TLN468 changes the pattern of tRNA used to readthrough premature termination codons in CFTR

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ABSTRACT

Nonsense mutations account for 12 % of the cases of Cystic fibrosis (CF). The gene inactivation resulting from premature termination codon (PTC) can be counteracted by the use of drugs stimulating PTC readthrough, thereby restoring production of the full-length protein. We recently identified a new readthrough inducer named TLN468, acting more efficiently than gentamicin.

Here, we measured readthrough induced by these two drugs on different cystic fibrosis transmembrane conductance regulator (*CFTR*) PTCs. Then we determined the amino acids inserted at the *CFTR* PTCs S1196X, G542X, W846X and E1418X during TLN468-mediated readthrough. Interestingly, TLN468 significantly promotes the incorporation of one specific amino acid at each stop codon, while gentamicin does not change the proportion of amino acids incorporated in basal conditions. Also, the function of the predicted recoded CFTR channels corresponding to these 4 PTCs was measured with or without potentiator. We found that, for some PTC, suppression induced by TLN468 treatment allows the expression of variants CFTR proteins with proper maturation and significant activity that can be potentiated by the presence of CFTR modulators.

These results suggest that TLN468 PTC suppression in combination with CFTR modulators may be beneficial for the treatment of CF patients with PTCs.

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Treatments, like CFTR modulators, have proven clinical benefits for patients carrying the F508del mutation [1]. Such treatments are however ineffective for patients carrying nonsense mutations introducing a premature termination codon (PTC). Those patients represent around 12% of all CF patients in Europe [2].

The appearance of an in frame stop codon (PTC) causes two major effects. First, this leads to the synthesis of truncated proteins that are often non-functional. In addition, PTC-containing mRNAs are also frequently unstable, resulting in a severe reduction in steady-state mRNA abundance. Then, these two PTC-induced events decrease the level of functional protein which can result in severe diseases. Some therapeutic approaches aim at promoting translational readthrough of the PTC, favoring the recruitment of a near-cognate tRNA in place of the termination complex, therefore enabling the expression of a full-length protein [3].

In the last decade, a lot of research has focused on in-frame PTCs as potential therapeutic targets. The best-characterized drugs active against PTCs are aminoglycosides. Their readthrough efficiency has been evaluated for different genes, including the cystic fibrosis transmembrane conductance regulator (CFTR) [4]. Encouraging results have been obtained in some cases, particularly for mutations displaying high levels of readthrough in the presence of gentamicin [5]. However, despite their medical value, aminoglycosides present side effects, with reports of various levels of ototoxicity and/or nephrotoxicity [6]-[7].

The limitations of aminoglycosides led to the development of other compounds with structures different from that of aminoglycosides. New molecules have been developed like Altaluren [8], negamicin [9]-[10], Clitocine [11], Escin [12] or the molecule, 2,6-diaminopurine [13] and more recently the small molecule SRI-41315 [14]. Further studies will be required to determine the true clinical potential of these molecules and the identification of new readthrough drugs is still important because a main factor limiting the impact of readthrough-inducing treatments is their highly variable efficacy [4]. Indeed, treatment response is highly influenced by the stop codon identity (UAG, UGA or UAA) [15] and its surrounding nucleotide sequence [15]-[16], restricting their potential clinical benefits to a limited number of patients. Another important factor is the activity of the recoded proteins. Indeed, It is possible

that several tRNAs are incorporated by readthrough at the stop codon. If these tRNAs do not allow the insertion of the amino acid present in the WT protein then there is a risk of obtaining a protein with little or no activity. Corresponding inserted amino acids have first been identified in yeast by mass spectrometry [18] and more recently in mammalian expressing systems [19]. Despite these early studies, there have never been any systematic studies of the identity of amino acids incorporated during treatment with a readthrough inducer.

Recently, we identified a new readthrough inducer named TLN468 [20], acting differently and more efficiently than gentamicin. We though to characterize the effect of this new drug onto 4 PTCs identified in CF patients: S1196X (UGA), G542X (UGA), W846X (UAG) and E1418X (UAA), to determine basal and drug-induced readthrough levels, the identity of incorporated amino acids, and activity of the restored proteins. Readthrough efficiencies were highly variable depending on the PTC, confirming that PTC suppression depends on the codon's nature and the nucleotide context. Then the amino acids identities and proportions where determined. Surprisingly, the amino acids respective proportions were significantly different in presence of TLN468 as compared to gentamicin treatment or basal condition. In fact, TLN468 highly promotes the incorporation of cysteine for the UGA codon, glutamine for UAG codon and tyrosine for the UAA codon. These results confirm that depending on the readthrough inducer used, frequency of the amino acids inserted during PTC suppression is different.

We also examined the functionality of variant CFTR proteins generated by PTC suppression induced by TLN468. We found that only some variants exhibited suitable maturation and activity, indicating the complexity of nonsense suppression therapy. Nevertheless, CFTR potentiator VX-770 (Ivacaftor) [21] can increase activity of recoded channels increasing the therapeutic potential of readthrough suppression therapies. These results suggest that combining the new nonsense suppression drug, TLN468, with CFTR modulators is a promising therapeutic approach to maximize CFTR function in CF patients that harbor nonsense mutations.

RESULTS

Basal and drug-induced readthrough levels

First, basal and TLN468 induced readthrough levels associated with 12 *CFTR* PTCs (listed in Table 1) were measured *in a human cell line (Figure S1)*. Four PTCs were then

selected for further studies based on their ability to respond to TLN468 and to represent the 3 different stop codons: S1196X for UGA; G542X for UGA; W846X for UAG; and E1418X for UAA) (Figure 1).

To measure the ability of TLN468 to stimulate readthrough in comparison with gentamicin, we used a dual reporter system to quantify stop codon readthrough efficiency [22]. This reporter system includes two enzymatic activities (β-galactosidase and firefly luciferase) and β-galactosidase is used as an internal control for the normalization of expression levels. We know that the local mRNA sequence context flanking a PTC is determinant for readthrough [23]. So, we introduced the stop codon, as well as the surrounding nucleotide context (three codons upstream and three codons downstream of the *CFTR* PTC position), at the beginning of luciferase coding phase into this reporter. For each molecule tested, at least 4 independent measurements were performed (Figure S1 &1).

Basal readthrough was variable, ranging from 0.004% for E1418X to 0.69% for S1196X. Gentamicin significantly increased readthrough to levels ranging from 0.06% to 10.5% and TLN468 is able to stimulate readthrough to levels ranging from 0.14% for to 19.5% for S1196X. Interestingly, with the exception of G542X mutation, TLN468 is much more efficient than gentamicin to induce readthrough. TLN468 is twice as effective as gentamicin for S1196X (UGA) PTC. Indeed, we go from a rate of readthrough about 10% with gentamicin to 20% with TLN468. For the W846X (UAG) PTC, TLN468 is twelve times more active as we go from a rate of about 0,3% with gentamicin to 3,5% with TLN468. And for the E1418X (UAA) PTC, we go from a rate of about 0,06% with gentamicin to 0,08% with TLN468, which means that the TLN68 is more effective as gentamicin.

Then, we investigated the identity of natural tRNAs suppressors of each PTC (Figure 2). PTC suppression occurs at relatively low frequencies making it difficult to analyze the amino acid inserted during PTC suppression. To overcome that, we chose to use a recently developed GST reporter allowing efficient purification, in a single step, of a protein synthesized during a stop codon readthrough event [18]. GST coding sequence has been humanized to favour its expression in human cells. Moreover, a frequent N-terminus protein degradation observed in Human cells prompted us to introduce a spacer region immediately downstream the AUG of GST but upstream of the cloning site for PTC sequences. GST is only produced if ribosomes readthrough the inserted stop codon.

We determine the amino acids inserted during basal readthrough of *CFTR* stop

mutations or after PTC suppression induced by gentamicin or TLN468 treatment. After the purification of readthrough GST proteins, peptides obtained were analyzed by LC-MS/MS and systematically normalized with the corresponding synthetic peptide.

Three amino acids were found to be inserted in different proportion at the *CFTR* S1196X UGA codon in each condition: arginine, cysteine and tryptophan (Figure 2.A). We found that the same tree amino acids, were inserted at the *CFTR* G542X UGA codon but surprisingly also serine and leucine (Figure 2.B). In parallel, we observed at the *CFTR W846X* UAG and E1418X UAA codon, the insertion of glutamine and tyrosine and also leucine at very low level (<1.2%) (Figure 2.C). We also find a small portion of lysine at E1418X UAA stop codon (Figure 2.D). Interestingly gentamicin modifies differently the ratio of the various amino acids inserted depending on the mutation considered, clearly suggesting that the stimulating effect of gentamicin is not independent of the PTC. Moreover, TLN468 has profound impact on tRNA incorporation by promoting the incorporation of one particular amino acid (>95%) for each tested mutation. Indeed, for the UGA PTCs (Figure 2.A & 2.B), TLN468 promotes the incorporation of cysteine (95%). For the UAG codon, it promotes the incorporation of glutamine at 97% (Figure 2.C) and tyrosine at 85% for the UAA codon (Figure 2.D).

Decreased dose of TLN468 does not affect amino acid incorporation frequency

Then, we wanted to know if a lower dose of TLN468 still promote the majority incorporation of cysteine at the *CFTR* S1196X PTC. First, we determined the readthrough efficiency of TLN468 at different doses (0, 20, 40, 60 and 80 µM). As expected, effect of TLN468 is dose-dependent with the highest readthrough efficiency of 59.6% obtained for 80 µM that decrease at 15.9% for 60 µM, 8.7% for 40 µM and 5.2% for 20 µM (Figure 3.A).

We chose to identify the amino acids incorporated at 40 µM because it's half the concentration to our reference dose (80 µM) and the readthrough efficiency is still high. We observed that there is no difference in cysteine incorporation between these two doses, respectively 96% and 95% of cysteine incorporation. This suggests that, whatever the dose used, TLN468 acts strongly on the cysteine tRNA to allow it to be incorporated more efficiently. To verify this effect was not the consequence of the overexpression of cysteine tRNAs isodecoders by TLN468, we performed a tRNA-seq experiment. Quantification of all tRNAs isodecoders in Expi293 in presence of TL468

shows no sign of change in cysteine tRNA abundance (Figure S2), ruling out this possibility.

Function of the predicted recoded channels

CFTR variants can have different levels of CFTR activity. To determine how incorporation of amino acids produced by suppression of the *CFTR* S1196X, G542X, W846X and E1418X mutation affects CFTR protein maturation and function, we respectively mutated a WT *CFTR* cDNA at codon S1196, G542, W846 and E1418 to create the CFTR variants identified by mass- spectrometry (Figure 2).

HEK293 cells were transfected with these CFTR variants, and we examined CFTR protein expression and maturation from band B (the immature, non-glycosylated form) to band C (the mature, fully glycosylated form) by western blotting (Figure 4.A). Most of cells transfected with each construct expressed similar levels of CFTR protein but the percentage of CFTR maturation differed among the different CFTR variants. The majority of WT CFTR was present as Band C (around 74%), indicating that most CFTR are fully-glycosylated. In contrast, a significant lower percentage of maturation was observed in cells expressing the E1418Q variant (55%) and G542W variant (40%) compared to the WT protein. Cells expressing the other variants showed almost the same % maturation as the WT form (Figure 4.B).

The function of the CFTR mutants was then measured using a halide-sensitive YFP-based assay. This assay is based on the sensibility of the YFP protein to the I^- ion. In fact, iodide anions highlight the activity of the channel by inducing the quenching of YFP fluorescence. Measurements revealed an iodide transport of 0.16 ± 0.02 mM·s^{-1} ($p < 0.0001$) in WT-transfected HEK293 cells (Figure 5). 9 channels had an activity comparable to WT channels (between 0.131 and 0.238 mM·s^{-1}), while 3 (G542W, G542R, G542C) had a significantly reduced transport rate (Figure 6). Cells were also incubated with the CFTR potentiator VX-770. WT-expressing cells showed a 1,5-fold increase of the iodide transport rate (0.24 ± 0.03 mM·s^{-1}; $p < 0.0001$) (Figure 5). A similar increase was observed with the channels presenting a normal basal activity except for W846Y and W846Q (respectively 1.2- and 1.1-fold increase). For the E1418K-expressing cells, the potentiator significantly enhanced channel function with a 3,3-fold increase of the iodide transport rate (0.36 ± 0.05 mM·s^{-1}). But for the G542W channel, which presents the most reduced amount of band C (Figure 4), the potentiator does not significantly enhanced channel function.

Discussion

Partial restoration of protein function by PTC suppression has been showed in several diseases caused by nonsense mutations, including CF [10], [24], [25]. Moreover, PTC suppression in combination with corrector, such as lumacaftor, has demonstrated to promote a further rescue of expression and function of CFTR PTC mutations [10], [19], [26].

This work represents the first systematic analysis of the amino-acids incorporated by stop codon readthrough in basal condition or in presence of either gentamicin or TLN468 in human cells. Although the mode of action of gentamicin is well documented, we do not know yet how TLN468 acts to stimulate stop codon readthrough. Understanding the effect of TLN468 on tRNAs incorporation can give us interesting leads to identify the molecular target of TLN468. In this context, besides measuring the effective protein production by quantitative methods, it is fundamental to prove protein functionality after readthrough. In the present study, we assess the efficacy of readthrough-inducing molecules TLN468 for four PTCs in the *CFTR* gene: S1196X PTC, G542X PTC, W846X PTC and E1418X PTC. First, we measured basal and drug-induced readthrough efficiency. We can clearly see that TLN468 overperforms gentamicin but not systematically. This indicates that both gentamicin and TLN468 are differently influenced by the nucleotide context surround the PTC, confirming that both drugs have different mode of action.

Interestingly the amino acids found in absence of drug closely resemble to the ones we previously identified in yeast, although for the G542X mutation we detect a greater variety of incorporated amino acids in humans than in yeast. Indeed, it's the first time that a serine is identified at a PTC readthrough. This clearly indicates that, contrary to initial assumptions, the nature of the amino acids incorporated is specific to each PTC and is probably influenced by the nucleotide environment of the PTC. We then asked the question of the amino acids incorporated in the presence of either drug. The effect of gentamicin is quite unexpected, addition of gentamicin has a clear effect on the incorporation Trp (W) on G542X but not on S1196X both being UGA codons. It stimulates the incorporation of tyrosine on W846X (UGA) but restricts it on E1418 (UAA). This is surprising because gentamicin acts through the binding of the decoding center promoting the incorporation of near-cognate tRNAs [25].

Here we clearly reveal a specificity of gentamicin depending on the tRNA and the stop codon considered. In contrast, the mode of action of TLN468 is totally unknown, and we observed that even at low concentration TLN468 strongly biases tRNA

incorporation, systematically allowing the incorporation of almost only one amino acid. However, the identity of this amino acid changes depending on the identity of the stop codon. It is currently rather difficult to identify a common point between the three tRNAs that are strongly stimulated by the presence of TLN468 (i.e Cys, Gln, Tyr). None of them has been found overexpressed in treated cells (Figure S2), excluding the possibility of a perturbation in tRNAs homeostasis. At least, these results indicate that the action of TLN468 is not specific to a single tRNA.

To a clinical point of view, the activity of the restored protein is a very important outcome. Our work clearly reveals that several amino-acids are inserted by readthrough at PTC, so it became important to determine whether the corresponding CFTR protein would display any activity. Our results indicate that for the position G542 the identity of the amino acid is crucial for the CFTR activity, however at the other position tested substitutions do not strongly impair CFTR activity especially in presence of VX-770, that is very interesting for a therapeutic perspective. In conclusion, work demonstrates that TLN468 is a very promising candidate drug for therapeutic treatment of cystic fibrosis due to a nonsense mutation. More importantly, it directs the incorporation of a single amino acid at the PTC which provides a convenient and efficient tool to specifically drive the incorporation of one of the three amino acids into the PTC.

MATERIALS AND METHODS

Cell Lines and media

Expi293F cells (Gibco) which are human cells derived from HEK293 cultivated in suspension were used to measure readthrough efficiency and relative incorporation of amino acids. They can grow to high density in Expi293™ Expression Medium (gibco) with addition directly in the media of queuine with a final concentration of 10nM. Cells were transfected with Expifectamin (gibco).

Hela cells were used to measure readthrough efficiency and relative incorporation of amino acids. These cells were cultured in DMEM medium supplemented with 10% fetal calf serum and were transfected with Jet Pei (Invitrogen).

HEK293 were used for Western blot analysis and functional assay. These cells were cultured in DMEM medium supplemented with 10% fetal calf serum and were transfected with with Lipofectamin 2000 (Life Technologies).

Readthrough quantification

For each nonsense mutation tested, complementary oligonucleotides corresponding to the stop codon and nine nucleotides on either side of the stop codon were ligated into the pAC99 dual reporter plasmid, as previously described (Bidou *et al.*, 2004). This dual reporter plasmid was to quantify stop codon readthrough through the measurement of luciferase and beta galactosidase (internal normalization) activities, as previously described (Stahl *et al*, 1995). Readthrough levels for nonsense mutations were analyzed in the presence or absence of the tested molecules. The cells were used to seed a six-well plate. The following day, they were transfected with the reporter plasmid in the presence of JetPei reagent (Invitrogen). They were incubated for 14 h and then rinsed with fresh medium, with or without potential readthrough inducers. Cells were harvested 24 hours later, with trypsin–EDTA (Invitrogen) and beta-galactosidase and luciferase activities were assayed as previously described (Stahl *et al.*, 1995). Readthrough efficiency was estimated by calculating the ratio of luciferase activity to beta-galactosidase activity obtained with the test construct, with normalization against an in-frame control construct.

Purification of readthrough proteins

Readthrough proteins were produced from pCDNA3.4-GST. This vector was constructed by adapting codon frequencies from the GST gene from pYX24-GST to human, and by the addition of a linker sequence of 171nt (H4) (Chen *et al*, 2013) at the AfeI immediately downstream the start codon.

Expi293F cells were transfected by pCDNA3.4-GST to express a readthrough GST protein and drug added the next day. Cells' extract was done 24 hours post treatments and GST purified on a 1 ml GSTrap HP column (GE Healthcare) with an AKTA purifier 10 FPLC system as previously described (Blanchet *et al*, 2014). The product of purification was then migrated on SDS-PAGE, allowing the isolation of S. japonicum GST from endogenous GST for mass spectrometry analyses.

Mass Spectrometry

Mass spectrometry analysis was performed on Coomassie-stained gel bands corresponding to the readthrough GST proteins (Blanchet *et al*, 2014). Briefly, bands were subjected to in-gel LysC digestion as previously described and readthrough peptides were further identified and quantified by nanoLC–MSMS using a nanoElute liquid chromatography system (Bruker) coupled to a timsTOF Pro mass spectrometer (Bruker). Before MS analysis, peptides were cleaned up with ZipTip C18 (millipore), dried under vacuum and then resuspended in 0.1% n-octylglucopyranoside (Sigma–Aldrich) to prevent the loss of hydrophobic peptides. Additionally, synthetic heavy isotope labeled peptides (AQUA, Thermo) were spiked in the mixture of their endogenous counterparts to take the relative ionization efficiencies of peptides and matrix effects into account. Peptides were loaded with solvant A and separated on an Aurora analytical column (ION OPTIK, 25 cm × 75 m, C18, 1.6 m) with a gradient of 0–35% of solvent B for 30 minutes. Solvent A was 0.1% formic acid and 2% acetonitrile in water and solvent B was acetonitrile with 0.1% formic acid. MS and MS/MS spectra were recorded from m/z 100 to 1700 with a mobility scan range from 0.6 to 1.4 V s/cm2. MS /MS spectra were acquired with the PASEF (Parallel Accumulation Serial Fragmentation) ion mobility-based acquisition mode using a number of PASEF MS/MS scans set as 10. MS and MSMS raw data were processed and converted into mgf files with DataAnalysis software (Bruker). Peptide and protein identifications were performed using the MASCOT search engine (Matrix Science, London, UK) against a homemade database (the unknown amino acid at stop codon is represented by « X » in the readthrough GST sequence). Database searches were performed using LysC cleavage specificity with two possible missed cleavages. Carbamidomethylation of cysteines was set as fixed modification and oxidation of methionines and tryptophan as variable modifications. Peptide and fragment tolerances were set at 10 ppm and 0.05 Da, respectively. Only ions with a score higher than the identity threshold and a false-positive discovery rate of less than 1% (Mascot decoy option) were considered.

The XIC-based relative quantification of readthrough peptides was corrected by dividing the XIC peak areas of endogenous peptides by their heavy isotope labeled counterparts.

Mutagenesis

All mutants were generated by site-directed mutagenesis using the QuickChange XL II site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) as previously described [28].

Western blot analysis

HEK293 cells were transfected with the appropriate constructs and harvested after 48 h. Cells were lysed with Ripa Buffer (Thermo Scientific) with 1% anti protease (Roche) and incubated during 30 min in ice. Total proteins were quantified with Bradford reagent (Biorad) and samples containing 30 µg total protein were analyzed by Western blot using 4-12% Bis-Tris Gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Biorad). Membranes were saturated by overnight incubation in 5% skim milk powder, 5% BSA in phosphate-buffered saline (PBS), and incubated for 1 h with the 596 anti-CFTR antibody (1/1000, CFFoundation). After three washes in PBS supplemented with 0.1% Tween, the membranes were incubated with the secondary antibody (horseradish peroxidase–conjugated anti-mouse IgG [1/2,500]) for 1H. The membranes were washed five times and chemiluminescence was detected with ECL Prime Western Blotting Detection Reagents (Amersham, GE Healthcare). The signal was quantified with ImageLab software.

YFP-based functional assay

CFTR activity was measured in transiently transfected HEK293 cells using the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L (kindly provided by L.J. Galietta; Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy). Transfected cells were incubated for 30 min with 100 µL of PBS containing CPT-AMPc (100 µM; Sigma Aldrich) and IBMX (100 µM) with and without VX-770 (10 µM; Selleckchem), and cell fluorescence was continuously measured before and after addition of 200 µL of PBS-NaI with a CLARIOStar (BMG Labtech). The resulting curves are then normalized to the initial fluorescence and the signal decay is fitted to an exponential function to derive the maximum slope using the equation F/F0 = span x k x e-k. Maximal slopes were converted to rates of change in intracellular iodide concentration (in mM·s−1).

tRNA-sequencing

Total RNA extractions were performed from EXPI293F treated or not by TLN468 80 µM during 24H. Extractions were done in triplicates for each condition. tRNA are extracted by gel purification from 5µg of total RNA by triplicate and sequenced by ArrayStar Inc. (USA). Bioinformatics analysis has been performed by ArrayStar Inc. by aligning tRNA reads onto high confidence tRNA sequences from GtRNAdb.

Statistics

Quantitative variables were described as mean±SD. Comparisons to wild-type (WT) conditions were made with one-way ANOVA followed by the Fisher test for p-value evaluation. CFTR treatment analysis, unpaired t-tests were used to calculate significance.

Legends of Figures and Table

Table 1 : Selected nonsense mutations and surrounding sequences inserted in the dual reporter plasmid. The inserted sequences corresponding to the indicated PTC are in red for the TGA codons, in blue for the TAG codons and in green for the TAA codons.

Figure 1 : Readthrough levels measured for 4 CFTR PTCs (S1196X, G542X, W846X, E1418X), in basal condition (NT) and after incubation with gentamicin (2,5 mM) or TLN468 (80 µM) during 24H in Expi 293F human cell line. Plots represents the mean with standard deviation, n≥4 for all samples.

Figure 2 : Relative frequencies of the readthrough amino acids incorporated at CFTR stop mutations in Expi293F human cell line established by MS-MS. Comparison of the amino acids inserted at basal state (NT), during Gentamicin and TLN468 suppression of the S1196X (A), G542X (B), W846X (C) and E1418X (D) PTCs. X/X: ratio between the peptide purified from human cells (X) and the corresponding AQUA peptide (X*). n=3 sample points.*

Figure 3 : Dose effect of TLN468 during readthrough. (A) Quantification of readthrough efficiency at the CFTR stop mutation S1196X with different concentration of TLN468 in Expi 293F human cell line. Plots represents the mean with standard deviation, n=5 for all samples. (B) Comparison of the amino acids inserted during TLN468 suppression of the S1196X PTC at 40 µM and 80 µM. X/X: ratio between the peptide purified from human cells (X) and the corresponding AQUA peptide (X*). n=3 sample points.*

Figure 4 : Maturation of recoded cystic fibrosis transmembrane conductance regulator (CFTR) channels. (A) Representative Western blot obtained from HEK293 cells expressing the indicated construct. CFTR bands C and B are indicated. (B) CFTR maturation was measured using the C/(B+C) ratio and WT CFTR was determined has the 100% maturation. Each band was normalized by GAPDH; # : significant difference compared with wild-type control conditions (p<0.01, ANOVA). N=3 minimum for all samples.

*Figure 5 : Function of recoded cystic fibrosis transmembrane conductance regulator (CFTR) channels. Transport rates measured under basal conditions and in the presence of the CFTR potentiator VX-770. *: significant difference compared with wild-type control conditions (p<0.01, ANOVA); #: significant effect of VX-770 (p<0.01, unpaired t-test). N=4 for all samples except for the WT (n=8 for basal conditions and n=10 with VX-770).*

Author contributions

SK, CS and LB performed readthrough and western-blot experiments; SK and DC

performed mass spectrometry analysis; SK, AH performed functional experiments, LB and OH designed the experiments and analyzed the results. All authors have participated to the redaction of the manuscript.

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Supplementary data

Figure S1 : Readthrough levels measured for 12 CFTR PTCs listed in table 1, at basal state (NT) and after incubation with TLN468 at 80 µM during 24H in Expi 293F human cell line. Plots represents the mean with standard deviation, n>4 for all samples.

Figure S2 : Impact of TLN468 on tRNA population. Volcano plot of variations in tRNAs isodecoders pools between Expi293F cells non treated vs TLN468 treatment. The Volcano plot was performed with the CPM (counts per million reads mapped) values calculated with multimapped reads. n=3 samples. Cystein tRNAs are highlighted in red, mitochondria tRNAs are in green, and asparagin tRNAs in orange.

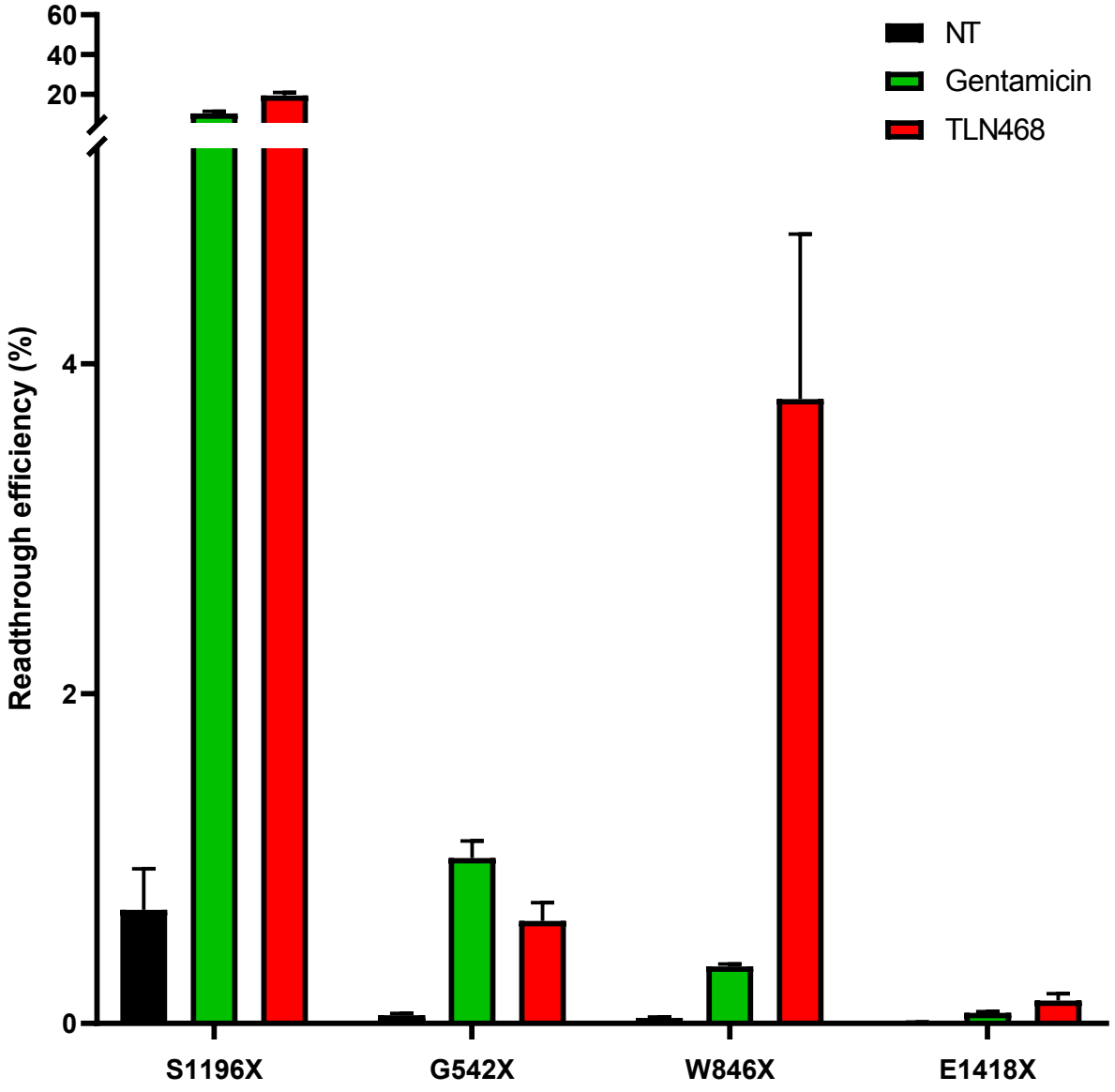

60
40
20
4
2
0
Readthrough efficiency (%)
NT
Gentamicin
TLN468
S1196X
G542X
W846X
E1418X

Figure 1

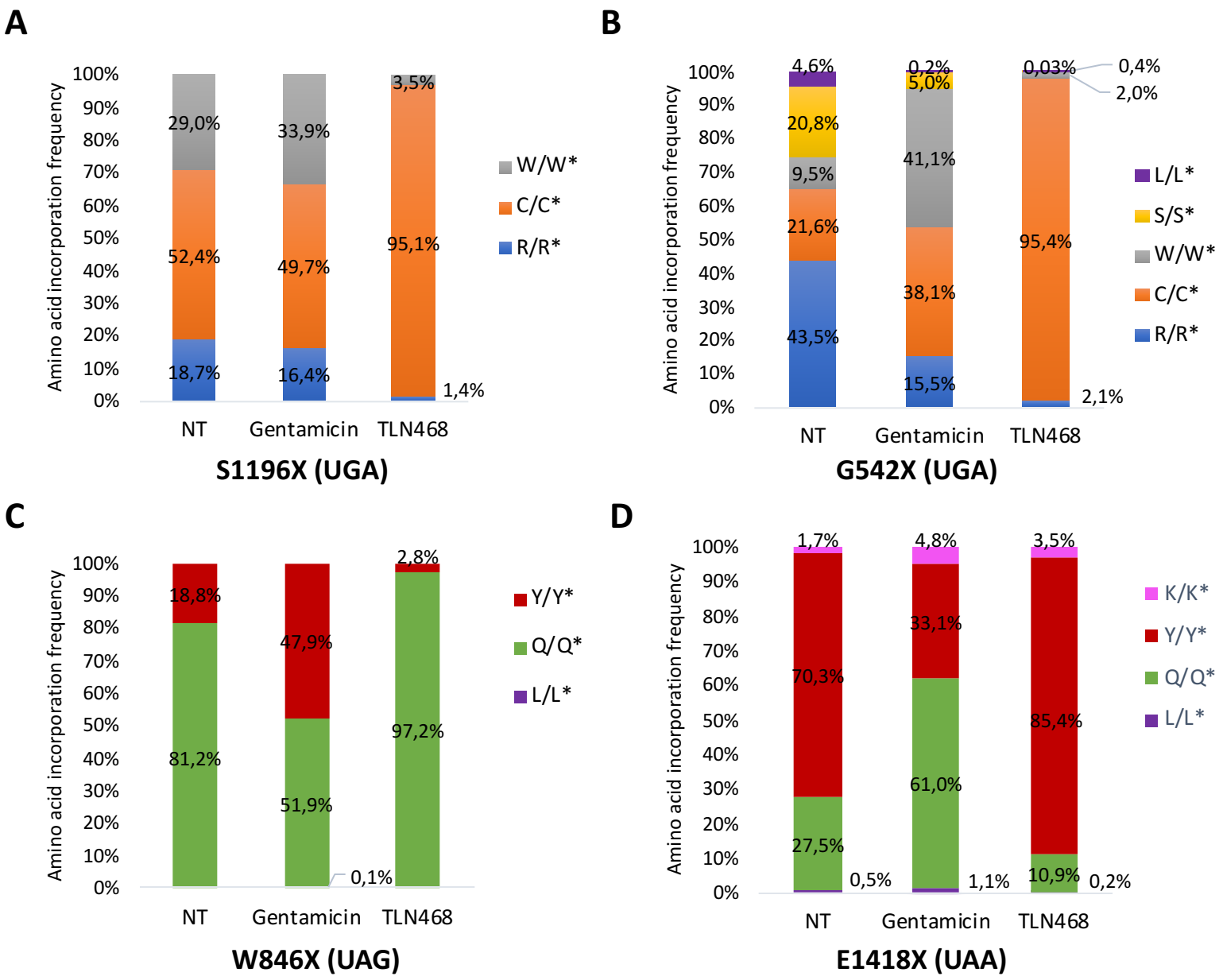
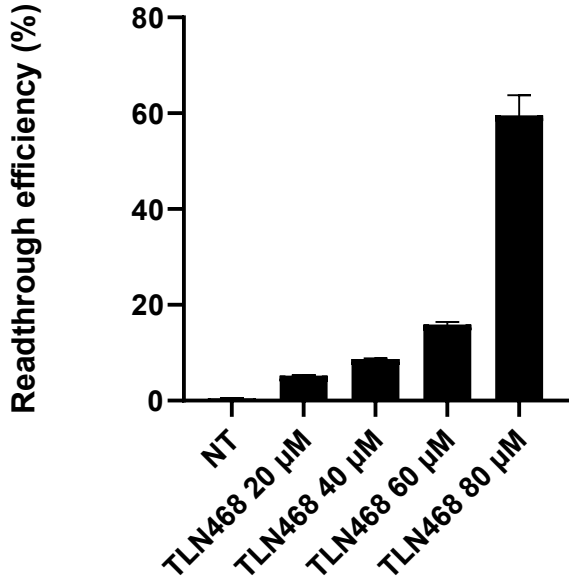

A
Amino acid incorporation frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
29,0%
52,4%
18,7%
33,9%
49,7%
16,4%
3,5%
95,1%
1,4%
NT
Gentamicin
TLN468
S1196X (UGA)
W/W*
C/C*
R/R*
B
4,6%
20,8%
9,5%
21,6%
43,5%
0,2%
5,0%
41,1%
38,1%
15,5%
0,03%
0,4%
2,0%
95,4%
2,1%
G542X (UGA)
L/L*
S/S*
W/W*
C/C*
R/R*
C
18,8%
81,2%
47,9%
51,9%
0,1%
2,8%
97,2%
W846X (UAG)
Y/Y*
Q/Q*
L/L*
D
1,7%
70,3%
27,5%
0,5%
4,8%
33,1%
61,0%
1,1%
3,5%
85,4%
10,9%
0,2%
E1418X (UAA)
K/K*
Y/Y*
Q/Q*
L/L*

Figure 2


Readthrough efficiency (%)
80
60
40
20
0
NT
TLN468 20 µM
TLN468 40 µM
TLN468 60 µM
TLN468 80 µM

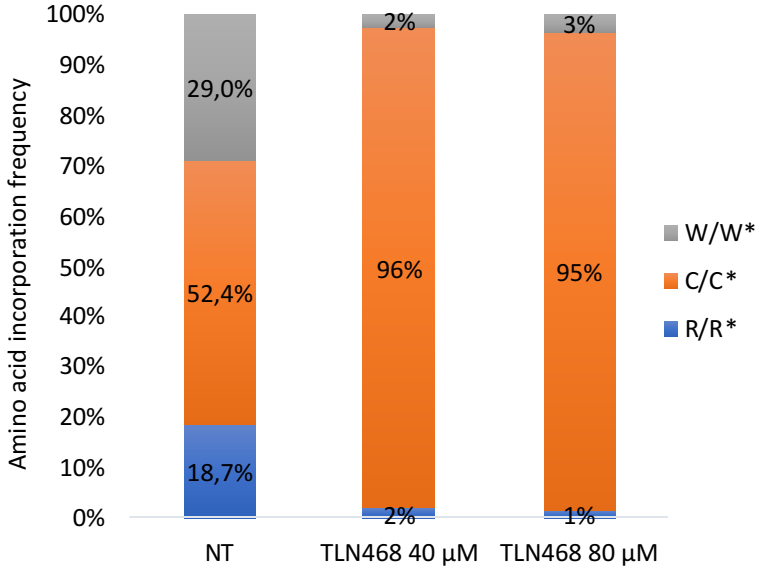
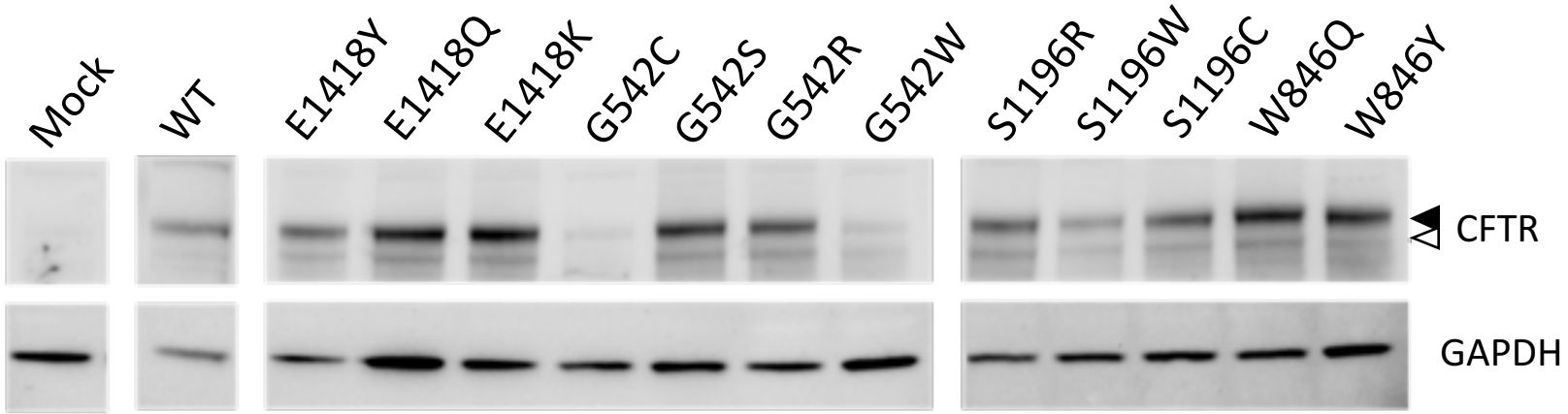

Amino acid incorporation frequency
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
29,0%
52,4%
18,7%
2%
96%
2%
3%
95%
1%
NT
TLN468 40 µM
TLN468 80 µM
W/W*
C/C*
R/R*

Figure 3


Mock
WT
E1418Y
E1418Q
E1418K
G542C
G542S
G542R
G542W
S1196R
S1196W
S1196C
W846Q
W846Y
CFTR
GAPDH

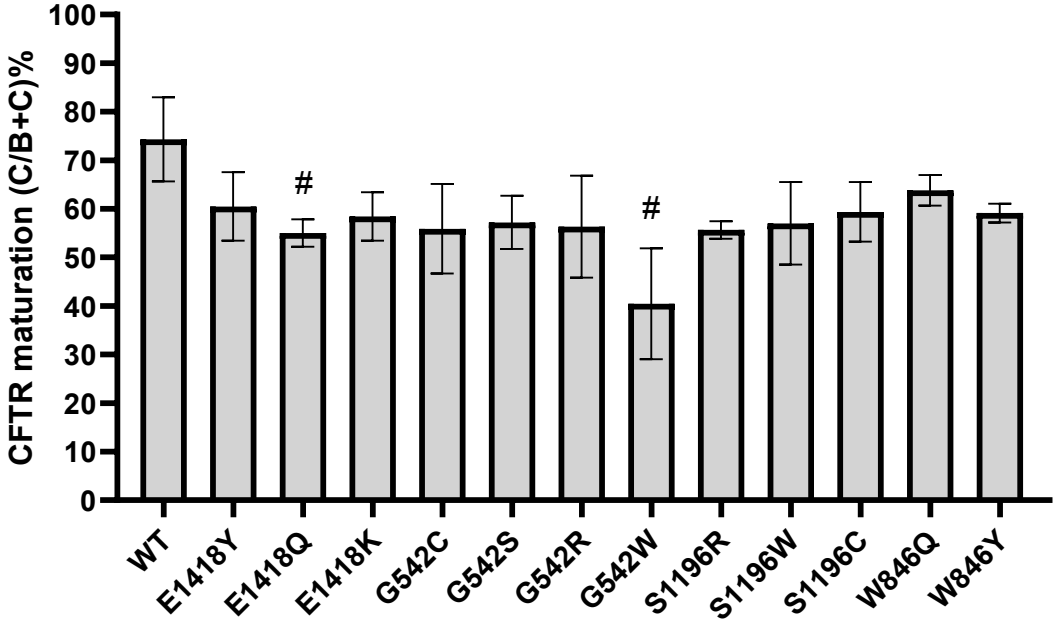
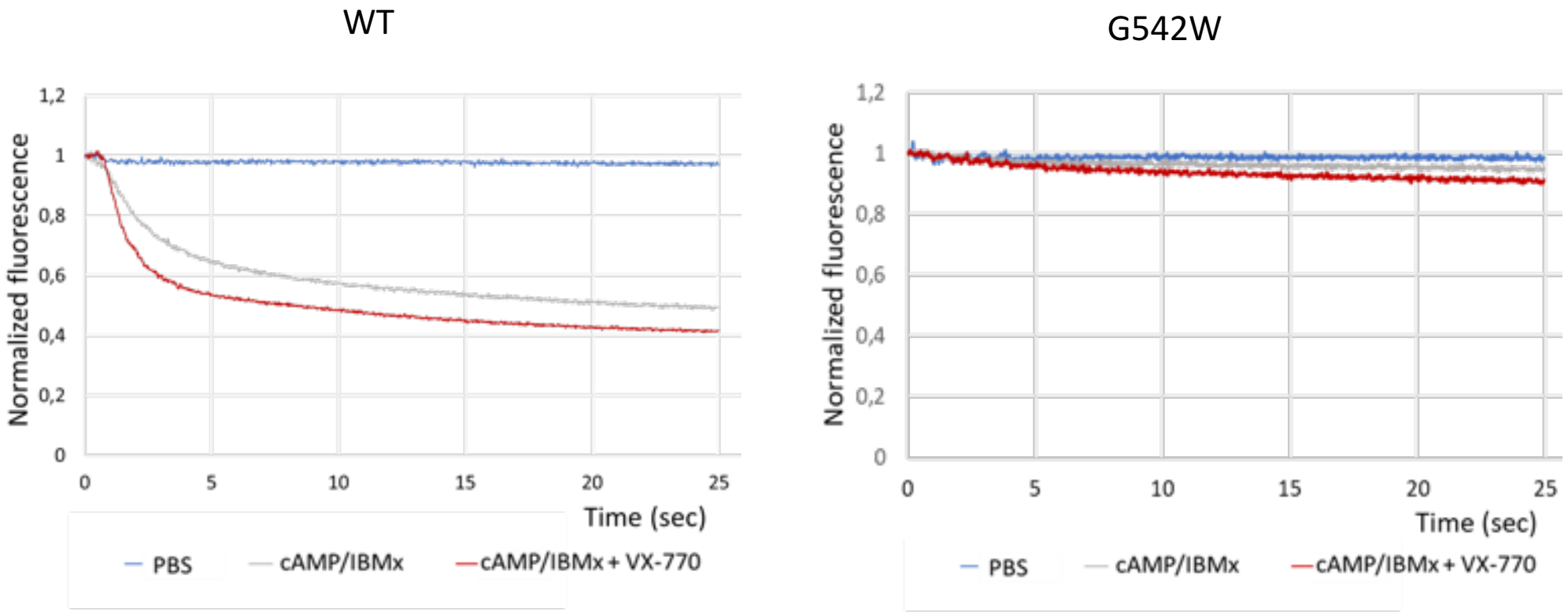

CFTR maturation (C/B+C)%
100
90
80
70
60
50
40
30
20
10
0
#
#
WT
E1418Y
E1418Q
E1418K
G542C
G542S
G542R
G542W
S1196R
S1196W
S1196C
W846Q
W846Y

Figure 4

A


WT
G542W
Normalized fluorescence
1,2
1
0,8
0,6
0,4
0,2
0
0
5
10
15
20
25
Time (sec)
PBS
cAMP/IBMx
cAMP/IBMx + VX-770

B

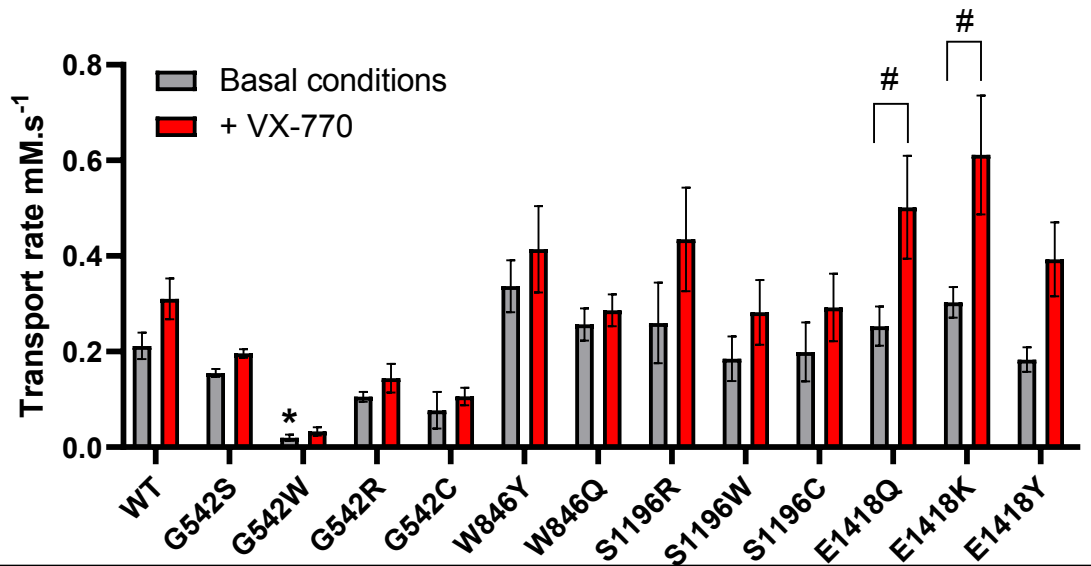

Transport rate mM.s-1
0.8
0.6
0.4
0.2
0.0
Basal conditions
+ VX-770
*
#
#
WT
G542S
G542W
G542R
G542C
W846Y
W846Q
S1196R
S1196W
S1196C
E1418Q
E1418K
E1418Y

Figure 5

Table 1

***CFTR* PTC**	**Inserted sequence**
S1196X	ATT GAG AAT **TGA** CAC GTG AAG
R1162X	TCT GTG AGC **TGA** GTC TTT AAG
W1282X	ATA GT TCTT **TGA** GAA GGT GGA
G542X	TTG CAA CAG **TGA** AGG AAA GCC
W846X	GTG ACT ACA **TAG** AAC ACA TAC
E60X	TGG GAT AGA **TAG** CTG GCT TCA
E831X	GAC TTA AAG **TAG** TGC TTT TTT
Q1268X	GGA GAA ATC **TAG** ATC GAT GGT
E1418X	TTG GTC ATA **TAA** GAG AAC AAA
Y1092X	TGG TTC TTG **TAA** CTG TCA ACA
K710X	TCT ATA CGA **TAA** AAA ACC ATT
Q290X	AAC TTA AGA **TAA** ACA GAA CTC