Early View

Original research article

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Single-cell RNA sequencing reveals that BMPR2 mutation regulates right ventricular function via *ID* genes

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Abstract:

Mutations in *bone morphogenetic protein type II receptor (BMPR2)* have been found in patients with congenital heart disease-associated pulmonary arterial hypertension (CHD-PAH). Our study aimed to clarify whether deficient BMPR2 signalling acts through downstream effectors, inhibitors of DNA-binding proteins (IDs), during heart development to contribute to the progress of PAH in CHD patients.

To confirm that IDs are downstream effectors of BMPR2 signalling in cardiac mesoderm progenitors (CMPs) and contribute to PAH, we generated Cardiomyocytes (CMs)-specific *Id 1/3* knockout mice (Ids cDKO), and 12/25 developed mild PAH with altered haemodynamic indices and pulmonary vascular remodelling. Moreover, we generated *ID1* and *ID3* double-knockout (IDs KO) human embryonic stem cells that recapitulated the BMPR2 signalling deficiency of CHD-PAH iPSCs.

CMs differentiated from induced pluripotent stem cells (iPSCs) derived from CHD-PAH patients with BMPR mutations exhibited dysfunctional cardiac differentiation and reduced Ca^{2+} transients, as evidenced by confocal microscopy experiments. Smad1/5 phosphorylation and ID1 and ID3 expression were reduced in CHD-PAH iPSCs and in *Bmpr2*^{+/-} rat right ventricles. Moreover, Ultrasound revealed that 33% of Ids cDKO mice had detectable defects in their ventricular septum and pulmonary regurgitation. CMs isolated from the mouse right ventricles also showed reduced Ca^{2+} transients and shortened sarcomeres. Single-cell RNA(scRNA)-seq analysis revealed impaired differentiation of CMPs and downregulated USP9X expression in IDs KO cells compared with wild-type (WT) cells.

We found that BMPR2 signals through IDs and USP9X to regulate cardiac differentiation, and the loss of ID1 and ID3 expression contributes to CM dysfunction in CHD-PAH patients with *BMPR2* mutations.

Key words: Congenital Heart Disease-associated Pulmonary Arterial Hypertension, Bone Morphogenetic Protein Type II Receptor, Inhibitor of DNA-binding Protein

Abbreviations:

scRNA-seq = Single-cell RNA Sequencing

CHD-PAH = Congenital Heart Disease-associated Pulmonary Arterial Hypertension

H/IPAH = Hereditary/Idiopathic Pulmonary Arterial Hypertension

IPSCs = Induced Pluripotent Stem Cells

CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats

CMPs = Cardiac Mesoderm Progenitors

CPCs = Cardiac Progenitor Cells

hESCs = human Embryonic Stem Cells

PDA = Patent Ductus Arteriosus

ASD = Atrial Septal Defect

VSD = Ventricular Septal Defect

SPAH = Spontaneous Pulmonary Arterial Hypertension

Introduction: Pulmonary arterial hypertension (PAH) is a progressive disease with pathological characteristics that include the thickening and narrowing of pulmonary microvessels, which leads to elevated pulmonary vascular resistance and right ventricular hypertrophy and often results in right ventricular failure [1, 2]. In the past two decades, mutations in components of the BMP signalling pathway have been found in patients with the genetic form of PAH [3]. For example, mutations in *BMPR2* have been identified in patients with congenital heart disease-associated PAH (CHD-PAH) [4]. To date, related studies have focused on pulmonary vascular

remodelling to understand the aetiology of PAH/CHD-PAH with *BMPR2* mutations [5, 6]. Whether the final heart failure in patients with PAH is related to a primary CM abnormality has not been elucidated.

Although a previous study provided evidence that cardiac defects, including VSD, might be caused by embryonic *BMPR2* mutations in mice, whether the mice had PAH was not reported because of the short lifespan associated with this mutation [7]. Moreover, a recent study demonstrated that rats with a monoallelic mutation in *Bmpr2* spontaneously developed PAH after 6 months and that their right ventricular CMs exhibited reduced Ca^{2+} responsiveness compared with those from WT rats [8]. Cardiac output was even reduced in nonspontaneous PAH rats, and further study suggested that the right ventricular CMs of *Bmpr2* mutant rats lacked the adaptation of CMs from wild-type rats. Before this report, evidence was available only for heart defects from subjects with CHD-PAH to support our hypothesis that mutated *BMPR2* affects the CMs in patients with PAH.

Inhibitors of DNA-binding proteins (IDs) are helix-loop-helix (HLH) transcription factors and have been reported to be important target downstream of BMP receptors during cardiogenesis [9, 10]. In our previous review, we speculated that *BMPR2* contributes to cardiac formation during development via the downstream effector gene *Id* based on the similar phenotypes of *Id* knockout (KO) mice and *BMPR2* *mox-cre* KO mice [11]. A recent study showed that *Id* genes are critical for cardiac development since quadruple KO of four subtypes of *Ids* (1-4) in mice led to the absence of cardiac development [12]. BMP receptors convey signalling through

phosphorylated Smad1/5 to induce IDs expression in most cell types, and the mutations of *BMPR2* and *BMP type I receptors* reported in PAH/CHD-PAH may affect IDs and thereby affect CM gene transcription.

In the present study, we aimed to clarify that ID1 and ID3 are important for PAH development and are responsible for the effect of mutant *BMPR2* on CMs. We found that Cardiomyocyte *Id 1/3* knockout mice developed PAH. Moreover, scRNA-seq analysis provided novel evidence of human embryonic stem cells (hESCs) lacking *ID1/3* genes differentiated less towards CMs and of the *BMPR2* downstream effectors *ID1/3* coordinately regulating heart development through *USP9X*. Cardiomyocyte *Id 1/3* knockout mice developed PAH, which further strengthens the knowledge of the impact of *BMPR2* signalling on heart development and the onset of PAH/CHD-PAH. The poor prognosis of these patients necessitates genetic screening for suspicious genes, including *BMPR2* and other signalling pathway components.

Methods:

The authors declare that all supporting data and methods are available within the article and the Online Supplementary material; the Expanded Materials & Methods section is available in the Online Supplementary material.

Establishment of patient-specific iPSC lines

Somatic reprogramming was used to generate iPSC lines from peripheral blood mononuclear cells (PBMCs) harvested from 4 patients with CHD-PAH and 4 healthy

subjects using the Sendai virus reprogramming protocol, as described previously [13]. More detailed descriptions are provided in the Online Supplementary material and Table S1.

Establishment of *ID1* and *ID3* double-knockout cell lines

The *ID1* and *ID3* CRISPR/Cas9 plasmids were generated according to a previous report (26). These two plasmids were transfected into H9 cells, and clones were selected with puromycin. A detailed description is provided in the Online Supplementary material.

Ethical considerations

Written informed consent was provided by all participants prior to the start of the study. 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.

Statistical analysis

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

Results:

***BMPR2* mutation impairs the differentiation of CHD-PAH iPSCs towards CMs and reduces the CM Ca^{2+} transients**

To examine the effects of *BMPR2* mutation on CMs in the context of CHD-PAH, we generated iPSCs from CHD-PAH patients with *BMP Receptor (BMPR)* mutations (CHD-PAH mut iPSCs), without *BMPR* mutation (CHD-PAH w/o iPSCs) and healthy donors (Control iPSCs) by reprogramming the PBMCs with the Sendai virus and characterized the mutations according to the clinical phenotypes of the CHD-PAH patients, including 3 mutation carrier with mild secundum ASDs and 1 mutation carrier with a patent ductus arteriosus (PDA) defect (Supplementary Figure 1a and Supplementary Table S1 and S9). All cell lines exhibited human pluripotent stem cells--like morphologies with flat and compact cell clones and pluripotency marker gene expression (Supplementary Figure 1b).

Control and CHD-PAH iPSCs (with/without *BMPR2* mutation) were then differentiated into CMs that spontaneously contracted rhythmically. Immunofluorescence staining showed decreased expression of EOMES on day 2 and the cardiogenic specific TBX5 and NKX2.5 on day 4 (Figure 1a). The effects on NKX2.5, MESP1 were more obvious in differentiated CHD-PAH mut cells on day 6 (Figure 1a and Supplementary Figure 2a). Both Control and CHD-PAH iPSC-differentiated cells expressed the CM-specific markers cTnT and α -Actinin on day 16 after differentiation (Figure 1a), but CHD-PAH mut cells had few expression of cTnT. A real-time PCR assay showed that the expression of Brachyury (T) and TBX5 was significantly reduced in mutant CHD-PAH cells compared with Control and CHD-PAH w/o cells on day 2. GATA4 and cTnT expression was significantly decreased in mutant cells on day 6 and day 8 (Figure 1b). On day 16 of differentiation,

FACS analysis showed that $90.84 \pm 0.26\%$ and $91.80 \pm 1.36\%$ of the cells differentiated from Control and CHD-PAH w/o iPSCs expressed cTnT, while significantly fewer cells differentiated from CHD-PAH iPSCs expressed cTnT ($67.32 \pm 2.91\%$, $p < 0.001$) (Figure 1c). Thus, iPSCs from CHD-PAH patients with *BMPR2* mutations differentiated into mature CMs that differed from the CMs differentiated from Control and CHD-PAH w/o iPSCs.

To determine the functional characteristics of the differentiated CMs, we next examined the spontaneous Ca^{2+} transients in 3 lines of mature Control CMs, 3 lines of CHD-PAH w/o CMs and 3 lines of CHD-PAH mut CMs via upright confocal microscopy. Both the Control and CHD-PAH CMs maintained rhythmicity based on the detection of store-operated Ca^{2+} entry (Figure 1d-f and Supplementary Figure 2b). Notably, the Ca^{2+} transient amplitude, decay tau and half decay time of CMs derived from CHD-PAH mut iPSCs were significantly reduced compared with those derived from the Control and CHD-PAH w/o iPSCs (Figure 1g, 1h and Supplementary Figure 2c); however, there were no significant differences in the half rising time or the time to peak (Supplementary Figure 2c), indicating that the Ca^{2+} storage in CHD-PAH mut CMs was impaired via either sarcoplasmic reticulum dysfunction or the efflux of Ca^{2+} through the Na^+ - Ca^{2+} exchanger. Additionally, the regularities of the Control and CHD-PAH mut CMs regarding the sarcomeric protein arrangement were similar when the signals via fast Fourier transformation were analysed, and there was no difference in the correlation coefficients based on the costaining of α -Actinin and cTnT (Supplementary Figure 2d and 2e). These results suggested that *BMPR2* mutation

impacted the differentiation and Ca^{2+} handling of CHD-PAH iPSC-CMs.

The ID1 and ID3 expression levels are reduced in CHD-PAH iPSCs with *BMPR2* mutation and exhibit coordinated regulation

BMP4 is important for cardiovascular developmental processes, including heart formation and vasculogenesis [14, 15]. Thus, we stimulated Control and CHD-PAH iPSCs with BMP4 to examine their responsiveness to BMPR2 signalling based on the phosphorylation of Smad1/5 and the expression of ID1 and ID3, which are major downstream effector genes of BMPR2 signalling in both humans and mice [16, 17]. After treatment with 30 ng/mL BMP4 for 2 h, immunoblotting showed that the ID1 levels were lower in the CHD-PAH mut iPSC lines than in the Control iPSC lines (Figure 2a and Supplementary Figure 3a). Similar results were found in the *BMPR1b*-mutated CHD-PAH line (Supplementary Figure 3b). CHD-PAH w/o iPSCs had intact pSmad1/5 and ID1 level (Supplementary Figure 3c). These results showed reduced BMPR2/ID signalling in CHD-PAH iPSCs with *BMPR* mutations.

We also evaluated the effects of the monoallelic deletion of *Bmpr2* (*Bmpr2*^{+/-}) on BMPRII protein expression and downstream signalling pathways in the right ventricles of rats. As expected, the protein expression of BMPRII was reduced by 50% in the right ventricular tissues of 6-month-old *Bmpr2* mutant rats (*Bmpr2*^{+/-}). We observed significant decreases in the protein levels of pSmad1/5 and Id1/Id3 in the right ventricles of *Bmpr2*^{+/-} rats compared with those of WT rats (Figure 2b and Supplementary Figure 3d). This result suggests that the monoallelic deletion of *Bmpr2*

also decreases the Id1 and Id3 protein expression levels in vivo.

We then studied the relationships between the BMPR2 signalling pathway and ID proteins in hESCs (the h9 cell line), which have the same differentiation potential and mutation generation characteristics as iPSCs, for gene function analysis. Immunoblotting showed that the protein levels of IDs (ID1, ID2, ID3 and ID4) were similar in the two conventional culture conditions. Specifically, the ID1 and ID3 proteins were more abundant than ID4, and no detectable expression of ID2 was observed (Supplementary Figure 3e). We examined the baseline ID1 expression with a luciferase/ID1-GFP reporter line (Supplementary Figure 3f) and found that the *ID1* promoter activity was significantly increased by BMP9, which was reported to activate BMPR2 (Supplementary Figure 3g). Immunoblotting analysis demonstrated that ID1 was induced, while ID3 expression was less affected by BMP4 treatment for 2 h (Figure 2c). We also showed the time course of ID1/3 induction by BMP4 stimulation for 36 h, and found the induction of ID3 reached maximum level at 6-12 h after BMP4 stimulation, thereafter ID1 was the major form downstream of BMPR2 signalling (Figure 2d).

To further investigate the regulation of ID1 and ID3 by BMP receptor, hESCs were treated with LDN193189 (LDN), a known inhibitor of activin receptor-like kinase (ALK2), the expression of ID1 was decreased as reported [25], while the protein expression of ID3 was simultaneously increased in the absence of BMP4 (Figure 2e). We established the ID1- and ID3-inducible overexpression cell lines H9-ESC-3F-ID1 and H9-ESC-3F-ID3, respectively, with a lentivirus tet-on 3G inducible expression

system (Supplementary Figure 3h and 3i). Decreased ID3 expression was observed when ID1 overexpression was induced with doxycycline (Dox) (Figure 2f). In contrast, reduced ID1 protein levels were observed when ID3 was overexpressed (Figure 2g). Taken together, our results suggested that the expression of ID1/ID3 was reduced in CHD-PAH iPSCs with BMPR2 mutations and that ID1 and ID3 exhibited coordinated regulation.

Ids cDKO mice are susceptible to PAH and pulmonary vascular remodelling

To investigate the roles of *Id* genes in the intrinsic alterations of the heart in PAH and bypass the limitation of the embryonic lethality of *Id1* and *Id3* double knockout [10], we crossed *Id1* KO mice (*Id1*^{-/-}) with mice harbouring a flox insertion in the *Id3* allele (*Id3*^{f/w}) and targeted *Id3* ablation in the precardiac mesoderm by utilizing CRE recombinase, which was driven by the earliest cardiovascular progenitor marker *Mesp1* (*Mesp1*-cre mice), thereby generating *Ids* conditional double-knockout mice (*Ids* cDKO) (Supplementary Figure 4a). After genotyping, we confirmed that the decreased protein expression of *Id1* and *Id3* in mouse hearts (E18.5) was consistent with the PCR results (Supplementary Figure 4b). Then, we performed ultrasonic scanning on 2-month-old mice (adult). Significant decreases in the right ventricular cardiac output (CO) and tricuspid annular plane systolic excursion (TAPSE) were detected in *Id1*^{-/-}*Id3*^{f/w};*Mesp1*-cre and *Id1*^{+/-}*Id3*^{f/f};*Mesp1*-cre mice compared to control mice (Supplementary Figure 4c). There were no significant differences in the left ventricular ejection fraction (LVEF) or left ventricular fractional shortening (LVFS)

among the Control, $Id1^{-/-}Id3^{f/w};Mesp1\text{-}cre$ and $Id1^{+/-}Id3^{f/f};Mesp1\text{-}cre$ mice (Supplementary Figure 4d), indicating that the *Ids* cDKO mice could develop PAH.

Next, cardiac catheterization was performed on 6-month-old mice to compare the haemodynamic indices of the 2 types of *Ids* cDKO mice and control mice. A substantial proportion of mice (5/12 of $Id1^{-/-}Id3^{f/w};Mesp1\text{-}cre$ and 7/13 of $Id1^{+/-}Id3^{f/f};Mesp1\text{-}cre$ mice) developed significantly elevated right ventricular systolic pressure (RVSP) and total pulmonary vascular resistance (tPVRI), referred to as spontaneous PAH (SPAH), while the other *Ids* cDKO mice did not develop PAH (non-SPAH) (Figure 3a and Supplementary Figure 4e). The mice with SPAH showed worse right ventricular function with a higher Fulton index and lower TAPSE, CO and cardiac index (CI) values than the control mice (Figure 3b-3d). Although the RVSP was not elevated in non-SPAH mice, the reduced CO and CI values suggested that heart function was also affected in these mice (Figure 3d). Notably, however, the unchanged LVEF and LVFS in all *Ids* cDKO mice indicated that the left heart function was not affected (Figure 3e).

Immunohistochemistry of mouse lung samples with α -smooth muscle actin (α -SMA) and CD31 staining revealed the increased muscularization of pulmonary microvessels in SPAH mice (with diameters smaller than 100 μ M) (Figure 3f and 3h). Elastic Van Gieson (EVG) staining demonstrating the significantly thickened pulmonary arterial wall in SPAH mice, but not in Non-SPAH mice compared with control mice, which suggested that pulmonary vascular remodelling occurred in SPAH mice (Figure 3g and 3i). These results suggested that the *Ids* cDKO mice were

susceptible to pulmonary vascular remodelling.

Ventricular defects and reduced Ca^{2+} transients in CMs compromise the right heart function in Ids cDKO mice

To assess the heart defects of Ids cDKO mice, we employed colour Doppler echocardiography. We observed abnormal blood flow in the hearts of 33.3% of the Ids cDKO mice at 2 months, including reflux of the tricuspid valve (1 mouse), VSDs (2 mice), and pulmonary valve defects (6 mice) (Figure 4a). Fifty percent of the mice with reflux had PAH at 6 months. Pulmonary valve regurgitation was detected during diastole in Ids cDKO mice but not in control mice (Figure 4b). Additionally, VSDs were clearly identified by ultrasonic scanning and in sequentially sliced heart sections (Figure 4c and 4d). The CMs of the middle and outer myocardial wall of the right ventricle from Ids cDKO mice were smaller than those from control mice (Figure 4e and 4f). Such differences were not found in CMs of the interventricular septum.

To examine the function of CMs, we isolated CMs from control and Ids cDKO mice. Significantly reduced Ca^{2+} transients and sarcomere length shortening were found in CMs isolated from the right ventricles of Ids cDKO mice but not in those isolated from the left ventricle compared to the control mice (Figure 4g and Figure 4h). Additionally, the regularities of the control and Ids cDKO CMs were similar in regards to the sarcomeric protein arrangement when the signals via fast Fourier transformation were analysed, and no differences were observed in the correlation coefficients determined based on the costaining of α -Actinin and cTnT

(Supplementary Figure 5a and 5b). Taken together, these results indicated that the loss of Id1 and Id3 protein expression in the heart impaired right ventricular function.

CM differentiation is impaired in *ID1/ID3* double-knockout hESC lines

To clarify the effects of *BMPR2/ID* deficiency on CHD-PAH development, we generated *ID1* and *ID3* double-knockout hESC lines (IDs KO) with an episomal CRISPR/Cas9 plasmid. We designed three small guide RNAs (sgRNAs) targeting *ID1* and *ID3* (Supplementary Table S5) and then transfected vectors into 293T cells to identify the sgRNA with the highest editing efficiency (Supplementary Figure 6a, 6b and 6c). We successfully subcloned 3 *ID1* and *ID3* double-knockout lines (IDs KOs), 2 *ID1*^{-/-} lines, 3 *ID3*^{-/-} lines, and 2 *ID3*^{-/-}*ID1*^{-/+} lines (Figure 5a, Supplementary Figure 6d and 6e). We next differentiated these cells into CMs. Using the differentiation protocol employed above, the population of cells positive for cTnT and α -actinin (a marker of mature CMs) was completely lost in the 3 IDs KO lines (Figure 5b and Figure 5c). Immunoblotting demonstrated that the protein levels of EOMES were reduced in the IDs KO lines compared to the WT cell lines (Figure 5d). Further characterization of mesoderm markers showed that the expression levels of Brachyury, MIXL1 and TBX5 were significantly reduced on day 2 of differentiation. On day 4, the expression of TBX5, GATA4 and ISL1 was further decreased (Figure 5e). This finding suggested that the ID double-knockout hESC lines also had impaired cardiac differentiation, similar to *BMPR2* mutant CHD-PAH iPSCs.

scRNA-seq analysis revealed the major affected populations of cardiac mesoderm progenitors in the IDs KO line

To examine the effect of *ID* knockout on hESC differentiation towards cardiac lineages, the single-cell transcriptomes of WT and IDs KO hESCs were examined after they were induced to differentiate towards CMs. We determined the *ID1* expression in WT hESCs during differentiation and observed peak mRNA expression on day 4 (Figure 6a). At the same time, we observed the expression of cardiac mesoderm progenitor (CMP) markers, including *EOMES* and *MESPI* (Figure 6a).

We then prepared single-cell samples for scRNA-seq according to the 10x Genomics general sample preparation method at day 4. After sequencing and data processing, we obtained high-quality transcriptomic data from 8690 single cells (4761 WT and 3929 ID KO cells) after stringent segregation and the removal of floating dead cells that detached during differentiation. Subsequently, unsupervised clustering analysis showed 6 clusters for the collected live cells and differential expression patterns of WT and IDs KO (Figure 6b). Then, we combined the original sample information and expression levels of well-known markers to determine the cell identity of each cluster and analysed their biological features by performing GO analysis of differentially expressed genes (DEGs). We identified 6 major cell clusters; C0 was the largest cell cluster, mainly containing neural progenitors with high expression of *STMN2*, *SOX11*, and *TUBB2B*. C1 cluster cells were mainly undefined and expressed *CITED2* and *FST*. C2 was a cluster of CMPs that expressed the cardiac mesoderm markers *MESPI*, *HAND1*, and *FOXC1*. The C3 cluster was composed of

cells expressing high levels of *MKI67*, *CDK1* and *PLK1*, which are involved in cell division and proliferation. Some C4 cluster cells expressed the pluripotency marker *POU5F1* and the metal ion-responsive genes *MT2A* and *MT1E*. C5 was a group of cells in the transition state from mesendoderm cells to CMPs that highly expressed *SOX17*, *GATA3*, and *ISL1* (Figure 6c and 6d). C2 and C5 were mainly composed of WT cells, whereas C0 and C1 were mainly composed of IDs KO cells. Taken together, these results suggest that ID1 and ID3 drive mesoderm differentiation towards the cardiac lineage and that the knockdown of *ID1* and *ID3* contributes to the emergence of neural marker gene-expressing cells.

***USP9X* is a downstream effector gene of ID1 and ID3 in CMPs**

Next, we used R-Seurat to analyse cells on day 4 of differentiation into CMs to identify differentially regulated genes in CMPs. As shown in the clustering analysis above, the majority of cells in CMP related clusters 2 and 5 were WT cells, as very few IDs KO cells were included in these clusters (Figure 7a). We further demonstrated that cardiogenesis-related markers (*MESP1*, *HAND1*, *EOMES*, *GATA4* and *BMP4*) and right ventricle-specific markers (*ISL1*) were widely expressed in cluster 2 or cluster 5, which mainly consisted of WT cells (Figure 7a). Surprisingly, we found that USP9X (ubiquitin-specific protease 9X) was one of the most differentially affected genes in WT cells in most clusters (C0, C1, C2, C3, and C5) compared with IDs KO cells (Figure 7b). USP9X, the homologue of *Drosophila* fat facets, is a deubiquitinating enzyme (DUB). Loss-of-function mutations in USP9X

were reported in 17 females that had distinct congenital malformations, including heart defects [18]. The expression of USP9X was highest in cluster 2 (Figure 7c), and DEG analysis showed that the USP9X levels were altered more extensively in CMPs than in other clusters after *ID1/ID3* knockout (Figure 7d). Thus, the expression levels of USP9X and cardiogenesis-related genes are reduced relative to those in WT cells.

Deficient BMPR2/ID signalling downregulates USP9X through E47 during CHD-PAH iPSC differentiation towards CMs

To further investigate whether the protein levels of ID1 and ID3 correlate with USP9X expression, we examined the effect of altered ID1/3 expression on USP9X in differentiated cells via immunoblot analysis. In the ID KO cells, USP9X expression was lower on day 4 of CM differentiation than in WT cells (Figure 8a). All three IDs KO lines had obviously reduced USP9X expression compared with that in the three WT lines on day 4 (Figure 8b). We next examined whether *BMPR2* mutations affected the CM differentiation in CHD-PAH iPSCs via USP9X. We differentiated CHD-PAH iPSCs with *BMPR2* mutations into beating CMs and examined downstream signalling. On day 4, Smad1/5 phosphorylation as well as ID1, ID3 and USP9X expression was reduced in these CHD-PAH cells compared to control cells (Figure 8c). Immunofluorescence staining also showed reduced USP9X expression in the cytoplasm of CHD-PAH mut differentiated cells (Figure 8d). We also overexpressed ID1 to confirm the correlation and found that USP9X expression was increased following the induction of ID1 protein expression, and a corresponding

reduction in *ID3* expression was observed (Figure 8e). During CM differentiation, the levels of *EOMES* and *TBX5* were significantly reduced in *IDs* KO cells compared with WT cells (Figure 5e and Figure 8f). Overexpression of *USP9X* in *IDs* KO cells enhanced the expression of *EOMES* and *TBX5* on days 2 and 4 of CM differentiation compared to that in *IDs* KO+Empty cells (Figure 8f). Therefore, the reduction of cardiogenesis-related marker expression caused by *ID1* and *ID3* knockout were rescued by *USP9X* overexpression.

To study the regulatory effects of *IDs* on downstream gene expression, we examined possible *ID* binding partners, such as E47, an E protein transcription factor that contains a basic DNA-binding region juxtaposed to a HLH domain. Without the DNA-binding region, *ID* proteins have only an HLH domain with which to form heterodimers with E47 and thereby compete for binding with other E proteins to inhibit the transcription of target genes. Based on a previous report that E47 represses cardiogenic mesoderm gene expression [12], we further found that the *USP9X* promoter region contains the E47 binding site sequence (CACCTG) by searching JASPAR (<http://jaspar.genereg.net>) (Figure 8g). A ChIP-PCR assay revealed that the binding of E47 to the *USP9X* promoter was increased in *IDs* KO cells (Figure 8h and Figure 8i). Furthermore, we overexpressed E47 and observed reduced *USP9X* levels as determined by qPCR (Figure 8j), indicating the inhibitory effect of E47 on *USP9X* gene transcription. Our results suggest that mutant *BMP2* signals via *ID1*, leading to the accumulation of E47 on the *USP9X* promoter, which negatively regulates *USP9X* gene expression during CM differentiation (Schematic summary).

Discussion:

Although pulmonary load is thought to be important for right ventricular function in patients with PAH, there are some genetic determinants for right ventricular failure. We are interested in the impact of individual components of the BMPR2 signalling pathway on mesoderm formation during gastrulation [12, 19]. Determining whether IDs contribute to right heart failure in PAH patients carrying *BMPR2* mutations continues to be challenging. Our results showed that in iPSC-CMs from CHD-PAH patients with BMP receptor mutations, the total ID protein level was reduced, and the CM differentiation efficiency and Ca^{2+} handling were affected. We detected reduced Ca^{2+} transients, tau decay and half decay time, but the half rising time and time to peak were not altered in mutant iPSC-CMs. We also confirmed reduced ID1/ID3 expression in the right heart samples from *Bmpr2*^{+/-} rats. By generating conditional knockouts, we circumvented the embryonic lethality in double KO Id1/3 mice and found that some Ids cDKO mice spontaneously developed PAH. Spontaneous PAH (SPA) was defined by a mPAP greater than or equal to 25 mmHg by Hautefort A. et al [8]. Their *Bmpr2*-deficient rats developed SPAH in an age-dependent manner, with the incidence rate ranging between 16.7% and 27.8%, perfectly mimicking the PAH incidence in patients with BMPR2 mutations. Additionally, susceptibility to SPAH was concurrent with the high levels of perivascular fibrillar collagen. In Figure 3 we demonstrated pulmonary vascular remodelling in SPAH group, whereas no obvious difference at remodelling rate of pulmonary arteries in “non-spontaneous PAH”

compared with control mice was detected. We found that a few of Ids cDKO mice had ventricular defects or pulmonary regurgitation. The reduced Ca^{2+} transients and sarcomere length shortening could be responsible for the reduction in CO in Ids cDKO mice, which was consistent with the compromised CM function in *Bmpr2*^{+/-} rats. However, many factors have not been clarified, such as the downstream tissue-specific targets of *IDs*.

To further study the heart-specific effects of ID1/3, we employed scRNA-seq analysis, which can advantageously classify mixed cells according to their gene expression and identify new cell types or marker genes [20]. In our study, the scRNA-seq results clearly demonstrated that the CM differentiation of IDs KO cells was impaired and that clusters 2 and 5, which expressed CMP related genes, were the major populations affected in IDs KO cells. DEG analysis revealed that USP9X expression was reduced in most clusters of IDs KO cells compared with the WT cell clusters. We also showed a reduction in USP9X expression during the CM differentiation of CHD-PAH patient-derived iPSCs. These findings advance the current understanding of the specific effects of *BMPR2* mutations on the heart.

It is worth to mention that hESCs and hiPSCs share the same characteristics, including their expression of pluripotent markers and their differentiation potential. About how BMPR mutations affect heart development should be investigated in the early stage of hESC differentiation when BMPs act via receptors to drive cardiac mesoderm formation. In the hESCs culture medium, the Essential 6 (E6) media does not contain FGF and TGF β , which are included in Essential 8 (E8) medium. E6 does

not maintain pluripotency in hESCs because of the lack of these two growth factors, which reduced the level of Smad1 phosphorylation. To reduce basal activation of smad1, signal transduction was assessed mainly in E8 medium, with which we found ID1 and ID3 were coordinately regulated downstream of BMPR2 in hESCs (Figure 2).

This study has several limitations. First, IDs are not exclusively regulated by BMPR2. Different mutations in BMPR2 signalling pathway components (which cause PAH), such as those of *BMPRI*B (CHD-PAH 4), also downregulate ID expression (Supplementary Table S1, Supplementary Figure 3). Other mutations in BMPR2 signalling components also converge at IDs to potentiate PAH [21], and VEGF can induce ID expression [22]. We consistently observed reduced ID1 expression in Sugena 5416 (VEGF inhibitor)/hypoxia PAH rats [23]. Although knocking out a single factor *IDs* recapitulated heart defects in *BMPR2^{flox/-};Mox2-cre* mice, comparable *BMPR2* CM-specific knockout animals have not been studied in the PAH setting. Second, the *Bmpr2^{+/-}* heterozygous rats had some phenotypic differences, such as differential RVSPs, which could be explained mainly by differences in the rat strains since the two groups targeted *BMPR2* to generate a frame shift in exon 1 [24]. It is worth examining abortive embryos to identify possible heart defects.

BMPR2 mutations occur among three shunt types—VSD, ASD and PDA— at similar frequencies in CHD-PAH patients [4]. For example, CHD-PAH mut 2 provided a rationale for determining the presence or absence of a *BMPR* mutation in a patient with CHD-PAH. The patient had a PDA defect with an mean pulmonary

arterial pressure (mPAP) of 79 mmHg, a systolic pulmonary artery pressure (sPAP) of 97 mmHg, a PVR of 19.5 wood units (higher than 5 wood units), and a QP/QS ratio of 0.85 (less than 1.5), which was not within the surgical closure indication. He developed much more severe PAH than that caused by a PDA defect. Another female ASD patient CHD-PAH mut 3 developed severe PAH at a younger age than most patients (mPAP of 31 mmHg, PVR of 9.38 wood units). She was shown to carry the c.1042G>A mutation in *BMPR2* exon 8, which prompted predictive genetic testing in unaffected relatives. We found that her father and sister had the same mutation despite having no symptoms thus far (Supplementary table S9).

Among the *BMPR2* mutations we detected in CHD-PAH patients, nearly all were missense mutations [4]. Five types of *BMPR2* mutations exists for H/IPAH: nonsense, deletion, frame shift, splice site and missense. The relationships between the phenotypes and mutation types are worthy of further exploration. The patients with CHD-PAH had much more severe clinical manifestations than those normally caused by the defects should undergo genetic testing to detect abnormalities of *BMPR2* and its downstream components. Mutation carriers are more at risk for PAH reoccurrence than those without *BMPR2* mutations and should be monitored even after surgical closure of the shunt. From our study it was recommended to include *USP9X* and *BMPR2* signalling components in the risk gene panel to screen CHD-PAH patients who have out-of-proportion PAH in relation to a (small) shunt (small ASD or VSD) or sustained/progressive PAH after shunt closure with PAH in the setting of cardiovascular left-to-right (LR) shunt. Targeted therapies or transplantation could be

considered for these patients in the early stage of the disease.

Conclusion:

By using a number of methods, including iPSC generation and differentiation, scRNA sequencing of CRISPR/CAS9-targeted hESCs and Ids cDKO mouse CM functional assays, we found that BMPR2 signals via IDs and USP9X to drive cardiac differentiation. The loss of ID1 and ID3 expression contributes to CM dysfunction in CHD-PAH patients with *BMPR2* mutations.

Schematic summary:

Acknowledgement:

Jun Yang designed the research studies and revised and finalized the manuscript; Mingxia Du conducted the animal and human stem cell experiments and drafted the

manuscript; Xin Zhao isolated mouse CMs and conducted the cellular Ca^{2+} transient assays; Haibin Jiang conducted the iPSC-related experiments and acquired the data; Hongxian Liu performed the data analysis; Fang Zhou conducted experiments and analysed the data; Frederic Perros provided the $\text{Bmpr}^{2+/-}$ rat heart samples. We thank Guoqiang Xu for providing the USP9X plasmids and Chunmei Piao for genetically screening the CHD-PAH patients, Feng Ma for providing iPSCs generation technique support. We thank Zhuang Tian for the recruitment and catheterization of patients. Bishen Ding and Jie Na provided the related transgenic mouse lines. Lihong Sun helped with the ultrasound scanning, and Wei Ying helped with the confocal microscopy. We are grateful for the advice and revisions provided by Professor Nick Morrell. Professor Hong Gu made the major contribution on providing the CHD-PAH patient samples and advice.

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Disclosures:

The authors have no conflicts of interest to declare.

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25. Identification of the role of bone morphogenetic protein (BMP) and transforming growth factor-beta (TGF-beta) signaling in the trajectory of serotonergic differentiation in a rapid assay in mouse embryonic stem cells in vitro.

Figure Legend

Figure 1. *BMPR2* mutation impairs the differentiation and Ca^{2+} transients of CMs derived from CHD-PAH iPSCs.

(a) Immunofluorescence of EOMES, TBX5, NKX2.5, MESP1, α -Actinin, cTnT and ID1 as determined using DAPI as a counterstain on days 2, 4, 6 and 16 of cardiomyocyte (CM) differentiation. Scale bars, 50 μm . (b) mRNA levels of Brachyury(T), GATA4, TBX5 and cTnT were measured with qPCR during CM differentiation from iPSCs, N=3. (c) FACS analyses (left) and quantification (right) of the percentages of cTnT+ cells on day 16 of CM differentiation from 3 Control, 3 CHD-PAH w/o and 3 CHD-PAH mut iPS cell lines; 4-6 repeats for each cell line. Cell line 1-1 and 1-2 in CHD-PAH w/o group were obtained from the same patient. N=12-14. (d-f) Representative Ca^{2+} imaging as determined by recording Fluo-4/AM traces in Control iPSC-derived CMs(d), CHD-PAH w/o iPSC-derived CMs(e) and CHD-PAH mut iPSC-derived CMs(f). (g) Comparison of Ca^{2+} transients of Control,

CHD-PAH w/o and CHD-PAH mut CMs in a single beating episode. **(h)** Statistical analysis of Ca^{2+} handling features, such as the amplitude of Ca^{2+} transients (left) and tau decay (right); 5-8 cells for each iPSC-CM line; N=18-24. CHD-PAH w/o, CHD-PAH without BMPR mutation; CHD-PAH mut, CHD-PAH without BMPR mutation. All results are presented as the mean $\pm$ SEM; **P < 0.01, ****P < 0.0001 vs the Control, CHD-PAH w/o or CHD-PAH mut group as determined using two-way ANOVA with post-hoc tests (tukey's multiple comparisons test) (b, c and h).

Figure 2. Reduced ID1/ID3 expression in CHD-PAH iPSCs with *BMPR2* mutations and the coordinative regulation between ID1 and ID3.

(a) Representative immunoblot analysis of the pSmad1/5, ID1 and ID3 levels in CHD-PAH mut and Control iPSCs treated with or without 30 ng/ml BMP4 for 2 h. α -TUBULIN was used as loading control. **(b)** Representative immunoblotting of BMPRII, pSmad1/5, ID1 and ID3 in the right ventricles of WT and BMPRII^{+/-} rats. The results from three rats (marked as 1/2/3) are presented simultaneously. Gapdh was used as the loading control. **(c)** Immunoblot analysis of hESCs cultured with or without 30 ng/ml BMP4 for 2 h. α -TUBULIN was used as the loading control. **(d)** Immunoblot time-course analysis of pSmad1/5, Smad1, ID1 and ID3 expression in hESCs stimulated without (0 h) and with 30 ng/ml BMP4 for 0.5-36 h. Smad1 and α -TUBULIN were used as loading controls. **(e)** Immunoblot analysis of the ID1 and ID3 levels in hESCs stimulated with DMSO or LDN193189 (LDN) at different concentrations for 24 h. Smad1 and α -TUBULIN was used as loading controls. **(f)** Immunoblot and densitometric analyses of total ID1 (endogenous (lower band) and exogenous (upper band)) and ID3 expression in the 3xFlag-ID1 inducible overexpression line h9-ESC-3F-ID1 treated with Dox (2 μ g/ml). α -TUBULIN was used as the loading control. **(g)** Immunoblot and relative densitometric analyses of total ID3 (endogenous (lower band) and exogenous (upper band)) and ID1 expression in the 3xFlag-ID3 inducible overexpression line h9-ESC-3F-ID3. α -TUBULIN was used as the loading control. All cells were cultured in E8 medium (a and c-g). E8,

Essential 8™ Medium, and the composition of it was shown in Supplementary Table S8; Dox, doxycycline.

Figure 3. Ids cDKO mice are susceptible to PAH and pulmonary vascular remodelling.

(a-e) Assessment of the right ventricular systolic pressure (RVSP) and total pulmonary vascular resistance index (tPVRI) ($tPVRI = RVSP/CI$) (a), Fulton index (right ventricular hypertrophy, $=RV/(LV+S)$) (b), tricuspid annular plane systolic excursion (TAPSE) (c), cardiac output (CO) and CI ($=CO/BW$) (d), and left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) (e) in control mice (Control, black) and Id cDKO mice without spontaneous arterial hypertension (non-SPAH, blue) or with SPAH (red) at 6 months of age. Mice were defined as having SPAH when their RVSP was higher than 25 mmHg. The numbers of mice that did or did not develop SPAH among the total mice in each group are shown on the x-axis. (f) Representative immunofluorescence staining of α -actin smooth muscle (α -SMA, green) and the endothelial cell marker CD31 (red) in lung sections revealed thickened arterial walls in Id cDKO mice with SPAH at 6 months of age. Nuclei were counterstained with DAPI (blue). Scale bars, 15 μ m. (g) Representative images of vascular remodelling in the distal arterioles stained with elastin and immunostained α -SMA, and representative images of pulmonary arteries stained with H&E. The lung sections were obtained from control and Id cDKO mice with non-SPAH or SPAH at 6 months of age. Scale bar, 25 μ m. (h) Vessel muscularization analysis based on α -SMA labelling on the lung sections from control and Id cDKO mice with SPAH at 6 months of age. (i) Pulmonary vascular remodelling rate based on Elastica van Gieson staining on the lung sections from control and Id cDKO mice with or without SPAH at 6 months of age. The vessels of 25-100 μ m in diameter in (f) and (g) were quantified and N=6-12 mice for each group in (h) and (i). CI, cardiac input; RV, right ventricle; LV, left ventricle; S, septum; BW, body weight; ns, no significant. All results are presented as the mean $\pm$ SEM; *P< 0.05,

****P < 0.01, ***P< 0.001 and ****P< 0.0001** vs the Control and other groups as determined using one-way ANOVA with post-hoc tests (dunnett's multiple comparisons test in panel a-e and h, and tukey's multiple comparisons test in panel i).

Figure 4. Ventricular defects and reduced Ca²⁺ transients in CMs compromised the right heart function in Ids cDKO mice.

(a) Types of blood reflux in the hearts of 2-month-old WT (n=20), Id1^{-/-}Id3^{f/w};Mesp1-cre (n=11) and Id1^{+/-}Id3^{f/f};Mesp1-cre (n=16) mice as detected by colour Doppler echocardiography. (b) Representative echocardiographic images (yellow arrow, left) and analysis (right) of Ids cDKO and control mice, revealing regurgitation across the pulmonary valve during diastole in Id cDKO mice. (c) Representative images of interventricular septum (IVS) defects (yellow arrow) in Ids cDKO mice based on colour Doppler echocardiography. (d) Haematoxylin and eosin (H&E) (left) and immunofluorescence (right) staining of the CM markers wheat germ agglutinin (WGA) and MF20 (myosin heavy chain 1, MYH1) in longitudinal heart sections from 6-month-old mice. Scale bars: 200 µm (black), 1000 µm (yellow) and 100 µm (white). (e-f) Immunofluorescence staining of WGA and MF20 (e) and quantification of the CM areas (f) in the hearts of control and Ids cDKO mice, revealing decreased CM areas in the middle and outer myocardial walls of the RVs from 6-month-old Ids cDKO mice. Scale bars: 1000 µm (yellow) and 20 µm (white); n≥60 cells. (g) Representative Ca²⁺ transient trace showing a decreased transient amplitude for the RV CMs of Ids cDKO mice but no significant differences in the LV CMs. (h) Representative raw traces of the sarcomere length showing decreased sarcomere length shortening in RV-CMs of Ids cDKO mice. The percentages of sarcomere length (SL) shortening were quantified in the CMs. N≥6 cells from each heart, 2 mice from each group. LV, left ventricle; RV, right ventricle; IVS, interventricular septum. All results are presented as the mean±SEM; *P< 0.05 and ****P< 0.0001 as determined using unpaired t tests (f-h).

Figure 5. Impaired cardiac differentiation in ID double-knockout hESC lines.

(a) Immunoblotting analysis of ID1 and ID3 in 3 IDs KO hESC lines (ID KO-#1/#2/#3) and 3 WT (WT-#1/#2/#3) cell lines. α -TUBULIN was used as a loading control. The experiment was performed 3 times for each cell line; n=3. (b) FACS analyses (left) and quantification (right) of the percentages of cTnT+ cells on day 16 of CM differentiation. n=3, 3 repeats for each cell line; N=3, 3 cell lines in each group. (c) Immunostaining of α -Actinin (green) and cTnT (red) in cells differentiated from WT-#1 and ID KO-#1 cell lines. DAPI was used as a counterstain and is shown in blue. Scale bars, 50 μ m. (d) Immunoblotting of EOMES/ID1/ID3 and α -TUBULIN (as a loading control) at different stages of CM differentiation in WT and IDs KO cell lines. (e) The mRNA levels of Brachyury (T), MIXL1, TBX5, GATA4 and ISL1 during CM differentiation were measured by qPCR. n=3, 3 technical replicates for one cell line. Dif., differentiation. All results are presented as the mean $\pm$ SEM; **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs WT and IDs KO as determined using unpaired t tests (b) and two-way ANOVA with post-hoc tests (sidak's multiple comparisons test) (e).

Figure 6. scRNA-seq analysis revealed the major affected populations of cardiac mesoderm progenitors in the IDs KO line.

(a) Schematic of the differentiation of CMs differentiated from hESCs. The mRNA levels of EOMES, MESP1 and ID1 at different stages were measured by qPCR and revealed that day 4 was the key point during differentiation. (b) t-Distributed stochastic neighbour embedding (t-SNE) with the R-Seurat package showing the different sources of cells analysed (right) and 6 clusters for WT and IDs KO lines (left) on day 4 of CM differentiation. (c) The Z score-scaled average expression levels of DEGs for 6 clusters are shown as a heatmap (top), with the selected lineage-specific markers shown on the right. Representative GO terms of biological processes are shown at the bottom. (d) The expression level distributions of representative marker genes are shown as violin plots across clusters. Cell lines and clusters are represented by different colours.

Figure 7. *USP9X* is a downstream effector gene of deficient IDs in CMPs.

(a) t-SNE visualization of the key marker genes (EOMES, GATA4, ISL1, BMP4, MESP1, IRX3 and HAND1) in clusters 2 and 5. Cells expressing the representative marker genes are denoted in red. (b) The Z score-scaled average expression levels of differentially expressed genes (DEGs) between WT cells (red line) and IDs KO cells (blue line) for 6 clusters are shown as heatmaps. (c) The expression level distributions of *USP9X* are shown by t-SNE (upper). Cells expressing representative marker genes are denoted in red. The expression level distributions of *USP9X* are shown as violin plots in various clusters (lower). Cell lines and clusters are represented by different colours. (d) Expression level alterations of the key markers (NANOG, ISL1, EOMES, MESP1 and GATA4) of clusters 2 and 5 and of *USP9X* are shown in different identity clusters as a dot plot. The size of the dot denotes the percentage of cells within a cluster, while the colour denotes the average expression level across all cells within a cluster.

Figure 8. Deficient BMPR2/ID signalling downregulates *USP9X* via E47 during CM differentiation.

(a-b) Immunoblot analysis and quantification of *USP9X*, ID1 and ID3 in WT and IDs KO cells at different stages of CM differentiation (a) and in different WT and IDs KO cell lines (3 cell lines in each group) on day 4 of CM differentiation (b). α -TUBULIN was used as the loading control. (c) Immunoblot analysis of *USP9X*, pSmad1/5, ID1 and ID3 expression and quantification of *USP9X* in Control and CHD-PAH mut iPSCs on day 4 of CM differentiation. Three cell lines, marked as 1, 2, and 3, were analysed in each group. Total Smad1 and α -TUBULIN were used as loading controls. (d) Representative fluorescence intensity of ID1 (red) and *USP9X* (green) (left) and the corresponding ImageJ-based quantifications (right) of the fluorescence intensity in the cells on day 4 of CM differentiation from Control or CHD-PAH mut iPSCs. n=5, 5 random fields. Scale bars, 50 μ m. (e) Immunoblot analysis of *USP9X*, ID1 (endogenous (lower band) and exogenous (upper band)) and ID3 expression in

h9-ESC-3F-ID1 cells with 3xFlag-ID1 overexpression induced by Dox (2 μ g/ml) at different time points. α -TUBULIN was used as the loading control. **(f)** The mRNA levels in WT, IDs KO+Empty (IDs KO overexpressing an empty vector in), and IDs KO+USP9X (overexpressing USP9X in IDs KO) cells on various days during CM differentiation as measured by qPCR. n=3, 3 technical replicates for one cell line. **(g)** Predicted DNA binding site motif of E47 in the *USP9X* promoter region. **(h-i)** The *USP9X* promoter region in WT and IDs KO cells pulled down with an E47 antibody was detected by ChIP-PCR (h) and ChIP-qPCR (i). n=3 in panel i. **(j)** The mRNA levels in WT cells after transfection with E47 or empty vector as determined by qPCR. n=3. All results are presented as the mean $\pm$ SEM; *P< 0.05, **P < 0.01, ***P< 0.001 and ****P< 0.0001 were determined using two-way ANOVA with post-hoc tests (sidak's multiple comparisons test) (a and j), two-way ANOVA with post-hoc tests (tukey's multiple comparisons test) (f) or unpaired t tests (b-d and i).

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. 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% CO₂ 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 $7-10 \times 10^4$ cells/cm² 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 NEOFECTTM 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™ 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 $\Delta\Delta C_t$ 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 μ L 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 μ L 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 μ L 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 μ L of wash buffer. Then, cTnT expression was evaluated by flow cytometry (BD, ACCURI C6, USA).

Spontaneous Ca²⁺ 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 μ M Fluo-4 AM (Beyotime, cat. no. S1060, China) in Tyrode's solution (ddH₂O containing 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 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 $7\text{-}10 \times 10^4$ cells/cm² 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 μ g of the USP9X overexpression vector and 1.5 μ g of the control vector, which did not encode USP9X, were diluted in 75 μ L of Opti-MEM (Gibco, cat. no.12040-077) separately to dilute the vectors, and 6 μ L of Lipofectamine Stem Transfection Reagent was diluted with 75 μ L 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- μ m-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 μ m) 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 μ mol/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 $\pm$ SEM. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.00005$.

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Supplementary Figure Legends

Supplementary Figure 1 Characterization of CHD-PAH with or without mutation iPSCs and Control iPSCs derived from PBMCs

(a) Sanger sequencing chromatograms of the forward strand 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 μ m. CHD-PAH w/o, CHD-PAH without BMPR mutation; CHD-PAH mut, CHD-PAH with BMPR mutation.

Supplementary Figure 2 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 μ m; y-axis, fluorescence intensity in arbitrary units). Nuclei were costained with DAPI. Scale bar, 5 μ m. (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 $\pm$ 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 3 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 μ m. (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, Blastidicin; 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 μ g/ml, 24 hours). All results are presented as the mean $\pm$ SEM; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were determined using unpaired t tests (a and d).

Supplementary Figure 4 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 $\pm$ 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 5 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 μ m; y-axis, fluorescence intensity in arbitrary units). Scale bar, 50 μ m (top); 10 μ m (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 $\pm$ SEM; ns=not significant as determined using unpaired t test (b).

Supplementary Figure 6 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-EF1 α , EF1 α 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 iPSC cell lines

Donor status			BMPR2 gene status		
iPSC lines	Sex	Clinical description	Nucleotide 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	Nucleotide 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 μ M
Dox	Sigma	D9891	2 μ g/ml
G418	Sigma	A1720	50-400 μ g/ml
BSD	Invitrogen	R201-01	2 μ g/mL
Puromycin	Sigma	8833	0.3-1 μ g/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
<i>Brachyury(T)</i>	TGCTTCCCTGAGACCCAGTT	GATCACTTCTTTCCTTTGCATCAAG
<i>cTnT</i>	AAGAGGCAGACTGAGCGGGAAA	AGATGCTCTGCCACAGCTCCTT
<i>EOMES</i>	CACATTGTAGTGGGCAGTGG	CGCCACCAAACCTGAGATGAT
<i>E47</i>	TGGGCAGCAGTGGAAGCAG	GGGCTGAGGAGAAGGAGGATG
<i>GAPDH</i>	TCATTTCTGGTATGACAACGA	TCTACATGGCAACTGTGAGG
<i>GATA4</i>	GTGTCCCAGACGTTCTCAGTC	GGGAGACGCATAGCCTTGT
<i>ID1</i>	GTTGGAGCTGAACTCGGAATCC	ACACAAGATGCGATCGTCCGCA
<i>ISL1</i>	AGATTATATCAGGTTGTACGGGATCA	ACACAGCGGAAACACTCGAT
<i>MIXL1</i>	CAGAGTGGGAAATCCTTCCA	TGAGTCCAGCTTTGAACCAA
<i>MESPI</i>	GAAGTGGTTCCTTGGCAGAC	TCCTGCTTGCCTCAAAGTGT
<i>TBX5</i>	CACATTGTAGTGGGCAGTGG	CGCCACCAAACCTGAGATGAT
<i>USP9X</i>	CATGATCTCCTGGCAAAATTGGC	GACGTATCAGCTCAAGTAGCTTT
<i>USP9X</i> (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	CCTGGAACCCACACATGAC	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	√	√
NaHCO ₃	√	√
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 = Right cardiac output (L/min); CI = Cardiac index (L/(min·m²)); 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	2.77	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

Kindly provided by The Capital Medical University Affiliated Beijing Anzhen Hospital.

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

a)

b)

a)