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Original research

BET proteins are associated with the induction of small airway fibrosis in COPD

Razia Zakarya,^{1,2} Yik L Chan,² Sandra Rutting,³ Karosham Reddy,^{1,2} Jack Bozier,^{1,2} Roy R Woldhuis,^{1,2,4} Dikaia Xenaki,¹ David Van Ly,⁵ Hui Chen,² Corry-Anke Brandsma ,⁴ Ian M Adcock,⁶ Brian G Oliver^{1,2}

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¹Respiratory Cell and Molecular Biology, Woolcock Institute of Medical Research, Glebe, New South Wales, Australia

²School of Life Sciences, University of Technology Sydney, Ultimo, New South Wales, Australia

³Airway Physiology and Imaging Group, Woolcock Institute of Medical Research, Glebe, New South Wales, Australia

⁴Pathology and Medical Biology, University Medical Centre Groningen, Groningen, Groningen, The Netherlands

⁵Genome Integrity Unit, Children's Medical Research Institute, The University of Sydney, Westmead, New South Wales, Australia

⁶Airways Disease, Respiratory Cell & Molecular Biology, Airways Disease Section, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, London, UK

Correspondence to

Dr Razia Zakarya, Respiratory Cell and Molecular Biology, Woolcock Institute of Medical Research, Glebe, NSW 2037, Australia; Razia.Zakarya@sydney.edu.au

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ABSTRACT

Rationale In COPD, small airway fibrosis occurs due to increased extracellular matrix (ECM) deposition in and around the airway smooth muscle (ASM) layer. Studies of immune cells and peripheral lung tissue have shown that epigenetic changes occur in COPD but it is unknown whether airway mesenchymal cells are reprogrammed.

Objectives Determine if COPD ASM cells have a unique epigenetic response to profibrotic cytokine transforming growth factor β 1 (TGF- β 1).

Methods Primary human ASM cells from COPD and non-COPD smoking patients were stimulated with TGF- β 1. Gene array analysis performed to identify differences in ECM expression. Airway accumulation of collagen 15 α 1 and tenascin-C proteins was assessed. Aforementioned ASM cells were stimulated with TGF- β 1 \pm epigenetic inhibitors with qPCR quantification of *COL15A1* and *TNC*. Global histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity were assessed. chromatin immunoprecipitation (ChIP)-qPCR for histone H3 and H4 acetylation at *COL15A1* and *TNC* promoters was carried out. Effects of bromoterminal and extraterminal domain (BET) inhibitor JQ1(+) on expression and acetylation of ECM target genes were assessed.

Measurements and main results COPD ASM show significantly higher *COL15A1* and *TNC* expression in vitro and the same trend for higher levels of collagen 15 α 1 and tenascin-c deposited in COPD airways in vivo. Epigenetic screening indicated differential response to HDAC inhibition. ChIP-qPCR revealed histone H4 acetylation at *COL15A1* and *TNC* promoters in COPD ASM only. ChIP-qPCR found JQ1(+) pretreatment significantly abrogated TGF- β 1 induced histone H4 acetylation at *COL15A1* and *TNC*.

Conclusions BET protein binding to acetylated histones is important in TGF- β 1 induced expression of *COL15A1* and *TNC* and maintenance of TGF- β 1 induced histone H4 acetylation in cell progeny.

INTRODUCTION

COPD is the fourth leading cause of death in the world and as the ageing population grows, has been projected to increase to the third by 2030.¹ COPD is characterised by a persistent loss in lung function precipitating as shortness of breath in patients, with small airways disease and emphysema being the pathological manifestations of the disease. Small airways disease is the predominant site of obstruction in COPD² and describes changes in the airway

Key messages

What is the key question?

► Is augmented extracellular matrix (ECM) deposition by small airway smooth muscle cells in COPD underpinned by a unique epigenetic mechanism?

What is the bottom line?

► Increased expression of ECM genes *COL15A1* and *TNC* in COPD is modulated by histone H4 acetylation.

Why read on?

► Further investigations demonstrate the link between bromoterminal and extraterminal domain proteins and the propagation of aforementioned histone H4 acetylation in COPD cells.

walls of 4th–16th generation bronchioles which are <2 mm in diameter. These changes include heightened inflammation^{3,4} and increased extracellular matrix (ECM) deposition in close proximity to the airway smooth muscle (ASM) layer⁵ within the airway wall.⁶ The overall effect of which being a thickened, more rigid, fibrotic airway wall wrapped around a lumen; subsequently decreasing lumen size and causing airway obstruction. Small airways disease has been shown to precede emphysematous changes,⁷ and thereby considered the primary pathological insult in COPD. Using primary mesenchymal cells, we have shown that cells from COPD smokers are hyperresponsive to cigarette smoke stimulation in vitro, depositing more ECM than those cells from non-COPD smokers.^{8,9}

Although cigarette smoking is an established risk factor for COPD, not all smokers develop COPD. The reasoning behind the same stimuli having variable outcomes on disease is the result of both genetic and epigenetic susceptibility. Epigenetic aberrancies in COPD have been described in epigenome-wide association studies describing differentially methylated CpG sites in leukocytes¹⁰ and small airway epithelial cells,¹¹ and we previously found that peripheral lung tissue and immune cells from patients with COPD had greater levels of histone H4 acetylation at the CXCL8 promoter and decreased HDAC activity.¹² However, no studies to date have investigated if epigenetic dysregulation

also occurs in genes involved in the pathogenesis of small airway fibrosis, namely ECM encoding genes.

Epigenetics is a wide-encompassing term that is best described by Riggs and Porter¹³ as mitotically or meiotically heritable changes to genetic function not attributable to gene sequence. The field encapsulates modifications made directly to DNA and post-translational modification of histones. In this study, we will explore DNA methylation and histone acetylation, in the context of ECM gene expression, in ASM cells. We posit that epigenetic factors are critical in small airways disease in COPD. The aim of this study is to identify and investigate the underlying epigenetic mechanisms of an aberrant ECM gene response to TGF- β 1 from primary human ASM cells from smokers with and without COPD. The study further delineates the role of epigenetic readers from the bromoterminal and extraterminal domain

(BET) protein family such as Brd2 and Brd4, which recognise and interact with acetylated histones to initiate and maintain transcriptional elongation.¹⁴

METHODS

Additional detail for all noted methods is provided in the online supplemental file.

Study subjects

All human lung tissue obtained following written informed consent. Human ASM cells were obtained from bronchial airways of subjects with COPD or no obstructive lung disease. Patient demographics are provided in online supplemental table E1.

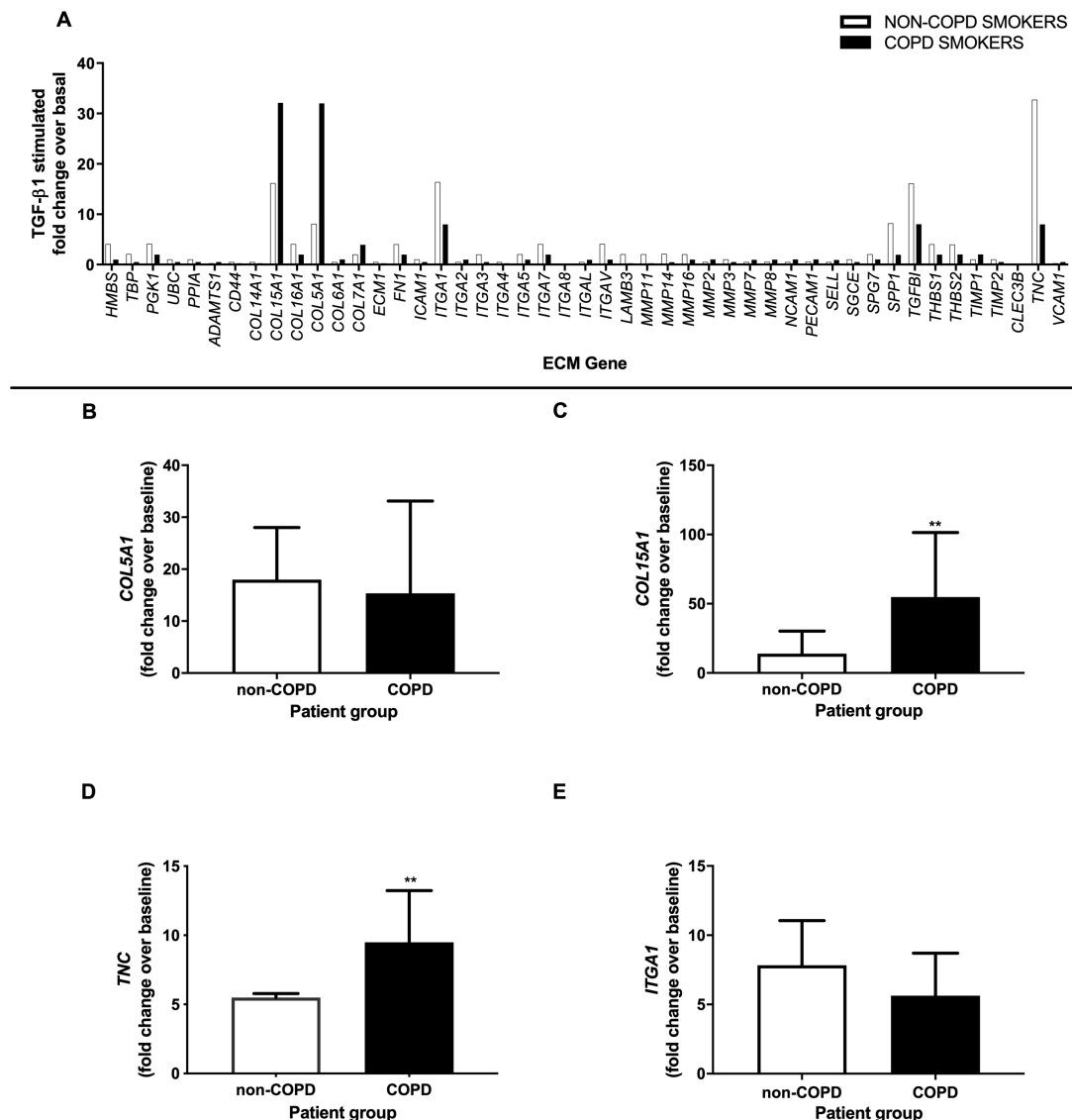


Figure 1 Focused microarray analysis and qPCR validation. (A) Primary human airway smooth muscle cells from COPD (n=7) and non-COPD (n=7) susceptible smokers were cultured \pm TGF- β 1 (10 ng/mL) for 48 hours. mRNA lysates were collected, and cDNA was reverse transcribed using RT-PCR. Resultant cDNA from treated and non-treated groups was pooled and ran on a microarray targeting a panel of 92 extracellular matrix genes of which those upregulated by TGF- β 1 are shown. Results are expressed as TGF- β 1 induced fold change over baseline (median \pm IQR). *COL15A1* and *COL5A1* expression was higher in COPD, while *TNC* and *ITGA1* expression in non-COPD smokers was shown to be higher. (B) Probe-based qPCR was carried out on *COL5A1* (i), *COL15A1* (ii), *TNC* (iii), and *ITGA1* (iv). Results were normalised to an endogenous gene (18s) and are expressed as TGF- β 1-induced expression over basal expression. Data were analysed using a Mann-Whitney test (** denotes $p < 0.01$). *COL15A1* and *TNC* expression were found to be significantly upregulated in COPD.

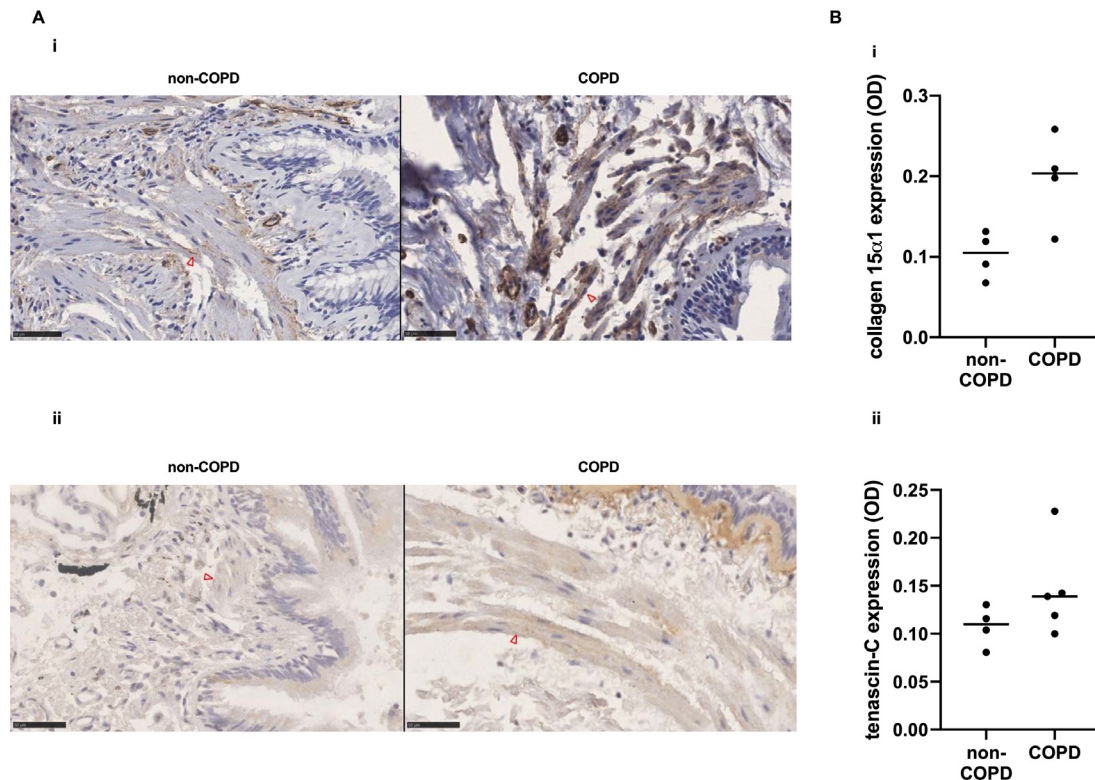


Figure 2 Immunohistochemical staining and quantification of extracellular matrix protein deposition in airway smooth muscle (ASM) layer. (A) Paraffin embedded human lung tissue from patient with COPD containing small airways and non-COPD airway was sectioned and stained with (i) anticollagen 15 α 1 antibody and (ii) anti-tenascin-C antibody. Δ denotes positive staining within the concentric ASM layer surrounding the small airway, black bar=50 μ m. (B) Airway section from control (n=4) and patients with COPD (n=4–5) were stained with (i) anticollagen 15 α 1 and (ii) antitenascin-C and quantified. Data were analysed using a Mann-Whitney test (median \pm IQR).

Cell isolation and culture

Airways were isolated from the lung and human ASM cells were isolated from the small airways (<2 mm) of patients undergoing lung transplantation or lung resection for thoracic malignancies and cells were isolated as previously described.¹⁵ These cells were used between passages 3–7.

Focused microarray analysis

cDNA from basal and TGF- β 1 treated cells from n=7 non-COPD and n=7 COPD subjects was pooled and a focused microarray analysis was performed for each group using Taqman Fast Array Plates targeting Human ECM Matrix & Adhesion Molecules (Life Technologies, Carlsbad, California, USA) at baseline and on stimulation.

Quantitative PCR

Quantitative PCR was performed as described previously.¹⁶ Data from the reactions were analysed using StepOne Software, V2.3 (Applied Biosystems).

Immunohistochemistry

Immunohistochemical analysis was performed as previously described.⁸ Airway was assessed in formalin-fixed paraffin-embedded sections using H&E, col 15 α 1, and tenascin-C staining (n=4–5). Imaging was conducted using a Hamamatsu NanoZoomer (40 \times) and stain intensity quantified using Fiji Image J.

Histone acetyltransferase and deacetylase activity

Histone acetyltransferase results are expressed as amount of catalytic byproduct (CoA-SH) per μ g protein. Histone deacetylase activity results are expressed as amount of deacetylated substrate per μ g protein.

ChIP assay

Chromatin immunoprecipitation (ChIP) assay was carried out as previously described¹⁷ using EZ-ChIP kit (Millipore, Bayswater, VIC, Australia) according to the manufacturer's protocol. Data are presented as fold enrichment compared with the IgG negative control.

RESULTS

COL15A1 and TNC are upregulated in COPD in vitro

Initially, we used a focused ECM microarray to analyse pooled cDNA (n=7 non-COPD; n=7 COPD) stimulated with TGF- β 1. TGF- β 1 induced greater COL5A1, COL15A1, ITGA1 and TNC expression (relative to baseline) in ASM cells from COPD smokers compared with cells from non-COPD smokers (figure 1A). We next used real-time PCR to validate these findings. TGF- β 1 induced heightened levels of COL15A1 expression (figure 1C) in COPD than in non-COPD smokers (p=0.0070; n=7; 54.86 \pm 69.74; range=15.75–131.4 versus n=7; 13.98 \pm 19.78; range=4.327–39.00). Similarly, TGF- β 1 induced TNC expression (figure 1D) was also significantly (p=0.0041; n=7) elevated in COPD (n=7; 9.491 \pm 4.698; range=8.095–35.06) compared with non-COPD smokers (n=7; 5.499 \pm 1.699; range=3.862–8.698). However, there was no statistically significant difference

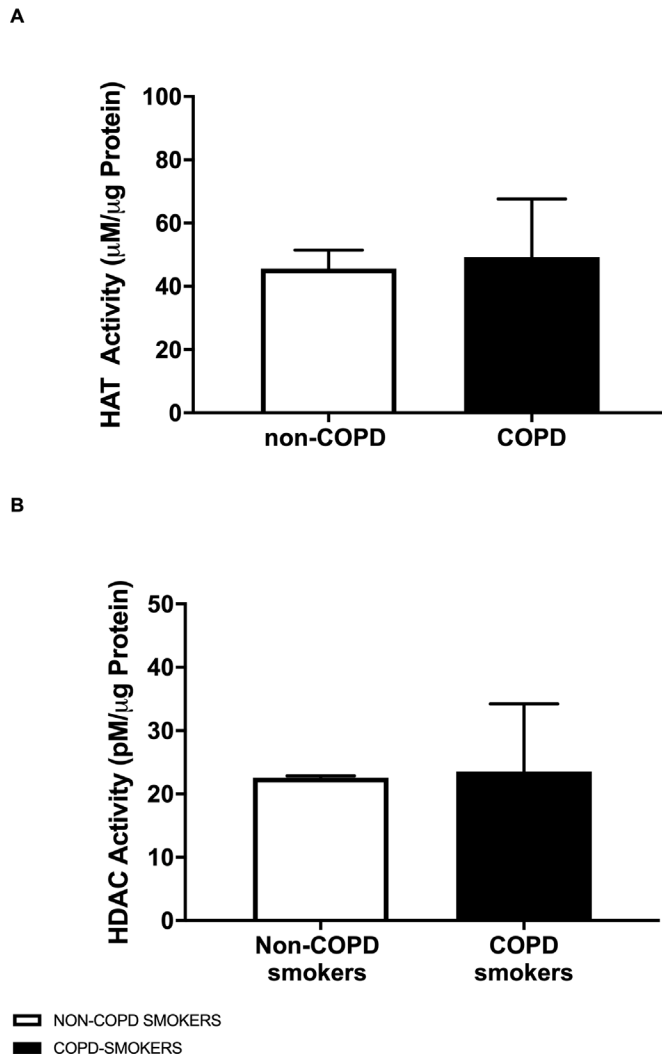


Figure 3 Acetyl histone modifying enzyme activity. Nuclear fractions isolated from primary human airway smooth muscle cells from COPD (n=4) and non-COPD (n=5) were used in a commercial enzyme activity assay. Results are expressed as amount of (A) catalytic byproduct (CoA-SH) per μg of protein for HAT activity, while HDAC activity is expressed as amount of deacetylated substrate per μg of protein (median \pm IQR).

between groups (n=7 per group) for *COL5A1* (figure 1B) and *ITGA1* (figure 1E). The data indicate a selective upregulation of *COL5A1* and *TNC* expression in COPD by TGF- β 1 rather than a global TGF- β 1-induced upregulation of ECM genes.

Collagen type XV α 1 and tenascin C proteins deposited in ASM layer in small airways of patients with COPD in vivo

To validate our in vitro mRNA findings, we performed immunohistochemical staining of bronchioles from non-COPD and COPD smokers. Collagen type XV α 1 (figure 2A) and tenascin c (figure 2B)—encoded for by genes *COL5A1* and *TNC*, respectively—are found within the smooth muscle layer in vivo. Of particular interest is the specific and exclusive localisation of collagen type XV α 1 to the ASM layer (figure 2A). Quantification of staining intensity showed trends towards higher levels of collagen type XV α 1 (p=0.0571; non-COPD: n=4; 0.1050 ± 0.055 , range=0.068–0.1313; COPD: n=4; 0.2036 ± 0.1052 , range=0.1219–0.2583; figure 2C) and tenascin-C (p=0.1905; non-COPD: n=4; 0.1098 ± 0.055 , range=0.081–0.1303; COPD: n=5; 0.1388 ± 0.076 ;

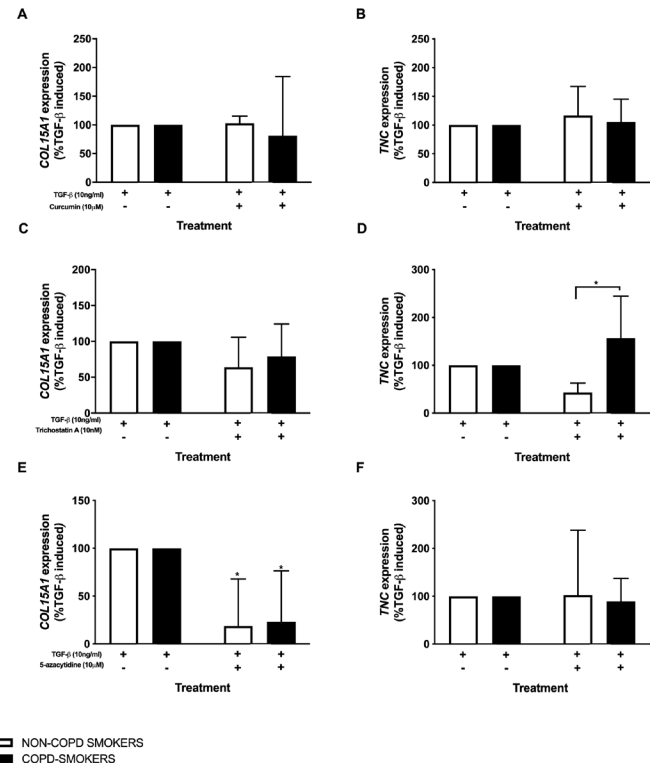


Figure 4 Effect of epigenetic inhibition with (A) curcumin (10 μM), (B) trichostatin-A (TSA; 100 nM), and (C) 5-azacytidine (10 μM) on (1) *COL5A1* and (2) *TNC* expression. Primary human airway smooth muscle cells from patients with COPD (n=6) and non-COPD (n=6–7) were treated with TSA or curcumin for 1 hour or grown in the presence of 5-azacytidine for 48 hours prior to stimulation with TGF- β 1 (10 ng/ml). mRNA was collected after 48 hours of treatment, and cDNA was reverse transcribed using RT-PCR. Fold change results were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method over baseline (normalised to 18s) and are expressed as percentage of TGF- β 1-induced expression (median \pm IQR). Data were analysed by Friedman test and Wilcoxon Sign Ranked post-hoc analysis (* denotes p<0.05 from relevant control, unless otherwise indicated; (D) significant non-COPD and COPD interaction $\chi^2=5.853$ (E) significant TGF- β 1 treatment interaction $\chi^2=14.827$).

range=0.100–0.2278; figure 2D) deposited in COPD airways in vivo.

Global HAT and HDAC activity unaltered in COPD ASM cells

To investigate if activity of epigenetic enzymes differed with disease, we carried out HAT and HDAC activity assays. We found no significant (p=0.7302) difference in HAT activity (figure 3A) in the nuclear fraction of non-COPD (n=5; 45.64 ± 21.0 ; range=29.2–54.8) and COPD susceptible (n=4; 49.18 ± 38.35 , range=30.3–72.0) smokers. Similarly, HDAC activity (figure 3B) did not differ (p=0.6857) between groups (non-COPD: n=4; 22.57 ± 5.230 , range=16.01–22.88. COPD: n=4; 23.56 ± 16.64 , range=16.06–37.36).

HDAC inhibition alters *TNC* expression in COPD suggests acetylation plays a role in gene expression

Although the overall HAT and HDAC activity did not differ between groups, we theorised that the differences may exist at a gene specific level. To investigate any acetylation differences at the *COL5A1* and *TNC* promoter region, we treated cells with the pan-HAT inhibitor, curcumin (10 μM) or the pan-HDAC

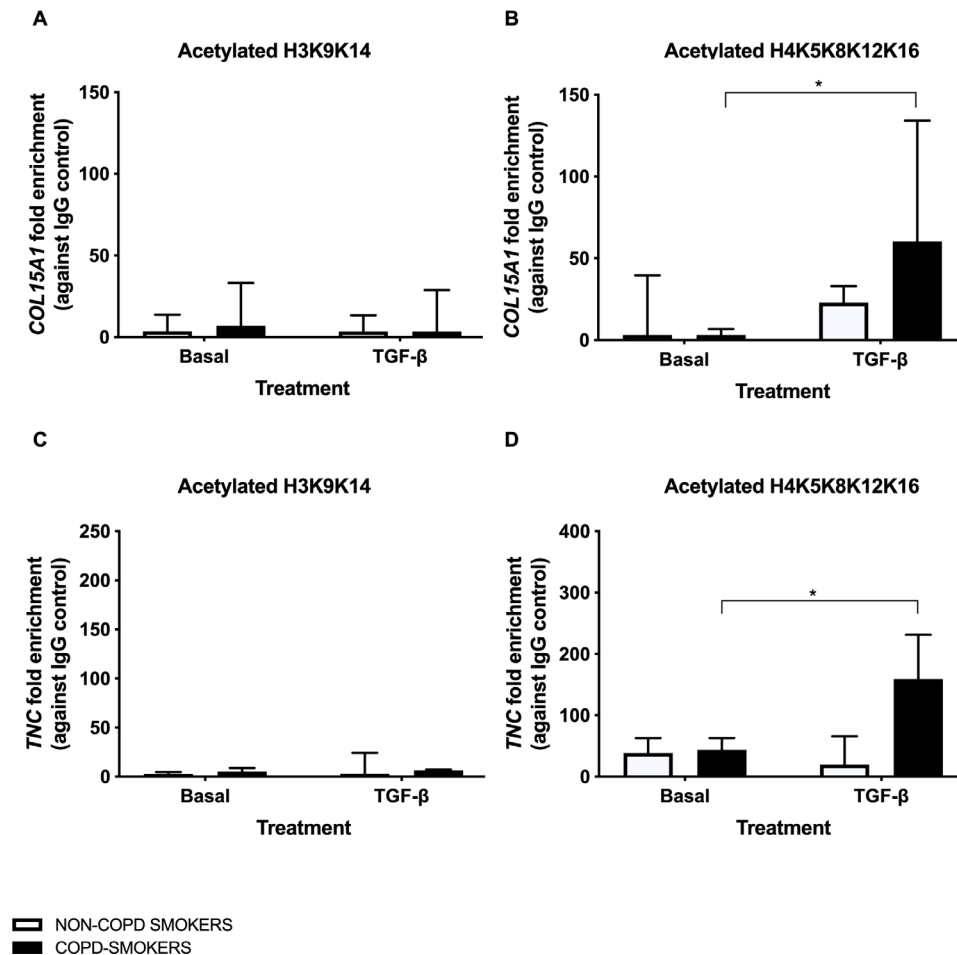


Figure 5 Chromatin immunoprecipitation (ChIP)-qPCR. Sonicated chromatin collected from non-COPD (n=4) and COPD (n=4) primary human airway smooth muscle cultured in Dulbecco's Modified Eagle's Medium (DMEM)±TGF-β1 (10 ng/mL) for 48 hours was immunoprecipitated against (A, C) anti-H3ac or (B, D) anti-H4ac antibody using ChIP assay. (A, B) *COL15A1* and (C, D) *TNC* template was subsequently quantified using SYBR qPCR. Results are expressed as fold change over negative IgG control (median ±IQR). It is evident that there is no significant difference between levels of *COL15A1* or *TNC* associated with acetylated H3 in either group basally or in response to stimulation. There is no significant difference between levels of *COL15A1* or *TNC* associated with acetylated H4 in either group basally while TGF-β1 stimulation induced histone H4 acetylation at the *COL15A1* and *TNC* promoters in COPD only. Results are expressed as fold change over negative IgG control (median ±IQR) and analysed by Friedman Test followed by Wilcoxon Signed Ranks post-hoc analysis (* denotes $p < 0.05$; (B) significant treatment interaction $\chi^2 = 6.90$ (D) significant treatment interaction $\chi^2 = 5.40$).

inhibitor, TSA (100 nM) prior to stimulation with TGF-β (10 ng/mL) for 48 hours. HAT inhibition using curcumin resulted in no significant difference in TGF-β1-induced *COL15A1* (figure 4A) or *TNC* (figure 4B) expression in cells from either patient group. In contrast, TGF-β1-induced *TNC* expression differed significantly ($p < 0.05$) between cells from non-COPD (n=7; 43.05 ± 41.48 , range=9.848–164.5) and COPD (n=7; 156.9 ± 189.3 , range=1.819–248.3) susceptible smokers in the presence of TSA (figure 4D). To investigate the role of methylation at the *COL15A1* and *TNC* promoter, cells were exposed to the DNMT1 inhibitor 5-azacytidine (10 μM) during cell division, resulting in significantly abrogated TGF-β1-induced *COL15A1* expression (figure 4E) in ASM cells from non-COPD (n=7; $p < 0.05$; 18.79 ± 53.61 , range=11.51–87.66) and COPD (n=7; $p < 0.05$; 23.11 ± 74.52 , range=4.752–80.88) susceptible smokers, with no difference between groups.

Histone 4 acetylated at *COL15A1* and *TNC* promoter in COPD

We used ChIP-qPCR to investigate acetylation of histones H3 and H4 at the *COL15A1* and *TNC* promoter regions. Results

show that histone H3 was not acetylated at the *COL15A1* (figure 5A) or *TNC* (figure 5C) promoters at baseline or in response to stimulation with TGF-β1. In contrast, histone H4 acetylation was significantly upregulated at the *COL15A1* (figure 5B; n=4; $p < 0.05$) and *TNC* (figure 5D; n=4; $p < 0.05$) promoters in COPD cells in response to TGF-β1 stimulation (*COL15A1* – non-COPD: 22.95 ± 25.02 , range=6.147–33.13; COPD: 60.26 ± 89.93 , range=40.07–117.7; *TNC* – non-COPD: 19.81 ± 56.03 , range=8.45–79.26; COPD: 159.0 ± 177.1 , range=47.81–243.7).

Acetyl-histone recognition underpins *COL15A1* and *TNC* expression in COPD

To investigate the direct effect of the acetylated histone marks defined in figure 3B and D, cells were treated with the BET mimic JQ1(+) at a range of concentrations (1 nM, 10 nM, 100 nM and 1 μM). JQ1(+) at 1 nM and 10 nM had no effect on TGF-β1-induced *COL15A1* or *TNC* expression; while expression of both *COL15A1* (figure 6A; n=6 non-COPD, n=5 COPD;

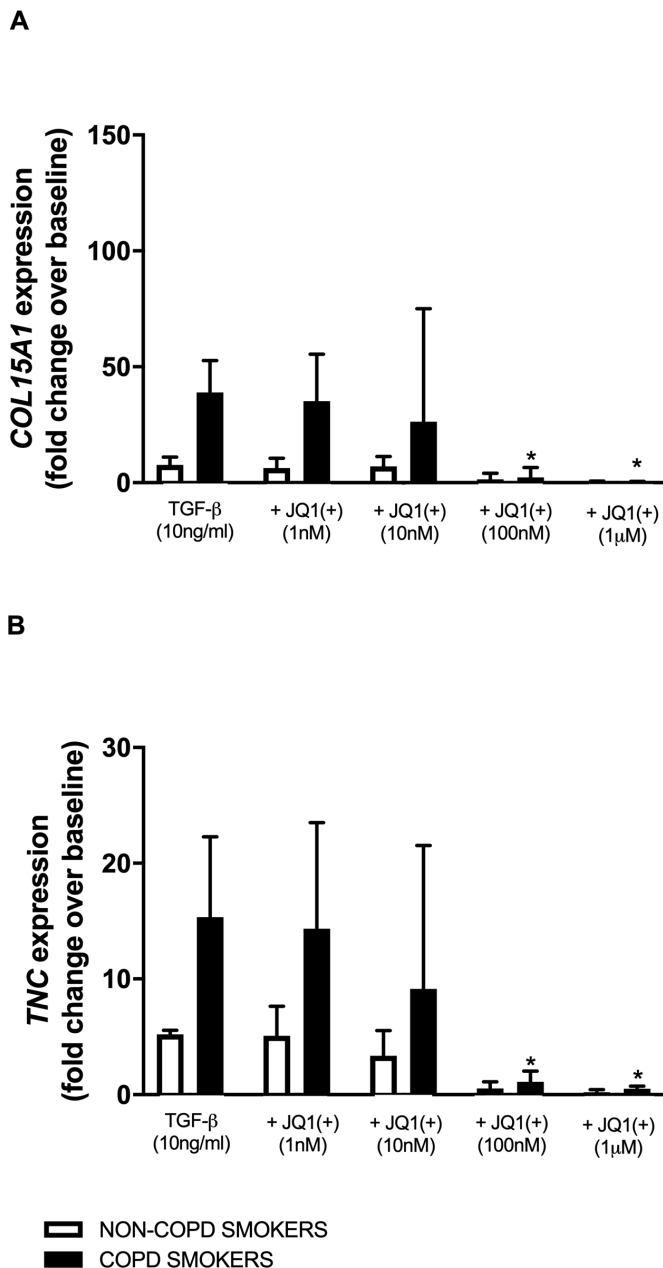


Figure 6 JQ1(+) dose response. Non-COPD (n=6) and COPD (n=6) human airway smooth muscle cells were stimulated with TGF- β 1 (10 ng/ml) \pm 1 hour JQ1 pretreatment (1 nM, 10 nM, 100 nM, 1 μ M). RNA lysates were collected at 48 hours and cDNA synthesised via RT-PCR. (A) *COL15A1* and (B) *TNC* template was quantified using probe-based PCR. All results were normalised against endogenous housekeeping gene 18s and are expressed as TGF- β 1 induced fold change over basal expression. Data are presented as median \pm IQR and analysed by Friedman test and Wilcoxon Sign Ranked post-hoc analysis (* denotes $p < 0.05$ from relevant control; (A) significant JQ1(+) treatment interaction $\chi^2 = 9.867$ (B) significant JQ1(+) treatment interaction $\chi^2 = 16.8$). The negative enantiomer JQ1(-) was used and had no inhibitory effect (data not shown).

100 nM: $p < 0.05$; non-COPD: 1.401 ± 2.83 , range = 1.205–4.24; COPD: 2.342 ± 4.03 , range = 1.735–6.594; 1 μ M $p < 0.05$; non-COPD: 0.322 ± 0.508 , range = 0.162–0.946; COPD: 0.2198 ± 0.176 , range = 0.195–0.512; and *TNC* (figure 6B; n=6 non-COPD, n=5 COPD; 100 nM: $p < 0.05$; non-COPD: 0.533 ± 0.707 , range = 0.305–1.864; COPD: 1.119 ± 1.631 ,

range = 0.1491–2.048; 1 μ M $p < 0.05$; non-COPD: 0.213 ± 0.30 , range = 0.1320–0.8889; COPD: 0.513 ± 0.340 , range = 0.2913–0.7589) was significantly abrogated in cells from volunteers with COPD at 100 nM and 1 μ M JQ1(+). These data demonstrate a role for histone H4 acetylation in aberrant ECM gene expression in ASM cells from patients with COPD.

BET proteins maintain pathological histone H4 acetyl mark

To elucidate the extent which BET proteins play a role in histone H4 acetylation, we carried out ChIP-qPCR on hASM cells treated with JQ1(+) at the effective concentrations (100 nM and 1 μ M) shown in figure 4A and C. The results show that Brd inhibition with 1 μ M JQ1(+) significantly ablated H4 acetylation at the *COL15A1* (figure 7A; n=4 per group; $p < 0.05$; non-COPD: 9.07 ± 11.64 , range = 1.46–13.99; COPD: 1.310 ± 1.043 , range: 0.7173–2.010) and *TNC* (figure 7B; n=4 per group; $p < 0.05$; non-COPD: 1.548 ± 4.647 , range = 0.8199–6.547; COPD: 6.797 ± 12.13 , range = 0.5539–13.79) promoter regions.

DISCUSSION

In this study, we investigated the epigenetic modulation of ECM gene expression in human ASM cells. We found that ASM cells expressed more *COL15A1* and *TNC* mRNA in COPD on stimulation with TGF- β 1 in vitro. The ECM proteins encoded by these genes, collagen XV α 1 and tenascin-C, were shown to be deposited within the ASM layer in human COPD airways in vivo, with trends towards higher deposition in COPD. HDAC inhibition significantly attenuated *TNC* expression in COPD only, while DNMT1 inhibition with 5-azacytidine and HAT inhibition with Curcumin lead to no difference in response between COPD and non-COPD cells. We found increased H4 acetylation at the *COL15A1* and *TNC* promoters in response to TGF- β 1 stimulation in COPD only. We competitively inhibited acetyl-lysine recognition by BET proteins with concentrations of JQ1(+) to show that *COL15A1* and *TNC* mRNA expression depends on BET protein recognition of acetylated histones. Finally, we carried out ChIP-qPCR on cells pre-treated with JQ1(+). Interestingly, 1 μ M JQ1(+) pretreatment lead to complete abrogation of TGF- β 1 induced histone H4 acetylation at the *COL15A1* and *TNC* promoter region. Thereby demonstrating that BET proteins play a role in maintaining TGF- β 1 induced histone H4 acetylation in COPD.

To induce ECM expression, cells from each cohort were stimulated with TGF- β 1; a known profibrotic cytokine shown to be elevated in COPD.¹⁸ We used cells from non-COPD and COPD smokers to delineate epigenetic aberrations relative to COPD rather than smoking status. Further, we controlled for age and gender to limit confounding factors. We found that COPD hASM cells expressed significantly higher levels of *COL15A1* and *TNC* mRNA in vitro and immunohistochemical staining demonstrated that these proteins are deposited within the ASM layer of small airways of patients with COPD in vivo. This increased ECM is consistent with other studies of COPD smokers,^{8,9,19} and contributes to small airway fibrosis which has been shown to positively correlate with COPD severity.⁵

ECM proteins have inherent bioactive properties in addition to providing structural support. Tenascin-C is a glycoprotein which induces myofibroblast formation, cell migration and ameliorates production of other ECM proteins in vivo and in vitro.^{20–22} This emphasises the critical role of tenascin-C in the onset and maintenance of fibrosis. Collagen XV α 1 is one of the lesser understood collagens but is expressed in basement membranes and in smooth and striated muscle of numerous organ systems.²³

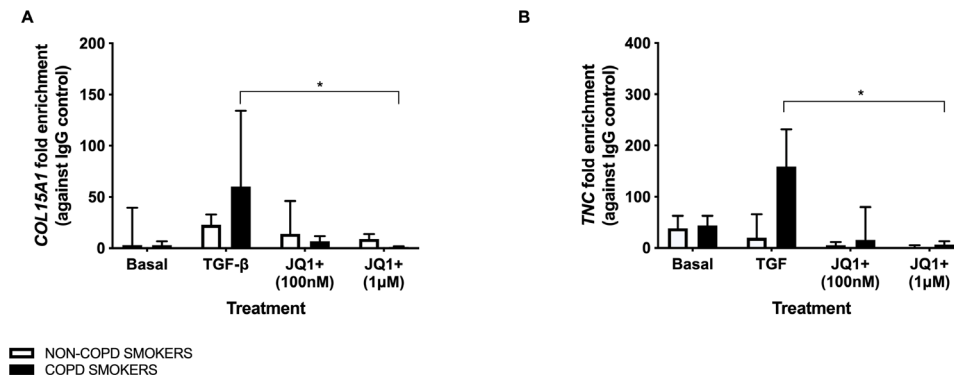


Figure 7 Chromatin immunoprecipitation (ChIP)-qPCR post-JQ1(+) inhibition. Sonicated chromatin collected from non-COPD (n=4) and COPD (n=4) primary human airway smooth muscle cultured in DMEM±TGF-β1 (10 ng/mL) for 48 hours±pretreatment with JQ1 (100 nM and 1 µM) was immunoprecipitated against anti-H4ac antibody using ChIP assay. (A) *COL15A1* and (B) *TNC* template was subsequently quantified using SYBR qPCR. Pretreatment with JQ1 at 100 nM and 1 µM significantly abrogates histone H4 acetylation at the *COL15A1* and *TNC* promoters. Results are expressed as fold change over negative IgG control (median ±IQR) and analysed by Friedman Test followed by Wilcoxon Signed Ranks post-hoc analysis (* denotes $p < 0.05$; (B) significant treatment interaction $\chi^2 = 17.25$ (D) significant treatment interaction $\chi^2 = 23.83$). The negative enantiomer JQ1(-) was used and had no inhibitory effect (data not shown).

Although increased levels of collagen XVα1 have been noted in other fibrotic diseases,²⁴ little is understood of its role in COPD. Its C-terminal fragment, restin, inhibits the migration of endothelial cells in vitro²⁵.

HAT and HDAC activity were similar between non-COPD and COPD susceptible smokers. These findings agree with previous data regarding HAT activity but different to macrophages where decreased HDAC activity in COPD is reported.¹² This suggests that the epigenetic aberrations revealed by this study are a consequence of targeted gene-specific and cell-specific mechanisms rather than epigenome-wide changes. Cells were treated with epigenetic inhibitors curcumin, TSA and 5-azacytidine to inhibit HATs, HDACs and DNMT1, respectively. Our results shown no effect of HAT inhibition on *COLXVA1* and *TNC* gene expression, while HDAC inhibition significantly attenuated *TNC* expression in ASM cells from COPD smokers, changes in *COL15A1* were not statistically significant but they did follow a similar trend.

DNMT1 inhibition with 5-azacytidine has been shown to inhibit the maintenance of cytosine methylation.²⁶ 5-azacytidine reduced *COLXVA1* expression in both groups. It is well established that DNA methylation represses gene transcription²⁷; given that our findings show that *COLXVA1* expression is decreased on DNMT1 inhibition, we surmise that our result is attributed to the increased expression of an upstream inhibitor of *COLXVA1* rather than an indication that DNA methylation plays a role in the increased expression in COPD. However, this requires further experimentation to confirm this. In light of these findings, we determined that the likely mechanism underpinning increased *COLXVA1* and *TNC* expression was local changes in histone acetylation status. The use of non-selective inhibitors of these epigenetic enzymes should be confirmed in future studies using more selective agents or specific knockdown/overexpression of the target enzymes. Coupling these data with RNA-sequencing will provide a greater overall view of the function of these epigenetic enzymes in the function of COPD ASM cells.

ChIP-qPCR demonstrated that the promoter regions for *COL15A1* and *TNC* were enriched for acetylated histone H4 in COPD. Histone acetylation leads to increased gene expression, with two mechanisms contributing to increased mRNA transcription. The first being that the addition of an acetyl moiety alters the charge of the histone, making it more negative and

therein repelling the negatively charged DNA. This conformational change from tightly bound heterochromatin to loosely bound euchromatin allows transcriptional activators, co-factors, and enzymes such as RNA Polymerase II (RNAP2) access to the transcriptional start site. With Brd2 being shown to facilitate the recruitment of RNAP2.²⁸ Second, the acetylated lysine residue acts as a tag for a BET protein, such as Brd4. Brd4 then subsequently recruits transcriptional elongation factor b (P-TEFb), which relieves transcriptional pause of RNAP2 to initiate mRNA transcription and elongation.²⁹ In light of our findings, we posit that histone H4 acetylation is the mechanism underpinning increased expression of *COL15A1* and *TNC* in COPD ASM cells.

To investigate whether acetylation played a direct role in the transcription of *COL15A1* and *TNC* mRNA, we used a small molecule competitive mimic or functional inhibitor of BET proteins' acetyl-recognition site, JQ1(+). Pretreatment of cells with 100 nM and 1 µM of JQ1(+) significantly attenuated increased *COL15A1* and *TNC* expression induced by TGF-β1 in COPD, while the negative enantiomer JQ1(-) did not. Thereby demonstrating the direct role acetylated histones play in increased *COL15A1* and *TNC* expression.

BET proteins contain two tandem acetyl-binding domains, bromodomain 1 (BD1) and (BD2). BD1 and BD2 hold high sequence homology among the BET protein family, thereby resulting in a paucity of inhibitors specific to each Brd. Often, BET inhibitors act via dual binding of BD1 and BD2, but selective inhibitors of BD2, such as RVX-208 have been developed.³⁰ The mechanism of action of JQ1(+) is well established³¹ and it does not discriminate between BET proteins or BDs. The classical understanding of BET protein-mediated transcription involves their constitutive capacity as an epigenetic reader; however, Brd2 and Brd4 can also modulate acetylation and subsequent mRNA transcription in a specific and targeted manner. Brd4 knockout is embryonically lethal; however, in vitro experiments have linked haploinsufficiency (*Brd4*^{-/+}) of Brd4 to reduced levels of H3K14 and H4K12 acetylation.³² Brd2 and Brd4 preferentially binds to acetylated H3 and H4,²⁹ with a higher affinity for the latter,³³ and continues to remain bound to those acetylated histones associated with transcriptional start sites of genes essential for establishing basic cellular functions during mitosis.^{14 29 34} It has been shown that at least half of the Brd4 'bookmarks' during mitosis are being carried over from interphase.³⁵ The capacity

of BET proteins to remain associated with transcriptional start sites throughout the cell cycle is unlike most other bromodomain containing proteins and transcription factors that are evicted from the chromatin during M-phase^{36,37} along with the displacement of most acetyl marks.^{36,38} The subsequent transcription of these 'marked' genes has been shown to commence early in G1³⁴ and indicate BET protein capacity to confer effective epigenetic memory of acetylated histones to daughter cells.

With this in mind, we carried ChIP-qPCR against acetyl-H4 on hASM pretreated with JQ1(+). JQ1(+) (1 µM) significantly abrogated TGF-β1-induced H4 acetylation of the *COL15A1* and *TNC* promoter region while the negative enantiomer, JQ1(-), did not exert such an effect. Thereby demonstrating that BET proteins play a role in maintaining an acetyl mark underpinning the overexpression of the aforementioned ECM genes. A study on Brd2 chromatin occupancy in a non-small lung cancer cell line H23³³ showed that JQ1(+) treatment depleted Brd2 chromatin association globally with no effect on global histone H4 acetylation. Interestingly, it was further shown that a small subpopulation of genomic regions that did not associate with Brd2 lost histone H4 acetylation in response to treatment with JQ1(+). The study did not investigate Brd4 occupation at regions of lost histone H4 acetylation in response to JQ1(+). Brd2 and Brd4 occupancy appears to be cell type specific as a separate study using murine Th17 cells found JQ1(+) treatment to displace global occupancy of Brd4, but not Brd2.²⁸ Our findings showing a loss of the acetyl mark post-JQ1(+) inhibition suggests that overexpression is likely linked to a BET protein, potentially Brd4, acting as a specific transcriptional modulator, rather than a classical epigenetic reader of acetylated lysine residues. Although its role as the latter would contribute to expression of the inherited mark during interphase.³⁴ Future studies should use experiments examining the effect of selective Brd2, Brd3 and Brd4 knockdown to confirm the exact function of these related proteins in the modulation of histone acetylation and subsequent ECM gene expression. These experiments may be combined with the use of the novel selective BRD4 specific inhibitors, ZL0420 and ZL0454.³⁹ We assert that our findings are bolstered by investigating genes directly, rather than overall global patterns, thereby allowing for detection of aberrancies that may otherwise be overlooked using bioinformatic methods such as peak calling and principal component analysis. We concede that our findings are based on antibody-based methods which may lead to bias in acetylated histone H4 detection post-JQ1(+) treatment which may contribute to epitope occlusion resulting from changes to nearby PTMs. Future studies may wish to bolster their findings by correlating ChIP-based methods with tandem mass spectrometry (LC-MS/MS) to confirm a decrease in acetylated histones. Recent research shows that Brd4 possesses inherent histone acetyltransferase activity⁴⁰ and our findings may be explained by this phenomenon. Although we did not observe any differences in HAT activity in COPD ASM cells, future studies should examine the activity associated with immunoprecipitated Brd4 and other HAT enzymes to confirm this. In addition, ATAC-seq linked with ChIP-seq analysis of these cells may provide greater insight into the epigenetic control of COPD ASMs.

Investigations into increased CXCL8 expression in asthmatic ASM showed increased histone H3 acetylation along with Brd3 and Brd4 binding at the CXCL8 promoter⁴¹; interestingly BET protein inhibition lead to significant abrogation of CXCL8 expression. Studies using primary human ASM from asthmatic patients showed pre-treatment with JQ1(+) prior to stimulation with TGF-β1 reduced Brd4 binding to the *IL6* and *CXCL8*

promoters, which correlated with a decrease in *IL6* and *CXCL8* protein expression.⁴² It has further been shown that the acetyl-lysine binding pocket of Brd2 associates with Stat3 to recruit Th17 factors and enhance recruitment of RNAP2 to inflammatory genes, at which point co-bound Brd4 activates RNAP2 to commence transcriptional elongation.²⁸ Thereby demonstrating the capacity of Brd2 and Brd4 to bind to the same gene with distinct roles in facilitating and sustaining gene expression. It is evident that BET proteins play an essential role in chromatin organisation, transcriptional elongation, and epigenetic memory yet there is a current paucity of studies delineating the distinct roles of BET proteins in transcriptional activation during interphase and/or mitosis. We have shown, for the first time, that mesenchymal cells from patients with COPD demonstrate an aberrant epigenetic response at the promoter region for ECM genes.

Central to all epigenetic investigations is the conundrum of which came first: the epigenetic insult predisposing a patient to disease or an epigenetic aberration as a consequence of modifiable risk factors, such as smoking. By controlling for smoking, we have demonstrated that ASM cells from patients with COPD have an inherent epigenetic predisposition to histone acetylation underpinning increased ECM gene expression in small airways. We posit that increased *COL15A1* and *TNC* expression is modulated by histone H4 acetylation; and that BET proteins act to maintain the acetylation mark in cell progeny. These novel findings shed light on a mechanism behind small airway fibrosis and highlight a new potential therapeutic avenue for the primary pathological insult in COPD.

Contributors RZ, SR, KR, YLC, JB, RRW and DX performed experiments. RZ analysed data, prepared figures and drafted manuscript. RZ, BGO and IA interpreted results of experiments. DVL, C-AB, IA and BGO edited and revised manuscript. RZ, SR, KR, YLC, JB, RRW, DVL, HC, C-AB, IA and BGO approved final version of manuscript. RZ, C-AB, IA and BGO conceived and designed research.

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ORCID iD

Corry-Anke Brandsma <http://orcid.org/0000-0001-8911-3658>

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