# Online Supplement

**S100A8 protects human primary alveolar type II cells against injury and emphysema**

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Running title: S100A8 protects against emphysema

# METHODS

## Human subjects

We selected donors with reasonable lung function with a $PaO_2/FIO_2$ ratio of >250, a clinical history and x-ray that did not indicate infection, and limited time on a ventilator. Non-smokers were individuals who never smoked and smokers smoked 10-20 cigarettes per day for at least 3 years. Lungs from patients with emphysema (GOLD IV) were obtained through Temple Biobank (Temple University, Philadelphia, PA). We used lung tissue from 3 - 12 individuals per group, 45-69 years old, both females and males. The study was performed in accordance with the Declaration of Helsinki protocols, and was approved by IRB at Partners Healthcare and Temple University.

## Isolation and culture of human primary ATII cells

ATII cells were isolated from non-smokers, smokers, and emphysema patients. Briefly, after instillation of 12.9 U/ml elastase (Worthington, Lakewood, NJ), the lung was minced followed by centrifugation to collect cell suspension. The cells were filtrated and purified by a density gradient made of Optiprep (Accurate Chemical Scientific Corp., Westbury, NY) and by negative selection with CD14-coated magnetic beads (Dynal Biotech ASA, Oslo, Norway). We used IgG-coated (Sigma Chemicals Inc., St. Louis, MO) dishes. The Committee for the Protection of Human Subjects at Temple University approved this research.

The isolated ATII cells were cultured as we reported previously (1). Briefly, we used DMEM medium (GE Healthcare, Bensalem, PA) supplemented with 10% fetal bovine serum (FBS; GE Healthcare, Bensalem, PA), 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 2.5 μg/ml amphotericin B and 10 μg/ml gentamicin (all from Thermo Fisher Scientific, Waltham, MA). ATII cell differentiated state was maintained by plating for 2 d with 10% FBS on millicell inserts (Millipore Corp., Bedford, MA) coated with 20% Matrigel

Matrix (Corning, Tewksbury, MA) and 80% rat-tail collagen in DMEM. ATII cells were cultured for 2 d with 1% charcoal-stripped FBS along with 10 ng/ml keratinocyte growth factor (KGF; R&D Systems Inc., Minneapolis, MN), and for 2 d with 1% charcoal-stripped FBS along with 10 ng/ml KGF, 0.1 mM isobutylmethylxanthine (Sigma Chemicals Inc., St. Louis, MO), 0.1 mM 8-bromo-cyclic AMP (Sigma Chemicals Inc., St. Louis, MO), and 10 nM dexamethasone (Sigma Chemicals Inc., St. Louis, MO).

**Chest computed tomography (CT) scans and tissue cores processing**

CT scans were subjected to a standard quality control procedure. Computerized image analysis was performed with 3D SLICER software (2). Emphysema was quantified by the percent of the lung voxels on inspiratory CT scan with attenuation $< -950$ HU (Insp−950) (3). It was considered absent in subjects with values for Insp−950 $< 4\%$ in smokers, to account for the fact that the increased lung density in smokers results in a decrease in emphysema index (4). Severe emphysema was defined by Insp−950 $> 14\%$ in smokers. Lung tissue cores were obtained from areas with mild and severe emphysema as previously described (5). Briefly, lungs were removed from the thorax, inflated with air and frozen by liquid nitrogen vapor followed by being cut into 2-cm thick slices in the same plane as the CT scan. Tissue cores were collected from areas with mild and severe emphysema using a sharpened steel cylinder diameter of 1 cm and processed for Western blotting analysis. Subjects provided written informed consent prior to surgery for use of these specimens and the relevant clinical and radiological data required for research. The study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Boards at Partners Healthcare and the Committee for the Protection of Human Subjects at Temple University.

**A549 cell culture**

The human alveolar epithelial cell line A549 was maintained in DMEM medium supplemented with 10% FBS, 125 U/mL penicillin and 125 U/mL streptomycin. Cells used for experiments were free of mycoplasma contamination as assessed by MycoSeq Mycoplasma Real-Time PCR Detection Kit (Invitrogen, Waltham, MA).

**Real-time PCR**

Total RNA was isolated and then reverse-transcribed into cDNA by using Superscript II Reverse Transcript kit (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions. Briefly, 1 μg of RNA was incubated with Oligo d(T)20 primers and dNTP mix at 65°C for 5 min. Reverse transcription was performed at 55°C for 10 min followed by 80°C for 10 min. Gene-specific primers were retrieved from PrimerBank (http:// pgamgh.harvard.edu/primerbank/) and ordered from Invitrogen (Waltham, MA, USA): human S100A8 forward 5'ATGCCGTCTACAGGGATGAC 3' and reverse 5' ACTGAGGACACTCGGTCTCTA 3'; human GAPDH forward 5' GGAGCGAGATCCCTCCAAAAT 3' and reverse 5' GGCTGTTGTCATACTTCTCATGG 3'. The SYBR Green Master Mix kit (Thermo Fisher Scientific, Waltham, MA) and StepOnePlus Real-Time PCR System were used (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: 95°C for 10 min and then 45 cycles of 95°C for 15 s, 58°C for 1 min, and 68°C for 20 s followed by 95°C for 15 s and 60°C for 15 s.

**Western blotting and immunoprecipitation**

Cells were lysed, and human lung tissue was homogenized in lysis buffer with protease and phosphatase inhibitor cocktail (Gold Biotechnology, Olivette, MO). The lysate was centrifuged at 14,000 rpm for 20 min. Proteins were resolved by SDS-PAGE (Thermo Fisher Scientific, Waltham, MA) and transferred to

nitrocellulose membranes. Western blotting was performed using the following antibodies: β-actin (Sigma; St. Louis, MO), multi-ubiquitin (MBL; Nagoya, Aichi, Japan), and 4-HNE (R&D Systems, Inc, Minneapolis, MN). The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti-human S100A8, S100A9, p-serine, p-tyrosine, p-threonine, p-PKAα, PKAα, SYVN1, p-PKCα, PKCα, casein Kinase II α, p-ERK2, ERK2, A-Raf, Akt1, Akt2, Akt3, casein Kinase II α‘, casein kinase II β, CaMKI, CaMKIδ, CaMKII, CaMKIIβ, CaMKIIγ, CaMKIV, ERK1, ERK3, ERK5, follistatin, IRAK-1, JNK, Raf-B, Raf-1, p38α/β, p38γ, PKAα/β/γ, PHKA2, Mosxe, and ZIP-kinase. Immunoprecipitation was conducted using human primary ATII cell and lung tissue lysates incubated with S100A8 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 18 h followed by adding G Mag Sepharose beads (GE Healthcare, Bensalem, PA) for 2 h at 4°C. The protein complex was washed with 0.05% Tween 20 in TBS, eluted and used for Western blotting. Densitometric analysis or relative protein expression were performed using Image J (NIH).

**Dihydroethidium (DHE) staining**

Paraffin-embedded human lung tissue sections were incubated with 0.1 mM DHE (Invitrogen) for 12 min at RT (6). Fluoroshield mounting medium with DAPI (Abcam) was used to detect nuclei. Images were obtained using a confocal laser-scanning microscope (Zeiss).

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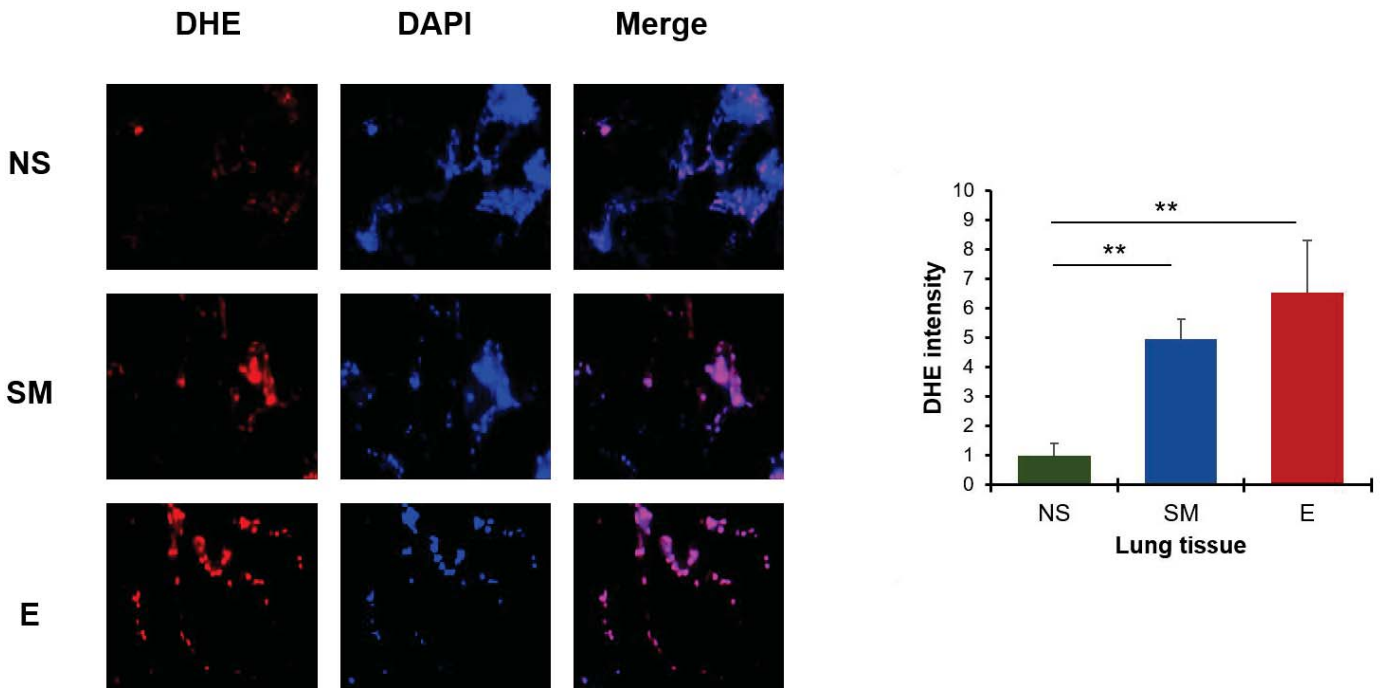



**Figure E1. ROS generation in human lung tissue as detected by DHE staining.** Representative images and relative fluorescence intensity are shown (n=4). $^{**}P < 0.001$. Data are shown as means ± s.e.m.

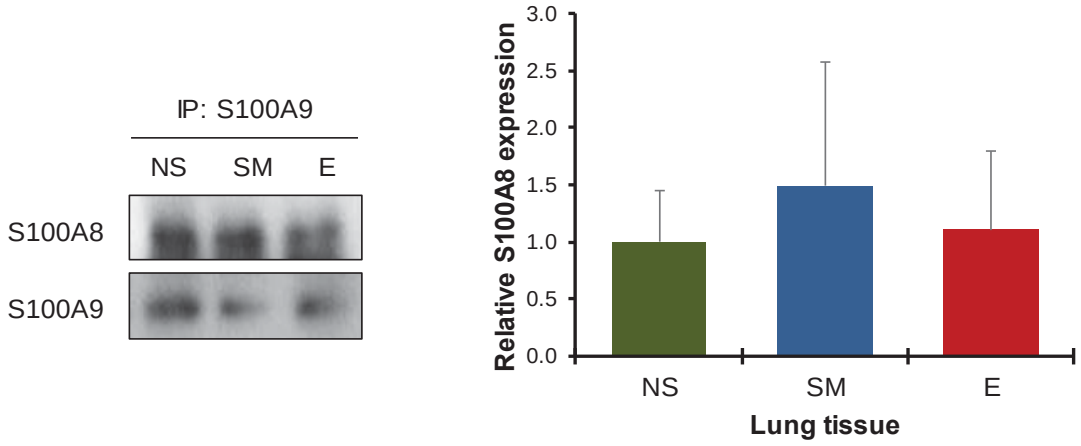



**Figure E2. S100A8 interaction with S100A9 in human lung tissue.** S100A9 was immunoprecipitated in lung tissue obtained from non-smokers (NS), smokers (SM), and patients with emphysema (E) followed by Western blotting to analyze S100A8 expression. Densitometric quantification is also shown (n = 4). The ratio of immunoprecipitated S100A9 to S100A8 was normalized to control non-smokers. Data are shown as means ± s.e.m.

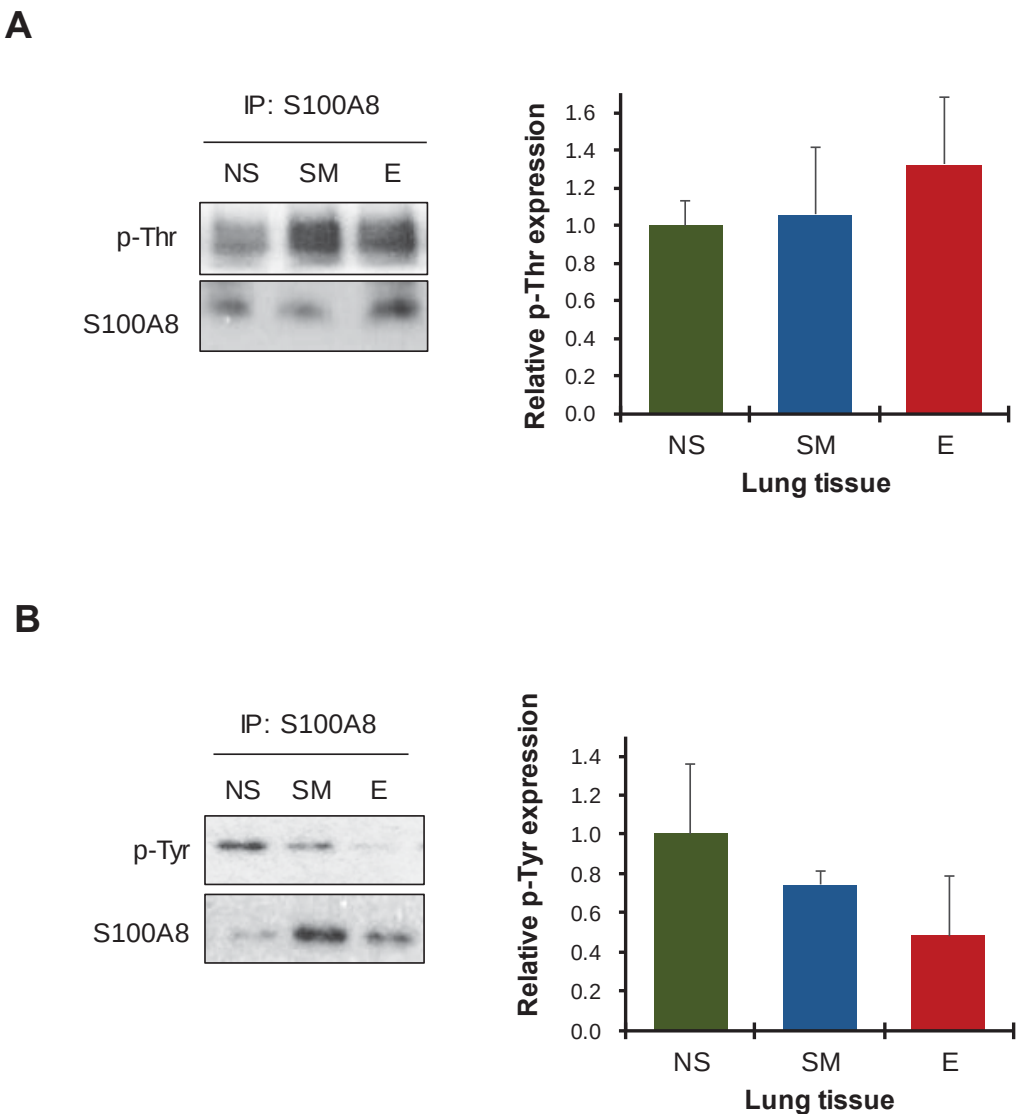
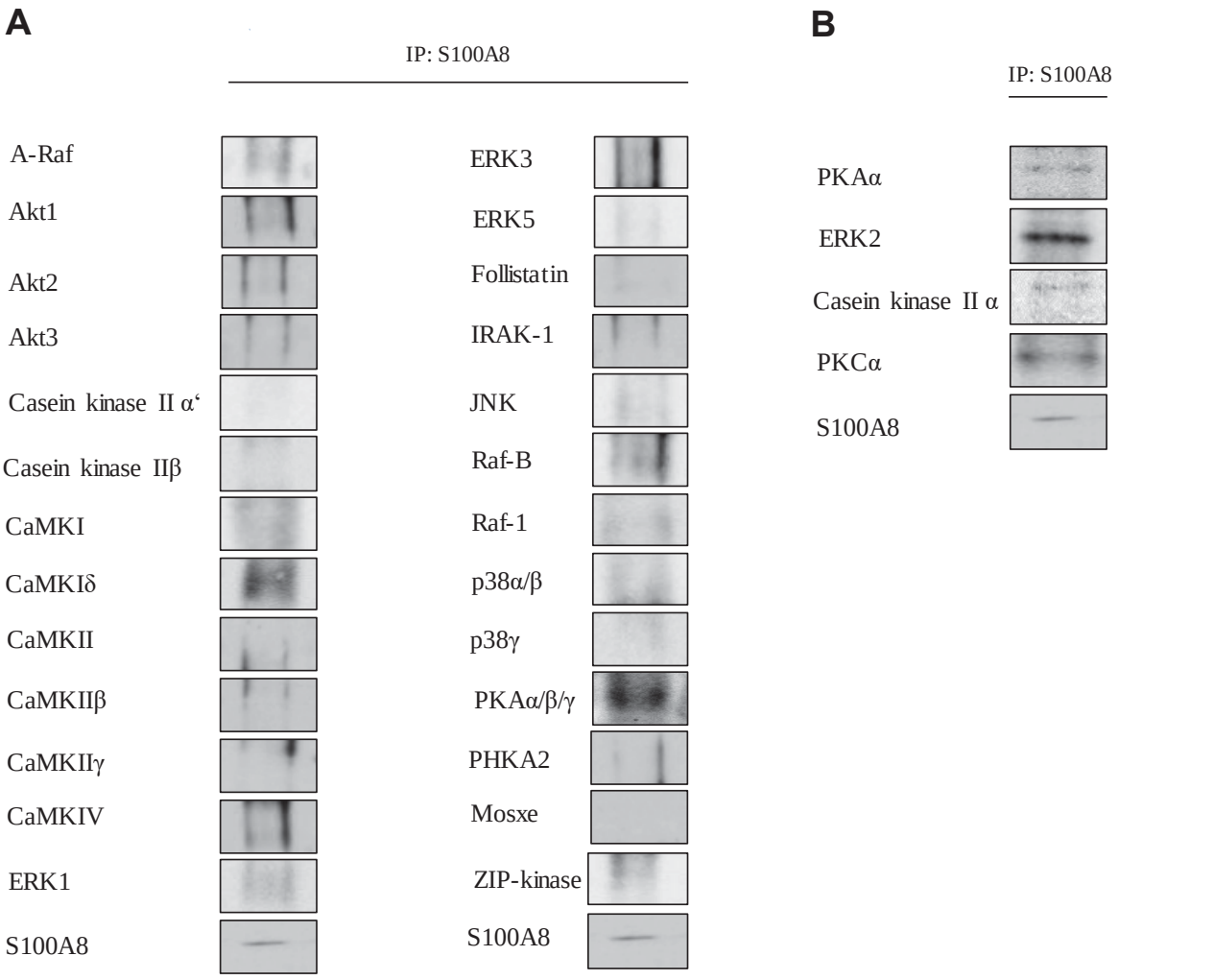



**Figure E3. S100A8 phosphorylation in human lung tissue.** (**A**) Immunoprecipitation of S100A8 in lung tissue obtained from non-smokers (NS), smokers (SM), and patients with emphysema (E) followed by Western blotting analysis to determine p-Thr expression. The ratio of immunoprecipitated S100A8 to p-Thr was normalized to control non-smokers. (**B**) S100A8 was immunoprecipitated in lung tissue lysate obtained from non-smokers, smokers and patients with emphysema followed by Western blotting to analyze p-Tyr expression. Densitometric quantification is also shown (n = 4). Data are shown as means ± s.e.m.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Akt2
Akt3
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CaMKIV
ERK1
S100A8
ERK3
ERK5
Follistatin
IRAK-1
JNK
Raf-B
Raf-1
p38α/β
p38γ
PKAα/β/γ
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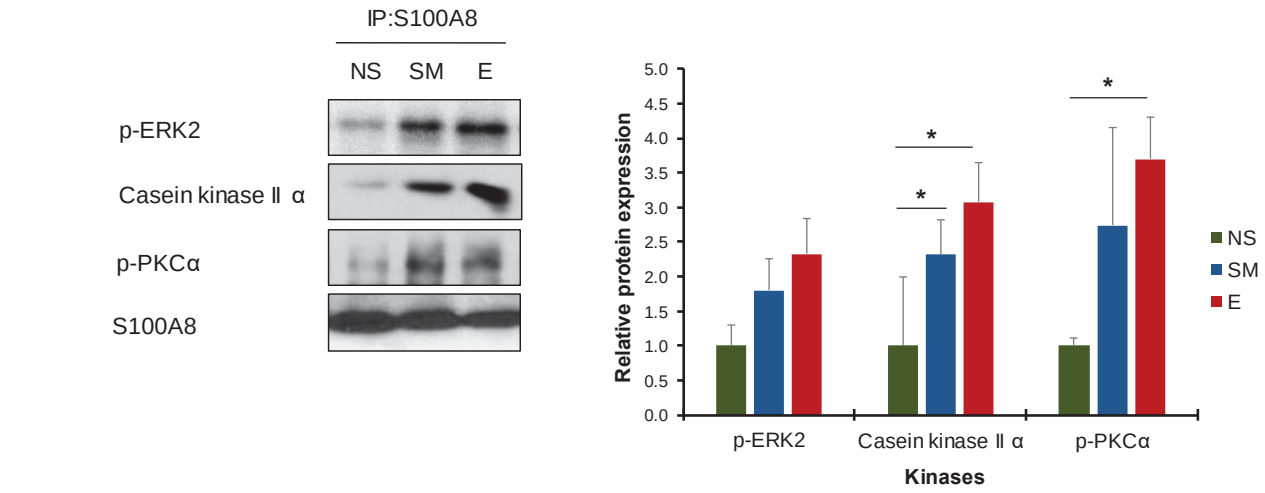
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Relative protein expression
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4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
p-ERK2
Casein kinase Ⅱ α
p-PKCα
Kinases
NS
SM
E

**Figure E4. Interaction of S100A8 with serine kinases in human lung tissue. (A)** S100A8 does not interact with: A-Raf, Akt1, Akt2, Akt3, casein Kinase II α‘, casein kinase II β, CaMKI, CaMKIδ, CaMKII, CaMKIIβ, CaMKIIγ, CaMKIV, ERK1, ERK3, ERK5, follistatin, IRAK-1, JNK, Raf-B, Raf-1, p38α/β, p38γ, PKAα/β/γ, PHKA2, Mosxe, and ZIP-kinase as determined by immunoprecipitation followed by Western blotting analysis. **(B)** S100A8 interacts with PKAα, ERK2, casein kinase II α, and PKCα. (**C**) Immunoprecipitation of S100A8 in lung tissue obtained from non-smokers (NS), smokers (SM), and patients with emphysema (E) followed by Western blotting to determine p-ERK2, casein kinase II α, and p-PKCα expression. Densitometric quantification normalized to control non-smokers is also shown. $P < 0.05$. Data are shown as means ± s.e.m.

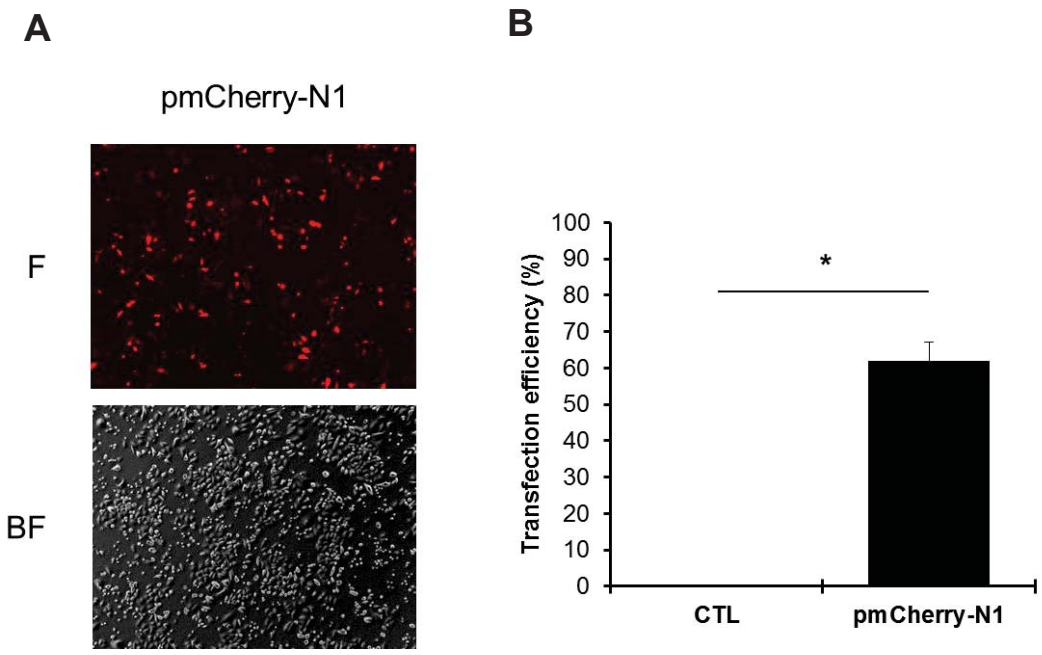


**Figure E5. Transfection efficiency of A549 cells. (A,B)** A549 cells were transfected with pmCherry-N1 plasmid for 48h. N-peptide-mCherry protein expression is shown on micrographs obtained using fluorescence (561 nm, F) and bright field (BF) microscopes. Quantification of transfection efficiency is also shown **(B)**. $P < 0.05$. Data are shown as means ± s.e.m.

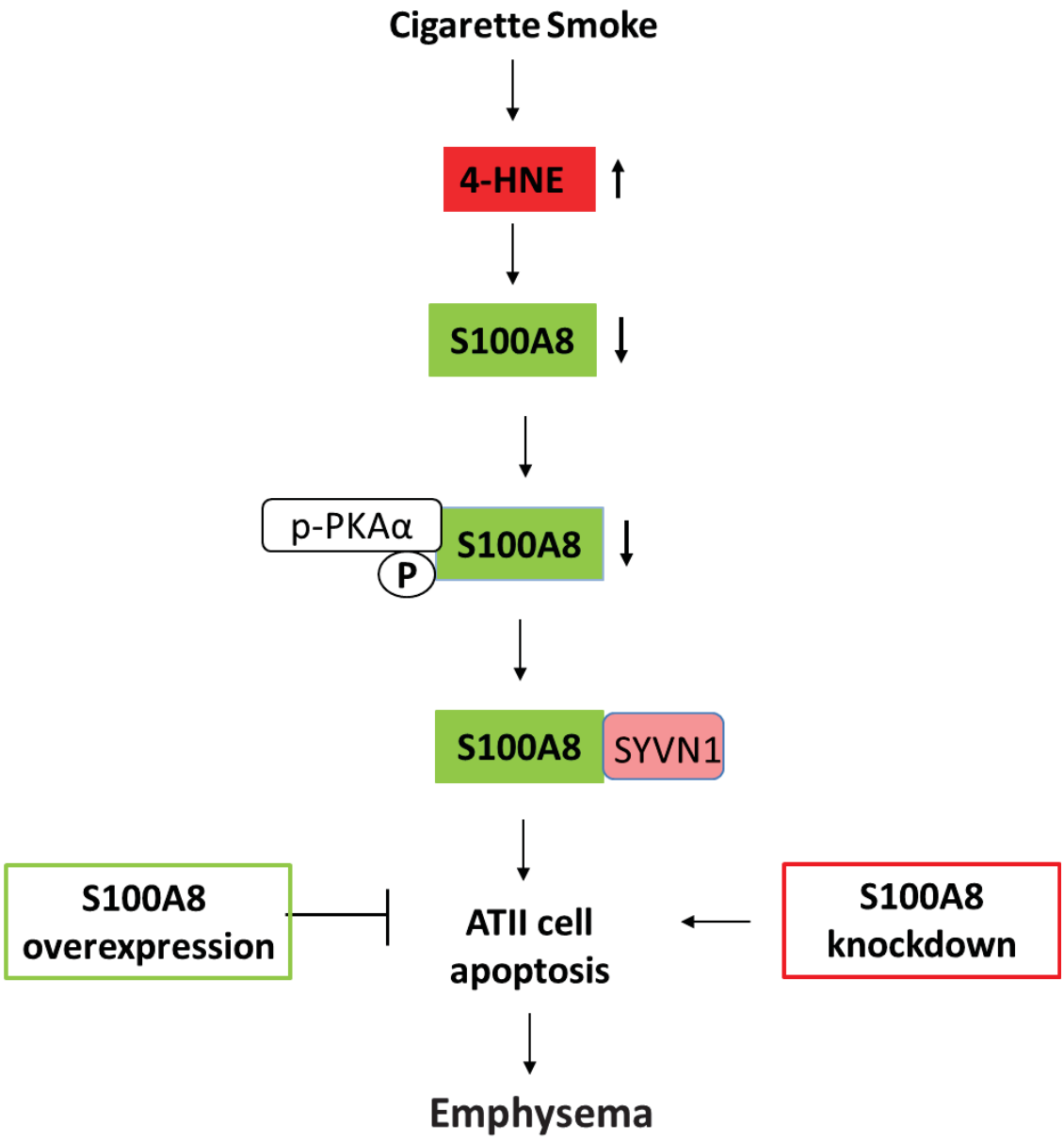


**Figure E6. The role of S100A8 in ATII cells.** High oxidative stress correlates with decreased S100A8 levels. Low S100A8 phosphorylation by p-PKAα and its ubiquitination by SYVN1 may contribute to emphysema development. S100A8 overexpression protects cells against injury induced by cigarette smoke extract and its knockdown sensitized cells to apoptosis.