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Review

Nonsense-mediated mRNA decay — Mechanisms of substrate mRNA recognition and degradation in mammalian cells ☆

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ABSTRACT

The nonsense-mediated mRNA decay (NMD) pathway is well known as a translation-coupled quality control system that recognizes and degrades aberrant mRNAs with truncated open reading frames (ORF) due to the presence of a premature termination codon (PTC). However, a more general role of NMD in posttranscriptional regulation of gene expression is indicated by transcriptome-wide mRNA profilings that identified a plethora of physiological mRNAs as NMD targets. In this review, we focus on mechanistic aspects of target mRNA identification and degradation in mammalian cells, based on the available biochemical and genetic data, and point out knowledge gaps. Translation termination in a messenger ribonucleoprotein particle (mRNP) environment lacking necessary factors for proper translation termination emerges as a key determinant for subjecting an mRNA to NMD, and we therefore review recent structural and mechanistic insight into translation termination. In addition, the central role of UPF1, its crucial phosphorylation/dephosphorylation cycle and dynamic interactions with other NMD factors are discussed. Moreover, we address the role of exon junction complexes (EJCs) in NMD and summarize the functions of SMG5, SMG6 and SMG7 in promoting mRNA decay through different routes. This article is part of a Special Issue entitled: RNA Decay mechanisms.

1. Introduction

The expression of genetic information in eukaryotes depends on the proper timing and spatial order of numerous chemical reactions and molecular interactions [1]. Errors inherent in each step along the elaborate pathway of mRNA synthesis and processing sum up to a considerable fraction of aberrant mRNAs that undermine the accuracy of gene expression. mRNA quality control processes therefore play an important, in vertebrates even an essential role, in recognizing and eliminating such problematic mRNAs [2–4]. So far, three different translation-coupled mRNA surveillance systems have been described in eukaryotes that all recognize and degrade mRNAs that cause problems during the process of translation. Two of these surveillance pathways, no-go mRNA decay and non-stop mRNA decay, act on mRNAs on which the ribosome halts unexpectedly either somewhere within the ORF or at the physical 3' end, respectively. In both cases, there is no stop codon present and hence no release factors engage with the stalled ribosome. No-go mRNA decay and non-stop mRNA decay share common factors and appear to be mechanistically related [4]. These two processes are described by Inada in another review in this issue (ref: this issue) and in other recent reviews [5,6]. In this review, we will focus on mechanistic aspects of NMD.

More than 30 years ago, it was discovered in *Saccharomyces cerevisiae* and in human bone marrow cells of β-thalassemia patients that the half-lives of mRNAs of which the ORFs are truncated by the presence of a PTC are reduced compared to the corresponding PTC-free mRNAs [7,8]. Over the years, destabilization of PTC-containing mRNAs has been reported in many other eukaryotic species, including the two highly divergent protists *Giardia lamblia* and *Trypanosoma brucei* [9,10], suggesting that NMD has evolved very early during the development of eukaryotes and exists in essentially all eukaryotes.

Because about 30% of all known disease-associated mutations result in the production of mRNAs with a PTC, NMD plays an important role as a modulator of the clinical manifestations of many genetic diseases (reviewed in [11–13]). NMD can be beneficial by preventing the production of C-terminally truncated proteins with a dominant-negative function, but there are also cases where the truncated protein encoded by the PTC-containing mRNA still has some residual function and the NMD-mediated reduction of mRNA abundance results in more

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severe clinical problems. A better understanding of the molecular mechanism of NMD will therefore also benefit the development of therapeutic approaches aiming at specifically manipulating NMD efficacy and substrate specificity.

Until the advent of transcriptome-wide identification of endogenous NMD targets, NMD has been merely perceived as a quality control system that evolved to rid cells of aberrant, PTC-containing mRNAs. However, it has meanwhile become clear that in addition to its quality control function, NMD also plays an important role in regulating gene expression by targeting many physiological mRNAs that code for full-length functional proteins, thereby influencing a wide variety of biological processes. In fact, mRNA profiling of NMD defective *S. cerevisiae* [14–16], *Caenorhabditis elegans* [17], *Drosophila melanogaster* [18] and human cells [19–23] revealed that NMD affects the levels of 3–10% of all cellular mRNAs. Moreover, there is new evidence for differential regulation of individual NMD factors and NMD activity in different cell types and tissues, and for an autoregulation of NMD factor abundance [22,24–26]. Together with the accumulating evidence for the existence of multiple branches of the NMD pathway [27,28] (see below), this reveals a so far underappreciated complexity of NMD regulation and suggests that the involvement of NMD in a wide spectrum of biological processes. Consistent with this view, more complex organisms generally are more sensitive to reduced or abolished NMD activity than simpler ones. For example, NMD is an essential process in mammals, zebrafish, and fruit flies, whereas NMD deficient mutants of *S. cerevisiae* and *C. elegans* are viable and have only mild phenotypes. Knockout of the NMD factors UPF1, UPF2 or SMG1 (see below) in mice leads to early embryonic death [29–31], knockdown of NMD factors in zebrafish disrupts brain development [32,33], and there is evidence for a function of NMD in mammalian brain development [25]. Furthermore, mutations in the human NMD factor UPF3B are associated with mental retardation, autism and schizophrenia [34–36]. For a comprehensive review of the emerging roles of NMD in gene regulation, development and cellular responses to environmental cues, we refer the reader to an accompanying review by Karam et al. (ref: this issue). In addition, the role of NMD in animal embryogenesis was recently reviewed by Hwang and Maquat [37].

While the phenomenon of NMD and its impact on gene expression and genetic diseases is well documented, the understanding of the underlying molecular mechanisms is still fragmented, in spite of a fair amount of genetic, structural and biochemical data that has been gathered during the years. Definitively, the lack of a functional in vitro system for NMD is hampering progress in elucidating the mechanism of NMD. In this review, we summarize the available data about the major known NMD factors and discuss current mechanistic models of NMD, thereby focusing on mammalian NMD and pointing out commonalities and differences to models derived from other species.

2. What defines an NMD substrate?

The discovery that not only the initially identified PTC-containing mRNAs but also many PTC-less mRNAs are targeted by NMD (re-)posed the question, which features render an RNA susceptible to NMD and pointed out our limited understanding of the mechanism of substrate selection. Besides the presence of an ORF-interrupting PTC, upstream ORFs (uORFs), introns in the 3′ untranslated region (UTR) and long 3′ UTRs are empirically identified features that can trigger NMD (Fig. 1). Furthermore, poly(A) site mutation or alternative polyadenylation can generate NMD-targeted mRNAs [38–40]. It should be emphasized however that about 28% of the annotated human mRNAs have 3′ UTRs of >1000 nucleotides (C.S., unpublished data), yet only a small percentage of them are NMD substrates.

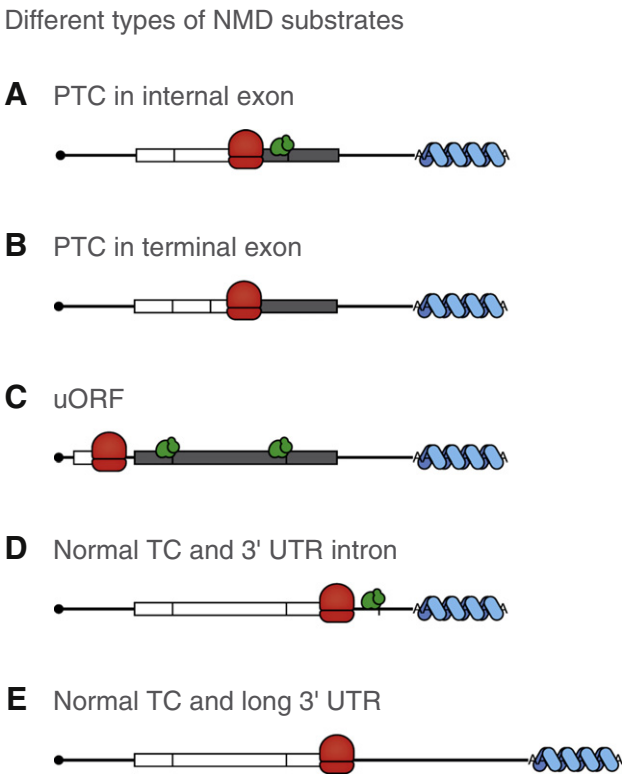

Fig. 1. Different types of mRNAs that can be substrates for NMD. (A) The classical mammalian NMD targets comprise mRNAs with a truncated ORF due to a premature termination codon (PTC) in an internal exon, on which one or several exon junction complexes (EJCs) are expected to remain after translation termination. (B) PTCs in terminal exons are the classical NMD substrates in *S. cerevisiae*, but many examples of this type have also been found in other organisms, including mammals. (C) Upstream ORFs (uORFs) 5′ of the main ORF are well-known NMD-inducers. (D) Introns in the 3′ UTR will in most cases lead to a remaining EJC after translation termination that can trigger NMD. (E) A long 3′ UTR can also function as an NMD-inducing feature in all investigated organisms. mRNAs are illustrated as black lines with a 5′ cap (black dot) and a 3′ poly(A) tail coated by poly(A)-binding protein (PABP, blue). White boxes denote translated coding sequence and gray boxes regions of the coding sequence that is not translated due to the presence of a PTC (A, B) or a uORF (C). Terminating ribosomes are illustrated in red, EJCs in green.

PTCs can arise at the DNA level by mutations or at the level of RNA by transcription errors or alternative pre-mRNA splicing. In fact, alternative splicing has been detected for at least 75% of human pre-mRNAs [41] and 45% of these alternatively spliced pre-mRNAs produce at least one mRNA isoform that might be degraded by NMD [42]. Consistently, splice isoforms of 30% of all expressed genes were found to be upregulated in mouse tissues ablated for the NMD factor UPF2 [43]. Hence, these unproductively spliced transcripts appear to constitute a substantial fraction of the NMD substrates in human cells.

Given that long 3′ UTRs can elicit NMD, the widespread differential poly(A) site usage observed under stress conditions [44,45], during mouse development [46], in proliferating cells [47], in cancer cells [48], by CF1 [49], and by U1 snRNP levels [50] are all predicted to potentially alter the half-lives of mRNAs and thereby influence gene expression.

3. mRNAs with a translation termination problem are targeted to NMD

What can we learn about the NMD mechanism from inspecting the different classes of NMD substrates depicted in Fig. 1? In contrast to mRNAs that are not affected by NMD, all different classes of NMD

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substrates have in common that translation terminates at an unusual position within the mRNP, either distant from the poly(A) tail or with an exon junction complex (EJC) located between the stop codon and the poly(A) tail (Fig. 1A–E). Several reports from different labs studying different organisms concluded that the exact arrangement of termination stimulating and antagonizing mRNP components downstream of a termination codon (TC) determines whether NMD ensues [51–57], reviewed in [58–60]. This basic concept is referred to as the *faux* 3′ UTR model [51] or the unified NMD model [61] and in essence posits that normal termination is a process that requires the presence of a set of termination stimulating factors. In contrast, translation termination in an mRNP environment devoid of these stimulating factors and/or in the vicinity of termination antagonizing factors is slow or even blocked, mechanistically distinct from normal termination, and leads to the formation of an NMD complex that finally recruits RNA degradation enzymes. On the following pages, we present in detail a version of this model, review the supporting evidence and point out open questions. For the sake of clarity, we first discuss how NMD substrate mRNAs are identified and then summarize our current knowledge about the degradation of these mRNAs.

4. EJCs in the 3′ UTR serve as assembly platforms for NMD factors

Early on, it was noticed that PTCs located >50–55 nucleotides upstream of the 3′ most exon–exon junction generally trigger NMD, whereas most PTCs located downstream of this 'boundary' appear to trigger no or a rather inefficient NMD response in mammals [62–64]. The discovery of the exon junction complex (EJC) [65], a multisubunit protein complex deposited on the mRNA during splicing 20–24 nucleotides upstream of exon–exon junctions (reviewed in [66,67]), provided a molecular explanation for the empirically detected '50–55 nucleotide boundary'. The EJC core, which consists of eIF4A3, MLN51 (also known as Barentsz, BTZ and CASC3) and the heterodimer MAGOH/Y14 [68,69], interacts through a composite surface formed by parts of Y14, MAGOH and eIF4A3 with the C-terminus of the NMD factor UPF3B [70] (Fig. 2). The UPF3B exon junction binding motif (EBM) is conserved in several other proteins, among them SMG6 (see below) [71]. Human UPF3 is a nucleo-cytoplasmic shuttling protein with orthologues from yeast to man, localizes mainly to the nucleus at steady state and is thought to bind the EJC before export of the mRNP to the cytoplasm [72,73]. In contrast to other species, the human genome harbors two UPF3 genes, UPF3A on chromosome 13 and UPF3B on the X chromosome (also known as UPF3X), each of which gives raise to two highly similar isoforms due to alternative splicing [73,74]. UPF3A is much less active than UPF3B in inducing NMD and stimulating translation [75]. Since UPF3A and UPF3B compete for binding to UPF2, high expression of UPF3A lowers cellular NMD activity. Under normal conditions however, UPF3B levels are high and UPF3A is rapidly turned over and is therefore only present at low levels [24]. In individuals harboring UPF3B mutations, UPF3A becomes stabilized and can support some NMD activity [24]. Whether the two splice isoforms of UPF3A and of UPF3B functionally differ from each other is currently not known, but there is evidence that the large UPF3A isoform associates with the early NMD complex and is exchanged with the small isoform at a later stage [76,77].

Like UPF3, UPF2 (also called RENT2) can also be considered a core NMD factor. It is conserved from yeast to man and functions as a bridge between UPF3 (i.e. the EJC) and the central NMD factor UPF1 [73,74,78,79] (Fig. 2). Furthermore, UPF2 is involved in SMG1-mediated phosphorylation of UPF1 [76] and it stimulates UPF1's helicase activity [80,81] (see below). In HeLa cells, UPF2 localizes perinuclear in the cytoplasm, although it harbors several putative nuclear localization signals [73].

Since EJCs positioned within the ORF are removed from the mRNA by elongating ribosomes [82] by a mechanism involving the ribosome-associated protein PYM [83], only mRNAs with a stop codon upstream of the '50–55 nucleotide boundary' are expected to still carry one or several EJCs when the first ribosome terminates. Such a leftover EJC can serve as an anchoring point for the assembly of the NMD complex and the subsequent degradation of the mRNA.

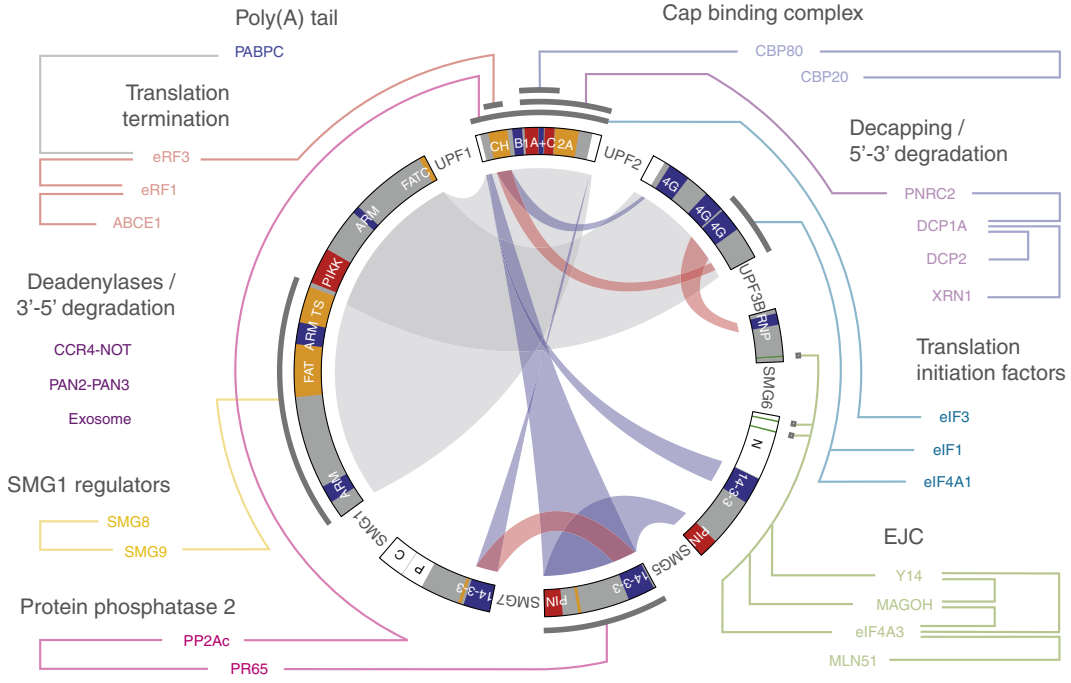

Fig. 2. NMD factor interaction map. The NMD factors UPF1, UPF2, UPF3B, SMG6, SMG5, SMG7, and SMG1 are schematically depicted on the central circle (clockwise, factor name always precedes corresponding schematic display). Specific motifs or domains in these proteins are indicated by colored boxes, labeled and discussed in the text. Reported interactions among these factors are depicted by graphical connections inside the circle. Interactions identified by co-immunoprecipitations with recombinant or in vitro translated proteins are shown in gray, interactions mapped by co-immunoprecipitations with exogenously expressed deletion or point mutants are shown in blue, and interactions derived from X-ray structures are shown in red. Interactions of additional proteins discussed in the text with the NMD factors in the circle are denoted by colored lines. Gray lines outside the circle indicate the region to which the interaction has been mapped.

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Further supporting this model, immunoprecipitation experiments and interaction studies with recombinant proteins showed that the EJC can assemble a complex together with UPF3, UPF2 and UPF1 [76,79,81] (Fig. 2). UPF1 is the most conserved NMD factor (48.5% amino acid identity between human and yeast) and plays a central role in NMD [84]. UPF1 belongs to the superfamily 1 (SF1) of ATP-dependent helicases [85]. As such, it has four major biochemical activities: ATP binding, ATP hydrolysis, single-strand polynucleotide binding, and 5′–3′ duplex unwinding [80,81,86–89]. In addition to the SF1 helicase domain, UPF1 contains a cysteine/histidine-rich (CH) domain that regulates the ATPase activity [80,81] and unstructured N- and C-terminal regions that are subjected to sequential phosphorylation/dephosphorylation cycles in *C. elegans* and mammalian cells [77,90–92]. A part of the C-terminal region also regulates the helicase [93]. Several lines of evidences suggest that in vivo formation of the UPF1-UPF2-UPF3 complex depends on translation and does either only form on NMD-targeted mRNAs or dissociates very quickly on mRNAs that do not undergo NMD [22,76,94–96]. To unravel the mechanism of NMD, it is therefore key to understand how NMD is coupled to translation.

5. Proper versus aberrant translation termination

Translation termination involves binding of the eukaryotic release factors (eRF) 1 and 3 to a ribosome stalling at a stop codon (UGA, UAG or UAA; reviewed in [97,98]). eRF3 is a GTPase that in its GTP-bound state forms a stable complex with eRF1 [99–103]. When binding to the A site of the ribosome, eRF1 adopts a tRNA-like structure, whereby a conserved groove of the N-terminal domain recognizes the stop codon, and the C-terminal domain interacts with eRF3 [104] (Fig. 3). The eRF1–eRF3 heterodimer binding into the A site leads to a two nucleotide shift of the ribosome, positioning the eRF complex probably closer to the P site [105]. eRF3 is an intrinsically repressed GTPase that requires two stimuli for efficient GTP hydrolysis, eRF1 interaction and binding to the ribosome [106]. GTP hydrolysis induces a conformational change that on the one hand moves the GGQ motif at the tip of the middle domain of eRF1 towards the peptidyl transfer center to induce hydrolysis of the polypeptide chain, and on the other hand leads to the dissociation of eRF3 [105,107]. Dissociation of eRF3 from the ribosome gives way for the termination factor ABCE1 (Rli1p in yeast) to interact with eRF1 and the stalled ribosome, which triggers dissociation and recycling of the ribosomal subunits [105,107–109] (Fig. 3). The otherwise not well conserved N-terminal region of eRF3 harbors two overlapping poly(A)-binding protein (PABP)-binding PAM2 motifs that are necessary for interaction of eRF3 with PABPC1 [110]. The functional consequence of the PABP-eRF3 interaction is not yet fully understood. On the one hand, it was reported that the N-terminal domain of *S. cerevisiae* eRF3 (Sup35p) is dispensable for translation termination [111]. On the other hand there is evidence that PABP facilitates translation termination [112–114], maybe by promoting eRF3-mediated GTP hydrolysis or by positioning the eRFs optimally for interacting with the stalled ribosome.

In contrast to this fast and efficient translation termination process (dubbed “proper translation termination”), during which ribosomes cannot be detected at the stop codon in toeprinting assays, termination at NMD-inducing stop codons mechanistically and kinetically differs, resulting in detectable ribosome toeprints both in *S. cerevisiae* cell extract and in rabbit reticulocyte lysate [51,115]. This prolonged pausing of the ribosome at the stop codon (dubbed “aberrant termination”) has been attributed to the absence of necessary termination stimulating factors/signals, for example PABP (reviewed in [116,117]). Since UPF1 has been found to interact with the release factors [76,118,119] (Fig. 2), it has been proposed that mRNAs that terminate translation aberrantly due to the absence of termination stimulating factors instead activate NMD by UPF1 binding to the stalled ribosome [51]. This means that at each termination event, competition between PABP and UPF1 for interacting with eRF3 would determine whether NMD ensues or not. Consistent with this competition model, Singh and colleagues showed that in vitro using human proteins, the UPF1–eRF3 interaction is outcompeted with substoichiometric amounts of PABPC1, which is the major cytoplasmic PABP species [55]. The model is further supported by the NMD suppression that can be achieved by artificial tethering of PABP near an otherwise NMD-triggering stop

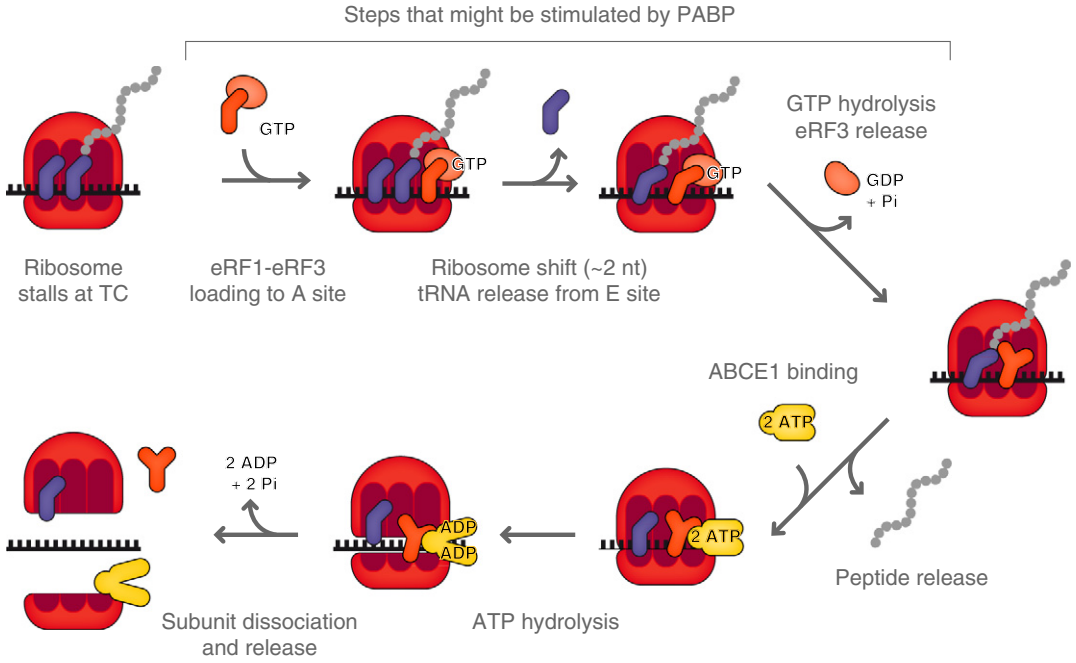

Fig. 3. Model for the sequential steps involved in proper translation termination and ribosome release. The ribosome (red) with its three tRNA binding sites E, P and A (dark red) is depicted at a stop codon. tRNAs are shown in purple, the nascent polypeptide chain as a string of gray dots, eRF1 in orange, the GTPase eRF3 in light orange, and the ABC-type ATPase ABCE1 in yellow. The proposed PABP-mediated promotion of termination could happen at one or several of the indicated steps: eRF1-eRF3 loading, 2 nucleotide ribosome shift, or eRF3-bound GTP hydrolysis. See text for details.

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codon [51–55,114] and by identification of long 3′ UTRs as an NMD-promoting feature in yeast, worms, Drosophila, mammals and plants [22,40,52,55,56,120–122]. However, there is data suggesting that the direct competition model (eRF-PABP vs. eRF-UPF1 complex formation) might oversimplify the actual mechanism of NMD substrate definition [114,123,124]. The binding assays with yeast proteins rather suggest a non-competitive inhibition of UPF1 interaction with eRFs in the presence of PABP [123], consistent with evidence for different binding sites on eRF3 for UPF1 and PABP: UPF1 interacts with the GTPase domain of eRF3, whereas PABP binds to the N-terminal 135 amino acids [110,112,114]. Irrespective of yet unsolved mechanistic details, the available data are consistent with UPF1 presence at the terminating ribosome preventing GTP hydrolysis and/or subsequent dissociation of eRF3 from the ribosome and as a consequence inhibition of ABCE1 access to eRF1, which precludes disassembly and recycling of the 40S and 60S ribosomal subunits. Importantly, a satisfying aspect of the *faux* 3′ UTR model is that it can explain NMD substrate selection in yeast as well as in humans and other species, reflecting the evolutionary conservation of the NMD factors UPF1-3 [61].

6. UPF1 recruitment and phosphorylation

How exactly UPF1 is recruited to the mRNP and whether this step confines the translation dependence of NMD remains to be elucidated and seemingly contradictory results have been reported. Consistent with UPF1 recruitment being the translation-dependent step in NMD, UPF1 can be immunoprecipitated in a complex together with the phosphatidylinositol 3 kinase-related protein kinase SMG1 [90,125] and with eRF1 and eRF3 (dubbed "SURF complex") [76] (Figs. 2 and 4). The SURF complex can be detected on mRNPs that contain the cap-binding complex (CBC, a heterodimer consisting of CBP80 and CBP20) and PABPC1 [126]. A transient interaction of UPF1 with CBP80 has been reported to stimulate recruitment of SURF to CBC-bound mRNPs [127]. Translation of CBC-associated mRNA, which has been termed 'pioneer round of translation', differs from subsequent rounds of translation occurring on eIF4E-bound mRNA by the involvement of different mRNP components [128].

In SURF, UPF1 is mainly unphosphorylated [76] and recent data indicates that SMG8 and SMG9, two SMG1 interactors that together allosterically inhibit SMG1's kinase activity, are also constituents of SURF [126,129] (Fig. 4). Phosphorylation by SMG1 of UPF1 at T28, S1096 and probably additional S/TQ sites [90,91] is considered to be a key step in NMD of metazoans, since it leads to the subsequent recruitment of RNA decay factors (see below).

Kashima and colleagues showed that UPF1 phosphorylation requires SURF to interact with the UPF2–UPF3–EJC complex downstream of the stop codon [76]. The interaction between the C-terminal half of SMG1 and UPF2 presumably induces the dissociation of SMG8 and thereby activates the kinase activity of SMG1, leading to phosphorylation of at least the C-terminal SQ motifs of UPF1 [76,129] (Fig. 4). Concomitant with UPF1 phosphorylation, binding of UPF2 to UPF1 causes the CH domain of UPF1 to swing around from the Rec2A subdomain to the Rec1A subdomain on the opposite side, a large conformational change that alleviates the inhibitory effect of the CH domain on ATP hydrolysis and hence triggers helicase activity of UPF1 [80,81,130]. The phosphorylation of UPF1 and its ATPase activity are both imperative for the NMD complex to induce decay in metazoans, but the functional relationship between UPF1 phosphorylation and ATP hydrolysis is currently not known. The ATPase activity appears to be independent of the phosphorylation status of UPF1 [76]. *Vice versa*, UPF1 mutants with defective ATPase or helicase activity accumulate as phosphoproteins, demonstrating that the ATPase activity is not required for UPF1 phosphorylation and that ATP hydrolysis precedes UPF1 dephosphorylation [76,131–133].

Although the C-terminal phosphorylation does not influence the UPF1 helicase, part of the SQ domain directly interacts with it and restrains ATP hydrolysis, tight RNA binding, and duplex unwinding [93]. It remains to be investigated at which point in the NMD mechanisms and by which effectors these SQ domain-mediated inhibition is relieved.

Based on the UPF2-induced conformational change that switches the helicase domain of UPF1 from its RNA-clamping mode to an RNA-unwinding mode [80,81], it seems unlikely that UPF1 would employ its helicase activity prior to UPF2 association to move along the mRNA from the ribosome to the EJC or to reel the mRNA to meet the EJC as has been recently suggested [134]. However, it is possible that after the interaction with UPF2 and UPF3, phosphorylated UPF1 together with its associated factors would start moving along the mRNA away from the ribosome. The crucial role that UPF2 appears to have for UPF1 phosphorylation and helicase activity is difficult to reconcile with data showing that a subpopulation of NMD targets can be degraded in cells depleted of UPF2 or expressing a UPF2 mutant that cannot interact with UPF1 [28,114,135]. Analogously, NMD activity was also found in cells depleted of UPF3A and UPF3B [27], indicating that there might exist two mechanistically distinct pathways to activate SMG1-mediated UPF1 phosphorylation.

Noteworthy, the view of UPF1 recruitment representing the translation-dependent NMD step has been challenged by a study providing evidence for translation-independent binding of UPF1 to 3′ UTRs [136]. By purifying tagged reporter mRNAs, Hogg and Goff demonstrated UPF1 association with mRNAs in a 3′ UTR length-dependent manner, even in the presence of the translation inhibitor puromycin. Translation-independent association of UPF1 with mRNA is difficult to reconcile with the model presented above and requires an alternative explanation for the translation dependence of NMD. Altogether, these seemingly inconsistent results emphasize that there are still many open questions regarding the mechanism of UPF1 recruitment to NMD target mRNAs and its subsequent phosphorylation. One important aspect to consider is that the different S/TQ sites in UPF1 could be differentially phosphorylated, a point that requires further investigations.

7. EJC-independent NMD and the role of EJCs in 3′ UTRs as NMD enhancers

In the working model outlined above, a residual EJC in the 3′ UTR of an mRNA appears to be a pre-requisite for phosphorylation of the ribosome-associated UPF1. However, there is a fast growing list of NMD-targeted mRNAs that lack an intron in the 3′ UTR and are therefore not expected to retain an EJC after the first ribosome has reached the stop codon [55,120,137–144]. A hallmark of these downstream EJC-independent NMD targets is that they typically contain a long 3′ UTR and thus their recruitment to the NMD pathway can basically be explained by the *faux* 3′ UTR model. However, one important question remains: how does UPF1 become phosphorylated in these cases? The EJC and its associated NMD factors UPF2 and UPF3 are required for SMG1-mediated UPF1 phosphorylation (see above) and they are also needed for downstream exon junction-independent NMD of β-globin and GPx1 reporter mRNAs [55,143] (A.Y. and O.M., unpublished data). Stalder and Mühlemann have speculated that in the absence of an EJC, UPF2 and UPF3 would bind to the ribosome-bound UPF1 and so facilitate UPF1 phosphorylation [117] (Fig. 4, right side). Presumably, UPF2 and UPF3 would have to find UPF1 by diffusion in this situation. Depending on the intracellular concentrations of these factors, this may be inefficient and result in rather inefficient NMD, which in fact is often observed for NMD targets lacking a 3′ UTR exon junction. In contrast, a 3′ UTR-bound EJC will serve as an enhancer of NMD, because the EJC-associated UPF2 and UPF3 are already in place to interact with ribosome-bound SURF complex,

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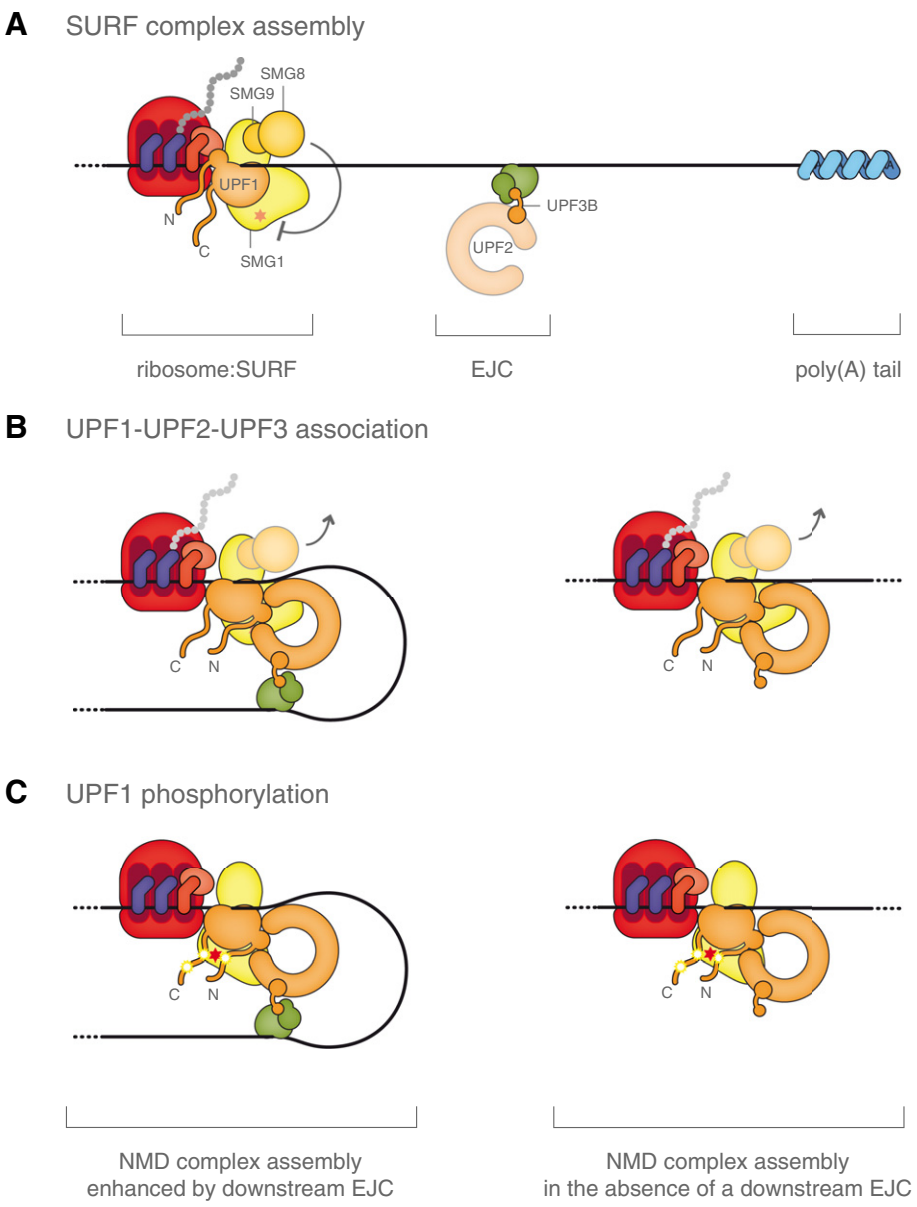

Fig. 4. Model for SURF recruitment to stalled ribosomes and subsequent UPF1 phosphorylation. (A) A complex consisting of SMG1, SMG9, SMG8, UPF1, eRF3 and eRF1 is believed to be recruited to or to form at the stalling ribosome. (B) UPF2 interaction with SURF induces conformational changes in UPF1 and SMG1. The UPF1-CH domain swings around to the other side of the helicase domain, and (C) SMG8-SMG9 dissociate from SMG1, leading to the activation of SMG1's kinase activity and the phosphorylation of S/TQ motifs in the unstructured N-and C-terminal parts of UPF1. We propose that this process occurs efficiently in the presence of a downstream EJC that has associated UPF3B and UPF2 (left side), but that it can even occur with reduced efficiency in the absence of a downstream EJC (right side). Because it is not known whether polypeptide chain release happens already before or during SURF complex assembly or only at a later stage, the polypeptide is depicted in faint gray in (A) and (B). See text for details.

resulting in UPF1 phosphorylation immediately after UPF1 recruitment. In this situation, chances for PABP to displace UPF1 from the terminating ribosome before it gets phosphorylated are reduced, which tilts the balance towards NMD.

The term "downstream exon junction-independent NMD" delineates NMD of mRNAs that are expected to be devoid of EJCs after the first ribosome has arrived at the stop codon. So far, the positions of EJCs on mRNAs have been predicted based on the positions of known introns in the pre-mRNA, with the assumption that i) every intron removal leads to the deposition of an EJC and ii) all EJCs are assembled at the defined position 20–24 nucleotides upstream of exon–exon junctions. Recent transcriptome-wide mapping of EJC binding sites in human cells however contradicted both of these assumptions [145,146]. The two studies using different methods consistently found that i) about 20% of exon–exon junctions do not detectably assemble EJCs, and that ii) about 50% of the detected EJCs mapped to non-canonical positions where no EJC binding sites were predicted to exist. In the light of this new data, it is likely that many NMD targets with 3′ UTR introns actually might not assemble a stable EJC in the 3′ UTR and hence be degraded by the EJC-independent pathway. At the same time, it cannot be excluded that on some NMD-targeted mRNAs without introns in the 3′ UTR, a non-canonical EJC might be present downstream of the TC. Notably, evidence for sequence-specific but splicing-independent binding of the core EJC factor eIF4A3 has been reported [147], and the formation of splicing-independent EJCs on specific sequences in 3′ UTRs can therefore formally not be excluded. In addition, the evidence for differential EJC deposition frequencies at specific EJC binding sites further complicates the picture [145,146,148]. Clearly, these two new studies demonstrate that the presence or absence of EJCs at a specific position in an mRNA cannot be reliably predicted, but has to be determined experimentally instead.

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8. Consequences of UPF1 phosphorylation: translation repression and recruitment of SMG6 and SMG5:SMG7

As outlined above, UPF1 phosphorylation is essential for NMD function in mammals. SMG1 phosphorylates more than 7S/TQ motifs of UPF1 in vitro [90]. Phosphorylation could also be detected in vivo for at least 4 of these sites: T28, S1078, S1096 and S1116 [77,90,91,149]. Contrary to the previously postulated mutually exclusive interaction of SMG6 and the SMG5:SMG7 heterodimer with the phosphorylated C-terminus of UPF1 [150], Okada-Katsuhata and colleagues showed that SMG6 and SMG5:SMG7 can simultaneously bind to two different phosphorylated S/TQ motifes located in the evolutionarily conserved N-terminal region and in the C-terminal SQ-rich region of UPF1, respectively [91] (Fig. 5). SMG5, SMG6 and SMG7 are conserved among metazoans (with the notable exception of *Drosophila melanogaster*, which apparently lack SMG7) [151], and share a 14–3–3-like domain that consists of a 14–3–3 fold fused to a C-terminal helical extension [152]. As is typical for 14–3–3 proteins, SMG6 and SMG7 have been found to bind to phospho-epitopes, specifically to the SMG1-catalyzed phospho-T28 and phospho-S1096 S/TQ motifs of UPF1, respectively [91]. Although SMG5 also associates preferentially with phosphorylated UPF1, no specific UPF1 phospho-epitope has been identified so far [77,91]. Instead, SMG5 was shown to bind directly to the unstructured conserved N-terminal region of UPF1, independently of the UPF1 phosphorylation status [77].

Genetic inactivation of SMG5, SMG6 or SMG7 in *C. elegans* inhibits NMD and leads to accumulation of phosphorylated UPF1, demonstrating that the cyclic phosphorylation and dephosphorylation of UPF1 is required for NMD and implying a role for SMG5, SMG6 and SMG7 in UPF1 dephosphorylation [133]. Indeed, the catalytic subunits of protein phosphatase 2A (PP2A) co-purified with the SMG5:SMG7 complex and a direct interaction of SMG5 with the PP2Ac–PR65 complex was detected [77,153]. Consistently, treatment of cell with the PP2A inhibitor okadaic acid suppresses NMD [114]. It is currently not known if SMG6 promotes UPF1 dephosphorylation by direct recruitment of a phosphatase or if this is an indirect effect. Furthermore, it

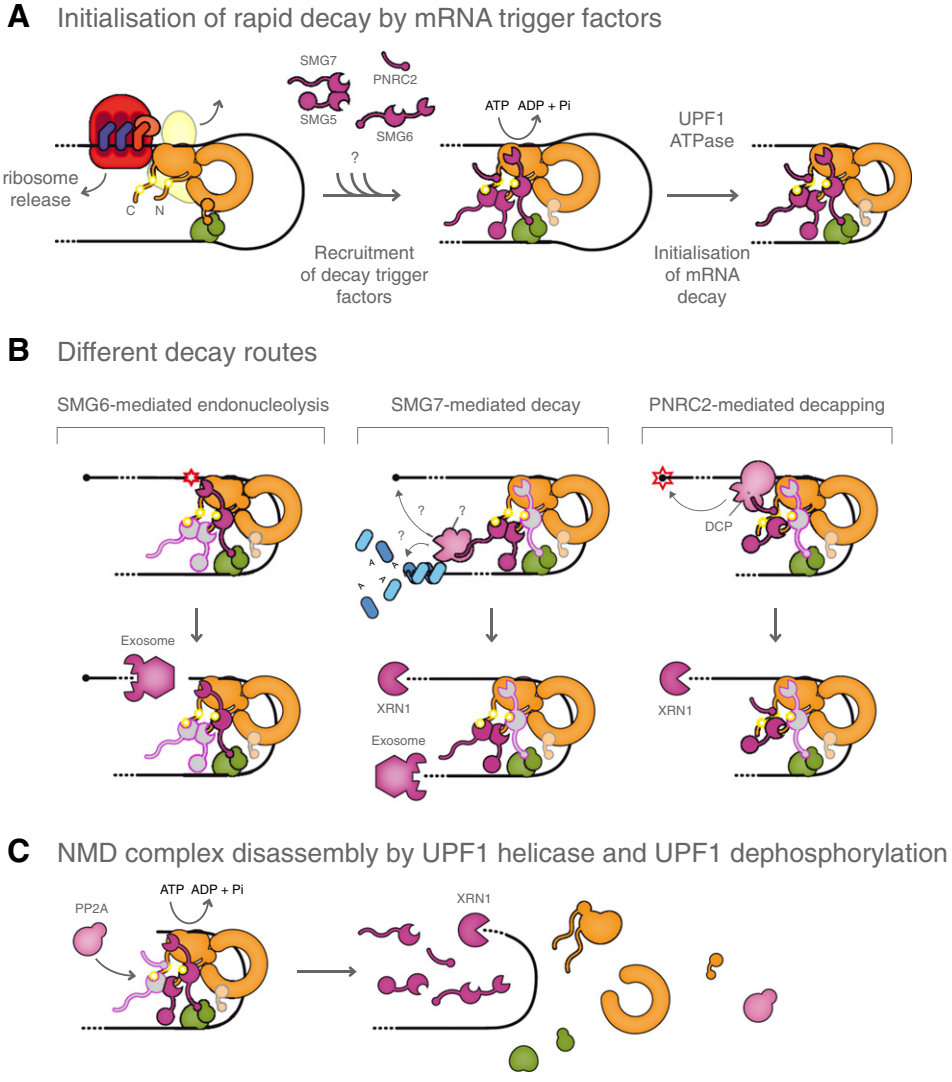

Fig. 5. Model depicting the mRNA degradation routes in mammalian NMD. (A) Upon phosphorylation of UPF1, SMG6 interacts with the phospho-T28 in the N-terminus of UPF1, the heterodimer SMG5:SMG7 binds to phospho-S1096 in the C-terminus, and PNRC2 has a preference to associate with a UPF1 region encompassing phospho-S1073 and phospho-S1078. (B) The degradation of NMD substrate mRNAs can be initiated by different decay routes: by SMG6-mediated endonucleolytic mRNA cleavage (red star) near the translation termination site (left side), by SMG7-mediated decay (middle), and/or PNRC2-mediated decapping (right side), followed by exonucleolytic degradation of the mRNA. PNRC2, which was found in a complex with SMG5 but without SMG7, has been shown to associate with the decapping complex (DCP), while the decay factors recruited by the C-terminus of SMG7 have not yet been identified (depicted by ?). Finally, deadenylated and decapped mRNA is exonucleolytically degraded by the 5′ to 3′ exonuclease XRN1 and the 3′ to 5′ acting exosome. (C) Degradation of the 3′ RNA fragment after endonucleolysis requires disassembly of the NMD complex, which involves UPF1's ATPase activity and its dephosphorylation. Whether the same activities are also needed for NMD complex dissociation in the exonucleolytic decay pathway is currently not known. See text for details.

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is not yet clear at which stage in the NMD pathway UPF1 dephosphorylation occurs. Analogous to the phosphorylation events, the possibility of temporally and/or spatially distinct dephosphorylation of the different UPF1 phospho-sites should be considered.

The SMG5:SMG7 complex is likely involved in the dissociation of the ribosome, the release factors, and UPF2 from UPF1, because inactivation of SMG5 leads to the enrichment of phospho-UPF1 together with these factors on CBC- and PABP-containing mRNPs [91], and in tethering assays, UPF1 and SMG5 were interdependent [154]. In contrast, SMG6 is dispensable for the dissociation of ribosomes, release factors and UPF2 from UPF1 [91].

Besides associating with SMG5, SMG6 and SMG7, phospho-UPF1 has been shown to interact with the multi-subunit translation initiation complex eIF3 (through its subunit eIF3a), thereby inhibiting the eIF3-dependent conversion of 40S/Met-tRNA^{iMet}/mRNA to translationally competent 80S/Met-tRNA^{iMet}/mRNA initiation complexes and repressing continued translation initiation [132]. Translation of PTC-containing mRNAs seems to be only moderately repressed, since cognate protein products accumulate along with phospho-UPF1 in cells with a knockdown of SMG5, SMG6 or SMG7, or in cells treated with the NMD inhibitor NMDI1 [40,76,133,155,156]. Alternatively, the presence of SMG5, SMG6 or SMG7 might be required for robust phospho-UPF1-mediated translation repression.

With regards to the nascent polypeptide encoded by NMD-subjected mRNAs, there is evidence that yeast UPF1 promotes the proteasomal degradation of these C-terminally truncated polypeptide chains [157] by a process that may involve the E3 ubiquitin ligase activity residing in the RING-related N-terminal CH domain of UPF1 [158]. Upon association with the E2 component Ubc3 (CDC34 in human) and UPF3, self-ubiqitination of UPF1 has been observed [158]. In human cell cultures, such a general destabilization of polypeptide chains encoded by NMD-targeted mRNAs was not observed. Instead, the stability of these prematurely terminated polypeptides appears to depend on mutated allele, with some alleles being stable and others being unstable [90,159,160].

9. Different nonsense-mediated mRNA degradation routes

A general concept in metazoan NMD seems to be that phosphorylated UPF1 induces various mRNA decay activities by recruiting decay factors or adaptor proteins for decay complexes through its N- and C-terminal phospho-sites. Among the NMD factors, SMG5 and SMG6 were candidates for having nuclease activity themselves, because both harbor a PIN domain at their C-termini, which is a common nuclease domain present in bacterial toxin–antitoxin systems. However, the SMG5 PIN domain lacks conserved Asp residues in its catalytic center and hence has no endonucleolytic activity in vitro [161,162]. The PIN domain of SMG6 on the other hand functions as an endonuclease in vitro and in vivo [163,164]. SMG6 PIN-dependent endonucleolytic cleavage was shown to occur in the vicinity of the PTC in NMD reporter transcripts, suggesting that SMG6 interacts with UPF1 phospho-T28 when UPF1 is still associated with or at least still near the stalling ribosome at the TC [163] (Fig. 5). The 5′ and 3′ RNA fragments generated by SMG6-mediated endonucleolysis are then quickly degraded by general cellular exonucleases, including XRN1 and presumably the exosome. The degradative activity SMG6 appears to be controlled because tethering SMG6 directly to a reporter mRNA elicits decay but in a UPF1-dependent manner [154]. Besides endonucleolysis, the SMG6 PIN domain is required for SMG6 to interact with SMG5 [71]. Furthermore, both SMG5 and SMG6 PIN integrity are required for UPF1 dephosphorylation [77,91], suggesting that the PIN domains are involved in the recruitment or activation of PP2A and thus structurally conserved in both factors.

As a second special feature, SMG6 has a N-terminal unstructured extension that contains two conserved EJC-binding motifs (EBMs) [71] that are similar to those found in UPF3A and UPF3B [70,75,135]. Mutations in the two partially redundant SMG6 EBMs that abrogated the SMG6:EJC interaction strongly stabilized TCR-β NMD reporter mRNAs, emphasizing the functional importance of this SMG6:EJC interaction [71]. Since the EBMs of SMG6 and UPF3B compete for binding to the same surface on EJCs (Fig. 2), with UPF3B having higher affinity [71], and taking into account that UPF3B is incorporated into EJCs already in the nucleus, a mechanism of active UPF3B dissociation of from EJCs presumably precedes the SMG6:EJC interaction. Consistent with this, less UPF3B co-immunoprecipitated with phospho-UPF1 upon overexpression of a dominant-negative C-terminally truncated SMG6 mutant [91].

Besides the endonucleolytic route for degrading NMD substrate mRNAs in mammalian cells [131,163,164], there is ample evidence for alternative decay pathways involving deadenylation, decapping and exonucleolysis [162,165–168] (reviewed in [169]; Fig. 5). This exonucleolytic decay pathway involves the SMG5:SMG7 complex (see below). Two observations suggest that the two decay routes might function in a partially redundant manner: i) mRNA decay induced by tethered SMG7 is not affected by SMG6 knockdown [162], and ii) co-depletion of SMG7 and SMG6 stabilizes PTC-containing NMD reporters much stronger than the single depletions [170,171].

SMG5 and SMG7 form a stable heterodimer by interaction of their N-terminal 14–3–3-like domains [77,91,152,153,171]. Tethering approaches showed that the ability to recruit exonucleolytic decay activities resides within the proline-rich, unstructured C-terminus of SMG7 [162]. Consistent with an exonucleolytic decay pathway, kinetic analysis of PTC-containing β-globin reporter transcripts in mammalian cells revealed an initial bi-phasic deadenylation of the 200–250 nucleotides long poly(A) tail [168,172]. Compared to deadenylation of normal mRNAs, the deadenylation rate of the first phase, which is mediated by the PAN2–PAN3 deadenylase and shortens the poly(A) tails to ~110 nucleotides, was strongly accelerated with NMD-targeted β-globin mRNA [168,172]. The second phase of deadenylation is carried out by the CCR4–CAF1 complex and concomitantly, DCP2-mediated decapping followed by XRN1-mediated 5′-to-3′ exonucleolysis occurs, leading to the rapid elimination of the mRNA [168,172]. The PAN2–PAN3 and the CCR4–CAF1 complexes are both recruited to the mRNP by direct interactions with PABPC1. In the case of CCR4–CAF1, this interaction occurs through the PAM2 motifs of TOB1 [172,173]. Interestingly, the PAM2 motif of eRF3 binds the same C-terminal region of PABPC1 and efficiently outcompetes the PAN3 and TOB interaction with PABPC1 because of its higher affinity to PABPC1 [174,175], which is the situation during a proper translation termination event. In contrast, as described above, the absence of proximal PABP is a hallmark of aberrant, NMD-inducing translation termination, during which UPF1 interacts with eRF3 instead [76,126]. Hence, the simplest interpretation for the accelerated deadenylation of PTC-containing transcripts is that the deadenylases PAN2–PAN3 and the TOB–CCR4–CAF1–NOT preferentially get recruited to mRNAs on which PABP is not engaged with eRF3 [169,176].

In addition to SMG5, SMG6 and SMG7, the human proline-rich nuclear receptor coregulatory protein 2 (PNRC2) has also been characterized as an adaptor that links UPF1 with RNA decay activity [177] (Figs. 2 and 5). PNRC2 is a largely disordered protein that contains three functional motifs in its C-terminal half (Arribas-Layton et al., this issue). The SH3-binding motif interacts with various nuclear receptors. The proline-rich sequence (PRS) motif, which is also found in EDC1 and EDC2, interacts with DCP1A and thus provides the molecular link to decapping. Finally, the short hydrophobic NR box motif at the very C-terminus of PNRC2 interacts directly with UPF1 (Fig. 2). Although this interaction is also observed with unphosphorylated recombinant UPF1, PNRC2 was reported to preferentially associate with a hyperphosphorylated UPF1 mutant [177,178], and the short LXXLL motif in the NR box was needed to specifically bind a UPF1 peptide encompassing phosphorylated S1073 and S1078 in vitro [179]. PNRC2 could also be precipitated in a complex with SMG5, but lacking

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SMG7. Moreover, destabilization of a β-globin reporter by tethered PNRC2 was dependent on SMG5, suggesting at least two SMG5-containing sub-complexes (or intermediates) triggering mRNA decay [154]. Hence, PNRC2 can be viewed as a decapping enhancer that recruits the DCP2-containing decapping complex through UPF1 to NMD targeted mRNAs (Fig. 5).

In summary, the presence of phosphorylated UPF1 on mammalian mRNAs appears to serve as a mark for NMD, and the RNA degradation can ensue by at least three different degradation routes: SMG6-mediated endonucleolytic cleavage near the TC, SMG5:SMG7-mediated decapping and/or deadenylation, and SMG5:PNRC2-mediated decapping. The relative contribution of the different pathways to the total NMD activity is not yet understood, and neither do we know the possible determinants that could preferentially activate one or the other decay pathway in a substrate-specific, tissue-specific or temporally regulated manner.

10. Concluding remarks — future directions

Over the last years, growing insight into the protein–protein and protein-RNA interactions involved in NMD have revealed important links of the 'NMD complex' to translation factors on one side and to RNA decay factors on the other side. Recent advances in expression methods of mammalian proteins and in cryo-electron microscopy even provided snapshots of NMD factors in different sub-complexes and conformations [79,129,180,181]. In addition, structures derived by X-ray crystallography and NMR gave insight into the regulation of UPF1 activities [80,86,130,182] and into the formation of other NMD-relevant sub-complexes [68–70,183]. Nonetheless, we still have only very limited and indirect information about the temporal order of all these molecular interactions. So, how can we gain insight in the dynamics of the NMD complex assembly? Currently, the lack of a reconstituted in vitro system to study NMD hampers progress towards a more detailed mechanistic understanding of NMD. The reason for the lack of information about the dynamics of the NMD mechanism is that most of the experimental approaches used so far examine static states, e.g. a trapped complex, or a large number of asynchronous assemblies. Microscopic observations of single molecules in real-time have been used to investigate divers biological processes, for example splicesome assembly [184] or miRNA-RISC assembly [185], and represent a promising way to also gain kinetic information about the assembly of the NMD complex from single factors.

As has been emphasized throughout this review, UPF1 is the key factor in NMD, yet we are still far from understanding all its functions. While it is well established that a cycle of UPF1 phosphorylation and dephosphorylation is essential for NMD, we lack a detailed understanding of the functional roles of each individual phosphorylation site. It is likely that the different phosphorylation sites form a switchboard-like phosphorylation pattern, for which the meaningful functional phosphorylation modules have yet to be identified. This will be an important task in the near future. It will be revealing to find out if UPF1 dephosphorylation serves primarily for recycling NMD factors [76] or if it acts as a phosphorylation code that orchestrates degradation activities. Currently, our insight is limited to only a few phosphorylation sites. An additional important unsolved question with regards to the NMD mechanism concerns the functional relationship between the UPF1 phosphorylation/dephosphorylation cycle and the ATPase and helicase activity of UPF1. In this context, more research is also needed to elucidate how UPF1 is recruited to mRNA and which mechanism confers the translation dependency of NMD. Furthermore, it needs to be clarified whether UPF1's ATPase activity is only needed at the very end of the NMD pathway to dissociate the 'NMD complex' on the 3′ RNA fragment after endocleavage [131] or whether ATP hydrolysis and helicase activity has additional functions.

Another area that needs further investigation is about the functional relationship between UPF1, SMG5, SMG6, SMG7 and the RNA decay factors. Here, much progress has been made in recent years, but a coherent picture is still missing. As all these open questions evidently document, there are still plenty of challenges in front of us before we can claim that we have figured out how NMD works.

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