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Variants in *CFTR* untranslated regions are associated with Congenital Bilateral Absence of  
the Vas Deferens

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## Abstract

**Background:** CBAVD (Congenital Bilateral Absence of the Vas Deferens), a frequent cause of obstructive azoospermia, is generated by mutations in the *CFTR* (Cystic Fibrosis Transmembrane conductance Regulator) gene. Despite extensive testing for point mutations and large rearrangements, a small proportion of alleles still remains unidentified in CBAVD patients. **Methods and Results:** Mutation scanning analysis of microsatellite variability in the *CFTR* gene identified two undescribed 4-bp sequence repeats (TAAA)<sub>6</sub> and (TAAA)<sub>8</sub> in intron 9 in two CBAVD patients heterozygote for either the -33G>A promoter transition or the classical [TG12T5] CBAVD mutation. In this report, we explored the putative impact of this promoter variant by using a combination of web-based prediction tools, reporter gene assays, and DNA/proteins interaction analyses. Results of transiently transfected vas deferens cells with either the -33G wild-type or the -33A variant *CFTR*-directed luciferase reporter gene confirmed that the -33A variant, which alters the FOXI1 (Forkhead Box II) binding, significantly decreases the *CFTR* promoter activity. We also investigated whether regulatory elements located within the intronic tetrarepeat might influence the *CFTR* expression. We evidenced that both the (TAAA)<sub>6</sub> and the (TAAA)<sub>8</sub> alleles modulate the *CFTR* transcription and the binding affinity for FOX transcription factors, involved in the chromatin architecture. **Conclusions:** As the vas deferens seems to be one of the tissues the most susceptible to a reduction in the normal *CFTR* transcripts levels, and as two mild mutations are sufficient to induce CBAVD phenotype, our findings raise the possibility that these uncommon variants may be a novel cause of CBAVD.

Key words: CBAVD, *CFTR* transcriptional regulation, microsatellite, unknown variant

## Introduction

Congenital bilateral absence of the vas deferens (CBAVD), an autosomal recessive disorder, is a frequent cause of obstructive azoospermia and accounts for approximately 2-3% of male infertility in Caucasian populations. In 90% of cases, CBAVD is caused by mutations in the *Cystic Fibrosis Transmembrane conductance Regulator (CFTR)* gene.<sup>1 2</sup> To date, more than 1,600 genetic changes have been identified in this gene. A combination of severe mutations on both alleles of a patient usually leads to severe forms of classical Cystic Fibrosis (CF).<sup>3 4</sup> Other mutations that retain residual CFTR function (above 10%), result in mild CF or incomplete phenotypes. CBAVD patients carry two mild mutations or are compound heterozygous for a severe and a mild alterations of the *CFTR* gene.<sup>1 5</sup> Despite extensive testing for point mutations and large rearrangements<sup>6-9</sup>, a small proportion of alleles still remains unidentified in CBAVD patients.<sup>1 10</sup> Interestingly, we have detected two novel rare (TAAA)<sub>n</sub> repeats in the IVS9 of the *CFTR* gene in two CBAVD patients, heterozygous for a variation in the minimal promoter (-33G>A)<sup>11</sup> or for the common CBAVD variant (TG12T5).

Microsatellites, also known as Short Tandem Repeats (STRs), are distributed throughout the genome<sup>12</sup>. Since the last decade, scientists suggested that alterations in the length of simple DNA repeat sequences might be responsible for diseases, such as myotonic dystrophy type 2<sup>13</sup> and gynecomastia.<sup>14</sup> Accumulating data evidenced that microsatellite variations including tetranucleotides affect regulation of gene expression by inhibiting the transcription of these genes implicated in diseases<sup>15 16</sup> or by altering RNA processing<sup>17</sup> for STRs detected close to some intron/exon boundaries.

Several microsatellites have been identified in non-coding regions of the *CFTR* gene.<sup>18-20</sup> Whereas most are dinucleotides, two tetranucleotides (TAAA)<sub>n</sub> and (GATT)<sub>n</sub> repeats have been reported in intronic regions.<sup>18 19</sup> Except for the (TG)mTn polymorphism

tracts in the *CFTR* IVS8, known to dramatically alter the *CFTR* exon 9 skipping<sup>18 21-25</sup>, the role of repeats in the regulation of *CFTR* function and expression remains poorly defined.

The aim of this study was to evaluate whether the uncommon -33G>A promoter variant and the (TAAA)<sub>6</sub> and (TAAA)<sub>8</sub> tetranucleotide repeat alterations located deeply within intron 9 may contribute to the CBAVD phenotype. We were also interested in understanding the molecular mechanism by investigating the potential regulatory function of Forkhead box (FOX) transcription factors in the presence of these variants.

## **Material and methods**

### *Haplotyping IVS9(TAAA)<sub>n</sub> repeats.*

PCR reactions were carried out on 100 ng of human DNA (see primers in Supplementary Table 1). Aliquots of the amplicons [(TAAA)<sub>n</sub> units] were separated by multicapillary electrophoresis on an ABI 3130xl Genetic Analyzer running GeneMapper v4.0 for allele identification as previously described.<sup>26</sup>

### *Expression plasmids, gene reporter vectors and hybrid minigene constructs.*

To perform gene reporter assays, the pGL3B-33A plasmid was generated from pGL3B-WT, pGL3-Basic reporter vector containing the wild-type human *CFTR* minimal promoter<sup>27</sup>, by site-directed mutagenesis (Stratagene).

The pcDNA3-FOXII expression vector was generously gift by Dr. S. Enerbäck (Center of Medical Genetics, Institute of Biomedicine, Göteborg University, Sweden). The AE4-promH1 and AE4-promH3 reporter constructs were kindly provided by Dr. C.A. Hübner (Department of Human Genetics, UKE-Hamburg, Hamburg, Germany).

For hybrid minigene assays, constructs containing the (TAAA)<sub>n</sub> repeats of the *CFTR* intron 9 were created as depicted in figure 3. The human *CFTR* genomic regions encompassing the

exons 9 and 10 with their flanking intronic sequences were amplified in two separated PCRs. The PCR products were subcloned into the *XhoI/NheI* digested pSPL3 plasmid, kindly gift by Dr. S. Tuffery-Giraud (Laboratory of Molecular Genetics, Montpellier, France). As positive controls, minigenes containing either a T5 or a T3 allele<sup>22</sup> were generated by directed mutagenesis (Stratagene).

To prepare the gene reporter vectors pGL3C-(TAAA)<sub>n</sub>, a 1222-base pair fragment spanning the *CFTR* intron 9 region was generated by PCR from genomic DNA of individuals with (TAAA)<sub>9</sub>, (TAAA)<sub>8</sub> or (TAAA)<sub>6</sub> repeat. The PCR products were restricted with *NheI* and *BglIII* endonucleases and then ligated into the pGL3-Control reporter vector (Promega) upstream the SV40 promoter.

To evaluate *CFTR* mRNA levels, expression plasmids containing either the wild-type or the mutated *CFTR* minimal promoter upstream the *CFTR* cDNA (pcDNA3.1-WT and pcDNA3.1-33A, respectively) were constructed. The full-length human *CFTR* cDNA was excised from the pTG5985 vector, generously given by Transgene SA, with *ApaI/XhoI* and ligated into the pcDNA3.1(-) expression vector. To drive the expression of the *CFTR* cDNA, the SV-40 promoter was replaced by the *CFTR* minimal promoter. For the (TAAA)<sub>n</sub> repeat, the (TAAA)<sub>n</sub> intron 9 region is inserted upstream the *CFTR* minimal promoter in the pcDNA3.1(-) expression vector containing the *CFTR* cDNA. All the primers are listed in Supplementary Table 1. All the constructs were verified by direct sequencing.

#### *Cell culture and transient transfections.*

HVP (SV40 ori-transformed epithelial vas deferens) cells, maintained in CMRL-1066 medium (Gibco, Invitrogen) and kindly provided by Dr. A. Harris (Weatherall Institute of Molecular Medicine, Oxford University, UK). HeLa (human cervical carcinoma) cells were cultured as previously reported.<sup>28</sup> For transient transfections, cells, seeded at a density of

about 10,000 cells/100  $\mu$ l of medium in 96-well plates, were transfected with 60 ng of indicated reporter vector and 6 ng of internal control pRL-SV40 containing Renilla luciferase (Promega), to normalize for transfection efficiency, by using Fugene®6 transfection reagent (Roche). In transient co-transfection experiments, 4 ng of either pcDNA3(-) or pcDNA3-FOXII vector were also transfected. Similar transfections were performed in 6-well plates for protein extracts. For siRNA assays, transfections of cells with 40 nM FOXII-targeting siRNAs (Santa Cruz, Clinisciences) were performed using Interferin reagent (PolyPlus, Ozyme). Non-targeting siRNA purchased from Santa Cruz (Clinisciences), served as a negative control. Dual Glo Luciferase Assay System (Promega) was used according to the manufacturer's recommendations.

To analyse the hybrid minigene expressions,  $1-1.5 \times 10^6$  cells were plated into Nunclon® 35 x 10 mm cell culture dishes. After 24 hours, cells were transiently transfected with 6  $\mu$ g of either pSPL3 empty vector or one minigene plasmid using Polyfect® transfection reagent (Qiagen). For the *CFTR* mRNA level analyses, 6  $\mu$ g of the indicated pcDNA3.1 expression vectors alone or with 2.5  $\mu$ g of FOXII expression vector or 40 nM of FOXII-targeting siRNA were transfected. After 48 hours, the cells were lysed for RNA extraction. All hybrid minigene, expression vector and gene reporter data represented the result of at least three independent experiments performed in triplicates with independently purified plasmid DNA preparations.

#### *Establishment of stable cell lines.*

For chromatin immunoprecipitation assays, HVP cells were seeded at a density of about 300,000 cells in 35-mm culture dishes and were co-transfected with 80 ng of pCI-neo vector and 2  $\mu$ g of indicated reporter vector by Polyfect® transfection reagent. Stably transfected

clones were selected in medium containing G418 antibiotic (Promega). Clones of G418-resistant cells were screened by direct sequencing.

#### *Reverse-transcriptase PCR and RT-qPCR.*

Total RNAs were extracted by using the RNeasy Plus Mini kit (Qiagen). RT were performed as previously described<sup>29</sup>, and cDNAs were amplified with either SD6/SA2/ $\beta$ -actin primers (hybrid minigene) or CFTR/GAPDH (expression vector), by using classical PCR or the LightCycler® 480 Real-Time PCR System (Roche), respectively. All PCR reactions were performed in triplicate in three independent experiments.

#### *Chromatin Immunoprecipitation assays (ChIP)*

ChIP assays were performed according to protocols previously described with minor modifications.<sup>29</sup> Prior to harvest, stably transfected HVP cells were cross-linked with formaldehyde. Purified crosslinked chromatin was immunoprecipitated using 3 $\mu$ g of either anti-FOXII antibody (AbCam) or anti-HA antibody (Roche), used to control for non-specific binding of DNA. Input and immunoprecipitated DNAs were analyzed using either primers specific of the *CFTR* minimal promoter or primers amplifying the *CFTR* intron 9 region containing the (TAAA)<sub>n</sub> repeat (Supplementary Table 1), with the LightCycler® 480 Real-Time PCR System (Roche). All PCR reactions were performed in triplicate. Experiments are repeated at least three times, averaged and expressed relative to the input signal and to negative control.

#### *Immunoblot analyses*

Total whole-cell proteins from transfected HVP cells were analyzed by western blot as previously described.<sup>29</sup> The membranes were incubated overnight with 1:1,000 diluted anti-



FOXI1 (Ab-Cam) primary antibody in 5% skim milk. The protein levels of the  $\beta$ -actin housekeeping gene were assayed for internal control of protein loading.

#### *Statistical analyses.*

Data are expressed as the mean  $\pm$  SE. Paired comparisons were made using student's t-test using InStat (GraphPad Software, version 3.0). Data were considered statistically significant at  $p < 0.05$ . Relative intensities of equal areas were compared using Quantity One<sup>®</sup> quantification analysis software (Bio-Rad).

## **Results**

### *The -33G>A transition in the CFTR minimal promoter leads to a decreased promoter activity*

We first investigated the putative effect of a rare promoter substitution, -33G>A, on both the *CFTR* transcriptional activity and mRNA levels. As this sequence variation has been identified only in a patient with CBAVD, wild-type (pGL3B-WT) and mutated (pGL3B-33A) promoter constructs were tested in epithelial cell lines derived from tissues where spatial and temporal regulation of the *CFTR* expression is functional (HVP: vas deferens) or not (Hela: cervix). Compared to the wild-type, the promoter activity of the mutant construct was significantly reduced by approximately 54% ( $p < 0.05$ ) in HVP cell line whereas no significant variation was observed in HeLa cells (Figure 1A). To ensure that the -33A variant is responsible for the observed inhibition, RT-quantitative PCR (qPCR) assays were performed from HVP and HeLa cells. Since RNA transcripts of the patient were not accessible, we transfected these cell lines with expression vectors containing the *CFTR* cDNA under the control of the *CFTR* promoter (pcDNA3.1-WT or pcDNA3.1-33A). The results confirmed a diminution in the *CFTR* mRNA level in HVP cell line whereas no difference was observed in

Hela cells (Figure 1B). Collectively, these findings established that the promoter -33A variant influences the *CFTR* transcription in a cell-specific manner.

*The -33A variant affects promoter activity through allele-specific binding of the FOXI1 transcription factor*

We then evaluated whether this single-nucleotide substitution perturbs the DNA binding affinity for transcription factors. *In silico* analyses, using TFsearch and Consite softwares, indicated that the *CFTR* minimal promoter region contains a binding site for FOX proteins overlapping the -33 position. Since FOXI1 (Forkhead Box I1) has been reported as a key regulator of genes involved in male fertility in epididymal epithelial cells<sup>30 31</sup>, we hypothesized that FOXI1 could participate to male infertility due to CBAVD by regulating the *CFTR* gene in HVP cell line. First, to test whether this factor is capable of binding *in vivo* to the endogenous *CFTR* promoter sequence, ChIP assays were carried out. The DNA immunoprecipitated with a non-specific antibody did not show any PCR signal (Figure 2A, lane 3). As negative control, we used the promoter of *Caspase 8* gene, which lacks FOX binding sites. As observed in figure 2A (lane 2), FOXI1 binds to the *CFTR* minimal promoter. We next employed quantitative ChIP analyses to obtain evidence that FOXI1 proteins bind differentially to the mutated *CFTR* promoter sequence. Because we could not have access to the patient's tissue, ChIP assays were performed using stable HVP cells expressing either the wild-type or the mutated *CFTR* promoter reporter construct (pGL3B-WT and pGL3B-33A). The qPCR results reveal that FOXI1 binds to the minimal *CFTR* promoter in HVP cells with a higher avidity for the mutated promoter (Figure 2B).

To assess the functional activity of the FOXI1 factor, we transiently co-transfected the FOXI1 expression vector with either the wild-type (pGL3B-WT) or the mutated (pGL3B-33A) reporter plasmid in HVP cells. Our results show that the over-expression of FOXI1

proteins reduces the activity of the *CFTR* promoter, which is more significant in mutated context than in wild-type context (51% versus 37%) (Figure 2C, grey bars). To confirm the role of FOXI1 in the regulation of the *CFTR* transcription, we transiently co-transfected the reporter plasmids along with a FOXI1-specific small interfering RNA (siRNA). This resulted in a weak but significant activation of the luciferase activity in HVP cells (Figure 2C, white bars). The efficiency of the over-expression and the siRNA knockdown of the FOXI1 proteins were verified by immunoblotting experiments. Transient transfections of FOXI1-siRNA reduced FOXI1 protein levels by approximately 50% compared to a non-specific siRNA control (Figure 2D). RT-qPCR experiments were performed to analyse the *CFTR* mRNA levels in both wild-type and mutated contexts after FOXI1 over-expression (Figure 2E). Although the effect of FOXI1 repression on the -33A allele *versus* the -33G allele is attenuated with RT-qPCR assays (Figure 2E), probably due to variation in constructs and approaches, both results suggested a markedly decrease of the *CFTR* expression for the mutated allele whereas only one nucleotide is changed. In addition, to confirm the negative role of FOXI1, we performed transient co-transfection experiments with either the AE4-promH1 or the AE4-promH3 reporter construct along with either the FOXI1 expression vector or FOXI1 specific siRNA in HVP cells. Our data demonstrated the strong activator role of FOXI1 on AE4-promH1 and AE4-promH3 transcription (Figure 2F) as previously reported.<sup>30 32</sup>

Collectively, these data established that FOXI1 inhibits the *CFTR* promoter activity with a marked effect on the -33A allele.

#### *Genetic analysis of previously undescribed (TAAA)<sub>n</sub> repeats sequence DNA motif in CFTR IVS9*

By scanning microsatellite variability in the *CFTR* IVS9, in a sample of 254 control

chromosomes and 86 chromosomes from CBAVD patients with 1 or 0 *CFTR* mutation, we identified five tetranucleotide (TAAA)<sub>n</sub> repeat tracts with number of motifs ranging from 9 to 13. This study showed that the 9-repeats allele is the most frequent (Table 1). In addition, analysis of DNAs, from CF patients homozygous for the p.Phe508del mutation and from CBAVD patients heterozygous for the p.Phe508del mutation and the [TG12T5] variant, revealed that the p.Phe508del mutations is mainly associated with the (TAAA)<sub>11</sub> tract (Table 1). Interestingly, we detected two previously undescribed variants, (TAAA)<sub>8</sub> and (TAAA)<sub>6</sub>, in two CBAVD patients carrying a (TAAA)<sub>9</sub> tract on the other chromosome. The patient with the 6-repeat allele is heterozygous for the -33G>A promoter variation<sup>11</sup>; the patient with the 8-repeat allele is heterozygous for the [TG12T5] variant. Accurate assignment of haplotypes was impossible (DNA from parents not available for segregation analyses). These two novel variants in IVS9 were not detected in a panel of 254 control chromosomes and 122 DNAs from the CBAVD subjects with the p.Phe508del and the TG12T5 alterations, and from the CF patients homozygous for the p.Phe508del mutation (Table 1).

**Table 1: (TAAA) allele frequencies in control, CF and CBAVD subjects**

Number of (TAAA) repeats	% control	% CBAVD patients		% CF patients
	n=254	0 or 1 identified mutation n=86	Mutations: p.Phe508del/ TG12T5 n=56	Mutations: p.Phe508del/ p.Phe508del n=66
13	2.75	1.20	0	0
12	2.36	1.20	0	0
11	8.66	10.46	50	94
10	11.40	9.30	3.60	0
9	74.80	75.60	46.40	6
8	0	1.20	0	0
6	0	1.20	0	0

'n' the total number of chromosomes

We hypothesized that these tetranucleotide repeats, located deeply within intron 9, could influence gene expression.<sup>15 16 33 34</sup> Also, we evaluated the functional importance of the new CBAVD (TAAA)<sub>6</sub> and (TAAA)<sub>8</sub> alleles compared to the most frequent (TAAA)<sub>9</sub> allele in general population.

*The (TAAA)<sub>n</sub> repeat motif does not influence the splicing of CFTR exon 10*

To investigate the putative impact of the IVS9 (TAAA) microsatellite on the RNA processing, *CFTR* exons 9-10 hybrid minigene constructs (Figure 3A) were transiently transfected into HVP and Hela cells, and the pattern of splicing was analyzed by RT-PCR with the SD6/SA2 primers. In the two tested cell lines, all PCR reactions produced one band of the expected size for wild-type mRNA transcript containing exons 9 and 10 (Figure 3B). As positive controls, hybrid minigenes containing either a T5 or a T3 allele showed a significant decrease of correctly spliced transcript with the appearance of an aberrantly-spliced transcript containing only the exon 10. As previously reported<sup>35</sup>, the same constructs with variable size containing (TAAA)<sub>n</sub> motif were also created to ensure that enclosed sequences to the motif repeats do not contain silencer or enhancer elements. No modulation of the spliced transcript has been observed (data not shown). Our findings indicated that the (TAAA)<sub>6</sub> and (TAAA)<sub>8</sub> repeats has no effect on exon 10 splicing.

*The (TAAA)<sub>n</sub> repeat motif alters the CFTR transcription*

In a second set of experiments, we speculated that the variation in the (TAAA) number might alter the activity of the *CFTR* promoter as previously reported for other genes.<sup>16 34</sup> To explore this possibility, we transfected in both HVP and Hela cells, pGL3-Control reporter vectors containing the (TAAA)<sub>9</sub>, (TAAA)<sub>8</sub> or (TAAA)<sub>6</sub> allele [pGL3C-(TAAA)<sub>n</sub>] as shown in Figure 4A. The results showed that in HVP cells, the pGL3C-(TAAA)<sub>6</sub> and pGL3C-(TAAA)<sub>8</sub>

plasmids significantly decreased the luciferase level. Interestingly, a decrease in repeat number from 9 to 8 and from 9 to 6 resulted in an inhibition of luciferase activity to 45 and 50% respectively (Figure 4A, left panel). In HeLa cells, the presence of either the (TAAA)<sub>6</sub> or (TAAA)<sub>8</sub> variants induced a significant decrease of the luciferase activity compared to the (TAAA)<sub>9</sub> allele (Figure 4A, right panel). To confirm the transient transfection results, RT-qPCR assays were performed from HVP and HeLa cells transfected with pcDNA3.1 expression vectors containing the (TAAA)<sub>n</sub> repeat and the *CFTR* cDNA. Results confirmed the repression of the *CFTR* transcripts level in HVP and HeLa cells although the data are less consistent (Figure 4B). Because the transient transfection results are not different in HVP and HeLa cells, further experiments were then performed only in HVP cells. Constructions with (TAAA)<sub>9</sub>, (TAAA)<sub>8</sub> or (TAAA)<sub>6</sub> allele located downstream the *luciferase* gene have also been tested as previously reported<sup>36</sup> to verify whether the orientation of the (TAAA)<sub>n</sub> repeats influences the *CFTR* expression. The results also indicated a decrease in luciferase activity similar to [pGL3C-(TAAA)<sub>n</sub>] constructs (data not shown). Similar results were also observed with constructs containing the minimal *CFTR* promoter instead of the SV-40 promoter with the motif repeat upstream the promoter or downstream the luciferase gene (data not shown). Because tissues from patients are inaccessible, we used artificial vectors even if the constructions differ from the *CFTR* gene organization, in order to evaluate the impact of the (TAAA)<sub>n</sub> repeat on the *CFTR* transcription. Our results showed that the (TAAA)<sub>8</sub> and (TAAA)<sub>6</sub> intronic variants significantly decrease the *CFTR* transcription suggesting the presence of a silencer element in the intron 9 (TAAA)<sub>n</sub> repeats of the *CFTR* gene. The decrease was not dependent on the location of the (TAAA)<sub>n</sub> repeat. We next hypothesized that this intronic transcriptional repression might reflect altered binding of one or more regulatory factors located within the (TAAA) microsatellite.

*The (TAAA)<sub>n</sub> motif corresponds to DNA binding motif for FOX family factors*

*In silico* analyses predicted binding sites for FOX proteins overlapping the (TAAA)<sub>n</sub> repeat. For the same reason that previously underlined in this paper, we assessed the possibility that FOXI1 factor could also regulate the *CFTR* transcription through these sequences in HVP cells. Transient co-transfection assays results indicated that enforced expression of FOXI1 protein induced a significant inhibition of the luciferase activity in the context of the *CFTR* intron 9 (Figure 5A). Results of siRNA assays confirmed the inhibitory role of the FOXI1 factor. We next confirmed the role repressor of FOXI1 at the *CFTR* mRNA level (Figure 5B).

To judge the FOXI1 binding ability on the (TAAA)<sub>n</sub> repeats, qChIP assays were performed using HVP cells stably transfected with (TAAA)<sub>9</sub>, (TAAA)<sub>8</sub>, or (TAAA)<sub>6</sub> allele. Quantitative PCR realized with specific primers for a 350 bp-region encompassing the (TAAA)<sub>n</sub> repeat, revealed that the FOXI1 proteins bind to these intronic sequences (Figure 5C). Interestingly, we observed an increasing binding activity associated with the loss of (TAAA) repeats. Although our findings evidenced that the FOXI1 transcription factor negatively influenced the *CFTR* transcription, this attenuation seems to be independent of the loss of (TAAA) repeats suggesting that the *CFTR* repression is mediated by another unknown molecular mechanism.

## **Discussion**

Because polymorphisms in the promoter region may modulate gene transcription by interacting with *trans*-acting elements, we set out functional analyses including *in silico* analyses, promoter reporter assays, and ChIP experiments, to explore the role of the -33G>A nucleotide change in promoter activity.

We demonstrated that the -33A allele inhibits significantly the *CFTR* promoter activity

and alters the binding of the FOXI1 *trans*-acting factor and that the enforced FOXI1 protein expression negatively modulates the *CFTR* transcription. As FOXI1 proteins are here described as repressors for the first time, we confirmed our data by using reporter vectors containing promoter sequences of genes known to be positively transactivated by FOXI1.<sup>30 32</sup> Interestingly, the inhibitory effect of FOXI1 is stronger on the -33A allele than on the wild-type allele. Our findings suggest the contribution of FOXI1 in the physiopathology of CBAVD disorder through alteration of the *CFTR* promoter activity. The winged helix/forkhead box (FOX) gene family encodes a large class of nuclear proteins, characterized by a highly conserved forkhead DNA-binding domain.<sup>37</sup> The FOX proteins are involved in a wide range of developmental processes through regulation of tissue-specific expression of target genes during morphogenesis and differentiation.<sup>38 39</sup> Among the FOX transcription factors, recent attention have been drawn to FOXI1 (also known as HFH3, Fkh10 and FREAC6). Although FOXI1 was originally identified as a kidney-expressed forkhead gene<sup>40-42</sup>, recent data indicated its pivotal regulatory role for normal sperm function.<sup>30 31</sup> Blomqvist and collaborators showed that mice lacking *FOXI1* fail to generate offspring. FOXI1 has been reported to function as a regulator of genes, important for the epididymal dependent maturation and storage of spermatozoa.<sup>30</sup>

In parallel, we provided experimental evidences showing that the (TAAA)<sub>n</sub> repeat sequence within the *CFTR* intron 9 has a functional role in the *CFTR* transcriptional regulation by using gene reporter assays, confirmed by *CFTR* mRNA expression. Microsatellites have been described to alter the activity of genes when located in the promoter<sup>15 34</sup> or in introns.<sup>16 33</sup> In addition, Phylactides *and coll.* have previously reported that *cis*-acting elements located deep within *CFTR* introns promote the *CFTR* expression.<sup>43</sup> To determine the molecular mechanism that might explain the transcriptional impact of the STR, we conducted *in silico* analyses and functional assays. The results of *in silico* analyses



revealed that the deletion of 1 or 3 (TAAA) repeats alter the putative binding for the tissue-specific FOX factors family. Using over-expression and RNA interference assays, we demonstrated that the FOXI1 factor inhibits the *CFTR* transcription, with similar extent in both wild-type and mutated context. However, ChIP analyses showed that the (TAAA) repeat shortening positively affects the affinity of FOXI1 transcription factor.

The role of the (TAAA)<sub>n</sub> alterations in regulating the *CFTR* transcription remains enigmatic. As the loss of (TAAA) repeat increased the ability of the FOXI1 binding and that enforced FOXI1 expression did not alter the *CFTR* promoter activity, a possible explanation is that microsatellite variation might form an alternative DNA structure that may affect gene transcription. FOX transcription factors regulate gene expression in virtue of their ability to bend or loop DNA, thereby facilitating communication between *cis*-element and *trans*-acting proteins.<sup>44</sup> Interestingly, FOXI1 is capable of remodelling chromatin structure and to maintain its DNA interactions in condensed chromatin.<sup>45</sup> In addition, the presence of the -33A nucleotide putatively creates another regulatory site through reinforcing the FOXI1 DNA binding that might affect the *CFTR* gene activity, with a markedly effect when both mutant alleles [-33A and (TAAA)<sub>6</sub>] are considered. Recent investigations demonstrated that the *CFTR* gene regulation is modulated by interactions between promoter and intronic sequences.<sup>46</sup>

To conclude, this work suggested that the -33G>A promoter variant and (TAAA)<sub>6</sub> and (TAAA)<sub>8</sub> repeats alter the *CFTR* transcription. Because familial segregation was not possible, we cannot rule out the possibility of the presence of another deep intronic mutations. In addition, as a relatively minor disruption of *CFTR* function appears to cause CBAVD and that two mild mutations are sufficient to induce a CBAVD phenotype, we propose that the combination of the -33A and the (TAAA)<sub>6</sub> allele perturbs an existing DNA loop (*in cis*) or the chromatin opening (*in trans*), and that the resulting impairment of the *CFTR* transcription

could be associated with the CBAVD phenotype.

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### **Conflict of Interest Statement**

None declared

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## Legends

*Figure 1. Analysis of allele-specific transcriptional effects of the -33G>A CFTR promoter sequence variation.*

(A) Transcriptional activities of the -33G and -33A alleles in HVP and Hela epithelial cell lines. Bars represent *Firefly/Renilla* luciferase ratios for the different constructs. Luciferase activity obtained from the pGL3-Basic plasmid was defined as 100%, and relative luciferase activities from both the wild-type construct (pGL3B-WT) and the mutant construct (pGL3B-33A), were expressed from this value. The asterisk (\*) indicates that the value is statistically significant ( $p < 0.05$ ) compared to the wild-type construct.

(B) RT-qPCR analysis of the -33G and -33A alleles in HVP and Hela epithelial cell lines. Cells transfected with either the pcDNA3.1-WT or the pcDNA3.1-33A constructs are harvested for RT-qPCR assays as described in Materials and Methods section. The expression level of the wild-type construct was then set as 100% for comparison with the mutant construct. The asterisk (\*) indicates that the value is statistically significant ( $p < 0.05$ ) compared to the wild-type construct.

*Figure 2. FOXI1 binds to the minimal CFTR promoter and inhibits the CFTR promoter activity in HVP cell line.*

(A) ChIP analysis of the FOXI1 binding to the *CFTR* minimal promoter. DNA from HVP cell line was immunoprecipitated with either an anti-FOXI1 or an irrelevant (NS) antibody. Classical PCRs were from both input (IN) and immunoprecipitate DNAs (IP FOXI1 and NS IP). The *Caspase 8* gene was amplified as negative control.

(B) Extracts from stable HVP cells expressing the pGL3B-WT or pGL3B-33A plasmid were immunoprecipitated with either an anti-FOXI1 or an irrelevant antibody. qPCR were performed from both input and immunoprecipitated DNAs (IP FOXI1 and IgG). The amplifications obtained from the FOXI1-immunoprecipitated DNA and the IgG were expressed from the value obtained from the input DNA. (\*  $p < 0.05$ ).

(C) Effect of FOXI1 on the *CFTR* -33G>A transcriptional activity. *Grey bars* show luciferase activities from HVP co-transfected with the FOXI1 expression vector. *White bars* show luciferase activities from HVP co-transfected with the FOXI-specific siRNA. Luciferase activity obtained from pGL3B-WT construct containing the wild-type allele (*black bars*) was defined as 100%. Other relative luciferase activities were expressed from this value. (\*  $p < 0.05$ ).

(D) FOXI1 protein expression levels. Immunoblots show either the FOXI1 or  $\beta$ -actin protein expression after either over-expression or knockdown (siRNA) of the FOXI1 protein. Densitometric analyses (DA) were performed as described in Materials and Methods section.

(E) Effect of FOXI1 on the *CFTR* -33G>A at the mRNA level. HVP cells were transfected with the pcDNA3.1-WT or the pcDNA3.1-33A constructs along with either the FOXI1 expression vector or the FOXI1-specific siRNA. (\*  $p < 0.05$ ).

(F) Effect of FOXI1 on the *AE4* transcriptional activity. Luciferase activities from HVP co-transfected with *AE4* promoter reporter constructs (*AE4-promH1* and *AE4-prom H3*) along

with either the FOXI1 expression vector or the FOXI-specific siRNA are shown. Luciferase activities obtained from AE4-promH1 and AE4-prom H3 constructs was defined as 100%. Other relative luciferase activities were expressed from this value. (\*  $p < 0.05$ ).

*Figure 3. Effect of deletion of (TAAA) motif repeats on the CFTR mRNA splicing.*

(A) Schematic representation of the minigene construct containing the *CFTR* exon 9 (with 139 bp of the intron 8 and 128 bp of the intron 9) and exon 10 (with 1842 bp of the intron 9 containing (TAAA)<sub>n</sub> repeats and 190 bp of the intron 10) . SD6 and SA2 primers were used to analyse the hybrid minigene expression.

(B) RT-PCR products generated from the splicing assay. For minigene containing either the (TAAA)<sub>9</sub>, the (TAAA)<sub>8</sub> or the (TAAA)<sub>6</sub>, only one product is observed on the agarose gel electrophoresis, a 638 bp corresponding to the RNA transcripts containing the *CFTR* exon 9 and 10. For the minigenes containing the mutated IVS8 polyT tract (T5 or T3), used as positive controls, an additional product is seen, the 455 bp band corresponding to the RNA transcript lacking *CFTR* exon 9. MW is the molecular weight marker. Schemas corresponding to the different RNA transcripts amplified are represented in the right panel.

*Figure 4. Analysis of allele-specific transcriptional effects of the (TAAA)<sub>n</sub> variants.*

(A) Transcriptional activities of the (TAAA)<sub>9</sub>, (TAAA)<sub>8</sub> and (TAAA)<sub>6</sub> alleles in HVP and Hela epithelial cell lines. Bars represent *Firefly/Renilla* luciferase ratios for the different constructs. Luciferase activity obtained from the pGL3-Control plasmid was defined as 100%, and relative luciferase activities from the constructs [pGL3C-(TAAA)<sub>9</sub>, pGL3C-(TAAA)<sub>8</sub> and pGL3C-(TAAA)<sub>6</sub>] were expressed from this value. The asterisk (\*) indicates that the value is statistically significant ( $p < 0.05$ ) compared to the pGL3C-(TAAA)<sub>9</sub> construct.

(B) RT-qPCR analysis of the (TAAA)<sub>n</sub> alleles. HVP and Hela cells were transfected with the pcDNA3.1-(TAAA)<sub>9</sub>, the pcDNA3.1-(TAAA)<sub>8</sub> or the pcDNA3.1-(TAAA)<sub>6</sub> constructs and the level of the *CFTR* mRNAs was measured. The expression level of the wild-type construct was then set as 100% for comparison with the mutant construct. (\* p < 0.05).

*Figure 5. FOXI1 inhibits the (TAAA)<sub>n</sub> transcriptional activity in HVP cell line.*

(A) Effect of FOXI1 on the *CFTR* (TAAA)<sub>n</sub> transcriptional activity. Grey bars show luciferase activities from HVP co-transfected with the FOXI1 expression vector. White bars show luciferase activities from HVP co-transfected with the FOXI-specific siRNA. Luciferase activity obtained from pGL3C-(TAAA)<sub>9</sub> construct (black bars) was defined as 100%. Other relative luciferase activities were expressed from this value. (\* p < 0.05).

(B) Effect of FOXI1 on the *CFTR* (TAAA)<sub>n</sub> alteration at the mRNA level. HVP cells were transfected with the pcDNA3.1-(TAAA)<sub>9</sub>, the pcDNA3.1-(TAAA)<sub>8</sub> or the pcDNA3.1-(TAAA)<sub>6</sub> constructs along with the FOXI1 expression vector. The *CFTR* mRNA values were normalized to the housekeeping *GAPDH* gene expression and expressed relative to the mRNA expression level of the wild-type construct (100%). (\* p < 0.05).

(C) ChIP analysis of FOXI1 binding to the *CFTR* intron 9. Extracts from stable HVP cells expressing the pGL3C-(TAAA)<sub>9</sub>, pGL3C-(TAAA)<sub>8</sub> or pGL3C-(TAAA)<sub>6</sub> plasmid were immunoprecipitated with either an anti-FOXI1 or a non-specific antibody. qPCR assays were performed from both input and immunoprecipitated DNAs (IP FOXI1 and IgG) as described in Materials and Method section. The amplifications obtained from the FOXI1-immunoprecipitated DNA and the IgG were expressed from the value obtained from the input DNA. (\* p < 0.05).

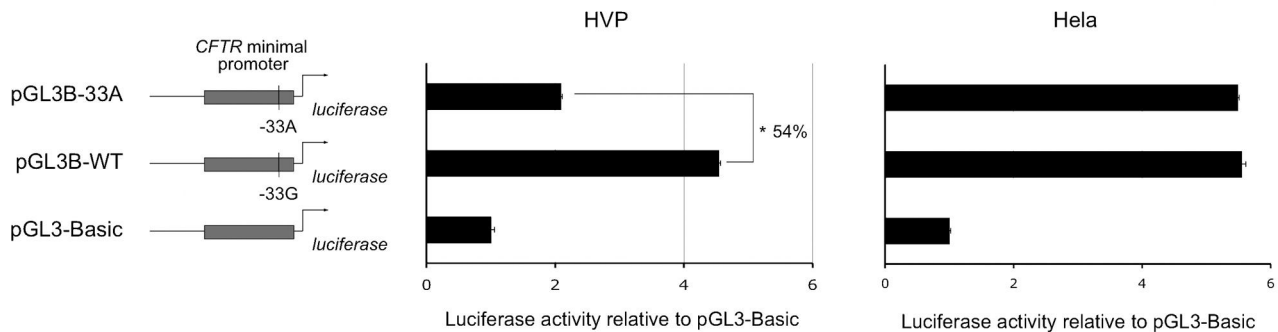
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Figure 1

A



B

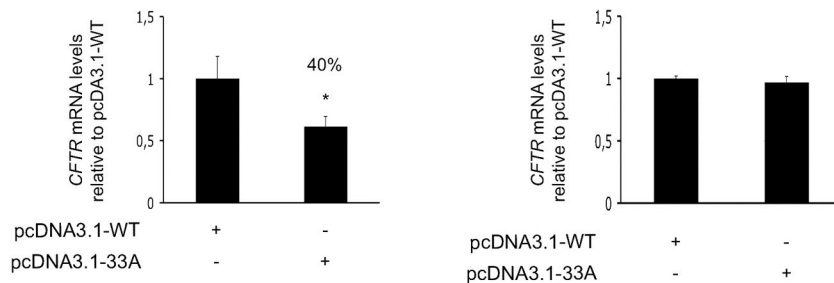


Figure 2

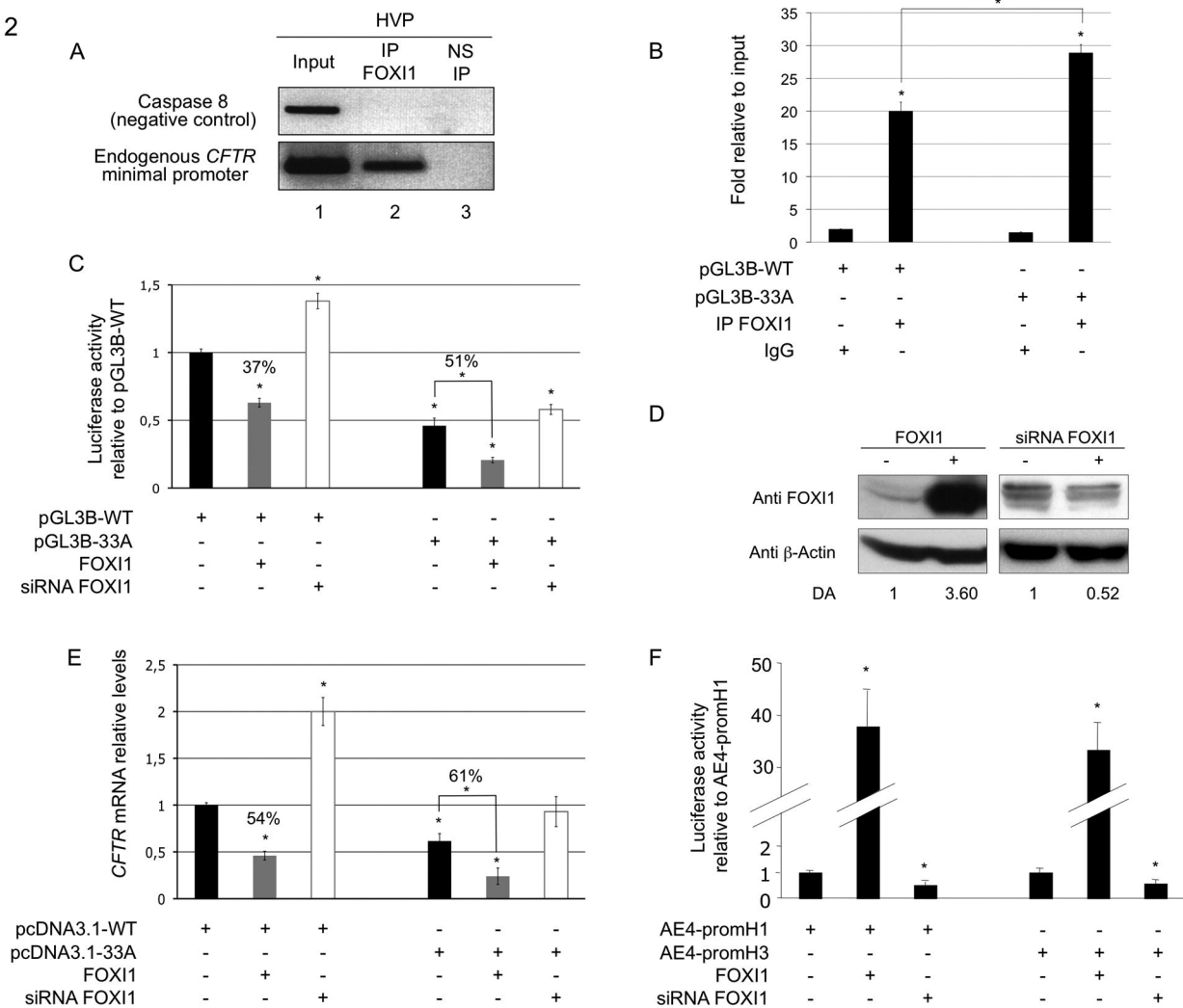
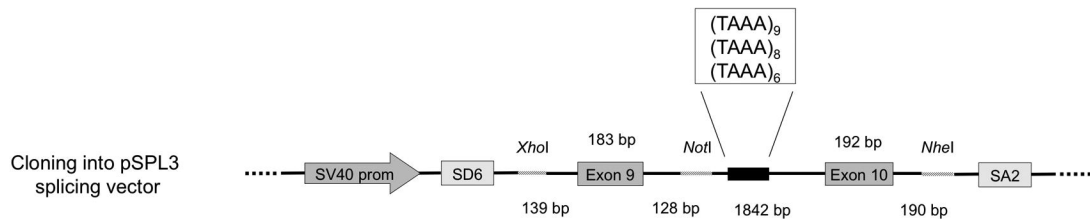


Figure 3

A



B

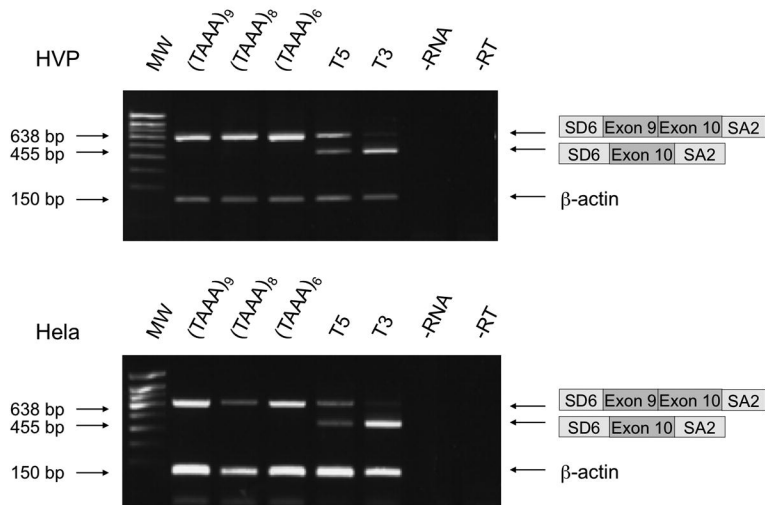
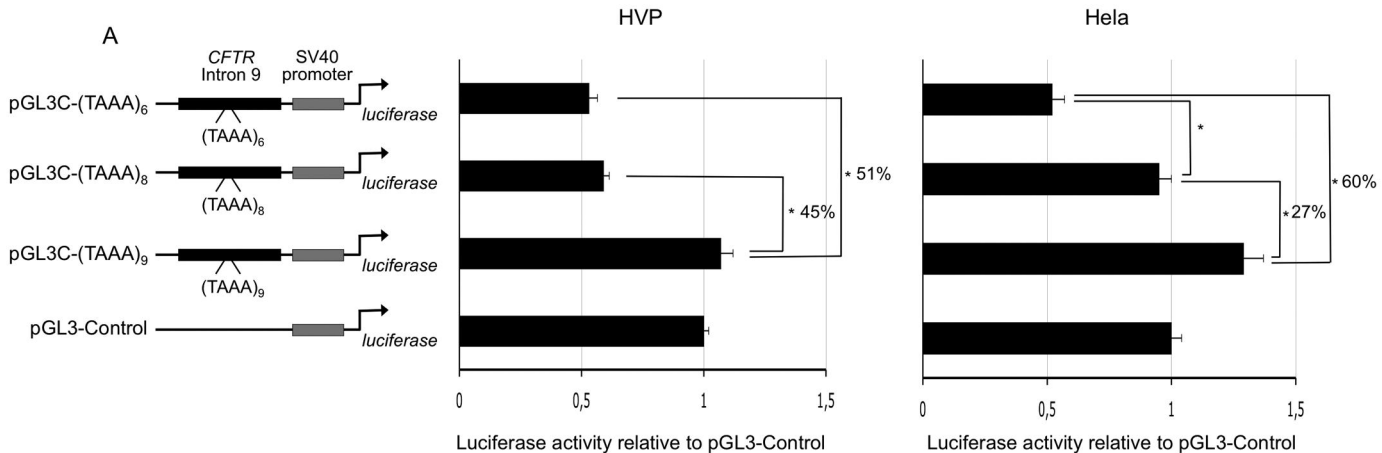


Figure 4



**B**

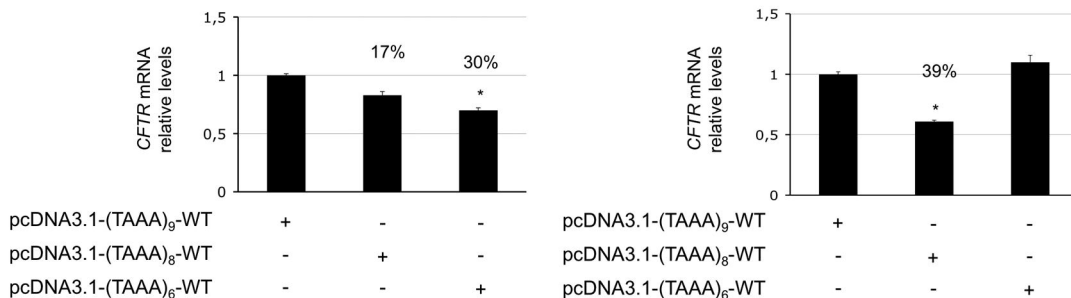
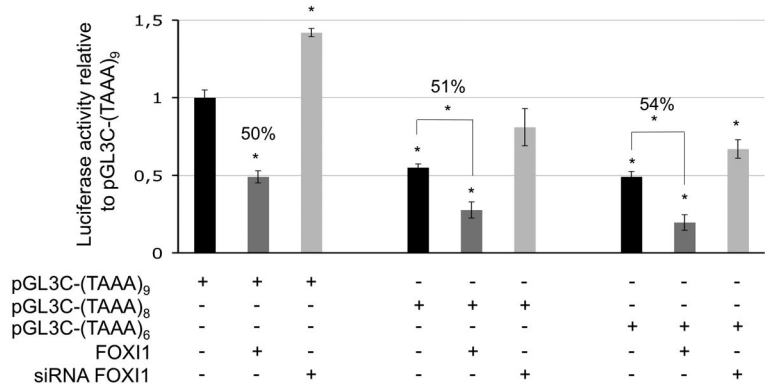
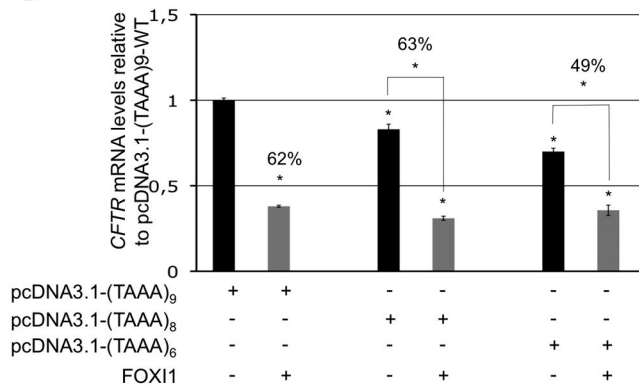


Figure 5

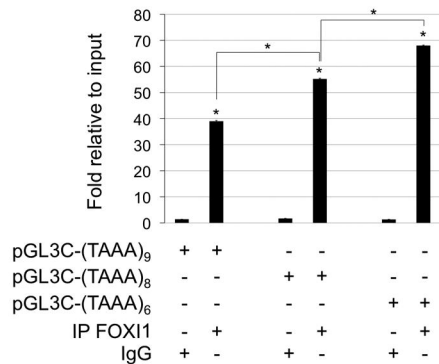
A



B



C



**Table**

**Table 1: (TAAA) allele frequencies in control, CF and CBAVD subjects**

Number of (TAAA) repeats	% control	% CBAVD patients		% CF patients
	n=254	0 or 1 identified mutation n=86	Mutations: p.Phe508del/ TG12T5 n=56	Mutations: p.Phe508del/ p.Phe508del n=66
13	2.75	1.20	0	0
12	2.36	1.20	0	0
11	8.66	10.46	50	94
10	11.40	9.30	3.60	0
9	74.80	75.60	46.40	6
8	0	1.20	0	0
6	0	1.20	0	0

'n' the total number of chromosomes