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Authors

Karam, Rachid
Lou, Chih-Hong
Kroeger, Heike
et al.

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The unfolded protein response is shaped by the NMD pathway

Rachid Karam[1,†], Chih-Hong Lou[1], Heike Kroeger[2], Lulu Huang[1,‡], Jonathan H Lin[2] & Miles F Wilkinson[1,3,*]

Abstract

Endoplasmic reticulum (ER) stress induces the unfolded protein response (UPR), an essential adaptive intracellular pathway that relieves the stress. Although the UPR is an evolutionarily conserved and beneficial pathway, its chronic activation contributes to the pathogenesis of a wide variety of human disorders. The fidelity of UPR activation must thus be tightly regulated to prevent inappropriate signaling. The nonsense-mediated RNA decay (NMD) pathway has long been known to function in RNA quality control, rapidly degrading aberrant mRNAs, and has been suggested to regulate subsets of normal mRNAs. Here, we report that the NMD pathway regulates the UPR. NMD increases the threshold for triggering the UPR *in vitro* and *in vivo*, thereby preventing UPR activation in response to normally innocuous levels of ER stress. NMD also promotes the timely termination of the UPR. We demonstrate that NMD directly targets the mRNAs encoding several UPR components, including the highly conserved UPR sensor, IRE1α, whose NMD-dependent degradation partly underpins this process. Our work not only sheds light on UPR regulation, but demonstrates the physiological relevance of NMD's ability to regulate normal mRNAs.

See also: **A Carreras-Sureda & C Hetz**

Introduction

Cells have evolved elaborate mechanisms to ensure the accuracy with which secreted and membrane proteins are folded and assembled [1]. In mammals, three ER transmembrane sensors—inositol requiring enzyme 1 (IRE1), eukaryotic translation initiation factor 2 alpha kinase (PERK, also known as PEK), and activating transcription factor 6 (ATF6)—serve to monitor ER lumen protein folding needs and, if necessary, initiate a set of intracellular signaling pathways, collectively termed the 'unfolded protein response' (UPR) [2]. These signaling pathways activate transcriptional and translational mechanisms that reduce global protein synthesis, increase ER protein-folding capacity, and promote the degradation of misfolded proteins [3]. If ER homeostasis is not achieved by these mechanisms in a timely manner, UPR triggers programmed cell death [3–7]. Because of the important role of UPR in regulating cell life/death decisions, it is critical that mechanisms are in place to prevent inappropriate UPR activation in response to innocuous or low-level stimuli. Little is known about how this is achieved. In this report, we demonstrate that an RNA regulatory mechanism—nonsense-mediated RNA decay (NMD)—serves in this capacity by raising the activation threshold of the UPR and promoting its timely attenuation.

NMD has two broad functions. It was originally identified as an RNA quality control pathway that degrades aberrant transcripts with premature translation termination codons generated as a result of mutations that cause disease [8–10]. Subsequently, it was discovered that NMD also degrades a subset of normal transcripts [11]. Approximately 3–10% of mRNAs are directly or indirectly regulated by NMD in eukaryotes spanning the phylogenetic scale [11]. While the 'rules' dictating whether a normal mRNA is targeted for decay by NMD have not been entirely elucidated, in general, RNA decay is triggered by stop codons that are in a premature context. For example, stop codons in middle exons typically trigger NMD by a mechanism dependent on a set of proteins collectively called the exon-junction complex (EJC) that is recruited just upstream of nearly all exon–exon junctions after RNA splicing [12]. Upstream ORFs (uORFs) can also trigger NMD, presumably as a result of translation termination at the 3′ end of the uORF [13]. Another NMD-inducing feature in mammalian mRNAs is a long 3′ untranslated region (UTR) [14,15]. The mechanism of how NMD targets mRNAs with long 3′ UTRs to decay is only partially understood; it may involve the capacity to load the NMD factor, UPF1, on the

1 Department of Reproductive Medicine, School of Medicine, University of California San Diego, La Jolla, CA, USA
2 Department of Pathology, School of Medicine, University of California San Diego, La Jolla, CA, USA
3 Institute of Genomic Medicine, University of California San Diego, La Jolla, CA, USA
*Corresponding author. Tel: +1 858 822 4819; E-mail: mfwilkinson@ucsd.edu
†Present address: Ambry Genetics, Aliso Viejo, CA, USA
‡Present address: ISIS Pharmaceuticals, Carlsbad, CA, USA

3′ UTR and/or the physical distance between the stop codon and the poly (A) tail [14–18].

While it is clear that NMD targets a subset of normal transcripts for decay, the physiological significance of this regulation is not known. This is a challenging problem, as the biological responses influenced by NMD are only just beginning to be understood. Furthermore, hundreds of mRNAs are regulated by NMD in all cell contexts that have been examined, making it difficult to assign functions to specific mRNAs [17,19–24]. Indeed, it is not known whether a given biological function of NMD depends on the regulation of a single mRNA or whole sets of mRNAs.

In this communication, we demonstrate that NMD has a critical role in shaping the UPR. It prevents inappropriate activation of the UPR and promotes timely termination of the UPR to protect cells from prolonged ER stress. We identify several mRNAs encoding UPR components that are targeted for decay by NMD and demonstrate that the mRNA encoding the most conserved UPR sensor, IRE1α [25], is a direct NMD target and has a role in NMD's ability to shape the UPR.

Results and Discussion

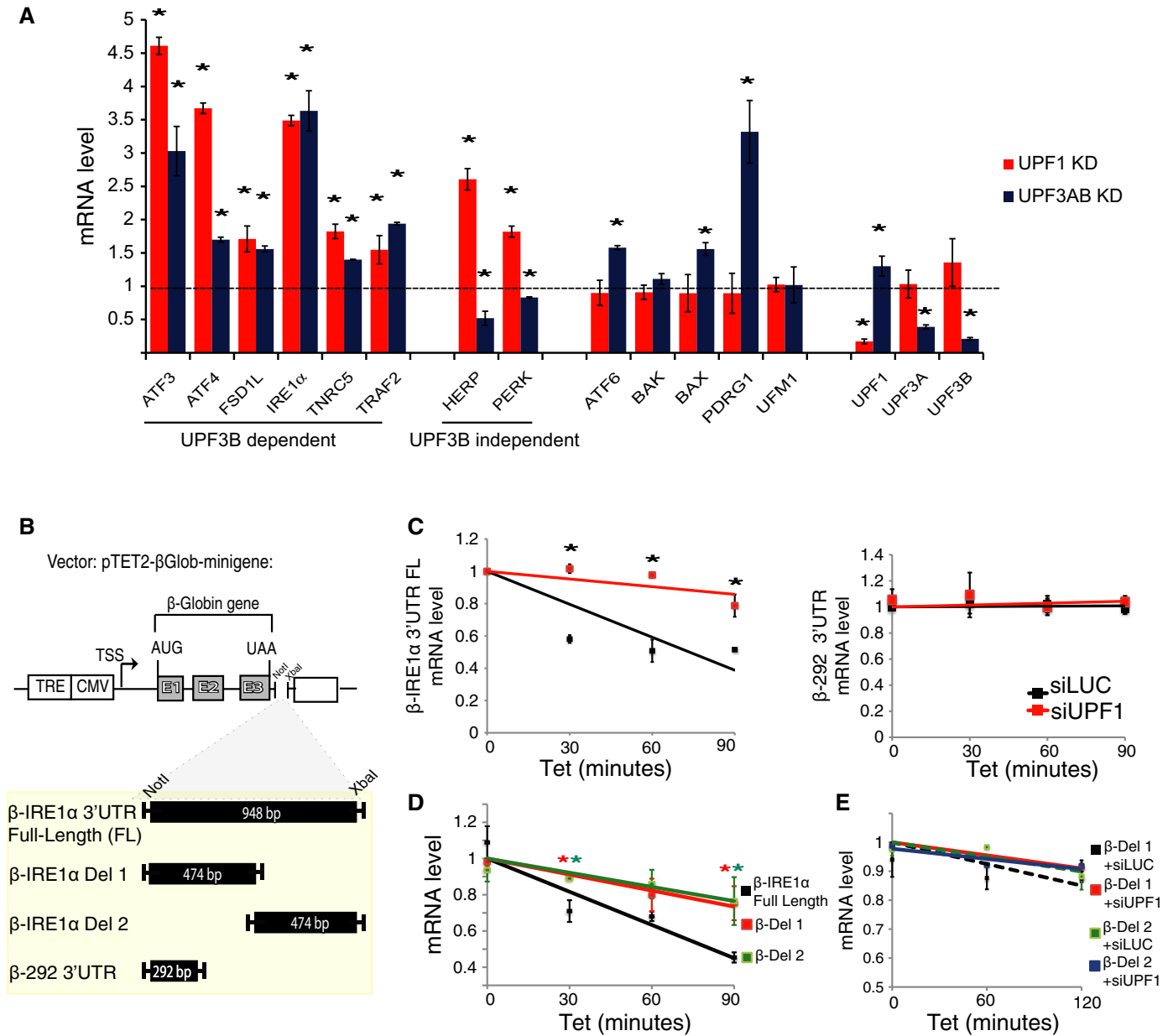### NMD targets transcripts encoding UPR components

Microarray analysis has shown that many mRNAs encoding proteins involved in stress–response pathways are upregulated in cell lines and mice depleted of NMD factors [19,23,26]. However, it has not been clear whether this is because NMD is important for regulating such mRNAs or because NMD factor depletion merely causes stress, which secondarily induces these transcripts. To assess the latter possibility that NMD perturbation triggers ER stress, we examined whether any of the three branches of the UPR were activated when NMD factors were depleted. We assessed this by measuring the following: (i) *XBP1* mRNA splicing, which is catalyzed by the UPR sensor, IRE1α, and thus is specifically induced by the IRE1 branch of the UPR [27], (ii) BIP transcriptional activation, which is primarily induced by the ATF6 branch of the UPR, but also by the IRE1 branch [28,29], and (iii) CHOP transcriptional activation, which is triggered by the PERK branch of the UPR [30]. As a positive control, we used tunicamycin (Tm), a potent ER stress inducer that inhibits N-linked glycosylation of nascent polypeptide chains [6]. We found that while spliced *XBP1* (*XBP1s*) was strongly induced by Tm, it was not detectably induced in response to depletion of the NMD factor, UPF3B, which is essential for a branch of the NMD pathway [19] (Supplementary Fig S1A). We also did not detect increased levels of *XBP1s* mRNA in response to depletion of both UPF3B and the central NMD factor UPF1 (Supplementary Fig S1B). In addition, neither *BIP* nor *CHOP* mRNA were statistically significant upregulated in NMD-deficient HeLa cells depleted of NMD factors (Supplementary Fig S1C). Together, these data suggest that depletion of NMD factors does not detectably induce any of the three branches of the UPR and thus perturbation of NMD elicits little or no ER stress, at least in the cells we tested.

Given that UPR component mRNAs are not upregulated by NMD perturbation because of ER stress, this raised the possibility that, instead, they are direct NMD target transcripts that are normally degraded by NMD. To test this possibility, we used quantitative polymerase chain reaction (qPCR) analysis to examine the effect of NMD factor depletion on 13 transcripts encoding UPR-related components, most of which have known NMD-inducing features (Supplementary Table S1). We found that eight of the 13 were significantly upregulated by in response to depletion of UPF1 (Fig 1A). Six of these 8 mRNAs—*ATF3, ATF4, FSD1L, IRE1α, TNRC1*, and *TRAF2* mRNA—were also upregulated in response to depletion of both UPF3A and UPF3B (Fig 1A), which we simultaneously depleted because of the evidence that these two NMD factors can act redundantly [19,31,32]. Three of these six mRNAs were also upregulated in response to UPF3B depletion alone (Supplementary Fig S1D). Two of these mRNAs—*ATF3* and *ATF4*—were previously suggested to be NMD targets, based on other lines of evidence [33,34]. The two mRNAs significantly upregulated by UPF1 depletion, but not in response to UPF3A/UPF3B depletion—*HERP* and *PERK* (Fig 1A)—are candidates to be targeted by the UPF3B-independent branch of NMD [19,35,36]. We also identified transcripts with the converse expression pattern; that is, significant upregulation in response to UPF3A/UPF3B depletion but not UPF1 depletion—*ATF6, BAX*, and *PDRG1* mRNA in Fig 1A. This was unexpected given that UPF1 is regarded as a central NMD factor required for all branches of the NMD pathway [36]; however, this has not been rigorously tested. These mRNAs may be either NMD target transcripts or regulated by an UPF3A/UPF3B-dependent mechanism not involving NMD. Another unexpected finding was that some mRNAs were downregulated, rather than upregulated, by UPF3A and/or UPF3B depletion (Fig 1A and Supplementary Fig S1D). These effects were usually modest. We do not know the underlying basis for the differential responsiveness of UPR transcripts to NMD factor depletion. Heterogeneous responses of NMD substrate mRNAs as a result of depletion of different NMD factors is a common occurrence [19,22,35–38] and is a subject of ongoing investigations in numerous laboratories.

Because the hallmark of NMD substrate RNAs is they are destabilized by NMD, we next performed RNA half-life analysis. If mRNAs encoding UPR components are direct NMD substrates, this predicts that perturbation of NMD will stabilize them. We observed that all UPR transcripts upregulated by UPF1 depletion (8 of 8) were also stabilized by UPF1 depletion (Supplementary Fig S1E). This stabilization effect was specific to UPR mRNAs that were upregulated upon NMD factor depletion; it was not exhibited by UPR mRNAs not upregulated by UPF1 depletion (*ATF6* or *PDRG1*; Fig 1A) or housekeeping transcripts we tested (*GAPDH* and *RPL13*) (Supplementary Fig S1E). Transcripts upregulated in response to UPF3A and UPF3B depletion (Fig 1A) were also stabilized by this double depletion (Supplementary Fig S1F). *HERP* and *UFM1* mRNA, which were not upregulated by this treatment (Fig 1A), were not stabilized (Supplementary Fig S1F).

Together, these data constitute strong evidence that 10 transcripts encoding UPR components—*ATF3, ATF4, ATF6, FSD1L, HERP, IRE1α, PERK, PDRG1, TNRC1*, and *TRAF2*—are NMD substrates. All but *HERP* have NMD-inducing features conserved in both humans and mice (Supplementary Tables S1 and S2). The field is still in the process of identifying the complete set of contexts that elicit NMD, and thus, further analysis may elucidate the molecular basis for why *HERP* mRNA appears to be targeted for decay by NMD.

Figure 1. NMD targets several mRNAs encoding UPR components.

A   qPCR analysis of HeLa cells stably depleted of UPF3A and UPF3B using shRNAs (as previously described [19]) or transiently depleted of UPF1 using a siRNA. A value of 1 (dotted line) indicates expression in control HeLa cells transiently transfected with a luciferase siRNA or stably transfected with a shRNA luciferase construct (shLUC). mRNA levels were normalized against the mRNA encoding ribosomal protein L19, which is not affected by the UPR [6] (n = 3).

B   Schematic representation of the Tet-off reporter, pTET2-βGlob-mini, containing the indicated sequences in the 3′ UTR region; β-292 is a NMD-resistant short 3′ UTR [15].

C   Pulse-chase mRNA half-life analysis of the indicated Tet-off reporter mRNAs. The reporter constructs were transiently transfected in HeLa Tet-off cells depleted of UPF1 (siUPF1) or luciferase (siLUC; control). Cells were treated with tetracycline to block transcription of the reporter promoter and samples were collected at the indicated time points. Values were normalized as in (A) (n = 6).

D   Pulse-chase mRNA half-life analysis of a Tet-off reporter mRNA harboring the full-length *IRE1α* 3′ UTR (β-IRE1α 3′UTR FL) versus the Tet-off reporter harboring the downstream and upstream deletions of the *IRE1α* 3′ UTR (β-IRE1α Del 1 and Del 2). Values were normalized as in (A) (n = 6).

E   Pulse-chase mRNA half-life analysis performed as in (D) in HeLa cells transiently depleted of UPF1 (siUPF1) or luciferase (siLUC; control) by RNAi. Values were normalized as in (A) (n = 3).

Data information: Statistical analysis by *t*-test (*$P < 0.05$). All error bars reflect SEM.

We elected to study the molecular basis for how the mRNA encoding IRE1α is targeted for decay by NMD. IRE1α is the most highly conserved sensor protein of the three UPR branches [2]. We noted that *IRE1α* mRNA has a conserved long 3′ UTR (958 nt and 922 nt in human and mice, respectively; Supplementary Tables S1 and S2), a feature that, as described above, can trigger the decay of

an mRNA by NMD [14–18]. To assess whether this is the case for *IRE1α* mRNA, we inserted its full-length 3′ UTR into a *β-globin* mini-gene reporter system controlled in a tetracycline (Tet)-regulated manner (β-IRE1α 3′UTR FL in Fig 1B). We transiently transfected this reporter construct into HeLa cells and performed pulse-chase analysis and found that NMD factor knockdown stabilized the transcribed reporter mRNA, but had no effect on a control mini-gene reporter mRNA containing a short (292 nt) *β-globin* 3′ UTR (Fig 1C). To test whether the length of the IRE1α 3′ UTR was the determining factor that dictated its destabilization by NMD, we made deletions in the 3′ UTR (β-IRE1α Del 1 and Del 2 in Fig 1B) and observed this stabilized the β-IRE1α Tet-reporter mRNA (Fig 1D) and rendered it unresponsive to depletion of UPF1 (Fig 1E). Together, these data suggest that *IRE1α* mRNA is a direct NMD target as a result of its long 3′ UTR. While the underlying mechanism by which long 3′ UTRs trigger NMD is not well understood, evidence suggests that this feature decreases the probability of translation release factors interacting with poly(A)-binding protein, thereby increasing their probability of interacting with UPF1, an event that promotes NMD [14–16]. We note that our results do not rule out that the IRE1α 3′ UTR confers sensitivity to NMD for another reason, such as essential *cis* elements in both its 5′ and 3′ halves.

NMD shapes the unfolded protein response

We reasoned that NMD may promote the decay of IRE1α and other UPR factor mRNAs to prevent activation of the UPR in response to low, non-pathogenic levels of ER stress. To test this, we treated NMD-deficient and control HeLa cells with different concentrations of Tm. We found that NMD-deficient cells had a dramatically lower UPR activation threshold in response to Tm than did control cells (Fig 2A). While NMD-deficient cells reached the same maximal response as control cells, they did this with much lower concentration of Tm. Similar results were obtained with another UPR-inducing drug, thapsigargin, which blocks calcium influx in the ER [6] (Supplementary Fig S2A–C). To evaluate whether this was also the case *in vivo*, we examined UPR activation in a classic UPR-responsive tissue—liver [39]—in NMD-deficient *Upf3b*-null mice [35]. Treatment of these NMD-deficient mice with different doses of Tm by intra-peritoneal (IP) injection revealed that they had a significantly lower UPR activation threshold than did control littermate mice. Thus, *Upf3b*-null mice exhibited significantly elevated UPR responses to the lower doses of Tm (0.1, 0.25, and 0.5 µg/g) (Fig 2B and Supplementary Fig S2D). In contrast, *Upf3b*-null mice responded normally (or almost normally) to the highest dose of Tm (1 µg/g), consistent with NMD raising the threshold for the UPR, but not affecting the potential to maximally respond to ER stress. Together, these data provided both *in vitro* and *in vivo* evidence that NMD increases the activation threshold of the UPR.

To further test whether NMD is responsible for dampening the UPR, we examined the temporal kinetics of the UPR in NMD-deficient cells in response to a low dose of Tm that only minimally triggers ER stress in normal cells. We observed that NMD-deficient cells exhibited more rapid and increased expression of *XBP1s* mRNA and stronger induction of the ATF6 downstream transcriptional target gene, *BIP*, relative to control cells (Fig 2C). *CHOP* mRNA was also rapidly and strongly induced in NMD-deficient cells, whereas it was only modestly induced at early time points in control cells

(Fig 2C). These results suggested that by dampening the UPR, NMD serves to prevent an over-exuberant response to innocuous ER stress.

Prolonged ER stress leads to attenuation of IRE1 signaling as a result of reduced IRE1α-mediated splicing of *Xbp1* mRNA [6]. The molecular mechanism responsible for this attenuation response is poorly understood. Because NMD destabilizes and downregulates *IRE1α* mRNA (Fig 1 and Supplementary Fig S1D–F), we hypothesized that NMD has a role in this attenuation response. As a first test of this hypothesis, we examined the effect of NMD deficiency on HeLa cells treated with a dose of Tm (2 µg/ml) sufficient to elicit a severe UPR at 12 h that wanes by 18 h in control cells (Fig 2D). Like control cells, NMD-deficient cells had elevated levels of *XBP1s*, *BIP*, and *CHOP* mRNA during the plateau phase (12 h), but cells depleted of either UPF3B alone or both UPF3A and UPF3B failed to normally downregulate these UPR mRNAs during the termination phase (Fig 2D and Supplementary Fig S2E–G). To determine whether NMD also promotes UPR termination *in vivo*, we examined NMD-deficient *Upf3b*-null mice. We found that *Upf3b*-null mice responded to a high dose of Tm virtually the same as control littermate mice during the peak phase of the UPR (12 h post treatment), but they had an impaired downregulatory response during the termination phase (24 h post treatment). *Upf3b*-null mice exhibited 1.8-, 1.8-, and 4.6-fold depressed downregulation of *Xbp1s, Bip,* and *Chop* mRNA, respectively, relative to littermate control mice (Fig 2E; note that *Bip* and *Chop* mRNA levels are depicted on a log scale because of their large induction). Together, these *in vivo* and *in vitro* data support the notion that NMD promotes the timely termination of the UPR, with the caveat that it is supported by *in vivo* data from only a single time point.

To further test the role of NMD in the UPR, we assayed activation of the UPR sensors, IRE1α and ATF6. A low dose of Tm was used to simulate innocuous stress. We examined phosphorylated IRE1α protein as a measure of IRE1 branch activation [40]. Control cells did not have detectable phosphorylated IRE1α at early time points and only trace levels of phosphorylated IRE1α at later time points (Fig 3A, shLUC lanes). In contrast, NMD-deficient cells exhibited rapid and strong induction of IRE1α phosphorylation after 2 h of treatment, which increased in level at later time points (Fig 3A, shUPF3B lanes). As another indication of UPR's IRE1 branch activation, NMD-deficient cells exhibited rapid and strong induction of *XBP1* splicing (Fig 3B), a reaction triggered by activated IRE1α [27]. Control cells exhibited only modest (and delayed) *XBP1* splicing (Fig 3B). The ATF6 branch of the UPR was also strongly induced in NMD-deficient cells, as shown by (i) the increased level of cleaved ATF6 (Fig 3C and E), a well-established marker of ATF6 activation [41] and (ii) stronger expression of the ATF6 downstream transcriptional target gene, *BIP*, than in control cells (Fig 3D). *CHOP* protein was also rapidly and strongly induced in NMD-deficient cells, not control cells (Fig 3D). Together, these results indicate that by dampening the UPR, NMD serves to prevent an over-exuberant activation of the response to innocuous ER stress.

Our finding that NMD promotes UPR attenuation raised the possibility that NMD protects cells from ER stress-induced apoptosis, a consequence of sustained UPR [6]. In support of this, we observed that UPF3B depletion strongly sensitized cells to undergo apoptosis 48 h after Tm treatment, a response that was exacerbated
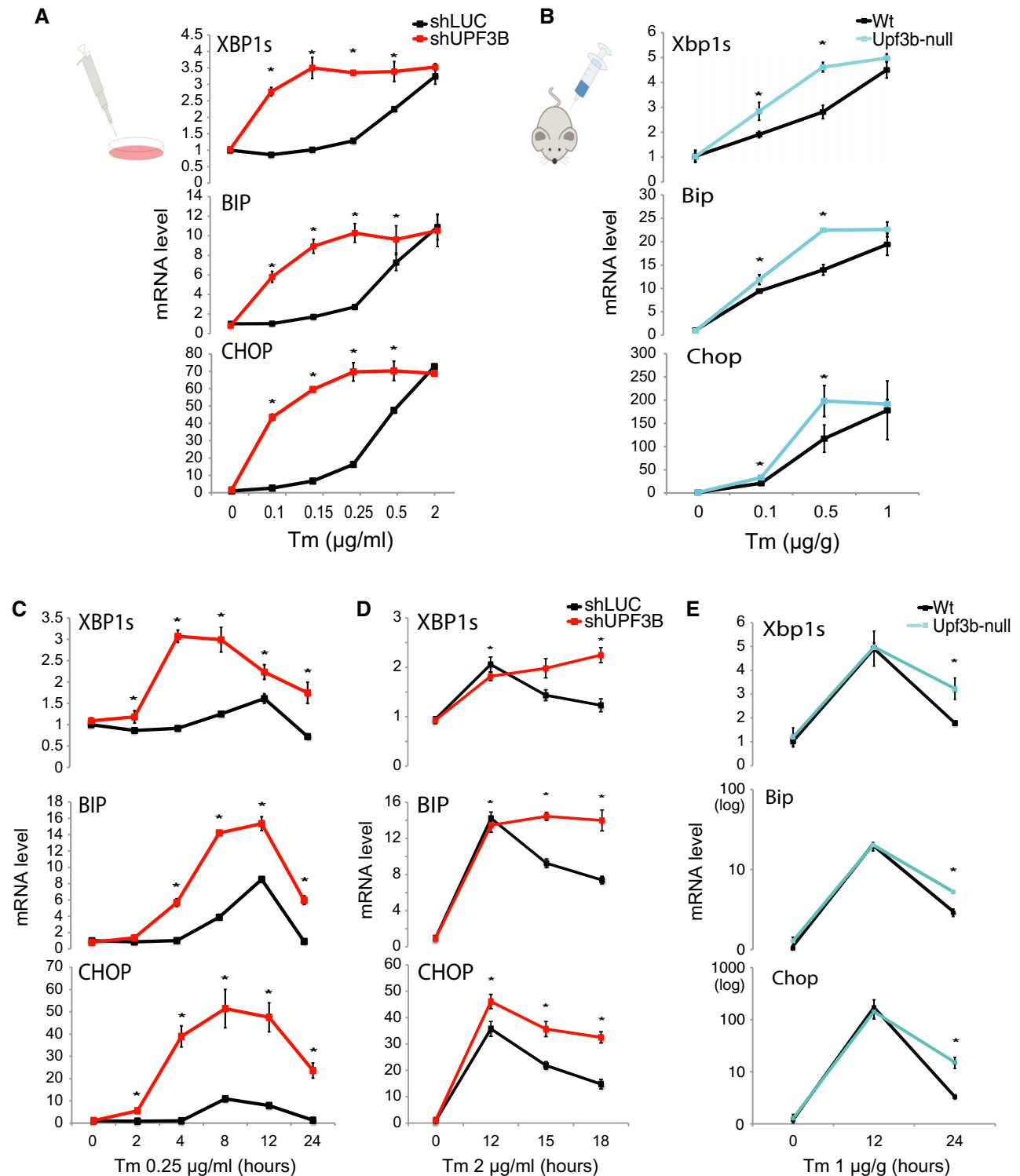
Figure 2. NMD raises the activation threshold of the UPR and promotes its attenuation.

A   qPCR analysis of HeLa cells stably depleted of the NMD factor UPF3B (shUPF3B) and treated with increasing concentrations of tunicamycin (Tm) for 4 h. HeLa cells stably transfected with a construct expressing an shRNA against luciferase (shLUC) serves as a negative control ($n = 6$).

B   qPCR analysis of liver from *Upf3b*-null ($n = 3$) and matched control littermate (Wt) mice ($n = 3$) injected IP with the indicated concentrations of Tm for 12 h.

C   qPCR analysis of the stably transfected HeLa cells described in (A) treated with a low dose of Tm (0.25 μg/ml) for the times indicated ($n = 6$).

D   qPCR analysis of the stably transfected HeLa cells described in (A) and incubated with a high dose of Tm (2 μg/ml) for the time points indicated ($n = 3$).

E   qPCR analysis of liver from *Upf3b*-null ($n = 3$) and control littermate (Wt) mice ($n = 3$) injected IP with Tm (1 μg/g) for the time points indicated.

Data information: Values were normalized as in Fig 1A and statistically analyzed by *t*-test (*$P < 0.05$). All error bars reflect SEM.

by additional depletion of UPF3A (Fig 4A). We also observed a modest increase in apoptosis in cells stably depleted of UPF3B or both UPF3A and UPF3B in the absence of ER stress.

We next tested whether NMD protects cells from ER stress-induced apoptosis *in vivo*. We analyzed the apoptosis of liver cells by TUNEL staining [5] and found greatly increased numbers of apoptotic cells in NMD-deficient mice, both 48 and 96 h after Tm treatment as compared to control mice (Fig 4B). In contrast, NMD-deficient mice liver cells did not exhibit significantly higher apoptosis than control mice in the absence of Tm treatment, providing evidence that NMD specifically protects cells from death in response to stress.

While our study demonstrated that the UPR is shaped by NMD, Sakaki *et al* provided evidence for another regulatory relationship between the UPR and NMD [42]. In particular, they found that debilitating mutations in the SMG1, 4, and 6 NMD factor genes in *C. elegans* triggered ER stress and the UPR, leading these investigators to suggest that loss of NMD causes the accumulation of misfolded proteins, which, in turn, activates the UPR. In contrast to these results in worms, we did not observe activation of the UPR when we depleted several different core NMD components in mammalian cells or knocked out the NMD factor UPF3B *in vivo* (Fig 2 and Supplementary Figs S1A–C and S2). While Sakaki *et al* showed that depletion of the NMD factor, SMG6, elicits UPR activation in HeLa cells, this may be because of loss of SMG6's non-NMD functions, such as telomere maintenance [43]. Indeed, Sakaki *et al* found that knockdown of another NMD factor, SMG1, did not significantly induce the UPR [42]. We suggest that the most parsimonious explanation for these seemingly divergent results is that the UPR and NMD pathways impact each other in varied ways, depending on context and species. In mammals, NMD is crucial for shaping the UPR so that it is not unnecessarily triggered by innocuous stimuli or undergoes prolonged activation, thereby avoiding toxicity. In *C. elegans*, NMD (or another process dependent on SMG6) is essential to avoid intrinsically activating the UPR response, perhaps because of the necessity of NMD's ability to sense genomic noise in worms.

**NMD acts, in part, through IRE1α to repress the UPR**

We next investigated the underlying mechanism by which NMD suppresses the UPR. This was challenging given that NMD regulates hundreds of mRNAs, any combination of which could contribute to its ability to suppress the UPR. Despite this likely complexity, several findings suggested that one molecule, IRE1α, was a particularly good candidate to be one player that acts downstream of NMD to suppress the UPR. First, our results indicated that *IRE1α* mRNA is among the UPR component transcripts most strongly upregulated by NMD (Fig 1A). Second, NMD-deficient cells not only had increased levels of *IRE1α* mRNA, but also increased IRE1α protein and IRE1α phosphorylation (Fig 3A). Third, we showed that *IRE1α* mRNA is a direct NMD target (Fig 1 and Supplementary Fig S1D–F), making it a more likely candidate to act in an NMD-based circuit. Finally, *IRE1α* mRNA levels were elevated in NMD-deficient cells in both human cells and mice, regardless of which NMD factor was depleted and whether ER stress was present or not (Figs 1A and 4C–E and Supplementary Fig S3A–C), suggesting that the ability of NMD to repress *IRE1α* expression is conserved and omnipresent in mammals.

To directly test whether NMD acts through IRE1α to repress the UPR, we first performed a gain-of-function experiment. We examined whether forced expression of *IRE1α* mRNA to a level similar to that in NMD-deficient cells (Supplementary Fig S3A–C) was sufficient to decrease UPR activation threshold. Indeed, we found that this modest overexpression of IRE1α increased the level of the spliced form of its direct substrate (*XBP1s* mRNA) and decreased the UPR activation threshold, as measured by both *BIP* and *CHOP* mRNA induction (Supplementary Fig 3D).

As a complementary approach to examine whether the ability of NMD to degrade *IRE1α* mRNA has a role in NMD's ability to suppress the UPR, we performed a rescue experiment. We first did titration studies to define a dose of siRNA against *IRE1α* that prevented the upregulation of *IRE1α* that normally occurs in response to NMD perturbation. This defined a dose of IRE1α siRNA that was effective in blocking NMD-deficient cells from expressing elevated levels of both *IRE1α* and *XBP1s*, a key direct product of IRE1α action (Fig 4F). To elucidate whether blocking IRE1α upregulation impacted the UPR, we examined the UPR effectors BIP and CHOP. We found that preventing *IRE1α* upregulation in NMD-deficient cells significantly suppressed BIP and CHOP induction in response to Tm (Fig 4F). This provided direct evidence that one mechanism by which NMD suppresses the UPR is by degrading *IRE1α* mRNA.

As another approach to address whether NMD acts through IRE1α to repress the UPR, we used a covalent inhibitor of IRE1α, STF-083010, which forms a selective Schiff's base with a catalytic lysine in the RNase active site of IRE1α, thereby blocking its function [44]. We identified a dose of STF-083010 that largely prevented the upregulation of *XBP1s* mRNA that normally occurs in response to ER stress specifically in NMD-deficient cells. This significantly reduced ER stress-induced UPR activation, as judged by both *BIP* and *CHOP* expression (Fig 4G). Indeed, *BIP* mRNA levels were similar in STF-083010-treated NMD-deficient cells as in control cells, suggesting that the ability of NMD to degrade *IRE1α* mRNA is a major mechanism by which it suppresses *BIP* gene activation. *CHOP* mRNA levels, while suppressed by STF-083010, did not reach control levels, consistent with the fact that CHOP is induced by PERK while BIP is induced by both the IRE1α and ATF6 branches of the UPR [28–30]. While these results indicate that NMD acts through its ability to degrade *IRE1α* mRNA to suppress the UPR, we found that NMD does not use this mechanism to suppress apoptosis. Thus, suppressing the upregulation of *IRE1α* mRNA that normally occurs in response to NMD perturbation did not significantly prevent the increased apoptosis caused by this perturbation (Supplementary Fig S3E).

The finding that *IRE1α* mRNA destabilization by NMD plays a role in shaping the UPR is important given that the physiological significance of NMD's ability to degrade normal mRNAs has not been clear since genome-wide approaches first revealed that NMD engendered this function [20,21,23]. Several studies have shown that loss or depletion of NMD factors causes developmental defects in organisms spanning the phylogenetic scale, which has led to the suggestion that NMD regulates normal transcripts to drive or modulate specific development processes and homeostatic mechanisms [13,45–47]. However, little is known regarding the specific transcripts NMD acts on to control such
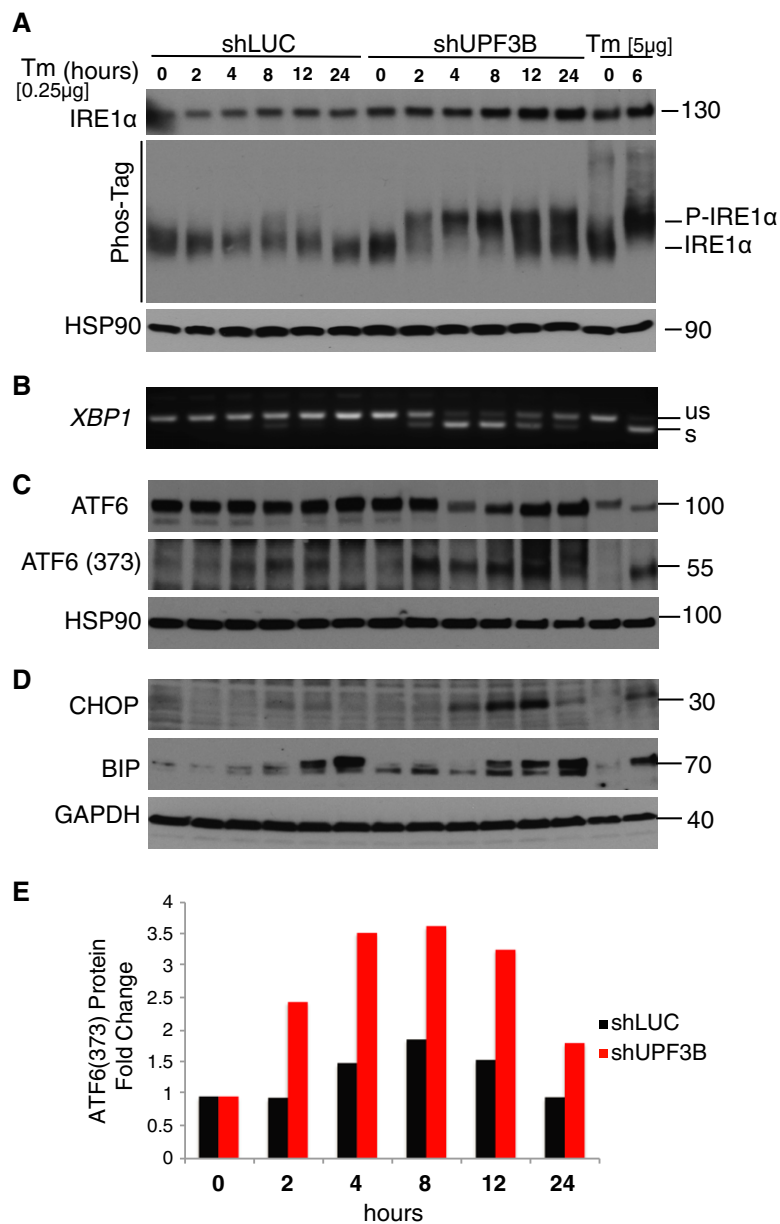
Figure 3. NMD suppresses UPR activation in response to endoplasmic reticulum stress.

A   Western blot analysis of total IRE1α (top) and phospho-IRE1α (bottom) in HeLa cells stably depleted of the NMD factor UPF3B (shUPF3B) treated with a low dose of Tm (0.25 μg/ml) for the times indicated. HeLa cells stably transfected with a luciferase shRNA construct (shLUC) serve as a negative control. Phospho-IRE1α was detected by a mobility shift assay, as described [60]. HSP-90 is the loading control, and the positive control is HEK293T cells treated with Tm (5 μg/ml) for 6 h.

B   RT–PCR analysis of *XBP1* splicing under the same conditions as in (A) (representative of three independent experiments).

C   Western blot analysis of total ATF6 (top) and its cleavage product (ATF6-373) in cells under the same conditions as in (A).

D   Western blot analysis of CHOP and BIP in cells under the same conditions as in (A).

E   Quantification of cleaved ATF6 protein [ATF6 (373)] relative to the loading control, HSP90, from the Western blot data in (C).

Data information: Data in (A, C and D) are representative of two independent experiments.

processes. Here, we showed using both gain- and loss-of-function approaches that a specific normal mRNA—*IRE1α* mRNA—is degraded by NMD to shape the UPR. This discovery strongly supports a recently advanced model that posits that IRE1α has a critical threshold concentration, which when reached, leads to UPR activation [40].

The UPR suppresses NMD to allow a strong ER stress response

While the ability of NMD to suppress the UPR provides the benefit of preventing UPR activation in response to innocuous ER stress, this attribute would be predicted to prevent an optimal UPR activation in response to *bona fide* ER stress. To avoid this,
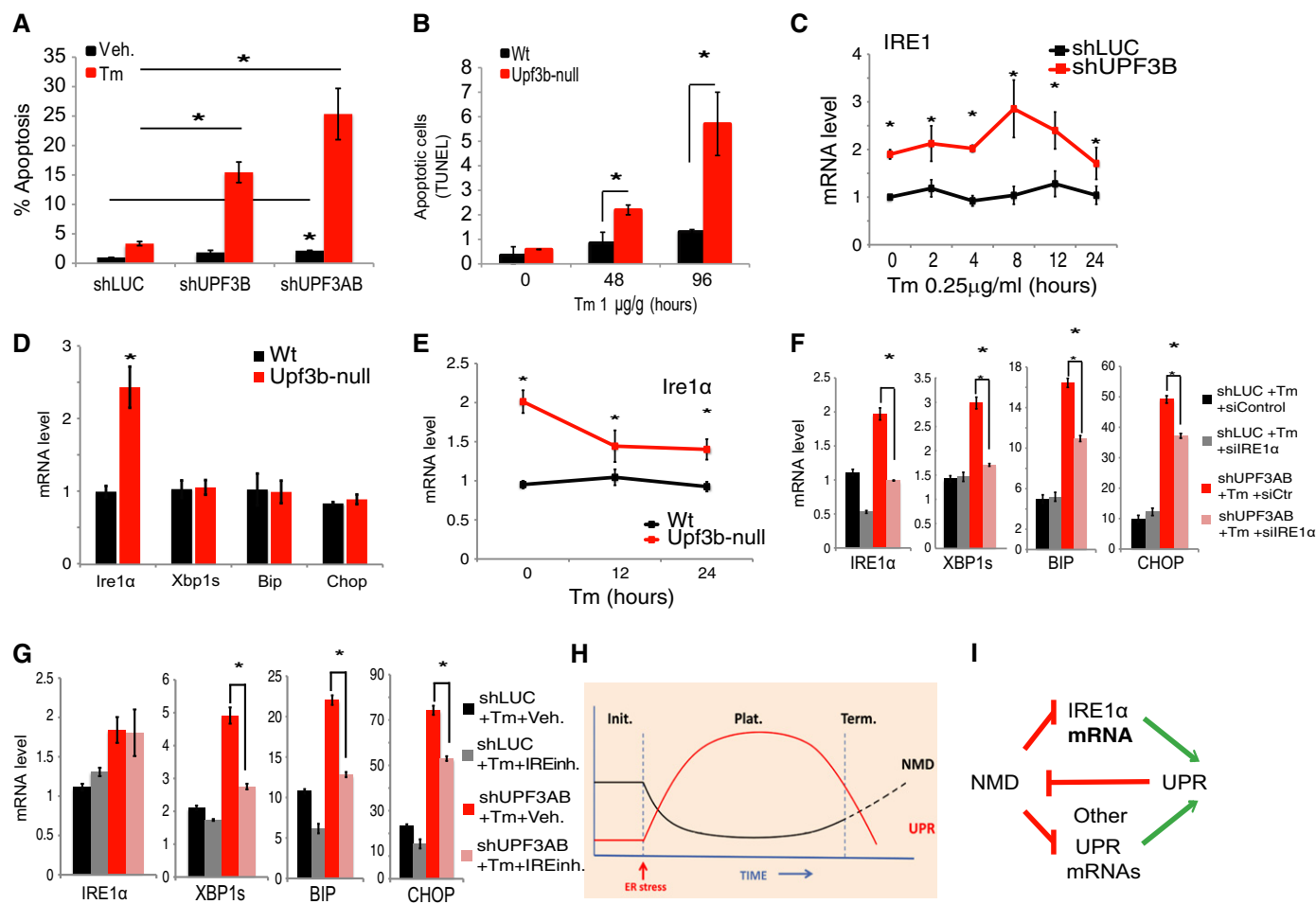
Figure 4. NMD acts through IRE1α to shape the UPR.

A FACS analysis indicating the percentage of apoptotic (annexin-V positive/PI negative) HeLa cells in response to Tm (2 μg/ml) treatment for 48 h. HeLa cells were stably depleted of UPF3B (shUPF3B) or both UPF3A and UPF3B (shUPF3AB). HeLa cells stably transfected with a luciferase shRNA construct (shLUC) serve as a negative control. The values shown are the average (mean ± SEM) from three experiments relative to control (shLUC). The percentage of apoptotic cells in non-Tm-treated shLUC, shUPF3B, and shUPF3AB cells was 1.0, 1.8, and 2.1%, respectively (n = 3).

B Quantification of apoptotic (TUNEL-positive) cells in liver tissue sections. Shown is the average number of TUNEL-positive cells in a field surrounding a hepatic portal area in *Upf3b*-null (n = 3) and control littermate (Wt) mice (n = 3) injected IP with Tm (1 μg/g) for the times indicated. Scoring was performed without knowledge of genotype.

C qPCR analysis of HeLa cells (described in A) treated with a single low dose of tunicamycin (0.25 μg/ml) for the time points indicated (n = 3).

D qPCR analysis of liver from *Upf3b*-null (n = 6) and control littermate (Wt) mice (n = 6).

E qPCR analysis of the liver of *Upf3b*-null (n = 3) and control littermate mice (n = 3) injected IP with Tm (1 μg/g) for the times indicated.

F qPCR analysis of the stably transfected HeLa cells described in (A) and transiently transfected with an *IRE1α* siRNA (siIRE1) or a control siRNA (siControl) and incubated with Tm (0.25 μg/ml) for 10 h (n = 3).

G qPCR analysis of the stably transfected HeLa cells described in (A) that were incubated with IRE inhibitor STF-083010 (60 μM) and Tm (0.25 μg/ml) for 10 h (n = 3).

H Model depicting the activity of the UPR and NMD pathways during the initiation (Init.), plateau (Plat.), and termination (Term.) phases of the UPR pathway.

I Model depicting the NMD-UPR circuit defined by our data that amplifies the signal-to-noise ratio of endoplasmic reticulum stress responses.

Data information: Statistical analysis by *t*-test (*$P < 0.05$). In (C–G), values were normalized as in Fig 1A. All error bars reflect SEM.

an optimal system would be one that suppresses NMD in response to strong ER stress so that *IRE1α* mRNA and other NMD-targeted UPR component mRNAs can be stabilized, allowing for strong UPR activation (Fig 4H and I). One potential means by which ER stress could achieve this is by downregulating NMD factors. Our analysis of 12 NMD factor mRNAs showed that they were either not affected or only modestly affected by Tm treatment of HeLa cells (Supplementary Fig S4A). The NMD factor

mRNA exhibiting the strongest decrease in expression (~60% decrease) was that encoding the core EJC factor eIF4AIII. The mRNAs encoding UPF2, UPF3B, and SMG1 were also modestly downregulated by Tm. While these downregulatory responses could have a role in downregulating NMD, it should be noted that this would only be the case if the degree of downregulation of a given NMD factor is sufficient to reach a rate-limiting level of that factor. Our previous overexpression analysis of eight

NMD factors showed that the endogenous level of only SMG1 was rate limiting for NMD in HeLa cells [35].

A mechanism that has been reported to suppress NMD in response to ER stress is eukaryotic initiation factor 2-α (eIF2α) phosphorylation. A wide variety of cellular stresses, not only ER stress, but also amino acid deprivation, oxygen deprivation, infection, reactive oxygen species, and double-stranded RNA, all are known to trigger the phosphorylation of eIF2α [33,48–50]. We confirmed that ER stress inhibits NMD activity in human and mice cells (Supplementary Fig S4B). We also found that NMD repression is abrogated by forced expression of the phosphatase GADD34 (Supplementary Fig S4B), which is known to dephosphorylate eIF2α [30,51], or by mutation of the eIF2α phosphorylation site (Supplementary Fig S4C). While the mechanism by which eIF2α phosphorylation inhibits NMD is not known, an obvious possibility is that it acts by inhibiting translation [52]. This follows from the fact that eIF2α phosphorylation is a potent inhibitor of translation [53] and NMD absolutely depends on translation [54]. However, some evidence suggests that eIF2α phosphorylation inhibits NMD independently of translation inhibition [48,50], and thus, the underlying mechanism remains to be determined.

Conclusions and future directions

Here, we report that the highly conserved RNA degradation pathway, NMD, is crucial for avoiding inappropriate and prolonged inactivation of the UPR. We identify several mRNAs encoding UPR components that are targeted for decay by NMD and demonstrate that the mRNA encoding the most conserved sensor of the UPR, IRE1α, plays a crucial role in the ability of NMD to regulate the UPR. We suggest that the NMD-UPR circuit we have uncovered is likely to impact many biological processes. For example, the finding that NMD is downregulated during brain and neural development [22,37] raises the possibility that this allows the UPR to be activated under low (physiologic) levels of ER stress that occur during neuron differentiation to accommodate fluctuations in the demand for protein synthesis and secretion [2]. The ability of NMD to suppress the UPR also has potential biomedical impact, as chronic ER stress occurs in many diseases, including cancer, diabetes, pro-inflammatory disorders, and diseases associated with neural degeneration [55].

Given that we showed that NMD promotes cell survival under UPR-inducing conditions, interventions that modulate the magnitude of NMD could reduce the severity of such diseases by increasing cell survival. Further therapeutic options stem from the fact that NMD is a branched pathway [19,31,38]. Thus, drugs that target a specific NMD branch have the potential to greatly increase specificity and decrease side effects. In this regard, we demonstrated herein that the UPF3B-dependent branch of NMD is specifically involved in regulating the UPR since we found that loss or depletion of UPF3B was sufficient to disrupt the normal activation threshold, magnitude, and attenuation of the UPR both *in vitro* and *in vivo*. This discovery, coupled with the fact that mutations in the *UPF3B* gene cause intellectual disability in humans and are associated with autism and schizophrenia [56,57], raises the possibility that the cognitive and psychiatric pathologies that occur in these *UPF3B* patients result from aberrant UPR activation. Because we showed that NMD greatly raises the threshold

for triggering the UPR in response to ER stress-inducing agents (Fig 2 and Supplementary Fig S2), these NMD-deficient individuals may suffer from far worse symptoms if they encounter ER stress. Indeed, we showed that NMD-deficient (*Upf3b*-null) mice had elevated liver apoptosis in response to external ER stress (Fig 4A and B). We note that loss of UPF3B only ablates a branch of the NMD pathway [19] and thus complete loss of NMD is likely to cause more severe UPR-related defects. In summary, our discovery that NMD serves as a post-transcriptional pathway that shapes the UPR provides a foundation for developing strategies for treating the many diseases characterized by chronic ER stress.

Materials and Methods

Animals and cell culture

Cell culture and transfections were performed as previously described [31]. Unless otherwise noted, cells were harvested 2 days after transfection. The siRNAs targeting UPF1 and Luciferase were obtained from Ambion (Carlsbad, CA, USA). The siRNAs targeting IRE1 and a scramble sequence were obtained from Thermo Scientific Dharmacon (Lafayette, CO, USA). Luminometry was performed as previously described [58]. Apoptosis was determined by FACS analysis of cells stained with annexin-V FITC/propidium iodide as previously described [5]. Tunicamycin and thapsigargin were obtained from Sigma (Saint Louis, MO, USA). All experiments with mice were performed in accordance with National Institutes of Health guidelines for care and use of animals. The *Upf3b*-null mice [35] and control littermate mice were given a single intraperitoneal injection of tunicamycin in 150 mM dextrose, as previously described [5,39]. At various times thereafter, the mice were killed by CO_2 narcosis, followed by cervical dislocation. Livers were removed and RNA was isolated for analysis, as described below. To detect liver apoptosis, the sections were stained with the DeadEnd fluorometric TUNEL system (Promega, Madison, WI, USA), following the recommendations of the manufacturer.

RNA analysis

Total cellular RNA was extracted from cells using TRIzol Reagent (Sigma), and cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). [γ^{32}P]-end-labeled RT–PCR analysis was done as previously described [59]. qPCR analysis was performed using the relative quantification method ($\Delta\Delta C_T$) in a ABI Step-One thermal cycler system (ABI). Endogenous mRNA half-life analysis and pulse-chase mRNA half-life analysis of the Tet-off reporter was done as previously described [35].

Protein analysis

Western blot analyses, including cell and tissue preparation, were done as previously described [6]. The following antibodies and dilutions were used for immunoblotting: anti-ATF6 at 1:1,000 (#ab122897; Abcam, Cambridge, MA, USA); anti-GRP78/BiP at 1:1,000 (#GTX113340; GeneTex, Irvine, CA, USA); anti-CHOP at

1:1,000 [#GTX112827 (N1C3); GeneTex]; anti-GAPDH at 1:5,000 [#sc-25778 (FL-335); Santa Cruz Biotechnologies, Dallas, TX, USA]; and anti-HSP90 at 1:5,000 (#GTX101448; GeneTex). In addition to conventional gels, Phos-tag gels were run that differed in that: (i) the 5% SDS–PAGE gel contained 25 μM Phos-tag and 50 μM $MnCl_2$ (Sigma); (ii) the gel was run at 100 V for 3 h, soaked in 1 mM EDTA for 10 min, and then transferred onto a PVDF membrane. Phos-tag™ Acrylamide AAL-107 was purchased from Wako (NARD Institute).

Oligonucleotides sequences

The primers used in this experiment are provided in Supplementary Table S3.

Author contributions

RK and MFW conceived, designed, and interpreted the main body of experiments. RK performed the bulk of the experiments. C-HL and HK performed additional experiments and greatly contributed to figure generation and data interpretation. LH generated the *Upf3b*-null mice and performed experiments in early project development. The manuscript was primarily written by RK and MFW, with contributions from others, including C-HL and JHL.

Conflict of interest

The authors declare that they have no conflict of interest.

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reports

Manuscript EMBOR-2014-39696

The unfolded protein response is shaped by the NMD pathway

Rachid Karam, Chih-Hong Lou, Heike Kroeger, Lulu Huang, Jonathan H. Lin and Miles F. Wilkinson

Corresponding author: Miles F. Wilkinson, UCSD

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Transaction Report:

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Editor: Nonia Pariente

1st Editorial Decision   28 October 2014

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although all the referees find the topic of interest and in principle suitable for us, referees 1 and 2 raise a number of issues that need to be addressed before the study can be considered for publication in EMBO reports.

Given these reports, I would like to give you the opportunity to revise your manuscript. As the concerns of referees 1 and 2 are all reasonable and pertinent, they would have to be experimentally addressed. Please note that we would require that you deplete UPF1 in some key experiments, as referee 2 requests, and not just discuss why this wasn't done, as referee 3 mentions. If the referee concerns are adequately addressed, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

From an editorial standpoint, I would suggest to move the results from supplementary figures 3B and 3C to the main manuscript. In addition, please merge the Results and Discussion sections, which we require, and which will help reduce the redundancy that is inevitable when discussing the same experiments twice. Although we have flexibilized our length limits, your manuscript text reads somewhat wordy at times and several things are explained more than once. I would urge you to go

through it during revision and provide a text as succinct as possible while maintaining the information.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

REFEREE REPORTS:

Referee #1:

REMARKS TO THE AUTHORS
The manuscript by Karam and colleagues described evidence of a novel mechanism by which NMD selectively targets UPR-target genes and specifically IRE1alpha to regulate and potentiate UPR. The authors demonstrate that NMD activity downregulates UPR-target genes. In addition, the authors report how NMD affects and shapes the UPR under ER stress conditions concluding that this effect is mainly due to a repression of IRE1 alpha mRNA impacting the UPR. Finally, the authors describe a novel idea where the UPR controls NMD, inhibiting it initially to promote a positive feedback in the proposed system. In addition, some of the data was corroborated in in vivo models. The work is well done, the controls are good and the story is novel.
Minor points
1. Due to the relevance of this new history I would like the author to discuss, (if needed with empirical data) the fact of the control of NMD during ER stress conditions. The NMD luciferase activity assay is good tools to demonstrate the effect of UPR on NMD activity, but, what is mechanism that regulates NMD activity during ER stress conditions? Could the authors discuss a bit more how UPR induction can impair the activity of UPF proteins? It looks it is downstream eif2a and GADD34 activities, but how this can be done?
2. In the same point, I would like to see the mRNA regulation of UPF genes involved in NMD. This would allow us to evaluate if there is another regulatory mechanism (a part of the differential activity of NMD). I think a time course expression analysis would be enough. (maybe in the GADD34 and eif2a background as controls in Figure 4S).
3. Using the concentrations on figure 4D, evaluate if there is an effect on cell death.
4. Due to the cartoon present in figure 4F, where a kinetic model is present, I would like to see a tracking of IRE in a time dependent manner. For this I would get a kinetic in the same conditions as 4G.
Points 2 and 4 could be done in the same cellular background, to be then correlated, validating, thus, more extensively the model purposed on figure 4F.

Referee #2:

The manuscript by Karam et al. describes a role for NMD in regulating the amplitude of the unfolded protein response. This is an interesting observation that sheds light on the physiological role of NMD and is therefore of broad interest.
The paper is well written and the conclusions are supported by well-controlled experiments.

I have a few suggestions to strengthen the manuscript:

1. While it is clear that the authors knocked down UPF3 in cells in order to compare the results with the in vivo studies, the choice of UPF3 is not optimal. Therefore it would be important to validate the results shown in Figure 1A as well in other experiments in cells depleted of UPF1, which is expected to have a stronger effect.
2. In Figure 1A, transcripts on the right of TNRC5 do not seem to be significantly regulated by NMD. These transcripts should be validated in UPF1 knockdowns or classified as non-targets.
3. For the real targets it will also be interesting to analyze mRNA half-lives in UPF1 depleted cells. In particular for the highly regulated transcripts such as IRE1 PDRG1, ATF3 and TRAF2.
4. Figures 2B and 2E are not particularly convincing. The curves differ only for one concentration/time point. The same is true for Figures S2B-F. More concentrations or later time points could be tested.

Referee #3:

SUMMARY
1. Does this manuscript report a single key finding? YES
This manuscript demonstrates a feed back interaction between NMD and the unfolded protein response, wherein NMD limits the response to low-level stressors, but high level stressors suppress the NMD effect.

2. Is the reported work of significance? YES

3. Is it of general interest to the molecular biology community? YES
By linking NMD to ER stress and the unfolded protein response this paper addresses questions of interest to a broad community of researchers.

4. Is the single major finding robustly documented using independent lines of experimental evidence? YES
One of the most impressive aspects of this work is the way the authors brought together in vitro and in vivo approaches that reached the same conclusion.

The underlying premise of the study by Karam et al. is that NMD has functions in regulating gene expression beyond surveillance of nonsense-containing mRNAs. They noted that loss of NMD factors in cell lines and in mice is associated with an increase in mRNAs for several of the effectors of the unfolded protein response (UPR), but not their downstream targets. A number of the UPR effector mRNAs have NMD-inducing features such as a long 3'-UTR, a uORF or a 3'-UTR intron. These were upregulated following knockdown of UPF3A, 3B or both, with the most striking effect seen for IRE1alpha, PDRG1 and ATF3. Actinomycin D chase and reporter assays confirmed that changes in these mRNAs resulted from NMD-mediated changes in half-life. In cultured cells and mouse liver the loss of UPF3B shifted the response curve for low-dose tunicamycin and thapsigargin to the left, and it reduced the ability of both to resolve UPR following high dose tunicamycin. The rapid and elevated induction of IRE1alpha phosphorylation, CHOP, BIP, XBP1 splicing and ATF6 cleavage in tunicamycin-treated cells knocked down of UPF3B indicated a role for NMD in buffering the UPR, and this was confirmed in vivo by increased TUNEL staining of apoptotic cells in livers of tunicamycin-treated UPF3B-null mice. IRE1alpha is the proximal effector of ER stress and the authors identify this as the principal target regulated by NMD. Finally, others had shown that various stressors can suppress NMD, but to the best of my knowledge this is the first study that provides mechanistic insight for this effect.

This is a well-written, carefully designed study that demonstrates a feedback interaction between NMD and ER stress. The the data are convincing, and the comprehensive approaches address each stage of UPR, from the activation of IRE1alpha to apoptosis in livers of UPF3B-null mice. Although the authors could have reached the same conclusions using cell lines, this paper stands out by the inclusion of in vivo experiments with UPF3B NMD null mice. Finally, I am particularly impressed by the way the authors brought together literature observations of stress-induced downregulation of NMD with NMD regulation of the UPR.

I have only a few comments, none of which require revision.

1. UPF1 knockdown is generally used as the lead approach for demonstrating a role for NMD in any particular process, and I was surprised to see the authors instead lead off with UPF3A/3B knockdown and only later address UPF1. No reason was given for this - presumably it is because UPF1 null mice are not viable and they had the UPF3B mouse. It would help to add some comment to this effect in the text.

2. The experiment in Fig. 1D is overinterpreted. I agree that shortening the 3'-UTR can be interpreted as changing their reporter from an NMD substrate to a non-substrate, but this approach does not rule out the loss of specific sequence elements, particularly since the target mRNA has such a long 3'-UTR.

3. Even though the authors note differences in scale used in Fig. 2E comparing the impact of high-dose tunicamycin in mice, it would be helpful to state the fold differences at the 24 hour time point in the text. On cursory examination these don't look that much different in the figure.

Below are our responses to the Reviewers' specific concerns:

Referee #1:

Minor points

1. "Due to the relevance of this new history I would like the author to discuss, (if needed with empirical data) the fact of the control of NMD during ER stress conditions. The NMD luciferase activity assay is good tools to demonstrate the effect of UPR on NMD activity, but, what is mechanism that regulates NMD activity during ER stress conditions? Could the authors discuss a bit more how UPR induction can impair the activity of UPF proteins? It looks it is downstream eif2a and GADD34 activities, but how this can be done?"

> Response: As we briefly discussed in the original submitted manuscript, previously published data, which was confirmed by us, demonstrated that ER stress triggers the phosphorylation of eIF2α, which leads to repressed NMD. We showed that this repression is reversed by GADD34, a phosphatase known to dephosphorylate eIF2α. Given that eIF2α phosphorylation is a well-established means to inhibit translation (Zheng *et al. Tumor Biol.* 35:6255, 2014) and translation is essential for NMD (Carter *et al. JBC 270:28995*, 1995), the most obvious means by which eIF2α phosphorylation inhibits NMD is through its ability to suppress translation. However, there is some evidence that eIF2α phosphorylation inhibits NMD by a translation-independent mechanism (Karam *et al. BBA*, 2013). These points are briefly discussed in the revised manuscript on pg. 16, line 1-6.

2. "In the same point, I would like to see the mRNA regulation of UPF genes involved in NMD. This would allow us to evaluate if there is another regulatory mechanism (a part of the differential activity of NMD). I think a time course expression analysis would be enough."

> Response: In the revised manuscript, we have included a new figure panel that provides a time course analysis of 12 NMD factors in response to ER stress in HeLa cells (**Supplementary Fig S4A**). Most NMD factor mRNA levels remain relatively constant after ER stress. An exception is eIF4AIII, a core factor of the EJC complex, whose mRNA is downregulated by ~60% 24 hr after tunicamycin treatment. *UPF2, UPF3B*, and *SMG1* mRNA levels are also reduced in response to tunicamycin, albeit by ≤40%. In cell types in which the endogenous level of these NMD factors is rate-limiting for NMD, these reductions could play a role in the downregulation of NMD in response to ER stress, as we indicate on pg. 15, lines 7-16 in the revised manuscript.

3. "Using the concentrations on figure 4D, evaluate if there is an effect on cell death."

> Response: We have performed this experiment and found that NMD does not act through IRE1α to protect cells from ER stress-induced cell death (new **Supplementary Fig. S3E**). In particular, we found that if we use RNAi to prevent IRE1α level from being upregulated in response to perturbation of NMD, this does not reduce apoptosis triggered by ER stress. Coupled with our other results, this indicates that while NMD's ability to downregulate IRE1α raises the threshold for triggering the

UPR in response to ER stress, it does not impact the magnitude of apoptosis triggered by ER stress.

4. *"Due to the cartoon present in figure 4F, where a kinetic model is present, I would like to see a tracking of IRE in a time dependent manner. Points 2 and 4 could be done in the same cellular background, to be then correlated, validating, thus, more extensively the model purposed on figure 4F."*

Response: In our previously submitted manuscript, we showed that depletion of the NMD factors, UPF3A and/or UPF3B, elevates *IRE1α* mRNA levels at all time points after ER stress (**Fig. 4C** and **Supplementary Fig. S3B** in the revised manuscript). This suggests that NMD suppresses IRE1α expression both constitutively and during all phases of the UPR. To definitively assess whether NMD is responsible, we tested the effect of depletion of the central NMD factor, UPF1, and obtained the same result (new **Supplementary Fig. S3A**).

Referee #2:

I have a few suggestions to strengthen the manuscript:

1. *"While it is clear that the authors knocked down UPF3 in cells in order to compare the results with the in vivo studies, the choice of UPF3 is not optimal. Therefore it would be important to validate the results shown in Figure 1A as well in other experiments in cells depleted of UPF1, which is expected to have a stronger effect."*

Response: We agree that it is important to complement our UPF3 depletion experiments with experiments depleting the central NMD factor, UPF1, to more clearly define NMD substrate mRNAs. We performed these experiments and new **Fig. 1A** provides the data. We found that 8 of 13 transcripts encoding UPR factors that we tested were significantly upregulated by UPF1 depletion. This supports our thesis that mRNAs encoding proteins involved in the UPR are commonly targeted by NMD. In the figure, we divided these 8 transcripts into 6 significantly upregulated by UPF3A/UPF3B depletion and 2 that were not. The latter group is consistent with previous *in vitro* and *in vivo* studies demonstrating the existence of a UPF3-independent pathway of NMD (Chan *et al. EMBO J.* 26:1820, 2007; Huang et al. *Mol Cell* 43:950, 2011; Metze *et al. RNA* 19:1432, 2013).

2. *"In Figure 1A, transcripts on the right of TNRC5 do not seem to be significantly regulated by NMD. These transcripts should be validated in UPF1 knockdowns or classified as non-targets."*

Response: New **Fig. 1A** delineates the different categories of transcripts that we defined, based on our new experiments explained in answer to the question above. The clear "non targets" in HeLa cells are *BAK* and *UFM1* mRNA.

3. *"For the real targets it will also be interesting to analyze mRNA half-lives in UPF1 depleted cells. In particular for the highly regulated transcripts such as IRE1 PDRG1, ATF3 and TRAF2."*

Response: We have performed RNA half-life analysis in response to UPF1 depletion. New **Supplementary Fig. 1E** shows this data. All the UPR transcripts we tested that were upregulated by UPF1 depletion (**Fig. 1A**) were also stabilized by UPF1 depletion, including *ATF3, IRE1α*, and *TRAF2* mRNA. This provides strong evidence that these mRNAs are direct NMD substrates. With regard to *PDRG1* mRNA, this is an unusual mRNA that we found was significantly upregulated in response to UPF3A/UPF3B depletion, but *not* UPF1 depletion (**Fig. 1A**). Consistent with this, we found it was

stabilized by UPF3A/UPF3B depletion, but not UPF1 depletion (**Supplementary Figs. 1E** & **1F**). We indicate in the revised text on pg. 6, lines 17–19 that it may be either a NMD target transcript or regulated by an UPF3A/UPF3B-dependent mechanism not involving NMD. There is precedent for transcripts upregulated by UPF3B but not UPF1; examples are *ASS1, FGF18,* and *MAP2K3* mRNA, as shown by microarray analysis of HeLa cells depleted of either UPF3B or UPF1 [Hug & Caceres, *Cell Reports* 8:1, 2014]). If deemed important, we can refer to this paper in the revised manuscript.

4. "Figures 2B and 2E are not particularly convincing. The curves differ only for one concentration/time point. The same is true for Figures S2B-F. More concentrations or later time points could be tested."

Response: The point of **Fig. 2B** is to show loss of *Upf3b in vivo* raises the threshold for the UPR. We regard this as convincing, as 2 of the 3 concentrations of tunicamycin (0.1 and 0.5 μg/g) elicit statistically significant effects. The only dose that did not have a statistical defect is the highest dose (1 μg/g), which is the predicted result if NMD raises the threshold for the UPR, one of the main points of our study. In the revised manuscript, we have also added data showing that loss of *Upf3b* also raises the threshold of the UPR at 12 hr in response to an intermediate dose (0.25 μg/g) of tunicamycin (new **Supplemental Fig. S2D**; note that the 12-hr time point is the same time point as examined in **Fig. 2B**).

Fig. 2E provides the *in vivo* evidence that NMD promotes the timely termination of the UPR. We agree with Reviewer #2 that we show an effect of 1 μg/g TM *in vivo* at only one time point (24 hrs). However we argue that this is convincing given that we observed the same effect for all 3 UPR effectors we tested (*XBP1S, BIP,* and *CHOP*). Furthermore, this *in vivo* result agrees with our *in vitro* data (**Fig. 2D** and **Supplementary Figs. S2E-G**). We tested a higher dose (2 μg/g) but found it caused mouse lethality. We feel that it is beyond the scope of this manuscript to conduct further time-consuming and expensive *in vivo* experiments. We have added a caveat in the revised manuscript, explaining that our *in vivo* data supporting the concept that NMD promotes the timely attenuation of the UPR is supported by only a single time point (pg. 10, lines 12-14).

Supplementary Figs. S2A-C shows that NMD raises the threshold of the UPR in response to the ER stress inducer, thapsigargin, just as it does in response to ER stress inducer tunicamycin. **Panel A** shows that UPF3A/UPF3B depletion significantly raises the levels of the UPR effector, *XBP1s*, at all doses of thapsigargin except the highest one, which strongly supports an effect on UPR threshold. Reviewer #2 is concerned about **panels B** and **C**, which show the response of the UPR effectors, *BIP* and *CHOP*. While we agree that the data is less striking for these effectors than for *XBP1s*, statistically significant effects were obtained at all the lower concentrations for *CHOP* (and 2 of 3 for *BIP*), which we regard as convincing. No significant effect was observed at the higher doses, as predicted if NMD raises the threshold of the UPR, as described above.

Previous **Supplemental Figs. S2D-F** (now **Supplemental Figs. S2E-G**) provide supporting evidence that NMD promotes the timely attenuation of the UPR *in vitro*. These supplementary figure panels show statistically significant effects at one time point from cells depleted of both UPF3A and UPF3B. This supplements data from cells depleted of only UPF3B—**Fig. 2D**—in which we show statistically significant effects on the UPR effectors at *several time points*. Thus, **Supplementary Figs. S2E-G** is included as data to back up **Fig. 2D** and thus we regard it as sufficient.

Referee #3:

"1. UPF1 knockdown is generally used as the lead approach for demonstrating a role for NMD in any particular process, and I was surprised to see the authors instead lead off with UPF3A/3B knockdown and only later address UPF1. No reason was given for this - presumably it is because UPF1 null mice are not viable and they had the UPF3B mouse. It would help to add some comment to this effect in the text."

> Response: As Reviewer #3 notes, we chose to use a UPF3 knockdown approach to allow us to make meaningful comparisons with our *in vivo* data from NMD-deficient mice lacking UPF3B. We agree that UPF1 knockdown is traditionally used to assess NMD substrates *in vitro*, and thus we have performed these experiments and added this data to the revised manuscript, as described in response to **Reviewer #2**, points 1-3.

2. "The experiment in Fig. 1D is overinterpreted. I agree that shortening the 3'-UTR can be interpreted as changing their reporter from an NMD substrate to a non-substrate, but this approach does not rule out the loss of specific sequence elements, particularly since the target mRNA has such a long 3'-UTR."

> Response: We agree that there are alternative interpretations of this result, including the one provided by Reviewer #3. In the revised manuscript, we have added a sentence saying "We note that our results do not rule out that the *IRE1α* 3' UTR confers sensitivity to NMD for another reason, such as essential *cis* elements in both its 5' and 3' halves" on pg. 8, lines 12-14.

3. "Even though the authors note differences in scale used in Fig. 2E comparing the impact of high-dose tunicamycin in mice, it would be helpful to state the fold differences at the 24 hour time point in the text. On cursory examination these don't look that much different in the figure."

> Response: We thank the Reviewer for this helpful suggestion. The revised text on pg. 10, lines 9-10 says: "*Upf3b*-null mice exhibited 1.8-, 1.8-, and 4.6-fold depressed downregulation of *Xbp1s, Bip,* and *Chop* mRNA, respectively, relative to littermate control mice."

2nd Editorial Decision   21 January 2015

Thank you for your patience while we have reviewed your revised manuscript. The study was seen by previous referees 1 and 2, who -as you will see from the reports below- are now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

- The information regarding the number of independent experiments performed, the type of error bars used and statistical test applied to the data is often confusing. In some instance, information regarding the "n" is missing, and in others information seems only to be included in the legend to one panel (for example figure 1E, and it is unclear if it applies to all of the panels of the figure. Please ensure that all figure legends include information on the number of independent experiments, the type of error bars used and -if applicable- the statistical test applied to the data and P values considered significant. The same information can apply to several panels in the same figure, but this should then be clearly stated at the end of the figure legend.

Once all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt

inclusion of your manuscript in our next available issue.

You may be pleased to know that I have commissioned a highlight to appear alongside your article in the same issue of the journal.

Thank you for your contribution to EMBO reports.

REFEREE REPORTS:

Referee #1:

I have read with great interest the answers of the authors and consider that this manuscript has been deeply improved. The authors have solved all my concerns and the study deserved publication in EMBO Reports.
The study of NMD factors under ER stress shows a complex crosstalk between both events, I think this story will be well received by the community.

Referee #2:

The authors have addressed the reviewers' comments and included additional data, the manuscript is now acceptable for publication in EMBO reports.

2nd Revision - authors' response	13 February 2015

Thank you for your e-mail of 21 January where you indicated our manuscript was provisionally accepted. Attached is a revised manuscript with the additional documents that you requested. The following revisions/additions were made:

- The figure legends have been extensively revised to indicate number of experimental repeats, statistics, etc., as you had requested. This is underlined.

- The source and description of the antibodies used in our study are indicated in the Materials and Methods section of the revised manuscript. This is underlined.

 We hope that our revised manuscript is deemed acceptable for publication in *EMBO Reports*.

3rd Editorial Decision	24 February 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

RNA metabolism: putting the brake on the UPR

Amado Carreras-Sureda[1,2] & Claudio Hetz[1,2,3]

The unfolded protein response (UPR) is a major signaling cascade that determines cell fate under conditions of endoplasmic reticulum (ER) stress. The kinetics and amplitude of UPR responses are tightly controlled by several feedback loops and the expression of positive and negative regulators. In this issue of *EMBO Reports*, the Wilkinson lab uncovers a novel function of nonsense-mediated RNA decay (NMD) in fine-tuning the UPR [1]. NMD is an mRNA quality control mechanism known to destabilize aberrant mRNAs that contain premature termination codons. In this work, NMD was shown to determine the threshold of stress necessary to activate the UPR, in addition to adjusting the amplitude of downstream responses and the termination phase. These effects were mapped to the control of the mRNA stability of IRE1, a major ER stress transducer. This study highlights the dynamic crosstalk between mRNA metabolism and the proteostasis network demonstrating the physiological relevance of normal mRNA regulation by the NMD pathway.

See also: R Karam *et al*

Approximately 30% of monogenic diseases are originated from nonsense mutations resulting from single nucleotide changes that introduce a premature termination codon (PTC). These mutations result in the translation of proteins that lack the carboxy-terminal region, leading to the expression of non-functional or even deleterious proteins. Nonsense-mediated RNA decay (NMD) operates as an efficient quality control mechanism to degrade nonsense-codon-containing mutant RNAs

prior to the production of aberrant products. NMD detects mRNA substrates at the pioneer-round of translation assisted by distinct NMD components, including SMG and UPF proteins [2]. NMD also functions as a post-transcriptional regulatory mechanism that controls the levels of a large variety of normal gene transcripts, between ~3 and 20% of total mRNAs—including a cluster of genes involved in the unfolded protein response (UPR) and stress-related genes [3]. The UPR is a major pathway that orchestrates cellular adaptation under conditions of endoplasmic reticulum (ER) stress [4]. Karam *et al* [1] now report a novel bidirectional crosstalk between NMD and the UPR, whereby NMD determines the threshold of UPR activation, shaping its timely termination to control cell fate under ER stress.

The UPR is initiated by three main stress sensors, IRE1α, PERK, and ATF6, which transduce information about the protein-folding status in the ER to the cytosol and nucleus, to induce an adaptation program [5]. Cell injury secondary to chronic ER stress is increasingly implicated in a wide range of human diseases, including diabetes, neurodegeneration, inflammatory and metabolic disorders, and cancer [6]. The activation of PERK leads to a global reduction of translation through the direct phosphorylation of the initiation factor eIF2α. This triggers the selective expression of the transcription factor ATF4, which controls a variety of genes involved in redox control, folding, and apoptosis [5]. IRE1α is a kinase and endoribonuclease that initiates the most conserved UPR signaling branch. Upon activation, IRE1α catalyses the splicing of the mRNA encoding for the transcription factor XBP1, leading to the expression of a stable

protein that upregulates a variety of UPR target genes [5]. IRE1α activity is also involved in RNA degradation (a process known as regulated IRE1-dependent decay or RIDD) of ER-localized mRNAs, ribosomal RNA, and micro-RNAs [4,5]. IRE1α is a central controller of cell fate under ER stress, since its downstream outputs engage both stress adaptation and cell death programs. The amplitude and kinetics of IRE1α signaling are tightly controlled through its binding to several negative and positive regulators [4].

NMD is emerging as a relevant pathway modulated by cellular stress. Reactive oxygen species, hypoxia, nutrient deprivation, ER stress, and viral infections, among other perturbations, can repress NMD [3]. A common mechanism that contributes to this inhibition is the phosphorylation of eIF2α in response to the activation of PERK and other related kinases [7]. Since NMD fully requires protein translation to recognize PTCs, the repression of translation under stress may explain the inhibitory activity on the pathway. The link between mRNA stability and UPR operates on a bidirectional mode. Genetic inhibition of NMD has been shown to stabilize a variety of mRNAs encoding for components of the PERK/eIF2α pathway, increasing the amplitude of downstream responses [7]. A pioneering study uncovered a functional link between the UPR and NMD *in vivo*. An RNAi unbiased screen in *C. elegans*, identified genes that are required for the development of *ire1* mutant worms, and uncovered an unexpected connection with the NMD pathway [8]. This study showed that targeting NMD results in spontaneous ER stress, in addition to sensitizing animals to the lethal effects of experimental
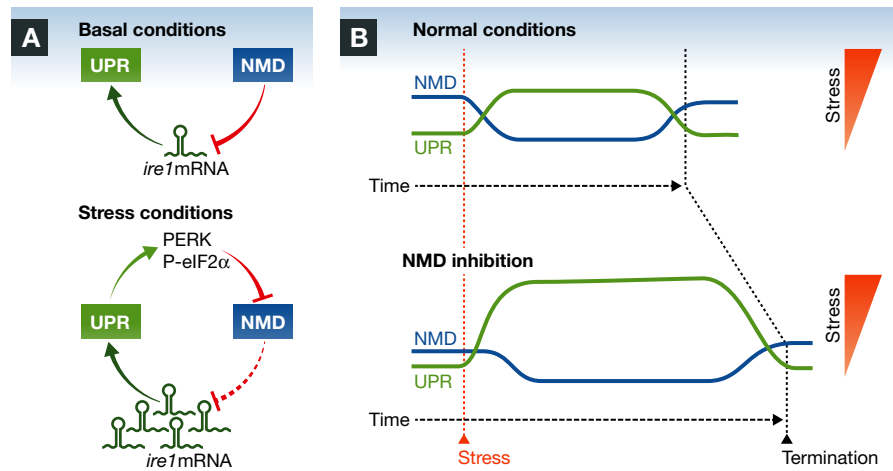
Figure 1. Bidirectional control between NMD and the UPR.
(A) In basal conditions, NMD constantly represses the UPR via the degradation of *ire1* mRNA and other components of the pathway. Under ER stress, the UPR inhibits NMD via eIF2α phosphorylation, which in turn promotes the accumulation of *ire1* mRNA, resulting in a robust UPR activation. (B) NMD determines the threshold for the establishment of a UPR reaction and thus impacts cell fate under ER stress. NMD modulates the amplitude and dynamics of UPR signaling, affecting the termination phase under chronic ER stress.

ER stress. In this context, the occurrence of ER stress in NMD-deficient cells may involve the accumulation of misfolded truncated proteins, suggesting that NMD operates as a translation-dependent surveillance mechanism to reduce basal levels of unfolded proteins at the ER.

Karam *et al* [1] propose a novel form of control of the UPR by NMD. The authors examined the mRNA levels of a variety of UPR components when the NMD pathway was altered. Remarkably, *Ire1α* was one of the most induced genes upon NMD disruption at the mRNA and protein levels. The authors were also the first to demonstrate that ER stress and NMD are interconnected *in vivo* in mammals, using mice lacking *Upf3b*. Gain- and loss-of-function assays indicated that NMD represses the UPR in an IRE1α-dependent manner. It is well known that under prolonged ER stress, IRE1 signaling is attenuated, leading to a reduction in XBP1 expression, which sensitizes cells to apoptosis [4,5]. Interestingly, NMD activity not only affected the threshold of UPR activation under low doses of ER stressors, but it also shaped its amplitude and kinetics,

having a significant role in attenuating XBP1 mRNA splicing (Fig 1). As a consequence, NMD inhibition has an impact on the susceptibility of cells to undergo ER stress-dependent apoptosis, both in cell culture and in mouse models. Overall, the Wilkinson lab describes a complex bidirectional circuit where UPR mRNAs are targeted for decay, whereas NMD activity is repressed by ER stress. The authors speculate that NMD may prevent UPR activation by innocuous ER stress, whereas ER stress signaling represses NMD to ensure the establishment of a robust adaptive program (Fig 1). In contrast to previous studies *in C. elegans* [8], no basal ER stress was observed in cells and animals depleted of NMD components, suggesting that NMD and the UPR are connected as a regulatory checkpoint involving signaling events, rather than altered ER physiology.

Perturbations to the NMD pathway, and more specifically of UFP3B, are involved in cognitive disorders, including autism and schizophrenia [9,10]. NMD promotes the degradation of mutant mRNA related to several disorders, including thalassemia, cystic fibrosis, and muscular dystrophy [2].

Recent drug discovery efforts led to the development of therapeutic approaches—including the use of aminoglycoside antibiotics and ataluren—to allow translational read-through of the PTC and generate full-length functional proteins [2]. Since ER stress is a salient feature of most neurodegenerative diseases involving abnormal protein aggregation [6], the alterations in the NMD pathway observed in cognitive disorders could be caused by abnormal ER stress levels. In this line, pharmacological modulation of NMD may also impact the adaptive capacity of a cell, increasing UPR responses. Based on these new studies, side effects related to ER stress signaling deregulation due to NMD inhibition are also predicted in the clinic.

Overall, a novel concept is emerging in which mRNA metabolism and protein quality control are merged into an interconnected pathway that globally monitors the proteostasis status of the cell, even before any protein is produced. Since IRE1α regulates mRNA and miRNA stability through RIDD, and NMD controls IRE1 levels, a interesting scenario arises where different layers of interactions occur between mRNA metabolism and protein homeostasis.

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