Supporting Information

Chen et al. 10.1073/pnas.1005574107

SI Methods

Preparation of CSE. For preparation of CSE, Kentucky 3R4F research-reference filtered cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) were smoked by using a peristaltic pump (VWR International). Before the experiments, the filters were cut from the cigarettes. Each cigarette was smoked in 6 min with a 17-mm butt remaining. Four cigarettes were bubbled through 40 mL of cell growth medium, and this solution, regarded as 100%-strength CSE, was adjusted to a pH of 7.45 and used within 15 min after preparation.

Total Body in Vivo CS Exposure. Total body CS exposure was performed in a stainless-steel chamber (71 cm × 61 cm × 61 cm) using a smoking machine (model TE-10; Teague Enterprises). The smoking machine puffs each 3R4F cigarette for 2 s, for a total of nine puffs before ejection, at a flow rate of 1.05 L/min, providing a standard puff of 35 cm^3. The smoke machine was adjusted to deliver 10 cigarettes at one time. Mice were exposed 5 d per week for 12 wk. The chamber atmosphere was periodically measured for total particulate matter concentrations of 100 to 120 mg/m^3. Carboxyhemoglobin levels in the C57BL/6 strains of mice after 2 wk of cigarette exposure were typically less than 8% immediately following exposure.

Lung Morphometry. Immediately following necropsy, the right lung was inflated by gravity with 4% paraformaldehyde and held at a pressure of 30 cm H_2O for 15 min. The left lung was removed and immediately stored in liquid nitrogen for subsequent protein studies. The right lung was gently dissected from the thorax and placed in 4% paraformaldehyde for a maximum of 8 h. The samples were cut parasagitally and embedded in paraffin. Modified Gills staining was performed and 12 random 1,300-pixel × 1,030-pixel images were acquired digitally for each lung sample using a light microscope (Axiophot; Carl Zeiss MicroImaging) equipped with a digital camera (Axiocam HRc; Carl Zeiss MicroImaging) at 20× magnification. Large airways, blood vessels, and other nonalveolar structures were manually removed from the images.

Airspace enlargement was quantified using a previously published, automated image processing algorithm (1), and also using the MLI method (2–4). Both these indexes are known to increase with the onset of emphysema. MLI was measured using modified ImageJ software (National Institutes of Health). The ImageJ software program automatically thresholded the images, and a median filter, set to a two-pixel radius, was run to smooth the image edges. The program laid a line grid comprised of 1,353 lines with each line measuring 21 pixels over the individual images. The software then counted the number of lines that ended on or intercepted alveolar tissue. These data were used to calculate the volume of air, the volume of tissue, the surface area, the surface area-to-tissue volume ratio. MLI was calculated according to methods adapted from Dunhill (2) as 4 divided by the surface area-to-volume tissue ratio. Airspace enlargement was also quantified by using an automatic image processing technique. Details of the algorithm are published elsewhere (1). Briefly, the method uses the watershed transform to segment digital images of stained histological sections into individual alveolar airspaces. The equivalent diameter (d_i) of an airspace with area A_i was then calculated using the following equation:

$$d_i = 2\sqrt{A_i/\pi} \quad \textbf{[S1]}$$

As with the MLI measurements, large airways and blood vessels were manually excluded from the analysis.

Transmission EM. Lung tissue sections were fixed in formalin and embedded in paraffin. Cells were fixed in 2.5% glutaraldehyde in PBS solution after experimental manipulations. These tissues or cells were photographed using a JEM 1210 transmission electron microscope (JEOL) at 80 or 60 kV onto EM film (ESTAR thick base; Kodak) and printed onto photographic paper.

Immunoprecipitation and Western Blot Analysis. Immunoprecipitation and immunoblotting were performed essentially as previously described (5–7). Antibodies against LC3B and β-actin were from Sigma. The cleaved caspase-3 antibody was obtained from Cell Signaling. All other antibodies were from Santa Cruz Biotechnology.

Sucrose-Gradient Subcellular Fractionation. Sucrose gradient-derived fractions were separated as described previously (8). In brief, cells were lysed in ice-cold MBS buffer (25 mM Mes, pH 6.5, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, and protease inhibitors). Lysates were adjusted to 4 mL of 40% sucrose by mixing with 2 mL of 80% sucrose and overlaid with 4 mL of 35% sucrose and 4 mL of 5% sucrose in MBS buffer. Samples were ultracentrifuged at 39,000 rpm for 18 h [SW41 rotor (Beckman Instruments, Palo Alto, CA)] and fractionated into 12 subfractions.

1. Parameswaran H, Majumdar A, Ito S, Alencar AM, Suki B (2006) Quantitative characterization of airspace enlargement in emphysema. *J Appl Physiol* 100:186–193.
2. Dunhill MS (1962) Quantitative methods in the study of pulmonary pathology. *Thorax* 17:320–328.
3. Weibel ER (1979) *Stereological Methods* (Academic Press, London).
4. Tomkeieff SI (1945) Linear intercepts, areas and volumes. *Nature* 155:24.
5. Chen ZH, et al. (2008) Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. *PLoS ONE* 3:e3316.
6. Park JW, et al. (2008) Protein kinase C α and ζ differentially regulate death-inducing signaling complex formation in cigarette smoke extract-induced apoptosis. *J Immunol* 180:4668–4678.
7. Wang XM, et al. (2006) Caveolin-1: A critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. *J Exp Med* 203:2895–2906.
8. Nakahira K, et al. (2006) Carbon monoxide differentially inhibits TLR signaling pathways by regulating ROS-induced trafficking of TLRs to lipid rafts. *J Exp Med* 203: 2377–2389.

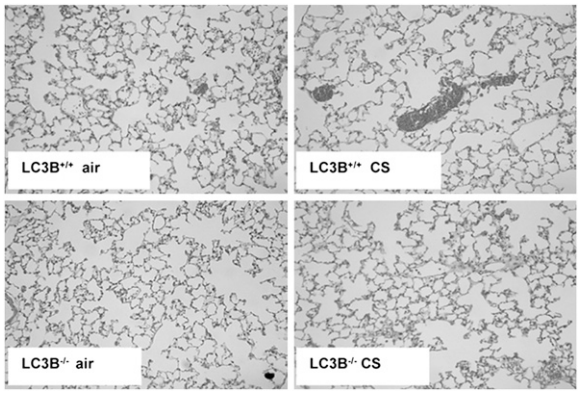

Fig. S1. WT C57BL/6, *LC3B*$^{+/+}$, or *LC3B*$^{-/-}$ mice were exposed to chronic CS exposure or air for 3 mo (C57BL/6, $n = 9$ for air, $n = 8$ for CS; *LC3B*$^{+/+}$, $n = 6$ for air, $n = 9$ for CS; *LC3B*$^{-/-}$ $n = 9$ for air, $n = 9$ for CS). Representative images of mouse lungs with H&E staining.

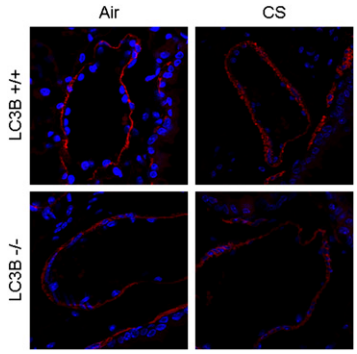

Fig. S2. CS alters vascular wall morphology in WT mice. *LC3B*$^{+/+}$ or *LC3B*$^{-/-}$ mice were exposed to chronic CS exposure or air for 3 mo ($n = 8$–11 for CS, $n = 6$–11 for air). Representative images of mouse lungs stained with α-smooth muscle actin to show vascular morphology. Sections were counterstained with DAPI. Images are at a magnification of 68×.

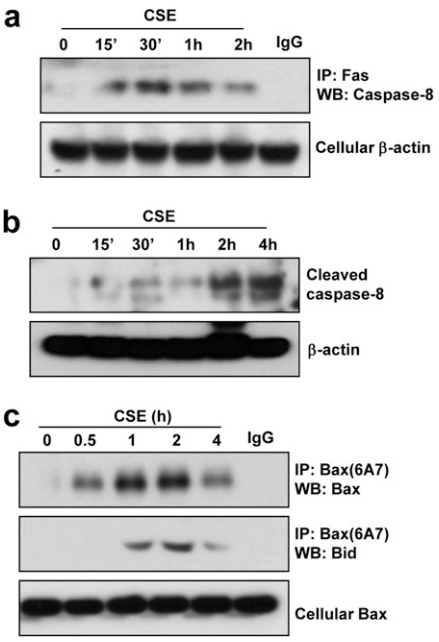

Fig. S3. CSE activates extrinsic apoptotic pathways in Beas-2B cells. Beas-2B cells were treated with 10% CSE for the indicated times and the cell lysates were subjected to IP and/or WB analyses for (*A*) Fas-mediated DISK formation, (*B*) caspase-8 activation, or (*C*) Bax activation by CSE in Beas-2B cells.

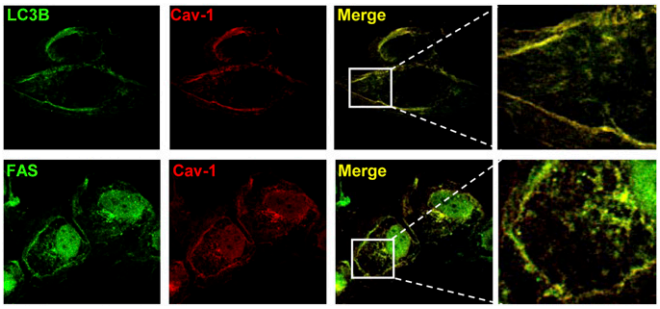

Fig. S4. LC3B colocalizes with Fas and Cav-1. Representative immunofluorescence images of LC3B, Fas, and Cav-1 staining under basal conditions in Beas-2B cells. Colocalization is indicated by the merged images (*Right*).

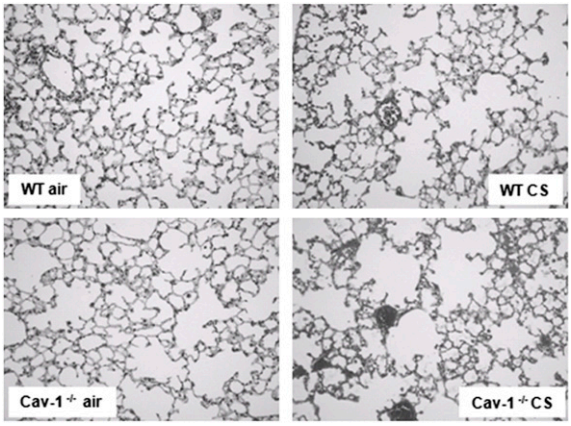

Fig. S5. WT C57BL/6 or *Cav-1*$^{-/-}$ mice were exposed to chronic CS or air exposure for 3 mo (each group, $n = 5$). Representative images of mouse lungs with H&E staining.

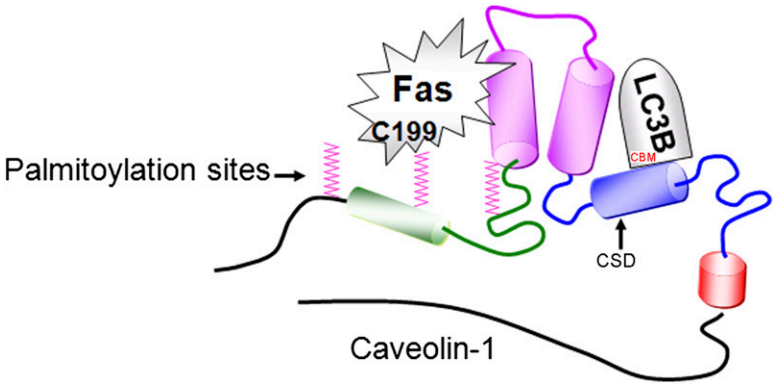

Fig. S6. Schemata showing proposed mode of binding of LC3 with the Cav-1 molecule through binding interactions at the LC3B CBM and CSD. Fas binds with Cav-1 through palmitoylation domains.

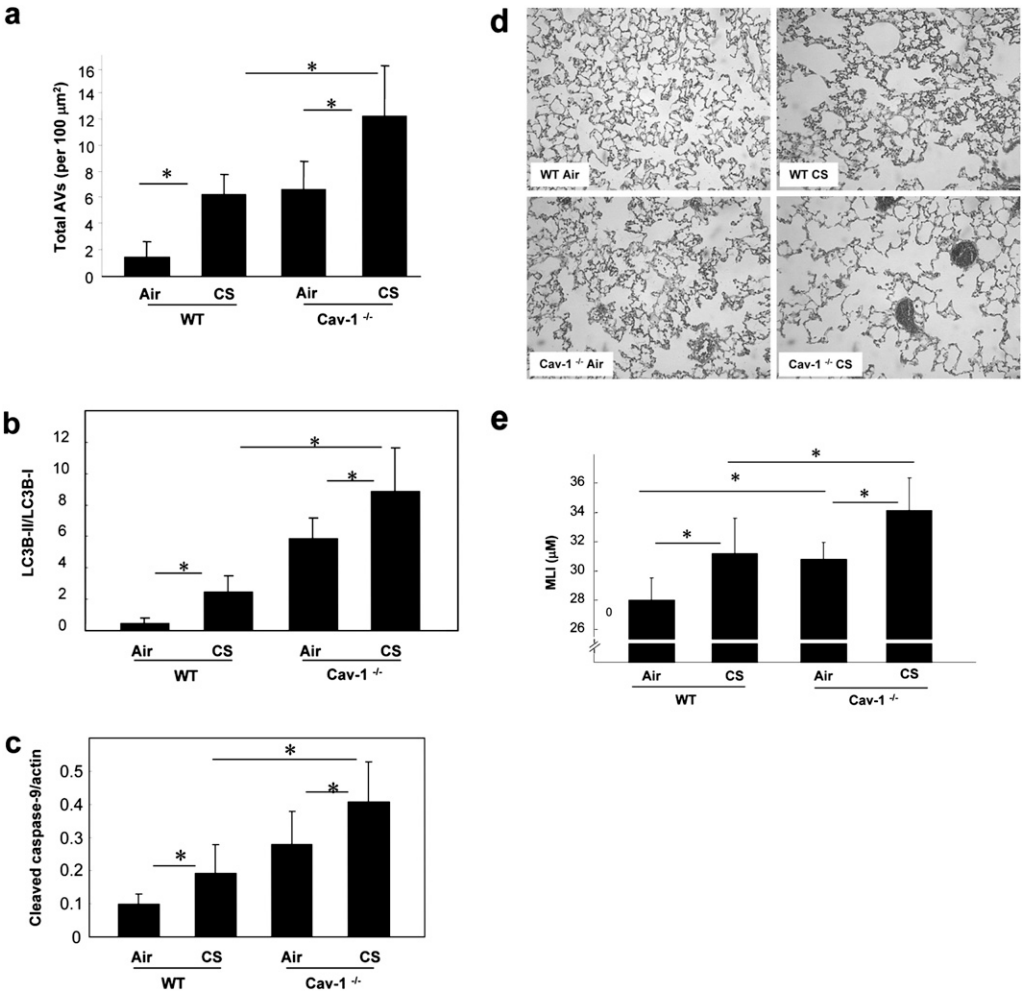

Fig. S7. Cav-1 regulates CS-induced autophagy, apoptosis, and emphysematous airspace enlargement in vivo, in the total body smoking model. WT C57BL/6 or *Cav-1*$^{-/-}$ mice were exposed to chronic CS exposure for 3 mo (n = 9–10 for each group). (*A*) EM images scored for number of AVs. The data are represented as AVs per 100 μm^2; n = 20 images representative of each group. (*B* and *C*) Western blot analysis and corresponding quantification for (*B*) LC3B-II/LC3B-I ratio and (*C*) cleaved caspase-9 in CS exposed mouse lungs. (*D*) Representative images of mouse lungs with H&E staining. (*E*) MLI scoring of mouse lungs exposed to CS. Data represent means ± SD; *$P < 0.05$.