Supporting Information

SI Methods

Chemicals and reagents

Collagen type 2 (Cat. No. NC9693955) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Dimethyl sulfoxide was from Sigma-Aldrich (Cat. No. D8418, Louis, MO, USA). 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) was from Merck Millipore (Burlington, MA, USA). Sugen5416 (Cat. No. A3847) was from APExBIO (Houston, TX, USA). Calcein-AM fluorescent dye was from Corning (Cat. No. 354216, New York, USA). Methanol was from Sigma-Aldrich (Cat. No. 179337, Louis, MO, USA). Hydrogen peroxide 30% (w/w) in water was from ACROS ORGANICS (Cat. No. AC41188, Radnor, PA, USA).

Generation of endothelial-specific *Pfkfb3* knockout mice

The animal use protocol was approved by the Institutional Animal Care & Use Committee of Augusta University. The *Pfkfb3-floxed* (*Pfkfb3*$^{flox/flox,}$ *Pfkfb3*^{WT}) mice were generated by Xenogen Biosciences Corporation (Cranbury, NJ, USA) (1). Mice with *Pfkfb3* deficiency selective in endothelial cells (*Pfkfb3*$^{\Delta VEC}$) were generated by cross-breeding *Pfkfb3*$^{flox/flox}$ (*Pfkfb3*^{WT}) mice with *Cdh5-Cre* transgenic mice (The Jackson Laboratory, Stk. No. 006137, Bar Harbor, ME, USA). Global heterozygous *Pfkfb3* (*Pfkfb3*$^{+/-}$) knockout mice were generated as previously described (2). All mice were on a C57BL/6J background.

For the inducible endothelial-specific *Pfkfb3* knockout mice, *Pfkfb3* floxed mice were crossed with *Cdh5-CreER*^{T2} mice (3) to generate *Cdh5-CreER*^{T2};*Pfkfb3*$^{flox/flox}$ mice (*Pfkfb3*$^{\Delta VEC-ERT2}$) and *Cdh5-CreER*^{T2} mice (*Pfkfb3*$^{WT-ERT2}$). The *Cdh5* promoter controls the activity of the tamoxifen-inducible Cre recombinase.

Hypoxic mouse PAH model

Pfkfb3$^{\Delta VEC}$ and *Pfkfb3*^{WT} mice were exposed to 10% oxygen or room air for four weeks. Anhydrous calcium sulfate and soda lime were used to keep the humidity and NH_3 within the chamber at normal levels. CO_2 concentration was controlled to less than 0.2% with *Litholyme*CO_2 absorbent (Allied Health Care Products, St. Louis, MO, USA). All of the experimental mice were fed the same chow diet and water. Four weeks later, mice were anesthetized, and the lung tissues collected for Western blot, quantitative real time PCR and histology analyses.

Rat Sugen5416/hypoxia pulmonary hypertension model

Male Sprague Dawley (SD) adult rats were purchased from Envigo RMS, Inc (Indianapolis, IN, USA). Rats were subcutaneously injected with 20 mg/kg of Sugen5416 (APExBIO), which was dissolved in 0.5% carboxymethylcellulose, 0.9% NaCl, 0.4% polysorbate and 0.9% benzyl alcohol in deionized water. Rats were placed in normobaric hypoxia (10% oxygen) for three weeks. Rats were then removed from the hypoxia chamber and randomly divided into two groups for vehicle or 3PO treatment (50 mg/kg/day, dissolved in 10% Tween-80, 10% DMSO and 80% saline) for five weeks. Rats were anesthetized for echocardiographic and hemodynamic assay.

Echocardiography

Transthoracic echocardiography was performed with a Visual Sonics Vevo 2100 ultrasound machine with a 40 MHz ultrasound probe (MS-400) for mice and 25 MHz ultrasound probe (MS-250) for rats. Briefly, mice or rats were anesthetized with continuous isoflurane inhalation (1.5-3.0%) and placed on a

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heated pad in a supine position. The fur on the chest of the mice or rats 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 and ejection time were obtained from the modified parasternal long axis view using pulsed Doppler mode. The LV ejection fraction (LV EF), LV fractional shortening (LV FS) and the cardiac output (CO) were 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.

Rat systolic arterial pressure measurement

The rat tail cuff pressure was taken as an estimate of systolic arterial pressure as previously described (4, 5). Rats were trained for 4 consecutive days before the beginning of the experiments. In brief, rats were placed in a chamber and occluding cuffs and pneumatic pulse transducers were placed on the rats' tails. During the programmed electrosphygmomanometer inflating and deflating, the signals were automatically obtained via a MacLab (ADInstruments, Milford, CT). A total of 12 readings were taken for each rat. The highest and lowest readings and any excess noise were removed, and the rest readings were averaged to determine the systolic arterial pressure.

Pulmonary hypertension and right ventricular hypertrophy assay

After establishment of the PH model, mice were anesthetized with ketamine hydrochloride (60 mg/kg, *i.p.*) and the trachea was intubated. The diaphragm was surgically exposed through the abdomen. A 23 gauge needle connected to a pressure transducer (ADInstruments) was inserted into the right ventricle (RV) through the diaphragm, and right ventricular systolic pressure (RVSP) was continuously monitored for 10-15 minutes. The diaphragm remains intact without opening the chest. Rats were anesthetized with ketamine hydrochloride (80 mg/kg, *i.p.*) and a rigid cannula containing a flexible catheter (inside diameter, 0.58 mm; outside diameter, 0.965 mm) were inserted into the right ventricle via the right jugular vein for the RVSP measurement, and further the catheter was advanced into the main pulmonary arteries for the mPAP measurement through a pressure transducer (6). Data were recorded by the PowerLab data-acquisition system (ADI Instruments). After hemodynamic measurements, the heart and lungs of mice and rats were rinsed with PBS through the pulmonary artery and then were collected. The hearts were dissected, and the right ventricular hypertrophy were calculated as the ratio of the right ventricular free wall weight to the weight of the left ventricle plus septum (RV/LV+S). The changed percentage of RVSP or (RV/LV+S) of *Pfkfb3* deficient mice (KO) over that of control mice (WT) exposure to hypoxia was determined by [(hypoxia/WT – normoxia/WT) - (hypoxia/ KO – normoxia/KO)]/(hypoxia/WT – normoxia/ WT) ×100%.

The left lungs were perfused with 4% paraformaldehyde (PFA) solution and embedded with paraffin or OCT for subsequent histological analysis. The right lungs were snap-frozen in liquid nitrogen for Western blot or quantitative real time PCR analyses.

Immunofluorescence staining

After PBS perfusion, the left lobe of lungs was immersed in OCT followed by OCT embedding for cryosectioning. Lung sections (10 μm) were then fixed with 4% PFA solution for 10 minutes and permeabilized with 0.5% TritonX-100 for 10 minutes at room temperature followed by blocking with 10% normal goat serum for 1 h at room temperature. Lung sections were then incubated with anti-α-SMA antibody (1:200, Cat. No. 180106, Abcam, for mouse lung sections, and 1:500, Cat. No. ab5694, Abcam, for rat lung sections), anti-Ki67 antibody (1:50, Cat. No.RM9106, ThermoFisher Scientific, for mouse lung sections, and 1:50, Cat. No.AB9260, Millipore, for rat lung sections), anti-F4/80 antibody (1:200, Cat. No. ab6640, Abcam, for mouse lung sections), anti-CD68 antibody (1:100, Cat. No. ab955, Abcam,

for rat lung sections), and anti-HIF2A antibody (1:100, Cat. No.NB100-122, Novus Biologicals) overnight at 4℃. The next day, the slides were incubated with AlexaFluor conjugated isotype-specific secondary antibodies (1:250, Invitrogen) at room temperature for 1 h. Nuclei were counterstained with DAPI (1 μg/mL, Cat. No. D1306, ThermoFisher Scientific) for 5 minutes at room temperature. After washing with PBST three times, the samples were mounted with the mounting media (Cat. No. H-1000, Vector Laboratories, Burlingame, CA, USA).

For paraffin-embedded sections, the left lobe of lungs was perfused with 4% PFA followed by paraffin processing. Lung sections (5 μm) were dewaxed and dehydrated. Antigen retrieval was done by boiling the slides in 10 mmol/L sodium citrate (pH 6.0) at 98℃ for 10 minutes. After cooling, the lung sections were blocked with 10% normal goat serum for 1 h at room temperature. Slides were then incubated with anti-PFKFB3 antibody (1:150, Cat. No. 13763-1-AP, ProteinTech), anti-CD31 antibody (1:200, Cat. No. DIA-310, for mouse lung sections, and 1:50, Cat. No. sc-374763, for rat lung sections), anti-α-SMA antibody (1:200, Cat. No.180106, Abcam), anti-ICAM1 antibody (1:100, Cat. No. sc-8394, Santa Cruz), and anti-vWF antibody (1:300, Cat. No. A008229-5, Dako) overnight at 4℃. The next day, the slides were incubated with AlexaFluor conjugated isotype-specific secondary antibodies (1:250, Invitrogen) at room temperature for 1 h. Nuclei were counterstained with DAPI (1 μg/mL, ThermoFisher Scientific) for 5 minutes at room temperature. After washing three times with PBST, the samples were mounted with mounting media (Vector Laboratories).

Immunohistochemistry

Rat paraffin-embedded lungs were sectioned and deparaffinized in xylene followed by rehydrating with gradient ethanol and water. The lung endogenous peroxidase activity was destroyed in methanol containing 30% H_2O_2 for 30 min at room temperature. Antigen retrieval was performed with 10 mM sodium citrate buffer (pH 6.0) at 98℃ for 10 min followed by blocking with avidin blocking solution for 1 hour at room temperature. Anti-α-SMA antibody (1:200, Cat. No. sc-56499, Santa Cruz) was diluted with biotin blocking solution according to manufacturer's instruction and incubated with slides at 4℃ overnight. A biotinylated goat anti-mouse IgG (H+L) secondary antibody (1:200, Cat. No.BA-9200, Vector Laboratories) was incubated with lung sections for 1 hour at room temperature, followed by incubation with ABC solution (Cat. No.PK-6100, Vector Laboratories) for 30 min at room temperature. Then the antibody was detected with the peroxidase substrate 3,3'-diaminobenzidine (Cat. No.3468, Dako, Santa Clara, CA, USA). The lung sections were counterstained with hematoxylin Ⅰ (Cat. No. GHS116, Sigma). Then slides were dehydrated and mounted with xylene-based mounting medium (Cat. No. 8312-4, Richard-Allan Scientific).

Morphometric analysis

Mouse or rat lung sections (5 μm in thickness) were immunostained with α-SMA as described above. Images of distal pulmonary arterioles, 50-100 μm in diameter, were taken from three different areas per animal with a Carl Zeiss 780 upright confocal microscope (Carl Zeiss Germany). The medial wall thickness was calculated with ImageJ software. The entire vessel area was identified as the total area. The medial wall thickness ratio was calculated as (total area – lumen area) / total area.

Pulmonary vascular muscularization analysis

Paraffin-embedded rat lung sections (5 μm in thickness) were labeled with α-SMA as described above. To assess the degree of pulmonary arteriolar muscularization, arteries with a diameter at 25-75 μm with positive α-SMA staining surrounding endothelial cells were identified and classified as fully muscularized (100~75%) and partially muscularized arteries (< 75%), whereas vessels with no significantly positive α-SMA staining were classified as non-muscularized ones. A minimum of 20

vessels were counted from sections of each rat. Statistical significance was determined by comparing the percentage of muscularized vessels between groups.

Mouse or rat pulmonary endothelial cell isolation

Lungs collected from euthanized mice or rats were minced, digested with 2 mg/mL of type 2 collagenase, and gently agitated for 40 minutes at 37℃. A 12-ml syringe was used to resuspend cells into a single-cell suspension, which was filtered through a 70-μm cell strainer and centrifuged at 600 g for 5 min at 4℃. To exclude non-ECs, cell samples were re-suspended in 500 μL of 0.1% bovine serum albumin, and incubated with 10μL of CD45 microbeads (Cat. No.130-052-301, Miltenyi Biotec, Bergisch Gladbach, Germany) for mouse cells and lung non-endothelial cell depletion cocktail (Cat. No.130-109-680, Miltenyi Biotec) for rat cells, respectively. After incubation for 15 min on ice, cell suspension were applied onto the LS columns (Cat. No.130-042-401, Miltenyi Biotec) and washed with 0.1% bovine serum albumin for three times. The effluents were collected and incubated with CD31 microbeads (Cat. No.130-097-418 for mouse cells, Cat. No.130-109-680 for rat cells, Miltenyi Biotec) for 15 min on ice. LS columns were used to collect the cells that were positively labeled by CD31 microbeads via magnetic separation according to the manufacturer's instructions.

Cell culture and treatments

Human PAECs (HPAECs, Cat. No. CC-2530) were purchased from Lonza (NJ, USA) and were used at passages 3 to 8. HPAECs were cultured in Vessel Cell Basal Medium (VCBM, ATCC, Manassas, VA, USA) supplemented with Microvascular Endothelial Cell Growth Kit-BBE (ATCC, Manassas, VA, USA), and 1% penicillin/streptomycin (Cat. No. 15-140-122, Fisher Scientific). For the hypoxia experiments, the culture medium was changed to 25% VCBM (25% VCBM complete medium + 75% VCBM basal medium) and cells were placed in the modular incubator chamber (Fisher Scientific, Waltham, MA, USA) with 1% O_2. The culture medium (conditioned media) was collected after 24 hours. Human PASMCs (HPASMCs) were purchased from Lonza (Cat. No. CC-2581) and used at passages 3 to 6. PASMCs were cultured in SBM-2 (Cat. No. CC-3181, Lonza) supplemented with SGM-2 (Cat. No. CC-4149, Lonza) and 1% penicillin/streptomycin. Before the proliferation assay, HPASMCs were starved for 16 hours with DMEM supplemented with 0.1% FBS, 10 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin, then replaced with the conditioned media and followed with incubation for 72 h for the WST-1 assay and 24 h for the EdU proliferation assay.

Mouse primary pulmonary artery smooth muscle cells (Ms PASMCs) were purchased from Cell Biologics (Cat. No.C57-6083, Chicago, IL, USA) and were used at passages 3 to 6. The culture medium and experimental treatment condition used for Ms PASMCs were the same as what have been used for human PASMCs.

Primary PAECs from peripheral type-III pulmonary arteries (<1 mm in diameter) of 4 IPAH patients (2 males and 2 females, 30-55 years of age) and 4 normal controls (2 males and 2 females, 26-54 years of age) were obtained from Pulmonary Hypertension Break-through Initiative (PHBI). The patients' identities were concealed. Exemptions of informed consents were approved by the Human Assurance Committee (HAC) of the Augusta University and by PHBI. These cells have been authenticated by PHBI to be endothelial cells using FACS analysis and immunofluorescence staining for von Willebrand factor. The cells were cultured and passaged in the same way as the Lonza PAECs. Third-to-five passage cells equilibrated in growth factor- and serum-free medium for 24 h were used for all experiments.

F-2,6-P2 level assay

For F-2,6-P2 level normalization, mouse and rat lung samples were weighed, and cells were lysed. Protein content was determined by BCA assay. Using a previously described method (7), lung samples were homogenized in 5 - 100 volume of 0.05 M NaOH whereas PAEC suspensions were mixed with 1 volume of 0.1 M NaOH; the resulting mixture was heated for 5 min at 80 ℃. After cooling, the samples were centrifuged and the obtained supernatants were neutralized with acetic acid. The mixture was again centrifuged and the levels of F-2,6-P2 in the supernatant were measured by the stimulation of PPi-PFK assayed in the presence of 0.5 mM pyrophosphate and 1 mM fructose 6-phosphate in the assay mixture as shown in the table below. Measurement was conducted at 340nm with Synergy H1 Hybrid Reader (BioTek, Gene Company). The OD values of F-2,6-P2 in these groups were calculated and normalized to protein content.

Supplemental Table 1. Reaction system for F-2,6-P2 level assay.

Substrate	Initial concentration	Final concentration
Tris-HCl (pH7.5)	50 mM	Make up the rest volume of 150 μL
NADH	20 mM	0.2 mM
DTT	1 M	5 mM
F6P	200 mM	1 mM
MgCl2	1 M	2 mM
Aldolase	700 U/mL	0.7 U/mL
GDH	450 U/mL	0.45 U/mL
TIM	1200 U/mL	0.6 U/mL
PPi-PFK		
PPi-Na	25 mM	0.5 mM

Note : PPi-PFK was extracted from sprouted potatoes and dissolved with 100 mM of Tris-HCl (pH8.4). The PPi-PFK solution was centrifuged at 14,000 rpm for 20 min at 4 ℃ and 10 μg PPi-PFK was used for each reaction

Lactate assay

The lactate levels in the PAECs were measured with the Lactate Assay Kit (Cat. No. MAK064, Sigma) according to the manufacturer's instructions.

Pyruvate assay

The pyruvate levels in the PAECs were measured with the Pyruvate Assay Kit (Cat. No. MAK071, Sigma) according to the manufacturer's instructions.

Adenovirus transduction of PAECs

HPAECs grown to 80% confluence were incubated with 500 μL basal medium containing adenoviral vectors of PFKFB3 knockdown (Ad-sh*PFKFB3*) or overexpression (Ad-*PFKFB3*) and their negative control adenovirus (Ad-sh*CTRL* or Ad-*CTRL*) for 2 hours. The medium was then replaced with fresh complete growth medium for continuous culture. After 48 hours, the cells were treated as indicated and collected for Western-blot and quantitative RT-PCR (qRT-PCR) analyses.

RNA interference

When HPAECs reached 70% confluence, they were transfected with 25 nM of siRNAs targeting human *PFKFB3* (si*PFKFB3*, Cat. No. L-006763-00-0005; Dharmacon, Lafayette, CO, USA) or *HIF1A* (si*HIF1A*, Cat. No. L-004018-00-0005; Dharmacon, Lafayette, CO, USA) or *HIF2A* (si*HIF2A*, Cat. No.

L-004814-00-0005; Dharmacon, Lafayette, CO, USA) or with a non-targeting negative control (si*CTRL*, Cat. No. D-001810-10-05; Dharmacon) using Lipofectamine RNAiMax reagent (Cat. No 13778-150; Invitrogen) according to the manufacturer's protocol. Four hours later, the medium was changed to complete growth medium for continuous culture. The cells underwent different treatments within 48 hours after siRNA transduction and were then collected for various assays.

Metabolic measurements

Human and isolated rat PAECs were seeded in the Seahorse XF96 cell culture plates at a density of 1.5×10^4 per well (Seahorse Bioscience, North Billerica, MA) in complete endothelial culture medium (VCBM). The medium was changed the next day to XF base assay medium (Seahorse Bioscience) supplemented with 2 mM glutamine with pH adjusted to 7.4. The plate was placed in a non-CO_2 incubator at 37℃ for 1 hour. The assay was run on the XF96 extracellular flux analyzer (Seahorse Bioscience).

Western blot analysis

Protein extracts were prepared from lung tissues and cells by lysing tissues and cells in RIPA buffer containing protease inhibitor cocktails (Cat. No. 05892970001, Roche, SC, USA). The lung tissues were grinded and tissue lysis was prepared. Extracts were centrifuged at 12000 rpm for 10 minutes. The supernatant was removed and the protein concentration was determined by BCA assay. The samples were separated by SDS-PAGE followed by transfer onto nitrocellulose membranes. After blocking in 5% skim milk for one hour, blots were probed using the below primary antibodies: PFKFB3 (1:1000, Cat. No. ab181861, abcam), PCNA (1:1000, Cat. No. sc-56, Santa Cruz), ICAM-1 (1:1000, cat. No. sc-8314, Santa Cruz), VCAM-1 (1:1000, Cat. No. sc-1504, Santa Cruz), HIF1A (1:1000, cat. No. AF1935-SP, R&D systerms), HIF2A (1:1000, Cat. No. NB100-122, Novus). Anti-β-actin (1:1000, Cat. No. sc47776, Santa Cruz) and GAPDH (1:1000, Cat. No.2118, CST) were used as loading controls.

RNA extraction and quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from cells and frozen mouse lung tissues with Trizol Reagent (Cat. No. 15596018, Invitrogen, Grand Island, NY). 1 μg of RNA was used for cDNA synthesis with the cDNA synthesis kit (Cat. No. 170-8891, BioRad) according to the manufacturer's instructions. qRT-PCR was performed with universal SYBR green mix (Cat. No. 172-5122, BioRad) on the StepOne Plus System (Applied Biosystems, Grand Island, NY). The sequences of gene-specific primers are provided in Supplementary Table 2. $2^{-\Delta\Delta CT}$ method was used to quantify the relative expression of target genes. 18S rRNA (for human and mouse genes) or cyclophilin A (CypA, for rat genes) were used as the internal controls.

ELISA assay

The levels of growth factors (PDGF-BB, FGF2 and TGFβ1) and cytokines (CXCL12, IL1β and TNFα) released in the culture supernatants of human and mouse PAECs were measured with R&D Quantikine ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Monocyte adhesion assay

THP-1 cells were used in this study. The THP-1 cells were firstly labeled with Calcein-AM (Thermo Fisher Scientific) according to the manufacturer's instructions. Then the confluent monolayer of PAECs were incubated with THP-1 cells for 20 min at 37℃. After incubation, PAECs were washed with RPMI-1640 three times followed by fixing with 4% PFA. Then images were taken under the fluorescence

microscope. Fluorescent-positive cells were counted with ImageJ sofeware and normalized with total cell numbers.

PASMC proliferation assay

For EdU staining assay, human PASMCs were seeded in 4-well culture slides (Corning) at a density of 5×10^4 in a volume of 1mL of smGM-2 complete medium. When the cell confluence reached 80%, human PASMCs were starved with DMEM supplemented with 0.1% FBS, 10 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin for 16 h followed by addition of the previously collected conditioned medium and 10 μM of EdU solution for 24 hours. The Click-iT EdU imaging kit (Cat. No. C10337, Invitrogen) was then used according to the manufacturer's instructions.

For WST-1 proliferation assay, human PASMCs were seeded in 96-well plates at a density of 2×10^4 in a volume of 100 μL of smGM-2 complete medium. When the cell confluence reached 80%, the human PASMCs were starved with DMEM supplemented with 0.1% FBS, 10 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin for 16 hours and then replaced with the previously collected conditioned medium. 72 hours later, 10 μL of WST-1 reagent was added per well and the plate incubated at 37℃ and 5% CO_2 for 3 hours. Absorbance was then read at OD450.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (LaJolla, CA, USA). The significance of the differences between two groups was assessed using unpaired Student's *t* test for $n > 5$, and Mann-Whitney *U* test for $n < 5$. Multiple comparisons were performed by one-way ANOVA followed by Bonferroni's *post hoc* test. All results are presented as mean ± SEM. $P < 0.05$ was considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All biological experiments were repeated at least three times using independent cell cultures or individual animals (biological replications).

Supplemental Table 2. Primer sequences used for QRT-PCR analyses.

Gene name	Forward primer	Reverse primer
(H/Ms/Rat)18s rRNA	CTTAGAGGGACAAGTGGCG	ACGCTGAGCCAGTCAGTGTA
Human *PFKFB3*	CTCGCATCAACAGCTTTGAGG	TCAGTGTTTCCTGGAGGAGTC
Human *PDGFB*	GCACACGCATGACAAGACGGC	AGGCAGGCTATGCTGAGAGGTCC
Human *FGF2*	ACGGCGTCCGGGAGAA	ACACTCCCTTGATGGACACAACT
Human *TGFB1*	TCCTGCTTCTCATGGCCA	CCTCAGCTGCACTTGTAG
Human *CXCL12*	AACTGTGCCCTTCAGATTGTAG	TCGAGTGGGTCTAGCGGAAAGT
Human *IL1B*	TTGTTGAGCCAGGCCTCTCT	ACCAAATGTGGCCGTGGTT
Human *ICAM-1*	GGCCGGCCAGCTTATACAC	TAGACACTTGAGCTCGGGCA
Human *VCAM-1*	TCAGATTGGAGACTCAGTCATGT	ACTCCTCACCTTCCCGCTC
Human *TNFA*	CCCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA
Mouse *Pfkfb3*	GATCTGGGTGCCCGTCGATCACCG	CAGTTGAGGTAGCGAGTCAGCTTC
Mouse *Pdgfb*	ATCCGCTCCTTTGATGATCT	GAGCTTTCCAACTCGACTCC
Mouse *Fgf2*	CAACCGGTACCTTGCTATGA	TCCGTGACCGGTAAGTATTG

Mouse *Tgfb1*	CCCTATATTTGGAGCCTGGA	CTTGCGACCCACGTAGTAGA
Mouse *Cxcl12*	CCAAGAGTACCTGGAGAAAGC	AGTTACAAAGCGCCAGAGCA
Mouse *Il1b*	TGTCTTGGCCGAGGACTAAGG	TGGGCTGGACTGTTTCTAATGC
Mouse *Icam-1*	GTGGCGGGAAAGTTCCTG	CGTCTTGCAGGTCATCTTAGGAG
Mouse *Vcam-1*	AGTTGGGGATTCGGTTGTTC	CATTCCTTACCACCCCATTG
Mouse *TNFα*	AGGGTCTGGGCCATAGAACT	CCACCACGCTCTTCTGTCTAC
Mouse *β-actin*	TGT CCA CCT TCC AGC AGA TGT	AGC TCA GTA ACA GTC CGC CTA G
Mouse *Rps3*	GCTGAAGATGGCTACTCTGGAG	CTGGACAACTGCGGTCAACTCT
Mouse *Arg1*	GGAATCTGCATGGGCAACCTGTGT	AGGGTCTACGTCTCGCAAGCCA
Mouse *Mt1*	AAGAGTGAGTTGGGACACCTT	GAGACAATACAATGGCCTCC
Mouse *Slc39a12*	ACCATCCAACTGAAAGGCCC	TGTGCAGGCTATCTCCAACC
Mouse *Egln3*	CAACTTCCTCCTGTCCCTCATC	CCTGGATAGCAAGCCACCATTG
Mouse *Serpine*	AGTCTTTCCGACCAAGAGCAG	GATGACAAAGGCTGTGGAGGA
Mouse *Pak6*	TCCGAAGCATGTTCCTGTCCAC	GCTACCAGGTTTGAAGAGGAGTC
Mouse *Il6*	TCTTGGGACTGATGCTG GTGA	GCAAGTGCATCATCGTT GTTCA
Mouse *Lox*	CATCGGACTTCTTACCAAGCCG	GGCATCAAGCAGGTCATAGTGG
Mouse *Bnip3*	GCTCCCAGACACCACAAGAT	TGAGAGTAGCTGTGCGCTTC
Mouse *Angptl4*	CTGGACAGTGATTCAGAGACGC	GATGCTGTGCATCTTTTCCAGGC
Mouse *Eno1*	TGCGTCCACTGGCATCTAC	CAGAGCAGGCGCAATAGTTTTA
Mouse *Nov*	GTCACCAACAGGAATCGCCAGT	GTAGGTGGATGGCTTTCAGGGA
Mouse *Pgk1*	GATGCTTTCCGAGCCTCACTGT	ACCAGCCTTCTGTGGCAGATTC
Mouse *Edn*	TTCCCAATAAGGCCACAGACC	TTGGGCCCTGAGTTCTTTTCC
Mouse *NOS2*	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Rat *CypA*	TATCTGCACTGCCAAGACTGAGTG	CTTCTTGCTGGTCTTGCCATTCC
Rat *Pfkfb3*	CACGGCGAGAATGAGTACAA	TTCAGCTGACTGGTCCACAC
Rat *Pdgfb*	ACACCTCAAACTCGGGTGAC	TCAGTGCCTTCTTGTCATGG
Rat *Fgf2*	ACGGCGTCCGGGAGAA	ACACTCCCTTGATGGACACAACT
Rat *Tgfb1*	ATACGCCTGAGTGGCTGTCT	TGAAGCGAAAGCCCTGTATT
Rat *Cxcl12*	TGTGCATTGACCCGAAATTA	TCCTCAGGGGTCTACTGGAA
Rat *Il1b*	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
Rat *TNFα*	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
Rat *Icam-1*	CTTCCGACTAGGGTCCTGAA	CTTCAGAGGCAGGAAACAGG
Rat *Vcam-1*	TGCACGGTCCCTAATGTGTA	TGCCAATTTCCTCCCTTAAA

Supplemental Table 3. List of antibodies used for Western blots

Primary antibodies	Company	Species	Type	Dilution (final concentration)	Cat. No
PFKFB3	Abcam	Rabbit	Monoclonal	1/1000	ab181861
PCNA	Santa Cruz	Mouse	Monoclonal	1/1000	sc-56
β-actin	Santa Cruz	Mouse	Monoclonal	1/2000	sc-47778
ICAM-1	Santa Cruz	Mouse	Monoclonal	1/1000	sc-8439
VCAM-1	Santa Cruz	Goat	Polyclonal	1/1000	sc-1504

HIF2A	Novus	Rabbit	Polyclonal	1/1000	NB100-122
HIF1A	R&D systems	Goat	Polyclonal	1/500	AF1935-SP
GAPDH	CST	Rabbit	Monoclonal	1/1000	2118

Supplemental Table 4. List of antibodies used for immunofluorescent assay

Primary antibodies	Company	Species	Type	Dilution	Cat. No
PFKFB3	ProteinTech	Rabbit	Polyclonal	1/200	13763-1-AP
CD31	Dianova	Rat	Monoclonal	1/200	DIA-310
PECAM-1	Santa Cruz	Mouse	Monoclonal	1/50	sc-376764
α-SMA	Sigma	Mouse	Monoclonal	1/200	C6198
α-SMA	Abcam	Rabbit	Polyclonal	1/500	Ab5694
F4/80	Abcam	Rat	Monoclonal	1/200	Ab6640
CD68	Abcam	Mouse	Monoclonal	1/100	Ab955
ICAM-1	Santa Cruz	Mouse	Monoclonal	1/100	sc-8439
vWF	Dako	Rabbit	Polyclonal	1/300	A008229-5
Secondary antibodies (Invitrogen Corp., Carlsbad, CA, USA)		Species	Type	Dilution	Cat. No
Alexa 488-conjugated anti-mouse IgG		Mouse	polyclonal	1/250	A11001
Alexa 488-conjugated anti-rabbit IgG		Rabbit	polyclonal	1/250	A11008
Alexa 488-conjugated anti-rat IgG		Rat	polyclonal	1/250	A11006
Alexa 594-conjugated anti-mouse IgG		Mouse	polyclonal	1/250	A11032
Alexa 594-conjugated anti-rabbit IgG		Rabbit	polyclonal	1/250	A11012
Alexa 594-conjugated anti-rat IgG		Rat	polyclonal	1/250	A21471

Supplemental Table 5. List of siRNAs used

	Target gene	Species	Company	Catalog number
siRNA	PFKFB3	Human	Dharmacon	L-006763-00-0005 5 nmol
siRNA	HIF1A	Human	Dharmacon	L-004018-00-0005 5 nmol
siRNA	HIF2A	Human	Dharmacon	L-004814-00-0005 5 nmol
siRNA	Non-targeting	Human	Dharmacon	D-001810-10-05

Supplemental Table 6. List of ELISA kits used

ELISA Kit name	**Cat. No.**	**Vendor**

Human PDGF-BB Quantikine ELISA kit	DBB00	R&D Systems
Human FGF basic Quantikine ELISA kit	DFB50	R&D Systems
Human TGF-β1 Quantikine ELISA kit	DB100B	R&D Systems
Human CXCL12 Quantikine ELISA kit	DSA00	R&D Systems
Human IL-1β Quantikine ELISA kit	DLB50	R&D Systems
Human TNF-α QuantiGlo ELISA kit	QTA00B	R&D Systems
Mouse PDGF-BB Quantikine ELISA kit	MBB00	R&D Systems
Mouse FGF basic Quantikine ELISA kit	MFB00	R&D Systems
Mouse TGF-β1 Quantikine ELISA kit	MB100B	R&D Systems
Mouse CXCL12 Quantikine ELISA kit	MCX120	R&D Systems
Mouse IL-1β Quantikine ELISA kit	MLB00C	R&D Systems
Mouse TNF-α Quantikine ELISA kit	MTA00B	R&D Systems

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Supplementary figures and figure legends

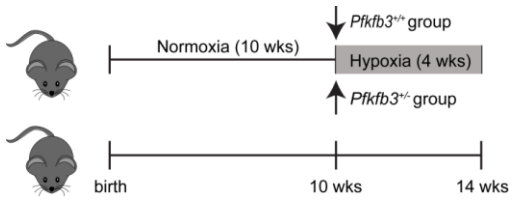

Figure S1. Experimental design for hypoxia-induced mouse PH. 10 weeks after birth, *Pfkfb3*$^{+/-}$ and control mice (*Pfkfb3*$^{+/+}$) were exposed to normoxia or hypoxia (10% O_2) for 4 weeks. Mice were anesthetized and analyzed for hemodynamic changes and other parameters.

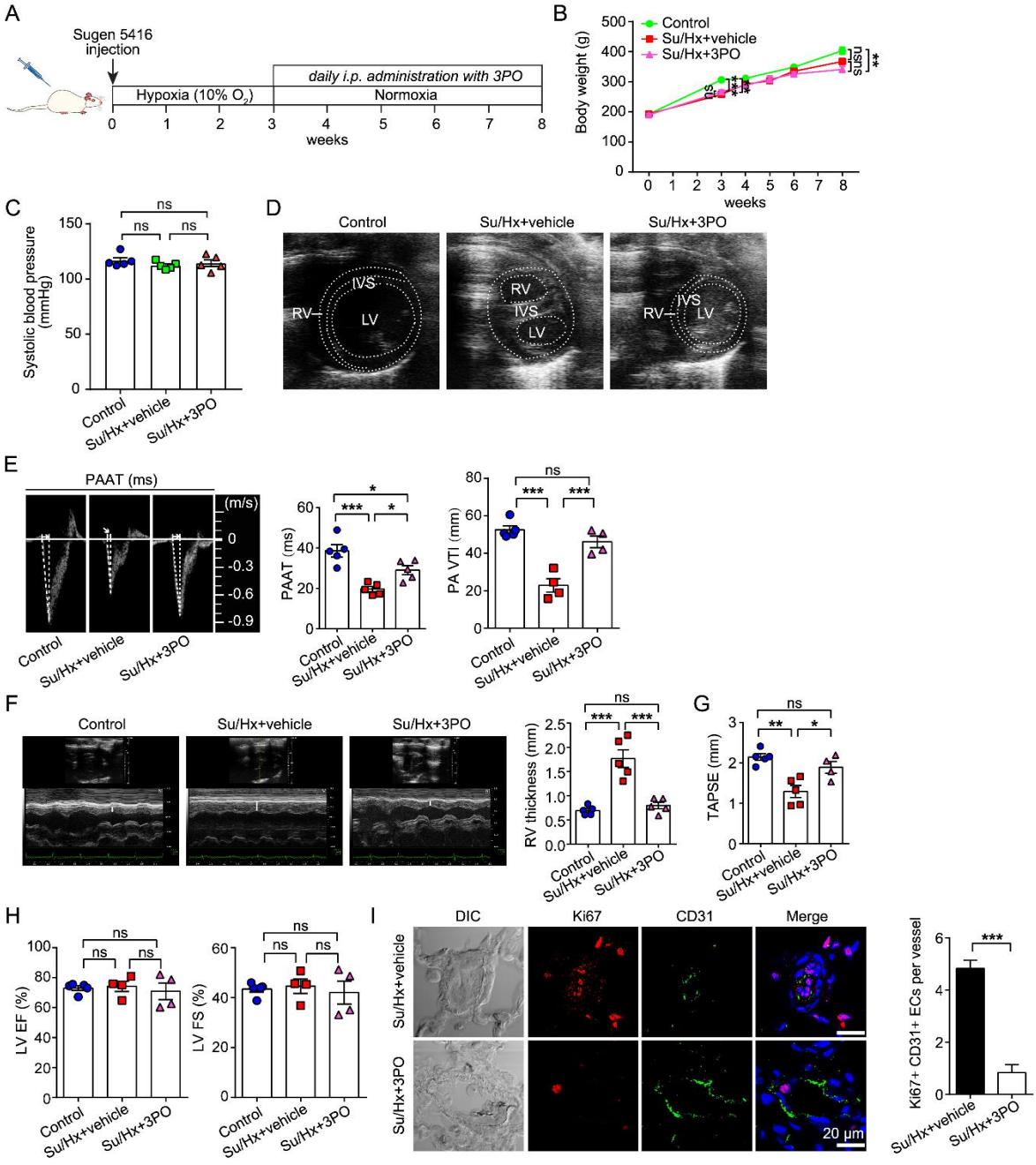
A
Sugen 5416 injection
Hypoxia (10% O2)
daily i.p. administration with 3PO
Normoxia
0 1 2 3 4 5 6 7 8
weeks
B
Control
Su/Hx+vehicle
Su/Hx+3PO
Body weight (g)
500 400 300 200 100 0
0 1 2 3 4 5 6 7 8
weeks
ns
**
C
Systolic blood pressure (mmHg)
150 100 50 0
ns ns ns
Control
Su/Hx+vehicle
Su/Hx+3PO
D
Control
Su/Hx+vehicle
Su/Hx+3PO
IVS
RV
LV
E
PAAT (ms)
(m/s)
0 -0.3 -0.6 -0.9
PAAT (ms)
60 40 20 0
* *** *
PA VTI (mm)
80 60 40 20 0
ns *** ***
F
Control
Su/Hx+vehicle
Su/Hx+3PO
RV thickness (mm)
2.5 2.0 1.5 1.0 0.5 0.0
ns *** ***
G
TAPSE (mm)
3 2 1 0
ns ** *
H
LV EF (%)
100 80 60 40 20 0
ns ns ns
LV FS (%)
60 40 20 0
ns ns ns
I
DIC
Ki67
CD31
Merge
Su/Hx+vehicle
Su/Hx+3PO
20 μm
Ki67+ CD31+ ECs per vessel
6 4 2 0
***

Figure S2. Treatment with 3PO improved hemodynamics in rats with Su/Hx-induced PH. (*A*) Experimental design for treatment of Su/Hx-treated rats with 3PO. (*B*) Rat body weight change during the whole experimental course. n = 9-10. (*C*) Systolic blood pressure of control rats and 3PO-treated or vehicle-treated Su/Hx rats. n = 5. (*D*) Representative echocardiograms show the relative normal RV chamber in 3PO-treated rats with PH compared with the enlarged RV chamber in rats with PH. (*E*) Representative echocardiographic images of pulsed wave Doppler of pulmonary artery flow and quantification of the pulmonary acceleration time (PAAT, ms) and PA VTI. (*F*) Representative echocardiograms and quantification results show the reduced RV wall thickness in 3PO-treated rats with PH compared with that of vehicle-treated rats with PH. (*G*) quantification of tricuspid annular plane systolic excursion (TAPSE, mm). (*H*) LV EF % and LV FS % analysis of 3PO-treated or vehicle-treated rats determined by echocardiography. (*I*) (*left*) Representative micrographs of proliferating endothelial cells in pulmonary vascular lesions of rat lung sections, and (*right*) quantification of Ki67 and CD31 positive endothelial cells (ECs) per vessel. The sections were co-stained with Ki67 (red), CD31 (green) and DAPI (blue). Scale bar, 20 μm. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test (for *B*-*C* and *E*-*H*) and unpaired Student's *t* test (for *I*). * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

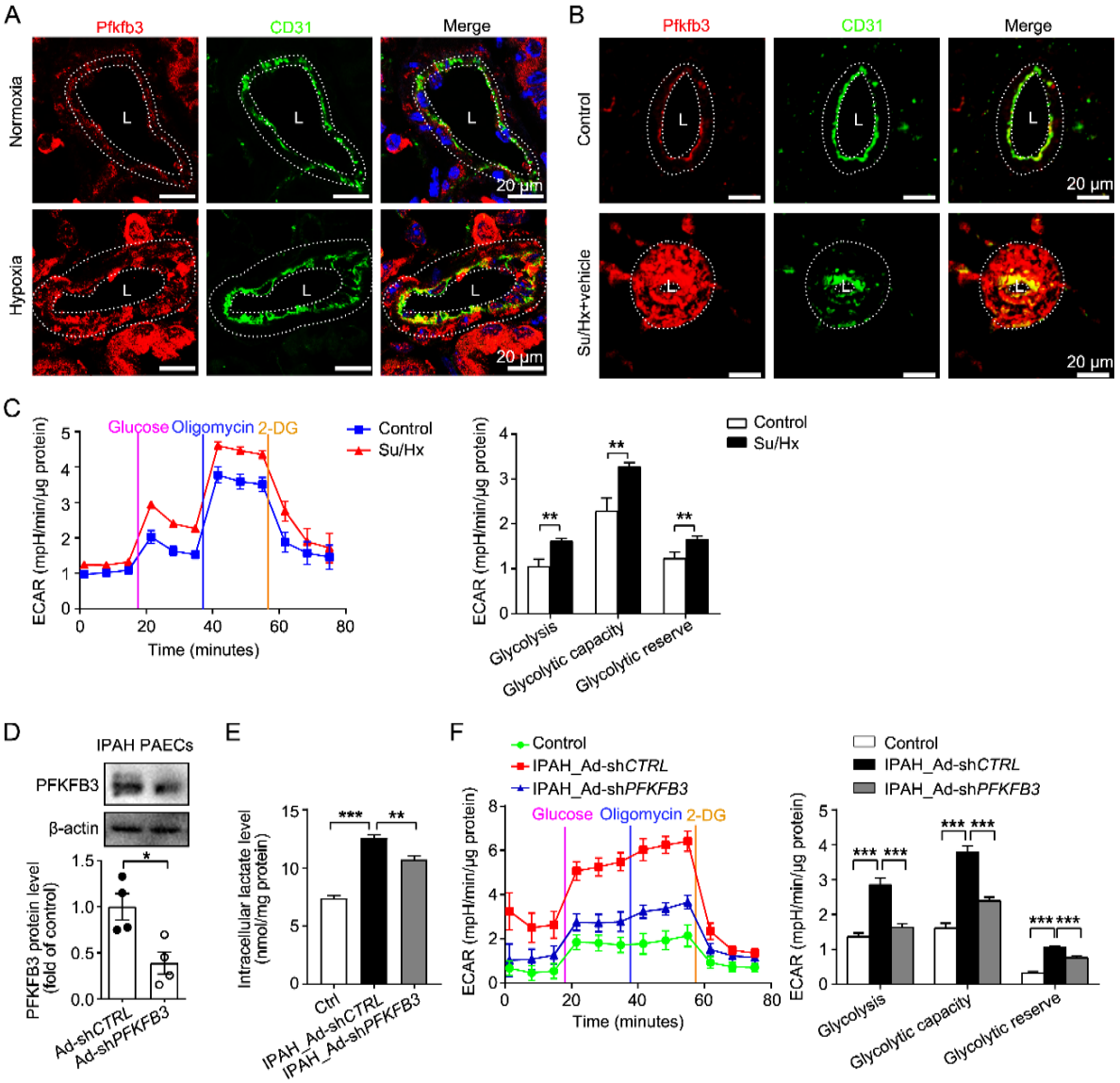

Figure S3. Pfkfb3 expression level is significantly increased in pulmonary endothelium of mice and rats with PH. (*A*) Representative micrographs of Pfkfb3 expression in the pulmonary endothelium of control (normoxia) or PH (hypoxia) mice. Sections were co-stained for Pfkfb3 (*red*) and CD31 (*green*). L, lumen. Scale bar, 20 μm. (*B*) Representative micrographs of Pfkfb3 expression in the pulmonary endothelium of control or Su/Hx PH rats. Sections were co-stained for Pfkfb3 (*red*) and CD31 (*green*). L, lumen. Scale bar, 20 μm. (*C*) (*left*) ECAR profile showing glycolytic activity in PAECs of control and Su/Hx rats. Vertical lines represent the time of addition of glucose (10 mmol/L), oligomycin (1 μmol/L), and 2-DG (50 mmol/L). (*right*) Quantification of glycolytic function parameters. (*D*) Western blot analysis and densitometric quantification of PFKFB3 protein levels in IPAH patient PAECs transfected with Ad-sh*CTRL* or Adsh*PFKFB3*. n = 4. (*E*) Intracellular lactate levels from Ad-sh*CTRL* and Ad-sh*PFKFB3*-transfected PAECs of control subjects or patients with IPAH. (*F*) (*left*) ECAR profile showing glycolytic activity in Ad-sh*CTRL* and Ad-sh*PFKFB3*-transfected PAECs of control subjects or patients with IPAH. Vertical lines represent the time of addition of glucose (10 mmol/L), oligomycin (1 μmol/L), and 2-DG (50 mmol/L). (*right*) Quantification of glycolytic function parameters. All data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t* test (for C), Mann-Whitney *U* test (for *D*) and one-way ANOVA followed by Bonferroni test (for *E* and *F*). * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$.

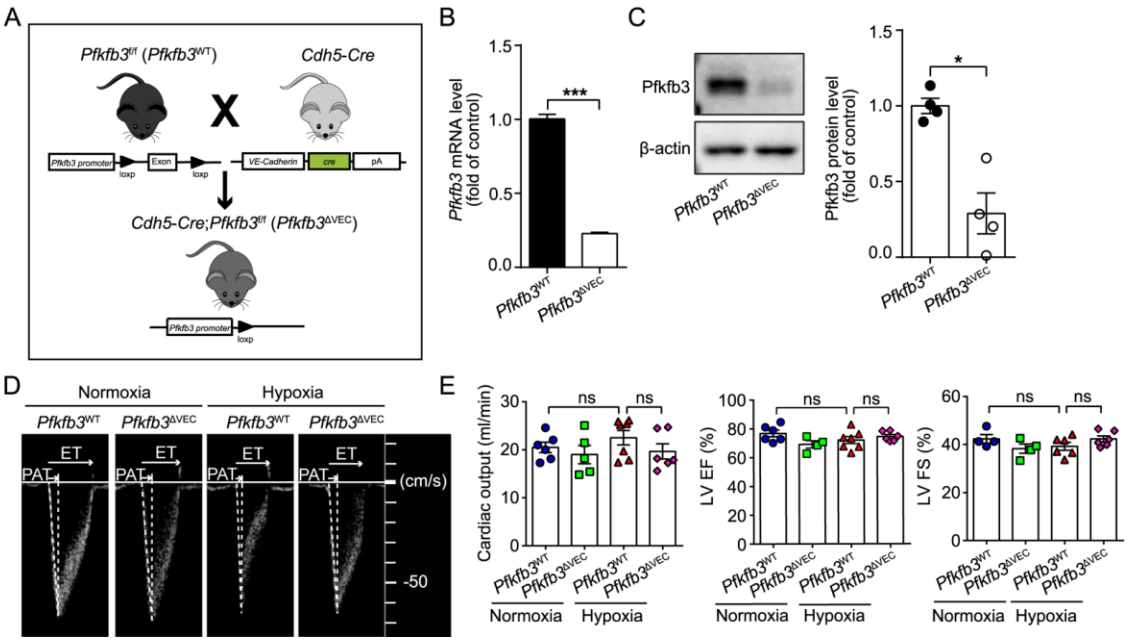

Figure S4. Endothelial specific *Pfkfb3* deficiency in mice ameliorates the development of hypoxia-induced PH. (*A*) Strategy for generating *Pfkfb3*$^{\Delta VEC}$ mice by crossing *Pfkfb3*$^{f/f}$ mice with *Cdh5-Cre* mice. (*B*) Real time-PCR analysis of mRNA levels of *Pfkfb3* in PAECs isolated from *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice. n = 6. (*C*) Western blot analysis and densitometric quantification of Pfkfb3 protein level in PAECs of *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice. n = 4. (*D*) Representative echocardiographic images of pulsed wave Doppler of pulmonary artery flow. (*E*) Echocardiography analysis of cardiac output, LV EF % and LV FS % of *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice exposed to normoxia or hypoxia (10% O_2) for 4 weeks. n = 4-7. All data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t* test (for *B*), Mann-Whitney *U* test (for *C*) and one-way ANOVA followed by Bonferroni test (for *E*). * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

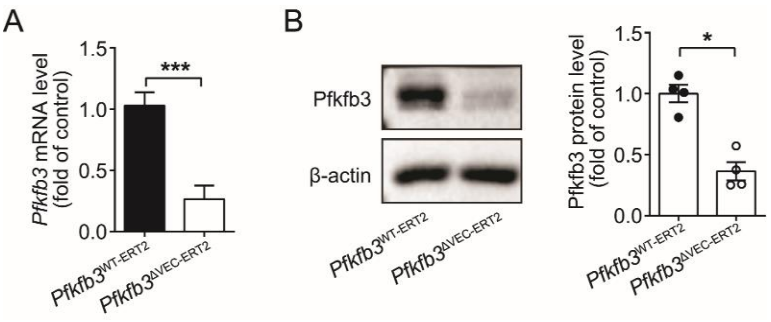

Figure S5. Endothelial *Pfkfb3* was successfully deleted in *Pfkfb3*$^{\Delta VEC\text{-}ERT2}$ mice after tamoxifen administration. (*A*) Real time-PCR analysis of mRNA levels of *Pfkfb3* in PAECs isolated from *Pfkfb3*$^{WT\text{-}ERT2}$ and *Pfkfb3*$^{\Delta VEC\text{-}ERT2}$ mice. n = 6. (*B*) Western blot analysis and densitometric quantification of Pfkfb3 protein level in PAECs of *Pfkfb3*$^{WT\text{-}ERT2}$ and *Pfkfb3*$^{\Delta VEC\text{-}ERT2}$ mice. n = 4. All data are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's *t* test (for *A*) and Mann-Whitney *U* test (for *B*). * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$.

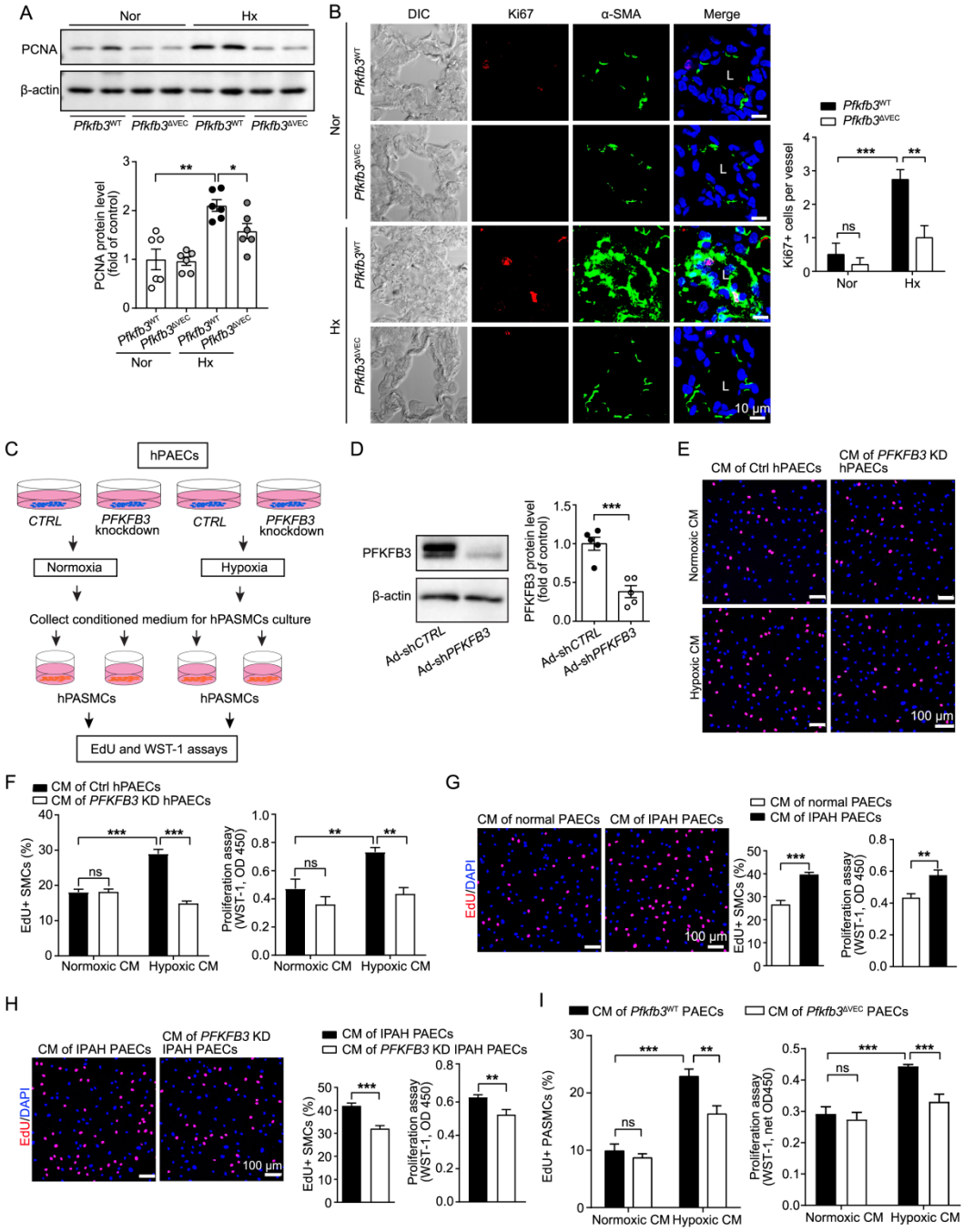

Figure S6. *PFKFB3/Pfkfb3* knockdown or deficiency in PAECs affect proliferation of PASMCs *in vitro*.(*A*) Western-blot analysis and densitometric quantification of PCNA protein levels of lung homogenates from *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice exposed to normoxia or hypoxia (10% O_2) for 4 weeks. n = 6. (*B*) (*left*) Representative images for Ki67 immunostaining of the distal pulmonary arteries of *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice under normoxic or hypoxic conditions for 4 weeks, and (*right*) quantification of Ki67 positive cells per vessel. Lung sections were costained with Ki67 (red), α-SMA (green) and DAPI (blue). L, lumen. Scale bar, 10 μm. n = 5-9. (*C*) Experimental design for analysis of human or mouse PASMC proliferation stimulated with CM of control or PFKFB3/Pfkfb3 knockdown or deficient human or mouse PAECs exposed to hypoxia (1% O_2) or normoxia. (*D*) Western blot analysis and densitometric quantification of PFKFB3 protein levels in human PAECs transfected with Ad-sh*CTRL* or Ad-sh*PFKFB3*. n = 5. (*E*) Representative images of EdU staining and (*F*) quantification of EdU staining and WST-1 assay in human PASMCs exposed to conditioned medium of Ad-sh*CTRL* or Ad-sh*PFKFB3* transfected PAECs exposed to hypoxia for 24 hours. Cells were costained for EdU (*red*) and DAPI (*blue*). Scale bar, 100 μm. (*G*) Representative images of EdU staining and quantification of EdU staining and WST-1 assay in human PASMCs exposed to conditioned medium from PAECs of normal controls

with IPAH. Cells were costained for EdU (*red*) and DAPI (*blue*). Scale bar, 100 μm. Cells were costained for EdU (*red*) and DAPI (*blue*). Scale bar, 100 μm. (*H*) Representative images of EdU staining and quantification of EdU staining and WST-1 assay in human PASMCs exposed to conditioned medium from Ad-sh*CTRL* or Ad-sh*PFKFB3* transfected PAECs of patients with IPAH. (*I*) Quantification of EdU staining and WST-1 assay in mouse PASMCs exposed to conditioned medium of *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ PAECs. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test (for A-B, F and I) and unpaired Student's *t* test (for D and G-H). * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

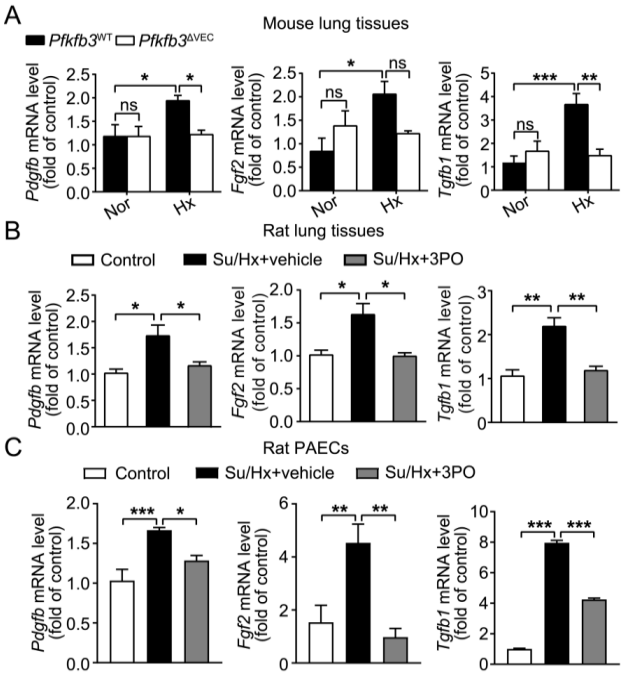

Figure S7. *Pfkfb3* deficiency/inhibition decreases gene expression of growth factors in lungs of mice and rats with PH. (*A*) Real time-PCR analysis of mRNA levels of *Pdgfb*, *Fgf2* and *Tgfb1* in lung homogenates of *Pfkfb3*$^{\Delta VEC}$ and *Pfkfb3*^{WT}mice exposed to normoxia or hypoxia (10% O_2) for 4 weeks. n = 6. (*B*) Real time-PCR analysis of mRNA levels of *Pdgfb*, *Fgf2* and *Tgfb1* in lung homogenates of control and vehicle- or 3PO-treated Su/Hx rats. n = 6. (*C*) Real time-PCR analysis of mRNA levels of *Pdgfb*, *Fgf2* and *Tgfb1* in PAECs isolated from control and treated rats (Su/Hx + vehicle and Su/Hx + 3PO). n = 5. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance. Nor, normoxia. Hx, hypoxia.

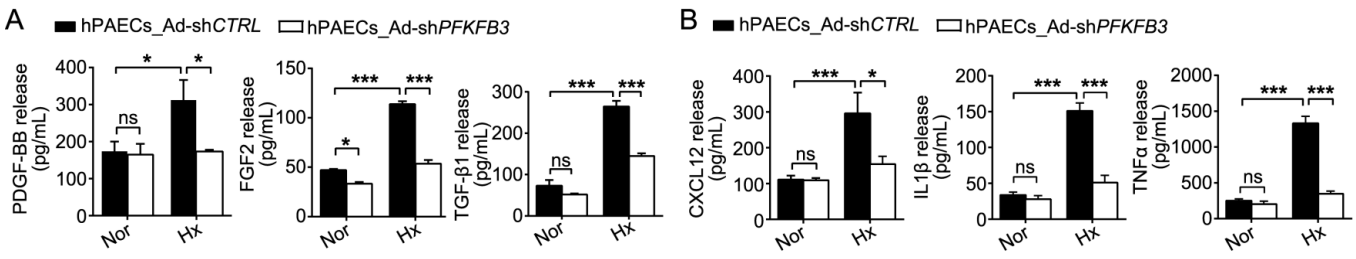

Figure S8. ***PFKFB3* knockdown decreases the hypoxia-induced growth factor and inflammatory cytokine release from hPAECs.** (*A*) Levels of released PDGF-BB, FGF2 and TGF-β1 in the culture supernatants of human PAECs transfected with Ad-sh*CTRL* and Ad-sh*PFKFB3* and exposed to normoxia or hypoxia (1% O_2) for 24 hours. n = 4-6. (*B*) Levels of released CXCL12, IL1β and TNFα in the culture supernatants of human PAECs transfected with Ad-sh*CTRL* and Ad-sh*PFKFB3* and exposed to normoxia or hypoxia (1% O_2) for 24 hours. n = 4-6. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance. Nor, normoxia. Hx, hypoxia.

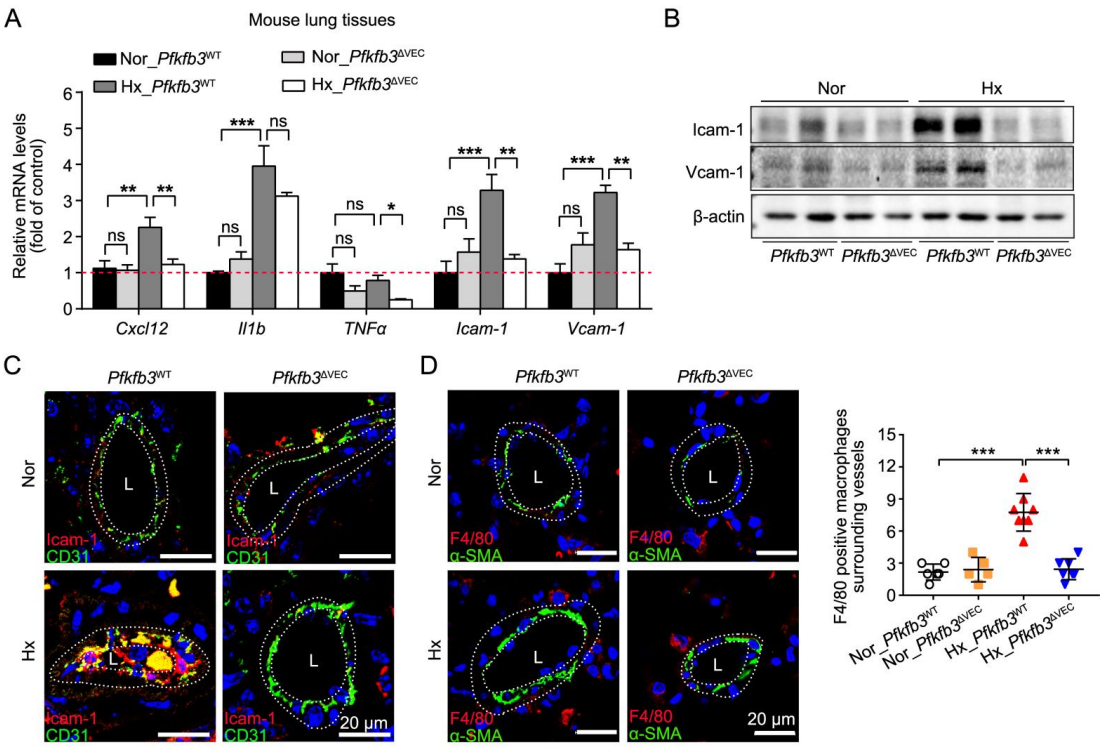

Figure S9. Endothelial *Pfkfb3* deficiency decreases the hypoxia-induced inflammation in mouse lungs. (*A*) Real time-PCR analysis of mRNA levels of *Cxcl12*, *Il1b*, *TNFα*, *Icam-1* and *Vcam-1* in lung homogenates of *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice exposed to normoxia or hypoxia (10% O_2) for 4 weeks. n = 6. (*B*) Western blot analysis of Icam-1 and Vcam-1 protein levels of lung homogenates from *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice exposed to normoxia or hypoxia (10% O_2) for 4 weeks. (*C*) Representative micrographs of *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mouse pulmonary vessels stained for Icam-1 (red), or CD31 (green), and counterstained with DAPI (blue). L, lumen. Scale bar, 20 μm. (*D*) Representative immunostaining (*left*) showing macrophages around pulmonary arteries in *Pfkfb3*^{WT} and *Pfkfb3*$^{\Delta VEC}$ mice exposed to normoxia or hypoxia (10% O_2) for 4 weeks. Sections were costained for F4/80 (red), or α-SMA (green) and counterstained with DAPI (blue). (*right*) Quantification of the infiltrated F4/80-positive macrophages around pulmonary arteries. n = 5-7. L, lumen. Scale bar, 20 μm. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance. Nor, normoxia. Hx, hypoxia.

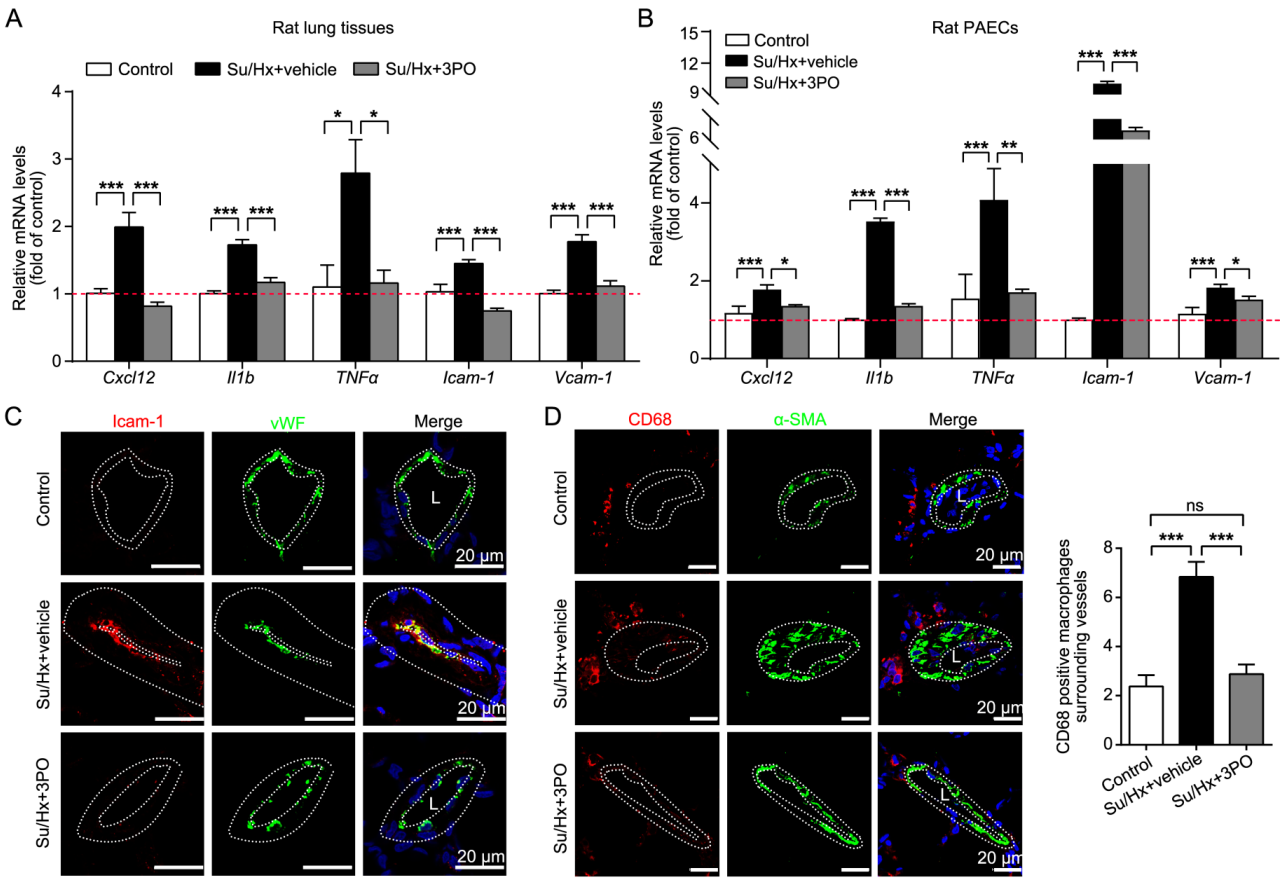

Figure S10. **Pfkfb3 inhibition reduces lung inflammation in Su/Hx rats.** (*A*) Real time-PCR analysis of mRNA levels of *Cxcl12*, *Il1b*, *TNFa*, *Icam-1* and *Vcam-1* in lung homogenates of vehicle or 3PO-treated Su/Hx rats. n = 6. (*B*) Real time-PCR analysis of mRNA levels of *Cxcl12*, *Il1b*, *TNFα*, *Icam-1* and *Vcam-1* in PAECs isolated from control rats and treated rats (Su/Hx + vehicle and Su/Hx + 3PO). n = 6. (*C*) Representative micrographs of pulmonary vessels stained for Icam-1 (red), vWF (green), and DAPI (blue). Sections were from vehicle and 3PO-treated Su/Hx rats. L, lumen. Scale bar, 20 μm. (*D*) (*left*) Representative immunostaining showing macrophages around pulmonary arteries in vehicle and 3PO-treated Su/Hx rats. Lung sections were co-stained with CD68 (red), α-SMA (green) and DAPI (blue). (*right*) Quantification of the infiltrated CD68-positive macrophages around pulmonary arteries. n = 6. L, lumen. Scale bar, 20 μm. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

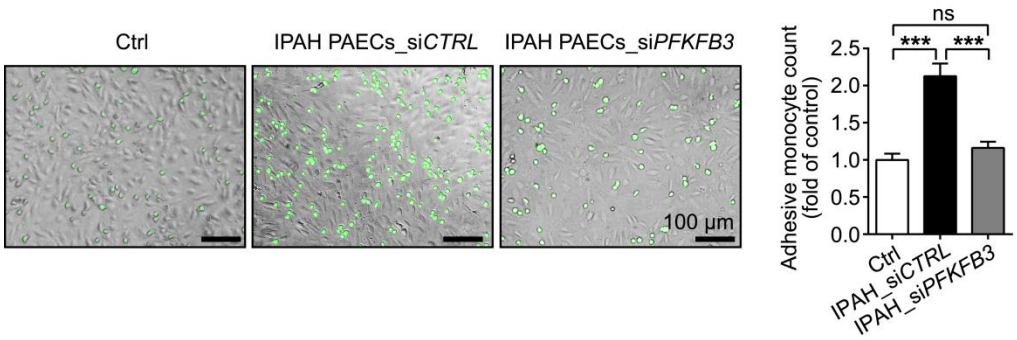

Figure S11. ***PFKFB3* KD in PAECs reduces monocyte adhesion to PAECs.** (*left*) Representative images and (*right*) quantification of monocyte adhesion to normal or si*CTRL* and si*PFKFB3* transfected PAECs of patients with IPAH. n = 4. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance.

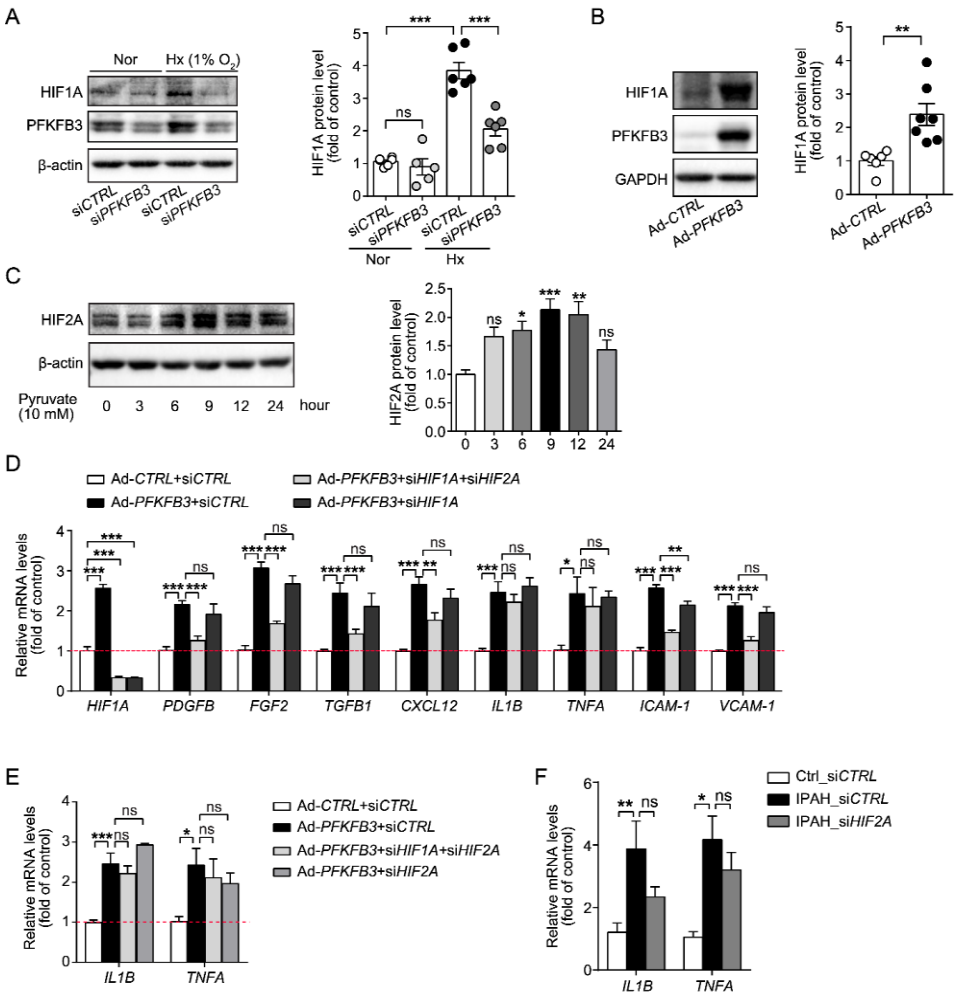

Figure S12. **The role of HIFs in PFKFB3-mediated production of growth factors and proinflammatory cytokines in PAECs.** (*A*) Western blot analysis and densitometric quantification of HIF1A protein levels in human PAECs transfected with si*CTRL* and si*PFKFB3* under normoxia and hypoxia (1% O_2) conditions for 6 hours. n = 5-6. (*B*) Western blot analysis and densitometric quantification of HIF1A protein levels in human PAECs transfected with Ad-*CTRL* and Ad-*PFKFB3*. n = 6 for Ad-*CTRL group*. n = 7 for Ad-*PFKFB3* group. (*C*) (*left*) Western blot analysis and (*right*) densitometric quantification of HIF2A protein levels in human PAECs stimulated with 10 mM pyruvate for time indicated. n = 5. (*D*) Real time-PCR analysis of mRNA levels of *HIF1A, PDGFB, FGF2* and *TGFB1*, *CXCL12*, *IL1B, TNFA, ICAM-1*, and *VCAM-1* in human PAECs transfected with Ad-*CTRL*-si*CTRL*, Ad-*PFKFB3*-si*CTRL*, Ad-*PFKFB3*-si*HIF1A*+si*HIF2A*, Ad-*PFKFB3*-si*HIF1A*. n = 6. (*E*) Real time-PCR analysis of mRNA levels of *IL1B* and *TNFA* in human PAECs transfected with Ad-*CTRL*-si*CTRL*, Ad-*PFKFB3*-si*CTRL*, Ad-*PFKFB3*-si*HIF1A*+si*HIF2A*, Ad-*PFKFB3*-si*HIF2A*. n = 6. (*F*) Real time-PCR analysis of mRNA levels of *IL1B* and *TNFA* in normal or IPAH PAECs transfected with si*CTRL* or si*HIF2A*. n = 6. Experiments were repeated 4 times independently with cells from 4 patients with IPAH or control subjects. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test (for *A* and *C*-*F*) and unpaired Student's *t* test (for *B*). * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance. Nor, normoxia. Hx, hypoxia.

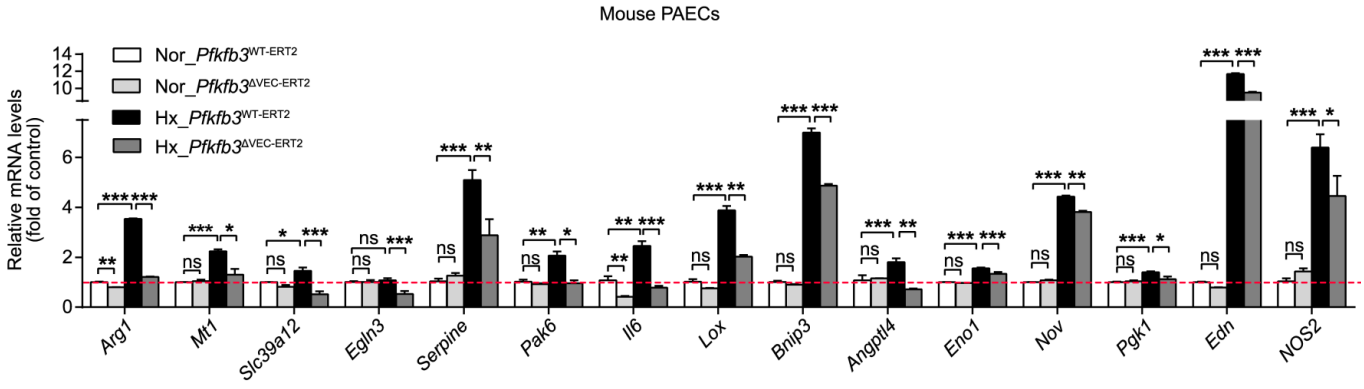

Figure S13. ***Pfkfb3*** **deficiency decreases expression of Hif2a-targeted genes in mouse PAECs.** Real time-PCR analysis of mRNA levels of Hif2a-targeted genes in PAECs of *Pfkfb3*$^{\text{WT-ERT2}}$ and *Pfkfb3*$^{\Delta\text{VEC-ERT2}}$ mice exposed to normoxia or hypoxia (10% O_2) for 6 weeks. n = 6. All data are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni test. * $p < 0.05$ was considered significant, ** $p < 0.01$, *** $p < 0.001$. ns, no significance. Nor, normoxia. Hx, hypoxia.

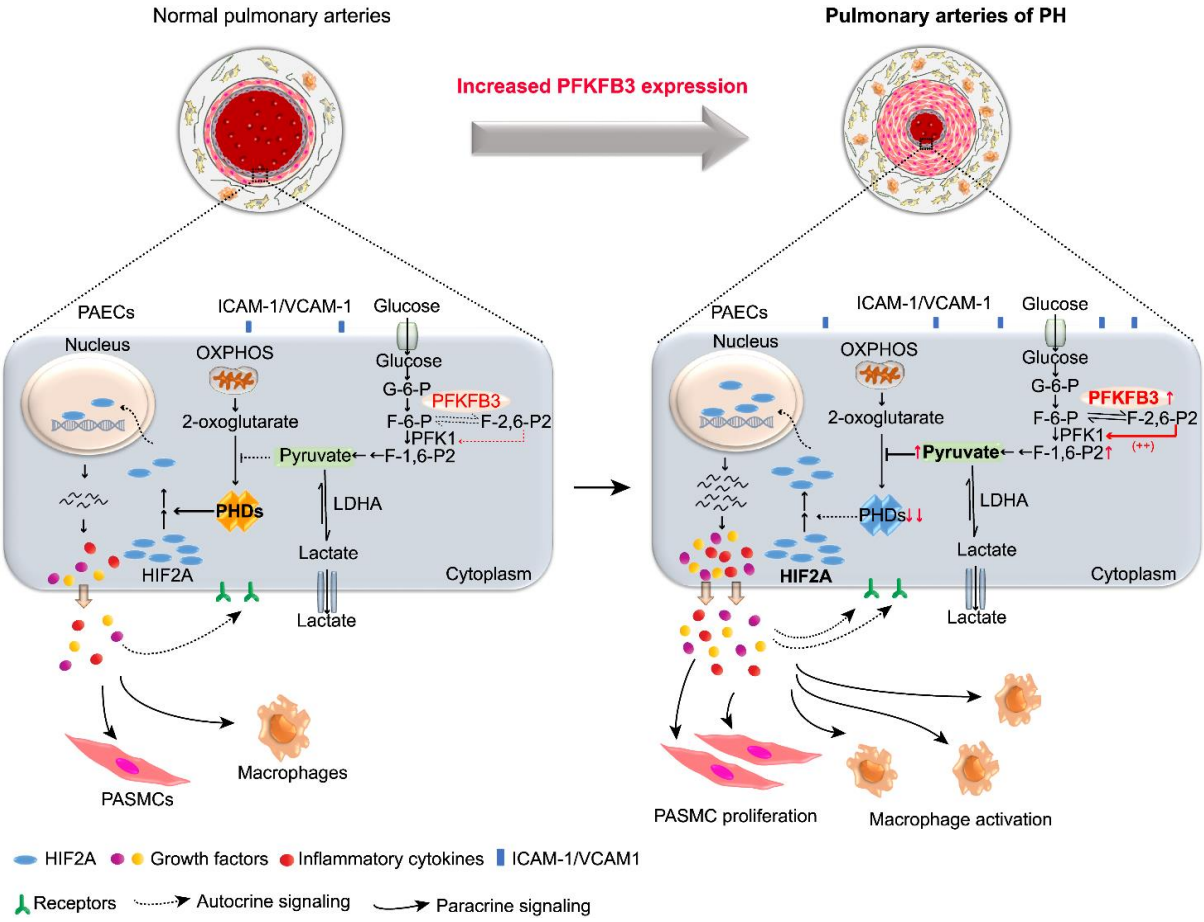

Figure S14. Schematic diagram indicates how endothelial-PFKFB3-mediated glycolysis affects the development and progression of PH. Enhanced expression and activity of endothelial PFKFB3 increase production of glycolytic metabolites including pyruvate. Prolyl hydroxylase domains (PHDs) are members of the 2-oxoglutarate-dependent family of dioxygenases, which require oxygen, ferrous iron, and 2-oxoglutarate as the substrates for catalytic activity. As pyruvate is structurally similar to 2-oxoglutarate, pyruvate can competitively bind to the 2-oxoglutarate site of PHDs, leading to decreased PHD activity and subsequent suppressed hydroxylation and degradation of HIF2A. An increased level of HIF2A enhances expression of many growth factors, proinflammatory cytokines, and adhesion molecules in endothelial cells. All these factors contribute to the development and progression of PH. G-6-P, glucose-6-phosphate. F-6-P, fructose-6-phosphate. PFK-1, phosphofructokinase 1. F-1,6-P2, fructose-1,6-bisphosphate. F-2,6-P2, fructose-2,6-bisphosphate. LDHA, lactate dehydrogenase A. OXPHOS, oxidative phosphorylation.