RNA surveillance is required for endoplasmic reticulum homeostasis

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The unfolded protein response (UPR) is an intracellular stress-signaling pathway that counteracts the accumulation of misfolded proteins in the endoplasmic reticulum (ER). Because defects in ER protein folding are associated with many pathological states, including metabolic, neurologic, genetic, and inflammatory diseases, it is important to understand how the UPR maintains ER protein-folding homeostasis. All metazoans have conserved the fundamental UPR transducers IRE1, ATF6, and PERK. In *Caenorhabditis elegans*, the UPR is required to prevent larval lethality and intestinal degeneration. Although *ire-1*-null worms are viable, they are particularly sensitive to ER stress. To identify genes that are required for development of *ire-1*-null worms, we performed a comprehensive RNA interference screen to find 10 genes that exhibit synthetic growth and intestinal defects with the *ire-1(v33)* mutant but not with *atf-6(tm1153)* or *pek-1(ok275)* mutants. The expression of two of these genes, *exos-3* and *F48E8.6*, was induced by ER stress, and their knockdown in a wild-type strain caused ER stress. Because these genes encode subunits of the exosome complex that functions in mRNA surveillance, we analyzed other gene products required for nonsense-mediated mRNA decay (NMD). Our results demonstrate that defects in *smg-1*, *smg-4*, and *smg-6* in *C. elegans* and *SMG6* in mammalian cells cause ER stress and sensitize to the lethal effects of ER stress. Although ER stress did not activate mRNA surveillance complex assembly, ER stress did induce SMG6 expression, and NMD regulators were constitutively localized to the ER. Importantly, the findings demonstrate a unique and fundamental interaction where NMD-mediated mRNA quality control is required to prevent ER stress.

endoplasmic reticulum quality control | premature termination codons

The endoplasmic reticulum (ER) is the site for the folding and modification of newly synthesized proteins that are destined for intracellular organelles, the plasma membrane, and the extracellular milieu. Protein folding in the ER is facilitated by molecular chaperones and is monitored by a stringent ER quality control system (ERQC) that allows only properly folded proteins to traffic to the Golgi apparatus. In ERQC, misfolded proteins are retained in the ER for an attempt to attain their appropriate conformation, and irreversibly misfolded proteins are targeted to ER-associated protein degradation (ERAD) or to autophagy (1).

The accumulation of misfolded proteins in the ER caused by alterations in ER homeostasis initiates signaling of the unfolded protein response (UPR) that attempts to resolve the protein-folding defect (2). In metazoans, the UPR is signaled through three ER transmembrane transducers, IRE1, ATF6, and PERK, that sense the accumulation of misfolded proteins and transmit signals to the cytosol and the nucleus. IRE1 is a protein kinase/endoribonuclease that initiates unconventional splicing of mRNA encoding the transcription factor X box-binding protein 1 (XBP1), to create a translational frameshift that produces an active transcription factor (sXBP1) (3, 4). ATF6 has a basic leucine zipper domain in its cytosolic N terminus. Upon accumulation of unfolded proteins in the ER, ATF6 transits to the Golgi apparatus, where it is processed by site 1 and site 2 proteases to release the cytosolic domain (ATFp50) for translocation to the nucleus to activate transcription (5, 6). sXBP1 and ATF6(p50) bind to the UPR element and the ER stress element (ERSE) to up-regulate the transcription of genes that function in ER protein folding, modification, trafficking, and degradation (7–9). The third UPR transducer, PERK, is a protein kinase that phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) to attenuate mRNA translation initiation, thereby reducing the unfolded protein load (10–12). In this manner, UPR signaling attempts to maintain the fidelity of protein folding and minimize the accumulation of unfolded proteins by ensuring a balance between the protein-folding load and the protein-folding capacity.

Recently, new mechanisms were described that ensure ERQC. ER stress induces autophagy, an intracellular degradation system for bulky aggregated proteins and dysfunctional organelles (13–16). Although the mechanisms that signal ER stress-induced autophagy remain unclear, it is thought that autophagy is part of ERQC to eliminate misfolded and/or aggregated proteins in the ER. In addition, preemptive ERQC was reported to degrade misfolded nascent polypeptides at the step of cotranslational translocation into the ER (17–19). Finally, it was proposed that regulated IRE1-dependent mRNA decay initiates the degradation of mRNAs encoding proteins that are translocated into the ER lumen (20, 21). Therefore, it appears that multiple mechanisms have evolved to prevent the accumulation of misfolded proteins in the ER.

In metazoans, newly synthesized mRNAs are subject to a pioneer round of translation that is necessary to ensure the presence of an intact ORF and provides an essential mechanism for mRNA quality control. Nonsense-mediated mRNA decay (NMD) is an mRNA surveillance mechanism that degrades mRNAs containing premature termination codons (PTCs) that would generate polypeptides prone to misfolding (22). The mechanism of NMD has been extensively characterized. The decay-inducing complex, including UPF1(SMG2), UPF2(SMG3), UPF3(SMG4), ribosome releasing factor (eRF)2, eRF3, and the PI3-kinase–like protein SMG1, captures the substrate mRNA during the pioneer round of translation. Subsequent to release of ribosomes and eRFs and SMG1-mediated UPF1 phosphorylation to repress translation initiation and promote decapping, SMG6 or SMG5/SMG7 binds to phosphorylated UPF1 and promotes protein phosphatase 2A-mediated dephosphorylation of UPF1 (23–26). SMG6 is an endoribonuclease that cleaves at a PTC-proximal site within the mRNA for subsequent 3′-5′ degradation by the exosome and 5′-3′ decay by XRN1 (27–29).

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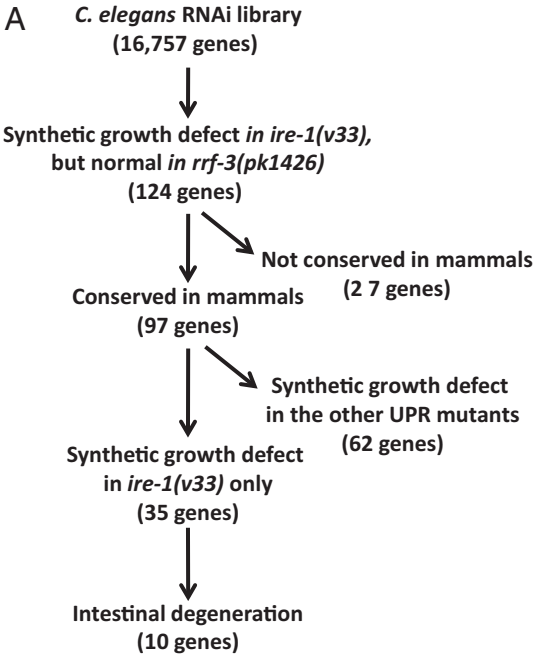
In this report, we demonstrate that genes required for NMD also contribute to ER homeostasis and are necessary for *Caenorhabditis elegans* larval development and mammalian cell proliferation in response to ER stress. In particular, SMG6 and two exosomal subunits, DIS3 and EXOSC3, were induced by ER stress. Although UPF1 phosphorylation and RNA surveillance complex assembly were not affected by ER stress, NMD regulators were constitutively localized to the ER. We propose that an effective RNA surveillance system on the ER membrane is required to maintain ER function and prevent the generation of misfolded proteins during the pioneer round of translation.

Results

***C. elegans* RNAi Screen Identifies Genes Required for Growth of *ire-1 (v-33)*.** Although the basic mechanisms of tripartite UPR signaling are conserved in all metazoan species, different species and cell types rely on different subpathways for their function and survival. Unfortunately, the presence of two *Ire1* homologs and two *Atf6* homologs in mammals has limited the analysis of their functional significance. Fortunately, *C. elegans* has a single gene for each of the key UPR regulators [PERK (*pek-1*), ATF6 (*atf-6*), IRE1 (*ire-1*), and XBP1 (*xbp-1*)]. However, previous studies revealed that the *ire-1*/*xbp-1* pathway regulates the vast majority of UPR transcriptional control in response to acute stress (induced UPR) and in larval development (constitutive UPR) (30). Although *C. elegans* with deletions of either single UPR sensor are viable, double deletion of either *pek-1* or *atf-6* in combination with *ire-1* produces the typical phenotype of UPR deficiency in *C. elegans*, which is L2/L4 larval lethality and intestinal degeneration. In contrast, the double mutant of *pek-1* and *atf-6* exhibits a normal phenotype (3). These findings suggest that the *ire-1*/*xbp-1* pathway is the most important UPR signaling pathway for ER functional homeostasis and embryonic development. The findings also suggest that the *ire-1(v33)* deletion mutant has adapted to constitutive ER stress to survive and propagate.

To identify additional genes that are required for development of the *ire-1(v33)* deletion mutant in *C. elegans*, we used feeding-mediated RNAi analysis to screen for genes that exhibit a synthetic larval lethal/severe growth defect and intestinal degeneration in the *ire-1(v33)* mutant but not in the *pek-1(ok275)* mutant. Screening of 16,757 genes covering 87% of the *C. elegans* genome did not identify any genes except for *ire-1* and *xbp-1* that exhibit synthetic lethality with only the *pek-1(ok275)* mutant. In contrast, this screen identified 97 genes that were evolutionally conserved in mammals and required for the normal growth of the *ire-1(v33)* mutant but did not affect growth of the control *rrf-3(pk1426)* mutant, which is hypersensitive to RNAi treatment (31) (Fig. 1*A* and Table S1). Of these 97 genes, knockdown of 62 genes displayed synthetic growth defects in both the *ire-1(v33)* and *atf-6(tm1153)* mutants, or all three UPR mutants [including *pek-1(ok275)*]. Within these 62 genes was a set of nuclear-encoded mitochondrial genes (*F59A3.3*, *nuo-2*, *F54C4.1*, *Y53G8AL.2*, *tag-313*, *phb-2*, *mrps-5*, *T22B11.5*, *Y43F8C.8*, *C47E12.2*, and *F53F4.10*), although their knockdown did not cause intestinal degeneration in any of the UPR mutants. Knockdown of these mitochondrial genes might deplete ATP or cause oxidative stress and inhibit worm development through a mechanism other than intestinal degeneration. Knockdown of 10 of these 62 genes caused intestinal degeneration in both the *ire-1 (v33)* and *atf-6(tm1153)* mutants (Table S2). This group includes master regulators of ER protein biosynthesis [*hsp-4* (GRP78) and *F08D12.1* (SRP72)]. Interestingly, this group also contained *nfyc-1*, which may encode a subunit of nuclear transcription factor Y (NF-Y) that binds with ATF6 to the mammalian ERSE (32, 33). It is



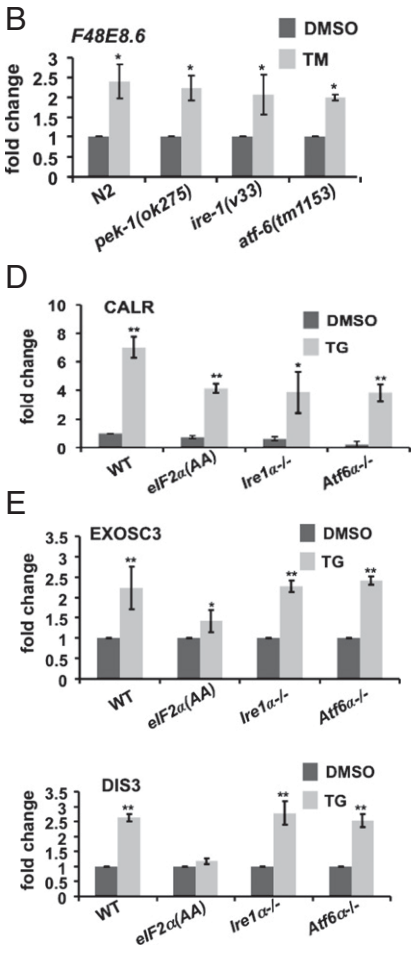


Fig. 1. *ire-1* interacts genetically with components of the exosome. (*A*) A comprehensive RNAi analysis identifies genes required for growth and intestinal proliferation in *ire-1(v33)* worms. Knockdown of 97 evolutionally conserved genes exhibited synthetic growth defects in the *ire-1(v33)* strain. Of these, 10 caused both a synthetic severe growth defect and intestinal degeneration in the *ire-1(v33)* mutant, but not in the other UPR mutants tested. (*B*) TM induction of *F48E8.6* does not require any single UPR pathway. Worms of each strain at the L2 larval stage were treated with 10 μg/mL TM for 3 h and analyzed by qRT-PCR. The expression of each gene was normalized to *act-2* (the *C. elegans* homolog of mammalian ACTβ). The relative mRNA abundance in each strain was normalized to the expression in wild type N2. (*C*) TG induces expression of CRT1, DIS3, and EXOSC3 in murine immortalized hepatocytes. Cells were treated with 1 μM TG for 8 h and analyzed by qRT-PCR. (*D* and *E*) Neither single UPR pathway is required for TG induction of CALR, whereas TG induction of DIS3 and EXOSC3 requires eIF2α phosphorylation. Cells were treated with TG and analyzed by qRT-PCR as described (*C*). Average values ± SD are shown (*B*, $n = 3$; *C*–*E*, $n = 4$). *P* values were calculated using the Student's *t* test. $*P < 0.05$; $**P < 0.01$; bars with no asterisks, $P > 0.05$.

Table 1. Genes that caused synthetic severe growth defect and intestinal degeneration by RNAi-mediated knockdown in *ire-1(v33)*

Gene name	Gene annotation	GFP level	Ratio of expression level to N2 (SD)		
		zcIs4	*ire-1(v33)*	*atf-6(tm1533)*	*pek-1(ok275)*
crt-1	Calreticulin	Induced	1.26 (±0.112)*	0.441 (±0.212)*	0.993 (±0.119)
arf-6	ADP ribosylation factor	Induced	1.71 (±0.159)**	1.09 (±0.186)	1.07 (±0.301)
4R79.2	GTP-binding protein SEC4	Induced	1.67 (±0.579)	0.969 (±0.0384)	0.981 (±0.355)
F48E8.6	Exosomal 3′-5′ exoribonuclease subunit Rrp44/Dis3	Induced	2.23 (±0.520)*	1.34 (±0.388)	1.39 (±0.169)*
exos-3	Exosomal 3′-5′ exoribonuclease subunit Rrp40/EXOSC3	Induced	2.78 (±0.392)**	0.978 (±0.0427)	1.08 (±0.281)
Y39G10AR.8	Translation initiation factor 2, γ-subunit	Reduced	1.35 (±0.323)	0.688 (±0.145)*	0815 (±0.0612)*
rpl-1	Large ribosomal subunit L 10a protein	Reduced	1.67 (±0.459)	0.991 (±0.102)	1.08 (±0.158)
ZK1098.4	Translation initiation factor 2B, α-subunit	Not changed	1.51 (±0.599)	1.32 (±0.282)	1.22 (±0.021)**
cpl-1	Cysteine proteinase Cathepsin L	Not changed	0.1.67 (±0.545)	1.11 (±0.299)	1.17 (±0.136)
K06A5.8	WD-repeat containing protein	Not changed	2.43 (±0.858)*	0.799 (±0.296)	0.870 (±0.312)

The 10 genes that exhibit synthetic lethality and intestinal defects upon RNAi knockdown in *ire-1(v33)* worms are listed. Gene annotations are from WormBase. ER stress induction upon RNAi knockdown was evaluated in the *zcIs4* strain (Fig. S1). The relative expression of each gene, analyzed by qRT-PCR, in the mutant strains is compared with the wild type (N2). Average values ± SD are shown ($n = 3$). *P* values were calculated using the Student's *t* test. $*P < 0.05$; $**P < 0.01$; bars with no asterisks, $P > 0.05$.

possible that NFYC-1 functions with XBP1 and/or ATF6 to activate UPR genes in *C. elegans*.

The synthetic lethal screen identified 35 genes that specifically exhibited growth defects in only the *ire-1(v33)* mutant but not in the *atf-6(tm1153)* and/or *pek-1(ok275)* mutants. RNAi-mediated knockdown of these 35 genes identified 10 genes that caused intestinal degeneration in the *ire-1(v33)* mutant (Fig. 1*A* and Table 1). In addition, this screen also identified *atf-6* and *pek-1*, which were used as positive controls for the synthetic lethal screen in *ire-1(v33)*. We assessed the effect of knockdown of these 10 genes on ER homeostasis by RNAi treatment in a *zcIs4* transgenic strain harboring the *hsp-4* promoter-driven GFP transgene that exhibits ER stress-dependent induction of GFP fluorescence. RNAi-mediated knockdown of five genes (*crt-1*, *arf-6*, *4R79.2*, *F48E8.6*, and *exos-3*) elevated GFP expression in intestinal cells (Table 1 and Fig. S1). This suggests that these five genes are required to maintain ER protein-folding homeostasis. In addition to *crt-1* encoding an ER chaperone, two of these genes, *4R79.2* and *arf-6*, encode vesicle trafficking-associated proteins. The reduced expression of these genes could directly disrupt ER function by perturbing ER protein folding and/or trafficking. Interestingly, the two other genes, *F48E8.6* and *exos-3*, encode a core subunit (RRP44/DIS3) and a regulatory subunit (RRP40/EXOSC3) of the 3′-5′ exoribonuclease (exosome) complex.

Quantitative RT-PCR (qRT-PCR) revealed that the basal expression of 3 of these 10 genes (*F48E8.6*, *exos-3*, and *K06A5.8*) was elevated more than twofold in the *ire-1(v33)* mutant compared with the wild type, whereas expression of *crt-1* was reduced to 44% in the *atf-6(tm1153)* mutant (Table 1). Because RNAi knockdown of these genes caused synthetic lethality in the *ire-1(v33)* mutant, the findings suggest up-regulation of *F48E8.6*, *exos-3*, and *K06A5.8* and that ATF6-mediated expression of *crt-1* may compensate for the loss of *ire-1* to ensure ER homeostasis during larval development. Furthermore, the expression of *F48E8.6* and *crt-1* was up-regulated more than twofold by tunicamycin (TM), which inhibits *N*-linked glycosylation and disrupts ER protein folding (Fig. 1*B* and Fig. S2), whereas the other genes were induced less than twofold or not at all (Fig. S3). TM induction of *F48E8.6* did not require *ire-1*, *atf-6*, or *pek-1*, suggesting that its induction did not require any single UPR pathway or that it uses an unconventional UPR pathway (Fig. 1*B*). On the other hand, the induction of *crt-1* required *ire-1* but not *atf-6*, indicating that *crt-1* requires *atf-6* for constitutive expression during larval development and requires *ire-1/xbp-1* for induction upon acute ER stress, consistent with previous findings (34).

We also analyzed ER stress induction of the murine homologs of these 10 genes in immortalized murine hepatocytes deleted in either *Ire1α* or *Atf6α* or harboring the serine 51 to alanine mutation at the PERK-mediated phosphorylation site in eIF2α [*eIF2α(AA)*] (10, 35, 36). Thapsigargin (TG) treatment, which depletes ER Ca^{2+} by inhibition of the Ca^{2+}-dependent ATPase in the ER, induced CALR (*crt-1*), DIS3 (*F48E8.6*), and EXOSC3 (*exos-3*) more than twofold in wild-type hepatocytes (Fig. 1*C* and Table S3). Similar to *C. elegans*, the basal expression of CALR was reduced to 22% in $Atf6\alpha^{-/-}$ cells compared with the wild type (Fig. S4), but its TG induction was not significantly reduced in any of the mutants, including the $Ire1\alpha^{-/-}$ hepatocytes (Fig. 1*D*). These findings suggest that the ATF6 requirement for basal expression of CALR is evolutionally conserved. However, no single UPR pathway is required for induction of CALR in response to acute ER stress in mammals, where only the *ire-1/xbp-1* pathway regulates *crt-1* induction in *C. elegans*. In contrast to the *C. elegans* homologs, the basal expression of DIS3 and EXOSC3 was not increased in $Ire1\alpha^{-/-}$ cells (Fig. S4), although their TG induction was reduced in *eIF2α(AA)* mutant cells (Fig. 1*E*). Therefore, ER stress induction of DIS3 and EXOSC3 apparently requires eIF2α phosphorylation in mammalian cells. Together, these findings indicate that DIS3 and EXOSC3 are important for ER functional homeostasis and larval development in *C. elegans*. The exosomal complex mediates mRNA decay in NMD surveillance. Consistent with this notion, we found that knockdown of either DIS3 or EXOSC3 caused accumulation of mutant protein derived from a PTC-containing mutant of the GABA receptor α 1 (GABRA1), which is degraded by ERAD (37) (Fig. S5). These findings suggest that a functional interaction exists between mRNA quality control and ER homeostasis.

NMD Genes Are Required for ER Homeostasis and Survival in Response to ER Stress. To assess whether ER protein-folding homeostasis requires NMD-mediated mRNA surveillance, we analyzed deletions of the NMD genes *smg-1*, *smg-4*, and *smg-6* in the *zcIs4*

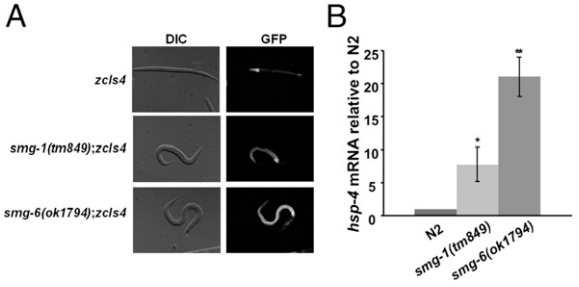


Fig. 2. The UPR is activated in *smg-1(tm849)* and *smg-6(ok1794)* mutant strains. (*A*) Expression of *hsp-4*-GFP is increased in *smg-1(tm849)* and *smg-6(ok1794)* mutants. Differential interference contrast (DIC) microscopy and fluorescence microscopy for GFP in L4 larvae from *smg-1(tm849);zcIs4*, *smg-6(ok1794);zcIs4*, and the parental *zcIs4* worms. (*B*) *hsp-4* mRNA expression is increased in *smg-1(tm849)* and *smg-6(ok1794)* mutants. L2-stage worms were analyzed by qRT-PCR for *hsp-4*. Error bars and asterisks are as described in Fig. 1 ($n = 3$).

transgenic *hsp-4* GFP reporter strain. All strains displayed greater GFP fluorescence compared the parental *zcIs4* strain (Fig. 2*A* and Fig. S6), and qRT-PCR revealed that the expression of endogenous *hsp-4* mRNA was constitutively elevated in the *smg-1(tm849)* and *smg-6(ok1794)* mutant strains compared the wild type N2 (Fig. 2*B*). The *smg-1*, *smg-4*, and *smg-6* mutants exhibited synthetic growth defects upon silencing of *ire-1* (Fig. S7). Although *smg-1*, *smg-4*, *smg-5*, and *smg-7* were represented in the bacterial RNAi library, they were not included in the 10 genes obtained in our screen, possibly because their knockdown was insufficient. *smg-6* was not included in the 10 genes because *smg-6* was not represented in the bacterial RNAi library. *smg-2* and *smg-3* were not tested by either RNAi treatment or gene disruption.

To analyze the NMD requirement for ER homeostasis in mammalian cells, we silenced SMG6 in HeLa cells. Analysis of an NMD reporter assay using *Renilla* luciferase fused to a minigene of β-globin demonstrated that siRNA-mediated knockdown of SMG6 caused accumulation of a PTC-containing mRNA (Fig. S8 *A* and *B*). Immunoblotting and qRT-PCR analysis revealed that SMG6 knockdown increased expression of CHOP and GRP78 (Fig. 3*A* and Fig. S9). The activity of the CHOP promoter was up-regulated by SMG6 knockdown, indicating that transcriptional activation of CHOP occurs upon NMD inhibition (Fig. S8*C*). Analysis using an ATF4 reporter in which the 5′ UTR of ATF4 is fused to GFP (38) demonstrated increased GFP expression upon SMG6 knockdown (Fig. S8*D*). In addition, knockdown of SMG6 increased *Xbp1* mRNA splicing compared with nonspecific siRNA-treated cells up to approximately threefold (Fig. 3 *B* and *C*). The silencing of SMG1 also slightly increased CHOP mRNA, although it did not alter expression of GRP78 or sensitize cells to ER stress, possibly due to inefficient knockdown (Fig. S10). These findings support the notion that NMD is required for ER homeostasis, namely that in the absence of NMD, cells activate the UPR.

To determine whether NMD is required for cell survival in response to ER stress, we analyzed cell survival in response to TM treatment. Whereas TM slightly reduced survival of HeLa cells treated with nonspecific siRNA, TM reduced survival to ~50% in cells treated with SMG6 siRNA (Fig. 4*A*). TM also reduced survival of immortalized murine hepatocytes treated with SMG6 siRNA compared with nonspecific siRNA (Fig. 4*B*). The overexpression of a dominant-negative SMG6 mutant lacking the PIN domain, which is required for endoribonuclease activity (28), also reduced cell survival compared with overexpression of wild-type SMG6 (Fig. 4*C*). Finally, overexpression of wild-type SMG6 increased survival of HeLa cells in response to ER stress (Fig. 4*D*). Importantly, overexpression of SMG6 attenuated CHOP induction upon long-term treatment with TM, whereas the induction of GRP78 was not affected (Fig. S11). Because CHOP encodes a proapoptotic transcription factor, this observation suggests that the proapoptotic response is reduced by overexpression of SMG6. These observations support the idea that NMD is critical for survival in response to ER stress.

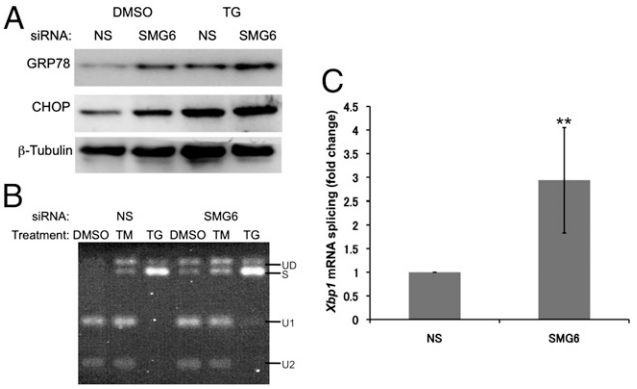


Fig. 3. Knockdown of SMG6 causes ER stress in HeLa cells. (*A*) Knockdown of SMG6 increases GRP78 and CHOP protein in HeLa cells. HeLa cells were transfected with SMG6 siRNA #2 (see Table S4) or nonspecific (NS) siRNA, and after 72 h were treated with 1 μM TG for 8 h. The expression of GRP78, CHOP, and β-tubulin was analyzed by immunoblotting. (*B*) Knockdown of SMG6 induces splicing of *Xbp1* mRNA. HeLa cells were transfected with SMG6 siRNA #2 or nonspecific siRNA. After 72 h, cells were treated with 1 μM TG, 10 μg/mL TM, or DMSO for 4 h. *Xbp1* mRNA splicing was analyzed as described in *Materials and Methods*. S, spliced *Xbp1* mRNA; U1 and U2, digested unspliced fragments 1 and 2; UD, undigested unspliced fragments, likely representing heteroduplex DNA. (*C*) The intensities of each band were determined as described in *Materials and Methods*, and efficiencies of *Xbp1* splicing were quantified by calculating the percentage of spliced *Xbp1* mRNA relative to total *Xbp1* mRNA (S, U1, U2 plus UD). Induction of *Xbp1* splicing by knockdown of SMG6 in comparison with nonspecific siRNA treatment is shown. Error bars and asterisks are as described in Fig. 1 (n = 10).

SMG6 Expression Is Induced in Response to ER Stress and Is Localized to the ER. An NMD reporter assay demonstrated that a PTC-containing mRNA accumulated in response to TM treatment (Fig. S12). Therefore, we analyzed the effect of ER stress on NMD activation by analysis of UPF1 phosphorylation and interaction with other components of the RNA surveillance complex in HeLa cells treated with TG. TG treatment did not alter UPF1 phosphorylation or coimmunoprecipitation with UPF3B or SMG7 (Fig. 5*A*). Because secretory proteins most frequently possess a signal peptide at their N terminus for targeting to the ER during the pioneer round of translation, we analyzed whether NMD regulators are localized to the ER. Immunofluorescence microscopy revealed colocalization of endogenous SMG6 and UPF3B with the ER protein chaperone GRP78/BiP (Fig. 5*B*). These observations suggest that NMD regulators constitutively reside at the ER.

We next asked whether the expression of NMD genes is regulated by ER stress. HeLa cells were treated with ER stress-inducing agents and the expression of SMG1, UPF1, UPF2, UPF3A, UPF3B, SMG5, SMG6, and SMG7 was analyzed by qRT-PCR. The results demonstrated that the expression of SMG6 was up-regulated approximately threefold by treatment with either TM or TG (Fig. 5*C*). The expression of SMG6 was also induced two- to threefold in response to TG treatment in wild type immortalized hepatocytes (Fig. 5*D*). However, ER stress-mediated induction of SMG6 was not reduced in any of the UPR mutant hepatocyte cell lines (Fig. 5*D*), indicating that any single UPR signaling pathway is not required for induction of SMG6 in response to ER stress.

Discussion

In this study, we discovered that genes encoding exosome and NMD machinery are required for ER functional homeostasis, cell survival upon ER stress, and normal development in worms lacking *ire-1*. Our findings suggest that one component of ER quality control relies on an intact mRNA surveillance system to minimize ER stress. The following observations support this conclusion: (*i*) The loss or reduced expression of NMD genes (*F48E8.6*, *exos-3*, *smg-1*, *smg-4*, and *smg-6* in *C. elegans*; *SMG6* in mammals) causes ER stress; (*ii*) *F48E8.6*, *exos-3*, *smg-1*, *smg-4*, and *smg-6* display synthetic growth defects with the *ire-1(v33)* deletion mutant; (*iii*) SMG6 knockdown in mammalian cells reduces and SMG6 overexpression increases cell survival in response to ER stress; (*iv*) DIS3 (*F48E8.6*) is induced by acute ER stress in *C. elegans* and mammals, and EXOSC3 (*exos-3*) and SMG6 are induced by acute ER stress in mammals; and (*v*) NMD factors are constitutively localized to the ER. Our findings lead us to propose that a defect in NMD would decrease the fidelity of mRNA quality control and increase the synthesis of proteins having aberrant carboxyl termini, which in the case of secretory proteins would misfold upon translocation into the ER. On the other hand, the accumulation of misfolded proteins in the ER would activate NMD as a preemptive quality control mechanism to degrade mRNAs before their cytoplasmic translation to attenuate protein misfolding. However, at this point, we cannot rule out that the requirement for exosomal components to survive ER stress reflects another exosome-dependent process, such as RNA processing or RNA degradation.

Remarkably, our findings indicate that ER stress induces the accumulation of PTC-containing mutant mRNAs and expression

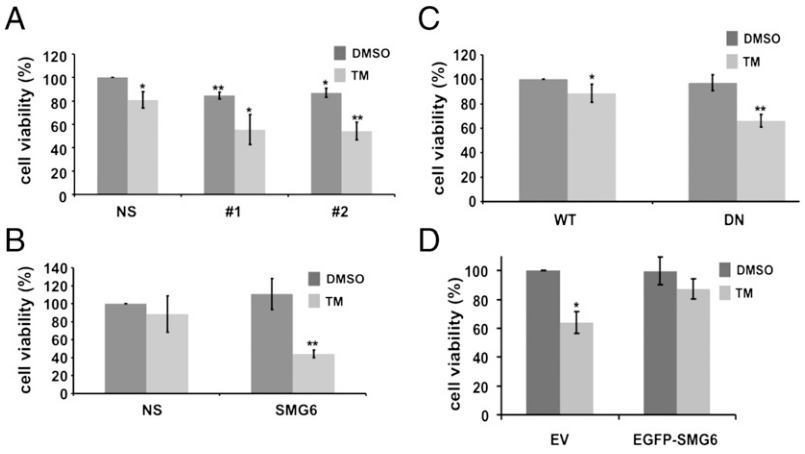


Fig. 4. SMG6 is required for cell survival in response to ER stress. (*A* and *B*) SMG6 siRNA reduces survival in ER-stressed cells. HeLa cells (*A*) and immortalized murine hepatocytes (*B*) were transfected with SMG6 siRNA. At 24 h after transfection, cells were treated with 250 ng/mL TM for 48 h, and cell viability was measured by MTS assay (*Materials and Methods*). Two different SMG6 siRNAs were analyzed in HeLa cells, and a mixture of four different siRNAs was analyzed in the hepatocytes. (*C*) Dominant-negative (DN) SMG6 reduces cell survival in response to ER stress. HeLa cells were transfected with plasmid DNAs encoding wild-type SMG6 (pEGFP-hSMG6) or a dominant-negative SMG6 mutant lacking the PIN domain (pEGFP-hSMG6ΔPIN). At 24 h after transfection, cells were treated with 300 nM TG for 48 h and analyzed for cell viability by MTS assay. (*D*) Overexpression of SMG6 improves survival of ER-stressed cells. HeLa cells were transfected with pEGFP-hSMG6 (SMG6) or empty vector (EV). At 24 h after transfection, cells were treated with 2 μg/mL TM for 48 h and analyzed for cell viability by MTS assay. Error bars and asterisks are as described in Fig. 1 (*A*, *B*, and *D*, $n = 3$; *C*, $n = 4$).

of SMG6 and exosomal subunits (DIS3 and EXOSC3), whereas the phosphorylation of UPF1 and assembly of the RNA surveillance complex are not affected (Figs. 1 and 5 and Fig. S12). In addition, the NMD regulatory proteins (SMG6 and UPF3B) localize to the ER (Fig. 5*B*). These observations suggest that NMD-mediated mRNA surveillance functions at the ER membrane, possibly in association with the translocon, to ensure quality control for mRNAs encoding secretory proteins. Induction of SMG6 and the exosomal subunits may be required to increase the efficiency of cleavage and degradation of PTC-containing mRNAs in NMD to attenuate the generation of misfolded proteins in response to ER stress.

Our findings show that the induction of exosomal subunits is regulated by eIF2α phosphorylation. In mammals, eIF2α is phosphorylated by four eIF2α kinases, including PERK. Because TM induction of *F48E8.6* was not altered by *pek-1* deletion (Fig. 1*B*), we speculate that eIF2α phosphorylation might result from other eIF2α kinases, such as *gcn-2*. In contrast, our findings indicate that neither of the UPR signaling pathways (IRE1α, ATF6α, or PERK) is required for ER stress induction of SMG6 in mammalian cells (Fig. 5*D*). To unravel the interplay between ER quality control and mRNA surveillance, further studies are required to investigate the mechanism by which NMD factors are recruited to the ER membrane, how SMG6 is induced upon ER stress, and how they coordinately function with ERQC to maintain ER functional homeostasis. The notion that NMD and ERAD cooperate to limit the production of abnormal proteins was recently suggested from analysis of nonsense mutations in the $GABA_A$ receptor GABRA1 (37) (Fig. S5).

It was also recently suggested that an IRE1-dependent surveillance system exists to degrade mRNAs associated with the ER membrane to limit the synthesis of proteins that require folding in the ER lumen (20, 21). The NMD machinery may provide a unique role to maintain ER homeostasis that does not involve NMD, namely contributing to the decay of mRNAs at the ER membrane that are initially cleaved by IRE1 in response to ER stress. To elucidate the molecular mechanism by which ERQC and the NMD machinery interact, future studies should investigate what mechanism(s) recruits NMD factors to the ER membrane and how their function is coordinated with processes of protein translocation and folding, ERAD, and UPR signaling to maintain ER protein-folding homeostasis.

NMD prevents the generation of misfolded proteins in many diseases caused by nonsense gene mutations. Recent studies suggest that inhibition of NMD or suppression of translation termination may be therapeutic in some of these human diseases. For example, a group of patients with Ullrich disease has nonsense mutations in the type II collagen IV [COLα2(IV)] gene, so that the mRNA is subject to NMD. Although the translation product of the mutant mRNA is truncated, it is structurally stable and functional at the cell surface. Inhibition of NMD or siRNA-mediated knockdown of SMG1 improved cell adhesion of cells from patients with Ullrich disease (39). Therefore, NMD inhibition may have clinical utility in management of these diseases. However, our findings show that NMD deficiency causes ER stress and reduces cell proliferation in response to ER stress. Therefore, NMD may be required for cells that are exposed to ER stress. As the UPR is required for differentiation and survival of cells that secrete large amounts of protein,

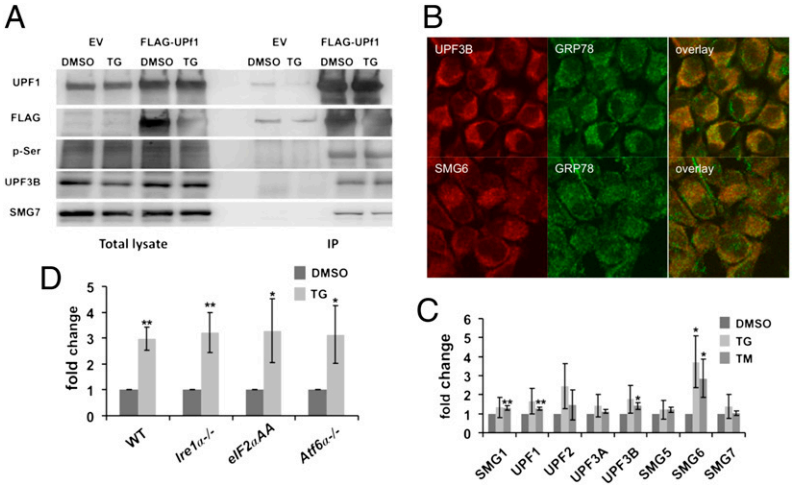


Fig. 5. ER stress does not alter NMD activation, but induces SMG6 expression. (*A*) ER stress does not alter UPF1 phosphorylation or assembly of the NMD complex. HeLa cells were transfected with pCMV5FLAG-hUPF1 or empty vector. After 36 h, cells were treated with 1 μM TG for 8 h. UPF1 phosphorylation and RNA surveillance complex formation were analyzed by immunoprecipitation (IP) with anti-FLAG antibody and Western blotting with antibodies as indicated. (*B*) SMG6 and UPF3B colocalize with GRP78. HeLa cells were stained with anti-UPF3B or anti-SMG6 and anti-GRP78 antibodies and observed by confocal microscopy. (*C*) ER stress induces expression of SMG6. HeLa cells were treated with 10 μg/mL TM or 1 μM TG for 8 h, and mRNA expression was analyzed by qRT-PCR. The expression of each gene was normalized to the expression of ACTβ, and the abundance relative to mock treatment is shown. (*D*) Induction of SMG6 by ER stress does not require any single UPR pathway. Wild type and *eIF2α(A/A)*, $Ire1\alpha^{-/-}$, and $Atf6\alpha^{-/-}$ immortalized hepatocytes were treated with DMSO or 1 μM TG for 16 h, and SMG6 expression relative to ACTβ was analyzed by qRT-PCR. Error bars and asterisks are as described in Fig. 1 ($n = 3$).

as well as for metabolic and inflammatory responses, there should be caution in treating patients with NMD inhibitors. We propose that the development of NMD inhibitors that do not disrupt ER homeostasis will be advantageous for the treatment of individuals with nonsense gene mutations, such as in muscular dystrophy.

Materials and Methods

Detailed procedures are available in *SI Materials and Methods*.

Nematode Strains. *smg-1(tm849)*, *smg-4(tm5409)*, and *atf-6(tm1153)* were obtained as described (40). All other strains were obtained through the *Caenorhabditis* Genetics Center. Genetic crosses were performed as described (41). The details of the mutant strains, except for *smg-4(tm5409)*, are shown in WormBase (http://www.wormbase.org). *smg-4(tm5409)* was generated in this study and lacks 338 bp in exon 2.

Feeding-Mediated RNAi Treatment in Worms. Comprehensive feeding-mediated RNAi analysis was performed as described (42) using the *C. elegans* RNAi library obtained from Source BioScience.

RNA Preparation, qRT-PCR, and XBP1 Splicing Analysis. cDNA synthesized from 1 μg of total RNA was analyzed by qPCR using POWER SYBR Green PCR Master Mix and the 7500 Real-Time PCR System (Applied Biosystems). Threshold cycles (CT) were normalized to *act-2* mRNA in worms or ACTβ mRNAs in mammals. XBP1 splicing was analyzed as described (43) and quantified using Image Gauge version 4.12 (Fujifilm).

Cell Proliferation and Luciferase Reporter Assays. Cell proliferation was analyzed by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay using the CellTiter 96 Aqueous One Solution Assay (Promega). Luciferase reporter assays were performed using the Dual Luciferase Reporter System (Promega).

Detection of UPF1 Phosphorylation and RNA Surveillance Complex Assembly. Human UPF1 cDNA was amplified by RT-PCR from total mRNA extracted from HeLa cells and inserted into pCMV5-FLAG to express FLAG-hUPF1 in HeLa cells. Detection of UPF1 phosphorylation and the RNA surveillance complex was performed as described (44).

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