Epithelial Endoplasmic Reticulum Stress and Apoptosis in Sporadic Idiopathic Pulmonary Fibrosis

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ONLINE DATA SUPPLEMENT

SUPPLEMENTARY METHODS

Handling and Storage of Biomaterials

At the time of explantation, lung biopsies were obtained from IPF, COPD or donor lungs and immediately shock frozen in liquid nitrogen for RNA analysis. The remaining lung tissue was flushed, cooled down to +4°C, and prepared according to a predefined algorithm. In this study, peripheral, subpleural lung samples from the lower lobe were aliquoted and taken for protein analysis as well as isolation of alveolar type II cells. Tissue samples for protein analysis were stored at -80°C.

Isolation of Human Alveolar Type II-Pneumocytes

Human type-II pneumocytes were isolated from fresh peripheral lung tissue obtained from explanted lungs of IPF patients and from non-utilized human donor lungs, by using dispase and DNase, mesh filtration, and panning over uncoated plastic plates to remove fibroblasts (E1).

In brief, 60-80 grams of human peripheral lung tissue were cut into pieces and put into steril 80 mL RPMI 1640 medium (Gibco, Invitrogen, Grand Island, NY) containing 10mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin and 292 µg/mL glutamine (Gibco), adjusted to pH 7.4. Then, 3.3 mg dispase (Roche Diagnostics) per gram lung tissue was added, mixed and incubated for 40 min at RT. Thereafter, 4.5 mg DNase (Sigma) per gram lung tissue was added to the mince, followed by incubation for 5 min at RT. The digested lung tissue suspension was then filtered through two bandages, followed by a filtration cascade through a 100 µm-, then 20 µm-, and finally a 10 µm mesh. The collected filtrate, containing single cells, was centrifuged, the supernatant removed, and the resulting type-II cell-enriched pellet

was resuspended in 100 mL isolation medium containing 100 µg/mL DNase, then plated into ten 100-mm plastic culture dishes (10 plates) and incubated for 45 min (37°C, 5% CO_2) to allow adhesion of contaminating leukocytes, fibroblasts and other cells. Thereafter, the supernatants containing non-adherent, type-II cell-enriched cells were collected, pooled and centrifuged at 300 × g for 10 min. The resulting pellet was resuspended in 100 mL isolation medium without DNase and plated again on new plastic culture dishes, followed by incubation for 45 min. This second panning step was performed at least 2 times. Finally, these supernatants containing enriched, non-adherent type-II alveolar epithelial cells were collected and pooled and centrifuged at 300 × g for 10 min.

Cells were counted and nile red staining was used to assess cell purity. According to this technique more than 95% of the cells were type II cells. The cells were aliquoted and used for isolation of protein or RNA.

Preparation of Lung Homogenate for Western Blot Analysis

Peripheral lung tissue samples (size 1 cm^3) were pulverized in liquid nitrogen by using mortar and pestle. The resulting powder was then transferred to 3 mL lysis buffer (50 mM tris-HCl [pH 7.5], 150 mM NaCl, 1% (w/v) triton X-100, 0.5% (w/v) Na-deoxycholate, 5 mM EDTA and 2 mM PMSF), and incubated on ice for 1 hour. Cell debris was removed from crude extracts by centrifugation at 10000 × g for 10 min. The resulting lung homogenates were divided into aliquots and frozen at −80°C until used. Protein concentrations were determined according to the BCA protein assay from Perbio Science. For SDS-polyacrylamide gel electrophoresis, equal amounts of protein extract (50 µg/lane) were transferred to SDS-sample buffer (final

concentration 2% (w/v) SDS, 10% (v/v) glycerol, 12.5 µM tris-HCl [pH 6.8], 0.1% (w/v) bromophenol blue) and immersed in boiling water for 10 min.

Western Blot Analysis

Lung homogenates (50 µg extracted protein per sample) or cell lysates (25 µg extracted protein per sample) were subjected to denaturating SDS-electrophoresis through 10%, 12% and 15% polyacrylamid gels followed by electroblotting onto PVDF membranes (Amersham Biosciences) and immunostaining for ATF-6α, ATF-4/CREB-2, CHOP, Bax, TTF-1, caspase 3, Bip/Grp78 and β-actin. In general, the immunoblots were blocked by incubating at room temperature for 2 h in blocking buffer (1 $\times$ tris-buffered saline (TBS; 50 mM tris-HCl, pH 7.5, 50 mM NaCl), 5% nonfat dried milk, and 0.1 % tween 20). The blots were incubated with primary antibody in blocking buffer overnight with gentle rocking at 4°C.

Primary antibodies were used at the following dilutions and obtained from the indicated sources: anti-human ATF-6α antibody (1:400, goat polyclonal) and anti-human ATF-4/CREB-2 (1:1000, rabbit polyclonal) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Two anti-human CHOP (DDIT3/GADD153) antibodies (1:250, rabbit polyclonal; 1:500 mouse monoclonal), another anti-human ATF-4 antibody (1:500, goat polyclonal) and anti-human β-actin (1:3000, mouse monoclonal) were all purchased from Abcam (Cambridge, UK). Anti-human Bax (1:1500, rabbit polyclonal) and anti-TTF-1 (1:2000, rabbit polyclonal) were from Upstate. Anti-human caspase 3 (1:120, mouse monoclonal) was obtained from dianova GmbH (Hamburg, Germany) and anti-human Bip/Grp78 (1:1000, rabbit polyclonal) was from Calbiochem (San Diego, CA).

The blots were washed four times in 1 × TBS-T (TBS containing 0.1 % tween 20) and incubated with respective horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Hamburg, Germany; anti-mouse-IgG, anti-goat-IgG, anti-rabbit IgG, 1:2000 diluted in blocking buffer). After four washes, blot membranes were developed with the ECL Plus chemiluminescent detection system (Amersham Biosciences), and band intensity of exposed film was analyzed by densitometric scanning and quantified using AlphaEase®FC Imaging System (San Leandro, CA).

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was prepared from human lung tissue frozen at the time of explantation and from freshly isolated type II-cells obtained from human LTX lungs by an acid phenol extraction method and quantified by spectrophotometry at 260 nm. Reverse transcription (RT) and PCR were performed sequentially in two separate steps. Complementary DNA (cDNA) was first synthesized by reverse transcription. The use of random primers allowed the quantification of a cDNA pool from one RT reaction. An aliquot of the finished RT reaction was then used for PCR. The complete list of primers used is given in **Table E1**.

Each 20-µL RT reaction contained (final concentration): 2 µg total RNA, 10 µM random hexamers, 10 units RNAse inhibitor (both Applied Biosystems, Foster City, CA), 500 µM of each dNTP and 4 units Omniscript Reverse Transcriptase (both Qiagen, Hilden, Germany). The RT reactions were incubated for 65 min at 37°C, directly followed by PCR. Each 25-µL PCR reaction contained (final concentration): 2 µL finished RT reaction (template cDNA), 0.2 µM each forward and reverse primer (metabion, Martinsried, Germany), 200 µM of each dNTP (FINNZYMES, Espoo, Finland) and 2.5 units HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). For

amplification of all described mRNA´s an optimized cycling protocol was performed: ″Hot-Start″ (initial activation step: 95°C for 20 min) followed by 3-step-cycling (30 cycles of amplification): denaturation: 94°C for 30 s; annealing: 55-60°C for 30 s (the annealing temperatures of each primer pair is given in **Table E1**), extension: 72°C for 1 min (but 10 min in the final cycle).

Equal aliquots of the PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide in 1× TAE buffer, and documented by scanning (AlphaEase®FC Imaging System, FluorChem™ IS 8900 software, V. 3.2.3.; San Leandro, CA).

RT-PCR Analysis of XBP-1 mRNA Splicing

Activation of IRE1p was analyzed by semiquantitative determination of the splicing of its substrate, the mRNA encoding the transcription factor XBP-1. To amplify human XBP-1 (NM_005080) mRNA, PCR was for 30 cycles (94°C for 30 s; 56°C for 30 s; and 72°C for 1 min (10 min in the final cycle) using 5´- GTT GAG AAC CAG GAG TTA AGA C - 3´ and 5´- CAG ACT CTG AAT CTG AAG AGT C - 3´ with *Taq* DNA Polymerase. Two hundred twenty-one and 195 bp fragments representing spliced ($XBP\text{-}1_S$) and unspliced ($XBP\text{-}1_U$) XBP-1 were documented after staining 2-4% agarose gels with ethidium bromide, and scanning photographs (shown in **Fig. 2**). In control experiments fragments were confirmed with a second primer pair, 5´- GAT GCC CTG GTT GCT GAA G - 3´ and 5´- GAG TCA ATA CCG CCA GAA TCC - 3´, which yielded a 143 bp fragment for $XBP\text{-}1_S$ and a 169 bp fragment for $XBP\text{-}1_U$ (as exemplarily shown in **Fig. 3A**).

Histology and Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were obtained from upper, middle and lower lung fields from IPF_{LTX} and IPF_{VATS} patients and COPD and from at least one area of donor lobes/lungs. They were routinely stained with hematoxyline and eosin (HE) and with a modified trichrome staining according to Masson and Goldner, in order to assess the degree of collagen deposition. A usual interstitial pneumonia (UIP) pattern was detected in all IPF_{LTX} and IPF_{VATS} subjects.

For immunohistochemistry, formalin-fixed, paraffin wax-embedded lung tissues from human lungs were cut into 3 µm sections which were layered onto glass slides and incubated overnight by 37°C. The tissue sections were dewaxed with xylene and rehydrated with graded dilutions of ethanol in water. For permeabilization, the sections were subjected to microwave irradiation (three times for 5 min, 500 W) in 0.1 M citrate buffer, pH 6.0. Following washing with phosphate-buffered saline (PBS, pH 7.4), immunohistochemistry was performed using the streptavidin-biotin-alkaline phosphatase (AP) method according to the manufacturer (ZytoChem-Plus AP Kit, Broad Spectrum; from Zytomed Systems, Berlin, Germany). Lung sections were incubated with SuperBlock-solution for 5 min, followed by incubation for 90 min at RT with the respective primary antibodies diluted in PBS containing 2% (w/v) bovine serum albumin (BSA). Primary antibodies were used at the following dilutions and obtained from the indicated sources: anti-human ATF-6α antibody (antibody directed against the aminoterminal 17 amino acids, 1:200, rabbit polyclonal), anti-human CHOP (mouse monoclonal, IgG2b) and anti-human ATF-4 antibody (1:100, rabbit polyclonal) were purchased from Abcam. Anti-human proSP-C antibody (rabbit polyclonal) was from Chemicon International, Inc. (Temecula, CA). The anti-Cleaved Caspase-3 rabbit polyclonal antibody was directed to a 13-amino acid peptide

sequence from carboxyl terminus of the human caspase-3 p20 subunit and was obtained from Trevigen (Gaithersburg, MD), and used in a dilution 1:100.

After three washes in PBS, a polyvalent secondary biotinylated antibody (rabbit, mouse, rat, guinea pig, provided by the ZytoChem-Plus AP Kit) was applied (15 min) followed by incubation with AP conjugated streptavidin (15 min). Sections were then developed with Fast Red substrate solution (10 min incubation at RT in the dark), and the reaction was terminated by washing in distilled water. The stained sections were counterstained with hemalaun (Mayers hemalaun solution, Roth, Karlsruhe, Germany) and mounted in Glycergel (DakoCytomation, Inc., Carpinteria, CA). As control experiments, the primary antibodies were replaced by the respective isotype control antibodies [mouse IgG2b or normal rabbit IgG, from Acris Antibodies GmbH (Hiddenhausen, Germany)]. Representative photomicrographs are shown in **Figs. E3** and **E4 online supplement.**

Lung tissue sections were examined histopathologically at 100 ×, 200 × and 400 × original magnification using a light microscope (Leica, Wetzlar, Germany), or scanned with a Mirax Desk slide scanning device (Mirax Desk, Zeiss, Göttingen, Germany).

Immunohistochemistry for proSP-C and CHOP was undertaken in all IPF_{LTX}, COPD and donor lung samples. Immunohistochemistry for proSP-C, p50ATF-6/ATF-6, ATF-4, CHOP and cleaved caspase-3 was undertaken in 10 IPF_{LTX}, COPD and donor lung samples.

In Situ Apoptosis Assay

For the *in situ* detection of apoptosis at the level of a single cell in lung tissue sections, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

(TUNEL) method was performed by using the *In situ* Cell Death Detection Kit, Fluorescein and AP (Roche Applied Science). The assay is based on the principle that terminaldeoxynucleotidyl transferase catalyzes a template-independent addition (polymerization) of fluorescein- conjugated dUTP to free 3´OH ends present in DNA breaks. This tailing reaction is especially sensitive to the type of DNA fragmentation occurring in apoptotic rather than necrotic cell death (E2).

Briefly, after deparaffinization and rehydration, lung tissue sections (3 µm) were permeabilized as described before followed by incubating with fluorescein-conjugated dUTP and terminal transferase enzyme for 1 h at 37°C in a humidified atmosphere in the dark according to the protocol of the manufacturer. After extensive washing with PBS, the fluorescein-labeled DNA fragments were detected by incubating the sections for 30 min at 37°C with an anti-fluorescein-antibody, conjugated with alkaline phosphatase (converter TUNEL-AP). The tissue sections were visualized with Fast Red substrate system (10 min incubation at RT in the dark) followed by counterstaining with methyl green (Vector Laboratories, Inc., Burlingame, CA), and mounted in Glycergel. Sections were analyzed under light microscope. The negative control (background control) had only the label solution without terminal transferase. TUNEL staining was performed in sections from five IPF_{LTX}, COPD and donor lungs, respectively. 10 fields were viewed per section.

Supplementary References

E1. Gonzales LW, Angampalli S, Guttentag SH, Beers MF, Feinstein SI, Matlapudi A, Ballard PL. Maintenance of differentiated function of the surfactant system in human

fetal lung type II epithelial cells cultured on plastic. *Pediatr Pathol Mol Med* 2001;20:387-412.

E2. Gold R, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab Invest* 1994;71:219-225.

Legends to Supplementary Figures

Figure E1. Typical Usual Interstitial Pneumonia (UIP) pattern observed in IPF_{LTX} tissues.

Representative photomicrographs of a paraffin-embedded lung tissue section from a patient with end-stage IPF undergoing transplantation. Staining was performed with HE. The typical temporal and spatial heterogeneity of histological changes in IPF is evident (**A**), with alternating areas of normal lung and fibrosis-defining changes. In (**B**), a typical fibroblast focus with hyperplastic epithelium is seen (**A**: × 1.25, **B**: × 10).

Figure E2. Expression of ER stress markers in IPF subjects at the time point of open lung biopsy (VATS).

Representative immunoblot lanes of LTX- as well VATS-lung tissue homogenates from patients with IPF and human donor lungs (HD) for p50ATF-6, ATF-4, CHOP, Bax, caspase-3 and β-actin. As can be seen, disturbed processing of hydrophobic

surfactant proteins and activation of apoptotic ER stress pathway occurred early in the disease (at the time of VATS) and does not represent an end-stage phenomenon.

Figure E3. Control IHC using normal rabbit IgG-isotype control.

Representative photomicrograph of a paraffin-embedded lung tissue section from an IPF patient stained with a proSP-C antibody (**A**) and a parallel section incubated with normal rabbit IgG isotype control. (**B**). No staining was observed in lung tissue sections incubated with the normal rabbit IgG (**B**).

Original magnification of photomicrographs **A** and **B**, 200 ×, bar = 100 µm.

Figure E4. Control IHC using mouse IgG2b-isotype control.

Representative photomicrograph of a paraffin-embedded lung tissue section from an IPF patient incubated with mouse IgG2b isotype control. No staining was observed in lung tissue sections incubated with the mouse IgG2b.

Original magnification of photomicrograph, 200 ×, bar = 100 µm.

Figure E5. Expression and immunohistochemical localization of ATF-6 in IPF.

Representative immunohistochemistry for proSP-C (**A**, **C**-**E**, red staining) and ATF-6 (**B, F**-**H**, violet staining) in paraffin-embedded lung tissue from an IPF lung. Staining of ATF-6 was stronger in AECIIs in areas of fibrotic remodeling (see **F** and **G**) when compared to AECIIs of thickened alveolar septae (**B**) and to AECIIs in histologically normal-appearing areas of IPF lung parenchyma (**F** and **H**).

Original magnification of photomicrographs **A**, **B**, **D**, **E**, **G**, **H**, 400 ×, bar = 50 µm; original magnification of photomicrographs **C** and **F**, 100 ×, bar = 200 µm.

Figure E6. No staining for ATF-6 in lung tissue sections from patients with COPD.

Representative immunohistochemistry for proSP-C (**A**, **B**) and ATF-6 (**C**, **D**) in paraffin-embedded lung tissue from a COPD lung. (**C**, **D**) No notable staining of the ATF-6 antibody with any cell of a COPD lung was observed. (**A, B**) Using a parallel section, proSP-C stained red the cytoplasm of type-II alveolar epithelial cells (see arrows).

Original magnification of photomicrographs **A** and **C**, 200 ×, bar = 100 µm; original magnification of photomicrographs **B** and **D**, 400 ×, bar = 50 µm.

Figure E7. No staining for CHOP in lung tissue sections from patients with COPD.

Representative immunohistochemistry for proSP-C (**A**) and CHOP (**B**) in paraffin-embedded lung tissue from a COPD patient. (**B**) No immunoreactivity of the CHOP antibody with any cell of a COPD lung was observed. (**A**) In a parallel section, proSP-C stained red the cytoplasm of type-II alveolar epithelial cells.

Original magnification of photomicrographs **A** and **B**, 200 ×, bar = 100 µm.

Figure E8. Co-localization of proSP-C, CHOP and the apoptotic TUNEL signal in AECIIs in areas of dense fibrosis in IPF.

Representative immunohistochemistry for proSP-C (**A**) and CHOP (**B**), as well apoptotic TUNEL staining (**C**) in paraffin-embedded lung tissue sections from an IPF lung.

Original magnification of photomicrographs **A**, **B** and **C**, 200 ×.

Figure E9. No measurable staining for cleaved caspase-3 p20 subunit in histologically normal-appearing areas of IPF lung parenchyma.

Representative immunohistochemistry for proSP-C (**A**, **B**) and cleaved caspase-3 (**C**, **D**) in a paraffin-embedded lung tissue section from an IPF patient. (**C**, **D**) In histologically normal-appearing areas of IPF lung parenchyma, immunostaining for cleaved (activated) caspase-3 was not virtually observed in any cell. (**A**, **B**) In a parallel section, proSP-C stained red the cytoplasm of type-II alveolar epithelial cells. Original magnification of photomicrographs **A** and **C**, 100 ×, bar = 200 µm; original magnification of photomicrographs **B** and **D**, 400 ×, bar = 50 µm.

Figure E10. No staining for cleaved caspase-3 p20 subunit in lung tissue sections from donor lungs.

Representative immunohistochemistry for proSP-C (**A**, **B**) and cleaved caspase-3 (**C**, **D**) in paraffin-embedded lung tissue from a donor lung. (**C**, **D**) Cleaved, activated caspase-3 was not virtually observed in any cell of donor lung tissues. (**A, B**) Using a parallel section, proSP-C stained red the cytoplasm of type-II alveolar epithelial cells. Original magnification of photomicrographs **A** and **C**, 200 ×, bar = 100 µm; original magnification of photomicrographs **B** and **D**, 400 ×, bar = 50 µm.

Figure E11. Localisation of cleaved caspase-3 p20 subunit in lung tissue sections from COPD lungs.

Representative immunohistochemistry for proSP-C (**A**-**C**, **G**, **H**) and cleaved caspase-3 (**D**-**F**, **I**, **J**) in paraffin-embedded lung tissue from a COPD patient. (**D**-**F**, **I**, **J**) Cleaved, activated caspase-3 was observed predominantly in alveolar macrophages of COPD lung tissue sections (see dashed arrows). Alveolar epithelial type II cells

indicated in (**A**-**C**, **G**, **H**) by proSP-C staining (red staining, marked by asterisks) didn´t reveal virtually an immunoreaction with the antibody directed against cleaved caspase-3.

Original magnification of photomicrographs **A** and **D**, 100 ×, bar = 200 µm; original magnification of photomicrographs **B** and **E**, 200 ×, bar = 100 µm; original magnification of photomicrographs **C**, **F**, **G**-**J**, 400 ×, bar = 50 µm.

Figure E12. Co-localization of proSP-C, activated caspase-3, CHOP, ATF-6 and ATF-4 in "hyperplastic" type-II alveolar epithelial cells (AECII) covering fibroblastic foci.

Representative immunohistochemistry for proSP-C (**A**, **D**), cleaved caspase-3 (**B**), CHOP (**C**), ATF-6 (**D**) and ATF-4 (**E**) in serial sections of IPF lung tissue.

Original magnification of photomicrographs **A**-**F**, 400 ×, bar = 50 µm.

Figure E13. No staining for ATF-4 in lung tissue sections from donor lungs.

Representative immunohistochemistry for proSP-C (**A**) and ATF-4 (**B**) in paraffin-embedded lung tissue from a donor lung. (**B**) No immunoreactivity of the ATF-4 antibody with any cell of a donor lung was observed. (**A**) Using a parallel section, proSP-C stained red the cytoplasm of type-II alveolar epithelial cells.

Original magnification of photomicrographs **A** and **B**, 400 ×, bar = 50 µm.

Table E1: Primers used in semiquantitative RT-PCR (homo sapiens).

Gene Symbol/ GenBank Accession (NCBI)	Product Size [bp]	Annealing-Temperature [°C]	Primers
SFTPB NM_198843	319	55	**Forward 5´- AAG TGC TTG ACG ACT ACT TCC - 3´** **Reverse 5´- GCT TGG ATC CGC TTG ATC AG - 3´**
SFTPC NM_003018	321	56	**Forward 5´- CTC ATC GTC GTG GTG ATT GTG - 3´** **Reverse 5´- CTG CAG AGA GCA TTC CAT CTG - 3´**
TITF1 NM_003317	311	54	**Forward 5´- GCT ACT GCA ACG GCA ACC - 3´** **Reverse 5´- CGC CGA CAG GTA CTT CTG - 3´**
DDIT3/CHOP NM_004083	220	56	**Forward 5´- ACT CTC CAG ATT CCA GTC AGA G - 3´** **Reverse 5´- GCC TCT ACT TCC CTG GTC AG - 3´**
XBP1 NM_005080	221 XBP-1 (u) 195 XBP-1 (s)	55	**Forward 5´- GTT GAG AAC CAG GAG TTA AGA C-3´** **Reverse 5´- CAG ACT CTG AAT CTG AAG AGT C-3´**
XBP1 NM_005080	169 XBP-1 (u) 143 XBP-1 (s)	56	**Forward 5´- GAT GCC CTG GTT GCT GAA G - 3´** **Reverse 5´- GAG TCA ATA CCG CCA GAA TCC - 3´**
ACTB NM_001101	220	55	**Forward 5´- ACC CTG AAG TAC CCC ATC G - 3´** **Reverse 5´- CAG CCT GGA TAG CAA CGT AC - 3´**

Primers were designed using “GeneFisher-Software Support for the Detection of Postulated Genes” (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). In the majority, intron-spanning primers were chosen. Abbreviations: XBP-1(u) = unspliced XBP-1; XBP-1(s) = spliced XBP-1.

Figure E1

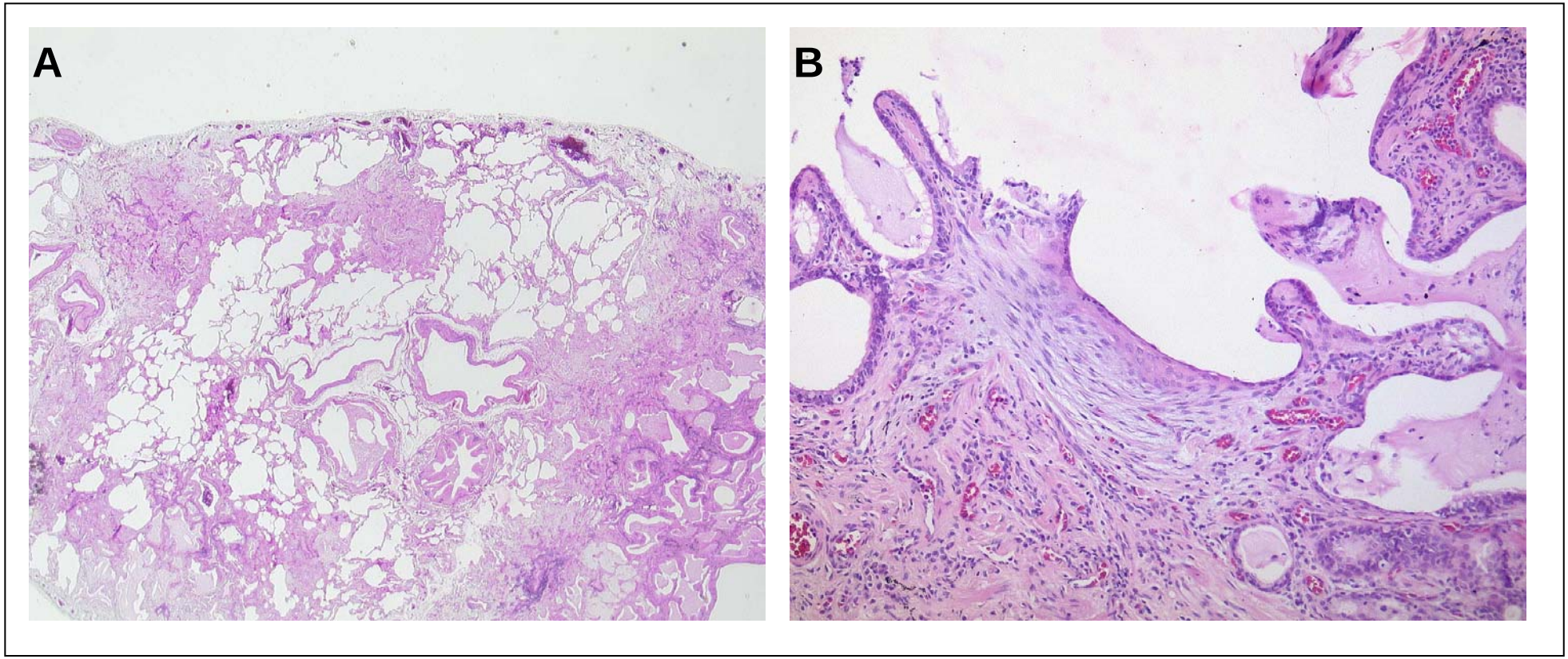
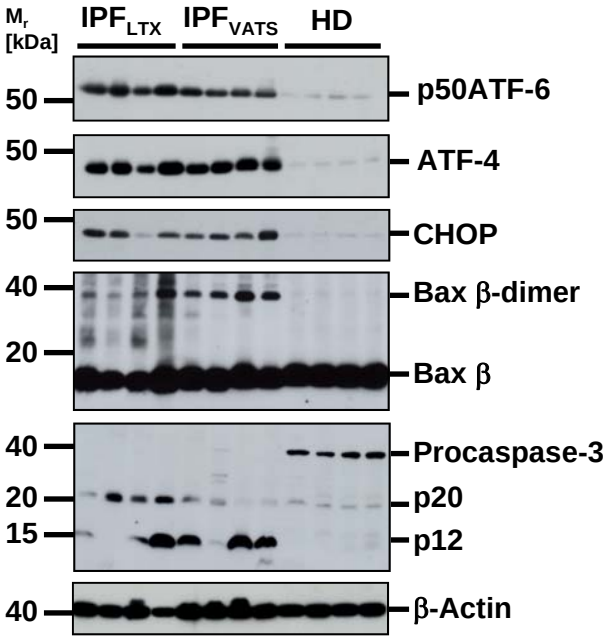


Figure E2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IPF VATS
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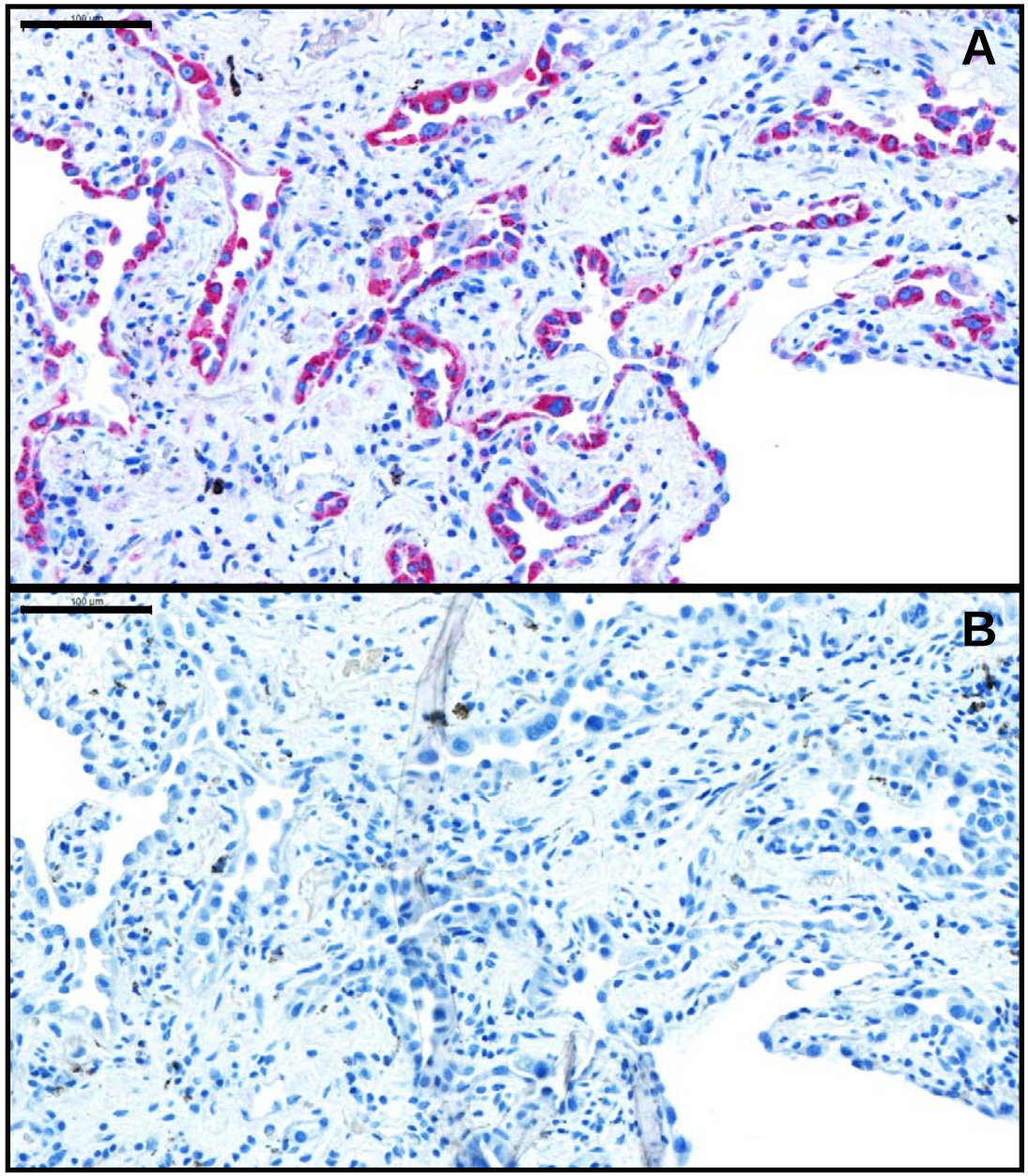
β-Actin

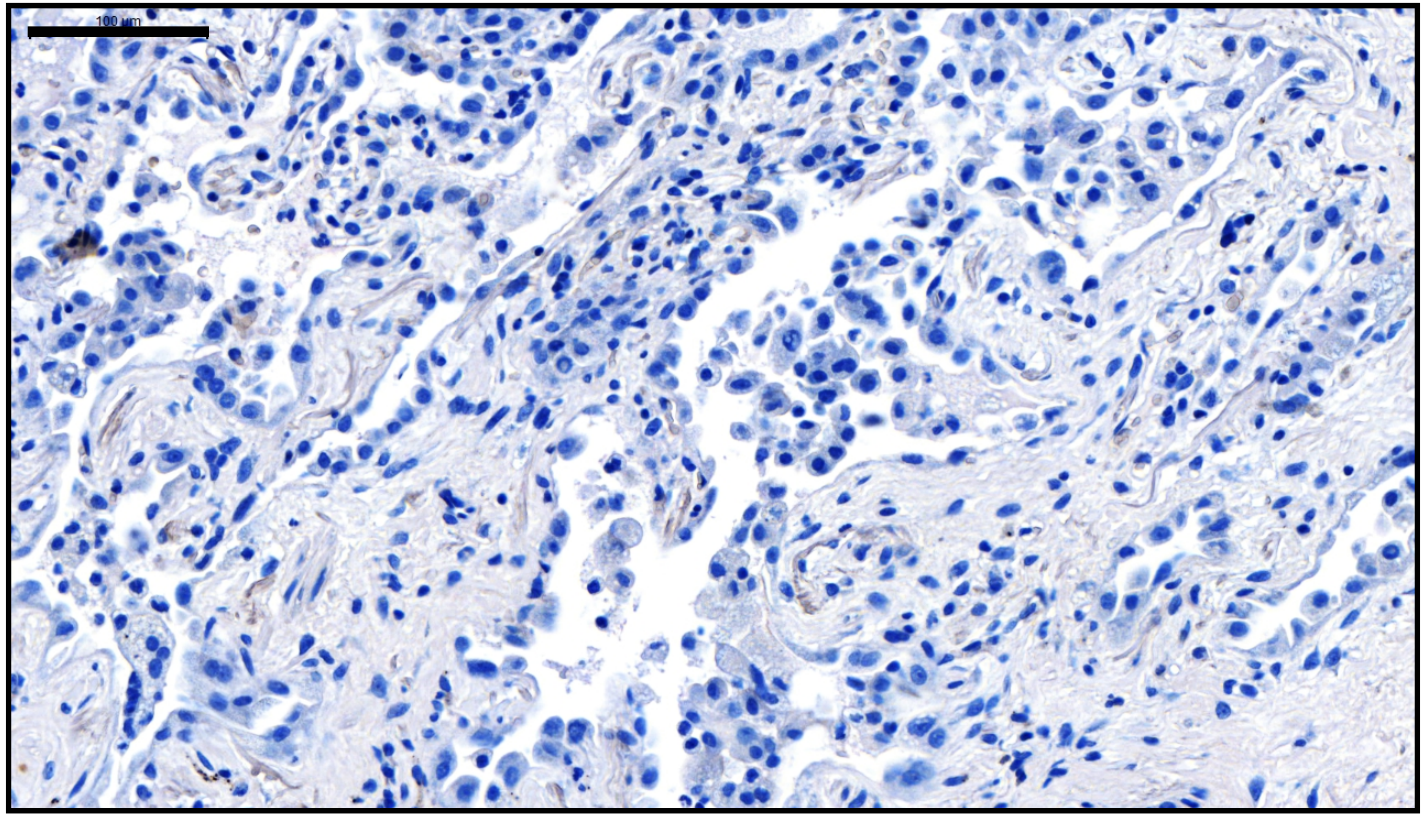
Figure E3



proSP-C

Isotype control antibody (normal rabbit IgG)

Figure E4



Isotype control antibody
(mouse IgG2b)

Figure E5

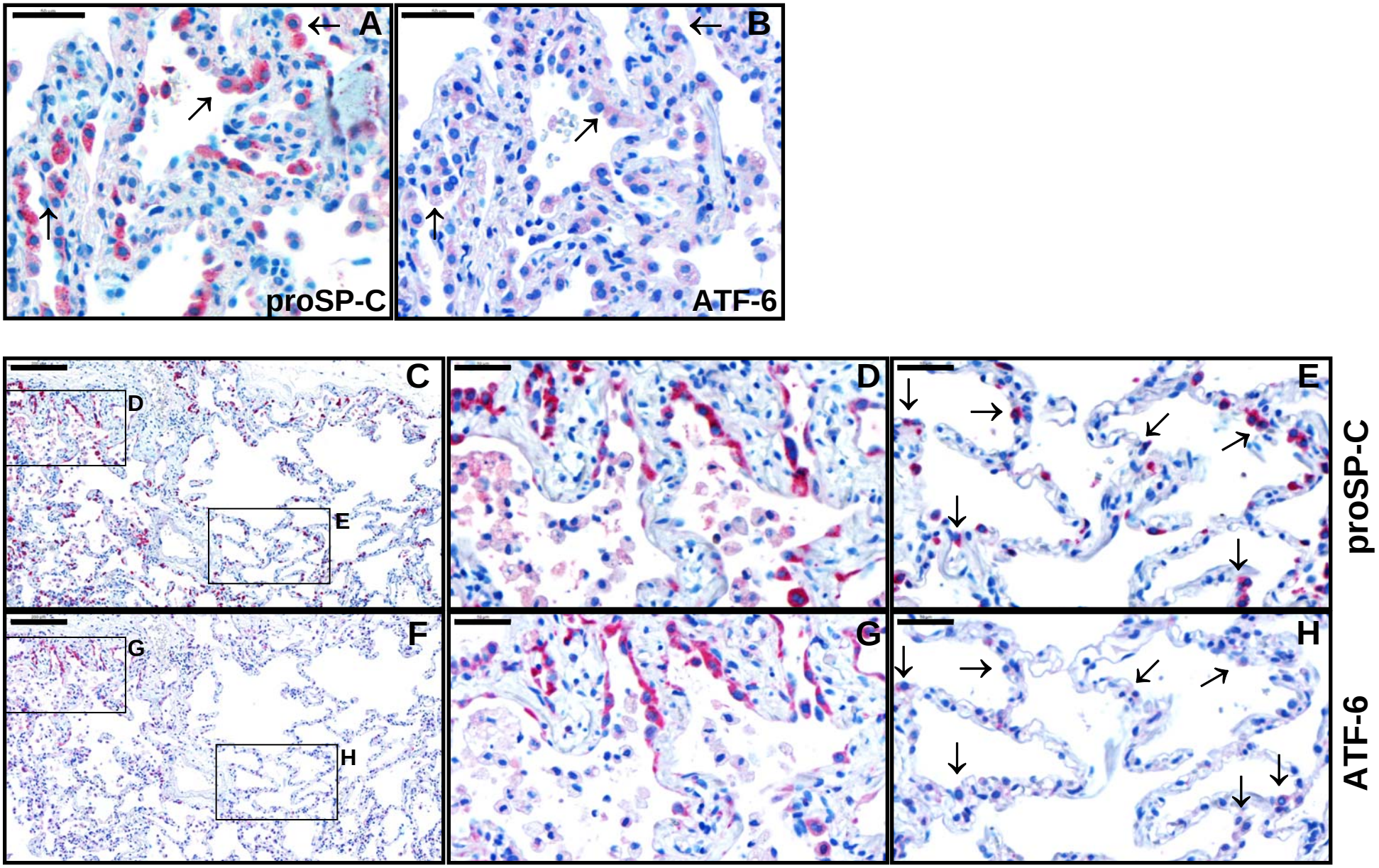
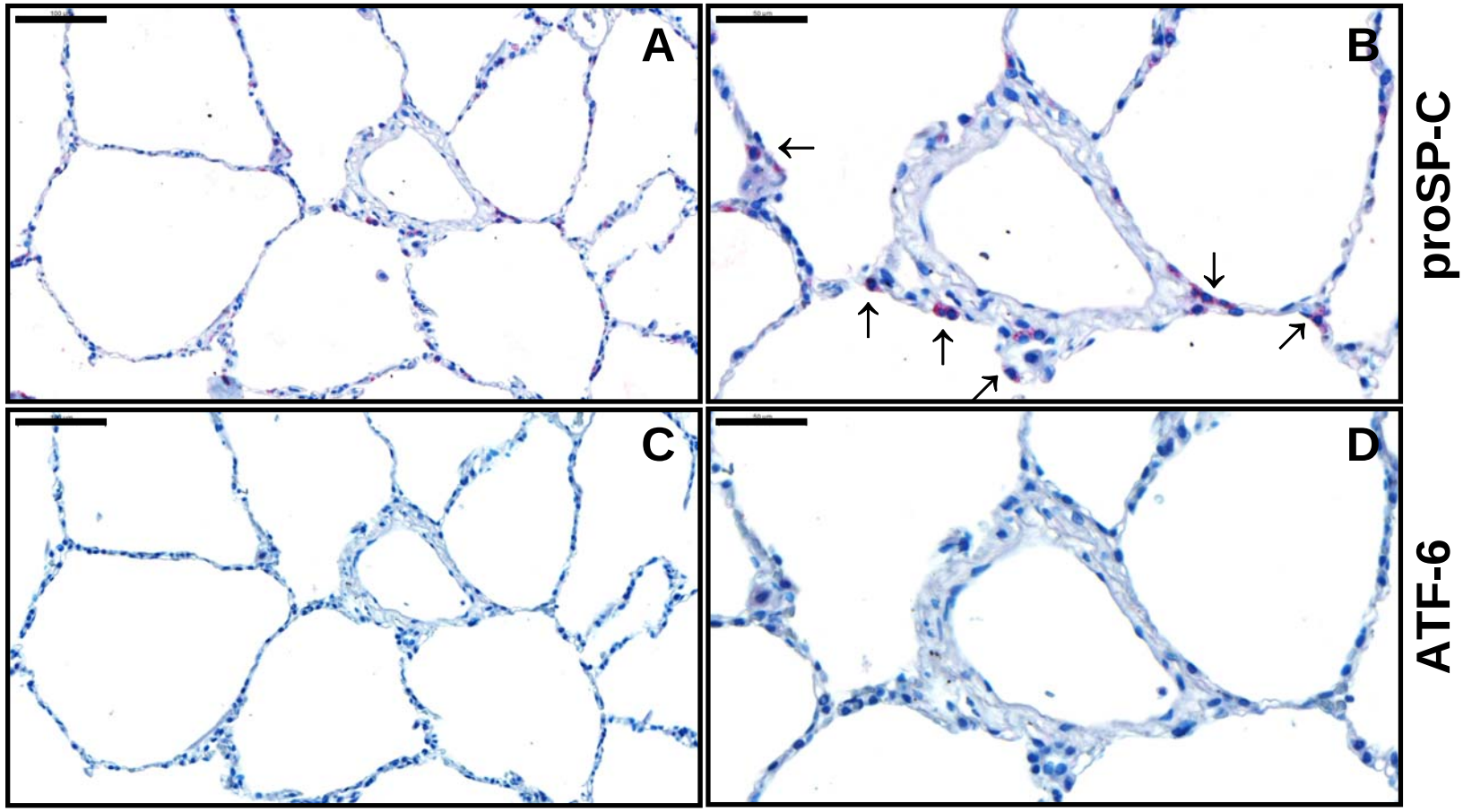


Figure E6



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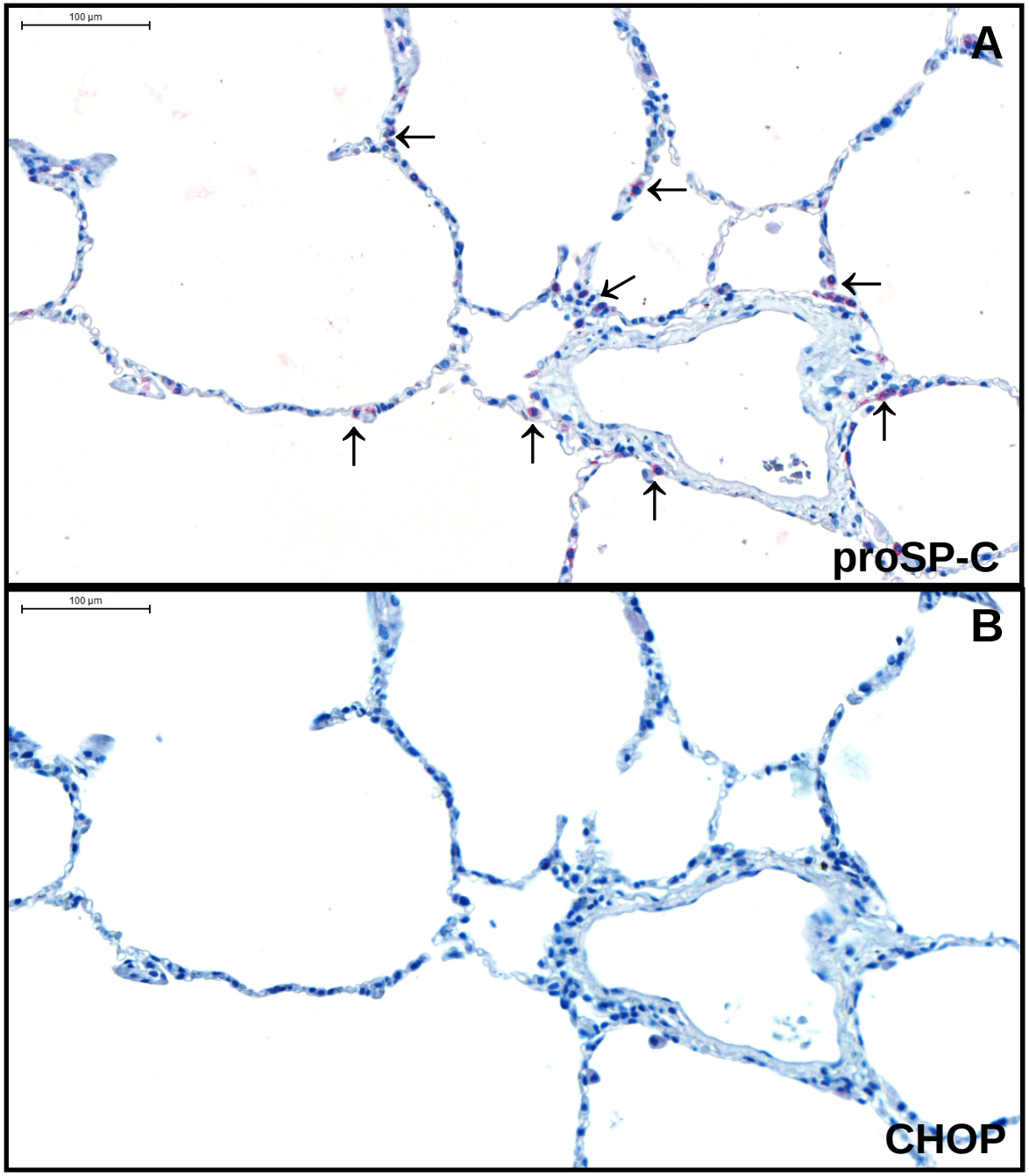
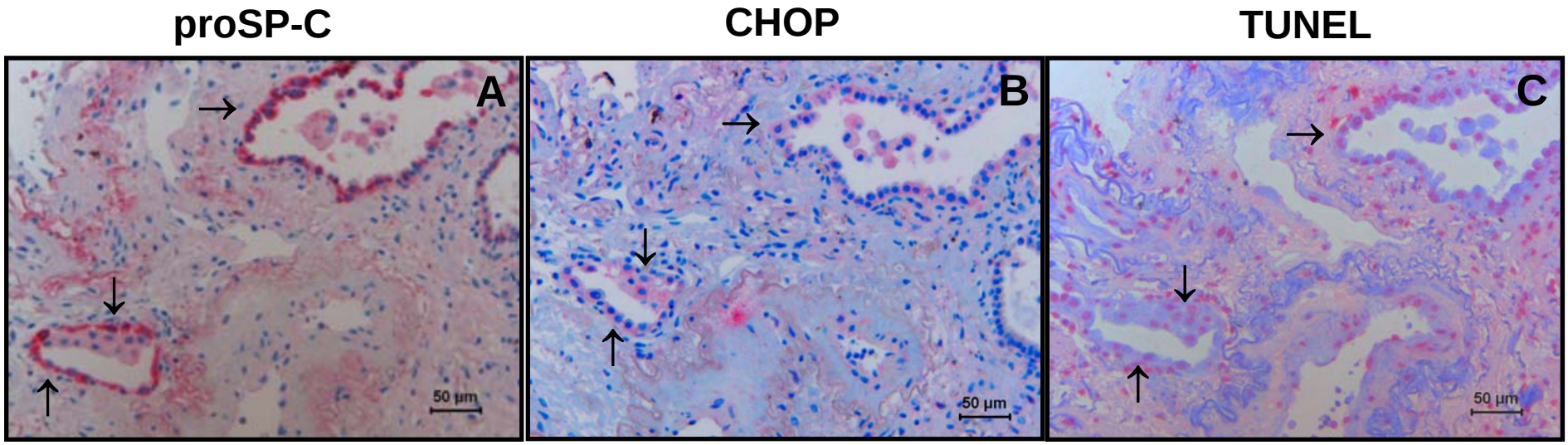


Figure E8



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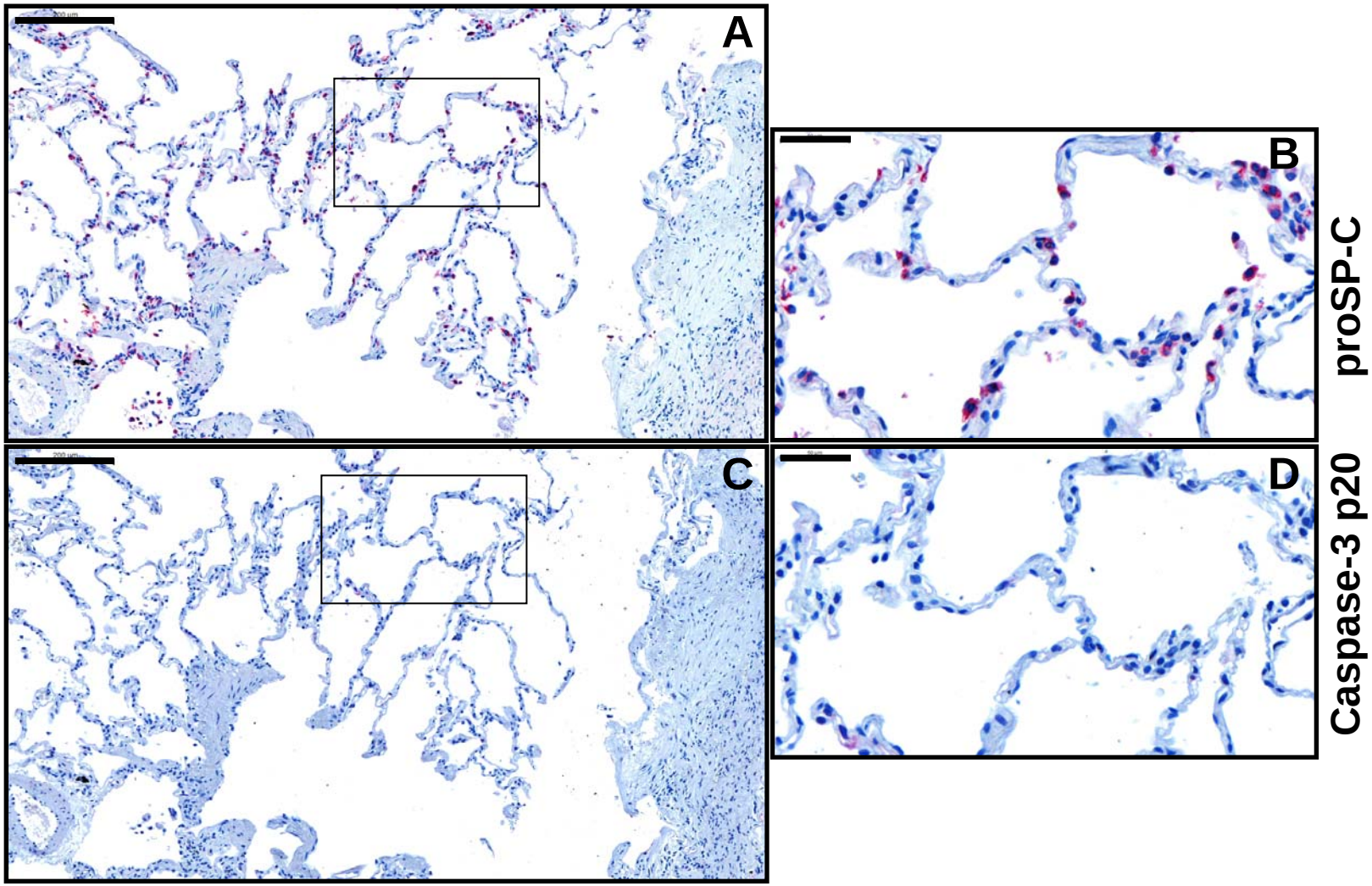

Figure E10

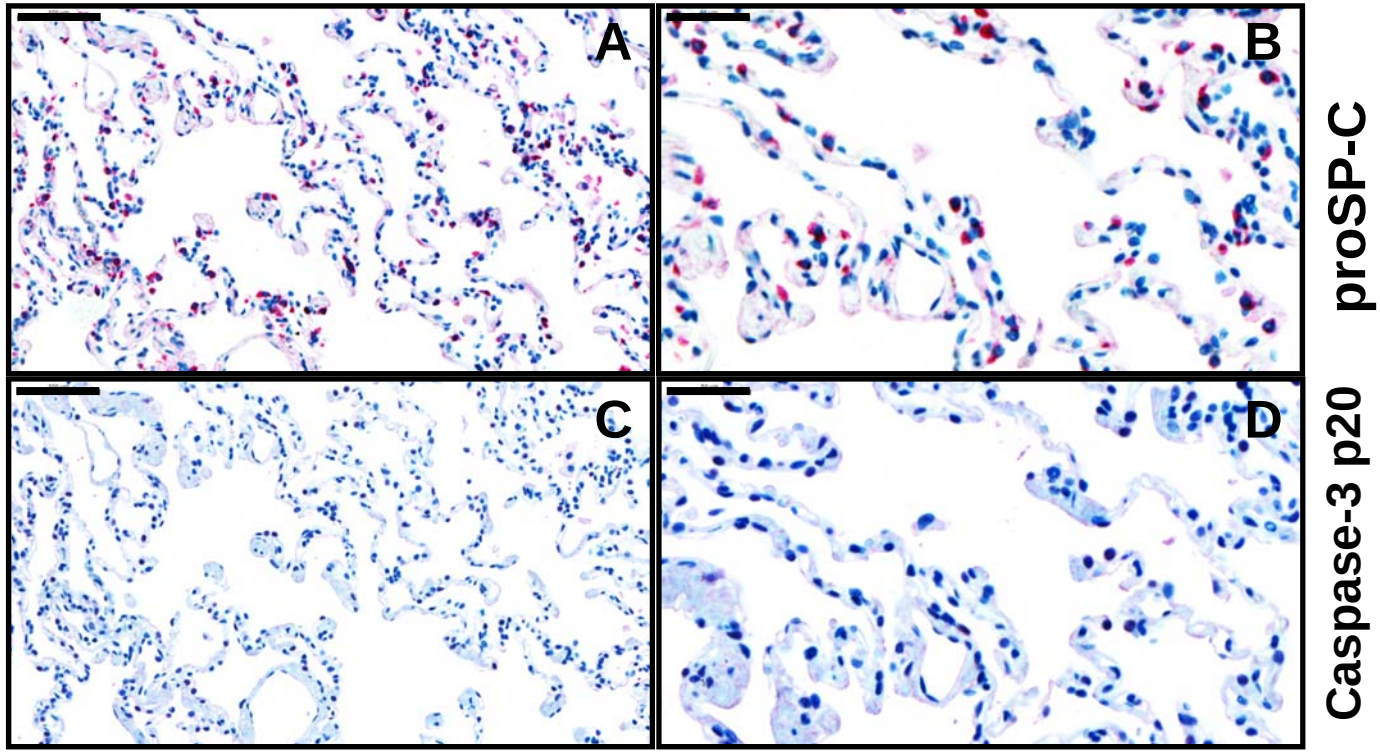

Figure E11

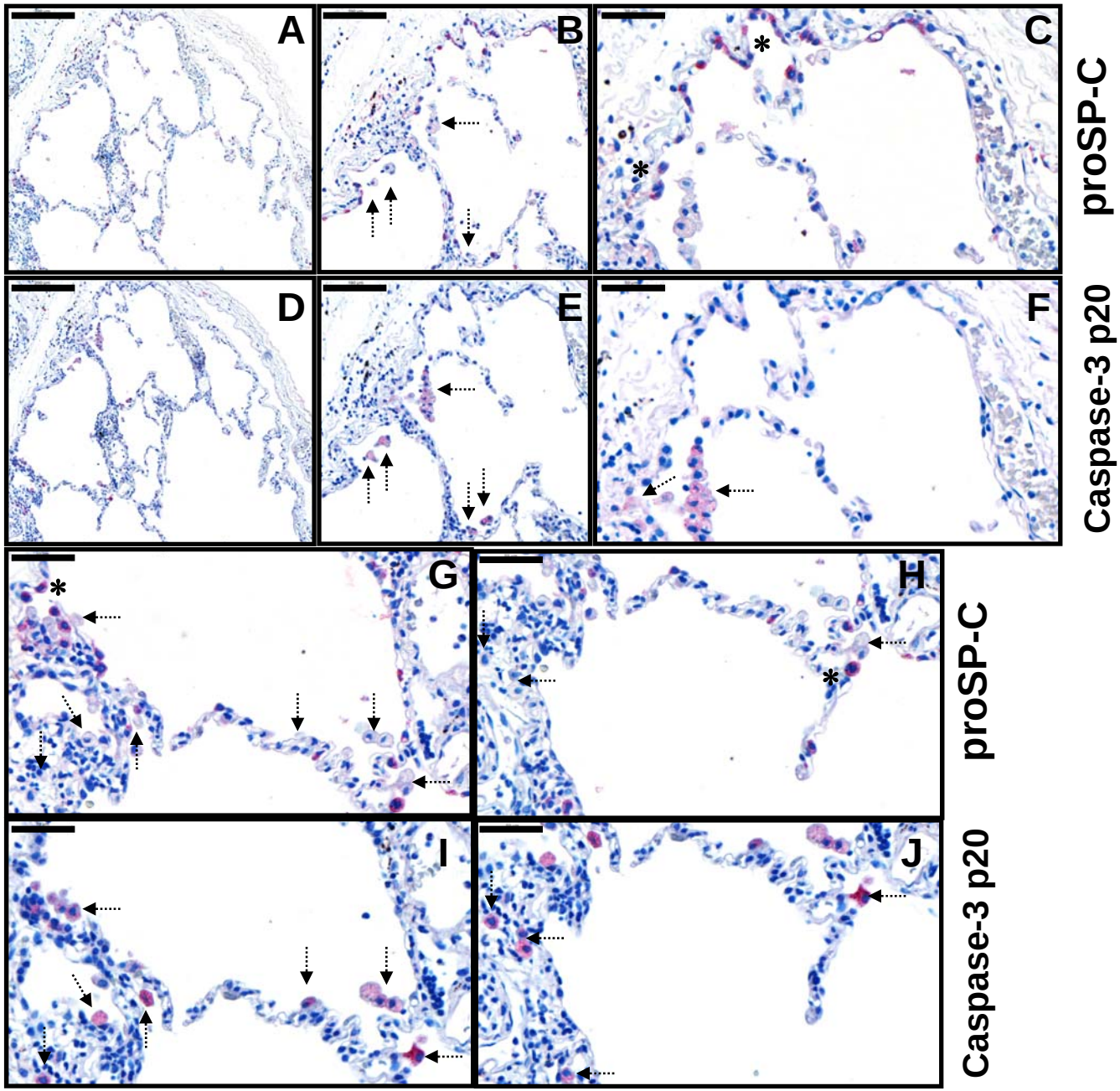

Figure E12

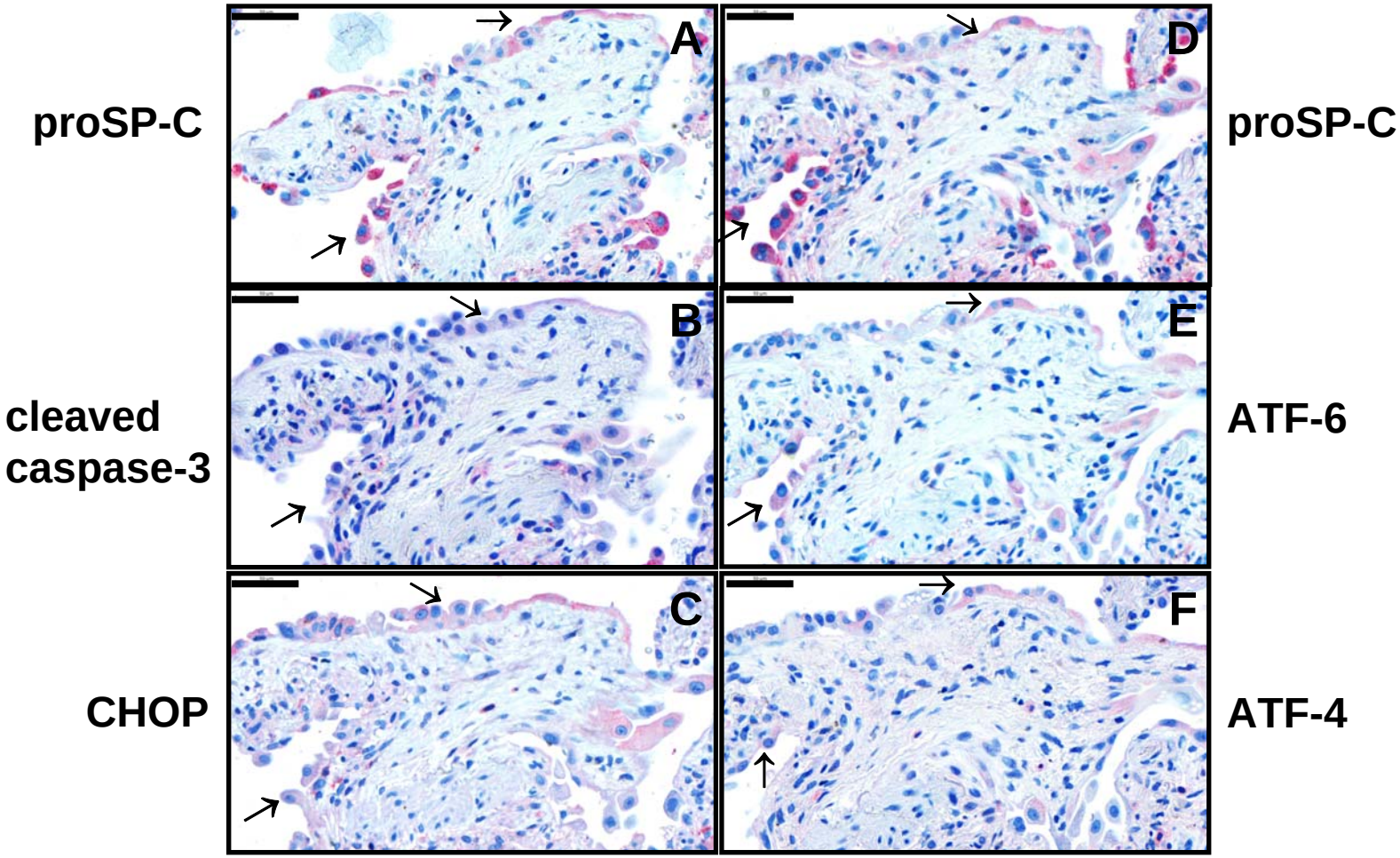

Figure E13