IUBMB Life

WILEY

SPECIAL ISSUE ARTICLE OPEN ACCESS

Therapeutic Potential of Translational Readthrough at Disease-Associated Premature Termination Codons From Tumor Suppressor Genes

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Received: 3 February 2025 | **Revised:** 27 March 2025 | **Accepted:** 14 April 2025

Funding: This work was partially supported by CIBERER and Instituto de Salud Carlos III through the project PI23/00959 (Spain and co-funded by European Union). LT has been the recipient of a predoctoral fellowship from Asociación Española Contra el Cáncer (AECC, Junta Provincial de Bizkaia, Spain). CEN-X is the recipient of a Miguel Servet Research Contract from Instituto de Salud Carlos III (grant number CP20/00008, Spain and co-funded by European Union). RP is funded by Ikerbasque, The Basque Foundation for Science, Spain.

ABSTRACT

Tumor suppressor genes are frequently targeted by mutations introducing premature termination codons (PTC) in the protein coding sequence, both in sporadic cancers and in the germline of patients with cancer predisposition syndromes. These mutations have a high pathogenic impact since they generate C-terminal truncated proteins with altered stability and function. In addition, PTC mutations trigger transcript degradation by nonsense-mediated mRNA decay. Suppression of PTC by translational readthrough restores protein biosynthesis and stabilizes the PTC-targeted mRNA, making a suitable therapeutic approach the reconstitution of active full-length tumor suppressor proteins by pharmacologically-induced translational readthrough. Here, we review the recent advances in small molecule pharmacological induction of translational readthrough of disease-associated PTC from tumor suppressor genes, and discuss the therapeutic potential of translational readthrough in specific groups of patients with hereditary syndromic cancers.

1 | Premature Termination Codons (PTC), Translation Termination, and Translational Readthrough

A large number of genetic disorders are caused by the introduction of a premature termination codon (PTC) in the protein coding sequence, directly caused by in-frame nonsense single-nucleotide substitutions or indirectly by frameshift small nucleotide insertions or deletions [1–3]. Mutations causing PTC are usually disruptive due to the generation of aberrant C-terminal truncated proteoforms, which likely display decreased protein stability and altered function. PTC may also cause nonsense mutation-dependent reinitiation of translation when ATG codons are present downstream in the vicinity of the PTC [4–6]. In addition, transcripts containing PTC are subjected to nonsense-mediated mRNA decay (NMD), a mRNA surveillance mechanism which leads to mRNA degradation under conditions of premature translation termination [7, 8]. In many genetic diseases, as well as in the case of tumor suppressors and cancer, an enrichment is found in pathogenic PTC that trigger NMD [9, 10], although many mRNA harboring PTC remain NMD-insensitive. NMD sensitivity of PTC-targeted mRNA is variable and depends on intrinsic and extrinsic molecular and cellular factors. For instance, mRNA with PTC located <50–55 nucleotides upstream of an exon-exon junction, or with PTC located in the last coding exon, escape NMD degradation [11–13]. A recent prediction based on a comprehensive in silico analysis

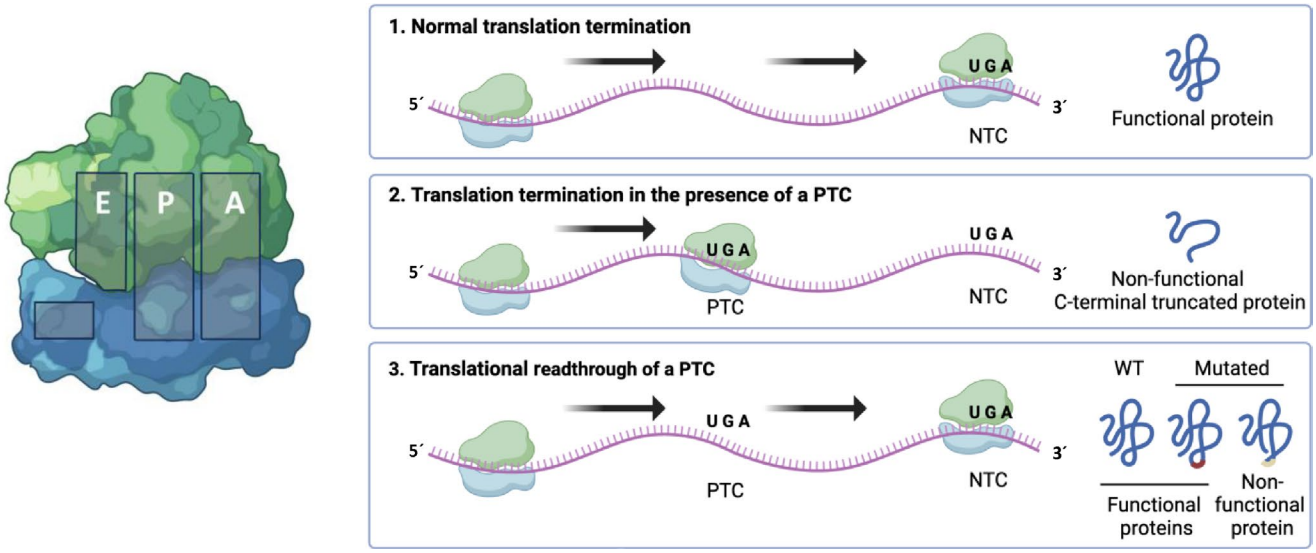

FIGURE 1 | Schematic illustration of the eukaryotic ribosome and translational readthrough of premature termination codons. In the left, a structural representation of the 80S eukaryotic ribosome is depicted, with indication of the exit site (E), the peptidyl binding site (P), and the aminoacyl binding site (A). In the right, the ribosome and the translating mRNA chain are depicted. (1) normal translation termination using the natural termination codon (NTC, UGA codon), generating a functional protein. (2) premature translation termination in the presence of a premature termination codon (PTC, UGA codon) in the protein coding sequence. The protein biosynthesis machinery recognizes the PTC and generates a non-functional or unstable C-terminal truncated protein. (3) translational readthrough of a PTC. The protein biosynthesis machinery miscodes the PTC and extends protein biosynthesis to the NTC, generating wild type (WT, functional) or mutated (functional or non-functional) full-length proteins.

indicates that about 30% of PTC variants associated with human disease can be classified as insensitive to NMD [14].

Translation termination occurs when the stop codon, either a PTC or a natural termination codon (NTC), enters the A site of the ribosome. In eukaryotes, two release factors are needed for stop codon recognition and protein translation termination: eukaryotic translation termination factors 1 and 3 (eRF1 and eRF3). Upon eRF1 recognition of any of the three stop codons (TAA, TAG and TGA in the DNA sequence) and binding to the GTPase eRF3, conformational changes are triggered in eRF1 by GTP hydrolysis. eRF1 then accommodates in the peptidyl transferase center of the ribosome, stimulating the release of the ester bond of the peptidyl-tRNA, the liberation of the polypeptide chain, and the dissociation of the ribosome subunits [15–17]. The competition for codon binding between aminoacyl-tRNAs and termination factors is continuously taking place during the elongation and termination phases of protein translation. In the case of PTC, the translating ribosome miscodes with very low frequency the stop codon by accommodating a near-cognate tRNA in the PTC position, which allows the progression of the elongation process until the ribosome reaches the next in-frame stop codon. This PTC miscoding event is known as translational readthrough (Figure 1). Stop codon basal readthrough has an overall frequency $< 0.1\%$ in NTC, whereas in PTC it occurs with frequency $< 1\%$ [18–20]. In the case of TGA PTC, the readthrough incorporated amino acids are mainly arginine, cysteine, and tryptophan, whereas for TAG and TAA, the incorporated amino acids are mainly glutamine, tyrosine, and lysine [21, 22]. This is relevant since in some cases the full-length reconstituted protein may have its functional properties affected (Figure 1).

The fidelity of translation termination and the readthrough efficiency is influenced by multiple factors, including cis-acting elements which are conserved in eukaryotes [23–25]. One of the critical factors is the identity of the stop codon, the efficiency of stop codon recognition being TAA>TAG>TGA, which results in an overall readthrough efficiency of TGA>TAG>TAA. In line with this, the stop codon with the highest termination fidelity, TAA, is enriched as the NTC in mRNAs from highly expressed genes [26], suggesting a negative selection of NTC readthrough to avoid the synthesis of C-terminally extended anomalous proteoforms. The importance of the nucleotide context surrounding the stop codon has been highlighted in several studies. In general, a cytosine (C) in the +4 position of the stop codon (the first nucleotide in the PTC being +1 position) significantly improves miscoding, whereas adenosine (A) or guanosine (G) at this position favors termination, likely because of a more robust union between eRF1 and the stop codon [27–30]. The proximity of the mRNA 3′ end and poly(A) region has also been proposed to influence negatively the efficiency of readthrough, due in part to the vicinity of poly(A)-binding proteins [31, 32].

Translational PTC readthrough is increased in the presence of several small molecules, mainly aminoglycoside antibiotics, raising therapeutic intervention opportunities for diseases with a high frequency of PTC mutations. The first demonstration of aminoglycosides as readthrough-inducing agents in mammalian cells was documented by Burke and Mogg in 1985 [33]. Since then, many studies have demonstrated the ability of aminoglycosides and other small molecules to induce PTC readthrough in a variety of mammalian cell models, and excellent reviews are available [18, 34–38]. In general, pharmacologically-induced readthrough does not increase the readthrough of NTC, as shown in in vitro and in vivo studies [39, 40]. G418/geneticin and gentamicin achieve the highest efficiencies among aminoglycoside readthrough inducers, although their toxicity excludes them as long-term therapeutic drugs, and extensive efforts are

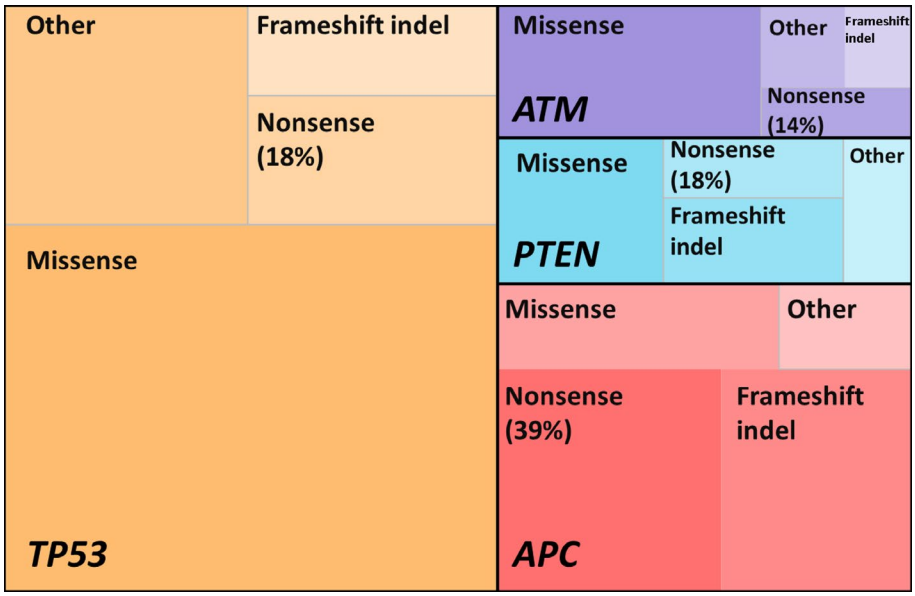

FIGURE 2 | TreeMap of the relative frequency of tumor-associated mutations found in TP53, APC, ATM, and PTEN tumor suppressor genes. The areas on the chart are proportional to the relative frequency of each type of mutation, and the percentage of nonsense mutations for each gene is indicated. The group named "Other" includes inframe indels, complex mutations, synonymous substitutions, and others, according to the COSMIC database (Catalogue of Somatic Mutations in Cancer), release v101 (Welcome Trust Sanger Institute; cancer.sanger.ac.uk [63]).

currently ongoing to search for low toxicity and high efficiency readthrough-inducing small molecules, alone or in combinations [41–44]. In addition, ribosome profiling studies indicate that G418 may induce readthrough of NTC [30]. To date, only the non-aminoglycoside oxadiazole readthrough inducer PTC124 (Ataluren; Translarna) has been conditionally approved in Europe for the treatment of Duchenne Muscular Dystrophy patients carrying PTC mutations in the DMD gene [45, 46], although non-renewal has been recommended by the European Medicines Agency due to lack of effectiveness. In addition, approaches based on the use of suppressor tRNAs [47–49], nucleotide analogs [50], eRF degraders or downregulators [51–53], sequence-specific oligonucleotides [54, 55], or NMD inhibitors [56–58], among others, are also under therapeutic consideration. Combinatorial use of readthrough-inducing drugs and NMD inhibitors is emerging as an alternative therapy for diseases with a PTC genetic basis [59–61], and therapeutic interventions of translational readthrough and NMD inhibition have been proposed in cancer syndromes [62].

2 | PTC in Tumor Suppressor Genes in Association With Disease

Tumor suppressor genes are frequently targeted by nonsense mutations causing PTC in association with sporadic cancer or hereditary cancer syndromes. Figure 2 shows the comparative relative frequency of mutation types in sporadic tumors from four major tumor suppressor genes: TP53, APC, ATM, and PTEN. As shown, nonsense mutations account for 10%–40% of the mutation load. A large number of these mutations come from arginine CGA codons (Figure 3), due to CpG to TpG transitions (CGA to TGA substitution) facilitated by methylation followed by spontaneous deamination, which are proportional to the stem cell division rate in the tissue [64–66]. As an example, TP53 CGA codons comprise less than 3% of the TP53 total codons, but they account for more than 70% of the TP53 mutations in colorectal cancers [67].

The distribution of PTC mutations along the nucleotide sequence of tumor suppressor genes is not random, independently of their frequency, as observed in sporadic tumor samples or in the germline of patients. This may be related to the contribution of the distinct regions of the encoded proteins on tumor suppression activity (Figure 3). For instance, few residues at the C-terminal portions of TP53, APC, or PTEN (but not of ATM) are targeted for PTC mutations, suggesting that these C-terminal regions are dispensable for the major tumor suppressive activity of those proteins. In addition, the N-terminal portion of TP53 is less targeted by PTC than the N-terminal portion of PTEN, in spite of having a similar potential PTCome (codons which can generate a PTC by one-nucleotide substitution) in this region. In the case of p53, translation reinitiation can occur at a downstream methionine (methionine 40) that generates a truncated but functional protein, suggesting that the p53 N-terminal region is not essential for protein function. This is illustrated by the existence of the physiologic Δ40p53 isoform, which maintains most of the functions of full-length p53 [5, 68]. On the other hand, PTEN amino acids at the very N-terminus are essential for PIP2 binding and PIP3 dephosphorylation in cells [69, 70].

3 | Pharmacologically-Induced PTC Readthrough in Tumor Suppressor Genes

Next, we summarize the current status of translational readthrough research on TP53, APC, ATM, and PTEN tumor suppressors, using small molecules as readthrough inducers. Table 1 illustrates a selection of studies on readthrough induction on these and other tumor suppressor genes. Figure 4 shows

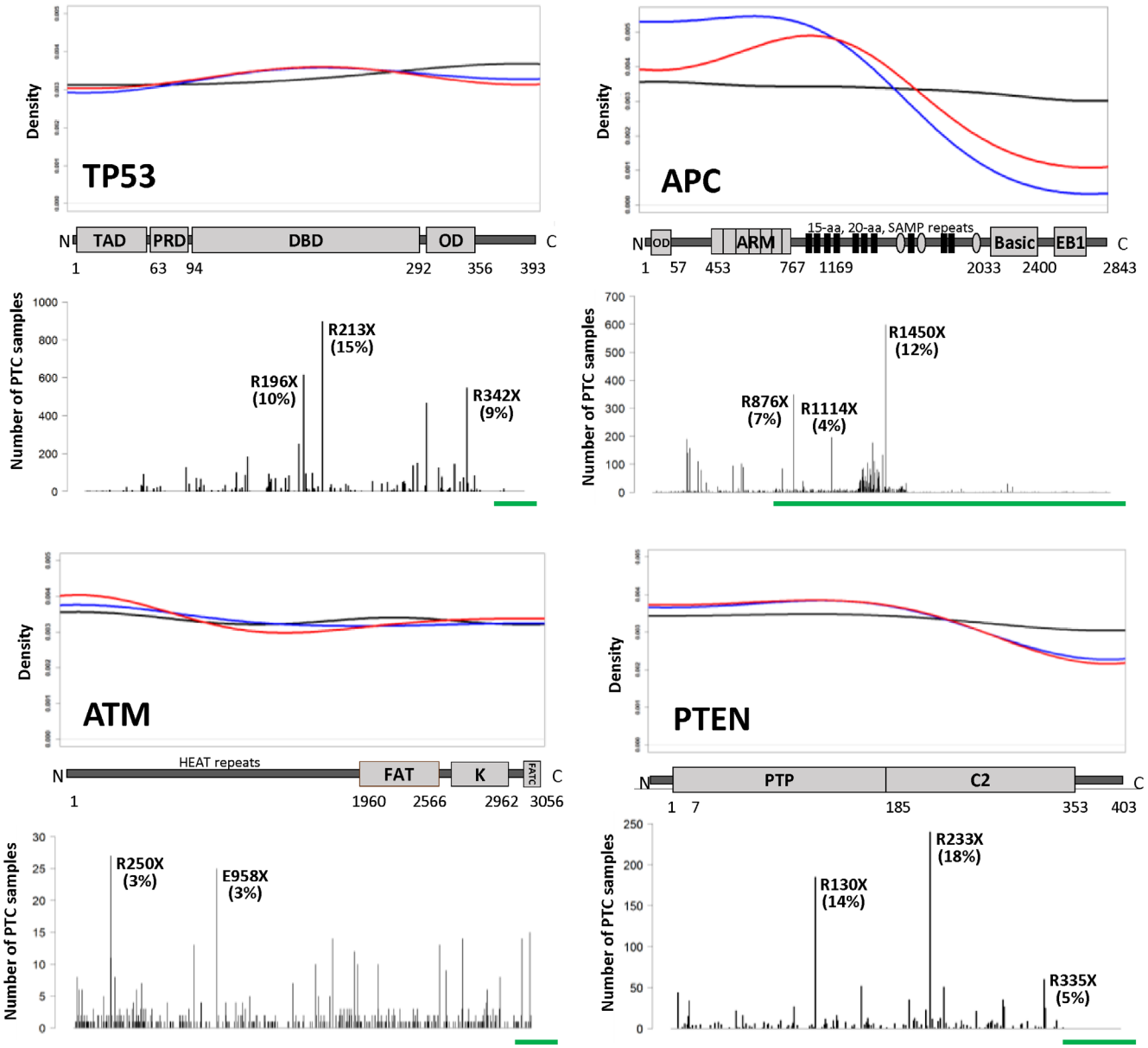

FIGURE 3 | Representation of the PTCome distribution of TP53, APC, ATM, and PTEN genes in association to disease. The plots at the top are qualitative Kernel density plots representing the density of PTC distribution along the protein sequence, independently of the frequency of each PTC. Black lines, potential PTCome (https://github.com/compneurobilbao/stop-codon-pulido-17). Blue lines, germline-associated PTCome (HGMD Professional database, release 2024.4 [3]). Red lines, somatic-associated PTCome (COSMIC database, release v101 [63]). At the bottom of each Kernel density plot, a schematic depiction of the domain composition of each protein is shown, with indication of amino acid numbering, according to the accessions TP53: NM_000546, NP_000537; APC: NM_000038, NP_000029; ATM: NM_000051, NP_000042; PTEN: NM_000314, NP_000305. ARM, armadillo repeats region; Basic, basic region; C2, C2-membrane binding domain; DBD, DNA-binding domain; EB1, EB1 binding domain; FAT, FRAP/ATM/TRRAP domain; FATC, FAT C-terminal domain; K, kinase domain; OD, oligomerization domain; PRD, proline rich domain; PTP, protein tyrosine phosphatase domain; TAD, transactivation domain. The bar plots are quantitative representations of the number of PTC samples that have been annotated in COSMIC database, with indication of the percentage of the more frequent PTC for each gene. The green lines under the bar plots indicate the extension of the last coding exon, non-sensitive to NMD, for each gene.

the chemical structure of the readthrough inducer compounds mentioned in the text and in Table 1.

3.1 | TP53

TP53 is a major tumor suppressor gene encoding p53, a 393 amino acid protein with transcription factor activity that inhibits cell division and survival. It contains a DNA-binding domain highly targeted by mutations in cancer (Figure 3). In the presence of DNA damage, p53 facilitates cell growth arrest or apoptosis, depending on the cell type and cell growth conditions [109, 110]. Most human cancers have either a TP53 loss or express functionally inactive (lacking tumor suppression activity) or gain-of-function (displaying oncogenic properties) mutated TP53 [111, 112]. Germline mutations at TP53 are the major cause

TABLE 1 | Translational readthrough studies on tumor suppressors.

Readthrough inducer	Cell line/model	Reference
TP53 (17p13.1), Li-Fraumeni syndrome, LFS, autosomal dominant, ORPHA:524		
Gentamicin, tobramycin, amikacin	TC	[71]
G418, gentamicin	TC, HDQ-P1	[72]
G418, NMD inhibitors	CALU-6, HDQ-P1, N417	[73]
G418, CDX5-1	HDQ-P1, MDA-MB-361, CAOV-3, SW900, NCI-H1688, CALU-6, EKVX, ESS-1, SK-MES-1, HCC1937, UACC-893	[74]
G418, gentamicin, NB124/ELX-02, other aminoglycoside derivatives	TC, HDQ-P1	[75]
G418, clitocine, gentamicin	CAOV-3, Calu-6, H520, xenografts	[50]
G418, *Lepista inversa* extract	CALU-6, CAOV-3, Caco-2	[76]
G418, gentamicin, proteasome inhibitors, p53-Mdm2 inhibitors	TC, HDQ-P1	[77]
G418, mefloquine	HDQ-P1, SW900, NCI-H1688, HCC1937	[78]
G418, aminothiazoles	HDQ-P1	[79]
G418, NB124/ELX-02	DMS114	[39]
G418, 2,6-diamonopurine (DAP)	Calu-6, CAOV-3, Caco-2, xenografts	[80]
G418, CC-885, CC-90009	TC, HDQ-P1, SW900, CAOV-3	[51]
G418, Y-320	TC, HDQ-P1	[81]
G418	TC	[82]
G418, 5-Fluorouridine (FUr)	TC, HDQ-P1	[83]
PTC124	TC	[84]
G418, C47, C61	TC, HDQ-P1	[85]
APC (5q22.2), Familial adenomatous polyposis, FAP, autosomal dominant, ORPHA:733		
G418, gentamicin, paromomycin, tylosin, josamycin, spiramycin	TC, SW1417, $Apc^{Min/+}$ mice, xenografts	[86]
G418, gentamicin, amikacin, negamycin	TC, LOVO	[87]
Gentamicin, erythromycin, azithromycin, PTC124	TC, SW1417, SW480, $Apc^{Min/+}$ mice	[88]
G418, gentamicin, NB124/ELX-02, other aminoglycoside derivatives	TC	[75]
G418, gentamicin, erythromycin, azithromycin, PTC124	TC, SW480, SW403, LOVO, Colo320, HT29, SW1417	[89]
Erythromycin	FAP patients	[90]
G418, gentamicin	TC, SW837, SW620, SW1417, SW403, DU4475, Colo320	[91]
ATM (11q22.3), Ataxia telangiectasia, A-T, autosomal recessive, ORPHA:100		
G418, gentamicin, paromomycin, tobramycin	TC, LCL from A-T patients	[92]
G418, gentamicin, paromomycin, RTC-13, RTC-14	TC, LCL from A-T patients, GM02052	[93]
G418, RTC-13	LCL from A-T patients	[94]
RTC-13, GJ071, GJ072, PTC124	TC, LCL from A-T patients	[95]

(Continues)

TABLE 1 | (Continued)

Readthrough inducer	Cell line/model	Reference
G418, RTC-13, RTC-229	hiPSC lines from A-T patients	[96]
Gentamicin, erythromycin, azithromycin	TC, lymphocytes from A-T patients	[88]
G418, GJ103	Explants from Atm^{R35X} KI mice	[97]
PTEN (10q23.31), PTEN hamartoma tumor syndrome, PHTS, autosomal dominant, ORPHA:306498		
G418, gentamicin, amikacin, tobramycin, erythromycin	TC	[98, 99]
G418, C47, C61	TC	[85]
G418, gentamicin, Y-320	TC	[100]
BRCA1 (17q21.31), Hereditary breast and/or ovarian cancer syndrome, autosomal dominant, ORPHA:145		
G418	TC, HCC1395	[101]
G418, antisense oligonucleotides	TC	[102]
NF1 (13q14.2), Neurofibromatosis type I, NF1, autosomal dominant, ORPHA:636		
PTC124	mES^{R683X}	[103]
G418, gentamicin, PTC124, NMD inhibitors	$NF1^{R1947X}$ pigs, primary $NF1^{R1947X}$ pig Schwann cells	[104]
PTC124	$NF1^{R683X}$ KI mice	[105]
RB1 (13q14.2), Retinoblastoma, RB, autosomal dominant, ORPHA:790		
G418, CC-90009	TC, SW1783	[106]
KDM6A (Xp11.3); *KMT2D* (12q13.12), Kabuki syndrome, KS, autosomal dominant, ORPHA:2322		
Gentamicin	TC, LCL from KS patients	[107]
BMPR1A (10q23.2), Juvenile polyposis syndrome, JPS, autosomal dominant, ORPHA:2929		
G418, PTC124	TC	[108]

Note: Gene name, chromosomal location, genetic disease associated to germline mutations, pattern of inheritance, and ORPHA numbers are indicated. TP53 PTC in cell lines: HDQ-P1, R213X; CALU-6, R196X; CAOV-3, Q136X; H250, W146X; SW900, Q167X; NCI-H1688, Q192X; HCC1937, R306X; Caco-2 E204X; DMS-114, R213X; N417, E289X; ESS-1, R213X; SK-MES-1, E298X; UACC-893, E342X; EKVX, E204X; MDA-MB-361, E56X. APC PTC in cell lines and mice: SW480, Q1338X; SW1417, R1450X; SW403, S1278X; LOVO, R1114X; Colo320, S811X; HT29, E853X; SW837, R1450X/R213X; SW620, Q1338X; DU4475, E1557X, Apc Min/+ mice, L850X. ATM PTC in cell lines: GM02052, R35X. BRCA1 PTC in cell lines: HCC1395, R1751X. RB1 PTC in cell lines: SW1783, R579X.
Abbreviations: hiPSC, human induced pluripotent stem cell; LCL, lymphoblastoid cell line; mES, mouse embryonic stem cell line; TC, transfected cells.

of Li-Fraumeni syndrome, an autosomal dominant inheritance syndrome associated with a high risk to develop multiple primary cancers [113, 114].

Around 10% of TP53 mutations are nonsense mutations that generate PTC and truncated p53 proteins, where R196X, R213X, and R342X are the more frequent PTC (Figure 2, Figure 3). In particular, R213X is present in about 1% of all human tumors [115]. Studies trying to overcome TP53 loss of function due to PTC mutations include NMD inhibition and consequent transcript stabilization, as well as translational readthrough studies [116].

Induction of PTC readthrough on TP53 with aminoglycosides was first demonstrated using reporter plasmids [71]. R210X, Q133X, and W143X TP53 PTC were tested for readthrough in rat embryo fibroblast cells and in a reticulocyte in vitro translation system, with gentamicin and amikacin showing a significant readthrough induction. This early study also highlighted the relevance of the nucleotide 3′ to the PTC (+4 position) for readthrough efficiency.

Floquet et al. tested aminoglycoside-induced readthrough on 11 TP53 PTC by transient transfections of reporter plasmids. Gentamicin, but especially G418/geneticin displayed good readthrough-inducing activity. The highest readthrough efficiency was obtained with R213X (*TGA*-C), the most frequent TP53 PTC [72]. R213X-containing human primary breast carcinoma cell line HDQ-P1 was also tested for readthrough, and full-length transcriptionally active p53 protein was detected upon G418 induction. In addition, G418 also stabilized TP53 mRNA [72]. Readthrough of TP53 R213X was further studied using new-generation aminoglycosides, produced by variations in G418 and paromomycin molecules [75]. Among these, NB124/ELX-02 was the most potent readthrough inducer, showing less cytotoxicity than gentamicin and G418. NB124/ELX-02 stabilized the R213X mRNA transcript and restored TP53 transcriptional activity and apoptotic functions [75]. NB124/ELX-02 readthrough efficiency on TP53 has also been shown for DMS-114 (R213X) small-cell lung cancer cell line, and this compound was tolerated in healthy subjects, without inducing readthrough of NTC [39]. NB124/ELX-02 has been tested in clinical trials for cystic fibrosis patients harboring

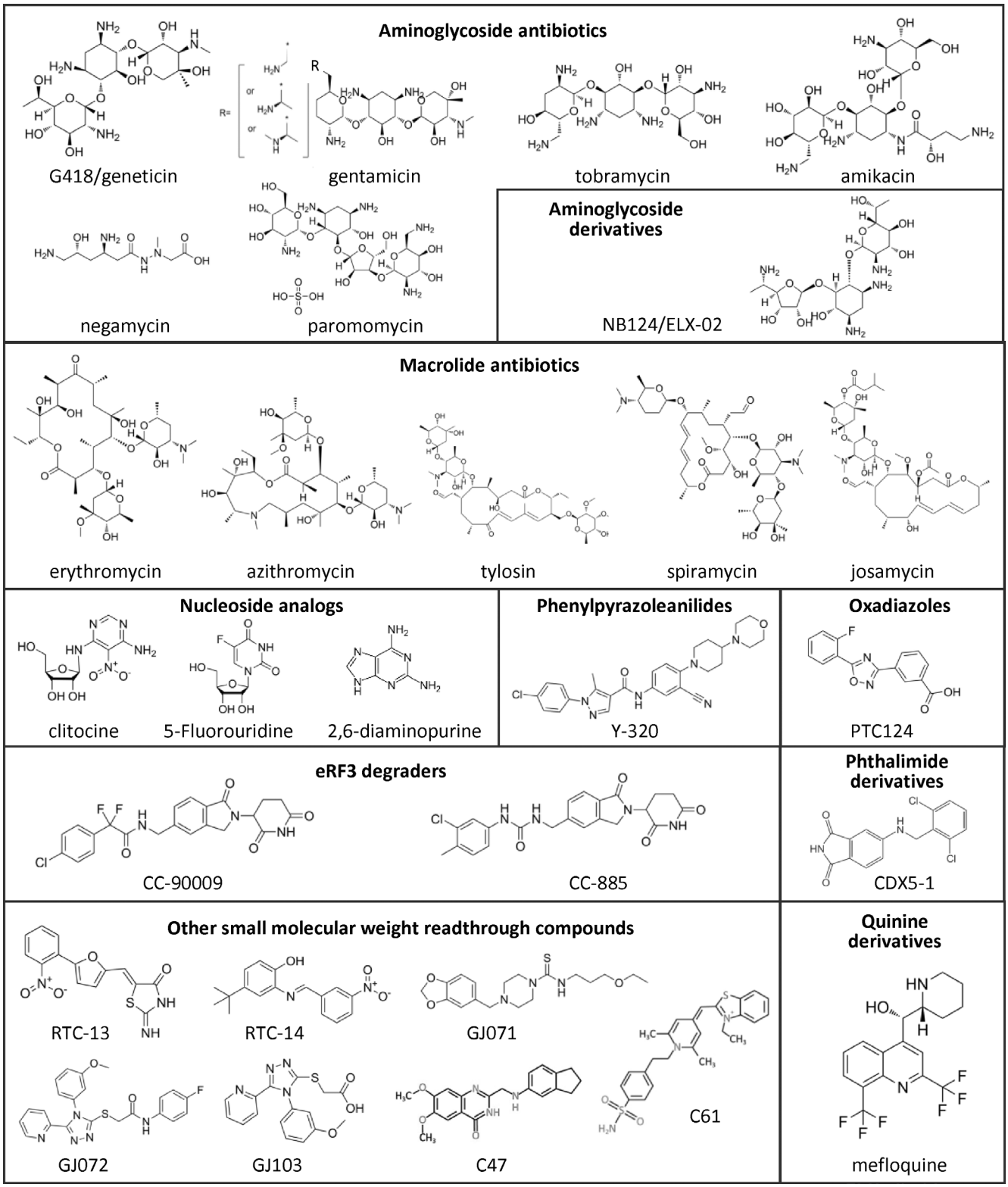

FIGURE 4 | Chemical structure of the readthrough inducer compounds mentioned in the text and in Table 1. The compounds are grouped in structural families or by their description in the literature.

PTC at CFTR gene, although it did not demonstrate a clinical benefit for patients.

The search for novel small compounds as TP53 readthrough inducers rendered the identification of CDX5 phtalimide derivatives and 2-aminothiazole-4-carboxamides as compounds that potentiate the readthrough efficiency of G418 in HDQ-P1 cells [74, 79]. In the case of CDX5-1, the findings were extended to other human cell lines harboring PTC at TP53 (Table 1), which showed distinctive PTC readthrough responses in line with the efficiency TGA>TAG>TAA. More recently, the compounds C47 and C61 were found to facilitate readthrough of TP53 R213X

PTC. C47 showed synergy in combination with G418, whereas C61 showed synergy with eRF3 degraders, indicating that combinatorial readthrough effects can be obtained targeting different protein translation effectors [85].

Clitocine, an adenosine nucleoside analog, has been proved to induce readthrough on endogenous TP53 mRNA from CALU-6 (R196X), CAOV-3 (Q136X) and H520 (W146X) cell lines. The full-length p53 produced by clitocine was functionally active, demonstrated by caspase activation and decrease in cell viability, and by p21 mRNA increase, a downstream target of p53. Clitocine is incorporated into the mRNA in place of adenosine during transcription, increasing spontaneous readthrough of the three stop codons with an efficiency TAA>>TGA>TAG, which is of interest since TAA is the less responsive PTC to most readthrough inducers. Distinct from aminoglycosides and their derivatives, clitocine did not stabilize TP53 mRNA harboring PTC [50].

The E3 ubiquitin ligase MDM2 is a major negative regulator of p53, and several MDM2 inhibitors are currently in clinical trials for cancer therapy [117, 118]. Synergistic activity for p53 functional rescue of readthrough inducers and MDM2 inhibitors or proteasome inhibitors has been analyzed in HDQ-P1 human breast cancer cells [77]. The TP53 readthrough effect of G418 and gentamicin was potentiated in the presence of bortezomib, nutlin-3a, or MI-773, and efficient reconstitution of full-length functionally active p53 was assessed by transcriptional activity and cell growth and survival assays [77]. The already approved antimalarial drug mefloquine has also shown to enhance G418-induced readthrough in TP53 [78]. Combination of mefloquine and G418 reconstituted functional full-length p53 expression in HDQ-P1 (R213X), SW900 (Q167X), NCI-H1688 (Q192X), and HCC1937 (R306X) cancer cell lines, as demonstrated by the increase in p53 Ser15 phosphorylation and in p21 transcript levels following radiation-mediated DNA damage. The mechanism by which mefloquine enhances G418 readthrough remains unclear, even though it is known that mefloquine binds to *Plasmodium falciparum* ribosomes, inhibiting protein synthesis [119]. These studies highlight the importance of exploring drugs currently approved or in clinical trials as translational readthrough enhancers.

Other compounds specifically inducing readthrough of TGA PTC have demonstrated efficacy in restoring p53 expression. 2,6-diaminopurine (DAP), extracted from the *Lepista inversa* mushroom, is a low toxicity compound that has shown readthrough activity in TP53 TGA PTC [76, 80]. DAP restored p53 expression and transcriptional activity in Calu-6 cells (R196X, TGA), but not in Caco-2 (E204X, TAG) cells, and decreased the tumor growth of Calu-6 xenografts in nude mice. DAP did not stabilize NMD targeted TP53 mRNAs, and the underlying mechanism of DAP readthrough activity could be explained by a more efficient TGA recognition by $tRNA^{Trp}$. The methyltransferase FTSJ1 methylates $tRNA^{Trp}$ and inhibition of this modification by DAP improves the ability of $tRNA^{Trp}$ to bind TGA codons.

5-Fluorouridine (FUr) is another compound that displayed translational readthrough activity in TP53 with efficiency TGA>TAG, as demonstrated using endogenous TP53 R213X from HDQ-P1 cells or by ectopic expression of TP53 R213X in H1299 cells [83]. FUr incorporates into mRNA and facilitates insertion of $tRNA^{Arg}$ at TGA PTC, which reconstitutes to the wild type amino acid the PTC nucleotide substitution CGA to TGA. In the case of TP53 R213X, functional restoration of p53 by FUr was achieved in cell cultures and in human tumor xenografts in mice. Additional studies addressing translational readthrough of TP53 are listed in Table 1.

3.2 | APC

Germline mutations in the APC gene are responsible for familial adenomatosis polyposis (FAP), the most common hereditary polyposis that predisposes to cancer. In addition, APC mutation is a key step in the malignant transformation of sporadic colorectal cancers (CRC), and a large proportion of sporadic CRC tumors carry mutations in both alleles of the APC gene [120, 121].

The APC gene is located in chromosome 5q22.2 and contains 15 coding exons that encode a large multi-domain scaffolding protein of 2843 amino acids (about 312 kDa) (Figure 3). APC is a key negative regulator of the Wnt/β-catenin signaling pathway and a promoter of cell–cell adhesion, playing major roles as a tumor suppressor [122]. APC regulates the distribution of β-catenin in the cytoplasm and cell membrane and forms part of the β-catenin destruction complex that targets β-catenin for proteasomal degradation. Through β-catenin degradation, APC indirectly regulates the transcription of critical cell proliferation genes [123, 124]. In addition, APC can prevent the formation and development of CRC independently of Wnt signaling, assisting in chromosome segregation and repressing DNA replication [125].

APC mutations associated with disease are not randomly distributed, with an enrichment in the Exon 15 region encoding amino acids at positions 1200–1600 (Figure 3), a protein segment involved in binding to β-catenin which has been named the mutation cluster region (MCR) due to its high mutational load. MCR mutations represent about 60% of the total APC mutations, whereas MCR only encodes for 15% of APC protein residues. The majority of APC germline and somatic mutations are either nonsense or frameshift mutations that generate PTC. In fact, APC is the tumor suppressor most targeted by nonsense mutations (about 40% of the total somatic mutations). The more frequent APC PTC is R1450X, which accounts for about 20% of nonsense and almost 10% of total APC somatic mutations (Figures 2 and 3) [126]. This enrichment in nonsense mutations is partially explained by the abrogation of β-catenin binding activity. In addition, APC truncated proteoforms are stable and may interact with different protein effectors, resulting in additional activation of Wnt signaling or in activation of transcription-independent pro-oncogenic pathways [124, 127].

APC PTC associated with disease are predominantly located in the final coding exon not sensitive to NMD degradation (Figure 2). This positions APC as a strong candidate for the development of readthrough-based therapeutic strategies. In this regard, a clinical trial with ten FAP patients treated with the macrolide erythromycin has been carried out, which provided proof-of-concept results in terms of decrease of adenoma burden

and lack of side effects [90]. Additional trials are guaranteed to reinforce the potential benefit of induced translational readthrough on specific groups of FAP patients.

APC PTC readthrough was first illustrated using ectopically expressed APC R1450X on HC116 CRC cells, as well as endogenous APC R1450X from SW1417 CRC cells, with aminoglycoside and macrolide antibiotics as readthrough inducers [86]. Full-length APC expression was partially restored, in correlation with a decrease in proliferation and colony formation. In addition, HT-29 (E853X) or SW1417 CRC cell xenografts exhibited up to a 50% decrease in tumor size after readthrough induction, and polyp reduction and increased life span were obtained with tylosin treatment in $Apc^{Min/+}$ mice, which carry the APC L850X mutation. A follow-up study extended these observations to other APC PTC [128]. The readthrough efficiency of the aminoglycoside derivative NB124/ELX-02 has also been documented in the case of transiently transfected L360X, R114X, and Q1131X APC PTC [75]. In addition, APC readthrough by aminoglycoside and macrolide compounds has been reported in a variety of CRC cell lines harboring distinct APC PTC. In some of these cell lines, readthrough was enhanced by serum starvation and by alterations in protein synthesis processes [89, 91]. These findings suggest the existence of cell-specific factors that facilitate the pharmacological induction of translational readthrough, which may be relevant in the case of tissue-specific readthrough efficiency associated with therapy.

3.3 | ATM

ATM (ataxia telangiectasia mutated) is a serine/threonine protein kinase that belongs to the PI3K-related (PIKK) protein family. Homozygous or compound heterozygous (different mutations in each allele) loss-of-function germline mutations in ATM are causative of ataxia-telangiectasia (A-T), an early onset progressive neurodegenerative disorder that is accompanied by predisposition to cancer [129]. ATM phosphorylates a wide variety of proteins (including p53) involved in DNA repair, cell cycle regulation, and apoptosis, orchestrating cell survival and the DNA damage response following oxidative stress, hypoxic conditions, and DNA double-strand breaks, among other genotoxic insults [130–132].

ATM is a large multi-domain, homodimeric kinase (3056 amino acids; about 350 kDa), with essential dimer-interaction residues and a kinase domain C-terminally located (Figure 3) [133]. The ATM gene is at chromosome 11q22.3 and contains a large number of coding exons, most of them of small size, which increases the possibilities of escaping NMD by ATM mRNA containing PTC [134]. ATM PTC associated with disease distribute all along the ATM amino acid sequence, without clear hotspot regions, with R250X and E958X as the PTC more frequently found in tumors (Figure 3). The high frequency of the E958X PTC (GAG to TAG) is remarkable since it does not involve a high mutability CpG to TpG transition, with the adjacent E597 residue (GAA) not being targeted for PTC. In addition, PTC located at the very C-terminus of ATM, such as R3047X, are found with relatively high frequency in tumors and A-T patients, suggesting an important functional role for the ATM C-terminal residues. In this regard, the acetyltransferase TIP60 binds to the FATC C-terminal domain of ATM and acetylates ATM at K3016, which regulates positively ATM activation [135, 136]. The N-terminal region of ATM is also crucial for ATM function, since it contains the kinase substrate binding site and it is required for nuclear localization and chromatin association [137].

Early work by Lai et al. provided experimental evidence of the potential of ATM translational readthrough to correct ATM PTC in A-T patients [92]. Consistent in vitro readthrough of several ATM PTC was achieved with the aminoglycosides G418, gentamicin, and paromomycin. Functional ATM PTC reconstitution, in terms of ATM autophosphorylation and cellular DNA synthesis in response to cell radiation, was verified upon G418 treatment. Other non-aminoglycoside small compounds (named as RTC and GJ compound series) were also found to reconstitute the function of distinct ATM PTC upon readthrough induction, including experiments on induced pluripotent stem cells from A-T patients, although their mechanism of action remains to be elucidated [93–96]. In addition, spleen and cerebellum explant tissues from a mouse model mimicking the human ATM R35X mutation have been used to prove the readthrough efficiency of aminoglycoside and non-aminoglycoside compounds [97].

3.4 | PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor gene frequently mutated in sporadic human cancers and in the germline of patients with PHTS (PTEN Hamartoma Tumor Syndrome). PTEN is a dual-specificity phosphatase whose major physiologic substrate is the lipid phosphatidylinositol (3–5) trisphosphate (PIP3). Dephosphorylation of PIP3 to phosphatidylinositol (4, 5) bisphosphate (PIP2) counteracts the activity of the pro-oncogenic PI3K and downregulates the signaling through the PI3K/AKT pathway [138–140]. In addition, PTEN dephosphorylates Ser/Thr and Tyr residues from proteins, and exerts tumor suppressive functions independent of its catalytic activity [141–143].

PTEN is a 403 amino acid protein that has two core domains: a N-terminal catalytic tyrosine phosphatase domain (PTP) and a C-terminal C2 domain that binds to membrane phospholipids. PTEN PTC associated with disease distribute along both the PTP and C2 domains, with mutations R130X, R233X, and R335X being the more frequent PTC both in somatic tumors and in PHTS patients. By contrast, the disordered C-terminal PTEN region (C-tail) is poorly targeted by PTC (Figure 3) [143, 144]. Alternative initiation of translation generates PTEN long isoforms with distinct N-terminal extensions and a variety of subcellular locations and different functions, although their specific role in human disease remains uncertain [145, 146]. In this regard, the unique N-terminal regions of the PTEN long isoforms are faintly targeted by PTC [100].

A comprehensive analysis of the therapeutic potential of translational readthrough of PTEN has been performed by our group [98, 99]. G418 readthrough induction was tested on the PTEN PTC associated with disease (115 different PTC), in parallel with functional studies of the reconstituted full-length PTEN proteins. Readthrough efficiency was provided for all PTEN PTC, taking into consideration both the full-length PTEN protein

synthesis and its PIP3-phosphatase activity in cells. The more frequent PTEN PTC (R130X, R233X, and R335X) showed either optimal or suboptimal readthrough efficiency upon G418 induction. Our comprehensive study adhered to the overall readthrough efficiency rule of TGA>TAG>TAA, with most TAA PTC displaying very low readthrough and most TGA PTC displaying optimal or suboptimal readthrough. This suggests that a substantial number of PHTS patients could obtain benefit from a readthrough-based therapy, highlighting the necessity of a precise patient stratification for clinical trials and therapy implementation. PTEN PTC upstream methionine 35 resulted in the translation of an N-terminal truncated PTEN proteoform (PTEN M35). In a more recent study, we have extended our findings on PTEN readthrough to PTEN-L, the longer PTEN isoform, and reported that protein synthesis inducers potentiate PTEN readthrough [100]. Another report has shown the induction of PTEN R130X, R233X, and R335X PTC readthrough and functional reconstitution by the compound C47, alone or in combination with G418, in parallel with a reduction in the levels of eRF1 [85]. Together, these findings suggest that appropriate combinations of low toxicity readthrough inducers and modulators of protein synthesis may show therapeutic benefits for PHTS patients harboring specific PTEN PTC.

3.5 | Other Tumor Suppressor Genes

Additional tumor suppressor genes that have been studied in terms of translational readthrough of PTC include BRCA1, NF1, and RB1, three major tumor suppressors frequently mutated in sporadic cancer and in the germline of patients with cancer predisposition syndromes (Table 1). The BRCA1 gene, whose mutations in the germline are associated with lifetime risk to familial breast and ovarian cancer, has been analyzed in detail for G418-induced readthrough, in terms of reconstitution of both full-length BRCA1 biosynthesis and function, providing proof-of-concept for the potential application of readthrough-based therapies for patients with BRCA-associated hereditary breast and ovarian cancer syndrome [101]. Of interest, Susorov et al. have recently reported the additive effect of G418 and antisense BRCA1 oligonucleotides on BRCA1 readthrough efficiency [102]. Readthrough of the NF1 gene, involved in neurofibromatosis type I, has been evaluated in cells from pig and mouse models harboring NF1 PTC. $NF1^{R1947X/+}$ minipig cells showed G418 readthrough induction, but only in the presence of NMD inhibitors [104]. In engineered mouse embryonic stem cells homozygous for NF1 R683X PTC, functional NF1 readthrough was achieved by PTC124 [103]. $NF1^{R683X/+}$(conditional) mice displayed decreased neurofibroma growth upon PTC124 food-treatment, and the effect was variable depending on the gender and the timing of the treatment [105]. Whether readthrough-based therapies could be influenced by sex in specific genetic diseases deserves analysis. Palomar-Siles et al. reported G418 readthrough induction of RB1, the tumor suppressor associated with retinoblastoma, on both endogenous and ectopically expressed RB1 harboring disease-associated PTC. The effect of G418 was potentiated by the eRF3 degrader CC-90009 [106]. Together, these findings give hope for novel therapies for patients with syndromic cancers and illustrate the existence of complex physiologic networks modulating the efficacy of readthrough-based therapies.

4 | Challenges and Hopes of PTC Readthrough in Tumor Suppressor Genes

PTC readthrough is emerging as a promising restoration therapy for many genetic diseases, including cancer predisposition syndromes, and several challenges need to be outpointed before clinical implementation. These include the necessity of a precise selection of the patients that will benefit from a particular readthrough therapy, as well as the identification and validation of readthrough-inducing drugs with a high safety/readthrough ratio, including effective drug combinations. A genome-wide quantification of translational readthrough of pathogenic PTC by small molecules has been reported that highlights the importance of the local sequence context on readthrough efficiency by specific compounds [147]. Evidence on the efficient readthrough of a PTC-targeted gene by a particular readthrough inducer will be determinant for the prediction of the effectiveness of PTC readthrough-based therapies. This needs to be addressed in the design of clinical trials based on therapeutic readthrough. In addition, information on the NMD sensitivity of the mRNA harboring the pathogenic PTC will also be of importance. In the case of cancer predisposition syndromes, malignancy usually manifests in the adult phase of the patients, making readthrough-based therapies an attractive option for preventive anti-cancer intervention. A relevant issue in the case of therapeutic readthrough of tumor suppressor genes is the necessity to reconstitute functionally active full-length tumor suppressor proteins, which feasibility has been demonstrated in several proof-of-concept studies. Together, this emphasizes the importance to perform dedicated studies on the biochemical and functional PTC readthrough response of tumor suppressor genes, and provides hopes for the implementation of translational readthrough-based therapies in cancer predisposition diseases.

Acknowledgments

We thank all personnel from Biobizkaia Health Research Institute for their expert assistance. This work was partially supported by CIBERER, and Instituto de Salud Carlos III through the project PI23/00959 (Spain and co-funded by European Union). L.T. has been the recipient of a predoctoral fellowship from Asociación Española Contra el Cáncer (AECC, Junta Provincial de Bizkaia, Spain). C.E.N-.X. is the recipient of a Miguel Servet Research Contract from Instituto de Salud Carlos III (grant number CP20/00008, Spain and co-funded by European Union). R.P. is funded by Ikerbasque, The Basque Foundation for Science, Spain.

Conflicts of Interest

The authors declare no conflicts of interest.

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