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SUPPLEMENTARY INFORMATION

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Quality and quantity control of gene expression by nonsense-mediated mRNA decay

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Supplementary Box 1: NMD avoidance by viruses and therapeutic approaches and possibilities

NMD avoidance — lessons from viruses

Many viruses produce RNAs with features that should subject them to host-cell nonsense-mediated mRNA decay (NMD). These viruses have evolved various strategies to subvert NMD in order to replicate, and these strategies offer windows through which the mechanism of NMD can be studied (see the table).

***Human Immunodeficiency Virus-1 (HIV-1*).** HIV-1 and other retroviruses use alternative splicing of a single primary transcript to generate the diverse mRNAs that are needed during their lifecycle. For proteins whose synthesis initiates toward the 3'-end of the transcript, splicing upstream of the coding region poses no risk of engaging NMD, as the corresponding termination codons are recognized as normal. However, for proteins encoded toward the 5'-end of the transcript, the virus must avoid splicing downstream of the termination codon in order to circumvent NMD. Thus, HIV-1 tightly controls splicing to ensure that intron removal proceeds in 5'-to-3' order to generate transcripts where the coding region is downstream of exon−exon junctions, and it uses the Rev accessory protein, which is produced early in the virus life-cycle to export to the cytoplasm incompletely spliced RNAs encoding proteins from their 5'-ends [1].

Human T-Lymphotropic Virus Type 1 (HTLV-1). HTLV-1 directly inhibits NMD by expressing the Tax protein, which antagonizes NMD by interacting with UPF1[2]. This interaction causes UPF1 phosphorylation and sequesters it in P-bodies. It also prevents UPF1 association with eIF3, which is critical for the step of translation repression that precedes mRNA decay. By preventing the return of UPF1 to its basal, hypo-phosphorylated state, it is thought that HTLV-1 prevents UPF1 from participating in further rounds of NMD. Consistent with this hypothesis, NMD is inhibited in cells expressing variants of SMG5 that are unable to recruit the PP2A holoenzyme, the phosphatase that dephosphorylates UPF1[3]. Furthermore, NMD is inhibited in cells that express an inactive variant of UPF1 or when cells are exposed to okadaic acid, which inhibits PP2A function[4-6]. Tax additionally inhibits NMD by plugging the RNA channel of UPF1, resulting in RNA-binding UPF1 translocation defects[7]. Another HTLV-1 protein, Rex, appears to inhibit NMD by unknown mechanisms[8].

Rous Sarcoma Virus (RSV). The retrovirus RSV also uses alternative splicing to generate multiple protein-coding mRNAs from a single pre-mRNA. However, the resulting unusually long ~7-kilobase 3' untranslated region (3'UTR; see *EJC-independent NMD*) fails to trigger NMD since the virus has evolved a Rous Stability Element (RSE), which is a 400-nucleotide sequence situated immediately downstream of the termination codon of *gag* mRNA (that is, in the unspliced pre-mRNA)[9]. Polypyrimidine tract-binding protein 1 (PTBP1) binds the RSE and shields the mRNA from NMD by preventing UPF1 loading and accumulation on the 3'UTR[10]. This mechanism is expected to exist in many cellular mRNAs that escape NMD but are computationally predicted to be NMD targets due to their long, unstructured 3'UTR that does not bring the termination codon sufficiently close to polyadenylate-binding protein 1, although the interfering protein can be other than PTBP1[11].

Hepatitis C Virus (HCV). Replication of HCV interferes with NMD[12]. The HCV core protein interacts with and sequesters the host protein partner of Y14 and mago (PYM1), which associates with exon junction complexes (EJCs) to promote their recycling[13]. Tethering PYM1 to the 3'UTR of a reporter mRNA results in mRNA degradation; for this and other reasons, PYM1 is thought to have a role in NMD[14]. Thus, it seems that sequestration of PYM1 by the viral core protein would attenuate NMD. How sequestration benefits the virus remains unknown. Although downregulating core NMD components did not boost viral titers, this experiment was done in a setting where the viral core protein–PYM1 interaction was fully intact so that NMD was already inhibited, possibly masking the effects of downregulating NMD factors on viral replication[12].

Semliki Forest Virus (SFV) and Sindbis Virus (SINV). Recently, it was shown that mammalian cells exploit NMD to restrict positive-strand RNA virus replication during infection by SFV and distantly related SINV family members[15]. The viral plus-strand RNA genomes act as an mRNA encoding non-structural proteins (nsPs) and also as template for the complementary negative-strand RNA that is used to direct viral genome replication. A small interfering RNA (siRNA) screen found that cellular UPF1 restricts SFV and SINV replication[15]. In support of the involvement of NMD in suppressing virus replication, downregulating SMG5 or SMG7 augmented SFV replication. When plus-strand RNA is used as mRNA, the nsPs are translated from one ORF at the 5'-end of the RNA, leaving a large 4000-nucleotide 3'UTR. This 3'UTR does not engage NMD factors as deletion of most of the 3'UTR still allowed suppression of viral replication by UPF1, leaving open the possibility that other *cis*-acting signals mediate UPF1 engagement. As expected, the virus attempts to protect its genome from UPF1 later during infection by sequestering its own replication intermediates within membranous replication factories that are devoid of protein synthesis factors (and thus NMD factors). Also, the viral replicase inhibits UPF1, possibly by displacing it from plus-strand RNA as point mutations that slow the replicase increase the sensitivity of the virus to UPF1

binding. Alternatively, nsP3 may directly inhibit NMD given that a mutant virus lacking the C-terminal domain of nsP3 is more sensitive to the presence of UPF1 than other viruses with nsP3 variants.

Zika Virus (ZIKV). As with SFV and SINV, NMD restricts ZIKV[16], which is a mosquito-borne plus-strand RNA virus. Experimental knockdown of UPF1 boosts viral titers, indicating that ZIKV has evolved a method for attenuating UPF1 activity during the course of infection. The ZIKV capsid forms a complex with UPF1 and directs its proteasomal degradation, thereby dampening NMD[16].

Therapeutic approaches and possibilities for targeting NMD

Translation read-through. During translation, aminoacylated tRNAs are recruited, one after another, into the translocating A site of the ribosome. The anticodon–codon base-pairing formed between each aminoacylated tRNA and the mRNA can be cognate (perfectly matched) or near-cognate (allowing one nucleotide mismatch). When the ribosome reaches a termination codon, the eRF1–eRF3 termination complex is recruited instead of an aminoacyl tRNA to facilitate peptidyl tRNA hydrolysis and release of the newly synthesized polypeptide. A small subset of near-cognate tRNAs can compete with the eRF1–eRF3 termination complex in occupying the ribosomal A site, thereby causing PTC read-through, or nonsense suppression[17]. As expected, the fidelity of translation is normally high — PTC read-through occurs in <1% of translation events and read-through at normal termination codons occurs one order of magnitude less than PTC read-through[17-19].

Nonsense mutations are estimated to account for ~11% of all genetic disease-causing mutations[20] and thus represent a fertile area for disease intervention using various nonsense suppressors. Nonsense suppressors, which include aminoglycosides, aminoglycoside derivatives (NB30, NB54, NB84, NB124), ataluren (PTC124), read-through compound 13 (RTC13), RTC14 and amlexanox, enhance ribosome read-through at PTCs to produce full-length proteins. Aminoglycosides, including gentamycin, paromomycin, G418 (geneticin), streptomycin, amikacin, lividomycin and tobramycin are the best-characterized nonsense suppressors to have been tested in individuals with genetic diseases such as cystic fibrosis[21-35], Duchenne muscular dystrophy[26,28,36-42], congenital muscular dystrophy[43], ataxia telangiectasia[44-46], Rett syndrome[47-50], type 1 User syndrome[51-54], Hurler syndrome[55-58], haemophilia A and B[59,60], cancer[26,57], Maroteaux–Lamy syndrome[61], carnitine palmitoyltransferase 1A deficiency[62],

methylmalonic acidura[63], neuronal ceroid lipofuscinosis[64,65], spinal muscular atrophy[66], peroxisome biogenesis disorders[67,68], obesity[69], aniridia[70], Hailey–Hailey disease[71], dystrophic epidermolysis bullosa[72] and Herlitz junctional epidermolysis bullosa[73].

The general therapeutic approach to using nonsense suppressors has the advantage of being transcript-unspecific and thus would be applicable to many diseases. However, they are only able to promote the production of a limited amount of PTC-containing transcripts and full-length proteins, in part owing to being toxic by targeting many cellular NMD targets. Thus, the therapeutic efficacy of nonsense suppressors depends on how much PTC-containing transcript is needed to ameliorate the disease phenotype. For example, aminoglycosides, which enter cells through endocytic receptors that are enriched in kidney cells and hair cells of the inner ear[74], trigger apoptosis and necrosis[75,76]. Thus, these drugs cause nephrotoxicity and ototoxicity in ~2–25% of patients[77]. In addition, since aminoglycosides target the bacterial ribosome and given the similarity of the bacterial ribosome to the mitochondrial ribosome, aminoglycosides cause mitochondrial dysfunction[78]. These and other problems have encouraged the search for safer yet adequately effective therapeutics.

Inhibition of NMD factors. An alternative therapeutic approach is to directly inhibit NMD by targeting NMD factors with small molecules. For example, wortmannin and caffeine inhibit a number of kinases, including SMG1[79,80]. Pateamine A binds eIF4A3 to inhibit its function in 3′UTR-EJC-mediated NMD[81]. Curcumin inhibits histone acetyltransferase (HAT) p300/CBP[82] and, among other effects, reduces *UPF1* gene transcription by reducing the amount of acetylated histone H3 Lys 27 and of RNA polymerase II at the *UPF1* promoter; pateamine A also reduces the levels of UPF2, UPF3 and UPF3X[83]. Cardiac glycosides inhibit the sodium–potassium ATPase in the plasma membrane, thereby elevating the intracellular calcium level. This inhibits NMD by an unknown mechanism[84]. NMD inhibitor 1 (NMDI1) blocks the interaction of UPF1 and SMG5, thereby transferring NMD-targeted mRNAs into translationally inactive cytoplasmic P-bodies[85]. NMDI14 binds to a pocket in SMG7 and disrupts its interaction with UPF1[86]. 5-azacytidine induces *MYC* gene expression[87], which inhibits NMD (see *Adaptation to stress and environment*). Cytoskeleton disrupting agents such as cytochalasin D, jasplakinolide, colchicine or taxotere can inhibit NMD, and cytochalasin D and jasplakinolide also induce PTC read-through[88].

A potential problem with using many of these small molecules is their specificity: some compounds may be either promiscuous in their targeting or they may target fundamental cellular pathways and have many effects secondary to NMD inhibition. For example, because curcumin targets the HAT p300[82], it changes the transcriptional output of a number of loci in addition to *UPF1*. Targeting such a general process such as transcription almost certainly leads to secondary effects. Therefore, more specific therapeutic siRNAs and chemically modified antisense oligonucleotides (ASOs) targeting NMD factors have been used to directly inhibit NMD[89,90]. These NMD-specific approaches are restricted to

diseases in which the mutated genes produces partially functional proteins (see *NMD modulates inherited diseases*); alternatively, they would require combination therapy with a nonsense suppressor to produce full-length, functional proteins. For most small-molecule NMD inhibitors, the molecular basis for inhibition and cellular toxicity remain to be thoroughly examined. In addition, since NMD not only eliminates aberrant mRNAs but also maintains proper expression levels of non-mutated cellular mRNAs, therapeutic doses of NMD inhibitors may cause unforeseen side effects.

Targeting NMD-substrate mRNAs. To address the above specificity concerns, several approaches to targeting PTC-containing mRNAs have been explored. Using chemically modified ASOs, EJC formation ≥50–55-nucleotides downstream of a PTC was physically blocked to inhibit 3'UTR-EJC-mediated NMD[91]. Because simple inhibition of NMD generally promotes stabilization of the PTC-containing mRNA, which may encode a toxic truncated protein, this ASO-based method would need to be combined with read-through therapy. Pseudouridylation, which is the most abundant post-transcriptional modification in cellular non-coding RNAs, can alter the recognition of nonsense codons to sense codons and has been experimentally directed to modify PTCs in yeast cells using artificial guide RNAs[92]. Specifically, pseudouridylated UAA or UAG stop codons encode serine or threonine, whereas pseudouridylated UGA encodes tyrosine or phenylalanine. Although this approach would not alter any other cellular process, i.e. NMD or global translation, functional analysis of the protein containing newly introduced missense amino acids would be required should the amino acid replacement not be synonymous with the original amino acid. Moreover, this target-specific approach must be examined in human cells. Recently, a high throughput screen identified anti-codon engineered transfer RNAs (ACE-tRNAs) that can supress PTCs by inserting the correct cognate amino acid needed to repair particular disease-causing mutations in human cells[93]. Importantly, ribosome profiling shows little evidence for readthrough of endogenous termination codons in cells expressing ACE-tRNAs[93].

Experiments have also focused on mRNAs harbouring NMD-insensitive mutations (see *NMD modulates inherited diseases*). Such mRNAs produce toxic truncated proteins and promote disease, for example in the case of certain cancer-causing adenomatous polyposis coli (*APC*) or *BRCA1* gene mutations[94]. Expression of these NMD-insensitive mRNAs was reduced using microRNAs. However, since microRNAs will also target the mRNA produced from the normal allele, it is important to identify microRNA concentrations that will sufficiently reduce the expression of the defective mRNA while maintain adequate levels of the wild-type mRNA and protein[95-100].

Table: Virus-mediated modulation of host-cell NMD

Virus	Mechanism	Purpose	References

HTLV-1	Cellular NMD is inhibited: - Viral Tax protein sequesters cellular UPF1 into P-bodies so that UPF1 cannot bind cellular eIF3 or undergo dephosphorylation; - Tax also plugs RNA channel of UPF1; - Viral Rex protein inhibits NMD by unknown means.	Promotes virus production	2,7,8
RSV	NMD of viral RNA is inhibited: Rous stability elements (RSEs) in viral transcripts recruit host PTBP1, which antagonizes UPF1 binding to viral RNA.	Promotes virus production	9,10
HCV	Cellular NMD is inhibited: Viral core protein sequesters PYM1, thereby preventing EJC recycling.	Unknown, presumably promotes the viral lifecycle	12
SFV	NMD of viral RNA is inhibited: - Viral replicase protein competes with UPF1 for binding to viral RNA, thereby preventing the NMD of viral RNA; - Viral non-structural proteins may directly inhibit UPF1, thereby inhibiting cellular NMD.	Promotes virus production	15
ZIKV	Cellular NMD is inhibited: Viral capsid protein binding to UPF1 leads to proteasome-mediated UPF1 degradation.	Promotes virus production	16

EJC, exon junction complex; PTBP1, polypyrimidine tract-binding protein 1; PYM1, partner of Y14 and mago.

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