CBX5/G9a/H3K9me-mediated gene repression is essential to fibroblast activation during lung fibrosis

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SUPPLEMENTARY MATERIAL

Figure S1

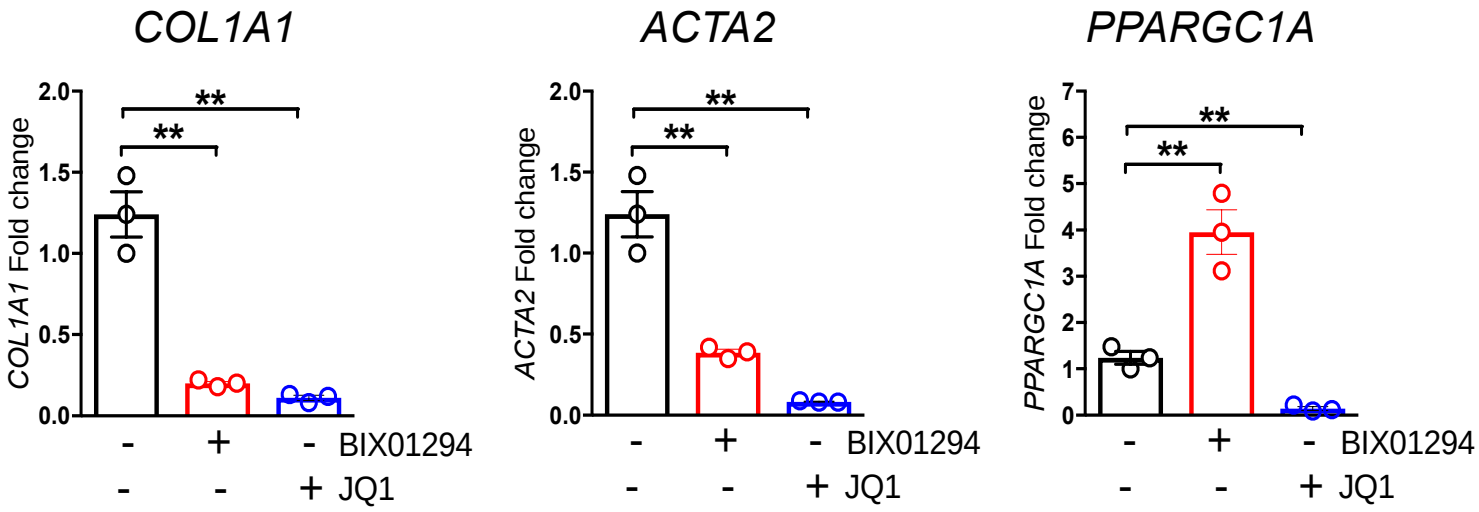

Figure S1. Inhibition of BRD4 in IPF-derived fibroblast reduced *PPARGC1A* gene expression. IPF-derived lung fibroblasts treated with the BRD4 inhibitor JQ1 (0.5μM) or G9a inhibitor BIX01295 (3μM) for 24 hours. Both BRD4 and G9a inhibition strongly reduced *ACTA2* and *COL1A1* gene expression compared to DMSO-treated fibroblasts. However, BIX01294 and JQ1 have opposite effects on *PPARGC1A* gene expression in IPF-derived fibroblasts with BIX01294 elevating and JQ1 reducing its gene expression compared to fibroblasts treated with DMSO (n=3). Data are shown as mean ± SEM of three independent experiments (**p<0.01 by 2-tailed, paired t-test).

Figure S2

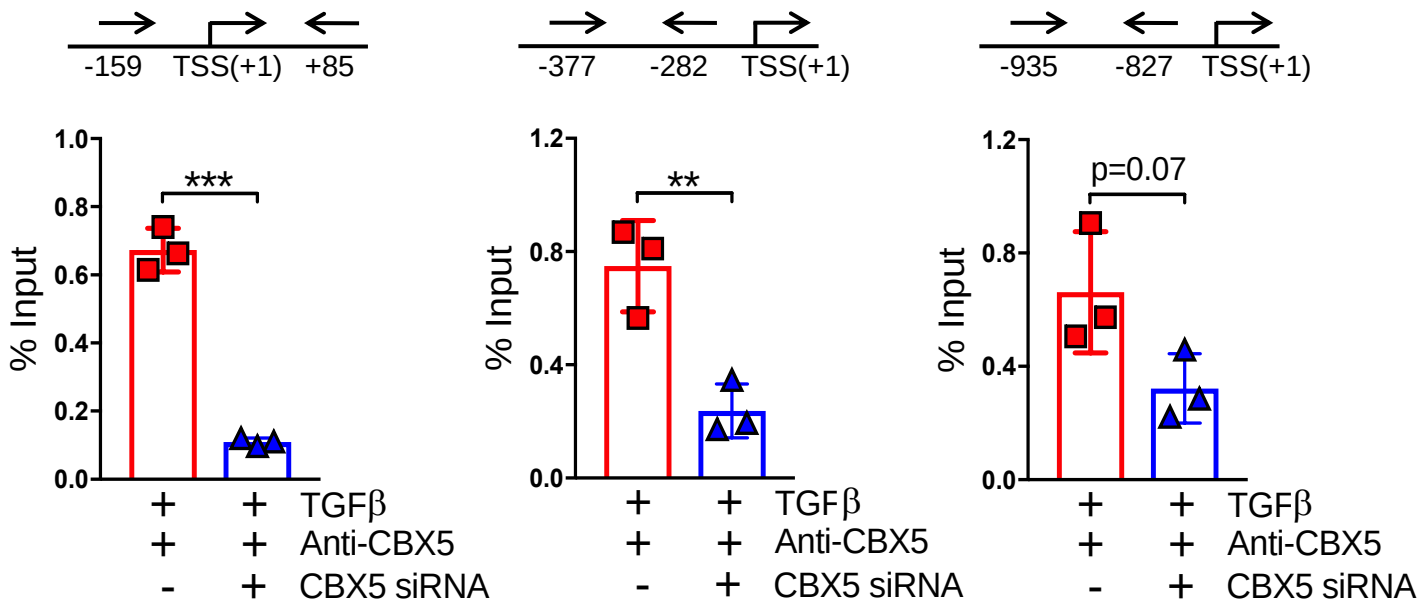

Figure S2. CBX5 binding on *PPARGC1A* gene promoter is significantly reduced in CBX5-silenced human lung fibroblasts. ChIP analysis shows reduced CBX5 enrichment at the proximal promoter of *PPARGC1A* gene in CBX5-silenced human lung fibroblasts compared to control siRNA . Three adjacent promoter regions were selected based on their affinity for transcription factors; starting from the TSS are CREB1, MEF2 and FoxO1 respectively (n=3). Data are shown as mean ± SEM (**p<0.01, **p<0.001 by 2-tailed, paired t-test).

Figure S3

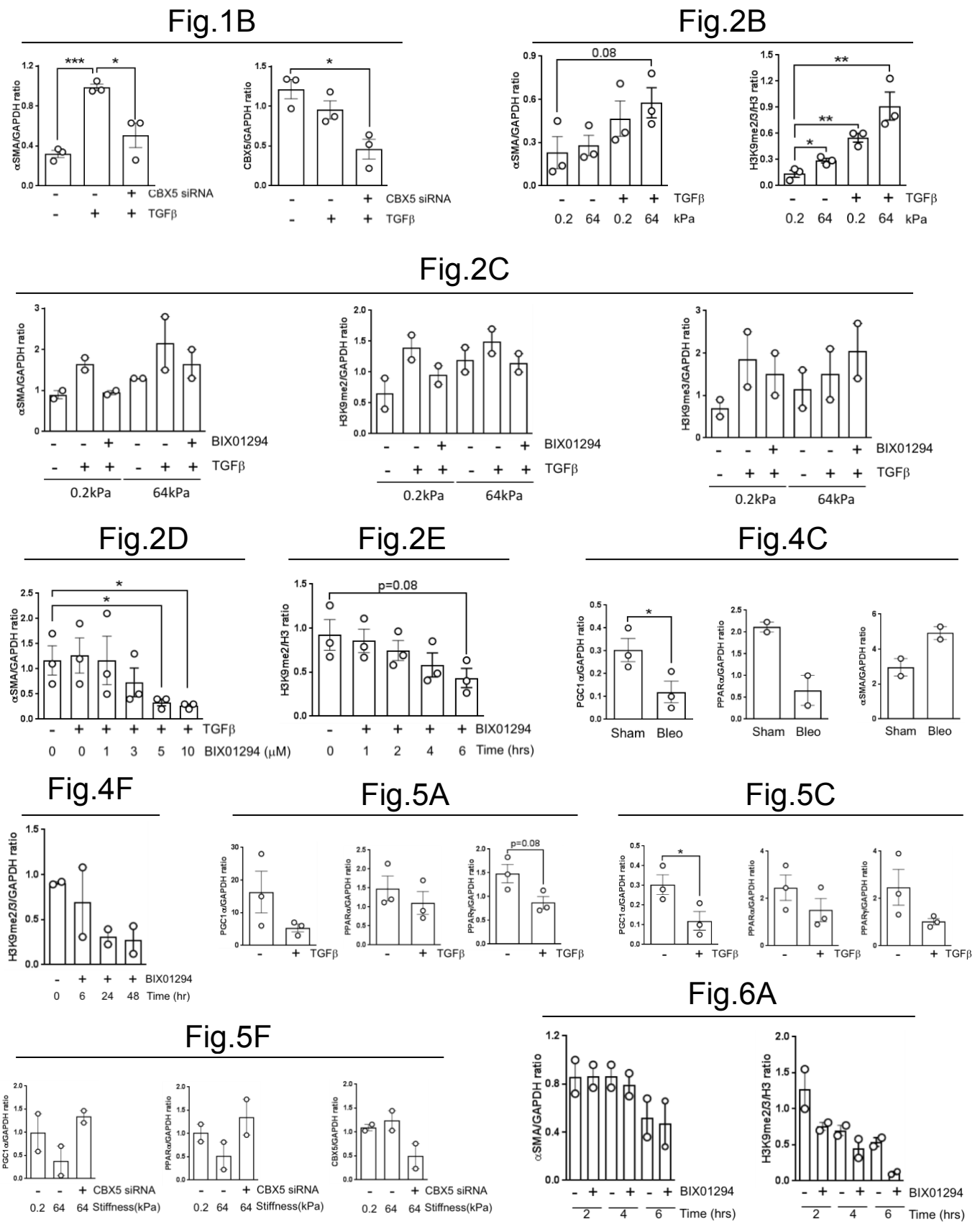

Figure S3. Densitometry analysis of all Western blots from which representative blots were selected and shown in the figures.

Figure S4

Primers	Forward (5'-3')	Reverse (5'-3')
Mouse *gapdh*	GTGGAGTCATACTGGAACATGTAG	AATGGTGAAGGTCGGTGTG
Mouse *Fn1*	TGTCAGTCAAAGCAAGCCCG	TTAGGACGCTCATAAGTGTCACCC
Mouse *Ctgf*	GTCCAGCACGAGGCTCA	TCGCCTTCGTGGTCCTC
Mouse *Ppara*	ACCACTACGGAGTTCACGCATG	GAATCTTGCAGCTCCGATCACAC
Mouse *Pparg*	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
Mouse *Ppargc1a*	CCCTGCCATTGTTAAGAC	TGCTGCTGTTCCTGTTTT
Human *GAPDH*	GGAAGGGCTCATGACCACAG	ACAGTCTTCTGGGTGGCAGTG
Human *PPARCG1A*	CTGTGTCACCACCCAAATCCTTAT	TGTGTCGAGAAAAGGACCTTGA
Human *PPARA*	AATGCACTGGAACTGGATGA	TTAGAAGGCCAGGACGATCT
Human *PPARG*	TGGAATTAGATGACAGCGACTTGG	CTGGAGCAGCTTGGCAAACA
Human *ACTA2*	GTGAAGAAGAGGACAGCACTG	CCCATTCCCACCATCACC
Human *COL1A1*	AAGGGACACAGAGGTTTCAGTGG	CAGCACCAGTAGCACCATCATTTC
Human *FN1*	TGTCAGTCAAAGCAAGCCCG	TTAGGACGCTCATAAGTGTCACCC
PPARGC1A ChIP (-159 to +85)	CACTGAAGCAGAGGGCTGCCTTT	AAGTAGGCTGGGCTGTCACTCAC
PPARGC1A ChIP (-377 to -282)	GGGAGCTTTGCCACTTGCTTGTTT	CTCTAAACGGAGCCCTGGCCATAT
PPARGC1A ChIP (-935 to -827)	GGGCACTAGGGTTGGAATTCAATG	CAGATCAGCTTTGATTCCCGGCTCC

Figure S4. List of human and mouse primers used in the qPCR experiments.

METHODS

RNA interference

RNA interference was performed using the following siRNA from Dharmacon specific for: CBX5 (D-004296-05-0002 and J-004296-09), G9a (D-006937-17 and J-006937-06), PGC1α (D-005111-01-0005), PPARα (J-003434-06), PPARγ (J-003436-09) or non-targeting siRNA (D-001810-10-05, D-001210-01-05). Transfection reagent/siRNA complexes were prepared by using Lipofectamine RNAiMAX (Life Technologies) and 10nmol/L of siRNA sequences were used in culture medium EMEM containing FBS 0.5%.

Immunofluorescence staining

Mouse and human tissues were immunostained following fixation with 3.7% formalin (Sigma-Aldrich), permeabilization with 0.05% Triton X-100 (Sigma-Aldrich) and blocking with 1% BSA. Tissue sections were incubated overnight with primary antibodies against Fibronectin I (Santa Cruz, sc-9068), H3K9me2 (Cell signaling, 4658S), αSMA (Sigma-Aldrich, F3777), and G9a (Abcam, ab40542) diluted in PBS containing 5% BSA, then washed and incubated with fluorescence-conjugated secondary antibodies (Invitrogen) diluted 1:1000. Tissues were then washed and nuclei were counterstained with DAPI (Thermo Fisher Scientific). Images were acquired using LSM 710 Zeiss confocal microscope. Nuclear G9a and H3K9me2 intensity were computationally measured using Image J software. After converting RGB image in binary images, the threshold was adjusted and kept at the same level for all the images.

Fibrosis evaluation

Seven µm thick sections were cut from Paraffin embedded lung tissues, and the sections were stained either with hematoxylin and eosin (H&E) or with Masson's Trichrome stain kit (Abcam). All H&E-stained slides and trichrome-stained slides were reviewed in a blinded fashion by a thoracic pathologist. The severity of the fibrosis was evaluated using Ashcroft score: 0 (no fibrosis), 1 (minimal interstitial and/or

peribronchiolar thickening due to fibrosis), 3 (moderate thickening without obvious architectural distortion), 5 (increased fibrosis with formation of fibrous bands and/or small masses), 7 (severe architectural distortion with large areas of fibrosis and areas of honeycomb changes), and 8 (total fibrous obliteration of the field). The mean of all scores was calculated for each mouse. Hydroxyproline content was measured using a hydroxyproline assay kit (Biovision). Briefly, left lung samples were transferred into glass tubes and hydrolyzed with 200 µl 6N HCL at 110°C for 48 h. The hydrolyzed samples were evaporated to remove excess HCL, reconstituted with 400 µl H2O and filtered in 1.5 ml centrifuge tubes equipped with a 0.45 µm semipermeable membrane filter. After samples were added to a 96 well micro-plate, Chloramine T solution was added and the plate was incubated at room temperature for 20 min. 100µl of Erlich's reagent was added to each well and the plate was incubated at 65°C for 18 min. This method gives an orange red color which is linear up to 6µg of hydroxyproline. OD 550 nm was obtained, and compared to a hydroxyproline standard curve.

Real-time PCR

For transcriptional analysis, total mRNA was isolated from cells, mouse lungs and fresh isolated FACS sorted mouse lung fibroblasts using RNeasy mini kit or RNeasy micro kit (Qiagen) followed by Nanodrop concentration and purity analysis. cDNA was synthesized using SuperScript VILO (Invitrogen); RT–PCR was performed using FastStart Essential DNA Green Master (Roche) and analysed using a LightCycler 96 (Roche). Primer sets were used are listed in Figure S4.

Protein extraction and Western Blotting analysis

Cell proteins were extracted using RIPA lysis buffer (ThermoFisher Scientific, Waltham, MA, USA). Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein lysates were separated by electrophoresis, transferred onto PVDF membranes and incubated overnight at 4 degrees with primary antibodies: GAPDH (Cell Signaling, 14C10), αSMA (Abcam, ab5694), H3K9me2 (Cell Signaling, 4658P), H3K9me3 (Abcam, ab8898), H3K9me2/3 (Cell Signaling, 5327S), H3 total (Cell Signaling, 4499P), CBX5 (Cell Signaling, 2616S), PGC1α (Novus Biologicals, NBP1-04676SS), PPARα

(Novus Biologicals, NB600-636S), PPARγ (Cell Signaling, 2435T). Blots were then washed and incubated with appropriate secondary antibodies for 1 hour at room temperature. Bands were visualized by using ChemiDoc Imaging System (Bio-Rad), according to the manufacturer's protocol. Quantitative densitometry analysis of all blots are shown in Figure S3.

FACS sorting

Fully anaesthetized Col1α1-GFP mice were perfused with cold PBS to remove blood cells. The lungs were immediately harvested and minced with a razor blade in cold DMEM medium containing 0.2 mg/ml Liberase DL and 100 U/ml DNase I (Roche, Indianapolis, IN). The mixture was transferred in 15 ml tubes and incubated at 37 °C for 30 min in a water bath to allow enzymatic digestion. After inactivation with DMEM medium containing 10% fetal bovine serum, the cell suspension was passed through a 40 µm cell strainer (Fisher, Waltham, MA) to remove debris. Cells were then centrifuged (1,300 rpm, 10 min, 4 °C), washed once in PBS and resuspended in 0.2 ml of FACS buffer (1% BSA, 0.5 mM EDTA pH 7.4 in PBS). The single cell suspension was then incubated with anti-CD45-PerCp-Cy5.5 (Biolegend, Cat# 103131), anti-CD31-PE (Biolegend, Cat# 102407), and anti-EpCAM-BV421 antibody (Biolegend, Cat# 118225) for 30 min on ice. DAPI staining was used to eliminate dead cells from the analysis. After incubation, cells were washed with ice-cold FACS buffer and resuspended in 0.5 ml of FACS buffer. FACS sorting was conducted using a BD FACS Aria II (BD Biosciences, San Jose, CA). FACS-sorted fibroblasts were collected in 1.5 ml Eppendorf tubes containing RLT lysis buffer (Qiagen, Hilden, Germany) and were subjected to mRNA isolation, cDNA synthesis and qPCR analysis.

ECM deposition assay

Cells grown in clear-bottom 96-well plates and treated as described in figure legends and then fixed in 3.7% formalin (Sigma-Aldrich). After, the cells were washed in PBS, blocked with Li-Cor Odyssey Blocking Buffer for 60 min and incubated with primary Collagen I (Novus NB600-408) and Fibronectin (Sigma sc-9068) antibodies (1:200 dilution in blocking buffer, overnight, 4°C). The day after, the cells were washed with PBS+Tween 20, stained with secondary goat anti-mouse IgG IRDye™ 800 antibody and a goat anti-rabbit IgG IRDye™ 680 antibody (1:750 dilution, 45 min, RT) and washed again before to read. The microplates

were scanned with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences), and the integrated fluorescence intensities were acquired using the software provided with the imager station (Odyssey Software Version 3.0, LI-COR Biosciences).