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Review

The broader sense of nonsense

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The term 'nonsense-mediated mRNA decay' (NMD) was initially coined to describe the translation-dependent degradation of mRNAs harboring premature termination codons (PTCs), but it is meanwhile known that NMD also targets many canonical mRNAs with numerous biological implications. The molecular mechanisms determining on which RNAs NMD ensues are only partially understood. Considering the broad range of NMD-sensitive RNAs and the variable degrees of their degradation, we highlight here the hallmarks of mammalian NMD and point out open questions. We review the links between NMD and disease by summarizing the role of NMD in cancer, neurodegeneration, and viral infections. Finally, we describe strategies to modulate NMD activity and specificity as potential therapeutic approaches for various diseases.

Highlights

Nonsense-mediated mRNA decay (NMD) serves as a quality control mechanism by degrading mRNAs with premature termination codons (PTCs), and it also regulates the abundance of physiological RNAs encoding functional proteins.

Recent work showed that NMD can be triggered with a certain probability by each translation termination event.

Readthrough of the PTC can render NMD-sensitive mRNA molecules immune to NMD, which can explain why a certain fraction of an otherwise NMD-sensitive mRNA population persists.

Accurate prediction of NMD-sensitive transcripts based on sequence information is currently not possible; experimental testing is necessary.

NMD modulates the severity of many genetic diseases and affects antigen presentation in cancer cells and the progression of developmental diseases.

NMD targets many viral RNAs, but viruses circumvent this by expressing NMD-inhibiting proteins and harboring NMD-suppressing sequence elements in their RNAs.

NMD in a nutshell

Eukaryotic cells can sense mRNAs with PTCs and stimulate their degradation, an observation that was termed 'nonsense-mediated mRNA decay' (NMD [1–5]). In doing so, NMD mitigates the consequences of genetic mutations by degrading the produced mRNA molecules and therewith preventing the production of C-terminally truncated and potentially deleterious proteins [6]. In addition to clearing cells from aberrant transcripts, NMD also targets endogenous mRNAs for degradation that encode full-length functional proteins, which implies that NMD also contributes to the regulation of gene expression (Box 1). As expected for a gene expression regulation mechanism, the extent of downregulation differs widely among NMD-targeted transcripts. The effect of NMD ranges from only slight reductions of steady-state mRNA levels (often observed with PTC-free transcripts) to almost complete elimination of aberrant, PTC-containing transcripts, depending on different features of the transcripts and their association with specific RNA-binding proteins [1–5].

Throughout the lifetime of an mRNA molecule, many proteins dynamically interact with the nucleic acid chain, forming **messenger ribonucleoproteins** (**mRNPs**; see Glossary) that largely determine the fate of the mRNA [7]. Aberrant mRNAs arise from DNA mutations or errors during transcription or splicing, leading from small indels or substitutions to extended insertions and deletions. Faulty mRNAs are also capped and polyadenylated, and cells face the challenge of distinguishing right from wrong. The presence or absence of NMD-stimulating proteins in mRNPs thereby regulates the half-life of an mRNA, among other factors regulating mRNA half-life. These mRNP components may act like 'ticking bombs', which, depending on how fast they initiate RNA degradation after undergoing specific activation steps, results in weak or strong downregulation of the cognate transcript. Moreover, the concentration of core NMD factors contributes to the overall NMD activity in different cells and tissues [3]. The wide range of RNAs targeted by NMD to various degrees poses a true challenge for understanding which features render an mRNA sensitive to NMD and deciphering the molecular mechanisms of NMD, two central and still incompletely solved questions in the field. Adding further complexity, increasing evidence suggests that NMD represents a branched network of interconnected RNA decay pathways involving an overlapping set of *trans*-acting factors rather than one linear biochemical pathway. Owing to this complexity, several working models for NMD proposed during the past 30 years

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Box 1. NMD degrades a wide range of RNA substrates

NMD targets aberrant and proper transcripts. Faulty transcripts contain PTCs that originate from DNA mutations, transcription, splicing errors, and unproductive genomic recombination. Regarding this category of transcripts, NMD acts as a quality control mechanism that proofreads the coding properties of the transcriptome and rids the cell of defective transcripts. However, it is meanwhile well established that NMD also fine-tunes the levels of many physiological mRNAs that encode full-length, functional proteins affecting a broad spectrum of biological processes, including development, neurogenesis, cell stress, and others [78].

Apart from mRNAs, NMD also targets many of the supposedly noncoding spliced transcripts of genes encoding small nucleolar RNAs (snoRNAs) in their introns and of other long noncoding RNAs (lncRNAs) [9]. This implies that many lncRNAs do engage with ribosomes that translate their short ORFs.

Recent evidence highlighted that NMD contributes to shaping the transcriptome through genetic compensation (also called 'genetic buffering') [110–112]. Genetic compensation describes the phenomenon that pathogenic mutations often do not exert the expected adverse phenotypes due to the compensatory expression of other genes fully or partially restoring physiological functions. The unexpected role of NMD in genetic compensation, along with the complex mechanism of NMD activation, blurs the ability to predict the impact of NMD-eliciting mutations and obscures the development of therapeutic schemes.

The finding that NMD targets a wide range of physiological transcripts raises the question what exact features determine whether NMD targets an RNA and how this NMD substrate specificity is achieved mechanistically. Deep sequencing has been used to identify and catalog endogenous NMD-sensitive transcripts and understand their NMD-triggering features [52,53].

Different NMD-sensitive reporter mRNAs have been used to study NMD. The extent of their NMD-mediated downregulation varies, and the variation depends mainly on the relative position of the termination codon and the mode of NMD inhibition in these experiments. Variable NMD activity was also observed in cell cultures, with the cell-to-cell variability depending on the levels of SMG1 [25] and the EJC component RNPS1 [113].

It should be noted that the substrate specificity of NMD differs considerably between yeast and metazoan cells. In *Saccharomyces cerevisiae,* NMD-targeted transcripts are characterized by long 3′UTRs [114], whereas metazoan NMD relies heavily on molecular signals deposited on mRNAs during splicing (e.g., EJC).

may seem contradictory, and it is indeed a challenging endeavor to arrange the different puzzle pieces of NMD research into a coherent picture. Nevertheless, the implication of NMD in human disease and its importance in diverse biological phenomena fuel the interest in elucidating how NMD functions at the molecular level in better understanding its diverse biological roles.

This review aims to provide an up-to-date outline of mechanistic insights into NMD and its impact on human disease. Focusing on NMD in mammalian cells, we summarize established principles supported by several lines of evidence and then highlight novel or contradictory findings that accompany several proposed models. We also provide a concise overview of the interplay between NMD and disease, focusing on carcinogenesis, neurodevelopmental conditions, NMD modulation during viral infection, and therapeutic approaches.

Mechanistic aspects of NMD

Key steps of NMD: activation and degradation

The process of NMD can be divided into two stages: (i) an activation step, during which a specific mRNA molecule is selected for degradation, and (ii) the subsequent degradation step, when the selected mRNA is degraded by NMD-specific nuclease activities or by the recruitment of general cytoplasmic RNases implicated in mRNA degradation (Figure 1). In brief, NMD can be described as a translation-dependent mRNA degradation pathway initiated by the activity of UPF1 (up-frameshift 1), a central NMD-inducing factor. UPF1 is an ATP-dependent RNA helicase that binds nonspecifically to mRNAs. NMD activation is marked by UPF1 hyperphosphorylation and consequent activation of its helicase and ATPase activities [8]. UPF1 becomes phosphorylated by the kinase SMG1 (suppressor with morphogenetic effect on genitalia 1), and this step is stimulated by UPF2 and UPF3B, two UPF1-interacting partners. Phosphorylated epitopes of UPF1

Glossary

Exon junction complexes (EJCs): EJCs are deposited on RNAs during splicing 20–24 nucleotides upstream of the exon–exon junctions. The core EJC consists of eIF4A3, MAGOH, and Y14 (RBM8A) and recruits additional factors that affect mRNP localization, translation, and surveillance. The presence of EJCs >30 nucleotides downstream of the translation termination codon in an mRNA is the best known NMD-stimulating feature.
Frameshift-creating indels: insertion or deletions in the ORF of a gene that shift the reading frame, because the number of inserted or deleted nucleotides is not a multiple of 3. Frameshifts will usually lead to occurrence of a termination codon within the 20 codons following downstream of the mutation site and therefore prematurely truncate the ORF.
Messenger ribonucleoproteins (mRNPs): dynamic complexes composed of mRNA and the proteins bound to it. mRNAs are always found in the form of mRNPs inside cells.
Nonsense mutations: mutations in the ORF that change an amino acid codon into one of the three termination codons (UGA, UAG, UAA).
PIN domain: an approximately 130-amino acid conserved protein domain with endonuclease activity. PIN domains cleave single-stranded RNA, and they contain four nearly invariant acidic amino acid residues in their catalytic center. PIN domain-containing proteins are typically found in prokaryotic toxin-antitoxin systems.
Upstream open reading frames (uORFs): sequences located in mRNAs upstream of the main protein-coding ORFs that code for short peptide chains. Translation of a uORF can regulate the translation of the main ORF and can trigger NMD.

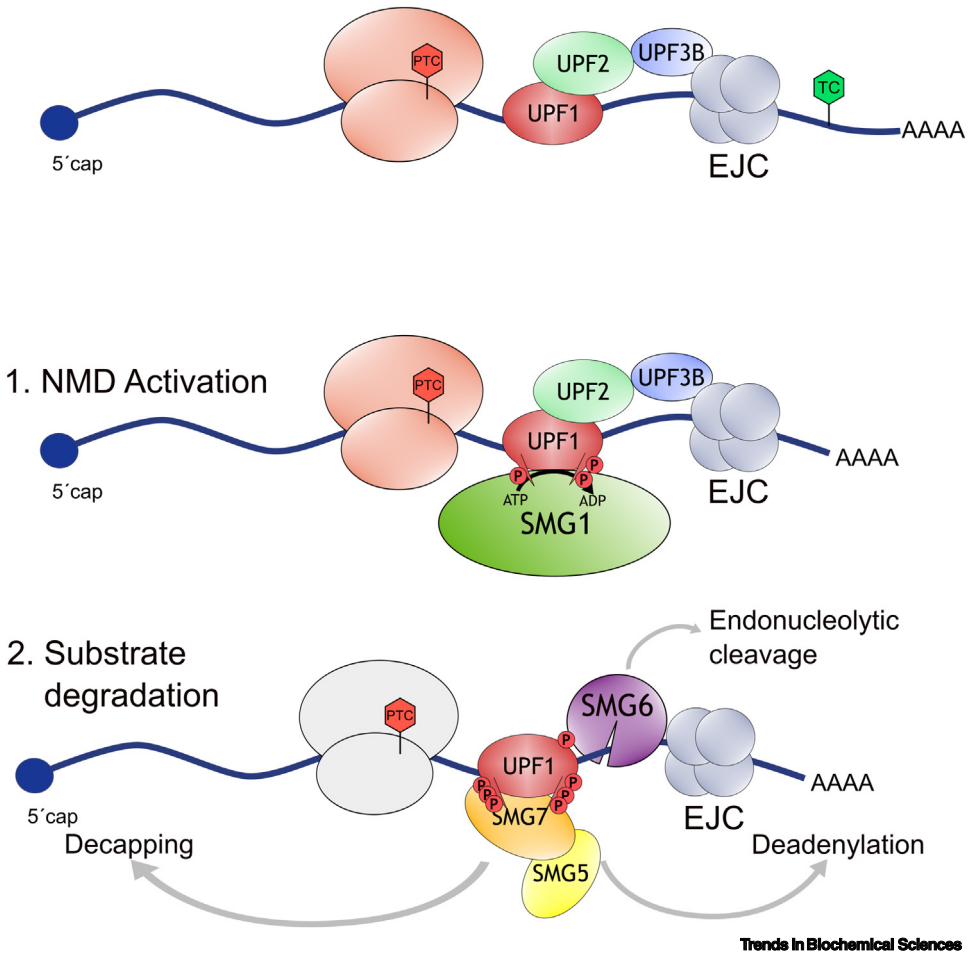

Figure 1. Nonsense-mediated mRNA decay (NMD) activation and mRNA substrate degradation is stimulated by the presence of an exon junction complex (EJC) downstream of a premature termination codon (PTC) after its recognition by a terminating ribosome. Translation termination at the PTC leads to SMG1-mediated hyperphosphorylation of UPF1, which is facilitated by UPF2, UPF3B, and the EJC. Hyperphosphorylated UPF1 recruits SMG6 and SMG5/7 to the RNA, leading to its degradation by endonucleolytic cleavage, deadenylation, and decapping. Abbreviations: SMG1, suppressor with morphogenetic effect on genitalia 1; UPF1, up-frameshift 1.

serve as binding platforms that recruit the endonuclease SMG6 [9–11] and the adaptor proteins SMG5 and SMG7 [12–14]. While SMG6 cleaves the mRNA in the vicinity of the termination codon, SMG5 and SMG7 promote deadenylation and decapping of the target mRNA [2].

NMD activation is linked to translation termination

NMD depends on translation. On a PTC-containing mRNA, each translation termination event appears to have a certain probability of triggering NMD [15], which depends on its mRNP composition. Depending on how high this probability is, NMD can occur during the first rounds of translation on mRNAs that are still associated with the nuclear cap-binding complex [16] or during later rounds, when the 5′ cap of the mRNA is bound by the eukaryotic initiation factor 4E (eIF4E) [15,17,18]. The mechanistic coupling between NMD and translation termination is supported by identifying protein complexes containing factors related to both processes [2,19,20].

Therefore, the decision whether NMD is triggered seems to happen during translation termination [3,21]. The process of translation termination starts when an elongating ribosome arrives at a termination codon (UGA, UAG, or UAA) and the eukaryotic release factor 1 (eRF1), together with GTP-bound eRF3, binds to the termination codon in the A site of the ribosome. Promoted by eRF3-mediated GTP hydrolysis, eRF1 hydrolyzes the peptidyl-tRNA bond of the nascent polypeptide residing in the P site, releasing the polypeptide chain from the ribosome. GTP hydrolysis further dissociates eRF3 from eRF1, and the ribosomal recycling factor ABCE1 (ATP-binding cassette subfamily E member 1) subsequently promotes dissociation of the ribosomal subunits. Data from *in vitro* translation experiments in yeast suggested that ribosomes may stall at PTCs (reviewed in [21]). However, *in vitro* and *in vivo* experiments using human cells showed similar ribosome occupancy on normal and NMD-eliciting termination codons, indicating that ribosome stalling is not a hallmark of NMD in human cells [22]. Using single-molecule imaging techniques, it was shown that every terminating ribosome at a PTC has an equal probability of eliciting NMD [15]. Therefore, the more often an NMD-sensitive mRNA is translated, the more likely NMD will degrade it. These results indicate that NMD is not the result of a kinetic dead end during translation termination but a more stochastic process that probably relies on the presence, combination, and concentration of specific proteins found in the vicinity of the termination codon [3]. According to this model, translation termination on an NMD-sensitive substrate may differ from regular termination in terms of the associated protein factors that stimulate UPF1 phosphorylation, an essential step of NMD activation.

Some NMD-sensitive mRNAs persist

Translation leads to mRNP remodeling because elongating ribosomes dissociate proteins from the open reading frame (ORF) and transiently disrupt secondary structures. Because translation termination at an early stop codon typically activates NMD due to NMD-promoting factors located further downstream on the mRNA [e.g., **exon junction complexes (EJCs)**], readthrough of ribosomes beyond the NMD-eliciting termination codon could dissociate such NMD-promoting mRNP components and render an NMD-sensitive mRNA immune to NMD. In principle, a single readthrough event on such an mRNA could confer NMD resistance. Supporting this hypothesis, two groups showed that stop codon readthrough caused by depletion of ABCE1 stabilized NMD-sensitive mRNAs, presumably by displacing NMD-activating proteins [23,24]. Consistently, in subpopulations of NMD-evading cells, approximately 20% of the protein produced from NMD-sensitive mRNAs that resist degradation originated from PTC readthrough. This rate is remarkably increased compared with the expected 1% readthrough rate for every single mRNA [25]. If indeed a single readthrough event is sufficient to stabilize an NMD-sensitive mRNA, this could explain why, even for the most sensitive NMD reporter genes, some transcripts appear to be resistant to NMD, a phenomenon also described as 'NMD escape' (observed, for instance, in [15,26]).

NMD relies on a set of core factors orchestrated by UPF1

Of the many factors associated with NMD, UPF1 plays a central role in orchestrating all NMD-related processes. UPF1 binds mRNAs without any pronounced sequence or secondary structure specificity and is found enriched in the 3′ untranslated regions (3′UTRs) of actively translated mRNAs, implying its displacement from the coding regions by elongating ribosomes [27,28]. UPF1 is an ATP-dependent RNA/DNA helicase with an unstructured N-terminal region, a cysteine-histidine-rich (CH) zinc-knuckle domain, a helicase region, and a C-terminal serine- and glutamine-rich (SQ) domain. ATP binds to a pocket between the RecA-like domains of the helicase core, consisting of RecA1 and RecA2. Two regulatory regions, 1B and 1C, can modulate the enzyme's processivity and affect its function [29]. Alternative splicing generates two UPF1 isoforms. Most UPF1 studies have been performed on the $UPF1_2$ isoform, the more abundant isoform in human cells. The $UPF1_1$ isoform harbors an additional 11-amino acid sequence in

the subdomain 1B that increases the binding and the helicase activity compared with the $UPF1_2$ isoform [30]. UPF1 helicase and ATPase activities are required for NMD and play a role in discriminating NMD-targeted transcripts from nontarget transcripts because ATPase-deficient UPF1 mutants fail to dissociate from nontarget transcripts [2].

The ATPase and helicase activities of UPF1 are regulated by its CH and C-terminal domains. When UPF1 is inactive, the helicase and ATPase centers are sterically inhibited, and UPF1 clamps to RNA [31,32]. This inhibition is alleviated by UPF2, which induces a conformational change of the CH domain that reduces RNA clamping and stimulates RNA unwinding, and by SMG1-mediated phosphorylation of UPF1. In its active form, UPF1 is a highly processive helicase and translocase essential for NMD. These activities have been linked to ribosome dissociation, mRNP remodeling, and recruitment of mRNA degradation factors ([32–34], reviewed in [2]).

In addition to UPF1 and UPF2, UPF3 is the third NMD factor that is conserved between metazoan and yeast. UPF3 also stimulates the activity of UPF1. There are two UPF3 paralogs in vertebrates, of which the generally more abundant UPF3B was shown to interact with the MIF4G-3 (middle domain of eIF4G) domain of UPF2 and with the EJC core factors MAGOH (mago nashi homolog) and eIF4A3 [2]. UPF3A, the second UPF3 paralog, is upregulated in cells lacking UPF3B and was recently reported to functionally substitute for UPF3B in promoting NMD [35,36].

As noted above, hyperphosphorylation of UPF1 by the phosphatidylinositol kinase-related kinase (PIKK) SMG1 is a key step for activating NMD in metazoan [37,38]. Recent publications provided structural details of the SMG1 complex, consisting of the Ser-Thr kinase SMG1 and two interacting proteins that regulate the enzymatic activity of the complex, SMG8 and SMG9 [39–43]. In the absence of UPF1, the C-terminal domain of SMG8 and the C-terminal insertion domain of SMG1 together repress SMG1 kinase activity [39] by inducing an autoinhibitory state of SMG1 [44].

Equally important as degrading NMD-sensitive mRNAs is protecting nontarget mRNAs from NMD. A hallmark for distinguishing targets and nontargets is the hyperphosphorylation of UPF1 [45]. Hyperphosphorylation is a common feature to regulate the activity of different proteins with crucial roles in gene expression, such as RNA polymerase II in transcription and SR (serine/arginine-rich) proteins in splicing [7]. The increasing phosphorylation of up to 19 different phosphorylation sites in the C- and N-terminal parts of transcript-bound UPF1 gradually increases UPF1 affinity for the degradation-inducing factors SMG6, SMG5, and SMG7 and renders the mRNA sensitive to NMD. This gradual charging of UPF1's potential to induce mRNA degradation reduces the risk of erroneously destroying nontarget mRNAs. UPF1 hyperphosphorylation has also been related to suppressing translation initiation, mRNP activation, remodeling, and dissociation from release factors. A series of protein interactions involving NMD factors, EJC components, and release factors [19,46] contribute to NMD initiation. A protein complex consisting of SMG1, UPF1, eRF1, and eRF3 is thought to interact with the EJC through UPF3, as well as through UPF2, which supports NMD activation [19,47,48].

Substrate degradation is mediated by NMD-specific and general degradation factors

UPF1 hyperphosphorylation attracts mRNA degradation activities in two ways. First, it recruits SMG6, an NMD-specific endonuclease [29]. The SMG6-mediated endonucleolytic cleavage of the RNA occurs in the vicinity of the termination codon through the catalytically active **PIN domain** (PilT N terminus) [10,11,49]. The resulting decay intermediates are rapidly further degraded by general exonucleolytic activities, including XRN1. A second degradation activity is brought about by the heterodimer SMG5-SMG7, which stimulates mRNA deadenylation by recruiting the CCR4-NOT complex (carbon catabolite repression–negative on TATA-less)

through the SMG7 proline-rich C-terminal domain followed by decapping and exonucleolytic degradation [14,50,51]. The two pathways seem to be largely redundant because they target the same transcripts and complement each other [52,53]. New evidence indicates that SMG5 not only is required for the SMG7/CCR4-NOT-mediated decay pathway but also plays a not yet fully understood but essential role in the SMG6-mediated decay pathway, revealing SMG5 as a gatekeeper for controlling the degradation steps of NMD [54,55]. UPF1 also interacts with PNRC2 (proline-rich nuclear receptor coactivator 2), which stimulates decapping, but its activity is not essential for NMD [14]. SMG6-induced mRNA degradation is stimulated by the presence of the EJC factors MAGOH and RBM8A (RNA-binding protein 8A) [56]. Recently, it was also shown that the EJC factor CASC3 (cancer susceptibility candidate 3) stimulates SMG6-mediated endonucleolysis when tethered to a reporter transcript [57].

Apart from UPF1, which is always required for NMD, the involvement of the other NMD factors can vary among different NMD-sensitive mRNAs, cell types, and species. Due to this apparent variability and complexity, NMD in metazoans is not considered a linear pathway but should rather be envisioned as a branched network of interconnected RNA decay pathways with overlapping, partially redundant activities [1].

RNA-binding proteins in the 3′UTR can trigger or inhibit NMD

That termination codons located >50 nucleotides upstream of the 3′-most exon–exon junction generally elicit NMD was noticed early on, and transcriptome-wide analyses confirmed that this so-called '50 nucleotides rule' is indeed the best predictor for human NMD-sensitive mRNAs [52,53]. EJCs are usually deposited during pre-mRNA splicing 20–24 nucleotides upstream of exon–exon junctions and removed by elongating ribosomes during translation from the coding sequence [58,59]. This finding provided a mechanistic explanation for the '50 nucleotides rule': EJCs bound in the 3′UTR are not removed from the mRNA by elongating ribosomes, and these persisting EJCs then stimulate NMD by interacting with and maybe even recruiting NMD factors [53,57]. Along these lines, recent reports showed that the total number of exon junctions of an mRNA correlates with its NMD sensitivity [15,53]. However, there are also exceptions of mRNAs with exon junctions in the 3′UTR that evade NMD, and genetic variants expected to induce NMD proved resistant to NMD [60].

Some of these exceptions can be explained by the presence of specific RNA-binding proteins in the vicinity of the termination codon that suppress NMD activation. For example, PABPC1 (polyadenylate-binding cytoplasmic protein 1) antagonizes NMD when it is tethered in the vicinity of an NMD-induction termination codon [61,62]. How PABPC1 suppresses NMD is not exactly understood. Although PABPC1's eRF3 binding domain is dispensable, the interaction of PABPC1 with eIF4G is required for NMD suppression [61,62]. The exclusion of UPF1 binding to the vicinity of the termination codon is mediated by other RNA-binding proteins such as PTBP1 (polypyrimidine tract-binding protein 1) [63] or hnRNP L (heterogeneous nuclear ribonucleoprotein L) [64]. Other NMD-evading mRNAs might be explained by the difficulty to accurately predict *a priori* the exact position of EJCs on RNAs. Attempts to experimentally identify EJC binding sites transcriptome-wide indicated that EJCs are not deposited on all exon–exon junctions, and some EJCs were detected in unexpected positions distant from exon–exon junctions [65,66]. Furthermore, the exceptions to the '50 nucleotides rule' highlight our limited understanding of NMD substrate specificity and suggest that additional features remain to be uncovered.

In human cells, 3′UTR length is a bad predictor of NMD sensitivity

Several studies on reporter mRNAs showed that long 3′UTRs can stimulate NMD [22,26,67–69], and initial studies aiming to identify endogenous NMD targets found an overrepresentation of mRNAs with long 3′UTRs among the NMD-targeted genes [70]. However, these early studies

based on microarrays or short-read sequencing impeded isoform-specific analysis of target mRNAs. More recently, an isoform-specific analysis was achieved using a combination of long- and short-read sequencing [53]. In this study, in which pairs of NMD-sensitive and -insensitive mRNAs from the same gene were analyzed, long 3′UTRs did not emerge as a predictive feature for NMD sensitivity. When transcripts with exon–exon junctions in the 3′UTR were excluded, the mean length of the 3′UTR was similar between NMD-sensitive and -insensitive mRNAs [53]. This finding implies that the mRNP composition and 3D architecture rather than the number of nucleotides of the 3′UTR contribute to the NMD sensitivity of RNAs.

Other NMD-modifying features

Some mRNAs with **upstream ORFs (uORFs)** are targeted by NMD, but which uORFs trigger NMD is not well understood [53,71]. Translation reinitiation often occurs downstream of a uORF, and, in this case, NMD is unlikely to ensue [5]. Reinitiation downstream of uORFs is enabled by initiation factors that remain bound to the ribosome and facilitate rescanning after translation of the uORF [72]. Evidence from cancer cells further indicated that long exons (>400 nt) and specific RNA motifs might also contribute to the sensitivity of physiological and aberrant mRNAs to NMD [73].

NMD and disease

Since its discovery, NMD has been linked to genetic diseases, and therefore, it is in the spotlight of biomedical research. The biological impact of **nonsense mutations** or splicing errors depends on the function of the gene and the position of the PTC. The production of truncated proteins originating from genetic loci with nonsense mutations can lead to loss of function of the cognate proteins, with severe consequences in homozygous carriers of such alleles. If truncated proteins adopt a dominant-negative function, even heterozygous nonsense mutations may cause severe disease symptoms if they outcompete the proteins coded from the healthy allele, preventing the formation of functional complexes. In such cases, NMD has a beneficial effect in limiting the production of such toxic truncated proteins by degrading the mRNAs that encode them, and pharmaceutical possibilities to specifically enhance NMD activity would be desirable. However, NMD can also aggravate disease phenotypes. This happens, for example, if NMD degrades mRNAs that code for a truncated protein that retains its full or partial function. In this case, NMD inhibition would be a promising therapeutic approach (Box 2). Moreover, recent advances have provided clear links between NMD and cancer, neurodegenerative diseases, and viral infections. Thus, NMD can impact human health in many different ways, and it is therefore not a surprise that NMD modulation is the focus of many therapeutic approaches (Box 2).

The impact of NMD on cancer

NMD impacts cancer progression and has been assigned both tumor-suppressing and tumor-enhancing roles [73–75]. By degrading mRNAs of transcripts from mutated alleles, NMD acts as a quality control system with tumor suppressor effects. Many mutations in tumor-suppressing genes, such as *BRCA1*, *ATM*, *VHL*, *p53*, *NF1*, and *NF2*, produce NMD-sensitive transcripts [76], and NMD prevents or reduces the pathological impact of some of these mutations. Besides nonsense mutations and **frameshift-creating indels**, splicing alterations leading to out-of-frame transcripts with PTCs are also NMD targets [77]. In line with its alleged antitumor function, there is evidence that NMD activity is repressed during tumor development. NMD repression occurs indirectly by the tumor microenvironment activating the integrated stress response [74], a stress condition known to inhibit NMD [78].

A second emerging link between NMD and cancer concerns neoantigens resulting from mutations accumulating in cancer cells. By degrading many of the mutated transcripts, NMD limits the production of neoantigens, which are immunogenic peptides that activate T cell responses

Box 2. Therapeutic approaches to modulate NMD

Given the many connections between NMD and disease, NMD modulation is the goal of several therapeutic approaches [115]. In the context of genetic mutations, an obvious way of alleviating the impact of a nonsense mutation is to promote readthrough of the PTC, which would allow the synthesis of the full-length polypeptide chains. During readthrough, the PTC is recognized by a near-cognate tRNA that competes with eRF1 to bind to the termination codon. Most efforts aim to suppress nonsense mutations by employing small molecules, suppressor tRNAs, RNA editing, RNA modification, or CRISPR technology. An apparent concern of these approaches is that a general induction of readthrough on essentially all mRNAs would be toxic for cells. For this reason, the therapeutic window of readthrough-promoting molecules needs to be carefully assessed.

Aminoglycoside antibiotics can stimulate PTC suppression. These molecules are thought to compete with translation termination, but the mechanistic details of this process remain obscure. Although aminoglycosides have shown promising results in disease models [116] and clinical trials [117], several drawbacks limit their application. Their efficiency depends on the stop codon identity and the nucleotide context in the vicinity of the PTC, and their use may affect the translation of essential genes such as histones, selenoproteins, and metabolic enzymes due to the lack of specificity [118]. Despite the aforementioned concerns, nonsense suppression is a promising therapeutic tool to promote selective readthrough. To this end, it is essential to delineate the factors that define readthrough to increase its specificity. The oxadiazole derivative ataluren, formerly known as PTC124, is another readthrough-promoting drug [119,120] that is clinically approved for treating Duchenne muscular dystrophy caused by nonsense mutations and has promoted readthrough in cells derived from patients with Usher syndrome [121]. Amlexanox, an antiallergic and anti-inflammatory oxadiazole derivative, was also shown to induce readthrough [83].

Several approaches attempt to correct nonsense mutations at the mRNA level by RNA editing or by using CRISPR-based genome editing to correct the mutations in the DNA. Adenosine deaminases that act on RNA (ADARs) are enzymes that convert adenosine to inosine in metazoan RNAs. In coding regions, inosine is read as guanosine [122]. ADAR-mediated RNA-specific editing of PTCs (UAA, UGA, UAG) could recode them to tryptophan (UGG), which has been attempted by different approaches [115].

against tumor cells [79,80] (Figure 2). Based on this, and because depletion of NMD factors such as UPF2 or SMG1 can reduce tumor growth [81], inhibition of NMD is expected to stimulate the production of neoantigens and reduce tumor progression. Interestingly, increased NMD activity was reported in a subgroup of colorectal cancers with widespread instability in microsatellite sequences [82]. NMD suppression in these cells stabilized PTC-containing mRNAs, and treatment with amlexanox, an oxadiazole-derived NMD inhibitor [83], stabilized several NMD targets and reduced the tumor size in subcutaneous xenografts [82]. In a similar cancer model, analysis of human leukocyte antigen class I-presented peptides revealed indel frameshift-derived neoepitopes [84,85], and their presentation increased upon NMD inhibition using 5-azacytidine [85], providing an additional link between NMD modulation and the possibility of enhancing the presentation of cancer-specific neoantigens to the immune system.

Consequently, the intricate link between NMD and cancer complicates the development of therapeutic approaches. The variability in the NMD pathway efficiency among different individuals, tissues, and single cells [25], the wide range of degradation efficiency for specific NMD targets, and the difficulty in accurately predicting NMD-eliciting mutations call for the development of personalized therapeutic approaches rather than a one-size-fits-all treatment.

The role of NMD in neuronal development and neurodegenerative diseases

Several mutations in core NMD factor genes are related to developmental and neurological syndromes, attributing to NMD a critical role in the development and function of the nervous system [86]. The most striking evidence for the importance of NMD in neuronal development is that point mutations and copy number variations in genes encoding NMD factors are associated with intellectual disability [78]. UPF3B has been associated with various conditions such as X-linked intellectual disability, schizophrenia, facial abnormalities, autism spectrum disorder, and attention-deficit/hyperactivity disorder. UPF3B-knockout mice showed a defect in the ability of neural stem cells to differentiate into functional neurons in cell culture and a defective

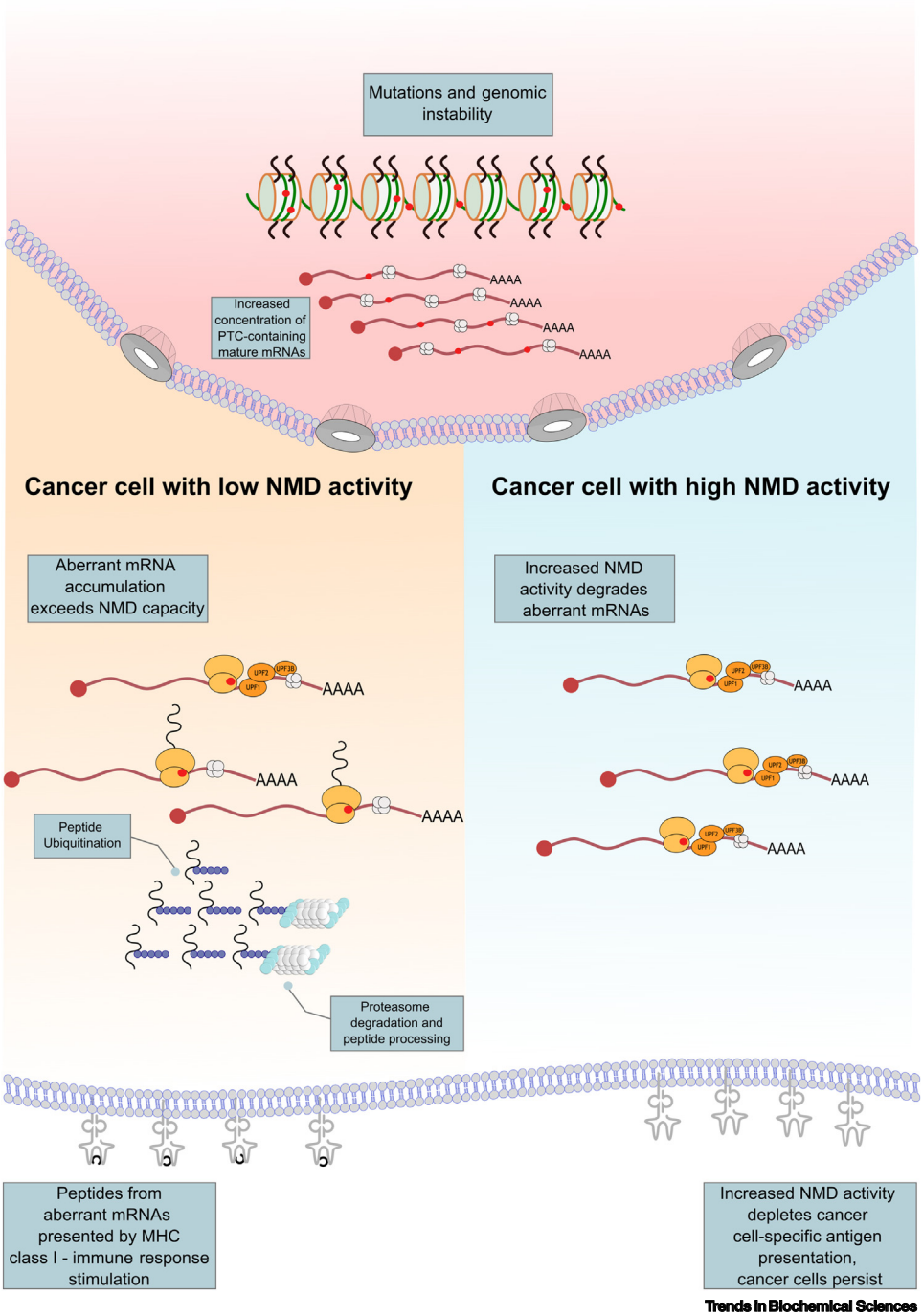

Figure 2. Nonsense-mediated mRNA decay (NMD) activity affects the presentation of neoantigens at the surface of cancer cells. Red dots on DNA (green) and mRNAs (red) represent premature termination codons that occur with increased frequency in cancer cells due to genome instability and hypermutation. Ubiquitin is represented as purple spheres.

maturation of dendritic spines affecting mainly pyramidal neurons in the prefrontal cortex [87]. That humans and mice without UPF3B are viable but have developmental defects implies that UPF3B has important tissue-specific roles. Similarly, neuron-specific disruption of UPF2 and

conditional knockouts in mice also caused cerebral defects that affected the cognitive capacities of the mice, and different causalities have been proposed [88–90]. Additionally, numerous cases suggest an essential role of SMG8 and SMG9 in neurodevelopment [91–93].

Considering the extensive alternative pre-mRNA splicing taking place in neuronal tissues, it is plausible that slight modulations of NMD activity that do not have a severe impact on other tissues wreak havoc on the neuronal transcriptome and lead to specific neuronal disease phenotypes.

How viruses protect their genome from NMD

NMD has been shown to act as the first line of defense against viral infections by degrading many viral RNAs [94,95]. Vice versa, viruses evolved different strategies to evade NMD, primarily by expressing viral proteins that antagonize NMD or by harboring sequence motifs on their RNAs that protect them from NMD (Figure 3).

Examples of viral proteins that inhibit NMD

In some cases, viral proteins can modulate the host cell NMD activity, and examples stem from different phylogenetically distant positive-sense single-stranded RNA viruses. For instance, in mouse hepatitis virus (MHV), which belongs to β-Coronaviridae [like severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome coronaviruses], the structural N protein (nucleoprotein), which packages the genomic RNA and enhances viral transcription and replication, was shown to inhibit NMD [96]. MHV infection led to the stabilization of NMD-sensitive transcripts independently of the virus-induced general translation inhibition, and co-expression of the N protein with NMD reporter RNAs led to a stabilization of the reporter RNA. Notably, the N protein is delivered to cells together with the MHV genomic RNA, probably offering protection from NMD. Intriguingly, N proteins from SARS coronavirus 2 (SARS-CoV-2) [97] and the avian infectious bronchitis virus [98] coprecipitate with UPF1, but the functional consequences of this interaction are still unclear.

Another example of a virus encoding an NMD-inhibiting protein is Semliki Forest virus (SFV). SFV is a mosquito-borne positive-sense single-stranded RNA alphavirus that causes lethal encephalitis in mice. NMD restricts replication of alphaviruses by degrading the viral genomic RNA [99], and the capsid protein, which is produced at very high amounts during viral replication, inhibits NMD [100].

In the case of the hepatitis C virus, another positive-sense single-stranded RNA virus, NMD is inhibited by an interaction between its core protein and the host cell protein PYM1 (PYM homolog 1). PYM1 can stimulate NMD [101] , but further details on the inhibition mechanism remain unclear. Proteomic data from West Nile virus (WNV), a flavivirus, indicated that the WNV capsid protein also interacts with PYM1 and interferes with EJC function and NMD [102].

The capsid protein of Zika virus (ZIKV) interacts with UPF1 and UPF3B and targets the nuclear fraction of UPF1 for proteasomal degradation, thereby inhibiting NMD. Additionally, the absence of UPF1 enhances the permissibility of ZIKV in neural progenitor cells [103]. However, how nuclear UPF1 contributes to ZIKV restriction is not yet understood. The T cell lymphotropic virus type I-encoded Tax factor also inhibits NMD. Tax reduces the affinity of UPF1 for RNA by interacting with its central helicase domain, inhibiting its ATPase and translocase activity [104].

NMD-evading viral RNA sequences

As mentioned above, host cell proteins such as PABPC1, PTBP1, and hnRNP L can confer immunity to NMD-sensitive transcripts when binding to the 3′UTR proximal to the termination codon. Similarly, the unspliced transcript of Rous sarcoma virus harbors an RNA stability element downstream of the termination codon, which binds multiple PTBP1 proteins that prevent binding

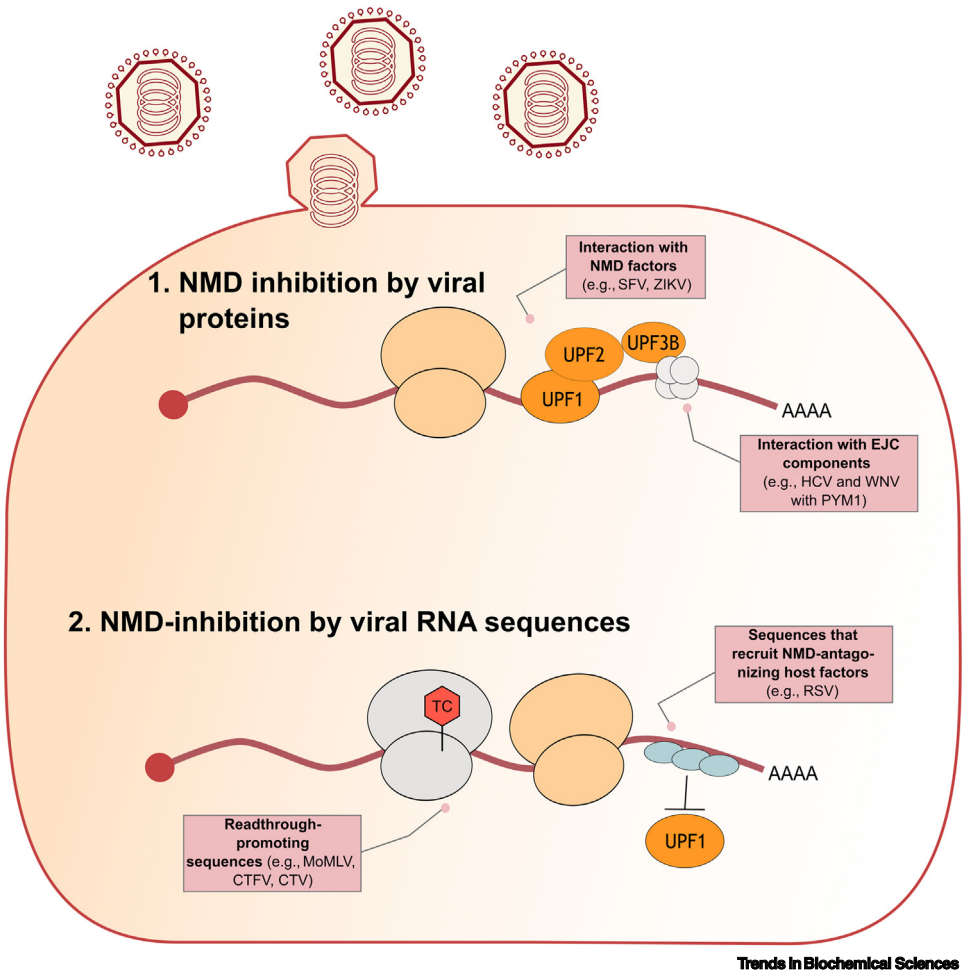

Figure 3. Viruses have evolved different strategies to protect their mRNAs from being targeted by nonsense-mediated mRNA decay (NMD). Viruses can inhibit NMD by expressing viral proteins that interact with NMD or exon junction complex (EJC) factors (upper part), or their mRNAs harbor sequence motives that promote readthrough or bind NMD-suppressing host cell factors (e.g., PTBP1) to evade NMD. Abbreviation: PTBP1, polypyrimidine tract-binding protein 1.

of UPF1 to this region, thereby preventing NMD of this RNA [105]. NMD-sensitive mRNAs can also evade NMD by readthrough of the early termination codon. At least three viruses, Moloney murine leukemia Virus), the human pathogenic Colorado tick fever virus, and the plant Turnip crinkle virus employ this strategy. Readthrough signals required to produce multiple proteins contribute to the transcript stability by protecting NMD activation [106–108]. As it is expected that more examples of NMD-inhibiting viral proteins will be discovered in the future, it would be of interest to explore the clinical implications of this inhibition and assess potential therapeutic opportunities.

Concluding remarks

In this review, we outline the hallmarks of mammalian NMD based on our current knowledge and, at the same time, highlight its complexity and the many still unresolved questions (Figure 4; see Outstanding questions). The lack of mechanistic understanding of why and how certain translation termination events activate NMD is one of the most significant knowledge gaps in the field.

Outstanding questions

What is the connection between translation termination and NMD activation?

Which factors determine the probability of an mRNA to undergo NMD?

How and when is UPF1 recruited in the mRNPs?

What is the series of events that lead to the activation of UPF1? Does SMG1 phosphorylation precede the allosteric conformation changes of UPF1 upon interaction with UPF2/UPF3B or vice versa?

What is the connection between UPF phosphorylation and its ATPase/helicase activity?

How and by which factors is UPF1 dephosphorylation regulated?

What is the exact functional relationship between UPF1, SMG5, SMG6, and SMG7? To what extent do the SMG6-mediated and the SMG7-mediated degradation activities overlap, and what is their relative contribution to NMD?

Can NMD inhibition improve the efficacy of cancer immunotherapies?

Can transcript-specific readthrough be achieved with small-molecule drugs?

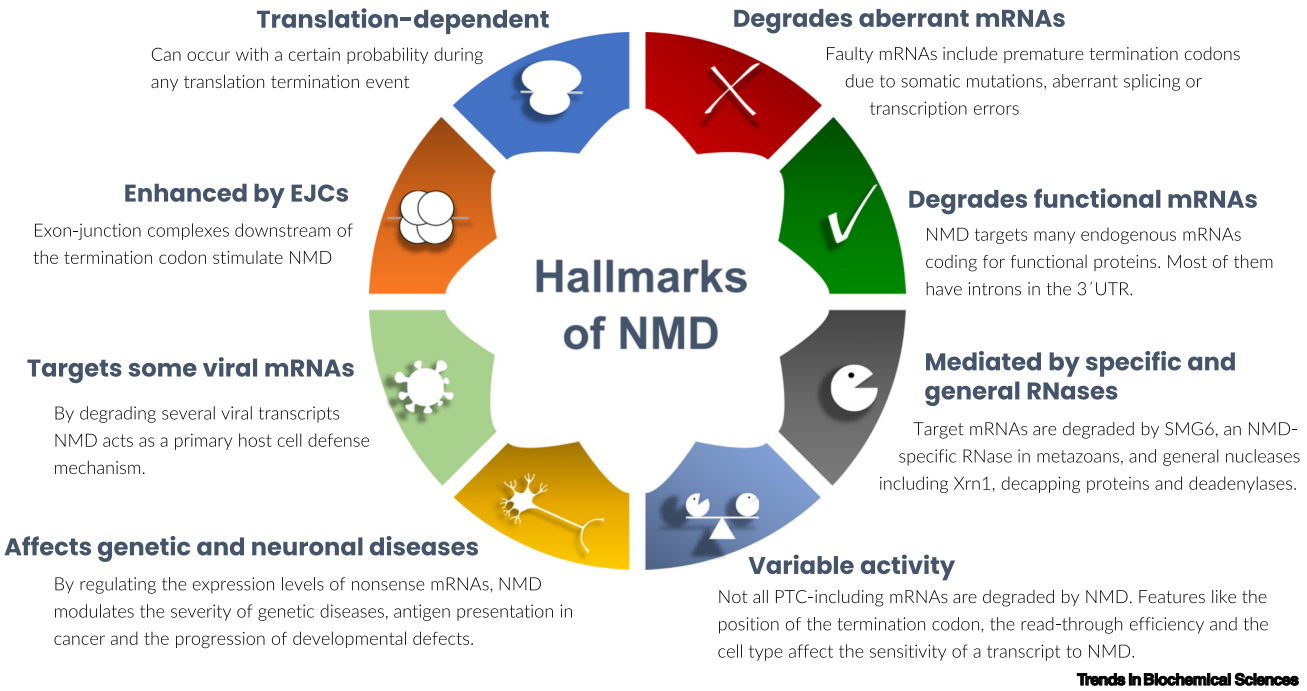

Figure 4. The hallmarks of mammalian nonsense-mediated mRNA decay (NMD). Abbreviation: EJC, exon junction complex.

Moreover, the sequence of molecular interactions between UPF1, UPF2, UPF3B, and SMG1 leading to NMD activation requires further clarification. Due to these gaps in understanding and the complexity of NMD, the consequences of PTC-causing mutations need to be carefully investigated. It cannot be assumed that NMD targets every PTC-containing RNA, and each specific case needs to be experimentally tested. Consequently, the frequently applied strategy of introducing PTCs to generate gene knockouts will not always lead to the desired inactivation of the gene, because these PTC-containing mRNAs may evade NMD and produce truncated and partially functional proteins, depending on the position of the termination codon [109]. Moreover, proteins originating from nonsense mRNAs that escape NMD can partially restore the function and confer phenotype plasticity that may give rise to new functions. Future research focusing on mechanistic studies with single-molecule approaches and in-depth analysis of NMD-related disease models will undoubtedly contribute to clarifying further the hallmarks and biological properties of this intricate molecular pathway.

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Declaration of interests

The authors have no interests to declare.

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