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RNA Homeostasis Governed by Cell Type-Specific and Branched Feedback Loops Acting on NMD

Lulu Huang[1,3,4,5], **Chih-Hong Lou**[1], **Waikin Chan**[4], **Eleen Y. Shum**[1], **Ada Shao**[1], **Erica Stone**[2], **Rachid Karam**[1,4,5], **Hye-Won Song**[1,3,4,5], and **Miles F. Wilkinson**[1,4,5,*]

[1]Department of Reproductive Medicine, School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

[2]Department of Cellular and Molecular Medicine, School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

[3]Genes and Development Program, The University of Texas Graduate School of Biomedical Sciences, Houston, TX 77033, USA

[4]Department of Biochemistry and Molecular Biology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77033, USA

SUMMARY

Nonsense-mediated mRNA decay (NMD) is a conserved RNA decay pathway that degrades aberrant mRNAs and directly regulates many normal mRNAs. This dual role for NMD raises the possibility that its magnitude is buffered to prevent the potentially catastrophic alterations in gene expression that would otherwise occur if NMD were perturbed by environmental or genetic insults. In support of this, here we report the existence of a negative feedback regulatory network that directly acts on seven NMD factors. Feedback regulation is conferred by different branches of the NMD pathway in a cell type-specific and developmentally regulated manner. We identify feedback-regulated NMD factors that are rate limiting for NMD and demonstrate that reversal of feedback regulation in response to NMD perturbation is crucial for maintaining NMD. Together, our results suggest the existence of an intricate feedback network that maintains both RNA surveillance and the homeostasis of normal gene expression in mammalian cells.

INTRODUCTION

The nonsense-mediated mRNA decay (NMD) pathway was originally identified as an RNA surveillance pathway that eliminates aberrant mRNAs harboring premature termination codons (PTCs) generated as a result of mutations or biosynthetic errors (Chang et al., 2007; Nicholson et al., 2010). NMD was shown to be crucial for determining disease outcome by

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*Correspondence: mfwilkinson@ucsd.edu.
[5]Present address: Department of Reproductive Medicine, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0864, USA

SUPPLEMENTAL INFORMATION
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protecting cells against dominant-negative effects of truncated proteins otherwise produced from disease gene alleles with frameshift and nonsense mutations (Maquat, 2004; Holbrook et al., 2004). The influence of NMD on disease phenotype is likely widespread, as one-third of human disease genes harbor frame-disrupting mutations that trigger NMD (Bashyam, 2009; Bhuvanagiri et al., 2010).

Recent evidence shows that NMD degrades not only aberrant transcripts from mutant genes, but also normal transcripts from genes that harbor a normal stop codon in a context that elicits NMD (Chang et al., 2007). Genome-wide analysis has revealed that NMD regulates 3%–20% of wild-type transcripts from a wide variety of species, including *S. cerevisiae, C. elegans, D. melanogaster*, plants, and mammals (Lelivelt and Culbertson, 1999; He et al., 2003; Mendell et al., 2004; Rehwinkel et al., 2005; Wittmann et al., 2006; Chan et al., 2007; Ramani et al., 2009). Many of the endogenous NMD substrates from these various species are conventionally spliced transcripts that encode normal proteins, but some are alternatively spliced transcripts that have a stop codon in a premature position, thus encoding a truncated protein that may or may not be functional (McGlincy and Smith, 2008; Nicholson et al., 2010). The finding that NMD regulates the expression of a large subset of genes in a wide range of species suggests that NMD has a crucial, conserved role in regulating gene expression (Neu-Yilik and Kulozik, 2008). In support of this notion, many species share common classes of NMD target transcripts (“NMD substrates”), including mRNAs encoding SR proteins and other RNA-binding proteins (McGlincy and Smith, 2008; Mühlemann et al., 2008).

The discovery that NMD has a dual role (i.e., RNA surveillance and regulation of gene expression) increases its biological importance, which in turn increases the probability that mechanisms have evolved to protect organisms from the deleterious consequences that would ensue if NMD were perturbed. Indeed, NMD is sensitive to many different forms of stress. For example, NMD is inhibited by environmental insults, including hypoxia, ultraviolet irradiation, other DNA damage-inducing agents, amino acid starvation, and conditions that activate the unfolded protein response (Sharifi and Dietz, 2006; Gardner, 2008; Gardner and Corn, 2008). Genetic perturbations have also been shown to downmodulate or completely ablate NMD. For example, it was discovered that naturally occurring mutations in the *UPF3B* gene in humans inhibit a branch of the NMD pathway and cause intellectual disability and other mental disorders (Chan et al., 2007; Tarpey et al., 2007; Laumonnier et al., 2009; Addington et al., 2011).

Because NMD regulates hundreds of normal transcripts (both direct and indirect targets) in all organisms studied, perturbing NMD would significantly alter transcript profiles in cells and thereby could potentially cause catastrophic consequences. In addition, by virtue of its role in RNA surveillance, NMD would no longer rapidly degrade aberrant PTC-bearing transcripts, leading to the expression of truncated, potentially toxic, dominant-negative proteins (Chang et al., 2007; Mühlemann et al., 2008). Consistent with this, knockout of the NMD factor genes *Upf1, Upf2*, or *Smg1* in mice elicits embryonic lethality (Medghalchi et al., 2001; Weischenfeldt et al., 2008; McIlwain et al., 2010). To protect against these negative consequences, we hypothesized the existence of buffering mechanisms that confer robustness to the NMD pathway. We previously reported that one such mechanism is the

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dramatic stabilization of the NMD factor UPF3A in response to loss of its paralog, UPF3B (Chan et al., 2009). We considered the possibility that this protein stabilization regulatory mechanism is complemented by a RNA stabilization regulatory mechanism, based on the finding that depletion of the NMD factor UPF1 in HeLa cells stabilizes the mRNA encoding the NMD factor SMG5 (Mendell et al., 2004; Chan et al., 2007; Singh et al., 2008a). Here, we report the identification of a large number of NMD factors upregulated in response to NMD perturbation in mouse and human cells. We examine the underlying mechanism for this regulatory response, demonstrate that it is exerted in a cell type-specific and developmentally regulated manner, and provide several lines of evidence that it serves a physiological role in mammalian cells. Taken together, our results support the existence of a conserved homeostatic pathway consisting of a series of negative feedback regulatory loops.

RESULTS

A Conserved Feedback Regulatory Network

To determine whether the NMD pathway negatively regulates NMD factors, we used RNAi to disrupt NMD. Depletion of the NMD factor UPF1 by RNAi reduced *UPF1* levels in HeLa cells by more than 90% and upregulated NMD target transcript *ATF3* (Mendell et al., 2004; Chan et al., 2007), indicating that NMD was in-hibited (Figure 1A). Transcripts encoding six NMD factors were significantly upregulated in response to UPF1 depletion (Figure 1A). To elucidate whether NMD represses UPF1 expression, we depleted UPF3B by RNAi and found that *UPF1* mRNA was upregulated (Figure 1B). Together, these data indicated that NMD inhibits the expression of seven NMD factors, a phenomenon that we will refer to as "feedback regulation" in accordance with a commonly accepted definition of this term (Lestas et al., 2010). Western blot analysis confirmed feedback regulation of UPF1 and SMG5 at the protein level (Figure S1A available online). This feedback regulatory response was specific, as the transcript encoding the uniquely weak NMD factor, UPF3A (Kunz et al., 2006; Chan et al., 2009), did not significantly increase in level in response to NMD perturbation, suggesting that it is not feedback regulated (Figure 1A). Also not significantly feedback regulated were the transcripts encoding the core exon-junction complex (EJC) factors (i.e.,Y14, MAGOH, CASC3, and eIF4A3), which all promote mammalian NMD but are not essential for NMD (Figure 1A) (Le Hir and Andersen, 2008; Chan et al., 2009; Rebbapragada and Lykke-Andersen, 2009). As an independent test of feedback regulation by NMD, we treated HeLa cells with the protein synthesis blocker cycloheximide (CHX), a potent inhibitor of the NMD pathway (Carter et al., 1996; Amrani et al., 2004). CHX upregulated the very same NMD factor transcripts that were up-regulated in response to NMD factor depletion (Figure S1B). Taken together, these results suggest that transcripts encoding seven NMD factors (UPF1, UPF2, UPF3B, SMG1, SMG5, SMG6 and SMG7) are specifically downregulated in HeLa cells by a feedback regulatory pathway triggered by NMD itself.

To determine whether this feedback response operates in another cell type, we depleted UPF1 in mouse neuronal stem cells. Transcripts encoding four NMD factors (UPF1, SMG1, SMG6, and SMG7) were upregulated (Figure S1C), indicating that the feedback regulatory response operates in stem cells and is conserved in mice and humans. Together, these results

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indicate that NMD negatively regulates a large fraction of NMD factors, suggesting the existence of a series of negative feedback regulatory loops acting on the mammalian NMD pathway.

NMD Factors Are Direct NMD Targets

The increased steady-state level of NMD factor transcripts in response to NMD perturbation could be the result of increased mRNA synthesis or stabilization. To test the first possibility, we examined the effect of NMD perturbation on the level of NMD factor pre-mRNAs, which are short-lived primary transcription products whose level is a reasonably accurate measure of their rate of synthesis (Ardehali and Lis, 2009; Imam et al., 2009). As a control, we tested the known NMD substrate *TBL2* mRNA, which, as previously reported (Viegas et al., 2007), increased significantly in level after UPF1 depletion. In contrast, *TBL2* pre-mRNA level did not significantly increase in response to depletion of UPF1 (Figure 1C). Likewise, we found that the seven NMD factor transcripts upregulated in response to NMD perturbation (*UPF1, UPF2, UPF3B, SMG1, SMG5, SMG6*, and *SMG7*) only exhibited a significant increase in mRNA level, not pre-mRNA level (Figures 1C and 1D).

Given that NMD perturbation is unlikely to upregulate NMD factor mRNAs at the transcriptional level, we next examined whether the feedback-regulated NMD factor mRNAs are stabilized, as predicted if they are direct NMD targets. A striking feature of all seven NMD factor mRNAs subject to feedback regulation is that they have long 3′ UTRs (Table S1), a feature that is known to elicit NMD (Bühler et al., 2006; Eberle et al., 2008; Singh et al., 2008a). *UPF2, SMG1, SMG5, SMG6*, and *SMG7* also have one or more upstream open reading frames (uORFs) (Table S1), which, by definition, have a stop codon in a premature position relative to the main ORF and thereby can trigger a NMD response (Oliveira and McCarthy, 1995; Mendell et al., 2004).

To directly determine whether NMD destabilizes NMD factor transcripts, we performed RNA half-life analysis. We restricted RNA half-life analysis to the three *UPF* mRNAs and the two most regulated *SMG* mRNAs (*SMG1* and *SMG5*). As an initial test of this assay, we verified that the known endogenous NMD target, *GADD45B* mRNA was stabilized in UPF1-depleted cells (Figure 2A) (Viegas et al., 2007). Likewise, *UPF2* and *SMG1* transcripts were stabilized in UPF1-depleted cells, and *UPF1* transcripts were stabilized in SMG1-depleted cells (Figure 2A). We could not determine whether NMD stabilizes *UPF3B* and *SMG5* transcripts, as these transcripts were relatively stable, even in control cells (Figure S2). To elucidate whether the putative NMD-inducing feature in *UPF1* (i.e., its long 3′UTR) is responsible for its downregulation, we adapted the tetracycline (Tet)-regulated β-*globin* reporter system used by Singh and colleagues (Singh et al., 2008a). We found that substituting the *UPF1* 3′ UTR for the β-*globin* 3′UTR in their β-*globin* reporter construct triggered a rapid degradation of the reporter in a UPF1-dependent manner (β*UPF1* in Figure 2B), indicating that the *UPF1* 3′UTR is sufficient for triggering mRNA degradation. Taken together, these data indicate that the mRNAs encoding UPF1 and at least a subset of other NMD factors are direct NMD targets.

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NMD Factors Are Regulated by Different Branches of the NMD Pathway

Rather than a single linear RNA surveillance pathway, NMD appears to consist of several distinct branches, each of which regulates different subsets of transcripts. Gehring and colleagues published evidence for two independent branches of the NMD pathway, one that requires EJC components but is not affected when RNPS1 or UPF2 are depleted by RNAi and another that requires RNPS1 and UPF2 but is not affected when EJC components are depleted by RNAi (Gehring et al., 2005). Both branches require the NMD factors UPF1 and UPF3B. We identified a branch that is not perturbed by depletion of either of the UPF3 paralogs: UPF3A or UPF3B (Chan et al., 2007). To elucidate whether different branches of the NMD pathway are responsible for regulating different NMD components, we depleted the factors that function in each of the known NMD branches. We found that depletion of the EJC core protein eIF4A3 in HeLa cells upregulated *UPF1* and *SMG5* transcripts but did not significantly upregulate *UPF2, UPF3B, SMG1, SMG6*, and *SMG7* transcripts (Figure 3A). This suggested that the EJC-dependent branch regulates *UPF1* and *SMG5* mRNAs and that the EJC-independent branch of NMD degrades the other five NMD factor mRNAs. In contrast, depletion of RNPS1 selectively upregulated *SMG1* transcripts, whereas others remain unchanged (Figure 3B), suggesting that the RNPS1-dependent NMD branch uniquely regulates *SMG1*. Finally, we tested the effect of UPF3B depletion and found that *UPF1* and *SMG7* transcripts were selectively upregulated, suggesting that the UPF3B-dependent branch of the NMD pathway regulates these two transcripts (Figure 3C). Because the *UPF3B* paralog, *UPF3A*, can substitute for *UPF3B* in downregulating a limited set of NMD substrates (Chan et al., 2009), we also depleted HeLa cells of both UFP3A and UPF3B. However, like UPF3B depletion, this only significantly upregulated *UPF1* and *SMG7* transcripts (Figure 3C). In summary, NMD factor mRNAs differ remarkably in their regulation by different branches of the NMD pathway in HeLa cells. The only common feature of all of the NMD factor mRNAs is that they are feedback regulated by UPF1.

Cell Type-Specific and Developmentally Induced Feedback Regulation

To begin to understand the biological significance of this feedback regulatory network, we examined whether its targets differ in different biological contexts. We focused on the NMD factors regulated by the UPF3B-dependent branch, as this branch was recently shown to have a role in human disease (Tarpey et al., 2007; Laumonnier et al., 2009; Addington et al., 2011). We first examined lymphoblastoid cell lines (LCLs) from three intellectually disabled patients with debilitating *UPF3B* mutations that result in undetectable expression of UPF3B protein (Tarpey et al., 2007). The LCL lines in all three patients had upregulated levels of *UPF1, UPF2*, and *SMG7* mRNA relative to control LCLs; the other NMD factor mRNAs were not significantly upregulated (Figure 3D). This suggested that patients with mutations in the *UPF3B* gene have compensatory increases in three NMD factors resulting from reversal of the feedback response. Comparison of the results from the LCLs (Figure 3D) with those of HeLa cells (Figure 3C) revealed that inactivation of UPF3B upregulated *UPF1* and *SMG7* transcripts in both, whereas *UPF2* transcripts were only upregulated in LCLs. Because HeLa cells are epithelial and LCLs are lymphoid, this suggested the possibility that the UPF3B-dependent feedback regulatory response acts in a cell type-specific manner. However, another possibility was that LCLs, but not HeLa cells, express a

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UPF2 mRNA isoform that is sensitive to the UPF3B-dependent NMD pathway. Using primers that distinguish between the three known isoforms of human *UPF2* mRNA (Figure S3A), we found that all three iso-forms are expressed in both LCL and HeLa cells, and all three are upregulated in *UPF3B* mutant LCLs relative to control LCLs, indicating that all three are NMD substrates (Figure S3B). We detected no other major alternative *UPF2* isoforms, including alternatively spliced isoforms or isoforms with alternative 5′ or 3′ ends (based on rapid amplification of cDNA ends [RACE] and reverse transcriptase-polymerase chain reaction [RT-PCR] anal- yses; data not shown). Together, these data strongly suggest that the cell type-specific difference in *UPF2* levels in response to perturbation of UPF3B results not from differential isoform expression, but rather from an intrinsic cell type-specific difference in feedback regulation.

To further address cell type-specific UPF3B-dependent feedback regulation, we generated *Upf3b* mutant mice from a gene-trap embryonic stem (ES) cell line harboring a debilitating insertion in the *Upf3b* gene. *Upf3b*$^{-/y}$ mice had > 100-fold lower expression of *Upf3b* mRNA than control littermate *Upf3b*$^{+/y}$ mice in 18 different tissues, indicating that these mutant mice have a null mutation in the *Upf3b* gene (Figure S4A). NMD was perturbed in these null mutant mice, as demonstrated by the upre-gulation of several previously identified endogenous NMD substrates (Weischenfeldt et al., 2008; Bruno et al., 2011) in *Upf3b*$^{-/y}$ versus *Upf3b*$^{+/y}$ mouse brain tissues (Figure S4B and Table S2). However, half of the previously identified NMD substrate that we tested were not upregulated in *Upf3b*$^{-/y}$ mouse brain (Figure S4B and Table S2), thereby verifying the existence of a UPF3B–independent branch of the NMD pathway. Its UPF3B independence was previously suggested to exist based on RNAi experiments in which UPF3 factors were only partially depleted (Chan et al., 2007).

As a first step to examine whether UPF3B-dependent feedback regulation is a cell type-specific response in mice, we generated mouse embryo fibroblasts (MEFs) from *Upf3b*$^{-/y}$ and *Upf3b*$^{+/y}$ mouse embryos. We found that *Upf3b*$^{-/y}$ MEFs did not express significantly elevated levels of any of the NMD factor transcripts (Figure 4A). In contrast, the mouse endogenous NMD substrate *Gas5* (Weischenfeldt et al., 2008) was upre-gulated (Figure 4A), indicating that the UPF3B-dependent branch of NMD is intact in MEFs. Furthermore, MEFs have an intact feedback regulatory response, as *Upf2, Smg1, Smg5*, and *Smg7* transcripts were upregulated in response to depletion of *Upf1* (Figure 4B). Thus, MEFs display an intrinsic and selective reliance on the UPF3B–independent branch of the NMD pathway for the feedback regulatory response. Because the mouse fibroblast cell line LMTK did not significantly upregulate any of the NMD factor transcripts that we tested in response to UPF3B depletion (knocked down by RNAi, as described in Chan et al. [2009]; data not shown), this likely represents a general feature of mouse fibroblasts.

To assess the role of UPF3B-dependent feedback regulation in development, we examined ES cells. We observed no significant differences in the expression of NMD factor transcripts between *Upf3b* mutant and control ES cells (Figure 4C), suggesting that, like fibroblasts, ES cells do not use the UPF3B–depen-dent branch of the NMD pathway for feedback regulation. To examine whether differentiation triggers use of the UPF3B-dependent branch, we cultured the ES cells under conditions that elicited their differentiation into embryoid

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bodies, as demonstrated by both morphology and several molecular markers (Singh et al., 2008b) (Figure S4C). We found that this triggered an upregulation of *Upf1, Smg1*, and *Smg5* transcripts in *Upf3b* mutant ES cells relative to control ES cells (Figure 4C), suggesting that differentiation of mouse ES cells activates the UPF3B-dependent feedback response.

To evaluate the in vivo role of the UPF3B-dependent branch of NMD on feedback regulation, we examined NMD factor mRNA levels in 14 tissues from *Upf3b*$^{-/y}$ and *Upf3b*$^{+/y}$ littermate mice. This revealed that loss of *Upf3b* had a remarkably selective effect, significantly increasing the level of NMD factor transcripts in only two tissues: brain and spleen. *Smg1* transcripts were upregulated in *Upf3b*$^{-/y}$ brain tissue, whereas *Upf1* and *Smg6* transcripts were upregulated in *Upf3b*$^{-/y}$ spleen tissue (Figure 5A). None of the other tissues had a statistically significant increase in the level of NMD factor mRNAs in *Upf3b*$^{-/y}$ relative to littermate *Upf3b*$^{+/y}$ mice (Figure 5A). To examine the possibility that this tissue-specific regulation results from differential isoform expression, we characterized *Upf1* and *Smg6* transcripts in spleen and brain (we did not characterize transcripts from the *Smg1* gene because of its large size [112 kb] and complexity [63 exons]). Extensive analysis using RACE, RT-PCR, and quantitative PCR (qPCR) did not reveal a pattern of isoform expression that could explain the selective downregulation of *Upf1* and *Smg6* transcripts by the UPF3B-dependent branch of NMD in spleen, but not brain (Figures S5A and S5B and data not shown). Rather, our data suggest that a tissue-specific NMD-dependent feedback mechanism controls *Upf1* and *Smg6* transcripts.

Spleen is composed of a complex array of different cell types, including lymphocytes and macrophages. We purified T lymphocytes, B lymphocytes, and macrophages from *Upf3b*$^{-/y}$ and *Upf3b*$^{+/y}$ littermate mice to determine whether any of these cell types exhibited an increase in NMD factor transcripts in response to loss of UPF3B. We found that only T cells displayed a statistically significant increase in NMD factor transcripts in response to loss of UPF3B (Figure 5B). Like whole spleen, T cells exhibited increased *Upf1* and *Smg6* mRNA levels, but they also had elevated *Smg1* and *Smg5* mRNA levels. We probably did not detect elevated *Smg1* and *Smg5* mRNA levels in *Upf3b* mutant spleen because T cells represent only a small fraction of the cells in the spleen. Characterization of *Smg5* transcripts by RACE and RT-PCR analyses identified only a single isoform of *Smg5* mRNA in T cells and B cells (Figure S5C and data not shown), suggesting that its differential regulation in T and B cells is not due to differential isoform expression but, rather, results from an intrinsic cell type-specific difference in feedback regulation. Table S3 summarizes our results on the cell type-specific and developmentally regulated UPF3B–depen-dent feedback regulatory response.

Physiological Role of the Feedback Regulatory Response

To begin to understand the physiological relevance of the NMD feedback pathway, we first examined whether any of the NMD factors that it acts upon are rate limiting for NMD. To address this, we determined whether the magnitude of NMD was enhanced in HeLa cells that modestly overexpress the NMD factors that we identified as being feedback regulated. We first performed dose-response experiments to determine the amount of expression plasmid required to elevate the level of each NMD factor by an amount approximating the

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degree of feedback regulation (Figures S6A and B). We found that, when overexpressed to a modest degree (~3-fold), SMG1 promoted NMD, as assessed by a well-characterized NMD reporter system (Boelz et al., 2006) (Figure 6A). The increase in NMD activity was corroborated when we assayed NMD activity using three well-characterized human endogenous NMD substrates: ATF3, GADD45B, and NAT9 (Figure 6B). In contrast, the NMD substrate *TBL2* mRNA (Viegas et al., 2007) (Figure 6C herein) was not significantly downregulated in response SMG1 overexpression (data not shown), suggesting that HeLa cells constitutively generate optimal levels of SMG1 for this particular NMD substrate. When overexpressed at higher levels (by >3-fold), SMG5 and SMG6 also increased NMD activity, as assessed with either endogenous NMD substrates or the NMD reporter (Figures S6C and S6D). This ability to augment NMD was specific,as overex-pression of other NMD factors (UPF1, UPF2, UPF3A, UPF3B, and SMG7) did not significantly affect the level of the NMD reporter or endogenous NMD substrates (Figures S6C and S6D). We conclude that SMG1, SMG5, and SMG6 are all rate limiting for NMD in HeLa cells. Only SMG1 may be of significance to the feedback response in HeLa cells, as we found that only a modest increase in its level was sufficient to improve the magnitude of NMD.

To determine whether the upregulation of SMG1 buffers the NMD pathway when NMD is perturbed, we performed double-depletion experiments in which UPF1 was strongly depleted to inhibit NMD and SMG1 was modestly depleted so that its level approximated that in untreated cells (Figure S6E). We reasoned that, if increased SMG1 serves to partially rescue NMD, then knocking SMG1 down to control levels should inhibit NMD. In agreement with this prediction, we found that the endogenous NMD substrates *ATF3, GADD45B, NAT9*, and *TBL2* mRNA, all of which increased when UPF1 was depleted, were further upre-gulated when we blocked SMG1 upregulation (Figure 6C). As a negative control, we knocked down UPF2 rather than SMG1, as UPF2 is not rate limiting in HeLa cells (Figures 6A and 6B and Figures S6A, S6C, and S6D). We found that knockdown of UPF2 to control levels (Figure S6E) did not significantly affect the level of the NMD substrates in UPF1-depleted cells (Figure 6C). Therefore, we conclude that the feedback response plays a crucial role in maintaining NMD activity in HeLa cells by virtue of its ability to upregulate the rate-limiting NMD factor SMG1.

In addition to serving as a feedback mechanism providing robustness, the downregulatory mechanism may also be important for holding NMD in check so that it does not cause unwanted physiological effects. To test this, we modestly overexpressed the rate-limiting factor SMG1 in HeLa cells (by 3-fold; Figure S6B) and found that this action significantly slowed cell growth (Figure 6D). Together with the previous finding that depletion of NMD factors also inhibits cell growth (Chan et al., 2007), these data suggest that maintaining the magnitude of NMD is critical for normal cellular functions.

DISCUSSION

Homeostatic mechanisms are crucial for the proper functioning of biological systems. An early proponent of this concept–Conrad Hal Waddington–proposed in the 1940s that cells have a tendency to follow favored paths by virtue of robustness mechanisms that buffer against genetic and environmental perturbations, a phenomenon that he called “canalization”

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(Jamniczky et al., 2010). Since then, many specific mechanisms that confer robustness have been identified. Many of these buffering mechanisms control the rate of transcription, e.g., at the level of chromatin, transcriptional enhancers, or signaling circuits (Chi and Bernstein, 2009; Goentoro and Kirschner, 2009; Frankel et al., 2010). Here, we focused on a posttranscriptional mechanism that confers robustness. In particular, we identified a multipronged negative feedback system that acts in a cell type-specific and developmentally regulated manner on the NMD RNA surveillance pathway. We propose that this system protects the NMD pathway, allowing it to maintain both its RNA surveillance and gene regulatory functions in the face of environmental and genetic insults (Figure 7). This feedback response may be conserved, as recent studies show that some NMD factor transcripts in lower eukaryotes are upregulated in response to loss or depletion of NMD (Rehwinkel et al., 2005; Riehs et al., 2008; Saul et al., 2009). However, the physiological role of feedback regulation in lower eukaryotes is not known, and it remains unclear whether it acts in as broad a manner as in mammals, as only *SMG5* and *SMG6* have been shown to be regulated in the *Drosophila melanogaster* S2 cell line (Rehwinkel et al., 2005) and *UPF3* and *SMG7* in *Arabidopsis thaliana* (Riehs et al., 2008; Saul et al., 2009). One advantage of a large number of NMD factors being subject to feedback regulation is that it increases the odds that NMD deficits will be corrected, regardless of which NMD factors are rate limiting. Although we found that only SMG1, SMG5, and SMG6 are rate limiting for NMD in HeLa cells (Figures 6A, 6B, S6C, and S6D), it is likely that other combinations of NMD factors will be rate limiting in some other cell types. This would explain why we found that distinct sets of NMD factors are feedback regulated in different cell types at different developmental stages (Figures 3, 4, and 5 and Table S3). However, some NMD factors may rarely be rate limiting for NMD and therefore not feedback regulated; e.g., we found that the weak NMD factor UPF3A (Kunz et al., 2006; Chan et al., 2009) is not significantly feedback regulated (Figure 1A). In other cases, specific NMD factors may not be subject to feedback regulation due to deleterious effects that would arise. For example, the EJC core factors may not be subject to feedback regulation (Figure 1A) because their overex-pression in response to NMD perturbation, though good formain- taining NMD, could negatively impact their non-NMD functions (e.g., regulation of mRNA splicing and cytoplasmic mRNA localization) (Tange et al., 2004; Ashton-Beaucage et al., 2010; Roignant and Treisman, 2010). Furthermore, such side effects may also explain why NMD factor mRNAs that are subject to feedback regulation are regulated only modestly (~1.5- to 3-fold; Figure 1A), as this minimizes misregulation of their non-NMD functions (Nicholson et al., 2010). Thus, by spreading moderate feed- back regulation to many NMD factors, physiological buffering is achieved with minimal deleterious consequences.

We found that NMD feedback regulation is a highly directed response that is mediated by different branches of the NMD pathway (Figures 3A, 3B, and 3C). Why? We suggest that, in some cases, NMD perturbation may selectively increase the levels of NMD factors that are in the same branch as the perturbed NMD factor, thereby maximizing NMD buffering and minimizing side effects. In other cases, partially redundant NMD factors may be upregulated to bolster NMD. Cell types may differ as to which sets of NMD factors are feedback regulated by different branches of the NMD pathway (Figures 3, 4, and 5 and Table S3) because of cell type-specific differences in rate-limiting and redundant NMD

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factors. Although it is not known why cell types differ in their NMD factor profile and sensitivity, a likely possibility is that cell types differentially require the proteins encoded by transcripts regulated by different NMD branches.

Future studies must decipher the control mechanisms for cell type-specific and developmental regulation of this feedback response. It could be dictated by various factors, including specific RNA-binding proteins and regulatory RNAs. Given that NMD depends on translation (Sato et al., 2008), another possibility is that feedback regulation is controlled by translation efficiency. Our initial studies have not supported this idea, as we found that the ratio of *Upf1* mRNA-to-UPF1 protein was virtually identical in a tissue that feedback regulates *Upf1* mRNA and one that does not (Figure S6F). However, this does not rule out that the magnitude of translation controls feedback regulation in other scenarios. For example, our finding that the UPF3B-dependent NMD feedback pathway is induced when ES cells undergo in vitro differentiation (Figure 4C) could be due, in part, to the increased translation rate that accompanies this ES cell differentiation event (Sampath et al., 2008). Regardless of the precise mechanisms involved, we propose that the net outcome of branch-specific feedback regulation is that different cell types will be able to respond to NMD perturbation in unique ways to optimize robustness.

We found that genetic insults, including both naturally occurring mutations in human NMD genes (Figure 3D) and those triggered by RNAi or a gene-trap (Figures 1, 3, 4, and 5), elicit reversal of feedback regulation and, consequently, the upregulation of specific NMD factors. We demonstrated that this upregulatory response helps maintain NMD in HeLa cells (Figure 6C), and thus it is reasonable to suppose that the upregulatory response that occurs in mentally deficient individuals lacking the UPF3B-dependent branch of NMD (Figure 3D) lessens their phenotypic symptoms. This buffering effect also provides an explanation for why strong depletion of NMD factors by RNAi (even by ~90%) fails to strongly inhibit NMD (Mendell et al., 2004; Wittmann et al., 2006; Chan et al., 2007). In the future, it will be interesting to determine which NMD factors are upregulated in response to commonly encountered stressors that inhibit NMD, such as hypoxia and DNA-damaging reagents, and whether this upregulatory response rescues NMD. Conversely, some stressors may upregulate or activate components of the NMD pathway, which if left unchecked could trigger increased NMD activity and thereby cause undesirable consequences, such as inhibited cell growth (Figure 6D). In principal, this increased NMD activity should elicit a stronger feedback response, which would reduce the level of NMD components and thereby dampen NMD (Figure 7).

Though the feedback regulatory system uncovered here is likely to be of selective value by virtue of its conferring robustness, this same quality has the potential to blunt the ability of NMD to be regulated. Given the growing evidence that the magnitude of NMD is modulated in response to various cues, this is a significant problem. For example, evidence suggests that NMD is downregulated during *D. melanogaster* embryogenesis in vivo and mammalian muscle cell differentiation in vitro (Alonso and Akam, 2003; Gong et al., 2009). NMD is also repressed during mammalian neural development, which allows a neural gene expression program to be activated that is accompanied by a more differentiated phenotype (Bruno et al., 2011). How can the magnitude of NMD be modulated in the face of a

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feedback control mechanism that counteracts such changes? One possibility is that some cell types shut off the feedback response at specific developmental stages, permitting regulation of NMD. To avoid losing the benefits of robustness, only a specific branch of the feedback response may be inactivated. Thus, we suggest that an intricate web of branched and regulated feedback control coupled with regulatory inputs permits NMD to perform specific biological roles in a buffered context.

NMD factors have roles in biological processes in addition to NMD (Isken and Maquat, 2008). For example, UPF1 has been shown to directly participate in mRNA decay pathways besides NMD and also to help maintain genome stability (Kaygun and Marzluff, 2005; Azzalin and Lingner, 2006; Kim et al., 2007). SMG1 functions directly in DNA surveillance by participating in genotoxic stress response pathways (Brumbaugh et al., 2004; Oliveira et al., 2008). Both SMG1 and UPF1, along with SMG6, have roles in telomere maintenance (Reichenbach et al., 2003). It is likely that strong selection has tightly regulated these non-NMD activities. As evidence, it is known that overexpressed SMG6 disrupts telomere maintenance, leading to telomere fusion and cell death (Reichenbach et al., 2003). We posit that the feedback pathways that we uncovered buffer these non-NMD activities from environmental and genetic insults, thereby maintaining normal DNA surveillance and RNA decay functions. We also suggest that the feedback mechanism might have a role in the evolution of the multifunctional nature of NMD factors. This follows from the fact that robustness systems allow an organism to be more “evolvable” by allowing accumulation of mutations that would be deleterious without the buffering system (Masel and Trotter, 2010). Some of these mutations may have positive selective value later during evolutionary time when a species “needs them” due to changes in the environment or the organism itself. Applying this principal to NMD, the robustness conferred by the feedback pathway may have permitted initially deleterious mutations in NMD factor genes to be fixed in the germline, which later drove the evolution and optimization of new functions.

In summary, we have identified a buffering system that fine-tunes the level of NMD factors to achieve optimal NMD activity and protect NMD from perturbation. This buffering system is controlled in a cell type-specific, developmentally regulated, and branch-specific manner, which we propose provides the versatile and subtle feedback control that is necessary to accommodate the diverse inputs and insults that impinge on NMD.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Molecular Analyses

HeLa cells were cultured and transfected with plasmids and siRNAs as described previously (Chan et al., 2007, 2009). All plasmid and siRNA sequences are available upon request. Primary mouse neural stem cells grown as neurospheres were isolated, cultured, and transfected as described previously (Tolias et al., 2007). MEFs were derived from E13.5 *Upf3b*$^{-/y}$ and *Upf3b*$^{+/y}$ mouse embryos from the same mother by standard methods (Wu et al., 2006). MEFs used for UPF1 depletion studies were derived from BL6 mice, as described above, and then immortalized at passage 2 by transfection with pBabeSV40LT (Deng et al., 2009). *Upf3b*$^{-/y}$ gene-trap and wild-type control ES cells (TIGM) were cultured with M15 media and feeder SNL 76/7 STO cells (MMRRC) and differentiated into EBs as described

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in the ATCC protocol. Total cellular RNA was isolated, and qPCR analysis was performed as described previously (Chan et al., 2007, 2009). All primer sequences are available upon request.

Generation of Upf3b$^{-/y}$ Mice and Cells

The *Upf3b* gene-trap ES cell clone B5 (TIGM), which contains the enhancer trap in intron 1, was injected into albino C57BL6/J blastocysts to generate chimeric mice, which were bred with albino C57BL6/J mice for germline transmission. The *Upf3b*$^{-/y}$ mice bred normally and had no overt phenotypic defects and will be described in detail in a later report. Mature T cells were purified from adult *Upf3b*$^{-/y}$ and *Upf3b*$^{+/y}$ mice spleen and lymph nodes by positive selection using CD4 and CD8 microbeads and an autoMACS Separator (Miltenyi Biotec), following the manufacturer's instructions. B cells were purified in the same manner using B220 microbeads (Miltenyi Biotec). Enriched macrophages were generated by seeding the negative fractions on tissue culture dishes for 30 min and harvesting the attached cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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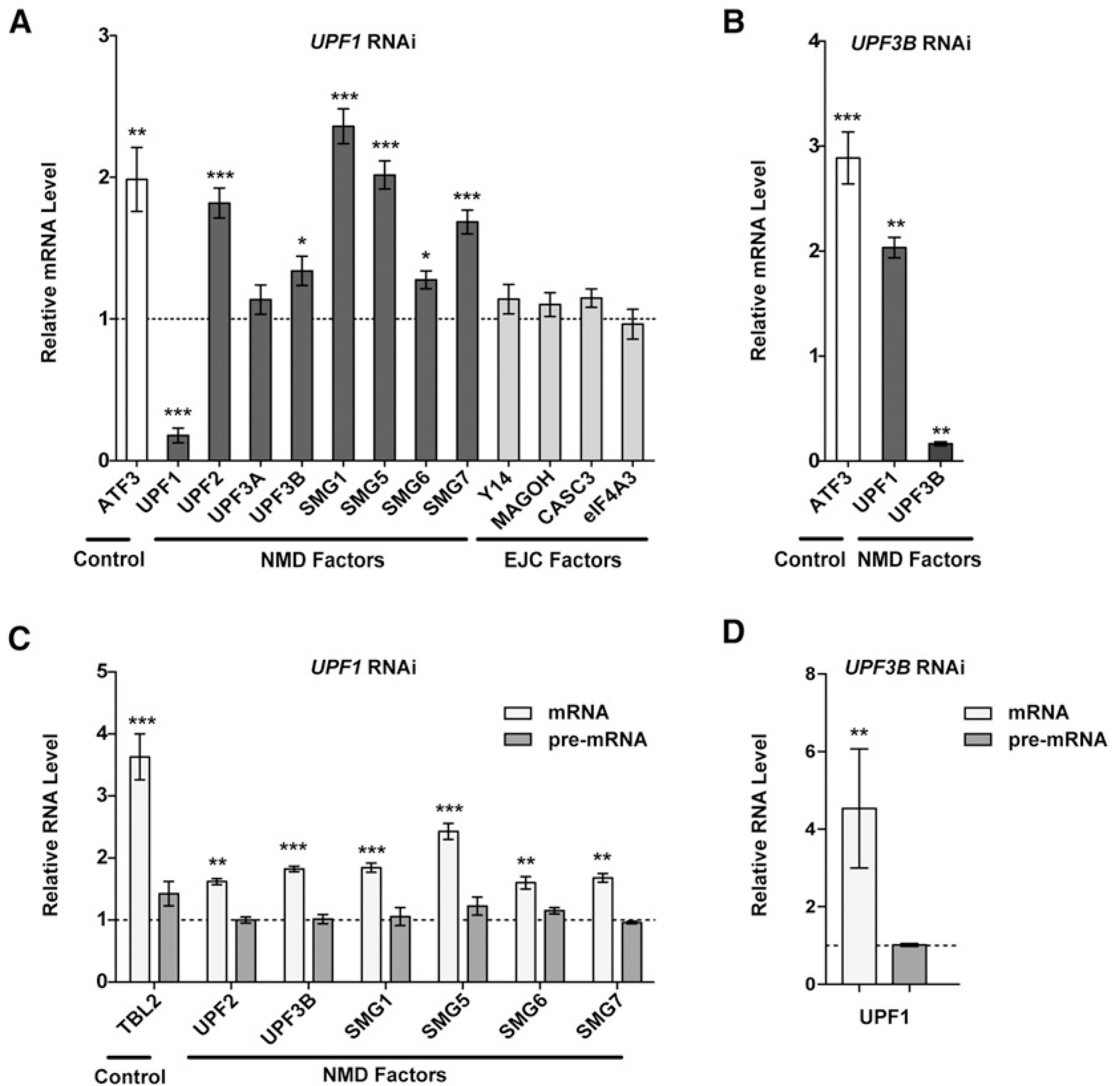

Figure 1. NMD Factor mRNAs Are Negatively Regulated by NMD

(A and B) Quantitative polymerase-chain reaction (qPCR) analysis of total cellular RNA from HeLa cells depleted of UPF1 (A) or UPF3B (B) using RNAi. The values shown are the average fold change (mean ± SEM) from three independent experiments relative to cells transfected with a negative-control siRNA (against luciferase [LUC]; a value of 1 indicates no change). Values were normalized against the housekeeping *GAPDH* and β-*ACTIN* transcripts. Statistical analysis was performed using the Student's t test. *p < 0.05; **p < 0.01; ***p < 0.001.

(C and D)) qPCR analysis of pre-mRNA and mature mRNA levels in HeLa cells depleted of UPF1 (C) or UPF3B (D). The results are from three independent experiments performed separately from those in (A) and (B). Data were quantified and statistically analyzed as in (A).

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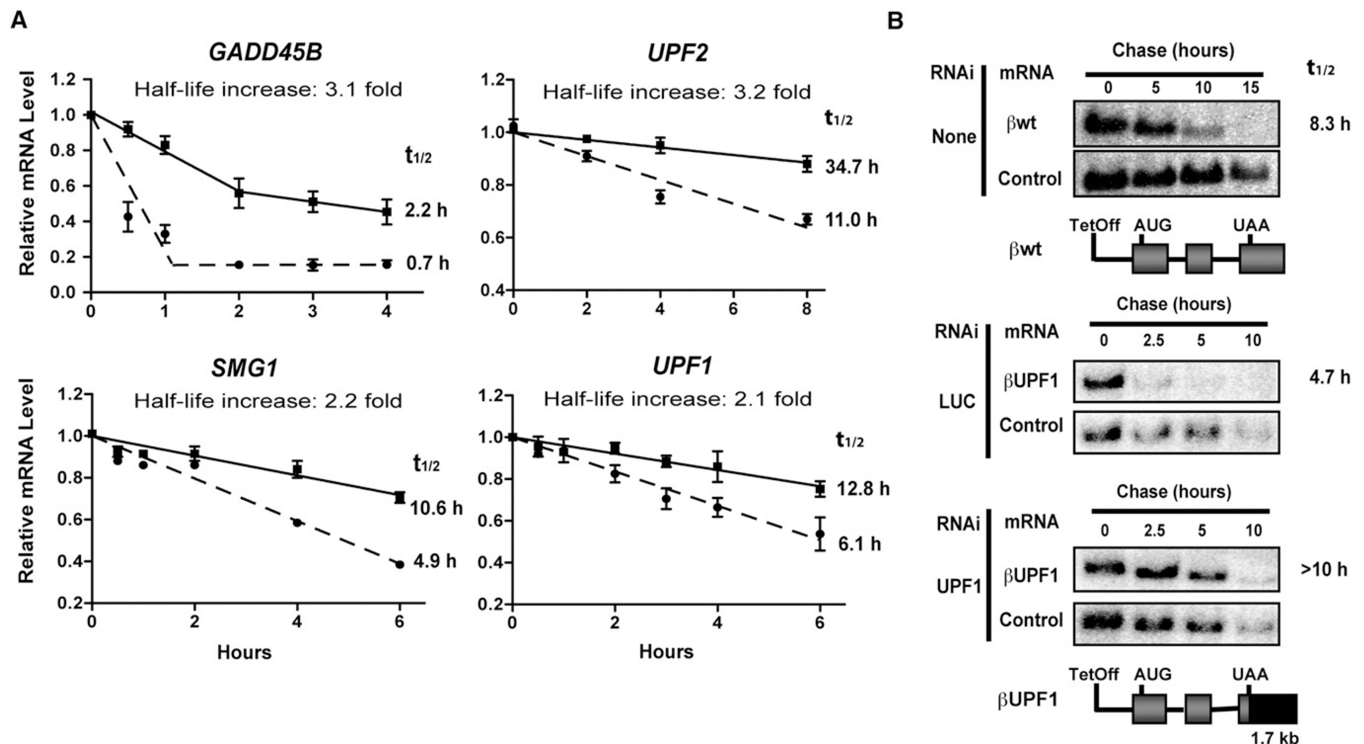

Figure 2. NMD Factor mRNAs Are Targeted for Decay by NMD

(A) qPCR analysis of total cellular RNA from HeLa cells incubated with actinomycin D (5 μg/ml) for the times shown. Cells were transfected with siRNAs and cultured for 40 hr prior to actinomycin D treatment. (Dashed line) Luciferase siRNA-treated cells. (Solid line) *UPF1* siRNA and *SMG1* siRNA-treated cells (the latter only for analysis of *UPF1* mRNA half-life). Data were quantified and statistically analyzed as in Figure 1A, and mRNA half-life was calculated by linear regression analysis.

(B) Northern blot analysis of total cellular RNA from HeLa Tet-off cells transiently transfected with Tet-regulated β-*globin* (β*wt*) (Singh et al., 2008a) and β*UPF1* reporter constructs, the latter of which was made by replacing the β-*globin* 3′UTR in β*wt* with the full-length human *UPF1* 3′UTR (1.7 kb; generated by RT-PCR from HeLa cell total RNA). The internal control is β-*globin* mRNA from a constitutively expressed construct (Singh et al., 2008a). mRNA half-life was calculated by linear regression analysis from two experiments. Data were quantified and statistically analyzed as in Figure 1A.

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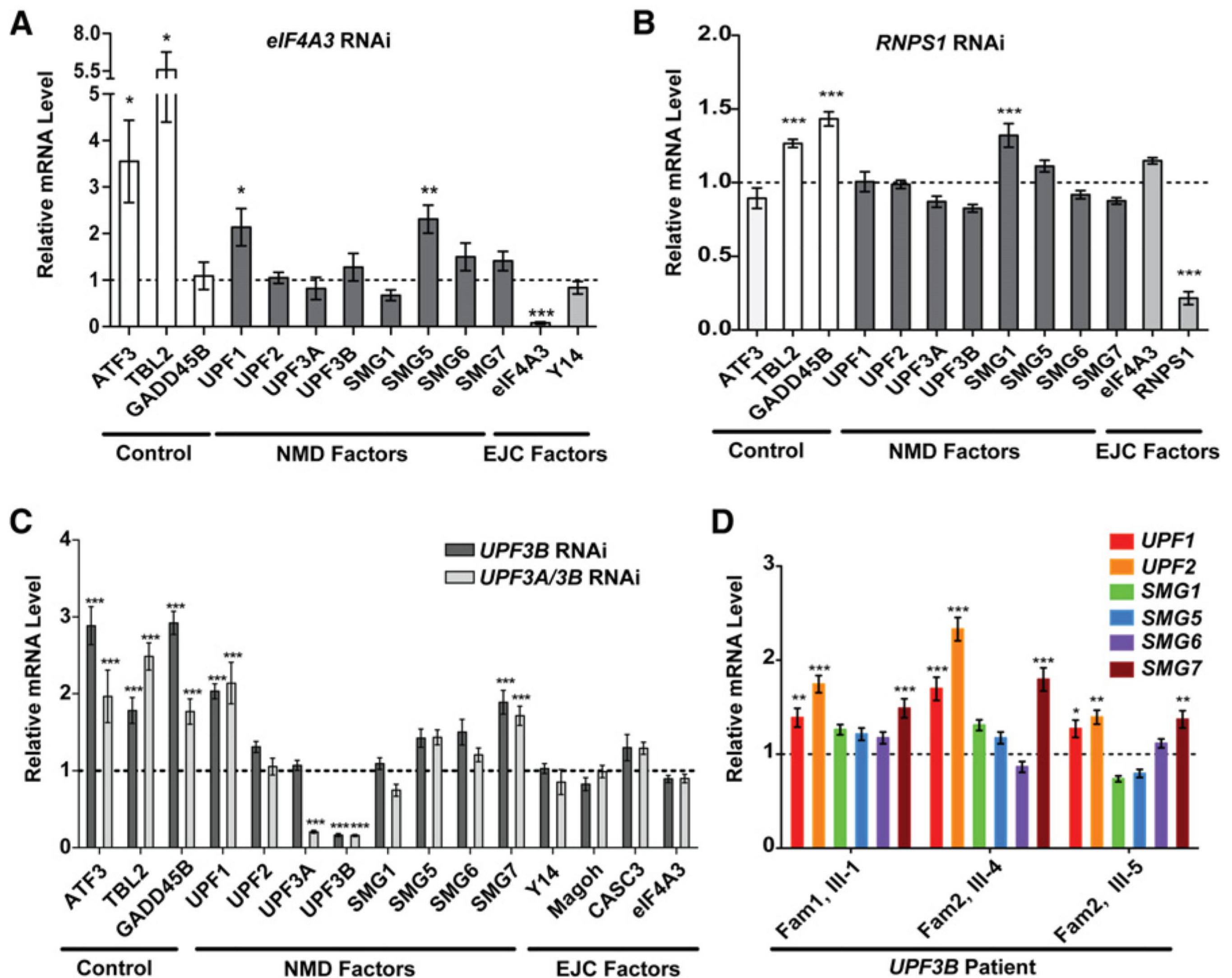

Figure 3. Different Branches of the NMD Pathway Participate in Feedback Regulation

(A–C) Level of NMD factor and NMD substrate transcripts in HeLa cells transfected with siRNAs against the indicated molecules versus luciferase (a value of 1 indicates no difference), as assayed by qPCR analysis. Data were quantified and statistically analyzed as in Figure 1A.

(D) Level of NMD factor transcripts in LCLs from individuals carrying *UPF3B* mutations (e.g., Fam1, III-1 is family 1, 3rd generation, patient 1) versus control individuals (a value of 1 indicates no difference). Data were quantified in triplicate by qPCR and statistically analyzed as in Figure 1A.

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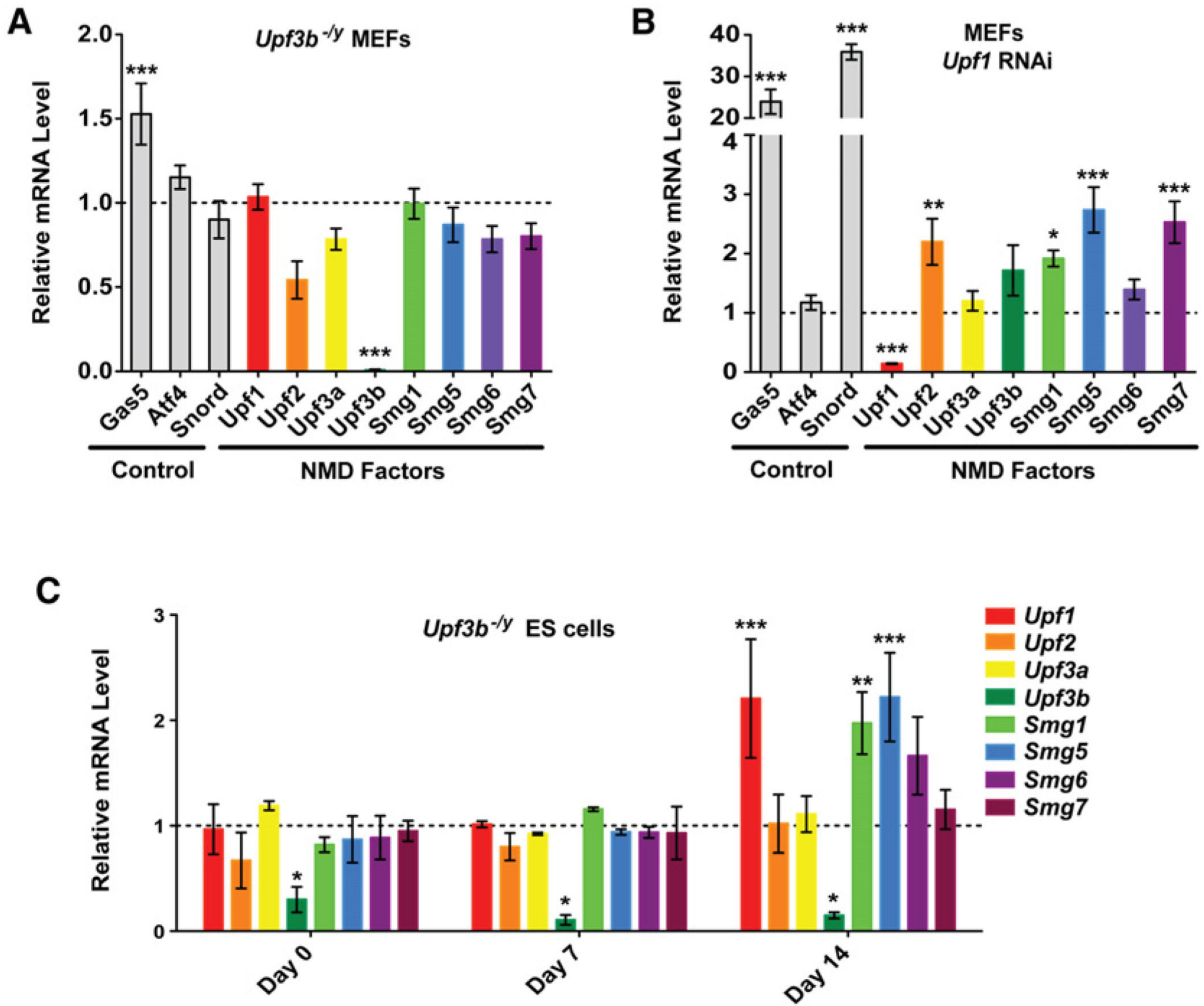

Figure 4. The UPF3B-dependent Feedback Pathway Is Cell Type Specific and Developmentally Regulated

All panels show the level of NMD factor and NMD substrate transcripts in the experimental sample versus the control (a value of 1 indicates no difference), as assayed by qPCR analysis, and are quantified and statistically analyzed as in Figure 1A using the housekeeping transcripts *Rpl19* and β-*Actin* for normalization.

(A) MEFs from *Upf3b*$^{-/y}$ (mutant) and *Upf3b*$^{+/y}$ (control) embryos (n = 3).

(B) *Upf3b*$^{+/y}$ MEFs transfected with *Upf1* siRNA versus luciferase siRNA (control); average values from three independent experiments.

(C) *Upf3b*$^{-/y}$ versus control *Upf3b*$^{+/y}$ ES cells, differentiated into EBs for the days shown; average values from two independent experiments.

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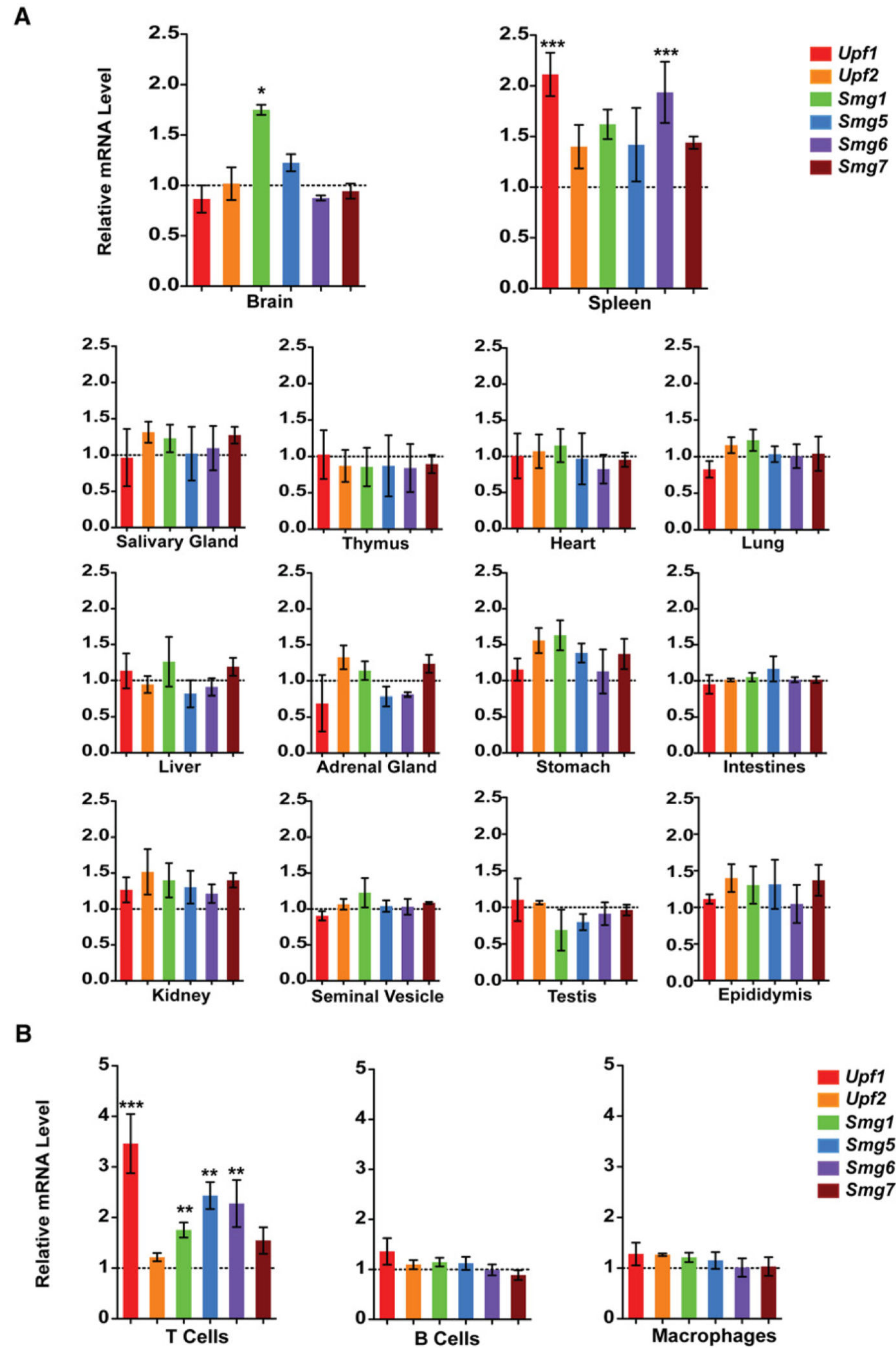

Figure 5. The UPF3B-dependent Feedback Pathway Is Tissue and Cell Type Specific In Vivo

All panels show the level of NMD factor and NMD substrate transcripts in the experimental sample versus the control (a value of 1 indicates no difference), as assayed by qPCR analysis, and are quantified and statistically analyzed as in Figure 1A using the housekeeping transcripts *Rpl19* and β-*Actin* for normalization.

(A) Adult *Upf3b*$^{-/y}$ versus control littermate *Upf3b*$^{+/y}$ mice tissues (n = 3).

(B) Mature hematopoietic cells purified from adult *Upf3b*$^{-/y}$ versus control littermate *Upf3b*$^{+/y}$ mice (n = 3).

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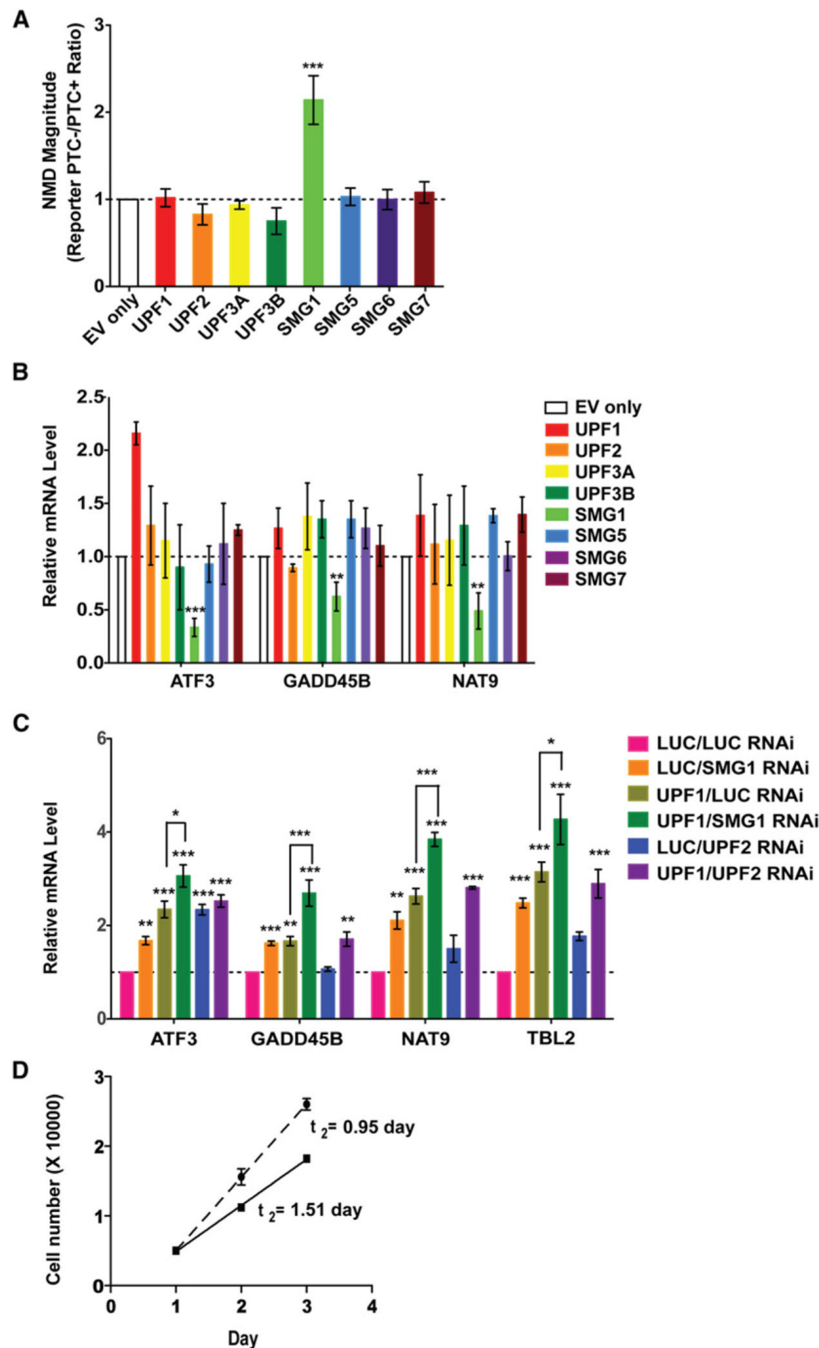

Figure 6. Physiological Role of the NMD Feedback Regulatory Pathway

(A) The magnitude of NMD as measured by luciferase activity (assayed as described previously [Bhardwaj et al., 2008]) in HeLa cells cotransfected with (1) PTC+ and PTC− versions of a Renilla-luciferase NMD reporter (50 ng) (Boelz et al., 2006), (2) the indicated expression vectors (EV is empty vector), and (3) a firefly luciferase construct as an internal control (50 ng). The ratio of luciferase activity from the PTC− and PTC+ reporters in EV-transfected cells is set as 1. To achieve modest (~2- to 3-fold) overexpression of the NMD factors, the following concentrations of NMD factor expression plasmids were transfected:

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UPF1 (2 ng), UPF2 (5 ng), UPF3A (5 ng), UPF3B (2 ng), SMG1 (100 ng), SMG5 (25 ng), SMG6 (25 ng), and SMG7 (25 ng) (this is based on the dose-response experiments shown in Figures S6A and S6B [the amount of EV control transfected for each factor was adjusted accordingly]). Data were quantified and statistically analyzed as in Figure 1A.

(B) qPCR analysis of total cellular RNA from HeLa cells transfected as in (A), quantified and statistically analyzed as in Figure 1A.

(C) qPCR analysis of total cellular RNA from HeLa cells transfected with UPF1 or luciferase (Luc) siRNAs, incubated for 24 hr, transfected with a dose of SMG1 or UPF2 siRNA sufficient to downregulate SMG1 or UPF2, respectively, to the endogenous level before treatment, followed by culture for 24 hr (see Figure S3E). UPF2, which is not rate limiting for NMD in HeLa cells (see A), serves as a negative control. Data were quantified and statistically analyzed as described in Figure 1 A.

(D) Cell counts of HeLa cells transfected with SMG1 expression vector (solid line) or EV (dashed line) (100 ng of vector was chosen to achieve ~3-fold SMG1 over-expression; see A and Figure S6B), calculated from three independent experiments. Cell doubling time was calculated by linear regression analysis. Data were quantified and statistically analyzed as described in Figure 1A.

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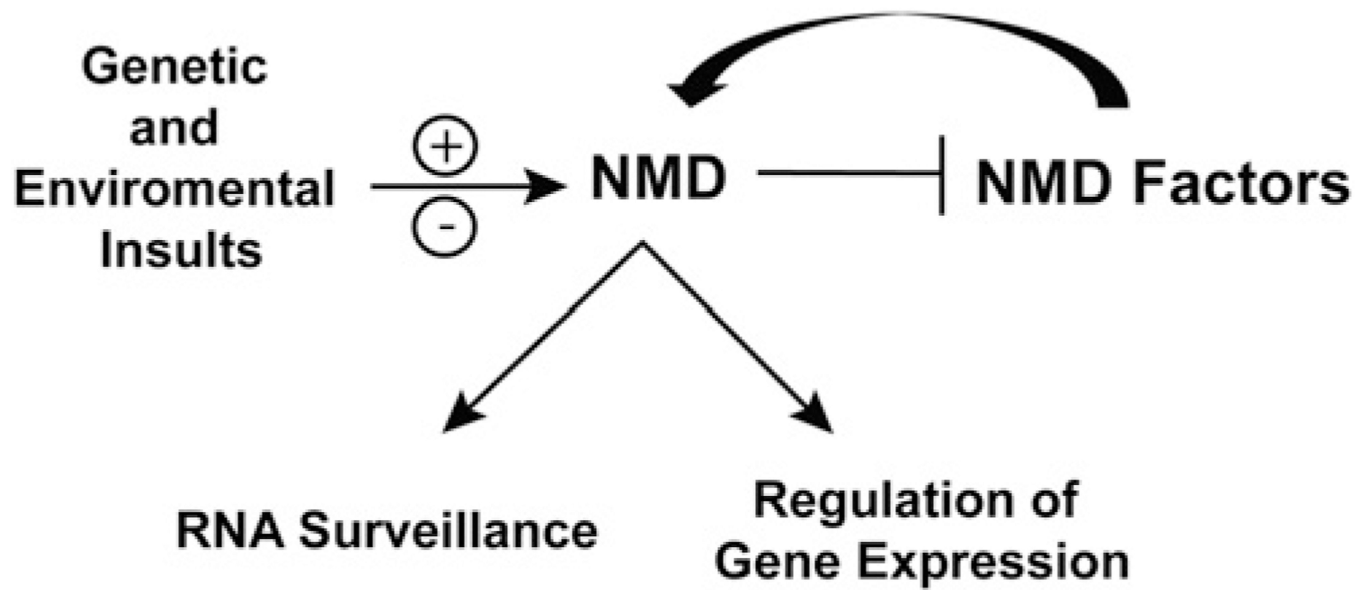

Figure 7. Model

Negative feedback regulatory loops buffer NMD from genetic and environmental perturbations, allowing NMD to maintain its functions in regulating gene expression and RNA surveillance.