C/EBP-Homologous Protein (CHOP) in Vascular Smooth Muscle Cells Regulates Their Proliferation in Aortic Explants and Atherosclerotic Lesions

**Rationale:** Myeloid-derived C/EBP-homologous protein (CHOP), an effector of the endoplasmic reticulum stress–induced unfolded protein response, promotes macrophage apoptosis in advanced atherosclerosis, but the role of CHOP in vascular smooth muscle cells (VSMCs) in atherosclerosis is not known.

**Objective:** To investigate the role of CHOP in SM22α VSMCs in atherosclerosis.

**Methods and Results:** Chopαβ mice were generated and crossed into the Apoe−/− and SM22α-CreKI−/− backgrounds. SM22α-CreKI causes deletion of floxed genes in adult SMCs. After 12 weeks of Western-type diet feeding, the content of α-actin–positive cells in aortic root lesions was decreased in ChopαβSM22α-CreKI Apo−/− versus control ChopαβApoe−/− mice, and aortic explant–derived VSMCs from the VSMC-CHOP–deficient mice displayed reduced proliferation. Krüppel-like factor 4 (KLF4), a key suppressor of VSMC proliferation, was increased in lesions and aortic VSMCs from ChopαβSM22α-CreKI Apo−/− mice, and silencing Klf4 in CHOP-deficient VSMCs restored proliferation. CHOP deficiency in aortic VSMCs increased KLF4 through 2 mechanisms mediated by the endoplasmic reticulum stress effector activating transcription factor 4: transcriptional induction of Klf4 mRNA and decreased proteasomal degradation of KLF4 protein.

**Conclusions:** These findings in SM22α-CHOP–deficient mice imply that CHOP expression in SM22α VSMCs promotes cell proliferation by downregulating KLF4. The mechanisms involve newly discovered roles of CHOP in the transcriptional and post-translational regulation of KLF4. (Circ Res. 2015;116:1736-1743. DOI: 10.1161/CIRCRESAHA.116.305602.)

**Key Words:** activating transcription factor 4 ■ atherosclerosis ■ transcription factor CHOP ■ unfolded protein response ■ vascular smooth muscle cells

The inflammatory and vascular cells that populate atherosclerotic lesions are exposed to chronic endoplasmic reticulum (ER) stress, leading to persistent activation of the unfolded protein response (UPR).1–3 A key UPR pathway involves activation of protein kinase RNA-like ER kinase (PERK), which phosphorylates eukaryotic initiation factor 2α (eIF2α) and thereby suppresses protein translation. However, activating transcription factor 4 (ATF4) escapes this translational inhibition and is preferentially synthesized when the PERK–eIF2α pathway is activated.4 ATF4 induces its transcriptional target C/EBP homologous protein (CHOP/Ddit3), and if CHOP expression is chronically elevated because of prolonged ER stress, apoptosis can ensue.5 CHOP also induces the phosphatase coactivator growth arrest and DNA damage-inducible protein 34 (GADD34), which helps mediate the feedback dephosphorylation of eIF2α and suppression of ATF4.6 Recent causation studies using holo-CHOP knockout mice and transplantation with CHOP-deficient-bone marrow in mouse models of atherosclerosis strongly support a key role for CHOP in macrophage apoptosis and plaque necrosis.

In This Issue, see p 1725
advanced atherosclerotic lesions. These findings are consistent with studies showing strong positive correlations among CHOP expression, lesional cell death, and clinical plaque stage in human coronary artery and carotid lesions. In addition, a reverse bone marrow transplantation study in atherosclerosis-prone mice suggested that CHOP in non–bone marrow-derived lesional cells also promotes atherosclerosis.9 However, neither the identity of the nonmyeloid-derived cell type(s) in which CHOP plays a role nor the mechanisms or atherosclerosis-related consequences have been elucidated. In this context, we sought to determine the role of CHOP in vascular smooth muscle cells (VSMCs) in atherosclerosis using a new mouse model of VSMC-specific CHOP deficiency.

Methods
An expanded Methods, including description of statistical analyses, is available in the Online Data Supplement.

Briefly, Chop/Ddit3 floxed mice were generated as described in Results and in the Online Data Supplement. The mice were then crossed onto Apoe−/− and SM22α-CreKI mice. All mice were on the C57BL/6j background (the strain of generation for the floxed mice and at least 10 backcrosses for the others). For atherosclerosis studies, the mice were placed on a Western diet for 12 weeks. For ex vivo cell culture studies, VSMCs were cultured from aortic explants from the mice.

Results
VSMC-Specific CHOP Deficiency Reduces the Content of α-Actin–Positive Cells in Apoe−/− Atherosclerotic Lesions
Chopfl/fl mice were generated as depicted in Online Figure IA–ID (details are available in the Online Data Supplement). The mice were bred with Apoe−/− mice to generate Chopfl/fl–Apoe−/− mice, which were further bred with SM22α-CreKI mice to generate VSMC-specific Chop-deficient mice (Chopfl/fl SM22α-CreKI–Apoe−/−; Online Figure IIA). Note that SM22α-CreKI deletes floxed genes in adult SMCs and not in embryonic SMCs, thus increasing cell-type specificity.11 The expression of CHOP was evaluated in SMCs, endothelial cells, and macrophages from Chopfl/−Apoe−/− and Chopfl/− SM22α-CreKI–Apoe−/− mice before and after treatment with the UPR inducer tunicamycin. Tunicamycin increased CHOP expression in all 3 cell types from control mice but only in endothelial cells and macrophages from Chopfl/−SM22α-CreKI–Apoe−/− mice (Online Figure IIB). Thus, CHOP is specifically deleted in SMCs among these 3 atherosclerosis-relevant cell types.

After 12 weeks of Western diet feeding, Chopfl/−Apoe−/− and Chopfl/− SM22α-CreKI–Apoe−/− mice did not differ significantly in terms of body weight, blood glucose, total plasma cholesterol, high-density lipoprotein cholesterol, plasma triglycerides, and lipoprotein profile, or blood pressure (Online Figure III). Thus, atherosclerotic lesions could be evaluated in the absence of major changes in systemic metabolism. Quantitative analysis of aortic root lesions revealed a ≈30% reduction in lesion area (P=0.003) and a similar decrease in necrotic area (P=0.029) in Chopfl/−SM22α-CreKI–Apoe−/− versus Chopfl/apoe−/− mice, consistent with decreased plaque progression (Figure 1A).

Both lesional cell content and extracellular matrix contribute to lesion size. To determine whether a decrease in one or more of these parameters contributed to the reduced lesion size in Chopfl/− SM22α-CreKI–Apoe−/− mice, aortic root sections were stained with DAPI (4’,6-diamidino-2-phenylindole) (nuclei) to measure total cell content; immunostained for smooth muscle α-actin, F4/80, and CD11c to assess lesional content of α-actin VSMCs, macrophages, dendritic-like cells, respectively; and stained with Masson trichrome to assess collagen. The number of cells positive for α-actin, F4/80, and CD11c and the collagen content were all significantly decreased in Chopfl/−SM22α-CreKI–Apoe−/− lesions (Online Figure IVA–IVC), consistent with an overall decrease in lesion progression. However, only the number of lesional α-actin VSMCs was significantly lower in the VSMC-CHOP-deficient cohort when corrected for the decrease in total lesion area, whereas the decreases in F4/80+ macrophages, CD11c+ cells, extracellular matrix, and plaque necrosis were proportional to the decrease in overall lesion area (Figure 1B). One possible explanation for this finding is a hierarchical relationship in which SMC-CHOP deletion, by decreasing lesional SMCs, leads to a secondary decrease in overall lesion progression. Lesion size and SMC content were also lower in aortic arch lesions of Chopfl/−SM22α-CreKI–Apoe−/− mice than in Chopfl/apoe−/− mice, and en face lesion area in the descending aorta of the SMC-CHOP–deficient mice was lower as well (Online Figure VVA–VD). In contrast, the content of aortic wall (media) SMCs in nonatherosclerotic chow-fed Chopfl/−SM22α and Chopfl/− SM22α-CreKI+ mice was similar between the 2 groups of mice (Online Figure VVE).

VSMC-CHOP Deficiency Decreases the Proliferation of Aortic Explant–Derived VSMCs
The lower content of α-actin+ VSMCs in the lesions of Chopfl/− SM22α-CreKI–Apoe−/− mice could be because of increased VSMC death. However, few apoptotic VSMCs were detected in the lesions of either group, and apoptosis did not differ between the 2 groups of mice and showed no correlation with lesional VSMC count (Online Figure VIA–VIC). Therefore, we explored the possibility that CHOP deficiency decreases VSMC proliferation and found that the lesions of Chopfl/− SM22α-CreKI–Apoe−/− mice showed less Ki67+ VSMCs (Figure 1C), which is a marker of proliferating cells. Because Ki67 is not a dynamic marker of cell proliferation, we compared the growth curves of aortic explant–derived VSMCs from the 2 groups of mice using a standard protocol, namely, incubation of serum-starved VSMCs in medium containing 10% FBS. The growth rate of CHOP-deficient VSMCs was significantly lower than that of control VSMCs (Figure 1D).
These combined data suggest that at least 1 mechanism for the decrease in VSMCs in the lesions of Chopsm22α-CreKI/Apoε−/− mice is decreased proliferation.

Decreased Proliferation in CHOP-Deficient VSMCs Is Causally Associated With the Upregulation of Krüppel-Like Factor 4

We interrogated a microarray data set derived from tunicamycin-treated wild-type and CHOP-deficient murine embryonic fibroblasts for mRNAs that might be linked to VSMC proliferation. One of the genes increased in CHOP-deficient murine embryonic fibroblasts was Klf4. Krüppel-like factor 4 (KLF4) inhibits VSMC proliferation, and mice lacking KLF4 exhibit enhanced neointimal formation in response to vascular injury. Enhanced neointimal formation in response to vascular injury.13

To explore the relevance of these findings to the atherosclerosis data described here, we assayed KLF4 and α-actin in lesions from Chopsm22α−/− and Chopsm22α-CreKI/Apoε−/− mice using immunofluorescence microscopy. KLF4 is expressed in multiple cell types in atherosclerotic lesions, and so the goal was to compare KLF4 expression in α-actin+ VSMCs versus other cells in the lesions of the 2 groups of mice. We found that the lesions of Chopsm22α-CreKI/Apoε−/− mice had an increased content of KLF4-positive cells in the α-actin+ cell population, whereas the number of KLF4-positive cells in the population of α-actin− cells was similar between the 2 groups (Figure 2A). We then asked whether ER-stressed SMCs in aortic explants have higher levels of KLF4 when CHOP is absent. We found that tunicamycin induced Klf4 mRNA in both Chopsm22α-CreKI/Apoε−/− and Chopsm22α−/− aortic VSMCs, but induction was higher in the CHOP-deficient cells (Figure 2B). These data suggest that ER stress induces Klf4 expression in VSMCs but that CHOP fine-tunes this expression in a suppressive manner. Klf4 mRNA and KLF4 protein were also elevated in CHOP−/− and Chopsm22α-CreKI/Apoε−/− aortic VSMCs, but induction was higher in the CHOP-deficient cells (Figure 2B). These data suggest that ER stress inducers Klf4 expression in VSMCs but that CHOP fine-tunes this expression in a suppressive manner. Klf4 mRNA and KLF4 protein were also elevated in CHOP-deficient VSMCs under the conditions of the proliferation assay, which involves serum repletion after a period of serum starvation (Figure 2C). Most importantly, CHOP-deficient VSMCs subjected to siRNA-mediated silencing of KLF4 no longer showed a defect in proliferation after 4 days in culture (Figure 2D, left). Note that KLF4 expression was higher in CHOP-deficient (Cre+) SMCs at day 0 and day 2, while by day 4 KLF4 expression was lower in all groups and not different between the Cre- and Cre+ group (Figure 2D, right). These data suggest that the effect of KLF4 silencing on cell proliferation at day 4 was a delayed effect from events occurring earlier in the time course. These combined data suggest that at least 1 mechanism for the decrease in proliferation in CHOP-deficient VSMCs is an increase in KLF4.

Figure 1. C/EBP-homologous protein (CHOP) deletion in vascular smooth muscle cells (VSMCs) in Apoε−/− mice reduces the content of α-actin-positive cells in atherosclerotic lesions and suppresses VSMC proliferation. A, Representative images and quantitative analyses of aortic root lesion area and necrotic area in Chopsm22α−/− and Chopsm22α-CreKI/Apoε−/− mice (n=14). The lesions are delineated with a black line, and necrotic areas are shown by asterisks. B, Representative images of α-actin staining (green) and quantification of proportion of the α-actin-positive area in Chopsm22α−/− and Chopsm22α-CreKI/Apoε−/− aortic root lesions (n=8). The lesions are delineated with a red line. C, Representative Z series projection images of Ki67 (red) and α-actin staining (green) in Chopsm22α−/− and Chopsm22α-CreKI/Apoε−/− aortic root lesions (n=8). The top left image shows the DAPI+ cells in this projection. Scale bar, 50 μm. D, Proliferation curve of Chopsm22α−/− and Chopsm22α-CreKI/Apoε−/− VSMCs cultured in 10% FBS medium 0 to 4 days (D0–D4) after serum starvation (n=3–5).
ATF4 Is Elevated in CHOP-Deficient VSMCs and Mediates Increased KLF4 Expression

To study how CHOP suppresses Klf4 expression, we analyzed murine embryonic fibroblast chromatin-immunoprecipitation-sequencing data and found that ATF4 but not CHOP directly associates with the Klf4 promoter region.\(^\text{14}\) Therefore, we reasoned that CHOP might suppress Klf4 by downregulating ATF4, which could occur through the CHOP-GADD34 negative-feedback pathway that decreases p-eIF2α and thereby decreases ATF4 translation.\(^\text{5}\) Consistent with the hypothesis, CHOP deficiency was associated with a decrease in Gadd34 mRNA after 12 hours of tunicamycin treatment, and after 16 hours of treatment, there were increases in p-eIF2α protein, ATF4 protein, and Klf4 mRNA (Figure 3A). Moreover, the levels of nuclear ATF4 and KLF4 in VSMCs were also elevated by CHOP deficiency (Figure 3B). To link to the atherosclerosis data, we analyzed nuclear ATF4-KLF4 coexpression and found that SMC-CHOP deficiency was associated with a higher percentage of total lesional cells that coexpressed nuclear ATF4 and KLF4 and a higher percentage that coexpressed nuclear ATF4 and α-actin (Online Figure VII).

We next verified by chromatin-immunoprecipitation analysis that ATF4 directly associates with the Klf4 promoter. Genomic fragments containing the ATF4-binding region in the Klf4 promoter were significantly enriched in tunicamycin-treated Atf4−/− versus Atf4+/− murine embryonic fibroblasts (Figure 3C), consistent with the conclusion that ATF4 regulates Klf4 mRNA transcription. Most importantly, silencing ATF4 significantly reduced both Klf4 mRNA and KLF4 protein in tunicamycin-treated CHOP-deficient VSMCs (Figure 3D). The protein data show that the level of KLF4 in siAtf4-treated CHOP-deficient VSMCs was restored to the

Figure 2. Krüppel-like factor 4 (KLF4) is increased in α-actin–positive cells in Chop−/− or CreKI−/− lesions and is a mechanism of decreased vascular smooth muscle cell (VSMC) proliferation. A, Representative Z series projection images of KLF4 staining (red) and α-actin staining (green) in Chop−/− or CreKI−/− lesions and quantification of KLF4+ α-actin+ cells or KLF4− α-actin− cells in this projection. Scale bar, 100 μm. B, Klf4 mRNA relative to Actb in Chop−/− and Chop−/− or CreKI−/− VSMCs treated with 2.5 μg/mL tunicamycin for 12 hours (n=3). C, Klf4 mRNA relative to Actb (n=5, graph) and immunoblot of KLF4 and CHOP protein (n=6, graph) and immunoblot of KLF4 and CHOP protein in tunicamycin-treated Chop−/− and Chop−/− or CreKI−/− VSMCs cultured in 10% FBS medium for 24 hours after serum starvation. β-actin was included as loading control. D, Left, Proliferation curve of scrambled (Scr) or siKlf4–transfected Chop−/− or Chop−/− or CreKI−/− or CreKI−/− VSMCs cultured in 10% FBS medium 0 to 4 days after starvation (n=3; at day 4, Cre+ siKlf4 value is different from the Cre+ ScrRNA value at P=0.017). Right, KLF4 protein expression in Scr or siKlf4–transfected Chop−/− and Chop−/− or CreKI−/− or CreKI−/− or CreKI−/− VSMCs.
level in scrambled RNA-treated WT SMCs. These combined data support the hypothesis that an increase in ATF4 in CHOP-deficient VSMCs mediates an increase in KLF4. The reason why ATF4 is higher in the face of CHOP deficiency may be because of the increase in p-eIF2α, which in turn could be explained by the decrease in CHOP-induced GADD34.

CHOP Deficiency Also Decreases Proteasomal Degradation of KLF4 Under ER Stress Conditions

In theory, CHOP deficiency could raise KLF4 by also increasing the stability of Klf4 mRNA or KLF4 protein. The former possibility does not appear to be the case, as Klf4 mRNA expression after actinomycin D (ActD) treatment was not affected by CHOP deficiency in VSMCs (Figure 4A). However, KLF4 protein turnover was more rapid in WT versus CHOP-deficient VSMCs after treatment with cycloheximide, an inhibitor of protein biosynthesis (Figure 4B). KLF4 protein is targeted for degradation by the proteasome, 15 which we confirmed by showing that MG132, a proteasome inhibitor, inhibited KLF4 protein degradation in VSMCs (Figure 4C). Moreover, less ubiquitin was associated with KLF4 in tunicamycin-treated CHOP-deficient VSMCs (Figure 4D). In contrast to the situation with KLF4, the turnover of 2 other VSMC proteins, HIF1α (hypoxia-inducible factor 1α) and
Figure 4. Activating transcription factor 4 (ATF4) prevents proteasomal degradation of Krüppel-like factor 4 (KLF4) in vascular smooth muscle cells (VSMCs) under endoplasmic reticulum (ER) stress conditions. A, Chop<sup>fl/fl</sup>Apoe<sup>−/−</sup> and Chop<sup>fl/fl</sup>SM22α-CreKI<sup>+</sup>Apoe<sup>−/−</sup> VSMCs were treated with 2.5 μg/mL tunicamycin (Tm) for 16 hours and then with 5 μmol/L actinomycin D (ActD) for 0, 1, 2, 4, 6, and 8 hours, followed by assay of Klf4 mRNA (n=2). B, Similar to A, but the cells were treated with 10 μg/mL cycloheximide (CHX) instead of ActD and assayed for KLF4 protein. A representative immunoblot and the KLF4 degradation curve for 3 separate experiments are shown. C, Similar to B, but the cells were treated with 10 μmol/L MG132 instead of CHX. D, VSMCs from the 2 groups of mice were treated in the absence or presence of Tm for 16 hours, and then the cells were treated for 6 hours with MG132. Cell extracts were subjected to immunoprecipitation with anti-KLF4 or control IgG and then immunoblotted for ubiquitin or KLF4 (input). Also shown is a β-actin immunoblot for whole extract that was used for the KLF4 immunoprecipitation. E, As in B, but all the cells were Cre+, and they were transfected with either Scr- or Atf4 siRNA. A representative immunoblot and the KLF4 degradation curve for 3 separate experiments are shown. F, Working model of how C/EBP-homologous protein (CHOP) deficiency increases KLF4 expression and thereby suppresses proliferation in VSMCs. Left, Under ER stress, protein kinase RNA-like ER kinase (PERK) activation induces eukaryotic initiation factor 2α (eIF2α) phosphorylation and translational activation of ATF4. In control Chop<sup>fl/fl</sup>Apoe<sup>−/−</sup> VSMCs (left), ATF4 activation induces CHOP expression, which in turn induces Gadd34. Growth arrest and DNA damage-inducible protein 34 (GADD34) dephosphorylates eIF2α, leading to decreased ATF4 translation (dotted lines). Right, The translational activation of ATF4 is preserved in Chop<sup>fl/fl</sup>SM22α-CreKI<sup>+</sup>Apoe<sup>−/−</sup> VSMCs because of lack of the GADD34 feedback pathway. Persistent ATF4 expression induces KLF4 by increasing its mRNA expression and reducing its proteasomal degradation, leading to inhibition of VSMC proliferation.
Nrf2 (nuclear factor erythroid-derived 2-related factor 2), was not altered by CHOP deficiency although both proteins are also targeted for degradation by the proteasome (Online Figure VIII). Most importantly, silencing ATF4 markedly increased KLF4 protein turnover in tunicamycin-treated CHOP-deficient VSMCs (Figure 4E). These data suggest that the increase in ATF4 in CHOP-deficient VSMCs raises KLF4 by 2 distinct mechanisms: transcriptional induction via ATF4 binding to the Klf4 promoter and protein stabilization via suppression of KLF4 proteasomal degradation. The molecular mechanism of the latter process remains to be determined.

**Discussion**

Using a newly created mouse model of cell-specific CHOP deletion, the data in this report show that CHOP deficiency in SM22α+ VSMCs leads to a decrease in α-actin+ VSMC content in atherosclerotic lesions in Apareo−/− mice. The proposed mechanism involves, in part, a fine-tuning module based on the suppression of ATF4 by the CHOP-GADD34-eIF2α dephosphorylation pathway and the increase in KLF4 by ATF4 (Figure 4F). Interestingly, there are examples of CHOP-ATF4 heterodimer affecting gene transcription,16 and additional work will be required to determine whether this complex plays a role in Klf4 regulation in ER-stressed SMCs. In addition, ATF4 suppresses the proteasomal degradation of KLF4 by a mechanism yet to be elucidated.

Decreased lesional SMCs in SMC-CHOP−/− mice was associated with features of decreased lesion progression, namely, lower numbers of inflammatory cells, plaque necrosis, and collagen. How a decrease in intimal SMCs might lead to lower inflammatory cells remains to be determined. Other studies have suggested that lesional VSMCs can regulate lesion development by affecting lipid content and by retaining and promoting the survival of lesional inflammatory cells.17 Moreover, a recent study provided evidence that VSMCs can be transformed into macrophage-like cells in atherosclerotic lesions,18 but whether VSMC-CHOP affects this transformation process remains to be determined. Nonetheless, the data herein suggest that uncontrolled elevation of VSMC content in lesions can contribute to lesion progression, whereas VSMC-CHOP deficiency may suppress this process by inhibiting VSMC proliferation.

The impact of the decrease in collagen content in the SMC-CHOP−/− lesions is an interesting topic. On the one hand, this decrease could lead to thinner fibrous caps, which is a sign of advanced plaque progression.19 However, fibrous cap thickness was similar in the aortic root lesions of the 2 groups of mice (data not shown). On the other hand, a decrease in lesional matrix might lessen the pathological process of arterial stiffening.20 Along these lines, it is interesting to note that silencing XBP1, a UPR effector that can have opposing actions to CHOP in the setting of prolonged ER stress, enhanced VSMC proliferation in vitro and promoted neointima formation in a murine wire injury model.21 Pending future studies to explore these and other issues, the findings in this report, together with previous studies showing the role of macrophage CHOP in advanced lesional apoptosis and plaque necrosis,7,9 help form a more complete picture of the cell-specific roles of the UPR and its effector CHOP in atherosclerosis.

**Acknowledgments**

We thank Dr Robert Clark, University of Michigan, for assistance in creating CreIAP mice; Dr Gary K. Owens, University of Virginia, for helpful discussions and for supplying the SM22α-CreKI mice (created by Zhang et al11); and Dr Michael S. Kilberg, University of Florida, for the activating transcription factor 4 antibody used in the chromatin-immunoprecipitation experiment.

**Sources of Funding**

This work was supported by a postdoctoral fellowship from the Swedish Research Council to A.-X. Zhou and National Institutes of Health grants HL75662 and HL57560 to I. Tabas and DK042394, DK088227, and HL052173 to R.J. Kaufman.

**Disclosures**

None.

**References**


Zhou et al. CHOP Regulates Smooth Muscle Cell Proliferation

What Is Known?

- Smooth muscle cells (SMCs) in atherosclerotic lesions are the major source of collagen and elastin that forms a protective fibrous cap, but they may also contribute to inflammation and plaque progression.
- A particular signaling molecule called Krüppel-like factor 4 acts as a suppressor of SMC proliferation in atherosclerotic lesions.
- Cells in human and murine atherosclerotic lesions are known to undergo chronic endoplasmic reticulum (ER) stress, which is probably because of their exposure to certain types of lipids that affect ER function and to factors that promote intracellular oxidative stress.

What New Information Does This Article Contribute?

- Using a new mouse model in which the ER stress effector C/EBP-homologous protein (CHOP) was specifically deleted in SMCs, the study shows for the first time that CHOP regulates SMC proliferation.
- Deletion of CHOP in SMCs in an atherosclerosis-prone mouse model resulted in atherosclerotic lesions that had less SMCs and less plaque progression.

Novelty and Significance

- CHOP deficiency lowers SMC proliferation through feedback induction of the ER stress effector ATF4, which then transcriptionally induces Krüppel-like factor 4 and decreases its degradation.

Cells in both human and murine atherosclerotic lesions undergo chronic ER stress, but the effect of ER stress in lesional SMCs was not known. We deleted the ER stress effector CHOP in SMCs in a mouse model of atherosclerosis and found a decrease in both lesional SMC content and overall lesion progression. CHOP deletion suppressed SMC proliferation through upregulation of Krüppel-like factor 4. The mechanism involved a feedback increase in ATF4, which both enhanced the transcription of Klf4 mRNA and stabilized Krüppel-like factor 4 protein. These findings reveal a role and novel mechanism of CHOP in the progression of atherosclerotic lesions.
C/EBP-Homologous Protein (CHOP) in Vascular Smooth Muscle Cells Regulates Their Proliferation in Aortic Explants and Atherosclerotic Lesions
Alex-Xianghua Zhou, Xiaobo Wang, Chyuan Sheng Lin, Jaeseok Han, Jing Yong, Marissa J. Nadolski, Jan Borén, Randal J. Kaufman and Ira Tabas

Circ Res. 2015;116:1736-1743; originally published online April 14, 2015; doi: 10.1161/CIRCRESAHA.116.305602

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/116/11/1736

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/04/14/CIRCRESAHA.116.305602.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Detailed Methods

**Generation of Chop^{fl/fl} (Ddit3^{fl/fl}) mice in the C57BL/6J background**

Using a bacteria artificial chromosome (BAC), exon 3 of the Ddit3 gene was flanked with a LoxP (L83) site and a Frt-Neo-Frt-Loxp (FNFL) cassette. The following three elements were then ligated into a gene-targeting vector carrying the DTA (diphtheria toxin alpha chain) negative-selection marker: a 2-kb short homology arm (5' to L83), the above LoxP-exon 3-FNFL sequence, and a 5-kb long homology arm (end of FNFL to 3'). The FNFL cassette conferred G418 resistance during gene targeting in CSL2J2 ES cells (derived from B6(Cg)-Tyr^{−/−}J mice; JAX Mice Stock number: 000058), and the DTA cassette provided an autonomous negative selection strategy to reduce random integration events during gene targeting. Several targeted ES cells were identified and injected into C57BL/6J blastocysts to generate chimeric mice. Male chimeras were bred to ACTB (Flpe/Flpe) females in the C57BL/6J background to transmit through germ line the floxed Ddit3 allele, i.e., L83/FL146 allele with neo cassette removed by Fple recombinase (Online Figure I). The ES cells and mice carrying the floxed Ddit3 allele were genotyped with the primers designated in Online Table I. Founder mice heterozygous for the Chop floxed allele were further cross-bred to generate littermates homozygous for Chop floxed gene (Chop^{fl/fl} mice).

**Verification of successful Chop gene deletion in vivo**

Chop^{fl/fl} mice were challenged with adenovirus encoding the Cre recombinase through tail vein injection, followed by treatment with Tm 2 mg/kg for 8 h. Protein and DNA were prepared from liver and/or spleen. Deletion of the exon 3 of Chop gene was verified by gel electrophoresis and sanger sequencing of the PCR product from liver genomic DNA using primers (Online Table I) amplifying the region flanked by the two LoxP sites; deletion of CHOP protein in liver was verified by western blotting.

**Generation of Chop^{fl/fl} Apoe^{−/−} and Chop^{fl/fl} SM22α-CreK1* Apoe^{−/−} mice and diets**

To generate Chop^{fl/fl}Apoe^{−/−} mice, the Chop exon 3-floxed (Chop^{fl/fl}) mice were bred with Apoe^{−/−} mice (in C57BL/6J background; JAX Mice Stock number: 002052) to introduce an atherosclerosis-prone genetic background. To generate SMC-specific Chop-deficient
mice, Chop\textsuperscript{fl/fl}Apo\textsuperscript{e−/−} mice were further bred with a SM22α-CreKI transgenic mice in C57BL/6J background.\textsuperscript{1} Distinct from endogenous SM22α expression, SM22α-CreKI mice express Cre recombinase in adult but not embryonic SMCs.\textsuperscript{1} Starting at 6 wks of age, male Chop\textsuperscript{fl/fl}Apo\textsuperscript{e−/−} and Chop\textsuperscript{fl/fl}SM22α-CreKI\textsuperscript{+}Apo\textsuperscript{e−/−} mice were fed a 21.2% fat, 0.2% cholesterol Western-type diet (Harlan Teklad, Madison, WI; catalog #TD88137) for 12 wks.

**Blood glucose and plasma lipid analysis**
At the end of the study, the mice were fasted overnight. A drop of blood was collected from mouse tail, and blood glucose was measured using a glucose meter (One Touch Ultra, Lifescan). Mice were then weighed, anesthetized using isoflurane, and euthanized. Blood was collected from the left ventricle for lipid analysis. Total plasma triglyceride and cholesterol and HDL-cholesterol were measured with a commercially available kit from Wako. Plasma lipoprotein profiles were determined by fast performance liquid chromatography (FPLC) gel filtration on a Superose 6 column at a flow rate of 0.2 ml per minute. Eluted fractions were assayed for cholesterol. All mouse protocols were approved by the Columbia University Institutional Animal Care and Use Committee.

**Atherosclerotic lesion analysis**
Mouse hearts were perfused \textit{in-situ} with saline and removed. The aortic root or aortic arch was placed in OCT medium and immediately frozen. Using a cryomicrotome, sections were cut serially at 8-μm intervals starting from the aortic sinus and then mounted on slides. For lesion area analysis, the sections were stained with eosin and hematoxylin, and intimal lesion area was quantified by averaging measurements from 6 sections, 50 μm apart, using a Nikon Labophot 2 microscope and Image Pro Plus image analysis software. To measure \textit{en face} aortic lesion area, the thoracic aorta was dissected from the heart and surrounding tissues and the adventitial fat tissue was cleaned and incubated overnight in 10%-buffered formalin. The aorta was then splayed open longitudinally under a dissecting microscope and pinned to silicone-coated plates, and Oil red O staining was performed. Images were acquired, and Oil red O–positive area was analyzed by applying a color threshold in Adobe Photoshop.
Measurement of Blood Pressure
Blood pressure was measured using a noninvasive computerized tail–cuff system (Kent Scientific Corporation, CT, USA). After the mice were placed in a plastic holder, the occlusion and sensor cuff were positioned on the base of the tail. After the mice were given time to adapt to the system, blood pressure was measured for at least three consecutive times in each mouse.

Immunochemistry and microscopy
Immunochemistry on frozen aortic root sections were performed as described previously. Briefly, frozen sections were fixed in ice-cold acetone for 5 min and then blocked with normal serum. Sections were labeled with unconjugated primary antibodies against smooth muscle α-actin (Sigma-Aldrich), CD11c, F4/80 (BD biosciences), Ki67 (Cell signaling), KLF4 (Millipore, or R & D Systems), ATF4 (Santa Cruz or Cell signaling), or activated caspase 3 (Cell Signaling) overnight, followed by fluorophore-conjugated secondary antibody for 1 h. The stained sections were mounted with DAPI-containing Vectorshield mounting medium (Vector) and then viewed using an Olympus IX 70 fluorescence microscope. The images were analyzed using ImageJ. Confocal microscopy was conducted using a Nikon Ti Eclipse inverted microscope. The Z series was obtained by imaging serial confocal planes (3 planes at 1.2-µm intervals) at 1024 x 1024 pixel resolution with a Nikon 20x objective. Projections of the Z-stacks were generated, and co-localization analysis was performed by NIS software (Nikon, NY, USA).

Masson’s trichrome staining
Paraffin-embedded aortic root sections were de-paraffinized and then stained with a Masson’s trichrome stain kit from Sigma-Aldrich. Collagen, which was stained with Aniline blue in the kit, was quantified using ImageJ.

Isolation and culture of mouse aortic vascular smooth muscle cells (VSMCs)
VSMCs were isolated from mouse thoracic aorta by an enzymatic digestion method. Cells were cultured in DMEM/F12 medium (GIBCO) supplemented with 20% FBS, penicillin/streptomycin 100U/ml before passage 6. After passage 6, cells were cultured with 10% FBS medium and used between passage 6 and 10. For serum starvation, cells were cultured with DMEM/F12 supplemented with L-glutamine 1.6 mM (Gibco), L-
ascorbic acid 0.2 mM (Sigma-Aldrich), transferrin 5 µg/ml (Sigma-Aldrich), insulin 2.8 µg/ml (Gibco), penicillin/streptomycin 100 U/ml (Gibco), and Na-selenate 6.25 ng/ml (Sigma-Aldrich) for 2-3 days.

**Isolation and culture of mouse lung endothelial cells (ECs)**
ECs were isolated from mouse lung after digestion with collagenase as described previously. Briefly, after two passages of total lung cell culture, ECs were isolated using magnetic Dynabeads (Invitrogen) conjugated with ICAM-2 antibody (BD Pharmingen). Cells were cultured in DMEM low glucose medium (GIBCO) supplemented with 10% horse serum, penicillin/streptomycin 100U/ml, L-glutamine 1.6 mM, ECGS 50 µg/ml (Biomedical Technologies), and heparin 100 µg/ml (Sigma-Aldrich). The first two passages of ECs were used in experiments.

**Isolation and culture of mouse bone marrow-derived macrophages**
Bone marrow-derived macrophages were separated as described previously. To prepare bone marrow-derived macrophages, both ends of femurs were removed, and the marrow was flushed with RPMI medium. The flushed cells were placed through a strainer to remove debris and then pelleted by centrifugation. The cells were re-suspended in DMEM containing 20% L-cell-conditioned medium, cultured for 4-5 days, and then used for the experiments.

**In vitro proliferation assay**
VSMC proliferation was measured as previously described. Briefly, serum-starved VSMCs were incubated with cell culture medium with 10% FBS at 37°C in a CO₂ incubator. After culture for 0-4 days after serum starvation, cells were collected by trypsin digestion and counted with a hemocytometer.

**Immunoblotting**
Cells were lysed in a buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% bromphenol blue and boiled at 100°C for 5 min. Lysate protein was separated on 4-20% gradient SDS-PAGE gel (Invitrogen) and electrotransferred to 0.4-µm PVDF membrane. The membranes were blocked for 1 h at room temperature in Tris-buffered saline and 0.1% Tween 20 (TBST) containing 5% (wt/vol) nonfat milk. The membranes were then incubated with primary antibody against
Chop (Santa Cruz), β-actin (Santa Cruz), KLF4 (Millipore), p-eIF2α and eIF2α (Cell Signaling), ATF4 (Santa Cruz), or GAPDH (Cell signaling) in TBST containing 5% nonfat milk at 4°C overnight, and the protein bands were detected with horse radish peroxidase-conjugated secondary antibodies (Cell signaling) and Supersignal West Pico enhanced chemiluminescence solution (Pierce).

**Quantitative RT-qPCR**

Total RNA was extracted from HCs using the RNeasy kit (Qiagen). cDNA was synthesized from 2 μg total RNA using oligo (dT) and Superscript II (Invitrogen). qPCR was performed in an 7500 Real time PCR system (Applied Biosystems) using SYBR green chemistry. QuantiTect primers for Klf4, Gadd34, Hprt, and Actb were used (Qiagen).

**siRNA-mediated gene silencing**

siRNA sequences against murine Klf4, Atf4 and scrambled RNA, were purchased from Qiagen; the target sequence of Klf4 siRNA was CCGGTTTATATTGAATCCAAA, the target sequence of Atf4 siRNA was GACUGAGAAAUUGGAAAGACTG. The scrambled RNA and siRNA were transfected into SMCs using the basic nucleofector kit for primary SMCs and Amaxa nucleofector (Lonza) according to the manufacturer’s instruction.

**Chromatin immunoprecipitation (ChIP)**

Atf4+/+ and Atf4-/- MEFs were treated with 2 μg/ml tunicamycin (Sigma-Aldrich) for 8 h, followed by cross-linking with 1% formaldehyde for 10 min and subsequent chromatin immunoprecipitation as described, using anti-ATF4 antibody (gift from Dr. Michael S. Kilberg, University of Florida College of Medicine). DNA immunoprecipitates were analyzed by RT-qPCR using the following primers: Klf4 intron1 (5'- CTC AGC TAA CAC CAA GGT AAG A-3' and 5'- TGT CCC AGT GTC CCA ATT C-3'), pKlf4 #1 (5'- AGT TCC AGC TCA CAA CTC ATC-3' and 5'- TAA CTC TTC TGG CCC TAC CT-3'), pKlf4 #2 (5'- CAA CAG CCT TCT GGA GGA TAA A-3' and 5'- GGA TGA GTT GTG AGC TGG AA-3'), pKlf4 #3 (5'- AGA TGA ATT GGA GAG CCA CTA TT-3' and 5'- AGA AGG CTG TTG GGA AAC TT-3'), and Trb3 (5'- aac tca acg atc cac ctg cct ct-3' and 5'- aac ctg agc taa tct gct gct gac-3'). Data were plotted as the ratio to the value obtained with 1:20 dilution of input DNA.
Ubiquitin assay
For immunoprecipitation, VSMCs from one 100-mm dish were infected with adenovirus encoding Klf4. After 3 days, the cells were treated with MG132 for 6 h and then lysed in IP buffer (1.2 ml of 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10% glycerol, 1% NP-40, 12.5 mM N-ethylmaleimide). The lysate was mixed with 5 µg anti-KLF4 antibody and rotated at 4°C overnight, followed by adding 100 µl protein G beads and rotating for additional 2 hours at 4°C. The beads were washed three times by vortexing in IP buffer, followed by centrifugation for 5 s at 10 000 x g. The IP pellet was boiled in 2X SDS sample buffer and subjected to immunoblot analysis.

Statistical Analysis
Data are displayed as mean ± S.E.M., with the n number given in the individual figure legends. After the data were shown to fit a normal distribution, the unpaired t-test was used to assess the statistical significance of the differences in experiments with two groups, and two-way ANOVA was used for analyses in multi-group experiments.

References
Online Table I. Primers used for genotyping and verification of Chop floxed mice.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ddit3screen1F</td>
<td>GCTTTTCCCCATTCCATTCCTAAACA</td>
</tr>
<tr>
<td>Ddit3screen1R</td>
<td>CCTCGAGGGACCTAATAACTTCGTA</td>
</tr>
<tr>
<td>Ddit3LNL5F</td>
<td>AAGTGCATCTTCATACACCACCACA</td>
</tr>
<tr>
<td>Ddit3LNL3R</td>
<td>TCCCAAGTGCTGGGACTAAAGGTAT</td>
</tr>
<tr>
<td>Ddit3FNFL5F</td>
<td>GAGGTGAGTGAGAATGCTGGTCCTCA</td>
</tr>
<tr>
<td>Ddit3FNFL3R</td>
<td>TTCTTCCTTGCTCTCCTCCTCTCTTC</td>
</tr>
<tr>
<td>PCR verification</td>
<td>Forward: CCTTCAGTCAGCTGGCAAT</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCATTTCCCTCGATGCTTA</td>
</tr>
<tr>
<td>Sanger sequencing</td>
<td>Forward: AGCCGATCATATTCAATAACCCTTA</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACCATTTCCCTCGATGCTA</td>
</tr>
</tbody>
</table>
Online Figure Legends

Online Figure I. Generation of Chop<sup>fl/fl</sup> mice and VSMC-targeted CHOP-deficient mice on the Apoe<sup>−/−</sup> background. A, Scheme of Chop DNA and insertion of 2 LoxP sites flanking exon 3 and Southern blots confirming genotypes of positive ES cells and the indicated mice. B, Deletion of Chop gene in liver by ectopic expression of Cre recombinase (Ad-Cre) efficiently abolished CHOP protein expression in response to tunicamycin (Tm) administration (i.p., with mice sacrificed 8 h post injection), while PBS-sham injected Chop<sup>fl/fl</sup> mice displayed CHOP protein induction at the predicted molecular weight of CHOP (~29 kDa). Lv, liver; Sp, spleen. C, A 1.2 kb PCR product corresponding to the LoxP-recombined genomic sequence was evident in the liver of adeno-Cre administered mouse, while the product was absent in the PBS sham-injected Chop<sup>fl/fl</sup> mice. D, Sanger sequencing of the PCR product confirmed LoxP insertion sites at the predicted locations, with the left arm inserted in the second intron of the Chop gene and the right arm inserted in the third intron. The underlined nucleotides represent the intronic region endogenous to the Chop gene, along with the adjacent nucleotides of the inserted LoxP sites (non-essential sequences not shown). The sequencing result also confirmed intact exon 4 sequence of the Chop gene demonstrating precise excision of exon 3 upon Cre recombinase expression (data not shown).

Online Figure II. Generation of VSMC-targeted CHOP-deficient mice on the Apoe<sup>−/−</sup> background. A, Breeding scheme for generating Chop<sup>fl/fl</sup> Apoe<sup>−/−</sup> and Chop<sup>fl/fl</sup> SM22α-CreKI<sup>+</sup> Apoe<sup>−/−</sup> mice. B, Immunoblots of CHOP in aortic VSMCs, lung ECs, and bone marrow-derived macrophages (Mφ) isolated from Chop<sup>fl/fl</sup> Apoe<sup>−/−</sup> and Chop<sup>fl/fl</sup> SM22α-CreKI<sup>+</sup> Apoe<sup>−/−</sup> mice before and after 12 h tunicamycin (Tm) treatment. β-actin was used as loading control.

Online Figure III. Body weight and systemic metabolic factors are similar in Chop<sup>fl/fl</sup> Apoe<sup>−/−</sup> and Chop<sup>fl/fl</sup> SM22α-CreKI<sup>+</sup> Apoe<sup>−/−</sup> mice. A-E, Body weight, fasting blood glucose, plasma triglyceride, plasma cholesterol, and HDL cholesterol of Western diet-fed male Chop<sup>fl/fl</sup> Apoe<sup>−/−</sup> and Chop<sup>fl/fl</sup> SM22α-CreKI<sup>+</sup> Apoe<sup>−/−</sup> mice (n=14). F, Pooled plasma samples were subjected to fast performance liquid chromatography gel filtration, and the fractions were assayed for cholesterol concentration. G-H, Systolic and diastolic blood pressure in 12-wk WD-fed Chop<sup>fl/fl</sup> Apoe<sup>−/−</sup> and Chop<sup>fl/fl</sup> SM22α-
CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} mice (n=5). None of the differences between the two groups of mice was statistically significant.

**Online Figure IV.** CHOP deletion in VSMCs in Apoe\textsuperscript{−/−} mice reduces the content of F4/80\textsuperscript{+} macrophage, CD11c\textsuperscript{+} cells, and collagen in aortic root lesions.

Aortic root lesions of Chop\textsuperscript{fl/fl} Apoe\textsuperscript{−/−} and Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} mice were immunostained for F4/80 or CD11c (A-B) or stained with Masson trichrome and analyzed for Aniline blue (collagen) staining (C). Total stained area and stained area per lesion area were quantified (n=8).

**Online Figure V.** CHOP deletion in VSMCs in Apoe\textsuperscript{−/−} mice reduces the content of α-actin\textsuperscript{+} cells in aortic arch lesions, but not non-atherosclerotic aorta, and decreases lesion size in both the aortic arch and descending aorta. A, Representative images of aortic arch cross-sections in Chop\textsuperscript{fl/fl} Apoe\textsuperscript{−/−} and Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} mice. B, Quantitative analyses of lesion area in the aortic arch (n=4). C, Quantitative analyses of α-actin\textsuperscript{+} cells in aortic arch lesions (n=4). D, Representative en face images and quantification of Oil Red O-stained descending aorta from Chop\textsuperscript{fl/fl} Apoe\textsuperscript{−/−} and Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} mice (n=3). E, Quantification of α-actin\textsuperscript{+} cells in the aortic wall of non-atherosclerotic chow-fed Chop\textsuperscript{fl/fl} and Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} mice (n=6; n.s., non-significant).

**Online Figure VI.** Apoptosis of α-actin\textsuperscript{+} cells is similar in aortic root lesions of Chop\textsuperscript{fl/fl} Apoe\textsuperscript{−/−} and Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} mice. A, Representative images of DAPI (blue), α-actin (green), and active-caspase 3 (red) staining in Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} lesions. Arrow, active caspase 3 staining that coincides with an α-actin\textsuperscript{+} cell. Bar, 20 μm. B, Quantification of α-actin/active caspase 3 double-positive lesional cells. C, Correlation analysis of α-actin/active Casp3 double-positive cells with total lesional VSMCs. For B-C, n=8 per cohort.

**Online Figure VII.** VSMC-CHOP deficiency is associated with a higher percentage of total lesional cells that co-stain for nuclear ATF4 and KLF4 and of α-actin\textsuperscript{+} lesional cells that stain for nuclear ATF4. A, Representative Z series projection images of KLF4 (Green), ATF4 (Red), and DAPI (blue) staining in Chop\textsuperscript{fl/fl} SM22a-CreKI\textsuperscript{+} Apoe\textsuperscript{−/−} lesions and percentage of KLF4/ATF4/DAPI triple-stained nuclei in 9
**Chop**\(^{-/-}\)\(\text{Apoe}^{-/-}\) and 11 **Chop**\(^{-/-}\)\(\text{SM22a-CreKI}\)\(\text{Apoe}^{-/-}\) lesions. Arrow, KLF4/ATF4/DAPI triple-stained nuclei. **B,** Representative Z series projection images of ATF4 (red), and α-actin (green), and DAPI (blue) staining in **Chop**\(^{-/-}\)\(\text{SM22a-CreKI}\)\(\text{Apoe}^{-/-}\) lesions and percentage of ATF4/α-actin/DAPI triple-stained cells in 10 **Chop**\(^{-/-}\)\(\text{Apoe}^{-/-}\) and 8 **Chop**\(^{-/-}\)\(\text{SM22a-CreKI}\)\(\text{Apoe}^{-/-}\) lesions. Arrow, ATF4/α-actin/DAPI triple-stained cells. Scale bar, 100 μm.

**Online Figure VIII.** Proteasome-mediated degradation of HIF1α and NRF2 is similar in control and CHOP-deficient VSMCs. **Chop**\(^{-/-}\)\(\text{Apoe}^{-/-}\) and **Chop**\(^{-/-}\)\(\text{SM22a-CreKI}\)\(\text{Apoe}^{-/-}\) VSMCs were pre-treated with 2.5 μg/ml tunicamycin (Tm) for 16 h and then incubated for 1-8 h with either 10 μg/ml cycloheximide (CHX) (**A, B**) or MG132 (**C, D**). Cell extracts were then immunoblotted for HIF1α (**A, C**) and NRF2 (**B, D**). β-actin was included as loading control.
Online Figure II
Online Figure III

A. Body Weight (g)

B. Glucose (mg/dl)

C. Plasma triglyceride (mg/ml)

D. Plasma cholesterol (mg/ml)

E. HDL cholesterol (mg/ml)

F. Cholesterol (mg/fraction)

G. Systolic blood pressure (mm Hg)

H. Diastolic blood pressure (mm Hg)
Online Figure IV
Online Figure V

**A**

Comparison of Cre- and Cre+ samples, showing differences in aortic lesions.

**B**

Bar graph showing lesion area with statistical significance (P<0.0001).

**C**

Bar graph showing percentage of α-Actin+ DAPI+ cells with statistical significance (P=0.004).

**D**

Comparison of Cre- and Cre+ samples, showing differences in Oil red O positive area (P=0.03).

**E**

Comparison of non-atherosclerotic aorta with no significant difference (n.s.).
Online Figure VI

A

\(\alpha\)-actin

DAPI

Active Casp3

Merge

B

\(\alpha\)-actin+/act\-Casp3+ cells per lesion

Cre- Cre+

C

\(\alpha\)-actin+ cells per lesion

\(\alpha\)-actin+/act-Casp3+ cells per lesion

Online Figure VI
Online Figure VII

(A) KLF4 and ATF4 expression in atheroma,merge and DAPI staining. 

(B) α-Actin and ATF4 expression in atheroma, merge, and DAPI staining.
Online Figure VIII