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Execution of nonsense-mediated mRNA decay: what defines a substrate?

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The nonsense-mediated mRNA decay (NMD) pathway targets mRNAs with premature termination codons as well as a subset of normal mRNAs for rapid decay. Emerging evidence suggests that mRNAs become NMD substrates based on the composition of the mRNP downstream of the translation termination event, which either stimulates or antagonizes recruitment of the NMD machinery. The NMD mRNP subsequently undergoes several remodeling events, which involve hydrolysis of ATP by the NMD factor Upf1 and in metazoans, a phosphorylation/dephosphorylation cycle of Upf1 mediated by Smg proteins. This leads to mRNA decay following translational repression. Recent evidence suggests that in Drosophila and human cells, decay is initiated by the endonuclease Smg6.

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Introduction

The expression of protein-coding genes in eukaryotes involves the orchestration of transcriptional and post-transcriptional processes. To ensure the fidelity of these processes, the eukaryotic cell has evolved several quality-control mechanisms. One such mechanism is the nonsense-mediated mRNA decay (NMD) pathway. NMD rids the cell of aberrant mRNAs that have acquired premature translation termination codons (PTCs) [1–7]. In addition, the NMD pathway serves to destabilize a subset of apparently normal mRNAs [8,9]. Several *trans*-acting factors critical for the NMD pathway have been identified. These include Upf1, a DEAD-box ATPase, Upf2, and Upf3, and in metazoans, Smg1, Smg5, Smg6, and Smg7. In this review we discuss recent insights into the mechanisms by which Upf and Smg proteins are specifically recruited to NMD substrates, and how they subsequently activate translational repression and mRNA decay.

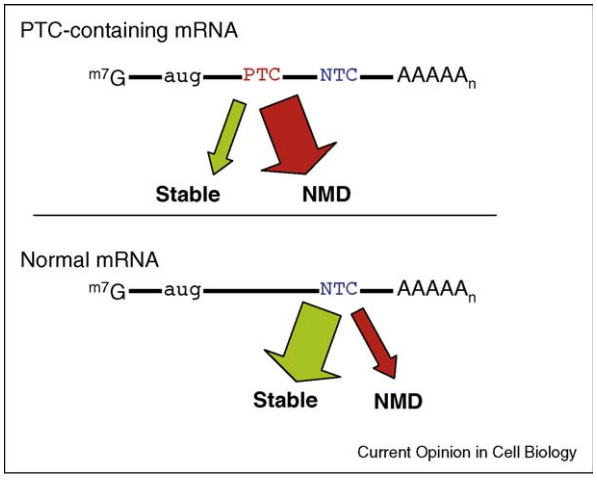
Which mRNAs are substrates of NMD?

Recent evidence suggests that the NMD pathway targets not only mRNAs that have acquired premature termination codons (PTCs), but also a subset of endogenous transcripts lacking PTCs (Figure 1). Global gene-expression profiling of several eukaryotes, where components of the NMD pathway were either transiently or stably inactivated, revealed an upregulation of about 1–10% of cellular transcripts [8,10–12,13••,14••,15••,16]. Although a subset of these transcripts may not be direct targets of NMD [16], some have been subsequently confirmed experimentally. Many of these endogenous NMD targets do not contain PTCs, although some have recognizable features such as upstream open reading frames (uORFs) that would make translation termination events appear as premature [8,9].

Although some mRNAs lacking PTCs are targets of NMD, mRNAs that have acquired PTCs likely constitute a major fraction of NMD substrates in the cell (Figure 1). PTC-containing mRNAs can arise from erroneous gene expression such as errors in transcription, faulty mRNA processing, somatic mutations, or nonproductive programmed DNA rearrangements, for example those that occur in the T-cell receptor (TCR) and immunoglobulin (Ig) genes [4–6,17]. PTCs can also be inheritable as in the case of genetic mutations in disease genes, expressed pseudogenes and small nucleolar RNA (snoRNA) host genes that have lost their protein-coding potential [17]. However, not all PTCs trigger NMD. For example, those PTCs positioned close to the normal termination or initiation codons often fail to trigger NMD [18–24].

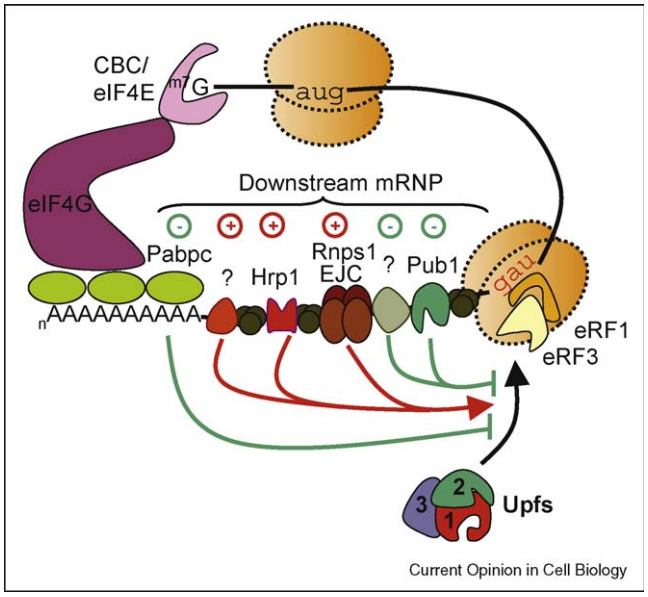
A major contributor of PTC-containing mRNAs is likely faulty or alternative splicing. For example, a recent study reported a strong bias for in-frame termination codons in introns in the ciliate *P. tetraurelia*, suggesting a reliance on NMD to eliminate inefficiently spliced transcripts [25••]. In mammals, the analyses of databases of expressed sequence tags suggested that one-third of alternative splicing events have the potential to generate PTC-containing mRNAs [26–28]. However, quantitative microarray splice variant profiling experiments revealed that many PTC-containing alternatively spliced transcripts are not significantly upregulated upon Upf1 knockdown [29]. Interestingly, several mRNAs encoding

Figure 1



Nonsense-mediated mRNA decay (NMD) can target both mRNAs that have acquired PTCs and normal mRNAs. NMD targets a majority of mRNAs that contain premature termination codons (PTCs) for rapid decay (top; red arrow), although some PTC-containing mRNAs escape NMD and are stable (top; green arrow). NMD also targets a subset of apparently normal mRNAs for NMD (bottom; red arrow).

Figure 2



Model for the mechanism of discrimination between a normal and an NMD-inducing translation termination event. The composition of the mRNP downstream of the terminating ribosome may dictate whether NMD occurs through a competition between stimulators and antagonists of Upf complex recruitment. NMD-stimulating factors, such as Hrp1p (red) in *S. cerevisiae* and the EJC and RNPS1 (brown) in human cells, stimulate NMD, likely by recruiting the Upf proteins to the mRNP downstream of the termination event. By contrast, PABPC (light green), and likely other factors including *S. cerevisiae* Pub1p (dark green), antagonizes NMD when in close downstream proximity of the termination event.

splicing-activator proteins are targets of NMD because of alternative splicing events that cause the inclusion of conserved stop codon-containing exons [27••,30–32]. This may provide a negative feedback loop that uses alternative splicing to autoregulate the levels of splicing factor proteins, while the NMD pathway depletes the cell of the PTC-containing alternatively spliced products that would otherwise produce truncated proteins.

How is a translation termination event that induces NMD discriminated from normal termination?

Evidence from multiple studies suggests that the composition of the messenger ribonucleoprotein (mRNP) downstream of the terminating ribosome is a key determinant for NMD. Whether the downstream mRNP triggers NMD may be decided by the arrangement of components of the mRNP that stimulate or antagonize NMD (Figure 2) [33••,34••,35••,36••,37,38••]. To allow for the observation that both normal termination codons and PTCs can elicit NMD we will here avoid the often used term 'PTC recognition' and instead refer to translation termination events that trigger NMD as NMD-inducing termination events.

Factors that stimulate NMD when part of the mRNP downstream of a termination event have been identified in both *Saccharomyces cerevisiae* and mammals. Early studies identified *S. cerevisiae* Hrp1p as a protein that coimmunoprecipitates (co-IPs) with Upf1, and associates with a downstream sequence element important for NMD of PTC-containing PGK1 mRNA [39]. Similarly, in mammalian cells the exon-junction complex (EJC), which is deposited upstream of exon–exon junctions after pre-mRNA splicing, and the EJC-associated splicing-activator RNPS1, both interact directly with the NMD factor Upf3 and stimulate NMD of a number of tested substrates when positioned downstream of a translation termination event [40–48,49••]. Interestingly, several splicing-activator SR proteins have been shown to stimulate NMD when overexpressed in human cells, although the mechanism is unknown [50,51]. Thus, factors such as Hrp1p, the EJC, and RNPS1 stimulate NMD, possibly by increasing the affinity of the Upf complex to the mRNP downstream of a termination event and would be predicted to be absent from normal 3′-UTRs. Consistently, introns are rarely found in the 3′-UTRs of mammalian genes [52]. Some EJC and SR proteins have also been observed to activate translation when located upstream of a termination event [53,54], but the relation between this activity and NMD remains unclear.

Recent evidence has identified cytoplasmic poly(A)-binding protein (PABPC) as an NMD antagonist in *S. cerevisiae*, *D. melanogaster*, and human cells. This includes observations that mRNAs with artificially extended

3′-UTRs, which positions the poly(A)-tail and PABPC distal to the translation termination event, are targeted for NMD, while artificial recruitment of PABPC in downstream proximity of the termination codon, by tethering or 3′-UTR looping, impairs NMD [33••,34••,35••,36••,38••,55••]. The mechanism by which PABPC antagonizes NMD remains unclear. However, direct interactions have been reported of both Upf1 and PABPC with eukaryotic release factor 3 (eRF3) [36••,56–61], and human PABPC was observed to outcompete the interaction between Upf1 and eRF3 *in vitro* [38••]. Additionally, overexpression of PABPC suppresses a yeast temperature-sensitive allele of eRF3 [56], while depletion of PABPC in human cell lines results in increased read-through of a dual luciferase reporter mRNA [36••], indicating that PABPC stimulates translation termination. Together these observations implicate PABPC as an NMD-antagonizing factor that when present in downstream proximity of a termination codon stimulates normal termination and prevents the recruitment of the Upf complex and NMD.

Factors other than PABPC that antagonize NMD likely exist. For example, evidence suggests that the NMD machinery of *S. cerevisiae* is capable of identifying NMD substrates even in the absence of PABPC or a poly(A)-tail [62••]. Pub1p has been identified as an *S. cerevisiae* factor that associates with a stabilizer element downstream of the uORF of GCN4 mRNA and antagonizes NMD when tethered at the same position [63]. Similarly, the 3′-UTR of the Rous sarcoma virus (RSV) gag gene contains a stability element that antagonizes NMD [64]. Future studies should reveal the relative importance of NMD-stimulating versus NMD-antagonizing factors in defining NMD substrates, and the molecular mechanisms by which these factors act. Moreover, an important question for future studies is whether the recruitment of NMD-modulating factors can be a regulated event, thereby using the NMD pathway to control gene expression upon cellular cues.

Does NMD take place during a pioneer round of translation?

Observations that the yeast nuclear cap-binding protein cbc1p co-IPs with eIF4G and is able to support translation *in vitro* led to the hypothesis that a newly exported mRNA can engage in translation before the exchange of the nuclear cap binding complex (CBC) for cytoplasmic translation initiation factor eIF4E [65]. The pioneer round of translation hypothesis for NMD posits that translation termination events that trigger NMD in mammalian cells can occur only during a pioneer round of translation, defined as the translation that takes place while the mRNA is associated with CBC. By contrast, according to this hypothesis, once eIF4E replaces the CBC the mRNA is immune to NMD [66].

Much of the evidence for the pioneer round of translation model comes from interaction studies. First, the ratio of an NMD substrate to its cognate wild-type mRNA that co-purifies from human cell extracts with CBP80 (the large subunit of CBC) or eIF4E was indistinguishable by RT-PCR assays [67]. Second, NMD factors, including Upf proteins, Smg1, and EJC components, were observed to co-IP in the absence of RNase with CBP80 but not with eIF4E. By contrast, components of the general translation machinery, co-IP with both CBP80 and eIF4E [68,69]. Third, knockdown of CBP80 was reported to impair the co-IP of Upf1 with Upf2, while far-western and pull down assays suggest that recombinant CBP80 interacts with Upf1 and stimulates Upf1–Upf2 interaction [69]. Functional evidence for the pioneer round of translation model comes from the observation that overexpression of eIF4E-binding protein 1 (4E-BP1) at levels that cause translational repression of reporter mRNAs in human cells does not show a corresponding increase in the steady-state levels of tested NMD substrates as measured by RT-PCR [68]. These observations suggest that eIF4E-initiated translation is not limiting for NMD of tested reporters, and that at steady state, NMD factors are primarily found on CBC-associated mRNA. This is consistent with the idea that NMD primarily takes place on CBC-associated mRNA. However, these observations do not provide evidence that eIF4E-associated mRNA is immune to NMD. The most direct evidence that NMD may be restricted to CBC-associated mRNA comes from reports that knockdown of endogenous CBP80 by RNAi causes increased steady-state levels of tested NMD reporters, which is rescued upon expression of exogenous CBP80 [69]. However, this observation leaves open the possibility that pleiotropic effects could interfere with NMD in an indirect manner. Interestingly, a PTC-containing mRNA in which translation is driven by an EMCV internal ribosome entry site was targeted for NMD, suggesting that cap-independent translation initiation can sustain NMD [70]. However knockdown of CBC components was reported to cause upregulation of this mRNA [71].

All evidence suggests that NMD in *S. cerevisiae* can take place on eIF4E-associated mRNA. First, NMD substrates expressed and translated under conditions of Upf protein depletion, are rapidly degraded upon restoration of the depleted Upf proteins at a time when CBC is presumed to have been replaced by eIF4E [72]. Second, an NMD reporter kept stably in polysomal fractions by drug-induced inhibition of translation elongation is rapidly degraded upon removal of the drug [73]. Third, the CBC is not limiting for *S. cerevisiae* NMD, as the depletion of cbc1p does not affect the abundance of tested NMD substrates [74]. Finally, analysis of the decay rates of a reporter mRNA engineered with programmed -1 ribosomal frame-shift signals' suggested that mRNAs

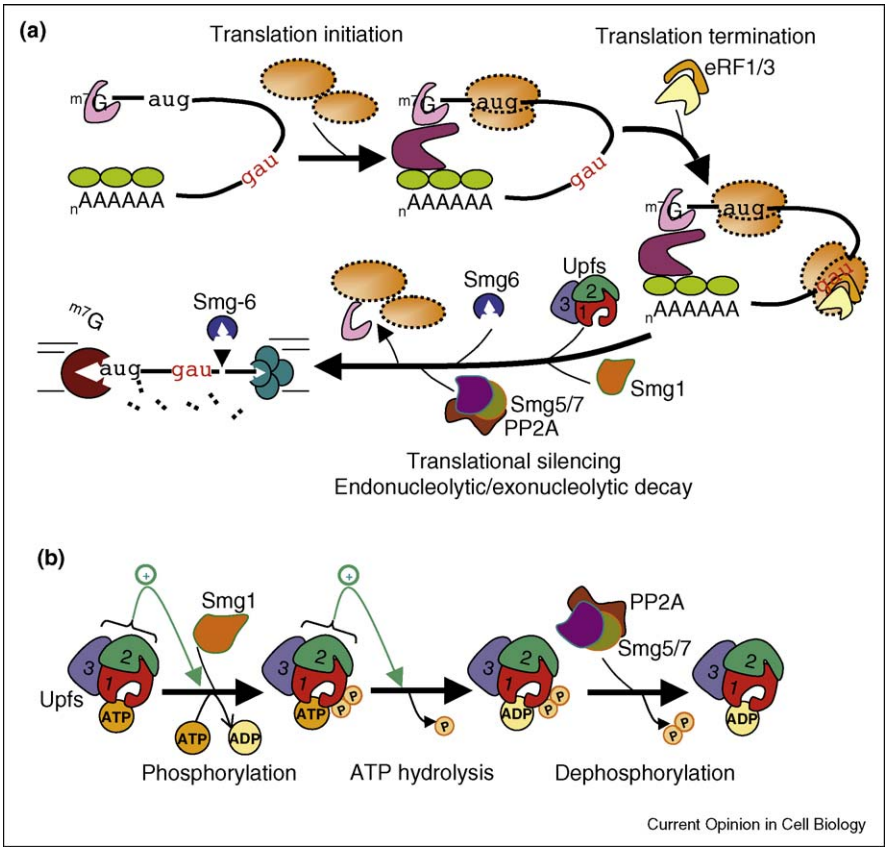
were available for NMD after the first ribosome has translated the mRNA [75]. While these lines of evidence suggest that eIF4E-associated mRNA is susceptible to NMD in *S. cerevisiae*, it has not been directly tested whether CBC-associated mRNA can also be targeted for NMD.

Several predictions from the pioneer round hypothesis of mammalian NMD remain to be tested. For example, the prediction that a fraction of NMD substrates should escape NMD by undergoing replacement of CBP80 with eIF4E before recognition by the NMD pathway, and thus should result in two-component decay kinetics has not been carefully tested. In previous experiments that indicated two-component NMD kinetics [76–78], it was not ruled out that the apparent slow phase of decay was not a result of incomplete transcriptional repression. Moreover, experiments monitoring decay of NMD substrates expressed under conditions of NMD repression followed by reactivation of NMD have not been performed in mammalian cells.

What happens to the NMD mRNP after translation termination?

Although the exact order of events downstream of an NMD-inducing termination event remains unknown, recent evidence suggests that at least two transitions in the NMD mRNP occur after the recruitment of the Upf proteins and before the degradation of the NMD substrate. These involve ATP hydrolysis by Upf1 and, at least in metazoans, a Upf1 phosphorylation/dephosphorylation cycle (Figure 3). Upf1 is a DEAD-box ATPase that shows RNA-dependent ATPase activity and ATP-dependent 5′–3′ RNA helicase activity *in vitro* [3–5]. Mutant Upf1 proteins that fail to bind or hydrolyze ATP are inactive in NMD and cause the accumulation of the NMD mRNP in P-bodies [79,80••]. This suggests that the ATPase activity of Upf1 is important for steps in NMD downstream of translation termination. Consistent with this, Upf2 and Upf3 stimulate ATP hydrolysis by Upf1 [49••] and NMD substrates accumulate in P-bodies upon depletion of Upf2 or Upf3 in *S. cerevisiae* [80••]. While it is thus clear that the ATPase activity of Upf1 is critical for

Figure 3



Transitions in the NMD mRNP leading to translation repression and decay. **(a)** For an mRNA to be targeted for NMD, it needs to undergo at least one round of translation, which results in the recruitment of Upf proteins to the terminating ribosome, followed by translational repression and degradation by both exonucleolytic and endonucleolytic processes. **(b)** After recruitment to the NMD mRNP and before decay, Upf1 undergoes Smg1-mediated phosphorylation and hydrolyzes ATP, followed by Smg5/Smg7-stimulated dephosphorylation by PP2A. Phosphorylation and ATP hydrolysis by Upf1 is stimulated by Upf2 and Upf3. See text for details.

NMD, the specific transition in the NMD mRNP that occurs upon ATP hydrolysis remains unknown.

Another critical transition in the metazoan NMD mRNP is a Upf1 phosphorylation/dephosphorylation cycle (Figure 3). The Upf1 kinase in metazoans is the phosphoinositol 3-kinase (PIK)-related protein kinase Smg1. Depletion of Upf2 or Upf3 inhibits the accumulation of phosphorylated Upf1, suggesting that Upf2 and Upf3 stimulate Smg1 activity [81••,82]. Biochemical and structural studies suggest that phospho-Upf1 interacts with Smg5 via the Upf1 N-terminus, and with Smg7 through interactions between the phosphorylated SQ-motifs of Upf1 and a 14-3-3 like domain of Smg7 [83–88]. Smg5 and Smg6 also contain predicted 14-3-3 like domains, suggesting that these proteins may also associate with Upf1 through the phospho-SQ-motifs [89]. Smg5 and Smg7 co-IP with protein phosphatase 2A (PP2A) and their depletion causes accumulation of phospho-Upf1, suggesting that Smg5 and Smg7 mediate dephosphorylation of Upf1 through the recruitment of PP2A. Phosphorylation and dephosphorylation of Upf1 is critical for metazoan NMD as evidenced by the impairment of NMD under conditions that inactivate these events [82,83,87,90••,91,92]. It is not clear whether phosphorylation of Upf1 is important for NMD in *S. cerevisiae*, although Upf1 has been observed to be a phospho-protein [93], and the protein Ebs1p has been identified as a putative *S. cerevisiae* ortholog of human Smg7 that interacts with Upf1 and inhibits NMD when overexpressed or depleted [94]. An important goal for future studies will be to understand the significance of the mRNP transitions that take place in the NMD pathway downstream of translation termination. It has been speculated that they may serve as a second step after translation termination to discriminate true NMD substrates from normal mRNAs [95].

How are NMD substrates translationally silenced and degraded?

The transitions in the NMD mRNP described above lead to decay of the NMD substrate, which is likely preceded by the inhibition of translation. Early studies in *S. cerevisiae* provided evidence that the translation of NMD substrates was downregulated several-fold more than their stability, suggesting that the recognition of an NMD substrate results in translational repression in addition to mRNA decay [96]. In mammalian cells, reduced expression of proteins from PTC-containing mRNAs has also been observed. However, this may at least in some cases be attributed to intrinsic instability of the truncated protein rather than that of translational repression [97,98]. Two-hybrid analyses identified translation initiation factor eIF4A1 as a weak interaction partner of eIF4G-homology (4GH) domains in *S. pombe* Upf2, and it was speculated that those interactions might contribute to translational repression [99]. More recent observations suggest that phospho-Upf1 interacts with translation initiation factor eIF3 and interferes with recruitment of the 60S ribosomal subunit to the 40S preinitiation complex [100,101••].

Several nuclease activities have been implicated in the decay of NMD substrates. Early observations in *S. cerevisiae* suggested that decapping is a rate-limiting step in NMD [102]. More recently, additional activation of deadenylation and 5′–3′ and 3′–5′ exonucleolytic activities by the NMD machinery has been observed in both *S. cerevisiae* and mammalian cells [3,4,6,66]. Interestingly, recent studies have implicated the NMD factor Smg6 as an endonuclease in NMD in *D. melanogaster* and humans [103••,104,105••]. The C-terminal PilT N-terminus (PIN) domain of Smg6 shows endonuclease activity *in vitro* [106] and catalytically inactive Smg6 fails to support NMD in human and Drosophila cells suggesting that endonucleolytic activity of Smg6 is important for NMD [103••,105••]. Thus both exonucleolytic and endonucleolytic activities are implicated in NMD. It remains to be determined which decay mechanism is predominant in various organisms.

Perspectives

The NMD pathway plays an important role in modulating gene expression by downregulating not only aberrant mRNAs that contain PTCs but also a subset of normal cellular mRNAs. Whether a translation termination event induces NMD seems to be dictated by the arrangement of the mRNP downstream of the termination event. Future studies should reveal the mechanisms and relative contributions to NMD in various organisms of NMD-stimulating and NMD-antagonizing mRNP factors. It will also be important to learn the specific mechanisms by which transitions in the NMD mRNP lead to translational repression and subsequent decay. Moreover, an important question for future studies is whether the NMD pathway can be regulated upon specific cellular cues to control expression of normal mRNAs. Given the rapid pace of the NMD field, the answers to many of these questions may be coming in the near future.

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