



# Rap1 promotes endothelial mechanosensing complex formation, NO release and normal endothelial function

Sribalaji Lakshminathan<sup>1</sup>, Xiaodong Zheng<sup>2,3,†</sup>, Yoshinori Nishijima<sup>2,3</sup>, Magdalena Sobczak<sup>1,‡</sup>, Aniko Szabo<sup>4</sup>, Jeannette Vasquez-Vivar<sup>5</sup>, David X Zhang<sup>2,3</sup> & Magdalena Chrzanowska-Wodnicka<sup>1,\*</sup>

## Abstract

Decreased nitric oxide (NO) bioavailability underlies a number of cardiovascular pathologies, including hypertension. The shear stress exerted by flowing blood is the main determinant of NO release. Rap1 promotes integrin- and cadherin-mediated signaling. Here, we show that Rap1 is a critical regulator of NO production and endothelial function. Rap1 deficiency in murine endothelium attenuates NO production and diminishes NO-dependent vasodilation, leading to endothelial dysfunction and hypertension, without deleterious effects on vessel integrity. Mechanistically, Rap1 is activated by shear stress, promotes the formation of the endothelial mechanosensing complex—comprised of PECAM-1, VE-cadherin and VEGFR2- and downstream signaling to NO production. Our study establishes a novel paradigm for Rap1 as a regulator of mechanotransduction.

**Keywords** mechanotransduction; nitric oxide; shear stress; small GTPase Rap1; vasodilation

**Subject Categories** Signal Transduction; Vascular Biology & Angiogenesis

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## Introduction

NO is a key regulator of endothelial homeostasis. A defect in NO production leads to endothelial dysfunction, a pathological condition linked to inflammation and atherosclerosis, perturbed endothelial barrier, progression of diabetes, and, in particular, vasomotor dysfunction and hypertension [1,2]. Endothelial NO synthase (eNOS) produces NO in the vasculature [3,4], and its activity is regulated by Ca<sup>2+</sup>/calmodulin [5], binding of regulatory cofactors

and posttranslational modifications, including phosphorylation events which sensitize eNOS to Ca<sup>2+</sup> and stimulate NO production [1]. Increased shear stress resulting from increased vascular smooth muscle-mediated vessel contraction is a potent stimulus of NO release, together with other relaxing factors, from endothelium [6]. Thus, smooth muscle contraction and endothelium-dependent vasodilation are two major mechanisms controlling vascular tone. Decreased NO, a hallmark of endothelial dysfunction, contributes to pathogenesis of hypertension [3,4].

Shear-stress-induced NO release from endothelium is regulated by mechanisms involving several mechanosensing cell-surface receptors [7–10]. Among those, shear-induced, ligand-independent transactivation of VEGFR2, acting as a component of endothelial junctional mechanosensing complex, triggers signaling culminating at eNOS phosphorylation at Ser1177 that leads to enhanced NO release [11,12]. Conversely, inhibition of VEGFR2 activation reduces shear-induced vasodilation *in vivo* [8,9]. While much progress has been made [6], the exact mechanisms of mechanotransduction underlying endothelial response are not fully understood [13].

Rap1, a ubiquitously expressed small GTPase, integrates signals from multiple receptors to control cell adhesion, polarity, and migration [14,15]. Two Rap1 isoforms, Rap1a and Rap1b, share 95% identity, and deletion of either gene leads to developmental cardiovascular defects [16,17], impaired adhesion of various blood cell populations [16,18,19] and altered angiogenesis [20,21] in mice. In cultured endothelial cells (ECs), Rap1 has been implicated in promoting cell–cell junction formation and vascular barrier [22–24], also via interactions with KRIT-1 [25], a gene product mutated in Cavernous Cerebral Malformations (CCM), a human neurological disorder leading to hemorrhage and stroke [26]. Therefore, Rap1 has been linked with human CV disease [17].

In addition to the described *in vivo* defects, our observation of frequently increased neonatal lethality and sudden death in endothelium-restricted Rap1 knockout mice suggested additional

<sup>1</sup> Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI, USA

<sup>2</sup> Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, USA

<sup>3</sup> Cardiovascular Center, Medical College of Wisconsin, Milwaukee, WI, USA

<sup>4</sup> Division of Biostatistics, Medical College of Wisconsin, Milwaukee, WI, USA

<sup>5</sup> Department of Biophysics and Redox Biology Program, Medical College of Wisconsin, Milwaukee, WI, USA

\*Corresponding author. Tel: +1 414 937 3890; Fax: +1 414 937 6284; E-mail: magdalena.chrzanowska@bcw.edu

<sup>†</sup>Present address: Department of Pathophysiology, Harbin Medical University, Daqing, China

<sup>‡</sup>Present address: Nencki Institute of Experimental Biology, Warsaw, Poland

underlying defects in cardiovascular function of these mice. In this manuscript, we report that endothelium-specific deletion of both Rap1 isoforms leads to a severe impairment of NO-dependent vasodilation and, significantly, is sufficient to induce hypertension *in vivo*. We show that, mechanistically, Rap1 activity is required for transmission of shear-induced signals from the endothelial mechanosensing complex leading to eNOS activation and normal NO release. Because shear stress from flowing blood is the main determinant of NO release, our novel findings position Rap1 as a key regulator of endothelial function. Furthermore, to our knowledge, this is the first report of Rap1 involvement in mechanotransduction *in vivo*.

## Results and Discussion

### EC-restricted Rap1-knockout mice develop hypertension

Endothelial lineage-specific deletion of both Rap1 isoforms leads to complete embryonic lethality due to hemorrhage around mid-gestation [17,27]. Deletion of all but one Rap1a allele (*Tie2-Cre<sup>+/0</sup>; Rap1a<sup>f/+</sup>Rap1b<sup>f/f</sup>*; Rap1-ECKO) reduces the level of total Rap1 to about 5% of that in WT ECs and has a milder defect with ~30% of animals surviving to adulthood with increased spontaneous lethality and poor survival following anesthesia, particularly in male mice. Upon dissection, we discovered enlarged hearts in Rap1-ECKO mice (Fig 1A), similar to what we have recently observed in total Rap1b-knockout mice [28]. Such pathological hypertrophy often results from increased vascular load and in Rap1b-knockout mice is accompanied by hypertension and increased smooth muscle contractility [28]. Because endothelium is an important regulator of vascular tone, we examined whether Rap1 deficiency in endothelium is sufficient for elevated blood pressure in mice and found that, indeed, Rap1-ECKO mice were hypertensive (Fig 1B). Rap1 has been implicated in promoting EC cell–matrix and cell–cell adhesion [29], and therefore, its significant reduction in ECs might be expected to lead to defects in EC junctions and endothelial barrier. However, we found that basal vascular permeability *in vivo* was not elevated in Rap1-ECKO mice (Fig 1C) and *ex vivo*, cell–cell junctions appeared normal (Fig 1D and Supplementary Fig S1A). Further, neither EC turnover (Fig 1D and Supplementary Fig S1A) nor apoptosis (Fig 1E and Supplementary Fig S1B) were altered in aortae of these mice. Therefore, we concluded that basal EC defects are not responsible for the observed phenotypes. Instead, we considered decreased NO, a hallmark of endothelial dysfunction, as an underlying cause of the elevated blood pressure in endothelium-specific Rap1 knockout mice.

### Endothelial dysfunction in EC-restricted Rap1-knockout mice

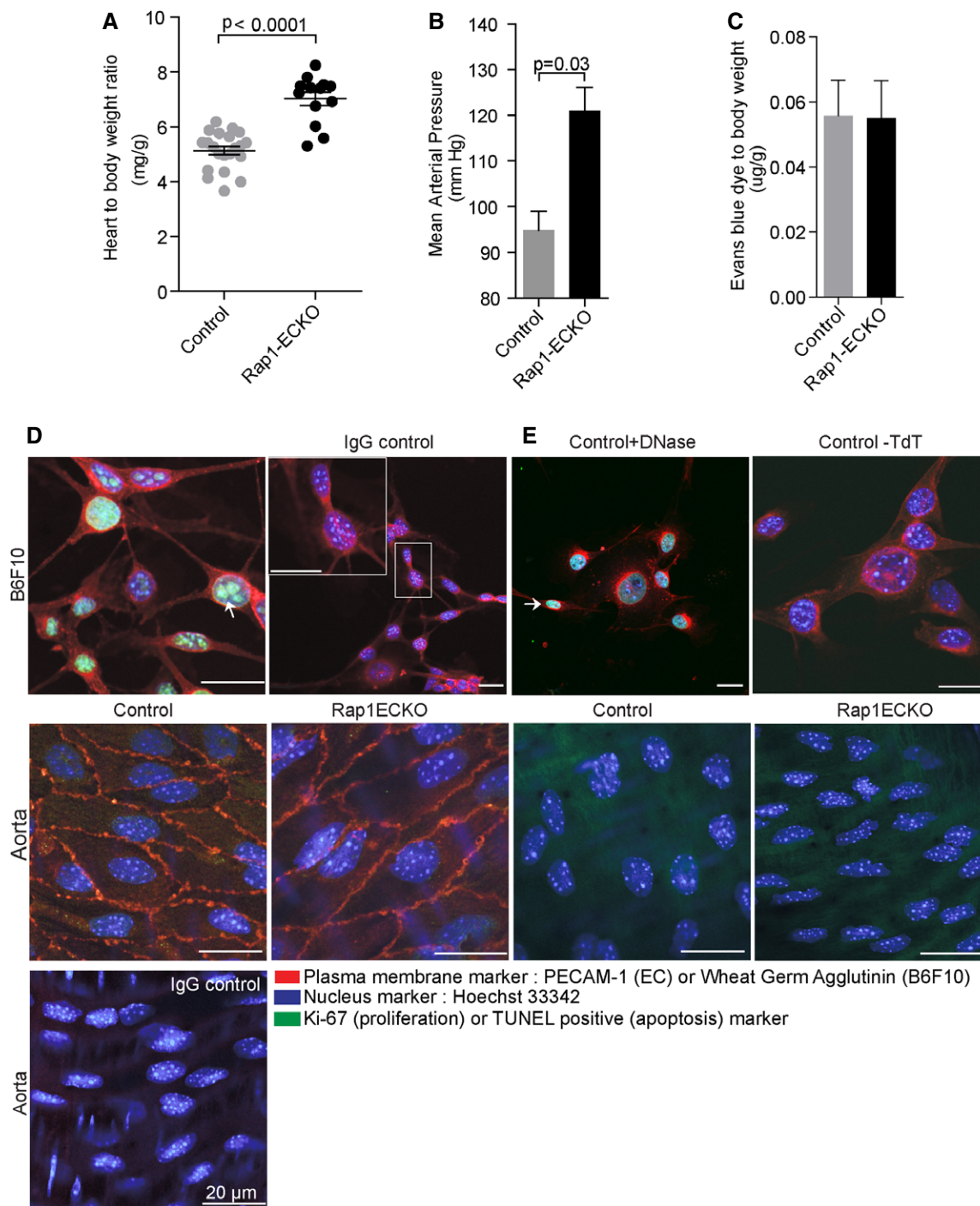
Because Tie2 promoter is expressed in the hematopoietic lineage [30] and Rap1 is excised in blood cells [27], it is possible that non-ECs may contribute to hypertension in Rap1-ECKO mice. Furthermore, vascular deficiency during development [17] may contribute to the vascular phenotype in adult mice. To avoid these complications, we generated inducible Rap1-knockout mice (*Cadh5(PAC)-CreERT<sup>+/0</sup>;Rap1a<sup>f/+</sup>Rap1b<sup>f/f</sup>*; Rap1<sup>iAEC</sup>), in which Rap1 is deleted in ECs with tamoxifen injections in neonates (Fig 2A). We found that blood pressure was significantly increased in Rap1<sup>iAEC</sup> mice

(Fig 2B), similar to that in Rap1-ECKO mice (Fig 1B). Because endothelial dysfunction leads to hypertension [3,4], we hypothesized that a defect in NO release underlies hypertension in endothelial Rap1-deficient mice. Therefore, we examined *ex vivo* NO-dependent vasodilation in aortic segments, where NO is the major vasodilating substance. We found that ACh-induced vessel dilation was significantly reduced in Rap1<sup>iAEC</sup> aortic segments precontracted with thromboxane analog U46619 (Fig 2C and Supplementary Table S1). To distinguish whether Rap1 regulates NO release from ECs or NO sensitivity of SM, we blocked NO release with L-nitro-arginine methyl ester (L-NAME) and measured vasodilation in the presence of sodium nitroprusside (SNP). We found that the extent of SNP-induced vasodilation of aortic segments was not affected by Rap1-deficiency (Fig 2D and Supplementary Table S2) compared to controls. Although SNP-induced vessel relaxation appeared decreased in Rap1<sup>iAEC</sup> versus WT segments at the lowest dose of ACh, the difference was not statistically significant (Supplementary Fig S2). These results indicate that deficiency of both Rap1 isoforms leads to a defect in NO bioavailability in an EC-endogenous manner and suggest that Rap1 is required for optimal NO release and normal vascular tone. In contrast, endothelial deletion of Rap1b isoform alone leads to a more moderate defect in vasodilation and is not sufficient for blood pressure elevation [28]. Cumulatively, these data show that genetic Rap1 deficiency in endothelium leads to endothelial dysfunction, which may be a primary mechanism explaining hypertension in these mice.

### Rap1 is required for fluid shear-induced signaling leading to optimal NO release

Blood flow-induced fluid shear stress is the main physiological stimulus regulating EC-dependent NO release *in vivo* [6]. Therefore, we examined the effect of Rap1 deficiency on EC-dependent NO release in response to shear stress. Since Rap1b is the main Rap1 isoform in ECs [27], we used ECs isolated from Rap1b-KO mice as a model of Rap1-deficient ECs. Rap1b-KO ECs were subjected to shear stress at 10–15 dynes/cm<sup>2</sup> using a cone viscometer, and NO release was quantified and compared to wild-type ECs treated in the same manner (Fig 3A). In the absence of shear stress stimulus, both wild-type and Rap1b-KO ECs showed negligible NO production that was enhanced with shear stress several fold in wild-type but not in Rap1b-KO ECs.

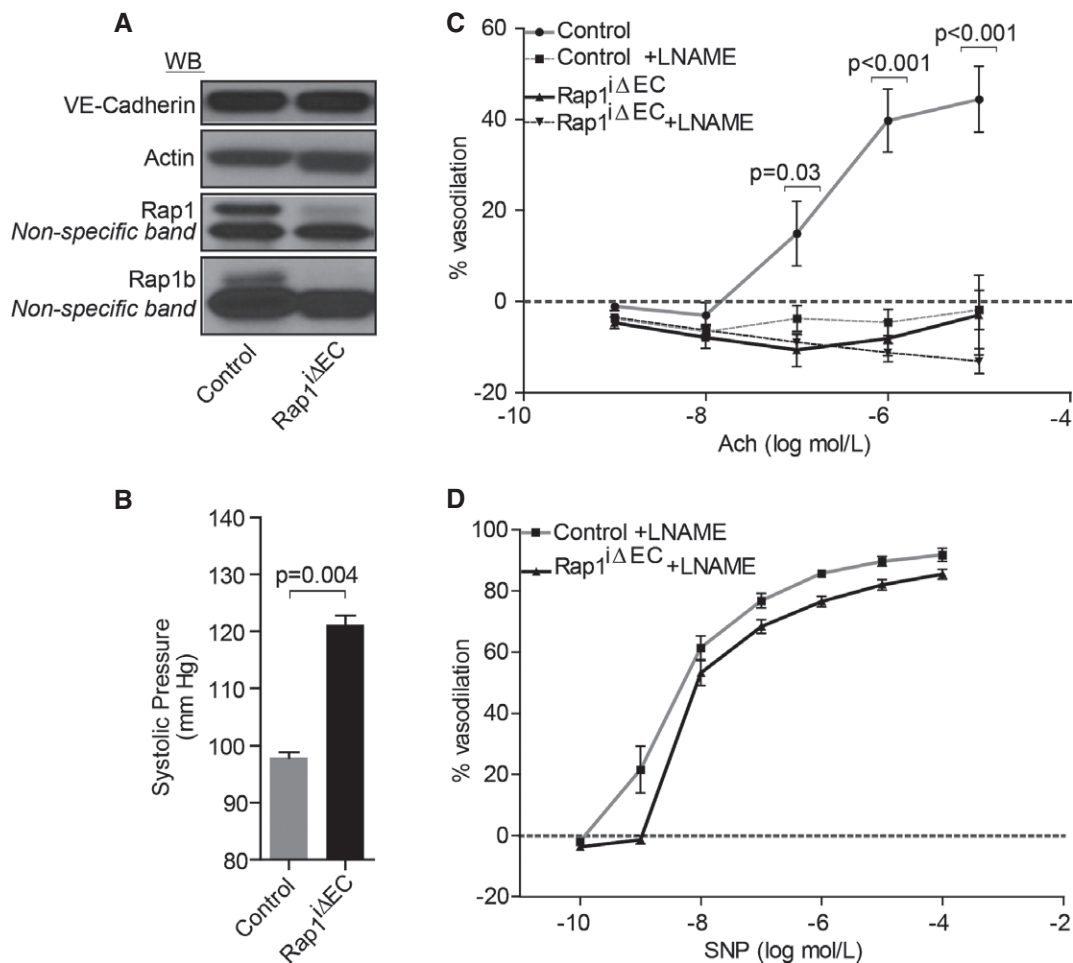
Sensed by a number of endothelial mechanoreceptors [6], including endothelial junctional mechanosensory complex comprising PECAM-1, VE-cadherin and VEGFR2, shear stress leads to Akt-dependent eNOS/Ser1177 phosphorylation [7–10]. Inhibition of VEGFR2 reduces shear-induced vasodilation *in vivo* [8,9]. Therefore, we examined shear-induced, ligand-independent VEGFR2 activation in a human EC model of Rap1 deficiency: HUVECs transfected with siRap1b [27]. Time-dependent pVEGFR2 was evident in control but not in Rap1-deficient human ECs, which failed to increase phosphorylation of the receptor and Akt-S473 and eNOS-S1177 (Fig 3B and C and Supplementary Fig S3A). Conversely, overexpression of a constitutively active mutant of Rap1b, Rap1b[G12V] led to an increased eNOS-S1177 phosphorylation in unstimulated HUVECs (Fig 3C) to the level observed in VEGF-stimulated control cells, consistent with Rap1 and VEGFR2 acting in the same pathway leading to eNOS phosphorylation.



**Figure 1. Endothelial-specific Rap1 deficiency induces hypertension and cardiac left-ventricular hypertrophy in *Tie2-Cre<sup>+/-</sup>; Rap1a<sup>+/+</sup>Rap1b<sup>-/-</sup>* (Rap1-ECKO) mice but no basal endothelial cell defects.**

**A** Quantification of heart-to-body weight ratios in controls and Rap1-ECKO mice ( $n \geq 13$ ).  
**B** Absolute values of telemetrically measured arterial blood pressure in Tie2-Cre-negative control and Rap1-ECKO mice ( $n = 4$ ).  
**C** Unchanged basal permeability in Rap1-ECKO mice. Permeability of vessels was measured following injection of Evans blue/PBS into mice. Shown is the quantification of Evans blue dye remaining in lung tissue following perfusion with PBS ( $n = 4$ ).  
**D, E** Lack of basal endothelial cell defects in Rap1-ECKO mice indicated by normal: PECAM-1 localization, Ki-67 staining (cell proliferation marker, D) and negative TUNEL reaction (apoptotic indicator, E) in Rap1ECKO aortic segments, stained *en face*. Top row: Representative images of B6F10 melanoma cells used as a positive control for proliferation and TUNEL reaction. Plasma membrane was stained with PECAM-1 or Wheat Germ Agglutinin (both shown in red). Nuclei are labeled with Hoechst 33342 (shown in blue). Goat and rabbit IgGs (D, top right) or cells without TdT labeling (E, top right) were used as negative controls. Representative images are shown ( $n = 3$ ). Arrows indicate nuclei and nuclear bodies. Scale bars, 20  $\mu$ m.

Source data are available online for this figure.



**Figure 2. Hypertension and decreased NO-dependent vasodilation in *Cadh5(PAC)-CreERT-Rap1* ( $Rap1^{IAEC}$ ) mice.**

**A** Representative immunoblot showing complete Rap1 excision in  $Rap1^{IAEC}$  mice, compared with Cre-negative controls. VE-cadherin is included as an EC marker (top blot).

**B** Tail-cuff measurements of systolic blood pressure in  $Rap1^{IAEC}$  and control mice. Values are means  $\pm$  s.e.m. ( $n = 3$ ).

**C, D** Decreased NO-dependent vasodilation of precontracted aortae from  $Rap1^{IAEC}$  mice. Dose-dependent relaxation of control and  $Rap1^{IAEC}$  aortic segments precontracted with U46619 in response to acetylcholine (ACh) in the absence or presence of L-NAME (**C**), and in response to sodium nitroprusside (SNP) in the presence of L-NAME (**D**) ( $n \geq 3$ ).

Source data are available online for this figure.

Consistent with decreased shear-induced VEGFR2 activation in Rap1-deficient ECs, association between VEGFR2 and p85 subunit of PI3K was decreased in these cells (Fig 4A), which may explain the decreased phosphorylation of Akt-S473. In contrast, VE-cadherin association with p85 was constitutive and not affected by Rap1-deficiency (Fig 4A). Together, these data show that Rap1 mediates fluid shear-induced recruitment of VEGFR2 to VE-cadherin-p85 complex and VEGFR2 activation promoting downstream signaling to phosphorylation-dependent eNOS activation and NO release from ECs.

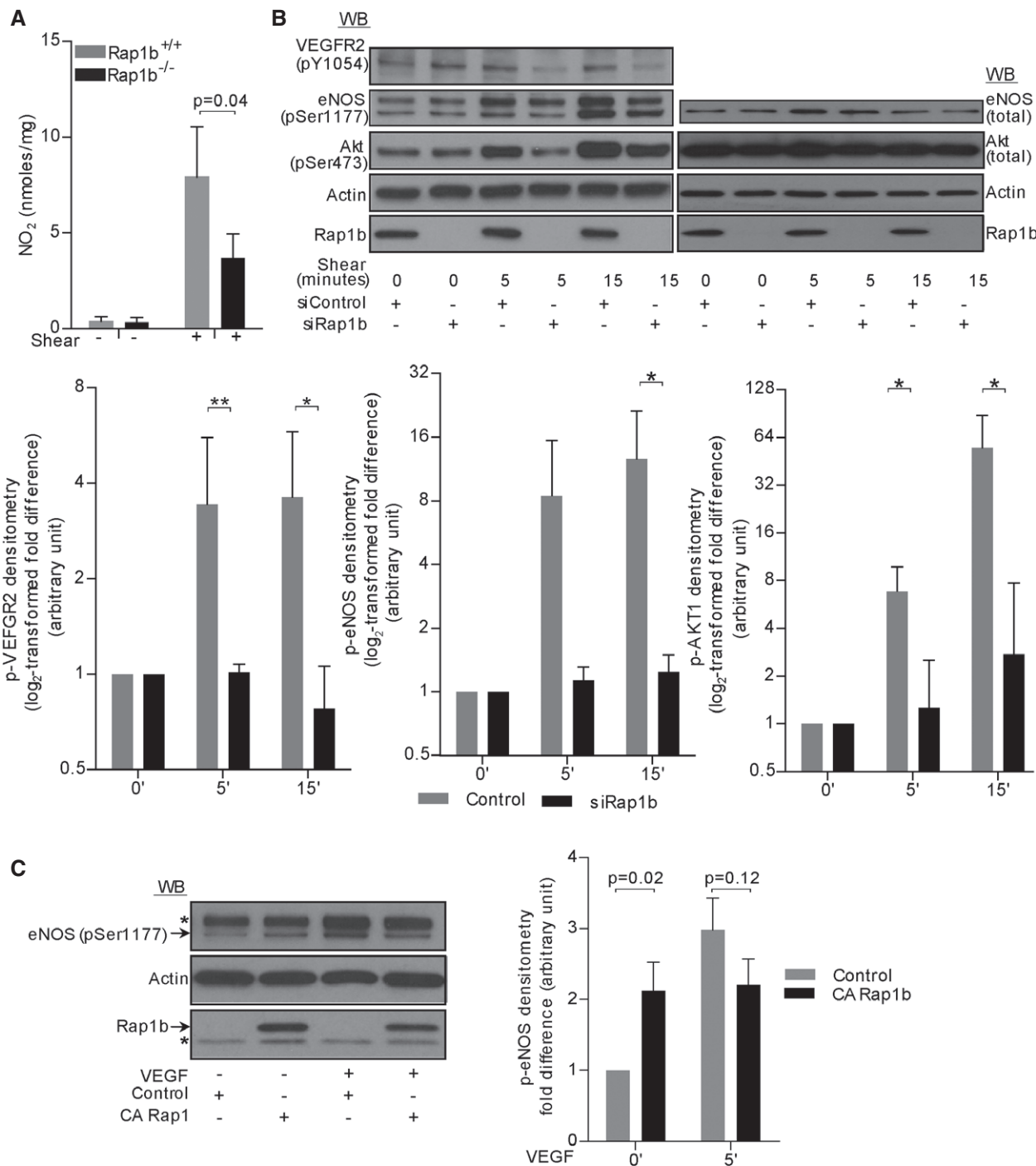
#### Function of Rap1 in the mechanosensing complex

Our results suggest that under conditions of shear, Rap1 acts upstream from VEGFR2 promoting its activation. VEGFR2 activation

in response to shear stress occurs as a result of association with PECAM-1, and, together with VE-cadherin, the three receptors form a mechanosensory complex sufficient to confer EC responsiveness to flow [9,10]. To determine the molecular basis of decreased shear-induced VEGFR2 activation in Rap1-deficient ECs, we analyzed association between PECAM-1 and VEGFR2 and found it was decreased in Rap1-deficient ECs compared with wild-type cells (Fig 4B). Phosphorylation of tyrosines within the PECAM-1 cytoplasmic domain by Src-family tyrosine kinase Fyn modulates shear stress-induced activation of eNOS [10,31]; however, it was unchanged in Rap1-deficient cells under shear stress (Fig 4B, top blot), suggesting that Rap1 acts downstream from PECAM-1.

To examine how Rap1 is involved in mechanosensing, we measured the effect of shear stress on Rap1 activity level. There is a considerable level of active Rap1 in quiescent ECs (Fig 4C,





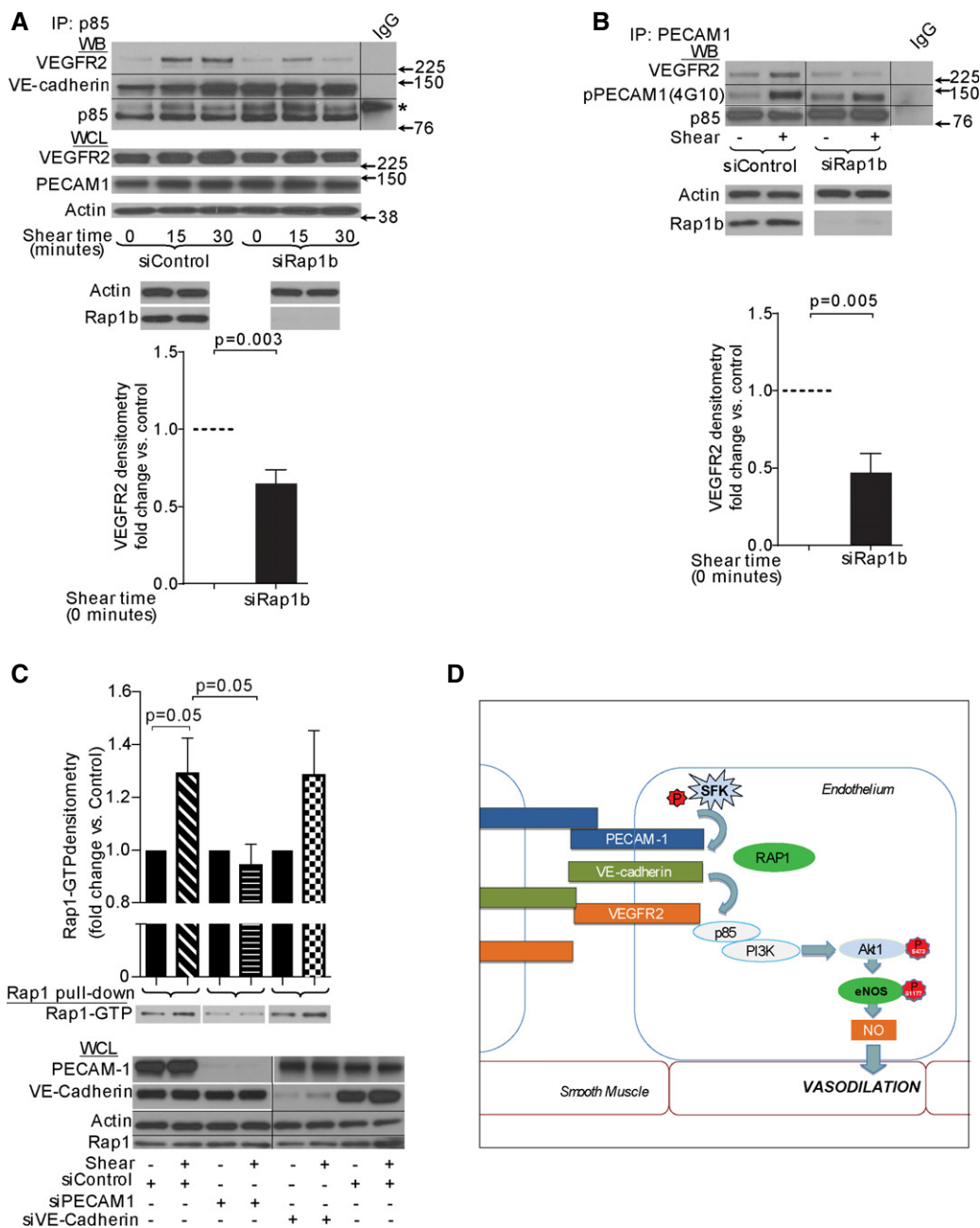
**Figure 3. Rap1 is required for shear-induced signaling leading to normal NO production.**

**A** Shear-induced NO release from cultured, quiescent or sheared ECs isolated from Rap1b-deficient or WT mice measured by nitrite-dependent chemiluminescence using an NO Analyzer. Shown are mean values of concentrations calculated using authentic standards that were normalized to total protein in each sample ( $n = 3$ ).

**B** Shear-induced VEGFR2 activation and signaling to NO production in cultured, HUVECs transfected with control, scrambled siRNA or Rap1b siRNA. Representative immunoblots of phosphorylated and total protein expression (top panel) and quantification of fold change in shear-induced phosphorylation of signaling molecules in Rap1b-deficient and control EC (bottom panel). Actin is shown as a loading control. Values are means  $\pm$  s.e.m. ( $n = 3$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ .

**C** Rap1 GOF experiment: overexpression of constitutively active (CA), Rap1b[G12V] mutant leads to elevated eNOS/Ser1177 phosphorylation in basal, but not VEGF-stimulated ECs. Representative immunoblot (top panel) and quantification of the phospho-eNOS band (bottom panel), normalized to actin. Exogenous expression of HA-tagged CA Rap1b was confirmed by Rap1b blot (top band) and HA immunoblot (data not shown). Values are means  $\pm$  s.e.m. ( $n = 3$ ). \* indicates a non-specific band.

Source data are available online for this figure.



**Figure 4. Rap1 is required for shear-induced formation of the junctional mechanotransduction complex and eNOS activation.**

A, B Interaction between VEGFR2 and p85-PI(3)K (A) or PECAM-1 (B) in HUVECs transfected with control, scrambled siRNA or Rap1b siRNA. Representative immunoblots (top panels) and quantification (bottom panels) of VEGFR2 in p85-PI(3)K (A), or PECAM-1 (B) immunoprecipitations from EC lysates. Basal and shear-induced (15-min treatment) levels are shown. Shear-induced PECAM-1 phosphorylation, detected by anti-phosphotyrosine mAb, 4G10, is not affected by siRap1b (B, middle row). p85 is shown as loading control. WCL: Immunoblots of EC lysates are shown to illustrate equal protein loading (actin, VEGFR2, PECAM-1 and VE-cadherin) or Rap1b knockdown efficiency and specificity. Values are means  $\pm$  s.e.m. ( $n \geq 4$ ). \* indicates a non-specific band. Black lines indicate where the PVDF membrane was cut to blot with specific antibodies, as indicated.

C Shear-induced activation of Rap1 is blocked by siPECAM-1, but not siVE-cadherin. Quantification (top panel) and representative Rap1 immunoblot (middle panel) of RalGDS-bound Rap1-GTP from lysates of HUVECs transfected with control, PECAM-1 or VE-cadherin-specific siRNA and sheared for 1 min. Bottom panel, top row: representative PECAM-1 and VE-cadherin immunoblot demonstrating siRNA knockdown efficiency in transfected HUVECs. Values are means  $\pm$  s.e.m. ( $n = 3$ ). Black lines indicate where the PVDF membrane was cut to blot with specific antibodies, as indicated.

D A model of Rap1 function in transducing shear stress signals leading to phosphorylation-dependent eNOS activation. Upon shear stress, activated downstream from Src-family kinase (SFK) and PECAM-1 [52], Rap1 promotes increased association between PECAM-1 and VEGFR2, and ligand-independent VEGFR2 transactivation in the junctional mechanosensing complex [9]. Such ligand-independent VEGFR2 activation promotes signaling to its downstream effectors, pAkt1 and eNOS, resulting in increased NO release and NO-dependent vasodilation [8].

Source data are available online for this figure.

Supplementary Fig S3A), which was transiently yet significantly increased in response to shear stress, with a peak within 30 s from initiation of flow (Fig 4C). Such rapid activation of Rap1 has been reported to occur in hematopoietic cells in response to turbulence [32]. In endothelium, this timeframe is consistent with flow-induced VEGFR2 transactivation [9]. Further, to investigate where in the sequence of events mediating EC mechanosensing Rap1 becomes activated, we analyzed Rap1 activity upon siRNA knockdown of PECAM-1 or VE-cadherin. We found that shear-induced Rap1 activation was attenuated in PECAM-1-deficient but not in VE-cadherin-deficient ECs (Fig 4C).

Based on our current report and previous findings in isolated cells, we propose the following model (Fig 4D). In response to shear stress, Rap1 is rapidly and transiently activated downstream from PECAM-1, and through a GEF likely distinct from Epac, as treatment of ECs with 8-pCPT-2'-O-Me-cAMP (007), a cyclic adenosine monophosphate analog that directly activates Epac, failed to induce eNOS activation (Supplementary Fig S3B) [33]. Active Rap1 promotes association of PECAM-1 with VEGFR2, which leads to ligand-independent VEGFR2 transactivation and triggers signaling for NO release to drive vasodilation. Rap1-dependent regulation of mechanotransduction pathways in the vasculature has not been previously considered. However, published data suggest that this novel paradigm of Rap1 involvement in transducing mechanical signals may also be applied to other cell types; Rap1 activation in response to turbulence has been reported in hematopoietic cells [32] and in response to stretching in permeabilized fibroblasts [34]. It will be important, however, to establish *in vivo* relevance of Rap1 mechanotransduction in these cell types. While exact molecular mechanisms of Rap1 mechanotransduction in endothelium need to be elucidated, our report reveals a physiological significance of this function: release of NO in response to shear stress of flowing blood.

### Novel paradigms of Rap1 signaling

Previous studies of Rap1 function in ECs have shown its importance in promoting integrin- and cadherin-mediated adhesion [29], functions important for angiogenesis and maintenance of vascular integrity [17,35]. *In vivo* analysis of endothelium-restricted Rap1-deficient mice demonstrates that Rap1 is particularly important for signaling by the adhesion molecules rather than their adhesive functions. Consistently, Rap1-deficient ECs are able to form grossly normal monolayers *in vivo* (Fig 1D and E and Supplementary Fig S1) without a deleterious effect on vessel permeability (Fig 1C) or gross vessel morphology. In contrast, other signaling functions of adhesion receptors are severely perturbed, as we demonstrate here in the context of shear stress sensing by the mechanotransduction complex; Rap1 acting downstream from PECAM-1 and upstream from VE-cadherin is critical for VEGFR2 transactivation and signaling to NO release. Interestingly, we have previously demonstrated that Rap1 acting upstream from ligand-dependent VEGFR2 activation promotes angiogenesis in a process dependent on integrin  $\alpha_v\beta_3$  [27]. Therefore, Rap1-dependent-signaling converging on VEGFR2 activation is required for multiple EC responses. In addition, our novel discovery of Rap1's involvement in transducing mechanical signals may help explain other Rap1-related phenotypes; specifically, the role of Rap1 in hemodynamic regulation of vessel stability

may be relevant for cranial hemorrhages observed in CCM and in a mouse model of Rap1 deficiency [17,26]. Furthermore, the implications of our findings may reach beyond the cardiovascular system as Rap1 is ubiquitously expressed and may be involved in transmitting mechanical forces in other tissues.

### Functional significance of our findings for human health

The significance of our findings is underscored by the phenotype of Rap1-deficient mice, which includes endothelial dysfunction and hypertension. The extent of the defect: severely diminished vasodilation in aortic segments from Rap1<sup>iAEC</sup> mice (Fig 2C) and sudden lethality of ~50% young male mice suggest that this may be physiologically the most critical Rap1 function in the cardiovascular system described so far [16,18–20,36,37]. Our studies with human ECs implicate Rap1 as an important regulator of NO signaling and suggest that perturbation of Rap1 activity may be linked with human disease. Interestingly, a genome-wide expression profiling study reported upregulated expression of a negative regulator of Rap1 activity, Rap1GAP, in lungs of patients with idiopathic pulmonary hypertension [38], a change that would be expected to lead to decreased Rap1 activity and increased airway resistance. Furthermore, endothelial dysfunction, in addition to hypertension, is linked with other endothelial pathologies, including inflammation, atherosclerosis and progression of diabetes. While additional studies exploring these functional consequences of endothelial dysfunction are required, and the exact Rap1 function in different vascular beds should be examined, our current study defines a novel framework for analysis of Rap1 function in the cardiovascular system and opens up the possibility for new therapeutic targets.

In conclusion, Rap1 critically regulates eNOS, NO release and endothelial function and plays a key role in maintenance of normal vascular tone. Granted the significance of NO for multiple cardiovascular functions, our findings suggest a broader impact of Rap1 signaling in the cardiovascular system. Further, by identifying mechano-effector functions of Rap1, our study also defines a novel conceptual framework for analysis of Rap1 biology.

## Materials and Methods

### Chemicals

Unless indicated, chemicals were purchased from Sigma-Aldrich. RNAiMAX was from Life Technologies. Antibodies used are described in Supplementary Methods.

### Genetic mouse models

All mouse procedures were performed according to approved Medical College of Wisconsin Institutional Animal Use and Care Committee protocol 00001206. Generation of endothelial lineage-restricted Rap1 knockout mice (*Tie2-Cre<sup>+/0</sup>;Rap1a<sup>+/+</sup>Rap1b<sup>fl/fl</sup>*; Rap1-ECKO) has been previously described [27]. Inducible endothelial-restricted (Rap1<sup>iAEC</sup>) mice were obtained by crossing mice containing floxed *Rap1a* and *Rap1b* alleles (*rap1a<sup>fl/fl</sup>rap1b<sup>fl/fl</sup>* mice) [39] with *Cdh5(PAC)-CreERT2* mice and inducing Cre activity with tamoxifen injections, as previously

described [40]. Mice were euthanized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) followed by cervical dislocation. Because estrogen is known to have protective effects on regulation of blood pressure and, in particular release of NO [41], 3- to 6-month-old Rap1-ECKO and 2- to 4-month-old Rap1<sup>iAEC</sup> male mice on mixed C57Bl6/129 Sv/Ev genetic background were used for blood pressure measurements and vasodilation studies.

### Heart isolation

Isoflurane-anesthetized mice were weighed, and hearts were perfused with PBS. Dissected hearts were thoroughly rinsed in PBS, blotted quickly and weighed, and heart-to-body weight ratio was calculated [42].

### Blood pressure measurements

Blood pressure and heart rate measurements were performed using telemetry and/or tail cuff, as described previously [28,43,44] and in Supplementary Methods. Tail-cuff measurement was performed because of the poor surgery survival of Rap1-deficient mice.

### In vivo lung vessel permeability

Vascular permeability to Evans blue-labeled BSA was determined essentially as described earlier [45]. Briefly, sterile 0.5% Evans blue/4% BSA in PBS was injected retro-orbitally into deeply anesthetized mice. After 1 h, mice were perfused with 5 mM EDTA/PBS, lungs were harvested and Evans blue dye was extracted with formamide at 60°C for 24 h. Concentration of the dye was determined by measuring absorbance at 620 nm, and the amount of Evans blue was calculated from the prepared standard curve.

### Analysis of endothelial proliferation and apoptosis in isolated vessels

*En face* immunohistochemical and TUNEL staining was performed as described in Supplementary Methods [46].

### Confocal microscopy

*En face* preparations and B6F10 cells were imaged using Laser Scanning Olympus FluoView1000 confocal microscope with 60×/1.2 water immersion objective lens. Images were recorded from five random fields within a sample, and at least 100 cells were analyzed using Olympus confocal software.

### Endothelium-dependent relaxation of aortic rings in response to acetylcholine

Vasodilation was measured as previously described [28,47]. Briefly, the thoracic aorta from control and Rap1-mutant mice was dissected and placed in Krebs physiological saline aerated with 21% O<sub>2</sub>/5% CO<sub>2</sub>. 1.5-mm segments were mounted on parallel pins in conventional myographs and maintained at optimal tension in Krebs buffer for 60 min at 37°C. The rings were precontracted with 10 nM U46619, exposed to increasing concentration of acetylcholine in the

absence or presence of 100 μM L-NAME, and changes in vascular tension were recorded.

### Cell culture, shear stress experiments and transfections

ECs isolated from neonatal Rap1b<sup>-/-</sup> mouse lungs [48] were used as cellular models of Rap1 deficiency in ECs, as Rap1b is the predominant Rap1 isoform in these cells [27]. To demonstrate relevance for human biology, human umbilical vein ECs (HUVECs) were used, as an established model to study NO release and signaling in response to shear [9]. HUVECs were isolated in-house from discarded umbilical cords obtained from local hospitals. Because the tissue was completely de-identified at the hospitals, our use does not constitute human subject research and therefore does not require approval of a local ethics review board. As the discard tissue came de-identified from multiple sources, we are unable to verify consent. Both mouse and human ECs were cultured in Vasculife EnGS Medium Complete (Lifeline Cell Technology). Mouse ECs were used for shear-induced NO release studies [49]. For biochemical analysis, 30–40% confluent cultures HUVECs, passage 3 or fewer, were transfected with 50 nM Rap1b, PECAM-1 or VE-cadherin siGENOME siRNA pool (Dharmacon) or with scrambled siRNA using Opti-MEM medium and RNAiMAX reagent (Life Technologies) for 6 h and cultured for additional 48 h in Vasculife EnGS complete medium, until cells reached confluency. ECs were then serum-starved for 4 h in Vasculife EnGS basal medium, and NO release or mechanosignaling was analyzed as follows. For analysis of mechanotransduction, HUVECs were placed in Vasculife EnGS basal medium with 5% dextran and, using a cone viscometer, subjected to shear stress at 10–15 dynes/cm<sup>2</sup> for indicated times. For a gain-of-function (GOF) experiment, HUVECs were transfected with lentiviral particles expressing constitutively active (CA) Rap1 (Rap1b[G12V] mutant) at five multiplicity of infection, cultured for 48 h including 6 h of starvation, as described above and lysed (no treatment) or stimulated with 40 ng/ml VEGF A, a potent inducer of eNOS phosphorylation [11] for 5 min. Experiments were terminated by cell lysis on ice followed by immunoprecipitations, Western blotting and Rap1 activity assay [50] as described in Supplementary Methods.

### Measurement of nitric oxide concentration

NO release from sheared mouse lung ECs was analyzed by monitoring 4,5-diaminofluorescein-2 triazole (DAF-2T) formation from DAF-2 diacetate (DAF-2DA) by chemiluminescence methodology using an NO Analyzer [51]. Detailed experimental procedure is described in Supplementary Methods.

### Statistical analysis

Mean values and standard error of mean (s.e.m.) were obtained from three or more independent experiments, as indicated. Statistical significance of group differences was determined using two-sample Student's *t*-test (Figs 1A–C and 2B), one-sample *t*-test (Figs 3C and 4A and B) and repeated measures ANOVA (Figs 2C and D and 4C), as appropriate for the experimental design. The Holm–Sidak adjustment for multiple comparisons was used within



each plot. The assumptions of the tests were evaluated using tests of normality and equal variance; in cases where data did not follow Gaussian distribution, biologically meaningful Box-Cox family transformations were examined. Specifically, densitometry values were log<sub>2</sub>-transformed before statistical analysis. For analysis of shear-stress-induced signaling data, within each experiment, fold change in phosphorylation induction between Rap1-deficient and control values were calculated for each condition and such obtained values were statistically analyzed as described above. All tests were two-sided, and a 5% overall significance level was used.

**Supplementary information** for this article is available online: <http://embor.embopress.org>

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### Author contributions

MC-W conceived of experiments. SL, MS, DXZ and MC-W designed the research. SL and MC-W analyzed the data. XZ, YN and DXZ performed and analyzed vasodilation experiments. SL and YN performed and analyzed blood pressure measurements. MS performed and analyzed *in vivo* permeability experiments. SL performed and analyzed endothelial cell experiments. SL and JV-V performed and analyzed nitric oxide measurement experiments. AS statistically analyzed data. MC-W wrote the manuscript. SL, AS, JV-V and DXZ critically read and edited the manuscript.

### Conflict of interest

The authors declare that they have no conflict of interest.

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