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ORIGINAL ARTICLE

Multicenter Preclinical Validation of BET Inhibition for the Treatment of Pulmonary Arterial Hypertension

Diederik E. Van der Feen[1]*, Kondababu Kurakula[2]*, Eve Tremblay[3]*, Olivier Boucherat[3], Guido P. L. Bossers[1], Robert Szulcek[4], Alice Bourgeois[3], Marie-Claude Lampron[3], Karima Habbout[3], Sandra Martineau[3], Roxane Paulin[3], Ewelina Kulikowski[5], Ravi Jahagirdar[5], Ingrid Schalij[4], Harm Jan Bogaard[4], Beatrijs Bartelds[1,6], Steeve Provencher[3], Rolf M. F. Berger[1‡], Sébastien Bonnet[3‡], and Marie-José Goumans[2‡]

[1]Center for Congenital Heart Diseases, Beatrix Children's Hospital, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; [2]Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, the Netherlands; [3]Pulmonary Hypertension and Vascular Biology Research Group of Quebec Heart and Lung Institute, Laval University, Quebec, Canada; [4]Pulmonary Medicine, Amsterdam Cardiovascular Sciences, Amsterdam University Medical Centers, Amsterdam, the Netherlands; [5]Reserverlogix Calgary, Calgary, Alberta, Canada; and [6]Division of Cardiology, Department of Pediatrics, Erasmus University Medical Center, Sophia Children's Hospital, Rotterdam, the Netherlands

Abstract

Rationale: Pulmonary arterial hypertension (PAH) is a degenerative arteriopathy that leads to right ventricular (RV) failure. BRD4 (bromodomain-containing protein 4), a member of the BET (bromodomain and extra-terminal motif) family, has been identified as a critical epigenetic driver for cardiovascular diseases.

Objectives: To explore the therapeutic potential in PAH of RVX208, a clinically available BET inhibitor.

Methods: Microvascular endothelial cells, smooth muscle cells isolated from distal pulmonary arteries of patients with PAH, rats with Sugen5416 + hypoxia– or monocrotaline + shunt–induced PAH, and rats with RV pressure overload induced by pulmonary artery banding were treated with RVX208 in three independent laboratories.

Measurements and Main Results: BRD4 is upregulated in the remodeled pulmonary vasculature of patients with PAH, where it regulates FoxM1 and PLK1, proteins implicated in the DNA damage response. RVX208 normalized the hyperproliferative, apoptosis-resistant, and inflammatory phenotype of microvascular endothelial cells and smooth muscle cells isolated from patients with PAH. Oral treatment with RVX208 reversed vascular remodeling and improved pulmonary hemodynamics in two independent trials in Sugen5416 + hypoxia-PAH and in monocrotaline + shunt-PAH. RVX208 could be combined safely with contemporary PAH standard of care. RVX208 treatment also supported the pressure-loaded RV in pulmonary artery banding rats.

Conclusions: RVX208, a clinically available BET inhibitor, modulates proproliferative, prosurvival, and proinflammatory pathways, potentially through interactions with FoxM1 and PLK1. This reversed the PAH phenotype in isolated PAH microvascular endothelial cells and smooth muscle cells *in vitro*, and in diverse PAH rat models. RVX208 also supported the pressure-loaded RV *in vivo*. Together, these data support the establishment of a clinical trial with RVX208 in patients with PAH.

Keywords: BET inhibition; BRD4 (bromodomain-containing protein 4); pulmonary arterial hypertension; vascular remodeling; right ventricle pressure load

At a Glance Commentary

Scientific Knowledge on the Subject: Pulmonary arterial hypertension (PAH) is a degenerative arteriopathy that leads to right ventricular failure. BRD4 (bromodomain-containing protein 4) has been identified as a critical epigenetic driver for cardiovascular diseases.

What This Study Adds to the Field: RVX208, a clinically available BET (bromodomain and extra-terminal motif) inhibitor, modulates proproliferative, prosurvival, and proinflammatory pathways. This reversed the PAH phenotype in isolated PAH microvascular endothelial cells (pulmonary origin) and smooth muscle cells *in vitro*, and in diverse PAH rat models. RVX208 also supported the pressure-loaded right ventricle *in vivo*. Together, these data support the establishment of a clinical trial with RVX208 in patients with PAH.

Pulmonary arterial hypertension (PAH) is characterized by occlusive vascular remodeling, vascular rarefaction, and sclerosis. This ultimately causes pulmonary vascular resistance (PVR) to rise and induces right ventricular (RV) failure. Loss of BMPR2 (bone morphogenic protein receptor 2) and accumulating DNA damage (1) are early pathogenic phenomena that seem to act in a vicious cycle (2). In different cardiovascular diseases and cancers, BRD4 (bromodomain-containing protein-4), a member of the BET (bromodomain and extra-terminal motif) family, has been identified as a critical transcriptional modulator in the context of DNA damage (3–6). BRD4 can inhibit apoptosis, the physiologic response to accumulating DNA damage, by promoting cell survival (7), and stimulate hyperproliferation (8). BRD4 can further regulate a cellular switch into a proinflammatory phenotype via increased transcription of cytokines, such as IL-6 and IL-8 (Figure 1) (9). In previous studies, we have shown that BRD4 is also increased in PAH (7) and associated with an aberrant DNA damage response (DDR) (10)

mediated by FoxM1, a downstream BRD4 effector (11). Specific BRD4 inhibition by JQ1 and siRNA has shown to reverse vascular remodeling and improve pulmonary hemodynamics in Sugen5416-hypoxia rats (SH-PAH), which was associated with reduced PAH smooth muscle cell (SMC) apoptosis resistance and proliferation (7). However, neither JQ1 nor siRNA can be used clinically. We therefore explored the therapeutic potential of RVX208, a clinically available BET inhibitor and BRD4 antagonist. The experiments were initiated and conducted independently by the Pulmonary Hypertension and Vascular Biology Research Group in Quebec, Canada, and by the PHAEDRA consortium in the Netherlands. Because of high similarity in experimental set up and outcome, the Canadian and Dutch preclinical trials were later unified.

Methods

Human Cell Culture and Treatments
Pulmonary microvascular endothelial cells (MVECs) and SMCs were isolated from idiopathic PAH (IPAH), and normal lung explant tissue. Cells were treated with vehicle (5% DMSO), or RVX208, thiostrepton (FoxM1 inhibitor), or BI6727 (PLK1 inhibitor) in the indicated doses for 16 hours, or with siRNA against BRD4, FoxM1, or PLK1. Cell proliferation, apoptosis, inflammation, and BMPR2 signaling were assessed. Detailed culture procedures and all assays are described in the online supplement.

Rat Models for PAH and RV Pressure Overload and Treatments
All experiments were performed according to recently published standards for methodologic rigor in preclinical PAH studies (12), as described in the online supplement. RVX208 was evaluated in two established rat models for progressive PAH: SH-PAH and monocrotaline/shunt (MS-PAH). The Canadian and Dutch *in vivo* experiments were initiated simultaneously, conducted and analyzed independently, and finally unified in this work. In the Canadian study, SH-PAH was induced in male Sprague-Dawley rats by a 20 mg/kg Su5416 injection followed by 3 weeks in 10% hypoxia and 3 weeks in normoxia. Saline-injected,

normoxia rats ($n = 6$) served as control animals. From Day 42 to Day 70, SH-PAH rats received daily oral treatment with vehicle (formulation EA006 [13]; $n = 14$), 90 mg/kg RVX208 ($n = 8$), or RVX208 + contemporary care (CC; tadalafil 10 mg/kg + macitentan 10 mg/kg; $n = 7$). In the Dutch studies, SH-PAH was induced similarly. Rats received daily oral treatment from Day 42 to Day 70 with vehicle (5% DMSO in drinking water; $n = 12$) or 100 mg/kg RVX208 ($n = 12$).

In the MS-PAH study, PAH was induced in 27 male Wistar rats by a 60 mg/kg monocrotaline injection followed by aortocaval shunt surgery at Day 7 as described previously (12, 13). Daily oral treatment with vehicle ($n = 12$) or 100 mg/kg RVX208 ($n = 10$) was given from Day 21 to Day 35 (MS35Veh or MS35RVX). The remaining five MS-PAH rats were killed at Day 21 to allow a baseline comparison at treatment initiation (MS21). RV pressure overload was induced in rats by pulmonary artery banding (PAB), as described previously (14, 15). Rats received daily oral treatment from Day 28 to Day 56 with vehicle (PAB56Veh; $n = 5$) or 100 mg/kg RVX208 (PAB56RVX; $n = 8$). The *in vivo* effects of RVX208 on hemodynamics, vascular morphology, proliferation, apoptosis, inflammation and BMPR2, FoxM1, and PLK1 signaling were assessed as described in the online supplement. Tissue exposure levels of oral 100 mg/kg RVX208 treatment were measured in the lung and heart of the PAB rats.

Results

BRD4 Regulates the DDR Protein FoxM1 and Its Transcriptional Target PLK1 in Vascular Cells in Human PAH
We confirmed increased BRD4 protein expression in MVECs and SMCs isolated from patients with IPAH compared with healthy control subjects (Figure 2A). In IPAH, BRD4 localized in the media of larger pulmonary arteries with medial hypertrophy, in neointimal fibrotic lesions, and lining the plexus channels within plexiform lesions. In control subjects, BRD4 localized predominantly in the endothelium (*see* Figure E1 in the online supplement). We then investigated the functional relationship of BRD4 with
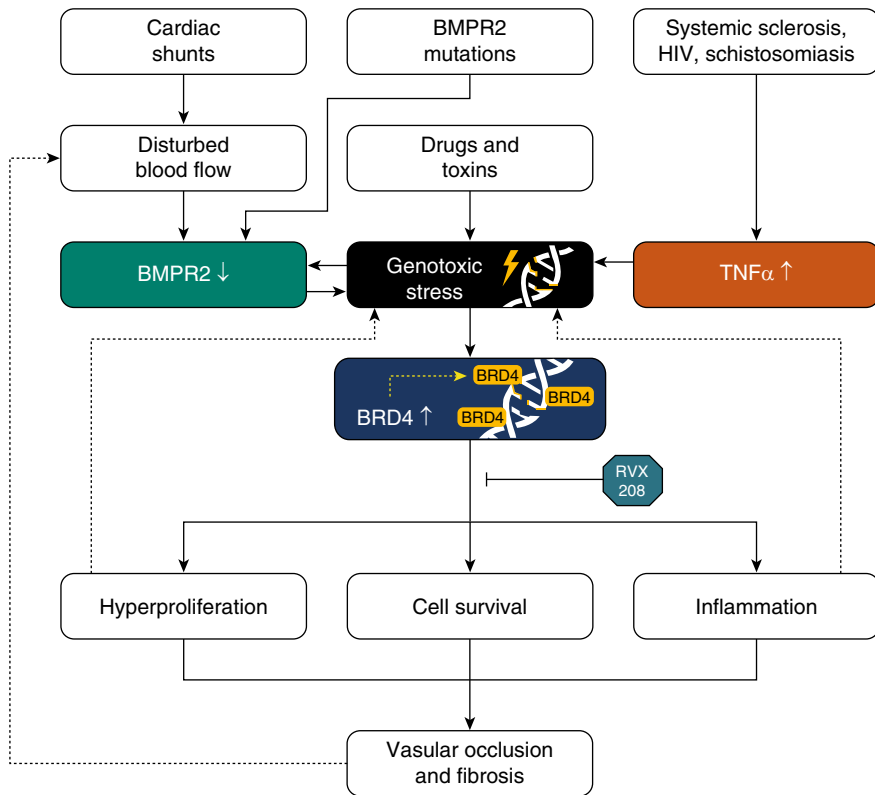
Figure 1. Hypothetical representation of the role of BRD4 (bromodomain-containing protein-4) in pulmonary arterial hypertension. DNA damage, induced by various genotoxic triggers associated with pulmonary arterial hypertension, leads to recruitment of BRD4 to the DNA, where it induces hyperproliferation, cell survival, and inflammation, which lead to vascular remodeling. BMPR2 = bone morphogenic protein receptor 2; TNFα = tumor necrosis factor-α.

FoxM1 and PLK1, respective downstream targets of BRD4 (11), in IPAH SMCs. In previous research, we have shown that expression of FoxM1 is increased in PAH-SMCs (10). FoxM1 is known to upregulate PLK1, an oncogene implied in hyperproliferation and apoptosis resistance in cancer (16). PLK1 expression was increased in IPAH, both in isolated PAH-SMCs (Figure 2B) and in the vessel wall (Figure 2C). To confirm that BRD4 regulates PLK1 via FoxM1 in PAH-SMCs, we inhibited BRD4 and FoxM1 by siRNA, which both decreased PLK1 protein expression (Figure 2D). Inhibition of BRD4 by RVX208 also decreased FoxM1 and PLK1 on the protein (Figure 2E) and transcriptional level in PAH-SMCs (Figure 2F). Finally, we showed that inhibition of FoxM1 by thiostreptone dose dependently decreased PLK1 protein expression (see Figure E2). In conclusion, we report that BRD4 expression is increased in MVECs and SMCs of patients with PAH, and induces PLK1 via FoxM1.

Reversal toward a Healthy Vascular Cell Phenotype by RVX208

Next, we assessed the effect of BRD4–FoxM1–PLK1 inhibition on principle features of vascular remodeling in PAH: proliferation, apoptosis resistance, TGFβ (transforming growth factor-β)/BMP (bone morphogenic protein) imbalance, and inflammation (Figures 3A, 3G, and 3K). We found that 10-μM RVX208 significantly reduced cell viability, a product of reduced proliferation and increased apoptosis, in control and PAH-MVECs and PAH-SMCs (Figure 3B). RVX208 reduced proliferative signaling in PAH-MVECs by decreasing cyclin-D1 promotor activity, and normalized the level of cyclin-D1 mRNA, which had been increased by stimulating healthy MVECs with 10-nM TNFα (tumor necrosis factor-α) (Figure 3C). We further showed that medium conditioned by PAH-MVECs induced proliferation of normal SMCs, whereas 10-μM RVX208 treatment of PAH-MVECs resulted in conditioned

medium that decreased SMC proliferation (Figure 3D), signifying that RVX208 modulates the proproliferative crosstalk of endothelial cells to SMCs. In isolated PAH-SMCs, siBRD4, or 50-μM RVX208 significantly reduced proliferation and apoptosis resistance, as shown by decreased Ki67 and increased annexin V expression (Figures 3E and 3F). Similar effects were observed in PAH–pulmonary artery smooth muscle cells exposed to siPLK1 or the PLK1 inhibitor BI6727 (see Figure E3).

Next, we assessed the effect of RVX208 (10 μM) on TGFβ/BMP imbalance, using ID1 and PAI1 as the respective readouts of BMP- and TGFβ-mediated signaling. In PAH-MVECs, RVX208 reduced the activity of the PAI1-derived TGFβ-responsive CAGA-Luc reporter and the ID1-derived BMP-responsive BRE-Luc reporter (Figure 3H). In control MVECs, RVX208 reversed the decrease in ID1 and increase in PAI1 mRNA that were induced by TNFα stimulation (Figure 3I). The increase in ID1 protein levels was confirmed in PAH-MVECs, likely explained by a concurrent increase in BMPR2 protein on RVX208 treatment (Figure 3J). Seeing the regulatory effects of an inflammatory stimulus, such as TNFα on the aforementioned processes, we finally assessed the effect of RVX208 on cytokine production. In control MVECs, RVX208 reversed the TNFα-mediated increase in IL-8, monocyte chemoattractant protein (MCP)-1, and CCL5 mRNA (Figure 3L). RVX208 also reduced IL-6 and MCP1 secretion by PAH-MVECs (ELISA) (Figure 3M), and reduced NFκB (nuclear factor-κB) promoter activity (Figure 3N). In conclusion, we report that RVX208 amended the TGFβ/BMP imbalance and reversed the proproliferative, apoptosis-resistant, and inflammatory PAH vascular cell phenotype.

RVX208 Reverses Pulmonary Vascular Remodeling and Improves Hemodynamics *In Vivo*

In the Canadian SH-PAH study all rats survived until the endpoint. Vascular occlusion, systolic RV pressure (sRVP), and mean pulmonary artery pressure (mPAP) were increased in vehicle-treated rats compared with control animals. Treatment with either RVX208 alone or RVX208 + CC in SH-PAH rats decreased sRVP, mPAP, and PVR and vascular occlusion by decreasing intimal thickness (Figure 4B; see Figure E4). Medial thickness was only
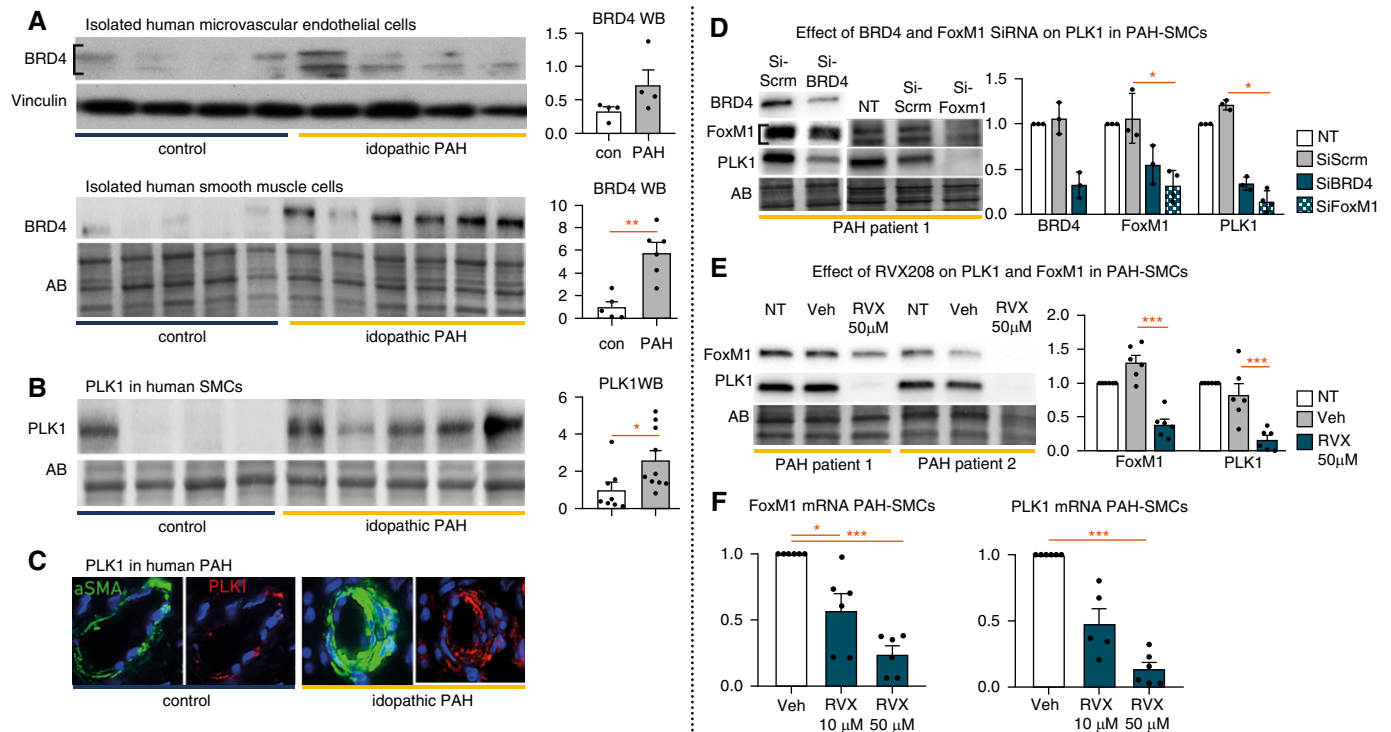
Figure 2. BRD4 (bromodomain-containing protein-4) regulates the DNA damage response protein FoxM1 and its transcriptional target PLK1 in vascular cells in human pulmonary arterial hypertension (PAH). (*A*) Western blot showing increased BRD4 protein in microvascular endothelial cells and pulmonary artery smooth muscle cells isolated from patients with idiopathic PAH versus control subjects. (*B*) Western blot showing increased PLK1 in idiopathic PAH smooth muscle cells (SMCs). (*C*) Increased expression of PLK1 (red) in the idiopathic PAH vessel wall (each image is 100 μm wide). SMCs are marked by α-SMA in green. (*D*) Western blot showing the effect of BRD4 and FoxM1 siRNA on PLK1 in PAH-SMCs. (*E*) Western blot showing the effect of RVX208 on PLK1 in FoxM1 in PAH-SMCs. (*F*) FoxM1 and PLK1 mRNA is reduced dose dependently by RVX208 in PAH-SMCs. Data are represented as mean ± SD. Statistical differences were assessed by Mann-Whitney *U* or Kruskall-Wallis test. Significant differences: *$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$. α-SMA = α-smooth muscle actin; AB = Amido black; NT = no treatment; siBRD4/FoxM1 = siRNA against BRD4/FoxM1; Si-Scrm = scrambled siRNA; Veh = vehicle (DMSO); WB = western blot.

decreased by RVX208 + CC (*see* Figure E4). The RV to left ventricular (LV) + intraventricular septal (IVS) weight ratio [RV/(LV + IVS)] was increased in SH-PAH compared with control subjects, but was not significantly decreased by treatment (*see* Figure E4).

Results were largely similar in the Dutch SH-PAH study. One rat died in each group (Figure 4). RVX208 significantly reduced sRVP, PVR, and vascular occlusion, also by reducing intimal thickness. No effects on the RV/(LV + IVS) weight ratio were observed (Figure 4E; *see* Figure E5). Pulmonary artery acceleration time, an echocardiographic parameter that decreases early during development of pulmonary hypertension (17), was increased by RVX208 treatment (*see* Figure E5).

In the MS-PAH study, three rats died before the endpoint at Day 35 in the vehicle group versus two in the RVX208 group. Vascular occlusion score and intimal

thickness were comparable in the MS21 and MS35veh rats, indicating initiation of treatment during end-stage pulmonary vascular disease. RVX208 significantly reduced vascular occlusion and intimal thickness (Figure 4H). Lung fibrosis, as assessed by Masson staining and quantified in the total lung (Figure 4J), was decreased by RVX208. Specifically, RVX208-treated lungs showed less fibrosis in the vessel wall (Figure 4K). Although mean pulmonary artery acceleration time was significantly improved by RVX208, the reduction in sRVP, mPAP, and PVR did not reach statistical significance and the RV/(LV + IVS) weight ratio was not affected by RVX208 (Figure 4H; *see* Figure E6).

In conclusion, we report that in three independent research groups, oral treatment with RVX208 reversed vascular remodeling in both the SH- and the MS-PAH rat, and improved pulmonary hemodynamics, particularly in the SH-PAH rat.

In Vivo Effects of RVX208 on FoxM1, PLK1, Proliferation, Apoptosis, Inflammation, and BMP Signaling

In the Canadian SH-PAH study, total lung PLK1 mRNA was increased in vehicle-treated rats versus normal control animals, and was decreased by RVX + CC (Figure 5A). FoxM1 expression in pulmonary artery smooth muscle cells was increased in SH-vehicle rats versus control animals, and decreased significantly by RVX + CC (Figure 5B). Cell proliferation was increased in SH-vehicle rats versus control animals, which was reversed by RVX + CC (Figure 5C). Apoptosis was not affected in SH-PAH, nor by RVX treatment (Figure 5D). Finally, mRNA levels of IL-6 and MCP1 were increased in SH-PAH versus control subjects and were decreased by RVX + CC (Figure 5E; *see* Figure E4).

In the MS-PAH study, FoxM1 and PLK1 protein expression tended to be higher in the total lung lysate of MS35Veh
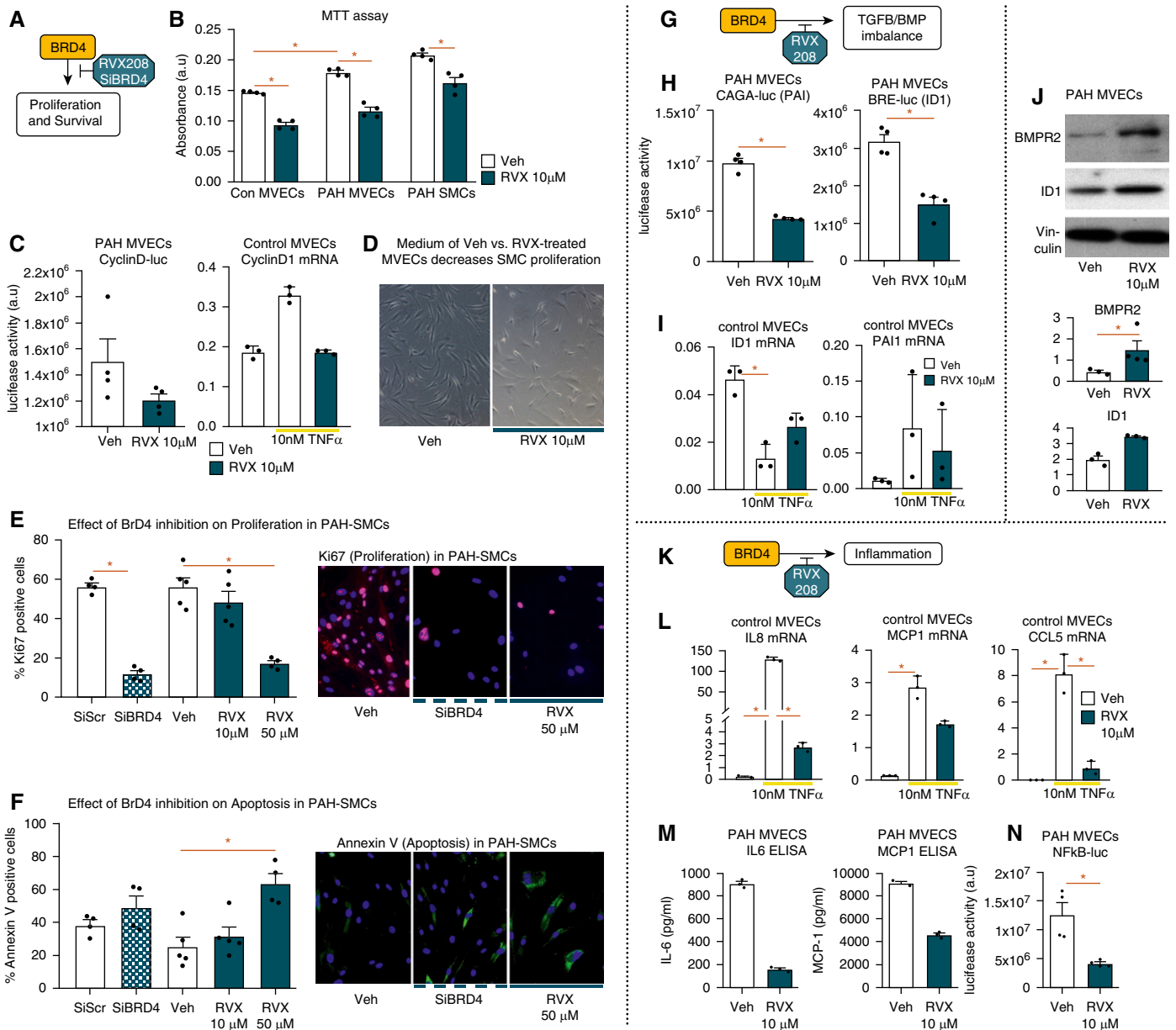
Figure 3. Reversal toward a healthy vascular cell phenotype by RVX208. (*A*) The effect of BRD4 (bromodomain-containing protein-4)–FoxM1–PLK1 inhibition on cell proliferation and survival (hypothesis). (*B*) MTT cell viability assays with 10-μM RVX208 in control and pulmonary arterial hypertension (PAH)–microvascular endothelial cells (MVECs) and PAH–smooth muscle cells (SMCs). (*C*) Luciferase assay showing decreased activity of the cyclin-D promoter by RVX208 in PAH-MVECs, and PCR for cyclin-D1 under TNFα (tumor necrosis factor-α) stimulation and RVX208 treatment. (*D*) SMCs cultured in medium conditioned by PAH-MVECs treated with vehicle or RVX208. (*E*) Ki67 staining and quantification showing the effect of BRD4 inhibition by siBRD4 and RVX208 on PAH-SMC proliferation. The dashed line denotes a different but similar treatment. (*F*) Annexin V staining and quantification showing the effect of BRD4 inhibition by siBRD4 and RVX208 on PAH-SMC apoptosis. (*G*) The effect of BRD4 inhibition on the TGFβ (transforming growth factor-β)/BMP (bone morphogenic protein) imbalance in PAH (hypothesis). (*H*) Luciferase assay for the BMP reporter (BRE-Luc) and TGFβ reporter (CAGA-Luc) in PAH-MVECs after BMP9 (1 ng/ml) or TGFβ (1 ng/ml) stimulation, respectively, in the absence or presence of RVX208 treatment. (*I*) PCR for ID1 and PAI1 under TNFα stimulation and RVX208 treatment. (*J*) Western blot (top) and quantification (bottom) showing increased BMPR2 and ID1 protein under RVX208 treatment in PAH-MVECs. (*K*) The effect of BRD4 inhibition on inflammation (hypothesis). (*L*) PCR for IL-8, monocyte chemoattractant protein (MCP)-1, and CCL5 under TNFα stimulation and RVX208 treatment in normal MVECs. (*M*) ELISA for IL-6 and MCP1 in PAH-MVECs. (*N*) Luciferase assay for the NFκB promoter in PAH-MVECs with RVX208 treatment. Data are represented as mean ± SD. Statistical differences were assessed by Mann-Whitney *U* or Kruskall-Wallis test. Significant differences: *$P < 0.05$. Con = control; MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor; NFκB = nuclear factor-κB; Veh = vehicle (DMSO).
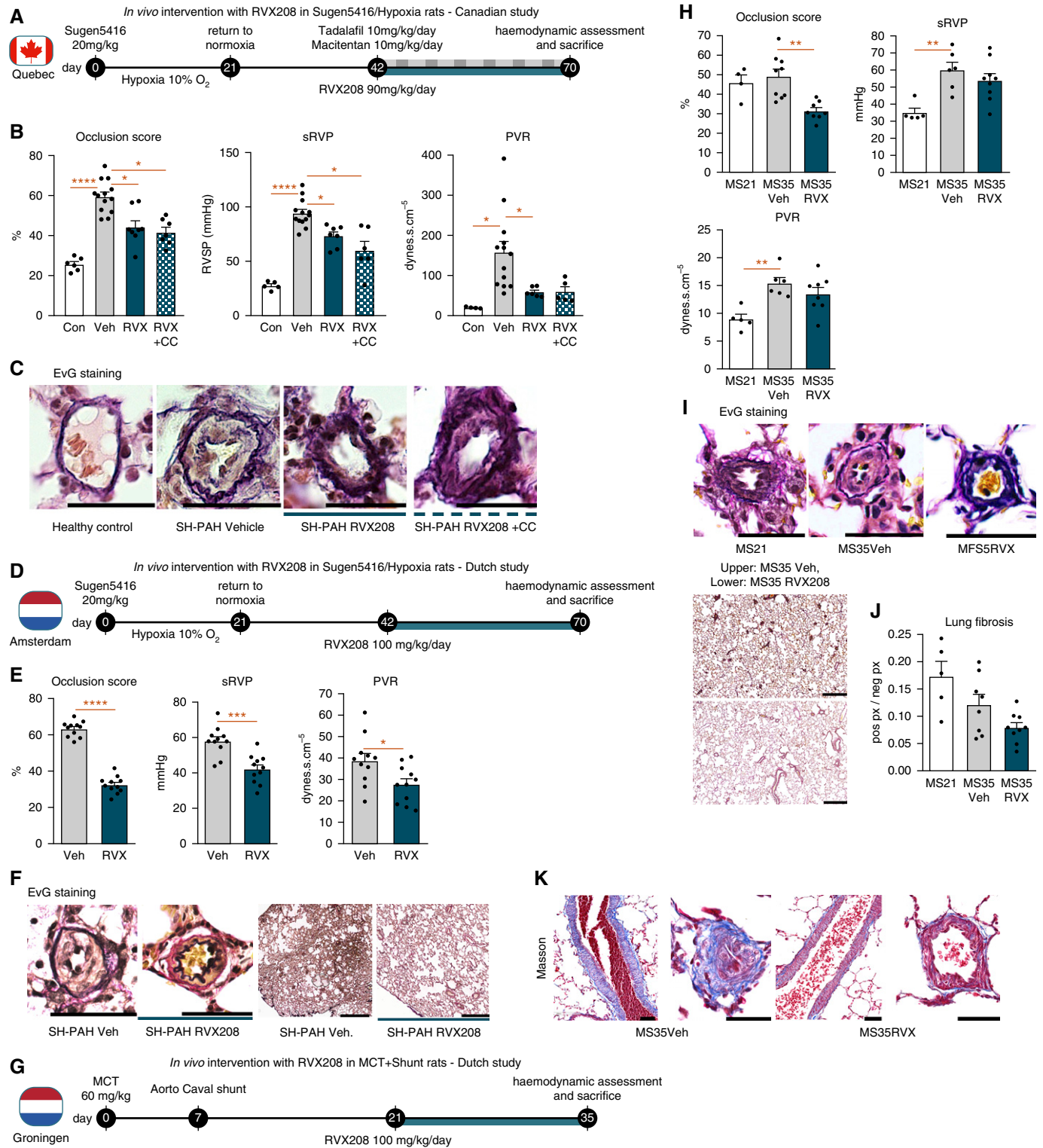
Figure 4. RVX208 reverses pulmonary vascular remodeling and improves hemodynamics *in vivo*. (*A*) Experimental design for the Canadian *in vivo* intervention study with RVX208 (± contemporary care) in Sugen-hypoxia rats (SH–pulmonary arterial hypertension [PAH]). (*B*) Quantification of vascular occlusion and hemodynamics. (*C*) Elastica-van Gieson (EvG) staining, representative examples of vascular lesions. The dashed line denotes RVX+ contemporary care. Scale bars, 20 μm. (*D*) Experimental design for the Dutch *in vivo* intervention study with RVX208 in SH-PAH. (*E*) Quantification of vascular occlusion and hemodynamics. (*F*) EvG staining. Left: representative examples of vascular lesions. Scale bars, 50 μm. Right: overview of the SH-PAH lung, showing fewer occluded vessels and inflammatory infiltrates in RVX208-treated rats. Scale bars, 500 μm. (*G*) Experimental design for

compared with normal control. RVX208 significantly decreased FoxM1 protein, and tended to decrease PLK1 protein (Figures 4F and 4G). Total lung PLK1 mRNA was decreased by RVX208 (Figure 5G). FoxM1 mRNA was not detected. Vascular FoxM1 expression, located mainly in the media, was increased in MS35Veh versus control subjects, which was blunted by RVX208 treatment (Figure 5H). Vascular PLK1 expression was largely absent in healthy control subjects, was increased in the endothelium of MS35Veh, and was reduced by RVX208 (Figure 5H). Contrary to the SH-PAH study, apoptosis (assessed by cleaved-caspase-3 staining, which located mainly in the intima) was increased by RVX208 treatment in MS35-PAH (Figure 5I). Ki67 staining is usually scarce in the vasculature of MS35Veh (18), and was therefore not assessed here. Furthermore, RVX208 treatment resulted in increased BMPR2 and ID3 protein (Figure 5J). IL-6 and MCP1 mRNA level was low in all MS35RVX rats, whereas high expression was observed in four of nine MS35Veh rats, and CCL5 mRNA was significantly reduced by RVX208 (Figure 5K; see Figure E6). In conclusion, we report a consistent downregulation of FoxM1 and PLK1 and a decrease in cytokine mRNA levels after RVX208 treatment in both the SH- and MS-PAH model. Model-specific effects of RVX208 treatment were observed regarding proliferation and apoptosis.

RVX208 Supports RV Function during Increased Pressure Load

We finally studied the effects of RVX208 treatment in RV pressure load induced by PAB in rats (14, 17) to confirm safety of the drug during RV compromise. Three rats died during surgery. At Day 14, we measured PAB pressure gradient using echocardiography to confirm effective and equal pressure load at baseline. In PAB56RVX rats, the pressure gradient increased from Day 14 to Day 56, indicating adaptation to pressure load

(Figure 6B). In line with this observation, cardiac output, RV stroke volume, stroke work, and RV power were also increased by RVX208 (Figure 6B). Tricuspid annular plane systolic excursion was normal in both groups. The relatively higher contractile force in PAB56RVX was also reflected in an increase in RV/(LV + IVS) mass. RV cardiomyocyte cross-sectional area showed a twofold increase compared with sham conditions (see Figure E7), but was similar in PAB56RVX and PAB56Veh (Figure 6C), indicating that the increased RV mass in PAB56RVX could be caused by a proportional increase in the length of cardiomyocytes, which is associated to higher contractility (19). The increased RV mass in RVX208-treated rats was not accompanied by evidence of adverse remodeling, as indicated by a favorable capillary/myocyte ratio and a low percentage of fibrosis (Figure 6D) (20). Furthermore, β-myosin heavy chain to α-myosin heavy chain ratio, typically increased in pathologic cardiac hypertrophy, tended to be lower in PAB56RVX. Together, these data indicated that RVX208 stimulates RV adaptation in response to increased pressure load without signs of adverse remodeling, suggesting a favorable safety profile or even therapeutic benefit for patients with RV dysfunction in advanced PAH. FoxM1 and PLK1 mRNA levels were also increased in the PAB56Veh RV compared with sham-operated animals, but were not decreased by RVX208 (Figures 6E and 6F). In conclusion, we report that RVX208 treatment support RV function in the context of increased RV pressure load.

As an additional verification of oral RVX208 bioavailability at the 100 mg/kg dose, we used pulmonary and LV tissue of the RVX-treated rats in the PAB study to determine tissue exposure levels. Mean pulmonary exposure was 2.1 ± 1.8 μM and mean LV exposure was 0.82 ± 0.4 μM (Figure 6G), which is in the same range as the maximum plasma concentration at the desired clinical dose.

Discussion

We described the union of two independent preclinical trials in three independent research laboratories that explored the therapeutic potential of the clinically available BET inhibitor RVX208 in PAH. Together, we confirmed that BRD4, the best studied member of the BET family, is upregulated in the remodeled pulmonary vasculature of patients with PAH, where it regulated the expression of the oncogene PLK1 and FoxM1, a DDR protein implied in vascular remodeling in PAH (10, 19, 20). BET inhibition by RVX208 normalized the hyperproliferative, apoptosis-resistant, and proinflammatory phenotype of MVECs and SMCs isolated from patients with PAH. At a clinically relevant dose, RVX208 reversed vascular remodeling in multiple complementary preclinical models of PAH, and could be combined safely with current PAH therapy. Finally, we showed that RVX208 supports the pressure-loaded RV in rats, indicating a beneficial, dual mode of action for patients with PAH-associated RV pressure overload.

BET proteins, such as BRD4, can be recruited to the DNA in a variety of pathologic circumstances, such as in cancer or inflammatory and cardiovascular diseases (5, 6, 21, 22). BRD4 recruitment has a common association with compromised DNA integrity (3) or genotoxic conditions, such as replicative, oncogenic, or oxidative stress (8, 23, 24). As an epigenetic reader, BRD4 is able to sense double strand breaks in the DNA where it elicits a DDR by stimulating γH2AX signaling (3), leading to survival rather than apoptosis of the cell. Although an effective DDR is crucial in normal physiology to repair DNA and prevent apoptosis after DNA damage, persistent DDR activation (e.g., by BRD4) during chronic genotoxic conditions can lead to aberrant DNA repair and to survival of cells with genomic abnormalities (2). In these cells, BRD4 can further modulate the chromatin landscape (3), and enable transcription of a context-dependent, yet

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Figure 4. (Continued). the *in vivo* intervention study with RVX208 in MCT + shunt rats (MS-PAH). (*H*) Quantification of vascular occlusion and hemodynamics. (*I*) EvG staining. Top: representative examples of vascular lesions. Scale bars, 50 μm. Bottom: overview of the MS-PAH lung, showing fewer occluded vessels in RVX208-treated rats. Scale bars, 500 μm. (*J*) Quantification of fibrosis in the whole lung. (*K*) Masson staining showing increased fibrosis in the vessel wall of vehicle-treated rats. Scale bars, 50 μm. Data are represented as mean ± SD. Statistical differences were assessed by Mann-Whitney *U* or Kruskall-Wallis test. Significant differences: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. CC = contemporary care (macitentan/tadalafil); Con = control; MCT = monocrotaline; PVR = pulmonary vascular resistance; sRVP = systolic right ventricular pressure; Veh = vehicle (5% DMSO).
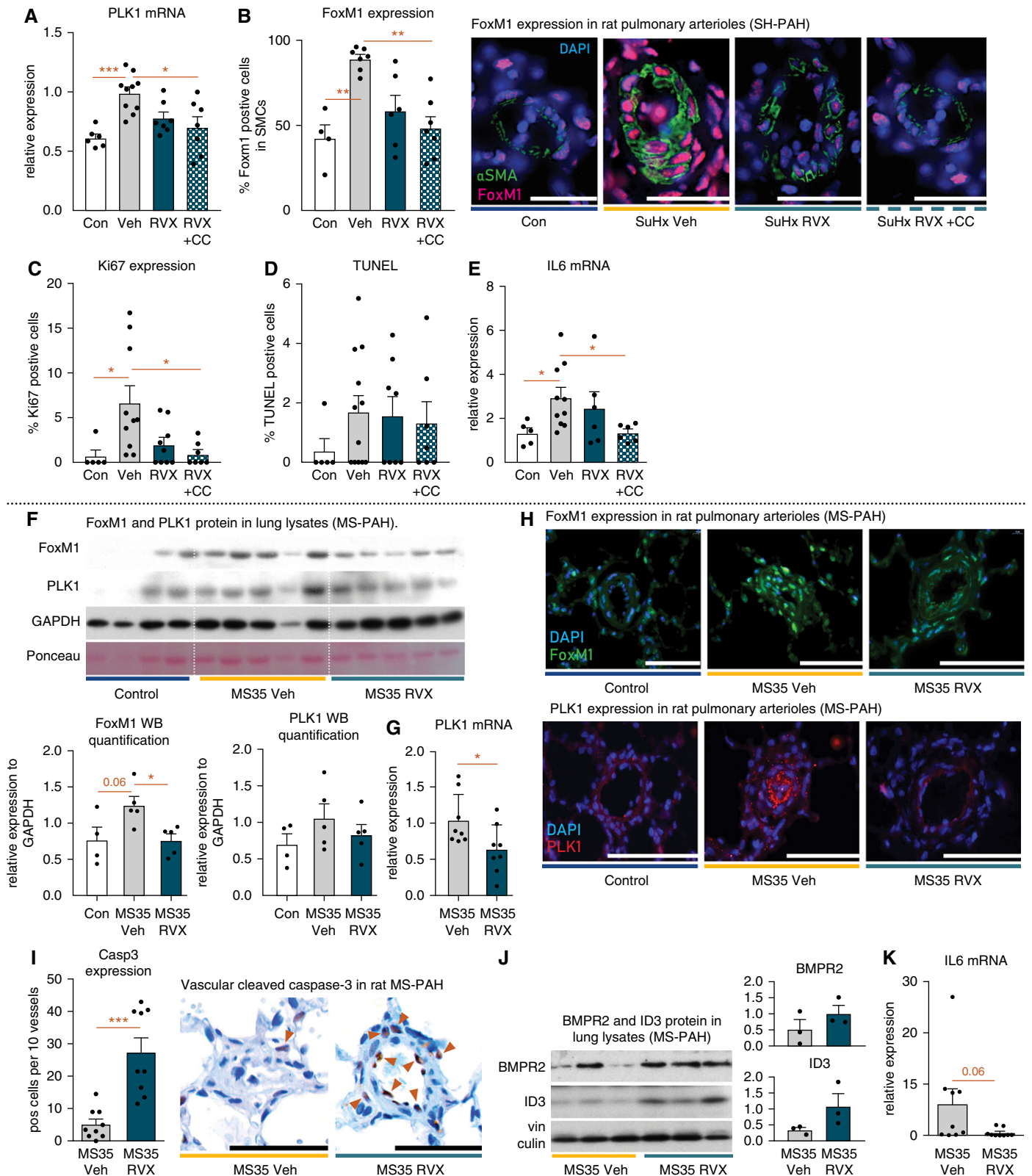
Figure 5. *In vivo* effects of RVX208 on FoxM1, PLK1, proliferation, apoptosis, inflammation, and bone morphogenic protein signaling. (*A–E*) Canadian Sugen-hypoxia rats–pulmonary arterial hypertension (PAH) study. (*F–K*) Dutch monocrotaline/shunt-PAH study. (*A*) PCR for PLK1 mRNA in whole-lung lysates. (*B*) FoxM1 staining (pink) and quantification of pulmonary vascular FoxM1 expression. Smooth muscle cells are stained in green. The dashed line denotes RVX+ contemporary care. (*C*) Quantification of pulmonary vascular Ki67 expression (proliferation). (*D*) Quantification of pulmonary vascular

characteristic profile of proproliferative, prosurvival (25), and/or proinflammatory factors (9). These data indicated BRD4 as an essential driver of DNA damage-associated pathologies. Substantial evidence indicates that DNA damage and prolonged DDR activation are also implied in PAH (26). DDR proteins, such as γH2AX, 53BP1, and PARP1, are upregulated in the pulmonary vasculature of patients with PAH (1), and many of the known triggers for PAH cause genotoxic stress. These include acute genotoxic hits, such as alkylating chemotherapy, toxins, or irradiation, or chronic genotoxicity as in inflammatory disease, such as systemic sclerosis, schistosomiasis, or HIV. Persistent BMPR2 downregulation, such as in BMPR2-mutant PAH or as occurs in situations of chronically disturbed shear stress (27), also compromises DNA integrity through loss of normal DNA repair mechanisms (2).

Taken together, these data suggested that modulating the DDR by BRD4 inhibition could be beneficial for patients with PAH. This was first established by inhibiting BRD4 specifically via JQ1 and siRNA in the SH rat (7). JQ1 and siRNA both improved pulmonary hemodynamics and reversed vascular remodeling, which was associated with reduced apoptosis resistance and proliferation (7). In other work, we showed that inhibition of FoxM1, another master regulator of the DDR associated with ineffective DNA repair (28), reversed SH and MCT-PAH in a similar fashion (10). This underlined the importance of the BRD4-FoxM1 axis in PAH. Here, we confirmed once more that BRD4 and FoxM1 are upregulated in PAH. As reported in cancer cells (11), and for the first time in PAH, we show that BRD4 and FoxM1 regulate the expression of PLK1, an oncogene involved in the proliferation-apoptosis (im)balance (Figure 2D). In cancer, where PLK1 is commonly overexpressed (16), it is thought that PLK1 promotes tumorigenesis by stimulating

survival, cell-cycle reentry, and proliferation (29). We hypothesized that this characteristic could also be relevant to the PAH-context, and confirmed PLK1 overexpression in isolated PAH-SMCs and in the remodeled PAH vasculature (Figures 2B and 2C). We further demonstrated that specific inhibition of PLK1 by siRNA or the PLK1 inhibitor BI6727 decreased the cancer-like phenotype of SMCs in PAH (see Figure E3).

Patients with PAH are in critical need of new, directly applicable treatment strategies that are able to reverse advanced vascular remodeling and are safe in context of RV failure. Contemporary treatment is aimed mainly at vasodilation and slows down the progression of vascular occlusion at best. The BRD4 inhibitor JQ1 held promise for treatment of end-stage PAH (7), but is not suitable for clinical use. We therefore chose RVX208 as a clinically relevant, orally available inhibitor of the BET-family that includes BRD4. In vitro, RVX208 reduced PAH-SMC proliferation and survival as effectively as BRD4- and PLK1-siRNA, or PLK1 inhibition by BI6727 (Figures 3E and 3F). RVX208 had a comparable effect on proliferation and survival in PAH-MVECs (Figures 3B–3D). Additionally, RVX208 was able to normalize the proinflammatory phenotype observed in PAH-MVECs (Figures 3L–3N), which is in line with the results of RVX208 treatment in proinflammatory endothelial cells and SMCs in atherosclerosis (30). Interestingly, inflammation and BRD4 were also recently implicated in coronary artery remodeling in patients with PAH, suggesting that BRD4 inhibition may have beneficial effects beyond the pulmonary circulation in PAH (31). A novel finding in this study was that RVX208 also increased BMPR2 signaling in PAH-MVECs, or restored the TGFβ–BMP balance in healthy MVECs after disturbance by TNFα, a genotoxic hit known to downregulate BMPR2 in

endothelial cells and predispose to PAH (Figures 3H–3J) (32). This was an unexpected positive contribution of RVX208 in restoring the PAH phenotype on top of the more commonly known actions of BRD4 inhibition.

In vivo, we have shown that a 4-week treatment with RVX208 at a clinically relevant dose (Figure 6G) significantly reduced vascular occlusion and improved hemodynamics in two independent rat studies with SH PAH (Figures 4A–4F), which was as effective as treatment with JQ1 or BRD4-SiRNA in previous work (7). To improve translatability to the clinic, we showed that RVX208 can be combined safely with contemporary standard of care (tadalafil and macitentan). However, RVX208 contributed to the most profound beneficial effects, particularly with regard to vascular remodeling (Figure 4B), indicating that RVX208 alone might also suffice. The MCT-shunt rat studies showed that short-term treatment with RVX208 already improves severe neointimal vascular remodeling, even with the shunt still in place (Figure 4H). Importantly, together these data demonstrated that the beneficial effects of RVX208 were not PAH model specific.

We finally investigated the effect of RVX208 treatment on the RV during pressure load, primarily to ensure safety of the drug in the context of RV dysfunction. Interestingly, FoxM1 and PLK1 mRNA were also significantly increased by pressure load in the rat RV, indicating a potential role for BRD4–FoxM1–PLK1 signaling in RV pathology as well (Figures 6E and 6F). We found that RVX208 treatment stimulated physiologic RV adaptation, which increased RV power (Figure 6B), suggesting that RVX208 treatment could be beneficial in patients with PAH-associated RV dysfunction, in addition to the effects on the pulmonary vasculature. Longer term studies may determine whether RVX208 can prevent or reverse the development of RV failure during pressure load. Our data are consistent with the results of BRD4 inhibition by JQ1 in physiologic LV

Figure 5. (Continued). TUNEL expression (apoptosis). (E) PCR for IL-6 mRNA in whole-lung lysates. (F) Western blots and quantification for FoxM1 and PLK1 protein in total lung lysates. (G) PCR for PLK1 mRNA in whole-lung lysates. (H) FoxM1 and PLK1 expression in rat pulmonary arterioles. Scale bars, 50 μm. (I) Staining (brown, arrowheads) and quantification of pulmonary vascular Cleaved Caspase-3 expression (apoptosis). (J) Western blot and quantification for BMPR2 and ID3 in whole-lung lysates. (K) PCR for IL-6 mRNA in whole-lung lysates. Data are represented as mean ± SD. Statistical differences were assessed by Mann-Whitney U or Kruskall-Wallis test. Significant differences: *$P < 0.05$, **$P < 0.001$, and ***$P < 0.0001$. α-SMA = α-smooth muscle actin; CC = contemporary care (macitentan/tadalafil); Con = control; MS = monocrotaline/shunt; SH = Sugen-hypoxia; SMC = smooth muscle cell; Veh = vehicle (DMSO).
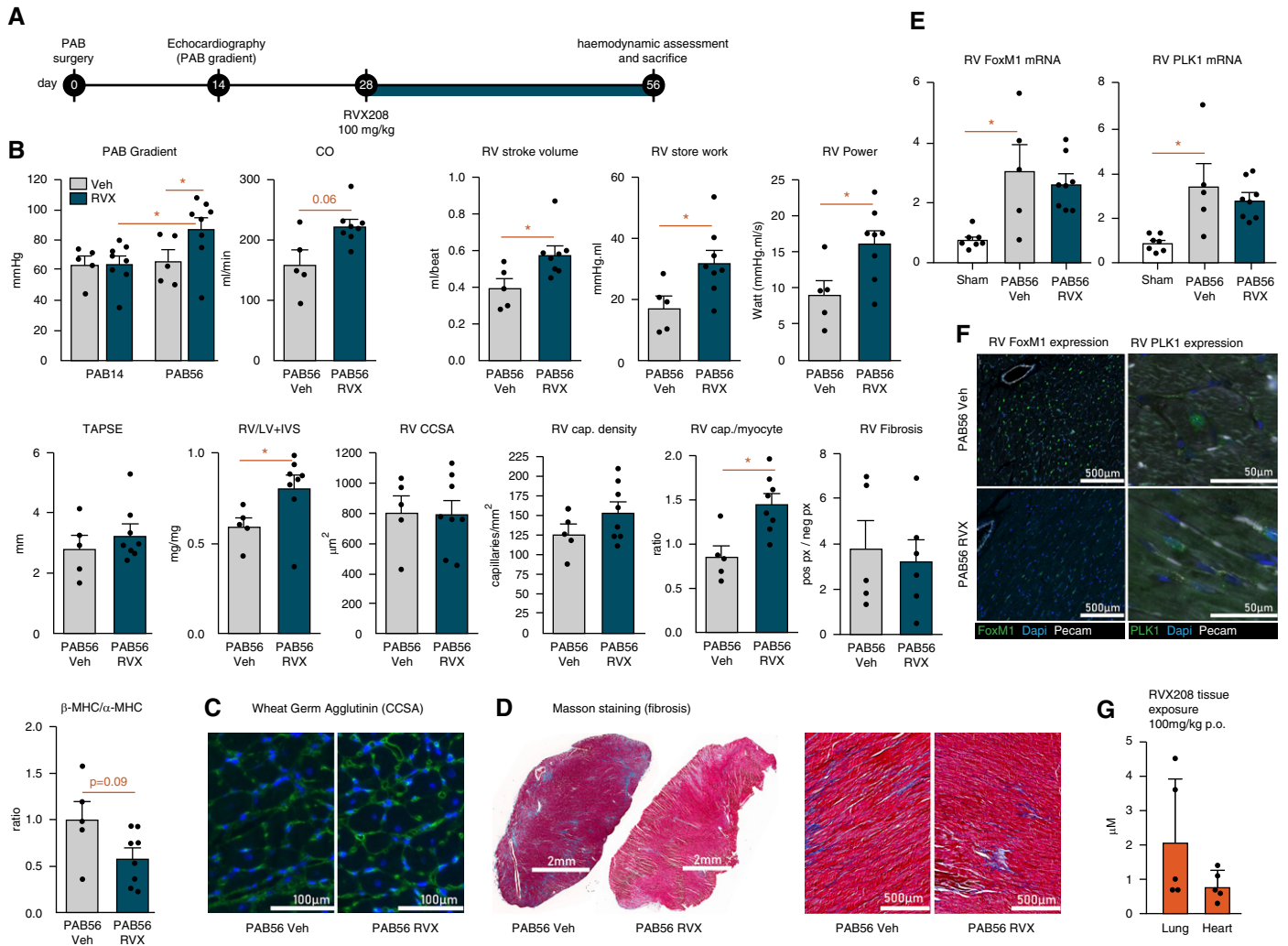
Figure 6. RVX208 supports right ventricular (RV) function during increased pressure load. (*A*) Experimental design for the *in vivo* intervention study with RVX208 in the rat pulmonary artery banding (PAB) model for isolated RV pressure load. (*B*) Hemodynamic and histologic evaluation. (*C*) Wheat germ agglutinin staining for cardiomyocyte cross-sectional area measurement. (*D*) Masson staining for fibrosis measurement. (*E*) PCR for FoxM1 and PLK1 mRNA in whole RV lysates. (*F*) Staining for FoxM1 and PLK1 (both in green) in RV tissue of PAB rats. (*G*) Tissue exposure levels for RVX208 at 100 mg/kg concentration administered via drinking water in 5% DMSO. The desired clinical dose is between 1 and 2 μM. Data are represented as mean ± SD. Statistical differences were assessed by Mann-Whitney U or Kruskall-Wallis test. Significant differences: *$P < 0.05$. Cap = capillary; CCSA = cross-sectional area measurement; CO = cardiac output; MHC = myosin heavy chain; RV/(LV + IVS) = right ventricular to left ventricular + intraventricular septal weight ratio; TAPSE = tricuspid annular plane systolic excursion; Veh = vehicle (DMSO).

remodeling, where treatment did not suppress LV adaptation because of high-intensity exercise in mice. In mice with severe LV failure caused by pressure load and myocardial infarction, JQ1 treatment improved LV function; reduced LV fibrosis; and repressed a spectrum of heart failure and fibrosis-associated genes, including TGFβ and NFκB (6). Overall, interfering with the BRD4 pathway does not limit the ventricular response to increased afterload, and may even enhance its adaptation.

The present study is the first to implement the methodologic rigor preconized in PAH preclinical confirmatory studies to increase the reproducibility and translatability of preclinical research, including independent replication, use of multiple animal models, treatment on top of CC, and assessment of direct effects on the RV, as published recently (12).

In conclusion, we describe the unified results of the first multicenter randomized preclinical trial performed in three independent laboratories that confirmed the therapeutic potential of the clinically available BET inhibitor RVX208 in various PAH rodent models. We also report that RVX208 modulates the proproliferative, prosurvival, and proinflammatory pathways by direct interaction with FoxM1 and PLK1. This reversed the PAH phenotype in isolated PAH-MVECs and SMCs, reversed vascular remodeling in two complimentary preclinical PAH models, and supported the pressure loaded RV. Taken together, these data support the establishment of a clinical trial with RVX208 in patients with PAH. ■

References

1. Meloche J, Pflieger A, Vaillancourt M, Paulin R, Potus F, Zervopoulos S, *et al*. Role for DNA damage signaling in pulmonary arterial hypertension. *Circulation* 2014;129:786–797.
2. Li M, Vattulainen S, Aho J, Orcholski M, Rojas V, Yuan K, *et al*. Loss of bone morphogenetic protein receptor 2 is associated with abnormal DNA repair in pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2014;50:1118–1128.
3. Floyd SR, Pacold ME, Huang Q, Clarke SM, Lam FC, Cannell IG, *et al*. The bromodomain protein Brd4 insulates chromatin from DNA damage signalling. *Nature* 2013;498:246–250.
4. Gilham D, Wasiak S, Tsujikawa LM, Halliday C, Norek K, Patel RG, *et al*. Corrigendum to "RVX-208, a BET-inhibitor for treating atherosclerotic cardiovascular disease, raises ApoA-I/HDL and represses pathways that contribute to cardiovascular disease" [Atherosclerosis 247 (2016) 48–57]. *Atherosclerosis* 2016;253:345.
5. Hajmirza A, Emadali A, Gauthier A, Casasnovas O, Gressin R, Callanan MB. BET family protein BRD4: an emerging actor in NFκB signaling in inflammation and cancer. *Biomedicines* 2018;6:16.
6. Duan Q, McMahon S, Anand P, Shah H, Thomas S, Salunga HT, *et al*. BET bromodomain inhibition suppresses innate inflammatory and profibrotic transcriptional networks in heart failure. *Sci Transl Med* 2017;9:eaah5084.
7. Meloche J, Potus F, Vaillancourt M, Bourgeois A, Johnson I, Deschamps L, *et al*. Bromodomain-containing protein 4: the epigenetic origin of pulmonary arterial hypertension. *Circ Res* 2015;117:525–535.
8. Zhang J, Dulak AM, Hattersley MM, Willis BS, Nikkilä J, Wang A, *et al*. BRD4 facilitates replication stress-induced DNA damage response. *Oncogene* 2018;37:3763–3777.
9. Tasdemir N, Banito A, Roe JS, Alonso-Curbelo D, Camiolo M, Tschaharganeh DF, *et al*. BRD4 connects enhancer remodeling to senescence immune surveillance. *Cancer Discov* 2016;6:612–629.
10. Bourgeois A, Lambert C, Habbout K, Ranchoux B, Paquet-Marceau S, Trinh I, *et al*. FOXM1 promotes pulmonary artery smooth muscle cell expansion in pulmonary arterial hypertension. *J Mol Med (Berl)* 2018; 96:223–235.
11. Zhang Z, Ma P, Jing Y, Yan Y, Cai M-C, Zhang M, *et al*. BET bromodomain inhibition as a therapeutic strategy in ovarian cancer by downregulating FoxM1. *Theranostics* 2016;6:219–230.
12. Provencher S, Archer SL, Ramirez FD, Hibbert B, Paulin R, Boucherat O, *et al*. Standards and methodological rigor in pulmonary arterial hypertension preclinical and translational research. *Circ Res* 2018; 122:1021–1032.
13. van Albada ME, Schoemaker RG, Kemna MS, Cromme-Dijkhuis AH, van Veghel R, Berger RM. The role of increased pulmonary blood flow in pulmonary arterial hypertension. *Eur Respir J* 2005;26: 487–493.
14. Borgdorff MAJ, Koop AMC, Bloks VW, Dickinson MG, Steendijk P, Sillje HHW, *et al*. Clinical symptoms of right ventricular failure in experimental chronic pressure load are associated with progressive diastolic dysfunction. *J Mol Cell Cardiol* 2015;79:244–253.
15. Borgdorff MA, Bartelds B, Dickinson MG, Steendijk P, de Vroomen M, Berger RM. Distinct loading conditions reveal various patterns of right ventricular adaptation. *Am J Physiol Heart Circ Physiol* 2013; 305:H354–H364.
16. Strebhardt K, Ullrich A. Targeting polo-like kinase 1 for cancer therapy. *Nat Rev Cancer* 2006;6:321–330.
17. Levy PT, Patel MD, Groh G, Choudhry S, Murphy J, Holland MR, *et al*. Pulmonary artery acceleration time provides a reliable estimate of invasive pulmonary hemodynamics in children. *J Am Soc Echocardiogr* 2016;29:1056–1065.
18. Dickinson MG, Kowalski PS, Bartelds B, Borgdorff MAJ, van der Feen D, Sietsma H, *et al*. A critical role for Egr-1 during vascular remodelling in pulmonary arterial hypertension. *Cardiovasc Res* 2014;103:573–584.
19. Kuo P-L, Lee H, Bray M-A, Geisse NA, Huang Y-T, Adams WJ, *et al*. Myocyte shape regulates lateral registry of sarcomeres and contractility. *Am J Pathol* 2012;181:2030–2037.
20. Reddy S, Bernstein D. Molecular mechanisms of right ventricular failure. *Circulation* 2015;132:1734–1742.
21. Dai Z, Zhu MM, Peng Y, Jin H, Machireddy N, Qian Z, *et al*. Endothelial and smooth muscle cell interaction via FoxM1 signaling mediates vascular remodeling and pulmonary hypertension. *Am J Respir Crit Care Med* 2018;198:788–802.
22. Dai J, Zhou Q, Tang H, Chen T, Li J, Raychaudhuri P, *et al*. Smooth muscle cell-specific FoxM1 controls hypoxia-induced pulmonary hypertension. *Cell Signal* 2018;51:119–129.
23. Zanconato F, Battilana G, Forcato M, Filippi L, Azzolin L, Manfrin A, *et al*. Transcriptional addiction in cancer cells is mediated by YAP/TAZ through BRD4. *Nat Med* 2018;24:1599–1610.
24. Das S, Senapati P, Chen Z, Reddy MA, Ganguly R, Lanting L, *et al*. Regulation of angiotensin II actions by enhancers and super-enhancers in vascular smooth muscle cells. *Nat Commun* 2017;8: 1467.
25. Belkina AC, Denis GV. BET domain co-regulators in obesity, inflammation and cancer. *Nat Rev Cancer* 2012;12:465–477.
26. Ranchoux B, Meloche J, Paulin R, Boucherat O, Provencher S, Bonnet S. DNA damage and pulmonary hypertension. *Int J Mol Sci* 2016;17: E990.
27. van der Feen DE, Bartelds B, de Boer RA, Berger RMF. Pulmonary arterial hypertension in congenital heart disease: translational opportunities to study the reversibility of pulmonary vascular disease. *Eur Heart J* 2017;38:2034–2041.
28. Zona S, Bella L, Burton MJ, Nestal de Moraes G, Lam EW-F. FOXM1: an emerging master regulator of DNA damage response and genotoxic agent resistance. *Biochim Biophys Acta* 2014;1839: 1316–1322.
29. Combes G, Alharbi I, Braga LG, Elowe S. Playing polo during mitosis: PLK1 takes the lead. *Oncogene* 2017;36:4819–4827.
30. Jahagirdar R, Zhang H, Azhar S, Tobin J, Attwell S, Yu R, *et al*. A novel BET bromodomain inhibitor, RVX-208, shows reduction of atherosclerosis in hyperlipidemic ApoE deficient mice. *Atherosclerosis* 2014;236:91–100.
31. Meloche J, Lampron MC, Nadeau V, Maltais M, Potus F, Lambert C, *et al*. Implication of inflammation and epigenetic readers in coronary artery remodeling in patients with pulmonary arterial hypertension. *Arterioscler Thromb Vasc Biol* 2017;37:1513–1523.
32. Hurst LA, Dunmore BJ, Long L, Crosby A, Al-Lamki R, Deighton J, *et al*. TNFα drives pulmonary arterial hypertension by suppressing the BMP type-II receptor and altering NOTCH signalling. *Nat Commun* 2017;8:14079.