



King's Research Portal

DOI:

[10.1161/CIRCRESAHA.117.311450](https://doi.org/10.1161/CIRCRESAHA.117.311450)

Document Version

Peer reviewed version

[Link to publication record in King's Research Portal](#)

Citation for published version (APA):

Zhao, G., Fu, Y., Cai, Z., Yu, F., Gong, Z., Dai, R., Hu, Y., Zeng, L., Xu, Q., & Kong, W. (2017). Unspliced XBP1 Confers VSMC Homeostasis and Prevents Aortic Aneurysm Formation via FoxO4 Interaction. *Circulation Research*, 121(12), 1331-1345. <https://doi.org/10.1161/CIRCRESAHA.117.311450>

Citing this paper

Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights

Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Research Portal

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Unspliced XBP1 Confers VSMC Homeostasis and Prevents Aortic Aneurysm Formation via FoxO4 Interaction

Guizhen Zhao¹, Yi Fu¹, Zeyu Cai¹, Fang Yu¹, Ze Gong¹, Rongbo Dai¹, Yanhua Hu², Lingfang Zeng², Qingbo Xu^{2,‡}, Wei Kong^{1,‡}

Running title: XBP1u Maintains VSMC Homeostasis

¹Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University; Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Beijing, 100191, P. R. China.

²Cardiovascular Division, King's College London BHF Centre, United Kingdom.

Address Correspondence to:

Dr. Wei Kong

Department of Physiology and Pathophysiology, Peking University Health Science Center

38 Xueyuan Rd, Haidian District, Beijing 100191, P.R. China

kongw@bjmu.edu.cn.

Or Dr. Qingbo Xu

Cardiovascular Division, King's College London BHF Centre

125 Coldharbour Lane, London SE5 9NU, United Kingdom

qingbo.xu@kcl.ac.uk

Abstract

Rationale: Although not fully understood, the phenotypic transition of vascular smooth muscle cells exhibits at the early onset of the pathology of aortic aneurysms. Exploring the key regulators that are responsible for maintaining the contractile phenotype of VSMCs may confer vascular homeostasis and prevent aneurysmal disease. X-box binding protein 1, which exists in a transcriptionally inactive unspliced form (XBP1u) and a spliced active form (XBP1s), is a key component in response to endoplasmic reticular (ER) stress. Compared to XBP1s, little is known about the role of XBP1u in vascular homeostasis and disease.

Objective: We aim to investigate the role of XBP1u in VSMC phenotypic switching and the pathogenesis of aortic aneurysms.

Methods and Results: XBP1u, but not XBP1s, was markedly repressed in the aorta during the early onset of aortic aneurysm in both angiotensin II-infused ApoE^{-/-} and CaPO₄-induced C57BL/6J murine models, in parallel with a decrease in SMC contractile apparatus proteins. *In vivo* studies revealed that XBP1 deficiency in SMCs caused VSMC dedifferentiation, enhanced vascular inflammation and proteolytic activity, and significantly aggravated both thoracic and abdominal aortic aneurysms in mice. XBP1 deficiency, but not an inhibition of XBP1 splicing, induced VSMC switching from the contractile phenotype to a proinflammatory and proteolytic phenotype. Mechanically, in the cytoplasm, XBP1u directly associated with the N-terminus of FoxO4, a recognized repressor of VSMC differentiation via the interaction and inhibition of myocardin. Blocking the XBP1u-FoxO4 interaction facilitated nuclear translocation of FoxO4, repressed SMC marker genes expression, promoted proinflammatory and proteolytic phenotypic transitioning *in vitro* and stimulated aortic aneurysm formation *in vivo*.

Conclusions: Our study revealed the pivotal role of the XBP1u-FoxO4-myocardin axis in maintaining the VSMC contractile phenotype and providing protection from aortic aneurysm formation.

Key words: XBP1u, VSMC phenotypic switching, aortic aneurysm, FoxO4

Non-standard Abbreviations and Acronyms:

XBP1	X-box binding protein 1
VSMCs	vascular smooth muscle cells
FoxO	Forkhead box protein O
TA	thoracic aorta
AA	abdominal aorta
SM22 α	smooth muscle 22 alpha
ApoE	apolipoprotein E
XBP1 ^{SMKO}	smooth muscle cell-specific XBP1 knockout
AngII	angiotension II
siRNA	small interfering RNA
MRTF	myocardin-related transcription factor
DN	dominant negative
Ad	adenovirus

Introduction

Aortic aneurysms, including thoracic aortic aneurysm (TAA) and abdominal aortic aneurysm (AAA), are life-threatening cardiovascular events that are characterized by a permanent dilatation of the aorta and have an extremely high mortality rate upon rupture. TAA and AAA differ in anatomic location of aortic dilatation, risk factors, genetic polymorphisms and histological characters.^{1, 2} For example, AAA represents strong positive association with aging, male gender, smoking and dyslipidemia.³ TAA is less common, can occur in all age groups, including young children, and is often associated with genetic risk factors and Mendelian syndromes affecting the connective tissue. Most recent genetic studies show no genetic overlap between AAA and TAA.¹ TAA exhibits strong correlation with genetic polymorphisms such as mutations in fibrillin-1 (*FBNI*)⁴, smooth muscle actin alpha 2 (*ACTA2*)⁵, smooth muscle myosin heavy chain 11 (*MYH11*)⁶, and TGF- β receptor II (*TGFBR2*)⁷, etc, whereas GWAS studies identified several susceptible loci for AAA including ANRIL, DAB2 interacting protein, and LRP1 (low-density lipoprotein receptor-related protein 1) etc.^{8, 9} Moreover, thoracic and abdominal aortas are also structurally different from one another and differ in embryology of VSMCs origin.¹⁰ The latter has been suggested to possibly contribute to the different pathogenesis of TAA and AAA.² Although recognized as distinct disease entities, both TAA and AAA are pathologically characterized by vascular extracellular matrix destruction/remodeling and a loss of vascular smooth muscle cell (VSMC) contractile function.^{11, 12} Although not fully understood, VSMCs are highly phenotypically plastic, and the loss of the contractile phenotype of VSMCs has been recognized as an early event in both thoracic and abdominal aortic aneurysms in a variety of animal models and human aneurysmal tissues.^{13, 14} However, the molecular linker connecting VSMC phenotypic transition and aortic aneurysm remains elusive.

X-box binding protein 1 (XBP1) is a key transcription factor controlling the ER-stress response and is crucial for cell survival under stress conditions.^{15, 16} There are three pathways in the ER-stress response: the PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 alpha (*IRE1 α*) pathways. Upon sensing the accumulation of unfolded proteins, *IRE1 α* cleaves unspliced XBP1 (XBP1u) mRNA and removes a 26-nt-long intron, resulting in the production of a spliced XBP1 (XBP1s).¹⁷ XBP1u and XBP1s have an identical N-terminal dimerization domain and internal DNA binding domain but differ in their C-termini.¹⁷ XBP1s then translocates to the nucleus and transcriptionally regulates genes that are involved in protein folding, glycosylation, ER-associated degradation (ERAD), autophagy, lipid biogenesis, and insulin secretion, among others.^{18, 19, 16} Compelling evidence has suggested an essential role of XBP1 splicing in angiogenesis²⁰, endothelial proliferation and apoptosis^{20, 21} and in VSMCs migration²² during the disease states of atherosclerosis and post-injury neointima formation. However, little is known regarding the physiological function of XBP1u. Previous studies have indicated that XBP1u counteracts XBP1s by shuttling between the nucleus and the cytoplasm and sequestering XBP1s for proteasomal degradation.²³ A recent study has revealed a role for XBP1u in controlling autophagy by recruiting FoxO1 to the 20S proteasome.²⁴ Intriguingly, global knockout of XBP1 causes embryonic lethality at E13.5 and leads to reduced vessel formation, thus indicating a pivotal role for XBP1 in vascular differentiation/development and function.²⁰ Previous research has indicated that XBP1u protects endothelial cells from disturbed flow-induced oxidative stress through interaction with histone deacetylase 3 in the cytosol.²⁵ In the current study, we sought to clarify

whether XBP1u is also involved in VSMC homeostasis and therefore modulates aortic aneurysm formation.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Materials and Methods

AngII-Induced Aortic Aneurysm in ApoE^{-/-} Mice

Sixteen-week-old XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} male mice were infused with AngII (1000 ng/kg/min, Sigma Aldrich Inc) via subcutaneous osmotic minipumps (Model 2004, ALZA Scientific Products) as described previously.^{26, 27} Systolic blood pressure was measured before and after AngII-infusion in conscious mice using the noninvasive tail-cuff BP-2000 system (Visitech Systems Inc., NC, USA). Aortic diameters were obtained by *in vivo* measurements using the Vevo 770 ultrasound system (Visualsonics, Toronto, Canada). In addition to *in vivo* measurements, aortas from saline- or AngII-infused mice were dissected following sacrifice and perfusion with 4% paraformaldehyde to measure the aortic diameters *ex vivo*. Aortic aneurysm in this model is mainly localized to the suprarenal abdominal aorta (distal to the renal arteries), followed by the thoracic aorta (ascending aorta and descending aorta) and defined as a localized dilation of aorta greater than 50% of its adjacent intact portion of aorta.^{28, 29} Pathological insights include elastin fragmentation, inflammatory cells infiltration particularly in the adventitia, and plasma inflammatory cytokines increase.^{11, 12}

Luciferase Reporter Assays

HEK293A, COS-7 cells or human VSMCs (T/G HA VSMC) were transiently co-transfected with luciferase reporter and β -galactosidase plasmids using Jet-PEI reagent according to the manufacturer's protocol. One day later, transfected cells were harvested for luciferase activity measurement using a luciferase assay kit (Promega, Madison, WI, USA) and β -galactosidase activity detection using the OPNG reaction (Promega, Madison, WI, USA). The transcriptional activity was presented as the ratio of luciferase activity to respective β -galactosidase activity.

Recombinant Adenovirus Construction and Infection in Mice

A murine FoxO4 aa 1-89 dominant negative fragment (FoxO4-DN) was subcloned into an adenovirus vector pDC316-mCMV-EGFP. Subsequently, the recombinant adenovirus plasmids pDC316-mCMV-FoxO4-DN-EGFP were co-transfected with pAV.Des1d vector into HEK293A cells to yield the final expression clone Ad-FoxO4-EGFP. Ad-FoxO4-EGFP was amplified by infecting HEK293A cells and purified by PD-10 Sephadex precipitation. An adenovirus vector carrying green fluorescence protein (Ad-EGFP) was applied as negative control. For *in vivo* studies, 1×10^9 pfu of adenovirus dissolved in 30% pluronic gel solution were perivascularly delivered to the suprarenal aortas as described previously.³⁰ The EGFP fluorescence signal was monitored by confocal laser scanning microscopy to demonstrate the infection of adenovirus to the medians of the aortas.

Statistical Analysis

All data are expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad prism 6.0 software (GraphPad Software, San Diego, CA, USA). For statistical comparisons, whether data were normally distributed was first evaluated. Then, a check was made for similar variances among normally distributed data, followed by Student's *t*-test for two-group comparisons and ANOVA for comparisons if evaluations of similar variances were passed. Non-parametric tests were used where data were not normally distributed. In all cases, statistical significance was concluded where the two-tailed probability was less than 0.05. The unpaired two-tailed Student's *t*-test was applied to compare the leukocyte infiltration, macrophage infiltration and cytokines in plasma between XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice after AngII infusion. The Chi-square test was used to compare the incidence of AAA and the percentage of phenotype transition SMCs after XBP1 knockdown or overexpression. Kaplan-Meier survival curve was used to analyze the survival percentage of saline- or AngII-infused mice. Nonparametric Kruskal-Wallis test with a Dunn's post-hoc test was applied for comparison of the elastin degradation grade between XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice after saline or AngII infusion. The Paired *t*-test (two-tailed) was performed for the relative gene-silencing efficiencies of the short interfering RNA (siRNA) on XBP1, IRE1 α and FoxO4. One-way ANOVA followed by Student-Newman-Keuls test for post hoc comparison was applied for the effects of FBS, TGF- β or AngII on XBP1 expression, XBP1 siRNA or XBP1 plasmid on SMC differentiation markers and transcription factor expression, as well as luciferase activity. Two-way ANOVA followed by the Bonferroni test was applied for comparisons of the expression of XBP1, SMC differentiation markers and ER-stress markers (GRP78, ATF-6 and p-eIF2 α) in the aorta between aneurysmal and nonaneurysmal tissues and the aortic diameter between XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice after saline or AngII infusion, as well as the effect of XBP and IRE1 α silencing on MCP-1 and IL-6 expression and secretion, MMP-2 and MMP-9 expression, and macrophage migration in the absence or presence of AngII.

Results

Unspliced XBP1 Reduction Correlates with VSMC Dedifferentiation During Early Stage of Aortic Aneurysm Formation

Systemic infusion of angiotensin II (AngII) via osmotic mini-pumps into ApoE^{-/-} mice for 28 days has been repeatedly demonstrated to cause aneurysmal pathology in both thoracic and abdominal aortas (Online Figure IA-C and Online Table I)^{26, 31, 12}. Therefore, we first compared the aortic expression levels of spliced and unspliced XBP1 as well as contractile apparatus proteins at early stages (3 and 7 days) in AngII-infused ApoE^{-/-} mice. As expected, VSMC-specific smooth muscle α -actin (SMA), calponin and SM22 α were significantly decreased as early as 3 days post infusion; the reduction persisted through 7 days (Figure 1A). Intriguingly, the protein level of XBP1u but not XBP1s was similarly reduced with the contractile proteins in both thoracic (including ascending and descending aortas) and abdominal aortas (Figure 1A). To further confirm these observations, we used another CaPO₄-induced AAA model in C57BL/6J mice (Online Figure ID-E). Similarly, XBP1u but not XBP1s was repressed in the abdominal aorta during different stages of aneurysm formation (3, 5 and 7 days), which is consistent with VSMC dedifferentiation (Online Figure IF). Immunohistochemical analysis further confirmed great reductions of XBP1u and VSMC-specific SM22 α in the medium tunica of both thoracic (including ascending and descending aortas) and abdominal aortic aneurysmal vessel wall in 7-day consecutively AngII-infused ApoE^{-/-} mice (Figure 1B), whereas no positive staining was detected by the species-matched IgG (data not shown). To determine the cellular localization of XBP1u, confocal fluorescence microscopy was performed on normal aortic tissue. XBP1u colocalized primarily in the medial SMCs (Figure 1C). Of interest, 10% FBS, which causes VSMCs dedifferentiation, significantly inhibited, whereas TGF- β (5 ng/ml) stimulation, which causes VSMCs differentiation, greatly upregulated, XBP1u expression in VSMCs (Online Figure IG). Taken together, XBP1u reduction may be involved in the phenotypic transition of VSMCs during the pathogenesis of aortic aneurysm.

In addition, to assess whether ER-stress response is activated at early stage of aortic aneurysms formation, the expression of ER-stress molecules including GRP78, ATF6, p-eIF2 α and XBP1s was detected by western blot (Online Figure IF, H-I and Figure 1A). Consistent with previous studies^{32, 33}, GRP78 and p-eIF2 α were increased and total ATF-6 was reduced in both AngII and CaPO₄ induced aneurysmal mice (Online Figure IH-I). In contrast, XBP1s was not altered whereas XBP1u was repressed at early stage of aneurysmal aortas (Figure 1A and Online Figure IF). Therefore, XBP1u reduction but not XBP1s-mediated ER-stress at early stage may be involved in aneurysm formation.

To ask why XBP1u was repressed during aortic aneurysms development, we further evaluated the regulatory mechanism of XBP1u. Firstly, real-time PCR results indicated that XBP1u mRNA was significantly decreased in AngII-infused thoracic and abdominal aortas (Online Figure IIA). As well, both XBP1u mRNA and protein levels were greatly downregulated in response to AngII *in vitro* (Online Figure IIB-C). Next, we found AngII did not transcriptionally affect XBP1u whereas ATF6³⁴ did as evidenced by luciferase reporter assay (Online Figure IID). Next, we applied actinomycin D (ActD) to test whether XBP1u was post-transcriptionally regulated by inhibition *de novo* synthesis of mRNA in VSMCs. Intriguingly, the half-life of XBP1u mRNA was markedly

reduced in response to AngII ($t_{1/2}$ from 90.3 min to 51.9 min) (Online Figure IIE), indicating that AngII may regulate XBP1u expression via destabilizing mRNA. Moreover, we applied cycloheximide (CHX) and MG-132 to inhibit *de novo* protein synthesis and proteasomal degradation respectively. As shown in Online Figure IIF-G, XBP1u protein level was not affected by CHX and MG132. Together, destabilization of XBP1u mRNA in VSMCs may account for the XBP1u reduction during aortic aneurysm development.

XBP1 Deficiency in VSMCs Causes Smooth Muscle Cell Dedifferentiation and Aggravates Aortic Aneurysms *in vivo*

Next, we investigated whether XBP1u reduction causes VSMC phenotypic transition and aortic aneurysm. At 4 months of age, the contractile apparatus proteins were greatly reduced in both thoracic (including ascending and descending aortas) and abdominal aortas in *SM22-Cre^{+/-}-XBP1^{loxp/loxp}* (XBP1^{SMKO}) mice compared to littermate *SM22-Cre^{+/-}-XBP1^{loxp/loxp}* (XBP1^{WT}) mice, as shown by western blot analysis and immunohistochemical staining (Online Figure IIIA-C), suggesting that XBP1 deficiency may facilitate VSMC dedifferentiation. In addition, the aortic rings from XBP1^{SMKO} and XBP1^{WT} mice were treated *ex vivo* in the absence or presence of AngII (1 μ M). The secretion of the proinflammatory cytokines MCP-1 and IL-6 and the protease activity of MMP-2 and MMP-9, which have been repeatedly demonstrated to contribute to the pathogenesis of aortic aneurysm,^{35, 36} were significantly elevated in the conditioned media from XBP1^{SMKO} aortic rings compared to XBP1^{WT} aortic rings (Online Figure IIID-E), suggesting that XBP1 deficiency may facilitate VSMC transition toward an inflammatory and proteolytic phenotype. Thus, the effect of XBP1 deficiency in the development of aortic aneurysm was determined by crossing VSMC conditional XBP1^{SMKO} with hyperlipidemic ApoE^{-/-} mice. After AngII (1000 ng/kg/min) infusion for 4 weeks, systolic blood pressure increased similarly in XBP1^{SMKO}ApoE^{-/-} and XBP1^{WT}ApoE^{-/-} mice, but no obvious difference was observed in body weight, plasma lipids or survival rates between the groups of mice (Online Table II and Online Figure IVA). These data suggest that the effects of SMC-derived XBP1 deficiency were independent on arterial hypertension elevated by AngII or plasma lipids. In the absence of AngII infusion, no mice developed spontaneous aortic dilations and aneurysms (Figure 2A-E). Nevertheless, in the presence of AngII, XBP1^{SMKO}ApoE^{-/-} mice exhibited a significant increase in maximal aortic diameter compared to XBP1^{WT}ApoE^{-/-} mice in the thoracic aortas (aortic root, ascending, and descending) by both ultrasound and *ex vivo* measurements (Online Figure IVB and Figure 2B). As well, XBP1 deficiency in VSMCs enhanced the AAA formation, and the incidence of abdominal aortic aneurysm at 28 days was markedly increased, from 55.55% in AngII-infused XBP1^{WT}ApoE^{-/-} mice compared to 100% in AngII-infused XBP1^{SMKO}ApoE^{-/-} mice ($P=0.0177$) (Figure 2C-E). Furthermore, AngII-infused XBP1^{SMKO}ApoE^{-/-} mice had significant increases in maximal aortic diameter compared with control mice (XBP1^{SMKO}ApoE^{-/-} + AngII, 2.50 ± 0.21 mm vs. XBP1^{WT}ApoE^{-/-} + AngII, 1.79 ± 0.22 mm; $P=0.0343$; Figure 2E). Similarly, further elastic Van Gieson (EVG) staining demonstrated dramatically increased elastin fragmentation in AngII-infused XBP1-deficient mice in both thoracic (including ascending and descending aortas) and abdominal aortas (Online Figure IVC). Moreover, leukocyte (CD45⁺) and macrophage (CD68⁺) infiltration were dramatically enhanced in XBP1^{SMKO}ApoE^{-/-} mice infused with AngII compared to AngII-infused XBP1^{WT}ApoE^{-/-} mice in the abdominal aorta (Online Figure IVD). In parallel, the upregulated proinflammatory cytokines MCP-1 and IL-6 were

observed in plasma (Online Figure IVE). Consistently, the aortas from XBP1^{SMKO}ApoE^{-/-} mice infused with AngII displayed a marked upregulation of MMP-2 and MMP-9 protein expression in both thoracic and abdominal aortas (Online Figure IVF). Meanwhile, no positive staining was detected by the species-matched IgG (data not shown). To further verify the role of XBP1 deletion in the pathogenesis of aortic aneurysm, we used a CaPO₄-induced AAA model in XBP1^{SMKO} and XBP1^{WT} mice. Consistent with the previous results, VSMC-specific XBP1 deficiency facilitated CaPO₄-induced abdominal aortic aneurysm formation (Online Figure IVG-H). Taken together, XBP1 deficiency in VSMCs causes VSMC dedifferentiation towards a proinflammatory phenotype and aggravates aortic aneurysms *in vivo*.

Unspliced XBP1 Maintains the Contractile Phenotype of VSMCs

To explore the definitive alteration of VSMCs due to XBP1 deficiency, we isolated primary VSMCs from XBP1^{SMKO} and XBP1^{WT} mice. As shown in Figure 3A, the expression of SMA, calponin and SM22 α were significantly decreased in XBP1-deficient VSMCs from both thoracic and abdominal aortas. Further siRNA knockdown of XBP1 in serum-starved differentiated VSMCs resulted in morphological changes from a spindle-like contractile phenotype to a polygonal synthetic phenotype (Online Figure VA-B). To further clarify whether XBP1u or XBP1s is involved in the VSMC phenotypic switching, siRNA targeting IRE1 α and the specific IRE1 α endoribonuclease inhibitor salicylaldehyde³⁷ were used to inhibit XBP1 splicing (Online Figure VC). Interestingly, IRE1 α silencing or salicylaldehyde application in serum-starved VSMCs displayed no effect on VSMC dedifferentiation, indicating XBP1u, rather than XBP1s, may contribute to maintaining the contractile phenotype of VSMCs (Online Figure VD and Figure 3B). Similar results were observed in primary cultured rat VSMCs (Online Figure VE). Moreover, upon AngII (1 μ M) stimulation, XBP1 silencing greatly induced the expression or secretion of proinflammatory cytokines MCP-1, IL-6, TNF- α and IL-1 β (Online Figure VF-G, I), upregulated MMP-2 and MMP-9 mRNA expression (Online Figure VH-I) and enhanced metalloproteinase activity (Online Figure VJ) in both aortic smooth muscle cell line and primary cultured VSMC, as well as facilitated increased macrophage recruitment towards VSMCs (Online Figure VK). In contrast, inhibiting XBP1 splicing did not affect the above changes (Online Figure VF-K). Accordingly, XBP1u but not XBP1s overexpressed in primary VSMCs isolated from XBP1^{SMKO} mice or in 10% serum VSMCs circumvented the dedifferentiated phenotype due to XBP1 deficiency (Figure 3C and Online Figure VL). Together, XBP1u but not XBP1s maintains the VSMC contractile phenotype and homeostasis. XBP1u deletion in VSMCs induced a proinflammatory and proteolytic phenotype in VSMCs.

Unspliced XBP1 Modulates VSMC Phenotype via FoxO4

To address the underlying mechanism by which XBP1u regulates VSMC contractile proteins expression, we first investigated whether XBP1u directly upregulated SMC contractile proteins transcription with a luciferase reporter assay (Figure 4A). Transfection of myocardin, a potent transcriptional activator of SMC marker genes, into COS-7 cells induced a significant increase in expression of luciferase reporters controlled by the promoters of SMA and SM22 α respectively. In contrast, transfection of XBP1u or XBP1s alone did not affect the luciferase reporter activity (Figure

4A), suggesting that XBP1u does not directly regulate SMC markers at the transcriptional level. Second, we hypothesized that XBP1u may affect serum response factor (SRF), a master transcriptional factor for SMC contractile marker genes, by binding to the CA_rG box of SMC marker gene promoters.³⁸ As shown by the ChIP assay in Figure 4B, silencing XBP1 did not affect the binding activity of SRF to the CA_rG box of SMA and calponin gene promoters. Moreover, the expression levels of a well-recognized transcriptional factor (SRF), coactivators (myocardin and myocardin-related transcription factor, MRTF)^{39, 40} or suppressors (Krüppel-like factor 4, KLF4 and Forkhead box O, FoxOs)^{41, 42} of VSMC markers were not altered at both the mRNA and protein levels (Online Figure VIA-B). Next, we investigated whether XBP1 modulates the subcellular translocation of SMC transcriptional cofactors, as compelling evidence indicates that cytoplasm-nuclear redistribution of SMC cofactors greatly affects the transcriptional program for SMC differentiation. For example, PI3K/Akt signaling promotes SMC differentiation, at least in part, by stimulating nuclear export of FoxO4, thereby releasing myocardin from its inhibitory influence.⁴¹ As shown in immunofluorescence assays, the silencing of XBP1 but not IRE α in serum-starved contractile VSMCs accelerated the nuclear translocation of FoxO4 (Figure 4C). In contrast, XBP1 and IRE α silencing did not affect the subcellular redistribution of myocardin (Figure 4C), which is consistent with constitutive nuclear localization of myocardin.⁴⁰ Concomitantly, ectopic overexpression of XBP1u, but not XBP1s, in serum-rich dedifferentiated VSMCs and primary cultured rat VSMCs facilitated FoxO4 shuttling from the nucleus to the cytoplasm in conjunction with morphological alteration of VSMCs from the synthetic to the contractile phenotype (Online Figure VIC-D). Both *in vitro* and *in vivo* analysis further revealed colocalization of XBP1u and FoxO4 in the cytoplasm of both primary VSMCs and aortic walls from thoracic and abdominal aortas in XBP1^{WT} mice, whereas there was a nuclear localization of FoxO4 in XBP1^{SMKO} mice (Online Figure VIE-F). To further assess whether XBP1u modulates VSMC differentiation by interfering with FoxO4, we performed a luciferase reporter assay. In accordance with previous reports, FoxO4 represses myocardin-induced transactivation of CA_rG-dependent SMA-luc and SM22 α -luc (Figure 4D). This effect could be effectively circumvented by XBP1u but not XBP1s (Figure 4D), suggesting that XBP1u may counteract FoxO4 activity. Accordingly, XBP1 silencing-induced VSMC dedifferentiation was abolished by additional FoxO4 knockdown (Online Figure VIG-H), thus reinforcing the idea that XBP1u maintains the contractile phenotype of VSMCs by negatively regulating VSMC marker repressor FoxO4.

Unspliced XBP1 Directly Interacts with FoxO4

Next, we investigated whether XBP1u directly interacts with FoxO4 and therefore affects its subcellular localization. Upon serum stimulation, FoxO4 was mostly retained in the nucleus, whereas XBP1u was most abundantly expressed in the cytoplasm (Figure 5A). With serum starvation, FoxO4 was enriched in the cytoplasm and colocalized with XBP1u (Figure 5A). Next, we tested whether endogenous XBP1u interacts with myocardin and FoxO4 by co-immunoprecipitation assays in differentiated VSMCs. As shown in Figure 5B, XBP1u specifically co-immunoprecipitated FoxO4 in differentiated VSMCs, and vice versa. In contrast, there was no direct interaction between XBP1u and myocardin, neither between XBP1u and FoxO4 in dedifferentiated SMCs (Online Figure VIIA-B). Meanwhile, we confirmed the interaction of XBP1u and FoxO4 in primary aortic SMCs isolated from XBP1^{WT} mice, but not in XBP1 deficiency SMCs

(Online Figure VIIC). Since XBP1u and XBP1s share the same N-terminal domain, we then tested whether spliced XBP1 interacts with FoxO4 in SMCs. Interestingly, the interaction of XBP1s with FoxO4 was not observed in differentiated SMCs (Figure 5C). Furthermore, a direct interaction was further confirmed by co-transfection of HEK293A cells with His-FoxO4 and Flag-XBP1u plasmids prior to co-immunoprecipitation. The anti-Flag antibody specifically immunoprecipitated His-tagged FoxO4 with XBP1u, whereas control IgG did not (Figure 5D), suggesting a direct association between XBP1u and FoxO4. Next, we tried to map the precise binding domain between XBP1u and FoxO4 by pull-down assays. GST-fused full-length, N-terminus (aa 1-101) and C-terminus (aa 89-261) constructs of XBP1u were incubated with purified MBP-FoxO4 protein *in vitro*. As shown in Figure 5E, both the full-length and N-terminus XBP1u constructs efficiently immuno-interacted with FoxO4, whereas the C-terminus XBP1u construct did not bind. In addition, we performed MBP-pulldown assays to determine the binding domain of FoxO4 with XBP1u. As shown in Figure 5F, the full-length and N-terminus of FoxO4 (aa 1-89), but not other domains, were required for the interaction with XBP1u. Together, our data provide the direct evidence that XBP1u directly associates with FoxO4 in contractile SMCs.

XBP1u-FoxO4 Cytoplasmic Association Prevents FoxO4 Nuclear Translocation and Myocardin Repression in VSMCs

As FoxO4 negatively regulates myocardin activity and represses SMC contractile marker genes transcription⁴¹, we hypothesized that an association between XBP1u and FoxO4 may interfere with the FoxO4-myocardin interaction and the subsequent release of myocardin coactivator activity. To address this issue, we carried out co-immunoprecipitation assays in HEK293A cells transfected with Flag-myocardin and His-FoxO4 in the presence or absence of XBP1u or XBP1s (Figure 6A). Indeed, XBP1u but not XBP1s inhibited the interaction between FoxO4 and myocardin (Figure 6A). Furthermore, the N-terminus of FoxO4 (aa 1-89) was used as a dominant-negative control (FoxO4-DN) to block the interaction between FoxO4 and XBP1u (Online Figure VIIIA). As a consequence, FoxO4 was translocated to the nucleus of differentiated VSMCs, the interaction between FoxO4 and myocardin increased and the SMC marker genes were markedly repressed at both the mRNA and protein levels (Online Figure VIIIB-C and Figure 6B-C). In parallel, the proinflammatory cytokines and MMPs expression levels were significantly upregulated, even in the absence of stimulus (Figure 6D). To reinforce the observation *in vivo*, an adenovirus flanking the dominant-negative domain (Ad-FoxO4-DN, 1×10^9 pfu per mouse) was periadventitially applied to the ApoE^{-/-} mice at the suprarenal abdominal aorta (Online Figure VIIID), and these mice were then exposed to AngII infusion for 4 weeks. As shown in Figure 6E-F, Ad-FoxO4-DN-infected mice had pronouncedly aggravated AngII-induced abdominal aortic aneurysm (Ad-FoxO4-DN + AngII, 50%, n=8 *vs.* Ad-EGFP + AngII, 100%, n=8, $P = 0.021$) *in vivo* without influencing the body weight, blood pressure or survival rate of mice (Online Table III and Online Figure VIIIE). Similarly, Ad-FoxO4-DN-infected aortas displayed enlarged aneurysm diameters, more elastin fragmentation, enhanced inflammatory cells infiltration and greater plasma inflammatory cytokines (MCP-1 and IL-6) (Figure 6G and Online Figure VIIIF-H), similar to the features of AngII-infused XBP1^{SMKO} ApoE^{-/-} mice. Taken together, XBP1u and FoxO4 association is essential for maintaining VSMC homeostasis and inhibiting aortic aneurysm

Discussion

The role of VSMC homeostasis during aortic aneurysm remains elusive. Herein, we have identified an essential role of XBP1u in maintaining the VSMC contractile phenotype and suppression of vascular inflammation and proteolytic degradation *in vitro* and *in vivo*. Intriguingly, our study revealed an XBP1u-FoxO4-myocardin network that regulates the VSMC phenotypic transition and vascular homeostasis, which may reflect some commonality in the pathophysiology of both AAA and TAA.

In the current study, we identified unspliced XBP1 as a molecular link between VSMC homeostasis and aortic aneurysm formation. Vascular SMCs are highly plastic and susceptible to phenotypic switching from a contractile phenotype toward a synthetic, inflammatory, or even osteochondrogenic phenotype upon various stimulation.^{13, 30, 43, 22} The dedifferentiation of VSMCs has been repeatedly observed during the early onset of a variety of vascular diseases, including Marfan syndrome, abdominal aortic aneurysm and dissection.^{13, 44-46} Dedifferentiated VSMCs are prone to migration^{47, 22}, produce more inflammatory cytokines⁴⁸, exhibit greater proteolytic activity^{13, 48} than contractile VSMCs and subsequently contribute to vascular remodeling. Thus, it is pivotal to uncover the endogenous molecules that maintain the contractile phenotype and homeostasis of VSMCs. Previous studies identified contractile proteins (smooth muscle α -actin, smooth muscle myosin heavy chain 11),^{5, 6} membrane receptor (TGF- β receptor II),⁴⁶ transcriptional factor or coactivator (SRF, myocardin),^{39, 38} signaling molecules (integrin ILK, AKT),^{49, 50} and ECM protein (fibrillin-1)^{4, 51} that maintain VSMC homeostasis and resist aortic aneurysm and dilation. Herein, we revealed that XBP1u-deficient VSMCs lost the contractile phenotype and were susceptible to both AngII-induced thoracic and abdominal aortic aneurysm and CaPO₄-induced abdominal aortic aneurysm. XBP1-deficient or silenced VSMCs expressed fewer contractile proteins, higher MMP-2/MMP-9 expression and activity, and increased inflammatory cytokines. These observations are in accordance with previous reports of MMP-2/9³⁶ and MCP-1/IL-6/IL-1^{52, 35} functioning in aortic aneurysm. Moreover, the observation of an essential role of XBP1u in maintaining the VSMC differentiated state is also consistent with a report that XBP-1 global knockout mice exhibit embryonic lethality at E13.5 with reduced vessel formation.²⁰

Notably, our study excludes the dependence of XBP1-splicing linked ER stress with regards to unspliced XBP1 for maintaining VSMC homeostasis and prevention of aortic aneurysm. In general, the accumulation and aggregation of misfolded proteins and sustained ER stress responses are common features of cardiovascular disorders. Previous studies have reported that ER stress molecules including GRP-78, GRP-94, p-eIF2 α , ATF6 α and XBP1s were elevated in aneurysmal areas of human AAA and Ang II-induced ApoE^{-/-} mice.³³ A deficiency of CHOP, a mediator of ER stress in mice, prevents thoracic aortic aneurysm/dissection (TAAD).⁵³ However, these markers were detected mostly at late stage of aneurysmal aorta and there is lack of direct evidence that which ER-stress signaling pathways participates in the pathogenesis of aortic aneurysms. In the current study, we observed no obvious alteration of XBP1s at the early stages of two different murine models of aortic aneurysm (Figure 1A and Online Figure IF), which may due to p-eIF2 α -mediated inhibition of translation.⁵⁴ In contrast, XBP1u was markedly decreased with time, in parallel with the repression of the smooth muscle contractile apparatus proteins (Figure 1A-B and Online Figure IF). Silencing of XBP1, but not an inhibition of XBP1 splicing or IRE1 α , facilitates VSMC

phenotypic switching towards synthetic and inflammatory phenotypes, showing that XBP1u protection of VSMCs overwhelmed XBP1s during the pathogenesis of aortic aneurysm. Of interest, VSMC dedifferentiation is also a hallmark of postinjury restenosis in vessels, and a previous study using the same SMC-specific XBP1 knockout mice has shown that XBP1 deficiency in VSMCs abrogates neointimal lesions of injured vessels.²² This discrepancy may be due to different injuries in different animal models. Indeed, the latter has reported that PDGF-BB activated XBP-1 splicing via interaction with the PDGF receptor, and therefore XBP1s increased SMC migration and contributed to neointima formation²², suggesting a context-dependent role for XBP1u and XBP1s in various vascular disease states.

Another interesting finding of the current study is the novel network of XBP1u-FoxO4-Myocardin in maintaining VSMC homeostasis (Figure 7). Compelling evidence has indicated that myocardin is an essential coactivator of serum response factor (SRF) and is a master regulator of VSMC differentiation.⁵⁵ SRF binds to CA₂G boxes and transcriptionally activates a variety of downstream muscle-specific genes, such as *SM22 α* , *ACTA2*, *MYH11*, and several other signaling pathways.⁵⁵ A reduction of myocardin availability for SRF by other myocardin-binding proteins, such as FoxO4, leads to decreased expression of VSMC contractile apparatus genes.^{41,55} In contrast, FoxO4 nuclear export to the cytoplasm leads to the release of myocardin and VSMC differentiation.⁴¹ In the current study, we unraveled a novel regulatory mechanism of myocardin activity and VSMC differentiation. In brief, XBP1u binding to FoxO4 in cytoplasm inhibits FoxO4 nuclear translocation and association with myocardin in the nucleus. XBP1 deficiency led to the nuclear accumulation of FoxO4, repressed myocardin activation, reduced SMC marker gene expression and enhanced inflammatory responses in the vessels. Disrupting XBP1u interaction with FoxO4 in contractile VSMCs or ApoE^{-/-} aorta mimicked XBP1-deficiency induced VSMC phenotypic switching and aneurysm formation, highlighting the importance of XBP1u-FoxO4-myocardin interactions in maintaining VSMC homeostasis. The XBP1^{-/-} mice recapitulated the phenotype of myocardin^{-/-} mice, which die at embryonic day 10.5 due to a lack of VSMC differentiation.³⁹ In addition, the enhanced inflammatory response of XBP1^{SMKO}ApoE^{-/-} mice in response to AngII infusion is also in line with a previous report that myocardin negatively regulates VSMC inflammatory activation.⁵⁶ Our data are also in line with a previous study showing that FoxO4 promotes SMC dedifferentiation in an animal model of restenosis.⁴¹ Of interest, a recent study of aging revealed that FoxO4 protects senescent cell viability by maintaining p53 sequestration in nuclear bodies and disrupting this interaction restores p53's apoptotic role, highlighting the importance of manipulation of FoxO4 nuclear-cytoplasm shuttling in the regulation of cell functions.⁵⁷

Of note, XBP1s and XBP1u share the same N-terminal, which is required for FoxO4 binding.²⁴ Therefore, XBP1s may also interact with FoxO4. Herein, we observed XBP1u mainly localized in cytoplasm whereas FoxO4 shuttled between nuclear and cytoplasm upon VSMCs phenotypic transition. We observed enhanced association in the contractile phenotype but decreased interaction upon dedifferentiation between XBP1u and FoxO4 in the cytoplasm of VSMCs. Interrupting XBP1u and FoxO4 cytoplasmic interaction in contractile VSMCs accelerates the nuclear interaction between FoxO4 and myocardin and causes cellular dedifferentiation. In contrast to XBP1u, XBP1s predominately localized in the nucleus. Although we did observe XBP1s-FoxO4 interaction in dedifferentiated SMCs (Online Figure VIID), we did not find XBP1s-FoxO4 association in

contractile VSMCs (Figure 5C). In accordance, XBP1s did not interfere with the interaction between FoxO4 and myocardin and did not affect the inhibition of FoxO4 on myocardin-induced transactivation. In addition, consistent with other studies, we found that inhibition of XBP1 splicing lead to enhanced SMC markers expression²², suggesting opposite effect of XBP1u and XBP1s on regulation of VSMCs phenotypic transition. XBP1u, but not XBP1s association with FoxO4 is pivotal for maintaining VSMC contractile phenotype and homeostasis. In addition to VSMC phenotypic switching, the aggravation of vascular inflammation and extracellular matrix degradation in aneurysmal XBP1^{SMKO} mice may involve the direct activation of MMP-9 transcription by FoxO4 during XBP1 deficiency. Targeting XBP1u-FoxO4 interactions may provide a novel strategy for the prevention of aneurysm-related disease.

Acknowledgments

We thank Prof. Weiguo Zhu from Peking University Health Science Center for kindly donating GST-XBP1u plasmids for our GST and MBP pull-down assays and Prof. Huihua Li from Capital Medical University for generously providing Flag-myocardin plasmid for our luciferase reporter assays.

Sources of Funding

This research was supported by funding from the International Cooperation and Exchanges National Natural Science Foundation of the P. R. China (NSFC, 81220108004), the key programs of NSFC (81730010, 91539203), the National Key R&D Program of China (2016YFC0903000), and the 111 Project of the Chinese Ministry of Education (No. B07001).

References

1. BK KHAS. Genetic and epigenetic regulation of aortic aneurysms *Biomed Res Int.* 2017;2017. doi: 10.1155/2017/7268521
2. Guo DC, Papke CL, He R, Milewicz DM. Pathogenesis of thoracic and abdominal aortic aneurysms. *Ann N Y Acad Sci.* 2006;1085:339-352. doi: 10.1196/annals.1383.013
3. Nordon IM, Hinchliffe RJ, Loftus IM, Thompson MM. Pathophysiology and epidemiology of abdominal aortic aneurysms. *Nat Rev. Cardiol.* 2011;8:92-102. doi: 10.1038/nrcardio.2010.180
4. Dietz HC, Cutting CR, Pyeritz RE, Maslen CL, Sakai LY, Corson GM, Puffenberger EG, Hamosh A, Nanthakumar EJ, Curristin SM, Stetten G, Meyers DA, Francomano CA. Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature.* 1991;352:337-339. doi:
5. Guo DC, Pannu H, Tran-Fadulu V, Papke CL, Yu RK, Avidan N, Bourgeois S, Estrera AL, Safi HJ, Sparks E, Amor D, Ades L, McConnell V, Willoughby CE, Abuelo D, Willing M, Lewis RA, Kim DH, Scherer S, Tung PP, Ahn C, Buja LM, Raman CS, Shete SS, Milewicz DM. Mutations in smooth muscle alpha-actin (*ACTA2*) lead to thoracic aortic aneurysms and dissections. *Nat Genet.* 2007;39:1488-1493. doi: 10.1038/ng.2007.6
6. Pannu H, Tran-Fadulu V, Papke CL, Scherer S, Liu Y, Presley C, Guo D, Estrera AL, Safi HJ, Brasier AR, Vick GW, Marian AJ, Raman CS, Buja LM, Milewicz DM. *MYH11* mutations result in a distinct vascular pathology driven by insulin-like growth factor 1 and angiotensin II. *Hum Mol Genet.* 2007;16:2453-2462. doi: 10.1093/hmg/ddm201
7. Pannu H, Fadulu VT, Chang J, Lafont A, Hasham SN, Sparks E, Giampietro PF, Zaleski C, Estrera AL, Safi HJ, Shete S, Willing MC, Raman CS, Milewicz DM. Mutations in transforming growth factor-beta receptor type II cause familial thoracic aortic aneurysms and dissections. *Circulation.* 2005;112:513-520. doi: 10.1161/CIRCULATIONAHA.105.537340
8. Bradley DT, Hughes AE, Badger SA, Jones GT, Harrison SC, Wright BJ, Bumpstead S, Baas AF, Gretarsdottir S, Burnand K, Child AH, Clough RE, Cockerill G, Hafez H, Scott DJ, Ariens RA, Johnson A, Sohrabi S, Smith A, Thompson MM, van Bockxmeer FM, Waltham M, Matthiasson SE, Thorleifsson G, Thorsteinsdottir U, Blankensteijn JD, Teijink JA, Wijmenga C, de Graaf J, Kiemeny LA, Wild JB, Edkins S, Gwilliam R, Hunt SE, Potter S, Lindholt JS, Golledge J, Norman PE, van Rij A, Powell JT, Eriksson P, Stefansson K, Thompson JR, Humphries SE, Sayers RD, Deloukas P, Samani NJ, Bown MJ. A variant in *LDLR* is associated with abdominal aortic aneurysm. *Circ Cardiovasc Genet.* 2013;6:498-504. doi: 10.1161/CIRCGENETICS.113.000165
9. Hinterseher I, Tromp G, Kuivaniemi H. Genes and abdominal aortic aneurysm. *Ann Vasc Surg* 2011;25:388-412. doi: 10.1016/j.avsg.2010.09.004
10. Wang G, Jacquet L, Karamariti E, Xu Q. Origin and differentiation of vascular smooth muscle cells. *J Physiol.* 2015;593:3013-3030. doi: 10.1113/JP270033
11. Daugherty A, Cassis LA, Lu H. Complex pathologies of angiotensin II-induced abdominal aortic aneurysms. *J Zhejiang Univ. Sci. B.* 2011;12:624-628. doi: 10.1631/jzus.B1101002
12. Dennis Bruemmer AD, Hong Lu and Debra L. Rateri. Relevance of angiotensin II-induced aortic pathologies in mice to human aortic aneurysms. *Ann. N. Y. Acad. Sci.* 2011:7-10. doi: 10.1111/j.1749-6632.2011.06332.x
13. Ailawadi G, Moehle CW, Pei H, Walton SP, Yang Z, Kron IL, Lau CL, Owens GK. Smooth muscle phenotypic modulation is an early event in aortic aneurysms. *J Thorac Cardiovasc Surg.*

- 2009;138:1392-1399. doi: 10.1016/j.jtcvs.2009.07.075
14. Martin-Alonso M, Garcia-Redondo AB, Guo D, Camafeita E, Martinez F, Alfranca A, Mendez-Barbero N, Pollan A, Sanchez-Camacho C, Denhardt DT, Seiki M, Vazquez J, Salaces M, Redondo JM, Milewicz D, Arroyo AG. Deficiency of MMP17/MT4-MMP proteolytic activity predisposes to aortic aneurysm in mice. *Circ Res.* 2015;117:e13-26. doi: 10.1161/CIRCRESAHA.117.305108
 15. He Y, Sun S, Sha H, Liu Z, Yang L, Xue Z, Chen H, Qi L. Emerging roles for XBP1, a sUPeR transcription factor. *Gene Expr.* 2010;15:13-25.
 16. Wu R, Zhang QH, Lu YJ, Ren K, Yi GH. Involvement of the IRE1 α -XBP1 pathway and XBP1s-dependent transcriptional reprogramming in metabolic diseases. *DNA Cell Biol.* 2015;34:6-18. doi: 10.1089/dna.2014.2552
 17. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell.* 2001;107:881-891. doi: [https://doi.org/10.1016/S0092-8674\(01\)00611-0](https://doi.org/10.1016/S0092-8674(01)00611-0)
 18. Lee AH, Heidtman K, Hotamisligil GS, Glimcher LH. Dual and opposing roles of the unfolded protein response regulated by IRE1 α and XBP1 in proinsulin processing and insulin secretion. *Proc Natl Acad Sci U S A.* 2011;108:8885-8890. doi: 10.1073/pnas.1105564108
 19. Margariti A, Li H, Chen T, Martin D, Vizcay-Barrena G, Alam S, Karamariti E, Xiao Q, Zampetaki A, Zhang Z, Wang W, Jiang Z, Gao C, Ma B, Chen Y-G, Cockerill G, Hu Y, Xu Q, Zeng L. XBP1 mRNA splicing triggers an autophagic response in endothelial cells through BECLIN-1 transcriptional activation. *J Biol Chem* 2013;288:859-872. doi: 10.1074/jbc.M112.412783
 20. Zeng L, Xiao Q, Chen M, Margariti A, Martin D, Ivetic A, Xu H, Mason J, Wang W, Cockerill G, Mori K, Li JY, Chien S, Hu Y, Xu Q. Vascular endothelial cell growth-activated XBP1 splicing in endothelial cells is crucial for angiogenesis. *Circulation.* 2013;127:1712-1722. doi: 10.1161/CIRCULATIONAHA.112.001337
 21. Zeng L, Zampetaki A, Margariti A, Pepe AE, Alam S, Martin D, Xiao Q, Wang W, Jin ZG, Cockerill G, Mori K, Li YS, Hu Y, Chien S, Xu Q. Sustained activation of XBP1 splicing leads to endothelial apoptosis and atherosclerosis development in response to disturbed flow. *Proc Natl Acad Sci U S A.* 2009;106:8326-8331. doi: 10.1073/pnas.0903197106
 22. Zeng L, Li Y, Yang J, Wang G, Margariti A, Xiao Q, Zampetaki A, Yin X, Mayr M, Mori K, Wang W, Hu Y, Xu Q. XBP1-deficiency abrogates neointimal lesion of injured vessels via cross talk with the PDGF signaling. *Arterioscler Thromb Vasc Biol.* 2015;35:2134-2144. doi: 10.1161/ATVBAHA.115.305420
 23. Yoshida H, Oku M, Suzuki M, Mori K. pXBP1(u) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(s) in mammalian ER stress response. *J Cell Biol.* 2006;172:565-575. doi: 10.1083/jcb.200508145
 24. Zhao Y, Li X, Cai MY, Ma K, Yang J, Zhou J, Fu W, Wei FZ, Wang L, Xie D, Zhu WG. XBP1u suppresses autophagy by promoting the degradation of FoxO1 in cancer cells. *Cell Res.* 2013;23:491-507. doi: 10.1038/cr.2013.2
 25. Martin D, Li Y, Yang J, Wang G, Margariti A, Jiang Z, Yu H, Zampetaki A, Hu Y, Xu Q, Zeng L. Unspliced x-box-binding protein 1 (XBP1) protects endothelial cells from oxidative stress through interaction with histone deacetylase 3. *J Biol Chem.* 2014;289:30625-30634. doi: 10.1074/jbc.M114.571984

26. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest.* 2000;105:1605-1612.
27. Lu H, Howatt DA, Balakrishnan A, Moorleggen JJ, Rateri DL, Cassis LA, Daugherty A. Subcutaneous angiotensin II infusion using osmotic pumps induces aortic aneurysms in mice. *J Vis Exp.* 2015:53191. doi: 10.3791/53191
28. Deng GG, Martin-McNulty B, Sukovich DA, Freay A, Halks-Miller M, Thinnies T, Loskutoff DJ, Carmeliet P, Dole WP, Wang YX. Urokinase-type plasminogen activator plays a critical role in angiotensin II-induced abdominal aortic aneurysm. *Circ Res.* 2003;92:510-517. doi: 10.1161/01.RES.0000061571.49375.E1
29. Kanematsu Y, Kanematsu M, Kurihara C, Tsou TL, Nuki Y, Liang EI, Makino H, Hashimoto T. Pharmacologically induced thoracic and abdominal aortic aneurysms in mice. *Hypertension.* 2010;55:1267-1274. doi: 10.1161/HYPERTENSIONAHA.109.140558
30. Du Y, Wang Y, Wang L, Liu B, Tian Q, Liu CJ, Zhang T, Xu Q, Zhu Y, Ake O, Qi Y, Tang C, Kong W, Wang X. Cartilage oligomeric matrix protein inhibits vascular smooth muscle calcification by interacting with bone morphogenetic protein-2. *Circ Res.* 2011;108:917-928. doi: 10.1161/CIRCRESAHA.110.234328
31. Daugherty A, Rateri DL, Charo IF, Owens AP, Howatt DA, Cassis LA. Angiotensin II infusion promotes ascending aortic aneurysms: Attenuation by CCR2 deficiency in apoE^{-/-} mice. *Clin Sci (Lond).* 2010;118:681-689. doi: 10.1042/CS20090372
32. Li Y, Lu G, Sun D, Zuo H, Wang DW, Yan J. Inhibition of endoplasmic reticulum stress signaling pathway: A new mechanism of statins to suppress the development of abdominal aortic aneurysm. *PLoS one.* 2017;12:e0174821. doi: 10.1371/journal.pone.0174821
33. Qin Y, Wang Y, Liu O, Jia L, Fang W, Du J, Wei Y. Tauroursodeoxycholic acid attenuates angiotensin II induced abdominal aortic aneurysm formation in apolipoprotein E-deficient mice by inhibiting endoplasmic reticulum stress. *Eur J Vasc Endovasc Surg.* 2017;53:337-345. doi: 10.1016/j.ejvs.2016.10.026
34. Guo FJ, Xiong Z, Lu X, Ye M, Han X, Jiang R. ATF6 upregulates XBP1s and inhibits ER stress-mediated apoptosis in osteoarthritis cartilage. *Cell Signal.* 2014;26:332-342. doi: 10.1016/j.cellsig.2013.11.018
35. Middleton RK, Lloyd GM, Bown MJ, Cooper NJ, London NJ, Sayers RD. The pro-inflammatory and chemotactic cytokine microenvironment of the abdominal aortic aneurysm wall: A protein array study. *J Vasc Surg.* 2007;45:574-580. doi: 10.1016/j.jvs.2006.11.020
36. Rabkin SW. The role matrix metalloproteinases in the production of aortic aneurysm. *Prog Mol Biol Transl Sci.* 2017;147:239-265. doi: 10.1016/bs.pmbts.2017.02.002
37. Volkmann K, Lucas JL, Vuga D, Wang X, Brumm D, Stiles C, Kriebel D, Der-Sarkissian A, Krishnan K, Schweitzer C, Liu Z, Malyankar UM, Chiovitti D, Canny M, Durocher D, Sicheri F, Patterson JB. Potent and selective inhibitors of the inositol-requiring enzyme 1 endoribonuclease. *J Biol Chem.* 2011;286:12743-12755. doi: 10.1074/jbc.M110.199737
38. Mack CP. Signaling mechanisms that regulate smooth muscle cell differentiation. *Arterioscler Thromb Vasc Biol.* 2011;31:1495-1505. doi: 10.1161/ATVBAHA.110.221135
39. Huang J, Wang T, Wright AC, Yang J, Zhou S, Li L, Yang J, Small A, Parmacek MS. Myocardin is required for maintenance of vascular and visceral smooth muscle homeostasis during postnatal development. *Proc Natl Acad Sci U S A.* 2015;112:4447-4452. doi: 10.1073/pnas.1420363112

40. Nakamura S, Hayashi K, Iwasaki K, Fujioka T, Egusa H, Yatani H, Sobue K. Nuclear import mechanism for myocardin family members and their correlation with vascular smooth muscle cell phenotype. *J Biol Chem.* 2010;285:37314-37323. doi: 10.1074/jbc.M110.180786
41. Liu ZP, Wang Z, Yanagisawa H, Olson EN. Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. *Dev Cell.* 2005;9:261-270. doi: 10.1016/j.devcel.2005.05.017
42. Yoshida T, Gan Q, Owens GK. Krüppel-like factor 4, Elk-1, and histone deacetylases cooperatively suppress smooth muscle cell differentiation markers in response to oxidized phospholipids. *Am J Physiol Cell Physiol.* 2008;295:C1175-C1182. doi: 10.1152/ajpcell.00288.2008
43. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res.* 2012;95:156-164. doi: 10.1093/cvr/cvs115
44. Crosas-Molist E, Meirelles T, Lopez-Luque J, Serra-Peinado C, Selva J, Caja L, Gorbenko Del Blanco D, Uriarte JJ, Bertran E, Mendizabal Y, Hernandez V, Garcia-Calero C, Busnadiego O, Condom E, Toral D, Castella M, Forteza A, Navajas D, Sarri E, Rodriguez-Pascual F, Dietz HC, Fabregat I, Egea G. Vascular smooth muscle cell phenotypic changes in patients with Marfan syndrome. *Arterioscler Thromb Vasc Biol.* 2015;35:960-972. doi: 10.1161/ATVBAHA.114.304412
45. Huang J, Davis EC, Chapman SL, Budatha M, Marmorstein LY, Word RA, Yanagisawa H. Fibulin-4 deficiency results in ascending aortic aneurysms: A potential link between abnormal smooth muscle cell phenotype and aneurysm progression. *Circ Res.* 2010;106:583-592. doi: 10.1161/CIRCRESAHA.109.207852
46. Inamoto S, Kwartler CS, Lafont AL, Liang YY, Fadulu VT, Duraisamy S, Willing M, Estrera A, Safi H, Hannibal MC, Carey J, Wiktorowicz J, Tan FK, Feng XH, Pannu H, Milewicz DM. TGFBR2 mutations alter smooth muscle cell phenotype and predispose to thoracic aortic aneurysms and dissections. *Cardiovasc Res.* 2010;88:520-529. doi: 10.1093/cvr/cvq230
47. Wang L, Zheng J, Bai X, Liu B, Liu CJ, Xu Q, Zhu Y, Wang N, Kong W, Wang X. ADAMTS-7 mediates vascular smooth muscle cell migration and neointima formation in balloon-injured rat arteries. *Circ Res.* 2009;104:688-698. doi: 10.1161/CIRCRESAHA.108.188425
48. Ali MS, Starke RM, Jabbour PM, Tjoumakaris SI, Gonzalez LF, Rosenwasser RH, Owens GK, Koch WJ, Greig NH, Dumont AS. TNF- α induces phenotypic modulation in cerebral vascular smooth muscle cells: Implications for cerebral aneurysm pathology. *J Cereb Blood Flow Metab.* 2013;33:1564-1573. doi: 10.1038/jebfm.2013.109
49. Duan C, Bauchat JR, Hsieh T. Phosphatidylinositol 3-kinase is required for insulin-like growth factor-I-induced vascular smooth muscle cell proliferation and migration. *Circ Res.* 2000;86:15
50. Kogata N, Tribe RM, Fassler R, Way M, Adams RH. Integrin-linked kinase controls vascular wall formation by negatively regulating rho/rock-mediated vascular smooth muscle cell contraction. *Genes & development.* 2009;23:2278-2283. doi: 10.1101/gad.535409
51. El-Hamamsy I, Yacoub MH. Cellular and molecular mechanisms of thoracic aortic aneurysms. *Nat Rev Cardiol.* 2009;6:771-786. doi: 10.1038/nrcardio.2009.191
52. Johnston WF, Salmon M, Pope NH, Meher A, Su G, Stone ML, Lu G, Owens GK, Upchurch GR, Ailawadi G. Inhibition of interleukin-1 β decreases aneurysm formation and progression in a novel model of thoracic aortic aneurysms. *Circulation.* 2014;130:S51-S59. doi: 10.1161/CIRCULATIONAHA.113.006800

53. Jia LX, Zhang WM, Zhang HJ, Li TT, Wang YL, Qin YW, Gu H, Du J. Mechanical stretch-induced endoplasmic reticulum stress, apoptosis and inflammation contribute to thoracic aortic aneurysm and dissection. *J Pathol.* 2015;236:373-383. doi: 10.1002/path.4534
54. Majumder M, Huang C, Snider MD, Komar AA, Tanaka J, Kaufman RJ, Krokowski D, Hatzoglou M. A novel feedback loop regulates the response to endoplasmic reticulum stress via the cooperation of cytoplasmic splicing and mRNA translation. *Mol Cell Biol.* 2012;32:992-1003. doi: 10.1128/MCB.06665-11
55. Zheng XL. Myocardin and smooth muscle differentiation. *Arch Biochem Biophys.* 2014;543:48-56. doi: 10.1016/j.abb.2013.12.015
56. Ackers-Johnson M, Talasila A, Sage AP, Long X, Bot I, Morrell NW, Bennett MR, Miano JM, Sinha S. Myocardin regulates vascular smooth muscle cell inflammatory activation and disease. *Arterioscler Thromb Vasc Biol.* 2015;35:817-828. doi: 10.1161/ATVBAHA.114.305218
57. Baar MP, Brandt RM, Putavet DA, Klein JD, Derks KW, Bourgeois BR, Stryeck S, Rijksen Y, van Willigenburg H, Feijtel DA, van der Pluijm I, Essers J, van Cappellen WA, van IWF, Houtsmuller AB, Pothof J, de Bruin RW, Madl T, Hoeijmakers JH, Campisi J, de Keizer PL. Targeted apoptosis of senescent cells restores tissue homeostasis in response to chemotoxicity and aging. *Cell.* 2017;169:132-147 e116. doi: 10.1016/j.cell.2017.02.031

Figure Legends

Figure 1. Unspliced XBP1 reduction occurs in parallel with VSMC dedifferentiation during early stage of aortic aneurysm formation. **A**, Representative western blot and quantification of XBP1 and smooth muscle cell (SMC) contractile proteins in ascending, descending and suprarenal abdominal aortas from ApoE^{-/-} mice (16-week-old, male; n=5 mice per group) infused with AngII (1000 ng/kg/min) for 0, 3 and 7 days. *P<0.05. Asc, ascending aortas; Des, descending aortas; Abd, suprarenal abdominal aortas; SMA, smooth muscle α -actin. NS, no significance. **B**, Representative immunohistochemical staining and quantification of XBP1u and SM22 α in ascending, descending and suprarenal abdominal aortas from ApoE^{-/-} mice (16-week-old, male) infused with saline or AngII (1000 ng/kg/min) for 7 days (n=3 mice per group). Scale bar, 50 μ m. *P<0.05. **C**, Representative immunofluorescent staining of XBP1u (green) and SM22 α (red) in normal ascending and suprarenal abdominal aortas of C57BL/6J mice. Nuclei stained by Hoechst were indicated in blue. Scale bar, 10 μ m.

Figure 2. XBP1 deficiency in VSMCs aggravates AngII-induced aortic aneurysms *in vivo*. **A-E**, 16-week-old XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} male mice were infused with saline or AngII (1000 ng/kg/min) for 28 days. XBP1^{WT}ApoE^{-/-} + saline, n=6; XBP1^{SMKO}ApoE^{-/-} +saline, n=6; XBP1^{WT}ApoE^{-/-} + AngII, n=9; XBP1^{SMKO}ApoE^{-/-} +AngII, n=10. **A**, Representative morphology of thoracic aortas from saline- or AngII-infused XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice. **B**, Quantification of maximal aortic diameters measured *ex vivo* from saline- or AngII-infused XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice.*P<0.05. NS, no significance. **C**, Representative morphology of aortas from saline- or AngII-infused XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice. **D**, Incidence of abdominal aortic aneurysm and dissection in saline- or AngII-infused XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice (XBP1^{WT}ApoE^{-/-} + saline, n=6; XBP1^{SMKO}ApoE^{-/-} +saline, n=6; XBP1^{WT}ApoE^{-/-} + AngII, n=9; XBP1^{SMKO}ApoE^{-/-} +AngII, n=10). *P<0.05 by Chi-square test. **E**, Quantification of maximal suprarenal abdominal aortic diameters from saline- or AngII-infused XBP1^{WT}ApoE^{-/-} and XBP1^{SMKO}ApoE^{-/-} mice. *P<0.05. NS, no significance.

Figure 3. Unspliced XBP1 maintains the contractile phenotype of VSMCs. **A**, Representative western blot and quantification of SMC contractile markers and XBP1 in primary VSMCs isolated from thoracic aortas (TA) and abdominal aortas (AA) of XBP1^{WT} and XBP1^{SMKO} mice (8- to 12-week-old, male). Five aortas were pooled in each sample. The experiments were performed four times independently. *P<0.05. NS, no significance. **B**, Western blot analysis of the expression of SMC contractile markers and XBP1 in treated A7r5 cells. The data were presented as means \pm SEM from 7 independent experiments in duplicate. *P<0.05. NS, no significance. **C**, Representative western blot and quantification of SMC contractile markers and XBP1 in primary VSMCs isolated from XBP1^{SMKO} mice (8- to 12-week-old, four aortas were pooled in each sample) after transfection of Flag-vector or Flag-XBP1u for 48 h. The experiments were performed four times independently. *P<0.05.

Figure 4. Unspliced XBP1 modulates VSMC phenotype via FoxO4. **A**, Luciferase reporter

analysis in COS-7 cells. SMA promoter-luc reporter- or SM22 α promoter-luc reporter-overexpressing COS-7 cells were transfected with Flag-myocardin, Flag-XBP1u or Flag-XBP1s plasmids. The luciferase activity normalized to β -galactosidase activity was presented as the means \pm SEM from three independent experiments performed in triplicate. * P <0.05. NS, no significance. **B**, Chromatin immunoprecipitation assay of SRF binding to SMA and calponin gene promoters in A7r5 cells with or without XBP1 silencing by siRNA transfection. The experiments were performed three times independently. NS, no significance. **C**, Representative immunofluorescent staining of myocardin (red, upper panels) and FoxO4 (green, lower panels) in A7r5 cells transfected with scramble siRNA, XBP1 siRNA or IRE1 α siRNA followed by serum-starvation for 24 h. Scale bar, 50 μ m. Three independent experiments were performed. **D**, Luciferase reporter analysis in HEK293A cells. SMA promoter-luc reporter- or SM22 α promoter-luc reporter-overexpressing HEK293A cells were transfected with Flag-myocardin, Flag-FoxO4, Flag-XBP1u or Flag-XBP1s plasmids. The luciferase activity normalized to β -galactosidase activity was presented as means \pm SEM from 4 independent experiments performed in duplicate. * P <0.05. NS, no significance.

Figure 5. Unspliced XBP1 directly interacts with FoxO4. **A**, Representative immunofluorescent staining of XBP1u (red) and FoxO4 (green) in A7r5 cells with or without 10% serum. Scale bar, 50 μ m. Three independent experiments were performed. **B**, Co-immunoprecipitation assay of XBP1u and FoxO4 in serum-starved A7r5 cells. Left, A7r5 cells were incubated with an anti-XBP1u or control IgG antibodies, followed by protein A/G agarose beads. The FoxO4 was measured by western blot analysis. Right, A7r5 cells were incubated with an anti-FoxO4 or control IgG antibodies. The XBP1u was analyzed by immunoblotting. The aliquots of whole-cell extracts prior to immunoprecipitation were applied as input controls. **C**, Co-immunoprecipitation assay of XBP1s and FoxO4 in serum-starved A7r5 cells. A7r5 cells were incubated with an anti-XBP1s or control IgG antibodies, followed by protein A/G agarose beads. The FoxO4 was measured by western blot analysis. **D**, Co-immunoprecipitation assay of XBP1u and FoxO4 in HEK293A cells. HEK293A cells, transfected with Flag-XBP1u and His-FoxO4, were incubated with an anti-His antibody or control IgG, followed by immunoblotting with anti-Flag antibody. **E**, Purified GST (negative control) or GST-fusion proteins of XBP1u full-length (aa 1-261), N-terminus (aa 1-101) and C-terminus (aa 89-261) constructs were immobilized on glutathione agarose and incubated with purified MBP-FOXO4. Trapped proteins were detected by immunoblotting with anti-MBP and anti-GST antibodies. The red arrows represent the target band. **F**, Purified MBP (negative control) or various MBP-FOXO4 fusion proteins were immobilized on amylose resin and incubated with purified GST-XBP1u. Trapped proteins were measured by immunoblotting with anti-GST and anti-MBP antibodies. The red arrows represent the target band.

Figure 6. Unspliced XBP1 but not spliced inhibites myocardin interaction with FOXO4. **A**, Co-immunoprecipitation analysis of FoxO4 and myocardin. Flag-Myo (Flag-myocardin) and His-FOXO4-overexpressing HEK293A cells, transfected with pcDNA3.1, pcDNA3.1-XBP1u or pcDNA3.1-XBP1s, were harvested for co-immunoprecipitation with an anti-Flag antibody and immunoblotting with an anti-His antibody, sequentially. The quantitative data of his after immunoprecipitation with flag antibody were from three independent experiments. * P <0.05. NS,

no significance. **B**, Co-immunoprecipitation analysis of FoxO4 and myocardin in serum-starved rat primary VSMCs infected with Ad-FoxO4-DN or Ad-EGFP. **C**, Western blot analysis of SMC contractile markers in serum-starved A7r5 cells transfected with Flag-vector or Flag-FoxO4-DN. Five independent experiments were performed in duplicate. $*P<0.05$. **D**, Quantitative real-time PCR analysis of mRNA levels of inflammatory cytokines and MMP-9 in serum-starved A7r5 cells transfected with Flag-vector or Flag-FoxO4-DN. The data were expressed as the means \pm SEM from 5 independent experiments performed in duplicate. $*P<0.05$. **E-G**, 16-week-old ApoE^{-/-} mice were infected periadventitally with adenovirus Ad-EGFP (n=8) or Ad-FoxO4-DN (n=8) in the suprarenal abdominal aortas. After 3 days, the mice were infused with AngII (1000 ng/kg/min) for 4 weeks. **E**, Representative morphology of AAA in ApoE^{-/-} mice after 28 days of AngII-infusion. **F**, Incidence of AngII-induced AAA in Ad-EGFP or Ad-FoxO4-DN infected mice. $*P<0.05$ by Chi-square test. **G**, Quantification of maximal suprarenal abdominal aortic diameters measured *ex vivo* from AngII-infused mice.

Figure 7. Schematic illustration of XBP1u-FoxO4-Myocardin in maintaining VSMC homeostasis.

Novelty and Significance***What Is Known?***

- The phenotypic switch of vascular smooth muscle cells (VSMCs) proceeds aortic aneurysms.
- X-box binding protein 1, which exists in a transcriptionally inactive unspliced form (XBP1u) and a spliced active form (XBP1s), is a key factor controlling the ER-stress response.

What New Information Does This Article Contribute?

- XBP1u is essential to maintain VSMCs homeostasis via ER-stress independent effect.
- XBP1u deficiency in VSMCs causes both thoracic and abdominal aortic aneurysms in mice.
- XBP1u-FoxO4-myocardin axis in SMCs plays a pivotal role in maintaining VSMCs phenotype and preventing aortic aneurysm formation.

The molecular link connecting VSMCs phenotypic switching and aortic aneurysm remains elusive. Our study revealed that XBP1u is pivotal to maintain the contractile phenotype of VSMCs and prevents aortic aneurysm formation. Mechanistically, XBP1u directly binds to cytoplasmic FoxO4 and prevents FoxO4 nuclear translocation and subsequent repression of myocardin-mediated contractile apparatus transcription in VSMCs. Interrupting the XBP1u-FoxO4 interaction promotes the proinflammatory and proteolytic phenotype of VSMCs *in vitro* and stimulates aortic aneurysm formation *in vivo*. Our study highlights the importance of XBP1u-FoxO4-myocardin interactions in modulating VSMC phenotypic transition during aortic aneurysm formation.