Benzo(a)pyrene Enhanced Dermatophagoides Group 1 (Der f 1)-Induced TGFβ1 Signaling Activation through the Aryl Hydrocarbon Receptor-RhoA Axis in Asthma

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The authors declare that they have no relevant conflicts of interest.

ABSTRACT

Background: We have previously demonstrated that benzo(a)pyrene (BaP) co-exposure with dermatophagoides group 1 allergen (Der f 1) can potentiate Der f 1-induced airway inflammation. We sought to investigate the molecular mechanisms underlying the potentiation of BaP exposure on Der f 1-induced airway inflammation.

Methods: BaP co-exposure with Der f 1-induced activation of TGFβ1 signaling was analyzed in airway epithelial cells (HBECs) and in asthma mouse model. The role of aryl hydrocarbon receptor (AhR) and RhoA in BaP co-exposure-induced TGFβ1 signaling was investigated. AhR binding sites in RhoA were predicted and experimentally confirmed by luciferase reporter assays. The role of RhoA in BaP co-exposure-induced airway hyper-responsiveness (AHR) and allergic inflammation was examined.

Results: BaP co-exposure potentiates Der f 1-induced TGFβ1 signaling activation in HBECs and in the airways of asthma mouse model. The BaP co-exposure-induced the activation of TGFβ1 signaling was attenuated by either AhR antagonist CH223191 or AhR knockdown in HBECs. Furthermore, AhR knockdown led to the reduction of BaP co-exposure-induced active RhoA. Inhibition of RhoA signaling with fasudil, a RhoA/ROCK inhibitor, suppressed BaP co-exposure-induced TGFβ1 signaling activation. This was further confirmed in HBECs expressing constitutively active RhoA (RhoA-L63) or dominant negative RhoA (RhoA-N19). Luciferase reporter assays showed prominently increased promoter activities for the AhR binding sites in the promoter region of RhoA. Inhibition of RhoA suppressed co-exposure-induced AHR, Th2-associated airway inflammation and TGFβ1 signaling activation in asthma.

Conclusions: Our studies identified a functional axis of AhR-RhoA that regulates TGFβ1 signaling activation, leading to allergic airway inflammation and asthma.

KEYWORDS

Benzo(a)pyrene (BaP), dermatophagoides group 1 allergen (Der f 1), Aryl hydrocarbon receptor, RhoA, TGFβ1.

Abbreviations

HDM	House dust mite
BaP	Benzo(a)pyrene
Der f 1	Dermatophagoides group 1 allergen
16HBEC	Human bronchial epithelial cell
DEP	Diesel exhaust particulates
AhR	Aryl hydrocarbon receptor
RhoA	Ras homolog family member A
BALF	Bronchoalveolar lavage fluid
AHR	Airway hyper-responsiveness
DRE	Dioxin-responsive element

INTRODUCTION

Air pollution, especially exposure to particulate matter, is one of the major risk factors for the increased prevalence of childhood asthma worldwide[1]. High levels of particulate matter [i.e., diesel exhaust particles (DEP)] can enhance the risk of atopic sensitization and exacerbation of asthma[2-4]. Especially, prenatal exposure to diesel exhaust particulates (DEP) or DEP-derived polycyclic aromatic hydrocarbons (PAHs) has been shown to be associated with atopic sensitization, early childhood wheeze, and asthma[5,6]. Most importantly, DEP co-exposure with house dust mite (HDM) can exacerbate allergic sensitization and induce key features of asthma[7-11]. So far, many epidemiological and clinical studies have suggested a significant link between exposure to environmental pollutants and allergens and allergic airway inflammation and asthma. However, the causal relationship and underlying molecular mechanisms are poorly characterized.

Benzo(a)pyrene (BaP) is one of the large number of polycyclic aromatic hydrocarbons (PAHs) that are formed during the incomplete combustion of organic matter[12]. Mixtures of PAHs including BaP are ubiquitous in the air because they are generally derived from cigarettes, barbecue grills, automobile exhausts, and industrial combustion[12,13]. Exposure to BaP alone has been shown to induce oxidative stress, bronchial epithelium injury, and inflammation[14-16]. Mechanistically, BaP can directly activate aryl hydrocarbon receptor (AhR) and induce IL-33 expression and eosinophil infiltration in a mouse model of allergic airway inflammation[17] and elicits T-helper 2-driven pro-inflammatory responses in a mouse model of allergic dermatitis[18]. AhR as a ligand-activated transcription factor can be activated by small molecules in various diets, metabolites, microorganisms, and pollutants[19-24] and plays an important role in the regulation of innate and adaptive immune responses[25-27]. Of note, we have recently made a novel finding that BaP co-exposure with dermatophagoides group 1 allergen (Der f 1) can potentiate Der f 1-induced airway

hyper-responsiveness (AHR) and lung inflammation[28]. Particularly, BaP co-exposure can promote Der f 1-induced oxidative stress (ROS) and IL-25, IL-33, and TSLP release from airway epithelial cells. Furthermore, BaP exposure activated AhR can promote Der f 1-induced oxidative stress and epithelial cytokine release that contribute to the enhanced allergic airway inflammation. However, the relationship between BaP activated AhR and airway inflammation is still poorly understood.

Transforming growth factor β1 (TGFβ1) is a pleiotropic regulator of immune responses and plays an important role in cell growth, differentiation, migration, and activation depending on environmental trigger, cell type, and microenvironment[29-34]. Airway epithelial cells are a major source of pulmonary TGFβ1 and activation of TGFβ1 signaling is essential in allergen-induced exacerbation of AHR and airway inflammation in asthma[33,35]. Thus, it is critical to determine the molecular mechanisms regarding the regulation of TGFβ1 signaling, which may provide novel insights into the mechanisms that lead to allergic airway inflammation. The ras homolog family member A (RhoA) of the Rho family GTPases has been considered to be one of the most promising and novel therapeutic targets for asthma[36]. Studies including ours have suggested a positive loop between RhoA/Rho-kinase signaling and TGFβ1 that drives airway constriction, airway hyper-responsiveness, and airway remodeling in asthma[37-40], raising the possibility that RhoA/Rho-kinase signaling may be one of the central pathways in regulating allergen-induced TGFβ1 activation.

In the current study, we demonstrate that BaP co-exposure exacerbates Der f 1 induced TGFβ1 signaling activation in airway epithelial cells and airways of asthma mouse model. Furthermore, the activation of AhR by BaP co-exposure plays a critical role in Der f 1-induced activation of TGFβ1 signaling. Importantly, we made novel findings that BaP activated AhR can bind RhoA and regulate RhoA/Rho-kinase activation, and inhibition of RhoA significantly suppresses co-exposure-induced AHR, Th2-associated airway inflammation, and TGFβ1 signaling in a mouse model of asthma.

MATERIALS AND METHODS

Animals

The experimental protocols in this study were reviewed and approved by the Animal Care and Use Committee in in Peking University Shenzhen Graduate School, and was in accordance with the guidelines and regulations of the institution. Both male and female C57BL/6 mice aged 6-8 weeks were purchased from experimental animal center of Guangdong province. Animals were maintained under specific pathogen-free conditions at the animal facility of Shenzhen University.

Bap co-exposure with Der f 1-induced mouse model of asthma

Generation of asthma mouse model was established as previously described[28]. Briefly, both male and female mice were sensitized and challenged every other week for 6 weeks with intranasal administrations of 25 μg Der f 1 (Indoor biotechnologies) under isoflurane anesthesia. BaP (Sigma-Aldrich) was dissolved in borate saline buffer (BBS, Sigma-Aldrich), and intranasally administered at a concentration of 20μM once every week during Der f 1 sensitization and challenge. In some cases, mice were pre-treated with Fasudil or Y-27632 at a dose of 30 mg/mL by intraperitoneal administration 1 h prior to every single BaP treatment. Age- and gender-matched control mice were treated with PBS.

Measurement of airway hyper-responsiveness

Airway hyper-responsiveness (AHR) was measured 24 h after the last challenge with whole-body plethysmography (Buxco Europe Ltd, Winchester, UK) as previously described[28]. Briefly, mice were exposed to increasing doses of methacholine (Mch) (Sigma-Aldrich, St Louis, USA) at 6.25, 12.5, 25, 50, and 100 mg/mL or PBS. Tests at two different concentrations were temporally separated to allow the respiratory intensity to drop back to baseline. The percentage curves for Penh values at different Mch doses were plotted, starting with PBS stimulation.

Broncheoalveolar lavage

Mice were sacrificed and broncheoalveolar lavage (BAL) was performed by instillation of 0.8 ml of PBS through a tracheal cannula. BAL samples were centrifuged at 1500 rpm for 5 min at 4℃. Supernatants were collected and stored at -80℃ for cytokine analysis, and cells were stained for the analysis of cellular compositions by Wright-Giemsa staining on microscope slides. The total number of eosinophils, neutrophils, and macrophages was determined by counting 200 leukocytes in randomly selected areas of the slides under a light microscopy.

Lung pathology

The whole lung was fixed in 4% formalin for 24 h and embedded in paraffin after dehydration in alcohol. Lung sections (3 μM) were stained with hematoxylin and eosin (H&E) and PAS solution for histopathological analysis following the protocols as previously described[28].

Cytokine determination

IL-4, IL-5, IL-13, IFNγ, IL-17A, IL-25, IL-33, TSLP, and TGFβ1 in BALFs were measured by ELISA (Sizhengbo Inc. Beijing, China) according to the manufacturer's instructions.

HDM-specific IgE, IgG1, and IgG2α detection

The levels of Der f 1-specific IgE, IgG1, and IgG2α in serum samples were determined by a standard ELISA as previously described[28]. Briefly, five-fold diluted serum was loaded in HDM-coated 96 well plate overnight at 4℃, followed by blocking and then adding biotinylated IgE, IgG1, IgG2α for 2 h, respectively. After washing, the plate was added the streptavidin-labeled horseradish peroxidase (HRP; 1:5000 dilution) and measured with a microplate reader at 450 nm and corrected by 540 nm.

Immunofluorescence staining

Sectioned lung tissues were first blocked using 5% w/v BSA for 1 hour, followed by incubation with

192 the primary antibodies against Smad3 (EP568Y, Abcam), phospho-Smad3 (ab52903; Abcam), TGFβ1
193 (Ab179695; Abcam), RhoA-GTPase (26904; New East Biosciences), AhR (Ab84833; Abcam) and
194 EpCAM (G8.8; ThermoFisher), respectively, overnight at 4°C. Sample sections were then incubated
195 with secondary antibodies conjugated with Alexa Fluor dyes (ThermoFisher) at room temperature for
196 1 hour. Isotype-matched negative control antibodies (R&D Systems) were used under the same
197 conditions. Nuclei were counterstained with 6-diamidino-2-phenylindole, dihydrochloride (DAPI,
198 Solarbio, Beijing, China). Sections were mounted with the ProLong Gold Anti-fade Kit (Molecular
199 Probes) and observed with a Nikon Eclipse Ti-U microscope equipped with a DS-Fi2 camera
200 (Nikon). To determine the fluorescence signal in tissue sections, fluorescent-positive cells in five
201 different high-power fields from each slide were quantified using ImageJ v1.50e (NIH) and
202 presented as mean fluorescence intensity per square micrometer. Two to three slides from each
203 sample were used for analysis.

204 **Western blotting**

205 Western blotting was performed as previously described[28]. Briefly, collected cells were lysed in RIPA
206 buffer (Sigma-Aldrich) containing Protease and Phosphatase Inhibitor Cocktails (Solarbio Inc.
207 Beijing China). Protein concentration was measured using a BCA Protein Assay kit (Pierce).
208 Aliquots of 30–50 μg protein samples were used for SDS-PAGE electrophoresis and then transferred
209 to a polyvinylidene difluoride membrane (Invitrogen). After blocking with 5% non-fat milk in TBST,
210 the membrane was incubated with anti-RhoA GTPase (New East Bioscience, PA), AhR (Abcam),
211 phosphor-Smad3 (Abcam) and total Smad3 (Abcam) or anti-β-actin antibodies. Detailed information
212 are included in the Online Repository (**Table S1**). Blots were visualized with an HRP-conjugated
213 secondary antibody (Sizhengbo Inc. Beijing, China) and ECL Western blotting detection system (GE
214 Life Sciences). Relative protein expression was determined by densitometric analysis using ImageJ

(NIH).

Cell culture and transfection

Human bronchial epithelial cells (16HBECs) were purchased from Haoge biological company (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37˚C in a humidified atmosphere at 5% CO_2. 16HBCEs were transfected with a plasmid expressing a constitutively active RhoA (RhoA-L63) or a dominant negative RhoA (RhoA-N19) or empty vector (EV) as previously described[37]. The efficiency of transfection was determined by Western blotting.

AhR activity determination

The AhR reporter plasmids expressing the GFP gene under the control of the dioxin-responsive element (DRE) sequence (PGL3-6xDRE (WT)-GFP, PGL3-6xDRE (mut)-GFP) were generated as illustrated in Fig E, 1. Both AhR reporter plasmids were transfected into 16HBECs and cultured for 24 h. The 16HBECs containing the Aryl hydrocarbon receptor DRE reporter plasmid were stimulated with different doses of BaP (0.01-10 μM) or Der f 1 (7.5-60 μg/mL) or combined BaP (1 μM) and Der f 1 (30 μg/mL) for 24 h, and then lysed. Supernatants were harvested and plated in a 96-well plate for the quantification of GFP with the Multi-Mode Microplate Reader (BioTek, US). The results were normalized on the basis of the negative GFP of the control.

Plasmid construction

The RhoA promoter construct was generated as previously described[41]. Briefly, five constructs containing different truncated lengths of the *RhoA* promoter regulatory sequences were generated with mouse genomic DNA and the forward and reverse primers incorporating MluI and XhoI sites at the 5′ and 3′ ends, respectively. Both the amplified DNA products and pGL3-Basic Vector (Promega) were digested by MluI and XhoI enzymes, and then linked by using ClonExpress II kit (Vazyme).

The QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate the constructs for site-directed mutation. All of the above constructs were verified by sequencing. All primers used are listed in Supplementary Table E2.

Luciferase reporter assays

For luciferase reporter assays of promoter activity, 16HBECs were transfected with RhoA promoter plasmids and pGL3-basic (RiboBio) using Lipofectamine 2000 (Thermo Fisher Scientific) for 24 h, and then treated with BaP (1 μM). After 24 h, these transfected and treated 16HBECs were lysed using RIPA buffer (Solarbio Beijing, China) and centrifuged. The supernatants were used for the measurement of luciferase activity in a Multi-Mode Microplate Reader (BioTek, US). The promoter activity was presented as a fold change in related to the luciferase activity of empty plasmid.

Statistical analysis

All data were analyzed with Graph Pad Prism version 5.1 software (GraphPad Software, La Jolla, CA) and are expressed as mean ± SEM. Statistical significance for normally distributed samples was assessed using an independent two-tailed Student's *t* test or with one-way ANOVA one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value <0.05 were considered statistically significant for all analyses. All experiments *in vitro* were performed two or more times independently under identical or similar conditions.

RESULTS

BaP co-exposure exacerbates Der f 1 induced TGFβ1 signaling activation

Airway epithelial cells are one of the major sources of TGFβ1 and activation of TGFβ1 signaling plays an important role in the allergen-induced AHR and airway inflammation in asthma[33,35]. Thus, we investigated whether BaP alone or co-exposure can induce or exacerbate TGFβ1 production in the airway epithelial cells. As expected, BaP exposure significantly induced TGFβ1 production in 16HBECs in a dose (Figure 1A) and time (Figure 1B)-dependent response. Furthermore, Der f 1 exposure can also induce TGFβ1 production, which was further potentiated by BaP co-exposure (20 pmol) in 16HBECs (Figure 1C). To confirm the BaP co-exposure-induced TGFβ1 expression in airway epithelial cells, we detected TGFβ1 in the airway epithelial cells of asthma mouse model by co-immunofluorescent staining TGFβ1 with epithelial cell marker EpCAM. As expected, a significant increased expression was observed for TGFβ1 in the airway epithelial cells of either BaP or Der f 1-treated mice (Figure 1D, E), which was further enhanced in mice when exposed to both BaP and Der f 1. Of note, the same pattern was observed for Smad3, downstream to TGFβ1 signaling (Figure 1D, F). Collectively, these results suggest that BaP co-exposure can potentiate Der f 1-induced epithelial TGFβ1 release and signaling activation.

BaP co-exposure promotes BaP-induced AhR activity

BaP is one of the ligands to AhR[17]. We have previously shown that BaP can induce expression of AhR and its major downstream gene *CYP1A1* in 16HBECs[28]. Here, we extended to examine whether BaP exposure alone or co-exposure can directly induce activation of AhR signaling by transfecting an AhR-DRE reporter plasmid (Figure 2A). The GFP gene under control of a tandem repeats of the dioxin-responsive element (DRE) (PGL3-6xDRE (WT)-GFP, PGL3-6xDRE (mut)-

GFP). The PGL3-6xDRE (WT)-GFP or PGL3-6xDRE (mut)-GFP plasmid was transduced in 16HBECs and cultured for 24 h, and then treated with different doses of BaP (0.01-10 μM), Der f 1 (7.5-60 μg/mL). BaP exposure induced AhR activation as assessed by luciferase activity in a dose-dependent manner (Figure 2B). By contrast, a dose-dependent AhR activation was not noted for Der f 1 exposure (Figure 2C). Of interest, the cultures treated with both BaP and Der f 1 showed significant increase in AhR activation in relative to those treated with BaP alone (Figure 2D), indicating that BaP co-exposure can enhance BaP-induced AhR activity in the airway epithelial cells.

AhR is critical in BaP co-exposure induced TGFβ1 signaling activation

We further investigated whether active AhR plays a role in TGFβ1 release from airway epithelial cells. 16HBECs were pre-treated with AhR antagonist CH223191 and then exposed to either BaP or Der f 1, or combined for 24 h, levels of TGFβ1 in supernatants were measured. Of note, BaP co-exposure-induced TGFβ1 production was almost completely blocked in CH223191 pre-treated 16HBECs (Figure 3A). To further understand the role of AhR in TGFβ1 secretion, AhR in 16HBECs was knocked down by siRNA and confirmed by Western blotting (Figure 3B). Consistent with the findings with AhR antagonist CH223191, levels of TGFβ1 were reduced in AhR deficient 16HBECs after exposed to BaP alone or BaP co-exposure with Der f 1 (Figure 3C). Furthermore, the Western blotting illustrated that BaP alone or co-exposure induced expression of phosphorylated Smad3 (p-SMAD3), which was reduced in 16HBECs with AhR knockdown (Figure 3D, E). These findings imply that AhR activation is essential in mediating BaP-induced airway epithelial TGFβ1 release and signaling activation.

RhoA mediates the regulation of AhR on BaP co-exposure induced TGFβ1 production

To explore the underlying mechanisms as for how AhR activation regulates TGFβ1 secretion and signaling activation, we specifically investigated whether RhoA/Rho-kinase signaling plays a role in BaP co-exposure-induced epithelial TGFβ1 release and signaling activation. Previous studies have suggested a feed forward connection between RhoA signaling and TGFβ1[37-40], raising the possibility that RhoA/Rho-kinase signaling may function as a central player in connecting the upstream BaP-induced AhR activation with downstream TGFβ1 production. To test whether AhR, upstream to RhoA, is involved in regulating BaP co-exposure-induced active RhoA, we performed western blot analysis to detect the expression of active RhoA in16HBECs with or without AhR knockdown. Increased expression of active RhoA (RhoA-GTP) was detected in 16HBECs after exposed to either BaP (1μM) or Der f 1 (30μg/mL) alone or in combination (Figure 4A, B). Of note, the increased expression of RhoA-GTP was clearly reduced in 16HBECs with AhR knockdown, particularly in BaP or co-exposure-treated 16HBECs, suggesting that AhR regulates BaP or co exposure-induced RhoA/Rho-kinase activation in 16HBECs. Next, we examined the role of active RhoA in BaP co-exposure-induced downstream TGFβ1 production by using fasudil, a selective RhoA/ROCK inhibitor[42]. Pretreatment of 16HBECs with fasudil showed a dose-dependent inhibition in BaP-induced TGFβ1 secretion (Figure 4C). The inhibition was observed not only for BaP, but also for Der f 1 or BaP co-exposure-induced TGFβ1 secretion (Figure 4D). To further confirm the role of active RhoA in regulating TGFβ1 secretion and signaling activation, we used 16HBECs expressing either a constitutively active RhoA (RhoA-L63) or dominant-negative (RhoA-N19) (Figure 4E). Compared to 16HBECs, 16HBECs expressing RhoA-L63 secreted higher levels of TGFβ1 in response to BaP alone or BaP co-exposure with Der f 1 (Figure 4F). In contrast, no clear change was noted for TGFβ1 secretion in 16HBECs expressing RhoA-N19 (Figure 4G).

The same patterns were observed for p-SMAD3 in 16HBECs expressing RhoA-L63 or RhoA-N19 (Figure 4H, I). Collectively, these results suggest that RhoA may play an important role in mediating the regulation of AhR on BaP co-exposure-induced TGFβ1 production and signaling activation.

AhR directly binds RhoA and participates the BaP-induced RhoA activity

To determine whether AhR regulates RhoA activation through a direct interaction with RhoA, we searched for the AhR-binding sites (T/CGCGTG, Figure 5A) in the promoter region of RhoA by using *in silico* analysis with BiBiServ RNAhybrid, a program that predicts multiple potential binding sites[43]. Eight putative AhR binding sites were predicted that may affect transcriptional activation (Figure 5B)**.** To confirm the significance of these binding sites, we performed luciferase reporter assays with luciferase reporter vector pGL-3 basic with various truncated fragments of the RhoA promoter containing AhR binding sites (Figure 5C). A total of five different truncated lengths of the *RhoA* promoter regulatory sequences were amplified and the PCR products were cloned into the pGL3-basic vector. All fragments showed a better response to BaP as compared to those with pGL3-basic vector. Of these, fragments containing AhR-binding site 6-8 and site 7-8 showed prominently increased promoter activity. To confirm the significance of binding sites in regulating promoter activity of RhoA, we mutated the binding site 6 or 7 and examined their effects on promoter activity. Compared to wild-type, the fragment containing mutated binding site 6 or site 7 showed significantly reduced promoter activity (Figure 5D). Furthermore, we examined the co-localization between AhR and RhoA in the lung tissues of asthma mouse model (Figure 5E) and found a clear overlapping between AhR and RhoA-GTP in the airways of either BaP or Der f 1 alone or co-exposure-treated mice, supporting that there might be an interaction between AhR and

RhoA. Collectively, these findings indicate that AhR may regulate BaP-induced RhoA activity in the airways via directly binding the promoter region of RhoA.

Inhibition of RhoA suppresses BaP co-exposure-induced AHR and airway inflammation

Given that AhR can regulate RhoA activation, we examined whether RhoA plays a role in BaP co-exposure-induced AHR and airway inflammation by using RhoA inhibitors fasudil and Y-27632 in our mouse model of asthma as illustrated in Figure 6A. BaP co-exposure induced an increased airway resistance when compared with either BaP or Der f 1 alone. Of note, the increased airway resistance was significantly inhibited by either fasudil or Y-27632 treatment (Figure 6B). Consistently, histological analysis demonstrated that BaP co-exposure increased airway inflammation as assessed by peribronchial inflammation (H&E, upper panel) and goblet cell hyperplasia (PAS, lower panel) (Figure 6C). Of interest, the increased lung inflammation was significantly abrogated when these BaP co-exposure challenged mice were pre-treated with either fasudil or Y-27632 treatment. The same pattern was also observed for total cell counts in BAL samples, particularly eosinophils and macrophages (Figure 6D), and serum levels of Der f 1 specific IgE, IgG1, and IgG2a (Figure 6E). The results suggest that inhibition of RhoA suppresses Bap co-exposure-induced AHR and airway inflammation.

Inhibition of RhoA suppresses BaP co-exposure-induced cytokine release

We have specifically investigated whether RhoA participates in the regulation of cytokine production. As compared to Der f 1 alone, we found that BaP co-exposure enhanced the production of IL-4, IL-5, IL13, IFNγ, IL-17, and IL-10 in BAL samples. As expected, the increase cytokine release was remarkably inhibited in mice pre-treated with either fasudil or Y-27632 as compared to

vehicle control (Figure 7A). Furthermore, we detected whether inhibition of RhoA can affect BaP co-exposure induced epithelial cytokine release. We found higher levels of IL-25, IL-33, TSLP, and TGFβ1 in BAL samples of BaP co-exposure-treated mice as compared to those pre-treated with Der f 1. Of note, all of these increased cytokines were remarkably inhibited by either fasudil or Y-27632 (Figure 7B), indicating that inhibition of RhoA suppresses BaP co-exposure-induced airway epithelial-derived cytokine release.

Inhibition of RhoA suppresses BaP co-exposure-induced TGFβ1 signaling in a mouse model of asthma.

We further confirmed the role of RhoA in TGFβ expression and signaling activation in the airway epithelium of asthma mouse model by co-immunostaining with epithelial marker EpCAM. Notably, BaP co-exposure enhanced TGFβ1 expression in the airway epithelial cells (Figure 7C, D). The increased expression was significantly reduced in those treated with either fasudil or Y-27632. Similar pattern was also observed for p-Smad3 (Figure 7C, E). BaP co-exposure promoted p-Smad3 expression, which was inhibited by either fasudil or Y-27632 treatment. Together, the data provide a mechanistic explanation for the role of AhR-regulated RhoA activation in epithelial cell TGFβ1 release, signaling activation, and subsequently Th2 –associated allergic airway inflammation.

DISCUSSION

It is evident that environmental pollutants can bind different allergens and facilitate them transporting into deep airways[44,45]. DEP exposure has been shown to increase allergen concentration and their potential for driving asthma attacks[7-11]. Particularly, epidemiological studies have suggested that allergic children with early-life exposure to high levels of DEPs showed a higher prevalence of asthma compared with those non-allergic children[46]. These findings were supported by studies from asthma mouse models that DEP co-exposure can exacerbate HDM-induced IgE, inflammatory cells, and Th2/Th17 cytokines[46,47]. However, the underlying mechanisms as for how DEP exposure enhances allergen-induced airway inflammation remain largely unknown. Airway epithelium lining the upper and lower respiratory tract forms the first line of defense of the airway and lungs against inhaled pathogens and environmental pollutants[48-50]. We and others have previously demonstrated that environmental pollutants can promote allergen-induced release of ROS and epithelial-derived cytokines (e.g., IL-25, IL-33, and TSLP) that can enhance DC maturation and type 2 innate lymphoid cells (ILC2) and Th2 responses[28,51,52]. Furthermore, AhR, as a dioxin receptor and environmental sensor, has been suggested to mediate DEP component-induced oxidative stress, epithelial cytokine release, Th17 immune responses, and allergic airway inflammation[28,47]. In the current study, we made a novel finding that BaP co-exposure can enhance Der f 1-induced TGFβ1 release and signaling activation that may contribute to the exacerbation of allergic airway inflammation. Most importantly, we identified a functional axis of AhR-RhoA that regulates epithelial TGFβ1 release and signaling activation.

TGFβ1 regulates fundamental cell biological functions, including cell growth, differentiation, migration, and activation[29-34]. There is increasing evidence to demonstrate that TGFβ1 is one of the major immune-regulatory cytokines from airway epithelial cells that recruits various immune cells

to the airways and interact with them to regulate AHR and airway inflammation in asthma[33,35]. On one hand, TGFβ1 can modulate lineage differentiation of T cells into different effector T cell subsets[53]. On the other hand, TGFβ1 is a cofactor of innate lymphoid cells to induce type-2 airway inflammation and key features of allergic diseases[33,54]. Thus, it is pivotal to determine how the increased TGFβ1 production is regulated, which offers novel insights into the mechanisms that lead to allergic airway inflammation. Here we demonstrated that BaP co-exposure could promote Der f 1-induce TGFβ1 production in the airway epithelial cells by using human primary bronchial epithelial cells and mouse models of asthma. Furthermore, the increased expression was also observed for p-Smad3, a downstream molecule of canonical TGFβ signaling, indicating that BaP co-exposure can potentiate Der f 1-induced epithelial TGFβ1secretion and signaling pathway activation.

Next, we explored how AhR regulates TGFβ1 activation and signaling pathway. AhR as a ligand-activated transcription factor senses and responds to environmental stimuli and regulates normal cell development and innate and adaptive immune responses[19-27]. Our previous studies have suggested that AhR controls mast cell growth and activation[55], cockroach allergen-induced immune responses in human fibroblast[56], and allergic airway inflammation in a mouse model of asthma[34]. Furthermore, we found that BaP exposure can activate AhR that subsequently leads to a promotion of Der f 1-induced oxidative stress and IL-25, IL-33, and TSLP release from epithelium[28]. Our findings were supported by some of other studies that BaP can directly induce AhR activation that contributes to the pro-inflammatory response in respiratory allergy through enhancing IL-33 expression and eosinophil infiltration[17]. Our current study further confirmed that BaP can directly activate AhR in 16HBECs as determined by a DRE-GFP reporter containing the GFP gene under control of a DRE tandem repeats[57]. GFP is only expressed when AhR ligands bind to DRE.

Intriguingly, we noted that BaP co-exposure showed clearly increased activation of AhR, suggesting a synergistic effect between BaP and Der f 1. However, the underlying mechanisms as for how both BaP and Der f 1 induce increased AhR activation still remain to be determined. Furthermore, we illustrated that AhR activated by BaP modulates TGFβ1 release from airway epithelial cells. In particular, BaP co-exposure enhanced Der f 1-induced levels of TGFβ1 in 16HBECs, but which were attenuated by AhR antagonist CH223191 or reduced in 16HBECs with AhR knockdown. The same result was observed for p-Smad3. Collectively, our findings provided evidence that active AhR is critical in controlling the activation of the TGFβ1/Smad3 signaling axis in airway epithelial cells.

To further address the relative contribution of AhR in the activation of the TGFβ1/Smad3 signaling axis, we studied its underlying mechanisms by focusing on RhoA/Rho-kinase signaling. RhoA is a nucleotide-dependent protein, switching between an inactive form, GDP-bound, and an active form, GTP-bound RhoA, and has been shown to regulate various biological functions, including cell differentiation, differentiation, recruitment, and apoptosis[36,58,59]. An increased expression and activation of RhoA have been associated with asthma[60-63]. Importantly, several studies including ours have suggested a feed forward circuit between RhoA signaling and TGFβ1 that drives airway constriction, airway hyper-responsiveness, and airway remodeling in asthma[37-40]. Thus, it is possible that RhoA/Rho-kinase signaling may function as a central player in connecting the upstream BaP-induced AhR activation with downstream TGFβ1 production and signaling activation. This was, indeed, supported by our several observations. First of all, we showed that BaP co-exposure induced increased activation of RhoA/Rho-kinase signaling when compared with either BaP or Der f 1 alone. Next, we provided evidence that BaP exposure induces activation of RhoA/Rho-kinase signaling through AhR. Eight putative AhR binding sites in the promoter region

462 of RhoA were predicted by using *in silico* analysis. These AhR binding sites were validated by
463 luciferase reporter assays with different truncated fragments of the RhoA promoter. All fragments
464 showed an increased promoter activity in response to BaP treatment. The relative role of AhR
465 binding sites in BaP-induced promoter activity was further confirmed by using the RhoA fragments
466 containing the mutated binding sites. Additionally, we found a co-locolization between AhR and
467 RhoA-GTP in the airways of asthma mouse model, indicating a possible interaction between AhR
468 and RhoA-GTP. Of interest, we demonstrated for the first time that Der f 1 alone can also induce
469 the activation of RhoA/Rho-kinase signaling. While the reason is unknown, further studies would
470 be of interest to determine whether Der f 1 either directly activate RhoA or induce RhoA activation
471 through its possible receptor PAR2, C-type lectin receptors (CLRs) or Toll-like receptors (TLRs).
472 Lastly, we provided evidence that active RhoA/Rho-kinase signaling is critical in BaP-induced
473 TGFβ1 production and signaling activation. RhoA/ROCK inhibitor fasudil significantly inhibited
474 BaP co-exposure induced TGFβ1 secretion from airway epithelial cells. This finding was further
475 confirmed in 16HBECs expressing a constitutively active RhoA-L63. 16HBECs expressing RhoA-
476 L63 secreted higher levels of TGFβ1 in response to BaP alone or BaP co-exposure with Der f 1.
477 These findings suggest that active RhoA/Rho-kinase signaling modulates BaP co-exposure-induced
478 TGFβ1 production and signaling activation. Together with the previous findings[37-40], our studies
479 suggest that the increased TGFβ1 signaling activation (e.g, p-Smad3) may, in turn, promote RhoA
480 activation. Collectively, these findings imply that BaP promotes RhoA activation through AhR,
481 thereby leading to the activation of the TGFβ1/Smad3 signaling axis. Importantly, our findings
482 provided further evidence that there may be a feed forward connection between RhoA activation
483 and TGFβ1 signaling activation (See graphic summary).

484 In light of the significance of active RhoA in the activation of the TGFβ1/Smad3 signaling

485 axis, our further study explored the potential in therapeutically translating these findings into the
486 treatment of allergic asthma. Thus, we specifically investigated the role of RhoA in BaP co-
487 exposure-induced AHR and allergic airway inflammation in asthma by using RhoA inhibitors.
488 Previous studies have demonstrated that RhoA/Rho-kinase plays an important role in regulating
489 airway inflammation, which may be through affecting differentiation, recruitment, and activation of
490 various inflammatory cells (e.g. eosinophils, macrophages, mast cells and T cells) in the
491 pathogenesis of asthma[36,62,64]. Of these, RhoA was showed to control Th17 cell differentiation and
492 house dust mite-induced allergic airway inflammation[62]. Our findings provided further evidence
493 that active RhoA/Rho-kinase signaling is pivotal in BaP co-exposure-induced AHR and Th2-
494 associated inflammation by using RhoA inhibitors fasudil and Y-27632. One of the striking
495 findings is that inhibition of active RhoA/Rho-kinase signaling suppresses the BaP co-exposure-
496 induced increased activation of the TGFβ1/Smad3 signaling pathway by both *in vitro* and *in vivo*
497 studies. These results provide a mechanistic explanation for the role of RhoA/Rho-kinase signaling
498 in AHR, Th2–associated allergic airway inflammation, and activation of the TGFβ1/Smad3
499 signaling pathway.

500 In summary, we have demonstrated that BaP co-exposure exacerbates Der f 1 induced
501 activation of the TGFβ1/Smad3 signaling axis in airway epithelial cells. Mechanistically, AhR is
502 critical in controlling the BaP co-exposure-induced activation of the TGFβ1/Smad3 signaling axis.
503 Furthermore, we provided novel evidence that RhoA/Rho-kinase signaling plays an important role
504 in connecting the upstream BaP-induced AhR activation with downstream TGFβ1 production and
505 signaling activation, and there is a feed forward connection between RhoA activation and TGFβ1
506 signaling activation. Our further study revealed that inhibition of active RhoA/Rho-kinase signaling
507 significantly suppressed BaP co-exposure-induced AHR, Th2-associated airway inflammation, and

airway epithelial cytokine release in a mouse model of asthma (Figure 8). Collectively, our studies identified a functional axis of AhR-RhoA that regulates the activation of the TGFβ1/Smad3 signaling, thereby leading to the development of allergic airway inflammation and asthma. Blockage of the AhR-RhoA axis represents a promising novel therapeutic approach for treatment of allergic asthma.

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Author contributions

E.W., W.T., D.D. performed experiments, analyzed data, and review the manuscript. E.W. and P.G. wrote the manuscript. S.H., Z. L., P.G. designed and supervised the study, and wrote the manuscript. L.Y., D.S., D.X. and P.Y. provided intellectual input and aided in the experimental design. All authors read and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. BaP co-exposure exacerbates Der f 1 induced TGFβ1 signaling activation. **A-B,** Levels of TGFβ1 in supernatants of 16HBECs treated with BaP at varying doses (0.001-1μM, **A**) and different times (6-24h, **B**). **C,** Levels of TGFβ1 in supernatants of 16HBECs treated with BaP (1μM) and Der f 1 (30μg/mL) alone or in combination. **D**, Representative immunofluorescence images of TGFβ1 (green) and p-SMAD3 (green) expression in the airway epithelial cells (EpCAM, red) of asthma mouse model. **E-F,** Quantitative analysis of TGFβ1 (**E**) and p-Smad3 (**F**) florescent signal (n=6 mice/group). (**A**-**C**). Data represent mean ± SEM of three independent experiments. **p*<0.05, ***p* <0.01, and ****p*<0.001.

Figure 2. BaP co-exposure promotes Der f 1 induced AhR activity. **A,** Schematic of experimental workflow for AhR reporter plasmid. **B-D**, Relative AhR activity in BaP (**B**), Der f 1 (**C**), and BaP + Der f 1 (**D**) treated 16HBECs that were transfected with AhR reporter plasmid. AhR activity was assessed by GFP quantification and expressed as fold changes in relative to control. Data represent mean ± SEM of three independent experiments. **p*<0.05, ***p* <0.01, and ****p*<0.001.

Figure 3. AhR mediates BaP co-exposure induced TGFβ1 signaling activation. **A,** Levels of TGFβ1 in supernatants of 16HBECs treated with BaP (1μM) and Der f 1 (30μg/mL) alone or in combination in the presence or absence of CH223191 (10μM). **B**, Western blot analysis of AhR knockdown in 16HBECs. **C**, Levels of TGFβ1 in supernatants of 16HBECs with or without AhR knockdown after exposed to BaP (1μM) and Der f 1 (30μg/mL) alone or in combination. **D**, Western blot analysis of p-Smad3 expression in BaP co-exposure treated 16HBECs with or without AhR knockdown. **E**, Quantitative analysis of western blots in (**D**). Data represent mean ± SEM of at least three independent experiments. **p*<0.05, ***p* <0.01, and ****p*<0.001.

Figure 4. RhoA mediates the regulation of AhR on BaP co-exposure induced TGFβ1 production

and signaling activation. **A,** Western blot analysis of RhoA expression in BaP (1μM) and Der f 1 (30μg/mL) or combination treated 16HBECs with or without AhR knockdown. **B,** Quantitative analysis of Western blots in (**A**). **C**, Levels of TGFβ1 in supernatants of BaP-treated 16HBECs with or without fasudil at varying doses (0.03 to 0.3μM). **D**, Levels of TGFβ1 in supernatants of BaP co-exposure-treated 16HBECs in the presence or absence of fasudil (0.3μM). **E**, Western blot analysis of RhoA expression in 16HBECs expressing either a constitutively active RhoA (RhoA-L63) or dominant-negative (RhoA-N19). **F-G**, Levels of TGFβ1 in supernatants of BaP (1μM), Der f 1 (30μg/mL), and co-exposure-treated 16HBECs expressing either RhoA-L63 (**F**) or RhoA-N19 (**G**). **H-J**, Western blot analysis of p-Smad3 expression in BaP (1μM), Der f 1 (30μg/mL), and co-exposure-treated 16HBECs expressing either RhoA-L63 (**H, I**) or RhoA-N19 (**H**, **J**). Data represent mean ± SEM of at least three independent experiments. *p<0.05, **p <0.01, and ***p<0.001.

Figure 5. AhR directly binds RhoA and participates the BaP-induced RhoA activity. **A,** Sequence of AhR binding site. **B**, Eight putative AhR binding sites in the promoter region of RhoA. **C**, Luciferase reporter assays with luciferase reporter vector pGL-3 basic (empty) or containing serially truncated fragments of the RhoA promoter (AhR reporter) in 16HBECs. **D**, Luciferase reporter assays with luciferase reporter vector pGL-3 basic containing wild-type or mutated truncated fragments in 16HBECs. **E**, Representative immunofluorescence images of co-localization of AhR and RhoA in lung tissues of mouse model. Data represent mean ± SEM of at least two independent experiments. *p<0.05, **p <0.01, and ***p<0.001.

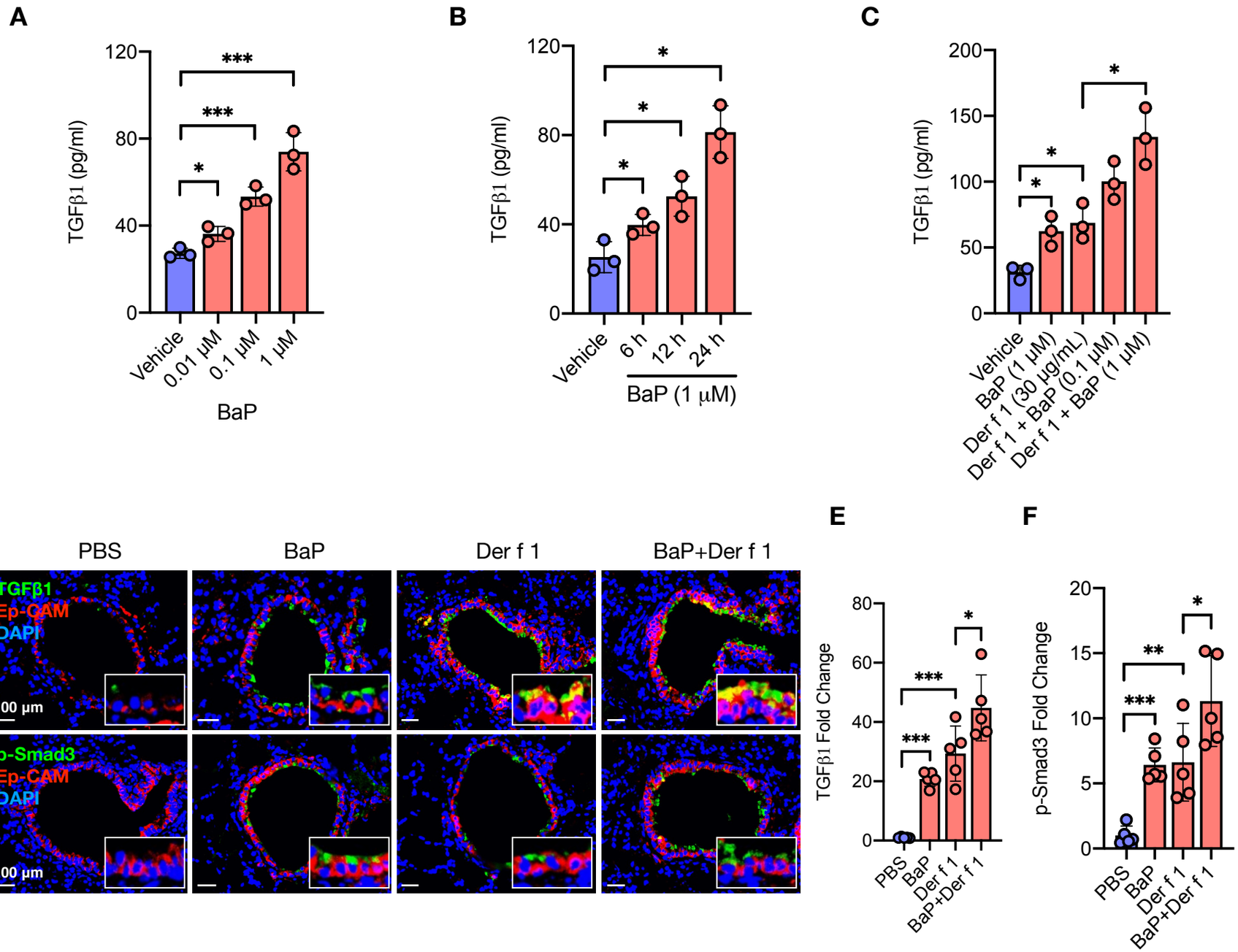
Figure 6. Inhibition of RhoA suppresses BaP co-exposure-induced AHR and Th2-associated airway inflammation. **A**, Protocol for BaP (20 μM) and Der f 1 (25μg) co-exposure induced mouse model of asthma in the presence or absence of Fasudil or Y-27632 (30 mg/mL, i.p.). **B**, Lung resistance in response to increasing concentrations of methacholine using the forced oscillation technique

(FlexiVent, SCIREQ). **C,** Histological examination of mouse paraffin lung sections stained with hematoxylin and eosin (H&E, upper panel) and Periodic acid–Schiff (PAS, lower panel) staining. **D,** Bronchoalveolar lavage (BAL) fluid total and differential (eosinophil, neutrophil, and macrophage) cell counts. **E**, Serum levels of HDM specific IgG1, IgG2a, and IgE. Data represent mean ± SEM (n=6/group). *p<0.05, **p <0.01, and ***p<0.001.

Figure 7. Inhibition of RhoA suppresses BaP co-exposure-induced cytokine production. **A-B**, **Levels of** Th1/Th2/Th17 inflammatory cytokines (**A**) and airway epithelial cell derived cytokines (**B**) in the BAL samples of mice treated with either Fasudil or Y-27632. **C,** Representative immunofluorescence images of TGFβ1 (green, upper panel) and p-Smad3 (Green, lower panel) expression in the airway epithelial cells (EpCAM, red) of asthma mouse model. **D-E,** Quantitative analysis of TGFβ1 (**D**) and pSmad3 (**E**) florescent signal. Data represent mean ± SEM (n=6/group). *p<0.05, **p <0.01, and ***p<0.001.

Figure 8. Graphic summary. BaP co-exposure exacerbates Der f 1-induced activation of TGFβ1/Smad3 signaling through the AhR-RhoA axis in epithelial cells.

Figure 1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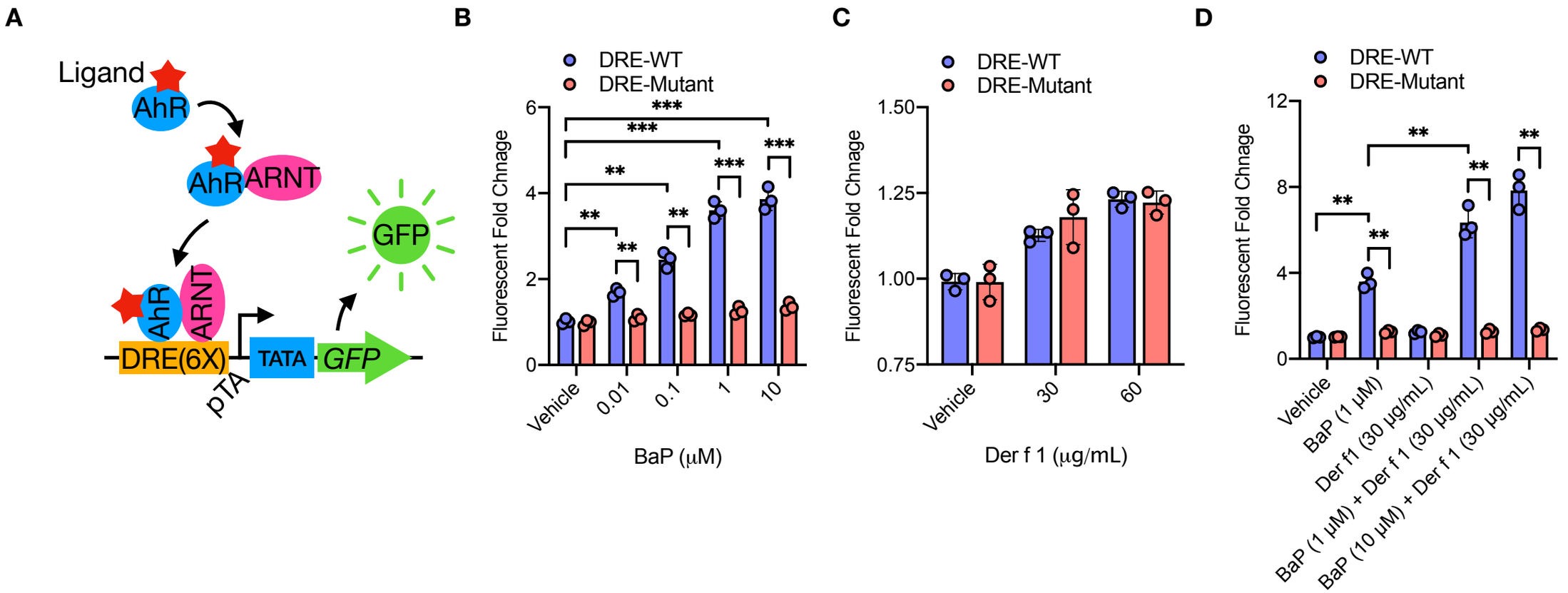
BaP+Der f 1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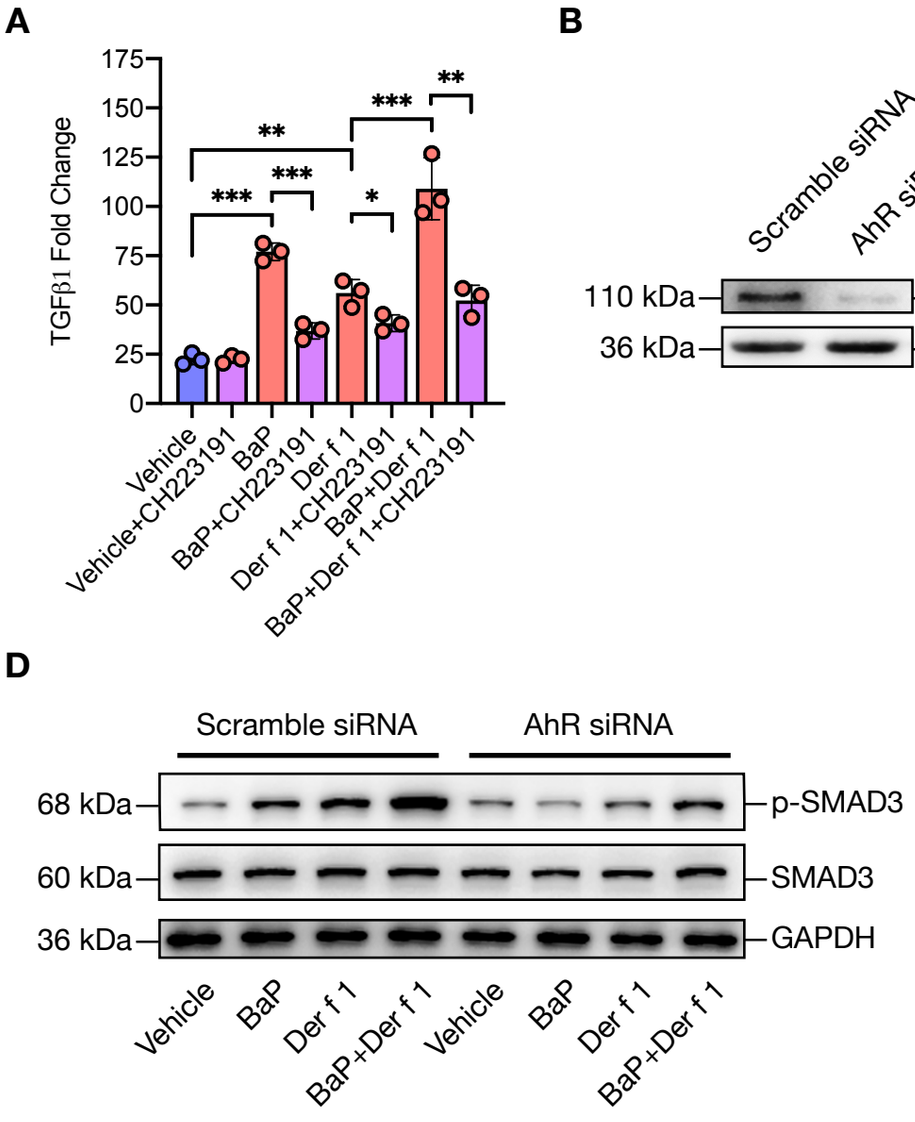
Vehicle
BaP (1 μM)
Der f1 (30 μg/mL)
BaP (1 μM) + Der f 1 (30 μg/mL)
BaP (10 μM) + Der f 1 (30 μg/mL)

Figure 3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AhR
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GAPDH
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Der f 1
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Vehicle
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BaP+Der f 1
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p-SMAD3/SMAD3 Fold Change
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Scramble siRNA
AhR siRNA
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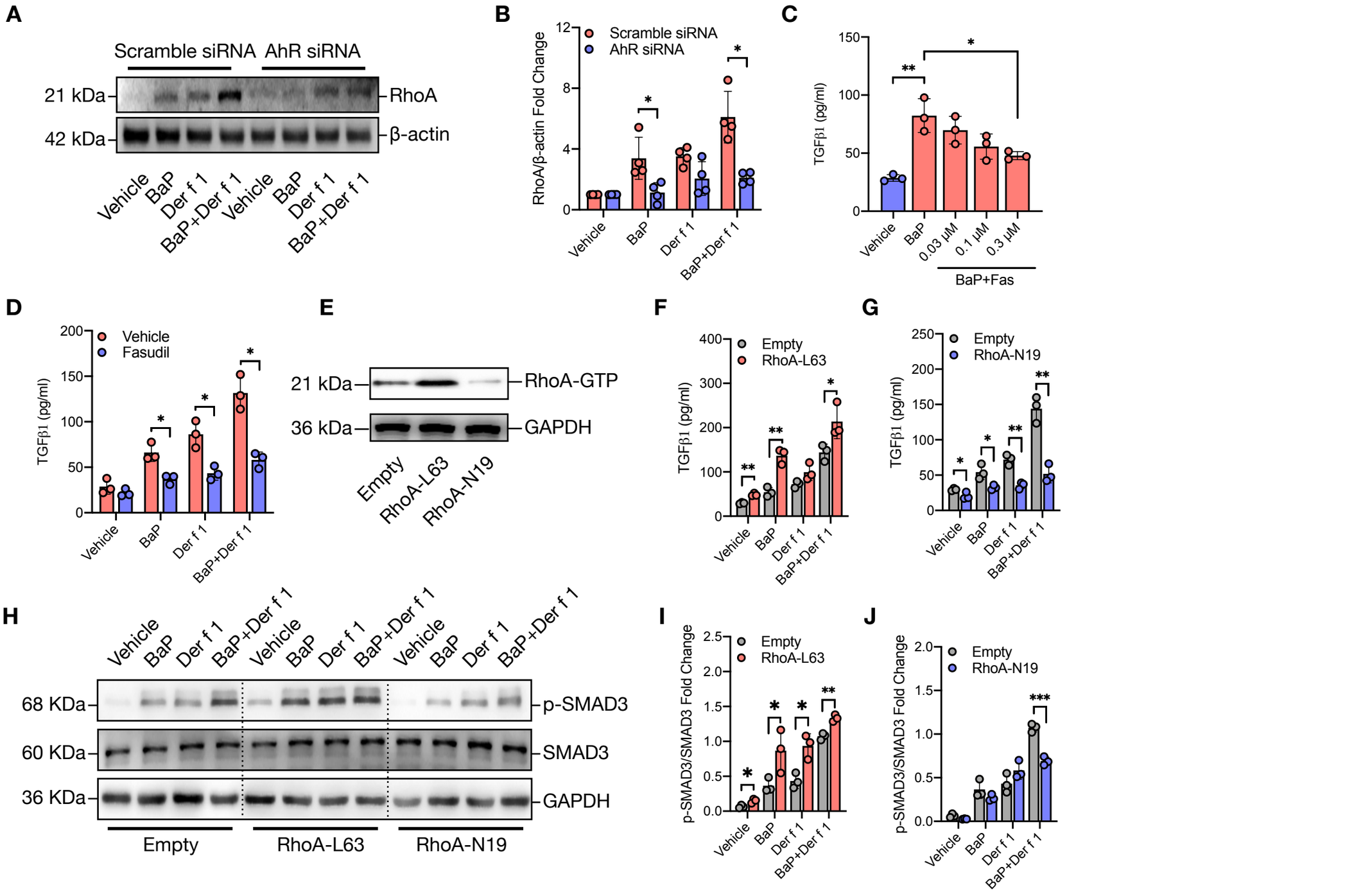
Der f 1
BaP+Der f 1


A
Scramble siRNA
AhR siRNA
21 kDa
RhoA
42 kDa
β-actin
Vehicle
BaP
Der f 1
BaP+Der f 1
B
RhoA/β-actin Fold Change
Scramble siRNA
AhR siRNA
C
TGFβ1 (pg/ml)
0.03 μM
0.1 μM
0.3 μM
BaP+Fas
D
Vehicle
Fasudil
E
RhoA-GTP
GAPDH
Empty
RhoA-L63
RhoA-N19
F
Empty
RhoA-L63
G
Empty
RhoA-N19
H
68 KDa
p-SMAD3
60 KDa
SMAD3
36 KDa
I
p-SMAD3/SMAD3 Fold Change
J

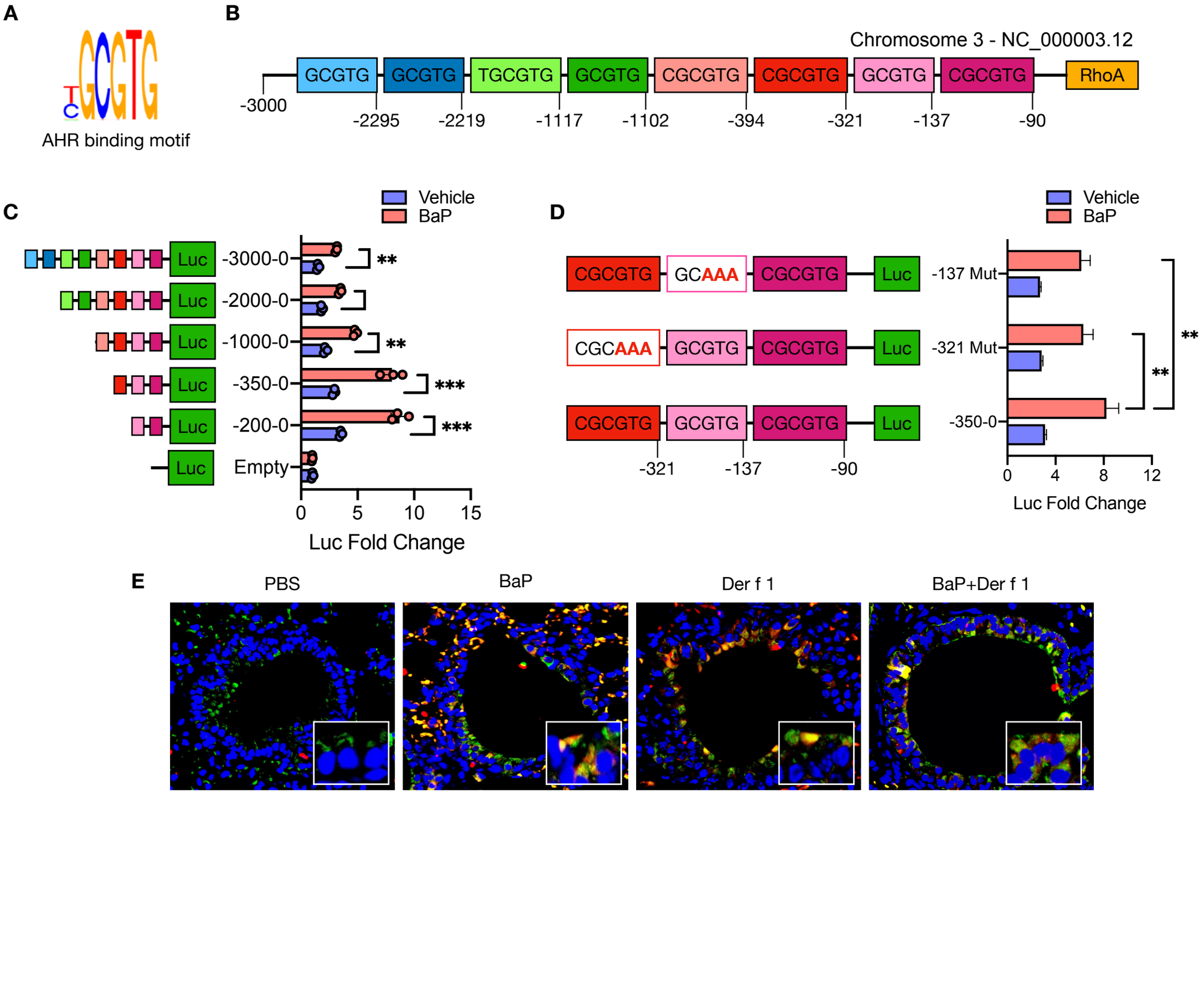
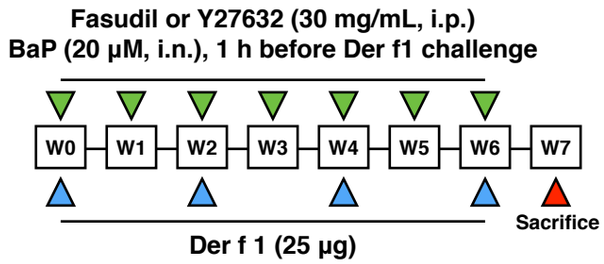
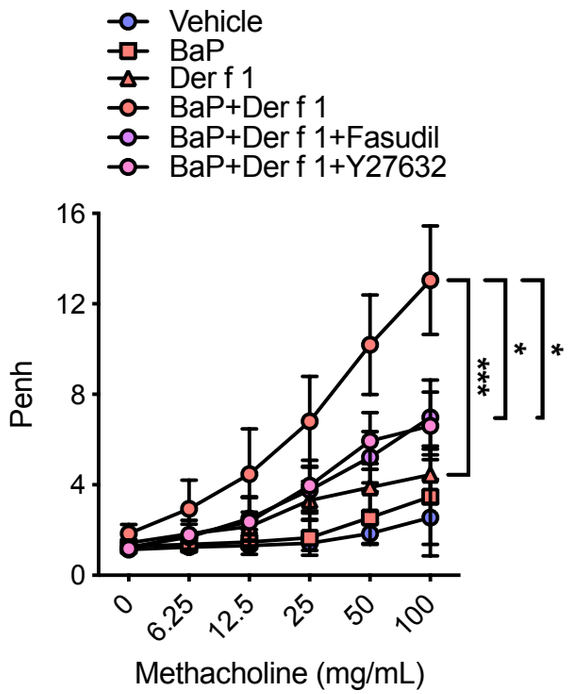

A
AHR binding motif
B
Chromosome 3 - NC_000003.12
GCGTG
GCGTG
TGCGTG
GCGTG
CGCGTG
CGCGTG
GCGTG
CGCGTG
RhoA
-3000
-2295
-2219
-1117
-1102
-394
-321
-137
-90
C
Vehicle
BaP
Luc
-3000-0
-2000-0
-1000-0
-350-0
-200-0
Empty
**
**
***
***
0
5
10
15
Luc Fold Change
D
Vehicle
BaP
CGCGTG
GCAAA
CGCGTG
Luc
CGCAAA
GCGTG
CGCGTG
Luc
CGCGTG
GCGTG
CGCGTG
Luc
-137 Mut
-321 Mut
-350-0
-321
-137
-90
**
**
0
4
8
12
Luc Fold Change
E
PBS
BaP
Der f 1
BaP+Der f 1

Figure 6

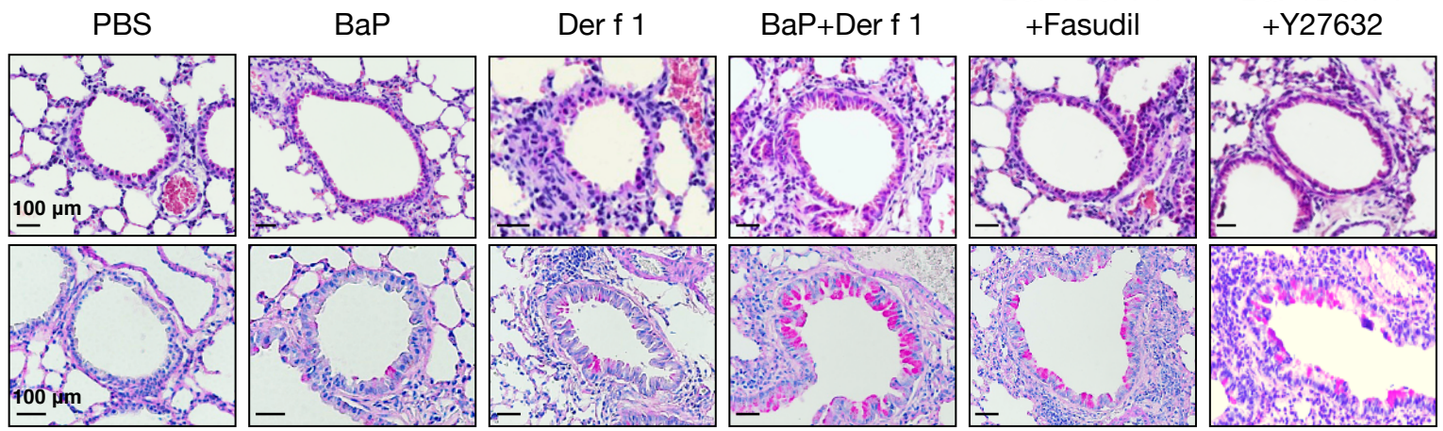
A



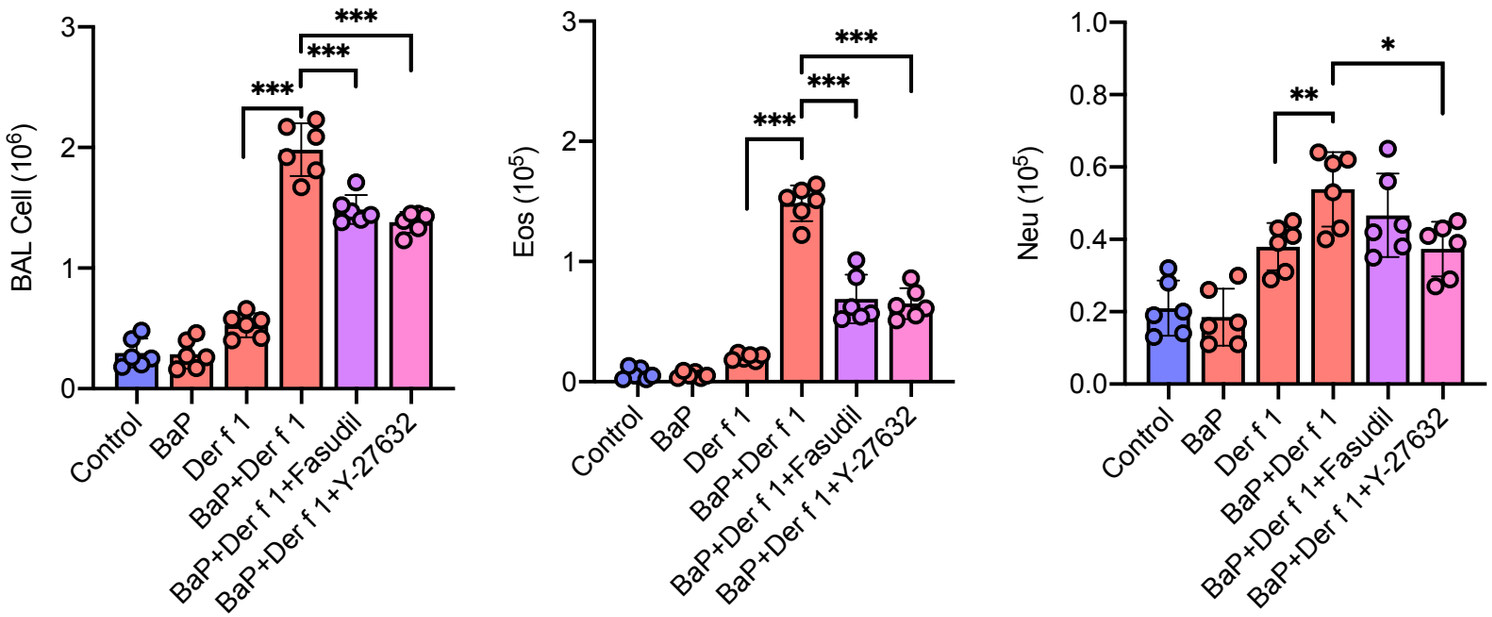
B



C



D



E

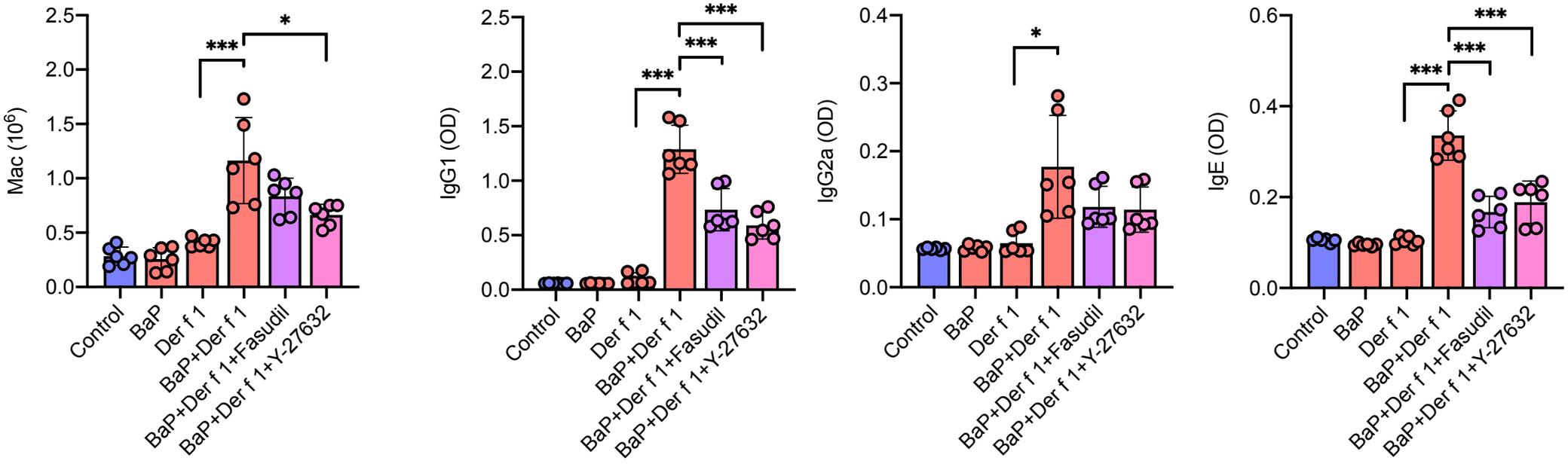
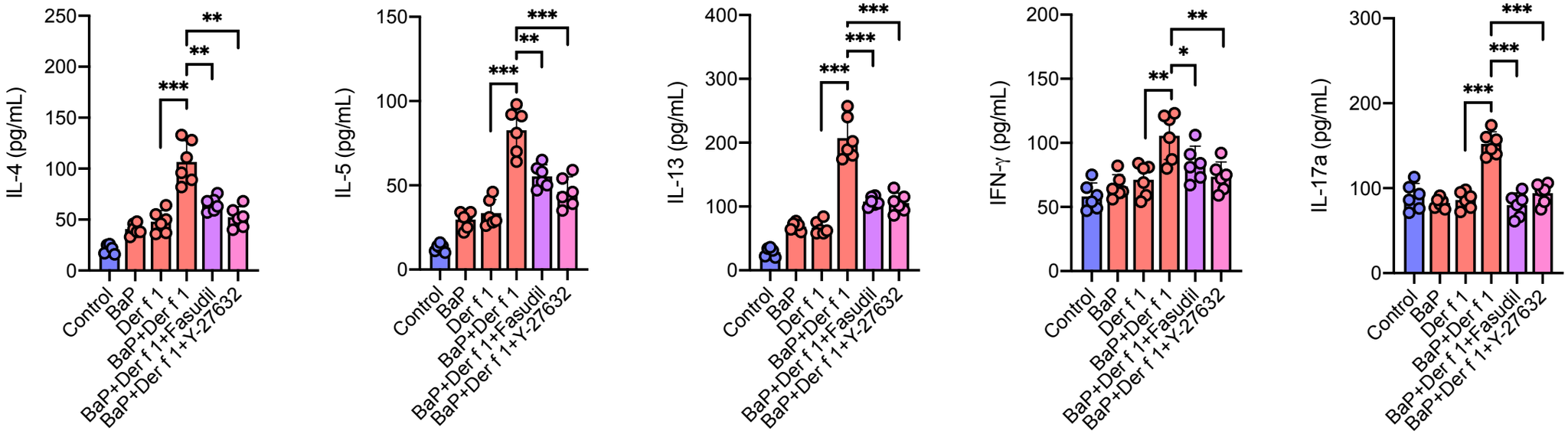
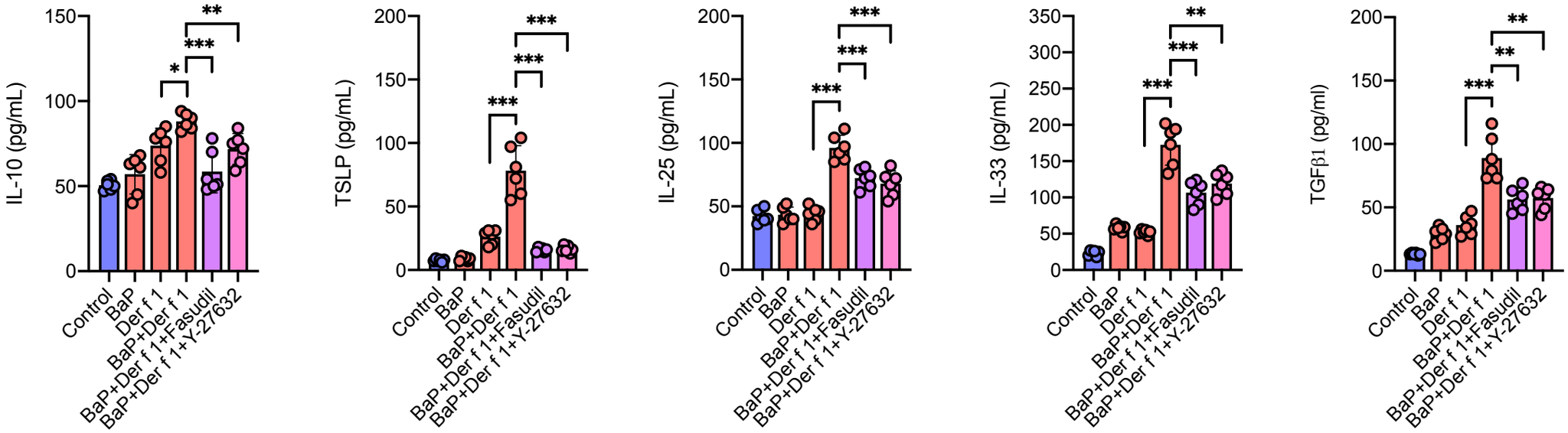


Figure 7

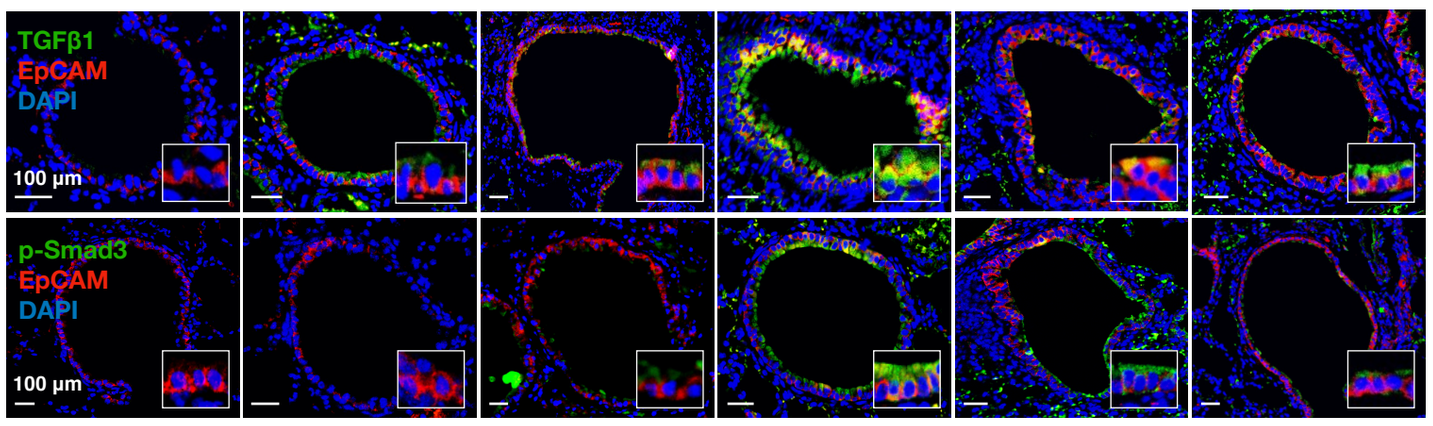
A



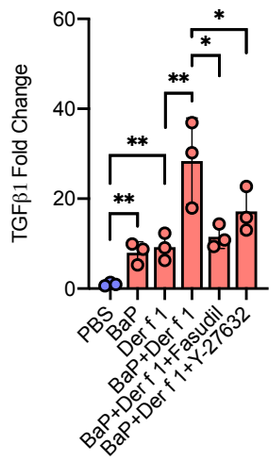
B



C



D



E

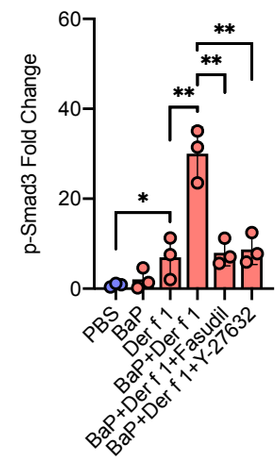


Figure 8

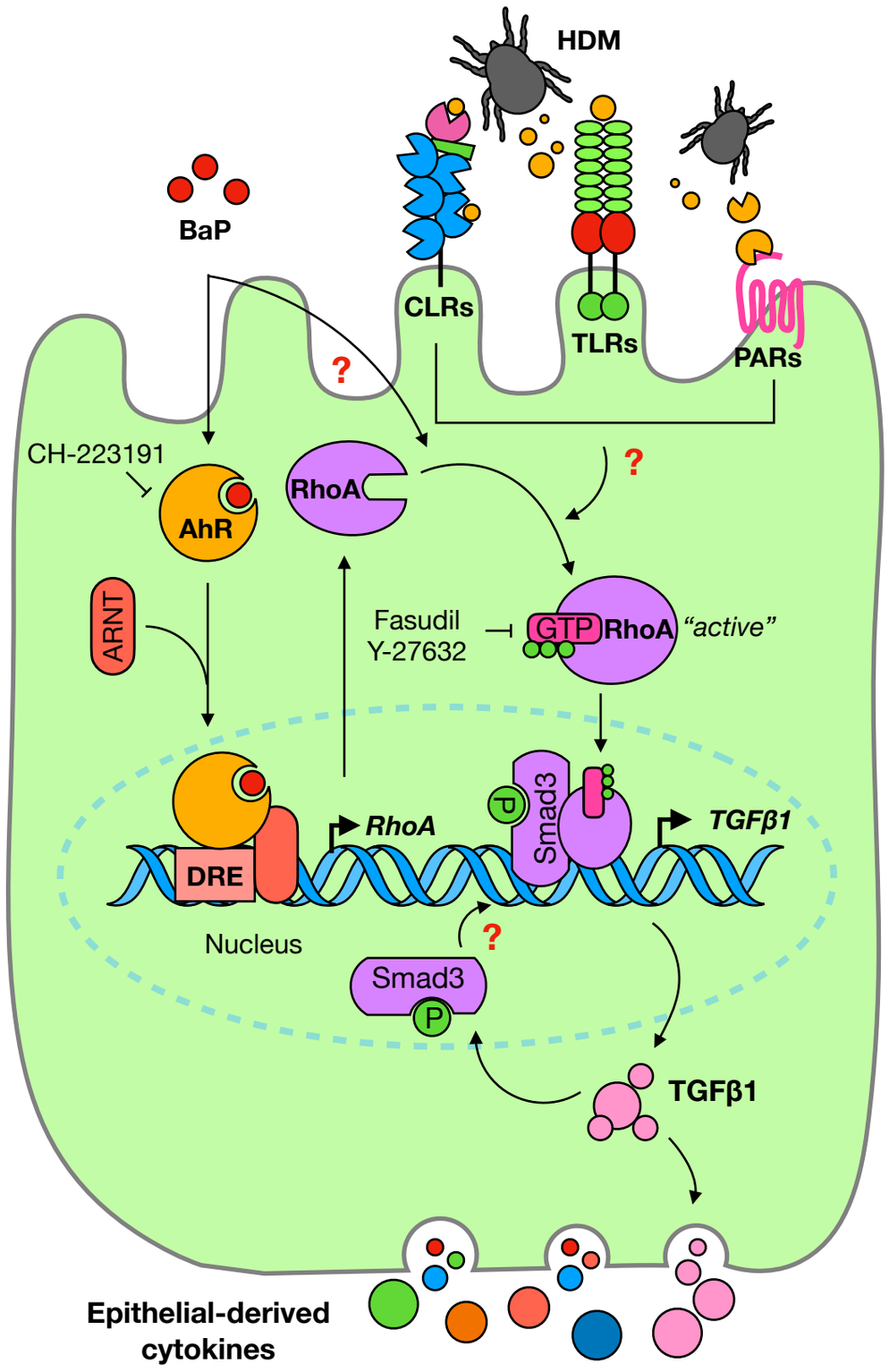
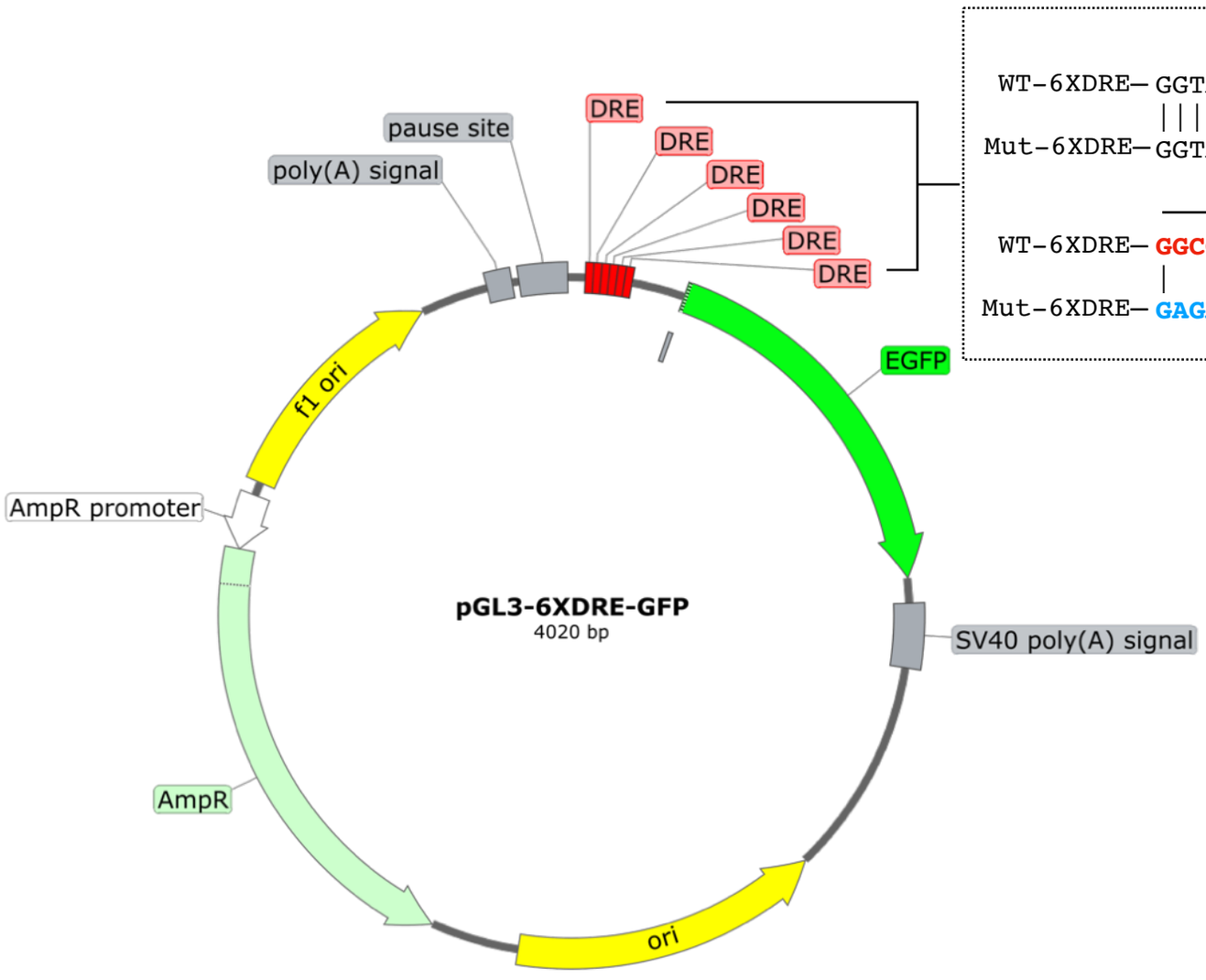

HDM
BaP
CLRs
TLRs
PARs
?
CH-223191
AhR
RhoA
?
ARNT
Fasudil
Y-27632
GTP
RhoA
"active"
DRE
RhoA
P
Smad3
TGFβ1
Nucleus
?
Smad3
P
TGFβ1
Epithelial-derived
cytokines

Figure E1


pause site
poly(A) signal
DRE
DRE
DRE
DRE
DRE
DRE
EGFP
f1 ori
AmpR promoter
pGL3-6XDRE-GFP
4020 bp
SV40 poly(A) signal
AmpR
ori

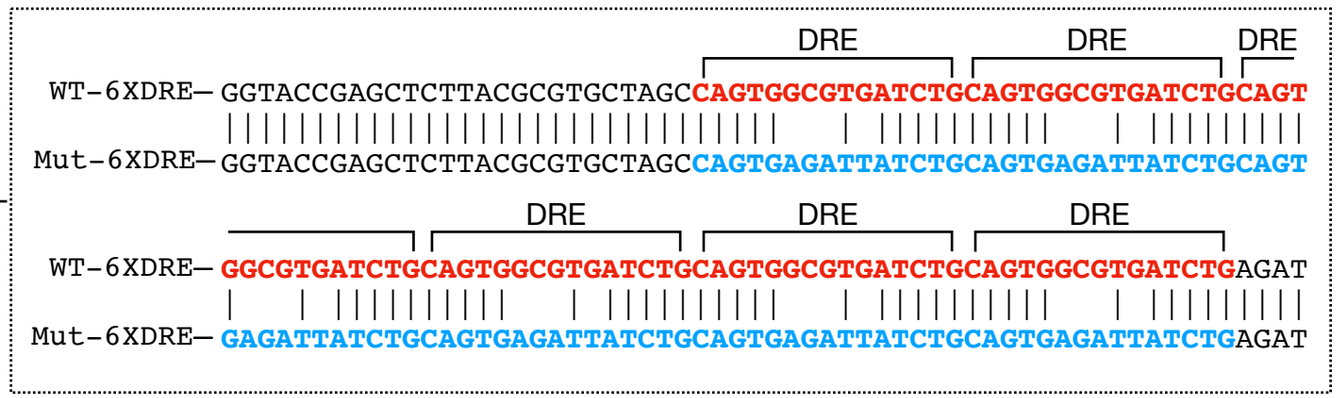

DRE
DRE
DRE
WT-6XDRE— GGTACCGAGCTCTTACGCGTGCTAGCCAGTGGCGTGATCTGCAGTGGCGTGATCTGCAGT
Mut-6XDRE— GGTACCGAGCTCTTACGCGTGCTAGCCAGTGAGATTATCTGCAGTGAGATTATCTGCAGT
DRE
DRE
DRE
WT-6XDRE— GGCGTGATCTGCAGTGGCGTGATCTGCAGTGGCGTGATCTGCAGTGGCGTGATCTGAGAT
Mut-6XDRE— GAGATTATCTGCAGTGAGATTATCTGCAGTGAGATTATCTGCAGTGAGATTATCTGAGAT

TABLE E1. Antibodies used for western blot and immunofluorescence

Antibody	Clone	Species	Application	Source
Epi-CAM	G8.8	Rat	IF (1:100)	ThermoFisher
RhoA-GTPase	26904	Mouse	IF (1:50)	New East
			WB (1:500)	Bioscience
AhR	Ab84833	Rabbit	IF (1:50)	Abcam
			WB (1:1000)	
p-Smad3	Ab52903	Rabbit	IF (1:50)	Abcam
			WB (1:1000)	
Smad3	Ab40854	Rabbit	WB (1:1000)	Abcam
TGFβ1	Ab170874	Rabbit	IF (1:50)	Abcam
GAPDH	5174S	Rabbit	WB (1:2000)	CST
β-actin	3700	mouse	WB (1:2000)	CST

GAPDH*, IF, Immunofluorescence; WB, Western blotting*

TABLE E2. Primers used for RhoA luciferase reporter assay

Primers	**Sequencing**
RhoA-F1	CGAGCTCTTACGCGTGCTAGCgagtagcagaacccagtgtag
RhoA-F2	CGAGCTCTTACGCGTGCTAGCagatcagaccacagccttgc
RhoA-F3	CGAGCTCTTACGCGTGCTAGCctggtgagggtcctaagggac
RhoA-F4	CGAGCTCTTACGCGTGCTAGCcgttagtgcgcacgcgtaaac
RhoA-F5	CGAGCTCTTACGCGTGCTAGCtgcttgcttaagggatgagtg
RhoA-R1	CTTACTTAGATCGCAGATCTCGAGgagggtagcgcgagagagc
R4-Mut-F	gtggggcctacttcgcgaacaagaagagttggcagttc
R4-Mut-R	gaactgccaactcttcttgttcgcgaagtaggccccac
R5-Mut-F	ggttttgcttttaggcaaagacgggctcctgagc
R5-Mut-R	gctcaggagcccgtctttgccctaaaagcaaaacc