Nonsense-Mediated RNA Decay Regulation by Cellular Stress: Implications for Tumorigenesis

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Abstract

Nonsense-mediated RNA decay (NMD) has long been viewed as an important constitutive mechanism to rapidly eliminate mutated mRNAs. More recently, it has been appreciated that NMD also degrades multiple nonmutated transcripts and that NMD can be regulated by wide variety of cellular stresses. Many of the stresses that inhibit NMD, including cellular hypoxia and amino acid deprivation, are experienced in cells exposed to hostile microenvironments, and several NMD-targeted transcripts promote cellular adaptation in response to these environmental stresses. Because adaptation to the microenvironment is crucial in tumorigenesis, and because NMD targets many mutated tumor suppressor gene transcripts, the regulation of NMD may have particularly important implications in cancer. This review briefly outlines the mechanisms by which transcripts are identified and targeted by NMD and reviews the evidence showing that NMD is a regulated process that can dynamically alter gene expression. Although much of the focus in NMD research has been in identifying the proteins that play a role in NMD and identifying NMD-targeted transcripts, recent data about the potential functional significance of NMD regulation, including the stabilization of alternatively spliced mRNA isoforms, the validation of mRNAs as bona fide NMD targets, and the role of NMD in tumorigenesis, are explored.

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Introduction

Cellular stress is a common feature of many physiologic and pathologic conditions including cancer, where tumor growth and an unorganized and faulty vascular system lead to significant hypoxia, amino acid deprivation, and reactive oxygen species (ROS) generation. The cellular response to these stresses includes dynamic alterations of gene expression, which is mediated by a variety of mechanisms. For example, a plethora of research over the last 2 decades has emphasized the importance of transcription factors not only in cancer etiology but also in the adaptive response of cancer cells to their microenvironment. However, the steady-state expression level of a gene is a product of its rate of not only transcription but also mRNA degradation. In fact, just as the complex regulation of a transcription factor is responsible for coordinating the expression of functional sets of genes, several distinct mechanisms of RNA decay are also responsible for degrading groups of transcripts with similar functions.

It is well established that one mechanism of RNA degradation, nonsense-mediated RNA decay (NMD), contributes to the rapid degradation of many mutated mRNAs, including mutated tumor suppressor transcripts (1). More recently, NMD has also been shown to degrade transcripts that participate in the adaptive response of cells to their microenvironment (2, 3). Furthermore, alternatively spliced mRNA isoforms, which are increasingly identified in cancer, may also be regulated by NMD (4). These functions take on further biological significance with the recent observations that NMD activity is inhibited by distinct forms of cellular stress that commonly occur in the tumor microenvironment (2, 3). Here, we review the regulation of NMD by the tissue microenvironment, focusing on cancer as a model.

NMD Is Carried Out by Several Multiprotein Complexes

NMD is an efficient mRNA surveillance process that selectively eliminates aberrant transcripts that contain premature termination codons (PTC). The identification and degradation of NMD transcripts are mediated by several multiprotein complexes. Over the last decade, these complexes have been at least partially characterized, and many individual proteins in these complexes have been validated as playing important roles in NMD. NMD is an evolutionarily conserved process and, for the most part, components of the NMD complex in mammalian cells have been identified by their homology to those in lower organisms and/or...
by their physical interaction to previously identified members of the NMD complex. Many of these proteins have been validated by knockdown, overexpression, and knock-out mouse model experiments. Experiments have also been carried out in which proteins are tethered to mRNA species to determine whether these proteins are sufficient to elicit NMD (5). These many experiments will not be detailed, as they are discussed extensively in several excellent recent reviews (6-10). Instead, the current prevailing model of NMD in mammalian cells will be briefly reviewed (Fig. 1), although it should be noted that this model is undoubtedly simplistic and additional/alternative models exist. It should also be noted that many of the proteins that play important roles in NMD, including those that are not extensively detailed in this review, also have seemingly independent roles in other diverse pathways, including DNA replication, and the protection against genomic instability (as reviewed in ref. 9).

During the processing of mammalian pre-mRNA, introns are excised and marked by a multiprotein complex termed the exon junction complex (EJC; Fig. 1, step 2). This EJC contains at least 10 proteins that are deposited 20 to 30 nucleotides upstream of the exon-exon junction and include the core NMD components UPF2/Rnt2 and UPF3 (11-14). Newly synthesized mRNAs are also capped at the 5′ terminus by the cap-binding complex (CBC). Later, the CBC will be replaced by eukaryotic initiation factor 4E (eIF4E), at which time mammalian transcripts seem to become immune to NMD (15-17), although some of the data supporting this model are indirect (as reviewed in ref. 10). mRNAs containing the CBC are thought to be translated through a pioneering round of translation, shortly after nuclear export, by a translation complex including eIF4G, eIF3, and eIF2, which is commonly overexpressed in some cancers and regulates protein translation in hypoxic and metabolically starved tumors, does not affect this pioneer round of translation or NMD (18, 20, 21).

The positional information provided by the EJC persists during export until the mRNA is translated; at least some components of the EJC are displaced by ribosomes during the pioneering round of translation (ref. 22 and reviewed in refs. 23-25). When the translation complex pauses at a PTC that is upstream of an EJC, eukaryotic release factors (eRF) physically bind to and recruit the RNA helicase UPF1/Rnt1, a vital component of the NMD mechanism (12, 26-28). Subsequently, the phosphatidylinositol 3-kinase SMG-1 is recruited to complete the formation of the SMG-1, UPF1, eRF (SURF) complex (29). This SURF complex then binds to the UPF proteins in the EJC (Fig. 1, step 3), thus bringing these two complexes and promoting the phosphorylation of UPF1 by SMG-1 (Fig. 1, step 4). Phosphorylated UPF1 then recruits SMG-5, SMG-6, and SMG-7 with the subsequent dephosphorylation of UPF1 by SMG-7 (Fig. 1, step 5; ref. 30). SMG-1 kinase activity can be inhibited by two other members of the SURF complex: SMG-8 and SMG-9 (31).

The phosphorylation and dephosphorylation of Upf1 are thought to be necessary and crucial steps in the NMD pathway (29, 32, 33). Interestingly, Upf1 phosphorylation has also recently been shown to repress translation by binding to eIF3 subunits and preventing the formation of active ribosomes (34). Whereas mRNA degradation by other mechanisms occurs via a variety of exonucleases and endonucleases, NMD-targeted transcripts are thought to be primarily degraded via removal of the 5′ 7-methylguanosine cap by the decapping enzymes dcp1 and dcp2 and the subsequent 5′→3′ exonuclease activity of xrn-1 (Fig. 1, step 6; ref. 35). Inhibition of decapping activity has been shown to interfere with the stability of NMD targets but not other transcripts (35). Phosphorylated Upf1 may help recruit decapping enzymes (34). A rapid deadenylation step and 3′→5′ nuclease activity may also take place (35, 36), although the exact contribution of each pathway is difficult to determine because of possible redundancies elicited when individual pathways are silenced. Recently, SMG-6 has been determined to contain endonuclease activity on single-stranded RNA and it has recently been proposed that NMD transcripts also undergo endonucleolytic cleavage in the vicinity of their PTCs by SMG-6 before terminal degradation by xrn-1 (37, 38).

The decapping enzymes dcp1 and dcp2 as well as xrn-1 are concentrated in cytoplasmic foci termed processing bodies (39). Processing bodies are distinct from stress granules, which form in response to a variety of cellular stresses found in tumors including hypoxia and metabolic starvation, although studies suggest that mRNAs can be transferred from stress granules to processing bodies (40, 41). In Saccharomyces cerevisiae, knockdown of xrn-1 leads to the accumulation of mRNA and Upf1 in processing bodies as do mutations in Upf1 that fail to bind or hydrolyze ATP and thus cannot participate in NMD (42, 43). Furthermore, tethering of Upf1/Rnt1 to an mRNA is sufficient to both target that mRNA to processing bodies and rapidly degrade the mRNA (43). These data supporting a model in which processing bodies are the sites of NMD-associated degradation, however, are primarily derived from yeast studies. Although mammalian Upf1/Rnt1 accumulates in processing bodies during the inhibition of NMD (it is not yet known if this is accompanied with NMD-targeted mRNAs), recent studies suggest that human ATP-deficient Upf1/Rnt1 accumulation in processing bodies is not accompanied by other components of the SURF complex, and that disruption of visible processing body formation does not affect the stability of NMD targets, suggesting that processing bodies may be a consequence of NMD and are not required for mammalian NMD (3, 44, 45).

As is obvious from this brief and simplified review, the mechanism by which NMD-degraded mRNAs are targeted and degraded involves many multienzyme complexes and an intricate process of phosphorylation and other enzymatic steps. NMD activity has been shown to differ among cell lines (46), and the absence or overexpression of a variety of...
proteins involved in the NMD pathway has been shown experimentally to either inhibit or accelerate the degradation of NMD transcripts, respectively, without interfering with other forms of RNA decay. Although Upf1/Rent1 has been noted to be upregulated in colon cancer tissue compared with adjacent normal colonic tissue (47), to date, there has been no systematic examination of the expression levels of key components of NMD in various tissues or...
pathologic conditions such as cancer. Similarly, it is not known if any members of the NMD complex are upregulated by oncogenes.

NMD Targets Share Distinct Characteristics and Include Mutated Transcripts Commonly Found in Cancer

The existence of a pathway that rapidly degrades mutated mRNAs was suggested ~30 years ago when studies of several common mutations in human genes, including the β globin gene, determined that PTCs do not always result in a truncated protein but rather a markedly diminished amount of mRNA (48, 49). Indeed, several years later, it was determined that mutations commonly responsible for thalassemia, including virtually all thalassemia in Sardinia, are caused by a PTC that results in unstable globin mRNA (50, 51). Since these early experiments, many additional NMD targets have been identified and similarities between their gene structures have been characterized. Many of these characteristics support the current model of the molecular mechanism of NMD, specifically the interaction between the SURF complex recruited at a PTC and the EJC that triggers NMD. Thus, several “rules” or algorithms have been derived in an attempt to identify NMD-targeted transcripts.

Authentic stop codons do not elicit NMD because they are typically present in the last exon of a gene, and mammalian cells require at least one intron downstream of a PTC in order for that transcript to be targeted for NMD. Generally, it is thought that for a transcript to be targeted by NMD the PTC must also be greater than ~55 nucleotides upstream of an exon-exon junction. Based on this algorithm, bioinformatic studies have estimated that up to 30% of all known mutations causing human disease generate mRNAs that are degraded by NMD (reviewed in ref. 1). These mutations include those responsible for many forms of thalassemia, Duchenne’s muscular dystrophy, and cystic fibrosis. Mutations predicted to lead to NMD are also commonly found in tumor suppressor genes, and several of these will be discussed in detail below as a way to review the strategies for identifying bona fide NMD targets, as well as an opportunity to critically evaluate several assumptions about the degradation, translation, and function of mRNAs predicted to be NMD targets.

Despite efforts to identify NMD targets with bioinformatic tools, the number of transcripts degraded by NMD may be underestimated because not every NMD target conforms to the above algorithm. For example, T-cell receptor gene rearrangements, which increase receptor diversity, often result in PTC-containing transcripts; these transcripts may be degraded by NMD despite having a PTC located within the last 50 nucleotides of the penultimate exon (52). Alternative mechanisms also exist to target some transcripts for NMD. In both Drosophila melanogaster and Saccharomyces cerevisiae (which do not have introns), a stop codon is recognized as premature if it occurs far upstream of a polyadenylyl [poly(A)] tail. Similarly, in mammalian cells, a long 3′-untranslated region (UTR) can also target a transcript for NMD; SMG-5 is one such example of a transcript regulated by NMD by virtue of its long 3′-UTR (53). This mechanism is thought to involve a disruption of the normal role of poly(A)-binding protein C1 (PABPC1) of inhibiting interaction between eRF3 and Upf1. In the absence of this inhibition, for example, when a transcript contains a long sequence between a stop codon and the 3′ poly(A) tail, Upf1 is recruited and NMD occurs (53, 54). This has led to the model that NMD is a function of a competition between components of the EJC, which stimulate the recruitment of the Upf/Rent complex to a stop codon, and PABPC1, which antagonizes the recruitment of the Upf complex to the ribosome at a stop codon (53). However, to complicate this model, there are also features of some long 3′-UTRs that confer immunity to NMD, at least in Drosophila (55).

As will be discussed subsequently, several nonmutated transcripts generated in the cell are also targeted by NMD (2, 3). Whereas some of these normal transcripts have recognizable features that could render them sensitive to NMD [e.g., long 3′-UTRs, upstream open reading frames (uORF), and alternatively spliced isoforms as discussed later in detail], many do not (2, 3, 53). Therefore, it is possible that other, as yet unknown, characteristics render still additional mutated transcripts sensitive to NMD. Conversely, many mutant transcripts presumed to be NMD targets have not been validated, and several transcripts and reporter constructs predicted to be NMD targets do not seem to be degraded by this pathway (53, 56). Thus, the full range of transcripts degraded by NMD remains largely elusive.

Several screening strategies have been designed and implemented to identify transcripts as bona fide NMD targets. A SNP2NMD database for human single nucleotide polymorphisms that encode PTCs upstream of EJCs has been constructed (57), although this database suffers from the issues mentioned above, specifically that some transcripts may fit the criteria of a NMD target but still not be degraded by NMD and still other transcripts may not fit these criteria and yet in fact be degraded by NMD. Many experimental strategies to identify transcripts degraded by NMD are based on early assays that chemically inhibited NMD via the global inhibition of protein translation and then amplified and in vitro translated stabilized NMD transcripts (58). The presence of a truncated protein generated only with the inhibition of NMD suggests that the corresponding transcript is an NMD target.

With the advent of expression array profiling, a protocol termed gene identification by NMD inhibition (GINI) has been developed to identify NMD transcripts in a high-throughput manner (59). In this assay, expression arrays are used to identify transcripts upregulated in the presence of emetine, an antibiotic that inhibits translation and thus NMD. Transcripts upregulated in the presence of emetine
are considered to be NMD targets. NMD inhibitors that may have less nonspecific effects in some cell lines, including caffeine that inhibits SMG-1, have also been used (60). GINI has also been modified to treat cells with the RNA synthesis inhibitor actinomycin D, which will lead to decreased expression of unstable NMD transcripts in the absence of NMD inhibitors but not in the presence of NMD inhibitors (60).

These strategies, as well as the systematic sequencing of common tumor suppressor genes, have led to the identification of several NMD-conferring mutations in genes that play a role in cancer. In fact, a systematic analysis of mutations in human genes revealed that whereas most mutations in oncogenes are missense mutations, tumor suppressor genes exhibit a disproportionate number of nonsense mutations, many of which are predicted to lead to NMD-targeted transcripts (61). Such mutated tumor suppressor transcripts include Wilms’ tumor 1 gene mutations, which result in truncated transcripts that are stabilized with emetine treatment and encode for proteins with dominant-negative properties (62). The classic tumor suppressor genes p53 and RB each contain PTC mutations in mantle cell lymphoma cell lines, and both these transcripts are stabilized in the presence of emetine, suggesting that they are NMD targets (63). In fact, one mutant p53 transcript found in breast cancer shows increased mRNA stability with the inhibition of NMD, but the resulting COOH-terminal truncated protein is more stable than wild-type p53 protein (64). In the familial form of colon cancer, familial adenomatous polyposis, many of the mutations found result in PTCs in early exons, including those found in 9 of 10 Scottish kindreds (65, 66). Most BRCA2 mutations also result in PTCs, and expression of the transcript generated from the PTC-mutated gene is decreased compared with transcript levels observed from other mutations in the gene, suggesting that this transcript is destabilized (64). Similarly, 80% of the PTC-containing transcripts found in BRCA1 result in reduction of mRNA abundance (67). Several PTC mutations in MRE11, a partner of ATM in the DNA damage response, can only be detected from the sequencing of genomic DNA, but not from cDNA unless RNA decay is inhibited, suggesting that these mutations destabilize the transcript (68, 69). Finally, in gastric cancer, 80% of the mutations in the E-cadherin gene result in a PTC predicted to be subjected to NMD, and indeed, the E-cadherin mRNA from PTC-containing alleles is downregulated compared with the nonmutated allele, and these mutated transcripts are also stabilized with emetine or with Rent1/Upf1 depletion (70).

In colon cancer cells with microsatellite instability, the mutator phenotype results in 1- or 2-bp insertions or deletions, frameshifts, and often PTCs. Validation studies of the GINI technique showed that ~40% of all transcripts were upregulated in several microsatellite colon cancer cell lines with emetine (59). A significant number of these transcripts were not altered in a control fibroblast cell line, suggesting that these transcripts were upregulated due to colon cancer-specific mutations. The DNA mismatch repair enzyme MLH1, which is known to carry a PTC mutation in several colon cancer cell lines, was strongly upregulated with NMD inhibition specifically in colon cancer cell lines, thus validating this approach. This finding led to an interesting modification and application of GINI, developed to rapidly identify mutations in the large and difficult to sequence MLH1 gene in patients suspected of having hereditary nonpolyposis colon cancer (HNPCC; ref. 71). Blood samples from three patients who fit the criteria of HNPCC were treated with a chemical inhibitor of NMD before RNA isolation from WBCs. Reverse transcription-PCR for the MLH1 transcript was done, and the amplicon was examined for upregulation or alternatively spliced isoforms that were stabilized in the presence of the NMD inhibitor. In two of the three cases, these analyses revealed transcripts with PTC mutations likely to cause NMD that were barely detectable in the absence of NMD inhibition.

Another study looking at several genes mutated specifically in microsatellite unstable colon cancer cell lines was able to validate ~50% of their NMD candidate transcripts, including several genes that play a role in cancer biology, by sequencing and identifying PTC mutations in these genes (60). Of 10 candidate genes, biallelic inactivating PTC mutations were found in 6. Many of these genes were mutated in several colon cancer cell lines, which exhibit microsatellite instability, and several were also found to be mutated in macrosatellite unstable colon cancer tumor samples. In addition, the transcript generated from the EP300 gene encoding p300, the coactivator that plays an important role in the transcriptional activity of several oncogenes, has also been found to be mutated and targeted by NMD in microsatellite unstable colon cancer cell lines (72). The mRNA of some, although not all, transcripts identified by GINI in these studies was upregulated when Upf1/Rent1 was downregulated, suggesting that these were indeed bona fide NMD targets (60).

Stable transcripts with a PTC, if translated, result in a truncated protein. Thus, NMD is thought to have evolved to selectively eliminate truncated proteins that might otherwise serve as either activating mutants or dominant negatives against an intact protein encoded by the nonaffected allele. Because many tumor suppressor genes mutated in cancer contain PTCs and are either predicted to be or have been validated to be NMD targets, NMD is commonly thought to prevent tumorigenesis. Although this is a popular and satisfying model, validation requires documentation that in the absence of NMD the transcript indeed encodes a truncated protein and that this truncated protein can affect the phenotype of the cell. In many cases, these criteria have not been satisfied.

For example, as discussed earlier, many mutations predicted to promote NMD do not, including multiple nonsense mutations described in the ATM gene that result in PTCs upstream of exon-exon junctions but do not destabilize the ATM transcript (73). In addition, even when NMD-targeted transcripts are abundant,
these transcripts are not necessarily translated. For example, even when a CHK2 transcript harboring an NMD-provoking mutation is stabilized, this does not result in a translated (truncated) CHK2 protein (64). Another study found that several transcripts with PTCs not predicted to elicit NMD (e.g., were in the last exon) were translated into truncated proteins, but several transcripts predicted to be degraded by NMD, even when stabilized, were still not translated (56). This may be related to the recently described ability of phosphorylated Upf1/Rent1 to repress translation (34).

Finally, the biological significance of truncated proteins, if translated, is often unknown. Although some of these truncated proteins (e.g., described Wilms' tumor 1 and p53 mutations) may indeed act as dominant negatives (62), the generation of some truncated proteins can function normally (as in other p53 mutations; ref. 74). Although several oncogenes have PTCs that are predicted and/or validated to elicit NMD, including the tyrosine kinase EPHB2 in both prostate cancer cell lines and >5% of primary prostate tumors, and Janus-activated kinase 1 mutations in prostate cancer transcripts (75, 76), it is unclear whether these transcripts (if they were not degraded by NMD) would result in truncated proteins with gain-of-function properties. Thus, experimental validation that NMD protects against deleterious mutations in cancer is still necessary.

Nonmutated Transcripts Targeted by NMD Targets: Characteristics and Implications for Alternatively Spliced Transcripts

Although NMD has long been appreciated to degrade many mutated transcripts, such as nonfunctional transcripts with retroviral or transposon insertions, recently, it has also been found to regulate many nonmutated transcripts, including, as discussed later, transcripts that play an important role in the cellular response to stress (2). This unexpected finding resulted from the small interfering RNA knockdown of Upf1/Rent1 in HeLa cells followed by microarray analysis for transcripts whose expression was altered. Although this study documented that NMD regulates up to 10% of the HeLa transcriptome, it should be noted that this type of analysis cannot distinguish between those transcripts directly stabilized by the inhibition of NMD and those indirectly stabilized by NMD. Transcripts directly targeted by NMD show increased stability with Upf1/Rent1 knockdown, whereas the indirect upregulation of a given transcript can occur via the stabilization and upregulation of either a transcription factor or an inhibitor of RNA degradation. Approximately half of the transcripts upregulated with Upf1/Rent1 knockdown do not have recognizable features that could make them sensitive to NMD. Because a large proportion of transcripts altered when NMD is disabled are downregulated, without an alteration in their stabilities (2), indirect processes clearly exist, as there is no obvious mechanism by which decreased NMD activity can directly result in the decreased expression of a transcript. Thus, NMD has the potential to regulate a marked number of transcripts in addition to those directly targeted by the pathway.

The observation that many cellular transcripts are degraded by NMD raises the question of how these are recognized by the NMD apparatus. Many mRNAs have uORFs that can result in ribosomal pausing upstream of a stop codon and theoretically trigger NMD (77). Bioinformatic studies have shown that, in general, transcripts with uORFs are expressed to a lower degree than transcripts without uORFs, suggesting either decreased transcription or increased degradation (78). Although many short uORFs are not sufficient to render a transcript sensitive to NMD, the uORF of one important NMD target discussed in detail later, activating transcription factor-4 (ATF-4), is sufficient to promote NMD (3, 79). As previously discussed, a second mechanism that can trigger NMD in normal transcripts requires long distances between eRFs deposited at a normal stop codon and PABPC1 bound to a distant 3′-UTR, which then permits the recruitment of Upf1 and subsequent NMD (53). Additional potential mechanisms include functional genes with insertions of retroviral or transposon and genes with introns in their 3′-UTR (2, 4, 80-82).

Yet, another mechanism that may lead to the degradation of a nonmutated transcript by NMD is the alternative splicing of that transcript (Fig. 2). Alternatively, spliced transcripts vary in different tissues, occur in cells exposed to stresses common in the tumor microenvironment, have been increasingly identified in cancer cells, and have been hypothesized to play a causal role in cellular phenotypes (83-88). Alternatively spliced isoforms that play a role in cancer, for example, include vascular endothelial growth factor and BCL-x, which has an isoform that inhibits apoptosis [Bcl-x(L)] and an isoform that promotes apoptosis [Bcl-x(s); ref. 89]. Up to 30% of all human genes may be affected by alternative splicing, although many of these do not affect coding sequences (52, 90-92). Many alternative splicing events can lead to altered reading frames and a PTC upstream of an exon-exon junction, thus rendering them susceptible to NMD. As opposed to transcripts with a nonsense mutation, these alternatively spliced transcripts can result in a new reading frame and thus a novel protein. Depending on the alternatively spliced transcript, the upstream protein sequence of the original protein may be maintained, and thus, a fusion protein may result. Using bioinformatics to assess expressed sequence tags, approximately 20% to 35% of alternatively spliced events are predicted to lead to a PTC that would render them targets of NMD (93-96). These alternatively spliced transcripts include several of those transcripts found to be upregulated in HeLa cells when NMD is disabled (2).

Many of alternatively spliced transcripts degraded by NMD are found in low abundance, are not conserved between mouse and human, result in frameshift mutations unlikely to reflect bona fide genes, and are thus considered to represent genomic noise generated from an error-prone splicing process (97, 98). However, when
othologous exons can be found, there is evidence that the NMD-specific isoform is under selective pressure, suggesting that these are indeed translated under at least some conditions and are in fact detrimental to survival (98). Although alternatively spliced transcripts degraded by NMD have been identified by experimental techniques, including the MLH gene in patients with HNPCC (71), experimental evidence to support a widespread role for NMD is controversial. Whereas one study using exon arrays, in which most gene exons are represented by multiple probes, found a limited effect of Rent1/Upf1 depletion, another study identified and validated >200 exons with altered expression on NMD inhibition (4, 99). Many of these NMD-degraded isoforms contained a PTC in all three reading frames and could be confirmed with conditional knockout of Upf2/Rent2 in mouse hematopoietic cells (82). These studies suggest that NMD can stabilize and increase the expression of alternatively spliced mRNA isoforms, which may play an important role in cancer, but more experimental work must be done to determine the extent and biological significance of NMD regulation in this process.

Cellular Transcripts Targeted by NMD Are Involved in the Cellular Stress Response

Although the identification and validation of an individual NMD transcript may in turn suggest a specific functional role for NMD, it is important that this be validated with a formal documentation of phenotype. In published studies, this documentation is often absent, as is even the evidence that with the inhibition of NMD protein expression of the targeted transcript is increased. It is clear, however, that gross genetic and molecular manipulation of NMD results in dramatic organismal and cellular phenotypes. For example, components of NMD (e.g., Upf1/Rent1 and Upf2) are necessary for zebrafish embryonic differentiation and survival (100). Upf1/Rent1 is necessary for mouse viability, and the knockout of Upf2/Rent2 in mouse hematopoietic cells leads to lethality within 10 days and a failure of definitive hematopoiesis through the loss of stem and progenitor populations (82, 101). Because differentiated cells are spared, it has been hypothesized that NMD is primarily essential for proliferating cells (82). NMD-targeted transcripts that are responsible for these described phenotypes, however, have not yet been identified. Although several of the proteins involved in NMD seem to play a role in diverse phenotypes such as cell cycle checkpoints and telomere maintenance in mammalian and lower organisms (reviewed in ref. 9), it is unclear if they affect these functions through NMD or through other pathways. For example, SMG-1 can respond to double-strand breaks and contribute to a G2 checkpoint, not via Upf1/Rent1 phosphorylation but through p53 phosphorylation (102). In addition, the inactivation of NMD surely results in the stabilization of mutant transcripts and results in truncated and mutant proteins, which, in turn, may nonspecifically elicit many of the described phenotypes. Thus, these phenotypes do not necessarily help in the identification of specific functional genes or pathways that are normally regulated by NMD.

The nonmutated mRNAs normally targeted by NMD, as identified by array studies, cover a wide range of ontologic categories, including those involved in cell cycle, differentiation, and signaling (2). A strong argument that NMD plays an important role in regulating normal gene expression comes from the appreciation that multiple transcripts from the same functional classes are targeted by

**FIGURE 2.** Transcript degradation by NMD can lead to stabilization of truncated and alternatively spliced isoforms. Left, during normal translation, a full-length protein is generated. Middle, a PTC normally triggers NMD, but if that transcript is stabilized via inhibition of NMD, then a truncated protein may be generated, which may then serve as dominant negative or may function as a wild-type protein. Right, if an alternatively spliced isoform (e.g., due to a skipped exon) generates a PTC, in the absence of NMD, this isoform may result in a protein with deletions or, if the alternatively spliced isoform results in a new reading frame, a novel protein.
NMD. For example, several of the alternatively spliced isoforms degraded by NMD are splicing-related factors, leading to the autoregulation, or regulated unproductive splicing and translation hypothesis (refs. 4, 82, 94 and reviewed in ref. 9). In this model, when genes that promote splicing (e.g., SR genes) are highly expressed, they then promote alternative splicing of their own transcripts. This alternative splicing leads to a transcript that is degraded by NMD and, thus, downregulation of mRNA expression. Conversely, genes that inhibit splicing (e.g., hnrNPs) repress the inclusion of the coding exon that creates a frameshift and triggers NMD.

In addition to splicing factors, a disproportionate number of reported NMD-regulated transcripts are involved in stress response and nutrient homeostasis pathways (2, 3, 82). Multiple studies in yeast have also shown an important role of NMD in regulating transcripts involved in amino acid transport and synthesis and oxidative stress (103, 104), suggesting that the targeting of mRNAs that can promote the cellular adaptation to hostile environments is a conserved feature of NMD. This, along with the assumption that when multiple transcripts with similar functions are predicted to be regulated by NMD, then these transcripts are likely bona fide NMD targets with important physiologic roles, makes it probable that the regulation of stress response/nutrient homeostasis is an important function of NMD in mammalian cells.

Specific mammalian stress/nutrient–related transcripts upregulated with the inhibition of NMD include those of two intimately related pathways: amino acid transport and synthesis (including asparagine synthetase, cystathionine γ-lyase, cysteinyl-tRNA synthetase, seryl-tRNA synthetase, glutamate/neutral, and dibasic/neutral amino acid transporters) and the endoplasmic reticulum (ER) stress response pathway known as the unfolded protein response (UPR; refs. 2, 3). Unfolded proteins accumulate in the ER when there is a marked increase in the synthesis of secreted or membrane-bound proteins and a decrease in the availability of ER chaperones to fold their client proteins and/or in the setting of ER microenvironmental perturbations including cellular hypoxia and/or generation of ROS (refs. 105-108 and reviewed in ref. 109). Activation of the UPR leads to the coordinated degradation of ER-associated proteins, the processing of the transcript for the xbp-1 transcription factor, and the phosphorylation of the α subunit of elf2α by the PKR-like ER kinase (110). In a mechanism termed the integrated stress response, elf2α can also be phosphorylated by a variety of cytoplasmic kinases that are activated by diverse forms of stress, including ROS generation and amino acid deprivation (105, 111).

Phosphorylation of elf2α leads to both a general suppression of protein translation as well as the paradoxical translational induction of the transcription factor ATF-4. NMD-targeted transcripts include ATF-4 and the ATF-4 targets CHOP (gadd153) and ATF-3 (2, 3, 82). The UPR plays a role in numerous phenotypes, including proliferation and differentiation (as reviewed in ref. 112). However, the best-delineated role for the upregulation of ATF-4 is the cellular adaptation to ER stress via the transcriptional upregulation of protein chaperones and other stress response genes. The UPR also protects against oxidative stress, which can be generated under conditions of ER stress, transformation with the Ras oncogene, hypoxia, as well as by by-products of O2-utilizing cellular processes (105, 111, 113-116).

There are several links between the UPR and the amino acid metabolism pathways. The UPR is activated by amino acid starvation (111). ATF-4 targets include many genes involved with amino acid import and metabolism, particularly ones that contain thiols, and UPR-deficient cells are more sensitive to amino acid deprivation (111, 117, 118). Regulation of both the UPR and amino acid metabolism also plays a particularly important role in cancer progression. Just as glucose metabolism is altered in many cancer cells, it is increasingly appreciated that the transport and metabolism of amino acids play an important role in tumor cell proliferation and survival (119, 120). Studies have also shown that the UPR is also activated in hypoxic areas of xenografted tumors and human tumors, and the generation of ATF-4 promotes the survival and growth of hypoxic tumors and induced angiogenesis (108, 121).

Many of the transcripts involved in amino acid transport and the UPR are directly targeted by NMD (2, 3). Importantly, transcripts involved in the UPR are not upregulated when NMD is inhibited simply because of the accumulation of mutant truncated unfolded proteins in the ER (3). Thus, although many stress response and amino acid transporter genes are upregulated by the NMD-targeted ATF-4, transcripts for these genes are also independently degraded by NMD. Specifically, these transcripts are (a) downregulated by the overexpression of Upf1/Rent1 (which is sufficient to drive NMD), (b) upregulated by the knockdown of Upf1/Rent1, and (c) stabilized by the knockdown of Upf1/Rent1 (2, 3). Many of the transcripts of the UPR (e.g., ATF-4 and CHOP), although necessary for survival to stress, are detrimental to cellular proliferation and survival when highly expressed in nonstressed cells, thus suggesting a potential reason for their rapid degradation by NMD. Thus, further work is required to determine the role of NMD in both normal physiology and cancer biology.

NMD Is Regulated by Stresses Commonly Found in the Tumor Microenvironment

The appreciation that NMD plays a role in degrading nonmutated, functional transcripts raises the question whether NMD might be regulated and thus stabilize both transcripts and alternatively spliced isoforms during physiologic or pathophysiologic conditions. Indeed, NMD activity is known to vary between different cell lines and even among distinct tissues (46). In patients with Schmid metaphyseal chondrodysplasia, collagen X PTC mutations lead to complete NMD in cartilage, but the mutant

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mRNA is not subjected to NMD in noncartilage (lymphoblasts and bone) cells (122). Experimentally in mice, when the prevalence of PTC-mutated Men1 mRNA is compared with mRNA from the nonmutated allele, there is a decrease in PTC versus wild-type mRNA in testis but not in lung, intestine, and thymus (123).

Because NMD has been found to degrade transcripts vital in amino acid transportation and transcripts necessary for the cellular response to ER stress, it is logical that amino acid starvation and ER stress might be two conditions that suppress NMD activity to upregulate these transcripts and allow a robust cellular response to these stimuli. Both amino acid deprivation and hypoxia (which activates the UPR) were found to inhibit the degradation of NMD reporter constructs (i.e., genomic sequences with PTCs) but not control constructs (2, 3). The inhibition of NMD in hypoxic cells and amino acid–deprived cells was found to increase the stabilities, and in many cases the steady-state expression level, of a variety of endogenous NMD transcripts (2, 3).

Insights into the mechanism by which NMD is inhibited are provided by the observation that a common link between amino acid starvation and hypoxia is that they both result in eIF2α phosphorylation. As discussed, eIF2α can also be phosphorylated by ROS, and intriguingly, in Schizosaccharomyces pombe, the upregulation of mRNAs in response to ROS also requires Rent1/Upf1 (104). To test whether eIF2α phosphorylation was necessary for the hypoxic inhibition of NMD, cells that could not phosphorylate eIF2α due to the genetic knock-in of an eIF2α mutant gene were rendered hypoxic and NMD activity was tested (3). In the absence of eIF2α phosphorylation, neither a PTC reporter construct nor endogenous NMD targets were stabilized in hypoxic cells, indicating that eIF2α phosphorylation is necessary for at least hypoxic inhibition of NMD. As discussed, ribosomal scanning during the pioneer round of protein translation is necessary for NMD, and although eIF2α phosphorylation attenuates protein translation, this is unlikely to be the mechanism by which eIF2α phosphorylation inhibits NMD for a variety of reasons, including the fact that many of the transcripts stabilized by the inhibition of NMD are actually robustly translated during this inhibition (3).

It is also possible that NMD is dynamically regulated by other mechanisms in addition to eIF2α phosphorylation. For example, radiation exposure has been reported to trigger Upf1/Rent1 phosphorylation by SMG-1 and ATM (102), and the fact that NMD requires multiple protein complexes and phosphorylation and dephosphorylation events suggests multiple potential steps for physiologic regulation. In addition, as discussed previously, experimentally the overexpression of several of these components can drive or inactivate NMD experimentally and it is possible that physiologic alterations of endogenous expression of these proteins (in different tissues, in cancer, or in other pathologic states) can alter NMD activity, although this has not yet been convincingly shown.

Thus, a model can be formed in which hypoxia, amino acid starvation, and ROS generation, which are all stresses commonly generated by the microenvironment, activate the UPR, phosphorylate eIF2α, and inhibit NMD. This inhibition then stabilizes transcripts necessary for amino acid transport and the cellular response to stress, many of which are also upregulated independently via other arms of the UPR. Thus, for example, eIF2α phosphorylation not only induces the translation of ATF-4 but also stabilizes the ATF-4 transcript. Although the ATF-4 transcript is not greatly induced in cells rendered hypoxic, studies have suggested that without the inhibition of NMD, the ATF-4 transcript is actually repressed in hypoxic cells (3, 107, 108). This redundant control of ATF-4 expression, together with the recent suggestion that ATF-4 protein stability is also increased in hypoxic cells in a mechanism similar to that of hypoxia-inducible factor–1α and hypoxia-inducible factor–1β (124), suggests that the tight upregulation of this protein is an important stress response mechanism but that the downregulation of ATF-4 in nonstressed cells is also crucial. The example of ATF-4 also highlights that the UPR truly invokes a wide range of responses ranging from transcription, to mRNA processing, to translation, and, with the inhibition of NMD, to the stabilization of transcripts.

In light of the relevance of the UPR and amino acid metabolism in cancer, the potential biological significance of this model can be examined by considering how NMD regulation by the tumor microenvironment may affect tumorigenesis (Fig. 3; refs. 108, 119, 121). During tumorigenesis, tumor growth and a leaky, disorganized vascular system lead to significant regions of hypoxia and amino acid deprivation. ROS are generated in these hypoxic cells, in addition to ROS generated via oncogenic Ras activity. All of these lead to eIF2α phosphorylation and activation of the integrated stress response, which in turn promote tumor cell survival and angiogenesis. eIF2α phosphorylation also inhibits NMD, which augments the integrated stress response and thus promotes tumorigenesis. NMD inhibition also leads to the stabilization of distinct alternatively spliced isoforms, normally degraded by NMD, encoding for both truncated proteins and novel proteins, which may also play a role in tumorigenesis. Finally, inhibition of NMD by the tumor microenvironment promotes the stabilization and upregulation of mutated tumor suppressor transcripts that would normally be degraded by NMD, leading to truncated dominant-negative proteins that can inhibit the remaining unmutated allele and promote tumorigenesis.

However, to date, the experimental evidence indicating that NMD activity is actually diminished in hypoxic and metabolically starved tumors in vivo is lacking. It is well documented that several other RNA degradative pathways are deregulated in cancer. For example, hypoxia is common in the tumor microenvironment and the vascular endothelial growth factor mRNA, which plays an important role in angiogenesis, is stabilized in hypoxic cells via the interaction of the RNA binding protein HuR with sequences.
contained in the 3′-UTR of vascular endothelial growth factor (125-127). HuR has also been shown to bind to p53 transcripts, in a manner dependent on the von Hippel-Lindau tumor suppressor, to increase p53 mRNA stability and translation (128, 129). There is strong circumstantial evidence that NMD regulation by the tumor microenvironment may occur in vivo and be important in tumorigenesis. Although it is clear that an active UPR, and specifically upregulation of ATF-4, is necessary for tumor growth (108, 121), it is unknown whether one contributing factor for this is the inhibition of NMD by eIF2α phosphorylation. Although several studies have recently suggested that alternatively spliced isoforms may be increased in cancer cell lines, studies thus far have focused on cis-mutations and the aberrant expression of splicing proteins found in cancer, not on the potential role of NMD in generating these isoforms (88, 130). Finally, it has recently been described that H-Ras has an alternatively spliced form that is degraded by NMD (131), but it has not yet been shown whether this alternatively spliced Ras transcript is translated and whether it serves a biological function. However, the knowledge that NMD is a regulated mechanism, and plays an important role in stress response, will undoubtedly provide the impetus for investigators to explore the role of NMD in tumorigenesis.

Summary and Conclusion

Thus, NMD, a process originally considered to be responsible only for degrading mutant transcripts, is a process regulated by stresses common in the microenvironment and is responsible for dynamically altering gene expression. The regulation of NMD has implications for the stabilization of mutated transcripts, the expression of alternatively spliced transcripts, and the augmentation of stress response pathways including the UPR. However, although eIF2α phosphorylation, necessary for the inhibition of NMD, and activation of the UPR clearly play important roles in many biological processes including tumor survival and growth, the documentation that NMD and/or the regulation of NMD alters cellular phenotype has not yet been clearly shown (106-108). Although conditional knockout animals for key components of the NMD pathway have been generated, studies to assess the role of NMD in the initiation and/or progression of tumors have been hampered by the observation that complete cessation of NMD seems to be incompatible with the survival of proliferating cells (82, 101). Future studies will need to explore the role of more moderate modulation of NMD activity on the tumorigenic role of this important RNA degradative pathway.
The very fact that NMD can be physiologically inhibited, has activity dependent on phosphorylation events, and may play a role in regulating many transcripts, including those relevant in cancer, does suggest that manipulation of NMD activity may be an achievable and novel target for the pharmacologic treatment of many diseases in addition to those genetic diseases caused by NMD-triggering mutations. Gentamicin and other compounds can promote ribosomal bypass of PTCs. Preclinical and clinical trials have shown that such treatments can result in expression of full-length proteins and ameliorate genetic diseases caused by NMD-provoking mutations, including cystic fibrosis and muscular dystrophy, although inexplicably at least some of these drugs do not seem to increase the stability of these transcripts (132). Other inhibitors of NMD have also been identified, including the natural product pateamine, which interacts with eIF4AIII but inhibits NMD independently of translation initiation inhibition (133).

Previous studies on NMD have primarily emphasized the determination of the complicated NMD mechanism and the identification of NMD targets. Little work has been done on validating and characterizing these targets. Future experiments will have to better define in vivo the role that NMD inhibition plays in biology, including tumorigenesis and other conditions (e.g., development), which are marked by hypoxia and other cellular stresses. Better insight into the mechanism by which eIF2α phosphorylation and potentially other events inhibit NMD will aid in the identification and testing of pharmacologic agents that can modulate NMD activity and perhaps be clinically useful in diseases caused by PTC-mutated transcripts and/or marked by cellular stress.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

17. Woeller CF, Gaspari M, Isken O, Maquat LE. NMD resulting from encephalomyocarditis virus IRES-directed translation initiation seems to be restricted to CBF80/20-bound mRNA. EMBO Rep 2008;9:446–51.


26. Gehring NH, Neu-Yilik G, Schell T, Hentze MW, Kulozik AE. Y14 and

27. Serin G, Gersappe A, Black JD, Aronoff R, Maquat LE. Identification and characterisation of human orthologues to Saccharomyces cer-

ance complex interacts with the translation release factors to en-
hance termination and degrade aberrant mRNAs. Genes Dev 1998;

Upf1-1-eRF1-eRF3 complex (SURF) to the exon junction complex
triggers Upf1 phosphorylation and nonsense-mediated mRNA de-

30. Fukuhara N, Ebert U, Untereholzner L, Lindner D, Izaurralde E, Conti
E. SMG7 is a 14-3-3-like adaptor in the nonsense-mediated mRNA

subunits of the SMG-1 complex, regulate remodeling of the
mRNA surveillance complex during nonsense-mediated mRNA de-

32. Yamashita A, Ohnishi T, Kashima I, Taya Y, Ohno S. Human SMG-
1, a novel phosphatidylinositol 3-kinase-related protein kinase, as-
sociates with components of the mRNA surveillance complex and
is involved in the regulation of nonsense-mediated mRNA decay.

induces formation of mRNA surveillance complexes containing

34. Chen Q, Kim YK, Hosoda N, Mayeur GL, Hershey JW, Maquat LE.
Upf1 phosphorylation triggers translational repression during non-

35. Lejeune F, Li X, Maquat LE. Nonsense-mediated mRNA decay in
mammalian cells involves decapping, deadenylating, and exonu-

36. Czaplinski K, Shyu AB. Rapid deadenylation triggered by a nonsense
codon precedes decay of the RNA body in a mammalian cytoplas-
mic nonsense-mediated decay pathway. Mol Cell 2003;23:

is the catalytic endonuclease that cleaves mRNAs containing non-

38. Eberle AB, Lykke-Andersen S, Muhlemann O, Jensen TH. SMG6
promotes endonucleolytic cleavage of nonsense mRNA in human

803–8.

40. Kederha N, Teockin G, Ayodele M, et al. Stress granules and pro-
cessing bodies are dynamically linked sites of mRNP remodeling.

41. Anderson P, Kederha N. RNA granules: post-transcriptional and

42. Cheng Z, Muhlrad D, Lim MK, Parker R, Song H. Structural and
functional insights into the human Upf1 helicase core. EMBO J
2007;26:253–64.

43. Sheth U, Parker R. Targeting of aberrant mRNAs to cytoplasmic

44. Stalder L, Muhlemann O. Processing bodies are not required for
mammalian nonsense-mediated mRNA decay. RNA 2009;15:
1265–73.

45. Eulalio A, Behm-Ansmant I, Schweizer D, Izaurralde E. P-body for-
mation is a consequence, not the cause, of RNA-mediated gene

46. Linde L, Boett S, Neu-Yilik G, Kulozik AE, Kerem B. The efficiency of
nonsense-mediated mRNA decay is an inherent character and

decay impacts MSI-driven carcinogenesis and anti-tumor

48. Chang JC, Kan YW. β 0 thalassemia, a nonsense mutation in man.

49. Joos R, Lacroute F. Interference of nonsense mutations with eu-
karyotic messenger RNA stability. Proc Natl Acad Sci U S A 1979;
76:5134–7.

50. Maquat LE, Kinniburgh AJ, Rachmilewitz EA, Ross J. Unstable β-
globin mRNA in mRNA-deficient β thalassemia. Cell 1981;27:
543–53.

51. Kan YW, Lee KY, Furbetta M, Angius A, Cao A. Polymorphism of
dNA sequence in the β-globin gene region. Application to prenatal
diagnosis of β 0 thalassemia in Sardinia. N Engl J Med 1980;302:
185–8.

transcriptome based on functional annotation of 60,770 full-length

53. Singh G, Rebbapragada I, Lykke-Andersen J. A competition be-
tween stimulators and antagonists of Upf complex regulates gene
processing in human nonsense-mediated mRNA decay. PLoS Biol
2006;4:e111.

54. Silva AL, Ribeiro P, Inacio A, Liebhaber SA, Romao L. Proximity of
the poly(A)-binding protein to a premature termination codon inhib-
its mammalian nonsense-mediated mRNA decay. RNA 2008;14:
563–76.

55. Behm-Ansmant I, Gatfield D, Rehwinkel J, Hilgers V, Izaurralde E.
A conserved role for cytoplasmic poly(A)-binding protein 1 (PABPC1)

56. You KI, Li LS, Kim NG, et al. Selective translational repression of
truncated proteins from frameshift mutation-derived mRNAs in tu-

57. Han A, Kim WY, Park SM. SNP2NMD: a database of human single
nucleotide polymorphisms causing nonsense-mediated mRNA de-

detection of premature termination mutations using a protein trun-
cation test designed to overcome problems of nonsense-mediated

59. Hasselbom E, Dietz HC. A strategy for disease gene identification
through nonsense-mediated mRNA decay inhibition. Nat Biotechn-

60. Ivanov I, Lo KC, Hawthorn L, Cowell JK, Ionov Y. Identifying candi-
date colon cancer tumor suppressor genes using inhibition of non-
sense-mediated mRNA decay in colon cancer cells. Oncogene
2007;26:2873–84.

61. Mort M, Ivanov D, Cooper DN, Chuzhanova NA. A meta-analysis of
nonsense mutations causing human genetic disease. Hum Mutat
2008;29:1037–47.

activation is inhibited by dominant negative mutant proteins. J Biol

63. Pinyol M, Bea S, Pla L, et al. Inactivation of RB1 in mantle-cell lym-

mRNA decay mechanism prevents the synthesis of truncated
BRCA1, CHK2, and p53 proteins? Hum Mutat 2008;
29:65–73.

65. Bala S, Kraus C, Wijnen J, Meera Khan P, Ballhausen WG. Multiple
products in the protein truncation test due to alternative splicing in
the adenomatous polyposis coli (APC) gene. Hum Genet 1996;99:
298–303.

66. Perrin-Vidot L, Sinilkovina OM, Stoppa-Lyonnet D, Lenoir GM, Ma-
zoyer S. The nonsense-mediated mRNA decay pathway triggers
degradation of most BRCA1 mRNAs bearing premature termination

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Nonsense-Mediated RNA Decay Regulation by Cellular Stress: Implications for Tumorigenesis

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