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The meaning of nonsense

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To ensure the accuracy of gene expression, eukaryotes have evolved several surveillance mechanisms. One of the best-studied quality control mechanisms is nonsense-mediated mRNA decay (NMD), which recognizes and degrades transcripts harboring a premature translation-termination codon (PTC), thereby preventing the production of faulty proteins. NMD regulates ~10% of human mRNAs, and its physiological importance is manifested by the fact that ~30% of disease-associated mutations generate PTCs. Although different mechanisms of PTC recognition have been proposed for different species, recent studies in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, plants and mammals suggest a conserved model. Here, we summarize the latest results and discuss an emerging model for NMD and its implications for the regulation of gene expression.

Introduction

Eukaryotic gene expression involves an intricate chain of complex biochemical reactions, starting with the synthesis of mRNA, followed by the production of encoded proteins, and ending with the degradation of both the mRNA and the protein. Tight control of and high accuracy within these processes are absolutely required to prevent inappropriate gene expression and to ensure cell survival, and cells have therefore evolved mechanisms to control many steps along the chain [1]. One of the best-studied quality control mechanisms is nonsense-mediated mRNA decay (NMD). NMD was initially described as a mechanism for recognizing and degrading faulty transcripts harbouring a premature translation-termination codon (PTC); such nonsense transcripts would otherwise result in the production of C-terminally truncated proteins with potentially dominant–negative effects. PTCs can arise either from mutations at the DNA level (e.g. nonsense mutations, frame-shifting deletions and insertions) or from altered splicing signals that induce production of alternatively spliced mRNA iso-forms with truncated reading frames [2]. It has been estimated that among the 60–70% of pre-mRNAs that undergo alternative splicing, 45% generate at least one splice form predicted to be an NMD substrate [3]. Over the last five years, it has become clear that NMD not only degrades faulty transcripts but also regulates the steady-state level of many physiological mRNAs involved in a variety of different cellular processes, such as DNA repair, the cell cycle, and metabolism [4–6]. Genome-wide screens in budding yeast, *Drosophila* and human cells have revealed that NMD regulates expression of ~3–10% of the transcriptome [4–6]. Furthermore, it has been estimated that ~30% of the known disease-associated mutations in humans generate a PTC-containing (PTC+) mRNA, and in many of these cases NMD influences the severity of the clinical manifestations caused by the mutation [7].

Despite intense investigation over the past two decades, the molecular mechanisms of NMD are still not fully understood. Indeed, different models to explain PTC recognition have emerged from studies in different organisms. However, recent data from *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, plants and mammals suggest that the basic mechanism for PTC recognition is much more conserved than previously thought [8–16]. On the basis of these new data, we discuss a 'unified NMD model', implicating a novel post-transcriptional mode of gene regulation. Furthermore, we highlight the implications of this NMD model for the clinical manifestation of genetic diseases.

Trans-acting factors involved in NMD

The NMD core factors Upf1p, Upf2p and Upf3p (for "up-frameshift 1-3") were initially identified in genetic screens in yeast, and SMG1-7 (for "suppressor with morphological effect on genitalia") were found to be NMD effectors in C. elegans (for review, see [17]). Sequence alignments revealed that SMG2 is homologous to Upf1p, SMG3 to Upf2p, and SMG4 to Upf3p, and that SMG1–7 are present in all higher eukaryotes analyzed to date (Figure 1), with the exception of *D. melanogaster*, which contains no clear homolog for SMG7 [17]. Additional NMD factors are very likely to exist, although they remain to be discovered. Recently, *smgl1* (also known as hNAG) and *smgl2* (also known as hDHX34) have been identified as NMD factors in *C. elegans* and humans [11].

Given that they are conserved from yeast to humans, UPF1, UPF2 and UPF3 are believed to function at the heart of NMD. UPF1 is the most highly conserved NMD factor, and elucidating its structure and function will provide the key to understanding the mechanism of NMD. UPF1 interacts with the eukaryotic release factors eRF1 and eRF3 (see Glossary) [8,18–20], it binds to UPF2 through its cysteine and histidine rich (CH-rich) region near the N-terminus [21], and it interacts with SMG1, SMG5, SMG6 and SMG7 [19,22–27]. Furthermore,

Glossary

3′ UTR: Untranslated region downstream of the termination codon.
eRF1 and eRF3: Eukaryotic release factors 1 and 3, which function in translation termination.
P bodies: Processing bodies, also called GW182-containing bodies, DCP1 foci, or XRN1 foci.
PABPC1: the major cytoplasmic form of poly(A)-binding protein in mammals.

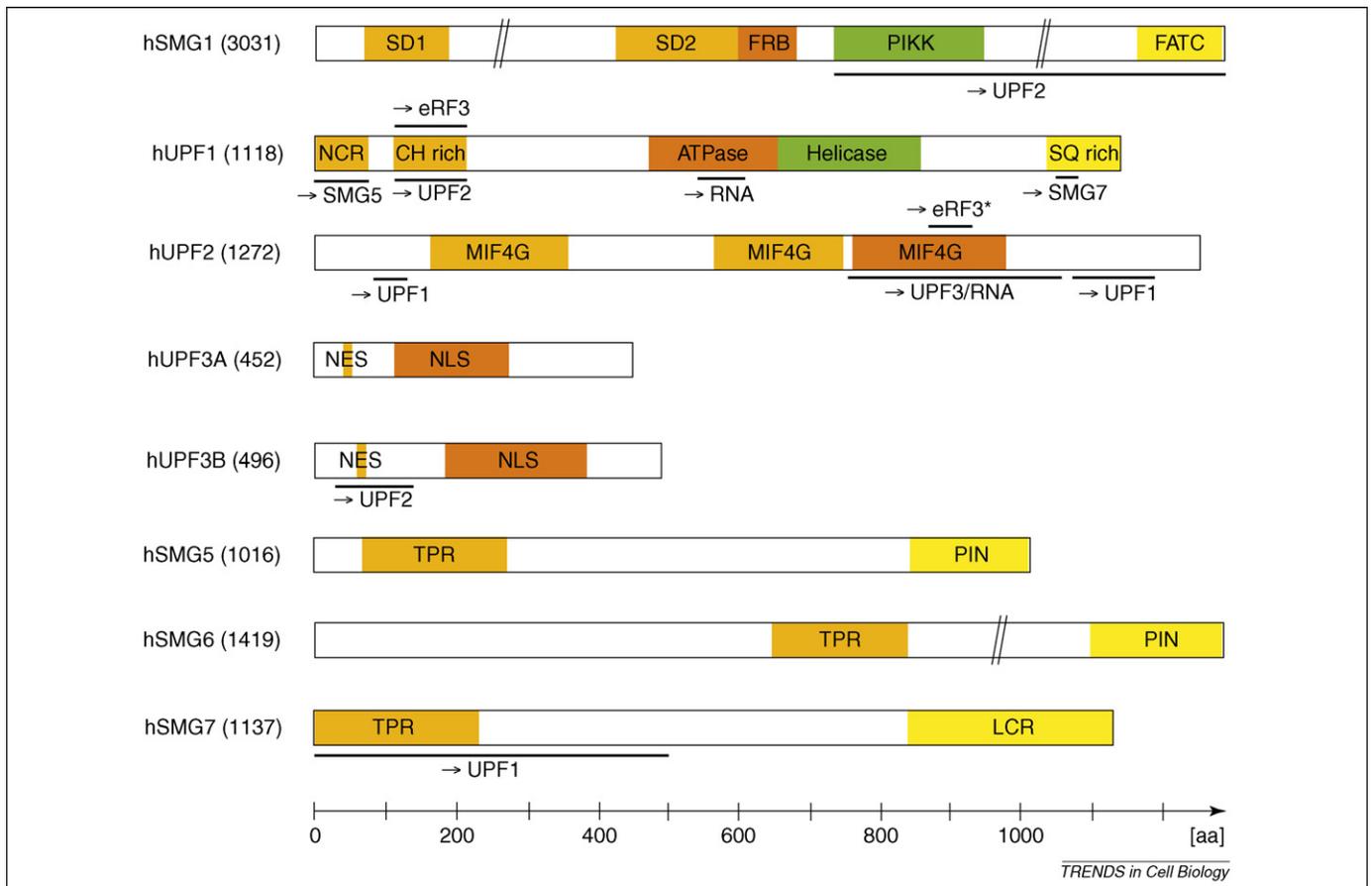*Corresponding author:* Mühlemann, O. (oliver.muehlemann@izb.unibe.ch).

Figure 1. Human NMD factors. The known human NMD factors are depicted schematically. The proteins are drawn to scale with respect to the number of amino acids that they have, indicated by the scale bar at the bottom. Direct interactions with other proteins or RNA are shown as black bars. Reported interactions for which the interaction domains are not yet mapped are not indicated (e.g. UPF1–SMG1, SMG5–SMG7, UPF1–SMG6 and UPF2–SMG1 [19,25,26]). Two SMG1 homology domains, SD1 and SD2, and an FKBP12-rapamycin binding site (FRB) have been mapped in hSMG1. hSMG1 also contains a FRAP–ATM–TRRAP C-terminal domain (FATC), which is found in the majority of PI3K-related kinases (PIKKs) [19,24,58]. hUPF1 contains an N-terminal conserved region (NCR), a CH-rich domain, a core domain comprising the ATPase and helicase, and an SQ-rich C-terminal domain [8,19,21,26,31,32]. hUPF2 contains three mammalian eIF4G-like (MIF4G) domains [19,20,32,59]. Regions containing nuclear export signals (NESs) and nuclear localization signals (NLSs) were found in hUPF3A and hUFP3B [20,23,59]. hSMG5, hSMG6 and hSMG7 contain two tetratricopeptide repeats (TPRs), which were shown to adopt a 14–3-3-like fold in hSMG7 [27]. The sequence similarity between the TPRs of hSMG5 and hSMG6 suggests that they also probably fold in a 14–3-3-like manner and bind to phosphorylated UPF1. hSMG5 and hSMG6 contain a C-terminal PIN domain (for PilT N terminus), which is present in proteins that have ribonuclease activity; a C-terminal low complexity region (LCR) was mapped in hSMG7, and this region is sufficient for the localization of the protein to P bodies and for its subsequent degradation, when tethered to a reporter mRNA [25–27,37,49,60]. The asterisk indicates regions that have only been mapped in *S. cerevisiae*.

UPF1 undergoes a cycle of phosphorylation and dephosphorylation, which is essential for NMD in metazoans and is regulated by the other NMD factors. SMG1 phosphorylates UPF1 at numerous serine residues in the C terminus [19,24]. This UPF1 phosphorylation was reported to depend on the presence of both UPF2 and UPF3b in humans [19], although recent studies provided evidence for UPF2- and UPF3b-independent NMD [8,28,29]. Phosphorylated UPF1 can be bound and dephosphorylated by SMG5, SMG6 and SMG7, all of which harbor two consensus tetratricopeptide repeat sequence motifs (TPRs) and recruit the phosphatase PP2A to UPF1 [22,25–27]. UPF1 exhibits RNA binding, RNA-dependent ATP hydrolysis, and 5′-to-3′ ATP-dependent RNA helicase activities, and inhibition of any of these activities suppresses NMD [19,30,31]. It has been shown *in vitro* that UPF1 dissociates from RNA after addition of ATP [30]. Interaction with eRF1 and eRF3 strongly reduces the ATPase and RNA binding activities of UPF1 [18]. Vice versa, RNA binding stimulates the ATPase activity and leads to a dissociation of UPF1 from eRF1 and eRF3. Furthermore, it was recently demonstrated that UPF2

and UPF3b cooperatively stimulate the ATPase and RNA helicase activity of UPF1 *in vitro* [32].

Proper versus aberrant translation termination
A central question to understand NMD is how a PTC can be distinguished from a natural termination codon (TC). Despite the conservation of the core NMD factors, remarkably different models have been proposed for different organisms [2,33,34]. In mammalian cells, the presence of an exon junction complex (EJC) downstream of the termination codon was found to be a crucial determinant for defining the termination codon as being premature (for review, see [2,34]). However, this model has been challenged by reports that NMD occurs even in the absence of a downstream EJC in mammals [15,16,35,36], and that NMD occurs independently of splicing in D. melanogaster and C. elegans, suggesting that the EJC is not involved in NMD in worms and flies [11,37].

An alternative model for PTC recognition invokes a 'faux 3′ untranslated region' (UTR; see Glossary). This model postulates that proper (or efficient) translation
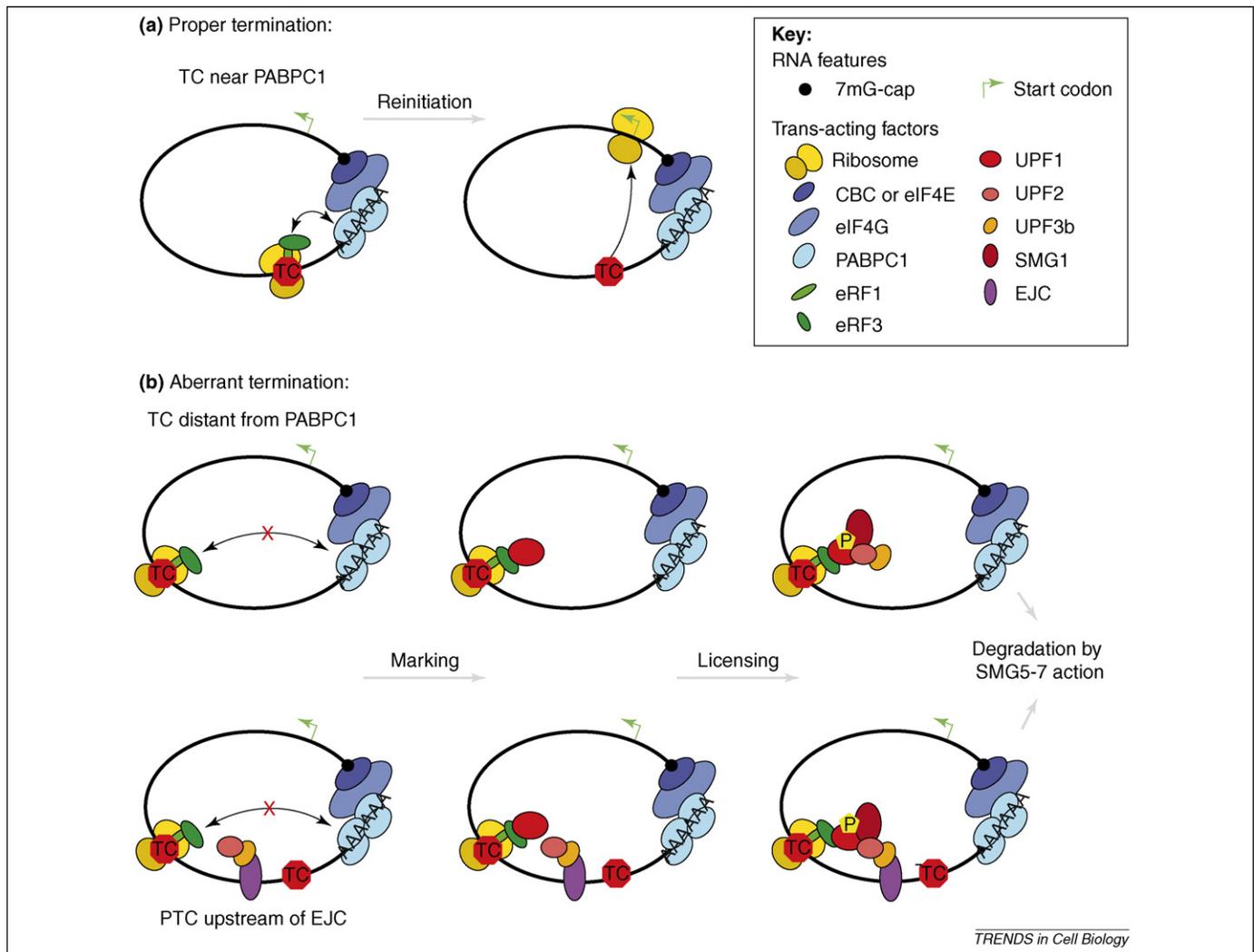
Figure 2. Model for PTC recognition. **(a)** The mRNP is thought to form a closed-loop structure through the interaction of cap-bound eIF4E or CBC (for ''cap-binding complex'') with eIF4G, which in turn interacts with the poly(A) tail-bound PABPC1. When the ribosome terminates at a TC in the vicinity of the poly(A) tail, a PABPC1-mediated signal promotes proper termination of translation, resulting in efficient reinitiation of the ribosome at the 5′ end of the mRNA, and the production of a stable mRNP. **(b)** If the ribosome terminates at a TC that is too far away from the poly(A) tail for it receive the PABPC1-mediated translation-termination-promoting signal, UPF1 binds to the stalled ribosome instead, thereby marking this TC as premature. Subsequently, UPF2 and UPF3b interact with UPF1, promoting SMG1-mediated phosphorylation of UPF1. This licensing step commits the mRNA to rapid degradation by as yet unknown pathways that involve the binding of SMG5–7 to the phosphorylated UPF1 (upper part). An EJC downstream of a TC functions as an NMD enhancer by shortening the time window between UPF1 binding and its phosphorylation by UPF2 and UPF3b (lower part).

termination requires a termination-promoting signal (Figure 2a), and that the absence of this signal typifies aberrant translation-termination at a PTC, which in turn leads to degradation of the mRNA (Figure 2b) [10,33]. Consistently, evidence for kinetic and mechanistic differences between aberrant and normal translation termination events has been obtained in *S. cerevisiae* [10]. Interestingly, tethering of poly(A) binding protein (Pab1p) into the proximity of a PTC suppressed NMD, suggesting that Pab1p or some factor bound to Pab1p might transmit the signal required for proper termination of translation [10]. Although the exact molecular events of translation termination remain to be elucidated, it seems that proper termination requires a certain mRNP structure and specific factors to promote efficient polypeptide release, ribosome disassembly, and recycling of the ribosome subunits to the 5′ end of the mRNA [10,33]. We speculate that the closed-loop structure of an mRNP, adopted by

juxtaposing the 5′ and 3′ ends through the eIF4E–eIF4G–Pab1p interaction, might represent such a structural environment for proper termination of translation [38]. The yeast data suggest that, if termination occurs too far away from this mRNP environment, disassembly of the ribosome is slow [10] and, as a result, Upf1p is recruited to the terminating ribosome through interaction with Sup35p and Sup45p, the yeast homologues of eRF3 and eRF1, respectively [18,33,39].

An evolutionarily conserved model for PTC recognition

Consistent with the *faux* 3′ UTR model, deletions that eliminate most of the sequence downstream of a PTC abolish NMD [36]. Furthermore, mRNAs with a long 3′ UTR were identified as being NMD substrates in *S. cerevisiae*, *D. melanogaster*, *C. elegans*, *Arabidopsis thaliana* and humans [9,11–13,15,16,36,40]. Recent studies in *D.*

melanogaster and humans demonstrate that tethering poly(A) binding protein (PABP) downstream of, but close to, the PTC suppresses NMD. This finding, also previously observed in yeast, further corroborates the idea of a common, evolutionarily conserved mechanism for PTC recognition [8–10,14–16]. Based on these recent reports from different organisms, we propose here a 'unified' model for PTC recognition. This model essentially extends the *faux* 3′ UTR model to all species and proposes that, during mammalian NMD, downstream EJCs act as an evolutionary adaptation to efficiently recognize nonsense mRNAs produced by extensive alternative splicing. Our laboratory recently showed that the physical distance, rather than the number of nucleotides, between a TC and the poly(A) tail is a crucial determinant in defining a TC as premature [15]. Specifically, NMD of PTC-containing immunoglobulin-μ reporter transcripts expressed in human cells was suppressed by bringing the poly(A) tail into the vicinity of the PTC by means of a secondary structure. Furthermore, it was shown that UPF1 and PABPC1 (see Glossary), the major cytoplasmic PABP, compete for the interaction with eRF3 in human cells *in vitro* [16]. In our study, we found that the extent of NMD suppression in the fold-back constructs gradually declines with increasing distance between the poly(A) tail and the TC. This is consistent with the notion that the balance of the competition between PABPC1 and UPF1 for interaction with eRF3 enables the cell to distinguish between correct and aberrant translation termination [15]. Thus, if a ribosome stalls at a TC that is too far away from the termination-promoting environment (i.e. distant from PABPC1), resulting in slow termination kinetics, the balance between the two antagonizing signals is tilted toward UPF1 binding ('marking' within Figure 2b). Notably, binding of UPF1 to the stalled ribosome in this context is EJC-independent. Based on data from *S. cerevisiae, C. elegans, D. melanogaster, A. thaliana* and humans, we propose that this marking of the aberrant mRNP by UPF1 is conserved among eukaryotes and represents the fundamental step in PTC recognition.

Different second signals have evolved
Although UPF1 has been found to preferentially associate with PTC-containing mRNA in *C. elegans*, some association with PTC-free mRNA was also observed [41]. Furthermore, in *S. cerevisiae*, Upf1p can target normal mRNAs to P bodies (see Glossary; for review, see [42]) without promoting the degradation of these mRNAs [43]. This suggests that simple binding of UPF1 to a terminating ribosome is not sufficient to elicit degradation, but rather a 'second signal' is required. The second signal might be the binding of UPF2 and UPF3 to UPF1, an event that is important for the SMG1-mediated phosphorylation of UPF1 in higher eukaryotes [19,44], and which stimulates the RNA helicase and ATPase activities of UPF1 [19,32]. As a consequence of ATP hydrolysis, binding of UPF1 to the RNA might be facilitated. Supporting this view, a UPF1 mutant that cannot interact with UPF2 was shown to accumulate in its unphosphorylated form in a complex with SMG1, eRF1 and eRF3 [19]. Furthermore, ATPase-defective UPF1 mutants show enriched co-immunoprecipitation with UPF2 and UPF3b

in mammalian cells [19]. In summary, we postulate that the phosphorylation of UPF1 and the stimulation of its ATP hydrolysis and helicase activity represent a 'point of no return' in the NMD pathway, and we define this as the 'licensing step' (Figure 2b). In contrast to the situation in mammals, ATP hydrolysis appears to be required for the recruitment of Upf2p and Upf3p after Upf1p-dependent targeting of the mRNAs to P bodies in yeast [43]. In conjunction with the absence of SMG1, SMG5, SMG6 and SMG7 homologs in *S. cerevisiae*, we suggest that the NMD pathway for *S. cerevisiae* diverges from the pathways of metazoans at this licensing step.

Degradation of UPF1-bound mRNA
After the licensing step in metazoans, SMG5, SMG6 and SMG7 bind to the phosphorylated UPF1 through their 14–3-3-like domain, leading to the degradation of the mRNA (Figure 2b) [25–27]. However, the exact molecular relationship between UPF1 and SMG5–7 is still unclear.

In yeast and mammals, nonsense transcripts appear to be degraded by exosome-mediated 3′–5′ decay and by decapping followed by XRN1-mediated (for "5′-3′ exonuclease 1") 5′–3′ decay [45,46]. Several interactions between NMD and mRNA decay factors have been mapped consistently. For example, UPF1 interacts with the decapping enzyme in *S. cerevisiae* and human cells [47]. In *D. melanogaster*, however, the degradation of PTC-containing transcripts is initiated by endonucleolytic cleavage, and the resulting cleavage-fragments are subsequently degraded by 5′–3′ and 3′–5′ exonucleases [48].

The cellular localization of the degradation is not yet clear. Recent studies have shown that P bodies are sites of NMD in *S. cerevisiae* [43]. P bodies are implicated in cellular degradation in mammalian cells too, because they contain decapping and degradation enzymes, NMD factors, and effectors of the RNA interference (RNAi) silencing pathway (for review, see [42]). SMG7 might provide the molecular link between NMD and the degradation machinery in mammalian cells. When overexpressed, SMG7 accumulates in P bodies, which also leads to accumulation of SMG5 and UPF1 there. Furthermore, when SMG7 is tethered to a reporter transcript, it is able to elicit mRNA decay independent of a PTC [49].

EJC has evolved as an enhancer of NMD in mammals
Contrary to the popular model for mammalian NMD, several studies have demonstrated that PTCs can trigger NMD in the absence of an EJC further downstream on the mRNA [15,16,34,36,50,51]. However, it is apparent that the extent of mRNA downregulation in these examples of EJC-independent NMD is lower than in corresponding examples of NMD of transcripts with EJCs in the 3′ UTR. Consistent with the idea that EJCs have an important role in NMD, knockdown of EJC core-factors in mammalian cells reduced the downregulation of many NMD reporter mRNAs [36,52–54]. In the light of this, we propose that, in mammals, the EJC has evolved as a specialized second signal to enhance mammalian NMD. Our unified NMD model provides a mechanistic explanation for the NMD-enhancing function of EJCs located downstream of a TC. As part of such a 3′ UTR-bound EJC, UPF2 and UPF3

are ideally positioned for immediate interaction with ribosome-bound UPF1 and SMG1. As a consequence, the time window between the binding of UPF1 to the terminating ribosome (the marking step) and its SMG1-mediated phosphorylation (the licensing step) would be shortened, and thus the competition between PABP and UPF1 for binding to the stalled ribosome would tilt toward NMD (Figure 2b). We hypothesize that, in mammals, under the evolutionary pressure to efficiently recognize and eliminate the large number of nonsense mRNAs produced by extensive alternative pre-mRNA splicing, the EJC as a spatial mark of previous splicing events has been incorporated into the mechanism of PTC recognition as an enhancer. Consistent with this view, proteins homologous to mammalian EJC core components are not involved in NMD in *D. melanogaster* and *C. elegans* [11,37], in which only a minor fraction of pre-mRNA is alternatively spliced. Notably, downstream sequence elements (DSEs) identified in *S. cerevisiae* might have NMD-enhancing functions similar to that of the EJC, by providing a binding platform for NMD enhancing factors [55].

NMD as a novel mode of translation-dependent post-transcriptional gene regulation

3′ UTRs, which in mammals can comprise several thousand nucleotides, contain binding sites for numerous factors that are known to regulate mRNA translation or stability [1]. As mentioned previously, we recently found that, by changing the spatial configuration of the 3′ UTR of a transcript by introducing intramolecular base pairing, the half-life of the transcript can be changed in an NMD-dependent manner. This suggests that NMD has a novel role as a post-transcriptional mechanism for gene regulation (Figure 3) [15]. We predict that many RNA-binding

factors alter the tertiary structure of the target transcript. Such structural rearrangements can, for example, change the physical distance between the TC and the poly(A) tail, and therewith change the local environment for translation termination; this in turn would affect the half-life of the mRNA and, as a consequence, have an effect at the protein level. RNA-binding factors can be either proteins or RNAs, and they can alter the 3-dimensional configuration of the 3′ UTR by masking mRNA sequences otherwise engaged in intramolecular base pairing, or by interacting with each other and thereby looping out mRNA sequences in-between. Importantly, such protein–protein and protein–RNA interactions can be regulated by environmental cues through signal transduction pathways that modify the involved RNA-binding proteins, for example by phosphorylation. In addition, transcript-specific RNA-binding proteins with intrinsic NMD-promoting or translation termination-promoting activities could directly modulate mRNA stability by binding near to the TC. To our knowledge, no physiological examples regulated by spatial remodeling of the 3′ UTR have been reported to date; however, there are likely to be many, because microarray analysis has revealed a surprisingly large number of physiological transcripts that rise in levels upon UPF1 knockdown [4–6,56]. Our NMD-mediated gene regulation model predicts that ongoing translation is required and that the population of transcripts affected by UPF1 depletion varies in a tissue-specific manner, during development and differentiation, and by environmental cues in general. Although experimental differences cannot be excluded, this might at least in part explain why the sets of transcripts affected by UPF1 depletion in the different microarray studies overlapped only very little [4–6,56,57]. Clearly, future work is needed to test the biological relevance of this model.
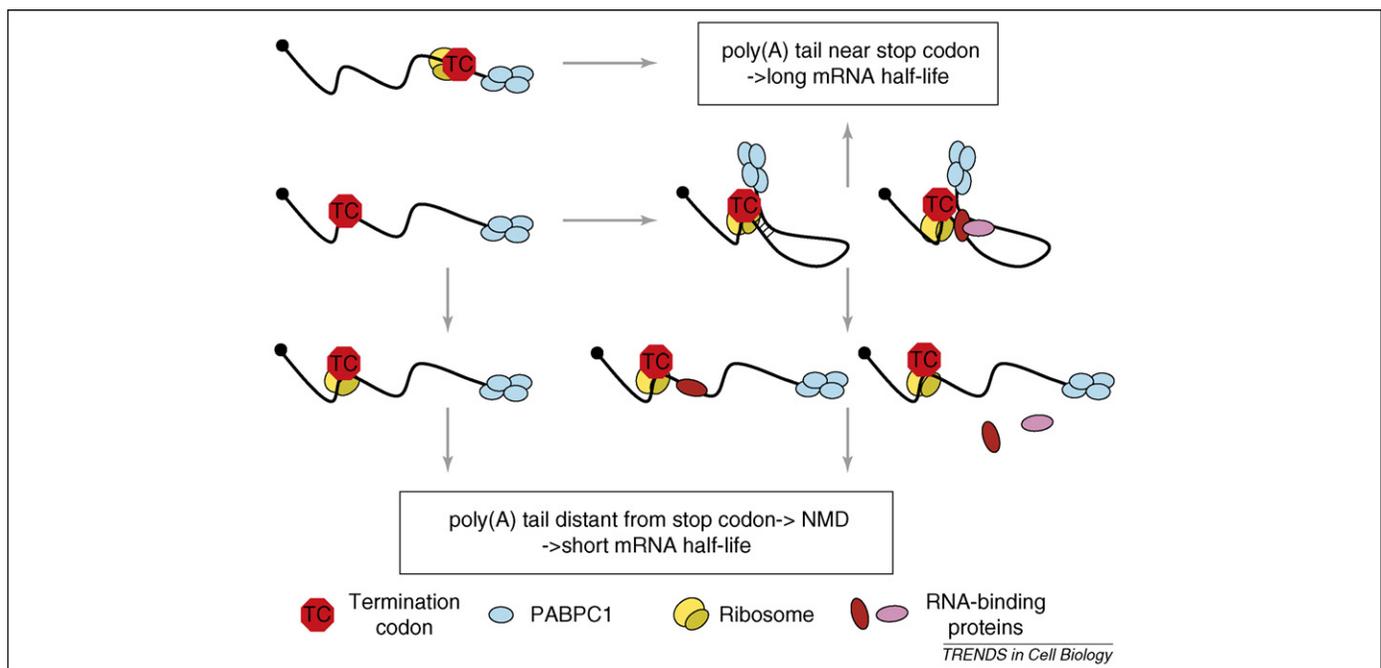

Figure 3. Post-transcriptional gene regulation by spatial rearrangement of the 3′ UTR. Many mammalian 3′ UTRs comprise thousands of nucleotides. They are most probably highly structured, and they provide binding sites for RNA binding proteins. We postulate that, by modulating the 3-dimensional configuration of the 3′ UTR and thereby the local environment for translation termination, the stability of mRNAs can be regulated in a translation-dependent manner. Such structural rearrangements can be mediated, for example, by RNA-binding factors that mask mRNA sequences that are otherwise involved in intramolecular base pairing, or by interacting with each other (Figure adapted from [15]).

Genetic diseases: implications of the unified NMD model

It has been estimated that ~30% of the known disease-associated mutations generate a PTC, which suggests that NMD has a widespread impact on the phenotype of numerous genetic diseases [7]. NMD is beneficial if it prevents the production of C-terminally truncated proteins that would have had dominant–negative effects. By contrast, NMD is detrimental if it prevents the production of truncated proteins that still have residual function, as has been described for frequent mutations causing cystic fibrosis [7]. In addition to the existence of these mutations in the open reading frame of a gene, the unified NMD model predicts the existence of more populations of NMD targets than were previously appreciated. On the one hand, many PTCs in the last exon have the potential to elicit NMD, especially when the last exon is long. On the other hand, various mutations in the 3′ UTR have the potential to alter the spatial relationship between the TC and the poly(A) tail: insertions into the 3′ UTR; mutations that destroy poly(A) sites or create cryptic ones; and modification of binding sites for RNA binding proteins. Any of these types of mutations could turn an mRNA into an NMD target. Collectively, this suggests that the total number of genetic diseases influenced by NMD has been underestimated.

Conclusions and open questions

Although NMD has been investigated intensively for at least 20 years, the molecular mechanisms are not yet fully understood. Here, we summarize some recent publications showing that proper translation termination requires an interaction of the terminating ribosome with poly(A) binding protein, that the physical distance between the TC and the poly(A) tail is a crucial determinant for recognition of PTCs, and that PAPBC1 and UPF1 compete for the interaction with eRF3. Accordingly, when a ribosome stalls at a PTC, it lacks the PABP-mediated termination-promoting signals, and therefore UPF1 can out-compete PABP for the interaction with eRF3. This unified NMD model is consistent with recent findings in the other eukaryotes and suggests a conservation of the basic mechanism of PTC recognition. According to this new view, spatial remodeling of the 3′ UTR provides a novel mechanism whereby cells can regulate gene expression post-transcriptionally and in a translation-dependent manner. Although physiological examples regulated by this mechanism have not been reported to date, it is consistent with the finding that NMD not only rids the cell of faulty transcripts but also is involved in the regulation of 'normal' transcripts. The mechanisms proposed in this review could explain why NMD regulates such a large number of transcripts, many of which would not be predicted to be NMD substrates according to the currently prevailing model. Furthermore, it provides a possible explanation for the poor overlap between the sets of NMD-regulated transcripts in different microarray studies on hUPF1-depleted cells, for the varying efficiency of NMD on different substrates, and for the physiological role of NMD. Finally, the unified NMD model predicts the existence of a population of NMD targets that, to date, has not been appreciated and which might be clinically important.

One important aspect of future research will be to test whether the postulated mode of gene regulation by spatial rearrangement of the 3′ UTR occurs *in vivo* under normal physiological conditions, and whether it is widespread among different tissues and cell types. Furthermore, the mechanistic details of PTC recognition and the steps leading to the degradation of the mRNA are still largely unknown. More structural and biochemical data of the factors and complexes involved in NMD will be required to finally understand the molecular mechanism of NMD.

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