Interaction between quality control systems for ER protein folding and RNA biogenesis

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The endoplasmic reticulum (ER) is the intracellular organelle responsible for the synthesis, folding and assembly of proteins destined for secretion and the endomembrane system of the cell. ER quality control (ERQC) is an intensively studied surveillance mechanism that selectively degrades misfolded proteins to ensure that only properly folded proteins exit the ER en route to the Golgi compartment. Proper protein folding is indispensable for the differentiation and function of cells that secrete high levels of protein and defects in protein folding are implicated in many pathologies, including metabolic, genetic, neurodegenerative and inflammatory diseases. Accumulation of misfolded proteins in the ER activates an adaptive set of signaling pathways, collectively known as the unfolded protein response (UPR), to resolve protein misfolding and restore ER homeostasis. Nonsense-mediated RNA decay (NMD) is an RNA surveillance system that selectively degrades nascent mRNAs containing premature termination codons (PTCs). Recently, we used a genetic screen to identify genes that interact with UPR signaling in *C. elegans*. These studies identified NMD-associated genes that are required for ER protein folding homeostasis. These findings link the quality control systems required for ER protein folding and RNA biogenesis, provide new insights into mechanisms of ERQC and have implications on diseases of ER dysfunction and therapeutic approaches based on NMD inhibition. Here, we discuss the biological significance of these findings and future directions for study.

C. elegans as a Genetic Model System for UPR Research

The ER is the center of protein biogenesis for secretory proteins and proteins localized to the secretory pathway in eukaryotic cells. Protein folding is the most error-prone step in gene expression. Maintaining ER protein folding homeostasis is essential for the numerous physiological processes associated with protein trafficking through the endomembrane system of the cell and protein secretion. Protein misfolding in the ER causes diverse pathological states including metabolic, genetic, neurodegenerative and inflammatory diseases. Therefore, eukaryotic cells have evolved mechanisms to ensure the fidelity of ER protein folding, which are known as ER quality control (ERQC). There are multiple defense systems that function to maintain ER protein folding homeostasis: (1) Only properly folded proteins are transported to the Golgi, and misfolded proteins are selectively retained in the ER for repeated attempts to fold properly; (2) Irreversibly misfolded proteins are re-translocated to the cytosol and degraded by the ubiquitin-proteasome pathway known as ER-associated protein degradation (ERAD);[1] (3) ER stress-induced autophagy (ERA) is induced under severe conditions of ER stress.[3-6] The biological significance of ERA is unclear, but it may be important for the bulk degradation of excessive or aggregated misfolded proteins in the ER and for supplying

energy sources derived from degradation products; (4) Accumulation of misfolded proteins interferes with ER function and eventually leads to apoptotic cell death. To counteract ER stress, the UPR is activated in an attempt to resolve the protein folding defect through reducing protein synthesis and increasing the capacity for ER protein folding and degradation;[2] and finally (5) Preemptive ER quality control degrades misfolded nascent polypeptides during their biogenesis at the step of co-translational translocation into the ER.[7-9]

The nematode *C. elegans* is an established genetic model system to study the UPR in metazoan species. In higher eukaryotes, the UPR consists of tripartite signaling pathways initiated by the ER stress sensor proteins IRE1, ATF6 and PERK. IRE1 is a bifunctional protein kinase and endoribonuclease that initiates unconventional splicing of the mRNA encoding XBP-1 to create a translational frame-shift to produce a potent transcription factor XBP-1s.[10,11] Upon accumulation of unfolded proteins in the ER, ATF6 traffics to the Golgi apparatus where cleavage by Site-1 and Site-2 processing enzymes release a cytoplasmic fragment containing a basic leucine zipper domain (bZIP)-containing transcription factor (ATF6p50).[12,13] XBP-1s and ATF6p50 bind to the UPR element (UPRE) and the ER stress element (ERSE), respectively, to upregulate expression of genes encoding functions in ER protein folding and degradation. Concomitantly, PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), to attenuate global protein biosynthesis to reduce the ER protein-folding load.[14-16] In this manner, the UPR maintains the fidelity of ER protein folding by coupling the capacity for ER protein folding/degradation with the ER protein-folding load.

The mechanism of activation the UPR sensors is conserved in all metazoan species, including *C. elegans*. In mammals, two homologs of *Ire1* and *Atf6* provide diversity for tissue-specific responses to ER stress, but this diversity has limited the analysis of their functional significance. Importantly, *C. elegans* possess single genes encoding IRE1 (*ire-1*), ATF6 (*atf-6*) and PERK (*pek-1*), making it a relatively simple model system in which to analyze

the significance of each signaling pathway. Recently, we revealed that the majority of ER stress response genes rely on the *ire-1/xbp-1* pathway in larval development (constitutive UPR: cUPR) and in response to acute stress (induced UPR: iUPR).[17] Furthermore, double deletion of either *atf-6* or *pek-1* in combination with *ire-1* causes a synthetic lethality and intestinal degeneration at the L2 larval stage, although each single deletion mutant displays a normal phenotype.[10] In contrast, worms with double deletion of *atf-6* and *pek-1* were apparently normal. These findings indicate that *ire-1/xbp-1* pathway is the most important pathway for ER homeostasis and embryonic development in *C. elegans*, raising question as to how worms that lack *ire-1* adapt and grow under conditions of chronic ER stress.

Nonsense-Mediated RNA Decay (NMD) in ER Homeostasis

Approximately 30% of single gene disorders are derived from nonsense mutations resulting from single nucleotide changes that introduce a premature termination codon (PTC). As a result of this nonsense mutation, a translation product that lacks the carboxy-terminus of the protein is generated that could misfold and produce a non-functional product or exhibit a dominant-negative effect to inhibit the function of the protein encoded by the wild-type allele. As a quality control mechanism to eliminate mRNAs containing premature termination codons, eukaryotic cells evolved nonsense-mediated RNA decay (NMD) to degrade nonsense-containing mutant RNAs prior to production of aberrant protein products.[18] PTC-containing mRNAs are detected at the pioneer-round of translation through a process in which a protein complex containing NMD regulators (SMG1, UPF1, UPF2 and UPF3) captures the mutant mRNA. SMG1, a member of the phosphoinositide 3-kinase (PI3K)-like kinase family, phosphorylates UPF1 causing a conformational change in the protein complex that recruits SMG6 and SMG5/7. Because SMG6 possesses an RNase H-like endoribonuclease activity, it cleaves the mutant mRNA at a site proximal to the PTC. Additionally, SMG7 is reported to cause dephosphorylation

of UPF1 that is mediated by protein phosphatase 2A (PP2A).[19-22] Finally, the cleaved RNA is degraded through 3'–5' degradation mediated by the exosome and 5'–3' degradation mediated by XRN1.[23-25]

We recently reported that NMD is required for ER homeostasis in *C. elegans* and mammals.[26] A comprehensive RNAi screen covering 87% of *C. elegans* genome was used to identify subunits of the 3'–5' exosomalribonuclease complex (F48E8.6 and exso-3; homologs of *DIS3* and *EXOSC3* in mammals) that are required for normal development of an *ire-1 C. elegans* deletion mutant. F48E8.6 and exso-3 were upregulated in the *ire-1* deletion mutant and their silencing caused ER stress in a wild-type strain. Furthermore, silencing of their mammalian homologs caused accumulation of translational products derived from a PTC-containing mRNA. *DIS3* and *EXOSC3* were induced in response to ER stress in an eIF2α phosphorylation-dependent manner. These findings demonstrate a requirement for exosome function in ER homeostasis, and suggest that NMD may function in ER homeostasis. Indeed, genetic disruption of *smg-1, smg-4* or *smg-6* in *C. elegans*, or silencing of SMG6 in mammals provoked ER stress. These *smg* mutants caused growth defects in in *ire-1* mutant worms, and silencing of SMG6 sensitized cells to pharmacological induction of ER stress. Although ER stress caused accumulation of a PTC-containing mRNA, the assembly of the NMD machinery and UPF1 phosphorylation were not altered by ER stress. However, we found that SMG6 was induced in response to ER stress, and NMD regulators were constitutively localized to the ER. The findings led us to conclude that NMD is required for ERQC to promote ER homeostasis.

What is the Interface Between NMD and ERQC?

Our findings reveal a novel interaction between NMD and ERQC. However, important questions arise. First, why do NMD defects provoke ER stress? The simplest explanation is that NMD functions as a "vanguard defense system" of ERQC to remove nonsense-mutated RNAs before steady-state translation. Our findings, as

well as those of Kang et al.,[27] demonstrate that NMD and exosomal deficiencies cause translation products derived from PTC-containing mRNAs to accumulate in the ER. Furthermore, NMD deficiency induces ER stress in intestinal cells of worms, which actively secrete proteins and hormones. Therefore, NMD may be important for the proliferation and function of secretory cells that are susceptible to chronic ER stress. ER stress can generate oxidative stress that could lead to gene mutations and transcriptional errors that would produce PTC-containing mRNAs.[28] Therefore, NMD deficiency would lead to accumulation of PTC-containing mRNAs and thereby generate more misfolded proteins to exacerbate ER stress in the intestine.

In addition to the decay of nonsense-containing mRNAs, NMD also regulates gene expression by degrading normal mRNAs that possess long 3'-UTRs, or PTC-containing alternatively-spliced mRNAs that are associated with NAS (nonsense-mediated alternative splicing).[29-32] It is also possible that defects in NMD cause ER stress by inhibiting NAS to generate PTC-type mRNA variants that encode defective ERQC-associated functions or products that act in a dominant-manner to disrupt ER homeostasis.

A second question that arises from our findings is how and why are NMD components regulated in response to ER stress? It is relevant that ER stress did not increase assembly of the NMD surveillance complex or UPF1 phosphorylation. As DIS3, EXSOC3 and SMG6 function in the cleavage and decay of PTC-containing mRNAs that are captured by the NMD surveillance complex, we propose that ER stress does not alter the capture of aberrant mRNAs, but rather, promotes their degradation. Although the mechanism of ER stress-mediated induction of *DIS3, EXSOC3* and *SMG6* remains unclear, the induction of DIS3 and EXOSC3 requires an eIF2α phosphorylation-dependent pathway in mammalian cells. Interestingly, the expression of F48E8.6, a *C. elegans* homolog of *DIS3*, was also increased by pharmacological induction of ER stress. However, F48E8.6 induction did not rely on any of single UPR pathway, including *pek-1*. Therefore, it is possible that other eIF2α kinases (e.g., GCN2), function in addition to PERK for ER stress-mediated induction of F48E8.6 in *C. elegans*.

Physiological and Clinical Significance of the Interaction of ERQC with NMD

Recently, a couple reports suggest therapeutic potential for approaches that inhibit NMD.[33,34] In Ullrich disease, a type of muscular dystrophy, a group of patients possess a PTC-mutation in the *COL(IV) A2* gene, which encodes the α2 subunit of Collagen IV. Ohno and colleagues revealed that NMD eliminates the PTC-containing *COL(IV)a2* mRNA, although this mutation produces a functional and stable truncated translation product.[33] Remarkably, silencing of SMG1 rescues the formation of functional extracellular matrix proteins in patient-derived tissue culture cells. Furthermore, Gilboa and colleagues reported that inhibition of NMD effectively induces tumor immunity to counteract tumor growth.[34] Because NMD inhibition can alter gene expression and increase the production of truncated proteins, the application of these therapeutic approaches should consider the adverse effect of NMD inhibition on increasing the production of misfolded proteins from stabilized PTC-containing mRNAs. Our findings demonstrate that NMD inhibition increases susceptibility to acute ER stress in mammalian cells, and also chronic ER stress in *ire-1* mutant worms. Furthermore, in *C. elegans*, NMD deficiency induces ER stress in intestinal cells, which require ER homeostasis for normal proliferation and function. These observations suggest that inhibition of NMD causes chronic ER stress in secretory cells. In patients with hereditary predisposition to ER stress, such as those with heterozygous mutations in genes that would produce proteins that cannot properly fold in the ER, NMD inhibition may disrupt cellular protein folding homeostasis interfere with proper cell function. Therefore, potential side effects of NMD inhibition on induction of ER stress should be evaluated. In considering the use of NMD inhibitors in the clinic, it would be advisable to develop avenues of NMD inhibition that would not disrupt cellular homeostasis. Studies are required to further understand the molecular mechanism and physiological significance of the interaction between NMD and ERQC.

Conclusion

NMD interacts with ER quality control to promote ER functional homeostasis and may be important to preserve ER function by eliminating PTC-containing mRNAs during the pioneer-round of translation (**Fig. 1A**). Defects in NMD stabilize PTC-containing mRNAs that would produce misfolded or dominant-negative truncated translation products that could cause ER stress (**Fig. 1B**). Under conditions of ER stress, the exosome subunits F48E8.6 and DIS3 are induced by an eIF2α phosphorylation-dependent pathway in mammalian cells (**Fig. 1C**). Because of their roles in NMD, induction of these gene products would increase cleavage and decay of PTC-containing mRNAs. Since protein misfolding in the ER causes oxidative stress,[28] protein misfolding could also increase DNA mutations that could increase the production of PTC-containing mRNAs (**Fig. 1C**). The interaction of NMD with ERQC may be important to minimize the generation of aberrant proteins to support the proliferation and function of secretory cells (such as intestinal cells of worms), which are susceptible to chronic ER stress. These findings suggest that the application of inhibitors in the clinic should proceed with caution as the approach may disrupt ER protein-folding homeostasis.
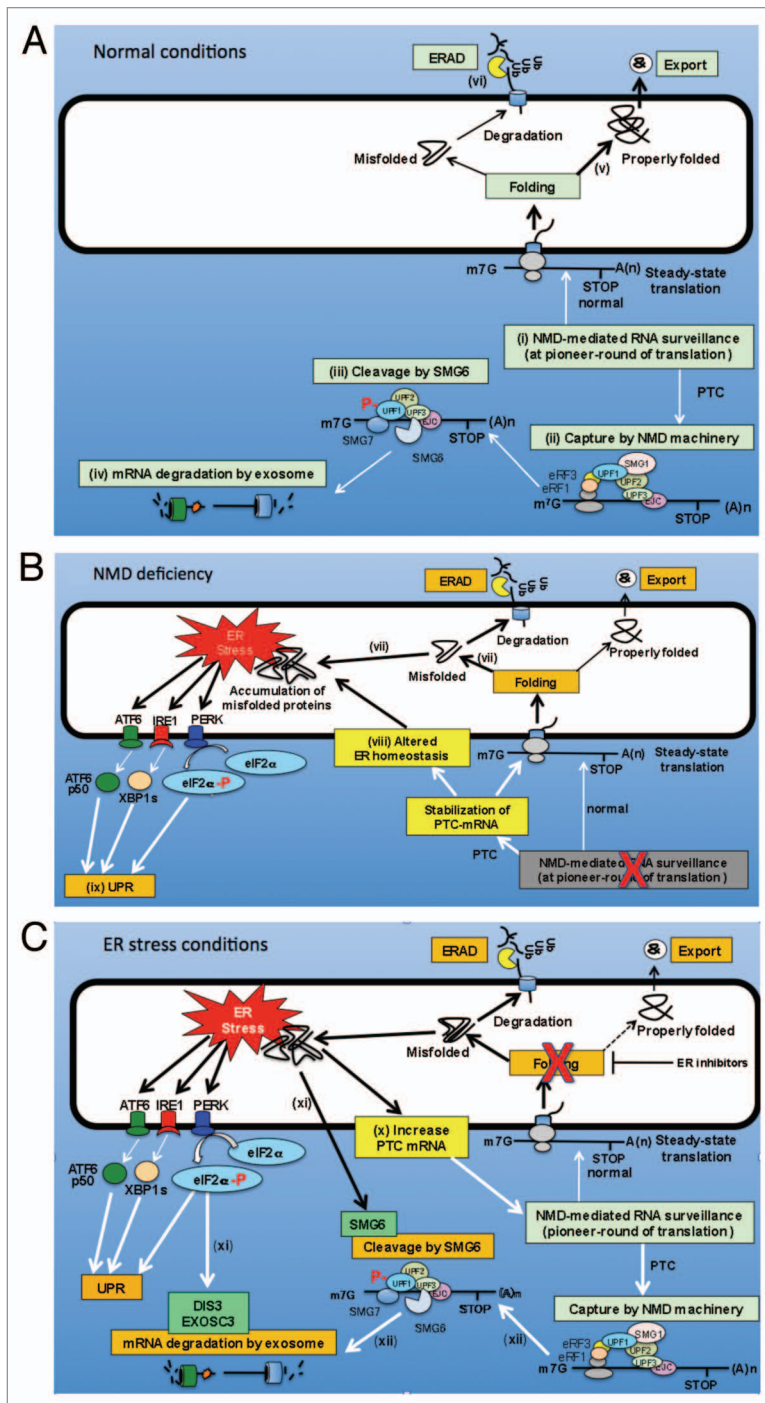
Figure 1. Model for the interaction of NMD and ERQC. (**A**) Under normal conditions, mature mRNAs are inspected at the pioneer-round of translation (i). Upon recognition of a PTC by the ribosome, the PTC-containing mRNA is captured by the NMD protein complex, cleaved by SMG6 and degraded by the exosome and XRN1 (ii)-(iv). Proteins translocated into the ER are modified and folded by ER protein chaperones (v). Properly folded proteins exit the ER *en route* to the Golgi, whereas misfolded proteins are degraded by ERAD or autophagy (vi). (**B**) Defects in NMD stabilize PTC-containing mRNAs that: (1) encode misfolded proteins to provoke ER stress (vii); or (2) disrupt ER function by altering gene expression or generating proteins that act in a dominant-negative manner (viii). The UPR is activated to maintain ER homeostasis (ix). (**C**) Under conditions of ER stress, the generation of PTC-containing mRNAs is increased, possibly due to oxidative stress-mediated DNA damage (x). In response to ER stress, the exosome subunits (DIS3 and EXOSC3) are induced by an eIF2α phosphorylation-dependent pathway and SMG6 is induced by an unidentified pathway (xi). Induction of these genes promotes cleavage and decay of PTC-containing mRNAs captured by NMD machinery (xii).

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