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Deciphering the molecular mechanism of stop codon readthrough

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ABSTRACT

Recognition of the stop codon by the translation machinery is essential to terminating translation at the right position and to synthesizing a protein of the correct size. Under certain conditions, the stop codon can be recognized as a coding codon promoting translation, which then terminates at a later stop codon. This event, called stop codon readthrough, occurs either by error, due to a dedicated regulatory environment leading to generation of different protein isoforms, or through the action of a readthrough compound. This review focuses on the mechanisms of stop codon readthrough, the nucleotide and protein environments that facilitate or inhibit it, and the therapeutic interest of stop codon readthrough in the treatment of genetic diseases caused by nonsense mutations.

***Key words*: s**top codon readthrough, mechanism, translation termination, premature termination codon, readthrough activators, molecules, screening.

CONTENTS

I. INTRODUCTION

For cells to function properly, genetic information must be faithfully expressed in RNAs or proteins. A key step in the gene expression pathway is translation from messenger RNA (mRNA) to protein. Translation obeys very specific rules, such as starting at an initiation codon (very often an AUG codon) and stopping at one of the three stop codons UAA, UAG,

or UGA, in order to ensure accurate protein length. Sometimes, however, rules can be bypassed, and translation is no exception. Under specific conditions, ribosomes may ignore stop codons, continuing with translation and extending the C-terminal part of the nascent protein. The C-terminally extended protein may acquire a new functional domain in this way. The absence of termination codon recognition is called stop codon readthrough. Although this can constitute a decoding error, stop codon readthrough can also be a means of expressing different protein isoforms and may represent a therapeutic solution for some pathologies. This review discusses the different molecular factors that regulate translation termination and stop codon readthrough, mainly in higher eukaryotes. It also outlines the potential of this mechanism in terms of basic science and clinical applications to advance understanding of pathways of gene expression and in the development of therapeutic approaches for nonsense-mutation-related genetic diseases. Potential clinical applications of a stop codon readthrough strategy have recently been discussed in several reviews (Morais, Adachi & Yu, 2020; Dabrowski, Bukowy-Bieryllo & Zietkiewicz, 2018; Sharma, Keeling & Rowe, 2020; Bezzerri *et al.*, 2020), hence we focus here on the molecular mechanisms leading to stop codon readthrough.

II. TRANSLATION TERMINATION

Translation is a process that promotes amino acid polymerization leading to a peptide sequence based on an mRNA sequence. The ribosome carries out translation with the help of cofactors, ensuring accurate decoding of the open reading frame (ORF) (Dever, Kinzy & Pavitt, 2016; Merrick, 1992; Kapur, Monaghan & Ackerman, 2017). Misincorporation of an amino acid occurs at a very low rate. For example, misincorporation of a lysine instead of an arginine during translation of protamine mRNA has been estimated at only 0.06% to 0.2% (Mori *et al.*, 1985). Site A of the ribosome is where the transfer RNA (tRNA) carrying an

amino acid hybridizes with a codon *via* its anticodon sequence, thus bringing to the growing polypeptide chain the next amino acid to be incorporated (Fig. 1A). tRNAs are subject to numerous post-transcriptional modifications that stabilize their tertiary and quaternary structures and also favour codon–anticodon interaction in the ribosome by changing the polarity of the modified base and allowing additional interactions with the ribosome (Grosjean & Westhof, 2016). It is established that post-transcriptional modifications of tRNAs play a role in the fidelity of codon recognition, and their absence can lead to increased codon recognition by near-cognate tRNAs (Blanchet *et al.*, 2018).

Translation termination occurs when site A of the ribosome reaches one of three stop codons (UAA, UAG or UGA) in frame with the translation initiation codon (Fig. 1A). In human cells, the only tRNA to recognize these stop codons is $tRNA^{[Ser]Sec}$, which carries the amino acid selenocysteine and pairs with UGA stop codons in a dedicated environment such as a selenocysteine insertion sequence (SECIS) combined with the presence of SECIS binding-protein 2 (SBP2) (Labunskyy, Hatfield & Gladyshev, 2014). When the ribosome reaches a stop codon, competition occurs between the translation termination complex and near-cognate tRNAs recognizing two of the three bases constituting the stop codon. Likely because of a lower energetic stability of stop codon recognition by a near-cognate tRNA, the translation termination complex is recruited in more than 99.9% of cases (Floquet *et al.*, 2012; Rajon & Masel, 2011). UAG and UAA stop codons share the same near-cognate tRNAs, which differ from those recognizing UGA stop codons. The amino acids glutamine, tyrosine, and lysine are incorporated during UAG or UAA readthrough, whereas arginine, cysteine, and tryptophan can be incorporated during UGA readthrough, consistent with their positions in the genetic code table (Feng *et al.*, 1990; Roy *et al.*, 2015). Yet our understanding of stop codon misdecoding by near-cognate tRNAs is far from complete, since other amino acids have also been found to be incorporated during readthrough, according to the nonsense mutation and its

nucleotide context. In particular, leucine is the main amino acid incorporated during readthrough of the UGA nonsense mutation at position 1282 (mutation W1282X) of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Xue *et al.*, 2017). It is important to note that the protein synthesized may or may not be functional according to the amino acid incorporated at the site of the nonsense mutation, if this position is crucial for the function or stability of the protein.

The translation termination complex is composed of at least two subunits, called eukaryotic release factors (eRFs) 1 and 3 (Fig. 1A). The eRF1 subunit mimics a tRNA and enters the A site of the ribosome to recognize the stop codon (Muramatsu *et al.*, 2001; Song *et al.*, 2000). In eukaryotic cells, interestingly, eRF1 recognizes all three stop codons, whereas in prokaryotic cells, two release factors (RFs) are necessary: RF-1 recognizes UAA and UAG stop codons, whereas RF-2 recognizes UAA and UGA stop codons. To understand how eRF1 recognizes all three stop codons, Brown *et al*. (2015) used cryo-electron microscopy and a catalytically inactive eRF1 to show that glutamic acid at position 55 and tyrosine at position 125 of eRF1 discriminate between purines and pyrimidines at the second and third base positions of a codon. They thus play a crucial role in the ability of eRF1 to recognize stop codons (Brown *et al.*, 2015). The eRF3 subunit is a GTPase whose activity is stimulated mainly by interaction with either the polyA-binding protein (PABP), when translation termination occurs at the physiological stop codon, or the nonsense-mediated mRNA decay (NMD) factor UPF3X (also called UPF3B), when translation termination occurs at a premature termination codon (PTC) (Neu-Yilik *et al.*, 2017). When eRF3 catalyses conversion of GTP to GDP, it induces a conformational change in the structure of eRF1, promoting translation termination through release of the nascent peptide chain and replacement of eRF3 by the ATPase ABCE1 (ATP Binding Cassette Subfamily E Member 1). ABCE1 will then hydrolyse ATP to ADP to promote recycling of the two ribosomal subunits

(Fig. 1A). In addition to the stop codon and to certain proteins located downstream of the stop codon (PABP or UPF3X for instance), the nucleotide environment around the stop codon, and particularly certain post-transcriptional modifications, may influence the translation termination process. A transcriptome-wide mRNA methylation analysis has revealed a high level of methylation at position 6 of adenosine (m6A) at the end of the ORF and the beginning of the 3' untranslated region (UTR), in the vicinity of the stop codon. This deserves further investigation and clarification, as it suggests a possible role of this mark in translation termination (Meyer *et al.*, 2012; Li *et al.*, 2014).

III. DIFFERENT TYPES OF STOP CODON READTHROUGH

Although translation termination must be a very efficient process to ensure the correct protein size, under certain conditions or at a very low rate, a near-cognate tRNA can be recruited to the A site of the ribosome when the latter reaches a stop codon. The consequence of this is that translation continues until a later stop codon that promotes translation termination. This event is called stop codon readthrough (Fig. 1B). Several types of readthrough can occur at stop codons, depending on the presence of regulatory elements or of readthrough-promoting molecules (Fig. 2).

(1) Non-programmed translational readthrough

In the absence of any readthrough molecules, readthrough of any physiological stop codon or PTC can occur at a basal level. This readthrough can be considered a translation error and is referred to here as non-programmed translational readthrough (Fig. 2A). This type of readthrough is a rare event, calculated as less than 0.1% in more than 80% of cases (Rajon & Masel, 2011; Floquet *et al.*, 2012; Fearon *et al.*, 1994). Interestingly, the likelihood of non-programmed translational readthrough depends on the identity of the stop codon. It is highest

at the UGA stop codon, whereas translation termination is most efficient at the UAA stop codon. In the case of PTCs, this very low readthrough rate is partly due to competition between near-cognate tRNAs and release factors, in favour of the former, but it is also due in part to activation of the surveillance mechanism provided by NMD, which results in silencing of the gene (Gupta & Li, 2018; Lejeune, 2017; Kurosaki & Maquat, 2016; He & Jacobson, 2015). Non-programmed translational readthrough thus occurs on the fraction of PTC-carrying mRNAs that escape NMD (Kuzmiak & Maquat, 2006).

Although the amount of protein generated by non-programmed translational readthrough is minute compared to the amount of protein produced without readthrough of a physiological stop codon or from the wild-type mRNA in the case of a PTC, the consequences of non-programmed translational readthrough can be considerable. For example, the phenotypes of patients suffering from the same pathology and carrying the same nonsense mutation may vary even though they should be identical due to silencing of the mutant gene. Some cystic fibrosis patients with a nonsense mutation that one would assume to cause a severe phenotype due to the absence of the CFTR protein actually show mild pulmonary damage (Cutting *et al.*, 1990; Kerem *et al.*, 1990). The level of non-programmed translational readthrough has been proposed to explain this mild phenotype, allowing some functional full-length CFTR protein to be synthesized.

(2) Programmed translational readthrough

The second type of readthrough is called programmed translational readthrough (Fig. 2B). It targets specific mRNAs (Loughran *et al.*, 2018; Freitag, Ast & Bolker, 2012; Dunn *et al.*, 2013) and is a proteome-expanding mechanism allowing synthesis of specific protein isoforms with particular functions. It is thus a way to synthesize two isoforms of a protein from one mRNA. A specific regulatory mechanism intervenes at the physiological stop codon

either to terminate translation at the first stop codon in phase with the initiation codon starting the ORF or to promote its readthrough so as to terminate translation at one of the downstream stop codons in phase with the initiation codon. This type of readthrough has been identified in viruses, fungi, *Drosophila* spp., and mammals. In human cells, programmed translational readthrough is thought to be a very rare event, reported to affect the expression of only a dozen genes: the opioid receptor Kappa 1 (OPRK1), opioid related nociceptin receptor 1 (OPRL1), aquaporin 4 (AQP4), mitogen-activated protein kinase 10 (MAPK10), peroxisomal lactate dehydrogenase B (LDHB), malate dehydrogenase (MDH1), vitamin D receptor (VDR), vascular endothelial growth factor A (VEGFA), myelin protein zero (MPZ), and beta-globin genes (Loughran *et al.*, 2014, 2018; Chittum *et al.*, 1998; Yamaguchi *et al.*, 2012; Eswarappa *et al.*, 2014; Schueren *et al.*, 2014; Geller & Rich, 1980). It appears more frequent in other species. In *Drosophila melanogaster*, for example, the expression of several hundred genes is regulated *via* this readthrough process (Lin *et al.*, 2007). Similarly, some 5% of yeast genes appear to be subject to programmed translational readthrough (Kleppe & Bornberg-Bauer, 2018). An *in silico* analysis aiming to identify physiological stop codons putatively subject to programmed translational readthrough suggests that this process may actually occur more frequently than previously thought in many species, including humans (Jungreis *et al.*, 2016; Dunn *et al.*, 2013). Consistent with these results, ribosome profiling performed on human foreskin fibroblasts revealed 42 genes as potentially subject to programmed translational readthrough. Interestingly, this process has been shown to generate C-terminally extended proteins very efficiently, with the amount of protein formed exceeding the amount of unextended product protein by up to 30% (Loughran *et al.*, 2014; Singh *et al.*, 2019). This suggests that specific translation termination regulation occurs at these physiological stop codons and that cis elements and trans-acting factors must be involved in promoting an

exceptionally high rate of readthrough. Some such elements have been identified and are described in Section IV.

(3) Induced translational readthrough

The third type of stop codon readthrough is PTC readthrough promoted by certain molecules (Fig. 2C), here termed induced stop codon readthrough. When the ribosome reaches a stop codon, the presence of such molecules favour recruitment of near-cognate tRNAs instead of the translation termination complex. In early studies, aminoglycosides were shown to facilitate this type of readthrough process in bacteria and yeast (Singh, Ursic & Davies, 1979). The first evidence of induced nonsense mutation readthrough in mammalian cells was reported a few years later in a study of G418 and paromomycin aminoglycosides (Burke & Mogg, 1985). Since then, both aminoglycoside and non-aminoglycoside molecules have been identified as readthrough molecules (see Section IV) that might potentially be used to treat nonsense-mutation-related pathologies.

Several lines of evidence indicate that induced translational readthrough occurs at PTCs and not at physiological stop codons. The nucleotide and protein environments around physiological stop codons have been evolutionarily selected to facilitate translation termination. This is not true of PTCs, since they appear through mutation in an environment selected to promote translation and not translation termination. While PTCs do promote translation termination, they are more sensitive to readthrough than physiological stop codons. Although several studies using readthrough molecules have shown the absence of readthrough at physiological stop codons (Benhabiles *et al.*, 2017; Trzaska *et al.*, 2020; Welch *et al.*, 2007), it remains necessary to demonstrate for each new molecule that it does not impact translation termination at physiological stop codons.

Interestingly, the efficiency of translational readthrough induced by a molecule may be related to the level of non-programmed translational readthrough (basal level of readthrough) occurring at the stop codon in the absence of that molecule: the higher the level of basal readthrough, the more efficient the readthrough promoted by molecules such as aminoglycosides (Floquet *et al.*, 2012), which should be taken into account when implementing a therapeutic approach for a given nonsense mutation.

IV. PARAMETERS INFLUENCING STOP CODON READTHROUGH

The basal readthrough level varies from one stop codon to another, as shown in various studies (Fearon *et al.*, 1994; Floquet *et al.*, 2012; Rajon & Masel, 2011). The identity of the stop codon influences this level, but other elements acting in cis or trans can also modulate the efficiency of PTC or physiological stop codon readthrough. Such elements can influence all three types of stop codon readthrough.

(1) Cis elements activating stop codon readthrough

Stop codon readthrough efficiency can be influenced by various factors, including the identity of the stop codon and the nucleotide sequence surrounding it (Bidou *et al.*, 2004; Howard *et al.*, 2000). Results from studies where a PTC was introduced into a reporter gene show that the UGA stop codon is the most permissive to readthrough and the UAA stop codon is the least permissive (Bidou *et al.*, 2004; Floquet *et al.*, 2012; Howard *et al.*, 2000; Wangen & Green, 2020; Manuvakhova, Keeling & Bedwell, 2000). However, these properties can be altered by the nucleotide context of the stop codon (Bonetti *et al.*, 1995; McCaughan *et al.*, 1995; Cassan & Rousset, 2001). In particular, counting the first nucleotide of the stop codon as position +1, the nucleotide at position +4 strongly influences translation termination efficiency. It appears that a purine at this position, as found in about 90% of the most highly

expressed human genes, favours translation termination (Tate & Mannering, 1996), whereas a pyrimidine facilitates readthrough (Brown *et al.*, 1990; McCaughan *et al.*, 1995; Tate & Mannering, 1996). In particular, the presence of a cytosine at position +4 has been shown to allow, in most cases, the most efficient stop codon suppression (Floquet *et al.*, 2012; Howard *et al.*, 2000; Phillips-Jones, Watson & Martin, 1993; Wangen & Green, 2020), although this is not an absolute rule. The nucleotide environment most favourable to efficient readthrough also depends on the identity of the stop codon. Manuvakhova *et al*. (2000) found cytosine to be the most favourable nucleotide at position +4 for promoting readthrough of UGA and UAA stop codons, but readthrough of UAG stop codons was most efficient when the other pyrimidine, uracil, is present at that position.

The nucleotide immediately following the stop codon is not the only nucleotide that can influence readthrough efficiency. Several studies have demonstrated that some downstream nucleotides can favour readthrough. Recently, a study measuring stop codon readthrough by ribosome profiling showed that the two nucleotides immediately downstream of the stop codon strongly influence readthrough efficiency (Wangen & Green, 2020). The authors concluded that enrichment in adenosines or uridines in the vicinity of the stop codon favours stop codon readthrough, whereas enrichment in guanosines or cytosines favours translation termination. Other studies have demonstrated that both upstream and downstream sequences influence the readthrough rate at a stop codon. In particular, CAA sequences upstream and downstream of a UAG stop codon in the *ste6* gene in yeast or in a reporter gene have been shown to act synergistically to promote readthrough (Fearon *et al.*, 1994; Manuvakhova *et al.*, 2000; Bonetti *et al.*, 1995; Xue *et al.*, 2014).

According to the reporter system used to investigate readthrough efficiency, the upstream and downstream consensus sequences favouring stop codon readthrough may differ slightly. For example, the downstream sequences CAR YYA (where R is a purine and Y is a pyrimidine)

and CAR NBA (where N is any of the four nucleotides and B can be U, C or G) seem to favour stop codon readthrough (Namy, Hatin & Rousset, 2001; Harrell, Melcher & Atkins, 2002; Beier & Grimm, 2001). These hexanucleotide sequences are found in several virus and cell genes associated with the regulation of programmed translational readthrough (Skuzeski *et al.*, 1991). The upstream sequence can also affect readthrough efficiency. For example, one study found that the nucleotide sequence spanning positions –6 to +9 influences readthrough rate. In particular, positions –1 and +4 were crucial for readthrough activation, and the sequence U STOP C has been reported as the consensus sequence for efficient readthrough (Floquet *et al.*, 2012). Supporting the idea of involvement of the upstream sequence in promoting readthrough, a recent study on the glycosyltransferase gene *B4GALNT1* has demonstrated that the base triplet AGC, immediately upstream and downstream of the PTC, is required for efficient basal readthrough generated by the M4 nonsense mutation at amino acid 228 (Yesmin *et al.*, 2020). Overall, all these studies indicate that stop codon readthrough efficiency is modulated by cis elements that have not yet been clearly identified and that are likely to differ among genes. It remains very difficult to predict with certainty the rate of readthrough of a stop codon without additional experimental data. For instance, when attempting to explain why a given readthrough molecule has variable effects on the same nonsense mutation located at different positions in a gene, one must consider the influence of the stop-codon-surrounding sequence on readthrough efficiency. This is especially true when the goal is to develop a therapeutic approach (Martorell *et al.*, 2020).

Besides the immediate primary sequence surrounding the stop codon, some secondary structures have been shown to facilitate stop codon readthrough. In the Moloney murine leukemia virus, a pseudoknot located eight nucleotides downstream of the stop codon separating the gag and pol ORFs promotes readthrough by about 5% of the ribosomes reaching this stop codon (Wills, Gesteland & Atkins, 1991). Since this discovery, efficient

readthroughs have been found to require other secondary structures (e.g. conserved hairpins) occurring at stop codons from various virus genomes (Firth *et al.*, 2011). The exact mechanism remains obscure, but these results suggest that certain proteins could be recruited by these secondary structures to promote readthrough.

(2) Trans elements activating readthrough

When the ribosome reaches a stop codon, it must terminate translation or ignore this translation termination signal by incorporating an amino acid and continuing translation of the ORF to a downstream stop codon. To understand the mechanisms underlying the 'decision' to stop translation or to continue, specific factors dedicated to stop codon readthrough have been sought. Some endogenous trans elements have been identified as proteins or RNAs required either for readthrough of specific stop codons or for the general readthrough mechanism. Molecules with the capacity to promote readthrough are of particular interest because of their potential in treating nonsense-mutation-related pathologies. Finally, the cell environment has been shown to influence readthrough efficiency, suggesting that regulation of the readthrough process may occur according to culture conditions.

(*a*) *Proteins and RNAs*

To date, only a few factors have been implicated in the readthrough process, some affecting all stop codon readthroughs and some that are specific to readthrough of one particular stop codon.

(*i*) *Factors involved in the general process of stop codon readthrough*

As readthrough opposes translation termination and *vice versa*, it is not surprising that loss of function of proteins involved in translation termination leads to increased levels of stop codon

readthrough. For example, the proteins termination and polyadenylation 1 (TPA1) and Ccr4 associated factor 1 (CAF1 also called POP2) involved in regulating the length of the poly(A) tail also participate in the translation termination process. In experiments using the firefly luciferase reporter gene carrying a PTC, loss of TPA1 or POP2 function in yeast resulted in increased basal readthrough levels (Keeling *et al.*, 2006). These proteins can thus be viewed as inhibitors of stop codon readthrough. However, in both yeast and human cells, the nucleotide context appears to determine whether TPA1 exerts a positive or a negative influence on stop codon readthrough (Loenarz *et al.*, 2014). In yeast, the balance between translation termination efficiency and stop codon readthrough efficiency can be altered by the expression levels of the inhibitor of translation termination 1 (ITT1) gene. This was demonstrated in experiments using a PGK1-STOP-LacZ reporter construct, in which overexpression of ITT1 caused increased basal levels of readthrough (Urakov *et al.*, 2001). Another example of the antagonism between translation termination efficiency and stop codon readthrough activation is illustrated by the dead-box RNA helicase Dbp5/DDX19, which is involved in translation termination. Dbp5/DDX19 interacts with eRF1, bringing it into contact with eRF3 so as to promote translation termination (Fig. 1). If the function of Dbp5/DDX19 is impaired, eRF1 interacts prematurely with eRF3 leading to failure of the translation termination process, thus allowing near-cognate tRNAs to enter the A site of the ribosome, recognize the stop codon, and promote readthrough (Gross *et al.*, 2007; Mikhailova *et al.*, 2017; Beissel *et al.*, 2019).

Although it makes sense that proteins involved in the translation termination process can interfere with stop codon readthrough, it is surprising to note the presence of translation initiation factors among the proteins that modulate stop codon readthrough. In both yeast and human cells, the eukaryotic initiation translation factor 3 (eIF3) seems to play a general role in programmed translational readthrough. The absence of functional eIF3 reduces the basal

level of readthrough of all three stop codons, provided they are in a readthrough-favourable nucleotide context (Beznoskova *et al.*, 2015). To promote readthrough, eIF3 appears to act as an inhibitor of eRF1 by interacting with the pre-termination complex and interfering with pairing between eRF1 and the third base of the codon. By preventing recognition of the stop codon by eRF1, eIF3 favours recognition of the stop codon by a near-cognate tRNA, thus promoting stop codon readthrough.

It is also possible to modulate the efficiency of stop codon readthrough by acting on the fidelity of the translation process. tRNAs are subject to various post-transcriptional modifications that prevent erroneous codon recognition by the tRNA. For example, the wobble effect, proposed to explain recognition of the first two bases of a codon by a near-cognate tRNA independently of the third nucleotide (Crick, 1966), is strongly increased when these post-transcriptional modifications are not present (Duechler *et al.*, 2016; Hagervall *et al.*, 1990; Bednarova *et al.*, 2017; Agris *et al.*, 2017). Interestingly, the rules governing recognition of a stop codon by a near-cognate tRNA appear less restrictive than proposed by the wobble effect, as more tRNAs than expected can recognize a stop codon (Roy *et al.*, 2015). Not only can codon recognition by a near-cognate tRNA occur *via* the first two bases of the codon independently of the third nucleotide, but it can also occur *via* the last two bases of the codon independently of the first. Similarly, the discovery that leucine is the amino acid most abundantly incorporated at the nonsense mutation W1282X in the CFTR gene suggests that a tRNA may recognize a stop codon with a mismatch at the central base of the triplet forming the codon (Xue *et al.*, 2017).

Note also that, before being substrates of stop codon readthrough, PTC-carrying mRNAs are substrates of NMD. It is therefore tempting to connect these two mechanisms and to hypothesize that they share factors in common. In humans at least, this is indeed the case for some NMD factors: downregulation of the NMD factors Up frameshift (UPF) 1, 2, or 3X/3B

impairs readthrough, indicating that these factors are necessary for this process (Jia *et al.*, 2017; Ivanov *et al.*, 2008). The exact roles of these NMD proteins in readthrough remain to be clarified, especially because contradictory reports have been published on this role of UPF proteins in other organisms such as yeast. It has notably been claimed that UPF proteins inhibit readthrough, since knockout of one UPF gene in yeast results in an increased readthrough rate (Salas-Marco & Bedwell, 2005; Wang *et al.*, 2001). However, another study failed to observe any effect of UPF gene knockout on the readthrough rate, suggesting that these proteins are not involved in readthrough (Harger & Dinman, 2004). The role of the UPF proteins in stop codon readthrough thus may differ between yeast and humans. This warrants in-depth investigations into the connection between NMD and stop codon readthrough.

Translation takes place *a priori* throughout the cytoplasm, and since stop codon readthrough shares the same translation machinery, it can be expected also to occur in the cytoplasm, without any dedicated sites. Yet a recent study demonstrated that the cytoskeleton influences PTC readthrough (Jia *et al.*, 2017). In particular, basal readthrough is activated when formation of actin filaments is impaired. It thus seems that actin filaments are neither required for readthrough nor favourable to it, meaning that they may participate in some form of readthrough inhibition. The same study showed that non-programmed translational readthrough occurs at specific cytoplasmic foci different from P-bodies and named readthrough bodies (Jia *et al*., 2017). Their results suggest that PTC-containing mRNAs are actively targeted either to degradation by NMD or to undergo PTC readthrough. Although UPF proteins have been found in readthrough bodies (unlike the P-body marker decapping protein 1a (DCP1a), which is involved in NMD), readthrough body characterization remains poor and no specific proteins have been identified.

(*ii*) *Factors involved in specific stop codon readthrough events*

To explain programmed translational readthrough on an mRNA, cis elements have been identified (see Section IV.1). Often these cis elements work together with or recruit factors to promote readthrough of a specific stop codon. For example, heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, an RNA-binding protein involved in primary microRNA (pri-miRNA) processing and in the trafficking and assembly of the human immunodeficiency virus (HIV) genome (Alarcon *et al.*, 2015; Beriault *et al.*, 2004; Levesque *et al.*, 2006), is involved in programmed translational readthrough on VEGFA mRNA (Eswarappa *et al.*, 2014; Houck-Loomis *et al.*, 2011). HnRNP A2/B1 interacts with an A2 response element (A2RE) located downstream of the physiological stop codon to promote stop codon readthrough. Mutating the A2RE sequence or downregulating hnRNP A2/B1 impairs readthrough of the physiological stop codon of VEGFA mRNA. Whether hnRNP A2/B1 interacts with VEGFA pre-messenger RNA (pre-mRNA) or mRNA only has not yet been investigated, but given the nuclear localization of this protein, it could be an early mark for specific programmed translational readthrough.

More recently, Lethal 7a (Let7a) microRNA (miRNA) has been shown to promote programmed translational readthrough on Argonaute 1 (Ago1) mRNA, generating a longer isoform called Ago1x. The miRNA binds a sequence downstream of the physiological stop codon and upstream of the subsequent in-phase downstream stop codon. This sequence is sufficient to promote readthrough when introduced into a heterologous 3' UTR (Singh *et al.*, 2019). This indicates that the mechanism is independent of the identity of the stop codon or of the mRNA, as long as a Let7a miRNA binding site is present downstream of the stop codon to be read through.

Both hnRNP A2/B1 and Let7a miRNA bind an RNA sequence located about 10 nucleotides downstream of the stop codon whose readthrough they promote. This suggests a possible interaction with the translation machinery pausing on the stop codon. In both cases,

interestingly, the rate of translational readthrough of the canonical stop codon reaches at least 20%, which can be considered very efficient stop codon readthrough (Eswarappa *et al.*, 2014; Singh *et al.*, 2019). Unfortunately, the precise mechanism remains to be determined in order to understand how these trans elements impair translation termination and efficiently promote synthesis of a C-terminally extended protein.

All factors involved in translation termination or stop codon readthrough constitute targets for the development of therapeutic approaches to treating genetic diseases caused by nonsense mutations. By inhibiting the synthesis of these factors or in some cases by overproducing them, stop codon readthrough is activated. Readthrough activation could thus represent a potential way to correct a nonsense mutation responsible for a pathology. Molecules capable of targeting the trans factors described in this section could be sought in the framework of developing a therapeutic approach. Yet to date, as discussed in Section V, this is not yet a common approach to identify readthrough molecules. The strategy used focuses on searching for molecules that target the readthrough mechanism as a whole, rather than targeting a specific factor.

(*b*) *Small molecules*

Because of their potential therapeutic interest, molecules activating PTC readthrough have been the focus of many studies. Historically, some members of the aminoglycoside family have shown the capacity to promote PTC readthrough (Burke & Mogg, 1985). This family of molecules is composed of a sugar substituted with one amino group. However, not all members of this family promote significant stop codon readthrough: gentamicin, geneticin (G418), paromomycin, neomycin, and lividomycin do have this effect (Table 1), but hygromycin, streptomycin, kanamycin, tobramycin, and amikacin do not (Manuvakhova *et al.*, 2000). Aminoglycosides promote stop codon readthrough by interacting with the 16S

449 ribosomal RNA located in the decoding centre of the ribosome (Carter *et al.*, 2000; Ogle,
450 Carter & Ramakrishnan, 2003; Prokhorova *et al.*, 2017; Zingman *et al.*, 2007). Besides
451 aminoglycosides, several non-aminoglycoside molecules have also been shown to promote
452 PTC readthrough (Table 2). PTC124/ataluren/translarna, an oxadiazole derivative, is the only
453 molecule to have reached clinical phase II/III trials for the treatment of genetic diseases
454 caused by nonsense mutations (Welch *et al.*, 2007; Kerem *et al.*, 2014). This molecule, which
455 can rescue expression of genes carrying UGA, UAG, or UAA nonsense mutations, has a
456 mode of action that remains to be clarified, but might either target the A site of the ribosome
457 as do aminoglycosides or interact directly with the stop codon to promote readthrough (Roy *et*
458 *al.*, 2016; Tutone *et al.*, 2019). Even though the efficacy of this molecule seems too low for
459 clinical development aimed at treating, for example, cystic fibrosis or Duchenne muscular
460 dystrophy (Haas *et al.*, 2015; Kerem *et al.*, 2014), it does illustrate the need to identify
461 readthrough molecules that might enhance the treatment of nonsense-mutation-related genetic
462 diseases (Kong *et al.*, 2019). Among other readthrough molecules, a dipeptide-like hydrazide
463 antibiotic negamycin, originally purified from *Streptomyces purpeofuscus*, appears more
464 potent than aminoglycosides and less toxic, as do negamycin derivatives (Arakawa *et al.*,
465 2003; Taguchi *et al.*, 2014; Hamada *et al.*, 2019) (Table 2).
466 For more than 10 years, various screening systems have been used to identify compounds
467 more potent than aminoglycosides and ataluren (Fig. 3). The readthrough compounds (RTCs)
468 RTC13, RTC14, RTC204, RTC219, GJ071, GJ072, NV2907, NV2909, NV2899 and NV2913
469 (Du *et al.*, 2009, 2013; Tutone *et al.*, 2020) (Table 2) have been identified and tested on
470 different constructs and cell models of nonsense-mutation-related genetic diseases. In most
471 cases, these molecules have shown a readthrough activity similar to that of ataluren or
472 aminoglycosides such as gentamicin (G418) (Du *et al.*, 2009; Tutone *et al.*, 2020; Gomez-
473 Grau *et al.*, 2015).

Among the compounds identified as promoting induced translational readthrough, some have remarkable characteristics. The anti-allergy and anti-asthma drug Amlexanox, for example, has shown the capacity to both inhibit NMD and activate readthrough of UGA, UAG, and UAA nonsense mutations (Atanasova *et al.*, 2017; Banning, Schiff & Tikkanen, 2017; Gonzalez-Hilarion *et al.*, 2012). The clinically approved molecule Escin has also been shown to exert this dual action (Mutyam *et al.*, 2016). In theory, such molecules should be more effective than molecules that only activate readthrough, since inhibiting NMD leads to an increased amount of RNA substrates for readthrough (Linde *et al.*, 2007; Gonzalez-Hilarion *et al.*, 2012). It seems, however, that this is not an absolute rule, as some readthrough activators with no NMD-inhibiting action promote greater synthesis of full-length proteins than do dual-action molecules. For instance, the readthrough activators *Lepista flaccida* extract H7 and 2,6-diaminopurine, which do not inhibit NMD, correct UGA and UAA (extract H7) or UGA only (2,6-diaminopurine) more effectively than dual-action G418 (Benhabiles *et al.*, 2017; Trzaska *et al.*, 2020; Correa-Cerro *et al.*, 2005).

Clitocine is another molecule with a high readthrough-promoting capacity. However, this molecule, found in various mushroom species (including *Leucopaxillus giganteus* and *Lepista flaccida*) (Trzaska *et al.*, 2020; Kubo *et al.*, 1986; Wilde *et al.*, 2007; Ren *et al.*, 2008; Benhabiles *et al.*, 2017; Fortin *et al.*, 2006) unfortunately shows high toxicity, which limits its potential therapeutic development (Fortin *et al.*, 2006; Sun *et al.*, 2012). The mode of action of clitocine has been studied. Interestingly, this molecule, whose structure resembles that of a nucleoside, is incorporated into RNA molecules during transcription, substituting for adenosines. Like adenosine, clitocine can preferentially pair with uracil, but it has been proposed that the translation termination factor eRF1 weakly recognizes stop codons containing clitocine, increasing the chance of near-cognate tRNA recruitment (Friesen *et al.*, 2017).

499

500 (*c*) *Cell environment*

501 Cell culture conditions can also influence readthrough efficiency. In ribosome profiling
502 experiments performed on the neural cell line PC12, oxygen and glucose deprivation were
503 shown to inhibit programmed translational readthrough of 18 mRNAs with a UGA
504 physiological stop codon followed by a cytosine: Fkbp1a, Hadhb, Hs1bp3, Klc1,
505 LOC102554884, Mdh1, Mrto4, Nedd8, Nudcd2, Plat, Polr2l, Ppp4c, Rfc2, Rnf111, Sec13,
506 Slc7a1, Ssna1 and Thy1 (Andreev *et al.*, 2015). Interestingly, this regulation occurs very
507 rapidly: less than 20 min after starting hypoxia and low glucose, programmed translational
508 readthrough was strongly reduced. Although the mode of action is not clear, it could be
509 related to the loss of protein hydroxylation at the decoding centre of the ribosome.
510 Other cell culture conditions that seem to potentiate PTC readthrough notably include serum
511 starvation. In a medium containing 1% serum, the PTC-readthrough efficacy of
512 aminoglycosides can increase two- to threefold without modifying the translation activity on
513 the mRNA substrates, as demonstrated using a dual-reporter green fluorescent protein (GFP)-
514 blue fluorescent protein (BFP) construct (Wittenstein *et al.*, 2019). How serum starvation
515 potentiates the readthrough activity of aminoglycosides is not yet understood, but given the
516 influence of serum on gene expression, it seems likely that specific gene products acting as
517 cofactors are overexpressed or repressed.

518

519 **V. THERAPEUTIC INTEREST OF STOP CODON READTHROUGH**

520 Favouring stop codon readthrough to correct a nonsense mutation represents an attractive
521 approach to the treatment of certain genetic diseases. About 11% of patients with a genetic
522 disease carry a nonsense mutation responsible for its pathology (Mort *et al.*, 2008). The use of
523 readthrough molecules was first tested in patients with cystic fibrosis caused by a nonsense

524 mutation in the CFTR gene. The aminoglycoside gentamicin was administered intravenously,
525 at 2.5 mg/kg every 8 h for seven days, to five patients carrying nonsense mutations and five
526 patients without nonsense mutation in CFTR (Clancy *et al.*, 2001). The results showed a mild
527 but encouraging rescue of CFTR expression and function. Another study on cystic fibrosis
528 patients demonstrated that rescue of CFTR function depends strongly on the nonsense
529 mutation: patients with the Y122X nonsense mutation seemed to respond better than patients
530 carrying G542X, R1162X, or W1282X (Sermet-Gaudelus *et al.*, 2007). Aminoglycosides
531 (particularly gentamicin) have also been tested in patients with Duchenne muscular dystrophy
532 (DMD) carrying a nonsense mutation in the *DMD* gene encoding dystrophin. Gentamicin has
533 been tested in several studies with different protocols. In one study, four DMD patients with a
534 nonsense mutation in the *DMD* gene were treated daily for 14 days with gentamicin at 7.5
535 mg/kg (Wagner *et al.*, 2001). This study concluded that the administered gentamicin treatment
536 was unable to promote the synthesis of full-length dystrophin protein. In another study,
537 positive results were obtained by intravenous administration of 7.5 mg/kg gentamicin, once or
538 twice a week for 6 months, to patients carrying a nonsense mutation in the *DMD* gene, as
539 compared to control patients carrying a frameshift mutation in the *DMD* gene (Malik *et al.*,
540 2010). The treatment was well tolerated by patients, without any sign of toxicity. The level of
541 dystrophin protein increased in gentamicin-treated patients carrying a nonsense mutation,
542 indicating that readthrough of the nonsense mutation occurred. However, the physical strength
543 of these patients was unaffected by the treatment, implying that dystrophin rescue was
544 insufficient.
545 The non-aminoglycoside molecule ataluren has also been tested on cystic fibrosis and DMD
546 patients carrying nonsense mutations. This molecule was well tolerated by patients. DMD
547 patients received ataluren orally three times daily at 4–4–8 mg/kg, 10–10–20 mg/kg, or 20–
548 20–40 mg/kg for 28 days. At the end of the study, dystrophin was detected in patients at the

549 lowest and middle doses but not in patients at the highest dose (Namgoong & Bertoni, 2016).
550 After a 48-week exposure to ataluren or placebo, drug-treated patients showed no significant
551 improvement in a 6-min walking-distance test as compared to the placebo group (Finkel *et al.*,
552 2013; Bushby *et al.*, 2014). For patients with cystic fibrosis caused by nonsense mutations,
553 several phase II and III clinical trials have been completed. Physical improvement was
554 observed in patients having received ataluren *versus* placebo, although this improvement
555 remained modest (Kerem *et al.*, 2008; Sermet-Gaudelus *et al.*, 2010; Lee & Dougherty, 2012).
556 The results of the clinical trials performed with gentamicin or ataluren clearly demonstrate
557 that it is possible to correct nonsense mutations by inducing PTC readthrough in patients.
558 Although no cure for patients with nonsense-mutation-related genetic diseases is yet available,
559 these encouraging results warrant both optimization of the tested protocols and a search for
560 new therapeutic approaches.
561

562 **VI. NEW DEVELOPMENTS AND THERAPEUTIC TARGETS**

563 As described below, aminoglycosides have high potential to correct nonsense mutations. Yet
564 because of their oto- and nephrotoxicity, their clinical development has been limited (Hock &
565 Anderson, 1995; Mingeot-Leclercq & Tulkens, 1999; Swan, 1997; Greenwood, 1959; Heck,
566 Hinshaw & Parsons, 1963; Matz, 1993; Wu *et al.*, 2001). To address this issue,
567 aminoglycoside derivatives have been synthesized and tested for the capacity to correct
568 nonsense mutations while showing lower toxicity. Several molecules meeting these criteria
569 appear to be good candidates for treating genetic diseases caused by nonsense mutations, their
570 efficacy having been demonstrated on cell lines and *in vivo* mouse models (Goldmann *et al.*,
571 2012; Nudelman *et al.*, 2009, 2006; Shulman *et al.*, 2014; Wang *et al.*, 2012; Xue *et al.*, 2014).
572 One of these molecules, named ELX-02 (from Eloxx Pharmaceuticals), has already
573 successfully passed clinical phase I (Leubitz *et al.*, 2019), and clinical phase II is ongoing.

574 ELX-02 is much less toxic than gentamicin or G418, and no nephrotoxicity has been detected.
575 Its readthrough efficiency is similar to that of G418, making this molecule an excellent drug
576 candidate (Brasell *et al.*, 2019).
577 Regarding ataluren (from PTC Therapeutics), the quest to identify derivative molecules with
578 higher efficacy has already begun. Molecules such as NV2445 and PTC414 seem to offer a
579 slightly higher readthrough efficiency. Provided their toxicity is no higher than that of
580 ataluren, they could represent optimized versions of this drug (Pibiri *et al.*, 2018; Moosajee *et*
581 *al.*, 2016).
582 One of the biggest challenges is to identify low-toxicity molecules with a significantly higher
583 readthrough efficiency than reference molecules such as G418. To identify readthrough
584 molecules, many screening systems have been devised (Fig. 3). They are based on the use of
585 either (*i*) one cDNA encoding a fluorescent protein or enzyme, in which a stop codon has
586 been introduced to interrupt the ORF and prevent synthesis of the functional protein (Fig. 3A)
587 (Du *et al.*, 2009; Manuvakhova *et al.*, 2000; Welch *et al.*, 2007; Burke & Mogg, 1985;
588 Sogaard, Jakobsen & Justesen, 1999) or (*ii*) two cDNAs encoding fluorescent proteins or
589 enzymes, separated by a stop codon (Fig. 3B) (Bidou *et al.*, 2004; Xue *et al.*, 2014; Cardno *et*
590 *al.*, 2009). In the latter case, the level of protein produced from the first cDNA is used to
591 normalize measurements between samples, and the level of protein produced from the second
592 ORF reflects the readthrough efficiency. The advantage of these constructs is that they are not
593 spliced downstream of the premature termination codon, so that the corresponding mRNA is
594 not subject to NMD (Lejeune & Maquat, 2005). This makes it possible to identify molecules
595 on the sole basis of their readthrough-promoting activity. However, with such constructs there
596 is a risk of selecting molecules with low readthrough efficiency (and hence low therapeutic
597 efficacy), as testing is done on mRNAs present in huge copy numbers. In addition, as the pre-
598 mRNAs of more than 90% of human genes are subject to splicing, the corresponding PTC-

599 carrying mRNAs will be substrates for NMD, which will strongly reduce the copy number of
600 substrates for readthrough. It is important to bear in mind that this is the prevailing situation in
601 patient cells. To identify molecules capable of correcting nonsense mutations in these cells,
602 screening systems should be designed with constructs subject to NMD. Such a screening
603 system has been described: it consists of a cDNA encoding the firefly luciferase interrupted
604 by an intron and an upstream PTC (Fig. 3C) (Benhabiles *et al.*, 2017). It has been shown that
605 the firefly luciferase pre-mRNA is spliced and the mRNA degraded by NMD. The luciferase
606 activity measured in this screening system thus depends on the efficiency of readthrough
607 occurring on the number of firefly luciferase mRNAs that have escaped NMD. This screening
608 system has been used successfully to identify *Lepista flaccida* fungus extract H7 and 2,6-
609 diaminopurine as two highly potent correctors of nonsense mutations in human cells
610 (Benhabiles *et al.*, 2017; Trzaska *et al.*, 2020).
611 Studies aiming to increase the effectiveness of readthrough compounds have identified some
612 molecules that potentiate the readthrough activity of aminoglycosides (Table 3). Interestingly,
613 these molecules do not promote readthrough by themselves. For example, the phthalimide
614 derivative CDX5-1 increases about 180-fold the readthrough efficiency of G418 in both yeast
615 and human cells (Baradaran-Heravi *et al.*, 2016). More recently discovered potentiators of
616 aminoglycosides include the antimalarial mefloquine (Ferguson *et al.*, 2019) and 2-
617 aminothiazole-4-carboxamides (Rabea *et al.*, 2019). How these molecules improve the
618 readthrough efficiency of aminoglycosides is unclear, but by making it possible to administer
619 lower doses of aminoglycosides, they should reduce aminoglycoside-linked toxic effects.
620 As the use of screening systems to identify molecules with readthrough activity takes time
621 and money, new targeted approaches are being or could be developed to promote efficient
622 readthrough. One such approach is based on the mechanism of action of 2,6-diaminopurine,
623 recently shown to promote readthrough of UGA premature stop codons by interfering with the

function of filamentous temperature sensitive J1 (FTSJ1). This enzyme is a methyltransferase targeting position C34 of the tRNA carrying tryptophan and recognizing the UGG codon (Trzaska *et al.*, 2020). Decreased C34 methylation allows this tRNA to recognize also the UGA stop codon (Trzaska *et al.*, 2020). Hence, tRNA-modifying enzymes appear as interesting targets. This readthrough-activating strategy seems more effective than targeting the ribosome, but it remains to be validated through identification of new molecules targeting other tRNA-modification enzymes. Another potential target in the search for readthrough-promoting molecules is the ATP-binding domain 3/cytosolic thiouridylase subunit 1 (ATPBD3/CTU1) protein. This tRNA-modifying enzyme notably acts at the anticodon loop of near-cognate tRNAs, enabling them to recognize a UAG or UAA stop codon (Blanchet *et al.*, 2018).

Pseudouridylation is another post-transcriptional modification occurring on RNA molecules that might be used to activate stop codon readthrough. Interestingly, pseudouridylation converts a uridine to a pseudouridine, which can pair with all four conventional bases (Kierzek *et al.*, 2014). Protocols have been designed to induce site-directed pseudouridylation at the uridine constituting the first base of all three stop codons (Wu, Huang & Yu, 2015). The idea is that the presence of a pseudouridine at this position will stabilize near-cognate tRNAs and increase basal readthrough of this stop codon.

A final approach worth mentioning is direct modification of tRNA anticodon sequences, enabling them to pair with stop codons. Such tRNAs, called suppressor tRNAs, have been investigated as potential therapeutics for almost 40 years (Temple *et al.*, 1982), and research is still ongoing. Very recently, investigators have described a high-throughput cloning system for identifying tRNAs that suppress nonsense mutations with high efficiency (Lueck *et al.*, 2019). Interestingly, a comparative study of suppressor tRNAs and readthrough molecules such as gentamicin and G418 demonstrated a superior nonsense-mutation-correcting

efficiency of the tested tRNAs. A legitimate question is whether such molecules affect translation termination at physiological stop codons. Lueck *et al*. (2019), using ribosome profiling, showed that suppressor tRNAs do read through physiological stop codons in rare cases, but that this readthrough is much less efficient than PTC readthrough. A limitation of this very promising approach remains delivery of the suppressor tRNA to the cells of patients liable to benefit from correction of a nonsense mutation responsible for a genetic disease.

VII. CONCLUSIONS

(1) Stop codon readthrough is a natural process resulting in continuation of translation beyond the first stop codon encountered in phase with the translation initiation codon. The mechanism determining whether the translation machinery recruits a tRNA to promote translational readthrough, or the translation termination complex to the stop codon remains poorly understood.

(2) Several types of stop codon readthrough can be identified. Non-programmed translational readthrough or basal stop codon readthrough arises as a translational error without any cis and/or trans facilitator elements and at a very low rate. Programmed translational readthrough involves cis and/or trans activator elements, and often reaches more than 20% efficiency. The cis and trans elements described herein constitute first steps in understanding of the molecular mechanisms involved. Induced translational readthrough involves small molecules that increase the recruitment of near-cognate tRNAs when the ribosome pauses at a stop codon.

(3) Several regulator elements have been reported to modulate stop codon readthrough. The nucleotide sequence in the vicinity of the stop codon has been reported strongly to influence the stop codon readthrough rate. RNAs, proteins and small molecules also play a role in stop codon readthrough efficacy. Given the therapeutic potential of activators of PTC-readthrough, regulator elements responsible for high efficiency of readthrough deserve in-depth study. It is

essential to fill the gaps in our knowledge of the molecular events leading to stop codon readthrough and its regulation. This should be accomplished by studying the molecular mechanism of stop codon recognition and the mode of action of readthrough molecules. (4) None of the therapeutic approaches under development to correct nonsense mutations responsible for genetic diseases has yet come to fruition. However, the diversity of strategies explored makes the field of nonsense mutation correction highly dynamic. Step by step, personalized therapeutic strategies based on stop codon readthrough are taking form. They should, in the near future, yield treatments for genetic diseases caused by nonsense mutations.

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Figure Legends

Fig. 1. Comparison of translation termination and stop codon readthrough mechanisms. (A) Translation termination. When the ribosome reaches a stop codon, in most cases the translation termination complex is recruited. When the A site of the ribosome covers a stop

codon, eRF3 and ABCE1 interact with the ribosome. ABCE1 recruits the Dbp5/DDX19-eRF1 complex, likely with the help of PABPC1. Dbp5/DDX19 then hydrolyses ATP to ADP, promoting its own departure. The energy supplied by eRF3-driven GTP hydrolysis favours a change in the conformational structure of eRF1, with subsequent dissociation of the synthesised peptide. ABCE1-driven ATP hydrolysis supplies energy for the release of the ribosome, which is then recycled. (B) Stop codon readthrough. At a very low rate or under certain conditions (drugs, eIF3, regulatory elements), a near-cognate tRNA, rather than the translation termination complex, is recruited when the ribosome reaches a stop codon. The elongation factor EF-Tu allows polymerization of the peptide, with GTP hydrolysis leading to continuation of translation to the next stop codon. ABCE1, ATP-binding cassette sub-family E member 1; DDX19, DEAD-box helicase 19B; EF-Tu, elongation factor thermo unstable; eIF3, eukaryotic initiation factor 3; eRF, eukaryotic release factor; GGQ, glycine-glycine-glutamine motif; mRNA, messenger RNA; PABP, polyA-binding protein; tRNA, transfer RNA.

Fig. 2. Different types of stop codon readthrough. (A) Non-programmed translational readthrough. This type of readthrough occurs at a basal level without any regulatory elements and without the presence of readthrough molecules. This readthrough happens at premature termination codons (PTCs) and physiological stop codons at a very low rate. (B) Programmed translational readthrough. Some mRNAs are subject to readthrough of the physiological stop codon, due to the presence of regulatory elements. This type of readthrough is more efficient than that shown in A. (C) Induced translational readthrough. The presence of molecules (aminoglycosides or non-aminoglycosides) promotes activation of readthrough. This type of readthrough is more efficient than that shown in A. DDX19, DEAD-box helicase 19B; eRF,

1188 eukaryotic release factor; GGQ, glycine-glycine-glutamine motif; mRNA, messenger RNA;
1189 PABP, polyA-binding protein; tRNA, transfer RNA; UTR, untranslated region.
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1191 **Fig. 3.** Strategies used to screen molecules for readthrough-promoting activity. (A) A
1192 premature termination codon (PTC) is introduced into a cDNA encoding an enzyme or a
1193 fluorescent protein. Measurement of the enzymatic activity or fluorescence related to this
1194 protein will indicate that readthrough has occurred. (B) Use of two cDNAs encoding an
1195 enzymatic activity or a fluorescence separated by a stop codon. The product of the first cDNA
1196 is used to normalize the signal. Measurement of the product of the second cDNA will indicate
1197 that readthrough has occurred. (C) Screening system with an intron introduced into a cDNA to
1198 promote a splicing event. The PTC is introduced more than 55 nucleotides upstream of the
1199 intronic sequence to activate nonsense-mediated mRNA decay (NMD) of the corresponding
1200 mRNA.
1201
1202 Table 1. Aminoglycoside molecules with known readthrough activity.
1203 Table 2. Non-aminoglycoside molecules with known readthrough activity.
1204 Table 3. Readthrough potentiator molecules.
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