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ORIGINAL RESEARCH

# Characterization of *Kcnk3*-Mutated Rat, a Novel Model of Pulmonary Hypertension

Mélanie Lambert, Véronique Capuano, Angèle Boet, Laurent Tesson, Thomas Bertero, Morad K. Nakhleh, Séverine Remy, Ignacio Aneón, Christine Pechoux, Aurélie Hautefort, Catherine Rucker-Martin, Boris Manoury, Valérie Domergue, Olaf Mercier, Barbara Girerd, David Montani, Frédéric Perros, Marc Humbert, Fabrice Antigny

**RATIONALE:** Pulmonary arterial hypertension is a severe lethal cardiopulmonary disease. Loss of function mutations in *KCNK3* (potassium channel subfamily K member 3) gene, which encodes an outward rectifier K<sup>+</sup> channel, have been identified in pulmonary arterial hypertension patients.

**OBJECTIVE:** We have demonstrated that *KCNK3* dysfunction is common to heritable and nonheritable pulmonary arterial hypertension and to experimental pulmonary hypertension (PH). Finally, *KCNK3* is not functional in mouse pulmonary vasculature.

**METHODS AND RESULTS:** Using CRISPR/Cas9 technology, we generated a 94 bp out of frame deletion in exon 1 of *Kcnk3* gene and characterized these rats at the electrophysiological, echocardiographic, hemodynamic, morphological, cellular, and molecular levels to decipher the cellular mechanisms associated with loss of *KCNK3*. Using patch-clamp technique, we validated our transgenic strategy by demonstrating the absence of *KCNK3* current in freshly isolated pulmonary arterial smooth muscle cells from *Kcnk3*-mutated rats. At 4 months of age, echocardiographic parameters revealed shortening of the pulmonary artery acceleration time associated with elevation of the right ventricular systolic pressure. *Kcnk3*-mutated rats developed more severe PH than wild-type rats after monocrotaline exposure or chronic hypoxia exposure. *Kcnk3*-mutation induced a lung distal neomuscularization and perivascular extracellular matrix activation. Lungs of *Kcnk3*-mutated rats were characterized by overactivation of ERK1/2 (extracellular signal-regulated kinase 1-/2), AKT (protein kinase B), SRC, and overexpression of HIF1- $\alpha$  (hypoxia-inducible factor-1  $\alpha$ ), survivin, and VWF (Von Willebrand factor). Linked with plasma membrane depolarization, reduced endothelial-NOS expression and desensitization of endothelial-derived hyperpolarizing factor, *Kcnk3*-mutated rats presented predisposition to vasoconstriction of pulmonary arteries and a severe loss of sildenafil-induced pulmonary arteries relaxation. Moreover, we showed strong alteration of right ventricular cardiomyocyte excitability. Finally, *Kcnk3*-mutated rats developed age-dependent PH associated with low serum-albumin concentration.

**CONCLUSIONS:** We established the first *Kcnk3*-mutated rat model of PH. Our results confirm that *KCNK3* loss of function is a key event in pulmonary arterial hypertension pathogenesis. This model presents new opportunities for understanding the initiating mechanisms of PH and testing biologically relevant therapeutic molecules in the context of PH.

**VISUAL OVERVIEW:** An online [visual overview](#) is available for this article.

**Key Words:** echocardiography ■ exon ■ extracellular matrix ■ hypoxia ■ rats

**Editorial, see p 696 | In This Issue, see p 659 | Meet the First Author, see p 660**

**P**ulmonary arterial hypertension (PAH) is an uncommon, progressive, and severe disease with an estimated prevalence of 15 to 50 per million.<sup>1</sup> PAH has been hemodynamically defined by an elevation of the mean pulmonary artery pressure >20 mmHg and

pulmonary vascular resistance >3 Wood units at rest.<sup>2</sup> PAH results from increased pulmonary vascular resistance because of remodeling of the small distal pulmonary arteries (PAs) and arterioles (diameter <500  $\mu$ m), causing adaptive right ventricular (RV) hypertrophy and

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## Novelty and Significance

### What Is Known?

- Loss of function mutations in the *KCNK3* (potassium channel subfamily K member 3) gene have been described in pulmonary arterial hypertension (PAH) patients and those who are carriers of *KCNK3* mutations are younger as compared with idiopathic PAH patients.
- Reduced *KCNK3* expression/function is common to heritable and nonheritable PAH and to experimental pulmonary hypertension.
- *KCNK3* is not functional in mouse pulmonary arterial smooth muscle cell limiting previous mechanistic exploration.

### What New Information Does This Article Contribute?

- By CRISPR/Cas9 technology, we generate *Kcnk3*-mutated rats to inactivate the *KCNK3* channel.
- At 4 months old, *Kcnk3*-mutated rats develop spontaneous elevation of right ventricular systolic pressure, are sensitized to induced pulmonary hypertension (monocrotaline or chronic hypoxia), and develop age-dependent pulmonary hypertension at 12 months.
- *Kcnk3* dysfunction leads to distal pulmonary vessels neomuscularization and perivascular collagen deposition associated with overactivation of proliferative pathways (ERK1/2 [extracellular signal-regulated kinase1-/2] and HIF1- $\alpha$  [hypoxia-inducible factor-1  $\alpha$ ]) and endothelial phenotype switch.
- We identify that an increase of HIF1- $\alpha$  and ERK1/2 are the primary molecular events linked to *Kcnk3*-dysfunction.

- Pulmonary arteries from *Kcnk3*-mutated rats are sensitized to constriction and are less responsive to vaso-relaxing stimuli.

Pulmonary arterial hypertension (PAH) is a progressive and severe disease. Since 2013, several loss of function mutations in *KCNK3* gene have been detected in select PAH patients. *KCNK3* encodes an outward-K<sup>+</sup> channel member of the 2-pore K<sup>+</sup> channel family. Patients with the *KCNK3* mutation are younger at diagnosis than idiopathic PAH patients. Loss of function and expression is a hallmark of PAH at pulmonary vascular and right ventricular levels. However, the mechanisms linking *KCNK3* dysfunction with PAH were unknown. Using unique *Kcnk3*-mutated rats and human pulmonary arterial smooth muscle cells we aimed to decipher the cellular mechanisms associated with *KCNK3*-dysfunction. Our results demonstrate that *KCNK3* loss of function is a key event in PAH pathogenesis. Using a combination of hemodynamic measurements, molecular biology, histological analyses, and electrophysiology approaches, we show that *KCNK3*-dysfunction leads to plasma membrane depolarization, pulmonary arterial smooth muscle cells overproliferation, ERK1/2 and HIF1- $\alpha$  over-activation, mitochondrial depolarization, pulmonary artery vasoconstriction, pulmonary vessels remodeling, endothelial cell phenotype switch, and reduction of RV cardiomyocytes excitability. These results highlight that *KCNK3* loss of function is consistent with human PAH pathobiology and demonstrate that *KCNK3*-dysfunction is a key events in PAH. This model presents new opportunities for understanding the initiating mechanisms of pulmonary hypertension.

### Nonstandard Abbreviations and Acronyms

<b><math>\alpha</math>-SMA</b>	$\alpha$ -smooth muscle actin
<b>CH</b>	chronic hypoxia
<b>ECM</b>	extracellular matrix
<b>EDHF</b>	endothelium-derived hyperpolarizing factor
<b>HIF1-<math>\alpha</math></b>	hypoxia-inducible factor-1 $\alpha$
<b>MCT</b>	monocrotaline
<b>NOS</b>	nitric oxide synthase
<b>PA</b>	pulmonary artery
<b>PAH</b>	pulmonary arterial hypertension
<b>PASMC</b>	pulmonary arterial smooth muscle cell
<b>PH</b>	pulmonary hypertension
<b>RVSP</b>	right ventricular systolic pressure
<b>TPR</b>	total pulmonary resistance
<b>VWF</b>	Von Willebrand factor
<b>WT</b>	wild type

when unchecked, right heart failure.<sup>3</sup> In 2013, whole-exome sequencing revealed 6 types of heterozygous mutations in *KCNK3* (potassium channel subfamily K member 3), representing the first channelopathy identified in PAH.<sup>4</sup> In 2016, Navas et al<sup>5</sup> identified 2 additional *KCNK3* mutations in PAH patients and described the first PAH patient with a homozygous *KCNK3* mutation associated with an aggressive form of PAH and early disease development.<sup>4</sup> Finally, in 2017, 2 new mutations were identified in an Asian and an American cohort of PAH patients.<sup>6,7</sup> *KCNK3* mutations carriers' patients are younger at diagnosis than idiopathic PAH patients and have a higher mean pulmonary artery pressure.<sup>8</sup>

*KCNK3* encodes an outward-K<sup>+</sup> channel member of the 2 pore K<sup>+</sup> channel family, characterized by 4 transmembrane domains and 2 pore domains per subunit.<sup>4</sup> *KCNK3* channel, also known as TASK-1 (Twik-related-acid-sensitive-K<sup>+</sup> channel) or K2P3.1<sup>9</sup> participates in regulating resting membrane potential in several cell types including human pulmonary arterial smooth muscle cells

(PASMCs).<sup>8</sup> Electrophysiological recording demonstrated a loss of function for all *KCNK3* mutations identified.<sup>4</sup> We recently determined that *KCNK3* dysfunction contributes to the development of heritable PAH (*BMPR2* mutated patients) and idiopathic PAH, indicating that *KCNK3* dysfunction is a hallmark of PAH at pulmonary vascular and RV levels.<sup>10,11</sup> However, the mechanisms linking *KCNK3* dysfunction with PAH are still unknown. Contrary to rats where *KCNK3* is functionally expressed in pulmonary vasculature,<sup>10</sup> mice are not a suitable models to study the role of *KCNK3* in pulmonary hypertension (PH) as *KCNK3* does not form a functional channel in mouse PSMCs<sup>12</sup> and is replaced by *KCNK6* channel.<sup>13</sup> Therefore, we developed the first *Kcnk3*-mutated rat line to decipher the role of *KCNK3* dysfunction in the context of PAH pathobiology, representing an interesting model to study the time-dependent cellular and molecular mechanisms involved in PH development.

Using a combination of hemodynamic measurements, molecular biology, histological analyses, and electrophysiology approaches, we characterized the first *Kcnk3*-mutated rat model, providing tools for developing specific therapeutic targets for PH.

## METHODS

The authors declare that all supporting data are available within the article and its [Online Data Supplement](#).

Because of space limitations, a detailed description of the Materials and Methods is presented in the [Online Data Supplement](#).

## RESULTS

### KCNK3 Current Is Absent in Freshly isolated PSMCs From *Kcnk3*-Mutated Rats

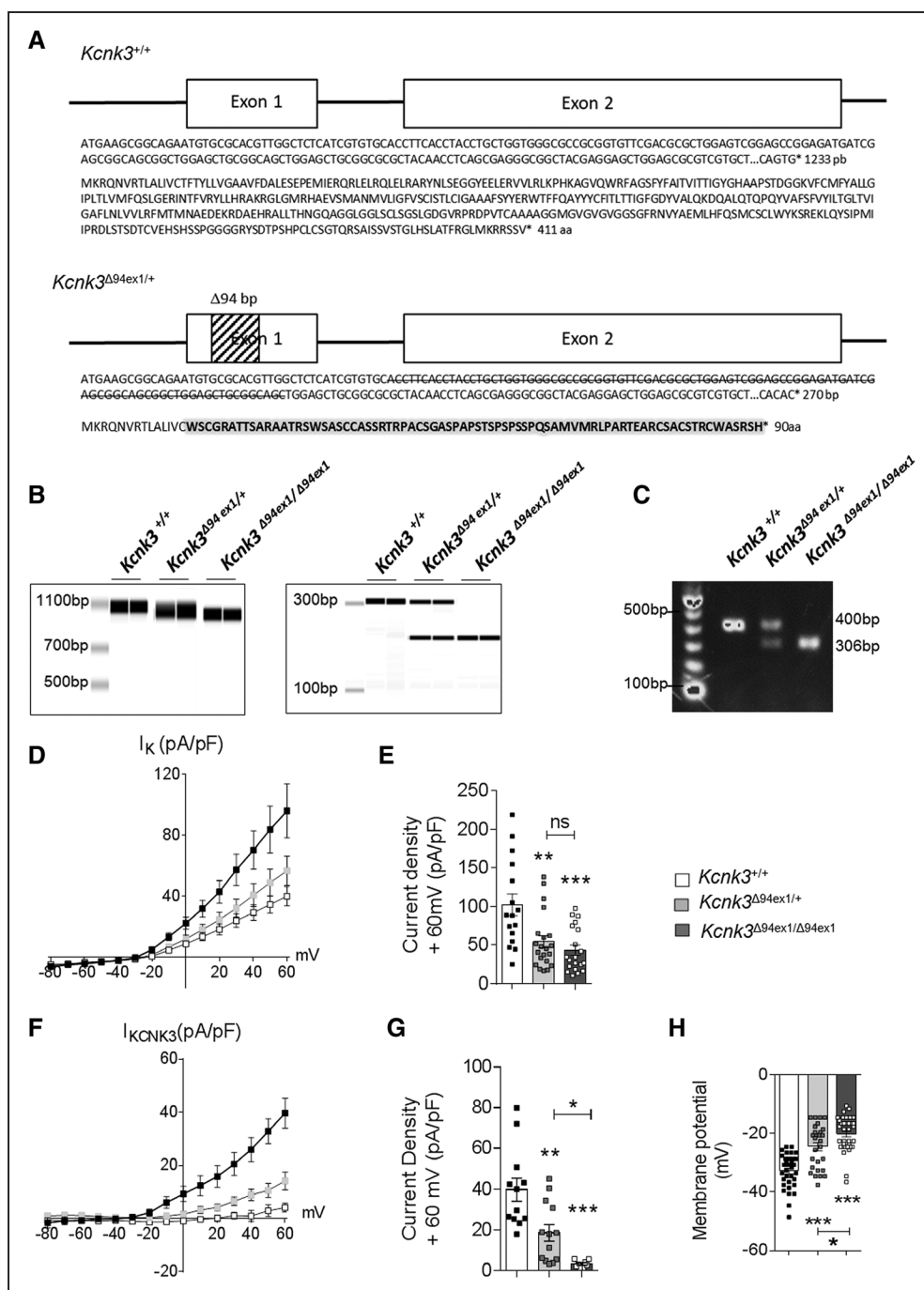
*Kcnk3*-mutated Sprague-Dawley rats were generated using CRISPR-Cas9 system with a specific sgRNA-r*KCNK3* and Cas9 mRNA,<sup>14</sup> targeting the first exon of *Kcnk3* gene. In one of the newborn rats, a deletion of 94 bp ( $\Delta 94\text{ex1}$ ) was found, which originated an out of frame shift in the open reading frame that leads to a premature stop codon and the generation of a completely different aa (amino acid) sequence (Figure 1A). Although premature stop codon may generate a degradation of the mRNA,<sup>15</sup> we showed the presence of a mutated mRNA deleted of 94 bp but not its absence (Figure 1B) suggesting an absence of nonsense mRNA degradation pathway as already mentioned elsewhere.<sup>15</sup> Nevertheless, a putative translation of the truncated mRNA could produce a truncated 90-aa protein instead of the 411 aa of the wild-type (WT) protein (Figure 1A) and share only the first 14 aa in common with the WT protein. The sequencing of the *Kcnk3* mRNA from *Kcnk3*<sup>+/+</sup> and *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rats confirmed the deletion of the 94

bp and an aberrant potential protein sequence with 8 potential premature stop codons (Online Figure I).

The founder animal with the  $\Delta 94\text{ex1}$  mutation was crossed with a WT partner, and the mutation was transmitted to the offspring as shown by genotype DNA analysis, demonstrating that the rats were either *Kcnk3*<sup>+/+</sup>, heterozygous *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup> , or homozygous *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  (Figure 1C). Unfortunately, there is no available specific antibodies for *KCNK3*,<sup>16,17</sup> thus avoiding any measurement of the protein expression of *KCNK3* in *Kcnk3*-mutated rats. We showed that all commercially anti-*KCNK3* antibodies tested were unusable because when tested in *Kcnk3*-knockout mice, they were either nonreactive or gave the same signal as in the WT mice (Online Figure II and IIb). The same unspecific staining occurred in *Kcnk3*-mutated rats. Thus, we could not assert the absence of *KCNK3* protein in *Kcnk3*-mutated rats. Nevertheless, whole cell patch-clamp measurement on freshly isolated PSMCs from WT and *Kcnk3*-mutated rats clearly showed a defect in *KCNK3* current in mutated rats. Indeed, the global-K<sup>+</sup> current was significantly reduced: 50% in *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup>  rats and 60% in *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rats compared with the WT (Figure 1D and 1E). We then specifically measured the *KCNK3* current ( $I_{\text{KCNK3}}$ ) after applying a specific *KCNK3* channel inhibitor (A293 at 200 nmol/L)<sup>10</sup> and defined it as the A293-sensitive K<sup>+</sup>-current.  $I_{\text{KCNK3}}$  was strongly decreased (60%) in freshly isolated PSMCs from *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup>  rats and was abolished (95%) in *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rats (Figure 1F and 1G). Consistent with the role of *KCNK3* in resting membrane potential, PSMCs isolated from *Kcnk3*-mutated rats were significantly depolarized compared with PSMCs from WT rats (Figure 1H). We performed RT-qPCR (quantitative reverse transcription polymerase chain reaction) experiments to evaluate the consequences of *Kcnk3*-dysfunction on other lung K<sup>+</sup> channels (Online Figure III). No expression of *KCNK4*, *KCNK9*,  $K_v1.4$ , or  $K_{Ca}4$  channels was detected. The mRNA expression levels of *KCNK2*, *KCNK6*,  $K_v1.5$ ,  $K_v2.1$ ,  $K_v9.3$ ,  $K_{Ca}1.1$ ,  $K_{Ca}2.1$ ,  $K_{Ca}2.2$ ,  $K_{Ca}2.3$ , and  $K_{Ca}4.2$  was unchanged in *Kcnk3*-mutated rats compared with WT. However, we observed a significant increase in the mRNA level of  $K_v1.2$  and  $K_{Ca}3.1$  in *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rat lungs. Yet, the A293-insensitive K<sup>+</sup>-current was similar in PSMCs isolated from WT and *Kcnk3*-mutated rats (not shown), suggesting that *Kcnk3*-dysfunction is not compensated by other K<sup>+</sup> currents.

### *Kcnk3*-Dysfunction Leads to an Increase in Right Ventricular Systolic Pressure in Males and Female

To determine whether *Kcnk3*-dysfunction leads to spontaneous PH, we performed closed chest right-heart catheterization at 4 months of age. We observed a slight but significant increase in right ventricular systolic pressure (RVSP) in *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rats compared with WT



**Figure 1. KCNK3 (potassium channel subfamily K member 3) is no longer functional in pulmonary arterial smooth muscle cells (PASCs) from *Kcnk3*-mutated rats.**

**A**, Schematic overview of the *Kcnk3* gene with wild-type (WT) and *Kcnk3*-mutant alleles. We generated *Kcnk3*-mutated rats with 94 bp deletion in exon 1 of the *Kcnk3* ( $\Delta 94\text{ex1}$ ). Below, *Kcnk3* DNA and  $\Delta 94$  bp DNA (strikethrough letter) and protein sequences in WT and  $\Delta 94\text{ex1}$  rats. Gray: aberrant/potential protein sequence. \*STOP codon. **B**, mRNA levels of *Kcnk3* analyzed by RT-PCR (reverse transcription polymerase chain reaction) in *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup>  rats, and *Kcnk3*<sup>+/+</sup> rats, using 2 oligo combinations r*Kcnk3*-17F/r*Kcnk3*-1146R (left; WT: 1148 bp/ $\Delta 94\text{ex1}$ : 1054 bp) and r*Kcnk3*-17F/r*Kcnk3*-303R (right; WT: 304 bp/ $\Delta 94\text{ex1}$ : 210 bp). **C**, Validation of the deletion by PCR, showing genomic DNA amplicons from rats carrying the *Kcnk3*<sup>+/+</sup> allele (400 bp) and  $\Delta 94\text{ex1}$  deletion allele (306 bp). **D**, Current-voltage relationship of global  $\text{K}^+$ -currents, in freshly isolated PASCs from *Kcnk3*<sup>+/+</sup>, *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup>  and *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup>  rats, corresponding to the depolarizing step protocol (+60 mV to -100 mV, holding potential -40 mV). **E**, The current was normalized by the capacitance of the cell and is represented in pA/pF (pico ampere/pico farad; WT=16 cells, 4 rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup> =21 cells, 4 rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup> =20 cells, 4 rats). After recording, the global  $\text{K}^+$ -current, KCNK3 current was isolated by applying a specific inhibitor (200 nmol/L, A293).  $I_{\text{KCNK3}}$  (A293 sensitive- $\text{K}^+$ -current) was then measured and presented in **(F)**, WT=12 cells, 4 rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup> =10 cells, 4 rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup> =13 cells, 4 rats). **G**, Current density of  $I_{\text{KCNK3}}$  measured at +60 mV. **H**, Using a current clamp, we measured the resting membrane potential of freshly isolated PASCs (WT=34 cells, 4 rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/+$</sup> =28 cells, 4 rats, *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup> =35 cells, 4 rats). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs *Kcnk3*<sup>+/+</sup>. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test for multiple comparisons. ns indicates nonsignificant.



in males (Figure 2A) and in females (Online Figure IVA). However, increased RVSP was not accompanied by RV hypertrophy (Fulton index) nor by a change in cardiac output or mean carotid artery pressures in males (Figure 2A) and in females (Online Figure IVA). Total pulmonary resistances (TPR) were significantly increased in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats compared with WT males (Figure 2A) and females (Online Figure IVA).

Using echocardiography, we examined the consequences of *Kcnk3*-dysfunction at 4 months old (males) on cardiac function. Pulmonary artery acceleration time was significantly reduced in *Kcnk3*-mutated rats (Figure 2B), indicating a higher resistive PA profile. In addition, heart rate was significantly increased in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats. We did not observe modification in the other parameters measured in *Kcnk3*-mutated rats (Online Table IV).

In mice, *Kcnk3* deletion causes primary hyperaldosteronism.<sup>18</sup> Conversely, in 4-month-old male *Kcnk3*<sup>A94ex1/Δ94ex1</sup>, serum aldosterone concentration significantly decreased, whereas no variations were observed in female (Online Figure V).

### **Kcnk3-Mutated Rats Were More Susceptible to Monocrotaline (MCT)-or Chronic Hypoxia (CH)-Induced PH**

As shown in Figure 2A, 4-month-old *Kcnk3*-mutated rats displayed a modest but significant increase in RVSP. Therefore, we exposed 4-month-old male and female rats to MCT (60 mg/kg). *Kcnk3*-dysfunction increased the severity of MCT-induced PH as demonstrated by higher RVSP in both males and females *Kcnk3*-mutated group, associated with higher TPR (Figure 2C; Online Figure IVB) with no modification of mean carotid artery pressure (not shown). These results demonstrate that *Kcnk3*-dysfunction sensitizes rats to develop PH under MCT-exposure.

In addition, male rats were exposed to CH (10% O<sub>2</sub> for 3 weeks). As shown in Figure 2D, *Kcnk3*-dysfunction increased the severity of CH-induced PH, as demonstrated by higher RVSP and TPR values in *Kcnk3*-mutated rats, compared with WT rats.

### **Kcnk3-Dysfunction Leads to Distal Pulmonary Vessel Neomuscularization and Increases Collagen Crosslinking**

We analyzed muscularization of small pulmonary vessels (<30 μm) by immunostaining against α-SMA (α-smooth muscle actin, smooth-muscle marker) and VWF (Von Willebrand factor, endothelium marker; Figure 3A). *Kcnk3*-mutated rats presented a significantly decreased percentage of nonmuscularized vessels along with significantly increased numbers of muscularized vessels (Figure 3B). Moreover, the level of fibrillar collagen

assembly was abnormally increased in pulmonary vessels from *Kcnk3*-mutated rats (Figure 3C).

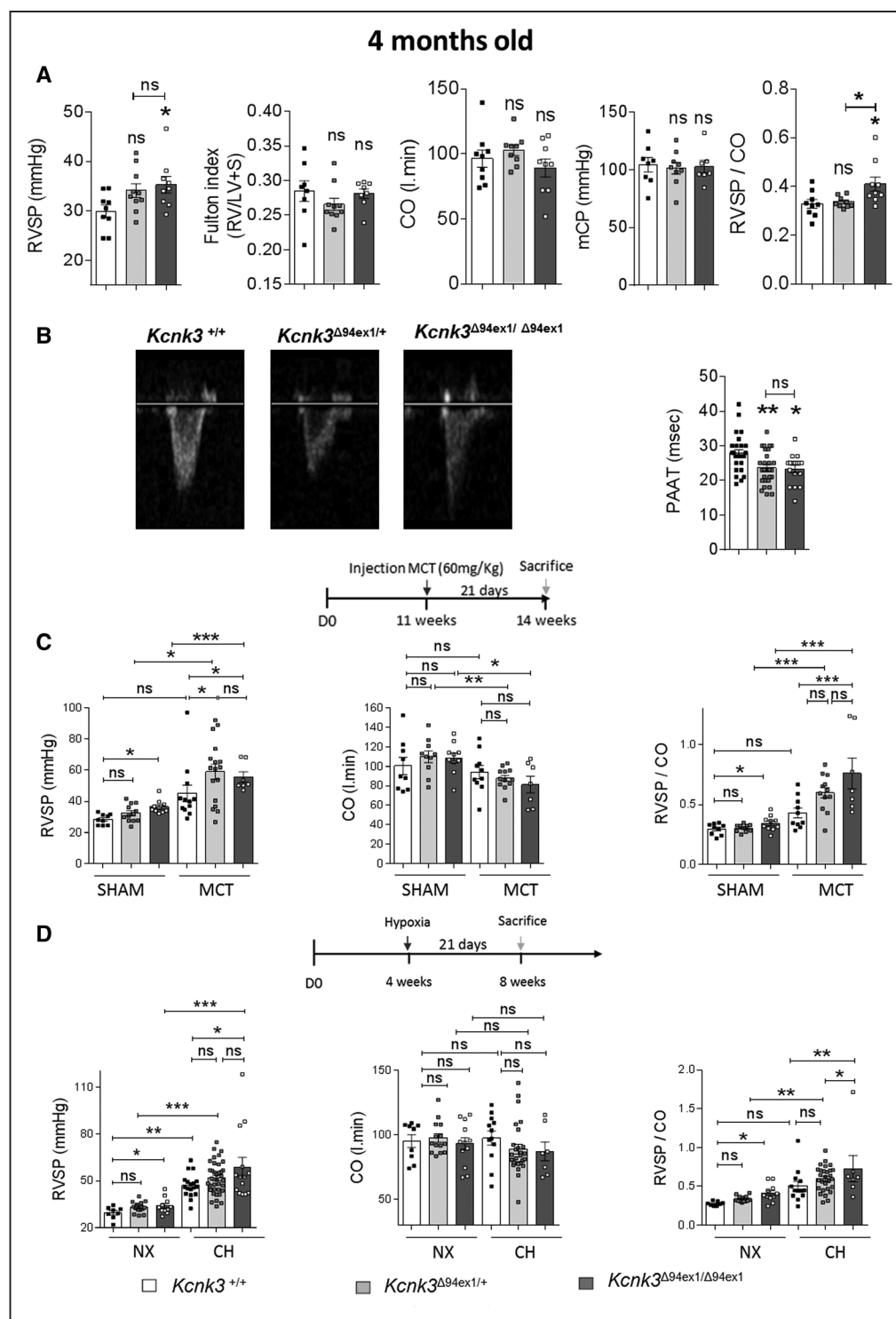
The increase in RVSP in *Kcnk3*-mutated rats could also be explained by the modification of pulmonary microvessel density. By counting the number of CD31<sup>+</sup> (cluster of differentiation-31) pulmonary vessels, we did not detect modification of microvessel density in *Kcnk3*-mutated rats compared with WT (Figure 3D). However, Western blot analysis showed significant decreases of CD31 and significant increases of VWF expression in lungs from *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Figure 3E). Both molecules are critical to maintaining endothelial integrity, suggesting that *Kcnk3*-dysfunction may result in an alteration of the endothelial functions.

### **Proliferation Pathways Were Activated in Kcnk3-Mutated Rat Lungs**

We analyzed the expression and activation of some proliferative or antiapoptotic proteins known to contribute to PAH pathobiology. Compared with WT, the phosphorylation state of ERK 1/2, and SRC significantly increased in lung from *Kcnk3*-mutated rats, without modification of P38 (Figure 4A). We found that HIF1-α (hypoxia-inducible factor-1 α) expression was also significantly increased in lungs from *Kcnk3*-mutated rats (Figure 4A), without alteration of *HIF1-α* mRNA expression (not shown). We also measured an increase in the survivin protein in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Figure 4A). AKT (protein kinase B) phosphorylation at Thr308 significantly increased in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Figure 4A). ERK1/2 and AKT are targets for serine-threonine PP2A (protein phosphatase 2A). As presented in Online Figure VIA, the expression of PP2A-A, PP2A-B, and PP2A-C proteins was unchanged. However, we showed that PP2A phosphatase activity was strongly reduced (50%) in PASMCs from *Kcnk3*<sup>A94ex1/Δ94ex1</sup> compared with PASMCs from WT rats, while PP2B (or calcineurin) activity was unchanged (Figure 4B).

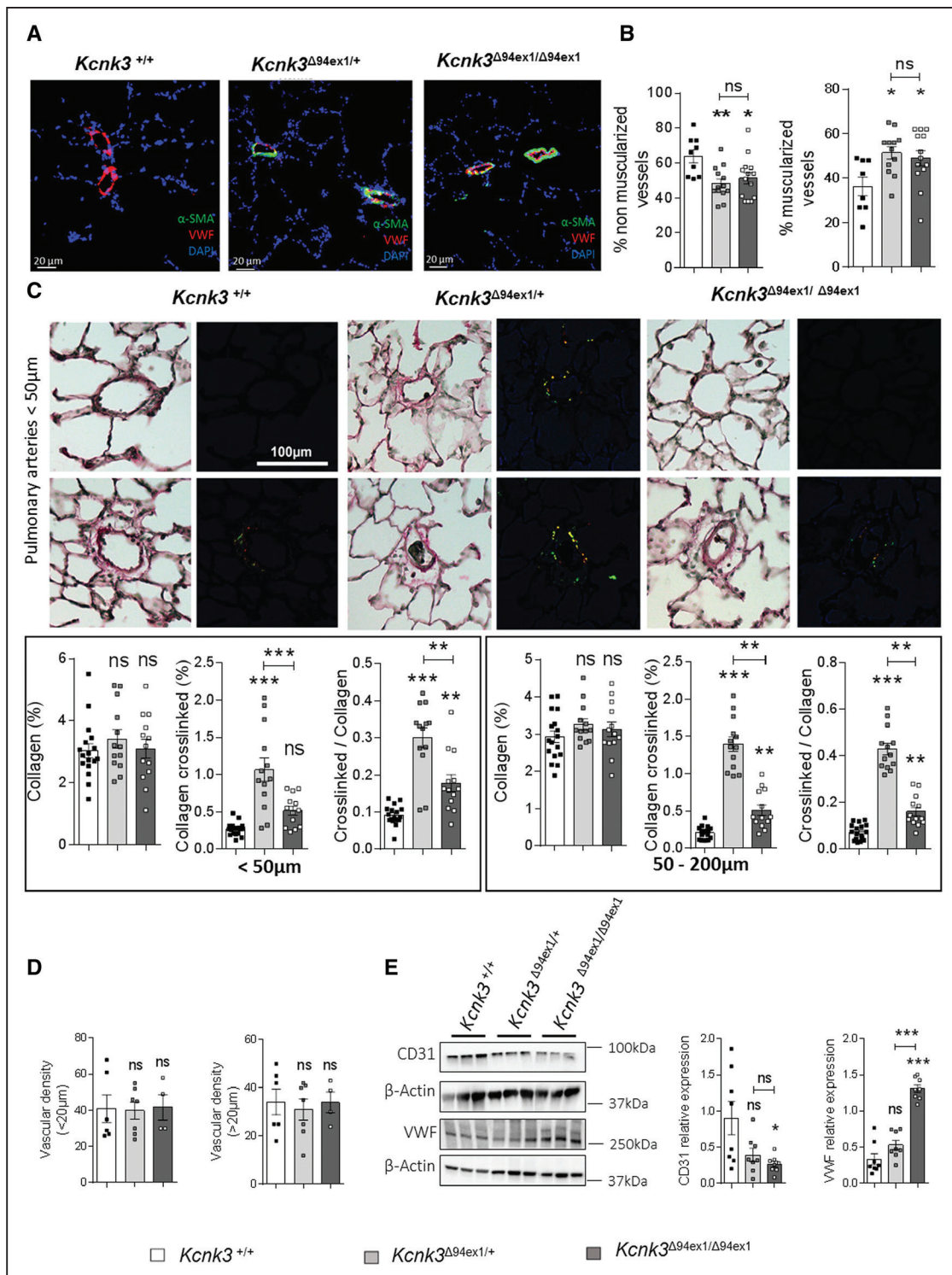
We also measured several other molecules known to be involved in PAH pathogenesis. We observed an increase in the expression of the endothelial-to-mesenchymal (endoMT) transcription factor TWIST1 (twist family bHLH transcription factor 1) and a decrease in the phosphorylation of CREB (cAMP response element-binding protein) in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Figure 4C), which are altered in PAH patients.<sup>19,20</sup> We measured an increase in the mRNA of *Nfatc1*, *Notch3*, *Mmp2*, and *Stat3* in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Online Figure VIB). No variation for BMPRII, phospho-SMAD1/5/8, phospho-SMAD2/3, and Caveolin-1 protein expression was observed in lungs from *Kcnk3*-mutated rats (Online Figure VII).

The mRNA expression of *IL-6*, IL-6 receptor (*GP130*), *IL-17Ra* and *Timp1* was unchanged in the lung from *Kcnk3*-mutated rats at 4 months old (Online Figure VIIIA).



**Figure 2. *Kcnk3* (potassium channel subfamily K member 3) dysfunction leads to a significant increase in right ventricular systolic pressure (RVSP) in males at 4 months of age.**

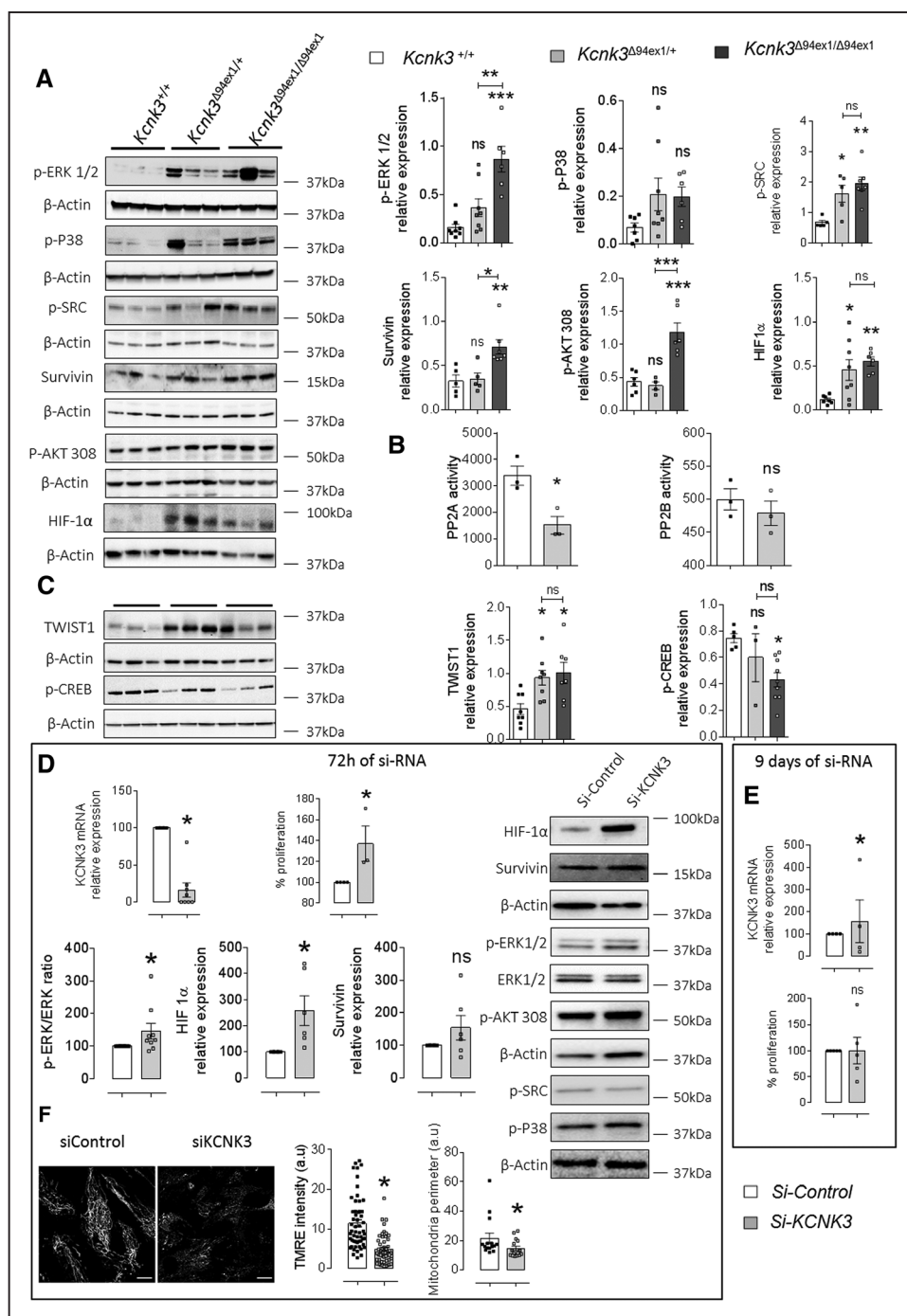
**A**, Quantification of right ventricular systolic pressure (RVSP), Fulton index (weight of right ventricle [RV]/left ventricle [LV]+septum [S]), cardiac output (CO), mean carotid pressure (mCP), and total pulmonary resistance (TPR evaluated by RVSP/CO; wild type [WT]=8 *Kcnk3*<sup>Δ94ex1/+</sup>=9, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=9 rats). **B**, Representative pulmonary artery (PA) flow through the pulmonary valve in *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats and quantification of the PA acceleration time (PAAT; WT=26, *Kcnk3*<sup>Δ94ex1/+</sup>=29, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=17 rats). **C**, At 4-month old, male *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats were exposed to 60 mg/kg of monocrotaline (MCT) or saline (SHAM). Three weeks later, we measured RVSP, CO, and TPR (SHAM; WT=9, *Kcnk3*<sup>Δ94ex1/+</sup>=12, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=10, MCT; WT=12, *Kcnk3*<sup>Δ94ex1/+</sup>=17, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=7 rats). **D**, Four-month-old male *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats were exposed 3 weeks to chronic hypoxia (CH, 10% O<sub>2</sub>) or normoxia (NX). Three weeks later, we measured RVSP, CO, and TPR (NX; WT=8, *Kcnk3*<sup>Δ94ex1/+</sup>=16, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=11, CH; WT=18, *Kcnk3*<sup>Δ94ex1/+</sup>=36, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=14 rats). \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test and experiments with 2 different categorical independent variables were analyzed with 2-way ANOVA completed by Tukey post hoc test for multiple comparisons. ns indicates nonsignificant.



**Figure 3. *Kcnk3* (potassium channel subfamily K member 3)-dysfunction leads to distal pulmonary vessel neomuscularization and increased collagen crosslinking.**

**A**, Immunolabeling and confocal imaging of  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin) in green, VWF (Von Willebrand factor) in red, and DAPI in blue on rat lung sections. Scale bar: 20  $\mu$ m. **B**, Percentage of nonmuscularized vessels and muscularized vessels (100 vessels counted per rat, wild type [WT]=9, *Kcnk3*<sup>Δ94ex1/+</sup>=13, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=14 rats). **C**, Upper, Picrosirius Red staining and below quantification of total collagen signal, collagen crosslinked, and crosslinked/collagen total ratio in small (<50  $\mu$ m) and medium (50–200  $\mu$ m) pulmonary artery from rats at 4 months old (WT=17, *Kcnk3*<sup>Δ94ex1/+</sup>=13, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=13 rats). Scale bar: 100  $\mu$ m. **D**, Pulmonary vessel density quantification based on CD31 (endothelium marker) labeling in paraffin-embedded lung sections, expressed as the percentage of small (<20  $\mu$ m) or large (>20  $\mu$ m) CD31<sup>+</sup> vessels measured in 20 fields of *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats at 4 months old (WT=6, *Kcnk3*<sup>Δ94ex1/+</sup>=7, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=4 rats). **E**, Representative Western blots and quantification of CD31 and VWF expression in lungs from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats (WT=8, *Kcnk3*<sup>Δ94ex1/+</sup>=8, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=8 rats).  $\beta$ -actin was used as the loading control. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs *Kcnk3*<sup>+/+</sup>. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test for multiple comparisons. ns indicates nonsignificant.





**Figure 4.** In vivo and in vitro *Kcnk3* (potassium channel subfamily K member 3)-dysfunction leads to activation of several proliferative and antiapoptotic pathways.

**A**, Representative Western blots and quantification of p-ERK1/2, p-P38, p-SRC, Survivin, HIF1- $\alpha$  (hypoxia-inducible factor-1  $\alpha$ ), and p-308 AKT (protein kinase B) expression in lungs from wild-type (WT) and *Kcnk3*-mutated rats (WT=7, *Kcnk3* $\Delta 94\text{ex}1/+$ =8, *Kcnk3* $\Delta 94\text{ex}1/\Delta 94\text{ex}1$ =6 rats). **B**, Quantification of PP2A and PP2B activity in pulmonary arterial smooth muscle cells (PASCs) from WT and *Kcnk3* $\Delta 94\text{ex}1/\Delta 94\text{ex}1$  (WT=3, *Kcnk3* $\Delta 94\text{ex}1/\Delta 94\text{ex}1$ =3 rats). **C**, Representative Western blots and quantification of TWIST1 (twist family bHLH transcription factor 1) and p-CREB expression in lungs from *Kcnk3* $^{+/+}$  and *Kcnk3*-mutated rats (WT=8, *Kcnk3* $\Delta 94\text{ex}1/+$ =8, *Kcnk3* $\Delta 94\text{ex}1/\Delta 94\text{ex}1$ =7 rats). **D**, Upper: left, *KCNK3* mRNA level in human control PASCs transfected 72 h with a si-RNA control (si-Control) or against *KCNK3* (si-KCNK3; n=8 patients), normalized by 18S mRNA expression. Right, percentage of proliferation of human PASCs (hPASCs) transfected with si-KCNK3 compared with Si-Control (n=6 patients). **D**, Below: representative Western blots and quantifications of HIF1 $\alpha$ , Survivin, p-ERK 1/2, ERK 1/2, p-AKT308, p-SRC, and p-P38 in hPASCs, 72 h after si-RNA transfection. **E**, Upper, *KCNK3* mRNA level in human control PASCs, 9 days after cell transfection with si-RNA (n=4 patients), normalized by 18S mRNA expression. Below, percentage of si-KCNK3 hPASC proliferation compared with si-Control (n=4 patients). **F**, confocal imaging of mitochondrial membrane potential ( $\Delta\psi_m$ ) using TMRE (Tetramethylrhodamine, ethyl ester) on hPASCs treated with si-RNA. Right, quantification of TMRE intensity and mitochondria perimeter. ns: nonsignificant. \* $P<0.05$ , \*\* $P<0.01$  vs *Kcnk3* $^{+/+}$  or si-Control. Experiments were analyzed using 2-tailed unpaired Student test. ERK1/2 indicates extracellular signal-regulated kinase1-2.

Likewise, the lung expression of CD45 (pan-leukocyte marker) was also unchanged (Online Figure VIII B).

### In Vitro KCNK3 Knockdown or Pharmacological KCNK3 Inhibition Increases Human PSMCs Proliferation

To evaluate the role of KCNK3 in human PSMCs (hPSMCs) and human pulmonary arterial endothelial cells proliferation, we abolished KCNK3 function using either si-RNA-mediated or specific pharmacological inhibition. Seventy two hours after transfection, *KCNK3* mRNA decreased in hPSMCs transfected with si-KCNK3 (by 85%). KCNK3 knockdown enhanced hPSMCs proliferation by 40% as measured by BrdU incorporation (Figure 4D). Pharmacological inhibition of KCNK3 by 24-hour exposure to A293 at 1  $\mu$ mol/L enhances the proliferation of control PSMCs from humans and rats (Online Figure IX A).

In human pulmonary arterial endothelial cells exposed to the KCNK3 inhibitor, we observed significantly decreased proliferation rate compared with the vehicle condition (Online Figure IX C). Using iCelligence™, we observed that specific KCNK3 inhibition enhances human pulmonary arterial endothelial cells adhesion without alteration of endothelial barrier opening by TNF $\alpha$  (tumor necrosis factor  $\alpha$ ) stimulation (5 ng/mL; Online Figure IX D and IX E).

Consistent to an enhancement of PSMCs proliferation by the knockdown of KCNK3, we found a significant increase in the expression of HIF1- $\alpha$ , an over-phosphorylation of ERK1/2, and a nonsignificant increase of survivin, without modification of P38, SRC, and AKT phosphorylation (Figure 4D). Nine days after si-RNA transfection, *KCNK3* expression returned to the normal, hPSMCs proliferation was normalized (Figure 4E) as well as HIF1- $\alpha$  expression and ERK1/2 activation (Online Figure IX B), demonstrating that loss of KCNK3 enhances PSMCs proliferation. In addition to HIF1- $\alpha$  and ERK1/2 overactivation in si-KCNK3-treated hPSMCs, we found that mitochondria membrane were depolarized (TMRE fluorescence intensity, tetramethylrhodamine, ethyl ester) and fragmented compared with mitochondria from si-Control hPSMCs (Figure 4F), without modification of the total expression of the regulating mitochondrial fission protein DRP1 (dynamin related protein-1; Online Figure X A). Likewise, we found that the mitochondria of PSMCs from *Kcnk3*<sup>A94ex1/A94ex1</sup> rats were also significantly depolarized and fragmented compared with WT. This strongly suggests that KCNK3 is involved in mitochondria integrity in human and rat PSMC (Online Figure X B and X C). We found that plasma membrane depolarization (induced by 138 mM KCl) strongly depolarized mitochondria membrane without modification of mitochondrial web (Online Figure X D and X E).

### Kcnk3-Dysfunction Predisposes PA to Constriction

We investigated the consequences of *Kcnk3*-dysfunction on PA contractility. Using isolated PA from 4-month-old WT and *Kcnk3*-mutated rats, we first assessed the consequence of *Kcnk3*-dysfunction on basal PA tone, measured as the extracellular Ca<sup>2+</sup>-dependent tone. The basal tone was unaltered in *Kcnk3*-mutated rats (Online Figure XI A). The contractile dose-response to increasing doses of KCl was unchanged in PA from *Kcnk3*<sup>A94ex1/+</sup> compared with PA from WT rats whereas the dose-response curve of PA from *Kcnk3*<sup>A94ex1/A94ex1</sup> rats was significantly shifted to the left as shown by the significant decrease in EC<sub>50</sub> (Figure 5A).

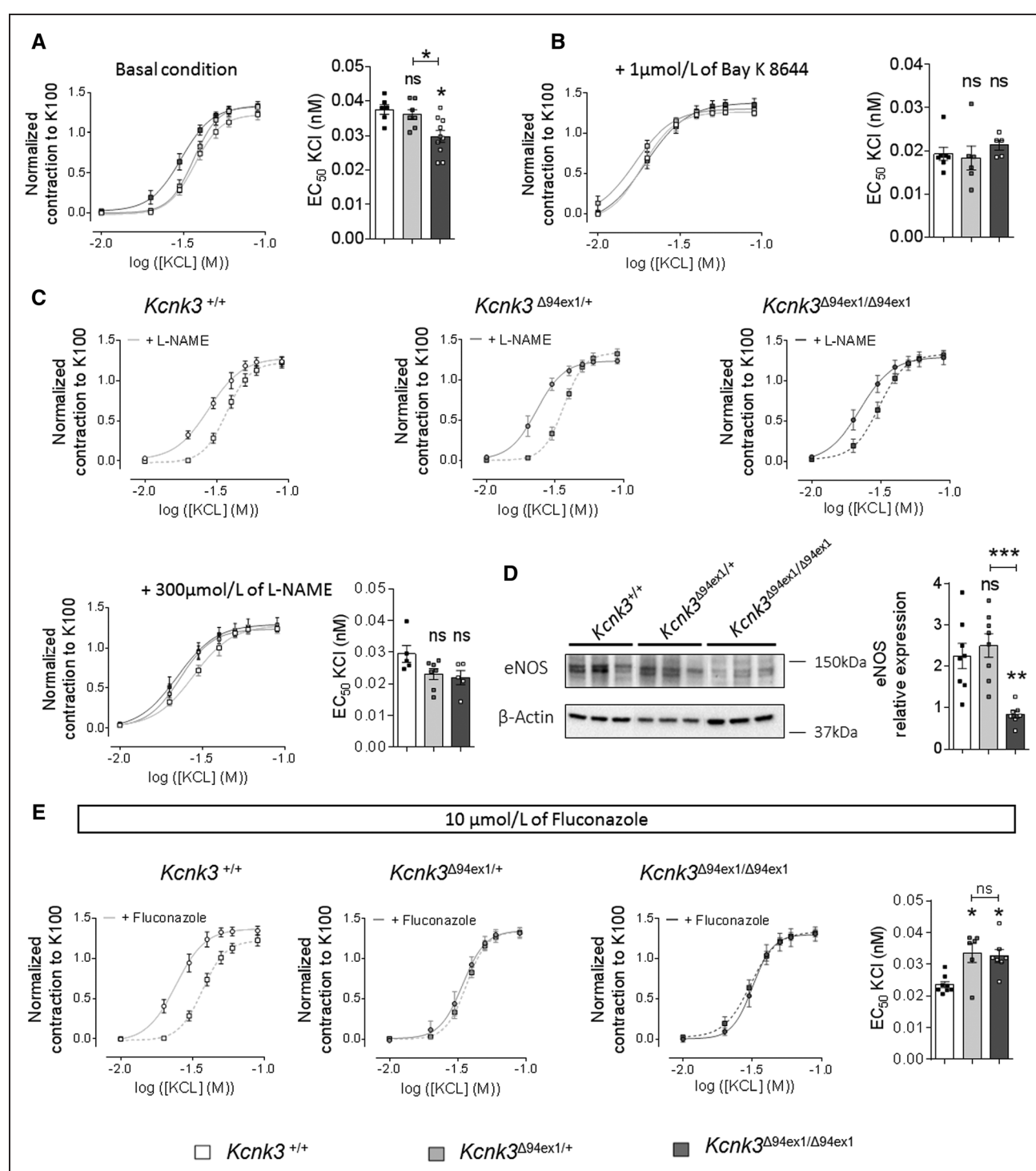
When PA were pretreated with Bay K 8644, a drug that potentiates voltage-sensitivity of L-Type-Ca<sup>2+</sup> channels, the contractile response was shifted to the left compared with the condition without Bay K 8644 (Figure 5B). Importantly, L-Type Ca<sup>2+</sup> channel potentiation abolished the previously observed differences in Figure 5A, consistent with the PSMCs depolarization (Figure 1H).

To rule out the contribution of endothelial-dependent PA relaxation via nitric oxide synthase (NOS) activities, we pretreated PA with L-NAME (L-N<sup>G</sup>-nitroarginine methyl ester; NOS inhibitor). With L-NAME, PA contracted at a lower concentration of KCl than in the absence of L-NAME, demonstrating the contribution of NOS activities and validating the efficacy of L-NAME treatment (Figure 5C). Interestingly, with L-NAME, in *Kcnk3*<sup>A94ex1/A94ex1</sup> rats, the difference in the KCl contractile dose-response was lost (Figure 5C). Western blot analyses showed that endothelial-NOS expression was significantly reduced in lungs from *Kcnk3*<sup>A94ex1/A94ex1</sup> rats (Figure 5D), whereas inducible-NOS expression was unchanged (Online Figure XI B).

Pulmonary vascular endothelium also releases EDHF (endothelium-derived hyperpolarizing factor). EDHF represents a family of endothelial factors as an arachidonic acid metabolite produced by cytochrome P450-2C9 that hyperpolarize and relax smooth muscles. To investigate the contribution of EDHF in PA tone, we used fluconazole, a cytochrome P450 epoxygenase inhibitor. With fluconazole, WT PA contracted at a lower KCl concentration than in the basal condition (Figure 5E, left), whereas fluconazole had no impact on PA contraction in *Kcnk3*-mutated rats (Figure 5E, middle and right). These data suggested that EDHF production is strongly reduced or inefficient in *Kcnk3*-mutated rats. All these parameters (PSMCs depolarization, reduced endothelial-NOS expression, reduced EDHF sensitivity) may contribute to enhanced PA vasoconstriction in *Kcnk3*<sup>A94ex1/A94ex1</sup> rats.

### Kcnk3-Dysfunction in Rats Reduced PA Relaxation

After inducing contraction with a thromboxane-A<sub>2</sub> mimetic (U46619), relaxation was stimulated using



**Figure 5. *Kcnk3* (potassium channel subfamily K member 3)-dysfunction in rats predisposes pulmonary arteries to constriction.**

**A**, Normalized dose-response curve (normalized to K100) established by applying an increasing concentrations of KCl on isolated pulmonary artery (PA) from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats and quantification of EC<sub>50</sub> (WT=6, *Kcnk3*<sup>Δ94ex1/+</sup>=7, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=10 rats). **B**, In presence of 1 μmol/L of Bay K 8644, normalized dose-response curve established by applying increasing concentrations of KCl on PA isolated from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats and EC<sub>50</sub> quantification (WT=7, *Kcnk3*<sup>Δ94ex1/+</sup>=6, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=5 rats). **C**, Comparison of PA contraction, in response to increasing dose of KCl, between PA pretreated with 300 μmol/L of L-NAME (L-N<sup>G</sup>-nitroarginine methyl ester) and PA nontreated (WT=5, *Kcnk3*<sup>Δ94ex1/+</sup>=7, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=5 rats). Bottom, normalized dose-response curve established by applying increasing concentrations of KCl in the presence of 300 μmol/L of L-NAME and EC<sub>50</sub> quantification (WT=5, *Kcnk3*<sup>Δ94ex1/+</sup>=7, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=5 rats). **D**, Representative Western blots and quantification of eNOS expression in lungs from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats (WT=8, *Kcnk3*<sup>Δ94ex1/+</sup>=8, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=7 rats). β-actin was used as the loading control. **E**, Normalized dose-response curve established by applying increasing concentrations of KCl on isolated PA from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats in presence of 10 μmol/L fluconazole and EC<sub>50</sub> quantification (WT=8, *Kcnk3*<sup>Δ94ex1/+</sup>=6, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=7 rats). \**P*<0.05, \*\**P*<0.01 vs *Kcnk3*<sup>+/+</sup>. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test for multiple comparisons. ns indicates nonsignificant.

acetylcholine to induce endothelium-dependent relaxation, an NO donor (sodium nitroprusside [SNP]) to induce endothelium-independent relaxation and a PDE5 inhibitor (sildenafil; Figure 6). PA relaxation induced by acetylcholine or SNP was similar between WT and *Kcnk3*<sup>A94ex1/+</sup> rats, whereas significantly reduced in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Figure 6A and 6B). Compared with WT, sildenafil-induced PA relaxation was reduced in *Kcnk3*-mutated rats, with a higher degree in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Figure 6C), suggesting that KCNK3 is critical for PDE5 inhibition-mediated PA relaxation. To explore this hypothesis, we preincubated PA from WT rats with a KCNK3 inhibitor (A293, 1 μmol/L) and measured the sildenafil-induced relaxation. Sildenafil-mediated relaxation was reduced compared with vehicle condition (Figure 6D). The amount of PDE5A (Online Figure XIC) and its localization was similar in *Kcnk3*-mutated rats (not shown). These results suggest that KCNK3 is involved in the PDE5 inhibition-induced PA relaxation.

In addition, we found a gain of mRNA expression of various genes known to be involved in SMC phenotype switch, including *Hexim1*, *MYL6*, and *PLN* in isolated PA from *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats and decrease expression in *Hrpt1* in isolated PA from *Kcnk3*-mutated rats (Online Figure XID).

### **Kcnk3-Dysfunction Did Not Alter Both Constriction and Relaxation of Aorta or Pulmonary Veins**

Interestingly, the contractile dose-response to KCl and the SNP-mediated relaxation were unchanged in aorta from *Kcnk3*-mutated rats (Online Figure XIIA and XIIB). Importantly, in isolated aorta, *Kcnk3*-dysfunction was not associated to an overactivation of MAP-kinase and antiapoptotic pathways (Online Figure XIIC). Finally, the contractile dose-response to KCl and the sildenafil-mediated relaxation in isolated pulmonary veins were unchanged in *Kcnk3*-mutated rats compared with WT (not shown), demonstrating specificity of the KCNK3 channel contribution in PAs. In line with normal aorta tone in *Kcnk3*-mutated rats, LV weights were similar in each group (Online Figure XIID and XIIE).

### **Kcnk3-Dysfunction Leads to Loss of Plasma Membrane Caveolae**

Using electron microscopy imaging of PA, we found in *Kcnk3*<sup>A94ex1/Δ94ex1</sup> a decrease in the number of caveolae per micrometer localized into the luminal ECs surface compared with WT, while the total number of caveolae was unchanged (Figure 6E and 6F). Morphometric analysis of the depth of these caveolae invaginations in *Kcnk3*-mutated rats revealed that they were shallower than caveolae from WT pulmonary arterial endothelial cells (Figure 6F).

### **Kcnk3-Dysfunction Induces Action Potential Prolongation in RV Cardiomyocytes**

In RV cardiomyocytes from *Kcnk3*-mutated rats, we recorded significant depolarization of resting membrane potential compared with WT (Figure 7A). Moreover, the action potentials were significantly prolonged in RV cardiomyocytes from *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats at −60 mV (Figure 7B). *Kcnk3*-mutated rats also showed some early after depolarization events (data not shown). Along with increased action potential duration, we measured a significant decrease in the outward-K<sup>+</sup>-transient current (*I*<sub>to</sub>; Figure 7C) and in an outward-K<sup>+</sup>-sustained current (*I*<sub>sus</sub>; Figure 7E). *I*<sub>to</sub> was reduced without modification of inactivation time constants (data not shown). At mRNA level, we observed a significant decrease in the expression of Kv4.3 channels, while Kv4.2, Kv1.5, Kv1.2 expressions remained unchanged (Figure 7D and 7F). The background-inward-K<sup>+</sup>-rectifier current (*I*<sub>K1</sub>) was also strongly reduced in isolated RV cardiomyocytes from *Kcnk3*-mutated rats (Figure 7G), while KCNJ12 mRNA expression remained unchanged (Figure 7H). Together, these results demonstrate that KCNK3 contributes to RV cardiomyocyte excitability. The expression of *Atp2a2*, *Myh6*, and *Myh7* were also unchanged (Online Figure XIIIA). About RV inflammation in *Kcnk3*-mutated rats, we measured a significant increase of *Gp130* (IL-6 receptor component) and *Timp-1* mRNA in RV from *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Online Figure XIIIB).

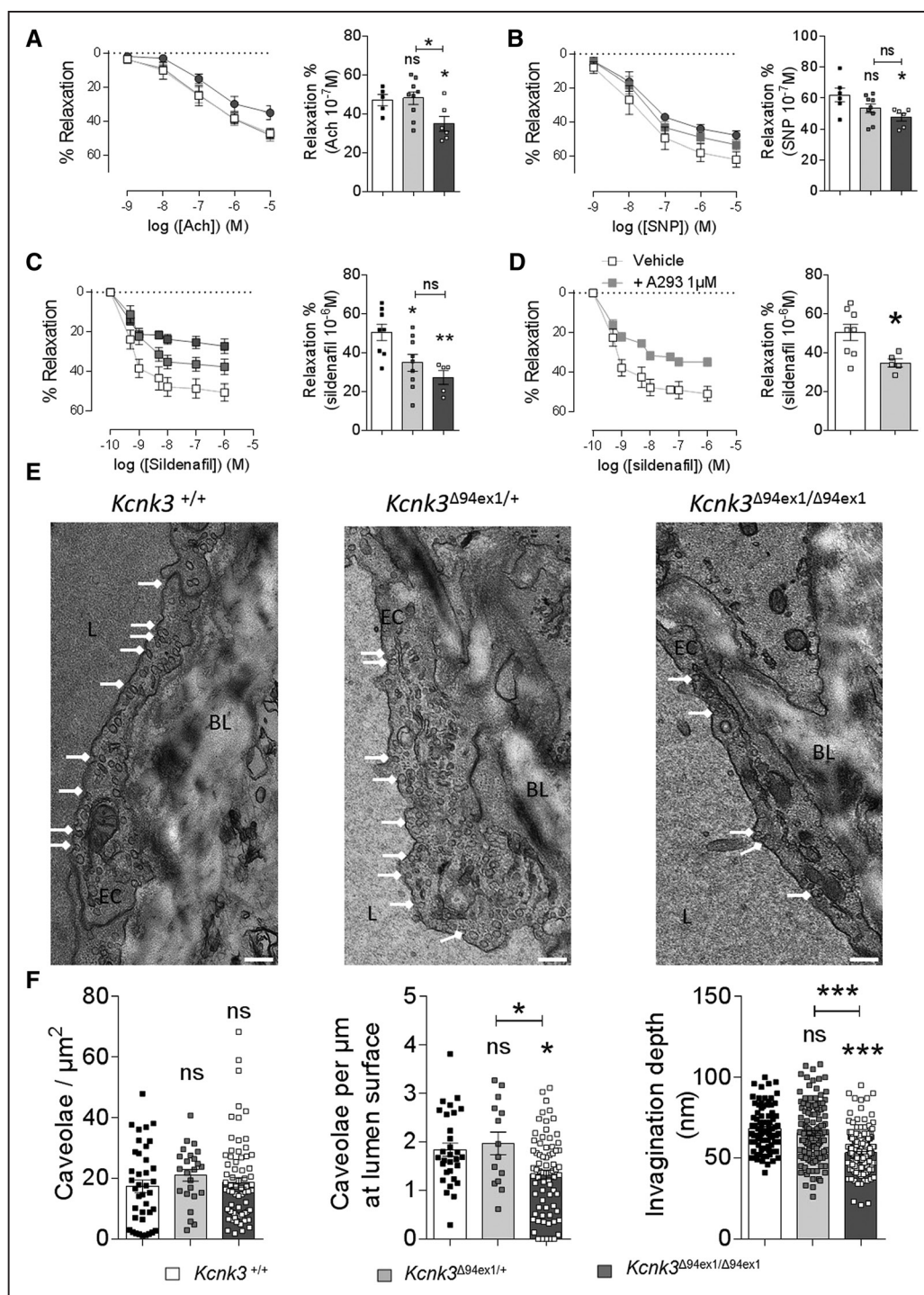
### **Both Male and Female Kcnk3-Mutated Rats Developed an Age-Related Increase in RVSP (12 Months)**

To determine whether PH development could be age-dependent, we performed right-heart catheterization in WT and *Kcnk3*-mutated rats at 12 months old (Figure 8A and Online Figure XIVA). PH is defined by an elevation of RVSP superior to 40 mmHg. In males, RVSP was superior to 40 mmHg in 25% of *Kcnk3*<sup>A94ex1/+</sup> rats and in 45% of *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats associated with a significant increase in TPR (Figure 8A). In females, RVSP was superior to 40 mmHg in 20% of *Kcnk3*<sup>A94ex1/+</sup> rats and in 36% of *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats, associated with a significant increase in TPR (Online Figure XIVA).

In males and females, the elevation of RVSP was not associated with the development of RV hypertrophy (Fulton index) or a reduction in cardiac output (Figure 8A; Online Figures XIV and XV) nor alteration of mean carotid artery pressure values (Figure 8A; Online Figure XIVA).

In addition, we observed an increase in the number of muscularized pulmonary vessels and a decrease in nonmuscularized pulmonary vessels in 12-month-old male *Kcnk3*-mutated rats (Figure 8B) associated with HIF1-α overexpression in lung from *Kcnk3*<sup>A94ex1/Δ94ex1</sup> rats (Online Figure XIVB). Significant higher level of fibrillary





**Figure 6. *Kcnk3* (potassium channel subfamily K member 3)-dysfunction in rats altered the relaxation of pulmonary artery (PA).**

**A**, Concentration-relaxation response curves of isolated segment of PA established by applying increasing concentrations of (A) acetylcholine (Ach; 1 nmol/L to 10  $\mu$ mol/L), (B) sodium nitroprusside (SNP; 10 nmol/L to 10  $\mu$ mol/L) and (C) sildenafil (1 nmol/L to 5  $\mu$ mol/L). Right, represent graph of the percentage of relaxation (WT=5, *Kcnk3* <sup>$\Delta$ 94Ex1/+</sup>=9, *Kcnk3* <sup>$\Delta$ 94Ex1/ $\Delta$ 94Ex1</sup>=7 rats). **D**, Concentration-relaxation response curves established by applying increasing concentrations of sildenafil (1 nmol/L to 10  $\mu$ mol/L) to PA segments isolated from *Kcnk3*<sup>+/+</sup> rats, treated with or without KCNK3 inhibitor (A293 1  $\mu$ mol/L), and percentage of relaxation to 10<sup>-6</sup> M of sildenafil (WT=6, *Kcnk3* <sup>$\Delta$ 94Ex1/+</sup>=9, *Kcnk3* <sup>$\Delta$ 94Ex1/ $\Delta$ 94Ex1</sup>=6 rats). **E**, Analysis of the ultrastructure of PA by electron microscopy in 4-month-old rats. Endothelial cells (ECs) were based on the basal lamina (BL). **F**, Caveolae density was quantified as the number of caveolae by micrometer square of total EC surface (Left; WT=34 cells, 2 rats, *Kcnk3* <sup>$\Delta$ 94Ex1/+</sup>=29 cells, 2 rats, *Kcnk3* <sup>$\Delta$ 94Ex1/ $\Delta$ 94Ex1</sup>=73 cells, 3 rats). Caveolae were counted as omega-shaped membrane profiles open at the lumen surface (L) and normalized per micrometer; WT=34 cells, 2 rats, *Kcnk3* <sup>$\Delta$ 94Ex1/+</sup>=29 cells, 2 rats, *Kcnk3* <sup>$\Delta$ 94Ex1/ $\Delta$ 94Ex1</sup>=73 cells, 3 rats; middle). Depths of the lumen caveolae (invagination depths) were quantified in right (WT=86 caveolae, 2 rats, *Kcnk3* <sup>$\Delta$ 94Ex1/+</sup>=131 caveolae, 2 rats, *Kcnk3* <sup>$\Delta$ 94Ex1/ $\Delta$ 94Ex1</sup>=234 caveolae, 3 rats). Bars= 400 nm. ns: nonsignificant. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001 vs *Kcnk3*<sup>+/+</sup>. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test for multiple comparisons.

collagen assembly was observed in small (<50  $\mu\text{m}$ ) and medium (50–200  $\mu\text{m}$ ) PA in *Kcnk3*-mutated rats (Figure 8C), while total collagen deposition was similar (Online Figure XIVC).

At 12-month-old rats (males), echocardiography analyses (Online Table V) revealed a reduction in pulmonary artery acceleration time in *Kcnk3*-mutated rats. LV diastolic and systolic diameters (normalized by weight) significantly increased in *Kcnk3*-mutated rats along with reduction in RV and LV thickness, demonstrating development of LV dilatation in *Kcnk3* <sup>$\Delta 94\text{ex1}/\Delta 94\text{ex1}$</sup> . LV dilatation seems to be compensated by higher RV and LV contractility as evaluated by RV and LV fractional shortening. At 12 months of age, *Kcnk3*-mutated rats showed chronic echography signs of left and right heart dilatation.

As low serum-albumin concentrations was associated with high mortality in PAH patients, serum-albumin was proposed as a marker of disease severity.<sup>21</sup> Interestingly, the serum-albumin level was unchanged in 4-month-old *Kcnk3*-mutated rats (Online Figure XIVD) but was significantly reduced in 12-month *Kcnk3*-mutated rats (Figure 8D), supporting the notion of age-dependent PH development in these animals.

## DISCUSSION

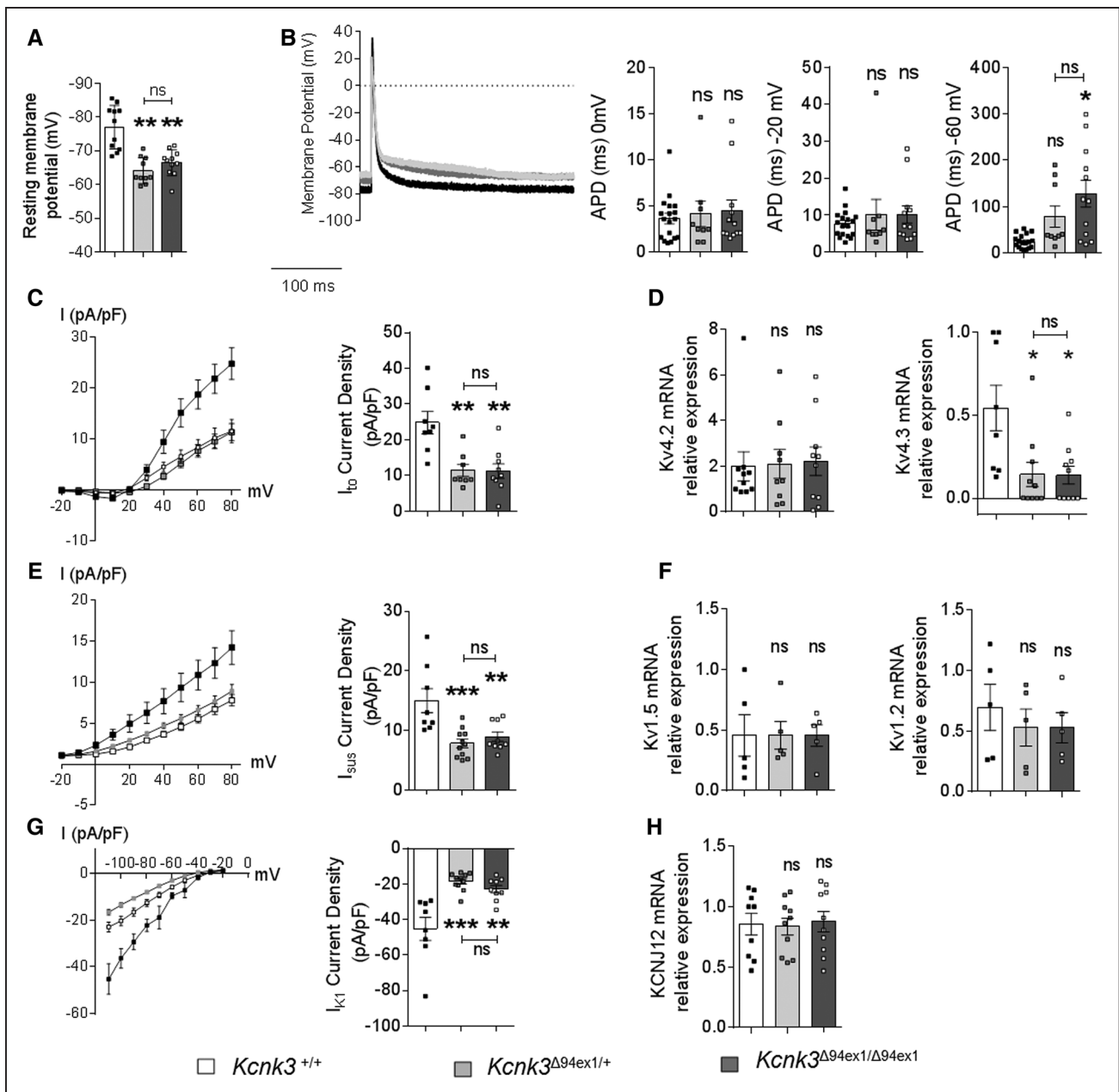
In this study, we performed electrophysiological, hemodynamic, histological, cellular, and molecular characterization of *Kcnk3*-mutated rat line ( $\Delta 94\text{ex1}$ ). First, we succeeded in generating a model of *Kcnk3*-inactivation with a decrease in  $I_{\text{KCNK3}}$  in PSMCs from *Kcnk3*-mutated rats. Second, at 4 months of age, RVSP are increased in *Kcnk3*-mutated rats and they are sensitized to PH induce by MCT-exposure or CH-exposure. Third, *Kcnk3*-dysfunction leads to increased pulmonary vessel muscularization and perivascular fibrillar collagen deposition. At the molecular level, *Kcnk3*-mutated rats are characterized by overactivation of proliferative pathways, with alteration of endothelial marker expression. Fourth, in human PSMCs, KCNK3 knockdown increase the amount of HIF1- $\alpha$  and overactivate ERK1/2 pathway as primary molecular events linked to *Kcnk3*-dysfunction. Fifth, PAs from *Kcnk3*-mutated rats are sensitized to constriction and are less responsive to vasorelaxing stimuli. Finally, *Kcnk3*-mutated rats develop spontaneous PH in an age-dependent manner with penetrance ranging between 30% and 60%. The variety of pulmonary vascular alterations observed in *Kcnk3*-mutated rats are summarized in Figure 8E is consistent with recent findings related to human PAH pathobiology.<sup>3</sup>

KCNK3 channels are commonly accepted as contributors to background- $\text{K}^+$ -currents that stabilize resting membrane potential.<sup>8</sup> In excitable cells including PSMCs, plasma membrane depolarization causes opening of L-type  $\text{Ca}^{2+}$  channels leading to influx of  $\text{Ca}^{2+}$ , PSMCs contraction, and proliferation via activation of

$\text{Ca}^{2+}$  sensitive pathways.<sup>22</sup> KCNK3 knockdown in mouse neuroblastoma cells increases proliferation rates by >25%.<sup>23</sup> In vivo chronic inhibition of KCNK3 induces aberrant pulmonary vascular cell proliferation.<sup>10</sup> We present evidence that pharmacological or genetic inactivation of KCNK3 promotes PSMCs proliferation, confirming that KCNK3 downregulation is an initial trigger for PAH pathobiology possibly via HIF1- $\alpha$  and ERK1/2 activation. In mice, *hif1- $\alpha$* -dysfunction reduces the development of PH after CH-exposure.<sup>24</sup> In PAH, there is normoxic activation of HIF1- $\alpha$  inducing a pseudo-hypoxic cell status leading to aberrant cell proliferation,<sup>25</sup> which might be mediated by an activation of specific kinases (like P38 or ERK1/2)<sup>26,27</sup> or phosphatases inactivation.<sup>26</sup> We found that *Kcnk3*-dysfunction leads to overactivation of ERK1/2, which could partly enhance HIF1- $\alpha$  expression. Mitochondrial membrane depolarization induced by the mitochondrial uncoupler (FCCP [carbonyl cyanide-4(trifluoromethoxy)phenylhydrazone]) leads to the activation of ERK1/2.<sup>28</sup> Moreover, the resting plasma membrane potential and  $\Delta\Psi\text{m}$  are intimately linked.<sup>29</sup> We found that extracellular high- $\text{K}^+$  concentration as well as si-KCNK3 induces mitochondrial membrane depolarization, which could also partly explain HIF1- $\alpha$  overexpression. Furthermore, mitochondrial dysfunction has been investigated in PAH, demonstrating that  $\Delta\Psi\text{m}$  from PAH PSMCs are more hyperpolarized compared with mitochondria from healthy PSMCs because of an alteration of SOD2 expression.<sup>30</sup> However, SOD2 protein expression was unchanged in *Kcnk3*-mutated rats (not shown). Finally, previous reports demonstrated that mitochondrial network is fragmented in iPAH PSMCs<sup>19</sup> in association with the development of PAH. Here, we also found that mitochondria network is fragmented in the context of *Kcnk3*-dysfunction.

Our results suggest that *Kcnk3*-dysfunction enhances the phosphorylation of pro-mitogen proteins ERK1/2, 308AKT, and SRC and decreases PP2A activity. Indeed, we recently showed in iPAH PSMCs that T-type- $\text{Ca}^{2+}$  channels signaling contributes to pathological hyperproliferation/cell survival and apoptosis resistance via phosphorylation of ERK1/2, 308AKT, and decreased PP2A activity.<sup>31</sup>

Higher levels of lung VWF have been associated with poor survival in PAH patients.<sup>32</sup> Mojiri et al<sup>33</sup> demonstrated that CH-exposure induced elevated lung VWF expression. We found that VWF expression is also severely increased in lung of *Kcnk3*-mutated rats, suggesting that *Kcnk3*-dysfunction alter also pulmonary endothelial function. CD31 is highly expressed at endothelial cell-cell junctions.<sup>34</sup> Here, we showed a decrease in CD31 expression in lungs from *Kcnk3*-mutated rats. Migrating endothelial cells lose specific endothelial markers such as CD31.<sup>35</sup> Increasing evidence demonstrates that EndMT contributes to PAH development.<sup>20</sup> EndMT is a process by which endothelial cells



**Figure 7. *Kcnk3* (potassium channel subfamily K member 3)-dysfunction leads to action potential prolongation in adult right ventricular (RV) cardiomyocytes.**

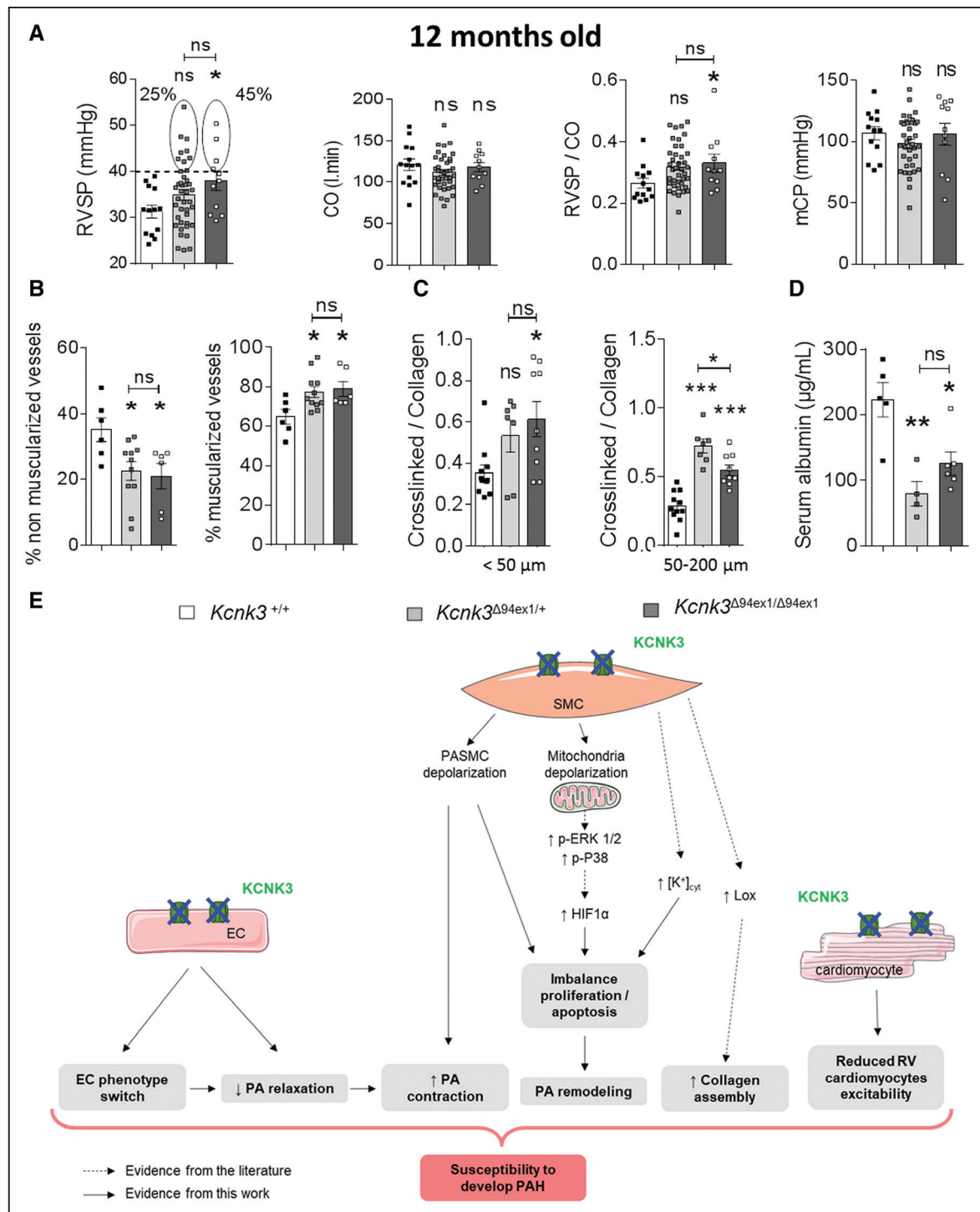
**A**, Average resting membrane potential in RV cardiomyocytes isolated from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats (wild-type [WT]=11 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/+</sup>=11 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=11 cells, 3 rats). **B**, Representative membrane potential of RV cardiomyocytes from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats and analysis of RV action potential duration (APD) at 0, -20, and -60 mV of membrane repolarization (WT=20 cells, 4 rats, *Kcnk3*<sup>Δ94ex1/+</sup>=11 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=11 cells, 3 rats). **C**, We analyzed the current-voltage relationship of the outward-K<sup>+</sup>-transient current (*I*<sub>to</sub>) or 4-AP-sensitive-K<sup>+</sup> current (WT=8 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/+</sup>=8 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=9 cells, 3 rats). **D**, mRNA expression of Kv4.2 and Kv4.3 in RV tissues (WT=11, *Kcnk3*<sup>Δ94ex1/+</sup>=11, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=11 rats). **E**, Outward-sustained-K<sup>+</sup>-current (*I*<sub>SUS</sub>) current (WT=11 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/+</sup>=11 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=11 cells, 3 rats). **F**, mRNA expression of Kv1.5 and Kv2.1 in RV tissues from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats (WT=5, *Kcnk3*<sup>Δ94ex1/+</sup>=5, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=5 rats). **G**, Inward-K<sup>+</sup>-rectifier-current (*I*<sub>K1</sub>; WT=8 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/+</sup>=11 cells, 3 rats, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=9 cells, 3 rats). **H**, mRNA expression of KCNJ12 in RV tissues from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats (WT=11, *Kcnk3*<sup>Δ94ex1/+</sup>=11, *Kcnk3*<sup>Δ94ex1/Δ94ex1</sup>=11 rats). \**P*<0.05 vs *Kcnk3*<sup>+/+</sup>. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test for multiple comparisons. ns indicates nonsignificant.

acquire a mesenchymal phenotype in association with expression of SMC genes or loss of endothelial marker expression.<sup>36</sup> We showed an overexpression of TWIST1, mainly defined as a master controller of EndMT and

epithelial-mesenchymal-transition,<sup>20</sup> suggesting *Kcnk3*-dysfunction contributes to EndMT pathway.

As caveolae are demonstrated to mediate albumin transcytosis in human ECs,<sup>37</sup> the loss of plasma-membrane





**Figure 8. *Kcnk3* (potassium channel subfamily K member 3)-mutated rats develop age-related pulmonary hypertension (12 mo).**

**A**, Right ventricular systolic pressure (RVSP), cardiac output (CO), total pulmonary resistance (TPR), and mean carotid pressure (mCP) were assessed at 12 months old (wild type [WT]=13, *Kcnk3* <sup>$\Delta$ 94ex1/+</sup>=41, *Kcnk3* <sup>$\Delta$ 94ex1/ $\Delta$ 94ex1</sup>=11 rats). **B**, Percentage of nonmuscularized and muscularized vessels (100 vessels count per rats; WT=6, *Kcnk3* <sup>$\Delta$ 94ex1/+</sup>=11, *Kcnk3* <sup>$\Delta$ 94ex1/ $\Delta$ 94ex1</sup>=6 rats). **C**, Collagen crosslinked to total collagen ratio in small (<50  $\mu$ m) and medium (50–200  $\mu$ m) pulmonary artery (PA) from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats at 12 months old (WT=11, *Kcnk3* <sup>$\Delta$ 94ex1/+</sup>=7, *Kcnk3* <sup>$\Delta$ 94ex1/ $\Delta$ 94ex1</sup>=9 rats). **D**, Serum-albumin concentration in plasma from *Kcnk3*<sup>+/+</sup> and *Kcnk3*-mutated rats at 12 months old (WT=5, *Kcnk3* <sup>$\Delta$ 94ex1/+</sup>=5, *Kcnk3* <sup>$\Delta$ 94ex1/ $\Delta$ 94ex1</sup>=6 rats). \* $P$ <0.05, \*\* $P$ <0.01 vs *Kcnk3*<sup>+/+</sup>. Experiments were analyzed using 1-way ANOVA followed by Tukey post hoc test. **E**, Proposed events arising from *Kcnk3*-dysfunction. Loss of KCNK3 results in plasma membrane depolarization, mitochondrial membrane depolarization, endothelial phenotype switch, reduces RV cardiomyocytes excitability. All the events leads to pulmonary arterial vasoconstriction, pulmonary vascular remodeling, and pulmonary arterial smooth muscle cells (PASMCs) proliferation (partly explained by ERK1/2 increased phosphorylation and overexpression of HIF1- $\alpha$  [hypoxia-inducible factor-1  $\alpha$ ]). Altogether, these events predispose for pulmonary vascular remodeling and pulmonary arterial hypertension (PAH). ns indicates nonsignificant.



caveolae in pulmonary arterial endothelial cells lacking KCNK3 could partly explain abnormal serum-albumin production in *Kcnk3*-mutated rats.

Moreover, increasing evidences suggest an important role of ECM (extracellular matrix) deposition in PAH. Numerous experimental PH models exhibit pulmonary vascular matrix deposition.<sup>38</sup> Interestingly at 4 months old, perivascular collagen cross-linking is markedly pronounced in heterozygous *Kcnk3*-mutated rats, which may contribute to abnormal RVSP elevation because perivascular collagen cross-linking is associated to PA stiffness.<sup>3</sup> Bertero et al<sup>39</sup> demonstrated in hPASMCs that the knock-down of KCNK3 promotes expression of the collagen crosslinking enzyme (lysyl oxidase) and promotes expression of miR130/301 that coordinate ECM remodeling.

In addition to PA remodeling and fibrosis, PAH patients have aberrant PA vasoconstriction mainly explained by the disruption of the NO pathway.<sup>3</sup> In PAH, endothelial-derived NO activity is decreased, promoting vasoconstriction and thrombosis.<sup>40</sup> As caveolae are shown to regulate endothelial-NOS activity,<sup>41</sup> the abnormalities observed in caveolae shape from pulmonary arterial endothelial cell *Kcnk3*-mutated rats could contribute to the decrease in NOS-mediated relaxation. Although EDHF is important to maintain normal vascular tone, the exact role of EDHF in PH pathogenesis is unclear. Alteration of EDHF-mediated responses has been reported with aging, hypertension, atherosclerosis, heart failure, and diabetes mellitus.<sup>42</sup> We showed that *Kcnk3*-dysfunction predisposes to PA vasoconstriction associated with reduced endothelial-NOS expression and alteration of EDHF pathway. Ouyang et al<sup>43</sup> have suggested that K<sup>+</sup>-channels regulate NO secretion in human pulmonary arterial endothelial cells.<sup>43</sup> Our results strongly suggest a crosslink between KCNK3 and NO or EDHF in PA.

We found that PA relaxation, induced by PDE5 inhibition, is reduced when KCNK3 is not functional. PDE5 inhibition increase intracellular levels of cGMP, thus increasing PKG activity and consequently relaxing smooth muscles.<sup>44</sup> KCNK3 contains multiple PKG phosphorylation sites. As PKG regulates KCNK3 activity,<sup>45</sup> we propose that KCNK3 channel is the end point of PDE5/GMP/PKG dependent signaling in PASMCs.

At cardiac level, we confirmed that KCNK3 contributes to RV cardiomyocyte excitability and action potential repolarization. Moreover, we recently demonstrated that pharmacological inhibition of KCNK3 (by A293) increased the action potential duration in adult RV cardiomyocytes from rats.<sup>11</sup> Previous reports showed similar results in *kcnk3*-knockout mice.<sup>46</sup> In human iPSC (induced pluripotent stem cells)-derived cardiomyocytes, *KCNK3* knock-down was found to prolong action potential duration.<sup>47</sup> *Kcnk3*-dysfunction should increase the susceptibility for early after-depolarizations and ventricular arrhythmias.

Collectively, our findings, summarized in Figure 8E, underscore the concept that genetic *Kcnk3*-inactivation

in rats leads to pulmonary vascular alteration, thus facilitating PH development in an age-dependent manner. Importantly, our results highlight that loss of KCNK3 function acts on PA compliance resulting in increased RVSP via 2 different ways. Heterozygous-*Kcnk3*<sup>A94ex1/+</sup> rats were characterized by PA remodeling and perivascular collagen crosslinking whereas PA remodeling and PA vasoconstriction (Online Figure XVI) characterize homozygous-*Kcnk3*<sup>A94ex1/A94ex1</sup> rats. These results confirm the importance of PA tone and mechanical cues at the early stages of PH development. This model provides new opportunities for understanding the initiating mechanisms of PH and represents a relevant tool for testing therapeutic targets against PH.

## Limitations

Our *Kcnk3*-mutated rats cannot be considered as knockout animals. Using CRISPR/Cas9 approach, we induced a deletion of 94 bp in the exon 1 of *Kcnk3* gene, shifting the open reading frame. This shift in the coding sequence results in an aberrant aa sequence and leads to a premature stop codon (8 potential premature stop codons; Online Figure I. Unfortunately, commercially available KCNK3 antibodies are not specific to KCNK3 preventing any evaluation of the expression of WT or truncated KCNK3 proteins. The putative translation of the truncated mRNA should produce a 90-aa protein (instead of a 411-aa for the WT protein) with only 14-aa in common with the WT protein, which could partly affect a signaling cascade induced by *Kcnk3*-mutation. However, we show that most of KCNK3-dependent signaling observed in *Kcnk3*-mutated rats were also detected in hPASMC in which KCNK3 is targeted by siRNA strategy. These similar results (ex vivo versus in vivo) indicate that the truncated protein negligibly affected the *Kcnk3*-mutated phenotype.

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M. Humbert has relationships with drug companies, including Actelion, Bayer, GSK, Novartis, and Pfizer. In addition to being investigators in trials involving these companies, other relationships include consultancy services and memberships to scientific advisory boards. The other authors report no conflicts of interest.

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