

Deletion of I κ B-Kinase β in Smooth Muscle Cells Induces Vascular Calcification Through β -Catenin–Runt-Related Transcription Factor 2 Signaling

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Background—Vascular calcification was previously considered as an advanced phase of atherosclerosis; however, recent studies have indicated that such calcification can appear in different situations. Nevertheless, there has been a lack of mechanistic insight to explain the difference. For example, the roles of nuclear factor- κ B, a major regulator of inflammation, in vascular calcification are poorly explored, although its roles in atherosclerosis were well documented. Herein, we investigated the roles of nuclear factor- κ B signaling in vascular calcification.

Methods and Results—We produced mice with deletion of IKK β , an essential kinase for nuclear factor- κ B activation, in vascular smooth muscle cells (VSMCs; KO mice) and subjected them to the CaCl₂-induced aorta injury model. Unexpectedly, KO mice showed more calcification of the aorta than their wild-type littermates, despite the former's suppressed nuclear factor- κ B activity. Cultured VSMCs from the aorta of KO mice also showed significant calcification in vitro. In the molecular analysis, we found that Runt-related transcription factor 2, a transcriptional factor accelerating bone formation, was upregulated in cultured VSMCs from KO mice, and its regulator β -catenin was more activated with suppressed ubiquitination in KO VSMCs. Furthermore, we examined VSMCs from mice in which kinase-active or kinase-dead IKK β was overexpressed in VSMCs. We found that kinase-independent function of IKK β is involved in suppression of calcification via inactivation of β -catenin, which leads to suppression of Runt-related transcription factor 2 and osteoblast marker genes.

Conclusions—IKK β negatively regulates VSMC calcification through β -catenin–Runt-related transcription factor 2 signaling, which revealed a novel function of IKK β on vascular calcification. (*J Am Heart Assoc.*2018;7:e007405. DOI: 10.1161/JAHA.117.007405.)

Key Words: atherosclerosis • IKK β • nuclear factor- κ B • vascular biology • vascular calcification

Vascular calcification was previously considered as a degenerative process seen in advanced atherosclerosis lesions, but recent studies have indicated that such calcification can appear in a different manner. Calcification not only accompanies with atherosclerosis but also accompanies diabetes mellitus or renal failure in the absence of atherosclerosis.^{1,2} Moreover, recent clinical studies have revealed that statins, a medicine widely used for atherosclerosis, promote coronary calcification,^{3–8} which clearly indicates that calcification stands on a different basis from atherosclerosis.⁹

However, the difference has not been fully explained because the mechanism of vascular calcification is poorly understood. For example, the roles of nuclear factor- κ B (NF- κ B) in the formation and progression of calcification have not been adequately assessed, although its roles in atherosclerosis have been well studied.^{10,11}

NF- κ B is a family of transcription factors that plays critical roles in inflammation and atherosclerosis, and reports have indicated that it is a target of statins.^{12,13} NF- κ B generally exists as a homodimer or heterodimer in the cytosol that is bound to the inhibitor of κ B (I κ B).¹⁴ In response to a wide variety of stimuli, including inflammatory cytokines, I κ B is phosphorylated and degraded via the ubiquitin pathway, which is followed by NF- κ B translocation to the nucleus and activation of transcription. The serine phosphorylation of I κ B is mediated by a large multiunit complex containing 2 catalytic subunits, IKK α and IKK β , and the regulatory subunit IKK γ or NF- κ B essential modulator (NEMO).¹⁵ Of these subunits, IKK β is the essential kinase that mediates I κ B phosphorylation.¹⁵

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Clinical Perspective

What Is New?

- Inhibition of IKK β , an essential kinase for nuclear factor- κ B activation, in vascular smooth muscle cells, augments vascular calcification.
- IKK β has a kinase-independent function, and it is involved in the suppression of vascular calcification.

What Are the Clinical Implications?

- IKK β is the target of aspirin, and statins suppressed the activity of nuclear factor- κ B, but both are reported to have no suppressing effect. They even accelerate vascular calcification.
- This study could account for the mechanism, at least in part.

In this study, we produced mice with a deletion of IKK β in vascular smooth muscle cells (VSMCs), a cell type contributing to calcification,¹⁶ and subjected them to CaCl₂-mediated aortic injury, which is an established animal model for vascular calcification.^{17,18} Unexpectedly, we observed more calcification in the aorta of IKK β -deleted mice and found the activation of β -catenin and Runt-related transcription factor 2 (Runx2) as a possible mechanism. Furthermore, we present, for the first time, that the kinase-independent function of IKK β has an effect on the inactivation of β -catenin, which leads to suppressing the calcification of VSMCs. These results indicate the protective effect of IKK β on vascular calcification, which at least partly explains the difference between the smooth muscle calcification and atherosclerosis observed in clinical settings.

Methods

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure because some materials are used for other unpublished projects.

Mice

All animal experiments were performed in accordance with the institutional guidelines of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Kyoto, Japan). For this study, transgenic mice were crossed onto a C57BL/6J background >10 times. To block smooth muscle cell NF- κ B signaling, we used IKK β knockout selectively in smooth muscle cells of mice. IKK β flox/flox mice were generously given by Professor Michael Karin and mated with Sm22 α , a protein that is specifically expressed in smooth muscle cells, and Cre⁺ mice (The Jackson

Laboratory, Bar Harbor, ME) to generate IKK β flox/wt Sm22 α -Cre^{+/-} mice, which were mated with IKK β flox/flox mice to generate IKK β flox/flox Sm22 α -Cre^{+/-} (KO) mice or IKK β flox/flox Sm22 α -Cre^{-/-} (wild-type [WT]) mice. To generate transgenic mice with constitutively active IKK β kinase (KA)¹⁹ selectively in smooth muscle cells, IKK β -KA flox/flox mice were mated with Sm22 α -Cre^{+/-} mice to generate IKK β -KA flox/wt Sm22 α -Cre^{+/-} (KA) mice or IKK β -KA flox/wt Sm22 α -Cre^{-/-} (WT) mice. To produce transgenic mice with kinase-dead IKK β (KD) in smooth muscle cells, IKK β -KD flox/flox mice were mated with IKK β flox/flox Sm22 α -Cre^{+/-} (KO) to generate IKK β -KD flox/flox IKK β flox/flox Sm22 α -Cre^{+/-} (KD) mice. These KD mice have complete knockout of endogenous IKK β from VSMCs, followed by knockin of kinase defective form of IKK β . In the mating protocol for all kinds of mice, we could have WT mice within littermate; therefore, we used the littermate WT as control mice in all experiments.

Generation of IKK β (K44A) (KD/IKK β flox/flox) Knock-In Mice

To generate conditional IKK β (K44A) expressing mice, a CAG promoter, a loxP flanked STOP cassette, and

IKK β (K44A) cDNA were inserted into the mouse Gt(Rosa)26Sor locus by homologous recombination. A 3.4-kb upstream DNA fragment and a 4.3-kb downstream DNA fragment from the unique XbaI site in intron 1 of Gt(Rosa)26Sor locus were amplified by polymerase chain reaction (PCR) from RENKA ES cell genomic DNA and used as the homology arms for the targeting vector. To construct the expression unit, a chemically synthesized DNA sequence containing a DYKDDDDK-tagged mouse IKK β (K44A), a rabbit β -globin polyadenylation signal, and a VloxP sequence were combined with an frt-flanked PGK_{neo} cassette (phosphoglycerate kinase 1 promoter-driven neomycin-resistant gene). At the 5' region of this expression unit, a CAG promoter and a loxP flanked STOP cassette containing chloramphenicol acetyltransferase cDNA and Vlox43L were inserted. Then, the promoter-expression unit was subcloned between 2 homology arms. As a negative selection marker, an MC1 promoter-driven diphtheria toxin A cassette was placed at the 5' region of the homology arm. The resulting knock-in targeting vector contains MC1 promoter-driven diphtheria toxin A, a 3.4-kb 5' homologous arm, a CAG promoter, the first loxP site, chloramphenicol acetyltransferase cDNA, polyA, Vlox43L, an frt-flanked PGK_{neo} cassette, the second loxP site, a DYKDDDDK tag, IKK2 (K44A) cDNA, bGH polyA, VloxP, and a 4.3-kb 3' homology arm. This vector was linearized and introduced into RENKA ES cells (C57BL/6 genetic background) by electroporation. After selection using geneticin, the resistant clones were isolated and their DNA

was analyzed to identify homologous recombinants by PCR using the following primer sets: sc_R10603, 5'-GTTACTCC ACTTTCAAGTTCCTTATAAGC-3'; neo_108r, 5'-CCTCAGAAGAA CTCGTCAAGAAG-3'; sc_5AF3, 5'-ATCCTAAGGTCACTTTTAAA TTGAGG-3'; and neo_G02, 5'-ATCAGGACATAGCGTTGGCT AC-3'. Genomic Southern hybridization was probed with a neomycin resistance gene. Homologous recombinant ES cell clones were aggregated with 8-cell embryos of ICR mice to generate chimeric mice. Chimeric mice with a high contribution from the RENKA background were bred with C57BL/6 mice. Germline transmitted mice were mated to B6;D2-Tg (CAG-FLP)18Imeg mice to eliminate the PGK_neo cassette from the genome through Flp/frt-mediated excision. The knock-in allele was identified by PCR with the following primers: 5AF1, 5'-CCATTGGCTCGTTCGTG-3'; and mutant_R, 5'-CCGGGGGATCCACTAGTA-3'. The WT allele was identified by PCR with the following primers: 5AF1 and wild_R, 5'-ACTCCCGCCCATCTTCTAGA-3'.

CaCl₂-Mediated Abdominal Aorta Injury

An abdominal aorta injury was induced in 8- to 14-week-old KO and WT male mice by periaortic application of CaCl₂, as described previously,^{17,18} with minor modifications. We started with a conventional protocol of 20 minutes CaCl₂ treatment, but too much dilatation and calcification seen in WT aorta interfere with the evaluation of the difference between WT and KO aorta. Therefore, we reduced the CaCl₂ incubation period to 10 minutes. Even in that protocol, 2 weeks after CaCl₂ application, the aortas in both types of mice were significantly dilated when compared with those in the sham-operated on mice. Mice were anesthetized by IP injection with pentobarbital (100 mg/kg) and then underwent a laparotomy. An incision of \approx 2 cm was made, and the small and large intestines were moved away to expose the infrarenal aorta. Small pieces of gauzes soaked with 0.6 mol/L CaCl₂ were placed circumferentially on the infrarenal aorta for 10 minutes. Then, the gauze was removed and the aorta was rinsed with 0.95% NaCl and wiped with a cotton swab. NaCl (0.95%) was substituted for CaCl₂ in sham-operated on mice. The incision was closed, and mice were placed on a warm plate until they had recovered from anesthesia. After the surgical procedure, the animals were allowed free access to food and water until the day of euthanasia.

Aorta Collection and Size Measurement

Two weeks after the operation, mice were euthanized with an overdose of pentobarbital. The vascular system was flushed with cold PBS from the left ventricle. The abdominal aorta was carefully dissected under a microscope, cleaned of extraneous tissue, and then photographed for diameter measurement.

Histology and Immunohistochemistry

Approximately 2 to 3 mm of infrarenal aorta was fixed with 4% paraformaldehyde overnight, embedded in paraffin, cut into 4- μ m cross sections. Then, cross sections were deparaffinized 3 times in xylene and dehydrated in 80%, 90%, and 100% ethanol for 5 minutes each. Elastin was visualized using the fluorescence properties of hematoxylin and eosin.²⁰ Alizarin Red staining was performed to detect calcification for both the in vivo and in vitro samples. For immunohistochemical staining, cross sections were boiled in 10 mmol/L citrate buffer for antigen retrieval, followed by avidin/biotin. Sections were then incubated with primary antibodies that recognize SM22 α , IKK β , Runx2, and active β -catenin overnight at 4°C. The secondary antibody IgG/biotin goat anti-rabbit or goat anti-mouse was incubated for 1 hour at room temperature, followed by diaminobenzidine. Sections were visualized using a BIOREVO BZ-9000 Keyence microscope.

Cell Culture

VSMCs from the aorta of littermate mice were isolated on the basis of a protocol described by Villa-Bellosta and Hamczyk.²¹ Briefly, aortas were perfused with cold PBS. The aorta from the aortic arch to the iliac bifurcation was isolated and incubated in collagenase type 2 solution for 15 minutes at 37°C in 5% CO₂. Then, adventitia was rolled off the medial layer. Medial layers were minced and further incubated for 2 hours at 37°C with agitation. Resuspended cells were centrifuged for 5 minutes at 300g. The supernatant was aspirated, and the cell pellet was resuspended in an appropriate volume of DMEM supplemented with 10% fetal bovine serum (FBS). Finally, cell suspensions were cultured in collagenase type 1 coated culture dishes and maintained in DMEM supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin in a 5% CO₂/water-saturated incubator at 37°C. Each kind of VSMCs was isolated from 1 mouse of KO, KA, KD, and littermate WT, and individual samples that used in vitro experiments were harvested from separately cultured dishes of cells.

Transient Transfection

KO VSMCs were transfected with 1 μ g of IKK β -KD or IKK β -KA plasmids using transfection reagent Effectene (Qiagen, Valencia, CA), according to the manufacturer's instructions. Two days after transfection, VSMCs were harvested and then used in Western blotting. Plasmids encoding IKK-2 K44M (IKK β -KD) and IKK-2 S177E S181E (IKK β -KA) were from Anjana Rao Laboratory and procured through Addgene (Addgene plasmid no. 11104 and no. 11105, respectively).¹⁹

Western Blotting

VSMCs were harvested from culture dishes using cell scraper tools. Protein extraction was performed on ice and using a cell lysis buffer (9803S; Cell Signaling) with a protease inhibitor cocktail. After the total protein concentration was measured, the same amount of extracted protein was loaded for SDS-PAGE immunoblot analysis and transferred to nitrocellulose membranes. Next, membranes were blocked with 5% nonfat milk or 5% BSA in Tris buffer solution containing 0.05% Tween-20 for 1 hour at room temperature, with gentle agitation. The membrane was immunoblotted with primary antibody overnight at 4°C. The membrane was washed 3 times with Tris buffer solution containing 0.05% Tween-20 for 10 minutes each. This was followed by a 1-hour incubation with a horseradish peroxidase-conjugated secondary antibody. Blots were visualized using the ECL Western Blotting Detection kit (GE Healthcare). Each band was normalized by the corresponding value of β -actin or GAPDH as an internal control. Densitometric analysis was performed by ImageJ Software. All experiments were performed at least in triplicate.

Quantitative Real-Time PCR

Total RNA was extracted from cultured VSMCs using an RNAqueous total RNA isolation kit (AM1912; Thermo Fisher Scientific K.K., Yokoyama, Japan) according to the manufacturer's instructions. cDNA was generated from total RNA using The High Capacity RNA-to-cDNA Kit (4387406). Quantitative PCR was performed using the QuantiTect SYBR Green PCR Kit and real-time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan). Relative mRNA level of osterix, alkaline phosphatase, and osteocalcin was normalized to the Rn18s mRNA level in the same sample.

Antibodies and Reagents

Antibodies used include anti-IKK β (Millipore, Billerica, MA), anti-Transgelin (for SM22 α ; Lifespan Bioscience, Seattle, WA), anti-GAPDH, Runx2, anti-mouse IgG horseradish peroxidase-linked antibody and anti-rabbit IgG horseradish peroxidase-linked antibody (CST), active β -catenin, total β -catenin (Santa Cruz, Dallas, TX), anti-p65 (Santa Cruz), monoubiquitinated and polyubiquitinated conjugate monoclonal antibody (ENZO), goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 594 donkey anti-goat IgG, and Alexa Fluor 488 donkey anti-goat IgG (Invitrogen, Foster City, CA). The reagents used included interleukin-1 β (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo).

Statistical Analysis

Experimental results are expressed as the mean \pm SD. Groups were compared using the 2-tailed Student *t* test or 1- and 2-way ANOVA, followed by the Tukey test. *P*<0.05 was considered statistically significant.

Results

Generation of Mice With Deletion of IKK β in VSMCs

To inactivate NF- κ B signaling in VSMCs, we produce mice with a deletion of IKK β selectively in smooth muscle cells (KO mice), as previously described. Mice were born according to mendelian frequency, and there was no significant difference in growth between WT and KO mice (Figure 1A). Immunohistochemistry analyses indicated that IKK β was successfully knocked out in SM22 α -positive VSMCs in IKK β knockout mice (Figure 1B). Furthermore, we performed cultured VSMCs from the aorta of KO and littermate control mice and confirmed that IKK β is deleted in VSMCs of KO mice (Figure 1C).

Suppression of NF- κ B Activity in IKK β -Deficient VSMCs

Next, we examined the NF- κ B activity in IKK β -deficient VSMCs. IKK β activates NF- κ B via the phosphorylation and degradation of I κ B and eventually phosphorylates p65, a major subunit of NF- κ B.¹⁵ VSMCs of the aorta from KO and control mice were stimulated by interleukin-1 β to activate NF- κ B signaling, and we found that the phosphorylation of p65 was significantly suppressed in KO cells (Figure 1D). These results confirm that NF- κ B is suppressed in VSMCs from KO mice.

Deletion of IKK β in VSMCs Promotes Aortic Calcification in CaCl₂-Induced Abdominal Aorta Injury

To elucidate the roles of IKK β /NF- κ B signaling in vascular calcification, we subjected WT and KO mice to a CaCl₂-induced aorta injury model. Unexpectedly, KO mice showed more significant calcification in aorta, and those aortas were more dilated compared with WT mice (Figure 2A through 2C). Histologically, the aorta of KO mice revealed extensive damage in the medial layer and more disruption and elongation of the medial elastic lamellae, possibly because of the significant calcification (Figure 2D). Consistent with these in vivo results, Alizarin Red staining of 4-week cultured VSMCs showed remarkable calcium deposition in KO VSMCs but not in WT VSMCs (Figure 2E).

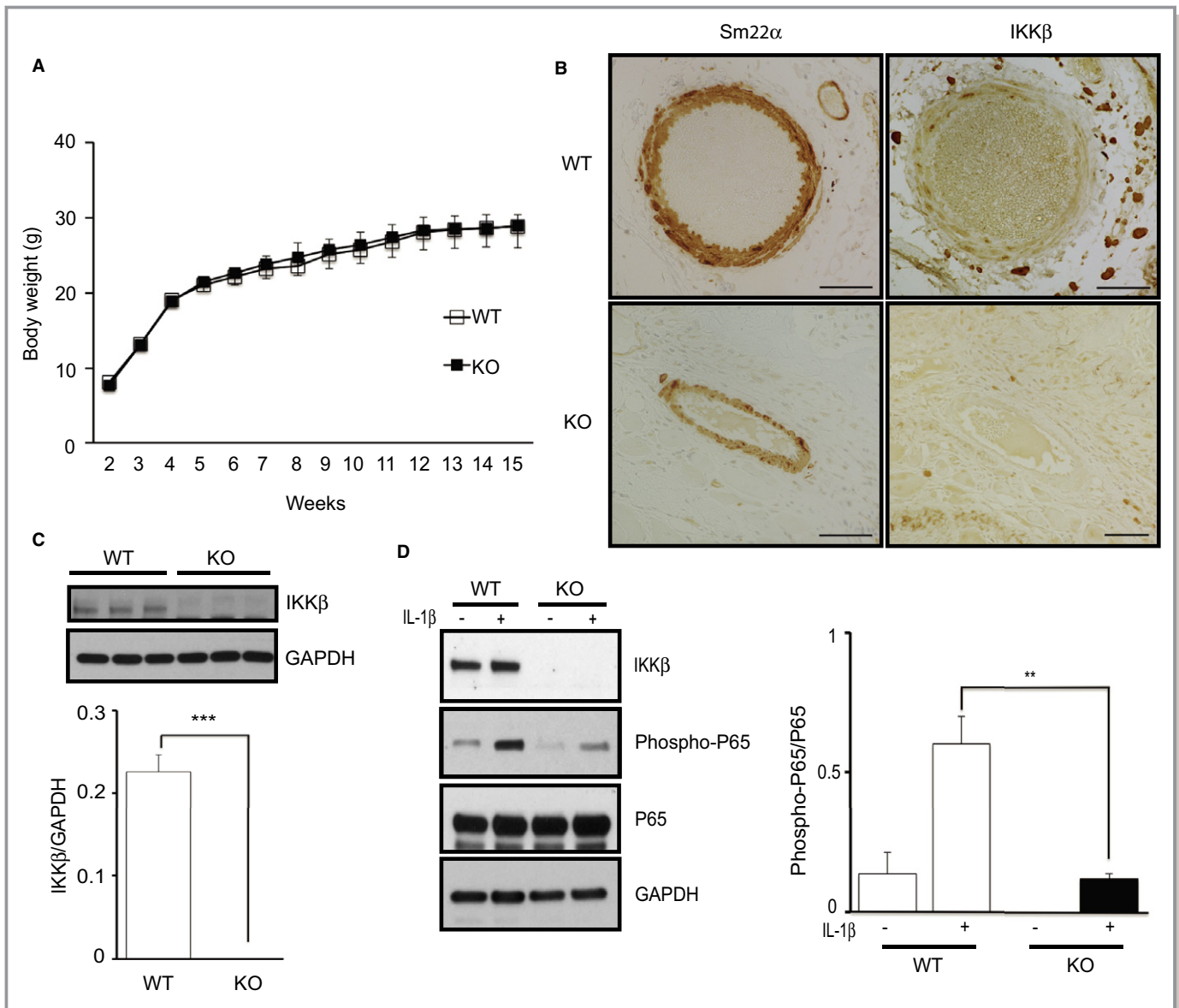


Figure 1. Making mice with deletion of IKK β in vascular smooth muscle cells (VSMCs). A, Body weight \pm SD of wild-type (WT) and IKK β knockout (KO) mice at the indicated age (n=5). B, Representative images of immunostaining of the aorta section with anti-SM22 α and anti-IKK β antibodies. Bar=50 μ m. C, Representative Western blots and densitometric analysis of IKK β expression in cultured VSMCs isolated from WT and KO littermate mice. Data represent the mean \pm SD (t test, n=3). D, Representative Western blots and densitometric analysis of phosphorylated p65/total p65 in cultured VSMCs with stimulation by interleukin (IL)-1 β (2.5 ng/mL) or the vehicle for 15 minutes. Data represent the mean \pm SD (2-way ANOVA, n=3). ** P <0.01, *** P <0.001.

Deletion of IKK β Induces Transdifferentiation of VSMCs to Osteoblast-Like Cells via Activating the β -Catenin–Runx2 Signaling Pathway

To determine the reason why injured aortic wall and cultured VSMCs of KO mice showed more calcium deposition, we investigated the underlying molecular mechanism. Recently, it is widely accepted that pathways controlling vascular calcification and bone remodeling are similar.^{22,23} Several studies have shown that the activation of β -catenin, which is the

prototypic mediator and marker of canonical Wnt signaling, is necessary for osteoblast formation and is involved in the calcification of VSMCs by promoting the expression of osteocyte genes, which leads to the transdifferentiation of VSMCs to osteoblast-like cells.^{24,25} Therefore, we investigated the activity of β -catenin in cultured VSMCs and found that the expression of the nonphosphorylated active form of β -catenin in the whole cell lysate of VSMCs was significantly augmented in KO cells when compared with that in WT cells (Figure 3A). We also assessed active β -catenin in nuclear

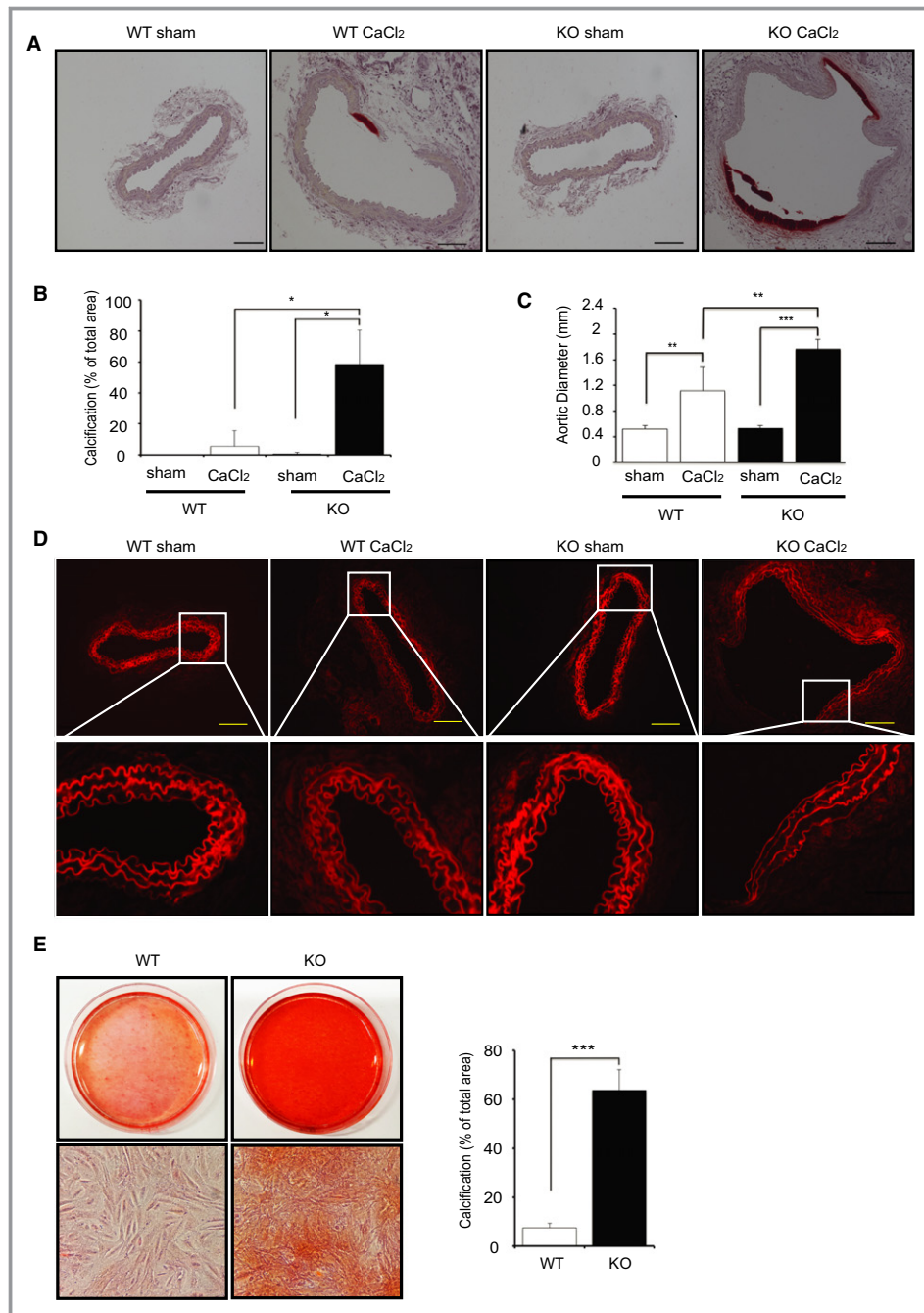


Figure 2. Deletion of IKK β in smooth muscle cells promotes calcification. A, Representative microscopy images of cross sections of infrarenal aortas stained with Alizarin Red from wild-type (WT) and IKK β knockout (KO) littermate mice 2 weeks after saline (sham) or CaCl₂ treatment. B, Quantification of vascular calcification. Calcification was quantified by ImageJ software. Graph presented is the percentage of positively stained medial layer areas in the total area of medial layer. Bars represent the mean \pm SD (2-way ANOVA, n=5). C, Graph is the maximal external diameter of the WT and KO infrarenal aorta 2 weeks after saline (sham) or CaCl₂ treatment. Bars represent the mean \pm SD (2-way ANOVA, n=5). D, Representative fluorescence microscopy images of medial elastic lamellae in the cross sections of infrarenal aortas 2 weeks after saline (sham) or CaCl₂ treatment. E, Representative microscopy images of Alizarin Red staining of 4-week cultured vascular smooth muscle cells (VSMCs) isolated from WT and KO littermate mice. Calcification was quantified by ImageJ software. Graph presented is the percentage of positively stained area in the total area randomly selected. VSMCs were cultured in normal medium with 10% fetal bovine serum. Bars represent the mean \pm SD (*t* test, n=6). **P*<0.05, ***P*<0.01, ****P*<0.001.

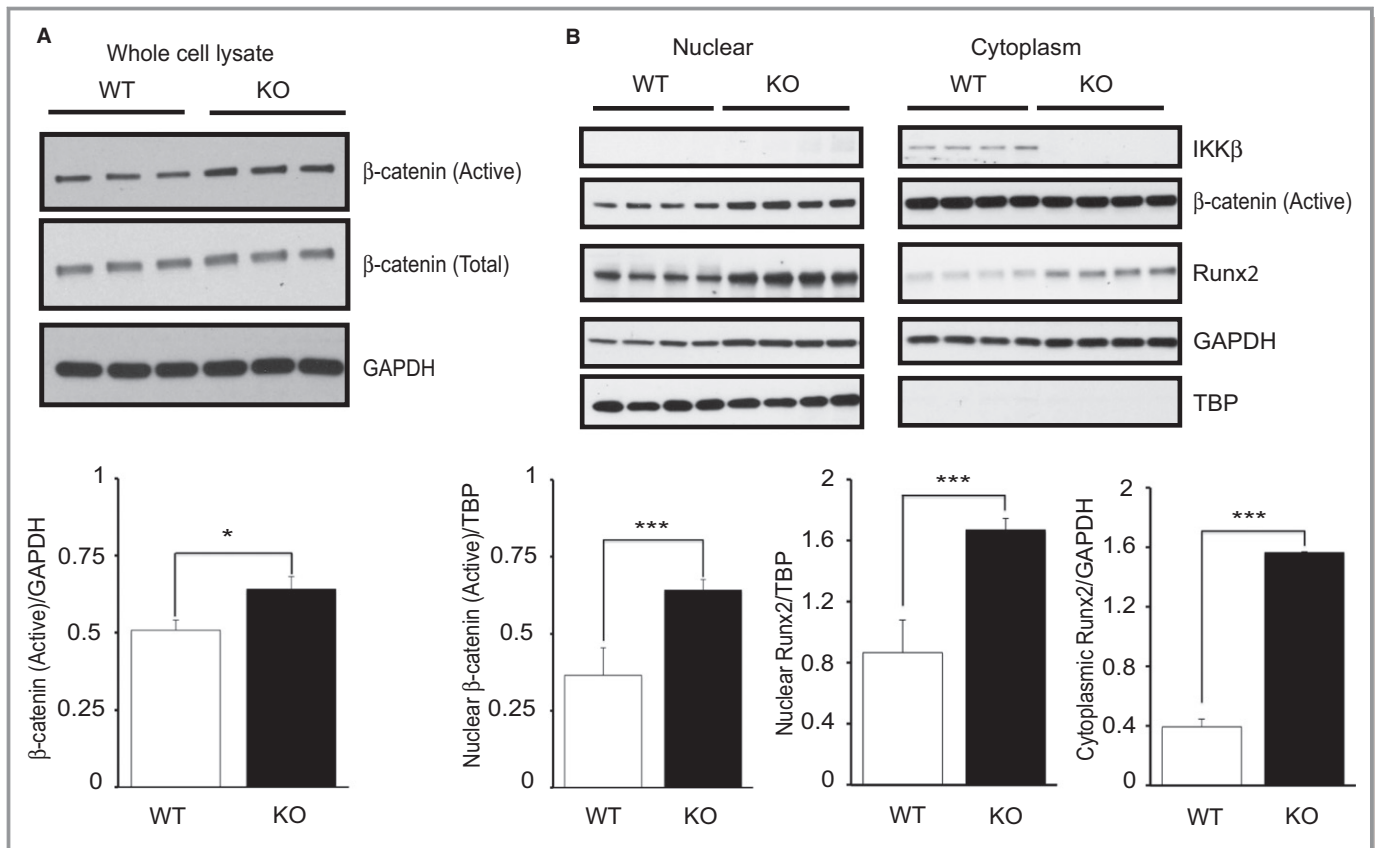


Figure 3. Activated β -catenin and Runt-related transcription factor 2 (Runx2) in IKK β -deficient vascular smooth muscle cells (VSMCs). A, Representative Western blots and densitometric analysis of active β -catenin/GAPDH in a whole cell lysate of cultured VSMCs isolated from wild-type (WT) and IKK β knockout (KO) littermate mice. Bars represent the mean \pm SD (*t* test, *n*=3). B, Representative Western blots and densitometric analysis of Runx2 and β -catenin expression in nuclear and cytoplasmic extractions normalized to tata-binding protein (TBP) or GAPDH, respectively. Bars represent the mean \pm SD (*t* test, *n*=4). **P*<0.05, ****P*<0.001.

extraction to confirm its translocation to the nucleus for osteocyte gene expression and found that more active β -catenin was located in the nucleus of KO VSMCs (Figure 3B). Next, we examined Runx2 expression in cultured VSMCs. Runx2 is a major transcription factor playing a crucial role in VSMC transdifferentiation into osteoblast-like cells^{26,27} by regulating osteoblast genes, and it is regulated by β -catenin.²⁴ Consistent with the β -catenin results, the expression of Runx2 was significantly more increased in KO VSMCs compared with that in WT VSMCs in both nucleus and cytoplasm (Figure 3B). These results indicate that the ablation of IKK β in VSMCs accelerates calcification through upregulation of β -catenin and Runx2 signaling.

Constitutively Active IKK β Protects VSMCs From Calcification via the Downregulation of β -Catenin and Runx2 Expression

To confirm the mechanism previously described, we examined the effect of kinase-active IKK β on vascular calcification as opposite situation. We produced mice with a knock-in of

kinase-active IKK β specifically in VSMCs (KA mice), as described previously,¹⁹ and subjected them to a CaCl₂-induced aorta injury model. Histologically, the aorta of KA mice showed an intact medial layer with negligible disruption in the medial elastic lamellae (Figure 4A), and no calcification was observed in the medial layer of the aorta after CaCl₂ (Figure 4B). Western blot analysis of cultured VSMCs from KA mice confirmed the upregulation of IKK β and phosphorylated p65 (Figure 4C and 4D), indicating the constitutive activation of NF- κ B in cultured VSMCs from KA mice. Consistent with *in vivo* results, Alizarin Red staining of 4-week cultured KA VSMCs showed an absence of calcium deposits (Figure 4E). Next, we examined β -catenin and Runx2 and found a significant decrease in both active and total β -catenin and Runx2 in KA-VSMCs when compared with that in WT (Figure 5A). Furthermore, nuclear location of Runx2 was significantly decreased in KA VSMCs (Figure 5B). These results indicated that overexpression of kinase-active IKK β negatively regulates β -catenin–Runx2 signaling in KA VSMCs, which were opposite to the results seen in KO VSMCs.

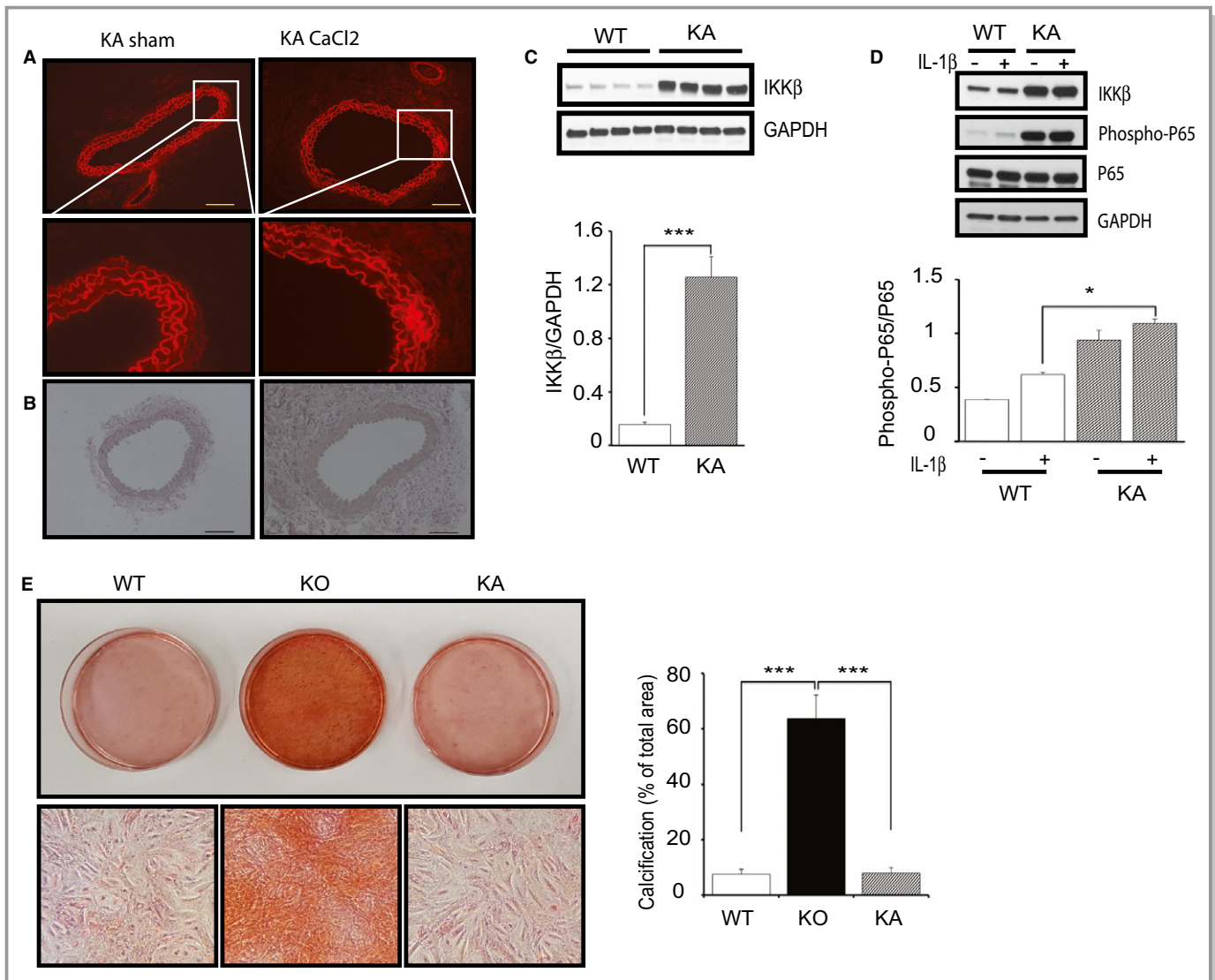


Figure 4. Constitutive activation of IKK β in vascular smooth muscle cells (VSMCs) inhibits calcification. A, Representative fluorescence microscopy images of medial elastic lamellae in the cross sections of infrarenal renal aortas from kinase-active IKK β (KA) mice 2 weeks after saline (sham) or CaCl₂ treatment. Bar=50 μ m. B, Representative microscopy images of cross sections of infrarenal aortas stained with Alizarin Red from KA mice 2 weeks after saline (sham) or CaCl₂ treatment. Bar=50 μ m. C, Representative Western blots and densitometric analysis of IKK β /GAPDH expression in cultured VSMCs isolated from wild-type (WT) and KA littermate mice. Bars represent the mean \pm SD (*t* test, n=4). D, Representative Western blots and densitometric analysis of phosphorylated p65/total p65 expression in cultured VSMCs isolated from WT and KA littermate mice with stimulation by interleukin (IL)-1 β (2.5 ng/mL) or the vehicle for 15 minutes. Bars represent the mean \pm SD (2-way ANOVA, n=3). E, Representative microscopy images of Alizarin Red staining of 4-week cultured VSMCs isolated from WT, IKK β knockout (KO), and KA mice and quantification of VSMC calcification. VSMCs were cultured in normal medium with 10% fetal bovine serum. Calcification was quantified by ImageJ software. Graph presented is the percentage of positively stained area in the total area randomly selected. Bars represent the mean \pm SD (1-way ANOVA, n=6). **P*<0.05, ****P*<0.001.

IKK β Modulates Ubiquitination of β -Catenin in VSMCs

According to previous reports, β -catenin is inactivated by ubiquitination via the ubiquitin-proteasome pathway after it is phosphorylated.²⁸ Thus, we examined ubiquitination of β -catenin on WT, KO, and KA-VSMCs. Cells were pretreated with proteasome inhibitor MG132 to block ubiquitin-induced

degradation by proteasomes and subjected to ubiquitination assay. The results showed that the deletion of IKK β in VSMCs reduced the ubiquitination of β -catenin and, conversely, it was augmented in VSMCs with constitutively active IKK β (Figure 5C). These results were consistent with the change of active β -catenin in KO and KA VSMCs. We also histologically examined the change of β -catenin and Runx2 in CaCl₂-injured aortas of WT, KO, and KA mice. As

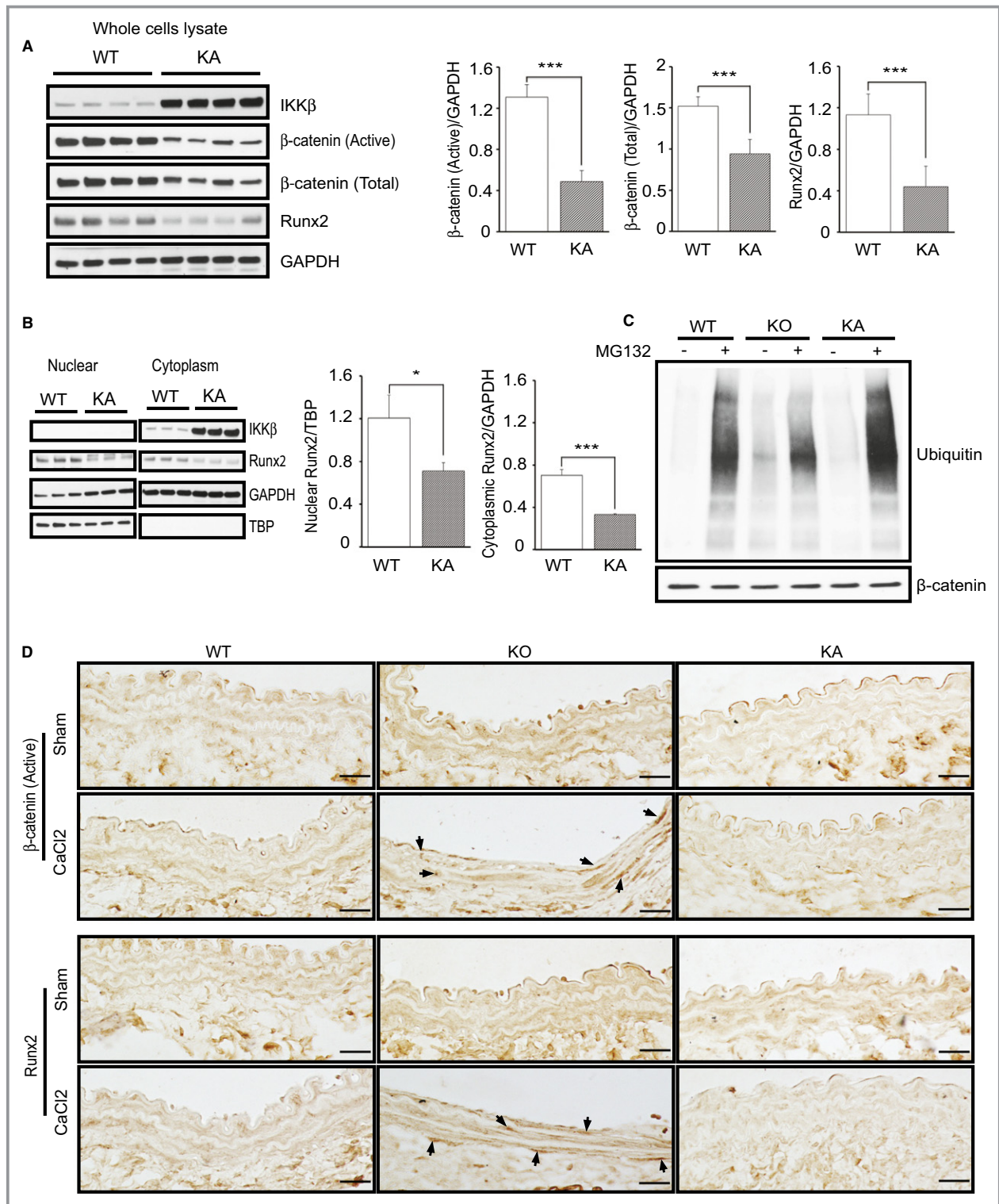


Figure 5. Constitutive activation of IKK β in vascular smooth muscle cells (VSMCs) inhibits β -catenin–Runx2-related transcription factor 2 (Runx2) signaling. **A**, Representative Western blots and densitometric analysis of active and total β -catenin/GAPDH and Runx2/GAPDH expression in a whole cell lysate of cultured VSMCs isolated from wild-type (WT) and kinase-active IKK β (KA) littermate mice. Bars represent the mean \pm SD (*t* test, *n*=4). **B**, Representative Western blots and densitometric analysis of Runx2 expression in nuclear and cytoplasmic extractions normalized to Tata-binding protein (TBP) or GAPDH, respectively. Bars represent the mean \pm SD (*t* test, *n*=3). **C**, Representative image of 3 independent ubiquitination assays in which β -catenin was precipitated and blotted with antiubiquitin. **D**, Representative images of immunostaining of the aorta section from WT, IKK β knockout (KO), and KA mice 2 weeks after saline (sham) or CaCl₂ treatment with inactive β -catenin and Runx2 antibodies. Bar=20 μ m. **P*<0.05, ****P*<0.001.

shown in Figure 5D, CaCl₂ induces staining of active β -catenin and Runx2 in nuclei of smooth muscle layers in KO aorta compared with that in sham group. In contrast, no apparent staining of active β -catenin and Runx2 was observed in both KA and WT aorta, even after CaCl₂-induced aortic injury. These results are consistent with observations seen in cultured VSMCs.

There is No Apparent Relationship Between Apoptosis and VSMC Calcification

Several reports have shown that apoptosis of VSMCs can induce vascular calcification.^{29,30} To investigate the involvement of apoptosis in VSMC calcification induced by IKK β deletion, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling staining on the aortas. There was no remarkable difference in number of apoptotic cells between

WT and KO aortas after CaCl₂ treatment. In contrast, terminal deoxynucleotidyl transferase dUTP nick end labeling staining revealed a remarkable increase of apoptotic cells after CaCl₂-induced aortic injury in KA mice, compared with WT and KO mice (Figure 6A). To understand the molecular mechanisms by which overexpression of kinase-active IKK β induced VSMC apoptosis, we examined caspase-9 and caspase-3, crucial mediators of apoptosis, in cultured VSMCs. Western blot analysis of cultured VSMCs demonstrated that expression of both cleaved caspase-9 and cleaved caspase-3 was significantly increased in KA VSMCs compared with those in WT and KO VSMCs (Figure 6B). Despite the increase of apoptosis at the medial layer of KA mice after CaCl₂ application, no remarkable calcification was observed, as previously shown. Collectively, these observations suggest that the apoptosis is not the primary cause of VSMC calcification, at least in these mouse models.

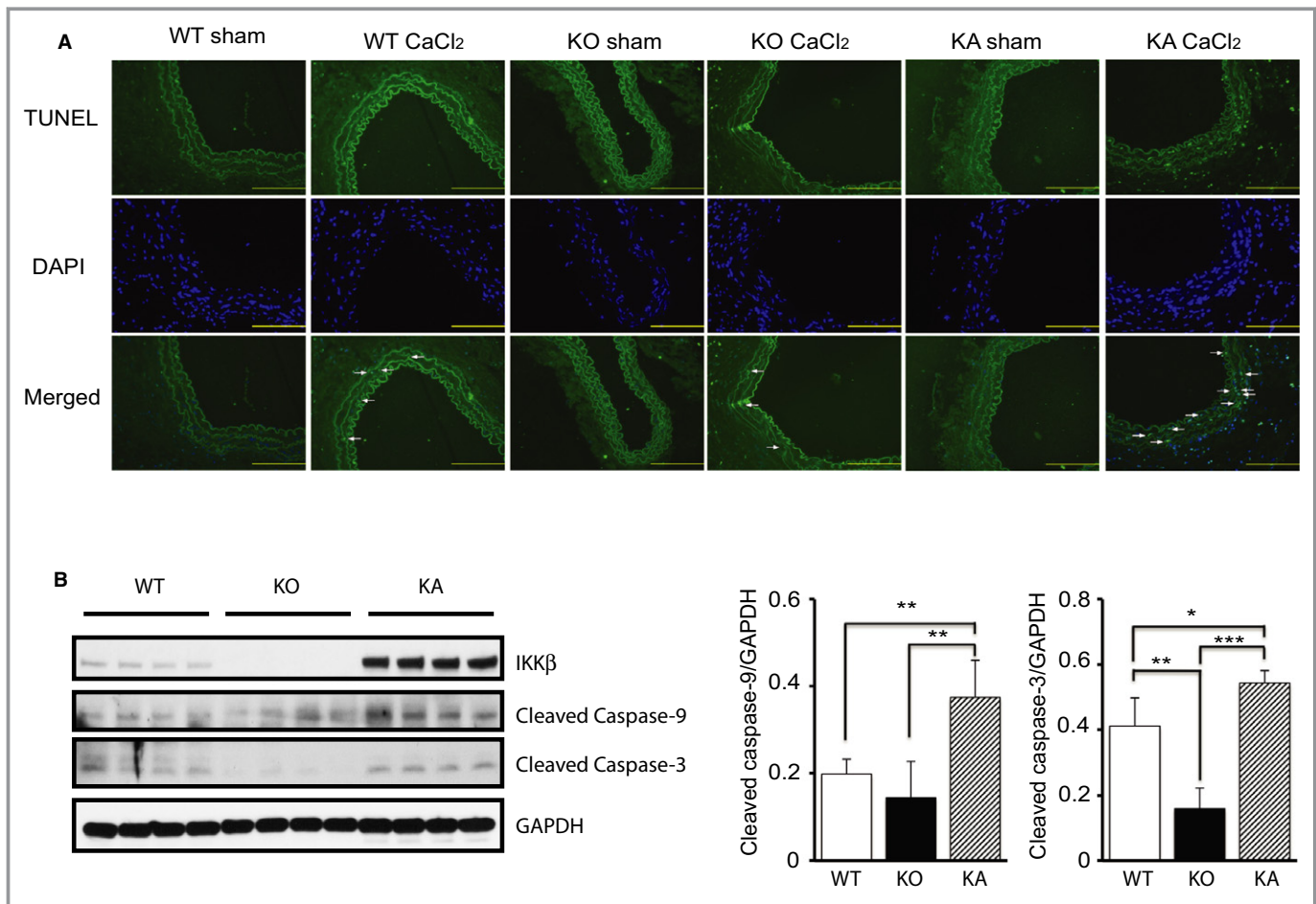


Figure 6. Apoptosis in injured aortas of IKK β knockout (KO) and kinase-active IKK β (KA) mice. A, Representative images of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay performed in cross sections of infrarenal aortas after 2 weeks CaCl₂ or saline application in wild-type (WT), KO, and KA mice. Bar=50 μ m. B, Representative Western blots and densitometric analysis of cleaved caspase-9 and caspase-3/GAPDH expression in cultured vascular smooth muscle cells isolated from WT, KO, and KA mice. WT mice were from littermates of KO mouse. Bars represent the mean \pm SD (1-way ANOVA, n=4). DAPI indicates 4',6-diamidino-2-phenylindole. * P <0.05, ** P <0.01, *** P <0.001.

Kinase-Independent Function of IKK β Is Involved in Regulating VSMC Calcification

Kinase-dependent function of IKK β in the activation of NF- κ B is well known; however, recent reports showed that IKK β has other regulatory functions, independently from NF- κ B activation.³¹ Furthermore, we previously reported that IKK β regulates other proteins, even in a kinase-independent manner.^{31,32} Results previously shown clearly indicated that IKK β negatively regulates calcification, but it is difficult to conclude whether either or both of kinase-dependent or kinase-independent function of IKK β is involved in it, as far as we compare among WT, KO, and KA-IKK β . Indeed, interleukin-1 β stimulation did not induce any change in Runx2 or β -catenin in WT cells (Figure 7A), which implicates the possible involvement of kinase-independent function of IKK β in regulating these molecules.

To make it clear, we produced mice expressing kinase-dead mutant (KD) of IKK β in VSMCs on the background of IKK β f/f sm22Cre^{+/-} (KO) mice, according to the previous report showing that point mutation of an amino acid inactivates kinase function¹⁹ (Figure 7B), and we examined cultured VSMCs from KD mice. As shown in Figure 7C, IKK β is highly more expressed in KD VSMCs compared with KO VSMCs. However, the level of phosphorylated p65 in KD cells is equivalent to that in KO cells, which indicated that the kinase activity is successfully lost in KD cells. Surprisingly, Alizarin Red staining showed no remarkable increase of calcium deposit in KD VSMCs compared with that in WT VSMCs (Figure 7D), contrary to KO VSMCs. Considering the significant calcification in KO VSMCs compared with that in WT VSMCs, it is clearly indicated that the kinase-independent function of IKK β has a suppressive effect on calcification. To confirm the difference in calcification among WT, KO, KA, and KD VSMCs, we performed real-time PCR to examine the expression of major osteoblast marker genes regulating transdifferentiation of VSMCs to osteoblast-like cells.^{33,34} As shown in Figure 7E, osterix, alkaline phosphatase, and osteocalcin were significantly upregulated only in KO VSMCs.

Next, we examined β -catenin and Runx2 signaling in KD VSMCs. Consistent with the previous results, active β -catenin and Runx2 in KD VSMCs were significantly downregulated compared with KO VSMCs, clearly indicating that the kinase-independent function of IKK β is involved in the downregulation of β -catenin (Figure 8A). We also noticed that active β -catenin and Runx2 in KD VSMCs were significantly more expressed than those in KA VSMCs, despite more expression of IKK β in KD cells, which indicated that kinase-dependent function of IKK β also has a suppressive effect on calcification. Furthermore, we performed β -catenin ubiquitination assay in KD VSMCs and found that β -catenin ubiquitination was upregulated in KD VSMCs (Figure 8B), which supported the

observation that β -catenin is inactivated in KD VSMCs. To confirm the observations further, KO VSMCs were transfected with IKK β -KD and IKK β -KA plasmids. KO VSMCs transfected with IKK β -KD or IKK β -KA plasmids showed a significant decrease in Runx2 expression (Figure 8C), which is consistent with results seen in transgenic VSMCs.

Discussion

Recent clinical studies have indicated that vascular calcification is different from atherosclerosis, but the mechanisms of vascular calcification have not been explored adequately. In this study, we show that the deletion of IKK β , an essential kinase for NF- κ B activation, in VSMCs promotes vascular calcification. We also show that it is mediated by the upregulation of β -catenin, Runx2, and transcription of osteoblast genes. Furthermore, we found that kinase-independent function of IKK β is involved in the regulation of such signaling. Considering that the accelerating roles of NF- κ B in atherosclerosis have been well analyzed, this finding would mechanistically explain the difference between atherosclerosis and calcification.

The difference between atherosclerosis and vascular calcification has been only empirically suggested, but recent clinical studies have clearly demonstrated it.⁴⁻⁷ For instance, Puri et al reported that statins, the most powerful medicine against atherosclerosis, unexpectedly accelerate vascular calcification.^{8,35} However, no mechanistic insights have been reported to explain this finding. Considering statin has the activating effect on β -catenin³⁶ and the suppressive effect on NF- κ B,^{12,37} the observations in this study would account for it, at least in part.

It was also an unexpected observation that more aortic dilatation was seen in KO mice. It has been reported that aortic calcification induces mechanical wall stress by decreasing biomechanical stability, which also associated with destruction and elongation of the elastic lamella in the medial layer.³⁸ In addition, mechanical wall stress itself causes expansion and dilatation of the aortic wall.³⁹ These mechanisms would account for the dilatation of calcified aorta in KO mice.

The molecular mechanisms of calcification in vessel walls and bone are reported to be similar, as indicated by the observation that the phenotypic transdifferentiation of VSMCs into the osteoblast-like cells is seen in vascular calcification.^{22,23} Indeed, β -catenin and Runx2, major regulators of bone-related genes, such as osterix (*OSX*), alkaline phosphatase (*ALP*), and osteocalcin (*OCN*), promote the transdifferentiation of VSMCs into osteoblast-like cells.²⁴⁻²⁷ Therefore, the changes of β -catenin, Runx2, and osteoblast marker genes seen in KO VSMCs fundamentally support the histological observation of vascular calcification in vivo and

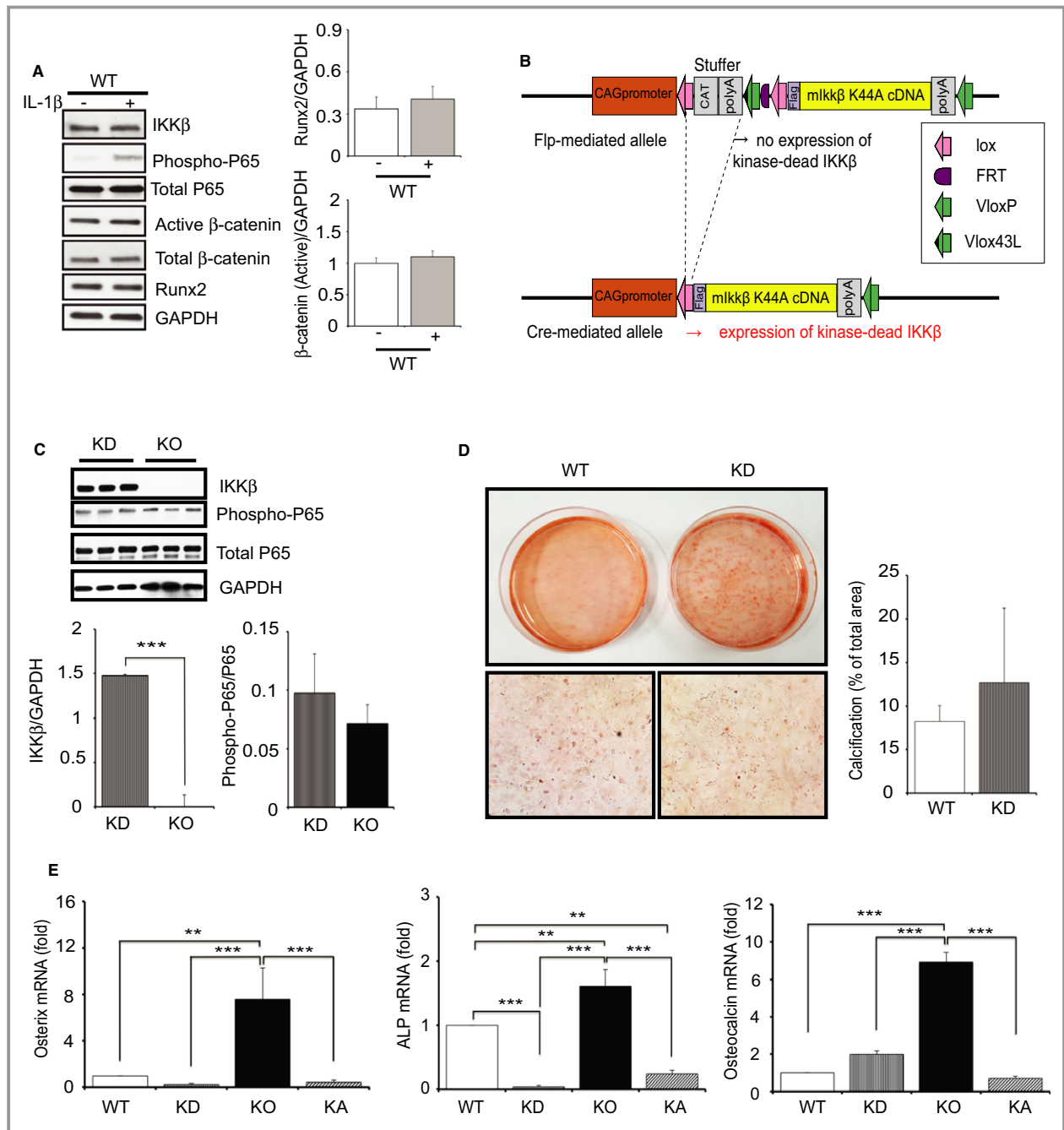


Figure 7. IKK β regulates vascular smooth muscle cells (VSMC) calcification through its kinase-independent function. A, Representative Western blots and densitometric analysis of active β -catenin/GAPDH and Runt-related transcription factor 2 (Runx2)/GAPDH expression in cultured VSMCs isolated from wild-type (WT) mouse with stimulation by interleukin (IL)-1 β (2.5 ng/mL) or the vehicle for 15 minutes. Bars represent the mean \pm SD (*t* test, *n*=3). B, Kinase-dead IKK β transgene construct. C, Representative Western blots and densitometric analysis of IKK β /GAPDH, phosphorylated p65/total p65 expression in VSMCs isolated from kinase dead (KD) and IKK β knockout (KO) mice. Bars represent the mean \pm SD (*t* test, *n*=3). D, Representative microscopy images of Alizarin Red staining of 4-week cultured VSMCs isolated from WT and KD mice and quantification of VSMC calcification. Calcification was quantified by ImageJ software. Graph presented is the percentage of positively stained area in the total area randomly selected. Bars represent the mean \pm SD (*t* test, *n*=6). E, Results of quantitative real-time PCR (qRT-PCR) for the expression of various osteogenic-related genes (osterix, alkaline phosphatase [ALP], and osteocalcin) in WT, KD, KO, and kinase active IKK β (KA) cells that were normalized to the Rn18s mRNA level. WT samples used in RT-PCR were from littermate of KO mouse. Cells used in qRT-PCR were cultured for 2 weeks in normal medium with 10% fetal bovine serum. Bars represent the mean \pm SD (1-way ANOVA, *n*=4). ***P*<0.01, ****P*<0.001.

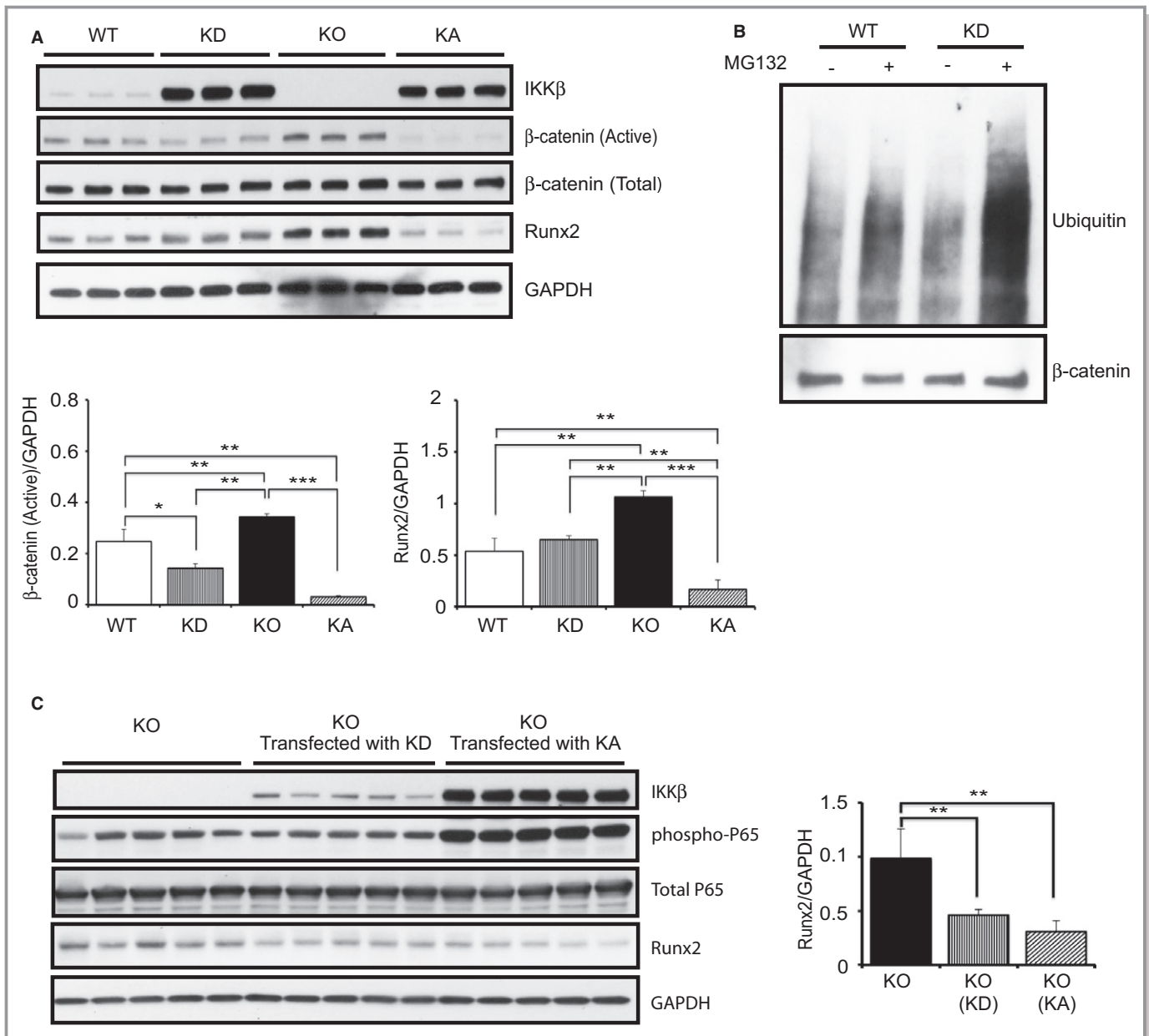


Figure 8. IKK β inhibits β -catenin–Runx2 signaling through its kinase-independent function. **A**, Representative Western blots and densitometric analysis of active β -catenin/GAPDH and Runx2/GAPDH expression in vascular smooth muscle cells (VSMCs) isolated from wild-type (WT), kinase dead (KD), IKK β knockout (KO), and kinase active IKK β (KA) mice. WT samples used in Western blot were from littermate of KO mouse. Bars represent the mean \pm SD (1-way ANOVA, n=3). **B**, Representative image of 3 independent ubiquitination assays in WT and KD VSMCs, in which β -catenin was precipitated and blotted with antiubiquitin. **C**, Representative Western blots and densitometric analysis of Runx2/GAPDH expression in KO VSMCs transfected with IKK β -KD and IKK β -KA plasmids. KO VSMCs were harvested after 2 days of transfection. Control KO VSMCs were transfected with no plasmid, but transfection reagent only. Bars represent the mean \pm SD (1-way ANOVA, n=5). * P <0.05, ** P <0.01, *** P <0.001.

in vitro from the molecular level. Furthermore, considering the similarity between bone formation and vascular calcification, it is interesting that previous reports on bone research showed that statin can enhance bone formation.^{40–42}

Although the roles of β -catenin in vascular calcification and bone formation have been well analyzed, as previously mentioned, those of IKK β /NF- κ B signaling have not been

adequately unveiled to date. In the bone research, it was reported that the deletion of IKK β significantly enhanced bone formation by activating β -catenin ubiquitination and degradation.⁴³ As its mechanism, Lamberti et al reported that IKK β interacts with and is able to phosphorylate β -catenin,⁴⁴ and Chang et al reported that IKK β promotes its ubiquitination.⁴³ In this study, the nonphosphorylated active form of β -catenin

is suppressed and its ubiquitination is augmented in KD VSMCs. The detailed mechanism of how IKK β regulates β -catenin in a kinase-independent manner is still unclear and is to be unveiled. However, this finding would be of high importance considering that kinase-independent function of IKK β has never reported, except by us.³² β -Catenin is the big target of interest, and it has been researched in many fields, including cancer, fibrosis diseases, and vascular calcification.

Considering that kinase-independent function of IKK β regulates calcification, as shown in this study, the regulation of protein expression of IKK β should be more carefully recognized. Interestingly, IKK β is the target of miR148a, miR503, and miR199a,^{45–47} which are upregulated microRNAs in vascular calcification.^{48,49} The suppression of such microRNAs targeting IKK β might be a new strategy against vascular calcification.

The findings in this study explain not only the difference between atherosclerosis and calcification but also the disappointing results reported in clinical studies that examined the effect of aspirin on calcification. Aspirin has a suppressive effect on inflammatory responses, including NF- κ B. Indeed, Yin et al reported that aspirin is a specific inhibitor of IKK β .⁵⁰ Therefore, people expect that aspirin use would have a protective effect against calcification; however, recent clinical studies have found that coronary calcification was associated with aspirin use⁵¹ and that aspirin had no protective effect on the onset of calcification in either coronary arteries or aortic valves. The anticalcification effect of IKK β revealed in this study might explain these results. Furthermore, we have previously reported that long-term and high concentration of aspirin can suppress the protein expression of IKK β ,⁵² which might indicate possible modification of kinase-independent function of IKK β by aspirin. These new insights can lead to unveiling a new relationship between vascular calcification and aspirin use.

There are strengths and limitations in this study. The strength is that kinase-independent function of IKK β in suppressing calcification is unveiled. The kinase-dependent functions of IKK β have been well analyzed; however, its kinase-independent function has been rarely reported, except in our previous study.³² Therefore, its roles in diseases are almost unknown. This study indicated its new function in vascular calcification, which would attract interest of researchers in the field of vascular diseases. On the other hand, the limitation of this study is that we have not evaluated the kinase-dependent roles of IKK β in the regulation of calcification. Considering that KD-IKK β works similarly with KA-IKK β in inhibition of calcification, kinase-independent function would have a major contribution to the inhibitory role of IKK β on calcification. However, it does not exclude the involvement of kinase-dependent function of IKK β on calcification. Some articles have reported that kinase activity of IKK β regulates β -catenin.^{43,44,53} Indeed, in Figure 8A, the

expression of β -catenin and Runx2 in KA-IKK β cells is less than that in KD-IKK β cells, despite less IKK β protein expression, which implicates the involvement of kinase activity of IKK β in regulation of these molecules. To explore it precisely, transient expression of constitutive active p65 in vitro would be not suitable because long-term cell culture is required for observing calcification. Future study of making CaCl₂ model on mice with modification of p65 or I κ B in VSMCs would make it clear.

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Disclosures

None.

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