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NMD: a multifaceted response to premature translational termination

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Abstract

Although most mRNA molecules derived from protein-coding genes are destined to be translated into functional polypeptides, some are eliminated by cellular quality control pathways that collectively perform the task of mRNA surveillance. In the nonsense-mediated mRNA decay (NMD) pathway premature translation termination promotes the recruitment of a set of factors that destabilize a targeted mRNA. The same factors also appear to play key roles in repressing the translation of the mRNA, dissociating its terminating ribosome and mRNP proteins, promoting the degradation of its truncated polypeptide product, and possibly even feeding back to the site of transcription to interfere with splicing of the primary transcript.

Gene expression must maintain a high level of fidelity to ensure cell function and viability. To tackle this challenge, cells use multiple decay pathways to eliminate nonfunctional transcripts[1,2]. At the level of mRNA, three pathways operate during translation to protect the cell from the possible accumulation of aberrant mRNAs and potentially toxic proteins. These include: Non-Stop Decay (NSD), which detects and degrades mRNAs lacking a stop codon[3-5], No-Go Decay (NGD), which targets mRNAs having ribosomes stalled in translation elongation[6,7], and Nonsense-mediated mRNA Decay (NMD), which promotes the degradation of mRNAs undergoing premature translation termination[8-11]. While these pathways were originally thought to simply promote accelerated mRNA decay, recent studies suggest more complex post-transcriptional regulation[12]. In NMD, the Upf proteins, a set of conserved factors of which Upf1 is the central regulator, recruit decay enzymes to promote endonucleolytic cleavage, 5’ to 3’ decay, or 3’ to 5’ decay of prematurely terminating mRNAs. However, they must also discriminate between normal and premature termination events and conduct ancillary processes involving translational repression of mRNAs containing premature termination codons (PTCs), dissociate and properly recycle poorly dissociable PTC-bound ribosomes, and unwind the PTC-containing mRNP, all in the interest of facilitating rapid decay of the mRNA and reutilization of the components of the translational machinery. Upf1 also promotes proteolysis of the nascent polypeptide, presumably to ensure a minimal opportunity for that protein fragment to interfere in cellular processes. In this Review, we provide an overview of the NMD pathway, seeking unified explanations for its broad range of activities in all eukaryotes.

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mRNA substrates for NMD

NMD targets transcripts in which translation is arrested by a premature termination codon (BOX 1). As such, the substrates of this pathway include transcripts derived from genes harboring nonsense mutations, inefficiently spliced pre-mRNAs that enter the cytoplasm with their introns intact, mRNAs in which the ribosome has bypassed the initiator AUG and commenced translation further downstream, some mRNAs that contain upstream open reading frames (uORFs), mRNAs that are subject to frameshifting or that are generated by certain alternative splicing events, bicistronic mRNAs, and transcripts of pseudogenes, transposable elements, and genes that are subject to programmed rearrangements[13-16]. NMD substrates also include mRNAs in which the translation termination codon occurs in an mRNA context characteristic of premature termination, e.g., at the 5’ end of an atypically long 3’-untranslated region (3’-UTR)[17]. The latter class includes mRNAs with normal or biologically regulated extensions of their 3'-UTRs.

Gene expression profiling of yeast, fly, and human cells show that from 1 to 10% of cellular transcripts are up-regulated by NMD inactivation[13,18-24]. These experiments have demonstrated that NMD not only controls the expression of aberrant transcripts, but also that of many apparently wild-type mRNAs. Accordingly, NMD appears to have regulatory functions beyond mRNA surveillance[25,26].

The role of translation termination

Destabilization of nonsense-containing mRNAs depends on recognition of the nonsense codon by the translational machinery[27-31] and the ability of that machinery to recognize a stop codon as ‘premature.’ Thus, a key question is how do normal and premature translation termination differ.

Normal translation termination

The termination of protein synthesis at UAA, UAG, or UGA stop codons occurs in multiple steps and requires two classes of release factors (RFs). Class I RFs act like tRNAs in that they first bind to the ribosomal **A site** in response to the presence of nonsense codons. However, instead of promoting peptidyl transfer they trigger hydrolysis of the polypeptide chain attached to the P site tRNA. Class II RFs are GTPases that stimulate class I RFs[32]. Translation termination in eukaryotes is mediated by the class I factor eRF1 and the class II factor eRF3 (Sup45 and Sup35 in yeast)[33-35]. Following stop codon recognition by eRF1, its conserved GGQ motif activates the peptidyltransferase center of the ribosome to mediate peptide release[36,37]. eRF3 GTPase activity is dependent on ribosome-bound eRF1 and couples eRF1 recognition of a nonsense codon to efficient polypeptide release[38]. Dissociation and recycling of the termination complex is facilitated by the ABCE1 ATPase and initiation factors eIF3, eIF1, eIF1A, and eIF3j [39-42]. ABCE1 interacts with eRF1 and this interaction is conserved by the ABCE1 homolog, Rli1, in yeast. Rli1 also interacts with subunit j of the initiation factor eIF3 (Hcr1p in yeast)[43].

Other factors have been assigned possible roles in translation termination because alterations in their activity (or presence) affect nonsense codon readthrough. Notably, normal termination is thought to be stimulated by the interaction of the poly(A)-binding protein (Pab1 in yeast) with eRF3[44,45]. Pab1 overexpression enhances termination efficiency in yeast[45] and, reciprocally, deleting PABPC1 increases nonsense codon readthrough in human cells[46]. Downstream of a normal termination codon (NTC) eRF3-Pab1 interaction may orchestrate formation of an mRNP complex favorable to termination[44-48]. Additional proteins implicated in termination include the mRNA export factors encoded by *NPL3*, *DBP5*, and *GLE1*[49-51]. While mutations in these genes lead to nonsense suppression and

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sensitivity to drugs affecting translation, it's possible that these are indirect consequences of their effects on mRNP structure.

The aberrant nature of premature termination

Premature termination does not appear to be the mechanistic equivalent of normal termination. Toeprint analyses assaying the precise position of single ribosomes on mRNA failed to yield any toeprinting signals from normal yeast termination codons unless eRF1 was inactivated by a temperature-sensitive lesion. In contrast, ribosomes at PTCs yielded toeprint signals consistent with A-site occupancy even without eRF1 inactivation[52]. Similarly, a codon 39 PTC that targets human β-globin mRNA for NMD was found to yield a toeprint whereas the normal termination codon of β-globin mRNA did not[53]. These data indicate that, in both yeast and human cells translation termination at a PTC is less efficient than normal termination and leads to a pause of the ribosome at the nonsense codon. Premature termination should thus be considered aberrant. Independent evidence for mechanistic differences between premature and normal termination follows from the identification of PTC124 (ataluren), a novel compound that promotes the **translational readthrough** of premature but not normal termination codons in human cells[54].

An unresolved question is which step in premature translation termination is affected. There is no evidence that peptide hydrolysis is slower at PTCs than at NTCs, thus implicating reductions in the rate of later steps in the termination process. Most evidence points to reductions in the efficiency of ribosome and mRNP dissociation subsequent to peptide hydrolysis. For example, the toeprinting experiments alluded to in the previous paragraph imply that ribosomes are dissociated much more slowly from PTCs than NTCs[52,53] and translational reinitiation after premature termination is markedly reduced *in vivo* or *in vitro* when any one of the three yeast Upf proteins is absent[52,55]. Further, following translation termination at a PTC, yeast *upf1Δ* extracts manifest a ribosome recycling defect that can be complemented by purified Upf1[55]. In human cells, the expression of ATPase- or helicase-deficient Upf1 leads to the accumulation of β-globin mRNA decay intermediates that are endonucleolytically cleaved by Smg-6, blocked in subsequent 5' to 3' exonucleolytic degradation of the 3'-cleavage product, and associated with Upf1 and the other Upfs in an mRNP that may also include the terminating ribosome[56]. Collectively, these results indicate that, in addition to promoting accelerated mRNA decay, the Upfs also play a role in disassembling the poorly dissociable post-premature termination mRNP[48].

Factors required for NMD

NMD is primarily carried out by the Upf proteins, but other factors are also necessary to recruit and regulate the Upfs and to activate NMD

Upf proteins

The proteins encoded by *UPF1* (also known as *SMG2*), *UPF2* (also known as *SMG3* and *NMD2*), and *UPF3* (also known as *SMG4*) are the principal NMD regulators in eukaryotes[57-62]. Mutations in the *UPF* genes, or silencing of their expression, stabilize nonsense-containing mRNAs, and increase their relative abundance, but generally have little or no effect on most wild-type transcripts[63]. The three Upf proteins interact (FIG. 1a) with Upf2 acting as a bridge between Upf1 and Upf3[63-65].

Upf1 is a large cytoplasmic protein (109 kDa in yeast; 130 kDa in humans) with two principal domains. A cysteine- and histidine-rich zinc-finger domain (CH domain) located near the Upf1 N-terminus is followed by a flexible linker segment and the conserved motifs common to superfamily I (SF1) helicases[9,66,67]. The Upf1 helicase core includes two RecA-

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like domains common to all SFI helicases, and two regulatory domains (1B and 1C) specific to Upf1[68]. Upf1 is able to bind RNA in the presence or absence of ATP[64,69,70]. Both the CH domain, and the RNA-dependent ATPase and RNA helicase activities are essential for NMD[69-72]. The CH domain also interacts with Upf2, an acidic 127 kD protein with three conserved MIF4G (middle portion of eIF4G) domains[59,62,73] (FIG. 1a). It utilizes the third of these domains to interact with Upf3, a basic 45 kD protein with an RNP-type RNA-binding domain (RBD)[63,74]. The nonspecific RNA-binding activity of the resulting Upf2:Upf3 complex is thought to be a property of a conserved basic region of Upf2.

UPF1 is the key effector of NMD, with Upf2 and Upf3 regulating Upf1 function. This conclusion follows from biochemical analyses showing that maximal activation of Upf1's ATPase activity *in vitro* requires both Upf2 and Upf3[64] and that overexpression of *UPF1* can compensate for *upf2Δ* and *upf3Δ* mutations, but not vice versa[75]. This functional hierarchy is consistent with mechanistic analyses. Upf1's CH domain can interact with a C-terminal domain of Upf2 and mutations affecting this interaction inhibit NMD[63,73,76,77]. In the absence of Upf2, Upf1 exists in a closed conformation in which the CH domain interacts with the RecA-like domains[78] (FIG. 1b). In this conformation domain 1B binds to RNA, thus increasing the overall extent of RNA binding by Upf1 and decreasing its ATPase and helicase activities[64,78,79]. Upon binding to Upf2, the Upf1 closed state undergoes substantial conformational change, with the CH domain switching from a position near the RNA binding site to a more distal position physically disconnected from both the RNA and nucleotide binding sites (FIG. 1b). Mediated by high affinity Upf2:CH domain interaction, this "open" form binds RNA less extensively and has increased levels of ATPase and helicase activities, i.e., it switches from a state in which it is tightly bound to RNA to one in which it has the capability for RNA unwinding[64,78]. The effects of Upf2 interaction on helicase and ATPase activation can be mimicked by deletion of the Upf1 CH domain[78].

Smg factors

In worms and other multicellular organisms, NMD is also regulated by Smg factors, proteins that are involved in a cycle of Upf1 phosphorylation and dephosphorylation (BOX 2). This cycle is required for NMD activation and regulates protein-protein interactions that recruit specific mRNA decay enzymes (see below). There's no evidence that Upf1 ATPase and helicase activities depend on the protein's phosphorylation status, but dephosphorylation has been shown to allow recycling of Upf1, including its exit from **P-bodies**[80] (BOX 2). The Upf1 phosphorylation/dephosphorylation cycle does not appear to be restricted to multicellular organisms. In yeast, Upf1 and Upf2 are also phosphorylated[81]. Ebs1 has been identified as a weak homologue to Smg-7 and has been implicated in the Upf1 phosphorylation/dephosphorylation cycle in yeast. However, deletion of *EBS1* has only a moderate effect on NMD[82].

The EJC

In mammalian cells, NMD is generally splicing dependent and usually requires that a PTC be localized at least 50-55 nt upstream of an exon-exon junction[8]. This phenomenon relies on the exon-junction complex (EJC), a group of proteins deposited on an mRNA during splicing, 20-24 nt 5' of an exon-exon boundary[83,84] (FIG. 2). The composition of the EJC is dynamic, and includes at least the core proteins Y14, Magoh, Barentz, and eIF4AIII, and one effector of NMD, Upf3. The EJC travels with an mRNA into the cytoplasm, where it acts as an effector for events in mRNA metabolism, including the activation of translation, mRNA localization, and NMD[85-87]. Upf3 binds to the EJC during splicing and Upf2 associates with EJC-bound Upf3 in the cytoplasm[85,86,88-90]. The EJC also plays a role in the recruitment of Smg-6, a factor implicated in Upf1's phosphorylation cycle as well in the endonucleolytic cleavage of PTC-containing mRNAs. Smg-6 and Upf3 share the same

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binding site and compete for EJC interaction, suggesting that the EJC is rearranged during NMD activation or that the NMD pathway can, in some instances, function independently of Upf3[91]. Indeed, NMD activation independent of Upf3, Upf2, or some EJC components has been reported, suggesting that NMD in mammals is not restricted to a single pathway[92,93].

Release factors

The dependence of NMD on translation termination is reinforced by experiments demonstrating that the Upf proteins bind the release factors. In humans and yeast, Upf1 interacts with eRF3[46,77,94,95], an interaction thought to recruit Upf1 to the termination complex. Support for this notion comes from identification of a complex between Upf1 and the release factors in human cells, i.e, the SURF (Smg-1-Upf1-Release Factors) complex (FIG. 2)[77,96]. In yeast, Upf2 and Upf3 also interact with eRF3, and Upf1 can also interact with eRF1. eRF3 seems to have a unique Upf1-binding site located in its GTPase domain[46,97], but Upf2, Upf3, and eRF1 all compete for binding to eRF3 and might share a common interaction domain[97]. Upf1 is thought to interact with eRF3 through its CH-domain[46], but this result is controversial[95,98]. Regardless of the definition of the respective interaction sites, the binding of eRF1 and eRF3 to Upf1 inhibits Upf1's ATPase activity[94], an observation consistent with the notion that Upf1 is initially recruited to the premature termination complex in an inactive form that must then be activated by the Upf2:Upf3 complex[64].

In yeast, mutations in the *UPF* genes promote nonsense suppression[66,70,75,97,99,100]. The initial interpretation of this observation, based in part on the interactions of the *UPF* proteins with the release factors, was that the Upfs played a direct role in maintaining the fidelity of translation termination. However, RNAi depletion of human Upf1 was shown to decrease nonsense codon readthrough, a surprising result considering the conservation of the termination and NMD machinery[46]. These discrepancies were resolved recently by the identification of mutations that reverse the readthrough phenotype in a yeast *upf1Δ* strain[101]. This study showed that the mRNA encoding yeast's principal Mg^{2+} transporter, Alr1, contained upstream ORFs and was an endogenous NMD substrate. When NMD was inactivated *ALR1* mRNA was stabilized, in turn raising cellular levels of Alr1. As a consequence, intracellular levels of Mg^{2+} also increased, thus reducing the fidelity of translation termination[101]. Thus, although Upf1 interacts with the release factors, the *ALR1* results suggest that, at least in yeast, its effects on termination fidelity are largely indirect.

NMD activation and regulation

As noted above, considerable information is available on the structures, activities, and interactions of the factors that regulate NMD. Below we discuss how these factors might operate during premature translation termination to activate NMD. We attempt to reconcile the different models of NMD activation while keeping in mind two key challenges to the initiation of this process: the specific targeting of the Upfs to an mRNA engaged in terminating translation prematurely and the subsequent activation of the ATPase and helicase activities of Upf1 by Upf2 and Upf3.

The mRNA marking models

Studies in yeast first suggested a mechanism for discriminating normal from premature termination by virtue of a protein mark that would be present only on prematurely terminated mRNAs[102-104]. Evidence that the Upf proteins interacted with eRF1 and eRF3[94,103,105] led to the notion of a termination 'surveillance complex' that could detect the presence of specific proteins 3' to the termination codon[102-104]. Yeast factors that interacted with the surveillance complex to activate NMD were thought to be mRNA-binding proteins

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such as Hrp1 that exited with the transcript from the nucleus, and were not removed by a translating ribosome because termination occurred prematurely[103,104]

A related model has also been proposed for mammalian cells, where NMD often depends on the presence of an intron downstream of the PTC, a link that reflects a role for the exon junction complex (EJC)[106-108]. This model[109] posits that Upf2 and Upf3 bind to the EJC core whereas Upf1 and its regulator Smg-1 are recruited to the termination complex by the release factors, with the latter forming the SURF complex with Smg-1 regulators Smg-8 and Smg-9[77,96] (BOX 2 and FIG. 2). If a ribosome stops at a PTC before it displaces the downstream EJC, then SURF-associated Upf1 is thought to be able to interact with Upf2:Upf3 bound to the EJC. In turn, this can activate Upf1 phosphorylation by Smg-1, forming the DECID (DECay InDucing) complex, and activate the Upf1 ATPase and helicase activities, leading to mRNA degradation through interaction of Upf1 with Smg 5-7 and Smg-6[110]. However, this proposed mechanism fails to be a suitable explanation for PTC-containing mRNAs derived from intron-less precursors or those mRNAs that are subject to NMD without prior splicing, proper EJC spacing, or EJC components[111-115].

The faux-UTR model

In yeast and *C. elegans*, mRNP immunoprecipitation experiments show that Upf1 preferentially associates with mRNAs that are NMD substrates[18,116]. However, the mechanism of its selective recruitment or selective retention on ribosomes engaged in premature termination is unknown. The same concern applies to mammalian SURF assembly. Thus, an alternate explanation for the initiation of NMD takes into account the nature of the mRNP 3’ to a **nonsense codon**. Studies in yeast have shown that deleting the coding region downstream of a PTC, thus bringing the normal 3’-UTR close to the premature termination codon, will stabilize an otherwise unstable mRNA, as well as eliminate the toeprint associated with premature termination[52,117,118]. Likewise, in human cells, an mRNA containing synthetic sequences allowing the 3’-UTR to fold back and be positioned near a PTC also becomes resistant to NMD[119]. Reciprocally, mRNAs with substantially extended 3’-UTRs are NMD subtrates in yeast, human, *Drosophila*, and plant cells[95,120-129]. Collectively, these observations indicate that normal rates of mRNA decay, and most likely normal termination, depend on the mRNP structure downstream of a termination codon. As postulated by the *faux*-UTR model, the 3’-UTR created by a PTC must lack termination regulatory factors present on a normal 3’-UTR, leading to aberrant translation termination and subsequent NMD activation[130]. In this model, proper translation termination and normal mRNA decay are postulated to depend in part on interactions between Pab1 and eRF3 (FIG. 3). Aspects of this model have been validated[46,52,95,119], but others have not. Notably, the cellular absence of Pab1 or the deletion of the respective interacting domains on Pab1 do not convert wild-type mRNAs to NMD substrates[131,132]. These results do not negate the general notions of the *faux*-UTR model, but they do indicate that the termination role of the 3’-mRNP is more complex than the sum of the functions of just Pab1 and eRF3.

In a variation of the *faux*-UTR model Hogg and Goff have reported the differential binding of human Upf1 as a direct function of mRNA 3’-UTR length[133]. They suggest that the apparent correlation of binding preference with NMD sensitivity implicates 3’-UTR length as a primary association determinant sensed by Upf1. This confirms the role of the 3’-UTR downstream of the PTC as a major determinant in NMD activation. Two aspects of the observed binding of Upf1 to 3’-UTRs are not easily reconciled with earlier studies. First, binding takes place when translation is inhibited, which is surprising because NMD depends on nonsense codon recognition by the ribosome (see above). Second, it is unclear how

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human Upf1, a protein quite closely related to its yeast counterpart, might be able to distinguish a completely different spectrum of UTR lengths than occur in yeast.

Reconciling mRNA targeting models

Although multiple aspects of the NMD pathway are understood in detail, there remains considerable controversy over its precise mechanism of action. This is particularly true if one attempts to reconcile models of NMD in lower and higher eukaryotes. That said, gene expression pathways in lower and higher eukaryotes are so substantially similar that it seems at least some unitary NMD characteristics should emerge.

A feature common to the prevailing models is that activation of NMD might depend on an inappropriate mRNP structure neighboring the PTC. This situation would hold whether Pab1 or other 3'-UTR factors are absent, or because an EJC is present, and could certainly accommodate organisms such as plants, in which NMD can be activated by either the presence of an exon-exon junction downstream of a PTC, or by an extended 3'-UTR[126]. An accommodating mRNP structure might provide sufficient specificity for Upf1 interaction with eRF3. The predicted competition of Upf1 and Pab1 for binding to eRF3 that was not manifested *in vitro*[132] may work in the context an RNP (FIG. 3). This would explain Upf1 specificity for premature termination, but leaves open the mechanism of Upf2 and Upf3 recruitment as well as the basis for Upf1 association with the 40S ribosomal subunit in yeast[55]. Clearly, 40S association could reflect a cause or consequence of ribosome dissociation. Another important consideration in any mRNP-centric model is that the presence or absence of certain proteins, for example the components of the EJC, could be seen not as crucial NMD factors but as activators of translation, a step critical to PTC recognition. In this regard, it should be noted that EJC proteins also have important roles in determining the ability of a mature mRNP to be translated[134-136].

A completely different way to consider this problem is from the perspective of the different efficiencies of termination. If normal termination is enhanced by interactions between the release factors and proteins associated with a normal 3'-UTR then perhaps the inefficiency of premature termination is initially attributable to poor release factor binding at the A-site of the ribosome or to slow dissociation of the termination complex after peptide hydrolysis. The latter is a more likely possibility because the toeprinting differences between normal and premature terminators indicate that a ribosome is stalled on the PTC and because there is no evidence for differences in peptide hydrolysis rates in normal and premature termination. However, either inefficiency might create a Upf1 binding site on the ribosome that is distinct from the A- or P-sites, but dependent on an atypical conformational state assumed by one of them; for example, the P site may retain a deacylated tRNA. Interaction between Upf1 and the eRFs could still occur, but the order of the hypothesized binding to the termination complex would now be reversed. This altered site model (FIG. 3) addresses specificity for premature termination, and Upf1:eRF and Upf1:ribosome interactions, but, as with the altered mRNP model discussed above, still leaves open the question of how Upf2 and Upf3 are recruited to activate Upf1. In cryoelectron microscopy reconstructions of a eukaryotic ribosome recycling complex only the structural homologue of eRF1 (Dom34) and the recycling factor ABCE1 (Rli1 in yeast) are present. This indicates that dissociation of eRF3 precedes ABCE1/Rli1 binding for ribosome recycling[42]. This result prompts us to propose that an aberrant step in premature translation termination could be the inefficient release of eRF3, possibly linked to the absence of Pab1. The presence of eRF3 on a terminating ribosome could recruit Upf1 to mediate an alternative mode of ribosome recycling.

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Consequences of activating NMD

Activation of NMD has multiple ramifications. After recognition of the termination event as premature, and formation of the trimeric Upf1:Upf2:Upf3 complex, several parallel and interrelated events ensue. The targeted mRNA is subject to translational repression, and activation of the Upf1 ATPase and helicase activities plays a critical role in promoting dissociation and recycling of the ribosome and other associated factors, steps that are likely to prepare the mRNA for additional events in decay. NMD activation also promotes overall downregulation of expression of the products of the targeted allele, including degradation of the nascent polypeptide fragment and inhibition of splicing of the respective pre-mRNA.

Translational repression

Muhlrad and Parker[123] observed that the presence of a PTC in a *CUP1* fusion mRNA led to an apparent decrease in its translational efficiency. This observation led to the hypothesis that nonsense-containing mRNAs must be translationally repressed prior to their degradation (FIG. 4 pathway A), a proposal for which P-body localization of NMD-targeted mRNAs was considered substantial support[137,138]. However, as discussed below, reductions in the level of polypeptides produced by a PTC-containing mRNA can also be explained by the existence of a proteasome-dependent proteolytic pathway that targets such proteins[139]. That said, independent evidence for translational repression of NMD substrates has been obtained in mammalian cells. Isken et al.[25] have observed that phosphorylated Upf1 can interact with eIF3 and interfere with translation initiation *in cis*. Strong support for this possibility came from experiments showing that comparable effects were not observed in mRNAs employing the eIF3-independent CrPV IRES[25].

Ribosome release and recycling

Activation of the Upf1 ATPase and helicase activities appears to play a critical role in preparing the mRNA for decay by dissociating the ribosome from the termination site, possibly starting with the 60S subunit, and unwinding other components of the mRNP[55,56] (FIG. 4 pathway B). This conclusion follows from an understanding of the aberrant nature of premature termination and from studies of the consequences of inactivating Upf1. Thus, addition of cycloheximide to yeast *in vitro* translation reactions was shown to allow detection of additional toeprints in close proximity to PTCs[52]. These toeprints were derived from post-termination ribosomes that failed to be released at premature terminators and were able to scan both 3’ and 5’ from the PTC and reinitiate translation at nearby AUGs. In eRF1-defective extracts, these toeprints were eliminated and replaced by toeprints corresponding to ribosomes stalled with the relevant stop codon in their A-sites. This indicated that, prior to any reinitiation event, a PTC must be recognized by eRF1 and peptide hydrolysis must be triggered. Importantly, the reinitiation toeprints were eliminated in extracts lacking one of the Upf proteins. This observation not only linked the reinitiation toeprints to NMD, but also reinforced the notion of inefficient premature termination and suggested that the Upf proteins might influence the extent to which prematurely terminating ribosomes remain associated with an mRNA. This possibility was underscored by the demonstration that reinitiation downstream of a PTC was also eliminated in *upf*-deletion strains *in vivo*[55].

A further understanding of the post-termination function of the Upf proteins followed from additional *in vitro* translation analyses in yeast extracts[55] and from studies in human cells expressing ATPase- or helicase-deficient Upf1[56]. A yeast *in vitro* assay that monitored ribosomes undergoing premature termination indicated that extracts lacking Upf1 not only fail to reinitiate, but also appear to have a 60S joining defect in conventional initiation. The latter defect is thought to be a consequence of inadequate dissociation and recycling of ribosomes at a prior premature termination event. The inability of *upf1Δ* extracts to recycle

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ribosomes efficiently from a nonsense-containing mRNA is consistent with other experiments indicating links between termination and initiation[40,43] and with prior studies showing genetic interactions between Upf1 and eIF1 or eIF3, initiation factors that stimulate the *in vitro* dissociation of post-termination 80S complexes[16,32,39]. In human cells Franks et al.[56] showed that endonucleolytically cleaved and partially degraded nonsense-containing β-globin mRNA accumulates in the presence of ATPase- or helicase-deficient Upf1. The decay intermediate appeared in a complex that included all three Upfs and, possibly, the ribosome. In short, a combination of experiments in yeast and human cells indicate that at least one inefficient step in premature termination occurs at post-termination ribosome release, and Upf1 (and presumably the other Upfs) plays an important role in the latter release event, including the activation of its ATPase and helicase activities to couple release and mRNP disassembly to a step enabling ribosome reutilization. A role for the Upfs in the disassembly of a poorly dissociable termination complex is reminiscent of the function of Dom34 and Hbs1 in No-Go Decay[6].

Accelerated mRNA decay

Upf1 interactions with several factors appear to play a key role in promoting the degradation of NMD substrates by multiple decay pathways in yeast and mammalian cells (FIG. 4, pathways C1 and C2, and FIG. 5)[13,140-142]. In yeast and human cells Upf1 interacts with the Dcp1/Dcp2 decapping enzyme, the exoribonuclease Xrn1, and various **exosome** components, suggesting a direct recruitment of the decay enzymes[59,140-143]. In addition, PNRC2 (Proline rich nuclear receptor co-regulatory protein 2) interacts with hyperphosphorylated Upf1 and Dcp1a, providing an additional link between the NMD and decapping machineries[144]. However, the notion of direct interactions between Upf1 and decay enzymes may be too simplistic. For example, it has been suggested that interactions between yeast Upf1 and Dcp2 may be indirect consequences of Upf1 interaction with the decapping activators Edc3 and Pat1[59,145,146].

As with the activation of Upf1 by Upf2:Upf3, the assembly of decay complexes in multicellular organisms appears to be a bit more straightforward and to include important roles for the non-Upf Smg factors. Two pathways have been described, which involve the interaction of phosphorylated Upf1 with either Smg-6 or Smg-5:Smg-7 (FIG. 2). Smg-6, by virtue of its **PIN domain**, has an endonuclease activity with which it initiates mRNA decay near the PTC in *Drososphila* and human cells[56,147,148]. The resulting 5’ and 3’ fragments are then degraded by the exosome and Xrn1, respectively[148,149]. Recruitment of Smg-5:Smg-7 is proposed to promote 5’ to 3’ and 3’ to 5’ decay of the targeted mRNA[150]. This is consistent with the absence or inactivity of PIN domains in both proteins and with experiments showing that tethering of Smg-7 to an otherwise normal mRNA is sufficient to promote its degradation, a function carried by its C-terminal domain[150-152]. Interestingly, it has been suggested that the choice between the two pathways in some organisms may reflect genomic differences[153]. For example, *D. melanogaster* lacks Smg-7 and its nonsense-containing mRNAs are thought to only initiate decay by Smg-6-mediated endonucleolytic cleavage[154], a conclusion rendered somewhat complicated by the recent observation that *Drosophila* NMD remains active in flies harboring SMG6 mutations[155].

Concomitant targeting of the nascent polypeptide

At least in yeast, NMD also activates the rapid degradation of the truncated polypeptide by the proteasome[139,156], underscoring the biological importance of minimizing the accumulation of potentially toxic partially-synthesized proteins (FIG. 4, pathway D). Proteolysis depends on Upf1, and utilizes the **ubiquitin-proteasome pathway**, but its timing relative to other steps in NMD is unknown. Upf1 function in nonsense-mediated polypeptide decay may include a role as an E3 ubiquitin ligase: its N-terminal **CH domain**

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is distantly related to classical E3-RING finger domains, and it could interact with an E2 (Ubc3), and Upf1 could self-ubiquitinate *in vitro* in a Upf3-dependent reaction[156]. Although it is tempting to speculate that such modification of Upf1 may be crucial to its role in promoting proteolysis, we cannot rule out the possibility that ubiquitination, like phosphorylation, controls the ability of Upf1 to interact with critical mRNA decay factors. The targeted degradation of the nascent nonsense polypeptide is reminiscent of both the co-translational decay of the polypeptide generated by mRNAs subject to NSD as well as the bacterial tmRNA system, which disassembles elongation-stalled ribosomes while adding an ssrA tag to the associated polypeptide to promote its rapid decay[157,158]. In all of these circumstances mRNA decay, peptide decay, and ribosome recycling appear to be highly coordinated.

Feedback to the site of transcription

The evidence is fairly strong that, in all genera, NMD is a cytoplasmic, translation-dependent process. However, an increasing number of studies has shown that pre-mRNAs derived from some PTC-containing genes, for example, those encoding Ig-μ and T cell receptor (TCR)-β, accumulate at or near their respective sites of transcription. This surprising down-regulation of expression is gene- and allele-specific, that is, it is not detected with frameshift or missense alleles of the same genes, it is Upf1- and Smg-6-dependent, and the pre-mRNAs which accumulate at the transcription site are unspliced[159-161] (FIG. 4, pathway E). As there is no current evidence for a direct link between NMD and gene-specific silencing mechanisms, it is useful to consider alternative, indirect explanations for this phenomenon. For example, it is well known that pre-mRNAs with defective 3' processing are often retained at their site of transcription[162]. Further, the Ccr4/Not complex, which is involved in cytoplasmic mRNA deadenylation and includes components that cycle to the nucleus, has been implicated in quality control mechanisms that stall transcripts in the nucleus[163]. Hence, it is possible that the rapid deadenylation of some mammalian NMD substrates may yield a post-translationally modified factor from the "accelerated" Ccr4/Not deadenylation complex in the cytoplasm that feeds back to its nuclear counterpart, with the latter stalling late events in nuclear processing and export[164]. Although the mechanism for allele specificity in such a model is unclear evidence that transcription may influence mRNA decay, and possibly vice versa, is starting to accumulate[165-167].

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, or termination that mimics at least some regulatory features characteristic of premature termination. The substrates of this pathway include the transcripts of genes harboring nonsense mutations or processing errors, pre-mRNAs that have entered the cytoplasm, mRNAs from rearranged genes, edited transcripts, mRNAs with uORFs, pseudogene mRNAs, and others. Collectively, NMD substrates may account for as much as 10% of the transcriptome in exponentially growing undifferentiated cells, including some mRNAs that encode normal proteins. It remains to be established whether all of these mRNAs contain PTCs or PTC-like features.

The three Upf proteins are the key NMD factors in all eukaryotes. Of these, Upf1, a superfamily I RNA helicase, is the central regulator. A detailed understanding of Upf1's specificity for binding to a ribosome translating a PTC-containing mRNA remains to be elucidated, but it may depend on interaction with eRF3 and/or eRF1, an accommodating mRNP structure downstream of the PTC, and the local absence of Pab1. In mammalian

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cells, activation of the Upf1 ATPase and helicase activities is often a consequence of interaction with Upf2 and Upf3 that is associated with a downstream EJC. In transcripts without EJCs the mode of Upf1 association with Upf2:Upf3 is presently unclear and raises the possibility that a unitary NMD model may be elusive. Interaction with Upf2:Upf3 not only activates Upf1's enzymatic activities, but also leads to its phosphorylation and its interaction with several other proteins including several Smg factors. Activated and phosphorylated Upf1 has multiple activities and much needs to be learned about the relative order of these activities and their coordinattion. Upf1 and its interactors appear to recruit decay enzymes that promote endonucleolytic cleavage, 5' to 3' decay, or 3' to 5' decay, but the mechanism of this recruitment as well as the means for selecting a particular decay pathway are far from clear. Upf1 can also repress the translation of a PTC-containing mRNA, dissociate and properly recycle its poorly dissociable PTC-bound ribosome, and unwind the PTC-containing mRNP, all in the interest of facilitating rapid decay of the mRNA and reutilization of the components of the translational machinery. Presumably to ensure that the nascent polypeptide has a minimal opportunity to interfere in cellular processes, Upf1 also promotes its proteolysis. It remains to be established whether all of these activities require all three Upfs, thus raising the question of whether mRNAs that associate with only one or two of these factors are subject to only a subset of their collective functions.

Almost all NMD-related activities are cytoplasmic. However, the extensive network of factors that carry out all the ramifications of NMD includes proteins that cycle between the cytoplasm and the nucleus. Accordingly, some cytoplasmic NMD events may provide feedback to nuclear steps in gene expression. Collectively, the set of events triggered by premature translational termination extend far beyond merely enhancing the rate of decay of a nonsense-containing mRNA. The resulting all encompassing shut-down of expression helps to explain why nonsense alleles are effectively null alleles and suggests potential avenues for enhancing the effects of therapeutic nonsense suppression[54,168].

Finally, studies of NMD have underscored how little is known about translation termination in eukaryotes, at least when compared to our understanding of initiation. Not very long ago termination appeared to depend on just a nonsense codon and the eRFs. This parts list has grown, but a thorough understanding of the termination mechanism awaits resolution of the complexities implied by the indirect effects of mRNP proteins, the dissimilarities of normal and premature termination, and the possible complications of sequence context.

Definitions

Nonsense codon—The original name for UAA, UAG, or UGA, the codons that provide a stop signal for the elongation of protein synthesis when the ribosome reaches the normal end of an mRNA's open reading frame. When they occur prior to that site they promote premature translational termination. Prevalent in all non-coding regions, such as introns, thus explaining why transcripts encompassing in-frame non-coding segments often become NMD substrates.

Upf (Up-frameshift) factors—The core regulators of the NMD pathway. These factors were first identified in yeast genetic screens seeking alleles that affected the consequences of frameshiting, the process wherein the ribosome can shift the translational reading frame during protein synthesis. Some yeast mRNAs undergoing frameshifting are known to be substrates for NMD.

Smg (Suppressor with morphogenetic effects on genitalia) factors—Additional effectors of the NMD decay pathway in multicellular organisms. Their name refers to the

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unusal phenotype they manifest in *Caenorhabditis elegans*, the organism in which they were first identified by genetic screens. *SMG2*, *SMG3*, and *SMG4* are orthologues of the *UPF1*, *UPF2/NMD2* and *UPF3* genes, respectively.

Zinc-finger domain (CH domain)—A protein structural motif that coordinates zinc ions with cysteine and histidine residues. This motif is often central to protein modules that serve as interaction domains or binding sites for DNA or RNA. In Upf1, this domain encompasses a unique combination of three zinc-binding motifs arranged into two tandem modules.

RNA helicase—An enzyme harboring ATPase and RNA unwinding activity. Cells contain many different RNA helicases that are respectively involved in a wide spectrum of gene expression functions, including pre-mRNA splicing, protein synthesis, and mRNA decay.

P (Processing) bodies—Cytoplasmic foci, also known as GW-bodies and decapping bodies, that contain high concentrations of factors involved in 5’ to 3’ mRNA decay, such as the decapping complex, Dcp1-Dcp2, and the exoribonuclease, Xrn1, as well as the machinery of miRNA-mediated mRNA decay in multicelular organisms. The role of P-bodies in mRNA decay has been controversial because these structures are not essential for mRNA decay or miRNA silencing, and mRNAs that enter P-bodies are not necessarily destined for degradation and can exit these foci and reinitiate translation.

Cycloheximide—An inhibitor of protein synthesis in eukaryotes that blocks the translocation of the ribosome during the elongation step. When added to cells or extracts in which translation is underway this drug will stall elongating ribosomes on the mRNA.

Ribosomal A, P, and E sites—Ribosomes, the cellular machines catalyzing protein synthesis from an mRNA template, harbor these three sites to accomodate tRNAs. Aminoacyl-tRNA binds in the A site, peptidyl-tRNA binds in the P site, and deacylated tRNA binds in the E site. During translational elongation, tRNAs move successively from the A to the P to the E site, always in response to specific codon:anticodon pairing.

PIN domain—A protein domain of approximately 130 amino acids that can function as a ribonuclease and cleave single stranded RNA. The name is derived from its identification in the N-terminus of the PilT protein.

Translational readthrough—Termination at a nonsense codon is not absolute. A near-cognate aminoacyl-tRNA, i.e., one which can base pair with a stop codon at two of the three positions of a codon–anticodon complex, is a competitor for eRF1 binding in the A site. If the stop codon is “read” by this competing tRNA, the ribosome will insert an amino acid residue and continue to translate sequences downstream of the nonsense codon, usually until it reaches the next stop codon. This readthrough event is also known as nonsense suppression and its frequency is often monitored as an indicator for the fidelity and efficiency of translation termination.

Toeprinting—This method identifies the position of ribosomes on an mRNA. The technique is based on the inhibition of primer extension by reverse transcriptase (RT). Using a labeled oligonucleotide that hybridizes 3' to the region of interest of an mRNA, reverse transcriptase extends the primer and synthesizes complementary DNA from the site of primer binding toward the 5' end of the mRNA. When the reverse transcriptase encounters the ribosome (or associated factor) bound to the mRNA, polymerization is halted and a cDNA toeprint fragment is generated. Its size is then determined on high-resolution polyacrylamide gels. At termination codons, the cessation of reverse transcription can be

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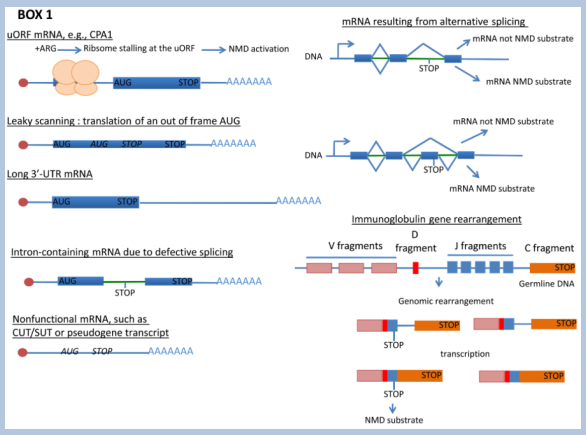
detected approximately 13 nt downstream of the U of the termination codon. This spacing is consistent with ribosomes that have paused with the termination codon in the ribosome's A site.

BOX 1

Substrates of the NMD pathway



In addition to mRNAs transcribed from genes harboring nonsense mutations, NMD also targets mRNAs in which translation termination occurs in a context that differs from conventional mRNAs. For example, some mRNAs containing one or more upstream ORFs (uORF), such as the yeast *CPA1* mRNA[31], are downregulated by NMD. With *CPA1* mRNA, the nascent Arginine Attenuator Peptide (AAP) encoded by the uORF stalls the ribosome at the termination codon in the presence of arginine[31]. mRNAs subject to leaky scanning, which can result in translation initiation at an out of frame AUG (and thus lead to premature termination), are also targeted by NMD. Termination that triggers NMD can also result from an unconventional 3'-UTR that extends significantly longer than those present on most mRNAs, either normally[127] or as a result of an mRNA processing error[17]. Intron-containing pre-mRNAs exported to the cytoplasm due to inefficient splicing, such as the *CYH2* pre-mRNA in yeast are also NMD substrates, as well as non-functional mRNAs transcribed from pseudogenes, transposons, or intergenic regions, as long as these transcripts are exported to the cytoplasm[169-171]. Finally, in mammalian cells where alternative splicing is widespread, mRNA isoforms containing PTCs are an important physiological class of NMD substrates, as are mRNAs transcribed from unproductively rearranged immunoglobulin mRNAs and T-cell receptor mRNAs.

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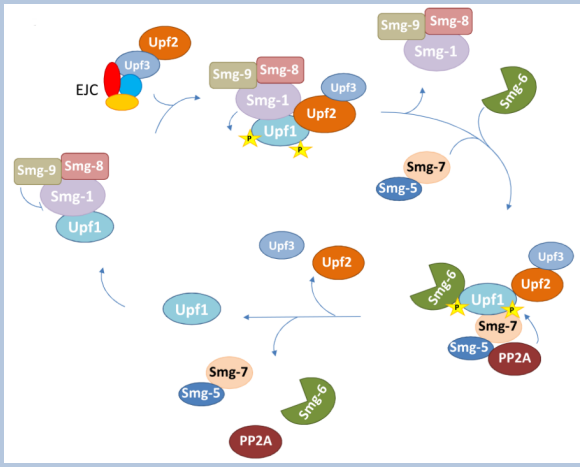
BOX 2

Phosphorylation cycle of UPF1



Factors Smg-1, Smg-5, Smg-6, Smg-7, Smg-8, and Smg-9, are regulators of the phosphorylation status of Upf1[96,172,173]. Phosphorylation of Upf1 on multiple serine residues present in N- and C-terminal domains of the protein is catalyzed by Smg-1, a protein kinase related to the phosphatidylinositol 3 kinase-related kinase (PIKK) superfamily[96,173-176]. Smg-1 activity is regulated by Smg-8 and Smg-9, which form a complex with Smg-1 and prevent inappropriate phosphorylation of Upf1[96,177]. In addition to Smg-1, phosphorylation of Upf1 also requires Upf2 and Upf3, confirming that the formation of a trimeric Upf1:Upf2:Upf3 complex triggers NMD[175]. Phosphorylated Upf1 can interact with the Smg-5:Smg-7 complex, and Smg-6, in turn promoting Upf1 dephosphorylation by recruiting the protein phosphatase PP2A[96,150,172,175,178-180]. The recruitment of Smg-5:Smg-7 and Smg-6 to phosphorylated Upf1 is thought to be direct, through a 14-3-3-like fold present in the three factors[110,150]. Additional interactions may also be pertinent as Smg-7 is detected in the Smg-1–Upf1–Upf2–EJC complex and Smg-6 interacts with the EJC complex[77,91]. Dephosphorylation has been shown to allow recycling of Upf1, including its exit from P-bodies[80].

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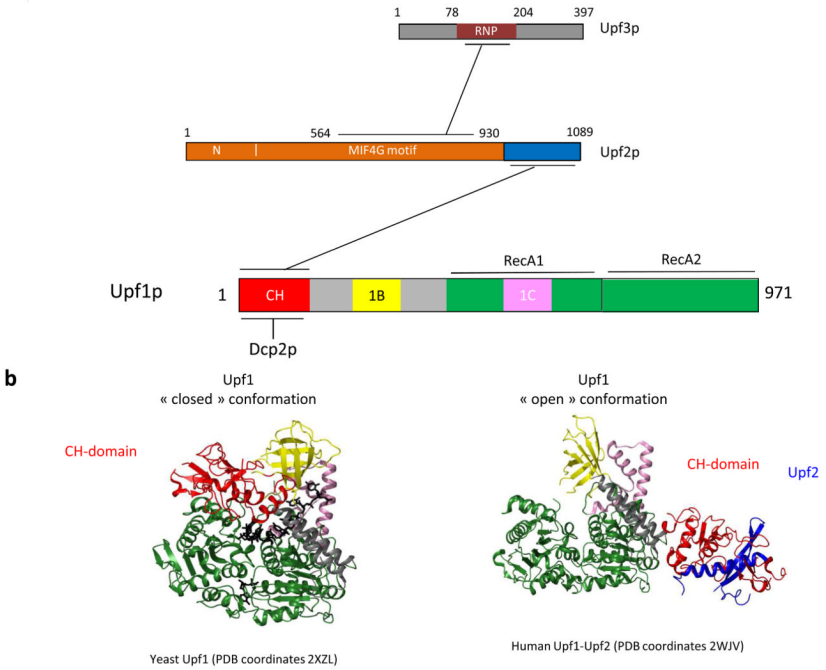

Figure 1. Structures and consequences of interactions between factors involved in NMD

a Interactions between the Upf proteins. Yeast amino acid numbering has been used to define the interaction domains and the size of the respective proteins. RNP= putative ribonucleoprotein domain, MIF4G= middle portion of eIF4G, CH= cysteine- and histidine-rich zinc-finger domain**,** 1B, 1C= additional regulatory domains. **b** Upf2 interaction switches Upf1 from closed (left) to open (right) conformation. The respective structures are of yeast Upf1 and human Upf1:Upf2, with coloring as indicated in the linear Upf1 model depicted in **a.** Yeast Upf1 (left) is shown bound to RNA-ADP:AIF4- (black). The Upf1:Upf2 heterodimer did not associate with RNA under the experimental conditions used for structure determination. Modified with permission from[78,79].

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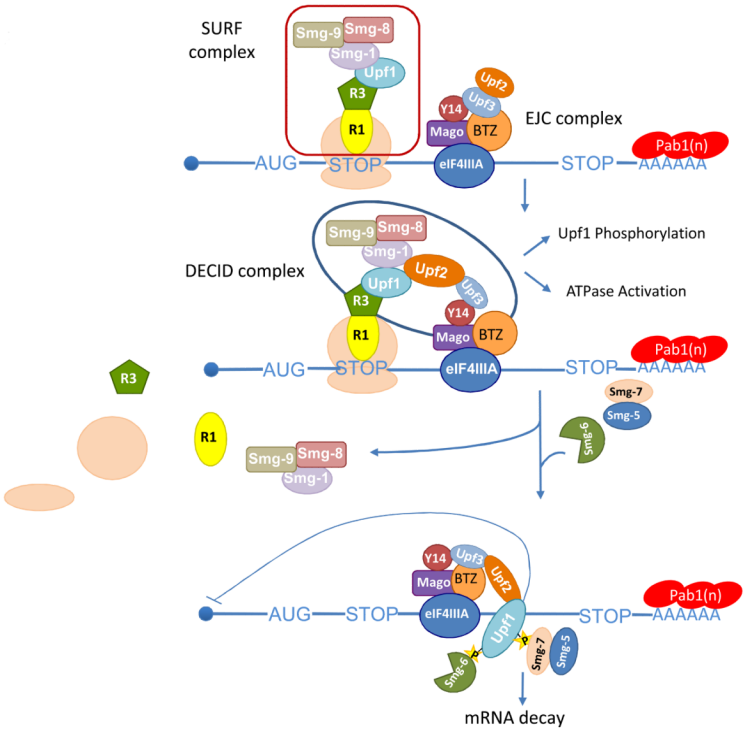

Figure 2. Activation of metazoan NMD by EJC-dependent interactions

The exon-junction complex (EJC) is a group of proteins deposited on an mRNA during splicing, 20-24 nt 5' of an exon-exon boundary[83,84,86,108]. The composition of the EJC is dynamic, and includes at least the core proteins Y14, Magoh, Barentz, and eIF4AIII, and one effector of NMD, Upf3[85,86,88,181-183]. In mammalian cells, Upf3 is loaded onto mRNAs during splicing and binds to a composite site comprised of parts of Y14, Magoh, and eIF4AIII[89]. Upf2 is thought to join the complex in the cytoplasm via Upf3 binding, after mRNA export from the nucleus (top image). In parallel, Upf1 associates with eRF1-bound eRF3, and Smg-1, Smg-8, and Smg-9 associate with Upf1, collectively forming the SURF complex[96,177]. If premature translational termination leads to retention of the downstream EJC then Upf1:Upf2 interaction is facilitated, leading to the formation of the DECID complex and to Upf1 phosphorylation and ATPase activation (middle image). Activation of NMD independently of Upf2, Upf3, or some EJC components has been described, suggesting that alternative pathways may also exist[92,93]. Upf1 phosphorylation inhibits translation in *cis* and promotes its interaction with Smg-6, an endonuclease that can cleave the mRNA, and with the Smg-5:Smg-7 complex, which appears to promote deadenylation and decapping (bottom image)[56,147,148,150,151,172,184]. Mago=Magoh; BTZ=Barentz; R1=eRF1; R3=eRF3. Modified with permission from[91]

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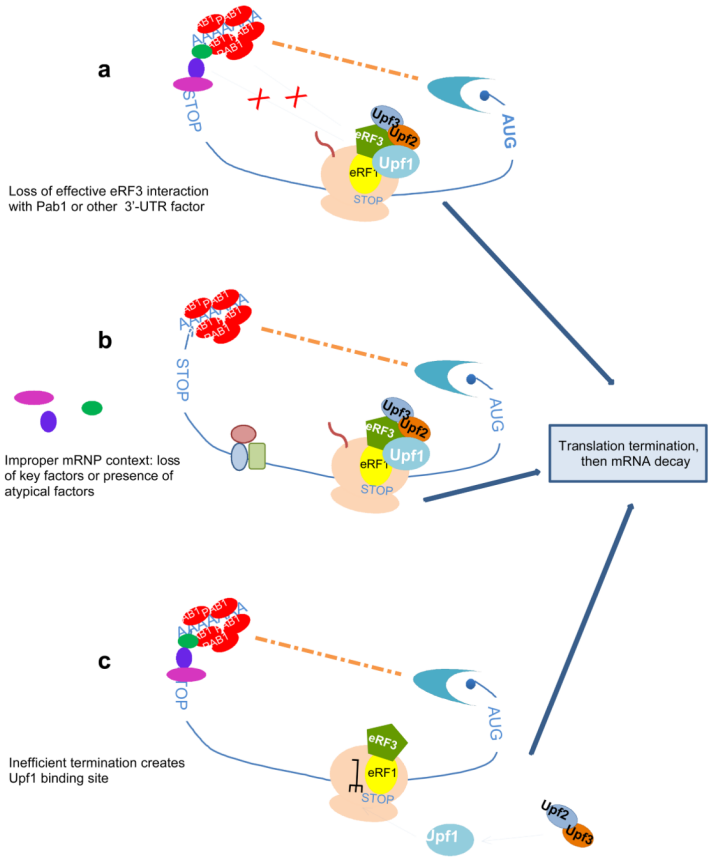

Figure 3. Alternative models for NMD activation by premature termination

Not all mRNAs require an EJC for NMD activation, particularly in lower eukaryotes. The Upf proteins may associate with a prematurely terminating ribosome because essential interactions between Pab1 (or other 3'-UTR-associated proteins) and eRF3 have been disrupted (**a**), the mRNP context is non-accommodating for termination, i.e., critical proteins have not been added to or removed from the mRNP (a variation of the EJC model) (**b**), or the inefficiency of premature termination has left the ribosome in an atypical conformation (**c**). In all three situations the altered state is thought to allow Upf1 association with the eRFs and the ribosome, followed shortly by binding of the Upf2:Upf3 complex to Upf1 and the activation of mRNA decay.

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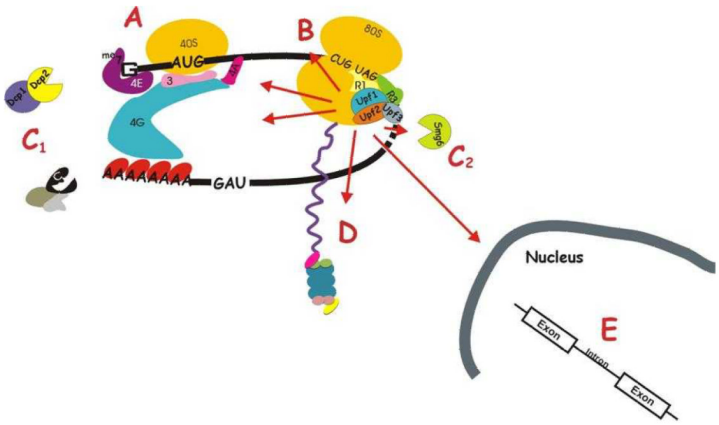

Figure 4. Ancillary processes that accompany NMD

The association of the Upf proteins with a prematurely terminating ribosome has multiple consequences for the expression of the respective mRNA, including: **A**, translational repression of the mRNA, most likely at the level of initiation; **B**, disassembly of the mRNP and dissociation of the inefficiently terminating ribosome; **C_1,** accelerated decapping and/or poly(A) shortening of the targeted mRNA or **C_2**, endonucleolytic cleavage of the targeted mRNA; **D,** degradation of the nascent polypeptide; and **E**, feedback to the site of transcription, leading to the inhibition of splicing of the nascent pre-mRNA. The order of the different events is arbitrary, but is intended to imply that translational repression and mRNP disassembly may be logical prerequisites to the onset of mRNA decay. 4E=eIF4E; P=proteasome; AAA=poly(A) tail; R1=eRF1; R3=eRF3; 4G=eIF4G; 3=eIF3; 4A=eIF4A; structure marked “C”=Ccr4/Not deadenylase complex; Dcp1/Dcp2=decapping enzyme.

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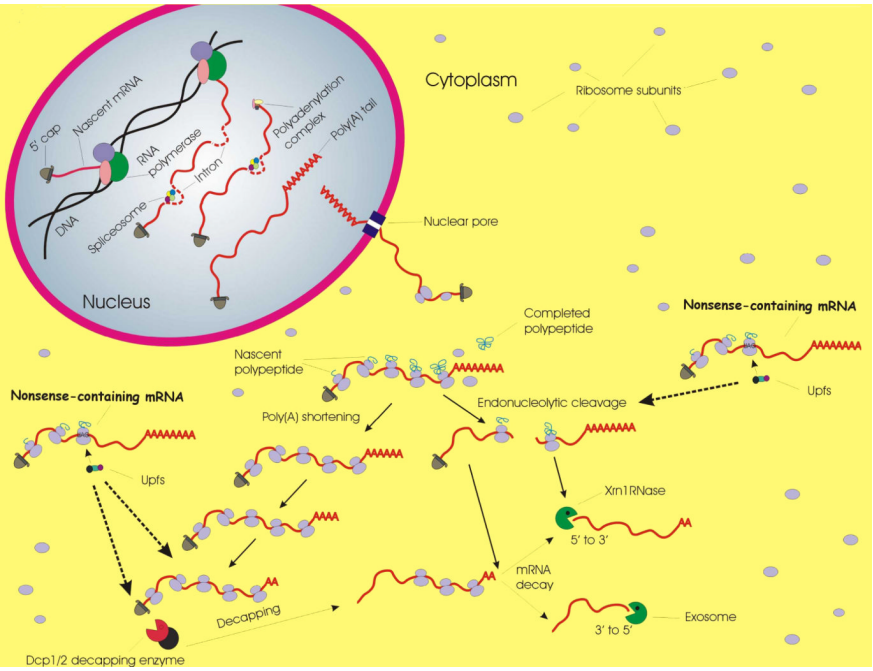

Figure 5. After interaction with the NMD and Smg factors nonsense-containing mRNAs are degraded by conventional cellular mRNA decay pathways

The nuclear biogenesis (upper left) and cytoplasmic translation and decay (center) of normal cellular mRNAs are depicted. Degradation of these mRNAs is initiated by exonucleolytic shortening of the poly(A) tail, catalyzed by the Ccr4:Caf1 and Pan2:Pan3 complexes[2,138,185-188] (complexes not shown), or by endonucleolytic cleavage. Subsequent to poly(A) shortening, mRNAs are usually degraded by the decapping-dependent 5' to 3' pathway or by the 3′ to 5′ exosome-dependent pathway. The latter pathways are also utilized to eliminate the 5' and 3' mRNA fragments resulting from endonucleolytic cleavage. Nonsense-containing mRNAs recruit the Upf factors (or the Upf and Smg factors) to the prematurely terminating ribosome, resulting in interactions that promote accelerated entry of the mRNA into the poly(A)-shortening or decapping pathways, or lead to endonucleolytic cleavage. Products of endonucleolytic are then degraded by the standard 5' to 3' and 3' to 5' pathways. Some steps of the 5' to 3' pathway, including translational repression, may take place in cytoplasmic P-bodies (GW-bodies in mammalian and *Drosophila* cells; not shown), sites where the Dcp1/Dcp2 decapping enzyme, the Xrn1 5'-3' exonuclease, and the Pat1, Dhh1, and Lsm1–7 decapping activators can accumulate[185,186]. Evidence showing that decapped decay intermediates can be polyribosome-associated[187] suggests that assembly of these structures is not essential for mRNA decay, at least in yeast. Moreover, in both yeast and metazoan cells, disruption of P bodies by depletion of core P body components failed to alter decay phenotypes for several different mRNAs[138,185,188].