Transcription Factor Runx2 Promotes Aortic Fibrosis and Stiffness in Type 2 Diabetes Mellitus

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**Rationale:** Accelerated arterial stiffening is a major complication of diabetes mellitus with no specific therapy available to date.

**Objective:** The present study investigates the role of the osteogenic transcription factor runt-related transcription factor 2 (Runx2) as a potential mediator and therapeutic target of aortic fibrosis and aortic stiffening in diabetes mellitus.

**Methods and Results:** Using a murine model of type 2 diabetes mellitus (db/db mice), we identify progressive structural aortic stiffening that precedes the onset of arterial hypertension. At the same time, Runx2 is aberrantly upregulated in the medial layer of db/db aortae, as well as in thoracic aortic samples from patients with type 2 diabetes mellitus. Vascular smooth muscle cell–specific overexpression of Runx2 in transgenic mice increases expression of its target genes, *Col1a1* and *Col1a2*, leading to medial fibrosis and aortic stiffening. Interestingly, increased Runx2 expression per se is not sufficient to induce aortic calcification. Using in vivo and in vitro approaches, we further demonstrate that expression of Runx2 in diabetes mellitus is regulated via a redox-sensitive pathway that involves a direct interaction of NF-κB with the Runx2 promoter.

**Conclusions:** In conclusion, this study highlights Runx2 as a previously unrecognized inducer of vascular fibrosis in the setting of diabetes mellitus, promoting arterial stiffness irrespective of calcification. (Circ Res. 2015;117:513-524.
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**Key Words:** arterial stiffness ■ diabetes mellitus ■ extracellular matrix ■ oxidative stress ■ smooth muscle cell

**Abstract**

Diabetes mellitus is a growing health problem throughout the world. The prevalence of diabetes mellitus was estimated to be 171 million people in the year 2000 and is predicted to more than double to over 366 million people by the year 2030.1 Largely unknown in the early 20th century, type 2 diabetes mellitus is now the 7th leading cause of death in the United States.2

Cardiovascular disease is the main cause of death in type 2 diabetes mellitus.3 One of the mechanisms linking diabetes mellitus to increased cardiovascular risk may be accelerated arterial stiffening that is frequently observed in diabetic patients.4 In fact, arterial stiffness predicts the development of cardiovascular disease and mortality in the general population and in type 2 diabetes mellitus.5,6

Arterial stiffening may occur as an adaptive response to hypertension, maintaining vascular mechanical stress homeostasis.7 Conversely, the lack of Windkessel function associated with aortic stiffening may generate elevated pulse pressure, thereby contributing to hypertension.8 Because of the high prevalence of coexistent hypertension in the diabetic population (≈70%),9 it is difficult to discern whether aortic stiffening is primarily caused by hypertension or occurs as an independent pathology and should therefore be diagnosed and treated independently. To this end, animal models may help study the relationship between arterial stiffness and hypertension.

The mechanical properties of the aorta, being a conduit vessel, are largely defined by its passive (structural) stiffness,
which in turn is mainly determined by the extracellular matrix (ECM) components of the vessel wall.\textsuperscript{10–12} Fibrillar collagen (type 1 and type 3) and elastin represent the major ECM proteins of the aorta. Although elastin is a relatively inert protein, collagen is readily remodeled in response to various stimuli.

The osteogenic transcription factor runt-related transcription factor 2 (Runx2; Cbfa1) may qualify as a mediator of ECM remodeling and aortic stiffening in the context of diabetes mellitus. Runx2 was initially identified as an activator of osteoblast differentiation during embryonic development.\textsuperscript{13} In postnatal life, however, it serves as a physiological transcriptional regulator of bone matrix protein deposition (such as type I collagen) in osteogenic tissues.\textsuperscript{14} More recently, Runx2 received attention in vascular biology, emerging as a marker of vascular smooth muscle cell (VSMC) transdifferentiation toward an osteogenic phenotype and contributing to the active vascular calcification found in athero- and arteriosclerosis.\textsuperscript{15–17} VSMC-specific knockout experiments demonstrated functional involvement of Runx2 in murine atherosclerotic calcification.\textsuperscript{17} However, the intermediate function of Runx2 in the vascular system—in particular as a potential regulator of matrix protein expression and arterial stiffness—is unknown.

This study tests the hypothesis that structural aortic stiffening occurs independently from arterial hypertension in a murine model of type 2 diabetes mellitus. We further use a VSMC-specific transgenic approach to investigate the potential of Runx2 to induce aberrant aortic matrix production, resulting in increased stiffness and hypertension. Finally, we delineate a novel signaling mechanism potentially leading to enhanced Runx2 expression in VSMC under diabetic conditions (reactive oxygen species [ROS]–nuclear factor kappa beta [NF-κB]–Runx2 pathway).

Methods

An expanded Methods section is available in the Online Data Supplement.

Animals

Male db/db mice (BKS.Cg-Dock7m+/+Leprdb/J) and their age-matched heterozygous nondiabetic controls (+/db) were purchased from the Jackson Laboratory (Bar Harbor, ME).

Transgenic mice with VSMC-specific Runx2 overexpression (Runx2-smTg; C57Bl/6 background) were generated at our institution by pronuclear injection of a transgene cassette consisting of the entire coding region of mouse type 1 Runx2 driven by the 445 bp form of the SM22α promoter.

Pressure Myography

Pressure myography was performed to directly assess the passive aortic mechanics according to adapted protocols.\textsuperscript{18} In brief, murine thoracic aortae were explanted and placed on specially designed stainless steel cannulas. Subsequently, the artery was pressurized from 0 to 180 mmHg in 18 mmHg increments, and the vessel's outer diameter was simultaneously tracked by continuous computer video analysis.

RNA Quantification

Total aortic RNA was isolated and processed for real-time quantitative reverse transcription polymerase chain reaction using standard protocols and methods.

Histology, Immunofluorescence, and In Situ Dihydroethidium Staining

Standardized protocols were used, with details available in the Online Data Supplement.

Elastic Imaging

Elastin autofluorescence was visualized in 30-μm-thick aortic cross-sections using confocal microscopy, and 3D structure was reconstructed from 28 layers using ImageJ (National Institutes of Health, Bethesda, MD). Elastin fragmentation was quantified in the histological images using elastin fluorescence morphometric analysis (ImageJ).

Aortic Collagen Quantification

Total aortic collagen was quantified using a hydroxyproline detection–based assay (QuickZyme Total Collagen Assay; Quickzyme) as per manufacturer’s protocol.

Western Blots

Standardized protocols were used, with details available in the Online Data Supplement.

Transfection of Cultured Cells

Transfection of human aortic smooth muscle cells (AoSMCs) was performed using Lipofectamine RNAiMAX (Invitrogen) reagent and siRNA targeting Runx2 or NF-κB subunits RelA (p65) and Nfkb1 (p50; all from Ambion).

Chromatin Immunoprecipitation Assay

AoSMC chromatin immunoprecipitation analysis was performed using the ChIP-IT High-Sensitivity Kit (Active Motif) as per manufacturer’s protocol.

NF-κB Activation Assay

To quantify cellular NF-κB activation in aortic tissue and cultured AoSMCs, nuclear extracts were obtained using the Nuclear Extract Kit (Active Motif) as per the manufacturer’s protocol. Subsequently, NF-κB activation (p65 DNA-binding activity) was measured using the TransAM NF-κB activation Kit ELISA (Active Motif).

RelA (p65)/DNA–Binding Assay

To analyze the NF-κB–binding capacity of the predicted RelA (p65)–binding motif within the Runx2 promoter, we used a DNA-binding ELISA approach (TransAM Flexi NF-κB p65 Kit; Active Motif) as previously described.\textsuperscript{19}

Luciferase Assay

The functional relevance of direct NF-κB binding for the Runx2 promoter activity was tested by transfecting high glucose (HG)-stimulated AoSMCs with SwitchGear GoClone luciferase constructs.

Statistics

Data are presented as mean±SEM. For comparison of 2 groups, Student’s t test (2-tailed) was performed; multiple groups (≥3 groups) comparison was accomplished by ANOVA with Bonferroni’s post test. For pressure myography analysis, 2-way repeated measures ANOVA followed by Bonferroni’s post test was used. Normality and homoscedasticity was tested to ensure that parametric testing was appropriate. A value of $P<0.05$ (2-sided) was considered statistically significant.
Results
Structural Aortic Stiffening Precedes the Onset of Hypertension in db/db Mice
To characterize the mechanical properties of db/db aortae, we first performed ex vivo mechanical testing of thoracic aortic segments using pressure myography. Aortic tissues derived from 20-week-old hyperglycemic db/db mice (Online Figure 1) exhibit significantly increased aortic stiffness compared with those from nondiabetic +/db controls (Figure 1A). Concomitantly, we found statistically significant elevations of systolic and mean blood pressure levels by noninvasive tail volume–pressure recording (Figure 1B). Importantly, pulse pressure, an indirect measure of aortic stiffness, was also increased in diabetic conditions (Figure 1B).

Further, investigating the temporal course of passive aortic stiffening and its relation to the onset of hypertension in diabetic db/db mice, we performed serial pressure myography measurements. Starting our measurements at 4 weeks of age (when db/db mice were mildly hyperglycemic; Online Figure I), we found progressive aortic stiffening at 10 and 20 weeks of age (Figure 1C). Blood pressure levels monitored at corresponding time points showed statistically significant elevations of systolic, diastolic, and mean blood pressure levels only at 20 weeks of age in db/db mice, but not at 10 weeks (Figure 1D). Pulse pressure was also increased only at 20 weeks of age (Figure 1D). Taken together, these results suggest that db/db mice display progressive mechanical stiffening of the aorta before development of hypertension.

db/db Mice Exhibit Enhanced Medial Collagen Production and Deposition Associated With Increased Runx2 Expression
Passive aortic stiffness largely depends on the matrix composition within the vessel’s medial layer. Thus, we next focused on structural ECM alterations as the putative basis for the observed aortic stiffening in db/db mice. Histological examination of aortic sections (by Picosiris Red, Masson’s Trichrome, Figure 2A; as well as HE staining, Online Figure II) revealed increased collagen deposition in the media from db/db aortae compared with controls. Additional immunofluorescence analysis confirmed enhanced Collagen I expression in the aortic medial layer (Figure 2A). These observations were reflected by an increased collagen content measured in db/db aortae (Figure 2B).

Runx2 is known as a transcriptional regulator of collagen deposition in bone. Investigating the potential contribution of Runx2 in the profibrotic response in db/db aortae, we detected increased protein expression of Runx2 that was largely confined to the medial layer of the aorta (Figure 2A). This was accompanied by significantly increased gene expression of Runx2 and several of its ECM target genes (collagen, type 1, alpha 1 [Col1a1], collagen, type 1, alpha 2 [Col1a2], fibronectin 1 [Fn1], and Spp1) in 10- and 20-week-old db/db mice compared with age-matched controls (Figure 2C–2G).

Aortic RUNX2 Expression Is Increased in Type 2 Diabetes Mellitus Patients
We sought to confirm the translational relevance of the increased aortic Runx2 expression found in our murine model. Therefore, we analyzed the gene expression in thoracic aortic samples that were collected during aortic-coronary bypass surgery (aortic plugs) from patients with type 2 diabetes mellitus (n=8; mean age 61.0±2.9 years) and from nondiabetic patients (n=10; mean age 61.2±4.1 years; F=ns versus diabetic patients). All patients were male, white, and nonsmokers. Although the patients (diabetic and nondiabetic) taking part in this study were not systematically screened for vascular calcification as a predefined category, there was no macroscopic evidence of gross thoracic aortic calcification that would complicate bypass anastomosis. We found RUNX2 and the target genes COL1A1, COL1A2, and FN1 to be significantly upregulated in aortae from diabetic patients compared with those from nondiabetic patients (Figure 2H; Online Figure III).

Runx2 Overexpression Induces Aortic Media Fibrosis, Increased Aortic Stiffness, and Elevated Pulse Pressure
To discern the functional impact of enhanced medial Runx2 expression on aortic stiffness, we generated Runx2-smTg mice expressing the entire coding region of murine Runx2 under control of the SM22α/ transgelin promoter, thereby achieving VSMC-specific overexpression of Runx2. VSMCs constitute the predominant cell type of the aortic medial layer that in turn largely defines the vessel’s mechanical stiffness. Runx2-smTg mice are viable and fertile and develop normally without any obvious phenotypic abnormalities. No differences in body weights were identified between Runx2-smTG mice and wild-type littermates in both genders (data not shown). Runx2-smTg mice exhibit uniform 15-fold upregulation of Runx2 gene expression that is similar to that in db/db mice compared with nondiabetic controls at 20 weeks of age. This is accompanied by significantly increased expression of matrix-related target genes (such as Col1a1, Col2a1, Fn1, Spp1, and Bglap3; Figure 3A). Induction of Runx2 protein expression in the aortic media was confirmed via fluorescent immunohistochemistry (Figure 3B) and Western blot (Figure 3C). According to its SM22α promoter–dependent regulation, Runx2 overexpression was not confined to the aorta, but was comparably expressed in carotid and femoral arteries (Online Figure IV). We further analyzed the expression levels of the matrix genes that are not under direct control of Runx2 as a transcription factor, such as Col3a1, Col5a1, as well as Eln. Expectedly, those genes were not upregulated in Runx2-Tg aortae (Online Figure VA). Similarly, diabetic aortae did not exhibit significant dysregulation of these aforementioned genes (Online Figure VB).

We next confirmed increased collagen expression histologically in the aortic medial layer of Runx2-smTg mice (Figure 3B; Online Figure VI), which was additionally reflected by increased total amounts of aortic collagen (Figure 3D). Further characterization of Runx2-smTg aortae revealed normally developed medial elastin lamellae without increased elastin breaks (Figure 3E and 3F; Online Video), as well as normal appearance of the smooth muscle layer positively stained with alpha-smooth muscle actin (Online Figure VII) without evidence of increased apoptosis (Online Figure VIII). Of note, Runx2-smTg mice exhibit normal blood glucose levels (Online Figure I).

Mechanical testing revealed markedly enhanced stiffness of Runx2-smTg aortae as compared with C57Bl/6 controls.
At the hemodynamic level, enhanced stiffness was paralleled by increased arterial pulse pressure levels (Figure 3H; Online Figure IX).

Given the known association between enhanced Runx2 expression and osteogenic transdifferentiation in VSMCs, we determined whether increased Runx2 expression per se was
sufficient to induce medial calcification in vivo, potentially augmenting arterial stiffness. However, we were unable to detect any substantial deposition of calcium (by Alizarin Red staining; Figure 3I) or inorganic phosphate (by von Kossa staining; Figure 3J) in the thoracic aortae of the Runx2 transgenic mice. Further, we assessed whether subtle calcification precursors, such as microcalcifications or enhanced CD63-positive exosome/matrix vesicle formation as a first nidus for mineralization,20 are present in Runx2-smTG aortae. Here, neither bisphosphonate-based microcalcification staining (OsteoSense; Online Figure X) nor CD63 immunofluorescence (Online Figure XI) was able to detect differences between Runx2-smTG aortic sections compared with the wild-type.

**Antioxidant Treatment Reduces Aortic Runx2 Expression, Medial Fibrosis, Aortic Stiffness, and Pulse Pressure in db/db Mice**

Diabetes mellitus is associated with increased generation of ROS in the vasculature. To determine whether diabetes mellitus–associated Runx2 expression is redox-sensitive, db/db mice were...
treated with the antioxidant agent TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) between 10 and 20 weeks of age.

We performed in situ dihydroethidium staining to monitor ROS formation in aortic sections. TEMPOL treatment resulted in a significant decrease in 2-hydroxyethidium generation in db/db mice, indicative of reduced oxidative stress (Figure 4A and 4B).

Gene expression analysis revealed that TEMPOL treatment dramatically attenuated Runx2 upregulation in db/db mice (Figure 4C). This was accompanied by downregulation of ECM target genes Col1a1 and Col1a2 (Figure 4C). We further detected decreased protein levels of Runx2 (by immunofluorescence and Western blot; Figure 4D and 4E) and collagen deposition (Figure 4D and 4F) in the aortic media of TEMPOL-treated db/db mice.

At the functional level, antioxidant treatment counteracted passive aortic stiffening as monitored by pressure myography and reduced pulse pressure in db/db mice (Figure 4G and 4H; Online Figure XII).
Runx2 Mediates a Profibrotic Response of Human AoSMCs to HG Stimulation

We investigated Runx2-dependent profibrotic mechanisms in vitro by exposing human AoSMCs to HG conditions. We found that HG (25 mmol/L) increased the expression of RUNX2 and target genes, including COL1A1, COL1A2, FN1, and SPP1. Also, ACTA2—a smooth muscle cell differentiation marker—was downregulated in HG conditions (Figure 5A). Focusing on the requirement of Runx2 in mediating HG-induced ECM gene upregulation, we found that siRNA-mediated knockdown of Runx2 abolished HG-induced upregulation of COL1A1 and COL1A2 (Figure 5B). Further, siRNA-mediated knockdown of Runx2 eliminated HG-enhanced production of soluble collagen in the supernatant of AoSMCs (Figure 5C).

NF-κB Is Activated in db/db Aortae and in AoSMCs Under HG Conditions and Serves as a Transcription Factor for Runx2

Given that aortic tissue from db/db mice display elevated ROS production, we next investigated the role of the redox-sensitive transcription factor, NF-κB, in the enhanced expression of Runx2. Indeed, we found increased Rela expression (Figure 6A), as well as increased NF-κB activation (by RelA (p65)/DNA–binding assay; Figure 6B), in db/db mice aortae, both of which are sensitive to antioxidant (TEMPOL) treatment. Similarly, we observed increased expression (Figure 6C) and nuclear translocation (Figure 6D) of Rela (p65) in AoSMCs in response to HG stimulation. Functionally, this resulted in increased NF-κB activity under HG stimulation that was reversible by antioxidant (TEMPOL) treatment (Figure 6E).

Figure 4. Antioxidant treatment (TEMPOL) reduces aortic Runx2 expression, medial fibrosis, aortic stiffness, and pulse pressure in db/db mice. A, In situ dihydroethidium (DHE) staining of thoracic aortic section from 20-week-old db/db mice, with and without 10 weeks of prior TEMPOL treatment. Reactive oxygen species (ROS) production was indicated by red fluorescence. Original magnification 400× and scale bar 50 μm. B, Average DHE intensity was quantified from 3 high-power fields of 3 different aortas per group. *P<0.05. C, Expression levels of Runx2 and its profibrotic target genes Col1a1 and Col1a2 in thoracic aortae of 20-week-old db/db mice with and without addition of TEMPOL treatment. Values are expressed as fold changes relative to the mean expression level of +/db controls (=1; dotted line). *P<0.05, n=5/group. D, Representative images of aortic cross section stained with Masson’s Trichrome (MT; collagen blue and muscle red), Representative immunofluorescent images of aortic cross section stained for collagen I (red) or Runx2 (red). Green depicts the autofluorescence of the elastic lamella. Nuclei are Hoechst stained (blue). Original magnifications are 400× and scale bars 50 μm. E, Aortic Runx2 protein expression in db/db mice with and without TEMPOL treatment as well as in +/db controls. F, Total collagen content per aortic dry weight. n=4/group. *P<0.05. G, Aortic pressure–diameter curves from 20-week-old db/db mice+TEMPOL treatment. #P<0.05 vs control for pressure levels ≥90 mmHg. n=5 to 7/group. H, Pulse pressure derived from 20-week-old db/db mice with and without TEMPOL treatment. *P<0.05. n=10/group. Col1a1 indicates collagen, type 1; Col1a2, collagen, type 1; alpha 1; and Runx2, runt-related transcription factor 2.
Knockdown of the NF-κB subunits RelA (p65) and Nfkb1 (p50) via siRNA completely inhibited the HG-stimulated upregulation of RUNX2, COL1A1, and COL1A2 in human AoSMCs (Figure 6F). To further delineate the mechanism behind NF-κB–dependent regulation of RUNX2, we searched for potential NF-κB–binding sites within the human RUNX2 promoter using TRANSFAC and JASPAR core databases via the ConTra v2 web server (http://bioit.dmbr.ugent.be/contra2/). We identified a potential RelA (p65) recognition motif (5′-GGAAAGGCCT-3′) 198 bp upstream of the transcription start site (Figure 6G).

To verify binding of the RelA (p65) subunit to the Runx2 promoter, we performed a quantitative chromatin immunoprecipitation assay detecting complexes between specific proteins and DNA sequences. Here, after immunoprecipitation with an anti-p65 antibody, we found significant (≈10-fold) enrichment of the Runx2 promoter under HG conditions relative to the IgG control (Figure 6H).

To further corroborate these findings, we tested the RelA (p65)-binding capacity of the predicted RelA (p65) recognition motif within the Runx2 promoter using a quantitative protein/solid-phase oligonucleotide–binding assay in HG-stimulated AoSMCs (TransAM assay). Immobilized oligos containing the predicted RelA (p65)-binding site were able to capture significantly more RelA (p65) protein compared with corresponding oligos with various sequence mutations (Figure 6G; Online Table I) of the RelA (p65)-binding motif (Figure 6I).

Finally, luciferase reporter constructs containing either the intact Runx2 promoter or mutations of the predicted RelA (p65)-binding site (Figure 6G; Online Table II) demonstrated that this binding site is critical for NF-κB–mediated activation of the Runx2 promoter in HG-stimulated AoSMCs (Figure 6J).

**Discussion**

Arterial stiffness has long been recognized as a feature of arterial aging.1 Over the last decade, arterial stiffness has received increasing attention in cardiovascular medicine, emerging as an independent risk factor for cardiovascular disease and mortality.2 This insight is particularly relevant in diabetic populations, which exhibit accelerated arterial stiffening and carry some of the highest risks of death from cardiovascular disease.6 Thus, there is a pressing need to characterize the mechanisms leading to arterial stiffness in diabetes mellitus, which may then help to establish targets for prophylactic or therapeutic intervention.
is primarily a function of concomitant hypertension or constitutes a distinct pathology—that may then require separate treatment. We addressed this question with the help of a murine model of diabetes mellitus, longitudinally screening aortic mechanical stiffness and blood pressure levels of diabetic db/db mice at different ages. Because the stiffness of conduit arteries is largely determined by their passive, structural properties, we performed ex vivo pressure myography to directly test the aortic mechanical properties under physiologically relevant loading conditions. We found that structural aortic stiffening in diabetic mice preceded the onset of arterial hypertension (Figure 1). In particular, aortic stiffening occurred before augmentation of pulse pressure (ie, the difference between systolic and diastolic blood pressure), which is frequently used as an indirect marker of aortic stiffness.

These findings demonstrate that passive aortic stiffening may occur independently of hypertension in this model of diabetes mellitus and are consistent with previous studies that highlight early aortic stiffness in association with decreased nitric oxide bioavailability that precedes arterial hypertension in a murine model of diet-induced obesity.

Delineating the structural basis for the observed passive aortic stiffening, we found enhanced medial fibrosis to be a prominent feature of aged db/db aortae (Figure 2A).
observation is consistent with enhanced aortic collagen deposition found in other models of diabetes mellitus.24–26

We next investigated the potential role of the transcription factor Runx2 as a central regulator of aberrant ECM accumulation and aortic stiffness in the setting of diabetes mellitus. Although not significantly expressed in the vasculature under normal conditions, Runx2 has recently emerged as a marker of pathological osteogenic VSMC transdifferentiation in the context of vascular calcification.27,28 However, the immediate transcriptional potential of Runx2 to induce vascular fibrosis resulting in arterial stiffness had never before been addressed experimentally.

Gene expression analysis revealed that Runx2 and ECM target genes were progressively upregulated in aging db/db animals compared with age-matched +/db controls (Figure 2C–2G). Further, we detected markedly upregulated expression of Runx2 protein in the aortic media of 20-week-old diabetic db/db mice as analogously reported in a streptozotocin-induced model of diabetes mellitus.29 Importantly, consistent with these animal model–derived data, we also found increased expression of RUNX2 and downstream genes COL1A1, COL1A2, and FN1 in thoracic aortic samples from patients with type 2 diabetes mellitus (Figure 2H).

To assess the functional impact of Runx2 on vascular matrix homeostasis, we generated Runx2-smTg mice expressing the entire coding region of murine Runx2 under control of the SM22α promoter, achieving VSMC–specific overexpression of Runx2.30,31 This approach enabled us to dissect direct downstream effects of increased Runx2 function in the VSMC–dominated aortic media. Runx2 overexpression histologically translated into marked collagen I accumulation in the aortic media (Figure 3). These structural alterations accompanied significantly increased material stiffness of the thoracic aorta, as well as increased arterial blood/pulse pressure levels.

Although we did not study Runx2 expression during embryonic development of Runx2-smTg mice directly, it is known that the SM22α promoter—regulating Runx2 expression in our mouse model—exhibits embryonic aortic activation (as early as E9.0).31 Consequently, we cannot exclude developmental abnormalities in our model per se. Therefore, it is important to exclude structural developmental defects of the aorta that may result in increased aortic stiffness unrelated to the profibrotic mechanisms under investigation. In contrast to the collagen matrix that readily undergoes lifelong remodeling in response to various stimuli, elastic fibers, being another major structural determinant of the mechanical properties of conduit arteries, are almost exclusively assembled during tissue development and function for the entire lifespan of the organism with low potential for postnatal repair.11 Therefore, elastin function is particularly susceptible to developmental defects. In this regard, we were unable to detect any irregularities of the medial elastin architecture in the Runx2-smTg by histological elastin 3D reconstruction and elastin break quantification (Figure 3E and 3F).

Further, in vitro experiments demonstrated that HG conditions lead to Runx2-dependent enhanced collagen production in human AoSMCs (Figure 5). Taken together, the data identify Runx2 as an inducer of aberrant medial fibrosis and aortic stiffening in the context of diabetes mellitus.

Interestingly, Runx2 overexpression per se did not induce enhanced mineralization/calcification in the aortic specimens taken from Runx2-smTg mice (Figure 3I and 3J; Online Figures X and XI). This was somewhat unexpected because Runx2 is not only considered a marker of osteogenic VSMC transdifferentiation in calcifying vascular disorders,15,17,28 but also shown to be of mechanistic significance for atherosclerotic calcification in a murine Runx2 knockout model.37 Thus, Runx2 may be necessary but not sufficient to promote vascular calcification, underlining the complex, multifactorial nature of vascular calcification processes.32,33 In this context, Runx2-smTg aortae do not exhibit increased elastin breakdown (Figure 3E and 3F) or VSMC apoptosis (Online Figure VIII), both factors associated with vascular (medial) calcification.32,33

Diabetes mellitus is generally associated with increased abundance of ROS in the cardiovascular system.34 We found increased ROS (superoxide) production in aortic sections from db/db mice (Figure 4A and 4B), consistent with other reports.35,36 Aberrant ROS-mediated signaling contributes to pathological vascular remodeling in various diseases (eg, atherosclerosis, arteriosclerosis, hypertension, and abdominal aortic aneurysm)37 and moreover has been shown to induce Runx2 expression in vitro.38 A recent study reported the utility of the antioxidant TEMPOL in preventing age-related arterial stiffening.39 In line with those findings, we demonstrated that 10 weeks of antioxidant treatment (TEMPOL) reduces ROS abundance in the aortic wall of db/db mice and reduces expression of Runx2 and the downstream collagen isoforms Colla1a1 and Colla2a1 (Figure 4C). Functionally, this results in decreased aortic stiffness and reduced pulse pressure in 20-week-old db/db mice (Figure 4G and 4H). Although TEMPOL is known to reduce blood pressure via multiple functional, vasodilatory mechanisms (including interference with nitric oxide metabolism and through sympatholytic properties),40 this study provides evidence for TEMPOL’s additional antihypertensive effects via modulation of diabetic arterial remodeling.

In contrast to a previous report,35 we did not find a significant effect of TEMPOL treatment on blood glucose levels in db/db mice, possibly because of the lower dose used in the present study (1 mmol/L versus 2 mmol/L).

To elucidate a mechanistic link between increased ROS abundance in the diabetic aorta and the enhanced expression of Runx2, we focused on the role of the oxidative stress-sensitive transcription factor, NF-κB.41,42 Indeed, previous studies have demonstrated increased aortic NF-κB expression and activation in diabetic mouse models43,44 and indicated that hyperglycemia can activate NF-κB in VSMCs.45–47 In line with those data, we find that NF-κB expression and activation is increased in a redox-sensitive fashion in db/db aortae (Figure 6A and 6B) and in human AoSMCs under HG conditions (Figure 6C and 6D).

In silico analysis revealed a potential RelA (p65)-binding site within the human Runx2 promoter, suggesting that Runx2 might be a direct transcriptionally activated target of NF-κB. Through an NF-κB–binding assay, we demonstrated that RelA (p65) binds to the predicted binding site within the Runx2 promoter (Figure 6I). Luciferase reporter constructs containing mutations of the RelA (p65)-binding site further confirm the
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Vascular smooth muscle cell–specific overexpression of RUNX2 is
RUNX2 induces aortic medial fibrosis and thereby increases aortic

• RUNX2 is upregulated under high glucose conditions in vitro and in vivo via a redox-sensitive activation of nuclear factor kappa beta.

• RUNX2 induces aortic medial fibrosis and thereby increases aortic stiffness.

• Vascular smooth muscle cell–specific overexpression of RUNX2 is insufficient to induce vascular calcification in vivo.
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SUPPLEMENTAL MATERIAL

Detailed Methods

Animals
All animal protocols were approved by the Administrative Panel on Laboratory Animal Care at Stanford University (http://labanimals.stanford.edu/) and followed the National Institutes of Health and U.S. Department of Agriculture Guidelines for Care and Use of Animals in Research. Male db/db mice (BKS.Cg-Dock7m +/+Leprdb/J) and their age-matched heterozygous non-diabetic controls (+/db) were purchased from the Jackson Laboratory (Bar Harbor, ME). Antioxidant treatment of 10 week-old db/db mice was performed with TEMPOL (1mmol/l; Sigma Aldrich) in drinking water freshly prepared every other day over a course of 10 weeks.

Transgenic mice with vascular smooth muscle cell (VSMC)-specific Runx2-overexpression (Runx2-smTg; C57Bl/6 background) were generated at our institution. Specifically, a transgene cassette consisting of the entire coding region of mouse type 1 Runx2 (a gift from Dr. Toshihisa Komori; Japan) driven by the 445 bp form of the SM22α promoter (a gift from Dr. Michael Parmacek, University of Pennsylvania) was initially produced and protein expression was confirmed in cultured VSMC. Among the two major Runx2 isoforms (type 1 and type 2) type 1 was studied as the relevant isoform whose expression (in contrast to type 2) is not restricted to osseous tissues1,2. Pronuclear injection and generation of founder mice was performed by the Stanford Transgenic Core Facility. Transgenic animals were screened by standard PCR of tail DNA and confirmed by Southern blotting. A single founder line that demonstrated modest Runx2 overexpression was used for the experiments pertaining to this study. Runx2-smTg mice are viable in normal litter sizes and have normal growth patterns compared to C57Bl/6 control mice.

Pressure myography
Pressure myography was performed to directly assess the passive aortic mechanics according to adapted protocols3. In brief, murine thoracic aortae were explanted, placed on specially designed stainless steel cannulas and secured with silk surgical suture (10-0). The vessel was mounted in the heated chamber of a pressure arteriograph system (Model 110P, Danish Myotechnology, Copenhagen, Denmark) and stretched to in vivo length. Physiological saline solution (PSS) at 37°C, aerated with 5% CO2/95% O2 was used to fill the vessel chamber and for aortic perfusion. After 3 preconditioning cycles, the aortic passive pressure-diameter relationship was determined by an automated protocol. The artery was pressurized from 0 to 180 mmHg in 18 mmHg increments, and the vessel’s outer diameter was simultaneously tracked by continuous computer video analysis (MyoVIEW software, Danish Myotechnology, Copenhagen, Denmark). The resting diameter d0 was quantified under 0 mmHg intraluminal (transmural) pressure and was not significantly different between the experimental groups tested (d0 ~1000 µm) and comparable to previous reports4.

Blood pressure measurements
Blood pressure measurements were performed using non-invasive VPR (Volume-Pressure Recording) tail cuff sensor technology (CODA System; Kent Scientific). All mice were acclimated to the restrainer for 10–20 min per day for at least 3 consecutive days before starting the study. Following this acclimation period, mice typically remained calm and still in the restrainer on the day of testing. To facilitate the acclimation process, the mice were handled gently and not forced to enter the restrainer, and the ambient temperature was maintained at warm room temperature (25–30°C).

Blood glucose measurements
Blood glucose levels were monitored from tail bleeds taken between 10 AM and noon using a glucometer (Accu-Check, Roche Diagnostics).

Preparation of mouse aortic tissue for RNA extraction
Mice were sacrificed with an inhalation overdose of isoflurane (Vet One, Meridian, ID, USA). Immediately following sacrifice the thoracic aorta was transected and flushed via the left ventricle with ice cold phosphate buffered saline (PBS; pH 7.4). The aorta was then dissected from fat and connective tissue under a microscope (Leica, Wetzlar, Germany). Specimens were snap-frozen individually in liquid nitrogen and stored at -80°C before further processing.

**Human tissue sample acquisition and preparation.**

Human thoracic aortic samples from patients who underwent aorto-coronary bypass surgery (aortic plugs derived from aortic punching) were collected during surgery, snap-frozen and stored at -80 °C.

**RNA quantification**

Total RNA was isolated using a TRIlol-based (Invitrogen) RNA isolation protocol. RNA was quantified by Nanodrop (Agilent Technologies), and RNA quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies). Samples required 260/280 ratios >1.8, and sample RNA integrity numbers >9 for inclusion. The iScript cDNA synthesis kit (Bio-Rad) was used to synthesize first-strand cDNA according to the manufacturer’s protocol. TaqMan qRT-PCR assay was performed using mouse and human specific primers (Life Technologies). All probes were normalized to 18S as internal control. Amplification took place on a QuantStudio12K Flex (Applied Biosystems). All fold changes were calculated by the method of ΔΔCt.

**Tissue preparation for histological analysis**

Mice were euthanized and perfused at a constant pressure of 100 mmHg via the left ventricle with saline followed by warm (37°C) agarose gel (Amresco) diluted in saline (3% w/v). After agarose solidification, the thoracic aorta was dissected free from the surrounding connective tissue and fixed in 4% formalin. The isolated thoracic aorta was then dehydrated through a graded sucrose series and subsequently embedded in OCT blocks. For in situ DHE oxidation aortae were dissected and OCT embedded without any prior fixation.

**Picrosirius Red, Masson’s Trichrome, Hematoxylin and Eosin, Alizarin Red, and von Kossa staining**

Aortic cross sections (7µm) were stained using the Picrosirius Red Stain kit, the Hematoxylin&Eosin (H&E) Stain kit, the Alizarin Red Stain kit, and the von Kossa Stain kit (all from American MasterTech, USA), as well as the Trichrome Stain Kit (Abcam) according to manufacturer’s instructions.

**Immunofluorescence**

Aortic cross sections (7 µm) were fixed for 15 minutes in 4% PFA and PBS. Primary antibodies against Runx2 (Abcam, ab23981), collagen I (Abcam, ab 34710), CD63 (Santa Cruz, sc-13363), alpha-smooth muscle actin (SMA) (Abcam, ab5694), cleaved caspase 3 (Cell Signaling, #9661) were applied overnight. Incubation with secondary antibodies (Alexa Fluor 594 goat anti-rabbit; Molecular Probes) was performed for 1 hour. Counterstaining was performed with Hoechst reagent (bisBenzide H 33258) (Sigma-Aldrich, St. Louis, MO, USA). Negative controls were performed with the omission of the primary antibody. Imaging was performed using a Leica DM4000B (Buffalo Grove, IL, USA).

**Elastin imaging**

Elastin autofluorescence was visualized in 30 µm thick aortic cross sections using confocal microscopy and 3D structure was reconstructed from 28 layers using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Elastin fragmentation was quantified in the histologic images using elastin fluorescence morphometric analysis (ImageJ). As previously described, each continuous set of pixels that formed a connected group was defined as an object. An elastin fragmentation index was defined as the ratio of the number of elastin objects to the area of elastin objects.
The number of elastin objects was defined by the count of elastin objects within the media region of interest (ROI). The area of elastin objects was equivalent to the total pixel count across all elastin objects within the media ROI.

**In situ DHE staining**
The amount of intracellular ROS production was determined using dihydroethidium (DHE) (Molecular Probes - red fluorescence). Frozen aortic cross sections (7 µm) were mounted on glass slides, rinsed in PBS, and incubated in 10 µM DHE (37°C, 30 minutes). Intensity was measured by fluorescence microscopy (Leica DM4000B: Buffalo Grove, IL, USA) using a Texas red filter (488-nm excitation, 610-nm emission). Autofluorescence from elastic lamellae was subtracted, and fluorescence was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

**Bisphosphonate-conjugated dye staining (OsteoSense)**
A bisphosphonate-conjugated fluorescent dye (OsteoSense 680EX, PerkinElmer) that binds to hydroxyapatite was used to assess vascular microcalcifications. Frozen aortic sections were incubated with OsteoSense680 for a minimum of 2 hours before imaging as previously described⁶. Human carotid plaque tissue served as positive control.

**Aortic collagen quantification**
Total aortic collagen was quantified using a hydroxyproline-detection based assay (QuickZyme Total Collagen Assay; Quickzyme) as per manufacturer’s protocol. In brief, dissected thoracic aorta (n=4/group) were dried after removal of all loosely attached perivascular connective tissue and dry weight was recorded. Subsequently, aortic tissue was hydrolyzed in 6 M HCl at 95°C for 20 hours. In the hydrolysate hydroxyproline content was quantified colorimetrically and correlated to a collagen standard curve. Aortic collagen content was expressed as % of dry weight.

**Western blots**
Aortae were homogenized in RIPA buffer, protease inhibitors, and 5 mM DTT. Equal amounts of protein were separated by 10% SFS-PAGE, transferred to PVDF membranes and stained with Runx2 (Abcam ab23981; 1:900) and beta-actin (Sigma AC-74; 1:10000), followed by incubation with HRP-conjugated secondary antibodies (Dako P0399, 1:2000 and P0447, 1:2000). Signals were visualized by chemiluminescence (Pierce, Rockford, IL).

**Cell culture**
Human aortic smooth muscle cells (AoSMCs) were propagated in growth media (SmGM-2; Lonza) with 5% fetal bovine serum (FBS) per standard protocols (Lonza; passage no. 4–5). Before experimental use, AoSMCs were serum-starved for 48h. For high glucose experiments AoSMCs were incubated in Dulbecco’s modified minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and D-glucose (25mM for high glucose conditions; 5mM glucose + 20mM D-mannitol as osmotic control for normal glucose controls) for 48h. TNF-α (10 nM) stimulation was performed for 15 min.

**Transfection of cultured cells**
Transfection of AoSMCs was performed using Lipofectamine RNAiMAX (Invitrogen) reagent, and siRNA targeting Runx2 or NF-κB subunits RelA (p65) and Nfkβ1 (p50) (all from Ambion). For each transfection, siRNA was diluted in Opti-MEM for a final transfection concentration of 50 nM. Cells were transfected with Lipofectamine/siRNA 24h before high glucose incubation for an additional 48 h, followed by harvest and RNA extraction using TRIzol (Invitrogen). Successful knockdown (>75%) was confirmed by qRT–PCR determining expression levels of RUNX2, RELA (p65) and NFKB1 (p50) (all primers from Life Technologies) (Online Figure XIV). Decreased nuclear NF-κB activity was additionally confirmed via TransAM NF-κ B activation ELISA (Active Motif) (Online Figure XV).
Immunofluorescence staining
AoSMCs were cultured in chamber slides under normal glucose or high glucose conditions (see above). The cells were then fixed in 4% paraformaldehyde, washed with PBS, permeabiled with 0.25% Triton-X and incubated with anti-RelA/p65 antibody (Abcam, ab7970) over night at 4°C. The cells were then washed and incubated with secondary antibodies (Alexa Fluor) and Hoechst nuclear counterstain.

Chromatin immunoprecipitation assay (ChIP)
ChIP analysis was performed using the ChIP-IT High-Sensitivity Kit (Active Motif) as per manufacturer’s protocol. In brief, following normal or high glucose treatment for 48h AoSMCs were fixed with formaldehyde buffer and sonicated to shear chromatin. Sonicated chromatin was incubated with ChIP-validated antibodies (4 µg per 30 µg chromatin) against NFkB p65 (Active Motif, 39369) and nonspecific IgG (Active Motif). The antibody-bound protein/DNA complexes were immunoprecipitated through the use of protein G agarose beads and washed via gravity filtration. Following immunoprecipitation, the DNA cross-links were reversed and the DNA was purified according to the ChIP protocol. The purified immunoprecipitated and input DNA were analyzed by PCR using SYBR Green PCR reagents and SimpleChIP Human RUNX2 Promoter Primers (Cell Signaling, 12376). Data were expressed as fold enrichment of the ChIP samples relative to the IgG samples (calculated by the method of ΔΔCt).

NF-κB activation assay
In order to quantify cellular NF-κB activation in aortic tissue and cultured AoSMCs, nuclear extracts were obtained using the Nuclear Extract Kit (Active Motif) as per manufacturer’s protocol. Subsequently NF-κB activation (p65 DNA-binding activity) was measured using the TransAM NF-κB activation Kit ELISA (Active Motif) as previously described7,8. In brief, activated NF-κB (p65) is captured on a 96-well plate via an immobilized oligo containing an NF-κB consensus motif (5'-GGGACTTTCC-3'). By using a horseradish peroxidase (HRP)-conjugated antibody against RelA (p65) (Active Motif, provided in the kit) the amount of bound NF-κB complex can then be quantified by spectrophotometry as per protocol.

RelA (p65)/DNA binding assay
In order to analyze the NF-κB binding capacity of the predicted RelA (p65) binding motif within the Runx2 promoter we used a DNA-binding ELISA approach (TransAM Flexi NFκB p65 Kit; Active Motif) as previously described9. Custom designed biotinylated 50 bp oligos (Stanford Protein and Nucleic Acid Facility), carrying either the intact predicted binding site or defined mutations thereof (Figure 6C and Online Table I for complete sequences) were bound to 96-well streptavidin-coated ELISA plate. Subsequently the solid-phase bound oligos were incubated with nuclear extract from high glucose stimulated AoSMCs. Using a horseradish peroxidase (HRP)-conjugated antibody against RelA (p65), the amount of bound NF-κB complex was then quantified by spectrophotometry as per protocol.

Luciferase assay
The functional relevance of direct NF-κB binding for the Runx2 promoter activity was tested by transfecting high glucose stimulated AoSMCs with SwitchGear GoClone luciferase constructs. Cells were transfected in 96-well white-bottomed plates at 40% confluence with plasmids (50 ng per well) containing either the intact human Runx2 promoter, custom mutations of the RelA (p65) binding site (Figure 6G and Online Table II for full sequence), a scrambled sequence, or an empty vector using FuGene (Switchgear) per manufacturer’s protocol. After 24 hours luciferase activity was determined by Lightswitch Assay (Switchgear) as per protocol.
References
Online Figure I: Blood glucose measurements. Blood glucose levels (fed) of (A) 4, 10 and 20 weeks old db/db mice and heterozygous +/db controls, (B) transgenic Runx2-smTg mice vs. WT (C57Bl/6) controls, and (C) 20 weeks old db/db mice ± 10 weeks of oral TEMPOL treatment. Values are mean ± SEM. * indicates p<0.05, n.s. non-significant.

Online Figure II: Aortic cross sections from 20 week-old mice stained with Hematoxylin and Eosin (HE). Muscle stains deep pink. Arrows (→) indicate medial fibrotic areas (pale pink). Nuclei stain blue. Original magnifications are 400x, scale bars 50 µm.

Online Figure III: Scatter blots depicting individual expression levels of RUNX2, COL1A1, COL1A2, FN1 and SPP1 in thoracic aortae of diabetic patients (n=8) compared to non-diabetic patients (n=10). Values are expressed as fold changes relative to the mean expression level in non-diabetic patients (=1; dotted line). * indicates p < 0.05 vs. non-diabetic patients.
Online Figure IV: Comparative analysis of arterial (aortic, carotid, femoral) Runx2 expression in Runx2-smTg mice. Runx2 expression levels in aortae, carotid arteries and femoral arteries harvested from Runx2-smTG mice. Values are expressed as fold changes relative to the mean aortic expression level (=1; dotted line). ns indicates no significant differences among groups.

Online Figure V: Aortic expression of Runx2-independent matrix genes in Runx2-smTg (A) and db/db (B) mice. Col3a1, Col5a1, and Eln expression is expressed as fold change relative to the mean expression level of the WT (A) or +/db (B) controls (=1; dotted line). ns indicates no significant differences vs. controls.

Online Figure VI: Aortic cross sections from 20 week-old mice stained with Hematoxylin and Eosin (HE). Muscle stains deep pink. Arrows (→) indicate medial fibrotic areas (pale pink). Nuclei stain blue. Original magnifications are 400x, scale bars 50 µm.

Online Figure VII: Representative immunofluorescent images of aortic cross sections stained for alpha smooth muscle actin (SMA)(red/orange). Green depicts the autofluorescence of the elastic lamella. Nuclei are Hoechst stained (blue). Original magnifications are 400x, scale bars 50 µm.
Online Figure VIII: Representative immunofluorescent images of aortic cross section stained for activated caspase-3 (red). Green depicts the autofluorescence of the elastic lamella. Nuclei are Hoechst stained (blue). Colon carcinoma tissue served as positive control (right panel). Original magnifications are 100x, scale bars 50 µm.

Online Figure IX: Blood pressure levels of Runx2-smTG mice. (A) Systolic, (B) diastolic, and (C) mean blood pressure levels of Runx2-smTg mice vs. WT (C57Bl/6) controls. n=10/group. Values are mean ± SEM. * indicates p<0.05, n.s. non-significant

Online Figure X: Representative images of aortic cross sections stained with bisphosphonate-conjugated fluorescent dye (OsteoSense) to detect microcalcifications (red). Green depicts the autofluorescence of the elastic lamella. Human carotid plaque tissue served as positive control. Original magnifications are 400x, scale bars 50 µm.
Online Figure XI: Representative immunofluorescent images of aortic cross sections stained for CD63 (red) to detect differences in exosome/matrix vesicle formation. Green depicts the autofluorescence of the elastic lamella. Nuclei are Hoechst stained (blue). Original magnifications are 400x, scale bars 50 µm.

Online Figure XII: Blood pressure levels of TEMPOL treated db/db mice. (A) Systolic, (B) diastolic, and (C) mean blood pressure levels of 20 weeks old db/db mice ± 10 weeks of oral TEMPOL treatment. n=10/group. Values are mean ± SEM. * indicates p<0.05, n.s. non-significant.
Online Figure XIII: Proposed mechanism of diabetes induced upregulation of Runx2 in VSMC leading to increased collagen deposition and aortic stiffening. Diabetes / hyperglycemia induces reactive oxygen species (ROS)-dependent NF-κB activation. NF-κB directly activates the Runx2 promoter resulting in enhanced expression of Runx2 and pro-fibrotic target genes Col1a1 and Col2a1 in vascular smooth muscle cells (VSMCs). Increased VSMC collagen production eventually leads to aberrant medial fibrosis and aortic stiffening.

Online Figure XIV: Confirmation of siRNA-mediated knockdown of RUNX2, RELA, and NFKB1, respectively in AoSMCS. Expression levels are depicted as fold changes relative to the mean expression level of the respective control siRNA (=1; dotted lines). * indicates p < 0.05 vs control siRNA

Online Figure XV: Confirmation of decreased NF-κB activity in AoSMCs nuclear extracts following siRNA-mediated NF-κB knockdown. * indicates p < 0.05 vs control siRNA
### Online Table I: List of biotinylated oligos used for the NF-κB binding assay.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Forward sequence</th>
<th>Complementary sequence (reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Runx2</td>
<td>ZAAAAATAAAAAGCTATAT CTTCTGGAATGCCAGGAG GGCCTTACCAAGGCTT</td>
<td>GGCTTGTGGTAAGGCCTTCTCCGGGAAAGCCTT</td>
</tr>
<tr>
<td>Runx2-mut1</td>
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<tr>
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<tr>
<td>Scrambled</td>
<td>ZAAAAATAAAAAGCTATAT CTTCTGGAATGCCAGGAG GGCCTTACCAAGGCTT</td>
<td>GGCTTGTGGTAAGGCCTTCTCCGGGAAAGCCTT</td>
</tr>
</tbody>
</table>

*Oligos were synthesized by MWG Biotech (Ebersberg, Germany).*
Online Table II: Sequence of luciferase constructs and custom designed mutants. Predicted RelA(p65) binding site / mutations underlined.