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Recognition and elimination of nonsense mRNA

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Introduction

Among the different cellular surveillance mechanisms in charge to prevent production of faulty gene products, nonsense-mediated mRNA decay (NMD) represents a translation-dependent posttranscriptional process that selectively recognizes and degrades mRNAs whose open reading frame (ORF) is truncated
by a premature translation–termination codon (PTC, also called „nonsense codon“). In doing so, NMD protects the cell from accumulating C-terminally truncated proteins with potentially deleterious functions. Transcriptome profiling
of NMD-deficient yeast, Drosophila, and human cells revealed that 3-10% of all mRNA levels are regulated (directly or indirectly) by NMD, indicating an important role of NMD in gene regulation that extends beyond quality control [1]. In this review, we focus on recent results from different model organisms that indicate
an evolutionarily conserved mechanism for PTC identification.

Sources of PTCs

PTCs arise from mutations in the DNA, but also on the RNA level (Table 1). Many DNA mutations within a gene will truncate the ORF. In addition to nonsense mutations, i.e. base substitutions that directly generate PTCs by changing an amino acid-encoding codon into one of the three termination codons (UAA, UAG, UGA), random nucleotide insertions and deletions shift in two of three cases the reading frame, where within the next 20 codons on average a termination codon will prematurely terminate translation. Often mutations also alter splicing signals and generate alternatively spliced mRNAs, many of which contain a PTC. Overall, it is estimated that about 30% of all known disease- associated mutations generate a PTC-containing (PTC+) mRNA [2, 3].

In addition to the sources described above, PTCs arise very frequently in genes belonging to the immunoglobulin superfamily (immunoglobulins, T-cell receptors) as a consequence of programmed V(D)J rearrangements during lymphocyte maturation [4]. During the joining of a V, a D (only in heavy chains), and a J fragment, non-templated nucleotides (N nucleotides) can be added by the enzyme terminal transferase and coding nucleotides from the opposite strand (P nucleotides) can be transferred to the coding strand at the junctions of the segments. The ORF can only be maintained when nucleotides in multiples of three (i.e. three or six) are added, but in two thirds of the rearrangements, a frameshift results in a nonproductive allele that encodes a PTC+ transcript.

Interestingly, it was observed that PTCs in genes belonging to the immunoglobulin superfamily cause a much stronger reduction of the steady-state mRNA level by NMD than in other genes [5-8].

On the RNA level, errors during transcription and alternative pre-mRNA splicing generate PTC+ mRNAs. Based on a misincorporation rate for RNA polymerase II in the order of 10^{-5} per nucleotide, and assuming 10^3 to 10^4 coding nucleotides in a typical gene, only 0.05% to 0.5% of all transcripts are estimated to acquire a PTC through transcription errors. In contrast, the fraction of PTC+ transcripts generated by unproductive alternative pre-mRNA splicing is much larger. Computational analysis of human EST databases revealed that among the 60%-

70% of human pre-mRNAs that are alternatively spliced, 45% had at least one splice form that was predicted to be a target of NMD [9]. Thus, about one third of all human protein-coding genes produce a PTC+ mRNA, and although their exact abundance is not known, they are likely to represent a significant fraction in the pool of the initially produced mRNAs.

***Trans*-acting factors involved in NMD**

The first *trans*-acting factors involved in NMD have been identified in genetic screens in *S. cerevisiae* and *C. elegans*. In screens for translational suppressors in *S. cerevisiae*, mutations in the three genes UPF1, UPF2/NMD2 and UPF3 (for Up-frameshift) were found to decrease decay rates of PTC+ mRNAs and to promote read-through of PTCs [10-13] (Table 2). Three labs had identified in independent screens in *C. elegans* loss-of-function mutations in seven genes called SMG1 to SMG7 (for suppressor with morphogenetic effects on genitalia)

that several years later were recognized to be defective in NMD [14-16]. Similarity searches revealed that SMG2 is homologous to yeast UPF1, SMG3 is homologous to UPF2, and SMG4 is homologous to UPF3, respectively. As genome sequencing projects progressed, orthologs of these NMD factors were identified in other eukaryotic organisms based on homology searches [17, 18]. All seven factors are present in *H. sapiens*, and *D. melanogaster* has orthologs for SMG1, UPF1, UPF2, UPF3, SMG5, and SMG6, but appears to lack an ortholog

for SMG7 [19-27]. It is likely that additional, yet unknown NMD factors exist in vertebrates. Notably, Longman and colleagues recently identified in *C. elegans* two additional proteins, called SMGL-1/hNAG and SMGL-2/hDHX34, which are required for NMD in worms and humans [28].

UPF1, UPF2, and UPF3 constitute the conserved core of the NMD system. The nucleic acid-dependent ATPase and RNA helicase UPF1 shows the highest sequence conservation among the UPF proteins in different species [10, 17, 29,

30] and understanding its structure, functions and regulation is key to elucidate the molecular mechanism of NMD. In the conserved region, seven group I helicase motifs can be found, and the ATPase activity of UPF1

resides in two of

these helicase motifs and is linked to the 5' to 3' helicase activity of the protein [30]. The ATPase activity is essential for NMD in all tested species [31-35] and the RNA-binding activity of UPF1 is modulated by ATP [30, 36]. UPF1 localizes predominantly to the cytoplasm [22], but has the capacity to shuttle between the nucleus and the cytoplasm [32]. UPF1 associates with the translation release factors eRF1 and eRF3 and with UPF2 [26, 31, 37, 38] (Figure 1). UPF1 interacts with UPF2 through its cysteine-histidine-rich domain (amino acids 115-272), which forms three Zinc-binding motifs arranged into two tandem modules, resembling the RING-box and U-box domains of ubiquitin ligases [39]. Multiple serine residues in the N- and C-terminal regions of UPF1 are targets for phosphorylation [33, 40]. Indeed, UPF1 activity in humans and worms is regulated by cycles of phosphorylation and dephosphorylation that depend on additional NMD factors. Phosphorylation of UPF1 is catalyzed by SMG1 and requires UPF2 and UPF3 [31, 41], whereas dephosphorylation of UPF1 is mediated by SMG5, SMG6, and SMG7, which are thought to recruit protein phosphatase 2A (PP2A) [25, 31, 33, 41-44]. Phosphorylation of human UPF1 seems to lead to a remodelling of the UPF1-containing surveillance complex.

Overexpression of a SMG1 mutant deficient in its kinase activity strongly increased UPF1 co-precipitation with eRF3, suggesting that phosphorylation of UPF1 induces the dissociation of eRF3 from UPF1 [31].

UPF2, which is also a phosphoprotein [43, 45], interacts with both UPF1 and UPF3, thereby serving as a bridge between the two [22, 24, 26, 38]. The interaction between human UPF2 and UPF3b (see below) involves the last of the three middle of eIF4G-like (MIF4G) domains of UPF2 and the RNA-binding domain (RBD) of UPF3b [46]. Human UPF2 interacts with the N-terminal Zinc- finger domain of UPF1 mainly through its C-terminal region (amino acids 1084-1272) [24, 26], but amino acids 94-133 from the N-terminal region also contribute to this interaction to some extent [26]. Even though the N-terminus of UPF2 contains several nuclear localization signals (NLS) and the N-terminal 120 amino acids can target a heterologous protein to the nucleus, UPF2 localizes at steady state to the perinuclear region of the cytoplasm [22, 24, 26].

UPF3 is the least conserved among the UPF proteins [17]. In contrast to yeast, worms, and flies, the human genome contains two UPF3-encoding genes, UPF3a on chromosome 13 and UPF3b on the X chromosome (also known as UPF3X), and two alternatively spliced mRNAs from each gene encode in total four UPF3 isoforms [22, 26]. The shorter form of UPF3a lacks exon 4 (amino acids 141-173) and the shorter form of UPF3b lacks exon 8 (amino acids 294-
306) [26]. Human UPF3 proteins contain putative NLS and NES motifs and shuttle between the nucleus and the cytoplasm, with predominant nuclear localization at steady state [22, 26]. UPF3 proteins are components of the exon junction complex (EJC, see below) and bind to mRNAs through interaction with the EJC core factor Y14 [47-50].

The serine-threonine kinase SMG1 belongs to the family of phosphatidylinositol 3-kinase-related protein kinases that also includes ATM, ATR, mTOR, and DNA- PK [51]. As mentioned above, SMG1 catalyzes the phosphorylation of UPF1, and this phosphorylation is essential for NMD [19, 27, 31, 33, 40, 41]. In contrast to the UPF proteins, no SMG1 ortholog has been identified in *S. cerevisiae* so far, and because yeast also appears to lack orthologs for SMG5, SMG6, and SMG7,
it seems likely that the regulation of UPF1's phosphorylation state during NMD is limited to metazoans.

The three proteins SMG5, SMG6, and SMG7 share common structural motifs and loss-of-function mutations in any of the three genes in *C. elegans* leads to accumulation of phosphorylated UPF1 [33]. All three proteins contain a 14-3-3 like domain through which they bind phosphorylated UPF1 [44]. In addition, SMG5 and SMG6 both contain a C-terminal PIN domain, which is found in many proteins with ribonuclease activity. Although the crystal structure of both PIN domains is very similar, only SMG6 has the canonical triad of acidic residues that are crucial in RNase H for activity and degrades single stranded RNA *in vitro*, whereas SMG5 has no nuclease activity [52]. SMG5 and SMG7 interact with each other and are part of a larger complex comprising protein phosphatase 2A (PP2A) and phosphorylated UPF1 [25, 42]. SMG6 also co-immunoprecipitates with PP2A and phosphorylated UPF1 [43], but this complex probably does not

contain SMG5 or SMG7. The C-terminal region of SMG7 is required for the localization of SMG7, SMG5, and associated UPF1 to P-bodies, large cytoplasmic structures enriched in mRNA degradation enzymes [44, 53]. In contrast, overexpressed SMG6 was detected in cytoplasmic foci different from P- bodies [53].

Eukaryotic release factors and poly(A) binding proteins play crucial roles in NMD

Early studies in *S. cervisiae* already indicated that NMD is mechanistically intertwined with the process of translation termination, and more recent data indicates that NMD is activated by the intrinsically aberrant nature of premature translation termination [54]. Thus it is not surprising that several translation factors play an important role in NMD. UPF1 interacts with eRF3 and with eRF1

[31, 37, 55]. Both eRF1, which recognizes all three stop codons in eukaryotes and triggers peptidyl-tRNA hydrolysis, and the GTPase eRF3 are essential for translation termination [56, 57]. GST pull-down experiments showed that in yeast UPF1, UPF2, UPF3, and eRF1 all interact with the GTPase domain of eRF3, whereby UPF2, UPF3, and eRF1 (but not UPF1) compete with each other for this

interaction [55]. Interestingly with regard to the requirement of the ATPase/helicase activity for NMD [35, 58, 59], eRF1 and eRF3 were found to inhibit the RNA-dependent ATPase activity of UPF1 [37].

Another protein that plays a role in translation termination is the poly(A) binding protein (PABP). In yeast, there is only one PABP present, whereas the human genome encodes for several cytoplasmic PABPs (PABPC1, testis PABP, inducible PABP, PABP5) and a mainly nuclear form [60]. The ubiquitously expressed PABPC1 is the only PABP whose function in mRNA translation and stability has been extensively studied. PABPC1 consists of four non-identical RNA recognition motifs (RRM), of which RRM 1 and RRM 2 bind to the poly(A) tail with high affinity, a linker region, and a carboxyl terminal domain that is involved in protein-protein interactions, for example with eRF3 [61-63]. Data from

yeast, flies, and humans revealed that PABP is a NMD antagonizing factor, since NMD can be suppressed by tethering PABP near the PTC [64-69].

Working model for an evolutionarily conserved NMD mechanism

One of the key questions about NMD is how a translation-termination codon (TC) is recognized as premature and distinguished from a natural, correct TC. Although the core NMD factors are conserved from yeast to humans (see above), remarkably different models of NMD have emerged from studies of
mammalian systems compared to studies in *S. cerevisiae*, *C. elegans*, *D. melanogaster*, and plants [70, 71]. However, the most recent data from investigations in different organisms have begun to reveal the existence of a basic, evolutionarily conserved mechanism for PTC recognition [28, 64, 65, 66,
69, 72, 73]. It remains a challenge for the future to thoroughly scrutinize this emerging “unified NMD model” and to understand the exact molecular basis for PTC definition.

The central feature of this “unified NMD model” is that the mechanism of translation termination at a PTC is intrinsically different from translation termination at a “correct” TC. In *S. cerevisiae*, it has been demonstrated that ribosomes do not efficiently dissociate from the mRNA when stalling at a PTC,
presumably because in that spatial environment they cannot receive the termination-stimulating signal from PABP [64, 74]. Likewise, in flies [65] and human cells [66-69], artificial tethering of PABP into close proximity of an otherwise NMD-triggering PTC efficiently suppresses NMD. Based on reported biochemical interactions (see above), this model proposes that the decision of whether or not NMD is triggered relies on a competition between UPF1 and PABP for binding to eRF3 bound to the terminating ribosome [69] (Figure 2). According to this model, a translation termination event is defined as “correct” if the ribosome stalls close enough from the poly(A) tail to efficiently interact with
PABP, which through yet unknown mechanisms leads to fast/efficient

polypeptide release and dissociation of the two ribosomal subunits (Figure 2A). If in contrast the spatial distance between the terminating ribosome and the poly(A)

tail is too big for this interaction to occur, UPF1 will bind to the ribosome-bound eRF3 instead. At this stage ("marking" step in Figure 2B), UPF1 might still be displaced by PABP and NMD would be prevented. However, when this signal is absent, UPF2 and UPF3 will eventually bind UPF1, which is required for SMG1

to phosphorylate UPF1. UPF1 phosphorylation is believed to definitively commit the mRNA for degradation by NMD, maybe by inducing a conformational change that causes UPF1 to bind the mRNA ("licensing" step in Figure 2B). Finally, the phosphorylated UPF1 will be bound by the 14-3-3-like phosphoserine-binding domains of SMG5, SMG6 and/or SMG7, ultimately leading to the degradation of the mRNA.

The molecular relationship between UPF1, SMG5-7 and the cellular degradation enzymes is still unclear. Evidence from yeast and mammals suggests that rapid degradation is achieved by shunting nonsense transcripts efficiently into the normal mRNA turnover pathway that proceeds through deadenylation, followed

by decapping and XRN1-mediated 5'-3' exonucleolytic decay as well as exosome-mediated 3'-5' exonucleolytic decay [75, 76] (Table 3). Consistent with this idea, several interactions between NMD and RNA decay factors have been reported. UPF1 protein co-immunoprecipitates with DCP1a and DCP2 in human cells, two subunits of the decapping complex [77]. Furthermore, UPF1, UPF2, and UPF3b have been reported to co-immunopurify with the decapping enzyme DCP2, 5'-3' exonuclease XRN1, exosomal components PM/Scl100 (RRP6), RRP4, and RRP41, and poly(A) ribonuclease (PARN) in African green monkey cells [78]. Interestingly and in apparent contrast to the data from other species, the decay of PTC+ transcripts is initiated by endonucleolytic cleavage near the PTC in *D. melanogaster*. The resulting fragments are rapidly exonucleolytically degraded in both directions, without undergoing deadenylation or decapping [79].

Mammalian exon junction complex as an NMD enhancer

During pre-mRNA splicing, a protein complex called exon junction complex (EJC) is deposited about 20-24 nucleotides upstream of exon-exon junctions in spliced mRNA [80]. The four core components of the EJC Y14, MAGOH, eIF4A3, and

Barentsz/MLN51 [81, 82] associate with the mRNA in the nucleus and escort it to the cytoplasm. The DEAD-box adenosine triphosphatase (ATPase) eIF4A3 binds the RNA with a footprint of 7-9 nucleotides in a sequence-independent manner
[47]. Additional factors associate with EJCs more dynamically and some already leave before mRNA export or only bind the EJC in the cytoplasm [80]. Identification of UPF2 and UPF3 as EJC components has immediately indicated a role for the EJC in NMD (Figure 1). Supporting this idea, RNAi-mediated knockdown of Y14, eIF4A3 and Barentsz/MLN51 in mammalian cells reduced the NMD-mediated downregulation of PTC+ reporter mRNAs [83-86]. In contrast, studies with *D. melanogaster* and *C. elegans* showed that the EJC is dispensable
for NMD in these organisms [21, 28], and *S. cerevisiae* do not possess an EJC at all. Thus it appears that in organisms producing a large number of nonsense mRNAs by extensive alternative pre-mRNA splicing, the EJC may have evolved
to facilitate efficient recognition and degradation of these “aberrantly spliced” transcripts.
It is believed that EJCs located within the ORF are removed from the mRNA by elongating ribosomes [87, 88], and hence only EJCs located downstream of the TC would still be present when the first ribosome terminates. According to the proposed model, the presence of an EJC downstream of a terminating ribosome functions as an enhancer of NMD by facilitating SMG1/UPF2/UPF3-dependent UPF1 phosphorylation (Figure 2C). As part of such a 3’UTR-bound EJC, UPF2 and UPF3 are positioned ideally for immediate interaction with ribosome-bound UPF1 and SMG1. As a consequence, the time window between UPF1 binding to the terminating ribosome and its SMG1-mediated phosphorylation would be shortened and the balance between PABP action (NMD suppression) and UPF1 action (NMD promotion) would tilt towards NMD. In contrast to previously proposed mammalian NMD models, the presence of an EJC downstream of the TC is not required for PTC identification [89], but rather functions as an NMD enhancer according to this new unified NMD model.

Evidence for restriction of NMD to CBC-bound mRNA in mammals

However, despite of the recent convergence of NMD models derived from different model organisms, one major difference appears to exist between mammalian and yeast NMD. In mammals, several lines of evidence suggest that NMD targets exclusively cap-binding complex (CBC)-bound mRNA ([90-92], reviewed in [93, 94]). CBP80, one of the two CBC constituents, interacts directly with UPF1, and this interaction was suggested to promote NMD during the so- called “pioneer round of translation” by increasing the binding of UPF1 to UPF2
[95]. In *S. cerevisiae* on the other hand, NMD is not restricted to CBC-bound transcripts and targets also eIF4E-bound, steady-state mRNA [96, 97]. It remains to be seen if eIF4E-bound mammalian PTC+ mRNAs are immune to NMD or if they can be NMD targets under experimental conditions that prevent their previous degradation during the CBC-bound state.

Different phenotypes of NMD deficiency in different organisms

The phenotypes observed after inactivation of the NMD pathway vary among different organisms. In *S. cerevisiae*, the NMD pathway is not essential for viability under laboratory conditions, null mutants of UPF1, UPF2, and UPF3 did not show any phenotype [54]. In *C. elegans*, mutations of SMG proteins lead to fertile worms with mild morphological defects of the genitalia [16], whereas UPF1 and UPF2 are required for larval development and cell proliferation in *D. melanogaster* [98, 99]. In *A. thaliana*, UPF1 and UPF3 are essential for the development and survival of the plant [100]. In mammals, NMD appears to be essential for viability. Homozygous Upf1 knockout mouse embryos do not survive after implantation and it was not possible to establish Upf1-/- embryonic mouse
stem cells in culture [101]. However, because UPF1 is also implicated in additional cellular processes apart from NMD (see below), it is currently not clear if the lethality is indeed due to NMD inactivation. Nevertheless, we would like to point out the apparent correlation between the severity of the phenotype caused by NMD deficiency in an organism and the extent of alternative pre-mRNA splicing occurring in that organism.

The physiological role of NMD

Until a few years ago, NMD has been seen merely as a quality control system that rids the cell of faulty mRNAs, but recent studies indicate that NMD represents a much more sophisticated tool serving multiple purposes in gene expression. The population of NMD substrates is not only restricted to faulty transcripts, but also comprises numerous endogenous, physiological transcripts. Among those are i) mRNAs containing short upstream ORFs (uORFs,) that play

a pivotal role regulating translation and turnover, ii) mRNAs encoding selenocysteine-containing proteins, iii) mRNAs harboring introns in their 3' UTR, iv) mRNAs with long 3' UTRs, and v) transposons and retroviruses [99, 102-106]. In addition, pseudogenes, bicistronic mRNAs, and mRNAs containing signals for programmed frameshifting have been identified as NMD targets in yeast [105] (Table 1). Genome-wide transcriptome profiling in yeast, Drosophila, and human cells revealed that 3 – 10 % of all mRNAs are regulated by NMD [99, 104, 105]. Curiously, the set of transcripts regulated by NMD varies a lot between species. And in contrast to flies, where knockdown of individual NMD factors affected essentially the same transcript population [99], the set of mRNAs regulated by

UPF1 in human cells only marginally overlapped with the set of mRNAs regulated by UPF2 [104, 107], which complicates interpretation of the data.

Based on our own recent data, we proposed a novel mode of translation-dependent posttranscriptional gene regulation that involves NMD ([66]; Stalder & Mühlemann, TICB, in press). According to this model, a given mRNA would be stable in a 3D configuration with the TC in physical proximity of the poly(A) tail, whereas the same mRNA could become an NMD substrate upon structural rearrangements that increase the distance between the TC and the poly(A) tail. Importantly, the protein-protein and protein-RNA interactions determining the 3D structure of the 3' UTR can be regulated in a tissue-specific manner, during development, and by environmental cues. Along the same lines, a recent study from Giorgi and colleagues indicates such a translation- and NMD-dependent

control of mRNA stability as a means to regulate spatially and temporally restricted protein synthesis, for example in synapses of neurons [108].

NMD is also involved in an auto-regulatory gene expression circuit termed "regulated unproductive splicing and translation" (RUST) [9]. For example, the *C. elegans* ribosomal proteins L3, L7a, L10a and L12 and the SR proteins SRp20 and SRp30b are regulated in this manner [109, 110]. By alternative splicing of their pre-mRNA, both a productive mRNA encoding the full-length protein and a nonproductive PTC+ mRNA are produced. At a certain concentration, the protein starts to alter the splicing of its own pre-mRNA towards production of more nonproductive PTC+ mRNA, which is rapidly degraded by NMD. In mammals, the SR protein SC35 has also been shown to auto-regulate its own expression using RUST. At high levels of SC35 protein, it binds its own pre-mRNA, inducing the production of alternatively spliced PTC+ mRNA [111]. Moreover, NMD can act in concert with alternative pre-mRNA splicing and miRNA silencing to regulate gene expression. In neuronal cells, miR-124 regulates the expression of PTBP1, which among others targets PTBP2 for alternative splicing, resulting in a PTC-containing PTBP2 mRNA. Upon expression of miR-124, PTBP1 protein levels are reduced, leading to an accumulation of correctly spliced PTBP2 mRNA and the production of PTBP2 protein [112]. In *A. thaliana*, the clock-regulated RNA-binding protein AtGRP7 regulates its abundance by an auto-regulatory negative feedback loop in a posttranscriptional manner. AtGRP7 targets its own pre-mRNA, leading to the production of alternatively spliced PTC+ transcripts

[113]. Thus, the coupling of NMD with alternative splicing seems to be widespread among eukayotes. Since NMD rarely downregulates a PTC+ transcript to less than 10%, physiological relevant levels of truncated proteins may still be produced from the remaining PTC+ transcript [114]. For example, the steady–state level of the PTC+ mRNA encoding the FcεRIβ receptor is very low, but nonetheless the truncated protein competes effectively with the full-length protein [115].

Interestingly, the overall NMD capacity can vary considerably between different cell lines, tissues, and individuals [116-121]. It has been proposed that these

variations of the NMD efficiency might contribute to the phenotypic variability of hereditary disorders [2, 122]. Recently, variability in NMD efficieny between cell lines has been reported to correlate with intracellular RNPS1 concentrations
[123].

Involvement of NMD factors in additional cellular processes

Given the intimate coupling of NMD to translation, it may not come as a surprise that NMD factors have been found to play a role in various aspects of translation. UPF1, UPF2, UPF3b, and the EJC factors Y14, MAGOH, and RNPS1 have been reported to stimulate translation [124]. These proteins enhanced translational yield and polysome association when tethered inside the ORF of an intronless
reporter mRNA, but the mechanism underlying this effect remains to be elucidated. In *S. cerevisiae*, NMD factors were shown to increase the fidelity of translation termination. Loss–of–function mutations in UPF1, UPF2 or UPF3 cause nonsense suppression, resulting in production of C–terminally extended proteins [55, 125]. The function of UPF1 – 3 in nonsense suppression might be attributed to their interaction with eRF1 and eRF3. However, knockdown of UPF1 and UPF2 in mammalian cells had no effect on nonsense suppression [32], and it remains to be shown if NMD factors are involved in nonsense suppression in other species than yeast.

More surprising was the finding that NMD factors are implicated in telomere maintenance. Est1p is involved in telomerase regulation in yeast [126]. Three Est1p-like proteins, EST1A, EST1B, and EST1C have been identified in humans and were found to be the homologs of *C. elegans* SMG6, SMG5, and SMG7, respectively [21, 25, 43, 127, 128]. Human SMG6 (hEST1A) associates with telomerase activity in HeLa cell extracts and over-expression of this protein leads
to accumulation of chromosome-end fusions [127]. Very recently, transcripts originating from telomeric repeat regions have been discovered in mammalian cells [129]. Intriguingly, UPF1, UPF2, SMG1, and SMG6 where all found to be enriched at telomeres, and they negatively regulate the association of the telomeric repeat-containing RNAs (TERRAs) with

chromatin [129]. Thus, these

NMD factors provide a molecular link between TERRA regulation and the maintenance of telomere integrity and therewith genome stability.

Furthermore and like ATM, another PI-3 kinase-related kinase, human SMG1 phosphorylates the cell-cycle checkpoint protein p53 [130]. SMG1 is required for optimal p53 activation upon genotoxic stress and SMG1 depletion causes spontaneous DNA damage and increased sensitivity to ionizing radiation [130]. UPF1 also gets phosphorylated under such conditions by ATR and associates with chromatin in discrete spots [131]. UPF1 depletion leads to a cell cycle arrest

in early S-phase and an ATR-depedent DNA-damage response [131], which together with the identification of UPF1 as the DNA polymerase δ-associated helicase [132] strongly suggests a function for UPF1 in DNA repair and corroborates a function for NMD factors in safeguarding genome stability.

Moreover, studies in *C. elegans* revealed that *SMG-2* (*C. elegans* UPF1), *SMG-5*, and *SMG-6* are required for establishing persistent RNAi-mediated gene silencing. Target genes could initially be silenced by dsRNA in mutant *smg*2, *smg*5, and *smg*6 animals, but the target mRNA levels recovered within 4 days, whereas the knockdown persisted in wild-type worms [133]. *Smg-3* and *smg-4* mutants gave a similar, but weaker phenotype, whereas RNAi persistence was not affected in *smg-1* mutants. Thus, the fast recovery from RNAi does not generally depend on inactivation of NMD. In addition to this genetic link between NMD and RNAi, a physical link between NMD and RNAi factors was recently

reported: UPF1 co-immunoprecipitates AGO1 and AGO2 in an RNAse A insensitive manner, and UPF1 was identified in AGO1-associated mRNPs [134].

Subcellular localization of NMD

A recurring topic in the NMD field has been the debate about the subcellular location of NMD in mammalian cells. Although it is undisputed that NMD depends on translation, a process that generally is thought to be confined to the cytoplasm, most examined nonsense mRNAs in mammalian cells are found to be degraded when still physically attached to the nucleus (i.e. in the RNA isolated from purified nuclei). Models to resolve this apparent paradox range from

proposing translation by cytoplasmic ribosomes associated with the outer nuclear membrane to postulating nuclear scanning of the reading frame at the site of transcription by a yet unknown nuclear frame reader (reviewed in [135, 136]).

The reported evidence for nuclear translation in human cells [137], the confinement of NMD to CBC-bound mRNA [90], and finding that NMD was not affected by inhibition of mRNA export [138] are consistent with the hypothesis of intranuclear NMD. On the other hand, Lykke-Andersen and colleagues showed

very recently that expression of polypeptides designed to inhibit various interactions between NMD factors specifically inhibited NMD when expressed exogenously in the cytoplasm [139]. The same polypeptides did however not inhibit NMD when they were confined to the nucleus by addition of a nuclear localization signal (NLS), suggesting that most if not all NMD occurs in the cytoplasm of mammalian cells [139].

For *S. cerevisiae*, evidence is accumulating that NMD occurs in the cytoplasmic processing bodies (P-bodies) [140], and nonsense mRNAs in mammalian cells were also proposed to be degraded in similar cytoplasmic foci that are enriched

in decapping and degradation enzymes, NMD factors, and effectors of the RNAi silencing pathway (reviewed in [141]). SMG7 might provide the molecular link between NMD and the degradation machinery in mammalian cells, through its

14-3-3 like domain in the N-terminus that interacts with phosphorylated UPF1 and SMG5. When overexpressed, SMG7 accumulates together with SMG5 and UPF1 in P-bodies. SMG7 elicits RNA degradation independently of a PTC when tethered to a reporter mRNA [53].

Nuclear aspects of NMD

Regardless of the controversy about nuclear versus cytoplasmic NMD, examples where the presence of PTC in the transcript affects nuclear processes such as pre-mRNA splicing and transcription have been reported. The crucial question with the examples of nonsense-associated altered splicing (NAS) is, if splicing is

altered specifically as a consequence of the ORF truncation, or if the PTC-causing mutation affects splicing directly. In many examples of NAS that were

thoroughly investigated, it was found that not only nonsense, but also some missense and silent mutations caused alternative splicing, and that these mutations disrupted exonic splicing enhancers critical for exon inclusion [142-
147]. However, evidence for frame-dependent NAS has also been reported [148-152]. The requirement for UPF1 is a distinction criteria for this second type of NAS that is believed to be specific for frame-disrupting mutations, whereas other NMD factors do not appear to be required [32, 152]. Interestingly, specific NMD- inactivating amino acid substitutions in UPF1 did still support frame-dependent NAS, indicating genetically separable functions of UPF1 in these two processes
[32]. That recognition of a PTC in an mRNA could generate a signal that alters splice site selection on its own pre-mRNA species is intriguing and somewhat paradoxical, and the underlying mechanism remains to be elucidated.
Yet another unexpected PTC-dependent nuclear effect was recently discovered in Ig-∝ and Ig-γ minigenes [153]. When these minigenes were stably integrated into the genome of HeLa cells, transcription of PTC-containing genes was silenced. This transcriptional silencing is PTC-specific and accompanied by chromatin remodeling, manifested by the loss of typical marks for transcriptionally active euchromatin (acetylated histone H3) and a concomitant accumulation of heterochromatin marks (H3K9 methylation). Consistently, this nonsense-mediated transcriptional gene silencing (NMTGS) can be reversed by treatment of the cells with histone deacetylase inhibitors. Remarkably, NMTGS was inhibited by overexpression of the siRNAse 3'hExo, which suggests that
small interfering RNAs (siRNAs) and hence the RNA interference (RNAi) pathway are involved [153]. However, attempts to detect Ig-∝-specific siRNAs have not been successful until now (O.M., unpublished results). The NMTGS pathway branches from the NMD pathway after translation of the PTC-containing mRNA and UPF1-dependent PTC recognition [154]. So far, NMTGS was only observed with PTC-containing Ig-∝ and Ig-γ minigenes, but not with an Ig-κ minigene or other classical NMD reporter genes [153], which led us to speculate that NMTGS might be important to silence expression of non-productively rearranged heavy chain alleles in B cells,

thereby constituting the second half of

the allelic exclusion system. However, investigation of clonal lines of immortalized murine pro-B cells did not reveal a difference of the transcriptional state between the productively and the non-productively rearranged heavy chain allele (A.B.E. and O.M., unpublished results). Thus, until now the biological significance of NMTGS remains enigmatic.

Clinical aspects of NMD and therapeutic approaches

It is estimated that approximately 30% of all human genetic diseases arise as a consequence of nonsense or frameshift mutations that disrupt the ORF of genes by the introduction of PTCs [122]. Expression of these mutant genes lead to the synthesis of nonsense mRNAs that if translated conduce to the production of truncated proteins with residual or no function, or in some cases with gain-of function or dominant negative activities (reviewed in [2, 122, 155]; see Table 4).

In most cases truncated proteins are not produced, because NMD detects and rapidly destabilize the PTC-containing mRNAs. By doing so, NMD acts as a “silencer” of PTC-containing genes and protect cells against the accumulation of potentially toxic C-terminally truncated proteins. Such a protective role of NMD

has been documented from studying β-thalassemic disorders, in which production of β-globin polypeptides and consequently of hemoglobin is compromised [156]. Many β thalassemias are caused by the introduction of PTCs in the β-globin gene. In most patients these diseases show a recessive mode of inheritance, but there are also a few cases in which the mode of inheritance is dominant. PTC that trigger NMD and hence lead to no or very little production of truncated β-globin are associated with the recessive mode of inheritance, but if the PTC is localized in the last exon of the β-globin gene, the transcripts produced are NMD-insensitive and are translated into truncated proteins with dominant-negative effects. PTC-generating mutations in the gene SOX10, which encodes a transcription factor essential for the development of cells in the neural crest lineage, provide another example of the protecting role of NMD against production of truncated toxic proteins [157]. Different nonsense mutations in SOX10 generate one of

two distinct neurological diseases. The

more severe one is a complex neurocristopathy called PCWH that is caused by the presence of PTCs in exon 5, the last exon of the gene. The less severe neurocristopathy (termed WS4) has a more restricted phenotype and PTCs in patients with WS4 reside principally in exons 3 and 4. Whereas SOX10 mRNAs with PTCs in exon 3 or 4 are degraded by NMD, resulting in the virtual absence

of truncated SOX10 protein and haploinsufficiency as a consequence, PCWH is the consequence of the activities of dominant-negative truncated proteins produced by the translation of NMD-insensitive SOX10 mRNAs with PTCs in the last exon.

In contrast to the two examples above, where the beneficial effect of NMD is evident, there are also cases in which NMD aggravates the disease phenotype, because the destruction of nonsense mRNAs prevents sufficient accumulation of truncated, yet still functional proteins. Mutant forms of the dystrophin gene provide an example of this detrimental NMD effect. PTCs in regions of the dystrophin gene where they trigger NMD are associated with the severe form of the disorder called Duchenne muscular distrophy (DMD), while the milder form, the so-called Becker muscular distrophy (BMD), is associated with nonsense mRNAs that are not recognized by NMD and hence serve as templates for synthesis of C-terminally truncated dystrophin protein [117].

For many genetic disorders caused by PTC-generating mutations, there are no effective treatments available. Because NMD plays an important role in modulating the clinical manifestations of such diseases, interfering with NMD represents a promising therapeutic strategy. For those cases where the prematurely truncated protein is still functional, inhibiting rapid degradation of the nonsense mRNA would in principle suffice to elevate the protein concentration and ameliorate the condition of patients. But in most cases, production of the full- length protein would be necessary to restore function, which can be achieved by promoting readthrough of the PTC [158, 159]. The first promising results were obtained with aminoglycoside antibiotics, which at very high concentrations

promote the readtrough of stop codons in eukaryotic mRNAs [158]. The aminoglycoside gentamicin suppresses stop codons in *in vitro* assays, and

beneficial effects of gentamicin treatment were reported from clinical trials with cystic fibrosis patients [160]. However, the high gentamicin concentrations also had toxic side effects on kidneys and ears, questioning the usefulness of its systemic application. More recently, a new readthrough-promoting compound
with no structural relationship to aminoglycosides has been developed. Remarkably, this small organic molecule called PTC124 selectively induces readthrough of premature but not normal termination codons [161]. PTC124 rescued striated muscle function in *mdx* mice expressing dystrophin nonsense alleles and the drug is currently being tested in clinical phase II trials on patients suffering from cystic fibrosis or DMD.

Open questions – future goals

Despite of intensive research and substantial progress during recent years, several fundamental questions about NMD are still waiting to be solved. Investigation of the underlying molecular mechanism would be greatly facilitated if an *in vitro* system could be developed that recapitulates the main features of NMD. To understand the NMD mechanism, understanding in more detail the regulation and function of the key NMD factor UPF1 will be crucial. Still very little
is also known about the molecular link between recognition of an aberrant translation termination event and the rapid degradation of this mRNA. And equally little is known about the actual degradation mechanism of nonsense mRNA. With regard to the various seemingly unrelated cellular processes that NMD factors affect, it will be interesting to learn, if these processes are somehow mechanistically coupled or if the NMD factors have a double life with two totally independent jobs. Furthermore, the role of NMD as a translation-dependent posttranscriptional regulator of many physiological mRNAs that are NMD targets needs to be explored. And finally, a detailed understanding of the mechanisms of PTC recognition and nonsense mRNA degradation will benefit the development
of highly specific tools for manipulating NMD and therewith gene expression. Such tools are not only promising for future therapies of diseases caused by

PTC-generating mutations, but would also offer various applications in biotechnology.

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Mühlemann et al.

Aberrant mRNAs	
Problem at DNA level	
Nonsense mutations	Base substitutions that directly generate PTCs.
Insertions and deletions	Random nucleotide insertions and deletions shift the reading frame in two of three cases, resulting in a PTC.
Mutations changing splicing signals	Mutations leading to aberrant splicing often result in a frameshift.
VDJ rearrangement	The immunoglobulin superfamily represents a special class of NMD targets that undergo very efficient NMD. Two of three rearrangements of the V, D, and J segments result in a frameshift.
Problem at RNA level	
Transcription errors	Frequency low, cause premature ORF truncation in <1% of transcripts.
Unproductive alternative splicing	45 % of alternatively spliced mRNAs are predicted to be an NMD target.
Problem at translation level	
Leaky scanning	Observed only in yeast. Ribosomes scan beyond the initiator AUG and initiate at a downstream AUG in a reading frame with a PTC.
Physiological mRNAs	
Programmed translational frame-shifting	Programmed +1 or -1 frameshifts lead into a PTC, if the ribosome fails to shift the reading frame properly.
mRNAs encoding selenoproteins	UGA can be recognized as codon for selenocysteine or as PTC, depending on endogenous selenium concentration.
mRNAs with uORFs	The termination codon of the uORF is likely to be interpreted as PTC, unless the mRNA harbors stabilizing elements nearby.
mRNAs with long 3' UTRs	Observed in all eukaryotes (including plants).
mRNAs with introns in the 3' UTR	Observed in yeast and mammals.
Transposons and retroviruses	Observed in yeast and mammals.
Bi-cistronic mRNAs	Observed only in yeast.
Transcribed pseudogenes	Observed only in yeast.

Table 1. Features and origins of NMD targets.

Mühlemann et al.

	S. cerevisiae	*C. elegans*	*D. melanogaster*	plants	mammals
NMD factors	UPF1	SMG2 (UPF1)	UPF1	UPF1	UPF1 (RENT1)
	UPF2 (NMD2)	SMG3 (UPF2)	UPF2	UPF2	UPF2
	UPF3	SMG4 (UPF3)	UPF3	UPF3	UPF3a, UPF3b (UPF3X)
	-	SMG1	SMG1	ND*	SMG1
	-	SMG5	SMG5	ND*	SMG5
	-	SMG6	SMG6	ND*	SMG6
	-	SMG7	-	ND*	SMG7
	-	SMGL-1	-	SMGL-1**	NAG (SMGL-1)
	-	SMGL-2	SMGL-2**	-	DHX34 (SMGL-2)
Translation termination	SUP45	T05H4.6	eRF1	ERF1	eRF1
	SUP35	H19N07.1, K07A12.4b	eRF3	ERF3	eRF3a, eRF3b
	PAB1	pab-1	pAbp	PAB	PABPC1
Exon junction complex (EJC)	No homologs	Present, not involved in NMD	Present, not involved in NMD	Present, role in NMD is still not clear	eIF4A3 Y14 MAGOH BARENTSZ (MLN51)

* ND = not determined
** role in NMD is not determined

Table 2. Homologous factors involved in NMD from different species.

Mühlemann et al.

Deadenylation: Removal of the poly(A) tail	
CCR4-POP2-NOT complex	Predominant deadenylase complex in yeast; the three factors are conserved in eukaryotes CCR4 and POP2: nuclease activity; NOT: accessory proteins.
PAN2/PAN3 complex	Conserved poly(A)-nuclease complex. PAN2: catalytic enzyme; PAN3 regulates and enhances PAN2 activity, and nteracts with MEX67 in yeast. PAN is involved in early steps of poly(A) metabolism (in the nucleus and during export).
PARN	Poly(A)-specific exonuclease; major deadenylases in mammals (*in vitro* studies). Found in mammals, plants, and *C. elegans*, but not in *D. melongaster* and *S. cerevisiae.*
Decapping: Removal of the cap structure	
DCP1-DCP2 complex	DCP2 is catalytic subunit. Decapping is inhibited by poly(A) tail and translation, and stimulated by a complex of LSM1-7 and additional proteins. DCP2 interacts with UPF1.
DCPS	Scavenger decapping enzyme. Mediates decapping of short cap oligonucleotides (e.g. produced by exosome-mediated degradation).
5' - 3' exonucleolytic degradation pathway	
XRN1	Major cytoplasmic. RNA with 5'-monophosphate (product of DCP2) is substrate for XRN1 *D. melongaster* homologue: Pacman
XRN2	5' - 3' exonuclease mainly involved in nuclear process. Yeast homologue: Rat1p
3' - 5' exonucleolytic degradation pathway	
Exosome	Large ring-like protein complex containing several 3' - 5' exonucleases. Highly conserved throughout evolution, involved in RNA degradation and processing.
SKI7	Adaptor between exosome and SKI2-SKI3-SKI8 complex. Homologous to GTP-binding elongation factor 1A. Required for non-stop decay. Interacts with UPF1.
SKI2-SKI3-SKI8 complex	Central component of the 3' – 5' cytoplasmic mRNA degradation pathway. Well analyzed in yeast, but little is known about the mammalian SKI complex. SKI2 is an ATP-dependent DEAD-box RNA helicase.

Table 3. Eukaryotic mRNA degradation factors.

Main references: Parker and Song, 2004 (NSMB); Wang, Lewis and Johnson, 2005 (RNA); Takahashi, Araki, Sakuno and Katada, 2003 (EMBO)

Table 4

Mühlemann et al.

Gene name	NMD efficiency	effect of mutation / phenotype
NMD beneficial		
β-globin	high	heterozygotes healthy; recessively inherited β-thalassemia major
	low	dominantly inherited β-thalassemia intermedia
SOX10	high	haploinsufficiency leads to less severe neurocristopathy (WS4: Waardenburg and Hirschsprung diseases)
	low	dominantly inherited complex neurocristopathy (PCWH: peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome and Hirschsprung disease)
Rhodopsin	high	heterozygotes have abnormalities on retinogram, but no clinical disease; recessively inherited blindness
	low	dominantly inherited blindness
Receptor tyrosine kinase-like orphan receptor 2	high	heterozygotes healthy; recessively inherited Robinow syndrome (orodental abnormalities, hypoplastic genitalia, multiple rib/vertebral anomalies)
	low	dominantly inherited brachydactyly type B (shortening of digits and metacarpals)
Cone-rod homeobox	high	mutation found in unaffected heterozygotes (no homozygotes found)
	low	dominantly inherited retinal disease
Coagulation factor X	high	heterozygotes healthy; recessively inherited bleeding tendency
	low	dominantly inherited bleeding tendency
NMD detrimental		
Dystrophin	high	severe form of muscular dystrophy (Duchenne muscular dystrophy)
	low	milder form of muscular dystrophy (Becker muscular dystrophy)
CFTR (cystic fibrosis)	high	severe cystic fibrosis
	low	milder form of cystic fibrosis

Table 4. Examples of genetic diseases where NMD modulates the phenotype. (adapted from [2])

Figure 1. Schematic illustration of NMD factors and their interactions.
NMD factors are shown in red, mRNA decay factors in blue, components of the EJC in green, proteins involved in translation in orange, and RNAi factors in purple. Apart from the exeptions below, black lines depict experimental evidence for direct protein-protein interactions.
* No direct physical interaction has been shown: a complex of the three UPF proteins co-immunoprecipitated with parts of the exosome, PARN (a deadenylase), XRN1, and DCP.
** The interaction between RNPS1 and the EJC is not yet mapped. And it is not clear if RNPS1 directly interacts with one of the three UPF proteins.
*** A complex of phosphorylated UPF1, SMG5, SMG7, and PP2A was found. But it is not clear whether SMG5 and SMG7 both interact with PP2A.

Mühlemann et al.

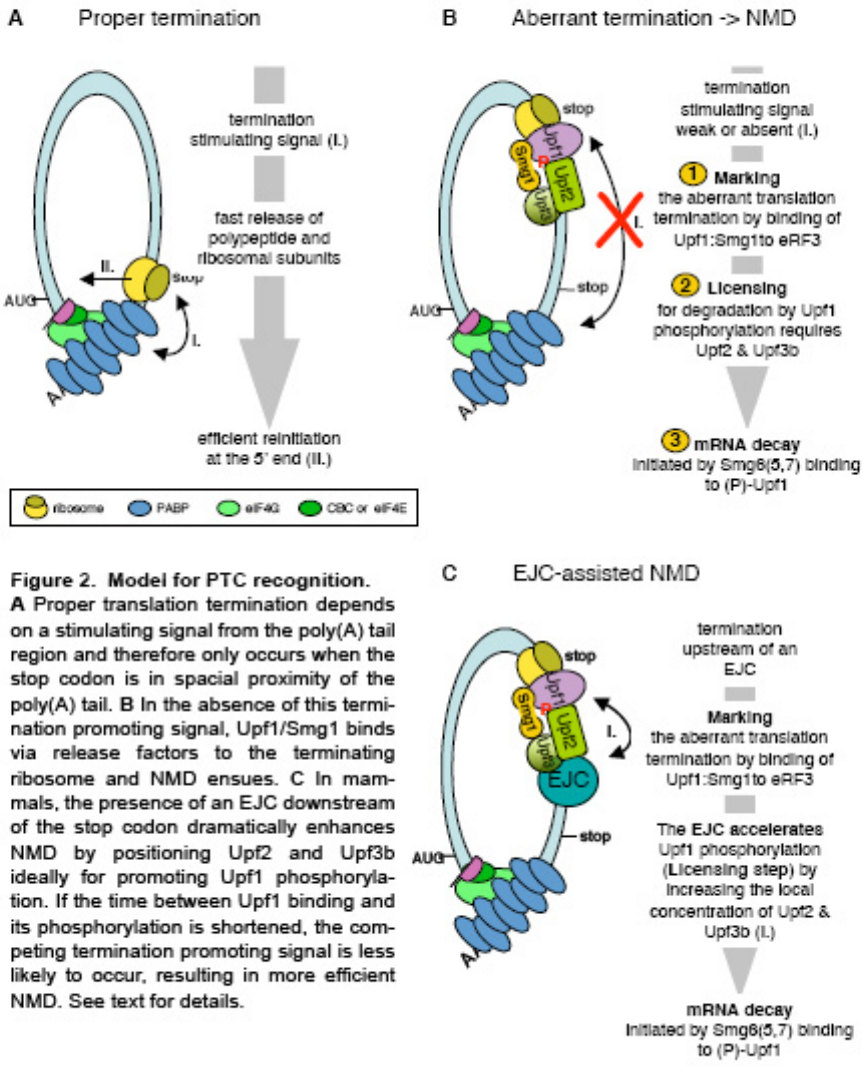

Figure 2. Model for PTC recognition. **A** Proper translation termination depends on a stimulating signal from the poly(A) tail region and therefore only occurs when the stop codon is in spacial proximity of the poly(A) tail. **B** In the absence of this termination promoting signal, Upf1/Smg1 binds via release factors to the terminating ribosome and NMD ensues. **C** In mammals, the presence of an EJC downstream of the stop codon dramatically enhances NMD by positioning Upf2 and Upf3b ideally for promoting Upf1 phosphorylation. If the time between Upf1 binding and its phosphorylation is shortened, the competing termination promoting signal is less likely to occur, resulting in more efficient NMD. See text for details.