Inhibition of Nonsense-Mediated RNA Decay by the Tumor Microenvironment Promotes Tumorigenesis††

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Received 25 May 2011/Accepted 20 June 2011

While nonsense-mediated RNA decay (NMD) is an established mechanism to rapidly degrade select transcripts, the physiological regulation and biological significance of NMD are not well characterized. We previously demonstrated that NMD is inhibited in hypoxic cells. Here we show that the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) translation initiation factor by a variety of cellular stresses leads to the inhibition of NMD and that eIF2α phosphorylation and NMD inhibition occur in tumors. To explore the significance of this NMD regulation, we used an unbiased approach to identify approximately 750 NMD-targeted mRNAs and found that these mRNAs are overrepresented in stress response and tumor-promoting pathways. Consistent with these findings, the inhibition of NMD promotes cellular resistance to endoplasmic reticulum stress and encourages tumor formation. The transcriptional and translational regulations of gene expression by the microenvironment are established mechanisms by which tumor cells adapt to stress. These data indicate that NMD inhibition by the tumor microenvironment is also an important mechanism to dynamically regulate genes critical for the response to cellular stress and tumorigenesis.

During tumorigenesis, a disorganized vasculature leads to amino acid and glucose deprivation, cellular hypoxia, the accumulation of reactive oxygen species (ROS), and various other stresses (5, 12). Cellular adaptation to the hostile tumor microenvironment requires the regulation of stress-induced genes (reviewed in reference 16). For example, the transcription factor ATF-4, upregulated in human tumors due to the stress-induced phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α), transactivates genes involved in amino acid metabolism, angiogenesis, and ROS attenuation (2, 3, 33). Cells that cannot phosphorylate eIF2α or that are deficient in ATF-4 and other stress-induced transcription factors do not form tumors in vivo (2, 13, 40), and therefore, a major goal in cancer biology has been to better understand and potentially target these adaptive mechanisms. However, while the translational and transcriptional responses that promote adaptation to the tumor microenvironment are well established, the role of mRNA stabilization in the cellular stress response has not been as thoroughly studied.

Nonsense-mediated RNA decay (NMD) degrades up to 30% of all mutated protein-coding mRNAs, including those responsible for many genetic disorders, such as thalassemia, cystic fibrosis, and muscular dystrophy (11). During the processing of mammalian pre-mRNA, introns are excised and marked by an exon junction complex, which contains core NMD components. Newly synthesized mRNAs are thought to undergo a pioneering round of translation by a complex that includes eIF2α (6). When this translation complex pauses at a premature termination codon (PTC) upstream of an exon junction complex, the RNA helicase UPF1/Rent1, an essential component of the NMD process, is recruited and then targets the transcript for degradation (reviewed in reference 15). The chemical inhibition or molecular suppression of NMD in a variety of cell lines has surprisingly revealed that a variety of nonmutated transcripts are also upregulated with the suppression of NMD, including genes involved in the cell cycle, differentiation, signaling, and RNA splicing (14, 28, 31).

Transcripts involved in stress response and nutrient homeostasis pathways have also been validated as bona fide direct NMD targets, and intriguingly, NMD is inhibited when cells are either deprived of amino acids or rendered hypoxic (14, 28). We therefore hypothesized that the inhibition of NMD may serve as an adaptive response to cellular stress. In this study we investigated whether NMD inhibition is a general response to microenvironment stress, whether NMD inhibition plays an important role in the adaptive response and survival of cells to these stresses, and whether the inhibition of NMD by the tumor microenvironment contributes to tumorigenesis.

MATERIALS AND METHODS

Cell lines and treatments. Cell lines and growth conditions were previously described (14, 29). β-Globin mRNA-expressing cells and Upf1/Rent1-depleted...
cells were generated by infecting cells with the lentiviruses described below, except for mouse embryonic fibroblasts (MEFs), which were transiently infected. Cells were rendered hypoxic and treated with chemicals as previously reported, or with 500 μM sodium arsenite or 2 nM AP20187, for 3 h prior to the assessment of RNA stability (14, 29).

Plasmids and virus production and infection. The human Upf1/Rent1 short hairpin RNA (shRNA) and overexpression plasmids were previously described (14). Mouse Upf1/Rent1 and Upf2/Rent2 shRNAs were obtained from Sigma (GenBank accession numbers NM_030680.1:41761c1 and NM_00108132.1-40182c1, respectively). Lentiviruses were constructed to express β-globin genomic DNA sequences (14) in a 3′- to 5′-orientation so that introns were not removed during viral processing, and genomic DNA globin sequences were integrated into the host genome (data not shown). Retroviruses were generated in 293T cells, and target cells were infected and selected as previously described (14). Vaccinia virus (VV) (Western Reserve strain) and E3L-deficient VV (VV2E3L) were amplified in BHK21 cells, collected, and sonicated, and titers were determined.

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Immunoblotting and RNA analysis. For the assessment of RNA stability (i.e., RNA expression in the absence of new RNA synthesis), RNA was collected after the initiation of the specific treatment (e.g., tunicamycin) for the time indicated, and cell RNA (1 μg/ml 5,6-dichlorobenzimidazole-1-D-ribofuranoside (DRB) was the added to suppress transcription. RNA was then serially collected at the indicated times, and gene expression was determined on cDNA by quantitative PCR. All time points are referenced to a normalized time zero. Typically, the starting expression of the wild-type β-globin mRNA was ~30 times that of the PTC 39 β-globin mRNA. RNA isolation, cDNA generation, real-time PCR, PCR (for quantitative PCR in the absence of reverse transcription (RT). Primer sequences not reported, or with 500 μM sodium arsenite or 2 nM AP20187, for 3 h prior to the addition of DBR and collection of RNA at time zero and at 1, 3, 5, and 4.5 h. The labeled target cRNA population was generated with the Affymetrix 3′-IVT Express kit and hybridized to HG-U133 Plus 2.0 Affymetrix GeneChips by using standard techniques recommended by the manufacturer. The raw data (.cel files) were normalized for probe level summarization by the robust multichip average (RMA) baseline normalized to time zero under each treatment condition and analyzed by using a combined approach of (i) fitting the normalized intensities of each probe set to an exponential decay curve and comparing the intensities under each condition and (ii) Pavlidis template matching (PTM) (at a P value of <0.05) to determine patterns of mRNA level stabilization under individual conditions and thresholding for a minimum change in the mRNA level of 67% at 3 of 4 time points in the control population. For these steps, we used Agilent Genepix GX11 software and TIGR Multichip Experiment Viewer of the TM-4 suite (34).

To identify potential NMD features in transcripts, transcripts from hgi8 were used to determine the length of the 3′ untranslated region (3′UTR) together with the genomic positions of the exon-exon boundaries and the genomic positions of the stop codons. For each group of transcripts belonging to the same gene, we computed the average 3′UTR length and the average distance between the stop codon and the nearest 3′ exon-exon junction (EEJ). A two-dimensional odds ratio map was generated by smoothing the average 3′UTR length and average distance between the stop codon and the 3′ exon-exon junction by using a Gaussian kernel with widths equal to 1/10 of the range of values in each dimension independently. The log odds ratio was then calculated as the point-wise log ratios between the two-dimensional smoothed maps, and a bootstrapping procedure was employed to assess the expected variance of the ratios.

For comparisons of NMD targets in normal and neoplastic tissues, expression levels of NMD targets were collected from a compendium of samples of both normal and tumor human tissues (breast, liver, lung, and esophagus) (Gene Expression Omnibus [GEO] accession number GSE5364 [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5364]). Each gene profile was mean centered across the compendium.

**RESULTS**

eIF2α phosphorylation regulates NMD. Because NMD is inhibited when cells are deprived of amino acids or rendered hypoxic, two cellular stresses known to result in eIF2α phosphorylation, and eIF2α phosphorylation by the endoplasmic reticulum (ER)-residing kinase PERK is necessary for the hypoxic inhibition of NMD (14, 28), we determined whether eIF2α phosphorylation is a general regulator of NMD. The exposure of mouse embryonic fibroblasts (MEFs) to sodium arsenite (which activates cytoplasmic eIF2α kinases) and Chinese hamster ovary (CHO) cells to the glycosylation inhibitor tunicamycin (a potent inducer of ER stress and an activator of PERK) led to eIF2α phosphorylation and the upregulation of ATF-4 and its targets ATF-3 and CHOP, as expected (Fig. 1A). We then measured the NMD activity in these treated cells by inhibiting transcription with DRB and using quantitative PCR to serially assess the expression of a mutated β-globin mRNA (PTC 39) degraded by NMD and a wild-type control β-globin mRNA. Both sodium arsenite and tunicamycin led to the stabilization of the PTC 39 β-globin mRNA without a significant effect on the wild-type β-globin mRNA stability, indicating the inhibition of NMD (Fig. 1B).

The MEFs utilized were deficient in endogenous PERK, as demonstrated by their lack of response to tunicamycin (Fig. 1A), supporting the conclusion that the ability of eIF2α kinases to inhibit NMD is not unique to ER stress and/or the activation of PERK. However, to rigorously uncouple eIF2α phosphorylation from ER stress, both these cell lines were engineered to express a cytoplasmic PERK construct that is dimerized and activated in the presence of the chemical ligand AP20187 (25). AP20187 treatment of both MEF and CHO cells led to a time-dependent phosphorylation of eIF2α and an upregulation of the ATF-4 target CHOP (Fig. 1C) in the absence of XBP-1.

**Microarray data accession number.** All microarray data were deposited to the NCBI GEO database under accession number GSE30499.
mRNA splicing, a surrogate marker of ER stress (Fig. 1D). This AP20187-induced phosphorylation of eIF2α in the absence of ER stress was accompanied by a stabilization of PTC 39 β-globin mRNA, but not wild-type β-globin mRNA, in MEF and CHO cells (Fig. 1B). Conversely, ER stress in the absence of eIF2α phosphorylation in tunicamycin-treated PERK-deficient MEFs (Fig. 1D and E) did not lead to a stabilization of the PTC 39 β-globin mRNA, in contrast to what was observed for wild-type MEFs (Fig. 1F), demonstrating that ER stress in the absence of PERK activation and eIF2α phosphorylation does not inhibit NMD.

We next assessed whether the activation of the eIF2α kinase PKR by double-stranded RNA, a component of the cellular defense mechanism against viral infections, affects NMD activity in MEFs (8). Vaccinia virus expresses the E3L protein, which attenuates PKR activation. We confirmed that the infection of MEFs with E3L-deficient vaccinia virus led to eIF2α phosphorylation (Fig. 1G). Consistent with this observation,
PTC β-globin mRNA was stabilized upon infection with E3L-deficient vaccinia virus and not upon infection with wild-type vaccinia virus, while wild-type β-globin mRNA stability was not significantly altered after infection with either virus (Fig. 1H).

Finally, we examined the stability of a number of endogenous NMD targets in HeLa cells under two conditions: eIF2α kinase activation with concomitant ER stress (tunicamycin and thapsigargin) and eIF2α kinase activation without ER stress (sodium arsenite) (Fig. 1I). The stress response effectors ATF-4, ATF-3, and CHOP are validated NMD targets (14, 28), and tunicamycin, thapsigargin, and sodium arsenite stabilized these and other NMD-targeted mRNAs without an effect on the stability of ORCL, a control transcript whose stability is not regulated by NMD (28) (Fig. 1J and see Table S1 in the supplemental material).

Taken together, these data indicate that eIF2α phosphorylation by diverse kinases activated by cellular hypoxia, amino acid and glucose deprivation, ROS generation, and other cellular stresses plays a central role in inhibiting NMD even in the absence of ER stress. The current mechanistic model of NMD requires that Upf1/Rent1 interact with the exon junction complex during a pioneering round of translation (6, 21). The phosphorylation of eIF2α diminishes global protein translation. Thus, the phosphorylation of eIF2α could theoretically suppress NMD activity by inhibiting the translation of NMD-targeted transcripts. However, the pulse-labeling of cells with [35S]methionine revealed that the treatments that we used to phosphorylate eIF2α and inhibit NMD attenuated global protein synthesis by only ~20 to 45% (see Fig. S1A in the supplemental material). This modest effect on protein translation contrasts with the effects of chemical inhibitors of protein translation, e.g., cycloheximide, and is similar to effects seen previously by other investigators (9, 38). In addition, several NMD-targeted transcripts increased their association with polysomes upon treatments that inhibit NMD, also consistent with previous reports (18) (Fig. S1B and S1C). A polysome association does not necessarily indicate robust translation, as ribosomes could theoretically be stalled and not actively translating mRNA, and the pioneering round of translation is not adequately assessed by this technique. However, both of these possibilities are less likely with the observation that several NMD targets, including ATF-4, ATF-3, and CHOP, are robustly translated despite cellular stress and eIF2α phosphorylation (Fig. 1A and C) (3, 14). Thus, although we do not yet know the detailed mechanism by which eIF2α phosphorylation inhibits NMD, it appears unlikely to be due to an inhibition of NMD-targeted transcript translation.

NMD is inhibited in three-dimensional tumors. Because hypoxia, ER stress, amino acid and glucose deprivation, and other cellular stresses that promote eIF2α phosphorylation are common in tumors, we investigated whether NMD is inhibited in tumors. We first examined the phosphorylation status of eIF2α in prostate cancer (PC3) cells grown as tumor explants in immunocompromised mice by using a validated antibody (see Fig. S2 in the supplemental material). There were areas of significant cytoplasmic eIF2α phosphorylation present in PC3 cells grown as tumors (Fig. 2A), consistent with previous studies documenting eIF2α phosphorylation and ATF-4 and CHOP induction in tumors (21, 40). We then generated PC3 cells that express either wild-type or PTC 39 β-globin genomic DNA and examined β-globin mRNA expression levels in cells grown as three-dimensional tumor explants in mice or grown in monolayers under standard tissue culture conditions (Fig. 2B). While wild-type β-globin mRNA expression levels were diminished in cells grown as three-dimensional tumors compared to cells grown in monolayers, the level of expression of the PTC 39 β-globin mRNA was significantly increased (approximately 2.5-fold) in tumors (Fig. 2B). Both β-globin constructs have identical promoters, and neither the β-globin DNA content (a measure of the β-globin copy number) nor β-globin pre-mRNA expression levels (a reflection of globin transcription) were increased when cells were grown as tumors as opposed to monolayers (Fig. 2C), indicating that the steady-state expression level of the PTC 39 β-globin mRNA is increased in three-dimensional tumors because of increased mRNA stability.

The mechanisms regulating steady-state endogenous gene expression in tumors are complex, as the tumor microenvironment may augment or repress the transcription of specific genes, in addition to altering their mRNA stabilities. For example, we previously showed that while the steady-state expression levels of the NMD-targeted ATF-4 transcript are similar in normoxic and hypoxic cells, the expression level of the ATF-4 transcript is diminished in hypoxic cells where the hypoxic inhibition of NMD is lacking (14). Despite this caveat, we identified several NMD-targeted mRNAs (transcripts upregulated with the depletion of Upf1/Rent1 and downregulated with the overexpression of Upf1/Rent1 [14, 28]) whose steady-state expression levels were increased when PC3 cells were grown as tumors compared to when these cells were grown as monolayers. Many of these increases in mRNA expression levels were associated with no evidence of transcriptional activation (as assessed by pre-mRNA levels) (Fig. 2D). Together, these data suggest that NMD can be inhibited by the tumor microenvironment.

NMD targets include a wide variety of transcripts, including those important for tumorigenesis. With the appreciation that NMD is a physiologically regulated process, we reasoned that the identification of additional transcripts stabilized by the inhibition of NMD might suggest biological functions impacted by NMD regulation. To identify additional bona fide NMD targets, we assessed global mRNA stability by inhibiting RNA synthesis with DRB and serially measuring mRNA expression levels with expression arrays containing 22,000 probe sets representing over 18,000 genes. mRNA stability was assessed in control cells and in cells in which NMD was inhibited chemically (with emetine, genetically (with Upf1/Rent1 depletion), or from ER stress (in cells rendered hypoxic or treated with tunicamycin). Because NMD is thought to rapidly degrade transcripts, we assessed stability during relatively short time points over 4.5 h to minimize the number of nondirect targets identified.

Over the time course of the experiment, 77% of the control probes changed less than 1.5-fold from the baseline. Thus, most transcripts are stable during this short time period. We identified transcripts whose expression levels decreased by >50% at each successive time point in control cells and which remained stable in our experimental sets, with a P value of 0.005. Using this algorithm, 17% of all probes were stabilized in emetine-treated cells, 9% were stabilized in cells depleted of Upf1/Rent1, 12% were stabilized in hypoxic cells, and 12% were
stabilized in tunicamycin-treated cells. Of the 4,852 probes stabilized with the Upf1/Rent1 knockdown, 71% were also stabilized with emetine treatment; an additional 5,829 probes were stabilized with emetine but not with the Upf1/Rent1 knockdown (Fig. 3A). This finding suggests that the inhibition of translation is a more efficient inhibitor of NMD than the 80% depletion of Upf1/Rent1 that we achieved (see Fig. S3 in the supplemental material) and/or that the inhibition of translation also leads to the stabilization of transcripts independently from NMD. There was a 50% overlap of the 6,465 probes that were stabilized in hypoxic cells and in tunicamycin-treated cells (Fig. 3B), suggesting a potential common mechanism for their stabilization.

To identify transcripts stabilized in hypoxic and tunicamycin-treated cells that were also NMD targets, we first examined the overlap between probes stabilized under both these conditions and also stabilized in emetine-treated cells and found 2,749 such transcripts (Fig. 3C). We then imposed the most stringent criteria and filtered these transcripts to identify those that were also stabilized with the Upf1/Rent1 knockdown and for which the levels decreased by at least 67% in control cells, and we identified 794 probes representing 749 annotated genes (see Table S2 in the supplemental material). Visualization of this set of transcripts either individually (Fig. 3D, top), as centroid-based stability (Fig. 3D, middle), or as a heat map (Fig. 3D, bottom) demonstrated that emetine inhibits NMD most effectively, followed by tunicamycin treatment. Real-time PCR validation validated the sensitivity of the array approach, in that 19 of 21 transcripts determined to be stabilized by the array were also stabilized by real-time PCR, and 3 of 5 transcripts determined not to be stabilized were not stabilized by real-time PCR (Fig. 3E and data not shown).

We next explored the structure and function of these NMD-targeted transcripts more closely. While it is unclear why many nonmutated transcripts are NMD targets, potential features include upstream open reading frames, introns in 3′ untranslated regions, and alternative splicing, which lead to a PTC upstream of an exon–exon junction complex and/or increase the distance from a stop codon to the 3′-bound poly(A)-binding protein (14, 28, 35, 37). We found that 45% of the transcripts identified as being NMD targets were classified as undergoing alternative splicing ($P = 2.4 \times 10^{-5}$). Furthermore, approximately 25% of our identified transcripts fit the criteria of having a PTC greater than 50 bp upstream of an exon-exon junc-

FIG. 2. NMD is inhibited in three-dimensional tumors. (A) PC3 cells grown as explants in mice were assessed for eIF2α phosphorylation. (B) Wild-type and PTC 39 β-globin mRNA expression levels were determined in PC3 cells grown as monolayers (n = 3 for wild-type β-globin and n = 3 for PTC 39 β-globin) or as three-dimensional tumors (n = 3 for wild-type β-globin and n = 3 for PTC 39 β-globin). Average expression levels ± SE are displayed. (C) Globin DNA expression levels and pre-mRNA expression levels were assessed, as described in Materials and Methods, in PC3 cells grown as monolayers and three-dimensional tumors. (D) Average expression levels ± SE (n = 3 for each tumor and each cell type). The mRNA and pre-mRNA expression levels of NMD-targeted transcripts (28) were determined in PC3 cells grown as monolayers and three-dimensional tumors. The expression levels of pre-mRNAs and mRNAs in tumors are displayed relative to the expression levels of pre-mRNAs and mRNAs in monolayer cells, which we define as 1. Average expression levels ± SE (n = 6 for tumors and n = 6 for cells) are displayed. Significance, determined by the Student t test, between tumor and monolayer conditions is indicated for each transcript (*, $P < 0.05$; #, $P > 0.2$).
tion (28). In addition, NMD targets (Fig. 4A, red histogram, horizontal axis) exhibit greater distances between an upstream stop codon and the closest EEJ than do non-NMD targets (green histogram, horizontal axis). There was also a trend for longer 3\'-UTRs in the NMD-targets group (Fig. 4A, red histogram, vertical axes), and these two characteristics were correlated (as shown by the red region in the top right of Fig. 4A).

Of the 749 annotated genes, 694 were recognized by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (19). Many functional categories involved in the promotion of tumorigenesis were significantly overrepresented in these 694 genes. In fact, 45% of the transcripts were involved in metabolic processes resulting in cell growth ($P < 3.4 	imes 10^{-6}$). Cellular pathways significantly ($P < 0.05$) overrepresented by these NMD targets include transcription, the cell cycle, cell growth, apoptosis, growth factor signaling, and cell migration (Fig. 4B and see Table S3 in the supplemental material). Other enriched categories are in agreement with previously known NMD functions, including RNA splicing and hematopoiesis (Fig. 4B) (30, 37), as were genes involved in the...
response to double-stranded RNA, suggesting that the inhibition of NMD by the PKR-induced phosphorylation of eIF2α may play a role in the cellular response to RNA virus infections (Fig. 1G and H).

Finally, we investigated whether these NMD-targeted transcripts are enriched in human tumors, with the appreciation that mRNA regulation in tumors is a complex process. Similar to our previous observations of prostate cancer cells grown as explants, and consistent with recent reports (2, 40), eIF2α phosphorylation was present in several human tumors, including breast, prostate, and melanoma (Fig. 4C). We then utilized a database that contains transcript expression levels in normal (n = 70) and matched tumorigenic (n = 270) breast, colon, liver, lung, thyroid, and esophagus tissue to determine the relative expression levels of the NMD targets (41). The database contained 385 probe sets of the 749 probe sets that are degraded by NMD and stabilized by the tumor microenvironment. There was a significant enrichment (P < 10^{-4}) of the expression level of these NMD targets in tumors compared to control tissues (Fig. 4D), whereas there was no significant differences in the expression levels of non-NMD targets between tumors and control tissues (P = 0.71), consistent with our hypothesis that NMD is inhibited in human tumors.

Inhibition of NMD promotes cell survival in response to ER stress. With the appreciation that NMD can dynamically regulate many transcripts, we next pursued the potential biological consequences of this regulation. Because transcripts critical for the response to ER stress are validated NMD targets, and the depletion of Upf1/Rent1 augments the mRNA and protein expression levels of many of these transcripts in stressed cells (Fig. 1J) (14, 28), we examined the contribution of NMD inhibition to the cellular response to ER stress. We reasoned that if an important component of the stress-induced phosphorylation of eIF2α is to inhibit NMD and stabilize stress-

FIG. 4. NMD-targeted transcripts have unique features and are involved in tumorigenesis. (A) Log odds ratio map of the distributions of two dimensions, the average 3' UTR lengths (horizontal axis), and the average distances between the stop codon and the nearest 3' exon-exon junction (vertical axis), as measured independently for NMD targets (red) and non-NMD targets (green) (P < 10^{-5}). Histograms of the marginal densities have been added for clarity, and a sample of the data is shown as dots on the map for visual aid. (B) Biological pathways in which NMD-targeted transcripts are significantly enriched compared to nonselected transcripts. The percentage of NMD-targeted transcripts in each group is displayed. dsRNA, double-stranded RNA. (C) eIF2α phosphorylation, noted by brown cytoplasmic staining, as assessed in a number of tumors. Control staining is demonstrated in Fig. S2 in the supplemental material. (D) Distribution of NMD target mean expression levels (top) and non-NMD target mean expression levels (bottom) derived from a large compendium of samples. The average expression level of NMD targets in each sample is demonstrated below (red bars, normal samples; black bars, tumor samples).
induced transcripts, then genetically inhibiting NMD should improve the cellular survival against ER stress in cells resistant to eIF2α phosphorylation. The depletion of Upf1/Rent1 in MEFs with knock-in eIF2α alleles which cannot be phosphorylated (eIF2α S51A) (Fig. 5A) led to NMD inhibition, as demonstrated by the stabilization of PTC 39 β-globin mRNA (Fig. 5B). Of note, the PTC 39 β-globin mRNA was not stabilized in tunicamycin-treated eIF2α wild-type cells, in contrast to what was observed for tunicamycin-treated eIF2α wild-type cells, confirming that eIF2α phosphorylation is necessary for the inhibition of NMD by tunicamycin (Fig. 5B).

Cell survival and plating efficiency were not significantly altered by the eIF2α status and/or expression levels of Upf1/Rent1 in unstressed cells. Cells were then exposed to ER stress for short periods, and their ability to survive and form colonies was assessed. As expected, eIF2α S51A cells were much more sensitive to tunicamycin than eIF2α wild-type cells (Fig. 5C, left). The suppression of NMD by Upf1/Rent1 depletion led to a only a mild increase in the survival of eIF2α wild-type cells compared to control cells (Fig. 5C, right). This observation is consistent with the notion that tunicamycin treatment inhibits NMD in eIF2α wild-type cells, and Upf1/Rent1 depletion does not further inhibit NMD and improve the cellular response to stress. However, in tunicamycin-treated eIF2α S51A cells in which NMD was molecularly inhibited by Upf1/Rent1 depletion, significant 2- to 3-fold increases in cell survival and colony growth compared to control eIF2α S51A cells were detected (Fig. 5C, right). Similar results were seen when another protein crucial for NMD, Upf2/Rent2, was depleted in these cells (Fig. 5D). These data indicate that the inhibition of NMD is an important component of the cell survival response mediated by eIF2α phosphorylation.

Inhibition of NMD promotes tumorigenesis. Because the response to ER and other cellular stresses is necessary for tumorigenesis (2, 40) and because NMD stabilizes transcripts involved in many tumor pathways (Fig. 4B), we next examined whether the microenvironmental inhibition of NMD impacts tumor formation. To assess the tumor-forming potential of cells that are refractory to the stress-induced inhibition of NMD, we made use of our observation that the overexpression of Upf1/Rent1 blocks the inhibition of NMD seen with eIF2α phosphorylation (Fig. 5B, compare PTC39 eIF2α wild-type and eIF2α S51A cells (as demonstrated by the immunoblot to the left) treated with tunicamycin for 2 h.

FIG. 5. Manipulation of Upf1/Rent1 regulates NMD and affects survival against ER stress. (A) Wild-type eIF2α and eIF2α S51A MEFs either were depleted of Upf1/Rent1, overexpressed Upf1/Rent1, or expressed a control plasmid (scr). (B) The stabilities of wild-type and PTC 39 β-globin mRNAs were determined for the cells described above (A), with and without tunicamycin (Tm) treatment. β-Globin mRNA stability was assessed in triplicate (average expression levels and standard errors are displayed). (C) eIF2α wild-type and eIF2α S51A cells (left) and the same cells with Upf1/Rent1 depletion (right) were treated with tunicamycin for the indicated time points, at which time tunicamycin was washed and cells were cultured for an additional 7 to 10 days. Colony formation was then assessed in triplicate in three independent experiments. The average values and standard errors of percent surviving cells (compared to no treatment) (left) and percent surviving Upf1/Rent1-depleted cells/percent surviving control cells are displayed. (D) Colony formation was similarly assessed in Upf2/Rent2-depleted eIF2α wild-type and eIF2α S51A cells (as demonstrated by the immunoblot to the left) treated with tunicamycin for 2 h.

We assessed anchorage-independent colony formation in soft agar, a model which leads to hypoxic conditions and should thus
demand a robust stress response (7). Upf1/Rent1-overexpressing PC3 cells formed significantly fewer and smaller colonies in soft agar than did control PC3 cells (Fig. 6D). There was no significant effect of the Upf1/Rent1 knockdown in these cells, suggesting that NMD may be maximally inhibited when PC3 cells grow as three-dimensional cultures. This hypothesis is supported by the finding that the depletion of Upf1/Rent1 did not improve survival against ER stress in eIF2α/H9251 wild-type cells, as NMD was already maximally inhibited by eIF2α/H9251 phosphorylation in these cells. To determine if NMD-targeted transcripts are required for the soft agar growth of other cell lines, we overexpressed Upf1/Rent1 in additional prostate cancer (DU145), colon cancer (HCT116), and melanoma (A375) cell lines (Fig. 6E). Similar to PC3 cells, the overexpression of Upf1/Rent1 in these cells selectively destabilized PTC 39/b-globin mRNA without affecting wild-type b-globin mRNA (Fig. 6F). While both HCT116 and A375 cells formed significantly fewer colonies when overexpressing Upf1/Rent1, there was no difference in the DU145 cell line (Fig. 6G), suggesting that the overexpression of Upf1/Rent1 either did not sufficiently activate NMD and/or that the activation of NMD has no effect in these cells (see Discussion).

We then assessed the role of Upf1/Rent1 manipulation in the growth of PC3 cells as tumor explants in nude mice, where eIF2α is phosphorylated and NMD is inhibited (Fig. 2). Similar to the observations with soft agar, we found robust tumor growth in both control and Upf1/Rent1 knockdown cells but almost no tumor growth under conditions of Upf1/Rent1 overexpression (Fig. 6H). Because there was not an alteration of PC3 growth under standard tissue culture conditions (Fig. 6B and C), together, these data suggest that the inhibition of NMD is important, specifically in three-dimensional tumor growth, where oxygen and nutrients are limited.

**DISCUSSION**

Almost half the alterations in gene expression induced by cellular stresses, including UV radiation and heat shock, are thought to be due to changes in RNA stability (10). It is becoming clearer that several mechanisms exist for altering the RNA stability in stressed cells. Indeed, we observed that unique transcripts are stabilized when cells are rendered hypoxic (e.g., the well-known hypoxia-inducible vascular endothelial growth factor [VEGF] and carbonic anhydrase IX [CA IX] transcripts) or exposed to ER stress (see Table S2 in the supplemental material). This observation confirms that several mechanisms exist for the hypoxic stabilization of transcripts (reviewed in reference 27) and suggests that ER stress also stabilizes transcripts via distinct mechanisms. However, our findings also demonstrate that a common response to a variety of cellular stresses, including hypoxic and ER stress, is the suppression of NMD. Because we show that the activation of a variety of eIF2α kinases leads to the inhibition of NMD and that the activation of these kinases in the absence of eIF2α phosphor-
ylation does not inhibit NMD, our model supports a central role of eIF2α phosphorylation in the inhibition of NMD.

This regulation of NMD has important biological consequences, many of which are dictated by the transcripts targeted by NMD. Previous strategies to identify NMD targets have involved primarily the suppression of NMD either with chemical inhibitors of protein translation or through genetic mechanisms, followed by the identification of upregulated transcripts (20, 28, 37). While these strategies have identified transcripts involved in the ER stress response, most of these transcripts have not been validated as direct NMD targets; i.e., the stability of these transcripts has not been assessed in the absence and presence of NMD. Transcripts upregulated with the suppression of NMD could be, for example, targets of a transcription factor that is stabilized by the suppression of NMD. Using stability as our readout, we identified 749 transcripts that are stabilized under all conditions that inhibit NMD. Of note, most of these mRNAs were not strongly upregulated upon a brief inhibition of NMD (only 29% were also upregulated greater than 1.5-fold after 3 h of emetine treatment, 16% were upregulated with Upf1/Rent1 depletion, 2% were upregulated in hypoxic cells, and 7% were upregulated with tunicamycin treatment) and thus could have been missed with the strategies used by previous studies.

We found that the overexpression of Upf1/Rent1 and blocking of the inhibition of NMD by cellular stress have important roles in suppressing tumorigenesis in several tumor models. Although Upf1/Rent1 is also thought to have non-NMD functions, including roles in Staufen-1-mediated mRNA decay (17; reviewed in reference 23), the observations that (i) Upf1/Rent1 and Upf2/Rent2 depletion had similar effects on gene regulation (28), (ii) we noted a similar augmentation of cellular survival in response to ER stress in Upf1/Rent1- and Upf2/Rent2-depleted cells, (iii) the majority of the genes that we found to be regulated by Upf1/Rent1 depletion are also regulated by the chemical inhibition of NMD, and (iii), the manipulation of Upf1/Rent1 does not lead to alterations in cellular proliferation or translation in unstimced cells all suggest that the effects that we noted for Upf1/Rent1 overexpression are due to NMD activation. NMD plays an established role in degrading transcripts for mutated tumor suppressor genes (reviewed in reference 15), and the rapid degradation of mutant dominant negative transcripts by NMD activation could potentially be responsible for diminishing tumorigenesis. However, it is unlikely that this mechanism is responsible for our observations, as similar effects were observed for three out of four disparate cancer cell lines tested. A more likely possibility for our observations is that NMD targets play crucial roles in promoting tumorigenesis, and the downregulation of a set of these transcripts prevents tumor growth. Because proliferation under nonstressed conditions is normal in NMD-hyperactivated cells, we favor the hypothesis that transcripts involved in the stress response pathways are critical transcripts regulated by NMD during tumor formation. However, many pathways involved in tumorigenesis, including metabolism, growth, and migration, are also potentially impacted by NMD regulation, and further studies will be required to determine if these pathways are also affected by NMD regulation.

The manipulation of Upf1/Rent1 expression did not affect the tumor-forming potential of all cell lines equally. Some tumors may be less responsive to NMD regulation by the overexpression of Upf1/Rent1. Indeed, it was noted previously that distinct cancer cell lines have different baseline NMD activities (24). Alternatively, in some cancers, tumorigenesis may not depend on transcripts regulated by NMD. For example, the activation of NMD could increase tumorigenesis if that cancer harbors a tumor suppressor gene that can be degraded by NMD. However, our data support the concept that taking advantage of the unique properties of the tumor microenvironment may provide an opportunity to combat cancer. For example, the eIF2α kinases PERK and PKR have both been shown to be necessary for tumorogenesis (2, 32), and the development of eIF2α kinase inhibitors has been proposed to be an effective means to blunt the cellular stress response in tumors. Our data suggest that the inhibition of eIF2α phosphorylation would have the additional effect of blocking the inhibition of NMD by the tumor microenvironment. Depending on the spectrum of mutations found in an individual tumor, this may be an effective therapeutic strategy and one that deserves further consideration.

Our findings have implications for conditions in addition to cancer. The cellular response to ER stress, which we show is augmented by NMD inhibition, plays an important role in many pathological conditions, including diabetes and atherosclerosis (26). NMD regulation may also play an important role in normal physiology and several pathological conditions marked by cellular stress. For example, because oxygen concentrations vary among tissues (4), the hypoxic regulation of NMD may play a role in the 2-fold differences in NMD efficiency noted for distinct tissues (42). It is also increasingly appreciated that various tissues, in both normal and cancerous states, express distinct alternatively spliced transcripts (36), some of which could be directly stabilized by NMD inhibition or indirectly stabilized by the regulation of splicing factors by NMD (30). The upregulation of alternatively spliced transcripts could lead to truncated proteins with dominant negative functions; mRNAs with altered reading frames, which generate novel proteins; and/or misfolded proteins, which could provoke a variety of cellular responses. Finally, 30% of all genetic diseases, including thalassemia, cystic fibrosis, and Duchenne muscular dystrophy, are caused by transcripts that are degraded by NMD (reviewed in reference 11). The stabilization of these transcripts by the tissue microenvironment (e.g., hypoxia in thalassemia and cystic fibrosis) could result in the generation of functional or dominant negative truncated proteins. Thus, the role of NMD regulation by the microenvironment in normal physiology and a number of pathological conditions requires further study, particularly since the manipulation of this regulation may offer an opportunity to affect these diseases.

ACKNOWLEDGMENTS

We appreciate the gifts of cell lines (E. Hernando and R. Kaufman) and reagents (S. Rivella, B. Jacobs, J. Lykke-Andersen, H.-M. Jack, and I. Aiffantis). We give particular appreciation to Luis Chiriboga and the NYU Cancer Institute Histology Core; the NYU Cancer Institute Genomics Core; and G. David, B. Dynlacht, and especially S. Tranquillo for review of the manuscript.

This work was supported by the NYU Cancer Institute Genomics, Cell Sorting, and Histology Core (NIH/NCI S P30CA16098-31). D.R. is supported by DK047115 and DK075311, and L.B.G. is supported by
DK08164 and the Saperstein Medical Fellowship. L.B.G. is the Saul J. Farber Assistant Professor of Medicine.

REFERENCES


