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Nonsense-mediated mRNA decay: target genes and functional diversification of effectors

Jan Rehwinkel[1], Jeroen Raes[1] and Elisa Izaurralde[1,2]

[1] EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany
[2] MPI for Developmental Biology, Spemannstrasse 35, D-72076 Tübingen, Germany

Recent genome-wide identification of nonsense-mediated mRNA decay (NMD) targets in yeast, fruitfly and human cells has provided insight into the biological functions and evolution of this mRNA quality control mechanism, revealing that NMD post-transcriptionally regulates an important fraction of the transcriptome. NMD targets are associated with a broad range of biological processes, but most of these targets are not encoded by orthologous genes across different species. Yeast and fruitfly NMD effectors regulate common targets in concert, but parallel pathways have evolved in humans, whereby NMD effectors have acquired additional functions. Thus, the phenotypic differences observed across species after inhibition of NMD are driven not only by the functional diversification of NMD effectors but also by changes in the repertoire of regulated genes.

NMD regulates gene expression post-transcriptionally

Regulation of gene expression at the post-transcriptional level is increasingly being recognized as a key mechanism by which cells can rapidly change their gene expression patterns. In eukaryotes, post-transcriptional processes, such as mRNA processing, export, translation and turnover, provide the means to regulate the expression of individual genes at many levels, but each step also increases the probability of introducing errors such as nonsense codons. The fidelity of gene expression is nevertheless ensured by robust mRNA quality control ('surveillance') mechanisms that detect and degrade aberrant mRNAs, thereby preventing the accumulation of potentially toxic protein fragments. Indeed, improperly processed mRNAs are degraded by surveillance mechanisms in the nucleus before being exported to the cytoplasm [1]. In the cytoplasm, additional mechanisms detect and further degrade mRNAs that do not encode a functional protein. For example, mRNAs with a nonsense codon or 'premature translation termination codon' (PTC) are degraded rapidly [1–4]. This degradation occurs via the nonsense-mediated mRNA decay (NMD) pathway – one of the best-characterized mRNA quality control systems in eukaryotes [2–4].

Recent analyses of gene expression profiles in cells in which NMD is inhibited have revealed that this pathway also has a crucial role in the post-transcriptional regulation of a considerable fraction of the transcriptome [5–9]. Here, we discuss new insights into the role of NMD in post-transcriptional gene regulation gained from the genome-wide identification of NMD targets in budding yeast (*Saccharomyces cerevisiae*), fruitfly (*Drosophila melanogaster*) and human cells. Among these species, the genes that have been so far identified as targets for regulation by NMD are largely non-orthologous – a finding that has implications for our understanding of the phenotypic differences observed across species after inhibition of the NMD pathway.

Effectors of the NMD pathway

The key effectors of the NMD pathway have been identified in genetic screens in yeast (*S. cerevisiae*) and more recently in nematode worms (*Caenorhabditis elegans*). These effectors include three yeast genes (*upf1–upf3*) and seven *C. elegans* genes (*smg-1–smg-7*) that each have an essential role in NMD (Table 1). The UPF1, UPF2 and UPF3 proteins (known as SMG-2, SMG-3 and SMG-4 in *C. elegans*) are conserved from yeast to human, whereas SMG-1 and SMG-5–SMG-7 have orthologs in multicellular organisms but not in yeast [2–4,10–12] (Table 1).

Deletion or silencing of the *upf1* gene results in stabilization of PTC-containing mRNAs in all organisms in which NMD has been investigated [2–4,10–14]. UPF1 is an RNA helicase; its activity is regulated by cycles of phosphorylation and dephosphorylation that require additional NMD effectors. Phosphorylation of UPF1 requires UPF2 and UPF3, and is catalyzed by SMG1 – a protein kinase related to phosphatidylinositol 3-kinase [13,15–17]. Dephosphorylation of UPF1 is mediated by SMG5, SMG6 and SMG7 – three related proteins that function as adaptors between phosphorylated UPF1 and protein phosphatase 2A [18–23].

Although direct interactions among NMD effectors have been observed *in vitro* (e.g. among UPF1, UPF2 and UPF3), assembly of these proteins into the so-called 'surveillance complex' seems to be regulated *in vivo* and occurs in a stepwise manner on mRNAs whose translation terminates prematurely [24,25] (Figure 1). Once assembled, the surveillance complex recruits mRNA decay

Corresponding author: Izaurralde, E. (elisa.izaurralde@tuebingen.mpg.de).
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Table 1. NMD effectors and phenotypes across species[a]

Organism	Effectors	Phenotype
Yeast: *S. cerevisiae*	Upf1 Upf2 (Nmd2) Upf3	Not essential
Worm: *C. elegans*[b]	SMG-2 (UPF1) SMG-3 (UPF2) SMG-4 (UPF3) SMG-1 SMG-5 SMG-6 SMG-7	Viable worms with morphological effects on genitalia
Fruitfly: *D. melanogaster*[c]	UPF1 UPF2 UPF3 SMG1 SMG5 SMG6	Required for cell-cycle progression and proliferation
Mammals: *Mus musculus*[d]	Upf1 (Rent1) Upf2 Upf3 Smg1 Smg5 Smg6 Smg7	*Upf1* KO: embryonic lethal; required for cell-cycle progression *Upf2* KD: no effect on cell viability

[a]NMD effectors in the organism for which NMD has been investigated are shown in addition to the phenotypes resulting from inhibition of the pathway by gene knockout (KO) or gene expression knock down (KD).
[b]Worms lacking a functional NMD pathway are viable [11,12].
[c]In fruitfly, phenotype has been analyzed only at the cellular level [8].
[d]In mouse, knockout of the *Upf1* gene leads to early embryonic death [58]. The effect of depleting human *UPF2* has been analyzed only at the cellular level [9,54].

enzymes, thereby coupling premature translation termination to mRNA degradation. Consistent with this, NMD factors have genetic, functional and/or physical interactions with both eukaryotic translation termination factors (i.e. eRF1 and eRF3) and mRNA degradation enzymes [2–4] (Figure 1).

Mechanisms of NMD

An essential step in the NMD pathway is the recognition of a stop codon as premature. How are premature stops discriminated from natural stops? A clue comes from the observation that the ability of a PTC to elicit NMD is position dependent: that is, the closer to the 3′ end of the transcript is the PTC, the smaller is the reduction in mRNA levels by NMD. Positional effects of PTCs are observed in all organisms and indicate that PTC recognition depends on the position of the stop codon relative to downstream RNA sequence elements and/or associated proteins [2–4,26].

Mammalian NMD

In mammals, these downstream sequences are represented by exon–exon boundaries (Figure 1). Indeed, natural stops are typically located in terminal exons, whereas destabilizing nonsense codons lie >50 nucleotides upstream of the last exon–exon boundary [2–4,26]. The position of exon–exon boundaries is marked by the exon junction complex (EJC), the core components of which interact with NMD effectors (Figure 1). At least five components of the EJC are required for NMD in human cells, supporting the idea that the EJC provides positional information to discriminate premature from natural stops in mammals. These components include the protein RNPS1, the Y14–MAGOH heterodimer, and additional binding partners such as the protein Barentsz and the RNA helicase eIF4AIII [27–38].

Yeast and fruitfly NMD

In yeast and fruitfly, PTC recognition occurs independently of splicing, and alternative models have been proposed to explain what distinguishes premature from normal translation termination in these organisms [14,39–41] (Figure 1). One model postulates that mRNAs contain loosely defined downstream sequence elements with a function analogous to that of mammalian exon–exon junctions [2–4,39,40]. Another, the 'faux 3′-UTR model', proposes that 3′ untranslated regions (UTRs) are marked by a specific set of proteins that influences translation termination. These proteins include the poly(A)-binding protein (termed Pab1p in yeast or PABPC1 in multicellular organisms), which binds the poly(A) tail of most cytoplasmic mRNAs. At natural stops, translation termination is efficient because terminating ribosomes can interact with these 3′-UTR-bound proteins [41]; at premature stops, by contrast, translation termination will be impaired or too slow, because the terminating ribosome cannot establish these interactions. In the latter case, the NMD complex is assembled, leading to rapid degradation of the mRNA. Thus, in the faux 3′-UTR model, premature translation termination is intrinsically aberrant because the stop codon is not in the appropriate context [41]. In support of this model, experiments in yeast have shown that tethering Pab1p to an mRNA mimics a normal 3′ UTR and suppresses NMD when the tethering site is located downstream of a PTC [41].

Thus, the distinguishing feature of natural stops in yeast seems to be their proximity to Pab1p. Consistently, the majority of yeast 3′ UTRs are homogeneous in length (~100 nucleotides) and aberrant transcripts with exceptionally long 3′ UTRs are substrates for NMD [42]. In fruitfly, tethering PABPC1 to a region downstream of a PTC also abolishes NMD (I. Behm-Ansmant and E.I., unpublished data) but, in contrast to those in yeast, the 3′ UTRs in fruitfly range from a few to several thousand nucleotides. Moreover, a large proportion of mRNAs with 3′ UTRs that are longer than average are not regulated by NMD [8]. This observation raises the question of whether PTCs in fruitfly are defined relative to PABPC1 and, if so, whether transcripts with exceptionally long 3′ UTRs have acquired features that antagonize the NMD pathway.

NMD regulates an important fraction of the transcriptome

Conservation of the NMD pathway suggests that its role is not restricted to the degradation of aberrant mRNAs containing PTCs, but instead might reflect additional roles in post-transcriptional gene regulation. Early studies in yeast and worms showed that NMD regulates the expression of a few naturally occurring transcripts [3,39,43,44]. More recently, gene expression profiling of yeast, fruitfly and human cells depleted of essential NMD factors has facilitated the identification of several transcripts that are regulated by NMD. These studies have shown that NMD modulates the expression of ~3–10% of the transcriptome [5–9].

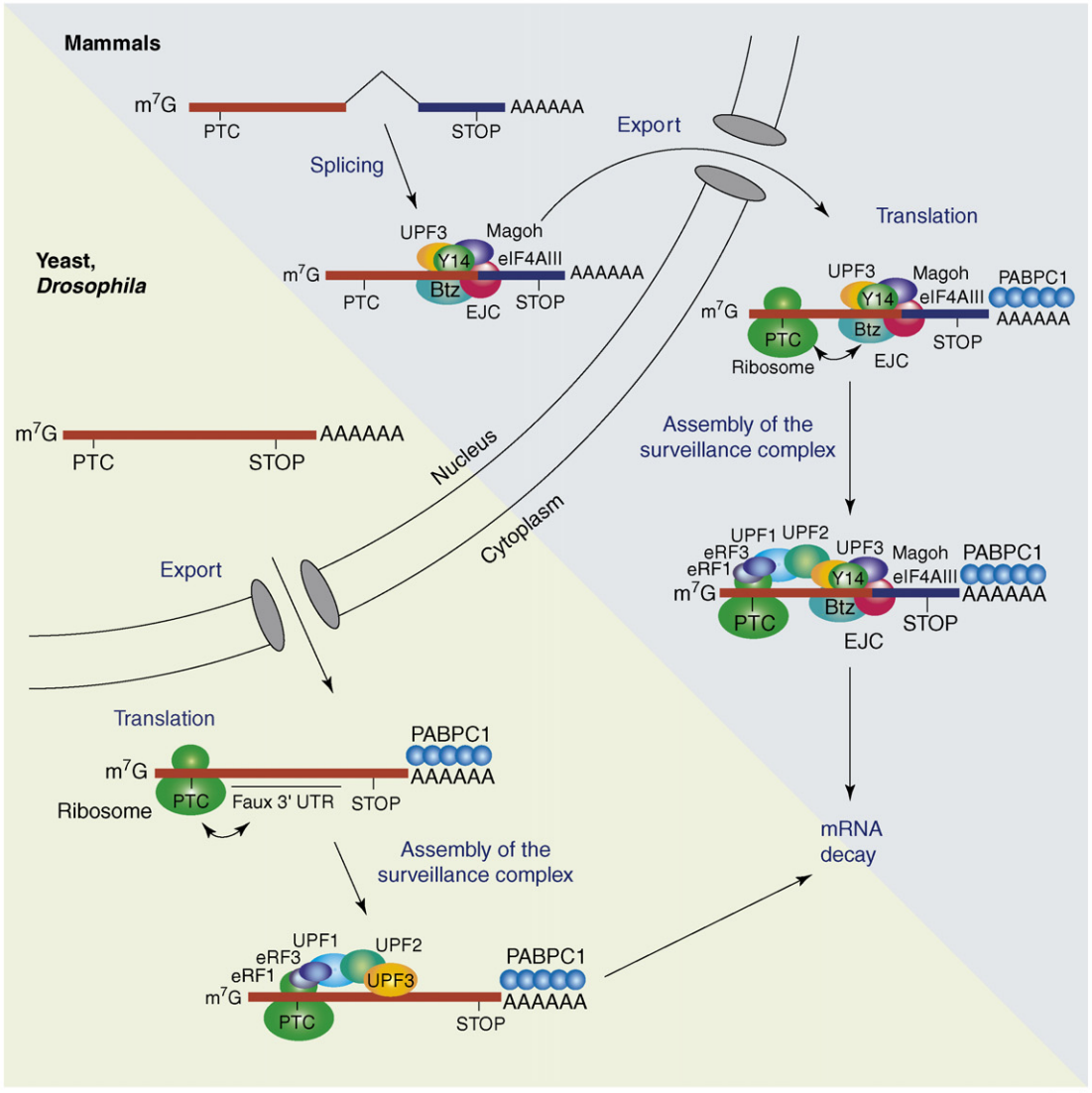

Figure 1. Mechanisms of NMD and cellular location. A PTC or nonsense codon is defined relative to the position of a downstream *cis*-acting signal that varies across species [2,3]. In mammals, the *cis*-acting signal is an exon–exon junction marked by a group of proteins that constitute the EJC. The EJC is deposited at exon–exon boundaries during splicing. A translation termination event upstream of an EJC leads to recruitment of UPF1 by the eukaryotic translation release factors eRF1 and eRF3. UPF1 then interacts with UPF2, UPF3 and additional EJC proteins bound to a downstream EJC (see Refs [24,25] and references therein). These interactions result in assembly of a 'surveillance complex' that targets the bound mRNA for degradation. In yeast and fruitfly, the *cis*-acting signal is a faux 3′ UTR [41]. Natural termination codons (STOP), in contrast to PTCs, are flanked by proper 3′ UTRs marked by a specific protein or set of proteins, including the cytoplasmic poly(A)-binding protein (PABPC1), which is thought increase the efficiency of translation termination (see Ref. [41] and references therein). Premature termination is thought to be inefficient because the PTC lacks proximal PABPC1 and/or other factors associated with a natural 3′ UTR. In this case, the NMD factors UPF1–UPF3 are recruited via interactions with the eRF proteins. As in mammals, this results in assembly of the surveillance complex and degradation of the nonsense mRNA.

In yeast, 545 mRNAs have been identified that are commonly regulated in strains lacking UPF1, UPF2 or UPF3 [6]. Similarly, 184 NMD targets identified in fruitfly represent an ensemble of transcripts commonly regulated by at least five of the six known NMD effectors (i.e. UPF1–UPF3 and SMG1, SMG5 and SMG6) [8].

Furthermore, 197 potential NMD targets have been identified (among 4000 detectable transcripts) in human cells depleted of UPF1 [7], whereas 37 of 2400 detectable transcripts are upregulated in those depleted of UPF2 [9]. There are, however, two main limitations associated with the identification of NMD targets in human cells. First, the arrays used in one of these studies cover only a fraction of the genes that have been annotated. Second, the expression profiles in cells depleted of UPF1 or UPF2 were analyzed in two independent studies [7,9], making the comparison of these profiles difficult. Because depletion of UPF2 might affect only a few genuine NMD targets (Ref. [34] and see later), in the following discussion we consider solely UPF1 targets.

Aberrant mRNAs with PTCs have been found among NMD targets in all organisms studied so far. These mRNAs include inefficiently spliced mRNAs that are exported to the cytoplasm with intronic sequences, alternatively spliced mRNA isoforms with PTCs, and transcripts derived from pseudogenes [5–9]. In vertebrates, mRNAs transcribed from unproductively rearranged immunoglobulin and T-cell receptor genes also represent an important physiological class of NMD substrates [45]. Indeed, approximately two-thirds of such recombination events

Table 2. Features of 'error-free' NMD targets

Features of NMD targets	Explanation	Refs
mRNAs with uORFs in the 5′ UTR[a]	Stalling of ribosomes at the uORF stop codon is likely to be the mechanism by which this class of mRNAs is recognized by the NMD machinery	[6,7,9,43]
mRNAs undergoing 'leaky scanning' for translation initiation	Leaky scanning refers to the situation whereby ribosomes skip an initiator codon that is not in the optimal context and initiate translation at AUG codons that are out of frame	[6,44]
mRNAs regulated by translational frame-shifting	Ribosomes that fail to shift the reading frame are likely to encounter a PTC that elicits NMD	[8,69]
mRNAs encoding selenoproteins	The UGA codon is interpreted as a selenocysteine codon or as a PTC	[7,70]
mRNAs regulated by stop codon read through	The efficiency of translation termination is reduced at the natural stop and ribosomes continue to translate the 3′ UTR sequences.	[6]
Bicistronic mRNAs	Observed among NMD substrates in yeast	[6]
Aberrant mRNAs with exceptionally long 3′ UTRs	Observed among NMD substrates in yeast	[42]
mRNAs with introns in the 3′ UTRs	In mammals, the natural stop is redefined as premature when located at least 50 nucleotides upstream of the intron	[7,9,26]

[a]Note that not all upstream open reading frames (uORFs) elicit NMD [68].

result in frame-shifted genes whose transcripts are degraded by NMD.

In addition to these PTC-containing mRNAs, numerous 'error-free' transcripts are also regulated by NMD [5–9]. These error-free NMD targets represent a heterogeneous group of mRNAs with various features (Table 2). Another important class of NMD substrates identified by microarray profiling comprises transcripts derived from transposable elements [7,8]. Expression of transposons, pseudogenes and repetitive sequence elements is also repressed by RNA silencing pathways in multicellular organisms [46,47]. This observation indicates that RNA silencing pathways and NMD are two independent mechanisms by which transcripts generated from these loci are degraded. Thus, NMD functions in parallel to RNA silencing pathways and helps to preserve the stability of the genome.

NMD targets function in diverse cellular processes

Analyses of the gene ontology terms associated with transcripts regulated by NMD in yeast, fruitfly and human cells show that, although some functional categories are overrepresented among NMD targets relative to their proportion in the genome, it is the diversity of functional categories that is most striking. This diversity is clearly visible among NMD targets in fruitfly (Figure 2), but a similarly broad range of functionalities is observed among yeast NMD targets and human UPF1 targets [6,7].

To compare functional categories in yeast, fruitfly and human NMD (UPF1) targets, we assigned proteins encoded by these transcripts to, respectively, 316, 159 and 170 eukaryotic orthologous groups of proteins ('KOGs') [8,48]. The KOG database includes proteins from seven eukaryotic genomes clustered into groups of orthologous genes [48], which facilitates a comparison of numerous transcripts across species. We found that 13 KOGs represented in both yeast and human NMD targets are also detectable in fruitfly cells but, remarkably, only two are regulated by NMD in the latter cells. One group of orthologous transcripts that is commonly regulated in all three organisms includes *Drosophila* SMG5 and SMG6, human SMG5 and yeast Ebs1p – a protein involved in telomere maintenance [8]. The regulation of SMG5 by NMD in both human and fruitfly suggests the existence of a conserved feedback mechanism [7,8].

Limited overlap of functional categories is also observed when only two species are compared. In addition to the above-mentioned categories, only 8 of the 136 KOGs that are represented in the list of yeast targets and detectable in fruitfly cells are regulated by NMD in fruitfly. Of these, one group includes yeast Tel1 and fruitfly ATM (also known as CG6535). These proteins are involved in DNA repair and

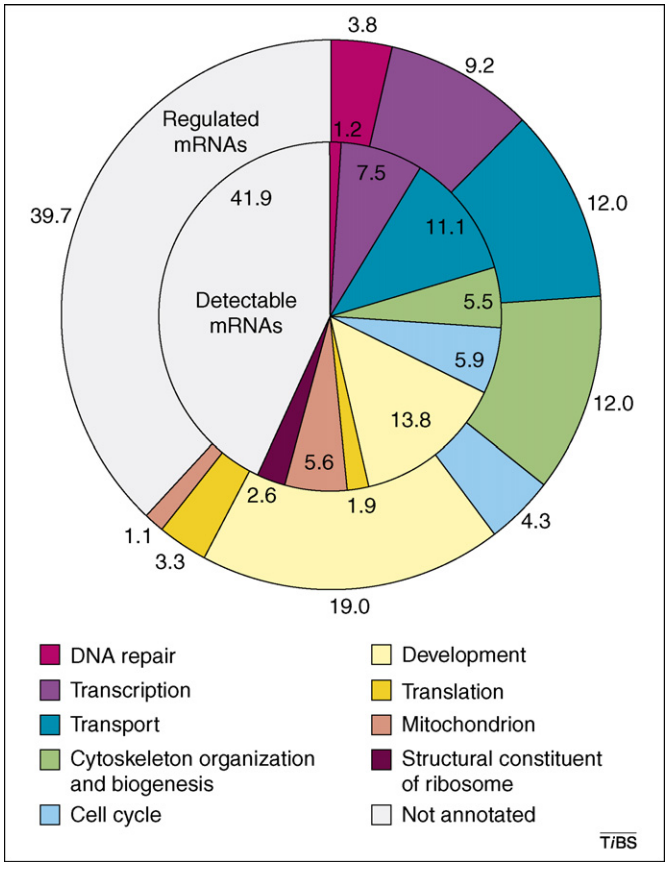

Figure 2. NMD regulates the expression of transcripts associated with diverse cellular processes. The percentage of genes associated with the indicated biological process or the cellular component ontology categories are given for all detectable transcripts (inner circle) and for NMD targets (outer circle) in fruitfly cells. A similar broad distribution of functional categories is observed among NMD targets in yeast and human cells. Figure was compiled with data from Ref. [8].

maintenance of telomere ends [49]. Finally, of the 93 KOGs represented among human targets and detectable in fruitfly cells, only 10 are regulated by NMD in fruitfly.

In summary, KOG analysis reveals that most NMD targets in fruitfly are not orthologs of genes regulated by NMD in yeast or human cells, indicating that the repertoire of genes targeted by NMD is not conserved. This lack of conservation might be explained by differences across species in the mechanism of PTC recognition. For example, human transcripts containing introns located at least 50 nucleotides downstream of the natural stop are degraded by NMD (Table 2); these transcripts are not substrates of the NMD pathway in yeast or fruitfly.

Conserved role of NMD in telomere maintenance and DNA repair

With few exceptions, NMD targets in yeast, fruitfly and human are not orthologous genes. Strikingly, the exceptions include mRNAs that encode orthologous proteins with roles in NMD and telomerase function such as human and fruitfly SMG5 and the yeast telomerase-associated proteins Ebs1p and Est1p. These proteins are clearly related and share a common domain organization [14,23].

Interestingly, in addition to *EBS1* and *EST1*, five genes involved in telomere maintenance are regulated by NMD in yeast: namely, *EST2*, *EST3, STN1, YKu70* and *TEL1* [6,50]. The regulation of genes involved in telomere maintenance by NMD has functional implications because, in yeast, inhibition of NMD results in telomere shortening and the derepression of silenced telomeric loci [50,51]. Furthermore, ATM (which is involved in telomere maintenance and DNA repair) is regulated by NMD in fruitfly [8]. This observation, together with recent reports implicating human UPF1, SMG5 and SMG6 in telomerase function [52,53,54], suggests that the role of NMD in regulating the expression of genes involved in telomerase function is conserved.

The role of NMD effectors in telomere maintenance and DNA repair seems to go beyond the indirect effects of the regulation of transcripts involved in these cellular processes [52–54]. Indeed, SMG5 and SMG6 are associated with telomerase in human cell extracts, and UPF1 associates with DNA polymerase δ, which is involved in DNA synthesis during replication and repair [52–54]. Furthermore, SMG1 is involved in cellular responses to DNA damage [55]. Further studies are required to elucidate the biological significance of these interactions and to determine whether these newly identified roles of NMD effectors are separable from their roles in the NMD pathway.

Functional diversification of the effectors of NMD

In human cells, Mendell *et al.* [7] have reported that UPF1 targets are also regulated by UPF2. By contrast, Gehring *et al.* [34] identified endogenous transcripts that are regulated by UPF1 but remain unaffected in cells depleted of UPF2. On the basis of this and additional observations, Gehring *et al.* [34] proposed the existence of parallel NMD pathways in human cells – namely, a UPF2-dependent and a UPF2-independent pathway [34]. This possibility would be in agreement with the analysis of expression profiles in human cells depleted of UPF2 [9]: it seems that UPF2 regulates only a few targets of UPF1 and vice versa [9].

The lack of genome-wide information on genes regulated by additional components of the NMD machinery in human cells leaves open the question of whether transcripts regulated exclusively by UPF1 or UPF2 represent NMD targets or instead reflect transcripts regulated by these proteins via mechanisms distinct from NMD, which would provide potential evidence for the functional diversification of these NMD effectors. The possibility that UPF1 and UPF2 might have partially non-overlapping functions is further supported by the observation that human cells depleted of UPF1 are arrested early in S phase, whereas depletion of UPF2 produces no apparent phenotype [9,54] (Table 1).

Some evidence suggests that UPF1 does indeed have additional roles. UPF1 can be recruited to the 3′ UTR of specific transcripts via interactions with Staufen1 (a double-stranded RNA-binding protein) and promotes mRNA degradation by a mechanism not requiring UPF2 or UPF3 [56]. Moreover, UPF1 is implicated in the degradation of histone mRNAs. In this case, UPF1 is recruited to the conserved stem-loop structure present at the 3′ end of histone mRNAs via interactions with the stem-loop-binding protein [57]. These observations raise the possibility that other RNA-binding proteins might function in a similar way.

In principle, the recruitment of any of the NMD effectors to a transcript by specific RNA-binding proteins could lead to mRNA decay in a process that might or might not require additional NMD components. The similarity of gene expression profiles in yeast and fruitfly cells depleted of NMD effectors indicates, however, that such a mechanism is not widespread in these organisms [6,8]. The contribution of these alternative pathways to post-transcriptional mRNA regulation in vertebrates will be elucidated only by comparing the gene expression profiles of human cells that have been individually depleted of all known NMD effectors. In addition, it remains possible that NMD effectors have acquired specialized functions that do not affect steady-state levels of mRNA. Indeed, the roles of UPF1, SMG1, SMG5 and SMG6 in DNA repair and telomerase function might not be discernable by microarray analysis.

Evolutionary diversification of the role of NMD

Components of the NMD pathway are not essential in yeast [10]. Similarly, inhibition of the NMD pathway in *C. elegans* leads to viable worms, albeit with defects in the male bursa and the hermaphrodite vulva [11,12]. Thus, NMD is not essential at the cellular level in yeast or worms (Table 1). By contrast, fruitfly cells depleted of UPF1 and UPF2 are arrested at the G2/M phase of the cell cycle, and cell proliferation is impaired by depletion of the other NMD effectors [8]. UPF1 is also required for cell viability in mice because *Upf1*-null mice die early in embryonic development and attempts to establish homozygous $Upf1^{-/-}$ embryonic stem cells have also failed [58]. Finally, depletion of UPF1, but not UPF2, from human cells inhibits progression of the cell cycle [9,54] (Table 1).

The identification of NMD targets in model organisms indicates that changes in the repertoire of genes regulated by NMD represent an important mechanism leading to phenotypic differences across species after inhibition of the NMD pathway. In addition to changes in targets, the different phenotypes can be explained by functional diversification of the NMD effectors.

Therapeutic relevance

Effectors of NMD are potential targets for therapeutic intervention aimed at regulating the expression of mutated genes [59,60]. Approximately 30% of inherited genetic disorders are caused by nonsense mutations or by frameshifts that generate nonsense codons. Transcripts derived from the mutant alleles are degraded by the NMD pathway, leading, in most cases, to a recessive mode of inheritance, because the wild-type protein product from the second functional allele can compensate, at least in part, for the absence of the nonsense-containing transcript. In some cases, the nonsense transcript escapes NMD, resulting in the production of a truncated protein that can lead to a dominant genetic defect. This phenomenon has been observed in the different forms of β-thalassemia, in which some mutations cause a recessive phenotype, whereas others result in dominantly inherited, severe disease owing to the accumulation and precipitation of insoluble globin chains [59,60]. Moreover, distinct phenotypes have been observed for the same mutation in different individuals, suggesting that NMD efficiency is subject to individual variation [59,60].

NMD can also protect an organism from the effects of acquired mutations, for example, during tumor progression. Transcripts of several mutant forms of the tumor suppressor proteins BRCA1, p53 and Wilms' tumor 1 (WT1) have been shown to be eliminated by NMD. These mutations would convert the tumor suppressors into dominant-negative oncoproteins if the transcripts were not eliminated by NMD. Indeed, it has been shown that expression of the corresponding truncated proteins from intron-less cDNAs, which are immune to NMD, leads to increased tumorogenicity [59–63]. Thus, NMD protects heterozygous carriers of these mutations from developing cancer.

In the examples mentioned above, increasing NMD efficiency would be beneficial. In others, however, loss of the protein product would be more deleterious to the cell than would expression of the truncated protein. This situation arises when the truncated protein does not have dominant-negative effects and is at least partially functional as occurs, for example, in some forms of cystic fibrosis. In this case, inhibition of NMD would be beneficial [59,60,64].

On the one hand, the results from microarray profiling are discouraging for the development of therapeutic strategies aimed at regulating NMD. Indeed, the observation that NMD regulates wild-type transcripts associated with a broad range of biological activities suggests that these strategies might be detrimental. On the other hand, NMD is not essential in yeast or worms. In mammals UPF1, but not UPF2, is required for cell viability. The possibility that parallel NMD pathways have evolved, and that not all NMD effectors have essential biological functions raises the potential for approaches targeting specific non-essential components of the NMD pathway.

Concluding remarks

Studies in model organisms such as yeast, worms and fruitfly have led to the identification of the effectors of the NMD pathway, but our understanding of the mechanisms by which premature and natural stops are discriminated remains at a rudimentary stage. Current evidence suggests that different mechanisms have evolved across species. In mammals, the position of the stop relative to the EJC has a crucial role, whereas in invertebrates it is the position of the stop relative to 3′ UTRs that is the primary determinant for triggering NMD. There are, however, examples in which PTCs are recognized in mammals in the absence of downstream EJCs [65–67]. Conversely, EJC components are present in most multicellular organisms. Whether these two mechanisms of NMD (i.e. EJC-dependent and 3′-UTR-dependent NMD) coexist in all eukaryotes, or whether EJC components have been co-opted only recently by the NMD machinery, remains to be determined. More studies are also needed to clarify whether alternative NMD pathways have indeed evolved in higher eukaryotes, and whether NMD effectors have additional functions.

Another important issue is whether distance from PABPC1 is the only determinant for defining a PTC in organisms in which exon–exon boundaries are thought not to have an essential role. If so, then why is it that not all mRNAs with exceptionally long 3′ UTRs are regulated by NMD? More generally, how has the increase in 3′ UTR lengths to accommodate regulatory elements and microRNA-binding sites in multicellular organisms emerged, despite the existence of a surveillance mechanism that targets transcripts with long 3′ UTRs for degradation? Have these 3′ UTRs acquired features that antagonize the NMD pathway? The current lack of mechanistic and comparative information makes it difficult to formulate a reasonable hypothesis about the impact of NMD on the evolution of eukaryotic 3′ UTRs.

Another important challenge for the future will be to investigate the consequences of perturbing NMD in animal models. In particular, it will be of great interest to analyze the phenotypes associated with specific NMD effectors to establish whether therapeutic approaches based on inhibiting specific NMD components are realistic.

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