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A Novel Angiotensin II Induced Long Non-Coding RNA *Giver* Regulates Oxidative Stress, Inflammation, and Proliferation in Vascular Smooth Muscle Cells

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Abstract

Rationale: Angiotensin II (AngII)-mediated vascular smooth muscle cell (VSMC) dysfunction plays a major role in hypertension. Long non-coding RNAs (lncRNAs) have elicited much interest, but their molecular roles in AngII actions and hypertension are unclear.

Objective: To investigate the regulation and functions of a novel lncRNA "Growth factor- and pro-Inflammatory cytokine-induced Vascular cell-Expressed RNA (*Giver*)", in AngII-mediated VSMC dysfunction.

Methods and Results: RNA-sequencing and RT-qPCRs revealed that treatment of rat VSMC with AngII increased the expression of *Giver* and *Nr4a3*, an adjacent gene encoding a nuclear receptor. Similar changes were observed in rat and mouse aortas treated *ex vivo* with AngII. RNA-FISH and subcellular fractionation showed predominantly nuclear localization of *Giver*. AngII increased *Giver* expression via recruitment of Nr4a3 to *Giver* promoter. Microarray profiling and RT-qPCR validation in VSMC showed that *Giver* knockdown attenuated the expression of genes

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DISCLOSURES

None declared.

involved in oxidative stress (*Nox1*) and inflammation (*Il6*, *Ccl2*, *Tnf*), but increased *Nr4a3*. Conversely, endogenous *Giver* overexpression showed opposite effects supporting its role in oxidative stress and inflammation. ChIP assays showed *Giver* overexpression also increased RNA-polymerase II (Pol II) enrichment and decreased repressive histone modification H3K27me3 at *Nox1* and inflammatory gene promoters. Accordingly, *Giver* knockdown inhibited AngII-induced oxidative stress and proliferation in rat VSMC. RNA pull-down combined with mass-spectrometry showed *Giver* interacts with nuclear and chromatin remodeling proteins, and corepressors including NONO. Moreover, NONO knockdown elicited similar effects as *Giver* knockdown on the expression of key *Giver*-regulated genes. Notably, *GIVER* and *NR4A3* were increased in AngII treated human VSMC, and in arteries from hypertensive patients, but attenuated in hypertensive patients treated with Angiotensin Converting Enzyme Inhibitors or Angiotensin Receptor Blockers. Furthermore, human *GIVER* also exhibits partial functional conservation with rat *Giver*.

Conclusions: *Giver* and its regulator *Nr4a3* are important players in AngII-mediated VSMC dysfunction and could be novel targets for anti-hypertensive therapy.

Keywords

Hypertension; Angiotensin II; long non-coding RNA (lncRNA); oxidative stress; inflammation; cell proliferation; vascular smooth muscle cells; ACE/Angiotension Receptors/Renin Angiotensin System; Basic Science Research; Cell Biology/Structural Biology; Hypertension; Vascular Biology

INTRODUCTION

Hypertension, a major contributor to global mortality, affects approximately 26% of the population worldwide, and is expected to rise to 30% by 2025.[1] The detrimental effects of high blood pressure (BP) are manifested through the development of cardio- and cerebro-vascular diseases, such as coronary heart disease and stroke.[2] The renin-angiotensin system (RAS) plays an important role in regulating BP in pathophysiological conditions. The primary effector of this system is angiotensin II (AngII), generated by angiotensin converting enzyme (ACE) in circulation and/or local vasculature. Under physiological conditions, AngII dynamically modulates fluid volume, sodium-potassium balance and vasoconstriction to maintain normal BP. Under pathological conditions, however, enhanced AngII production and AngII type 1 receptor (AT_1R) signaling promotes contraction and growth of vascular smooth muscle cells (VSMC), resulting in arterial wall remodeling and elevated BP.[3]

VSMC, the major components of the arterial wall, are responsible for maintaining vascular tone in response to hemodynamic changes and humoral stimulation. Dysfunction of VSMC, including increased oxidative stress, inflammation, migration, hyperplasia, and hypertrophy, all of which are induced by AngII and other growth factors, plays critical roles in the pathogenesis of hypertension, restenosis and atherosclerosis.[3, 4]

NR4A3 (NOR1) belongs to the orphan nuclear receptor 4a (NR4A) subfamily which includes two other members NR4A1 (NUR77) and NR4A2 (NURR1). It is an important

immediate early response gene and promotes VSMC proliferation by regulating key cell cycle-related genes[5]. NR4A members share a common structure consisting of two transactivation domains and one highly conserved DNA binding domain, but lack ligand binding domains. Thus, the function of these nuclear receptors are mostly regulated via changes in expression and posttranslational modifications.[5] Once induced, NR4A members activate target gene transcription by binding as monomers or homodimers to a canonical DNA sequence, the nerve growth factor-induced protein B-responsive element (NBRE), consisting of an octanucleotide AAAGGTCA sequence. In particular, NR4A homodimers preferentially bind to NBRE-related sequence, an inverted repeat of AAAT(G/A)(C/T)CA.[5] NR4A receptors are induced by diverse stimuli, including growth factors and inflammatory cytokines.[5, 6] However, little is known about the regulatory effects of AngII on *NR4A3* expression or its downstream signaling pathways.

In recent years, there is heightened interest in gene regulatory mechanisms mediated by non-coding RNAs (ncRNAs) and their connections to the pathogenesis of cardiovascular diseases (CVD).[7–11] The ncRNAs can be further divided into several subclasses, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Compared to miRNAs (18–22 nt) that have distinct roles in targeting mRNAs, lncRNAs (>200 nt) can act via multiple mechanisms to regulate genes associated with diverse biological processes.[7–11] Recent studies show that vascular cell expressed lncRNAs, such as *SENCR*, *MALAT1*, *SMILR*, *MYOSLID*, and *Paupar*, can regulate transcription of both closely (*cis* action) and remotely located (*trans* action) protein-coding genes, and have important functions in vascular endothelial cells and VSMC.[8–10, 12–16] However, the detailed regulatory mechanisms and functional roles of lncRNAs in cardiovascular pathology are still not well understood.

We previously showed that miRNAs play key roles in AngII actions in VSMC.[17] Furthermore, using RNA-sequencing (RNA-seq) and chromatin immunoprecipitation linked to high throughput sequencing (ChIP-seq), we identified, for the first time, 466 AngII-regulated lncRNAs (*Lnc-Angs*) in rat VSMC (RVSMC). Moreover, we demonstrated that one of the AngII-induced novel lncRNAs (*Lnc-Ang362*) functions as the host transcript for *miR-221* and *miR-222* to further augment RVSMC proliferation.[13] Recently, we also showed functional role for *lnc-Ang383* that overlaps with enhancers/super-enhancers in regulating AngII-induced genes in VSMC.[18] However, the functions of other AngII-regulated novel lncRNAs are not known. Furthermore, lncRNAs are poorly conserved among different species[19], and potential AngII-regulated human lncRNAs have not yet been characterized in human VSMC or arteries.

In the current study, we investigated the upstream regulatory mechanisms, downstream target genes, and functional roles of a novel AngII-induced lncRNA, *Lnc-Ang164*, in VSMC. This lncRNA was worthy of investigation because it is located adjacent to *Nr4a3*, whose function in AngII-mediated vascular pathology is not yet known. Notably, our results show a highly coordinated spatiotemporal interplay between *Lnc-Ang164* and *Nr4a3* in AngII-mediated signaling and VSMC dysfunction. Because *Lnc-Ang164* was induced not only by AngII, but also other growth factors and cytokines, we renamed it as Growth factor- and pro-Inflammatory cytokine-induced Vascular cell-Expressed lncRNA (*Giver*). Interestingly, we found that human orthologs *GIVER* and *NR4A3* were upregulated in

human VSMC treated with AngII, and in mammary arteries from hypertensive patients versus normals, and were reduced in hypertensive patients treated with RAS inhibitors, suggesting *GIVER* could be a novel therapeutic target for hypertension.

METHODS

The authors declare that all data supporting the findings of this study are available within the article and its Online supplementary files.

Detailed Methods and Statistics are available in the Online Supplement. RVSMC were isolated from thoracic aortas of 12-week-old male Sprague-Dawley rats. Gene expression was analyzed from VSMC, rat and C57BL/6 mice aortas, and non-hypertensive and hypertensive patients' samples. Details of PCR and ChIP-PCR primers are provided in Tables I-III in the Online Supplement. Results are presented as Means + SEM.

RESULTS

AngII induces Giver and Nr4a3 expression in a time-dependent manner.

Evidence shows that many lncRNAs can regulate expression of neighboring genes on the same chromosome through *cis*-acting mechanisms.[20, 21] Therefore, we analyzed RNA-seq data from AngII treated RVSMC[13] to identify differentially expressed lncRNAs located within ± 100 kb of AngII-induced coding genes. We found that a novel lncRNA, *Lnc-Ang164*, which we renamed as *Giver*, and a nearby orphan nuclear receptor coding gene *Nr4a3*, located ~99 kb from *Giver* on rat chromosome 5, were both significantly increased at 3h after AngII treatment (Figure 1A). H3K4me3 peaks depict start sites of these genes. To verify the regulation of *Giver* and nearby *Nr4a3*, RVSMC were treated with AngII for 1–6h and gene expression analyzed by RT-qPCR. *Giver* expression was significantly upregulated as early as 1h and peaked at 3h, while *Nr4a3* expression peaked at 1h and remained elevated up to 6h after AngII treatment (Figure 1B-C).

We next examined the effects of other growth factors, cytokines and diabetic conditions (high glucose, HG) known to promote VSMC dysfunction. Both 20% fetal bovine serum (FBS) and 10ng/ml platelet-derived growth factor-BB (PDGF-BB) elicited marked induction of *Nr4a3* expression as shown before[22]. Interestingly, they also induced significant increases in *Giver* expression (Figure 1D-G). 30mmol/L HG, 5ng/ml transforming growth factor-beta1 (TGF-β), 20ng/ml tumor necrosis factor-alpha (TNF-α) and 100 ng/ml lipopolysaccharide (LPS) also significantly induced both *Giver* and *Nr4a3* with similar patterns (Figure 1H-O). However, 20 ng/ml IL-4 failed to induce *Giver*, and elicited only a moderate increase in *Nr4a3* at 1h (Online Figure IA-B).

Together, these results demonstrate that *Giver* and its nearby gene *Nr4a3* exhibit similar expression patterns in VSMC (suggesting co-regulation) in response to AngII and other stimuli implicated in the pathogenesis of CVD.

AngII induces Giver and Nr4a3 in cultured human vascular cells, and in rat and mice aortas.

To assess whether *Giver* is conserved across species in human and mouse, we first used the LiftOver tool[23] to identify transcripts with similar genomic organization on human (hg19) and mouse (mm9) reference genomes. Potential human (h*GIVER*) and mouse (m*Giver*) orthologs were located ~99 kb upstream of corresponding *NR4A3* gene similar to the rat genome (Figure 1A and Online Figure IIA-B). Sequence comparison using NCBI BLAST tool[24] revealed that rat *Giver* shares 75% and 51% homology with predicted mouse and human genes respectively. Subsequent RT-qPCR analysis showed that *GIVER* and *NR4A*3 were significantly induced by AngII in human VSMC (HVSMC) (Figure 2A-B), similar to RVSMC.

To further clarify whether other vascular cells and monocytes can also contribute to the increase of *NR4A3* and *GIVER* in aortas, we analyzed their expression in human umbilical vein endothelial cells (HUVEC) and THP-1 monocytes. AngII increased *NR4A3* and *GIVER* RNA expression in HUVEC in a time-dependent fashion (Figure 2C-D). In contrast, although *NR4A3* RNA was increased at 1h, AngII did not alter *GIVER* expression in THP-1 human monocytes (Figure 2E-F). Together, these results show that *GIVER* RNA is markedly increased by growth factors and pro-inflammatory cytokines in vascular cells, but not monocytes, supporting the nomenclature ***G****rowth factor- and pro-****I****nflammatory cytokine-induced* ***V****ascular cell-****E****xpressed lnc****R****NA* (***GIVER***).

We next investigated the expression of *Giver* and *Nr4a3* in aortas treated *ex vivo* with AngII. As shown in Figure 2G-H, both *Giver* and *Nr4a3* expressions were increased in rat aortas 1h and 3h after AngII treatment respectively. AngII also increased both *Giver* and *Nr4a3* transcripts at 1h and 3h after treatment in mouse aortas (Figure 2I-J). In addition, PDGF-BB treatment significantly increased the expression of *Giver* and *Nr4a3* transcripts at 3h in mouse aortas (Online Figure III A-B). Overall, these results suggest co-regulation of *Giver* and *Nr4a3* by AngII and PDGF also in *ex vivo* rodent models.

Nr4a3 mediates AngII-induced transactivation of Giver promoter via NBRE sequences.

Data in the previous sections suggested a close temporal relationship between the expression of *Giver* and *Nr4a3* in response to a variety of mitogens and cytokines (Figure 1). Evidence shows that NR4A3 transactivates target gene promoters by binding to different canonical NBRE sequences.[5] TRANSFAC and Jasper[25, 26] motif analysis of the *Giver* promoter spanning −3026 to +1bp verified the presence of several NBRE motifs (Figure 3A). Hence, we hypothesized that Nr4a3 may regulate *Giver* transcription in RVSMC via promoter NBRE *cis*-elements. To evaluate whether *Nr4a3* is required for AngII-induced *Giver* expression, we performed siRNA mediated knockdown or overexpression of Nr4a3. Transfection of RVSMC with siRNAs targeting *Nr4a3* (si*Nr4a3*) reduced *Nr4a3* protein and RNA in both control and AngII treated cells relative to control siNTC oligonucleotides (Figure 3B-C, respectively). Notably, NR4a3 knockdown also significantly reduced AngII-induced *Giver* expression by ~30% (Figure 3D). Conversely, transfection of phNR4A3 vector expressing human NR4A3 (gift from Dr. Dennis Bruemmer, University of Pittsburgh)

increased *NR4A3* RNA and protein levels, and also enhanced *Giver* transcript expression (~1.5 fold) versus empty vector (Figure 3E-G).

To further identify the specific NBRE binding sites involved (Figure 3A), we constructed luciferase reporter constructs driven by a series of *Giver* WT promoter fragments spanning from −3026, −1581, −1456, and −202 to +160 bp (Figure 3H). Transient transfections and subsequent luciferase assays showed that AngII transactivated the −3026 promoter, but the highest increase (~4 fold) in transcriptional activity was observed with the −1581 WT construct. TRANSFAC analysis of −3026 and −1581 promoter fragments revealed nine ZNF333 binding sites between −3026 and −1581. ZNF333 is a Zinc finger protein which contains KRAB domain associated with transcriptional repression. This might explain why the −3026 WT construct showed lower transcriptional activity compared to −1581 WT. Activity of −1581 WT was lost with the −1456 and −202 WT deletions (Figure 3H), suggesting the presence of important positive regulatory sites between −1581 and −1456 bp. Interestingly, an overlapping inverted AAATACCA repeat (−1527/−1515 bp) was found within this 125 bp (Online Figure IV). Accordingly, deletion of this potential NBRE-related motif in −1581-MUT construct significantly attenuated AngII response compared to −1581 WT (Figure 3I). Moreover, si*Nr4a3* significantly inhibited AngII-induced transcriptional activity of −1581 WT, but had no significant effect on −1581 MUT promoter (Figure 3J). Conversely, Nr4a3 overexpression mimicked AngII actions by increasing basal transcriptional activity of −1581 WT, which was significantly attenuated in 1581 MUT (Figure 3K). In addition, ChIP assays with Nr4a3 antibody revealed that Nr4a3 overexpression significantly increased Nr4a3 enrichment at the *Giver* promoter compared to control transfected cells (Figure 3L). *Skp2* promoter served as positive control for Nr4a3 binding (Figure 3M).[27]

Collectively, these data demonstrate that Nr4a3 mediates AngII-induced *Giver* transcription via NBRE-related site located 1515 bp upstream of *Giver*'s transcription start site (TSS).

Giver is nuclear enriched and negatively modulates NR4a3 expression.

Next, we employed Rapid amplification of cDNA ends (RACE) to construct full length *Giver* transcript. The 5'- and 3'-RACE showed *Giver* transcript is 1,225 bp long with three exons confirming our RNA-seq annotation (Online Figure VA-B). We also determined that *Giver* was polyadenylated by oligo-dT column purification and RT-qPCR (Online Figure VC-D). DNA sequencing revealed an alternative poly A+ signal (TATAAA; 1193/1198), 27 bp prior to the start of actual poly A+ sequence (Online Figure VE), indicating potential regulation by post-transcriptional mechanisms. We also verified that *Giver* does not have protein coding potential by performing *in vitro* transcription/translation assays (Online Figure VF).

Because sub-cellular localization of lncRNAs is closely associated with their functions, we next examined levels of *Giver* transcripts in nuclear and cytoplasmic fractions prepared from both untreated and AngII-treated (3h) RVSMC. Using U6 as nuclear marker and tRNAf-met as a cytoplasmic marker, we confirmed purity of sub-cellular fractions (Figure 4A-B). Furthermore, RT-qPCR showed that *Giver* transcript was highly enriched in the nuclear fractions with or without AngII stimulation (Figure 4C). We further confirmed nuclear

localization by RNA-FISH after hybridization with probes targeting *Giver* (Figure 4D). These results suggest that *Giver* is polyadenylated and functions mainly in the nucleus. Having demonstrated that *Giver* is predominantly nuclear, we next examined its role in gene regulation. Because *Nr4a3* is an AngII-regulated nearby gene, and Nr4a3 could also regulate *Giver*, we examined whether *Giver* also reciprocally regulates *Nr4a3*. We knocked down *Giver* with Dicer substrate siRNAs targeting *Giver* exon sequence (si*Giver*) in RVSMC. Compared to the non-targeting control siRNA (siNTC), *Giver* transcript was reduced by ~80% and ~50% in si*Giver*-transfected RVSMC with or without AngII treatment, respectively (Figure 4E). Surprisingly, *Giver* knock-down did not reduce *Nr4a3* expression, but instead enhanced *Nr4a3* expression in basal and AngII treated cells (Figure 4F), suggesting that AngII-induced *Giver* might suppress *Nr4a3* expression, likely in a negative feedback regulatory loop.

To further verify this negative effect of *Giver* on *Nr4a3*, we first cloned full-length *Giver* into pcDNA3.1 (+) vector (p*Giver*) (Online Figure VIA-B). Transient transfection of RVSMC with p*Giver* significantly increased *Giver* expression (Figure 4G). But, exogenously overexpressing *Giver* (ectopic) did not significantly reduce *Nr4a3* expression, despite a trend towards decrease (Figure 4H). Furthermore, we also used the CRISPR-on method to overexpress endogenous *Giver* (Online Figure VIC). We designed two groups of sgRNAs, targeting either the promoter (PR) or exonic regions (EX) of *Giver*, and cloned these sgRNAs into pAC154-dual-dCas9VP160-sg vector. Of note, the target DNA sequences of PR sgRNAs were located within 200 bp upstream of TSS, while EX sgRNAs spanned 3 different exons (Figure 4I). As shown in Figure 4J, *Giver* expression was enhanced ~2 fold in PR group but not significantly altered in EX, compared to control vector (EV). In contrast, *Nr4a3* was decreased in PR sgRNA-transfected RVSMC but unaffected in EX (Figure 4K). These results, suggest that *Giver* negatively modulates *Nr4a3* expression at the transcriptional level.

We next examined whether *Giver* modulates other nearby genes besides *Nr4a3* (−99 kb). The expression of five other genes within ±500 kb of *Giver* was not affected by *Giver* knockdown (Online Figure VID-I). Together, these data strongly suggest that AngII-induced *Giver* mainly suppresses *Nr4a3*, without affecting other proximal genes.

GIVER promotes inflammatory gene expression, oxidative stress, and proliferation in RVSMC.

In order to determine the putative functional roles of *Giver* in VSMC, we first performed unbiased microarray profiling of RVSMC transfected with si*Giver* to identify potential target genes. We found substantial differences in gene expression in RVSMC transfected with si*Giver* versus control siNTC (Online Figure VIIA-B) in untreated and AngII treated cells. We identified a total of 565 differentially expressed transcripts in untreated (257 down-regulated and 308 up-regulated) and 1214 transcripts in AngII-treated RVSMC (570 down-regulated and 644 up-regulated), respectively after *Giver* knockdown (FDR <0.005, log2 fold change ≥1.25). As expected, *Nr4a3* was increased after r*Giver* knockdown in AngII treated cells. Interestingly, NADPH oxidase1 (*Nox1*) was among the top 10 downregulated genes in both untreated and AngII-treated RVSMC transfected with *Giver* siRNA (Online

Table IV-VII). Moreover, pro-inflammatory genes, such as *C-C motif chemokine ligand 2* (*Ccl2*) and *Interleukin 6* (*Il6*), were also among the downregulated genes in both untreated and AngII-treated RVSMC (Online Figure VIIC-D). Furthermore, Ingenuity Pathway Analysis (IPA) revealed that inflammatory response, cell cycle, and cell growth were strongly associated with *Giver* knockdown (Online Figure VIIIA-D). These results indicate that *Giver* may regulate oxidative stress, inflammation, and proliferation in RVSMC.

Giver knockdown differentially regulated (up- and downregulated) the expression of genes not only on chr5 on which it is located, but also those present on other chromosomes (Figure 5A). Using RT-qPCR we validated that *Giver* knockdown significantly reduced the expression of *Ccl2*, *Il6*, and *Tnf-* (*Tnf*) (Figure 5B-D), confirming that AngII inducible *Giver* can indeed upregulate inflammatory genes. Furthermore, *Giver* knockdown inhibited AngII-induced *Nox1* expression at both RNA (Figure 5E) and protein levels (Figure 5F). Conversely, exogenously (Figure 5G-J) and endogenously (by CRISPR-on) (Figure 5K-N) overexpressing *Giver* markedly enhanced expression of *Ccl2*, *Il6*, *Tnf*, and *Nox1.* Together, these results indicate that AngII inducible *Giver* enhances the expression of distally located target genes (Online Table VIII) that are closely associated with inflammation (*Ccl2*, *Il6*, and *Tnf*) and oxidative stress (*Nox1*).

Next, to investigate whether epigenetic mechanisms are involved in *Giver*-mediated regulation of these genes, we performed ChIP assays with antibodies specific to RNA Polymerase II (Pol II) and repressive chromatin mark H3K27me3 after *Giver* overexpression in RVSMC. *Giver* overexpression increased Pol II enrichment but decreased H3K27me3 enrichment on *Ccl2*, *Tnf*, and *Nox1* promoters (Figure 5O-Q and S-U). *Rplpo* promoter was used as negative control (Figure 5R and V).

Since *Nox1* expression was enhanced, we next examined whether *Giver* can functionally promote oxidative stress. We measured intracellular reactive oxygen species (ROS) with fluorescent probes DHE and H_2DCFDA after transfection with siNTC or si*Giver*. *Giver* knockdown attenuated AngII-induced intracellular ROS production in RVSMC, as determined by decreased red fluorescence (Figure 6A-B). Furthermore, quantification of H_2DCFDA fluorescence intensity showed similar reduction after *Giver* knockdown (Figure 6C), thus confirming *Giver* can mediate AngII-induced ROS production. We also performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and 5-bromo-2'-deoxyuridine (BrdU) incorporation assays to investigate the effects of *Giver* on cell proliferation. As shown in Figure 6D and 6E, *Giver* siRNA also attenuated AngII-induced proliferation in RVSMC. Together, these data demonstrate that *Giver* can mediate AngII-induced inflammatory gene expression, oxidative stress, and proliferation in VSMC.

Giver interacts with the nuclear paraspeckles and splicing factor, non-POU domain-containing octamer-binding protein (NONO).

To evaluate potential mechanisms by which *Giver* RNA regulates key VSMC genes and functions, we performed RNA-pull down assays to identify *Giver*-interacting proteins. *Giver* sense and anti-sense RNAs were synthesized by *in vitro* transcription and then biotinylated. RNA integrity and biotinylation efficiency were checked by denaturing gel electrophoresis

and dot blots (Online Figure IXA-B). Biotinylated sense and anti-sense probes were incubated with RVSMC nuclear extracts and RNA-bound complexes separated on an SDS-PAGE gel (Figure 7A). Protein bands from four different regions were subjected to mass-spectrometry analysis from sense and anti-sense lanes. Results revealed that multiple proteins are specifically associated with *Giver* sense strand but not anti-sense (Online Figure X). STRING database was used to identify the common protein complexes enriched among the *Giver* interacting proteins and their key functions (Figure 7B). *Giver*-interacting proteins were enriched in RNA binding proteins like NONO, and proteins involved in various RNA functions such as splicing and ribosome biogenesis. Interestingly, proteins in chromatin modification and remodeling complexes were also bound to *Giver* (Figure 7B). Specifically, RuvB Like AAA ATPase 1 (RUVBL1) and WD repeat-containing protein 82 (WDR82) are subunits of the INO80 chromatin remodeling complex and SET1 methyltransferase complex respectively (Online supplement Figure XIA). Moreover, histone-binding protein (RBBP7), AT-rich interactive domain-containing protein 4B (ARID4B) and chromodomain-helicase-DNA-binding protein 3(CHD3) are subunits of repressor complexes such as Sin3A-HDAC complex (Online supplement Figure XIB) suggesting *Giver* RNA can have positive and negative effects on gene expression. To validate some of these interactions, we performed RNA Immunoprecipitation (RIP) assays with NONO, WDR82, RBBP7 antibodies and control IgG. Results confirmed specific interaction of *Giver* with NONO, but not with other two interacting partners, possibly due to unavailability of rat-specific antibodies for these two proteins (Figure 7C, Online Figure XII). NONO did not co-precipitate with control *Ppia*. Interaction with NONO was also confirmed by RNA-pull-down followed by western blot (Figure 7D). Together, these data confirm NONO interacts with *Giver* and may mediate some molecular functions of *Giver*.

NONO is an RNA-binding protein belonging to the Drosophila behavior human splicing (DBHS) family of proteins including SFPQ and paraspeckle protein 1 (PSPC1). NONO regulates gene expression by multiple mechanisms.[28] NONO, together with SFPQ, PSPC1, hnRNPs, pre-mRNA splicing factors and lncRNA *Neat1,* forms paraspeckles[29] and is also involved in miRNA processing.[30] To evaluate the function of *Giver*-NONO complex, we examined effects of NONO knockdown in RVSMC (Figure 7E). NONO siRNA did not alter *Giver* expression under basal or AngII stimulated condition (Figure 7F), but, interestingly, it enhanced *Nr4a3* expression under AngII induction and attenuated *Il6* expression in both basal and AngII conditions (Figure 7G and H), showing that NONO knockdown phenocopies the effect of *Giver* knockdown on these genes (Figure 4F, 4K and 5C). Thus, *Giver*-NONO complex might be functionally involved in *Giver*-mediated gene regulation. Interestingly *Giver* overexpression decreased H3K9ac and Pol II enrichments on the *Nr4a3* promoter (Figure 7I-J) H3K27me3 enrichment was unaltered (Figure 7K).

GIVER and NR4a3 transcripts are increased in arteries collected from patients with hypertension.

To investigate the clinical *in vivo* significance of *GIVER* in human vascular pathologies, we measured its levels in arteries from patients with hypertension versus controls, as well as hypertensive patients on ACE inhibitors (ACEI) or AngII receptor blocker (ARB) therapy (Online Table IX). Interestingly, *GIVER* expression was significantly increased in untreated

hypertensive patients compared with non-hypertensive control subjects. In contrast, *GIVER* expression was markedly reduced in hypertensive patients treated with ACEIs or ARBs (Figure 8A). *NR4A3* expression followed a similar pattern, with increases in untreated hypertensives and decreases in treated hypertensive subjects (Figure 8B). p47phox, a positive control for increased oxidant stress, especially in human hypertension[31] also followed a similar pattern (Figure 8C). These data collectively implicate AngII induced *GIVER* and *NR4A3* as important players in the pathogenesis of human hypertension likely through augmenting vascular oxidant stress, growth and inflammation.

Putative hGIVER shows functional conservation with rGiver.

To assess whether human ortholog of *GIVER* can elicit similar phenotype as r*Giver*, we identified putative h*GIVER* transcripts (*GIVER1-4*) using RNA-seq data from HVSMC treated with AngII (unpublished) and publicly available HVSMC data[16] aligned to the hg38 genome (See Online Methods and Figure XIIIA). We verified that AngII induces the expression of these putative h*GIVER* transcripts in HVSMC by RT-qPCR by using two different sets of primers (P1 and P2) (Online Figure XIIIB-C) similar to what we observed with the LiftOver identified h*GIVER* transcript. Interestingly, *GIVER* (P1) expression was also significantly increased in hypertensive patients and this was reduced in hypertensive patients treated with ACEIs or ARBs (Online Figure XIIID).

In order to further investigate the functional conservation between r*Giver* and h*GIVER*, we generated a putative h*GIVER1* construct in pcDNA3.1 (pGIVER1) and overexpressed it in RVSMC, and examined candidate genes that we had found to be altered by *rGiver* in this system. Our data clearly showed human *GIVER1* was overexpressed in RVSMC (Figure 8D) and also increased the expression of *Ccl2* and *Nox1* without affecting *Tnf* and *Il6* (Figure 8E-H). Interestingly, ectopic *GIVER1* overexpression in RVSMC did not alter *Nr4a3* (Figure 8I). Together, these results suggest h*GIVER* shares some functional properties with r*Giver*.

DISCUSSION

In this study, we investigated the mechanisms of regulation and functional roles of a novel AngII-regulated rat lncRNA, *Giver*. We found that AngII induces the expression of *Giver* and its nearby protein-coding gene *Nr4a3* in VSMC. AngII-induced Nr4a3 transactivated the expression of *Giver* through binding to the NBRE-related sequence 1515 bp upstream of *Giver* TSS. In turn, *Giver* transcript partially suppressed *Nr4a3* expression but simultaneously up-regulated several target genes associated with inflammation and oxidative stress (*trans* actions), including *Il6*, *Ccl2*, *Tnf*, and *Nox1,* likely via interactions with key RNA binding proteins and chromatin remodeling complexes. In addition, *Giver* functionally promoted oxidative stress and cell proliferation in AngII-treated RVSMC. We further showed that *GIVER* was increased in AngII-treated HUVEC and HVSMC, but not in human THP-1 monocytes. Furthermore, *GIVER* and *NR4A3* were markedly increased in arteries from human hypertensive patients.

LncRNAs are important epigenetic regulators that have recently been shown to be associated with vascular cell functions and vascular pathologies, acting via various mechanisms.[8, 9, 12–16] In particular, nuclear lncRNAs can modulate the transcriptional activation or

suppression of local (in *cis*) and/or distal (in *trans*) target genes through alterations in chromatin modifications including enhancers and super-enhancers at their loci and other mechanisms.[13, 18, 19]

Here, we found that *Giver* is a 1.2 kb polyadenylated nuclear lncRNA located ~99 kb upstream of *Nr4a3*. Both *Giver* and *Nr4a3* were upregulated in VSMC by various growth factors and pro-inflammatory cytokines in addition to AngII. Interestingly, *Giver* expression always peaked with or after that of *Nr4a3*, supporting a correlation between these neighboring genes. Their cross-talk regulation was also evident from the observation that *rGiver* promoter (−3026 to +1 bp) had several NBRE-related motifs. Further studies revealed that an NBRE-related sequence containing inverted repeat AAATACCA (−1527/−1515 bp)[6] was involved in promoter transactivation, implying that Nr4a3 homodimers may transactivate *Giver* in AngII-treated RVSMC. These data also reveal a novel nuclear-receptor mediated regulation of a vascular lncRNA. On the other hand, treatment with si*Giver* enhanced *Nr4a3* expression in AngII-treated RVSMC, whereas endogenous *Giver* overexpression (with CRISPR-on) inhibited *Nr4a3*. These data collectively suggest that *Giver* forms a negative regulatory loop with *Nr4a3*, with AngII induced Nr4a3 promoting *Giver* expression, and then *Giver* partially suppressing *Nr4a3* expression in AngII-treated RVSMC (Figure 8J).

NR4a receptors function as constitutively active and ligand independent receptors and their transactivation activity is primarily regulated by extracellular stimuli that alter their expression and posttranslational modifications.[5, 6] However, to our knowledge, there are no reports showing lncRNAs regulating the expression of NR4a receptors. Therefore, our observation that AngII inducible *Giver* partially suppressed *Nr4a3* transcription reveals new lncRNA-dependent regulatory mechanisms of these genes.

In addition to the effects of *Giver* on its neighboring gene *Nr4a3*, we also explored its *trans*-effects on other target genes through unbiased microarray analysis. Compared with siNTC-transfected RVSMC, *Nox1* was among the top ten differentially expressed genes in si*Giver*-transfected cells with or without AngII stimulation. ROS production in VSMC dysfunction and hypertension is well established, including the role of AngII as a major inducer of ROS in VSMC. Many of the pleiotropic vascular effects of AngII, such as inflammation and cell proliferation, are mediated through NAD(P)H oxidases (NOXs), including Nox1.[32] Nox1 can mediate the hypertensive response to AngII,[33] again emphasizing the significance of the AngII-Nox1 axis. Our data suggests that Nox1 may be one of the key effectors of *Giver*-mediated signaling and vascular pathology. In support of this, IPA analysis showed significant enrichment of genes and pathways associated with oxidative stress, inflammation, and cell proliferation networks among those altered by si*Giver*. Importantly, we demonstrated the functional roles in VSMC pathology, because *Giver* knockdown attenuated AngII-induced ROS generation (likely due to Nox1 downregulation), and decreased cell proliferation in RVSMC. Thus, *GIVER* may promote cell proliferation and inflammation in VSMC through Nox1-mediated signaling pathways.

Examination of protein interacting partners of *Giver* RNA provided valuable clues about the potential mechanisms by which *Giver* promotes the expression of inflammatory genes and

Nox1, while also suppressing *Nr4a3*. RNA-pull down coupled to mass-spectrometry revealed several proteins related to RNA function such as splicing, nuclear paraspeckles, ribosome biogenesis and translation as *Giver* interacting partners. We validated the interaction of the nuclear paraspeckle protein NONO with *Giver*. Interestingly, NONO knockdown had similar effects on *Nr4a3* and *Il6* gene expression as that of *Giver* knockdown, suggesting *Giver*-NONO complex may regulate these genes. NONO and SFPQ are known corepressors that interact with the well-known Sin3A-HDAC repressive complex.[34] *Giver* interacting partners also included RBBP7, CHD3 and ARID4B which are subunits of the Sin3A-HDAC complexe. Since we observed *Giver* negatively regulates *Nr4a3* expression, after upregulation by AngII via Nr4a3, *Giver* might recruit these repressive complexes to the *Nr4a3* promoter though interactions with NONO, SPFQ and other proteins, as part of an adaptive negative feedback loop (Figure 8J). Interestingly, leucine-rich repeat flightless-interacting protein1 (LRRFIP1), also identified as a *Giver* interacting protein, is an RNA binding protein that binds to GC rich sequences and functions as transcriptional repressor by binding to EZH2 in the PRC2 complex.[35, 36] LRRFIP1 is induced by PDGF and TNF and can downregulate TNF, PDGF and epidermal growth factor receptor (EGFR) expression after initial burst of signaling.[36, 37] As LRRFIP1 is also induced by AngII, similar mechanisms may downregulate *Nr4a3* in VSMC after its initial surge (1h) of induction by AngII. *Nr4a3* promoter harbors GC rich sequences that could be bound by LRRFIP1, *Giver* and repressive complexes to repress *Nr4a3* expression. Ectopic *Giver* overexpression increased *Giver* transcript levels to a much greater magnitude (almost 60 fold) compared to its endogenous overexpression by CRISPR-on or AngII treatment (2–3 fold) (Figure 4G versus 4J and 1B). This high expression of *Giver* might facilitate the binding of such repressive complexes to *Giver* RNA and subsequent recruitment to the *Nr4a3* locus, resulting in decreased H3K9ac and Pol II enrichment (Figure 7I-J), but not sufficient enough to significantly decrease *Nr4a3* expression (Figure 4H).

We also found key proteins related to active chromatin complexes such as SET1 (Online Figure XIA) among *Giver* interacting proteins. LncRNAs can regulate transcription by recruiting regulatory protein complexes in *trans* by RNA-protein interactions.[38] Such interactions are possibly involved in *Giver* mediated increases *Nox1* and inflammatory gene expression via chromatin remodeling and epigenetic mechanisms (Figure 8J). This is indirectly supported by ChIP data showing increased Pol II and decreased H3K27me3 enrichment at these genes in *Giver* overexpressing cells (Figure 5O-V). Therefore, *Giver* might have negative effects on *Nr4a3* via *cis* action, but positive effects on the expression of other genes in *trans,* through interactions with key proteins (like NONO) and other mechanisms including histone modifications. Additional experiments are needed to test these mechanisms systematically. Of note, we recently showed lncRNA *Dnm3os* has *trans*-actions on inflammatory genes in macrophages through RNA-protein interactions with nucleolin.[39]

ACEIs and ARBs, among the first-line of anti-hypertensive drugs, directly alter the production and functions of AngII.[40] However the epigenetic mechanisms of action of ACEI/ARBs remain largely unknown. We found correlations between lncRNA *GIVER* levels and ACEI/ARB therapy. Expressions of *GIVER, NR4A3* and *p47phox* (positive control for oxidant stress in human hypertension[31]) were increased in mammary arteries

collected from hypertensive patients relative to controls, but were restored to normal levels in hypertensive patients on ACEIs or ARBs. To our knowledge, this is the first study showing correlation between lncRNAs and antihypertensive therapy. In addition, h*GIVER* was induced by AngII in HVSMC and HUVEC, but not in THP-1 monocytes, indicating specific regulation in vascular cells. Importantly, alterations in *GIVER* transcript was always accompanied by prior or simultaneous changes of *NR4A3* RNA, implying that *GIVER* may also be regulated by NR4A3 in human vasculature. Notably, we used a combination of *in silico* transcriptome data analyses coupled with experimental approaches, including cloning and functional assays, to identify putative *hGIVER* transcripts showing functional conservation, at least in part, with *rGiver* (Online Figure XIII and Figure 8D-I).

In summary, we report several new findings, including the mechanisms of regulation of a novel AngII-induced lncRNA *GIVER* by a nuclear receptor, its functional roles in promoting inflammatory gene expression (via epigenetic mechanisms), oxidative stress and proliferation, identification of *Giver* interacting proteins and association of the *GIVER-NR4A3* pathway with hypertension as well as RAS-associated antihypertensive therapy. The in-depth molecular mechanisms by which *Giver* interacting proteins modulate its functions needs further investigation. Notwithstanding, the current observations enhance our understanding of the complex interplay between lncRNAs and nuclear receptors such as NR4A3 in the pathogenesis of hypertension and CVDs, which, in turn, could lead to new therapeutic interventions for hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

ACEI	angiotensin converting enzyme inhibitor
AngII	angiotensin II
ARB	angiotensin receptor blocker
AT_1R	AngII type-1 receptor
BP	blood pressure
BrdU	5-bromo-2'-deoxyuridine

CABG	coronary artery bypass grafting
CVD	cardiovascular diseases
DHE	dihydroethidium
FBS	fetal bovine serum
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HG	high glucose
IPA	Ingenuity Pathway Analysis
lncRNA	long non-coding RNA
miRNA	microRNA
MTS	3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NBRE	nerve growth factor-induced protein B-responsive element
ncRNA	non-coding RNA
PDGF-BB	platelet-derived growth factor-BB
RAAS	renin-angiotensin-aldosterone system
ROS	reactive oxygen species
VSMC	vascular smooth muscle cell
RIP	RNA immunoprecipitation
NONO	non-POU domain-containing octamer-binding protein
Pol II	RNA Polymerase II
H3K27me3	histone H3 trimethylation on lysine 27

REFERENCES

1. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. Lancet 2005;365:217–223. [PubMed: 15652604]
2. Poulter NR, Prabhakaran D, Caulfield M. Hypertension. Lancet 2015;386:801–812. [PubMed: 25832858]
3. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am. J. Physiol. Cell Physiol 2007;292:C82–97. [PubMed: 16870827]
4. Bennett MR, Sinha S, Owens GK. Vascular Smooth Muscle Cells in Atherosclerosis. Circ Res 2016;118:692–702. [PubMed: 26892967]
5. Zhao Y, Bruemmer D. NR4A orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. Arterioscler Thromb Vasc Biol 2010;30:1535–1541. [PubMed: 20631354]

6. Martinez-Gonzalez J, Badimon L. The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. Cardiovasc Res 2005;65:609–618. [PubMed: 15664387]
7. Leung A, Stapleton K, Natarajan R. Functional Long Non-coding RNAs in Vascular Smooth Muscle Cells. Curr. Top. Microbiol. Immunol 2016;394:127–141. [PubMed: 25910717]
8. Sallam T, Sandhu J, Tontonoz P. Long Noncoding RNA Discovery in Cardiovascular Disease: Decoding Form to Function. Circ Res 2018;122:155–166. [PubMed: 29301847]
9. Leung A, Amaram V, Natarajan R. Linking diabetic vascular complications with LncRNAs. Vascul Pharmacol 2018
10. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 2009;458:223–227. [PubMed: 19182780]
11. Freedman JE, Miano JM, National Heart L, Blood Institute Workshop P. Challenges and Opportunities in Linking Long Noncoding RNAs to Cardiovascular, Lung, and Blood Diseases. Arterioscler Thromb Vasc Biol 2017;37:21–25. [PubMed: 27856459]
12. Bell RD, Long X, Lin M, Bergmann JH, Nanda V, Cowan SL, Zhou Q, Han Y, Spector DL, Zheng D, Miano JM. Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. Arterioscler Thromb Vasc Biol 2014;34:1249–1259. [PubMed: 24578380]
13. Leung A, Trac C, Jin W, Lanting L, Akbany A, Saetrom P, Schones DE, Natarajan R. Novel long noncoding RNAs are regulated by angiotensin II in vascular smooth muscle cells. Circ Res 2013;113:266–278. [PubMed: 23697773]
14. Vance KW, Sansom SN, Lee S, Chalei V, Kong L, Cooper SE, Oliver PL, Ponting CP. The long non-coding RNA Paupar regulates the expression of both local and distal genes. EMBO J 2014;33:296–311. [PubMed: 24488179]
15. Zhao J, Zhang W, Lin M, Wu W, Jiang P, Tou E, Xue M, Richards A, Jourd'heuil D, Asif A, Zheng D, Singer HA, Miano JM, Long X. MYOSLID Is a Novel Serum Response Factor-Dependent Long Noncoding RNA That Amplifies the Vascular Smooth Muscle Differentiation Program. Arterioscler Thromb Vasc Biol 2016;36:2088–2099. [PubMed: 27444199]
16. Ballantyne MD, Pinel K, Dakin R, et al. Smooth Muscle Enriched Long Noncoding RNA (SMILR) Regulates Cell Proliferation. Circulation 2016;133:2050–2065. [PubMed: 27052414]
17. Jin W, Reddy MA, Chen Z, Putta S, Lanting L, Kato M, Park JT, Chandra M, Wang C, Tangirala RK, Natarajan R. Small RNA sequencing reveals microRNAs that modulate angiotensin II effects in vascular smooth muscle cells. J. Biol. Chem 2012;287:15672–15683. [PubMed: 22431733]
18. Das S, Senapati P, Chen Z, Reddy MA, Ganguly R, Lanting L, Mandi V, Bansal A, Leung A, Zhang S, Jia Y, Wu X, Schones DE, Natarajan R. Regulation of angiotensin II actions by enhancers and super-enhancers in vascular smooth muscle cells. Nat Commun 2017;8:1467. [PubMed: 29133788]
19. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A 2009;106:11667–11672. [PubMed: 19571010]
20. Engreitz JM, Haines JE, Perez EM, Munson G, Chen J, Kane M, McDonel PE, Guttman M, Lander ES. Local regulation of gene expression by lncRNA promoters, transcription and splicing. Nature 2016;539:452–455. [PubMed: 27783602]
21. Wang KC, Yang YW, Liu B, et al. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature 2011;472:120–124. [PubMed: 21423168]
22. Martinez-Gonzalez J, Rius J, Castello A, Cases-Langhoff C, Badimon L. Neuron-derived orphan receptor-1 (NOR-1) modulates vascular smooth muscle cell proliferation. Circ Res 2003;92:96–103. [PubMed: 12522126]
23. Tyner C, Barber GP, Casper J, et al. The UCSC Genome Browser database: 2017 update. Nucleic Acids Res 2016
24. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. NCBI BLAST: a better web interface. Nucleic Acids Res 2008;36:W5–9. [PubMed: 18440982]

25. Wingender E, Dietze P, Karas H, Knuppel R. TRANSFAC: a database on transcription factors and their DNA binding sites. Nucleic Acids Res 1996;24:238–241. [PubMed: 8594589]
26. Mathelier A, Zhao X, Zhang AW, et al. JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. Nucleic Acids Res 2014;42:D142–147. [PubMed: 24194598]
27. Gizard F, Zhao Y, Findeisen HM, Qing H, Cohn D, Heywood EB, Jones KL, Nomiyama T, Bruemmer D. Transcriptional regulation of S phase kinase-associated protein 2 by NR4A orphan nuclear receptor NOR1 in vascular smooth muscle cells. J. Biol. Chem 2011;286:35485–35493. [PubMed: 21868379]
28. Knott GJ, Bond CS, Fox AH. The DBHS proteins SFPQ, NONO and PSPC1: a multipurpose molecular scaffold. Nucleic Acids Res 2016;44:3989–4004. [PubMed: 27084935]
29. Fox AH, Lam YW, Leung AK, Lyon CE, Andersen J, Mann M, Lamond AI. Paraspeckles: a novel nuclear domain. Curr. Biol 2002;12:13–25. [PubMed: 11790299]
30. Jiang L, Shao C, Wu QJ, Chen G, Zhou J, Yang B, Li H, Gou LT, Zhang Y, Wang Y, Yeo GW, Zhou Y, Fu XD. NEAT1 scaffolds RNA-binding proteins and the Microprocessor to globally enhance pri-miRNA processing. Nat. Struct. Mol. Biol 2017;24:816–824. [PubMed: 28846091]
31. Guzik TJ, Sadowski J, Guzik B, Jopek A, Kapelak B, Przybylowski P, Wierzbicki K, Korbut R, Harrison DG, Channon KM. Coronary artery superoxide production and nox isoform expression in human coronary artery disease. Arterioscler Thromb Vasc Biol 2006;26:333–339. [PubMed: 16293794]
32. Nguyen Dinh Cat A, Montezano AC, Burger D, Touyz RM. Angiotensin II, NADPH oxidase, and redox signaling in the vasculature. Antioxid. Redox Signal 2013;19:1110–1120. [PubMed: 22530599]
33. Dikalova A, Clempus R, Lassegue B, et al. Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. Circulation 2005;112:2668–2676. [PubMed: 16230485]
34. Mathur M, Tucker PW, Samuels HH. PSF is a novel corepressor that mediates its effect through Sin3A and the DNA binding domain of nuclear hormone receptors. Mol. Cell. Biol 2001;21:2298–2311. [PubMed: 11259580]
35. Shi L, Song L, Fitzgerald M, Maurer K, Bagashev A, Sullivan KE. Noncoding RNAs and LRRFIP1 regulate TNF expression. J. Immunol 2014;192:3057–3067. [PubMed: 24567534]
36. Suriano AR, Sanford AN, Kim N, Oh M, Kennedy S, Henderson MJ, Dietzmann K, Sullivan KE. GCF2/LRRFIP1 represses tumor necrosis factor alpha expression. Mol. Cell. Biol 2005;25:9073–9081. [PubMed: 16199883]
37. Khachigian LM, Santiago FS, Rafty LA, Chan OL, Delbridge GJ, Bobik A, Collins T, Johnson AC. GC factor 2 represses platelet-derived growth factor A-chain gene transcription and is itself induced by arterial injury. Circ Res 1999;84:1258–1267. [PubMed: 10364563]
38. Long Y, Wang X, Youmans DT, Cech TR. How do lncRNAs regulate transcription? Sci Adv 2017;3:eaao2110.
39. Das S, Reddy MA, Senapati P, Stapleton K, Lanting L, Wang M, Amaram V, Ganguly R, Zhang L, Devaraj S, Schones DE, Natarajan R. Diabetes Mellitus-Induced Long Noncoding RNA Dnm3os Regulates Macrophage Functions and Inflammation via Nuclear Mechanisms. Arterioscler Thromb Vasc Biol 2018
40. James PA, Oparil S, Carter BL, et al. 2014 evidence-based guideline for the management of high blood pressure in adults: report from the panel members appointed to the Eighth Joint National Committee (JNC 8). JAMA 2014;311:507–520. [PubMed: 24352797]

NOVELTY AND SIGNIFICANCE

What Is Known?

- Angiotensin II (AngII), a key player in hypertension and other cardiovascular diseases, promotes growth and inflammatory responses in vascular smooth muscle cells (VSMC).
- AngII exerts its pathological effects in VSMC via the AngII type 1 receptor and several downstream signaling pathways, transcription factors, and protein-coding genes.
- AngII also regulates microRNAs, long noncoding RNAs (lncRNAs), enhancers and super-enhancers in VSMC. These epigenetic regulators contribute to inflammatory and growth promoting effects of AngII.

What New Information Does This Article Contribute?

- AngII induces a novel lncRNA *Giver* in VSMC which negatively regulates its nearby protein coding gene *Nr4a3* by interacting with non-pou domain-containing octamer-binding protein (NONO).
- *Giver* also regulates inflammation, oxidative stress and proliferation in VSMC by altering the expression of key genes involved in these processes via epigenetic *trans* mechanisms.
- Human *GIVER* expression was increased *in vivo* in hypertensive patients and attenuated in hypertensive patients treated with Angiotensin Converting Enzymes Inhibitors or Angiotensin Receptor Blockers. Rat *Giver* and human *GIVER* showed functional similarities.

LncRNAs are emerging as novel epigenetic regulators of gene expression and disease states. Although recent studies show that AngII induces the expression of several lncRNAs in VSMC, their functions and mechanisms of action are unclear. Here, we characterized a novel AngII regulated lncRNA *Giver* in VSMC and examined the molecular mechanisms by which it promotes VSMC dysfunction. We found that *Giver* and its nearby nuclear receptor gene *Nr4a3* are coordinately altered by AngII both *in vitro* and *ex vivo,* and *Giver* is transcriptionally regulated by Nr4a3. Using gain- and loss-of-function approaches, we discovered that lncRNA *Giver* suppresses the expression of *Nr4a3* via negative feedback mechanisms, but increases the expression of oxidative and inflammatory genes involved in VSMC dysfunction via chromatin remodeling and epigenetic mechanisms. We found that *Giver* interacts with nuclear paraspeckles, splicing factor NONO and other corepressors to inhibit *Nr4a3* expression. Notably, we demonstrate that rat *Giver* shares functional conservation with the human *GIVER* ortholog in regulating VSMC dysfunction. Furthermore, we found *GIVER* levels are enhanced in arteries of hypertensive subjects, and attenuated in hypertensive patients treated with angiotensin receptor blockers. These results illustrate new lncRNA-*GIVER* dependent molecular mechanisms of AngII action that could serve as novel targets for anti-hypertensive therapy.

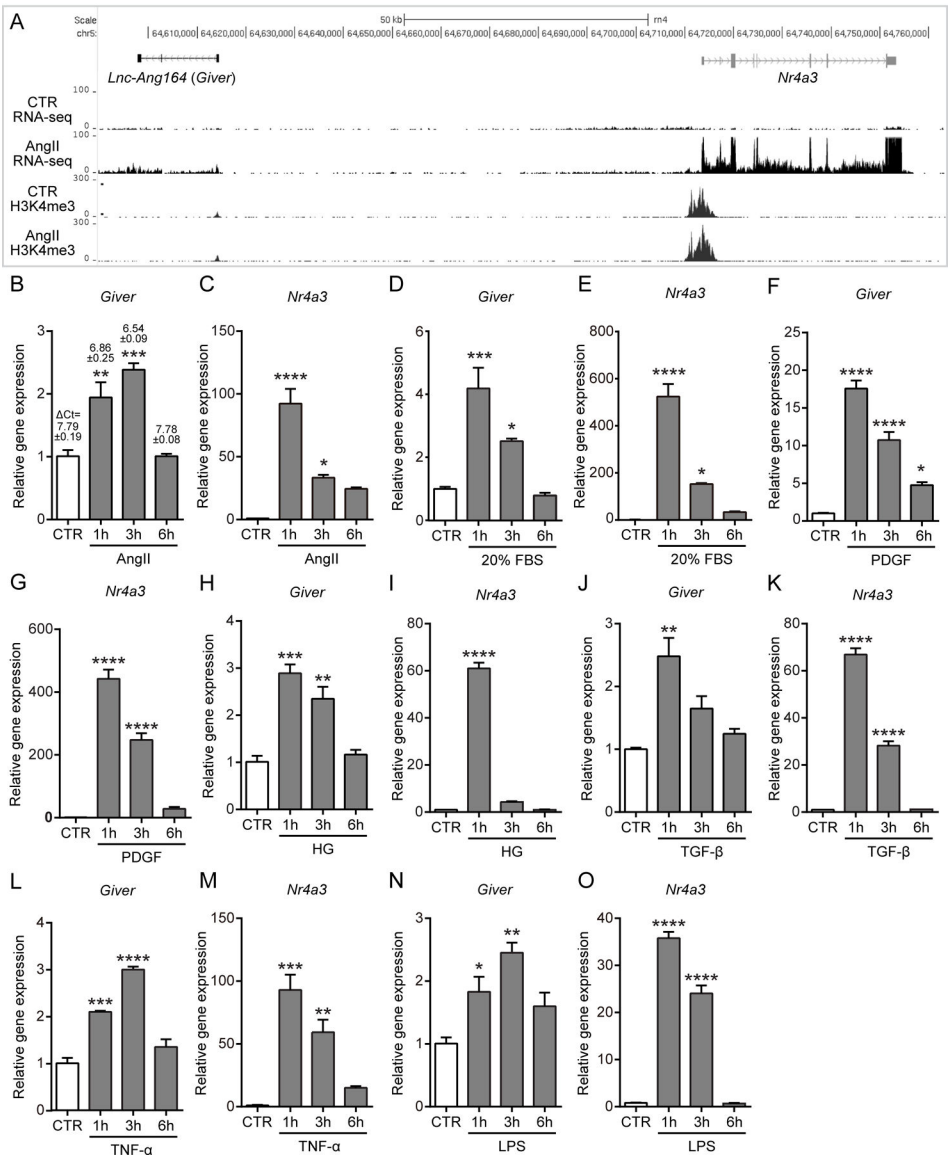

Figure 1. Rat *Giver* and *Nr4a3* expression is induced by AngII, mitogenic and pro-inflammatory factors in RVSMC.

(**A**), Normalized RNA-seq and H3K4me3 ChIP-seq tracks at *Lnc-Ang164* (*Giver)* and *Nr4a3* genomic loci. (**B-O**), Expression of *Giver* and *Nr4a3* in response to indicated treatments in RVSMC using RT-qPCR. Delta *Ct* (*Giver-Cypa*) values are shown for *Giver* expression (**B**). Mean+SEM; n=3; **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 using one-way ANOVA Dunnett's multiple comparison tests.

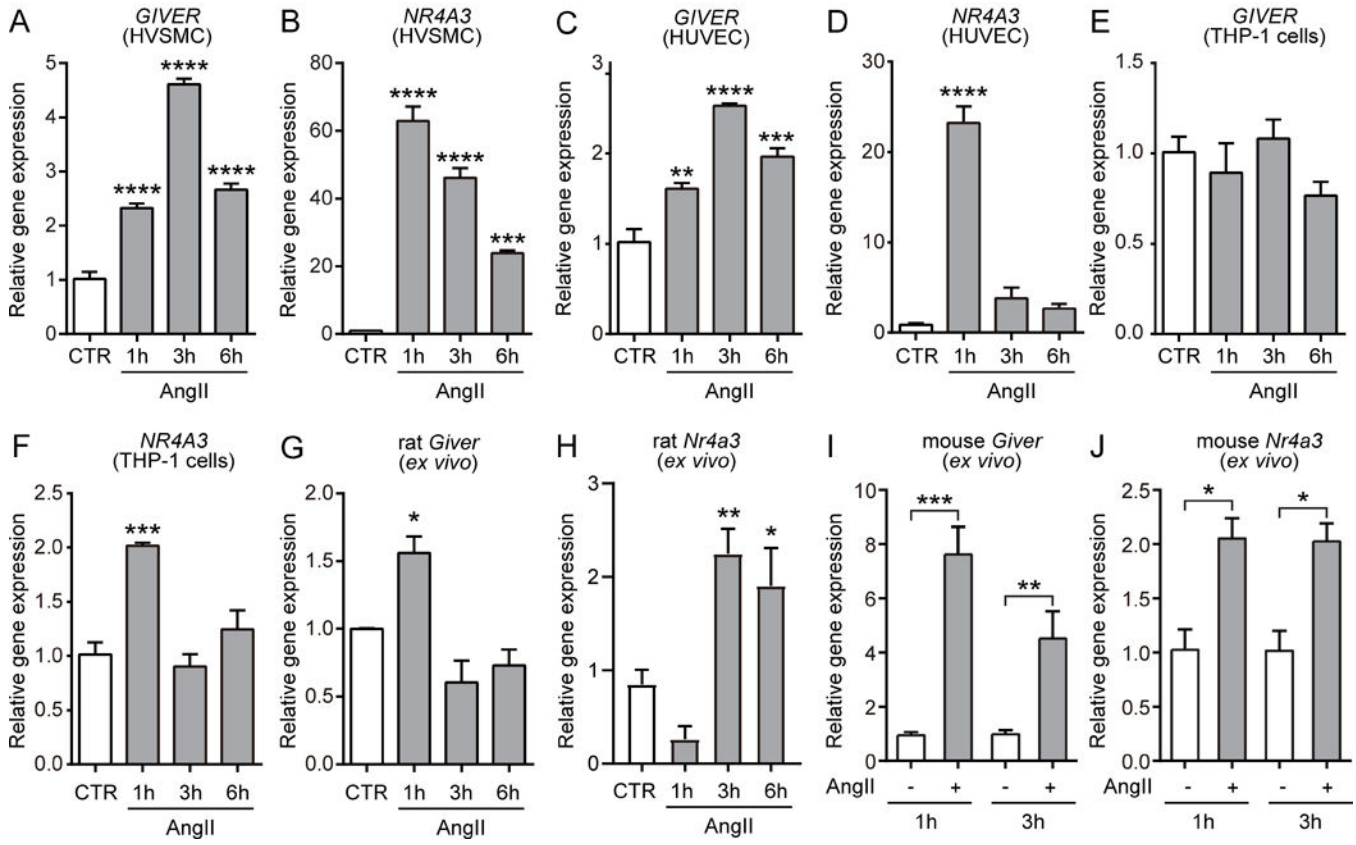

Figure 2. AngII-regulated *GIVER* expression in human cells (*in vitro*) and rodent aortas treated *ex vivo*.

(**A-J**), Gene expression of *GIVER* and *NR4A3* in response to AngII in HVSMC (**A-B**), HUVEC (**C-D**), THP-1 cells (**E-F**), and *ex vivo* in rat (**G-H**) and mouse aortas (**I-J**). Mean +SEM; n=3 (for **A-F**); n=5 rat aortas (for **G-H**); n=3–6 mouse aortas (for **I-J**); **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 using one-way ANOVA Dunnett's multiple comparison tests (for **A-H**) and unpaired two-tailed t-tests (for **I-J**).

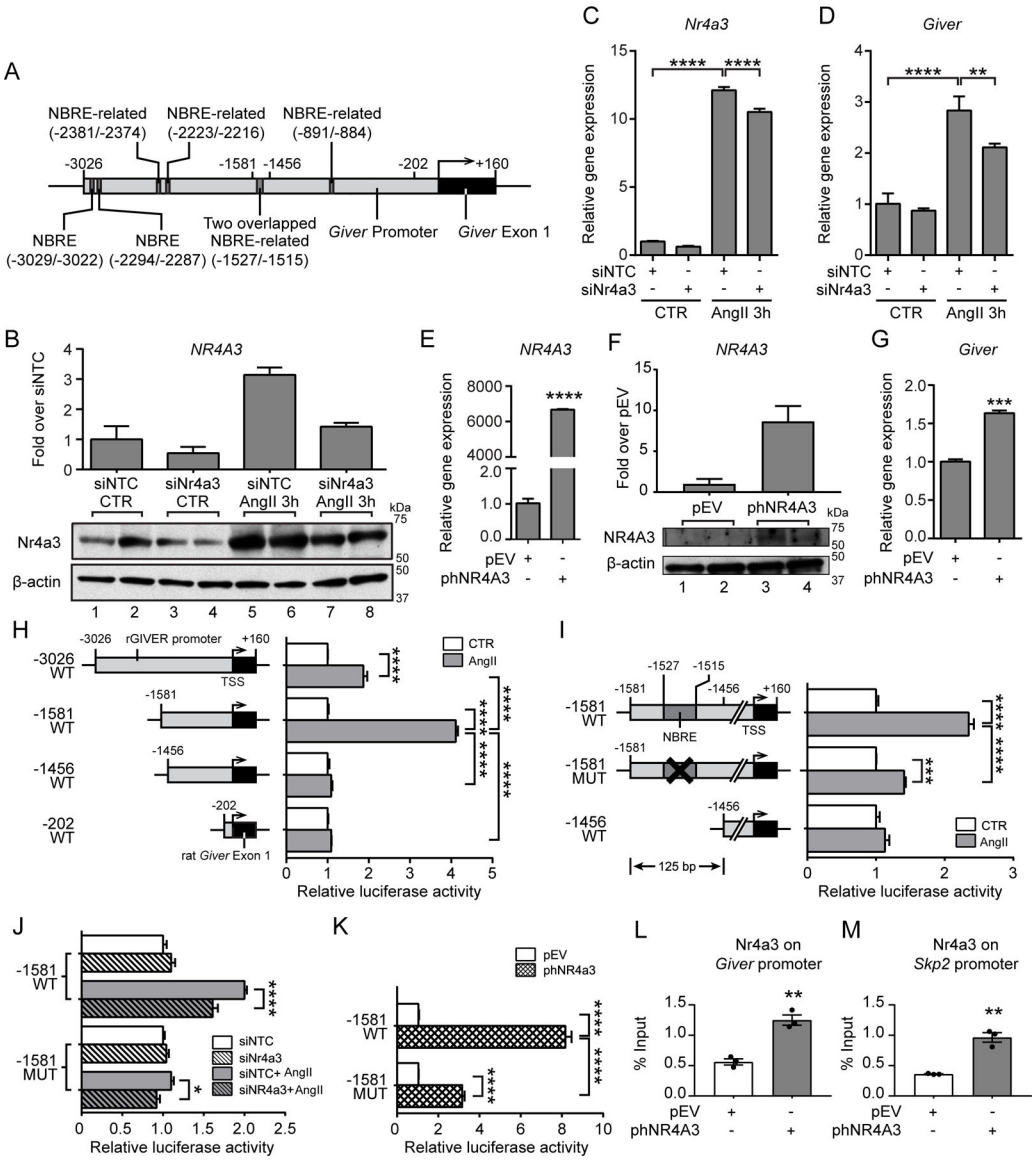

Figure 3. Nr4a3 regulates *Giver* by binding to NBRE-elements on the *Giver* promoter.

(**A**), Schematic diagram of NBRE and NBRE-related motifs relative to *Giver* TSS. (**B-D**), Nr4a3 protein levels quantified by western blot (**B**), and RNA levels of *Nr4a3* (**C**) and *Giver* (**D**) by RT-qPCR in RVSMC transfected with siRNA targeting NR4a3 (siNR4a3), or control (siNTC) and treated ± AngII for 3h. (**E-F**), Relative gene expression of human *NR4A3* (**E**) and its protein (**F**) in RVSMC transfected with human NR4A3 expression vector (phNR4a3) and empty vector (pEV). (**G**), RT-qPCR of *Giver* in RVSMC transfected with phNR4a3 and pEV. (**H-I**), Relative luciferase activity of wild type (WT) *Giver* promoter constructs (**H**), and NBRE-site deletion mutant (MUT) (**I**), treated ± AngII. (**J-K**), Relative luciferase activity of −1581 WT and −1581 MUT *Giver* promoter in RVSMC transfected with siNR4a3 or siNTC and treated ± AngII (**J**), and with phNR4a3 or pEV (**K**). (**L-M**), ChIP assays showing Nr4a3 enrichment at NBRE binding site on *Giver* (**L**) and *Skp2* (**M**) promoters in RVSMC transfected with phNR4A3 and pEV. Mean+SEM; n=2 (for **B** and **F**); n=3 (for **C-**

D, **E**, **G-H** and **L-M**); n=6 (for **I-J**; 3 biological replicates with 2 technical replicates for each); n=5 (for **K**; 3 biological replicates with 2 technical replicates for 2 of them); **P*<0.05; ***P*<0.005; ****P*<0.001; *****P*<0.0001 using one-way (for **C-D**), two-way ANOVA (for **H-K**) Tukey's multiple comparison tests, and unpaired two-tailed t-tests (for **E**, **G**, **L-M**).

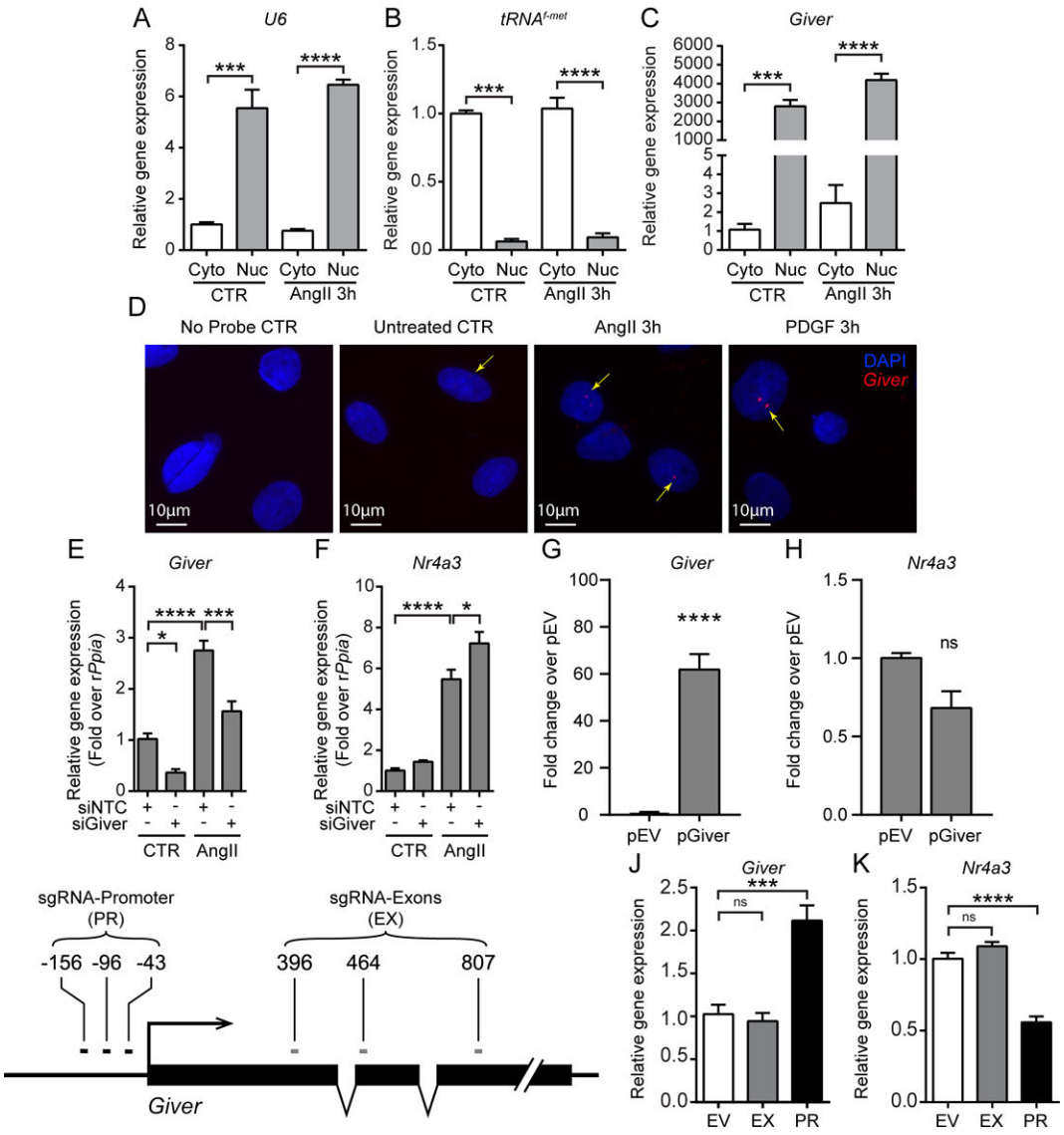

Figure 4. *Giver* is nuclear enriched and regulates *Nr4a3* expression through a negative feedback loop upon AngII signaling.

(**A-C**), RT-qPCR analysis of indicated RNAs from cytoplasmic (Cyto) and nuclear (Nuc) fractions from RVSMC treated ± AngII for 3h. (**D**), RNA-FISH analysis of *Giver* in RVSMC treated ± AngII or PDGF. Blue represents nuclear staining with DAPI. (**E-F**), RT-qPCR analysis of *Giver* (**E**), *Nr4a3* (**F**) after siNTC or siGiver transfection ± AngII treatment. (**G-H**), RT-qPCR analysis of *Giver* (**G**) and *Nr4a3* (**H**) 48h after transfection with empty vector (pEV) or rat *Giver* expression plasmid (pGiver). (**I**), Schematic depicting the location of sgRNAs targeting promoter (PR) and exons (EX) relative to *Giver* TSS for CRISPR-dCas9 mediated overexpression. (**J-K**), RT-qPCR analysis of *Giver* (**J**) and *Nr4a3* (**K**) 48h after transfection with pAC154-dual-dCas9VP160-sg expression vectors expressing indicated sgRNAs. Mean+SEM; n=3 (for **A-C**); n=6 (for **E-F** and **J-K**); n=5 (for **G-H**); *P<0.05; ***P<0.001; ****P<0.0001; using one-way ANOVA Tukey's (for **A-C** and **E-F**) and

Dunnett's (for **J**-**K**) multiple comparison tests, and unpaired two-tailed t-test (for **G**) and Mann-Whitney test (for **H**).

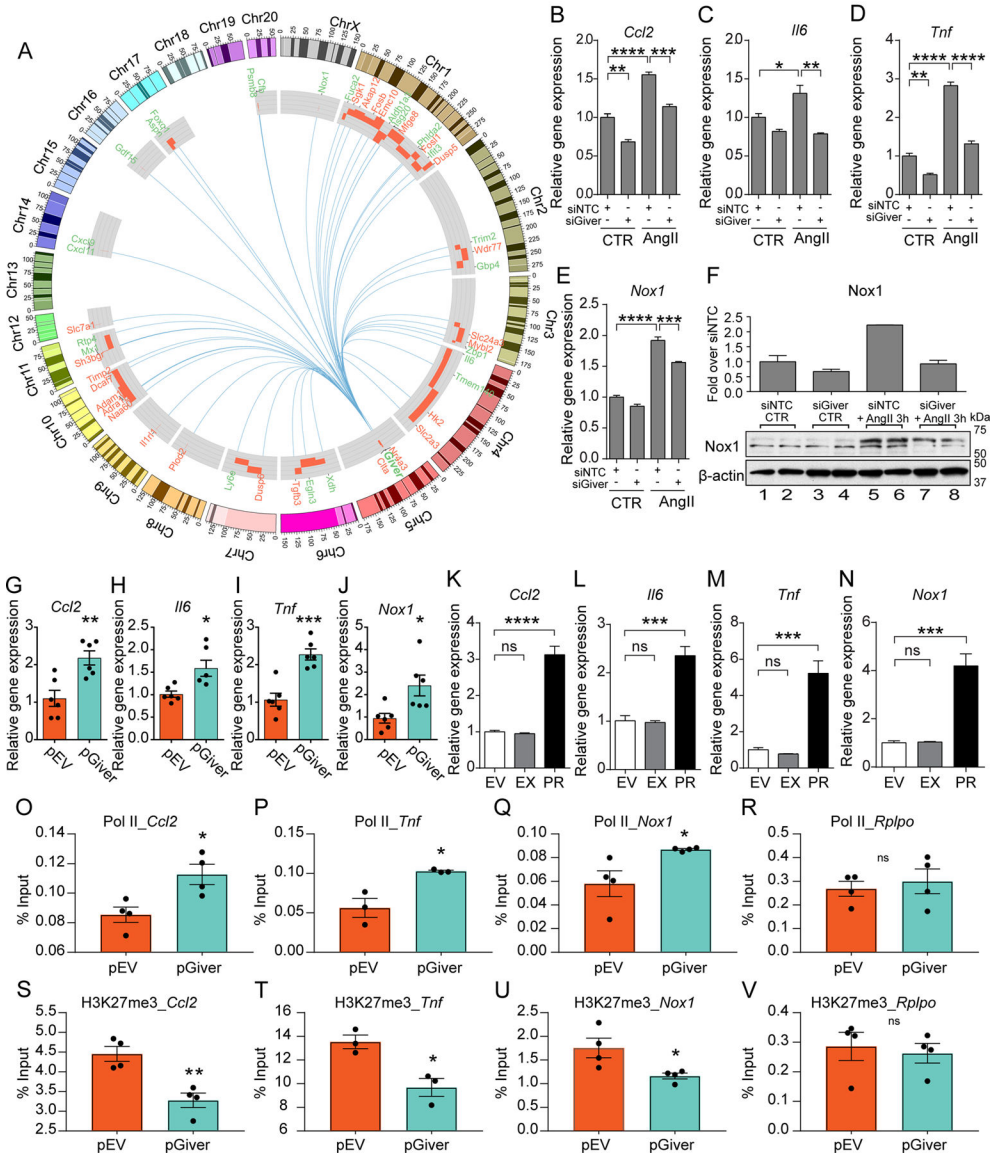

Figure 5. *Giver* regulates pro-inflammatory and oxidative stress genes by altering chromatin states.

(**A),** Circos plot showing genes either upregulated (red) or downregulated (green) after *Giver* knockdown in RVSMC. Upregulated and downregulated genes are connected to the *Giver* locus on chr5 by blue lines. Outer circle represents chromosome ideograms and inner circles show histograms (red color) representing log2FC of each gene. Only the top 25 downregulated and upregulated genes are shown. (**B-F**), RT-qPCR validation of indicated genes (**B-E**) and Nox1 protein levels quantified by western blot (**F**) in siNTC- or siGiver-transfected RVSMC treated ± AngII. (**G-J**), RT-qPCR validation of indicated genes in RVSMC transfected with *Giver* expression vector (pGiver) versus empty vector (pEV). (**K-N**), RT-qPCR validation of indicated genes in RVSMC transfected with pAC154-dual-dCas9VP160-sg vector without sgRNA (EV), or vectors expressing sgRNAs targeting *Giver* promoter (PR) or exons (EX). (**O-V**), Bar graphs represent Pol II and H3K27me3 enrichments on the *Ccl2* (**O** and **S**), *Tnf* (**P** and **T**), *Nox1* (**Q** and **U**), and *Rplpo* (control) (**R**

and **V**) promoters respectively in pEV and pGiver transfected RVSMC. Data represented as percent input. Mean+SEM; n=3 (for **B-E** and **K-N**); n=2 (for **F**); n= 6 (for **G-J**); n=3–4 (for **O-V**); **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 using one-way ANOVA Tukey's multiple comparison tests (for **B-E**) and Dunnett's multiple comparison tests (**K-N**) and unpaired two-tailed t-tests (for **G-J** and **O-V**).

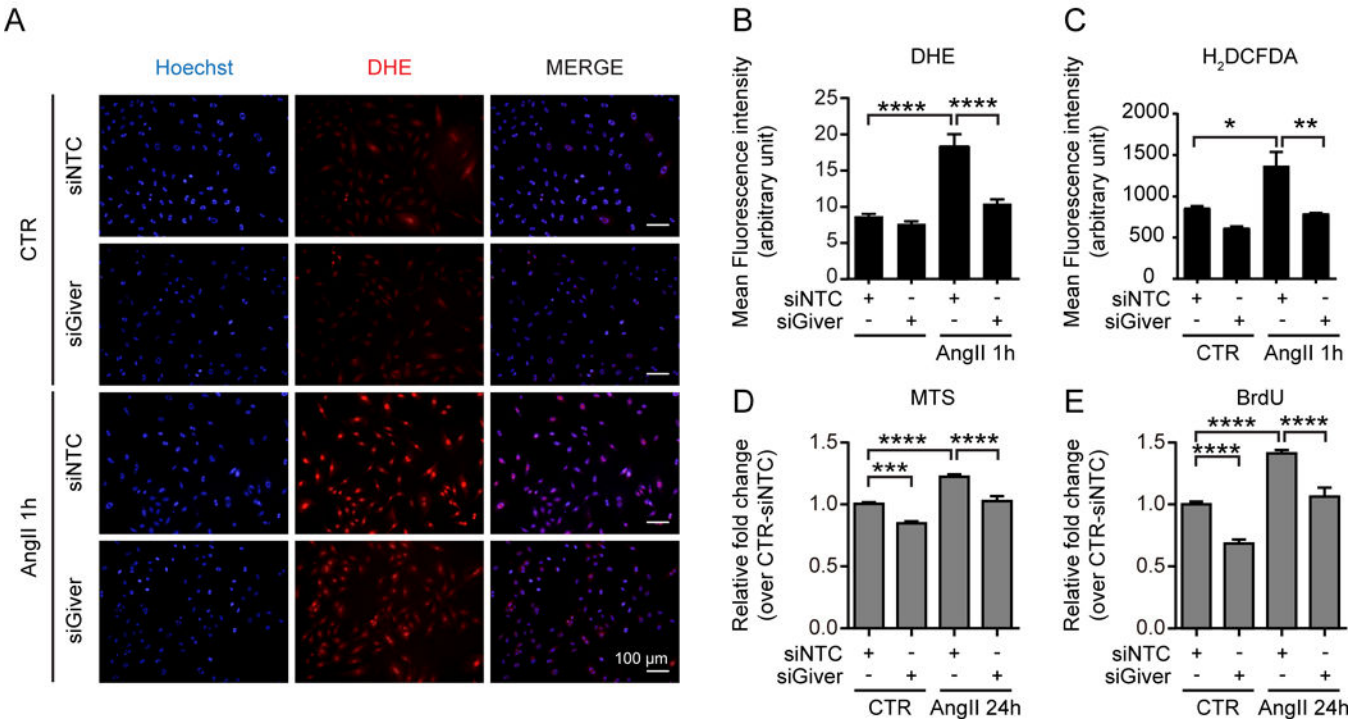

Figure 6. *Giver* knockdown inhibits AngII-induced oxidant stress and cell proliferation.

(**A**), Representative images of DHE staining in RVSMC with indicated treatments. Hoechst and DHE shown in blue and red respectively. (**B**), Quantification of DHE fluorescent signals using Image J Pro software. (**C**), Mean fluorescence intensity of H_2DCFDA staining quantified by flow cytometry. (**D-E**), Cell proliferation of RVSMC was quantified by MTS (**D**) and BrdU incorporation (**E**) assays. Mean+SEM; n=4–6 (for **A-B**); n=3 (for **C**); n=12 (for **D-E**); **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 using one-way ANOVA Tukey's multiple comparison tests.

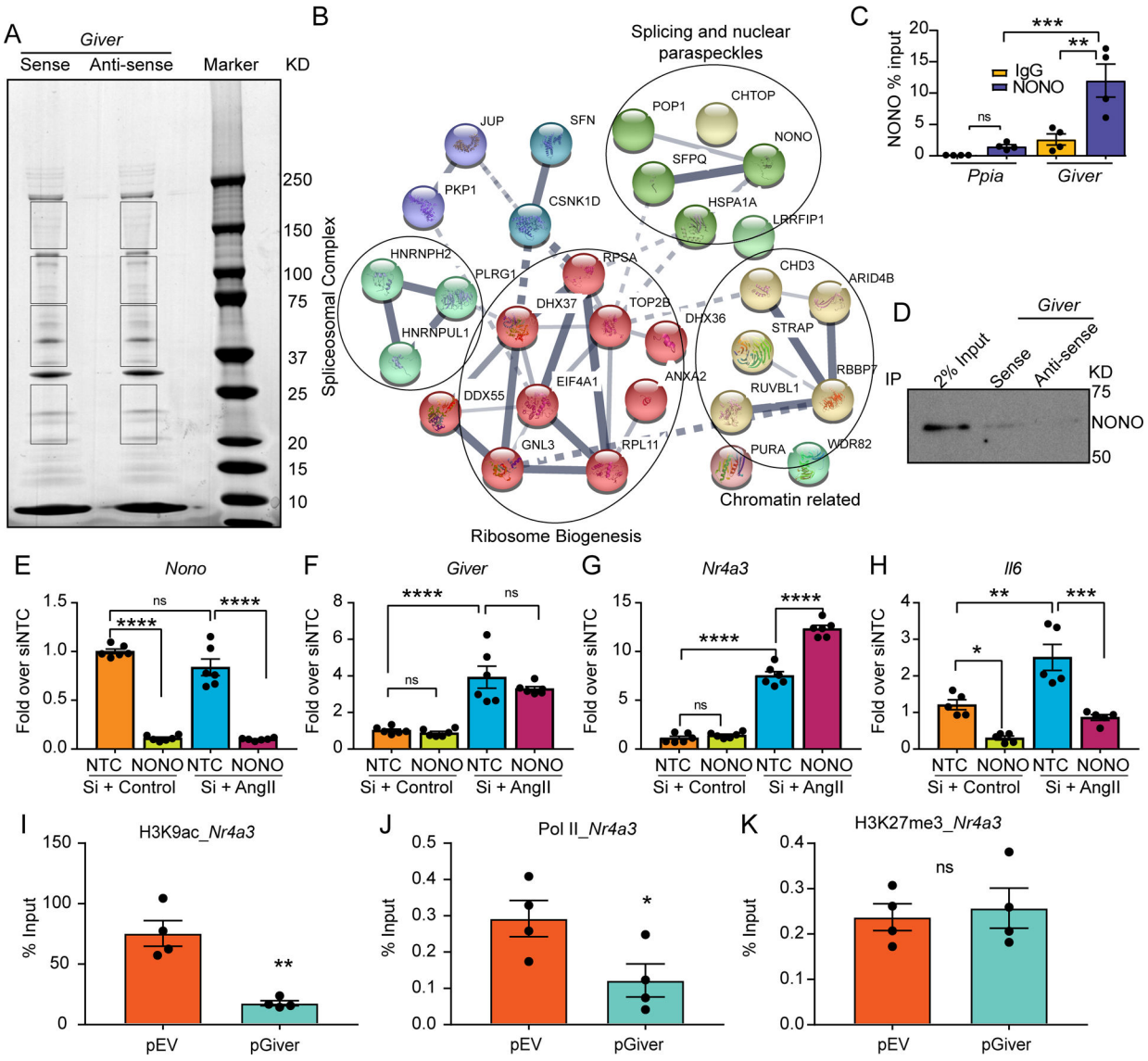

Figure 7. *Giver* interacts with nuclear proteins in RVSMC.

(**A**), Gel image showing proteins pulled down by biotinylated *Giver* sense and anti-sense RNA from RVSMC nuclear lysates. Box marked regions subjected to mass-spectrometry analysis. (**B**), Network generated using STRING database from *Giver* sense-strand-specific interacting proteins identified by mass-spectrometry. Human protein symbols for the indicated proteins were used to generate the network. Major enriched complexes are highlighted by circles. Solid lines indicate interactions within each complex. Thickness of the lines indicate confidence of interaction from literature. (**C**), NONO was immunoprecipitated from UV-crosslinked RVSMC nuclear lysates, and associated RNAs detected by RT-qPCR. (**D**), RNA pulldown followed by western blot with NONO antibody. (**E-H**), Bar graphs represent expression of indicated genes after siRNA-mediated depletion of NONO followed by AngII treatment in RVSMC. (**I-K**), Bar graphs represent H3K9ac, Pol II and H3K27me3 enrichment respectively on the *Nr4a3* promoter in pEV and pGiver

transfected RVSMC. Data represented as percent input. Mean+SEM; n=4 (for **C** and **I-K**); n=5–6 (for **E-H**); **P*<0.05, ***P*<0.01, ***P<0.001, *****P*<0.0001 using one-way ANOVA Tukey's multiple comparison tests (for **C** and **E-H**) and unpaired two-tailed t-tests (for **I-K**).

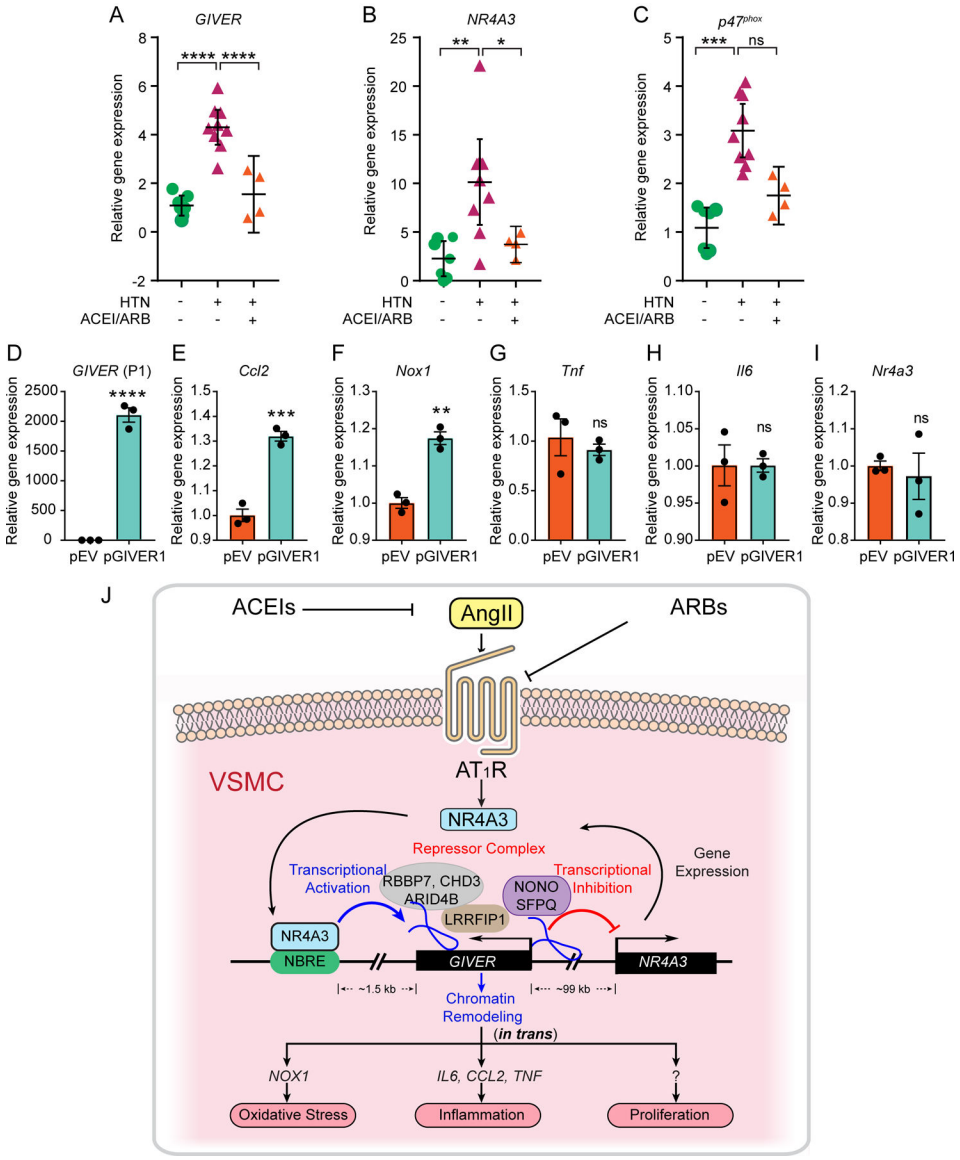

Figure 8. Functional role of h*GIVER* in VSMC and in AngII-mediated hypertension. The *Giver*-Nr4a3 axis in VSMC dysfunction.

(**A-C**), *GIVER, NR4A3* and *p47phox* are differentially expressed in arteries collected from hypertensive patients ± ACEI or ARB therapy. RNAs isolated from mammary artery trimmed residuals of patients undergoing CABG were subjected to RT-qPCRs to detect indicated genes. Mean with 95% CI; n=7 for non-hypertensive subjects, n=9 for hypertensive patients not on ACEI or ARB therapy, and n=4 for hypertensive patients on ACEIs or ARBs. (**D-I**), RT-qPCR validation of indicated genes in RVSMC transfected with human *GIVER1* expression vector (pGIVER1) versus empty vector (pEV). Human *GIVER1* transcript was characterized as described in Online Figure XIII. Mean±SEM; n=3 (for **D-I**); **P*<0.05; ***P*<0.01, ****P*<0.001, *****P*<0.0001 using one-way ANOVA Tukey's (for **A-B**) and Dunn's (for **C**) multiple comparison tests; and unpaired two-tailed t-tests (for **D-I**). (**J**), Schematic of the role of *Giver* in AngII-induced VSMC dysfunction. Activation of AT_1R by AngII induces *NR4A3* in VSMC. NR4A3 induces *GIVER* via NBRE-element in its

promoter. *GIVER* in turn inhibits *NR4A3* indicating a negative feedback mechanism, but increases oxidative stress and inflammatory genes associated with VSMC dysfunction. *Giver* binding partners NONO as well as LRRFIP1, CHD3, RBBP7 and ARID4B might be involved in *Giver*-mediated repression of *Nr4a3*. On the other hand, *GIVER* increases *NOX1* and inflammatory genes (likely via protein binding factors, Pol II recruitment, and histone modifications involved in chromatin remodeling and consequent chromatin relaxation) to promote oxidative stress, VSMC proliferation and inflammatory responses.