**Supplemental Material**

# Poldip2, a novel regulator of Nox4 and cytoskeletal integrity

Alicia N. Lyle[1], Nita N. Deshpande[1], Yoshihiro Taniyama[2], Bonnie Seidel-Rogol[1], Lily Pounkova[1], Pingfeng Du[1], Christopher Papaharalambus[1], Bernard Lassegue[1] and Kathy K. Griendling[1]

## Extended Materials and Methods

### Cell Culture

VSMCs from rat thoracic aorta were grown in Dulbecco's Modified Eagle's Media (DMEM; Invitrogen) as described previously.[1] HEK293 cells (Clontech) were cultured in DMEM with 10% Fetal Bovine Serum (Invitrogen). Human aortic smooth muscle cells (HASMCs) were from Lonza. Rat VSMCs stably transfected with empty vector (Vector) or antisense p22phox (p22AS) vector were generated and cultured in 10% calf serum DMEM supplemented with 400 µg/mL G418. Cells at passages 6 to 12 were used for all experiments.

### Nox1 knockout (Nox1 y/-) cells

Nox1 y/– mice were generated by Dr K.H. Krause.[2, 3] VSMCs were isolated from mouse aortas by enzymatic digestion, as described previously, and used between passages 3 to 10.[4]

**Antibodies**

p22phox antibodies were provided by Dr. Mark Quinn (Montana State University) or from Santa Cruz, Nox4 antibodies were provided by Dr. David Lambeth (Emory University) or custom made by Sigma. Poldip2 Goat antibody was custom made by GenScript Corporation (Piscataway, NJ) against the peptide sequence NPAGHGSKEVKGKTC. When available, commercial antibodies were used: p22phox, Nox1, and RhoA antibodies were purchased from Santa Cruz, the Vinculin antibody was from Sigma, the Paxillin antibody was from BD Biosciences, the HA-tag antibody was obtained from Abcam, and Myc-tag antibodies were purchased from Cell Signaling**.**

**Yeast Two-Hybrid Assay**

We utilized the Matchmaker LexA yeast two-hybrid system (Clontech) and a VSMC cDNA library constructed in pB42AD. The hydrophilic cytosolic tail of rat p22phox (nt 360-579 in accession number FJ515740, corresponding to the C-terminal 73 amino acids), used as bait, was inserted into pLexA vector such that it is expressed as a fusion protein containing an N-terminal DNA binding domain that binds to the LexA operator sequence. The plasmid was co-transformed with the pB42AD vector and a vector (p8op-lacZ) containing the lacZ reporter gene into yeast (strain EGY48). Co-transformants containing all three plasmids (pLexA-BD-p22phox, pB42AD-cDNA, and p8opLacZ) were amplified on minimal medium (-His/-Trp/-Ura). Colonies were scraped

and replated on Leu induction medium. X-gal was included in this selection medium to confirm expression of hybrid proteins by assaying β-galactosidase activity conferred by the LacZ reporter and to help to eliminate false positives. False positives were further removed by mating independent positive clones with YM4271 yeast bearing the pLexA containing the BD domain alone.

**siRNA**

For transfection with siRNA, VSMCs were trypsinized and plated at 40-50% confluence on collagen-coated substrate. After 4-6 h, cells were washed with serum-free OPTI-MEM, and incubated with siRNA + Oligofectamine complexes for 48 h. Cells were incubated in serum free OPTI-MEM for an additional 2-4 days. A stealth siRNA for human and rat Poldip2 (siPoldip2; primer 1: 5'GCCCACAUAUAUCUCAGAGAUCUCA3', primer 2: 5'UGAGAUCUCUGAGAUAUAUGUGGGC3') and the stealth control siRNAs (siControl) of the corresponding GC content were purchased from Invitrogen. A second siPoldip2 sequence was used in some experiments and gave similar results (Supplemental Fig. 1b). Cells were transfected with a final siRNA concentration of 15 nmol/L. Nox4 siRNA (siNox4; 25 nmol/L) was used as described previously,[5] with the Allstars Negative Control (Qiagen).

**Constructs**

HA-tagged Nox1 (Nox1-HA) and V5-tagged p22phox (V5-p22phox) were prepared as described previously.[6] To prepare an adenovirus for overexpression of N-terminal myc-tagged rat Poldip2, the coding region of Poldip2 was first amplified by PCR and subcloned into the pAdTrackCMV vector at Kpn I and Xba I sites.

**Adenoviruses**

The AdEasy System was used to prepare viruses with either no insert (AdGFP), hemagglutinin (HA)-tagged Nox1 (AdNox1HA), antisense Nox1 (AdNox1AS),[7] antisense Nox4 (AdNox4AS),[5] and myc-tagged Poldip2 (AdPoldip2). The LacZ control (AdLacZ), HA-tagged constitutively active RhoA (AdRhoAGV), and HA-tagged dominant negative RhoA adenoviruses (AdRhoTN) were kind gifts of Dr. Aviv Hassid (University of Tennessee). VSMCs were transduced with recombinant adenoviruses for 2 h at 37°C in serum-free DMEM, followed by incubation for 48 h-4 days in serum-free DMEM without virus.

**GST-Pulldown**

VSMCs were transfected with AdGFP or with AdPoldip2 and labeled with $^{35}$S-methionine (20 µCi; 3 h). Lysates were incubated with GST fusion proteins (GST-vector or GST-p22phox) were prepared using the TNT T7 Quick coupled transcription/translation system (Promega). Binding partners were detected by autoradiography.

**Immunoblotting and Immunoprecipitation**

VSMCs were lysed in standard lysis buffer, as described previously,[6, 8] for Fig. 1b, or in Hunter's buffer for all additional experiments (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors). Whole cell lysates were utilized for Western blot and immunoprecipitation, as described previously.[5, 6] For all immunoprecipitation experiments, lysis buffer (LB) alone is incubated with primary antibody and beads as a

negative control. Proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies. Proteins were detected by ECL (Amersham). Band intensity was quantified by densitometry using ImageJ 1.38 software.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was extracted from cells using the RNeasy kit (Qiagen), per the manufacturer's recommendations. Superscipt II (Invitrogen) and random primers were used for reverse transcription. Message expression of poldip2 (primer sequences: GTATGAGACGGGACAGCTATTTCTCCA and CTGACATAGTCCAAGCCTGGGATG), nox1, nox4, p22phox, 18S rRNA, were measured by amplification of rat VSMC cDNA using the LightCycler (Roche) real-time thermocycler and SYBR green dye.[5] Specific rat poldip2, nox1, nox4, or p22phox primers were used to measure mRNA and normalized to 18S rRNA. Copy number was calculated by the instrument software from standard curves of genuine templates.

**Immunocytochemistry and Confocal Microscopy**

VSMCs were plated on collagen-coated glass coverslips and either serum deprived for 48 h, transiently transfected with siRNA for 72 h, or grown to 50-60% confluence before treatment with adenovirus. The cells were prepared as described previously [8], followed by incubation with primary antibodies for vinculin or paxillin (focal adhesions), Poldip2, Nox4, p22phox, Myc-tag, HA-tag, or with Phalloidin (stress fibers) or DAPI (nuclei) for 1 hour. Secondary antibodies conjugated to specific fluorophores were used for detection. Vectashield mounting medium (Vector Laboratories, Inc.) was used for all confocal expierments. Images were acquired with a Zeiss LSM 510 META Laser

Scanning Confocal Microscope System using a Plan-Apo 420782-9900 63x oil objective lens (numerical aperture: 1.40) and either Zeiss LSM 510 or Zeiss ZEN acquisition software. Controls with no primary antibody showed no fluorescence and single label controls were performed in all multiple labeling experiments. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant.

**Amplex Red Assay**

Hydrogen peroxide ($H_2O_2$) measurements in intact cells were made by measuring the oxidation of Amplex Red (100 µmol/L) in the presence of horseradish peroxidase as described previously.[9] $H_2O_2$ production was calculated and normalized to cellular protein, measured by the Bradford Assay (Bio-Rad).

**Lucigenin-enhanced Chemiluminescense**

NADPH oxidase activity in membrane fractions was assessed by measuring the reduction of 5 µmol/L Lucigenin by $O_2^{\bullet}$- in the presence of NADPH (100 µmol/L, reduced form) as described previously.[10] Briefly, cells were collected in cold HBSS and centrifuged at 1000 x g at 4°C for 10 minutes prior to resuspension in 200 µL of Hypotonic Lysis Buffer (20 mmol/L Phosphate Buffer (100 mmol/L EGTA, pH to 7.4) with 10 µg/mL Aprotinin, 0.5 µg/mL Leupeptin, and 0.5 mmol/L PMSF). Cells were sonicated on ice at a power of 4 watts for 10 seconds, followed by centrifugation at 28,000 x g at 4°C for 15 min to isolate the membrane fraction. Membrane fractions were resuspended in 60 µL of Assay Buffer (53 mmol/L Potassium Phosphate Buffer, sucrose, 100 mmol/L EGTA, pH to 7.4). Measurements were normalized to protein concentration, measured using the Bradford Assay.

**Detection of Superoxide using DHE-HPLC**

To evaluate intracellular production of $O_2^{\bullet}$-, the conversion of 2-hydroxyethidium from DHE using high-performance liquid chromatography (HPLC).[5] In some samples, polyethylene glycol (PEG)–SOD (50 U/mL) was added prior to the addition of dihydroethidium. Results are expressed as PEG-SOD-inhibitable signal.

**Detection of $H_2O_2$ using Electron Spin Resonance**

ESR was used to measure $H_2O_2$ production in membrane fractions as described previously.[11, 12]

**Immunohistochemistry**

Tissues from rats were harvested and prepared for immunohistochemistry as described previously.[5] The Poldip2 staining patterns were visualized by the streptavadin-catalyzed color reaction and 3', 3'-diaminobenzidine (DAB).

**Rho Activity Assay**

Rho activity was measured by two independent methods. The first method was a pulldown assay in which Rho activity was measured by immunoprecipitating active Rho from cell lysates using beads bound to the Rho-GTP binding peptide Rhotekin (MilliPore). Active RhoA was assessed by western blot analysis and normalized to total RhoA. The second method is a G-LISA method in which the amount of active GTP-bound Rho from cell lysates that binds to the Rho-GTP-binding protein is detected with a RhoA specific antibody and luminometry (Cytoskeleton, Denver, CO).

**Migration Assay**

Migration was measured using Boyden Chamber assays as described previously.[13] Briefly, 5 x $10^4$ cells from each treatment group were seeded in a transwell plate with

6.5 mm inserts with 8-μm pores that were coated with 0.5 mg/mL Collagen. Cells were subsequently allowed to migrate through the transwell membrane. Cells that did not migrate were removed from the upper surface of the membrane, and cells that migrated were fixed and stained with DAPI (1 μg/mL). The number of migrated cells was quantified using a Zeiss Axioskop microscope where 5 images from 5 random fields were quantified from each of 3 independent experiments. The number of migrated cells was then quantified using ImageJ 1.38 software.

**Statistical Analysis**

Results are expressed as mean ± S.E.M. from at least three independent experiments. Statistical comparisons significance was assessed using analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison post-hoc test. A value of $p<0.05$ was considered significant.

## Online Figure Legends

**Online Figure I.** ***a.*** Western blot analysis of Poldip2 expression in protein extracts isolated from rat VSMCs and immunoblotted with goat anti-Poldip2 antibody (Ab) alone or with 1:5 ratio of goat anti-Poldip2 antibody (Ab) to blocking peptide (BP). ***b.*** Western blot analysis of Poldip2 expression in protein extracts isolated from rat VSMCs transiently transfected for 4 days with 15 nmol/L of either control siRNA (siCont), siRNA against Poldip2 (siPoldip2) sequence 1 (seq.1), or siPoldip2 sequence 2 (seq. 2). CDK4 levels (lower blot) were included as a loading control. ***c.*** Rat VSMCs were transduced with either control adenovirus (AdGFP) or adenovirus to express Myc-tagged Poldip2 (AdPoldip2) for 72 h before using Western blot analysis to verify the expression of Myc-Poldip2 with an anti-Myc antibody. Myc-Poldip2 can be detected at 42 kDa and 37 kDa. ***d.*** Western blot analysis of rat VSMCs stably transfected with antisense p22phox (p22AS) or vector control (vector) to verify knockdown of p22phox. ***e.*** Western blot analysis of rat VSMCs transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to express antisense Nox4 (AdNox4AS) for 48 h prior to transducing cells with either control adenovirus (-; AdGFP) or adenovirus to overexpress Myc-tagged Poldip2 (+; AdPoldip2) for an additional 72 h to verify the knockdown of Nox4 by AdNox4AS. ***f.*** Western blot analysis of human VSMCs transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to overexpress Myc-tagged Poldip2 (AdPoldip2) for 72 h to verify that Poldip2 overexpression does not increase NADPH oxidase activity by increasing Nox4 or p22phox protein expression. ***g.*** Immunocytochemistry images were acquired using confocal microscopy of rat VSMCs either untreated or transiently transfected with 15

nmol/L of either siControl or siPoldip2 before single labeling with an anti-Poldip2 antibody (red). Images acquired at the focal adhesion plane of the cell are depicted.

**Online Figure II. a.** Confocal images of mouse nox1 wild-type (nox1 y/+) or mouse nox1 knockout (nox1 y/-) VSMCs transduced with AdGFP or AdPoldip2 (detected as green in GFP panels) before labeling with vinculin (red) or with phalloidin (pseudo-colored cyan). **b.** Confocal images of mouse nox1 wild-type (nox1 y/+) or mouse nox1 knockout (nox1 y/-) VSMCs either untreated or transiently transfected with 15 nmol/L of either siCont or siPoldip2 before labeling with vinculin (green) or with phalloidin (purple). Nuclei are labeled with DAPI (blue). Scale bars in ***a*** and ***b*** are 10 μm.

**Online Figure III.** Rat VSMCs stably transfected with empty vector (Vector) or antisense p22phox (p22AS) vector were transduced for 72 h with AdPoldip2 or AdGFP prior to using ESR to detect $H_2O_2$ levels, *$p<0.01$ vs. Vector–Poldip2, n.s. vs. p22AS–Poldip2.

## Supplemental References


**1.** Griendling KK, Taubman MB, Akers M, Mendlowitz M, Alexander RW. Characterization of phosphatidylinositol-specific phospholipase C from cultured vascular smooth muscle cells. *J Biol Chem.* 1991; 266: 15498-15504.

**2.** Gavazzi G, Banfi B, Deffert C, Fiette L, Schappi M, Herrmann F, Krause KH. Decreased blood pressure in NOX1-deficient mice. *FEBS Lett.* 2006; 580: 497-504.

**3.** Gavazzi G, Deffert C, Trocme C, Schappi M, Herrmann FR, Krause KH. NOX1 deficiency protects from aortic dissection in response to angiotensin II. *Hypertension.* 2007; 50: 189-196.

**4.** Lee MY, San Martin A, Mehta PK, Dikalova AE, Garrido AM, Datla SR, Lyons E, Krause KH, Banfi B, Lambeth JD, Lassegue B, Griendling KK. Mechanisms of vascular smooth muscle NADPH oxidase 1 (Nox1) contribution to injury-induced neointimal formation. *Arterioscler Thromb Vasc Biol.* 2009; 29: 480-487.

**5.** Clempus RE, Sorescu D, Dikalova AE, Pounkova L, Jo P, Sorescu GP, Schmidt HH, Lassegue B, Griendling KK. Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arterioscler Thromb Vasc Biol.* 2007; 27: 42-48.

**6.** Hanna IR, Hilenski LL, Dikalova A, Taniyama Y, Dikalov S, Lyle A, Quinn MT, Lassegue B, Griendling KK. Functional association of nox1 with p22phox in vascular smooth muscle cells. *Free Radic Biol Med.* 2004; 37: 1542-1549.

**7.** Lassegue B, Sorescu D, Szocs K, Yin Q, Akers M, Zhang Y, Grant SL, Lambeth JD, Griendling KK. Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ Res.* 2001; 88: 888-894.

**8.** Hilenski LL, Clempus RE, Quinn MT, Lambeth JD, Griendling KK. Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2004; 24: 677-683.

**9.** Weber DS, Rocic P, Mellis AM, Laude K, Lyle AN, Harrison DG, Griendling KK. Angiotensin II-induced hypertrophy is potentiated in mice overexpressing

p22phox in vascular smooth muscle. *Am J Physiol Heart Circ Physiol.* 2005; 288: H37-42.

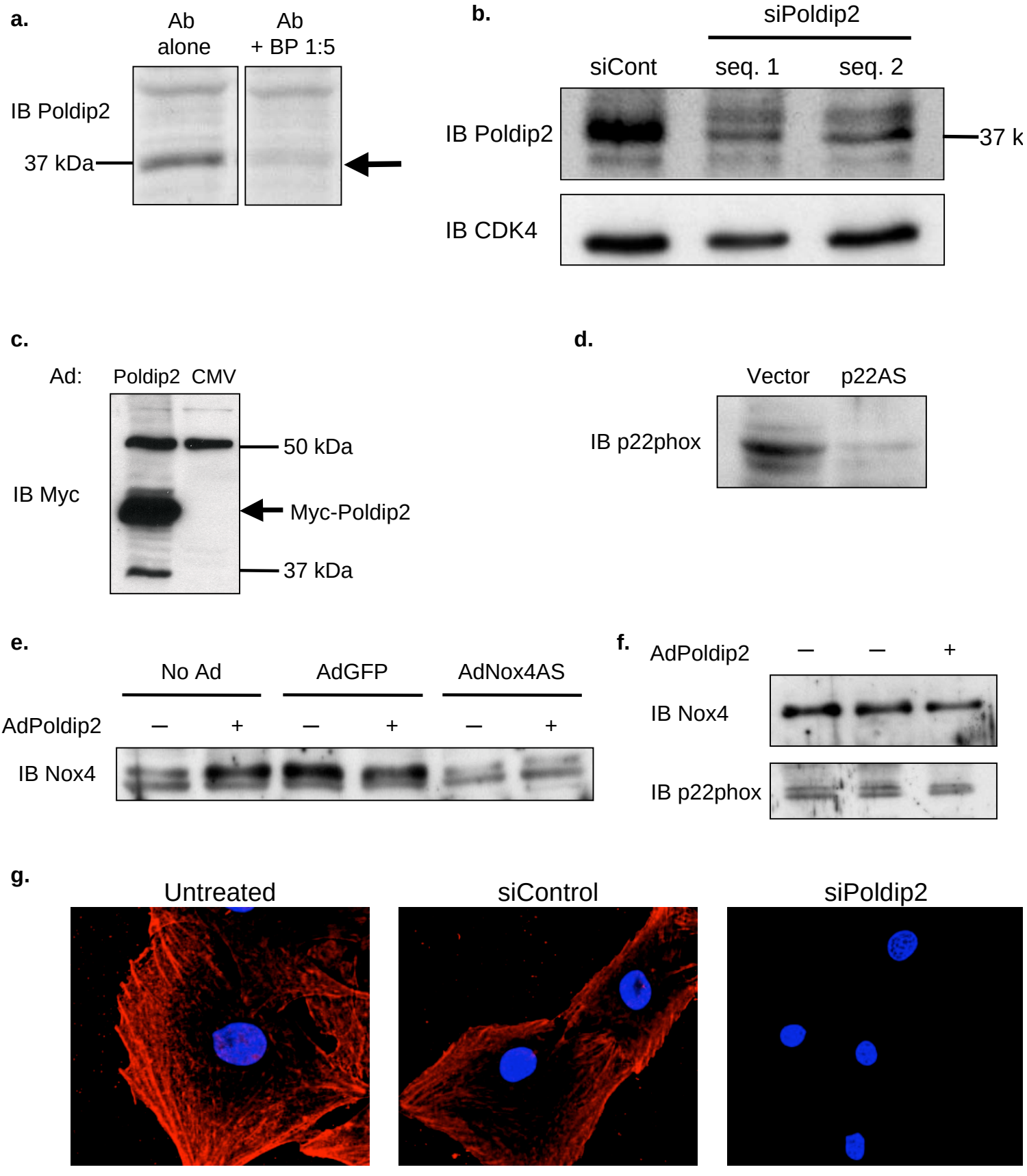
**10.** Sorescu D, Somers MJ, Lassegue B, Grant S, Harrison DG, Griendling KK. Electron spin resonance characterization of the NAD(P)H oxidase in vascular smooth muscle cells. *Free Radic Biol Med.* 2001; 30: 603-612.

**11.** Dikalov S, Griendling KK, Harrison DG. Measurement of reactive oxygen species in cardiovascular studies. *Hypertension.* 2007; 49: 717-727.

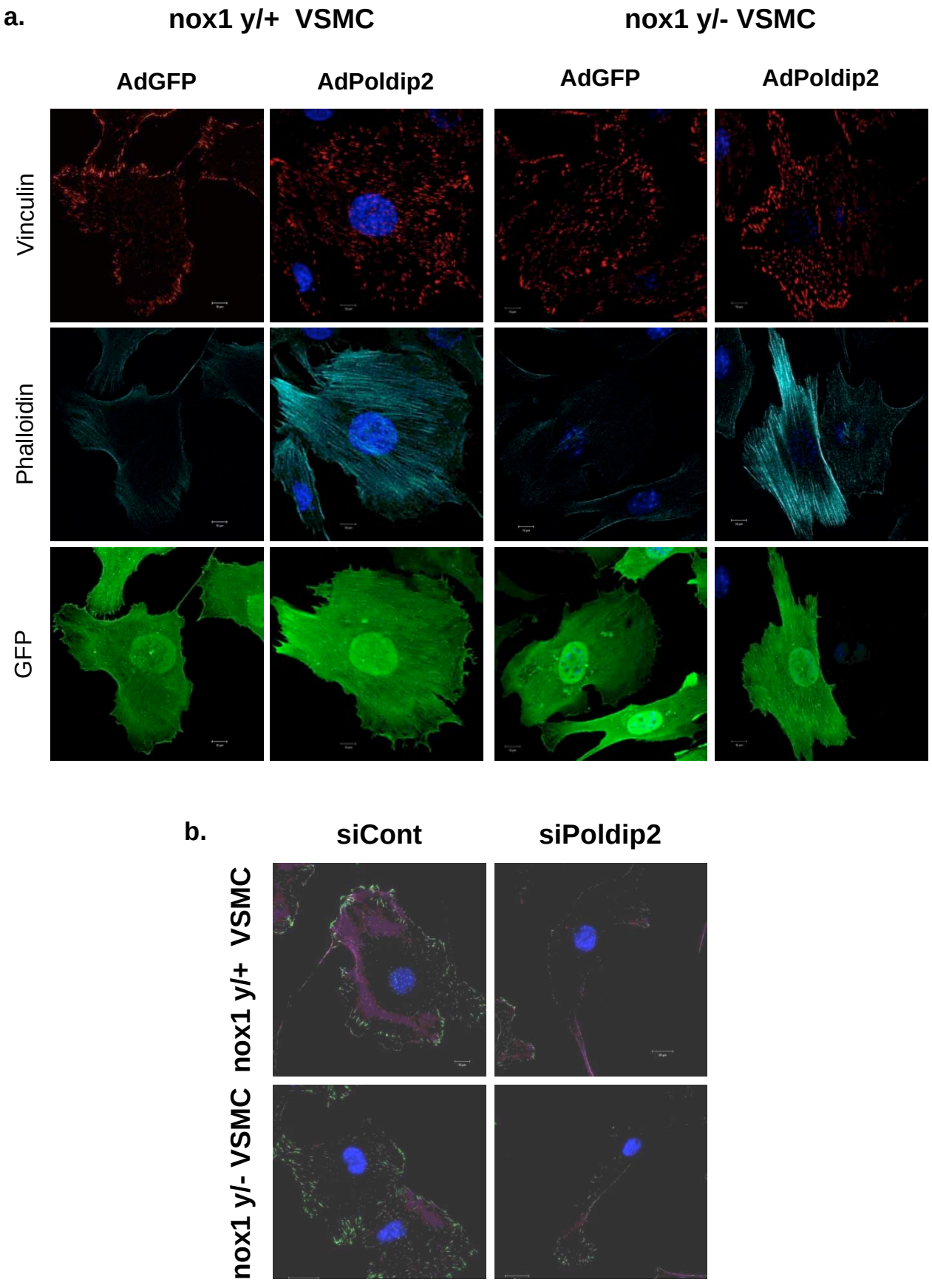
**12.** Dikalov SI, Dikalova AE, Bikineyeva AT, Schmidt HH, Harrison DG, Griendling KK. Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free Radic Biol Med.* 2008; 45: 1340-1351.

**13.** Weber DS, Taniyama Y, Rocic P, Seshiah PN, Dechert MA, Gerthoffer WT, Griendling KK. Phosphoinositide-dependent kinase 1 and p21-activated protein kinase mediate reactive oxygen species-dependent regulation of platelet-derived growth factor-induced smooth muscle cell migration. *Circ Res.* 2004; 94: 1219-1226.

# Online Figure I



# Online Figure II



# Online Figure III