Identification of rare sequence variation underlying heritable pulmonary arterial hypertension

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Pulmonary arterial hypertension (PAH) is a rare disorder with a poor prognosis. Deleterious variation within components of the transforming growth factor-β pathyway, particularly the bone morphogenetic protein type 2 receptor (BMPR2), underlies most heritable forms of PAH. To identify the missing heritability we perform whole-genome sequencing in 1038 PAH index cases and 6385 PAH-negative control subjects. Case-control analyses reveal significant overrepresentation of rare variants in ATP13A3, AQP1 and SOX17, and provide independent validation of a critical role for GDF2 in PAH. We demonstrate familial segregation of mutations in SOX17 and AQP1 with PAH. Mutations in GDF2, encoding a BMPR2 ligand, lead to reduced secretion from transfected cells. In addition, we identify pathogenic mutations in the majority of previously reported PAH genes, and provide evidence for further putative genes. Taken together these findings contribute new insights into the molecular basis of PAH and indicate unexplored pathways for therapeutic intervention.
idiopathic and heritable pulmonary arterial hypertension (PAH) are rare disorders characterised by occlusion of arterioles in the lung, leading to marked increases in pulmonary vascular resistance. Life expectancy from diagnosis averages 3–5 years, with death ensuing from failure of the right ventricle.

Mutations in the gene encoding the bone morphogenetic protein type 2 receptor (BMPR2), a receptor for the transforming growth factor-beta (TGF-β) superfamily, account for over 80% of families with PAH, and approximately 20% of sporadic cases. Mutations have been identified in genes encoding other components of the TGF-β/bone morphogenetic protein (BMP) signalling pathways, including ACVRL1 and ENG. On endothelial cells, BMPR2 and ACVRL1 form a signalling complex, utilising ENG as a co-receptor. Case reports of rare sequence variation in BMPR2-negative kindreds revealed heterozygous mutations in genes not directly impacting on the TGF-β/BMP pathway, including CAV1, and the potassium channel, KCNK3. Deletions and loss of function mutations in TRX4, an essential regulator of embryonic development, were identified in childhood-onset PAH. A clinically and pathologically distinct form of PAH, known as pulmonary veno-occlusive disease or pulmonary capillary haemangiomatosis (PVOD/PCH), was shown recently to be caused by biallelic recessive mutations in EIF2AK4, a kinase in the integrated stress response.

The purpose of the present study was to identify additional rare sequence variation contributing to the genetic architecture of PAH, and to assess the relative contribution of rare variants in genes implicated in prior studies. A major finding is that rare likely causal heterozygous variants in several previously unidentified genes (ATP13A3, AQP1 and SOX17) were significantly overrepresented in the PAH cohort, and we provide independent validation for GDF2 as a causal gene.

Results

Description of the PAH cohort. In total, 1048 PAH cases (1038 index cases and 10 related affected individuals) were recruited for WGS. Of these, 908 (86.7%) were diagnosed with idiopathic PAH, 58 (5.5%) gave a family history of PAH and 60 (5.7%) gave a history of drug exposure associated with PAH. Twenty two cases (2.1%) held a clinical diagnosis of PVOD/PCH (Fig. 1a). Demographic and clinical characteristics of the PAH cohort are provided in Supplementary Table 1. An additional UK family was recruited separately for novel gene identification studies. Briefly, the proband was diagnosed at 12 years with a persistent ductus arteriosus and elevated pulmonary arterial pressure. Explant lung histology following heart-lung transplantation revealed the presence of plexiform lesions. Two of the proband’s offspring were also diagnosed with childhood-onset PAH, one of which had an atrial septal defect. The proband’s parents, siblings and a third child showed no evidence of cardiovascular disease.

Pathogenic variants in previously reported PAH disease genes. Our filtering strategy detected rare deleterious variation in previously reported PAH genes in 19.9% of the PAH cohort. For BMPR2, rare heterozygous mutations were identified in 160 of 1046 cases (15.3%). The frequency of BMPR2 mutations in familial PAH was 75.9%, in sporadic cases 12.2%, and 8.3% in anorexigen-exposed PAH cases. Forty-eight percent of BMPR2 mutations were reported previously, and the remainder were newly identified in this study. Fourteen percent of BMPR2 mutations resulted in the deletion of larger protein-coding regions ranging from 5 kb to 3.8 Mb in size. Supplementary Data 1 provides the breakdown of BMPR2 SNVs and indels, and the larger deletions are shown in Fig. 2a–c with a detailed summary in Supplementary Table 2.

Of the other genes previously reported in PAH we identified deleterious heterozygous rare variants in ACVRL1 (9 cases, 0.9%), ENG (6 cases, 0.6%), SMAD9 (4 cases, 0.4%), KCNK3 (4 cases, 0.4%), and TRX4 (14 cases, 1.3%). We identified one case with

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**Fig. 1** Flow diagrams illustrating a the composition of the NIHR BioResource—Rare Diseases (NIHR BR-RD) PAH study and b the analysis strategy to identify novel PAH disease genes. a The study comprised 1048 adult cases (aged 16 or over) attending specialist pulmonary hypertension centres from the UK (n = 731), and additional cases from France (n = 142), The Netherlands (n = 45), Germany (n = 82) and Italy (n = 48). A series of case-control comparisons including and excluding cases with variants in previously reported disease genes were undertaken using complementary filtering strategies.
**Fig. 2** Analysis of copy number deletions. **a** Deletions affecting the BMPR2 locus in 23 PAH cases. Genes are indicated in orange and labelled with their respective gene symbol. Deletions are drawn as blue boxes above the genome axis (grey) showing the genomic position on chromosome 2. The grey box highlights the location of BMPR2. **b** Locus zoom on BMPR2 highlighting the focal deletions affecting one or more exons. **c** WGS coverage profiles of a selected set of smaller and larger deletions, visualised with the Integrative Genomics Viewer (IGV)\(^5\), with deletions highlighted by red bars. **d** and **e** Manhattan plots of the genome-wide case-control comparison of large deletions. In **d**, all subject are considered. In **e**, subject with larger deletions affecting the BMPR2 locus are excluded. The adjusted P value threshold of \(5 \times 10^{-8}\) for genome-wide significance is indicated by the red line.
highly deleterious variants in both BMPR2 (p.Cys123Arg) and SMAD9 (p.Arg294Ter). Details of consequence types, deleteriousness and conservation scores, and minor allele frequencies are provided in Supplementary Data 2. Fourteen cases (1.3%) with biallelic EIF2AK4 mutations were found. No pathogenic coding variants in CAV1, SMAD1 or SMAD4 were identified. Taken together, rare causal variation in non-BMPR2 disease genes (TBX4, ENG, ACVR1I, SMAD9, KCNK3 and EIF2AK4) accounted for 4.7% of the entire PAH cohort. The clinical characteristics of cases with variants in these previously reported genes are shown in Supplementary Table 3.

In a case-control comparison of the frequencies of deleterious variants confined to the previously reported PAH genes, we observed significant overrepresentation of rare variants in BMPR2, TBX4, ACVR1I and biallelic variants in EIF2AK4 only (P < 0.05; Supplementary Table 4).

Identification of novel PAH disease genes. The strategy to identify novel causative genes in PAH employed a series of case-control analyses (Fig. 1b). To identify signals that might be masked by variants in previously reported PAH genes, we excluded subjects with rare variants and deletions in BMPR2, EIF2AK4, ENG, ACVR1I, TBX4, SMAD9 and KCNK3. A genome-wide comparison of protein-truncating variants (PTVs), representative of high impact variants, identified a higher frequency of PTVs in ATPI3A3 (six cases) (P_adj = 0.0346). Moreover, we identified additional PTVs in several putative PAH genes, including EV5 (5 cases, 1 control) and KDR (4 cases, 0 controls; Fig. 3a), that require further validation to evaluate their contribution to PAH pathogenesis (Supplementary Table 5).

We next analysed rare missense variants overrepresented in the PAH cohort, again excluding subjects with variants in the previously reported PAH genes. This revealed significant over-representation of rare variants in GDF2 after correction for multiple testing (P_adj = 0.0023), followed by AQP1 (Fig. 3b and Supplementary Table 6). Next, in a combined analysis of rare missense variants and PTV, only GDF2 remained significant (P = 0.001). Rare variants in additional putative genes occurred at higher frequency in cases compared to controls, including AQP1, ALPL2, ATPI3A3, OR8U1, IFT74, FLNA, SOX17, ATPI3A5, C3orf20 and PIWIL1 (uncorrected P value < 0.0005), but were not significant after correction for multiple testing (Fig. 3c and Supplementary Table 7).

In order to increase power to detect rare associations, we deployed SKAT-O on filtered rare PTVs and missense variants. Excluding previously reported genes, this analysis revealed an association with rare variants in AQP1 (P_adj = 4.28 × 10^{-6}) and SOX17 (P_adj = 6.7 × 10^{-5}; Fig. 3d). AQP1 and SOX17 were both also nominally significant in the combined burden tests, described above. Association was also found with rare variants in MFRP (P_adj = 1.3 × 10^{-3}). However, we consider MFRP a false-positive finding for reasons given in the Discussion. Supplementary Table 8 shows the top 50 most significant genes identified by SKAT-O, providing further candidates to be evaluated in future studies. Details of rare variants in novel PAH genes (GDF2, ATPI3A3, AQP1, SOX17) identified in cases are provided in Supplementary Data 3.

Notably, a genome-wide assessment of larger structural variation did not identify any additional large deletions after exclusion of subjects harbouring deletions in BMPR2 (Fig. 2d, e).

The proportion of PAH cases with mutations in the new genes was 3.5%. The clinical characteristics of PAH cases with mutations in these genes are provided in Supplementary Table 3b.
Of note, cases with mutations in SOX17 and AQP1 were significantly younger at diagnosis (32.8 ± 16.2 years \(P = 0.002\)) and 36.9 ± 14.3 years \(P = 0.013\), respectively) compared to cases with no mutations in the previously established genes (51.7 ± 16.6 years).

**Fig. 4** Pedigree structures and analysis of familial transmission of variants in AQP1 and SOX17. a Individual II.1 harbours a heterozygous de novo SOX17 c.411 C > G (p.Y137*) PTV resulting in a premature termination codon, which has been transmitted to the affected male (III.1). No unaffected family members carry the variant. No sample was available from subject III.2. b Proband E011942 has inherited a heterozygous AQP1 c.583 C > T (p.R195W) missense variant from her affected father. No sample was available from the affected sister of the proband. The younger healthy uncle of the index case also carries the AQP1 variant. No samples or further clinical information was available for the grandparents, who were not known to have cardiopulmonary disease. c Both the proband E012415 and her father are affected and carry the rare AQP1 c.527 T > A (p.V176E) missense variant. There was no further information available about the siblings of the father. d Subject E010634 has inherited the heterozygous AQP1 c.583 C > T (p.R195W) missense variant from her affected father. No rare variants in previously reported PAH genes were identified in any of these families. Index cases are highlighted in red. d death, mo months old, yo years old

**Non-coding variation around PAH disease genes**. An initial analysis for enrichment of variants in the non-coding sequence surrounding previously reported and newly identified PAH disease genes, including upstream gene regions, 5' UTRs, intronic sequence, 3' UTRs and downstream gene regions, did not detect
an significant overrepresentation in the PAH cohort. Details of the non-coding variants that passed the filtering strategy are provided in Supplementary Data 4.

Independent validation and familial segregation analysis. To provide further validation of the potentially causal role of mutations in the new genes identified, we examined whole-exome data from an independent UK family with three affected individuals across two generations. Microsatellite genotyping across chromosome 2q33 had previously demonstrated non-sharing of haplotypes in affected individuals, consistent with exclusion of linkage to the BMPR2 locus. No pathogenic variants were identified in the protein-coding regions of the BMPR2 gene or other TGF-β pathway genes. Analysis of exome sequence data from individual II-1 identified a novel heterozygous c.411 C > G (p. Y137*) PTV in the SOX17 gene. Segregation analysis in the extended family demonstrated that the mutation had arisen de novo in the affected father (II-1) and was transmitted to the affected offspring (III-1). All unaffected family members were confirmed as wild-type (Fig. 4a).

Three HPAH subjects harbouring rare variants in AQP1, identified in the NIHR BR-RD WGS study, were also selected for familial co-segregation analysis (Fig. 4b–d). No pathogenic variants in any of the previously reported genes were identified in these families. The first pedigree comprised three affected individuals across two generations. Sanger sequencing confirmed the presence of the heterozygous AQP1 c.583 C > T (p.R195W) missense variant in the proband (E011942), the affected father (E011942.f) and the healthy younger paternal uncle (E011942.u1). An additional unaffected uncle did not carry the AQP1 variant. These results indicate likely incomplete penetrance in the unaffected carrier, as observed in BMPR2 families. No additional clinical information was available for the deceased grandparents (Fig. 4b). The remaining two families comprised affected parent-offspring individuals. By Sanger sequencing we independently confirmed a heterozygous AQP1 c.527 T > A (p. Val176Glu) missense variant in proband (E012415) and his affected father (Fig. 4c), as well as a heterozygous AQP1 c.583 C > T (p.R195W) missense variant in proband (E010634) and her affected father (Fig. 4d). These results highlight recurrent AQP1 variation across unrelated families and demonstrate co-segregation with the phenotype.

Predicted functional impact of variants in novel PAH genes. To evaluate the potential functional impact of rare variants identified in the likely causative new genes we performed structural analysis of GDF2, ATP13A3, AQP1, and SOX17. In addition we undertook a functional analysis of the GDF2 variants identified.

![Fig. 5 Structural analysis of GDF2 mutations.](image-url)
Heterozygous mutations in GDF2 exclusive to PAH cases comprised 1 frameshift variant and 7 missense variants. GDF2 encodes growth and differentiation factor 2, also known as bone morphogenetic protein 9 (BMP9), the major circulating ligand for the endothelial BMPR2/ACVRL1 receptor complex. Amino acid substitutions were assessed against the published crystal structure of the prodomain bound form of GDF2 (Fig. 5). Variants clustered at the interface between the prodomain and growth factor domain. Since the prodomain is important for the processing of GDF2, it is likely that amino acid substitutions reduce the stability of the prodomain-growth factor interface. In keeping with these predictions, HEK293T cells transfected with GDF2 variants exclusive to PAH cases, demonstrated reduced secretion of mature GDF2 into the cell supernatants (Fig. 5d), compared with wild-type GDF2.

Fig. 6 Structural analysis of ATP1A3 mutations. a) Topology of ATP1A3, plotted according to UniProtKB Q9H7F0. Frameshift and stop-gained mutations identified in PAH cases are shown as khaki circles, and missense mutations as red circles. Frameshift/stop-gained mutations are predicted to truncate the protein prior to the catalytic domain and essential Mg binding sites, leading to loss of ATPase activity. b) Sequence alignment of ATP1A3 with ATP1A1 (P05024), of which the high resolution structure was used for the structural analysis in c. The conserved regions of ATP1A3 and ATP1A1, essential for ATPase activity, show good alignment (data not shown). Only regions containing the missense PAH mutations are shown, with positions of the four missense mutations highlighted in yellow above the sequences. c) Structural analysis of the 4 PAH missense mutations plotted on the ATP1A1 crystal structure based on the sequence alignment in b (PDB: 3wgu). Green: α subunit (P05024), cyan: β subunit (P05027), grey: γ-subunit transcript variant a (Q58k79). Y535, Y677, R685 and I787 are the numbering in ATP1A1. Positions of the four missense mutations found in PAH are labelled and highlighted by red circles. d) Magnified view of the cytoplasmic region of the ATPase, showing the presence of ADP at the active site. The conserved regions essential for ATPase activity are shown in light pink. The L675V and R858H mutations are located close to the ATP catalytic region.

Heterozygous mutations in GDF2 exclusive to PAH cases comprised 1 frameshift variant and 7 missense variants. GDF2 encodes growth and differentiation factor 2, also known as bone morphogenetic protein 9 (BMP9), the major circulating ligand for the endothelial BMPR2/ACVRL1 receptor complex. Amino acid substitutions were assessed against the published crystal structure of the prodomain bound form of GDF2 (Fig. 5). Variants clustered at the interface between the prodomain and growth factor domain. Since the prodomain is important for the processing of GDF2, it is likely that amino acid substitutions reduce the stability of the prodomain-growth factor interface. In keeping with these predictions, HEK293T cells transfected with GDF2 variants exclusive to PAH cases, demonstrated reduced secretion of mature GDF2 into the cell supernatants (Fig. 5d), compared with wild-type GDF2.
We identified three heterozygous frameshift variants, two stop gained, two splice region variants in ATP13A3, which are predicted to lead to loss of ATPase catalytic activity (Fig. 6a). In addition, we identified 4 heterozygous likely pathogenic missense variants in PAH cases, two near the conserved ATPase catalytic site and predicted to destabilise the conformation of the catalytic domain (Fig. 6b–d). The distribution of variants (Fig. 6a) suggests that these mutations impact critically on the function of the protein.

The majority of rare variants identified in ATP13A3, which encodes aquaporin-1, are situated within the critical water channel (Fig. 7). In particular the p.Arg195Trp variant, identified...
in five PAH cases, locates at the hydrophilic face of the pore. This arginine at position 195 helps define the constriction region of the AQP1 pore structure and is conserved across the water-specific aquaporins. Rare variants in SOX17, included four nonsense variants (including the PTV identified in the additional UK family) predicted to lead to loss of the beta-catenin binding region, and six missense variants predicted to disrupt interactions with Oct4 and beta-catenin (Fig. 8).

GDF2 is known to be secreted from the liver, but the cellular localisation of proteins encoded by the other novel genes is less...
well characterised. Thus we employed immunohistochemistry to examine localisation in the normal and hypertensive human pulmonary vasculature. Figure 9 shows that AQP1, ATP13A3 and SOX17 are predominantly localised to the pulmonary endothelium in normal human lung and to endothelial cells within plexiform lesions of patients with idiopathic PAH. In addition, we determined the relative mRNA expression levels of AQP1, ATP13A3 and SOX17 in primary cultures of pulmonary artery smooth muscle cells (PASMCs), pulmonary artery endothelial cells (PAECs) and blood outgrowth endothelial cells (BOECs). AQP1 was expressed in PASMCs and endothelial cells, with a trend towards higher levels in PASMCs (Fig. 10a). ATP13A3 was highly expressed in both cell types (Fig. 10b), whereas SOX17 was almost exclusively expressed in endothelial cells (Fig. 10c). Although AQP1 and SOX17 are known to play roles in endothelial function, the function of ATP13A3 in vascular cells is entirely unknown. Thus, we determined the impact of ATP13A3 knockdown on proliferation and apoptosis of BOECs. Loss of ATP13A3 led to marked inhibition of serum-stimulated proliferation of BOECs, and increased apoptosis in serum-deprived conditions (Fig. 10d–f).

**Discussion**

We report a comprehensive analysis of rare genetic variation in a large cohort of index cases with idiopathic and heritable forms of PAH. Whilst we utilised WGS, the main goal was the identification of rare causal variation underlying PAH in the protein-coding sequence. The approach involved a rigorous case-control comparison using a tiered search for variants. First, we searched for high impact PTVs overrepresented in cases, having excluded previously established PAH genes. This revealed PTVs in ATP13A3, a poorly characterised P-type ATPase of the P5 subfamily. There is little information regarding the function of the ATPase, ATP13A3, which appears widely expressed in mouse tissues. Although, the precise substrate specificity is unknown, ATP13A3 plays a role in polyamine transport. Based on available RNA sequencing data, ATP13A3 is highly expressed in human pulmonary vascular cells and cardiac tissue. We confirmed that ATP13A3 mRNA is expressed in primary cultured pulmonary artery smooth muscle cells and endothelial cells, and provide preliminary data that loss of ATP13A3 inhibits proliferation and increases apoptosis of endothelial cells. These findings are consistent with the widely

**Fig. 9** Immunolocalisation of AQP1, ATP13A3 and SOX17 in normal and PAH lung. The typical histological findings (haematoxylin and eosin staining) of concentric vascular lesions with associated plexiform lesions are shown (a). Higher magnification images of plexiform lesion (b), with frequent endothelialised channels (c, anti-CD31) surrounded by myofibroblasts (d, anti-SMα). Additional high magnification images demonstrating endothelial expression of ATP13A3 (e), AQP1 (f) and SOX17 (g) in PAH lung. Controls lung sections demonstrating predominantly endothelial expression of ATP13A3 (h), AQP1 (i) and SOX17 (j). (Scale bars = 50 µm)
accepted paradigm that endothelial apoptosis is a major trigger for the initiation of PAH28,29. It will be of considerable interest to determine the role of ATP13A3 in vascular cells and whether it is functionally associated with BMP signalling, or represents a distinct therapeutic target in PAH.

Analysis of missense variation, and a combined analysis of all predicted deleterious variation, revealed that mutation at the GDF2 gene is also significant determinant of predisposition to PAH. Of the new genes identified, GDF2 provides further evidence for the central role of the BMP signalling pathway in PAH. GDF2 encodes the major circulating ligand for the endothelial BMPR2/ACVR1 receptor complex20. Taken together, the genetic findings suggest that a deficiency in GDF2/BMPR2/ACVR1 signalling in pulmonary artery endothelial cells is critical in PAH pathobiology. The majority of GDF2 variants detected in our adult-onset PAH cohort were heterozygous missense variants, in contrast to a previous case report of childhood-onset PAH due to a homozygous nonsense mutation20. The finding of causal GDF2 variants in PAH cases, associated with reduced production of GDF2 from cells, provides further support for investigating replacement of this factor as a therapeutic strategy in PAH31.

To maximise the assessment of rare variation in a case-control study design, we deployed the SKAT-O test. This approach revealed a significant association of rare variation in the aquaporin gene, AQPI, and the transcription factor encoded by SOX17. Of note, both AQPI and SOX17 were within the top 8 ranked genes in our combined PTV and missense burden test analysis (Supplementary Table 7), providing further confidence in their causative contribution to PAH.

Aquaporin-1 belongs to a family of membrane channel proteins that facilitate water transport in response to osmotic gradients32, and AQPI is known to promote endothelial cell migration and angiogenesis32. Thus, approaches that maintain or restore pulmonary endothelial function could offer new therapeutic directions in PAH. Conversely, AQPI inhibition in pulmonary artery smooth muscle cells ameliorated hypoxia-induced pulmonary hypertension in mice33, suggesting that further studies are required to determine the key cell type impacted by AQPI mutations in human PAH, and the functional impact of these AQPI variants on water transport. The demonstration of familial segregation of AQPI variants with PAH provides further support for the potentially causal role of these mutations in disease. However, we also identified an unaffected AQPI variant carrier consistent with reduced penetrance, which is well described for other PAH genes, including BMPR2.

Although functional studies are required to confirm the mechanisms by which mutations in SOX17 cause PAH, this finding provides additional support for the vascular endothelium as the major initiating cell type in this disorder. SOX17 encodes the SRY-box containing transcription factor 17, which plays a fundamental role in angiogenesis34 and arteriovenous
diffuse collagen degeneration in the pulmonary interstitium28. Moreover, SHOX transcripts are largely conserved across species, and therefore likely to have a similar role in humans and mice. Our findings suggest that SHOX mutations play a causal role in PAH, and that this gene could be an important therapeutic target.

Methods

Ethics and patient selection. Cases were recruited from the UK National Pulmonary Hypertension Centres, Universite Sud Paris (France), the VU University Medical Centre Amsterdam (the Netherlands), the University Medical Center Utrecht (the Netherlands), the Marburg (Germany), San Matteo Hospital, Pavia (Italy), and Medical University of Graz (Austria). All cases had a clinical diagnosis of idiopathic PAH, heritable PAH, drug-associated and toxin-associated PAH, or PVOD/PCH established by their expert centre. The non-PAH cohort comprised 6385 unrelated controls recruited to the NIHR BR-RD study. All PAH and non-PAH patients provided written informed consent (UK Research Ethics Committee: 13/EE/0325), or local forms consenting to genetic testing in deceased patients and non-UK cases. An additional UK family diagnosed with HPAH was ascertained as described previously25. Blood and saliva samples were collected under written informed consent of the participants or their parents for use in genetic identification studies (UK Research Ethics Committee: 08/H0082/32).

High-throughput sequencing. DNA extracts from venous blood underwent whole-genome sequencing using the Illumina TruSeq DNA PCR-Free Sample Preparation kit (Illumina Inc., San Diego, CA, USA) and Illumina HiSeq 2000 or HiSeq X sequencer, generating 100–150 bp reads with a minimum coverage of 15× for ~95% of the genome (mean coverage of 35×). Whole-exome sequencing was conducted for individual II-1 (Fig. 4a) using genomic DNA extracted from peripheral blood. Paired-end sequence reads were generated on an Illumina HiSeq 2000.

Generation of analysis-ready data sets. Sequencing reads were pre-processed by Illumina with Isaac Aligner and Variant Caller (v2, Illumina Inc.) using human genome assembly GRCh37 as reference. Variants were normalised, merged into multi-sample VCF files by chromosome using the gVCF aggregation tool agg (https://github.com/Illumina/agg) and annotated with Ensembl’s Variant Effect Predictor (VEP). Following read alignment to the reference genome (GRCh37), variant calling and annotation of whole-exome data for individual II-1 were performed using GATK UnifiedGenotyper46 and ANNOVAR45, respectively. Annotations included minor allele frequencies from other control data sets (i.e. ExAC42, 1000 Genomes Project43 and UK10K45) as well as deleteriousness and conservation scores (i.e., CADD45, SIFT46, PolyPhen247 and GERP48) enabling further filtering and assessment of the likely pathogenicity of variants. To take forward only high quality calls, the pass frequency (proportion of samples containing alternate alleles that passed the original variant filtering) and call rate (proportion of samples with the minor or alternate genotypes) were combined into the overall pass rate (OPR: pass frequency × call rate) and variants with an OPR of 80% or higher were retained.

Estimation of ethnicity and relatedness. We estimated the population structure and relatedness based on a representative set of SNPs using the R package GENESIS to perform PC Air49 and PC Relate50, respectively. The selected 35,114 autosomal SNPs were present on Illumina genotyping arrays (HumanCoreExome129×1, HumanCoreExome-24v1.0, HumanOmni2.5–8v1.1), do not overlap quality control excluded regions or multiallelic sites in the 1000 Genomes (1000 G) Phase 3 data set40, do not have any missing genotypes in NIHR BR-RD, had a MAF of 0.3 or above and LD pruning was performed using PLINK52 with a window size of 50 bp, window shift of 5 bp and a variance inflation factor threshold of 2. The 1000 G Project including the European (EUR), African (AFR), South Asian (SAS) and East Asian (EAS) populations (excluding the admixed American population) were filtered for the selected SNPs and the filtered regulatory regions to PAH aetiology remains to be determined. This will require functional annotation of regulatory and other non-coding regions specific for relevant cell types, further case-control analyses of these regions and ultimately functional studies of gene regulation to assess the pathogenicity of non-coding variants. Our findings to date provide support for a central role of the pulmonary vascular endothelium in disease pathogenesis, and suggest new mechanisms that could be exploited therapeutically in this life-limiting disease.
data were used to perform a principal component analysis (PCA) using PC Air. We modeled the scores of the leading five principal components as data generated by a population-specific multivariate Gaussian distribution and estimated the corre-
sponding mean and covariance parameters. Genotypes from the NHIR BR-RD samples were projected onto the loadings for the leading five principal components from the 1000 G PCA and we computed the likelihood that each sample belonged to each subpopulation under a mixture of multivariate Gaussians models. Each sample was allocated to the population with the highest likelihood, unless the highest likelihood was similar to likelihood values for other populations, as might be expected for example under admixed ancestry or if the sample came from a population not included in 1000 G. Such ambiguous samples were labelled as “other”. Variants were filtered to identify relatives in NHIR BR-RD. We used the first 20 PCs from PC Air to adjust for relatedness and extracted the pairwise Identity-By-State distances and kinship values. The pairwise information was used by Primus to infer family networks and calculate the maximum set of unrelated samples.

Of the final 63042 BR-RD samples, we assigned 80.2% to Non-Finish European (n = 7307), 7.2% to South Asian (n = 649), 2.3% to African (n = 2133, 0.08% to East Asian (n = 78), 0.02% to Finnish-European (n = 19) and 9.2% to Other (n = 844) and retrieved a maximum set of 7,493 unrelated individuals (UWGS10K), representing 82.2% of the entire NHIR BR-RD cohort.

Cohort definition and allele frequency calculation. Based on the relatedness analysis, we defined the following sample subsets: (a) the maximum number of unrelated non-PAH controls (UPAHc, n = 6385), (b) all affected PAH cases (PAHAAF, n = 1048), and (c) all unrelated PAH index cases (PAHIX, n = 1938). These subsets were used to annotate the variants in the multi-sample VCF file with calculated minor allele frequencies using the fill-tags extension of BCFTools.

Rare variant filtering. Filtering of rare variants was performed as follows: (1) variants with a MAF less than 1 in 10,000 in UPAHc subjects, UK10K and ExAC were retained (adjusted for X chromosome variants to 1 in 8000); (2) variants with a combined annotation dependent depletion deleteriousness (CADD) score of less than 15 were excluded. CADD scores were calculated using the CADD web service (http://cadd.gs.washington.edu) for variants lacking a score; (3) premature truncating variants (PTVs) or missense variants of the canonical transcript were retained; 4) missense variants predicted to be both tolerated and benign by SIFT and PolyPhen-2, respectively, were removed. To identify likely causative mutations (as reported in Supplementary Table 3), variants in previously reported and putative genes, identified in this study, were examined in more detail to exclude variants that did not segregate in families (where data available). Furthermore, variants shared between cases and non-PAH controls, as well as variants of uncertain significance that co-occurred with previously reported causative mutations or high impact PTVs were also excluded.

Burden analysis of protein-truncating and missense variants. Filtered variants were grouped per gene and consequence type (predicted PTV/missense) and subjects with at least one variant were counted (no double counting) per group and tested for association with disease. We applied a one-tailed Fisher’s exact test with post hoc Bonferroni correction to calculate the P value for genome-wide significance.

Rare variant analysis using SKAT-O. To further investigate the aggregated effect that rare variants contribute to PAH etiology, we applied a Sequence Kernel Association test (SKAT-O). SKAT-O increases the power of discovery under different inheritance models by combining variance-component and burden tests. To further investigate the aggregated effect that rare variants contribute to PAH etiology, we applied a Sequence Kernel Association test (SKAT-O). SKAT-O increases the power of discovery under different inheritance models by combining variance-component and burden tests. To further investigate the aggregated effect that rare variants contribute to PAH etiology, we applied a Sequence Kernel Association test (SKAT-O). SKAT-O increases the power of discovery under different inheritance models by combining variance-component and burden tests.

Confirmation of variants. Variant sequencing reads for SNVs, indels and deletions were visualised for validation on Integrative Genomes Viewer (IGV)47, and were confirmed by diagnostic high-throughput DNA sequencing, if the IGV inspection was not satisfactory. For the familial segregation analysis, linkage to the BMPR2 locus was first examined by microsatellite genotyping analysis. Mutation screening of the BMPR2, ACTR1L, ENG, ACAP1 and SOX17 genes was conducted by capillary sequencing using BigDye Terminator v3.1 chemistry. All DNA frag-
maments were resolved on an ABI Fragment Analyzer (Applied Biosystems). All primer sequences are listed in Supplementary Table 9. The family trees were drawn using the R package FamAbb58.

Structural analysis of novel variants. The domain structures and the functional domains of the novel PAH genes were plotted according to the entry in UniProtKB. Clustal Omega was used for sequence alignment. Structural data were obtained from RCSB Protein Data Bank and analysed according to published reports. Fig-
ures were generated using PyMOL Molecular Graphics System.

Production of pGDF2 wild type and variant proteins. The cloning of human wild-type pro-GDF2 (pGDF2) in pcDNA4 has been described previously59. Site-
directed mutagenesis was performed according to the manufacturer’s instructions (QuickChange Site-Directed Mutagenesis Kit, Agilent Technologies). Mutations were confirmed by Sanger sequencing. HEK-EBNA cells were transfected with plasmids containing either wild-type or mutant pGDF2 for 14 h. The transfecting supernatant was removed and replaced with CDCHO media (Invitrogen) for 5 days to express the proteins. The conditioned media containing GDF2 and the variant were collected and stored on dry-ice before being stored at –80 °C. For each variant, conditioned media from three independent transfections were collected for further characterisation.

GDF2 ELISA. High binding 96-well ELISA plates (Greiner, South Lanarkshire, UK) were coated with 0.2 µg/well of mouse monoclonal anti-human GDF2 antibody (R&D Systems, Oxfordshire, UK) in PBS (0.1 M phosphate pH 7.4, 0.137 M NaCl, 2.7 Mm KCl, Sigma) overnight at 4 °C in a humidified chamber. Plates were washed with PBS containing 0.05% (v/v) Tween-20 (PBS-T), followed by blocking with 1% bovine serum albumin in PBS-T (1% BSA/ PBS-T) for 90 min at room temperature. Recombinant human GDF2 standards (1–3000 pg/ml) or conditioned media samples (100 µl/well of 1:30, 1:100, 1:1000, 1:3000 and 1:10,000 dilutions) were then added and incubated for 2 h at room temperature. After washing, plates were then incubated with 0.04 µg/ml biotinylated goat anti-human GDF2 (R&D Systems) in 1% BSA/ PBS-T for 2 h. Plates were washed, then incubated with ExtrAvidin(r)- Alkaline phosphatase (Sigma) diluted 1:400 in 1% BSA/ PBS-T for 90 min. Plates were washed with PBS-T followed by water. The ELISA was developed with a colorimetric substrate comprising 1 mg/ml 4-Nitrophenyl phos-
phate disodium salt hexahydrate (Sigma) in 1 M Diethanolamine, pH 9.8 con-
taining 0.5 mM MgCl₂. The assay was developed in the dark at room temperature and the absorbance measured at 405 nm.

Cell culture and treatments. Distal human pulmonary artery smooth muscle cells (PASMCs) were cultured from explants dissected from lung resection specimens. Segments 1.2 mm diameter (0.5 to 2 mm diameter) were dissected and divided into small pieces before plating in T25 flasks. Explants were left to adhere for 2 h and then incubated in DMEM/20% FBS plus amino acids at 37 °C in 95% air/5% CO2 until PASMCs had confluent monolayers. Cells were then trypsinized, and for subsequent passages cells were maintained in DMEM supplemented with 10% FBS. The cellular phenotype of PASMCs was confirmed by positive immunofluorescence staining with anti-smooth muscle specific alpha-actin (Clone IA14 Sigma-Aldrich; 1:100 dilution). The derivation of human tissues and cells was approved by Papworth Hospital ethical review committee (Ref 08/H0304/56 + 5) and all subjects provided informed and written consent.

Human blood growth endothelial cells (BOECs) were derived from 40–80 ml of peripheral venous blood isolated from healthy subjects. The study was approved by the Cambridge Children’s Research Ethics Committee (Ref 11/EE/00297), and all subjects provided informed and written consent. BOECs were cultured in 10% FBS supplemented with EGM-2MV (Life Technologies, Carlsbad, CA). Cells were used between passages 4 and 8th. The endothelial phenotype of BOECs was determined by flow cytometry for expression of endothelial surface markers, as described previously55. Cells were routinely tested to exclude mycoplasma infection.

Human pulmonary artery endothelial cells (PAECs) were purchased from Lonza (Cat. No. CC-2530; Basel, Switzerland). Cells were maintained in EGM-2 with 2% FBS (Lonza). PAECs were used for experiments between passages 4 and 8. For experiments cells were cultured in the presence of EBM-2 containing 10% FBS. The cellular phenotype of PAECs was confirmed by positive immunofluorescence staining with anti-smooth muscle specific alpha-actin (Clone IA4 Sigma-Aldrich; 1:100 dilution). The derivation of human tissues and cells was approved by Papworth Hospital ethical review committee (Ref 08/H0304/56 + 5) and all subjects provided informed and written consent.

RNA preparation and quantitative reverse transcription-PCR. Total RNA was extracted using RNeasy Mini Kit with DNase digestion (Qiagen, West Sussex, UK), according to the manufacturer’s instructions. cDNA was prepared from 1 µg of
RNA using High Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). Quantitative PCR reactions employed MicroAmp optical 96-well reaction plates (Applied Biosystems). 29.9 ng µl−1 DNA was used with SYBR Green Jumpstart Taq ReadyMix (Sigma-Aldrich), ROX reference dye (Invitrogen) using custom made sense and anti-sense primers (all 200 nmol l−1). Primers for human ACTB (encoding β-actin), AQP1, ATRIP3A3, B2M, HPRT and SOX17 were designed using Primer3 (http://www.primer3.sourceforge.net). Supplementary Table S9. Reactions were amplified on a Quantstudio 6 Real-Time PCR system (Applied Biosystems). The relative abundance of each target gene in different cell lines was compared using the equation 2−(CtGOI−Ct3HK), where Ct3HK corresponded to the arithmetic mean of the Ct's for ACTB, B2M and HPRT for each sample. For expression analysis of siRNA knockdown, the 2−(ΔΔCT) method was used and fold expression determined relative to the D16 control.

siRNA transfection. Prior to transfection, cells were preincubated in Opti-MEM-I reduced serum media (Invitrogen) for 2 h before transfection with 10 nM siRNA that had been lipoplexed for 20 min at RT with DharmaFECT1 (GE Dharmacon, Lafayette, CO). Cells were then incubated with the siRNA/DharmaFECT1 complexes for 4 h at 37 °C before replaced by full growth media. Cells were kept in growth media for 24 h before further treatment. Knockdown efficiency was confirmed by mRNA expression or immunoblotting. For proliferation assays, parallel RNA samples were collected on the day of the experiment and analysed using FlowJo software, with Annexin-V

Flow cytometric apoptosis assay. BOECs were plated 150,000/well into 6-well plates and transfected with siATP1A3 or siCIP lipoplexed with DharmaFECT1. Cells were then serum-starved in EB2-2 (Lonza) containing 0.1% FBS and A/A for 8 h before treating with EB-2 and A/A containing either 0.1%FBS or 5% FBS for another 24 h. Cells were then trypsinized and after washing with PBS, stained using the FTTC Annexin V Apoptosis Detection Kit I (BD Biosciences). For each condition, dual-staining of 5 µl FITC conjugated Annexin V and 5 µl propidium iodide (PI) were added and incubated at room temperature for 15 min. For the single staining controls for compensation, either 5 µl FITC or 5 µl PI was added into non-transfected cells. All samples were analysed on BD Accuri® C6 Plus platform (BD Biosciences). Data were collected and analysed using FlowJo software, with AnnexinV+/PI− cells defined as early apoptotic (Treestar).

Caspase-Glo 3/7 assay. BOECs were seeded at a density of 150,000/well into 6-well plates and transfected with siATP1A3 or siCIP lipoplexed with DharmaFECT1. For each condition, cells were trypsinized from 6-well plates and reseeded in triplicates into a 96-well plate at a density of 15,000–20,000/well and left to adhere overnight. Cells were quiesced in EB2-2 containing 0.1% FBS for 24 h before treating with or without EB-2 and A/A containing either 0.1% FBS or 5% FBS or 5% FBS for 16 h. For measuring caspase activities, 100ul Caspase-Glo® 3/7 Reagent (G8091 Promega) was added into each well, incubated and mixed on a plate shaker in the dark for 30 min at room temperature. The lysates were transferred to a white-walled 96-well plate and luminescence was read in a GloMax® luminometer (Promega).

Data availability. WGS data of PAH cases included in this manuscript and eligible registries.

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Author contributions
S.G., N.W.M. and W.H.O. conceived and designed the research. S.G., M.H., M.B. and C.H. processed the data and performed the statistical analysis. S.G., M.H., M.B., C.H. and N.W.M. drafted the manuscript. L.S., R.D.M. and R.C.T. conducted the SOX17 familial segregation analyses. W.L. performed the structural analysis of the rare variants. R.S. generated the mutant cells. J.H., R.M.S., B.L. and P.D.U. conducted the functional experiments on the novel disease genes. M.S. performed the immunohistochemistry for novel gene products. L.C.D. helped with the assessment of pertinent findings. O.S. was involved with data analysis. D.W. participated in DNA extraction, sample QC and plating. L.S., R.D.M., S.H., M.A., C.J.R., W.H.O., N.S., A.L., R.C.T. and M.R.W. helped with data analysis and interpretation and made critical revision of the manuscript for important intellectual content. J.M.M., C.M.T. and K.Y. coordinated data collection. N.W.M. and W.H.O. handled the funding for the study. All other authors were responsible for data acquisition and recruitment of subjects to the study and helped to draft the final version of the manuscript.

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