NOTCH3 SIGNALING IS REQUIRED FOR THE DEVELOPMENT OF PULMONARY ARTERIAL HYPERTENSION

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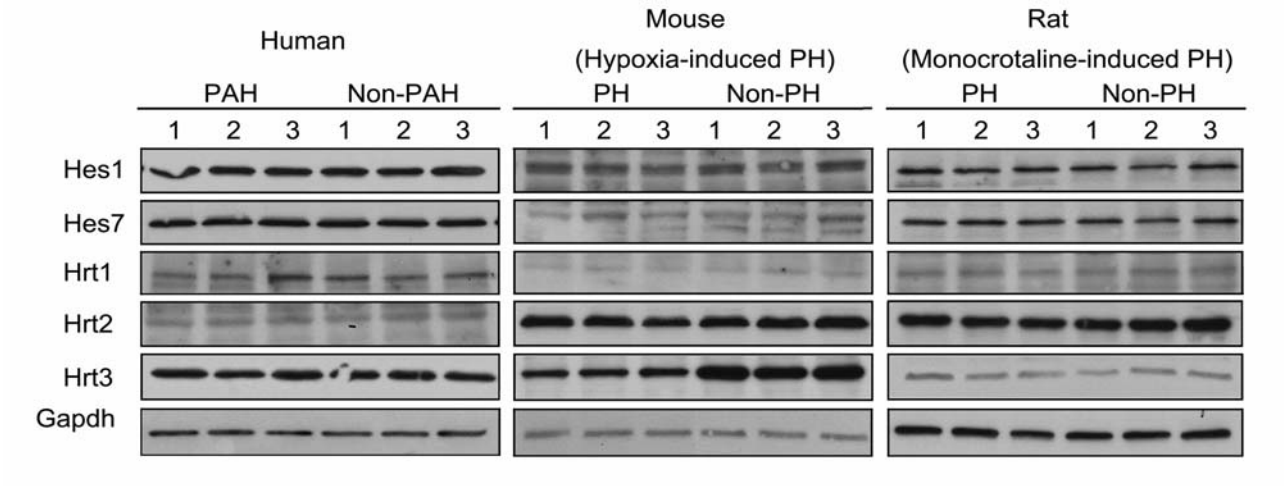
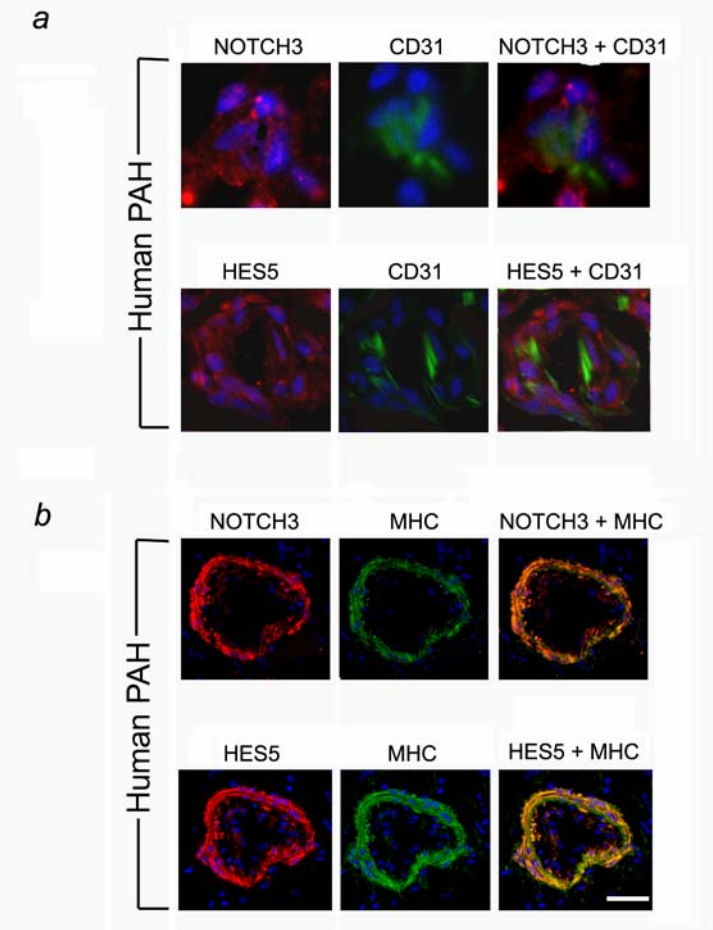
Supplementary Figure 1.



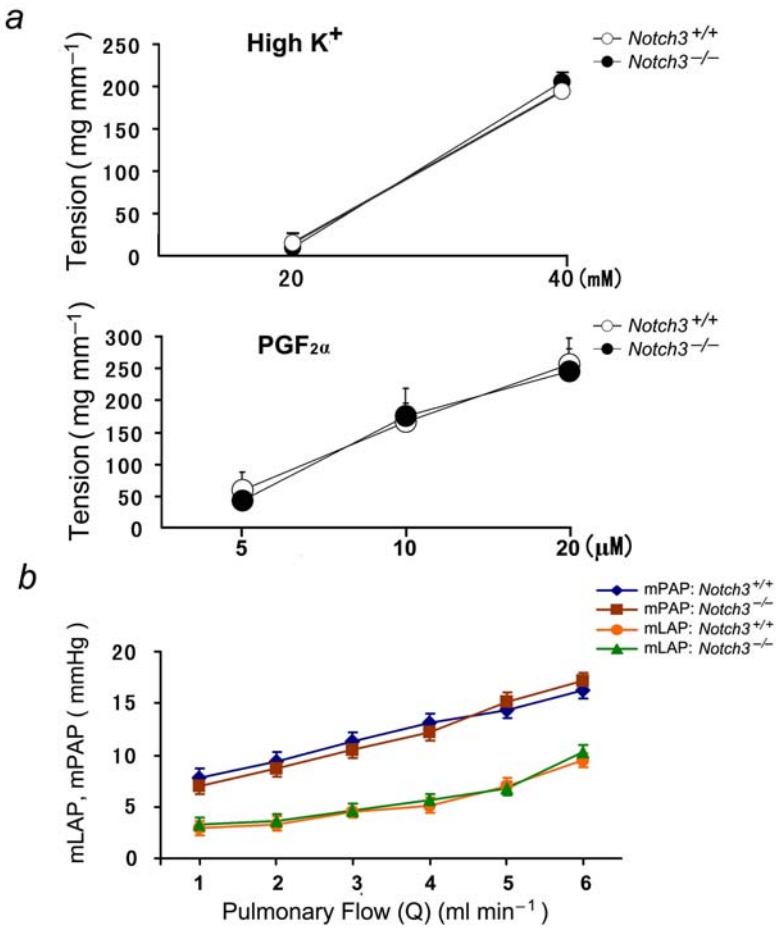
Supplementary Figure 1. Expression of Notch3 target genes (other than Hes5) in pulmonary hypertensive and normotensive lung tissue from human and rodents: representative results. (a) Western blot analysis of Hes1, Hes7, Hrt1, Hrt2, and Hrt3 in lung tissue from humans with idiopathic PAH (three human subjects – left panel), mice with hypoxia-induced PH (three animals – middle panel), and rats with monocrotaline-induced PH (three animals – right panel) compared to normotensive human and rodent lung tissue, relative to Gapdh expression. Sample protein concentrations were normalized to Gapdh, then five separate gels were run with the same quantity of protein in each lane, with each membrane treated with a different Hrt or Hes antibody. Hes1 and Gapdh were measured on the same gel shown. (b) Relative expression values obtained by densitometric analysis of Hes1, Hes7, Hrt1, Hrt2, and Hrt3 expression in: left panel, the lungs of 20 human subjects with PAH compared to the lungs of 20 human subjects without PAH, each lane normalized to GAPDH; middle panel, the lungs of 20 mice with hypoxia-induced (6 weeks) PH compared to the lungs of 20 mice without PH, each lane normalized to Gapdh; and right panel, the lungs of 20 rats with monocrotaline-induced PH compared to the lungs of 20 rats without PH, each lane normalized to Gapdh. Results are presented as mean ± SEM.

Supplementary Figure 2



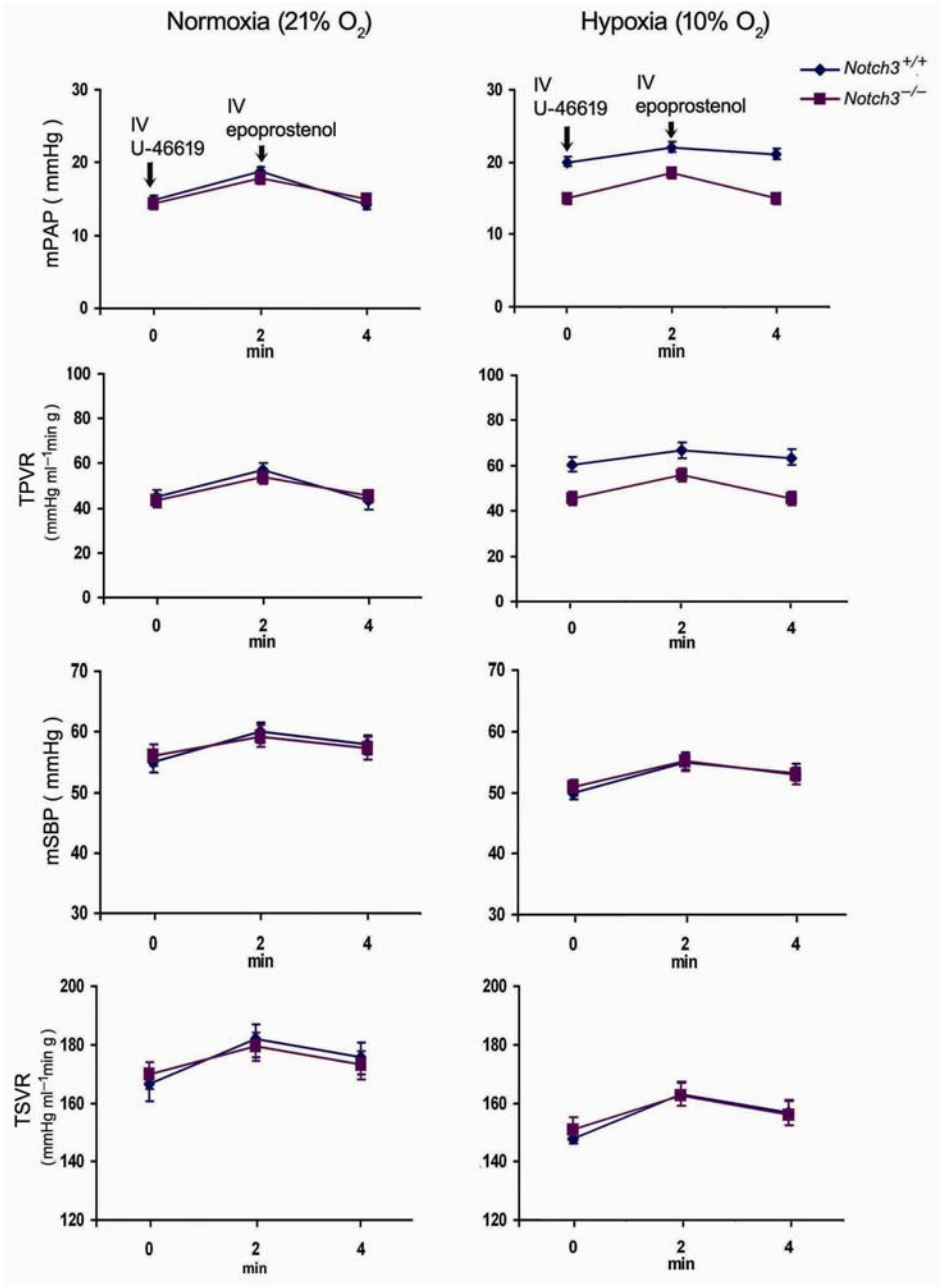
Supplementary Figure 2. NOTCH3 and HES5 expression are predominantly localized to sPASMCs in the media in vessels with neointimal thickening. **(a)** NOTCH3 (red) and CD31 (green) immunofluorescence staining in small arteries 75–125 μm in diameter in lung tissue from human subjects with PAH (top panel). HES5 (red) and CD31 (green) immunofluorescence staining in similar vessels (bottom panel). Neointima stains with CD31 and sparsely with NOTCH3; media stains with NOTCH3 and HES5. Scale bar = 50 μm. **(b)** NOTCH3 (red) and myosin heavy chain (MHC; green) immunofluorescence staining in small arteries 75–125 μm in diameter in lung tissue from human individuals with PAH (top panel). HES5 (red) and MHC (green) immunofluorescence staining in similar vessels (bottom panel). NOTCH3, HES5, and MHC staining predominates in media, with sparse staining in the neointima. Scale bar = 50 μm.

Supplementary Figure 3



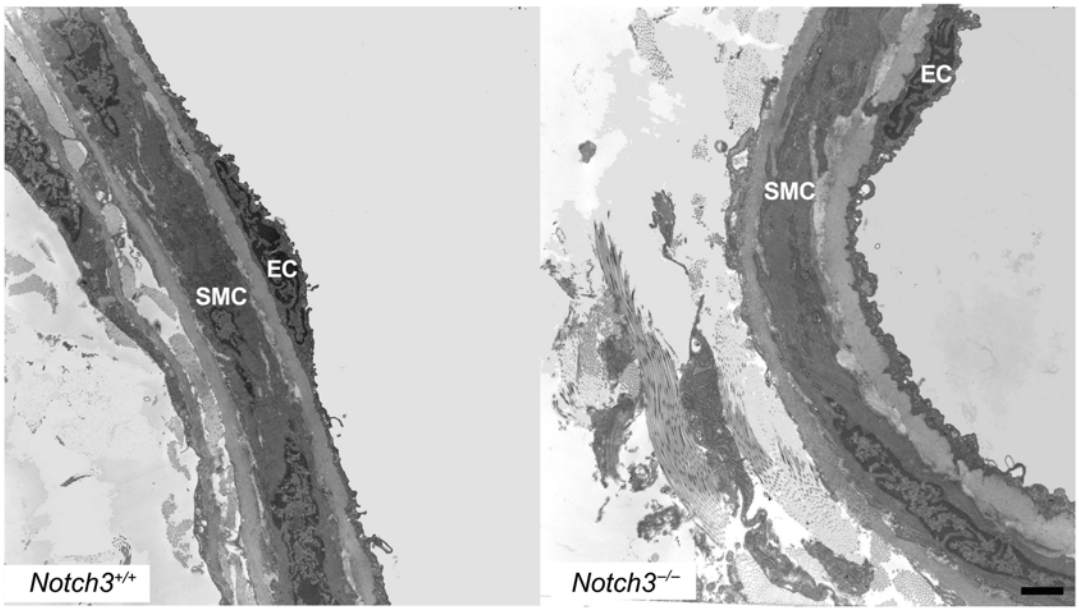
Supplementary Figure 3. Agonist-mediated vasoconstriction in isolated small pulmonary arteries and pulmonary artery pressure as a function of flow in an isolated lung perfusion system are not affected by Notch3 deletion. (**a**) Active tension induced by high K^+ and prostaglandin F2α (PGF2α) in PA rings from 4th–5th order small intrapulmonary arteries. Summarized active tension (mean ± SEM) in wild type (*Notch3*$^{+/+}$, open circles, *n* = three mice, two vessels per animal, three measurements per drug concentration) and knockout mice (*Notch3*$^{-/-}$, solid circles, *n* = three mice, two vessels per animal, three measurements per drug concentration) in PA rings exposed to 20 or 40 mM K^+ (upper panel), as well as to 5, 10 or 20 μM PGF2α in the presence of extracellular Ca^{2+}. The high K^+- or PGF2α-induced pulmonary vasoconstriction is indistinguishable in isolated intrapulmonary arteries between *Notch3*$^{+/+}$ and *Notch3*$^{-/-}$ mice. (**b**) Pulmonary artery pressure as a function of flow in isolated lung perfusions from normoxic *Notch3*$^{+/+}$ and *Notch3*$^{-/-}$ mice. Mean pulmonary artery pressure (mPAP) (blue line for *Notch3*$^{+/+}$ mice; red line for *Notch3*$^{-/-}$ mice) and mean left atrial pressure (mLAP) (orange line for *Notch3*$^{+/+}$; green line for *Notch3*$^{-/-}$ mice) versus pulmonary flow rate (Q) for mouse lungs under normoxic conditions (*n* = ten independent animal lung perfusion systems for each *Notch3*$^{+/+}$ and *Notch3*$^{-/-}$ group; five hemodynamic measurements taken over ten min for each Q; data presented as mean ± SEM).

Supplementary Figure 4



Supplementary Figure 4. Response to intravenous vasodilators in normoxic and chronically-hypoxic *Notch3*$^{+/+}$ and *Notch3*$^{-/-}$ mice. Mean pulmonary artery pressure (mPAP), mean systemic blood pressure (mSBP), total pulmonary vascular resistance (TPVR), and total systemic vascular resistance (TSVR) in normoxic (left panel) or chronically-hypoxic (right panel) *Notch3*$^{+/+}$ and *Notch3*$^{-/-}$ mice at baseline and after 2 min of continuous intravenous infusion of U46619 (50 ng kg^{-1} min^{-1}; continued for the duration of the experiment) followed 2 min later by continuous intravenous infusion of epoprostenol (4 ng kg^{-1} min^{-1}) (ten animals per group, five independent readings at each timepoint).

Supplementary Figure 5



Supplementary Figure 5. Electron micrographs of 5th–6th order small intrapulmonary arteries 100 μM in diameter from *Notch3*$^{+/+}$ (left panel) and *Notch3*$^{-/-}$ (right panel) mice. Scale bar = 8 μm.

Supplementary Table 1

Pulmonary and Systemic Arterial Pressures in Mouse and Rat PH

	Mouse			Rat		
	Weeks of 10% oxygen			Weeks of monocrotaline		
	0	4	6	0	2	4
PA Systolic[a]	19.3 ± 1.5	29.0 ± 1.0	32.0 ± 1.4	18.9 ± 2.0	28.3 ± 1.8	45.9 ± 2.4
PA Diastolic[a]	2.0 ± 2.0	8.0 ± 2.0	10.0 ± 2.0	9.1± 2.1	15.4 ± 1.0	26.6 ± 2.7
Systemic BP[a]	92.5 ± 5.2	89.2 ± 4.2	86.6 ± 6.7	95.2 ± 4.7	90.7 ± 3.4	87.0 ± 5.1

Supplementary Table 1. Pulmonary and systemic arterial pressures in mice developing hypoxic-induced PH or rats developing monocrotaline-induced PH, showing time course and severity of disease progression. 20 animals per timepoint, ten averaged readings per animal.
[a] values given as mean ± SEM in mmHg.

Supplementary Table 2

a Semiquatitative Morphometric Analysis of Pulmonary Vascular Lesions in *Notch3*$^{-/-}$ mice

Vessel Morphology at 6 weeks hypoxia	*Notch3*$^{-/-}$ *n* = 20	*Notch3*$^{+/+}$ *n* = 20	*Notch3*$^{+/-}$ *n* = 20
Vessels with medial thickening (%)[a]	6.9 ± 1.1	38.7 ± 4.3	36.9 ± 3.4
Vessels with > 50% luminal stenosis[a]	8.6 ± 2.1	20.7 ± 2.0	22.5 ± 4.1
Mean no. of myocytes/ vessel wall[b]	6.0 ± 3.8	11.3 ± 6.4	12.0 ± 5.8
Vessel/alveoli ratio (%)[a]	4.5 ± 0.1	4.4 ± 0.2	4.2 ± 0.3

Ten sections per animal lung reviewed
[a] Four fields/section examed per mouse at 25x. [b] Four fields/section examed per mouse at 50x

b Semiquatitative Morphometric Analysis of Pulmonary Vascular Lesions in DAPT-treated hypoxic mice

Vessel Morphology at 6 weeks hypoxia	DAPT-treated *n* = 20	DMSO-treated *n* = 20
Vessels with medial thickening (%)[a]	17.5 ± 13.7	50.0 ± 13.8
Vessels with > 50% luminal stenosis[a]	9.0 ± 7.9	29.0 ± 12.1
Mean no. of myocytes/ vessel wall[b]	6.1 ± 1.8	8.8 ± 1.9
Vessel/alveoli ratio (%)[a]	4.6 ± 0.2	4.4 ± 0.3

Ten sections per animal lung reviewed
[a] Four fields/section examed per mouse at 25x. [b] Four fields/section examed per mouse at 50x

Supplementary Table 2. (**a**) Semiquantitative morphometric analysis of pulmonary vascular lesions in *Notch3*$^{-/-}$, *Notch3*$^{+/+}$, and *Notch3*$^{+/-}$ mice after 6 weeks of hypoxia (*n* = 20 animals for each group, ten lung sections/animal reviewed; values presented as mean ± SEM). (**b**) Semiquantitative morphometric analysis of pulmonary vascular lesions in DAPT- and placebo-treated mice after 6 weeks of hypoxia (*n* = 20 animals for each group, ten sections per lung reviewed; values presented as mean ± SEM).

SUPPLEMENTARY METHODS

Probes for Northern Analysis. The *Notch3* probe (671 bp) for rat and mouse was generated via PCR with primers 5'-TACTCTTGGTGACAGTTGTGAGGAT-3' and 5'-GTGTTAGTAGCTCCAGAGGGTGAC-3'. The *NOTCH3* probe (439 bp) for human was generated via PCR with primers 5'-GAGAGACTGCACCAGGACATC-3' and 5'-GGCTGAGTACACATCCTCCAG-3'.

Primers for qRT-PCR. We used the following primers for qRT-PCR: for *NOTCH3*, P1 5'-AGGCCATGGTCTTCCCTTAC-3' and P2 5'-CAGAGCCGGTTGTCAACTTC-3'; for *HES5*, P1 5'- GCCCGGGGTTCTATGATATT-3' and P2 5'-GAGTTCGGCCTTCACAAAAG-3'; for *α-SMOOTH MUSCLE-ACTIN* (α-SM-ACTIN), P1 5'-CAACCGGGAGAAAATGACTC-3' and P2 5'- GCGTCCAGAGGCATAGAGAG-3'; for *MYOSIN HEAVY CHAIN*, P1 5'- CTCCGTGCTACACAACCTGA-3' and P2 5'-CGAGTAGATGGGCAGGTGTT-3'; for *CALPONIN*, P1 5'-GGAGCTGAGAGAGTGGATCG-3' and P2 5'- CCTGGCTGCAGCTTATTGAT-3'; for *SMOOTHELIN*, P1 5'- GGGATCTCACCAGAAAGGAA-3' and P2 5'-CAGATCTGCTGTGACCTCCA-3', and for 18S RNA, P1 5'-GTCTGTGATGCCCTTAGATG-3' and P2 5'- AGCTTATGACCCGCACTTAC-3'.

Antibodies used for Western analysis and Immunofluorescence Experiments. Goat polyclonal antibody to Notch3 ICD, Hes1, Hes7, and Hrt3 as well as mouse monoclonal antibody to PCNA were obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to Hes5, Hrt1, and Hrt2 were obtained from Millipore Biosciences,. Mouse

monoclonal antibody to α-SM-actin was obtained from Abcam Inc. Rabbit monoclonal antibody to Gapdh was obtained from Cell Signaling Technology. Mouse monoclonal antibody to Tubulin was obtained from Sigma. Apoptag apoptosis detection kit (TUNEL stain, Serologicals) was used as previously described (Bonnet *et al.,* 2005).

Amino Acid Sequence Adeno-Notch3 ICD. The amino acid sequence of Notch3 ICD encoded in our Adeno-*Notch3 ICD* vector spans amino acids 1667–2318 and is: M R R K R E H S T L W F P E G F A L H K D I A A G H K G R R E P V G Q D A L G M K N M A K G E S L M G E V V T D L N D S E C P E A K R L K V E E P G M G A E E P E D C R Q W T Q H H L V A A D I R V A P A T A L T P P Q G D A D A D G V D V N V R G P D G F T P L M L A S F C G G A L E P M P A E E D E A D D T S A S I I S D L I C Q G A Q L G A R T D R T G E T A L H L A A R Y A R A D A A K R L L D A G A D T N A Q D H S G R T P L H T A V T A D A Q G V F Q I L I R N R S T D L D A R M A D G S T A L I L A A R L A V E G M V E E L I A S H A D V N A V D E L G K S A L H W A A A V N N V E A T L A L L K N G A N K D M Q D S K E E T P L F L A A R E G S Y E A A K L L L D H L A N R E I T D H L D R L P R D V A Q E R L H Q D I V R L L D Q P S G P R S P S G P H G L G P L L C P P G A F L P G L K A V Q S G T K K S R R P P G K T G L G P Q G T R G R G K K L T L A C P G P L A D S S V T L S P V D S L D S P R P F S G P P A S P G G F P L E G P Y A T T A T A V S L A Q L G A S R A G P L G R Q P P G G C V L S F G L L N P V A V P L D W A R L P P P A P P G P S F L L P L A P G P Q L L N P G A P V S P Q E R P P P Y L A A P G H G E E Y P A A G T R S S P T K A R F L R V P S E H P Y L T P S P E S P E H W A S P S P P S L S D W S D S T P S P A T A T N A T A S G A L P A Q P H P I S V P S L P Q S Q T Q L G P Q P E V T P K R Q V M A.

Isometric Tension Measurement of Pulmonary Arterial Ring. The lungs were isolated from *Notch3*$^{-/-}$ mice and age-matched *Notch3*$^{+/+}$ littermate controls and placed in Krebs-Henseleit solution for dissection. The Krebs solution contained 138 mM NaCl, 1.8 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5 mM HEPES, and 10 mM glucose (pH 7.4). Fourth or fifth-order small intrapulmonary arteries (PAs) were isolated from the lung tissue. After removal of adherent tissues (including lung parenchymal, bronchial, and connective tissues), the isolated PA ring was cut into 0.8–1.3 mm segments which were then mounted onto 20 μm wires in a myograph (DMT-USA, Inc.). The isometric tension was continuously monitored and acquired using a PowerLab system (AD Instruments, Inc.). The resting tension was set at 100 mg and the rings were allowed to equilibrate for 60 min at the resting tension with intermittent perfusion of Krebs solution. The PA rings were then challenged three times with 40 mM K^+-solution to obtain a stable contractile response. In the high K^+ solution, NaCl was replaced by equimolar KCl to maintain osmolarity. Vascular contraction was described as net increase in tension (mg mm^{-1}) when high K^+ solution or PGF2α-containing solution were applied to the PA rings (Xu et al., 2008). All experiments were performed at 37 °C.

Isolated Lung Preparation with Hemodynamic and Flow Rate Measurements.
The isolated, ventilated, perfused lung preparation was performed as previously described by Tuchscherer et al. (2007). Briefly, the animals were intubated and ventilated with room air at 90 breaths min^{-1} with peak inspiratory and expiratory pressures of 10 and 1 cm H_2O respectively, and an inspiratory/expiratory ratio of 1:1. The main pulmonary artery was

cannulated through an incision in the right ventricle and secured in place by an 8-0 polypropylene suture around the right ventricular outflow tract. The left atrium was cannulated through an incision in the left ventricle. The left atrial cannula was positioned and held in place by the mitral valve ring without suture. The lung vasculature was perfused with 3.5% Ficoll (Sigma-Aldrich) in RPMI 1640 cell culture medium (Cellgro, Mediatech, Inc.) preheated to 37 °C. Pulsatile flow was driven by a high frequency oscillatory piston pump (EnduraTEC Systems Corporation,) and passed through a heat exchanger at 37 °C before reaching the pulmonary artery. Flow exited the left atrial cannula into a small diameter tubing (internal diameter 1.03 mm), which then exited to the atmosphere at the same elevation as the left atrial cannula. Mean pulmonary artery pressure (mPAP) and mean left atrial pressure (mLAP) were measured by pressure transducers; pulmonary vascular flow rate (Q) was measured by an in-line flow meter (T106; Transonic Systems). All pressures and flows were monitored by continuous display on digital monitor (Hewlett Packard).

In order to obtain initial steady-state flow rate measurements of mPAP, mLAP, and Q, ten *Notch3*$^{+/+}$ and ten *Notch3*$^{-/-}$ lungs were perfused at 1 ml min^{-1}, based on previously reported flow rates for isolated perfused mouse lungs (Tuchscherer et al., 2006). Steady-state flow rate was then increased from 1 ml min^{-1} to 6 ml min^{-1} in 1 ml min^{-1} increments, with five readings taken at each flow rate.

Measurement of Hemodynamics in Mice. Mice were anesthetized with an intraperitoneal injection of 250 ng g^{-1} fentanyl and 50 μg kg^{-1} ketamine. A left anterior thoracotomy was used to expose the thoracic organs. A right neck incision was used to expose the right carotid

artery. Catheters (PE-10, Becton Dickinson) were inserted into the main pulmonary artery and into the right carotid artery to measure mean pulmonary artery pressures (mPAP) and mean systemic blood pressure (mSBP) respectively. Cardiac output was measured by measuring the lower thoracic aortic flow (TAF = CO) with a small-vessel flow probe connected to a flow meter (T106; Transonic Systems) (Beppu *et al.,* 2004). All hemodynamic data was recorded using biomedical amplifiers while mice were mechanically ventilated with room air or 10% oxygen. Total pulmonary vascular resistance (TPVR) and total systemic vascular resistance (TSVR) were calculated by dividing mPAP and mSBP respectively, by the CO. Ten *Notch3*$^{+/+}$ mice and ten *Notch3*$^{-/-}$ mice were studied under normoxic conditions; ten *Notch3*$^{+/+}$ mice and ten *Notch3*$^{-/-}$ mice were studied after 6 weeks of hypoxia (10% oxygen), while mechanically ventilated with a fraction of inspired oxygen (FiO_2) of 10%. Hemodynamic measurements were made at baseline, 2 min following continuous IV infusion of the pulmonary vasoconstrictor U46619 (thromboxane A_2 analog; Sigma Aldrich; dose 50 ng kg^{-1} min^{-1} based on dose-response experiments done in Tabuchi *et al*., 2007; infusion continued for the duration of the experiment), followed by 2 min of continuous IV infusion of the pulmonary vasodilator epoprostenol (4 ng kg^{-1} min^{-1}).

Electron Microscopy. Mice were euthanized and injected with 50 U intravenous heparin. A left anterior thoracotomy was used to expose the heart. The left atrial appendage was removed. The pulmonary arterial circulation was perfused through the right ventricle with phosphate-buffered saline (PBS), followed by 10 ml fixative solution (Karnowsky's solution: 2% paraformaldehyde, 2.5% glutaraldehyde in 0.10 $NaPO_4$ buffer, pH 7.4). The right atrium was opened and the systemic circulation perfused with PBS, followed with 20 ml fixative

solution. Tail artery and the 5^{th}–6^{th} order small intrapulmonary arteries were dissected out, cut into 1 mm segments, and kept in fixative solution for 4 h. Tissues were post-fixed in 1% OsO4 in 0.01M $NaPO_4$ buffer for 60 min at 22 °C, then dehydrated in graded series of ethanol and propylene oxide. Tissue was embedded in Quetol resin (Ted Pella, Inc.). Ultrathin sections of small pulmonary arteries were stained with uranyl acetate and bismuth subnitrate. Electron micrographs were recorded using a Zeiss 10E electron microscope (Zeiss Optics) operated at an accelerating voltage of 60 kV.

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