Polymerase Delta Interacting Protein 2 Sustains Vascular Structure and Function


Objective—On the basis of previous evidence that polymerase delta interacting protein 2 (Poldip2) increases reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (Nox4) activity in vascular smooth muscle cells, we hypothesized that in vivo knockdown of Poldip2 would inhibit reactive oxygen species production and alter vascular function.

Approach and Results—Because homozygous Poldip2 deletion is lethal, Poldip2−/− mice were used. Poldip mRNA and protein levels were reduced by ≈50% in Poldip2−/− aorta, with no change in p22phox, Nox1, Nox2, and Nox4 mRNAs. NADPH oxidase activity was also inhibited in Poldip2−/− tissue. Isolated aortas from Poldip2−/− mice demonstrated impaired phenylephrine and potassium chloride–induced contractions, increased stiffness, and reduced compliance associated with disruption of elastic lamellae and excessive extracellular matrix deposition. Collagen I secretion was elevated in cultured vascular smooth muscle cells from Poldip2−/− mice and restored by H2O2 supplementation, suggesting that this novel function of Poldip2 is mediated by reactive oxygen species. Furthermore, Poldip2−/− mice were protected against aortic dilatation in a model of experimental aneurysm, an effect consistent with increased collagen secretion.

Conclusions—Poldip2 knockdown reduces H2O2 production in vivo, leading to increases in extracellular matrix, greater vascular stiffness, and impaired agonist-mediated contraction. Thus, unaltered expression of Poldip2 is necessary for vascular integrity and function. (Arterioscler Thromb Vasc Biol. 2013;33:2154-2161.)

Key Words: blood vessel ■ extracellular matrix ■ hydrogen peroxide ■ Nox4 ■ Poldip2

Vascular remodeling occurs in response to elevated blood pressure, vessel injury, endothelial dysfunction, as well as leukocyte infiltration, and is exacerbated in aging. Documented changes in vascular structure in the adult include vascular smooth muscle hypertrophy and hyperplasia, accumulation of extracellular matrix, and loss of elasticity. Excess matrix can lead to loss of contractility and arterial stiffening, both of which have important consequences for vascular function. During the past decade, it has been suggested that aortic stiffness can be considered an important tissue biomarker risk factor for cardiovascular disease. Indeed, a recent meta-analysis of 17 clinical studies showed that aortic stiffness, as measured by pulse wave velocity, is a strong predictor of future cardiovascular events and all-cause mortality. It is therefore of critical importance to understand the mechanisms contributing to the regulation of vascular compliance.

One newly appreciated regulator of aortic stiffness is oxidative stress. Zhou et al found that aged mice deficient in superoxide dismutase 2 with reduced H2O2 exhibit increased pulse wave velocity, increased collagen I expression, impaired integrity of elastic lamellae, and enhanced medial smooth muscle cell (SMC) apoptosis. Of interest, a similar phenotype was not observed in mice deficient in p47phox, a component of the Nox1 and Nox2 reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. However, Maillaro-Rafferty et al found that apolipoprotein E–deficient mice crossed with smooth muscle–specific catalase overexpressing mice have increased aortic stiffness and greater collagen content. Other work suggests that reactive oxygen species (ROS) derived from Nox4 NADPH oxidase can also affect extracellular matrix composition and structure. Nox4 increases collagen expression in cardiac myofibroblasts, matrix metalloproteinase-2 (MMP-2) activity in human ovarian cancer cells, activation of MMP-1 by diesel exhaust fumes in lung epithelial cells, and urotensin II–induced activation of MMP-2 in vascular SMCs (VSMCs). Moreover, genetic deletion of Nox4 leads to increased interstitial cardiac fibrosis in response to suprarenal aortic constriction. These...
observations suggest that H₂O₂ derived from Nox4 may impinge on arterial structure.

We recently reported that polymerase delta interacting protein 2 (Poldip2) binds to p22phox and enhances Nox4 activity. Poldip2 overexpression in VSMCs increases ROS production in a Nox4-dependent manner, and knockdown of Poldip2 leads to a loss of focal adhesions and impaired migration. Moreover, overexpression of Poldip2 prevents focal adhesion dissolution in response to platelet-derived growth factor. These observations, together with the known effects of Nox4 on matrix integrity, led us to postulate that Poldip2 may influence vessel contractility and compliance. To test this hypothesis, we generated mice with reduced Poldip2 expression and investigated vascular structure and function. We found that loss of Poldip2 markedly alters aortic extracellular matrix, impairs contractility, and increases stiffness, suggesting that the Poldip2/Nox4 axis may be an important regulator of vascular physiology.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Characterization of Poldip2-Deficient Mice

Mice were produced at the Texas A&M Institute for Genomic Medicine from a clone of embryonic stem cells with a gene trap construct inserted into the first intron of Poldip2 (Figure IA in the online-only Data Supplement). To verify the location of the insert in vivo, we amplified genomic DNA from a heterozygous mouse, using polymerase chain reaction primer pairs P1-P3 and P4-P2 (Figure IA in the online-only Data Supplement), surrounding both junctions of the construct. Sequencing of the polymerase chain reaction products confirmed the successful interruption of the Poldip2 gene at the first intron and the presence of splice acceptor and β-geo cassette in the correct orientation (not shown).

Aiming to study the physiological effects of a complete Poldip2 ablation in vivo, we crossed heterozygous mice, but obtained very few of the desired homozygous pups (Figure IB in the online-only Data Supplement). After genotyping 227 weanlings, we only observed 7 live Poldip2−/− mice, rather than 56, as expected from classical Mendelian genetics. Furthermore, these few survivors were small and had to be weaned later than their siblings. This observation suggests that the gene trap effectively blocks expression and that Poldip2 is essential for survival. Because homozygous mice could not be produced in significant numbers, all additional studies were conducted in heterozygotes, which seemed to be healthy and were indistinguishable from their wild-type (WT) littermates in size and weight.

To verify the effectiveness of the gene trap and to detect possible changes in Nox subunits, we measured the expression of selected genes by quantitative reverse transcriptase–polymerase chain reaction. Whereas Poldip2 mRNA was decreased by 50% in whole aortas from Poldip2+/− mice, there was no change in p22phox, Nox1, Nox2, and Nox4 (Figure 1A). Reduction in Poldip2 protein expression was confirmed in lysates from whole aortas by Western blotting (Figure 1B), whereas p22phox and Nox4 proteins were not affected (Figure II in the online-only Data Supplement). Poldip2 protein expression was similarly decreased in lung (not shown). These results suggest that Poldip2 expression is directly dependent on the number of functional genes,
and further support the idea that the Poldip2 gene trap allele is null. Moreover, because no compensatory upregulation of Nox enzymes was observed, the partial knockdown of Poldip2 can be expected to have detectable physiological consequences.

**NADPH Oxidase Activity in Poldip2+/− Mice**

Because Poldip2 enhances Nox4 activity, the effect of Poldip2 reduction on NADPH oxidase activity was measured using an electron spin resonance assay. Because of the small amount of aortic tissue available, these measurements were made in kidney slices. As shown in Figure 2, Poldip2 knockdown in heterozygous mice significantly decreased NADPH-dependent production of $O_2^-$ and $H_2O_2$. As expected, the latter ROS was generated at a much higher rate than the former, because $O_2^-$ produced by Nox4 is mostly converted to $H_2O_2$, rather than being released. Importantly, these results suggest for the first time that Poldip2 contributes to Nox4 activity in vivo.

**Contraction of Poldip2+/− Aortas Ex Vivo**

Because vascular contractility is strongly influenced by $H_2O_2$, the impact of Poldip2 knockdown on vascular contraction was investigated. Isometric force per cross-sectional area was measured in isolated aortic rings exposed to increasing concentrations of the $\alpha$-adrenergic agonist, phenylephrine. Although sensitivity (EC50) to the stimulus was unchanged, maximal force was reduced in vessels from Poldip2+/− mice (48±17% inhibition; Figure 3A). This effect was not caused by an inhibition of agonist signaling, because it was also observed when contraction was induced by depolarization with exogenous potassium chloride (44±17% inhibition; Figure 3B). The inhibition of contraction persisted, although to a lesser degree, after removal of the endothelial layer, suggesting that both smooth muscle and endothelium contribute to the observed phenotype (Figure III in the online-only Data Supplement). The inhibition of contraction in Poldip2+/− aortas was also observed when vessels were preincubated for 30 minutes with 1000 U of polyethylene glycol–catalase, indicating that it is not mediated by an acute effect of $H_2O_2$ (Figure III in the online-only Data Supplement). Furthermore, because the aortic cross-sectional area was not affected by the genotype (Figure IV in the online-only Data Supplement and data not shown), we explored vascular biomechanics in greater detail. Stiffness seemed to be increased in maximally dilated aortic rings from Poldip2+/− mice subjected to stepwise extensions.

![Figure 2](image_url)

### Figure 2

NADPH oxidase activity is inhibited in Poldip2+/− mice. Superoxide ($O_2^-$, left) and hydrogen peroxide ($H_2O_2$, right) were measured in kidney slices from wild-type (black bars) and Poldip2+/− (gray bars) mice by electron spin resonance. Data represent average±SEM from 5 to 10 mice in each group; **P<0.01.

![Figure 3](image_url)

### Figure 3

Aortic contraction and compliance are reduced in Poldip2+/− mice. Isometric force normalized to cross-sectional area (CSA) was measured in isolated aortic rings from wild-type (black symbols) and Poldip2+/− mice (gray symbols) exposed to indicated concentrations of phenylephrine (A) or potassium chloride (B). To evaluate stiffness, aortic segments were incrementally elongated (C) or inflated (D and E). Data represent averages±SEM from 5 to 9 vessels. ***P<0.001, *P<0.05 +/+ versus +/−. Poldip2 indicates polymerase delta interacting protein 2.
(Figure 3C). This result was confirmed by measuring compliance in aortic segments incrementally inflated with cell culture medium (Figure 3D and 3E).

Structure of Poldip2+/− Vessels
To investigate the possibility that the observed changes in compliance were a consequence of structural alterations, we examined transverse aortic sections using transmission electron microscopy. In aortas from WT mice, elastic lamellae divide the vascular media in regular concentric rings (Figure 4A and 4C). In marked contrast, elastic lamellae from Poldip2+/− aorta were frequently fragmented and irregular both in thickness and orientation (Figure 4B and 4D, Figure IV in the online-only Data Supplement). Furthermore, the percentage of interlamellar area occupied by fibrillar (Figure 4F) or amorphous (Figure 4D) extracellular matrix was significantly increased in Poldip2+/− mice, compared with WT mice (Figure 4E). These alterations in structural organization are consistent with the decrease in vascular compliance observed in Figure 3 and may relate to abnormal matrix deposition as well as excessive protein secretion by SMCs.

Collagen I Secretion in VSMCs
To examine the role of Poldip2 in matrix production, collagen secretion was measured in VSMCs isolated from WT and Poldip2+/− mice. As shown in Figure 5, collagen I released into the medium was increased in Poldip2+/− compared with WT cells. A similar result was obtained in rat VSMCs treated with Poldip2 siRNA, as extracellular fibronectin and collagen were both increased (Figure V in the online-only Data Supplement). Furthermore, neither the mRNAs of collagens I, III, IV, and XVIII, elastin, fibronectin (Figure VI in the online-only Data Supplement) nor intracellular collagen protein were affected (not shown), suggesting that in healthy vessels Poldip2 inhibits secretion, rather than synthesis, of extracellular matrix. To determine whether this effect of Poldip2 on collagen secretion is mediated by ROS, mouse VSMCs were treated with exogenous glucose oxidase, which produces H₂O₂ continuously in the presence of glucose in the medium (Figure VII in the online-only Data Supplement). As shown in Figure 5, glucose oxidase abolished the increase in collagen I observed in Poldip2+/− VSMCs. Thus, collagen I secretion seems to be inversely related to H₂O₂ production and Poldip2 expression.

To begin investigating other possible changes that may be associated with impaired contraction and elevated extracellular matrix production, the mRNA expression of candidate genes was measured by quantitative reverse transcriptase–polymerase chain reaction. As shown in Figure VI in the online-only Data Supplement, none of the measured inflammatory markers were affected. In contrast, MMP-2 and MMP-9 were upregulated, whereas their inhibitors TIMP1 and TIMP2 were unchanged. This result suggests that compensatory mechanisms mitigate the elevation in extracellular matrix deposition in the vessel wall. Further studies will be required to determine which signaling pathways are responsible for this effect.
Blood Pressure in Poldip2+/− Mice
Impaired vascular contraction and compliance in vitro would be expected to affect blood pressure regulation in vivo. Although basal blood pressure was not changed, Poldip2+/− mice presented a slight reduction in angiotensin II–induced hypertension (Figure VIII in the online-only Data Supplement). This result suggests that Poldip2 can regulate vascular function, at least when animals are exposed to a chronic challenge.

Aortic Dilation in Poldip2+/− Mice
Because alterations in extracellular matrix deposition would be expected to affect vascular remodeling, aortic dilation was induced in Poldip2+/− mice by surgical application of CaCl2 to the abdominal aorta in a model of experimental aneurysm.16 As expected in vessels with excess matrix, Poldip2+/− mice were protected against vascular dilation compared with WT mice (Figure 6). Collectively, these findings suggest that a reduction in Poldip2 with a corresponding decrease in Nox4 activity enhances aortic matrix deposition, thereby disrupting vascular structure and leading to reduced agonist-induced contraction and decreased susceptibility to experimental aortic dilation.

Discussion
This is the first study addressing the functions of Poldip2 in vivo. Although Poldip2 has multiple cellular targets, here we focused on its ability to regulate ROS. We found that Poldip2 ablation is embryonically lethal, whereas a reduction of Poldip2 levels in heterozygous animals decreases NADPH oxidase activity and alters arterial structure. Aortas from Poldip2 heterozygous animals exhibit disordered elastic lamellae and excess collagen, the latter of which seems to result directly from lower H2O2 levels. In consequence, smooth muscle contraction and vascular compliance are reduced, which leads to protection against experimental aortic dilation. Thus, Poldip2 represents a novel mechanism to regulate aortic stiffness, which is a major cardiovascular risk factor.

Poldip2 has been implicated in several signaling pathways essential to life, explaining why Poldip2 knockout is embryonically lethal, whereas Nox4 knockout is not.9,17 Poldip2 is a 42-kDa protein with a mitochondrial targeting sequence that is cleaved in some tissues, including smooth muscle, to create a functional 37-kDa protein.10,18 It was first identified as a protein that binds to the p50 subunit of DNA polymerase delta and proliferating cell nuclear antigen, suggesting that it has a role in DNA repair.18,19 Poldip2 was then shown to interact with cell–cell adhesion receptor (CEACAM-1), which enables it to shuttle between the cytoplasmic and nuclear compartments where it has a role in mitotic spindle organization and chromosomal separation.20 Recently, we found that Poldip2 also interacts with p22phox and increases Nox4 activity, leading to enhanced stress fiber and focal adhesion formation.18 These latter functions of Poldip2 suggest that it has an important role in cytoskeletal dynamics, which may be related to its ability to interact with Nox4. In this study we found that Poldip2 deletion enhances collagen secretion in a redox-sensitive manner, implying that it may have a concerted effect on matrix–cytoskeletal interactions and vascular contraction.

The clear effect of Poldip2 on vascular structure and extracellular matrix strongly suggested that vascular compliance would be affected. The mechanical properties of isolated aortas were assessed using 2 different methodologies, which produced similar results. In the first method, force is measured while incrementally stretching aortic rings between 2 wires. The data indicate that Poldip2+/− arteries are stiffer than those of WT, because they require greater force to dis- tend to the same degree (Figure 3C). In the second method, diameter is measured at a fixed length while inflating aortic lamellae and excess collagen, thereby disrupting vascular structure and leading to reduced agonist-induced contraction and decreased susceptibility to experimental aortic dilation.
relevant because native vessel geometry is better preserved in the latter model.22–24

Although the decrease in compliance in Poldip2+/− mice seems modest, clinical studies have reported that changes in arterial stiffness can be used as an accurate predictor of risk for cardiovascular events.25,26 In other studies, measurements of the incremental modulus of elasticity, distensibility and compliance showed that markers of stiffness were elevated in patients with end-stage renal disease who died from cardiovascular disease, compared with those who had no events.27 It has also been reported that the elastic modulus and Young’s modulus were approximately 25% and 11% greater, respectively, in hypertensive as compared with normotensive patients,28 and a similar study found that the carotid distensibility in hypertensive patients was ≈24% lower than in normotensive control patients.29 Thus, fairly modest changes in arterial stiffness have been associated with cardiovascular disease. The changes in vascular compliance reported in our study (10%–14%) are numerically similar to those reported in humans with cardiovascular disease (11%–25%). Importantly, it was also shown that the carotid distensibility in hypertensive patients was associated with a high Framingham risk score.30 Thus, we suggest that even though modest, the changes in compliance we observed represent a significant and important role for Poldip2+/− in arterial mechanics corroborated by the decrease in angiotensin II–induced hypertension observed in Poldip2+/− mice (Figure VIII in the online-only Data Supplement).

Although excessive oxidative stress has been linked to fibrosis and increased collagen synthesis in other settings,30–34 in the present study extracellular matrix and collagen are increased as a result of lower Poldip2 and H2O2. Because Poldip2 deletion affects neither the mRNAs of major extracellular matrix components (Figure VI in the online-only Data Supplement), such as collagen I, nor intracellular mature procollagen protein, our findings suggest that reduced H2O2 favors procollagen I secretion (Figure 5 and Figure V in the online-only Data Supplement), thereby impairing vascular contraction. This interpretation is supported by the observation that supplementation with H2O2 normalized collagen secretion from VSMCs over 3 days, whereas a short preincubation with catalase had minimal effects on vascular contraction. In addition, we observed an increase in MMP-2 and MMP-9 mRNAs in Poldip2+/− aortas, with no change in TIMP1 and TIMP2 (Figure VI in the online-only Data Supplement), suggesting feedback regulation of the excess extracellular matrix deposition. Future studies will be required to verify that the activity of these proteases is indeed upregulated and to analyze their regulatory pathways in Poldip2+/− tissues.

Poldip2 knockout also has a dramatic effect on the integrity of the elastic lamellae. Mature elastin fibers are composite structures, consisting of an inner amorphous core of cross-linked elastin and a meshwork of more than 30 elastin-associated molecules,35 including fibrillins, fibulins, microfibril-associated glycoproteins, and proteoglycans.36 Although the amount of elastin in the vessel does not change after birth,36 elastin fiber assembly is an ordered, hierarchical sequence of events in which microfibrillar and other proteins serve as templates for the deposition of soluble tropoelastin molecules, which then become highly cross-linked and aggregated into insoluble filamentous structures. Progressive aggregation and fibrillogenesis have been linked to the dynamic motility of cells as they move and organize fibrillar material within the mechanically coupled cell–extracellular matrix environment.37,38 The proposed correlation between cell/tissue motion and the organizational patterning of the extracellular matrix scaffold could explain why Poldip2+/− mice, whose VSMCs exhibit impaired focal adhesion and actin cytoskeleton formation and migration,39,40 have altered elastin fiber architecture leading to reduced vessel elasticity and also potentially to homozygous lethality.

It is not surprising that the contractility and stiffness of vessels from Poldip2 heterozygous mice are compromised, given these major alterations in extracellular matrix composition, structure, and previously observed reductions in focal adhesions. However, H2O2 can also affect contractility of smooth muscle directly or indirectly via release of endothelium-dependent factors.41 For example, H2O2 increases calcium flux through L-type calcium channels, leading to contraction of cerebral arteries,39 and ROS derived from Nox2 activate protein kinase C to induce contraction in coronary arteries.40 The combination of increased cytosolic calcium concentration and activation of other signaling kinases can lead to activation of myosin light chain kinase, favoring contraction.41 On the basis of these reports, one could predict that the reduction of H2O2 in Poldip2+/− animals would lead to less force generation, which is in fact what we observed. Because our results (Figure III in the online-only Data Supplement) implicate both endothelium and smooth muscle, further studies using tissue-specific Poldip2 knockout animals will be required to dissect the contributions of these potential mechanisms to the reduced force generation observed in Poldip2 heterozygote aortas.

The relative amounts and organization of elastin, collagen, and VSMCs in the media determine the mechanical properties of the tissue. Elastin is generally thought to endow distensibility to tissues over low loading, whereas collagen provides a stiffener response at higher loads.41 A mechanistic link between ROS and arterial stiffness has recently emerged. Reduction of vascular H2O2, either by deletion of 1 allele of superoxide dismutase 2 or by overexpression of catalase,4 leads to increased collagen deposition and increased aortic stiffness. These results are similar to those observed here in Poldip2+/− aortas, and are consistent with alterations in ROS levels playing a causal role in this animal model. Poldip2+/− animals also exhibit fragmented and disordered elastic lamellae and increased collagen content, which is consistent with increased arterial stiffness. The observed increase in aortic stiffness in these animals suggests that genetically based reductions in Poldip2 expression or activity in humans may be a risk factor for future cardiovascular disease.

However, loss of Poldip2 can also be protective. Here, we demonstrate that heterozygosity of Poldip2 slightly reduces angiotensin II–induced hypertension and protects animals against aortic dilatation. Both elevations in ROS42,43 and dysfunctional elastin and collagen cross-linking44 promote aneurysm formation. Moreover, alteration in the
microarchitecture of adventitial collagen fibrils can contribute to aneurysms.55 As Poldip2 heterozygote animals have both reduced ROS and increased collagen, either or both pathways could contribute to protection against experimental aortic dilatation.

In summary, we have shown that Poldip2 regulates ROS production in vivo and that deletion of Poldip2 leads to a profound structural alteration in the vessel wall. The induction of collagen secretion by loss of Poldip2 suggests that one perhaps unexpected function of Poldip2 and possibly Nox4 is to repress extracellular matrix formation. This previously unidentified function of the enzyme complex has important implications not only for the development of vascular lesions, but also for a number of connective tissue diseases such as rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis.

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Disclosures
None.

References
Polymerase delta interacting protein 2 was previously shown to enhance NADPH oxidase 4 activity and regulate cytoskeletal organization in vascular smooth muscle cells. We now demonstrate that in vivo knockdown of polymerase delta interacting protein 2 inhibits NADPH oxidase activity and induces an unexpected vascular phenotype. It has been well documented that oxidative stress, resulting from a pathological increase in reactive oxygen species production, can induce fibrosis in various tissues. In contrast, our results suggest that a moderate chronic impairment in H_2O_2 production increases collagen secretion by vascular smooth muscle cells and enhances extracellular matrix deposition in the vascular wall, thereby contributing to increased vascular stiffness, reduced contractility, and protection against aortic dilation. Our data thus support the view that subtle changes in redox regulation, regardless of their direction, can have a broad effect on the whole organism.
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In the article by Sutliff et al, which appeared in the September 2013 issue of the journal (Arterioscler Thromb Vasc Biol. 2013;33:2154–2161. DOI: 10.1161/ATVBAHA.113.301913), the legend for Figure 4 should have appeared as:

Disrupted elastic lamellae (EL) and increased extracellular matrix in Poldip2<sup>−/−</sup> aorta. Transmission electron micrographs of transverse aortic sections from wild-type (top) and Poldip2<sup>−/−</sup> (bottom) mice at increasing magnifications (left to right). The vascular lumen is visible at the top of images A to D. EL appear dark after poststaining with tannic acid in A and B. Breaks in elastic lamellae in Poldip2<sup>−/−</sup> are marked with arrows in B and D. Morphometric measurements of interlamellar amorphous (C and D) or fibrillar (F) extracellular matrix (ECM) areas in 195 images from 3 wild-type and 3 Poldip2<sup>−/−</sup> mice are expressed as average±SEM in E; *P<0.05. Poldip2 indicates polymerase delta interacting protein 2; and VSMC, vascular smooth muscle cell.

The online version of the article has been corrected and is available at http://atvb.ahajournals.org/content/33/9/2154.full.
**Poldip2 sustains vascular structure and function**


**Supplemental Table I. Primer sequences and reaction conditions used for quantitative RT-PCR**

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Ta is the annealing temperature.
Supplemental Figure I. Homozygous disruption of Poldip2 is lethal

A. Map of the Poldip2 mutant allele. Mice were produced from an embryonic stem cell clone with a gene trap construct (top) inserted into the first intron of the Poldip2 gene (bottom, exons are gray boxes). Transcription of the Poldip2 gene is expected to end near the polyadenylation signal (polyA) of the β-geo cassette, spliced as a new second exon, thus omitting endogenous exons 2-11. The construct is surrounded by retroviral long terminal repeats (LTR) to favor random insertion into the genome. The native Poldip2 coding sequence encompasses exons 1-11, between the indicated start and stop codons. Arrows labeled P1-P4 represent PCR primers mentioned in the text.

B. Poldip2 genotype distribution. Observed counts (gray bars) of mouse pups produced by crossing Poldip2+/− parents were significantly different from the expected Mendelian 1:2:1 (+/+: +/- : −/−) distribution (black bars), but not from a theoretical 1:2:0 distribution in which the homozygous genotype is lethal (white bars). Statistical significance of distribution differences: *** P < 0.001; NS not significant. Error bars represent 90% confidence intervals on observed counts.
Supplemental Figure II. Nox4 and p22phox proteins are unchanged in Poldip2+/- aorta

Samples from whole Poldip2+/- aortas and wild-type control mice were lysed and processed for Western blot analysis using Nox4, p22phox and β-tubulin primary antibodies (three upper panels). Densitometric data (lower panels) represent Nox4 and p22phox average ± SEM, normalized to tubulin, from 3 animals in each group. P > 0.05 +/- vs. +/+.
Supplemental Figure III. Inhibition of contraction persists in Poldip2+/- aortas after removal of the endothelium

The endothelium was removed from aortic rings before measuring isometric force in response to indicated concentrations of phenylephrine (A, B) or potassium chloride (C). Vessels were additionally preincubated for 30 minutes with 1,000 units of PEG catalase before stimulation with phenylephrine (B). Data represent the average ± SEM from 8 to 14 vessels. * P < 0.05 +/- vs. +/+ and ** P < 0.01 +/+ vs. +/-.
Supplemental Figure IV. Elastic lamellae are altered in Poldip2+/− aortas

Elastin was visualized in transverse sections of paraffin-embedded Poldip2+/− thoracic aortas and wild-type controls using Verhoeff-Van Gieson stain (top two panels). Microphotographs and Image J software were used to count breaks and forks in elastic lamellae and measure the media area used for normalization (bottom). Measurements were performed in duplicate sections from 3 mice in each group. Data are average ± SEM. * P < 0.05.
Supplemental Figure V. Poldip2 knockdown increases ECM proteins secreted by rat VSMC

Cultured rat VSMC were transfected with 15 nM non-silencing (siNeg) or Poldip2 (siPoldip2) siRNA. Media incubated with cells from days 2 to 5 post-transfection were collected for Western blot analysis of extracellular matrix proteins (top). Densitometric data represent average ± SEM from 3-4 independent experiments (bottom); ** P < 0.01.
Supplemental Figure VI. MMP2 and MMP9 are upregulated in Poldip2+/- aortas

Quantitative RT-PCR was performed using RNA from whole Poldip2+/- aortas and wild-type controls. Indicated primers are specific for extracellular matrix components, matrix metalloproteases and their inhibitors, as well as cytokines and inflammation markers. VCAM1, interleukins and iNOS were close to the detection limit and osteopontin was only detected in a few samples. IL-8 and MCP-1 were not included in the figure because they were below the detection limit. Data are expressed as % change relative to wild-type and represent average ± SEM from 4-5 animals in each group. * P < 0.05.
Supplemental Figure VII. Exogenous glucose oxidase compensates for decreased H₂O₂ production from Poldip2+/− VSMC

Cultured VSMC from wild-type and Poldip2+/− mice were incubated without (Control) or with 2 ng glucose oxidase for 3 days before measurement of H₂O₂ in the medium using the amplex red assay. Data represent average ± SEM from 5-7 independent experiments; * P < 0.05 vs. +/+ Control, ** P < 0.01 vs. +/+ Control, ° Not significant vs. +/+ Glc oxidase.
Supplemental Figure VIII. Poldip2 knockdown reduces Ang II-induced hypertension

Systolic blood pressure (BP) was measured by tail cuff plethysmography in male wild-type (black bars) and Poldip2+/− (gray bars) mice.

A. Blood pressure was measured at indicated ages in the absence of additional treatment. Data represent the average ± SEM of measurements from 4-5 mice in each group.

B. 6 month-old mice were infused without or with angiotensin II (Ang II, 0.7 mg/kg/day), using osmotic mini-pumps, for 2 weeks before measuring blood pressure. Data represent the average ± SEM of measurements from 5 mice in each group; * P < 0.05 vs. wild-type in the presence of Ang II.
Materials and Methods

**Poldip2 gene trap mice**

Mice with a gene trap insertion in the first intron of Poldip2 (chromosome 11, NCBI Gene ID: 67811) were produced at the Texas A&M Institute for Genomic Medicine (College Station, TX). Mice were generated from a clone (IST12080D9) of C57BL/6N embryonic stem cells selected from the OmniBankII library made by Lexicon Pharmaceuticals (The Woodlands, TX). The library was made by high throughput methods including transduction of stem cells with a recombinant retrovirus carrying the gene trapping vector VICTR76, clone selection by neomycin resistance, and identification of the genome insertion site for each clone. Following quality control, stem cells were injected into blastocysts which were implanted in albino pseudopregnant mice. A high percentage chimera crossed with C57BL/6 mice produced heterozygote founders that were further bred with C57BL/6. All animal protocols complied with IACUC requirements from Atlanta Veterans Affairs Medical Center, Georgia Institute of Technology and Emory University School of Medicine.

**Poldip2 genotyping**

Tissue samples from ear punches, collected at the time of weaning (21 days of age), were used for preparation of genomic DNA by overnight digestion with proteinase K in DirectPCR Lysis Reagent (Viagen Biotech, Los Angeles, CA). A standard 3 primer PCR method was developed for genotyping, with one pair of forward 5’-CGAGGGAGAACAAGTAACGCTTC-3’ and reverse 5’-CCATCTGGCTACACAGCATGATTC-3’ primers (300 nM each) annealing to the first intron of Poldip2, around the gene trap insertion site and an additional forward 5’-ATAAGTTGCTGCCAGCTTACCTC-3’ primer (100 nM) annealing to the gene trap construct. Following amplification with Taq DNA polymerase, wild-type (613 bp) and mutant (720 bp) bands were separated by agarose gel electrophoresis.

**cDNA preparation**

Immediately after CO₂ euthanasia, male mice were perfused at physiological pressure from the left ventricle with a solution of 26 mM sodium citrate in 0.9% sodium chloride (pH 7.4) to flush blood from the aorta. Whole thoracic aortas were rapidly harvested and stored in RNA later solution (Ambion, Austin, TX) at 10°C overnight before careful removal of adherent fat. Aortas were homogenized with a motorized rotor/stator device and total RNA was purified with the RNeasy kit (Qiagen, Chatsworth, CA), including digestion with DNase I. Following reverse transcription (RT) with random primers and Superscript II enzyme (Invitrogen, Carlsbad, CA), cDNA was purified with the QIAquick kit (Qiagen).
**Real-time PCR**

Quantitative PCR was carried out with a LightCycler instrument (Roche Applied Science, Indianapolis, IN) in glass capillaries, using PlatinumTaq DNA polymerase (Invitrogen) and SYBR green (Invitrogen) dye. Primer sequences and reaction conditions are indicated in Supplemental Table I. Data analysis was performed using the mak3 module of the qpcR software library in the R environment.\(^2,4\) When absolute quantification was desired, standard curves generated with plasmid DNA were used to calculate the correspondence between the mak3.\(D_0\) parameter and copy numbers for each gene by linear regression, following log transformation.

**Poldip2 Western blotting**

To measure Poldip2 expression in vessels, aortas harvested from 2 mice of the same genotype were pooled per sample. Aortas were homogenized in lysis buffer (20 mM Tris pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 10 mM NaF, 1 mM Na\(_3\)VO\(_4\), 1% Triton X-100, 0.1% SDS, 1% Na deoxycholate, 1 tablet/10 ml EDTA-free complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 1 mM β-glycerolphosphate, 2.5 mM Na pyrophosphate] on ice and centrifuged at 14,000 x g for 10 minutes. Protein concentration in supernatants was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Desired amounts of protein were added to sodium dodecyl sulfate sample buffer solution and loaded into bis-tris-PAGE precast mini gels (Invitrogen). Following electrophoresis, proteins were transferred to nitrocellulose membranes. Primary antibodies, diluted 1:1,000 in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween, pH 7.4) containing 5% powdered non-fat dry milk, were either a custom-made PolDip2 goat polyclonal, described previously\(^5\) or a CDK4 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary anti-goat or anti-rabbit peroxidase-coupled antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted 1:5,000 in 5% milk/TBST. Blots were washed 3 x 10 min with TBST after primary and secondary antibody incubations. Bands were visualized by enhanced chemiluminescence using SuperSignal West Pico reagents (Pierce Biotechnology) and quantified with a ChemiDoc XRS/HG system (Bio-Rad Laboratories, Hercules, CA).

**p22phox, Nox4, Collagen I and Fibronectin Western blotting**

Samples were lysed in Hunter’s buffer (25 mM HEPES, 150 mM NaCl, 1.5 mM MgCl\(_2\), 1 mM EGTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1 mM Na-orthovanadate, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 10% Glycerol, and protease inhibitors), as described previously.\(^5\) Aortas were minced with scissors and homogenized by douncing. Protein lysates were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. In the case of Nox4, sonication and boiling prior to gel loading was omitted. Following blocking, blots were incubated with primary antibodies: anti-p22phox rabbit polyclonal (Abcam, Cambridge, MA), anti-Nox4 rabbit monoclonal (Epitomics, Burlingame, CA), anti-collagen I rabbit polyclonal (Fitzgerald, Acton, MA or
Abcam), anti-fibronectin 1 mouse monoclonal (Sigma, St Louis, MO) or anti-β-tubulin (Sigma). After incubation with HRP-conjugated secondary antibodies, bands were visualized using enhanced chemiluminescence (Amersham, Sunnyvale, CA) and quantified by densitometry with ImageJ software.

**NADPH oxidase activity in kidney slices**

ROS were measured from a 30 mg wet weight slice taken in the middle of the kidney. Both superoxide and hydrogen peroxide were measured in membrane fractions using an EMX electron spin resonance spectrometer (Bruker BioSpin, Billerica, MA) with a super-high Q microwave cavity and CPH as a spin probe, as described previously. Superoxide formation was assayed as NADPH-dependent, SOD-inhibitable (95-98% inhibition) formation of 3-carboxyproxyl radical. Hydrogen peroxide was measured by peroxidase-mediated oxidation of CPH in the presence of acetamidophenol and was blocked by catalase. Results were normalized to milligram protein measured using the Bradford assay.

**Contractility measurements**

Aortas harvested from 2- to 3-month-old wild-type and Poldip2+-/ male mice were prepared for contractility measurements as described previously. Briefly, 5-mm aortic rings were mounted isometrically at 20 mN to approximate an in vivo aortic pressure of ~100 mm Hg. Vascular contractility was assessed by generating concentration-response curves to phenylephrine and potassium chloride. Data were acquired and analyzed using Powerlab hardware and Chart software (AD Instruments, Mountain View, CA). Isometric contractility was normalized to cross-sectional area (CSA). Because force is measured perpendicular to the vessel axis, CSA = 2 x volume/circumference, with volume calculated from wet weight.

In a separate series of experiments, the endothelium was removed by rubbing aortic rings between thumb and forefinger. Denudation was confirmed for each vessel by a lack of endothelium-dependent relaxation in response to 30 mM acetylcholine.

**Stiffness and compliance measurements**

Vascular stiffness was determined as described previously. Aortic rings were mounted between two wires in an organ chamber and maximally dilated with 30 µM sodium nitroprusside. The distance between the wires was increased until a deflection in the force measurement was observed (distance \( L_0 \)). The distance between the wires was then further increased in 10 µm increments while monitoring tension. Vascular compliance was measured in separate experiments. Excised aortas were maintained in culture medium (Dulbecco’s modified Eagles medium containing 4.5 g/L glucose and sodium pyruvate, without L-glutamine or phenol red, Invitrogen) containing sodium nitroprusside to ensure that the arteries were fully dilated. Branches within the
suprarenal region were ligated using 10-0 silk sutures and the aortas were mounted on two glass cannulae using 8-0 sutures. The vessel and cannulae were suspended in a bath on the mechanical testing device. This computer-controlled device has the capability of maintaining precise luminal pressure and axial length of the vessel, while recording the outer diameter and axial force during testing. Following preconditioning, fixed length pressure-diameter tests were performed under quasistatic loading conditions. Vessels were cyclically inflated from 0 to 160 mmHg at a series of fixed axial stretches of $\lambda = 1.3, 1.4, 1.5, 1.6, 1.7, 1.8,$ and $1.9$ ($\lambda$ is loaded vessel length/unloaded vessel length), with three loading/unloading cycles for each axial stretch. The compliance of the vessels was calculated as the slope of the diameter-pressure curve ($\Delta D/\Delta P$), normalized to the outer diameter ($D$); namely, $C = (\Delta D/\Delta P)/D$ at any given pressure.

Transmission electron microscopy

Immediately after CO$_2$ euthanasia, 3 month-old male mice were perfused at physiological pressure from the left ventricle with saline for 2 minutes, followed by 2.5% glutaraldehyde (EM grade, Electron Microscopy Sciences, Hatfield, PA) in 0.1 M phosphate buffer for 2 minutes. Samples of 2-3 mm sections rapidly excised from the thoracic aorta were fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by postfixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydration in a graded ethanol series followed by propylene oxide, and embedding in Epon-812 resin. Ultrathin, transverse sections taken from the thoracic aorta were poststained with uranyl acetate (or, alternatively, with tannic acid-uranyl acetate to enhance electron density of elastin$^{11}$), and lead citrate. Images were taken on a Hitachi H-7500 electron microscope operated at 75 kV, digitized and imported into Image-Pro Plus v. 6.2 (MediaCybernetics, Bethesda, MD) for morphometric area measurements. Images were prepared for publication using Adobe Photoshop CS3 Extended and Photoshop Elements 6.0.

Electron microscopy morphometric measurements

Two types of 2-dimensional area measurements using Image-Pro Plus software were made on 195 digitized images of thoracic aorta transverse sections from 3 Poldip2+/+ and 3 Poldip2+-/ mice: (a) total interlamellar area (area in the media between two elastin lamellae) and (b) fibrillar/amorphous areas within these interlamellar zones, identified morphologically as extracellular matrix (ECM) surrounding vascular smooth muscle cells. ECM area/total interlamellar area was expressed as % ECM.

Light microscopy morphometric measurements

Elastin was visualized in transverse sections of paraffin-embedded mouse thoracic aortas using Verhoeff-Van Gieson stain. Microphotographs and Image J
software were used to count breaks and forks in elastic lamellae and measure the media area used for normalization.

**Isolation of VSMC**

VSMC were isolated from thoracic aortas of wild-type and Poldip2+/- mice$^{12}$ or Sprague Dawley rats.$^{13}$ The vessels were excised and dissected to remove adherent connective tissue and fat. After a brief digestion with collagenase, adventitia and endothelium were mechanically removed. A second digestion with collagenase and elastase was used to dissociate isolated media. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (mouse cells) or calf serum (rat cells), 4.5 g/L glucose, 2mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and used between passages 3 to 10 (mouse cells) or 6 to 15 (rat cells).

**Collagen I secretion from cultured VSMC**

VSMC from WT and Poldip2+/- mice were grown in 35-mm dishes and made quiescent by incubation with serum-free DMEM for 24 hours. Cells were then incubated for 3 days with 1 ml of serum-free medium containing 50 µg/ml ascorbic acid, 50 µg/ml β-aminopropionitrile (an inhibitor of lysyl oxidase, which is responsible for collagen cross-linking) and vehicle or 2 ng (≥ 30 µ units) freshly prepared glucose oxidase from Aspergillus niger (Sigma). Collagen I released in cell culture medium was measured at the end of this incubation period by Western blot analysis, using primary anti-collagen I antibody (Fitzgerald or Abcam).$^{12}$ To correct for possible differences in extracellular matrix generation caused by differences in cell growth, gel loading was adjusted for total protein concentration in media as determined by Bradford assay (Bio-Rad Laboratories). Cell lysates were then collected to measure intracellular collagen by Western blotting.

Extracellular collagen, secreted from rat VSMC, was measured in similar experiments after transfection with Poldip2 stealth siRNA or non-silencing siRNA control (Invitrogen).$^5$ To allow efficient transfection, cells were first plated at 40% confluence and incubated after 4 hours with oligofectamine/siRNA complexes for 48 hours. Equal numbers of siRNA-treated cells were replated on 35 mm dishes. After 8 hours, cells were made quiescent as described above for mouse cells and media were collected after 3 days for Western blot analysis.

**Amplex red assay in mouse VSMC**

Mouse VSMC were grown in DMEM without phenol red (Sigma) and deprived of serum for 24 hours. Following incubation with or without glucose oxidase for 4 hours, 100 µl of culture medium were collected and used to measure H$_2$O$_2$ with a fluorometric assay detecting the oxidation of 100 µM amplex red (Invitrogen) in the presence of horseradish peroxidase, which is abolished by catalase. H$_2$O$_2$ concentration was
calculated from a standard curve and normalized to cellular protein, measured with the Bradford assay (Bio-Rad Laboratories).\(^\text{14}\)

Aortic dilatation by surgical application of CaCl\(_2\)

Male, 9-10 week-old mice were anesthetized by inhalation, using isoflurane in oxygen (5% induction, followed by 2% maintenance). After removal of abdominal fur and disinfection with betadine, a midline incision was performed using sterile instruments. The bowels were retracted to the upper left quadrant and wrapped in a sterile gauze moistened with saline. The bladder and seminal vesicles were gently pushed deep into the pelvis to expose the bifurcation of the aorta. The abdominal cavity was kept moist with sterile saline. The aorta was isolated from the inferior vena cava using fine forceps. A sterile gauze soaked in 0.25 M sterile CaCl\(_2\) was applied to the external surface of the aorta for 15 minutes before rinsing with saline.\(^\text{15}\) The bowels, bladder and seminal vesicles were moved back to their correct anatomic position and the abdominal incision was closed with 2 layers of 4-0 sutures. Buprenorphine was given for post-operative analgesia at 0.05 mg/kg. The mice were placed on a heating pad during recovery. The aortic diameter was measured with a caliper and by analysis of a photograph with Image J software (NIH) on the day of surgery (day 0) and at the end of the experiment (week 8).

Blood pressure measurements

Systolic blood pressure was measured in 3 to 12 month-old wild-type and Poldip2+/- male mice, using computer-assisted tail cuff plethysmography (Visitech Systems, Apex, NC). Mice were habituated to the procedure by daily measurements for 5 successive days. Only the last 3 days of data were included in the final results. Ten successive measurements from each animal were used to calculate the average blood pressure. In addition, 6 month-old mice were infused with 0.9% sodium chloride without or with Ang II (0.7 mg/kg/day) for two weeks, using subcutaneously implanted osmotic mini-pumps (Alzet, Cupertino, CA), as described previously.\(^\text{16}\) Systolic blood pressure was measured as above for the last 5 days of the experiment and data collected only during the last 3 days.

Statistical analysis

Normalization was used to correct differences in exposure between duplicate Western blots. Correction factors were calculated as the average intensity of all blots, divided by the average intensity of each blot.

Counts of genotypes produced by crosses of heterozygote breeders were analyzed using the \(\chi^2\) method. All other data were expressed as mean ± SEM and analyzed using 1 or 2-way ANOVA, followed by Bonferroni’s multiple comparison test, when applicable. A P value < 0.05 was considered significant.
Supplemental References


