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NMD: at the crossroads between translation termination and ribosome recycling

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Abstract

Nonsense-mediated mRNA decay (NMD) is one of three regulatory mechanisms that monitor the cytoplasm for aberrant mRNAs. NMD is usually triggered by premature translation termination codons that arise from mutations, transcription errors, or inefficient splicing, but which also occur in transcripts with alternately spliced isoforms or upstream open reading frames, or in the context of long 3'-UTRs. This surveillance pathway requires detection of the nonsense codon by the eukaryotic release factors (eRF1 and eRF3) and the activities of the Upf proteins, but the exact mechanism by which a nonsense codon is recognized as premature, and the individual roles of the Upf proteins, are poorly understood. In this review, we highlight important differences between premature and normal termination. Based on our current understanding of normal termination and ribosome recycling, we propose a similar mechanism for premature termination events that includes a role for the Upf proteins. In this model, the Upf proteins not only target the mRNA and nascent peptide for degradation, but also assume the role of recycling factors and rescue a ribosome stalled at a premature nonsense codon. The ATPase and helicase activities of Upf1, with the help of Upf2 and Upf3, are thus thought to be the catalytic force in ribosome subunit dissociation and ribosome recycling at an otherwise poorly dissociable termination event. While this model is somewhat speculative, it provides a unified vision for current data and a direction for future research.

Keywords

Nonsense mediated mRNA decay; Premature translational termination; Ribosome recycling

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Three cytoplasmic surveillance pathways monitor the integrity of the translation process in eukaryotes and target aberrant transcripts that affect ribosomal progression. These pathways, No-Go Decay (NGD) [1, 2], Non-Stop Decay (NSD) [3, 4], and Nonsense-mediated mRNA Decay (NMD) [5, 6]

Substrates of the NMD pathway

NMD targets polysome-associated mRNAs [7–9] that account for as much as 15% of the complexity of an organism's transcriptome [10–18]. The premature termination codons (PTCs) that are characteristic of NMD-targeted mRNAs can arise from genetic “errors” such as mutations in genomic DNA, alternative pre-mRNA processing, or non-productive DNA rearrangements, but they are also inherent to a subset of normal transcripts. For example, some mRNAs with upstream open reading frames (uORFs) are degraded by the NMD pathway [10, 12, 19–21], with the distinction between those mRNAs that do or do not trigger NMD reflecting the extent of uORF nonsense codon occupancy by the ribosome [19, 22] or the presence of downstream sequences that inactivate NMD [23, 24]. Notably, termination codons that trigger NMD do not need to be premature. Termination codons associated with atypically long 3'-UTRs are also sufficient to elicit NMD [25–29], an observation which led to the notion that termination context dictates an mRNA's susceptibility to NMD [30]. The broad range of transcripts encompassed as NMD substrates has substantial biological impact: not only does NMD ensure removal of “junk” (e.g., some byproducts of alternate splicing, intron-containing transcripts that enter the cytoplasm, or pseudogene mRNAs [10]), but it also effectively renders most nonsense alleles as null alleles [31, 32] and has been co-opted to regulate the levels and locations of specific proteins [33, 34].

Regulation of NMD by the *UPF* genes

The proteins encoded by the *UPF1* (*SMG2*), *UPF2* (*NMD2/SMG3*), and *UPF3* (*SMG4*) genes are the key factors controlling NMD [35–41]. Inactivation of their function by mutation, complete deletion, or down regulation stabilizes and increases accumulation of nonsense-containing mRNAs while having no significant effects on the abundance and stability of most wild-type transcripts [10, 41, 42]. These three proteins are mostly cytoplasmic (Upf3 shuttles between the nucleus and the cytoplasm) [43, 44] and interact with each other, the ribosome, and multiple mRNA decay and translation factors (including the Dcp2 subunit of the decapping enzyme and the eukaryotic release factors (eRFs) [37, 45–48]). In yeast, overexpression of Upf1 can compensate for mutations in Upf2 and Upf3, but not *vice versa* [49], indicating that Upf1 is NMD's key regulator. Upf1 is a superfamily I RNA helicase with two major domains: a cysteine- and histidine-rich (CH) domain at its N-terminus and a helicase domain towards its C-terminus [50–54]. The CH domain encompasses two zinc-knuckle modules similar to those of ubiquitin ligases [55] and the RNA helicase domain includes two core helicase segments (RecA1 and RecA2) and two regulatory segments (1B and 1C) [53, 54, 56]. The Upf1 helicase, RNA binding, and ATP hydrolysis activities are critical for several steps in NMD, i.e., association with the 40S ribosomal subunit, disassembly of a terminating mRNP, and recycling of components of the protein synthetic and NMD machineries. These activities are regulated by Upf2 and Upf3 by

interactions between the Upf1 CH-domain and the C-terminus of Upf2 [40, 54, 55, 57]. In the absence of Upf2, the Upf1 CH domain interacts with the helicase domain, yielding a closed conformation [53, 58] that increases RNA binding by the helicase domain and decreases ATPase and helicase activities. Upf2 binding opens the structure of Upf1, moving the CH domain away from the helicase domain, reducing RNA binding, and switching Upf1 from RNA clamping to RNA unwinding capability [53, 59]. In addition to manifesting intramolecular interactions, in yeast the CH domain of Upf1 is also involved in interaction with the ribosomal protein Rps26 [60], self-association [61], and binding to the decapping enzyme [37, 62], and possibly to eRF3 in human cells [46], suggesting that it may play a role in sequential molecular interactions during execution of NMD.

Upf2 contains three mIF4G domains (structures comparable to the middle domain of eukaryotic initiation factor eIF4G) in its N-terminal two-thirds [63–66], the most C-terminal of which (mIF4G-3) interacts with the central RRM (RNA Recognition Motif) in Upf3 [40, 64]. In addition to its roles as a scaffolding protein that bridges Upf1 and Upf3 [40, 43, 59, 67] and a switch for the Upf1 RNA helicase activity [53], Upf2 also recruits the Smg-1 kinase that phosphorylates Upf1 in metazoans [66]. In yeast, the Upf2 mIF4G-1 domain binds RNA and at least one other protein (Dbp6, a DEAD-box helicase) that may have a role in NMD [68]. Upf3 is a basic protein with an RNP-type RNA-binding domain (RBD) [40, 64]. The nonspecific RNA-binding activity of Upf2-Upf3 is thought to be a property of a conserved basic region of Upf2 [64]. However, *in vitro* studies challenge the capacity of the Upf2:Upf3 complex to bind RNA [59], thus leaving open the possibility that the Upf1 interaction domain on Upf2 is the principal contributor to the assembly of the trimeric Upf1:Upf2:Upf3 surveillance complex.

Other regulators of NMD

Upf proteins interact with the release factors. Upf1 interacts with both eRF1 and eRF3 [26, 45, 46, 69], and in human cells this leads to formation of the SURF (Smg-1-Upf1-Release Factors) complex [69, 70]. In addition, in yeast, Upf2 and Upf3 interact with eRF3 and could compete with eRF1 for a specific eRF3 interaction domain [71]. Importantly, Upf1 binding to eRF1 and eRF3 inhibits Upf1's ATPase activity [45], a result supporting the notion that Upf1 is inactive when it is first recruited to a prematurely terminating ribosome and that subsequent steps in NMD require Upf1 activation by interaction with a Upf2:Upf3 complex, as seen with the human factors [59].

In many eukaryotes other than yeast the Smg-1 kinase and the Smg-8 and Smg-9 proteins, as well as the PP2A phosphatase, regulate NMD by controlling Upf1's phosphorylation status [69, 70, 72, 73], while Smg-5 and Smg-7 form a complex that promotes mRNA deadenylation and decapping, and Smg-6 is an endonuclease thought to cleave NMD substrates [74–77]. Smg-8 and -9 repress the Smg-1 kinase activity, maintaining Upf1 in a dephosphorylated state in the SURF complex until it has interacted with Upf2 and Upf3. The latter interaction promotes Smg-1-mediated phosphorylation of Upf1, an apparent requirement for subsequent Upf1 interaction with the Smg-6 endonuclease and the Smg-5/7 complex. Upf1 and Upf2 are also phosphorylated in yeast, but there are no significant yeast homolog to any of the Smg proteins that regulate phosphorylation, nor is there good

evidence that the yeast phosphorylation events regulate NMD [78, 79]. In mammalian cells, and to some extent in other eukaryotes, NMD is often enhanced by an exon-exon junction at least 50–55 nt downstream of a nonsense codon, a phenomenon reflecting the involvement of the exon-junction complex (EJC), a dynamic structure deposited during splicing whose core proteins include eIF4AIII, Y14, Magoh, and MLN51 [80, 81]. Upf3 has been shown to associate with the EJC core [67, 81–85], and this observation led to a popular model for EJC regulation of NMD [86] (see below).

Normal translation termination

NMD and translation termination appear to be linked because nonsense codon recognition by the translation apparatus is a requirement for NMD [8, 19, 87–90]. In eukaryotes, termination at in-frame UAA, UAG, or UGA codons requires the release factors eRF1 and eRF3 (Sup45 and Sup35 in yeast) (Figure 1). eRF1 recognizes nonsense codons in the ribosomal A site, after which a conformational change in eRF1 allows its conserved GGG motif to activate the peptidyltransferase center of the ribosome and mediate peptide release [91–93]. eRF3 is a GTPase that stimulates eRF1 activity on the ribosome, conferring GTP dependency upon the termination process [94–97]. Subsequent to peptide hydrolysis, the termination complex is dissociated and recycled by the combined activities of the ABCE1 (Rli1 in yeast) ATPase, the essential initiation factors eIF3, eIF1, eIF1A, and the non-essential eIF3 subunit eIF3j [93, 98–100]. ABCE1 interacts with eRF1 and also with yeast eIF3j (Hcr1p) [101]. Cryo-EM reconstitution of the ribosome-eRF1-Rli1 complex shows that Rli1 is bound to the complex at the same position as eRF3, and the presence of non-hydrolyzable GTP analogs inhibits the subunit splitting activity of Rli1 [99], suggesting that ABCE1 and eRF3 cannot bind the ribosome-eRF1 complex simultaneously [93].

In addition to eRF1 and eRF3 several other proteins have been shown to influence translation termination, including poly(A)-binding protein (Pab1 in yeast; PABPC1 in humans). Poly(A)-binding protein interacts with eRF3 and overexpressing or deleting its gene respectively leads to enhanced or reduced termination efficiency in yeast or human cells [46, 102–104]. eRF3-Pab1 interaction has been proposed to play a role in the formation of an mRNP complex favorable to normal translation termination [102, 103, 105, 106]. Purification of full-length human and yeast eRF3 has proved challenging, thus limiting *in vitro* translation termination systems to the use of a truncated version of eRF3 lacking its Pab1 interaction domain [92, 107]. Thus, the formal role of eRF3-Pab1 interaction in translation termination remains an open question [108–111]. It is possible that Pab1 is not directly involved in mediating efficient termination, but promotes appropriate post-termination events to ensure recycling of the ribosome and the termination factors.

Mutations in the *NLP3*, *DBP5*, and *GLE1* genes, all of which encode mRNA export factors, have been shown to promote nonsense codon readthrough and to alter cellular sensitivity to compounds that affect translation [112–114]. While these results suggest that the respective export factors may regulate the termination process, it's more likely that the observed effects on readthrough are indirect consequences of their effects on mRNP structure.

Premature translation termination is aberrant

Although NMD typically begins when a nonsense codon occupies the ribosomal A site, the ensuing events appear to be mechanistically different from normal translation termination. In addition to the fact that most normal termination events do not trigger NMD two key lines of evidence support this contention. First, toeprint analyses in yeast cell-free translation systems fail to yield any toeprinting signals from ribosomes at normal termination codons (NTCs) unless eRF1 is inactivated by a temperature-sensitive lesion [30]. In contrast, ribosomes at premature termination codons (PTCs) yield toeprint signals consistent with A-site occupancy even without eRF1 inactivation [30]. Similar results have been obtained with the NTC and a codon 39 PTC of human β -globin mRNA [115]. Second, although nonsense codon readthrough can occur at some NTCs [116, 117], direct comparisons demonstrate that PTCs are considerably more amenable to readthrough than NTCs. This conclusion is most clearly drawn from experiments with ataluren (PTC124), a small molecule drug developed to promote therapeutic nonsense suppression [118, 119]. Ataluren promotes readthrough at PTCs present in both reporter mRNAs and mRNAs derived from *bona fide* PTC-containing alleles, but fails to yield detectable readthrough at the NTCs of an equally diverse set of transcripts [118–126]. These observations indicate that at least one step in termination is proceeding considerably slower at PTCs than at NTCs, a difference that may well be attributable to termination-enhancing factors associated with a normal 3'-UTR that are absent from the “*faux*” 3'-UTR created by a PTC.

Consistent with the notion that Upf proteins play a role in recognition of such inefficiency in termination, deletion of any of the *UPF* genes further increases PTC readthrough [33, 49, 71, 127]. However, such nonsense suppression can be attributed at least in part to an increase in intracellular Mg^{2+} concentration caused by stabilization of the uORF-containing *ALR1* and *ALR2* mRNAs that encode yeast's principal Mg^{2+} transporters [33]. Adding complexity to the issue, the ATPase and subunit splitting function of ABCE1 is inhibited when Mg^{2+} concentration is increased [100, 128]. Since the same ATPase domain is also responsible for interacting with eRF1 [101], it is possible that the increase in intracellular Mg^{2+} levels and the increase in readthrough at a PTC when NMD is inhibited could also reflect a decrease in ABCE1 function.

Further indications of the aberrant nature of premature termination are observations that reinitiation of translation after premature termination is markedly reduced in yeast cells or extracts when any one of the three Upf proteins is absent or inactive [30, 129], and that yeast cell-free extracts derived from a *upf1* mutant manifest a defect in ribosome recycling that can be complemented by purified Upf1 [129]. Similarly, in human cells, ATPase- or helicase-deficient Upf1 leads to the accumulation of mRNPs containing endonucleolytically cleaved β -globin mRNA decay intermediates that are blocked in subsequent exonucleolytic degradation, and the latter mRNPs may also include the terminating ribosome [130]. All these observations suggest that, like the NGD and NSD pathways [1, 2, 4], one ancillary role for NMD and its Upf factors is the dissociation of an otherwise poorly dissociable ribosome:mRNP complex [5, 106, 129], a function that could require Pab1 and other associated factors at a normal termination codon [30, 131–133].

mRNA targeting by Upf1

All NMD studies in yeast, worms, flies, and mammals point to a central role of Upf1 in targeting an mRNA for degradation after premature termination, promoting the recruitment of the decay enzymes, and recycling of the premature termination complex. An important question, then, is how Upf1 is recruited to a premature termination event. mRNP immunoprecipitation experiments show that Upf1 preferentially associates with NMD substrate mRNAs in yeast and worms [11, 134], a non-trivial observation in light of the fact that NMD substrates comprise no more than 5% of the total mRNA mass [10]. Two possibilities for the selective association of Upf1 with NMD substrates are likely. In the first, Upf1 routinely associates with mRNA or ribosomes and a ribosomal encounter with a PTC stabilizes this association, activates Upf1, and launches the NMD process. In essence, this is the gist of the pioneer round/EJC model, where Upf1 is thought to associate with any ribosome that has been pre-bound by the eRF1 and eRF3 release factors, only to be activated if a downstream EJC can contribute Upf2 and Upf3 to the formation of a complex that will then incorporate the Smgs, phosphorylate Upf1, and recruit decapping and/or endonucleolytic enzymes [86, 135, 136]. However, the proposed role for a retained EJC in Upf1 activation clearly does not explain how mRNAs derived from intron-less precursors, e.g., most yeast mRNAs or those metazoan mRNAs that are subject to NMD without prior splicing, proper EJC spacing, or EJC components [137–141]. Recent studies in human cells suggested a variation of this model. Instead of binding directly to the ribosome Upf1 might occupy all translationally active mRNAs, presumably poised to be activated when a ribosome encounters a PTC and otherwise removed by elongating ribosomes and thus restricted to 3'-UTRs [142–146]. This model could explain how mRNAs without EJCs might be targeted by NMD.

The second possibility is that Upf1 only associates with ribosomes when termination is aberrant or inefficient (Figure 2). The *faux*-UTR model posits that efficient translation termination and proper recycling of the termination complex depends on interactions between the release factors and proteins bound to a normal 3'-UTR and that the mRNP structure associated with the 3'-UTR created by a PTC must lack at least one critical factor that normally enhances termination [5, 30, 106]. Accordingly, Upf1 association with the prematurely terminating ribosome could be favored by the novel mRNP context of a *faux* 3'-UTR, the loss of effective interaction between one of the release factors and a protein normally associated with the 3'-UTR, or the slow dissociation of the termination complex after peptide hydrolysis [5]. Three observations favor the second model: a) mRNA marking models like the pioneer round/EJC model need to invoke immunity to NMD for any mRNA not targeted for degradation in its initial round of translation, but experiments exploiting inducible NMD in yeast [19, 147], and demonstrating NMD susceptibility of eIF4E-associated mRNAs in mammalian cells [148, 149], argue that NMD immunity is unlikely; b) EJC enhancement of NMD may be indirect [80, 150–152]; and c) in yeast, the number of Upf1 (and Upf2 and Upf3) molecules per cell is approximately 1/30th to 1/100th the number of ribosomes [49, 153], thus precluding stoichiometric association with every mRNA or ribosome. However this is not the case in mammalian cells where the numbers are more comparable [154, 155]. These lines of evidence suggest that the promotion of normal termination might rely more on an mRNP structure organized by multiple factors that

include eRF3 and Pab1, and Upf proteins can recognize the absence of these factors marked by an inefficient termination event.

Conclusions

The NMD pathway, one of several cytoplasmic quality control mechanisms ensuring the fidelity of gene expression, minimizes the accumulation of potentially toxic polypeptides that arise as products of premature translation termination. NMD is regulated in all eukaryotes by the three Upf proteins, Upf1, Upf2, and Upf3. Of these, Upf1, a superfamily I RNA helicase, is the central regulator whose activation depends on Upf2 and Upf3 binding. NMD is triggered by premature translation termination, an event mechanistically different from normal termination. Premature termination appears to be an inefficient termination event, and this may reflect different mRNP complexes at PTCs and NTCs. Since eRF1 and eRF3 are required for activation of NMD events after stop codon recognition by eRF1 and GTP hydrolysis by eRF3 may encompass the differences between premature and normal termination. Normal termination and ribosome recycling appear to depend on interactions between the release factors and proteins associated with a normal 3'-UTR. We propose that the inefficiency of premature termination is triggered by the mRNP structure downstream of a PTC which, in turn, may lead to poor release factor binding at the A-site or to slow dissociation of the release factor(s) after peptide hydrolysis (Figure 2). These shortcomings are thought to be resolved by the recruitment of Upf1 to the premature termination complex, activation of its ATPase and helicase to promote ribosome reutilization, and interaction with the decapping enzyme and the proteasome to initiate NMD and nascent polypeptide degradation [156]. Premature termination could lead to a unique mRNP structure that might provide sufficient specificity for Upf1 interaction with eRF3 or the small ribosomal subunit, and Upf1 activation might dissociate the 60S subunit, similar to ATPase activity of Rli1. However the mechanism of Upf2 and Upf3 recruitment, the timing of peptide hydrolysis (before or after Upf association), and Upf1 association with the 40S ribosomal subunit in yeast [60, 129] remain to be understood.

In short, two ribosome recycling pathways could exist. One promoted by termination at a normal stop codon, which requires a “proper” mRNP structure that includes Pab1, eRF1-eRF3, and ABCE1/Rli1 among other factors, and another at a premature termination codon, in which the ribosome is stalled, possibly because eRF3 is not able to leave the complex, and which leads to recruitment of the Upf proteins and the onset of the events of NMD. Both pathways would thus rely on ATP hydrolysis to promote subunit splitting. Clearly, this model is highly speculative and will require experimental validation.

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- Nonsense-mediated mRNA decay (NMD) targets anomalous translation termination events
- Premature termination differs mechanistically from normal termination
- Premature termination leads to a poorly dissociable mRNP complex
- The UPF proteins recruit mRNA decay factors
- The UPF factors may also promote ribosome dissociation and recycling

Figure 1. Translation termination at a normal stop codon

The figure depicts a current understanding of the different steps occurring between recognition of a stop codon by eRF1 and eRF3 and complete dissociation of the ribosome. Some steps remain to be clarified, such the possible stimulatory role of Pab1 on the efficiency of translation termination and the dissociation of eRF3 after GTP hydrolysis.

Figure 2. Proposed mechanism for NMD in yeast

This model is speculative and aims to account for multiple observations on NMD in a uniform scenario. **1.** eRF1 and eRF3 are recruited to a ribosome paused at a stop codon in an inappropriate 3' mRNP context. This leads to improper termination, possibly linked to the inability of eRF3 to leave the complex, and could be favored by the absence of Pab1. Upf1 in an inactive form is recruited to eRF3-eRF1. Two alternative outcomes are then considered: **2a.** After its recruitment to the termination complex, Upf1 binds to Rps26 and is joined by Upf2 and Upf3 and “opened” for activation. This step promotes peptide degradation (as indicated by the arrow). **2b.** Upf2-Upf3 binds to Upf1 on the termination

complex and activates Upf1. The complex then associates with Rps26 through the Upf1 CH domain, and promotes peptide degradation (as indicated by the arrow). **3.** Upf1 is active, associated with Upf2-Upf3, and bound to the ribosome via Rsp26. These components promote dissociation of the termination complex, a step that requires the Upf1 helicase activity and leaves the 40S subunit still bound to the mRNA (the events leading to 40S removal are unknown). This step is different from the “normal” recycling event involving Rli1 (see Figure 1). **4.** The presence of Upf1 promotes mRNA decapping by Dcp1-Dcp2 (as indicated by the arrow), via Upf1 interaction with Dcp2. Destabilization of the mRNA closed-loop structure, subsequent to improper recycling, could also promote mRNA decay.