Supplemental Materials:

Supplemental Methods:

Quantitative RT-PCR Analysis. The following primer sets were used for analyses: mouse $p21^{Cip1}$, 5'-GACAAGAGGCCCAGTACTTCCT-3' and 5'-CAATCTGCGCTTGGAGTGATA-3'; mouse IGF-1, 5'-ACAGGCTATGGCTCCAGCAT-3', and 5'-GCTCCGGAAGCAACACTCAT-3'; mouse VEGF-A, 5'-TCCAAGATCCGCAGACGTGTAA-3', and 5'-TGGCTTGTCACATCTGCAAGTAC-3'; mouse nNOS, 5'-ACCGAATACAGGCTGACGATGT-3' and 5'-GCACGGATTCATTCCTTTGTGT-3'; mouse PKG-1, 5'-CTGTCACAGATCCAGGAGATTG-3' and 5'-ATCGCCTTCCTTGATGATGCAG-3'; and mouse PKG-2, 5'-TTCAGTGTGGATTTCTGGTCCC-3' and 5'-GTCATCATTTGGTCTATCCCAG-3'. All gene expression was normalized to cyclophilin (21) as an internal control.

sGC Enzyme Activity and Lung cGMP Measurement. sGC enzyme activity was measured as previously described by Mittal. Briefly, lung tissue was homogenized in buffer containing 50 mM Tris.HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethyl sulfonyl fluoride, 0.5 mM 3-isobutyl-1-methylxanthine, and protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 100,000g for 1 h at 4^0C. Supernatants (50 μg in 30 μl lysis buffer) were incubated for 10 min at 37°C in a reaction mixture containing 50 mM Tris.HCl (pH 7.5), 5 mM MgCl2, 0.5 mM 3-isobutyl-1-methylxanthine, 7.5 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 1 mM GTP with (Basal) or without 1 mM sodium nitroprusside. The total reaction volume was 100 μl. The reaction was terminated by 0.1 N HCl. The cGMP in the reaction

mixture following dilution with assay buffer (1:5 dilutions for the basal reaction mixture and 1:50 dilution for the sodium nitroprusside-stimulated reaction mixture) was measured using a commercial cGMP enzyme immunoassay kit (GE Healthcare) following manufacture's instruction. sGC activity is expressed as picomoles of cGMP produced per min per milligram of lung extract supernatant. To determine the lung cGMP levels, mouse lung tissue was homogenized in cold 6% (w/v) trichloroacetic acid and supernatant was collected following centrifugation at 2000 g for 15 min at 4°C. Following 4 washing of the supernatant with 5 volumes of water saturated diethyl ether, the aqueous extract of cGMP was lyophilized and resuspended in 220 μl of assay buffer. cGMP levels were measured with the same cGMP enzyme immunoassay kit following manufacture's instruction. Mouse lung cGMP levels were expressed as pmoles/g wet lung.

***In Vitro* PKG Kinase Assay.** Mouse lung tissue was homogenized in 800 μl lysis buffer containing 10mM HEPES, 0.5 mM EDTA, 10mM dithiothreitol, 1mM 3-isobutyl-1-methylxanthine, 125 mM KCl, 35 mM β-glycerophosphate, 0.1 mg/ml trypsin inhibitor, 1 μM antipain, 1 μM E64, and 0.4 mM PMSF. The lysate was then sonicated for 10s for 3 times following centrifugation at 14,000 rpm for 15 mi at 4°C . The supernatant (7.5 μg in 20 μl of lysis buffer) was used for PKG activity measurement. The assay was carried out in a total volume of 50 μl containing 150 μM BPDEtide (Calbiochem), 10 mM HEPES, 35 mM β-glycerophosphate, 4 mM magnesium acetate, 5 μM PKI (a synthetic protein kinase A inhibitor, Calbiochem), 0.5 mM EDTA, 200 μM ATP, and 2 μCi of [γ-^{32}P]ATP (specific activity 3,000Ci/mmol, GE Healthcare) with or without addition of 2.5 μM cGMP. The mixture was incubated at 30^{o}C for 12 min and terminated by spotting 40

μl aliquots of mixture on phosphocellulose papers (P81, Whatman). Following 4 washing with 75mM of ice-cold phosphoric acid solution, the papers were then dried and counted in a liquid scintillation counter. 5 μl of the reaction mixture was directly spotted on the phosphocellulose paper without washing for determination of the γ-^{32}P-ATP specific activity. PKG activity is expressed as picmoles of ^{32}P incorporated into PKG substrate per min per microgram protein.

NO Measurement. eNOS-derived NO from human lung samples was also determined using the three-electrode system (1). Briefly, with the aid of a micromanipulator, the NO sensor was carefully placed on the surface of the lung slice and the baseline was recorded. To determine the lung eNOS activity, the samples were subsequently incubated in a bath containing HBSS, and iNOS and nNOS inhibitors for 20 min. After addition of 1mM L-Arginine, NO release was recorded and the maximal current at 20s was calculated for eNOS-derived NO production. Similarly, iNOS-derived NO was also determined with the three-electrode system. Following 20 min incubation of eNOS and nNOS inhibitor (L-NNA, 4 μM), L-Arginine was added and NO release was recorded. The maximal current at 20 min was calculated for iNOS-derived NO production.

Detection of Protein S-nitrosylation. S-nitrosylation was detected with the S-Nitrosylated Protein Detection Assay kit (Cayman Chemical) following manufacture's instruction. Briefly, freshly isolated mouse lung tissue under weak fluorescent light condition was homogenized in Buffer A containing blocking reagent and precipitated with ice-cold acetone. The precipitates were then resuspended in Buffer B containing reducing and labeling reagents and incubated for 1 h at room temperature. All these procedures were performed under weak fluorescent light condition. And then, the biotin-

labeled proteins were precipitated with ice-cold acetone and resuspended in washing buffer. 10 μg of each sample was used for direct Western blotting analysis of total S-nitrosylation. The same blot was blotted with anti-GAPDH for loading control. To detect PKG-1 S-nitrosylation, the samples (300 μg each) were immunoprecipitated with anti-PKG-1 (2μg) overnight and then detected for S-nitrosylation by Western blotting analysis. The same blot was blotted with anti-PKG-1 for detection of PKG-1 expression.

***In Vivo* Gene Delivery to Lungs.** WT and *Cav1*$^{-/-}$ mice were randomized into two groups for AdvPKG and AdvLacZ, respectively, and anesthetized for delivery of recombinant adenovirus. While breathing spontaneously, each mouse was nebulized with 75 μl of sterile PBS solution containing 1.5 X 10^8 pfu of recombinant adenovirus expressing either human PKG-1 (a generous gift from Dr. K.D. Bloch at the Cardiovascular Research Center and Department of Anesthesia, Massachusetts General Hospital) or LacZ by use of an intratracheal microspray device through the mouth (MicroSprayer, Penn-Century Inc.). 7d after nebulization, mice were anesthetized for measurements of RVSP and PVR. Lungs were collected for Western blot analysis.

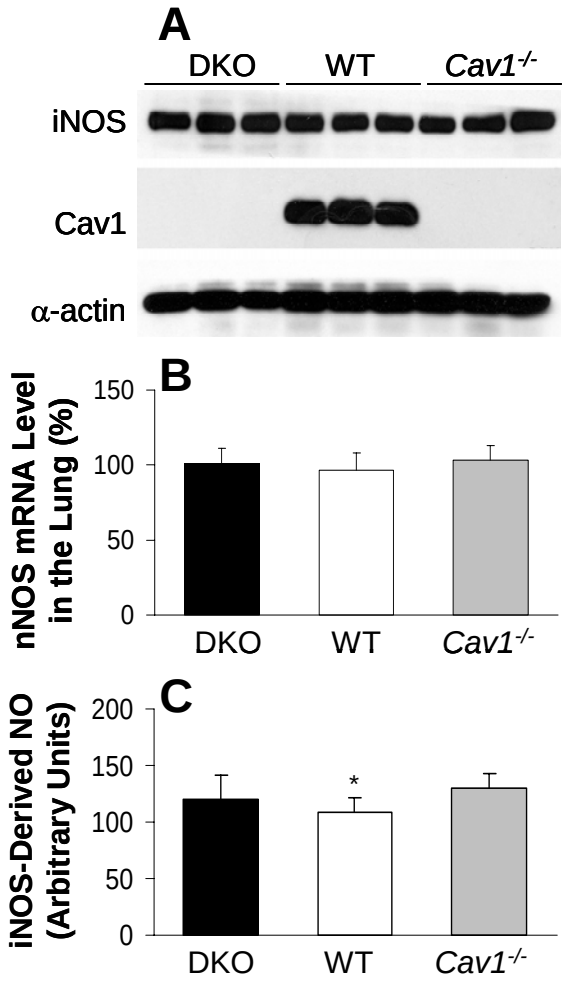
Histology and Imaging. Lung tissues were fixed and processed for H & E staining and immunofluorescent staining as described previously () and in detail in Supplement Methods. for 5 min by instillation of 10% PBS-buffered formalin through trachea catheterization at a transpulmonary pressure of 15 cm H_2O, and then overnight at 4^{o}C with agitation. After paraffin processing, the tissues were cut into semi-thin 4 to 5 μm thick, and stained with H & E for histological analysis. For immunofluorescent staining, antigen retrieval was performed by incubating the slides in 10 mM sodium citrate (pH 6.0) at 95°C for 10 min. After 1h incubation at room temperature in a blocking solution

containing 2% bovine albumin serum, 0.1% Triton X-100, and 2% normal goat serum, the sections were incubated for 2h at room temperature with anti-smooth muscle α-actin mAb (1:400, Sigma-Aldrich) and then 1h with FITC-conjugated goat anti-mouse IgG (1:250, Sigma-Aldrich). Nuclei were counterstained with DAPI. The anti-α-SMA-positive pulmonary arterial vessels per field (200X) were counted based on the diameter (< 40μm versus > 40μm). Twenty fields per section were randomly identified and counted. To examine tyrosine nitration in the pulmonary vasculature, cryosections of mouse lung tissues were fixed with 4% paraformaldehyde and then immunostained with anti-nitrotyrosine (mouse monoclonal antibody, 1:80, Cayman Chemical) to detect nitration (green) and anti-smooth muscle α-actin (rabbit polyclonal antibody, 1:250, Abcam) to detect muscularized vessels (red). The Nuclei were counterstained with DAPI.

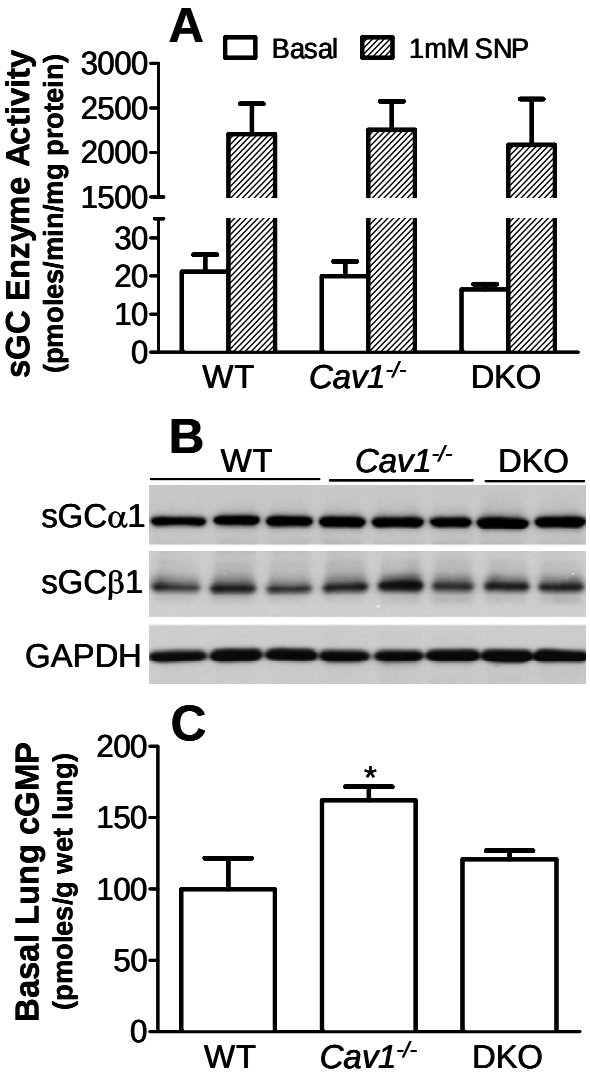
Supplemental References:

1. Skidgel, R.A., et al. 2002. Nitric oxide stimulates macrophage inflammatory protein-2 expression in sepsis. *J. Immunol.* **169**:2093-2101.

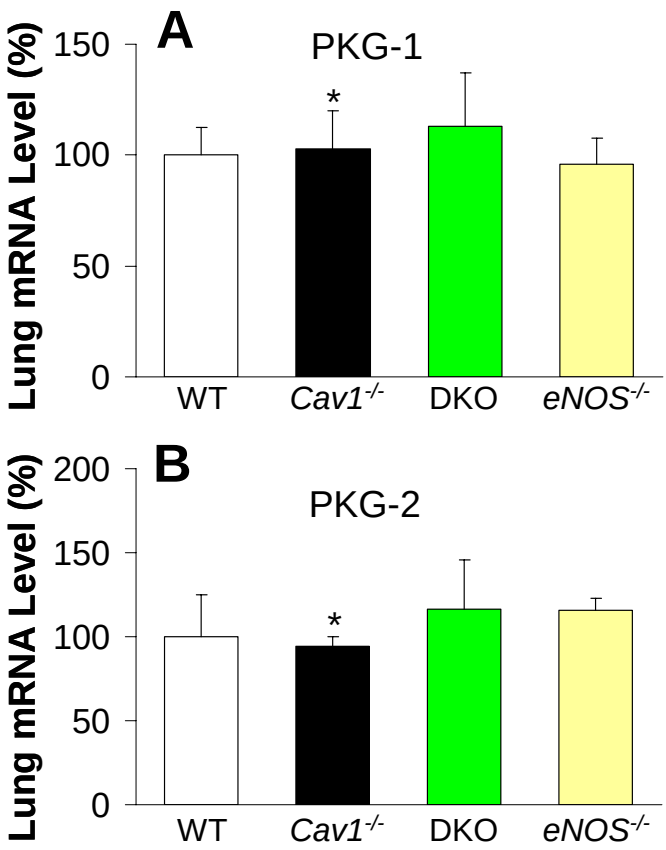
Supplemental Data:



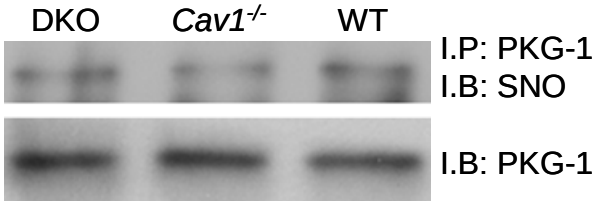
Supplemental Figure 1. Similar iNOS and nNOS expression and iNOS-derived NO production in WT and *Cav1*$^{-/-}$ mouse lungs. (**A**) Western blot analysis of iNOS expression in mouse lungs. (**B**) Quantitative RT-PCR analysis of nNOS expression in mouse lungs. (**C**) iNOS-derived NO production in mouse lungs. Mouse lung tissues were incubated with eNOS and nNOS inhibitor (L-NNA, 4 μM) for 20 min, L-arginine was then added and NO release was recorded with a three-electrode system. The maximal current at 20 min was calculated for iNOS-derived NO production. Data are expressed as mean ± SD (n=4-5). *, $P > 0.05$ WT versus *Cav1*$^{-/-}$.



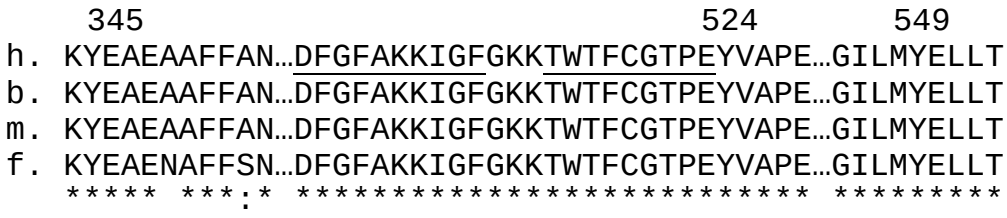
Supplemental Figure 2. Normal sGC activity and cGMP production in *Cav1*$^{-/-}$ mouse lungs. (**A**) sGC activity in mouse lungs. Each lung lysate (50 μg), partially purified by 1h centrifugation at 100,000g, was used for sGC activity assay at either basal state or after addition of sodium nitroprusside (SNP) at 1mM, a high dose for maximal activation. Data are expressed as mean ± SD (n=3-5). There is no difference in sGC activities at either basal or under maximal stimulation among WT, *Cav1*$^{-/-}$ and DKO lungs. (**B**) Expression of sGC subunits in mouse lungs. Each lung lysate (20 μg) was used for Western blot analysis of the α1 subunit (sGCα1) and the same blot was blotted with anti-sGCβ1 and GAPDH. We observed no differences in protein levels of either subunit in mouse lungs. (**C**) Increased cGMP production in *Cav1*$^{-/-}$ lungs. Lungs were lysed with 6% (w/v) trichloroacetic acid and then cGMP concentations were determined with a cGMP Enzyme Immunoassay Kit (GE Healthcare) following manufacturer's instruction. Data are expressed as mean ± SD (n=3-4 per group). *, $P < 0.05$ versus either WT or DKO.



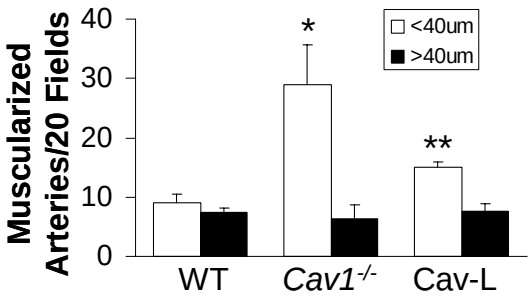
Supplemental Figure 3. Similar PKG1 and PKG2 mRNA expression in WT and *Cav1*$^{-/-}$ mouse lungs. Total RNA isolated from lungs collected from 2 mo old mice and mRNA levels of PKG-1 (**A**) and PKG-2 (**B**) were analyzed with quantitative RT-PCR assay. mRNA levels of cyclophilin were used for normalization. Data are expressed as mean ± SD (n=3-5). *, $P > 0.5$ *Cav1*$^{-/-}$ versus either WT, or DKO, or *eNOS*$^{-/-}$. mRNA levels of PKG-1 was approximately 2 times of PKG-2 in mouse lungs.



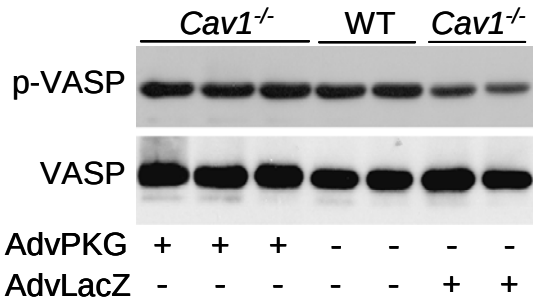
Supplemental Figure 4. Basal low levels of PKG-1 S-nitrosylation in mouse lungs. Biotin-labeled lysates (300 μg per sample) of freshly isolated mouse lung tissue was immunoprecipitated with anti-PKG-1 overnight, and then S-nitrosylation (SNO) was detected by Western blot analysis with avidin-coupled reagents. The same blot was also immunoblotted with anti-PKG-1. Low and similar levels of S-nitrosylation of PKG-1 were detected in lung tissues from WT, *Cav1*$^{-/-}$, and DKO.



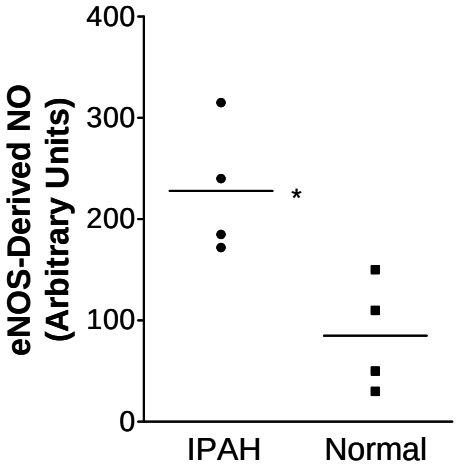
Supplemental Figure 5. Sequence alignment analysis demonstrating conserved tyrosine residues. Underlined regions indicating the activation loop. h, human, b, bovine, m, mouse, f, zebra fish.



Supplemental Figure 6. L-NAME-treated *Cav1*$^{-/-}$ mice had significantly fewer muscularized distal pulmonary arteries. *Cav1*$^{-/-}$ mice received water *ad libitum* (*Cav1*$^{-/-}$, control) or water with 1 mg/ml of L-NAME (Cav-L) or its inactive analog D-NAME (Cav-D) for 5 wk. Data are expressed as mean ± SD (n=5-7). *, $P < 0.001$ *Cav1*$^{-/-}$ versus WT; **, $P < 0.05$ Cav-L versus *Cav1*$^{-/-}$.



Supplemental Figure 7. Normalization of PKG-mediated phosphorylation of VASP in *Cav1*$^{-/-}$ lungs transfected with AdvPKG. 7d post-recombinant adenovirus administration, mouse lung tissues were collected and lysated for Western blot analysis. Anti-VASP phosphoSer239 (p-VASP) was used to detect PKG-mediated phosphorylation of VASP at residue Ser239, and the same blot was immunoblotted with anti-VASP. Decreased VASP phosphorylation was detected in *Cav1*$^{-/-}$ lungs compared to in WT lungs although similar VASP expression was expressed. AdvPKG administration restored PKG-mediated VASP phosphorylation in *Cav1*$^{-/-}$ lungs to the levels similar to in WT lungs.



Supplemental Figure 8. eNOS-derived NO production in human lung tissue determined by a three-electrode system. Human lung tissues were incubated in a solution containing iNOS and nNOS inhibitors for 20 min. L-arginine (1mM) was then added and NO release was recorded with the three-electrode system. The maximal current at 20s was calculated for eNOS-derived NO production. *, $P < 0.05$ (n=4).