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ORIGINAL ARTICLE

Whole-Exome Sequencing Reveals *TopBP1* as a Novel Gene in Idiopathic Pulmonary Arterial Hypertension

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Abstract

Rationale: Idiopathic pulmonary arterial hypertension (IPAH) is a life-threatening disorder characterized by progressive loss of pulmonary microvessels. Although mutations in the bone morphogenetic receptor 2 (BMPR2) are found in 80% of heritable and ~15% of patients with IPAH, their low penetrance (~20%) suggests that other unidentified genetic modifiers are required for manifestation of the disease phenotype. Use of whole-exome sequencing (WES) has recently led to the discovery of novel susceptibility genes in heritable PAH, but whether WES can also accelerate gene discovery in IPAH remains unknown.

Objectives: To determine whether WES can help identify novel gene modifiers in patients with IPAH.

Methods: Exome capture and sequencing was performed on genomic DNA isolated from 12 unrelated patients with IPAH lacking BMPR2 mutations. Observed genetic variants were prioritized according to their pathogenic potential using ANNOVAR.

Measurements and Main Results: A total of nine genes were identified as high-priority candidates. Our top hit was topoisomerase DNA binding II binding protein 1 (TopBP1), a gene involved in the response to DNA damage and replication stress. We found that TopBP1 expression was reduced in vascular lesions and pulmonary endothelial cells isolated from patients with IPAH. Although TopBP1 deficiency made endothelial cells susceptible to DNA damage and apoptosis in response to hydroxyurea, its restoration resulted in less DNA damage and improved cell survival.

Conclusions: WES led to the discovery of TopBP1, a gene whose deficiency may increase susceptibility to small vessel loss in IPAH. We predict that use of WES will help identify gene modifiers that influence an individual's risk of developing IPAH.

Keywords: pulmonary hypertension; vascular biology; high-throughput nucleotide sequencing; bioinformatics; DNA injury

Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by abnormally elevated pulmonary pressures, severe right heart failure, and decreased exercise tolerance (1, 2). It is currently estimated that the mean time between onset of symptoms and diagnosis is 2 years and the mean survival of untreated patients with PAH is 2.8 years (3–5). Given the poor outcome of untreated patients, there is much interest in developing diagnostic strategies that can help identify high-risk

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At a Glance Commentary

Scientific Knowledge on the Subject: Although there is a strong association between mutations in the bone morphogenetic protein receptor 2 and heritable and idiopathic forms of pulmonary arterial hypertension (PAH), the low penetrance of these mutations suggests that other genetic and environmental modifiers may be required for disease development. Genome sequencing techniques, such as whole-exome sequencing (WES), have recently been used to identify novel genes in patients with heritable PAH lacking bone morphogenetic protein receptor 2 mutations, but whether this approach can also be used to identify susceptibility genes in idiopathic PAH (IPAH) has not been tested.

What This Study Adds to the Field: WES combined with a bioinformatics-based approach was capable of identifying novel candidate genes in a population of 12 unrelated patients with IPAH and led to the discovery of topoisomerase DNA binding II binding protein 1 as a key gene involved in protecting the pulmonary endothelium against injury. We believe that WES could help future efforts to personalize the care of patients with IPAH by allowing clinicians to screen for genetic variants that could impact a patient's prognosis and response to therapy.

patients either before or during the early phase of the disease.

PAH is thought to develop in high-risk individuals following an unknown genetic and/or environmental injury that results in both progressive loss of pulmonary microvessels and severe obliterative vasculopathy (6, 7). The genetic basis for PAH remained a mystery until 2000 when two gene linkage studies independently reported that mutations in the bone morphogenetic receptor protein 2 (BMPR2) were prevalent in some cases of heritable PAH (HPAH), a form of the disorder characterized by an autosomal-dominant pattern of inheritance (8, 9). Since then, it has been estimated that BMPR2 mutations are present in approximately 75% of HPAH and approximately 15% of sporadic cases of PAH (10, 11). However, despite their prevalence, BMPR2 mutation carriers may not necessarily develop PAH given the low penetrance (~20%) of these mutations (2, 12). Moreover, the fact that BMPR2 mutations are not present in all cases of HPAH and sporadic PAH suggests the need for other unidentified genetic modifiers to trigger clinical manifestations in susceptible individuals.

Efforts to discover the identity of these unknown PAH genetic modifiers have been aided by availability of next-generation sequencing technologies capable of screening the whole genome for genetic variants relevant to the pathogenesis of common and mendelian disorders (13). In particular, whole-exome sequencing (WES) has been successfully applied to identify novel candidate genes in mendelian disorders, such as hypertrophic cardiomyopathy, and disorders with nonmendelian inheritance (14, 15). More recently, use of WES in patients with BMPR2-negative familial PAH has identified caveolin 1 and KCNK3 as two new candidate genes that, despite the absence of BMPR2 mutations, may increase susceptibility for PAH in carriers (16, 17). However, although these studies have shed light on the genetic landscape of HPAH, no studies have yet attempted to apply WES to study patients with idiopathic (i.e., nonfamilial) PAH (IPAH).

In the present study, we used WES to screen the genome of 12 patients with IPAH with the goal of discovering genetic variants predicted to contribute to IPAH by increasing susceptibility and/or altering cellular response to vascular injury. Our findings led us to focus on topoisomerase DNA binding II binding protein 1 (TopBP1), a novel gene that promotes cell survival by ensuring a proper response to DNA damage and replication stress. We propose that WES could help accelerate the understanding of the pathobiology of IPAH and could assist future efforts to develop a personalized approach to the diagnosis and management of IPAH.

Some of the results of these studies have been previously reported in the form of an abstract (18).

Methods

WES

Samples were prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced in an Illumina HiSeq 2000 Sequencer (Illumina Inc., San Diego, CA) using paired-end 75- to 100-bp sequences. Samples were sequenced to at least 125-fold (×125) sequence coverage. Raw sequence reads were aligned to human reference sequence hg19 using SAM/BAM. Sequencing data were analyzed using ANNOVAR software (openbioinformatics.org) (19). After filtering synonymous (i.e., not altering protein sequence) variants and those with estimated minor allele frequency greater than 15%, two independent investigators screened the resulting list for genes predicted to have nonsynonymous (i.e., altering protein sequencing) variants with known heart and lung expression and with association to human disease based on annotation in public databases (GeneCards, KEGG, PubMed, and OMIM). To further estimate the impact of selected variants on protein structure and function we used a set of metrics that take into consideration functional impact on evolutionary conserved domains: Polyphen2 (20), SIFT (21), MutationTaster (21), LRT (22), and GERP (19). All clinically relevant candidate variants were validated using Sanger capillary sequencing methods. Details can be found in the online supplement.

Results

Patient Characteristics

We chose to study 12 unrelated patients with IPAH who had undergone a complete diagnostic work-up in our Pulmonary Hypertension Clinic (Stanford University Medical Center, Stanford, CA) over the past 5 years, none of which had any family history of PAH (Table 1). Our patient population was composed predominantly of females (n = 7; 58%) with a mean age of 41 ± 14.1 years and a body mass index of 24.9 ± 2.5. On presentation, most patients where categorized as New York Heart Association functional class III (41.6%) and had documented mean 6-minute-walk distance of 540 ± 108 m and serum N-terminal pro B-type natriuretic peptide levels of 378.4 ± 763.5 pg/ml. All

Table 1: Patient Characteristics

	IPAH Cohort (*N = 12*)
Age, yr	41.4 ± 14.1
Sex, M, F (%)	7, 5 (58%)
BMI, kg/m^2	24.9 ± 2.5
NYHA, n (%)	
I	3 (25%)
II	2 (16.7%)
III	5 (41.6%)
IV	2 (16.7%)
6MWD, m	540 ± 108
NT-pro BNP, pg/ml	378.4 ± 763.5
Therapies, n (%)	
Prostacycline	6 (50%)
ERA	5 (41.6%)
PDE-I	7 (58%)
CCB	4 (33.3%)
Hemodynamics	
mRA, mm Hg	7.1 ± 2.4
mPAP, mm Hg	47.9 ± 14
PCWP, mm Hg	10.3 ± 2.5
CO, L/min	3.8 ± 0.9
PVR, WU	11.1 ± 6.3

Definition of abbreviations: 6MWD = 6-minute-walk distance; BMI = body mass index; CCB = calcium channel blocker; CO = cardiac output; ERA = endothelin-1 receptor antagonist; IPAH = idiopathic pulmonary arterial hypertension; mPAP = mean pulmonary artery pressures; mRA = mean right atrial pressure; NT-pro BNP = N-terminal pro B-type natriuretic peptide; NYHA = New York Heart Association symptom class; PAH = pulmonary arterial hypertension; PCWP = pulmonary capillary wedge pressure; PDE-I = phosphodiesterase inhibitor; PVR = pulmonary vascular resistance; WU = Wood units.
Values represent mean ± SD.

patients underwent right heart catheterization that showed an average mean right atrial pressure of 7.1 ± 2.4 mm Hg, a mean pulmonary artery pressure of 47.9 ± 14 mm Hg, mean pulmonary artery wedge pressure of 10.3 ± 2.5 mm Hg, mean cardiac output of 3.8 ± 0.9 L/min, and pulmonary vascular resistance of 11.1 ± 6.3 Wood units.

WES Identifies Candidate Genes in Patients with IPAH

Table 2 summarizes the variants found in our IPAH population. By selecting variants with an estimated minor allele frequency of less than 15% (23, 24), we found a total of 54,439 rare variants including 297 nonsense and 968 insertion-deletions variants (*see* Table E1 in the online supplement). Taken together, these variants were predicted to affect an estimated total of 3,251 candidate genes.

To identify candidate genes with relevance to pulmonary biology, we reviewed the available biologic information for each gene using several annotated public genomic databases (GeneCards [25], PubMed, ExPASY, OMIM) and prioritized those genes with known expression in both heart and lungs and known association to human disease. Using this approach, we were able to prioritize nine candidates (Table 3). Although we found no BMPR2 mutations in any of our patients, we identified variants in several genes associated with the BMP signaling pathway (*see* Table E2), some of which appeared clustered in several patients (*see* Table E3).

Relevance of Top Three WES Hits to IPAH Pathobiology

Using the data obtained from our database review, we created a model predicting a possible role for each of our top three candidates in IPAH pathogenesis to guide future studies (Figure 1). Histidine Rich Glycoprotein codes for a protein found in plasma thought to promote thrombosis, inhibit angiogenesis, and regulate immunity (26–30). Although no studies have yet reported a link between Histidine Rich Glycoprotein and IPAH, it is possible that altered protein function could predispose to *in situ* thrombosis, a pathologic feature of IPAH. Versican is an extracellular matrix protein that is highly expressed by smooth muscle cells of the lung and in myocardium (31, 32). Recent studies have found that excessive Versican deposition may predispose smooth muscle cell response to local growth factors in vascular disorders, such as coronary atherosclerosis and peripheral vascular disease (33, 34). TopBP1 is a protein with eight BRCA repeats involved in the initiation of DNA replication and the response to DNA damage (35–37). Mutations that impair the ability of TopBP1 to bind and/or activate ataxia-telangiectasia and Rad 3–related protein kinase can lead to deleterious somatic mutations, including loss or fragmentation of chromosomes (38, 39). This was of particular interest to us given recent reports alluding to the presence of abnormalities in the DNA repair machinery found in both pulmonary endothelial (40) and smooth muscle cells (41) from patients with IPAH. Furthermore, TopBP1 has been shown to interact with E2F1 (42), a gene reported to promote pulmonary smooth muscle cell proliferation in the setting of hypoxia (43, 44). In our patient population, we found three single-nucleotide variants (SNVs) (Figure 2A) located in close proximity to both the TopBP1 transactivation domain (rs55633281) and the topoisomerase II interacting domain (rs17301766 and rs10935070) (Figure 2B). Application of five different predictive algorithms to estimate potential deleteriousness (MutationTaster, SIFT, LRT, PolyP2, and GERP) revealed mixed results for each of the three TopBP1 SNVs (Figure 2C), whereas protein sequence alignment using MUSCLE (45) demonstrated that all three SNVs occur within highly conserved regions of the protein (Figure 2D). On account of its critical role in the DNA damage response (40, 41, 46–48), we postulated that reduction in TopBP1 expression and/or activity could act as a risk factor for IPAH.

TopBP1 Expression Is Reduced in Vascular Lesions and Pulmonary Microvascular Endothelial Cells from Patients with IPAH

We sought to compare the levels of TopBP1 expression in lung sections from healthy donors and patients with IPAH via immunohistochemistry. Compared with healthy donors, vascular lesions of patients with IPAH demonstrated reduced TopBP1 nuclear staining (Figure 3A). Because TopBP1 expression seemed to be higher in the endothelium compared with the other vascular compartments, we decided to measure TopBP1 mRNA and protein levels in nuclear extracts of pulmonary microvascular endothelial cells (PMVECs) purified from lungs of five unrelated healthy

Table 2: Genetic Variants Identified in IPAH Population Using WES

Variant Type	IPAH
Patients	12
All SNPs	226,864
Synonymous	172,425
Nonsynonymous	54,439
Missense variants	54,142
Nonsense variants	297
Rare (<5% MAF) nonsynonymous	15,477
All Indels	968
Coding Indels	654
Frameshift Indels	314
Rare (<5% MAF) Indels	57
Candidate genes	3,251

Definition of abbreviations: Indel = insertion/deletion; IPAH = idiopathic pulmonary arterial hypertension; MAF = minor allele frequency; SNP = single-nucleotide polymorphism; WES = whole-exome sequencing.

Table 3: Candidate Genes Identified via WES in the IPAH Population

Gene ID	Chromosome Location	Gene Name	Gene Ontology	Gene Function	Disease Association	Possible Link to PAH
11073	3q22.1	TOPBP1	DNA repair (GO:0006281) DNA metabolism (GO: 0006259)	Rescues stalled replication forks	Cancer, mutagen sensitivity	Predicted to interact with BMPR2 and E2F1
1462	5q14.3	VCAN	Cell recognition (GO:0008037) Cell adhesion (GO: 0007155)	Extracellular matrix, regulates cell growth and motility	Wagner syndrome, cancer Atherosclerosis	Present in plexiform lesions
3273	3q27	HRG	Angiogenesis (GO: 0001525) Platelet degranulation (GO: 0002576)	Regulates coagulation and fibrinolysis	Thrombophilia	*In situ* thrombosis of pulmonary lesions
3084	8p12	NRG1	Cell communication (GO: 0007154) Myocardium morphogenesis (GO: 0003222)	Activates NEU tyrosine kinase receptors	Schizophrenia Breast cancer	Regulates right ventricular cardiomyocyte function
140690	20q13.31	CTCFL	Gene regulation (GO: 0010628) Histone methylation (GO: 0016571)	Regulates DNA methylation	Cancer	Increased cell proliferation in vessel wall
350	17q24.2	APOH	Angiogenesis (GO: 0016525) Coagulation (GO: 0007597)	Regulates coagulation and antiphospholipid antibodies	Antiphospholipid antibody syndrome	*In situ* thrombosis and thromboembolism
114803	1p32.1	MYSM1	Histone deubiquitination (GO: 0016578) DNA transcription (GO: 0006351)	Regulates histone acetylation	Lung cancer Retinopathy	Increased cell proliferation in vessel wall
2208	19p13.3	FCER2	Nitric oxide synthase (GO: 0051000) Immune response (GO: 0002925)	Regulates B-cell differentiation	Lymphoma Leukemia Allergy	Regulation of inflammation
1543	15q24.1	CYP1A1	Response to hypoxia (GO: 0001666) Response to stress (GO: 0006950)	Detoxification	Cancer	Susceptibility to environmental insults

Definition of abbreviations: IPAH = idiopathic pulmonary arterial hypertension; WES = whole-exome sequencing.

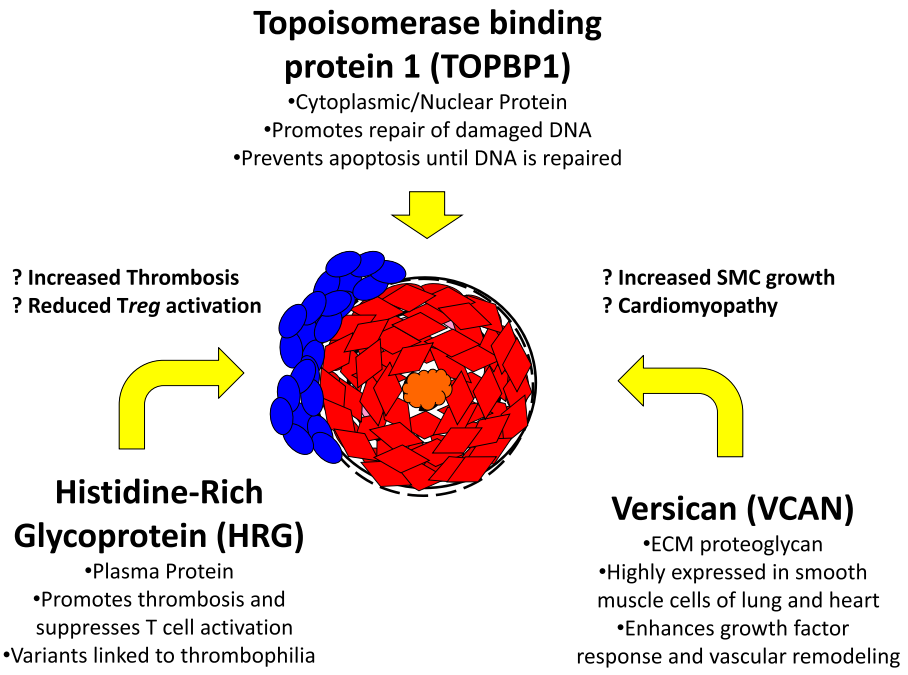

Figure 1. Proposed role of top three candidate genes identified by whole-exome sequencing in the pathogenesis of idiopathic pulmonary arterial hypertension. ECM = extracellular matrix; SMC = smooth muscle cell.

donors and patients with IPAH via quantitative polymerase chain reaction and Western blot, respectively. It is important to point out that these samples were not obtained from any of the patients included in the WES study because the latter are still alive and have not undergone transplant. We found that both levels of TopBP1 mRNA (Figure 3B) and protein (Figure 3C) were reduced in nuclear extracts of IPAH PMVECs compared with healthy control subjects. Although none of the healthy donors carried any of the candidate TopBP1 SNPs, allelic discrimination assays demonstrated that all IPAH samples carried some of the TopBP1 SNVs (*see* Table E4).

TopBP1 Deficiency Increases Susceptibility to DNA Damage and Apoptosis in PMVEC Exposed to Hydroxyurea

TopBP1 is thought to play a role in sensing and responding to DNA damage encountered during DNA replication and its deficiency could predispose IPAH PMVECs to accumulate somatic mutations that may impair cell function and survival. To test whether IPAH PMVECs are more susceptible to DNA replication stress and apoptosis, we treated cells with hydroxyurea (HU), a chemotherapeutic agent that interferes with DNA replication by reducing production of deoxyribonucleotides and that is known to induce TopBP1 recruitment to stalled replication forks (49, 50). To document DNA replication stress, we stained for phosphorylated histone 2AX (p-H2AX), a marker of DNA strand breaks (51). At baseline, we found that IPAH PMVECs displayed more p-H2AX foci per nuclei compared with healthy cells, which inversely correlated with levels of TopBP1 in each cell type (Figure 4A). When exposed to HU for 24 hours, we observed a greater increase in p-H2AX in healthy cells that correlated with increased caspase 3/7 apoptosis rate (Figures 4A–4B).

Next, we sought to determine whether the observed SNVs had any impact on the capacity of TopBP1 to protect against HU-induced replication stress. To do this, we generated various TopBP1 mutant constructs for each of the candidate SNVs and transfected them into healthy donor PMVECs followed by HU exposure for 24 hours. Compared with cells transfected with the wild-type (WT) construct, we found that PMVECs carrying the rs55633281 TopBP1 mutant demonstrated higher replication stress suggesting a dominant negative effect (Figure 5). Interestingly, although cells transfected with either the rs17301766 or the rs10935070 TopBP1 mutant also demonstrated an increase in p-H2AX foci compared with WT cells, the number of foci was less compared with that seen with the rs55633281 TopBP1 mutant (Figure 5).

Finally, we sought to determine whether reduction of TopBP1 in healthy PMVEC could increase susceptibility to DNA replication stress and apoptosis. We transfected healthy PMVECs with either a nontargeting or a TopBP1-specific siRNA, which led to a more than 50% reduction in TopBP1 expression 72 hours after transfection (Figure 6A). Similar to their IPAH counterparts, TopBP1 siRNA–treated PMVECs demonstrated a higher number of p-H2AX nuclear foci (Figure 6B) and a higher rate of apoptosis (Figure 6C).

Previous studies have suggested that small vessel loss in IPAH may result not only from accelerated endothelial cell loss (52–55) but also from an inability to regenerate lost pulmonary microvessels (6, 56). This is supported by studies showing that PMVECs isolated from patients with IPAH form much smaller vascular networks when seeded in matrigel scaffolds (57). To determine whether TopBP1 deficiency could reduce the angiogenic potential of PMVECs, we seeded cells transfected with either nontargeting or TopBP1-specific siRNA in matrigel scaffolds and quantified the number of tubes formed over a period of 6 hours. Compared with control subjects, TopBP1 siRNA–treated PMVECs formed fewer tubes and gave rise to a smaller vascular network similar to what has been described in IPAH PMVECs (Figure 6D) (57).

Restoration of TopBP1 Protects IPAH PMVEC against HU-mediated DNA Injury and Apoptosis

Our studies support a critical role for TopBP1 in protecting against PMVEC loss and promoting angiogenesis. To determine whether restoring TopBP1 could improve IPAH PMVEC survival and angiogenesis, we transfected cells with either an empty vector or a plasmid containing WT human TopBP1 followed by measurement of nuclear TopBP1 (Figure 7A). As predicted, restoration of TopBP1 in IPAH PMVECs resulted in significantly less HU-induced apoptosis (Figure 7B) and improved tube formation in matrigel (Figure 7C).

Discussion

Since the first reports of HPAH, great efforts have been undertaken to understand the genetic basis of PAH. One of the major

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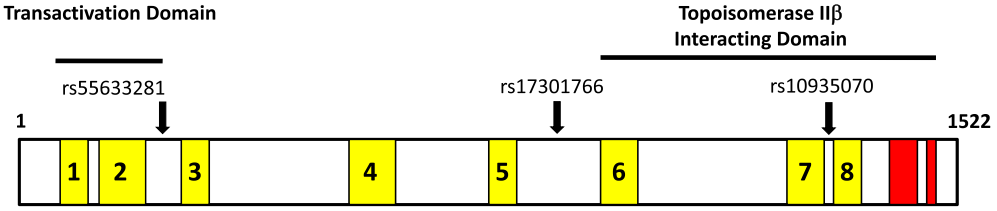

B

SNV	Predicted Allele Freq	Allele Frequency in IPAH Cohort	Function	AA Switch	AA Position	Affected Carriers
rs55633281	4.5%	8.3%	Missense	Arg ⇒ Cys	309	1
rs17301766	10%	25%	Missense	Ser ⇒ Leu	817	3
rs10935070	15%	33.3%	Missense	Asn ⇒ Ser	1042	4

C

SNV	MutationTaster	SIFT	LRT	PolyP2	GERP
rs55633281	N	0.06	0.00092	B	5.70
rs17301766	P	0.11	0.003	B	6.03
rs10935070	P	0.80	0.97	B	5.82

D

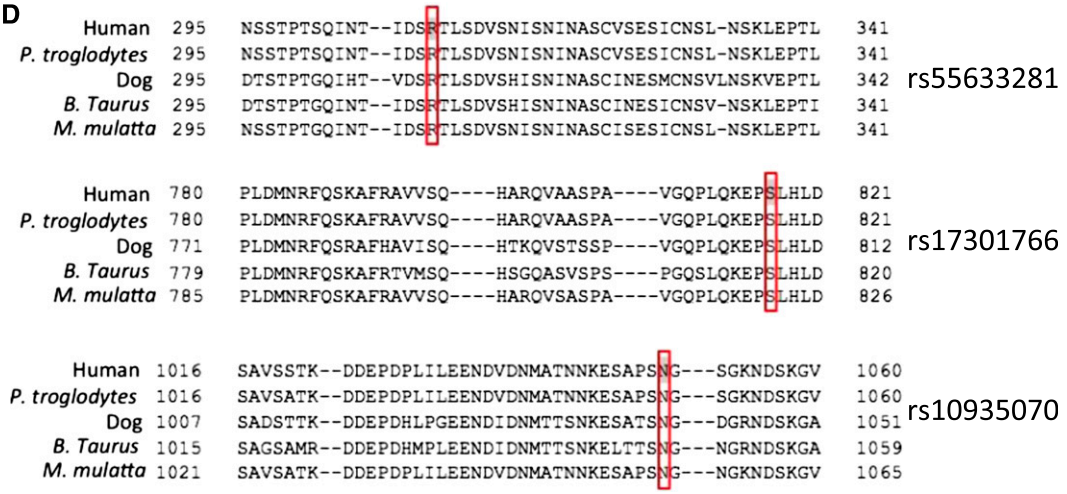

Figure 2. Topoisomerase DNA binding II binding protein 1 (TopBP1) variants identified in patients with idiopathic pulmonary arterial hypertension (IPAH) are located within conserved protein domains. (*A*) Primary sequence of TopBP1 showing location of three identified variants. (*B*) Predicted and actual allele frequency of three TopBP1 SNVs in whole-exome sequencing cohort with the predicted amino acid (AA) switch. (*C*) Predicted functional impact of the three observed TopBP1 variant using MutationTaster, SIFT, LRT, Polyphen2 (PolyP2), and GERP. (*D*) Variants target conserved amino acids as seen when human TopBP1 protein sequence is aligned with that of other related organisms using MUSCLE. SNV = single-nucleotide variants.

breakthroughs was the discovery of BMPR2 as the major cause of HPAH using gene linkage analysis (8, 9). However, because of the low penetrance (~20%) of BMPR2 mutations, most carriers do not develop PAH during their lifetime. At present, the lack of other well-defined genetic risk factors limits the ability to truly measure the contribution that DNA mutations may have in most IPAH cases seen in clinical practice. To gain insight into the identity of these unknown genetic modifiers, we screened the genome of 12 individuals with IPAH using WES, a technique that provides detailed information in gene coding regions of the genome (58). Our analysis of high-risk variants led to the validation of TopBP1, a DNA damage response gene that exerts a protective effect in the pulmonary endothelium.

Gene ontology analysis of the top variants present in our patient population presented us with a list of novel gene candidates with potential relevance to IPAH pathobiology. Our choice to focus on TopBP1 was motivated by its known association to DNA damage response and cell survival (59, 60) and its predicted interaction with genes known to be involved in PAH pathobiology (e.g., E2F1 [42]). It is worth pointing out that use of the STRING (61) tool to identify relevant gene interactions also suggested a possible

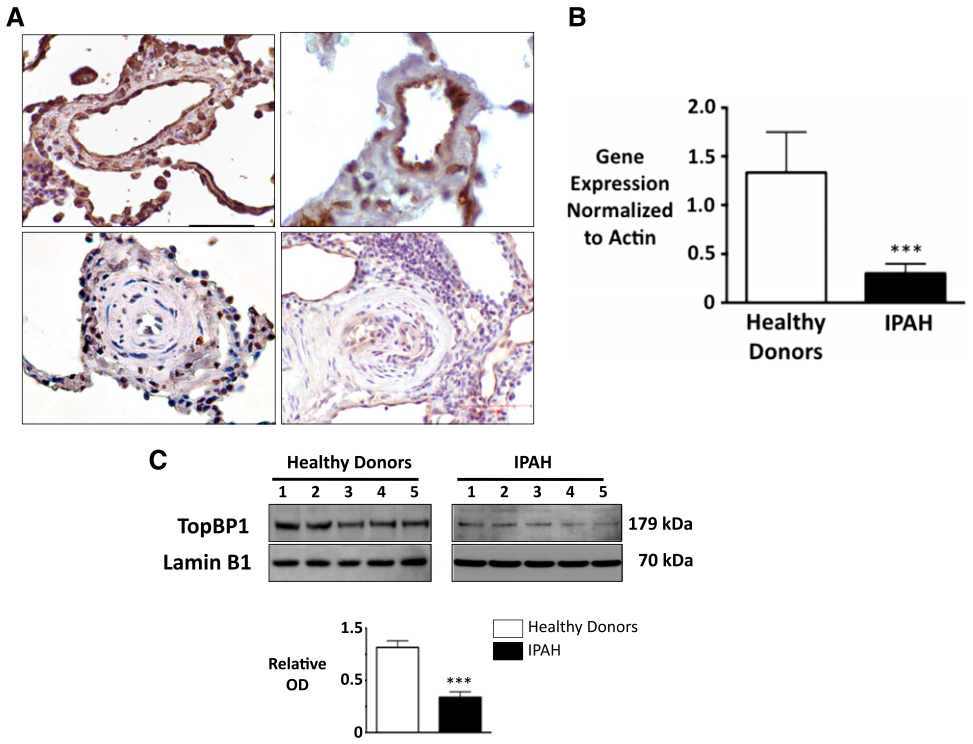

Figure 3. Topoisomerase DNA binding II binding protein 1 (TopBP1) nuclear abundance is reduced in idiopathic pulmonary arterial hypertension (IPAH). (*A*) Representative immunohistochemistry images of lung sections obtained from healthy donor (*top panels*) and patients with IPAH (*bottom panels*). Scale bar = 25 μm. (*B*) Quantitative polymerase chain reaction of TopBP1 mRNA expression in healthy donor and IPAH pulmonary microvascular endothelial cells (PMVECs). *Bars* represent mean SEM from experiments involving five patients per group. ***$P < 0.0001$, unpaired *t* test. (*C*) Representative nuclear extraction studies demonstrating TopBP1 expression in PMVECs from PMVECs purified from five healthy donors and patients with IPAH. Distribution of all three TopBP1 SNVs in IPAH PMVECs can be found in Table E4. *Bars* represent mean SEM from experiments involving five patients per group. ***$P < 0.0001$, unpaired *t* test. SNV = single-nucleotide variants.

interaction between TopBP1 and BMPR2 on account of one publication (62) but we found this to be inaccurate and have alerted the curators of this online resource of this error. Also, although occurrence of TopBP1 SNV in our WES patients was sporadic (*see* Table E3), we found that PMVECs purified from lungs of patients with IPAH contained more TopBP1 SNVs that would have been predicted based on the WES data (*see* Table E4). This is relevant when we consider that TopBP1 expression is impaired in IPAH PMVECs and correlates with reduced survival following exposure to HU, a drug that interferes with DNA replication, suggesting that abnormalities in other regulatory mechanisms (e.g., epigenetic, post-translational) may be involved in regulation of TopBP1 expression. We propose that TopBP1 is required for the DNA damage response in the setting of injury and its absence may predispose to cell death and impaired angiogenesis (Figure 8). This is important when we consider recent evidence describing a high incidence of chromosomal abnormalities, including loss of whole chromosomes in clones of endothelial cells purified from the lungs of patients with PAH (46). The lifetime risk of a carrier of germline BMPR2 mutations of developing PAH may be increased in the presence of somatic mutations that target other genes involved in the BMP pathway. In their study, Aldred and coworkers (46) found that a carrier of germline BMPR2 mutations also exhibits complete loss of chromosome 13, which houses SMAD9, a gene critical for the transduction of BMP signaling and the regulation of miRNA-mediated growth in both pulmonary endothelial and smooth muscle cells (48). The contribution of abnormal DNA repair mechanisms to PAH pathogenesis has been underscored by recent publications demonstrating that BMPR2-deficient PMVECs have increased susceptibility to DNA damage (40), whereas pulmonary arterial smooth muscle cells from patients with PAH also seem to demonstrate increased expression of DNA damage markers (41). Taken together, these observations bring to mind the "cancer paradigm" concept introduced by Voelkel and coworkers (63–65) and lends support to the idea that DNA injury may play a crucial role in increasing an individual's risk of developing PAH.

Although our study focuses exclusively on non-HAH, other groups have used genome-wide association studies (GWAS) and WES to screen patients with HPAH and sporadic PAH for novel candidate genes. A recently published multinational GWAS using genetic data from 625 patients diagnosed with IPAH and familial PAH reported the discovery of a novel susceptibility locus in chromosome 18q22.3, which houses the cerebellin 2 precursor (CBLN2) gene (66). A challenge inherent to GWAS is the need to use large (>1,000) sample sizes to increase the discovery rate of true-positive variants and adequate analysis of rare variants (67–69). This is caused by the fact that GWAS analyses are dominated by common SNVs found within

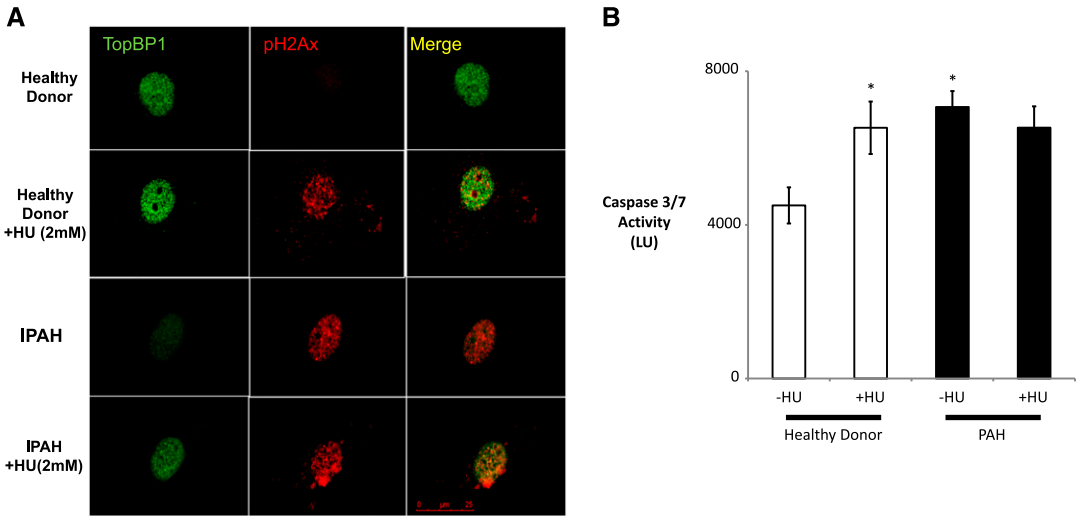

Figure 4. Idiopathic pulmonary arterial hypertension (IPAH) pulmonary microvascular endothelial cells (PMVECs) demonstrate evidence of increased DNA damage and apoptosis in response to hydroxyurea (HU). (*A*) Representative immunofluorescence studies demonstrating nuclear topoisomerase DNA binding II binding protein 1 (TopBP1) (*green*) and p-H2AX (*red*) in PMVECs from healthy donors (*upper six panels*) and patients with IPAH (*lower six panels*) at baseline and after exposure to HU (2 mM) for 24 hours. Scale bar = 25 μm. (*B*) Caspase 3/7 activity assays of healthy donor and IPAH PMVECs at baseline and after HU exposure for 24 hours. *Bars* represent mean SEM from experiments involving five patients per group. LU = luminescence intensity. *$P < 0.05$ versus healthy donor −HU, one-way analysis of variance with Bonferroni post-test unpaired *t* test.

coding and noncoding regions of the genome whose relevance to a disease state may not be immediately evident. Use of WES circumvents some of these limitations by limiting the analysis to the coding regions of the genome where substitutions or structural changes in the nucleic acid sequence can result in alterations in protein function and pathology (58). To date, WES has been applied to the discovery of novel gene candidates in families with HPAH where mutations in BMPR2 are absent. The discovery of two new genes linked to PAH (caveolin 1 and KCNK3 [16, 17]) confirms that use of WES could complement other established approaches for genetic studies (i.e., gene linkage) to help us achieve a greater understanding of the genetic basis of PAH.

Although most published studies to date have used WES to study familial disorders, it is reasonable to think that this approach could also be used to study disorders affecting unrelated individuals. Our study is the first to our knowledge that has applied WES exclusively to patients diagnosed with IPAH. It is pertinent to note that none of our patients had evidence of BMPR2 mutations based on WES and traditional Sanger sequencing. However, we found that some patients in our cohort had high-risk variants in genes belonging to the BMP signaling pathway and in genes associated with risk of PAH in hereditary hemorrhagic telangiectasia (*see* Table E2). Among these, variants in the BMP co-receptor BMPR1A were found in almost 50% of our patients and seem to predict a missense mutation that could affect protein abundance and/or function. This is

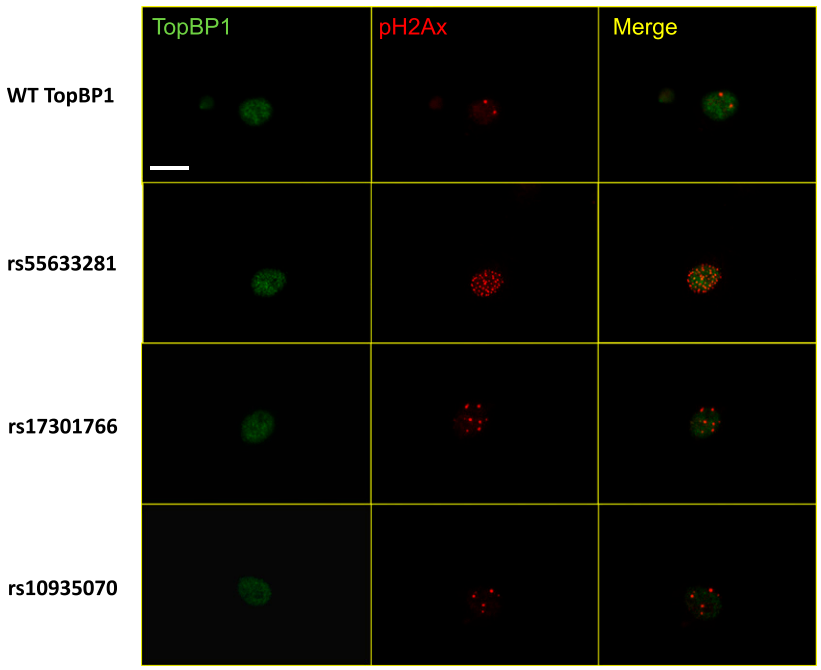

Figure 5. Impact of candidate topoisomerase DNA binding II binding protein 1 (TopBP1) SNVs on susceptibility to hydroxyurea-mediated replication stress in healthy pulmonary microvascular endothelial cells. Representative immunofluorescence studies demonstrating nuclear TopBP1 (*green*) and p-H2AX (*red*) in healthy pulmonary microvascular endothelial cells transfected with either wild-type (WT) or mutant constructs containing each of the three TopBP1 candidate SNVs (rs55633281, rs17301766, and rs10935070) after exposure to hydroxyurea (2 mM) for 24 hours. Scale bar = 10 μm. SNV = single-nucleotide variants.

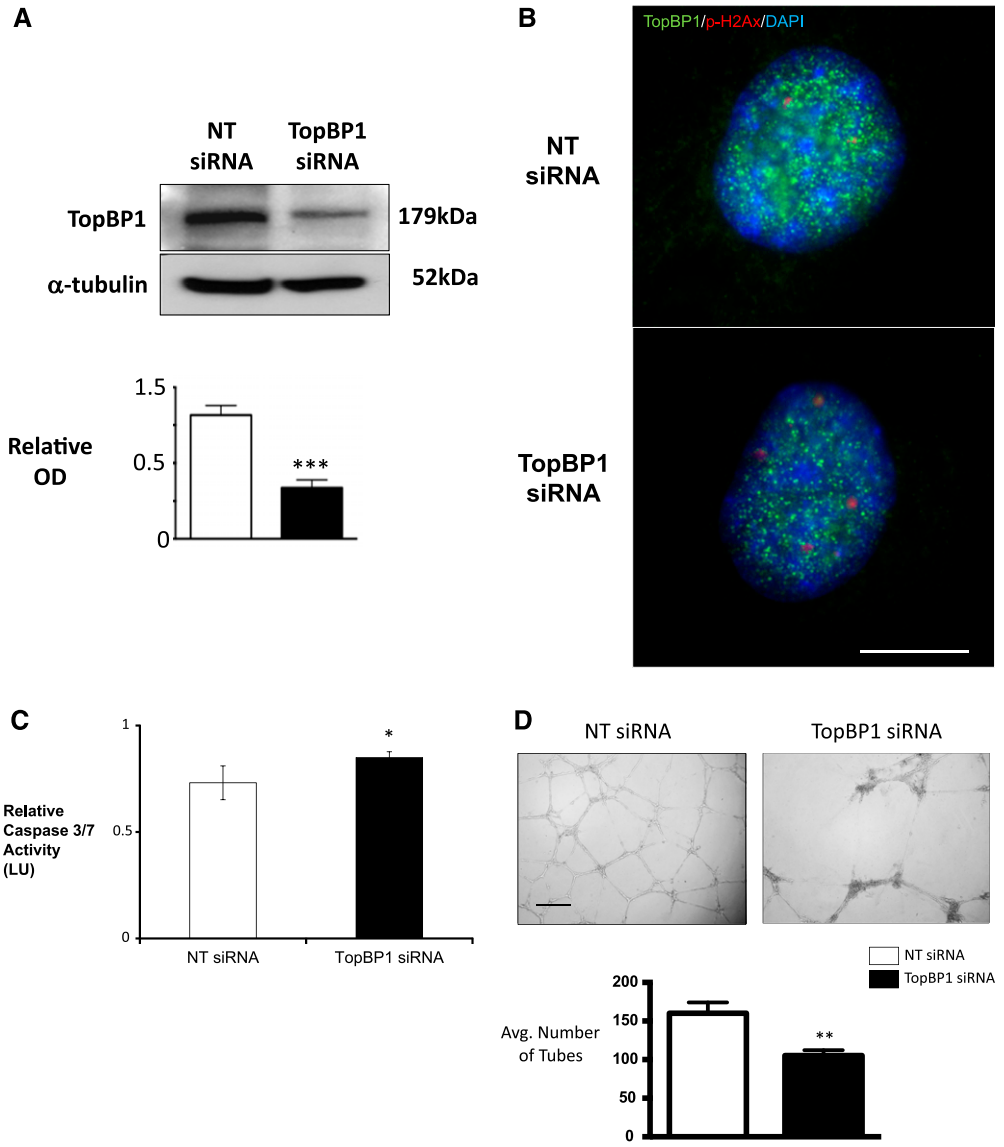

Figure 6. Topoisomerase DNA binding II binding protein 1 (TopBP1) siRNA knockdown increases susceptibility to DNA damage and apoptosis in healthy pulmonary microvascular endothelial cells (PMVECs). (*A*) Western blot showing TopBP1 expression in PMVECs transfected with nontargeting (NT) or TopBP1-specific siRNA. Densitometry is measured relative to α-tubulin as a loading control. ***$P < 0.0001$, unpaired *t* test. (*B*) Representative immunofluorescence studies demonstrating nuclear TopBP1 (*green*) and p-H2AX (*red*) in PMVECs transfected with NT (*top*) or TopBP1 siRNA (*bottom*) at baseline after 24 hours. Scale bar = 10 μm. (*C*) Relative caspase 3/7 activity assays of NT and TopBP1 siRNA transfected PMVECs at baseline for 24 hours. *Bars* represent mean SEM from experiments performed in triplicate. *$P < 0.05$, unpaired *t* test. (*D*) Matrigel tube formation assay comparing NT and TopBP1 siRNA transfected PMVECs. Tube number was quantified 6 hours after seeding the cells. **$P < 0.001$, unpaired *t* test. Scale bar = 150 μm.

of potential interest to future studies in PAH genetics in light of previous reports demonstrating that BMPR1A expression is reduced in lung tissues of patients with PAH (70) and patchy deletion of BMPR1A in smooth muscle cells can predispose to pulmonary vascular remodeling in a transgenic mouse model (71).

There are several limitations to our study. First, our analysis was limited to only 12 patients as a result of the cost of running a WES on each sample, a circumstance that limits the power of most bioinformatic statistical approaches for identifying causative genes in this data set. Also, it is worth pointing out that most of our patients were from a diverse ethnic background, a fact that could influence the frequency in which certain variants are observed in our population. Second, recognition of gene variants in our WES study was performed using genetic data from public databases, such as the 1,000 Genomes, as a reference. A major advantage of this approach is that these public databases include whole genome sequencing data from a large group (>2,000) of ethnically diverse individuals, but because of the anonymity of the individuals included, there are no data on clinical phenotype associated with each sequenced genome. Furthermore,

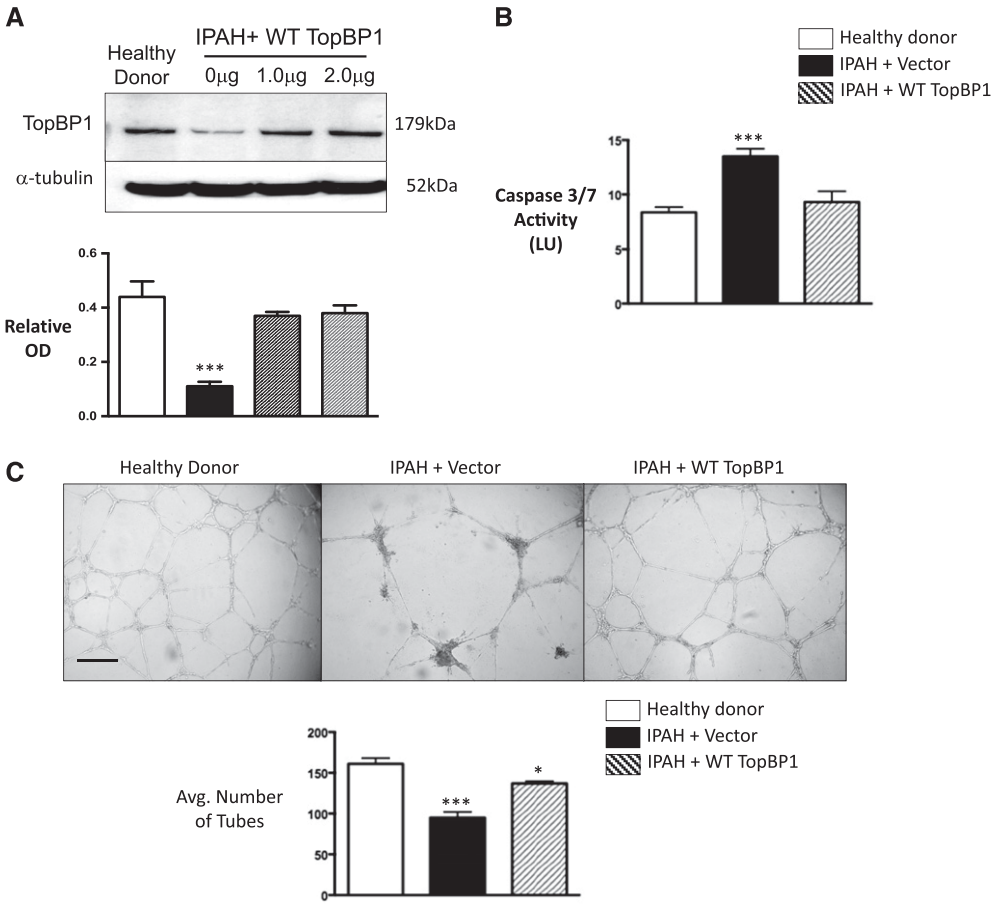

Figure 7. Restoration of topoisomerase DNA binding II binding protein 1 (TopBP1) levels protect idiopathic pulmonary arterial hypertension (IPAH) pulmonary microvascular endothelial cells (PMVECs) against hydroxyurea-induced apoptosis and improve tube formation. (*A*) Western blot showing TopBP1 expression in IPAH PMVECs transfected with wild-type (WT) TopBP1 expression construct (0, 1, and 2 μg). Densitometry is measured relative to α-tubulin as a loading control. ***$P < 0.0001$ versus healthy donor. One-way analysis of variance (ANOVA) with Bonferroni post-test, n = 3. (*B*) Caspase 3/7 activity assays of IPAH PMVECs transfected with either empty vector or WT TopBP1 expressing construct following hydroxyurea exposure for 24 hours. *Bars* represent mean SEM from experiments performed in triplicate. ***$P < 0.0001$ versus healthy donor, one-way ANOVA with Bonferroni post-test. (*C*) Matrigel tube formation assay comparing healthy donor, IPAH+vector, and IPAH+WT TopBP1 PMVECs. Tube number was quantified six hours after seeding the cells. *$P < 0.05$ versus healthy donor, ***$P < 0.0001$ versus healthy donor, one-way ANOVA with Bonferroni post-test. Scale bar = 150 μm.

systematic sequencing bias may exist in different control data sets that can confound the quantification of rare variant burden. These limitations underscore the importance of having access to sequencing data derived from the same population from which cases were sampled. To overcome this limitation, our group has recently begun assembling a WES database of healthy individuals who undergo health surveillance at our institution. We anticipate that this resource will be of benefit to the medical community in general and will accelerate the understanding of the genetic basis of other pulmonary disorders.

In conclusion, we demonstrate that WES can be used to expand the understanding of the genetic basis of IPAH and may help accelerate the discovery of possible biomarkers and therapeutic targets. The data obtained from WES can be used to map relevant gene interactions and identify signaling networks that may influence a patient's risk for disease progression or response to a particular treatment strategy. It is important to point out that the wealth of genetic information provided by WES and other next-generation sequencing technologies can only be of assistance if it is balanced against the ability to properly diagnose and recognize the clinical manifestations of PAH. We anticipate that learning to apply genetic data to patient care will allow clinicians to personalize their treatment plans and it is hoped improve the outcomes and quality of life of those who suffer from this devastating disease. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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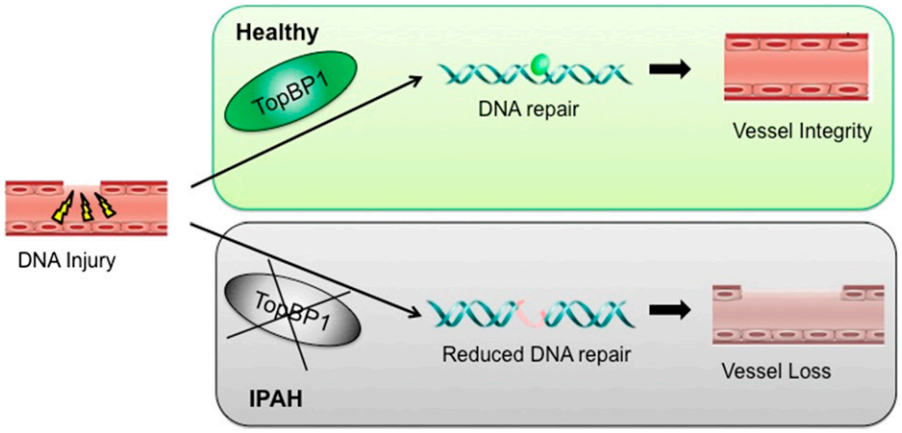

Figure 8. Proposed model. Topoisomerase DNA binding II binding protein 1 (TopBP1) helps protect pulmonary microvascular endothelial cells against injury and promotes angiogenesis (*top*). Reduced TopBP1 may contribute to idiopathic pulmonary arterial hypertension (IPAH) by increasing susceptibility to DNA damage resulting in loss of pulmonary microvascular endothelial cells and impaired angiogenesis (*bottom*).

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