	HSP16-2
T27E4.2	2.48 ± 0.22	0.29 ± 0.41	HSP-16
T27E4.9	1.97 ± 0.08	−0.57 ± 0.32	HSP16-49
C12C8.1	2.08 ± 0.15	0.54 ± 0.17	Member of the heat shock HSP70 protein family
T14G8.3	1.35 ± 0.05	−0.17 ± 0.13	HSP70-like ATPase
C14B9.2	1.83 ± 0.16	0.19 ± 0.15	Member of the protein disulfide isomerase protein family
C07A12.4	1.38 ± 0.10	0.42 ± 0.17	PDI-2
B0403.4	1.66 ± 0.12	0.46 ± 0.10	Member of the thioredoxin protein family
C53B4.7	2.28 ± 0.44	−0.09 ± 0.14	Putative GDP-mannose 4,6-dehydratase
Y41C4A.11	3.27 ± 0.21	−0.40 ± 0.13	Member of the WD repeat protein family
F45E4.1	3.05 ± 0.34	0.67 ± 0.15	ARL-6
F20D1.5	2.80 ± 0.51	0.68 ± 0.20	ARL-7
B0285.9	3.50 ± 0.30	−0.28 ± 0.11	Member of the choline/ethanolamine kinase protein family
K01D12.11	4.21 ± 0.31	0.85 ± 0.13	Member of the GST protein family
C55B6.2	1.85 ± 0.17	0.29 ± 0.14	Putative orthologue of human protein kinase inhibitor p58
ZK662.4	3.10 ± 0.31	0.67 ± 0.02	Negative regulator of the LET-23 receptor tyrosine kinase LIN-15
F20D6.4	2.69 ± 0.10	0.04 ± 0.31	SRP-7, serine protease inhibitor
B0222.9	2.05 ± 0.20	1.00 ± 0.08	Similar to xanthine dehydrogenase
Y40B10A.G	1.40 ± 0.13	0.27 ± 0.16	Similar to *O*-methyltransferase
R07B1.10	1.34 ± 0.10	0.19 ± 0.09	Member of the galectin family
F41B4.3	2.86 ± 0.45	0.06 ± 0.09	Unknown
K02D7.4	1.70 ± 0.05	0.38 ± 0.14	Unknown

Shown is the mean ± SEM ($n = 3$) of the $base_2$ log–fold increase in tunicamycin-treated animals over untreated animals for each genotype. The induction of *lin-15* (ZK662.4) is likely to be an artifact of the *hsp-4::gfp;lin-15* array present in both wild-type and mutant animals.

pathway has retained its essential role in upregulating expression of many UPR target genes that are similarly upregulated by the homologous pathway in yeast. We also discovered a novel family of highly related genes that protect against ER stress when the *ire-1* and *xbp-1* signaling pathway is defective.

Results

We compared the gene expression profile in unstressed and ER-stressed wild-type young adult *C. elegans* with the profile in an *xbp-1* mutant strain that is defective in the *ire-1* and *xbp-1* signaling pathway (Calfon et al., 2002). Exposure of wild-type animals to the ER stress–inducing glycosylation inhibitor tunicamycin resulted in a 2.5-fold or greater statistically significant activation ($P < 0.05$) of 34 of the ~18,000 genes on the array. Of these 34 genes, 26 were uninduced or induced to significantly lower levels ($P < 0.05$) in the *xbp-1* mutant strain. All but 2 of the 26 *xbp-1*–regulated genes encode familiar proteins such as ER or cytosolic chaperones, proteins involved in vesicular transport or other aspects of posttranslational processing of ER client proteins (Table I). Therefore, in *C. elegans*, as in yeast, the UPR entails induction of genes involved in many different aspects of posttranslational protein processing in the secretory pathway. We confined our presentation only to genes whose induction withstood stringent statistical criteria that took into account both variation among replicate points in our experiments (so-called local variation) and historical information on variation between replicates in other experiments performed on the same array (so-called global variation). Among the genes whose induction passed these stringent statistical criteria, <1/4 were *xbp-1* independent. These observations suggest that robustly induced UPR genes are significantly controlled by *xbp-1*, however we have not addressed the role of *xbp-1* in the activation of genes with more variable levels of induction by ER stress.

19 genes were induced to higher levels in *xbp-1* mutant animals than in wild-type animals (Table II). Nine of these encode highly similar, novel proteins with a hydrophobic NH_2-terminal signal sequence, a potential transmembrane domain, and a short COOH-terminal cytoplasmic domain (Fig. 1, A, B, and C). We refer to members of this family as activated in blocked UPR (*abu*). Two other similarly regulated genes encode proteins that are more distantly related to the nine ABU family members (Table II). A representative family member, *abu-1* (*AC3.3*), was chosen for further study based on its central location in the family tree (Fig. 1 A). Northern blot analysis confirmed the induction of *abu-1* by tunicamycin treatment of *xbp-1* mutant animals but not wild-type animals (Fig. 1 D).

ABU proteins have a distant similarity to a mammalian scavenger receptor of endothelial cells and to the *C. elegans* cell corpse engulfment protein CED-1, which are transmembrane cell surface proteins (Adachi et al., 1997; Zhou et al., 2001). To study the subcellular localization of ABU-1, the protein was tagged at its COOH terminus with GFP and expressed in the intestine of *C. elegans* from a transgene driven by the *ges-1* promoter. *ges-1::abu-1–gfp* transgenic animals had a punctate pattern of green fluorescence. Confocal mi-

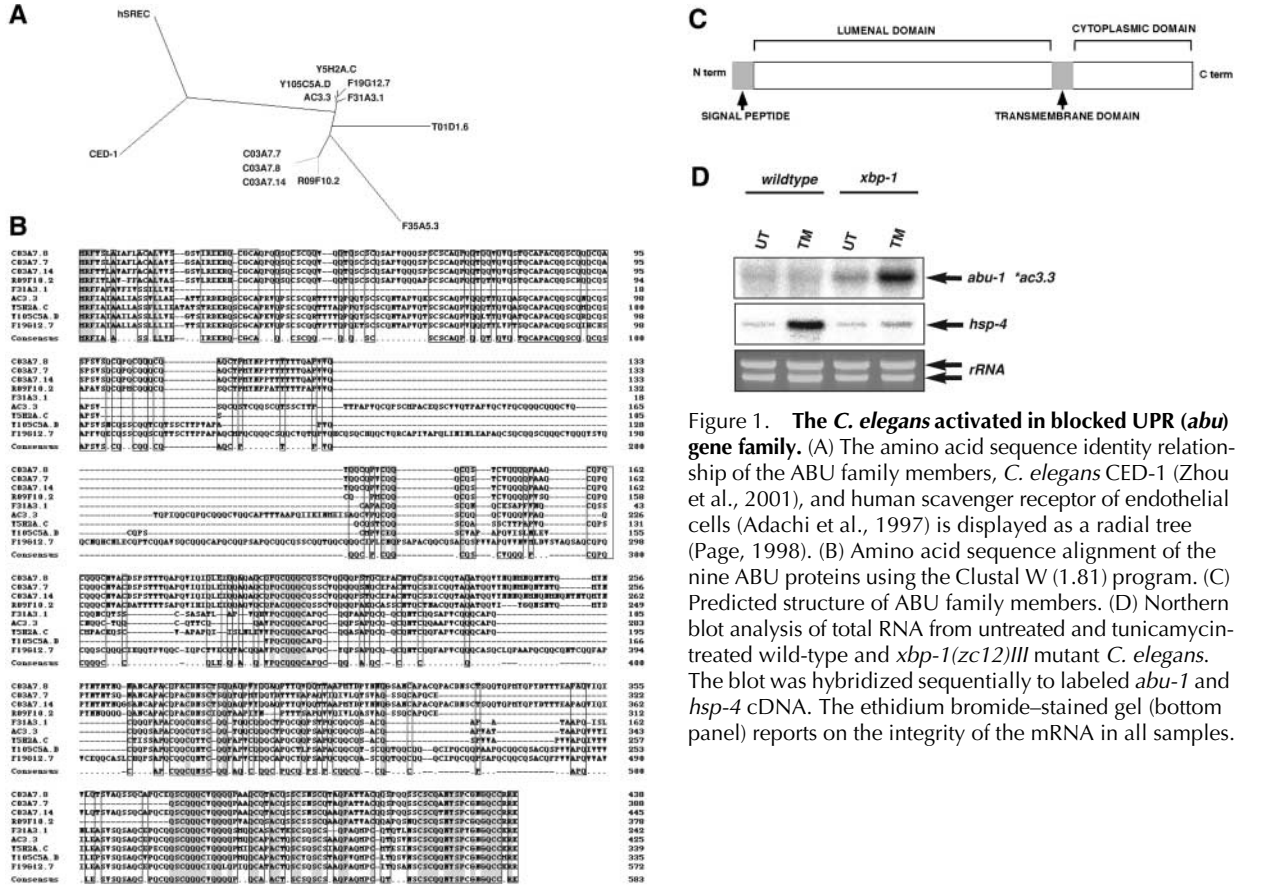


Figure 1. **The *C. elegans* activated in blocked UPR (*abu*) gene family.** (A) The amino acid sequence identity relationship of the ABU family members, *C. elegans* CED-1 (Zhou et al., 2001), and human scavenger receptor of endothelial cells (Adachi et al., 1997) is displayed as a radial tree (Page, 1998). (B) Amino acid sequence alignment of the nine ABU proteins using the Clustal W (1.81) program. (C) Predicted structure of ABU family members. (D) Northern blot analysis of total RNA from untreated and tunicamycin-treated wild-type and *xbp-1(zc12)III* mutant *C. elegans*. The blot was hybridized sequentially to labeled *abu-1* and *hsp-4* cDNA. The ethidium bromide–stained gel (bottom panel) reports on the integrity of the mRNA in all samples.

croscopy showed that the puncta corresponded to vesicular structures that were present throughout the large intestinal cell and tended to cluster near its apical surface (Fig. 2 A). No cell surface staining was noted. In contrast, *ges-1::gfp* fluorescence was observed throughout the cytoplasm and nucleus of the intestinal cell (Fig. 2 B) and nontransgenic animals had only background signal from autofluorescence (Fig. 2 C). To expand on these observations, we expressed the ABU-1–GFP fusion protein from its endogenous *abu-1* promoter. The expression level of this transgene was much lower than that of *ges-1::abu-1–gfp* and was noted in the pharynx and not the intestine (also see Fig. 5 A). ABU-1–GFP ex-

Table II. **Genes whose activation by tunicamycin treatment is enhanced in *xbp-1(zc12)III* mutant animals**

Genes	Induction in *xbp-1*	Induction in N2	Protein	Chromosome
F19G12.7	1.90 ± 0.26	−0.11 ± 0.11	ABU (see below)	X
Y5H2A.C	1.89 ± 0.28	0.24 ± 0.28	ABU (see below)	V
F31A3.1	1.52 ± 0.13	0.12 ± 0.27	ABU (see below)	X
AC3.3	1.45 ± 0.29	−0.16 ± 0.46	ABU-1 (see below)	V
Y105C5A.D	1.42 ± 0.37	−0.35 ± 0.27	ABU (see below)	IV
C03A7.14	1.77 ± 0.29	−0.39 ± 0.15	ABU (see below)	V
C03A7.7	1.75 ± 0.28	−0.39 ± 0.13	ABU (see below)	V
R09F10.2	1.29 ± 0.40	−0.39 ± 0.10	ABU (see below)	X
C03A7.8	1.22 ± 0.49	−0.67 ± 0.22	ABU (see below)	V
T01D1.6	1.59 ± 0.30	−0.07 ± 0.08	ABU (see below)	II
F35A5.3	1.44 ± 0.30	−0.25 ± 0.12	ABU (see below)	X
Y38C1BA.G	1.60 ± 0.39	0.02 ± 0.08	Collagen	
F53B1.4	1.24 ± 0.13	−0.18 ± 0.10	Thymidine metabolism	
T06E4.8	1.81 ± 0.13	−0.43 ± 0.08	Unknown	
Y73F8A.G	1.66 ± 0.32	−0.29 ± 0.16	Unknown	
F41E6.11	1.65 ± 0.13	−0.46 ± 0.14	Unknown	
W08E12.G	1.51 ± 0.36	−0.43 ± 0.08	Unknown	
W02A2.3	1.49 ± 0.31	−0.41 ± 0.12	Unknown	
D2096.6	1.47 ± 0.15	−0.27 ± 0.30	Unknown	

Shown is the mean ± SEM (n = 3) of the base$_2$ log–fold increase in hybridization signal in tunicamycin-treated animals over untreated animals for each genotype. The linkage group for the nine *abu* genes is also shown.

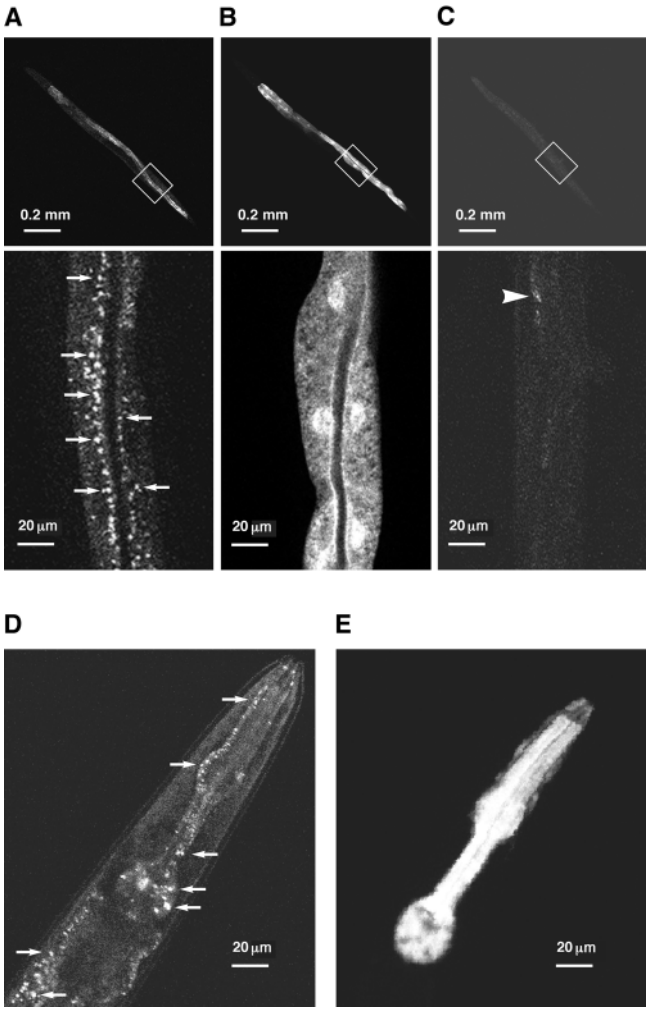
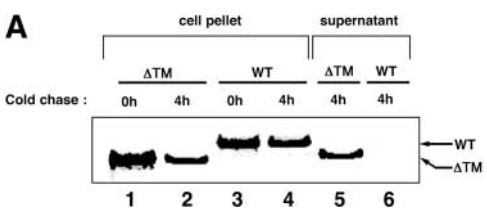


Figure 2. **Confocal fluorescence micrographs of *C. elegans* expressing fluorescent fusion proteins.** (A) *ges-1::abu-1::gfp(zcEx7)* expressing ABU-1 with GFP fused to its COOH terminus in the intestinal cell. (B) *ges-1::gfp(zcEx6)* expresses unmodified GFP in the intestinal cell. (C) Animals with no transgene. The arrows point to the vesicular structures positive for ABU-1–GFP and the arrowheads point to the background autofluorescence that is present in worms with no transgene. Shown are representative images obtained using the same settings. (D) The *abu-1::gfp(zcEx8)* transgene expresses an ABU-1–GFP fusion protein from the endogenous *abu-1* promoter. The arrows point to the vesicular structures positive for ABU-1–GFP protein. (E) The *abu-1::gfp(zcIs8)X* reporter expresses GFP from the abu-1 promoter.

pressed at these low levels was also associated with vesicular structures (Fig. 2, D and E), suggesting that this pattern of expression is not an artifact of unphysiologically high expression levels of the fusion proteins. However, given that we were not able to detect the endogenous ABU-1 protein, it remains possible that the apparent localization to intracellular vesicles is due to misfolding of the fusion proteins.

The fluorescence pattern of ABU-1–GFP suggested that the protein was associated with the intracellular endomembrane system. To extend these observations, we studied the subcellular localization of *C. elegans* ABU-1 in mammalian cells, an experimental system in which we could make use of established markers for the various organelles and compartments. In transfected mammalian COS1 cells, metabolically-labeled ABU-1 tagged at its COOH terminus with the FLAG epitope remained associated with the cell pellet, from which it could be extracted only by detergent solubilization (Fig. 3 A). Deletion of the predicted transmembrane domain led to secretion of the protein into the culture media (Fig. 3 A). Immunostaining of the FLAG-tagged ABU-1–expressing COS1 cells with anti-FLAG antibodies showed a diffuse reticular pattern that colocalized with the ER marker ribophorin I (Fig. 3 B). These results suggest that ABU-1 encodes an integral membrane protein that is retained in the ER by its transmembrane domain. The secretion of transmembrane domain–deleted ABU-1 suggests that the lumenal portion of the protein folds properly in mammalian cells and that the full-length ABU-1 is unlikely to be retained in the ER as a consequence of malfolding in mammalian cells.

The protein-coding region of the nine *ABU* genes is >89% identical at the nucleotide level over a stretch of ~100 residues in the 3′ end of the genes. Therefore, we considered it possible to simultaneously inactivate several members of this gene family by RNA-mediated interference (RNAi), using sequences derived from this conserved region. To study the impact of this genetic manipulation, we first identified a pharmacological treatment that would cause ER stress but could be tolerated by both wild-type and *ire-1* or *xbp-1* mutant animals. The heavy metal cadmium activated the *hsp-4::gfp* reporter (a marker of ER stress) in an *ire-1*– and *xbp-1*–dependent manner (Fig. 4 A), indicating that it causes ER stress. Both wild-type and *xbp-1* mutant animals tolerated cadmium exposure for extended periods of time (Fig. 4 B). Feeding of double-stranded *gfp* RNA (*gfp[RNAi]*)



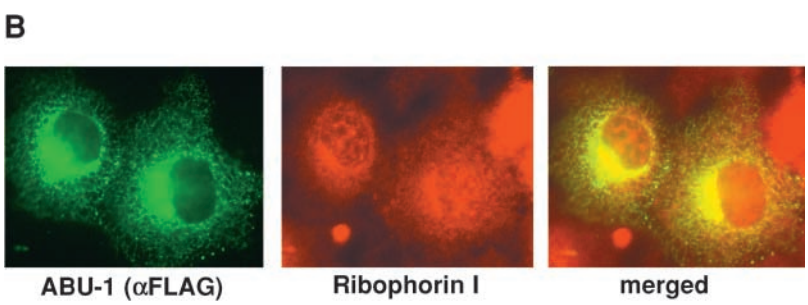


Figure 3. **ABU-1 is retained in the ER of mammalian cells by its transmembrane domain.** (A) Autoradiogram of metabolically-labeled wild-type ABU-1 (WT) or ABU-1 lacking the transmembrane domain (ΔTM) expressed in COS1 cells and immunoprecipitated immediately after the labeling period (lanes 1 and 3) or after 4 h of cold chase (lanes 2 and 4–6). The protein in lanes 1–4 was extracted by detergent from disrupted cells, whereas the protein in lanes 5 and 6 was recovered from the culture supernatant. (B) Immunocytochemical staining of FLAG-tagged ABU-1 expressed in COS1 cells using an anti-FLAG monoclonal antibody. Staining of the same cells with ribophorin I outlines the ER and the merged image reveals the colocalization of ABU-1 (anti-FLAG) and ribophorin 1.

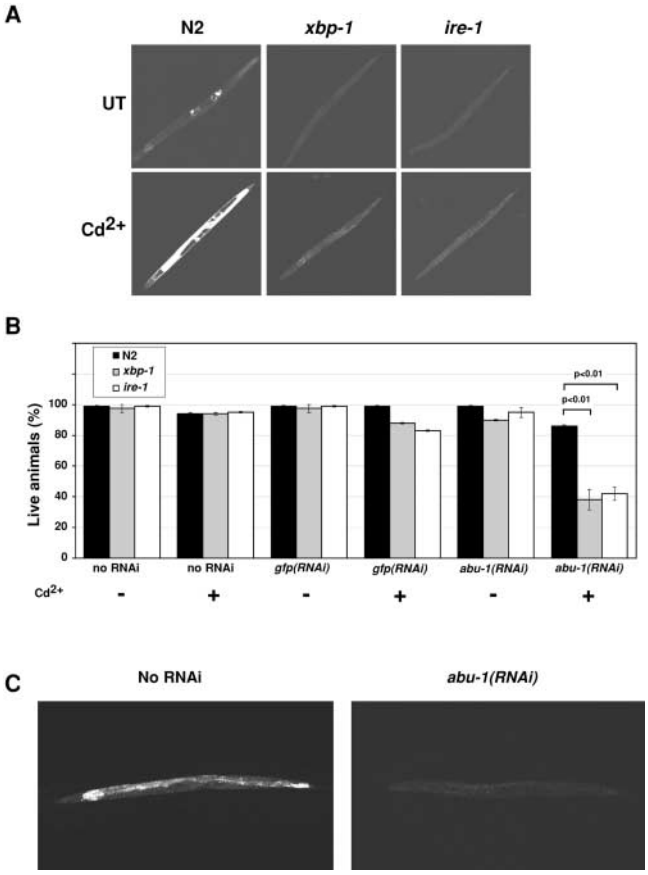


Figure 4. **Inactivation of *abu-1* by RNAi reduces the viability of *C. elegans* with a blocked UPR.** (A) Fluorescence micrographs of untreated (UT) or cadmium-treated (Cd^{2+}) wild-type, *xbp-1(zc12)III* mutant, or *ire-1(zc14)II* mutant *C. elegans* transgenic for the ER stress reporter gene *hsp-4::gfp(zcIs4)V*. (B) Survival at 48 h of untreated (UT) or cadmium-treated (Cd^{2+}) wild-type, *xbp-1(zc12)III* mutant, or *ire-1(zc14)II* mutant *C. elegans* at the L4 stage that had been subjected to *abu-1(RNAi)*, *gfp(RNAi)*, or no RNAi. Shown are mean ± SEM of a typical experiment performed in duplicate on 30 animals in each group and reproduced four times. (C) Fluorescence micrograph of untreated (UT) and *abu-1(RNAi)*–treated *ges-1::abu-1::gfp(zcEx7)* animals.

had little impact on the viability of wild-type or mutant animals whether exposed to cadmium or not (<5% lethality). However, feeding of double-stranded *abu-1* RNA markedly impaired survival of cadmium-treated *xbp-1* and *ire-1* mutant animals, but had minimal impact on survival of cadmium-treated wild-type animals or on survival of untreated *xbp-1* and *ire-1* mutant animals (Fig. 4 B). The inhibition of ABU-1 protein expression by *abu-1(RNAi)* was revealed by the observation that feeding double-stranded *abu-1* RNA to *ges-1::abu-1–gfp* animals strongly inhibited GFP fluorescence (Fig. 4 C). These observations are consistent with a role for *abu-1* (and possibly other *ABU* genes) in protecting animals with a defective UPR against ER stress.

To determine the expression pattern of *abu-1* in ER-stressed animals, we generated an *abu-1::gfp* transcriptional reporter *C. elegans* strain. *abu-1::gfp* was expressed constitutively in the pharynx and head region of wild-type and *xbp-1* mutant adult animals. A low level of basal expression was observed in the gut. Intestinal expression was induced by ER stress, most notably in *xbp-1* mutant animals (Fig. 5 A). The induction of *abu-1::gfp* in the intestine of cadmium- and tunicamycin-treated animals is consistent with observations that the intestine is active in protein secretion and is a major target for ER stress in *C. elegans* (Fig. 4 A) (Shen et al., 2001; Calfon et al., 2002). *abu-1(RNAi)* upregulated the ER stress reporter gene *hsp-4::gfp* in the intestine (Fig. 5 B), implying that *abu-1* (and possibly other *ABU* genes) may also be active in the intestine under basal conditions and that interfering with ABU function causes ER stress.

Genes active in degradation of malfolded ER proteins (ER-associated degradation [ERAD] genes) are not essential for yeast viability under normal growth conditions but are essential during ER stress or when the UPR is blocked. Furthermore, disruption of ERAD genes in yeast activates the UPR (Casagrande et al., 2000; Friedlander et al., 2000; Ng et al., 2000; Travers et al., 2000). We considered the possibility that *abu* genes might interact with the process of malfolded protein disposal from the *C. elegans* ER. First we confirmed that in *C. elegans*, like yeast, inactivation of ERAD genes causes ER stress. RNAi of *sel-1*, the *C. elegans* homologue of the yeast ERAD gene *HRD3* (Hampton et al., 1996), resulted in marked upregulation of the ER stress indicator *hsp-4::gfp* (Fig. 5 B). The induction of *hsp-4::gfp* by *sel-1(RNAi)* and by RNAi of a homologue of another yeast ERAD gene, *HRD1* (encoded in *C. elegans* by *F55A11.3*), were both *xbp-1* dependent (unpublished data).

Inactivation of *sel-1* by RNAi had no impact on the viability of wild-type animals and only modestly reduced the viability of *xbp-1* mutants. However, when combined with inactivation of *abu-1*, *sel-1(RNAi)* increased lethality of *xbp-1* mutant animals (Fig. 5 C). Combined inactivation of *sel-1* and *abu-1* also affected the appearance of *xbp-1* mutant animals. Viewed under Nomarski optics, the intestine of wild-type, *xbp-1* mutant, *abu-1(RNAi)*, or *sel-1(RNAi)* young adults had a similar, fine, granular appearance. Animals with compound *xbp-1;abu-1(RNAi)* or *xbp-1;sel-1(RNAi)* genotypes had more dark staining large intestinal granules. The *xbp-1;abu-1(RNAi);sel-1(RNAi)* animal had a marked increase in such granules (Fig. 5 D). These observations suggest that *abu-1* (and possibly other *abu* genes) and *sel-1* perform partially redundant functions in animals with a blocked UPR.

Discussion

Our search for genes activated in the *C. elegans* UPR revealed similarities to the yeast *S. cerevisiae*. In both species the UPR induces genes that function at many levels of the secretory pathway and encode proteins with diverse and unrelated biochemical activities. In both species, the signaling pathway initiated by IRE1 exerts nearly complete control over the induction of well-characterized components of ER client protein processing machinery, such as BiP (*hsp-4*), protein disulfide isomerases, and 4-prolyl hydroxylases. We confined our analysis to genes whose induction by tunicamycin passed stringent statistical criteria. The application of these stringent statistical criteria eliminated many genes that probably are true UPR targets and therefore the list presented in Table I is only representative of the most reliably

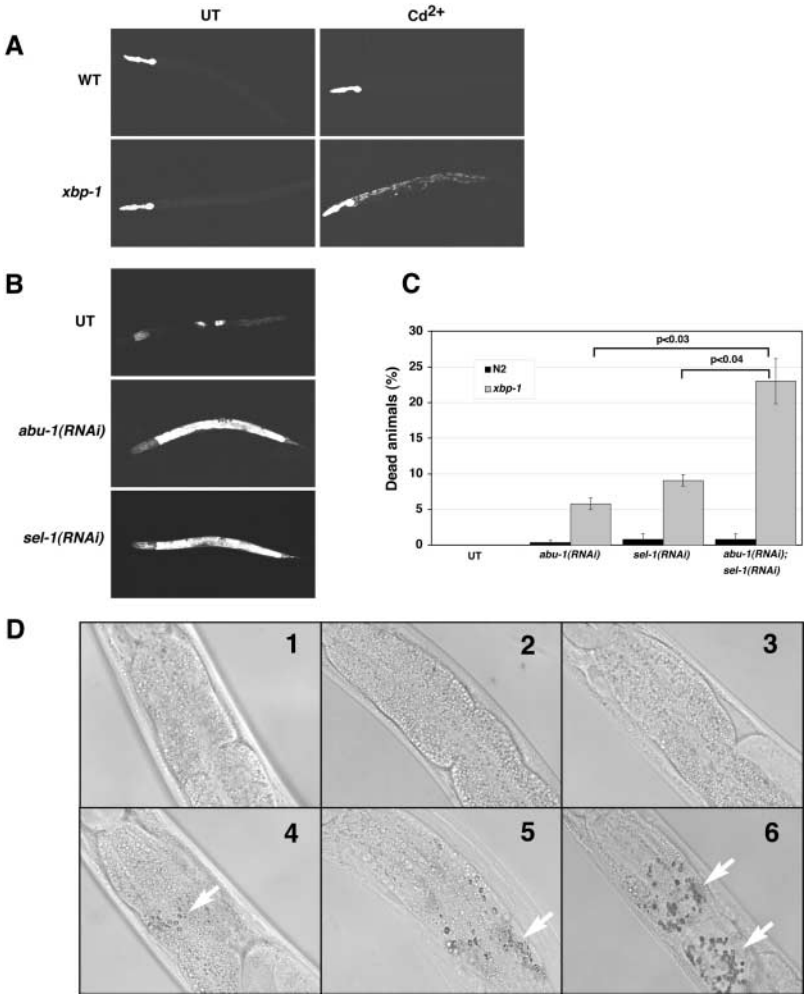


Figure 5. **Inactivation of *abu-1* causes ER stress and enhances the phenotype of *C. elegans* with defective ER-associated protein degradation.** (A) Fluorescence micrographs of untreated (UT) or cadmium-treated (Cd^{2+}) wild-type or *xbp-1(zc12)III* mutant *C. elegans* transgenic for an *abu-1::gfp(zcIs8)X* reporter. (B) Fluorescence micrographs of untreated (UT), *abu-1(RNAi)*, or *sel-1(RNAi)* animals transgenic for the ER stress reporter gene *hsp-4::gfp(zcIs4)V*. (C) Lethality at 72 h of wild-type and *xbp-1(zc12)III* mutant *C. elegans* untreated (UT), *abu-1(RNAi)*, *sel-1(RNAi)*, or compound *abu-1(RNAi)*; *sel-1(RNAi)*. Shown are mean ± SEM of a typical experiment performed in duplicate on 30 animals of each genotype and reproduced four times. (D) Photomicrographs using Nomarski optics (X400) of the intestine of living young adult *C. elegans* of the following genotypes: (1) wild type, (2) *abu-1(RNAi);sel-1(RNAi)*, (3) *xbp-1(zc12)III*, (4) *xbp-1(zc12)III; abu-1(RNAi)*, (5) *xbp-1(zc12)III; sel-1(RNAi)*, and (6) *xbp-1(zc12)III;abu-1(RNAi);sel-1(RNAi)*. The arrow points to the dark large granules that accumulate in the cytoplasm of the compound mutant animals.

induced UPR genes in *C. elegans*. Heterogeneity in developmental stage and variation in uptake of drug stand out as likely contributors to the lower power of the whole animal array performed here compared with yeast arrays (Casagrande et al., 2000; Travers et al., 2000), and as Fig. 1 D shows, the microarray hybridization procedure is also less sensitive than Northern blot analysis in detecting fold changes in expression of *hsp-4*. Despite these limitations, we can firmly conclude that most of the reliably induced UPR genes in *C. elegans* are *xbp-1* dependent.

Our genome-wide survey also identified 19 genes that were activated by ER stress in *xbp-1* mutant animals but not in wild-type animals. Among these were nine genes predicted to encode a family of highly related ABU proteins. These *abu* genes are coregulated across different physiological and developmental perturbation; clustering together in a single, small, isolated and well-defined "expression mountain," mount 29, in the recently established gene expression map of *C. elegans* (Kim et al., 2001). GFP driven by the promoter of a representative family member, *abu-1::gfp*, was expressed strongly in the pharynx and head region of transgenic animals from larval stages L3–L4 to young adult (Fig. 5 A). A similar expression pattern was also observed in animals transgenic for a transcriptional fusion of *gfp* to a different *abu* gene F19G12.7 (unpublished data). Furthermore, the basal expression pattern of the *abu-1* transcriptional reporters correlated nicely with the in situ hybridization pattern of another *abu* family member, C03A7.7. Together, these observations suggest that the *abu-1::gfp* reporter reflects the activity of the endogenous gene. In older animals, we noted low levels of basal expression of *abu-1::gfp* in the intestine that was markedly upregulated by ER stress in *xbp-1* mutant animals (Fig. 5 A). This last observation is consistent with the Northern blot analysis of *abu-1* expression (Fig. 1 D). Although the significance of basal expression of *abu* genes in the pharynx is currently not understood, the intestinal expression correlates with the phenotype of animals in which *abu-1* had been inactivated by RNAi. We do not know if *abu-1* induction by ER stress in *xbp-1* mutant animals is dependent on PERK (*pek-1*) or ATF6 (*atf-6*) signaling, as compound *xbp-1;pek-1* and *xbp-1;atf-6(RNAi)* mutant animals arrest at early larval stages before we could reliably examine *abu-1::gfp* expression in the gut (Shen et al., 2001; unpublished data).

Inactivation of *abu-1* by RNAi led to marked induction of the ER stress indicator *hsp-4::gfp* in the intestine (Fig. 5 B). This induction was completely blocked by the *xbp-1* mutation (unpublished data), indicating the development of ER stress in the gut of *abu-1(RNAi)* animals and implying that *abu-1* plays a role in an ER function, which, when

inactivated, causes ER stress. This ER stress appeared to be well tolerated, as the *abu-1(RNAi)* animals developed at a normal rate and survived exposure to Cd^{2+}, a toxin that induced further ER stress. However, in *xbp-1* mutant animals, *abu-1(RNAi)* had serious consequences, significantly reducing their viability after exposure to additional ER stress (Fig. 4 B). The *abu* genes have a very high level of sequence identity at the nucleotide level, and the double-stranded RNA used to inactivate *abu-1* could also initiate RNA inactivation of other family members. It is therefore possible that the strong phenotype observed in the *abu-1(RNAi)* animals reflects the simultaneous inactivation of other *abu* family members. Collectively, these results point to a role for *abu-1*, and possibly other *abu* genes, in some aspect of ER function that is also active under basal conditions. Reduced levels of this *abu*-dependent activity can be compensated by a normal UPR. However, when the UPR is compromised, loss of this *abu*-dependent activity becomes limiting, killing the affected animals.

In yeast, the ability to dispose of misfolded proteins in the secretory pathway (ERAD) plays an important role in adapting to high levels of ER stress, but is dispensable for viability under normal conditions (Casagrande et al., 2000; Friedlander et al., 2000; Ng et al., 2000; Travers et al., 2000). Given that *abu-1(RNAi)* is well tolerated under normal conditions, but not in animals experiencing high levels of ER stress, we considered the possibility that the *abu* genes may interact genetically with components of the *C. elegans* ERAD apparatus. An interaction was uncovered between *sel-1*, a *C. elegans* gene encoding an ER-localized component of the ERAD apparatus, and *abu-1*. Animals with diminished *xbp-1*, *sel-1*, and *abu-1* activity had reduced viability and accumulated unusually large vesicles with dark content in their intestinal cells (Fig. 5 D). *sel-1* and *abu-1* are unlikely to function in the exact same pathway, as *abu-1(RNAi)* did not affect the phenotype of a hypomorphic allele of *lin-12*, whereas *sel-1(RNAi)* suppressed the egg-laying defect of *lin-12(n676; n930)III* (unpublished data). However, the observation that inactivating either gene leads to ER stress and the synthetic interactions between them suggest that *sel-1* and *abu-1* function in interacting cellular processes.

ABU-1 is a transmembrane protein that likely functions within the endomembrane system (Figs. 2 and 3). The lumenal domain of some ABU proteins resembles the extracellular domains of a mammalian scavenger receptor and *C. elegans* CED-1. Both the scavenger receptors and CED-1 are believed to serve as cell surface receptors for chemically modified macromolecules, oxidized lipoproteins, and other altered plasma proteins in the case of the scavenger receptors (Van Berkel et al., 2000) and corpses of apoptotic cells in the case of CED-1 (Zhou et al., 2001). Endocytosis and lysosomal degradation follow binding of these abnormal ligands to their receptors. Scavenger receptors and CED-1 are therefore believed to play a role in recycling the building blocks of damaged macromolecules that are present outside the cell. It is possible therefore that the ABU proteins may be playing a similar role within the endomembrane system, perhaps by binding to altered ER client proteins and modulating their intracellular fate.

Materials and methods

RNA isolation, cDNA synthesis, microarray hybridization, and data analysis

Whole genome hybridization experiments were conducted as previously described (Reinke et al., 2000). In brief, RNA was harvested from untreated and tunicamycin-treated (5 μg/ml, 5 h) *hsp-4::gfp(zcIs4)V* (wild type) and *hsp-4::gfp(zcIs4)V; xbp-1(zc12)III* (mutant) animals. Total RNA was isolated from populations of stage-synchronized young adult hermaphrodite animals. Poly(A)$^+$ RNA was purified from total RNA by oligo (dT) affinity chromatography. Labeled cDNA probe for DNA microarray hybridization was prepared from 10 μg of poly(A)$^+$ RNA. cDNA from untreated and tunicamycin-treated animals was labeled with Cy5 and Cy3, respectively. Microarrays containing 17,871 *C. elegans* genes were used. Microarrays were hybridized for 20 h, scanned using an Axon scanner, and the expression level for each gene was determined using GenePix software. The $base_2$ log of the ratio of Cy3/Cy5 signal for each gene was determined in each of three hybridization experiments with a pair of untreated and tunicamycin-treated mRNA samples with wild-type or *xbp-1(zc12)III* genotypes (for a total of six hybridization experiments). A *t* test was used to determine the probability that differences in treatment-induced levels of mRNA between the wild type and *xbp-1* mutant were due to chance. A 95% confidence limit was applied. The data were analyzed twice, first using the standard deviation resulting from the experiments presented in this paper and then using the global standard deviation derived from the large set of combined experiments in the Stanford Microarray Database. Differences in levels of expression were reported only if they were significant using both measurements of standard deviation.

Transgenic *C. elegans*

A 1.3-kb fragment of *C. elegans* genomic DNA immediately 5′ of the predicted initiation ATG codon of *abu-1* (*AC3.3*) was amplified by PCR using the oligonucleotides AC3.1S (5′-GGCATTGTGGCACGCATTGAACTG-3′) and AC3Bam.2AS (5′-GATAGGATCCATTGTTAATATGCTTGAAGAGC-TGC-3′) and ligated in frame with the GFP coding region in the plasmid of pPD95.75 (gift of Andy Fire, Carnegie Institute of Washington, Baltimore, MD). The *abu-1::gfp(zcEx8)* strain was created by coinjecting the *ac3.3*.pPD95.75 plasmid (25 μg/ml) with a *lin-15* rescuing plasmid, pSK1 (25 μg/ml), into *lin-15(n765ts)* strain. The extrachromosomal array was integrated into the chromosome with ultraviolet/trimethylpsoraren treatment, yielding the *abu-1::gfp(zcIs8)X* reporter strain.

To produce the *ges-1::gfp(zcEx6)* strain, the *ges-1* promoter was amplified by PCR from *C. elegans* genomic DNA using the primers ges-1.1S (5′-CTCATACATCATTGTCAAGTGACG-3′) and ges-1.Pst.2S (5′-AGTACTG-CAGAGACAAGGAATATCCGCATCT-3′) and ligated into the HindIII-PstI sites of pPD95.81 (gift of Andy Fire). The *abu-1* coding region fragment encoding ABU-1 amino acids 7–425 was ligated in frame with GFP into PstI-BamHI sites of this plasmid to produce the *ges-1::abu-1::gfp(zxEx7)* strain. The promoter and coding region of *abu-1* was ligated in frame with GFP to produce *abu1::abu-1::gfp(ZcEx8)* strain. The animals containing transmissible extrachromosomal arrays were analyzed using a 510 laser scanning confocal microscope (ZEISS). Digital images were processed with Adobe Photoshop® 5.0.

RNAi feeding and activation of ER stress by pharmacological means

Interference with gene function by RNAi followed an established protocol (Timmons et al., 2001). In brief, double-stranded RNA was produced in HT115 strain of *Escherichia coli* transformed with pPD129 plasmids containing cDNA fragments of genes being studied (nucleotides 1032–1278 of *abu-1* cDNA and nucleotides 478–1631 of *sel-1* cDNA). IPTG (1mM) was added to the bacterial growth media to induce transcription of the double-stranded RNA, and L4-staged animals of defined genotype were added to plates individually and produced their brood. RNAi phenotypes were evaluated in these progeny. Where indicated, 30 L4-stage progeny from the RNAi plate or from a control plate were transferred to a new plate seeded with fresh *E. coli* where they were exposed to tunicamycin (5 μg/ml) for 5 h or $CdCl_2$ (10 mM) for the indicated period. Animals were scored as dead when pharyngeal pumping had ceased and they failed to move after being tapped on the nose with a platinum wire.

Cell culture, transfection, immunocytochemistry, and pulse-chase analysis

COS-1 cells were obtained from American Type Culture Collection. All cells were grown in DME containing 10% FBS (Cellgro®) at 5% CO_2 and 37°C.

ABU-1 was tagged with a Flag epitope at its COOH terminus by overlapping PCR using the primers AC3.Bam.7S (5′-GCGCGGATCCGCAATGCGCTTTATCGCAATTGCAG-3′), AC3.Flag.16AS (5′-ATCGTCGTCCTTGTAGTCCTTTCTCTTGCAACACTG-3′), and Flag3′U.Not (5′-CTAGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTC-3′). The full-length coding region of *abu-1* with the COOH-terminal Flag tag was cloned as a BamHI-NotI fragment into pcDNA3 (Invitrogen). COS-1 cells were transfected with this plasmid and stained with anti-Flag antibody (Sigma-Aldrich). Rabbit anti–ribophorin I antibody (a gift from Dr. Gert Kreibich, New York University [NYU] School of Medicine) was used to visualize the ER staining pattern.

The *abu-1* coding region fragment encoding ABU-1 amino acids 22–425 was amplified by PCR using primers AC3.EcoRI.5S (5′-GGCAACAAGAATTCGCGATAAACG-3′) and AC3.XbaI.4AS (5′-GCGCTCTAGATGTCTACTTTCTCTTGCAAC-3′). The *abu-1* coding region fragment coding ABU-1 amino acids 22–331 was amplified by PCR using AC3.EcoRI.5S and AC3.XbaI.6AS (5′-GGCATCTAGAGCTTGGCATGCAGATTGGC-3′), deleting the transmembrane domain and COOH terminus of ABU-1. These fragments were cloned into pcDNA3 containing a Flag epitope with signal peptides. COS-1 cells were transfected with wild-type ABU-1 or ABU-1 lacking the transmembrane domain with a Flag tag. Newly synthesized proteins were labeled in vivo with ^{35}S-methionine and ^{35}S-cysteine (500 μCi/ml; ICN Biochemicals). After removal of the labeling media, cells were washed and incubated in complete media for cold chase for the indicated times. Cells were washed and lysed with 1% Triton buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 4 mg/ml aprotinin, and 2 mg/ml pepstatin A). Samples were clarified by centrifugation at 14,000 *g* for 10 min and by preincubation for 1 h with 10 μl protein A–Sepharose. Soluble proteins from the cells and secreted proteins in the media were immunoprecipitated by anti-Flag antibody bound to 10 μl protein A–Sepharose and washed three times with RIPA buffer. Bound proteins were resolved by 10% SDS-PAGE under reducing conditions and revealed by autoradiography.

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Note added in proof. It has recently been noted that ABU genes are predicted to contain glutamine- and asparagine-rich ("prion") domains (Wormbase web site, http://www.wormbase.org, release WS77, April 12, 2002). Such domains are predicted to form polar zipper protein–protein interactions with similar domains on the same protein or on other proteins. This raises the interesting possibility that ABU proteins may recognize similar domains exposed on abnormally folded ER client proteins through such polar zipper interactions and thereby target them for destruction.

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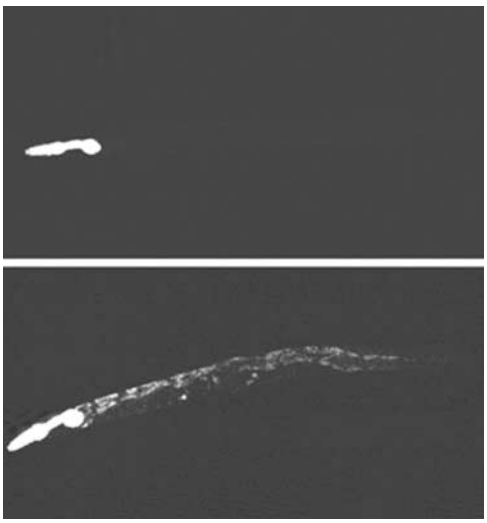
ABU to perform under stress

A report on page 639 identifies a family of proteins that are important when worms are unable to deal with ER stress. The results from Urano et al. suggest that these proteins are receptors for incorrectly folded proteins at the ER.

In worms and other eukaryotes, conditions that stress the ER, such as those that inhibit glycosylation, activate the unfolded protein response (UPR). The UPR comprises several signaling pathways that increase the protein folding capacity of the ER through the induction of chaperones and other protein processing and trafficking components.

The UPR does not, however, explain everything. Using microarrays, Urano et al. identified a default pathway activated in worms under ER stress when the UPR is blocked. Among the genes identified were nine members of a family they named *abu*, for activated in blocked UPR. RNAi inhibition of one or more ABU proteins in UPR-deficient mutants severely impaired their survival during ER stress. In wild-type worms, interference with ABU function by RNAi induced ER stress genes, indicating that the ABU proteins may also function under nonstress conditions.



ER stress induces *abu-1* (white) when UPR pathways are blocked (bottom).

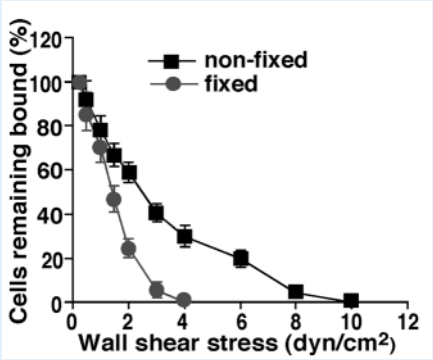
Localization experiments indicated that ABU-1 is an ER-localized integral membrane protein. The ABU family resembles mammalian scavenger receptors, plasma membrane proteins that bind extracellular proteins that are oxidized or otherwise chemically modified. ABU proteins may serve a similar function at the ER, although a direct interaction with improperly folded proteins has yet to be shown. Mammals do not have obvious orthologues of ABU proteins. But recent results showing that the ER and plasma membrane may fuse during phagocytosis suggest that the distinction between plasma membrane proteins and ER proteins may not be as clear as previously suggested. Thus, the scavenger receptors may be able to fill in for ABU proteins in the ER. ■

Selectin flexible cells

Leukocytes use glycoprotein ligands to grab onto selectins on blood vessel walls and roll along with the blood flow. The longer the cell can roll without detaching, the greater its chances are of responding to chemoattractants telling it to traverse the endothelium at a site of inflammation. Now, on page 787, Yago et al. show that flexibility helps a cell avoid detachment when circulatory forces are high.

As shear force increases with higher blood pressure and smaller vessel size, leukocytes can maintain a stable rolling velocity, an ability known as an automatic braking system (ABS). Artificial microspheres containing certain ligands also roll on selectins, but lack an ABS—under high forces, their rolling velocity increases, and they are more apt to detach. Recent discussion in the field has centered on whether this difference reflects a problem with ligands or their carriers: either the microsphere ligands may not be optimal for binding under high forces, or the microspheres may lack some structural feature of cells.

Yago et al. address this issue by using the same ligands on both microspheres and a hematopoietic cell type that does not express selectin ligands. Their results show that ABS is a function of the ability of the cell to change its shape, a feature not found in rigid microspheres. Cells made more flexible by depolymerizing actin rolled even more efficiently. Making cells stiffer by fixation or addition of a cholesterol chelator made cells roll more like microspheres. ABS efficiency may be improved through the flattening of a cell, placing more of its ligands in contact with selectin on endothelial cells. Rolling leukocytes are also known to extend long thin tethers at their trailing edge; these structures may reduce the force exerted on the rest of the cell. ■



Flexible cells (squares) are less likely to detach as shear stress increases.