**Volonte et al., Supplemental Material**

## SUPPLEMENTAL METHODS

*Materials.* Antibodies and their sources were as follows: anti-caveolin-1 IgG (pAb N-20), anti-p21 (pAb), anti-p53 IgG (pAb FL-393), and anti-ATM IgG (mAb 2C1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser1981-ATM IgG (mAb) was from Cell Signaling Technology (Beverly, MA). All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

*Immunoblotting.* Cells were collected in boiling sample buffer. Cellular proteins were resolved by SDS-PAGE (12.5% acrylamide) and transferred to BA83 nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Blots were incubated for 2 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 2% powdered skim milk and 1% bovine serum albumin (BSA). After three washes with TBST, membranes were incubated for 2 h with the primary antibody and for 1 h with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG. Bound antibodies were detected using an ECL detection kit (Pierce, Rockford, IL).

*Immunofluorescence Microscopy.* Cells grown on glass coverslips were washed three times with PBS w/$Ca^{++}$/$Mg^{++}$ and fixed for 30 min at room temperature with 2% paraformaldehyde in PBS w/$Ca^{++}$/$Mg^{++}$. Fixed cells were rinsed with PBS w/$Ca^{++}$/$Mg^{++}$and permeabilized with 0.1% Triton X-100, 0.2% bovine serum albumin for 10 min. Then cells were treated with 25 mM $NH_4Cl$ in PBS w/$Ca^{++}$/$Mg^{++}$ for 10 min at room temperature to quench free aldehyde groups. Cells were rinsed with PBS w/$Ca^{++}$/$Mg^{++}$ and incubated with the primary antibody (diluted in PBS with 0.1% Triton X-100, 0.2% bovine serum albumin) for 2h at room temperature. After three washes with PBS w/$Ca^{++}$/$Mg^{++}$ (10 min each), cells were incubated with the secondary antibody for 1h at room temperature: lissamine rhodamine B sulfonyl chloride-conjugated goat anti-rabbit antibody (5 μg/ml) and fluorescein isothiocyanate-conjugated goat anti-mouse antibody (5 μg/ ml). Finally, cells were washed three times with PBS w/$Ca^{++}$/$Mg^{++}$ (10 min each wash) and slides were mounted with slow-Fade anti-fade reagent (Molecular Probes, Inc., Eugene, OR) and observed using a Zeiss Confocal Microscope (LSM 5 Pascal).

*Acid β-galactosidase staining.* Cells were subjected to acid β-galactosidase staining using the Senescence-Associated β-galactosidase Staining Kit (Cell Signaling), according to the manufacturer's recommendations. Briefly, cells were washed twice with PBS and fixed with the fixative solution for 15 minutes. Then, cells were washed twice with PBS and incubated overnight at 37$^{o}$C with the staining solution. Cells were then examined for the development of blue color using a BX50WI Olympus Optical light microscope (Tokyo, Japan).

*Fibroblast Isolation and Cigarette Smoke Extract (CSE) Treatment*. Fibroblasts were cultured by harvesting lungs and placing them in 100-mm tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum. After excising the large airways, lungs were transferred to a fresh 100-mm tissue culture dish containing the same medium as above and minced with sterile scissors. The plates were then placed in a 37°C incubator with 5% $CO_2$. Adherent fibroblasts were cultured in DMEM + 10% fetal bovine serum. CSE solutions were prepared as described previously (9). Through one opening of a stopcock, 10 ml of sterile DMEM were drawn into a 50-ml plastic syringe. Subsequently, 40 ml of cigarette smoke were drawn into the syringe and mixed with the medium by vigorous shaking. One cigarette was used for each 10 ml of medium. The generated CSE solution was filtered (0.22 μm) to remove large particles. The resulting solution was designated a 100% CSE solution. The CSE solution was used immediately after generation.

*Cigarette Smoke Exposure*. 8-week-old caveolin-1 null mice were used in these experiments ($n$ = 6). Age- and sex-matched wild type (WT) littermates were used as controls ($n$ = 6). Mice were put into a standard smoking chamber, and they received a total of four unfiltered cigarettes (University of Kentucky) per day (5 days a week) for either 6 weeks or 6 months. Mice were placed in a smoking chamber with nose and mouth enclosed in a small compartment with eyes protected. 20 ml of cigarette smoke were drawn out of a cigarette with syringes and then exhausted, by means of a two-way valve, into a compartment surrounding the noses and mouths of mice. Smoke was delivered two puffs/min with a 10-min rest between each cigarette for a maximum of two cigarettes. There was a 4-6 h rest, and then another two cigarettes were administered as described above to yield a total of four cigarettes/day. Initially, mice were placed in the chamber without difficulty. After ~2 weeks of exposure, they ran in the chamber themselves. Following this protocol, mice have been previously shown to well tolerate the treatment without evidence of toxicity (carboxyhemoglobin levels ~10%) (24). Mice were then euthanized by $CO_2$ narcosis, and their lungs were removed and analyzed.

## SUPPLEMENTAL FIGURE LEGENDS

Supplemental Fig. 1. Oxidative stress activates ATM, upregulates p21, and induces premature senescence in lung fibroblasts. A-B. WI-38 cells were treated with 450 μM $H_2O_2$ for 2 hours. Cells were washed and recovered in growth medium for 24 hours. Untreated cells were used as controls. Cells were then collected and subjected to immunoblotting analysis using antibody probes specific for phospho-Ser1981 ATM (A), total ATM (A), and p21 (B). Immunoblotting with anti-β-actin IgGs was performed to show equal loading (B). C-D. WI-38 cells were treated with 450 μM $H_2O_2$ for 2 hours in the presence or absence of the ATM inhibitor caffeine (200 nM). Cells were washed and recovered in growth medium for 5 days in the presence or absence of caffeine. Untreated cells were used as controls. Cells were then collected and cellular senescence determined by staining for senescence-associated (SA) β-galactosidase (β-gal) activity. Representative fields are shown in C. Quantification of the staining from three independent experiments is shown in D. Values represent means ± SEM. *$P$<0.005.

Supplemental Fig. 2. Caveolin-1 promotes the translocation of PP2A-C into caveolar membranes after oxidative stress. (A) WI-38 cells were treated with 450 μM $H_2O_2$ for 2 hours. Cells were washed and recovered in growth medium for 24 hours. Untreated cells were used as controls. Cells were then collected and detergent-resistant microdomains (DRMs) separated from the bulk of cellular membranes and cytosolic proteins (Non-DRMs) by equilibrium sucrose density gradient centrifugation. Expression of PP2A-Cα, caveolin-1 (Cav-1), and ATM were examined by immunoblotting analysis using specific antibody probes. (B) WI-38 cells were treated with 450 μM $H_2O_2$ for 2 hours. Cells were washed and recovered in growth medium for 24 hours. Untreated cells were used as controls. Cells were then collected, lysed, and caveolin-1 immunoprecipitated using anti-caveolin-1 IgGs. Immunoprecipitates were then subjected to immunoblotting analysis using an antibody probe specific for PP2A-C (Upper panel). Lower panels show total expression of both PP2A-C and caveolin-1 before immunoprecipitation.

Supplemental Fig. 3. PP2A-C partially co-localizes with caveolin-1 after oxidative stress. Wild-type (WT) and caveolin-1 null (Cav-1 KO) MEFs were treated with 150 μM $H_2O_2$ for 2 hours. Cells were washed and recovered in growth medium for 24 hours. Untreated cells were used as controls. Cells were then collected and subjected to immunofluorescence analysis using antibody probes specific for PP2A-C (green) and caveolin-1 (red) in A and ATM (red) and DAPI (blue) in B.

Supplemental Fig. 4. Accumulation of inflammatory cells in response to cigarette smoking is reduced in caveolin-1 null lungs. Two-month-old wild type (WT) and caveolin-1 null (Cav-1 KO) mice were exposed to cigarette smoking for 6 weeks (Sm). Non-smoking WT and Cav-1 null mice were used as

controls (NS). Bronchoalveolar lavage (BAL) total cell counts were determined on NS and Sm WT and Cav-1 null mice. Values represent means ± SEM (n=2). $*,^{\#}P<0.005$.

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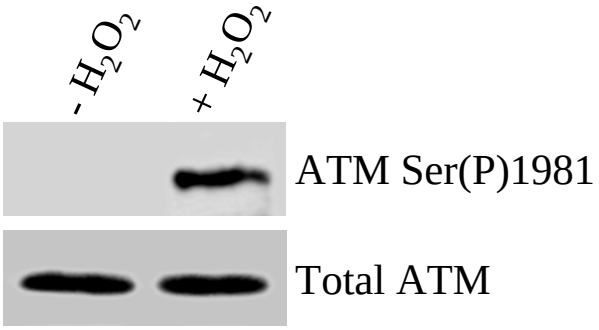
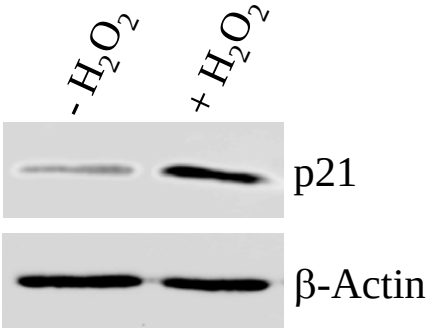
Suppl. Figure 1A, B

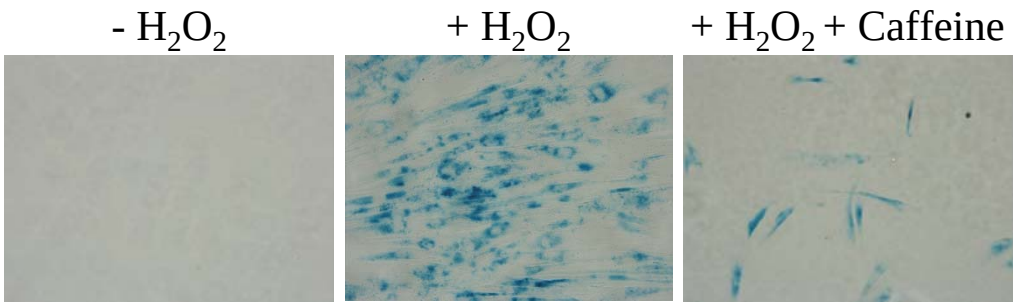
**A**



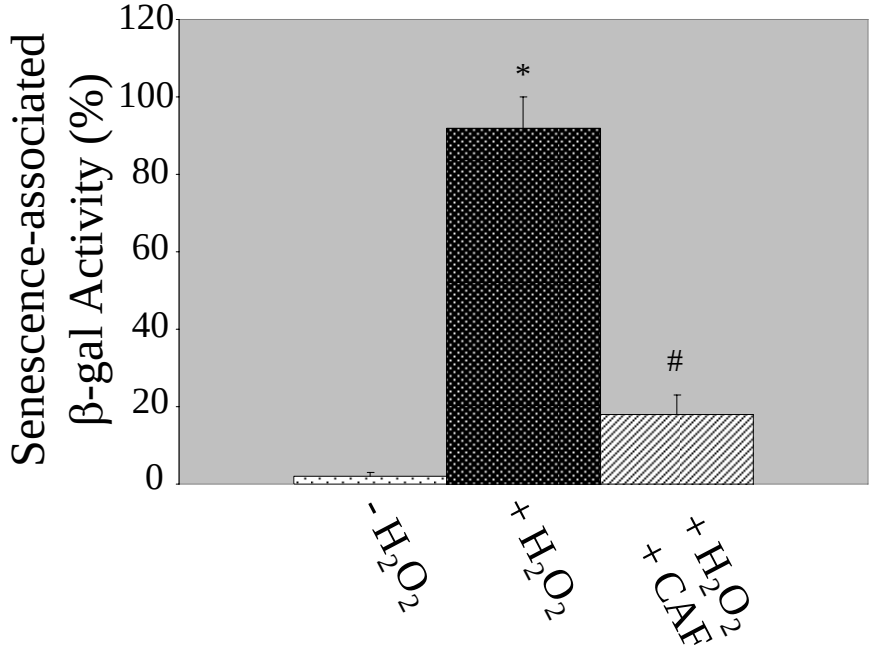

**B**



**C**

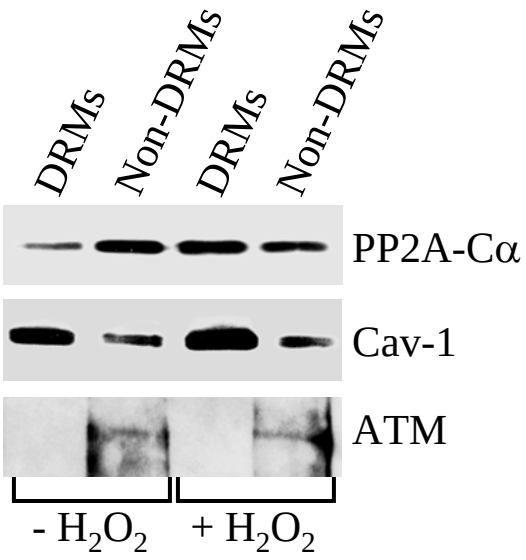

- $H_2O_2$
+ $H_2O_2$
+ $H_2O_2$ + Caffeine

**D**


Senescence-associated
β-gal Activity (%)
120
100
80
60
40
20
0
*
#
- $H_2O_2$
+ $H_2O_2$
+ $H_2O_2$
+ CAF

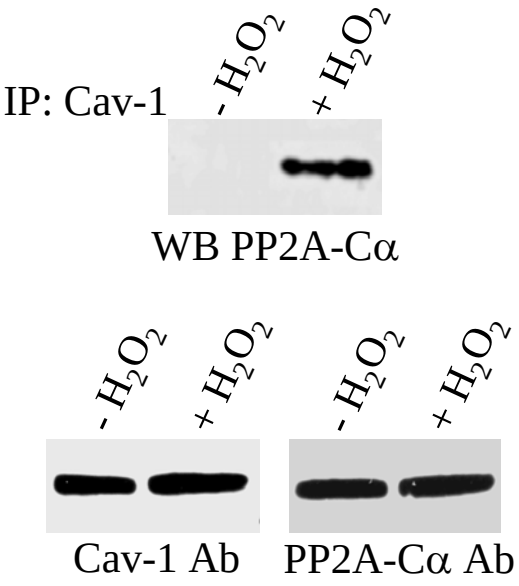
Volonte et al.,
Suppl. Figure 2A

**A**



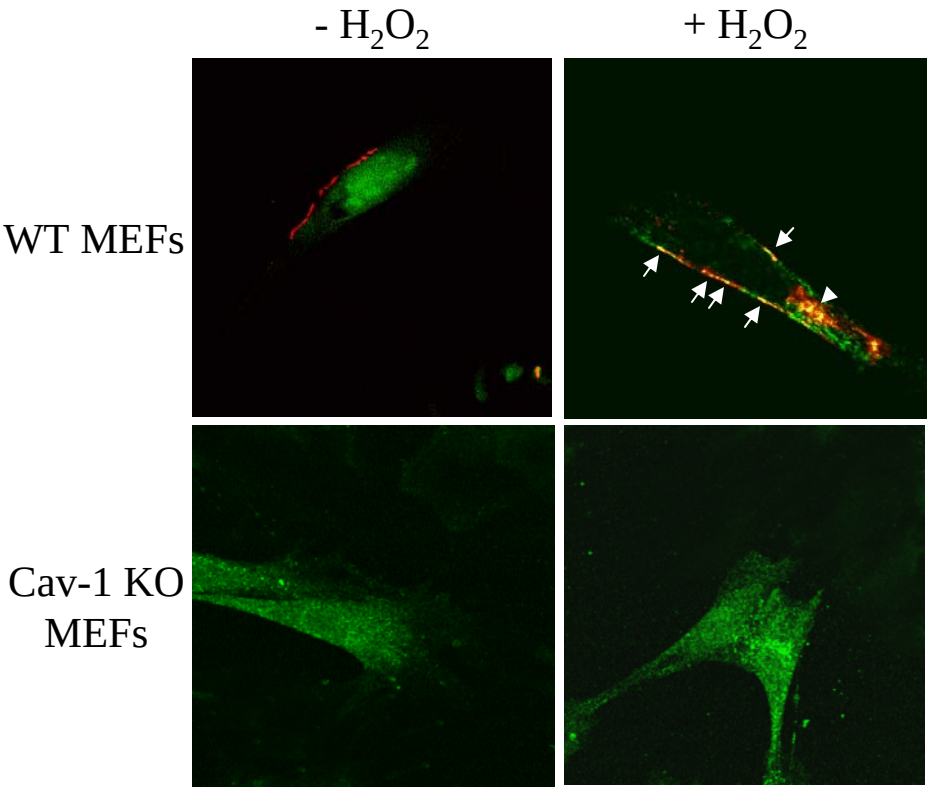
Volonte et al.,
Suppl. Figure 2B

**B**



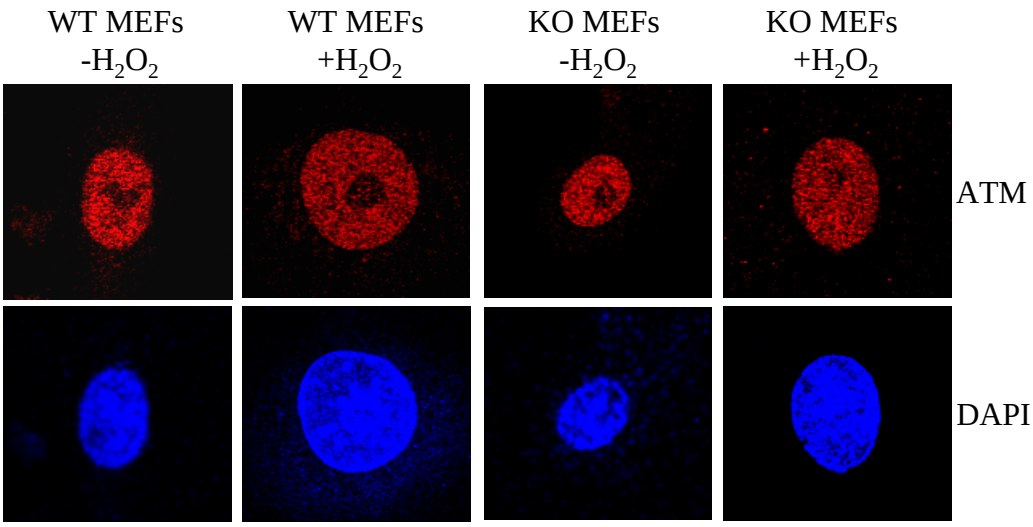
Volonte et al.,
Suppl. Figure 3A

**A**



Volonte et al.,
Suppl. Figure 3B

**B**



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Suppl. Figure 4