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**Induction of translational readthrough on protein tyrosine phosphatases targeted by premature termination codon mutations in human disease**

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**Abstract**

Nonsense mutations generating premature termination codons (PTC) in a variety of genes are frequently associated to somatic cancer and hereditary human diseases, since PTC commonly generate truncated proteins with defective or altered function. Induced translational readthrough during protein biosynthesis facilitates the incorporation of an amino acid at the position of a PTC, allowing the synthesis of a complete protein. This may evade the pathological effect of the PTC mutation and provide new therapeutic opportunities. Several protein tyrosine phosphatase (PTP) genes are targeted by PTC in human disease, the tumor suppressor PTEN being the more prominent paradigm. Here, using PTEN and Laforin as examples, two PTP from the dual-specificity phosphatase subfamily, we describe practises/methodologies to analyze *in silico* the distribution and frequency of pathogenic PTC in PTP genes. We also summarize laboratory protocols and technical notes to study in cellular models the induced translational readthrough reconstitution of the synthesis of PTP targeted by PTC in association with disease.

**Key words:** protein translation, translational readthrough, nonsense mutation, premature termination codon, PTEN, Laforin

**1. Introduction**

Nonsense mutations by single-nucleotide substitution, generating premature termination codons (PTC) in the protein coding region of the targeted genes, are frequently found in association with human disease. These include germline mutations linked to hereditary diseases as well as tumor somatic mutations associated with cancer (1, 2). For instance, the *APC* gene, coding for the Adenomatous polyposis coli tumor suppressor protein, is heavily targeted by PTC in the germline of patients with familial adenomatous polyposis and in sporadic colorectal cancers (3). Another emblematic example is the *DMD* gene, which encodes the dystrophin protein and whose alterations cause Duchenne muscular dystrophy. many of the disease-associated DMD mutations found are nonsense mutations generating truncated dystrophin proteoforms (4). The high potential pathogenicity of PTC is explained by the generation of truncated proteins, as well as by the degradation of the PTC-targeted mRNA by nonsense-mediated mRNA decay (NMD) (5). In the case of cancer-related diseases, nonsense mutations are relatively abundant in most tumor suppressor genes, frequently due to the combination of the pathogenicity of the mutation and to the presence of DNA sequences prone to mutation (6, 7). In addition, generation of PTC by nonsense mutations during evolution has also been proposed as a mechanism of allele variability in human populations (8).

Translational readthrough of termination codons consists on the biosynthetic incorporation of an amino acid in the position corresponding to the termination codon (UAA, UAG, and UGA). In mammals, translational readthrough occurs because of competition to enter the ribosome acceptor site (A-site) between near-cognate tRNAs and the release factors eRF1 and eRF3. These near-cognate tRNAs have a sufficiently stable interaction to allow the translation machinery to continue reading the next in-frame codon, incorporating a non-random amino acid and facilitating translation until the natural termination codon, thus generating a full-length protein. Under normal conditions, basal readthrough of natural termination codons in mammalian cells takes place with a very low frequency, within the range of 0.001-0.1%, mainly due to the influence of downstream molecular signals in the mRNA that mark bona fide natural termination of protein translation. Basal readthrough of PTC, caused by nonsense mutations in gene coding sequences, is higher than readthrough of natural termination codons, and it can be efficiently increased by readthrough inducers. Several readthrough inducer compounds directly bind to the A-site of the ribosomes and induce conformational changes in the ribosome decoding center, which facilitates the incorporation of near-cognate tRNAs. This is the case of some aminoglycoside antibiotics such as G418/geneticin and gentamicin (9-13). Non-aminoglycoside compounds can also facilitate translational readthrough, although their mechanisms of action are less understood (14). Translational readthrough of PTC results in the biosynthesis of a full-length non-truncated protein with variable efficiency, mainly depending on the type of PTC and its nucleotide context, which can rescue the pathogenicity of the PTC mutation. This makes important the experimental analysis of the readthrough responses of genes of interest. Since the possibility of generation of non-functional full-length proteins by translational readthrough exists, due to the incorporation of non-wild type residues at the PTC site (15), it is also important to verify experimentally the reconstitution of the function of the readthrough protein products (16, 17). Thus, the potential benefits of induced readthrough as a therapeutic alternative for specific genetic diseases needs to be explored in detail for each individual PTC mutation (18, 19)

Protein tyrosine phosphatases (PTP) are major regulators of cell and tissue homeostasis and development, and their involvement in human disease has been widely documented (20-22). **Table 1** contains information on the PTP family genes that are targeted by PTC germline mutations, as reported in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>), with indication of the disease conditions associated to the mutations. **Figure 1** illustrates the abundance of different PTC found in these PTP. Several PTP from the dual-specificity phosphatase (DUSP) subfamily display a relatively high number of residues targeted by PTC, including some myotubularins (*MTM1*, *SBF2*; causing X-linked myotubular myopathy and Charcot-Marie-Tooth disease 4B2, respectively), Laforin, and the PTEN tumor suppressor. Laforin is a glycogen phosphatase encoded in the *EPM2A* gene; whose mutations cause Lafora disease, a progressive myoclonus epilepsy (23). *PTEN* is the PTP gene more abundantly targeted by PTC in human disease, both in the germline of patients causing PTEN Hamartoma Tumor Syndrome (PHTS) and in sporadic tumors (17, 24). *PTPN11* is the only gene from the classical PTP subfamily showing a relevant number of residues targeted by PTC, which are associated with metachondromatosis. The presence of PTC in *FIG4* (associated with Charcot-Marie-Tooth disease 4J), and *EYA1* (associated with Branchio-oto-renal syndrome) *and EYA4* (associated with nonsyndromic hearing loss) stand out from the SAC and EYA PTP subfamilies, respectively.

In this chapter, we show methodologies to analyse the collection of PTC (PTCome) associated to disease from PTP genes of interest, and laboratory protocols to study the experimental induction of translational readthrough of PTP targeted by PTC, taking PTEN and Laforin as two representative examples.

**2. Materials**

All solutions are prepared in double-distilled, deionized MilliQ filtered water. Plasticware is autoclaved or sterilized by ethylene oxide. Cell culture and transfection procedures require sterile conditions.

**2.1 *In silico* analysis of the PTCome distribution on PTP**

1. An updated web browser, such as Mozilla Firefox, Google Chrome or Apple Safari.
2. R and RStudio programs, version 1.1.463+.
3. *Stringr*, *dplyr* and *stats* R packages.
4. PTCMAKER Phyton program (https://github.com/compneurobilbao/stopcodon-pulido-17) (*see* **Note 1**).
5. xxx

**2.2 Assessing the induction of translational readthrough (by immunoblot)**

1. Tissue-culture plates.
2. Simian kidney COS-7 cells, suitable for transfection and transient overexpression of recombinant proteins (*see* **Note 1**).
3. Complete medium: Dulbecco’s Modified Eagle Medium (DMEM) containing high glucose supplemented with 5% heat-inactivated FBS, 1mM l-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin.
4. Trypsin-EDTA solution.
5. cDNAs of PTP wild type and PTC mutations of interest, cloned into suitable mammalian expression vectors (*see* **Note 2**).
6. Transfection reagents (*see* **Note 3**).
7. Translational readthrough inducers (*see* **Note 4**)
8. Lysis buffer: *Mammalian Protein Extraction Reagent* (M-PER, Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (*cOmplete EDTA-free* and *PhosSTOP* [Roche, Switzerland], respectively.
9. Sample buffer: *NuPAGE™ LDS (4X)* (Thermo Fisher Scientific), containing 5% 2-mercaptoethanol.
10. Polyvinylidene fluoride (PVDF) protein transfer membranes.
11. Transfer buffer: 48 mM Tris base, 39 mM glycine, 0.037% SDS, 20% methanol.
12. Prestained molecular weight standard protein markers.
13. Primary antibodies against the recombinant protein under study or recognizing a suitable artificial tag, and primary antibodies against an endogenous reference protein (*see* **Note 5**).
14. Fluorochrome-conjugated secondary antibody diluted, compatible with the detection system used (*see* **Note 6**).
15. Immunoblot blocking buffer: OBB buffer (*Odyssey Blocking Buffer*, LI-COR Biosciences) diluted 1:1 in PBS.
16. Immunoblot incubation and washing buffer: NET-gelatin buffer, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, 0.25% gelatin.

**3. Methods**

The implementation/incorporation of genetic studies as standard procedures for patient diagnosis is providing a wealth of information on the molecular basis of human disease. Many genetic alterations associated to disease, including alterations targeting the PTP gene family, consist of nonsense single-nucleotide substitutions generating premature termination codons (PTC) in the coding region of specific proteins. This makes important the experimental analysis of these PTC to develop efficient precision therapies for the carrier patients. The *first method* (*see* subheading 3.1) that we present in this chapter consists on *in silico* methodology to visualize and analyse the qualitative and quantitative distribution of the collection of PTC (PTCome) found on genes of interest in association to disease (PTEN and Laforin dual-specificity phosphatases are used as examples), as annotated in the literature and in gene mutation and gene variant databases (*see* **Note xx**). The qualitative analysis of PTC distribution along the domains of the protein under study may provide predictive genotype/phenotype information and potential attributes of pathogenicity to the distinct protein regions, whereas the quantitative analysis of PTC informs on the presence of mutational hotspots, which are influenced by both the functional output of the PTC mutation and the mutagenic chemical properties of specific nucleotide sequences (*see* **Figure 1** and comments in the legend). In many cases, proteoforms generated by PTC are unstable and their functional output is similar to gene loss. In addition, PTC truncated proteoforms can exist in the cell displaying pathogenic altered functions. In conclusion, qualitative and quantitative distribution of the PTCome associated to disease display gene-specific patterns, which need to be analysed individually in the context of pathogenicity caused by loss or alteration of specific protein functions.

The *second method* (*see* subheading 3.2) illustrates methodology to study the induction of biosynthetic translational readthrough of genes targeted by PTC, as monitored by immunoblot with specific antibodies of cell lysates from mammalian cells transiently transfected with cDNAs encoding the PTC gene variants of interest. Translational readthrough consists on the incorporation of an amino acid in the position of the PTC, which evade translation termination and permits the biosynthesis of a full-length protein. The efficiency of induced readthrough in these experiments mainly depends on the efficient translation of the protein product, the properties of the readthrough inducer, and the type of PTC and its nucleotide context (*see* **Figure 2** and **Figure 3**). This protocol is intended to detect the translation, upon readthrough induction, of the full-length protein under study, in contrast to the protocols that detect the activity of a reporter enzyme (such as luciferase) fused with the peptide of interest. The protocol can also detect the truncated proteoforms generated from the PTC. Our method has the advantage of determining the readthrough efficiency in the presence of the complete protein coding nucleotide sequence and in the absence of secondary effects of the readthrough inducers on the reporter protein (25). This facilitates the testing of the functional properties from the reconstituted protein (see **Note xx**). Since cDNA is used, which lacks exon-intron junction nucleotide sequences, the effect of NMD is not manifested in this protocol, providing reliable information on the efficiency of the induced readthrough reconstitution of the full-length protein, independently of PTC-dependent mRNA decay.

**3.1 *In silico* analysis of the PTCome distribution on PTP (Kernel plots)**

* + 1. Obtaining the potential PTCome (PTC that can be generated by single‐nucleotide substitutions from a protein coding nucleotide sequence)

1. To obtain the potential PTCome, extract from Gene database (NCBI, https://www.ncbi.nlm.nih.gov/gene/) the cDNA sequence of the desired gene. Copy it in a text document.
2. Import the text document in the PTCMAKER program and run the online code what is the online code? (*see* **Note xx**).
3. Run the code in (xx) to obtain the list of potential PTC residues.
   * 1. Cancer-associated PTCome (PTC generated by single‐nucleotide substitution somatic mutations found in tumors) (see **Note xx** databases)
4. To obtain the cancer-associated PTCome (somatic mutations), browse for the desired PTP in the COSMIC database (https://cancer.sanger.ac.uk/cosmic). Go to the mutation distribution section and click on nonsense substitution. Download/Export the desired dataset in a comma delimited (.csv) format. For this step you need to be logged in with a user and a password. Reliable cancer-associated PTCome can be obtained only for PTP whose function (usually lack-of-function) is related with cancer and display a significant number of PTC mutations distributed along their sequence (see **Note xx**).
5. Import the downloaded file in RStudio by File > Import Dataset > From text (readr) or using the *read\_csv(xx)* function, specifying comma as a delimiter.
6. Run the code in (*xx*) to obtain unique PTC mutated residues.
   * 1. Germline-associated PTCome
7. To obtain the germline-associated PTCome, browse for the desired PTP in the HGMD database (https://www.hgmd.cf.ac.uk). HGMD displays a data frame with missense and nonsense mutations together (*see* **Note xx**). ClinVar xxx
8. Import the downloaded file in RStudio by File > Import Dataset > From Excel, or using the *read\_excel()* function. Add additional PTC manually from the literature
9. Run the code in () to obtain unique PTC mutated residues.

ClinVar?

* + 1. PTCome qualitative representation: Kernel density plot

1. Insert where? the amino acid length and the name of your protein of interest.
2. Run the code in (*xx*) to obtain a kernel plot representation. The three vectors (potential PTCome, cancer-associated PTCome and germline-associated PTCome) are rescaled to 100 to facilitate comparisons between proteins. Mirror vectors are generated to avoid bias at boundaries and a density is calculated of sum of the parental vector and the mirror vectors. The final plot contains curves representing the potential PTCome, the cancer-associated PTCome and the germline-associated PTCome.

3.1.5 PTCome quantitative representation: Histogram of PTC frequency

Examples of *in silico* analysis of the PTCome from the dual-specificity phosphatases PTEN and Laforin (*EPM2A* gene) are provided in **Figure 2**. A larger number of mutations are found in the *PTEN* gene, which gives more robustness to the interpretation of the plots.

**3.2 Assessing the induction of translational readthrough**

1. Plate cells on 6 well-culture plates (1.5 x 104 cells/cm2; about 1.5 x 105 cells/well) (*see* **Note 7** and **Note 8**).
2. After 24 h of culture, transfect each well with 1 g of plasmid containing cDNA coding for the PTP under study, wild type or PTC mutations, or with empty vector (*see* **Note 2**, **Note 3**, and **Note 5**). When designing the experiment, consider that you will always need a control well for each cDNA, that will not be treated with the readthrough inducer.
3. After 24 h of culture, add the readthrough inducer (*see* **Note 4**).
4. After 24-48 h of culture, lyse the cells directly adding 150 L of ice-cold lysis buffer to each well. Keep the plate on ice for 10 min under balancing and transfer the lysate from each well to an Eppendorf tube.
5. Centrifuge the lysate 10000g, 10 min, 4°C. Transfer the supernatant, containing the cellular protein extract, to a clean Eppendorf tube. Cell lysates can be frozen at this step.
6. Mix 50 L of cell lysate with 15 L sample buffer (*see* **Note 8**).
7. Boil for 3 min, spin and load the mix in a 10% SDS-PAGE gel. We routinely run 10% or 12% SDS-PAGE gels, depending on the size of the proteins to be resolved. Include a lane with prestained molecular weight standard protein markers.
8. Run the gel and transfer to a PVDF membrane. Cut a piece of the membrane in the range of the migration of your full-length protein, the PTC-generated truncated protein, and the reference endogenous protein (*see* **Note 9**).
9. Perform standard immunoblot procedure, incubating the membrane with primary and secondary antibodies according to the specifications of the antibodies used (*see* **Note 5**).
10. Quantify the intensity of the full-length band of the PTC protein variant under study, upon the readthrough induction conditions, in comparison with the wild type protein. Efficiency of readthrough can be shown/represented as the percentage of expression with respect to the wild type protein (*see* **Note 6** and **Note 10**).

**4. Notes**

1. PTCMAKER lists the PTC which can be generated by single‐nucleotide substitutions from a protein coding nucleotide sequence, which we call the potential PTCome. In principle, the potential PTCome should show a relatively homogeneous distribution (straight line in Kernel representation) of potential PTC along the sequence of interest.
2. COS-7 simian kidney cells are suitable for high transfection efficiency and high levels of overexpression, but other mammalian or human cell lines can be used if transfection and overexpression conditions are optimized.
3. A high efficiency mammalian expression vector is recommended. We routinely use the pRK5 mammalian expression vector, which contains the SV40 origin of replication and works efficiently in COS-7 cells expressing the SV40 large T-antigen. In the case of using antibiotics as readthrough inducers, note that the expression vector cannot contain the corresponding antibiotic resistance gene. Accordingly, when using the mammalian selectable antibiotic G418/geneticin as the inducer, the commonly used neomycin resistance gene (whose protein product is active against geneticin) cannot be carried in the expression vector (*see* **Figure 3**).
4. There are different suitable protocols for transfection of adherent mammalian cells, which need to be optimized for specific cell lines. In our experience, *GenJetTM* (SignaGen*®* Laboratories) works efficiently as a transfection reagent with several cell lines, but any other commercial lipid transfection reagent can also be used. In addition, COS-7 cells are transfected with good efficiency using the DEAE-dextran/DMSO/chloroquine method (26).
5. A wide variety of aminoglycoside and non-aminoglycoside readthrough inducers exist. G418/geneticin is widely documented as a high efficiency aminoglycoside readthrough inducer in a 100-200 g/mL (about 150-300 M) concentration range, and it is recommended as an inducer in the first tests, although its toxicity excludes its long-term experimental use or its potential therapeutic use.
6. Dilution and washing conditions need to be individually tested for each antibody. High affinity antibodies are recommended to achieve efficient detection of the recombinant protein of interest upon translational readthrough. Take into consideration that the location of the epitope recognized by the antibody determines the possibility of detecting truncated stable proteins generated by the PTC (*see* **Figure 4**). The use of artificial epitope tags (such as Hemagglutinin or Flag epitope tags), which can be located N-terminal or C-terminal in the recombinant protein sequence, is helpful to visualize truncated and non-truncated protein forms under readthrough induction. Note that when a C-terminal tag is incorporated to the recombinant protein under study, the reconstitution of a full-length protein upon readthrough induction is monitored both by the size of the protein and by the recognition by the anti-tag antibody (*see* **Figure 3**). As endogenous reference antibodies we use routinely anti-GAPDH or anti--actin antibodies.
7. We use the *Odyssey® CLx Imaging System* (LI‐COR Biosciences)fluorescence detection system, which permits reliable quantification of the protein bands from the immunoblot experiments. For band quantification, we use the *Image Studio™* (LI‐COR Biosciences)software.
8. Optimal cell confluency for transfection depends on each cell line, but in our experience, it is not convenient to transfect cells with more than 50% confluency. As an example, COS-7 cells are optimally transfected when they are at about 20-25% confluency.
9. Final loading volumes about 40-80 L are convenient, depending on the size of your SDS-PAGE wells.
10. To detect truncated forms of the protein under study, because of the PTC, do not cut away the bottom part of the membrane.
11. The efficiency of the readthrough induction depends on several factors (*see* heading 3). A range of 0-40% of efficiency can be achieved using G418/geneticin as the readthrough inducer. Although the readthrough of each PTC needs to be evaluated individually, the readthrough efficiency of the three types of PTC, when considered globally, follows the order UGA>UAG>UAA (TGA>TAG>TAA when considering the cDNA sequence).

**Acknowledgements**

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**Legends to the Figures**

**Figure 1.** Representation of the number of different PTC identified in PTP genes in association with human disease, as reported in ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar). The top panel shows the total number of different PTC annotated for each gene. The bottom panel shows the percentage of different PTC with respect to the total number of amino acids from each protein. Note that these are qualitative data, and they do not represent the number of times each PTC has been annotated or reported.

**Figure 2.** Kernel etc

**Figure 3.** Translational readthrough induction of PTEN PTC (premature termination codon) mutations. (a) Readthrough‐induced expression of the disease‐associated PTEN R233X [p.(Arg233Ter), TGA] mutation. COS‐7 cells were transfected with pRK5 plasmids containing untagged PTEN (PTEN) or C-terminal hemagglutinin-tagged PTEN (PTEN-HA), wild type (WT) or the R233X mutation, and 24 h after transfection cells were kept untreated or incubated in the presence of G418/geneticin (200 μg/ml) for an additional 24 h, as indicated. EV, empty vector. Proteins were resolved in 10% sodium dodecyl sulfate‐polyacrylamide gel electrophoresis (SDS‐PAGE) gels and were detected by immunoblot using the anti‐PTEN 6H2.1 mAb, which recognizes a PTEN C-terminal epitope (residues 392-398) (27) (top panel), or the anti-HA 12CA5 mAb (bottom panel). The arrow (→) indicates the migration of full‐length PTEN or PTEN-HA. (b) Induced expression of PTEN R233X mutation by different readthrough inducer compounds. COS-7 cells were processed as in (a) and treated for 24 h with the indicated compounds (-, no treatment; G418, 200 μg/ml; gentamicin, 800 μg/ml; amikacin, 2 mg/ml; tobramycin, 800 μg/ml; erythromycin, 175 μg/ml). Proteins were detected by immunoblot using the anti‐PTEN 6H2.1 mAb and an anti-GAPDH antibody. The arrow (→) indicates the migration of full‐length PTEN. (c) Schematic of PTEN domains, with indication of the PTC illustrated in the Figure and the methionine residue at position 35 (Met35), which is used as an alternative initiation Met in the presence of upstream PTC, generating the PTEN‐N‐terminal‐truncated protein PTEN M35 (17). (indicated with an asterisk (\*) in the top panel) (d) Readthrough‐induced expression of the disease‐associated PTEN R11X [p.(Arg11Ter), TGA], R15X [p.(Arg15Ter), TGA], and R130X [p.(Arg130Ter), TGA] N-terminal mutations. COS-7 cells were processed as in (a) and treated for 24 h with G418 (200 μg/ml), as indicated. Proteins were detected by immunoblot as in (b). In the top panel, a representative immunoblot is shown. The arrow (→) indicates the migration of full‐length PTEN. The asterisk (\*) indicates the migration of PTEN M35. In the bottom panel, the quantification of the readthrough‐induced expression of the PTEN PTC mutations is shown. The readthrough efficiency is represented as the percentage of full-length PTEN expression from each PTEN PTC mutation with respect to PTEN wild type. PTEN amino acid numbering is according to NP\_000305.

**Figure 4.** Translational readthrough induction of Laforin premature termination codon (PTC) mutations. (a) Schematic of Laforin domains, with indication of the PTC illustrated in the Figure. (b) Readthrough‐induced expression of the disease‐associated Laforin Y86X [p.(Tyr86Ter), TAG] and R241X [p.(Arg241Ter), TGA] mutations. COS-7 cells were processed as in Figure 2 and treated for 24 h with G418 (200 μg/ml), as indicated. Proteins were detected by immunoblot using anti-Laforin antibody recognizing Laforin residues 131-144 (Sigma Aldrich, SAB2500581), followed by anti-GAPDH antibody, as indicated. The arrow (→) indicates the migration of full‐length Laforin. The asterisk (\*) indicates the migration of Laforin R241X truncated proteoform. (c) Induced expression of Laforin R241X mutation by different readthrough inducer compounds. COS-7 cells were processed as in (a) and treated for 24 h with the indicated compounds (-, no treatment; G418, 200 μg/ml; gentamicin, 800 μg/ml; amikacin, 2 mg/ml; PTC124/Ataluren, 1 μg/ml). Proteins were detected by immunoblot using the anti‐Laforin and anti-GAPDH antibodies, as in (b). The arrow (→) indicates the migration of full‐length Laforin. The asterisk (\*) indicates the migration of Laforin R241X truncated proteoform. Laforin amino acid numbering is according to NP\_005661.

**Figure 5.** context etc