



REVIEW

**REVISED** **The evolution and diversity of the nonsense-mediated mRNA decay pathway [version 2; referees: 4 approved]**

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**Abstract**

Nonsense-mediated mRNA decay is a eukaryotic pathway that degrades transcripts with premature termination codons (PTCs). In most eukaryotes, thousands of transcripts are degraded by NMD, including many important regulators of developmental and stress response pathways. Transcripts can be targeted to NMD by the presence of an upstream ORF or by introduction of a PTC through alternative splicing. Many factors involved in the recognition of PTCs and the destruction of NMD targets have been characterized. While some are highly conserved, others have been repeatedly lost in eukaryotic lineages. Here, I detail the factors involved in NMD, our current understanding of their interactions and how they have evolved. I outline a classification system to describe NMD pathways based on the presence/absence of key NMD factors. These types of NMD pathways exist in multiple different lineages, indicating the plasticity of the NMD pathway through recurrent losses of NMD factors during eukaryotic evolution. By classifying the NMD pathways in this way, gaps in our understanding are revealed, even within well studied organisms. Finally, I discuss the likely driving force behind the origins of the NMD pathway before the appearance of the last eukaryotic common ancestor: transposable element expansion and the consequential origin of introns.

**Keywords**

RNA, NMD, evolution, UPF1, SMG1, transposable element, RNA decay

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**REVISED Amendments from Version 1**

Minor changes were made to the text and figures throughout this article. In particular new information relating to the protein-protein interactions of UPF1 and SMG5/6/7 family members was added in yeast and animals because evidence for direction, phosphorylation-independent interactions informs us of how to view the mechanism and evolution of NMD, especially regarding the role of SMG1 and how it could have been independently lost multiple times throughout eukaryotic evolution.

**See referee reports**

## What is nonsense-mediated mRNA decay?

Gene expression is controlled by a variety of mechanisms, sometimes in unexpected ways. Analysis of mutant screens and genetic diseases identified mutations that introduced nonsense mutations, but surprisingly, these premature termination codons (PTCs) lead to a reduction in mRNA stability<sup>1,2</sup>. This increase in RNA decay is the result of an active translation-dependent process<sup>1,3</sup>. This pathway was termed nonsense-mediated mRNA decay (NMD) and is now known to regulate hundreds to thousands of transcripts in plants, animals, fungi and ciliates<sup>4-10</sup>. Many of the NMD targeted transcripts are not the result of nonsense mutations, but are instead the result of alternative splicing events that introduce PTCs or the presence of an upstream open reading frame (uORF). Many such splicing events are not the result of splicing errors, but are in fact highly conserved events<sup>11,12</sup>. Therefore, NMD has a major role in shaping the transcriptome of diverse eukaryotes. However, the exact molecular nature of the NMD pathway varies between organisms. Most eukaryotes share the core NMD factors (see below), but an impressive number of modifications to the NMD pathway exist. In this review, I will examine the factors known to act in NMD, discuss the diversity of these factors in eukaryotes, and explore the different mechanisms that explain how a PTC is differentiated from an authentic stop codon. Finally, I will discuss how the NMD pathway may have evolved and some remaining key questions in our understanding of the NMD pathway.

## The factors that read nonsense

Early mutant screens in baker's yeast and *Caenorhabditis elegans* identified three conserved factors that could suppress a nonsense mutation<sup>13,14</sup>. These factors were named UP-frameshift (UPF) 1, 2 and 3 in baker's yeast and Suppressors with Morphological defects on Genitalia (SMG) 2, 3 and 4 in *C. elegans*. The baker's yeast names of these factors are used throughout this review. UPF1 is a highly conserved RNA helicase<sup>15</sup> that interacts with UPF2, which is an MIF4G domain-containing protein<sup>16</sup>, that in turn binds to UPF3 (Figure 1)<sup>17,18</sup>. The initial mutant screens in *C. elegans* also revealed four additional factors: the kinase SMG1 and the 14-3-3-like domain proteins SMG5, SMG6 and SMG7<sup>13,19</sup>. In animals, SMG1 is known to phosphorylate UPF1 after a PTC is been recognised (Figure 1)<sup>20-22</sup>. From these early studies in *C. elegans*, the different NMD factors were defined by their role in the phosphorylation of UPF1. UPF2 and UPF3 support the phosphorylation of UPF1 by creating a complex compatible for phosphorylation by SMG1<sup>22</sup>, while also acting to activate

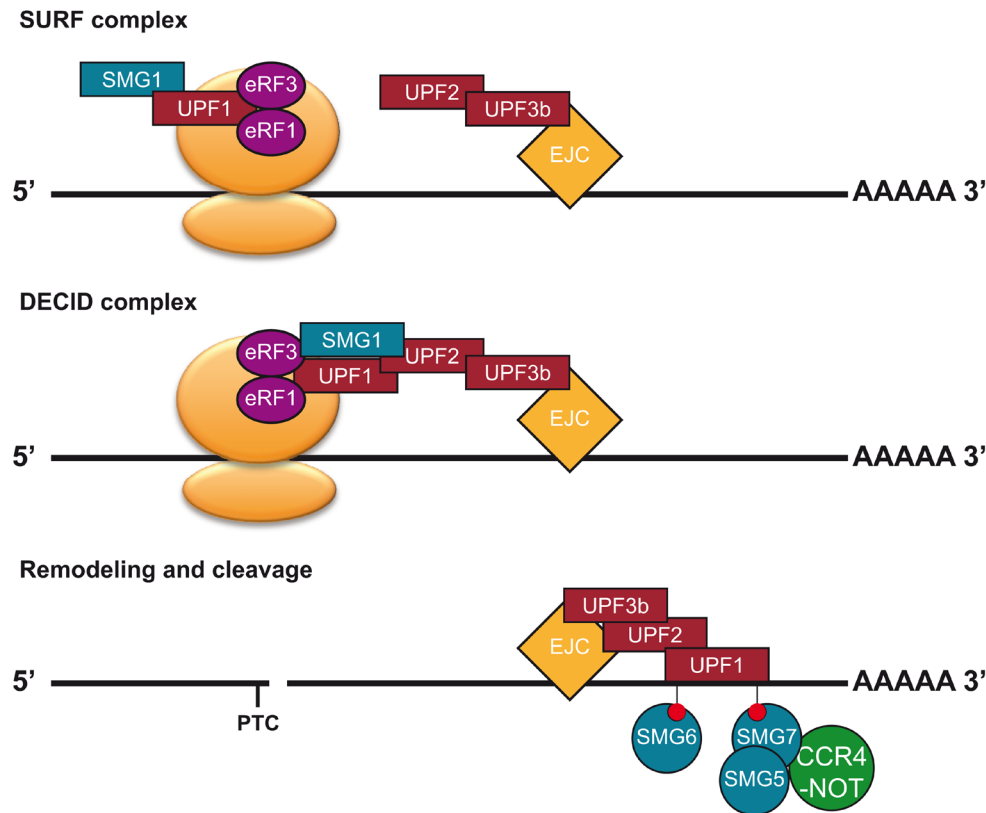
the RNA helicase activity of UPF1<sup>23</sup>. SMG5/6/7 bind to phosphorylated UPF1<sup>24</sup> and are active in the dephosphorylation of UPF1 by recruiting the PP2A phosphatase<sup>25-27</sup>. However, it is now clear that their primary role is in acting at various stages of RNA decay. SMG5/6/7 have a central role in recruiting the degradation machinery to degrade the NMD target<sup>28-31</sup> (Figure 1). SMG5 and SMG7 act to recruit exonucleases<sup>29</sup>, while SMG6 is an endonuclease, cutting the transcript near the PTC<sup>30,31</sup>. Over time, many more NMD factors have been identified through further genetic and biochemical screens<sup>32-35</sup>. Of these, SMG8 and SMG9 are of particular interest. First identified in human cells as SMG1-interacting proteins, they act in the NMD pathway of humans and possibly *C. elegans*<sup>34,36</sup> through the inhibition of the kinase SMG1. Curiously, studies in mammals have revealed that many NMD targets do not require the involvement of all NMD factors. Many NMD targets are degraded by specific "branches" of the NMD pathway that do not require UPF2<sup>37</sup> or UPF3b<sup>38</sup> in mammals. However, all branches do involve UPF1, highlighting its central importance to the NMD pathway.

Together these studies, mostly using animal systems, paint a picture where multiple factors (UPF2, UPF3, SMG1, SMG8, and SMG9) assist in the activation of UPF1, while other factors (SMG5/6/7) act to degrade an NMD target and dephosphorylate UPF1.

## Variations on a common pathway

Despite the deduction of a basic schematic of the NMD pathway in animals (Figure 1), many of the factors involved in this classical model of NMD vary between different organisms (Figure 2 and Figure 3). The most highly divergent NMD pathways are those found in the excavata (Figure 2 and Figure 3). The excavata have been suggested to be the most basal group of eukaryotes<sup>39</sup>, although other work places them within the same supergroup as plants<sup>40,41</sup>. Although the NMD pathways of the parasites *Giardia lamblia* and *Trypanosoma brucei* have been studied, it is unclear if a functional NMD pathway exists in these organisms<sup>42,43</sup>. They contain heavily reduced complements of NMD factors: the genome of *G. lamblia* only harbors UPF1, and the genome of *T. brucei* only harbors UPF1 and UPF2<sup>42,43</sup>. Over-expression of UPF1 in *G. lamblia* caused an NMD reporter to further decrease, suggesting that *G. lamblia* might have an active NMD pathway<sup>42</sup>. In contrast, the knockdown of UPF1 in *T. brucei* did not increase NMD reporter construct expression, or endogenous genes<sup>43</sup>. However, tethering of UPF1 in *T. brucei* did decrease reporter expression<sup>43</sup>. Therefore, it is difficult to definitively conclude the status of the NMD pathway in excavata. However, it is worth noting that parasites are known to have reduced genomes relative to free-living relatives<sup>44</sup>, and that the non-parasitic excavata *Naegleria gruberi* does harbor the additional NMD factors of SMG1 and SMG9<sup>45</sup>. This indicates that a complex NMD pathway involving the kinase SMG1 likely existed in the last eukaryotic common ancestor.

Further support for a complex NMD pathway existing in the last eukaryotic common ancestor comes from the examination of plants. Plants, which diverged from animals and fungi early in eukaryotic evolution (Figure 2), do have functional homologues

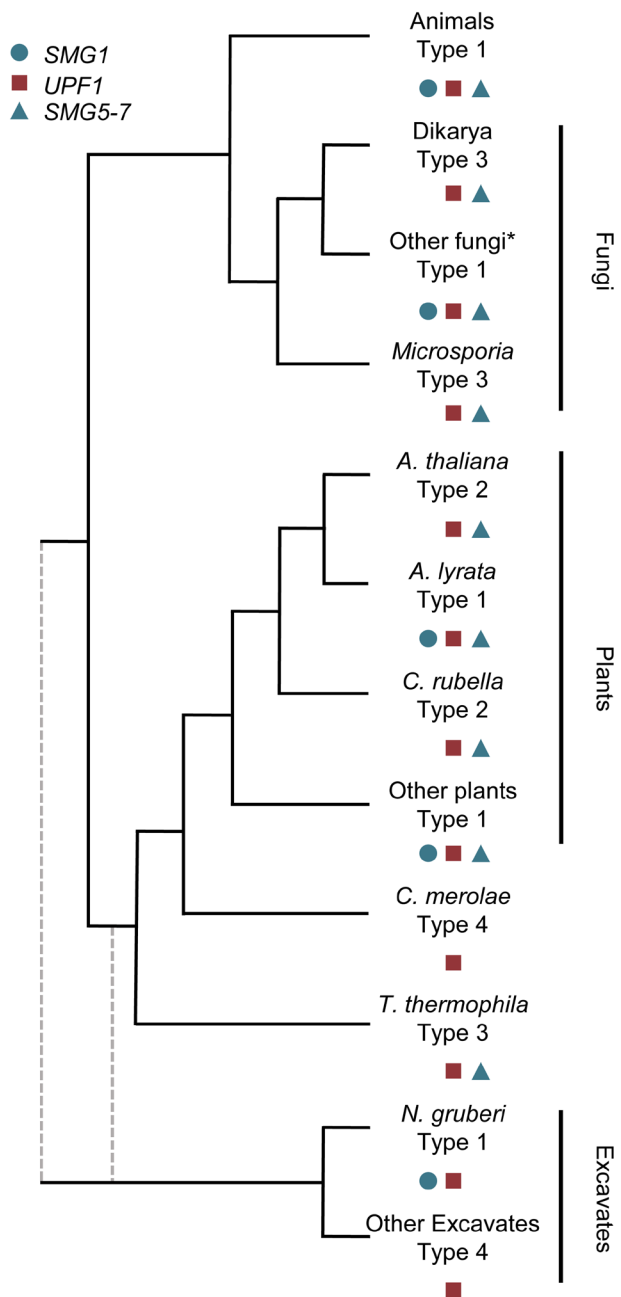


**Figure 1. The model of NMD activation in animals.** At termination events, UPF1 and SMG1 are recruited to termination events by eRF1 and eRF3, leading to the formation of the SMG1-Upf1-eRF1-eRF3 (SURF) complex<sup>22</sup>. Further recruitment of UPF2 and UPF3 (UPF3b in mammals) leads for the formation of a decay-inducing (DECID) complex<sup>20,46</sup>. This will lead to the phosphorylation of UPF1 by SMG1. Then the ribosome will disassociate and SMG5/6/7 will be recruited to transcript through phos-UPF1 binding. The transcript is degraded by endonucleolytic cleavage by SMG6 and the CCR4-NOT complex is recruited by SMG7/5. UPF2 and UPF3 can be recruited to NMD targeted transcripts by the EJC, although many transcripts a degraded without the presence of an EJC<sup>47,48</sup>.

of the NMD holy trinity: UPF1-3<sup>47,49</sup>. Plants also have homologues of SMG5/6/7, known as SMG7 and SMG7-like<sup>50</sup>, and SMG1 homologues<sup>45,51</sup>. SMG1 has been repeatedly lost throughout eukaryotic evolution, including two losses in land plants (*Arabidopsis thaliana* and *Capsella rubella*) and multiple losses in fungi (Figure 2)<sup>45,51</sup>. The repeated loss of SMG1 raises some interesting questions about the mechanism of NMD activation. In animals, and presumably in most plants, SMG1 phosphorylates SQ and TQ dipeptides at the N- and C-termini of UPF1<sup>20,52,53</sup>. Species, such as baker's yeast, with an ancient loss of SMG1 (Figure 2), have UPF1 sequences depleted of S/TQ dipeptides relative to species with SMG1<sup>45</sup>. Species that lost SMG1 more recently, such as *A. thaliana*, have UPF1 proteins that are rich in S/TQ dipeptides<sup>45</sup>. The repeated losses of SMG1 in eukaryotes suggests that there is a genetic buffer, another factor/mechanisms that allows SMG1 to be lost but the NMD pathway to be activated<sup>45,51</sup>. In support of this notion, the experimental perturbation of SMG1 in fruit flies and zebrafish has little or no effect on the NMD pathway of these organisms<sup>54-56</sup>, suggesting that a backup UPF1-activation mechanism is already present in these species. One possibility is that an alteriave kinase has replaced SMG1 and might

even be ancestral and operational in many species, allowing for the loss of SMG1<sup>51</sup>. However, this does not explain why the putative phosphorylation sites are lost in many species<sup>45</sup>. One exciting possibility is that direct interactions between SMG5/6/7 family proteins is sufficient for NMD to be activated in some species (see below).

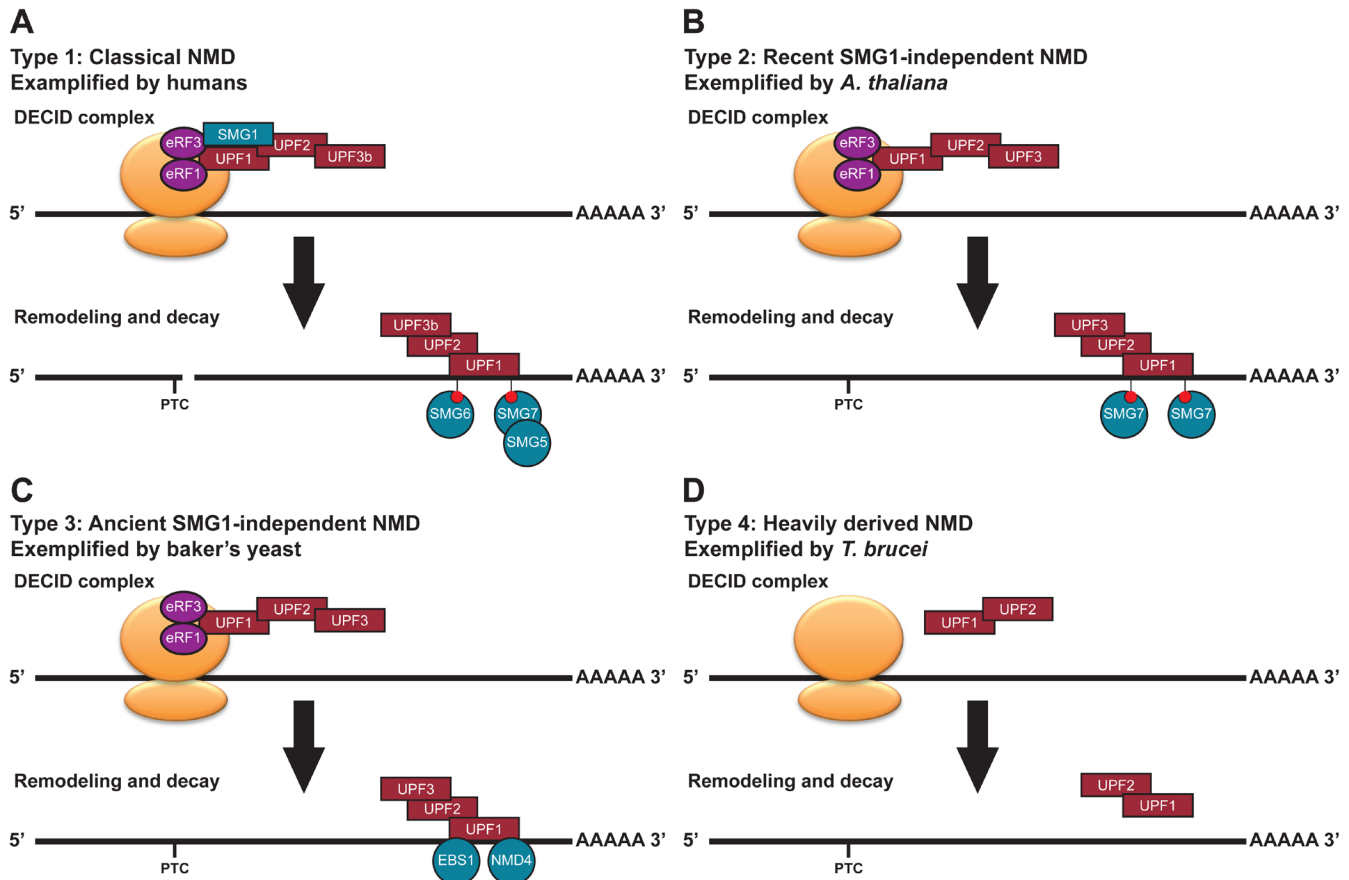
The SMG5/6/7 family split and diversified in the animal lineage, with the acquisition of the PIN domain in SMG5 and SMG6<sup>27,57,58</sup>. The PIN domain of SMG6 gives it the ability to act as an endonuclease, cutting the NMD targeted transcript near the PTC<sup>30,31</sup>. The SMG5/6/7 family also have a role in regulating telomere length<sup>59</sup>. SMG5/6/7 homologues in plants are known as SMG7, given they lack the PIN domain of SMG5 and SMG6<sup>50</sup>. SMG5/6/7 family members of baker's yeast, EBS1 and EST1, also lack the PIN domain<sup>60</sup>. In baker's yeast, EST1 is implicated in telomere regulation but not NMD, while a knockout of EBS1 reveals a mild NMD phenotype<sup>60,61</sup>. Given that baker's yeast lacks SMG1<sup>21,45,51</sup>, it is not clear why EBS1/SMG7 would be required for NMD. The UPF1 of baker's yeast is depleted of S/TQ dipeptides<sup>45</sup>, which once phosphorylated by SMG1,



**Figure 2. The various NMD types across diverse eukaryotic lineages.** The distribution of the key NMD factors, UPF1, SMG1 and a member of the SMG5-7 family define the NMD pathway type. All NMD types have arisen multiple times within eukaryotic evolution. NMD pathways can be classified into four types, Type 1: classical SMG1 dependent NMD, Type 2: recent loss of SMG1 with S/TQ rich UPF1, Type 3: ancient loss of SMG1 with S/TQ depleted UPF1, Type 4: Heavily derived NMD (Figure 3). To date, no SMG5/6/7 family member has yet been identified in *N. gruberi* but given the presence of SMG1<sup>45</sup>, I am currently classifying it as a type 1 NMD pathway. The branch lengths do not reflect the relatedness of any species, but represent the order of separation between the lineages. The root of eukaryotes is unclear, so branches representing an Excavata early and late divergence are represent in grey, dashed-lines. \*SMG1 appears to have been lost in other fungal lineages as well, representing repeated losses in multiple fungal lineages<sup>45</sup>.

normally act as binding site for SMG5/6/7<sup>24</sup>. The lack of S/TQ dipeptides suggest that classical phosphorylation of UPF1 is not required for the activation of NMD in baker's yeast. Tyrosine phosphorylation of UPF1 in baker's yeast has been observed and appears to regulate the RNA helicase activity of UPF1<sup>62</sup>, although the role in NMD, if any, and kinase responsible is still unknown. It is possible that these or other phosphorylated sites could act to recruit decay factors like S/TQ dipeptides do. However, given the differences between S and T residues from Y, it seems unlikely that EBS1/SMG7 would be involved. It could be that RNA decay enzymes are recruited directly to UPF1, alternative mechanism to the phosphorylation-mediated recruitment<sup>61,63,64</sup>. Recently, the yeast EBS1 and NMD4 proteins were found to interact directly with UPF1 during NMD<sup>61</sup>. NMD4, like SMG6, contains a PIN domain<sup>61</sup>. Transcripts responsive to the deletion of UPF1 also increased in deletions of EBS1 and NMD4, however, to a lesser extent<sup>61</sup>. Interestingly, the importance of EBS1 and NMD4 became more pronounced when yeast cells expressed a truncated UPF1<sup>61</sup>; when the truncated UPF1 was expressed alone, NMD efficiency was about 30% of wild-type, in contrast, when either EBS1 and NMD4 were deleted in the truncated UPF1 lines, NMD efficiency was close to zero<sup>61</sup>. This suggests that EBS1 and NMD4 become essential in NMD limiting conditions. This raises the possibility that in species lacking SMG1, the phosphorylation checkpoint of NMD is not required and SMG5/6/7 family proteins directly interact with UPF1 when at a PTC. SMG1 mutants in fruit flies have been found to have a lesser effect on NMD than the mutation of other NMD factors<sup>54,55</sup>. SMG5 was found to be essential for NMD<sup>28</sup>, and when a mild disruption of SMG5 is introduced, mutations of SMG1 enhanced the severity of the NMD phenotype<sup>28</sup>. This supports the notion that NMD can be activated without phosphorylation and that phosphorylation simply enhances decay under limiting conditions<sup>28,65</sup>. Interestingly, mammalian SMG6 has also been found to bind UPF1 independent of phosphorylation<sup>66,67</sup>, suggesting some level of conservation of phosphorylation-independent recruitment of decay factors in NMD. However, it is not clear why a phosphorylation checkpoint is needed for NMD in some organisms like mammals<sup>20,52</sup> and plants<sup>51,53</sup>, but likely not others such as yeast, but direct interaction seems likely to be the mechanism. Recently, another member of the SMG5/6/7 family was characterized in the ciliate *Tetrahymena thermophila*<sup>9</sup>, despite the loss of the SMG1 kinase from *T. thermophila*<sup>9,45</sup>. The SMG5/6/7 family member of *T. thermophila* was named SMG6-like (SMG6L) due to the presence of the C-terminal NYN nuclease domain, potentially taking on the same role as the PIN domain of animal SMG6 proteins<sup>9</sup>. SMG6L appears to work with UPF1 in the NMD pathway of *T. thermophila* and is conserved in many other protozoa<sup>9</sup>. However, it is unclear if SMG6L directly interacts with UPF1 or if it is via phosphorylation, but there is no SMG1 and classical phosphorylation sites on UPF1<sup>9</sup>.

The kinase activity of SMG1 is regulated in part by SMG8 and SMG9<sup>34</sup>. These factors have been identified but not characterized outside of the animal kingdom<sup>45</sup>; a curious finding which indicates they may have a role in NMD in diverse eukaryotes. When SMG1 is lost from a genome, SMG8 and SMG9 are generally also lost<sup>45</sup>. Further work will be needed to reveal the extent of any conserved role in NMD for these factors.



**Figure 3. Models of evolutionarily diverse NMD pathways.** (A) Classical NMD, exemplified by humans (modified from Figure 1). (B) Recent SMG1-independent NMD, exemplified by *A. thaliana*. *A. thaliana* lost SMG1 within the last 5–10 million years<sup>45,51</sup>. *A. thaliana* requires SMG7 for a functional NMD pathway<sup>50</sup>, retains a S/TQ rich UPF1<sup>45</sup> and its UPF1 needs to be phosphorylated to function in NMD in tobacco leaves<sup>53,68</sup>. This suggests an alternative kinase may have replaced SMG1. (C) Ancient SMG1-independent NMD, exemplified by baker's yeast. The NMD pathway of baker's yeast was the first to be characterised. UPF1, UPF2 and UPF3 have central roles in this pathway. Reverse genetics revealed a potential lesser role for EBS1, a SMG7 homologue, in NMD<sup>60</sup> but its UPF1 is depleted in S/TQ dipeptides<sup>45</sup>. (D) Heavily derived NMD, exemplified by *T. brucei*. It is unclear if a functional NMD pathway exists in these organisms. In *T. brucei*, it has been shown that UPF1 and UPF2 interact, but their interaction with the ribosome and potential NMD targets is unclear<sup>43</sup>. Tethering of UPF1 a transcript can decrease its abundance<sup>43</sup>.

Taken together, a diverse set of NMD pathways with varying levels of classically defined NMD factors been identified. Generally speaking, these can be split into four major types and a spread across many unrelated eukaryotic lineages (Figure 2 and Figure 3):

- 1) Classical SMG1-dependent NMD (As exemplified by humans, worms, and moss)
- 2) Recent SMG1-independent NMD (As exemplified by *A. thaliana*)
- 3) Ancient SMG1-independent NMD (As exemplified by baker's yeast and *T. thermophila*)
- 4) Heavily derived NMD (As exemplified by *G. lamblia*, *T. brucei* and *Cyanidioschyzon merolae*)

Type 1 NMD pathways (classical SMG1-dependent NMD; Figure 3A) are known to exist in both animals and plants<sup>20,21,51</sup>

and is likely to be the ancestral state of NMD<sup>45,51</sup>. However, even here, the dependence on SMG1 is not always clear: SMG1 mutants in fruit flies have much milder phenotypes than mutations in other NMD factors<sup>55,69</sup> and knockdown of SMG1 in zebrafish revealed no phenotype<sup>56</sup>. It is possible that the NMD pathways of some species with a type 1 NMD pathway in appearance might better resemble type 2 NMD (recent SMG1-independent NMD).

Type 2 NMD pathways (recent SMG1-independent NMD; Figure 3B), such as those of the land plants *A. thaliana* and *C. rubella*, appear very much like those of type 1, with the exception of SMG1 being absent from the genome, likely with the accompanying loss of SMG8 and SMG9<sup>45,51</sup>. However, UPF1 still maintains the relatively high level of phosphorylatable S/TQ motifs<sup>45</sup>, and phospho-UPF1 binding protein SMG7<sup>8,50</sup>. It would be tempting to speculate that a kinase related to SMG1 replaced it in the NMD pathway<sup>51</sup>. ATM and ATR are two kinases from the same family as SMG1 that are conserved in plants and are

involved in DNA repair. However, in *A. thaliana*, the reported mutant phenotypes of ATM and ATR<sup>70</sup> do not overlap with the classical NMD phenotypes<sup>49</sup>, so this seems unlikely to be the case. TOR is the only other related kinase in *A. thaliana*, and is involved with the regulation of translation, although the phenotype of TOR knockdown lines do not appear to match those of NMD factors in *A. thaliana*<sup>71</sup>.

A type 3 NMD pathway (ancient SMG1-independent NMD; **Figure 3C**), was the first to be characterized by a mutant screen in baker's yeast<sup>14,72</sup>. These ancient losses of SMG1 lead to an NMD pathway without SMG1, without SMG8 and SMG9<sup>45</sup>, with a UPF1 depleted in S/TQ dipeptides<sup>45</sup>, but a potential role for SMG5/6/7 proteins<sup>9,60,61</sup>. Future work (see below) will be needed to better understand the exact molecular role of SMG5/6/7 proteins in type 3 NMD pathways, and to understand how the NMD pathway functions without the SMG1 activating UPF1.

Type 4 NMD pathways (heavily derived NMD; **Figure 3D**) are the most variable group and are found throughout the eukaryotic tree of life. These pathways often lack SMG1, but also core NMD factors (UPF2 and UPF3). Although UPF3 is hard to identify with homology searches<sup>73</sup>, it does appear to be missing from the genomes of a number of species<sup>45</sup>. These include the excavata parasites *G. lamblia* and *T. brucei*<sup>42,43</sup> but also the red algae *C. merolae*<sup>51</sup>. *C. merolae* has a very reduced genome, with only 27 introns in total<sup>74</sup>. *C. merolae* and *G. lamblia* also lack homologues of UPF2. It is certainly possible that the presence of these factors do not represent a fully functional form of an NMD pathway and instead reflect the molecular reminance of a former NMD pathway whose factors have now been co-opted for other functions. NMD factors do function in other pathways, for example, UPF1 is known to be involved with mammalian DNA replication<sup>75</sup>. Although in mammals, some NMD transcripts only require a subset of NMD factors<sup>37,38,76</sup>, these branches of the NMD pathway support the notion that a more reduced NMD pathway may exist.

In any of these species, additional NMD factors are likely to have arisen. The only non-type 1 species to have had a forward genetics screen performed for is the baker's yeast, so we have limited unbiased studies to draw from. Protein-protein interaction studies in yeast have revealed the species specific factor NMD4<sup>61,77</sup>. Performing similar work in other species is likely to reveal more species/lineage specific factors. This will be especially exciting in type 4 species, with the most heavily reduced NMD pathways. This framework of NMD types based on presence/absence of conserved NMD factor is aimed at aiding the comparison and discussion of NMD pathways from diverse organisms. Thinking of all NMD pathways as being fundamentally the same at the molecular level is wrong. There is certainly an overlap, but more focused studies are needed to understand when homologous NMD factors do have the same molecular role in NMD and do not.

### Defining NMD targets

So far I have discussed the molecular processes that link the recognition of a PTC to transcript destruction. However, a lot of

work has also been focused on understanding the mechanism of how a PTC is differentiated from an authentic stop codon. Multiple models for how this is achieved have been proposed. One of the most well characterized models centres around the exon junction complex (EJC), a protein complex deposited on an mRNA after two exons are ligated together during splicing<sup>78,79</sup>. While most EJCs are removed from the transcript by the translating ribosome<sup>80</sup>, EJCs associated with exon-exon junctions  $\geq 50$  nt downstream of a stop codon are not removed and can elicit NMD<sup>81,82</sup>. Early work showed that the EJC was not involved in the NMD pathways of fruit flies<sup>58</sup>, but more recent work proved the contrary, revealing a role for the EJC in fruit fly NMD<sup>83</sup>. The EJC has been lost from baker's yeast and so cannot have a role in its NMD pathway, but the EJC is involved in the fungi *Neurospora crassa*'s NMD pathway<sup>84</sup>. The EJC mode has even found support in plants, with reporter genes and transcriptome-wide studies supporting a role for exon-exon junctions in 3' UTRs eliciting NMD<sup>47,85-87</sup>. These findings would suggest that the EJC mode is an ancient mechanism for targeting transcripts to NMD. A surprising version of the EJC mode is the finding that some NMD targets in *T. thermophila* appear to be dependent on splice junctions downstream of the stop codon, but not on the EJC itself<sup>9</sup>. Knockout of the core EJC component Mago nashi did not alter the expression levels of NMD targets identified by knockout of UPF1 and SMG6L<sup>9</sup>. This indicates that an alternative mechanism might maintain an EJC-like mode of NMD in *T. thermophila*.

Another well-explored system used in defining PTCs is the long 3' UTR mode. Transcripts with abnormally long 3' UTRs have been found in reporter genes<sup>47,85,88</sup> and transcriptome-wide studies<sup>5,86,89</sup> to target transcripts to NMD, although some recent transcriptome-wide studies found little to no trend across the transcriptome, when the presence of 3' UTR introns were taken into account<sup>9,87,90,91</sup>. One proposed mechanism is the increased distance between the stop codon (PTC) and the polyA-binding protein, bound to the polyA tail<sup>92,93</sup>. This physical separation between the polyA tail and the terminating ribosome might lead to aberrant termination and the recruitment of NMD factors<sup>92,93</sup>. Although some transcripts appear to be targeted due to their length independent of the polyA tail in yeast<sup>94</sup>. An alternative, but not mutually exclusive model posits that longer 3' UTRs are able to recruit more UPF1 directly bound to the 3' UTR<sup>95</sup>. It has been found that UPF1 coats transcripts but translation displaces UPF1 from all regions, except the 3' UTRs<sup>96</sup>. This model suggests that a higher level of UPF1 binding increases the chances of NMD being triggered during the termination of translation; naturally long 3' UTRs that are resistant to NMD have been observed to bind less UPF1 than susceptible long 3' UTR transcripts<sup>95</sup>. In fact some naturally long 3' UTR transcripts in mammals appear to be protected from NMD by various features such as a recently identified cis-sequence element in the TRAM1 gene<sup>97</sup> or the many genes found to bind PTBPI near the stop codon to prevent NMD<sup>46</sup>. In yeast, the RNA binding protein Pub1 binds to sequence elements and protects some uORF-containing transcripts from NMD<sup>98</sup>. Such features protecting long 3' UTR transcripts from NMD might explain why transcriptome-wide studies find so few long 3' UTR transcripts that are targeted to NMD.

The mechanisms used to define PTCs in the last eukaryotic common ancestor are unclear. While the EJC mode has been identified in plants, fungi, and animals, suggesting an ancient origin, there are many eukaryotic lineages where it has not been characterized, or does not function<sup>9,99</sup>. The long 3' UTR mode of NMD has also been characterized in many diverse eukaryotes (plants, animals and fungi), but the mechanism underlying this mode, and failure to observe a strong signal for this feature in transcriptome-wide studies, does raise questions.

### The origins of NMD

Today, eukaryotes appear to utilize NMD in a variety of ways to achieve the same aim, degrading PTC-containing transcripts from a variety of sources. It appears that a rather complex NMD pathway, belonging to the type 1 group, existed in the last eukaryotic common ancestor (see above). In extant diploid eukaryotes, NMD can prevent some mutations from being dominant, protecting heterozygous individuals by turning these alleles recessive<sup>13,100,101</sup>. However, NMD also increases the severity of some genetic disorders<sup>102</sup>, creating a double-edged sword: protecting some mutation-carrying individuals while exacerbating the conditions of others. Therefore, it is unlikely that protecting the genome from nonsense mutations was the driving force behind the origin of the NMD pathway. Early eukaryotes did face a particular selective pressure not present in prokaryotes: rapidly multiplying transposable elements (TEs). The origin of sex in eukaryotes allowed for TEs to expand in copy number, which is not possible in prokaryotes with their primarily asexual reproductive system<sup>103,104</sup>. With the advent of sex, eukaryotes faced the expansion of many TE classes, including the self-splicing (group II) introns. Expansion of group II introns has been proposed to have driven the evolution of the spliceosome to enhance the splicing of these selfish elements<sup>105</sup>, the nucleus evolved to physically separate the processes of transcription and translation and allow for intron removal before translation<sup>106</sup>, and NMD evolved to degrade intron-retaining transcripts that escaped the nucleus<sup>106,107</sup>. These adaptations ensure that transcripts with retained introns do not undergo multiple rounds of translation. More recent expansions of introns in some eukaryotic lineages are due to the expansion of DNA transposons<sup>108</sup>, indicating the importance of these mechanisms to protect the genome from TE expansions in extant eukaryotes, and suggests multiple origins for introns from TEs throughout eukaryotic evolution. NMD has been proposed as a general protection mechanism against RNA viruses and TE expansion<sup>109</sup>. Once functional as a TE-intron protection pathway, NMD appears to have been co-opted to control gene expression. Today, in addition to repressing the expression of uORF-containing genes, pseudogenes and the products of alternative splicing, NMD may allow for the evolution of new introns. The presence of NMD may act as a buffer for novel introns with weak splice sites<sup>107,110</sup>. In fact, the red algae *C. merolae* only has 27 introns<sup>74</sup> and is missing all of the classical NMD factors with the exception of a UPF1 homologue<sup>51</sup>. It is possible that *C. merolae* lacks a functional NMD pathway and this limits the acquisition of new introns, at least partly explaining its intron depleted genome.

### Unanswered questions

Many years of study have revealed diverse NMD pathways, centering on UPF1. However, there are a number of fundamental questions remaining in the field regarding the mechanisms and evolution of NMD.

- 1) Why is SMG1 repeatedly lost in different lineages? Is there a backup mechanism to activate UPF1 and is this conserved between the lineages that have recently lost (eg *A. thaliana*) and more anciently lost (eg baker's yeast and *T. thermophila*) SMG1? Or are there multiple SMG1 replacement mechanisms?
- 2) What recruits the RNA degradation machinery to UPF1 when SMG1 is lost and S/TQ dipeptides are depleted? Does it rely on the direct interactions of SMG5/6/7 family proteins with UPF1 or another mechanism?
- 3) If phosphorylation of UPF1 represents a checkpoint in the activation of NMD, what explains the variability of the presence of this checkpoint between species?
- 4) Can the EJC mode of PTC recognition exist without the involvement of the EJC, potentially in *T. thermophila*? If so, what is the molecular basis for this and does it exist in other species?
- 5) What is the precise mechanistic roles of UPF2/UPF3 in relation to EJC mode and non-EJC mode NMD pathways? How do UPF2/UPF3 get recruited to NMD targets independently of the EJC?
- 6) To understand the discrepancies between transcriptome-wide and reporter construct approaches to the long 3' UTR mode of NMD and to uncover the molecular mechanism(s) behind the long 3' UTR mode.
- 7) To identify what precisely determines the accumulation of UPF1 on some transcripts, and why this appears to be dependent UPF1 ATPase activity<sup>111</sup>.
- 8) What is the mechanism leading to NMD of uORF transcripts? Is it EJC mode, long 3' UTR mode, both or neither? This will need to be done for each uORF transcript of interest.

Hopefully future research efforts can resolve these and other unknowns surrounding NMD.

### Conclusion

Here I have discussed the NMD pathway in the context of evolution and the many shapes the NMD pathways takes. I have proposed a classification system with four types of NMD pathway, based on the presence/absence of conserved NMD factors. I propose that the classical (type 1) NMD involves UPF1-3, the UPF1-kinase SMG1 and the SMG5/6/7 family. The recent (type 2) and ancient (type 3) loss of SMG1 define the next two types of NMD, while loss of all but UPF1 and perhaps UPF2 define the final type (type 4), where NMD might not actually function at all.



It is highly likely that species specific NMD factors have been co-opted in many, if not all, of these types of NMD pathway and are waiting to be discovered. Discussing the evolution and mechanism of NMD within this framework will hopefully aid in the communication of ideas between different model systems used to study NMD and therefore help in knowledge acquisition. Finally, I outline key outstanding questions regarding the mechanism and evolution of the NMD pathway. Focused research efforts to address these issues will certainly help in our overall understanding of the NMD pathway and for us to at last appreciate the true fundamental nature of NMD.

### Data availability

No data are associated with this article.

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# Open Peer Review

Current Referee Status:



Version 1

Referee Report 25 September 2018

<https://doi.org/10.5256/f1000research.17330.r37784>



**J. Robert Hogg** 

Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA

The nonsense-mediated mRNA decay pathway is found throughout eukaryotes, where it performs important quality control and regulatory functions. How the pathway originally emerged and has subsequently adapted to diverse eukaryotic transcriptomes is an important but poorly understood question. This manuscript represents a helpful summary of the evidence for compositionally- and mechanistically-distinct NMD pathways in diverse eukaryotes and raises interesting questions that deserve future study.

Major points:

1. The review raises the question of how RNA decay machinery is recruited to NMD substrate mRNAs in organisms that have lost SMG1. Work from yeast and human cells give two possible answers to this question. First, the Jacobson and Parker labs have presented evidence that budding yeast UPF1 directly interacts with components of the decapping complex, enabling SMG1- and SMG6-independent decay <sup>1,2</sup>. Second, the Conti and Muhlemann groups have <sup>3,4</sup> shown that SMG6 can interact with UPF1 in a phosphorylation-independent manner. Their data suggest that UPF1 phosphorylation may be dispensable for SMG6 recruitment but may contribute to activation of its endonucleolytic activity, raising the possibility that a SMG-1 independent pathway could rely on phosphorylation-independent SMG6-UPF1 interactions coupled with a distinct mechanism for activation of SMG6.
2. Figure 3: The schematics in the figure suggest that there is a universal step of mRNP remodeling that involves ribosome displacement from target mRNAs prior to initiation of decay, but it is not clear that this is the case. Previous work from the Baker lab has indicated that budding yeast NMD can initiate on polysome-bound mRNAs, rather than those stripped of ribosomes <sup>5</sup>. In addition, this figure and Figure 1 should acknowledge that there is evidence that “classical” NMD can also proceed through deadenylation, decapping, and exonucleolytic decay, not just SMG6-mediated cleavage. For other organisms, “cleavage” implies an endonucleolytic step, which in organisms lacking SMG6 is not known to occur. As referenced above, yeast NMD proceeds through decapping, and this should be made clear in the figure.
3. It would be helpful to note that in *Drosophila*, a much more significant role for SMG1 is uncovered in SMG5 mutants <sup>6</sup>. This is consistent with the idea that organisms such as *Drosophila* have developed redundant pathways for NMD. However, this also means that the extent to which “the

dependence on SMG1 is not always clear” may be overstated. The fact that SMG1 has been maintained in this organism and can function in at least some contexts should carry greater weight than the failure to observe a strong phenotype in the limited experimental contexts in which it has been examined.

4. Page 6, second paragraph: It is somewhat misleading to state that “transcriptome-wide studies find so few long 3’UTR transcripts that are targeted to NMD.” It is true that there have been differing reports of the extent to which 3’UTR length correlates with decay susceptibility transcriptome-wide, but this is not the same as saying that these studies did not find evidence that substantial numbers of long 3’UTR-containing transcripts are subject to NMD. In addition, it is important to recognize that the Lindeboom *et al.* study cited here examined apparent NMD susceptibility of mRNAs with nonsense mutations, not the scope of long 3’UTR-mediated decay among normal transcripts <sup>7</sup>.
5. Page 6, second paragraph, continued: The poly-A binding protein-centric model and the UPF1 length-sensing model are not necessarily exclusive. It has been reported that Pab1 and poly-A tails are dispensable for accurate NMD target discrimination in yeast <sup>8</sup>, but it is possible that UPF1 binding contributes to competition between poly-A binding protein and NMD factors for binding to release factors at the terminating ribosome. Another emerging possibility is that PABP antagonizes UPF1 binding to 3’UTRs, as proposed by Lee *et al.* <sup>9</sup>.

Minor points:

1. It would be helpful to the reader to reference recent evidence that PNRC2 may function in general decapping but contribute minimally to NMD in vertebrates <sup>10</sup>.
2. Page 2, second paragraph: Since UPF2 and UPF3 were identified and initially characterized in yeast, in which UPF1 phosphorylation is not known to play a key role in decay, the basis for the statement that “initially, NMD factors were defined by their role in the phosphorylation of UPF1” is unclear.
3. Page 2, first paragraph: The Maquat *et al.*, 1981 paper was not based on a “mutant screen” but instead observations in human genetic disease <sup>11</sup>.
4. Figure 1 implies that the roles of UPF2 and UPF3 are dependent on the EJC, but this is not the case — these proteins have been found to be required for decay of NMD substrates lacking 3’UTR introns <sup>12,13</sup>.
5. At several points, it is not clear whether the authors use the nomenclature ‘SMG5-7’ to refer to SMG5, 6, and 7, or just SMG5 and 7, as is the more standard usage. For example, on Page 3, first paragraph, it should be made clear that SMG6 does not function in “recruiting the degradation machinery” but is itself an endonuclease.
6. Page 5, fifth paragraph. It is possible that other PIKK-type kinases other than ATM and ATR are re-purposed to phosphorylate UPF1.

7. Discussion of the Tetrahymena data should acknowledge that at this point the evidence for a role for exon junctions is correlative and remains to be mechanistically investigated. This is an important caveat to the classification system offered by the author <sup>14</sup>.
8. Page 6, third paragraph: More recent investigations of yeast NMD have not uncovered evidence for a downstream sequence element that contributes significantly to decay target discrimination <sup>8, 15</sup>.

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**Is the topic of the review discussed comprehensively in the context of the current literature?**

Yes

**Are all factual statements correct and adequately supported by citations?**

Partly

**Is the review written in accessible language?**

Yes

**Are the conclusions drawn appropriate in the context of the current research literature?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 12 Nov 2018

**James Lloyd**, University of Western Australia, Australia

Thank you for your thoughtful and insightful reading of my review. You raised a number of important points that I have tried to address in version 2 of my review article. Some of the points I directly address are:

Second, the Conti and Muhlemann groups have <sup>3,4</sup> shown that SMG6 can interact with UPF1 in a phosphorylation-independent manner.

It would be helpful to note that in *Drosophila*, a much more significant role for SMG1 is uncovered in SMG5 mutants <sup>6</sup>.

*This is a very important point and I think that this points to a possible mechanism to explain how some species can have an active NMD pathway without SMG1. When we combine this with recent yeast work finding direct interactions between EBS1 and NMD4, and UPF1, this suggests that in many cases, phosphorylation is not needed for NMD. This supports a model that in mammals phosphorylation of UPF1 increases if NMD is stalled, suggesting that it acts to increase the recruitment of degradation factors (<https://www.nature.com/articles/ncomms12434>), potentially in NMD limiting conditions or for tricky to degrade transcripts.*

The poly-A binding protein-centric model and the UPF1 length-sensing model are not necessarily exclusive. It has been reported that Pab1 and poly-A tails are dispensable for accurate NMD target discrimination in yeast <sup>8</sup>, but it is possible that UPF1 binding contributes to competition between poly-A binding protein and NMD factors for binding to release factors at the terminating ribosome. Another emerging possibility is that PABP antagonizes UPF1 binding to 3'UTRs, as proposed by Lee et al. <sup>9</sup>.

*This is a fair point and I did not mean to suggest that the two models were mutually exclusive in the first version. Also I think that Lee et al. is a great reference for me to include, thank you for bringing it to my attention!*

It would be helpful to the reader to reference recent evidence that PNRC2 may function in general decapping but contribute minimally to NMD in vertebrates <sup>10</sup>.

*Thank you for raising this point about PNRC2, as did other referees and I have removed mention of it from my review for the sake of simplicity.*

Figure 1 implies that the roles of UPF2 and UPF3 are dependent on the EJC, but this is not the case — these proteins have been found to be required for decay of NMD substrates lacking 3'UTR introns <sup>12,13</sup>.

*Yes this is a very important point and I did not mean to give that impression with this figure. I have removed the EJC from Figure 3 and I have mentioned in the legend of Figure 1 that NMD can happen without an EJC present in the now submitted version 2 of this manuscript.*

It is possible that other PIKK-type kinases other than ATM and ATR are re-purposed to phosphorylate UPF1.

*The only other kinase active PIKK in Arabidopsis is TOR, which I have now included in the submitted version 2 of this review.*

More recent investigations of yeast NMD have not uncovered evidence for a downstream sequence element that contributes significantly to decay target discrimination <sup>8,15</sup>.

*I have now removed the section of DSE from the submitted version 2 of this review.*

**Competing Interests:** No competing interests were disclosed.

Referee Report 24 September 2018

<https://doi.org/10.5256/f1000research.17330.r38016>



**Niels H. Gehring** 

Institute for Genetics, Department of Biology, University of Cologne, Cologne, North Rhine-Westphalia, Germany

In this review article James P. B. Lloyd summarizes the evolution of NMD in various eukaryotic organisms. He describes the process of NMD in general as well as the detection of NMD substrates by the NMD machinery and discusses the functions of several NMD factors. As a novelty, he introduces a new classification of NMD pathways based on the presence of NMD factors in different organisms.



In my view, this is a well-written review that takes a refreshing new look at evolutionary aspects of NMD. However, one should keep in mind that the absence of a certain NMD pathway does not necessarily mean that it really does not exist in a particular organism. A good example for this is NMD in *Drosophila*, which was thought to be EJC-independent based on initial publications. However, there is now evidence that certain NMD substrates in *Drosophila* are degraded in an EJC-dependent manner. It is therefore possible that the classification of NMD pathways will change with future publications.

Additional comments:

Page 4, last paragraph on the right side: The author probably does not mean ETS1, but EST1 (Reichenbach *et al.*, 2003<sup>1</sup>). Furthermore, a second SMG6 homologue in yeast may be NMD4, which has been originally identified in a yeast-2-hybrid screen (He and Jacobson, 1995<sup>2</sup>) and was recently described by Dehecq *et al.* (Dehecq *et al.*, 2018<sup>3</sup>) to be associated with UPF1.

Page 4, last paragraph on the right side and page 5, first paragraph on the left side: Wang *et al.* (Wang *et al.*, 2006<sup>4</sup>) and de Pinto *et al.* (de Pinto *et al.*, 2004<sup>5</sup>) have reported that UPF1 and UPF2 are phosphoproteins in yeast. However, it is not clear which kinase is responsible for the phosphorylation of UPF1 and UPF2 and whether the phosphorylation plays a functional role during NMD in yeast.

Page 5, penultimate paragraph on the right side: It is strongly debated whether PNRC2 is an NMD factor (Nicholson *et al.*, 2018<sup>6</sup>). Therefore, PNRC2 is not a good example for a vertebrate-specific NMD factor.

Page 6, last paragraph on the right side: the meaning of the sentence “These adaptations ensure that retention of these efficiently-spliced introns would not be repeatedly translation” is not clear to me.

Page 7, question 2: It is known that SMG6 can interact with UPF1 also in a phosphorylation-independent manner (Nicholson *et al.*, 2014<sup>7</sup> and Chakrabarti *et al.*, 2014<sup>8</sup>). This could explain how NMD substrates are degraded in the absence of SMG1 or when S/TQ dipeptides are depleted.

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**Is the topic of the review discussed comprehensively in the context of the current literature?**

Yes

**Are all factual statements correct and adequately supported by citations?**

Yes

**Is the review written in accessible language?**

Yes

**Are the conclusions drawn appropriate in the context of the current research literature?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** mRNA turnover (nonsense-mediated mRNA decay)

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 12 Nov 2018

**James Lloyd**, University of Western Australia, Australia

I am truly grateful for your careful reading and thoughtful feedback of my review. I have now submitted a version 2 of this article that updated the text and figures with many of the insights you and the other referees offered. Below are some highlights of things that I address in the updated manuscript:

one should keep in mind that the absence of a certain NMD pathway does not necessarily mean that it really does not exist in a particular organism. A good example for this is NMD in *Drosophila*, which was thought to be EJC-independent based on initial publications. However, there is now evidence that certain NMD substrates in *Drosophila* are degraded in an EJC-dependent manner. It is therefore possible that the classification of NMD pathways will change with future publications.

*I completely agree with this point and I hope that the readers will take this message away with them after reading my review.*

The author probably does not mean ETS1, but EST1

*Yes, thank you for catching this.*

Furthermore, a second SMG6 homologue in yeast may be NMD4, which has been originally identified in a yeast-2-hybrid screen (He and Jacobson, 1995<sup>2</sup>) and was recently described by Dehecq et al. (Dehecq et al., 2018<sup>3</sup>) to be associated with UPF1.

*Thank you for bringing Dehecq et al. to my attention! I think that this article represents very important work and I have now included much discussion of it and its implications in the submitted version 2 of my article.*

penultimate paragraph on the right side: It is strongly debated whether PNRC2 is an NMD factor

*Thank you for raising this point about PNRC2, as did other referees and I have removed mention of it from my review for the sake of simplicity.*

It is known that SMG6 can interact with UPF1 also in a phosphorylation-independent manner (Nicholson et al., 2014<sup>7</sup> and Chakrabarti et al., 2014<sup>8</sup>). This could explain how NMD substrates are degraded in the absence of SMG1 or when S/TQ dipeptides are depleted.

*Yes, I think that this is a really great point and I discuss this at length in the submitted version 2 of the review. Thank you for raising this point.*

**Competing Interests:** No competing interests were disclosed.

Referee Report 21 September 2018

<https://doi.org/10.5256/f1000research.17330.r37786>



**Damien Garcia** 

Institute of Molecular Biology of Plants (IBMP), University of Strasbourg, Strasbourg, France

In this review, the author suggests a novel NMD classification system based on the evolutionary conservation of known NMD factors among different species. The author also discusses a possible driving force for the apparition of NMD. This is a well-written and structured manuscript considering NMD under an interesting evolutionary point of view. It is of broad interest for the NMD community, and it mentions several fundamental questions currently unanswered in the NMD field. Nevertheless, a few modifications of the figures and the addition of some very important references and concepts could make it easier to read and broaden the general interest of the review, as detailed in the following paragraphs.

#### Major comments:

1. In Figure 1, the model of mammalian NMD indicates cleavage of the NMD target downstream of the PTC. It doesn't indicate the distinct roles of SMG6 and SMG5/7; it would be important here to indicate the distinct involvement of SMG6 in the cleavage activity and the role of SMG5/7 in decay factors recruitment. These two routes for decay should clearly appear in Figure 1.

2. In Figure 2, the definition of Type 1/2/3/4 NMD should already be mentioned in the figure legends (e.g. Type 1: classical SMG1 dependent NMD, Type 2: recent loss of SMG1 with conserved phosphorylation, Type 3: ancient loss of SMG1 with loss of UPF1 S/Q phosphorylation, Type 4: Heavily derived NMD).
3. In the paragraph 'Defining NMD targets', the author cites the 2016 paper on the NMD protection effect of PTB1; an earlier study describing similar protection effect of Pub1 in yeast should also be cited (Ruiz-Echevarria and Peltz, 2000<sup>1</sup>).
4. In the same paragraph, the author focuses on Baker's yeast DSE, stimulating NMD. The author should also mention a recent paper in yeast describing that poor translation efficiency is a major criteria for NMD targeting (Celik et al., 2017<sup>2</sup>). This major result could potentially explain not yet understood deregulations observed in several other species upon NMD knockdown.
5. It was proposed that invading RNAs of external origin, including TEs and viruses, could be a driving force for NMD apparition and evolution - this should be mentioned in the paragraph on the possible origin of NMD (Hamid and Makeyev, 2016<sup>3</sup>).

#### Minor comments:

1. UPF1 might have other essential functions beyond NMD, as observed for UPF3b in mammals, involved in translation termination (Neu-Yilik et al., 2017<sup>4</sup>), which could explain its presence in some species without any other known NMD factors. This could be mentioned in the corresponding section.
2. As branches of NMD exist without the need of UFP2/UPF3, it suggests that NMD could be active with only UPF1. This possibility should be discussed/mentioned when describing Type 4 species depleted of UPF2 and UPF3.
3. In addition to ATR/ATM, the author could mention TOR or TRRAP kinases as described in (Lloyd and Davies, 2013<sup>5</sup>), as possible kinase replacements for SMG1.
4. In Figure 3, the author should add precisions on endonucleolytic cleavage and decay factor recruitment for **(A)**, and decay factor recruitment only for the others. The different types of NMD defined in Figure 2 should be mentioned again here in Figure 3, namely: Type 1, Type 2, Type 3 and Type 4.

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**Is the topic of the review discussed comprehensively in the context of the current literature?**

Yes

**Are all factual statements correct and adequately supported by citations?**

Yes

**Is the review written in accessible language?**

Yes

**Are the conclusions drawn appropriate in the context of the current research literature?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 12 Nov 2018

**James Lloyd**, University of Western Australia, Australia

I am thankful for you carefully reading my review and giving great suggestions for its improvement. I have now submitted a version 2 of this article that updated the text and figures with many of the insights you and the other referees offered. Below are some highlights of things that I address in the updated manuscript:

involvement of SMG6 in the cleavage activity and the role of SMG5/7 in decay factors recruitment. These two routes for decay should clearly appear in Figure 1.

*This is a great point and I have tried to address this in the submitted version 2 of the review.*

In Figure 2, the definition of Type 1/2/3/4 NMD should already be mentioned in the figure legends

*I have now added this in the submitted version 2 of this review.*

In the paragraph 'Defining NMD targets', the author cites the 2016 paper on the NMD protection effect of PTB1; an earlier study describing similar protection effect of Pub1 in yeast should also be cited

*Thank you for bringing this to my attention and it is now included in the submitted version 2 of this review.*

It was proposed that invading RNAs of external origin, including TEs and viruses, could be a driving force for NMD apparition and evolution - this should be mentioned in the paragraph on the

possible origin of NMD (Hamid and Makeyev, 2016<sup>3</sup>).

*I have now cited this work in the submitted version 2 of this review.*

UPF1 might have other essential functions beyond NMD, as observed for UPF3b in mammals, involved in translation termination (Neu-Yilik et al., 2017<sup>4</sup>), which could explain its presence in some species without any other known NMD factors. This could be mentioned in the corresponding section.

*Great point and I have now mentioned this in the submitted version 2 of this review.*

As branches of NMD exist without the need of UFP2/UPF3, it suggests that NMD could be active with only UPF1. This possibility should be discussed/mentioned when describing Type 4 species depleted of UPF2 and UPF3.

*Agreed and this is now mentioned in the submitted version 2 of this review.*

In addition to ATR/ATM, the author could mention TOR or TRRAP kinases as described in (Lloyd and Davies, 2013<sup>5</sup>), as possible kinase replacements for SMG1.

*Good point, I have now added TOR to the submitted version 2 of this review. I did not add TRRAP because TRRAP has been reported to be a kinase dead member of the family (<https://www.sciencedirect.com/science/article/pii/S0092867400814798?via%3Dihub>).*

**Competing Interests:** No competing interests were disclosed.

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### Wei Miao

Key Laboratory of Aquatic Biodiversity and Conservation, Institute of Hydrobiology (IHB), Chinese Academy of Sciences (CAS), Wuhan, China

In this manuscript, the author discussed the diversity of the NMD pathway according to the variation of NMD factors in different eukaryotes, and briefly summarized some popular NMD models. Furthermore, the author also discussed the relationship between intron gain/loss and NMD. Several interesting and unsolved questions in this field were also mentioned by the end of the manuscript. Interestingly, the author came up with a novel classification system (although it needs to be further modified, see below) to classify NMD mechanisms into different types based on the new criterion. Namely, it classifies NMD mechanisms according to the presence or absence of key factors that related to the well-characterized Upf1 C-terminus phosphorylation events. The manuscript is clearly written, and this work is likely to be of

general interest to the NMD field. My main criticism is about the novel classification system.

#### Major

1. As it is still unclear whether excavates have functional NMD, the type 4 NMD may not need to be taken into account. Or question marks should be included in the figure 2 and figure 3D, and in the main text.
2. Type 1 NMD seems to be SMG1-dependent and EJC-dependent (see figure 3A). Hence it cannot be exemplified by *C. elegans* (EJC-independent NMD). The author could modify the figure a little. For example, one could draw EJC with a dashed line to indicate it is dispensable for NMD in some organisms, like *C. elegans*. Besides, the presence of Smg1 and Upf1 orthologs in *N. gruberi* does not necessarily mean it possesses a functional NMD pathway, thus I suggest not consider it has type 1 NMD. Or, as I mentioned above, excavates need not be discussed too much.
3. As mentioned by the author, some organisms (e.g., *D. melanogaster* and *D. rerio*) are considered to have SMG1-dependent type 1 NMD, yet their SMG1 proteins are dispensable for NMD activation and therefore similar to the type 2 NMD. I am wondering whether this issue can be solved by defining another type of NMD, namely in between type 1 and type 2. Besides, an additional figure (e.g., figure 3 in (Lareau and Brenner, 2015<sup>1</sup>)) could be provided to show the evolutionary relationship between different types of NMD.
4. Figure 2 needs to be modified. Clearly, solely based on the pattern of these icons (NMD proteins), readers may get confused why organisms with the same pattern are not classified into the same group. For example, like *C. rubella*, *Tetrahymena* also has a red square and a blue triangle. However, they are classified into different types. Although, in this case, it can be easily solved by adding another icon to indicate Upf1 with phosphorylatable S/TQ motifs. Additional modifications are required to let readers understand, for example, why *Dikarya* and *Mirosporia* are considered as type 1, but not type 2.

#### Minor

1. Page 2, left panel, lane 10. Ciliates should be mentioned here as well, because NMD was also proved to be required for regulating many of their transcripts (Jaillon et al. 2008<sup>2</sup>, Tian et al. 2017<sup>3</sup>).
2. Page 2, right panel, lanes 19 – 20. I suggest deleting this sentence. Or, I would rather say that, even in animals, NMD factors were simply defined by their requirement for NMD. Because phosphorylation of Upf1 seems to be not essential for eliciting NMD in some animals (e.g., SMG1-independent NMD in fruit flies and zebrafish).
3. Page 2, figure 1, the bottom panel. I suggest moving Upf2, Upf1 and its associated proteins (especially the endonuclease Smg6) to the left side of the EJC, close to the endonucleolytic cleavage site.
4. Page 2, figure 1, the title of figure legend. The word “Animals” is probably too general, hence the author may want to replace it with another word (e.g., vertebrates). Because it is known that EJC is not required or dispensable for NMD in some invertebrates (Longman et al. 2007<sup>4</sup>, Gatfield et al. 2003<sup>5</sup>).

5. Page 2, figure 1, the last sentence of the figure legend. Replace “phoso-UPF1” with “phosphor-UPF1” or “phos-UPF1”.
6. Page 3, right panel, lane 8. Smg7 recruits CCR4–NOT deadenylase complex instead of exonuclease (Loh et al. 2013<sup>6</sup>).
7. Page 3, left panel, lane 14. Reference 34 (Rosains and Mango, 2012<sup>7</sup>) suggests that Smg8 is not required for NMD in *C. elegans*.
8. Page 3, left panel, lane 28. Change the word “animals”, see comments #3.
9. Page 3, left panel, the first paragraph of the section “Variations on a common pathway”. Since it is still unclear whether NMD exists in excavates, it is inappropriate to say that they have “the most divergent NMD pathway”. Some following sentences in this paragraph are also against the existence of NMD pathway in excavates. To support the hypothesis “a complex NMD pathway involving... in the last eukaryotic common ancestor”, the author could start the discussion from plant NMD mechanism, and then use the existence of orthologs of NMD core factors in excavates as a supporting evidence.
10. Page 4, figure 3 legend. “In *T.*, it has been shown” should be “In *T. brucei* ...”.
11. Page 5, left panel, lanes 2 – 3 and lanes 20 – 21. The author should mention that the interaction between Upf1 and Smg6 can also be phosphorylation-independent (Chakrabarti et al. 2014<sup>8</sup>).
12. Page 5 and many other places. It is better to use a unified way to indicate different organisms (groups). For example: change “*C. elegans*, humans, and moss” to “*C. elegans*, *H. sapiens* and *P. patens*” or “worms, humans and moss”.
13. Page 5, right panel, lane 5. Is there experimental evidence to support that S/TQ sites have undergone phosphorylation? If not, I suggest replacing “S/TQ dipeptide phosphorylation sites” with “phosphorylatable S/TQ motifs”.
14. Page 5, right panel, “PNRC2 is a vertebrate-specific NMD factor”. The author may want to remove this sentence because a recent study has shown that PNRC2 may not be required for NMD (Nicholson et al. 2018<sup>9</sup>).
15. Page 6, left panel, lanes 19 – 21. The author should modify this sentence a little. Despite the splice junctions downstream of the stop codon (DSJ) are enriched in potential *Tetrahymena* NMD targets (Tian et al. 2017<sup>3</sup>), it is still insufficient to conclude that “NMD in *T. thermophila* is dependent on DSJ”.
16. Page 6, left panel, bottom. The description of DSE model could be removed because evidence from a few studies are against this model (for example (Meaux et al. 2008<sup>10</sup>)).
17. Page 6, right panel, lane 12. The fungus *N. crassa* should also be mentioned here, due to the requirement of EJC for its NMD pathway.



18. Page 6, right panel, section “The origins of NMD”, the second sentence. The word “clear” is too strong here.
19. Page 6, right panel, section “The origins of NMD”. Firstly, the author may want to change the subtitle of this section. In this section, a clear answer to origins of NMD is not given, and discussions are mainly about the relationship between NMD and the intron evolution. Secondly, when discussing the relationship between intron evolution and (the evolution of) NMD, an earlier study from Michael Lynch and Avinash Kewalramani is deserved to be mentioned here (Lynch et al. 2003<sup>11</sup>).
20. Page 7, left panel, section “unanswered questions”. Regarding the question 2, the author should consider the existence of phosphorylation-independent interaction between Upf1 and Smg6. The question 4 should be modified as the existence of “an EJC mode of PTC recognition” in *Tetrahymena* has not been proven yet.

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**Is the topic of the review discussed comprehensively in the context of the current literature?**

Yes

**Are all factual statements correct and adequately supported by citations?**

Partly

**Is the review written in accessible language?**

Yes

**Are the conclusions drawn appropriate in the context of the current research literature?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 12 Nov 2018

**James Lloyd**, University of Western Australia, Australia

Thank you for reading my review and giving so many insights into how I can improve my manuscript. I have now submitted a version 2 of this article that updated the text and figures with many of the insights you and the other referees offered. Below are some highlights of things that I address in the updated manuscript:

As it is still unclear whether excavates have functional NMD, the type 4 NMD may not need to be taken into account

*Certainly and I have tried to make this clear in the submitted version 2 of this review.*

Type 1 NMD seems to be SMG1-dependent and EJC-dependent (see figure 3A). Hence it cannot be exemplified by *C. elegans* (EJC-independent NMD).

*I did not mean to give that impression so I have removed the EJC from Figure 3 of the submitted version 2 of this review. Also, while no reports of EJC involvement in *C. elegans* are published, I do not think we have enough data to state whether NMD in *C. elegans* is really EJC-independent and I look forward to future work that might add to this.*

Figure 2 needs to be modified. Clearly, solely based on the pattern of these icons (NMD proteins), readers may get confused why organisms with the same pattern are not classified into the same group. For example, like *C. rubella*, *Tetrahymena* also has a red square and a blue triangle. However, they are classified into different types. Although, in this case, it can be easily solved by adding another icon to indicate Upf1 with phosphorylatable S/TQ motifs. Additional modifications are required to let readers understand, for example, why *Dikarya* and *Mirosporia* are considered as

type 1, but not type 2.

*Thank you for raising some issues with Figure 2. I have now corrected some of my mistakes of misclassification in this figure. I was not satisfied with any of my attempts to differentiate between UPF1 proteins of Type 2 and 3 so that I have left unchanged but I was a good point that you raise.*

“PNRC2 is a vertebrate-specific NMD factor”. The author may want to remove this sentence because a recent study has shown that PNRC2 may not be required for NMD

*Thank you for raising this point about PNRC2, as did other referees and I have removed mention of it from my review for the sake of simplicity.*

The description of DSE model could be removed because evidence from a few studies are against this model

*This is a great point and I have now removed the section of DSE from the submitted version 2 of this review.*

**Competing Interests:** No competing interests were disclosed.

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