

## Online Supplemental Materials

### PFKFB3 in Smooth Muscle Promotes Vascular Remodeling in Pulmonary Arterial Hypertension

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## Online Supplemental Methods

### Reagents and Materials

Human pulmonary artery smooth muscle cells (PASMCs) and culture medium were obtained from Lonza (Walkersville, MD, USA). Calpain inhibitor MDL28170, PFKFB3 inhibitor 3PO and fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC were from Calbiochem (La Jolla, CA, USA). The Dynabeads Protein G and siPORT Amine Transfection Agent were purchased from Life Technologies (Grand Island, NY, USA). The phosphatase and protease inhibitor tablets were purchased from Roche Applied Science (Indianapolis, IN, USA). Antibody against collagen-I (#NBPI-30054) were from Novus Biologicals (Littleton, CO, USA). Antibody against PFKFB3 (#13763-1-AP) were from Proteintech Group, Inc (Rosemont, IL, USA). Anti-calpain-1 (#RP3-calpain-1) and calpain-2 (#RP1-calpain-2) antibodies were purchased from Triple Point Biologics (Forest Grove, OR, USA). Anti-von Willebrand factor antibody (#Ab6994) was from Abcam. The siRNAs against ERK1/2 (#6560), the anti p-ERK1/2 (#4370) and total ERK1/2 (#4695) antibodies and GAPDH antibody (#21185) were from Cell Signaling Technologies (Danvers, MA, USA). Ki67 antibody (#RM-9106-S0) was from Thermofisher.  $\alpha$ -actin antibody (#SC-56499) and PECAM antibody (#SC-376764) were from Santa Cruz. The MAPK inhibitor PD98059 were from Selleck Chemicals (Houston, TX, USA). Primary antibodies against phospho-Ser50 and phospho-Ser369 of calpain-2 were designed and produced by Pacific Immunology (Ramona, CA, USA). The specificity of these antibodies have been confirmed by site-directed mutation of serine 50 and serine 369 of calpain-2 in our previously studies. The siRNAs against calpain-1, -2 and the control siRNA were from Qiagen (Valencia, CA, USA). PDGF-BB, sodium-L-lactate, antibody against calpastatin (#C270), lactate assay kit, VEGF receptor antagonist SU5416 and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

All animal studies were performed using unbiased approaches (Bonnet S, Provencher S, Guignabert C, Perros F, Boucherat O, Schermuly RT, Hassoun PM, Rabinovitch M, Nicolls MR, Humbert M. Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension. *Am J Respir Crit Care Med* 2017; 195: 583-595 and Provencher S, Archer SL, Ramirez FD, Hibbert B, Paulin R, Boucherat O, Lacasse Y, Bonnet S. Standards and Methodological Rigor in Pulmonary Arterial Hypertension Preclinical and Translational Research. *Circ Res* 2018; 122: 1021-1032).

### Knockout mice

To create an inducible smooth muscle-specific knockout of PFKFB3 in mice (PFKFB3<sup>flox/flox</sup>;SMMHC-CreER), the PFKFB3<sup>flox/flox</sup> mice were crossed with B6.FVB-Tg(Myh11-cre/ERT2)1Soff/J mice (Jax Mice, Bar Harbor, Maine). Deletion of PFKFB3 was induced by administration of tamoxifen (20 mg/kg/day, i.p.) for 5 days and was confirmed by analysis of PFKFB3 using QRT-PCR and Western blotting of isolated pulmonary artery smooth muscle and fluorescence confocal microscopy of pulmonary arteries. Control male mice were PFKFB3<sup>flox/flox</sup> or PFKFB3<sup>flox/+</sup> or SMMHC-CreER mice treated with the same tamoxifen regimen. Mice between 12 and 14 weeks old were used for the hypoxic PAH model.

### Hypoxic PAH model

Smooth muscle-specific PFKFB3 knock-out mice and their littermate control mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) in a clear plastic polypropylene chamber (30''x20''x20''). The oxygen concentration (10%) was maintained using Proox

Oxygen Controller (BioSpherix, Lacona, NY). The chamber has ventilation holes and a small quiet fan to provide forced circulation and instant homogenization of gases. Litholyme CO<sub>2</sub> absorbent (Allied Health Care Products, St. Louis, MO) kept the CO<sub>2</sub> concentration at <0.2%. Boric acid was used to keep minimal NH<sub>3</sub> levels within the chamber. Relative humidity within the chamber was kept at <60% with anhydrous CaSO<sub>4</sub>. All animals had access to standard mouse chow and water ad libitum at both normoxic and hypoxic conditions. After 4 weeks, pulmonary hypertension and pulmonary vascular remodeling were assessed.

### **MCT-induced PAH rat model**

Male Sprague-Dawley rats 8 weeks old were purchased from Envigo Rms, Inc. (Indianapolis, IN). Pulmonary hypertension was induced by a single subcutaneous injection of MCT (60 mg/kg). Control rats received saline (0.5 ml, subcutaneously). After 2 weeks, control rats and MCT injected rats (MCT 2wks) were subjected to echocardiography and determination of pulmonary hypertension and pulmonary vascular remodeling. At same time (the beginning of third week), another groups of MCT injected rats started to receive the PFKFB3 inhibitor 3PO (i.p.) (MCT 4wks+25mg/kg and MCT 4wks+50mg/kg) or saline (MCT 4wks+Vehicle) once a day and 5 days per week. Echocardiography, pulmonary hypertension and pulmonary vascular remodeling were accessed 2 week later (4 weeks after MCT injection).

### **Sugen/Hypoxia PAH rat model**

Male Sprague-Dawley rats 8 weeks of age were injected subcutaneously with SU5416 (20 mg/kg) and were exposed to 10% oxygen (hypoxia) for 3 weeks and to 21% oxygen (normoxia) for additional 2 weeks in a clear plastic polypropylene chamber as described above. The oxygen concentration (10%) was maintained using Proox Oxygen Controller (BioSpherix, Lacona, NY). Control age-matched male rats were maintained under normoxia for 5 weeks. All animals had access to standard rat chow and water ad libitum at both normoxic and hypoxic conditions. After 5 weeks, groups of control rats (Norm + PD98) and Sugen/Hypoxia-exposed rats (SuHx + PD98) were injected intraperitoneally with ERK1/2 inhibitor PD98059 (10 mg/kg) 5 days/week for 3 weeks. This dose is chosen according to previous experiments (30). Second groups of control rats (Normoxia) and SuHx-exposed rats (SuHx) received the same volume of vehicle (DMSO/0.9% NaCl [1:10 v/v] administered i.p. at 1 ml/kg). Echocardiography, pulmonary hypertension and pulmonary vascular remodeling were assessed 3 week later.

### **Echocardiography**

Transthoracic echocardiography was performed with a Visual Sonics Vevo 2100 ultrasound machine with a 40 MHz ultrasound probe (MS400D). Briefly, mice and rats were anesthetized with continuous isoflurane inhalation (1.5-3.0%), and placed on a heated pad in a supine position. The fur on the chest was removed with a chemical hair remover. The RV wall thickness during diastole was obtained from the parasternal long axis view using M-mode. The pulmonary artery (PA) acceleration time (PAAT) were obtained from the modified parasternal long axis view using pulsed Doppler mode. The cardiac output (CO) was obtained from the parasternal short axis view using M-mode. The tricuspid annulus plain systolic excursion (TAPSE) was measured by M-mode from the parasternal short axis view at the aortic valve level.

### **Assessment of pulmonary hypertension and histological analysis**

Mice and rats were anesthetized with ketamine hydrochloride (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.) and the trachea was intubated. The diaphragm was surgically exposed through the abdomen. A 25 gauge needle connected to a pressure transducer (AD Instruments) was inserted into the right ventricle (RV) through the diaphragm, and RV pressure continuously monitored for

10-15 minutes. Data were recorded using a PowerLab data-acquisition system (AD Instruments, Colorado Springs, CO). With this approach, the diaphragm remains intact without opening the chest.

After hemodynamic measurements, mice and rats were euthanized by thoracotomy. The blood in the pulmonary circulation was removed by infusing of PBS through the pulmonary artery and the heart and lungs were removed. The free wall of the RV, left ventricle (LV) and septum (S) were then carefully dissected and individually weighed to calculate the Fulton index (ratio of RV/ (LV +S)) as a parameter of right ventricular hypertrophy.

The right lungs were removed and snap-frozen in liquid nitrogen for preparing homogenates and the left lungs were filled with 4% paraformaldehyde (PFA) solution with 0.5% agarose at 25 cm H<sub>2</sub>O and fixed in 4% PFA for 24 h. The fixed lungs were then sliced mid-sagittally and embedded in paraffin. The slides (7 μm thickness) were stained with hematoxylin and eosin for morphometric analysis and were examined by Olympus BX41 microscope. Olympus DP72 digital camera and Image J software (<http://rsbweb.nih.gov/ij/>) were used to analyze slides. A minimum of 10 microscopic fields was examined for each slide. To quantitate pulmonary arterial wall thickness, the lumen area at the level of the basement membrane and total vascular area at the adventitial border in 20 muscular arteries with diameter of 50-100 μm per lung section were outlined and area sizes were measured using ImageJ. The vascular wall thickness was calculated as follows: wall thickness = (total vascular area – lumen area) / total vascular area. The percentage of the occluded vessels were calculated as total number of occluded vessels divided by the total number of vessels. At least 40 vessels below 100 μm were studied in 10–15 fields of view per subject.

### **Immunofluorescence confocal microscopy**

To determine the content of PFKFB3, p-ERK1/2, p-calpain-2, SBDP, and collagen-I in pulmonary arterioles, double immuno-staining of PFKFB3/ $\alpha$ -actin, p-ERK1/2/ $\alpha$ -actin, p-calpain-2/ $\alpha$ -actin, SBDP/ $\alpha$ -actin, collagen-I/ $\alpha$ -actin and Ki67/ $\alpha$ -actin was performed on lung tissue slides from PAH animal models and from normal and PAH patients. Human lung slides were obtained from lung tissues of patients undergoing lung transplantation for idiopathic PAH (n=6) and from unused donor lungs (normal lungs, n=6). These samples were provided by the Pulmonary Hypertension Breakthrough Initiative (PHBI). The lung slides were incubated first with a rabbit polyclonal antibody against PFKFB3 (dilution 1:100), p-ERK1/2 (dilution 1:100), P-Ser50 of calpain-2 (dilution 1:20), SBDP (dilution 1:100), collagen-I (dilution 1:100), Ki67 (dilution 1:100) and mouse monoclonal antibody against  $\alpha$ -actin (dilution 1:700) overnight and then with goat anti-rabbit IgG Alexa Fluor 488 and goat anti-mouse IgG Alexa Fluor 594 (dilution 1:500). The slides were sealed with mounting solution containing antifade reagent and were examined using a Zeiss LSM 510 laser scanning confocal microscope. The yellow color indicates co-localization of PFKFB3, p-ERK1/2, p-calpain-2, SBDP, and collagen-I and  $\alpha$ -actin. The area of yellow color was measured using ImageJ.

### **Cell culture**

Primary human PASMCs were purchased from Lonza, Inc (#CC-2581, Walkersville, MD) and cultured in smooth muscle growth medium-2 (SmGM-2, Lonza). These PASMCs were originally isolated from proximal pulmonary arteries of 5 normal subjects. The cell authentication has been established by the company. All cells were maintained in a sterile, humidified cell culture incubator at 37°C with 5% CO<sub>2</sub>. After reaching confluence, cells were passaged by using 0.25% trypsin-EDTA. Third-to-eighth passage cells equilibrated in growth factor- and serum-free SmGM-2 for 24 h were used for all experiments.

## **PAH patient tissues and PSMCs**

Lung sections and PSMCs of unused donor and IPAH were provided by the pulmonary hypertension breakthrough initiative (PHBI). Funding for the PHBI is provided under an NHLBI R24 grant, #R24HL123767, and by the Cardiovascular Medical Research and Education Fund (CMREF). The clinical information of these patients are shown in Supplemental Table E1. These patient cells are primary PSMCs from peripheral type-III pulmonary arteries (<1 mm in diameter). These cells have been authenticated by PHBI to be smooth muscle cells using FACS analysis and immunofluorescence staining for smooth muscle actin and SMMHC. The cells were cultured and passaged in the same way as the Lonza PSMCs. Third-to-five passage cells equilibrated in growth factor- and serum-free SmGM-2 for 24 h were used for all experiments.

## **Western blot analysis**

The lysates from lung tissue or PSMCs were centrifuged at 12,000 rpm for 10 min to collect the supernatant, then mixed with Laemmli sample buffer. Samples (15–20 µg protein) were boiled for 5 min and separated by SDS-PAGE using polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membranes. The membranes were incubated in blocking solution (5% BSA) for 1 h at room temperature, and then hybridized with primary antibodies against PFKFB3 (1:5000), calpain-1 (1:5000), calpain-2 (1:5000), collagen-I (1:1000), calpastatin (1:1000), p-ERK1/2 (1:2000), total ERK1/2 (1:1000), PCNA (1:1000) and GAPDH (1:10000) overnight at 4°C. The protein bands were visualized by chemiluminescence method and quantified by densitometry using Quantity One 1-D Analysis Software.

## **Lactate measurements**

The levels of intracellular and secreted lactate were determined using a lactate assay kit (Sigma) according to the manufacturer's instructions. Data were normalized to mg protein.

## **Calpain activity assay**

Calpain activity in PSMC was measured using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC as a substrate with a SpectraMax M2e microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) as described previously. Briefly, cells were cultured and treated in 24-well plates, after which were washed with PBS, and the fluorogenic Suc-Leu-Leu-Val-Tyr-AMC peptide substrate was added to a final concentration of 80 µM in the presence and absence of MDL28170. Fluorescence was immediately recorded at 2-min intervals for 20 min at excitation 360 nm and emission 460 nm. Calpain activity was calculated as fluorescence units in the absence of MDL28170 subtracted by those in the presence of MDL28170.

## **Cell proliferation assay**

PSMCs were cultured in 96 well plates for 24 h and serum-starved for 12-16 h. Proliferation of PSMCs was determined using a colorimetric BrdU ELISA kit (Cell Signaling Technology, Danvers, MA) that monitors the BrdU incorporation into cellular DNA during cell proliferation. Incorporated BrdU was detected with an anti-BrdU–peroxidase conjugate antibody according to the manufacturer's instructions. Absorbance at 450 nm was measured by using a Spectra-Max M2e microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

## **In vitro cell proliferation and apoptosis measurements.**

After treatment of the PSMCs, cells were fixed with 4% paraformaldehyde at room temperature for 10 min. PSMC proliferation was measured by Ki67 staining and apoptosis was measured by TUNEL assay according to the manufacturer's instruction. The percentage of the Ki67- or TUNEL-positive cells were calculated as total number of positive cells divided by the

total number of nucleus (DAPI). At least 400-500 cells were counted in 5-7 fields of view per subject. All experiments were done for at least in triplicate.

### **Adenoviral transduction of PSMCs**

The GFP-labeled-PFKFB3 shRNA adenovirus targeting the 3' UTR sequence of human PFKFB3 and the control adenovirus were constructed by Vector Biolabs. These adenoviruses were expanded inside HEK293 cells, and the virus concentration was determined using an Adeno-XTM rapid titer kit (Clontech) as described previously (20). PSMCs at 70-80% confluence were transduced with the adenovirus (10 pfu/cell) and were used for experiments 36 h after the transduction.

### **Determination of changes in calpain-2 phosphorylation status**

After the treatments, PSMCs were washed with ice cold PBS buffer and lysed in RIPA buffer containing phosphatase and protease inhibitor cocktail. The cell lysates were centrifuged at 12,000 rpm for 15 min. The clear lysates (~ 500-700 µg total proteins) were incubated with phospho-specific antibodies against P-Ser50 or P-Ser369 of calpain-2 antibodies (5 µg/IP reaction) for 4-5 h, respectively, after which 30 µl of Dynabeads Protein G were added and samples were further incubated overnight at 4 °C with gentle rotation. Immunoprecipitates were pulled down by a magnet and washed three times with RIPA buffer. The precipitated proteins were eluted by boiling for 10 min in Laemmli sample buffer and then analyzed by Western blot using antibody directed against calpain-2.

### **RNA interference in PSMCs**

The expressions of calpain-1, calpain-2 and ERK1/2 were silenced using siRNA technology. Calpain-1 siRNA (target sequence: 5'-AAGCTAGTGTTCGTGCACTCT-3'), calpain-2 siRNA (target sequence: 5'-CTGGAACACTATAGACCCAGA-3') and the non-targeting siRNA (target sequence: 5'- AACGTACGCGGAATACTTCGA-3') were synthesized by Qiagen. The sequences of ERK1/2 siRNAs were not disclosed by the Cell Signaling Technologies. The siRNAs were transfected into PSMCs with siPORT amine transfection reagent according to the manufacturer's instruction. 3 days after transfection, the medium was changed to serum-free medium for 24 h followed by the treatments of PSMCs.

### **Study approval**

All animal experiments were performed in accordance with the guiding principles of the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Augusta University. In the studies of human lung tissues and PSMCs, patient identifiers were concealed. Waiver of informed consent was approved by the IRB of Augusta University and by PHBI.

### **Statistical analysis**

Within each experiment, cells were matched for number of passages to avoid differences related to tissue culture variables. Results are shown as means ± SE for n experiments. One way ANOVA and t test analysis (2-tailed) were used to determine the significance of differences between the means of experimental and control groups. A value of  $P < 0.05$  was considered significant.

Supplemental Table E1. Clinical information of PAH patients and controls

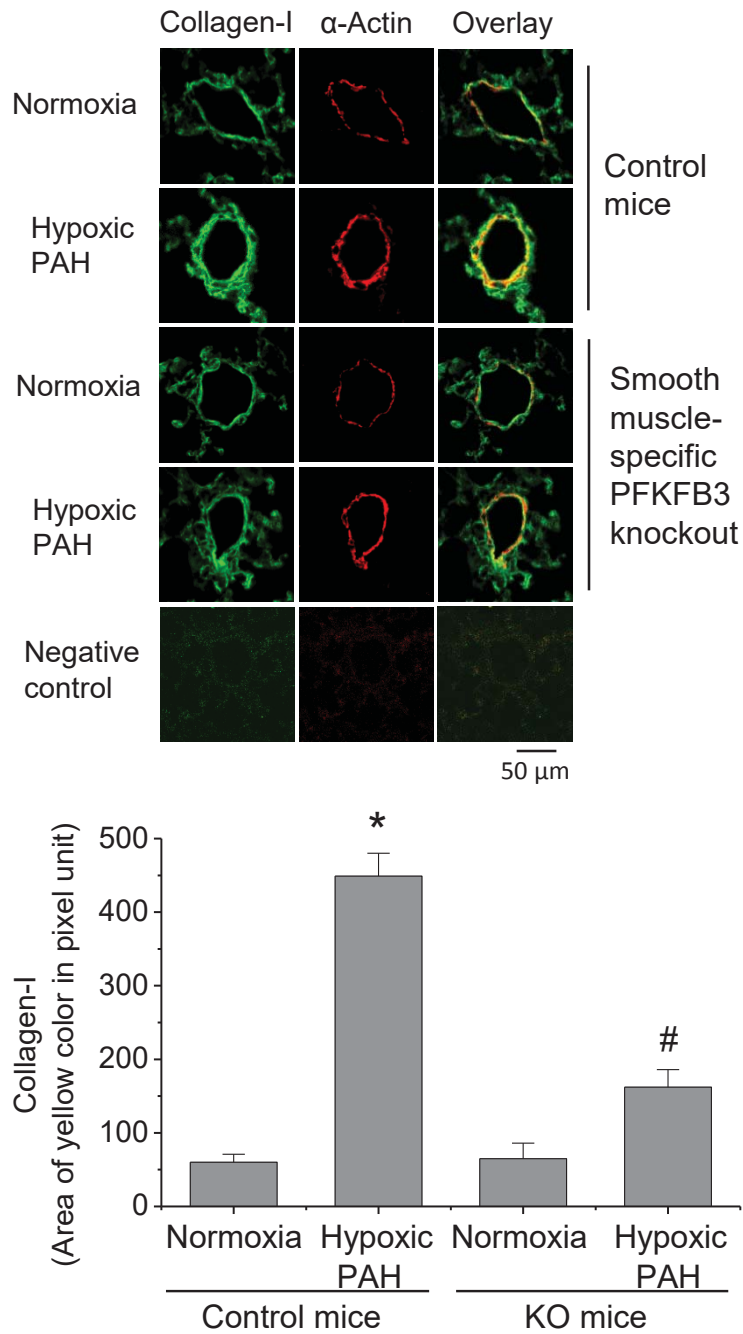
	Sex	Age (y)	PAP (s/d/m)	PVR (WU)	PAH medications
IPAH-1	Female	27	110/49/69	23.27	Ilopros, treprostiniil
IPAH-2	Male	51	73/36/48	27.04	Sildenafil, epoprostenol
IPAH-3	Male	25	94/42/59	17.8	Treprostiniil, sildenafil
IPAH-4	Male	7	85/40/55	21.5	Sildenafil, treprostiniil
IPAH-5	Male	40	89/46/60	16.77	Sildenafil, iloprost
IPAH-6	female	16	68/38/48	27.12	Tadalafil, epoprostenol

	Sex	Age (y)	Cause of death
Normal-1	Female	57	Stroke
Normal-2	Female	64	Blunt injury to head
Normal-3	Female	28	Intercranial hemorrhage
Normal-4	Male	62	Intercranial hemorrhage
Normal-5	Male	47	Car accident-head trauma
Normal-6	Male	52	Cerebrovasclar-Stroke

	Cell type	Sex	Age (y)	mPAP (s/d/m)	PVR (WU)	PAH medications
IPAH cell-1	SMCs from type-III PA	Female	56	83/39/57	23.27	Treprostiniil, sildenafil
IPAH cell-2	SMCs from type-III PA	Male	56	100/45/65	11.94	Bosentan, tadalafil
IPAH cell-3	SMCs from type-III PA	Male	25	94/42/59	17.8	Treprostiniil, sildenafil
IPAH cell-4	SMCs from type-III PA	Male	40	89/46/60	16.77	Sildenafil, iloprost
IPAH cell-5	SMCs from type-III PA	Female	32	68/38/48	27.12	Tadalafil, epoprostenol
IPAH cell-6	SMCs from type-III PA	Female	46	60/38/45	12.94	Epoprostenol, bosentan

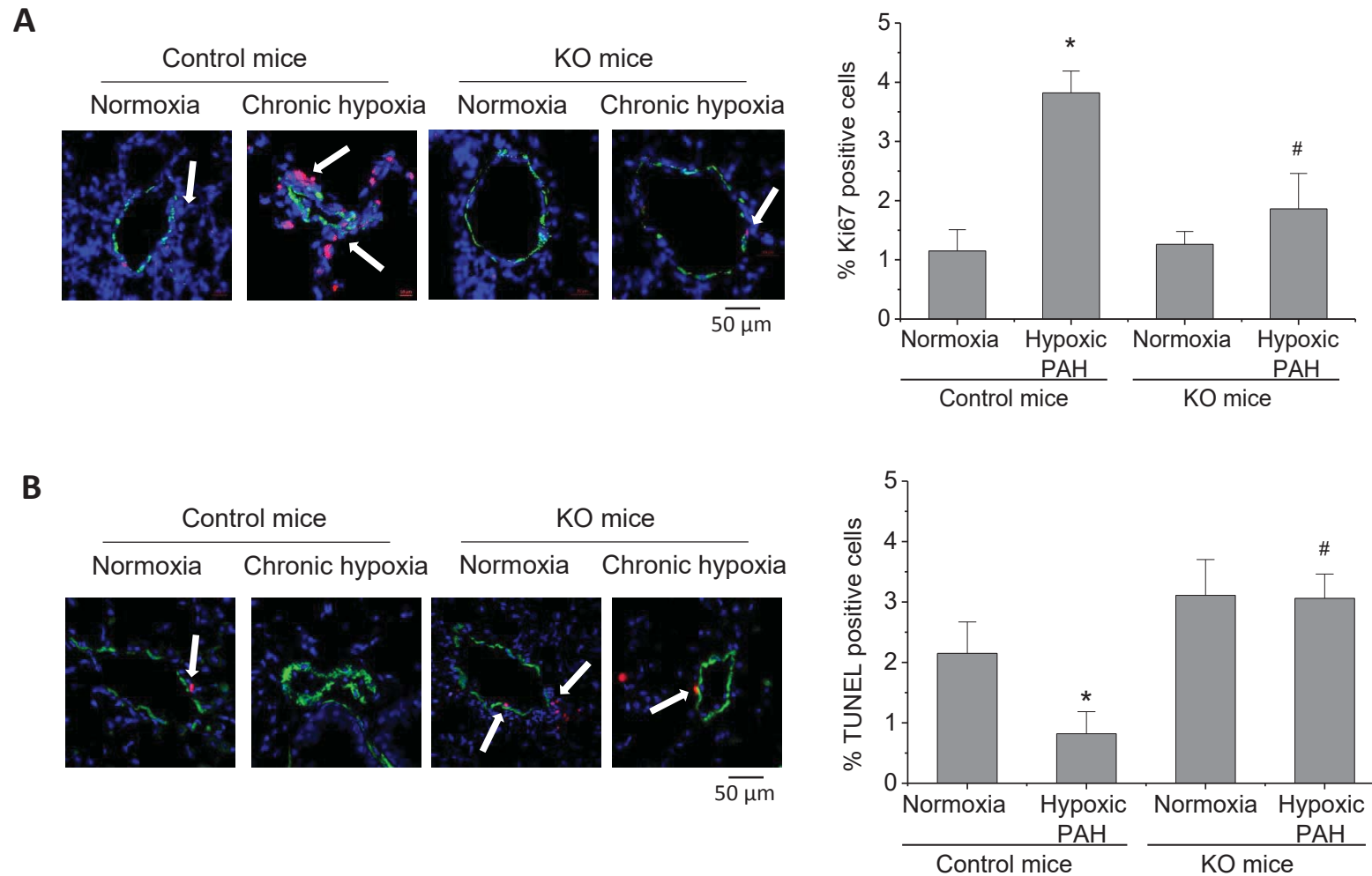
	Cell type	Sex	Age (y)	Cause of death
Normal cell-1	SMCs from type-III PA	Female	20	Seizure disorder
Normal cell-2	SMCs from type-III PA	Female	36	Subarachnoid hemorrhage
Normal cell-3	SMCs from type-III PA	Male	26	Gunshot wound to the head
Normal cell-4	SMCs from type-III PA	Male	11	Anoxia-hanging
Normal cell-5	SMCs from type-III PA	Male	25	Car accident-head trauma
Normal cell-6	SMCs from type-III PA	Female	60	Head trauma-intracranial hemorrhage

## SUPPLEMENTAL FIGURE AND FIGURE LEGENDS

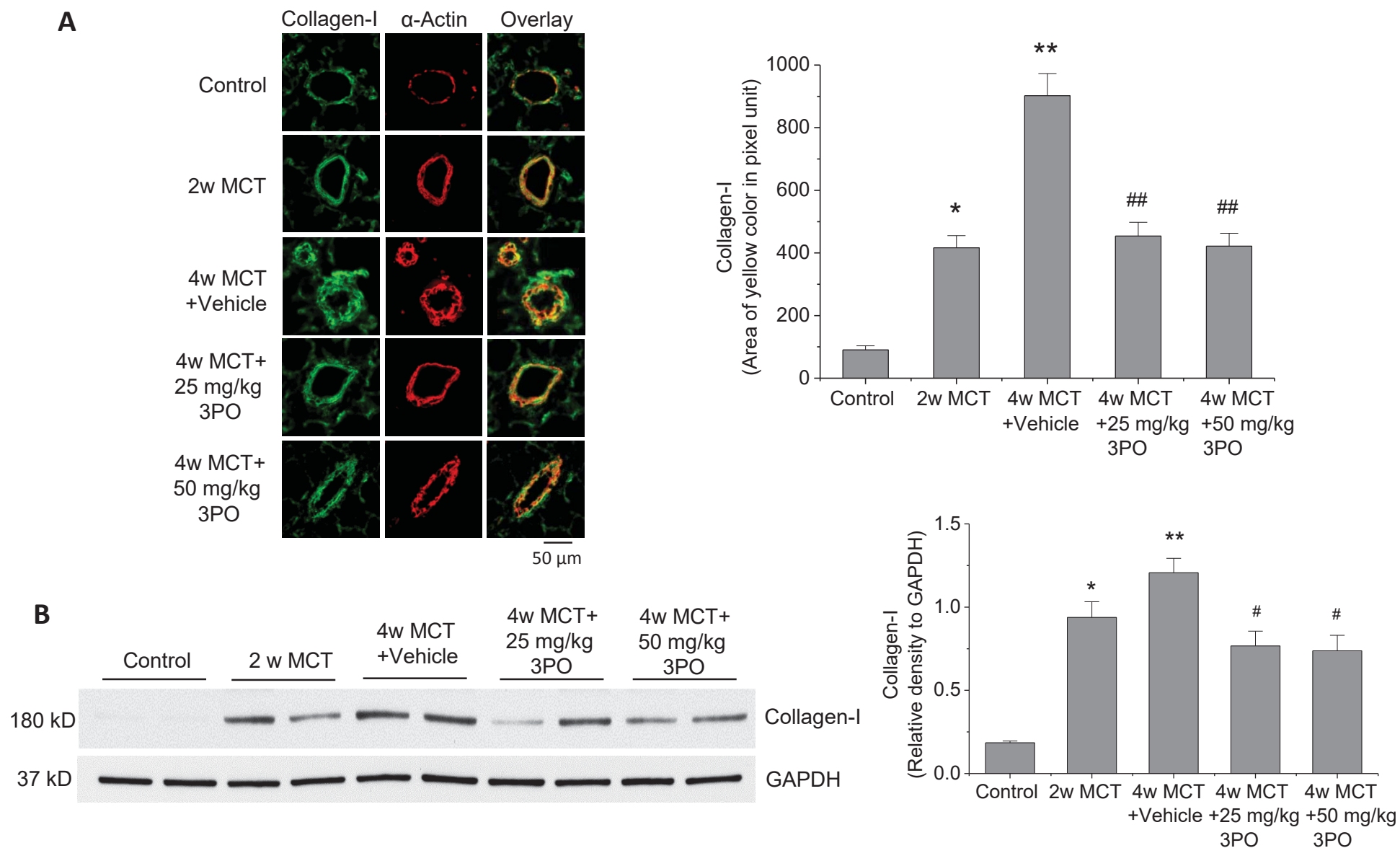


**Figure E1. Effect of smooth muscle-specific knockout of PFKFB3 on collagen-I in the smooth muscle of pulmonary arterioles of mice with hypoxic PAH.** 5 days after regimen of tamoxifen administration, control mice and PFKFB3<sup>flx/flx</sup>;SMMHC-CreER mice were exposed to room air (normoxia) or 10% oxygen (chronic hypoxia) for 4 weeks. Lung slides from PFKFB3<sup>flx/flx</sup>;SMMHC-CreER and control mice exposed to normoxia or chronic hypoxia were double-stained for  $\alpha$ -actin (red) and collagen-I (green). The images are representative images of 6 independent experiments. Results are expressed as mean  $\pm$  SE; n = 6. \*  $P < 0.01$  vs. normoxia; #  $P < 0.01$  vs. hypoxic PAH of control mice.

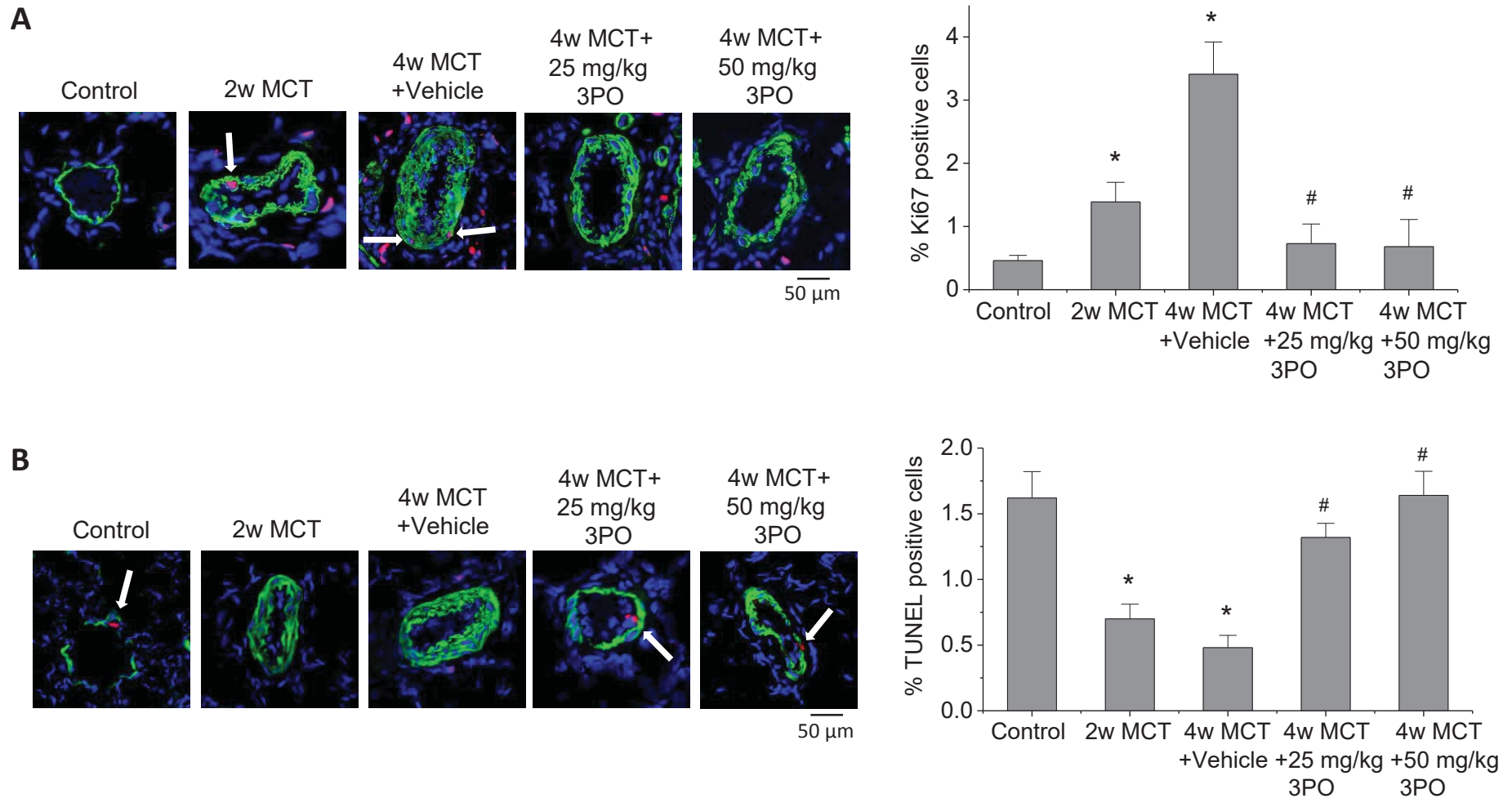




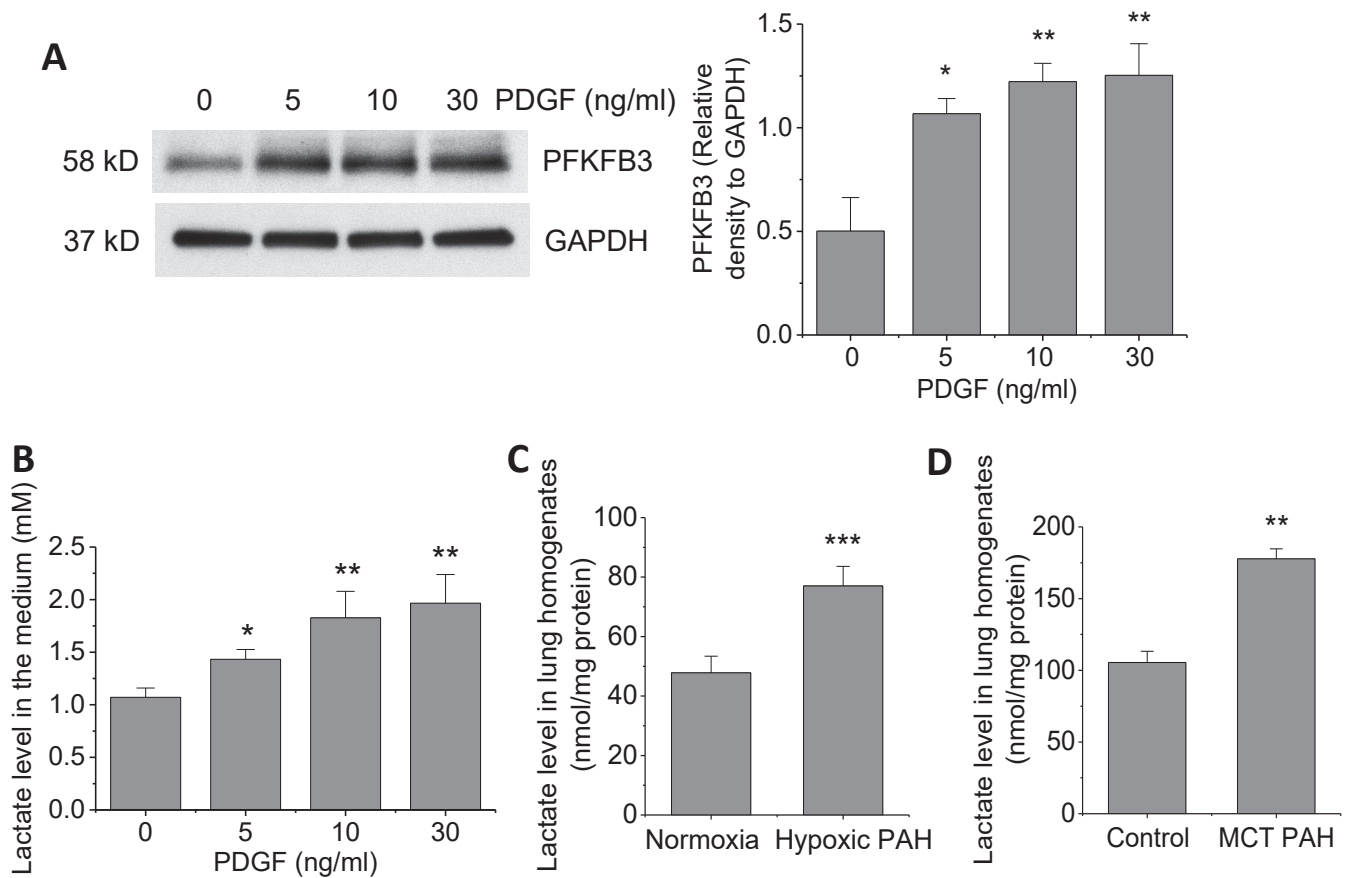
**Figure E2. Smooth muscle-specific knockout of PFKFB3 prevents PASMC proliferation (A) and induces PASMC apoptosis (B) in pulmonary arteries in hypoxic PAH.** 5 days after regimen of tamoxifen administration, control mice and PFKFB3<sup>flx/flx</sup>;SMMHC-CreER mice were exposed to room air (normoxia) or 10% oxygen (Chronic hypoxia) for 4 weeks. Lung slides from PFKFB3<sup>flx/flx</sup>;SMMHC-CreER and control mice exposed to normoxia or chronic hypoxia were stained for  $\alpha$ -actin (green), Ki67 (red) or TUNEL (red) and DAPI (blue). Arrows indicate positive cells. Results are expressed as mean  $\pm$  SE; n = 6. \*  $P < 0.05$  vs. normoxia; #  $P < 0.05$  vs. hypoxic PAH of control mice.



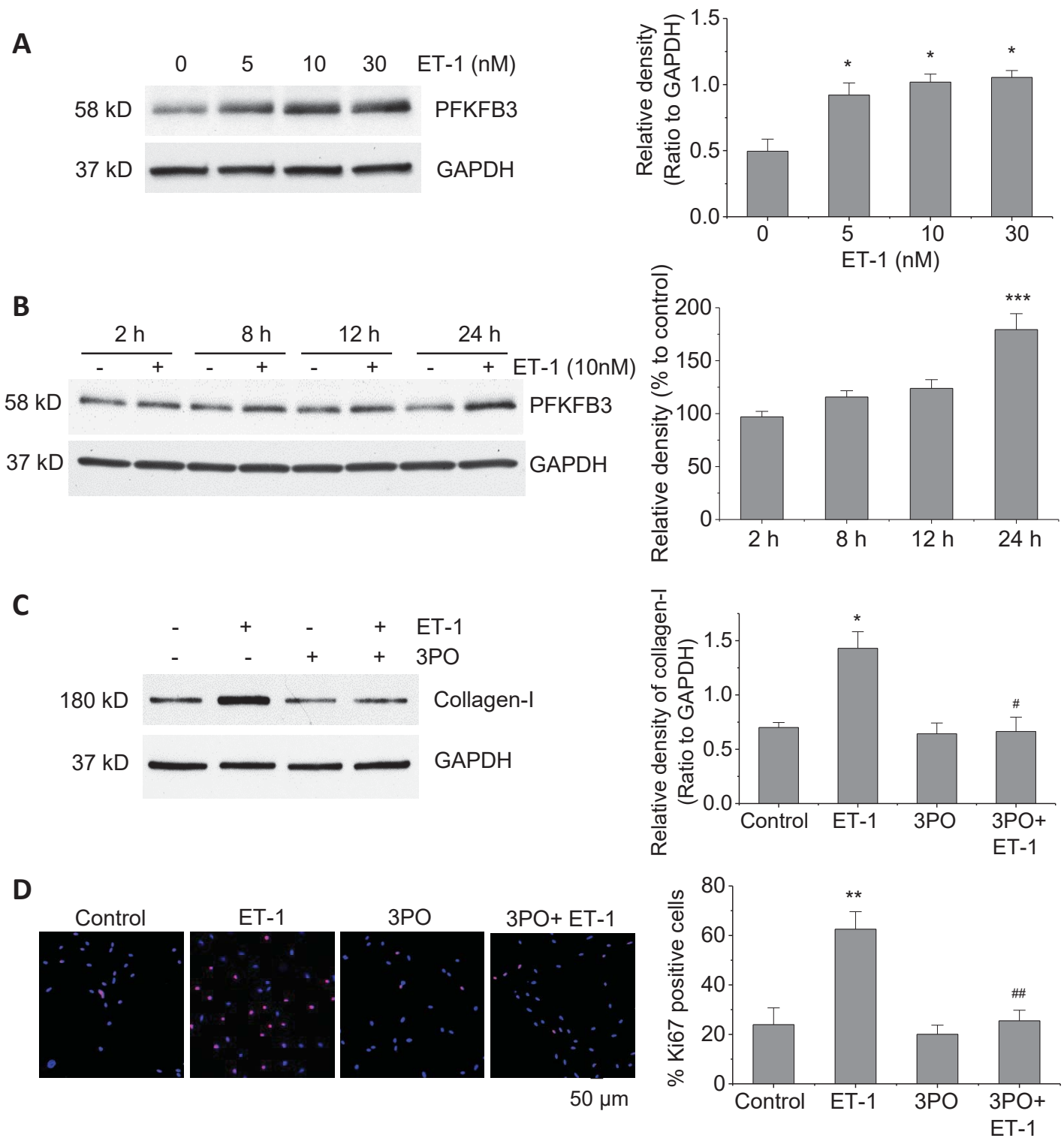
**Figure E3. Inhibition of PFKFB3 reduces increases in collagen-I protein levels in the MCT PAH rat model.** (A): Lung slides were double-stained for  $\alpha$ -actin (red) and collagen-I (green). The images are representatives of 5 independent experiments. (B): Protein levels of collagen-I in lung homogenates of MCT PAH rat model were measured by using Western blot. The blots are representative immunoblots of 5 independent experiments. Results are expressed as mean  $\pm$  SE; n = 5. \* $P$  < 0.001, \*\* $P$  < 0.0001 vs control; # $P$  < 0.05, ## $P$  < 0.01 vs 4wk MCT+Vehicle.



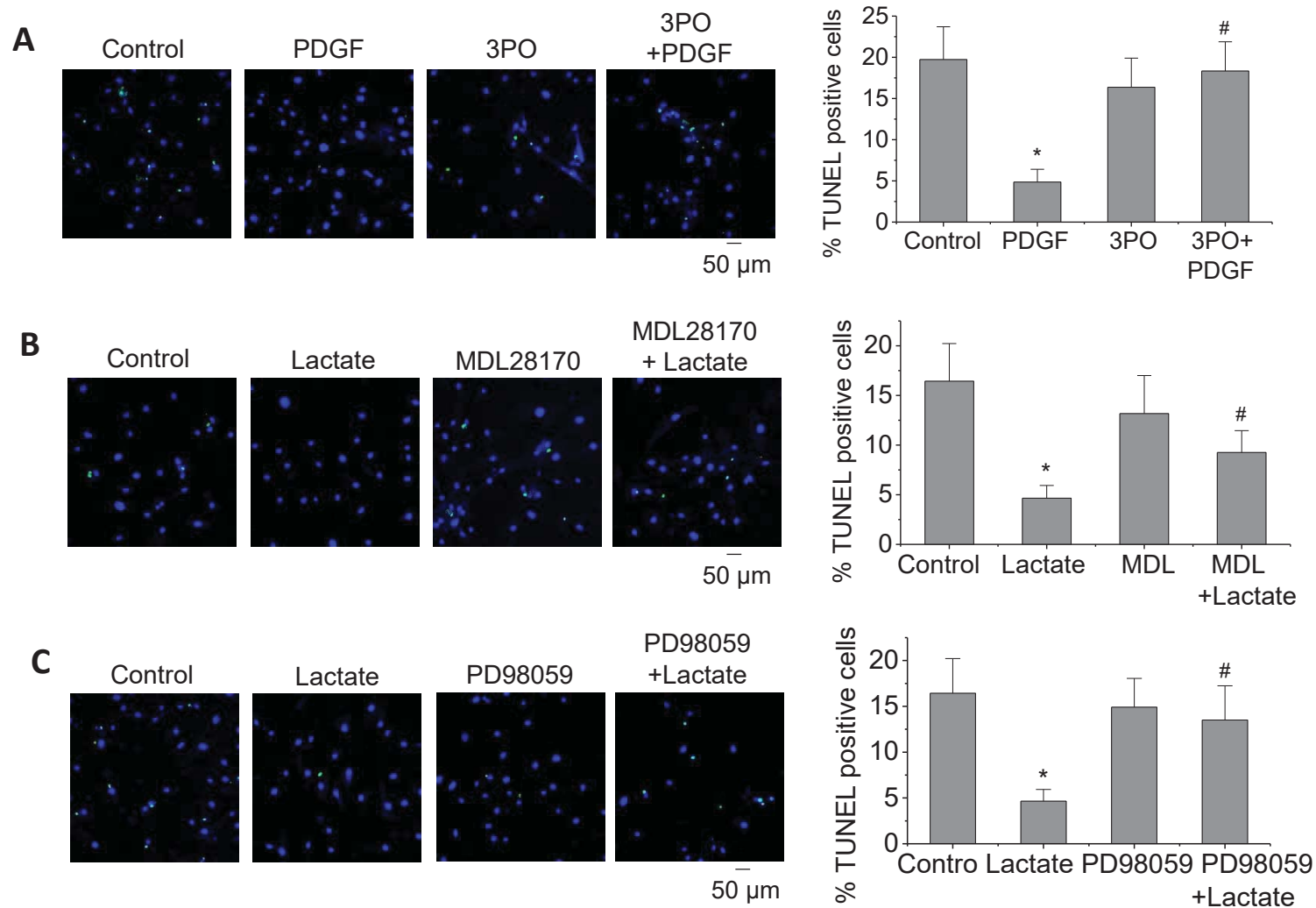
**Figure E4. Inhibition of PFKFB3 using 3PO inhibits PASMC proliferation (A) and induces PASMC apoptosis (B) in pulmonary arteries in MCT PAH rats.** Lung slides from control and MCT rats receiving vehicle or 3PO were stained for  $\alpha$ -actin (green), Ki67 (red) or TUNEL (red) and DAPI (blue). Arrows indicate positive cells. Results are expressed as mean  $\pm$  SE;  $n = 5$ . \* $P < 0.05$  vs Control; # $P < 0.05$  vs 4w MCT+Vehicle.



**Figure E5. PDGF increases protein levels of PFKFB3 and lactate production in PSMCs, and higher lactate levels in lungs of PAH rodents.** (A and B): PSMCs were incubated with PDGF-BB (10-30 ng/ml) for 24 h, then protein levels of PFKFB3 (A) and lactate concentration in culture medium (B) were measured. (C and D): Lactate levels in lung homogenates of hypoxic PAH mice and MCT PAH rats were measured. Results are expressed as mean  $\pm$  SE; n = 4. \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. control (or 0), \*\*\*  $P < 0.05$  vs. normoxia.

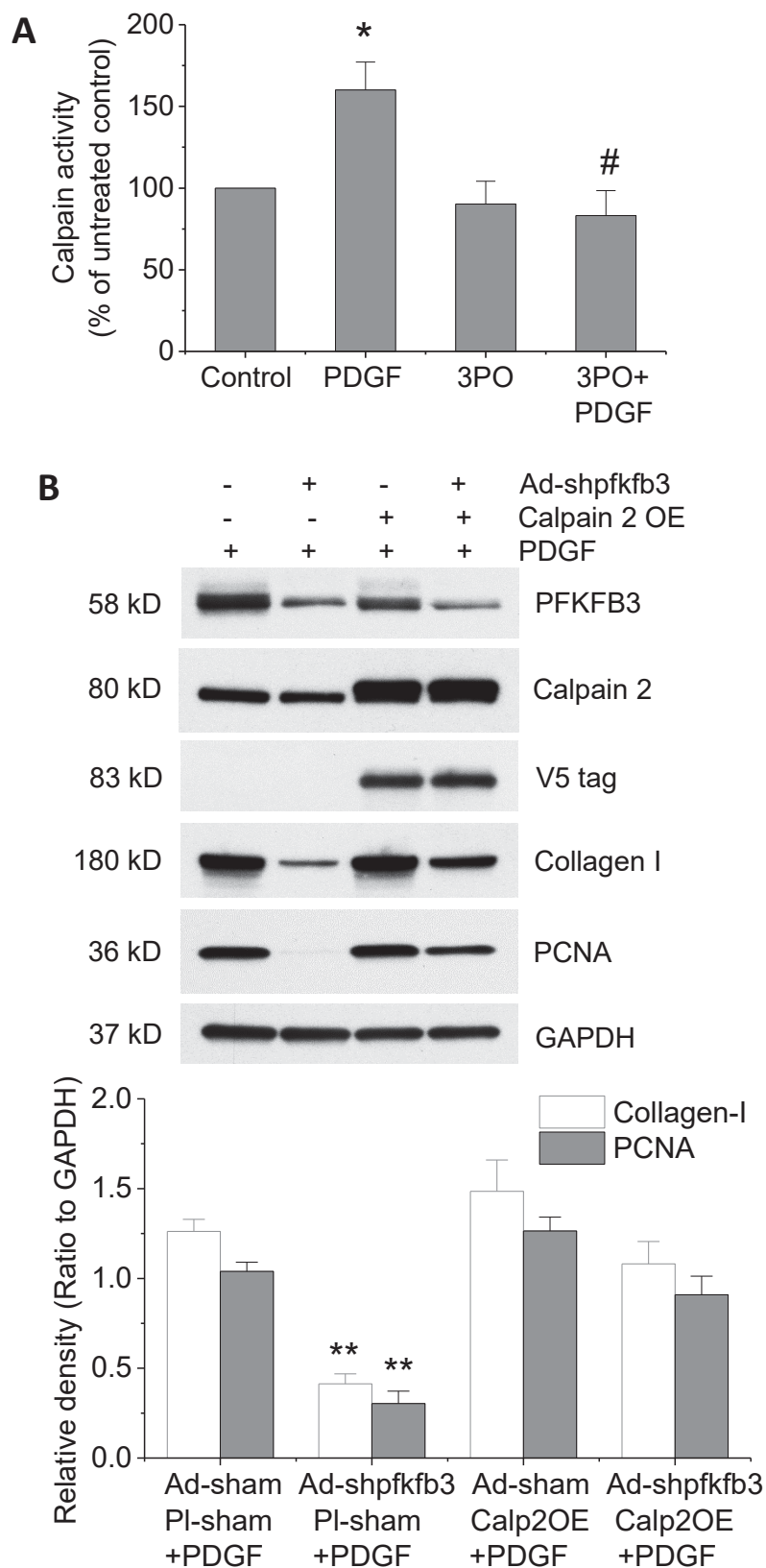


**Figure E6. ET-1 increases PFKFB3 protein levels, and inhibition of PFKFB3 attenuates ET-1-induced collagen synthesis and proliferation of PSMCs.** (A and B): PSMCs were incubated with ET-1 (0-30 nM) for 24 h (A), and with ET-1 (10 nM) for 2-24 h (B), then protein levels of PFKFB3 were measured. (C and D): PSMCs were incubated with ET-1 (10 nM) in the presence and absence of PFKFB3 inhibitor 3PO (10  $\mu$ M) for 24 h, after which collagen-I protein levels (C) and cell proliferation by Ki67 labeling (D) were measured. Results are expressed as mean  $\pm$  SE;  $n = 4$ . \*  $P < 0.05$ , \*\*  $P < 0.01$  vs. control. \*\*\*  $P < 0.05$  vs. 2 h; #  $P < 0.05$ , ##  $P < 0.01$  vs. ET-1.

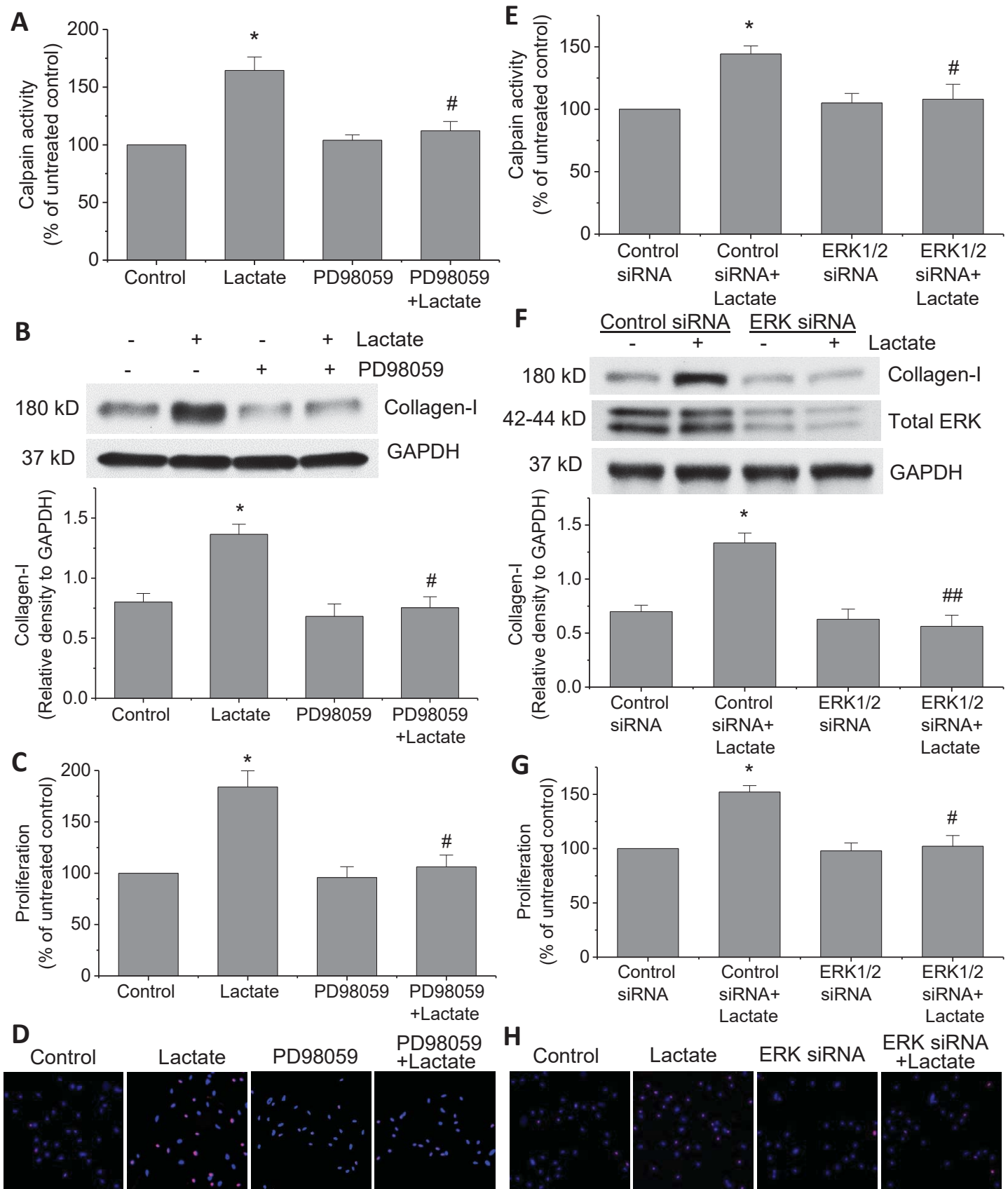


**Figure E7. Inhibition of PFKFB3-ERK1/2-calpain signaling reduces the PDGF- and lactate-induced decreases in apoptosis of PSMCs.** PSMCs were incubated with PDGF (10 ng/ml) or lactate (10 mM) in the presence and absence of PFKFB3 inhibitor 3PO (10  $\mu$ M) (A) or calpain inhibitor MDL28170 (20  $\mu$ M) (B) or ERK1/2 inhibitor PD98059 (10  $\mu$ M) (C) for 24 h, then apoptosis was measured by TUNEL assay. Representative images shown labeling of apoptotic TUNEL-positive cells (green) and DAPI (blue). Results are expressed as mean  $\pm$  SE; n = 4. \*  $P < 0.05$  vs. control. #  $P < 0.05$  vs. PDGF or lactate.



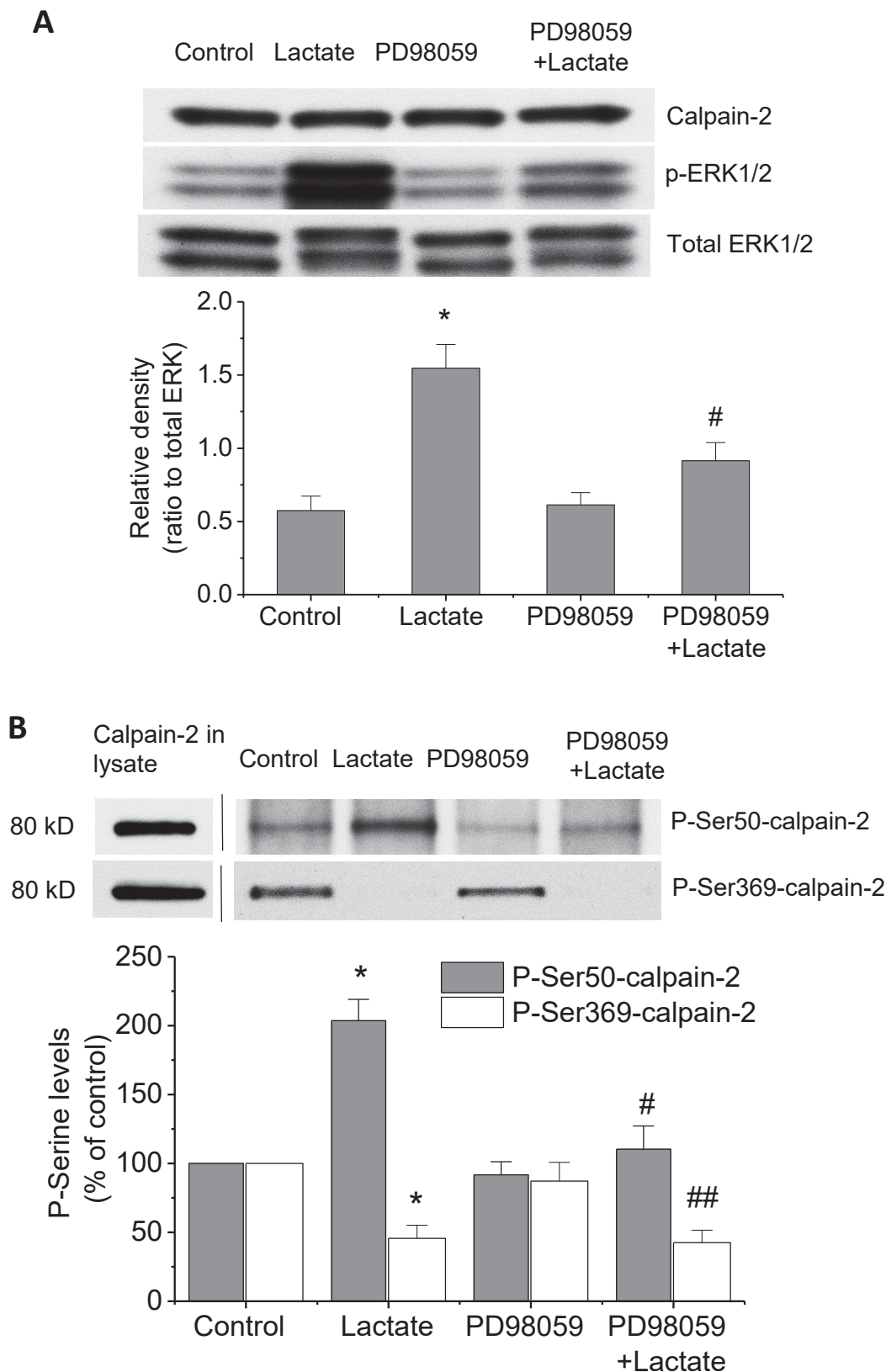


**Figure E8. inhibition of PFKFB3 attenuates PDGF-induced calpain activation in PSMCs and overexpression of calpain-2 restores the PDGF-induced collagen synthesis and cell proliferation in PFKFB3-deficient PSMCs.** (A): PSMCs were incubated with PDGF-BB (10 ng/ml) in the presence and absence of PFKFB3 inhibitor 3PO (10  $\mu\text{M}$ ) for 10 min, after which calpain activity was measured. Results are expressed as mean  $\pm$  SE;  $n = 6$ . \*  $P < 0.01$  vs. control; #  $P < 0.01$  vs. PDGF. (B): PSMCs at 70-80% confluence were transduced with the control and GFP-labeled-PFKFB3 shRNA adenovirus (10 pfu) and empty sham adenovirus (10 pfu) and incubated for 48 h, then cells were transfected with sham and pcDNA3.1/V5-His-human calpain-2 wild type plasmids. After additional 48 h, the cells were incubated with PDGF-BB (10 ng/ml) for 24 h and then protein levels of PFKFB3, calpain-2, V5-tag, collagen-I and PCNA were measured. Results are expressed as mean  $\pm$  SE;  $n = 4$ . \*\*  $P < 0.01$  vs. PDGF.

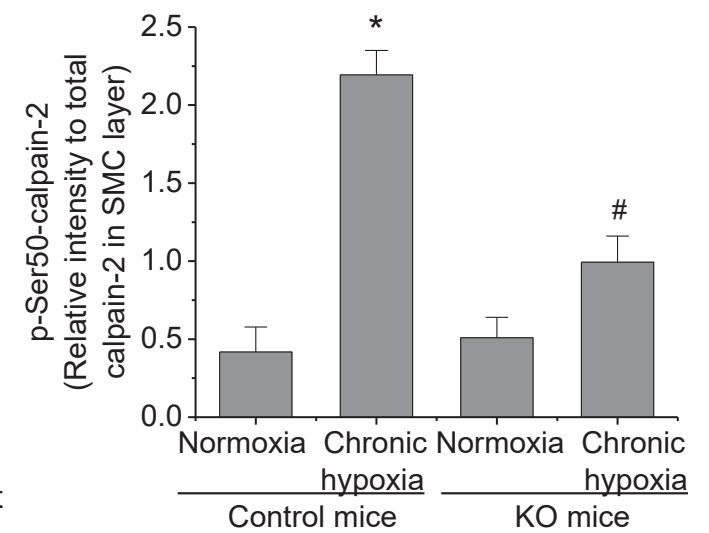
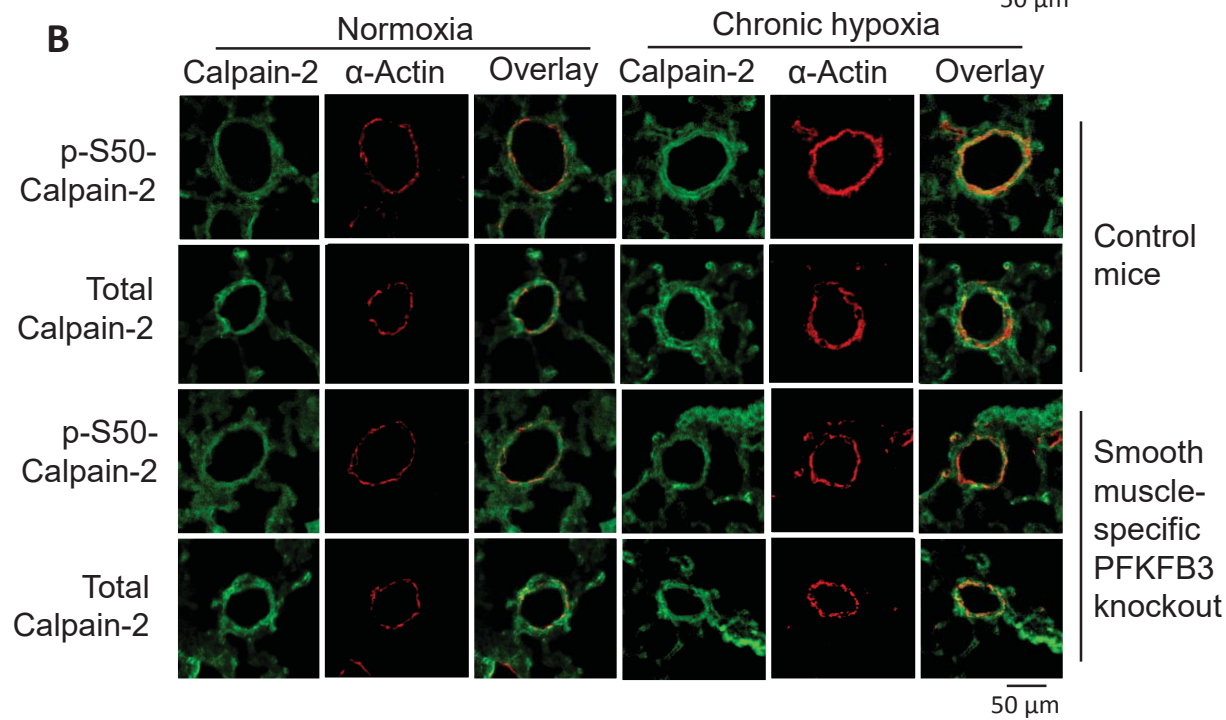
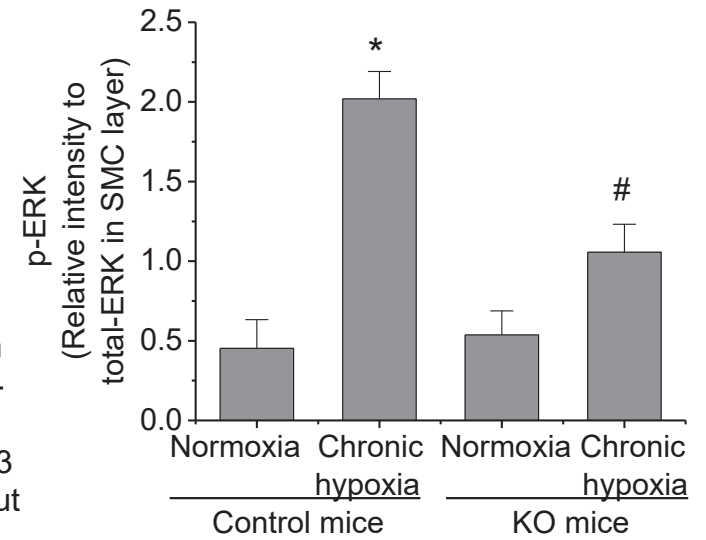
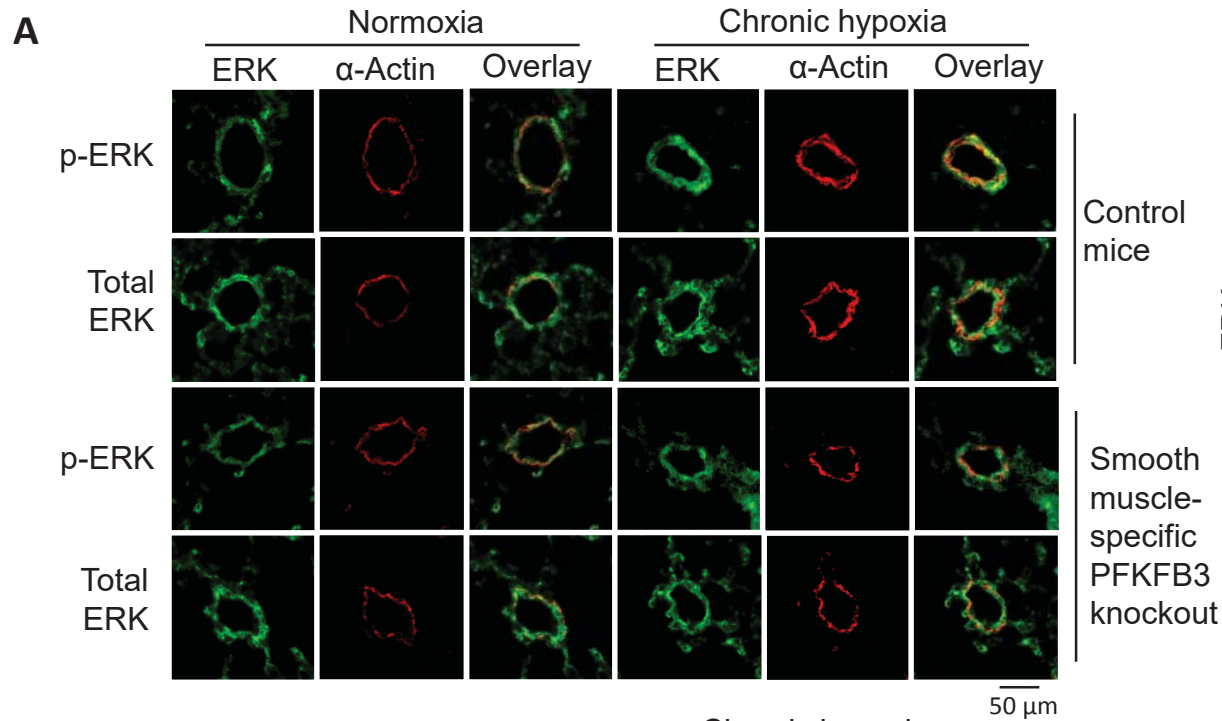


**Figure E9. Inhibition of ERK1/2 prevents the lactate-induced calpain activation, collagen synthesis and proliferation of PSMCs.** (A, B, C and D): PSMCs were incubated with sodium lactate (10 mM) in the presence and absence of ERK1/2 inhibitor PD98059 (10  $\mu$ M) for 10 min to 24 h, after which calpain activity (A), collagen-I protein levels (B), and cell proliferation using BrdU assay (C) and Ki67 staining (D) were measured. (E, F, G and H): PSMCs were transfected with siRNAs against ERK1/2, or control siRNA, respectively. After 72 h, the cells were incubated with sodium lactate (10 mM), then calpain activity (E), protein levels of intracellular collagen-I and total ERK1/2 (F), and cell proliferation using BrdU assay (G) and Ki67 staining (H) were measured. Results are expressed as mean  $\pm$  SE; n = 4. \*  $P$  < 0.05 vs. control, #  $P$  < 0.05, ##  $P$  < 0.01 vs. lactate.

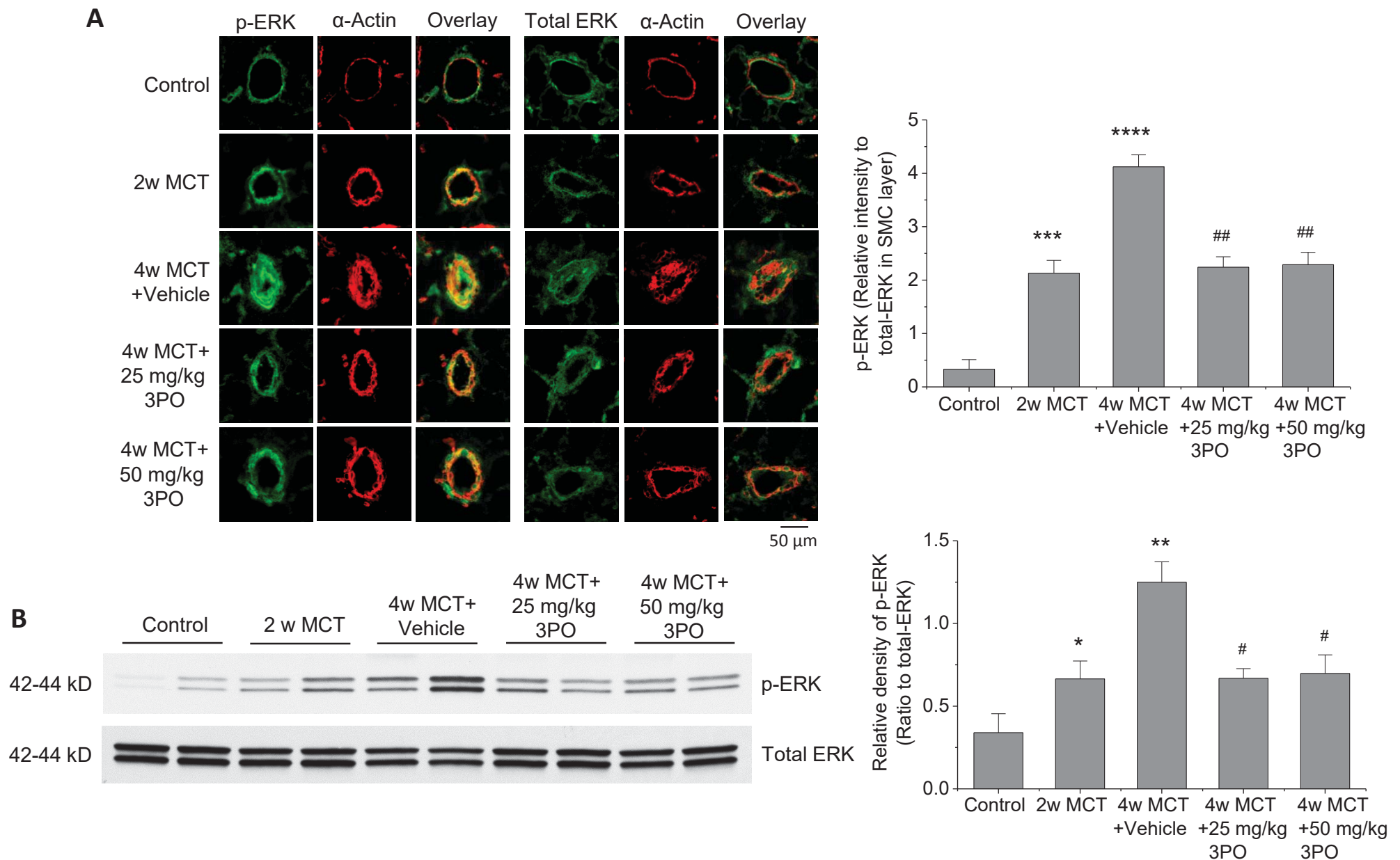




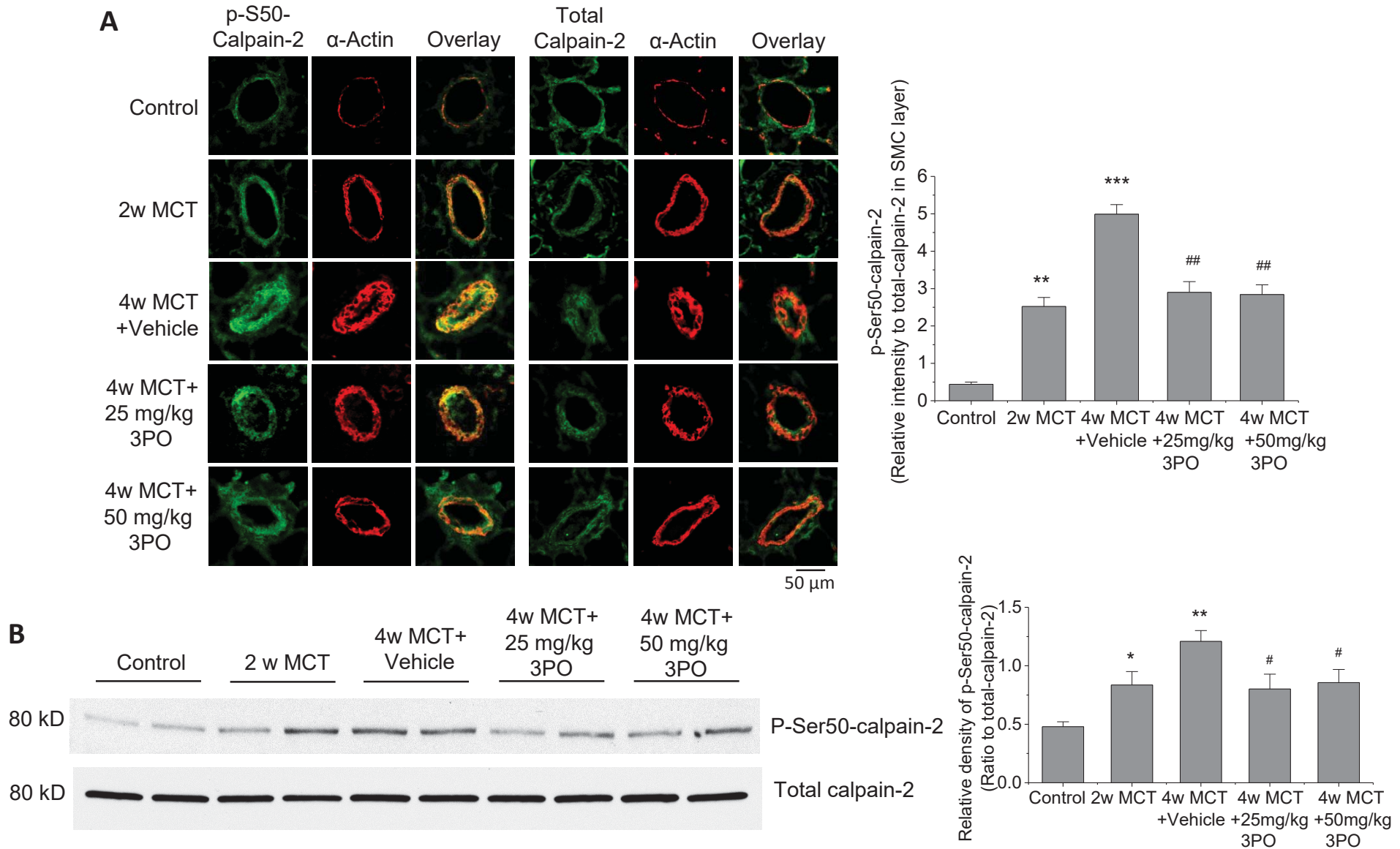
**Figure E10. Inhibition of ERK1/2 blocks the phosphorylation of calpain-2 at serine 50 (p-Ser50) in lactate-treated PSMCs.** PSMCs were incubated with sodium lactate (10 mM) in the presence and absence of the ERK1/2 inhibitor PD98059 (10  $\mu$ M) for 10 min after which cells were lysed and protein levels of calpain-2, p-ERK1/2 and total ERK1/2 were measured (A). Some cell lysates were immunoprecipitated using specific antibodies against P-Ser50-calpain-2 or P-Ser369-calpain-2. The immunoprecipitates were subjected to immunoblots against calpain-2 (B). Results are expressed as mean  $\pm$  SE; n = 3. \*  $P < 0.01$  vs. control; #  $P < 0.01$  vs. lactate; ##  $P < 0.001$  vs. PD98059 without lactate.



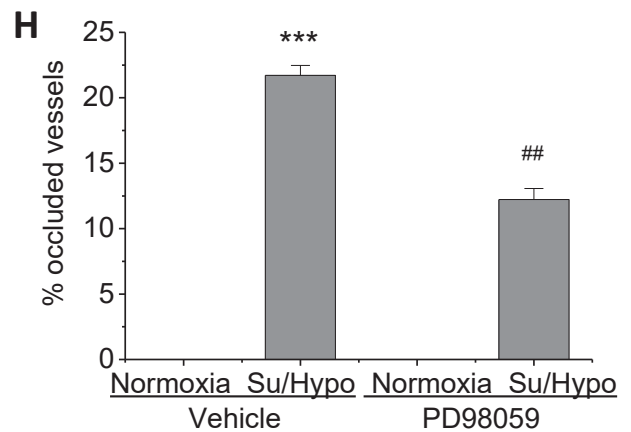
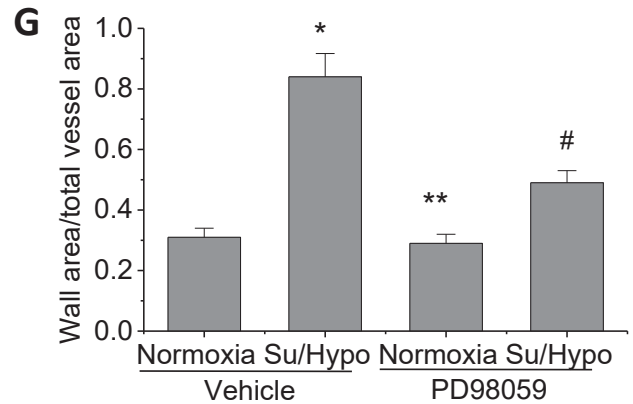
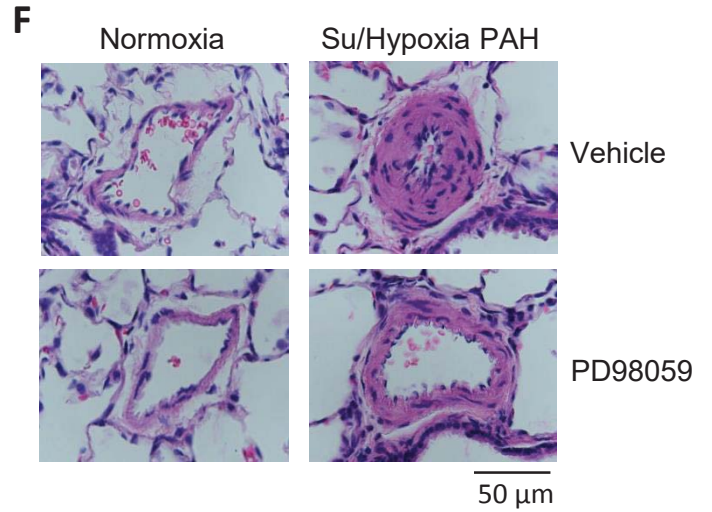
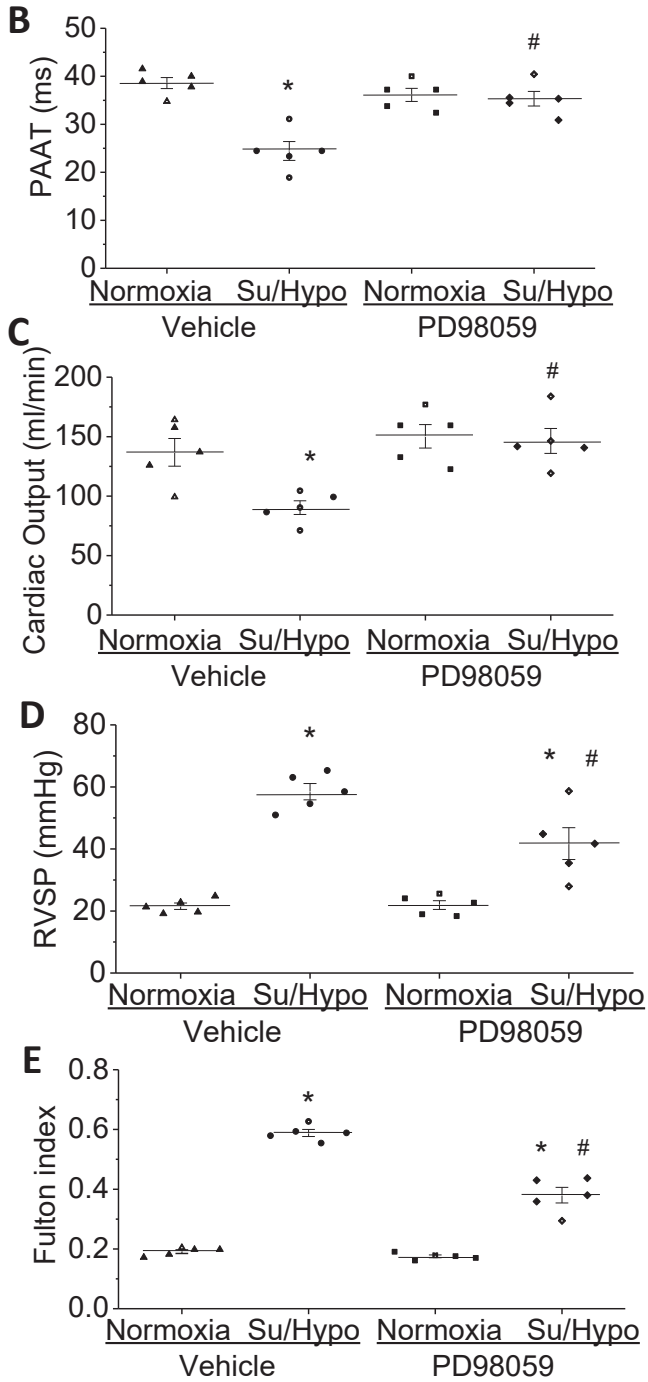
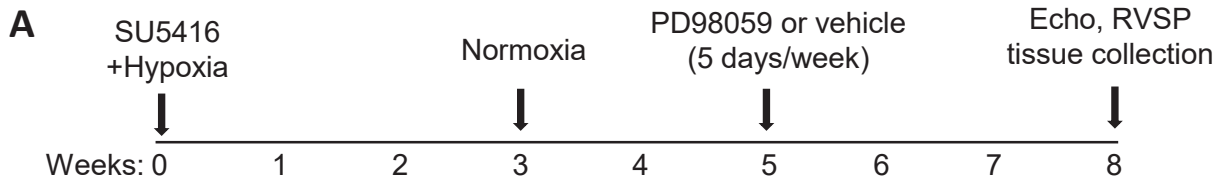
**Figure E11. SMC-specific knockout of PFKFB3 attenuates ERK1/2 phosphorylation and calpain-2 phosphorylation at Ser50 in chronic hypoxia-induced PAH.** 5 days after regimen of tamoxifen administration, control mice and PFKFB3<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>;SMMHC-CreER mice were exposed to room air (normoxia) or 10% oxygen (chronic hypoxia) for 4 weeks. Lung slides from PFKFB3<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>;SMMHC-CreER and control mice exposed to normoxia or chronic hypoxia were double-stained for  $\alpha$ -actin (red) and p-ERK1/2, total ERK1/2, p-S50-calpain-2 and total calpain-2 (green). Results are expressed as mean  $\pm$  SE; n = 6. \*  $P < 0.01$  vs. normoxia; #  $P < 0.01$  vs. chronic hypoxia of control mice.



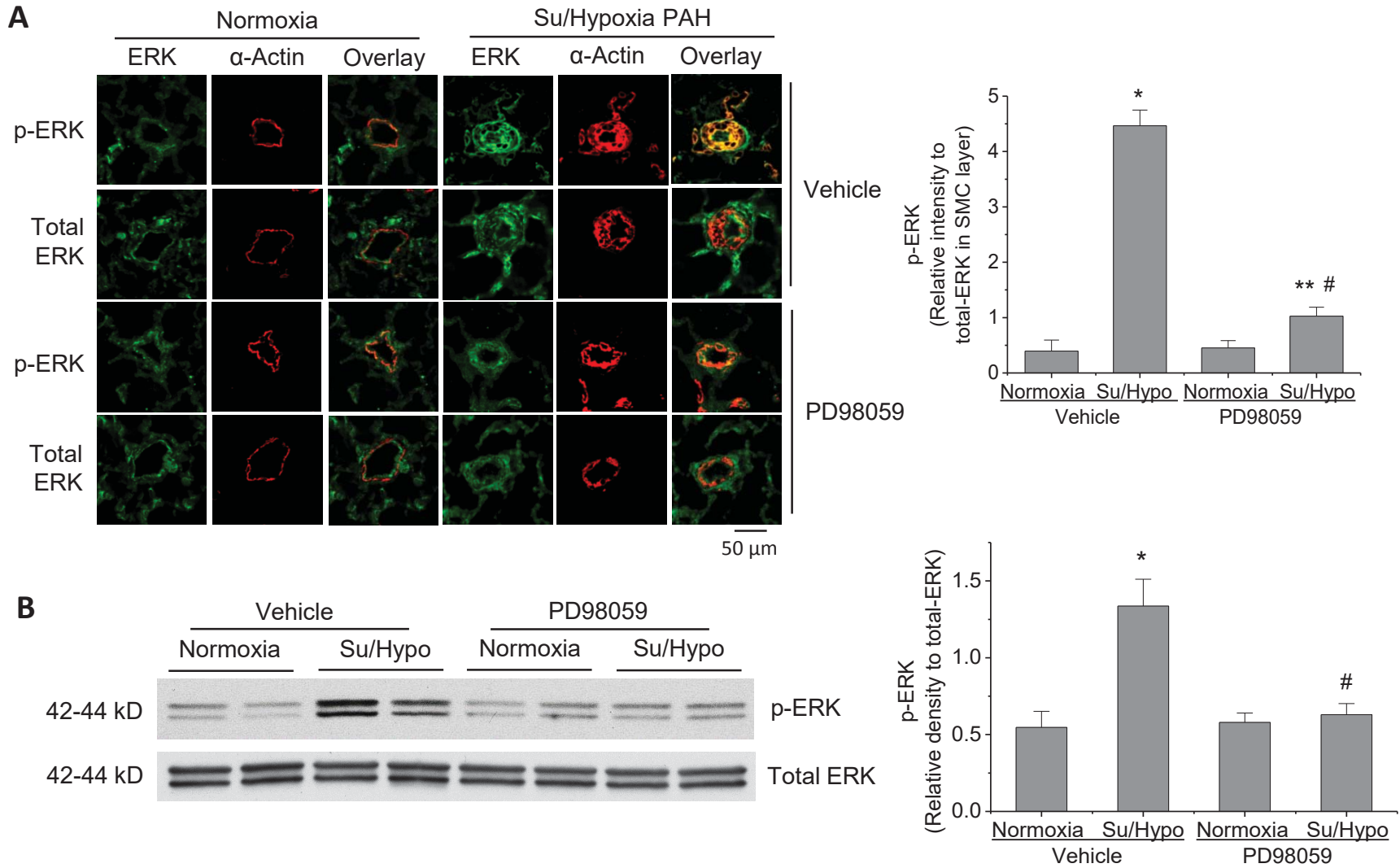
**Figure E12. Inhibition of PFKFB3 reduces ERK1/2 phosphorylation in the MCT PAH rat model.** (A): Lung slides were double-stained for  $\alpha$ -actin (red) and p-ERK1/2 (green) and total ERK1/2 (green). (B): Protein levels of p-ERK1/2 and total ERK1/2 in lung homogenates of MCT PAH rat model were measured by using Western blot. Results are expressed as mean  $\pm$  SE; n = 5. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001 vs control; # $P$  < 0.05, ## $P$  < 0.001 vs 4 w MCT+Vehicle.





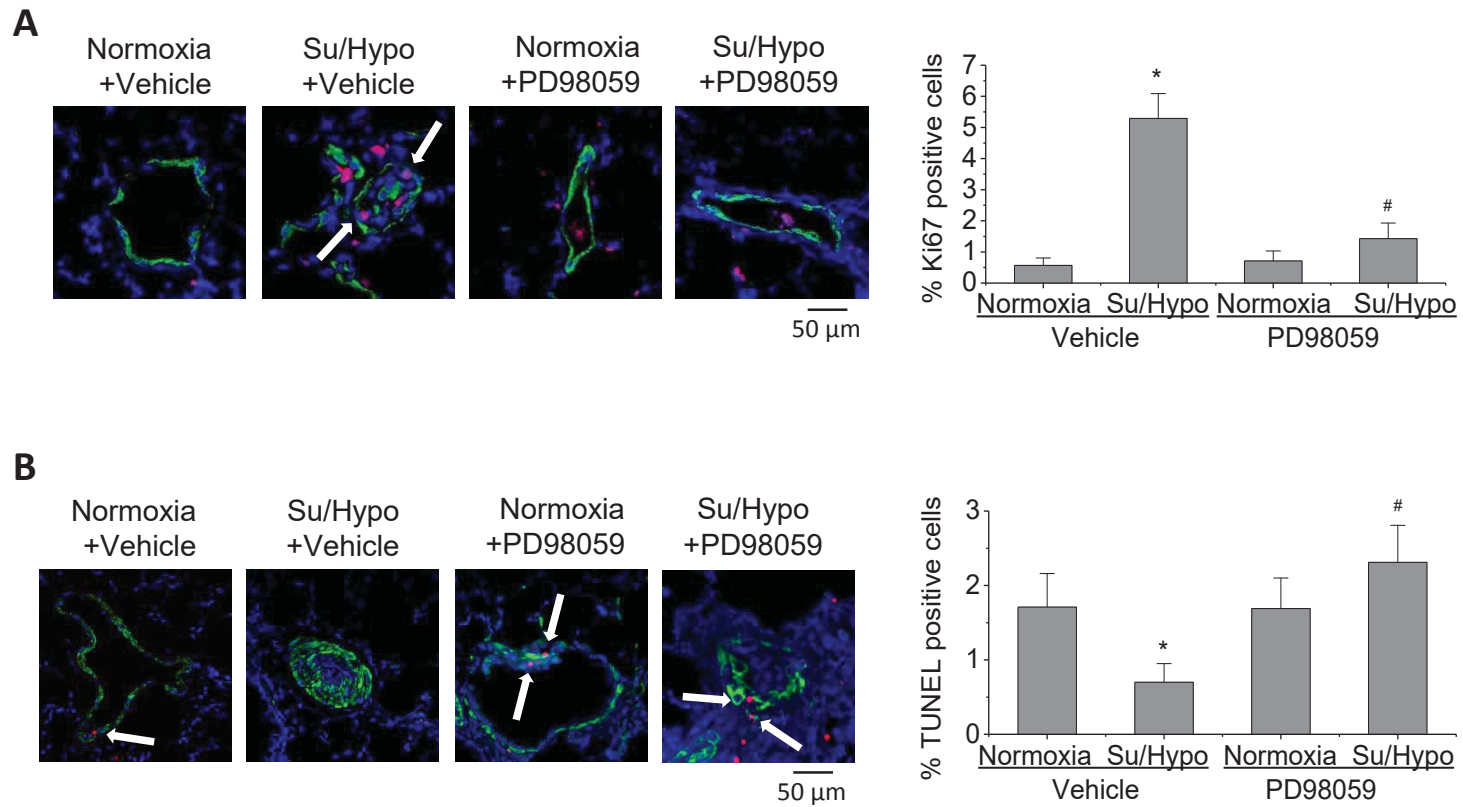


**Figure E14. Inhibition of ERK1/2 attenuates progression of pulmonary hypertension and pulmonary vascular remodeling in the Sugden/Hypoxia PAH rat model.** Male Sprague-Dawley rats 8 weeks of age were given single subcutaneous injection with SU5416 (20 mg/kg) and were exposed to hypoxia (10% O<sub>2</sub>) for 3 weeks and then housed for 2 weeks under normoxia (21% O<sub>2</sub>). Control age-matched male rats were maintained under normoxia. After that, groups of control rats (Normoxia+PD98059) and Sugden/Hypoxia-exposed rats (Su/Hypo+PD98059) began receiving PD98059 (10 mg/kg, i.p.) 5 days/week for 3 weeks. Second groups of control rats (Normoxia+Vehicle) and Su/Hypo-exposed rats (Su/Hypo+Vehicle) received the same volume of vehicle. Pulmonary hypertension and pulmonary vascular remodeling were assessed 3 week later (8 weeks after PAH induction). (A) is the protocol for time course of the experiment. (B and C): Echocardiography showing PAAT and CO. (D) shows the changes in RVSP. (E) shows the changes in Fulton index (RV/LV+S). (F) is representative images of lung sections of rats. (G) shows the changes in ratio of vascular wall area to total vessel area in the lung sections. (H) shows percentage of occluded vessels. Results are expressed as mean ± SE; n = 5. \**P* < 0.001 vs normoxia; \*\**P* < 0.05 vs normoxia+PD98059; # *P* < 0.01 vs Su/Hypo in vehicle.

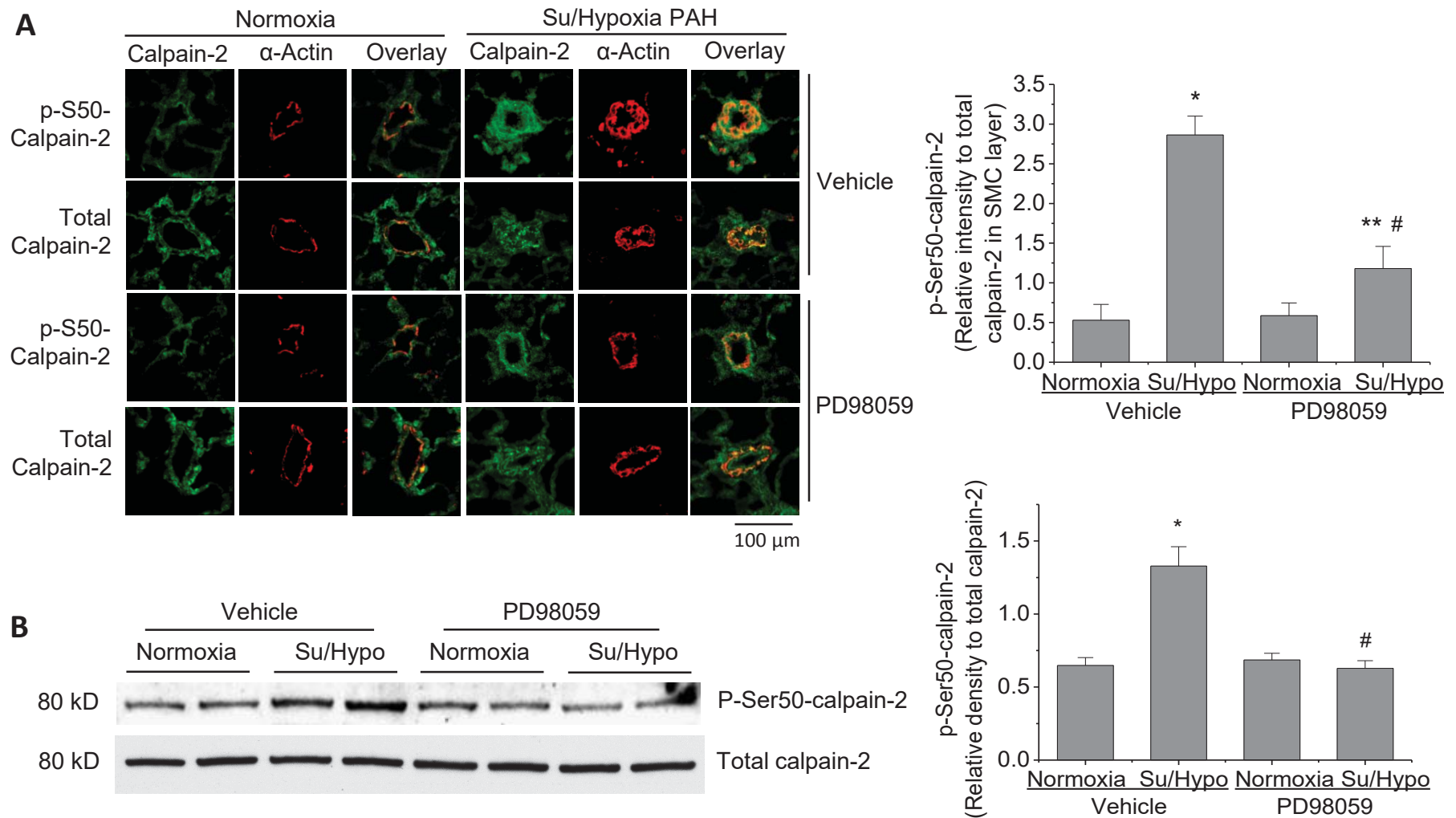


**Figure E15. Inhibition of ERK1/2 reduces ERK1/2 phosphorylation in the Sugden/Hypoxia PAH rat model.** (A): Lung slides were double-stained for  $\alpha$ -actin (red) and p-ERK1/2 (green) and total ERK1/2 (green). (B): Protein levels of p-ERK1/2 and total ERK1/2 in lung homogenates of Sugden/Hypoxia PAH rat model were measured by using Western blot. Results are expressed as mean  $\pm$  SE; n = 5. \* $P$  < 0.001 vs normoxia; \*\* $P$  < 0.05 vs normoxia+PD98059; # $P$  < 0.01 vs Su/Hypo+Vehicle.

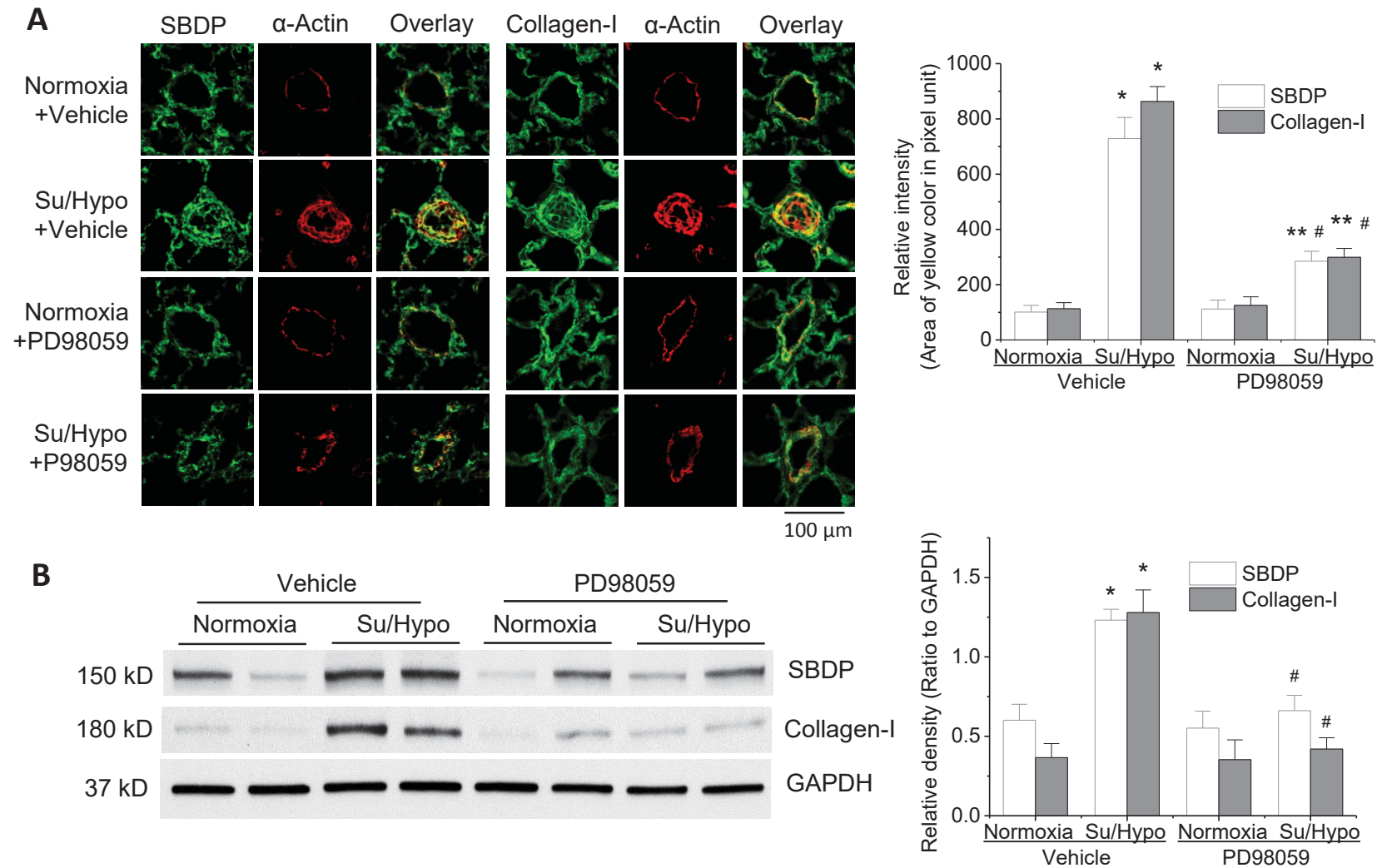




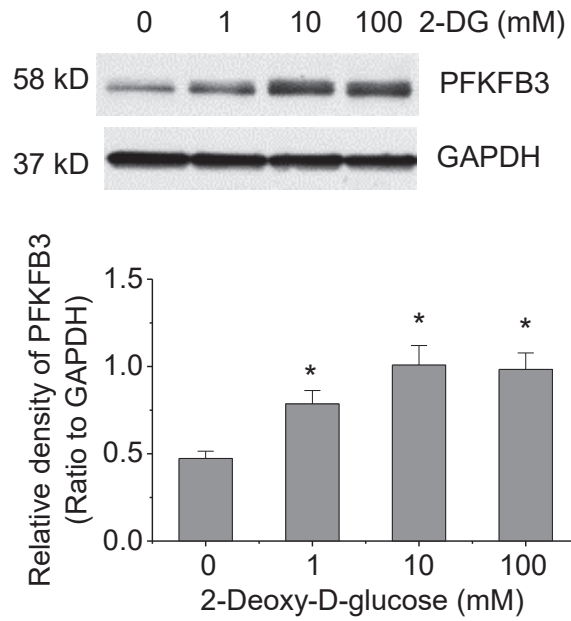
**Figure E16. Inhibition of ERK1/2 inhibits PASM proliferation (A) and induces PASM apoptosis (B) in pulmonary arteries in Sugen/hypoxia PAH.** Lung slides from rats receiving vehicle or PD98059 exposed to normoxia or Sugen/hypoxia were stained for  $\alpha$ -actin (green), Ki67 (red) or TUNEL (red) and DAPI (blue). Arrows indicate positive cells. Results are expressed as mean  $\pm$  SE; n = 5. \* $P$  < 0.05 vs normoxia; # $P$  < 0.05 vs Su/Hypo+Vehicle.



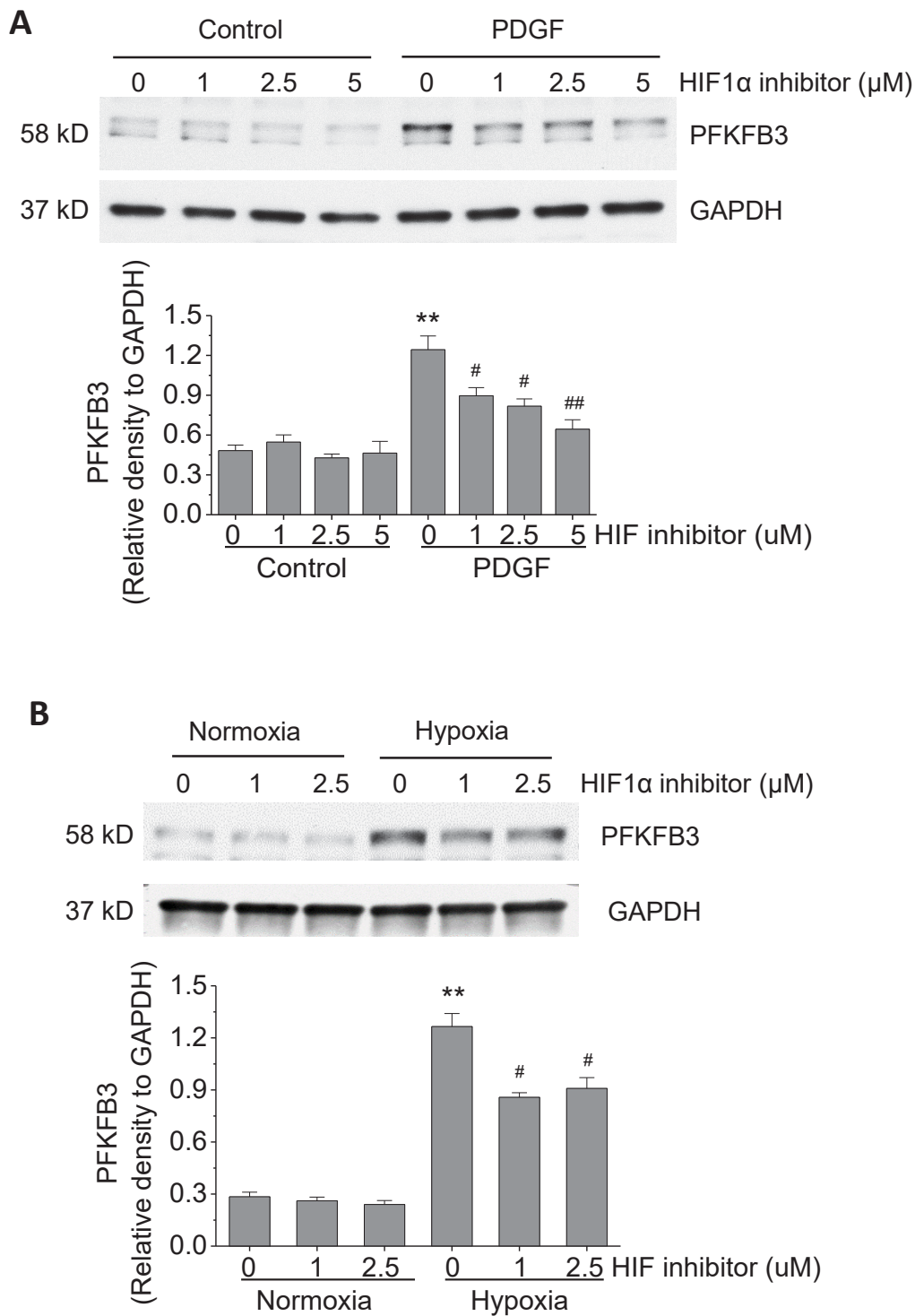
**Figure E17. Inhibition of ERK1/2 reduces calpain-2 phosphorylation at Ser50 in the Sugen/Hypoxia PAH rat model.** Male Sprague-Dawley rats 8 weeks of age were given single subcutaneous injection with SU5416 (20 mg/kg) and were exposed to hypoxia (10% O<sub>2</sub>) for 3 weeks and then housed for 2 weeks under normoxia (21% O<sub>2</sub>). Control age-matched male rats were maintained under normoxia. After that, groups of control rats (Normoxia+PD98059) and Sugen/Hypoxia-exposed rats (Su/Hypo+PD98059) began receiving PD98059 (10 mg/kg, i.p.) 5 days/week for 3 weeks. Second groups of control rats (Normoxia+Vehicle) and Sugen/Hypoxia-exposed rats (Su/Hypo+Vehicle) received the same volume of vehicle. Pulmonary hypertension and pulmonary vascular remodeling were assessed 3 week later (8 weeks after PAH induction). (A): Lung slides were double-stained for  $\alpha$ -actin (red) and p-S50-calpain-2 (green) and total calpain-2 (green). (B): protein levels of p-S50-calpain-2 and total calpain-2 in lung homogenates were assayed by Western blot. Results are expressed as mean  $\pm$  SE; n = 5. \**P* < 0.001 vs normoxia; \*\**P* < 0.05 vs normoxia+PD98059; #*P* < 0.01 vs Su/Hypo in vehicle.



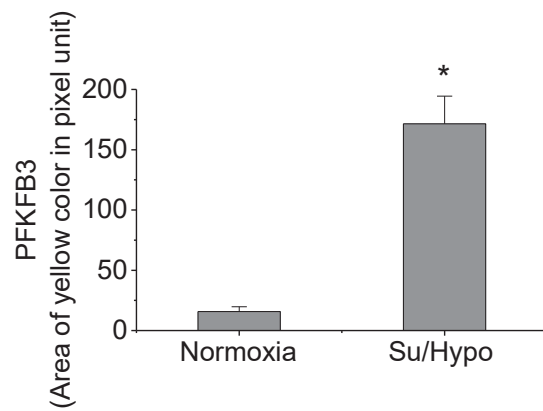
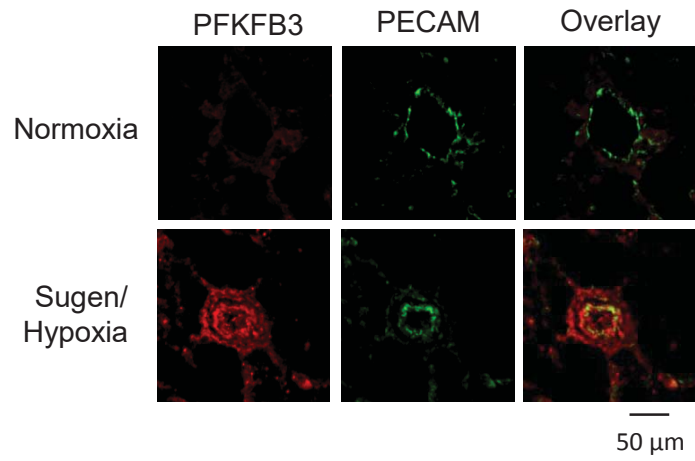
**Figure E18. Inhibition of ERK1/2 reduces calpain-2 phosphorylation at Ser50, calpain activation and collagen-I protein levels in the Sugen/Hypoxia PAH rat model.** Male Sprague-Dawley rats 8 weeks of age were given single subcutaneous injection with SU5416 (20 mg/kg) and were exposed to hypoxia (10% O<sub>2</sub>) for 3 weeks and then housed for 2 weeks under normoxia (21% O<sub>2</sub>). Control age-matched male rats were maintained under normoxia. After that, groups of control rats (Normoxia+PD98059) and Sugen/Hypoxia-exposed rats (Su/Hypo+PD98059) began receiving PD98059 (10 mg/kg, i.p.) 5 days/week for 3 weeks. Second groups of control rats (Normoxia+Vehicle) and Sugen/Hypoxia-exposed rats (Su/Hypo+Vehicle) received the same volume of vehicle. Pulmonary hypertension and pulmonary vascular remodeling were assessed 3 week later (8 weeks after PAH induction). (A): Lung slides were double-stained for  $\alpha$ -actin (red) and SBDP (green) and collagen-I (green). (B): protein levels of SBDP and collagen-I in lung homogenates were assayed by Western blot. Results are expressed as mean  $\pm$  SE; n = 5. \**P* < 0.001 vs normoxia; \*\**P* < 0.05 vs normoxia+PD98059; #*P* < 0.01 vs Su/Hypo in vehicle.



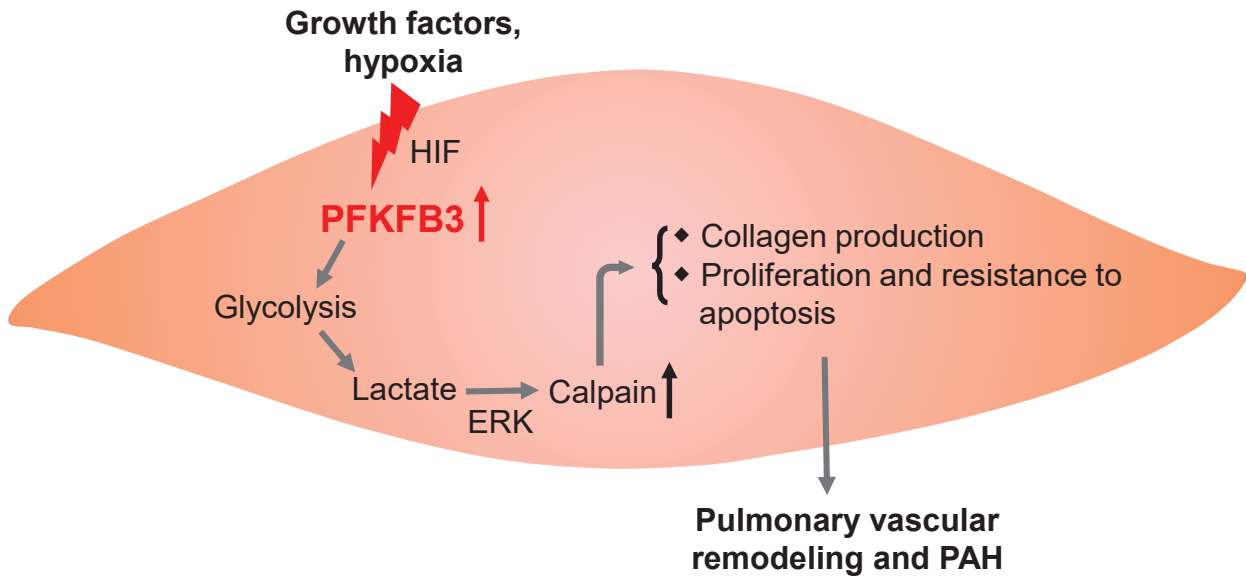
**Figure E19. Hexokinase inhibitor does not reduce the protein levels of PFKFB3 in PSMCs.** PSMCs were incubated with hexokinase inhibitor 2-Deoxy-D-glucose (2-DG) (1-100 mM) for 24 h, then protein levels of PFKFB3 were measured. Results are expressed as mean  $\pm$  SE; n = 4. \*  $P < 0.01$  vs. control (0).



**Figure E20. Inhibition of HIF1 $\alpha$  attenuates PDGF- and hypoxia-induced increases in protein levels of PFKFB3 in PASCs.** PASCs were incubated in the presence and absence of HIF1 $\alpha$  inhibitor methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate (1-5  $\mu$ M) with PDGF (10 ng/ml) for 24 h (A), or under normoxia (21 % O<sub>2</sub>) and hypoxia (1% O<sub>2</sub>) for 12 h (B), then protein levels of PFKFB3 were measured. Results are expressed as mean  $\pm$  SE; n = 4. \*\*  $P$  < 0.01 vs. control or normoxia. #  $P$  < 0.05, ##  $P$  < 0.01 vs. PDGF or Hypoxia.



**Figure E21. Higher levels of PFKFB3 in PAECs of Sugene/hypoxia PAH rats.** Lung slides of Sugene/hypoxia PAH rats were double-stained for PFKFB3 (red) and PECAM (green). Results are expressed as mean  $\pm$  SE;  $n = 5$ . \*  $P < 0.001$  vs. normoxia.



**Figure E22. A schematic pathway illustrating the role of PFKFB3 in pulmonary vascular remodeling of PAH.**