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Review

Translation-coupled mRNA quality control mechanisms

Laura Monaghan, Dasa Longman & Javier F Cáceres*

Abstract

mRNA surveillance pathways are essential for accurate gene expression and to maintain translation homeostasis, ensuring the production of fully functional proteins. Future insights into mRNA quality control pathways will enable us to understand how cellular mRNA levels are controlled, how defective or unwanted mRNAs can be eliminated, and how dysregulation of these can contribute to human disease. Here we review translation-coupled mRNA quality control mechanisms, including the non-stop and no-go mRNA decay pathways, describing their mechanisms, shared trans-acting factors, and differences. We also describe advances in our understanding of the nonsense-mediated mRNA decay (NMD) pathway, highlighting recent mechanistic findings, the discovery of novel factors, as well as the role of NMD in cellular physiology and its impact on human disease.

Keywords RNA quality control; No-go mRNA decay; Non-stop mRNA decay; Nonsense-mediated mRNA decay; UPF1
Subject Categories Chromatin, Transcription & Genomics; RNA Biology
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Introduction

Translation-coupled RNA quality control mechanisms sense ribosome stalling, or premature translation stops and elicit mRNA degradation and ribosome recycling. In eukaryotic cells, there is an intricate relationship between mRNA turnover and active translation (Fig 1A). This was recently confirmed in mammalian cells in culture, with the use of single-molecule imaging approaches that revealed translation-dependent destabilization of mRNA (Dave *et al*, 2023). In addition, the presence of a series of non-optimal codons can negatively influence protein production by decreasing ribosome translocation rates leading to ribosome collisions that have the potential to trigger RNA quality control pathways and lead to mRNA decay (Hanson & Coller, 2018; Wu & Bazzini, 2023). This process, known as codon optimality-mediated mRNA decay (COMD), allows the cell to distinguish between variation in normal translation speeds and terminal ribosome stalling, which triggers alternative RNA quality control pathways (Fig 1B) (Wu *et al*, 2019; D'Orazio & Green, 2021).

Two major pathways of co-translational mRNA surveillance, no-go mRNA decay (NGD) and non-stop mRNA decay (NSD), sense aberrant translation by monitoring the stalling of ribosomes and/or ribosomes that fail to encounter a stop codon, respectively (Fig 1C and D). A common feature of both NGD and NSD is that upon sensing defective ribosome translocation, these pathways activate mechanisms leading to mRNA and nascent peptide degradation, and ribosome recycling (Brandman & Hegde, 2016; Powers *et al*, 2020). NGD and NSD use similar factors, but differ mainly in their substrate mRNAs and initial triggering mechanism (Simms *et al*, 2016). In both pathways, ribosome stalling is followed by recruitment of nucleases leading to degradation of mRNA (Powers *et al*, 2020). By contrast, the nonsense-mediated mRNA decay (NMD) pathway senses inappropriate translation termination (Kervestin & Jacobson, 2012; Hug *et al*, 2016) (Fig 1E). NMD eliminates mRNAs that harbor premature termination codons (PTCs), thus, preventing the synthesis of truncated proteins. Significantly, NMD has a more global role in post-transcriptional regulation of gene expression and also regulates the stability of many cellular non-mutated transcripts, which do not harbor PTCs. These non-canonical functions of the NMD pathway, which do not represent RNA quality control per se, are important for the regulation of many cellular pathways, including differentiation, neurogenesis, synaptic control, as well as the response to viral infections and stress (Jaffrey & Wilkinson, 2018; Kurosaki *et al*, 2019).

In this review article, we will focus primarily on three translation-coupled RNA quality control mechanisms: NGD, NSD, and NMD. We will review similarities and differences among these pathways and cover recent advances related to their mechanisms, targets, and trans-acting factors that ensure a tight control of aberrant RNAs that fail to be properly translated.

No-go mRNA decay

Initially discovered in *S. cerevisiae*, the no-go mRNA decay (NGD) pathway is triggered when ribosomes move slowly or stall during translation elongation, leading to ribosome collisions with trailing ribosomes, forming disomes (Doma & Parker, 2006; Harigaya & Parker, 2010). Ribosome stalling can be triggered by a number of

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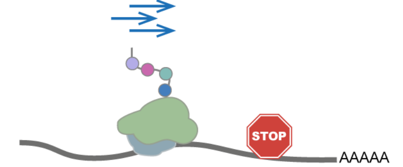
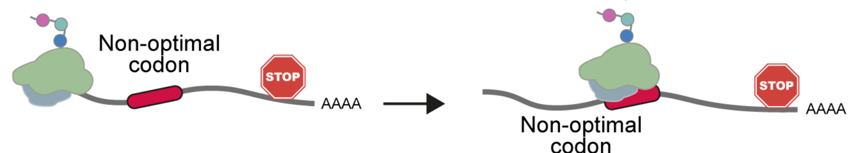
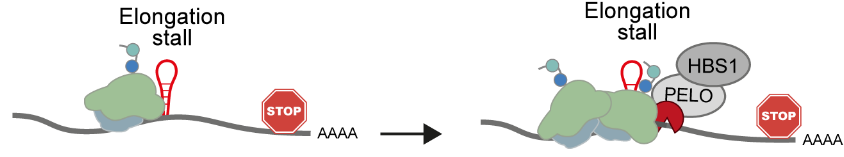
A Stability of mRNA is affected by translation



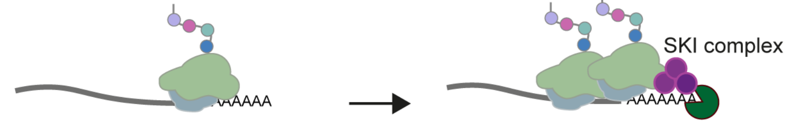
B Codon optimality-mediated mRNA decay (COMD)



C No-go mRNA decay (NGD)



D Non-stop mRNA decay (NSD)



E Nonsense-mediated mRNA decay (NMD)

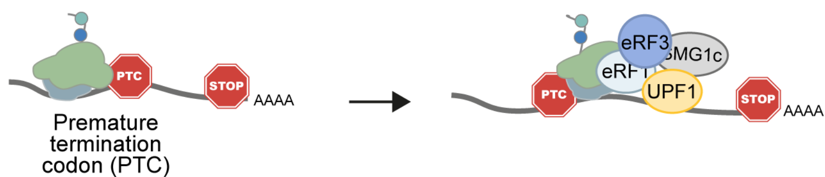


Figure 1. Translation-coupled mRNA quality control mechanisms.

(A) Stability of mRNAs is affected by translation. Several layers of regulation monitor the efficiency of mRNA translation, including the translation rate, amino acid composition, and mRNA secondary structures. (B) The mRNA translation rate is slowed down when the ribosome encounters sub-optimal codons leading to a decrease in mRNA stability. (C) No-go mRNA decay (NGD) is triggered by the presence of mRNA secondary structures leading to ribosome stalling. (D) The absence of a stop codon results in slowing of the ribosome reading through the poly (A) tail triggering the non-stop mRNA decay (NSD) pathway. (E) Recognition of a PTC sets in motion a cascade of event involving the UPF family of proteins, resulting in mRNA degradation by the nonsense-mediated mRNA decay (NMD) pathway.

stimuli, such as the presence of stable stem loops, pseudoknots, GC-rich sequences, or damaged RNA bases (Shoemaker & Green, 2012). Persistent collisions trigger a two-pronged cellular response that aims to inhibit translation re-initiation on the problematic mRNA, and simultaneously remove faulty mRNAs and nascent peptides, and recycle the ribosomes (Fig 2).

Initially, translational repression is mediated by EDF1, which binds to collided ribosomes and recruits the translational repressor complex GIGYF2-4E2 (Syh1-Smy2 in *S. cerevisiae*) (Hickey *et al*, 2020; Sinha *et al*, 2020; Veltri *et al*, 2022). Upon ribosome collision, leading and trailing ribosomes form a 'rotated' interface of 40S subunits recognized by the E3 ubiquitin ligase ZNF598 (Hel2 in yeast) that ubiquitinates ribosomal proteins eS10 and uS10 and acts independently of the GIGYF2-4E2 branch (Juszkiewicz *et al*, 2018; Ikeuchi *et al*, 2019) to initiate a degradation pathway. Ubiquitination of the 40S subunit is critical for the recruitment of an endonuclease, which cleaves mRNAs in the middle of a stalled disome, exposing fragmented mRNA to further degradation by XRN1 and potentially

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Figure 2. Steps and factors involved in no-go and non-stop mRNA decay.
Flow chart depicting steps and trans-acting factors shared or unique to these two RNA quality control pathways from initial ribosome collision, ubiquitination and endonucleolytic cleavage to ultimate mRNA degradation and ribosome recycling. *S. cerevisiae* homologs are indicated in brackets.

the exosome. A genetic screen in yeast recently identified the endonuclease Cue2 as a component of the NGD pathway (D'Orazio *et al*, 2019). Cue2 is a low-abundance protein that cleaves mRNA at the A site of the rotated collided ribosome in a Hel2-dependent manner and has homologues in *C. elegans* (NONU1) and in mammalian cells (N4BP2; D'Orazio *et al*, 2019; Glover *et al*, 2020; Monem

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et al, 2023). As a consequence of mRNA cleavage, stalled ribosomes appear near the 3′ end of mRNA, which favors the binding of an evolutionarily conserved complex formed by PELOTA (PELO) and its GTPase cofactor HBS1 (Dom34/Hbs1 in yeast; Doma & Parker, 2006; Pisareva *et al*, 2011). Despite structural similarities with release factors eRF1/eRF3, instead of detecting a stop codon, the PELO/HBS1 complex preferentially binds an empty ribosomal A site. This induces ribosome splitting into 40S bound to the mRNA, and 60S with the attached nascent peptide, and is mediated by the ribosome recycling factor ABCE1 (Rli1 in yeast; Shoemaker *et al*, 2010). The nascent peptide is subsequently targeted for Listerin-mediated ubiquitination and degraded by the proteasome as part of the ribosome quality control (RQC) pathway (Shao *et al*, 2013). However, despite widespread conservation of the Cue2-Dom34/Hbs1 pathway, recent evidence suggests that this represents a secondary decay method in NGD and may only be required when the capacity of the XRN1-mediated degradation pathway is saturated (Juszkiewicz *et al*, 2020). In the majority of mRNAs with ribosome stalls within the open reading frame (ORF), Hel2-mediated 40S ubiquitination of disomes recruits the ribosome quality control trigger (RQT) complex containing the RNA helicase Slh1 in yeast, with the homologous complex ASC-1 performing the same role in mammals (Matsuo *et al*, 2017). Upon 40S ubiquitination of collided ribosomes, the RNA helicase ASCC3 (part of the ASC-1 complex) selectively disassembles the lead ribosome in an ATP-dependent manner, in a process not requiring PELO/HBS1 activity (Juszkiewicz *et al*, 2020; Matsuo *et al*, 2020). After removing the roadblock of the lead stalled ribosome, normal elongation can resume, or in case of persistent stalls, subsequent ribosome collisions induce further quality control and mRNA is subjected to XRN1-mediated degradation, likely with a contribution of the PELO/HBS1 branch of NGD.

Non-stop mRNA decay

The non-stop mRNA decay pathway (NSD) degrades mRNAs that lack a stop codon, ensuring the degradation of mRNAs that will fail to be properly translated (Frischmeyer *et al*, 2002; van Hoof *et al*, 2002). It can be initiated by a number of aberrant translation events which promote readthrough into the poly(A) tail resulting from lack of an in-frame stop codon or polyadenylation within the ORF. Poly(A) translation within the coding region slows down when the nascent poly(lysine) peptide (encoded by poly(A)) interferes with the ribosomal exit tunnel. This attenuation of translation elongation allows stretches of poly(A) to adopt a conformation that impedes further mRNA decoding at the ribosome A site resulting in stalling of the translating ribosome (Ito-Harashima *et al*, 2007; Chandrasekaran *et al*, 2019; Tesina *et al*, 2020). Interestingly, hindered elongation can induce ribosome sliding on poly(A) sequences causing frameshifting that may lead to an out-of-frame PTC and subsequent triggering of NMD-mediated mRNA degradation (Koutmou *et al*, 2015). Importantly, elongation stalling causes collisions with trailing ribosomes and the resulting disomes are recognized and ubiquitinated by the ZNF598 E3 ubiquitin ligase with subsequent Cue2-mediated endonucleolytic cleavage, steps that are also common to NGD, as described above (Juszkiewicz & Hegde, 2017; Powers *et al*, 2020).

In yeast, 80S ribosomes stalled at the 3′end of an mRNA lacking a stop codon are bound by the SKI complex, leading to recruitment of the exosome by the exosome-associated factor SKI7, resulting in degradation of the aberrant mRNA in a 3′ to 5′ direction. SKI7 binds the empty A site of the stalled ribosome at the 3′ end of mRNA and directs mRNA degradation through an eRF3-like domain (Saito *et al*, 2013). This process also requires the RNA helicase SKI2 (SKIV2L in mammals) to guide RNA molecules to the exosome complex, triggering mRNA degradation upon ribosome stalling on A-rich sequences (Tuck *et al*, 2020). In mammalian cells where SKI7 is not present, bridging the SKI complex to the exosome is carried out by HBS1, its closest homologue. This triggers the interaction with Dom34/Pelota-Hbs1 complex shared with NGD and acts to dissociate the stalled ribosome with the help of ribosome recycling factors Rli1 (yeast)/ABCE1 (mammals; Tsuboi *et al*, 2012; Saito *et al*, 2013).

Fundamentally, NGD and NSD are parallel mRNA quality control pathways that share many similarities, such as ubiquitination of crucial ribosome sites, Cue2-mediated endonucleolytic cleavage, activation of mRNA decay, and ribosome recycling; however, they have some key differences (Fig 2). The NSD helicase SKI2 is highly related to the NGD helicase Slh1 in yeast/ASCC3 in mammals; however, the Ski complex directly recruits the exosome, thus directly linking mRNA degradation and ribosome recycling. Conversely, in NGD, the Slh1/ASCC3 complex removes stalled ribosomes first, resulting in mRNA degradation mediated by XRN1. NSD has been implicated in the development of several human diseases, highlighting the physiological importance of this RNA quality control pathway. For example, non-stop mutations in the Dysferlin gene (*DYSF*) lead to degradation of its mRNA and a subsequent reduction in *Dysferlin* expression, contributing to the progression of muscular dystrophy (Cacciottolo *et al*, 2011). Insufficient NSD activity can also lead to disease, such as mutations which eliminate the stop codon in the skeletal muscle alpha actin (*ACTA1*) gene. Here, incomplete NSD fails to remove mRNAs encoding 47 additional amino acids that are translated within the 3′UTR, leading to large protein aggregates that are responsible for the development of severe skeletal myopathy (Wallefeld *et al*, 2006).

Nonsense-mediated mRNA decay

NMD is an RNA quality control mechanism that targets mutated mRNAs harboring PTCs for degradation, but also regulates the stability of many cellular transcripts. As such, NMD modulates the phenotypic outcome of genetic disorders caused by frameshift or nonsense mutations that generate PTCs (Bhuvanagiri *et al*, 2010; Karousis & Mühlemann, 2022). In contrast to NGD and NSD, which are triggered by the collision of elongating ribosomes, NMD initiates mRNA degradation in response to faulty translation termination events. Similar to NGD and NSD, NMD degrades mRNAs co-translationally, leading to production of truncated nascent peptides. In the case of NGD and NSD, these potentially toxic peptides are rapidly degraded by the RQC pathway. By contrast, peptides produced from PTC-containing transcripts are targeted by an ubiquitin proteasome system, which specifically targets peptides remaining tethered to the ribosome following decay of a nonsense mRNA (Inglis *et al*, 2023).

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The function of NMD in preventing the accumulation of truncated proteins can have a positive or negative cellular effect, depending on whether the truncated protein has a deleterious dominant negative function, or whether it retains at least partial function, elimination of which by NMD could be detrimental. For example, inhibition of NMD by antisense oligonucleotides (ASOs) targeting the W1282X mutation in the CFTR gene increases production of a partially functional protein that enhances the CFTR-mediated chloride current in human bronchial cells (Kim *et al*, 2022). On the other hand, failure of NMD to degrade β-globin transcripts harboring a PTC in the last exon leads to expression of truncated, dominant-negative protein causing severe beta-thalassemia (Hall & Thein, 1994). The effect of NMD must be carefully considered in the development of any therapeutic approach for diseases caused by PTC mutations. Importantly, NMD also has a role in many physiological processes, for example in the regulation of the stress response and as a modulator of neural development (Jaffrey & Wilkinson, 2018; Kurosaki *et al*, 2019).

In mammals, NMD is linked to the process of pre-mRNA splicing via the exon junction complex (EJC), a multi-subunit protein complex that is deposited 20-24 nucleotides upstream of most exon-exon junctions (Le Hir *et al*, 2000a, 2000b). The splicing factor CWC22 interacts with the core EJC factor eIF4AIII and links splicing with EJC deposition, leading to NMD activation (Alexandrov *et al*, 2012; Barbosa *et al*, 2012; Steckelberg *et al*, 2012). Mechanistically, the NMD response is coupled to mRNA translation since EJCs remain bound to mRNAs until they are displaced by the translation machinery. Initially, it was suggested that NMD activation occurs as a consequence of ribosome stalling at the termination codon (TC). However, recent data challenge this model and revealed that NMD activation in humans is not necessarily linked to stable stalling of ribosomes at TCs (Karousis *et al*, 2020). A ribosome terminating prematurely at a PTC located ≥ 50–55 nucleotides upstream of the final exon–exon junction will not remove an EJC (Nagy & Maquat, 1998; Thermann *et al*, 1998) and this initiates the NMD response (Metze *et al*, 2013; Kurosaki *et al*, 2019).

Core NMD factors

A central player in the NMD pathway is the ATP-dependent RNA helicase of the SF1 superfamily, Upstream Frameshift 1 (UPF1), which harbors an amino-terminal cysteine-and histidine-rich (CH) domain and a carboxy-terminal RNA helicase domain (Fig 3; Kim & Maquat, 2019).

UPF1 is activated by phosphorylation carried out by the SMG1c complex, comprised of the phosphoinositide 3-kinase (PI3K)-like kinase, SMG1, and two additional subunits, SMG8 and SMG9, that negatively regulates its activity (Yamashita *et al*, 2001, 2009; Fernández *et al*, 2011; Langer *et al*, 2021). SMG1 phosphorylates UPF1 at multiple SQ and TQ motifs located in the amino- and carboxy-terminal domains (Yamashita *et al*, 2001). Until recent years it was widely accepted that initially, the association of UPF1 with SMG1 and the eukaryotic release factors eRF1 and eRF3 form the surveillance complex (SURF) in the vicinity of the PTC (Kashima *et al*, 2006) (Fig 4). Subsequently, interaction of the SURF complex with UPF2 and UPF3B and an EJC downstream of the PTC leads to the assembly of a decay-inducing complex (DECID), where UPF1 is phosphorylated and eRF1 and eRF3 are released (Kashima *et al*, 2006; Chamieh *et al*, 2008; López-Perrote *et al*, 2016). In addition to this, a central role for UPF3B in translation termination has been highlighted. A fully reconstituted *in vitro* translation system showed the predominance of the interaction of UPF3B with ribosome release factors, to delay translation termination and dissociate post-termination ribosomal complexes that are devoid of the nascent peptide. UPF1 was shown to interact transiently with the termination factors and UPF3B to initiate the subsequent mRNA decay (Neu-Yilik *et al*, 2017). Despite the lack of mammalian *in vivo* data, a recent *in vivo* study in yeast re-established the central role of the UPF1:80S interaction for translation termination and NMD initiation (Ganesan *et al*, 2022).

Under normal conditions UPF1 has low basal helicase activity; however, upon recognition of a PTC and subsequent binding of UPF1 to UPF2, this activity is greatly increased. Alongside this, a large conformational change of the CH inhibitory domain modifies the RNA-binding properties and the catalytic activity of UPF1, causing a switch from an RNA-clamping mode to an RNA-unwinding mode (Chakrabarti *et al*, 2011). The active UPF1 helicase functions as an RNPase translocating along the mRNA with a 5′ to 3′ polarity, acting to resolve secondary structures, remove proteins from mRNA, and provide access to nucleases (Franks *et al*, 2010; Fiorini *et al*, 2015). Importantly, NMD can also be elicited on mRNAs that do not have a downstream EJC, although the mechanism of non-EJC dependent NMD is less well defined (Bühler *et al*, 2006; He & Jacobson, 2015). Interestingly, alternative branches of the NMD pathway that act independently of UPF2, UPF3B, or the EJC have also been described (Gehring *et al*, 2005; Chan *et al*, 2007; Ivanov *et al*, 2008). In mammals, there are two highly related UPF3 paralogs, UPF3A and UPF3B (also called UPF3X due to its location in chromosome X). Recent studies have shown that UPF3A and UPF3B perform redundant functions and can activate NMD without EJC binding, suggesting that UPF3 paralogs play a more active role in NMD than simply bridging the EJC and the UPF complex. UPF1 almost exclusively associates with UPF3B and only minimally with UPF3A; however, when UPF3B is mutated or removed, the association of UPF1 with UPF3A is enhanced 4-6 times, independent of RNA. Thus, UPF3A seems almost dispensable for NMD; however, it performs a compensatory role and can maintain NMD in the absence of UPF3B (Wallmeroth *et al*, 2022; Yi *et al*, 2022; Chen *et al*, 2023). In cells lacking both UPF3 paralogs, although NMD is not completely abrogated, its activity is reduced significantly (Bufton *et al*, 2022; Yi *et al*, 2022).

A current model postulates that UPF1 binding to mRNAs does not inevitably mark mRNAs for NMD-mediated degradation. Use of CLIP (cross-linking and immunoprecipitation) revealed that UPF1 binds target RNAs prior to mRNA translation. Once translating ribosomes are engaged, they displace UPF1 from coding sequences, leading to UPF1 enrichment at 3′UTRs (Hurt *et al*, 2013; Zünd *et al*, 2013). By contrast, binding of phosphorylated UPF1 (P-UPF1) marks mRNAs for NMD-mediated degradation, since P-UPF1 is enriched on endogenous transcripts degraded by NMD, whereas unphosphorylated UPF1 is released from non-targeted transcripts in an ATP-dependent manner (Kurosaki *et al*, 2014; Lee *et al*, 2015). Interestingly, UPF1 mutants with substantially impaired processing and slower unwinding rates are still functional in NMD and still have the capacity to restore NMD functionality upon loss of WT UPF1 (Fig 3) (Chapman *et al*, 2022).

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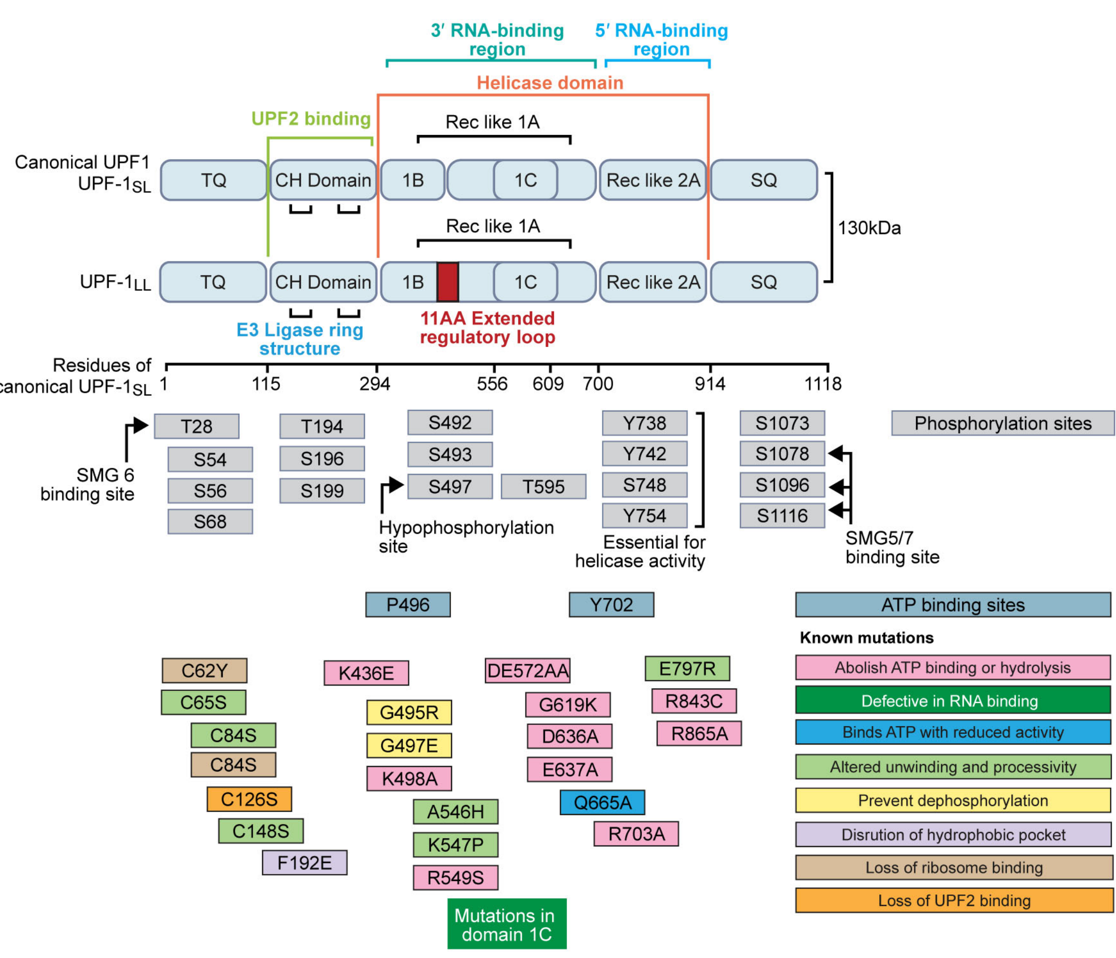

Figure 3. UPF1 structure, binding sites, and known mutations.
Diagram depicting the domain structure of the canonical $UPF1_{SL}$ isoform, highlighting key residues for its phosphorylation and ATP binding capacity. An isoform generated via alternative splicing, UPF_{LL}, includes an extra 11 amino acid extended regulatory region, while all other structural elements remain the same. Mutations and their effect on UPF1 function are indicated by the colored boxes. Some mutations in UPF1 have the capacity to eliminate NMD function, whilst some mutations sustain functional NMD. All residue numbers relate to the canonical $UPF1_{SL}$ isoform.

In addition to a rapidly increasing number of NMD regulators, it was recently shown that $UPF1_{LL}$, an alternative mammalian-specific isoform of the core NMD factor UPF1 ($UPF1_{SL}$), harbors a regulatory loop that is 11-residues longer and preferentially binds and down-regulates a different subset of NMD targets through a reduction in the RNA dissociation potential (Fig 3) (Gowravaram *et al*, 2018; Fritz *et al*, 2022). Canonical NMD is mediated by $UPF1_{SL}$ that represents >75% of the total UPF1 pool and is inhibited by moderate translational repression. By contrast, the $UPF1_{LL}$ isoform triggers NMD in response to activation of the integrated stress response (ISR), and repression of translation, targeting novel mRNA, including stress response genes (Fritz *et al*, 2022). Interestingly, $UPF1_{LL}$ requires translation termination events; however, due to its improved RNA-binding capacity in the presence of ATP compared to $UPF1_{SL}$, its sustained RNA interaction favors a reduced frequency of termination events in conditions of attenuated translation. Therefore, under conditions of moderate translation inhibition where NMD is inhibited, $UPF1_{LL}$ activity is enhanced, changing the specificity of NMD in response to stress conditions (Fritz *et al*, 2022).

UPF1 phosphorylation represents a non-reversible point in NMD progression during which the transcript is committed for degradation, leading to repression of further translation initiation, a key step in the NMD pathway (Isken *et al*, 2008). This phosphorylation event leads to the recruitment of additional NMD factors, namely the phospho-binding proteins SMG6 and/or SMG5/SMG7, which function in two independent, yet overlapping pathways, and lead to further recruitment of nucleases to elicit mRNA degradation. The SMG6 endonuclease cleaves NMD targets in the vicinity of the PTC (Huntzinger *et al*, 2008; Eberle *et al*, 2009) and generates a 5′ cleavage product that is most likely degraded 3′ to 5′ by the exosome and/or DIS3L2 (DIS3-like exonuclease 2; Kurosaki *et al*, 2018). The resulting 3′ cleavage product is cleared of protein components by UPF1 to provide access to the exoribonuclease XRN1 (Kurosaki *et al*, 2019). Alternatively, the heterodimer SMG5/SMG7 binds to P-UPF1 and recruits the CCR4/NOT complex to promote deadenylation, leading to 3′ to 5′ decay (Loh *et al*, 2013) and decapping, resulting in XRN1-catalyzed 5′ to 3′ degradation (Unterholzner & Izaurralde, 2004). Transcriptome profiling revealed that SMG6 and SMG7 act on

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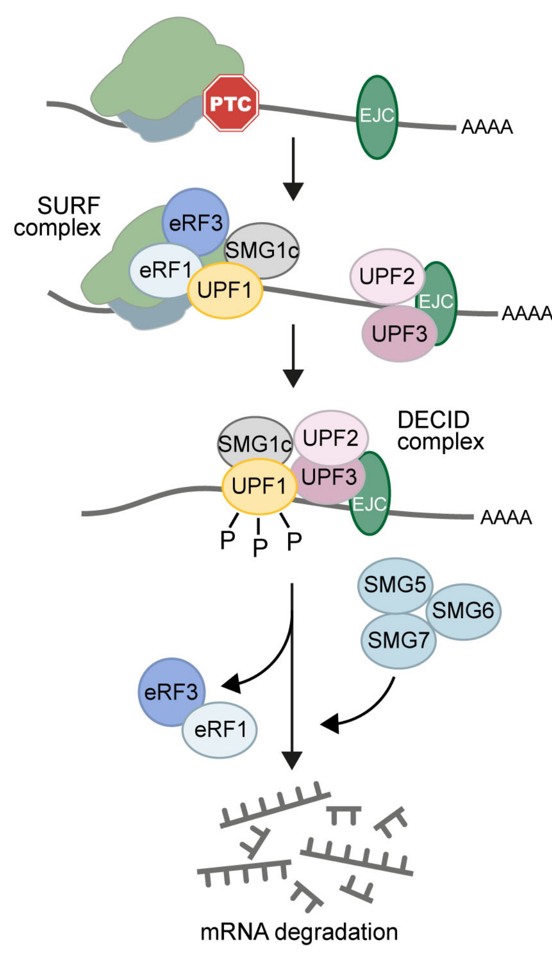

Figure 4. Mechanism of NMD activation.
Schematic depicting the widely accepted molecular events leading to the assembly of the surveillance complex (SURF), its transition to the decay-inducing complex (DECID) leading to UPF1 phosphorylation and recruitment of SMG6 and/or SMG5/SMG7 that elicit mRNA degradation.

essentially the same transcripts, suggesting extensive redundancy between the endo- and exonucleolytic decay pathways (Colombo *et al*, 2017). There is a tight regulation between these two RNA degrading pathways, since inactivation of the SMG5-SMG7 pathway also abrogates the SMG6-dependent mechanism, supporting a mechanism that involves UPF1 phosphorylation and SMG5-SMG7 recruitment to access SMG6 activity (Boehm *et al*, 2021).

Additional NMD factors

Core NMD factors SMG1-7 were initially discovered using genetic screens in *C. elegans* and in *S. cerevisiae* and were later found by homology searches in other species, including *Arabidopsis*, *Drosophila*, and mammals. Since then, a variety of experimental approaches have been employed to identify novel regulators of this pathway, most of which are still being investigated and therefore do not feature in models of NMD (Table 1) (Hug *et al*, 2016).

We developed RNAi screens in *C. elegans* that resulted in the identification of novel NMD factors, including *smgl-2/DHX34* and *smgl-1/NBAS* that are conserved throughout evolution and function in NMD in nematodes, zebrafish, and human cells (Longman *et al*, 2007, 2013; Anastasaki *et al*, 2011). DHX34, a DExH/D box RNA helicase, forms a complex with SMG1 and UPF1 and activates NMD by promoting the transition from the surveillance to the decay-inducing complex (Hug & Cáceres, 2014; Melero *et al*, 2016). Heterozygous mutations in *DHX34* were identified in four families affected with inherited acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). These mutations map to different domains of DHX34, and all germline variants identified in these families abrogated NMD activity (Rio-Machin *et al*, 2020). DHX34 is also associated with the human spliceosomal catalytic C complex and regulates a large number of alternative splicing (AS) events in mammalian cells in culture, establishing a dual role for DHX34 in both NMD and pre-mRNA splicing (Hug *et al*, 2022).

Neuroblastoma amplified sequence (*NBAS*) encodes a protein localized to the endoplasmic reticulum (ER) that is a component of the Syntaxin 18 complex and fulfils a role in Golgi-to ER retrograde transport, which is independent of its function in NMD (Aoki *et al*, 2009; Longman *et al*, 2020; discussed below in the "Localized NMD and the stress response" section).

Interactome studies, RNAi screens, and newer CRISPR screens in mammalian cells in culture have led to the identification of novel NMD regulators, which are still being characterized (Table 1). The RNA helicase MOV10, a member of the UPF1-like group helicase superfamily 1 (SF1), interacts with UPF1 and promotes degradation of UPF1-regulated mRNA transcripts (Gregersen *et al*, 2014). An interactome of the SMG1 protein kinase identified the AAA ATPases, RuVB-like 1 (RUVBL1) and RuVB-like 2 (RUVBL2). These proteins are involved in a variety of cellular functions, including transcription and DNA repair, and were previously shown to have a role in the early stages of NMD (Izumi *et al*, 2010). We recently showed that RUVBL1/2 also interact with DHX34, coupling their ATPase activity to the assembly of factors required to initiate the NMD response (López-Perrote *et al*, 2020).

A genome-wide RNAi screen in human cells identified ICE1, an EJC-associated factor that promotes the interaction of UPF3B with the EJC and activates NMD (Baird *et al*, 2018). CRISPR screens identified several candidate NMD genes (Alexandrov *et al*, 2017) and highlighted a role for the ribosome recycling factor ABCE1 in NMD (Annibaldis *et al*, 2020; Zhu *et al*, 2020). A CRISPR screen in K562 cells identified the translational repressors GIGYF2 and EIF4E2, suggesting a model wherein recognition of a stop codon as premature leads to its translational repression mediated by GIGYF2 and EIF4E2, a process shared with the NGD pathway (Zinshteyn *et al*, 2021). Finally, a haploid-cell genetic screen for NMD effectors identified several components of the AKT signaling pathway. It was shown that AKT-mediated phosphorylation of the UPF1 CH domain at T151D overcomes auto-inhibition of UPF1 helicase activity, which is critical for NMD and decreases the dependence of helicase activity on ATP. AKT also promotes formation of EJCs that contain AKT at the expense of UPF2, potentially facilitating a UPF2 independent branch of NMD (Cho *et al*, 2022). Interestingly, AKT1 had been independently shown to phosphorylate UPF1 and activate NMD (Palma *et al*, 2021; Table 1).

NMD factors have also been shown to display additional cellular functions. This is particularly prominent in the case of UPF1 that not only functions in genome stability (Azzalin & Lingner, 2006), but also contributes to several RNA decay pathways (Kim &

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Table 1. Factors involved in nonsense-mediated mRNA decay.

Protein	Role in nonsense-mediated mRNA decay	References
SMG1	A phosphoinositide 3-kinase (PI3K)-like kinase that phosphorylates UPF1	Yamashita *et al* (2001), Kashima *et al* (2006)
SMG8/9	Two subunits of the SMG-1 complex which negatively regulate SMG-1 kinase activity	Yamashita *et al* (2009)
UPF1	An ATP-dependent RNA helicase of the SF1 superfamily, which undergoes cycles of phosphorylation and dephosphorylation	Sun *et al* (1998), Bhattacharya *et al* (2000), Kim and Maquat (2019)
UPF2	Recruited following recognition of a PTC leading to conformational changes in UPF1 and formation of the DECID complex	Serin *et al* (2001), Chamieh *et al* (2008)
UPF3A/B	UPF3 paralogs that bridge the EJC and the surveillance complex	Serin *et al* (2001), Neu-Yilik *et al* (2017)
SMG5/7	A heterodimer that binds to phosphorylated UPF1 and recruits the exonucleolytic RNA degrading machinery	Unterholzner and Izaurralde (2004), Loh *et al* (2013)
SMG6	An endonuclease that cleaves NMD targets in the vicinity of the PTC	Huntzinger *et al* (2008), Eberle *et al* (2009)
DHX34	A DExH/D box helicase that activates NMD by promoting a transition from the SURF to DECID complex	Hug and Cáceres (2014)
NBAS	NMD factor localized at the membrane of the ER that recruits UPF1 to activate a local NMD response	Longman *et al* (2007, 2020)
MOV10	A 5′ to 3′ RNA helicase that contributes to UPF1-mediated mRNA target degradation	Gregersen *et al* (2014)
RUVBL1/2	AAA+ adenosine triphosphatases involved in the early stages of NMD that interact with SMG-1 and promote the formation of the SURF complex	Izumi *et al* (2010)
ICE1	An EJC-associated protein that promotes UPF3B recruitment to the EJC and provides a link between splicing and NMD	Baird *et al* (2018)
GIGYF2/EIF4E2	Mediate translational repression following recognition of a PTC	Zinshteyn *et al* (2021)
AKT	AKT signaling leads to formation of alternative EJCs where AKT replaces UPF2. AKT-mediated phosphorylation of UPF1 activates NMD.	Palma *et al* (2021), Cho *et al* (2022)
CWC22	Essential splicing factor which interacts with eIFA3 to activate NMD	Alexandrov *et al* (2012), Barbosa *et al* (2012), Steckelberg *et al* (2012)
ABCE1	Responsible for ribosome recycling necessary to initiate translation termination and initiation of NMD via SMG6-mediated endonucleolytic pathway	Annibaldis *et al* (2020), Zhu *et al* (2020)

Maquat, 2019). These include the Staufen-mediated decay pathway (SMD), which involves UPF1 recruitment to stem-loops in the 3′UTRs of mRNAs bound by the RNA-binding proteins STAUFEN1 and its paralog STAUFEN 2 (Kim *et al*, 2005; Park *et al*, 2013), and in the degradation of histone mRNAs, where UPF1 is recruited to target mRNAs via the stem-loop binding protein (SLBP) (Kaygun & Marzluff, 2005). Interestingly, UPF1 has also been proposed to act as an E3-ubiquitin ligase and promote degradation of the truncated polypeptide produced by translation of a PTC-containing transcript (Takahashi *et al*, 2008; Feng *et al*, 2017), establishing a link between RNA degradation and protein decay (Kim & Maquat, 2019; Inglis *et al*, 2023). In *S. cerevisiae*, UPF1 facilitates proteasome degradation of truncated polypeptides in a ubiquitin dependent manner. The truncated protein product gets released from the ribosome following NMD-mediated mRNA degradation, but remains associated with UPF1 which directs it to the proteasome for removal, thus leading to a protein and mRNA turnover coupled process (Kuroha *et al*, 2013).

NMD targets

Despite decades of research, it is still not entirely clear what constitutes a *bona fide* NMD target. The introduction of a mutation, such as a single nucleotide variant, which gives rise to a PTC, represents the most obvious target for NMD. However, transcriptome profiling to identify NMD targets in cells of different species revealed that the majority of NMD-sensitive transcripts do not contain PTCs but are rather mRNAs coding for full-length proteins. This led to the hypothesis that a combination of NMD-inducing and NMD-antagonizing features will contribute to determine NMD susceptibility for any given mRNA. Some of the common features that render mRNAs susceptible to NMD include the presence of a PTC located at least 50-55 nucleotides upstream of an EJC (Colombo *et al*, 2017), mRNAs with upstream ORFs (uORFs; Calvo *et al*, 2009), and the presence of a long 3′UTR (Hogg & Goff, 2010). However, recent use of cDNA Nanopore sequencing combined with short RNA-seq allowed the detection of full-length NMD substrates that are highly unstable and only display an increase in RNA levels when NMD is inhibited. This analysis identified NMD target mRNAs derived mainly from alternative exon usage, yet it did not identify long 3′UTRs as a common feature for NMD regulated mRNAs (Karousis *et al*, 2021). RNA-seq allows for the analysis of steady-state changes, which are influenced by stability, degradation, and transcription rates. Recently, SH-linked alkylation for the metabolic sequencing of RNA (SLAM-seq) using 4-thiouracil pulse-chase labeling (Herzog *et al*, 2017) was used to accurately measure changes in RNA half-lives and to identify new targets of the NMD pathway in *S. cerevisiae* (Alalam *et al*, 2022). SLAM seq analysis of *Smg*5-7 genetic knockouts in mouse ESCs revealed that NMD controls expression levels of the translation initiation factor *Eif4a2* and its alternative splicing

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isoform that harbors a PTC-encoding isoform ($Eif4a2^{PTC}$). Upon NMD inhibition, aberrant expression of the $eIF4A2^{PTC}$ elicits increased mTORC1 activity and translation rates and causes differentiation delays, highlighting a role of RNA stability regulation in development (Huth *et al*, 2022).

NMD regulation

The NMD pathway is dynamic and subject to regulation and impacts several physiological processes, such as the stress and immune responses. A correct magnitude of NMD activity is particularly important for proper brain function and indeed, NMD activity is extensively regulated during neural development (Jaffrey & Wilkinson, 2018). It was shown that NMD activity in human neuroblastoma cells is attenuated by fragile X protein FMRP, which is recruited to NMD targets by UPF1. FMRP acts as an NMD repressor in neural cells and in its absence, NMD is hyperactivated, leading to widespread transcriptome changes which contributes to intellectual disability and autism (Kurosaki *et al*, 2021). Recently, a conditional *Smg6* mutant mouse model revealed that the NMD pathway has a role in controlling circadian clock regulation (Katsioudi *et al*, 2023). The NMD response varies within and across cell lines (Sato & Singer, 2021), in different cell tissues (Zetoune *et al*, 2008), and even among individuals (Nguyen *et al*, 2014; Rivas *et al*, 2015). NMD is tightly regulated by a negative feedback network that leads to a large proportion of core NMD factors being regulated by NMD itself in several organisms, including mammalian cells, nematodes, zebrafish, and plants (Huang *et al*, 2011; Yepiskoposyan *et al*, 2011; Longman *et al*, 2013). NMD can occur with equal probability during each round of translation of an mRNA molecule; however, this probability is variable and linked to sequence features, including the exon sequence downstream of the PTC, the PTC-to-intron distance, and the number of introns both upstream and downstream of the PTC. Furthermore, a subpopulation of mRNAs can escape NMD, further contributing to variation in NMD efficiency (Hoek *et al*, 2019). Use of a single-cell approach comprising a bi-directional NMD reporter expressing two β-globin genes with or without a PTC in the same cell, allowed the characterization NMD efficiency in individual cells. This revealed a broad range of NMD efficiencies in the population (where some cells degraded essentially all mRNAs and others escaped NMD almost completely) and was correlated to the differential level of SMG1 expression and P-UPF1. Mechanistically, this escape occurred either by translational read-through at the PTC or by inefficient mRNA degradation following translation termination at the PTC (Sato & Singer, 2021).

Localized NMD and the stress response

The decay of NMD reporters in mammalian cells occurs in the cytoplasm (Trcek *et al*, 2013) and is closely linked to mRNA translation (Kervestin & Jacobson, 2012). We previously identified two novel NMD factors, *NBAS* and *SEC13*, which localize to the ER, raising the possibility that they could be involved in a localized NMD pathway (Longman *et al*, 2007; Casadio *et al*, 2015). There are precedents for a localized NMD response in neurons that regulate the expression of dendritic and axonal mRNAs upon the activation of their localized mRNA translation (Colak *et al*, 2013). It has been shown that mRNAs coding for secreted or transmembrane proteins are translated only when they encounter the ER (Wu *et al*, 2016). However, it remained largely unknown how NMD regulates the stability of

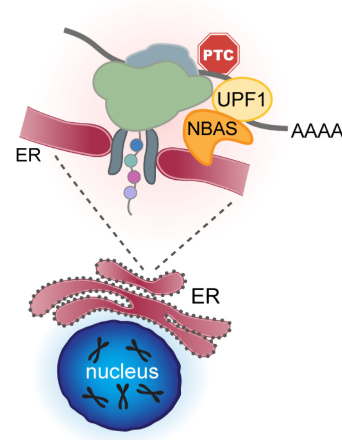

Figure 5. Localized NMD response at the ER.
NBAS localizes to the outside membrane of the endoplasmic reticulum (ER), in the vicinity of the translocon, where it recruits the core NMD factor UPF1 to activate a local NMD response at the ER (Longman *et al*, 2020).

RNAs translated at the ER, which due to their intrinsic localized translation, will not have sufficient exposure to cytoplasmic NMD surveillance. NBAS is a member of the Syntaxin 18 complex as part of the COPI vesicle and is involved in Golgi-to-ER retrograde transport (Aoki *et al*, 2009). We showed that NBAS fulfils a second, independent function and recruits the core NMD factor UPF1 to the membrane of the ER and activates a local NMD response that regulates RNAs associated with cellular stress and membrane trafficking (Fig 5; Longman *et al*, 2020). Loss-of-function mutations in NBAS have been found in diseases affecting bone, connective tissue, and acute liver failure (Hug *et al*, 2016; Staufner *et al*, 2020). We identified compound heterozygous variants in *NBAS* as a cause of atypical osteogenesis imperfecta (Balasubramanian *et al*, 2017). It remains to be seen whether the phenotype of *NBAS* mutations is due to faulty NMD response and/or defective Golgi-to-ER retrograde transport (Haack *et al*, 2015).

The Unfolded Protein Response (UPR) senses and responds to excessive amounts of misfolded proteins in the ER, that cause ER stress (Walter & Ron, 2011). Inappropriate UPR activation contributes to many human pathologies, most notably related to neurodegeneration (Hetz & Mollereau, 2014), therefore the fidelity of UPR activation must be tightly regulated. Accordingly, a role for NMD in regulating the UPR has been proposed (Karam *et al*, 2015; Goetz & Wilkinson, 2017). In particular, mRNAs encoding UPR components, including the UPR sensor IRE1α, as well as ATF-4 and CHOP that are activated by PERK branch signaling, are regulated by NMD and thus control the threshold of cellular stress that is necessary to activate the UPR (Gardner, 2008; Karam *et al*, 2015; Sieber *et al*, 2016). A feed-back loop mechanism operates, wherein NMD ensures a correct activation threshold for the UPR response and the activity of NMD is in turn down-regulated by the UPR (Karam *et al*, 2015). It is likely that limiting the chronic activation of the UPR has a protective effect in neurodegenerative diseases. Along those lines, a protective role for UPF1 was shown in rodent primary neuronal models of amyotrophic lateral sclerosis (ALS) and frontotemporal

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dementia (FTD; Barmada *et al*, 2015) and in a rat ALS paralysis model (Jackson *et al*, 2015).

It is also possible that modulation of ER-NMD activity could be crucial to regulate ER stress (Longman *et al*, 2020). In such a scenario, NMD-mediated degradation of RNA targets would decrease ER load by suppressing aggregation of truncated and/or misfolded proteins in the ER, limiting UPR activation. The newly identified $UPF1_{LL}$ that, upon ISR activation and impaired translation, regulates a new subset of NMD targets, can be a key to the appropriate cellular stress response (Fritz *et al*, 2022).

NMD in human disease

In vertebrates, NMD activity is essential for proper development, and loss of NMD leads to embryonic lethality (Chousal *et al*, 2022). There are several reports of mutations in core NMD factors that lead to human disease. Two consanguineous families with homozygous loss-of-function mutations in SMG9, a component of the SMG1c complex, display a multiple congenital anomaly syndrome, that includes heart and eye defects, and brain malformation (Shaheen *et al*, 2016). Four consanguineous families with four deleterious homozygous variants in SMG8 display a phenotype that resembles that found in patients with SMG9 mutations, including global developmental delay, microcephaly, facial dysmorphism, and variable congenital heart and eye malformations. These patients display increased phosphorylation of UPF1, which most likely reflect the loss of SMG8-mediated inhibition of SMG1 kinase activity (Alzahrani *et al*, 2020). NMD has also an important role in neuronal development as demonstrated by the fact that mutations, as well as copy number variations in UPF2 and UPF3B, lead to intellectual disability and/or neurodevelopmental disorders in humans, including schizophrenia and autism spectrum disorder (Jolly *et al*, 2013; Nguyen *et al*, 2013; Jaffrey & Wilkinson, 2018). Altered levels of NMD activity were also implicated in the pathogenesis of C9orf72-linked ALS/FTD (Xu *et al*, 2019; Ortega *et al*, 2020; Sun *et al*, 2020) and other neurodegenerative diseases (Kurosaki *et al*, 2021). For example, loss of the fragile X protein FMRP leads to intellectual disability and autism, and FMRP deficiency results in a hyperactivated NMD response in human cells (Kurosaki *et al*, 2021). UPF3B mutations cause intellectual disability with impairment of neural stem cell differentiation and reduction in neuronal branching, through a loss of UPF2 interaction leading to NMD abrogation (Bufton *et al*, 2022). Altogether, these data suggest that an imbalance in NMD activity could lead to neurodegeneration. Interestingly, NGD and NSD components *Pelo* and *HBs1l* are critical for cerebellum neurogenesis in mice but expendable for survival of these neurons after development (Terrey *et al*, 2021). Similar effects were observed upon deletion of the core NMD factor, Upf2. This suggests that several RNA quality control pathways may interact or have compensatory roles to drive early development.

The role of NMD in cancer is complex, since it can display both tumor suppressing and tumor enhancing roles (Wang *et al*, 2011; Tan *et al*, 2022). Cancer cells can exploit the NMD response through introduction of selective tumor suppressor mutations that initiate mRNA decay or via the introduction of NMD-insensitive mutations in oncogenes to prevent their targeting (Lindeboom *et al*, 2016). For example, tumor cells are known to hijack the NMD system to suppress the expression of potent tumor suppressors such as WT1, BRCA1/2 and p53, inducing uncontrolled cell growth (Mort *et al*, 2008; Nogueira *et al*, 2021). A therapeutic potential for the treatment of cancer was suggested in a number of studies. Abrogating NMD in cancer may lead to the expression of tumor-specific proteins that can increase natural immune responses directed against the tumor (Pastor *et al*, 2010). Furthermore, attenuation of NMD facilitates the response to cancer therapeutics, as shown in human breast cancer cells, subject to NMD inhibition. Combining this with the front-line chemotherapeutic doxorubicin promotes a faster and more robust cancer-cell killing by apoptosis (Popp & Maquat, 2015).

Conclusions

Translation-coupled RNA quality control pathways play a central role in ensuring accurate gene expression. There is a great variety of such mechanisms that link defective mRNA translation with mRNA decay and degradation of truncated proteins. We have highlighted here three main pathways, NGD, NSD, and NMD. The first two share common features and trans-acting factors and mainly differ in features present in the target mRNAs that trigger these pathways. NMD is the most studied mRNA surveillance system, both in terms of mechanism and trans-acting factors, mostly due to its high impact as a potent buffering system for human disease. Given that the NMD pathway affects the phenotype of approximately one third of all genetic diseases (Holbrook *et al*, 2004; Mort *et al*, 2008), a better understanding of its regulation and factors that influence its function *in vivo* could be of importance for designing strategies to modulate the NMD response for therapeutic use. Challenges for the future involve the identification of all factors required for NMD regulation in a physiological setting, as well as a better understanding of what constitutes an NMD target.

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Author contributions

Javier F Caceres: Conceptualization; writing – original draft; writing – review and editing. **Laura Monaghan:** Conceptualization; writing – original draft; writing – review and editing. **Dasa Longman:** Conceptualization; writing – original draft; writing – review and editing.

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