Cathepsin K-Mediated Phenotypic Switching of Pulmonary Smooth Muscle Contributed to the Development of Chronic Stress-related Pulmonary Hypertension in Mice

SUPPLEMENTARY MATERIALS

Suppl. Tables S1-S3

Suppl. Figures S1-S7

Data Set

Major Resources Table

Materials

Commercial antibodies were used. The following antibodies were purchased from R&D Systems (Minneapolis, MN): anti-mouse IgG (#14709) and anti-rabbit IgG (#14708) antibodies. Anti-α-SMA (AF1032), anti-goat IgG (SA00001-3) and goat anti-rabbit IgG (H+L)–Alexa Fluor 594 (S0006) were obtained from Affinity Biosciences (Cincinnati, OH, USA). Goat anti-mouse IgG (H+L)–Alexa 488 (K1204) and goat anti-rabbit IgG (H+L)–Alexa 488 (K1206) were purchased from APExBIO Technology (Houston, TX). Anti-phospho-SMAD2/3 (#8828) and anti-transforming growth factor-beta (TGF-β, #3711) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-tubulin (#ab15246), CD31 (ab7388), BMPR2 (ab96826), Collagen I (ab260043), goat anti-rat IgG (H&L)–FITC (#ab6840), goat anti-rabbit IgG (H&L)–Alexa 555 (ab150078, Abcam, 1:500), and goat anti-mouse IgG H&L-Alexa 647 (#ab150115) were purchased from Abcam (Cambridge, MA). Anti-CTSK (#sc-48353), TGF-βR2 (#sc-17792), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The RIPA lysis buffer (R0010) and the Pierce BCA Protein Assay Kit was purchased from Solarbio (Beijing, China). The polyvinylidene difluoride (PVDF) membranes were obtained from Merk Millipore (Carrigtwohill, Ireland), and the Amersham ECL Prime Western Blotting Detection kit was from GE Healthcare (Freiburg, Germany). The TRIZOL Reagent, the Super Script III First Strand, Permeabilization Buffer and IC Fixation Buffer (#0082249) and Permeabilization Buffer (#0083356) were purchased from Invitrogen (Carlsbad, CA). The RNeasy Micro Kit and SYBR™ Green Master Mix were from Qiagen (Hilden, Germany). PASMCs were purchased from Fuheng Biotechnology

Co. (Shanghai, China). The anti-fluorescence quenching solution including DAPI (P0131) was purchased from Beyotime (Shanghai China). The enzyme-linked immunosorbent assay (ELISA) kit for cathepsin K (CTSK, cat. no. E-EL-M0250), intercellular adhesion molecule-1 (ICAM-1, cat. no. E-EL-M3037) and vascular cell adhesion molecule-1 (VCAM-1, cat. no. E-EL-M1233) were purchased from Elabscience Biotechnology (Wuhan, China).

Suppl. Table S1. The experimental groups

Experiment	Mouse	Model 1. Hypoxia (4 wks) 2. Chronic stress (3 wks)	Group
Exp. 1	$CTSK^{+/+}$ (wildtype)	Normoxia	$CTSK^{+/+}$-Nor
		Hypoxia	$CTSK^{+/+}$-Hy
		Hypoxia+SU-5416	$CTSK^{+/+}$-HySU
		Hypoxia+SU-5416+Stress	$CTSK^{+/+}$-HySU-S
Exp. 2	$CTSK^{+/+}$	Normoxia	$CTSK^{+/+}$-Nor
	$CTSK^{-/-}$	Normoxia	$CTSK^{-/-}$-Nor
	$CTSK^{+/+}$	Hypoxia	$CTSK^{+/+}$-Hy
	$CTSK^{-/-}$	Hypoxia	$CTSK^{-/-}$-Hy
Exp. 3	$CTSK^{+/+}$	Hypoxia+SU-5416+Non-stress	$CTSK^{+/+}$-HySU-NS
	$CTSK^{-/-}$	Hypoxia+SU-5416+Non-stress	$CTSK^{-/-}$-HySU-NS
	$CTSK^{+/+}$	Hypoxia+SU-5416+Stress	$CTSK^{+/+}$-HySU-S
	$CTSK^{-/-}$	Hypoxia+ SU 5416+Stress	$CTSK^{-/-}$-HySU-S

$CTSK^{+/+}$: C57BL/6J, $CTSK^{-/-}$: CTSK knockout, M: male, SU: SU-5416, wks: weeks.

Suppl. Table S2. The mouse primer sequences used for the quantitative real-time polymerase chain reaction (PCR)

Gene	Forward primer	Reverse primer
ATAC2	CAACCGGGAGAAAATGACTC	GCGTCCAGAGGCATAGAGAG
CNN1	GGAGCTGAGAGAGTGGATCG	CCTGGCTGCAGCTTATTGAT
Collagen I	AGGCGAAGGCAACAGTCG	GTTCCGGTGTGACTCGTGC
CTSK	AGCAGGCTGGAGGACTAAGGT	TTTGTGCATCTCAGTGGAAGACT
FN	ACAAGCATGTCTCTCTGCCA	GCAATGTGCAGCCCTCATTT
SM22α	ATCATAGTGCAGTGTGGCCC	CACCTGCTCCATCTGCTTGAQQQ
TGF-β1	TGGCGTTACCTTGGTAACC	GGTGTTGAGCCCTTTCCAG
VCAN	CCAGATGCCTACGCAAACAC	TCCACTTCCACAGCATCAAC
VIM	GTGGACCAGCTAACCAACGA	AAGGTCAAGCGTGCCAGAG
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCT

CNN1: calponin-1, Collagen I: collagen type I, CTSK: cathepsin K, FN: fibronectin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, SM22α: smooth muscle protein 22-alpha, TGF-β1: transforming growth factor-beta 1, VCAN: versican, VIM: vimentin.

Suppl. Table S3. Plasmid sequences used for gene transfection

Gene	Forward primer	Reverse primer
CTSK siRNA	CCUCUCGAUCCUACAGUAATT	UUACUGUAGGAUCGAGAGGTT
CTSK pc-DNA	Gene ID: 13038, NM_007802.4	

MAJOR RESOURCES TABLES

Animals (in vivo studies)

Species	Vender or Source	Background Strain	Sex	Persistent ID/URL
Wild-type C57BL/6J	Institutional Animal Care and Use Committee of Yan Bian University	C57BL/6J	M	Laboratory animals, Yan Bian University
CTSK knockout mouse	Shanghai Biomodel Organism Science & Technology Development Co.	C57BL/6J	M	Laboratory animals, Yan Bian University

Cultured Cells

Name	Vender or Source	Sex (F, M, or unknown)	Persistent ID/URL
PASMCs	Fuheng Biotechnology Co.	N/A	https://www.fudancell.com/sys-pd/4794.html

Other

Product	Source/Repository	Persistent ID or URL
RIPA lysis buffer	Solarbio	
Pierce BCA Protein Assay Kit	Solarbio	
FACS Lysing Solution	BD	
PowerUp SYBR Green	ThermoFisher	A25742
SuperScript III First-Stand Synthesis System	ThermoFisher	18080-501
RNasy Micro Kit	QIAGEN	74904
Polyvinylidene difluoride (PVDF) membranes	Merk Millipore	
Amersham ECL Prime Western Blotting Detection Kit	GE Healthcare	
TRIZOL Reagent	Invitrogen	
Super Script III First Strand	Invitrogen	
The RNeasy Micro Kit and SYBR^{TM} Green Master Mix	Qiagen	
Lipofectamine RNAiMAX Transfection Kit	ThermoFisher	13778150

Lipofectamine 3000 Transfection Kit	ThermoFisher	L3000015
Cathepsin K (CTSK) (ELISA) Kit	Elabscience Biotechnology	
Intercellular Adhesion Molecule-1 (ICAM) (ELISA) Kit	Elabscience Biotechnology	
Vascular Cell Adhesion Molecule-1 (VCAM) (ELISA) Kit	Elabscience Biotechnology	

ARRIVE guidelines: No information for sample size estimation, inclusion/exclusion criteria, randomization or blinding is available. No samples were excluded from the analysis in these studies.

Supplementary Figure Legends

Suppl. Fig. S1. Unsupervised clustering of single-cell transcriptomes from lung tissue and cell annotation based on hallmark genes. **A:** The UMAP dimensionality reduction visualization revealing the 30 transcriptionally distinct cell clusters obtained through unsupervised clustering. **B:** Dot plots showing the expression patterns of representative hallmark genes across different clusters. *Dot size:* the proportion of cells expressing the gene. *Color intensity:* the average expression levels. **C:** Bar charts displaying the average expression levels of representative extracellular matrix (ECM) and fibrosis-related genes in lung tissue from healthy controls and patients with idiopathic pulmonary hypertension (IPAH). D–G: Differential gene set activity between the healthy controls and IPAH patients. Violin plots show the biological processes (GO) including **D:** the hypoxia response (Hallmark hypoxia), **E:** the TGF-β signaling pathway (Hallmark TGF-β signaling), and **F:** the epithelial-mesenchymal transition (Hallmark EMT). **G:** Boxplots showing the median and interquartile range. Statistical significance was assessed using the two-tailed Wilcoxon signed-rank test. $p<0.0001$. **H:** The mouse study design and experimental groups.

Suppl. Fig. S2. Chronic restraint stress (CPS) further elevated the expression of CTSK and promoted the development of pulmonary hypertension (PAH) in Hypoxia ± SU-5416-induced PAH. **A:** The protocol for the mice exposed to hypoxia ± SU-5416 with or without CPS at the indicated time points. **B:**The plasma concentration of CTSK (n=6/group). **C:**Representative H&E and Masson images (scale bars: 50 μm) showing increased medial thickness and fibrosis (n=6/group). **D,E:** Quantitative data showing the ratio of the area of vascular medial thickness to the total vessel area of pulmonary arteries (H&E), the percentage of fibrosis area (Masson), and the percentage of the $CTSK^+$ to α-SMA^+ in lung tissues from the four groups (n=6/group). **F,J:** Representative images and quantitative data showing the RVSP, PAT/PET, TAPSE, and RV/(LV+S) in the four experimental groups (n=6/group). **K:** The changes in body weights during the 28-day follow-up in three groups (n=8/group). **L,M**: Quantitative data showing the weight data of the subcutaneous white adipose tissue (SWAT) and lung in the three experimental groups (n=8/group). **N–P:** qPCR data showing the levels of CTSK, TGF-β1, and collagen I. VCAN, FN, VIM, ATAC2, CNN1 and SM22α mRNAs in the lungs of the three experimental groups (n=6/group). Data are mean ± SEM. For the data in panels B, D, E, and G–P, the significance of differences was assessed by a one-way ANOVA with Tukey's post hoc tests.

Suppl. Fig. S3. CTSK deletion prevented the hypoxia-induced progression of inflammation in mouse models and the proliferation of primary pulmonary arterial smooth muscle cells (PASMCs). **A:** The changes in body weights during the 28-day follow-up in both groups (n=8/group).

B,C: Quantitative data showing the weight data of the SWAT and lung in the four experimental groups (n=8/group). **D,E:** The plasma concentrations of ICAM-1 and VCAM-1 determined by ELISA (n=7/group). **F:** The extraction of primary PASMCs. **G:** Primary PASMCs were identified by α-SMA with immunofluorescence staining. Scale bar: 50 μm. **H:** Representative EdU staining images and quantitative data showing the numbers of EdU^+ cells (*red*) in the four experimental groups (n=6/group). Scale bar: 100 μm. Data are mean ± SEM. NS: not significant. For the data in panels A–E and H, the significance of differences was assessed by a one-way ANOVA with Tukey's post hoc tests.

Suppl. Fig. S4. The CTSK levels were elevated in PASMCs under the Hypoxia condition. **A:** The protocol for PASMCs cultured in the hypoxia (1% O_2) or normoxia (21% O_2) condition for 24 hr. **B,C:** Representative EdU staining images and quantitative data show the numbers of EdU^+ cells (*red*) in the four groups (n=5/group). Scale bar: 100 μm. Data are mean ± SEM. For the data in panels C, the significance of differences was assessed by an unpaired Student's *t*-test.

Suppl. Fig. S5. CTSK silencing mitigated the proliferation of PASMCs and increased the mitochondrial real-time oxygen consumption rate in response to hypoxia with S-serum. Following transfection with a non-targeting negative control (NC) and si-CTSK for 48 hr, the PASMCs

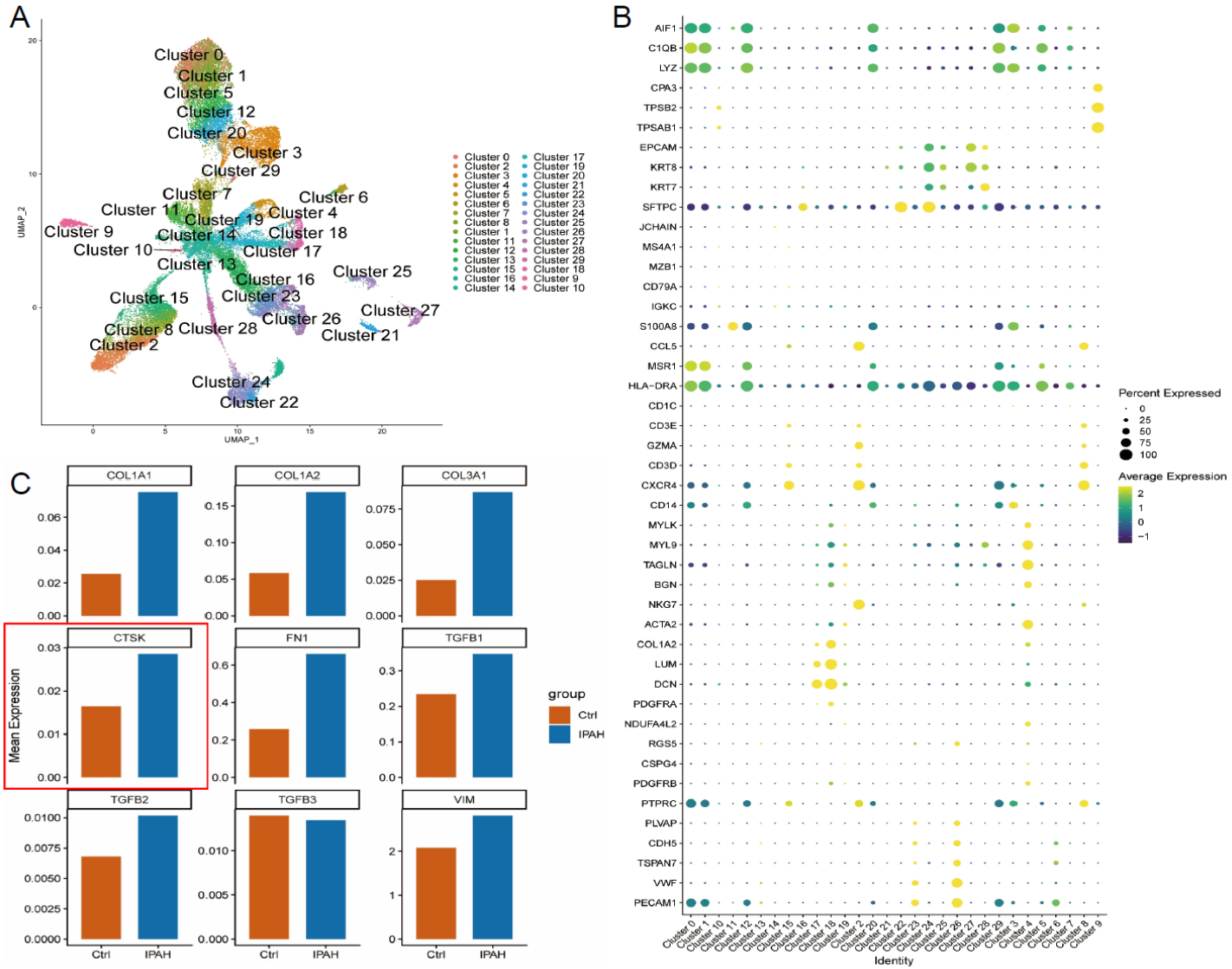
were treated with 5% stress serum (S-serum) for 24 hr. **A:** Fluorescence verification of siCTSK transfection (scale bar: 75 μm). **B:** Western blot analysis of the CTSK protein levels in the NC group and siCTSK group (n=4/group). **C,D:** Representative EdU staining images and quantitative data show the numbers of EdU^+ cells (*red*) in the four groups (n=6/group). Scale bars: 100 μm. **E,F:** Quantitative data showing p-$SMAD^+$/α-SMA^+ cells and collagen I^+/α-SMA^+ cells in the four groups (n=6/group). **G–J:** The mitochondrial real-time oxygen consumption rate (OCR) measured in PASMCs successively to measure the basal respiration, proton leak, ATP production, and maximum respiration in the four experimental groups (n=3/group). Data are mean ± SEM. For the data in panel B. the significance of differences was assessed by an unpaired Student's *t*-test. For the data in panels D–J, the significance of differences was examined by a one-way ANOVA with Tukey's post hoc tests.

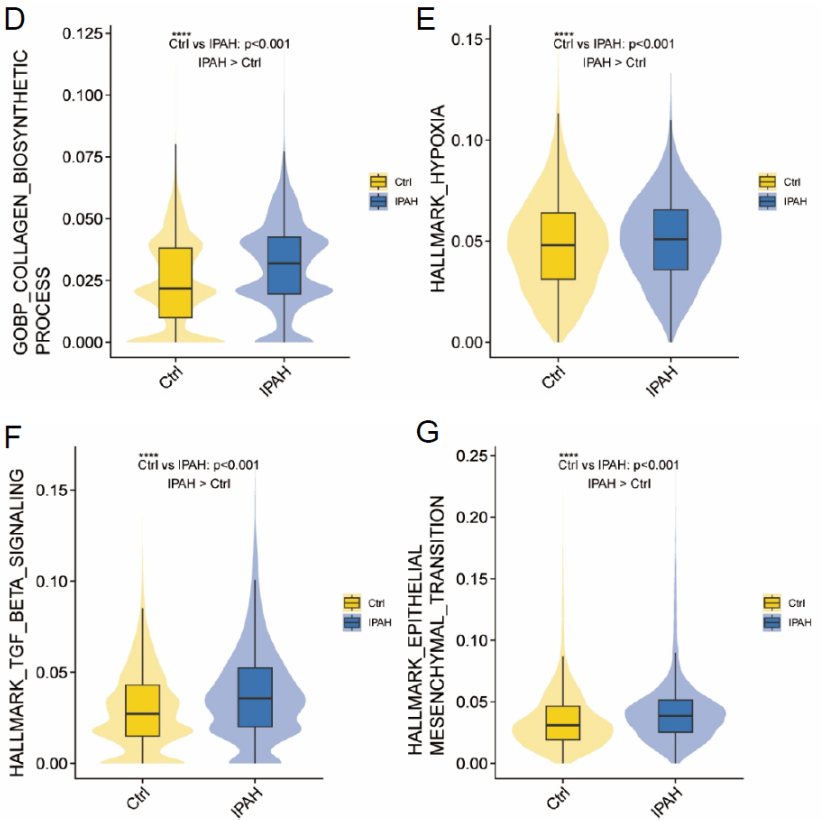
Suppl. Fig. S6. CTSK overexpression enhanced the hypoxia with S-serum-induced proliferation and downregulated the oxygen consumption rate of PASMCs. Following transfection with a non-targeting negative control (NC) and pl-CTSK for 48 hr, the PASMCs were treated by 5% stress serum (S-serum) stimulations for 24 hr. **A:** PASMCs were transfected with GFP-plCTSK for 6 hr, and the fluorescence was observed under a microscope for the confirmation of successful plCTSK transfection. Scale bar: 75μm. **B:** The western blot analysis of the investigated molecular protein levels (CTSK) in the NC group and plCTSK group (n=4/group). **C,D:** Representative EdU staining images and quantitative

data showing the numbers of EdU^+ cells (*red*) in the four groups (n=6/group). Scale bar: 100 μm. **E,F:** Quantitative data showing p-$SMAD^+$/α-SMA^+ cells and collagen I^+/α-SMA^+ cells in the four groups (n=6/group). **G–J:** The mitochondrial real-time oxygen consumption rate (OCR) measured in PASMCs successively to measure the basal respiration, proton leak, ATP production, and maximum respiration in the four experimental groups (n=3/group). Data are mean ± SEM. For the data in panel B. the significance of differences was assessed by an unpaired Student's *t*-test. For the data in panels D–J, the significance of differences was examined by a one-way ANOVA with Tukey's post hoc tests.

Suppl. Fig. S7. The CTSK inhibitor and TGF-β inhibitor effectively suppressed TGF-β signaling in PASMCs. **A:** Protocol design: PASMCs pretreated ± the CTSK inhibitor balicatib (1 μM, 30 min) prior to 24 hr of hypoxia (1 % O_2) plus 5% S-serum. **B:** Representative immunoblotting images and quantitative data for CTSK, TGF-β, and PPAR-γ proteins in the two experimental groups (n=3/group). **C:** Quantitative data for CTSK, TGF-β, and PPAR-γ proteins in both groups with or without the TGF-β receptor inhibitor (LY2109761) (n=3/group). **D:** Quantitative data showing the p-$SMAD^+$/α-SMA^+ areas in both experimental groups (n=6/group). **E:** Quantitative data presenting the collagen I^+/α-SMA^+ areas in the two experimental groups (n=6/group). **F:** Quantitative data showing the numbers of EdU^+ cells (*red*) in both groups (n=6/group). Data are mean ± SEM. For the data in panels B–F, the significance of differences was assessed by an unpaired Student's *t*-test.

Supplement Figure 1





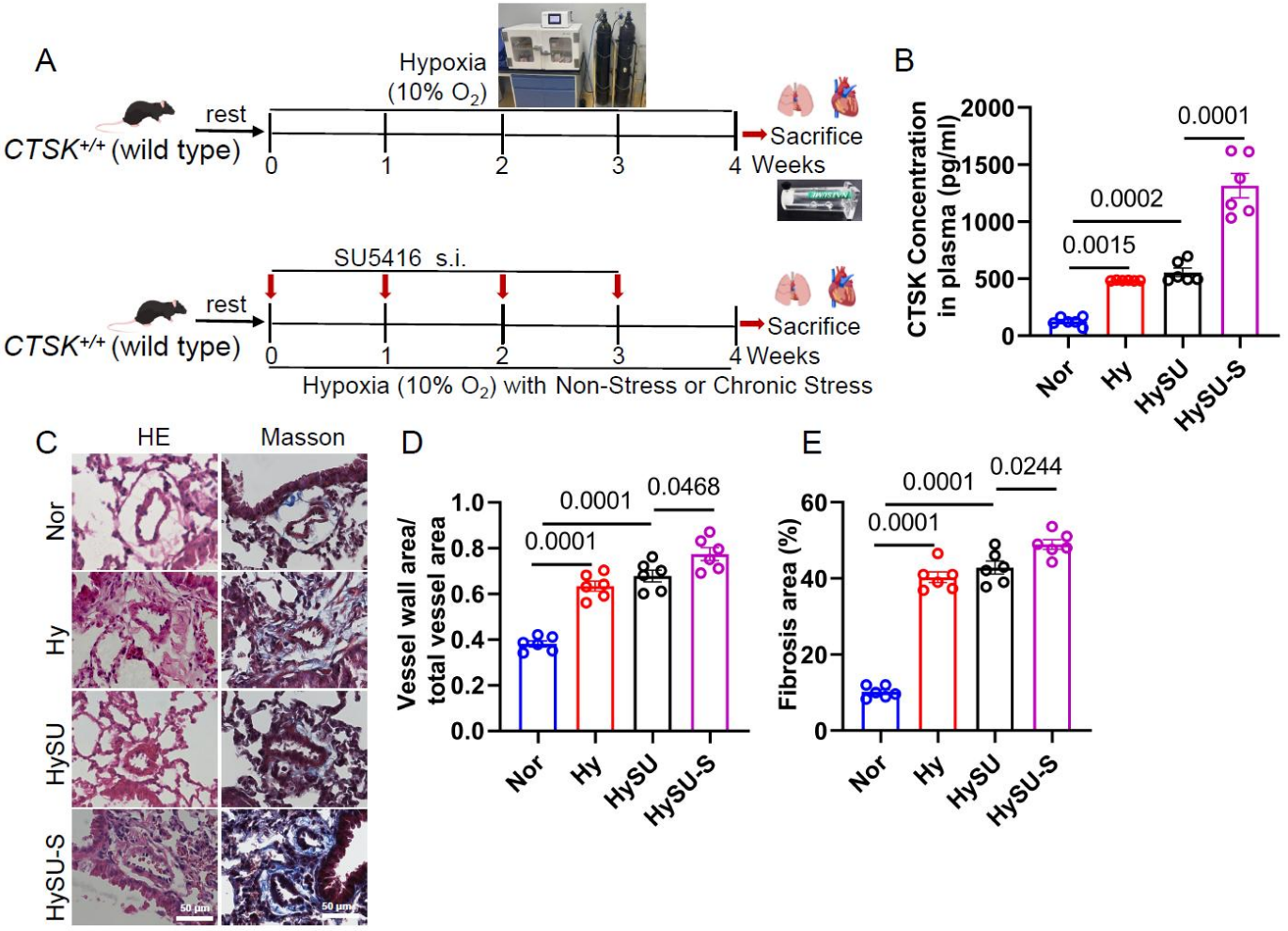
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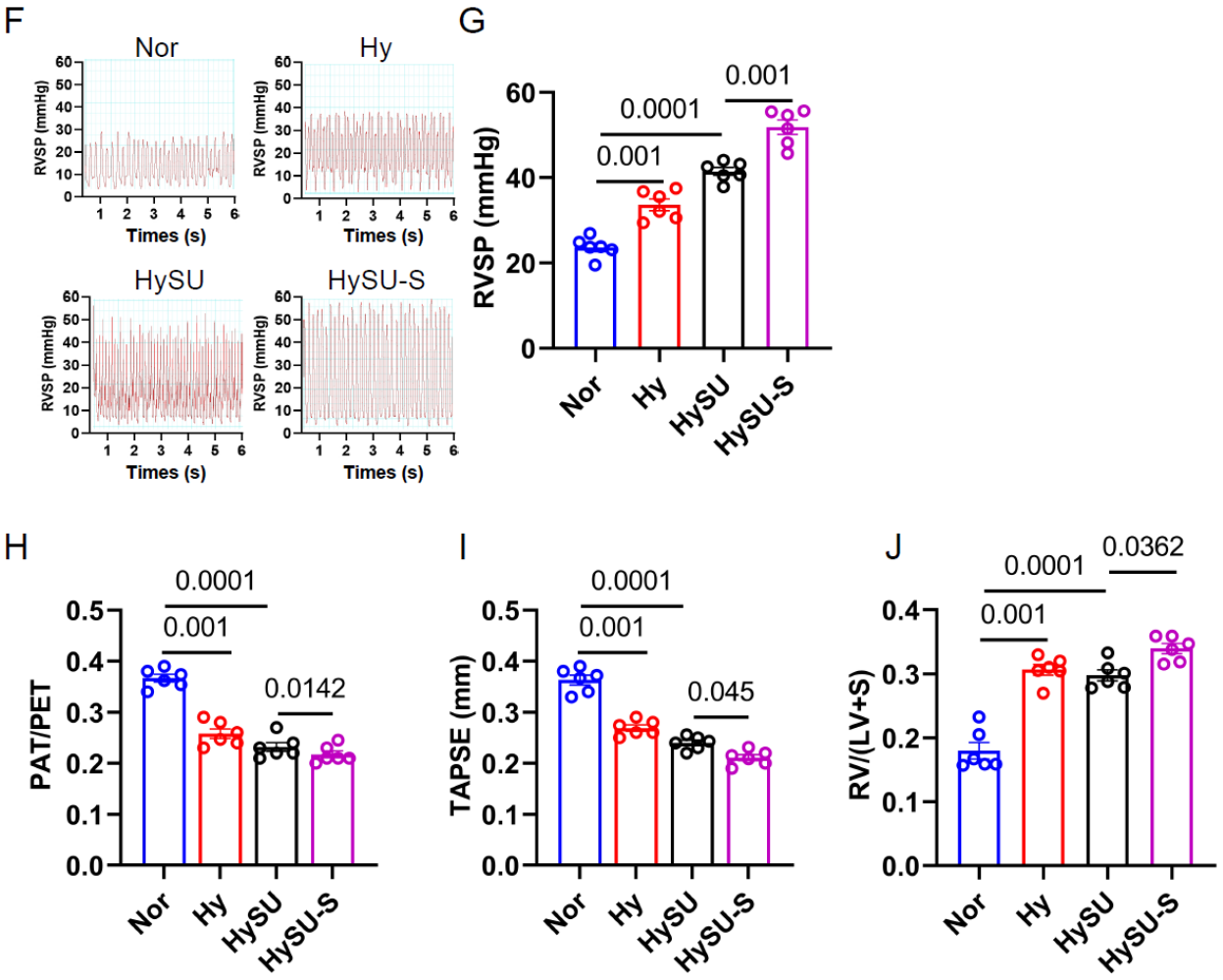
Suppl. Table S1. The experimental groups

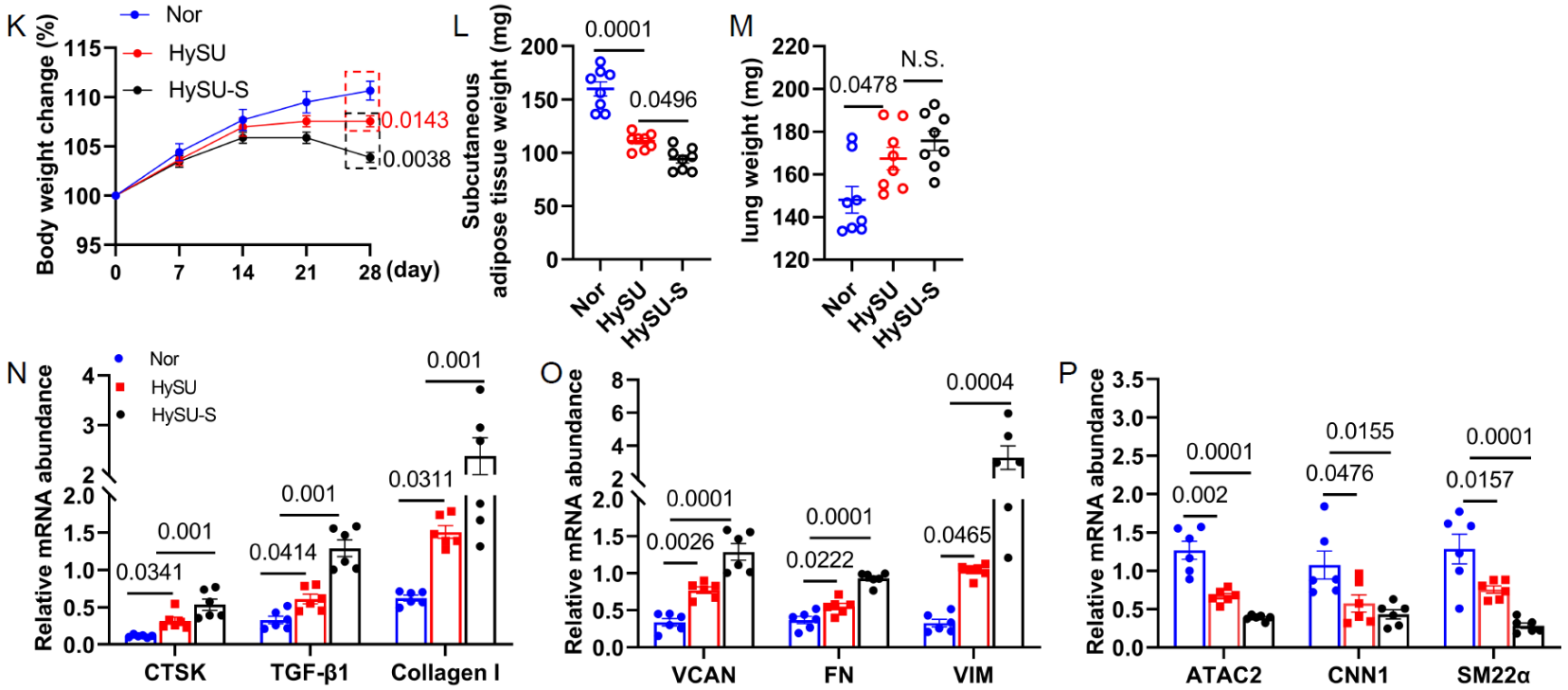
Experiment	Mouse	Model 1. Hypoxia (4 wks) 2. Chronic stress (3 wks)	Group
Exp. 1	$CTSK^{+/+}$ (wildtype)	Normoxia	$CTSK^{+/+}$-Nor
		Hypoxia	$CTSK^{+/+}$-Hy
		Hypoxia+SU-5416	$CTSK^{+/+}$-HySU
		Hypoxia+SU-5416+Stress	$CTSK^{+/+}$-HySU-S
Exp. 2	$CTSK^{+/+}$	Normoxia	$CTSK^{+/+}$-Nor
	$CTSK^{-/-}$	Normoxia	$CTSK^{-/-}$-Nor
	$CTSK^{+/+}$	Hypoxia	$CTSK^{+/+}$-Hy
	$CTSK^{-/-}$	Hypoxia	$CTSK^{-/-}$-Hy
Exp. 3	$CTSK^{+/+}$	Hypoxia+SU-5416+Non-stress	$CTSK^{+/+}$-HySU-NS
	$CTSK^{-/-}$	Hypoxia+SU-5416+Non-stress	$CTSK^{-/-}$-HySU-NS
	$CTSK^{+/+}$	Hypoxia+SU-5416+Stress	$CTSK^{+/+}$-HySU-S
	$CTSK^{-/-}$	Hypoxia+ SU 5416+Stress	$CTSK^{-/-}$-HySU-S

$CTSK^{+/+}$: C57BL/6J, $CTSK^{-/-}$: CTSK knockout, M: male, SU: SU-5416, wks: weeks.

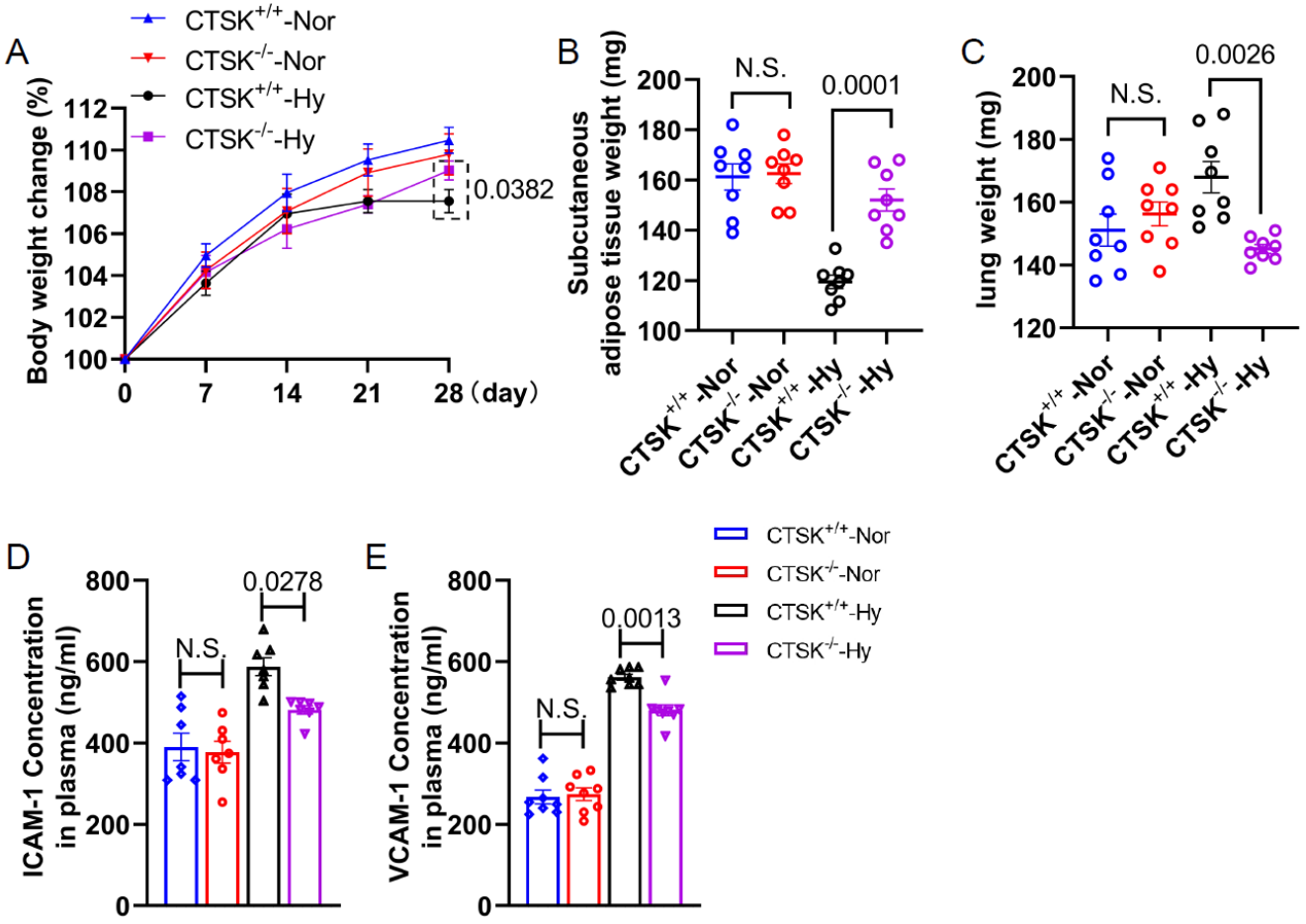
Supplement Figure 2

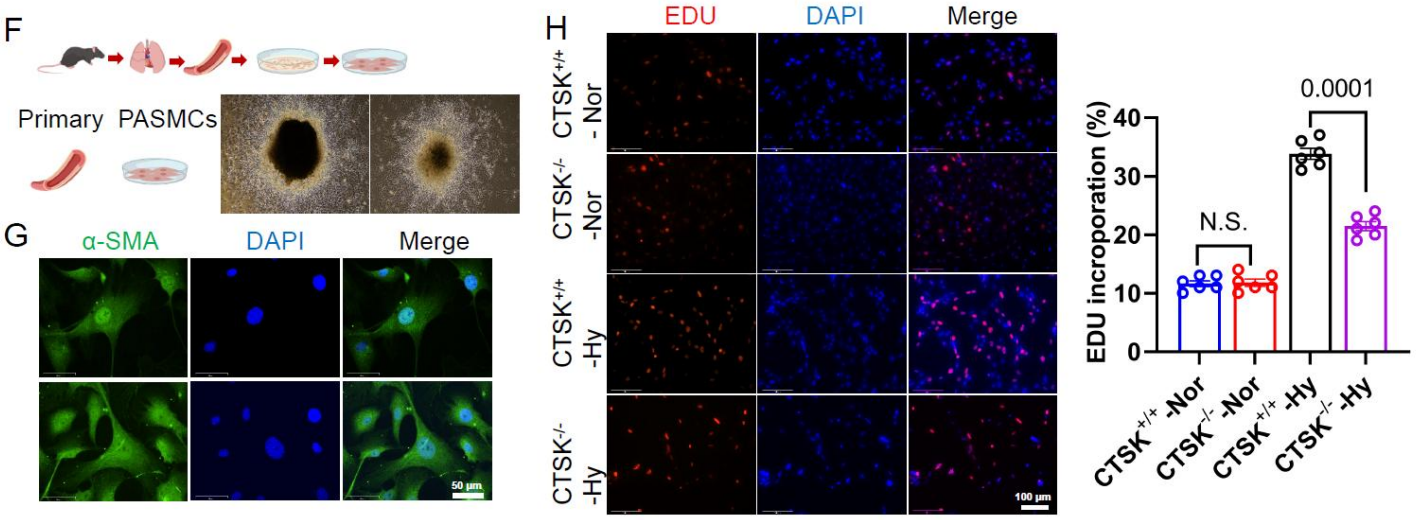



F
Nor
Hy
HySU
HySU-S
RVSP (mmHg)
Times (s)
G
RVSP (mmHg)
0.001
0.0001
0.001
Nor
Hy
HySU
HySU-S
H
PAT/PET
0.0001
0.001
0.0142
I
TAPSE (mm)
0.0001
0.001
0.045
J
RV/(LV+S)
0.0001
0.0362
0.001

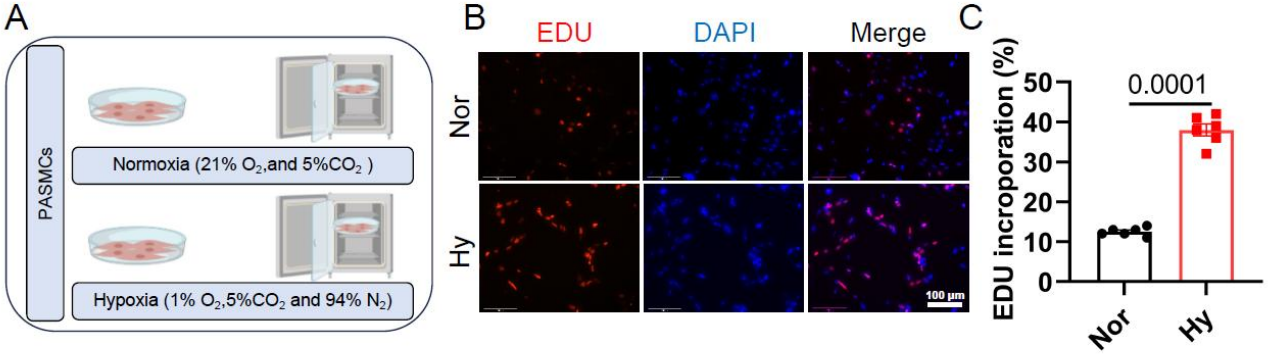

K
Nor
HySU
HySU-S
Body weight change (%)
115
110
105
100
95
0
7
14
21
28 (day)
0.0143
0.0038
L
Subcutaneous adipose tissue weight (mg)
200
150
100
50
0
0.0001
0.0496
Nor
HySU
HySU-S
M
lung weight (mg)
220
200
180
160
140
120
0.0478
N.S.
Nor
HySU
HySU-S
N
Nor
HySU
HySU-S
Relative mRNA abundance
4
3
2
2.0
1.5
1.0
0.5
0.0
0.0341
0.001
0.0414
0.001
0.0311
0.001
CTSK
TGF-β1
Collagen I
O
Relative mRNA abundance
8
6
4
2
2.0
1.5
1.0
0.5
0.0
0.0026
0.0001
0.0222
0.0001
0.0465
0.0004
VCAN
FN
VIM
P
Relative mRNA abundance
3.5
3.0
2.5
2.0
1.5
1.0
0.5
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0.0001
0.0476
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0.0001
ATAC2
CNN1
SM22α

Supplement Figure 3

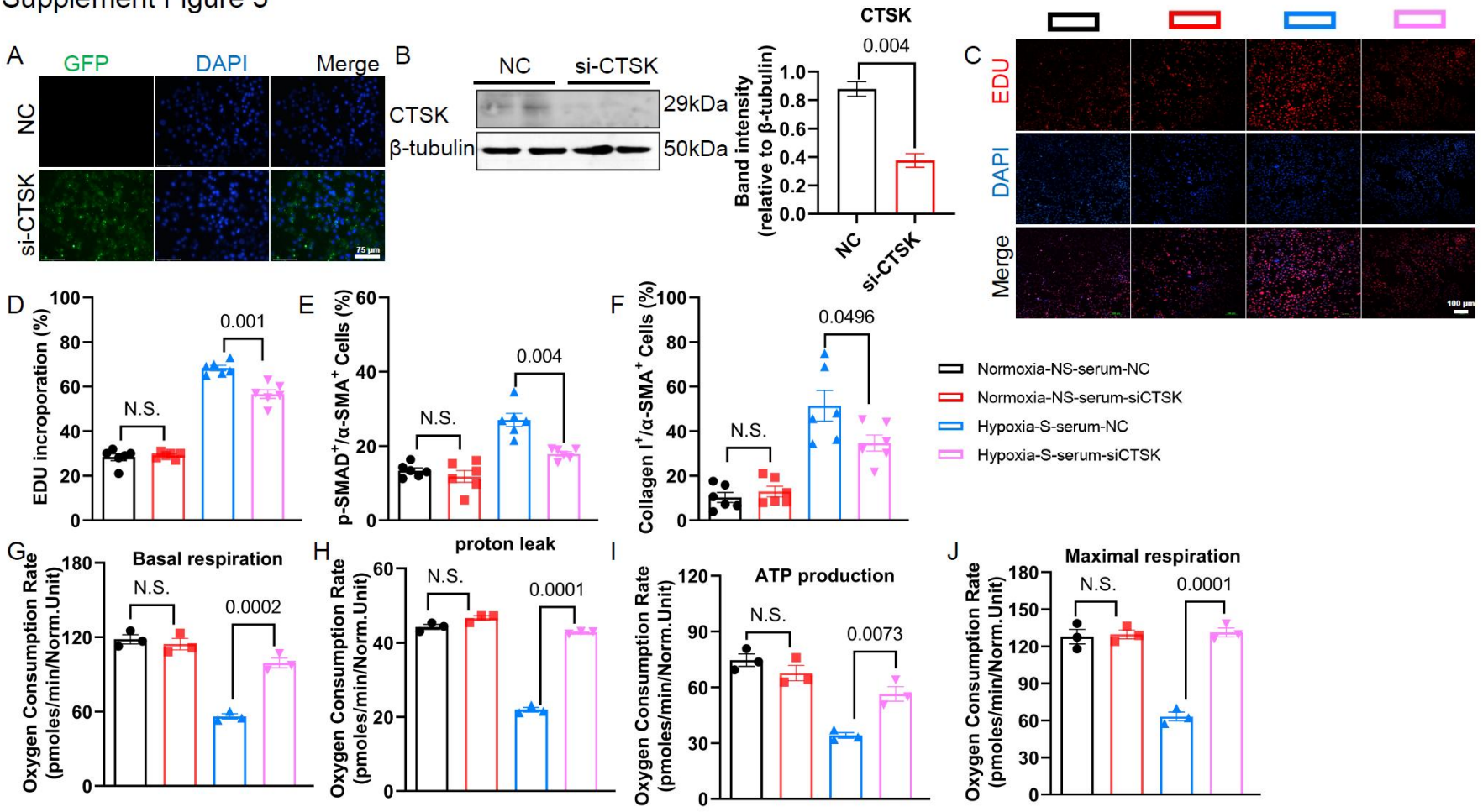



F
Primary PASMCs
G
α-SMA
DAPI
Merge
50 μm
H
EDU
DAPI
Merge
CTSK+/+ -Nor
CTSK-/- -Nor
CTSK+/+ -Hy
CTSK-/- -Hy
100 μm
0.0001
N.S.
EDU incroporation (%)
0
10
20
30
40
CTSK+/+ -Nor
CTSK-/- -Nor
CTSK+/+ -Hy
CTSK-/- -Hy

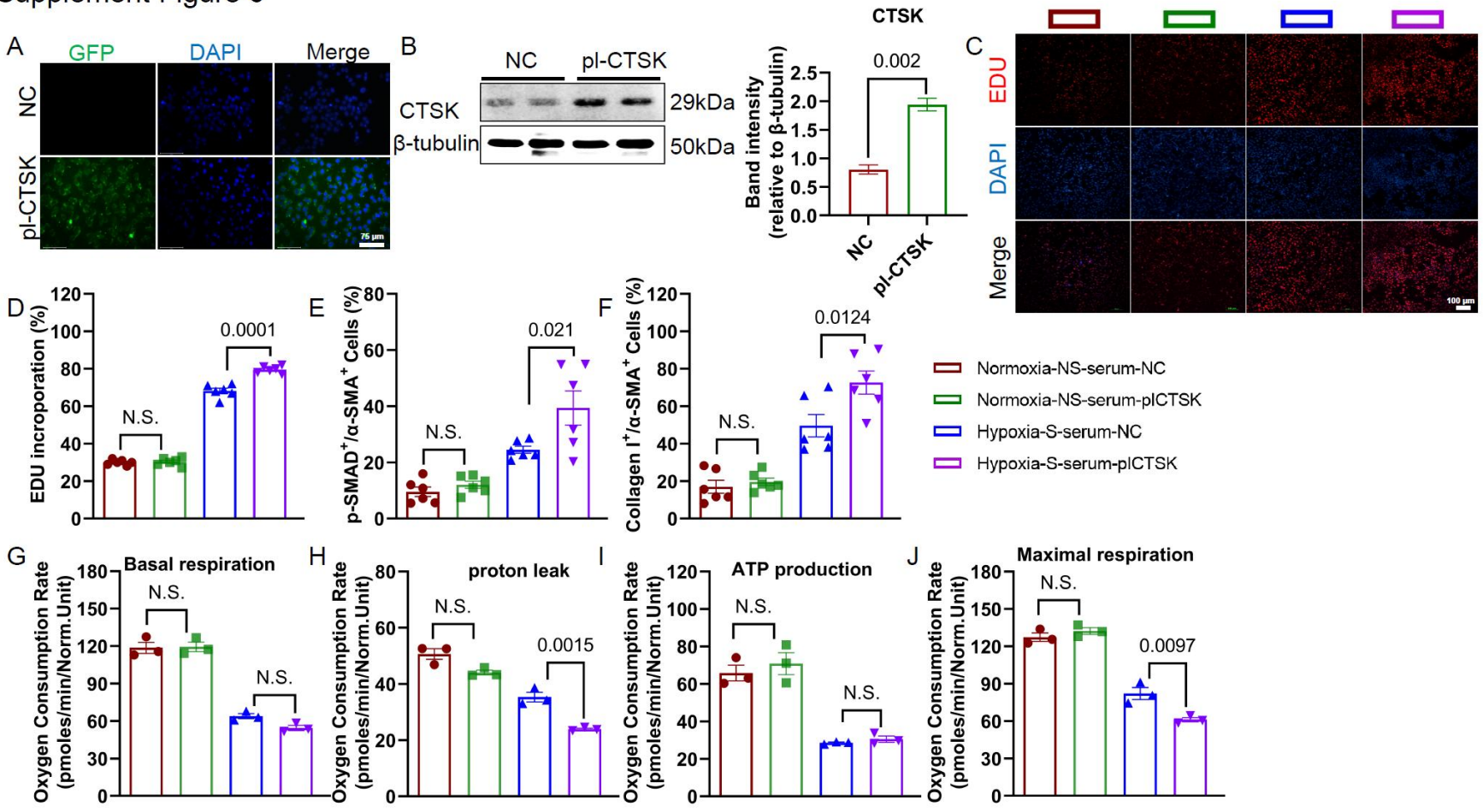
Supplement Figure 4



Supplement Figure 5



Supplement Figure 6



Supplement Figure 7

