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Functional analysis of a promoter variant identified in the *CFTR* gene in *cis* of a  
frameshift mutation

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## **Abstract**

In monogenic diseases, the presence of [several sequence variations in the same allele](#) may complicate our understanding of genotype-phenotype relationships. We described [new alterations](#) identified in a cystic fibrosis patient harboring a 48C>G promoter sequence variation [associated](#) in *cis* of a 3532AC>GTA mutation [and in \*trans\* with the F508del mutation](#). Functional analyses including *in vitro* experiments confirmed the deleterious effect of the 3532GTA frameshift mutation through the creation of a premature termination codon. The analyses also revealed that the 48G promoter variant has a negative effect on both transcription and mRNA level, thus demonstrating the importance of analyzing all mutations or sequence variations with potential impact on cystic fibrosis transmembrane conductance regulator (CFTR) processing, even when the two known disease-causing mutations have already been detected. Our results emphasize the need to perform, wherever possible, functional studies that may greatly assist the interpretation of the disease-causing potential of rare mutation-associated sequence variations.

## **Keywords**

CFTR, promoter sequence variation, frameshift mutation, functional analysis

## Introduction

Cystic fibrosis (CF, MIM# 219700) is the most common lethal autosomal recessive disease affecting the Caucasian population with an average prevalence of 1 in 3000 births. Birth prevalence varies from country to country and with ethnic background<sup>1</sup>. While in its classical form, CF affects the physiology of several organs including the pancreas and the gastrointestinal and reproductive tracts, the morbidity is mainly due to pulmonary defects<sup>2</sup>. It is caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*; MIM# 602421) gene, for which over 1850 sequence variations have been reported to date (<http://www.genet.sickkids.on.ca/cftr/>). The pathogenicity of several types of mutations such as nonsense, frameshift and splicing mutations are obvious while the interpretation of the molecular consequences of others such as silent mutations<sup>3,4</sup> or those identified in non coding regions<sup>5,6</sup> is arduous. In addition, the *CFTR* gene is characterized by the presence of a number of complex alleles, i.e. at least two mutations in *cis* on the same chromosome that generally hinder the establishment of genotype-phenotype correlations. Mutations found in *cis* can modulate the effect of the principle mutation. The mutation R117H occurring in *cis* with the 5-thymidine (5T) tract variant in intron 8 generally results in pancreatic sufficient CF and as such is considered as a mild mutation, whereas R117H in *cis* with the 7T is mainly considered as a *CFTR*-related disease (*CFTR*-RD) associated mutation with low penetrance<sup>7</sup>. Complex alleles may also involve alterations in the *CFTR* promoter region, as is the case for the [-102T>A;S549R]+[F508del] genotype for which the promoter sequence variation is associated with an increase in *CFTR* expression and a moderate clinical phenotype<sup>8</sup>.

Here we report the functional analysis of a promoter variant associated in *cis* with a frameshift mutation [48C>G;3532AC>GTA]+[F508del] identified in a patient with a classic form of CF as characterized by a positive sweat test, pulmonary symptoms, digestive manifestations and pancreatic insufficiency. The two alterations 48C>G and 3532AC>GTA

are rare since they have been identified in only one patient and none have been found to occur alone elsewhere. In our continuing efforts to assist in the interpretation of uncharacterized mutations, we assessed the contribution of each alteration towards the disease phenotype. Although the molecular defect induced by the 3532AC>GTA mutation (frameshift mutation) seems clear, we also evaluated whether the 48C>G promoter sequence variation modulates the effect of the 3532AC>GTA mutation.

## **Material and methods**

### *Nomenclature of mutations*

For convenience to readers, we used legacy nomenclature as previously reported to the International Consortium Mutation Database (<http://www.genet.sickkids.on.ca/cftr/>). According to the Human Genome Variation Society (HGVS) nomenclature, the two described alterations 48C>G and 3532AC>GTA correspond to c.-85C>G and c.3400\_3401delACinsGTA respectively (GenBank NM\_000492.3), where nucleotide number 1 corresponds to the A of the ATG translation initiation codon (previously the A of the ATG was numbered 133).

### *Plasmid constructs*

To study the impact of the mutations, several *CFTR* constructions were created by direct mutagenesis (QuickChange II site directed mutagenesis kit, Agilent). The 48C>G sequence variation was introduced either in the *CFTR* promoter upstream of the *Luciferase* cDNA (pGL3b vector), or in the *CFTR* promoter upstream of the wild-type (WT) *CFTR* cDNA (pcDNA3-1 vector). The 3532AC>GTA was introduced in the *CFTR* cDNA under the control of either the *CFTR* promoter (WT or altered) or the CMV promoter in the pcDNA3-1 vector. The 3532AC>GTA was also introduced in a pSPL3 hybrid minigene containing WT *CFTR*

exon 18 and flanking introns (150bp). All constructs were verified by sequencing. All the oligonucleotides used for generating the constructs are available on demand.

#### *Transfection assays*

The A549 pulmonary epithelial cell line was grown in DMEM/F12 and the Cos7 simian fibroblast cell line in DMEM at 37°C under 5% CO<sub>2</sub>. Each medium was completed with 5% SVF (Eurobio), 1% Ultrosor G (Pall), 1% antibiotics (Invitrogen) and 1% L-glutamine (Invitrogen). All transient transfections were realized with Polyfect® (Qiagen) in 6-well plates (BD falcon) with 1500 ng of indicated vector. For the reporter assay, cells were transfected in 96-well plates (BD falcon) using Fugene6® (Roche Applied Science), with 60 ng of indicated reporter vector and 6 ng of pRL-SV40 (Renilla Luciferase) to normalize for transfection efficiency. Samples were harvested 48h after transfection. A stable A549 cell line, containing WT or 48G altered *CFTR* promoter was generated as previously described <sup>6</sup>.

#### *Protein truncation test*

In the first step, the *CFTR* fragment of interest was amplified from *CFTR* cDNA using specific primers with the addition of the T7 promoter at the 5'end <sup>9</sup>. To generate WT and mutated peptides encompassing the 3532 position labeled with <sup>35</sup>S-methionine, we used the TNT® T7 Quick Coupled Transcription/Translation System (Promega). Produced peptides were then separated by migration on a 12% denaturing acrylamide gel. After fixation and amplification, the signal was exposed on a Maximum Resolution film (Kodak) during 12 hours. Molecular weights were calculated with compute pI/Mw in ExPASy tools.

#### *Western blot*

Whole protein extracts were directly collected in Laemmli buffer and sonicated before their loading on a 7% SDS-polyacrylamide gel. After migration, proteins were transferred onto a PVDF membrane (Westran Clear Signal, Whatman), which was blocked with 5% skimmed milk in PBS-Tween for 1 h. Membranes were incubated overnight with anti-CFTR primary antibody (1:400, clone MM13-4 recognizing an N-terminus epitope, Millipore) or anti-Lamin A/C primary antibody (1:10000, Upstate). After 1h of incubation with the anti-mouse secondary antibody, proteins were revealed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) on a chemiluminescence-detection film (Kodak).

#### *Quantification of transcript level*

Total mRNAs were purified with RNeasy plus Mini Kit (Qiagen) as recommended by the manufacturer. Genomic DNA was removed with DNase I amplification grade (Invitrogen). After treatment with RNasin (Promega), RT-PCR was performed on 1 µg of RNA with random primers and MML-V reverse transcriptase (Invitrogen). Quantitative PCR was realized with 1:50 diluted cDNA and amplified with the LightCycler® 480 SYBR Green I Master (Roche Applied Science). *CFTR* cDNA was quantified and normalized by the quantification of *GAPDH* and *β-actin* housekeeping genes. Controls with no template or reverse transcriptase were also included. All PCR reactions were performed in triplicate in at least three independent experiments.

#### *Minigene assay*

Total mRNA extraction and RT-PCR were performed as described above for the quantification of transcript level. PCR was then performed using specific primers of pSPL3 plasmid and PCR Master Mix (Promega). Fragments were separated on a 1.5% agarose gel.

### *Reporter assay*

The experiment was performed as previously described <sup>6</sup>. Firefly and Renilla Luciferase activities were measured 48h after transfection by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's recommendations. Luciferase activities are representative of at least three independent experiments with each construct tested in triplicate per experiment.

### *Quantitative chromatin immunoprecipitation assays (Q-ChIP)*

Q-ChIP was performed as previously described, except for minor modifications <sup>6</sup>. Purified crosslinked chromatin was immunoprecipitated using 3 µg of anti-E2F, anti-MZF1, or anti-Sp1 antibodies. As a control for non-specific binding of DNA, 3 µg of anti-HA, anti-goat or anti-mouse antibodies were used. All antibodies were obtained from Santa Cruz, Clinisciences except for anti-HA which was bought from Roche Applied Science. Input and immunoprecipitated DNAs were analyzed after their amplification on the LightCycler® 480 real-time PCR system using the LightCycler® 480 Probes Master, specific primers for the *CFTR* minimal promoter region encompassing the 48C>G sequence variation and probe #62 (Roche Applied Science). All PCR reactions were performed in triplicate. Experiments were performed at least three times and expressed relative to the input signal and to non-specific immunoprecipitated chromatin.

### *Statistical analyses*

Data are expressed as the mean ± SE. Paired comparisons were made using Student's t-test with InStat (GraphPad Software, version 3.0). Data were considered statistically significant at  $p < 0.05$  and extremely significant at  $p < 0.0001$ .

## Results

### *Confirmation of the deleterious effect of the 3532AC>GTA frameshift mutation.*

We firstly hypothesized that the probable change in the open reading frame (ORF) induced by the 3532GTA frameshift mutation generates a premature termination codon (PTC) (Fig1A). We therefore performed a protein truncation test (PTT) by transcribing and translating *in vitro* the WT and mutated *CFTR* fragments. Results of electrophoresis showed a shift in the molecular weight of the synthesized peptides between CFTR-WT and CFTR-3532GTA conditions (Fig1B). The presence of a band with the expected molecular weight confirmed the creation of a PTC, twenty-one codons after the mutation, at the end of the last transmembrane segment.

To investigate further the several additional bands in the CFTR-3532GTA compared to the WT conditions revealed by our PTT, we assessed the effect of the 3532GTA mutation on CFTR maturation by western blot. Total proteins from cells transfected with an expression vector containing WT or mutated *CFTR* cDNA downstream of the CMV promoter, were used for immunoblot assay. Total proteins from cells transfected with the construction containing WT *CFTR* cDNA resulted in typical bands including band C (mature fully-glycosylated protein, 170 kDa) and band B (immature core-glycosylated protein, 140 kDa) (Fig1C). As a control, we used the F508del mutation, known to induce protein misconformation and in turn its premature degradation. No bands were detected however for the 3532GTA mutation as in the non-transfected (NT) condition whereas protein was present, as confirmed using anti-Lamin A/C primary antibody. The mutated sequence therefore prevents protein synthesis, suggesting that transcripts bearing this PTC are potential targets for degradation through nonsense-mediated mRNA decay (NMD) and/or for exon skipping through nonsense-associated alternative splicing (NAS).

We next assessed the impact of the 3532GTA allele on the mRNA level or splicing process.

Evaluation of mRNA amounts revealed that the 3532GTA mutation induces a decrease in *CFTR* transcript levels of more than 80%, compared to the WT condition in Cos-7 cells (Fig1D). The same results were obtained in A549 lung-specific cells (data not shown). Because this frameshift mutation might also produce both correctly and aberrantly spliced mRNA, we used a minigene assay to investigate whether the second control mechanism NAS could account for the observed inhibited synthesis. Hybrid minigene assay showed no difference between WT and mutated contexts (Fig1E). As alternative splicing events have been proposed to differ between cell lines<sup>10</sup>, we verified that the same results were obtained in the other tested cell line A549 (data not shown). These results indicate that degradation of the transcript bearing the PTC in exon 18 is the result of NMD rather than NAS.

#### *Characterization of the pathogenicity of the 48C>G promoter sequence variation*

To evaluate the effect of the 48G variant on transcriptional regulation, we performed a reporter assay. Luciferase activity, reflecting *CFTR* promoter control, indicated a decrease of about 40% in the 48G variant context compared to WT (Fig2A). We next assessed differences in *CFTR* mRNA amounts and found that the 48G allele induces a decrease of 75% in the *CFTR* transcript level when compared to the WT (Fig2B). These results suggest that the 48G promoter variant, in addition to its deleterious effect on transcription, may also disrupt the stability of the *CFTR* transcript. When associated in *cis* of the 3532GTA frameshift mutation, the 48G variant did not however increase the impact of 3532GTA alone on the mRNA level. Finally, we investigated which regulatory motifs, encompassing the 48C>G promoter sequence variation, were involved. Bioinformatics analyses predicted a change in binding capability of E2F, MZF1 (Myeloid zinc finger 1) and Sp1 (Specific protein 1) transcription factors. We therefore performed quantitative chromatin immunoprecipitation (Q-ChIP) on WT or sequence varied promoters using specific anti-E2F, anti-MZF1 and anti-Sp1

antibodies. Results show that none of the three transcription factors bound *in vivo* to the tested region of the WT promoter (Fig2C) whereas E2F, MZF1 and Sp1 proteins bound to the sequence varied promoter with high affinity (14, 15 and 23 fold enrichment relative to input respectively), suggesting their putative involvement in the 48G promoter variant effect on transcriptional control.

## **Discussion**

Here we have described the contribution of each [alteration](#) (48C>G and 3532AC>GTA) to CFTR function. The creation of a PTC with the 3532GTA frameshift mutation has been confirmed using PTT assay. This mutation was shown to have a deleterious effect at both the mRNA and protein levels with results suggesting the involvement of NMD in the recognition and degradation of aberrant transcripts. While no band C was observed by western blot, indicating 100% degradation, residual CFTR mRNA was obtained. Previous studies have shown the concomitant nature of the translation and folding of CFTR<sup>11,12</sup>. Our data suggest the role of an additional quality control mechanism that rapidly directs the elimination of the truncated protein (lacking NBD2) by ER-quality control systems.

We next explored the functional impact of the 48C>G sequence variation found in the promoter in *cis* of the frameshift mutation. Study of the 48G variant shows its deleterious effect at the transcriptional and mRNA levels. This outcome could be in part explained by the higher affinity of the altered sequence for the three transcription factors E2F, MZF1 and Sp1. MZF1 is well known for its role in regulating cell proliferation<sup>13</sup> but along with Sp1, it is also involved in regulating the expression of the human *MUC1* gene, expressed in several epithelial tissues<sup>14</sup>. Furthermore, MZF1 has previously been identified as a putative inhibitor of the *Cftr* gene, through its binding in the -834 to -524 region of the mouse promoter<sup>15</sup>. Here we have shown that MZF1 only binds to the altered *CFTR* allele (48G), suggesting its at least

partial role in decreasing promoter activity. E2F transcription factors are largely involved in cell cycle regulation <sup>16,17</sup> and along with MZF1, regulate genes that are developmentally regulated during cystogenesis <sup>18</sup>. Moreover, E2F and Sp1 can control the initiation of transcription in TATA-less promoters <sup>19,20</sup> through their interaction with other proteins. For instance, E2F could superactivate Sp1-dependant transcription in *DHFR* TATA-less promoter <sup>21</sup>. Transcription factors, through their binding to regulatory regions, either activate or repress the transcription of their target genes by promoting or inhibiting the recruitment of RNA polymerase II, through TSS use. Indeed, E2F and Sp1 have been shown to interact with several proteins of the general transcription machinery, such as TFIID and TFIIF <sup>22-25</sup>. These data suggest that the binding of these factors putatively modifies the use of the major TSS essential for *CFTR* transcription during lung development <sup>26,27</sup>.

The identification of [alterations in non-coding regions](#) and the determination of the impact of each allele component have several important consequences for recessive disorders such as CF. They permit a better understanding of the complex genotype-phenotype relationships and the opportunity to offer personalized genetic counseling or adapted therapy. These data reinforce the necessity to screen wherever possible for additional alterations even when two allelic mutations known to be disease-causing are found, and especially when a discrepancy between phenotype and genotype is observed.

### **Acknowledgments**

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The authors declare having no conflict of interest.

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## **Titles and legends to figures**

Figure 1: *Confirmation of the deleterious effect of the 3532AC>GTA frameshift mutation.*

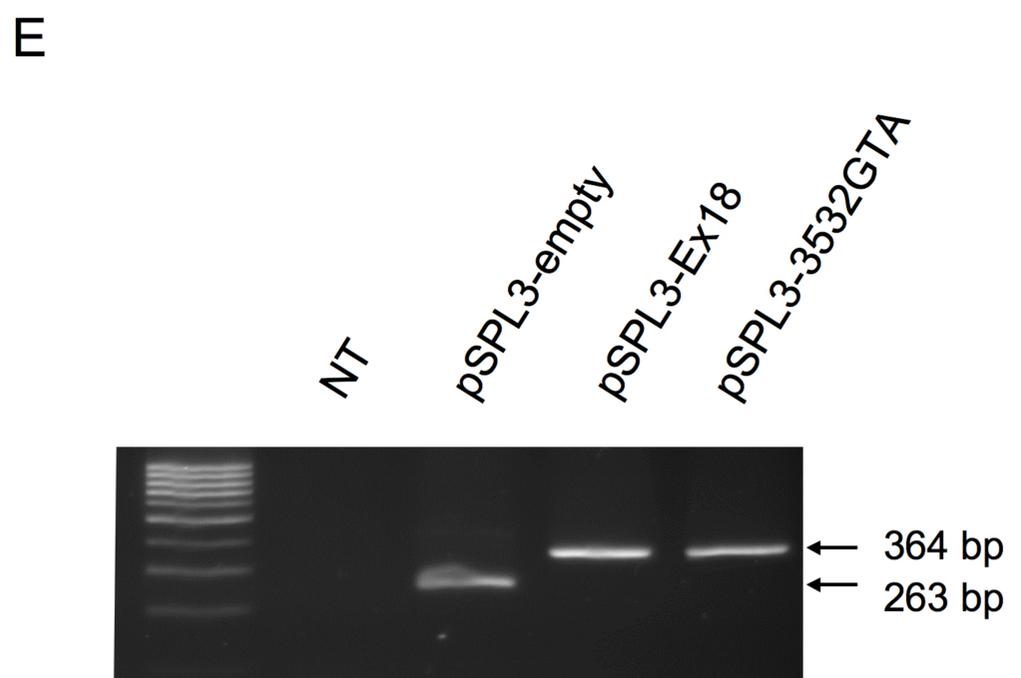
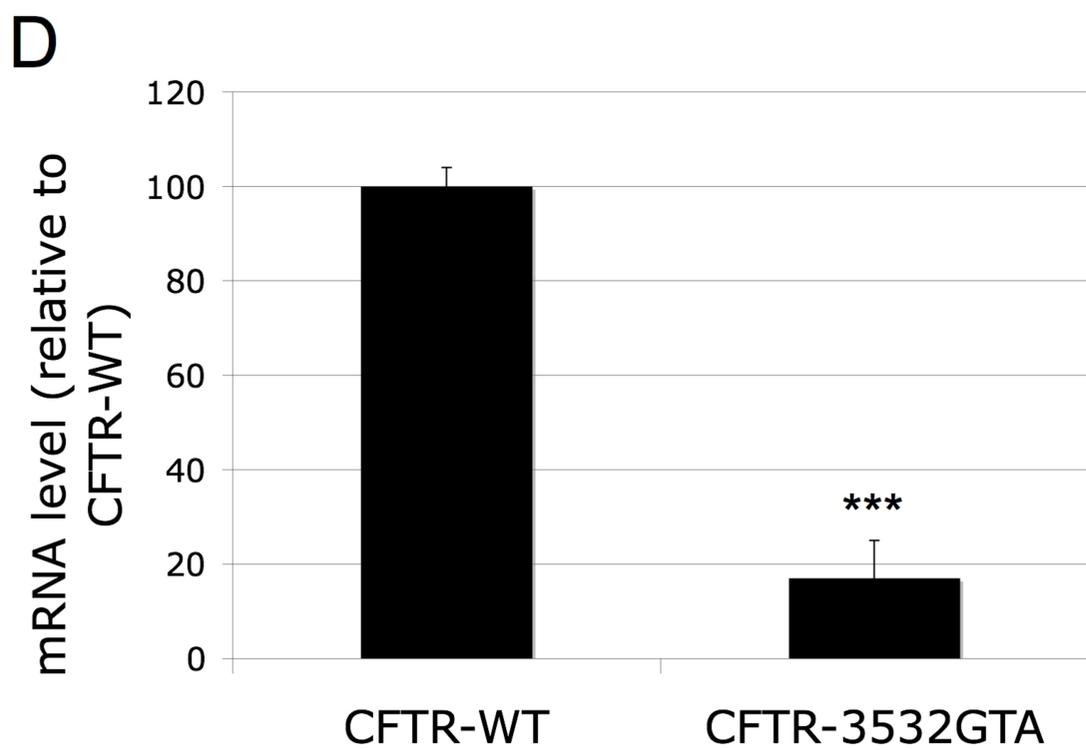
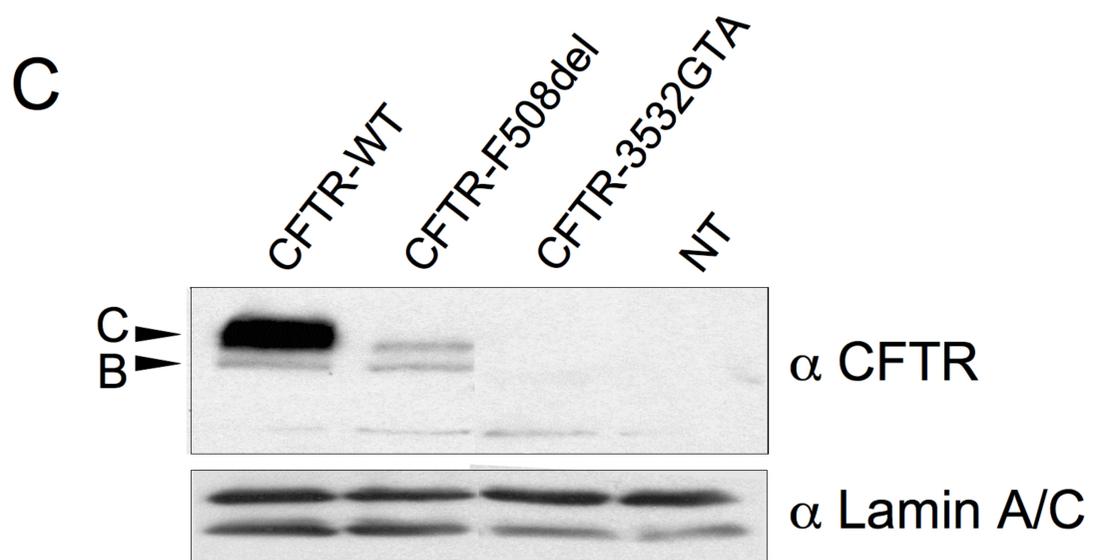
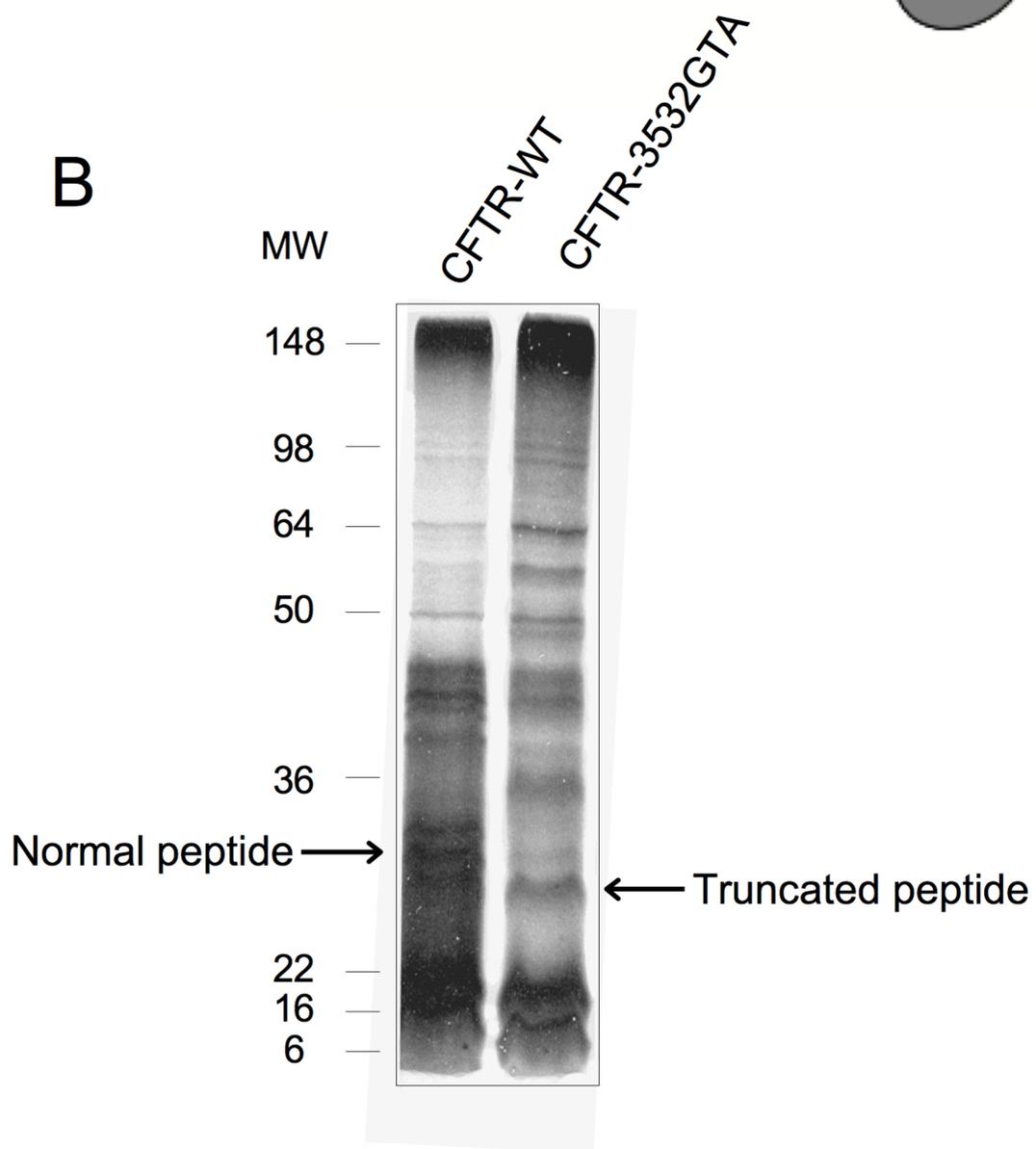
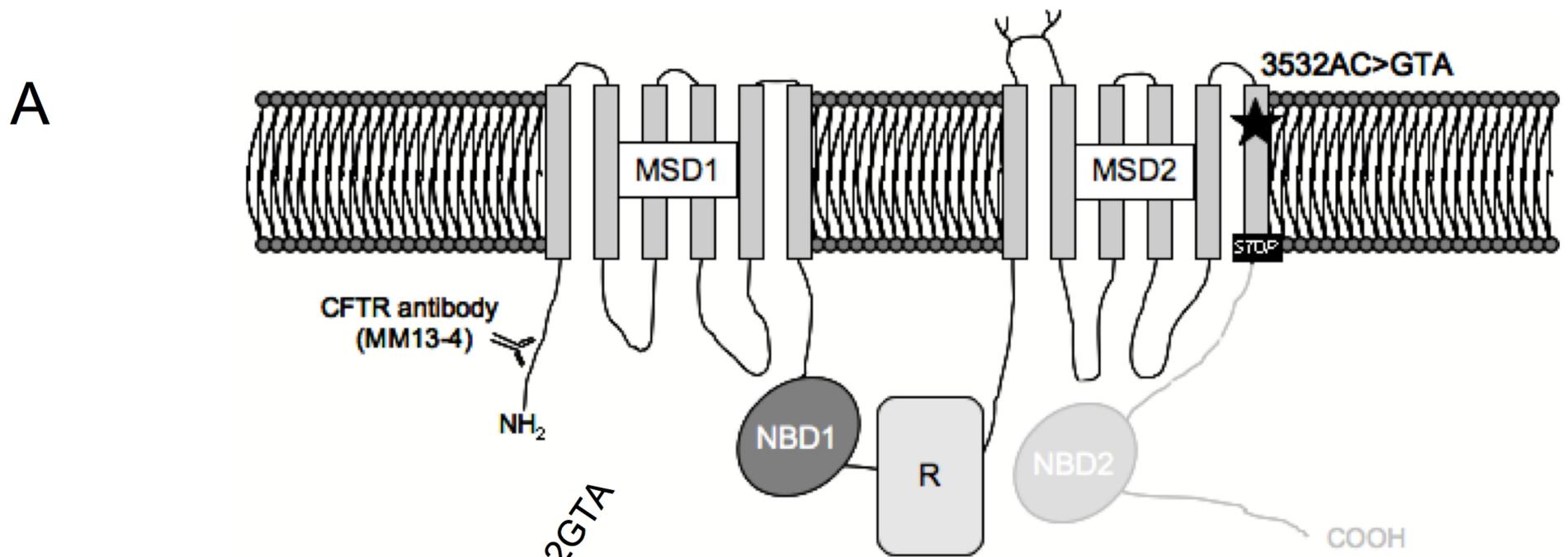
**A. Schematic representation of the location of the frameshift mutation, premature stop codon and anti-CFTR primary antibody.** The star represents the location of the frameshift mutation in exon 18 coding for the last transmembrane region of MSD2, STOP in the square shows the position of the PTC. **B. Analysis of the impact of the 3532GTA mutation by protein truncation test (PTT).** Confirmation of the creation of a PTC in the mutant context (CFTR-3532GTA) compared to wild-type (CFTR-WT). The presence of the normal or truncated peptide was defined by expected molecular weight. **C. Analysis of the impact of the 3532GTA mutation on protein maturation by western blot assay.** Proteins were extracted from Cos-7 cells non transfected (NT) or transfected with either WT (CFTR-WT), or mutated (CFTR-F508del or CFTR-3532GTA) constructs. Immunoblots were performed using either anti-CFTR, or anti-LaminA/C antibodies. **D. Assessment of the frameshift mutation at the CFTR mRNA level.** mRNA was extracted from Cos-7 cells transfected with wild-type (CFTR-WT) or mutated (CFTR-3532GTA) constructs and analyzed by RT-quantitative PCR. The data are normalized to the *GAPDH* and *β-actin* transcript level. **E. Analysis of the effect of 3532GTA on splicing by hybrid minigene.** Total mRNAs were extracted from Cos-7 cells, transfected with either empty vector (pSPL3-empty) or constructs with exon 18 containing or not the 3532GTA mutation. Values are extremely significant at  $p < 0.0001$  (\*\*\*) when compared to the WT context.

Figure 2: *Characterization of the pathogenicity of the 48C>G promoter sequence variation.*

**A. Impact of the 48G variant at the transcriptional level by reporter assay.** Luciferase was measured in cell lines derived from human lung (A549 cells) and transfected with empty plasmid or with the constructs containing either the 48C WT allele (CFTR-WT) or the 48G sequence varied allele (CFTR-48G). The Firefly Luciferase activity was normalized to Renilla

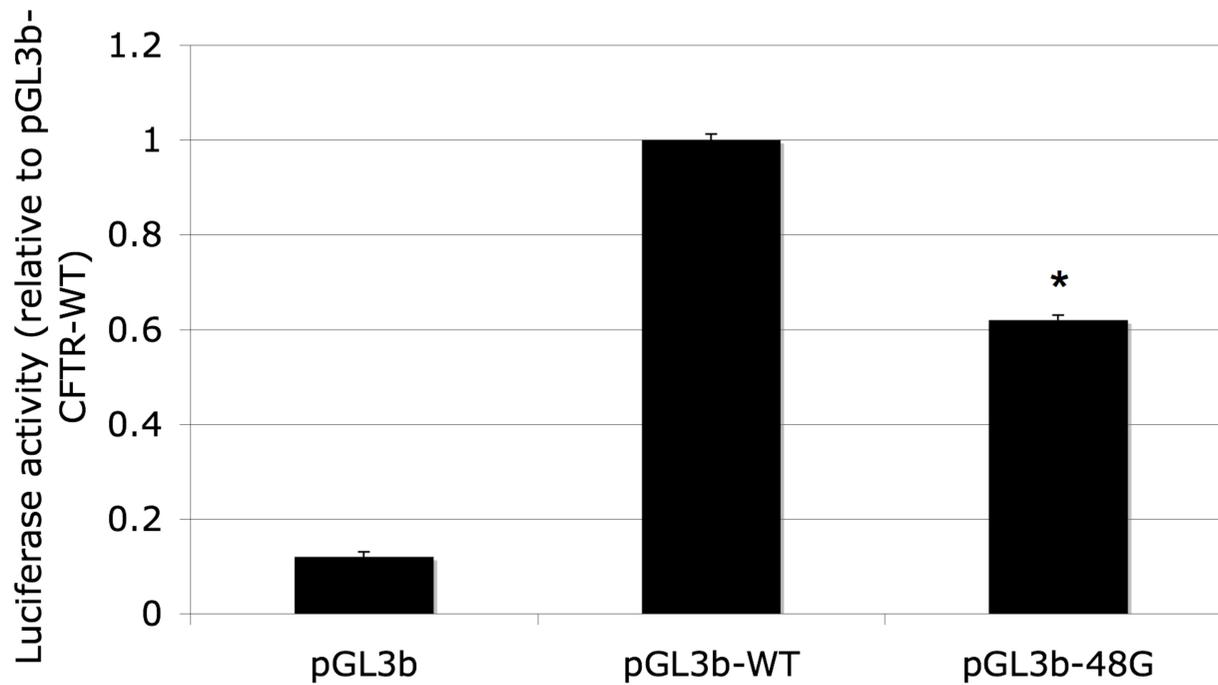
Luciferase. **B. Impact of the alterations at the *CFTR* mRNA level.** mRNA was extracted from A549 cells transfected with WT (CFTR-WT) or altered constructs: either 48G alone (CFTR-48G) or in combination with the 3532GTA mutation (CFTR-48G/3532GTA), and then analyzed by RT-quantitative PCR. The data are normalized to *GAPDH* and  *$\beta$ -actin* transcript level. **C. Binding affinity of E2F, MZF1 and Sp1 transcription factors.** Chromatin from A549 cells, that stably express constructs containing either wild-type (CFTR-WT) or sequence varied (CFTR-48G) promoter, was used for ChIP (Chromatin Immunoprecipitation) assay. Chromatin was immunoprecipitated (IP) with specific E2F, MZF1 and Sp1 antibodies. The data are presented relative to non immunoprecipitated chromatin (input). The results are also normalized to chromatin immunoprecipitated by non-specific antibodies. Values are significant at  $p < 0.05$  (\*) and extremely significant at  $p < 0.0001$  (\*\*\*) compared to the WT context.

# Figure 1

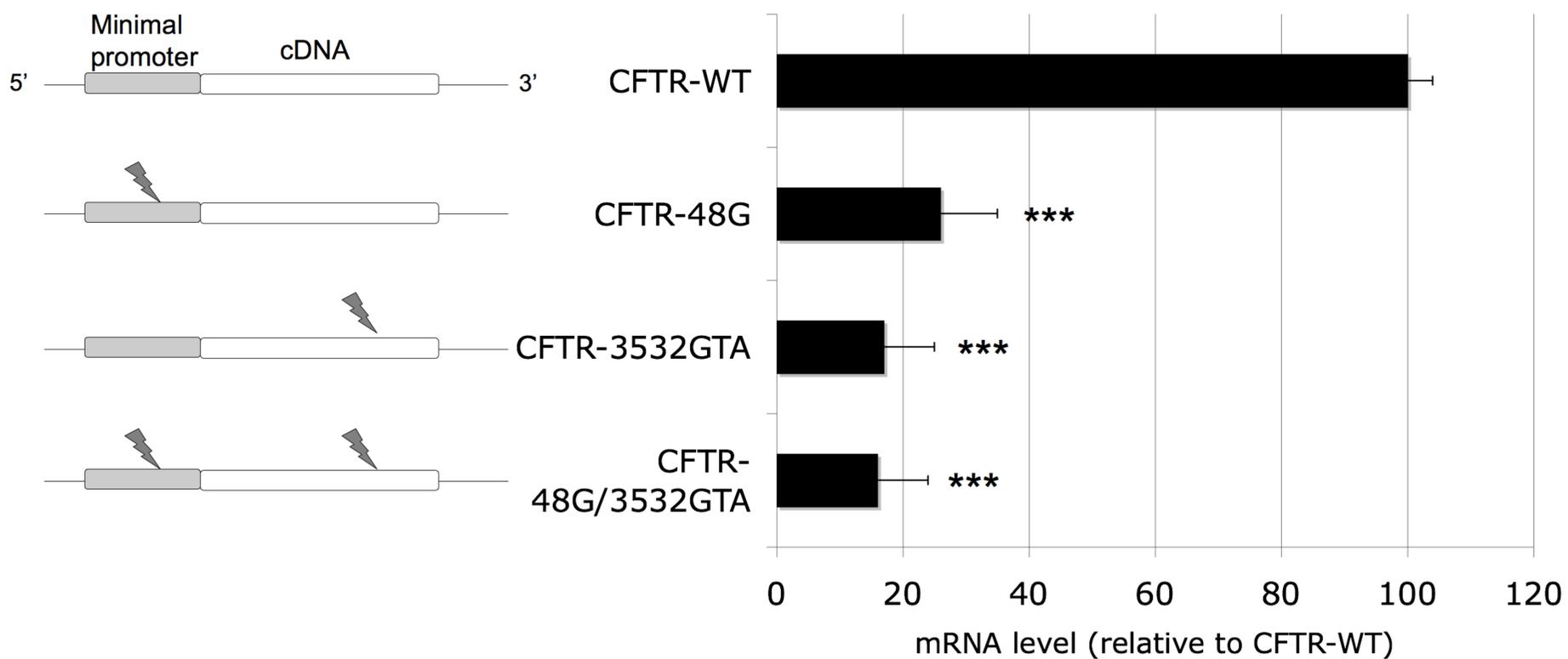


# Figure 2

## A



## B



## C

