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

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SUPPLEMENTARY METHODS

Antibodies. Antibodies to Rap1 (rabbit polyclonal; #4938), Rap1B (clone: 36E1; rabbit mAb; #2326), eNOS (rabbit polyclonal; #9572) and phospho-eNOS-Ser1177 (clone: C9C3; rabbit mAb; #9570), phospho-Akt-Ser473 (clone: D9E; rabbit mAb; #5012), VEGFR2 (clone: 55B11; rabbit mAb; #2479) were from Cell Signaling. Antibodies to actin (I-19; goat polyclonal; #sc-1616), agarose-conjugated VEGFR2 (clone: A-3; mouse mAb; #sc-6251), p85 (clone: D-9; mouse mAb; #sc-374534) and VE-cadherin (clone: C-19; goat polyclonal; #sc-6458) were from Santa Cruz Biotechnology. Antibody to phospho-VEGFR2-Tyr1054/1059 (I-19; rabbit polyclonal; #44-1047G) was from Life Technologies. Antibody to phosphotyrosine (clone: 4G10 platinum; mouse mAb; #05-1050) was from EMD Millipore. Antibody to PECAM-1 (clone 1.3) was a generous gift from P. Newman (Blood Research Institute). PECAM-1 goat polyclonal antibody (#AF-3628) was from R&D systems. *Ki-67* (rabbit polyclonal; #AB-15580) was from Abcam. All HRP-conjugated secondary antibodies were from Jackson ImmunoResearch.

Blood pressure and heart rate measurements were performed using telemetry and/or tail cuff, as described previously [1-3]. For telemetric blood pressure measurement, mice were pre-anesthetized with isoflurane and then deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.). An implantable telemetric device (TA11PA-C20 BP; Data Sciences International) was implanted in the carotid artery. Both antibiotic (30 mg/kg cefazolin) and analgesic (0.1 mg/kg Buprenex, s.c. or 5mg/kg Carprofen, s.c.) were administered post surgically, and the mice were allowed to fully awaken from anesthesia on a temperature-controlled pad. The mice were allowed to recover for 3 days following surgery. Blood pressure and heart rate measurements were obtained on post surgical days 4–6. For tail cuff blood pressure measurements, systolic blood pressure was determined bi-weekly in conscious 8- to 12- wk old animals using IITC Life Sciences Blood Pressure System. Mice were acclimated to the blood pressure chamber three times a week starting at 6 wk of age. Recordings were taken after 30 minutes of acclimation. Three to six successful measurements were averaged as a single data point. For some groups of animals, we validated this method by comparing the values obtained with those from telemetry, performed in the same animals.

Analysis of endothelial proliferation and apoptosis in isolated vessels. *En face* immunohistochemical staining was performed as previously described [4]. Briefly, mouse thoracic aorta was perfusion-fixed with 100 ml of freshly prepared 4% paraformaldehyde at 70 mm Hg. The aorta was placed in Krebs physiological saline and carefully dissected under the microscope. The aortic segments were cut longitudinally

and pinned flat with EC facing up on silicone elastomer (Sylgard 184)-coated Petri dish. The segments were briefly washed in PBS, permeabilized with 0.3% Triton® X-100 for 40 minutes at RT and blocked with 10% FBS/PBS for 60 min at RT. The segments were incubated overnight at 4ºC with anti-mouse CD31 (goat polyclonal; R&D systems; 1:100 dilution) and anti-*Ki-67* (rabbit polyclonal, Abcam, 1:100 dilution in 1% bovine serum albumin (BSA), 1% normal FBS, 0.3% Triton X-100 in PBS) antibodies. Anti-rabbit and anti-goat IgGs (Jackson Immunoresearch) were used as IgG controls. Following primary antibody treatment, segments were washed trice in PBS and incubated with donkey anti-goat Alexa Fluor 594- or anti rabbit Dylight 647-conjugated secondary antibodies for 1 hour at RT. Following two washes, aortic strips were incubated with Hoechst 33342 dye (1:1000; Invitrogen) for 15 minutes. The segments were carefully transferred to superfrost microscopic slide (Fischer Scientific) and mounted with coverslip and ProLong Gold anti-fade reagent (Invitrogen).

As a positive control for proliferation, fast growing B6F10 melanoma cells, a kind gift of Dr. Sam Hwang, cultured as previously described [5] were plated onto sterilized coverslips. After 24 hours of culture, the cells were washed twice with PBS and fixed for 20 minutes at RT with freshly prepared 4% paraformaldehyde. Cells were washed twice in wash buffer (0.1% BSA in 1X PBS) and incubated with blocking buffer (10% normal FBS, 0.3% Triton® X-100) for 45 minutes at RT. Cells were incubated overnight with rabbit polyclonal *Ki-67* antibody, prepared as described above, at 4ºC. Anti-rabbit IgG was used as secondary-antibody-only control. Cells were washed trice with wash buffer and incubated with Dylight 647 goat anti-rabbit IgG at 1:200 dilution for 1 hr at RT protected from light. Following two washes, cells were incubated with Hoechst 33342 and wheat germ agglutinin (1:1000; Invitrogen) for 15 minutes. Finally, cells were rinsed with 1X PBS and briefly in distilled water. Coverslips were carefully mounted on superfrost microscope slide with ProLong Gold anti-fade reagent.

For analysis of apoptosis, *en face* aortic preparations were subjected to TUNEL assay using In Situ Cell Death Detection Kit, following manufacturer's protocol (Roche). As a positive control for TUNEL reaction, B6F10 melanoma cells were treated with *DNase I* (Invitrogen; 20 units/ml) for 10 minutes. B6F10 cells treated without TdT labeling served as negative control. At completion of TUNEL reaction, cells or aortic segments were incubated with Hoechst 33342 and carefully mounted on microscopic slides as described earlier in *Ki-67* staining procedure.

Western blotting, immunoprecipitations and Rap1 activity assay. Following stimulation with Ach or bradykinin, exposure to shear stress, VEGF or 8-pCPT-2′-O-Me-cAMP (007), a cyclic adenosine monophosphate analog specific for Rap1 guanine nucleotide exchange factor, Epac, ECs were rapidly washed once with ice cold PBS and lysed with lysis buffer (0.001M EDTA, 0.15M NaCl, 1% NP-40, 5% glycerol in 0.025M Tris-pH 7.4, 1X protease/phosphatase inhibitor cocktail). Cell lysates were spun at 13K rpm for 5 minutes at 4ºC and supernatants were stored at -80ºC. Total lysates were resolved on 4-12% gradient gel and blotted using specific antibodies, as indicated. Band intensity was determined by densitometry of X-ray film, values obtained for phosphoproteins were normalized to actin in the same sample and phosphorylation fold induction over the basal phosphorylation level was determined. For immunoprecipitation, cell lysates were pre-cleared with mouse IgG (2µg/100µg of

protein), followed by protein A/G sepharose for 1 h at 4ºC and incubated with mouse monoclonal p85, a PI(3)K subunit, antibody or mouse monoclonal PECAM-1 (2µg/100µg of protein) for 3 h followed by protein A/G sepharose for 1 h at 4ºC. Immune complexes were washed three times in lysis buffer and eluted in 1X LDS sample buffer followed by Western blot analysis. p85-associated VEGFR2 was detected by VEGFR2 antibody and normalized to total p85. PECAM-1 phosphorylation was assessed by anti-phosphotyrosine blot in PECAM-1 specific immune complex. Active, GTP-bound fraction of Rap1 in lysates of HUVECs subjected to shear stress was measured using a RalGDS-GST pull-down method, as previously described [6].

Measurement of Nitric Oxide Concentration. NO release from Ach- or bradykinin-stimulated HPAECs or sheared mouse lung ECs was analyzed by monitoring 4,5-diaminoflourescein-2 triazole (DAF-2T) formation from DAF-2 diacetate (DAF-2DA) by HPLC and by chemiluminescence methodology using an NO Analyzer, respectively [7]. For analysis of Ach- and bradykinin- stimulated NO release, HPAECs were incubated with 5 µM DAF-2DA in culture medium for 30 min and Ach or bradykinin were added to stimulate NO production for additional 30 min. Cells were washed twice, pelleted and suspended in 150µl phosphate buffer (10 mM, pH 7.5) with 0.1 mM DTPA and 0.1% Triton X-100. Cell were syringe-lysed and an aliquot was taken for protein determination and the remaining lysate was loaded onto a Centricon spin column (10,000 MWC, Millipore) and centrifuged for 1hr at 10,000 rpm at 4ºC. The eluent (50 µl) was loaded onto a Synergi fusion analytical column (Phenomenex) and isocratic eluted using an aqueous mobile phase consisting of potassium phosphate buffer (pH 7.5, 10 mM) and acetonitrile (2% by volume) at a flow rate of 1 ml/min. Authentic DAF-2T was used as a standard for quantification using fluorescent detection at 490 nm excitation and 515 nm emission. It is important to note that while the exact mechanism of reaction between NO and DAF-DA is not fully understood, radical recombination mechanisms between DAF-2 anilinyl radical intermediate and NO is a likely mechanism [7]. The accumulation of NO-derived NO_2- (nitrite) over 6h of shear from mouse ECs allowed the use of chemiluminescence for detection of NO. Briefly, monolayers of mouse lung ECs were grown in 100 mm tissue culture-treated dishes. Before applying shear stress, cells were rinsed 3 times with basal EC medium to remove growth factor supplements followed by an addition of 15 ml fresh basal EC media containing 5% dextran and incubated at 37ºC. Cell media samples (1 mL) were collected from cell culture dishes before stimulation by shear stress (for measurement of NO without stimulation) and after shear for 6h (for measurement of NO formation with stimulation). Blank samples, i.e., without cells, were prepared following the same protocol to account for nitrite contamination in the reagents. Samples were analyzed immediately after collection by detecting NO-dependent chemiluminescence after reduction of nitrite (NO_2^-), an oxidation product of NO, with triiodide reducing reagent (50 mg/ml KI in water mixture with ~4ml glacial acetic acid) at room temperature. Calibration plots were built using nitrite (1-5µM) and were examined at the beginning and end of the analysis. In both methods, concentrations of NO were calculated using calibration plots and values normalized to total protein in each dish.

Statistical analysis. For analysis of vasodilation a Gompertz dose response curve with ACh/SNP concentration on the log10 scale was fitted to the data from each strip, and the estimates of EC_{50}, the slope coefficient, and the maximum response were obtained.

Each parameter was analyzed over the experiments using a linear mixed effects model with a fixed genotype effect and random animal effects [8].

SUPPLEMENTARY REFERENCES

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Legends

Figure S1 - Lack of basal endothelial cell defects in *Tie2-Cre*$^{+/0}$; *Rap1a*$^{f/+}$ *Rap1b*$^{f/f}$ (Rap1-ECKO) aortae indicated by normal PECAM-1 localization and negative TUNEL reaction staining for apoptosis (B). Note: neither *Tie2-Cre*-negative control nor Rap1-ECKO aortic segments stain with Ki67 proliferation marker (A), indicating quiescent status of ECs. Endothelial junctions are visualized with PECAM-1 (shown in *red*); nuclei are labeled with Hoechst 33342 (shown in *blue*). IgG-only stained cells were used as negative controls. Representative images are shown. (n=3). Scale bar, 20 μm.

Figure S2 - Dose-dependent relaxation responses in U46619-preconstricted WT and Rap1$^{i\Delta EC}$ (KO) aortae in response to SNP. Relaxation is expressed as a percent maximal dilation relative to U46619 constriction. Plotted are actual values; lines are fit plots obtained using 3-parameter Gompertz or logistic models. Dashed lines show the ED50 estimates obtained on each experimental day. (n=3).

Figure S3 – eNOS activation in HUVECs.

A Rap1 activation precedes that of pAkt and eNOS in sheared ECs. HUVECs were exposed to shear stress for indicated times and protein phosphorylation (two top blots) was determined by WB, as indicated. Rap1 activity was determined using RalGDS-GST pulldown; Rap1 immunoblot of EC lysates is shown as a loading control (bottom blots).

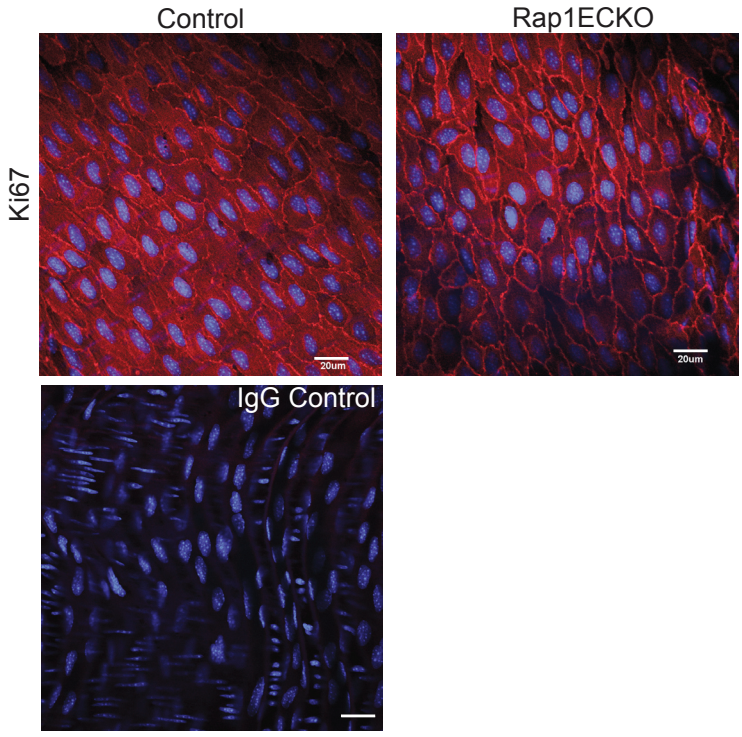
B 8-pCPT-2′-O-Me-cAMP (007), a cyclic adenosine monophosphate analog specific for Rap1 guanine nucleotide exchange factor, Epac, activates Rap1 but fails to induce eNOS activation in HUVECs. Top panel: the kinetics of Rap1 activation by 007 in HUVECs, determined by RalGDS-GST pulldown. Bottom panel: eNOS/P-Ser1177A induction occurs in response to VEGF (40 ng/ml, 5 min, a positive control), but not in response to Epac treatment. Equal protein loading is shown by actin WB (top panels). Shown are representative Western blots and a quantification of fold change (n=4).

Table S1 – Relaxation of control and Rap1$^{i\Delta EC}$ aortic segments preconstricted with U46619 in response to acetylcholine (ACh) in the absence L-NAME. Actual force values recorded by the myography transducer and normalization calculations are shown. Normalized data is presented in Figure 2C.

Table S2 – Relaxation of control and Rap1$^{i\Delta EC}$ aortic segments preconstricted with U46619 in response to sodium nitroprusside in the presence of L-NAME (SNP-l). Actual force values recorded by the myography transducer and normalization calculations are shown. Normalized data is presented in Figure 2D.

Figure S1

A


Control
Rap1ECKO
Ki67
20um
20um
IgG Control

B

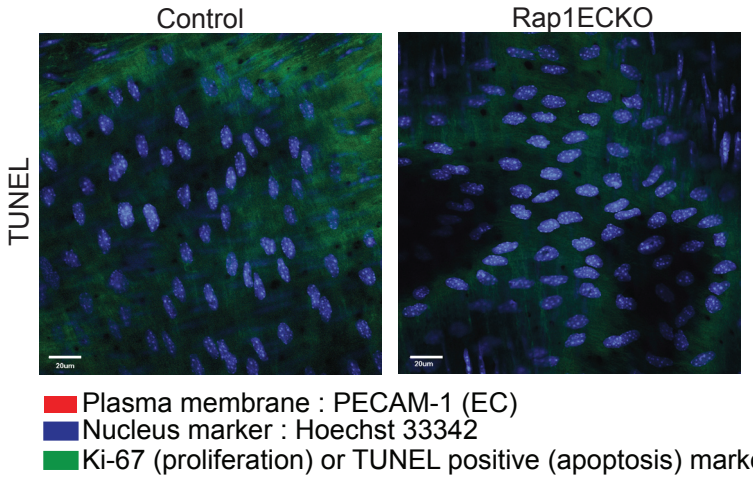

Control
Rap1ECKO
TUNEL
20um
20um
Plasma membrane : PECAM-1 (EC)
Nucleus marker : Hoechst 33342
Ki-67 (proliferation) or TUNEL positive (apoptosis) marker

Figure S2

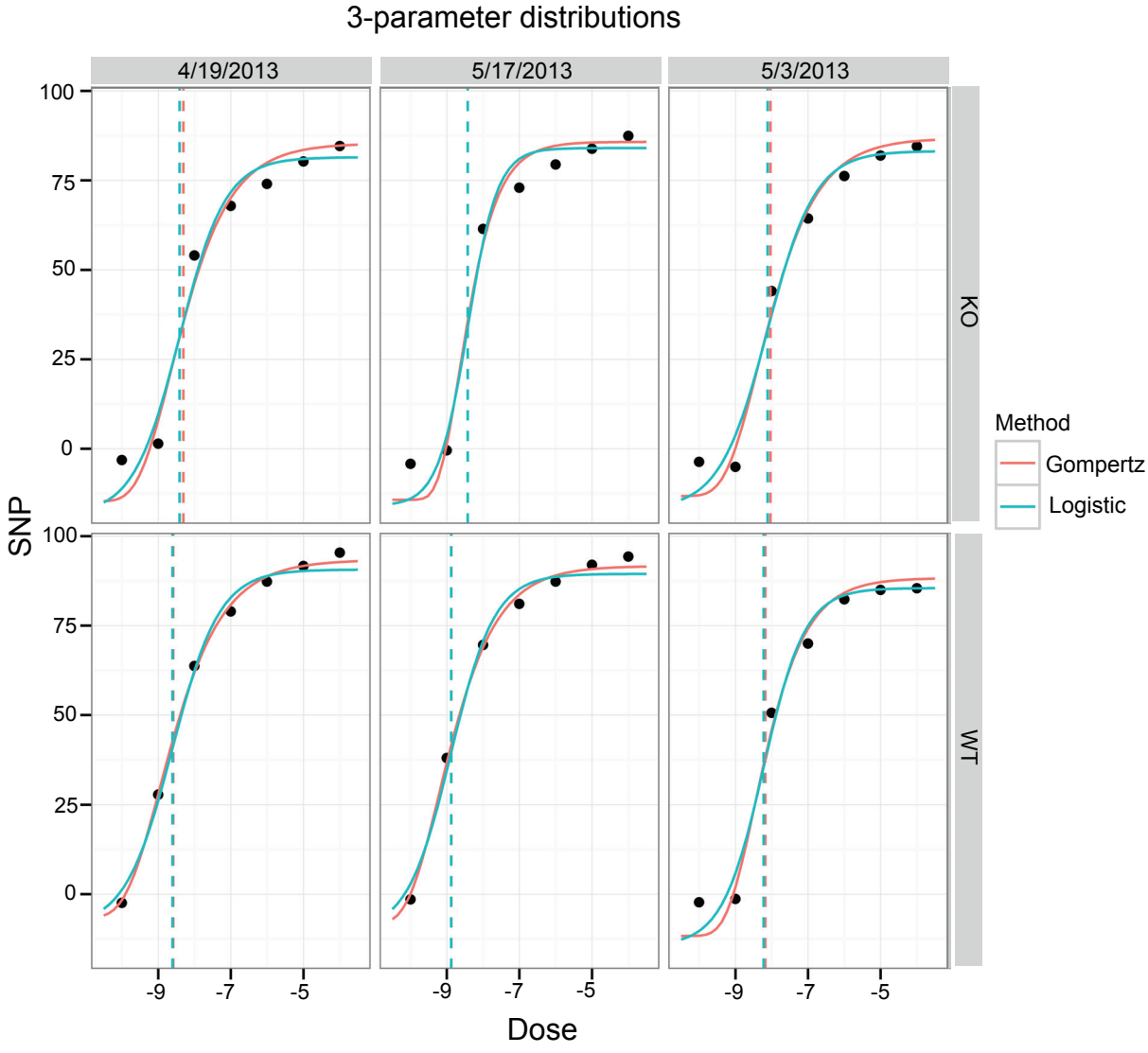
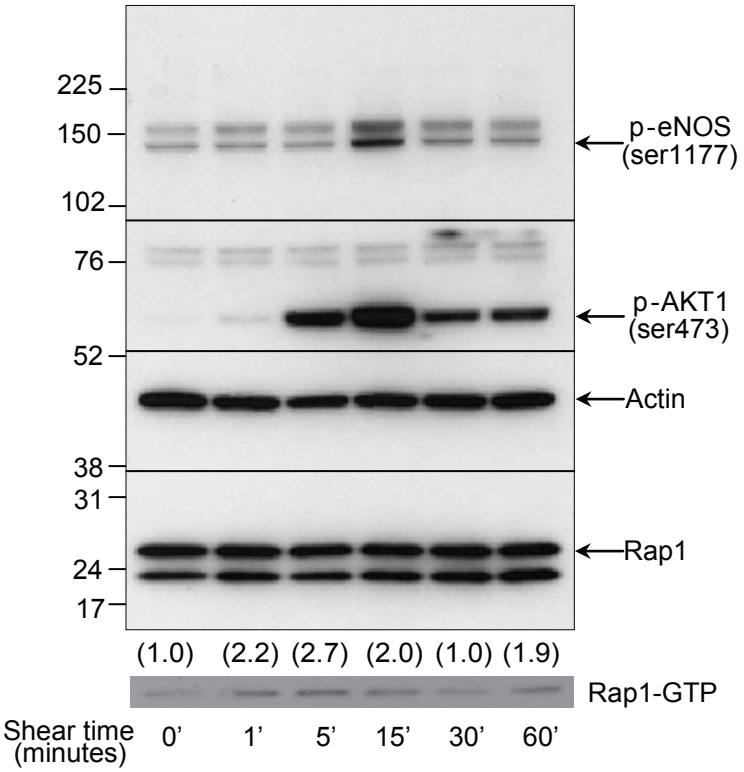

3-parameter distributions
4/19/2013
5/17/2013
5/3/2013
KO
WT
SNP
Dose
100
75
50
25
0
-9
-7
-5
Method
Gompertz
Logistic

Figure S3

A


225
150
102
76
52
38
31
24
17
p-eNOS (ser1177)
p-AKT1 (ser473)
Actin
Rap1
(1.0) (2.2) (2.7) (2.0) (1.0) (1.9)
Rap1-GTP
Shear time (minutes) 0' 1' 5' 15' 30' 60'

B

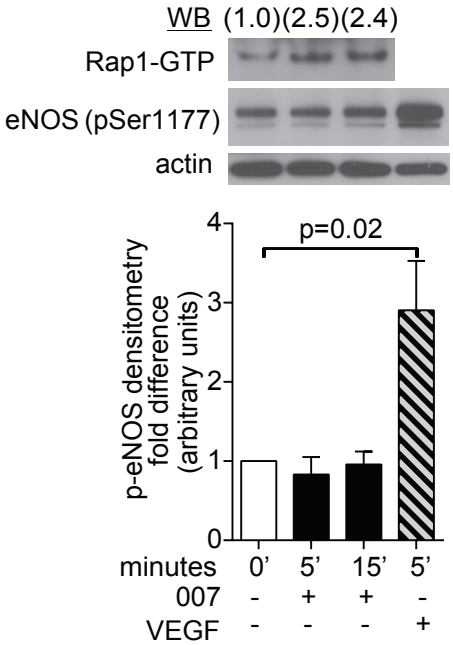

WB (1.0)(2.5)(2.4)
Rap1-GTP
eNOS (pSer1177)
actin
p=0.02
p-eNOS densitometry fold difference (arbitrary units)
0
1
2
3
4
minutes 0' 5' 15' 5'
007 - + + -
VEGF - - - +

Table S1 – Relaxation of control and Rap1iΔEC aortic segments preconstricted with U46619 in response to acetylcholine (ACh).

ACH

			RAW DATA						Preconstriction		Dose response curve				
Experiment Date	SAMPLE ID		U46 nM	**Max Tens.**	**Min Tens.**	Distend. Tens.	KCl PRE	KCl POST	U46 PRE	U46 POST	Ach 10-9M	Ach 10-8M	Ach 10-7M	Ach 10-6M	Ach 10-5M
CONTROL															
4/19/2013	1	N110+/+	30.0	9.81	1.12	1.50	1.43	3.50	1.12	5.88	5.96	6.10	5.96	3.23	2.99
	2	N110+/+	30.0	10.18	1.39	1.50	1.72	3.73	1.39	6.13	6.05	5.68	3.90	3.18	3.07
5/3/2013	1	N163+/+	20.0	16.40	1.13	1.50	1.50	4.56	1.21	8.79	9.01	9.22	8.48	6.98	6.93
	2	N163+/+	20.0	18.99	1.36	1.50	1.36	4.56	1.60	9.55	9.88	10.28	9.01	5.91	5.17
5/17/2013	1	N213+/+	20.0	14.51	1.49	2.00	1.96	4.81	1.49	8.88	8.88	8.88	7.82	7.22	7.07
	2	N213+/+	20.0	12.86	1.80	2.00	2.08	4.98	1.89	7.54	7.54	8.04	6.48	5.88	5.38

								% Constriction			% Dilation				
DATE				MAX (ΔmN)	**Basal (mN)**	CONSTR (ΔmN)	CONSTR (%)	KCl (%)		U46 (% max)	Ach 10-9M	Ach 10-8M	Ach 10-7M	Ach 10-6M	Ach 10-5M
4/19/2013		N110+/+		8.70	1.12	4.76	54.8%	23.8%		54.8%	-1.7%	-4.7%	-1.6%	55.6%	60.7%
		N110+/+		8.80	1.39	4.74	53.9%	22.8%		53.9%	1.7%	9.5%	46.9%	62.2%	64.6%
5/3/2013		N163+/+		15.26	1.21	7.58	49.7%	20.0%		49.7%	-2.9%	-5.6%	4.2%	23.9%	24.6%
		N163+/+		17.63	1.36	8.19	46.4%	18.1%		45.1%	-4.1%	-9.0%	6.6%	44.4%	53.5%
5/17/2013		N213+/+		13.02	1.18	7.70	59.1%	21.9%		56.8%	0.0%	0.0%	13.8%	21.6%	23.5%
		N213+/+													
											Ach	Ach	Ach	Ach	Ach
											10-9M	10-8M	10-7M	10-6M	10-5M
										Mean	-1.1%	-3.1%	14.8%	39.5%	44.1%
										SEM	0.9%	2.8%	7.1%	7.0%	7.4%
										N	6	6	6	6	6

			RAW DATA						Preconstriction		Dose response curve				
Experiment Date	SAMPLE ID		U46 nM	**Max Tens.**	**Min Tens.**	Distend. Tens.	KCl PRE	KCl POST	U46 PRE	U46 POST	Ach 10-9M	Ach 10-8M	Ach 10-7M	Ach 10-6M	Ach 10-5M
Rap1iΔEC															
4/19/2013	3	N112-/-	10.0	7.42	1.65	1.50	1.67	2.75	1.67	5.88	5.88	5.92	5.93	6.01	5.02
	4	N112-/-	10.0	8.50	2.27	1.50	2.33	3.62	2.27	6.76	6.84	6.75	6.65	6.57	5.48
5/3/2013	3	N164-/-	20.0	19.81	1.39	1.50	1.39	4.70	1.43	8.45	8.93	9.24	9.71	9.51	9.95
	4	N164-/-	20.0	14.13	1.36	1.50	1.53	4.11	1.36	7.55	7.97	8.33	8.55	7.63	8.63
5/17/2013	3	N214-/-	20.0	13.16	1.82	2.00	1.98	3.87	1.82	7.49	7.80	8.07	8.42	8.41	8.27
	4	N214-/-	20.0	19.48	2.00	2.00	2.24	4.94	2.33	10.52	11.14	11.57	11.76	11.97	11.69

								% Constriction			% Dilation				
DATE				MAX (ΔmN)	**Basal (mN)**	CONSTR (ΔmN)	CONSTR (%)	KCl (%)		U46 (%)	Ach 10-9M	Ach 10-8M	Ach 10-7M	Ach 10-6M	Ach 10-5M
4/19/2013		N112-/-		5.77	1.60	4.27	74.0%	18.6%		72.7%	-0.2%	-1.0%	-1.2%	-3.1%	20.0%
		N112-/-		6.23	1.88	4.88	78.3%	20.6%		72.1%	-1.5%	0.4%	2.4%	4.0%	26.3%
5/3/2013		N164-/-		18.42	1.39	7.07	38.4%	18.0%		38.1%	-6.7%	-11.1%	-17.8%	-14.9%	-21.2%
		N164-/-		12.77	1.36	6.19	48.5%	20.2%		48.5%	-6.8%	-12.6%	-16.2%	-1.3%	-17.4%
5/17/2013		N214-/-		11.34	1.18	6.31	55.6%	16.6%		50.0%	-4.9%	-9.2%	-14.8%	-14.6%	-12.4%
		N214-/-		17.48	1.83	8.70	49.8%	15.4%		46.8%	-7.1%	-12.0%	-14.2%	-16.6%	-13.5%

	Ach 10-9M	Ach 10-8M	Ach 10-7M	Ach 10-6M	Ach 10-5M
Mean	-4.5%	-7.6%	-10.3%	-7.8%	-3.0%
SEM	1.2%	2.4%	3.5%	3.6%	8.4%
N	6	6	6	6	6

Table S2 – Relaxation of control and Rap1iΔEC aortic segments preconstricted with U46619 in response to SNP.

SNP (+L-NAME)

			RAW DATA						Preconstriction		Dose response curve							
Experiment Date	SAMPLE ID		U46 [nM]	**Max Tens.**	**Min Tens.**	Distend. Tens.	KCl PRE	KCl POST	U46 PRE	U46 POST	SNP-i 10-11M	SNP-i 10-10M	SNP-i 10-9M	SNP-i 10-8M	SNP-i 10-7M	SNP-i 10-6M	SNP-i 10-5M	SNP-i 10-4M
CONTROL																		
4/19/2013	1	N110+/+	30.0	13.85	1.50	1.50	1.96	4.73	2.07	13.34		13.54	9.78	5.91	4.03	3.11	2.56	2.23
	2	N110+/+	30.0	15.20	1.16	1.50	1.80	4.75	2.32	14.77		15.20	11.51	6.46	4.58	3.44	2.90	2.30
5/3/2013	1	N163+/+	10.0	16.40	1.13	1.50	1.50	4.56	1.59	16.00		16.27	16.40	9.28	6.11	4.09	3.80	3.83
	2	N163+/+	10.0	18.99	1.36	1.50	1.36	4.56	2.23	18.50		18.97	18.52	8.96	6.00	4.12	3.55	3.36
5/17/2013	1	N213+/+	10.0	14.51	1.49	2.00	1.96	4.81	2.03	13.87		14.15	9.97	5.72	4.25	3.38	2.75	2.51
	2	N213+/+	10.0	12.86	1.80	2.00	2.08	4.98	2.32	11.10		11.17	7.11	4.53	3.53	3.04	2.66	2.42

				% Constriction						% Dilation							
DATE				MAX (ΔmN)	**Basal (mN)**	CONSTR (ΔmN)	CONSTR (%)	KCl (%)		U46 (%)	SNP-i 10-11M	SNP-i 10-10M	SNP-i 10-9M	SNP-i 10-8M	SNP-i 10-7M	SNP-i 10-6M	SNP-i 10-5M	SNP-i 10-4M
4/19/2013	1	N110+/+		12.34	1.60	11.74	95.1%	22.4%		91.3%		-1.6%	30.4%	63.3%	79.4%	87.2%	91.8%	94.7%
	2	N110+/+		14.04	1.80	12.98	92.4%	21.0%		88.7%		-3.3%	25.2%	64.1%	78.6%	87.4%	91.5%	96.1%
5/3/2013	1	N163+/+		15.26	1.50	14.50	95.0%	20.0%		94.4%		-1.9%	-2.7%	46.4%	68.2%	82.2%	84.1%	83.9%
	2	N163+/+		17.63	1.36	17.14	97.2%	18.1%		92.3%		-2.7%	-0.1%	55.7%	72.9%	83.9%	87.2%	88.3%
5/17/2013	1	N213+/+		13.02	1.96	11.90	91.4%	21.9%		90.9%		-2.4%	32.7%	68.4%	80.8%	88.1%	93.4%	95.4%
	2	N213+/+		11.07	2.08	9.02	81.5%	26.1%		79.4%		-0.7%	44.3%	72.9%	84.0%	89.4%	93.6%	96.3%

	SNP-i 10-10M	SNP-i 10-9M	SNP-i 10-8M	SNP-i 10-7M	SNP-i 10-6M	SNP-i 10-5M	SNP-i 10-4M
Mean	-2.1%	21.6%	61.8%	77.3%	86.3%	1.1875576	142.0%
SEM	0.4%	7.7%	3.9%	2.3%	1.1%	0.0191593	1.5%
N	6	6	6	6	6	6	6

			RAW DATA						Preconstriction		Dose response curve							
Experiment Date	SAMPLE ID		U46 [nM]	**Max Tens.**	**Min Tens.**	Distend. Tens.	KCl PRE	KCl POST	U46 PRE	U46 POST	SNP-i 10-11M	SNP-i 10-10M	SNP-i 10-9M	SNP-i 10-8M	SNP-i 10-7M	SNP-i 10-6M	SNP-i 10-5M	SNP-i 10-4M
Rap1iΔEC																		
4/19/2013	1	N112-/-	10.0	15.89	1.99	1.50	1.99	4.81	2.57	15.42		15.89	15.25	7.85	5.89	5.02	4.13	3.64
	2	N112-/-	10.0	13.70	0.66	1.50	0.75	3.12	2.87	13.34		13.70	13.15	6.84	5.19	4.45	3.71	3.08
5/3/2013	1	N164-/-	10.0	19.81	1.39	1.50	1.39	4.70	1.75	14.94		15.58	15.86	7.75	5.58	4.05	3.21	2.82
	2	N164-/-	10.0	14.13	1.36	1.50	1.53	4.11	2.51	13.61		13.93	14.02	9.37	6.40	4.90	4.27	3.99
5/17/2013	1	N214-/-	10.0	13.16	1.82	2.00	1.98	3.87	2.96	12.69		13.16	12.86	5.69	4.64	4.22	3.78	3.40
	2	N214-/-	10.0	19.48	2.00	2.00	2.24	4.94	3.78	18.42		19.08	18.32	9.04	6.87	5.40	4.65	4.05

				% Constriction						% Dilation							
DATE				MAX (ΔmN)	**Basal (mN)**	CONSTR (ΔmN)	CONSTR (%)	KCl (%)		U46 (%)	SNP-i 10-11M	SNP-i 10-10M	SNP-i 10-9M	SNP-i 10-8M	SNP-i 10-7M	SNP-i 10-6M	SNP-i 10-5M	SNP-i 10-4M
4/19/2013	1	N112-/-		13.90	1.99	13.44	96.7%	20.3%		92.4%		-3.5%	1.3%	56.4%	70.9%	77.4%	84.0%	87.7%
	2	N112-/-		13.04	0.75	12.59	96.6%	18.2%		80.3%		-2.8%	1.5%	51.6%	64.7%	70.6%	76.5%	81.5%
5/3/2013	1	N164-/-		18.42	1.39	13.55	73.6%	18.0%		71.6%		-4.7%	-6.8%	53.0%	69.1%	80.4%	86.6%	89.4%
	2	N164-/-		12.77	1.52	12.09	94.7%	20.2%		87.0%		-2.6%	-3.4%	35.1%	59.6%	72.0%	77.2%	79.5%
5/17/2013	1	N214-/-		11.34	1.88	10.81	95.3%	16.6%		85.8%		-4.4%	-1.5%	64.8%	74.5%	78.4%	82.5%	85.9%
	2	N214-/-		17.48	2.24	16.18	92.5%	15.4%		83.7%		-4.1%	0.6%	58.0%	71.4%	80.5%	85.2%	88.9%

	SNP-i 10-10M	SNP-i 10-9M	SNP-i 10-8M	SNP-i 10-7M	SNP-i 10-6M	SNP-i 10-5M	SNP-i 10-4M
Mean	-3.7%	-1.4%	53.2%	68.4%	76.5%	0.8198266	85.5%
SEM	0.3%	1.3%	4.1%	2.2%	1.7%	0.0172036	1.7%
N	6	6	6	6	6	6	6