


Review Article

The long and short of EJC-independent nonsense-mediated RNA decay

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Nonsense-mediated RNA decay (NMD) plays a dual role as an RNA surveillance mechanism against aberrant transcripts containing premature termination codons and as a gene regulatory mechanism for normal physiological transcripts. This dual function is possible because NMD recognizes its substrates based on the functional definition of a premature translation termination event. An efficient mode of NMD target recognition involves the presence of exon-junction complexes (EJCs) downstream of the terminating ribosome. A less efficient, but highly conserved, mode of NMD is triggered by long 3' untranslated regions (UTRs) that lack EJCs (termed EJC-independent NMD). While EJC-independent NMD plays an important regulatory role across organisms, our understanding of its mechanism, especially in mammalian cells, is incomplete. This review focuses on EJC-independent NMD and discusses the current state of knowledge and factors that contribute to the variability in the efficiency of this mechanism.

Introduction

Nonsense-mediated RNA decay (NMD) is a quality control process that degrades transcripts that contain a premature termination codon (PTC) arising from nonsense, frameshift, or splice-site mutations. By degrading PTC-containing transcripts, NMD protects the cell from the accumulation of potentially deleterious truncated protein products. NMD also acts as an additional layer of post-transcriptional gene regulation for transcripts that mimic NMD substrates during normal biological processes [1–4]. Discrimination of PTC-containing transcripts relies on the recognition of a translation termination event as premature due to an abnormal termination environment. An abnormal termination environment may be defined by factors such as increased distance from the poly(A) tail and the presence of RNA binding proteins (RBPs) not typically present in the 3' UTR, such as exon junction complexes (EJCs) [5]. Once a PTC is recognized in a translation-dependent manner, the key NMD factors UPF1, UPF2, and UPF3A/B, which are conserved from yeast to humans, assemble at the PTC [6]. In mammals, the SURF complex forms at the PTC, consisting of UPF1, eukaryotic release factors 1 and 3, and the UPF1-specific kinase SMG1 [5]. This assembly promotes the hyperphosphorylation of UPF1, which serves as the trigger for the decay of the PTC-containing mRNA by recruited mRNA decay factors [2,5–7].

There are two variations of the NMD pathway that recognize and degrade PTC-containing transcripts: (1) EJC-dependent NMD, which relies on the retention of EJCs near splice sites that are at least 50–55 nt downstream of the PTC (Figure 1A) [8,9] and (2) EJC-independent NMD, where a long 3' UTR is recognized (Figure 1B) [10–12]. Both variations of the NMD pathway rely on UPF1 and involve UPF2 and UPF3. However, some evidence suggests that EJC-independent NMD may be affected more by the depletion of UPF2 and UPF3B compared with EJC-dependent NMD [5]. EJC-dependent NMD has been observed in *Caenorhabditis elegans*, *Drosophila melanogaster*, and

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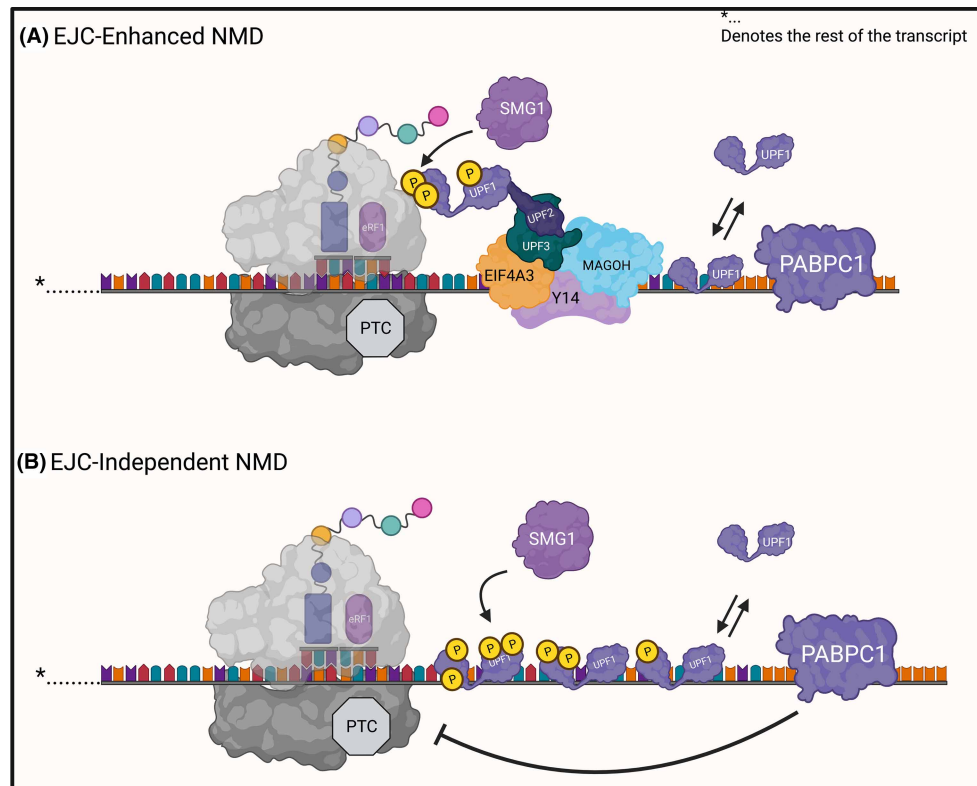


Figure 1. EJC-dependent versus EJC-independent NMD Mechanisms.

(A) EJC-dependent NMD requires an exon junction complex (EJC) downstream of the terminating ribosome. When this condition is met, UPF1–3 and eRF1 bind the EJC. (B) EJC-independent NMD relies on the recognition of an abnormal termination context in the absence of downstream EJCs. The proximity of PABPC1 to the termination codon may inhibit EJC-independent NMD. Nucleases degrade the released transcript after ribosomal dissociation. Both pathways require UPF1 surveillance and hyperphosphorylation of UPF1 by SMG1, which causes the ribosome to release from the transcript and subsequent transcript degradation by recruited nucleases.

mammals and is the more efficient form of NMD, enhanced by the sustained presence of EJCs downstream of the PTC [5,13,14]. EJC-dependent NMD has been extensively studied in mammalian systems and has been reviewed in detail in the following articles [15–20].

EJC-independent NMD is a conserved mechanism of mRNA decay that relies on the recognition of long 3' UTRs independently of retained EJCs. Long 3' UTRs place the stop codon in an abnormal termination context that is sufficient to trigger NMD even in the absence of downstream EJCs. Despite higher eukaryotes having evolved EJC-dependent NMD as an additional level of regulation of gene expression that is lacking in *Saccharomyces cerevisiae*, the basic mechanism of long 3' UTR discrimination in EJC-independent NMD remains conserved across eukaryotes [13,14,21,22]. This form of NMD relies on the length of the 3' UTR to detect the PTC, which may occur in part due to the increased distance to the poly(A) tail and differences in sequence composition, structure, and RBP occupancy on the long 3' UTR [10,12,23–25]. While this mechanism makes sense as a surveillance process in yeast, where the mean normal 3' UTR length is only 121 nt [26], the significantly longer 3' UTRs of mammalian transcripts (~1278 nt on average [27]) makes the EJC-independent distinction of PTCs from normal stop codons more challenging. This review will delineate the current state of knowledge on EJC-independent NMD, which is more poorly understood than EJC-dependent NMD, especially in mammalian cells. We will first describe EJC-independent NMD in yeast, where it was discovered and is more well characterized, followed by outlining the current knowledge on the more complex regulation of this pathway in mammalian cells. We will then address the many ways in which the efficiency of EJC-independent NMD can be modulated by *cis* and *trans*-acting factors, discuss the physiological importance of this form of NMD, and highlight open questions in this field.

Features that trigger EJC-independent NMD

Across eukaryotes, PTCs can trigger NMD if a PTC creates an extended 3' UTR, even if this 3' UTR lacks EJCs. In *S. cerevisiae*, whose genes contain few introns and lack EJCs, EJC-independent NMD triggers the degradation of PTC-containing transcripts [10,25]. Studying NMD in *S. cerevisiae* led to the 'faux-UTR' model of NMD, where long 3' UTRs are sufficient to trigger NMD [10,25,28]. The significance of a longer 3' UTR for EJC-independent NMD appears in part to be the increased distance between the termination event and poly(A)-binding protein (PABPC1), as positioning PABPC1 closer to the PTC antagonizes NMD [10,12,13,23]. Though studies in multiple organisms indicate that the proximity of PABPC1 to the PTC can regulate NMD efficiency, it should be noted that neither PABPC1 nor the poly(A) tail is necessary for NMD in *S. cerevisiae* [29]. Thus, although EJC-independent NMD is conserved among many eukaryotes, species-specific regulation adds nuance to the distinction between EJC-independent and dependent NMD. In *Schizosaccharomyces pombe*, for instance, NMD is enhanced by the presence of PTC-proximal introns in a splicing-dependent but EJC-independent manner [30].

In support of the long 3' UTR model of EJC-independent NMD, transcripts containing the same open reading frame (ORF) but different 3' UTR lengths are discriminated between by the *S. cerevisiae* NMD machinery, with a 3' UTR of 1170 nt triggering decay but 3' UTRs < 285 nt evading NMD [28]. This NMD pathway is largely conserved in the early branching eukaryote *Tetrahymena thermophila*, which lack a functional EJC and carry out EJC-independent NMD of PTC-containing transcripts, primarily relying on the UPF protein family [31]. Similarly, in *D. melanogaster*, extending the 3' UTR length from 198 nt to 396 nt following a previously normal termination codon redefines that transcript as an NMD target [13], and in *C. elegans*, NMD occurs independently of exon-exon junctions and increases in strength as the PTC is moved farther from the 3' end of the transcript [21]. EJC-independent NMD is also conserved in *Arabidopsis thaliana* [22] and in mammalian cells, where 3' UTRs > 300 nt or 420 nt, respectively, can promote NMD in a length-dependent manner [12,23,24]. However, the regulation of EJC-independent NMD is more complex than UTR length alone, with RBP interactions and UTR sequence and structure often playing a significant regulatory role in the efficiency and evasion of NMD, as discussed in the next section in more detail [12,32,33].

In conjunction with 3' UTR length, factors including the sequence and structural composition of the UTR and RNA-RBP interactions can regulate sensitivity to EJC-independent NMD in both yeast and mammals. In yeast, the downstream sequence element (DSE), 5'-TGYYGATGYYYYY-3', following a PTC can trigger EJC-independent NMD when it is bound by protein factors including Hrp1/Nab4 and UPF1 (Upf1p in *S. cerevisiae*) following premature translation termination [34-36]. In mammalian cells, genome-wide analyses of transcripts stabilized by NMD inhibition have shown that NMD-triggering 3' UTRs are enriched for GC-rich sequence motifs compared with non-NMD target 3' UTRs and are often predicted to be structured [37,38]. In support of a role for 3' UTR secondary structure in NMD, inserting a stem loop near the DSE in yeast NMD targets enhances NMD [35].

Differences in length, sequence, and structural composition of 3' UTRs can also influence RNA-RBP interactions to influence NMD efficiency [12,24,38,39]. The RNA helicase UPF1 is a highly conserved NMD factor that binds RNA tightly but nonspecifically [6]. Studies in mammalian cells have shown that the association of UPF1 with transcripts is dependent on 3' UTR length and that 3' UTRs of NMD targets are enriched for UPF1 [23]. The GC-rich motifs present in NMD-targeted mammalian 3' UTRs also enhance UPF1 association. Fusing the GC-rich long 3' UTR of an NMD target to a reporter gene decreased the reporter mRNA stability in a translation and UPF1-dependent manner, and this effect was absent when four GC motifs were substituted with adenine [38,40]. It should be noted that this enhanced association of UPF1 may rely on as yet undiscovered cofactors and not solely sequence motifs. This increased UPF1 occupancy on long 3' UTRs prepares the transcript for rapid commitment to NMD after the hyperphosphorylation of UPF1 by SMG1 and the recruitment of NMD effector enzymes [6,23].

The recognition of a stop codon as premature requires translation termination at the PTC [24,41]. Recent research using EJC-dependent NMD reporters has shown that each termination event has an equal probability of triggering NMD, hence each termination event at a PTC on a single transcript increases the probability of NMD [42]. Therefore, any factor that enhances translation efficiency, including splicing and EJC deposition on a transcript irrespective of the position of the EJCs, can enhance NMD efficiency [43,44]. Previous research has suggested that the increased distance to the poly(A) tail and UPF1 binding on NMD targets prohibit the interaction of PABPC1 and eRF3, decreasing termination efficiency at PTCs of transcripts with long 3' UTRs

[24,41]. Further influencing premature termination, the increased GC content in NMD-triggering 3' UTRs is predicted to increase RNA secondary structure around the PTC, which may also affect termination and hence, NMD efficiency [37,38,45]. However, more recent studies have challenged the idea that translation termination at PTCs is less efficient, as no measurable difference could be found in termination codon ribosome occupancy [46,47]. One caveat to consider when studying EJC-independent NMD is that characteristics of the 3' UTR can affect mRNA stability through other mechanisms, such as miRNAs that bind longer 3' UTRs and RBPs that recognize AU-rich elements in the 3' UTR. These alternatives need to be carefully investigated as confounding factors when altering 3' UTR composition [32,48]. Inhibition of NMD using small molecules such as the SMG1 kinase inhibitor, SMG1i [49], can be used to confirm that observed differences in mRNA stability following 3' UTR modulation are due to EJC-independent NMD.

Factors that allow evasion of EJC-independent NMD

Although long 3' UTRs can trigger NMD in mammalian cells by a similar mechanism as they do in yeast, substrate discrimination in EJC-independent NMD is more complex where many functional transcripts contain long 3' UTRs [27]. Exactly how mammalian cells protect the majority of their genes containing long 3' UTRs from NMD is not completely understood, but it is thought that certain *cis*- and *trans*-acting mechanisms of NMD evasion may allow for transcript and environment-specific regulation of EJC-independent NMD in higher eukaryotes (Figure 2).

Instances of long 3' UTR NMD substrate protection in *trans* by RBPs highlight a key protective mechanism — inhibiting UPF1 surveillance [12,33,50]. The first and most well-characterized example is PTBP1, a splicing factor that inhibits exon inclusion and shuttles in and out of the nucleus to facilitate RNA metabolism [51]. An *in vitro* study finds that PTBP1 interacts with UPF1's regulatory loop 1B, inhibiting NMD surveillance by triggering release from transcripts [52]. Additionally, hnRNPL, also an exon inclusion inhibitor, protects long 3' UTRs from being targeted by UPF1, reducing the likelihood of transcript degradation [53]. While positioning PABPC1 near the PTC antagonizes NMD *in vitro* [13,54], whether natural NMD-evading transcripts with long

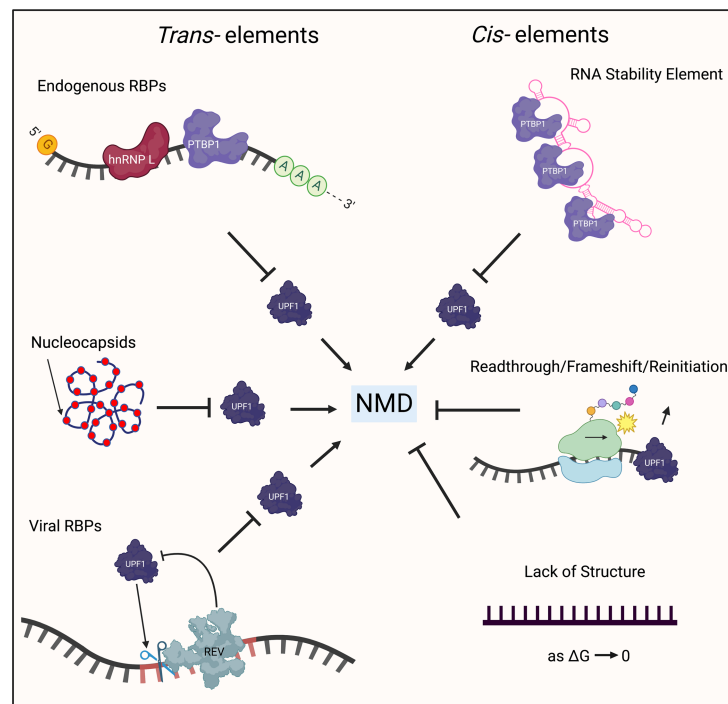


Figure 2. Factors that allow evasion of EJC-independent NMD.

Trans elements that inhibit NMD include endogenous and exogenous RBPs such as PTBP1 and REV, which encumber UPF1 surveillance. *Cis* elements inhibit NMD by sequestering NMD-inhibiting RBPs with their structure and sequence, by promoting readthrough and frameshifts and reinitiation, or by the lack of structural stability.

3' UTRs use this strategy *in vivo* is unknown and difficult to predict. Endogenous NMD evasion might require specialized scaffolding, facilitating specific RBP binding or localizing PABPC1 closer to a normal termination codon, inhibiting UPF1 transcript scanning [12]. The activities of these RBPs may hold the key to understanding how 3' UTRs might leverage transcript protection to stabilize themselves under normal conditions.

Viruses offer an additional platform to understand potential mechanisms of EJC-independent NMD escape. Due to the compact nature of their genome, viruses often produce polycistronic mRNAs. Upon translation of a 5'-proximal ORF, such transcripts mimic NMD substrates containing long 3' UTRs. Hence, viruses have adapted specialized *cis* and *trans* mechanisms to evade NMD. The best-understood retroviral NMD evasion strategy functions in *cis* in the Rous sarcoma virus (RSV). Immediately downstream of the gag gene of RSV, a highly conserved 150 nt-long structure recruits the host PTBP1 with high specificity, inhibiting UPF1 and its ability to surveil the viral transcript [33,55,56]. The Moloney Murine Leukemia Virus, an RNA virus, uses a readthrough sequence downstream from its gag coding sequence to evade NMD despite having a ~6000 nt long 3' UTR [57]. This strategy is also used by the plant-targeting turnip crinkle virus (TCV), where the long 3' UTR's ability to evade NMD is primarily dependent on the presence of readthrough elements and an unstructured region at the beginning of the TCV long 3' UTR [58]. Viral strategies that involve *trans* factors in NMD avoidance generally inhibit core NMD factors to stabilize the viral transcript or outright degrade NMD factors. For example, coronaviruses such as SARS and MERS use the nucleocapsid protein, N, to stabilize their ~10 kb long 3' UTRs [59]. The pea enation mosaic virus 2 also uses an NMD protective protein, p26, to transport the viral genome through the plant vascular system, concomitantly protecting highly structured, long, and endogenously GC-rich 3' UTRs [60]. It is tempting to speculate that a subset of endogenous long UTR-containing mammalian transcripts might use similar mechanisms to circumvent EJC-independent NMD in a context-dependent manner, although such mechanisms are yet to be deciphered.

Significance of EJC-independent NMD modulation

About one-third of the annotated transcripts in the human genome have 3' UTRs that are longer than 1 kb (GENCODE annotation V42; [61]). Yet, most transcripts with long 3' UTRs in humans are not subject to EJC-independent NMD [62–64]. In fact, a recent study that utilized long-read sequencing to exclude transcripts with more than one exon in the 3' UTR did not find a correlation between UTR length and NMD efficiency [64]. The same study also found that NMD-sensitive transcripts that lack exon–exon junctions in the 3' UTR are enriched for upstream ORFs (uORFs), suggesting that uORFs may be an additional trigger for EJC-independent NMD [64]. Our current understanding of factors that trigger EJC-independent NMD in mammalian cells and the evasion of this decay mechanism is insufficient to allow an accurate prediction of targets of this pathway and how their expression is modulated under different cellular conditions. Additionally complicating the identification and prediction of EJC-independent NMD targets in mammalian cells is the finding that not all splicing reactions result in the deposition of an EJC near the junction or at the expected canonical position [65,66]. Given the variability in EJC deposition and location, identifying long 3' UTRs that trigger NMD completely independently of EJCs is difficult [66].

A better understanding of EJC-independent NMD and its regulation is critical for several reasons. EJC-independent NMD shapes the cellular response to stress and differentiation. For example, in yeast, copper homeostasis is regulated through differential 3'-end processing that affects 3' UTR length; therefore, this pathway allows for isoform-specific regulation of mRNA by altering NMD sensitivity [67,68]. In mammalian cells, binding of the long loop isoform of UPF1 (UPF1_{LL}) to transcripts with long 3' UTRs makes them recalcitrant to RBPs that otherwise promote NMD evasion, and this binding is exploited during the integrated stress response to down-regulate a new set of targets that are normally immune to NMD [69]. Similarly, alternative polyadenylation (APA) that changes the 3' UTR of a transcript can also modulate its NMD susceptibility, as UPF1 preferentially binds and down-regulates the products of APA with long 3' UTRs [70,71]. Thus, when APA usage is altered during cell differentiation or other physiological changes, the stability of the resulting transcripts is dictated by NMD. During spermatogenesis, EJC-independent NMD eliminates long 3' UTR transcripts, allowing a shift to a testis-specific transcriptome containing predominantly short UTR transcripts [72]. Interestingly, the differentiation of naïve cell types to more mature counterparts is often associated with a down-regulation of NMD activity [73], suggesting that there may be added layers of regulation that allow long UTR transcripts to become more stable in such circumstances. Additionally, PTBP1 has been shown to bind to certain long UTR products of APA to promote their stability [70], again suggesting the delicate balance that exists between long and short UTR isoforms of transcripts depending on the cell state and *trans* factor activity.

Understanding NMD evasion by viral long 3' UTRs as well as some mammalian long 3' UTRs is necessary to inform research on therapeutic targeting of NMD activity. In the case of viral infections, antisense oligonucleotides (ASOs) could be used to prevent the binding of protective RBPs to specific regions of viral transcripts. For genetic diseases caused by nonsense mutations that trigger efficient NMD and cause loss of gene function, artificial tethering of RBPs that promote NMD evasion might offer a novel avenue for therapeutic development. Similarly, better understanding of NMD evasion may also lead to therapeutic interventions to enhance NMD on specific transcripts to counter gain-of-function genetic diseases. An example of such a disease is facioscapulo-humeral muscular dystrophy (FSHD), where NMD is broadly inhibited leading to aberrant stabilization of NMD targets *en masse* [74]. While it is unclear to what extent loss of NMD contributes to this complex genetic disease, strategies to enhance the NMD capacity in affected tissues would be an interesting avenue to pursue. As our capability to develop RNA-based therapeutics improves, modulating RBP binding to 3' UTRs could hold great promise for transcript-specific regulation of NMD.

Perspectives

- EJC-independent NMD is a pervasive and highly conserved mechanism of RNA quality control that shapes the transcriptome.
- Our current understanding of EJC-independent NMD and the precise mechanisms of its regulation across species is incomplete.
- Investigations directed at deciphering the rules and exceptions to EJC-independent NMD may yield novel ways to combat human genetic diseases and viral infections.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

O.M. and M.L. wrote the paper with input from S.J.; O.M. generated the figures.

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Abbreviations

APA, alternative polyadenylation; DSE, downstream sequence element; EJCs, exon-junction complexes; NMD, nonsense-mediated RNA decay; PABPC1, poly(A)-binding protein; PTC, premature termination codon; RSV, Rous sarcoma virus; TCV, turnip crinkle virus; uORFs, upstream ORFs.

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