A MUC5B polymorphism associated with Idiopathic Pulmonary Fibrosis mediates overexpression through decreased CpG methylation and C/EBPβ transcriptional activation

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Short title: CEBPβ and CpG methylation regulate *MUC5B* in idiopathic pulmonary fibrosis

Abstract

Idiopathic pulmonary fibrosis is a progressive and fatal lung disease of unknown aetiology. The strongest genetic risk factor associated with IPF development is a *MUC5B* promoter polymorphism (*rs35705950*). However, the mechanism underlying its effects remains unknown. In this study we have focused on the molecular consequences of the polymorphism on the regulation of MUC5B expression. We have identified a combined mechanism involving both methylation and direct transcriptional regulation mediated by the polymorphic variant on MUC5B overexpression. Our results demonstrate that the minor allele (T) associated with *rs35705950* disturbs a DNA methylation site, directly increasing MUC5B expression. Furthermore, this same variant also creates a novel binding site for the transcription factor C/EBPβ leading to transcriptional activation of *MUC5B*. Our findings provide a novel insight into the regulatory effects of the IPF risk allele, *rs35705950* and identifies C/EBPβ as an important regulatory factor in the development of IPF.

Keywords

CEBPB, Idiopathic pulmonary fibrosis, methylation, MUC5B, *rs35705950* polymorphism

Introduction

Idiopathic pulmonary fibrosis (IPF) is an irreversible interstitial lung disease characterized by a progressive scarring of lung parenchyma often leading to a fatally declining lung function. IPF incidence has been estimated to be around 75/100,000, affecting 5 million people world-wide [1-3]. Furthermore, IPF is believed to be an under diagnosed disease [4-6].

Due to the complexity and progressive nature of the disease, available treatments are limited and have only a modest impact on IPF progression. Until recently, only lung transplant has been proved to increase survival [1]. In recent years, improvement in development of new therapies has met some success, as two novel drugs have been introduced, pirfenidone and nintedanib. Both drugs are believed to target profibrotic signalling pathways in IPF. Pirfenidone inhibits TGF-β1 production possibly by inhibiting the upregulation of HSP47 and Col1 RNA in fibroblasts [7]. Nintedanib is a potent small-molecule receptor tyrosine kinase inhibitor targeting platelet-derived growth factor receptors *(PDGF-R)*, fibroblast growth factor receptors *(FGFR)* and vascular endothelial growth factor *(VEGF)* -family tyrosine kinase receptors [8].

Both genetic and environmental factors are believed to contribute to the onset and progression of the disease. Several environmental factors have been associated with IPF, including exposure to metal and wood dust [9-12], viruses [13-15], drugs [16-18], and cigarette smoke [19-21]. Recent findings point to genetic factors as major triggers of IPF. Rare mutations found in 6 genes (*TERT* [22, 23], *TERC* [22, 23], *RTEL1* [24, 25], *PARN* [25], *STPC* [26, 27] and *SFTPA2* [28]), with associated variants in 11 different loci [29] indicate

that the telomerase pathway and surfactant protein genes play a key role in IPF pathogenesis. However, these mutations only explain a small proportion of IPF cases.

Two large genome-wide association studies (GWAS) have been conducted on pulmonary fibrosis (familial and sporadic) [30, 31]. Both studies showed the most important genetic risk to be conferred by a common G-to-T risk variant in the upstream region of *MUC5B* (*rs35705950*). The frequency of the risk allele (T) is ~35% among European ancestry cases, compared with ~9% of European ancestry controls [32, 33]. These studies furthermore identified other common variants, including three polymorphisms in the *TOLLIP* gene and a desmoplakin (*DSP*) intron variant [31]. These studies concluded that the genome-wide variants account for 30-35% of the IPF risk, suggesting an important role for these common genetic variations in the disease aetiology [33].

Deciphering the molecular and cellular effects of non-coding SNP´s has proven to be notoriously difficult. With the thousands of common SNPs associated with many common diseases only a few have led to a clear molecular understanding [34-36]. There are many possible explanations for this. Most often there is a complex network of SNPs in varying degrees of linkage disequilibrium all associated with the phenotype or disease [36]. Resolving the true informative SNP within this complex network of variants can be challenging. Another explanation for the difficulty is connecting a non-coding variant to a target gene or regulatory region mediating the effects of the genetic association. Even though the closest gene may be a good candidate, the effects can be on genes further away. This can sometimes be resolved through expression-quantitative trait loci (eQTL), i.e. if the sequence variant influences the expression level of one or more genes.

The *MUC5B* promoter variant *rs35705950* is the strongest known risk factor (genetic and otherwise) for the development of IPF [32, 33]. This variant is unusual as it is relatively simple, with no other variants in strong linkage disequilibrium and carries a strong eQTL association with MUC5B expression in lung. The odds ratio (OR) associated with carrying one allele of the variant is 4.5-6.6, while homozygosity leads to an OR of 9.6-20.2 [30, 31, 37-41]. Furthermore, carriers of the *MUC5B* variant are also at risk of getting subclinical interstitial lung disease based on screening with high-resolution CT [4]. Interestingly, recent studies have examined the relevance of *MUC5B* variation in other ethnic groups, showing that the frequency of *rs35705950* minor allele (a G-to-T SNP) is 11, 8 and 1% among European, South Asian and East Asian populations, respectively [38, 42, 43], and it is almost non-existent in Africans [44]. Despite the different prevalence, the evidence indicates that the risk of developing IPF associated with the variant is comparable to the risk observed in European population [4, 31].

MUC5B (Mucin 5B) is a highly glycosylated protein, expressed throughout the upper and lower respiratory tract. In human upper airways MUC5B is predominantly expressed in nasal and oral gland secretions, while in tracheobronchial conducting airways it is expressed in surface epithelium and submucosal glands, where it is less predominant than the related MUC5AC in surface epithelia. In bronchioles MUC5B predominates, while MUC5AC is less expressed. Under disease conditions, the MUC5B expression pattern changes dramatically, it becomes overexpressed, showing significantly higher expression than MUC5AC [1, 45, 46]. The pattern suggests an important role for MUC5B as a host defence barrier. The G-to-T *rs35705950* variant is in a presumed regulatory region 3 kb upstream of the transcription start site in the *MUC5B* gene on chromosome *11p15*. A DNase I hypersensitivity site overlaps the variant location suggesting an

105 important regulatory role. Furthermore, the *MUC5B* variant lies within a cluster of CpG
106 sites and CpG DNA methylation has been shown to affect *MUC5B* expression [47].

107 Even though the *MUC5B* variant has a strong and consistent genetic association with IPF
108 and the variant seems to positively regulate MUC5B expression, the mechanism
109 underlying its role in IPF is poorly understood. It has been hypothesized that, due to the
110 large size of the MUC5B protein, its production may carry a significant metabolic stress,
111 which can interfere with differentiation of airway stem cells [33]. It should also be
112 emphasized that due to the increased expression level there could also be secondary
113 problems with post-translational modifications, such as glycosylation. In a mouse model
114 of intestinal inflammation aberrant mucin assembly has been shown to cause ER-stress
115 through activation of the unfolded protein response leading to inflammation, apoptosis,
116 and wound repair [48]. ER-stress has been shown to be involved in IPF, specifically in
117 cases caused by mutations in surfactant proteins, another protein that is highly
118 glycosylated [49]. Other proposed mechanisms consider the possibility that IPF is a
119 mucociliary disease caused by recurrent injury/inflammation/repair at the
120 bronchoalveolar junction, as MUC5B overexpression might cause a reduction in
121 mucociliary function and retention of particles leading to lung injury [33].

122 In this study, we aim to define the molecular mechanisms involved in mediating the
123 effects of the *MUC5B* variant (T allele) on *MUC5B* expression. We use two immortalized
124 human bronchial epithelial cell lines (BCi_NS1.1 and VA-10) and the well-established
125 adenocarcinoma cell line A549 to corroborate the positive effects of the variant on
126 MUC5B expression. With our models, we show that the T allele is associated with a
127 twofold mechanism affecting *MUC5B* expression, leading to both the disruption of a

DNA methylation site that directly increases *MUC5B* expression and the creation of a novel C/EBPβ transcription factor binding site that also positively regulates *MUC5B* expression.

Material and methods

Allele-specific match for DNA binding proteins

Motifs for DNA binding proteins were retrived through MotifDB, a bioconductor package for R, wherein we made use of the Catalog of Inferred Sequence Binding Preferences (CIS-BP) database containing information on sequence specificities for 313 human DNA binding proteins [50]. We then used matchPWM implemented in Biostrings for R to calculate PWM (position weight matrix) scores for DNA sequences with and without the *rs35705950* minor allele. P-values were assessed by scoring each of the 313 *CIS*-BP motifs on 100 thousand DNA sequences randomly selected from regulatory regions in the human genome (ChromHMM 25-state regions assigned as promoter, tss, or enhancer). We then calculate two P-values for each motif as the area under the null-curve above the observed score for the corresponding motif match against the minor and major alleles, respectively. P-values less than 0.01 were then considered as ´candidate binding sites´ and, as an indicator of biological relevance, we considered only sites wherein the *rs35705950* minor allele alters an important nucleotide in the matching of the motifs to the DNA sequence defined here as nucleotides accounting for a mininum of 0.70 (or 70%) at a given position in the probability position matrices.

Cell culture

The BCi_NS1.1 cell line is a human bronchial epithelial cell line kindly provided by Dr. Matthew S. Walters, Weill Cornell Medical College, New York NY, USA [51]. It was established by immortalization with retrovirus expressing human telomerase (hTERT). The bronchial epithelial cell line VA-10 was previously established by retroviral transduction of primary bronchial epithelial cells with E6 and E7 viral oncogenes [52]. Both cell lines were cultured in bronchial epithelial growth medium, BEGM (Lonza, Walkersville, MD) supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin (Gibco, Burlington, Canada).

The human lung adenocarcinoma derived alveolar epithelial cell line A549 (American Type Culture Collection, Rockville MA) was cultured in DMEM-Ham's-F12 basal medium supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin (Gibco). All cell lines were cultured at 37°C, 5% CO_2. All three cell lines are WT (homozygous G-allele) at *rs35705950*.

Air-Liquid interface culture

To establish an air-liquid interface cultures (ALI), cells were seeded on the upper layer of Transwell cell culture inserts (Corning®Costar®) pore size 0.4 μm, 12 mm diameter, polyester membrane) (Sigma-Aldrich, St. Louis, USA) at density of 2×10^5 cells per well. The cultures were maintained on chemically defined bronchial epithelial cell medium (BEGM, Cell Applications, San Diego) for 5 days, 0,5 ml in the upper chamber and 1.5 ml in the lower chamber. After 5 days, medium was changed to DMEM/F-12 (Invitrogen), supplemented with 2% UltroserG (Cergy-Saint-Christophe, France) for additional 5 days. For ALI culture, the medium was aspired from the apical side and the cell layer rinsed 1x with PBS.

Goblet cell differentiation by IL-13 treatment

Cells were cultured for 5 days on BEGM and then for 5 days on DMEM/UG in a submerged culture. After 5 days of ALI culture, IL-13 (Peprotech, London, UK) was added to the basal side to a final concentration of 25 ng/ml and cultured for 14 days.

Immunofluorescence staining

Cells were rinsed twice with chilled PBS. The fixation of cells was performed using 100% methanol at -20°C overnight. Subsequently, cells were submerged in 100% acetone for one minute. Staining was performed using immunofluorescence buffer, (IMF) (0.1% TX-100, 0.15M NaCL, 5nM EDTA, 20mM HEPRES, pH 7.5, 0.02% NaN3 as preservative). Cells were incubated with primary antibody overnight at +4°C, and then rinsed three times for 15 min with IMF buffer. Cells were then incubated with a secondary antibody and DAPI for two hours at room temperature, followed by four times washing with IMF buffer. Cells were mounted using ProLong Antifade (Thermo Fisher Scientific). Antibodies used for these experiments are listed in S1 Table.

DAB staining

Paraffin-embedded tissue samples of control and IPF lung biopsies were obtained from the Department of Pathology, Landspitali University Hospital. The samples were deparaffinized, antigen retrieved by boiling in TE buffer for 20 minutes and stained with EnVisionH+ System-HRP kit (Dako) according to the manufacturer's instructions. Primary antibodies were incubated at RT for 30 min. The immunofluorescence staining of the paraffin embedded samples (IF-P) were done as stated above with the following modifications: After antigen retrieved, samples were rinse in PBS. Primary antibody was

194 incubated overnight. Samples were washed in PBS prior the incubation with secondary
195 antibody and DAPI for 2 hours.

196 Antibodies used for these assays are listed in S1 Table. Immunofluorescence was
197 visualized and captured using laser scanning Fluoview® FV1200 Confocal Microscope
198 (Olympus Life Science).

199 **Transient transfection**

200 Cells were grown at 70% confluence one day before transfection. FuGENE® HD
201 Transfection Reagent (Promega) was used on BCi_NS1.1 and VA10 cells, while
202 Lipofectamine (Thermo Fisher Scientific) was used on A549 cells. All transfection was
203 performed following manufacturer's instructions. The results were analysed 48h after
204 transfection. Plasmids used for C/EBPβ overexpression was generously donated by Joan
205 Massague: C/EBPβ LAP isoform (addgene #15738) and C/EBPβ LIP (addgene #15737)
206 [53]. Plasmids used for this assay are listed in S2 Table.

207 **Production of lentiviral and cell transduction**

208 To produce lentiviral cell lines containing pGreenFire1™ Pathway Reporter lentivector
209 (Cat#TR010PA-N and Cat#TR000PA-1, System Bioscience) expressing the *MUC5B*
210 promoter region and controls, we followed the general guideline provided by System
211 Bioscience. Briefly, 70% confluent HEK-293T cells were cultured for 24 h w/o antibiotics
212 and transfected (Lipofectamine, Thermo Fisher Scientific) with lentiviral transfection
213 constructs and packaging plasmids (psPAX2 and pMD2.G) (Addgene plasmids #12260
214 and #12259, respectively). Culture medium containing the virus was harvested 24 and
215 48 hours post transfection and centrifuged at 1250 rpm at 4°C for 5 minutes and filtered
216 through 0,45 μm filter. Lentiviral particle solution was added to culture medium

(containing 8 μg/ml polybrene) and then added to culture flasks of 70% confluent cells (BCi_NS1.1, VA10 and A549) at a low multiplicity of infection (MOI) and incubated for 20 hours. cells were then cultured further for 24 hours in fresh culture media. Infected cells were then selected with puromycin or neomycin as appropriate for 48hs. Plasmids used for this experiment are listed in S2 Table.

Luciferase Assay

Each cell type was seeded at 70% confluence one day before transfection in a 96 well plate. To perform the luciferase assay, the Dual-Glo® Luciferase Assay System kit supplied by Promega was used, following the general guidelines provided with the kit. Luminosity was measured in a microplate reader Modulus™ II (Turner BioSystem). Luciferase measurement was normalized using Renilla co-transfection. Plasmids used for this experiment are listed in S2 Table.

Real Time qPCR

RNA was isolated using Tri-Reagent® solution (Ambion) and cDNA preparation was carried out using RevertAid™ First strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. Real-time PCR using Power SYBR Green PCR Master mix (Applied Biosystems) was used to detect the relative quantity of each cDNA. *GAPDH* was used as the endogenous reference gene. Data were analysed using 7500 Software v2.0 (Applied Biosystems). All primers used are listed in S3 Table.

Western Blot

Protein lysates were acquired using RIPA buffer supplemented with phosphatase and protease inhibitor cocktails (Life Technologies). For western blots, 5μg of protein was

used per lane, unless otherwise stated. Samples were denatured using Laemli buffer, 10% β-mercaptoethanol at 95°C for 5 min and run on NuPage 10% Bis-Tris gels (Life Technologies) in 2-(N-morpholino) ethanesulfonic acid (MES) running buffer. Samples were then transferred to Immobilon FL PVDF membranes (Millipore). Membranes were blocked in Li-cor blocking buffer and primary antibodies were incubated overnight at 4 °C. Near-infrared fluorescence visualization was measured using Odyssey CLx scanner (Li-Cor, Cambridge, UK). Antibodies used are detailed in S1 Table.

In Vitro Methylation Assay

DNA fragments were cut out of the pGL3-*MUC5B*pr vector using XbaI-EcoRI restriction enzymes, generating 4.1Kb of the *MUC5B* promoter region, with WT or T allele. Fragments were gel-purified using GeneJET PCR Purification Kit (ThermoFisher Scientific) following the manufacturer's instructions and subsequently methylated with M.SssI methyltransferase (New England Biolabs) overnight at 37°C. The methylated fragments were then ligated into the pGL3 basic vector. DNA concentration were measured at 260nm before being used in transfection as described by *Vincent et al* [47]. Differential influence of methylation in the 4.1kb *MUC5B* promoter was measured by luciferase activity in three individual experiment performed in triplicates for each transfected cell line. All plasmids used are listed in S2 Table.

Bisulfite sequencing

$1x10^5$ cells were used to extract DNA from each cell line. Extraction was performed with PureLink Genomic DNA MiniKit (Invitrogen) following the manufactures instruction. Bisulfite conversion of DNA was performed with EZ DNA Methylation-Gold™ Kit (ZymoPURE™, Germany). Amplification of the region of interest was done with the

EpiMark® Hot Start Taq DNA Polymerase (New England Biolab, UK) and the resulting product was sequenced by Sanger sequencing (Beckman Coulters Genomics, GENEWIZ, UK.) DNA methylation analyses of bisulfite PCR amplicons were performed using Sequence scanner V1.0. DNA methylation level was scored as percentage methylation of individual CpG units in each sample. Primers used for bisulphate sequencing are listed in S3 Table.

5-aza-2'-deoxycytidine DNA Methylation Inhibition

After 10 days of ALI culture, 5-aza-2'-deoxycytidine (Peprotech, London, UK) was added to basal side to a final concentration of 10μM and the cells further cultured for 3 days before being analysed. DMSO was used as a control.

siRNA transfection

Small-interfering RNAs (siRNA) targeting human C/EBPβ were purchased from Sigma Aldrich (St Louis, MO, USA). siRNA ID: SASI_Hs02_00339146, SASI_Hs01_00236023, SASI_Hs02_00339148, SASI_Hs01_00339149, 10nM each. siRNA transfection was performed following manufactures instructions. MISSION siRNA Universal Negative Control (Sigma Aldrich) was used as a control. Cells were seeded in 96 well plates for Luciferase Assay, or in a 12 well plate to be analysed by a RT-qPCR. siRNA was transfected using Lipofectamine 2000 reagent (Invitrogen) in OPTI-MEM medium (GIBCO). Twenty-four hours later, the transfected cells were transferred to complete medium. After 48h, the cells were harvested and used for luciferase measurement and RT-qPCR.

CRISPR

Three independent pairs of oligos targeting the *rs35705950* sequence was cloned separately into the MLM3636 guide RNA mammalian expression vector (Addgene # 43860) using the restriction enzyme BsmBI. As a control, 20 nucleotides long scrambled sgRNA was cloned into the same MLM3636 vector. gRNA vectors were corroborated by sanger sequencing. Cells were then seeded in a 12 well plate prior transfection (protocol stated above) of the spCas-9 expression vector (Addgene #44758), gRNA and homologous region and a non-homologous recombination inhibitor (Sigma-Aldrich), at 70% confluence. Selection was performed with Blasticidin. Single cell cloning assays were performed to select individual clones. Sequencing of individual clones was performed to corroborate the genotype (S3c Fig).

To generate the CRISPR pooled cells, BCi_NS1.1 and A549 cell lines selected using Blasticidin but prior single cell cloning (named pooled CRISPR cells) was used to analyze *MUC5B* expression. Sanger sequencing was done to corroborate the presence of positive edited cells in the CRISPR pool.

CRISPR control cell line was generated transfecting the spCas-9 expression vector, the scrambled gRNA and homologous region and the non-homologous recombination inhibitor. gRNA and repair template sequences are included in S3 Table.

Statistical Analysis

Data are presented as mean and s.d. (error bars) from number of at least 3 independent experiments. Statistical differences between samples were assessed with Student two – tailed T-test. P-values below 0.05 were considered significant (***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq .0.05$).

Results

Rs35705950 risk allele for IPF is associated with higher expression of MUC5B.

The common polymorphism *rs35705950*, where a G is replaced by a T nucleotide, has been shown in two GWAS studies to be correlated with a predisposition to develop IPF [30, 31]. The polymorphism is located in a mucin gene cluster, three kilobases upstream of the transcription start site of *MUC5B* on chromosome 11 (S1 Fig). The molecular mechanism explaining how this polymorphism affects the aetiology of IPF has not been elucidated, although both hetero- and homozygote carriers have been shown to express higher levels of MUC5B in the lung epithelium (S1 Fig, S2 Fig) [32, 54]. To study the direct effects of this polymorphism on *MUC5B* gene regulation, a 4.1 kb of the *MUC5B cis*-regulatory region was cloned into a luciferase lentiviral reporter vector and the G of the wild type allele was replaced with the risk associated T allele using site directed mutagenesis (S3 Fig).

The resulting lentiviral vectors containing the luciferase gene under the control of 4.1kb *MUC5B* upstream region containing the promoter and either the wild type or the T variant (*rs35705950*), were used to stably transduce two human bronchial-derived basal epithelial cell lines, BCi_NS1.1 and VA10, and a human lung adenocarcinoma derived alveolar epithelial cell line A549. After stable selection, the cell lines were cultured at confluency and luciferase reporter activity measured. As shown in Fig 1a the IPF risk associated T allele consistently increased the luciferase reporter signal in all three cell lines, with the effect being most pronounced in BCi_NS1.1 cells.

To directly asses the effect of the T allele in the endogenous *rs35705950* site, we used CRISPR/Cas9 genome editing. The bronchial basal cell lines did not survive single cell

cloning and thus we were only able to generate A549 edited cell lines. Five heterozygous A549^{CRISPR} clones (1-5) were generated, carrying the human [G/T] genotype (S4 Fig). The T allele had a very significant effect on the expression levels of both, MUC5B mRNA (Fig 1b) and protein (Fig 1c), showing a direct effect of the T allele on *MUC5B* expression. The above data support the previous findings [55] that the T allele confers an increased promoter/enhancer activity to the *MUC5B* upstream *cis*-regulatory sequence.

The *rs35705950* T allele disrupts a repressive CpG DNA methylation site

It has been previously reported that the methylation of several CpG islands in the 4.1kb promoter region of *MUC5B* affects the expression of the MUC5B protein [47]. Interestingly, the presence of the T allele disrupts a CpG site that was previously characterized as methylated in the wild type. We hypothesized that the effects of the T allele could be mediated, at least in part, through epigenetic regulation. To analyse the effect of DNA methylation on *MUC5B* expression we used an air-liquid interphase (ALI) culture of BCi_NS1.1 cells. Under these conditions the cells differentiate into both, goblet cells producing mucins and ciliated cells [56]. We treated these cultures with 5'aza2'-deoxycytidine (5'AZA2'), a DNA methylation inhibitor. 5'AZA2' treatment resulted in increased expression of *MUC5B* and *MUC5AC* indicating that DNA methylation either directly or indirectly impacts *MUC5B* expression (Fig 2a). To directly address the potential effects of DNA-methylation at the T allele, we used *M.SssI* CpG methyltransferase to *in vitro* methylate the 4.1 kb *MUC5B* promoter region. The promoter (with T allele or WT) was restriction cut from the plasmid, and the fragments was *in vitro* methylated using M.SssI CpG methyltransferase prior to ligating back into a luciferase reporter plasmid and transiently transfected into the three cell lines. We

353 compared the effects of the mutation with (Fig 2b, bottom) or without (Fig 2b, top)
354 direct *in vitro* methylation. In Fig 2b without *in vitro* methylation no difference is seen in
355 luciferase activity in VA10 or A549 cells between T allele and WT while after *in vitro*
356 methylation a significant increase is seen in the T allele carrying the luciferase plasmid
357 compared to WT in all three cell lines. This supports the idea that differential CpG
358 methylation of the T allele and wild type allele is at least partially involved in regulating
359 *MUC5B* expression.

360 Targeted bisulfite sequencing of the region in our cell lines further revealed that
361 methylation was present in all cell lines at the WT genotype although the BCi_NS1.1 cells
362 is less methylated (21-40%), than in both VA10 and A549 cells (81-100%) under normal
363 cell culture conditions (Fig 2d). All the cell lines are WT (G allele) homozygous carriers at
364 the *rs35705950* site, as stated above. The CRISPR/Cas9 edited A549 cell lines harbouring
365 the heterozygous [G/T] genotype showed less methylation (41-60%, clones 1-2, 21-40%
366 clone 3) at the risk allele (Fig 2e) compared to $A549^{WT}$ (81-100%), confirming that the
367 presence of the T allele reduces methylation in the polymorphic site. This suggests that
368 direct DNA methylation might at least partially explain the increased *MUC5B* expression
369 in individuals carrying IPF risk associated T allele.

370 **C/EBPβ mediates MUC5B overexpression through *rs35705950* T allele.**

371 Even though our data support a role of differential DNA methylation to explain the
372 effects of T allele on *MUC5B* expression, additional mechanisms might also be at play.
373 To further study the potential effects of the T allele of *rs35705950* we used *Match,* a
374 weight matrix-based program for predicting transcription factor binding sites in DNA
375 sequences using the DNA sequence flanking *rs35705950* polymorphism. The program

uses a library of positional weight matrices from TRANSFAC® Public 6.0. Fig 3a shows the predicted transcription factor binding motif. Binding motifs for PAX2 and PAX4 overlap the polymorphism in the WT sequence while the T allele leads to the loss of the PAX4 motif. The T allele furthermore leads to a gain of a novel *CCAAT*/enhancer-binding proteins (C/EBPs) motif (Fig 3a, S6a,S6b Fig, S3 Table). Due to the low expression levels of PAX2 and PAX4 in lung tissue (S2b Fig) they are not likely to be causative of the T allele increased promoter activity. On the other hand, in the C/EBP family, C/EBPβ has a documented role in inflammation [57, 58] and C/EBPβ is highly expressed in lung tissue (S2a Fig), making it an ideal candidate to further studies. As shown in S2c Fig, the eQTL effects of the *rs35705950* polymorphism is primarily seen in the lung, suggesting lung specific effects. To further address whether the T allele might lead to a novel C/EBPβ binding site we carried out a direct comparison of a predicted C/EBPβ binding using motifbreakR [59]. S6a Fig shows that there is a predicted consensus binding site for C/EBPβ on the negative strand in the minor allele. On the negative strand the T-allele is now A and the WT allele a C. The PWM (position weight matrix) score for the A allele is 89% (P = 0.0041) compared to a PWM score of 71% (P = 0.103) for the C allele (the major allele), novel binding of C/EBPβ to the negative strand of the IPF risk allele.

C/EBPβ has three isoforms generated by alternative splicing (S1 Fig). Two of these isoforms (LAP and LAP*) mediate transcriptional activation through the transactivation domain, while the third isoform (LIP) has an inhibitory role due to the lack of the transactivation domain [60].

Immunofluorescence staining on four IPF lung samples show that C/EBPβ and MUC5B are co-expressed in airway epithelial cells (Fig 3b, S3 Fig). Furthermore, in healthy lung

399 samples C/EBPβ is highly expressed in alveolar cells resembling alveolar macrophages
400 and they also express high levels of MUC5B (S3 Fig).

401 In addition to the aforementioned co-expression, C/EBPβ might act through a common
402 signalling pathway potentially driving *MUC5B* overexpression through the T allele. IL-13
403 has been previously used to induce goblet cell hyperplasia in asthma models and has
404 also been used to induce *MUC5AC* expression in cell culture models [56]. We cultured
405 both VA10 and BCi_NS1.1 cell lines, under ALI culture conditions with or without IL-13.
406 In both cell lines, IL-13 induced C/EBPβ as well as *MUC5B* expression at both the protein
407 (Fig 3c) and mRNA level (Fig 3d), suggesting a co-regulatory pathway.

408 To analyse specifically the differential effects of C/EBPβ on *MUC5B* *cis*-regulatory
409 domain on the wild type and T alleles, we overexpressed the C/EBPβ isoforms in the
410 BCi_NS1.1 (Fig 4a), A549 (Fig 4b) and VA10 (S6c Fig) cell lines harbouring the stably
411 integrated luciferase reporter associated with the 4.1kb *MUC5B* promoter region. The
412 C/EBPβ LAP* isoform increased *MUC5B* promoter regulated luciferase activity 2.5-fold
413 when the T allele was present, compared to the wild type in both BCi_NS1.1 and A549.
414 This relationship is dose-dependent, as shown in S6d Fig. Overexpression of the C/EBPβ
415 LIP isoform reduced *MUC5B* reporter activity when the T allele was present in both cell
416 lines (Fig 4 a, b). When both isoforms were co-expressed in equimolar concentrations
417 an increased luciferase signal was seen in the BCi_NS1.1 cell line.

418 To further corroborate the role of C/EBPβ in regulating *MUC5B* expression through the
419 risk allele, we knocked down C/EBPβ expression using siRNAs targeting either the
420 LAP/LAP* or all isoforms. Stable cell lines carrying the luciferase reporter vector and
421 $A549^{CRISPR}$ clones were used to analyse the effect of C/EBPβ knock-down on *MUC5B*

422 expression by RT-qPCR (Fig 4c, S6e Fig) and luciferase reporter assay (Fig 4d, S6f Fig).
423 Our results show that siRNAs targeting C/EBPβ lowers *MUC5B* expression in T allele
424 genotype to a similar level as wild type.

425 The correlation between C/EBPβ expression and the strong T allele specific positive
426 expression suggests that this transcription factor is a candidate key regulator of *MUC5B*
427 expression in carriers of the T allele in IPF.

428

Discussion

The results presented in this study suggest that the IPF associated polymorphism, *rs35705950* (T allele), has a direct and major effect on *MUC5B* expression. We have shown that it is mediated through a combination of effects on both epigenetic and transcriptional regulatory mechanisms. Specifically, we have shown that the T allele directly reduces DNA methylation and that this increases the *MUC5B* expression in our cell culture models. Furthermore, we have shown that the T allele creates a novel C/EBPβ binding site that strongly positively regulates *MUC5B* expression.

CpG DNA methylation has previously been identified as an important regulatory mechanism for mucins. The human genes *MUC2*, *MUC5AC*, *MUC5B* and *MUC6* are clustered on chromosome *11p15* and their promoters show a GC-rich structure [47]. Hypermethylation of *MUC5B* promoter is the major mechanism responsible for its silencing, combined with histone acetylation while the expression of *MUC5AC* was rarely influenced by epigenetic marks. In this study, we confirm a general role of methylation in *MUC5B* repression. Furthermore, we show that the *rs35705950* T allele disrupts a CpG methylation site and this disruption relieves a DNA methylation-dependent negative regulation site. Furthermore, the *in vitro* methylation assay corroborates the differential methylation pattern between alleles.

Data from the ENCODE project suggest that the area surrounding the *MUC5B* polymorphism is located within a *cis*-regulatory element, based on both, the DNAse hypersensitivity cluster and the high number of transcription factors binding in the area. Our data, based on *in silico* binding analysis, predicts a C/EBPβ binding site overlapping *rs35705950* when the IPF associated T allele is present. Here, we directly show that

452 *rs35705950* polymorphism leads to a specific response to C/EBPβ positively regulating
453 MUC5B expression. C/EBPβ would thus be implicated as an important regulator of
454 MUC5B expression in carriers of the *rs35705950* T allele.

455 Mucin regulating signalling pathways are complex and poorly understood. It has been
456 shown that activation of MEK1/2, PI3K, SPhk1, and MAPK14 (p38α-MAPK) are implicated
457 in IL-13 induced mucus production [61, 62]. Furthermore, through PMA signalling PKC,
458 EGF/TGF-a, Ras/Raf, Mek, ERK and Sp-1 signalling pathways have also been associated
459 with *MUC5AC* and *MUC5B* expression [63]. Interestingly, C/EBPβ is also overexpressed
460 after PMA stimulus [64] and, in our results, we show a common signalling pathway
461 between MUC5B and C/EBPβ involving IL-13, suggesting common signalling pathways.
462 C/EBPβ has been previously associated with inflammation and immune responses [58,
463 65-67]. Similarly, MUC5B has been implicated in innate immune response in the lung
464 [68], although C/EBPβ has not been implicated so far. C/EBPβ is also expressed in
465 macrophages [69, 70]. Recently, Satoh *et al*.[71] identified a monocyte derived cell,
466 termed segregated-nucleus-containing atypical monocyte (SatM). These SatM cells
467 were shown to be crucial for bleomycin induced fibrosis in a mouse model and C/EBPβ
468 was shown to be a key transcriptional regulator in their differentiation. Interestingly, the
469 SatM-termed monocytes, induce a pro-fibrotic signalling pathway linking them directly
470 to the fibrosis. Furthermore, macrophages have been shown to be related to goblet cell
471 hyperplasia and in that context to induce *MUC5B* but not *MUC5AC* in human bronchial
472 epithelial cells [72]. Whether macrophages play any role in dysregulation of *MUC5B* in
473 IPF remains to be tested.

Other potential transcriptional binding sites are present in the region of the *rs35705950* polymorphic site. Highly preserved HOX9 and a FOXA2 binding domains have been described [55, 73]. The binding of FOXA2 transcription factor was shown to be 32bp upstream of *rs35705950* polymorphism. The proximity to the polymorphism may suggest a co-regulatory site that could through, e.g. interaction with the novel C/EBPβ binding site affect *MUC5B* expression. Whether the overexpression is caused by a combination of FOXA2 and C/EBPβ needs to be clarified. The eQTL data showing that the positive expression effects of the *rs35705950* polymorphic site is primarily seen in the lung (S2d Fig) could be related to a lung specific pattern of expression of transcription factors, e.g. C/EBPβ with or without other coregulators such as FOXA2. Other tissues that express high levels of MUC5B, such as salivary glands, stomach, small and large intestine are not shown to have a positive eQTL with the polymorphic site.

In our results, we have also shown that C/EBPβ is co-expressed with MUC5B. Its presence is predominantly in basal cells in the pseudostratified epithelium under normal conditions, while in fibrotic lungs, it is also expressed in ciliated and goblet cells. This could be related with the role of C/EBPβ previously associated with fibrosis development [74, 75].

To summarize, we have shown that T allele associated with the *rs35705950* polymorphism strongly induces *MUC5B* overexpression. Interestingly, this appears to be through two independent mechanisms. Firstly, it leads to disruption of a CpG methylation site that naturally represses *MUC5B*, resulting in an overexpression. Secondly, the same T allele creates a potential novel C/EBPβ binding site that positively regulates *MUC5B* expression. These results identify the C/EBPβ transcription factor as a

candidate target to study in fibrosis associated with IPF. Further studies are needed to decipher how C/EBPβ and MUC5B can result in fibrosis.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Conception and design: AUA, MKM;

Analysis and interpretation: AUA, AJA, OAS, GG, TG, MKM;

Drafting the manuscript for important intellectual content: AUA, MKM

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821

822 ## Figure legends

823 **Fig 1. Risk allele (T) is associated with a higher expression of *MUC5B*.** a) Luciferase
824 activity on stable cell lines transduced with 4,1Kb of *MUC5B* *cis*-regulatory region.
825 Luminescence was measured after 24h in a monolayer culture. b) Wild type (WT) allele
826 was replaced on A549 cells by CRISPR editing technique to get five heterozygous cell
827 lines [G/T]. RT-qPCR shows *MUC5B* relative expression (mRNA) in different cell lines.
828 Culture was performed on monolayer, 48h before RNA extraction. c) IF staining on
829 $A549^{CRISPR}$ clones (1-5) shows higher *MUC5B* expression levels compared to $A549^{Cas9}$
830 Control (WT) cell line. Scale bars = 50µm. (*p<0.05 **p<0.01 and ***p <0.001 with error
831 bars representing SD).

832

833 **Fig 2. Methylation plays a role in MUC5B upregulation driven by T allele.** a) IF staining
834 shows an increased expression of MUC5B (left) and MUC5AC (right) after 48h with
835 5'AZA'2 treatment compare to the untreated sample under ALI conditions. Scale bar =

10μm. b) Transient transfection luciferase assay with (lower panel) or without (upper panel) *in vitro* methylation of the 4.1kb cis-regulatory region. Without in vitro methylation no difference is seen in VA10 or A549 cells between T-allele and WT while after in vitro methylation a significant increase is seen in the T-allele carrying luciferase plasmid compared to WT in all three cell lines. c) Schematic representation of a bisulfite sequencing experiment shows the differential methylation on the *rs35705950* region between cell lines (c) and how it changes with the presence of the T allele on CRISPRed cells (d). Legend indicates the range of methylation (*p<0.05 and **p <0.01 with error bars representing SD).

Fig 3. C/EBP is predicted to bind rs35705950 MUC5B cis-regulatory region only in presence of the T allele and C/EBPβ is co-expressed with MUC5B in IPF. a) Results of a weight matrix-based program (Match) for predicting transcription factor binding sites in *MUC5B* promoter sequence using the DNA sequence flanking *rs35705950* using TRANSFAC©. ** indicates a novel binding motif in the T-variant allele. * indicates the loss of a transcription factor binding motif, while (+) and (-) indicates the strand where the bindings occurs. b) IF-P in an IPF sample shows the co-expression of MUC5B (green) and C/EBPβ (red). c) IL-13 (20ng/mL) increases MUC5B (green) and C/EBPβ (red) expression at protein (c) and at RNA level (d), analysed by RT-qPCR, compared to the untreated sample. Scale bars = 50μm.

Fig 4. C/EBPβ mediates the MUC5B upregulation by the rs35705950 T allele. a) The three C/EBPβ isoforms were transfected individually into BCi_NS1.1 (a) and A549 (b) luciferase stable cell lines carrying the 4.1kb *MUC5B* promoter for 24h. The activatory

isoform (LAP) increases the *MUC5B* reporter activity only in presence of the T allele, while the inhibitory isoform (LIP) inhibits the differential activity between WT and T allele. c) siRNA silencing C/EBPβ isoforms, LAP and LIP together or C/EBPβ LAP alone, was performed on $A549^{CRISPR}$ Clone 1 compared to $A549^{Cas9}$ Control cell line. Inhibition of C/EBPβ restores differential *MUC5B* expression driven by the T allele to the WT associated *MUC5B* expression levels while no effect is seen in $A549^{Cas9}$ Control cell line. d) siRNA was used to knowck down C/EBPβ (LAP and LIP or only LAP isoform) in A549 luciferase stable cell line carrying the 4.1kb *MUC5B* promoter. Inhibition of C/EBPβ restores *MUC5B* expression driven by the T allele to the WT associated *MUC5B* expression levels (*p<0.05 and **p <0.01 with error bars representing SD).

Figure 1

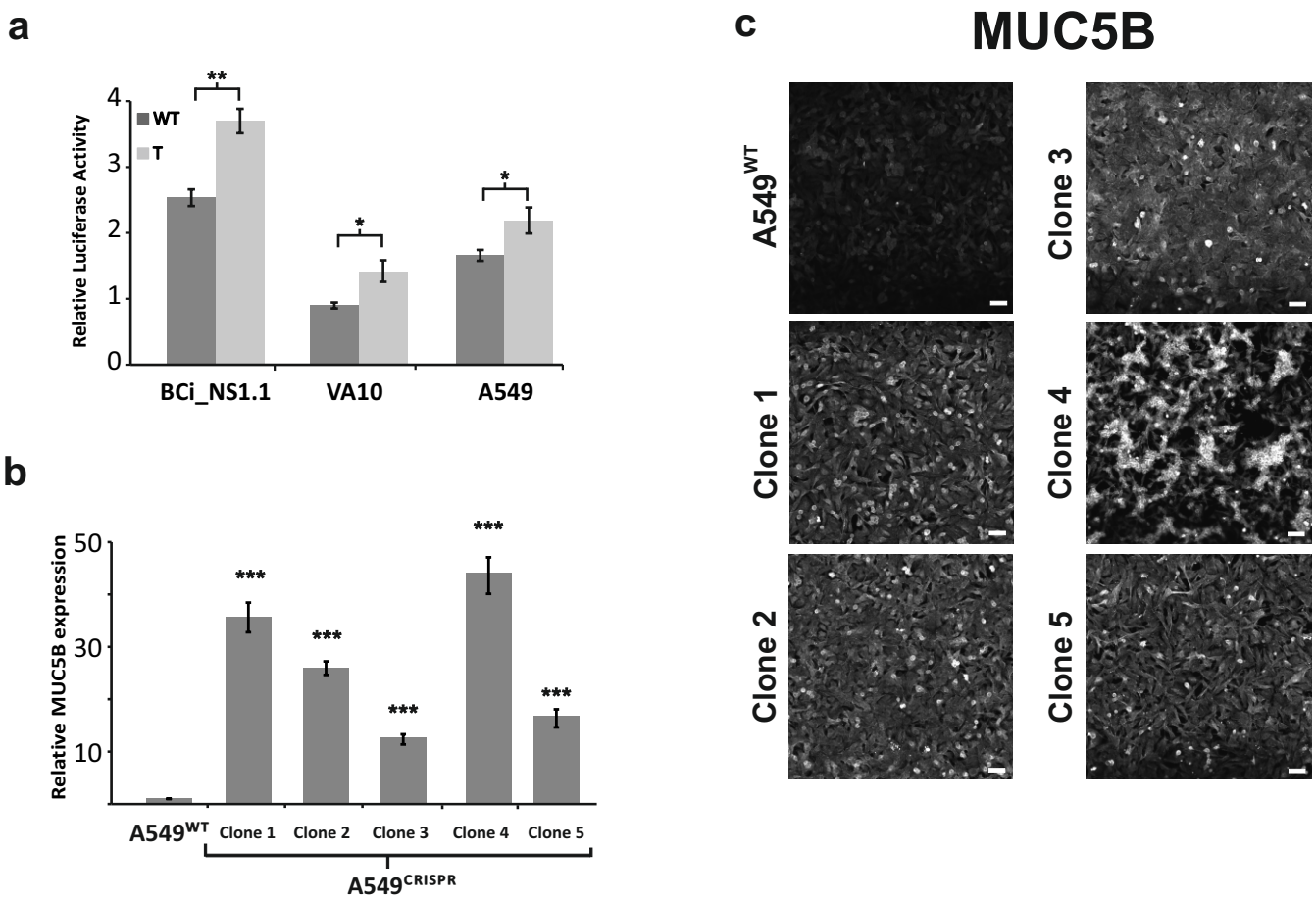


Figure 2

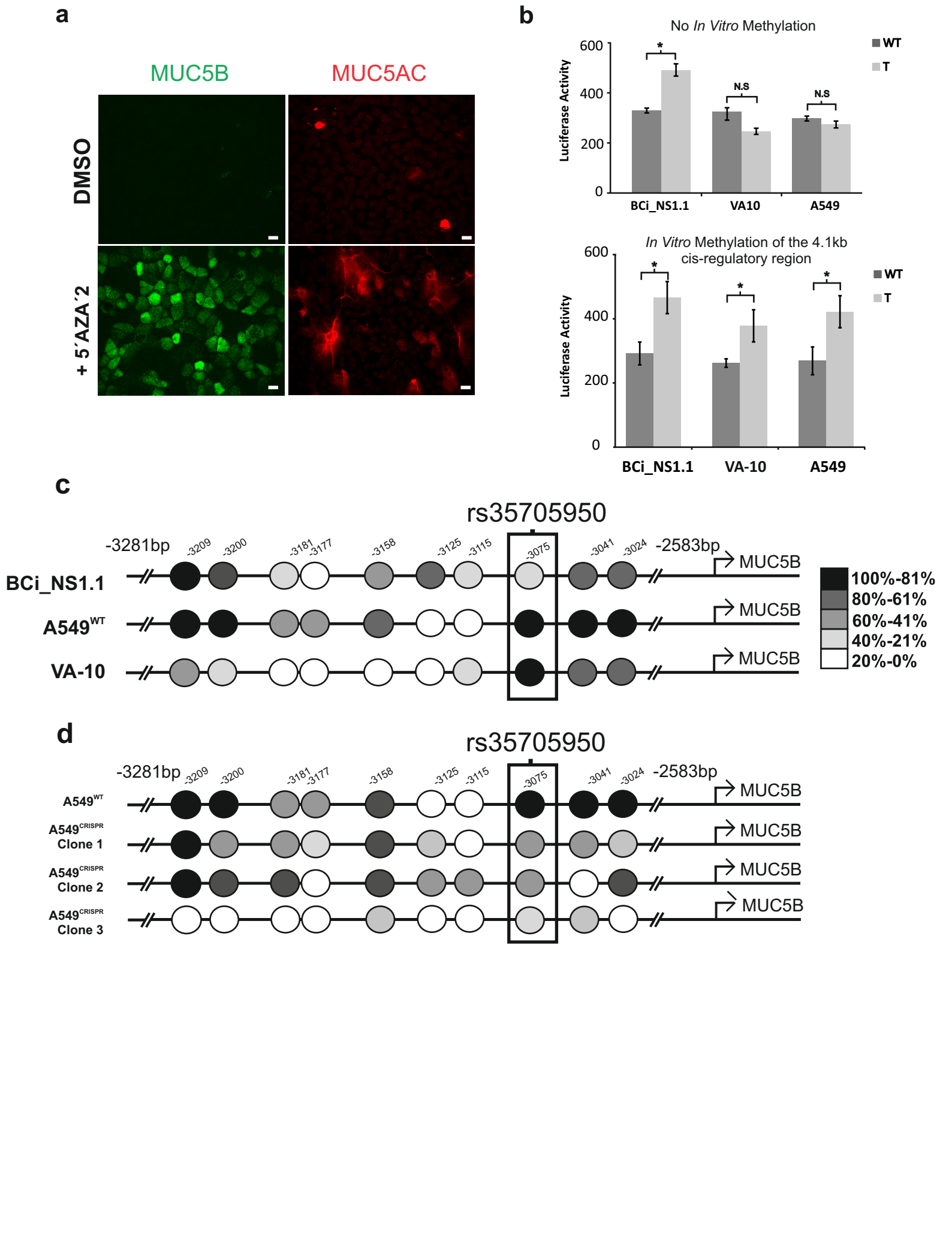


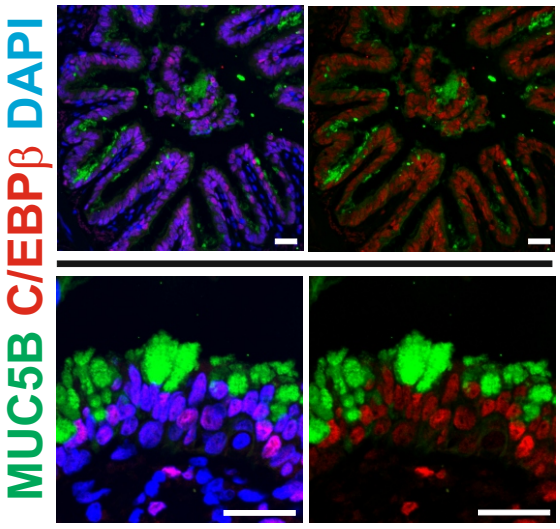
Figure 3

a

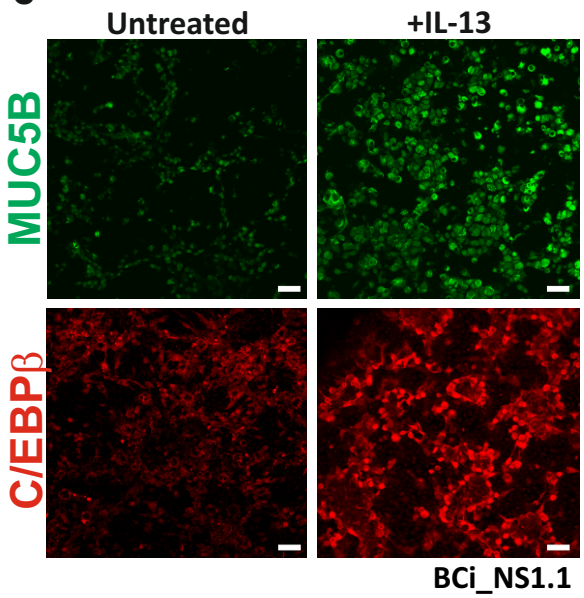
```
                                        gaagAGGTGaac      ZEB-1      (-)
                                     acTGTGAagaggt        C/EBP      (+)
                                   caactgtgaAGAGG         KLF4       (+)
                                 ttcAACTGtg               c-Myb      (+)
                    tcTGTTT                               SRY        (-)
               tATCTTctgttttca                            Evi-1      (-)
          ctTTATCttc                                      GATA-3     (-)
CCTTCCTTTATCTTCTGTTTTCAGCGCCTTCAACTGTGAAGAGGTGAACTCTTCAAACA
                         T
                   agctcctTCAACtg                         C/EBP      (-) **
                     cgccTTCAActgtgaagag                  PAX2       (+)
                     cgcctTCAACtgtgaagaggt                 PAX4       (+) *
                      gccttcaactGTGAAgagg                  PAX2       (-)

* (disappears with minor allele)
**(appears with minor allele)
```

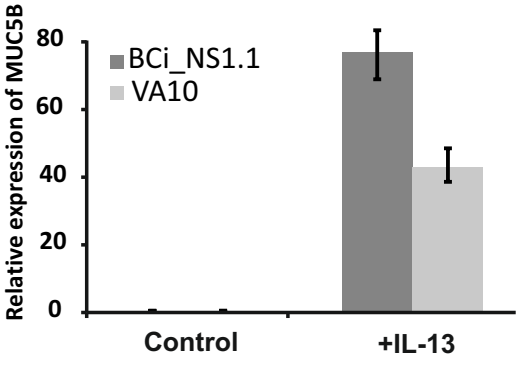
b



c



d



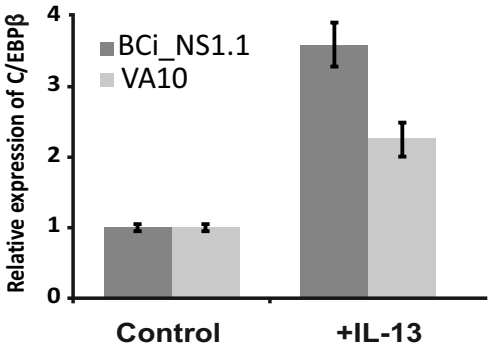


Figure 4