Supporting Information

SI Materials and Methods

Readthrough Drugs

PTC Therapeutics, Inc. supplied ataluren for this study. Ataluren was dissolved in dimethylsulfoxide (DMSO) and cultured cells were treated such that the final DMSO concentration was 0.3% (vol/vol). Tobramycin, geneticin (G418), and gentamicin were purchased from Sigma and were administered in PBS.

Plasmid Construction

a) Yeast expression plasmids

i. *LUC* reporters. Construction and use of epitope-tagged *LUC*-based PTC_{20} reporters has been described previously (1).

ii. *HIS3* reporter. The wild-type HA-*HIS3*-SF and the PTC-containing HA-*HIS3*$_{(UAA100)}$-SF constructs consist of the yeast *TPI1* promoter followed by an N-terminal 3X Hemaglutinin (HA) tag fused in-frame with the *HIS3* gene (with/without an in-frame stop codon at *HIS3* position 100), followed by in-frame StrepII and FLAG tags and the *TPI1* 3' UTR. The plasmids harboring either the wild-type or the PTC-containing *HIS3* cassette were generated by PCR and standard molecular cloning techniques, and fragments were inserted into yEplac181 after digestion of the respective restriction sites.

b) Mammalian expression plasmids

i. NanoLuc reporters. The pFN[Nluc/CMV/Neo] plasmid containing the NanoLuc open reading frame was purchased from Promega Corporation (CS181701). Using site-directed mutagenesis and oligonucleotides DB4084 and DB4085 (Supp. Table 7), a point mutation was introduced into NanoLuc at codon 12, which changed the tryptophan codon (UGG) to a UGA PTC. Using standard techniques, the WT and W12X NanoLuc open reading frames were then subcloned into the NheI and XhoI sites of pcDNA3.1Zeo(-) (Invitrogen). These plasmid constructs were subsequently transfected into 293H cells and stable transfectants were selected using 0.2 mg/ml zeocin (Invitrogen) for three weeks.

ii. p53 reporters. Alleles of the p53 ORF were synthesized with TGA nonsense mutations at positions 196 or 213 and then cloned into pcDNA3.1/Hygro(+) using standard techniques. The plasmids were then transfected into either CALU-6 or HDQ cell lines.

iii. H2B-GFP reporter. This reporter encompasses a histone H2B coding sequence fused to that of GFP, with the TGG codon at nucleotide positions 172-174 of the GFP ORF mutated to TGA. Construction of the vector was as described by Lentini *et al*. (2)

iv. *LUC* reporters. The luciferase reporter system used for readthrough analyses in mammalian cells is similar to the set of yeast luciferase readthrough reporters used in Roy *et al.* (1), with a few modifications. Intron 6 from the yeast *TPI1* gene was introduced into the *LUC* coding sequence at nucleotide 239, downstream of the PTC_{20} insertion site. The HA-*LUC*2-SF cassette (wild-type or PTC-containing) was then cloned into corresponding restriction sites in the pSELECT Zeo MCS using standard molecular biology techniques.

v. TGFP reporter. *CFTR-G542X* (UGA) or the corresponding wild-type along with the G542X context (three codons upstream and downstream) was fused to TurboGFP (TGFP) along with HA and 6XHis tags. This fragment was inserted into the pcDNA 3.1 Zeo(+) expression vector (Invitrogen). CFTR-G542R, G542C, and G542W were obtained through site-directed mutagenesis of a wild-type CFTR cDNA. The mutated fragment of CFTR was inserted into the CFTR cDNA in pcDNA 3.1 Zeo(+) and sequences were confirmed.

Yeast Cell Methods

a) Protein purification and analyses. Cells expressing HA-*LUC*-SF or HA-$LUC_{(PTC20)}$-SF were grown in synthetic complete (SC) medium lacking leucine with or without any drug treatment for readthrough induction. Readthrough products were purified as described previously (1). Briefly, cells were collected by rapid filtration and resuspended in Buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA; supplemented with 0.1 mM DTT, 1 mM PMSF, and 1X protease inhibitor cocktail (Roche)). Cells were lysed using a cryoMill (Retsch) at 10 hertz for 20 min with constant liquid nitrogen cooling. Cell lysates

were clarified and luciferase was purified using Strep-Tactin resin according to the manufacturer's protocol (IBA, Germany). The efficiency of luciferase purification was monitored by analyzing fractions for luciferase activity, as well as by western blot analyses using anti-FLAG antibody (Sigma). Elution fractions from Strep-Tactin purification were concentrated using Amicon Ultra-15 30K NMWL filters (EMD Millipore) followed by analysis on 8% SDS-PAGE to resolve the readthrough product. Gels were silver stained (Proteosilver, Sigma) and processed for MS analysis. Three independent experiments were performed for all (one codon under one readthrough-inducing condition) except G418 treatment for which only two independent experiments were performed.

b) RNA analyses. Total RNA isolation and northern analyses were performed as described previously (1). Cells expressing either HA-*LUC*-SF or HA-*LUC*$_{(PTC20)}$-SF were grown in synthetic complete (SC) media lacking leucine. Random-primed DNA probes made from the 1.6 Kb NcoI-XhoI luciferase fragment or 129 bp XhoI-XbaI Strep-FLAG fragment were used to detect *LUC* mRNA and *HIS3* mRNA, respectively. Random-primed DNA probes made from the 0.6 Kb EcoRI-HindIII *CYH2* fragment, or the 0.5 Kb EcoRI *SCR1* fragment were used to detect the *CYH2* pre-mRNA, *CYH2* mRNA, and the *SCR1* RNA as controls for NMD and normalization, respectively. Transcript-specific signals were determined with a FUJI BAS-2500 analyzer.

For tRNA expression analysis, total tRNA was isolated from wild-type cells and cells overexpressing either tRNA-Arg-UCG (DL869) (3), tRNA-Arg-UCU (ECB1451), or tRNA-Glu-UUC (ELH239) as described previously (4). Isolated tRNAs (10 µg per sample) were resolved on a 6.5% polyacrylamide-8 M urea-0.1 M NaAc (pH 5.0) gel and electroblotted to Zeta-Probe membranes (Bio-Rad). ^{32}P-labeled oligonucleotides complementary to the tRNA of interest were used as probes. tRNA-specific signals were determined with a FUJI BAS-2500 analyzer.

c) Western analyses. Yeast cells (0.4 OD_{600} units per sample) were collected and processed as described previously (1). Samples were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore) membranes, and incubated with anti-HA antibody (Sigma), anti-FLAG antibody (Sigma), or a monoclonal anti-Pgk1 antibody (Molecular Probes).

d) Protein half-life measurements. Readthrough product half-life was quantified as described previously (5). Yeast cells expressing HA-*HIS3*-SF or HA-*HIS3*$_{(PTC100)}$-SF reporters were treated with ataluren and were grown in synthetic complete (SC) media lacking leucine to log phase. Cycloheximide (50 µg/mL) was added to terminate protein synthesis followed by collection of equal number of cells at various time points and subjected to western blot analyses.

Mammalian Cell Methods

a) Protein purification and activity assays

i. NanoLuc activity assay. Prior to performing NanoLuc assays, zeocin was omitted from the media of the stable NanoLuc 293H cell lines for two passages due to its inhibitory effect on cell growth. WT and W12X reporter cells were then seeded into 96-well culture plates at a density of 4 x10^4 per well. When cells became ~50% confluent, they were treated with drugs for a period of 48 hours prior to assay. NanoLuc activity was measured using the Nano-Glo Luciferase Assay (Promega, N1110). All cells expressing the NanoLuc constructs were lysed in 50µl of 1X Passive Lysis Buffer (PLB) (Promega, E1941). However, the lysate of WT NanoLuc expressing cells was subsequently diluted 1:1000 with 1X passive lysis buffer. In a separate 96-well plate (Fisher 12-566-04), 5µl of the Nano-Glo Reagent were mixed with 5µl of each cell lysate and then incubated for 10 min at room temperature. Luciferase activity readings were then measured using a GloMax Multi Detection System (Promega). The data are expressed as the NanoLuc activity generated normalized to micrograms of total protein.

ii. Purification and analysis of *LUC* readthrough products. Wild-type or PTC-containing *LUC* expression plasmids were transfected into 293H cells (ThermoFisher Scientific) with FuGene 6 (Promega) and stable clones were selected using 300µg/mL Zeocin (ThermoFisher Scientific). Cells were harvested 72 hours after 30 µM ataluren treatment and 24 hours after 150 µM G418 (geneticin) treatment. Tandem purification of the readthrough products utilized C-terminal Strep or FLAG tags, followed by selection for the N-terminal HA tag. Briefly, cells were harvested in lysis buffer (FLAG purification: 0.025 M Tris, pH 7.4, 0.15 M NaCl, 0.001 M EDTA, 1% NP40, 5% glycerol; StrepTactin purification:100 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) and the clarified lysates

were bound to either StrepTactin (Qiagen) or FLAG (Sigma) magnetic beads at 4ºC overnight according to the manufacturer's protocol. The eluates from the first purification were then bound to anti-HA magnetic beads for one hour and eluted by boiling the beads in 1X Laemmli Buffer (BioRad) for 10 min. The elution profile was monitored by measuring luciferase activity at each step in the purification process, using Bright-Glo (Promega) as a substrate and measured using a Perkin Elmer EnVision plate reader. The purified readthrough products were then analyzed on 10% SDS-PAGE, and gels were then silver stained (ProteoSilver, Sigma) and processed for mass spectrometry. At least three independent experiments were performed for each condition (each codon under one specific readthrough-inducing condition).

iii. Purification of GFP-CFTR readthrough products. 293H cells were harvested and lysed using three freeze and thaw cycles and a lysis buffer (pH 8.0, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) containing a protease inhibitor cocktail tablet (Roche). The lysate was centrifuged and the supernatant was transferred to a new tube and incubated with Ni-NTA Superflow resin (Qiagen) overnight at 4ºC. The next day, the resin was washed three times with lysis buffer and eluted with elution buffer (pH 8.0, 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole) four times.

b) Western analyses

i. Luciferase polypeptides. For detection of the readthrough products, 293H whole-cell lysates were electrophoresed through 10% (w/v) Bis-Tris polyacrylamide gels (ThermoFisher Technologies), transferred to a nitrocellulose membrane (BioRad), and incubated with anti-HA (1:1,000; ThermoFisher Technologies), anti-FLAG (1:1,000; Sigma), or anti- Firefly Luciferase (1:2,000; Rockland) antibodies. An anti-GAPDH antibody (1:10,000; Abcam) was used for normalization.

ii. GFP polypeptides. For the TGFP readthrough reporters, 293H cells were washed with 1X PBS, harvested, and lysed with lysis buffer. The lysate was centrifuged, boiled, and fractionated by electrophoresis on SDS-PAGE gels. Gels were transferred to Immobilon-FL PVDF membranes (EMD Millipore), which were then blocked using 5% (w/v) milk in PBST (0.3% Tween 20, v/v), incubated with anti-HA (Covance Research), anti-Tubulin (Abcam or DHSB), and anti-TGFP (Pierce) antibodies, followed by incubation with secondary antibodies IRDye 680RD and IRDye 800CW (Li-Cor) before imaging

using a Li-Cor Odyssey® CLx Infrared Imaging System. For CFTR western blots, cells were lysed with RIPA buffer containing a protease inhibitor tablet (Roche). The lysate was collected, incubated at 37°C for 30 min before electrophoresis, and then transferred and blotted as was done for TGFP proteins. CFTR antibodies were a 1:1 mixture of #570 and #596 monoclonal anti-CFTR antibodies from the University of North Carolina (http://www.unc.edu/~tjjensen/CFADP/index.html).

For the H2B-GFP readthrough products, 293H cell lysates were electrophoresed through 10% (w/v) Bis-Tris polyacrylamide gels (ThermoFisher Technologies), transferred to a nitrocellulose membrane (BioRad), and incubated with anti-GFP (1:1,000; Life Science Technologies) antibodies. An anti-GAPDH antibody (1:10,000; Abcam) was used for normalization.

iii. p53 polypeptides. The p53 readthrough products from cell lines expressing p53 nonsense alleles were detected using p53 MSD/ELISA (Meso Scale Discovery) using DO1 SC-126X (SantaCruz) and ab59243 (Abcam).

c) RNA analyses

i. *LUC* transcripts. Total RNA was extracted using RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions. cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). RNA samples were analyzed by Taqman-based RT-qPCR using luciferase specific primers (Table S7).

ii. GFP transcripts. Total RNA was extracted using RNeasy Mini Kit (Qiagen) as per the manufacturer's directions. cDNA was obtained through reverse transcription using iScript™ Reverse Transcription Supermix (Biorad). Quantitative real-time PCR was performed using the CFX96 Real-Time PCR Detection System (Biorad). Primer efficiencies for human CFTR discovered in Primerbank (http://pga.mgh.harvard.edu/primerbank/index.html) (5'-CCTATGACCC GGATAACAAG GA-3' and 5'-GAACACGGCT TGACAGCTTT A-3') and rat tubulin15 (5'-CAACACCTTC TTCAGTGAGA CAGG-3' and 5'-TCAATGATCT CCTTGCCAAT GGT-3') were 93.4% and 95.8%, respectively.

d) Functional assays in FRT monolayers

CFTR constructs were transfected into Fischer Rat Thyroid (FRT) cells and cell lines that stably express each CFTR cDNA were selected. Cells were seeded on Costar

24 well 0.4 µM permeable supports (Corning). After four days, confluent monolayers with tight junctions had formed, as measured by transepithelial resistance using an epithelial volt-ohm meter (WPI). Isc was measured in Ussing Chambers (Physiologic Instruments) using an 8-channel voltage clamp, while Gt was measured using a 24-channel voltage clamp (6).

Sample Preparation for Mass Spectrometry Analyses

a) Analyses of luciferase readthrough products. Full-length luciferase readthrough products resolved on SDS-polyacrylamide gels were excised after silver staining and the destained gel slices were subjected to endoproteinase Lys-C (Roche Diagnostics) digestion followed by LC-MS/MS analyses as described previously (1). Raw data files were subjected to database searching with Mascot Server (version 2.5) against the SwissProt index of *S. cerevisiae* (for yeast samples) and Human **(**for mammalian samples) that contain the sequences of all 20 potential codon 20 mutations of the *LUC* gene. The relative abundance of each amino acid at position 20 of luciferase was calculated by adding the corresponding precursor intensity of individual endo Lys-C peptides containing a codon 20 amino acid to yield a total peptide abundance that was then used to calculate the percentage of insertion of each amino acid (1).

b) Analyses of GFP-CFTR readthrough products. Full-length TGFP was purified using Ni-NTA Superflow resin (Qiagen). Protein fractions were resolved on an SDS Bis-Tris gel (Invitrogen) and gel bands corresponding to the molecular weight of interest were reduced, carbidomethylated, dehydrated, and digested with Trypsin Gold (Promega). Peptides were extracted, concentrated under vacuum, and resolubilized in 0.1% formic acid prior to analysis by 1D reverse phase LC-ESI-MS2 (7). Peptide digests were injected onto a Surveyor HPLC plus (Thermo Scientific) using a split flow configuration on the back end of a Jupiter C-18 column (Phenomenex). This system runs in-line with a Thermo Orbitrap Velos Pro hybrid mass spectrometer, equipped with a nano-electrospray source (Thermo Scientific), and all data were collected in CID mode. Data were searched using SEQUEST with a precursor mass window of 20ppm, trypsin digestion, variable modification C at 57.0293, and M at 15.9949. Searches were performed with a human subset of the UniRef100 database, which included common contaminants such as

digestion enzymes and human keratin, in addition to sequences specific to these experiments. A list of peptide IDs was generated based on SEQUEST search results, and which was then filtered using Scaffold Q+ (Protein Sciences). The filter cut-off values were set with peptide length (>5 AA's), no peptides with a MH+1 charge state were included, peptide probabilities were calculated and set to >90% C.I., with the number of peptides per protein set at two or more, and protein probabilities set to >99% C.I. Relative quantification across experiments was performed via spectral counting, and spectral count abundances were then normalized between samples. All spectra covering the sequences of interest were manually interpreted as a final quality control.

PTC124-AMP

a) Synthesis of PTC124-AMP. The compound was prepared as described by Auld *et al*. (8) (see their SI methods, compound 6). Briefly, a mixture of 3-(5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl)benzoic acid (0.2 g (0.70 mM) and ethyl-(N',N'-dimethylamino) propylcarbodiimide (EDC) 0.67 g (3.52 mM) in anhydrous DMF (4 mL) was stirred at room temperature for 10 min. Then adenosine-5-monophophate disodium salt 0.275 g (0.70 mM) and 4-dimethylamino-pyridine (DMAP) 9 mg (0.07 mM) were added and stirred vigorously at room temperature for one hour. The crude product was purified by preparative HPLC and lyophilized to get the free acid as a white powder. The free acid was then dissolved in 0.1 M ammonium bicarbonate solution and lyophilized to obtain the ammonium salt of the PTC124-AMP adduct.

b) Stability of PTC124-AMP. The stability of PTC124-AMP was assessed under conditions previously described for a cell-free translation system (9) except that 293H cells were used to prepare the lysate. Synthetic *LUC* mRNA containing a PTC at position 190 was prepared using the MegaScript® *in vitro* transcription kit (Ambion, Austin, TX). The reaction mixtures (20 µl) contained 16.5 mM HEPES (KOH) pH 7.4, 85 mM potassium acetate, 1.48 mM magnesium acetate, 0.56 mM ATP, 0.075 mM GTP, 18.75 mM creatine phosphate (di-Tris), 1.275 mM dithiothreitol (DTT), amino acids, creatine kinase, cell extract (60% of the reaction volume), 100 ng RNA, and 0.25% DMSO. Stock solutions of PTC124 (ataluren) and PTC124-AMP were prepared in 100% DMSO and PTC124-AMP at a final concentration of 5uM was added to the *in vitro* reaction. At various

time points following the addition of PTC124-AMP, aliquots were removed and the levels of PTC124-AMP and PTC124 (ataluren) were determined by LC/MS-MS.

Supplementary references

1. Roy B, Leszyk JD, Mangus DA, & Jacobson A (2015) Nonsense suppression by near cognate tRNAs employs alternative base-pairing at codon positions 1 and 3. *Proc. Natl. Acad. Sci. USA* 112:3038-3043.
2. Lentini L*, et al.* (2014) Toward a Rationale for the PTC124 (Ataluren) Promoted Readthrough of Premature Stop Codons: A Computational Approach and GFP-Reporter Cell-Based Assay. *Mol. Pharm.* 11:653-664.
3. Letzring DP, Wolf AS, Brule CE, & Grayhack EJ (2013) Translation of CGA codon repeats in yeast involves quality control components and ribosomal protein L1. *RNA* 19(9):1208-1217.
4. Johansson MJ & Bystrom AS (2004) The Saccharomyces cerevisiae TAN1 gene is required for N4-acetylcytidine formation in tRNA. *RNA* 10(4):712-7199.
5. Belle A, Tanay A, Bitincka L, Shamir R, & O'Shea EK (2006) Quantification of protein half-lives in the budding yeast proteome. *Proc. Natl. Acad. Sci. USA* 103(35):13004-13009.
6. Xue X*, et al.* (2014) Synthetic aminoglycosides efficiently suppress cystic fibrosis transmembrane conductance regulator nonsense mutations and are enhanced by ivacaftor. *Am. J. Respir. Cell Mol. Biol.* 50(4):805-816.
7. Ludwig MR*, et al.* (2016) Surveying the serologic proteome in a tissue-specific kras(G12D) knockin mouse model of pancreatic cancer. *Proteomics* 16(3):516-531.
8. Auld DS*, et al.* (2010) Molecular basis for the high-affinity binding and stabilization of firefly luciferase by PTC124. *Proc. Natl. Acad. Sci. USA* 107(11):4878-4883.
9. Welch EM*, et al.* (2007) PTC124 targets genetic disorders caused by nonsense mutations. *Nature* 447(7140):87-91.

Supplementary figure legends

Fig. S1. Key features of reporter genes expressed in yeast and mammalian cells. (*A*) The yeast *HA-HIS3$_{(UAA100)}$-SF* reporter contained an in-frame 3X HA tag at the N-terminus of its ORF, adjacent StrepII and FLAG (SF) tags at the ORF C-terminus. (*B*) The NanoLuc reporter for mammalian cells that encoded a UGA stop codon at ORF position 12 (W12X). (*C*) The construction of four distinct yeast *LUC* alleles, one wild-type and three with each of the possible PTCs inserted at codon 20 of the *LUC* ORF, was described previously (1). The *HA-LUC$_{(PTC20)}$-SF* reporter set for expression in 293H cells was similar to that used in yeast (1), but contained an intron insertion (site denoted in figure). (*D*) The Turbo GFP-*CFTR*-G542X reporter used to identify amino acids inserted at the PTC during readthrough. The *CFTR* context included in the construct is shown. (*E*) The H2B-GFP reporter originally described by Lentini et al. (2). (*F*) The p53 reporters used to detect ataluren-mediated readthrough encode the p53 ORF with UGA at codons 196 or 213.

Fig. S2. Nonsense-containing reporter transcripts are substrates for NMD. Northern analyses of the *(A) HA-LUC$_{(PTC20)}$-SF* and *(B) HA-HIS3$_{(UAA100)}$-SF* mRNAs expressed in wild-type [*PSI*-] yeast cells without/with drug treatment and in *upf1Δ* cells (as a control). Blots were re-probed for *CYH2* transcripts as an internal NMD control. *SCR1* RNA was used as a loading control. *(C)* RT-qPCR analyses of *HA-LUC-SF* wild-type and nonsense-containing mRNAs in 293H cells after cycloheximide treatment, showing drug-dependent stabilization of the transcripts derived from PTC alleles.

Fig. S3. Readthrough efficiencies of different reporters expressed in mammalian cells. (*A*) Ataluren and G418 increase readthrough of the p53-UGA-196 allele in CALU-6 cells and the p53-UGA-213 allele in HDQ cells. The respective reporters are shown in Fig. S1 and the data depict fold increase over DMSO as quantified by MSD/ELISA (n=3). (*B*) Ataluren and G418 increase readthrough from the H2B-GFP-UGA$_{58}$ reporter depicted in Fig. S1. Western blot of readthrough products in transiently transfected 293H cells. H2B-GFP is the full-length product expressed from a construct lacking a PTC. (*C*) Readthrough efficiency measured as luciferase activity from HA-*LUC*-SF and HA-

LUC$_{(PTC20)}$-SF reporters expressed in 293H cells (n=3; error bars represent standard deviation from the mean). (*D*) Western analyses showing full-length readthrough products expressed from HA-*LUC*$_{(UGA20)}$-SF reporters after ataluren or G418 treatment of 293H cells.

Fig. S4. Ataluren treatment of yeast cells promotes readthrough of HA-*LUC*$_{(PTC20)}$-SF reporter mRNA. Western analyses showing full-length readthrough products expressed from HA-*LUC*$_{(UGA20)}$-SF reporters after ataluren or G418 treatment.

Fig. S5. Half-life of the His3 protein.

Top: representative western blot depicting decay of the WT His3 protein after cycloheximide treatment of yeast cells at t=0. Bar graphs: upper and middle panels depict quantitation of results from untreated and cycloheximide-treated cultures expressing wild-type His3 protein; lower panel depicts quantitation of full-length His3 protein derived from ataluren-mediated readthrough after cycloheximide treatment of yeast cells at t=0. The levels of wild-type or PTC readthrough His3 protein were determined by western blotting and normalized to levels of the Pgk1 protein also determined by western blotting in the same experiments. T15, 30, 45, 60=15, 30, 45, and 60 min after cycloheximide addition.

Fig. S6. PTC124-AMP is rapidly converted to the active readthrough molecule PTC124 (ataluren) in an *in vitro* translation extract. PTC124-AMP at a final concentration of 5uM was added to an *in vitro* readthrough assay. At various time points following the addition of PTC124-AMP (dissolved in DMSO), aliquots were removed and the levels of PTC124-AMP and PTC124 (ataluren) were determined by LC/MS-MS.

Fig. S7. Purification of luciferase readthrough products.

(A) Western blot analysis of the full-length readthrough products purified on Strep-Tactin resin from wild-type [*PSI-*] yeast cells after treatment with ataluren. Anti-FLAG antibody was used for detection. Ec depicts elution fractions (E3-E7) that were pooled and concentrated. *(B)* Silver-stained SDS/PAGE gel showing the results of FLAG - HA

purification of the luciferase readthrough protein (marked with an asterisk) from ataluren-treated 293H cells expressing the HA-*LUC*$_{(UGA20)}$-SF reporter.

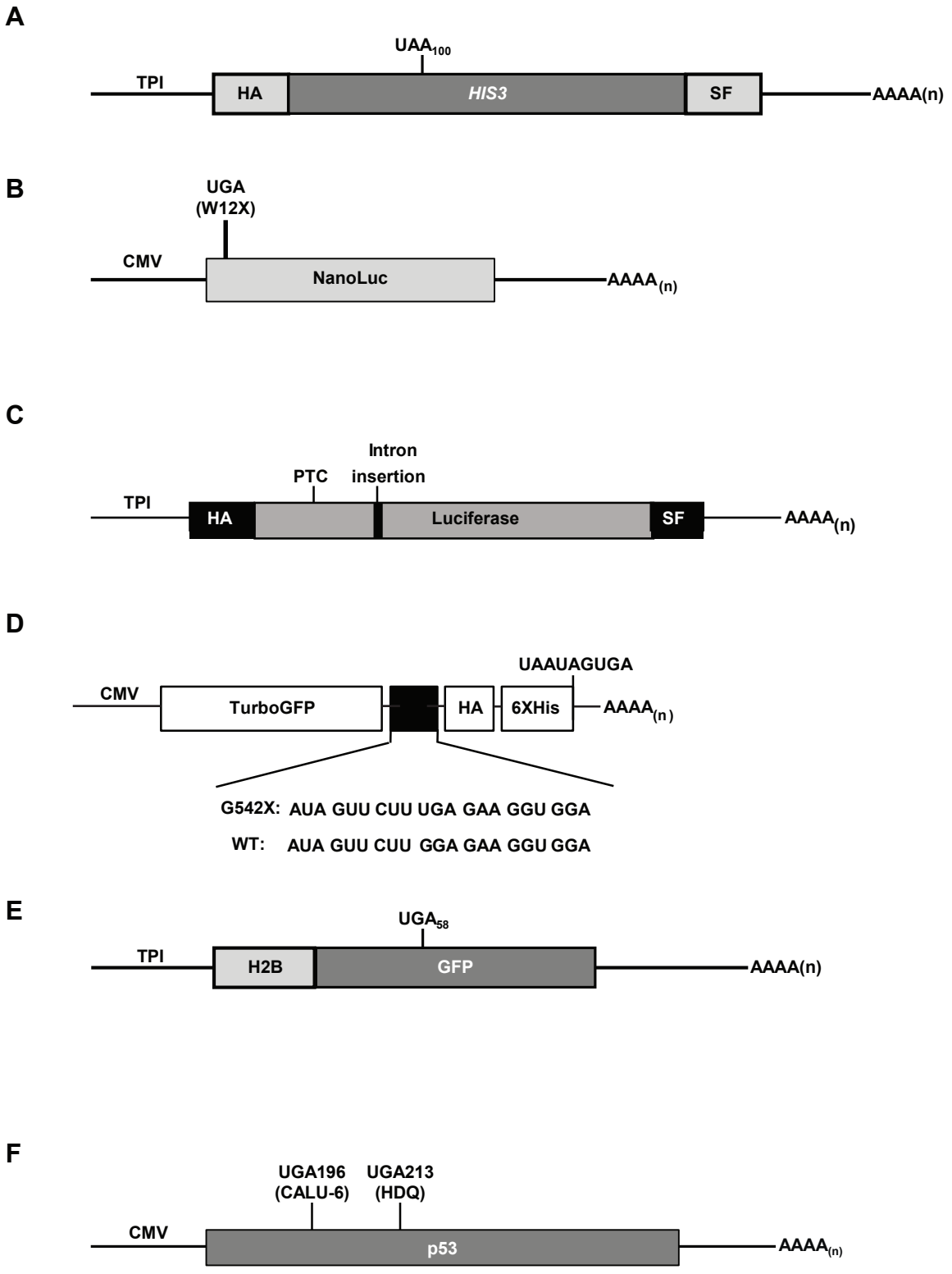
Fig. S8. Ataluren-induced His3 and luciferase readthrough products are functional. *(A)* Growth of wild-type cells expressing the HA-*HIS3*$_{(UAA100)}$-SF allele in media lacking histidine with or without drug treatment. *(B)* Luciferase activity from HA-*LUC*-SF reporter with missense mutations introduced at position 20 of luciferase. Luciferase activity is expressed as relative luciferase units/µg of protein (n=3; error bars represent standard deviation from the mean).

Fig. S9. Representative mass spectrometry spectra in CFTR G542WT and G542X samples. Tandem MS2 spectra are illustrated for the CFTR wild-type tryptic peptides and those from three CFTR mutants analyzed in our study; *(A)* G542G; *(B)* G542R; *(C)* G542W; and *(D)* G542C.

Fig. S10. tRNA overexpression analyses. *(A)* Northern analyses of total RNA isolated under acidic conditions from wild-type cells and cells overexpressing tRNA-Arg-UCU or tRNA-Arg-UCG. After polyacrylamide gel electrophoresis and transfer, blots were probed for tRNA-Arg using a ^{32}P-labeled oligonucleotide. The blots were re-probed for tRNA-Glu as a control for equal loading. Lane 1: Wild-type; Lane 2: Wild-type + HA-*LUC*$_{(UGA20)}$-SF; Lane 3: Wild-type + HA-*LUC*$_{(UGA20)}$-SF + tRNA-Arg-UCU; Lane 4: Wild-type + HA-*LUC*$_{(UGA20)}$-SF + tRNA-Arg-UCG. *(B)* Readthrough efficiency measured as luciferase activity from the HA-*LUC*$_{(UGA20)}$-SF reporter from wild-type [*PSI*-] cells or cells overexpressing either tRNA-Arg-UCU or tRNA-Arg-UCG. Luciferase activity is expressed as relative luciferase units/µg of protein/RNA units (n=3; error bars represent standard deviation from the mean). Lane 1: Wild-type + HA-*LUC*$_{(UGA20)}$-SF; Lane 2: Wild-type + HA-*LUC*$_{(UGA20)}$-SF + tRNA-Arg-UCU; Lane 3: Wild-type + HA-*LUC*$_{(UGA20)}$-SF + tRNA-Arg-UCG; Lane 4: Wild-type + HA-*LUC*-SF**.** (*C*) Comparison of amino acid insertion at UGA during termination readthrough of HA-*LUC*$_{(UGA20)}$-SF reporter in yeast cells with either endogenous levels tRNA-Arg-UCU, or overexpression of tRNA-Arg-UCU or tRNA-Arg-UCG. HA-Luc$_{(PTC20)}$-SF proteins purified from yeast cells were subjected to mass

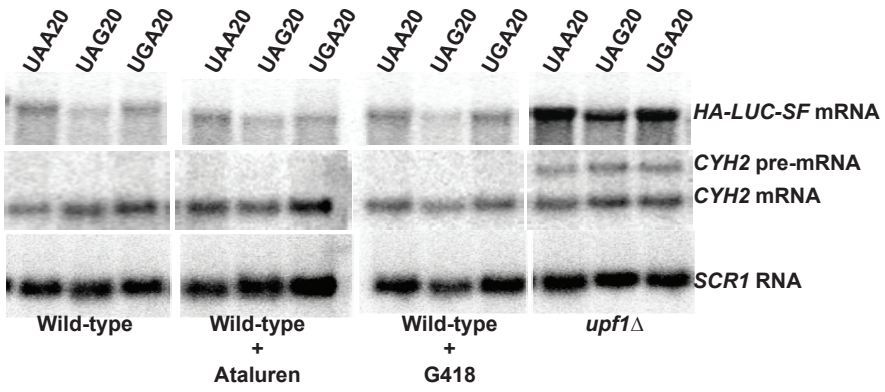
spectrometry analyses. The numbers denote the frequency (± standard deviation of the mean) of insertion of the amino acids at UGA (n=3). The type and the position of mispairing in the codon are depicted for each amino acid inserted. *(D)* Northern blot analyses of total RNA isolated under acidic conditions from wild-type yeast cells and cells overexpressing tRNA-Glu-UUC. After polyacrylamide gel electrophoresis and transfer, blots were probed for tRNA-Glu using a ^{32}P-labeled oligonucleotide. The blots were re-probed for tRNA-Arg-UCU as a control for equal loading. Lanes 1-3 were all derived from the same gel. *(E)* Readthrough efficiency measured as luciferase activity from the HA-*LUC*$_{(UAA20)}$-SF reporter in wild-type yeast cells and cells overexpressing tRNA-Glu-UUC. Luciferase activity is expressed as relative luciferase units/µg of protein/RNA units (n=3; error bars represent standard deviation from the mean*). (F)* Amino acids inserted at UAA during termination readthrough in yeast cells overexpressing tRNA-Glu-UUC. HA-*LUC*$_{(UAA20)}$-SF protein products were purified and subjected to mass spectrometry analyses. The numbers denote the average frequency of insertion of the amino acids at UAA (from two independent experiments).

Supplementary Figure 1



Supplementary Figure 2

A



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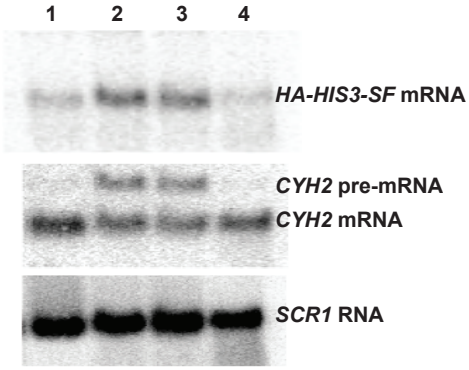
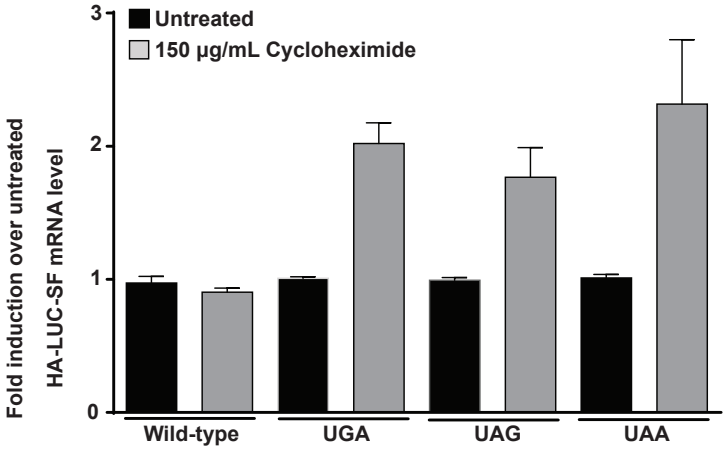
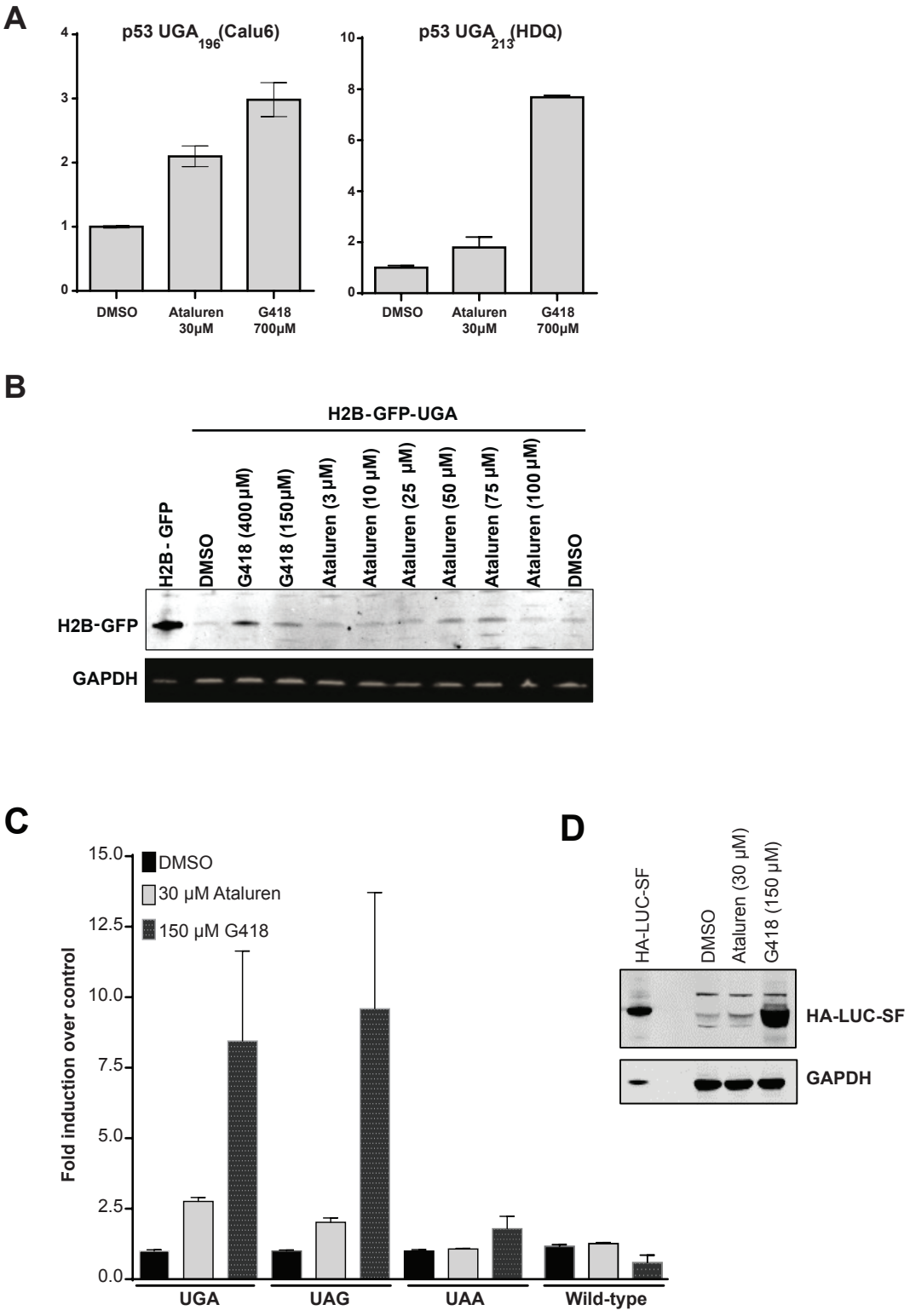


1. Wild-type + HA-HIS3(UAA100)-SF
2. *upf1*Δ + HA-HIS3(UAA100)-SF
3. *upf1*Δ + HA-HIS3(UAA100)-SF + Ataluren
4. Wild-type + HA-HIS3(UAA100)-SF + Ataluren

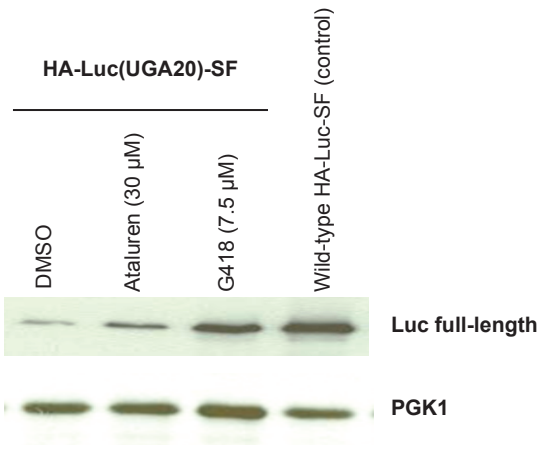
C



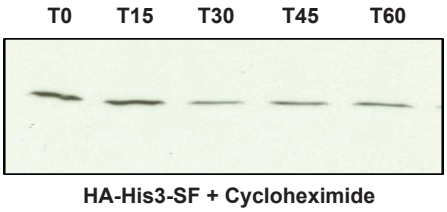
Supplementary Figure 3



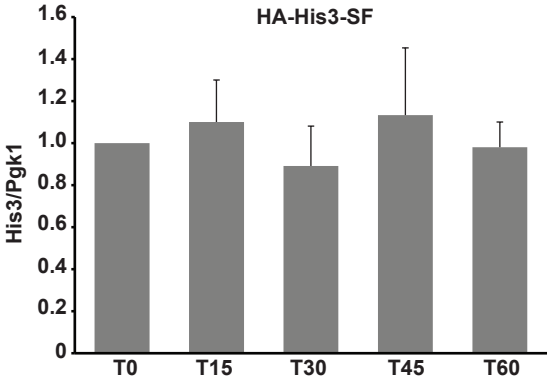
Supplementary Figure 4

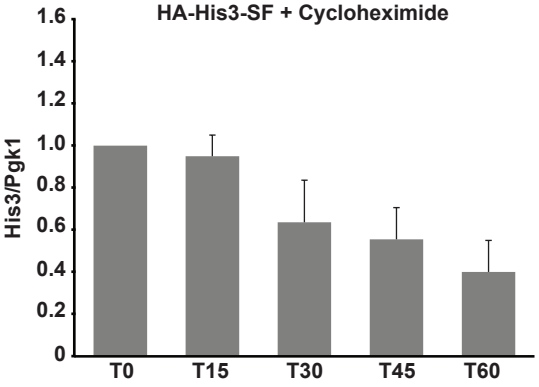


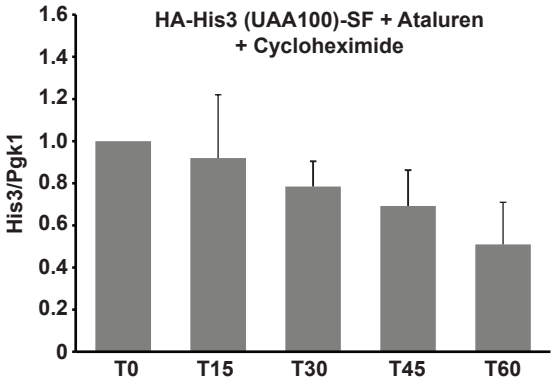
Supplementary Figure 5



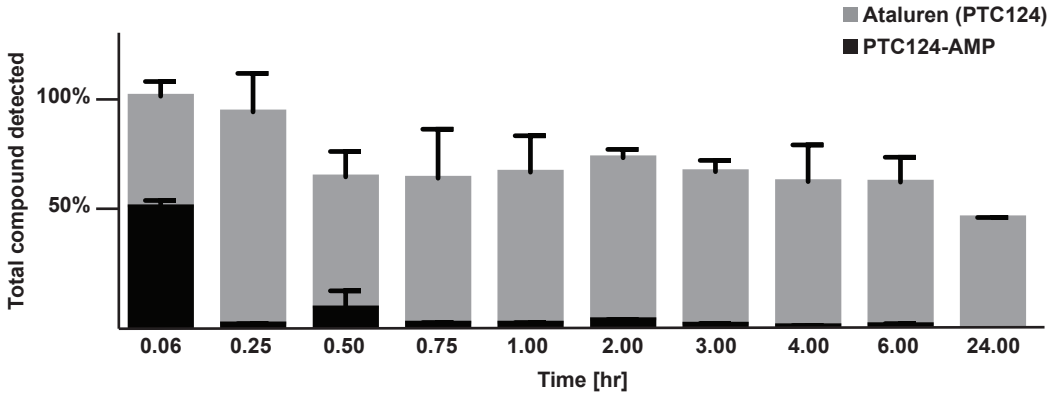
HA-His3-SF + Cycloheximide



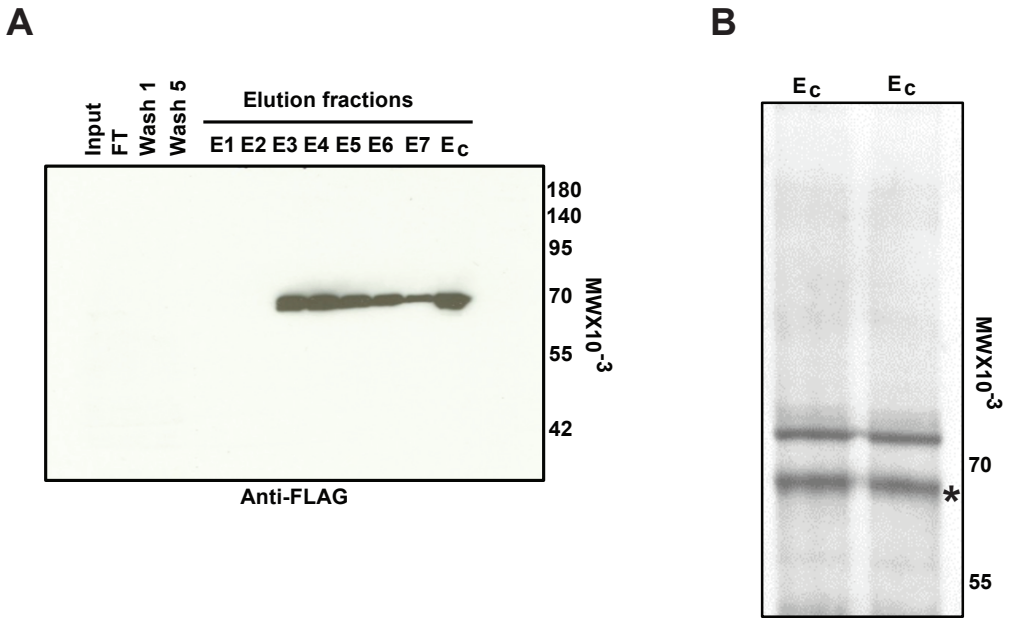




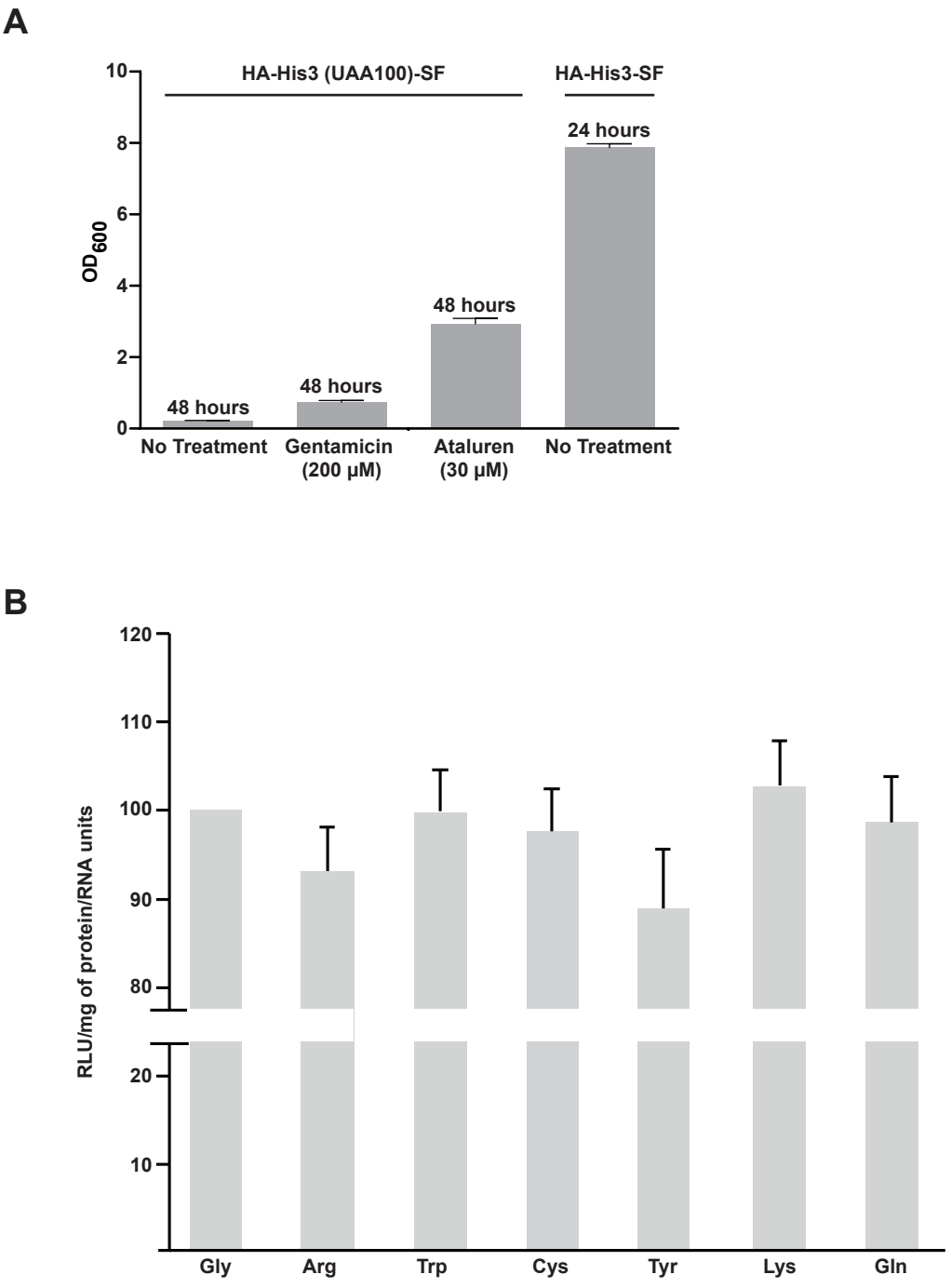
Supplementary Figure 6



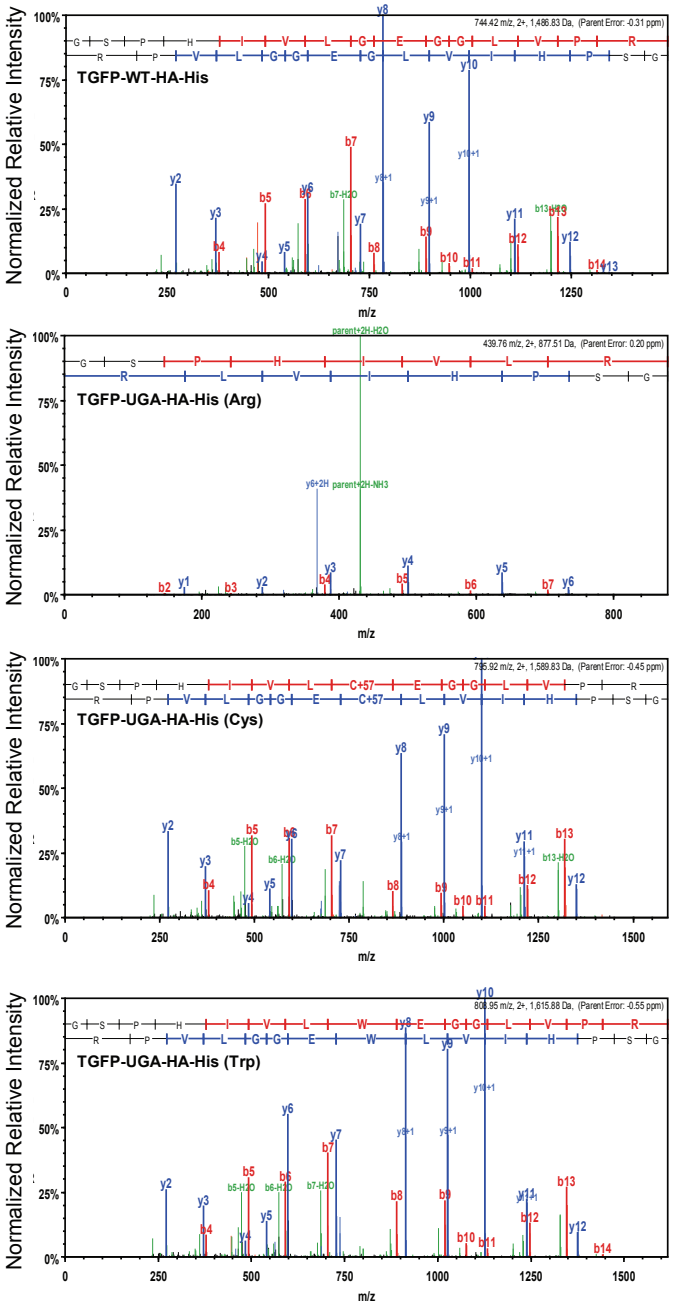
Supplementary Figure 7



Supplementary Figure 8



Supplementary Figure 9



Supplementary Figure 10

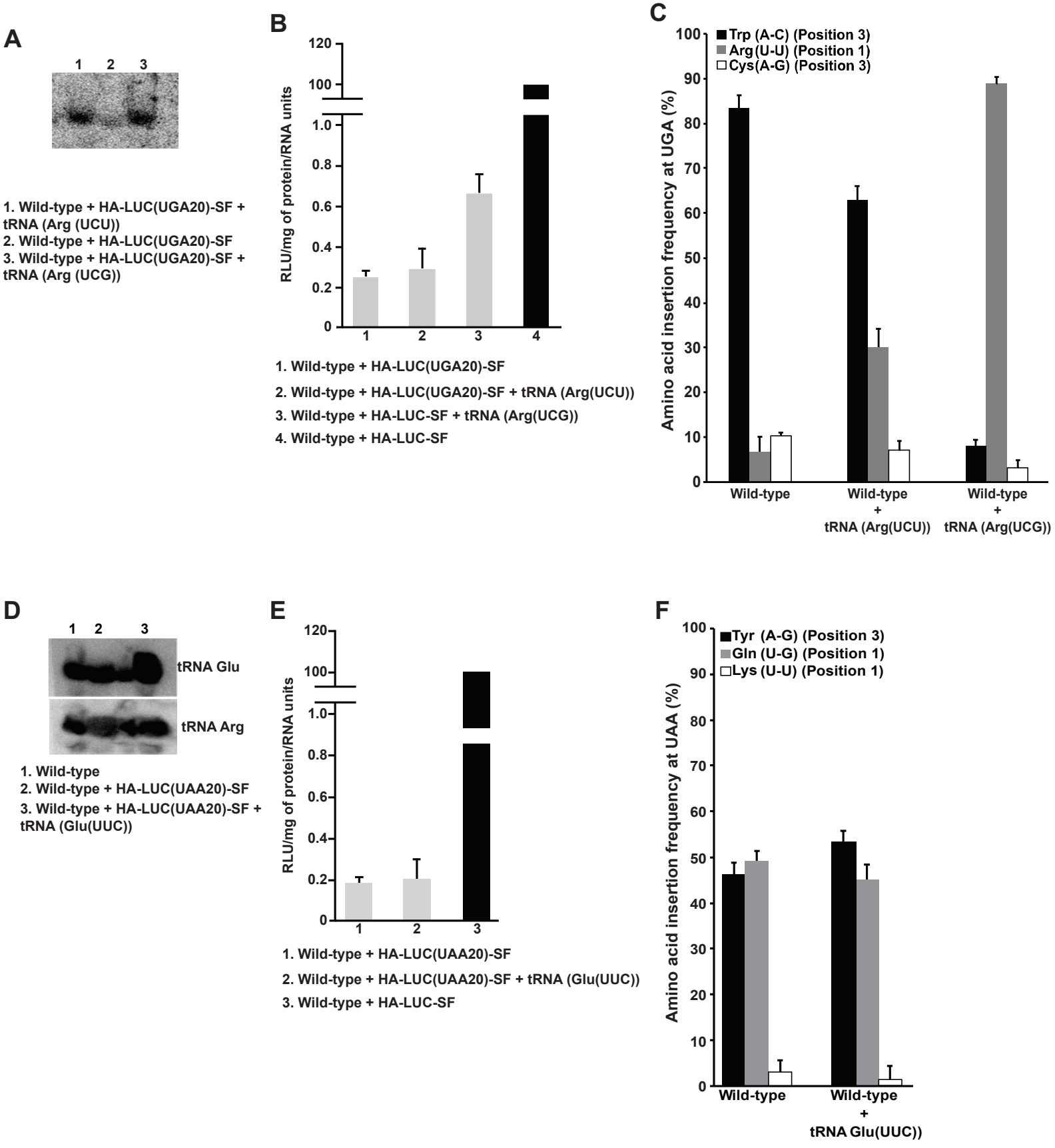


Table S1. Amino acids inserted at PTCs after treatment of wild-type [*PSI-*] yeast cells with 7.5 µM G418.

Codon	Amino acid inserted	Frequency of insertion (%)		Mispairing event
		Experiment 1	Experiment 2	
UAA	Tyr	51.1	52.6	Position 3 (A-G)
	Gln	46.9	45.4	Position 1 (U-G)
	Lys	1.1	2.0	Position 1(U-U)
UAG	Tyr	1.59	1.55	Position 3 (G-G)
	Gln	4.35	5.1	Position 1 (U-G)
	Lys	90.9	90.8	Position 1(U-U)
	Trp	1.55	0.85	Position 2 (A-C)
	Arg	0.87	0.79	Non-cognate tRNA employing position 1 (U-G) and position 2 (A-C) mispairing
	Cys	0.26	0.18	Non-cognate tRNA employing position 2 (A-C) position 3 (G-A/G-G) mispairing
	Ser	0.02	0.02	Position 2 (A-G); Non-cognate tRNA employing position 2 (A-G) and position 3 (G-A/G-G/G-U) mispairing
	Gly	0.18	0.53	Non-cognate tRNA employing position 1(U-C) and position 2 (A-C) mispairing
	Glu	0.03	0.03	Position 1 (U-C); Non-cognate tRNA employing position 1 (U-C) and position 3 (G-U) mispairing
	Thr	0.11	0.08	Non-cognate tRNA employing position 1(U-U) and position 2 (A-G) mispairing
	Phe	0.02	0	Non-cognate tRNA employing position 2(A-A) and position 3 (G-A/G-G) mispairing
	Val	0	0.02	Non-cognate tRNA employing position 1(U-C) and position 2 (A-A) mispairing
UGA	Trp	61.0	57.3	Position 3 (A-C)
	Arg	11.0	16.2	Position 1 (U-U)
	Cys	27.9	26.5	Position 3 (A-G)

Table S2. Amino acids inserted at PTCs after treatment of wild-type [*PSI-*] yeast cells with 15 µMG418.

Codon	Amino acid inserted	Frequency of insertion (%)	Mispairing event
UAA	Tyr	72.9	Position 3 (A-G)
	Gln	24.2	Position 1 (U-G)
	Lys	2.5	Position 1(U-U)
	His	0.16	Non-cognate tRNA employing position 1 (U-G) and position 2 (A-A/A-G) mispairing
	Trp	0.11	Non-cognate tRNA employing position 1 and 2 (A-C) mispairing
UAG	Tyr	2.25	Position 3 (G-G)
	Gln	4.36	Position 1 (U-G)
	Lys	90.5	Position 1(U-U)
	Trp	0.98	Position 2 (A-C)
	Arg	0.81	Non-cognate tRNA employing position 1 (U-G) and position 2 (A-C) mispairing
	Cys	0.39	Non-cognate tRNA employing position 2 (A-C) and position 3 (G-G/G-A) mispairing
	Ser	0.05	Position 2 (A-G); Non-cognate tRNA employing position 2 (A-G) and position 3 (G-A/G-G/G-U) mispairing
	Gly	0.42	Non-cognate tRNA employing position 1(U-C) and position 2 (A-C) mispairing
	Glu	0.02	Position 1 (U-C); Non-cognate tRNA employing position 1 (U-C) and position 3 (G-U) mispairing
	Thr	0.15	Non-cognate tRNA employing position 1(U-U) and position 2 (A-G) mispairing
	Val	0.02	Non-cognate tRNA employing position 1(U-C) and position 2 (A-A) mispairing
UGA	Trp	47.9	Position 3 (A-C)
	Arg	26.1	Position 1 (U-U)
	Cys	24.4	Position 3 (A-G)
	Tyr	0.81	Non-cognate tRNA employing position 2 (G-U) and position 3 (A-A/A-G) mispairing
	Lys	0.56	Non-cognate tRNA employing position 1 (U-U) and position 2 (G-U) mispairing

Table S3. Possible codon-anticodon base pairings during PTC readthrough.

Codon Position	Mispaired tRNA sequence (3'-5')	AA encoded	BP created
1 UAA	UUU	Lys	U-U
	UUG	Gln	U-G
	UUC	Glu	U-C
	UUA	STOP	U-A
2 UAA	UUA	STOP	A-U
	UGA	Ser	A-G
	UCA	STOP	A-C
	UAA	Leu	A-A
3 UAA	UUA	Stop	A-U
	GUA	Tyr	A-G
	AUA		A-A
	CUA	Stop	A-C

Codon Position	Mispaired tRNA sequence (5-3')	AA encoded	BP created
1 UAG	CUU	Lys	U-U
	CUG	Gln	U-G
	CUC	Glu	U-C
	CUA	STOP	U-A
2 UAG	CUA	STOP	A-U
	CGA	Ser	A-G
	CCA	Trp	A-C
	CAA	Leu	A-A
3 UAG	UUA	STOP	G-U
	GUA	Tyr	G-G
	AUA		G-A
	CUA	Stop	G-C

Codon Position	Mispaired tRNA sequence (5'-3')	AA encoded	BP created
1 UGA	UCU	Arg	U-U
	UCG		U-G
	UCC	Gly	U-C
	UCA	Stop	U-A
2 UGA	UUA	Stop	G-U
	UGA	Ser	G-G
	UCA	Stop	G-C
	UAA	Leu	G-A
1 UGA	UCA	Stop	A-U
	GCA	Cys	A-G
	ACA		A-A
	CCA	Trp	A-C

Table S4. Amino acids inserted at UGA after treatment of HEK293cells with Gentamicin.

Codon Position	Mispaired tRNA sequence (5'-3')	AA encoded	BP created	Gentamicin (average ± stdev)
1 UGA	UCU	Arg	U-U	48.4±6.8
	UCG		U-G	
	UCC	Gly	U-C	
	UCA	Stop	U-A	
2 UGA	UUA	Stop	G-U	
	UGA	Ser	G-G	
	UCA	Stop	G-C	
	UAA	Leu	G-A	
1 UGA	UCA	Stop	A-U	
	GCA	Cys	A-G	27.0±9.8
	ACA		A-A	
	CCA	Trp	A-C	24.2±3

Table S5. Amino acids inserted at G542X after treatment of HEK293 cells with G418

Sample	Peptide Sequence	m/z (z, ppm)	RT (min)	R-NSC Exp 1	R-NSC Exp 2	Ave R-NSC
WT	GSPHIVLGEGGLVPR	496.62 (3, -0.36)	66.5	100	100	100
G542X	GSPHIVLR	439.76 (2, 0.20)	41.2	26	15	20
	GSPHIVLCEGGLVPR (IAA modified)	530.95 (3, -0.15)	67.4	41	46	44
	GSPHIVLWEGGLVPR	539.64 (3, 0.16)	81.9	33	38	36

Table S6. Amino acids inserted at PTCs after treatment of 293H cells with ataluren or G418.

Codon Position	Mispaired tRNA sequence (3'-5')	AA encoded	BP created	DMSO	DMSO	DMSO	Ataluren	Ataluren	Ataluren	G418	G418	G418	DMSO average ± stdev	Ataluren average ± stdev	G418 average ± stdev
1 UAA	UUU	Lys	U-U												
	UUG	Gln	U-G	23.4	32.6	41.2	39.2	51.9	28.6	37	53.6	65.3	32.4 ± 8.9	39.9 ± 11.7	52 ± 14.2
	UUC	Glu	U-C												
	UUA	STOP	U-A												
2 UAA	UUA	STOP	A-U												
	UGA	Ser	A-G												
	UCA	STOP	A-C												
	UAA	Leu	A-A												
3 UAA	UUA	Stop	A-U												
	GUA	Tyr	A-G	76.5	67.3	58.7	59.9	45.7	68.1	62.7	46.4	34.6	67.5 ± 8.9	57.9 ± 11.3	47.9 ± 14.1
	AUA		A-A												
	CUA	Stop	A-C												

Codon Position	Mispaired tRNA sequence (5-3')	AA encoded	BP created	DMSO	DMSO	DMSO	DMSO	Ataluren	Ataluren	Ataluren	Ataluren	Ataluren	Ataluren	G418	G418	G418	G418	DMSO average ± stdev	Ataluren average ± stdev	G418 average ± stdev
1 UAG	CUU	Lys	U-U											3	1.1	1.5	2.2			2.0 ± 0.8
	CUG	Gln	U-G	34.9	39	15	11.9	81.4	33.7	30.2	81.4	45.1	47.3	80	97.7	87.9	80.5	25.2 ± 13.7	53.2 ± 20.3	86.5 ± 8.3
	CUC	Glu	U-C																	
	CUA	STOP	U-A																	
2 UAG	CUA	STOP	A-U																	
	CGA	Ser	A-G																	
	CCA	Trp	A-C	2.4	2.6		0.19	2.1	0.49	0.2	1.7	4.9	1.2					1.3 ± 1.4	1.8 ± 1.9	
	CAA	Leu	A-A																	
3 UAG	UUA	STOP	G-U																	
	GUA	Tyr	G-G	61.6	58.8	84	87.8	15.6	64.2	69.3	15.7	48.9	49.9	16	1.1	10.1	16	73.1 ± 15.0	43.9 ± 20.9	10.8 ± 7.0
	AUA		G-A																	
	CUA	Stop	G-C																	

Codon Position	Mispaired tRNA sequence (5'-3')	AA encoded	BP created	DMSO	DMSO	DMSO	Ataluren	Ataluren	Ataluren	Ataluren	Ataluren	G418	G418	G418	G418	DMSO average ± stdev	Ataluren average ± stdev	G418 average ± stdev
1 UGA	UCU	Arg	U-U	56	60.5	59.24	63.7	65.3	57.5	86	76	51.7	66.7	79.7	60	58.6 ± 2.3	69.7 ±11.3	64.5 ± 11.8
	UCG		U-G															
	UCC	Gly	U-C															
	UCA	Stop	U-A															
2 UGA	UUA	Stop	G-U															
	UGA	Ser	G-G															
	UCA	Stop	G-C															
	UAA	Leu	G-A															
1 UGA	UCA	Stop	A-U															
	GCA	Cys	A-G				0.5	0.5	2	0.06	0.2	28.8	11.2	12.4	18.3		0.7 ± 0.78	17.7 ± 8.0
	ACA		A-A															
	CCA	Trp	A-C	44	39.5	40.76	35.7	34.2	40.2	13	21	19.5	22.1	7.8	22	41.4 ± 2.3	28.8 ± 11.4	17.9 ± 6.8

Table S7. Oligonucleotides used in this study.

Position 20 Arg For	CTACCCACTCGAAGACAGAACCGCCGGCGAGCAG
Position 20 Arg Rev	CTGCTCGCCGGCGGTTCTGTCTTCGAGTGGGTAG
Position 20 Trp For	CTACCCACTCGAAGACTGGACCGCCGGCGAGCAG
Position 20 Trp Rev	CTGCTCGCCGGCGGTCCAGTCTTCGAGTGGGTAG
Position 20 Cys For	CTACCCACTCGAAGACTGCACCGCCGGCGAGCAG
Position 20 Cys Rev	CTGCTCGCCGGCGGTGCAGTCTTCGAGTGGGTAG
Position 20 Tyr For	CTACCCACTCGAAGACTACACCGCCGGCGAGCAG
Position 20 Tyr Rev	CTGCTCGCCGGCGGTGTAGTCTTCGAGTGGGTAG
Position 20 Lys For	CTACCCACTCGAAGACAAAACCGCCGGCGAGCAG
Position 20 Lys Rev	CTGCTCGCCGGCGGTTTTGTCTTCGAGTGGGTAG
Position 20 Gln For	CTACCCACTCGAAGACCAAACCGCCGGCGAGCAG
Position 20 Gln Rev	CTGCTCGCCGGCGGTTTGGTCTTCGAGTGGGTAG
DB4084	CGTTGGGGACTGACGACAGACAGCC
DB4085	GGCTGTCTGTCGTCAGTCCCCAACG

For= Forward primer
Rev= Reverse primer