SUPPLEMENTARY MATERIAL

Materials and Methods

All cell lines, mice and rats with genotypes are summarized in Supplementary Table S7.

Patient samples and ethical approval

CHD-PAH patients and healthy control individuals provided written informed consent before being included. This study was approved by the Institutional Review Board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing (014-2015) and Zhejiang University (2021-020), China. Blood samples were collected from CHD-PAH patients at Beijing Anzhen Hospital. Human genomic DNA was extracted with a blood genomic DNA extraction kit (TIANGEN Biotech, cat. DP348-02, China) according to the manufacturer's instructions. A panel screening did not reveal mutations in genes other than *BMPR2* and *BMPR1B* (shown in Supplementary Table S1) except for CHD-PAH 2, which also had ARHGAP31 mutations. Mutations of *BMPR2* or *BMPR1B* were verified by sequencing after PCR amplification.

Peripheral blood mononuclear cell (PBMC) isolation and reprogramming

PBMCs were isolated from peripheral blood (PB) using Ficoll-Paque Premium (GE Healthcare, cat. 17-1440-03, UK) according to the method published by Bin-Kuan Chou et al. (1). By loading onto a 15 mL layer of Ficoll-Paque Premium, PBMCs were obtained from the mononuclear cell layer after being washed and seeded in low-attachment 6-well plates in MNC medium consisting of 50% IMDM (Gibco, cat. 21056-023, USA), 50% Ham's F12 medium (Gibco, cat. no. 31765-027), ITS-X (Gibco, cat. no. 51500-056), lipid concentrate (Gibco, cat. no. 11905031), 5 mg/mL BSA (Sigma-Aldrich, cat. no. A-9418, USA), 50 μg/mL L-ascorbic acid (Sigma, cat. no. A4403), 2 mM Glutamax (Gibco, cat. no. 35050), 200 μM 1-thioglycerol (Sigma, cat. no. no. M6145), 10 ng/mL interleukin (IL)-3 (PeproTech, cat. no. AF-200-03, USA), 100 ng/mL human stem cell factor (PeproTech, cat. no. AF-300-07), 40 ng/mL insulin-like growth factor 1 (PeproTech, cat. no. AF-100-11), 2 U/mL erythropoietin (R&D, cat. no.287-TC, USA), 100 mg/mL human holo-transferrin (R&D, cat. no. 2914-HT), and 20 mM dexamethasone (Sigma, cat. no. D4902). Cells were maintained in MNC medium for approximately 10 days before reprogramming.

Human induced pluripotent stem cell (iPSC) lines were generated via the overexpression of 4 Yamanaka factors using a Sendai Reprogramming Kit (Invitrogen, cat. no. A16517, USA) in PBMCs according to the manufacturer's instructions. Single iPSC colony was selected and transferred onto fresh Matrigel (Corning,

cat. no. 354277, USA)-coated dishes in Essential 8™ medium (E8, Gibco, cat. no. A1517001, USA) after emergence at approximately 15-21 days after infection. Usually, 12 colonies were picked for each cell line, and pluripotency and differentiation potential analyses were performed after passage 15 in culture. We generated iPSC lines from 3 patients with ASDs and 1 patient with a PDA defect. There were no clinical suspicions of genetic syndromes.

Cell culture

Human pluripotent stem cells (hPSCs), including human IPSCs and hESCs, were maintained routinely in E8 medium or mTeSR1 medium (STEMCELL, cat. no. 85850, Canada) on tissue culture plates coated with Matrigel. For passage, a 12-well tissue culture plate coated with Matrigel (incubated at 37°C, 5% CO_2 for at least 1 hour) was utilized, and hPSC colonies were then incubated with 0.5 mM EDTA (Gibco, cat. 15575-038) for 5 min at 37°C and dissociated by pipetting up and down 5-6 times using 1 mL of fresh E8 medium with a 1 mL tip after discarding the EDTA diluent. The cells were immediately harvested and plated on culture plates at 1:20 to 1:50 in medium. hPSCs were typically passaged every 4-5 days. To study the relationships between BMP signalling pathway components and ID proteins, hPSCs were cultured in E8 or Essential 6 (E6, Gibco, cat. A1517001) medium induced by 30 ng/ml BMP4 (R&D, cat. no. 314-BP-01M, USA) or by LDN193189 (LDN, MCE, cat. no. HY-12071) for 24 hours. For luciferase assays, luciferase/ID1-GFP reporter hESCs were treated with or without different concentrations of BMP4 after cytokine starvation in E6 for 12 hours. The information and dilutions for all the stimulants used are listed in Supplementary Table S2. HEK293T cells were cultured according to the protocols recommended by American Type Culture Collection (ATCC).

Myocardial differentiation of hPSCs

The differentiation of hPSCs into cardiomyocytes (CMs) was induced via the STEMdiff™ Cardiomyocyte Differentiation and Maintenance Kits (STEMCELL, cat. no. 05010 and 05020). In brief, hPSCs were dissociated into single cells by Accutase (Gibco, cat. A11105-01) and seeded on 12- or 24-well plates precoated with Matrigel at a density of 7-10x10^4 cells/cm^2 in E8 medium supplemented with 5 μM Y-27632 (Sigma, cat. no. Y0503) the seeding densities of the different cell lines may have varied slightly. Approximately 2-3 days later, the differentiation protocol was started when the cells reached > 95% confluence. After washing the cells with Dulbecco's phosphate-buffered saline (DPBS, Gibco, cat. 14190), the culture medium was replaced with differentiation medium A/B/C or maintenance medium on days 0, 2, 4, and 6 of differentiation. Standard assays were performed after at least 16 days of differentiation. For Ca^{2+}

transient analysis, differentiated hiPSC-CMs were maintained in CM maintenance medium (STEMCELL, cat. no. 05020) until day 18.

Establishment of inducible ID1/ID3-overexpressing hESC lines

For the overexpression of ID1 or ID3 in hESCs (h9), lentivirus-based tetracycline inducible tet-on expression vectors containing 3×Flag-ID1 or 3×Flag-ID3 (with a 3×Flag fusion at the N-terminus) were constructed. The vector construction strategy is shown in Supplementary Figure S3h. The insertion of the mNeonGreen-P2A-blasticidin (BSD) fragment facilitated the selection of a single hESC colony. The plasmids used for overexpression were donated by Prof. Baoyang Hu at the Institute of Zoology, Chinese Academy of Sciences. The vector carrying target fragments was transfected into 293T cells together with the two packaging vectors using NEOFECT^{TM} DNA transfection reagent (Neofect Biotech, China). The supernatant was collected at 24, 36 and 48 hours after transfection, and the concentrated virus was obtained after the supernatant was centrifuged in an ultrafiltration centrifuge tube (Millipore, cat. no. UFC910024) at 4°C. hESCs were treated with Y27632 for 1 day after 1 day of passage before infection with the concentrated virus. Fresh E8 medium was provided after the addition of polybrene (Sigma, cat. no. 107689) to the cells and lentiviral vectors, and the cells were incubated for 24 hours. Two days later, the cells were treated with BSD (2 g/ml, Invitrogen, cat. no. R201-01) and neomycin (50 g/ml, Sigma, cat. no. A1720) to select drug-resistant clones. Then, the hESCs were dissociated into single cells with Accutase and sorted by FACS to acquire mNeonGreen-positive cells that could grow into a single colony. Stable 3F-ID1- and 3F-ID3-inducible overexpression cell colonies were picked and identified after 5-10 days of cultivation.

Establishment of *ID1 and ID3* homozygous gene double-knockout hESC lines with the CRISPR-Cas9 system

To generate loss-of-function mutations of *ID1* and *ID3* in hESCs, the small guide RNA (sgRNA), respectively targeting the human *ID1* and *ID3* genes, were designed using CRISPR Design software (http://crispr.mit.edu/) (2); genotyping primers and off-target detecting primers were also designed. The sgRNA sequences were cloned into an episomal CRISPR/Cas9 (epiCRISPR) plasmid (3) and the pHS-AVC plasmid. The sgRNA with the highest editing efficiency was selected according to the positive ratio of mKate after the contransfection of the epiCRISPR and pHS-AVC plasmids into 293T cells. *ID1* sgRNA epiCRISPR and *ID3* sgRNA epiCRISPR were transfected into hESCs (H9) using the P3 Primary Cell 4D-Nucleofector® X Kit (Lonza, V4XP-3024) with a 4D-Nucleofector System (Lonza). Clones were selected with puromycin (Sigma, cat. no., 8833) and expanded to isogenic cell lines with defined mutations. The cell lines were

verified by the direct sequencing of PCR products and by Western blotting. The sgRNA sequences, genotyping primers and off-target detection primers are shown in Supplementary Table S5.

RT-qPCR

Total RNA was isolated using TRIzol (sigma, cat. no. T9424-200) according to the protocol provided by the manufacturer. The RNA was subsequently quantified and qualified according to the standard qPCR protocol and then treated with gDNA Eraser to remove genomic DNA. Complementary DNA (cDNA) produced using the PrimeScript^{TM} RT reagent kit (TaKaRa, cat. no. RR047A) was used to evaluate the expression of specific genes in different cells. All qPCRs were performed on a CFX Connect Real-Time System (RIO-RAD, cat. no. CFX96 Connect) with TransStart Tip Green qPCR SuperMix (TransGen, cat. no. AQ141, China). Gene expression was determined relative to GAPDH using the ΔΔCt method. All of the primers used are listed in Supplementary Table S4.

Immunofluorescence

To analyse the expression of specific marker genes, hPSCs or iPSC-CMs were seeded on glass coverslips precoated with Matrigel. The cells were fixed in 4% paraformaldehyde (PFA, Sigma, cat. no. P6148) in phosphate buffered saline (PBS) for 15 min at room temperature (RT), permeabilized with 0.3% Triton X-100 (Sigma X100) in DPBS 3 times for 5 min each, and blocked with DPBS containing 5% donkey serum (Solarbio, SL050, China) for 1 hour at RT. The cells were incubated with primary antibodies overnight at 4°C, washed three times with DPBS and then incubated with secondary antibodies for 1 to 2 hours at RT. Finally, nuclei were counterstained with the fluorescent dye DAPI (1 μg/mL, Sigma, cat. no. D9542), washed three times with DPBS, and then mounted on a coverslip with anti-quenching mounting medium (ZSGB-BIO, cat. no. ZLI-9556, China). The information and dilutions for all the antibodies used are listed in Supplementary Table S3. Images were acquired with an LSM 780 confocal microscope (Zeiss, LSM 780, Germany).

Western blot analysis

For protein level analysis, cells were lysed with RIPA buffer (Sigma-VETEC, cat. no. V900854) supplemented with a protease inhibitor (Roche, cat. no. 04693132001) and a phosphatase inhibitor (Roche, cat. no. 04906845001) and quantified by a BCA protein assay kit (Thermo Pierce, cat. 23225) according to the manufacturer's instructions. The supernatant was separated by SDS–PAGE, and proteins were subsequently transferred onto nitrocellulose (NC) membranes (Pall Gelman Laboratory, USA). The membranes were incubated with primary antibodies overnight at 4°C after being blocked with 5% milk in

TBS/0.1% Tween 20 (TBST) for one hour. The following primary antibodies were used in this study: ID1 (Biochech, cat. no. BCH-1/195-14), ID2 (Biochech, cat. no. BCH-3/9-2-8), ID3 (Biochech, cat. no. BCH-4/17-3), ID4 (Biochech, cat. no. BCH-9/82-12), EOMES (CST, cat. no. 81493), NANOG (CST, cat. no. 3580), pSmad1/5 (CST, cat. no. 9516), SMAD1 (CST, cat. no. 6944), USP9X (Abcam, cat. no. Ab180191), NANOG (CST, cat. no. 3580), BMPRII (ABclonal, cat. no. A18079), GAPDH (TransGen, cat. no. HC301), and α-TUBULIN (Sigma, cat. no. T9026). The information and dilutions for all the antibodies used are listed in Supplementary Table S3. The membranes were further incubated with secondary antibodies labelled with horseradish peroxidase, followed by detection with a Tanon Imaging System (Tanon, 5800, China). Relative quantification was carried out with ImageJ, and the P-values were calculated using a t test or ANOVA for data from at least 3 biological replicates.

Flow cytometry analysis of intracellular proteins

Cells were disassociated into single cells with Accutase and centrifuged for 5 min at 350 g. Then, the cells were fixed with 300 μL of 4% PFA for 15 mins at RT after being washed with wash buffer (2% BSA in DPBS). Then, 2 mL of cold wash buffer was added to the cells, and the tube was centrifuged for 5 min at 350 g. The cell pellet was resuspended in 300 μL of 0.2% Triton X-100 (aMReSCO, cat. no. 0694) in DPBS for permeabilization. Fifteen minutes later, the cells were washed and evenly divided into three tubes (100 μL per tube) in blocking buffer (5% mouse serum in washing buffer) and incubated for another 15 mins. Three tubes containing cells were labelled as the unstained control, PE-IgG isotype control (BD, 551436, USA) and PE-Cardiac Troponin T (BD, 564767, USA) and incubated for 30 min at 4°C in blocking buffer. Finally, the cells were washed 3 times with cold washing buffer and resuspended in 100 μL of wash buffer. Then, cTnT expression was evaluated by flow cytometry (BD, ACCURI C6, USA).

Spontaneous Ca^{2+} transient imaging of iPSC-CMs

Differentiated iPSC-CMs were dissociated by STEMdiff™ Cardiomyocyte Dissociation Medium (STEMCELL, cat. no. 05025) and re-seeded onto a glass-bottom cell culture dish precoated with Matrigel (SORFA, cat. no. 201100, China). Next, after the cells had recovered for 4-6 days after seeding, 5 μM Fluo-4 AM (Beyotime, cat. no. S1060, China) in Tyrode's solution (ddH_2O containing 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH=7.4 with NaOH at RT) was added for 15 mins at 37°C until normal beating was observed. The cells were washed 3 times with Tyrode's solution and then incubated for more than 10 mins in Tyrode's solution to ensure that Fluo-4 AM was completely converted into Fluo-4 before imaging. An upright confocal microscope (Olympus, Olympus

BX61, Japan) equipped with a 63X water immersion objective was used to observed spontaneous calcium signalling in iPSC-CMs. Ca^{2+} transients were recorded in fast 2-dimensional scanning mode (512 pixels X 512 pixels) at a speed of 25 ms-40 ms/image. Usually, 100-200 images were collected continuously without a time interval for one region of interest (ROI). Time series image analysis was performed using the plugin Time Series Analyser V3 based on the ImageJ Fuji programme. Ca^{2+} variation was normalized to the average resting fluorescence intensity (F0) to first obtain F-F0. The Ca^{2+} transient properties were evaluated from (F-F0)/F0.

Single-cell capture and scRNA-seq data analysis

We used a 10× Genomics system to perform the single-cell capture and to construct the library as described in the manufacturer's instructions (Novogene provided related technical support). A total of 3000 cells were loaded onto chromium microfluidic chips and barcoded with a 10× chromium controller. A Chromium Single-Cell V2 reagent kit was used to convert all polyadenylated messenger RNA (mRNA) into cDNA with cell-specific barcodes. After reverse transcription and preamplification, cDNAs were prepared as samples for next-generation sequencing using library tagmentation and 3' end enrichment. Illumina sequencing was performed with NavaSeq.

The sequenced reads were mapped against the reference GRCh38 using STAR v2.5.2a. scRNA-seq expression data, quantified by counts via featureCounts v1.5.1, were analysed with Seurat v3.0.1 (PCA, Cluster, t-SNE and cluster). In brief, the Seurat object was generated from digital gene expression matrices. The parameter "Filtercells" is nGene (2000 to 8800) and transcripts (-Inf to 6e + 05). In the standard preprocessing workflow of Seurat, we selected 8706 variable genes for subsequent principal component analysis (PCA). Then, we performed cell clustering and t-Distributed stochastic neighbour embedding (t-SNE). Fifteen principal components were used in the cell cluster, with the resolution parameter set at 1.5. The marker genes for each cell cluster were subjected to Gene Ontology (GO) and KEGG analyses, which were used to define the cell types. Cell clusters were annotated with information on the cell types and germ layers. Digital gene expression matrices with annotations from Seurat were analysed by Monocle v2.3.6 (pseudotime analysis). TFs from animal transcription factor databases(TFDBs) and surface genes were used to filter the gene lists. The cell–cell interactions were assessed by igraph v1.12 as previously reported. We used DAVID to perform the GO and KEGG analyses. GO terms were visualized by REVIGO and Cytoscape. Bulk RNA-seq data, quantified by FPKM via RSEM v0.4.6, were analysed with DEseq2 v1.14.1.

Overexpression of USP9X during CM differentiation

The USP9X overexpression vector was donated by Prof. Guoqiang Xu at Soochow University. WT and ID KO hESCs were passaged at a density of 7-10x10^4 cells/cm^2 in E8 medium according to the protocol described above. When the cells reached 60-70% confluence, Lipofectamine Stem Transfection Reagent (Invitrogen, cat. no. STEM00003, USA) was used to transfect hESCs according to the instructions provided by the manufacturer. Briefly: (1) Spent medium was aspirated and replaced with 0.5 mL of fresh E8 medium in each well of a 12-well plate before transfection. (2) Next, 1.5 μg of the USP9X overexpression vector and 1.5 μg of the control vector, which did not encode USP9X, were diluted in 75 μL of Opti-MEM (Gibco, cat. no.12040-077) separately to dilute the vectors, and 6 μL of Lipofectamine Stem Transfection Reagent was diluted with 75 μL of Opti-MEM for each vector. (3) The diluted vector was added to the diluted transfection reagent, mixed in tubes and incubated for 10 mins at RT. (4) The vector complex was added to the cells, and the plate was gently swirled to ensure even distribution of the complex across the entire well. (5) Finally, the culture plates were returned to the incubator and cultured at 37°C and 5% CO2 overnight before replacing the medium with the vector complex. After 24 hours of transfection, the cells underwent CM differentiation, and undifferentiated transfected cells served as the D0 control. RNA and protein samples were collected on days 2 and 4 of differentiation.

Animal and genotyping

Mice with mutations in the *Id1* genes ($Id1^{-/-}$) were provided by Prof. Bingsheng Ding, while mice with a floxP insertion in the *Id3* allele ($Id3^{f/w}$) were obtained from Jackson Laboratories, and Mesp1-cre transgenic mice were obtained from Prof. JieNa at Tsinghua University. $Id1^{-/-}$ mice and $Id3^{f/w}$ mice were crossed to generate $Id1^{+/-}Id3^{f/w}$ mice, which were crossed with Mesp1-cre mice to generate $Id1^{+/-}Id3^{f/w}$;Mesp1-cre mice. Control and Id conditional double-knockout (Id cDKO) mice were obtained by self-insemination of $Id1^{+/-}Id3^{f/w}$;Mesp1-cre mice and mutual mating of the offspring. The mice were genotyped by PCR analysis using toe DNA with a Mouse Direct PCR Kit (Bimake, cat. no. B40015, USA) and 2x Super PCR Mix (TsingKe, cat. no. TSE101, China); all of the primers used are provided in Supplementary Table S6. All of the mice used were congenic and of the C57BL/6J genetic background, and all mouse experiments were approved by the Animal Care and Use Committee of the Institute of Basic Medical Sciences, Peking Union Medical College and Chinese Academy of Medical Sciences (PUMC & CAMS No: ACUC-A01-2017-002).

Right ventricular tissues from WT and $Bmpr2^{+/-}$ rats were provided by Professor Frédéric Perros; $Bmpr2^{+/-}$ rat strain has a monoallelic deletion of 71 bp in the first exon of Bmpr2 (4). Tissues were genotyped by PCR analysis using heart DNA, and the primers are shown in Supplementary Table S6.

Echocardiography and haemodynamic measurements

Transthoracic echocardiography was performed on 2- and 6-month-old mice by an experienced sonographer using a VisualSonics Vevo2100 ultrasound biomicroscope (VisualSonics, Canada) with a 30-MHz linear array ultrasound transducer. All mice were anaesthetized with 1.0% isoflurane (Yipin Pharmaceutical, cat. no. H19980141, China) until their heart rate (HR) was stabilized at approximately 300 beats/min. Parasternal long-axis images and the maximum LV length were acquired in B-mode with appropriate positioning of the scan head. Wall thickness and chamber dimensions were measured from M-mode images. An apical four-chamber view was acquired to identify the presence or absence of cardiac blood reflux, and the tricuspid annular plane systolic excursion (TAPSE) parameter was measured by placing the M-shaped sampling line on the side annulus of the tricuspid valve. The right ventricular systolic pressure (RVSP) was monitored after echocardiography. The polyethylene (PE) catheter was filled with heparinized saline and connected to a micropressure transducer (PT-102, TECHMAN, China), which was mounted on a BL-420S physiological experiment system (TECHMAN, China). A PE catheter was inserted into the right ventricles of mice through the external jugular vein, and the chest was closed. Id cDKO mice were divided into two groups, without spontaneous arterial hypertension (non-SPAH) and with SPAH (SPAH), according to whether the RVSP was higher than 25 mmHg, LVEDP were measured with a Millar system and catheter SPR-839.

Mouse heart morphometry

The mice were exsanguinated after RVSP measurements, and their hearts were excised quickly. Some of the mouse hearts in each group were separated into the right ventricle (RV) and the left ventricle (LV) plus the septum (S); these parts were used to assess right ventricular hypertrophy as determined by the ratio of RV weight to LV+S weight (RV/(LV+S), Fulton index). Another part of the fresh mouse heart with auricles and vessels was fixed in 4% PFA overnight for histological analysis. Fixed hearts were embedded in paraffin, cut longitudinally into 5-μm-thick sections, and staining with haematoxylin and eosin (H&E) or cardiac myocyte markers, including MF20 (DSHB, cat. no. MF20, USA) and wheat germ agglutinin (WGA).

Mouse lung morphometry

At the same time the heart was removed, the right lung was excised and placed into a 4% PFA solution for fixation. Five-micrometre-thick paraffin-embedded lung sections were subjected to H&E and immunohistochemical staining for α-smooth muscle actin (α-SMA; Sigma, cat. A2547) and CD31 (Abcam, cat. no. ab182981). Two vessel diameters (25-100 μm) were used to calculate the muscularization of

pulmonary vessels, which was the ratio of the α-SMA-positive area relative to the total vessel area. The images of vessels costained with CD31 and α-SMA were sampled with an Olympus high-resolution confocal microscope (Olympus, Olympus IX83-FV3000-OSR, Japan). The remodelling rate was calculated by Elastica van Gieson staining.

Isolation of cardiac myocytes from adult mouse hearts

CMS were isolated from adult mice according to the method published by Matthew Johnson (5). In brief, after the mouse was anaesthetized completely, the thoracic cavity was opened to expose the heart. Then, the descending aorta and inferior vena cava were cut, and EDTA buffer (7 ml) was injected into the base of the RV. The ascending aorta was then clamped, and the heart was dissected and placed into a 60 mm dish. Afterwards, 10 ml of EDTA buffer, 3 ml of perfusion buffer, and 20 ml of collagenase buffer were successively injected into the LV. Then, we separated the LV and RV of the heart, and the two parts were completely digested by gently trituration. After the digestion was terminated, the cells were washed by gravity sedimentation. CMs were obtained from the LV and RV separately, immediately followed by Ca^{2+} transient imaging or by seeding on coverslips precoated by laminin (Thermo, cat. 23017-15) for immunofluorescence analysis.

Measurement of Ca^{2+} transients in adult mouse ventricular myocytes

CMs from the LV and RV were loaded with 1 μmol/L Fura-2-AM (Beyotime, cat. S1052, China) before microanalysis. Myocytes were stimulated at different frequencies (1-4 Hz) using an external stimulator (Grass Technologies, USA). Dual excitation (at 360 and 380 nm; F1 and F0) was delivered using OptoLED light sources (Cairn Research, UK), and emission light was collected at 510 nm (sampling rate 1 kHz). Simultaneous changes in calcium transients and sarcomere length were recorded using IonOptix software. The parameters measured included the calcium amplitude and the percentage of sarcomere length (SL) shortening.

Statistical analysis

Student's t test, one-way ANOVA, and two-way ANOVA were used for the statistical analyses, which were carried out using GraphPad Prism 6.0. All data are presented as the mean ± SEM. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.00005$.

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Supplementary Figure Legends

Supplementary Figure S1 Characterization of CHD-PAH with or without mutation iPSCs and Control iPSCs derived from PBMCs

(a) Sanger sequencing chromatograms of the forward strain show *BMPR2* mutations detected in CHD-PAH mut 1/2/3 and *BMPR1B* mutations in CHD-PAH mut 4. The yellow arrows show the amino acid sites of the mutations. **(b)** Confocal images of a single iPSC clone reprogrammed from PBMCs. iPSCs stained positive for pluripotency markers, including the nuclear markers OCT4, NANOG, SOX2 as well as the surface markers TRA-1-81 and SSEA4. Nuclei were costained with DAPI. Scale bar, 50 μm. CHD-PAH w/o, CHD-PAH without BMPR mutation; CHD-PAH mut, CHD-PAH with BMPR mutation.

Supplementary Figure S2 The Ca^{2+} transient half decay time was decreased in CHD-PAH mut iPSC-CMs

(a) The quantification of fluorescence intensity for TBX5 at day 4, NKX2.5 and MESP1 at day 6 in the cells during CHD-PAH with or without mutation iPSCs and control iPSCs differentiate into CMs corresponding to Figure 1a. N=5-8, 5-8 random fields. **(b)** Representative recording traces of Ca^{2+} transients obtained by fast 2-dimensional scanning. **(c)** Statistical analyses of calcium handling properties, such as the half rising time, time to peak and half decay time; 4-10 iPSC-CMs from each cell line and N=18-24. **(d)** Immunofluorescence and corresponding fast Fourier transformation (FFT) analyses of the sarcomeric protein alpha-actinin (α-Actinin) and cardiac troponin T (cTnT) in control and CHD-PAH mut iPSC-CMs; quantifications were performed with ImageJ. Striation patterns for the α-Actinin and cTnT sarcomeric distributions are shown as line scans in the right panels (x-axis, distance in μm; y-axis, fluorescence intensity in arbitrary units). Nuclei were costained with DAPI. Scale bar, 5 μm. **(e)** Pearson's correlation analysis of α-Actinin and cTnT indicated a normal negative correlation in CHD-PAH mut iPSC-CMs. 3-4 iPSC-CMs from each cell line and N=10-12. ns=not significant. All results are presented as the mean±SEM; *P < 0.05, ****P < 0.0001 vs the control, CHD-PAH w/o and CHD-PAH mut group as determined using two-way ANOVA with post-hoc tests (tukey's multiple comparisons test) (a and c) or unpaired t tests (e).

Supplementary Figure S3 Reduced expression of ID1/ID3 in *BMPR*-mutated cells and establishment of inducible 3×Flag-ID1/ID3 hESC lines

(a) Densitometry quantification of ID1 in the presence or absence of BMP4 corresponding to Figure 2a; 4-8 technical replicates for one cell line, N=13-18. The statistical significance only exists in BMP4+. **(b-c)** Immunoblot analysis of pSmad1/5, ID1 and ID3 in iPSCs from CHD-PAH mut 4 and Control 4 (b) and Control, CHD-PAH w/o or CHD-PAH mut treated with or without 30 ng/ml BMP4 for 2 hours(c). Total Smad1 and α-TUBULIN were used as loading controls. **(d)** Densitometry quantification of the Bmpr2, pSmad1/5, Id1 and Id3 expression levels in right ventricles from WT and $Bmpr2^{+/-}$ rats corresponding to Figure 2b. N=5 rats in each group. **(e)** Immunoblot analysis of NANOG (pluripotent marker), ID1, ID2, ID3 and ID4 in hESCs cultured in E8 or mTeSR medium. α-TUBULIN was used as the loading control. **(f)** Bright field (bright) and fluorescence (GFP) images of the Id1-Venus-Luc-GFP dual reporter cell line (hESCs), in which luciferase and GFP expression was driven by the ID1 promoter. Scale bar, 100 μm. **(g)** Relative luminescence units corresponding to the Id1-Venus-Luc activity in cells treated without (Control) or with different concentrations of BMP9. N=3. **(h)** Vector for the inducible expression of 3xFLAG-ID1 (3F-ID1) and 3xFLAG-ID3 (3F-ID3) in the pLVX-TRE3G plasmid. BSD, Blasticidin; mNG, mNeonGreen. **(i)** Immunoblot analysis of NANOG, ID1 (exogenous) and ID3 showed inducible expression of 3F-ID1 in 13 cell lines (i-1) and inducible expression of 3F-ID3 in 9 cell lines (i-2) with Dox (2 μg/ml, 24 hours). All results are presented as the mean±SEM; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were determined using unpaired t tests (a and d).

Supplementary Figure S4 Acquisition and characteristics of Id conditional double-knockout (Ids cDKO) mice

(a) Schematic diagram of mouse mating and reproduction. **(b)** PCR validation of Id1 fragment deletions, insertion of floxP at the edges of Id3 gene sequences and insertion of the Cre gene after the Mesp1 promoter in the heart of embryo on day 18.5 of pregnancy (E18.5) (top). Verification of Id1 knockout and Id3 monoallelic deletion at the protein level in the heart on E18.5 (bottom). **(c)** Cardiac output (CO) and tricuspid annular plane systolic excursion (TAPSE) values in control (n=17), $Id1^{-/-}Id3^{f/w}$;Mesp1-cre (n=10) and $Id1^{+/-}Id3^{f/f}$;Mesp1-cre (n=14) mice at 2 months of age. **(d)** Left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) values in control (n=14), $Id1^{-/-}Id3^{f/w}$;Mesp1-cre (n=7) and $Id1^{+/-}Id3^{f/f}$;Mesp1-cre (n=12) mice at 2 months of age. **(e)** Representative abnormally high right ventricular systolic pressure (RVSP) in Id cDKO mice at 6 months of age. All results are presented as the mean±SEM; *P < 0.05, **P < 0.01 and***P < 0.001 using one-way ANOVA with post-hoc tests (tukey's multiple

comparisons test) (c-d); ns=not significant.

Supplementary Figure S5 Normal sarcomere protein arrangement in RV CMs of Ids cDKO mice as determined by the analysis of α-Actinin and cTnT signals via fast Fourier transformation

(a) Immunofluorescence and corresponding fast Fourier transformation (FFT) analyses of the sarcomeric protein alpha-actinin (α-Actinin) and cardiac troponin T (cTnT) in RV CMs isolated from control and Id cDKO mice; data were quantified with ImageJ. The striation patterns for α-Actinin and cTnT sarcomeric distributions are shown as line scans (x-axis, distance in μm; y-axis, fluorescence intensity in arbitrary units). Scale bar, 50 μm (top); 10 μm (bottom). **(b)** Pearson's correlation analysis of α-Actinin and cTnT indicates a normal negative correlation in RV CMs isolated from Id cDKO mice; N=5. The results are presented as the mean±SEM; ns=not significant as determined using unpaired t test (b).

Supplementary Figure S6 Genetic editing with CRISPR/Cas9 to knock out ID1 and ID3 in hESCs

(a) Schematic diagram of the epiCRISPR plasmid (a-1) and vector used to verify the editing efficiency of the gRNAs (a-2). P-U6, U6 promoter; p-EF1α, EF1α promoter; Puro, puromycin; SF, sequence fragment. **(b)** The editing efficiencies of the sgRNAs are shown as the percentages of mKate+ cells. **(c)** Schematic diagram of ID1-sgRNA3 for ID1 knockout and ID3-sgRNA3 for ID3 knockout. **(d)** Immunoblotting confirmed the deletion of ID1 or ID3, ID1 and ID3 in 10 knockout hESC lines (ID1+/+ID3+/+ as wild-type (WT): C39/C98/C46, named WTs-#1/#2/#3; ID1-/-ID3-/-: C5/C16/C72, named IDs KOs-#1/#2/#3; ID1-/-ID3+/+: C26/C34; ID1+/-ID3-/-: C20/C86; ID1+/+ID3-/-: C3/C68/C59). All hESCs were cultured in E8 medium. **(e)** DNA sequence for homozygous ID1 and ID3 double knockout (IDs KOs-#1/#2/#3).

Supplementary Table S1-9

Supplementary Table S1: Genotype-phenotype information of patients with CHD-PAH patients and normal people with associated iPS cell lines

Donor status			BMPR2 gene status		
iPSC lines	**Sex**	**Clinical description**	**Nuleotide change**	**AA change**	**Location**
CHD-PAH mut 1	F	CHD-PAH (ASD)	Exon8, c.1042G>A	p.V348I	KD
CHD-PAH mut 2	M	CHD-PAH (PDA)	Exon11, c.1481C>T	p.A494V	KD
CHD-PAH mut 3	F	CHD-PAH (ASD)	Exon8, c.1042G>A	p.V348I	KD
CHD-PAH w/o 1	F	CHD-PAH (VSD)	No		
CHD-PAH w/o 2	M	CHD-PAH (ASD)	No		
Control 1	F	Normal			
Control 2	F	Normal			
Control 3	M	Normal			
Control 4	F	Normal			
Donor status			**BMPR1B gene status**		
iPSC lines	**Sex**	**Clinical description**	**Nuleotide change**	**AA change**	**Location**
CHD-PAH mut 4	F	CHD-PAH (ASD)	Exon10, c.1024A>G	p.K342E	KD

M, male; F, female; ASD, atrial septal defect; PDA, patent ductus arteriosus; KD, kinase domain;

CHD-PAH, congenital heart disease-associated pulmonary arterial hypertension;

CHD-PAH mut, CHD-PAH with BMPR mutation;

CHD-PAH w/o, CHD-PAH without BMPR mutation.

Supplementary Table S2: Sources and dilutions of the factors and drugs

Soluble Factors	Source	Cat. No.	Final Concentration
Human BMP4	R&D	314-BP-0	10, 30 ng/ml
Human BMP9	R&D	3209-BP-010	2, 10, 50 and 100 ng/mL
LDN193189 (LDN)	MCE	HY-12071	0.1, 0.5, 1 μM
Dox	Sigma	D9891	2 μg/ml
G418	Sigma	A1720	50-400 μg/ml
BSD	Invitrogen	R201-01	2 μg/mL
Puromycin	Sigma	8833	0.3-1 μg/ml

Supplementary Table S3: Sources and dilutions of the antibodies utilized in this study

Soluble Factors	Source	Cat. No.	Final Concentration
α-Actinin	Sigma	a7732	1:300 (IF)
α-TUBULIN	Sigma	T9026	1:5000 (WB)
α-SMA	Sigma	A2547	1:200 (IF)/1:500 (IHC)
Alexa Fluor 488 donkey anti-rabbit	Invitrogen	A21206	1:2000 (IF)
Alexa Fluor 488 donkey anti-mouse	Invitrogen	A21202	1:2000 (IF)
Alexa Fluor 594 donkey anti-rabbit	Invitrogen	A21207	1:2000 (IF)
Alexa Fluor 594 donkey anti-mouse	Invitrogen	A21203	1:2000 (IF)
BMPRII	ABclonal	A18079	1:100 (IF)
BMPRII	Abcam	Ab130206	1:500 (WB)
CD31	Abcam	Ab182981	1:800 (IHC-IF)
cTnT	Abcam	Ab45932	1:500 (IF)
EOMES	CST	81493	1:100 (IF)/1:1000 (WB)
GAPDH	TransGen	HC301	1:5000 (WB)
ID1	Biochech	BCH-1/195-14	1:2500 (WB)
ID1	Santa Cruz	sc-133104	1:200 (IF)
ID2	Biochech	BCH-3/9-2-8	1:2500 (WB)
ID3	Biochech	BCH-4/17-3	1:2500 (WB)
ID4	Biochech	BCH-9/82-12	1:2500 (WB)
MESP1	Bioss	bs-9471R	1:200 (IF)

MF20	DSHB	MF20	1:70 (IHC-IF)
NANOG	CST	3580	1:500 (IF)/1:1000 (WB)
NKX2.5	ABclonal	A5651	1:100 (IF)
OCT4	Abcam	ab18976	1:100 (IF)
PE Mouse Anti-cTnT	BD	564767	3 μl/sample
PE Mouse IgG1 Isotype Control	BD	551436	12 μl/sample
pSmad1/5	CST	9516	1:1000 (WB)
SMAD1	CST	6944	1:1000 (WB)
SOX2	Abcam	ab97959	1:600 (IF)
SSEA4	Abcam	Ab16287	1:100 (IF)
TBX5	Proteintech	13178-1-AP	1:50 (IF)
TRA-1-81	Abcam	Ab16289	1:100 (IF)
USP9x	Abcam	Ab180191	1:2000 (WB)/1:150 (IF)
WGA	Sigma	L4895	1:200 (IHC-IF)

IF: Immunofluorescence; WB: Western blot; IHC: Immunohistochemistry

Supplementary Table S4: Quantitative Real time PCR primers

Genes	Forward Primer	Reverse Primer
Brachyury(T)	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG
cTnT	AAGAGGCAGACTGAGCGGGAAA	AGATGCTCTGCCACAGCTCCTT
EOMES	CACATTGTAGTGGGCAGTGG	CGCCACCAAACTGAGATGAT
E47	TGGGCAGCAGTGGAAGCAG	GGGCTGAGGAGAAGGAGGATG
GAPDH	TCATTTCCTGGTATGACAACGA	TCTACATGGCAACTGTGAGG
GATA4	GTGTCCCAGACGTTCTCAGTC	GGGAGACGCATAGCCTTGT
ID1	GTTGGAGCTGAACTCGGAATCC	ACACAAGATGCGATCGTCCGCA
ISL1	AGATTATATCAGGTTGTACGGGATCA	ACACAGCGGAAACACTCGAT
MIXL1	CAGAGTGGGAAATCCTTCCA	TGAGTCCAGCTTTGAACCAA
MESP1	GAAGTGGTTCCTTGGCAGAC	TCCTGCTTGCCTCAAAGTGT
TBX5	CACATTGTAGTGGGCAGTGG	CGCCACCAAACTGAGATGAT
USP9X	CATGATCTCCTGGCAAAATTGGC	GACGTATCAGCTCAAGTAGCTTT
USP9X (ChIP-qPCR)	GTGACGCGGGTCAGCTGCAC	GTAGCCCCGCCGACAAGAAC

Supplementary Table S5: Primers used for CRISPR sgRNA sequences and genotyping

A. sgRNA sequences

Primer	Sequence 5'-3'
ID1-sgRNA1	AACCGCAAGGTGAGCAAGG
ID1-sgRNA2	GGGTGGGCACCAGCTCCTTG
ID1-sgRNA3	AAGGCCGGCAAGACAGCGAG
ID3-sgRNA1	TGTCGTCCAGCAAGCTCAG
ID3-sgRNA2	GTGCGCGGCTGCTACGAGG
ID3-sgRNA3	GGGTACCAGTTCCCGCAGGC

B. Primers used for genotype amplification

Gene	Forward Primer	Forward Primer
ID1	TTTCCGTATCTGCTTCGGGC	GGATCTGGATCTCACCTCGG
ID3	GTCACTGTAGCGGGACTTCT	GCCGTTTAAACCTCCCTCTC

C. Primers used for the detection of off-targets (OFs)

Site	Forward Primer	Forward Primer
ID1-OT1	ATGTCCAGCTTGGGCTGC	GGAGTTGTGGCAGCAGGTAA
ID1-OT2	CAGGTCCTGAATGAGGTGGTG	GCACAGGATGGCCCTAGGAT
ID1-OT3	ACCCGAGTCAGGGACGATAA	GCAGACTGCCACCCTACAAA
ID1-OT4	CCTGGAACCCCACACATGAC	CCGAACACAGCGTCAACAAT
ID1-OT5	CTGTCCCAGCCGTCCTGTC	GAGGCAGCCCTGGGGA

ID3-OT1	TACCAGGCTGAGATGGACGA	GCAGCTTCTCCTTGCACTTG
ID3-OT2	GCGGTGCTTACAGCCTGA	GCGCGGCCTGCCAAG
ID1-OT3	GAAAGGGCTCGGGTCATCC	TTAGGTATCGTCCCGGAGGG
ID3-OT4	AGGAGCCCGGCGCGCGAAA	TGCTGTCCCCGAGCCTCCTCCT
ID3-OT5	CTGAGGCAGAAAAGTGCCCC	AATTGATCTCGGACGCCAAC

Supplementary Table S6: Primers used for rat and mouse genotyping

Primer	Sequence 5'-3'
Rat-Bmpr2-F	CACGGACATGCCTTCAGTTTG
Rat-Bmpr2-R	AGGGAATACGGGAGTAGGGAC
Mice-Id1-F	GGTTGCTTTTGAACGTTCTGAACC
Mice-Id1-R1	CCTCAGCGACACAAGATGCGATCG
Mice-Id1-R2	GCACGAGACTAGTGAGACGTG
Mice-Id3-F	ATTCCTGACGCCAGTGAGTC
Mice-Id3-R	CCCACAGCTCTGAGGTCAT
Mice-Mesp1-cre-F	GCCTGCATTACCGGTCGATGC
Mice-Mesp1-cre-F	CAGGGTGTTATAAGCAATCCC

Supplementary Table S7: Summary of cell lines, mice and rats with genotypes

	BMPR mutation		**IDs knockout**			**IDs overexpression**
Species	**Human**	**Rat**	**Human**	**Mice/genotype**		**Human**
Control group	Control 1	WT 1	WT-#1	**Control**	$Id1^{+/+}Id3^{w/w}$	
	Control 2	WT 2	WT-#2		$Id1^{+/+}Id3^{f/w}$	
	Control 3	WT 3	WT-#3		$Id1^{+/+}Id3^{f/f}$	
	Control 4	WT 4			Mesp1-cre	
	CHD-PAH w/o 1	WT 5				
	CHD-PAH w/o 2					
Experimental group	CHD-PAH mut 1	$Bmpr2^{+/-}$ 1	IDs KO-#1	**Ids cDKO**	$Id1^{-/-}Id3^{f/w}$;Mesp1-cre	hESC-3F-ID1
	CHD-PAH mut 2	$Bmpr2^{+/-}$ 2	IDs KO-#2		$Id1^{+/-}Id3^{f/f}$;Mesp1-cre	hESC-3F-ID3
	CHD-PAH mut 3	$Bmpr2^{+/-}$ 3	IDs KO-#3			
	CHD-PAH mut 4	$Bmpr2^{+/-}$ 4				
		$Bmpr2^{+/-}$ 5				

CHD-PAH: congenital heart disease-associated pulmonary arterial hypertension;

CHD-PAH w/o, CHD-PAH without BMPR mutation;

CHD-PAH mut, CHD-PAH with BMPR mutation;

IDs KO: ID1 and ID3 double knockout hESCs;

Ids cDKO: Id1 and Id3 conditional double knockout mice.

Supplementary Table S8: Components of E8 medium and E6 medium

Components	**E8 medium**	**E6 medium**
DMEM F-12	√	√

L-scorbic acid	√	√
Selenium	√	√
Transferrin	√	√
NaHCO3	√	√
Insulin	√	√
FGF2	√	
TGFβ1	√	

Supplementary Table S9: CHD-PAH BMPR2 mutant carrier summarized information

Blood pressure = Ascending aorta pressure; mPAP = mean pulmonary arterial pressure (mmHg); RAP = Right atrial pressure (mmHg); CO = cardiac output (L/min); CI = Cardiac index (L/(min·m^2)); TPR = Total pulmonary resistance (WOOD); SaO2 = Arterial oxygen Saturation (%); SvO2 = Mixed venous oxygen saturation (%).

	AZ1		AZ3	
Gender	Female		Female	
Age	1982（39y）		2016(5y)	
BMI	24.14		13.52	
Type of mutations	Exon8, c.1042G>A, ASD		Exon8, c.1042G>A, ASD	
	At diagnosis 2008	After surgery (4.5 year later)	At diagnosis 2019	After Iloprost inhalation （30min later）
Blood pressure	/	137/87	88/54	80/46
mPAP	39	33	31	31
RAP	4	9	10	11
CO	4.54	4.3	2.56	2.01
CI	3.11	2.62	5.22	4.10
TPR	6.23	7.7	6.7	7.56
SaO2	96.1	95	96	66
SvO2	81.3	68	79	75

	AZ2
Gender	Male
Age	1999(22y)
BMI	20.41
Type of mutations	c.1481C>T(0/24), PDA

	At diagnosis 2010	After Iloprost inhalation (30 min later)
Blood pressure	93/72	99/76
mPAP	79	80
RAP	3	8
CO	4.3	2.9
CI	3.36	2.27
TPR	16.72	12.03
SaO2	92	93
SvO2	72	82

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