Review

Introducing sense into nonsense in treatments of human genetic diseases

Liat Linde and Batsheva Kerem

Department of Genetics, The Life Sciences Institute, Givat Ram Campus, The Hebrew University, Jerusalem 91904, Israel

Approximately one-third of alleles causing genetic diseases carry premature termination codons (PTCs), which lead to the production of truncated proteins. The past decade has seen considerable interest in therapeutic approaches aimed at readthrough of in-frame PTCs to enable synthesis of full-length proteins. However, attempts to readthrough PTCs in many diseases resulted in variable effects. Here, we focus on the efforts of such therapeutic approaches in cystic fibrosis and Duchenne muscular dystrophy and discuss the factors contributing to successful readthrough and how the nonsense-mediated mRNA decay (NMD) pathway regulates this response. A deeper understanding of the molecular basis for variable response to readthrough of PTCs is necessary so that appropriate therapies can be developed to treat many human genetic diseases caused by PTCs.

Therapeutic approaches for correcting PTCs

Premature termination codons (PTCs) originate from either mutations, such as nonsense mutations, frame-shift deletions and insertions, or from aberrant splicing that generates mRNA isoforms with truncated reading frames. These mutations can lead to the production of truncated, nonfunctional or deleterious proteins, owing to dominant-negative or gain-of-function effects [1]. In the past few years, there has been an attempt to develop mutation-specific pharmacological approaches aimed at achieving sufficient levels of functional proteins.

In this review, we focus on therapeutic approaches for mutations generating in-frame PTCs. These therapies are aimed at promoting translational readthrough of the PTCs, to enable the synthesis and expression of full-length functional proteins. Readthrough of PTCs can be achieved by suppressor transfer RNAs (tRNAs), factors that decrease translation-termination efficiency, such as small-interfering RNAs (siRNAs) directed against the translation-termination factors, and RNA antisense that targets the nonsense mutation region. Another extensively studied approach that has reached clinical trials is readthrough by drugs affecting the ribosome decoding site, such as aminoglycoside antibiotics [2–7]. However, aminoglycosides have severe side effects when used at high concentrations and/or used long-term. More recently, a high-throughput screening revealed a new small molecule, PTC124, which can readthrough PTCs without severe side effects.

Many readthrough studies using various aminoglycosides and PTC124 have been performed in the past decade, mainly in cystic fibrosis (CF) and Duchenne muscular dystrophy (DMD). Interestingly, these studies revealed a wide variability in the response to these readthrough treatments. Here, we focus on the molecular basis for this variability. Furthermore, the contribution of different factors to successful readthrough is reviewed and re-evaluated and the importance of the nonsense-mediated mRNA decay (NMD) pathway in regulating the response to readthrough treatments is discussed.

Factors affecting the response to readthrough by aminoglycosides

It has long been known that the antimicrobial activity of aminoglycosides results from their ability to inhibit bacterial protein synthesis in high doses. In low doses, however, some of these antibiotics cause translational misreading in prokaryotes and eukaryotes by binding to the decoding site of the 16S or 18S ribosomal RNA (rRNA), respectively [8–11] (Figure 1). This binding induces a local conformational change that mimics the change in the 16S or 18S rRNA induced by a correct codon–anticodon pair during translation [12]. In prokaryotes, the binding of aminoglycosides to ribosomes is efficient and, thus, leads to inhibition of protein synthesis. However, in eukaryotes, the binding is less efficient, owing to subtle differences in the sequence of the rRNA decoding site, which results in translational readthrough by the insertion of an amino acid at the stop codon [13–19]. Readthrough of in-frame PTCs enables the protein synthesis to continue to the end of the transcript, thus, generating a full-length protein with either the correct or an abnormal amino acid (bound to a tRNA that is a near-cognate of the stop codon) at the PTC. Because the binding of aminoglycosides is inefficient, both full-length and truncated proteins will be synthesized. Aminoglycosides have minimal effects on normal translation termination, because the normal stop codons of eukaryote genes are surrounded by upstream and downstream sequences, which enhance the efficiency of translation termination, whereas nonsense mutations are usually not surrounded by these sequences [20–23]. The proximity of normal stop codons to the poly(A) tail also contributes to the efficiency of translation termination [24]. All these factors have led to the hypothesis that aminoglycosides might be beneficial for treatment of diseases caused by PTCs, in which low levels of physiologically functional proteins are sufficient to restore their function.

The potential of aminoglycosides to readthrough disease-causing PTCs was first investigated >10 years ago in CF. Briefly, Howard *et al.* [25] and Bedwell *et al.* [26] used a

Corresponding author: Kerem, B. (kerem@cc.huji.ac.il).

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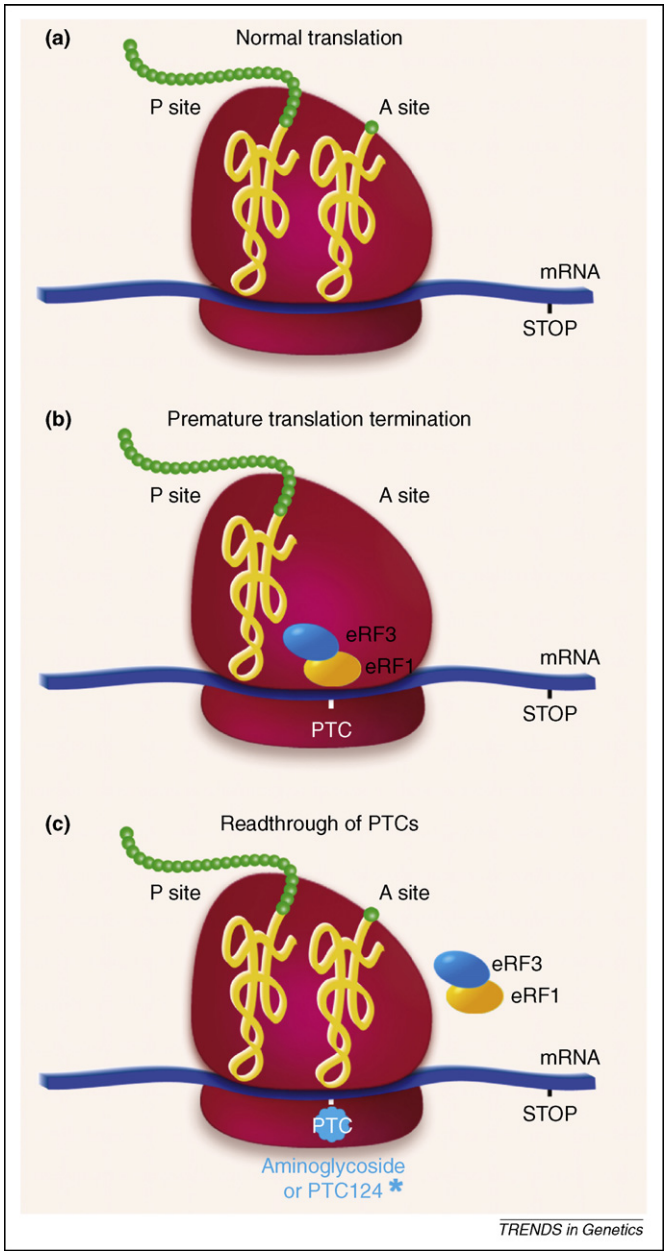

Figure 1. The effect of readthrough drugs on protein translation. **(a)** Normal protein synthesis. In the A site of the rRNA, recognition occurs between the codon of the mRNA and the anticodon of the amino-acyl-tRNA and, in the P site, the peptidyl tRNA is bound. During translation, the nascent polypeptide binds to the amino acid in the A site and the ribosome moves along the mRNA three nucleotides at a time, transferring the tRNA polypeptide from the A site to the P site. **(b)** Premature termination of protein synthesis owing to a PTC. When the ribosome encounters a PTC, there is no corresponding tRNA and the translation is stalled. This leads to the binding of the release factors (eRF1 and eRF3), resulting in translation termination and release of the polypeptide. At this stage, NMD might be elicited (Box 1, Figure I). **(c)** Readthrough of PTC by aminoglycosides or PTC124. The aminoglycosides can bind the A site of the rRNA. This binding alters the RNA conformation and the accuracy between codon–anticodon pairing is reduced. The reduced accuracy occasionally enables readthrough of the PTC by incorporation of an amino acid, generating full-length proteins. The specific binding location of PTC124 is not known but probably occurs at a different location on the ribosome than gentamicin (*).

cell line transfected with CF-transmembrane-conductance regulator (*CFTR*) constructs carrying different nonsense mutations (Table 1). Full-length CFTR proteins were detected after treatment with the aminoglycoside G418. Moreover, some of the readthrough experiments, using the R1162X, G542X and R553X constructs, led to CFTR-mediated anion efflux comparable to that found when a wild-type CFTR construct was transfected. These 'proof of principle' studies were followed by additional studies in cells of CF patients carrying nonsense mutations (Table 1). Treatment with either G418 or gentamicin resulted in stimulation of cyclic adenosine monophosphate (cAMP)-dependent chloride currents and localization of CFTR proteins on the apical cell surface that could not be seen in cells carrying mutations of non-stop codons [26–28].

Although the potential of aminoglycosides to readthrough disease-causing PTCs was demonstrated, variability in the response was found in many studies of different inherited diseases (Tables 1–3). For example, in three different clinical trials on CF patients carrying PTCs, response to gentamicin treatment was observed in most of the patients (7 out of 9 [29]; 17 out of 19 [30]; 4 out of 5 [27]), whereas in another study, no response was observed in 11 CF patients treated with gentamicin or tobramycin [31]. Variable response was also observed among studies in *mdx* mice (a model for DMD) carrying a PTC in the dystrophin (*DMD*) gene. 300 $\mu g\ ml^{-1}$ gentamicin treatment for 14 days in primary muscle cells from *mdx* mice led to the synthesis of full-length dystrophin protein and proper localization in these cells. This full-length protein was completely missing in muscle cells of *mdx* mice with no treatment or with lower gentamicin concentrations (100 $\mu g\ ml^{-1}$ and 200 $\mu g\ ml^{-1}$). When higher gentamicin concentrations were used, the size and number of the muscle cells diminished (400 $\mu g\ ml^{-1}$) or did not develop (500 $\mu g\ ml^{-1}$), thereby indicating an interference in translation [32]. Gentamicin treatment in the *mdx* mice also resulted in the synthesis and localization of dystrophin in muscles [32] and correction of the structural and functional vascular defects [33], leading to functional restoration. In contrast to these two studies, Dunant *et al.* [34] reported that gentamicin treatment in *mdx* mice in the same concentrations, for the same time and using the same method of application, failed to increase the dystrophin expression in their muscles. Variability in response was also found among gentamicin clinical trials in DMD patients. In the first study, two DMD (severe disease form) and two Becker muscular dystrophy (BMD; a milder disease form) patients were treated with intravenous gentamicin for 14 days. After treatment, no full-length dystrophin was observed in the muscles of these patients [35]. However, in a more recent study, in which four DMD patients carrying PTCs were treated with intravenous gentamicin for two six-day cycles at an interval of seven weeks, muscle biopsy revealed restoration of dystrophin expression in three of the patients [36].

Several factors were shown to affect the response to readthrough treatment. In some studies, the variable response was found to associate with the identity of the PTC and its sequence context. The readthrough efficiency inversely correlated with the translation-termination efficiency. The highest readthrough efficiency is of the UGA stop codon, followed by UAG and, to a lesser extent, UAA [37,38]. For example, variable response to gentamicin treatment, which was found among four DMD patients carrying PTCs, was associated with the sequence of the PTC. A response was found only in the patients with UGA

Table 1. Readthrough studies in tissue cultured cells and in mice models in CF and DMD[a]

Disease	Gene	Mutation	Stop codon	Readthrough drug	System	Response	Refs
CF	*CFTR*	G542X R553X	UGA G UGA C	G418[d]	cDNA overexpression in a cell line	Yes	[25]
CF	*CFTR*	W1282X R1162X	UGA A UGA G	G418	cDNA overexpression in a cell line	Yes	[26]
		W1282X	UGA A	G418, Gentamicin	A cell line from a patient carrying the mutation	Yes	
CF	*CFTR*	G542X	UGA G	Gentamicin	Primary nasal cells from a patient carrying the mutation	Yes	[27]
CF	*CFTR*	G542X	UGA G	Gentamicin	Primary bile duct cells from a patient carrying the mutation	Yes	[28]
CF	*CFTR*	Y122X W1282X R1162X G542X	UAA C UGA A UGA G UGA G	Gentamicin	Overexpression of a dual reporter vector in a cell line	Yes Y122X > W1282X, R1162X, G542X	[45]
CF	*CFTR*	W1282X R1162X	UGA A UGA G	G418	cDNA stable expression in cell lines	Yes	[67]
CF	*CFTR*	G542X	UGA G	Gentamicin, Tobramycin	*In vitro* transcription–translation and a human transgene in a *Cftr*$^{-/-}$ mouse	Yes, Gentamicin > Tobramycin	[42]
CF	*CFTR*	G542X	UGA G	Amikacin, Gentamicin	*In vitro* translation	Yes, Gentamicin > Amikacin	[43]
		G542X	UGA G	Amikacin, Gentamicin	A human transgene in a *Cftr*$^{-/-}$ mouse	Yes, Amikacin > Gentamicin	
CF	*CFTR*	G542X	UGA G	PTC124, Gentamicin	A human transgene in a *Cftr*$^{-/-}$ mouse	Yes	[53]
DMD	*DMD*	mdx (3185C→T)	UAA A	Gentamicin	Primary skeletal muscle cells from *mdx* mice	Yes	[32]
		mdx (3185C→T)	UAA A	Gentamicin	*mdx* mice	Yes, variability among mice	
DMD	*DMD*	–[b]	UGA N[c] UAG N[c] UAA N[c]	G418, Gentamicin, Paromomycin	Overexpression of a dual luciferase reporter vector in cell lines	Yes 1. UGA > UAG > UAA N = C > U > A ≥ G 2. G418 > Gentamicin, Paromomycin	[38]
DMD and BMD	*DMD*	9G→A 4240C→T 4250T→A 4693C→T 6868A→T 7720C→T 8608C→T 9337C→T	UGA U UAA U UAG A UAG U UAA C UAG U UGA A UGA A	Gentamicin, Amikacin, Paromomycin	Overexpression of a dual luciferase reporter vector in cell lines	Yes 1. UGA > UAG > UAA +4 C > U > G ≥ A 2. Gentamicin, Paromomycin > Amikacin	[40]
		All the above	All the above	Tobramycin	Overexpression of a dual luciferase reporter vector in cell lines	No	
		mdx –[b]	UAA A UGA C	Gentamicin	Overexpression of a dual luciferase reporter vector in cell lines	Yes, but very small Yes	
DMD and congenital muscular dystrophy (CMD)	*DMD*	E1593X Q60X Q988X mdx Q1240X Q1143X E2726X Q1437X Q2125X Q3149X W651X L1417X Q2522X E931X Q2264X Q673X R1326X	UAA U UAA A UAA C UAA A UAA U UAA C UAA G UAG A UAG U UAG A UAG G UAG A UAG A UAG C UAG G UAG C UGA G	Gentamicin	Overexpression of a dual luciferase reporter vector in a cell line	Yes UGA > UAG > UAA	[39]

Table 1 (*Continued*)

Disease	Gene	Mutation	Stop codon	Readthrough drug	System	Response	Refs
		C967X	UGA A				
		R3085X	UGA G				
		R744X	UGA G				
		R3190X	UGA A				
		R1549X	UGA C				
		R1967X	UGA A				
		R3381X	UGA A				
		R2098X	UGA U				
		R145X	UGA C				
		S319X	UGA C				
		S319X	UGA C	Gentamicin	Overexpression of a dual luciferase reporter vector in mice	Yes	
DMD	*DMD*	mdx	UAA A	Gentamicin	*mdx* mice	Yes	[33]
DMD	*DMD*	mdx	UAA A	Gentamicin	*mdx* mice	No	[34]
DMD	*DMD*	A PTC in exon 28	UGA	PTC124	Primary muscle cells from a patient carrying the mutation	Yes	[51]
		mdx	UAA	PTC124	*mdx* mice	Yes	
CMD	*LAMA2*	C1546X	UGA A	Gentamicin, Amikacin	Overexpression of a dual luciferase reporter vector in a cell line	Yes 1. The stop codon and flanking sequences	[68]
		1326c	UGA				
		1437c	UAG				
		1240c	UAA				
DMD	*DMD*	mdx	UAA			2. Gentamicin > Amikacin	
		319d	UGA				
CMD	*LAMA2*	C1546X	UGA A	Gentamicin, Amikacin	Primary muscle cells from a patient carrying the mutation	No	

[a]The table includes only results from disease-causing PTCs. Owing to possible polymorphisms, the fourth nucleotide that immediately follows the stop codon is specified only in cases in which it was specified in the study. Explanations for variable response, if given in the study are mentioned in the 'Response' column.
[b]Not specified in the study.
[c]N was either A, C, G or U.
[d]G418 = geneticin.

as the PTC [36]. The sequences upstream and downstream of the stop codon also have an important role in determining its susceptibility to readthrough. In particular, the importance of the fourth nucleotide immediately after the stop codon (position +4) was shown. A cytosine (C) residue after either UGA or UAA is correlated with high levels of readthrough [37,38]. A more recent study showed that the impact of the +4 nucleotide is largely affected by the surrounding context [39]. A C at this position is associated with some of the highest readthrough levels but, also, with very moderate ones depending on the flanking sequences.

Another factor that can affect the response to aminoglycoside treatment is their chemical composition. Comparison of readthrough efficiency that is induced by several aminoglycosides in human cells expressing reporter constructs showed lower readthrough efficiency by tobramycin and neomycin than by gentamicin, amikacin and paromomycin [2,40,41]. Studies in a *Cftr*$^{-/-}$ mouse, stably expressing the human CFTR–G542X transgene (Table 1) found that, after treatment for seven days using a high gentamicin dose (34 $\mu g\, g^{-1}$ body weight), human CFTR proteins were detected at the apical surface of intestinal tissues and a modest increase in the *in vitro* chloride current through the CFTR channel was observed. However, after tobramycin treatment at the same conditions, a weaker CFTR staining and a half magnitude of chloride current was observed [42]. Later, high dose of another aminoglycoside, amikacin (170 $\mu g\, g^{-1}$ body weight), was found to readthrough the human CFTR–G542X more effectively than gentamicin in the same transgenic mice, as measured by CFTR-protein staining and chloride efflux. The same difference between amikacin versus gentamicin readthrough efficiency was observed when lower doses of both aminoglycosides were used [43]. It is important to note that studies in patients used gentamicin (and not amikacin) because the effect of amikacin has only recently been reported.

The brand and composition of gentamicin can also contribute to different readthrough efficiencies. Commercially available gentamicin usually contains three major components: gentamicin C1, C1a and C2, which have only slight differences in their chemical structure but differ in their propensity to induce readthrough. Gentamicin C1a and C2 bind to the decoding site of the rRNA with similar affinities, which are higher than that of gentamicin C1 [44]. Hence, the variable response found among different studies in CF patients [27,29–31,45], DMD mice models [32–34] and DMD patients [35,36] (Tables 1,3) might be attributed to different brands, which might contain different relative concentration of each gentamicin component.

The duration of the readthrough treatment between studies, or a different method of application, could also contribute to the variable response as well as the variability in drug metabolism between individuals.

However, these factors cannot explain the variability in response among patients who participated in the same clinical study and carried the same PTC. For example, intravenous gentamicin was recently shown to readthrough the Y122X mutation, one of the most frequent nonsense mutations in the French population, in six out of

Table 2. Readthrough studies in tissue cultured cells and in mice models in other diseases[a,b]

Disease	Gene	Mutation	Stop codon	Readthrough drug	System	Response	Refs
Hurler syndrome (mucopolysaccharidosis Type I)	*IDUA*	Q70X W402X	UAG C UAG G	Gentamicin	*In vitro* transcription–translation and primary fibroblasts from a patient carrying the mutations	Yes	[69]
Hurler syndrome (mucopolysaccharidosis Type I)	*IDUA*	W402X	UAG G	Gentamicin, Tobramycin, Amikacin	*In vitro* transcription–translation	Yes Amikacin, Gentamicin > Tobramycin	[70]
Mucopolysaccharidosis Type I	*IDUA*	Q70X W180X Q400X W402X R628X	UAG C UGA A UAG C UAG G UGA C	Gentamicin	Fibroblast extracts from patients carrying the mutations	Yes The stop codon and flanking sequences	[71]
		Y343X	UAG G	Gentamicin	Fibroblast extracts from patients carrying the mutations	No	
		Q70X W402X 	UAA UAG UGA UAA UAG UGA	Gentamicin	cDNA overexpression in a cell line	Yes For both mutations the smallest readthrough was with UAG	
Spinal muscular atrophy (SMA) type II and III	*SMN1*	W102X	UAG T	G418[d]	Primary fibroblasts and lymphoblastoid cells	Yes	[72]
SMA type I	*SMN2*	SMNΔ7	A PTC four amino acids into exon 8	Amikacin, Gentamicin, G418, Lividomycin, Streptomycin, Tobramycin	Fibroblasts from a patient carrying the mutation	Yes Amikacin, Tobramycin > Gentamicin, G418, Lividomycin, Streptomycin	[73]
X-linked nephrogenic diabetes insipidus (XNDI)	*AVPR2*	W200X R337X	UGA UGA	G418	cDNA overexpression in a cell line	Yes	[74]
XNDI	*AVPR2*	E242X	UAG C	G418, Gentamicin, Paromomycin	Mouse cDNA overexpression in cell lines	Yes G418 > Gentamicin, Paromomycin	[75]
		E242X	UAG C	Amikacin, Hygromycin B, Kanamycin A, Neomycin, Streptomycin, Tobramycin	Mouse cDNA overexpression in cell lines	No	
		E242X	UAG C	G418	Primary kidney cells from a mouse model carrying the mutation and in mutant mice	Yes	
X-linked retinitis pigmentosa	*RP2*	R120X	UGA G	Gentamicin	Lymphoblastoid cell lines from patients carrying the mutation	No	[76]
		R120X –[c]	UGA G UGA C	Gentamicin	Overexpression of a dual luciferase reporter vector in a cell line	Yes +4 C > G	
Nephropathic cystinosis	*CTNS*	W138X	UGA	Gentamicin	Fibroblast cell lines from patients carrying the mutations	Yes	[77]
		73X 65-kb deletion	–[c] –[c]	Gentamicin	Fibroblast cell lines from patients carrying the mutations	No	

Ataxia-telangiectsia	*ATM*	AT185LA TAT51 AT187LA AT153LA	UAA G UGA C UAA G UGA A	G418, Gentamicin, Paromomycin	*In vitro* protein truncation testing	Yes 1. UGA C, UGA A > UAA G 2. G418 > Gentamicin, Paromomycin	[78]
		All the above	All the above	Tobramycin	*In vitro* protein truncation testing	No	
		AT185LA AT160LA AT187LA	UAA G UGA A UAA G	G418, Gentamicin	Lymphoblastoid cell lines from patients carrying the mutations	Yes G418 > Gentamicin	
Polycystic kidney disease	*PKD2*	R186X Y386X	UAA UAA	Gentamicin, Isepamicin	Lymphoblastoid cell lines from patients carrying the mutations	Yes	[79]
Factor VII deficiency	*FVII*	K316X W364X	UAG G UGA G	G418, Gentamicin	cDNA overexpression in a cell line	Yes	[80]
		K316X W364X	UAG G UGA G	Netilmicin	cDNA overexpression in a cell line	No	
Familial atrial fibrillation	*KCNA5*	E375X	UAA C	Gentamicin	cDNA overexpression in a cell line	Yes	[81]
Hemophilia B	*F9*	R338X	UGA T	G418, Gentamicin	Human minigenes in a *F9*$^{-/-}$ mouse	Yes 1. G418 > Gentamicin 2. Variability among mice	[82]
		R29X	UGA G	G418, Gentamicin	Human minigenes in a *F9*$^{-/-}$ mouse	No	
McArdle disease	*Myophosphorylase*	R49X	UGA G	Gentamicin	Primary muscle cells from a patient carrying the mutation	No	[83]
Usher syndrome type 1	*PCDH15*	R3X R245X R643X R929X	UGA C UGA A UGA G UGA A	G418, Gentamicin, Paromomycin	*In vitro* transcription–translation	Yes 1. The stop codon and flanking sequences 2. G418 > Gentamicin, Paromomycin	[84,85]
		R245X	UGA A	G418, Gentamicin, Paromomycin	Partial cDNA overexpression in a cell line	Yes G418 > Gentamicin > Paromomycin	
		R3X	UGA C	Gentamicin, Paromomycin	Overexpression of a dual luciferase reporter vector in a cell line	Yes	

[a]Diseases other than CF and DMD.
[b]The table includes only results from disease-causing PTCs. Owing to possible polymorphisms, the fourth nucleotide that immediately follows the stop codon is specified only in cases in which it was specified in the study. Explanations for variable response, if given in the study, are mentioned in the 'Response' column.
[c]Not specified in the study.
[d]G418 = geneticin.

Table 3. Readthrough studies in patients[a]

Disease	Gene	Mutation	Stop codon	Readthrough drug	Method of application	Responders	Refs
CF	*CFTR*	W1282X	UGA A	Gentamicin	Intranasal	7 out of 9	[29]
		G542X	UGA G				
		3849+10 kb C→T	UAA U				
CF	*CFTR*	W1282X	UGA A	Gentamicin	Intravenous	4 out of 5	[27]
		G542X	UGA G				
		R553X	UGA C				
CF	*CFTR*	W1282X	UGA A	Gentamicin	Intranasal	17 out of 19	[30,47]
		G542X	UGA G			Variability was	
		3849+10 kb C→T	UAA U			explained by the	
						transcript levels	
CF	*CFTR*	Y122X	UAA C	Gentamicin	Intravenous	6 out of 9	[45]
		W1282X	UGA A	Gentamicin	Intravenous	0 out of 4	
		R1162X	UGA G				
		G542X	UGA G				
		R553X	UGA C				
CF	*CFTR*	W1282X	UGA A	Gentamicin,	Intranasal	0 out of 11	[31]
		G542X	UGA G	Tobramycin			
		R553X	UGA C				
		R1162X	UGA G				
		Y1092X	UAA C				
		E60X	UAG C				
CF	*CFTR*	W1282X	UGA A	PTC124	Oral	17 out of 23 Variability	[52]
		G542X	UGA G			was explained by	
		3849+10 kb C→T	UAA U			the transcript levels	
DMD	*DMD*	Q625X	UAA G	Gentamicin	Intravenous	0 out of 2	[35]
		Q2198X	UAG C				
BMD	*DMD*	S757X	UGA G	Gentamicin	Intravenous	0 out of 2	
		W3294X	UGA C				
DMD	*DMD*	_[b]	UGA	Gentamicin	Intravenous	3 out of 3	[36]
		_[b]	UAA			0 out of 1	
Hemophilia A	*FVIII*	S1395X	_[b]	Gentamicin	Intravenous	1 out of 1	[86]
		R2116X	_[b]			0 out of 1	
		R427X	_[b]			0 out of 1	
Hemophilia B	*FIX*	R333X	_[b]	Gentamicin	Intravenous	1 out of 1	
		R252X	_[b]			0 out of 1	
Factor VII deficiency	*FVII*	K316X	UAG G	Gentamicin	Intravenous	0 out of 2	[87]
		W364X	UGA G			Minimal	
						sub-therapeutic effects	
Hailey–Hailey disease	*ATP2C1*	R468X	UGA	Gentamicin	Topical	1 out of 1	[88]
McArdle disease	*Myophosphorylase*	R49X	UGA G	Gentamicin	Intravenous	0 out of 4	[83]
		R269X	UGA G				

[a]Owing to possible polymorphisms, the fourth nucleotide that immediately follows the stop codon is specified only in cases in which it was specified in the study. Explanations for variable response, if given in the study, are mentioned in the 'Responders' column.
[b]Not specified in the study.

nine CF patients [45]. After gentamicin treatment, the CTFR protein was detected at the membrane of the nasal epithelial cells and the CFTR-dependent chloride secretion reached normal values in the responders. A variable response to gentamicin intranasal treatment was also observed among nine CF patients all carrying at least one W1282X nonsense mutation, the most frequent mutation in the Ashkenazi Jewish population [29]. This mutation generates the stop codon UGA, followed by an adenine (A) and, owing to a founder effect, the mutation resides on the same haplotype in all patients [46]. Specifically, in 80% of the patients, a correction of the CFTR electrophysiological abnormalities was observed. Variability in response was also found in another study among 19 CF patients, all carrying at least one allele with the W1282X mutation [30]. After the gentamicin treatment, there was a complete normalization of the electrophysiological abnormalities caused by the CFTR defect in 17 out of 19 patients. In addition, a significant peripheral and surface staining of full-length CFTR proteins was observed in the nasal epithelial cells of responders. This staining pattern was similar to that observed in non-CF individuals. The variability found in each of these studies indicates that another factor might contribute to the response to gentamicin treatment. Given that the PTC-bearing transcripts are the target for readthrough, their level might vary among individuals and be a limiting factor in the response. This hypothesis was recently tested and the results clearly showed no response to gentamicin in patients with markedly reduced levels, whereas response was only found in patients with higher levels of CFTR nonsense transcripts [47]. Because the level of PTC-bearing transcripts was not investigated in other readthrough studies (such as in Sermet-Gaudelus *et al.* [45]), the possibility that variable levels of PTC-bearing transcripts contributed to the reported variability in the response of patients cannot be excluded. Altogether, these results indicate that analyzing the level of mRNA before the readthrough treatment might be beneficial to the identification of patients with a potential to respond to the treatment.

Aminoglycosides have been used in tissue-culture cells and mouse models for a variety of diseases, among

Box 1. Nonsense-mediated mRNA decay

NMD is a quality-control process found in all eukaryotic organisms studied to date [89–92]. It degrades transcripts carrying disease-causing PTCs and a variety of physiologic transcripts, among which are transcripts with upstream open-reading frame (uORF), transcripts containing introns in the 3′ untranslated region (UTR) and transcripts derived from alternative splicing [93–96]. The NMD physiologic substrates function in a broad range of cellular processes including transcription, telomere maintenance, DNA repair, cell growth, intracellular transport and NMD itself [93,97–100]. Thus, NMD has an important role in regulating many cellular functions. NMD usually reduces the level of PTC-bearing transcripts but does not eliminate them completely. Hence, reduced levels of the encoded truncated proteins are observed.

NMD occurs in mammalian cells after pre-mRNA splicing and, in most cases, is mediated by the exon-junction complex (EJC), which comprises at least ten proteins that are deposited 20–24 nucleotides upstream of exon–exon junctions [101,102]. This complex is usually displaced during the pioneer round of translation. According to current NMD models (Figure I), for most mRNAs, stop codons that are located >50–55 nucleotides upstream of an exon–exon junction are recognized as premature during the pioneer round of translation, because EJC is found downstream to the stop codon [103]. Translation termination is triggered by recognition of the stop codon by the eukaryotic release factors eRF1 and eRF3, which are recruited to stalled ribosomes. These release factors recruit the key NMD factor, UPF1, which recruits the kinase SMG1. These four proteins generate on the stalled ribosome at stop codons a complex known as SURF. In the case of a PTC, there is a downstream EJC and UPF1 at the termination site can interact with the EJC-associated protein, UPF2. This interaction leads to phosphorylation of UPF1 by SMG1 and dissociation of the release factors. The phosphorylated UPF1 recruits additional factors (SMG5 and SMG7), leading to NMD triggering. Once NMD is stimulated, the targeted transcripts are degraded by both 5′–3′ exoribonucleases such as hXRN1 and by 3′–5′ decay involving de-adenylation and the exosome [104–106].

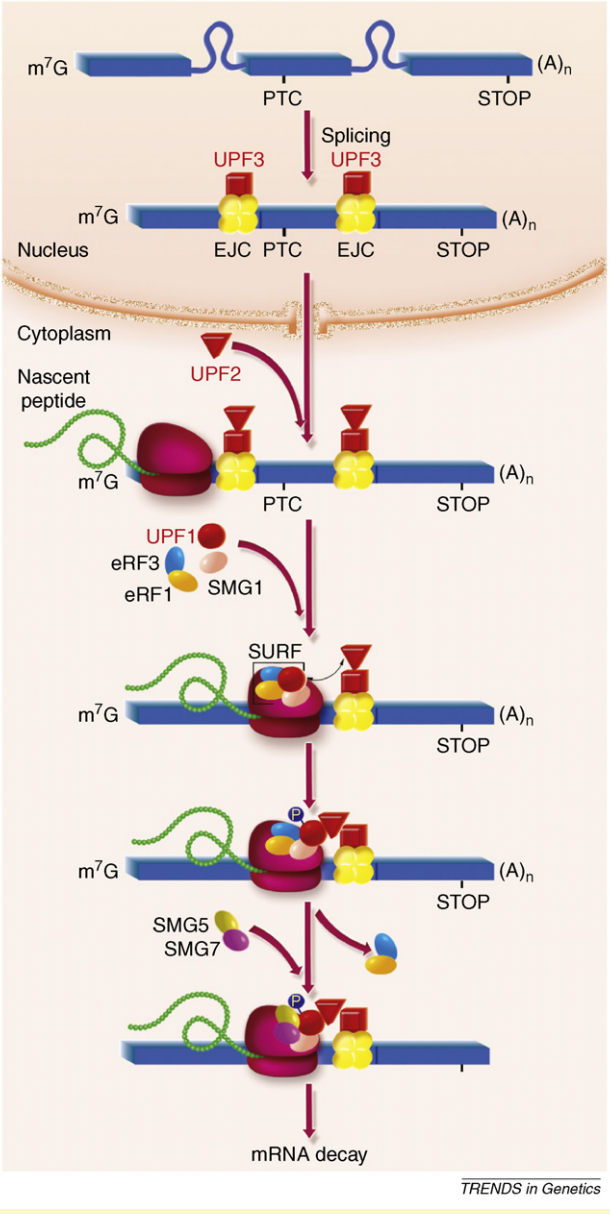

Figure I. The mammalian NMD pathway. After pre-mRNA splicing in the nucleus, transcripts with EJC that includes the NMD core protein UPF3 are exported to the cytoplasm. In the cytoplasm, the NMD core protein UPF2 binds to UPF3. Ribosomes start to translate the mRNA until reaching a PTC. At these stalled ribosomes, four proteins (SMG1, UPF1, eRF1 and eRF3) generate a complex known as SURF. In cases in which there is a downstream EJC, UPF1 (at the termination site) can interact with the EJC-associated protein, UPF2, thereby leading to phosphorylation of UPF1 by SMG1 and dissociation of the release factors. The phosphorylated UPF1 recruits SMG5 and SMG7 factors, eliciting NMD. Color code: exons, blue rectangles; introns, blue loops.

which are spinal muscular atrophy and usher syndrome (summarized in Table 2). However, very few of these studies have led to clinical trials and, in those that did (e.g. hemophilia A and B), the numbers of patients included was very small (n ≤4) and had different nonsense mutations (Table 3). All these studies showed that aminoglycosides can promote readthrough of disease-causing PTCs; however, their efficiency considerably varied.

In summary, among the tested aminoglycosides, gentamicin was the most promising drug that showed both *in vitro* and *in vivo* efficient readthrough of PTCs that cause many genetic diseases. However, the clinical benefit of gentamicin is limited because high concentrations and/or long-term treatments require intravenous administration and can cause severe side effects such as kidney damage and hearing loss [48,49].

PTC124 – a new readthrough drug

High-throughput screens identified PTC124, a small organic molecule with no antibiotic properties, that can promote readthrough of disease-causing PTCs and does not affect termination at stop codons located at the end of coding sequences [50,51] (Figure 1). Unlike aminoglycosides, PTC124 has no serious adverse side effects [50,52] and, therefore, has great potential to treat genetic disease. PTC124 probably functions at a different location on the ribosome than aminoglycosides because it is part of a distinct structural class of drugs [50].

PTC124 promoted dose-dependent readthrough of all three stop codons in cell lines expressing luciferase nonsense alleles in low concentrations (0.01–10 μM), whereas 1000 μM gentamicin did not show readthrough of the three stop codons [51]. Moreover, PTC124 oral administration to *mdx* mice led to the production of full-length dystrophin in

the membrane of primary muscle cells and partially rescued the functional strength deficit [51]. The potential of PTC124 to restore *in vivo* the functional activity of PTC-bearing transcripts was also analyzed in the CF mouse model expressing the human CFTR–G542X transgene in a *Cftr*$^{-/-}$ background [53]. The results showed that both subcutaneous and oral administration of PTC124 to these mice led to a strong expression of full-length CFTR proteins and to 24–29% of the average cAMP-stimulated transepithelial chloride currents observed in wild-type mice (Table 1).

Recently, PTC124 clinical trials have been initiated for CF patients (Table 3). 23 adult CF patients carrying different nonsense mutations were orally treated with low and high doses of PTC124 for two weeks each [52]. Improvement in the electrophysiological abnormalities caused by CFTR dysfunction was found in many patients, whereas some of them showed end-of-treatment values in the normal range. This indicated that PTC124 can induce *in vivo* synthesis of full-length functional CFTR proteins. Furthermore, improvements in lung function, CF-related neutrophilia and body weight were observed in these patients. As in the gentamicin clinical trial [30], most of the patients in this PTC124 clinical trial [52] carried the W1282X mutation and, thus, shared the same sequence context; the readthrough compound was of the same batch and the method of application and treatment period was the same for all patients. Nevertheless, variable response to PTC124 was observed, such that a response was found only in patients with sufficient levels of CFTR nonsense transcripts [52]. Interestingly, different PTCs demonstrated a different threshold of the level of PTC-bearing transcripts required for response. For transcripts carrying the W1282X mutation, $\geq$20% of wild type was required for normalization of the electrophysiological abnormalities, whereas for those carrying the G542X mutation very low levels (<10%) of wild type were sufficient for the response. This is probably a result of different readthrough efficiencies for the two PTCs. Indeed, PTC124 showed that readthrough of UGA–G (the sequence of G542X) is more efficient than that of UGA–A (the sequence of W1282X) [51].

There are currently several ongoing PTC124 clinical trials for CF patients in which the treatment period has been extended to three months (phase IIa) and, in another trial, pediatric patients (6–18 years old) were studied. Although the results of these studies are not yet published, the preliminary results are promising (http://www.ptcbio.com). A clinical trial in DMD patients using PTC124 is currently ongoing and the results are being evaluated (http://www.ptcbio.com).

NMD efficiency can modify the response to readthrough

Several mechanisms can regulate the level of transcripts including transcription, pre-mRNA processing and mRNA degradation. One post-transcriptional mechanism that specifically regulates the level of PTC-bearing transcripts is NMD (Box 1 and Figure I). It detects and degrades such transcripts to prevent the synthesis of truncated proteins that might be nonfunctional or deleterious owing to dominant-negative or gain-of-function effects. Because transcripts carrying PTCs are the template for readthrough treatment, it was hypothesized that NMD might regulate the readthrough response. Indeed, inhibition of NMD by siRNA directed against its key factors UPF1 (regulator of nonsense transcripts homolog [yeast] 1) or UPF2, led to an improvement in the CFTR chloride efflux in response to gentamicin in all tested concentrations (50 $\mu g\,ml^{-1}$, 100 $\mu g\,ml^{-1}$ and 200 $\mu g\,ml^{-1}$) [47]. In one cell line (IB3–1) carrying the W1282X mutation, a response to gentamicin was found only after NMD inhibition, together with 100 $\mu g\,ml^{-1}$ or 200 $\mu g\,ml^{-1}$ gentamicin. In another cell line, also carrying the W1282X mutation (CFP15a), a concentration-dependent CFTR activation was observed already after gentamicin treatment. Downregulation of UPF1 or UPF2 increased the level of CFTR nonsense transcripts and led to a higher CFTR chloride efflux than that found after gentamicin treatment alone, in all concentrations. After UPF1 downregulation, together with 200 $\mu g\,ml^{-1}$ gentamicin, the CFTR function was similar to that found in human epithelial cells that carry normal CFTR alleles (T84). Furthermore, inhibition of NMD enabled a treatment with lower gentamicin concentrations to achieve comparable or even higher CFTR chloride efflux than that achieved with higher gentamicin concentrations [47]. For example, downregulation of UPF1 in CFP15a cells treated with 50 $\mu g\,ml^{-1}$ gentamicin resulted in chloride efflux comparable to that achieved with 100 $\mu g\,ml^{-1}$ gentamicin alone. Additionally, downregulation of UPF1 in cells treated with 100 $\mu g\,ml^{-1}$ resulted in higher activation compared with that achieved with 200 $\mu g\,ml^{-1}$ gentamicin alone. Altogether, therapeutic approaches aiming to specifically increase the level of transcripts carrying a disease-causing PTC are expected to enable a response in patients with markedly reduced levels of PTC-bearing transcripts. This might be achieved by developing an antisense oligonucleotide directed against a region in the PTC-bearing transcripts that will perturb the ability of NMD factors to bind and degrade these transcripts. This should be designed specifically to the targeted PTC-bearing transcripts; otherwise, many other transcripts might be affected, leading to changes in many cellular processes.

In several studies, variability in NMD efficiency was attributed to tissue-specific differences. For example, indirect inhibition of NMD by cycloheximide resulted in a different increase in the level of mRNA transcribed from *TCR-β* (β subunit of T-cell antigen receptor) constructs in HeLa and T cells carrying the same PTC [54]. An additional study found variability in NMD efficiency for collagen type X α-1 (*COL10A1*) transcripts carrying a PTC among cells derived from different tissues of a Schmid metaphyseal chondrodysplasia patient [55]. Similarly, variable NMD efficiency of *ESCO2* (establishment of cohesion 1 homolog 2) transcripts carrying a PTC was found between cells derived from different tissues of two fetuses with Roberts syndrome [56]. Recently, NMD efficiency was shown to vary even among cells derived from the same tissue [47]. This variability was found for transcripts carrying disease-causing PTCs in different genes such as the W1282X *CFTR* and NS39 *β-globin*, and for different NMD physiologic substrates such as *RPL3*, *SC35 1.6 kb*, *SC35 1.7 kb*, *ASNS* and *CARS* [47,57]. There were cells (CFP22a, CFP15a and HeLa) in which NMD was highly

efficient for all analyzed transcripts and cells (CFP15b and MCF7) in which NMD was less efficient, thereby indicating that the NMD efficiency is a general phenomenon and an inherent character of cells [47,57]. Importantly, such variability in NMD efficiency might lead to variable levels of nonsense transcripts available for readthrough. When NMD is efficient, the level of nonsense transcripts is markedly reduced such that there are not enough nonsense transcripts to make sufficient proteins, even if a readthrough drug is provided. By contrast, when NMD is less efficient, the level of nonsense transcripts is higher and is expected to enable a response.

There are >30 known proteins participating in NMD and there might be additional, as yet unknown, NMD factors and/or regulators. These factors might lead to variable NMD efficiency owing to sequence differences that affect the level and function of mRNAs and/or proteins. Recently, Viegas *et al.* [58] searched for the molecular basis of variable NMD efficiency among three different HeLa strains. The NMD was less efficient in strains B and C than in strain A. The results showed different levels of one of the NMD factors, RNA-binding protein S1 (RNPS1), which is part of the exon–junction complex (EJC), among the strains. In strain B, the level was ~30% of that in strains A or C. Increasing the amount of RNPS1 in strain B led to higher NMD efficiency, indicating that RNPS1 might be the limiting factor in NMD efficiency in this strain [58]. Future proteomic studies can contribute to the identification of additional factors that might affect NMD efficiency. Combining a proteomic approach with readthrough treatments might shed new light on the genetic factors regulating the readthrough response.

NMD has effects on other genetic aspects such as the phenotype and mode of inheritance in patients carrying PTC-causing diseases (for a review see Refs [59,60]). In cases that the mutant protein has a residual activity associated with a mild phenotype, degradation of the PTC-bearing transcripts by NMD leads to a more severe phenotype. For example, PTCs near the 3′ end of the dystrophin gene result in a mild phenotype (BMD), thus, indicating that these truncated proteins can partially rescue the phenotype. However, PTCs in the 5′ part of the gene were associated with a severe phenotype (DMD). Although it was not directly shown, this severe phenotype is probably caused by the function of NMD [61]. In cases in which the mutant protein has a dominant-negative effect, degradation of the PTC-bearing transcripts by NMD leads to a mild phenotype. For example, PTCs in the final exon of the SRY–BOX 10 (*SOX10*) gene escape NMD and, as a result, large amounts of truncated proteins are produced. These truncated proteins have a dominant-negative effect that leads to a severe and extended neurological phenotype. However, PTCs in *SOX10* gene that trigger NMD reduce the dominant-negative expression, leading to a relatively milder phenotype [62]. Similarly, different PTCs in the myelin protein zero (*MPZ*) gene are responsible for distinct clinical phenotypes that affect the myelin of the peripheral nervous system. These neuropathies include severe early-onset congenital neuropathy, and the milder adult-onset phenotype. PTCs in the *MPZ* gene that do not elicit NMD cause the more severe phenotype, owing to dominant-negative effects, whereas PTCs that are subject to NMD cause the milder adult-onset phenotype owing to loss-of-function effects [62]. Another example is the R1014X nonsense mutation in the human ether-a-go-go-related (*hERG*) gene, which causes long QT syndrome [63]. In cases in which the mutant protein has a dominant-negative effect and the NMD is highly efficient, a disease phenotype is found only in patients carrying two defective alleles, thereby changing the mode of inheritance from dominant to recessive. PTCs in the 5′ part of the *β-globin* gene elicit NMD very efficiently, leading to a recessive β-thalassemia. PTCs in the 3′ part of the gene, which escape NMD, result in an atypical dominant form of the disease owing to a dominant-negative effect [64,65]. Myotonia congenita provides another example for the role of NMD as a modifier of the mode of inheritance. PTCs in the last exon of the chloride channel 1 (*CLCN1*) gene escape NMD and lead to Thomsen disease, which is the dominant form of the disease. However, PTCs in an upstream exon of *CLCN1* gene efficiently trigger NMD and lead to Becker disease, the recessive form of the disease [66]. Future studies should investigate the effect of NMD efficiency on disease severity and mode of inheritance among patients carrying the same PTC.

Concluding remarks

This review has discussed the molecular basis for variability in response to readthrough of disease-causing PTCs by aminoglycosides and the recently identified readthrough drug, PTC124. This includes the identity of the PTC and its sequence context, the chemical composition of the readthrough drug and the level of PTC-bearing transcripts. Several mechanisms can regulate the level of transcripts including transcription, pre-mRNA processing and mRNA degradation. Recent studies indicate a role for NMD in regulating the response to readthrough treatment. Understanding the mechanisms regulating the response is important for the development of safe and efficient treatments for patients carrying PTCs. This might be achieved by identification of functional single nucleotide polymorphisms in candidate NMD genes, comparison of the transcriptome and proteome of responders and non-responders patients and identification of functional modifications of NMD proteins, among others. This is expected to reveal inter-ethnic and individual genetic differences that will facilitate rational drug discovery and development and to distinguish 'good responders' from 'poor responders' before therapy. This should lead to a deeper understanding of the pharmacogenomics of readthrough-based therapies and to the development of readthrough 'tailor made' therapies based on the sequence profile of each individual for many human genetic diseases caused by PTCs.

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