4-Phenylbutyric acid treatment rescues trafficking and processing of a mutant surfactant protein C

Gareth A. Stewart
Ross Ridsdale
Emily P. Martin
Cheng-Lun Na
Yan Xu
Karunyakanth Mandapaka
Timothy E. Weaver

ONLINE DATA SUPPLEMENT

Materials and Methods

RNA isolation, RT-PCR and Microarray Analyses

RNA was extracted from triplicate samples using the RNeasy Plus Mini Kit (Qiagen), followed by cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed using the ABI 7300 system with TaqMan Assays specific for human SP-C (Hs00161628_m1) and human ACTB (actin, beta) endogenous control (Applied Biosystems). Relative quantitation data was obtained using the SDS Software and statistical analysis was completed as described in the Statistics section. Microarray analyses were performed exactly as previously described (1).

Immunogold labeling of HEK293 cells

For immunogold labeling, HEK 293 cells were fixed *in situ* with 4% periodate-lysine-paraformaldehyde, 0.1% glutaraldehyde, and 0.1% $CaCl_2$ in 0.2M HEPES, pH 7.2, cryoprotected with polyvinyl-pyrrolidone/sucrose and processed for cryoimmunogold labeling (2, 3). Cryosections were incubated with proSP-C antibodies described above and 10 nm protein A gold probes (Dept Cell Biology, U. Utrecht Medical School, Utrecht, The Netherlands). Immunoelectron micrographs were acquired using a Hitachi H-7650 TEM (Hitachi High Technologies America, CA) equipped with a 2,000 x 2,000 pixel AMT TEM CCD camera (Advanced Microscopy Techniques, MA).

REFERENCES

E1. Dong M, Bridges JP, Apsley K, Xu Y, Weaver TE. ERdj4 and ERdj5 Are Required for Endoplasmic Reticulum-associated Protein Degradation of Misfolded Surfactant Protein C. *Mol Biol Cell* 2008;19:2620-2630.

E2. Wang M, Bridges JP, Na CL, Xu Y, Weaver TE. Meckel-Gruber syndrome protein MKS3 is required for endoplasmic reticulum-associated degradation of surfactant protein C. *J Biol Chem* 2009;284:33377-33383.

E3. Ridsdale R, Na CL, Xu Y, Greis KD, Weaver. T. Comparative Proteomic Analysis of Lung Lamellar Bodies and Lysosome-Related Organelles. *PLoS ONE* 2011;6:e16482.

E4. Stevens PA, Pettenazzo A, Brasch F, Mulugeta S, Baritussio A, Ochs M, Morrison L, Russo SJ, Beers MF. Nonspecific interstitial pneumonia, alveolar proteinosis, and abnormal proprotein trafficking resulting from a spontaneous mutation in the surfactant protein C gene. *Pediatr Res* 2005;57:89-98.

Figure Legends

Figure S1. Posttranslational modification of SP-C.

A. HEK293 cells stably expressing SP-C^{WT} were grown in the absence of doxycycline. Cells were labeled with ^{35}(S) cysteine/methionine for 10 min in the presence of brefeldin A (BFA, 10 μM). The chase was initiated by replacing the labeling media with media containing cycloheximide (100 μg/ml), unlabeled cysteine (1 mM) and unlabeled methionine (5 mM). Cells were harvested at the indicated timepoints and lysates immunoprecipitated with proSP-C antibody followed by SDS-PAGE/autoradiography. proSP-C isoforms 1 and 2 are indicated.

B. A pulse-chase experiment was performed with HEK293 cells stably expressing SP-C^{I73T} as described above. proSP-C isoforms 1-3 are indicated.

C. HEK293 cells were transiently transfected with plasmids encoding WT or I73T, I73S, I73K or E66K mutants of SP-C. Cells were harvested 48h later and analyzed by SDS-PAGE/western blotting with proSP-C antibody. SP-C^{E66K} is a disease-associated mutation with a phenotype that is similar to patients carrying the I73T mutation (4). proSP-C isoforms 1-3 are indicated.

Figure S2. PBA treatment stabilizes SP-C^{L188Q} proprotein and enhances processing to mature peptide in multiple cell lines.

A. Three independent HEK293 cell lines stably expressing SP-C^{L188Q} at different levels were treated with PBA for 48h. Cell lysates were analyzed by SDS-PAGE/western blotting. Blots were sequentially probed for proSP-C, mature SP-C and actin. The inset within the middle panel is a longer exposure of mature peptide in the L188Q line #30. L188Q line #22 is the cell line that was used in all the experiments described in this study.

B. MLE12 cells were cultured in HITES media and plated at $2X10^5$ cells/well in 6-well plates. Twenty-four hours later, cells were transfected with pcDNA3.1+ (vector control) or SPC ^{L188Q} (cloned into pcDNA3.1+) using Lipofectamine2000. Twenty-four hours following transfection, cells were fed with fresh HITES media containing 0 or 1 mM PBA. Cells were harvested 24 hours after PBA treatment and analyzed by SDS-PAGE followed by sequential western blotting, as described above.

Figure S3.

A. SP-C^{I73T} does not engage ERAD chaperones.

HEK293 cells expressing SP-C^{WT}, SP-$C^{\Delta exon4}$, SP-C^{L188Q} or SP-C^{I73T} were cotransfected with plasmids encoding HA-tagged ERdj4 or ERdj5. Cell lysates were immunoprecipitated with proSP-C antibody and analyzed by SDS-PAGE western blotting. Blots were sequentially probed with HA antibody (to detect ERdj4, top panel or ERdj5, middle panel) and proSP-C antibody (lower panel).

B. SP-C^{I73T} does not induce expression of ER stress genes.

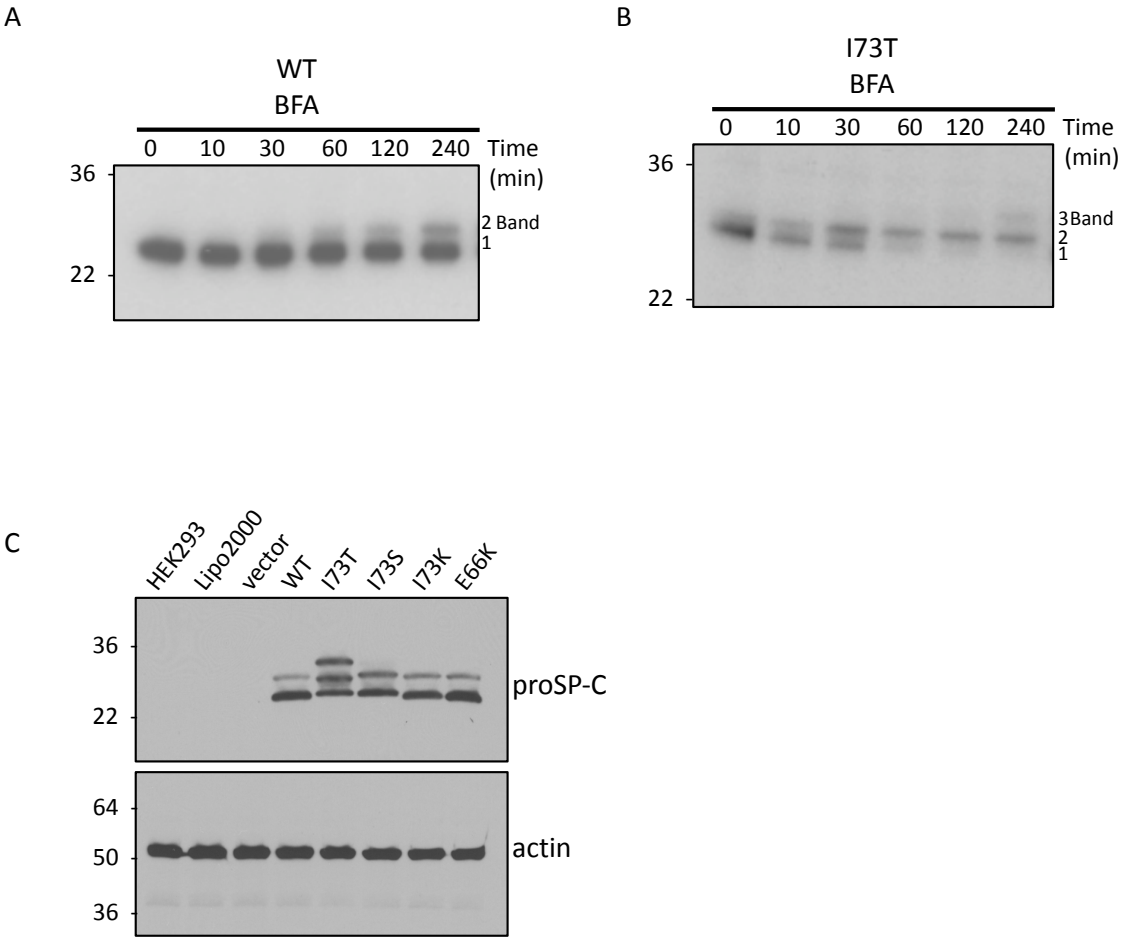
HEK293 cells were transiently transfected with constructs encoding SP-C^{WT}, SP-$C^{\Delta exon4}$ or SP-C^{I73T} cloned into pIRES2-EGFP. Twenty-four hours after transfection, EGFP-positive cells were recovered by fluorescence-activated cell sorting. RNA was isolated as described above and microarray analyses performed exactly as previously described (1). Differentially expressed genes were identified by ANOVA (P value < 0.05, fold change >1.2).

Figure S4. Localization of SP-C^{I73T} at the plasma membrane.

Cells expressing SP-C^{WT} (A) or SP-C^{I73T} (B) were grown in the absence of doxycycline and prepared for electron microscopy and immunogold labeling, as described above. Robust immunogold labeling was detected on the plasma membrane of cells expressing SP-C^{I73T} (B) but not in cells expressing SP-C^{WT} (A); in contrast, gold particles were readily detected in the endolysosomal pathway of cells expressing SP-C^{WT}. LYS = lysosome, MVB = multivesicular body, V = endocytic vesicle.

Figure S5. Extended residence of SP-C^{I73T} at the cell surface.

Cells expressing SP-C^{I73T} were grown in the presence of doxycycline for the indicated amount of time. Fixation, staining and confocal microscopy was performed as described in Figure 6.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BFA
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36
22
2 Band
1
B
I73T
BFA
0 10 30 60 120 240 Time (min)
36
22
3 Band
2
1
C
HEK293
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vector
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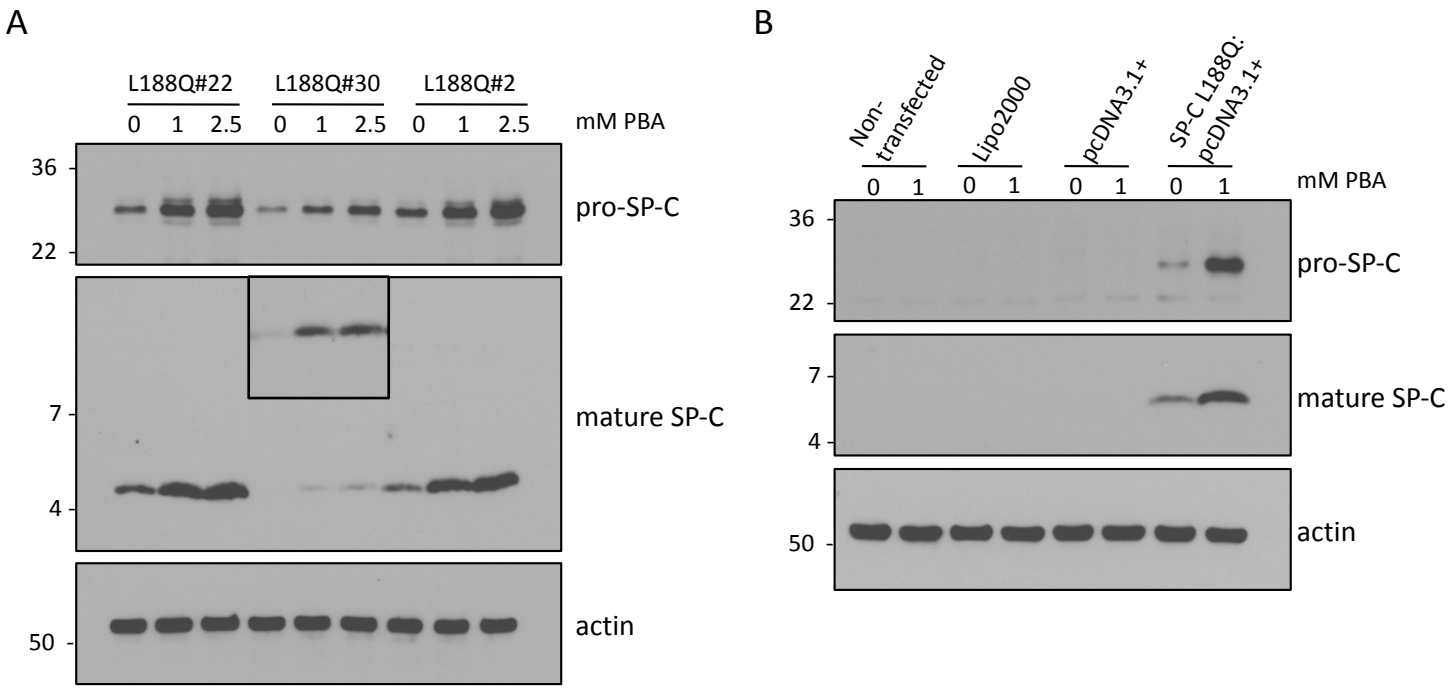
I73T
I73S
I73K
E66K
36
22
proSP-C
64
50
36
actin

Figure S1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L188Q#30
L188Q#2
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22
pro-SP-C
7
4
mature SP-C
50
actin
B
Non-transfected
Lipo2000
pcDNA3.1+
SP-C L188Q: pcDNA3.1+
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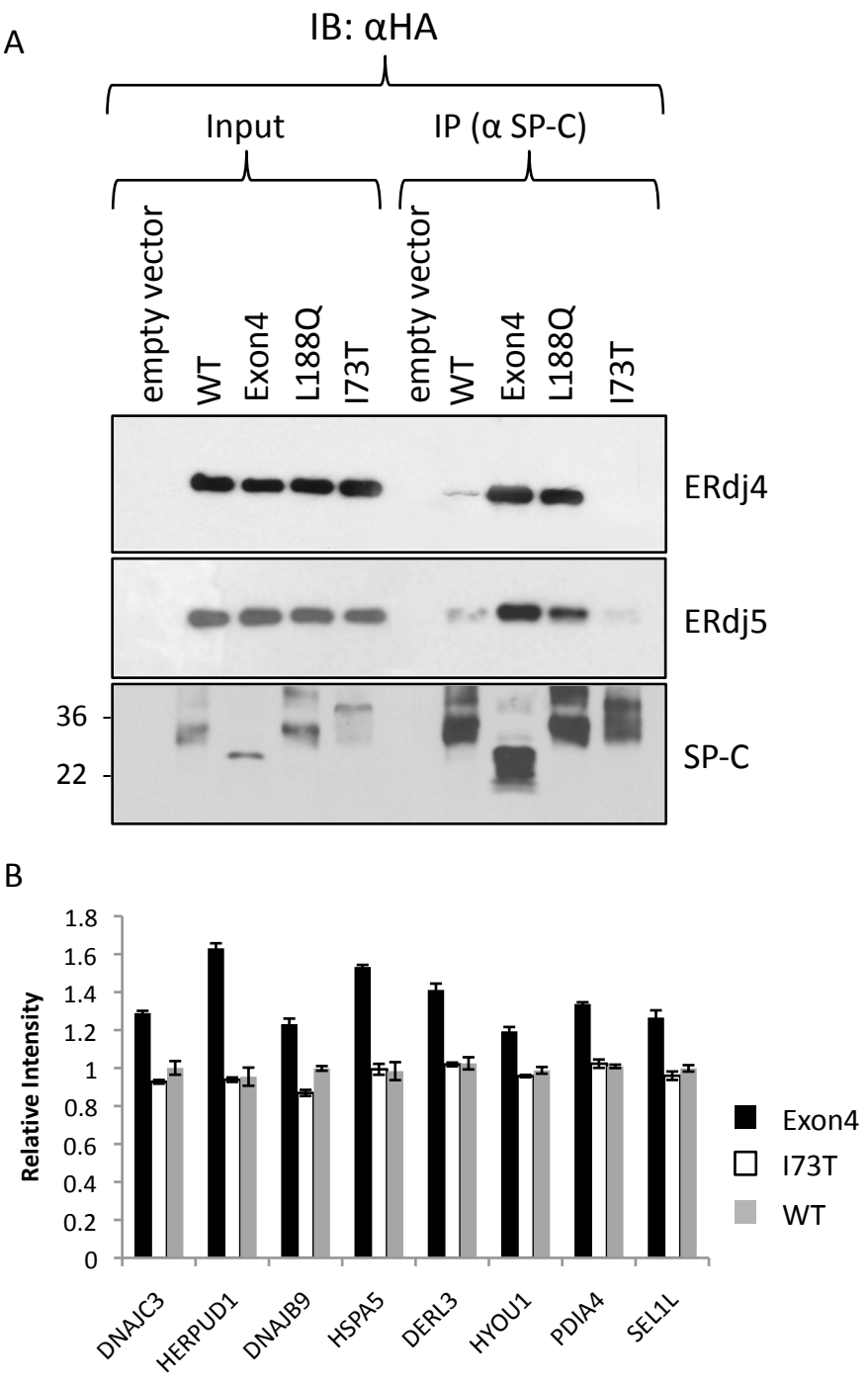
22
pro-SP-C
7
4
mature SP-C
50
actin

Figure S2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IP (α SP-C)
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Exon4
L188Q
I73T
empty vector
WT
Exon4
L188Q
I73T
ERdj4
ERdj5
36
22
SP-C
B
Relative Intensity
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
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DERL3
HYOU1
PDIA4
SEL1L
Exon4
I73T
WT

Figure S3

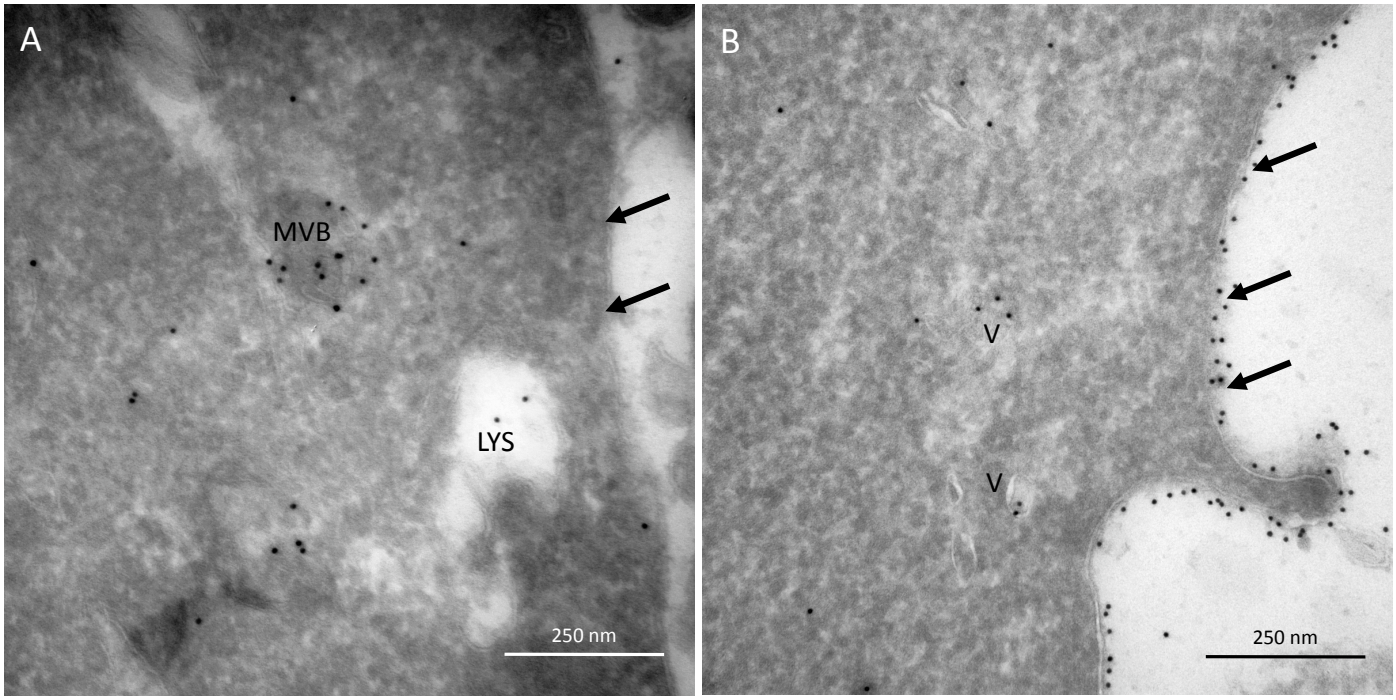

Figure S4

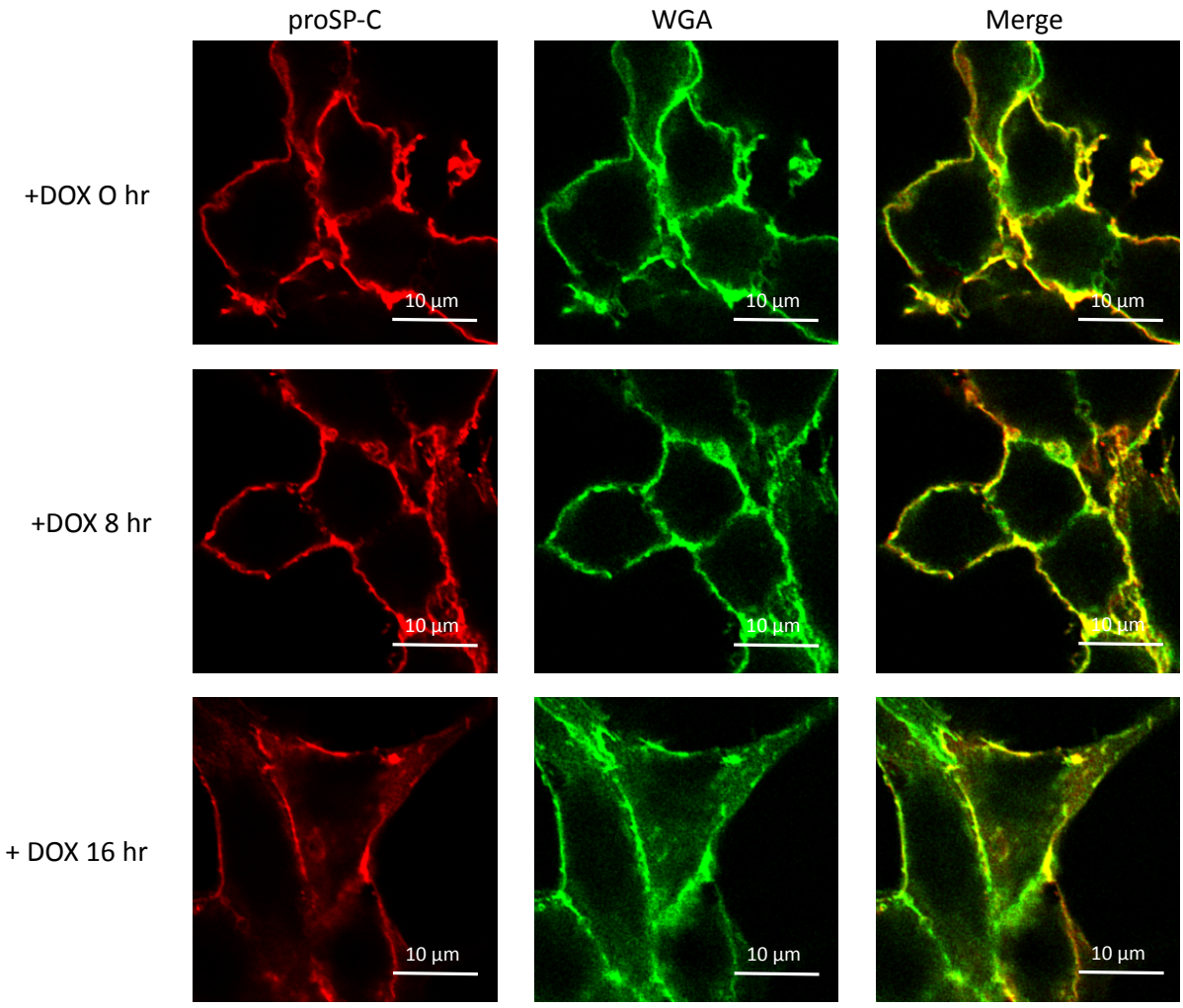

Figure S5