REVIEWS

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 decay (NMD) pathway premature translation termination promotes the recruitment of a set of factors that destabilize a targeted mRNA. The same factors also seem to have key roles in repressing the translation of the mRNA, dissociating its terminating ribosome and messenger ribonucleoproteins (mRNPs), 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 non-functional 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 bound by ribosomes that are stalled in translation elongation[6–8], and nonsense-mediated decay (NMD), which promotes the degradation of mRNAs undergoing premature translation termination[9–12]. Although these pathways were originally thought to simply promote accelerated mRNA decay, recent studies suggest more complex post-transcriptional regulation[13].

In NMD, 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, UPF proteins must also discriminate between normal and premature termination events and conduct ancillary processes that involve translational repression of mRNAs containing premature termination codons (PTCs), dissociate and properly recycle poorly dissociable PTC-bound ribosomes and unwind the PTC-containing messenger ribonucleoprotein (mRNP), all in the interest of facilitating rapid decay of the mRNA and recycling the components of the translational machinery. Upf1 in yeast also promotes proteolysis of the nascent polypeptide, presumably to ensure a minimal opportunity for that protein fragment to interfere in cellular processes[14].

In this Review, we provide an overview of the NMD pathway and discuss the mechanisms by which NMD factors discriminate between normal and premature termination events, recruit mRNA decay enzymes and conduct ancillary processes involving ribosome and mRNP dissociation and nascent polypeptide degradation. We also seek unified explanations for its broad range of activities in all eukaryotes.

mRNA substrates for NMD

NMD targets transcripts in which translation is arrested by a PTC. As such, the substrates of this pathway include transcripts derived from genes harbouring nonsense mutations, inefficiently spliced pre-mRNAs that enter the cytoplasm with intact introns, mRNAs in which the ribosome has bypassed the initiator AUG codon 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, transcripts of pseudogenes, transposable elements and genes that are subject to programmed rearrangements[15–21]. NMD substrates also include mRNAs in which the translation termination codon occurs in an mRNA context characteristic of premature termination, for example at the 5′ end of an atypically long 3′ untranslated region (3′ UTR)[22,23]. Such NMD substrates include mRNAs with normal or biologically regulated extensions of their 3′ UTRs.

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Gene expression profiling of yeast, fly and human cells showed that 1–10% of cellular transcripts are upregulated by NMD inactivation[15,24–30]. 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 seems to have regulatory functions beyond mRNA surveillance[31,32], including possible roles in cell cycle progression and telomere maintenance.

Translation termination

Destabilization of nonsense-containing mRNAs depends on the recognition of the nonsense codon by the translational machinery[33–37] and the ability of that machinery to recognize a stop codon as 'premature'. Thus, a key question is what distinguishes normal from premature translation termination.

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. Class I release factors 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 release factors are GTPases that stimulate class I release factors[38]. Translation termination in eukaryotes is mediated by the class I release factor eRF1 and the class II release factor eRF3 (Sup45 and Sup35 in yeast, respectively)[39–41]. Following stop codon recognition by eRF1, its conserved Gly-Gly-Gln motif activates the peptidyl transferase centre of the ribosome to mediate peptide release[42,43]. eRF3 GTPase activity depends on ribosome-bound eRF1 and couples eRF1 recognition of a nonsense codon to efficient polypeptide release[44]. Dissociation and recycling of the termination complex is facilitated by the ABCE1 (ATP-binding cassette subfamily E member 1) ATPase and the eukaryotic translation initiation factors eIF3, eIF1, eIF1A and eIF3J[45–48]. ABCE1 interacts with eRF1, and this interaction is conserved in yeast, with eRF1 interacting with the ABCE1 homologue, Rli1 (RNase L inhibitor 1). Rli1 also interacts with Hcr1, the yeast homologue of subunit J of the initiation factor eIF3 (REF. 49).

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 poly(A)-binding protein (Pab1 in yeast and PABPC1 in metazoans) with eRF3 (REFS 50,51). Pab1 overexpression enhances termination efficiency in yeast[51] and, reciprocally, deleting PABPC1 increases nonsense codon readthrough in human cells[52]. Downstream of a normal termination codon (NTC) the interaction of the release factor eRF3 with PABPC1 may orchestrate the formation of an mRNP complex that is favourable to translation termination[50–54]. Additional proteins that have been implicated in translation termination include the mRNA export factors encoded by *NPL3* (nuclear protein localization 3), *DBP5* (DEAD box protein 5) and *GLE1* (Gly-Leu-Phe-Gly lethal 1)[55–57]. Although mutations in these genes lead to nonsense suppression and sensitivity to drugs affecting translation, it is possible that these are indirect consequences of their effects on mRNP structure.

The aberrant nature of premature termination. Premature termination does not seem 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 yeast NTCs, indicating that there was no ribosome pausing and the process was efficient, unless eRF1 was inactivated by a temperature-sensitive lesion. By contrast, ribosomes at PTCs yielded toeprint signals consistent with A-site occupancy even without eRF1 inactivation[58]. Similarly, human β-globin mRNA with a PTC at position 39 that targets the mRNA for NMD was found to yield a toeprint signal, whereas the NTC of β-globin mRNA did not[59]. 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 has been obtained from the identification of PTC124 (ataluren), a compound that promotes nonsense suppression of PTCs but not NTCs in human cells[60].

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 decreased efficiency of ribosome and mRNP dissociation subsequent to peptide hydrolysis. For example, the toeprinting experiments discussed above imply that ribosomes are dissociated slower from PTCs than NTCs[58,59], and that translational reinitiation after premature termination is markedly reduced *in vivo* and *in vitro* when any one of the three yeast UPF proteins is absent[58,61]. Furthermore, following translation termination at a PTC, extracts from yeast lacking Upf1 have a ribosome recycling defect that can be complemented by the addition of purified Upf1 (REF. 61). In human cells, the expression of ATPase-deficient UPF1 leads to the accumulation of β-globin mRNA decay intermediates that are endonucleolytically cleaved by SMG6, a factor implicated in the UPF1 phosphorylation cycle (BOX 1) (see below). The 3′ cleavage product is stabilized and associates with UPF1 and other UPF proteins in an mRNP complex that may also include the terminating ribosome[62]. Collectively, these results indicate that, in addition to promoting accelerated mRNA decay, UPF proteins also have a role in disassembling the poorly dissociable post-premature termination mRNP complex[54], a step required for NMD completion.

Factors required for NMD

NMD is primarily carried out by UPF proteins, but other factors are also necessary to recruit and regulate UPF proteins and to activate NMD.

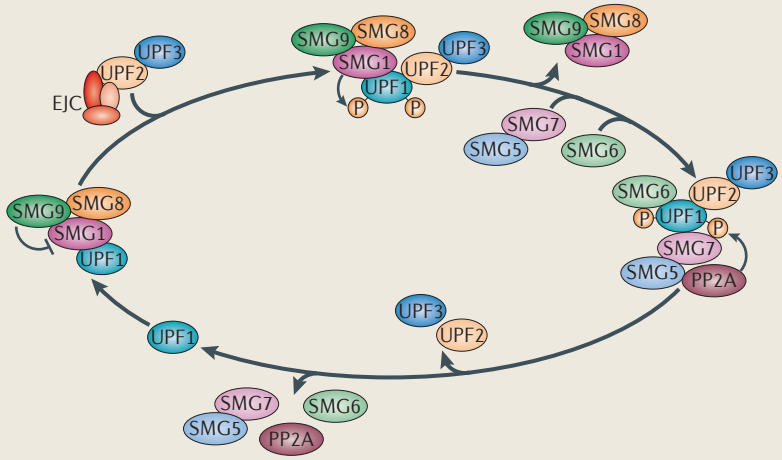
Nonsense codon
A codon located at the normal end of an open reading frame of an mRNA that provides a stop signal for the elongation of protein synthesis by the ribosome. When a nonsense codon occurs before that site it promotes premature translation termination and nonsense-mediated mRNA decay activation.

A-site
A ribosomal site that binds aminoacyl-tRNAs. During translational elongation, tRNAs move successively from the A-site to the P-site (which binds peptidyl-tRNA) to the E site (which binds deacylated tRNA), always in response to specific codon–anticodon pairing.

Nonsense suppression
(also known as translational readthrough). If a stop codon is 'read' by a near-cognate aminoacyl-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 is known as nonsense suppression. Its frequency is often monitored as an indicator for the fidelity and efficiency of translation termination.

Toeprint analysis
An *in vitro* method that identifies the position of ribosomes on an mRNA, on the basis of the inhibition of primer extension by reverse transcriptase from a labelled oligonucleotide hybridized 3′ to the region of interest. Termination codons usually yield a toeprinting fragment that terminates approximately 13 nucleotides downstream of the uracil of the termination codon, spacing that is consistent with ribosomes that have paused with the termination codon in their A-site.

Box 1 | **Phosphorylation cycle of UPF1**



SMG1, SMG5, SMG6, SMG7, SMG8 and SMG9 are regulators of the phosphorylation status of UPF1 (REFS 102,173,174) (see the figure). Phosphorylation of UPF1 on multiple Ser residues present in its amino-terminal and carboxy-terminal domains is catalysed by SMG1, a protein kinase related to the phosphatidylinositol 3-kinase-related kinase (PIKK) superfamily[102,174–177]. SMG1 activity is regulated by SMG8 and SMG9, which form a complex with SMG1 and prevent inappropriate phosphorylation of UPF1 (REFS 102,178). In addition to SMG1, phosphorylation of UPF1 requires UPF2 and UPF3, confirming that the formation of a trimeric UPF1–UPF2–UPF3 complex triggers nonsense-mediated decay (NMD)[176]. Phosphorylated UPF1 can interact with the SMG5– SMG7 complex and with SMG6, which promotes UPF1 dephosphorylation by recruiting the protein phosphatase PP2A[102,154,173,176,179–181]. The recruitment of SMG5–SMG7 and SMG6 to phosphorylated UPF1 is thought to be direct via a 14-3-3-like fold present in the three factors[116,154]. Additional interactions may also be pertinent, as SMG7 is detected in the SMG1–UPF1–UPF2–exon junction complex (EJC), and SMG6 interacts with the EJC[83,97]. Dephosphorylation has been shown to allow recycling of UPF1, including its exit from P-bodies[86].

UPF proteins. The proteins encoded by *UPF1* (also known as *smg-2* in *Caenorhabditis elegans*), *UPF2* (also known as *smg-3* and *NMD2* in *C. elegans* and yeast, respectively) and *UPF3* (also known as *smg-4* in *C. elegans*) are the principal NMD regulators in eukaryotes[63–68]. 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[69]. The three UPF proteins interact (FIG. 1a), with UPF2 acting as a bridge between UPF1 and UPF3 (REFS 69–71).

UPF1 is a large cytoplasmic protein (109 kDa in yeast and 130 kDa in humans) with two principal domains. A Cys- and His-rich zinc-finger domain (CH domain) located near the UPF1 amino terminus is followed by a flexible linker segment and the conserved motifs common to superfamily I (SFI) helicases[10,72,73]. The UPF1 helicase core includes two recombinase A (RecA)-like domains common to all SFI helicases, and two UPF1-specific regulatory domains (1B and 1C)[74]. UPF1 can bind RNA in the presence or absence of ATP[70,75,76]. Both the CH domain and the RNA-dependent ATPase and RNA helicase activities of UPF1 are essential for NMD[75–78]. The CH domain also interacts with UPF2, an acidic 127 kDa protein with three conserved MIF4G (middle portion of eIF4G) domains[65,68,79] (FIG. 1a). UPF2 uses the third MIF4G domain to interact with UPF3, a basic 45 kDa protein with an RNP-type RNA-binding domain (RBD)[69,80]. The nonspecific RNA-binding activity of the resulting UPF2–UPF3 complex is thought to be mediated by a conserved basic region in 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 the UPF1 ATPase activity *in vitro* requires both UPF2 and UPF3 (REF. 70), and that overexpression of *UPF1* can compensate for mutations in *UPF2* and *UPF3* but not vice versa[81]. This functional hierarchy is consistent with mechanistic analyses. The UPF1 CH domain can interact with a carboxy-terminal domain of UPF2, and mutations affecting this interaction inhibit NMD[69,79,82,83]. In the absence of UPF2, UPF1 exists in a closed conformation in which the CH domain interacts with the RecA-like domains[84] (FIG. 1b). In this conformation, domain 1B of UPF1 binds to RNA, thus increasing the overall extent of RNA binding by UPF1 and decreasing its ATPase and helicase activities[70,84,85]. Upon binding to UPF2, UPF1 undergoes substantial conformational change, with the CH domain switching from a position near the RNA-binding site to a more distal position, thus physically disconnecting UPF1 from both the RNA- and nucleotide-binding sites (FIG. 1b). Mediated by the high affinity of the UPF2– UPF1 CH domain interaction, this open form binds RNA less extensively and has increased levels of ATPase and helicase activities; that is, it switches from a state in which it is tightly bound to RNA to one in which it can unwind RNA[70,84]. The effects of the interaction with UPF2 on the UPF1 helicase and ATPase activation can be mimicked by deleting the UPF1 CH domain[84].

SMG factors. In worms and other multicellular organisms, NMD is also regulated by SMG factors, which are proteins that are involved in a cycle of UPF1 phosphorylation and dephosphorylation (BOX 1). This cycle is required for NMD activation and regulates protein–protein interactions that recruit specific mRNA decay enzymes (see below). There is no evidence that UPF1 ATPase and helicase activities depend on the phosphorylation status of the protein, but dephosphorylation has been shown to allow recycling of UPF1, including its exit from P-bodies[86] (BOX 1). The UPF1 phosphorylation–dephosphorylation cycle is also conserved in yeast[87]. In this case, Ebs1, which is a weak homologue of SMG7, has been implicated in the Upf1 phosphorylation–dephosphorylation cycle. However, deletion of *EBS1* has only a moderate effect on NMD[88].

The EJC. In mammalian cells, NMD generally depends on splicing and usually requires a PTC to be localized at least 50–55 nucleotides upstream of an exon–exon junction[9]. This phenomenon relies on the exon junction complex (EJC), a group of proteins deposited on an mRNA during splicing at 20–24 nucleotides 5′ of an exon–exon boundary[89,90] (FIG. 2). The composition of the EJC is dynamic and includes at least the core proteins Y14, MAGOH, barentsz (BTZ; also known as CASC3 and MLN51) and eIF4AIII, as well as one effector

Zinc-finger domain
(CH domain). A protein structural motif that coordinates zinc ions with Cys and His 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 harbouring ATPase and RNA unwinding activity. Cells contain many different RNA helicases that are involved in a wide range of gene expression functions, including pre-mRNA splicing, protein synthesis and mRNA decay.

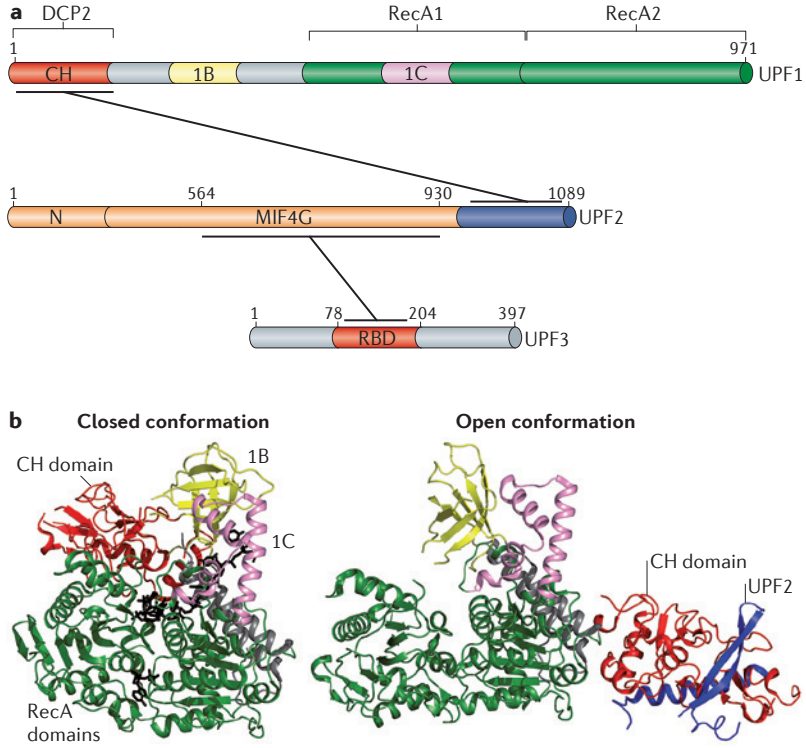


Figure 1 | **Structures and consequences of interactions between factors involved in NMD. a** | Interactions between UPF proteins. Yeast amino acid numbering has been used to define the interaction domains and the size of the respective proteins. **b** | UPF2 interaction switches UPF1 from a closed (left) to an open (right) conformation. The respective structures are yeast Upf1 (Protein Data Bank identifier (PDB ID): 2XZL) and human UPF1–UPF2 (PDB ID: 2WJV). Yeast Upf1 (left) is shown bound to mRNA–ADP–AlF4 (black). The UPF1–UPF2 heterodimer did not associate with mRNA under the experimental conditions used for structure determination. CH, Cys- and His-rich zinc-finger domain; DCP2, mRNA-decapping enzyme 2; MIF4G, middle portion of eIF4G; N, amino; RBD, ribonucleoprotein-binding domain; RecA, recombinase A; 1B and 1C are additional regulatory domains. Images in part **a** and part **b** (left) are modified, with permission, from REF. 84 © (2011) Cell Press. Image in part **b** (right) is modified, with permission, from REF. 85 © (2009) Macmillan Publishers Ltd. All rights reserved.

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[91–93]. UPF3 binds to the EJC during splicing, and UPF2 associates with EJC-bound UPF3 in the cytoplasm[91,92,94–96]. The EJC also has a role in the recruitment of SMG6. UPF3 and SMG6 share the same binding site and compete for interaction with the EJC, suggesting that the EJC is rearranged during NMD activation or that the NMD pathway can, in some instances, function independently of UPF3 (REF. 97). Indeed, activation of NMD independently of UPF3, UPF2 or some EJC components has been reported, suggesting that NMD in mammals is not restricted to a single pathway[98,99].

Release factors. The dependence of NMD on translation termination is reinforced by experiments demonstrating that UPF proteins bind to release factors. In humans and yeast, UPF1 interacts with eRF3 (REFS 52,83,100,101), an interaction that is thought to recruit UPF1 to the termination complex. Support for this notion comes from the identification of a complex between UPF1 and release factors in human cells, which together constitute the SURF (SMG1–UPF1–release factor) complex[83,102] (FIG. 2). In yeast, Upf2 and Upf3 also interact with eRF3, and Upf1 can associate with eRF1. eRF3 seems to have a unique Upf1-binding site located in its GTPase domain[52,103], but Upf2, Upf3 and eRF1 all compete for binding to eRF3 and might share a common interaction domain[103]. UPF1 is thought to interact with eRF3 through its CH domain[52], but this result is controversial[101,104]. Regardless of the definition of the respective interaction sites, the binding of eRF1 and eRF3 to UPF1 inhibits the UPF1 ATPase activity[100], which is 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[70].

In yeast, mutations in *UPF* genes promote nonsense suppression[72,76,81,103,105,106]. The initial interpretation of this observation, based in part on the interactions of UPF proteins with release factors, was that UPF proteins have a direct role in maintaining the fidelity of translation termination. However, RNA interference (RNAi)-mediated depletion of human *UPF1* was shown to decrease nonsense suppression, a surprising result considering the conservation of the termination and NMD machinery[52]. These discrepancies were resolved recently by the identification of mutations that reverse the readthrough phenotype in yeast *upf1* deletion mutants (*upf1*Δ)[107]. This study showed that the mRNA encoding the yeast principal Mg^{2+} transporter, Alr1, contained uORFs and was an endogenous NMD substrate. When NMD was inactivated, *ALR1* mRNA was stabilized, which in turn increased the cellular levels of Alr1. As a consequence, intracellular levels of Mg^{2+} also increased, thus reducing the fidelity of translation termination[107]. Thus, although Upf1 interacts with release factors, these 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 with regard to the initiation of this process: the specific targeting of UPF proteins to an mRNA engaged in prematurely terminating translation; 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[108–110]. Evidence that UPF proteins interact with eRF1 and eRF3 (REFS 100,109,111) suggested the existence of a termination 'surveillance complex' that could detect the presence of specific proteins that are located 3′ to the

P-bodies
Cytoplasmic foci that contain high concentrations of factors involved in 5′ to 3′ mRNA decay. The role of P-bodies in mRNA decay has been controversial because these structures are not essential for mRNA decay or microRNA silencing, and mRNAs that enter P-bodies are not necessarily destined for degradation and can exit these foci and reinitiate translation.

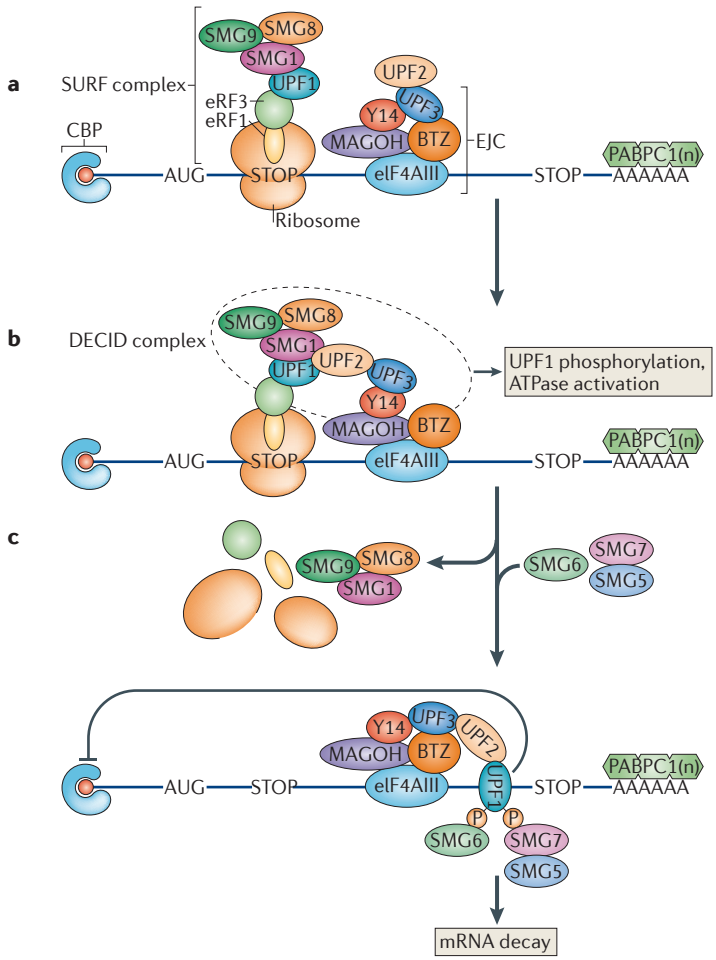


Figure 2 | **Activation of metazoan NMD by EJC-dependent interactions. a** | The exon junction complex (EJC) is a group of proteins deposited on an mRNA during splicing, 20–24 nucleotides 5′ of an exon–exon boundary[89,90,92,114]. The composition of the EJC is dynamic and includes at least the core proteins Y14, MAGOH, barentsz (BTZ) and eIF4AIII, and one effector of nonsense-mediated decay (NMD), UPF3[91,92,94,182–184]. In mammalian cells, UPF3 is loaded onto mRNAs during splicing and binds to a composite site comprised of parts of Y14, MAGOH and eIF4AIII[95]. UPF2 is thought to join the complex in the cytoplasm by binding to UPF3 after mRNA export from the nucleus. In parallel, UPF1 associates with eukaryotic release factor 1 (eRF1)-bound eRF3, SMG1, SMG8 and SMG9, collectively forming the SURF (SMG1–UPF1–release factor) complex[102,178]. RNA cap-binding proteins (CBPs) bind to the cap structure and may include the CBP80–CBP20 complex or eIF4E (not shown). **b** | Premature translational termination leads to retention of the downstream EJC on the transcript, which facilitates interaction of UPF1 with UPF2, leading to the formation of the DECID (decay inducing) complex and to UPF1 phosphorylation and activation of its ATPase activity. Activation of NMD independently of UPF2, UPF3 or some EJC components has been described, suggesting that alternative pathways may also exist[98,99]. **c** | UPF1 phosphorylation inhibits translation in *cis* and promotes its interaction with SMG6, an endonuclease that can cleave the mRNA, and with the SMG5–SMG7 complex, which seems to promote mRNA deadenylation and decapping[62,151,152,154,155,173,185]. PABPC1, poly(A)-binding protein. The figure is modified, with permission, from REF. 97 © (2009) Cold Spring Harbor Laboratory Press.

termination codon[108–110]. Yeast factors that interact with the surveillance complex to activate NMD were thought to be mRNA-binding proteins, such as the helicase Hrp1, that exit with the transcript from the nucleus and are not removed by a translating ribosome because termination occurred prematurely[109,110].

A related model has also been proposed for mammalian cells, in which NMD often depends on the presence of an intron downstream of the PTC, a link that reflects a role for the EJC[112–114]. This model[115] posits that UPF2 and UPF3 bind to the EJC core, whereas UPF1 and its regulator SMG1 are recruited to the termination complex by release factors and form the SURF complex together with the SMG1 regulators SMG8 and SMG9 (REFS 83,102) (BOX 1;FIG. 2). If a ribosome stops at a PTC before it displaces the downstream EJC, SURF-associated UPF1 is thought to be able to interact with UPF2–UPF3 bound to the EJC. In turn, this can trigger UPF1 phosphorylation by SMG1, which results in the formation of the DECID (decay inducing) complex. UPF1 phosphorylation promotes the activation of its ATPase and helicase activities, leading to mRNA degradation through interaction of UPF1 with SMG5, SMG6 and SMG7 (REF. 116). However, this proposed mechanism cannot explain how NMD is activated by PTC-containing mRNAs derived from intron-less precursors or mRNAs that have not undergone prior splicing or lack proper EJC spacing or EJC components[117–121].

The* faux-*UTR model. In yeast and *C. elegans*, mRNP immunoprecipitation experiments have shown that UPF1 preferentially associates with mRNAs that are NMD substrates[24,122]. However, the mechanism of its selective recruitment or selective retention on ribosomes that are 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 located 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 PTC, will stabilize an otherwise unstable mRNA and eliminate the toeprint associated with premature termination[58,123,124]. Likewise, in human cells, mRNA containing synthetic sequences that allow the 3′ UTR to fold back and be positioned near a PTC also become resistant to NMD[125]. Reciprocally, mRNAs with substantially extended 3′ UTRs are NMD substrates in yeast, human, *Drosophila melanogaster* and plant cells[23,101,126–134]. 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 that are present on a normal 3′ UTR, leading to aberrant translation termination and subsequent NMD activation[135]. In this model, proper translation termination and normal mRNA decay are postulated to depend in part on interactions between PABPC1 and eRF3 (FIG. 3). Some aspects of this model have been validated[52,58,101,125], but others have not. Notably, the cellular absence of Pab1 or the deletion of the eRF3-interacting domains of Pab1 do not convert wild-type mRNAs into NMD substrates in yeast[136,137]. These results do not negate the general notions of the *faux*-UTR model, but they do indicate that the termination role of the

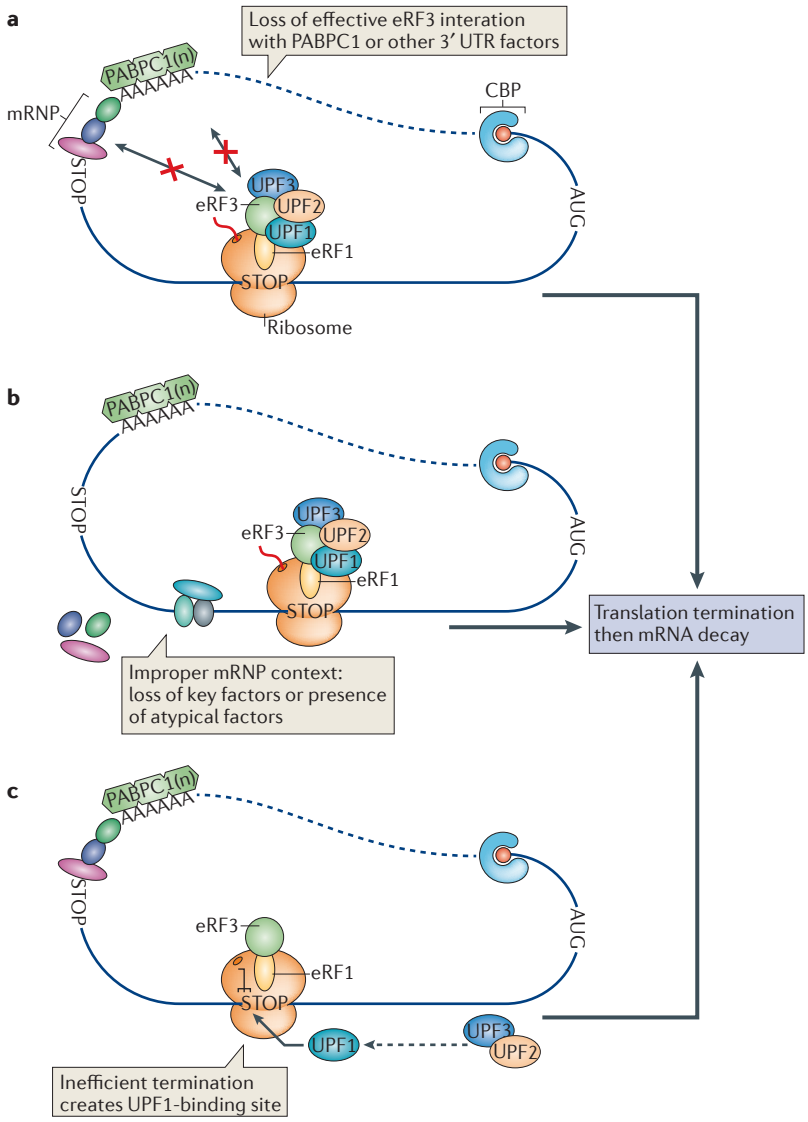


Figure 3 | **Alternative models for NMD activation by premature termination.** Not all mRNAs require an exon junction complex (EJC) for nonsense-mediated decay (NMD) activation, particularly in lower eukaryotes. UPF proteins may associate with a prematurely terminating ribosome because: essential interactions between poly(A)-binding protein (PABPC1; or other 3′ untranslated region (UTR)-associated proteins) and eukaryotic release factor 3 (eRF3) have been disrupted (**a**); the messenger ribonucleoprotein (mRNP) context is not accommodating for termination (that is, crucial 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 release factors and the ribosome, followed shortly by binding of the UPF2–UPF3 complex to UPF1 and the activation of mRNA decay.

3′ mRNP is more complex than the sum of the functions of just Pab1 and eRF3.

In a variation of the *faux*-UTR model one study reported that human UPF1 binding to mRNA varies directly as a function of mRNA 3′ UTR length[138]. The authors 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 that the 3′ UTR downstream of the PTC is 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 human UPF1, a protein that is closely related to its yeast counterpart, might be able to distinguish a completely different range of UTR lengths to the ones found 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 similar that it seems that at least some common NMD characteristics should emerge.

A feature common to the prevailing models is that activation of NMD might depend on an inappropriate mRNP structure neighbouring the PTC. This situation would hold true when PABPC1 or other 3′ UTR factors are absent, or when an EJC is present. Such a model 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[132]. An accommodating mRNP structure might provide sufficient specificity for UPF1 to interact with eRF3. In yeast, the predicted competition of Upf1 and Pab1 for binding to eRF3 was not observed *in vitro*[137], but it might be revealed in the context of an mRNP (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[61]. Clearly, 40S association of Upf1 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 components of the EJC, could be seen not as crucial NMD factors but as activators of translation, a step that is integral for 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[139–141].

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. Slow dissociation of the complex is the more likely possibility because the toeprinting differences between normal and premature termination 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-site or P-site, but dependent on an atypical conformational state assumed by one of them; for example, the

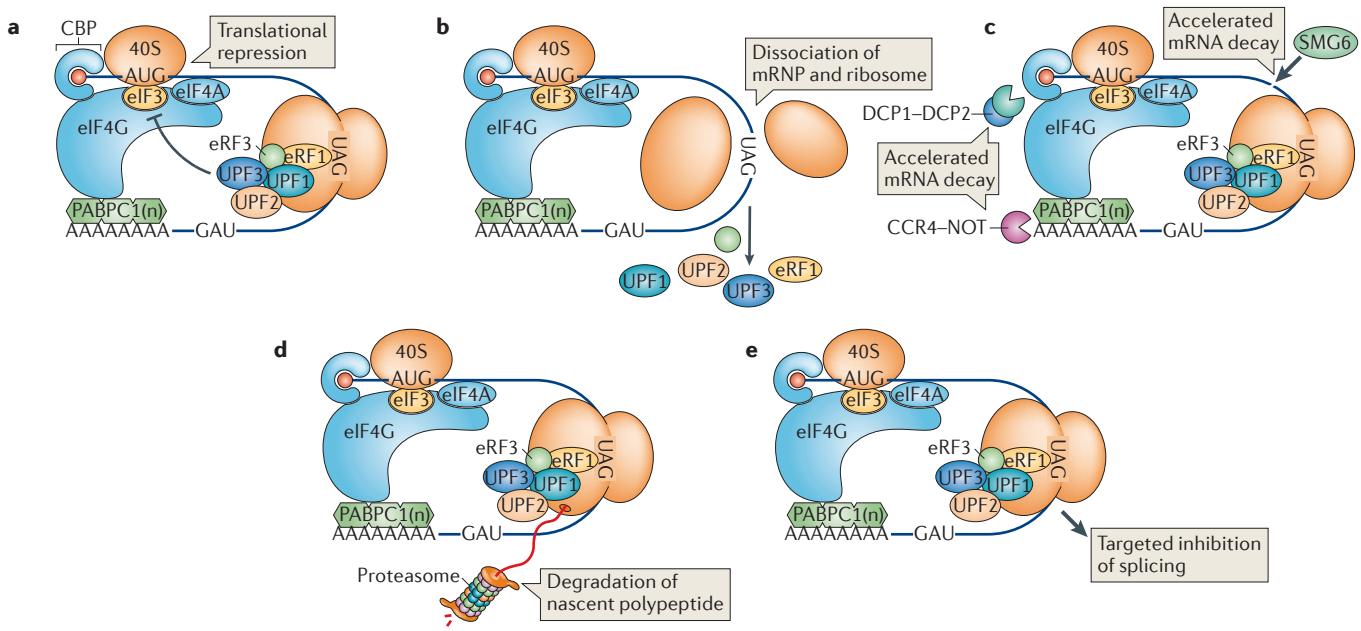


Figure 4 | **Ancillary processes that accompany NMD.** The association of UPF proteins with a prematurely terminating ribosome has multiple consequences for the expression of the respective mRNA, including: translational repression, most likely at the level of initiation (**a**); disassembly of the messenger ribonucleoprotein (mRNP) complex and dissociation of the inefficiently terminating ribosome (**b**); accelerated decapping (by the mRNA-decapping enzyme complex DCP1–DCP2) and/or poly(A) tail shortening of the targeted mRNA (by the CCR4–NOT complex) or endonucleolytic cleavage of the targeted mRNA (by SMG6) (**c**); degradation of the nascent polypeptide through the ubiquitin–proteasome system (**d**); and feedback to the site of transcription, leading to the inhibition of splicing of the nascent pre-mRNA (**e**). 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. CBP, RNA cap-binding proteins; eIF, eukaryotic translation initiation factor; eRF, eukaryotic release factor.

P-site may retain a deacylated tRNA. Interaction between UPF1 and release factors 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–release factor 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 cryo-electron microscopy reconstructions of a eukaryotic ribosome recycling complex only the structural homologue of eRF1 (Dom34 in yeast, which, together with Hbs1, functions in NGD) and the recycling factor ABCE1 are present. This indicates that dissociation of eRF3 precedes ABCE1 (or Rli1 in yeast) binding for ribosome recycling[48]. This result prompts us to propose that an aberrant step in premature translation termination could be the inefficient release of eRF3, which is possibly linked to the absence of PABPC1. The presence of eRF3 on a terminating ribosome could recruit UPF1 to mediate an alternative mode of ribosome recycling (FIG. 3).

Consequences of activating NMD

The activation of NMD has many ramifications (FIG. 4). 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 has a crucial 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. The presence of a PTC in a *CUP1* fusion mRNA was found to lead to an apparent decrease in its translational efficiency[129]. This observation led to the hypothesis that nonsense-containing mRNAs must be translationally repressed before their degradation (FIG. 4a), a proposal for which P-body localization of NMD-targeted mRNAs was considered substantial support[142,143]. However, as discussed below, reductions in the levels of polypeptides that are produced by a PTC-containing mRNA can also be explained by the existence of a proteasome-dependent proteolytic pathway that targets such proteins[14]. That said, independent evidence for translational repression of NMD substrates has been obtained in mammalian cells. Moreover, it was hypothesized that phosphorylated UPF1 can interact with eIF3 and interfere with translation initiation in *cis*[31]. Strong support for this possibility came from experiments showing that comparable effects were not observed in mRNAs using an eIF3-independent internal ribosome entry site (IRES)[31].

Ribosome release and recycling. Activation of the UPF1 ATPase and helicase activities seems to have a crucial 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[61,62] (FIG. 4b). As mentioned above, ribosomes at PTCs yield toeprint signals, which is indicative of an inefficient termination process. The addition of the protein synthesis inhibitor cycloheximide to yeast *in vitro* translation reactions was shown to allow detection of additional toeprints in close proximity to PTCs that were derived from post-termination ribosomes which failed to be released at premature terminators and could scan both 3′ and 5′ from the PTC and reinitiate translation at nearby AUG codons[58]. In eRF1-defective extracts, these toeprints were eliminated and replaced by toeprints corresponding to ribosomes that are stalled with the relevant stop codon in their A-sites. This indicated that a PTC must be recognized by eRF1 and peptide hydrolysis must be triggered before any reinitiation event can occur. Importantly, the reinitiation toeprints were not detected 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 UPF proteins might influence the extent to which prematurely terminating ribosomes remain associated with an mRNA. Consistent with this, reinitiation downstream of a PTC was also eliminated in *upf*Δ strains *in vivo*[61].

A further understanding of the post-termination function of UPF proteins followed from additional *in vitro* translation analyses in yeast extracts[61] and from studies in human cells expressing ATPase-deficient UPF1 (REF. 62). Results from a yeast *in vitro* assay that monitored ribosomes undergoing premature termination indicated that extracts lacking Upf1 not only fail to reinitiate translation but also seem to have a 60S joining defect in conventional initiation. This joining defect is thought to be a consequence of inadequate dissociation and recycling of ribosomes at a prior premature termination event. The inability of *upf1*Δ extracts to recycle ribosomes efficiently from a nonsense-containing mRNA is consistent with other experiments indicating links between termination and initiation[46,49] and with prior studies showing genetic interactions between Upf1 and eIF1 or eIF3 (which are initiation factors that stimulate the *in vitro* dissociation of post-termination 80S complexes)[18,38,45]. In human cells, endonucleolytically cleaved and partially degraded nonsense-containing β-globin mRNA was shown to accumulate in the presence of ATPase-deficient UPF1 (REF. 62). The decay intermediate was found in a complex that included all three UPF proteins 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 during post-termination ribosome release, and UPF1 (and presumably the other UPF proteins) has an important role in this release event via its ATPase and helicase activities, which couple release and mRNP disassembly to a step enabling ribosome recycling. A role for UPF proteins in the disassembly of a poorly dissociable termination complex is reminiscent of the functions of Dom34 and Hbs1 during NGD in yeast[7].

Accelerated mRNA decay. The interaction of UPF1 with several factors seems to play a key part in promoting the degradation of NMD substrates by multiple decay pathways[15,144–146] (FIGS 4c,5). In yeast and human cells, UPF1 interacts with the DCP1–DCP2 mRNA decapping enzyme complex, the 5′ to 3′ exoribonuclease XRN1 and various exosome components, which suggests a direct recruitment of mRNA decay enzymes[65,144–147]. In addition, PNRC2 interacts with hyperphosphorylated UPF1 and DCP1A, thereby linking the NMD and decapping machineries[148]. 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 association with the decapping activators Edc3 and Pat1 (REFS 65,149,150).

As with the activation of UPF1 by UPF2–UPF3, the assembly of decay complexes in multicellular organisms seems to be a bit more straightforward than that in yeast and to include important roles for SMG factors. Two pathways have been described that involve the interaction of phosphorylated UPF1 with either SMG6 or SMG5–SMG7 (FIG. 2). SMG6, by virtue of its PIN domain, has an endonuclease activity with which it initiates mRNA decay near the PTC in *D. melanogaster* and human cells[62,151,152]. The resulting 5′ and 3′ fragments are then degraded by the exosome and XRN1, respectively[152,153]. In the second pathway, there is no endonucleolytic cleavage, and recruitment of SMG5–SMG7 has been proposed to promote 5′ to 3′ and 3′ to 5′ decay of the targeted mRNA by DCP1–DCP2, XRN1 and the exosome[154]. This is consistent with the absence or inactivity of PIN domains in both proteins and with experiments showing that tethering of SMG7 to an otherwise normal mRNA is sufficient to promote its degradation, a function carried out by the C-terminal domain of SMG7 (REFS 154–156). Interestingly, it has been suggested that the choice between the two pathways in some organisms may reflect genomic differences[157]. For example, *D. melanogaster* lacks SMG7, and its nonsense-containing mRNAs are thought to initiate decay only by SMG6-mediated endonucleolytic cleavage[158], a conclusion complicated by the recent observation that *D. melanogaster* NMD remains active in flies harbouring SMG6 mutations[159].

Concomitant targeting of the nascent polypeptide. At least in yeast, NMD also activates the rapid degradation of the truncated polypeptide by the proteasome[14,160], underscoring the biological importance of minimizing the accumulation of potentially toxic, partially synthesized proteins (FIG. 4d). Proteolysis depends on Upf1 and is mediated by 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, as its N-terminal CH domain is distantly related to classic E3 RING finger domains, so it is possible that

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.

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

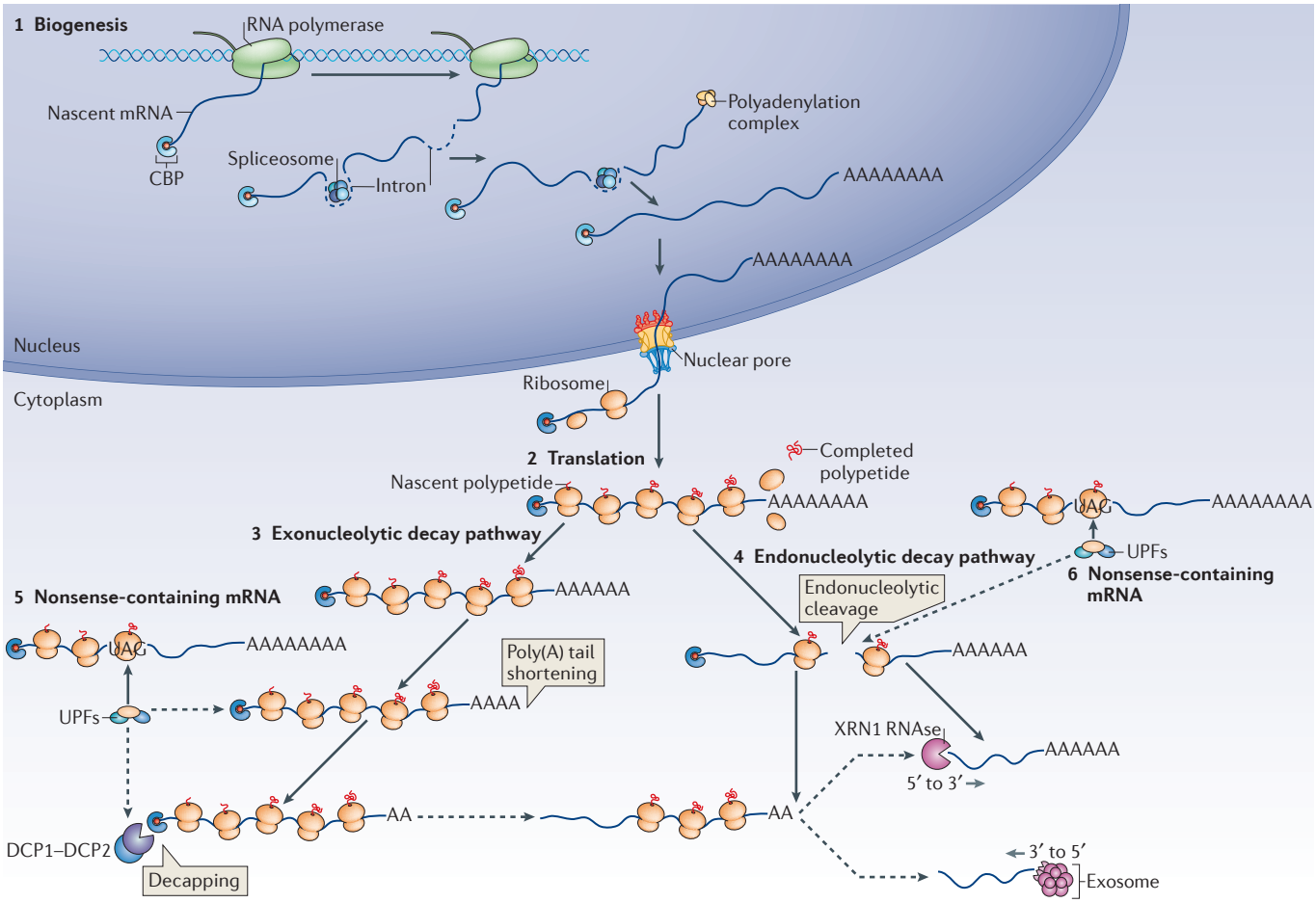


Figure 5 | **mRNA biogenesis and decay.** mRNAs are generated in the nucleus (**1**) and then translocate to the cytoplasm, where they associate with ribosomes and become translated (**2**). mRNAs that are to be degraded are first subjected to poly(A) shortening (catalysed by the CCR4–NOT and PAN2–PAN3 complexes[2,142,186–189]; complexes not shown) and then degraded by the exonucleolytic 5′ to 3′ decay pathway (**3**). Alternatively, they are degraded by the exonucleolytic 3′ to 5′ exosome decay pathway (**4**). These pathways are also used to eliminate the 5′ and 3′ mRNA fragments resulting from endonucleolytic cleavage. Nonsense-containing mRNAs recruit UPF proteins (or UPF proteins 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 (**5**) or lead to endonucleolytic cleavage (**6**). Products of the endonucleolytic pathway 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 melanogaster* cells; not shown), which are sites where the mRNA-decapping enzyme complex DCP1–DCP2, the XRN1 5′ to 3′ exoribonuclease, and PAT1, DHH1 and LSM1–LSM7 (not shown) decapping activators can accumulate[186,187]. Evidence showing that decapped decay intermediates can be associated with polyribosomes[188] 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[142,186,189].

it interacts with the ubiquitin-conjugating enzyme Ubc3 (also known as Cdc34) and self-ubiquitylates *in vitro* in a Upf3-dependent reaction[160]. 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 ubiquitylation, like phosphorylation, controls the ability of Upf1 to interact with key 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 transfer-messenger RNA (tmRNA) system, which disassembles elongation-stalled ribosomes while adding a small stable 10S RNA (ssrA) tag to the associated polypeptide to promote its rapid decay[161,162]. In all of these circumstances mRNA decay, peptide decay and ribosome recycling seem to be highly coordinated.

Feedback to the site of transcription. There is strong evidence 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 immunoglobulin-μ and T cell receptor-β (TCRβ), accumulate at or near their respective sites of transcription. This surprising downregulation 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 SMG6-dependent, and the pre-mRNAs that accumulate at the transcription site are unspliced[163–165] (FIG. 4e). As there is currently no 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[166]. Furthermore, 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[167]. Therefore, it is possible that the rapid deadenylation of some mammalian NMD substrates may yield a post-translationally modified component of the CCR4–NOT deadenylation complex participating in accelerated mRNA decay in the cytoplasm that feeds back to its nuclear counterpart, which stalls late events in nuclear processing and export[168]. 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[169–171].

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 of termination that mimics at least some regulatory features characteristic of premature termination. Collectively, NMD substrates may account for as much as 10% of the transcriptome in exponentially growing undifferentiated cells, including some mRNAs that encode normal, full-length 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, with UPF1 acting as the central regulator. A detailed understanding of the specificity of UPF1 for binding to a ribosome that translates a PTC-containing mRNA remains to be obtained, but it may depend on interaction with eRF3 and/or eRF1, an accommodating mRNP structure downstream of the PTC and the local absence of PABPC1. In mammalian cells, activation of the UPF1 ATPase and helicase activities is often a consequence of interaction with the UPF2–UPF3 complex that is associated with a downstream EJC. In transcripts that are not bound by EJCs the mode of UPF1 association with UPF2–UPF3 is currently unclear and raises the possibility that a common NMD model may be elusive.

Interaction with UPF2–UPF3 not only activates the enzymatic activities of UPF1, but also leads to its phosphorylation and its interaction with several other proteins, including SMG factors. Activated and phosphorylated UPF1 has multiple functions, and much remains to be learnt about the relative order of these activities and their coordination. UPF1 and its interactors seem to recruit decay enzymes that promote endonucleolytic cleavage, 5′ to 3′ decay or 3′ to 5′ decay, but the mechanism of this recruitment and 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 reuse 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 UPF proteins, 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 shutdown of expression helps to explain why nonsense alleles are effectively null alleles and suggests potential avenues for enhancing the effects of therapeutic nonsense suppression[60,172].

Finally, studies of NMD have underscored how little is known about translation termination in eukaryotes, at least when compared with our understanding of translation initiation. Until recently, termination seemed to depend on just the presence of a nonsense codon and release factors. 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.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

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