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Unusual SMG suspects recruit degradation enzymes in nonsense-mediated mRNA decay

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Abstract

Degradation of eukaryotic RNAs that contain premature termination codons (PTC) during nonsense-mediated mRNA decay (NMD) is initiated by RNA decapping or endonucleolytic cleavage driven by conserved factors. Models for NMD mechanisms, including recognition of PTCs or the timing and role of protein phosphorylation for RNA degradation are challenged by new results. For example, the depletion of the SMG5/7 heterodimer, thought to activate RNA degradation by decapping, leads to a phenotype showing a defect of endonucleolytic activity of NMD complexes. This phenotype is not correlated to a decreased binding of the endonuclease SMG6 with the core NMD factor UPF1, suggesting that it is the result of an imbalance between active (e.g., in polysomes) and inactive (e.g., in RNA-protein condensates) states of NMD complexes. Such imbalance between multiple complexes is not restricted to NMD and should be taken into account when establishing causal links between gene function perturbation and observed phenotypes.

KEYWORDS
NMD, premature termination codon, RNA degradation, RNA translation, RNA-protein complexes

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is a conserved degradation pathway initiated by translation termination at premature termination codons (PTC). PTCs are abundant in the transcriptome of eukaryotic cells and are present also, for example, in viral-related transcripts.[1–3] In addition to limiting viral replication, NMD also cleans up the transcriptome from numerous RNA isoforms synthesized with unusual transcription initiation or termination sites, e.g.,[4] or by alternative or inefficient splicing ([5] for a review). Due to its ancient origin and presence in most eukaryotes, NMD participates to the regulation of gene expression for processes requiring rapid RNA turnover, such as embryo development.[6]

Despite the conservation of NMD factors, the proposed molecular mechanisms of NMD are surprisingly diverse (reviewed by[7,8]). This divergence of mechanisms can be real, but some of it also comes from technical limitations of the experiments used to study NMD in yeast, worm, plant or human cells. RNA sequencing, the use of a variety of new reporter systems and improved genetic manipulation tools are affecting the picture of NMD factors and mechanisms. Some of the new results, discussed here, challenge established NMD models about the order of recruitment of late NMD factors and their role in triggering RNA degradation. They also suggest, for example, that affecting the balance of distinct NMD complexes by depletion of an NMD protein can indirectly affect NMD because factors critical for RNA degradation are present in low, limiting amounts in cells.[9]

Since NMD mechanism affect host pathogen interactions, tumor progression or monogenic disease potential treatments ,e.g., cystic fibrosis mutations,[10] they are of interest for a broad category of scientists. It is thus important to point out new results and how they challenge previously proposed models, explored in depth in a recent review.[7] The available data challenges established views, including the role of NMD factors in translation termination, the redundancy of RNA degradation pathways in NMD and the importance of protein phosphorylation in the dynamics of NMD complexes. Fresh data should lead to an adjustment of the accepted views on NMD mechanisms. This

should be similar to how past ideas, such as the nuclear localization of NMD or its restriction to the first round of mRNA translation, were invalidated by later studies.[11–14]

We start by presenting the diversity of RNA molecules degraded through NMD and why cytoplasmic noncoding RNAs are excellent NMD substrates. Next, we provide a brief overview of NMD factors conserved in eukaryotes and present a compilation of data on the protein-protein interactions in this pathway. Finally, once the context is set up, we discuss recent results about how RNA degradation is initiated during NMD, specifically the role of the SMG5/7 heterodimer. These results suggest that phenotypes of cells in which NMD factors were depleted can be interpreted in terms of an imbalance between NMD complexes active or inactive for RNA degradation. Similar considerations probably apply to other processes involving populations of RNA and DNA molecules, such as chromatin modification, DNA repair, DNA transcription or RNA splicing and intracellular transport.

TRANSLATION OF NONCODING RNAS INITIATES THEIR DEGRADATION THROUGH NMD

Knowing what makes an RNA unstable through NMD is essential to understand the origin and diversity of this class of transcripts. Two complementary strategies identified NMD substrates, one measured changes in cellular RNA levels and another tested artificial reporter molecules in cells in which NMD was removed. The obtained results were also correlated with data on the physical association of NMD factors to both reporters and cellular transcripts, for example.[15–19] These studies allowed a better description of PTCs, considered as the defining elements of NMD-sensitive RNAs. The idea of a PTC originated from initial studies in which inclusion of a stop codon upstream the normal translation termination signal led to degradation of a reporter mRNA. The PTC denomination can be misleading when there is no obvious coding sequence present in an RNA. This is the case for thousands of noncoding RNAs that are produced in most eukaryotic cells. Noncoding RNAs lack a long open reading frame and, most often, are not conserved among eukaryotes.[20] However, if they reach the cytosol, random short open reading frames present in their sequence are translated,[21] making noncoding RNAs excellent targets for NMD, for example.[4] In yeast, such noncoding RNA sensitive to NMD include several populations of RNAs issued from pervasive transcription. A class of these transcripts was named SUT, for stable unannotated transcripts,[22] but the "stable" refers only to the fact that the SUT levels are generally unaffected when nuclear RNA degradation is defective. The SUT population of transcripts partially overlaps with RNAs observed to accumulate in the absence of the major 5′–3′ exonuclease XRN1.[23] Since XRN1 is responsible for the degradation of RNAs recognized by the NMD machinery, pervasive transcripts escaping the nucleus are an important source of NMD substrates in yeast.[24]

Thus, situations that create PTCs, initially considered to be restricted to rare cases of nonsense point mutations or infrequent splicing defects, are diverse and exist for many different types of transcripts, as depicted in Figure 1A. They all have in common the presence, downstream of the PTC, of a long untranslated RNA region or of an exon-exon junction at more than 50 nucleotides from the stop.[25,26] While the presence of a long 3′ UTR region drives RNA instability through NMD in all eukaryotes, downstream exon-exon junctions and the associated exon junction complex (EJC) enhance NMD only in some organisms, where it might have evolved as a supplementary control mechanism (for a discussion on NMD evolution see [27]). Similar to how a long 3′ UTR does not automatically lead to instability of RNA through NMD in yeast,[28] the presence of an exon junction downstream a stop codon may not be followed by instability of a reporter mRNA in human cells.[29]

In addition to noncoding RNAs, a surprising source of NMD substrates was found when RNA sequencing methods that could differentiate between alternative 5′ ends of transcripts became available. For example, stop codons that function as PTCs are frequently added to transcripts since RNA polymerase II is promiscuous about start site selection. RNA isoforms generated by transcription that begins upstream, and even downstream, the canonical start, can contain short open reading frames (uORFs) that end in PTCs.[4,35] Start codons on such RNAs are followed by randomly positioned stops, usually close to the transcript end. The RNA region situated downstream such translation termination is a potentially long 3′ UTR. The short coding sequence followed by a long 3′ UTR is typical for NMD substrates that are efficiently degraded in eukaryotes. Of note, alternative transcription termination sites, downstream the canonical ones, also produce RNAs with longer 3′ UTRs.

Being an excellent NMD substrate is not necessarily a bad thing. Pro-NMD features can even be selected and are desirable to avoid the production of truncated proteins, toxic through aggregation, for example.[36] A nice example of the imprint of NMD on evolution of genome sequence is the analysis of the frequency by which intron retention occurs in transcripts of the ciliate *Paramecium tetraurelia*. An advantage of this organism is that its introns are very short, between 20 and 35 nucleotides, making intron retention identification by sequencing straightforward. In line with the idea that NMD plays an important error correction function but needs a PTC to be effective, retained introns show an excess of in-frame stop codons.[37] NMD is thus intimately linked with splicing efficiency and imposes constraints on genome sequence evolution.[38]

Intriguingly, when no uORF or no long 3′ UTR are present, cellular RNAs can still be subject to premature termination leading to NMD due to a ribosomal frame shift long before reaching the canonical stop codon (Figure 1A). In this case, the frame shifting ribosome encounters a random stop codon, temporarily transforming a region of the annotated coding sequence in a 3′ UTR, thus creating conditions that are favorable for NMD.[39] The frequency at which such frame shifts followed by NMD occur is, for the moment, unknown.

VIRUSES FIGHT NMD

The SARS-CoV2 subgenomic RNA that serves as messenger for the synthesis of the structural viral protein spike (S) has a 3′ UTR regions

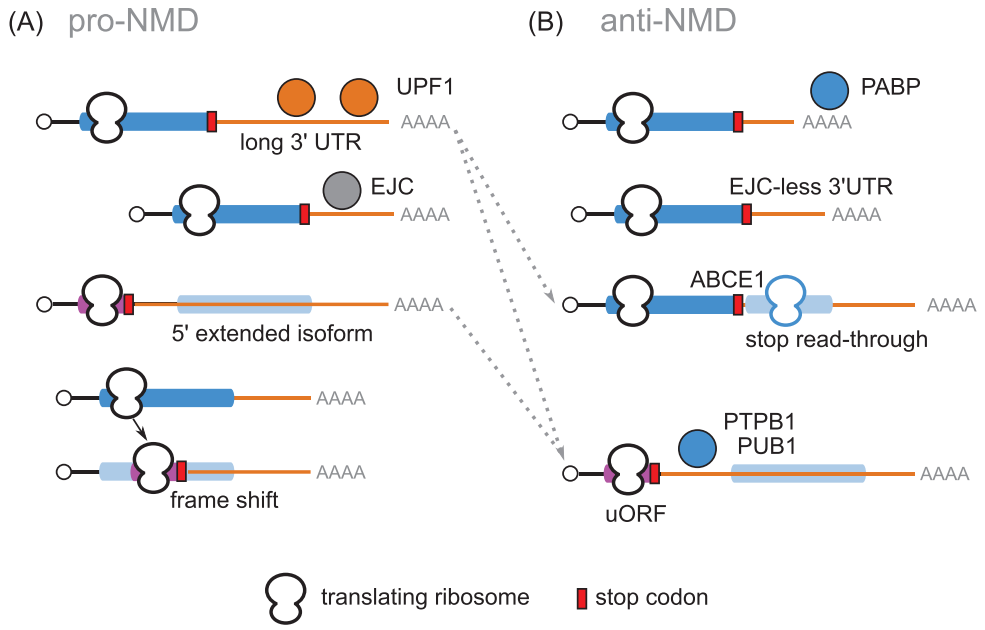

FIGURE 1 Configuration and origin of NMD substrates. (A) Conditions that favor degradation of RNA through NMD include the presence of a long 3′ UTR (due to transcription termination or to the inclusion of an early stop codon via defective splicing), of an exon junction downstream the stop codon, of short upstream ORFs, or ribosome frame shifts. (B) Conditions that allow immunity to NMD in specific cases include the opposite of those favoring NMD, such as a short 3′ UTR or lack of an exon exon junction downstream the stop codon, but also binding of specific factors or stop codon read-through. Arrows indicate relationships between various scenarios, depending on the origin of the NMD signal. Other proteins indicated and involved in NMD are the poly(A) binding protein (PABP), the exon junction complex (EJC),[30] the ABCE1 factor[31,32] affecting translation termination, or RNA binding PTPB1, in mammals [33] and PUB1, in yeast.[34]

with a length of about 5000 nucleotides.[40] Compared with cellular mRNA, with a median length of the 3′ UTRs of about 500 nucleotides, such a long 3′ UTR might induce RNA degradation through NMD. Whether subgenomic RNA instability is a limiting factor for SARS-CoV2 replication is unclear, but inactivation of NMD benefits the related murine hepatitis virus, at least in cell culture.[41] Thus, viral proteins that inhibit NMD mechanisms are likely to benefit viral replication. This hypothesis has received experimental evidence from studies done, for example, on human and animal coronaviruses, hepatitis C virus, West Nile, Dengue, Semliki Forest, Zika or human T-lymphotrophic type 1, viruses (reviewed in [1–3]).

Viral infections are not the only situations in which inhibition of NMD occurs. Anti-NMD features can be present in the architecture of many transcripts, as depicted in Figure 1B. Improved gene expression through immunity to NMD occurs in the absence of introns in the 3′ UTR region, through progressive loss of upstream open reading frames, to avoid uORFs, or by preferential use of early transcription termination sites for shorter 3′ UTR regions. When uORFs are essential parts of translation regulation, as in the case of the GCN4 transcription factor,[42] recruitment of anti-NMD factors ensures stability of the RNA. In the case of GCN4, it is not clear how the RNA binding protein Pub1 protects the RNA from NMD in yeast.[34] A reasonable hypothesis is that PUB1 interferes with translation termination at the potential PTC, leading to increased read-through of the stop codon of the uORF with removal of destabilizing elements from downstream region. Alternatively, Pub1 could also directly affect the binding of the NMD factor UPF1 to the RNA. Since the presence of UPF1 on 3′ UTRs correlates with degradation of the RNA through NMD,[15,16] its removal could inhibit NMD. This type of mechanism has been proposed for the NMD inhibition effects of RNA binding proteins PTBP1[33,43] and hnRNP L[44] in mammals.

Since various RNA features can lead to immunization against NMD, they can also give clues about how a PTC is detected as an RNA destabilization feature. A short 3′ UTR, for example, leads to the proximity of the stop codon to the poly(A) binding protein, Pab1 in yeast,[45] or PABPC1 in mammals.[46] This proximity was proposed to ensure normal translation termination, which would be perturbed if the 3′ UTR is long and led to the "faux 3′UTR" model for the identification of NMD substrates. Here, "faux," "false" in French, refers to the fact that some 3′ UTRs might not fulfill their role in ensuring normal RNA export, stability, and transport and, instead, promote RNA degradation via NMD. This model is appealing because it is simple: a PTC is a stop codon far from the downstream poly(A) tail and for which Pab1, the poly(A) binding protein, cannot assist translation termination, as it would normally do.[45] However, ribosome stalling at the stop codon might not be the only explanation for the role of poly(A) distance to stop in NMD, since ribosome stalling on NMD substrates was not detected in a different experimental system.[47] While it remains unclear whether ribosome stalling at a stop codon marks it as a PTC, translation termination defects, and especially stop codon read-through, decrease NMD degradation of a reporter in cells depleted for the ribosome recycling factor ABCE1.[31,32]

Altogether, both pro- and anti-NMD mechanisms depend on factors that are either dedicated to this process, such as the RNA helicase UPF1, or are essential participants, such as the ribosome.

NMD FACTORS AND COMPLEXES ARE SIMILAR ACROSS SPECIES

Recent screens for NMD factors in mammalian cells[31,48–50] confirmed that previous studies in *S. cerevisiae* and *C. elegans* identified most if not all the most conserved genes dedicated to this pathway in eukaryotes. Additional NMD factors were discovered through genetic screens in *C. elegans*, for example,[51] and at least one of them, the Nbas protein, might be involved in an ER-specific metazoan RNA degradation version of the NMD pathway.[52] What a "dedicated to NMD" factor means is not straightforward because many proteins are required for the process but have a primary role elsewhere. An example of the latter case of a NMD-related factor is the exonuclease XRN1 that degrades any RNA with an available 5′ monophosphate group.[53] XRN1's action occurs late in the NMD pathway and is thus dependent on earlier RNA degradation events that remove the 5′ cap structure or cleave the RNA. Similar to XRN1, translation termination factors eRF1 and eRF3 are important for triggering NMD, but are difficult to be considered primarily as NMD factors due to their major role in ribosome function. Finally, EJC components are also NMD-associated factors even if their primary role is related to premRNA splicing.

To help with the nomenclature of NMD factors, in practice two abbreviations indicate if a gene is involved in NMD: UPF and SMG. UPF is short for "up frameshift" and indicates that mutations in yeast UPF factors could increase the expression levels for a HIS4 gene having a +1 frame shift mutation leading to the presence of an out of frame PTC.[54,55] SMG genes were identified in *C. elegans* based on the ability of mutants to correct a defect in myosin class II heavy chain, UNC-54, production. This defect is the consequence of the loss of normal transcription termination for the r293 allelle of *unc-54*, leading to the probable use of a downstream termination signal and creating an unusually long 3′ UTR region. Since suppressor mutations also led to defects in the formation of male worm bursa and hermaphrodite vulva, the "suppressor with morphogenetic effect on genitalia," SMG, abbreviation was proposed.[56] There are three *UPF* genes, *UPF1*, *UPF2*, and *UPF3* and several *SMG* genes: *SMG1*, with cofactors *SMG8* and *SMG9*, *SMG6*, *SMG5 and SMG7*.

A unique nomenclature of NMD factors might have rendered life of scientists easier, but the numbers of *SMG* and *UPF* genes were not matched, with SMG2 being the equivalent of UPF1, SMG3, the equivalent of UPF2 and SMG4, the equivalent of UPF3. A reason for maintaining these two types of names for *NMD* genes is also the observation that UFP1, UPF2, and UPF3 are universally conserved from yeast to humans, while *SMG* genes, 1, 5, 6, and 7, were only found initially in multicellular organisms. As illustrated in Figure 2A, the probable conservation of these different NMD factors spans all eukaryotes, with factors missing in some organisms or having minimal roles in others. While the equivalent of the protein kinase SMG1 in yeast remains elusive, unrelated protein kinases, including the casein kinase two complex (CKA1, CKA2, CKB1, CKB2) and HRR25, are associated with NMD complexes[57] and mutant UPF1 can accumulate as a phosphoprotein[58] in this organism. Analysis of the interaction domains of UPF1 with other NMD factors suggests that the equivalent of SMG6 in yeast is NMD4, even if it is likely that the yeast protein has lost the endonucleolytic activity that characterizes SMG6 in other species.[59–61] SMG5 and SMG7, whose interaction to form a heterodimer is important for their role in human NMD[62] have probably a single equivalent in yeast, the EBS1 protein.[57,63] In *Arabidopsis thaliana* there is no equivalent of SMG5, and SMG1 has been recently lost[64] but the NMD pathway is present and active in this model organism.[65]

Since core NMD factors are present in most eukaryotes, NMD mechanisms are likely to be conserved. These mechanisms depend on molecular interactions that are under active investigation. We compiled a set of interactions among NMD factors based only on recent results using only affinity and proximity labeling approaches followed by mass-spectrometry analysis (Figure 2B).

The interactions among human NMD factors depicted in Figure 2B are not entirely compatible with the current NMD models. For example, translation termination factors and their interaction with SMG1 and UPF1, proposed to be part of the SURF complex,[72] were not detected. It is possible that the mass-spectrometry based approaches are not yet sensitive enough or were not performed under conditions that allow the accumulation of specific NMD complexes. It is also possible that transient interactions of NMD factors with the translation termination machinery are specific to mutant forms of UPF1[73] or depend on the action on translation termination of other NMD factors, such as UPF3B.[74]

Among the interactions that were not recovered by large-scale approaches are those between SMG1, a protein kinase crucial for mammalian NMD, and UPF2 and UPF3B, even if a complex containing all these factors and UPF1 was proposed to exist. Identification of NMD mechanisms is a challenging task since some NMD factors, such as UPF2 and UPF3, have low abundance. Potential interactions might be too weak to resist purification conditions and involve a heterogeneous population of RNA molecules. Finally, catalytically active NMD complexes involved in decapping or endonucleolytic cleavage might not be easily distinguished from complexes of identical composition but having no enzymatic effect on RNA molecules.

THE CURIOUS CASE OF UPF1 PHOSPHORYLATION CYCLE

UPF1 phosphorylation, particularly in the C-terminal domain of the protein, is an important event in NMD and SMG1, the protein kinase responsible for this reversible modification, is required for NMD in many organisms. Even in *D. melanogaster*, where SMG1 mutants have a mild effect on NMD,[75] this protein kinase becomes important when the function of another NMD factor, SMG5, is perturbed.[76] SMG5 and SMG7 contain tetratricopeptide repeat (TPR) domains

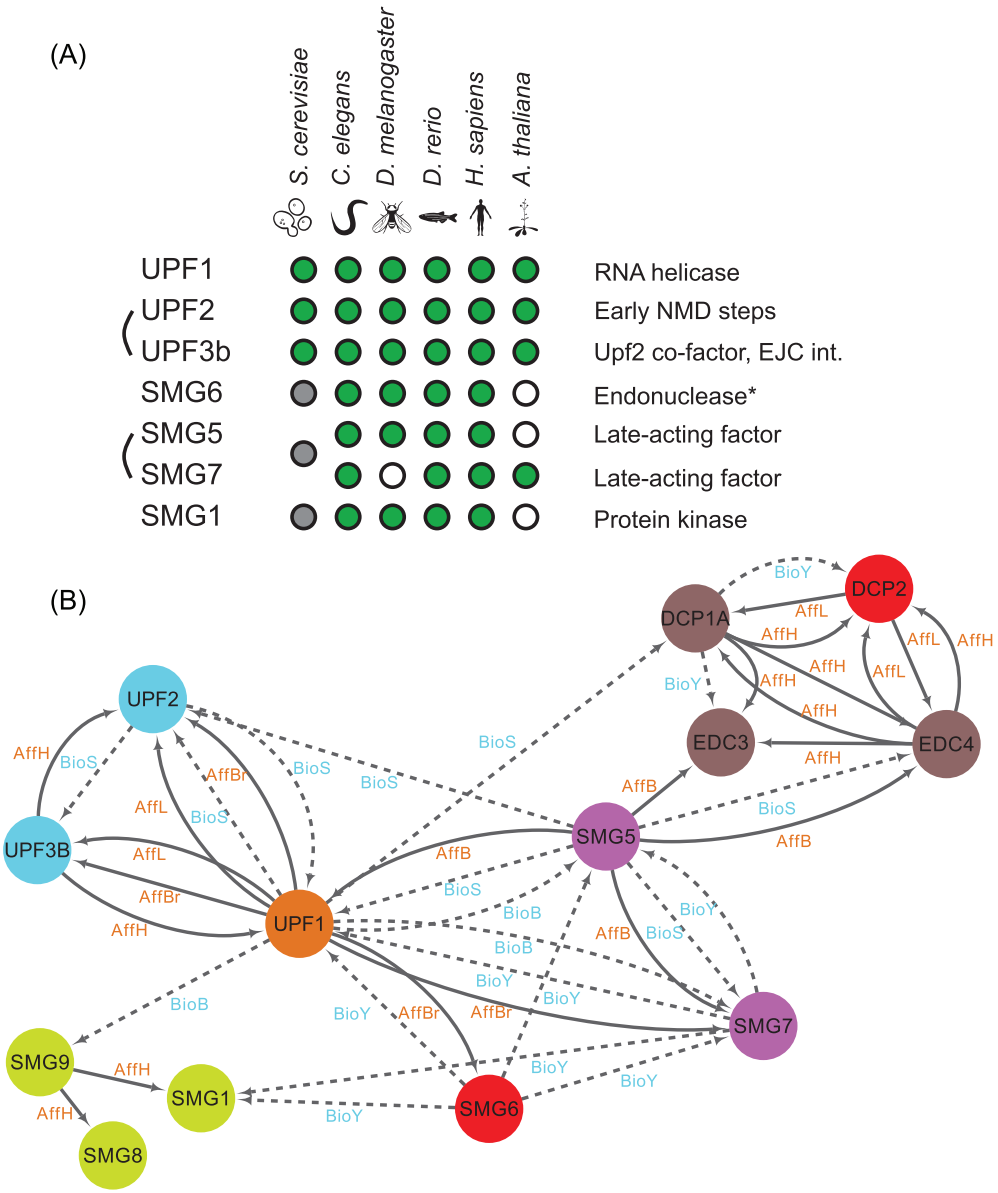

FIGURE 2 Conservation and interactions of core NMD factors. (A) Presence of equivalents for the core NMD factors in various species is indicated with green dots. Gray dots indicate partial similarity and white dots indicate the absence of a clear equivalent. SMG8 and SMG9, not shown, are SMG1 partners that follow the same conservation pattern.[66] For SMG6, an asterisk indicates that in some organisms the enzymatic activity of the protein is probably absent. (B) Interactions of human NMD factors detected through approaches based on affinity purification (solid lines, labeled "Aff") or proximity labeling (dashed lines, labeled "Bio"). The codes of the affinity-based interactions correspond to initials of first author in the corresponding publications: "AffB" Boehm,[9] "AffBr" Brannan,[67] "AffH" Hein,[68] "AffL" Li [69] and for the biotin labeling interactions correspond to: "BioB" Boehm,[9] "BioS" Schweingruber,[70] "BioY" Youn.[71] The arrows orientation indicate which proteins were used as "bait". SMG6 and DCP2, RNA cleavage enzymes, are depicted in red

that allow specific binding to phospho-UPF1.[77,78] Purification of SMG5 and SMG7 shows preference to the phosphorylated form of UPF1.[79] While the relative amounts of phosphorylated Upf1 under steady-state conditions are relatively low, they increase when the function of SMG5, SMG6, and SMG7 is perturbed in *C. elegans*[80] or human cells.[81] SMG5 and SMG7 form a heterodimer and were proposed to act as recruiters of the protein phosphatase PP2A.[82] These observations integrate into a sequence of events occurring at a PTC, in which activation of SMG1, potentially in a UPF1/2/3 complex, creates the conditions for UPF1 phosphorylation and binding of SMG/SMG7 and of the endonuclease SMG6.

What if UPF1 phosphorylation is just a mark of transient, but crucial, association of UPF1 with SMG1 during NMD ? This alternative interpretation fits with the fact that the interaction of the SMG6 endonuclease with UPF1 is only partially dependent on UPF1 phosphorylation.[78,83] It can also explain surprising recent results about SMG7. Mutants in the TPR domains of SMG7 (K66E/R163E), which remove the ability of the protein to bind phospho-UPF1,[77] also

perturb its association with nonphosphorylated UPF1. However, and unexpectedly, these mutations do not cancel the ability of SMG7 to function in NMD, when overexpressed.[9] By comparison, the formation of the SMG5/7 heterodimer turned out to be crucial for NMD, as studied in a cell line in which endogenous SMG7 was knocked out. Contrary to previous observations, proposing that a role of SMG5 and SMG7 is to recruit the protein phosphatase complex PP2A,[82] SMG5 was not found to be specifically associated with components of this phosphatase. Moreover, a specific association of SMG5, SMG7 or any other NMD factor with a protein phosphatase was not revealed by mass-spectrometry approaches (Figure 2B, references in the figure legend).

In light of the current results, the proposed molecular events at the PTC, including the formation of a SURF complex (SMG1, UPF1, eRF), might need to be re-evaluated. SURF includes, in addition to the core NMD factor UPF1, SMG1 and translation termination factors eRF1 and eRF3. SURF was proposed to form at PTCs and transition to a decay inducing complex (DECID), in which UPF1 interacts with UPF2 and UPF3 for the next step in NMD.[72] As discussed above, other scenarios are possible. Phosphorylation of UPF1, for example, might be not a cause, but a consequence of other molecular events involved in NMD, specifically the association of SMG1 to NMD complexes. This association might require the kinase activity of SMG1 for autophosphorylation,[84] to allow further remodeling of the NMD complex. Persistent association between UPF1 and SMG1, in the absence of the later SMG5-7 factors, would lead to UPF1 hyperphosphorylation, an idea that has been already suggested.[85] Similarly, the absence of UPF2, for example, which leads to decreased phosphorylation of UPF1, would be a sign that SMG1-UPF1 association was diminished, placing SMG1 association to UPF1 downstream the interaction of UPF1 with UFP2, and probably UPF3B. Finally, UPF1 dephosphorylation, proposed to be mediated by the recruitment of the serine/threonine phosphatase 2A,[79,82] might occur only on specific conformations of the NMD complexes, when the phosphorylated residues are accessible. Thus, the hyperphosphorylation of UPF1 after depletion of SMG5 and SMG7 could be interpreted as an increased insulation from highly active and relatively nonspecific cellular phosphatases.[86] The phosphorylation cycle of UPF1 in NMD needs further evaluation, to distinguish correlations from mechanistic cause and effect.

SMG5 AND SMG7, RNA DECAPPING AND ENDONUCLEOLYTIC ACTIVATORS ?

RNA degradation in NMD is initiated by either endonucleolytic cleavage through the activation of SMG6 catalytic activity in proximity of a PTC or by stimulation of RNA decapping by the DCP2 complex. UPF1 coordinates these events as it interacts with both the endonuclease SMG6 and with the decapping complex (Figure 2B). But is the recruitment of SMG6 and decapping factors in proximity of an RNA through UPF1 enough to trigger RNA degradation ? Tethering experiments, in which SMG6 was bound to a reporter RNA, showed that its RNA destabilization activity requires UPF1 and SMG1, but not UPF2, SMG5 or SMG7, when individually knocked-down. Strangely, the interaction of SMG6 with UPF1 occurs in the helicase domain of UPF1 and is independent on the phosphorylation of the protein,[83] which occurs on both N and C-terminal regions of UPF1. How the SMG1 protein kinase assists the activation of the endonucleolytic activity of SMG6 if its interactions do not seem to depend on UPF1 phosphorylation,[78,85] remains unclear.

Contrary to the lack of effect of individual SMG5 and SMG7 depletion on SMG6 activity, concomitant inactivation of these SMG genes leads to a loss of a SMG6-dependent RNA fragment from an NMD reporter.[9] This observation led to the proposal that SMG6 abundance might be limiting for the NMD process and can be viewed in the context of the equilibrium established among various forms of NMD complexes (Figures 3A, B). The persistence of SMG6 in UPF1 complexes in the absence of SMG5 and SMG7 indicates that biochemical analysis of NMD complexes and their dynamics in mutant cells is only partially informative of molecular mechanisms.[9]

Removal of both SMG5 and SMG7 decreases NMD to a large extent and leads, in addition to SMG6 inactivation, to the apparent accumulation of full-length NMD reporter RNA, at least for a studied transcript.[9] This observation is consistent with previous proposals for a mechanism of action of the SMG5 and SMG7 in RNA decay to occur through activation of RNA decapping. Decapping activation seems to be a universal feature of NMD mechanisms and predominates in yeast[87] and plant NMD.[88] However, how exactly the decapping is activated during NMD in all organisms remains unclear. Is it dependent on an acceleration of deadenylation[62] or could it be promoted by a direct interaction with UFP1[89] or some of its cofactors ?

The idea that in mammalian cells SMG5/7 would be responsible for accelerated deadenylation, which in turns activates decapping, comes from the study of the C-terminal region of SMG7 and on its ability to induce degradation of RNA in tethering experiments. This effect was independent on other NMD factors and was correlated with an interaction of the SMG7 domain with a component of the CCR4-NOT deadenylation complex, the deadenylase POP2.[62] The absence of the C-terminal domain of SMG7 does not abolish its ability to assist tethered SMG5 in destabilizing an RNA, as long as UPF1 is present.[90] Correlated with this result, removal of the C-terminal domain of SMG7 does not affect the ability of SMG7 to complement the NMD phenotype generated by the absence of the protein in SMG7 knock-out cells.[9] Moreover, deadenylases are not among the proteins associated with NMD complexes (Figure 2B), making it unlikely that accelerated deadenylation plays a major role in NMD.

If deadenylation acceleration is not responsible for the action of SMG5 and SMG7 in NMD, these proteins could participate to recruitment of the decapping complex. Decapping activation was proposed to occur through the interaction between SMG5 and PNRC2, a decapping cofactor. While PNRC2 was required in a tethered UPF1 RNA degradation assay, the observed interaction between PNRC2 and SMG5[91,92] was not robustly detected in more recent work.[90] Further studies will be required to understand whether PNRC2 is an essential component linking UPF1 with activation of decapping during NMD. Of note, in addition to the N and C-terminal regions of UPF1, the CH domain

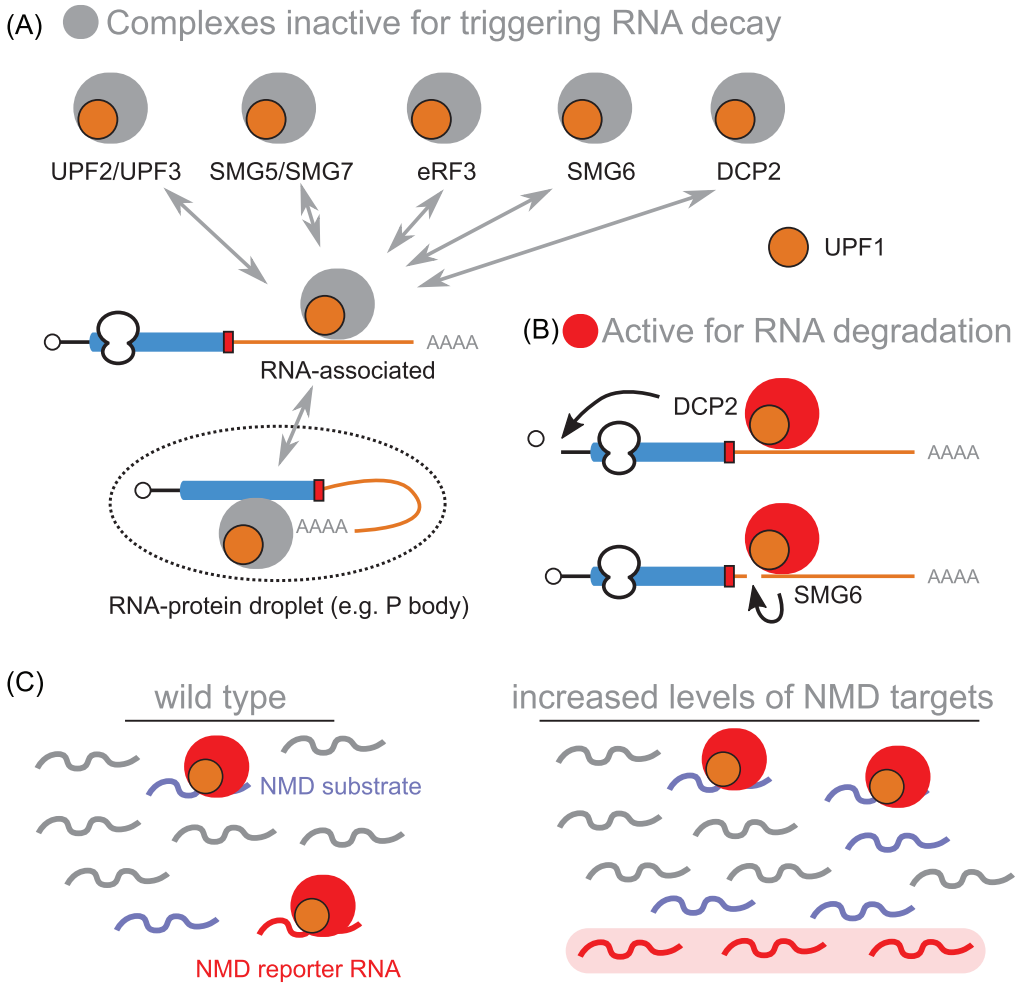

FIGURE 3 Challenges in the study of NMD mechanisms. Global evaluation of NMD complexes composition can be ineffective to distinguish active from inactive complexes in RNA degradation. Schematic representation of NMD complexes containing the core factor UPF1 in inactive (gray, A), or active for RNA degradation (red, B) configurations. Changes in the balance between the various forms might not be reflected by changes in the average composition of NMD complexes. (C) Low abundance NMD factors can become limiting for NMD by indirect mechanisms. A hypothetical scenario in which large amounts of RNAs that are NMD substrates are produced in a cell, bind available NMD factors, leading indirectly to accumulation of an NMD reporter molecule

was identified as important for the interaction with DCP2 in human cells.[62] This is surprisingly similar to the requirement for the CH domain of UPF1 for its interaction with the decapping machinery in yeast.[57] Thus, UFP1 could directly recruit the decapping machinery in mammalian NMD through a conformational change that depends on the late acting NMD factors SMG5 and SMG7. This hypothesis implies that recruitment of SMG6 and decapping activation are dependent on the association of the heterodimer SMG5/7 with UPF1[9] and is consistent with RNA level changes observed following NMD factors depletion, in which both decapping and endonucleolysis are involved.[93,94]

A coordination of decapping and endonucleolytic cleavage of NMD substrates shows surprising similarities with RNA degradation in bacteria. For example, in *E. coli*, endonucleolysis via RNAse E is partially dependent on the presence of a mono-phosphate group at the 5′ end of the RNA.[95] One of the enzymes responsible for the removal of phosphates from 5′ triphosphate and biphosphate RNA is rppH,[96] which is also active *in vitro* in removing the cap structure of eukaryotic RNA.[97] Even if the molecular players are different in bacteria and eukaryotes, removal of 5′ end protection of RNA and endonucleolytic cleavage are probably coordinated.

Since SMG5/7 can affect the function of SMG6 without an apparent loss of interaction of the protein with NMD complexes,[9] an alternative for the role of this heterodimer in NMD was proposed. Based on the observation that SMG6 is the lowest abundance NMD protein in human cells, its levels are probably a limiting factor and trapping the protein in dead-end complexes should decrease global NMD activity (Figure 3C). This scenario suggests that depletion of NMD factors can shift the balance between, for example, those with an active endonucleolytic activity and those, with a similar composition, that are not associated with RNA or are trapped in RNA-protein condensates (Figure 3A). The presence of proteins and RNA in liquid separated droplets can affect the catalytic activity of the decapping enzyme, DCP2.[98] Moreover, some of the best known phase separated granules, the yeast P bodies, contain high concentrations of NMD factors, such as UPF1.[99]

However, it is not clear to what extent affinity purification methods have access to complexes located in liquid droplets since these structures have been mostly explored through microscopy and fluorescence activated cell sorting.[100]

Changing the levels of RNAs that are substrates for NMD can affect NMD complexes and availability of SMG6 for RNA degradation. This is probably why depletion of the XRN1 exonuclease, which leads to accumulation of RNA degradation intermediated affects the observed composition of NMD complexes, with an increase in the association of SMG6 with UPF1.[85]

Distinguishing direct from indirect effects in NMD is crucial for understanding its molecular mechanisms. NMD has global effects on hundreds or thousands of RNA molecules. Low abundance, limiting NMD factors, could be effectively diluted if the amount of NMD substrates in a cell increases. This is not a hypothetical scenario, as similar situations have been already described. For example, flooding cells with high levels of a "decoy" RNA that contains motifs bound by the Nrd1:Nab3 complex, decreases its ability to assist transcription termination in yeast.[101] This relatively simple mechanism can explain why defects in nuclear RNA degradation, leading to accumulation of noncoding RNA in the nucleus and a decrease of Nrd1:Nab3 availability, are followed by transcription termination defects.[102] Interestingly, even relatively abundant proteins, such as the poly(A) binding protein (e.g., PABPC1) can be limiting under specific situations. The difference in the amount of protein produced from a short tail reporter mRNA (29 As) and a long tail reporter (139 As) in frog oocytes can be erased by overexpressing PABPC1. Thus, long poly(A) tail RNAs are able to restrict the availability of PABPC1 for shorter tail RNAs, thus limiting translation of these transcripts.[103]

We expect a decrease in NMD efficiency, as observed using a reporter molecule, if large amounts of NMD substrates are generated in a cell. This could occur, for example, in cells with splicing defects and could explain why EJC component depletion can, probably indirectly, affect the stability of an NMD reporter that has no exon-exon junction downstream its stop codon.[48] Thus, NMD phenotypes can be observed through indirect effects that provoke an imbalance in the population of NMD complexes.

Altogether, variations of in the amounts of proteins associated with NMD complexes need careful examination and, if possible, dissection based on RNA association or presence in aggregates (Figure 3). This is a technical and conceptual challenge for future studies of NMD and RNA degradation mechanisms.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated during the current study.

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