Published in final edited form as:

Biochim Biophys Acta. 2013 ; 1829(0): 624–633. doi:10.1016/j.bbagr.2013.03.002.

Regulation of nonsense-mediated mRNA decay: Implications for physiology and disease

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Abstract

Nonsense-mediated mRNA decay (NMD) is an mRNA quality control mechanism that destabilizes aberrant mRNAs harboring premature termination (nonsense) codons (PTCs). Recent studies have shown that NMD also targets mRNAs transcribed from a large subset of wild-type genes. This raises the possibility that NMD itself is under regulatory control. Indeed, several recent studies have shown that NMD activity is modulated in specific cell types and that key components of the NMD pathway are regulated by several pathways, including microRNA circuits and NMD itself. Cellular stress also modulates the magnitude of NMD by mechanisms that are beginning to be understood. Here, we review the evidence that NMD is regulated and discuss the physiological role for this regulation. We propose that the efficiency of NMD is altered in some cellular contexts to regulate normal biological events. In disease states—such as in cancer—NMD is disturbed by intrinsic and extrinsic factors, resulting in altered levels of crucial NMD-targeted mRNAs that lead to downstream pathological consequences.

Keywords

NMD; NMD regulation; microRNA; miR-128; cancer; UPR; eIF2 α ; ATF4; c-myc; e-cadherin; UPF1; UPF3B

1. Introduction

First discovered in *S. cerevisiae* over three decades ago [1], NMD is a conserved quality-control mechanism that rapidly degrades abnormal mRNAs harboring premature termination codons (PTCs) [2]. Most early studies focused on the ability of NMD to degrades mRNAs transcribed from mutated genes in human diseases, as approximately one-third of congenital disorders are caused by genes harboring PTCs [3,4]. Efforts also were directed towards understanding the underlying mechanisms of NMD, which led to the identification of key

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components required for this pathway, including the UPF and SMG proteins [2]. Together with a set of factors that are deposited just upstream of exon-exon junctions after RNA splicing—the exon junction complex (EJC)—these proteins orchestrate a mechanism that identifies PTCs and targets the mRNAs containing them for rapid decay [5].

Recently it has become clear that NMD is not only an RNA surveillance mechanism that degrades mutant gene transcripts, but also a regulatory pathway that downregulates many normal gene transcripts. Indeed, ~3–20% of mRNAs transcribed from protein-coding genes in organisms ranging from *S. cerevisiae* to mammals have been estimated to be regulated—either directly or indirectly—by NMD [6–10]. Some of its direct target mRNAs (i.e., NMD substrates) are conserved, including transcripts encoding RNA-binding proteins, ribosomal proteins, and stress-related genes [6,11–15]. Many of the “NMD-inducing features” in normal transcripts that cause them to be degraded by NMD have been elucidated, most of which place stop codons in a premature context [5]. For example, one NMD-inducing feature is an exon-exon junction downstream of a stop codon. Exon-exon junctions recruit the EJC, which elicits NMD by interacting with proteins recruited during translation termination, including the UPF proteins UPF1, UPF2, UPF3A, and UPF3B [2,16]. mRNAs that harbor a stop codon in the final exon avoid NMD because the EJCs recruited upstream of the stop codon appear to be displaced by the translocating ribosome during the first round of translation [2,16,17]. A common event that leads to EJC-dependent NMD is alternative splicing, since alternatively spliced exons typically have one or more in-frame stop codons [5]. Another NMD-inducing feature is an upstream open reading frame (uORF). While the mechanism of action of uORFs is not clearly understood, a likely scenario is that translation termination at uORFs recruits NMD factors, which, in turn, interact with downstream EJCs, thereby eliciting NMD. Long 3′ untranslated regions (UTRs)—generated either constitutively or by alternative polyadenylation [18]—can also trigger NMD [19–23].

The discovery that NMD regulates many normal transcripts raises the possibility that NMD itself is subject to regulation. Indeed, in recent years, several mechanisms have emerged that tightly regulate NMD and thus have the potential to dictate the expression of the large repertoire of NMD target mRNAs. In this review, we summarize the recent evidence that NMD is a highly regulated process and the possible mechanisms behind it. We will also discuss and speculate about the role of NMD regulation in physiological processes and in disease.

2. Molecular mechanisms of NMD regulation in physiological contexts

2.1. MicroRNA-mediated regulation of NMD

One example of a regulatory mechanism that modulates NMD activity is direct targeting of NMD factors by microRNAs. MicroRNAs are small (~22-nt) non-coding RNAs that bind and repress the expression of specific target mRNAs through translational repression and/or rapid mRNA decay [24]. With this in mind, Bruno *et al.* searched for miRNAs that might target NMD factors [25]. Using bioinformatic algorithms that identify putative miRNA targets, they discovered that the 3′ UTRs of the pivotal NMD factor UPF1 and of the EJC core protein MLN51 are complementary with the seed sequence in miR-128-1 and -2 (which we will refer to hereafter as simply “miR-128” since they are identical in sequence unless alternatively processed). These investigators then went on to demonstrate that miR-128 directly regulates these two NMD factors in human and mouse cells. They also showed that miR-128 potently represses NMD activity, and that it does so through its ability to repress the expression of UPF1 and MLN51 (Figure 1A). Of note, however, miR-128 does not eliminate NMD, it merely reduces its level, thereby allowing NMD to continue to function in RNA surveillance and other activities. Finally, they demonstrated that miR-128 is dramatically upregulated during both brain development and neuronal maturation, raising

the possibility that this leads to repressed NMD and hence stabilization of its of mRNA targets. Thus, this miRNA-NMD circuit has the potential to serve as a switch to induce gene expression during development.

miR-128 is identical in sequence in many vertebrates, as is its seed complementary region in UPF1 [25]. Coupled with the fact that NMD is a highly conserved RNA decay pathway [26,27], this raised the possibility that the miR-128/NMD circuit is also conserved. Indeed, Bruno *et al.* found that miR-128 both represses UPF1 expression and upregulates NMD target transcripts in both chick neural tubes and *Xenopus laevis* embryos [25]. While the physiological role of this circuit in birds and amphibians is not known, Bruno *et al.* obtained evidence that this miR-128/NMD circuit upregulates the levels of transcripts encoding crucial neuronal proteins in a temporally regulated manner during brain development in mammals [25]. It remains to be determined whether the increase in the level of these mRNA levels is accompanied by corresponding increases in the encoded proteins and, if so, whether it is physiologically significant. Nonetheless, since the brain-selective pattern of miR-128 expression is conserved in many species [28,29], this suggests that the miR-128/NMD regulatory circuit could be widely used to control the development and functioning of the nervous system.

The ability of miR-128 to regulate gene expression through NMD may not be restricted to neural cells. For example, miR-128 is also expressed in the thymus [25], indicating that the miR-128/NMD circuit could also be involved in T-cell development. Relevant to this, Frischmeyer-Guerrero *et al.* recently demonstrated that thymocyte development is arrested in transgenic mice that ubiquitously express a dominant-negative form of UPF1 [30]. While the mechanism behind this is not known, Frischmeyer-Guerrero *et al.* obtained evidence that the arrested development occurs because the NMD deficiency permits accumulation of T-cell receptor (TCR) transcripts from non-productively rearranged (out-of-frame) TCR genes, which, in turn, engages a rearrangement-blocking feedback response that normally is only engaged after a productive (in-frame) rearrangement. The consequence is an accumulation of immature T cells lacking TCR on their surface, all of which die. miR-128's role in this may be to suppress NMD in the most immature T cells in the thymus—CD4⁺ CD8⁺ thymocytes—since they express high levels of germline (non-rearranged) TCR mRNAs that could prematurely activate the feedback response. Consistent with this, the host gene of miR-128-2—*ARPP-21 (RCS)*—is specifically expressed in CD4⁺ CD8⁺ thymocytes [31].

miRNAs in addition to miR-128 may be identified in the future that regulate NMD. If expressed in the nervous system, such miRNAs could collaborate with miR-128 to promote stronger downregulation of NMD. If such miRNAs were also developmentally regulated, this could provide both dynamic and robust control of gene expression in neurons. Alternatively, if expressed in cell types other than neurons, NMD-regulatory miRNAs would have the potential to influence additional biological events through their ability to regulate NMD. It will be interesting to determine the biological consequences of such regulation, as different cell types have different sets of NMD substrates [32]. A further level of complexity arises from the fact that NMD is a multi-branched pathway regulated by distinct factors [8,33] (Figure 1A). This opens the opportunity for distinct subsets of mRNAs to be regulated through miRNAs targeting different NMD factors.

Because NMD is a translation-dependent mechanism [2], this raises the possibility that another means to regulate the magnitude of NMD is through translation control circuits. For example, since microRNAs inhibit the translation of many of their targets (Figure 1B) [34], microRNAs would be predicted to quench NMD. Thus, by inhibiting NMD, microRNAs could potentially *stabilize* NMD substrate mRNAs, instead of miRNA's usual mode of

action, which is to trigger rapid mRNA decay. Indeed, Choe *et al.* recently provided evidence for this scenario [35]. First, they showed that one of the catalytic components of the RNA-induced silencing complex (RISC) required for miRNA action—Argonaute 2 (AGO2) [36]—preferentially targets mRNAs harboring EJC and the cap-binding complex (CBC). This is significant since several lines of evidence suggest that NMD targets mRNAs bound by both the EJC and CBC [17], the latter of which binds to 5' caps of mRNAs when they are first transcribed and processed in the nucleus. Evidence for the functional significance of AGO2 binding to these mRNAs was Choe *et al.*'s finding that artificially tethering AGO2 to the 3' UTR of a NMD reporter inhibited both its translation and its ability to be degraded by NMD [35]. This AGO2-NMD circuit is likely to have important downstream consequences, as microarray analyses revealed the existence of many NMD mRNA targets that were stabilized as a consequence of AGO2 tethering [35]. In the future, it will be interesting to identify specific microRNAs that stabilize NMD substrates by this mechanism. By both translationally silencing and stabilizing NMD substrates, such microRNAs would serve as supervisors that direct this class of mRNA to be placed in “cold storage” (i.e., not degraded) until needed later.

In a follow-up paper, Choe *et al.* provided more mechanistic insight into how miRNA-mediated translation repression might regulate NMD [37]. They obtained several lines of evidence that human AGO2 directly competes with CBC for cap association, thereby inhibiting CBC-dependent translation and NMD. For example, they showed that a mutant form of AGO2 that lacks cap-association activity was not able to inhibit NMD when tethered to the 3' UTR of a PTC-containing mRNA. This mutant AGO2 also failed to inhibit the binding of CBC to the cap structure, as measured by immunoprecipitation [37]. Together, these two studies provided molecular insight into the cross talk between miRNA-mediated mRNA silencing, CBC-dependent translation, and NMD. Coupled with the discovery that specific miRNAs directly target NMD components [25], a picture emerges in which regulation of NMD by miRNAs has the potential to be a crucial post-transcriptional mechanism to broadly modulate gene expression in different biological contexts.

2.2. Regulation of NMD by competition with the SMD RNA decay pathway

Mammalian Staufen-1 (STAU1) is an RNA-binding protein that functions in mRNA transport and translational control [38]. STAU1 has also been demonstrated to participate in an mRNA decay mechanism named STAU1-mediated mRNA decay (SMD) [38]. While the requirement for STAU1 is unique to SMD, SMD shares with NMD the requirement for UPF1 and a termination codon. UPF1 participates in SMD by directly binding to STAU1, which, in turn, elicits mRNA decay when tethered downstream of a termination codon [38]. But unlike NMD, SMD does not involve pre-mRNA splicing and is independent of other NMD factors, such as UPF2 or UPF3B [38].

Since both NMD and SMD require UPF1, Gong *et al.* examined whether NMD and SMD are competitive pathways [39]. They found that the STAU1- and UPF2- binding sites within UPF1 overlap and that STAU1 and UPF2 compete for binding to UPF1. As a consequence of this, these investigators found that down-regulating the cellular abundance of STAU1, which decreased the magnitude SMD, increases the efficiency of NMD, whereas down-regulating the cellular abundance of UPF2, which inhibits NMD, increases the efficiency of SMD. To examine the role of their competitive relationship in a developmental context, these investigators turned to the C2C12 cell line, which differentiate from myoblast-like proliferative cells into post-mitotic myotubes *in vitro*. They found that induction of differentiation in these cells increased SMD efficiency and decreased NMD efficiency, consistent with their finding that more STAU1, but less UPF2, bound to UPF1 in myotubes compared with myoblasts. They also identified target transcripts that could have a developmental role in this system; *PAX3* mRNA is an SMD target encoding an anti-

myogenic factor that is downregulated during myogenesis, whereas *myogenin* mRNA is an NMD target encoding a protein required for myogenesis that is upregulated during myogenesis. Complicating this simple scenario, these investigators obtained evidence that one arm of the NMD pathway—the UPF3B-dependent branch—is upregulated, rather than downregulated, during myogenesis [39]. This exemplifies the need to take all branches of NMD into account when considering the consequences of NMD regulation in a given biological setting. It will be interesting to determine in the future if the UPF3B-dependent branch of NMD targets mRNAs encoding anti-differentiation factors in muscle cells, as predicted if it was contributing to the muscle lineage differentiation pathway.

Another physiologically relevant example of a “battle” between SMD and NMD occurs during adipogenesis. Choi *et al.* found that STAU1 abundance increases during adipogenesis, which decreases NMD efficiency [40]. They also observed that UPF1 becomes hyperphosphorylated during adipogenesis, which increases its association with STAU1 and consequently leads to a further repression in the magnitude of NMD. This NMD downregulatory response appears likely to exert a causal role in adipogenesis since these investigators found that depletion of NMD components by RNAi promoted adipogenesis.

2.3. Autoregulation of NMD

Many factors that affect the cellular microenvironment, such as hypoxia or gene deregulation in cancer, can have a profound negative impact on biological processes [41–44]. In order to avoid the deleterious effect of such insults, cells have developed buffering mechanisms that confer robustness for processes that are fundamental for cell and organismal viability [45,46]. Given that NMD has a dual role—serving not only as a quality control mechanism but also as a regulator of normal gene expression—NMD is a prime candidate to be one of the processes that are buffered by robustness-conferring mechanisms. If NMD were *not* buffered from inhibitory insults, the outcome would likely be extremely deleterious. For example, there would be upregulation of truncated proteins from mutant and cryptically spliced transcripts that normally would be degraded by NMD. Some of these mutant proteins are likely to possess dominant-negative activity and thus be toxic for cells. Additionally, inhibition of NMD could also result in misregulation of normal proteins encoded by both endogenous NMD substrates and mRNAs indirectly regulated by NMD. As described below, two buffering mechanisms that preserve the integrity of NMD in response to insults have recently been discovered (Figure 2).

The first hint of a buffering mechanism came from Mendel *et al.*, who performed microarray-profiling analysis on NMD-deficient HeLa cells depleted of the NMD factor UPF1. Among the many upregulated transcripts they identified was the mRNA encoding the NMD factor, SMG5 [6]. Subsequently, *SMG5* mRNA was also shown to be upregulated in response to depletion of another NMD factor, UPF3B, raising the possibility that its levels are controlled by feedback regulatory response dependent on the UPF3-dependent branch of NMD [8]. Later studies demonstrated that the regulation of *SMG5* mRNA by NMD is a conserved response and that other NMD factor mRNAs are negatively regulated by NMD in *D. melanogaster* and *Arabidopsis thaliana* [27,47,48]. Together, these studies suggested the possible existence of a feedback mechanism that protects NMD from insults.

Two recent studies—Yepiskoposyan *et al.* and Huang *et al.*—have characterized this ability of NMD to regulate its own factors in greater detail and have obtained evidence that NMD is regulated by a complex autoregulatory feedback system [14,49]. Together, these two studies demonstrated that the steady state levels of the mRNAs encoding seven NMD factors—UPF1, UPF2, UPF3B, SMG1, SMG5, SMG6 and SMG7—are upregulated in response to NMD knockdown in HeLa cells. This indicated that these NMD factors are all negatively regulated by NMD, which, in turn, suggested a remarkably simple mechanism that is

responsible for their autoregulation: the mRNAs that encode them are NMD substrates themselves (Figure 2A). Supporting this hypothesis, all seven of these NMD factor transcripts have long 3' UTRs (>1 kb) downstream of the stop codon, a feature that commonly elicits NMD [21,22]. Some of them have upstream open reading frames (uORFs), which can also trigger NMD [49]. Direct evidence for their role came from experiments in which the candidate NMD-inducing sequences were cloned into reporter constructs that allowed measurement of RNA half-life. This approach was first used in an earlier study from Singh *et al.* to show that the long 3' UTR in *SMG5* mRNA is sufficient to elicit degradation by NMD [21]. Yepiskoposyan *et al.* confirmed this and also demonstrated that the uORF in *SMG5* conferred weak NMD activity. The long 3' UTR in *UPF1* and *SMG7* were also found to elicit NMD [14,49]. To determine whether the endogenous mRNAs encoding NMD factors are NMD substrates, RNA half-life studies were performed. These experiments demonstrated that NMD inhibition stabilized several of the NMD factor transcripts, consistent with them being direct NMD substrates [14,49]. As another assay to detect NMD targets, Yepiskoposyan *et al.* performed RNA immunoprecipitation experiments to determine whether these transcripts were bound by the NMD factor UPF1, a property of transcripts subject to NMD [19,20]. Indeed, they found that most NMD factor mRNAs subject to autoregulation are bound to UPF1 [49].

Huang *et al.* obtained evidence that this NMD feedback network is a highly directed set of responses controlled by different branches of the NMD pathway acting in a tissue- and cell type-specific manner. Using cell lines of different tissue origins, they demonstrated that different sets of NMD factors are regulated by NMD in different cell types. These investigators confirmed this *in vivo* using NMD-deficient *Upf3b*-null mice. For example, they found that *UPF1*, *SMG1*, *SMG5* and *SMG6* mRNA are feedback regulated by the UPF3B-dependent NMD pathway in T cells, but not other lymphoid cells in the spleen [14]. They also demonstrated that NMD feedback regulation is developmentally regulated, based on experiments in embryonic stem cells. Finally, they determined that depletion of NMD factors required for different branches of the NMD pathway had different impacts on feedback regulation. In particular, their results suggested that UPF1 and SMG7 are feedback regulated by the UPF3B-dependent NMD branch, UPF1 and SMG5 are regulated by EJC-dependent NMD branch, and only SMG1 is regulated by the UPF2/RNPS1-dependent NMD branch.

To test the physiological relevance of NMD autoregulation, Huang *et al.* examined the feedback role of SMG1, which they found is a rate-limiting factor for NMD in HeLa cells. They determined that if they did not allow SMG1 to be upregulated in response to NMD perturbation (by using RNAi), this resulted in a stronger depression of the magnitude of NMD than NMD perturbation alone. This demonstrated that reversal of SMG1 feedback regulation protects NMD from insults. They also showed that the downregulation of SMG1 by feedback regulation is also important under normal circumstances (i.e., when NMD is not perturbed), as moderate overexpression of SMG1 inhibited HeLa cell growth [14]. Together these results suggested that it is critical that the level of SMG1 is tightly regulated, as either modest up- or down-regulation of SMG1 has negative consequences for cells.

Another NMD robustness-conferring mechanism that was recently discovered is one that acts at the level of protein stability to upregulate an NMD factor to compensate for loss of its stronger paralog. Chan *et al.* found that depletion of the NMD factor UPF3B leads to dramatic upregulation of the protein encoded by its paralog *Upf3a* [10]. Using various approaches, they demonstrated that the upregulation of UPF3A protein is the result of protein stabilization, not mRNA stabilization or increased gene transcription. Several lines of evidence indicated that UPF3A is normally unstable because it is outcompeted for binding to the NMD factor UPF2 by UPF3B. When UPF3B is depleted, this allows more

UPF3A to bind to UPF2, and thus UPF3A is stabilized. While UPF3A is a weak NMD factor, at least *in vitro* [50], Chan *et al.* found it is able to at least partially functionally compensate for UPF3B when the latter factor is depleted. This cross-regulatory mechanism has the potential to influence human disease (Figure 2B), as UPF3A protein levels are also elevated in intellectually disabled patients with germline silencing mutations in the *UPF3B* gene [10,51]. Indeed, it was shown that the level of UPF3A protein in *UPF3B*-deficient patients is inversely correlated with the severity of the patients' phenotype. This discovery is consistent with the possibility that the UPF3A-UPF3B cross-regulatory mechanism exerts a protective role in intellectual disability patients with mutations in the *UPF3B* gene [51].

2.4. Regulation in response to stress

In addition to repression in response to microRNAs and NMD itself, NMD can be repressed by stress (Figure 3). The first evidence for this was reported by Mendell *et al.*, who showed that amino-acid deprivation potentially inhibited NMD [6]. Later it was demonstrated that a variety of cellular stresses, including oxygen deprivation (hypoxia), nutrient deprivation, infection, reactive oxygen species (ROS), and double stranded RNA could also inhibit NMD [52]. A likely common mechanism by which these stresses inhibit NMD is through their ability to activate kinases that phosphorylate eukaryotic initiation factor 2- α (eIF2 α). eIF2 α is phosphorylated in response to many stimuli, including amino-acid deprivation (through the GCN2 kinase), ROS (through PERK and HRI kinases), and viral infection (through the PKR kinases) (Figure 3) [53–55]. The phosphorylation of eIF2 α by these many stresses has been termed the integrated stress response (ISR) [56]. With regard to NMD, eIF2 α phosphorylation has been shown to be an essential intermediary step by which stress inhibits NMD [53,54]. Indeed, if eIF2 α phosphorylation is uncoupled from stress, NMD is not inhibited [53,54]. For example, NMD is not inhibited in hypoxic cells depleted of the endoplasmic reticulum (ER)-resident kinase PERK, which is normally activated when hypoxia hampers ER protein folding and unfolded proteins accumulate in the ER [57]. Conversely, ectopic activation of PERK by chemical ligand-induced dimerization, which elicits eIF2 α phosphorylation in the absence of stress, is sufficient to inhibit NMD.

The mechanism by which eIF2 α phosphorylation inhibits NMD activity is unknown. An obvious possibility is that the ability of eIF2 α phosphorylation to suppress protein translation is responsible since NMD absolutely requires translation for PTC recognition [58,59]. However, suppression of translation is unlikely to be the only mechanism by which eIF2 α phosphorylation represses NMD. There are several reasons for this, including the finding that eIF2 α phosphorylation does not completely block translation and that stress does not completely prevent NMD mRNA targets from being translated [53,57].

3. Functional consequences of NMD Regulation

3.1 Augmentation of stress responses

What are the consequences of NMD being inhibited in response to stress? Several transcripts that assist in the cellular adaptation to stress, and are stress-induced via transcriptional and translational mechanisms, are also NMD targets. mRNA half-life analysis indicated that the inhibition of NMD can stabilize and hence augment the upregulation of these transcripts to better allow cells to respond to stress. For example, the mRNA encoding a key transcription factor in the unfolded protein response, ATF4, appears to be a direct NMD target, based on its stabilization in response to knockdown of the NMD factor UPF1 [6,41]. In addition, cells depleted of UPF1 have higher levels of ATF4 protein, and respond with stronger upregulation of ATF4 protein in response to chemically induced ER stress [41]. ATF4 has many important roles in cellular stress responses, including the upregulation of genes encoding protein chaperones and oxidative stress protection. As a consequence, the

inhibition of NMD has the potential to augment the ISR and increase cell survival in response to ER stress [53,57]. In addition to upregulating stress-response components such as ATF4, inhibition of NMD may influence stress responses by other means. One potential mechanism derives from the finding the NMD factors UPF1 [60] and SMG1 promote genome stability [61]. This leads to the prediction that depletion of UPF1 or SMG1 would not only reduce the magnitude of NMD, but it would elicit stress adaptation responses as a consequence of the loss of genome stability. It will be important for future studies to determine the relative contributions of the direct and indirect actions of NMD on stress responses.

3.2 An NMD-based feedback circuit regulating amino-acid metabolism

Similar to stress response factors, many of the transcripts important for amino-acid metabolism are encoded by mRNAs that are NMD targets. In fact, these transcripts represent a major subset of NMD substrates; microarray analysis of HeLa cells depleted of UPF1 revealed that ~15% of all upregulated transcripts fell into this category [6]. These mRNAs encode proteins involved in many aspects of amino-acid metabolism, including amino-acid transport and channels. One of these, the dibasic/neutral amino acid transporter—SLC3A2—appears likely to be a direct NMD target since *SLC3A2* mRNA is stabilized by depletion of UPF1 and has an uORF, a well-established motif that triggers NMD [6].

NMD may participate in feedback circuits important for upregulating amino-acid transporters to compensate for low levels of amino acids or to increase amino-acid levels when there is a need. This is supported by two findings. First, NMD is inhibited by amino-acid deprivation [6]. This is likely to be due, at least in part, to the activation of GCN2 kinase which, in turn, phosphorylates eIF2 α [62]. Second, as described above, many transcripts encoding proteins involved in amino-acid metabolism are upregulated when NMD is inhibited. However, it remains to be determined whether amino-acid transport proteins or amino-acid transport itself are upregulated in response to NMD inhibition. Of note, many amino-acid transporters are also upregulated by the NMD target ATF4 [55], suggesting the possibility that many of these amino-acid transporters may be regulated by NMD indirectly through the action of ATF4.

3.3 Global regulation of splicing

Among the most conserved NMD substrates are those encoding splicing regulators [12,63,64]. This raises the possibility that NMD plays a central and widespread role in splicing regulation [65]. A major class of splicing regulators targeted by NMD is serine-arginine (SR) proteins, a large family of proteins that typically bind to exonic enhancers and stimulate exon inclusion. In particular, NMD targets alternatively splice variants of SR protein mRNAs that contain PTCs, thereby creating negative-feedback circuits that allow SR proteins to autoregulate their own expression [12]. As an example, excessively high expression of the SR splicing factor SC35 promotes alternative splicing of its own mRNA to an isoform that acquires a PTC (and has a unique 3' UTR) and thus is rapidly cleared from the cell by NMD [66]. SC35 achieves this by virtue of its ability to bind to specific sites within its own pre-mRNA. By the same means, SC35 regulates the alternative splicing of many other mRNAs, and thus the ability of NMD to impact SC35 feedback regulation is likely to have global effects on alternative splicing. NMD-based autoregulatory feedback loops are employed by virtually all SR proteins, thereby further expanding the potential effects of NMD on alternative splicing [12].

Another example of splicing autoregulation revolving around NMD involves two RNA-binding proteins that have opposing roles in neuronal differentiation [67]. High expression of one of these, polypyrimidine-tract-binding-protein-1 (PTBP1), triggers alternative

splicing of its own pre-mRNA, leading to generation of an exon-skipped mRNA harboring a PTC that is degraded by NMD [68]. Among the targets of PTBP1 is a critical cassette exon in the pre-mRNA of PTBP2 (nPTB), a nervous system-enriched PTBP1 homolog. When this exon is skipped, *PTBP2* mRNA is also subject to NMD. During neuronal differentiation, the microRNA miR-124 has been shown to downregulate PTBP1 levels, leading to the accumulation of correctly spliced *PTBP2* mRNA and a dramatic increase in PTBP2 protein. These events culminate in the transition from non-neuronal to neuronal-specific alternative splicing patterns and promotion of neural differentiation [67,69]. Because PTBP2 is a pro-neural molecule, this is an example of a circuit revolving around alternative splicing, microRNAs, and NMD that dictate the decision as to whether a neuron differentiates or not.

In the examples given above, NMD appears to act as a biological switch that degrades alternatively spliced mRNAs in scenarios when the protein product is not needed. NMD also has other roles. For example, in some cases, NMD appears to operate as a quality control mechanism that clears cells of constitutively generated aberrantly spliced mRNAs [70,71]. NMD probably also regulates the steady-state levels of alternatively spliced transcripts that encode *functional* proteins. In support of this, a recent RNA-seq analysis study identified a large array of alternatively transcripts encoding normal proteins that were dysregulated in response to loss of the NMD factor UPF2 [65]. While some of these dysregulated alternatively spliced transcripts have known NMD-inducing features and thus are likely to be direct targets, many did not, suggesting this latter group includes alternative spliced transcripts indirectly regulated by NMD, such as through SR proteins. Regardless of the precise role of NMD in each individual case, it is very likely that NMD has a major impact on the “alternative splicing transcriptome” given that one-third of all splice variants generated by human genes contain a PTC and thus are predicted to be targeted by NMD for decay [9,13,65,72,73].

Not only does NMD impact splicing, but splicing regulates NMD. For example, it has been demonstrated that splicing efficiency dictates the magnitude of NMD. Thus, reducing splicing efficiency reduces the magnitude of NMD and enhancing splicing efficiency stimulates the magnitude of NMD [74]. This raises the possibility that regulatory loops connecting splicing pathways with NMD exist that serve to fine-tune both of these processes in a mutually dependent manner.

4. NMD regulation and disease

4.1 Aberrant mRNAs targeted by NMD impact cancer

Increasing evidence suggests that the magnitude of NMD is often inhibited in tumors. One mechanism by which this occurs is through eIF2 α phosphorylation, which, as described above, is an event triggered by a variety of cellular stresses in normal cells. Indeed, a variety of cellular stresses prevalent in tumors, including hypoxia, can trigger eIF2 α phosphorylation [53,57] (Figure 4). “Oncogenic stress” has also been shown to inhibit NMD through eIF2 α phosphorylation. Wang *et al.* showed that elevated expression of the c-myc oncogene, a common event in tumors, triggers eIF2 α phosphorylation through c-myc’s ability to activate the PERK kinase [54]. As a consequence, NMD is suppressed and there is an upregulation of NMD-targeted transcripts [54]. c-myc expression also triggers the generation of ROS, which also contributes to the suppression of NMD [54].

The role of NMD in tumors is likely to be complex. NMD can have either a beneficial or detrimental effect, depending on the nature of the mutation triggering NMD and the nature of the disease itself. This “Jekyll and Hyde” nature of NMD has been well documented by several studies examining NMD’s effects in human inherited genetic diseases [3,4,75]. NMD reduces the severity of genetic diseases caused by truncating dominant-negative

mutations since NMD degrades the mRNAs encoding such toxic proteins. In contrast, NMD increases the severity of genetic disorders when the truncating mutation does not completely abrogate normal function; i.e., by degrading the mRNA encoding a partially functional protein, NMD worsens disease symptoms.

As with other hereditary disorders, cancer is impacted by NMD in distinct ways depending on the particular transcript it targets and the location of the PTC. For example, one class of NMD substrates is mRNAs from mutant genes encoding dominant-negative truncated forms of tumor-suppressor proteins. By degrading this class of mutant mRNA, NMD protects heterozygous carriers with such mutations from developing cancer as long as the other tumor-suppressor allele remains intact [4]. A well-studied example is the tumor suppressor gene *BRCA1*, which causes familial cases of breast and ovarian cancer when mutated in the germline. NMD is believed to provide a protective role in these cancers by triggering the degradation of mutant *BRCA1* mRNAs that encode truncated dominant-negative forms of BRCA1 [76]. The decay of these mutant transcripts is crucial since carboxyl-terminal truncated BRCA1 proteins can confer chemoresistance, decreased susceptibility to apoptosis, and decreased ability to suppress *in vivo* tumor growth. Truncated BRCA1 proteins can also block the ability of ectopically expressed wild-type BRCA1 to induce chemosensitivity and inhibit “protective” estrogen receptor transcriptional activity, suggesting that truncated BRCA1 proteins function by inhibiting the function of wild-type BRCA1 [77]. By degrading the mRNAs encoding such dominant-negative tumor suppressor proteins, NMD exerts anti-cancer effects.

On the other hand, NMD has the potential to *increase* the severity of cancer syndromes when the tumor suppressor gene harboring the nonsense or frameshift mutation encodes a truncated protein retaining some or all of its beneficial activity. An example is the adhesion protein E-cadherin, which when truncated at the carboxyl-terminal region retains partial function [78]. Thus, in hereditary diffuse gastric cancer (HDGC), the vast majority of germline mutations in the gene that encodes E-cadherin—*CDH1*—lead to the generation of PTCs, most of which elicit NMD, thereby greatly reducing the level of the “protective” E-cadherin protein that is expressed from these gene alleles [79]. Evidence suggests that this reliance on the mutant *CDH1* allele occurs because of inactivation of the wild-type allele of *CDH1* by a second-hit event [80]. Clinical relevance is suggested by the finding that people harboring *CDH1* mutations that allow escape from NMD (because they are at the extreme 3' end of the gene) have a lower cumulative risk of developing HDGC than people harboring *CDH1* mutations that elicit NMD [81]. Indeed, a retrospective study revealed that mutations predicted to elicit NMD are associated with an earlier age of onset of HDGC [79].

There is some evidence that the multi-step selection process that generates tumors as a result of mutations in tumor-suppressor genes and oncogenes [82] results from selection events that take advantages of NMD for the benefit of the tumor. Thus, tumor-suppressor genes exhibit a disproportionate number of nonsense mutations, most of which would be predicted to trigger NMD and therefore lower the level of beneficial tumor suppressor proteins [83]. In contrast, most mutations in oncogenes are missense, which would not elicit NMD and thus permit high levels of expression of the encoded oncoproteins, some of which would be more active as a result of the missense mutations [83]. Below, we discuss how regulation and modulation of NMD in the tumor microenvironment can impact the fate of the tumor.

4.2 Normal, non-mutated mRNAs targeted by NMD impact cancer

As described above, the inhibited magnitude of NMD in tumors can either benefit the patient or the tumor, depending on the genes mutated and the location of the nonsense or frameshift mutations within these genes. An additional layer of complexity is the changes in levels of the vast repertoire of *normal* transcripts targeted by NMD in tumor cells. This was revealed

by an unbiased genome-wide approach taken by Wang *et al.* in osteosarcoma cells [57]. They identified ~750 mRNAs that were statistically upregulated in osteosarcoma cells in response to *every* condition they tested in which NMD was inhibited: RNA-mediated depletion of either the NMD factor UPF1 or UPF2, incubation with the protein synthesis inhibitor emetine, exposure to hypoxia, or incubation with the ER stress inducer tunicamycin. Intriguingly, over-represented among the upregulated mRNAs were those encoding proteins involved in tumor-promoting pathways. Several of these transcripts encode proteins with important roles in tumorigenesis, including ATF4, which has been shown to be required for tumor growth [84], and proteins involved in amino acid metabolism, a pathway increasingly appreciated to have an important role in cancer progression [85,86]. Many of these NMD-targeted transcripts have been shown to be upregulated in three-dimensional tumor models *in vivo*, as well as in Burkitt lymphoma, melanoma, breast, and prostate cancers. Consistent with these findings, there is evidence that inhibition of NMD encourages tumor formation. For example, prostate cancer cells that overexpress UPF1, and are thus no longer susceptible to NMD inhibition by cellular stress, do not grow as three-dimensional spheres or as tumor explants in nude mice, though their growth in standard non-stressed tissue culture conditions is unaffected [57]. Together, these studies strongly suggest that NMD inhibition by the tumor microenvironment is an important mechanism to dynamically regulate genes critical for tumorigenesis and cellular stress genes important for dictating the outcome of malignancy.

4.3. Regulation of NMD as a therapeutic approach

Nonsense mutations account for ~11% of all gene lesions known to cause human inherited disease and ~20% of disease-associated single-base pair substitutions affecting gene coding regions [83]. The knowledge we have acquired about how NMD is regulated has the potential to translate into therapeutic approaches to treat some of these genetic diseases. For diseases caused by mutant genes that encode functional proteins, the goal is to generate therapies that inhibit NMD so as to stabilize the mutant mRNAs encoding these functional proteins. Some progress has been made using this approach *in vitro*. Wortmannin and caffeine, which inhibit the essential NMD factor SMG1, have both been shown to upregulate the abundance of PTC-containing mutant collagen VI $\alpha 2$ mRNA, which encodes a truncated but partially functional protein. This resulted in the formation of partially functional extracellular matrix in cultured fibroblasts from patients with Ulrich's disease [87,88]. It will be important to determine whether sufficiently high doses of wortmannin and caffeine can be provided to patients to confer benefits without undue side effects. Other examples of diseases that evidence suggest are caused by downregulation of transcripts containing PTCs include cystic fibrosis (CF), a common autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, as well as ataxia-telangiectasia-like disorder, Hurler's syndrome, Frasier syndrome, and Duchenne muscular dystrophy [89]. Thus there is a considerable need for therapies that inhibit NMD.

Strategies targeting specific NMD factors could also be tested for efficacy. For example, screens could be done to identify small molecules that inhibit NMD factors. One particularly attractive target is SMG1; not only would inhibition of this phosphoinositide-3-kinase-related protein kinase (PIKK) be potentially broadly useful for the class of genetic diseases described above, but it might have other applications such as in acute myeloid leukemia (AML), which overexpresses SMG1 in some patients [90]. Furthermore, AML cells from some patients are sensitized to apoptosis in response to PIKK inhibition [90]. While agents that broadly inhibit NMD are potentially promising, a major concern is the side effects that might be caused by this. NMD is a branched pathway [8,10,16,33] and thus an approach to reduce side effects is to inhibit only a single branch of the NMD pathway. For example, the UPF3B-dependent branch of NMD is a viable target for NMD-inhibition therapy since this

branch can be completely eliminated in both mice and humans, even during early development, without causing lethality [14,91]. In contrast, complete loss of the pan-NMD factors, UPF1 and SMG1, cause embryonic lethality, at least in mice [72,92]. While loss of UPF3B causes intellectual and behavioral abnormalities [91], these defects may be the result of abnormal embryonic brain development and thus would not be triggered by inhibition of UPF3B after birth. Even if this was not the case, UPF3B-inhibition therapy could be specifically directed to diseased *non*-neuronal cells to avoid neurological toxicity.

Recent discoveries have led to the possibility of using other approaches to inhibit NMD. Thus, for example, delivery of the NMD-inhibitory neuron-expressed microRNA, miR-128 [25], to diseased non-neuronal cells would be predicted to inhibit NMD and thus be useful to treat the symptoms of diseases aggravated by NMD. Given that miR-128 acts by repressing UPF1, miR-128 therapy could also be useful to treat MSI colorectal tumors, which often overexpress UPF1 [93]. In the future, it will be important to determine whether there are microRNAs that target branch-specific NMD factors, as they would likely have less side effects than miRNAs targeting pan-NMD factors.

5. Final Remarks

It is likely that NMD regulatory networks are far more complex than we currently appreciate. It will be important to discover other inputs—in addition to miRNAs and environmental stress factors—that dictate the magnitude of NMD. The intricate nature of the NMD mechanism, which requires the dynamic association of several multi-protein complexes, suggests that there will probably be many modes of regulating NMD activity. Another means to control NMD is to regulate the decay of its components. For example, in yeast, Upf1 undergoes self-ubiquitination in response to its association with the NMD factor Upf3 [94]. Mutations of Cys and His residues in the CH domain impair both the ubiquitination of Upf1 and NMD, raising the possibility that ubiquitination-mediated degradation of Upf1 controls the magnitude of NMD. Another potential site of regulatory control is Upf1 phosphorylation [95]. Several lines of evidence from different organisms indicate that a cycle of phosphorylation and dephosphorylation of UPF1 is required for NMD [75]. However, it remains to be determined whether the rate of phosphorylation or dephosphorylation of UPF1 is subject to regulation.

The biological significance of NMD regulation is not well understood. Given the hundreds of mRNAs regulated by NMD, the consequences of NMD regulation are likely to be complex and depend on the extent of the regulation, the mechanisms by which it is exerted, the cell type and tissues involved, and the context in which NMD is regulated. For example, in the case of cancer, inhibition of NMD may be beneficial for some poorly vascularized tumors and allow them to adapt to cellular stress generated by the tumor microenvironment, but may be maladaptive for other tumor cells that harbor NMD-provoking mutations in detrimental genes. In summary, further investigation of how the magnitude of NMD is regulated has the potential to greatly impact both our basic understanding of organismal physiology and how to treat both genetic and non-genetic diseases in the future.

Acknowledgments

This work was supported by the National Institutes of Health grants RO1-GM058595 and RO1-DK08164.

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Highlights

- Comprehensive summary of known molecular mechanisms of NMD regulation.
- Discussion of the functional consequences of NMD regulation.
- Modulation of NMD regulation as a therapeutic approach for malignancy.

Fig. 1. MiRNA-mediated regulation of NMD

(A) The miRNA miR-128 directly represses the level of the NMD factor UPF1 and the EJC core component MLN51, which leads to low NMD activity. (B) AGO2, which is recruited to the 3' UTR of mRNAs by miRNAs, displaces the cap-binding complex (CBP) from the 5' cap, thereby inhibiting the translation of exon-junction complex (EJC)-bound mRNAs, which, in turn, inhibits the decay of these mRNAs by NMD.

Fig. 2. Autoregulation of NMD

(A) The mRNAs encoding seven NMD factors—UPF1, UPF2, UPF3B, SMG1, SMG5, SMG6 and SMG7—are degraded by NMD. This provides a buffering mechanism to preserve the integrity of NMD. In response to a stress that inhibits NMD, these mRNAs are stabilized, allowing higher levels of NMD factors to be translated for compensation. (B) Evidence suggests that the NMD factor UPF3A is highly unstable unless it binds to the NMD factor UPF2. Because UPF3A is outcompeted by its paralog, UPF3B, for binding to the NMD factor UPF2, UPF3A levels are normally low. However when UPF3B is depleted or lost, this allows much more UPF3A to bind to UPF2, leading to a dramatic increase in UPF3A levels, which permits a partial rescue of NMD. This cross-regulatory mechanism has the potential to influence the intellectual disability and psychiatric symptoms of patients with mutations in UPF3B.

Fig. 3. Mechanisms that inhibit NMD in response to stress

Many kinds of stress activate protein kinases that phosphorylate eukaryotic initiation factor 2α (eIF2α), an event that, in turn, inhibits NMD. In part, NMD may be inhibited because eIF2α phosphorylation suppresses protein translation, a process absolutely required for NMD. However, suppression of translation is unlikely to be the only mechanism by which eIF2α phosphorylation represses NMD, as eIF2α phosphorylation does not completely block translation and stress does not prevent NMD mRNA targets from being translated.

Fig. 4. Inhibited NMD impacts tumorigenesis

A variety of cellular stresses typically present in tumors inhibit eIF2 α phosphorylation, which, in turn, represses NMD. Suppressed NMD can, in theory, either promote or inhibit tumors (see text). Shown is a scenario that promotes tumor formation: stabilization of aberrant NMD target mRNAs encoding mutant dominant-negative tumor suppressor proteins (e.g., DN-BRCA1) and normal NMD target mRNAs that encode pro-tumor proteins and proteins that promote cellular adaptation to stress (e.g., ATF4).