Molecular Determinants and Therapeutic Targeting of Stop Codon Readthrough in Eukaryotic Translation ☆

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Abstract

Accurate translation termination is essential for proteome integrity and in eukaryotes is primarily governed by the release factors eRF1 and eRF3, which ensure precise recognition of stop codons and efficient release of nascent polypeptides. However, proteome integrity is challenged by mutations that generate premature termination codons (PTCs), leading to truncated, nonfunctional proteins and degradation of the aberrant transcript via nonsense-mediated mRNA decay (NMD). Collectively, these events account for ∼1800 human genetic diseases. Translational readthrough, the process by which near-cognate tRNAs decode stop codons and allow ribosomes to continue elongation beyond the stop codon, represents a possibility to suppress PTCs and restore full-length protein synthesis. Initially discovered in viruses as a mechanism to expand coding capacity, readthrough is now recognized as a regulated feature of eukaryotic gene expression influenced by both *cis*-acting sequence elements and *trans*-acting factors. Recent evidence highlights the remarkable context dependence of readthrough, revealing variation across transcripts, tissues, and developmental stages. In this review, we examine the molecular determinants that define stop codon recognition and readthrough efficiency, with particular emphasis on nucleotide context. We further discuss the mechanisms and binding sites of small molecules that promote PTC readthrough, and summarize the clinical development landscape of readthrough-inducing compounds for the treatment of diseases caused by nonsense mutations.

Introduction

In eukaryotic cells, translation termination is triggered when one of the three stop codons (UAA, UAG, or UGA) enters the ribosomal A-site. These codons are specifically recognized by the class I release factor eRF1, whose tRNA-mimicking structure enables direct decoding of stop signals [1]. Upon stop codon recognition, eRF1 associates with eRF3 and GTP to form a ternary complex that catalyzes hydrolysis of the peptidyl-tRNA bond, releasing the nascent polypeptide from the ribosome [2]. Under normal conditions, eRF1 efficiently discriminates against near-cognate tRNAs, ensuring accurate termination [3]. Occasionally, however, a near-cognate tRNA pairs with the stop codon, leading to the incorporation of an amino acid instead of peptide release, a process termed translational readthrough (TR) [4]. The ribosome then continues elongation until encountering the next in-frame stop codon. TR was first identified in viruses, where it acts as a strategy to extend coding capacity within compact genomes [5,6].

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Similar mechanisms have since been observed in higher eukaryotes, producing C-terminally extended protein isoforms that often remain functional [7]. Although basal readthrough frequencies in mammalian cells are typically below 0.1% [8], certain contexts support levels exceeding 10% [9,10], indicating that the process is selectively tunable rather than purely stochastic. Comparative genomics and ribosome profiling have revealed hundreds of conserved readthrough events in *Drosophila*, yeast, and mammals, establishing that stop codon readthrough is far more widespread than commonly appreciated, with efficiencies spanning over two orders of magnitude among transcripts [9,11]. These findings collectively establish that TR constitutes a regulated layer of gene expression. Both *cis*-acting mRNA features, including the identity of the stop codon and its surrounding nucleotide context, and *trans*-acting factors that influence termination fidelity act in concert to define the likelihood of readthrough [4,8,12].

The biomedical relevance of stop codon suppression is underscored by the prevalence of premature termination codons (PTCs) in human genetic diseases. Nonsense mutations account for ~11% of all inherited disorders, including cystic fibrosis, Duchenne muscular dystrophy, and hemophilia [13]. These mutations prematurely halt translation, yielding truncated, nonfunctional, or even toxic proteins that lead to loss-of-function or gain-of-function pathologies. Pharmacological approaches that promote PTC suppression, collectively termed nonsense-suppression therapeutics [8,14], aim to restore the synthesis of full-length, functional proteins by inducing selective readthrough at PTCs while maintaining accurate termination at natural stop codons. Achieving this balance remains a central challenge for therapeutic development and underscores the need for a deeper mechanistic understanding of the difference between translation termination at PTCs (i.e. stop codons that trigger NMD) and at "normal" stop codons.

In this review, we examine the molecular determinants that govern stop codon recognition and readthrough, emphasizing the influence of mRNA sequence context, tissue specificity, and age-dependent regulation. We also summarize current progress in the identification and optimization of PTC readthrough agents, highlighting their mechanistic diversity, therapeutic potential, and limitations.

Sequence context governing TR

The +4 nucleotide and stop codon-specific regulation of readthrough

Structural and biochemical studies have provided detailed insight into how eRF1 recognizes stop codons and distinguishes them from sense codons [15]. Crosslinking and mutational analyses have identified several conserved motifs within the N-terminal domain of eRF1, most notably the TASNIKS (NIKS), YxCxxxF, and GTS motifs, which together form a composite pocket that accommodates the stop codon [16,17]. High-resolution cryo-electron microscopy (cryo-EM) structures of mammalian ribosomes with eRF1 bound at the A-site revealed that termination involves an atypical configuration in which four mRNA nucleotides, the three bases of the stop codon plus one additional 3′ nucleotide, occupy the decoding centre (DC) [18–20]. This extended conformation, stabilized by a network of eRF1–rRNA interactions, establishes a four-base termination signal rather than the triplet readout typical of sense codons. Within this architecture, the hydroxylated lysine of the NIKS motif specifically recognizes the uridine at the first (+1) position, enforcing the requirement for U at the start of all stop codons. The YxCxxxF motif interacts primarily with the second and third bases (+2 and +3), conferring selectivity among UAA, UAG, and UGA [18], while the GTS loop closes the recognition pocket to stabilize the complex [19]. The binding of eRF1 also induces a conformational change in the mRNA, drawing the fourth nucleotide downstream of the stop codon into the A-site [18–20]. These structural insights explain long-standing biochemical observations that the nucleotide immediately following the stop codon (the +4 position) has a profound influence on termination efficiency [18,20]. Among the four possible bases, cytosine at +4 is most consistently associated with elevated basal readthrough [21,22]. Genome-wide analyses further confirm a consistent relationship between the identity of the +4 nucleotide and readthrough efficiency, following the order C > U > A > G (Figure 1) [23]. In mammalian cells, the highest readthrough frequencies occur at UGA followed by cytosine, while similar but weaker effects are observed for UAA and UAG with a cytosine at the position +4 [24,25]. Nucleotides extending beyond the stop codon therefore fine-tune termination efficiency, likely by modulating mRNA–ribosome interactions and stabilizing the eRF1–stop codon complex. Consistent with this interplay between sequence and ribosomal architecture, post-translational modification of the ribosome itself can influence readthrough outcomes. Specifically, hydroxylation of a conserved proline residue in ribosomal protein uS12, which contacts the mRNA backbone near the +4 position, alters readthrough efficiency in a manner dependent on the +4 base (Figure 1) [26]. This interplay between local RNA sequence and ribosomal chemistry indicates that termination fidelity arises not only from mRNA signals but also from the structural adaptability of the DC, highlighting the regulatory role of the +4 nucleotide.

Beyond the +4 base, comparative ribosome profiling and reporter assays across species have

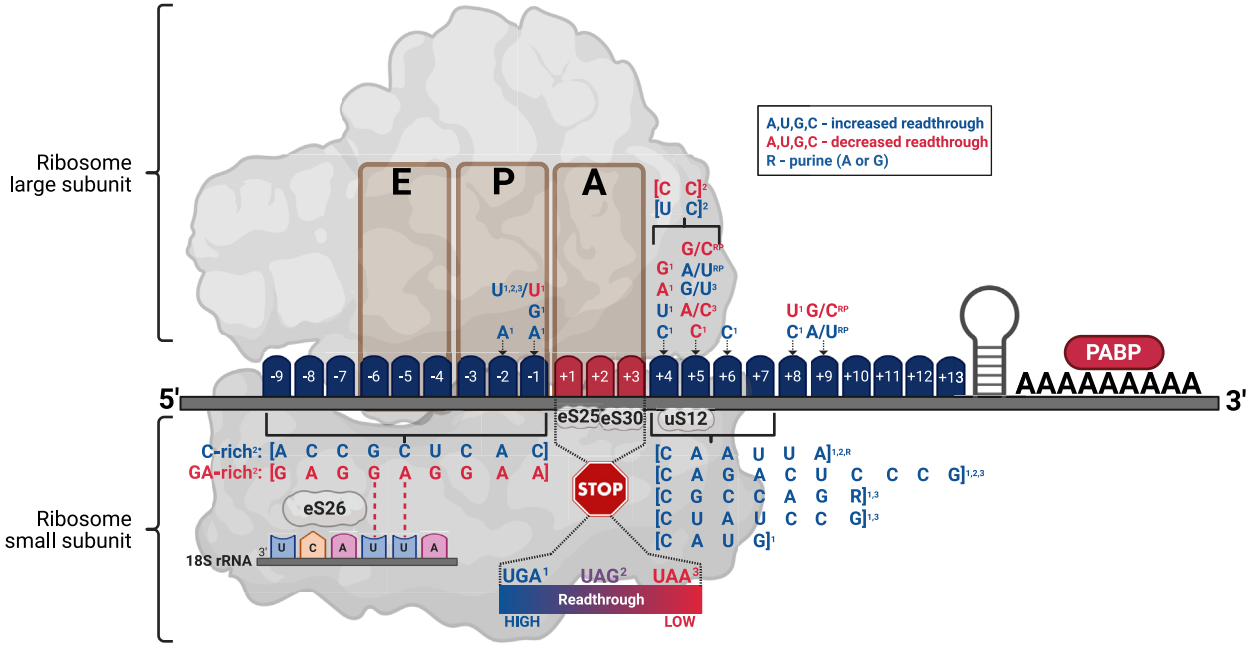

Figure 1. Sequence and structural determinants of translational readthrough (TR). Cumulative analysis of sequence-dependent readthrough events in eukaryotes across different stop codon contexts. The figure summarizes how nucleotide composition surrounding the stop codon influences the efficiency of TR. Nucleotides that decrease TR when present in the respective positions are shown in red, nucleotides that enhance TR efficiency are shown in blue. Superscript numbers denote in which stop codon context the nucleotides were tested: [1], UGA; [2], UAG; [3], UAA. [RP] indicates that the information is based on ribosome profiling data. Specific sequence motifs whose influence on TR was tested are surrounded by [ ]. Additional indicated elements contributing to context-dependent control of translation termination are the ribosomal proteins eS25, eS26, eS30, and uS12, regions of the 18S rRNA, stem–loop structures located downstream of the stop codon, and the poly(A)-binding protein (PABP).

revealed a consistent hierarchy of stop codon susceptibility to readthrough: UGA is the most prone, followed by UAG, whereas UAA confers the highest termination fidelity (Figure 1) [11]. This pattern likely reflects intrinsic differences in eRF1 recognition combined with the competitive dynamics of near-cognate tRNAs. For example, tRNAs decoding tryptophan (UGG) can readily mispair with UGA because the first two nucleotides are identical and both codons terminate with a purine, facilitating wobble recognition [27]. In contrast, cysteine and tyrosine tRNAs (whose codons UGC/UGU and UAC/UAU end in pyrimidines) pair less efficiently, while glutamine tRNAs (CAA/CAG) show limited complementarity at the first codon position, reducing misreading of UAA and UAG [27]. Notably, UAA is enriched in mRNAs encoding highly expressed or essential proteins, consistent with selective pressure to minimize readthrough and prevent accumulation of aberrant C-terminally extended polypeptides [21,28].

Collectively, these findings illustrate that stop codon identity and its immediate sequence context act in concert to define termination efficiency, reflecting the interplay between eRF1 specificity, local mRNA architecture, and near-cognate tRNA competition.

Downstream sequence context: the +5 to +9 positions

Beyond the immediate +4 nucleotide, additional downstream bases play a role in modulating translation termination efficiency [29]. Systematic reporter assays testing all 64 tetranucleotide combinations for each stop codon in mammalian cells revealed that nucleotides at the +5 and +6 positions can either stabilize or weaken the termination signal depending on their identity and surrounding context [30]. For the most efficient stop codon, UAA, termination was enhanced when adenine or cytosine occupied the +5 position, whereas guanine or uracil tended to reduce efficiency (Figure 1) [30]. In contrast, UAG and UGA displayed distinct response patterns, suggesting codon-specific interactions with eRF1. For UAG, combinations of dissimilar purines or pyrimidines at +4 and +5 (e.g., +4U followed by +5C) generally weakened termination, whereas identical base pairs (e.g., +4C +5C) promoted more efficient translation termination (Figure 1) [30]. UGA, the most readthrough-prone codon [21,25], was particularly sensitive to both +5 and +6 nucleotides: a +5C could modestly improve termination, while a +6C often exacerbated readthrough (Figure 1) [30].

Reporter-based assays have been instrumental in defining the contribution of these downstream nucleotides, yet they are inherently limited by experimental throughput [31]. Furthermore, synthetic constructs may not fully recapitulate the complexity of endogenous termination environments [23]. Ribosome profiling has addressed these limitations by providing genome-wide measurements of stop codon readthrough in human cells. These analyses revealed that the presence of adenine or uracil at the +5 position increases the likelihood of readthrough, whereas cytosine and guanine promote efficient termination (Figure 1) [23]. The broad enrichment of cytosine and guanine nucleotides within the first 20–30 nucleotides of 3′ untranslated regions (3′UTRs) across eukaryotes likely reflects evolutionary pressure to maintain termination fidelity [23].

The influence of the downstream context extends beyond the immediate +4 to +6 nucleotides into the +7 to +9 region, which interacts with the outer segments of the mRNA channel, where the transcript contacts ribosomal proteins rather than rRNA [30,32]. Experimental variation of these distal bases revealed substantial effects on termination efficiency, consistent with observations in yeast and plant viral systems, where mutations at similar positions modulate readthrough [6]. Among these residues, the +7 and +8 nucleotides proved most influential, with +8 exerting the strongest effect on signal strength. At this position, cytosine failed to restore efficient termination, whereas uracil markedly enhanced it, and adenine or guanine produced intermediate outcomes (Figure 1) [30].

Recent genome-wide and predictive analyses further highlighted the contribution of distal downstream nucleotides to translational fidelity. In a comprehensive study combining ribosome profiling data with machine-learning models, the +9 position was identified as a significant determinant of readthrough efficiency in human cells [33]. Nucleotide identity at +9 ranked among the most predictive features for readthrough propensity, comparable in weight to the +4 site. In particular, adenine or uracil at +9 correlated with increased readthrough, whereas cytosine and guanine were associated with more efficient termination (Figure 1). These trends were conserved across eukaryotes, including yeast [7], indicating that the regulatory influence of +9 is evolutionarily conserved. Mechanistically, the +9 nucleotide likely resides within the ribosomal mRNA channel, where it may affect mRNA positioning or tRNA dynamics rather than directly altering eRF1 recognition [33]. Functionally, variants carrying readthrough-permissive +9 bases, such as those identified in specific Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) nonsense mutations, exhibit enhanced stop codon suppression in cellular assays, highlighting the potential clinical relevance of distal sequence determinants [33].

Downstream TR motifs

Evidence from several experimental systems indicates that specific sequence motifs positioned immediately downstream of stop codons can influence TR efficiency. Systematic *in vitro* selection, ribosome profiling, and cell-based assays have identified several high-efficiency readthrough motifs conserved across viral and mammalian mRNAs. A classic example is provided by the tobacco mosaic virus (TMV), which achieves 5–10% readthrough through the hexanucleotide sequence CAAUUA positioned directly downstream of a UAG stop codon (Figure 1) [6]. Independent ribosome profiling confirmed these findings, revealing the TMV CAAUUA element as the strongest readthrough-promoting hexanucleotide signal downstream of a stop codon [23]. Comprehensive analyses by Anzalone and colleagues [34] systematically explored how downstream sequences and structures regulate readthrough using a combination of *in vitro* selection and cell-based validation. In their mammalian mRNA display screen, performed in rabbit reticulocyte lysate with a UAG stop codon library, several potent readthrough-promoting motifs emerged, including the TMV-like signal. Notably, CAGACUCCCG, CGCCAGR, and CUAUCCG emerged as among the most potent sequences (Figure 1) [34]. When tested in yeast, these motifs displayed codon-specific activity, following the hierarchy UGA > UAG > UAA, with CAGACUCCCG sustaining robust readthrough across all three codons. Other motifs, such as CGCCAGR and CUAUCCG, were most active in the context of UGA and UAA (Figure 1) [34]. In human cells, several endogenous transcripts harboring these motifs, including *CDKN2B*, *LEPROTL1*, *PVRL3*, and *SFTA2*, demonstrated measurable readthrough, producing C-terminally extended protein isoforms [34]. Complementary *in silico* regression analyses identified TR-promoting nucleotide contexts in more than 50 human genes, with experimental validation confirming TR in six of them across multiple cell types. Among these, the CUAG motif downstream of a UGA stop codon conferred particularly high readthrough propensity, and deletion experiments demonstrated that this element is a determinant of TR efficiency (Figure 1) [9,35].

Collectively, these findings establish that the stop codon and its extended downstream region operate as an integrated structural and regulatory unit. The mRNA context extending at least nine to twelve nucleotides beyond the stop codon fine-tunes the balance between termination fidelity and readthrough efficiency, acting through a

coordinated network of sequence, structural, and positional cues that modulate eRF1 engagement and ribosomal dynamics.

Upstream sequence context: the −1 to −9 positions

In mammalian cells, the nucleotides immediately upstream of the stop exert a measurable influence on TR. Experiments in mouse fibroblasts (NIH3T3) and HEK293T cells showed that TR was highest when the first upstream position (−1) was occupied by adenine or guanine, whereas uracil at this site correlated with reduced readthrough (Figure 1) [9,36]. Similar sequence preferences have been observed in yeast, where adenine at positions −1 and −2 enhances TR, particularly for the UAG, but also for UAA and UGA (Figure 1) [28,37]. These findings indicate that purine enrichment directly 5′ adjacent to the stop codon promotes readthrough across eukaryotic species, likely by stabilizing near-cognate tRNA pairing or modulating eRF1 recognition efficiency. However, contrasting results have been reported under drug-induced conditions. In an analysis of 66 mRNA sequences containing stop codons, gentamicin treatment enhanced readthrough most strongly when uracil occupied the −1 position, with the consensus U–stop–C motif systematically associated with induced readthrough levels exceeding 0.5% (Figure 1) [38].

Beyond the −1 and −2 positions, the broader upstream region (extending to −9) also shapes termination kinetics. High-resolution ribosome profiling and reporter assays revealed that distinct nucleotide compositions in this region modulate ribosome pausing, eRF1 engagement, and readthrough propensity. GA-rich motifs immediately upstream of the stop codon tend to suppress downstream translation, whereas C-rich sequences promote ribosomal sliding past the stop codon, leading to translation within the 3′ UTR (Figure 1) [39]. Reporter analyses further indicated that a C triplet directly preceding the stop codon is among the least effective contexts for triggering 3′UTR translation, suggesting that sequence specificity in this region likely operates upstream of the ribosomal E-site [39]. Notably, out-of-frame stop codons are disproportionately enriched for C-rich upstream sequences, implying an evolutionary advantage to promoting termination pausing at annotated stop codons, presumably to minimize readthrough and ensure translational fidelity [39].

Mechanistically, structural and mutational results suggest that nucleotides located immediately upstream of the stop codon (positions −9 to −3) can base-pair with the 3′ end of 18S rRNA within the mRNA channel of the 40S subunit (Figure 1) [39]. This transient interaction aligns the mRNA for accurate engagement of eRF1 and stabilizes the ribosome during the final decoding step. Mutational disruption of the U-rich tail of 18S rRNA alters this communication, reversing sequence preferences for ribosome pausing and shifting termination fidelity [39]. These findings reveal that the mRNA:rRNA interface acts as a checkpoint that transiently slows mRNA translocation after decoding, effectively prolonging ribosome dwell time at stop codons and providing an extended window for eRF1 loading and peptide release [39]. The ribosomal protein eS26 further modulates this process by bridging the mRNA segment upstream of the E-site with the 3′ end of 18S rRNA (Figure 1). Structural modeling shows that eS26 occupies a position that restricts direct mRNA:rRNA pairing. Consequently, loss or depletion of eS26 enhances complementarity-driven mRNA:rRNA contact and increases termination pausing, whereas its overexpression suppresses pausing at GA-rich contexts [39].

Together, these findings establish the upstream stop codon context as an integral *cis*-regulatory module that modulates termination kinetics, eRF1 access, and the likelihood of readthrough or ribosome sliding into the 3′ untranslated region.

Influence of the 5′ sequence on TR

Although downstream nucleotides following the stop codon play a role in modulating TR, recent systematic analyses reveal that the upstream (5′) sequence can exert an even greater influence. Using dual-reporter assays, Smoljanow and colleagues [40] compared the effects of interchanging 5′ and 3′ regions among the functional readthrough genes *MDH1*, *AQP4*, and *LDHB*, whose transcripts share the TR-promoting motif UGA-CUAG. Despite this common motif, reporter constructs containing these sequence contexts display markedly different readthrough efficiencies. Swapping upstream regions between constructs consistently produced more pronounced changes in TR than exchanging downstream segments, demonstrating that nucleotides preceding the stop codon act as dominant regulators of termination fidelity [40]. In particular, the 5′ context of *MDH1* strongly promoted readthrough when transferred to other reporter constructs, whereas replacing it with sequences from *AQP4* or *LDHB* reduced TR. This asymmetry suggests that upstream nucleotides modulate termination efficiency through mechanisms consistent with those described by Jia and colleagues [39]: the −9 to −3 region can transiently base-pair with the 3′ end of 18S rRNA within the mRNA channel, influencing ribosomal alignment, the rate of translocation, and the window available for eRF1 engagement. Variations in the 5′ context may therefore alter the stability or geometry of this mRNA–rRNA interface, shifting the balance between accurate termination and near-cognate tRNA accommodation. Together, these findings identify the 5′ region as a major determinant of readthrough efficiency and underscore that the regulatory landscape of stop codon recognition extends well beyond downstream motifs alone.

Context-dependent effects of nucleotide substitutions

Among the three analyzed genes, *MDH1* displayed the highest readthrough efficiency, followed by *AQP4*, whereas *LDHB* exhibited the lowest [40]. *MDH1* and *AQP4* share identical nucleotides downstream of the stop codon (+8, +11, and +12) that differ from those in *LDHB* (Figure 2). Variations are also present upstream at position -9, -8, -6, and -5 (Figure 2), prompting investigation into whether these sequence differences account for their contrasting TR efficiencies. To dissect these contributions, Smoljanow and colleagues [40] systematically exchanged single nucleotides and nucleotide pairs between the high-TR *MDH1* and low-TR *LDHB* contexts.

Mutational analyses revealed that even minimal sequence changes can alter readthrough efficiency, often in a non-linear and transcript-specific manner. Substituting pairs of nucleotides in the upstream region produced disproportionately large, and sometimes opposing, effects compared with single-base changes. For example, replacing the *MDH1* dinucleotide TC → AA at positions −9 and −8 markedly increased TR, whereas single substitutions at either position reduced or had no effect, indicating strong positional interdependence (Figure 2). Similarly, the exchange TC → GA at positions −6 and −5 sharply decreased readthrough in *MDH1*, while the reciprocal GA → TC substitution in *LDHB* enhanced TR, underscoring the context-specific influence of these upstream bases (Figure 2) [40].

Comparable trends were observed downstream of the stop codon: double substitutions at positions +11 and +12 substantially enhanced readthrough in both contexts, whereas single changes at these sites had a negligible impact (Figure 2) [40]. The effect was even more pronounced in *MDH1*: substitution of adenine with thymine at +8 further increased TR, and reciprocal changes at +8 and +11/+12 in *LDHB* produced comparable enhancements (Figure 2) [40]. Moreover, combining TR-promoting substitutions from both upstream and downstream regions did not further increase efficiency, suggesting non-additive or compensatory interactions between these elements.

Collectively, these findings underscore the combinatorial and interdependent nature of the stop codon context, where coordinated interactions among adjacent upstream and downstream nucleotides fine-tune termination fidelity and ultimately shape the propensity for TR.

Sequence rather than amino acid identity determines TR

Several studies have demonstrated that the nucleotide composition surrounding a stop codon, rather than the identity of the encoded amino acid, plays a central role in determining TR efficiency [36,39,40]. Analysis of the *MDH1* gene showed that altering nucleotides at positions –9 to –7 from TCC to TCT (both coding for serine) produced a significant increase in readthrough when the TCT codon was present (Figure 2) [40]. In contrast, substitutions that changed the encoded amino acid did not produce consistent effects on readthrough levels [40]. These observations suggest that codon identity, rather than the amino acid incorporated before termination, is the main determinant of readthrough

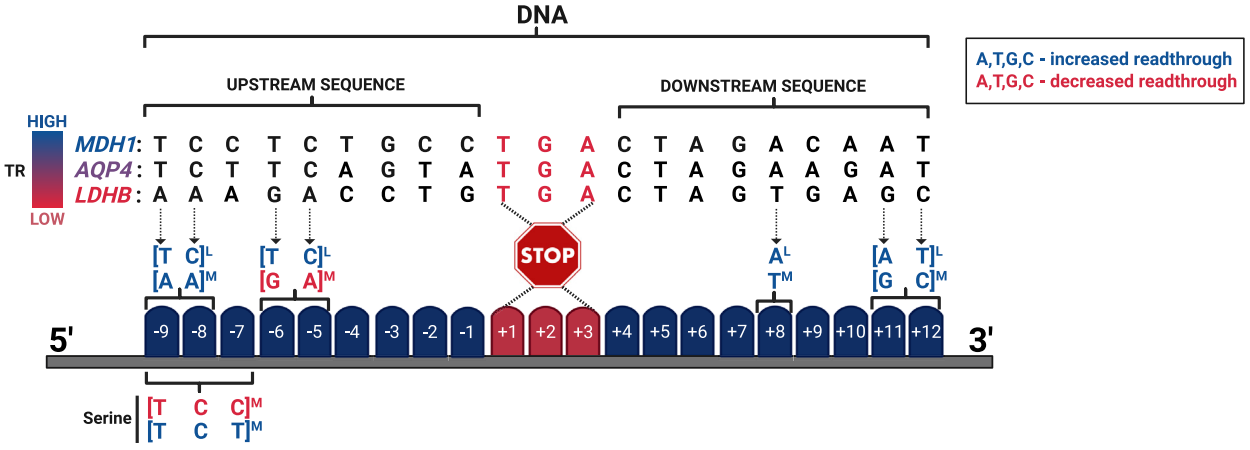

Figure 2. Context-dependent effects of nucleotide substitutions in transcripts with naturally high TR levels. Comparative analysis of *MDH1*, *AQP4*, and *LDHB*, three genes that exhibit intrinsically high levels of TR, illustrates how specific nucleotide substitutions in the vicinity of the stop codon modulate readthrough efficiency in a sequence- and transcript-dependent manner. Superscript labels indicate the experimental background in which a given nucleotide was tested: ^{M}, sequence elements from *LDHB* inserted into the *MDH1* context; ^{L}, sequence elements from *MDH1* inserted into the *LDHB* context. Nucleotides surrounded by [ ] denote specific sequence motifs evaluated in mutational analyses.

efficiency. This interpretation aligns with previous work in mouse cells, which found that the codon immediately upstream of the stop codon exerted no amino acid–dependent influence on readthrough [36]. Supporting evidence from Jia and colleagues [39] further indicates that mRNA sequence composition, rather than the translated peptide, governs ribosome dynamics near stop codons. Their analysis showed that (i) positional effects of surrounding sequences do not follow codon triplet boundaries, and (ii) fusion-protein assays reveal minimal contribution of the translated amino acid to termination behavior [39]. Collectively, these findings support the view that local mRNA sequence context, rather than peptide composition, shapes ribosomal kinetics at stop codons and thereby modulates the efficiency of TR.

While the amino acid sequence before the stop codon seems to have little effect on TR, the amino acid immediately preceding a PTC has recently been shown to strongly affect NMD [41]. Glycine codons were found enriched upstream of PTCs and associated with increased NMD efficiency, while they are depleted before normal termination codons [41]. Furthermore, there is evidence that specific amino acids preceding stop codons influence translation termination efficiency. For example, proline encoded by the CCC codon suppresses translation termination in eukaryotes during the synthesis of long proteins [42]. Although these studies did not directly examine the effect of the last amino acid codon on PTC readthrough, it would be interesting to determine whether increased NMD activity or altered termination efficiency associated with specific amino acids correlates with changes in readthrough levels.

Tissue- and age-dependent TR

Emerging evidence indicates that regulated TR in eukaryotic gene expression is far more widespread and biologically significant than previously recognized. High-resolution ribosome profiling in mice revealed cell-type-specific patterns of readthrough, demonstrating that termination fidelity is not uniform across tissues but can be modulated in a physiological context [43]. These findings suggest that TR is integrated into broader tissue-specific translational control programs that operate across diverse metazoan cell types.

This phenomenon is most extensively documented in *Drosophila*, where numerous genes, among them many with neural functions, have been predicted or experimentally validated to undergo developmentally regulated stop codon readthrough [44–46]. Most *Drosophila* genes predicted to undergo TR encode regulatory or signaling proteins, implying that the addition of a C-terminal extension could offer context-dependent functional flexibility [47]. Computational studies further indicate that these readthrough-derived isoforms tend to be large, multidomain proteins whose extended regions are intrinsically disordered and compositionally simple [48,49]. Such unstructured C-terminal tails may enhance conformational adaptability, enabling the extended isoforms to engage in new molecular interactions or adopt alternative functions without disrupting the core structure of the native protein [47].

Functional and reporter-based assays have revealed cell- and tissue-specific differences in TR. Neurons display robust readthrough activity, whereas glial cells and endocrine tissues such as the prothoracic gland show little or no detectable TR [50]. These observations are consistent with the notion that the central nervous system (CNS) supports high-efficiency, developmentally controlled readthrough, potentially expanding proteomic diversity during neural differentiation [51]. This variability is further refined at the level of neuronal subtypes, with cholinergic neurons exhibiting the highest TR and dopaminergic neurons the lowest [52]. In contrast, tissues of the reproductive system generally display minimal readthrough activity [50]. A notable example is the *kelch* mRNA, which is abundantly expressed in ovarian cells yet shows virtually no readthrough, despite possessing a downstream sequence context that is permissive for termination bypass in other cellular environments [50].

TR variability between neuronal and reproductive tissues is further exemplified by a well-characterized case of tissue-specific readthrough in *Drosophila*: the *traffic jam* (*tj*) gene, which produces the high-efficiency readthrough isoform *tj-TR* [47]. This extended isoform is generated in a spatially restricted and translationally regulated manner. Its expression is advantageous in the nervous system, where it may contribute to neuronal differentiation or function, yet it is detrimental in the gonads, where misexpression leads to developmental abnormalities [47]. These findings underscore the importance of maintaining tissue-specific control of translational fidelity. Variations in the abundance of termination factors appear to underlie these differences. Notably, the splice variant eRF1H, an isoform of eRF1, shows tissue-specific expression patterns that correlate with enhanced readthrough at leaky stop codon contexts [47]. This association points to a possible mechanism by which alternative eRF1 isoform usage contributes to the modulation of termination efficiency in distinct cellular environments.

Comparable principles appear to govern termination dynamics in mammals. Ribosome profiling across mouse tissues demonstrated striking tissue-specific differences in ribosome behavior at stop codons: testis displayed pronounced ribosome pausing, whereas termination peaks were barely detectable in heart, liver, or brain, despite similar mRNA sequence contexts [39]. Independent studies likewise

reported a markedly reduced stop codon peak in mouse liver [53]. These observations indicate that termination kinetics vary across tissues and are shaped not solely by the local mRNA sequence but also by *trans*-acting regulatory mechanisms. This difference has been attributed to the ribosomal protein eS26. In testis, eS26 is strongly depleted, and its absence is predicted to strengthen the mRNA–rRNA interaction interface, thereby stabilizing the terminating ribosome and promoting prolonged pausing at the stop codon. This extended dwell time enforces accurate termination and suppresses readthrough. By contrast, higher eS26 abundance in somatic tissues restricts the mRNA–rRNA contact surface, reducing termination pausing and consequently increasing the likelihood of readthrough [39].

Recent studies further suggest that translation fidelity declines with age. In mice [54], *in vivo* and *ex vivo* analyses demonstrate an age-related increase in stop codon readthrough, particularly in post-mitotic tissues such as muscle and brain, but not in liver [54]. Similar findings have been reported in *Drosophila*: readthrough rates vary across life [52,55] and are highest in older adults [52]. These observations imply that age-associated ribosomal errors, including stop codon readthrough, contribute to a proteostatic decline and may play a role in the aging process. The molecular basis of this decline remains unresolved but is likely multifactorial, involving cumulative changes in ribosomal composition, tRNA modification, and translational factor stoichiometry.

Overall, findings from *Drosophila* and mammalian systems demonstrate that TR is subject to tissue-, developmental-, and age-dependent regulation. This variation indicates that *cis*-acting sequence signals alone are insufficient to dictate readthrough efficiency and that *trans*-acting factors, including release factor isoforms, RNA-binding proteins, tRNA pools, and ribosomal protein composition, play central roles in modulating termination fidelity. Understanding how these factors converge to shape cell- and age-specific translation outcomes remains a major challenge for future research.

Other factors influencing TR

Compared with the basal level of TR at natural stop codons, readthrough at PTCs can be an order of magnitude higher, underscoring the notion that premature termination differs mechanistically from normal translation termination [24,36,56]. In the preceding sections, we discussed several parameters that contribute to this difference, including mRNA sequence context and tissue-dependent modulation of termination fidelity. However, accumulating evidence indicates that a variety of additional molecular and structural factors also influence readthrough efficiency. These determinants include the length and composition of the 3′ UTR, ribosome pausing dynamics at stop codons, availability of near-cognate tRNAs, and levels of eRF1 and eRF3. Interestingly, some of these parameters, particularly 3′ UTR length, have been associated with opposing effects on readthrough across studies, reflecting the complexity and context dependence of termination control. In Table 1, we summarize the major *cis*- and *trans*-acting factors reported to modulate TR under physiological and experimental conditions.

TR as a therapeutic approach for diseases caused by nonsense mutations

PTCs account for approximately 11% of all pathogenic single-base substitutions documented in the Human Gene Mutation Database [13], and their number continues to rise as genome sequencing efforts expand [69,70]. Well-characterized examples include cystic fibrosis (CF) and Duchenne muscular dystrophy (DMD), where nonsense variants in the *CFTR* and *DMD* genes, respectively, result in truncated, non-functional proteins [14,71]. Although each individual disorder is rare, cumulatively, nonsense mutations affect millions of patients worldwide [8], contributing to a broad spectrum of inherited metabolic, neuromuscular, and neurodevelopmental diseases, as well as to malignant transformation when they occur in tumor-suppressor genes [13,72].

A central therapeutic strategy to counteract these mutations is the induction of TR. Multiple approaches have been developed to achieve PTC readthrough, including suppressor tRNAs. Of these, small molecules have emerged as the most intensively pursued avenue, owing to their pharmacological tractability, suitability for systemic administration, and potential to be optimized for potency and selectivity. Despite notable progress, achieving efficient and selective PTC readthrough in human cells remains a formidable challenge. Many compounds that promote readthrough also perturb normal termination or induce off-target effects on global translation, limiting their therapeutic window and clinical applicability [73].

To address these limitations, recent efforts have focused on elucidating the molecular mechanisms by which readthrough-inducing agents interact with the eukaryotic ribosome and translation machinery [74–76]. Structural and biochemical studies are beginning to clarify how distinct chemical scaffolds influence decoding fidelity, ribosomal conformational dynamics, and release factor engagement. Such mechanistic insights will be essential for the rational development of next-generation readthrough therapeutics with improved efficacy and reduced toxicity. In the following section, we summarize recent high-resolution structural

Table 1 Molecular factors influencing stop codon readthrough, including *cis*-acting sequence features and *trans*-acting regulators.

FACTORS INFLUENCING TR	
Trans-acting factors	Function/effect
eRF1 concentration/availability	Reduced eRF1 levels preferentially increase readthrough (particularly UGA), indicating that release factor availability can be rate-limiting for efficient termination [30].
Distribution of a release factor splice variant, eRF1H,	In *Drosophila*, the eRF1H isoform plays a critical role in modulating differential TR at leaky stop codon contexts. eRF1H is most abundant in head tissues, where it constitutes roughly one-third of the total eRF1 pool, coinciding with the highest readthrough levels observed *in vivo*, reaching ~20% for the *tj* gene [47].
eRF3 concentration/availability	Depletion of eRF3 exerts minimal effect on readthrough in HEK293 cells, but in HeLa cells it enhances readthrough at all three stop codons [57].
Initiation factor eIF3	Enhances readthrough specifically at weak termination contexts, likely by promoting the accommodation of near-cognate tRNAs at the A-site [58,59].
	eIF3 induces conformational changes in the ribosomal A-site that facilitate accommodation of either eRF1, promoting efficient termination, or near-cognate tRNA, enabling readthrough when eRFs are limiting. Reduced eRF availability, together with eIF3-mediated A-site remodeling, therefore favors near-cognate tRNA accommodation [60].
RNA-binding proteins (RBPs)	Defined *cis*-acting elements downstream of the stop codon (e.g., the 63-nt Ax element in VEGFA) recruit RNA-binding proteins such as hnRNP A2/B1 to program TR and produce functional C-terminally extended isoforms [61].
ABCE1	ABCE1 knockdown enhances the rate of readthrough [62].
Ribosomal proteins	uS12, which contacts the mRNA backbone near the +4 position, modulates readthrough efficiency in a base-dependent manner [26].
	eS26 prevents excessive readthrough by restricting 18S rRNA interactions with upstream GA-rich mRNA sequences [39].
	Arg10 of eS30 appears to sense the 28:42 base pair within the anticodon stem of the near-cognate $tRNA^{Gln}_{CUG}$. Substitution of Arg10 with neutral or acidic residues (Ala or Asp) abolishes UAG readthrough, whereas the conservative Arg to Lys change maintains it. These observations suggest that a positively charged side chain at this position is required to form stabilizing electrostatic contacts with the 28:42 base pair, thereby facilitating accommodation of the near-cognate tRNA in the A-site and enabling readthrough [63].
	eS25 deletion or truncation of its N-terminal residues prevented $tRNA^{Tyr}_{GUA}$ from stimulating readthrough [63].
Availability/identity of near-cognate tRNAs	Increased abundance or favorable pairing properties of near-cognate tRNAs enhance the likelihood of stop codon decoding; specific near-cognate tRNAs (e.g., Cys-tRNA recognizing UGA) can efficiently promote TR [56,58,64].
tRNA backbone	A $tRNA^{Trp}_{CCA}$ with a shortened anticodon stem (4 bp instead of the canonical 5), enhances UGA readthrough [65].
***Cis-acting* factors**	**Function/Effect**
RNA secondary structures downstream of the stop codon	3′-proximal stemloop or pseudoknot structures downstream of the stop codon enhance TR in multiple endogenous transcripts (e.g., *Drosophila kelch* [50] and *headcase* [51]), illustrating a conserved structural mechanism of termination modulation.
RNA modifications	Pseudouridylation [the conversion of uridine to pseudouridine (Ψ)] within stop codons suppresses translation termination and promotes readthrough both *in vitro* and *in vivo* [66].
3′ UTR stem-loop motifs	Unbiased *in vitro* selection identified 3′ UTR stem-loop motifs that enhance TR across diverse eukaryotic systems [34].
3′ UTR length	Longer 3′ UTRs correlate with higher TR [33].
	Genome-wide analyses of *Drosophila* readthrough genes showed no association between 3′ UTR length and readthrough efficiency. For instance, the *kelch* mRNA, which is highly expressed in ovarian cells, exhibits minimal readthrough despite possessing a 3′ UTR longer than 3000 nucleotides, whereas high-efficiency readthrough is observed predominantly in neurons [50].
mRNA:rRNA	The mRNA:rRNA interface functions as a regulatory checkpoint that transiently slows mRNA translocation after decoding, prolonging ribosome dwell time at stop codons and thereby extending the window for eRF1 recruitment and peptide release [39].
Proximity to the polyA tail	Proximity of the stop codon to the mRNA 3′ end suppresses readthrough [67,68].

advances that illuminate how these compounds bind to the eukaryotic ribosome and modulate TR.

Aminoglycosides

The discovery that aminoglycosides can modulate the fidelity of translation marked the first major advance toward targeted suppression of PTCs. Historically characterized as antibacterial drugs, these compounds exert their antimicrobial activity by binding with high affinity to the bacterial ribosomal DC, where they promote codon misreading. In contrast, their effects on eukaryotic ribosomes are considerably weaker [77]. A central determinant of aminoglycoside binding specificity is the identity of 16S rRNA nucleotide 1408 (*Escherichia coli*) in the decoding site. In bacteria, this position is occupied by A1408, a residue that forms critical contacts with many aminoglycosides. By contrast, the equivalent position in the eukaryotic DC contains a guanine, which disrupts these interactions [78]. Consistent with this structural divergence, introducing an A1408G substitution into the bacterial ribosome markedly reduces aminoglycoside affinity [77]. This key nucleotide difference is therefore thought to contribute substantially to the selective inhibition of bacterial, but not eukaryotic, ribosomes, underpinning the clinical utility of aminoglycosides as antibiotics [79]. Early proof-of-concept experiments established that aminoglycosides stimulate nonsense suppression in eukaryotes, restoring partial expression of full-length proteins in several genetic disease models, including CF [69–73].

Geneticin 418

Among the known aminoglycosides, geneticin (GEN) acts as a PTC readthrough promoter, although it also acts as a general translation inhibitor by blocking elongation [80]. GEN efficiently restores expression of genes harboring nonsense mutations [23,81], but its clinical use is limited by dose-dependent toxicity [82]. Structural analyses using *Candida albicans* ribosomes, whose DC is highly conserved with those of *Saccharomyces cerevisiae* and humans [83], have revealed the precise mode of GEN binding [75]. At a concentration of 250 μM, and resolved at 3.0 Å, GEN inserts deeply into the DC pocket of the small ribosomal subunit [75]. The compound stacks against nucleotide A1741 of the 18S rRNA and forms seven hydrogen bonds with G1629, A1743, G1744, and U1745, creating a tightly stabilized network of interactions with 18S rRNA. Upon binding, GEN induces several local rearrangements: nucleotides A1742 and A1743 in helix h44 flip outward and stack together, while A1741 rotates by about 20° toward the drug, strengthening its π–π interaction [75].

These conformational changes closely mimic those that naturally occur during tRNA decoding. In the unbound ribosome, two highly conserved adenosines, specifically A1755 and A1756 in *Saccharomyces cerevisiae* [84] (A1492 and A1493 in bacteria [32,85]), act as molecular sensors that toggle between "in" and "out" positions depending on whether a tRNA occupies the A-site [86]. When flipped "out," these adenosines stabilize correct codon–anticodon pairing through A-minor interactions with the mRNA–tRNA helix [86,87], which promotes domain closure of the small subunit, a hallmark conformational change that locks the DC around a cognate tRNA [88]. This structural rearrangement is specific to the correct tRNA selection and is absent when eRF1 engages a stop codon [20]. Similarly, by stabilizing a decoding-competent conformation through the outward flipping of A1742 and A1743, GEN effectively lowers the fidelity threshold of the DC, rendering the ribosome more permissive to near-cognate tRNAs that can mispair with stop codons (Figure 3). This structural mimicry allows the ribosome to insert an amino acid at a PTC and resume elongation, thereby promoting readthrough.

Paromomycin

Paromomycin (PAR) is a well-characterized aminoglycoside originally developed as an antimicrobial agent against *Leishmania* infections [89]. Beyond its antiparasitic activity, PAR has attracted attention for its ability to promote PTC readthrough in genetic diseases [23,81]. Although its readthrough potency is lower than that of GEN, PAR is distinguished by its substantially lower toxicity in humans [90], making it a valuable scaffold for the development of safer next-generation readthrough inducers [91]. Recent high-resolution crystal structures of PAR bound to eukaryotic ribosomes from *Candida albicans* have provided insight into its mode of action. It revealed that PAR binds at multiple locations, primarily within the large ribosomal subunit rather than the DC. At concentrations below 250 μM, no binding was detected, whereas at 250 μM, PAR occupied several sites along the rRNA backbone, contacting phosphate groups and bases between helices H66 and H75 and replacing a Mg^{2+} ion via multiple hydrogen bonds [75]. Binding to the DC was observed only at higher concentrations (≥500 μM), where PAR formed stacking interactions with A1741 and seven hydrogen bonds with nearby nucleotides, inducing the flipping of adenosines A1742 and A1743 (h44) from their "in" to "out" conformations, similar to the rearrangements caused by GEN (Figure 3) [75]. These findings suggest that PAR stimulates readthrough by promoting conformational changes in the ribosome that favor near-cognate tRNA accommodation and altered subunit dynamics. However, the precise mechanism remains unresolved. It is not yet clear whether the observed conformational shifts originate directly from PAR binding at the DC or involve additional

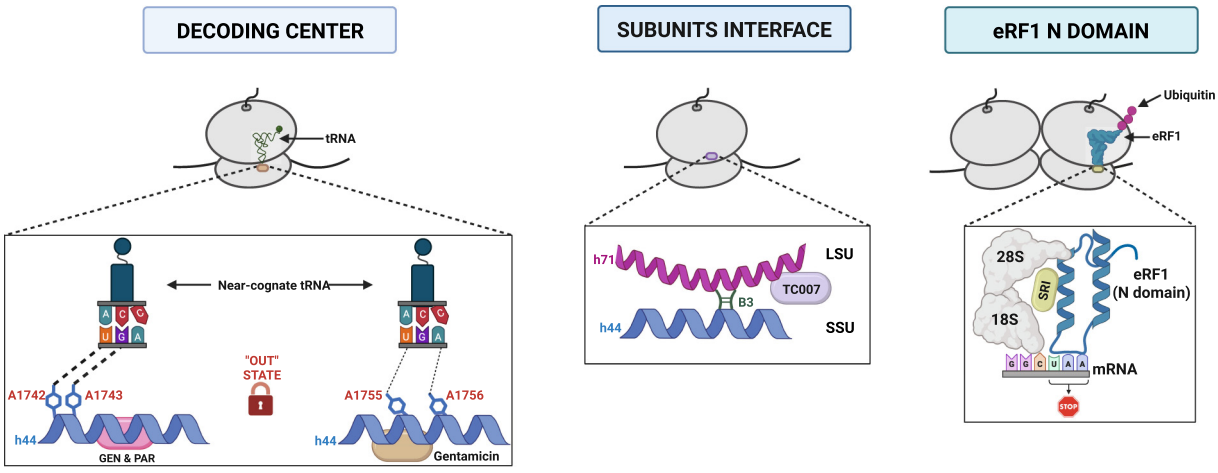

Figure 3. Illustration of the binding sites of various PTC readthrough-promoting molecules in the ribosome. The distinct binding sites and modes of action of small molecules that enhance PTC readthrough on the eukaryotic ribosome are schematically depicted. (Left panel) Geneticin (GEN) and paromomycin (PAR) bind deeply within the decoding center, flipping out the highly conserved A1742 and A1743 (numbering based on human 18S rRNA) and thereby facilitating near-cognate tRNA accommodation. Gentamicin binds a similar region of the decoding center but induces only a partial flipping of the 18S rRNA bases A1755 and A1756, resulting in weaker miscoding activity. (Middle panel) The synthetic aminoglycoside TC007 binds near the interface of the small (SSU) and large ribosomal subunits (LSU), close to bridge B3, where it slows intersubunit rotation and stabilizes conformations that favor tRNA over eRF accommodation. (Right panel) SRI-41315 occupies a pocket between the N domain of eRF1 and the 18S and 28S rRNAs, effectively "gluing" eRF1 to the ribosome and promoting its ubiquitylation and proteasomal degradation, thereby enhancing readthrough through release factor depletion.

secondary binding sites that collectively influence ribosomal structure and readthrough efficiency [75].

Gentamicin

Among all aminoglycosides, gentamicin is the most extensively studied compound *in vitro* [92]. In preclinical models, gentamicin has been shown to promote readthrough of *COL17A1* and *LAMB3* nonsense mutations associated with epidermolysis bullosa, partially restoring protein expression and function [92,93]. High-resolution structural studies of the *Saccharomyces cerevisiae* 80S ribosome complexed with gentamicin [76], solved at 3.4 Å resolution, revealed that the drug binds to the DC of the small ribosomal subunit but in a slightly shifted orientation compared with other aminoglycosides such as PAR and GEN. The unique 6′-amino substituent on gentamicin introduces steric and electrostatic repulsion with G1645 of 18S rRNA, preventing the compound from inserting deeply into the DC pocket [76]. As a result, gentamicin cannot form the extensive stacking interactions that normally stabilize the adenosines A1755 and A1756 in their fully "flipped-out" positions, the configuration that supports accurate tRNA decoding and promotes readthrough. In gentamicin-bound ribosomes, these adenosines are only partially extruded (Figure 3) [76], producing a less stable and less "decoding-ready" conformation of the DC. Functionally, this means that the ribosome is only weakly biased toward accepting near-cognate tRNAs, explaining gentamicin's lower readthrough potency compared with GEN or PAR, which engage more deeply and fully stabilize the decoding pocket. In addition to its primary binding site, gentamicin interacts with secondary ribosomal regions, including the inter-subunit bridges B2c and B4 and areas adjacent to the mRNA–tRNA complex [76]. These auxiliary interactions likely reflect the high gentamicin concentration (4 mM) required to capture the complex structurally, which may enhance nonspecific or low-affinity binding events.

TC007

Spinal muscular atrophy (SMA) is one of the leading genetic causes of infant mortality and results from the loss of full-length Survival of Motor Neuron (SMN) protein, for which no curative treatment currently exists [94,95]. Screening for small molecules capable of suppressing PTCs identified TC007 as a potential therapeutic candidate. In preclinical studies, TC007 exhibited a favorable safety profile, extended survival in SMA mouse models, and partially mitigated disease severity [96]. Structural analysis of the *Saccharomyces cerevisiae* 80S ribosome in complex with TC007 [76], resolved at 3.7 Å, revealed an unusual binding mode distinct from that of other aminoglycosides. Even at high concentrations (4 mM), TC007 does not bind within the DC. Instead, positive electron density was observed beneath the DC, at the interface between helix h44 of 18S rRNA and helix H71

of 25S rRNA, near bridge B3 [76], a structural contact that mediates rotational movement between the small and large ribosomal subunits (Figure 3) [97,98]. This peripheral location indicates that TC007 acts allosterically, altering inter-subunit communication rather than directly contacting the decoding nucleotides. Functionally, TC007 appears to stabilize a partially rotated conformation of the ribosome, a transient intermediate that normally exists just before the release factor binds [99]. During normal termination, the ribosome must transition from the rotated state to the unrotated conformation that enables proper termination factors accommodation and peptide release [100]. By hindering this rotational transition, TC007 effectively delays eRF1 binding and extends the lifetime of the rotated state. In this configuration, the A-site remains accessible, allowing near-cognate tRNAs to compete more successfully with eRF1 for decoding of the stop codon. As a result, the ribosome incorporates an amino acid in place of termination, promoting TR.

Limitations of aminoglycosides

Despite their clear mechanistic importance in understanding TR, conventional aminoglycosides exhibit significant limitations that restrict their clinical application. Their ability to promote stop codon suppression in eukaryotic systems is modest, and long-term use is hindered by dose-dependent toxicity and poor selectivity for target sites [101,102]. These drawbacks underscore the pressing need for next-generation nonsense suppression agents that can achieve higher efficacy with improved safety profiles, particularly for diseases such as CF and other PTC-associated disorders [103–105]. One of the principal causes of aminoglycoside-induced cytotoxicity lies in their off-target inhibition of mitochondrial translation [106]. Because mitochondrial ribosomes share structural homology with bacterial ribosomes [76], aminoglycosides inadvertently bind to the mitochondrial DC, impairing respiratory chain function and leading to ototoxicity [106]. GEN also functions as a translation inhibitor, blocking elongation and overall protein synthesis [80]. At a concentration of 5 μg/ml, it reduces the amount of the translated protein by more than 60% [107]. Its therapeutic utility is therefore limited, as clinical dosing required for efficient readthrough overlaps with levels that cause irreversible cellular damage [108]. The relatively low efficiency of PTC readthrough (from less than 1% to 28% depending on the stop codon context [107]) and its high toxicity threshold have constrained the therapeutic window of aminoglycosides [31,109]. Nevertheless, their potential clinical benefit has prompted several proof-of-concept trials. Aminoglycoside-based treatments have been evaluated for CF [110], DMD [111], and dystrophic epidermolysis bullosa [112]. Experimental studies have suggested possible applications in Werner syndrome [113] and certain cancers [72]. Although results demonstrated some degree of phenotypic improvement, none of these compounds has achieved an acceptable balance between readthrough potency and safety. To address these challenges, structural analogs and derivatives have been explored as safer alternatives. The neomycin derivative TC007, for example, has shown efficacy in SMA models, improving survival and motor function in mice and demonstrating favorable toxicity profiles in human fibroblasts [96,109,114]. Yet even with such advances, broad-spectrum aminoglycosides remain constrained by their limited specificity, off-target effects, and poor pharmacokinetic properties. These limitations continue to drive the development of novel, structure-guided molecules designed to selectively modulate eukaryotic ribosomes while minimizing interference with mitochondrial or cytoplasmic translation.

An alternative to aminoglycosides: SRI-41315

High-throughput screening for compounds capable of suppressing *CFTR* nonsense mutations identified two heterocyclic small molecules, SRI-37240 and its derivative SRI-41315 [115]. Unlike the sugar-rich scaffolds of aminoglycosides, these compounds feature a compact pyrimidoquinoline core. The optimized analog, SRI-41315, displayed improved physicochemical stability and greater readthrough potency than its parent compound [115]. Notably, SRI-41315 acted synergistically with GEN, further enhancing PTC suppression efficiency [115]. Mechanistically, SRI-41315 promotes readthrough by reducing the cellular abundance of eRF1 [115]. Studies on two chemically related readthrough-promoting molecules, NVS1.1 and NVS2.1, revealed that this class of compounds induces eRF1 degradation directly from the ribosomal A-site through a surveillance pathway involving the ribosome-collision sensor GCN1 and the E3 ubiquitin ligases RNF25 and RNF14 [116]. In this cascade, RNF25 first ubiquitylates the ribosomal protein eS31 (RPS27A), which in turn triggers the recruitment of RNF14 to the stalled ribosome. Once positioned, RNF14 catalyzes the ubiquitylation of eRF1, targeting it for proteasomal degradation [116]. The resulting intracellular reduction of eRF1 abundance then leads to increased stop codon readthrough [116]. Recent cryo-EM structures of 80S ribosomes from rabbit reticulocyte lysate in complex with SRI-41315 [74] have provided detailed insight into this process. The compound anchors the N-terminal domain of eRF1 at the ribosomal subunit interface, near the stop codon DC, occupying a pocket formed jointly by eRF1, 28S rRNA, and 18S rRNA (Figure 3). SRI-41315 sits within a cavity framed by the α2, β1, and β4 helices of eRF1, the phosphate backbone of U1827 and C1828 of 18S rRNA, and nucleotides

A3759 and 2′-O-methyladenosine 3760 (A2M-3760) of 28S rRNA [117]. By effectively "gluing" eRF1 to the ribosome, SRI-41315 prevents its release and promotes its ubiquitination and subsequent proteasomal degradation. This mechanism is distinct from classical release factor inhibitors, which typically act near the ribosomal exit tunnel [118–120]. The eRF1-degrader function of SRI-41315 parallels that of Ternatin-4, a small molecule that traps the elongation factor eEF1A in the A-site of ribosomes, also triggering RNF14- and RNF25-dependent degradation of the trapped protein [121]. However, Ternatin-4 binds allosterically and does not contact the ribosome directly [122]. Ribosome-profiling data further demonstrated that, unlike GEN, SRI-37240 and SRI-41315 act specifically at PTCs, without affecting termination at normal stop codons [115]. This specificity represents a major pharmacological advantage, as it preserves global translational fidelity [115]. However, despite their potent readthrough activity, SRI-41315 and SRI-37240 display off-target effects on the epithelial sodium channel (ENaC), enhancing sodium transport and potentially exacerbating airway dehydration in cystic fibrosis, which limits their immediate therapeutic use [115]. Nonetheless, the absence of widespread translational toxicity supports the concept that targeted modulation of eRF1 abundance represents a viable and mechanistically distinct strategy for nonsense suppression [115].

eRF3a degraders: CC-885 and CC-90009

An alternative strategy for targeting eRF1 is via the degradation of eRF3a, a core component of the translation termination complex. Two recently characterized small molecules, CC-885 and CC-90009, exhibit potent tumoricidal activity in acute myeloid leukaemia (AML) models [123,124]. Both compounds function as cereblon (CRBN) E3 ubiquitin ligase modulators: they promote the recruitment of eRF3a to CRBN and induce its ubiquitination and proteasomal degradation [123,124]. CC-885 induces eRF3a degradation but also affects additional CRBN substrates, whereas CC-90009 appears more selective, with minimal impact on the broader proteome [125]. CC-90009 was the first eRF3a degrader to enter clinical trials in patients with relapsed or refractory AML [126]. However, it did not advance beyond early-phase evaluation (NCT04336982 and NCT02848001). With respect to TR, neither CC-885 nor CC-90009 significantly increased PTC readthrough when administered alone [125]. Similarly, siRNA-mediated depletion of eRF3a or eRF3b did not induce substantial readthrough in *TP53* nonsense models. However, both compounds enhanced GEN-induced readthrough [125], indicating that depletion of eRF3a sensitizes cells to aminoglycoside-mediated suppression of PTCs rather than acting as an effective monotherapy.

PTC readthrough enhancer: mefloquine

The antimalarial drug mefloquine (MFQ) has recently been identified as a potent enhancer of aminoglycoside-induced PTC readthrough. Initially developed to target the ribosome of *Plasmodium falciparum* [127,128], MFQ was later shown to markedly potentiate the readthrough activity of GEN in mammalian cells [129]. Ferguson and colleagues [129] demonstrated that MFQ alone does not affect translation termination fidelity but significantly enhances GEN-induced readthrough of *TP53* nonsense mutations in human cancer cells [129]. This synergistic effect enables the use of lower aminoglycoside concentrations and reduces associated cytotoxicity, positioning MFQ as a clinically approved and orally bioavailable compound that represents a particularly promising candidate for combination nonsense-suppression therapy.

High-resolution cryo-EM analyses of *Candida albicans* ribosomes revealed MFQ binding sites on the ribosome [75]. The primary site lies in the inter-subunit space between the C-terminal domain of eL43 and helix h23 of 18S rRNA, corresponding to bridge B7b/c, which mediates rotational motion between the small and large ribosomal subunits. MFQ binding at this site shifts the ribosome toward a rotated conformation, altering its normal rotational equilibrium. However, Kolosova and colleagues [75] showed that modulation of ribosomal rotation alone is not sufficient to induce readthrough, as MFQ does not alter the DC. In contrast, TC007, which similarly affects the rotational state of the ribosome, is capable of promoting readthrough on its own. This difference likely reflects the distinct spatial positioning of the two compounds. TC007 binds closer to the DC, near bridge B3, where it is mechanically coupled to the DC through helix h44 [76]. This proximity allows TC007 to subtly influence the local geometry of the DC and delay eRF1 accommodation, thereby extending the time window for near-cognate tRNA competition. MFQ, by contrast, acts further from the DC and thus affects only global rotational dynamics without altering local decoding fidelity. Although both compounds share a broadly similar mode of action, their distance from the DC might account for their differing abilities to induce readthrough.

NMD variability across tissues modulates therapeutic response

Early clinical evaluations [130–134,111] demonstrated restoration of dystrophin or CFTR expression and partial functional correction in subsets of patients in response to gentamicin treatment. However, subsequent studies reported no detectable production of functional full-length protein despite treatment [135–137]. This variability cannot be explained solely by differences in drug exposure or stop codon context. Since many of the disease-

causing mutations lead to the production of mRNAs that contain PTCs, NMD imposes another layer of regulation by reducing the abundance of PTC-containing transcripts and thereby limiting the substrate available for pharmacological readthrough [70].

Evidence for the impact of NMD emerged from studies of patients with CF carrying the W1282X mutation in the *CFTR* gene, a severe allele associated with greatly reduced channel activity [138]. Following topical gentamicin administration to the nasal epithelium, a subset of patients exhibited improved electrophysiological parameters and detectable full-length CFTR protein. In others, no functional correction was observed. Notably, the same W1282X mutation elicited variable NMD efficiency in patient-derived epithelial cells. Similar variability was observed for endogenous NMD substrates, including *RPL3*, *SRSF2* (*SC35* isoforms), *ASNS,* and *CARS1* [138]. Importantly, attenuation of NMD stabilized CFTR nonsense transcripts and enhanced gentamicin-induced chloride transport, demonstrating that transcript abundance can be a limiting determinant of therapeutic efficacy [138].

Tissue-specific variation in NMD efficiency has also been documented in other genetic contexts. In patients carrying PTCs in *COL10A1*, efficient NMD was observed in cartilage but not in non-cartilage cells [139]. Similarly, in fetuses with Roberts syndrome homozygous for PTCs in *ESCO2*, distinct tissues exhibited differential NMD efficiency [140]. Evidence from animal models supports these observations [141]. In a mouse model carrying a truncated version of *MEN1* generated by the deletion of exon 3, which introduces a PTC in exon 4, tissues were segregated into two groups based on NMD efficiency. Testis, ovary, brain, and heart displayed strong degradation of the mutant transcript, whereas lung, intestine, and thymus showed more moderate decay [141]. Notably, these differences did not correlate with expression levels of core NMD factors or with *MEN1* transcript abundance, suggesting that additional regulatory mechanisms underlie tissue-specific NMD activity [142]. Genome-wide analyses have yielded partly divergent conclusions. Some studies report lower NMD efficiency in nervous and reproductive tissues compared with tissues such as the digestive tract [143], whereas others estimate relatively high NMD efficiency across most brain regions, with exceptions including cerebellum, anterior cingulate cortex, hypothalamus, and spinal cord [144]. In contrast, additional investigations, including recent large-scale analyses, found NMD efficiency to be largely consistent across tissues [145,146]. Inter-individual variability in NMD activity has also been described in some cohorts [142,144], although this has not been uniformly observed [146]. Together, these data indicate that NMD efficiency can vary across tissues and individuals, but the extent, determinants, and physiological relevance of this variability remain incompletely resolved.

NMD attenuation as a therapeutic strategy for PTC-associated diseases

Mucopolysaccharidosis type I (MPS I) is a severe lysosomal storage disorder caused by a deficiency of α-L-iduronidase, encoded by *IDUA* [147]. This enzyme is required for the degradation of the glycosaminoglycans heparan sulfate and dermatan sulfate. Nonsense mutations represent the predominant class of pathogenic mutations in *IDUA* (50–70%) and are strongly associated with the most severe form of MPS I, the Hurler phenotype [148]. In a mouse model of MPS I, pharmacological attenuation of NMD using NMDI-1 [149] increased steady-state levels of PTC-containing IDUA transcripts [150]. Although gentamicin-mediated readthrough alone produced modest restoration of α-L-iduronidase activity, co-administration with NMDI-1 significantly enhanced enzymatic activity compared with gentamicin alone [150]. This combinatorial approach resulted in a more pronounced reduction of glycosaminoglycan storage across multiple tissues, including the central nervous system [150]. This represents another example of mRNA abundance being rate-limiting for TR efficacy *in vivo*.

A similar synergy between NMD inhibition and TR has been observed in models of CF [151]. In primary cells from mice carrying the *CFTR* G542X nonsense mutation, inhibition of the NMD kinase SMG1 enhanced the functional rescue achieved with aminoglycosides such as GEN and gentamicin [151]. Functional assays of CFTR channel activity revealed synergistic effects, consistent with increased availability of target transcripts [151].

Collectively, these findings indicate that NMD efficiency is an important determinant of the efficacy of therapeutic PTC suppression approaches. Importantly, subsequent studies suggested that partial, moderate attenuation of NMD is generally tolerated in most somatic tissues [152], raising the possibility that controlled NMD modulation could be used therapeutically. However, given the fundamental role of NMD in transcriptome surveillance and gene regulation [153], the long-term consequences of systemic inhibition must be carefully assessed. Conversely, when the presence of a PTC causes the production of a dominant-negative or toxic truncated protein, maintaining or even enhancing NMD is beneficial, as it limits the synthesis of the toxic protein [154]. In this context, NMD acts as a protective mechanism. Therefore, NMD modulation is broadly applicable but must be tailored to the specific mutation and the functional impact of the truncated protein.

Readthrough-inducing molecules in clinical applications

The development of effective pharmacological agents that promote the readthrough of PTCs remains a major therapeutic objective. One illustrative example is the restoration of the tumor suppressor function through TR in colorectal tumorigenesis. Germline mutations in *APC* cause familial adenomatous polyposis (FAP), the most common hereditary polyposis syndrome predisposing to colorectal cancer [155]. Importantly, most pathogenic *APC* PTCs reside in the final coding exon and therefore evade NMD, preserving mutant transcript abundance [156]. This molecular context renders *APC* an attractive candidate for readthrough-based therapeutic intervention. Among the compounds under clinical evaluation for FAP are the macrolide antibiotics azithromycin and erythromycin, both currently in phase IV trials (Table 2). These agents bind within the ribosomal nascent peptide exit tunnel and inhibit translation in prokaryotes [157]. In the context of PTC suppression in eukaryotes, macrolides are proposed to facilitate readthrough by weakening release factor binding or by interfering with peptide release dynamics [158].

Among the non-aminoglycoside drugs, the most studied drug at present is PTC124 (ataluren; Translarna™), which was discovered through a high-throughput luciferase reporter screen [159]. Numerous preclinical studies have reported PTC-specific suppression of translation termination and restoration of full-length protein expression in various disease models [8,12,160]. However, other studies concluded that PTC124 does not significantly increase readthrough in cellular reporter assays or in fibroblasts derived from a patient carrying a nonsense mutation [161,162]. Moreover, PTC124 was reported to bind and stabilize the firefly luciferase used for the initial drug screen, indicating that part of the initially attributed TR activity was an experimental artefact [163,164]. Nevertheless, PTC124 received conditional marketing authorisation from the European Medicines Agency (EMA) in 2014. However, following a review by the European Commission in 2023, the EMA issued a negative opinion on the renewal of its approval in 2024 [165]. As of 2026, the drug has also not been approved by the US Food and Drug Administration (FDA).

One of the most advanced synthetic aminoglycoside derivatives developed to promote TR is ELX-02 (NB124). In isogenic models of CF, ELX-02 demonstrated greater efficacy than gentamicin in restoring protein expression [166]. Beyond CF, ELX-02 induced readthrough of PTC-containing transcripts in the tumor suppressor genes *TP53* and *APC*, achieving readthrough efficiencies of up to 20% and inducing apoptosis in cancer cell models [167,168]. In a clinical safety study, single doses of ELX-02 were well tolerated in healthy volunteers, with no evidence of nephrotoxicity or ototoxicity [169]. Notably, ELX-02 exhibits more than 100-fold greater selectivity for eukaryotic over prokaryotic ribosomes compared with gentamicin or GEN, a property that likely contributes to its improved safety profile [170]. ELX-02 is currently being evaluated in two phase II clinical trials for the treatment of CF and Alport syndrome (Table 2). A summary of completed and ongoing clinical trials of readthrough-promoting compounds is provided in Table 2.

Closing remarks

TR is not a stochastically occurring translation mistake, but a finely tuned regulatory process shaped by the interplay of *cis*-acting sequence features and *trans*-acting factors, determined by the cellular context, including tissue type, developmental stage, and age. When a PTC arises within an open reading frame, it is embedded in a sequence environment that differs from that surrounding natural stop codons, conferring altered sensitivity to the translation-termination machinery. At the canonical stop codon of the main open reading frame, eRFs are efficiently recruited and activated through interactions with PABP, eIF3j, and additional cofactors [171,172]. Stop codons within upstream open reading frames are similarly supported by eRF recruitment mediated by eIF3j and eIF3 [172]. In contrast, a PTC located within the coding region lacks proximity to key architectural features, such as the poly(A) tail and the 5′ cap-associated machinery, that facilitate efficient loading and activation of eRFs [27]. As a consequence, the local concentration and activity of release factors at a PTC are reduced. This relative depletion of termination-promoting factors creates a competitive window in which near-cognate or suppressor tRNAs can more effectively compete with eRFs for stop codon recognition [27]. These contextual differences help explain why PTC readthrough is generally more permissive than readthrough at natural termination sites.

A central challenge for the therapeutic development of readthrough-promoting drugs is therefore to selectively stimulate PTC readthrough without compromising normal termination. Achieving this precision will require a deeper mechanistic understanding of how stop codon context, ribosomal dynamics, and regulatory factors converge to shape termination fidelity. A comprehensive view of these determinants will be essential for guiding the rational design of next-generation nonsense-suppression strategies. Recent genome-wide analyses [173] indicate that individual readthrough-inducing compounds promote suppression of only a subset of PTCs. Drug responsiveness is strongly influenced by local

Table 2 Readthrough compounds in clinical trials.

Molecule	Disease	Study type[a]	Status[b]	Trial ID[c]
NMD inhibition/PTC readthrough molecules (not specified)	Cystic Fibrosis	Observational	Recruiting	NCT03670472
Azithromycin	Familial Adenomatous Polyposis	Phase 4	Unknown	NCT04454151
Erythromycin	Familial Adenomatous Polyposis	Phase 4	Unknown	NCT02354560
Symdeko Ivacaftor and Symdeko	Cystic Fibrosis	Phase 4	Terminated *Terminated* prematurely, ineffective intervention *and the trial was terminated prematurely. No formal statistical analysis was performed*	NCT03624101
Gentamicin	Junctional Epidermolysis Bullosa	Phase 2	Recruting	NCT03526159
Gentamicin	Junctional Epidermolysis Bullosa	Phase 2	Unknown	NCT04140786
Gentamicin	Junctional Epidermolysis Bullosa	Phase 2	Completed	NCT02698735 NCT04644627
Gentamicin	Cystic Fibrosis	Phase 2	Terminated	NCT00376428
NPC-14	Duchenne Muscular Dystrophy	Phase 2	Unknown	NCT01918384
Ataluren	Duchenne Muscular Dystrophy	Phase 3	Terminated *Sponsor decision due to commercial availability of ataluren.*	NCT02090959
Ataluren	Cystic Fibrosis	Phase 3	Terminated *Based on the results of study PTC124-GD-021-CF (NCT02139306), clinical development of ataluren in cystic fibrosis was discontinued and this study was closed.*	NCT02107859
Ataluren	Amino Acid Metabolism, Inborn Errors	Phase 2	Terminated *Terminated due to low enrollment and unclear pharmacologic effect in available pharmacodynamic data (not due to any safety concerns).*	NCT01141075
Ataluren	Hemophilia A and B	Phase 2	Terminated *Study was terminated early due to Sponsor decision and not reflective of adverse safety findings.*	NCT00947193
ELX-02	Alport Syndrome	Phase 2	Unknown	NCT05448755
ELX-02 Ivacaftor	Cystic Fibrosis	Phase 2	Completed	NCT04126473
ELX-02	Cystinosis	Phase 2	Terminated *Due to study design limitations, this study has been discontinued and will not proceed with the 2nd cohort as contemplated in the original protocol*	NCT04069260

[a] **Study type**: Describes the nature of the clinical study, including interventional studies (clinical trials) and observational studies.

[b] **Status:** Recruiting – The study is actively enrolling participants. Terminated – The study has been stopped early and will not resume. Completed – The study has concluded as planned; no further participant follow-up is ongoing. Unknown – The study status has not been recently verified and may be outdated.

[c] **ID**: The unique identification code assigned to a clinical study upon registration at ClinicalTrials.gov. It consists of the prefix "NCT" followed by an eight-digit number.

sequence context, enabling predictive models of readthrough specificity. Consequently, no single agent is likely to effectively suppress all disease-causing PTCs within a given gene [173]. Instead, successful clinical implementation will require a repertoire of compounds, with drug selection tailored to the specific PTC carried by each patient [173]. Accurate genome-scale prediction of drug-induced readthrough may therefore improve clinical trial design and support the development of personalized nonsense-suppression therapies.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT-5.2 to make the final manuscript more concise. After using this tool/service, the authors reviewed and edited the wording as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Wojciech Teodorowicz: Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Oliver Mühlemann:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

DATA AVAILABILITY

This is a review article summarizing already published data by others

DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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