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ILK-Dependent Modulation of DPP4 Prevents Progression of Calcific Aortic Valve Disease

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BACKGROUND: Calcific aortic valve disease (CAVD) is characterized by endothelial dysfunction, fibrosis, and osteogenic calcification, yet the molecular mechanisms driving disease progression remain incompletely understood, and no pharmacological therapies are currently available. Reduced endothelial ILK (integrin-linked kinase) expression has been implicated in CAVD, but its downstream effectors remain undefined.

METHODS: To explore the direct role of ILK in CAVD development, we used a mouse model in which ILK is conditionally deleted from endothelial cells (endothelial cell-specific ILK conditional knockout). DPP4 (dipeptidyl peptidase 4) expression and activity were examined in human aortic valve tissue and plasma from patients with and without CAVD as a potential therapeutic target. Mechanistic studies were performed in human valvular endothelial cells subjected to ILK silencing, with or without pharmacological or genetic DPP4 inhibition. In vivo, endothelial cell-specific ILK conditional knockout mice were treated with the DPP4 inhibitor sitagliptin, and valvular, cardiac function, and remodeling were assessed.

RESULTS: DPP4 expression was increased in circulation and in the aortic valves of patients with CAVD and was inversely correlated with ILK levels, with predominant localization to valvular endothelial cells. ILK silencing in human valvular endothelial cells increased DPP4 expression and enzymatic activity, promoted endothelial-to-mesenchymal transition, and induced osteogenic reprogramming; these effects were attenuated by DPP4 inhibition. Endothelial ILK deletion in mice recapitulated CAVD features, including inflammation, valve thickening, calcification, and cardiac remodeling, all of which were associated with increased DPP4. Sitagliptin treatment mitigated disease severity. Mechanistically, sitagliptin inhibited NF- κ B (nuclear factor kappa B)-driven DPP4 upregulation caused by ILK loss.

CONCLUSIONS: These findings identify DPP4 as a key downstream effector linking endothelial ILK deficiency to inflammation, endothelial-to-mesenchymal transition, and calcific remodeling in CAVD and support DPP4 inhibition as a potential disease-modifying strategy to slow disease progression.

GRAPHIC ABSTRACT: A [graphic abstract](#) is available for this article.

Key Words: aortic valve ■ cardiovascular diseases ■ endothelial-mesenchymal transition ■ inflammation ■ scaffold protein ILK

Human life expectancy has increased in recent decades, leading to a higher prevalence of age-associated cardiovascular diseases, including calcific aortic valve disease (CAVD). CAVD is characterized by fibrocalcific remodeling of the aortic valve, leading to aortic stenosis and accounting for approximately half of all valvular heart disease. In developed countries, it is the third most common cardiovascular condition after

coronary artery disease and hypertension.^{1,2} Established risk factors include congenital valve abnormalities, aging, smoking, hypercholesterolemia, hypertension, and diabetes. CAVD also exhibits marked sex-related differences, with men showing a higher incidence and greater calcific burden, whereas women more often develop a fibrotic phenotype despite comparable hemodynamic severity, differences that extend to myocardial remodeling and may

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Nonstandard Abbreviations and Acronyms

ALP	alkaline phosphatase
CAVD	calcific aortic valve disease
CM	conditioned media
Col	collagen
DPP4	dipeptidyl peptidase 4
ecILK cKO	endothelial cell-specific integrin-linked kinase conditional knockout
EndMT	endothelial-to-mesenchymal transition
FBS	fetal bovine serum
FN	fibronectin
GFP	green fluorescent protein
GLP-1	glucagon-like peptide-1
hVEC	human valvular endothelial cell
IB4	isolectin B4
ICAM	intercellular adhesion molecule
IGF-1	insulin-like growth factor-1
ILK	integrin-linked kinase
LDL	low-density lipoprotein
LV	left ventricle
MCP-1	monocyte chemoattractant protein-1
NF-κB	nuclear factor kappa B
OSPN	osteopontin
RUNX2	runt-related transcription factor 2
SDF-1α	stromal cell-derived factor-1α
VCAM	vascular cell adhesion molecule
VEC	valvular endothelial cell
VE-cadherin	vascular endothelial cadherin
VIC	valvular interstitial cell

affect imaging-based assessments of disease severity.³ In addition, systemic conditions contribute to accelerated CAVD progression. Chronic kidney disease, particularly in the context of bone-mineral disorders, is increasingly recognized as a major driver of valvular calcification through sustained alterations in calcium-phosphate homeostasis, inflammation, and osteogenic signaling.⁴ Untreated severe aortic stenosis is associated with rapid myocardial deterioration and high mortality, approaching 50% within 2 years of diagnosis. Although surgical and transcatheter aortic valve replacement reduce mortality in high-risk patients, they do not modify disease progression, and no medical therapies are currently available, highlighting an urgent unmet clinical need.⁵

CAVD progresses through distinct phases: initial endothelial dysfunction and lipid buildup, followed by inflammation, fibrosis, osteogenic transformation, and calcification. The early stages are characterized by lipid accumulation and inflammation, while later stages involve fibrosis and osteogenic changes that thicken and calcify

What Are the Clinical Implications?

These findings identify endothelial dysfunction, specifically ILK (integrin-linked kinase) deficiency-driven upregulation of DPP4 (dipeptidyl peptidase 4), as an early and potentially actionable mechanism in calcific aortic valve disease, shifting therapeutic focus to upstream processes before irreversible valve calcification occurs. The observation that DPP4 is increased in the circulation and aortic valves of patients with calcific aortic valve disease, together with its mechanistic link to endothelial osteogenic reprogramming, supports its role as both a biomarker and a therapeutic target in disease progression. Pharmacological inhibition of DPP4 attenuates endothelial inflammation, valve calcification, and cardiac dysfunction in experimental models, suggesting that clinically approved DPP4 inhibitors could be repurposed to slow calcific aortic valve disease progression, particularly at early disease stages. The widespread clinical use of DPP4 inhibitors in patients with diabetes provides a strong rationale for retrospective and prospective studies to evaluate their impact on aortic valve disease outcomes.

the valve leaflets, worsening the condition. Concurrent myocardial fibrosis impairs heart function, contributing to heart failure.^{6,7}

Despite mechanistic similarities with atherosclerosis, large statin trials in established aortic valve stenosis have shown no clear benefit,⁸ possibly due to late intervention and limited follow-up; in contrast, emerging evidence suggests that early LDL (low-density lipoprotein) lowering may delay CAVD progression and the need for valve replacement.⁹ Recent studies have indicated that endothelial dysfunction in the aortic valve increases DPP4 (dipeptidyl peptidase 4) expression, leading to the degradation of IGF-1 (insulin-like growth factor-1) and subsequent osteogenic differentiation of valvular interstitial cells (VICs).¹⁰ DPP4 is an enzyme that degrades incretin hormones, thereby regulating insulin secretion, blood glucose levels, and exerting effects on vascular biology, inflammation, and thrombosis in the cardiovascular system. DPP4 is ubiquitously expressed on the surface of various cell types and circulates in a soluble, enzymatically active form in serum/plasma.¹¹ Several DPP4 inhibitors used to treat type 2 diabetes have been shown to slow the progression of aortic valve calcification in CAVD animal models.^{12,13} This suggests that DPP4 inhibitors may prevent or delay the progression of CAVD.

Previous work has shown that ILK (integrin-linked kinase) is associated with aortic valve calcification, increasing the expression of procalcific markers such as RUNX2 (runt-related transcription factor 2) in patients with CAVD and an aged mouse model.^{14,15} Specifically, decreased ILK expression in valvular endothelium is

associated with a reduction in NO synthesis, the initiation of an endothelial-mesenchymal transition program, and the occurrence of calcification. In addition, ILK may also play a role in the higher incidence of CAVD in chronic kidney disease patients through a mechanism dependent on the uremic toxin indoxyl sulfate.^{16,17}

A murine model of endothelium-specific conditional ILK deletion has demonstrated myocardial dysfunction, fibrosis, and microvascular remodeling driven by endothelial-to-mesenchymal transition (EndMT).¹⁸ Previous studies indicated that the DPP4 inhibitor, linagliptin, inhibited kidney fibrosis in diabetic mice, with antifibrotic effects associated with EndMT suppression.¹³ Thus, DPP4 may represent a therapeutic target influenced by ILK expression in CAVD through EndMT inhibition.

In this article, we aim to investigate the direct role of ILK in valvular calcification using a mouse model in which ILK is conditionally deleted from endothelial cells. We will also explore DPP4 inhibition as a potential therapeutic strategy to slow the progression of CAVD associated with ILK loss.

MATERIALS AND METHODS

Data and materials related to this study are available from the corresponding author upon reasonable request.

A detailed listing of the reagents, antibodies, and primers used throughout the study is listed in the [Major Resources Table](#) in the [Supplemental Material](#).

Human Calcified Aortic Valves

This study was designed as an observational case-control analysis of human aortic valve specimens obtained from patients undergoing valve replacement surgery at Ramón y Cajal University Hospital (Madrid, Spain) between 2019 and 2025. A total of 22 patients with CAVD and 16 control subjects were included.

Patients in the CAVD group underwent surgical aortic valve replacement for severe calcific aortic stenosis, as confirmed by standard echocardiographic criteria. The control (non-CAVD) group consisted of patients undergoing surgery for isolated aortic regurgitation without echocardiographic or macroscopic evidence of calcification. Patients with active infection, autoimmune or systemic inflammatory diseases, or malignancy were excluded.

Clinical variables were extracted from electronic medical records and analyzed in a deidentified manner. All enrolled patients completed the study protocol, and no missing data were identified for the variables included in the analyses. Baseline clinical characteristics are summarized in [Table S1](#).

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Ramón y Cajal Hospital. Written informed consent was obtained from all participants before inclusion.

This observational case-control study is reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology guidelines, and the completed checklist is provided in the [Major Resources Table in the Supplemental Material](#).

Blood samples were obtained at the time of valve replacement, and human valve samples were collected as surgical residues. Of the 3 leaflets collected, one was processed in 10% formalin for histological study, the second was stored at -80°C for protein analysis, and the third was stored at -80°C for RNA isolation.

Human Valve Endothelial Cell and Human Valve Interstitial Cell Isolation

Valves from patients without CAVD were kept in DMEM/F12 culture medium supplemented with 10% fetal bovine serum (FBS) from Gibco (Waltham, MA) and 0.05-mg/mL penicillin/streptavidin (Lonza, Basel, Switzerland), as described previously.¹⁵ Briefly, the valve leaflets were delicately sectioned into 2-mm fragments and cultured in a Matrigel-coated dish (Corning, Madrid). The valve explants were grown in endothelial basal medium-2 supplemented with an EGM-2 kit (CC4176, Lonza) for 2 weeks. The explants were extracted, and the endothelial cells were dissociated from the Matrigel using a 2-U/mL Dispase II solution (Roche, Madrid) at 37°C for 1 hour. The cell suspension was centrifuged at 900 rpm for 5 minutes, and the pellet was resuspended and seeded onto a gelatin-coated plate. Afterward, the remaining explanted material was pooled and further digested with collagenase type II in VIC media overnight at 37°C . The cell suspension was filtered through a 40- μm filter, centrifuged twice at 1500 rpm for 5 minutes, and then plated on a nongelatinized plate to obtain VICs. Subsequent characterization of these cells using confocal microscopy revealed that 97.7% of the valvular endothelial cell (VEC) primary cell culture expressed CD31 and 98% of the VIC primary cell culture expressed α -SMA (alpha smooth muscle actin), thereby confirming the isolation of both cell types.

Cells were cultured in a controlled, humidified CO_2 environment, and cells from passages 2 to 5 were used in subsequent experiments, ensuring the reliability and integrity of the research. In all cell-based experiments, each value of n corresponds to a different patient.

Animals

Endothelial-specific ILK conditional knockout mice (genotype VE-Cadherin-Cre⁺/ILK^{fl/fl}) were generated as previously described.¹⁸ Ai40 (B6.Cg-Gt[Rosa]26Sortm40.1[CAG-aop3/eGFP (enhanced green fluorescent protein)] Hze/J) mice were purchased from Jackson Laboratory 021188 (The Jackson Laboratory, United States). Ai40 conditionally expresses an improved archaerhodopsin-3/eGFP fusion protein (ArchT [archaerhodopsin-TCT]-eGFP) from the endogenous Gt(Rosa)26Sor locus. Following Cre (Cre recombinase)-mediated removal of the floxed-STOP cassette, ArchT-eGFP expression is observed in the Cre-expressing cells. Ai40 mice were crossed with the VE-cadherin (vascular endothelial cadherin)-Cre/ILK lox/lox mice, generating the endothelial cell-specific ILK conditional knockout (ecILK cKO)-eGFP mice. To control, the parental VE-cadherin-CreERT2 (Cre recombinase-estrogen receptor T2 fusion protein) mice crossed with Ai40 mice were used (further referred to as CT [control]-eGFP mice). When tamoxifen is administered intraperitoneally for 5 days, CT-eGFP mice will express GFP (green

fluorescent protein) in endothelial cells with intact ILK expression. In contrast, eILK cKO-eGFP mice will express eGFP and lack endothelial ILK.

The animals were housed in dedicated rooms at the Animal Experimentation Center of the University of Alcalá. Mice were maintained under specific pathogen-free conditions in individually ventilated cages (3–5 animals per cage) with ad libitum access to food and water. Animals were fed a standard rodent maintenance diet (Teklad Global 2014, Inotiv, West Lafayette, IN) containing 14% protein, 3.7% fat, and 38% carbohydrate. We have included this information in the Materials and Methods section. The facility maintained a controlled environment with a 12-hour light/dark cycle, temperature of $22 \pm 2^\circ\text{C}$, and relative humidity of 50% to 60%. Environmental enrichment (nesting material and shelters) was provided in all cages. All experimental procedures complied with EU Directive 2010/63/EU and Recommendation 2007/526/EC, as implemented in Spanish law by Royal Decree 53/2013. Protocols were approved by the institutional animal use committee and the Animal Protection Office of the Autonomous Community of Madrid (PROEX 131.5/24). Twelve-week-old male mice, weighing ≈ 25 g, were used in the study. Animals were randomly assigned to control and treatment groups using a computer-generated randomization schedule. To induce gene deletion, tamoxifen (for eILK cKO mice) or corn oil (for control mice) was administered intraperitoneally at 1.5 mg/d for 5 consecutive days. Mice were then evaluated either 4 weeks or 3 months after induction. Tamoxifen-inducible CreERT2 experiments were performed exclusively in male mice to ensure consistent recombination efficiency and to minimize confounding effects of endogenous estrogen on tamoxifen responsiveness and fibrosis-related outcomes.^{19–21} This approach enhances data comparability and experimental reproducibility across individuals and experimental groups.

A separate subgroup of animals received daily sitagliptin treatment (15 mg/kg per d) delivered in drinking water, starting on the first day of tamoxifen injection and continuing until the end of the study. The drinking water was refreshed every 7 days.

A subset of experiments was performed in eILK cKO-eGFP mice. To generate these mice, Ai40 (B6.Cg-Gt[ROSA]26Sortm40.1[CAG-aop3/eGFP]Hze/J; RRID:MG1:5461353) mice were purchased from Jackson Laboratory 021188 (The Jackson Laboratory). Ai40 conditionally expresses an improved archaerhodopsin-3/eGFP fusion protein (ArchT-eGFP) from the endogenous Gt(ROSA)26Sor locus. Following Cre-mediated removal of the floxed-STOP cassette, ArchT-eGFP expression is observed in the Cre-expressing cells. Ai40 mice were crossed with the VE-cadherin-Cre/ILK lox/lox mice, generating the eILK cKO-eGFP mice. To control, the parental VE-cadherin-CreERT2 mice crossed with Ai40 mice were used (further referred to as CT-eGFP mice). When tamoxifen is administered intraperitoneally for 5 days, CT-eGFP mice will express GFP in endothelial cells with intact ILK expression. In contrast, eILK cKO-eGFP mice will express eGFP and lack endothelial ILK.

Animals were housed in cages distributed across different shelf levels and rack positions in a balanced manner to minimize potential environmental effects, such as light, temperature, and noise, across the animal room.

All measurements were conducted at the same time of day to avoid diurnal variation affecting the results. No specific

criteria were established a priori to exclude animals during the experiment or analysis. All animals and data collected were included in the final analysis. The exact number of animals (n) per experimental group is reported in the corresponding figure legends.

A single experienced operator performed echocardiographic assessments and blood pressure measurements under blind conditions. Group allocation was known to the individual(s) responsible for assigning animals to treatment groups and conducting the experiment; however, data analysis was conducted in a blinded manner throughout the experiment. In total, 90 mice were used for these studies. Group sizes were determined based on our prior experience,¹⁸ ensuring at least 5 animals per experimental condition and per time point. However, outcome assessment and data analysis were performed by investigators who were blinded to group allocation to reduce the risk of bias.

All experimental sample numbers are indicated in the figure legends. After the completion of experiments, mice were deeply anaesthetized through isoflurane inhalation ($>4.5\%$) and subsequently euthanized via cervical dislocation.

Echocardiography

Mouse hearts were routinely imaged by echocardiography using a Vivid Q ultrasound system equipped with a 12.5-MHz transducer. Mice were anesthetized with 2% isoflurane, maintaining a heart rate of ≈ 350 to 400 bpm.

A comprehensive echocardiographic analysis, including valve function, was performed using a Vevo3100 (Visualsonics, Toronto, ON, Canada) equipped with an MX550 transducer. The images obtained were measured offline using the cardiac measurement package integrated into the VevoLab software (version 5.7.1). Cardiac function and left ventricular (LV) morphology were evaluated using M-mode at the mid-papillary level from the parasternal short-axis view. The M-mode gate was positioned perpendicular to the interventricular septum and LV walls to derive systolic function parameters, including stroke volume, ejection fraction, fractional shortening, and cardiac output. LV wall thickness and chamber dimensions, such as end-systolic volume, end-diastolic volume, LV mass, and anterior and posterior wall thickness, were also assessed from this view.

The aortic route, aortic valve, and part of the right ventricular outflow tract were visualized using a modified right parasternal long-axis view. The aortic diameter (mm) was measured at diastole at both the ascending aorta, just above the aortic valve, and at the sinus using B-mode imaging. Peak aortic velocity (mm/s) and pressure gradient (mm Hg) were measured using the pulsed-wave Doppler mode, with calipers placed at 2 locations: the proximal ascending aorta just above the aortic valve and the descending aorta, between the left carotid and left subclavian artery branches. Finally, the length of the aortic arch was measured from the sinotubular junction to the end of the descending aorta along its central axis using B-mode. All measurements were averaged over at least 5 cardiac cycles.

Micro-Computed Tomography Analysis

Full chest 3-dimensional tomographic images were acquired using an X-ray Quantum GX (Perkin Elmer, Waltham) with the following parameters: 90-kVp x-ray source voltage, 88- μA current, and the high-resolution scanning protocol for a total

acquisition time of 4 minutes and a gantry rotation of 360°. Heart and diaphragm motion-derived artifacts were reduced using retrospective 2-phase respiratory gating (inspiration/expiration) on each acquisition. The obtained 3-dimensional tomographic images had 919 projections with an isotropic 72- μ m voxel size and a resolution of 512×512 pixels per slice.

Noninvasive Blood Pressure

Noninvasive blood pressure was recorded in conscious mice using a tail-cuff sphygmomanometer (LE 5001 Pressure Meter; Letica Scientific Instruments, Hospitalet, Spain) following the protocol described previously.²² To minimize stress, animals underwent a 5-day training period before measurements and were prewarmed to 30°C using a heating device. Blood pressure was assessed multiple times between 9:00 and 12:00 AM, and data were considered valid once 10 consecutive readings were obtained. This approach has been validated for accuracy against radiotelemetric measurements using implanted catheters, as demonstrated by Feng et al.²³ In addition, 3 acclimatization sessions were conducted before baseline measurements to habituate the animals, reducing stress-related effects on vascular tone and resting blood pressure.

Histology

The heart was washed with PBS and fixed in 10% formalin solution for 48 hours. After that time, they were dehydrated in ethanol and embedded in paraffin. Histological sections (5 μ m) were deparaffinized, rehydrated, and stained with Masson trichrome (EMD Millipore Corporation, Billerica, MA) and Von Kossa staining kit (Abcam) or Alizarin Red staining for quantification of fibrosis and calcium, respectively. All images were visualized with a Nikon brightfield microscope. The leaflet area and Von Kossa/Alizarin Red-positive area were quantified in ImageJ (RRID:SCR_003070) software from at least 3 sections per aortic valve.

Immunohistochemistry

Heart sections were boiled in citrate buffer for 20 minutes after deparaffinization with xylene for antigenic retrieval. A mouse- and rabbit-specific HRP/DAB (ABC) IHC kit (Abcam) was used according to the manufacturer's instructions. After incubation overnight at 4°C with the corresponding primary antibodies, sections were washed and counterstained with Harris hematoxylin, dehydrated, and mounted with DPX (Casa Alvarez, Madrid). Images were taken (2–3 sections; n=5 animals/group) using a Nikon brightfield microscope. Data quantification was performed using ImageJ.

Morphometric Analysis

Quantification of myocardial fibrosis was performed on sections stained with Masson Trichrome, where fibrotic regions appear blue. Imaging was performed at 10× magnification using a Nikon microscope, capturing 6 nonoverlapping fields that included the LV free wall, interventricular septum, and right ventricle, thereby encompassing the entire myocardium. For each heart, 4 cross-sections were analyzed. The proportion of fibrotic tissue was determined using the color-threshold plugin in ImageJ (<https://imagej.net/ij/>; RRID:SCR_003070), which calculates the area stained blue relative to the total myocardial

area. Fibrosis was expressed as the mean percentage of fibrotic area relative to total heart area for each mouse.

Coronary arterial wall thickness was assessed on Masson trichrome-stained sections and further validated on sections stained with α -SMA as previously described.¹⁸ To analyze microvascular remodeling, wall thickness (encompassing intima and media) was averaged from measurements taken at 4 distinct points around each arteriole. The results were presented as the ratio of vessel wall thickness to lumen area for both arteriolar size categories.

Valve dimensions in murine tissue sections stained with Masson trichrome and Sirius Red were imaged at ×5 magnification on a Nikon microscope, capturing 4 distinct valve sections per animal. In ImageJ, morphometric measurements of each leaflet, annulus, and lumen were obtained across these sections, and the reported values represent the mean of up to 4 measurements per structure for each animal. All morphometric measurements were performed blindly.

Alizarin Red S Staining for Calcium Deposition in Valves/Tissue

Calcium deposition was assessed using Alizarin Red S staining. Human VECs (hVECs) and human VICs were fixed with 15% paraformaldehyde and stained with 40-mmol/L Alizarin Red S solution (pH 4.2) for 30 minutes at room temperature. Excess dye was removed by rinsing with distilled water, and the extent of calcium mineralization was determined based on the intensity of Alizarin Red S retained in the well. For control and calcified valves embedded in paraffin, samples were rehydrated through a graded series of decreasing alcohol concentrations, finishing with distilled water.¹⁵ Rehydrated valves were subsequently processed following the same Alizarin Red S staining protocol as the cultured cells, ensuring methodological consistency across samples.

Osteogenic Differentiation Assay In Vitro

hVECs and valve interstitial cells (VICs) were cultured for 15 days in a pro-osteogenic medium consisting of low-glucose DMEM with L-glutamine, sodium pyruvate, supplemented with 10% FBS, 0.05-mg/mL penicillin/streptomycin, and 2.5- μ g/mL amphotericin, as well as 10-nmol/L β -glycerolphosphate, 50- μ mol/L ascorbic acid, and 10- μ mol/L dexamethasone to create an osteogenic environment. Control cultures were maintained in DMEM with antibiotics and 10% FBS.

A subset of human VICs was incubated with conditioned media (CM) from hVECs. For CM generation, hVECs were cultured under pro-osteogenic conditions for a total of 15 days. During this period, ILK silencing and sitagliptin treatment were performed according to the experimental condition. On day 13, FBS was withdrawn, and the culture supernatant was collected after 48 hours (day 15). The CM was cleared of cellular debris by low-speed centrifugation (500g for 5 minutes at 4°C) before use, following standard CM preparation protocols.²⁴ For CM experiments, VICs were cultured for 15 days using VEC-derived CM supplemented with FBS to a final concentration of 10%. The medium was refreshed every 4 days using newly collected CM under identical experimental conditions.

Following the experimental period, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After fixation, cells were rinsed with PBS.

Mineralization was assessed by staining with 0.02-mg/L Alizarin Red S (Sigma-Aldrich, St. Louis, MO) at a concentration of 40 nmol/L.

The positive Alizarin Red S staining area was standardized to quantify mineralization based on cell count. For each experimental condition, at least 4 images were captured using a Leica DMI8 inverted microscope (Leica Microsystems, Wetzlar, Germany) and analyzed with ImageJ 2.0.0 software (National Institutes of Health, Bethesda, MD).¹⁷

Confocal Microscopy

The slices were deparaffinized and, after antigenic retrieval, incubated with primary antibodies as described above.¹⁸ After washing with PBS, they were incubated with fluorescein isothiocyanate-conjugated secondary antibodies, Alexa-488, or Alexa-647 for 1 hour at room temperature. The sections were washed twice with PBS and mounted with Fluor Save Reagent (Merck). Images were taken for data quantification using a Leica TCS SP5 confocal microscope (UAH-NANBIOSIS-CIBER-BNN). Fluorescence-conjugated Alexa Fluor 647 conjugated Goat anti-mouse secondary antibody (ab150115) was used. Nuclei were stained with Hoechst. Fluorescent images were captured at $\times 60$ magnification.

Cell Transfection

hVECs were transfected with 25 nmol/L of scrambled control siRNA (SiSc), ILK-targeting siRNA (siILK), and DPP4-targeting siRNA (Si-DPP4; Santa Cruz Biotechnology, Santa Cruz, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in OptiMEM (Gibco) medium. After transfection, cells were cultured in EGM-2 medium (Lonza) for 4 days.

For gain-of-function experiments, a plasmid overexpressing ILK under the cytomegalovirus promoter was used; ILK plasmid: mIFP12-ILK-C-14 was a gift from Michael Davidson (Addgene plasmid 56255; <http://n2t.net/addgene:56255>; RRID: Addgene_56255).²⁵ hVECs were transfected with 1 μ g of ILK plasmid or control plasmid using Lipofectamine RNAiMax in OptiMEM, 36 hours after siRNA transfection. After transfection, cells were cultured in EGM-2 medium (Lonza) for 3 additional days. Transfection efficiency was studied by flow cytometry; 79% of cells transfected expressed mIFP12 fluorescence.

Cell Treatment

hVECs were maintained in complete endothelial growth medium and treated with 100- μ mol/L sitagliptin (Sigma-Aldrich) or Linagliptin (100 μ mol/L; Sigma-Aldrich) for either 5 days or 14 days according to the experimental design. To ensure a constant drug concentration throughout the assay, the treatment medium was refreshed every 48 to 72 hours.

hVECs were incubated with 10- μ mol/L BAY 11-7082 (Sigma-Aldrich) following transfection for 12 hours to ensure sustained NF- κ B (nuclear factor kappa B) inhibition throughout the incubation period.

Western Blotting

Protein extracts were obtained following standard procedures.²⁶ In brief, tissue specimens were homogenized in a lysis buffer,

and the resulting protein mixtures were resolved via 4% to 12% sodium dodecyl sulfate-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Following blocking, membranes were incubated with specific primary antibodies at 4°C overnight, followed by incubation with the appropriate secondary antibodies. Immunoreactive bands were visualized using the Pierce Western blotting Substrate, following the manufacturer's guidelines (Pierce, Waltham).

Quantitative Real-Time Polymerase Chain Reaction

RNA extraction from hVECs, human, and mouse valve tissue was performed using TRIzol reagent (Invitrogen Corporation, Carlsbad) following the manufacturer's instructions. For murine valves, aortic valves from at least 3 animals were pooled. To synthesize cDNA, 2 μ g of total RNA was used in a 20- μ L reaction volume, utilizing the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad). Quantitative polymerase chain reaction was conducted using the SYBR Select Master Mix (Life Technologies). The quantitative polymerase chain reaction protocol adhered to standard cycling conditions for primers with melting temperatures $\geq 60^\circ\text{C}$, starting with UDG activation at 50°C for 2 minutes, followed by AmpliTaq DNA Polymerase and UP activation at 95°C for 2 minutes, 15 seconds of denaturation at 95°C, and annealing/extension at 60°C for 1 minute. Finally, the $\Delta\Delta\text{Ct}$ method was used to compare mRNA abundance.

Flow Cytometry

hVECs were detached from culture plates by enzymatic dissociation, collected by centrifugation at 300g for 5 minutes, and washed with PBS. Cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by permeabilization with 0.1% Triton X-100 in PBS for 10 minutes. After permeabilization, cells were incubated with primary antibodies for 1 hour at 4°C. Following incubation, cells were washed with PBS and subsequently incubated with the fluorophore-conjugated secondary antibodies for 30 minutes at 4°C in the dark. Cells were then washed, resuspended in PBS, and analyzed by flow cytometry using a MACSQuant Analyzer (Miltenyi Biotec). Data acquisition and analysis were performed with MACSQuantify Software (Miltenyi Biotec).

Cytokine Array

The Proteome Profiler Human Cytokine Array Kit (Catalog No. ARY005B, Bio-Techne, United States) was utilized to evaluate cytokine secretion by hVEC. Briefly, hVECs were transfected with scramble SiRNA or siILK as described and treated or not with sitagliptin and harvested at the indicated times. Protein concentration of the hVEC was determined using a bicinchoninic acid assay, and 100 μ g of hVEC protein was used for the cytokine array. The assay was performed following the manufacturer's protocol. Results were quantified using ImageJ software and normalized to the internal control spots.

DPP4 Activity Assay

DPP4 activity was quantified using the DPP4 Activity Assay Kit (ab204722) according to the manufacturer's instructions.

Experiments were conducted on cultured hVECs transfected with either nontargeting control siRNA or ILK-specific siRNA, as well as on aortic valve tissue harvested from eILK cKO mice and their controls after 12 weeks of ILK deletion. For each assay, 50 µg of total protein was used; to achieve this amount, valve lysates were pooled from up to 3 valves per sample. Protein concentration was determined by bicinchoninic acid assay before DPP4 activity measurement.

DPP4 ELISA

Plasma DPP4 concentrations were quantified by ELISA using species-specific kits: the Mouse DPP4 SimpleStep ELISA Kit (ab264630) for murine samples and the Human DPP4 ELISA Kit (ab252365) for human samples.

Citrate blood was collected from control and eILK cKO mice at 12 weeks postdeletion and centrifuged to obtain platelet-poor plasma. Murine plasma samples were assayed in duplicate according to the manufacturer's instructions.

Human platelet-poor plasma samples from non-CAVD controls and patients with CAVD were similarly analyzed using the human DPP4 ELISA kit according to the manufacturer's protocol. All measurements were performed in duplicate.

Statistical Analysis

Statistical analyses were carried out using R (version 4.3.2) and GraphPad Prism (RRID:SCR_002798; version 10.0). In animal experiments, the number of animals analyzed is indicated in the corresponding figure legends. Data distribution was evaluated using the Shapiro-Wilk test before applying parametric statistical methods. Differences between 2 independent groups were analyzed using an unpaired 2-tailed Student *t* test when data followed a normal distribution. When normality assumptions were not met, or the sample size was <30, the Mann-Whitney *U* test was used. Comparisons among >2 groups were corrected using 1-way ANOVA with the Bonferroni adjustment for multiple comparisons.

Baseline clinical characteristics were analyzed separately. Continuous variables are reported as mean±SD, and we checked the hypothesis with the 2-tailed Welch *t* test. Dichotomous clinical variables are presented as absolute numbers, and percentages and were analyzed using the Fisher exact test.

For Western blotting, gene expression analyses, flow cytometry, calcification assays, and DPP4 enzymatic activity measurements, experiments were performed in technical duplicates and independently repeated at least 3×. Sample sizes (*n*) for each analysis are indicated in the corresponding tables and figure legends. No *a priori* power calculations were performed due to the exploratory nature of the study and the limited availability of human samples. A 2-sided *P*<0.05 was considered statistically significant.

For all experiments, biological replicates were defined as independent samples from human and mouse sources. Cell culture experiments were performed using independent cell culture preparations from different donors. Technical replicates were performed within each experiment as indicated. Data are presented as the mean±SEM of biological replicates, unless otherwise specified. Exact sample numbers for each experiment are indicated in the corresponding figure legends.

RESULTS

Reduced Endothelial ILK Is Associated With Increased DPP4 in Human CAVD and Human Valve Endothelial Cells

To assess the expression of both membrane-bound and soluble DPP4, we analyzed plasma and aortic valve samples from patients undergoing valve replacement surgery with CAVD and without calcific lesions (non-CAVD).

Circulating DPP4 protein levels were significantly elevated in patients with CAVD compared with non-CAVD controls (Figure 1A). The same result was observed in the aortic valves, where DPP4 mRNA expression was also increased in CAVD (Figure 1B). Immunostaining revealed marked upregulation of DPP4 in VECs of patients with CAVD, as evidenced by its colocalization with the endothelial marker CD31 (Figure 1C). As previously documented,¹⁵ ILK decreased in the aortic valves of patients with CAVD (Figure 1D). DPP4 valve expression was inversely associated with ILK levels in the aortic valve, as assessed by the Spearman rank correlation analysis (Figure 1E). Reduced DPP4 expression was independent of sex, with no significant difference between male and female patients with CAVD (Figure S1A and S1B). Because DPP4 expression may increase under hyperglycemic and diabetic conditions, we next assessed whether a similar pattern of regulation occurs in aortic valves. Stratification by diabetes status revealed no difference in increased DPP4 levels observed in CAVD between patients with diabetes and patients without diabetes (Figure S1C).

Based on these findings, we hypothesized that loss of endothelial ILK would increase DPP4 expression in hVECs. To test this, we silenced ILK in hVECs and evaluated DPP4 expression. As shown in Figure 1F and 1G, DPP4 protein and mRNA levels increased 3 days after ILK silencing in hVECs, along with RUNX2, a key osteogenic marker. We further verified by flow cytometry that the percentage of endothelial cells expressing membrane-bound DPP4 increased after ILK silencing (Figure 1H). Curiously, ILK knockdown led to the appearance of a significant population of DPP4-expressing cells, but the percentage of VE-cadherin-positive cells decreased compared with control conditions (Si scramble). Because ILK silencing has been reported to induce EndMT,^{15,18} this population may represent endothelial cells that have undergone transition and express DPP4 on their plasma membrane. DPP4 silencing confirmed the specificity of the results. In addition to its cell-surface presence, we investigated whether ILK levels affect DPP4 proteolytic activity. Using a DPP4 activity assay, we observed higher DPP4 peptidase activity in siILK hVECs (Figure 1I).

Together, these data indicate that both circulating DPP4 levels and aortic valve DPP4 expression increased in aortic valves from patients with CAVD, concomitant with

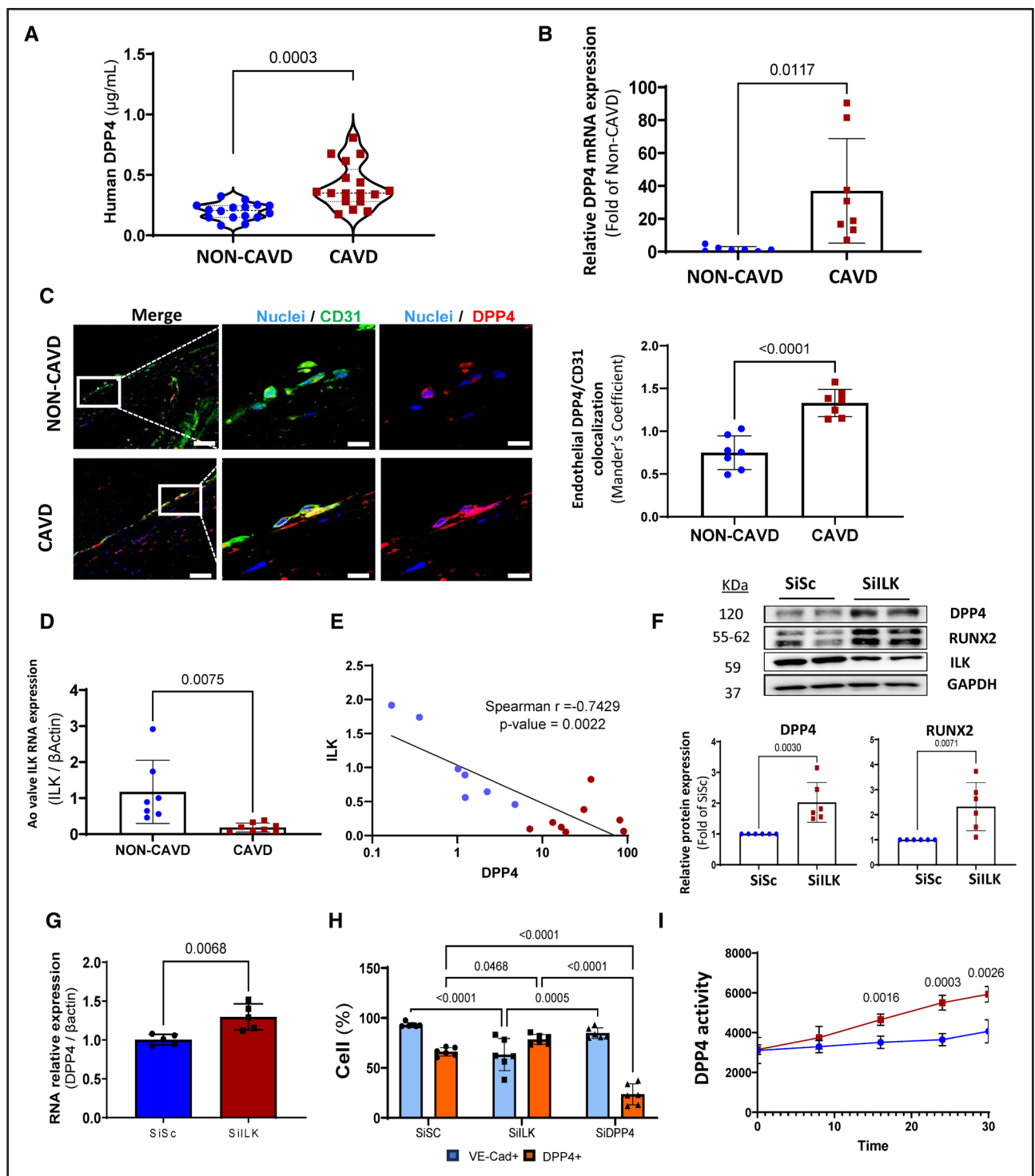


Figure 1. Increased DPP4 (dipeptidyl peptidase 4) in the plasma and aortic valves of patients with calcific aortic valve disease (CAVD) correlates with lower ILK (integrin-linked kinase) levels and is associated with higher RUNX2 (Runt-related transcription factor 2) expression. **A**, Plasma DPP4 levels measured by ELISA in patients without (non-CAVD) and with CAVD. **Right**, Densitometric quantification (non-CAVD: $n=16$; CAVD: $n=17$). **B**, Aortic valve mRNA expression in human patients of DPP4 (non-CAVD: $n=7$; CAVD: $n=8$). **C**, Representative immunofluorescence staining of aortic valve for DPP4 (red), the endothelial marker CD31 (green), and nuclei stained with Hoechst (blue), demonstrating colocalization of DPP4 with CD31⁺ endothelial cells as indicated by the Mander colocalization analysis on the **right** ($N=7$). Scale bar left, 50 and 10 µm in the magnifications. **D**, Aortic valve ILK mRNA expression in human patients (non-CAVD: $n=7$; CAVD: $n=8$). **E**, Spearman correlation of ILK and DPP4 levels shown in **B** and **D**, revealing a significant inverse association ($r=-0.6835$; $P=0.0088$; non-CAVD: $n=7$; CAVD: $n=8$). **F**, Immunoblot and densitometric analysis of DPP4 and RUNX2 in human valvular endothelial cells (hVECs) transfected with scrambled SiRNA (SiSc) or ILK-targeting siRNA (siILK; $N=4$). **G**, Quantitative real-time polymerase chain reaction (RT-qPCR) analysis of DPP4 transcript levels in scrambled (SiSc) vs ILK knockdown (siILK) in VECs ($n=6$). **H**, Flow cytometric analysis of (Continued)

reduced ILK levels. In addition, whole-cell DPP4, cell-surface DPP4, and DPP4 enzymatic activity increased in ILK-silenced hVECs, linking decreased endothelial ILK to enhanced DPP4 and to CAVD.

Inducible ILK Deletion in the Endothelium Led to CAVD in Mice

To study the role of endothelial ILK in CAVD and its relationship with DPP4 expression *in vivo*, we used the endothelium-restricted ILK deletion (ecILK cKO) mice, previously described.¹⁸ We treated a group of 12-week-old male mice with 1.5 mg/d per animal of tamoxifen (ecILK cKO) or vehicle (CT) for 7 days to induce ILK deletion. We studied cardiac and valve function at 4 and 12 weeks after ILK deletion, as indicated in Figure 2A. The efficiency and specificity of ILK deletion in valve endothelium were confirmed by double immunofluorescence of the endothelial marker CD31 (green) and ILK (red; Figure 2B).

Cardiac function parameters were analyzed by echocardiography in both CT and ecILK cKO mice. The ecILK cKO mice exhibited a progressive reduction in ejection fraction and shortening fraction (Figure S2A and S2B) at 4 and 12 weeks compared with controls, both indicators of decreased cardiac contractility. In addition, we observed a decrease in cardiac output accompanied by an increase in LV mass (Figure S2C and S2D), suggesting myocardial dysfunction and remodeling in ecILK cKO mice. Blood pressure rose significantly after 4 weeks of deletion but did not increase further at 12 weeks (Figure S1E).

Next, we examine valvular function by echocardiography. ecILK cKO mice exhibited progressively higher valve peak velocities than CT mice (Figure 2C). Analysis of the valve structure revealed significant thickening of the leaflets and a notable reduction in the aortic valve opening area in ecILK cKO compared with CT, with the difference becoming significant 12 weeks after deletion (Figure 2D). The aortic leaflet area of ecILK cKO animals at 12 weeks was 2× greater than that of controls ($6.25 \pm 0.54 \times 10^3$ versus $16.27 \pm 2406 \times 10^3 \mu\text{m}^2$). We found extensive areas of calcification in animals with endothelial ILK deletion, which increased over time, as detected by Alizarin Red staining (Figure 2E). Accordingly, there was enhanced expression of the calcification markers, OSPN (osteopontin) and osterix (Figure 2F). Interestingly, we observed increased RUNX2 expression in both the valvular interstitium and the endothelium,

further supporting the hypothesis that ILK deletion in hVEC contributes to the calcific phenotype (Figure 2G). Western blot analysis of RUNX2 and ALP (alkaline phosphatase) proteins also showed increased expression in ecILK cKO mouse valve tissue (Figure S3A and S3B). These results suggest that endothelium-specific ILK deletion directly contributes to the calcifying phenotype by promoting the osteogenic switch in resident valve cells. The procalcific effect of endothelial ILK deletion was further documented by micro-computed tomography at 12 weeks post-ILK deletion, confirming that advanced deposition of calcium hydroxyapatite occurred in ecILK cKO (Figure 2H). Furthermore, the histological analysis of the myocardium showed progressively increased fibrosis, as anticipated,¹⁸ along with microvascular remodeling, indicating that maladaptive myocardial changes aligned with increased afterload (Figure S3C through S3E).

To directly evaluate the contribution of endothelial ILK-deficient cells to the procalcific phenotype, we generated an endothelial-specific ILK-eGFP conditional knockout mouse (ecILK cKO-eGFP) by crossing VE-cadherin-Cre/ILK^{lox/lox} mice with Ai40D reporter mice, in which Cre recombination induces eGFP expression in endothelial cells (Figure S4A). Control reporter mice (CT-eGFP) were generated by crossing VE-cadherin-Cre mice with Ai40D mice. Immunofluorescence analysis confirmed that robust eGFP expression was confined to the valvular endothelium, validating this model for endothelial lineage tracing. As expected, CT-eGFP mice retained endothelial ILK expression, whereas ecILK cKO-eGFP mice exhibited complete loss of ILK in eGFP-positive endothelial cells (Figure S4B). α -SMA expression was detected in eGFP-positive cells within the endothelial layer, and a subset of eGFP+/ α -SMA+ cells was also observed within the valve interstitium, indicating that endothelial-derived cells contribute to the myofibroblast population. Furthermore, strong RUNX2 immunoreactivity was detected in eGFP-positive cells, consistent with endothelial transdifferentiation toward an osteogenic phenotype. Collectively, these data demonstrate that ILK deletion promotes endothelial cell transdifferentiation, contributing to both myofibroblastic and osteogenic cell populations within the valve, thereby supporting a direct role for endothelial cells in the procalcific phenotype.

Interestingly, DPP4 levels were increased in the aortic valve of the ecILK cKO mice, as revealed by confocal microscopy using an endothelial marker (IB4 [isolectin B4]) and an anti-DPP4 antibody (Figure 3A).

Figure 1 Continued. DPP4 and VE-cadherin (vascular endothelial cadherin) coexpression in hVEC. Graph shows the percentage of DPP4+ and VE-Cad+ cells in control (SiSc), ILK knockdown (siILK), and DPP4 silencing (siDPP4) conditions (n=3, per triplicate). Blue columns: VE-Cad+ cells. Orange columns: DPP4+ cells. **I**, DPP4 enzymatic activity in VECs transfected with scramble (Sc) or ILK-targeting siRNA (siILK; n=3 with triplicate in each condition). For all figures, results are expressed as mean±SD; P values were calculated using the unpaired Student *t* test (**A–G** and **I**) and 1-way ANOVA with the Bonferroni post hoc test (**H**). Spearman correlation coefficient (*r*) was calculated, and statistical significance was determined using a 2-tailed *t* test.

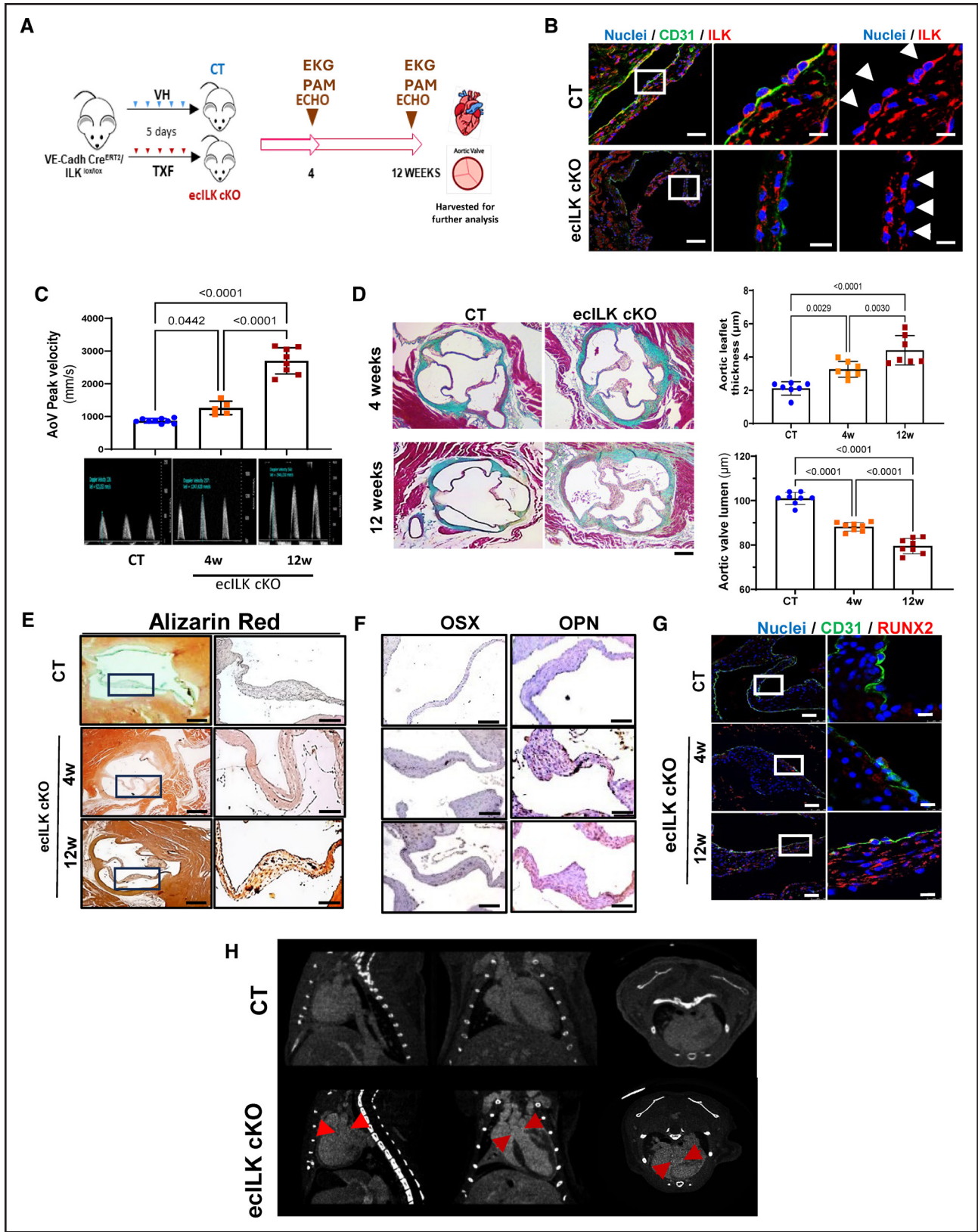


Figure 2. Endothelial ILK (integrin-linked kinase) deletion leads to aortic valve dysfunction and calcification in mice.
A, Schematic figure of the experimental design. **B**, Representative confocal microscopy image of mouse aortic valves 4 weeks after treatment with tamoxifen (endothelial cell-specific ILK conditional knockout [ecILK cKO]) showing endothelial ILK deletion or vehicle (control [CT]). Endothelial cells are labeled with IB4 (isolectin B4; green), ILK (red), and nuclei (blue). Arrows point to endothelial cells. Scale bar, 25 and 10 μ m in the magnification (N=6). **C**, Peak aortic velocity (mm/s) measured by echocardiography in CT (12 weeks) and ecILK cKO mice at 4 and 12 weeks (CT: n=8; 4 weeks: n=5; and 12 weeks: n=8). **Bottom**, Representative images of transvalvular peak velocity. (Continued)

The analysis of DPP4 mRNA levels in the valve also indicates an increase in DPP4 transcription after 12 weeks of endothelial ILK deletion (Figure 3B). We also studied DPP4 expression in coronary microvascular vessels and found a strong induction in both endothelial and smooth muscle cells compared with CT mice (Figure 3C). In the aortic root and ascending aorta, this was accompanied by upregulation of RUNX2 and Col (collagen) 1, along with a marked reduction in ILK levels. The rise in RUNX2 and Col 1 suggests activation of osteogenic and fibrotic pathways, which may contribute to vascular stiffening and structural remodeling in the aortic arch, likely driven by valvular stenosis (Figure 3D).

In this model, circulating DPP4 levels and DPP4 activity were also increased relative to CT mice, reinforcing the link between the endothelial ILK deficit, aortic valve calcification, and DPP4 (Figure 3E and 3F).

DPP4 Inhibition Limits EndMT and Blocks Osteogenic Reprogramming of VECs After ILK Silencing

Given that reduced ILK expression is associated with EndMT in human CAVD,¹⁵ we investigated whether DPP4 contributes to this process. hVECs were subjected to ILK silencing, and DPP4 activity was inhibited either pharmacologically with sitagliptin or genetically using siRNA-mediated DPP4 knockdown (siDPP4). EndMT, fibrotic, and calcific markers were subsequently assessed. Sitagliptin did not affect VEC viability or ILK expression levels (Figure S5A and S5B), excluding non-specific cytotoxic effects or feedback regulation of ILK.

ILK silencing induced a marked EndMT response, characterized by increased expression of mesenchymal markers (SM22 α [smooth muscle protein 22- α] and N-cadherin) and a concomitant reduction in endothelial markers (VE-cadherin and CD31; Figure 4A; Figure S5C). A similar pattern was observed for fibrotic markers, including FN (fibronectin) and Col I. Both pharmacological inhibition of DPP4 and DPP4 gene silencing significantly attenuated EndMT and fibrosis induced by ILK depletion. Notably, siDPP4 provided only a modest additional reduction beyond that achieved with sitagliptin alone. However, neither sitagliptin nor DPP4 silencing restored CD31 or VE-cadherin levels to basal levels.

ILK silencing also increased the expression of osteogenic and calcific markers, including RUNX2, OSPN, and ALP. These effects were significantly reduced by both DPP4 inhibition and DPP4 silencing (Figure 4B; Figure S5D).

Next, to determine whether the observed changes in DPP4 expression were directly attributable to ILK loss, we performed ILK gain-of-function experiments. DPP4 expression was upregulated following ILK silencing and restored to control levels upon ILK re-expression (Figure 4C; Figure S5E). EndMT and calcific marker expression showed a corresponding reversal, including endothelial cell markers, confirming that reduced ILK levels directly drive increased DPP4 expression and the associated osteogenic and mesenchymal reprogramming of VECs (Figure 4C; Figure S5E and S5F).

To determine whether other DPP4 inhibitors shared these effects, we compared sitagliptin with linagliptin, a DPP4 inhibitor with a better cardiac profile.²⁷ Linagliptin similarly reduced the expression of EndMT, fibrotic, and calcific markers induced by ILK silencing. However, its effects were less pronounced than those observed with sitagliptin (Figure S6).

Silencing ILK promoted calcification in hVECs cultured under osteogenic conditions, as evidenced by increased mineral deposition. This effect was significantly reduced by pharmacological DPP4 inhibition (Figure 4D). These findings indicate that ILK silencing-induced osteogenic differentiation of VECs is, at least in part, dependent on DPP4 activity.

Because VICs are the primary cell type responsible for executing the calcific program in the aortic valve, we next examined whether ILK-deficient VECs influence VIC osteogenic activation through paracrine signaling. CM from control VECs, Si-Sc-VECs, and siILK-VECs treated with sitagliptin was applied to VICs. CM derived from siILK-VECs significantly increased osteogenic marker expression and enhanced VIC calcification, whereas these effects were abolished when siILK-VECs were treated with sitagliptin (Figure 4E).

Taken together, these findings demonstrate that the loss of ILK in valvular endothelial cells induces DPP4 upregulation, driving EndMT and fibrotic and osteogenic reprogramming of VECs, thereby promoting paracrine activation of VICs. While DPP4 inhibition attenuates ILK-dependent pathological remodeling, these data suggest that additional ILK-regulated mechanisms may be required to fully restore endothelial homeostasis,

Figure 2 Continued. **D**, Masson trichrome staining of mouse aortic valves 4 and 12 weeks after treatment with tamoxifen (ecILK cKO) or vehicle (CT). Scale bar, 100 μ m. **Right**, Quantification of aortic leaflet area (μ m²) and aortic valve lumen (mm) of ecILK cKO vs CT mice (N=7). **E**, Representative images of calcium deposition staining by Alizarin Red in CT and ecILK cKO mice at 4 and 12 weeks. Scale bar, 100 and 10 μ m in the magnification (N=7). **F**, Representative immunostaining of the osteogenic markers OPN (osteopontin) and OSX (osterix) in mice treated as in **E**. Scale bar, 10 μ m (n=7). **G**, Double immunofluorescence of endothelial marker CD31 (green) and RUNX2 (runt-related transcription factor 2; red). Scale bar, 25 and 10 μ m in the magnification (N=6). **H**, Representative images of micro-computed tomography (micro-CT) of 12-week CT and ecILK cKO mice in sagittal, coronal, and transverse planes. Red arrows indicate aortic valve calcification (N=4). For all figures, results are expressed as mean $\pm$ SD. *P* values were calculated using 1-way ANOVA with the Bonferroni post hoc test.

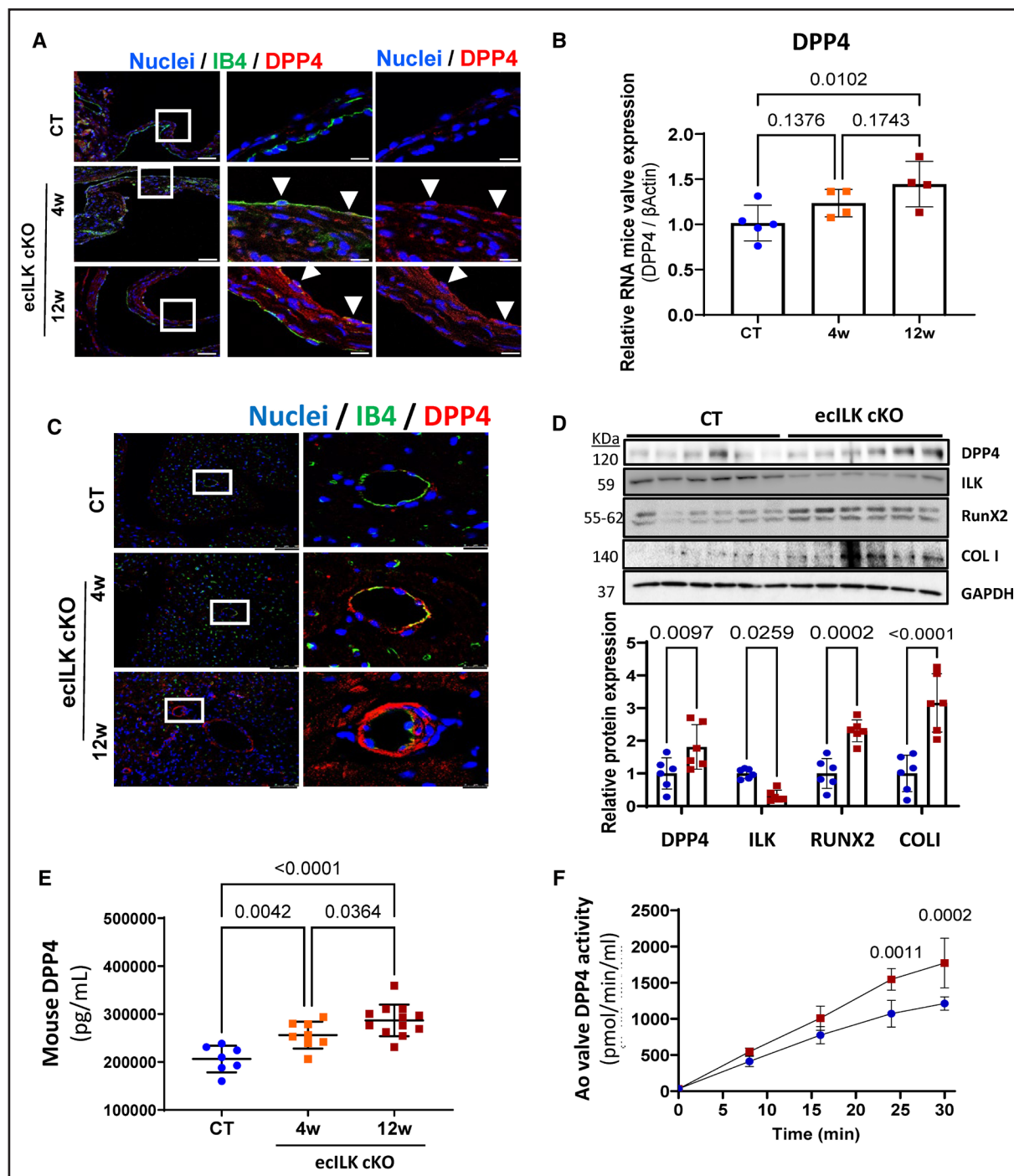


Figure 3. ILK (integrin-linked kinase) deletion in murine endothelium enhances both vascular and valvular DPP4 (dipeptidyl peptidase 4) expression and activity.

A, Representative immunofluorescence of aortic (Ao) valve leaflets stained for endothelial marker IB4 (isolectin B4; green) and DPP4 (red), showing DPP4 signal markedly increased in the endothelium and in the valvular interstitium in the Ao valves of endothelial cell-specific integrin-linked kinase conditional knockout (ecILK cKO) mice. Nuclei were stained with Hoechst (blue). Scale bar, 50 and 10 μ m in the amplification. Arrowheads mark endothelial cells positive for DPP4. **B**, Quantitative real-time polymerase chain reaction (RT-qPCR) analysis of DPP4 mRNA in valve tissue of control (CT) and ecILK cKO mice at 12 weeks; each point represents an independent pool of 4 animals (N=4). **C**, Representative immunofluorescence of the coronary endothelium of CT and ecILK cKO mice at 4 and 12 weeks, stained as in **A**. Scale bar, 50 and 10 μ m in the magnification. **D**, Representative immunoblot of Ao root lysates from CT and ecILK cKO mice at 12 weeks probed for DPP4, ILK, RUNX2 (runt-related transcription factor 2), and COL I (collagen I; N=6). **E**, Plasma DPP4 levels measured by ELISA in mice. ecILK cKO animals display significantly higher circulating DPP4 levels than CT (N=6–12). **F**, DPP4 enzymatic activity assay performed on valve lysates from CT (blue dots) and ecILK cKO mice at 12 weeks (red squares); each data point represents a pool of 4 animals (N=3). For all figures, data are presented as mean $\pm$ SD. *P* values were calculated using 1-way ANOVA with the Bonferroni post hoc test and the unpaired Student *t* test in **F**.

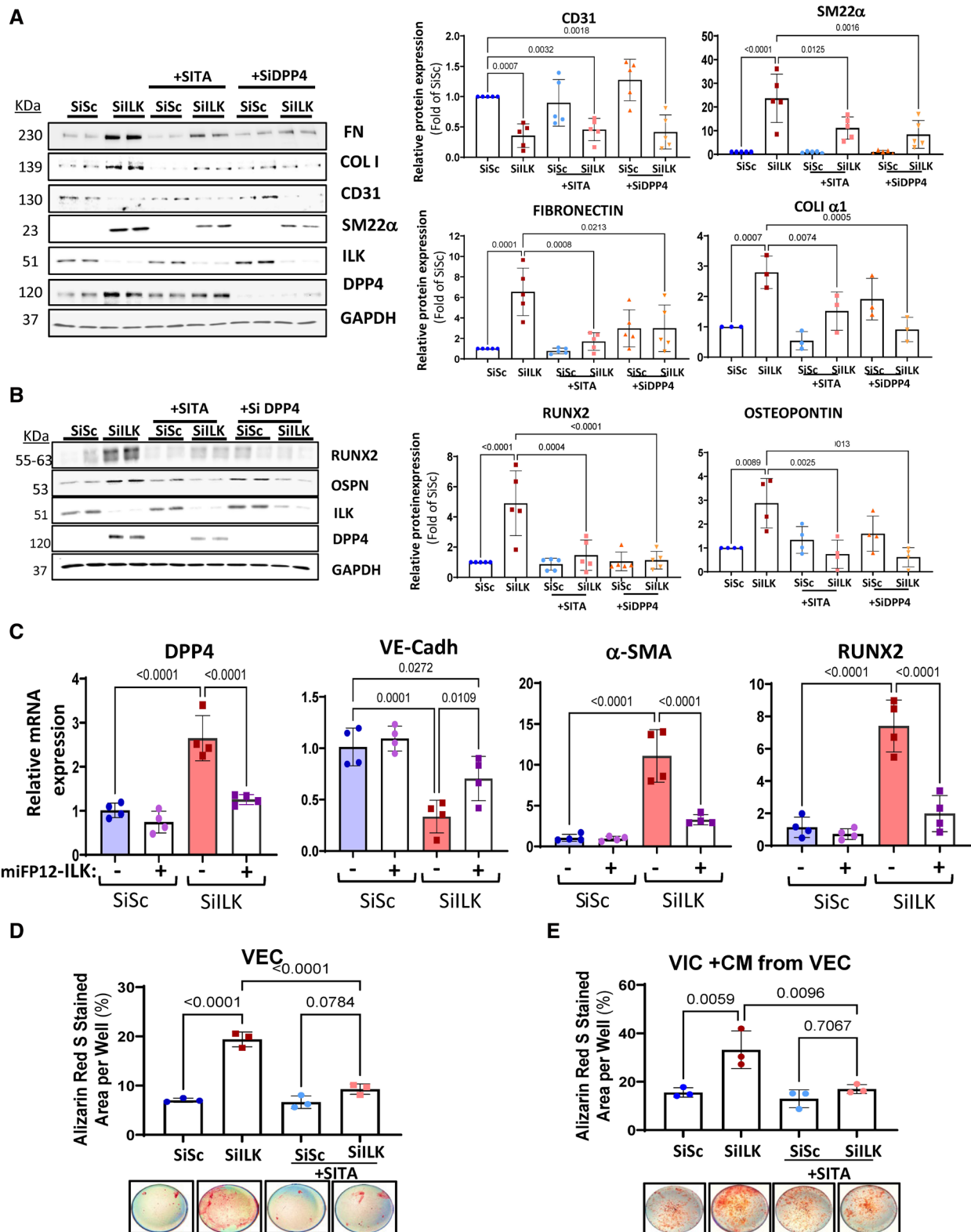


Figure 4. DPP4 (dipeptidyl peptidase 4) inhibition reduces the endothelial-to-mesenchymal transition (EndMT) and calcifying phenotype induced by ILK (integrin-linked kinase) downregulation in valvular endothelial cells (VECs).

A, Representative immunoblot of fibronectin, collagen I, CD31, and SM22 α (smooth muscle protein 22-alpha) in human VECs (hVECs) transfected with control siRNA (SiSc) or ILK-targeting siRNA (siILK) in the absence or presence of sitagliptin or siRNA to DPP4. Densitometric analysis of fibrogenic and EndMT marker expressions is presented on the right (N=5 per duplicate). **B**, Representative immunoblot of RUNX2 (runt-related transcription factor 2) and osteopontin in hVECs transfected with control siRNA (SiSc) or ILK-targeting siRNA (siILK) in the absence or presence of sitagliptin or siRNA to DPP4. Densitometric analysis of osteogenic protein expression is presented on the (Continued)

positioning DPP4 as a key, but not exclusive, mediator linking endothelial dysfunction to calcific remodeling in CAVD.

Sitagliptin Treatment Improves Cardiac Function and Reduces Valve Calcification in the *ecILK* cKO Model by Targeting the EndMT Program

Next, we investigated whether sitagliptin could inhibit aortic valve calcification in *ecILK* cKO mice. We first assessed its effects on EndMT, as the earliest pathogenic process in CAVD. Consistent with our *in vitro* findings, 12 weeks of sitagliptin treatment significantly reduced α -SMA expression in the valve endothelium and decreased expression of osteogenic markers, including RUNX2 and ALP (Figure 5A through 5C). Correspondingly, sitagliptin markedly reduced calcium deposition induced by endothelial ILK deletion (Figure 5D and 5E).

Despite these robust effects on EndMT and calcific markers, sitagliptin only partially attenuated valvular dysfunction and did not fully prevent valve thickening (Figure 5F and 5G), suggesting that structural and hemodynamic alterations may be less responsive to DPP4 inhibition than early molecular and cellular changes.

We next assessed myocardial remodeling and LV function in *ecILK* cKO mice treated with sitagliptin. Sitagliptin partially alleviated LV dysfunction, improving both ejection fraction and shortening fraction (Figure S7A and S7B). In addition, sitagliptin treatment attenuated the remodeling observed in *ecILK* cKO mice, reducing myocardial fibrosis and microvascular remodeling (Figure 6A through 6C; Figure S7C and S7D). DPP4 circulating levels increased after ILK deletion but did not significantly change with sitagliptin treatment (Figure S7E).

A positive association between aortic valve thickness and myocardial fibrosis across experimental groups was identified using the Spearman correlation analysis (Figure 6D). In sitagliptin-treated *ecILK* cKO mice, this relationship was characterized by less pronounced valvular and myocardial remodeling. Although sitagliptin did not fully prevent structural remodeling of either the valve or the myocardium, the extent of these changes was consistently attenuated compared with untreated *ecILK* cKO mice, with values clustering toward less advanced remodeling, suggesting that sitagliptin may prevent disease progression.

ILK Deletion Promotes Endothelial Inflammation and Increases DPP4 Expression via NF- κ B Activation, Processes That Are Reduced by Sitagliptin Treatment

Chronic inflammation and EndMT are intimately linked in a vicious cycle during CAVD development, in which inflammation promotes EndMT, and transformed cells perpetuate inflammation and fibrosis, contributing to leaflet thickening and calcification.²⁸ We have already found that ILK silencing increases nitrosative and oxidative stress in hVECs, thereby activating the proinflammatory transcription factor NF- κ B.¹⁵ Because the DPP4 promoter contains NF- κ B consensus sequences, we examined DPP4 transcriptional expression in ILK-silenced VECs in the presence of an NF- κ B inhibitor (BAY 11-7082). NF- κ B inhibitor treatment effectively blocked I κ B (inhibitor of κ B) phosphorylation and p65 nuclear translocation caused by ILK silencing. More importantly, BAY treatment prevented DPP4 overexpression induced by ILK silencing, indicating that NF- κ B transcriptionally activates DPP4 expression in response to decreased ILK levels (Figure 7A).

To further investigate the anti-inflammatory effect of sitagliptin on siILK-VECs, we conducted a cytokine profiling analysis, which revealed that ILK silencing triggered a strong proinflammatory response, increasing the expression of MCP-1 (monocyte chemoattractant protein-1), RANTES (regulated upon activation, normal T-cell expressed and secreted), and ICAM (intercellular adhesion molecule), among others. Conversely, sitagliptin treatment reduced this response, shifting the cytokine profile toward an anti-inflammatory state (Figure 7B). MCP-1 and ICAM protein levels increased after ILK silencing and were dependent on NF- κ B activity, confirming the cytokine array results (Figure 7C). Moreover, sitagliptin treatment of *ecILK* cKO mice reduced VCAM (vascular cell adhesion molecule) and ICAM expression in VECs after ILK deletion, demonstrating the ability of sitagliptin to modulate endothelial inflammatory activity in valvular disease *in vivo* (Figure 7D).

In summary, our results reveal that sitagliptin not only mitigates EndMT and valvular osteogenesis, thereby reducing fibrosis and calcification in a CAVD model, but also significantly modulates the endothelial inflammatory environment, interrupting the pathological cycle that perpetuates valvular damage.

Figure 4 Continued. right (N=5 per duplicate). **C**, Quantitative real-time polymerase chain reaction (RT-qPCR) assessing DPP4, VE-cadherin (vascular endothelial cadherin), α -SMA (alpha smooth muscle actin), and RUNX2 mRNA expression in hVECs transfected with scrambled siRNA (SiSc), ILK-silenced (siILK), and an ILK overexpression plasmid (miFP12-ILK; N=4 per duplicate). **D**, Representative Alizarin Red staining of scramble SiRNA (SiSc) and ILK-silenced (siILK) hVECs, either untreated or treated with sitagliptin, 14 days in osteogenic medium; each point represents an experiment performed in triplicate (N=3). A representative staining is displayed below. **E**, Alizarin Red staining and quantitation of VICs cultured for 14 days in conditioned media (CM) harvested from VECs in the 4 conditions described in **C**. A representative staining is displayed below (N=3). For all figures, results are expressed as mean $\pm$ SD. *P* values were calculated using 1-way ANOVA with the Bonferroni post hoc test.

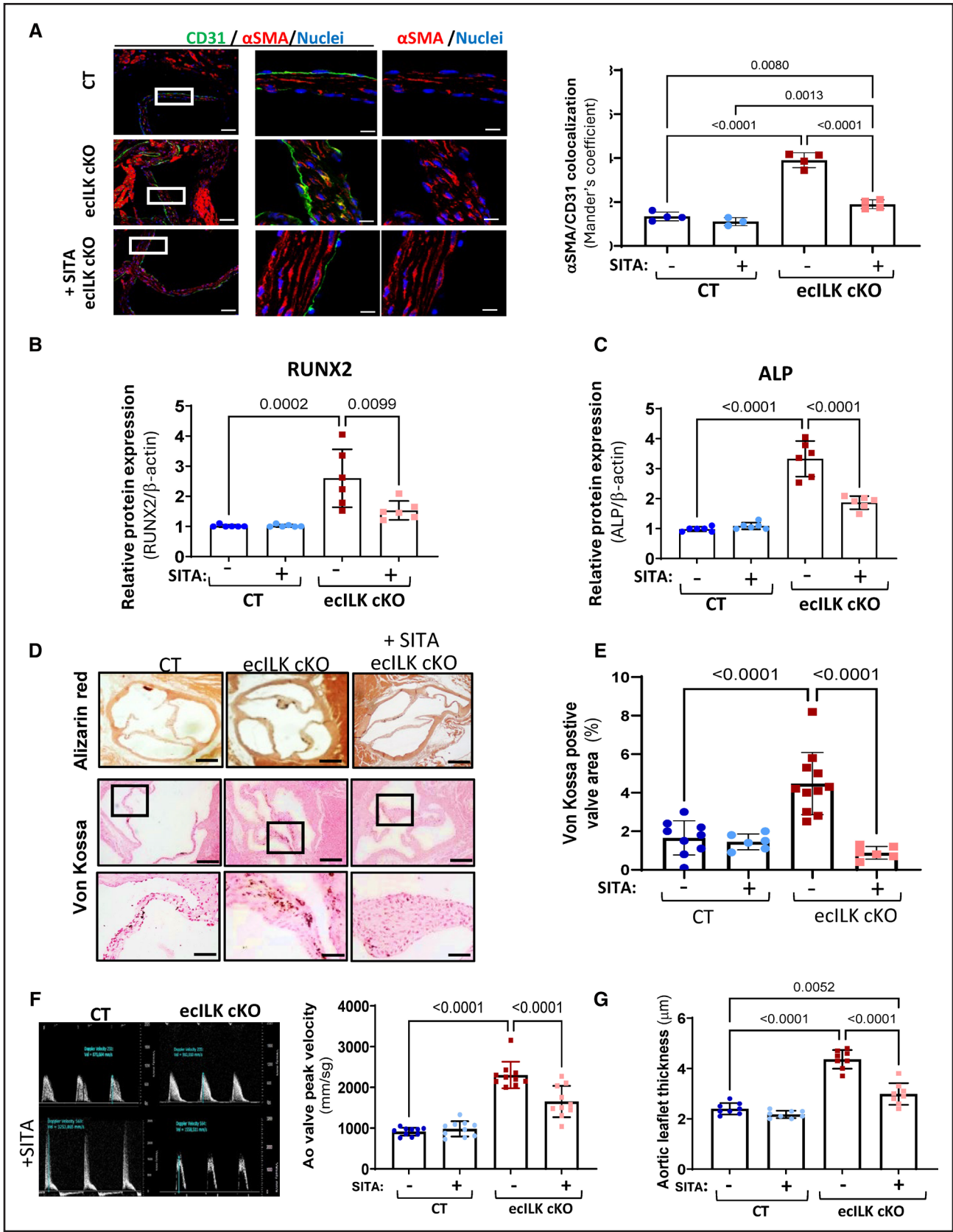


Figure 5. Sitagliptin treatment reduces valvular endothelial-to-mesenchymal transition (EndMT) and calcification in vivo. **A**, Representative confocal images of aortic valves from endothelial cell-specific integrin-linked kinase conditional knockout (ecILK cKO) or control (CT) mice treated with and without sitagliptin (SITA) for 12 weeks, stained with α -SMA (alpha smooth muscle actin; red); endothelial cells were stained with CD31 (green), and nuclei were counterstained in blue with Hoechst. Scale bar, 50 and 10 μ m in (*Continued*)

DISCUSSION

This study identifies endothelial ILK as a pivotal regulator of valvular endothelial homeostasis and a key upstream driver of CAVD. Pharmacological inhibition of DPP4 with sitagliptin partially counteracted ILK-driven endothelial dysfunction, reducing inflammation, limiting EndMT, and decreasing osteogenic differentiation, thereby revealing an endothelial ILK-DPP4 axis contributing to CAVD pathogenesis.

DPP4 inhibitors are under investigation for CAVD in addition to their established use in diabetes. Retrospective clinical studies suggest that selected DPP4 inhibitors are associated with slower progression of aortic stenosis in patients with diabetes and mild-to-moderate disease.^{29,30} Consistent with these observations, we detected increased DPP4 expression and activity in calcified human valves, elevated circulating soluble DPP4, and higher DPP4 levels in valvular endothelial cells from patients with CAVD showing ILK reduced expression levels. Importantly, this ILK-dependent upregulation was observed independently of sex or diabetes, supporting a previously unrecognized ILK-DPP4 regulatory axis.

The eILK cKO mouse recapitulates key features of human CAVD, including leaflet thickening, calcification, and adverse cardiac remodeling. Notably, significant valvular and cardiac alterations developed within 12 weeks of ILK deletion, even in the absence of a pro-atherogenic diet, offering a rapid and reproducible alternative to traditional models that rely on genetic predisposition, mechanical injury, or dietary manipulation.^{31–34} Although this model does not recapitulate dyslipidemia typical of human CAVD,⁵ it could be combined with a pro-atherogenic background to better reflect lipid-driven early disease.

Mechanistically, endothelial ILK deficiency promoted EndMT, increased osteogenic marker expression (RUNX2 and ALP), activated NF- κ B, and elevated expression of inflammatory mediators, contributing to calcific remodeling and activating interstitial cells through paracrine signaling. These effects were attenuated by sitagliptin, consistent with reports implicating DPP4 inhibition in modulating EndMT and fibrosis across cardiovascular and renal disease models.^{35–37} Chronic inflammation is a known initiator and amplifier of EndMT,³⁸ and EndMT-derived cells can perpetuate inflammatory signaling, creating a feed-forward loop that exacerbates valvular damage.³⁹ Accordingly, ILK-silenced

endothelial cells exhibited a proinflammatory cytokine profile that shifted toward an anti-inflammatory state with sitagliptin treatment.

In vivo, sitagliptin improved aortic valve hemodynamics and myocardial contractility, and attenuated calcification while only partially attenuating valve remodeling in the eILK cKO phenotype. This partial efficacy mirrors heterogeneous associations reported among individual DPP4 inhibitors in clinical studies,^{12,40} suggesting that DPP4 inhibition should not be considered a uniform therapeutic strategy. Differences in tissue penetration, target engagement, or off-target effects may underlie these discrepancies,^{27,29} as supported by our in vitro findings showing reduced Linagliptin efficacy compared with sitagliptin. Genetic silencing of DPP4 produced slightly more pronounced protective effects than pharmacological inhibition, further indicating that partial enzymatic blockade may be insufficient to fully recapitulate DPP4 loss. In this context, the ongoing Evogliptin trial (Unique identifier: NCT04521452) highlights the need for mechanistic models to guide therapeutic selection.

The eILK cKO mouse provides a robust and clinically relevant model for studying endothelial-driven mechanisms of CAVD and for testing therapeutic strategies targeting inflammatory and metabolic pathways. However, several limitations should be acknowledged. First, all in vivo experiments were performed in male mice to ensure efficient recombination; therefore, potential sex-specific differences in ILK-DPP4 signaling or disease progression cannot be excluded. Although ILK-DPP4 associations were observed in human valve samples independent of sex, future studies using sex-balanced cohorts and alternative recombination strategies will be required to address sex as a biological variable more directly.

Second, the interpretation of myocardial and coronary microvascular remodeling in this model warrants caution, particularly at early time points after endothelial ILK deletion.¹⁸ Although significant valve thickening and calcium deposition are detectable as early as 4 weeks after ILK loss, early myocardial and microvascular alterations may not be fully attributable to valvular hemodynamic changes alone. These findings suggest that endothelial ILK deficiency may contribute to pathological remodeling in the valve and coronary microvasculature through partially overlapping, endothelial-driven processes, including endothelial inflammation and EndMT. At later stages, progressive valve calcification and stenosis are

Figure 5 Continued. magnification. **Right**, A bar graph shows the quantification of α -SMA colocalizing with CD31, indicating partial EndMT (N=3–6). **B** and **C**. RUNX2 (runt-related transcription factor 2) and ALP (alkaline phosphatase) levels in aortic valves from eILK cKO mice treated or not with SITA for 12 weeks (n=6). **D**, Representative histological staining of aortic valves with Alizarin Red and Von Kossa showing calcification. Scale bar, 100 μ m and 10 mm in amplification. **E**, Quantification of Von Kossa aortic valve area (N=6–11). **F**, Representative images of transvalvular peak velocity from eILK cKO or CT mice treated with and without SITA for 12 weeks. Quantitative analysis of valvular peak velocity (N=10). **G**, Quantification of aortic valve leaflet thickness (μ m) in experimental groups as **F** (N=8). For all figures, results are expressed as mean $\pm$ SD. All *P* values were calculated using 1-way ANOVA with the Bonferroni post hoc test.

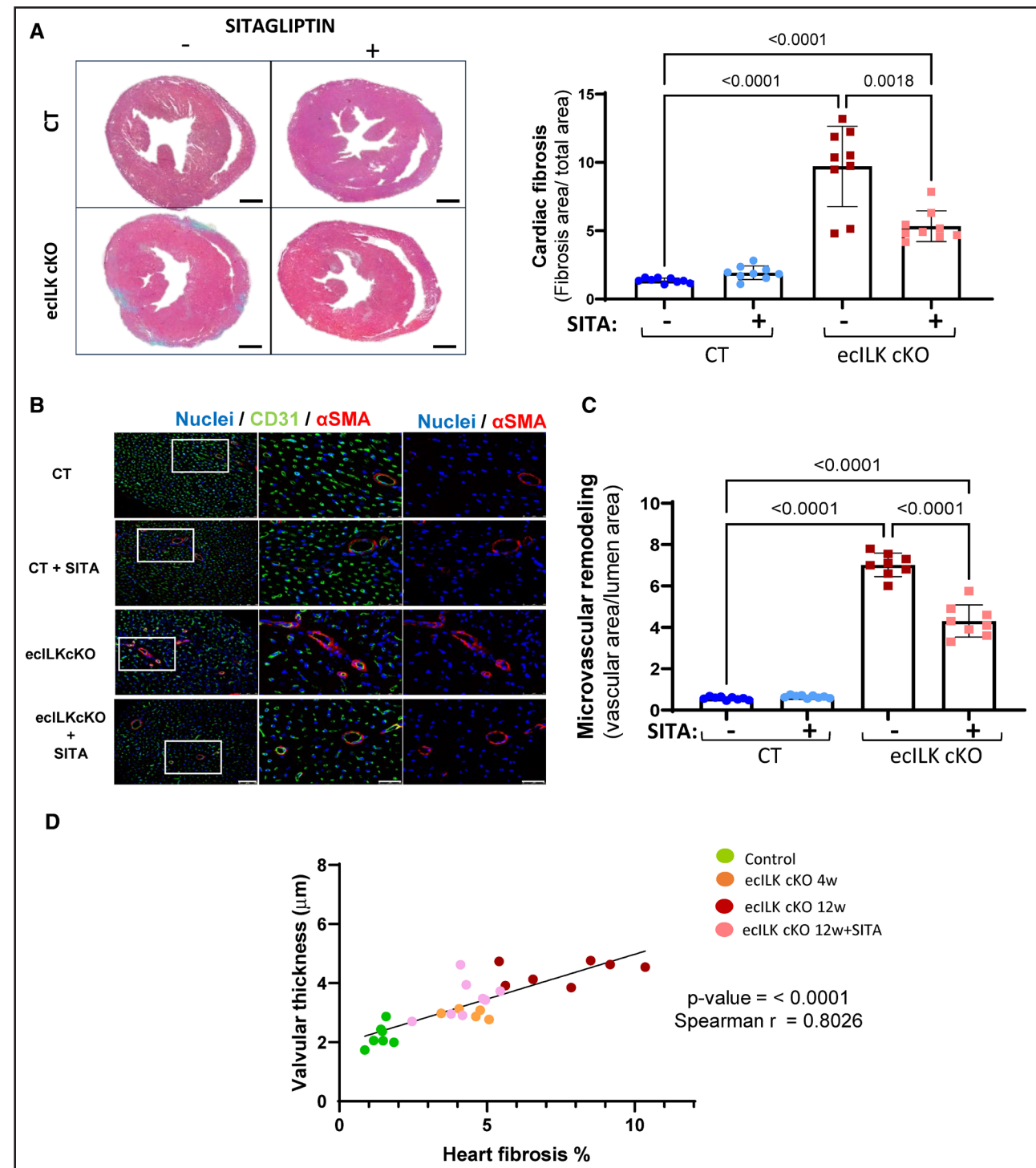


Figure 6. Sitagliptin (SITA) partially attenuates myocardial remodeling in endothelial cell-specific integrin-linked kinase conditional knockout (ecILK cKO) mice.

A, Representative Masson trichrome staining showing cardiac fibrosis in ecILK cKO and control (CT) mice treated with and without SITA for 12 weeks. Scale bar, 1 mm. **Right**, Quantitative analysis of heart fibrosis (N=9). **B**, Representative confocal images of coronary microvessels from ecILK cKO or CT mice treated with and without SITA for 12 weeks, stained with α -SMA (alpha smooth muscle actin; red); endothelial cells were stained with CD31 (green), and nuclei were counterstained in blue with Hoechst. Scale bar, 50 and 10 μ m in magnification. **C**, Quantitative analysis of microvascular remodeling in animals treated as in **A** (N=9). **D**, Spearman correlation analysis between aortic valve thickness and cardiac fibrosis. Each data point represents an individual mouse (n=5–7). For all figures, results are expressed as mean $\pm$ SD. All *P* values were calculated using 1-way ANOVA with the Bonferroni post hoc test. **G**, Spearman correlation coefficient (*r*) was calculated, and statistical significance was determined using a 2-tailed *t* test.

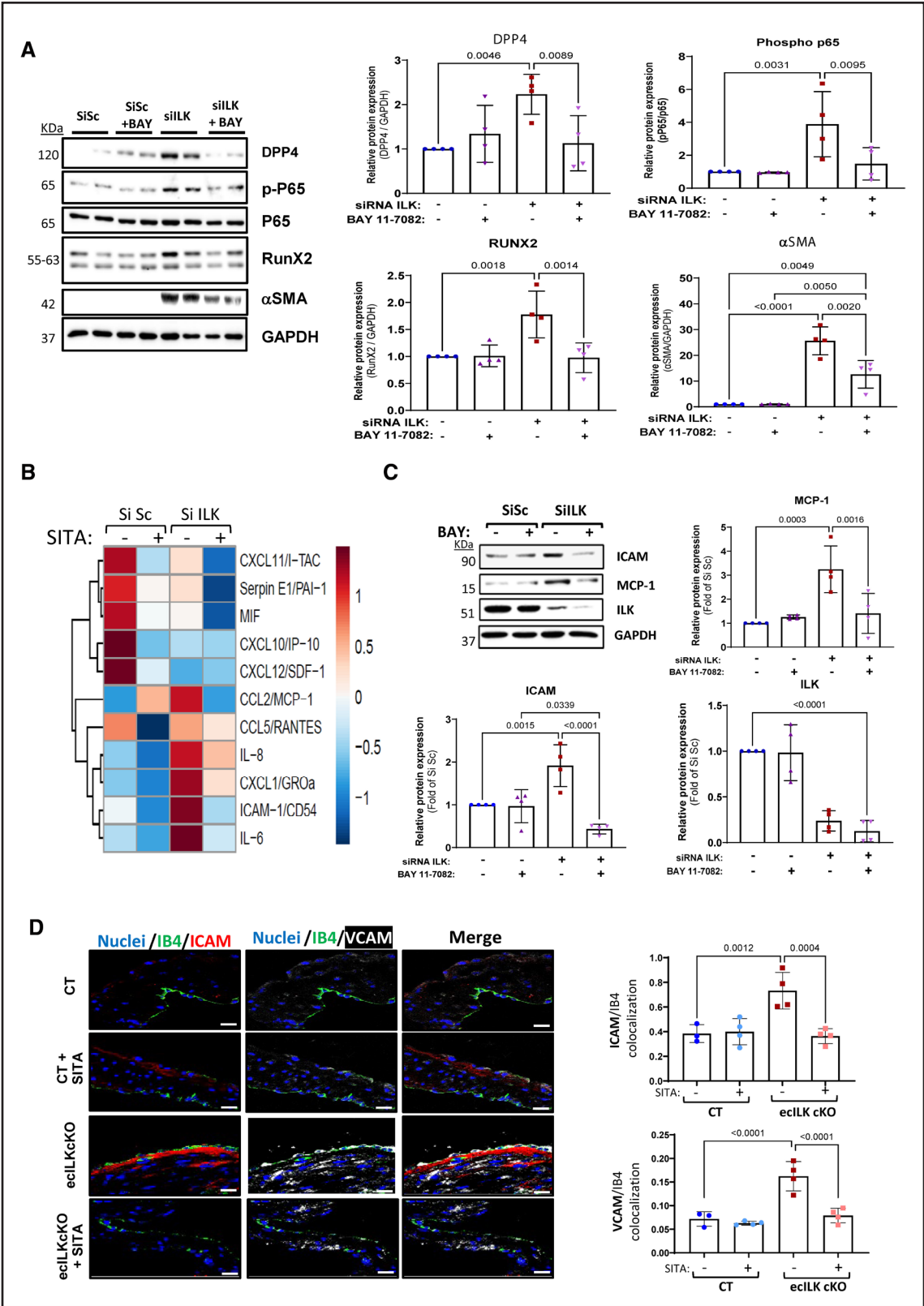


Figure 7. Sitagliptin reversed NF-κB (nuclear factor kappa B)-dependent DPP4 (dipeptidyl peptidase 4) expression and inflammatory signaling induced by ILK (integrin-linked kinase) loss in both valvular endothelial cells (VECs) and eILK cKO (endothelial ILK conditional deleted mice) mouse model.
A, Representative immunoblot of human VECs (hVECs) transfected with scramble (SiSc) and siILK and treated with BAY 11-7082. Blots were probed for DPP4, phospho-NF-κB p65 (p-p65) and total p65, RUNX2 (runt-related transcription factor 2), and α-SMA (alpha smooth muscle actin). **Right**, Densitometric quantification (N=4 in duplicate). **B**, Heatmap of Z score-normalized levels of (Continued)

Figure 7 Continued. endothelium-expressed cytokines (ICAM [intercellular adhesion molecule]-1/CD54, IL [interleukin]-6, IL-8, CCL2/MCP-1 [monocyte chemoattractant protein-1], CCL5 [CC chemokine receptors-5]/RANTES [regulated upon activation, normal T-cell expressed and secreted], CXCL1 [C-X-C motif chemokine ligand]/GRO α [growth-regulated oncogene- α], CXCL10/IP-10 [interferon- γ -inducible protein 10], CXCL11/I-TAC [interferon-inducible T cell α chemoattractant], CXCL12/SDF-1 [stromal cell-derived factor-1], MIF [macrophage migration inhibitory factor], and serpin E1/PAI-1 [plasminogen activator inhibitor 1]) measured by cytokine assay in hVEC silenced with SiSc or ILK (siILK) and treated or not with sitagliptin for the duration of the experiment (N=3). **C**, Representative immunoblot of VECs SiSc and siILK transfected and treated with or without BAY 11-7082. Blots were probed for MCP-1, ICAM, and ILK. Densitometric analyses of ICAM-1, MCP-1, and ILK protein expressions are shown (N=4). **D**, Representative immunofluorescence of aortic valve leaflets from control (CT) and endothelial cell-specific ILK conditional knockout (ecILK cKO) mice, untreated or treated with sitagliptin for 12 weeks. Endothelial cells are stained with IB4 (isolectin B4; green), ICAM-1 (red), or VCAM-1 (vascular cell adhesion molecule-1; white), and nuclei with Hoechst (blue). **Right**, Quantification of ICAM-1/IB4 and VCAM-1/IB4 colocalization by Manders overlap coefficient. Scale bars, 25 μ m. Each dot represents 6 independent measurements from each animal (N=3). All results are expressed as mean $\pm$ SD. *P* values were calculated using ANOVA with the Bonferroni post hoc test.

expected to further influence myocardial remodeling by increasing afterload, limiting the clear separation of primary endothelial effects from secondary hemodynamic consequences. In this context, DPP4 inhibition attenuated inflammatory activation and reduced mesenchymal and osteogenic marker expression in both the valve and coronary microvessels but did not restore endothelial marker expression. Such incomplete recovery may contribute to residual structural remodeling despite improvements in inflammatory and calcific end points, consistent with prior observations indicating that partial reversion of EndMT may be insufficient to fully reverse established endothelial alterations.⁴¹

Based on these observations, we propose a working model in which endothelial ILK deficiency initiates early inflammatory and EndMT-associated changes in the aortic valve and myocardial microvasculature, with progressive valve calcification and stenosis subsequently amplifying myocardial remodeling through hemodynamic mechanisms. Thus, while sitagliptin confers partial benefit, it is insufficient as monotherapy to fully rescue valvular function in this model. These findings underscore the multifactorial nature of ILK-associated valvulopathy and suggest that earlier intervention or combination approaches may be necessary to achieve full therapeutic efficacy.

Mechanistically, DPP4 inhibition may influence valvular remodeling by modulating NO-dependent pathways and altering the availability of downstream substrates. Reduced NO bioavailability has been implicated in the pathogenesis of CAVD, as shown in eNOS (endothelial nitric oxide synthase)-deficient models.^{42,43} Consistent with prior evidence that ILK supports endothelial homeostasis via NO production, we observed reduced NO bioavailability in ILK-deficient valves.^{15,44} Although it remains unclear whether restoration of NO signaling is sufficient to normalize DPP4 expression, these findings raise the possibility that increased DPP4 activity under ILK-deficient conditions may be, at least in part, NO-dependent. In support of this concept, recent work by Choi et al⁴⁵ linked increased VIC-DPP4 activity to aortic valve calcification in both patients with CAVD and eNOS KO mice, conditions characterized by impaired NO signaling. In addition, DPP4

inhibition may modulate valvular remodeling by increasing the availability of downstream substrates, such as GLP-1 (glucagon-like peptide-1) and SDF-1 α (stromal cell-derived factor-1 α), attenuating DPP4-mediated immune activation, and modulating circulating soluble DPP4. These pathways have been implicated in suppressing osteogenic signaling, promoting endothelial repair, and limiting fibrotic and inflammatory responses^{46–50} but were not directly tested in the present study and warrant further mechanistic investigation.

CONCLUSIONS

This study identifies endothelial ILK as a central regulator of valvular endothelial homeostasis and an upstream contributor to CAVD. Using a rapid and reproducible endothelial-specific ILK knockout model, we show that ILK deficiency promotes endothelial inflammation, EndMT, and calcific remodeling of the aortic valve, accompanied by myocardial alterations. We further identify DPP4 as a functionally relevant downstream effector of ILK signaling and demonstrate that pharmacological DPP4 inhibition with sitagliptin partially attenuates ILK-dependent pathological remodeling. Collectively, these findings support an endothelial ILK-DPP4 axis as a disease-modifying pathway in CAVD and highlight endothelial-targeted modulation of inflammatory and metabolic signaling as a potential strategy to slow disease progression.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Tables S1–S3

Figures S1–S7

Major Resources Table

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