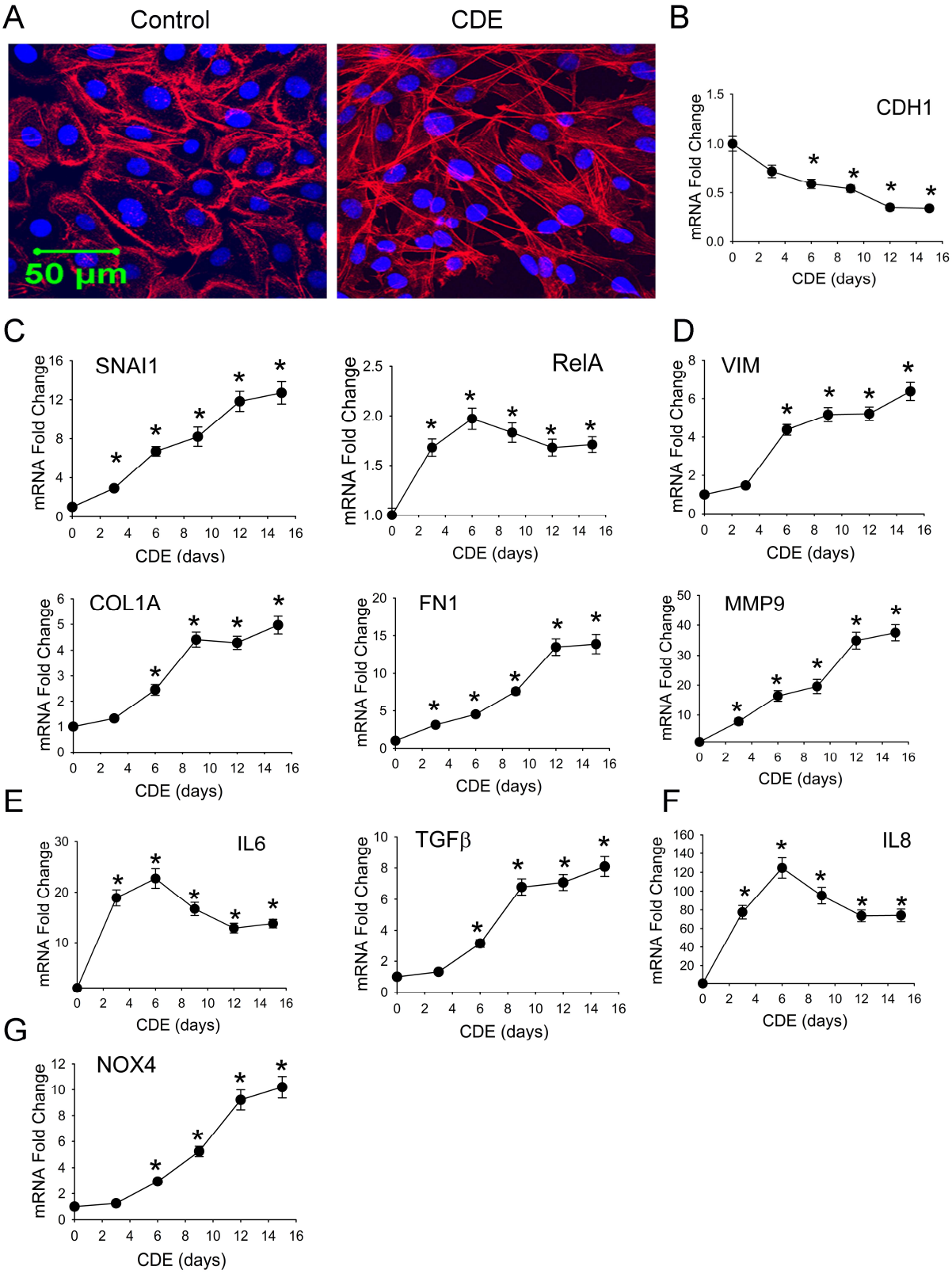
A
Control
CDE
50 µm
B
CDH1
mRNA Fold Change
CDE (days)
C
SNAI1
RelA
D
VIM
COL1A
FN1
MMP9
E
IL6
TGFβ
F
IL8
G
NOX4

Fig E1.

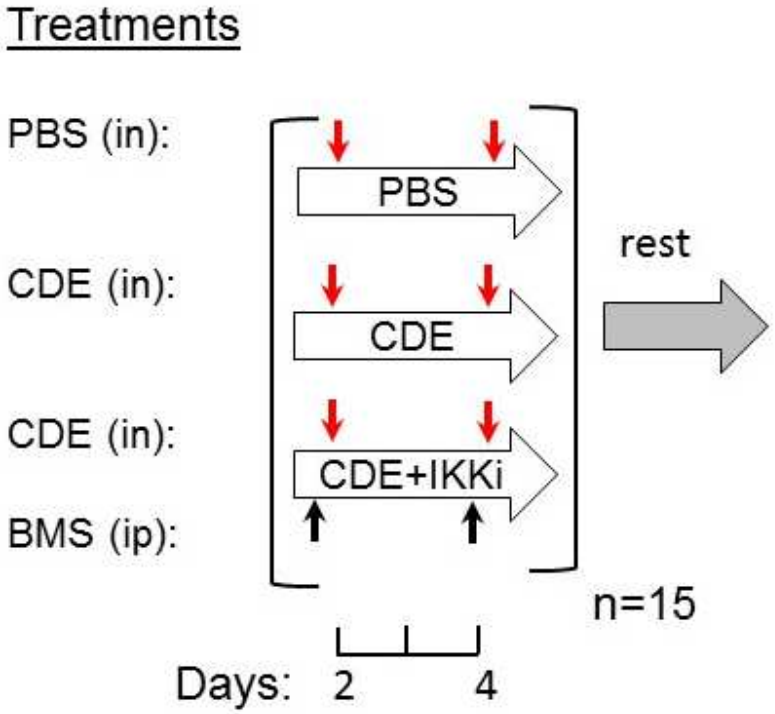
Treatments
PBS (in):
CDE (in):
CDE (in):
BMS (ip):
PBS
CDE
CDE+IKKi
rest
n=15
Days: 2 4

Fig E2

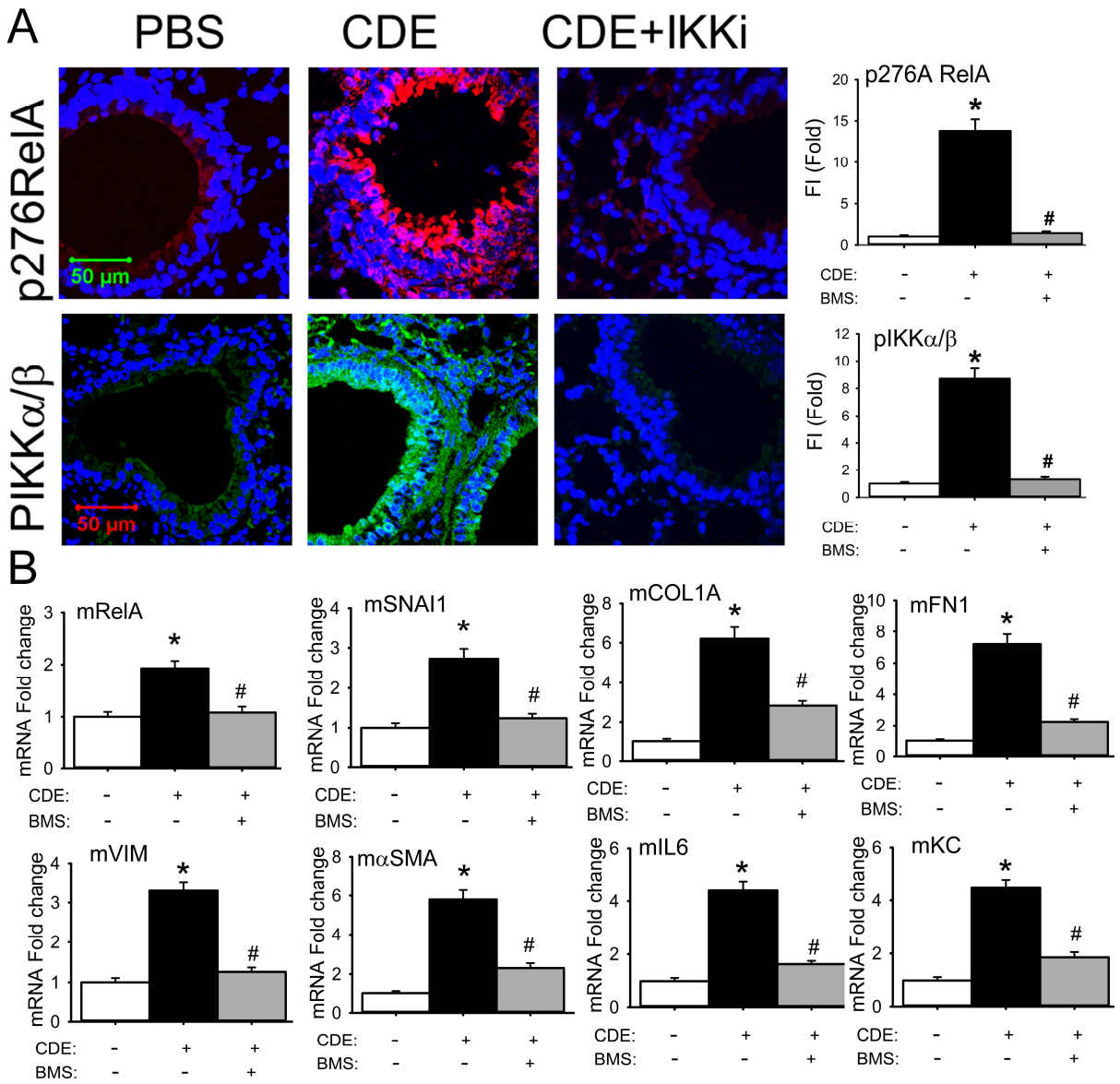
A
PBS
CDE
CDE+IKKi
p276RelA
PIKKα/β
50 μm
50 μm
p276A RelA
FI (Fold)
pIKKα/β
FI (Fold)
CDE:
BMS:
B
mRelA
mSNAI1
mCOL1A
mFN1
mVIM
mαSMA
mIL6
mKC
mRNA Fold change

Fig E3

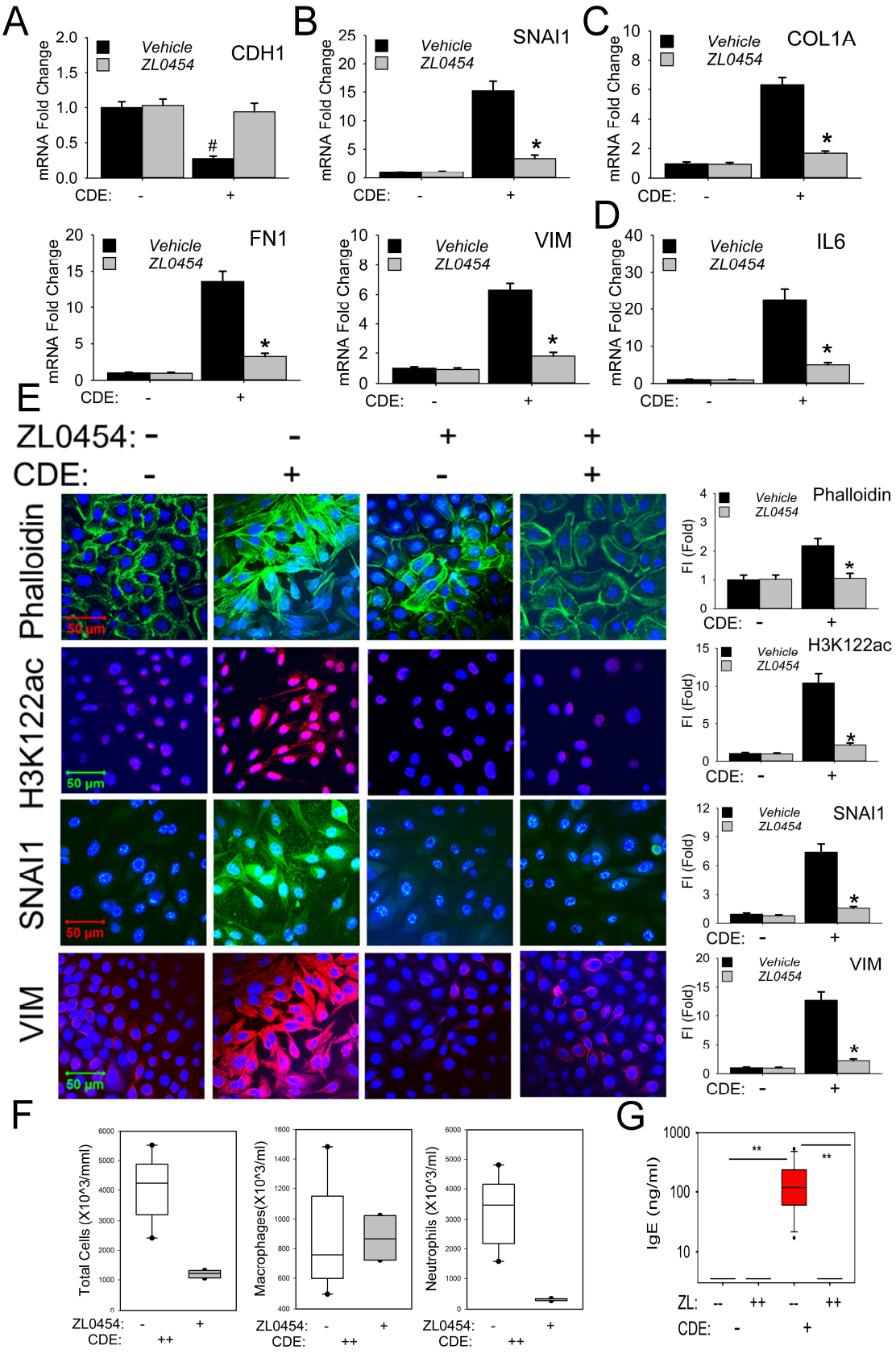
A
B
C
D
E
F
G
Vehicle
ZL0454
CDH1
SNAI1
COL1A
FN1
VIM
IL6
mRNA Fold Change
CDE:
ZL0454:
Phalloidin
H3K122ac
SNAI1
VIM
50 µm
FI (Fold)
Total Cells (X10^3/ml)
Macrophages(X10^3/ml)
Neutrophils (X10^3/ml)
IgE (ng/ml)
ZL:

Fig E4

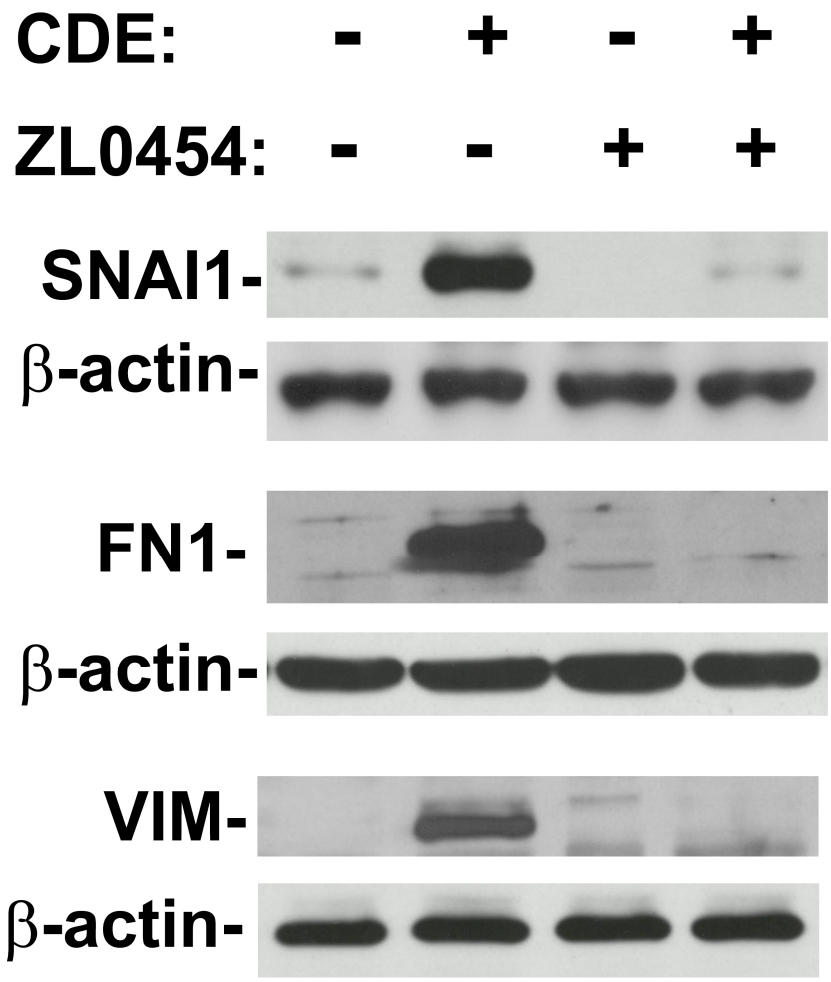

Fig E5

Methods

Human subjects

Human subjects were enrolled after providing informed consent to a protocol approved by the UTMB IRB. Demographic information was obtained to include duration of asthma, age at diagnosis, current medications, and history of exacerbations, in a manner consistent with our previous US SARP or US ACRN2 study protocols. Bronchial mucosal biopsies were obtained from the right middle and right lower lobes, and were stored in RNA Later ®. Extracted total RNA from mucosal biopsy samples was analyzed for mRNA expressions of EMT genes using Q-RT-PCR.

Cell culture and treatment

Immortalized human small airway epithelial cells (hSAECs) were previously described (25). hSAECs were grown in SAGM small airway epithelial cell growth medium (Lonza, Walkersville, MD) in a humidified atmosphere of 5% CO_2. BMS345541 was purchased from Sigma Aldrich. CDE was purchased from Greer Laboratories. The BRD4 selective small molecule inhibitor ZL0454 [(E)-4-((2-Amino-4-hydroxy-5-methylphenyl)diazenyl)-N-cyclopentylbenzenesulfonamide] was synthesized and characterized by instrumental analyses including NMR, mass spectrometry and, HPLC as described [1]. ZL0454 was used at 10 µM concentrations in cell culture medium and 10 mg/kg body weight *in vivo*.

hSAECs expressing a doxycycline (Dox)-regulated shRNA were produced by lentiviral transduction. TRIPZ Tet-on inducible lentiviral RelA shRNA and TRIPZ Inducible lentiviral empty vector shRNAs plasmids were commercially obtained (Dharmacon, GE Life Sciences, Lafayette, CO) and packaged after transfection of BOS23 cells. hSAECs were infected with collected virus-containing supernatants and selected for puromycin resistance (4 µg/ml). Puromycin resistant hSAECs were pooled and characterized. RelA depletion was produced by addition of doxycycline to the culture medium (2 µg/ml, 5 d).

Animal studies

Animal experiments were performed according to the NIH Guide for Care and Use of Experimental Animals and approved by the University of Texas Medical Branch (UTMB) Animal Care and Use Committee (approval no. 1312058A). Mice were housed under pathogen-free conditions with food and water ad libitum.

Bronchoalveolar lavage and tissue processing

Animals were anesthetized, bronchoalveolar lavage fluid (BALF) was obtained and the mice sacrificed. Lung tissues were taken for total RNA extraction or fixed for histological examination. For histological examination, lungs were inflated under 25 cm H2O pressure with 10% (v/v) neutral buffered formalin through the tracheal cannula and immersed in 10 % buffered formalin for at least 24 h. After being processed into paraffin blocks, the lungs were cut into 5-µm sections and stained with Masson Trichrome to assess fibrotic changes. Microscopy was performed on a NIKON Eclipse Ti System [2].

Periodic acid-Schiff (PAS) staining (pink color) was performed in parallel to demonstrate mucin secretion in airway epithelium [3, 4]. Quantification of accumulated mucin was assessed by 2 investigators who were blind to the treatment groups on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked, or severe mucin deposition, respectively. Data were expressed as means of scores recorded by 2 blinded investigators [3, 4].

BALF analysis of cellular inflammation

Cellular recruitment into the airway lumen was assessed in the collected bronchoalveolar lavage fluid (BALF) of mice. Lungs were perfused twice with 1 mL of sterile PBS (pH 7.4) and total cell counts determined by trypan blue staining and counting using a hemocytometer. Differential cell counts were performed on cytocentrifuge preparations

(Cytospin 3; Thermo Shandon, Pittsburgh, PA) stained with Wright-Giemsa. A total of 300 cells were counted per sample using light microscopy.

Quantitative Real-Time PCR (Q-RT-PCR)

For gene expression analyses, 0.1 µg of cDNA product from reverse transcription of total RNA was amplified using SYBR Green Supermix (Bio-Rad) and gene-specific primers as previously described [2, 5]. Quantification of relative changes in gene expression was calculated using the ΔΔCt method [6, 7] and expression as the fold change between experimental and control samples was normalized to internal control peptidylprolyl isomerase A (PPI1A)/cyclophilin A.

Confocal Immunofluorescence Microscopy

hSAECs were incubated ± CDE (20 µg/mL) for 15 d, re-plated on glass cover slips pretreated with rat tail collagen (Roche Applied Sciences), and fixed with 4% paraformaldehyde in PBS. Afterwards, the fixed cells were stained with Alexa Fluor® 488- or 568- phalloidin (Life Technologies) for cytoplasmic distribution of F-actin (green or red color) and also counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) for nuclear staining (blue color). The cells were visualized with a Nikon fluorescence confocal microscope at a magnification of 63X [2, 5].

For immunofluorescence staining, hSAECs were plated on rat tail collagen-treated cover glasses and stimulated with CDE for the indicated times. The cells were fixed with 4% paraformaldehyde in PBS and incubated with 0.1 M ammonium chloride for 10 min. Cells were permeabilized with 0.5% Triton-100, followed by incubation in blocking buffer (5% goat serum, 0.1% IGEPAL CA-630, 0.05% NaN3, and 1% BSA) and incubated with primary antibodies of RelA (Santa Cruz. 1:300 dilution), VIM, SNAI1, CDH1, p267 RelA, H3K122ac, and pPol II Ser2 (Abcam, 1:200 dilution) in incubation buffer (0.1% IGEPAL CA-630, 0.05% NaN3, and 2% BSA) overnight at 4 ℃. After washing, cells were staine d with Alexa Fluor 488- or 568--conjugated

goat anti-rabbit IgG (Life Technologies) respectively in incubation buffer for 1 h, then visualized with a LSM510 fluorescence confocal microscope, magnification 63X [2, 5].

Confocal Immunofluorescence assays of lung sections were performed on formalin-fixed, paraffin-embedded sections after rehydration using serial concentrations of ethanol. Antigen retrieval was performed in Tris-EDTA buffer (pH 9.0). Lung sections were blocked using 0.1% Triton-X, 5% normal goat serum in phosphate buffered saline (PBS) and incubated with primary antibodies of rabbit anti-p276RelA, pIKKα/β, SNAI1, H3K122ac, COL1A, VIM, FN1, and αSMA (Abcam, 1:200 dilution) overnight at 4℃. Norm al anti-rabbit IgG were used as staining specificity controls. After washing, lung sections were stained with Alexa Fluor 488- or 568- conjugated goat anti-rabbit IgG (Life Technologies) in incubation buffer for 1 h. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (5 μg/ml in PBS, 20 min) and mounted slides visualized with a LSM510 fluorescence confocal microscope, magnification 63X.

IgE measurement

Total IgE in serum were measured using Sandwich ELISA. 96-well plates 4 HBX (Thermo Scientific, Hudson, NH, USA) were coated with the purified rat anti-mouse IgE capture antibody (BD Biosciences, San Jose, CA, USA) for 2 hours at room temperature. After three washing, the plates were blocked with Sea Block blocking buffer (Pierce Biotechnology, Inc, Rockford, IL, USA). Serum were added and incubated overnight. After washing, the plates were incubated with biotin-conjugated rat IgE (BD Biosciences, San Jose, CA, USA) for 2 hours at room temperature, then washed and incubated with avidin-conjugated alkaline phosphatase for 45 minutes at 4℃. After washing, fluorometric valu es for each well were measured after addition of AttoPhos substrate solution (Promega, Madison, WI, USA) [3, 4].

Cat dander-specific IgE measurement was performed on 96-well plates. Plates were coated with 100 μg/ml of cat dander protein overnight at room temperature and blocked for 2 hour with sea block buffer. Plates were applied with serum overnight. After washing, the plates

were incubated with biotin-conjugated rat IgE (BD Biosciences, San Jose, CA, USA) for another 2 hours at room temperature, then washed and incubated with avidin-conjugated alkaline phosphatase for 45 minutes at 4℃. After washing, f luorometric values for each well were measured after addition of AttoPhos substrate.

Analysis of collagen content

To estimate amount of collagen in the lung tissue and BALF, hydroxyproline content was measured colorimetrically using a hydroxyproline assay kit (Sigma-Aldrich, St. Louis, MO) with minor modifications (24, 25). Briefly, the lungs were weighed, homogenized in liquid nitrogen with 2 ml PBS, after which 2.0 ml of 12 N HCl was added, and the samples were hydrolyzed at 120 ℃ within a PTFE-lined capped pressure-tight vi al for 6 h. Separately, 100 µl of BALF was hydrolyzed with 12 N HCl as above. Afterwards, 10 µl hydrolyzed samples were mixed with 100 µl of chloramine T/oxidation buffer at room temperature for 5 min and later incubated with the 4-(Dimethylamino) benzaldehyde (DMAB) reagent for 90 min at 60 ℃. The absorbance of oxidized hydroxyproline was determined by absorbance 560 nm (Infinite M200 PRO multimode microplate reader, Tecan Instruments). Standard curves were generated for each assay using hydroxyproline standards. The amount of collagen was expressed in micrograms per milligram lung tissue while it was expressed in nanograms per milliliters in BALF. The data shown are the means ± S.D. from n=5 experiments.

Stable isotope dilution (SID)-selected Reaction Monitoring (SRM)-mass spectrometry (MS)

The selection of the signature peptides for targeted MS-based quantification of BRD4, Pol II, H3, and H4 used a published workflow[8]. Stable isotope-labeled signature (SIS) peptide standards were chemically synthesized incorporating isotopically labeled [13C615N4] Arginine or [13C615N2] Lysine to a 99% isotopic enrichment (Thermo Scientific). BALF proteins were

digested with trypsin. Afterwards, an aliquot of 5 µL of stable isotope-labeled signature peptides was added to each tryptic digest. These samples were desalted with a ZipTip C18 cartridge before MS analysis. The SRM transitions for the signature peptides and their stable isotope label standard peptides are tabulated in **Table E2**. All data are shown as the ratio of target to SIS peptide and represent a mean ± S.D. from n=5 animals with technical replicates.

In Situ Proximity Ligation Assay (PLA)

Paraffin embedded lung section slides were subjected to antigen retrieval, permeabilized with 0.1% Triton X-100, and incubated with IgG or primary rabbit Ab to RelA (Santa Cruz), and mouse Ab to BRD4 (Sigma Aldrich). Slides were then subjected to PLA using the Duo-link PLA kit from O-Link Bioscience (Uppsala, Sweden) according to the manufacturer's instructions. The nuclei were counterstained with DAPI, and the PLA signals were visualized in a LSM510 fluorescence confocal microscope at 63X Magnification.

References

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Table E1. Clinical demographics of human subjects. MMA, mild-moderate asthma; FEV1, forced expiratory volume in 1 sec; FVC, forced vital capacity; %, percent predicted. ICS, inhaled corticosteroid, LABA, long-acting β2 agonist, SABA, short-acting β2 agonist.

	AGE:	SEX	Treatment:	STATUS	FEV1	FVC
1.	33	M	None	Normal	4.72	5.70
2.	28	F	None	Normal	3.37	3.75
3.	30	M	None	Normal	3.67	4.61
4.	31	M	None	Normal	4.07	4.95
5.	26	F	None	Normal	3.67	4.20
				average	3.9	4.642
6.	56	F	ICS, SABA	Mild Moderate	2.52	3.05
7.	28	M	ICS, SABA	Mild Moderate	2.71	4.30
8.	55	F	ICS, SABA	Mild Moderate	2.07	2.76
9.	29	M	SABA , LABA, ICS	Mild Moderate	4.31	6.95
10.	56	F	ICS, SABA	Mild Moderate	2.16	2.87
11.	46	F	LABA	Severe	2.53	3.02
12.	51	F	ICS, SABA	Severe	0.78	1.74
				average	2.44	3.53

Table E2. SID-SRM-MS parameters for airway remodeling protein

Protein Name	Uniprot #	Gene Name	Sequence	Q1, m/z	Q3, m/z	Precursor ion Z	Product ion Z	ion type	CE (V)
Fibronectin	P11276	Fn1	VFAVHQGR	457.2537	596.3257	2	1	y5	18.8606
			VFAVHQGR	457.2537	667.3629	2	1	y6	18.8606
			VFAVHQGR	457.2537	814.4313	2	1	y7	18.8606
			VFAVHQGR	457.2537	913.4997	2	1	y8	18.8606
			VFAVHQGR[13C615N4]	462.2579	606.334	2	1	y5	19.0308
			VFAVHQGR[13C615N4]	462.2579	677.3712	2	1	y6	19.0308
			VFAVHQGR[13C615N4]	462.2579	824.4396	2	1	y7	19.0308
			VFAVHQGR[13C615N4]	462.2579	923.508	2	1	y8	19.0308
SPARC-like protein 1	Q14515	Sparcl1	ILTHSELAPLR	625.3667	698.419	2	1	y6	24.5765
			ILTHSELAPLR	625.3667	785.451	2	1	y7	24.5765
			ILTHSELAPLR	625.3667	922.5099	2	1	y8	24.5765
			ILTHSELAPLR[13C615N4]	630.3709	708.4272	2	1	y6	24.7466
			ILTHSELAPLR[13C615N4]	630.3709	795.4593	2	1	y7	24.7466
			ILTHSELAPLR[13C615N4]	630.3709	932.5182	2	1	y8	24.7466

Fig E1. Chronic CDE stimulation induces mesenchymal transition of airway epithelial cells. A, Confocal immunofluorescence micrographs of human small airway epithelial cells (hSAECs) incubated in the absence or presence of CDE (20 µg/mL) for 15 days. Cells were stained with Alexa568-conjugated phalloidin (red color) and DAPI (a nuclear DNA stain, blue color). Graphs are shown at 63X magnification. **B,** Q-RT-PCR assays of total RNA extracted from a time course of CDE-stimulated hSAECs for the epithelial marker *CDH1* (E-cadherin). Shown as fold-change mRNA abundance normalized to *PPIA* (*cyclophilin* A). * p<0.01, n=3. **C,** Q-RT-PCR of *SNAI1* and *RelA* mRNAs. **D,** Q-RT-PCR for vimentin (*VIM*), collagen 1A (*COL1A*), fibronectin (*FN1*), and *MMP9*. **E,** Q-RT-PCR for expression of paracrine growth factor *IL6* and *TGFβ* mRNA. **F,** Q-RT-PCR for *CXCL8/IL8*. **G,** Q-RT-PCR for inducible NADPH oxidase, *NOX4*. All Q-RT-PCR data are the means ± S.D. from n=3 experiments. *, p<0.05, compared to without CDE, t-test.

Fig E2. Repetitive CDE (rCDE) exposure *in vivo*. Schematic of experimental strategy. 18 week-old C57BL/6 mice were pretreated IKK inhibitor BMS345541 (10 mg/kg body wt, ip; n = 5) or vehicle and were subjected to n=15 challenges with intranasal (in) CDE (20 µg/dose) every other day for a total of 30 d. Shown is the timing of the administration for each of the treatment groups. Red vertical arrows, administration of PBS or CDE via the in route; black arrows, administration of CDE via the ip route. 12 days after the last CDE challenge, mice were sacrificed and analyzed.

Fig E3. rCDE exposure activates NFκB pathway and mesenchymal program in the airway mucosa. A, Confocal immunofluorescence micrographs of phospho-Ser 276 RelA (p276RelA, red) or phospho-IKK α/β(green), counterstained with DAPI in representative lung sections from PBS, CDE or BMS+CDE treated mice. Images were acquired at 63X magnification. At right are quantifications of relative fluorescence intensity in 5 independent images. *, p<0.05, compared to without CDE; #, p<0.05 compared to CDE alone. **B,** Q-RT-PCR of total RNA from lung tissues of PBS, CDE or BMS+CDE treated mice. Shown is fold change in mouse (m) mRNA expression for each gene normalized to PPIA. *, p<0.01, compared to without CDE; #, p<0.01 compared to CDE alone, n=5, t-test.

Fig E4. BRD4 pathway mediates CDE-induced EMT of airway epithelial cells. WT hSAECs were treated with CDE (20 µg/mL) for 0 or 15 d in the presence or absence of BRD4 inhibitor ZL0454 (10 µM) before harvesting for Q-RT-PCR and performing confocal immunofluorescence microscopy. A, Q-RT-PCR for *CDH1* mRNA expression. #, p<0.01, compared to without CDE, n=3, T-test. B, Q-RT-PCR for *SNAI1* mRNA expression. *, p<0.01, compared to CDE alone, n=3. C, Q-RT-PCR for *COL1A*, *FN1*, and *VIM* mRNA expression. D, Q-RT-PCR for *IL6* mRNA expression. E, Confocal immunofluorescence microscopy for phalloidin (green color), H3K122-Ac (red color), SNAI1 (green color), and VIM (red color) counterstained with DAPI (blue color) in WT hSAECs in absence or presence of CDE (20 µg/mL, 15d) stimulation. Images were acquired at 63X. At right are quantifications of relative fluorescence intensities of phalloidin, H3K122ac, SNAI1, and VIM, * p<0.01, n = 5, t-test. F, Mice were treated in absence or presence of ZL0454 (10 mg/kg) prior to acute intranasal challenge of CDE. Shown are total, macrophage, and neutrophil counts in BALF 24 h later after CDE challenge. G. Total serum IgE was quantitated for each chronic treatment group, **, p<0.01, n=5, t-test.

Fig E5. BRD4 inhibitor blocks rCDE-induced SNAI1, FN1, and VIM accumulation in lung tissue. 300 μg homogenized whole lung extracts of mice were fractionated by SDS-PAGE and subjected to western immunoblot with SNAI1, FN1, and VIM Abs, β-actin was probed as loading controls. Similar results were found in experiments repeated three times independently.