To NMD or not to NMD: nonsense-mediated mRNA decay in cancer and other genetic diseases

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Abstract

The nonsense mediated mRNA decay (NMD) pathway degrades some but not all mRNAs bearing premature termination codons (PTCs). Decades of work have elucidated the molecular mechanisms of NMD. More recently, statistical analyses of large genomic datasets have allowed the importance of known and novel 'rules of NMD' to be tested and combined into methods that accurately predict whether PTC-containing mRNAs are degraded or not. Here we discuss these genomic approaches and how they can be applied to identify diseases and individuals that may benefit from the inhibition or activation of NMD. We also discuss the importance of NMD for gene editing and tumor evolution, and how inhibiting NMD may be an effective strategy to increase the efficacy of cancer immunotherapy.

Keywords

loss-of-function variants, transcriptomics, genetic disease, genetic variant interpretation, tumor suppressor genes, cancer immunotherapy

NMD: the linchpin bridging gene regulation and transcriptome quality control

Nonsense-mediated decay (NMD) is a quality control pathway that removes transcripts bearing **premature termination codons (PTC)** (see Glossary). Many comprehensive reviews cover the biochemistry of NMD in mammals and other organisms [1–3] so our aim here is not to re-cover this mechanistic work. Rather, we will focus on recent genomic analyses that have tested, refined and extended the rules governing how NMD chooses which PTC-bearing transcripts to degrade and which to ignore. We will then discuss the implications and applications of these rules to understanding and treating cancer and other genetic diseases (Figure 1, Key Figure). Some of the NMD rules that we will discuss are well-established and mechanistically characterized, others have been proposed more recently and the underlying biochemical mechanisms are still unclear.

PTC-bearing transcripts can be caused by single nucleotide mutations in coding regions but also by mutations in splice sites and insertions or deletions (indels) resulting in frameshifts that generate a downstream stop codon [4,5]. These mutations are by default often considered to be loss-of-function (LoF) events for the protein-coding genes that harbor them, in part because of the assumption that NMD will degrade transcripts bearing PTCs and no protein will be produced. However, genomic analyses show that this assumption is surprisingly frequently invalid and that many PTCs – including known disease-causing variants – actually completely or partially evade NMD detection and mRNA degradation [6–8], likely resulting in the production of truncated and frameshifted proteins. According to the NMDetective model that provides genome-wide predictions of NMD efficacy, approximately 50% of all possible PTC variants that can occur in human would evade NMD to some extent [8]. This is highly variable across genes, though. In 17% of human genes, >¾ of the coding regions will allow NMD to be fully triggered if a PTC occurs therein, while in 36% of genes >¾ of the coding sequence will allow PTCs to at least partially evade NMD [8].

It is important to stress that NMD not only degrades mutated transcripts: NMD is also a quality-control mechanism that removes aberrantly spliced transcripts, such as those resulting from retained introns or skipped exons. Moreover, NMD has been estimated to regulate approximately 10% of the normal transcriptome[9–11], thus having an important impact on physiological gene expression. This is because many transcripts have NMD-inducing features even in the absence of PTCs [12]. Additionally, alternative splicing can be coupled with NMD as a means for gene regulation: tissue-specific inclusion of a PTC-bearing cassette exon in a transcript will silence expression of a gene in that tissue [13]. Gene regulation by NMD is important for organismal development, and particularly critical for differentiation in some tissues and for cellular stress responses. This physiological regulation of gene expression by NMD has been covered in recent review articles [1–3], and so we do not cover these topics here.

Testing the rules of NMD through genomic analyses

In mammals, the principal mechanism by which NMD distinguishes between PTCs and normal stop codons is thought to be via a coupling to the splicing machinery: upon removal of introns, a protein assembly (**the exon-junction complex, or EJC**) usually remains deposited on the mRNA near the splice site. During translation, the elongating ribosome strips off the EJCs from the mRNA. EJCs after the stop codon will therefore not be removed and serve as a signal to initiate NMD [14–16]. It follows from this molecular mechanism that PTCs in the last exon will not be seen by NMD, which we refer to as the *last-exon* rule of **NMD evasion**. Additionally, PTCs in the last approximately 50 nt of the penultimate exon will also cause the last EJC to be removed from the mRNA because of the footprint of the ribosome and the positioning of the EJC

[17]. Again, NMD will be prevented; we refer to this as the *50nt-rule* of NMD evasion. The last-exon rule and the 50 nt-rule were also jointly referred to as the "50–55 nt rule" previously [17,18]. These two 'canonical' NMD rules mean that NMD is blind to PTCs in the 3' end of the transcript in mammals; this was very robustly observed in experimental work on individual PTCs or small sets thereof [14,15,19].

The validity of the canonical NMD rules have been tested in multiple large-scale analyses of human genomic sequences, matched with RNA sequencing (RNA-Seq) of transcriptomes from certain tissues, commonly blood, in the same individuals. Early analyses examined PTCs resulting from heterozygous single-nucleotide germline variants in tens of human individuals [20,21], and confirmed that NMD is indeed less efficient for nonsense variants in the last exon. An analysis of transcriptomes of blood cell lines from approximately a hundred individuals again reported loss of expression of the PTC-bearing allele in NMD-sensitive regions (considering last-exon and 50nt rules) [22], but also noted that many of the variants predicted to trigger NMD did not have detectable effects on gene expression. Barring issues with statistical power in detecting allelic imbalance, this suggested that the canonical last-exon/50 nt NMD rules may not be a complete description of how NMD selects transcripts for decay. This was mirrored in later, more extensive analyses of hundreds of paired human genomes and transcriptomes from the GTex and Geuvadis projects [23,24]. Not only did many of the PTC variants predicted to trigger NMD appear to escape NMD (i.e. no allele-specific expression was seen), but also the predicted NMD-escaping PTC variants appeared to have higher allelic imbalance than synonymous variants suggesting some of them did not escape detection. Overall, deviations from the canonical NMD rules appeared common [22–24], implying that additional rules remained to be discovered.

Learning new rules of NMD from cancer genomes

Cancer genomics presents an opportunity for large-scale data analysis to better understand NMD, because of the abundance of data (the Cancer Genome Atlas, or TCGA, provided approximately 10,000 matched tumor exomes and transcriptomes) that boosts statistical power. An additional benefit is that the signatures of **negative selection** acting upon genetic variation in tumors are less strong than in the germline [25]: most somatic mutations are 'passengers' thus making it less likely that their downstream effects on mRNA expression (for instance via triggering NMD) are shaped by selection, which can confound analyses that aim to discover NMD rules. The very clear signal reflecting the canonical, well-established NMD rules validated the use of somatic mutations for defining NMD rules (Box 1).

<<< BOX 1 >>>

Discovering NMD rules from paired cancer exomes and transcriptomes

Influences on the efficiency of NMD can be discovered by comparing gene expression levels between tumors harboring a somatic PTC mutation in a certain gene and those tumors that do not harbor PTCs in that gene to estimate the efficiency of NMD acting upon that PTC [26]. By eschewing the allele-specific gene expression analysis (which requires a substantial sequencing coverage of the specific locus bearing the PTC by RNA-Seq reads and is thus applicable to the more highly expressed genes only) it was possible to examine NMD effects across a broad set of genes of various expression levels. A challenge in such an analysis, however, is to compensate for influences on gene expression in *trans* which arise e.g. from global tissue-specific gene expression patterns [27] and also from the impact of copy number alterations (CNA). Upon stringent filtering of CNA-affected regions and defining subgroups of tumors that were relatively uniform by global gene expression patterns, a set of ~2,800 high-confidence nonsense somatic mutations was available for systematic discovery of NMD rules -- a data set substantially larger than prior efforts based on germline variation. The rules were validated in a set of ~3,100 PTC-inducing frameshifting indel mutations from the same TCGA tumor data set, and additionally in an independent set of ~1,800 nonsense germline variants in the Geuvadis data set.

This confirmed a very strong effect of the canonical *last-exon rule* and the *50-nt rule* of NMD evasion in cancer data: tumors with PTCs positioned in those 3' transcript regions, on average, did not exhibit changed gene expression compared to tumors without a PTC [26]. These two rules stem from the standard EJC model of NMD. An interesting addendum to this is that in transcripts with intron-bearing 3' UTRs, PTCs in the penultimate exon may also strongly evade NMD despite the presence of a downstream EJC [26], suggesting the EJC in 3' UTRs may be less potent in initiating NMD.

The '*faux* 3' UTR' model is an EJC-independent NMD mechanism demonstrated in yeast (which has few introns) and *Drosophila*. Here a long 3' UTR, caused by a PTC far upstream from the 3' gene end, is proposed to promote NMD by hampering the interaction between poly-A tail binding protein PABP and the terminating ribosome. A related NMD mechanism was reported in mammalian cells [28–31]. Thus far, however, systematic genomic analyses of tumors suggest this mechanism appears not to commonly act on many transcripts in mammalian cells, because PTCs far from the transcript 3' end, overall, tend toward a reduced NMD efficiency [26].

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By testing the ability of many different genomic features to predict NMD efficiency in the cancer data, we proposed additional, 'non-canonical' rules of NMD (Figure 1). Most

salient is the *start-proximal rule* of NMD evasion, where NMD efficiency is decreased in the 5'-most approximately 150 nt of the coding region of a transcript, with a gradual increase in efficiency from 5' to 3' in this segment. This rule was anticipated by a known example 5' terminal PTCs in the beta-globin gene and the triosephosphate isomerase gene which evaded NMD, suggesting an approximately 25 nt start-proximal NMD evasion region [32–34] with the mechanism underlying this being re-initiation of translation on a downstream start codon. Based on this, several individual genetic reports have described how translation reinitiation affects disease severity when NMD was evaded by start-proximal PTCs [35–38]. The cancer data analyses [26] provides systematic evidence that this rule indeed applies broadly, but with the evading region longer than 25 nt. The cancer genomic data also supports that the reinitiation mechanism is commonplace (although not necessarily universal), because having an in-frame start codon nearby reduces NMD efficiency three-fold [26].

The cancer data also suggested other non-canonical NMD rules that were, to our knowledge, not anticipated. First, the long-exon rule: very long exons (>400 nt) tend to have lower NMD efficiency than shorter ones, with an additional corollary that PTCs in such long exons that are further away from the 3' end of the exon trigger NMD less efficiently [26]; this was later supported by experimental work [39] as well as by analyses of CRISPR gene editing data [8]. Speculatively, this may be due to the (known) EJC-dependent mechanism, where the stalled ribosome at the PTC needs to make physical contact with the downstream EJC to initiate NMD, which would presumably be less efficient if the distance between the ribosome and EJC were large. Second, it was found that PTC that are very far from the normal stop codon have somewhat reduced NMD efficiency (the *PTC-to-normal-stop* rule) [26], which is the opposite of what the "faux-3' UTR" model of NMD (of yeast and Drosophila) would predict (Box 1). Third, it was observed that mRNAs which normally have shorter-half-lives also have lower NMD efficiency, presumably due to competition between NMD and other mRNA degradation processes [26]. Fourth, the presence of certain motifs in the mRNA, which could be located either near to the PTC or in the natural UTR of the transcript, is associated with altered efficiency of NMD [26], with strong evidence to support four motifs corresponding to the SRSF1, PABPN1, SNRPB2 and ACO1 binding motifs (Figure 1).

<<< BOX 2 >>>

Validation of the NMD rules using genomics, single-molecule microscopy and gene editing

Some of the non-canonical NMD rules proposed from analyses of cancer genomic data [26] were subsequently validated in independent work [8,39,40]. Analysis of the allele-specific expression of 2,000 tumors in the TCGA [40] confirmed that the distance of

PTC to 3' exon end is important (see long-exon rule above), that there is common NMD evasion in start-proximal PTCs (especially if <100nt to start codon), and finally that also penultimate exon (even before the 50nt) may sometimes evade NMD(these samples overlap those used in the original analyses [26] but the method of estimating NMD efficacy, based on allelic imbalance in RNA-Seq, is orthogonal to the original method). Experimental work using single-molecule microscopy [39] supported that the distance between the PTC and a downstream EJC affects NMD efficiency, and also that the number of downstream EJCs of the PTC -- rather than simply having any or none -- is relevant, which may be related to the rule involving the penultimate exon (see above). This study further suggests that the sequence adjacent to a PTC can have a large effect on NMD efficiency [39], however no specific motifs were proposed.

Additional work [8] has further validated these rules by analyzing the effects of CRISPR/Cas9 gene editing on protein expression [41] and cellular fitness [42]. In both cases, the non-canonical *start-proximal* NMD evasion rule was verified: edits targeting start-proximal sites did not decrease protein levels at maximum efficiency, nor did they elicit the same **fitness loss** when targeting essential genes, as edits in predicted NMD-sensitive regions [8]. Translation re-initiation, downstream of the gene editing site, coupled to an evasion of NMD, may be a common reason for failed attempts to inactivate genes using CRISPR-Cas9 editing [43,44]. Additionally, the non-canonical *long-exon* rule was validated in the fitness data [8] .

These analyses provide guidelines to refine existing CRISPR/Cas9 reagents and libraries, where incorporating the knowledge of the NMD rules was proposed to be helpful [18]. In existing CRISPR/Cas9 genetic screening libraries, typically, approximately half of the targeted sites may lie in NMD-evading regions, according to a canonical or to a non-canonical NMD rule [8], highlighting how more attention needs to be paid to incorporating NMD rules into CRISPR reagent design. Shunting edited mRNAs to the NMD pathway helps to 'cleanly' inactivate a gene by avoiding generation of **truncated proteins** where the loss-of-function is only partial, or which might possibly have gain-of-function or be toxic to cells.

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The non-canonical NMD rules – which have been further supported by subsequent studies (Box 2) – cover substantial parts of genes and thus apply to a large number of PTCs that may occur [8]. This means that they are quantitatively important predictors of NMD activity. Considered jointly in a predictive model, the canonical NMD rules (*last-exon* and *50 nt* rule) can explain about 50% of the systematic variation in NMD efficiency across PTCs observed in cancer genomes and the non-canonical rules, an additional 25% variation [26]. In other words, a single large-scale genomic analysis was

able to substantially increase our understanding of how NMD identifies substrates to degrade. The remaining ~25% of the systematic variability in NMD activity is currently not explained by the proposed NMD rules. Larger data sets with more statistical power may be useful to discover additional rules that are likely to individually have only subtle effects, or are applicable rarely, but collectively help explain the remainder of the NMD activity. In addition, further insight into the unexplained NMD rules might be gained via experimental work in model systems, or by use of new or improved sequencing technologies. For instance, long-read sequencing has the potential for characterizing transcriptomes in more detail, revealing novel isoforms that may be targeted by NMD, or isoforms where certain PTC variants may have different ability to elicit NMD than in the common isoforms. Moreover, the increasing throughput and resolution of spatial transcriptomics [45] and single cell multi-omics technologies [46,47] will enable further investigation on how cell fate, cellular micro-environment and cell-to-cell variability impact the efficacy of NMD and the outcome of PTCs.

The implications of NMD evasion for genetic disease

A lesson learned from the work comparing matched genomes and transcriptomes in human populations and human cancers [23,24,26] is that – perhaps contrary to previous expectations – many PTC variants in the human germline and soma result in transcripts that are not fully cleared by NMD. Many PTC containing transcripts are therefore likely to be translated into truncated and/or frameshifted proteins, and thus should not be automatically considered as complete LoF (null) variants. Instead, they may retain partial function (hypomorphic alleles), dominant-negative (antimorphic), gain-of-function (GoF, neomorphic), or effectively silent. It is intuitively clear how NMD is critical for determining whether the PTC results in a null mutation: transcripts efficiently destroyed by NMD cannot be translated into protein and represent complete LoF alleles. However, NMD-escaping PTC variants can have a variety of effects, where full LoF is only one possible outcome. In particular, truncated versions of disease genes may surprisingly often retain partial function [48] and algorithms that predict the pathogenicity of truncating variants such as ALoFT and LOFTEE [49,50] typically incorporate features that predict NMD escape based on the canonical NMD rules (last-exon and 50 nt rule). Including the non-canonical NMD rules improves the prediction of PTC variant pathogenicity, complementing other conventionally recognized features (e.g. if a PFAM domain is affected) [8].

Different effects of truncation on the biochemical functions of a protein may translate into different phenotypes, such as variable disease severity. Truncations resulting in dominant negative proteins can result in more severe disease phenotypes than LoF mutations. In contrast, truncations that retain partial function can result in less severe phenotypes than NMD-triggering PTCs (Figure 2). For example, in beta-thalassemia

truncated forms of the beta-globin protein can be toxic and so mRNA degradation by NMD ameliorates the disease. In contrast, in Duchenne muscular dystrophy, C-terminal truncations of the DMD protein can retain activity such that mRNA degradation by NMD aggravates the disease. Additional examples of disease genes conforming to one or the other paradigm are known (reviewed in [51,52]).

The preconception that the primary role of the NMD pathway is to clear nonsense variants from the transcriptome suggests that PTCs are usually harmful even when heterozygous and that NMD protects against them. This logic has shaped thinking about the role of NMD genetic disease where NMD tends to be considered as a protective mechanism. However, genomic analyses actually suggest that the opposite is often the case. An analysis of known disease-causing nonsense mutations found an enrichment of NMD-triggering variants over NMD-escape variants, suggesting that NMD actually promotes disease and that dominant-negative effects are a less common mechanism by which pathogenic nonsense variants cause disease [6]. Considering disease genes individually and including the non-canonical NMD rules clarifies this conclusion, classifying many known disease variants as NMD-escapers [8]. In the majority of disease genes, at least a quarter of PTCs reported in ClinVar are predicted to escape NMD. This is because the non-canonical *start-proximal* and *long-exon* rules apply to a substantial proportion of gene sequence: both rules cover ~12% of human protein-coding sequence, in sum, similar to the ~18% and ~3% covered by the canonical *last-exon* and *50-nt* rules, respectively (note that the *last-exon* rule also encompasses intronless genes). Quantifying the enrichment of pathogenic variants in NMD-evading and NMD-triggering regions for each disease gene identified 49 disease genes with a two-fold or higher excess of pathogenic PTCs in NMD-evading regions and 155 disease genes with an excess of pathogenic PTCs in NMD-triggering regions [8]. Thus, for a majority of human disease genes, NMD actually more frequently aggravates the disease (Figure 2A). Analyses of the prevalence of truncating variants in the general human population support the conclusion that, overall, NMD tends to increase the detrimental effects of truncating variants (Box 3). Distinguishing whether NMD aggravates or counteracts the effects of disease mutations is important for designing therapeutic approaches to alleviate disease phenotypes: whereas in many patients inhibiting NMD is likely to be beneficial, in others activating NMD to remove a PTC-containing transcript would be the correct therapeutic strategy.

<<< BOX 3 >>>

Negative selection on NMD-eliciting variants in human populations

Variants that are deleterious to fitness are depleted from the common variants in the human population and are therefore rare. Early analyses of transcriptomes from hundreds of individuals reported a higher fraction of NMD-eliciting truncating variants

(estimated by allele-specific expression) in the rare standing variation in the general population [23,24], thus the rare germline PTCs are more likely to trigger NMD than the common PTCs.

The expanded set of NMD rules [8] and a greatly enlarged dataset of human population genetic variation [49] allowed the occurrence of PTC variants to be compared to a random baseline obtained by simulating mutational processes [8]. The fraction of NMD-triggering PTC variants among all variants was lower than expected, suggesting they are under stronger negative selection than NMD-evading variants. Moreover, within the rare variants (allele frequency, AF=[10^{-5}, 10^{-4})) this depletion of NMD-triggering variants was modest, while in the common variants (AF=[10^{-1}, 1]) this depletion was considerably stronger [8], consistent with truncating variants seen by NMD being more effectively purged by selection. This is in line with the notion that overall, the effects of NMD acting upon PTC variants appears to be detrimental rather than beneficial (notwithstanding the key roles of NMD in gene regulation, which are essential for correct organismal development).

A further application of the NMD rules to population genomic data was to identify genes in which truncations would yield dominant-negative effects [7]. The usual measures of PTC depletion in population data (e.g. the pLI metric, or LOEUF [49,53]) are intended to test for intolerance to heterozygous LoF variation – without specifying whether this results from haploinsufficiency or from dominant-negative effects. Activity of NMD upon variants in such genes can resolve the two scenarios: an “NMD-escape intolerance score” nominated 252 genes with a depletion for truncating variants specifically in NMD-escape regions in human genetic variation databases [7], suggesting dominant-negative effects of the truncations. This illustrates how NMD rules may be applied to learn about gene function from population genomics analyses of ‘human knockouts’; other examples are provided in the section “NMD directs cancer evolution”.

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NMD directs cancer evolution.

Tumorigenesis is a Darwinian evolutionary process where positive selection, negative selection and drift determine the frequency of genetically heterogeneous clones within the tumor mass. Recent work confirms that, as anticipated, NMD plays an important role in determining the selective benefit of somatic mutations that result in PTC-bearing transcripts: frameshifting indels, nonsense mutations and splice site mutations. The category of genes where NMD is most relevant are tumor suppressor genes (TSGs): abolishing the function of TSGs such as *TP53*, *RB1* or *PTEN* releases the ‘breaks’ on

tumor growth, for example by overriding cell cycle controls, and thus null mutations in TSGs confer a fitness advantage to the cancer cells that bear them.

Overall, truncating mutations in TSGs that trigger NMD are under stronger positive selection than those which escape NMD [26], consistent with NMD resulting in a complete LoF of that allele (Figure 2B). Such NMD-eliciting mutations are very prevalent and are associated with lower gene expression in tumors [54,55]. It is important, therefore, to consider the effects of NMD when evaluating the likely cancer-driving effects of truncating mutations in tumors. While most work on cancer NMD genomics has focused on nonsense mutations and frameshifting indels [26,54,55], splice site mutations are also an important cause of truncations: they commonly lead to intron retention events in TSGs, often generating out-of-frame transcripts that bear PTCs enriched in NMD-sensitive regions [56]. Splice site mutations can also be exonic (particularly the 3'-most nucleotide of an exon [56]), meaning their effects could be mis-interpreted as missense or synonymous, rather than LoF, as is observed in the TP53 tumor suppressor [57]. NMD inhibition by pharmacological means, alone or in combination with stop-codon readthrough agents, is considered as means of reactivating mutated TSG to treat tumors [58,59].

Many TSGs are thought to conform to the 'two-hit' paradigm, where both alleles need to be inactivated. Jointly considering the occurrence of NMD-eliciting versus NMD-escaping nonsense mutations with the occurrence of copy number alterations provides a classification scheme for TSGs [26], depending upon whether they are more often two-hit (classical) or one-hit (haploinsufficient or dominant-negative) TSGs, and whether the 'hits' derive from truncating variants or from copy-number alterations. While NMD generally enhances the positive selection acting upon mutated TSGs, individual examples of dominant-negative truncated variants of TSG are known (e.g. germline variants in *WT1*, or in *BRCA1* [60,61]) and for these NMD may confer a fitness penalty for the tumor. These examples however appear rare, thus missense mutations would likely be a more frequent cause of the dominant-negative effects on TSGs (for example, these appear common in the *TP53* tumor suppressor [62]).
Finally, although positive selection on driver mutations seems to dominate the evolution of tumors, the application of the rules of NMD provides evidence that negative selection against mutations in genes essential for tumor growth may also be occurring in human tumors (Figure 2B; Box 4).

<<< BOX 4 >>>
Using the rules of NMD to detect negative selection in tumor genomes.

Because NMD activity results in full LoF alleles, it would be expected that NMD increases the fitness penalties incurred by truncating mutations in genes essential for viability or proliferation.
Negative selection is detectable on very few genes in cancer genomes [25,63,64], possibly because many genes are haplosufficient in tumors, requiring both alleles to be inactivated to incur a substantial fitness cost. However, focusing only on more disruptive nonsense mutations and pooling them by whether they are likely to trigger or evade NMDs reveals a significant deficit of mutations in the NMD-eliciting regions of oncogenes (genes that normally promote tumor growth and thus would not be expected to tolerate LoF mutations in a tumor) and cell-essential genes (whose inactivation should incur a fitness penalty to most cell types) [26]. Additionally, negative selection may operate on certain pathways: regulation of cell proliferation, the spliceosome, and, intriguingly, cell migration genes [26]. The significance of this result for understanding tumor evolution is that it supports the notion that some subclones are eliminated during tumorigenesis because they carry deleterious mutations [65]. Furthermore, detecting negative selection on cancer genomes is of high interest because it identifies therapeutic vulnerabilities - protein targets that are essential for tumor growth or survival.

Frameshifting indels in NMD-escaping regions have also been suggested to be under negative selection in tumors because they are underrepresented compared to stop-gain mutations in NMD-escaping regions [55]. This suggests that the resulting proteins are detrimental to cancer cells, for example by generating dominant-negative activities [66] or by provoking an immune response against **neoantigens**. Consistently, longer frameshifted neopeptides were more likely to be recognized by the immune system [55]. Pharmacologically inhibiting NMD may be a therapeutic strategy to reactivate expression of such toxic or immunogenic polypeptides, which are likely to be prevalent in heavily mutated tumor cells but rare in healthy cells.
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NMD and the immune reactivity of tumors
Frameshifting indels are important determinants of immune infiltration of tumors and the tumoral response to **immunotherapy** [8,55,67,68]. Immune checkpoint blockade is now one of the most successful approaches to cancer therapy; principles and modalities of cancer immunotherapy were reviewed recently (see for instance [69,70]).
The high mutational load of many tumors means that they carry multiple indel mutations that, if translated, will result in frameshifted proteins with tens of altered amino acids. Such neopeptides can act as neoantigens to trigger an immune response against a tumor. However, frameshifting indels also often introduce PTCs into transcripts. If these PTCs are detected by NMD no neoantigens will be produced (Figure 2C). Thus,

whether PTCs introduced by frameshifts trigger or evade NMD may be critical for whether frameshifts result in neoantigen production and an important influence on the recognition of tumor cells by the immune system [8,71,72]. Consistent with this hypothesis, a high number of frameshifting indels that evade NMD – but not a high number of frameshifting indels that trigger NMD – predicted the infiltration of lymphocytes into tumors, as estimated by lymphocyte-specific gene expression [8]. Moreover, in uterine cancer, deleterious mutations in UPF1 – a key factor in NMD – were also associated with higher immune infiltration [8]. Increased NMD-evading frameshift burden also predicts less aggressive disease in kidney cancer where the presence of even a single NMD-escaping frameshift in coding regions is associated with better survival [8]. Somatic copy number alterations in multiple NMD genes were noted to co-occur in cancer genomes, and this was associated with the global burden of NMD-detectable mutations [68], suggesting the intriguing possibility that tumors may boost NMD capacity to deal with increased **mutation burden**. Consistently, inhibiting NMD was toxic to hypermutating, microsatellite-instable cells [66]. One possible mechanism involved mutations in the HSP110 gene that induce exon skipping and encode a dominant-negative protein product [73,74] whose transcript can be cleared by NMD [66].

Most importantly, in various cohorts of melanoma, lung cancer and kidney cancer patients (and smaller numbers of patients of other tumor types) the burden of NMD-evading frameshift indels predicted patient response to immune checkpoint blockade [8,55]. In contrast, the burden of NMD-triggering frameshifts did not predict immunotherapy response, highlighting the critical role of NMD in circumventing the surveillance of tumors by the immune system. We note that measuring levels of mutated proteins would provide additional confidence that the NMD-evading mutations are in fact those responsible for the immunogenicity.

One well-recognized marker for immunotherapy response is the overall tumor mutation burden (TMB; indels are normally just a small fraction of this), presumably because tumors with more mutations typically produce neoantigens. In a joint model, the NMD-escaping frameshifting indels were predictive of immunotherapy response independently of TMB [8,55]. Consistently, NMD-escaping frameshifts could help predict responders among low-TMB patients [55]; at a specificity of approximately 90%, a TMB+NMD-escape frameshift model achieved a 10 percentage points increase in sensitivity over a TMB-only model [8], meaning that many additional immunotherapy responders could be identified by examining specifically the NMD-escape frameshifts than by the TMB alone.

The implications of these studies [8,55] are two-fold. First, the extent to which frameshifting indels evade NMD should be included in models to predict patients that will respond to immunotherapy. Second, they suggest that inhibition of NMD may be an effective strategy to improve the number of patients that respond to immunotherapy (Figure 2D). Indeed the burden of NMD-evading frameshifts [8,55] and perhaps also variation in the efficacy of NMD across tumors [75] should help predict the patients most likely to respond to such an adjunct therapy. Pharmacological inhibitors of the NMD pathway (reviewed in [72]) can be well tolerated, and have shown efficacy in some cancer models [66,71].

Concluding remarks

The conventional understanding of the role of NMD in genetic disease tends to assume that NMD has a protective role because it prevents translation of harmful protein products (e.g. examples of dominant-negative protein truncations). Cases where NMD is harmful and aggravates disease have been recognized, but they tend to be seen as the exception rather than the rule. Based on large-scale, systematic analyses of human genomic data, we posit that this assumption should be revised. In many disease genes, perhaps the majority, NMD activity appears to more often enhance rather than relieve the deleterious effects of PTCs. In genome-wide analyses, NMD-triggering mutations appear to be effectively purged from human populations. Moreover, in cancer, NMD frequently contributes to full inactivation of tumor suppressor genes thus driving cancer. Finally, NMD also protects tumor cells by silencing the expression of immunogenic and/or toxic peptides resulting from frameshifting indel mutations. Overall, the patterns of selection in human genomic data could be interpreted to mean that the primary role of the NMD pathway is not to buffer the effects of deleterious stop codons, since it often does not succeed at the task and might in fact have the opposite effect. Instead, the *raison d'etre* of NMD would be its established roles as quality control for mRNA splicing and/or as a global gene regulation mechanism. These important roles likely explain why a functional NMD pathway is essential for correct development of organisms and their loss is commonly embryonic lethal or results in neurodevelopmental phenotypes.

Thus, although in some genetic diseases activating NMD (to silence a detrimental protein) may be beneficial, inhibition of the pathway is likely to be beneficial for many more diseases. We are therefore optimistic that there are abundant opportunities for inhibiting NMD in treating tumors and for alleviating the symptoms of many genetic diseases (Figure 2D). Indeed, we would encourage a more concerted effort to identify novel and more specific NMD inhibitors to be tested in a wide range of genetic diseases. In particular, the inhibition of NMD may be a quite general strategy to increase the number of patients responding to cancer immunotherapy and experimental

work and clinical trials are needed to further evaluate the efficacy of this approach. In both cancer and other genetic diseases, genomic predictors of NMD efficacy will be key for classifying patients into those most likely to benefit from NMD inhibition.

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Glossary

Copy number alterations (CNA): Changes in the number of genomic copies of chromosomal segments, resulting from duplication or deletion of DNA.

Exon junction complex (EJC): A protein assembly that usually remains deposited on the mRNA near the splice site after splicing.

Fitness loss/gain: An decrease/increase in the ability of cells or organisms to survive or to reproduce.

Immunotherapy: A type of cancer treatment that boosts the ability of the immune system to clear cancerous cells.

Negative/positive selection: Decrease/increase of the frequency of a genotype in a population due to a fitness loss/gain to cells or individuals carrying that genotype.

Neoantigens: Antigens expressed on tumor cells but not on normal cells that may trigger an immune response and derive from mutated or aberrantly expressed proteins.

NMD evasion: A passive process in which NMD fails to recognize and degrade a PTC-bearing transcript.

Premature termination codon (PTC): A stop codon that occurs 5’ of the normal stop codon in the transcript, due to a mutation or due to altered splicing.

Truncated protein (also, protein truncation): A protein that is shortened because a mutation interrupted its translation, which can impair its function.

Tumor mutation burden (TMB): The total number of mutations in the tumor genome or exome.

Figure legends

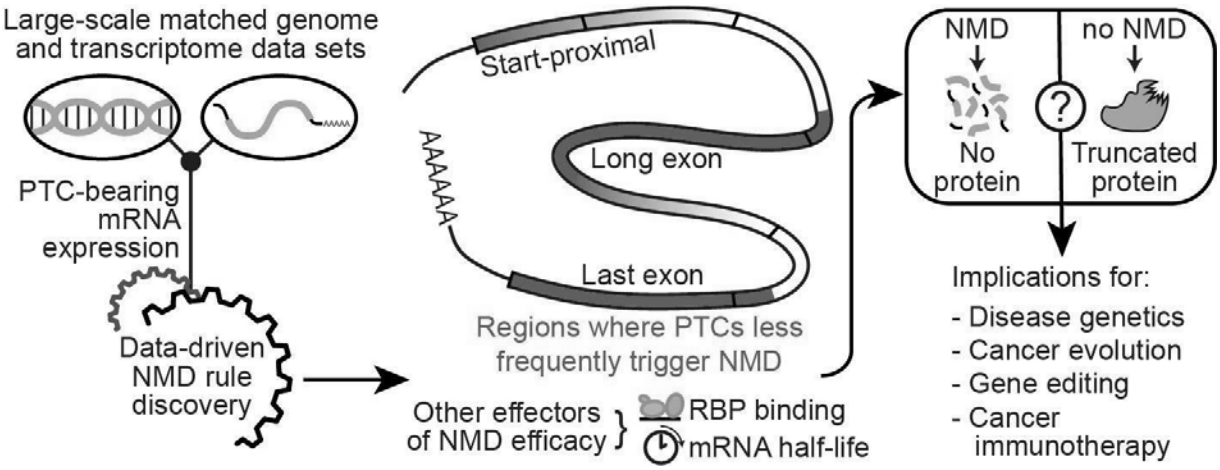

Figure 1, Key Figure: Using genomics data to uncover the rules and implications of NMD. Matched genome and transcriptome data can be used to quantify the downregulation of mRNA expression that is induced by premature termination codon (PTC) introducing mutations through NMD. Associating the effect of NMD to the PTC location helped reveal the molecular features that determine when a PTC can trigger NMD. These molecular determinants are called the rules of NMD, and predict the functional outcome of PTC-introducing mutations, which has wide implications for disease biology. The regions in a mRNA transcript in which PTCs are less likely to trigger NMD are highlighted in blue. RBP: RNA binding protein.

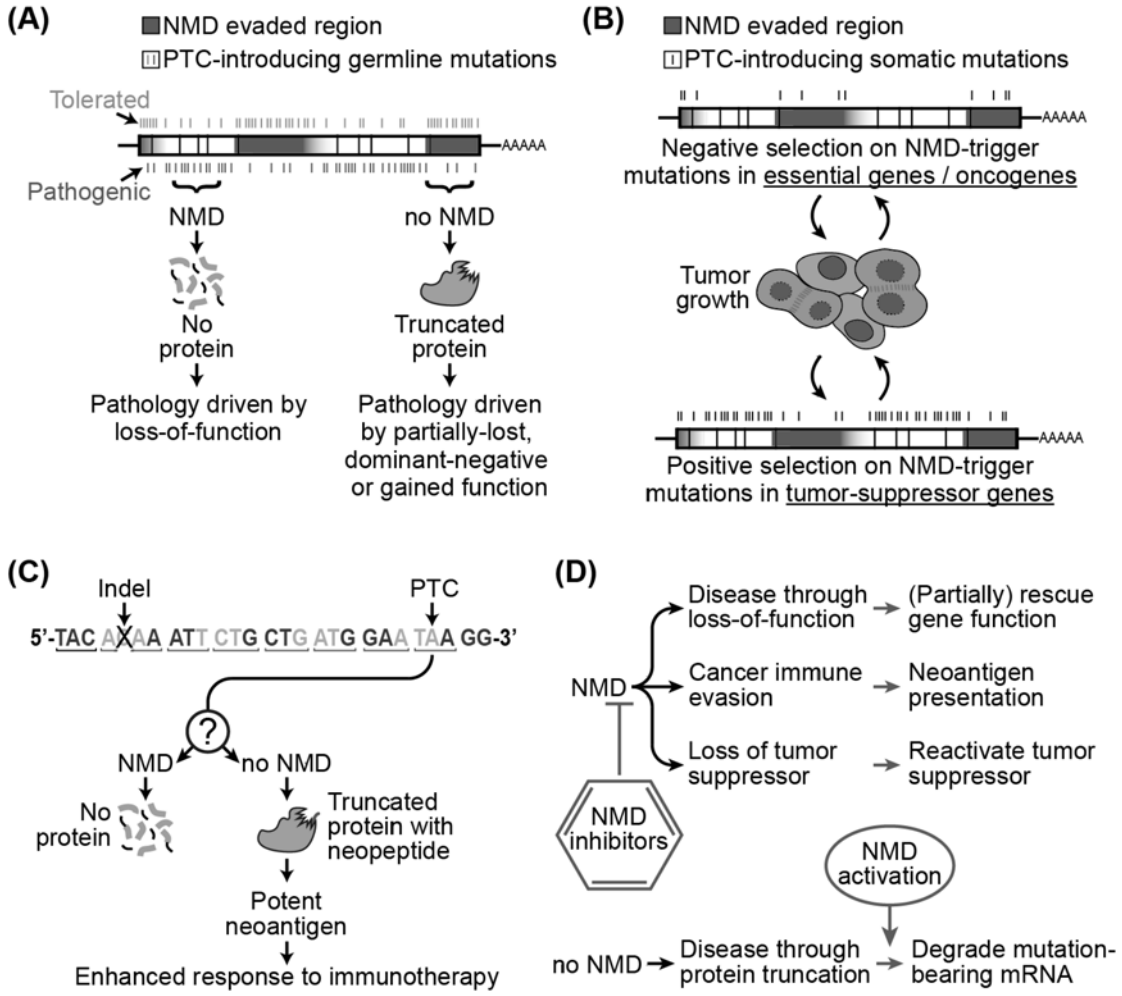

Figure 2: Schematic overview of the implications of NMD on disease. (A) Pathogenic premature termination codon (PTC) introducing mutations are enriched in regions that can efficiently trigger NMD. Whether or not NMD is triggered by a pathogenic PTC gives insight in the molecular mechanism by which the affected gene contributes to the disease phenotype. **(B)** In cancer evolution, there is selective pressure for mutations that trigger NMD on tumor suppressor genes, and against mutations that trigger NMD on essential genes and oncogenes. **(C)** Only when NMD is not triggered, somatic frameshifting mutations can produce potent neoantigens that elicit the anticancer immune response and increase the efficiency of immunotherapies against cancer. **(D)** Pharmaceutical modulation of NMD holds potential for alleviating a wide range of diseases.