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How and where are nonsense mRNAs degraded in mammalian cells?

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The nonsense-mediated mRNA decay (NMD) pathway is responsible for the rapid degradation of eukaryotic mRNAs on which ribosomes fail to terminate translation properly. NMD thereby contributes to the elimination of aberrant mRNAs, improving the fidelity of gene expression, but also serves to regulate gene expression at the post-transcriptional level. Here we discuss recent evidence as to how and where mRNAs targeted to NMD are degraded in human cells. We discuss accumulating evidence that the decay step of human NMD can be initiated by two different mechanisms: either by SMG6-mediated endonucleolytic cleavage near the aberrant stop codon, or by deadenylation and decapping. While there is evidence that mRNAs targeted for NMD have the capacity to accumulate with other translationally repressed mRNAs in P-bodies, there is currently no evidence that this is required for the degradation of the NMD substrate. It therefore remains an open question whether NMD in human cells is restricted to a particular cellular location or whether it can be initiated wherever translation of the NMD substrate takes place.

Key words: Nonsense-mediated mRNA decay, mRNA turnover, endonuclease, PIN-domain, decapping, deadenylation, P-bodies

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Introduction

Given the complex chain of biochemical reactions involved in transforming the genetic information of an organism into gene products, the overall accuracy of gene expression is quite astonishing. At the mRNA level, the nonsense-mediated mRNA decay (NMD) pathway contributes to the high fidelity of gene expression in eukaryotes by recognizing and eliminating aberrant mRNAs on which ribosomes terminate translation prematurely. The truncation of the open reading frame (ORF) as a result of gene expression errors that lead to introduction of premature termination codons (PTCs), represents a well-studied feature that targets an mRNA for NMD, but transcriptome-wide profiling also revealed many seemingly normal, physiological mRNAs as NMD substrates. Thus, one of the key questions remains how the NMD machinery identifies its substrates. This topic has been extensively covered in several recent reviews.[1-6] Here, we discuss another key question about the NMD pathway: once identified as an NMD substrate, how and where is the target mRNA degraded? We focus our discussion on recent findings in mammalian cells and compare and contrast these to previous observations in the yeast *Saccharomyces cerevisiae* and the fruitfly *Drosophila melanogaster*.

Nip the End or Slice in Half? Different Species—Different Answers

Eukaryotic mRNAs are protected from mRNA decay by the 5' N7-methylguanosine cap and the 3' poly(A) tail. The initiation of mRNA decay requires an event that exposes the mRNA ends to 5'-to-3' or 3'-to-5' exonucleases. Depending on the specific mRNA decay pathway, this is achieved by removal of either the cap by the process of decapping, or the poly(A) tail by deadenylation. Alternatively, mRNA decay can be initiated by an endonuclease cutting the mRNA into two pieces, which also generates unprotected ends accessible to exonucleases.[7]

NMD requires the UPF proteins, UPF1, 2 and 3, which are conserved

among known eukaryotes. In addition, a UPF1 kinase, SMG1, and phospho-UPF1-interacting proteins, SMG 5, 6 and 7, are important for NMD in metazoans (reviewed in refs. 5 and 8). How do these NMD factors engage the mRNA decay machinery to degrade mRNAs targeted to NMD (hereafter called nonsense mRNAs)? This has been most thoroughly studied in the yeast *S. cerevisiae*, where the rapid decay of NMD substrates seems to be initiated primarily through an accelerated rate of mRNA decapping. This conclusion was based on observations that no deadenylated NMD substrate intermediate could be observed in pulse-chase mRNA decay assays, and that inhibition of 5'-to-3' exonucleolytic decay in cis by a guanosine-tract or in trans by depletion of decapping or 5'-to-3' exonuclease enzymes resulted in accumulation of polyadenylated decay intermediates.[9,10] In addition, deadenylation followed by exosome-mediated 3'-to-5' exonucleolysis has also been reported to contribute to NMD in yeast.[11,12] This is based on the observations (1) that nonsense mRNAs were stabilized in yeast cells lacking components of the 3'-to-5' exonuclease complex (the exosome), (2) that degradation in the 3'-to-5' direction proceeded more rapidly in a Upf- and exosome-dependent manner on nonsense mRNAs than on normal mRNAs, and (3) that an interaction of Upf1p with the exosome-associated Ski7p promoted exosome-mediated 3'-to-5' degradation. Kinetic analyses suggested that NMD speeds up 3'-to-5' decay primarily by accelerating the deadenylation rate, and that the initiation of NMD by decapping or deadenylation is dependent on the relative position of the PTC to the cap and poly(A) tail.[9] Thus all evidence in yeast suggests that NMD is initiated from the end, by the removal of the cap and/or poly(A) tail.

It came therefore as a surprise when it was discovered that in *D. melanogaster* S2 cells, degradation of nonsense mRNA begins with an endonucleolytic cleavage in the vicinity of the PTC, followed by rapid exonucleolytic degradation of the two resulting RNA fragments.[13] These decay intermediates are highly unstable and were only detected when the respective exonuclease activities were sufficiently inhibited: detection of the 5' fragment required simultaneous knockdown of three exosome components, RRP4, CSL4 and SKI2, while the 5' fragment was detectable after knockdown of the 5'-to-3' exonuclease XRN1.[13] Endonucleolytic cleavage was observed for several exogenously expressed nonsense mRNAs as well as for an endogenous mRNA, suggesting that it represents a major general decay route for NMD substrates in flies.

In cultured mammalian cells, seemingly contradictory observations have been reported and the situation appears to be more complicated than in yeast and flies. On the one hand, evidence for initiation of nonsense mRNA decay by deadenylation and decapping has been reported.[14-17] NMD targets showed enhanced deadenylation as compared to normal mRNAs, and accumulated as full-length transcripts upon knockdown of enzymes involved in decapping, deadenylation, or in 5'-to-3' or 3'-to-5' exonucleolysis.[14-17] Specifically, RNAi-mediated downregulation of DCP2, the catalytic subunit of the decapping complex, of XRN1, and of RRP6/PM-Scl100/EXOSC10 (a nucleolus-enriched homolog of the nucleus-specific yeast exosome component Rrp6p[18]) increased nonsense mRNA abundance and slowed their decay rate.[16] With regards to deadenylation, one study reported increased nonsense mRNA abundance upon knockdown of PARN,[16] a deadenylase that primarily localizes in the nucleus. By contrast, a separate study reported no effect on NMD of PARN overexpression or knockdown, whereas inhibition of NMD was observed upon knockdown, or upon overexpression of inactive mutants, of the CCR4-CAF1 and PAN2-PAN3 deadenylase complexes.[17] The ability of the NMD pathway to activate decapping and deadenylation is supported by reports that UPF1 interacts with the decapping complex through a recently discovered bridging factor, and that UPF proteins co-purify with PARN.[16,19-21] Moreover, UPF proteins have been reported to exist in complex with the 5'-to-3' exonucleases XRN1 and XRN2/RAT1, and several exosome components.[16] These observations are consistent with the idea that NMD in human cells, as in yeast, can be initiated by decapping and deadenylation.

On the other hand, recent reports have provided evidence for endonucleolytic cleavage near the PTC in human cells.[22,23] As in Drosophila cells, nonsense mRNA-specific polyadenylated 3' fragments could be observed in XRN1-depleted human cells.[22] The appearance of these 3' endocleavage fragments was dependent on UPF1, but was not affected by knockdown of DCP2.[22] Moving the position of the PTC in three different reporter transcripts led to a corresponding change in the size of the 3' fragment, indicating that endonucleolytic cleavage occurs in the vicinity of the PTC, but apparently not at a specific site, since cloning and sequencing of 3' fragments suggested that the cleavage can occur without any apparent sequence preference within a range of 40 nucleotides upstream or downstream of the PTC.[22]

What is the endonuclease in mammalian and Drosophila NMD? The identification of a PIN domain in the NMD factor SMG6 with in vitro nuclease activity,[24] made SMG6 the prime candidate. Indeed, bacterially expressed PIN domain of human SMG6 degrades circular RNA in vitro, demonstrating its endonuclease activity.[22] Notably, the PIN domain of the exosome component DIS3/RRP44 has also recently been identified as an endonuclease,[25,26] suggesting that other proteins with PIN domains might also exhibit endonuclease activity, provided they contain critical aspartic acid residues in the catalytic center.[24] In vivo knockdown and reconstitution experiments further demonstrated that SMG6 is the factor responsible for the endonucleolytic cleavage of NMD substrates both in mammals and in flies.[22,23] Earlier studies suggested that in erythrocytes, PTC-containing β-globin mRNA, and to a lesser extent also the wild-type mRNA, undergoes endonucleolytic cleavage by a PMR-like enzyme,[27,28] but these cleavages occurred at specific sites (preferentially UG dinucleotides) and independently of the PTC position, suggesting that this phenomenon was caused by a different mechanism than the UPF1- and SMG6-dependent cleavage near the PTC.

How can the seemingly contradictory evidence for initiation of NMD by decapping and deadenylation versus endonucleolytic cleavage in human cells

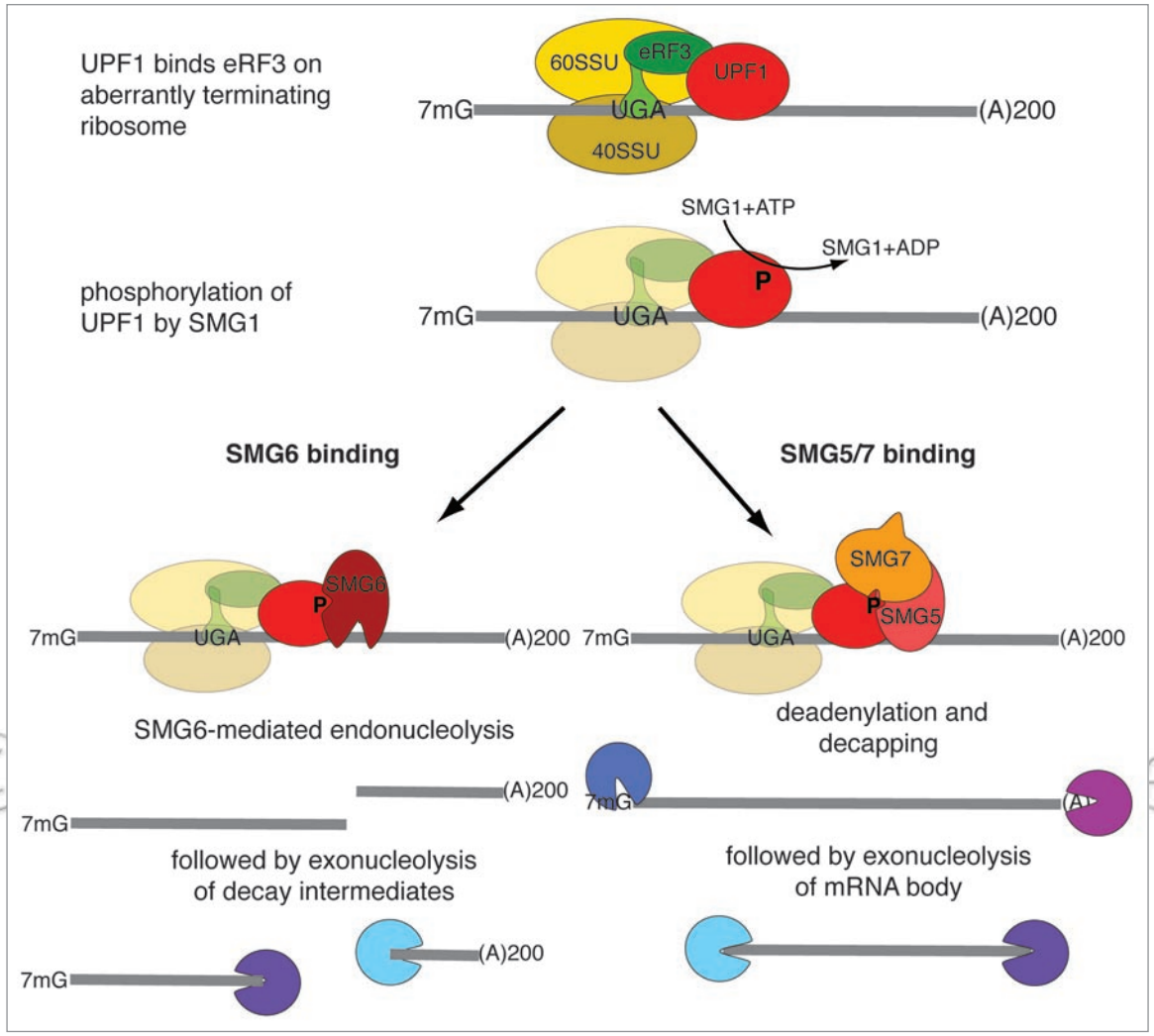

Figure 1. Model of the decay step(s) of human NMD. Aberrant translation termination gives time for UPF1 to bind to the stalled ribosome through interaction with eRF3. Subsequent phosphorylation of UPF1 induces a conformational change that increases UPF1's affinity for RNA and marks the mRNA for degradation. Phosphorylated UPF1 can either be bound by SMG6 or the heterodimer SMG5/SMG7. Binding of the SMG6 endonuclease will cleave the RNA near the aberrant termination codon and the resulting decay intermediates will be rapidly degraded by 5'-to-3' (light blue PacMan) and 3'-to-5' exonucleases (violet PacMan). In contrast, binding of SMG5/SMG7 results in recruitment of deadenylases (purple PacMan) and decapping enzyme (dark blue PacMan). The decapped and deadenylated body of the mRNA is subsequently degraded by exonucleases.

be reconciled? The simplest hypothesis is that after PTC recognition, a transcript can be subjected to either degradation route: it is either degraded through the "normal" mRNA turnover pathway starting with deadenylation and/or decapping or through endonucleolytic cleavage. In either case, this is followed by exonucleolytic decay of the resulting RNA fragments from the unprotected ends (**Fig. 1**). If so, what determines which pathway is activated? Interestingly, SMG5, SMG6 and SMG7 are all proteins of similar structure that, at least in part, interact through 14-3-3-like domains with the phosphorylated C-terminal domain of UPF1.[29] However, whereas the PIN domain of SMG6 possesses endonuclease activity,[22] the PIN domain of SMG5 lacks important catalytic residues and SMG7 has no PIN domain at all.[24] SMG7 on the other hand activates mRNA decay in a DCP2- and XRN1-dependent manner, but independently of SMG6, when tethered to a reporter mRNA.[30] Is it possible that the identity of the SMG5-7 protein(s) recruited to the NMD complex determines the mechanism of decay? This idea may explain why in flies, which lack a paralog of SMG7,[31] NMD appears to rely on SMG6-mediated endocleavage, whereas in yeast, which lack SMG6 but possess Ebs1p, a protein homologous to SMG7,[32] NMD initiates by decapping and deadenylation.

This "branched NMD pathway model" also raises several questions. First, are there transcript-specific preferences for one or the other pathway, or is the activity of each decay route regulated developmentally and in a tissue-specific manner? Second, if the mechanism of nonsense mRNA decay is determined by the identity of the recruited SMG5-7 protein, could this be regulated, for example through differential phosphorylation of UPF1. Third, what would be the biological significance of multiple pathways to decay? Are they

required for efficient decay under varying conditions? Finally, the possibility that PTC recognition is not a 100% efficient process and that therefore a population of escaping nonsense mRNAs is present in cells raises the somewhat heretic question of whether the nonsense mRNAs observed to undergo "normal" mRNA degradation could represent such NMD escapees. All these questions remain to be addressed in future studies.

Where does the Decay of NMD Substrates Occur?

Since PTC recognition is tightly coupled to translation, it was a priori expected that NMD must occur in the cytoplasm. Observations based on cell fractionation assays, that in human cell lines the levels of most tested NMD substrates are reduced not only in the cytoplasmic fraction but also in the nuclear fraction, were interpreted to suggest that human NMD takes place either in the nucleus, or during or immediately after nuclear export while the mRNA is still associated with the nucleus.[33] A report presenting evidence for translation within the nucleus of Hela cells[34] combined with multiple observations that linked mammalian NMD to nuclear processes further invigorated the idea of nuclear NMD.[35] On the other hand, more recent evidence that dominant negative peptides inhibit NMD in human cells only when localized in the cytoplasm suggests that the majority of HeLa cell NMD occurs in the cytoplasm,[36] and the issue about the existence of intranuclear NMD remains controversial and unsolved to date.

More recently, NMD factors and substrates have been observed in cytoplasmic mRNP granules called processing bodies (P-bodies),[37,38] suggesting a link between P-bodies and NMD. P-bodies are dynamic structures of translationally repressed, ribosome-free mRNAs and their associated proteins. These include a number of proteins involved in general and specific translation repression and in decapping and 5'-to-3' mRNA decay (reviewed in refs. 39–41). The association of an mRNP with the P-body appears to require at least two steps: (1) the mRNP needs to be released from translating ribosomes, and (2) the mRNP needs to associate with factors that enable its multimerization with other repressed mRNPs. Consistent with this, several lines of evidence suggest that the extent of cellular P-body formation correlates with the levels of ribosome-free mRNPs in the cytoplasm (reviewed in refs. 39–41). Moreover, recent studies have begun to identify protein domains, including protein dimerization domains and prion-like glutamine/asparagine (Q/N)-rich domains, that direct mRNP multimerization and P-body formation.[42,43]

What does the localization of NMD factors and substrates in P-bodies tell us about the cellular location of NMD? Early reports suggested that the formation of P-bodies might be important for translational repression and/or mRNA turnover. However, a more recent study has provided evidence that mRNA decay can be initiated while the mRNA is still engaged with elongating ribosomes in yeast.[44] In addition, recent studies failed to observe major defects in translation or mRNA turnover of tested mRNAs under conditions where formation of microscopically detectable P-bodies was prevented by depletion of factors important for generating translationally repressed mRNPs[45-47] or for the multimerization of mRNPs into P-bodies.[42,43] Similarly, no evidence for a defect in NMD was observed in HeLa cells when microscopically detectable P-bodies were ablated by depletion of the decapping enhancer EDC4/HEDLS/Ge-1.[48] Thus, mRNPs targeted to NMD have the capacity to multimerize with other repressed mRNPs into P-bodies, but no evidence exists that this is critical for the degradation of the NMD substrate. NMD factors and substrates are only strongly observed in P-bodies when late steps in the NMD pathway are inhibited, for example by the inhibition of UPF1 ATPase activity or UPF1 dephosphorylation.[37,38,48] This is predicted to lead to the accumulation of high levels of translationally repressed NMD mRNPs, which in turn accumulate in P-bodies. The observation of NMD factors and substrates in P-bodies therefore does not necessarily reflect a specific cellular location of NMD since the repressed NMD mRNP could in principle have been generated anywhere in the cell prior to its association with other mRNPs in P-bodies. Thus, whether NMD in human cells is restricted to a particular cellular location or whether it can be initiated wherever translation takes place remains a question for future study.

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