Online Data Supplement

Phospho-Id3 antibody generation and analysis:

A 10mer peptide mimicking the phosphorylated N-terminal residues 1-10 of Id3 was synthesized by the University of Virginia Biomolecular Research Facility. The peptide was protected by C-terminal amidation and N-terminal acetylation, and sequence verified by mass spectroscopy following synthesis. Phospho-Id3 peptide was injected into rabbits, followed by boosts at 14, 21 and 49 days. Bleeds were performed before injection and at 35 and 56 days and purified by fractionation. Immunoreactivity of the 35-day bleed was confirmed by Western blotting lysate from VSMC transfected with vector expressing a serine 5 to alanine mutation in Id3 (pAdId3 S5A), wild-type Id3 (pAd Id3) in duplicate, and a control vector (pAd empty) (fig. 1a). The control pre-bleed (taken before peptide injection) did not display any immunoreactivity Post-immune serum demonstrated immunoreactivity with wildtype Id3 but not Id3S5A (fig. 1a). Anti-phospho-Id3 antiserum was further purified by affinity purification over a phospho-Id3 column. Purified anti-phospho-Id3 antiserum was examined for specificity to Id3 by Western blotting VSMC lysate from $Id3^{+/+}$ and $Id3^{-/-}$ mice. $Id3^{+/+}$ lysate showed positive immunoreactivity with our anti-phospho-Id3 antiserum at the appropriate molecular weight, while $Id3^{-/-}$ lysate did not (fig. 1b). Both $Id3^{+/+}$ and $Id3^{-/-}$ lysate reacted with an antibody against Id2 (Santa Cruz), which contains a similar N-terminal sequence, including a cdk-2 phosphorylation site at Ser5 (fig. 1b), providing evidence that our phosphoId3 antibody does not recognize endogenous phosphoId2. Moreover, the phopshoId3 antibody stained VSMC in the vessel wall of wildtype but not $Id3^{-/-}$ mice

providing evidence that the *in vivo* staining is specific for phosphoId3. To further confirm the specificity of our purified antiserum, we performed Western blot analysis on lysate from VSMC over-expressing wild-type Id3 or Id3S5A. Results indicate that lysate from cells transfected with wild-type Id3, but not Id3S5A, displayed positive immunoreactivity with our purified antiserum (fig. 1c).

Effect of other mitogens on Id3 phosphorylation:

In order to examine whether specific mitogens induce Id3 protein expression and phosphorylation in VSMC, we quiesced cultured cells for 72 hours in serum-free media, followed by stimulation with either 10% FBS, 10 ng/mL PDGF-BB or 5 μM angiotensin II. Cell lysates were harvested 0, 1, 3, 5, 18 and 24 hours following stimulation for Western blot analysis of Id3 and phospho-Id3 protein expression. Results indicate that PDGF-BB induces Id3 expression in a biphasic fashion similar to that observed with FBS stimulation (fig. 2). Interestingly, and unlike FBS, phosphorylated Id3 was not observed through 24 hours. Angiotensin II has been shown in the literature to transiently induce Id3 expression in VSMC approximately 1 hour following stimulation [1]. As with PDGF-BB, however, there was no immunoreactive band when lysates from Angiotensin II-treated cells at any time point were probed with the Id3 serine 5 phosphospecific antibody although the serum-stimulated 24 hours time point used as positive control did demonstrate a band at the predicted size (data not shown).

Expanded materials and methods:

Cell culture: Primary aortic VSMC were obtained from adult male C57BL6 mice, $Id3^{-/-}$, $Id3^{+/-}$ and $Id3^{+/+}$ littermate control mice by enzymatic digestion and grown in Dulbecco's modified Eagle's medium/F12 (1:1) (Gibco) with 20% FBS (HyClone). Separate primary harvests of VSMC from 6 independent groups of animals for both the $Id3^{+/+}$ littermate controls and the $Id3^{-/-}$ mice were performed. Cell number assays in all primaries demonstrated consistent results as depicted in figure 1a (i.e. loss of Id3 results in significant inhibition of growth). Following attachment, cells were cultured in 10% FBS. Cultured mouse cells were used between passages 5 and 10. Growth arrest was accomplished using serum-free medium for 72 hours. S-phase arrest was performed by adding 2.5mM thymidine to the culture medium overnight. Primary aortic rat VSMC were obtained from adult male Sprague-Dawley rats as above. Rat VSMC were used between passages 5 and 10.

Cellular proliferation assays: Cultured VSMC were plated into 96 well plates at an initial density of 1,000 cells per well. At 3, 24 and 48 hours following plating, cells were assayed for proliferation. Proliferation was measured using an MTS assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay, Promega) according to the manufacturer's protocol.

FACS analysis: Cells were trypsinized, centrifuged and washed in PBS prior to fixation in 70% EtOH overnight at $4^{o}C$. Cells were stained for DNA content using a propidium iodide (PI) staining solution (20 μg/mL PI, 0.1% Triton X-100 and 0.2 mg/mL RNAse in

PBS) at37^{o}C for 15m and analyzed for cell cycle content by FACS analysis on a FACSCalibur dual laser benchtop cytometer (Becton Dickinson).

*Western blotting***:** Lysates were collected in mPER lysis buffer (Pierce, Rockford, Illinois). Samples were electrophoresed with 4-20% SDS gradient gels (NuPAGE, Invitrogen) and transferred to PVDF membranes (Sigma). Western blotting was performed with antibodies to Id3 (0.5 μg/mL, Santa Cruz Biotechnologies), p21 (0.5 μg/mL, PharMingen), α-tubulin (0.5 μg/mL, Sigma) or purified phospho-Id3 antiserum (1:1000 dilution).

*Transient transfection***:** Transfections were performed using Fugene transfection reagent (Roche) according to the manufacturer's instructions.

Generation of Id3S5A: A point mutation was introduced into the coding region of Id3 using PCR. 5' PCR primers contained a single nucleotide substitution changing the residue 5 codon from AGC→GGC (Ser→Ala). PCR product was TA cloned into PGEM-T vector (Promega) and subsequently subcloned into the pAdLox mammalian expression vector.

*Promoter-reporter assays***:** Cultured rat VSMC were transfected with 0.8 μg of pAdLox (pAd)-empty vector, pAd-Id3 or pAd-Id3S5A together with 0.1 μg of pCDNA-E47 or PCDNA-empty vector and 0.1 μg of p21-Luc. 48 hours following transfection, cells were

harvested in luciferase lysis buffer, incubated with luciferase substrate (Promega) and measured for luciferase activity.

Mammalian two-hybrid assay: Mammalian two-hybrid assays were performed using the Mammalian Matchmaker Two-Hybrid Assay Kit (Clontech). Cultured rat VSMC were transfected with 0.2 μg pG5CAT reporter construct together with 0.4 μg pVP16-Pan1 bait construct and 0.4 μg pM-empty vector, pM-Id3 or pM-Id3S5A. 48 hours following transfection, lysates were harvested and analyzed for CAT activity using ELISA (CAT ELISA, Roche).

Effect of Id3 and Id3S5A on cell proliferation: Cultured rat VSMC were transiently co-transfected with 0.5 μg pAd-GFP and 0.5 μg of either pAd-empty vector, pAd-Id3 or pAd-IdS5A. 24 hours following transfection, cells were harvested in a minimal volume of accutase (Innovative Cell Technologies) and GFP-positive cells were sorted using a Becton Dickinson FACSVantage SE Turbo Sorter directly into 96-well plates at a density of 1,000 cells per well. At 3 and 48 hours following plating, cell number was assayed using an MTS assay (Promega).

*Mouse immunohistochemistry***:** ApoE-/- mice were fed a Western atherogenic diet containing 21% fat by weight (0.15% by weight cholesterol and 19.5% by weight casein without sodium cholate) for 5weeks before sacrifice. Mice were given an overdose of ketamine/xylazine and pressure-perfused with 4% paraformaldehyde. Injured arteries

were removed, postfixed in 4% paraformaldehyde overnight at 4 ^{o}C, dehydrated in a graded alcohol series and paraffin-embedded for thin sectioning. Five-micron arterial sections were stained using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Utah) using a 1:1000 dilution of purified anti-phospho-Id3 antiserum. Slides were then incubated for 30m in 0.3% hydrogen peroxidase substrate (Sigma) for 2m, counterstained with hematoxylin and mounted with Vectashield mounting medium (Vector Laboratories).

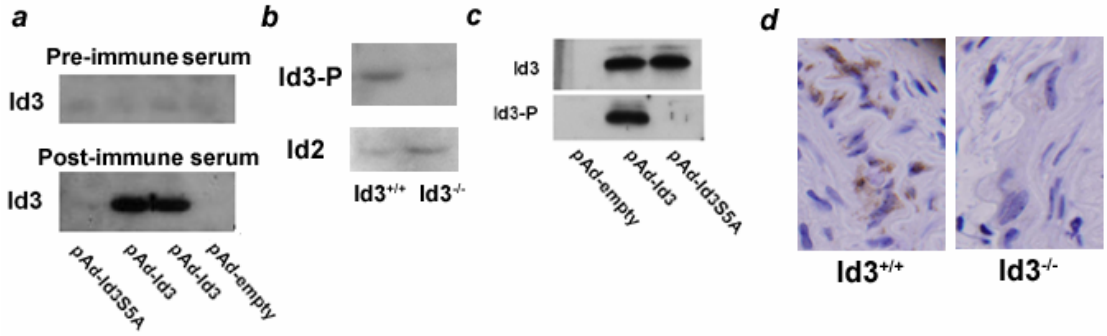
1. Mueller C, Baudler S, Welzel H, Bohm M, Nickenig G. Identification of a novel redox-sensitive gene, Id3, which mediates angiotensin II-induced cell growth. *Circulation*. 2002;105:2423-2428.

Online Figure Legends:

Online Figure 1: *A*—Analysis of pre-and post-immune serum from rabbits injected with phospho-Id3 peptide. Serum was harvested from rabbits before (pre-immune) or 35 days after immunizing with a synthetic 10-mer corresponding to the Ser5-phosphorylated N-terminus of Id3. VSMC were transfected with cDNA encoding Id3S5A (pAd Id3S5A), wild-type Id3 (pAd Id3) or empty vector control (pAd empty) and harvested for Western blot analysis 48 hours later using a 1:1000 dilution of the indicated sera. *B*—Purified anti-phospho-Id3 antiserum is specific for Id3. Lysate from $Id3^{+/+}$ and $Id3^{-/-}$ VSMC was analyzed by Western blotting using affinity purified anti-phospho-Id3 antiserum, then stripped and re-probed using a commercial Id2 antibody. *C*—The specificity of the anti-phospho-Id3-specific antiserum was verified by blotting lysates from COS cells over-expressing wild-type Id3 or the non-phosphorylatable mutant, Id3S5A. *D*—The specificity of anti-phospho-Id3 antiserum in immunohistochemical analysis was verified by staining the media of carotid arteries from $Id3^{+/+}$ and $Id3^{-/-}$ mice using a 1:1000 dilution of purified antiserum.

Online Figure 2: The effect of PDGF-BB on Id3 protein expression and phosphorylation. Cultured VSMC were serum-starved for 72 hours and then stimulated with either 10% FBS or 10 ng/mL PDGF-BB. Lysates were harvested at the indicated time points for Western blot analysis using purified anti-phospho-Id3 antiserum or a commercial Id3 antibody.

Online Figure 1



Online Figure 2