Supplemental Materials

Altered Smooth Muscle Cell Histone Acetylome and its Regulation in Pulmonary Hypertension

Materials & Methods:

The primary antibodies Ac-H3K9 (9649T), total H3 (9715S) and KLF4 (4038S) were from Cell Signaling Technology (CST) (Danvers, MA). Two SPHK2 antibodies, SPHK2 (32346) was purchased from Cell Signaling Technology (CST) (Danvers, MA) and SPHK2 (17096-1-AP) was purchased from Proteintech (Rosemont, IL). The phospho-SPHK2 (Thr614) antibodies from PA5-39812 (Thermo Fischer Scientific) and ab212750 (Abcam) were utilized. Alexa fluorescent labeled secondary antibodies and phalloidin were purchased from Thermo Fischer Scientific (Waltham, MA). Alpha-tubulin was from Abcam and Lamin B was purchased from Santa Cruz (Dallas, TX). HRP-conjugated rabbit and mouse secondary antibodies were from CST. ABC294640 (iSPHK2: 10 μM for 1 hour) was purchased from Echelon Biosciences Inc. (Salt Lake City, UT). Knockdown experiments were performed using ON-TARGETplus siRNAs specific for SPHK2 or non-targeting control (GE Dharmacon, Lafayette, CO) and INTERFERin transfection reagent from Polyplus transfection (New York, NY) per manufacturer's instructions. CUT&RUN assay was performed using SNAP-ChIP validated antibodies and the kit from EpiCypher (Durham, NC).

Human iPAH (Group 1 PH) and FDL lung tissues and EFPE slides

All studies involving human lung tissues and cells were conducted according to the Institutional review board of Indiana University School of Medicine. Deidentified archived tissue obtained from the Pulmonary Hypertension Breakthrough Initiative (PHBI) tissue biorepository from patients with iPAH at time of lung transplantation and patients without PH that were failed donor lungs (FDL) (Table 1) included snap-frozen peripheral lung tissues and Ethanol Fixed Paraffin embedded (EFPE) slides. According to the PHBI

biorepository (https://ipahresearch.org/phbi-research/), tissues were obtained in the operating room and immediately snap frozen at the collection site, cataloged, and stored to maintain tissue integrity. According to the PHBI biorepository (https://ipahresearch.org/phbi-research/), cells were isolated from the left lower lung, phenotypically characterized, and frozen in aliquots for distribution to PHBI biorepository requests. Tissue for histology were ethanol fixed paraffin embedded (EFPE) slides as per the PHBI biorepository(https://ipahresearch.org/phbi-research/).

Experimental PH Mouse model

Animal experimental procedure was performed in accordance with the guidelines issued by the University of Notre Dame/Indiana University Institutional Animal and Use Committee. Mice were maintained in a controlled pathogen free animal facility at all times with 12h light/dark cycles at a temperature of 22-24° Celsius with routine check on animal status and were provided standard irradiated mouse chow ad lib. In the experimental rodent pulmonary hypertension (PH) model, 12-14 wk old mice of SPHK2-deficient [*SPHK2 KO*] mice in C57BL/6NJ background[1] and age-matched C57BL/6NJ[2] (WT) control mice were exposed to hypoxia (10% O_2) in a ventilated chamber or normoxia for 21 days. To avoid possible hormonal issues, only male mice were used. Randomization on 12-14 week littermate cohorts was performed by using a coin flip methodology and distribution of littermates across all experimental groups and none of these animals were excluded.

Echocardiography of PAH mice to non-invasively assess pulmonary vascular resistance and pulmonary acceleration time:

The mice were lightly anesthetized using isoflurane anesthesia at 3-4% induction and 1-3% isoflurane maintenance to maintain the heart rate at 300-500 beats/minute to perform transthoracic echocardiography. Mice were placed in the supine position on a temperature-controlled mouse pad. Any hair on the anterior chest was removed. Cardiac function was analyzed via echocardiography using the VisualSonics Vevo 770 ultrasound machine (FUJIFILM VisualSonics, Inc, Toronto, Ontario, Canada). The transducer probe (45 MHz, VisualSonics Model 707B) was applied to the anterior chest first in a parasternal long axis view, and in B mode setting of the ultrasound machine, a full view of the left ventricle (LV) was obtained. Then, the ultrasound was switched to M mode to assess the movement of structures over time. The ultrasound was placed back in B mode and the parasternal short axis view was obtained by placing the transducer probe 90° rotated in clockwise from the parasternal long axis view. The right atrium was focused, and the ultrasound was switched to the color Doppler mode. The pulse wave (PW)-line of the ultrasound was placed over the tricuspid valve to measure flow through the tricuspid valve.

All procedures and analysis were performed in a blinded manner. Blind-analysis was performed to assess the maximum velocity of tricuspid regurgitation (TR_{max} velocity) and the velocity time integral of the right ventricular outflow tract (VTI_{RVOT}) using Vevo 770 protocol-based measurements and calculations software. Pulmonary vascular resistance (PVR) was calculated using the following equation[3].

$$PVR = TR_{max} \text{ velocity}/VTI_{RVOT}$$

The mean pulmonary arterial pressure (MPAP) in mice cannot be accurately measured using the Vevo ultrasound using human clinical equations due to the drastic heart rate differences between two species. Therefore, the pulmonary acceleration time (PAT) was assessed which inversely and linearly correlates with MPAP[3,4].

Assessment of Fulton Index in PAH mice

Right ventricular hypertrophy was calculated using Fulton index by the weight ratio of the right ventricle (RV) divided by the sum of left ventricle and septum (LV + S).

$$\text{Fulton Index} = RV / [LV + S]$$

Mouse Lung Microscopy and Morphometry Analysis

Lung tissue sections of 5 microns were prepared as described previously[5]. Five-micron sections were subjected to elastin staining and images were taken at 100 micrometer scale. The peripheral vessel muscularization is expressed as the number of partially or fully muscularized vessels (< 50 microns) at alveolar duct per field of 100 alveoli in 3 fields in each animal.

The vascular wall thickness was calculated from elastin–stained sections using the following equation.

$$\text{Wall thickness (\%)} = (2 \times \text{medial wall thickness} / \text{external diameter}) \times 100$$

Human Lung Microscopy Analysis

Tissue for histology were ethanol fixed paraffin embedded (EFPE) slides as per the PHBI biorepository (https://ipahresearch.org/phbi-research/) and sectioned at 5 microns. Following deparaffined and rehydrated protocol: slides were incubated at 65°C for 30 min. followed by Xylene (5 min x 2), 100% EtOH (1 min x 2), 95% EtOH (1 min x1), 70% EtOH (1 min x1), and 50% EtOH (1 min x1) in deionized water, sections were washed with PBS x 3, incubated with peroxidase blocking reagent (3% H_2O_2 solution or 10 min), alkaline phosphatase blocking reagent (2 mM levimasole in PBS for 10 min) followed by rinsing the slides x2 with distilled water. Antigen retrieval was performed using a 6.0 pH Citrate buffer solution as per manufactures protocol (Life technologies Cat# 005000) and then rinsed with TBS-T. Following blockade for non-specific proteins with CAS-block™ solution (Life Technologies #00-8120) for 20 min, the TripleStain IHC kit R&R&M on human (Abcam Ab183290) was utilized per manufactures protocol employing only the R&M components. Secondary antibody only controls were used to assess background and selectivity of staining. pSPHK2 (1:50, ThermoFischer Scientific PA5-39812) and EMAP II (1:100, Santa Cruz Sc-32723) incubation overnight in CAS block at 4°C, followed by rinsing with TBS-T, incubation with Rabbit AP Polymer and Mouse HRP polymer mixture for 30 min, TBS-T rinse followed by revealed with Permanent Red Substrate, rinsing with distilled water, and incubation with Emerald Chromogen for 5 minutes. Slides were washed in tap water for 1 minute, rinsed with distilled water, air dried, and dehydrated with 85% EtOH (1 x 20 seconds), 95% EtOH (1 x 20 seconds), 100% EtOH (3 x 30 seconds), and 100% xylene (1 x 20 seconds). Samples were immediately mounted in

non-aqueous mounting medium. Images were obtained on an BX50 light / fluorescent microscope with DP-70 digital capture system.

EMAP II preparation

6x-His tagged EMAP II was prepared as previously described[35]. The endotoxin levels in all preparations used for this study were below detectable limits, containing <0.1 ng/EU (GenScript).

RNA extraction and quantification

Total RNA was extracted with TriZol from human or mouse lung tissues using Direct-zol™ RNA MiniPrep kit (Zymo Research, R2050) as per manufacturer's instructions and from human pulmonary arterial smooth muscle cells (hPASMC) using standard RNA extraction protocol. Extracted RNA (around 0.5 µg) was reverse transcribed with SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, 18080044) as per manufacturer's instructions. *SPHK2* (PrimePCR™ SYBR® Green Assay, BioRad, Human (qHsaCED0037773), Mouse (qMmuCED0039969)), *KLF4, SOX2, 18S rRNA* and *Hprt1* transcripts were quantified using SsoAdvanced™ Universal SYBR® Green Supermix from BioRad. *18S rRNA* was used as the internal control for human data and results were also validated with *Actin* as an internal control. *Hprt1* was used as the internal control for mouse tissue data and results were also validated with *Ppia* as an internal control. Primers are given in supplementary Table 2. Denaturation condition of 95^0C for 15 sec and annealing condition of 60^0C for 30-60sec were used. 40 cycles were performed. dC_t

was calculated against the internal control for each target gene and dCt was normalized to the average dC_t of each group to obtain ddC_t.

Cell culture and treatments

Primary human pulmonary artery smooth muscle cells (hPASMCs), human pulmonary microvascular endothelial cells (hPMVECs) were purchased from Lonza (Walkersville, MD) and iPAH: PASMCs were cultured in complete growth medium or conditioned media in a humidified atmosphere or 1% O_2 (BioSpherix ProOx110) with 5% CO_2 at 37° C. For all studies, passages 5–10 were used for hPASMCs, hPMVECs and iPAH: PASMCs. For treatment studies, subconfluent cells plated in multi-well plates were serum starved and if required, pretreated with inhibitors before stimulation with 2 µg/mL recombinant EMAP II[6]. All the controls were handled similar to the test samples. Cells were treated with EMAP II, vehicle or pretreatment with SPHK2 inhibitor (iSPHK2: ABC294640 (Echelon Biosciences Inc., Salt Lake City, UT)) of 10 µM for 1 hour prior to EMAPII treatment, rinsed at the desired time point, and lysis buffer was applied.

ELISA for S1P quantification

Equal number of hPASMCs (15000/ cm^3) were plated and serum starved overnight. Cells were pretreated with inhibitors if needed and stimulated during a series of time points and collected. Cells were pelleted and washed with PBS. After the extraction of nuclear proteins, protein concentration was measured. S1P levels were measured using S1P

ELISA kit (MyBioSource, MBS069092) following manufacturer's instructions. S1P levels were normalized against protein levels.

Transfection with Small Interfering RNA

Knockdown of endogenous SPHK2 was carried out by transfecting with 25 nM final concentration of ON-TARGETplus siRNAs specific for SPHK2 or non-targeting control (GE Dharmacon, Lafayette, CO) using INTERFERin (Polyplus, NY) per manufacturer's instructions. Cells were stimulated with EMAP II for desired time post-transfection. Non-targeting controls and SPHK2 knocked-down samples were washed, treated and handled similar to each other.

Immunoblotting

After appropriate treatments, protein lysates were prepared by using RIPA buffer (for cells) or home-made lysis buffer (tissue) supplemented with phosphatase and protease inhibitors (Thermo Fisher Scientific). Alpha-tubulin, actin or vinculin was used as a loading control. The nuclear and cytoplasmic fractionation was performed using NE-PER kit from Thermo Fischer Scientific for cells and Minute™ Cytosolic and Nuclear Extraction Kit from Invent Biotechnologies, MN for tissues. Western blots (8% homemade gels or 4-12% NuPAGE (NP0321BOX) gels were used) or dot blots were performed according to standard methods and quantified using densitometry using Image Studio Lite software from LI-COR Biosciences.

Immunofluorescence staining

Primary hPASMCs cells were seeded in Nunc Lab-Tek™ II 4-well imaging plates (Thermo Fisher Scientific) and cultured in growth medium. After the serum starvation, cells were stimulated with EMAP II. Treated cells were rinsed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.1% Triton X-100 at room temperature for 10 min. After washing with PBS three times, the cells were incubated with pSPHK2 antibody (1:50) at 4° C overnight. The cells were then rinsed with PBS three times and subsequently incubated with respective secondary antibody conjugated with Alexa Fluor 647 and Phalloidin 488 at room temperature for 1 h. The cells were rinsed with PBS three times and coverslips were mounted with SlowFade Gold Antifade Mountant with DAPI (Thermo Fischer Scientific). Secondary antibody only controls were used to assess background and selectivity of staining. The cells were examined under Olympus microscope with 40× water objective lens for pSPHK2.

NanoString nCounter gene expression

Transcripts levels were analyzed in 100 ng of total RNA from human iPAH or non-iPAH lung tissues that were hybridized for 24 hours with probes from the nCounter custom stem cell panel containing 31 target genes and 5 reference genes (NanoString Technologies, Seattle, WA, USA) using nCounter SPRINT profiler (NanoString Technologies) according to the manufacturer's instructions. The raw data were quality controlled and normalized using nSOLVER 4.0 (NanoString Technologies) by performing background subtraction, positive control normalization and normalization to housekeeping.

HDAC assay

Equal number of hPASMCs (15000/ cm^3) were plated and serum starved overnight. Cells were pretreated with inhibitors, stimulated for the desired time points and then collected. Cells were pelleted and then washed with PBS. After the extraction of nuclear proteins, then protein concentration was measured. HDAC levels were measured using HDAC activity flurometric kit (Abcam, ab156064) following manufacturer's instructions. Free unbound HDAC levels were normalized against the protein levels.

CUTANA CUT&RUN, Illumina sequencing, and data analysis

Cleavage Under Targets and Release Using Nuclease (CUT&RUN) was performed with female hPASMCs using CUTANA® protocol v1.6 [www.epicypher.com]. For each condition, nuclei from two biological replicates were extracted by incubating cells on ice for 10 min in Nuclei Extraction buffer (NE: 20 mM HEPES–KOH, pH 7.9; 10 mM KCl; 0.1% Triton X-100; 20% Glycerol; 0.5mM spermidine; 1x complete protease inhibitor (GoldBio). The cell pellet was collected by centrifugation (600 g, 3 min, 4ºC), the supernatant discarded, and the pellet resuspending [100 µl / 500K nuclei] in NE buffer. For each target 500K nuclei were immobilized onto Concanavalin-A beads (EpiCypher #21-1401) and incubated overnight (4º C with gentle rocking) with 0.5 µg of antibody IgG (negative control), trimethylated H3K4 (positive control), Ac-H3K9 (all validated to SNAP-ChIP nucleosome standards). CUT&RUN enriched DNA was purified and used to prepare sequencing libraries with the Ultra II DNA Library Prep kit (New England Biolabs #E7645S). Libraries were sequenced on the Illumina NextSeq 550, obtaining ~8 million paired end reads on average. The CUT&RUN paired reads were used for quality control using FASTQ Groomer (Galaxy Version 1.1.5) and paired ends were aligned against the

GRCh38/hg38 reference genome assembly for human using bowtie2 (Galaxy Version 2.4.2+galaxy0). After quality-based filtering with SAM or BAM tools (Galaxy Version 1.8+galaxy1), MACS2 (Galaxy Version 2.1.1.20160309.6) was applied for CUT&RUN peak calling of Ac-H3K9 against the IgG control with genome size of 2.7e9 and then, DiffBind (Galaxy Version 2.10.0) was used to analyze the differential binding sites between the control and rEMAP II treated samples. Gene Ontology studies were performed using ShinyGO v0.75 (http://bioinformatics.sdstate.edu/go/). ChiPseeker (Galaxy Version 1.18.0+galaxy1) was used for peak annotation and visualization to identify gene sets associated with differentially acetylated sites in different conditions. Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) was used to compare gene lists of differentially acetylated sites.

Data availability

CUT&RUN data are at NCBI-SRA [http://www.ncbi.nlm.nih.gov/bioproject/1010000] with BioSample accessions SAMN37177214, SAMN37177215, SAMN37177216, SAMN37177217.

Statistical analysis

The data are presented as means ±1 standard error of mean (SEM) from at least three independent experiments, if not mentioned otherwise. Each biological replicate has been normalized against the average of control or ‘0’ time point if it is possible. In time-course studies, all the time points were performed on the same day and repeated at least three times over different days. Statistical significance was determined with unpaired Student's

t-test or one-way or two-way ANOVA for experiments with $n \geq 6$ and normality confirmed data sets by Shapiro-Wilk test after log transformation or otherwise Kruskal-Wallis or Kolmogorov-Smirnov non-parametric test for experiments with $n < 6$ using GraphPad Prism software.

Condition	**Gender**	**Number of samples**	**Age range**
FDL	Male	10	41±14
FDL	Female	10	48±9
IPAH	Male	10	35±17
IPAH	Female	10	47±10

Table S1. Human Lung tissue subjects: FDL (n=20) and IPAH (n=20).

Condition	**Gender**	**Age**
Non-iPAH	Male	51
Non-iPAH	Female	50
Non-iPAH	Female	30
iPAH	Male	40
iPAH	Female	40

Table S2. Human Lung Cells utilized: Non-iPAH:PASMCs and iPAH:PASMSc.

Table S3. Primer sequences for quantitative real-time PCR.

Gene	Forward	Reverse	Size
Human *SPHK2*	qHsaCED0037773, BioRad		114
Human *KLF4*	TCGCCTTGCTGATTGTCTATT	AATTGGCCGAGATCCTTCTTC	125
Human *18S rRNA*	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA	70
Mouse *SPHK2*	qMmuCED0039969, BioRad		76
Mouse *KLF4*	TGGTGAGTCGTGGTTCTAAAG	CCTGGCTTAGGTCATCAATGTA	119
Mouse *HPRT1*	CCCCAAAATGGTTAAGGTTGC	AACAAAGTCTGGCCTGTATCC	76

Supplementary Figures

Figure S1. **H3K9 acetylation and SPHK2 expression show a potential correlation in PH patients' lungs both in male and female sexes.** (A) Sex-based quantitation of Ac-H3K9/ Total H3 in protein lysates of human idiopathic pulmonary arterial hypertension (iPAH: type of Group 1 PH) lung or FDL, n=10/sex/group. (B) *SPHK2* expression levels normalized against *18S rRNA* in iPAH lung and FDL tissues in sex-based (n=10) manner. (C) Sex-based quantitation of SPHK2/ Tubulin in protein lysates of human iPAH lung or FDL, n=10. *P* values are calculated two-way ANOVA, Tukey's multiple comparisons test, and results are shown as means ± SEM.

Figure S2. **Hypoxia-induced experimental PH mouse model.** (A) Right: Representative histology of non-muscularized and muscularized pulmonary arteries (PAs) and left: Percentage of muscularized PAs at alveolar duct and wall level is quantified per field of 100 alveoli in 3 fields in each animal. n=5-6/group. (B) Cardiac output n≥8/ group in

SPHK2 KO or wild type (WT) control mice (C57BL/6NJ) subjected to 3 wks of hypoxia (10% O2) or normoxia (room air). *P* values are calculated using one-way ANOVA, Tukey's multiple comparisons test. Results are shown as means ± SEM.

Figure S3. **EMAP II, nuclear SPHK2 activation, histone acetylation in PH.** (A) Sex-based quantitation of AIMP1/ Tubulin in protein lysates of human iPAH lung or FDL, n=9-10/sex/group. (B) Representative images of pSPHK2 (pink) and EMAP II (green) coimmunostaining in vessel areas in lung sections of failed donors and iPAH patients. Scale bar is 10 μm and n=6/group. (C) Representative immunoblot probed for Ac-H4K5 and total histone H3 in hPASMCs following EMAP II treatment for 0, 1. 2, 4 and 6 hours. (D) Representative immunoblot probed for pSPHK2, Ac-H3K9 and total histone H3 (nuclear marker) in lung nuclear extracts of mice exposed to 10% O2 or room air for 1 wk and quantification of pSPHK2 against total H3 (E) Representative immunoblot probed for pSPHK2 and tubulin following EMAP II treatment with without EMAP II neutralizing antibody and quantification of pSPHK2 against tubulin in whole cell lysates. (F) Representative immunoblot probed for pSPHK2, SPHK2, tubulin and lamin B in cytoplasmic and nuclear fractions of hPMVECs following EMAP II treatment for 0, 2 and 4 hours and (G) Representative immunocytochemistry images of pSPHK2 (pink), actin (green, cytoplasmic marker) and DAPI (blue, nuclear) coimmunostaining in EMAP II treated (2 hr) or vehicle treated fixed hPMVECs, scale bar is 20 μm. (G) Representative immunoblot probed for AIMP1 and actin of hPMVECs whole cell lysate with 125 μM $CoCl_2$ chemical hypoxia treatment, n=2. n≥3/ group if not mentioned. Unpaired t-test or one or two-way ANOVA, Tukey's multiple comparisons test. Results are shown as median and inter-quartile range.

Figure S4. **EMAP II inhibits HDAC activity through SPHK2 in pulmonary vascular SMCs.** (A) HDAC activity normalized against 1 µg of nuclear proteins in the nuclear fractions of hPASMCs following EMAP II for 150 minutes with or without SPHK2 inhibitor. (B) Representative immunoblot probed for HDAC1 and tubulin in hPASMCs following EMAP II treatment for 0, 1. 2, 4 and 6 hours and quantitation of HDAC1/ Tubulin. (C) Representative immunoblot probed for HDAC2 and tubulin in hPASMCs following EMAP II treatment for 0, 1. 2, 4 and 6 hours and quantitation of HDAC2/ Tubulin. Following Kruskal-Wallis or Kolmogorov-Smirnov non-parametric test. Results are shown as median and inter-quartile range or means ± SEM and n=3/group.

Figure S5. **Pulmonary expression of KLF4 is upregulated in PH.** (A) Normalized mRNA counts of stem cell markers in pulmonary hypertension or control lung tissues that reported to have acetylation in candidate Cis-Regulatory Elements (cCREs) by EMAP II compared to control and regulated by SPHK2 inhibitor or Yamanaka factors profiled by Nanostring, n=3. (B) Representative immunoblots probed for KLF4 and Tubulin in protein lysates from SPHK2 KO or wild type (WT) control mice (C57BL/6NJ) were subjected to 3 wks of hypoxia (10% O2) or normoxia (room air) and, (C) quantification of KLF4/Tubulin. (D*) KLF4* expression levels normalized against *Hprt1* utilizing cDNA synthesized from RNA extracts of SPHK2 KO or wild type (WT) control mice that were exposed to 3 wks of hypoxia (10% O2) or normoxia (room air). (E) *KLF4* expression levels normalized against *18S rRNA* in human iPAH or FDL, n=20/group. (F) Representative immunoblot probed for KLF4 and Tubulin in protein lysates of human iPAH or FDL tissue specimens and (G) quantitation of KLF4/ Tubulin in protein lysates of human iPAH or FDL, n=20/group. (H) Representative immunoblot probed for OCT4A and tubulin in hPASMCs following EMAP

II treatment for 0, 1. 2, 4 and 6 hours and quantitation of OCT4A/ Tubulin. (I) Representative immunoblot probed for SOX2 and tubulin in hPASMCs following EMAP II treatment for 0, 1. 2, 4 and 6 hours and quantitation of SOX2/ Tubulin. Following Kruskal-Wallis or Kolmogorov-Smirnov non-parametric test or one-way ANOVA, Tukey's multiple comparisons test, *p<0.05, ****p<0.0001. Results are shown as means ± SEM and n≥3/ group.

Figure S6. **SPHK2 mediated histone H3K9 acetylation in PH vascular PASMCs.** (A) *SPHK2* expression levels normalized against *18S rRNA* in iPAH:PASMCs and non-iPAH:PASMCs. (B) Quantification of SPHK2/ Tubulin in Figure 8H (C) *SPHK1* expression (log of counts per million) in iPAH:PASMCs compared to non-iPAH:PASMCs by RNA-seq in GSE144274. Following Kruskal-Wallis or Kolmogorov-Smirnov non-parametric test, and results are shown as median and inter-quartile range. n≥3/ group.

Figure S1

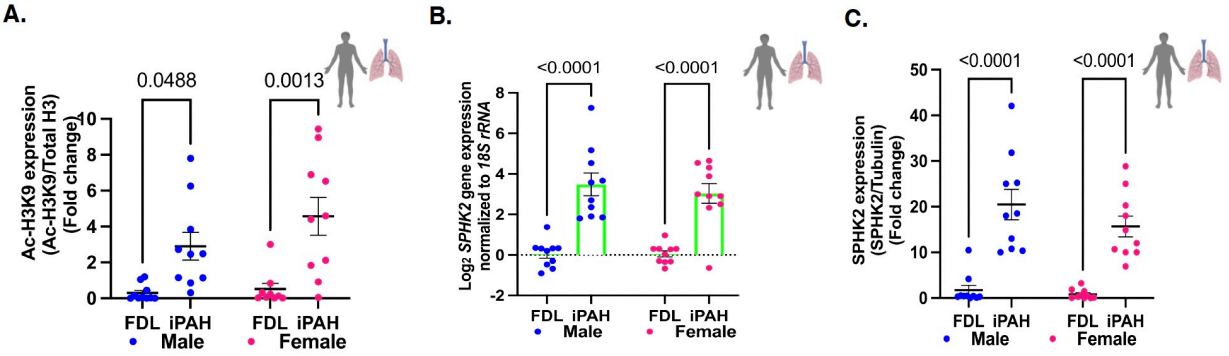

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Figure S2

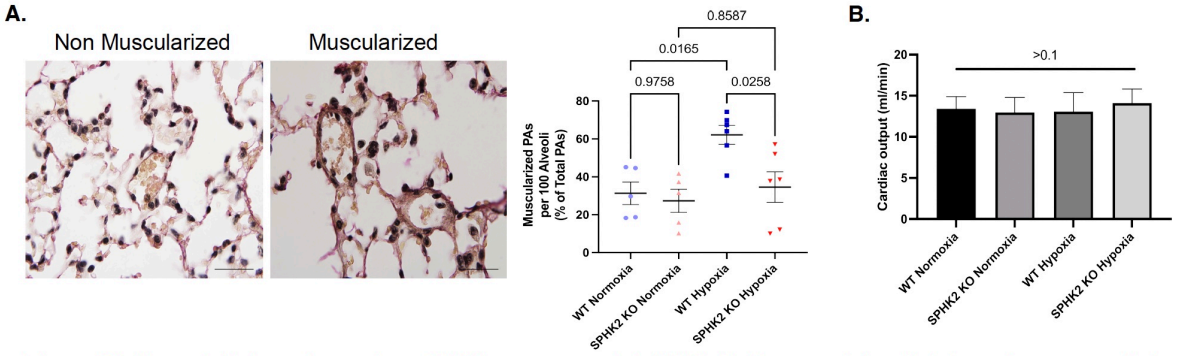

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Figure S3

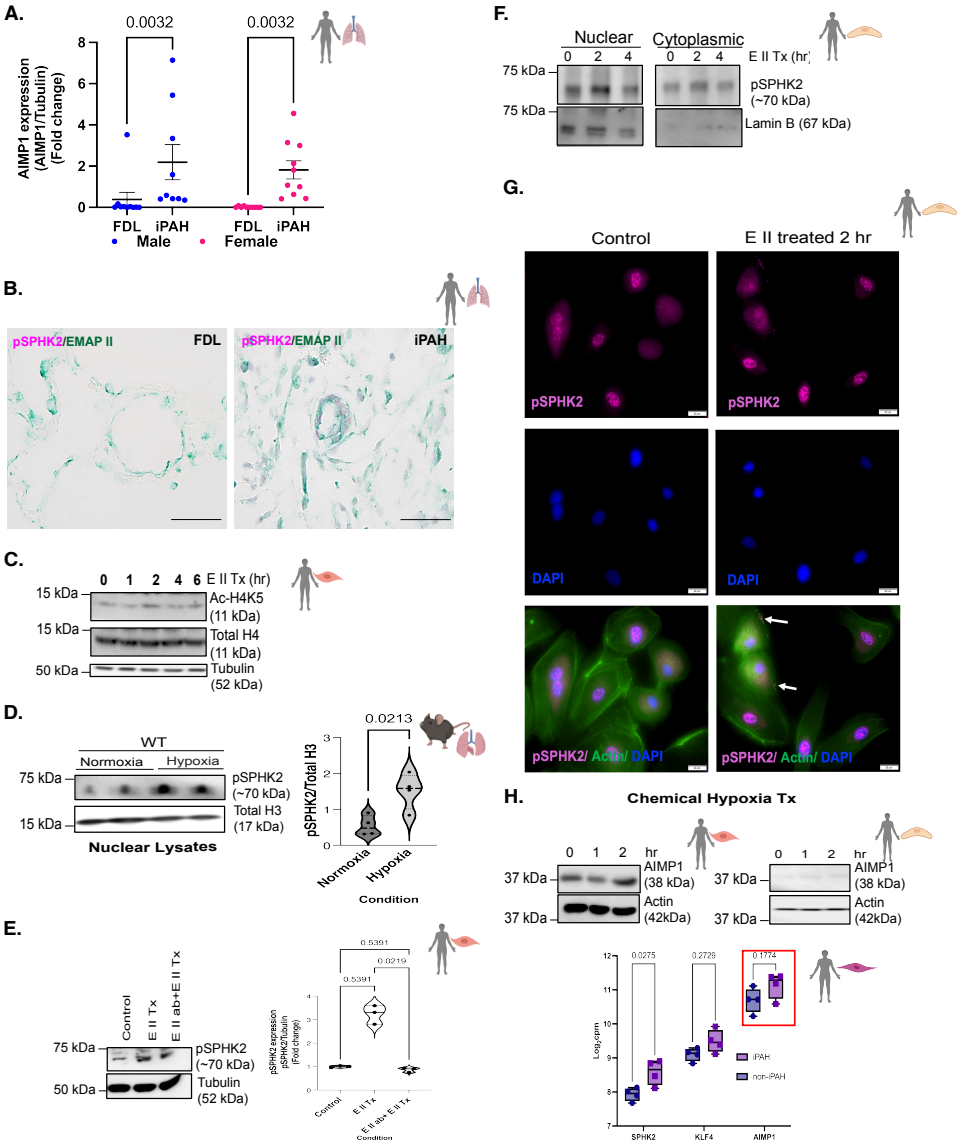

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Figure S4

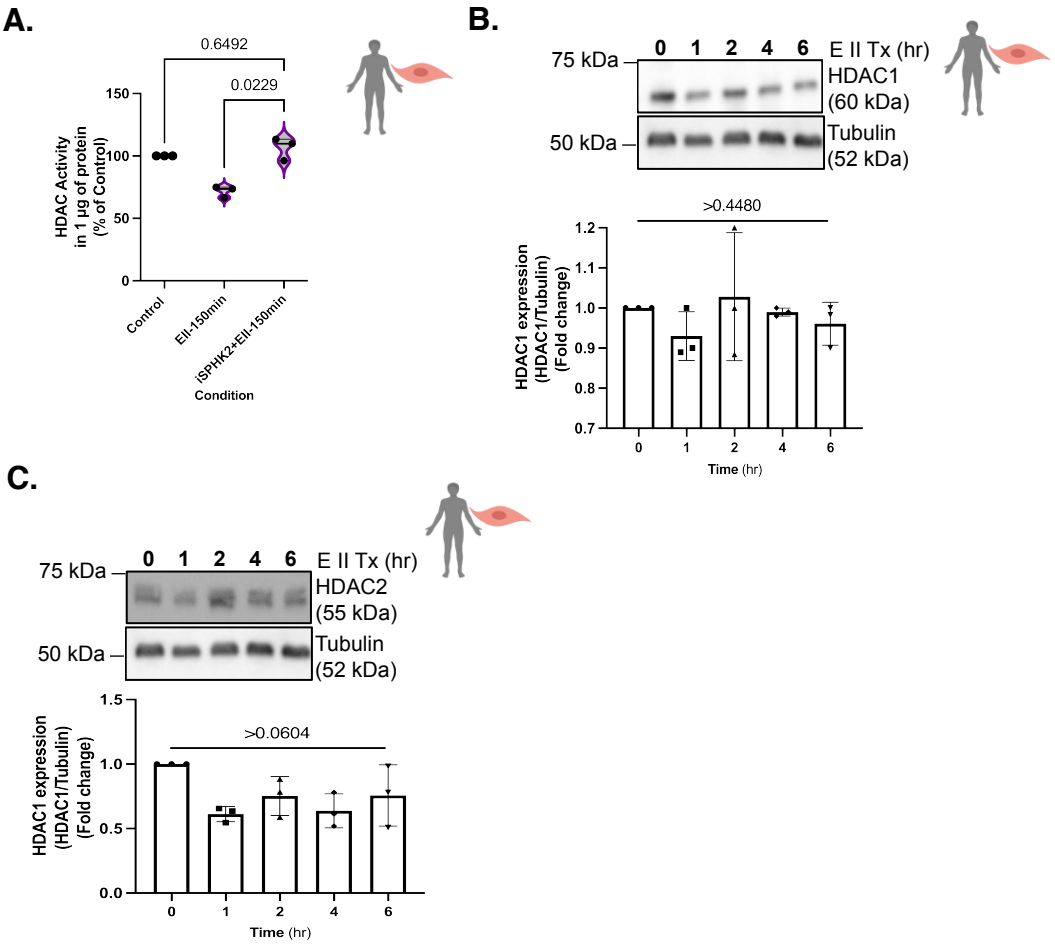

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Figure S5

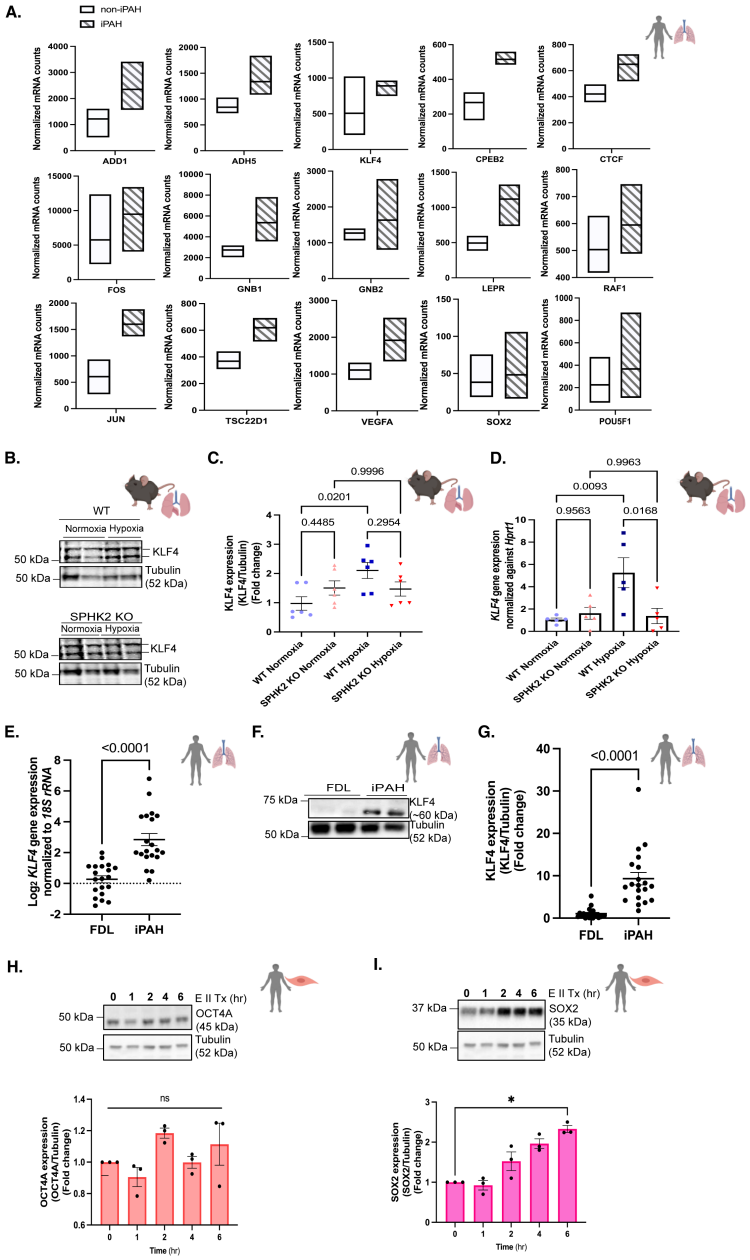

Figure S5. **Pulmonary expression of KLF4 is upregulated in PH.** (A) Normalized mRNA counts of stem cell markers in pulmonary hypertension or control lung tissues that reported to have acetylation in candidate Cis-Regulatory Elements (cCREs) by EMAP II compared to control and regulated by SPHK2 inhibitor or Yamanaka factors profiled by Nanostring, n=3. (B) Representative immunoblots probed for KLF4 and Tubulin in protein lysates from SPHK2 KO or wild type (WT) control mice (C57BL/6NJ) were subjected to 3 wks of hypoxia (10% O2) or normoxia (room air) and, (C) quantification of KLF4/Tubulin. (D) *KLF4* expression levels normalized against *Hprt1* utilizing cDNA synthesized from RNA extracts of SPHK2 KO or wild type (WT) control mice that were exposed to 3 wks of hypoxia (10% O2) or normoxia (room air). (E) *KLF4* expression levels normalized against *18S rRNA* in human iPAH or FDL, n=20/group. (F) Representative immunoblot probed for KLF4 and Tubulin in protein lysates of human iPAH or FDL tissue specimens and (G) quantitation of KLF4/ Tubulin in protein lysates of human iPAH or FDL, n=20/group. (H) Representative immunoblot probed for OCT4A and tubulin in hPASMCs following EMAP II treatment for 0, 1. 2, 4 and 6 hours and quantitation of OCT4A/ Tubulin. (I) Representative immunoblot probed for SOX2 and tubulin in hPASMCs following EMAP II treatment for 0, 1. 2, 4 and 6 hours and quantitation of SOX2/ Tubulin. Following Kruskal-Wallis or Kolmogorov-Smirnov non-parametric test or one-way ANOVA, Tukey's multiple comparisons test, *p<0.05, ****p<0.0001. Results are shown as means ± SEM and n≥3/ group.

Figure S6

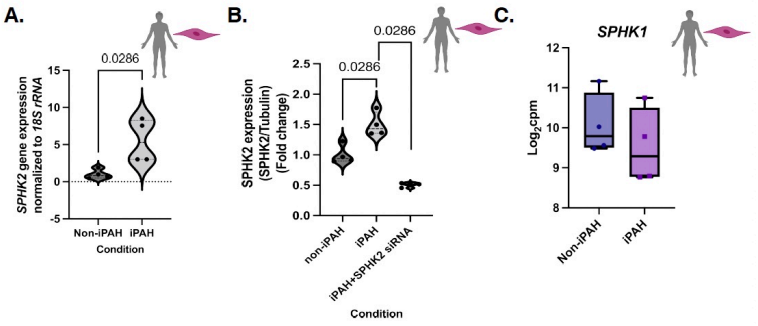

Figure S6. **SPHK2 mediated histone H3K9 acetylation in PH vascular PASMCs.** (A) *SPHK2* expression levels normalized against *18S rRNA* in iPAH:PASMCs and non-iPAH:PASMCs. (B) Quantification of SPHK2/ Tubulin in Figure 8H (C) *SPHK1* expression (log of counts per million) in iPAH:PASMCs compared to non-iPAH:PASMCs by RNA-seq in GSE144274. Following Kruskal-Wallis or Kolmogorov-Smirnov non-parametric test, and results are shown as median and inter-quartile range. n≥3/ group.