SUPPLEMENTAL MATERIAL

Detailed Methods

Mice

Human Caveolin-1 (Cav-1) in pcDNA3.1 was mutated (F92A) by site directed mutagenesis, the C-terminus was PCR tagged with HA, before *Sal*I/*Eco*RV cloned into pTet-Splice (TetO7 promoter). The linearized plasmid was injected into fertilized eggs of C57BL/6J mice (The Jackson Laboratory, #000664). A male founder was backcrossed 5 times before breed to Cdh5-tTA mice (tTA), a kind gift from *Laura Benjamin*[1]. Single transgenic mice from the same litter were used as controls. For suppression of Cav-1-F92A expression 2 mg/ml doxycycline with 5 % sucrose was given in drinking water. The NOS inhibitor L-NAME was given at 1 mg/ml in the drinking water. The Institutional Animal Care Use Committee of Yale University approved all the experiments.

Isolation of Primary Mouse Lung Endothelial Cells (MLECs)

MLECs were isolated using a modified protocol published before[2]. In brief, after lethal dose administration i.p. of ketamine/xylazine the whole lungs from 6-8 wk old mice were isolated, digested in collagenase I and sorted using CD31-dynabeads and a magnetic separator. The cell fraction bound to the CD31 beads ($CD31^+$) and the non-bound fraction ($CD31^-$) were lysed and analyzed by immunoblotting. A detailed step-by-step protocol (~6 pages) for the isolation of MLECs can be requested**.**

Immunofluorescence Staining of Vessels

Cross sections of thoracic aorta: Mice were euthanized using ketamine/xylazine. Mice were PBS and PFA (4 %) perfused by catheter placement in the left ventricle and punching the right atrium. The thoracic aorta was isolated, fixed O/N in PFA/PBS (4 %), then dried with sucrose (30 %) O/N before embedding in OTC media. Cryosections of 6 µm were stained with antibodies listed in table IV using a regular immunofluorescence protocol and imaged with a CLSM.

Whole-mount staining of the mesenteric artery: Mesenteric arteries (primary branch) were isolated from mice upon euthanization. Mesenteric arteries were isolated and fixed in 4 % PFA/PBS for 15 min at RT. Enface samples were blocked and incubated with primary antibodies in TNB blocking buffer[3]. Respective secondary antibodies were applied prior to DAPI staining and subsequent imaging, as previously described.

Blood Pressure Measurement

Male mice, 10–12 wk old, were maintained under 1.75% (v/v) isoflurane anesthesia. The carotid artery was catheterized and a pressure transmitter (PA-C10, Data Sciences International) was implanted. After recovery the blood pressure was monitored continuously (one reading per minute) by oscillometric blood pressure measurement for 9 days. The first two days the baseline blood pressure was recorded, before the drinking water was switched to either doxycycline or L-NAME.

Electron Paramagnetic Resonance for Nitric Oxide Hemoglobin (NO-Hb)
Mice, 10–12 wk old, were euthanized by CO_2 (10 – 30% volume displacement per min). Whole blood was withdrawn from the inferior vena cava into 1-ml syringes and frozen immediately in liquid N_2. The frozen blood was transferred to a 77 K cold finger dewar. The three-line hyperfine spectrum of 5-coordinate nitrosyl hemoglobin was recorded at X-band with a Bruker ELEXYS E500 spectrometer equipped with a SHQ cavity in a similar setup published before[4]. ESR spectrometer settings were as follows: microwave frequency, 9.35 GHz; microwave power, 25 mW; modulation frequency, 100 kHz; modulation amplitude, 10 G; receiver gain, 70 dB; conversion time, 41 ms; time constant 20.5 ms; sweep time, 42 s. Quantification was performed by summing the peak-to-trough heights of the first two bands followed by normalization to the sample mass.

Adenoviral Reconstitution of Caveolin-1 WT and F92A mutant
Immortalized (retrovirus encoding the polyoma middle T-antigen) mouse lung endothelial cells (MLECs) isolated from WT and Cav-1 KO mice[5] were maintained in EBM-2 medium supplemented with EGM-2 MV SingleQuots. The adenovirus for Cav-1 WT (myc-tagged) and Cav-1-F92A have been generated by sub cloning into the adenovirus shuttle plasmid. The sub cloning, production and titering of the virus was done at the Viral Vector Core of the University of Iowa.

Sucrose Gradient Fractionation
Mice were euthanized using ketamine/xylazine. Four lungs from double and single transgenic animals per experiment were isolated in MBS (25 m*M* MES, 150 m*M* NaCl, pH 7.5) with protease/phosphatase inhibitors (Roche cOmplete/Pefabloc). After mincing and homogenizing the tissue triton-x-100 was added to a final concentration of 1 % and incubated for 30 min under continuous rotation. The so prepared tissue lysate was then mixed 1 : 1 with 85 % sucrose (→ 42.5 %), aliquoted in ultracentrifugation tubes (14 x 89 mm: 12 ml) and overlaid with 35 % sucrose, followed by 15 % sucrose. The tubes were centrifuged for 18 h at 35,000 rpm in a SW40 rotor (Beckman Coulter) at 4°C.

Immunogold Transmission Electron Microscopy
Immortalized MLECs (as described before) were cultured in 10-cm dishes until confluency. The cells were fixed in 4 % PFA, followed by 0.1 % glutaraldehyde in PBS. Samples were pelleted and were cryoprotected by sucrose when frozen in liquid nitrogen. Thin sections (60 nm) were cut in liquid N_2 by a cryo-microtome. The imaging was performed on a FEI Tecnai Biotwin TEM.

Histology
Aged mice, 20 – 22 wk old, were euthanized using ketamine/xylazine and perfusion fixed as described before. Hearts and lungs were isolated and fixed O/N in PFA (4 %) before stored in 70 % EtOH. Specimens were paraffin embedded, sectioned and stained with hematoxylin/eosin.

Echocardiography
Aged mice, 20 – 22 wk old, were maintained under 1.75% (v/v) isoflurane anesthesia on a heat pad for maintaining the body temperature. Echocardiography was performed on a Vevo® 2100 (Visual Sonics). In brief, anatomical M-mode was used and all measurements were made during multiple consecutive cardiac cycles, and the average values were used for analysis. LV end-diastolic (LVD,d) and end-systolic (LVD,s) dimensions, as well as the thickness of the intraventricular septum wall (IVSW) and posterior wall (PW) were measured from the M-mode tracings. Diastolic measurements were taken at the point of maximum cavity, and systolic measurements were made at the point of minimum dimension.

Genotyping
Cdh5-tTA and Cav-1-F92A transgenes were genotyped by PCR with the primers and the PCR program (same for both transgenes) described in Table III.

Immunoblotting
For immunoblotting, tissues or cells were placed in lysis buffer (50 m*M* Tris-HCl, 1 % NP-40, 0.1 % SDS, 0.1 % Na-deoxycholate, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 200 µ*M* NaF, 20 µ*M* Na-pyrophosphate, 2 mg/ml cOmplete protease inhibitor, 0.3 mg/ml Pefabloc phosphatase inhibitor, 40 m*M* β-glycerophophate, 2 m*M* Na_3VO_4). Tissues were homogenized before sonication. Cell lysates were centrifuged for 15 min at top-speed at 4°C. Protein concentrations were determined (Bio-Rad Protein Assay). Equal amounts of protein were separated by 10 % SDS-PAGE or 4 -20 % gradient gels (Bio-Rad) and transferred to nitrocellulose membranes. After Ponceau S staining, the membranes were blocked with 1 % casein for 1 hr at RT under continuous rotation before incubation with the primary antibody O/N at 4°C. Table IV lists all primary antibodies used in this study. LI-COR compatible secondary antibodies (680 or 800 nm) were used based on species origin of the primary antibody.

Densitometry Analysis of Immunoblots
Densitometry Analysis of band intensities was performed using the Li-COR software Image Studio.

Reagents
Unless otherwise stated all reagents were obtained in the highest grade of purity from Sigma-Aldrich.

Statistics
Bar graphs represent the mean and their SEM. Statistical analysis was performed using Prism 6 (GraphPad Software). Means were compared with the Student's *t* test. Differences with $p < 0.05$ were considered as statistically significant (indicated by a *).

Supplemental Figures with Figure Legends

Figure IA
Mouse model: Endothelial specific promotor Cdh5 of transgene 1 (Tg 1) drives the expression of the tetracycline-controlled transactivator (tTA) protein. In the absence of doxycycline (- DOX) tTA binds to the TetO7 promoter of Tg 2 and drives the expression of Cav-1-F92A HA (F92A) expression. In the presence of doxycycline tTA cannot bind the TetO7 promoter (Tet-off system).

Figure IB
Reporter Mouse for tTA. Cdh5-tTA was bred to a tTA reporter mouse[6] (TetO7-GFP) as an independent readout, that the Cdh5-tTA is mainly responsible for the mosaic pattern and not the transgene (TetO7-Cav-1-F92A) itself. Whole-mount staining of mesenteric artery. En-face preparations were stained for the nucleus (blue), PECAM-1 (red) and GFP (green). Top panel, GFP alone. Bottom panel, merged.

Figure II
A, Left panels, Averaged diastolic blood pressure and heart rate from 8 control and 8 Cav-1-F92A animals. The first two days baseline was recorded, before drinking water was switched to doxycycline (2 mg/ml) with 5 % sucrose. Dotted lines show the changes of the systolic blood pressure (slopes of the curves are listed in Table I). Right panels, bar graph presentation of the same results. The bars present the average diastolic blood pressure and heart rate without and with doxycycline. B, Left panels, Averaged diastolic blood pressure from 3 control and 3 Cav-1-F92A animals. The first two days baseline was recorded, before drinking water was switched to L-NAME (1 mg/ml). Dotted lines show the changes of the diastolic blood pressure and heart rate (slopes of the curves are listed in Table II). Right panels, bar graph presentation of the same results. The bars present the average diastolic blood pressure and heart rate without and with L-NAME.

Figure III
Representative EPR traces for single (WT) and double transgenic (WT + Tg) animals.

Figure IV
As controls venous blood from eNOS KO, eNOS S1176A and eNOS S1176D mice was isolated and measured in the same way described before and compared to the WT. Plasma and blood cells (mostly erythrocytes) were isolated from WT mice. The cell fraction was incubated with an excess of $NaNO_2$ and $Na_2S_2O_4$ to generate nitric oxide *in situ* to proof that the three hyperfine EPR bands are occurring from NO-Hb.

Figure V
Immunoblotting for p-VASP (S239) and t-VASP in the whole aorta lysate as a readout of bioavailability of nitric oxide. Cav-1 and HA immunoblotting as a control for the expression of the transgene. HSP90 was used as loading control. Each lane represents one animal (3 per group).

Figure VI
Immunoblotting for nitrotyrosine (see Table IV) on lung and heart lysate from 3 control and 3 Cav-1-F92A mice (one lane per animal). 50 µg were loaded.

Figure VII

Model of Cav-1-F92A membrane integration and signaling. A, domain structure of Cav-1. B, Cav-1 WT integrates in the membrane in a horseshoe loop structure (aa 102 – 134), whereas F92 is localized cytosolic and inhibits eNOS. C, Cav-1-F92A integrates exactly the same way into the plasma membrane as the WT, but the cytosolic A92 cannot inhibit eNOS, which leads to an increased NO release.

Supplemental Tables

Table I
Slopes of the changes of blood pressure and heart rate after doxycycline administration.

	Control	Cav-1-F92A
systolic blood pressure	- 0.009 mmHg/hr	+ 0.028 mmHg/hr
diastolic blood pressure	- 0.008 mmHg/hr	+ 0.012 mmHg/hr
heart rate	+ 0.007 BPM/hr	- 0.067 BPM/hr

Table II
Slopes of the changes of blood pressure and heart rate after L-NAME administration.

	Control	Cav-1-F92A
systolic blood pressure	+ 0.103 mmHg/hr	+ 0.096 mmHg/hr
diastolic blood pressure	+ 0.099 mmHg/hr	+ 0.051 mmHg/hr
heart rate	- 0.311 BPM/hr	- 0.182 BPM/hr

Table III
Genotyping primers and PCR program

Cdh5-tTA		
forward	5′-GAC GCC TTA GCC ATT GAG AT-3′	
reverse	5‘-CAG TAG TAG GTG TTT CCC TTT CTT-3‘	
amplicon size	333 bp	
Cav-1-F92A-HA		
forward	5′-AGG TCG ACC CTC CTC ACA G-3′	
reverse	5′-TTC GAT ATC TTA AGC GTA GTC TGG GAC GTC GTA TG-3′	
amplicon size	590 bp	
PCR program (both primer sets)		
step 1	94°C	3:00 min
step 2	94°C	1:00 min
step 3	56°C	1:00 min
step 4	72°C	0:40 min
step 5	go to 2, 34 x	
step 6	72°C	10:00 min
step 7	4°C	forever

Table IV
Antibodies used in the study

protein	ordering number	company	dilution
Immunoblotting			
p-AKT (S473)	4060	Cell Signaling	1 : 1,000
t-AKT1	2938	Cell Signaling	1 : 1,000
Cav-1	610060	BD Biosciences	1 : 10,000
Cav-2	610685	BD Biosciences	1 : 1,000
Cav-3	610421	BD Biosciences	1 : 1,000
Cavin-1	A301-270A	Bethyl Laboratories	1 : 1,000
GAPDH	2118	Cell Signaling	1 : 1,000
p-ERK (T202/Y204)	9101	Cell Signaling	1 : 1,000
t-ERK	4696	Cell Signaling	1 : 1,000
HA	11-867-423-001	Roche	1 : 1,000
HSP90	sc-13119	Santa Cruz	1 : 1,000
Nitrotyrosine	189542	Cayman Chemical	1 : 1,000
p-VASP (S239)	3114	Cell Signaling	1 : 1,000
t-VASP	3112	Cell Signaling	1 : 1,000
Immunofluorescence			
HA (cross sections)	3724	Cell Signaling	1 : 100
HA (whole-mount)	ab1208	Abcam	1 : 200
PECAM-1/CD31	MAB1398Z	Millipore	1 : 250
α-SMA	M0851	Dako	1 : 600

Table V
Other readouts of the echocardiography. Mean and SEM is listed.

Parameter	WT	WT + Tg
Volume in systole (ml)	25 ± 3	26 ± 4
Volume in diastole (ml)	68 ± 8	63 ± 4
Stroke Volume (ml)	43 ± 5	37 ± 1
Ejection Fraction (ml)	63 ± 2	59 ± 2
Fractional Shortening (ml)	34 ± 1	31 ± 2
Cardiac Output (ml)	21 ± 2	17 ± 1
LV Mass (mg)	137 ± 9	118 ± 10

Supplemental References

1. Sun JF, Phung T, Shiojima I, Felske T, Upalakalin JN, Feng D, Kornaga T, Dor T, Dvorak AM, Walsh K, Benjamin LE. Microvascular patterning is controlled by fine-tuning the akt signal. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:128-133
2. Allport JR, Lim YC, Shipley JM, Senior RM, Shapiro SD, Matsuyoshi N, Vestweber D, Luscinskas FW. Neutrophils from mmp-9- or neutrophil elastase-deficient mice show no defect in transendothelial migration under flow in vitro. *Journal of leukocyte biology*. 2002;71:821-828
3. Pitulescu ME, Schmidt I, Benedito R, Adams RH. Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. *Nature protocols*. 2010;5:1518-1534
4. Dikalov S, Fink B. Esr techniques for the detection of nitric oxide in vivo and in tissues. *Methods in enzymology*. 2005;396:597-610
5. Lin MI, Yu J, Murata T, Sessa WC. Caveolin-1–deficient mice have increased tumor microvascular permeability, angiogenesis, and growth. *Cancer Research*. 2007;67:2849-2856
6. Krestel HE, Mayford M, Seeburg PH, Sprengel R. A gfp-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse. *Nucleic acids research*. 2001;29:E39

Figure IA

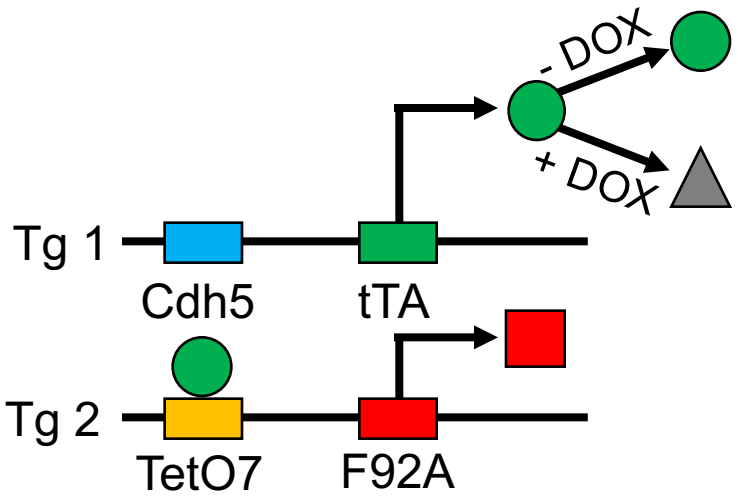


Figure IB

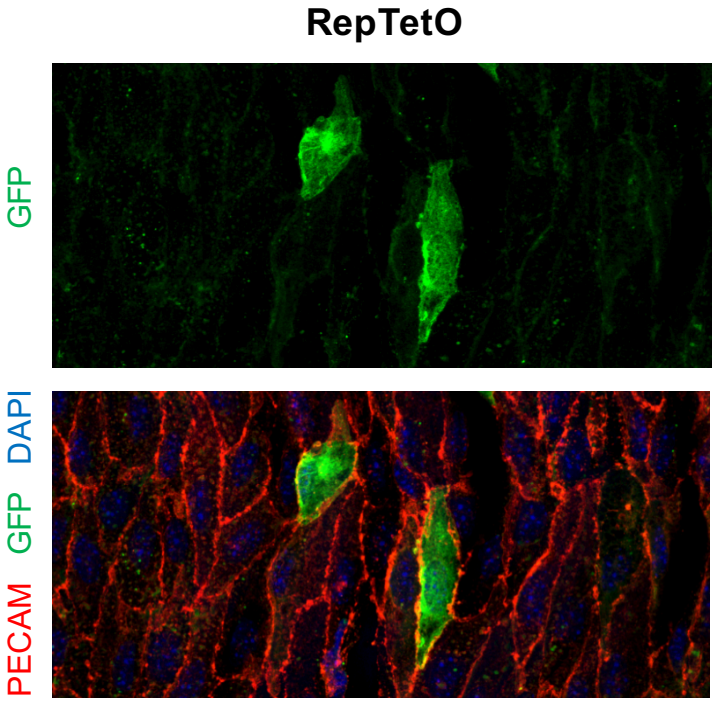


Figure II

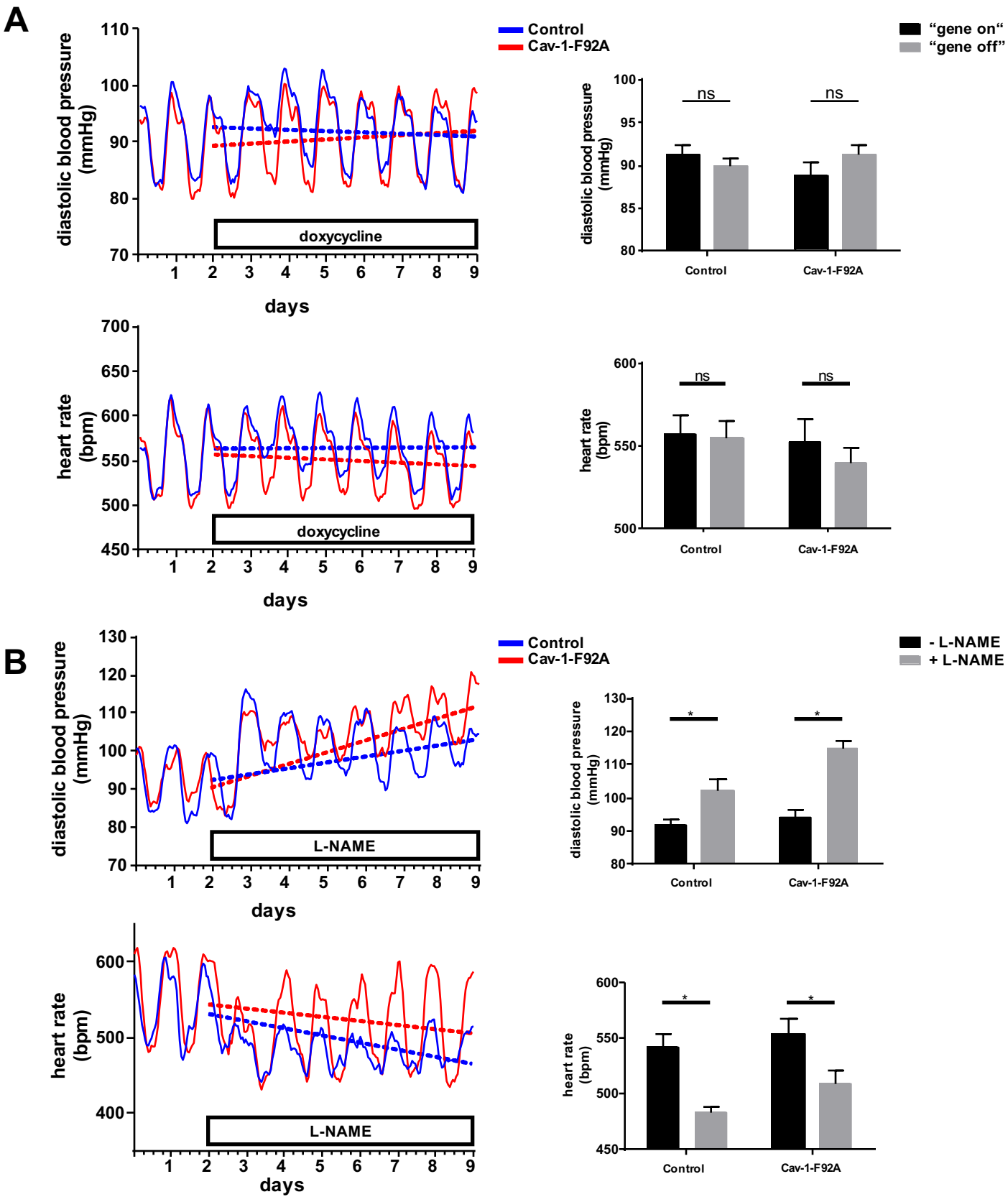

A
Control
Cav-1-F92A
"gene on"
"gene off"
diastolic blood pressure (mmHg)
doxycycline
days
ns
Control
Cav-1-F92A
heart rate (bpm)
B
- L-NAME
+ L-NAME
L-NAME
*

Figure III

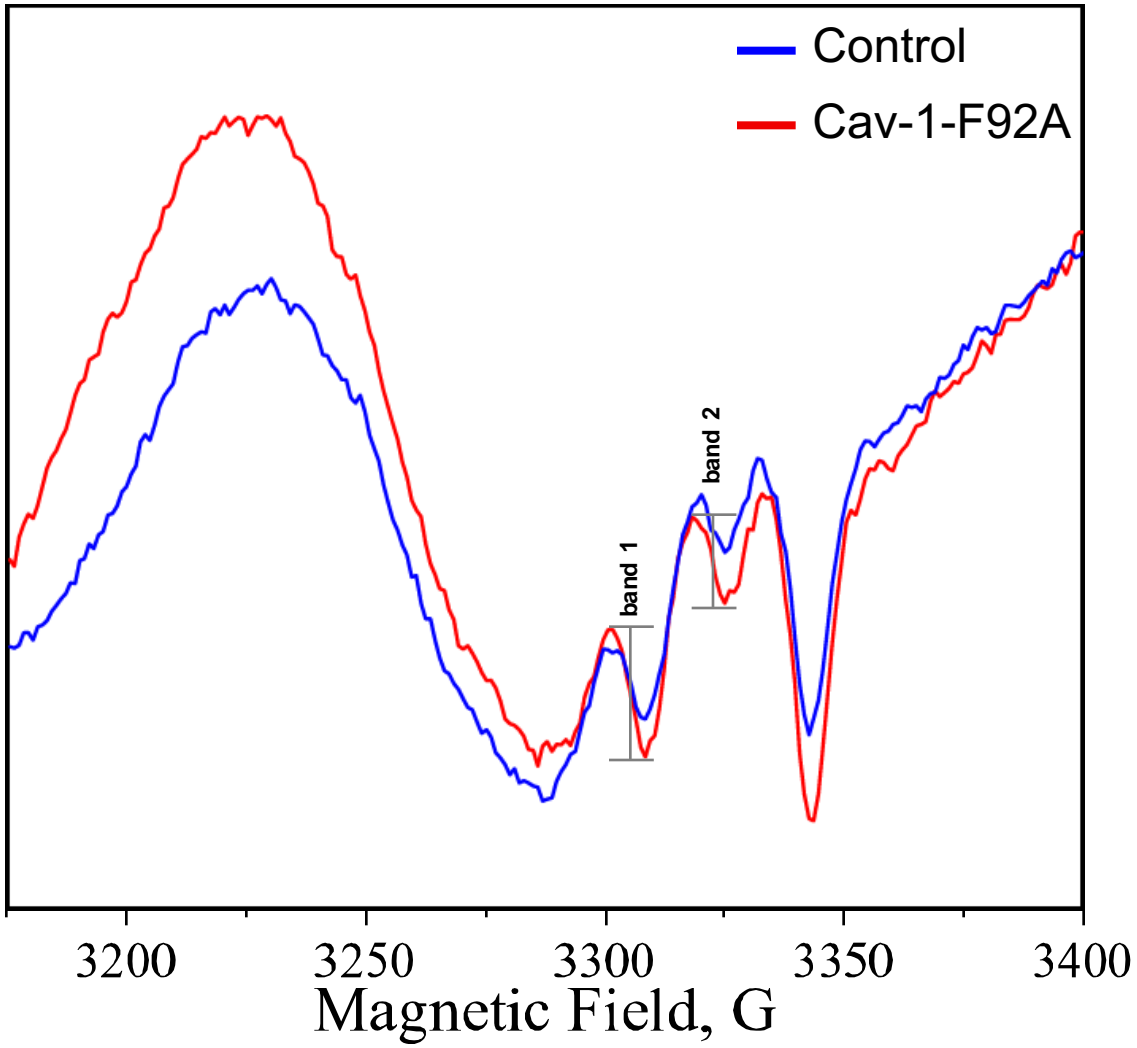
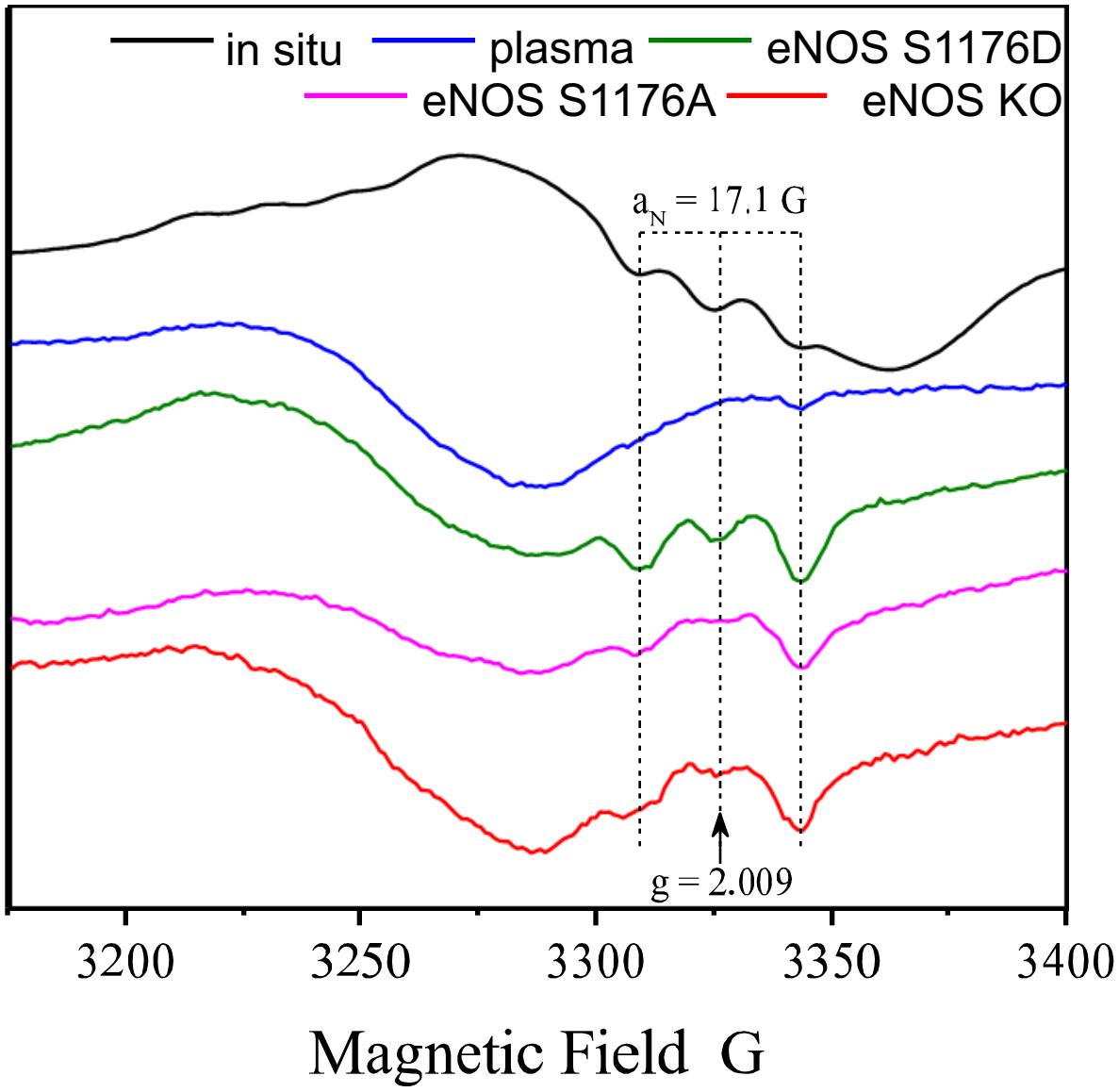

Control
Cav-1-F92A
band 1
band 2
3200
3250
3300
3350
3400
Magnetic Field, G

Figure IV


in situ
plasma
eNOS S1176D
eNOS S1176A
eNOS KO
a_N = 17.1 G
g = 2.009
3200
3250
3300
3350
3400
Magnetic Field G

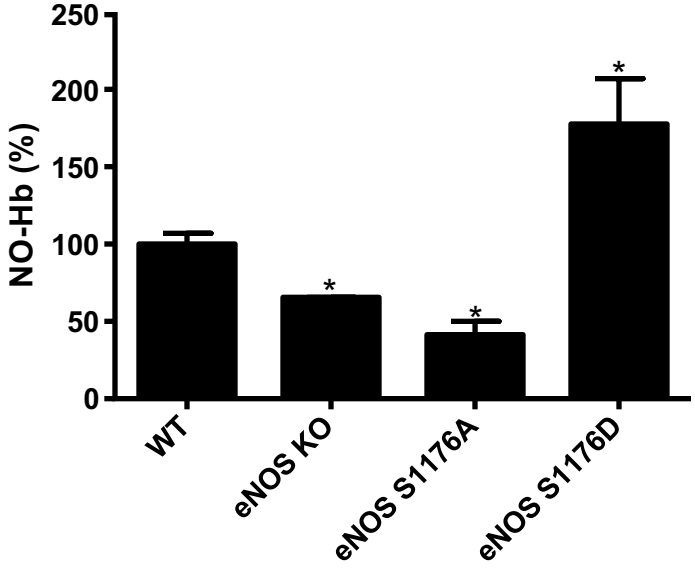

NO-Hb (%)
250
200
150
100
50
0
*
*
*
WT
eNOS KO
eNOS S1176A
eNOS S1176D

Figure V

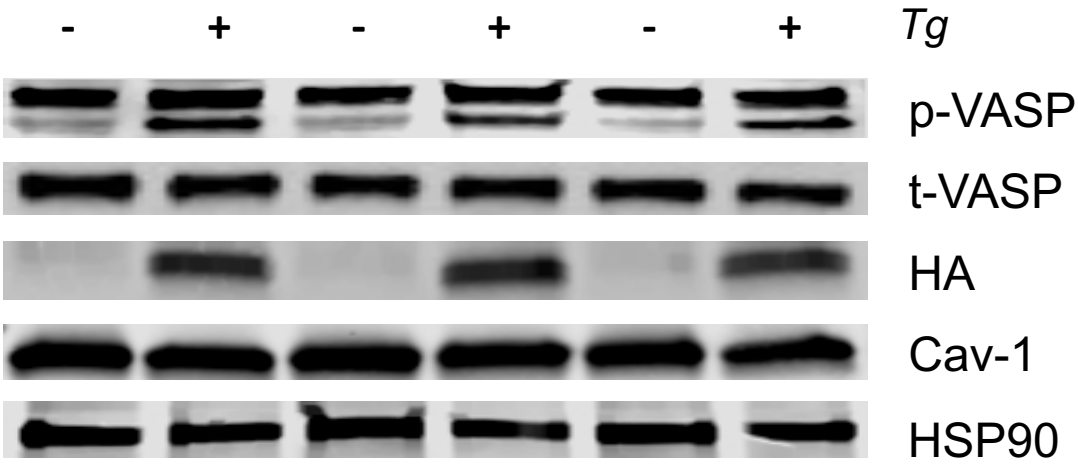


Figure VI

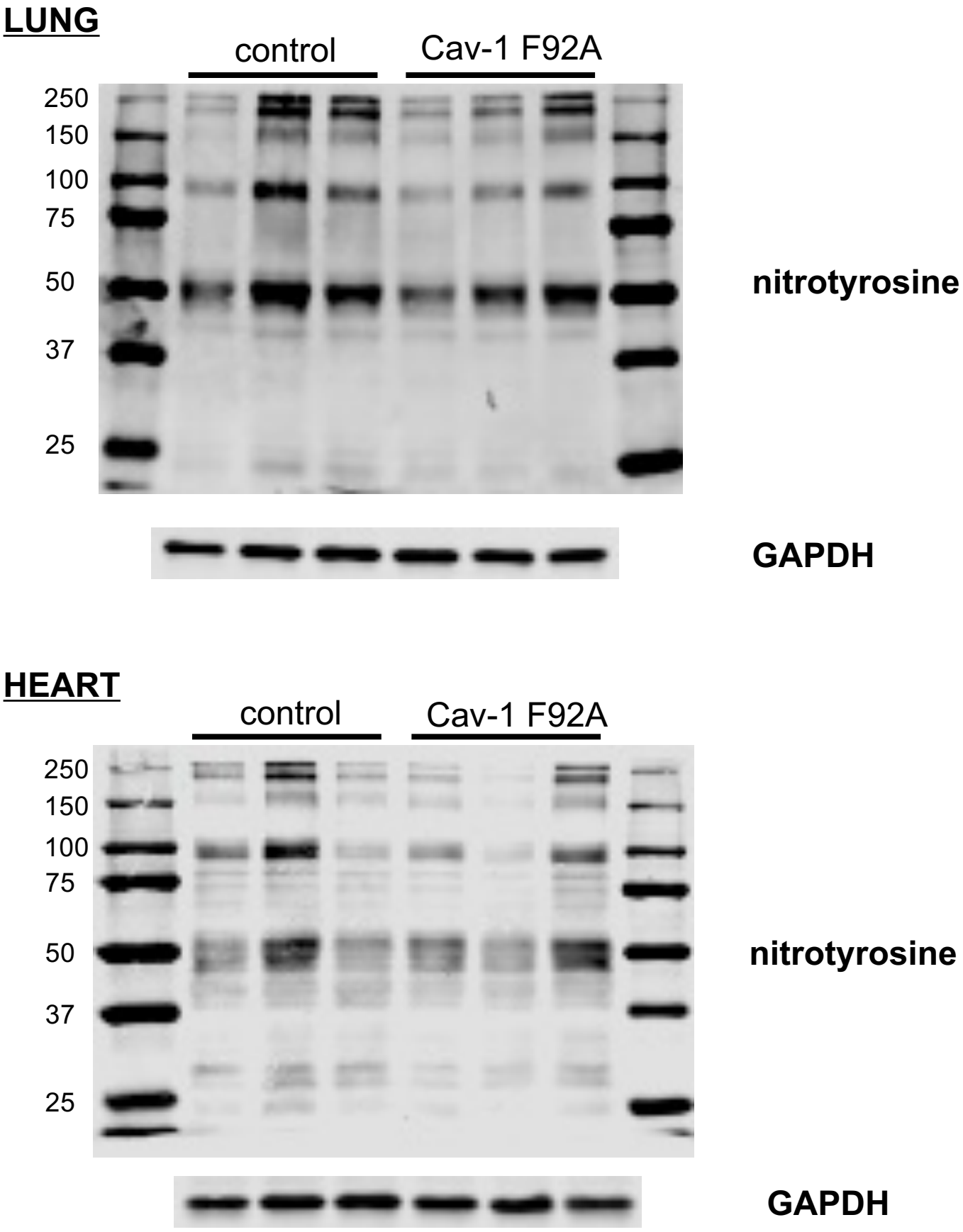
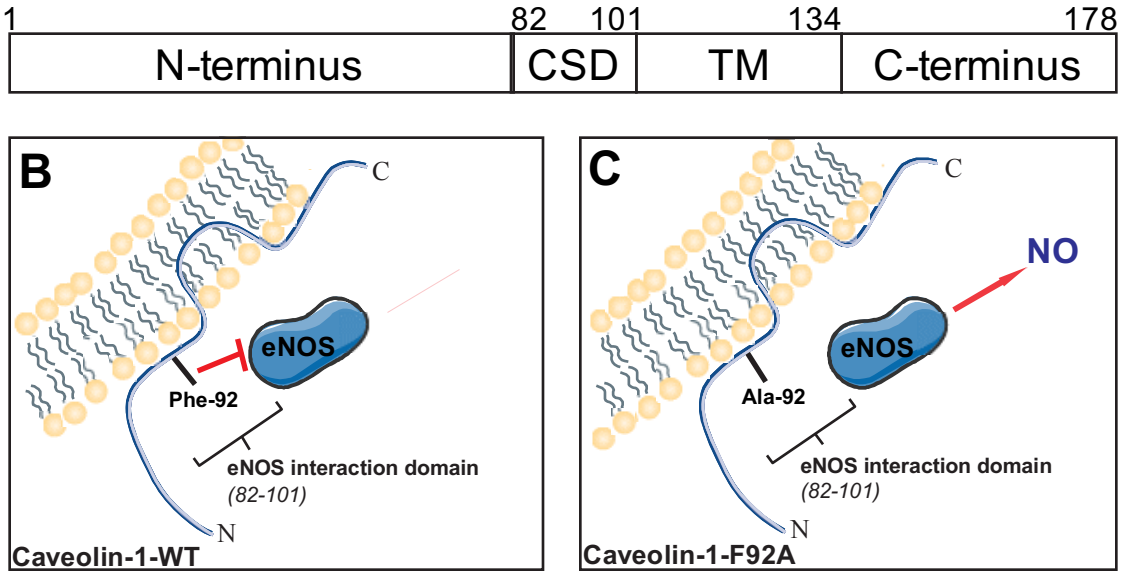

LUNG
control
Cav-1 F92A
250
150
100
75
50
37
25
nitrotyrosine
GAPDH
HEART
control
Cav-1 F92A
250
150
100
75
50
37
25
nitrotyrosine
GAPDH

Figure VII


A
1
82
101
134
178
N-terminus
CSD
TM
C-terminus
B
C
eNOS
Phe-92
eNOS interaction domain
(82-101)
Caveolin-1-WT
C
N
C
NO
eNOS
Ala-92
eNOS interaction domain
(82-101)
Caveolin-1-F92A
C
N