Mutation of *Plekha7* attenuates salt-sensitive hypertension in the rat

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**PLEKHA7** (pleckstrin homology domain containing family A member 7) has been found in multiple studies as a candidate gene for human hypertension, yet functional data supporting this association are lacking. We investigated the contribution of this gene to the pathogenesis of salt-sensitive hypertension by mutating *Plekha7* in the Dahl salt-sensitive (SS/JrHsdMcwi) rat using zinc-finger nuclease technology. After four weeks on an 8% NaCl diet, homozygous mutant rats had lower mean arterial (149 ± 9 mmHg vs. 178 ± 7 mmHg; *P* < 0.05) and systolic (180 ± 7 mmHg vs. 213 ± 8 mmHg; *P* < 0.05) blood pressure compared with WT littermates. Albumin and protein excretion rates were also significantly lower in mutant rats, demonstrating a renoprotective effect of the mutation. Total peripheral resistance and perivascular fibrosis in the heart and kidney were significantly reduced in *Plekha7* mutant animals, suggesting a potential role of the vasculature in the attenuation of hypertension. Indeed, both flow-mediated dilation and endothelium-dependent vasodilation in response to acetylcholine were improved in isolated mesenteric resistance arteries of *Plekha7* mutant rats compared with WT. These vascular improvements were correlated with changes in intracellular calcium handling, resulting in increased nitric oxide bioavailability and the response of the vasculature to endothelial cells.

**Significance**

**Zinc-finger nuclease (ZFN)-mediated mutagenesis has now enabled researchers to manipulate specific genes to test their function in animal models other than mice. Applying ZFNs to rats, we can now test the role of specific human genome-wide association studies (GWAS)-nominated genes for hypertension in a well-characterized hypertensive rat model, the Dahl salt-sensitive rat. This study provides the first functional evidence that the GWAS-nominated gene *Plekha7* plays an essential role in blood pressure regulation and cardiovascular function by modulating vascular function. Our results indicate that *Plekha7* plays a role in the regulation of intracellular calcium, nitric oxide bioavailability, and the response of the vasculature to increased flow.**


The authors declare no conflict of interest.

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**Hypertension** is a complex disease that is characterized by increased blood pressure, renal damage, and vascular dysfunction which collectively increase risk of atherosclerosis, stroke, heart disease, and renal failure in one-quarter of all adults worldwide (1–3). Because there is strong evidence of heritability in hypertension (2, 4, 5), considerable effort has been put toward identifying novel candidate genes and their molecular mechanisms. Genome-wide association studies (GWAS) have identified many potential hypertension loci, which shed light on the genetic complexity of this disease (5–8) but have provided little mechanistic insight. As such, validation and elucidation of the functional roles and disease mechanisms for these genes candidates are the next important challenges (4).

Because hypertension is a complex disease (i.e., multiple variants of small effect sizes contributing to disease risk), we hypothesized candidate gene targeting on a genetically sensitized background would reveal functional role(s) of genetic disease modifiers. The Dahl salt-sensitive (SS) rat is an inbred genetic model of salt-sensitive hypertension that displays hypertension-induced renal damage, cardiac hypertrophy and vascular dysfunction (9–11). These phenotypes are induced by exposing SS rats to a high-salt diet, which results in rapid induction of hypertensive phenotypes that closely resemble salt-induced hypertension seen in humans (12–15). Knockout of specific genes in this disease model using zinc-finger nuclease (ZFN) technology have revealed the importance of key mechanisms contributing to hypertension risk, such as the protection from salt-induced hypertension and renal injury by selective ablation of adaptive immune cells in the SS-Rag2\(^{em1Mcwi}\) and SS-Cd247\(^{em1Mcwi}\) knockout rats (16, 17) and reduced hypertension and renal injury in the SS-Ncf2\(^{em1Mcwi}\) (p67phox) null model exhibiting reduced medullary oxidative stress (18). Additionally, we have recently demonstrated multiple genes at a single hypertension GWAS-nominated locus (*Agrap-Plod1*) can have additive or subtractive effects on blood pressure and renal function when mutated in the SS rat (19). These previous studies highlight the utility of this model system for testing the roles of GWAS candidate human disease genes by disrupting their specific rat orthologs using ZFN technology (20).

A single-nucleotide polymorphism (SNP) (rs381815, minor allele frequency 0.26) in intron 1 of the pleckstrin homology domain containing family A member 7 (*PLEKHA7*) gene, was identified by five independent GWAS to be associated with elevated systolic blood pressure and hypertension in multiple populations (5, 6, 8, 21, 22). The associated locus contains only the *PLEKHA7* gene (5); however, the genetic mechanism(s) underlying this locus have not yet been functionally characterized. *Plekha7* is highly expressed in the kidney and heart, where it may be involved in formation and maintenance of the apical junction complex of epithelial cells (23). However, limited data on *Plekha7* function are available to extrapolate its potential role(s) in the pathogenesis of hypertension. Here we used ZFN mutagenesis to obtain the first evidence to our knowledge in any model system that *Plekha7* has a functional role in several hypertension-associated phenotypes in the rat. We found that mutation of *Plekha7* in the SS rat attenuated salt-induced hypertension.
hypertension, reduced renal damage, and improved cardiac function. We also show that Plekha7 modulates calcium handling and nitric oxide (NO) bioavailability, both of which are required for normal vascular health. Collectively, these studies provide significant mechanistic insight to the role of Plekha7 in salt-sensitive hypertension.

Results
Targeting Plekha7 for Mutation Using ZFNs. To investigate the role of Plekha7 in hypertension, we used ZFNs to target genomic sequences encoding the pleckstrin homology (PH) functional domain of Plekha7 in the SS rat (Fig. S1A). Injection of ZFNs targeting exon 8 of the Plekha7 gene into SS embryos resulted in a 19-bp deletion mutation in the coding sequence. This frame-shift mutation is predicted to cause the translation of 18 nonsense amino acids and introduce a premature stop codon, yielding a truncated, nonfunctional protein consisting of 231 amino acids that lacks the full PH domain (Fig. S1D). Verification of the mutation in homozygous SS-Plekha7 mutant animals was confirmed in cDNA by RT-PCR and sequencing (Fig. S1 B and C). Quantitative PCR revealed an approximated 50% reduction in Plekha7 expression within the mutant animals compared with WT, indicating nonsense-mediated decay of the transcript in the mutant rats (Fig. S1B). Homozygous mutant and WT control experimental rats were generated by heterozygous brother-sister mating. There were no clear developmental abnormalities in the mutant animals compared with WT littermates, and mutant rats were similar in weight to WT at 9 wk of age (264 ± 9 vs. 250 ± 8 g, respectively).

Protection Against Salt-Sensitive Hypertension in Plekha7 Mutant Rats. To test whether mutation of Plekha7 affects the development of hypertension in the SS rat, blood pressure (BP) was measured by telemetry in conscious male WT and SS-Plekha7 mutant littermates on a 0.4% NaCl diet for 31 d; however, there was a significant attenuation in 8% NaCl-induced increases in MAP in the mutant animals compared with WT but was not statistically significant. *P < 0.05. Error bars represent SEM.

SS-Plekha7 Mutant Rats Have Reduced Renal Damage Compared with WT Littermates. To examine whether renal damage under pathogenic conditions was affected by Plekha7 mutation, urine was collected and analyzed in male 9-wk-old WT and mutant rats (n = 10 per group) that were initially fed a 0.4% NaCl diet and then switched to an 8% NaCl diet for a total of 28 d. Urinary albumin and protein excretion did not differ on the 0.4% NaCl diet (Fig. 2). After 28 d of the 8% NaCl diet, mutant rats had significantly lower urinary excretion of albumin (110 ± 10 mg/d; P < 0.05) and protein (190 ± 16 mg/d; P < 0.05) compared with WT levels of albuminuria (211 ± 27 mg/d) and proteinuria (381 ± 31 mg/d) (Fig. 2). In contrast, no differences in urine flow rate, urinary sodium, or urinary potassium concentrations between the mutant and WT rats were detected at the measured time points (Table S1). Body weight of WT (371 ± 5 g) and SS-Plekha7 mutant rats (379 ± 4 g) on the 8% NaCl diet for 4 wk was not statistically different, suggesting that food and salt intake were similar between WT and mutant rats. Collectively, these data indicate that the reduction in albuminuria and proteinuria in the mutant rat are not due to differences in salt consumption or natriuresis.

Fig. 1. Blood pressure was measured by telemetry in WT and mutant rats fed a 0.4% NaCl diet for 31 d (n = 4 per group) or in WT and mutant rats fed a 0.4% NaCl diet for 3 d followed by a 8% NaCl diet for 28 d (n = 8–10 per group). (A) There was no difference in BP between WT and mutant rats fed a 0.4% NaCl diet for 31 d; however, there was a significant attenuation in 8% NaCl-induced elevations in MAP in the Plekha7 mutant animals from day 11 to day 28 compared with WT littermates. (B) Plekha7 mutant rats also displayed an attenuation in 8% NaCl-induced elevations in systolic BP from day 3 to day 28 compared with WT. (C) Diastolic BP was lower in the Plekha7 mutant animals compared with WT but was not statistically significant. *P < 0.05. Error bars represent SEM.
SS-Plekha7 mutant rats exhibit reduced renal and vascular damage that are likely secondary to changes in BP.

SS-Plekha7 Mutant Rats Exhibit Increased Cardiac Output and Reduced Systemic Vascular Resistance. To determine whether reduced BP and renal damage coincided with changes in cardiac function, the hearts of WT and SS-Plekha7 mutant rats on the 8% NaCl diet for 28 d were examined by echocardiography and histology. Compared with WT, the hearts of mutant rats had significantly increased stroke volume and cardiac output (Fig. 4 A and B), which corresponded to a 44% (P < 0.05) decrease in systemic vascular resistance in mutant rats (Fig. 4C). The improved cardiac function in mutant rats also coincided with a reduction in interstitial fibrosis and a decrease in perivascular fibrosis compared with WT rats (Fig. 4 D–F). Moreover, mutant rats had significantly reduced heart weight (1.54 ± 0.08 vs. 1.71 ± 0.04g; P < 0.05) and mean septal wall thickness (0.17 ± 0.01 vs. 0.25 ± 0.01cm; P < 0.001) compared with WT. Taken together, these results indicate that the SS-Plekha7 mutant rats have improved cardiac function and significantly lower vascular resistance after the 8% NaCl diet treatment compared with WT rats.

Plekha7 Modulates Vascular Dilation. Because Plekha7 has been shown to localize to the vasculature (24) and SS-Plekha7 mutant rats had decreased systemic vascular resistance (Fig. 4C), we hypothesized that Plekha7 might directly mediate vascular function. Vasoreactivity was examined in isolated second-/third-order mesenteric resistance arteries from male rats fed a 0.4% NaCl diet at a point where there were no differences in MAP between WT and mutant rats (108 ± 1 vs. 107 ± 2 mmHg, respectively). Compared with WT, mutant arteries demonstrated a greater sensitivity to the endothelium-dependent relaxation agonist acetylcholine (Ach), whereas no changes were observed in log EC\textsubscript{50} values for sodium nitroprusside or contractile responses to either phenylephrine (PE) or serotonin (5-HT) (Fig. 5A). Additionally, we found no differences in maximal dilation responses to Ach or sodium nitroprusside or maximal contractile responses to PE or 5-HT between WT and mutant arteries. Interestingly, however, we found that Plekha7 mutant arteries dilated by ~7% in response to increased flow, whereas WT arteries did not dilate at all (Fig. 5B). The lack of flow-mediated dilation (FMD) in WT animals is consistent with the impaired endothelium-dependent dilation reported previously in the SS rat (25, 26). Moreover, the observed FMD response in Plekha7 mutant arteries could be blocked by the eNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) (Fig. 5B). Because L-NAME blocked FMD, whereas sodium nitroprusside (a NO donor) had no differential effect between WT and Plekha7 mutant (Fig. 5A), these data suggest that Plekha7 mediates endothelial NO production rather than downstream NO effects on the vascular smooth muscle.

SS-Plekha7 Mutant Vessels Generate More NO. Because mutation of Plekha7 improved FMD in SS rats and increased sensitivity to ACh-mediated vessel dilation, we next examined levels of NO, a downstream mediator of FMD and ACh signaling (27), in isolated aortas of WT and mutant rats fed a 0.4% NaCl diet. Compared with WT in normal physiological salt solution (PSS) buffer, DAF-FM diacetate staining revealed a 1.65-fold (P < 0.05) increase in NO production in mutant aortas stimulated with ACh, which was completely abrogated by L-NAME treatment (Fig. 6 A and B), suggesting that mutation of Plekha7 improves NO bioavailability.

Previous studies suggest that NO production is Ca\textsuperscript{2+}-dependent (28, 29) and that PH domain-containing proteins interact with phosphatidylinositol members to regulate intracellular Ca\textsuperscript{2+} signaling (30, 31). Because Plekha7 contains a PH domain, we hypothesized that this protein influences NO production by modulating endothelial-specific Ca\textsuperscript{2+} signaling. To test this hypothesis, we examined ACh-mediated release of intracellular Ca\textsuperscript{2+} in isolated vessels of SS-Plekha7 mutant and WT rats, using the fluorescent Ca\textsuperscript{2+} indicator (Fluo-4) and two-photon confocal microscopy. As shown in Fig. 6C, endothelial cells of the bisected aorta exhibit a strong green fluorescence, indicating sufficient loading of the Fluo-4 dye. In response to ACh in normal PSS, SS-Plekha7 mutant endothelial cells displayed a marked increased Ca\textsuperscript{2+} response compared with WT as measured by fluorescent intensity (Fig. 6 D and E). To determine whether this effect exhibited by the mutant endothelial cells was due to extracellular Ca\textsuperscript{2+} influx or intracellular Ca\textsuperscript{2+} storage, we carried out similar studies in Ca\textsuperscript{2+}-free solution. In Ca\textsuperscript{2+}-free buffer, we found that the mutant endothelial cells continued to exhibit a 2.24-fold (P < 0.05) increased Ca\textsuperscript{2+} response to ACh compared with WT, indicating that intracellular Ca\textsuperscript{2+} storage was higher in the mutant cells (Fig. 6F). Collectively, these data indicate that mutation of Plekha7 results in improved Ca\textsuperscript{2+} handling leading to increased NO production within the vasculature.

Fig. 2. Renal function analysis of WT and mutant animals. (A) Urinary microalbumin excretion rate and (B) protein excretion rate was measured in Plekha7 WT and mutant rats (n = 10 per group) throughout the BP measurement protocol. There was a significant attenuation in 8% NaCl-induced increases in urinary albumin excretion rate and total protein in the mutant animals compared with the WT. *P < 0.05. Error bars represent SEM.

Fig. 3. Analysis of renal damage. Kidneys were sectioned and stained with trichrome stain. (A) Histological sections demonstrate that mutant rats are less damaged than WT littermates. (B) There was a significant reduction in tubular damage as assessed by protein casting and (C) a significant reduction in glomerular sclerosis in the mutants compared with WT. (D) Representative images of renal vessels from WT and mutant animals stained with alpha-smooth muscle actin. (E) Vessel wall thickening (vessel media, square micrometers) was calculated by measuring the outer circumference of the vessel minus the inner circumference of the lumen (20 random images within the cortex per rat; n = 3 rats per group). There was a significant reduction in vessel media of the Plekha7 mutant renal arteries compared with WT. ***P < 0.001. Error bars represent SEM.
and fibrosis (Figs. 3D and 4F) and reduced renal damage (Fig. 3A). We drew this conclusion because isolated vessels from normotensive SS-Plekha7 mutant rats had an increased ability to dilate in response to ACh (Fig. 5A) and flow (Fig. 5B) and exhibited elevated bioavailability of NO (Fig. 6A), a key vasodilator and currently the primary indicator of peripheral vascular health in humans (34–36). We also observed decreased peripheral fibrosis in hypertensive mutant rats compared with WT (Fig. 4F), suggesting that decreased vascular stiffening may be an additional factor in reducing systemic vascular resistance in the SS-Plekha7 mutant rat (37). Multiple other BP mediators have effects on vascular remodeling that may contribute to hypertension [e.g., AngII (38) and ET-1 (39)], suggesting that the Plekha7-mediated BP effects are not mechanistically unique, but rather one of the few known genetic risk factors to date that contribute to hypertension risk by affecting vascular function and remodeling. Future studies using an endothelial-specific knock-out of Plekha7 will be necessary to demonstrate whether it mediates hypertension risk solely through the vascular endothelium or has other nonendothelial roles in BP regulation.

**Plekha7 Modulates Vasodilator Responses via Endothelial Calcium Signaling.** Our whole-animal phenotyping suggested that Plekha7 has a vascular role in BP regulation, prompting us to begin to elucidate the downstream pathways that might mediate Plekha7 signaling. Plekha7 is a cell-surface protein with a PH domain that we hypothesize interacts with phospholipids to affect intracellular levels of Ca$^{2+}$ (30, 31, 40), the central mediator of vascular tone (41). Knockdown of HADP1, the closest known orthologous protein to PLEKHA7 in zebrafish, was shown to regulate intracellular Ca$^{2+}$ signaling by interaction with the phosphatidylinositol 4-kinase pathway (31). Furthermore, other PLEKHA family members directly interact with phosphoinositide members and influence Ca$^{2+}$ release from intracellular stores, suggesting that Plekha7 may be working through similar mechanisms (42, 43). Our data now provide evidence that Plekha7 is involved in Ca$^{2+}$ signaling and intracellular Ca$^{2+}$ storage in vertebrate mammals as well. Using the fluorescent Ca$^{2+}$ indicator (Fluo-4) and two-photon confocal microscopy, we observed a significant increase in intracellular Ca$^{2+}$ release in response to ACh in isolated vessels from SS-Plekha7 mutant rats compared with WT littermates in both normal PSS buffer and Ca$^{2+}$-free buffer (Fig. 6 D–F). The increased influx of Ca$^{2+}$ in mutant vessels coincided with elevated NO bioavailability (Fig. 6A and B), fitting with previous reports of Ca$^{2+}$-mediated regulation of NO bioavailability in the endothelium (28, 29, 44, 45). Because endothelial NO synthase (eNOS) activity is highly dependent on Ca$^{2+}$$++$ and inhibition of eNOS with L-NAME blocked FMD and ACh-mediated NO release in the mutant rats, we propose that Plekha7 is signaling through this pathway; however, additional research is needed to determine the mechanistic details.

**Role of the Vasculature in PLEKHA7-Mediated Hypertension Risk.** The role of vascular resistance in hypertension is well established (32, 33), but the genetic mechanism(s) underlying this relationship have not been fully elucidated. Here, we demonstrated that mutation of Plekha7 in the rat attenuated salt-sensitive hypertension (Fig. 1), which was likely due to reduced systemic vascular resistance (Fig. 4C), leading to decreased vascular damage and fibrosis (Figs. 3D and 4F) and reduced renal damage (Fig. 3A). We drew this conclusion because isolated vessels from normotensive SS-Plekha7 mutant rats had an increased ability to dilate in response to ACh (Fig. 5A) and flow (Fig. 5B) and exhibited elevated bioavailability of NO (Fig. 6A), a key vasodilator and currently the primary indicator of peripheral vascular health in humans (34–36). We also observed decreased peripheral fibrosis in hypertensive mutant rats compared with WT (Fig. 4F), suggesting that decreased vascular stiffening may be an additional factor in reducing systemic vascular resistance in the SS-Plekha7 mutant rat (37). Multiple other BP mediators have effects on vascular remodeling that may contribute to hypertension [e.g., AngII (38) and ET-1 (39)], suggesting that the Plekha7-mediated BP effects are not mechanistically unique, but rather one of the few known genetic risk factors to date that contribute to hypertension risk by affecting vascular function and remodeling. Future studies using an endothelial-specific knock-out of Plekha7 will be necessary to demonstrate whether it mediates hypertension risk solely through the vascular endothelium or has other nonendothelial roles in BP regulation.

**Discussion**

Our goal was to test whether mutation of the GWAS-nominated gene Plekha7 affected the development of hypertension in a rat model of salt-sensitive hypertension. Compared with WT, Plekha7 mutation altered MAP, systolic BP, vasoreactivity, and total peripheral resistance, suggesting that the vasculature is a major contributor to Plekha7-mediated BP reduction. This was supported by in-depth analysis of renal and cardiac vascular histology, isolated vascular contractility, and FMD experiments, which indicated that Plekha7 mutation increases endothelial NO bioavailability driven by increased intracellular storage of Ca$^{2+}$. We also examined multiple other parameters of hypertension risk (e.g., natriuresis and nephron number) that did not change between WT and Plekha7 mutant animals. However, in the case of natriuresis, daily measurements of sodium handling might provide additional insights that were potentially missed by our weekly measurements. Collectively, these findings provide the first functional evidence to our knowledge that Plekha7 modulates BP by mediating systemic vascular resistance in a hypertensive rat strain. Moreover, to our knowledge these are the first mechanistic data to support multiple GWAS that have nominated PLEKHA7 as a hypertension risk gene (5, 6, 8, 21, 22) but had yet to demonstrate causality.

**Role of the Vasculature in PLEKHA7-Mediated Hypertension Risk.** The role of vascular resistance in hypertension is well established (32, 33), but the genetic mechanism(s) underlying this relationship have not been fully elucidated. Here, we demonstrated that mutation of Plekha7 in the rat attenuated salt-sensitive hypertension (Fig. 1), which was likely due to reduced systemic vascular resistance (Fig. 4C), leading to decreased vascular damage and fibrosis (Figs. 3D and 4F) and reduced renal damage (Fig. 3A). We drew this conclusion because isolated vessels from normotensive SS-Plekha7 mutant rats had an increased ability to dilate in response to ACh (Fig. 5A) and flow (Fig. 5B) and exhibited elevated bioavailability of NO (Fig. 6A), a key vasodilator and currently the primary indicator of peripheral vascular health in humans (34–36). We also observed decreased peripheral fibrosis in hypertensive mutant rats compared with WT (Fig. 4F), suggesting that decreased vascular stiffening may be an additional factor in reducing systemic vascular resistance in the SS-Plekha7 mutant rat (37). Multiple other BP mediators have effects on vascular remodeling that may contribute to hypertension [e.g., AngII (38) and ET-1 (39)], suggesting that the Plekha7-mediated BP effects are not mechanistically unique, but rather one of the few known genetic risk factors to date that contribute to hypertension risk by affecting vascular function and remodeling. Future studies using an endothelial-specific knock-out of Plekha7 will be necessary to demonstrate whether it mediates hypertension risk solely through the vascular endothelium or has other nonendothelial roles in BP regulation.

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studies will be needed to determine the connection between Plekh7 and eNOS. Collectively, these data show for the first time to our knowledge that Plekh7 regulates Ca2+ signaling in mammals and modulates endothelium-dependent vasodilator responses via the Ca2+ and NO signaling axis in a disease model of salt-sensitive hypertension. Because Ca2+ can also regulate PG12 and a number of different processes, more studies are needed to identify the contribution of Plekh7 and Ca2+ to other endothelium-dependent control mechanisms, such as prostanooid release.

**Perspectives.** Although GWAS can nominate candidate genes, they are unable to establish causality owing to the difficulty in investigating disease mechanism(s) for candidate genes directly in humans, especially for complex physiological phenotypes such as the control of blood pressure. As such, we have undertaken identifying and functionally testing candidate genes on disease-susceptible rat models. The foundation of this approach has been the newly developed gene-editing techniques (e.g., ZFN, TAL-ENS, CRISPR/Cas9, etc.) that have recently been applied to the rat (20, 46, 47). The advantages of this approach are multifold: (i) Disease-susceptible strains already harbor multiple disease alleles that are necessary to manifest the modest effect sizes of complex disease alleles; (ii) >10 disease-susceptible strains have now been modified (see http://rgd.mcw.edu/wg/physgenknockouts), demonstrating that current gene-editing approaches are directly applicable to different backgrounds enabling candidate risk genes to be tested in multiple disease models and under various experimental conditions; and (iii) gene editing is possible on pure inbred strains with uniform genetic backgrounds, limiting confounding genetic heterogeneity. These considerations, combined with a controlled experimental setting and ability to perform mechanistic studies, have enabled significant advances in characterizing complex disease candidates, such as PLEKHA7.

Based on the success of testing Plekh7 function in the SS-Plekha7 mutant rat model, we propose that the next step to translating these data to human hypertension will be to better understand the role of specific risk alleles in regulating Plekh7 expression or function. With the growing number and sophistication of gene manipulation technologies now available and applicable to both whole-animal and human cell model systems, we will now be able to specifically test how individual candidate SNPs regulate gene function using multiple approaches.

**Materials and Methods.**

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

**Generation of Mutant Rats.** ZFN constructs were designed, assembled, and validated by Sigma-Aldrich. mRNA encoding the Plekh7 ZFNs was diluted in microinjection buffer (1 mM Tris and 0.1 mmol/L EDTA, pH 7.4) and injected into one-cell SS/JrHsdMcwi rat embryos as described previously (48). See SI Materials and Methods and Fig. S1.

**Blood Pressure, Renal Function, Urine/Serum Biochemistry, and Echocardiography.** All rats were initially fed a 0.4% NaCl diet (113755; Dyets Inc.). Urine volume, Na+, creatinine, protein, and microalbumin were measured as described previously (49). BP was measured as described previously (49). Urine measurements were taken weekly. Echocardiography was performed on experimental rats using a GE Vivid 7 ultrasound machine (GE Healthcare) as described previously (50). Cardiac output was calculated in anesthetized rats by multiplying stroke volume (measured by echocardiography) by the HR (measured by telemetry). Total peripheral resistance was calculated by dividing the MAP (measured by telemetry) by the cardiac output. See SI Materials and Methods.

**Kidney and Heart Histology.** Kidneys and hearts were sectioned, mounted on microscope slides, and stained with trichrome. Immunohistochemistry (IHC) was performed using an antibody against alpha-smooth muscle actin using standard IHC methodology (49). Vessel media was calculated by measuring the outer circumference of the vessel minus the inner circumference of the lumen (20 random vessels within the cortex). See SI Materials and Methods.

**Vascular Reactivity Studies and NO and Ca2+ Measurements.** Mesenteric arteries were either mounted on a wire myograph (DMT) as described previously (51) or were mounted on a wire myograph (DMT) as described previously (51) or were incubated in the NO labeling indicator DAF-FM DA. Mutant aortas displayed an increased fluorescent response to the endothelium-dependent dilator ACh. The fluorescent response was abolished in WT and mutant cells by incubating the vessels with L-NAME (100 nM). (C) Aortas from WT and mutant rats (n = 3–5 per group) were extracted, opened longitudinally, labeled with the Ca2+ dye, Fluo-4, and imaged using a two-photon confocal microscope. Endothelial cells exhibited a strong green fluorescent signal indicating that Ca2+ was present in these cells and the cells were loaded properly. (D) Endothelial cells were imaged every 1.11 s in a normal PSS buffer and in response to ACh. (E and F) Mutant endothelial cells displayed an increased response to ACh in normal PSS and Ca2+-free buffer compared with WT. Arrows point to the endothelial cells. *P < 0.05. Error bars represent SEM.
cannulated as described previously (25, 26). For vasodilation studies, vessels were preconstricted with 10 μM PE and drug-response curves to either ACh or sodium nitroprusside were measured. Drug-response curves for contractile force were performed for PE and 5-HT. Flow studies were carried out as described previously (52). NO levels were measured in aortas using DAF-FM diacetate and imaging the fluorescent response to the addition of 2 mM ACh, as described previously (45). Ca2+ transient studies were performed on aortas loaded with Fluo-4. Fluorescence was imaged in vessels (n = 3–5 rats per group) after addition of 2 mM ACh. Average Ca2+ transients of endothelial cells (n = 25–50 cells per group) were calculated. See SI Materials and Methods.
