RE: Submission #69636-RG-1

Supplemental Material (Lam *et al.*)

Supplemental Methods

Chemicals and Reagents. Filtered research-reference cigarettes (3R4F) were from the Tobacco Research Institute (University of Kentucky). Ham's F-12 with L-glutamine and DMEM/F-12 without L-glutamine and HEPES were from Cellgro by Mediatech. Deoxyribonuclease I from bovine pancreas, retinoic acid, HEPES 1 M in H_2O (200 mM L-glutamine, insulin from bovine pancreas, human apo-transferrrin, SigmaFast protease inhibitor cocktail tablets (EDTA free), leupeptin hemisulfate salt and cholera toxin from *Vibio cholerae* were from Sigma-Aldrich. Pronase from *Streptomyces griseus* was from Roche. Complete protease inhibitor cocktail tablets with EDTA were from Roche Diagnostics. Collagen I, mouse epidermal growth factor, bovine pituitary extract, Nu-Serum and Primaria Falcon 100 mm cell culture dishes were from BD Biosciences. Bovine serum albumin, amphotericin B/fungizone, 12 mm Transwell® with 0.4 μm pore polycarbonate membrane and 12 mm Transwell® with 0.4 μm pore polyester membrane were from Corning Inc. Costar. Cell culture antibiotics penicillin/streptomycin (10,000 U) were from Lonza. Dulbecco's Modified Eagle Medium with High Glucose (DMEM), phosphate buffered saline (1X), and gentamicin reagent (50 mg/ml) were from Invitrogen-Gibco. TEM and SEM grade paraformaldehyde and glutaraldehyde were from Electron Microscopy Sciences. Histology grade formaldehyde, 37% solution, was from Mallinckrodt Baker, Inc. The following antibodies were from Santa Cruz Biotechnology: acetyl α-tubulin (sc-23950), Beclin-1 (sc-11427), Bcl-2 (sc7382), BaxΔ21 (sc-6236). The following antibodies were from Sigma-Aldrich: LC3B (L7543), p62 (P0067), β-actin (A2228). The following antibodies were from Cell

Signaling Technology: LC3B (27735S), cleaved caspase 3 (9661S). Beclin-1 (612112) C-terminal specific antibody was from BD Transduction Laboratories™. Centrin 1 (ab11257) antibody was from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibodies for immunoblot analysis were from Santa Cruz: goat anti-rabbit IgG-HRP (sc-2004), goat anti-mouse IgG-HRP (sc-2005). Immunofluorescence reagents and secondary antibodies Cy™3-conjugated donkey anti-rabbit (711-165-152) or anti-mouse (715-165-150) were from Jackson ImmunoResearch Laboratories, Inc. Alexa Fluor® 488 (A12379) and 647 (A22287) conjugated to phalloidin and Alexa Fluor® 488 secondary antibody anti-rabbit (A11034) and anti-mouse (A10667) and were from Invitrogen, Molecular Probes. All other reagent chemicals were from Sigma-Aldrich.

Animals. Becn1$^{+/-}$ mice were from Beth Levine (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX). The *Hdac6*$^{-/Y}$ mice were from Bin Shan (Tulane University, New Orleans, LA). *Map1lc3b*$^{-/-}$ mice were from Marlene Rabinovitch (Stanford University, Stanford, CA), and backcrossed into a pure C57BL/6 background in our laboratory. The GFP-LC3B mice were from Riken BioResource Center (Ibaraki, Japan). WT C57BL/6 were purchased from Jackson Laboratories. All animal experimental protocols were approved by the Harvard Standing Committee for Animal Welfare.

In vivo CS exposure and harvest protocol. Age and sex matched mice starting at 6-12 weeks of age were exposed to total body CS in a stainless steel chamber (71 cm X 61 cm X 61 cm) using a smoke machine (Model TE-10 Teague Enterprises) 5 days per week for 1 week, 2 months, and 6 months or room air. The CS treatment required approximately 2 hours each day as the mice were

exposed to the mainstream and sidestream smoke of 100 3R4F cigarettes with an average TPM of 150 mg/m^3. The carboxyhemoglobin levels were typically less than 8% following exposure. At the end of the exposure regimen, mice were euthanized by CO_2. The left lung was isolated with a suture, dissected and flash frozen in liquid nitrogen. The mouse was then cannulated through the trachea and the right lungs were inflated with 2% formalin in PBS at 25 cm of H_2O pressure, dissected from the mouse and fixed in 4% formalin at 4°C for 2 days. The proximal trachea up to the larynx and a portion of proximal portion of the left lung were fixed for TEM analysis.

Cell culture and CSE treatment. Beas 2B cells, a human lung epithelial cell line, and mouse lung fibroblasts were maintained in complete media DMEM supplemented with 10% FBS and gentamicin (50 μg/ml). CSE was prepared using a peristaltic pump (VWR International) to bubble mainstream smoke from four 3R4F cigarettes with filters removed through 40 mL DMEM. Each cigarette was smoked within 6 minutes until approximately 17 mm remained. The extract was adjusted to a pH of 7.5 filter sterilized, stored at -80°C, and used immediately upon thawing. The CSE generated in this fashion was considered 100% strength was diluted in complete DMEM media for cell treatment. Cigarette smoke condensate (CSC) was prepared and added to culture media as previously described (1).

Generation and CS exposure of MTEC cultures. Methods for MTEC isolation and culture generation were described previously (2, 3). For each isolation, age and sex matched mice 6-12 weeks were euthanized by CO_2 necrosis. The tracheae were isolated and stored on ice in Ham's F12 containing antibiotics (1X penicillin/streptomycin and fungizone). Careful dissection was

used to remove all connective tissue and the tracheae were cut longitudinally to expose the luminal surface for overnight digestion at 4°C in 0.15% pronase in Ham's F-12 with antibiotics. Six tracheas were digested in 10 mL of solution in a 50 mL conical tube. The next day the tracheal digests were inverted 12 times and allowed to incubate for an additional hour at 4°C. The enzymatic activity was stopped by adding 10 mL of Ham's F-12 containing 20% FBS and antibiotics and gently inverted 12 times. The tracheae were spooled onto a pasture pipette and transferred to a new 15 mL conical tube containing 10 mL of Ham's F-12 with 20% FBS and antibiotics and inverted 12 times. This mechanical disruption of the tracheas was repeated two times more using fresh 15 mL conical tubes and media. Finally, the tracheae were discarded and the contents of the three 15 mL tubes were collected in the original 50 mL conical tube. The cells were pelleted at 350 x *g* for 10 minutes at 4°C. The media was aspirated and the cells were incubated for five minutes on ice in 100-200 μL per trachea of DNase solution (0.5 mg/mL DNase, 10 mg/mL BSA, in Ham's F12 with antibiotics). The cells were then pelleted at 350 x *g* for 5 minutes at 4°C and then the media was aspirated. The cells were seeded onto 100 mm Primaria plates in MTEC Basic Media (1 M Hepes, 200 mM Glutamine, 7.5% $NaHCO_3$, 0.25 ìg/mL Fungizone, 102 U/mL Penecillin Streptomycin in DMEM:Ham's F-12) containing 10% FBS and incubated for 5-6 hours in a humidified incubator at 37°C, 5% CO_2. Cells that did not attach to the plate were collected by centrifugation at 350 x *g* for 10 minutes at 4°C and resuspended in MTEC proliferation media (10 μg/μL insulin, 5 μg/mL transferrin, 0.1 μg/mL cholera toxin, 25 ng/mL epidermal growth factor, 30 μg/mL bovine pituitary extract, 10 X 10^{-8} M retinoic acid, and 5% FBS in MTEC basic media). In the 12 well transwell dish 1.5 mL of MTEC proliferation media was added to the basal compartment and 75-100 X 10^3 cells/well were seeded in 500 μL onto the transwells. The transwells were precoated with 400 μL of 100

mg/mL type 1 rat tail collagen in 0.02 N acetic acid and incubated overnight. The next day excess solution was aspirated and allowed to dry before washing twice with PBS. Cells proliferated for 10 days in submerged culture conditions with media changes every other day and fresh media made every fifth day. Cultures with a resistance over 1000 Ω/cm^2, as measured using an ohmvoltometer (EVOM™, World Precision Instruments), were put at an ALI by removing all apical media and replacing the basal media with 750 μL of MTEC differentiation media (2% NuSerum and 10 X 10^{-8} M retinoic acid in MTEC basic media) in the basal compartment. The cultures then differentiated for 14 days at ALI with media changes every other day. CS treatment was conducted in a custom designed humidified chamber (EMI Services) at 37°C by exposing the cells for 10 minutes to 50 or 100 mg/m^3 of mainstream CS from 3R4F research-reference filtered cigarettes.

Protein extract preparation and immunoblot analysis. All protein extractions were done on ice with ice-cold homogenization buffer. Protein extracts were made by lysing and scraping cells using cell RIPA buffer (400 mM HEPES, 5 M NaCl, 0.5 M EDTA, 1 M Na_3VO_4, 1 M NaF, 100 mM glycerol 2-phosphate, 3% Chaps w/v) or for tissues, tissue RIPA buffer (300 mM NaCl, 50 mM Tris (pH 7.6), 1% NP-40, 10% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4) made with a fresh complete protease inhibitor tablet (Roche Diagnostics). The samples were homogenized by sonication 5 times for 10 seconds each time (60 Sonic Dismembrator, Fisher Scientific). Cells were spun once for 20 minutes at 14,000 x *g* while tissue protein extracts were prepared by centrifuging 5 times 10 minutes each time at 14,000 x *g* and transferred to a new eppendorf tube for each consecutive centrifugation. The supernatants were collected after the final centrifugation and the protein concentrations were determined by a Coomassie Plus

(Bradford) protein assay (Pierce Biotechnology Inc.) by measuring the absorbance at 595 nm and linear regression form a BSA standard curve. The protein samples were then normalized using homogenization buffer. Approximately 10-50 μg of the protein samples were subjected to electrophoresis using a 4-12% SDS-polyacrylamide gel (Invitrogen). The proteins were electro-transferred onto polyvinylidene diflouride membrane (PVDF; Invitrogen). After the membranes were blocked in 5% nonfat milk in 0.2% Tris-buffered saline with 0.1% Tween 20 (TTBS) for 1 hour at room temperature, the blots were incubated overnight at 4°C in primary antibodies diluted in TTBS. Membranes were washed three times for 5-7 minutes with TTBS, and incubated at room temperature for 2 hours in the corresponding HRP-conjugated secondary antibody. Membranes were washed three times for 5-7 min again in TTBS and developed with ECL reagent (Amersham Biosciences).

Tissue and cell homogenization to obtain a lysosome-enriched fraction. Subcellular fractionation for the LE fraction was previously described in detail[5]. Briefly, tissues were flash frozen upon harvesting and stored at -80°C until processing, which was all done at 4°C or on ice. Cells were trypsinized and pelleted at 300 x *g* for 8 minutes at 4°C. Lysates were made with homogenization buffer (10 mM Tris, pH 8.0, 5 mM EDTA, 250 mM sucrose, protease inhibitor tablet without EDTA). Cells were mechanically disrupted by Dounce homogenization ten times with the loose and ten times with the tight pestle. The tissue was mechanically homogenized (7X95 mm Saw Teeth Generator, Omni International, The Homogenizer Company). The homogenate was then centrifuged at 700 x *g* for 10 minutes and transferred to a new tube. If debris was still present an additional 700 x *g* spin was implemented. The protein was then normalized to 1-2 mg/ml using the homogenization buffer. An aliquot was saved for the whole

cell fraction. The sample was then centrifuged at 20,000 x *g* for 30 minutes. Supernatant was saved for the cytoplasmic fraction. The pellet was washed twice with homogenization buffer and resuspended in sample buffer. All cell fractions were prepared with 1X NuPage LDS sample buffer (NP007, Invitrogen) boiled for 10 minutes and subjected to subsequent immunoblot analysis. Due to the labile nature of the LC3B II, the samples were stored at -80 °C.

In vivo autophagic flux assay. A detailed protocol for *in vivo* autophagic flux has been described previously (4). Briefly, mice exposed to CS or RA were given an intraperitoneal injection of 40 mg/kg leupeptin in pharmaceutical grade saline at 1 hour or 24 hours following the last CS treatment. Control mice received an equal volume of the vehicle. The leupeptin treated RA and CS mice were then harvested 2 hours later in parallel. Tissues were flash frozen and LC3B turnover was assessed in the lysosome-enriched (LE) fraction. To quantify LC3B levels we performed Western blot analysis, with a standard curve consisting of purified GST-LC3B protein run alongside in the same gel (range 10 ng–0.1 ng protein per lane). The blots were analyzed by densitometry and LC3B band intensity was converted to protein quantity by extrapolation from the standard curve using regression analysis tools in Microsoft Excel 2003 (Microsoft Corporation). GST-LC3B content was converted to endogenous LC3B content by multiplying by a factor of 2.5, and this value was then normalized to mg of total protein. We defined autophagic flux as the difference in LC3B-II quantity on Western blots obtained in the presence versus the absence of inhibitor (4).

In vitro autophagic flux assay. Following exposure of MTEC cultures to CS (50 mg/m^3), or Beas-2B cells to 20% CSE, cells were treated for 10 minutes later with chloroquine (25 μM). Control cells were treated with chloroquine in the absence of CS or CSE treatment. The samples

were then harvested in RIPA buffer from control and treated cells at the same time: 0 and 1 hrs post-treatment for MTECs; and 0, 1, 4, or 8 hrs post-treatment for Beas-2B cells. Lysates were then analyzed by standard immunoblot assays for LC3B and p62.

Isolation of autophagosomes from LC3B-GFP mice. GFP-LC3B autophagosomes were isolated using a modified version of a previously described method (5). Briefly, lungs from LC3B-GFP mice exposed to room-air or CS for 3 weeks were perfused with PBS, harvested and homogenized in ice cold Homogenization buffer (250 mM Sucrose, 10 mM Tris pH 8.0, 5 mM EDTA supplemented with protease and phosphatase inhibitors). Autophagosome/lysosomal enriched fractions were generated by pelleting (20,000 *x g*) the 3000 *x g* supernatants. LC3B positive autophagosomes/lysosomes were isolated by incubating equal amounts of protein with μMACS microbeads (Millentyi Bioyech) conjugated to an epitope specific GFP antibody for 1 hour rotation at 4°C. Following immunoprecipitation, samples were loaded onto a μMACS Column and placed in the magnetic field of a μMACS Separator. The magnetically labeled GFP-tagged and associated proteins retained on the column were eluted using HB buffer. The Beads were pelleted by centrifugation, washed in HB, resuspended in sample buffer and heated to 99°C for 5 min. Beads were pelleted by centrifugation and supernatants analyzed by SDS electrophoresis followed by Western immunoblot analysis.

Cell viability and cytotoxicity assays. Cytotoxicity was assessed by measuring LDH activity in the basal media of the MTEC cultures according to the manufacturer's protocol (Cytotoxicity Detection KitPLUS, Roche Diagnostics). This assay measures LDH released into the media upon plasma membrane permeability, the assay determines LDH content by a coupled enzymatic

reaction in which the tetrazolium salt is reduced to formazan dye, which is measured at 500 nm. The assay was performed in duplicate in a 96-well format and the absorbance was measured using a Multiskan EX Microplate Photometer from Thermo Scientific. The Annexin V FITC Apoptosis Detection Kit was purchased from BioVision. The assay was performed according to the manufacturer's protocol. The proportion of viable and apoptotic cells were assessed using a FACS Canto II (BD Bioscience) and FlowJo analytical software (Tree Star, Inc.).

Immunofluoresence staining. Cells were fixed with 4% paraformaldehyde in 1X PBS directly on transwells for 1 hour at 4°C then washed with PBS and stored at 4°C. Staining was done following a standard protocol. The cells were permeabilized for 15 minutes in 0.01% Triton-X 100 then washed in PBS twice and 0.5% bovine serum albumin (BSA) in PBS twice. The cells were blocked for 45 minutes with 2% BSA in PBS. The cells were washed once in 0.5% BSA and then incubated for 1 hour at room temperature in primary antibodies, LC3B (1:50) and acetylated α-tubulin (1:250). The samples were washed 5 times with 0.5% BSA and then incubated for 1 hour at room temperature in secondary antibody, Alexa 488 anti-rabbit (1:1000), Cy3 anti-mouse (1:500) and or phalloidin conjugated to Alexa-647 or Alexa-488 (1:1000). Misfolded protein aggregates were stained using a recently developed dye, ProteoStat Aggresome Detection kit for flow cytometry and microscopy (ENZO® Life Sciences). The cells were washed 3 times with 0.5% BSA and then twice with PBS. Nuclei were then stained with Hoechst for 30 seconds and washed 3 times with PBS. The samples were then mounted onto slides using and imaged using an epifluoresence (Leica DM LB microscope and DFC 480 3CCD Color Vision Module) or confocal (Zeiss LSM 510 with two-photon).

Quantification of fluorescent puncta. A macro was created for ImageJ in which the threshold was set and mask file was created using a Gaussian blur with a "filter radius" followed by a background subtraction and "rolling ball radius." A binary "watershed" was created from which particle analysis was completed by counting objects with size between the "puncta min size" and "puncta max size".

Transmission electron microscopy. Cells were fixed for 1 h and tissues were fixed overnight at 4°C using TEM grade fixative solution of 2% formaldehyde and 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The samples were washed and stored in 0.1 M sodium cacodylate buffer and kept at 4°C until processing. Sample embedding was performed using a standard protocol, in which all steps were done at room temperature unless otherwise indicated. The samples were post-fixed for one hour in the dark in 1% osmium tetroxide/1.5% potassium ferrocyanide in H_2O. The samples were washed 3 times in H_2O or maleate buffer pH 5.15, then incubated in 1% uranyl acetate in H_2O or maleate buffer for 30 min. The samples were washed three times in H_2O and dehydrated by a graded series of ethanol: 70% for 15 min, 90% for 15 min, then 100% twice for 15 min. The samples were then further dehydrated in propylene oxide for 1 h. Infiltration was done with a 1:1 ratio of Epon:propylene oxide for 2-3 h. The samples were then embedded in freshly mixed Epon and polymerized 24-48 h at 60°C. Ultrathin sections were then absorbed onto hydrophilic formvar/carbon coated grids and negatively stained with 1-2% aqueous uranyl acetate, dried and imaged using a Technai™ G2 Spirit BioTWIN transmission electron microscope.

Immunogold labeling for transmission electron microscopy. Cells were fixed in 4% paraformaldehyde in 0.1 N sodium phosphate (pH 7.4) for 30 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min and then blocked with 1% BSA in PBS. The cells were incubated overnight at 4°C with Polaris primary antibody (1:20) in 1% BSA in PBS. The samples were washed 3-4 times over 30 min and then labeled with Protein A-gold-5 nm in 1% BSA for 1-2 h. The samples were washed 5 times over 1 h then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for an additional hour at 4°C and stored in 0.1 M cacodylate buffer at 4°C. The samples were embedded as described in the TEM protocol.

Scanning electron microscopy. MTEC cultures were washed with PBS twice with gentle agitation on a vortex to disrupt mucus coating then fixed for 1 h at 4°C in 4% paraformaldehyde in 1X PBS (pH 7.4). The samples were then washed thoroughly in three changes of PBS and stored at 4°C. The tissue was post fixed in 1% osmium tetroxide for 1 hour then washed three times with PBS. The samples were dehydrated in a graded series of ethanol by 15 min incubations with: 30%, 50%, 70%, 90%, and three times at 100%. The samples were then critical point dried and mounted onto studs with the epithelial cell layer facing upwards and then sputter coated and stored in the desiccator. Specimens were imaged with a JEOL 9355 Field Emission Zeiss Gun scanning electron microscope with backscatter detector.

Immunohistochemistry staining. Formalin-fixed, paraffin-embedded 5-micron-thick lung sections were deparaffinized through graded alcohols and washed in PBS. Heat-activated antigen retrieval was performed in a microwave oven using a citrate buffer (DAKO Cytomation, pH 6.0) for 10 min. Endogenous peroxidases were blocked using a peroxidase blocker (DAKO

Cytomation), and the slides were blocked for nonspecific protein binding by incubating in 10% normal horse serum for 45 minutes, and the primary antibody, goat anti-HDAC6 (Santa Cruz Biotechnology, used at 1:250 for the murine slides and 1:100 for the human samples) was applied at 4ºC overnight. The following morning, the slides were washed with PBS, and biotinylated donkey anti-goat (Jackson ImmunoResearch, used at 1:2500) was applied for 2 hours at 4ºC. The slides were washed, and strepavidin-horseradish peroxidase (Jackson ImmunoResearch, used at 1:1000) was applied for 30 min at room temperature. The slides were then incubated with 0.025% diaminobenzidine to identify immunopositive cells. The slides were then counterstained with 2% methyl green (Sigma), dehydrated and coverslipped. The negative control consisted of substituting PBS for the primary antibody.

The threshold feature in Image J was used to measure differences in HDAC6 staining intensity. All images were taken at the same settings and magnification using an Olympus FSX100 Fluorescent Microscope. Briefly, all images were converted to RGB stacks to separate the red, green and blue channels. Regions of interest were drawn over airways or parenchyma respectively. Using the threshold function in the green channel, the Brightness bar was adjusted to a point where all cells/tissue were selected. With the ROI selected, the intensity of all the cells was recorded using the Analyze>measure function of Image J. Using the threshold function in the red channel the Brightness bar was adjusted to a point were only the IHC stain was selected. With the ROI selected, the intensity of all the cells was recorded using the Analyze>measure function of Image J. The percentage intensity of HDAC6 staining was calculated by (IHC stained area)/(Total area) * 100%.

Patient samples. Patient samples were classified based on the guidelines of the Global Initiative for Obstructive Lung Disease (6). Patient samples in Figure 9 were obtained from the Lung Tissue Research Consortium (LTRC) and are described in Supplementary Table 1.

Assessment of mucociliary clearance in vivo via 3D μSPECT imaging of the whole animal. Mucociliary clearance was quantified using a non-invasive, oropharyngeal aspiration procedure described previously (7-9). Mice were anesthetized by i.p. injection of a mixture of ketamine (100 mg/kg) and xylazine (16 mg/kg) and suspended from their upper incisors at a 45º incline. 50 μl of normal saline containing approximately 0.3-0.5 mCi of the radiopharmaceutical ^{99m}Technetium-sulfur colloid (^{99m}Tc-SC) was introduced into the distal part of the oropharynx and aspirated. Mouse lungs were imaged immediately after aspiration (time 0h), at 1 and 3 hours, thereafter, with mice being re-anesthetized approximately 10 min before the 1 hour and the 3 hour images were obtained. Whole mouse 3 D μ-SPECT images were obtained using the Harvard Medical School μSPECT scanner (HMS-μSPECT), based on a TRIAD XLT-20 triple head-detector SPECT system [Trionix], equipped with custom-built high-magnification tungsten pinhole collimators which provide a reconstructed spatial resolution of ~0.8 mm within the mouse (10). Ninety dual-pinhole projection images were acquired using 30 angular steps during a 360° rotation of the three gamma cameras; at each position, photon counts were acquired by all detectors into a 256x256 image matrix for 20 sec, yielding a total acquisition time of ~10 min for each mouse. Three-dimensional (3D) image volumes were reconstructed using a 3D ordered-subsets expectation-maximization (OSEM) algorithm (11), modified for the geometry of the HMS μSPECT system; two iterations of OSEM were utilized with 10 angular subsets, of 9 projections each, per iteration. The reconstructed image volume consisted of 80 x 80 (transaxial) x 200 (axial) isotropic voxels, each of dimensions 0.4 x 0.4 x 0.4 mm^3. A syringe containing a

known amount of ^{99m}Tc was acquired at the start of each imaging day and reconstructed for two iterations of OSEM to check the linearity of counts/pixel to activity over the course of the study.

It is known that the site of aerosolized radiotracer deposition can affect MCC. For example, insoluble particles that deposit in ciliated airways are cleared from the respiratory tract with half-times between 3–12 hours in both animals and man (7-9, 12) while removal from non-ciliated lung regions is typically not by MCC mechanisms and requires more than 24 hours. To explore the possibility of differences in MCC due to variations in regional lung deposition, we analyzed the initial aerosol distribution within the lungs of all mice in this study. This method is adapted from a previous method to include both the right and left lung, and a detailed explanation for distribution determination has been described previously (9). Briefly, the combined right and left lung image of each animal was divided into 9 smaller squares. The square that was most central and closest to the trachea was identified as the central region (C). The remaining squares were collectively identified as the peripheral region (P). See Figure 1 Bhashyam, A.R., *et al.* (2012) (8). Distribution was quantified in terms of a central to peripheral ratio (C:P ratio). High C:P ratios indicate greater radiotracer deposition within the large airways, while low C:P ratios indicate greater deposition in the smaller airways and alveoli.

Reconstructed image volumes from the 0, 1, and 3 hour time points were analyzed using tools in ImageJ (http://rsbweb.nih.gov/ij/). A composite image created from a "sum slices" z-projection over 80 coronal images was used to analyze clearance of ^{99m}Tc via MCC. Regions of interest (ROI) were drawn to encompass both lungs (excluding the trachea, determined by anatomical location at the carina, and GI tract) and distributed activity was measured at 0, 1 and

3 hours after instillation. Counts in both lungs at 1 and at 3 hours were background-corrected followed by decay-correction to time 0 hour counts. Decay-corrected counts were then divided by time 0 hour counts. Retention at each time point was subtracted from 1.00 and multiplied by 100% to obtain percent removed by mucociliary clearance[7]. Radioisotope aerosol distribution was qualitatively assessed between all groups for differences in central to peripheral distribution. Biodistribution studies post 3 hour μSPECT imaging were performed by sacrificing the animals using CO_2 asphyxiation. This was immediately followed by dissection and collection of all major organs of interest in pre-weighed tubes. Radioactivity in the various organs was determined in an automated gamma-counter in the preset energy windows for 142 keV gamma-emission of ^{99m}Tc isotope. Tubes containing known ^{99m}Tc activity were also counted as standards along with the harvested tissues to standardize cpms/μCi in the harvested tissues. The retention of the ^{99m}Tc-SC activity 3 hour post pulmonary instillation in the lungs and any redistribution of the ^{99m}Tc to other major organ was then calculated and expressed as % of instilled dose/g of organ weight (%ID/g). Anatomic localization of the lungs was obtained by acquiring a ^{99m}Tc-MDP bone SPECT prior to a ^{99m}Tc-SC lung instillation. Separately acquired skeletal and lung images were co-registered to the simultaneously acquired ^{99m}Tc-MDP/^{99m}Tc-SC image and fused using the Align Stacks plugin for ImageJ (http://www.med.harvard.edu/JPNM/ij/plugins/AlignStacks.html) **(Figure. 8A).**

Statistical analysis. Statistical analysis was conducted using GraphPad Prism software (GraphPad Software). Data are presented as the mean ± SEM from at least three independent experiments. Differences in measured variables between experiment and control groups were assessed using the Student's t test and between multiple groups and conditions using one-way

and two-way ANOVAs and Bonferroni post tests. Statistically significant differences *P<0.05, highly significant differences **P<0.01 and very highly significant differences ***P<0.005 are indicated.

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Supplementary Figure Legends

Supplemental Figure 1. 100mg/m^3 CS disrupts MTEC culture integrity and induces cytotoxicity. (**A**) Time- and dose-dependent changes in TER following exposure to 50 and 100 mg/m^3 CS (N=3). (**B**) LDH activity was assayed in the basal media of MTECs following treatment with 50 and 100 mg/m^3 CS (N=3). (**C**) ATP levels were measured in MTEC cultures 24 h after exposure to 50 and 100 mg/m^3 CS. (**D**) Early Apoptotic cells (annexin V positive and propidium iodide negative) of MTEC cultures 24 h after exposure to 50 and 100 mg/m^3 were identified by flow cytometry (N=3). (**E**) Scanning Electron Microscopy (SEM) morphology of MTEC cells 24 h after exposure to 50 and 100 mg/m^3 CS. Arrows indicate intact ciliated cells, arrowheads indicate cells with apoptotic morphology, characterized by cells rounding up and losing cell-cell contacts. Number of MTEC cells with apoptotic morphology (rounding up) / field by SEM (N=2 / group, 10 fields from each sample) (*bottom right*). All data are mean $\pm$ SEM. **P*<0.05, ***P*<0.01,****P*<0.001, compared to controls by Student's unpaired *t* test or one-way ANOVA followed by Bonferroni post tests.

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Western blots are representative of 3-4 independent experiments. β-Actin served as the standard. All data are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, one-way or two-way ANOVA and Bonferroni post tests.

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Supplemental Figure 11. CS induces autophagic turnover of cilia components in vivo. (**A**) Autophagosome/lysosome-enriched fractions of lungs from LC3B-GFP mice exposed to RA (N=8) or CS (N=9) for 3 weeks immunoprecipitated with Anti-GFP. Mice were injected with PBS (N=4) or Leupeptin (N=4) for 2 hours prior to harvesting. GFP immunoprecipitated LC3B positive autophagosomes and lysosomes were immuoblotted for LC3B and HDAC6. Bar charts are representative of average densitometry for N=3-4 immunoblots normalized to RA PBS controls. All data are mean + SEM. *P<0.05, **P<0.01, student's unpaired t test.

Supplemental Figure 12. Representative Hematoxylin and eosin (H&E) staining of mouse airways exposed to RA, CS (3 weeks) or 3 weeks CS followed by 1 week recovery. Arrows indicate cilia. Scale is 10μM. N=3 mice/group.

Supplemental Figure 13. HDAC6 expression is regulated by smoking and in COPD and cilia disassembly markers correlate with COPD disease severity. (**A**) HDAC6 expression was assessed by Western Immunoblotting of lung homogenate samples of never smokers, ever (former) smokers, and COPD patients (G2, G4). β-Actin served as the standard. N=4-5 patient samples/group (**B**) Expression of the cilia regulatory proteins phospho-GSK 3α/β and Aurora A in never smokers (NS), ever smokers (ES) and COPD patient lung tissue (G2, G4) was assessed by immunoblotting. p38 MAPK served as the standard and densitometry was calculated (*right*) by normalizing to p38. N=2 patients/group. All data are mean + SEM. *P<0.05, one-way ANOVA.

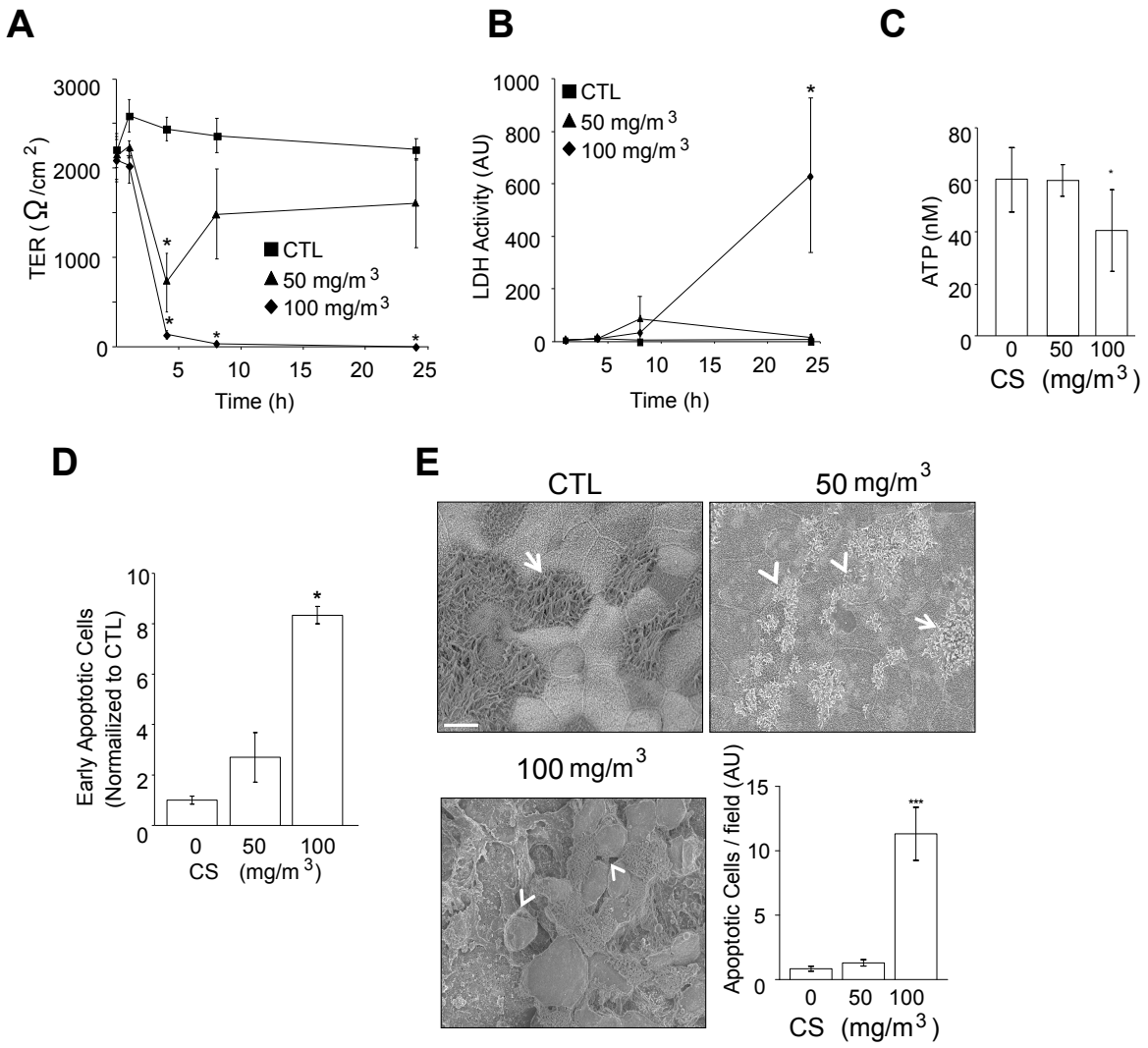
Supplemental Figure 14. Representative immunostaining from airway tissues stained with HDAC6. HDAC6 expression was assessed in mouse airways after 6 months exposure to RA or CS. Arrows denote HDAC6 positive staining. N=3 mice/group.

Supplemental Figure 15. Protein aggregates in the parenchyma of Never smokers (NS), ever smokers (ES) and COPD patients with GOLD stage 2 (G2) or 4 (G4) disease severity. Red staining representative of protein aggregates (PA), green staining representative of acetylated α-tubulin and blue Hoechst staining of nucleated cells. Representative of N=3 patients/group. Scale bars: 10 μm, arrows indicate protein aggregates and inclusion bodies.

SUPPLEMENTAL TABLE 1. Clinical information of human lung specimens.

	Never-Smokers (*N* = 5)	Ever-Smokers (*N* = 5)	COPD GOLD 2 (*N* = 5)	COPD GOLD 4 (*N* = 5)
Age, yr	79 ± 3	72 ± 3	70 ± 3	55 ± 2*#§
Smoking index at entry, pack-years	0 ± 0	52 ± 10*	52 ± 4*	50 ± 7*
Lung function				
FVC, % predicted	99.8 ± 5.2	85.4 ± 2.1*	91.3 ± 2.8	46.0 ± 3.7*#§
FEV_1, % predicted	102.4 ± 5.7	85.8 ± 1.7*	66.3 ± 1.9*#	17.0 ± 0.4*#§
FEV_1/FVC	0.76 ± 0.02	0.74 ± 0.02*	0.53 ± 0.01*#	0.30 ± 0.04*#§
Emphysema score	0.0 ± 0.0	0.6 ± 0.2*	1.3 ± 0.3	3.2 ± 0.1*#§

Definition of abbreviations; COPD = chronic obstructive pulmonary disease; GOLD = The Global Initiative for Obstructive Lung Disease; FVC = forced vital capacity; FEV_1 = forced expiratory volume in one second.
Data expressed as mean ± standard error of the mean (SEM).
* $p < 0.05$, compared with the never-smoker group.
# $p < 0.05$, compared with the ever-smoker group.
§ $p < 0.05$, compared with the COPD GOLD 2 group.



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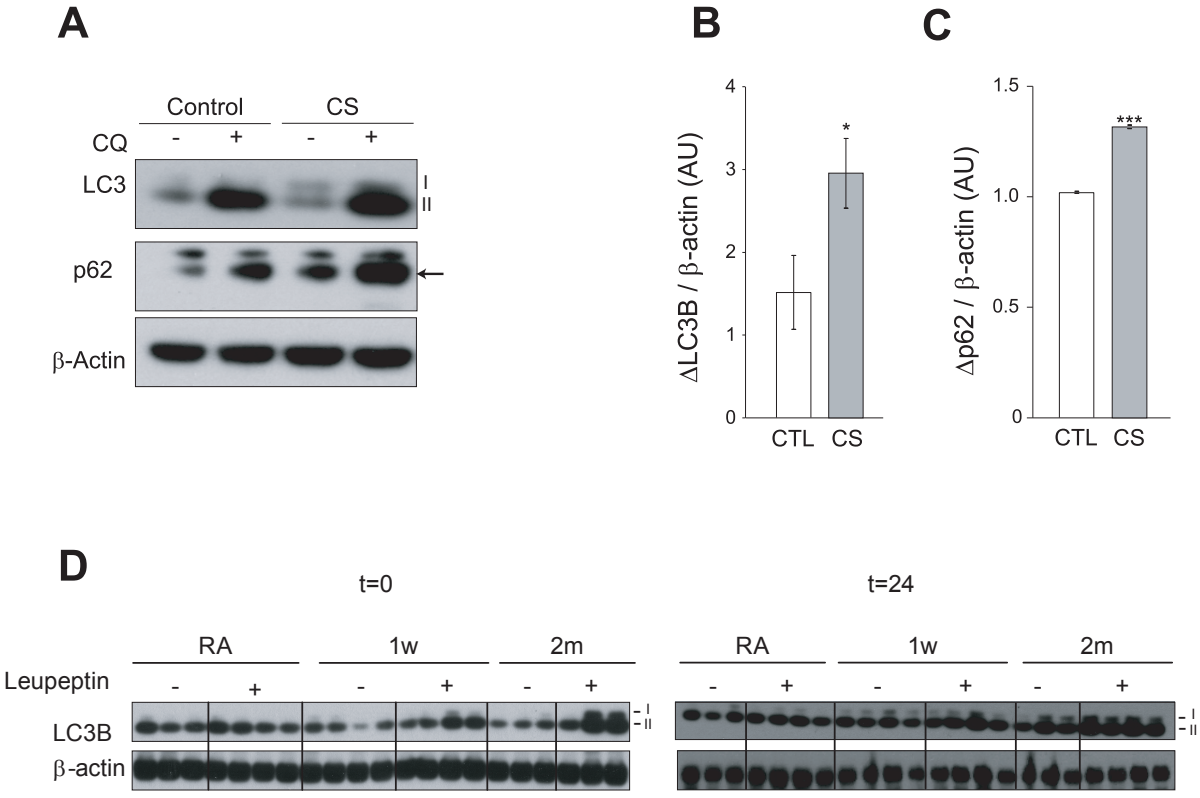

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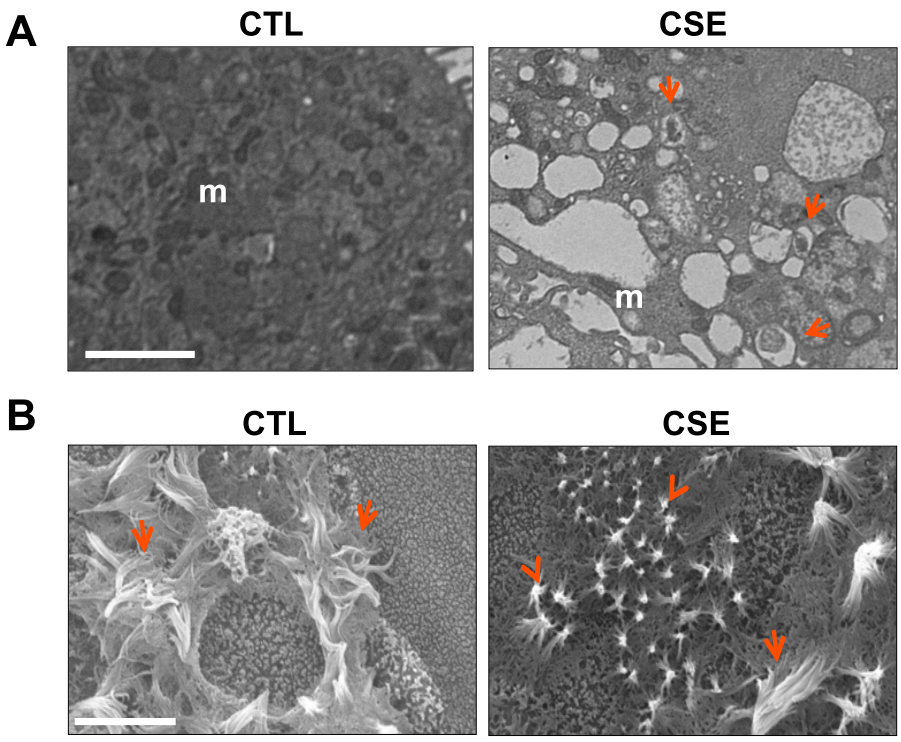

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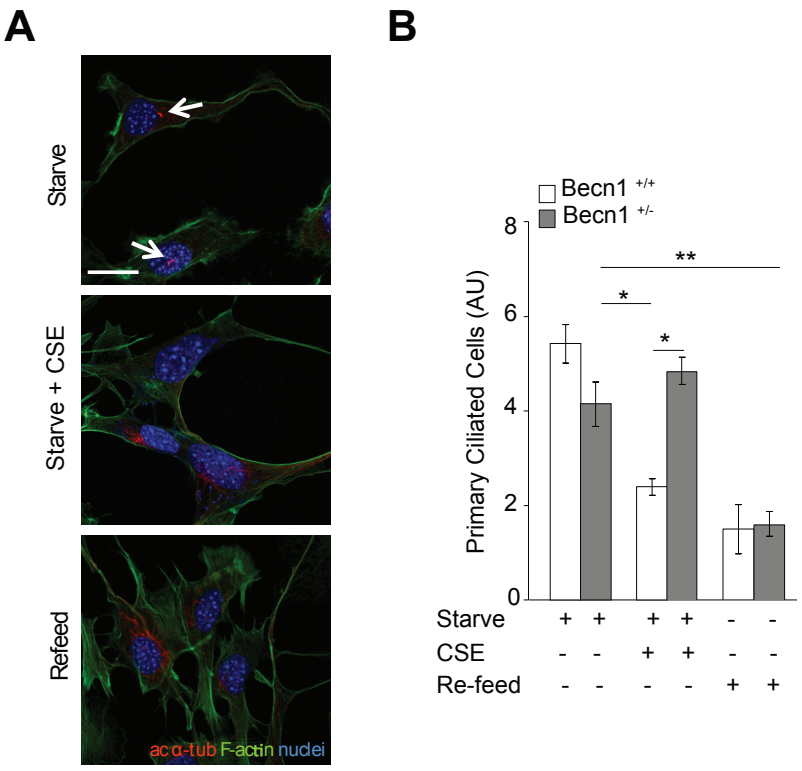

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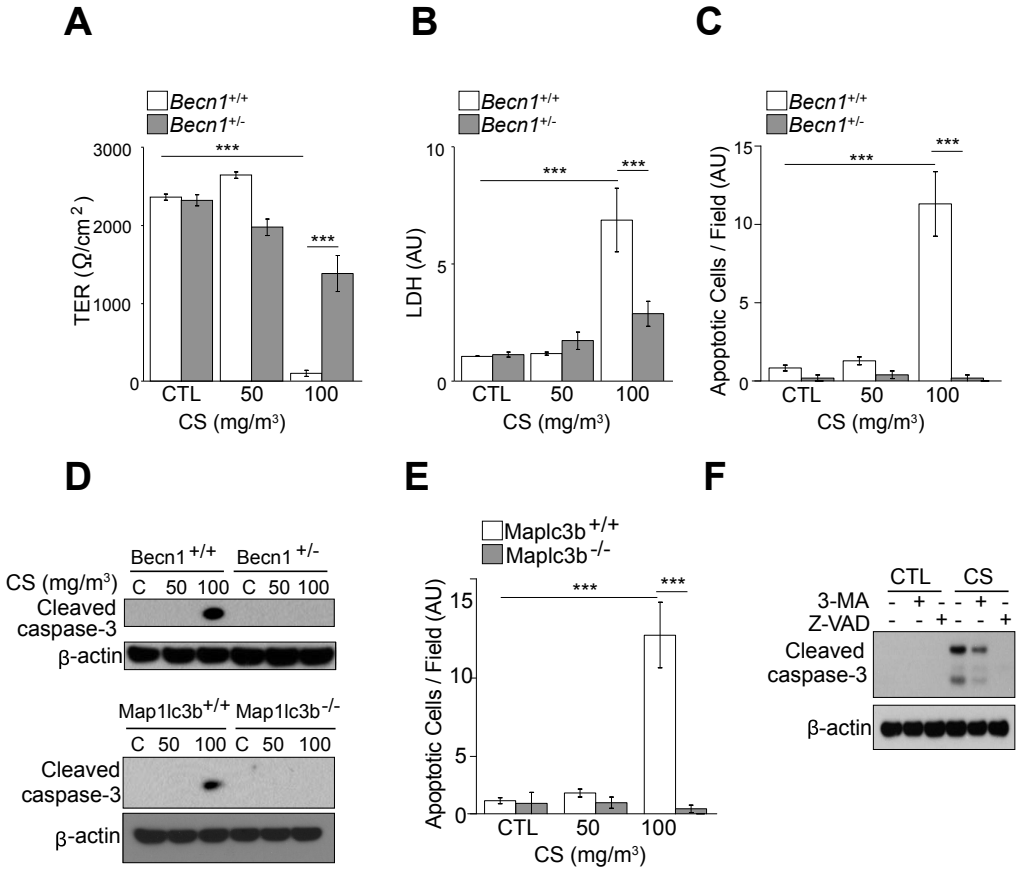

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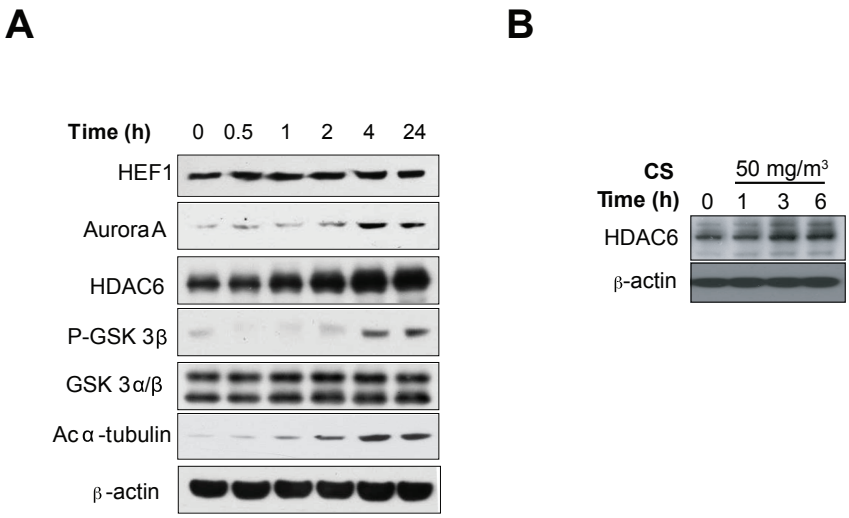

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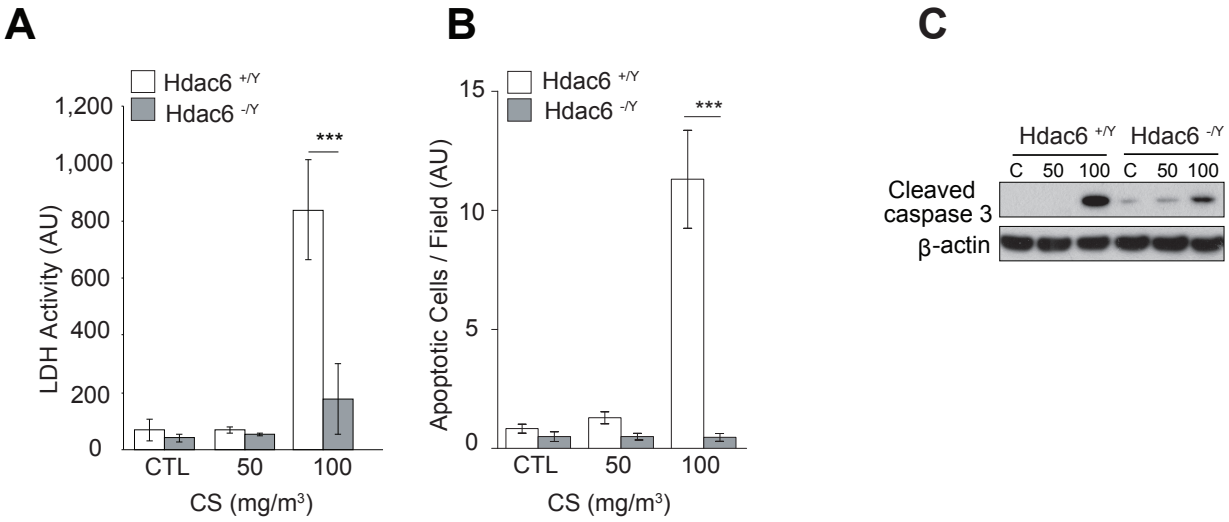

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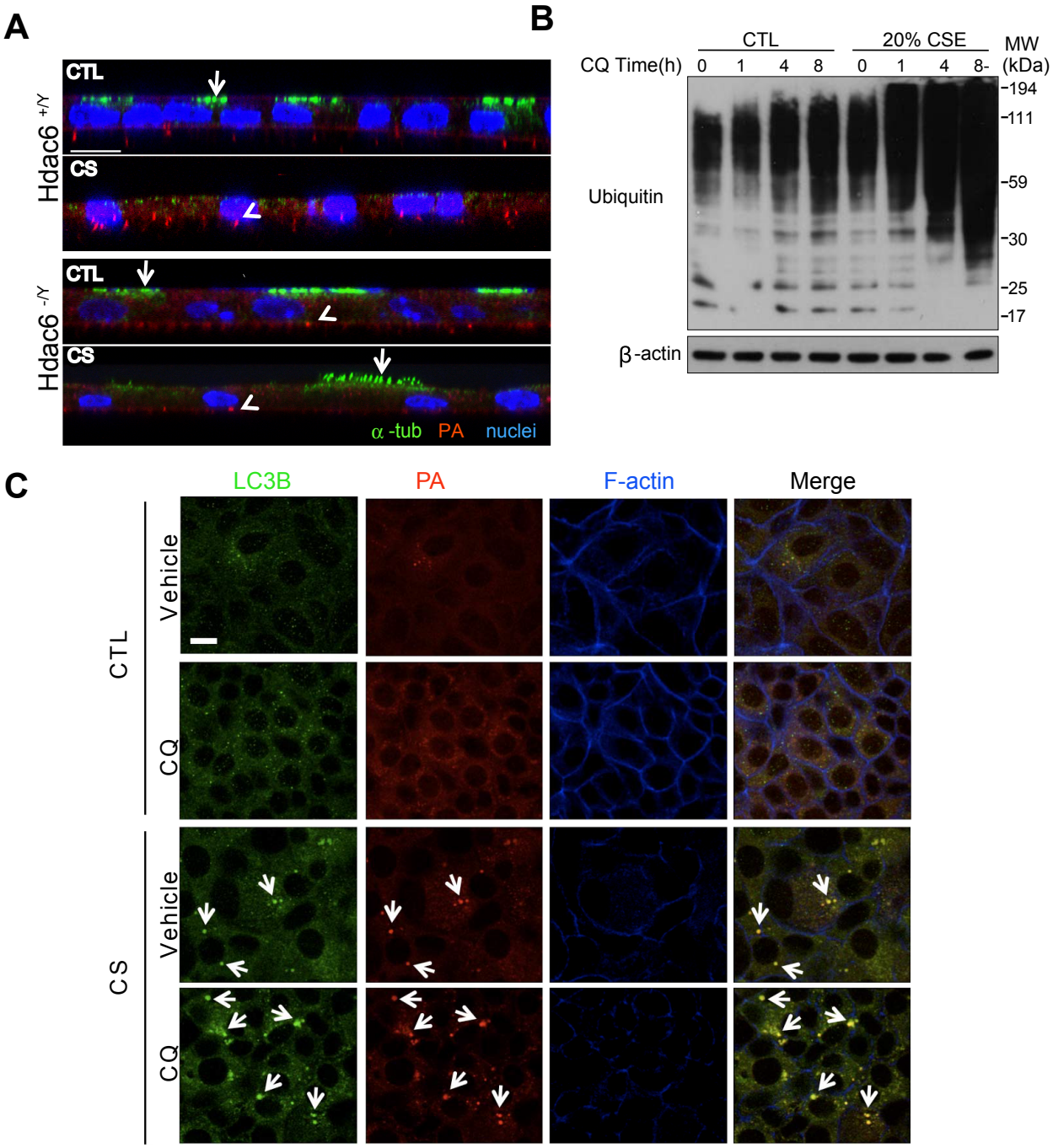

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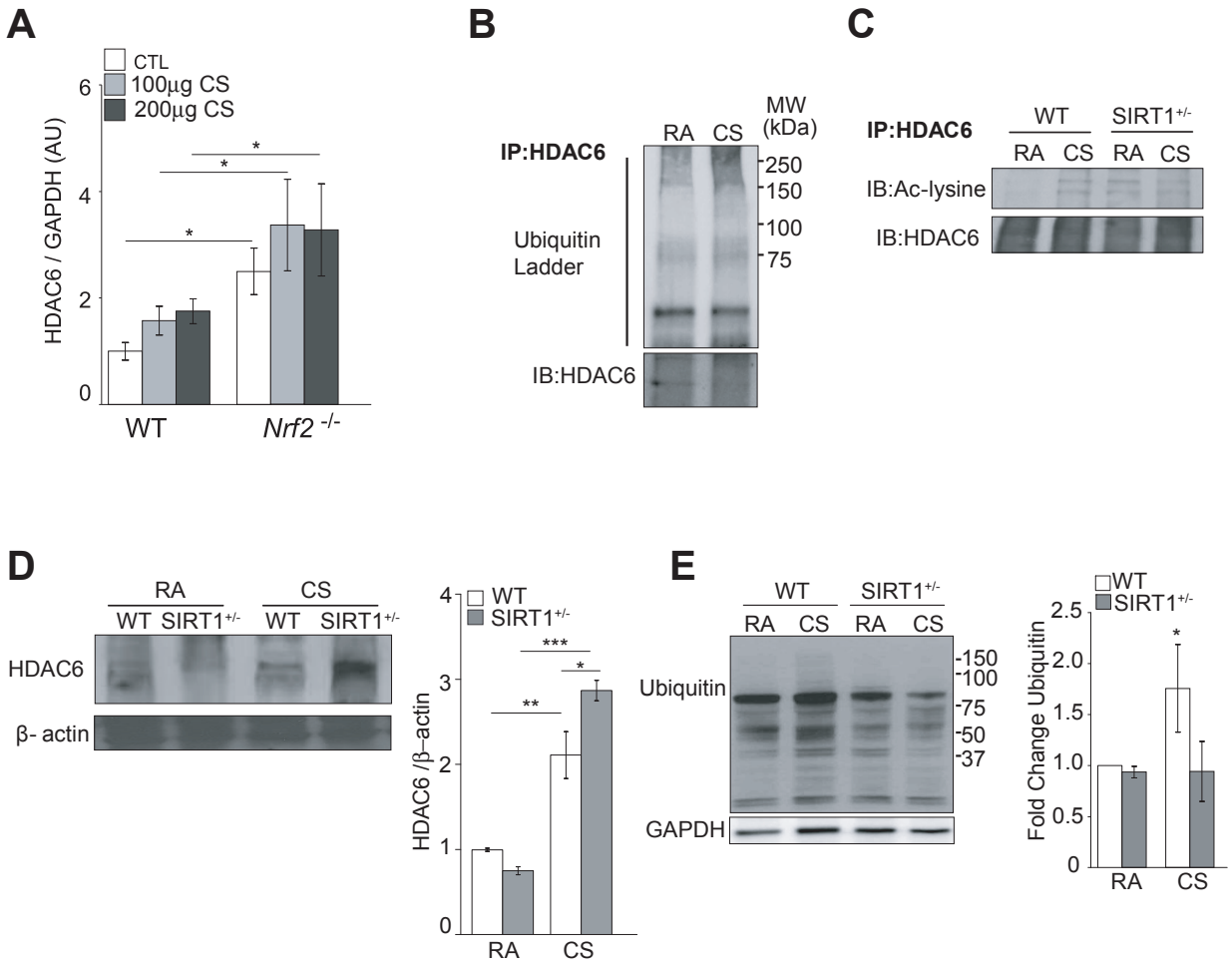

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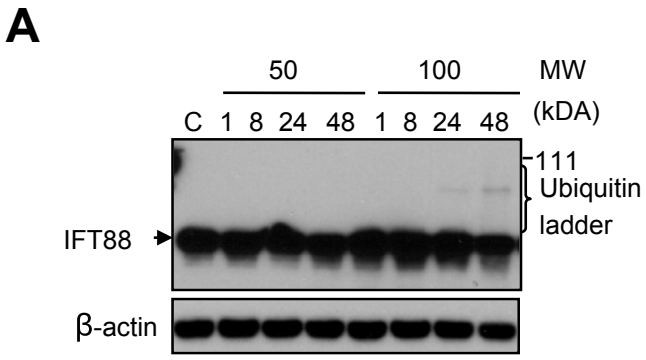

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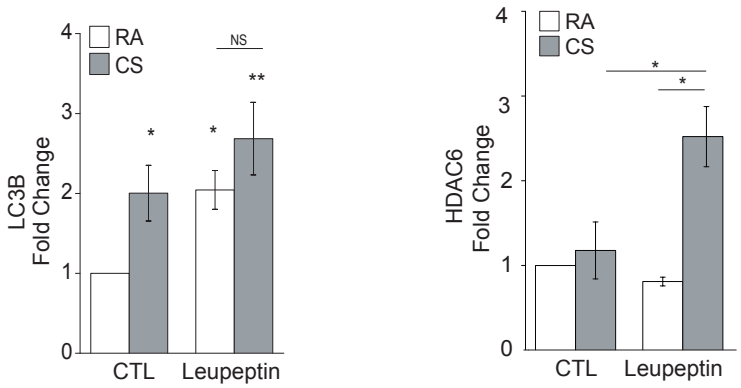

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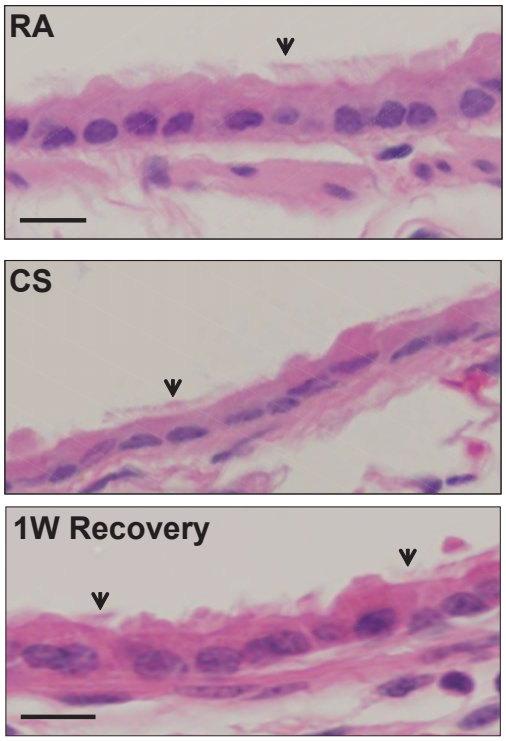

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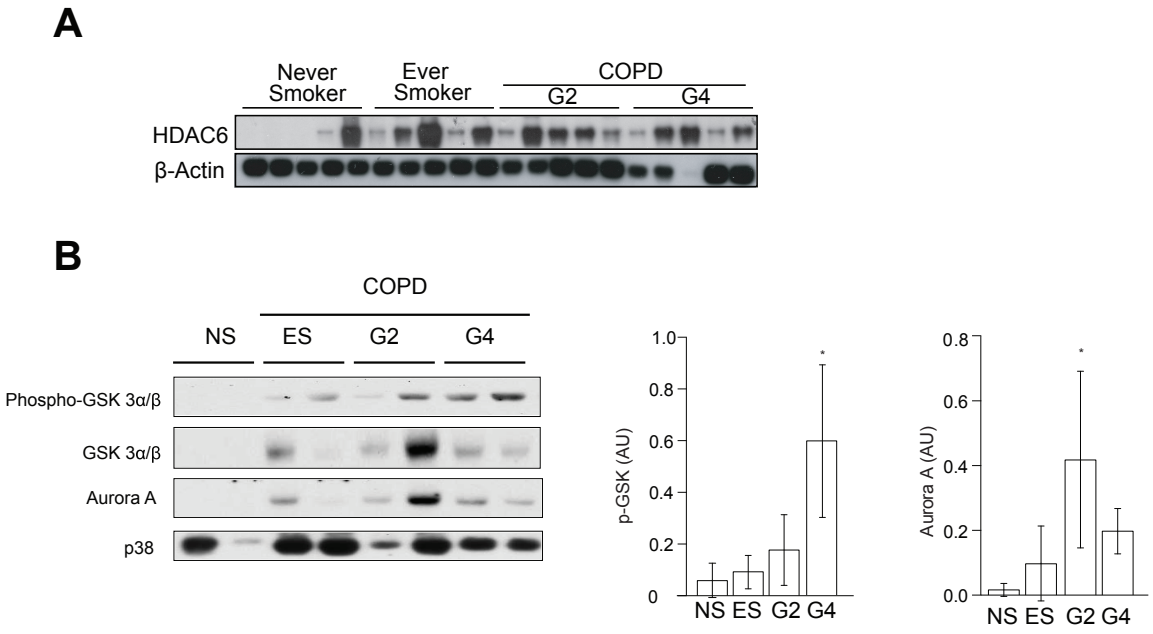

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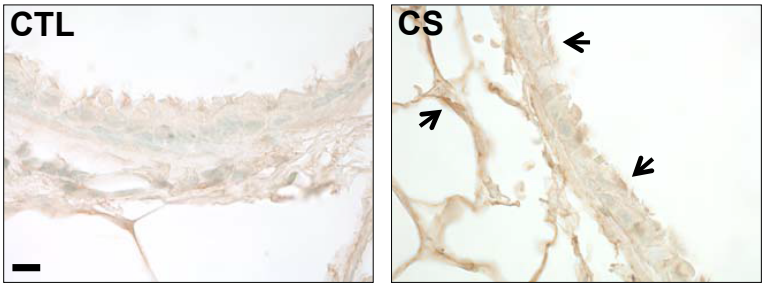
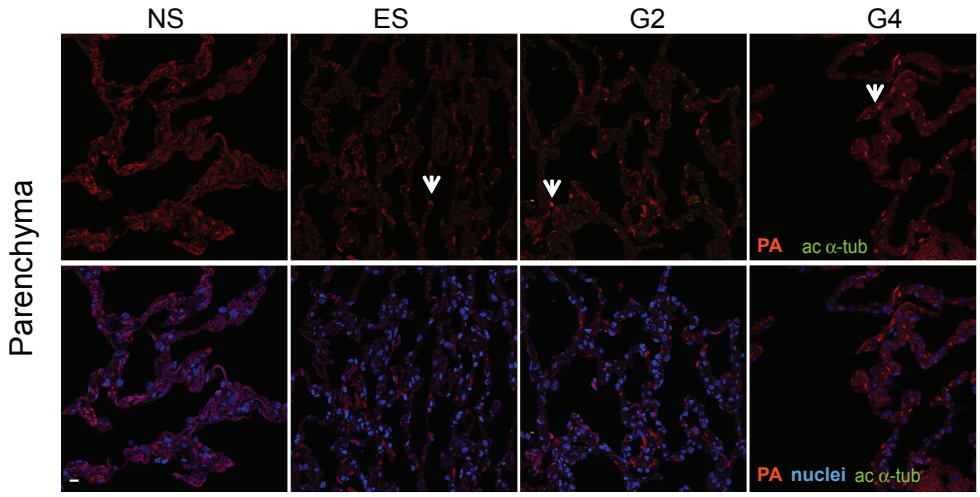


Supplemental Figure 14. Representative immunostaining from airway tissues stained with HDAC6. HDAC6 expression was assessed in mouse airways after 6 months exposure to RA or CS. Arrows denote HDAC6 positive staining. N=3 mice/group.



Supplemental Figure 15. Protein aggregates in the parenchyma of Never smokers (NS), ever smokers (ES) and COPD patients with GOLD stage 2 (G2) or 4 (G4) disease severity. Red staining representative of protein aggregates (PA), green staining representative of acetylated α-tubulin and blue Hoechst staining of nucleated cells. Representative of N=3 patients/group.Scale bars: 10 μm, arrows indicate protein aggregates and inclusion bodies.