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How to get away with nonsense: Mechanisms and consequences of escape from nonsense-mediated RNA decay

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Abstract

Nonsense-mediated RNA decay (NMD) is an evolutionarily conserved RNA quality control process that serves both as a mechanism to eliminate aberrant transcripts carrying premature stop codons, and to regulate expression of some normal transcripts. For a quality control process, NMD exhibits surprising variability in its efficiency across transcripts, cells, tissues, and individuals in both physiological and pathological contexts. Whether an aberrant RNA is spared or degraded, and by what mechanism, could determine the phenotypic outcome of a disease-causing mutation. Hence, understanding the variability in NMD is not only important for clinical interpretation of genetic variants but also may provide clues to identify novel therapeutic approaches to counter

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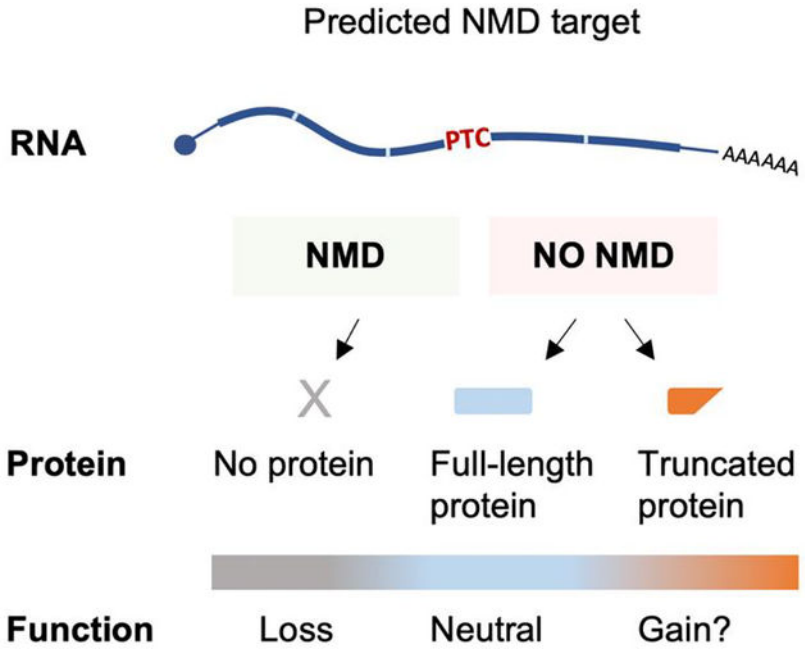
genetic disorders caused by nonsense mutations. Here, we discuss the current knowledge of NMD variability and the mechanisms that allow certain transcripts to escape NMD despite the presence of NMD-inducing features.

Graphical Abstract



Variation in RNA quality control by NMD can lead to different gene expression outcomes of nonsense-containing transcripts.

Introduction

Nonsense-mediated RNA decay (NMD) safeguards cells against aberrant mRNAs and their translation products (Brogna et al., 2016; Celik et al., 2017). In mammalian cells, a ribosome translating a normal transcript evicts exon junction complexes (EJCs), which are protein marks left behind by the splicing machinery, and other RNA binding proteins (RBPs) as it makes its way through the transcript to the normal stop codon (Figure 1A). However, in the case of an aberrant transcript that contains a premature termination codon (PTC), the PTC causes the ribosome to terminate before it reaches the normal stop codon, thus failing to evict downstream EJCs. The continued presence of an EJC or other RBPs downstream of the terminating ribosome acts as a signal to recruit NMD machinery and initiate destruction of the aberrant mRNA (Figure 1B; (Fatscher et al., 2015; Hug et al., 2016)). As a result, nonsense mutations often inactivate genes and cause loss of function (Kurosaki and Maquat, 2016; Miller and Pearce, 2014).

One-third of all inherited human diseases are caused by nonsense or frameshift mutations that introduce a premature stop codon in a transcript (Mendell and Dietz, 2001; Stenson et al., 2003). Prominent examples include β-thalassemia, Duchenne muscular dystrophy, and

cystic fibrosis (Miller and Pearce, 2014). Studying such diseases has offered a wealth of information on how NMD works and how aberrant transcripts are recognized by NMD. For example, nonsense mutations in early exons of β-globin (*HBB*) cause a recessive form of β-thalassemia. Here, mutant HBB mRNA is to degraded via NMD, which leads to insufficient levels of β-globin protein and disease (Neu-Yilik et al., 2011). Conversely, mutations in part of the penultimate exon or the last coding exon cause a dominant form of the disease because the mutant *HBB* mRNA escapes NMD and results in production of a C-terminally truncated β-globin protein (Neu-Yilik et al., 2011). Studies that identified mutations where the resulting transcript either underwent or escaped led to the proposal of the "50–55 nt rule" (Hentze and Kulozik, 1999; Li and Wilkinson, 1998; Maquat, 1995), where only those nonsense mutations that are at least 50–55 nucleotides upstream of the final exon-exon trigger efficient NMD (Figure 1B).

Several exceptions to the 50–55 nt rule have been observed. For example, nonsense mutations found close to the start codon often escape NMD because translation can reinitiate downstream of the PTC and EJCs that might otherwise trigger NMD are evicted (Inacio et al., 2004; Pereira et al., 2015; Zhang and Maquat, 1997). Stop codon readthrough also contributes to the escape of some nonsense variants from NMD for the same reason (Hogg and Goff, 2010; Keeling et al., 2004). Finally, NMD efficiency varies across cell types (Linde et al., 2007), tissues (Bateman et al., 2003; Zetoune et al., 2008), and individuals (Kerr et al., 2001; Nguyen et al., 2014), offering potential explanations for tissue tropism and varying penetrance across the population of disease-causing nonsense variants. Thus, NMD variability can compromise the accuracy of variant interpretation in clinical genetic diagnostics settings and, for this reason, is important to understand.

NMD machinery can also target normal transcripts that contain upstream open reading frames (uORFs), spliced introns in 3' untranslated regions (UTRs), or long 3' UTRs (Lykke-Andersen and Jensen, 2015; Mendell et al., 2004; Nasif et al., 2018; Nickless et al., 2017a). These features cause a transcript to engage the NMD machinery due to the continued presence of EJCs or other RBPs downstream of a terminating ribosome. The expression level of such transcripts can be dialled up or down by varying the efficiency with which they undergo NMD (Nasif et al., 2018). Moreover, depending on the context and mode of NMD inhibition, different subsets of physiological NMD substrates are stabilized, adding another layer of complexity to this regulatory mechanism. How and why are some NMD targets degraded and some spared? How does cellular context influence this choice? How do cells cope with regulated NMD inhibition given its role as a quality control process? These are open questions that can only be answered through better understanding of both the rules and exceptions of the NMD process. In this review, we will discuss the current state of knowledge of NMD variability and identify gaps that need to be filled through further investigations.

NMD MECHANISMS

Over the years, several cues that allow NMD to distinguish normal and aberrant transcripts have been discovered (Carter et al., 1996; Hentze and Kulozik, 1999; Thermann et al., 1998; Zhang et al., 1998). The presence of a premature stop codon >50–55 nucleotides upstream

of an exon-exon junction is a strong predictor of NMD (Hsu et al., 2017), and induces a phenomenon called the EJC-dependent NMD (Ivanov et al., 2008; Kashima et al., 2006). The length of the 3' UTR is another factor that also influences NMD, with longer 3' UTRs inducing an EJC-independent mode of RNA surveillance termed long UTR-mediated NMD (Buhler et al., 2006; Eberle et al., 2008; Hogg and Goff, 2010; Hurt et al., 2013; Singh et al., 2008). However, it is worth noting that not all long 3' UTRs induce NMD and additional factors exist that may modulate the efficiency with which a long UTR elicits NMD (Boehm et al., 2014; Toma et al., 2015). Yeast, which lacks introns in the majority of its genes, is thought to primarily utilize an EJC-independent NMD mechanism termed the "faux 3' UTR model", wherein premature termination causes the downstream RNA segment to function as a "faux" long UTR and trigger NMD via the long-UTR model (Amrani and Jacobson, 2013).

At the core of NMD substrate recognition via all mechanisms is the non-specific binding of UPF1, an RNA helicase, to RNA molecules (Hogg and Goff, 2010; Kurosaki and Maquat, 2013; Singh et al., 2008; Zund et al., 2013). When translation termination occurs close to the natural end of transcripts, the presence of the polyA binding protein, PABPC1, is thought to prevent the interaction between UPF1 and the eukaryotic release factors, eRF1 and eRF3, as well as increase the efficiency of translation termination (Amrani et al., 2004; Behm-Ansmant et al., 2007; Eberle et al., 2008; Singh et al., 2008). In the case of the EJC-dependent mode of NMD, a ribosome terminating >50–55 nucleotides upstream of an exon-exon junction fails to evict the downstream EJC. The combined presence of a terminating ribosome and the downstream EJC, as well as the efficient interaction of UPF1 with eRF1/3, is thought to induce the sequential recruitment and assembly of UPF2/3 and SMG1/5/6/7 factors on to the aberrant transcript, ultimately causing degradation of the RNA (Ivanov et al., 2008; Kashima et al., 2006). In long UTR-mediated NMD, UPF1 again interacts efficiently with eRF1/3, though UPF2/3 recruitment occurs unassisted by the EJC, with further assembly of SMGs and other degradation factors following similar mechanism as the EJC-dependent NMD (Eberle et al., 2008; Hogg and Goff, 2010; Singh et al., 2008). Finally, the combined action of the endonuclease SMG6, exonuclease XRN1, and the exosome complex, together with decapping and deadenylation factors, ensures efficient clearance of the aberrant RNA. In both of these models of NMD, the kinetics of premature termination appear distinct from that of normal termination in a way that facilitates the formation of various protein complexes in the context of the terminating ribosome to then recruit the NMD machinery (Celik et al., 2015; Ghosh et al., 2010).

For a more detailed discussion of the molecular mechanisms of NMD, we refer the reader to several excellent reviews on the subject (Brogna et al., 2016; Celik et al., 2017; Fatscher et al., 2015; Hug et al., 2016; Karousis et al., 2016; Kervestin and Jacobson, 2012; Kurosaki and Maquat, 2016; Lykke-Andersen and Jensen, 2015; Miller and Pearce, 2014; Nasif et al., 2018; Nickless et al., 2017a).

MECHANISMS OF ESCAPE FROM NMD

Nonsense variants can escape NMD via many routes (Buisson et al., 2006; Hamid et al., 2010; Harries et al., 2005; Hulsebos et al., 2014; Kerr et al., 2001; Krempely and Karam,

2018; Loughran et al., 2018; Poli et al., 2018; Sciezynska et al., 2017; Stump et al., 2012; Stump et al., 2013; Yamaguchi and Baba, 2018; Yamaguchi et al., 2012). *Cis* elements in the mRNA that modify splicing or translation as well as *trans* factors that affect these processes can influence the fate of a predicted NMD target. For example, if an exon containing the stop codon is excluded from the transcript via alternative splicing, the presumed target will no longer undergo NMD. Similarly, if a sole intron downstream of a PTC is inefficiently spliced, there will be no EJCs downstream of the PTC to signal NMD. Any variability in EJC deposition can also influence NMD efficiency. An unbiased, transcriptome-wide analysis of the RNA binding sites of four EJC components—eIF4A3, BTZ, UPF3B, and RNPS1— identified high-confidence EJC binding sites and revealed that EJC composition and deposition are largely homogenous with the notable exception of transcripts that encode ribosomal proteins (Hauer et al., 2016). While it is unknown whether the lack of EJC deposition on ribosomal transcripts is associated with a higher degree of escape from NMD, it is possible that they do. In fact, an anti-sense oligonucleotide (ASO) targeting the downstream EJC deposition site of a PTC-containing transcript was shown to block EJC assembly and thus inhibit NMD of the corresponding transcript (Nomakuchi et al., 2016), demonstrating that lack of EJC deposition on specific transcripts does allow such mRNAs to escape decay by NMD (reviewed here (Nasif et al., 2018)). Though Hauer *et al* only evaluated EJC deposition in healthy cells, it is possible that other transcripts might exhibit variability in EJC deposition during disease or cell stress, thus potentially escaping NMD in particular cellular contexts.

Other factors that can modify NMD efficiency include the sequence and architecture of the 3’ UTR, as well as the associated protein complexes and their relative abundance in different contexts (Singh et al., 2008). For example, some long 3’ UTRs contain *cis* elements that contribute to NMD evasion (Toma et al., 2015). It has also been shown in yeast that longer open reading frames desensitize transcripts with long 3’ UTRs to NMD (Decourty et al., 2014). As NMD is a translation-dependent process, *cis* and *trans* factors that influence translation can modulate NMD efficiency. Several stress response pathways culminate in phosphorylation of eIF2α that leads to inefficient translation and inhibition of NMD (Goetz and Wilkinson, 2017; Karousis et al., 2016; Li et al., 2017; Wang et al., 2011). At the mRNA level, inefficient translation termination at the PTC (a process termed stop codon readthrough or nonsense suppression), or reinitiation of translation downstream of the PTC, evicts downstream EJCs and causes NMD evasion by the PTC-containing transcript. Nonsense suppression via readthrough inducing small molecules is being investigated as a therapeutic strategy in several genetic disorders (Asiful Islam et al., 2017; Keeling et al., 2014; Lee and Dougherty, 2012; Mah, 2016; Wang and Gregory-Evans, 2015), highlighting the role of the ribosome and its interpretation of a stop codon in eliciting efficient NMD. In the following sections, we will separately consider gene-specific and context-specific modifiers of NMD, their mechanisms of action, and their molecular and phenotypic consequences.

GENE-SPECIFIC MODIFIERS OF NMD EFFICIENCY

Molecular studies of genetic disorders have led to the identification of hundreds of isolated instances of nonsense variants that escape NMD. However, we have only recently

begun systematically assessing the fraction of nonsense variants that escape NMD, the mechanisms responsible for NMD evasion, and the quantity of protein produced from a nonsense allele (Jagannathan and Bradley, 2016; Lindeboom et al., 2016). Analyses of publicly available functional genomics datasets to assess the NMD efficiency of a subset of highly common nonsense mutations (*i.e.* those that have not been purged by negative selection during evolution) found that 52.3% of these common nonsense variants escape NMD (Jagannathan and Bradley, 2016). Reporter-based molecular validation further showed that translation reinitiation and stop codon readthrough, among other post-transcriptional mechanisms, contribute to NMD escape, enabling protein production from the nonsense alleles (Jagannathan and Bradley, 2016). Another study used 2,840 high-confidence somatic nonsense mutations found in the Cancer Genome Atlas (TCGA) and asked how well the known rules for NMD explain the observed NMD efficiency (Lindeboom et al., 2016). Surprisingly, only ~50% of the variance in NMD efficiency could be explained by the 50–55 nt rule (Lindeboom et al., 2016). These results highlight our incomplete understanding of the range of molecular mechanisms that could rescue or modify gene function in the presence of a predicted loss-of-function mutation (Figure 2). While the proteins produced from nonsense alleles that escape NMD may not be fully functional, plasticity in RNA processing and translation has the potential to convert loss-of-function variants from genetic nulls into hypomorphic, silent, or even neomorphic alleles (Jagannathan and Bradley, 2016).

Co-transcriptional mechanisms that prevent the production of NMD isoforms

Co-transcriptional events, including alternative promoter usage, alternative splicing, and alternative polyadenylation, can all result in the exclusion of an exon containing a premature stop codon, and hence introduce variability of expression of a predicted NMD target in a gene-specific manner. Alternative splicing is perhaps the best understood mechanism that allows gene expression despite the presence of a nonsense mutation or genetic variant with numerous examples in the literature (da Costa et al., 2017; Lewis et al., 2003). For example, alternative-splicing coupled NMD (AS-NMD) serves as a homeostatic regulatory mechanism to regulate the expression of different mRNA isoforms produced from a gene (Lareau et al., 2007; Ni et al., 2007). In one prominent example, members of the SR-family splicing factors autoregulate their levels by modulating inclusion or exclusion of a PTC-containing exon (referred to as “poison exon”) by binding to an exonic splicing enhancer (ESE) within the poison exon. In the presence of high levels of an SR protein, its binding to the ESE promotes inclusion of the PTC-containing exon and leads to NMD of the transcript, and thus lowers the level of the protein (Lareau et al., 2007; Ni et al., 2007).

Several nonsense variants found in cassette exons (i.e. those that can be included or excluded in a regulated manner) appear to induce alternative splicing, particularly when the exon skipping preserves the translation reading frame (da Costa et al., 2017). With increasing use of CRISPR/Cas9 genome editing, there is accumulating evidence for nonsense mutations resulting in observed changes in in the abundance of mRNA isoforms generated by alternative splicing (Lalonde et al., 2017; Mou et al., 2017; Sui et al., 2018), though it is unclear if the splicing decision itself is affected in these cases or if it is simply a case of differential isoform targeting by NMD (i.e. preferential degradation of PTC-inclusion versus -exclusion isoform). Finally, tissue-specific and developmentally-regulated alternative

splicing introduces yet another layer of complexity to NMD wherein a transcript containing a PTC is only produced in some tissues, thus allowing the PTC-containing gene to escape NMD-mediated loss of function in other tissues (Barberan-Soler et al., 2009; Jagannathan and Bradley, 2016; Yeo et al., 2004).

Post-transcriptional mechanisms that allow an NMD isoform to evade degradation

Sequence-specific RBP binding—Several RNA binding proteins are capable of coordinating with NMD factors to protect specific transcripts from NMD. For example, binding of cytoplasmic poly(A)-binding protein, PABPC1, to long 3' UTRs effectively suppresses NMD activation (Fatscher et al., 2014). More broadly, short peptides consisting of PABP-interacting motif 2 (PAM2) found in a variety of proteins bound to long 3' UTRs also suppress NMD activity (Fatscher and Gehring, 2016). Some long 3' UTRs contain an RNA stability Element (RSE) that recruits polypyrimidine-tract-binding protein 1 (PTBP1) to form an mRNP structure that inhibits NMD (Ge et al., 2016). A recent study also found that some normal mRNAs with long 3' UTRs and multiple 3' UTR introns that contain features bound by the protein hnRNPL can evade NMD-mediated degradation (Kishor et al., 2019). Importantly, in many B cell lymphomas with BCL2:IGH translocations, hnRNPL recruitment protects and stabilizes aberrant BCL2 fusion mRNAs, which contributes to increased BCL2 expression (Kishor et al., 2019). Thus, 3' UTR sequence elements regulate the extent of NMD-mediated degradation and gene expression.

Translation reinitiation downstream of a PTC—Reinitiation occurs when a post-termination ribosome is not recycled but continues to scan and then initiate at downstream start codons (Gunisova et al., 2018). Despite being well accepted as a cause for escape from NMD (Buisson et al., 2006; Hulsebos et al., 2014; Neu-Yilik et al., 2011; Paulsen et al., 2006; Stump et al., 2012; Stump et al., 2013; Zhang and Maquat, 1997), the mechanism that enables efficient reinitiation of a subset of nonsense variants is poorly understood. Transcripts with uORFs are often destabilized via NMD due to the presence of EJCs that are not evicted due to the uORF reducing translation of the downstream ORF (Colombo et al., 2017). However, there are several examples of transcripts with start-proximal nonsense codons that are not subject to NMD due to efficient reinitiation of translation at downstream start codons (Buisson et al., 2006; Hulsebos et al., 2014; Paulsen et al., 2006; Pereira et al., 2015; Stump et al., 2012; Stump et al., 2013; Zhang and Maquat, 1997), leading to the proposal of a "boundary" within each transcript where reinitiation is efficient (Pereira et al., 2015). In support of this model, by analyzing common nonsense variants in the human population, we observed a statistically significant enrichment of downstream in-frame methionine within 50 codons of the nonsense variants (Jagannathan and Bradley, 2016). Specifically, we found that 57% of rare and 85% of common nonsense variants contained a downstream in-frame methionine within 50 codons of the variant, compared to only 52% of synonymous variants (Jagannathan and Bradley, 2016). This observation only held, however, in the first 10% of a coding sequence, consistent with studies that have found that reinitiation is most efficient when a PTC is close to the start codon (Inacio et al., 2004; Kozak, 2001; Stump et al., 2012). These observations suggest that the post-termination ribosome may need to be close enough to the canonical start codon in

order to be reinitiation-competent, as well as encounter a downstream start relatively quickly to reinitiate efficiently.

Nonetheless, this reinitiation boundary varies even across closely related genes such as alpha and beta globin (Pereira et al., 2015), which suggests transcript-specific features modulate reinitiation. Prior studies have shown that the eukaryotic Initiation Factor complex 3 (eIF3), but not other initiation factors, remains transiently associated with the ribosome through elongation and termination (Hronova et al., 2017; Mohammad et al., 2017; Munzarova et al., 2011). Those ribosomes that retain eIF3 are then able to resume scanning for downstream start codons and reinitiate post-termination. In some cases of reinitiation downstream of short uORFs, the association of eIF3 with the post-termination ribosome is facilitated by specific *cis* regulatory sequences (Hronova et al., 2017; Mohammad et al., 2017; Munzarova et al., 2011). Thus, the distance between the start codon and the premature stop, as well as the presence of specific sequence elements or RNA modifications, may influence whether or not the ribosome is able to reinitiate and define the “window of opportunity” for reinitiation.

Stop codon readthrough—A key step translation termination the competition between the release factor and a non-cognate tRNA, to bind to the A site in the ribosome. The outcome of this competition determines whether translation terminates prematurely to trigger NMD or continues to proceed via readthrough and incorporation of a non-cognate amino acid in the place of the PTC (Celik et al., 2015). Nonsense suppression therapies for genetic diseases target this step of NMD using suppressor tRNAs, aminoglycosides, and non-aminoglycosides such as ataluren—all of which induce stop codon readthrough (Asiful Islam et al., 2017; Keeling and Bedwell, 2011). However, the efficiency and specificity of nonsense suppression drugs for premature versus canonical stop codons has been debated and the drugs themselves are known to have significant side effects (Namgoong and Bertoni, 2016).

Decades of work on termination efficiency at “normal” stop codons has shown that readthrough is a rare and inefficient phenomenon in mammalian cells (Cassan and Rousset, 2001; Loughran et al., 2014; Loughran et al., 2018). Previous work on canonical stop codon readthrough in viral, yeast, *Drosophila*, and mammalian genes has identified several factors that influence the efficiency of readthrough, including stop codon identity and context, and RNA secondary structures such as pseudoknots (Brown et al., 1996; Cassan and Rousset, 2001; Green and Goff, 2015; Liu and Xue, 2004; Loughran et al., 2014; Loughran et al., 2018; Napthine et al., 2012; Stiebler et al., 2014; Xu et al., 2018). Moreover, termination at PTCs has been shown to be kinetically distinct from termination at canonical stop codons in yeast even though the underlying mechanism is unclear (Amrani et al., 2004; Amrani and Jacobson, 2013), and a recent study in mammalian cells found that PTC readthrough occurs in specialized cytoplasmic foci upon NMD inhibition (Jia et al., 2017). With the discovery of a subset of mRNAs with PTCs that are naturally read through with high efficiency (as indicated by the uninterrupted ribosome footprint density across a PTC and/or near-normal levels of full-length protein product from a homozygous NMD allele; (Jagannathan and Bradley, 2016), it is clear that there may be sequence contexts or other factors that facilitate natural PTC readthrough – especially since canonical stop codons are readthrough very poorly (Cassan and Rousset, 2001; Loughran et al., 2014; Loughran et

al., 2018). For instance, RNA modifications can also influence stop codon readthrough. A recent study found that pseudouridylation of the 'U' in stop codons by sequence-specific pseudouridine synthases promotes readthrough (Karijolich and Yu, 2011). Therefore, the mechanism(s) that allow readthrough of a normal stop codon may also be different from that of readthrough of a premature stop codon, and understanding how to coax ribosomes into reading through PTCs can have an immense practical benefit in targeting genetic diseases caused by nonsense mutations.

While both co-transcriptional and post-transcriptional events contribute to NMD variability, the relative fractional contribution of these mechanisms to escape from NMD is yet to be determined. As we accumulate more ribosome footprinting and proteomics datasets to complement RNA-seq data on nonsense variants, quantitative estimates of the contribution of translational recoding to NMD escape may be derived.

CONTEXT-SPECIFIC MODIFIERS OF NMD EFFICIENCY

Because NMD functions in part to regulate normal gene expression, overall NMD efficiency and specific mRNAs targeted for degradation can vary based on genetic background, cell type, and developmental stage, among other contexts (Lykke-Andersen and Jensen, 2015; Nasif et al., 2018). As an example, studies in yeast have found that the background strain can influence levels of accumulated NMD substrate mRNAs, which suggests that many genes influence NMD efficiency (Kebaara et al., 2003). In addition, the recognition of cytoplasmic downregulation of normal mRNAs with PTCs as part of normal physiological processes (Li and Wilkinson, 1998) led to several investigations into the role of NMD in diverse cell types and during development.

Physiological variation of NMD during differentiation and development

Several cellular contexts exist in which NMD efficiency is modulated to affect specific physiological consequences (Figure 3). NMD is key for myogenic differentiation and yet plays seemingly contradictory roles. On the one hand, NMD is essential for downregulating c-Myc mRNA, which in turns drives myogenic differentiation (Yeilding et al., 1998). Importantly, initial studies showed that c-myc mRNA downregulation occurs in the cytoplasm, is translation-dependent, and is mediated by exons 2 and 3 (Yeilding and Lee, 1997; Yeilding et al., 1998). Recent reports in embryonic stem cells suggest the NMD pathway is responsible for forced down-regulation of c-Myc mRNA, which promotes cellular differentiation (Li et al., 2015; Lou et al., 2015). Here, genetic deletion of Smg6, as well as transient knockdown of other NMD factors, leads to increased levels of c-Myc mRNA and a differentiation block. Restoring NMD function by expressing NMD-proficient Smg6 constructs rescues differentiation. On the other hand, during differentiation of mouse C2C12 myoblasts to myotubes, the efficiency of NMD decreases due to preferential recruitment of UPF1 to a competing pathway called Staufen 1-mediated mRNA decay (SMD) (Gong et al., 2009). In this context, reduced NMD efficiency permits increased expression of myogenin—a muscle-specific transcription factor involved in muscle differentiation—and consequently, myogenesis (Gong et al., 2009). Furthermore, consistent with the idea that reduced NMD, or NMD factors, in this case, is critical

for myogenesis, it was recently demonstrated that the E3 ligase function of the UPF1 protein (Kuroha et al., 2013; Kuroha et al., 2009) targets MYOD, a protein that promotes myogenesis, for ubiquitination and degradation (Feng et al., 2017; Takahashi et al., 2008; TD and Wilkinson, 2018). Knockdown of UPF1 mRNA leads to increased MYOD protein and increased myogenesis.

NMD also plays a prominent role in neural development and in stem cell biology. These topics have been reviewed extensively elsewhere (Han et al., 2018; Jaffrey and Wilkinson, 2018; Lykke-Andersen and Jensen, 2015). Briefly, UPF1 is downregulated via a neuronally-expressed microRNA, miR-128, causing a decrease in NMD activity during mouse embryonic development and when neural progenitor cells undergo differentiation (Karam and Wilkinson, 2012; Lou et al., 2014). Here, the authors demonstrate that in mouse neuronal stem cells, UPF1 negatively regulates components of the TGF-b family of proteins, and that transient knockdown of UPF1 leads to increased levels of factors that promote differentiation. Further evidence that the NMD pathway is important for neural development is the finding that human genetic mutations in UPF3B are associated with intellectual disability (Nguyen et al., 2012; Tarpey et al., 2007): Upf3b-null mice exhibit a learning deficit (Huang et al., 2018), and cultured stem cells from Upf3b-null mice have an impaired ability to differentiate (Jolly et al., 2013).

Recent data suggest the composition of the assembled NMD protein complex also varies in certain developmental scenarios and helps determine the NMD susceptibility of specific substrates. In one example, researchers investigated the expression level of UPF3A mRNA in adult mouse tissues and found that it is highly expressed in spermatocytes and is developmentally regulated across different germ cell subsets. UPF3A is a paralog of UPF3B, and has a unique, antagonistic role in NMD (Chan et al., 2009; Shum et al., 2016). When bound by UPF3B, NMD function is maintained and the target transcript is degraded; when bound by UPF3A, the NMD complex is repressed and the target transcript remains stable (Shum et al., 2016). In addition, UPF3A exhibits variable expression in many adult mouse tissues (Shum et al., 2016) and is increased in cell lines derived from human patients with loss-of-function mutations in UPF3B (Nguyen et al., 2012). Taken together, these examples show the importance of NMD for development of many different tissues and that regulated modulation of NMD efficiency is also critical.

Physiological variation of NMD during cell stress

Accumulation of misfolded proteins in the lumen of the endoplasmic reticulum activates a signaling cascade called the unfolded protein response (UPR). Several groups have shown that the UPR is shaped by mRNA surveillance as well (Goetz and Wilkinson, 2017; Nasif et al., 2018). Early studies investigating the link between ER stress, UPR activation, and NMD activity found that the loss or deletion of many NMD factors cause ER stress (Sakaki et al., 2012). Notably, NMD factors are also known to colocalize with the ER, though whether their localization has any significance to NMD is poorly understood (Sakaki et al., 2012). These data support a model in which NMD functions to eliminate aberrant transcripts that could encode polypeptides capable of inducing ER stress. Later studies discovered that NMD functions to fine tune regulation of the ER stress response and

UPR activation. Under mild ER stress conditions, NMD activity prevents full-scale UPR activation by raising the activation threshold and quickly resolving mild stress (Karam et al., 2015). Conversely, NMD is inhibited during the plateau phase of the UPR and during chronic activation of ER stress (Karam et al., 2015; Li et al., 2017), which allows for strong activation of the UPR. Furthermore, some transcripts encoding key mediators of the UPR—such as the transcription factors ATF3, ATF4, and CHOP—are NMD substrates and increase as a consequence of NMD inhibition (Oren et al., 2014). Following a strong ER stress response, reviving ER homeostasis and translation depends on restored NMD activity, which is evidenced by findings that chronic depletion of NMD reduces mammalian cell proliferation and survival (Karam et al., 2015; Sakaki et al., 2012).

During ER stress, the levels of mRNAs encoding key NMD factors do not change (Karam et al., 2015). However, global translation is inhibited by a mechanism that involves phosphorylation of eIF2α. As NMD is a translation-dependent process, reduced global translation results in increased stability of NMD substrates. UPR activation has also been shown to attenuate NMD by enhancing ribosome readthrough of PTCs (Oren et al., 2014). It is worth noting that most stress response pathways, including oxidative stress, anti-viral response, and heat shock response, lead to eIF2α phosphorylation and thus, can inhibit NMD. In fact, NMD inhibition was observed in the tumor microenvironment and found to promote tumorigenesis by stabilizing mRNAs that are involved in stress response and tumor-promoting pathways (Wang et al., 2011). Given the chronic activation of cell stress in many human diseases, pharmacologic therapies to restore NMD activity may provide clinical benefit and address unmet medical needs (Goetz and Wilkinson, 2017).

Pathological variability of NMD in disease

Aside from the regulated inhibition of NMD in specific physiological contexts, *en masse* inhibition of NMD is quite rare in pathological contexts, perhaps due to the critical role that many NMD factors play in mammalian embryogenesis (Han et al., 2018; Li et al., 2015; Lou et al., 2014; Nasif et al., 2018). Therefore, germline mutations that cause loss of function of an NMD factor do not survive past early embryonic stages (Han et al., 2018). One exception to this general trend is UPF3B, in which loss of function mutations are viable, but cause severe neurodevelopmental defects and intellectual disability (Jaffrey and Wilkinson, 2018). Recent studies have reported somatically acquired point mutations in UPF1 that are frequently found in pancreatic adenosquamous carcinoma (Liu et al., 2014) and in inflammatory myofibroblastic tumors (Lu et al., 2016). In most of these cases, inactivation of an NMD factor has been shown to affect a specific subset of endogenous NMD targets that are consistent with the observed pathology, rather than a general inhibition of NMD with a strong effect on all endogenous targets (Lykke-Andersen and Jensen, 2015). While robust knockdown of individual NMD factors does affect cell growth and viability, partial knockdowns often have modest effects on cell physiology, consistent with the idea that NMD factors are under an autoregulatory feedback loop that protects the NMD pathway from perturbations (Chan et al., 2009; Huang et al., 2011; Yepiskoposyan et al., 2011).

The only known example of *en masse* NMD inhibition playing a key role in a disease process was recently discovered in a genetically inherited muscular dystrophy called

facioscapulohumeral muscular dystrophy (FSHD). FSHD is caused by epigenetic de-repression of a macrosatellite repeat array in chromosome 4q and the resulting activation of an early embryonic transcription factor, DUX4, in skeletal muscle cells (Lemmers et al., 2010). In addition to turning on expression of direct target genes (Geng et al., 2012), skeletal muscle DUX4 also leads to an unprecedented degree of inhibition of NMD (Feng et al., 2015). Interestingly, the DUX4 transcript is an endogenous target of NMD due to the presence of two introns in the 3' UTR (Feng et al., 2015). Consequently, when DUX4 expression rises slightly, a feed-forward loop initiates because DUX4 expression decreases NMD function, which then relieves NMD-mediated inhibition of DUX4 and causes a characteristic burst pattern of DUX4 expression. This burst of DUX4 expression is observable in FSHD muscle cells and causes cell death and muscle deterioration in FSHD (Rickard et al., 2015). In addition to stabilizing DUX4 mRNA, the systemic inhibition of NMD also causes accumulation of hundreds of NMD-substrate mRNAs in DUX4-expressing cells and in the FSHD muscle (Feng et al., 2015; Jagannathan et al., 2019). Dramatically reduced NMD in DUX4-expressing skeletal muscle cells raises the possibility that the proteins translated from NMD-substrate mRNAs contribute to skeletal muscle deterioration in FSHD. It also raises the possibility that reduced NMD efficiency may play a role in other skeletal muscle diseases.

Mechanisms of NMD inhibition and their contribution to muscle cell death in FSHD are beginning to be explored. Interestingly, global inhibition of NMD in FSHD skeletal muscle cells is caused by post-translation degradation of core NMD factors. The first support of such a post-translational mechanism came from the finding that increased DUX4 expression causes decreased levels of UPF1 and SMG7 protein, but UPF1 and SMG7 mRNAs were either unchanged or increased (Feng et al., 2015). A recent unbiased proteomics study from FSHD myoblasts revealed a discordance between the protein and mRNA levels for many core NMD factors, including UPF1, UPF3B, SMG6, and the exonuclease XRN1 (Jagannathan et al., 2019). Despite recent progress, the upstream events that are responsible for targeted, post-translational depletion of core NMD factors are not known. Additional studies focused on identifying these upstream triggering events will lead to a better understanding of the underlying molecular pathology of FSHD and may lead to new therapeutic strategies.

NMD also varies between individuals (Nguyen et al., 2014), a finding especially important to consider in the context of human diseases and interpreting potential disease-causing mutations. For instance, a recent study showed that an identical nonsense mutation known to cause Choroideremia exhibited up to 40% variability in the RNA level (Sarkar et al., 2019). It is thus possible that genetic variation in NMD factors that affect their level or activity could modify the penetrance and the severity of diseases even if they do not cause diseases by themselves.

In summary, NMD activity is modulated in a number of different ways to affect specific physiological or pathological outcomes (Figure 3). Some of the mechanisms that lead to NMD inhibition include downregulation of NMD factors by microRNAs (Jin et al., 2016; Karam and Wilkinson, 2012; Wang et al., 2013) or proteasomal degradation (Feng et al., 2015; Jagannathan et al., 2019), cleavage of specific NMD factors to produce

dominant negative products (Popp and Maquat, 2015), competition between alternative decay pathways (Gong et al., 2009), translation inhibition (Li et al., 2017), and activation of signaling pathways such as p38 MAPK (Nickless et al., 2017b). In physiological scenarios, NMD inhibition leads to the increased abundance of one or more physiological NMD targets that confer a benefit to the cell. In pathology, NMD inhibition often promotes or exacerbates the disease, again by stabilizing a subset of substrates. How the regulatory arm of NMD is balanced with the quality control arm, and how different subsets of NMD targets are stabilized under different scenarios are some of the biggest questions facing NMD researchers.

Future Directions

Understanding the rules and exceptions that govern NMD is essential for accurate clinical interpretation of genetic data (Popp and Maquat, 2016). For example, an NMD target may degrade and generate no protein or escape degradation and result in the production of a full-length or truncated protein (Anczukow et al., 2008), each scenario associated with a potentially different disease outcome (Figure 4). We have discussed numerous examples—both in normal physiology and in disease—of NMD targets escaping degradation and having a functional consequence. Further studies are needed to quantify the cumulative impact of gene-specific and context-specific NMD modifiers on NMD efficiency of a given transcript containing a PTC in a given scenario. Given the numerous factors that can alter the fate of a transcript containing a PTC, clinical genetic pipelines need to account for factors other than the 50–55 nt rule in order to make an accurate prediction. For example, if a gene carrying a nonsense variant also carries a sequence element known to induce escape from NMD, a pipeline could be trained to identify and report the variant accordingly. Thus, understanding gene-specific NMD escape mechanisms has immense practical value.

With sufficient data, we can build better predictive models of NMD that consider the location of a PTC within a gene, the sequence context of a PTC, distal elements in the transcript that may modify NMD efficiency, and the abundance of various NMD factors in a relevant tissue and even individual, in order to accurately predict the fate of the transcript. If a transcript is predicted to escape NMD, the mechanism by which it does so can be used to predict the protein the transcript is most likely to make, which would be most useful for clinical interpretation of a disease-causing mutation. Finally, better understanding of the many routes to NMD escape may lead to novel therapeutics approaches that leverage these mechanisms to restore full function of gene despite the presence of a nonsense mutation.

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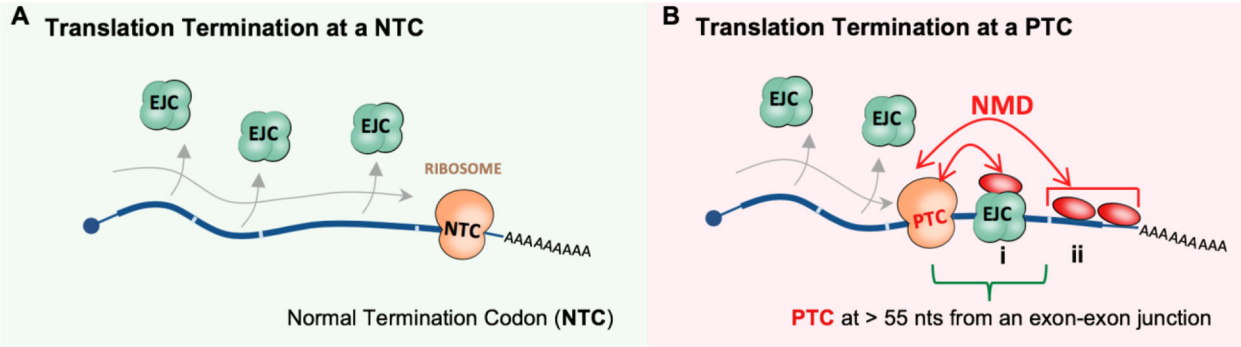

Figure 1. Mammalian model of NMD activation.

(A) During translation of a normal mRNA the ribosome dislodges EJCs as it traverses the RNA molecule up until the NTC. (B) Activation of NMD on a PTC-containing mRNA can occur by at least two mechanisms: i) During EJC-dependent NMD, a PTC found more than 50–55 nucleotides upstream of a downstream exon-exon junction triggers NMD. ii) EJC-independent NMD relies on proteins other than the EJC found downstream of the PTC, including UPF1. Red ovals represent NMD-associated factors.

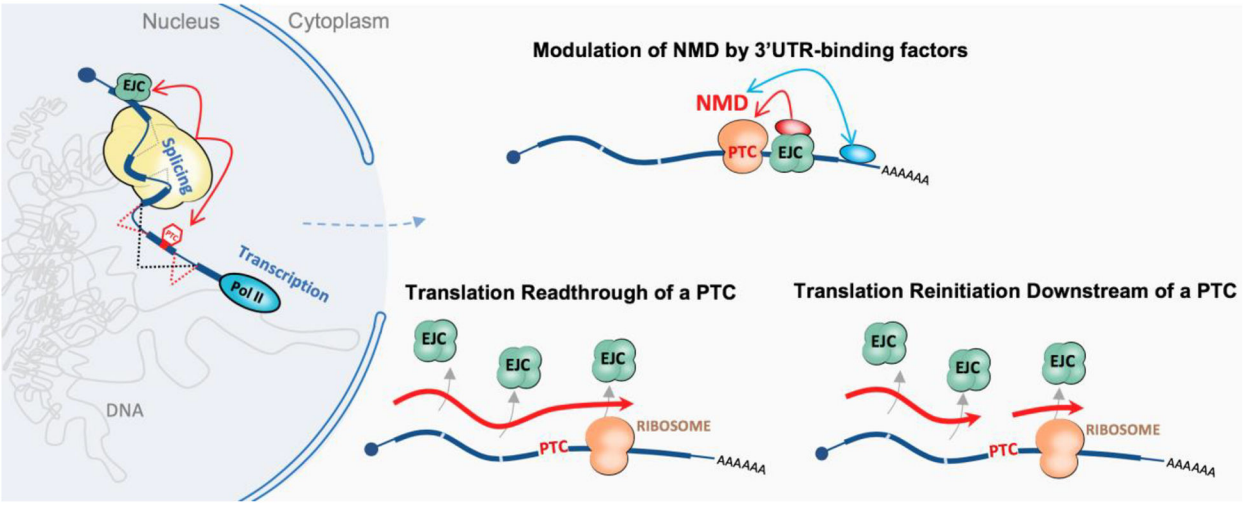

Figure 2. Graphical illustration of the different gene-specific molecular events that promote escape from NMD.

PTC-containing mRNAs can be generated via multiple mechanisms. Among co-transcriptional mechanisms that prevent the production of an NMD isoform, an alternative splicing decision that leads to the exclusion of an exon containing an in-frame PTC is shown. Not shown are alternative transcription initiation and alternative polyadenylation, both of which can allow exclusion of a PTC-containing. Post-transcriptional escape from NMD can be mediated by gene-specific elements such as nucleotide sequences or mRNA structure and may facilitate mRNA escape from NMD by two potential mechanisms: 1) facilitating recruitment of RNA-binding factors that can modulate NMD, or 2) by stimulating non-canonical translation at the PTC which causes removal of downstream EJCs and inhibition of NMD, including translation readthrough and translation reinitiation. Red oval represents NMD-associated factors; Blue oval represents NMD-modifying factors.

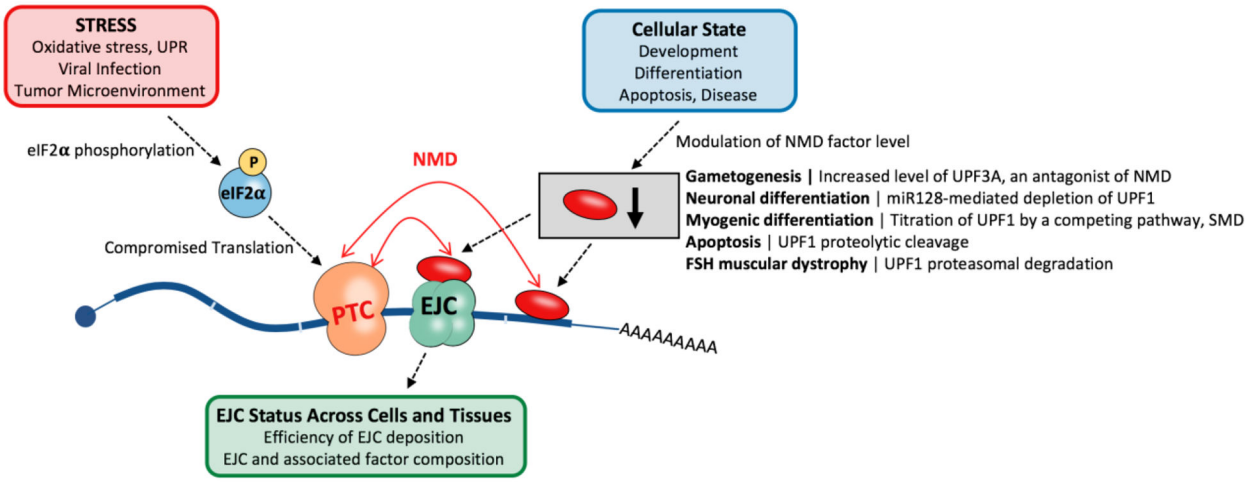

Figure 3. Cellular contexts that induce variability in NMD.

A) Cell stresses including oxidative stress, unfolded protein response (UPR), viral infection and the tumor microenvironment can induce phosphorylation of the translation initiation factor, eIF2α, and lead to translation attenuation and consequently, NMD inhibition. B) Variability in the level of EJC and associated factors as well as the efficiency of EJC deposition can vary NMD. C) Cellular states such precursor versus differentiated cells, cells undergoing apoptosis, and cells derived from disease states (e.g. FSHD) show characteristic modulation of NMD factor levels that allows variation in NMD efficiency. Red oval represents NMD-associated factors

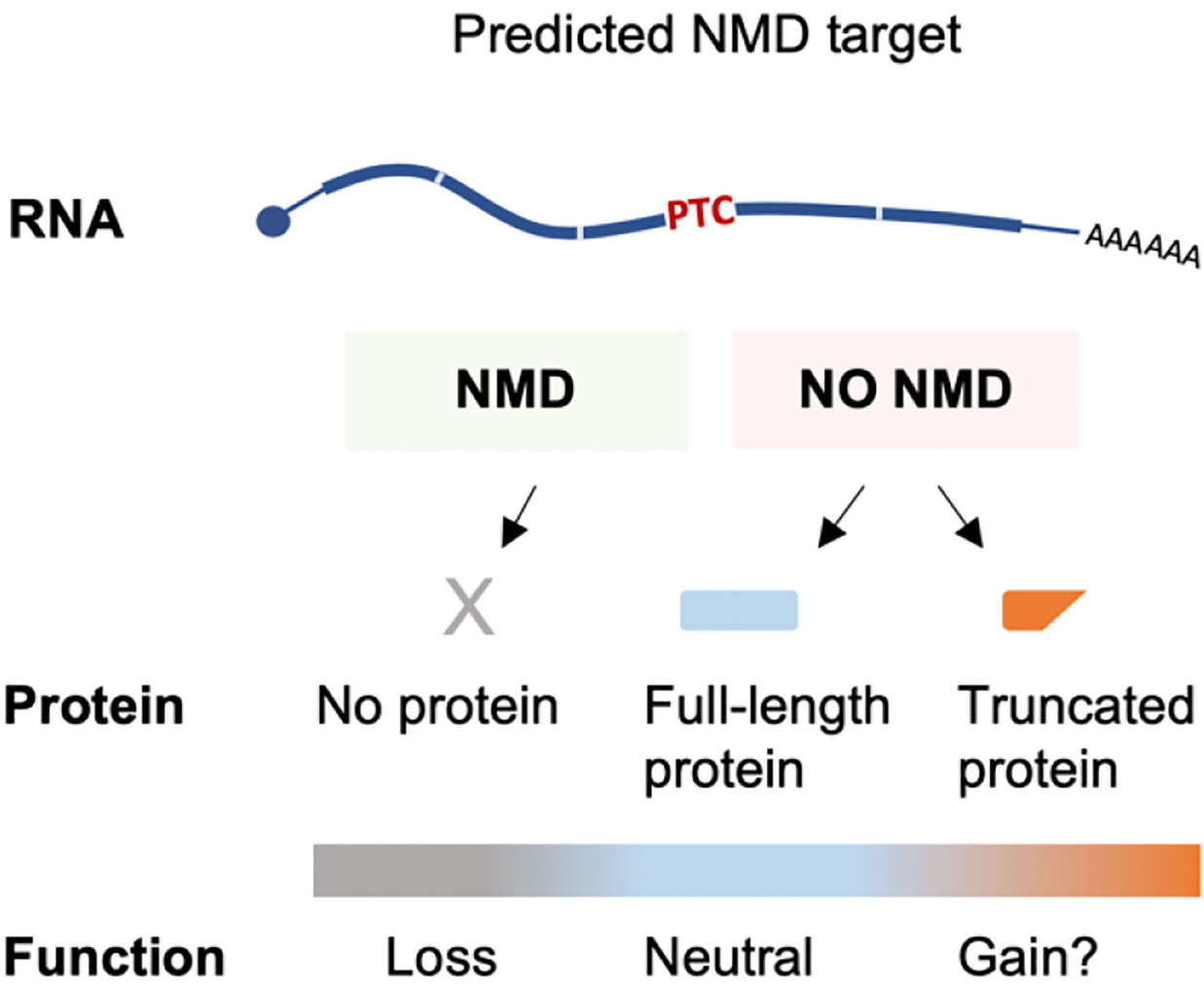

Figure 4.
Varying RNA quality control efficiency modulates expression of protein-truncating variants.