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Nonsense-mediated mRNA decay: inter-individual variability and human disease

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Abstract

Nonsense-Mediated mRNA Decay (NMD) is a regulatory pathway that functions to degrade transcripts containing premature termination codons (PTCs) and to maintain normal transcriptome homeostasis. Nonsense and frameshift mutations that generate PTCs cause approximately one-third of all known human genetic diseases and thus NMD has a potentially important role in human disease. In genetic disorders in which the affected genes carry PTC-generating mutations, NMD acts as a double-edge sword. While it can benefit the patient by degrading PTC-containing mRNAs that encode detrimental, dominant-negative truncated proteins, it can also make the disease worse when a PTC-containing mRNA is degraded that encodes a mutant but still functional protein. There is evidence that the magnitude of NMD varies between individuals, which, in turn, has been shown to correlate with both clinical presentations and the patients' responses to drugs that promote read-through of PTCs. In this review, we examine the evidence supporting the existence of inter-individual variability in NMD efficiency and discuss the genetic factors that underlie this variability. We propose that inter-individual variability in NMD efficiency is a common phenomenon in human populations and that an individual's NMD efficiency should be taken into consideration when testing, developing, and making therapeutic decisions for diseases caused by genes harboring PTCs.

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Conflict of interest

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Keywords

Nonsense-mediated mRNA decay; inter-individual NMD efficiency; regulation of NMD; expression quantitative trait loci; copy number variation; Staufen-mediated mRNA decay; *miR-128*; *miR-125*

1 Introduction

NMD is an RNA degradation pathway originally discovered by virtue of its role as a quality control mechanism that we now know also serves to regulate normal patterns of gene expression. With regard to its role in quality control, NMD is an RNA surveillance pathway that degrades transcripts harboring PTCs. Such aberrant transcripts can arise as a consequence of mutations (nonsense or frameshift), alternative or aberrant mRNA splicing, errors in transcription, and programmed gene rearrangements. NMD is a crucial RNA surveillance mechanism since it rapidly degrades mRNAs that would otherwise translate truncated proteins with potentially harmful dominant-negative effects (Nicholson et al., 2010).

Approximately one third of all human genetic disorders of known etiology are caused by genes with germline or *de novo* mutations that generate PTCs (Frischmeyer and Dietz, 1999). Thus, NMD has the potential to influence the outcome of a large fraction of human diseases. In most positions, PTCs trigger NMD, which is beneficial for disease outcome if the mRNA encodes a truncated deleterious protein. But some mutant mRNAs with PTCs encode truncated proteins that retain partial function and therefore, by degrading such mRNAs, NMD can actually worsen disease outcome (Khajavi et al., 2006). Thus, NMD is a double-edged sword that can either benefit the patient or promote disease.

Recent evidence suggests that variation in the efficiency of NMD can potentially lead to different clinical outcomes in patients, even if they carry the same PTC-generating mutation (Kerr et al., 2001; Nguyen et al., 2012; Resta et al., 2006). For example, two patients who carry the same PTC-generating mutation in the X-chromosome linked *DMD* gene exhibit markedly different phenotypes: one has Duchene Muscular Dystrophy (DMD) (Mendelian Inheritance in Man, MIM 310200) whereas the other has much less severe Becker Muscular Dystrophy (BMD) phenotype (MIM 300376) (Kerr et al., 2001). When examining muscle biopsy taken from the patient with BMD, the investigators noted a moderate expression of DMD protein, suggesting that NMD was weak in this patient, allowing accumulation of PTC-containing mRNA and hence translation of the truncated, but still functional DMD protein (Kerr et al., 2001). As another potential example of this phenomenon, embryonic tissues from two spontaneously aborted foetuses with Roberts Syndrome (MIM 268300) differed significantly in their ability to downregulate the same mutant PTC-containing transcript emanating from the causative gene, *ESCO2,* in a manner that inversely correlated with the length of the survival of the two foetuses (Resta et al., 2006). As these examples illustrate, NMD efficiency appears to vary from individual to individual, which may be an important modifier of some disease phenotypes.

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While the mechanism and regulation of NMD have been studied extensively (Huang and Wilkinson, 2012; Karam et al., 2013; Nicholson et al., 2010; Schoenberg and Maquat, 2012; Schweingruber et al., 2013), relatively little is known about the factors underlying variable NMD efficiency. This review aims to focus on this less well-explored aspect of NMD. We will first briefly recapitulate the general functions of NMD and its underlying mechanism. We will then discuss its involvement in human genetic diseases, particularly with regard to the role of inter-individual variation in NMD efficiency. Known factors that influence NMD efficiency, including stress, microRNAs, and feedback regulation will be detailed. We will close by discussing how recent findings concerning NMD may lead to the generation of modalities that suppress or enhance NMD for therapeutic purposes.

2 Nonsense-Mediated RNA Decay

NMD requires the action of several proteins, many of which are in well-defined complexes. The first NMD factors discovered—up-frameshift-1 (Upf1), Upf2, and Upf3—were identified in a genetic screen in *Saccharomyces cerevisiae* (Leeds et al., 1991; Leeds et al., 1992). Orthologs of these three NMD genes, as well as four other NMD genes—*SMG1, SMG5, SMG6*, and *SMG7—*were later discovered in *Caenorhabditis elegans* through another genetic screen. Mutations in these NMD factor genes in *C. elegans* resulted in morphological defects in the male bursa or the hermaphrodite vulva, leading them to be named suppressor of morphological defects on genitalia (*Smg*) genes (Cali et al., 1999; Hodgkin et al., 1989; Pulak and Anderson, 1993). Subsequently, human orthologs of these genes were identified based on sequence conservation, as well as new NMD genes (*SMG8*, *SMG9* and *DHX34*) using other methods (Applequist et al., 1997; Denning et al., 2001; Longman et al., 2007; Lykke-Andersen et al., 2000; Mendell et al., 2000; Ohnishi et al., 2003; Perlick et al., 1996; Serin et al., 2001; Yamashita et al., 2009; Yamashita et al., 2001) (Table 1). The UPF1, UPF2, and UPF3 proteins are considered to be the core NMD factors and are highly conserved (Applequist et al., 1997; Serin et al., 2001).

In many eukaryotes, including mammals, the UPF2 and UPF3 proteins associate with the exon junction complex (EJC), a set of four proteins—Y14, MAGOH, eIF4AIII, and MLN51 (Table 1)—that are deposited ~20 to 24 nucleotides (nt) upstream of the exon-exon junctions during pre-mRNA splicing (Kim et al., 2001a; Le Hir et al., 2001; Le Hir et al., 2000; Lejeune et al., 2002). Recent studies have shown that most, but not all, spliced exon-exon junctions have an EJC (Sauliere et al., 2010; Sauliere et al., 2012; Singh et al., 2012). The position of the stop codon relative to the last exon-exon junction (i.e., where the most 3′ EJC is deposited) dictates whether NMD will be triggered or not in many mammalian mRNAs. If the stop codon lies in the last exon, this allows the ribosome to strip off all EJCs before translation termination and thereby prevent EJC-dependent NMD. However, if a natural stop codon or a PTC generated by mutation lie in a middle exon, the EJC(s) downstream of the stop codon will typically escape removal by the EJC. As a consequence, these EJCs will have an opportunity to interact with NMD factors recruited upon translation termination (e.g., UPF1), leading to mRNA decay by mechanisms that are still under investigation (Figure 1). A notable exception to this “downstream exon-exon junction” rule are mRNAs in which the stop codon is ~55 nt or closer to the last exon-exon junction (Nagy and Maquat, 1998). Most of these mRNAs are immune to NMD, most likely because the dimensions of

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the EJC and the ribosome lead the former to be stripped off by the latter. For a more complete discussion of models of NMD action, see recent reviews (Huang and Wilkinson, 2012; Karam et al., 2013; Nicholson et al., 2010; Schoenberg and Maquat, 2012).

Notable exceptions to the 55-nt boundary rule exist. Two examples are *Immunoglobulin* (*Ig*) and *T-cell receptor* (*TCR*) transcripts, both of which are transcribed from genes that undergo programmed rearrangement events, a process that frequently generates frameshifts. As a consequence of these frameshifts, non-productively rearranged *Ig* and *TCR* genes give rise to transcripts with PTCs that are degraded by NMD (Baumann et al., 1985). The downregulation of PTC-bearing *Ig* and *TCR* transcripts by NMD is typically extremely robust (>50-fold) when the stop codon is >55 nt upstream of the last exon-exon junction, however stop codons closer than this can also trigger NMD in some transcripts, *albeit* not as efficiently (Buhler et al., 2004; Wang et al., 2002a). In further support of this, an *in vivo* study was recently published that showed that a knock-in allele of a *TCR-β* gene that lacks all introns in the region that acquires PTCs as a result of programmed rearrangement gives rise to mRNAs that are more modestly downregulated by naturally derived PTCs than does a knock-in *TCR-β* gene allele that is identical except that it contains these introns (Mahowald et al., 2011). These data suggest that there is fail-safe EJC-independent mechanism that collaborates with an EJC-dependent NMD mechanism to degrade aberrant *TCR-β* transcripts.

Further support for the existence of different NMD branches with different co-factor requirements comes from *in vitro* studies. Gehring et al. (2005) uncovered two NMD branches that are not affected by RNAi-mediated knockdown of UPF2/RNPS1 or EJC components, respectively, suggesting that there are alternative NMD branches independent of these factors (Gehring et al., 2005). Using the same approach, Chan et al. (2009) obtained evidence for a UPF3-independent branch of NMD that is not affected by depletion of UPF3A (an autosomal gene also known as UPF3) and UPF3B (an X-chromosome linked gene also known as UPF3X), the two known UPF3 paralogs that exist in mammals (Chan et al., 2009; Chan et al., 2007). Confirmation of a UPF3B-independent branch of NMD comes from recent studies in *Upf3b*-null mice (Huang et al., 2011). Little is known about the specificity of these alternative NMD branches, but it is likely that they sometimes act independently and sometimes in concert to regulate the expression of different subsets of genes in different tissue types and developmental stages.

3 NMD as a crucial regulator of the transcriptome

Over the past decade, it has become clear that the role of NMD goes well beyond its originally defined function as quality control mechanism that degrades aberrant mRNAs. NMD is now considered a fundamental developmental regulator of the transcriptome. Genome-wide analysis of cell lines depleted of NMD factors, as well as NMD-deficient mice, have shown that 3 to 20% of the transcriptome is deregulated as a consequence of compromised NMD (Chan et al., 2009; He et al., 2003; Mendell et al., 2004; Rehwinkel et al., 2005; Tani et al., 2012; Weischenfeldt et al., 2008; Weischenfeldt et al., 2012; Wittmann et al., 2006). It is not clear yet what proportion of these are direct NMD targets. One example of a direct NMD target is *PTBP2* mRNA, which encodes a RNA-binding protein

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that promotes the neural differentiation program. In neural progenitor cells, *PTBP2* mRNA contains a PTC introduced by the exclusion of exon 10 and thus it is rapidly degraded by NMD. However, upon differentiation of progenitor cells into neurons, the expression of the splicing repressor PTBP1 that inhibits exon 10 inclusion is lost, which as a consequence restores the reading frame of *PTBP2* transcript so that it is now immune to NMD. This allows functional PTBP2 protein to be made, which in turn activates a cascade of events required for neuronal maturation (Makeyev et al., 2007). Other notable examples of conserved NMD substrates are the mRNAs encoding most SR protein family members (Lareau et al., 2007). SR proteins are RNA-binding proteins that regulate many post-transcriptional events, including RNA splicing, mRNA export, and translation, and thus the ability of NMD to regulate their levels is likely to have profound effects on fundamental processes, including growth and development. Consistent with this, knockout (KO) mice of *Upf1, Upf2*, *Magoh,* and *Smg1* NMD genes all suffer from early embryonic lethality and marked developmental abnormalities (McIlwain et al., 2010; Medghalchi et al., 2001; Silver et al., 2010; Weischenfeldt et al., 2008). Partial and complete loss of function mutations in NMD factor genes in man that do not lead to embryonic lethality are associated with various forms of neuro-developmental conditions and congenital anomalies (Albers et al., 2012; Laumonnier et al., 2010; Lynch et al., 2012; Nguyen et al., 2013; Tarpey et al., 2007; Xu et al., 2013), a topic that will be described in greater detail in Section 4.

What are the features in normal mRNAs that prompt them to be degraded by NMD? The current consensus is that the context of the stop codon defining the end of the main open reading frame (ORF) dictates whether NMD is triggered or not (Nagy and Maquat, 1998). These features include (i) a natural stop codon >55 nt upstream of an exon-exon junction (Nagy and Maquat, 1998), (ii) alternative splicing (AS) events that cause a frameshift that results in the generation of an in-frame stop codon >55 nt upstream of an exon-exon junction (Horikawa et al., 2000; Wang et al., 2002b), (iii) leaky translation due to differential usage of internal ribosomal entry sites (Welch and Jacobson, 1999), and (iv) long 3′ UTRs (Kebaara and Atkin, 2009). In addition, ORFs 5′ of the main reading frame—so-called upstream ORFs (uORFs)—can, in some cases, elicit NMD; perhaps because their stop codons recruit termination factors that can, in turn interact with downstream NMD-promoting factors, such as the EJC (Ruiz-Echevarria and Peltz, 2000; Silva et al., 2006).

4 NMD factors associated with genetic diseases

The most significant evidence supporting the involvement of NMD in human genetic disorders comes from patients with neuro-developmental disorders and intellectual disability (ID) that carry mutations in the *UPF3B* gene (MIM 300676). Studies of several families with multiple affected individuals have demonstrated that mutations in *UPF3B* cause ID in these patients (Addington et al., 2011; Laumonnier et al., 2010; Lynch et al., 2012; Tarpey et al., 2007; Xu et al., 2013). Many of the patients, some of them also singletons, present with comorbidities including schizophrenia or autism, suggesting that loss of *UPF3B* might contribute to these psychiatric disorders (Addington et al., 2011; Laumonnier et al., 2010; Lynch et al., 2012; Tarpey et al., 2007). A summary of the clinical features associated with *UPF3B* mutations and an in-depth discussion of the role of NMD in psychiatric disorders has recently been compiled by Laumonnier et al. (2013).

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In addition to *UPF3B* mutations, heterozygous deletions of genomic regions that include *UPF2* were recently shown to be associated with ID and neuro-developmental disorders (Nguyen et al., 2013). In these patients, compromised NMD led to deregulation of 5–10% of the transcriptome, as determined using their lymphoblastoid cell lines (LCL). Several of these deregulated genes were further validated using neuronal cell models (Jolly et al., 2013), and as such, they may explain at least some aspects of the patients' clinical phenotypes (Nguyen et al., 2012; Nguyen et al., 2013). Further support for the involvement of NMD in psychiatric disorders comes from the identification of a *de novo* missense mutation in *UPF2* in a patient with schizophrenia, although the functional consequences of the mutation have not been experimentally tested (Gulsuner et al., 2013).

NMD has also been linked with another human disease: Thrombocytopenia with Absent Radius (TAR) Syndrome (MIM 274000). This is a debilitating disease that was recently shown to be caused by mutations in the *RBM8A* gene, which encodes the core EJC factor RBM8A; also known as Y14 (Albers et al., 2012). TAR typically results from compound heterozygosity for a deletion (*de novo* or inherited) of *RBM8A* (on one allele) and a deleterious regulatory single nucleotide polymorphisms (SNP) in its promoter or its intron (on the other allele) (Albers et al., 2012). The main clinical hallmarks of patients with TAR syndrome are reduction in the number of platelets and skeletal anomalies with variable severity, ranging from absence of radii to upper limbs, with or without lower limb defects. Intriguingly, a small proportion of these patients (~7%) have compromised cognitive function, which may be due to the thinning of the corpus callosum and cysts, as identified by MRI (MacDonald et al., 1994; Rosenfeld et al., 2012). The small genomic region deleted in these patients, also termed the 1q21.1 proximal region, was found to be significantly associated with developmental delay and congenital anomalies without hallmarks of TAR syndrome (Rosenfeld et al., 2012). This suggests that at least some of the 16 genes in the region, including *RBM8A*, may play role in normal cognitive development. The evidence supporting the notion that *RBM8A* has such a role is that it encodes a protein that is part of the same protein complex as UPF3B, which causes intellectual disability when absent or mutated (Tarpey et al., 2007).

A recent survey of copy number variations (CNVs) encompassing 18 known NMD and EJC genes in ~57,000 patients and ~20,000 controls identified several of these genes as being significantly associated with neurological diseases. Both copy number gains and losses of regions encompassing *UPF2* or *RBM8A* were associated with various forms of neuro-developmental disorders, with or without congenital anomalies (Nguyen et al., 2013). Also associated with these conditions were copy number losses of genomic regions encompassing *UPF3A* and copy number gains in genomic regions encompassing *SMG6*, *EIF4A3* or *RNPS1*. While the molecular pathology of these CNVs have not yet been investigated in humans, it is worth noting that overexpression of *Rbm8a* in a mouse model has been shown to increase anxiety-like behavior, impair social skills and decrease immobile time (Alachkar et al., 2013). Mice overexpressing *Rbm8a* also display enhanced frequency of miniature excitatory postsynaptic currents (Alachkar et al., 2013). Together, these studies suggest that either too much or too little of a given NMD factor can cause neurological or psychiatric disorders. However, it remains to be determined whether overexpression of NMD factors

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causes disease because it affects the magnitude of NMD or because this triggers NMD-independent deleterious effects.

The clinical presentations of known patients with NMD factor mutations and CNVs vary considerably. For example, there has been no consistent diagnosis for males from the ten families identified with *UPF3B* mutations, apart from ID (Addington et al., 2011; Laumonnier et al., 2010; Lynch et al., 2012; Tarpey et al., 2007; Xu et al., 2013). Broad and variable clinical expressivity, even due to identical mutations in the same gene, is not unusual, but the underlying mechanism in most cases is not known. Pertaining to this, molecular analysis of a brother pair who carry exactly the same mutation in *UPF3B* revealed that the UPF3B protein paralog, UPF3A, is expressed at a level that inversely correlates with the extent of their transcriptome deregulation and the severity of their neurological symptoms (Nguyen et al., 2012). This is potentially important since UPF3A has been shown to be stabilized and to compensate for the loss or depletion of UPF3B (Chan et al., 2009; Nguyen et al., 2012). Thus, UPF3A could be a modifier of the clinical phenotype of *UPF3B* patients. What determines differential UPF3A protein levels in these patients remains to be elucidated.

Similar to patients with *UPF3B* mutations, patients carrying CNVs encompassing other NMD and EJC genes show a wide range of clinical presentations. Of note, some of these CNVs are also found in apparently normal individuals (at much lower frequencies) or were, in some instances, inherited from unaffected parents, suggesting that they are incompletely penetrant (Nguyen et al., 2013). Such variation in penetrance is not unusual and is observed in many genetic disorders (Girirajan and Eichler, 2010). In the case of NMD factor genes, we hypothesize that their variable copy number alters the magnitude of NMD efficiency, which can make a non-penetrant individuals fall below the “clinically relevant” threshold and thus present with a disease, as will be further discussed in Section 7.

5 Inter-individual variation in NMD efficiency may affect phenotype

Several lines of evidence suggest that differences in NMD efficiency between individuals can modify the outcome of genetic diseases. In addition to the above-mentioned examples of variable responses to PTC-eliciting mutations in *DMD* or *ESCO2* (Section 1), NMD efficiency has also been suggested to vary among individuals with nonsense mutations in the *CTFR* gene (Kerem et al., 2008; Linde et al., 2007; Linde and Kerem, 2008). Two “PTC read-through” drugs, Gentamicin and PTC124, have been used with some success in clinical trials to inhibit reading of the PTCs in the *CFTR* mRNA in these patients and thereby restore translation of some full-length CFTR protein (Keeling and Bedwell, 2011). Of note, there has been some controversy as to whether PTC124 is a *bona fide* read-through inhibitor; several studies have obtained evidence that while PTC124 is active in the luciferase-based reporter screen used to identify it (Welch et al., 2007), this is due to an off-target effect (McElroy et al. (2013) and references therein). Regardless of this concern, it is interesting that patients who had higher basal level of the mutant transcript responded better to treatment with either Gentamicin or PTC124 (Kerem et al., 2008; Linde et al., 2007; Linde and Kerem, 2008). While not explicitly tested, it is possible that such patients had intrinsically inefficient NMD, which resulted in higher amounts of mRNA available to be

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translated, and hence higher levels of functional CFTR protein (Kerem et al., 2008; Linde et al., 2007; Linde and Kerem, 2008).

Variable NMD efficiency also appears to be an important factor contributing to the diversity of normal phenotypic traits in humans. In a genome-wide survey of loss-of-function (LOF) variants in human protein coding genes, MacArthur et al. (2012) found on average ~100 heterozygous LOF variants per personal genome, with ~20 genes being completely inactivated (MacArthur et al., 2012). A large proportion of these LOF variants consisted of naturally occurring mutations that introduce a PTC as a result of a deletion or a small duplication and/or aberrant splicing leading to frameshift. However, they observed only a small proportion (25%) of the mRNAs predicted to be degraded by NMD were consistently downregulated by NMD in most individuals, at least as determined by qPCR analysis of 119 lymphoblastoid cell lines from individuals with and without LOF variants (MacArthur et al., 2012). This experimental evidence suggested considerable inter-individual variability in NMD efficiency, especially since there was a large standard deviation in many of the mRNA levels. It is possible that the PTC-containing mRNAs escaping NMD in these individuals could produce truncated proteins with dominant-negative effects, which could in turn contribute to phenotypic variation. In further support of inter-individual variability of NMD, nonsense and frameshift mutations that generate NMD-immune PTCs in the nuclear factor 1 X-type (*NFIX*) gene are responsible for the lethal Marshall-Smith Syndrome (MIM 602535), whereas mutations in *NFIX* that generate NMD-sensitive PTCs cause Sotos-like Syndrome (MIM 614753), which is a milder disease (Malan et al., 2010). Likewise, a patient with a mutation causing the generation of an NMD-immune PTC in the latent *TGFβ-binding protein-4* (*LTBP4*) gene exhibited severe malformation of the gastrointestinal tract, a symptom not typically associated with *LTBP4* mutations, as they normally cause a mild disease called Type-1 Recessive Cutis Laxa (MIM 613177) (Callewaert et al., 2013). There are several other examples of disease severity being modified by NMD through downregulation of mRNAs leading to production of dominant-negative truncated proteins, as discussed in several reviews (Holbrook et al., 2004; Khajavi et al., 2006).

Taken together, these studies demonstrate that inter-individual variation in NMD efficiency might be relatively common and thus is relevant for health and disease (Figure 2). In addition to point mutations and CNVs that impact NMD genes (Addington et al., 2011; Albers et al., 2012; Laumonnier et al., 2010; Lynch et al., 2012; Nguyen et al., 2013; Tarpey et al., 2007; Xu et al., 2013), there are several other factors that have been discovered that can influence NMD efficiency, which will be discussed below.

6 Sources of inter-individual variation in NMD efficiency

6.1 Expression quantitative trait loci

Expression quantitative trait loci (eQTL) are important factors that contribute to the phenotypic diversity within and among different human population (Dixon et al., 2007; Stranger et al., 2012). These loci typically are SNPs or CNVs that are associated with gene expression changes. The current SNP-eQTL map of the human genome has been extensively drawn from several large-scale studies involving hundreds of samples (Dixon et al., 2007; Stranger et al., 2012). With regard to NMD, multiple SNPs have been found to be highly

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associated with the expression of *SMG7* ($P < 1.0e^{-8}$, Table 2) (Dixon et al., 2007; Stranger et al., 2012), which encodes a protein essential for NMD that promotes the dephosphorylation of UPF1 (Page et al., 1999). Likewise, a frequent SNP rs2428212 with minor allele frequency (MAF) of 0.23 (i.e., the frequency of the less common allele is 23% in the population) was found to be associated with the expression of *UPF3B* ($P < 1.0e^{-8}$, Table 2) (Castagne et al., 2011). These SNP-eQTL map *in cis* with *SMG7* and *UPF3B*, suggesting that they directly regulate their expression, but it cannot be excluded that they are in linkage disequilibrium (i.e. close by) with the *bona fide* regulatory SNPs. While these studies suggest that *cis*-regulatory SNPs cause only a few cases of inter-individual variation in NMD efficiency, the actual number of NMD factors subjected to this type of regulation is likely to be higher than reported so far, as published studies have been conducted on a limited number of cell types (e.g., LCLs and blood monocytes) and only examined common SNPs with frequencies greater than 5%. Since SNP-eQTL are often tissue- and population-specific (Fu et al., 2012; Stranger et al., 2012), it would not be surprising if there were many additional layers of NMD factor eQTL variability that are responsible for NMD factor variability among different individuals.

While, as described above, most studies have focused on relatively common SNPs, two rare SNPs associated with *RBM8A* expression have been reported: rs139428292 (MAF = 0.03) and the intronic rs201779890 (MAF = 0.0042), both of which were found to be significantly enriched in patients with TAR syndrome (Table 2). Since inheritance of either one of these SNPs predisposes the carrier to TAR syndrome, there is tremendous interest in defining their molecular mechanism (Albers et al., 2012). Allele A of rs139428292 was experimentally shown to decrease the binding of transcription factor EVI1 to the promoter of *RBM8A*, thereby providing a likely explanation for reduced *RBM8A* expression (Albers et al., 2012). Allele C of rs201779890 is also associated with a reduction in *RBM8A* expression, but the functional role of this SNP, if any, is not known. More such low frequency SNPs will hopefully be captured as more personal genomes are generated. Together, this may provide a more complete picture of how mutations can alter the expression of NMD and EJC factors, and thereby influence the transcriptome under the control of the NMD pathway.

In addition to being associated with gene expression, SNPs associated with protein levels—referred to as SNP protein-QTL (SNP-pQTL)—are beginning to be defined. In a recent study, the level of ~5000 proteins in LCLs from 95 HapMap individuals were examined alongside with their genotypes (Wu et al., 2013). Among the few hundred SNP-pQTL identified, one [rs6673692 (MAF = 0.46)] was found to be significantly associated with the expression of MAGOH, a core member of the EJC (Table 1 and 2). While the variation in MAGOH level was only modest (~10%), it is possible that this elicits sizeable physiological effect, based on the observed defects in neural stem cell division and brain size in heterozygous *Magoh* mice (Silver et al., 2010).

When interpreting the effect of simple nucleotide and CNV variation on inter-individual variability in NMD efficiency, it is important to bear in mind that NMD efficiency does not necessarily correlate with the level of NMD factors. If a given NMD factor is *not* rate limiting in a given cell type at a particular developmental stage, then mutations that increase its expression or modestly reduce its levels would be predicted to have no impact on the

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magnitude of NMD. On the other hand, if a NMD factor is rate limiting, then even a modest increase or decrease in its level could lead to major changes in the transcriptome. For example, Huang et al. (2011) found that modest overexpression of SMG1 increased NMD efficiency in HeLa cells, while modest overexpression of several other NMD factors did not, suggesting that SMG1 is the only rate-limiting NMD factor in these cells (Huang et al., 2011). UPF1 overexpression increases the magnitude of NMD in U2OS osteosarcoma cells, indicating that UPF1 is rate limiting for NMD in these cells (Gardner, 2008). Evidence that many NMD factors are *not* rate limiting *in vivo* was observed by Zetoune et al. (2008), who observed that while the magnitude of NMD differed significantly between different mouse tissues (as indicated by the ratio of PTC+ to PTC-*Men1* transcripts in a *Men1* heterozygote), this did not correlate in an obvious way with NMD factor mRNA levels (Zetoune et al., 2008).

6.2 Competition with the Staufen-mediated mRNA decay pathway

Similar to NMD, Staufen-mediated mRNA decay (SMD) pathway is an RNA decay pathway used by cells to regulate gene expression. SMD shares with NMD the requirement for UPF1, however, SMD differs from NMD in other respects, including that it is independent of splicing and the EJC (Hosoda et al., 2005; Kim et al., 2005). SMD is a translation-dependent mechanism that is triggered when the RNA-binding protein, Staufen, binds to the 3′ UTR of its target mRNA. This leads to recruitment of UPF1, followed by rapid degradation of the transcript via mechanisms that may be similar to that which attacks NMD substrates (Kim et al., 2005; Kim et al., 2007; Maquat and Gong, 2009).

SMD and NMD are competitive pathways by virtue of the fact that both depend on UPF1. One line of evidence for this is that the SMD factors, STAU1 and its paralog STAU2, bind to the same region of UPF1 protein as the NMD factor UPF2 (Gong et al., 2009; Park et al., 2013). As a result of this, STAU1 and STAU2 bind to UPF1 in a mutually exclusive manner with UPF2. Another line of evidence is that down-regulating STAU1 enhances NMD efficiency, whereas down-regulating UPF2 increases SMD efficiency (Gong et al., 2009). This is believed to be an important mechanism utilized by cells to fine tune the expression of specific transcripts that drive specific developmental fates. As evidence for this, differentiation of C2C12 myoblast-like proliferative cells into multinucleated myotubes was shown to reciprocally regulate the efficiency of SMD and NMD, leading to decreased expression of the anti-muscle differentiation factor *PAX3* and increased expression of the pro-muscle differentiation factor *MYOGENIN*, respectively (Gong et al., 2009).

Interestingly, available evidence suggests that nucleotide variations in *STAU2* may be common in normal human populations. Seven partial CNVs (3 gains and 4 losses) spanning various exons of *STAU2* have been recorded in the Database of Genomic Variants (DGV), which houses CNV data obtained from large number of controls from published studies (http://dgv.tcag.ca/dgv/app/home). These partial CNVs have breakpoints within the coding region of *STAU2* and, therefore are likely to alter the reading frame of *STAU2* transcripts, leading to haploinsufficiency. Although no SNP-eQTL was found to be associated with *STAU2* mRNA expression, there are also over 50 small CNVs that reside within the introns of *STAU2*, which could also affect its transcription or processing by disrupting

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transcriptional enhancers or splicing *cis* elements, respectively. While disruption of STAU2 expression or function may be partially compensated by STAU1, it is known that depletion of STAU2 disrupts both SMD and NMD, even in the presence of STAU1, at least in cell lines (Park et al., 2013). Given the competitive relationship of SMD and NMD, it will be important to determine how and to what extent mutations of SMD genes impact NMD and whether this has clinically measurable and significant effects.

6.3. MicroRNA-mediated regulation of NMD

Micro-RNAs (miRNAs) are small (~22 nucleotide) non-coding RNAs that typically repress gene expression via complementary base-pairing of their "seed" sequence with the 3′ UTR of their target mRNAs. Transcripts bound by miRNAs are targeted for degradation, translationally repressed, or both (Okamura, 2012). Currently, there are over 2000 human miRNAs recorded in the miRbase database (Kozomara and Griffiths-Jones, 2011). Together, these miRNAs could target a large portion of the genes in the transcriptome; however, due to the high false positive rates of different prediction programs, experimental validations are necessary to determine the exact number of genes affected (Thomson et al., 2011). To identify miRNAs that regulate NMD, Bruno et al. (2011) performed a bioinformatics search for miRNAs predicted to target NMD factors. They identified two identical miRNAs generated from independent gene loci, *miR-128-1* and *miR-128-2*, as potential regulators of the core NMD factor UPF1 and the EJC factor MLN51, which they confirmed experimentally (Bruno et al., 2011). The ability of *miR-128* to repress UPF1 and MLN51 was functionally relevant, as *miR-128* was shown to be capable of regulating the magnitude of NMD (Bruno et al., 2011). *miR-128* is highly upregulated during both brain development and neuronal maturation, which raised the possibility that this downregulates NMD efficiency, which, in turn, would increase the stability of NMD substrate mRNAs in neurons. In support of this, the investigators identified several NMD substrates encoding neural-relevant proteins that are upregulated during brain development *in vivo* (Bruno et al., 2011). That this is physiologically relevant is supported by finding that forced expression of *miR-128* in neural stem cells upregulates scores of mRNAs encoding proteins with functions in neural differentiation and function (Bruno et al., 2011).

Indirect genomic evidence suggests the existence of inter-individual variation in the expression of *mir-128*. This comes from the host genes of *mir128-1* and *mir128-2*, *R3HDM1* and *ARPP21*, respectively. There are several partial CNVs spanning regions of both *R3HDM1* (2 gains, 3 losses) and *ARPP21* (4 gains, 2 losses) (as recorded in the DGV). Since *mir128-1* and *mir128-2* are located in the introns of these genes, it is reasonable to speculate that alterations in the copy number of these loci will lead to alterations in the levels of these miRNAs. Intriguingly, it was recently reported that there are 20 CNV gains (complete duplications) of *ARPP21* in a large cohort of both controls and patients with neuro-developmental conditions, though not being significantly enriched in any particular population (Cooper et al., 2011). Recently, another miRNA—*miR-125*—was shown to regulate NMD through its ability to repress the expression of the NMD factor SMG1 (Wang et al., 2013). Like *miR-128*, *miR-125* is transcribed and processed from two independent loci. Genomic evidence suggests variability in the expression of these two miRNAs, as there are 11 CNVs (6 gains, 5 losses) encompassing one of them (*miR-125a*) and 5 CNVs (3

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gains, 2 losses) encompassing the other (*miR-125b*), based on the DGV and published cohorts of patients and controls (Cooper et al., 2011). In coming years it will be interesting to elucidate the complexity of the networks involving NMD and the miRNAs controlling neural differentiation and function.

6.4 Negative feedback loops

Given the importance of NMD in maintaining cellular homeostasis, it is not surprising that self-regulatory feedback mechanisms have evolved to govern this process. The first hint of NMD feedback regulation in mammalian cells was noted by Mendel et al. (2004) and Chan et al. (2007), who found that *SMG5* mRNA was significantly upregulated in HeLa cells upon depletion of UPF1 or UPF3B, respectively. This raised the possibility that the upregulation of NMD factors could rescue NMD function when NMD is perturbed. More recently, two other studies confirmed this hypothesis and provided further details of this regulation (Huang et al., 2011; Yepiskoposyan et al., 2011). These studies showed that not only *SMG5*, but also *UPF1, UPF2, UPF3B, SMG1, SMG6* and *SMG7* are up-regulated in HeLa cells upon inhibition of NMD. Interestingly, all of these NMD factor mRNAs contain "NMD-inducing features" (long 3′ UTRs and/or uORFs), some of which where empirically shown to trigger NMD (Huang et al., 2011; Yepiskoposyan et al., 2011). Together, these data suggest that a broad-acting buffering mechanism exists that stabilizes the mRNAs encoding most NMD factors in response to NMD perturbation.

Studies conducted by Huang et al. (2011) revealed that this NMD feedback loop acts in a cell- and tissue-specific manner and is also developmentally regulated. For example, the investigators found that loss of the NMD factor *Upf3b* in mice only upregulated NMD factors mRNAs in specific tissues and cell types. Among the tissues tested, the spleen was particularly active in "compensating" for the loss of *Upf3b*, as evidenced by it upregulating the NMD factor transcripts *Upf1*, *Upf2*, *Smg1*, *Smg6* and *Smg7*. This is interesting in light of the fact that the spleen houses T cells and B cells, which express antigen receptors generated from mRNAs that frequently contain PTCs as a result of error-prone programmed gene rearrangements, as described in Section 2. The brain was also found to be subjected to NMD feedback regulation, as *Smg1* mRNA levels were increased in response to loss of *Upf3b* (Huang et al., 2011).

The NMD feedback regulatory response is highly conserved. Evidence suggests that this mechanism extends to *Drosophila melanogaster*, *Arabidopsis thaliana*, and humans (Chan et al., 2009; Huang et al., 2011; Kerenyi et al., 2008; Nguyen et al., 2012; Rehwinkel et al., 2005; Saul et al., 2009). This may be clinically relevant since such feedback response could alleviate environment toxicants that are known to inhibit NMD (see the next Section). In addition, such feedback regulation may be the reason why patients lacking a functional *UPF3B* gene are able to survive and maintain some behavioural and cognitive functions (Chan et al., 2009; Nguyen et al., 2012). While the selective forces that led to the generation of this feedback regulation is not known, it is reasonable to suspect that one functional benefit that led to its formation was limiting the variability in NMD function. By providing a buffer to ensure that NMD efficiency is within an acceptable range, this feedback system maintains transcriptome homeostasis (Figure 3).

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6.3 Environmental factors

The efficiency of NMD can also be influenced by external factors. It has been shown that cellular stress elicited by amino-acid starvation, oncogene overexpression and hypoxia can rapidly inhibit NMD activity (Gardner, 2008; Mendell et al., 2004; Wang et al., 2011a). A common downstream consequence of these cellular stresses is increased phosphorylation of the translation initiation factor eIF2α, which has been empirically shown to inhibit NMD (Gardner, 2008). An obvious explanation for why eIF2α phosphorylation inhibits NMD is that it reduces the ability of nonsense codons to be recognized by the translation apparatus, but there is also evidence that this is not the case (Gardner, 2008, 2010; Wang et al., 2011a). Importantly, NMD inhibition upregulates many transcripts necessary for stress response, including the mRNAs encoding ATF3, ATF4, and CHOP, all of which are known to allow cells better cope with stress (Gardner, 2008). While an increased capability of dealing with stress is beneficial for cells, NMD inhibition may also have negative consequences. For example, there is evidence that NMD normally serves to repress tumor formation and thus inhibition of NMD would be predicted to promote tumor progression (Wang et al., 2011b). Indeed, inhibition of NMD leads to upregulation of many transcripts necessary for tumour progression (Wang et al., 2011b). It will be important in the future to determine whether inter-individual variability in NMD efficiency influences the likelihood of tumor progression. While the notion that NMD inhibits tumor formation is intriguing, we note that very few mutations have been reported in NMD genes in tumours in the Database of Somatic Mutations in Cancer, or Cosmic Database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/), (unpublished observation). Thus, it is unclear whether changes in NMD function or efficiency are major contributing factors that drive tumor formation in humans.

7 Perspective

As we have discussed in this Review, it is becoming apparent that NMD efficiency varies between individuals and that this efficiency can be influenced by both genetic and non-genetic actors. Both the full spectrum of these factors and the interplay between these factors is likely to dictate a given individual's net magnitude of NMD (Figure 3). We propose that variable NMD efficiency provides a plausible explanation for the variable clinical presentations and drug responses of patients carrying the same PTC mutation (Section 5). It may also explain apparently normal clinical presentations of some carrier parents (Section 4). Thus, we suggest that inter-individual differences in NMD efficiency should be taken into consideration when treating human diseases caused by genes with PTC-generating mutations and in particular those treatments incorporating PTC read-through drugs.

More studies are needed in order to fully appreciate the impact of inter-individual variability in NMD efficiency. Firstly, it is necessary to determine the extent of inter-individual variability in NMD efficiency in disease and non-disease cohorts. As readout, the levels of established canonical NMD targets ought to be determined in well-defined sets of tissue types from different population groups. This can be facilitated by the large number of microarray and RNA-Seq datasets currently available from the HapMap depository (http://hapmap.ncbi.nlm.nih.gov/) and expression studies of different disease cohorts, e.g. autism

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(Voineagu, 2012). Secondly, it will be necessary to establish the physiological consequences (both functional and clinical) of copy number gains or losses of NMD factor genes in patients with neuro-developmental conditions (Section 4). Thus far, the evidence in support of a cause-and-effect relationship has been limited to studies in manipulated cell lines; e.g., HeLa cells. In the future, it will be important to perform whole transcriptome and proteome analyses on primary cells, iPS-derived neurons and other cell types from individuals with such CNVs (Nguyen et al., 2013).

Although it may or may not be possible to establish a broadly applicable and "clinically relevant" threshold of NMD, the information on individual's NMD efficiency will form another facet of personalized therapy approaches, including those aimed at suppressing NMD and others aimed at enhancing the magnitude of NMD. At present, only NMD-inhibitory drugs are being tested. This should be expanded to include NMD-enhancing drugs since it is clear that compromised or 'weak' NMD efficiency can also be clinically relevant (section 4). We suggest that one target of such NMD-enhancement therapy be the NMD factor, UPF3A, since its levels positively correlate with less severe symptoms in ID patients (Nguyen et al., 2012). The recent evidence for the intricate and dynamic mechanisms that regulate NMD (Karam et al. (2013) and references therein) should be taken into account when developing and clinically applying both NMD-enhancing and –inhibiting drugs. Thus, it will be important to develop tissue- and NMD factor-specific approaches to reduce side effects and maximize therapeutic efficacy. This follows from the evidence that (i) NMD efficiency is variable, (ii) different NMD components are probably rate-limiting factors in different tissues, and (iii) mRNAs differ with regard to the branch of NMD they respond to (Chan et al., 2007; Gehring et al., 2005; Metze et al., 2013). Because "out-of-range" NMD efficiency can impair neuronal development and function (Section 4), drug dose should be carefully titrated to adjust the efficiency of NMD to be within the "normal" range (Figure 3). Thus, future clinical trials should include comprehensive mental assessments of the participants after both short and long term administration of the drugs.

Research into the existence, scale and implications of inter-individual variation in NMD efficiency is in its infancy. This subject represents both an opportunity and a considerable challenge. An increased understanding of the factors controlling NMD efficiency will be essential for the better management and treatment of human diseases caused by genes with protein truncating mutations, and thus provide another example of "personalized medicine." Given that NMD is, to one extent or another, involved in about one-third of all currently known human genetic disorders, manipulation of the efficiency of NMD has the potential to have a large impact on human disease and human well being, in general.

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Highlights

- Nonsense-mediated mRNA decay (NMD) is a crucial regulator of normal development.
- We examine evidence supporting inter-individually variable NMD and its relevance to disease.
- Sources of variable NMD include eQTL, miRNA, SMD and negative feedback loop.
- NMD efficiency should be considered when manipulating it for therapeutic purposes.

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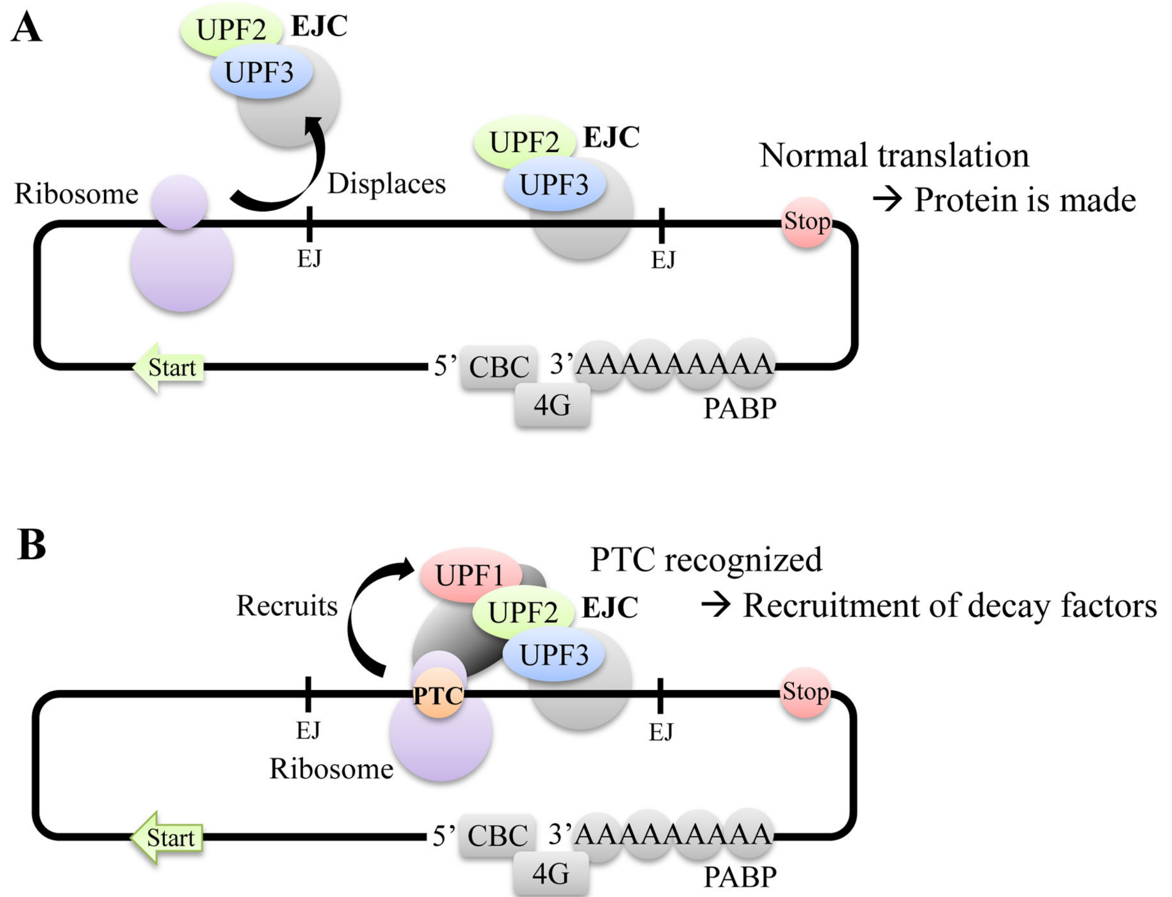

Figure 1. Current simplified model of canonical NMD

In the nucleus and during pre-mRNA splicing process, the EJCs are deposited by the spliceosomes ~ 20–24 nt upstream of the exon-exon junction (EJ). EJC is made up of several proteins and is associated with the UPF3 proteins. The mRNP (mRNA-protein complex) is then exported to the cytoplasm, at which point the EJC-UPF3 complex acquires UPF2 via direct interaction with UPF3 proteins.

A. During the translation process, the ribosome reads along the mRNA and displaces all downstream EJCs before terminating at the normal stop codon. Full-length protein is made.

B. If a stop codon is introduced in a context that would be considered premature, the translating ribosome stalls at the PTC and recognizes downstream EJC. Here, another complex, which contains UPF1, is recruited, bringing together the interaction of the three "core" components of NMD UPF1, UPF2 and UPF3. This subsequently leads to UPF1 being phosphorylated, recruiting further downstream exo- and endonucleotic decay factors to degrade the mRNA.

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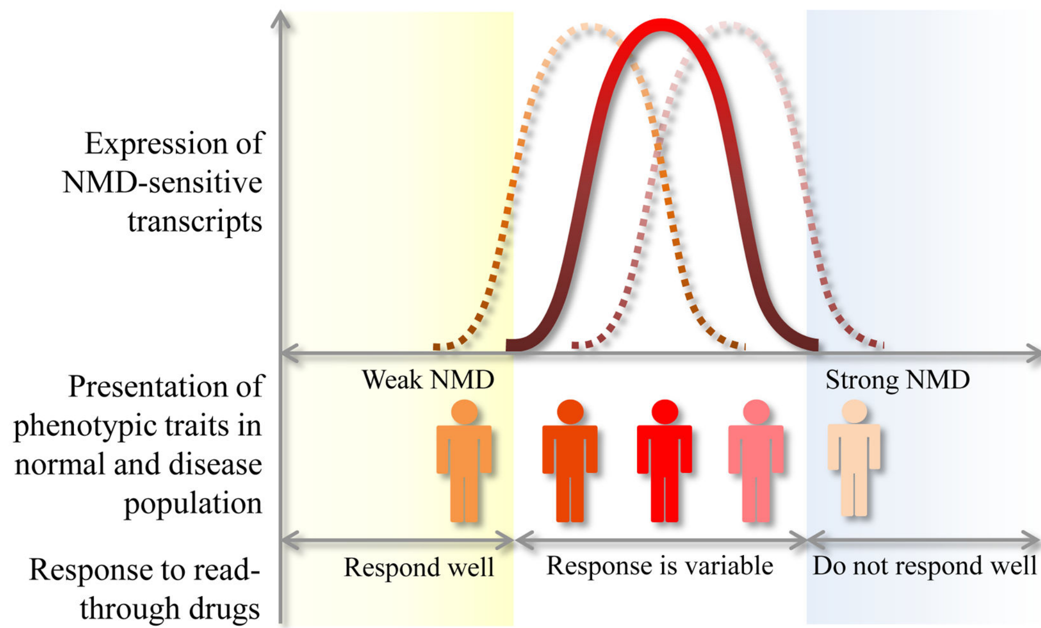

Figure 2. Variable NMD efficiency modifies phenotypic traits

Variation in NMD efficiency could modify the presentation of clinical phenotypes and response to PTC read-through drug treatment.

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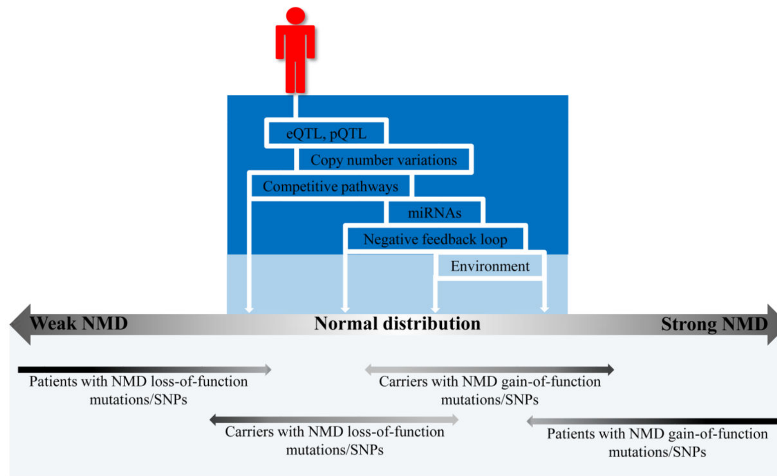

Figure 3. Multiple factors influencing NMD efficiency of each individual

Different combination of genetic and non-genetic factors determines the NMD efficiency of each individual (top panel). Individual's NMD efficiency may lie outside the normal distribution, in which case it will result in a detectable phenotype i.e. ID as seen in patients with NMD or EJC CNVs (Bottom panel).

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Table 1

Proteins involved in the formation and function of the NMD and EJC complexes.

Group	Factor	Aliases	MIM #	Cellular localization	Role in NMD	References
NMD factors	UPF1	RENT1	601430	Shuttling protein, but mainly in cytoplasm	Joins to EJCs when PTC was recognized, mediates translational repression and recruitment of downstream degradation machinery	(He and Jacobson, 1995; Hosoda et al., 2005; Isken et al., 2008; Kashima et al., 2006; Maderazo et al., 2000; Nazarenus et al., 2005)
	UPF2	RENT2	605529	Perinuclear (cytoplasmic)	Joins EJC when the mRNP is exported to the cytoplasm, recruits and promotes phosphorylation of UPF1	(Hosoda et al., 2005; Le Hir et al., 2001; Lykke-Andersen et al., 2000; Mendell et al., 2000; Serin et al., 2001)
	UPF3B	RENT3B, UPF3X	300298	Shuttling protein, mainly in nucleus	Joins EJC via a contiguous surfaced formed by EIF4A3/Y14/MAGOH, recruits UPF2 to EJC	(Gehring et al., 2005; Gehring et al., 2003; Kadlec et al., 2004; Kim et al., 2001a; Le Hir et al., 2001; Serin et al., 2001)
	UPF3A	RENT3A, UPF3	605530	Shuttling protein, mainly in nucleus	Similar to UPF3B but less efficient, compete for binding to UPF2 for stabilization	(Chan et al., 2009; Kim et al., 2001a; Kunz et al., 2006; Le Hir et al., 2001; Serin et al., 2001)
	SMG1	ATX	607032	Cytoplasm and nucleus	Promotes phosphorylation of UPF1	(Denning et al., 2001; Kashima et al., 2006; Morita et al., 2007; Yamashita et al., 2001)
	SMG5	EST1B	610962	Cytoplasm and nucleus	Involved in dephosphorylation of UPF1, mediates exonucleotic decay pathway	(Chiu et al., 2003; Lejeune et al., 2003; Ohnishi et al., 2003; Okada-Katsuhata et al., 2012; Unterholzner and Izaurralde, 2004)
	SMG6	EST1A	610963	Cytoplasm	Mediates endonucleotic decay pathway	(Eberle et al., 2009; Huntzinger et al., 2008; Kashima et al., 2010)
	SMG7	EST1C	610964	Cytoplasm	Involved in dephosphorylation of UPF1, mediates exonucleotic decay pathway	(Chiu et al., 2003; Fukuhara et al., 2005; Lejeune et al., 2003; Ohnishi et al., 2003; Okada-Katsuhata et al., 2012; Unterholzner and Izaurralde, 2004)
	SMG8		613175	n.a.	Forms complex with SMG1 to inhibit its kinase activity before SMG1 joins EJC	(Yamashita et al., 2009)
	SMG9		613176	n.a.	Forms complex with SMG1 to inhibit its kinase activity before SMG1 joins EJC	(Yamashita et al., 2009)
	NBAS	NAG, SMGL1	608025	n.a.	Required for NMD in *C. elegans* and *D. rerio* through unknown mechanism	(Longman et al., 2007)
	DHX34	SMGL2	n.a.	n.a.	Required for NMD in *C. elegans* and *D. rerio* through unknown mechanism	(Longman et al., 2007)
EJC factors	RBM8A	Y14	605313	Shuttling protein, mainly in nucleus	Directly interacts with EIF4A3 and MAGOH to form the contiguous surface for UPF3B interaction	(Fribourg et al., 2003; Gehring et al., 2003; Kataoka et al., 2000; Kim et al., 2001b; Lau et al., 2003; Shi and Xu, 2003)
	MAGOH		602603	Shuttling protein, mainly in nucleus	Binds strongly to RBM8A, EIF4A3 and mRNA, interacts directly with UPF3B	(Kataoka et al., 2001; Lau et al., 2003; Shi and Xu, 2003; Zhao et al., 2000)

Group	Factor	Aliases	MIM #	Cellular localization	Role in NMD	References
	MAGOHB		n.a.	n.a.	Paralog of MAGOH with almost identical sequence conservation. Binds directly to RBM8A, EIF4A3 and UPF3B to activate NMD.	(Singh et al., 2013)
	CASC3	BTZ, MLN51	606504	Mostly cytoplasm	Binds to mRNA directly and interacts with EIF4A3	(Baguet et al., 2007; Degot et al., 2004; Gehring et al., 2005; Gehring et al., 2009a; Palacios et al., 2004)
	EIF4A3		608546	Nucleus	Binds to mRNA directly, provides anchor for the remaining EJC components	(Chan et al., 2004; Gehring et al., 2005; Gehring et al., 2009a; Palacios et al., 2004)
	RNPS1		606447	Nuclear speckles	Binds to mRNA directly, mediates an alternative NMD pathway independent of some EJC factors	(Gehring et al., 2005; Le Hir et al., 2001; Le Hir et al., 2000; Lykke-Andersen et al., 2001)
	WIBG	PYM	n.a.	Cytoplasm	Forms complex with MAGOH-RBM8A, acts to disassemble EJC and negatively regulates NMD	(Bono et al., 2004; Gehring et al., 2009b)

Abbreviations: n.a., not analyzed, not available; MIM, Medelian Inheritance in Man.

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Table 2

SNP-eQTL and SNP-pQTL associated with the expression and efficiency of NMD.

NMD Factors	SNPs	MAF	Reference	Effects	Experimentally validated
SMG7	rs1044879, rs10911353, rs2702180, rs2275675, rs2296164, rs2274064, rs6662844, rs4047801, rs4652800	>0.05	Dixon et al. (2007)	Either increased or decreased *SMG7* expression	No
	rs2702182, rs2702178, rs2702180, rs10911353, rs12117885, rs12032165, rs12144253	>0.05	Stranger et al. (2012)	Either increased or decreased *SMG7* expression	No
UPF3B	rs2428212	0.23	Castagne et al. (2011)	Either increased or decreased *UPF3B* expression	No
RBM8A	rs139428292	0.03	Albers et al. (2012)	Allele A - Reduced *RBM8A* expression	Yes
	rs201779890	0.0042	Albers et al. (2012)	Allele C - Reduced *RBM8A* expression	No
MAGOH	rs6673692	0.46	Wu et al. (2013)	10% change in MAGOH protein expression	No

Abbreviation: MAF, Minor Allele Frequency