Supplemental File

METHODS

Animals. All procedures with animals in this study were performed according to approved protocols under the supervision of the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus (Aurora, CO). All mice were C57bl6/J background. The experiments in mice began when they were 12 weeks old. Sprague Dawley (SD) male rats weighing 210-245 grams were used for rat experiments.

Transgenic mice and genotyping.

Hif1α floxed mice (1), *Hif-2α* floxed mice (2) were constructed wherein exon 2 (coding DNA binding domain) of the Hif1α or Hif2α gene was floxed with LoxP sites. *UbcCreERT*$^{+}$ mice express a fusion protein consisting of cre recombinase and the human estrogen receptor binding domain under control of a ubiquitously active Ubiquitin-C gene promoter, in which Cre recombinase is activated upon tamoxifen exposure. Crossing of Hif1α$^{fl/fl}$ or Hif2α$^{fl/fl}$ mice with UbcCreERT^{+} mice generated *Hif1α$^{fl/fl}$;UbcCreERT*$^{+}$mice, *Hif2α$^{fl/fl}$;UbcCreERT*$^{+}$ mice and *Hif2α$^{fl/WT}$;UbcCreERT*$^{+}$ mice, in which tamoxifen treatment completely or partially deleted *Hif1α* or *Hif2α* gene in all cell types in adult mice (See Suppl Fig 1A-C for breeding information and Suppl Table 1 for Hifa reduction efficiency). Endothelial cell–specific knockout of *Hif2α* was generated by crossing *Hif2α$^{fl/fl}$* mice to mice expressing cre recombinase under the control of the VE-cadherin promoter (from the Jackson laboratory, kindly provided by Dr. Eltzschig's laboratory, University of Colorado, Denver, CO, USA) (See Suppl Fig 1D for breeding information)(3). In all cases, mouse genotypes were determined using real time PCR of tail genomic DNAs by a

commercial vendor (Transnetyx, Cordova, TN, USA). Age-matched (about 12 wk old), male and female mice with the desired genotypes were used for experimentation.

Tamoxifen treatment in mice. To delete Hif1α or Hif2α gene in adult mice, *UbcCreERT*$^{+}$ or *UbcCreERT*$^{-}$ (as control) mice were injected with 100 µl of tamoxifen solution (in peanut oil at 10mg/ml) i. p. once daily over five consecutive days. Mice were then allowed to rest for one week before being exposed to hypobaric hypoxia or normoxia.

Antisense oligonucleotide treatment in mice*. Hif1α* antisense oligonucleotide (henceforth *Hif1α*-ASO; 75mg/kg; 298745-11) or *Hif2α-ASO* (50mg/kg; 589983-5) or non-specific ASO (control-ASO; 549148-18) was injected intraperitoneally twice per week, beginning one week before the hypoxia exposure and continued during the hypoxia exposure. ASOs were provided by Ionis Pharmaceuticals, Inc. (Carlsbad, CA, USA). See Suppl Table for HIF1α and HIF2α mRNA reduction rate.

Rats treated with HIF2 inhibitor PT2567. Sprague Dawley (SD) male rats weighing 210-245 grams (Charles River Laboratories) were housed in chambers under normoxia or hypoxic (high altitude ~ 18,000 feet) conditions for four days or four weeks. Rats were dosed with vehicle methylcellulose (0.5%)/Tween-80 (0.5%) or PT2567 (300 mg/kg/day) (4), beginning the day they were placed in chambers. PT2567 was provided by Peloton Therapeutics, Inc.

Exposure to chronic hypoxia*.* Mice or rats were housed for indicated times (5 weeks for mice, 4 days or 4 weeks for rats) in chambers equipped for continuous hypobaric hypoxia (0.5 atm, equivalent to 5,486m/18,000 feet altitude and corresponding to FiO_2 0.1). Chambers were vented to room atmosphere weekly for cage maintenance (twice weekly in case of ASO administration). Control mice were housed in identical chambers

at sea level atmosphere (1.0 atm, 0m altitude, FiO_2 0.21).

Measurement of right ventricular systolic pressure in mice and mean pulmonary arterial pressure in rats. RV pressure measurements were performed essentially as previously described (5). Briefly, mice were anesthetized with isoflurane. The RV was directly catheterized with a fluid-filled catheter interfaced with a pressure transducer connected to a dedicated computer. Trains of successive pressure waveforms were analyzed and averaged over three seconds to determine RV systolic pressure (RVSP). MPAP measurements were performed as described (6). Additional measurements were performed in ASO-experiments utilizing a 1.4 French Pressure Volume Conductance System SPR-839 (Millar Instruments, Houston, TX) inserted into the RV via a surgical transdiaphragmatic approach.

Tissue preparation, hematocrit measurement, Fulton index and weight ratios. Animals were deeply anesthetized. Blood samples were taken by left ventricular puncture and the hematocrit was measured using glass capillaries and a suitable centrifuge (IEC, model MB micro-capillary centrifuge). Lung vasculature was flushed with PBS through the RV after incision of the left atrium. For immunohistochemical analysis, left lungs were inflated with agarose in situ and fixed in paraformaldehyde (4%) after excision. Left lungs were then paraffin embedded and cut into 5 µm sections. Right lung lobes were excised, snap frozen in liquid nitrogen and stored at -80°C for RNA extraction to conduct pulmonary gene expression analysis. To determine right ventricular hypertrophy, the atria were removed from the heart. Subsequently, the heart was dissected into sections of RV free wall and LV free wall including the septum (LV+S) or LV only. Heart tissue was patted dry, weights were taken and the Fulton index (Fulton index = $Weight_{RV}/Weight_{LV+S}$) or

$Weight_{RV}/Weight_{LV}$ and the ratio of $weight_{RV}$/bodyweight were calculated. After taking weights, heart tissue was snap frozen in liquid nitrogen and stored at -80°C for RNA extraction to conduct right ventricle gene expression analysis.

Immunohistochemistry of lung tissue. Vascular remodeling was quantified by immunohistochemical staining with specific antibody and hematoxylin counterstaining as previously described (5). Alpha-smooth muscle actin antibody (αSMA) (Abcam ab 66133 rabbit polyclonal antibody; dilution 1:400) was used to assess vessel muscularization. Using a Nikon microscope, twelve fields (1μm^2 each) per lung were inspected and muscularized vessels (αSMA+) were counted. Anti-ED1 antibody, anti-Ki67 antibody, or anti-Tenascin C antibody was used to assess monocyte/macrophage accumulation, cell proliferation, or vessel remodeling respectively.

Cell culture and treatments. Normal human pulmonary artery endothelial cells and smooth muscle cells were provided by Pulmonary Hypertensive Breakthrough Initiative. Human ECs were cultured in endothelial cell basal medium plus supplements (Cat#: CC-3202, Lonza Walkersville, MD USA). Human SMCs were cultured in smooth muscle cell basal medium plus supplements (Cat#: CC-3182, Lonza Walkersville, MD USA). Human pulmonary artery Fibs are from control donors undergoing lobectomy or pneumonectomy at Papworth Hospital, Cambridge, UK and were cultured in fibroblast growth medium (DMEM) (15-018-CV, Corning, VA USA) supplemented with fetal bovine serum (FBS) (10%, Cat#: 100-106, Gemini, CA USA) and Penicillin-Streptomycin (P/S) (30-002-CI, Corning, VA USA), MEM Nonessential Amino Acids (25-025-CI, Corning, VA USA) and L-Glutamine (4mM, 25-005-CI, Corning, VA USA). When hypoxia treatment was needed, cells were placed in normoxia (Nx, 21% O2) or hypoxia (Hx, 1.5% O2) for 16 h for HIF

target gene studies, cultured under regular 10% FBS medium. To assess the role of HIF1 or HIF2 in the increased expression of HIF target genes in hypoxic EC cells, ECs were treated with HIF2 inhibitor PT2567 at concentration of 0.1, 1. or 10 μM or targeted with siRNA against Hif2*α* or Hif1*α* mRNA.

Gene expression analysis*.* After tissue disruption and homogenization using a bead-mill system, RNA from organs such as lung and heart, or cells was isolated using RNeasy column kits (Qiagen). Synthesis of cDNA was performed using a commercially available kit according to the manufacturer's instructions (iScript Advanced cDNA Synthesis Kit, Bio-Rad, Hercules, CA, USA). Quantification of messenger RNA levels was performed by reverse transcription quantitative PCR (RT–qPCR) using iQ Universal SYBR Green Supermix (Bio-Rad) and CFX384 Real Time System (Bio-rad). All primer sets for RT–qPCR were validated for their specificity and amplification efficiency (85% to 110%) using melt curve analysis, RT–qPCR product sequencing and standard dilution analysis. Primer sequences are listed in Suppl. Table 6. The qPCR results were analyzed using the ΔΔCT method using 18S ribosomal RNA and beta actin messenger RNA as reference genes and presented in relative to samples from controls. For Fig. 3A) and Suppl. Fig. 8), commercially TaqMan probes and reagents were used (Applied Biosystems, Grand Island, NY), according to the manufacturer's instructions. At least three independent experiments were performed for all in vitro results presented in this paper.

Statistics*.* In general, data is reported as mean +/- SEM. Statistical differences were evaluated by 2-way ANOVA test as well as an unpaired, two-tailed t-test. Figure legends specified the statistical analysis used for the data in each panel. Star (*) is used to indicate the difference between hypoxia versus normoxia in the same genotype (or treatment)

group. # is used to show the differences between genotypes or treatments under hypoxic condition while ^ is used to mark the differences between genotypes or treatments under normoxic condition. Survival curves were compared by log-rank test. $P<0.05$ (* or # or ^) was considered statistically significant, other levels of statistical significance are reported as $p<0.01$ (** or ## or ^^) and $p<0.001$ (*** or ### or ^^^). Group size (n) is reported in the corresponding figure legends. Data was logged using Excel (Microsoft Corp.), graphing and calculations were performed using Graphpad Prism (GraphPad Software Inc.) and figures were designed using Powerpoint (Microsoft Corp.).

References

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