SUPPLEMENTARY ONLINE REPOSITORY

ATF6α regulates airway hyperreactivity, smooth muscle proliferation, and contractility

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SUPPLEMENTARY METHODS

Mouse model of house dust mite (HDM) induced asthma

ATF6α deficient mice (kindly provided by Dr Mori)[E1] on a C57Bl6 background and WT control C57Bl6 mice aged approximately 12 weeks were either challenged with HDM (Greer)(HDM group)(n=8 mice/ group), or not challenged with HDM (no HDM group) (n=8 mice/ group) as previously described[E2]. The HDM used in this study contains 1,145 µg Der p 1 per 25 ml vial (Greer product certification of analysis sheet). In brief in the HDM group, ATF6α deficient or WT mice were intranasally administered 100 µg HDM in 50 µl PBS on day 0, 7, 14, and 21. On day 24, AHR was measured, mice were sacrificed and bronchoalveolar lavage (BAL) fluid and lungs collected to quantitate levels of airway inflammation and airway remodeling as described below. All experimental mouse protocols were approved by the UCSD Institutional Animal Care and Use Committee.

Measurement of airway hyperresponsiveness

Airway responsiveness to methacholine was assessed in intubated and ventilated mice aged 12 wk (n = 8 mice/group) (flexiVent ventilator; Scireq) using Scireq software on day 24 of the HDM mouse protocol. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. and exposed to nebulized PBS and methacholine (0, 3, 24, and 48 mg/ml) as previously described[E3]. The following ventilator settings were used: tidal volume (10 ml/kg), frequency (150/min), and positive end-expiratory pressure (3 cmH_2O).

Lung processing

Lungs were processed for immunohistology (paraffin-embedded lung sections) as previously described in this laboratory[E3,E4]. In brief, lungs were equivalently inflated with an intratracheal injection of the same volume of 4% paraformaldehyde solution (Sigma Chemicals, St. Louis, MO) to preserve the pulmonary architecture.

Assessment of thickness of airway smooth muscle layer

The thickness of the airway smooth muscle layer was measured by α-smooth actin immunohistochemistry as previously described[E3,E4]. Lung sections were immunostained with an anti-mouse α-smooth muscle actin Ab to detect peribronchial smooth muscle. The area of peribronchial α-smooth muscle actin staining in paraffin-embedded lungs was outlined and quantified under a light microscope (Leica DMLS, Leica Micro systems) attached to image analysis system (image-Pro plus, Media Cybernetics, Bethesda MD) as previously described[E3,E4]. Results are expressed as the area of peribronchial α-smooth actin staining per micrometer length of basement membrane of bronchioles 150-200 μm internal diameter.

Assessment of peribronchial fibrosis

The area of peribronchial trichrome staining in paraffin-embedded lungs was outlined and quantified under a light microscope attached to image analysis system as previously described[E3,E4]. Results are expressed as the area of peribronchial trichrome staining per micrometer length of basement membrane of bronchioles 150-200 μm internal diameter.

Airway mucus expression.

To quantitate the level of mucus expression in the airway, the number of periodic acid-Schiff $(PAS)^{+}$ cells in similar sized individual bronchioles was counted as previously described[E3,E4]. Results are expressed as the number of PAS^{+} cells per bronchiole.

Assessment of airway inflammation

BAL cell counts. BAL fluid was collected by lavaging the lung with 1 ml PBS via a tracheal catheter as previously described[E3,E4]. BAL total and differential cell counts were quantified in Wright Giemsa stained slides.

Peribronchial lung eosinophils, neutrophils, macrophages and lymphocytes.

Levels of lung eosinophils, neutrophils, CD4+ lymphocytes, and F4/80 positive macrophages were quantitated in the peribronchial space in lung sections processed for immunohistochemistry using an anti-mouse MBP Ab (rabbit polyclonal Ab kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, Arizona), an anti-mouse neutrophil elastase Ab (rat monoclonal Ab SC-71674, Santa Cruz, Dallas, Tx), an anti-mouse CD4 Ab (rat monoclonal Ab, GTX85525, GeneTex), and an anti-mouse F4/80 Ab (rat monoclonal Ab, SC-52664 Santa Cruz Biotechnology) as previously described[E3,E4]. The number of individual cells staining positive in the peribronchial space was counted using a light microscope. Results are expressed as the number of peribronchial cells staining positive per bronchiole with 150–200 μm of internal diameter. At least five bronchioles were counted in each slide.

BAL cytokines

Levels of IL-5 (R&D Systems, Minneapolis, MN), IL-13 (R&D Systems, Minneapolis, MN), and TGF-β1 (R&D Systems, Minneapolis, MN) were quantitated by Elisa in BAL fluid obtained from WT and ATF6α deficient mice.

Mouse airway smooth muscle (mASM) cell culture

Mouse airway smooth muscle was obtained from WT and ATF6α deficient mice as previously described[E5]. In brief, after mouse sacrifice, the tracheas were excised, cut longitudinally with a scalpel to expose the lumen and then transferred to 0.14% Pronase (Roche Applied Science) in Ham's F-12 nutrient medium and incubated at 4°C overnight. The tracheas were then brushed with a cotton swab to remove the remaining adherent epithelial cells, cut into small pieces (~30 per trachea), and cultured in DMEM/F-12 and 10% FBS for attachment and outgrowth. Four days later, smooth muscle cell outgrowth was apparent, and the cells were left to multiply for 2 more days, after which they were trypsinized and plated into a 175-cm^2 flask. Confluency was reached within about 3-5 days. Experiments were done on the second passage, which was split 1:2 from the first passage.

Human airway smooth muscle (hASM) culture and transfection with ATF6α siRNA

Primary hASM were obtained from Sciencell (Carlsbad, CA)[E6] .hASM were maintained and cultured in flasks or plates (6, 24 and 96 well) using smooth muscle cell media with smooth muscle growth supplement (ScienCell) and 2% fetal bovine serum. hASM were transfected with either control siRNA or ATF6α siRNA (100nM) by using transfection reagents siTran1.0 (OROGENE) and Opti-MEM (Thermo Fisher Scientific) according the manufacturer's

instructions. The transfected hASM cells were used 48 hrs after the transfection in all experiments. We assessed the efficiency of ATF6α gene knockdown by qRT-PCR as previously described in this laboratory[E3,E4]. In brief, total RNA was extracted with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). qRT-PCR was performed with TaqMan PCR Master Mix and ATF6α primers (all from Life Technology). The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA and compared between control siRNA transfected samples and ATF6α siRNA transfected samples by the ΔΔ cycle threshold method as previously described in this laboratory[E3,E4]. In addition, we quantitated levels of ATF6α protein in hASM cells transfected with either control siRNA or ATF6α siRNA by western blot as previously described in this laboratory[E4]. In brief, proteins were separated on a SDS/PAGE gel and transferred to a PVDF membrane. Membranes were blocked in 5% (wt/vol) milk in 1x Tris-buffered saline with Tween for 1 h and then incubated with primary antibody overnight at 4 °C. The primary antibodies used in this study were mouse monoclonal anti-ATF6α (Abcam), and rabbit monoclonal anti-GAPDH (Genetex).

Eotaxin-1

Levels of eotaxin-1 were quantitated by ELISA (R&D Systems, Minneapolis, MN) in the supernatants of hASM transfected with either control siRNA or ATF6α siRNA and stimulated with IL-13 (100 ng/ml) for 24 hrs.

mASM and hASM contraction assay

Either mouse ASM (WT vs ATF6α deficient) or human ASM (control vs ATF6α siRNA transfected) were used in an in vitro smooth muscle gel contraction assay as previously described in this laboratory[E7]. ASM ($2x10^5$ cells/well) were cultured in basal medium without growth factors for 24 hours before seeding in collagen gels free of LPS (Advanced BioMatrix, San Diego, Calif). After overnight incubation in collagen gels, ASM cells were cultured in the presence or absence of either diluent (control), methacholine (100 μM) or histamine (200 μM) for varying time periods (0, 15, and 30 min).With agonist-induced ASM contraction, the area of the gel decreases significantly, as described in studies of ASM[E7]. The area of the gels was quantitated by using a Bio-Rad ImageDR transilluminator and Versadoc scanner (Bio-Rad Laboratories, Hercules, Calif) with an accompanying image-capture and analysis program to generate the area in square millimeters. Results are expressed as % contraction, which compares the area of the gel at the time point studied to the area of the gel at baseline (0 min).

mASM and hASM Proliferation

mASM and hASM proliferation was assessed by BrdU incorporation using a BrdU ELISA (Exalpha Biological). BrdU incorporation in either mouse ASM (WT vs ATF6α deficient) or human ASM (control vs ATF6α siRNA transfected) was assessed in ASM ($2x10^4$ cells/well) stimulated to proliferate with either 2% FBS (low concentration) or 10% FBS (high concentration), or as a control no FBS in a 96-well microplate. BrdU was added 24hrs after incubating ASM with FBS, and 24hrs later BrdU incorporation was quantitated according to the manufacturer's instructions.

mASM and hASM apoptosis.

We used a Caspase-Glo 3/7 apoptosis assay (Promega) to detect caspase-3/7 activity in either mouse ASM (WT vs ATF6α deficient) or human ASM (control vs ATF6α siRNA transfected). In these experiments ASM cells ($2x10^4$ cells/well) were incubated with either 0% FBS, 2% FBS, or 10% FBS in 96-well microplate. The Caspase-Glo 3/7 apoptosis assay utilizes a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD to detect caspase-3/7 activity. Incubation of ASM with 1μM staurosporine a known inducer of apoptosis was used as a positive control. Staurosporine was added 24hrs after incubating ASM with FBS, and 24hrs later caspase-3/7 activity was quantitated according to the manufacturer's instructions.

Statistical analysis

All results are presented as mean $\pm$ SEM. A statistical software package (Graph Pad Prism, San Diego, CA) was used for the analysis. A t test was used for analysis of two groups. ANOVA analysis was use when more than two groups were compared. P values of < 0.05 were considered statistically significant.

SUPPLEMENTARY DISCUSSION

Our in vivo studies used global ATF6α$^{-/-}$ mice, and thus we are not able to determine whether a deficiency of ATF6α in ASM, or in cell types other than ASM, could have influenced ASM contraction in vivo. It is possible that ATF6α$^{-/-}$ mice have a developmental defect in ASM, although baseline levels of ASM in WT and ATF6α$^{-/-}$ mice are similar. The increase in ASM mass noted in this study in WT mice with four HDM challenges in vivo over a 3 week period may either be due to ASM hypertrophy and/or ASM proliferation and requires further study. Although our in vitro studies demonstrate that ATF6α is required for methacholine or histamine induced ASM proliferation in vitro, further studies are needed to determine the role of ATF6α in ASM proliferation induced by other important stimuli in asthma including leukotrienes and HDM. In this study we only performed experiments on ASM and thus we are not able to state whether ATF6α regulates the function of other lung structural cells such as fibroblasts or myofibroblasts. Further human studies are also needed to determine whether ORMDL3 regulates ATF6α in human ASM as it does in human bronchial epithelial cells[4].

In addition to our studies of the ER demonstrating that ORMDL3 regulates ATF6α in mouse and human lung cells[E3,E4], previous studies have demonstrated that the ER resident protein 57 (ERp57) is upregulated in allergen challenged mouse and human lung epithelial cells[E8]. Studies of HDM challenged mice selectively deficient in ERp57 in epithelial cells demonstrated reduced airway inflammation (in particular neutrophils and lymphocytes), fibrosis, and AHR[E9]. Interestingly, in epithelial cells in vitro, ATF6α knockdown decreased HDM induced upregulation of ERp57 and decreased apoptosis[E8]. In contrast, ATF6α does not regulate levels of apoptosis in ASM, suggesting that the ATF6α pathway to ERp57 differs in functional outcomes

in epithelial cells and ASM. As ATF6α is a transcription factor that regulates expression of at least 60 genes[E1], further study is needed to determine which downstream pathway from ATF6α in ASM is important to ASM contractility. One candidate pathway downstream of ATF6α is sarco/endoplasmic reticulum Ca^{2+} ATPase 2b (SERCA2b) which like ORMDL3 and ATF6α is also localized in the ER. We have previously demonstrated that in vitro and in vivo, increased expression of ORMDL3 leads to increased activation of ATF6α and increased expression of SERCA2b[E3,E4]. In addition, knockdown of ATF6α reduces levels of SERCA2b expression[E3,E4]. Thus, further study is needed to determine whether SERCA2b or other downstream pathways in ASM mediate the effect of ATF6α on ASM contractility.

SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure E1. No difference in airway inflammation in ATF6α$^{-/-}$ vs WT mice.

ATF6α$^{-/-}$ or WT mice (n = 8 mice/group) were challenged with HDM intranasally on day 0, 7, 14, 21. On day 24, levels of BAL cellular inflammation was quantitated, and lung tissues were processed for immunohistology and image analysis. The number of Wright-Giemsa stained **A,** BAL eosinophils, **B,** BAL lymphocytes, **C,** BAL neutrophils, and **D,** BAL macrophages were quantitated by light microscopy. **E,** The number of peribronchiole MBP^{+} eosinophils, **F,** CD4+ lymphocytes, **G,** neutrophil elastase^{+} (NE^{+}) neutrophils, and **H,** F4/80^{+} macrophages per bronchiole of 150-200μm internal diameter was quantitated by immunohistochemistry and image analysis. Levels of BAL **I,** IL-5, **J,** IL-13, and **K,** TGFβ1 were quantitated by ELISA. **L,** Levels of eotaxin-1 were quantitated by ELISA in the supernatants of hASM stimulated with IL-13. $^{*}P$ <0.05, $^{***}P$ <0.001, and NS (not significant).

Supplementary Figure E2. Comparison of ASM contraction in non-siRNA treated hASM cells and control siRNA treated hASM cells in vitro.

Levels of smooth muscle contraction in non-siRNA treated hASM cells and control siRNA treated hASM cells were assessed at baseline (time 0 min), as well as 15 and 30 min after incubation with either **A,** methacholine, or **B,** histamine using an in vitro smooth muscle gel contraction assay. $^{*}P$ <0.05, $^{***}P$ <0.001 and NS (not significant).

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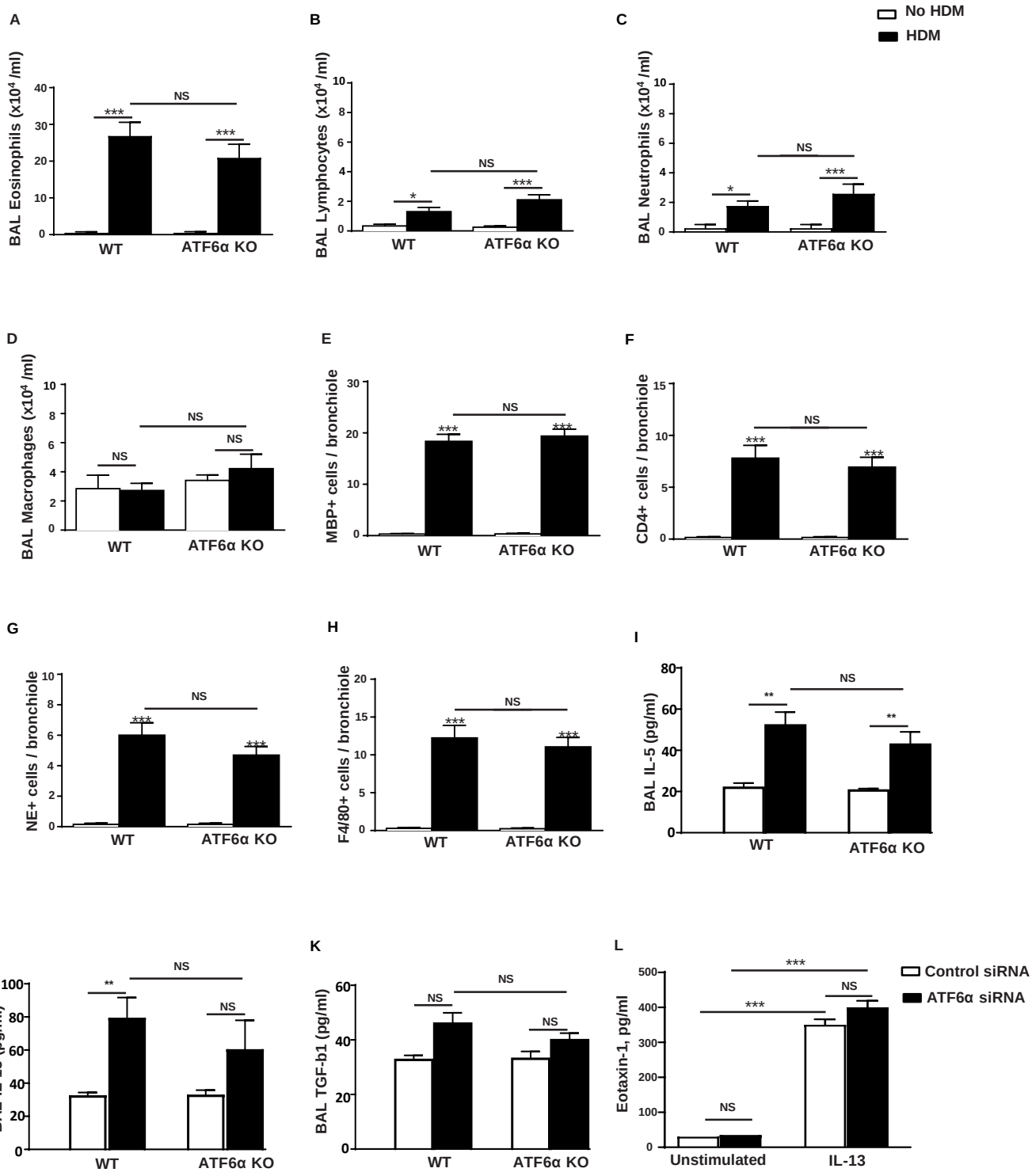
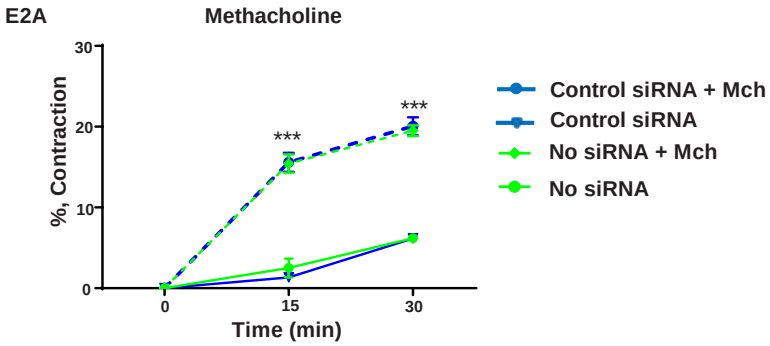


Supplementary Figure E1

E2A



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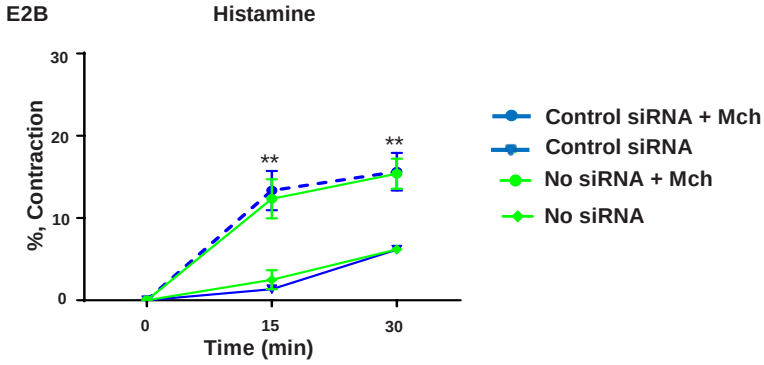

Supplementary Figure E2