

SUPPLEMENTARY INFORMATION

Caldesmon-1–Mediated Actin Dynamics is Essential for Osteogenic Differentiation of Aortic Valve Interstitial Cells

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Supplementary Tables

Supplementary Table S1. Characteristics of patients with aortic stenosis included in immunohistochemical analysis

Aortic stenosis (n = 7)	
Age, years	75 ± 6
Male, n (%)	6(86)
Body mass index	18 ± 3.2
Hypertension, n (%)	67(100)
Smokers, n (%)	2 (29)
Dyslipidaemia, n (%)	2 (29)
Diabetes, n (%)	1 (14)
Coronary artery disease, n (%)	1 (14)
Mean gradient (mmHg)	66 ± 15.5
Tricuspid aortic valves, n (%)	4(57)

Data are given as n (%) or means ± standard deviations (SD).

Supplementary Table S2. Characteristics of autopsy cases corresponding to the samples used in immunohistochemical staining

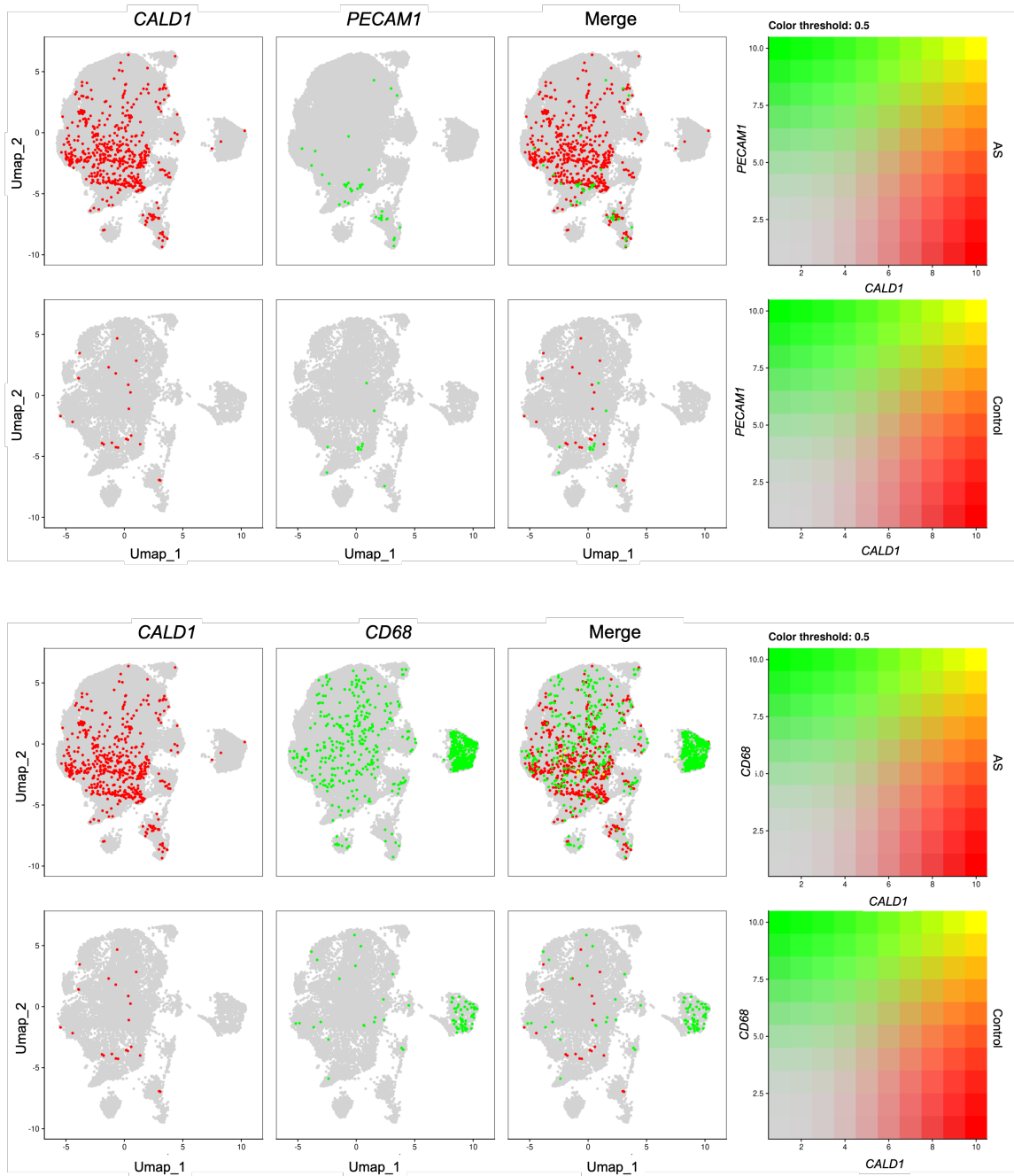
Autopsy cases (n = 7)	
Parameters	% (n)
Age, years	
mean ± SD	77.29 ± 15.00
Sex	
Male	42.86 (3)
Female	57.14 (4)
Smoking history	
Yes	14.29 (1)
No	71.42 (5)
unknown	14.29 (1)
Hypertension	
Yes	57.14 (4)
No	42.86 (3)
Diabetes mellitus	
Yes	0 (0)
No	100 (7)
Dyslipidaemia	
Yes	0 (0)
No	100 (7)
Ischaemic heart disease	
Yes	42.86 (3)
No	57.14 (4)

Supplementary Table S3. Characteristics of patients with aortic regurgitation included in cell growth analysis

Aortic regurgitation (n = 4)	
Age, y	55 ± 14.9
Male, n (%)	4(100)
Body mass index	22 ± 1.8
Hypertension, n (%)	2 (50)
Smokers, n (%)	1 (25)
Dyslipidaemia, n (%)	0 (0)
Diabetes, n (%)	0 (0)
Coronary artery disease, n (%)	0 (0)
Tricuspid aortic valves, n (%)	4(100)

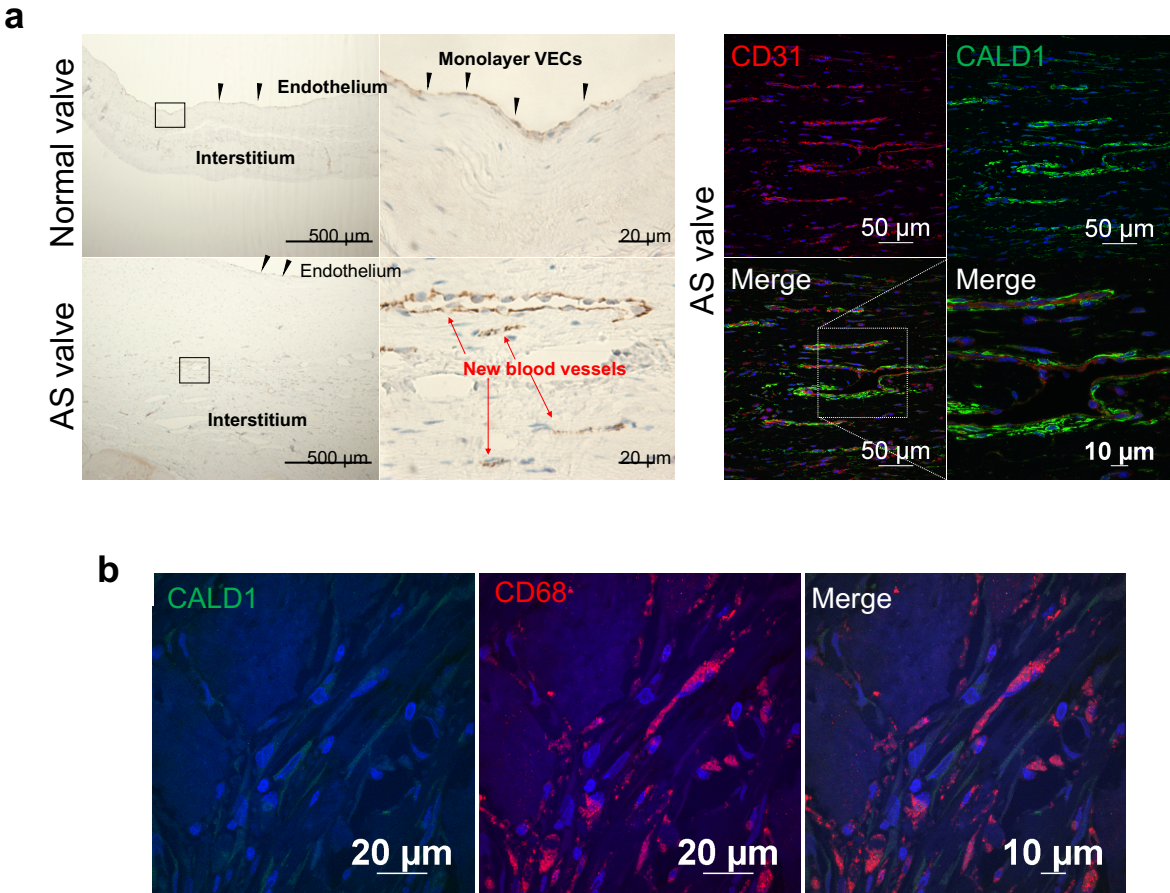
Data are given as n (%) or means ± standard deviations.

Supplementary Figures



Supplementary FigS1.

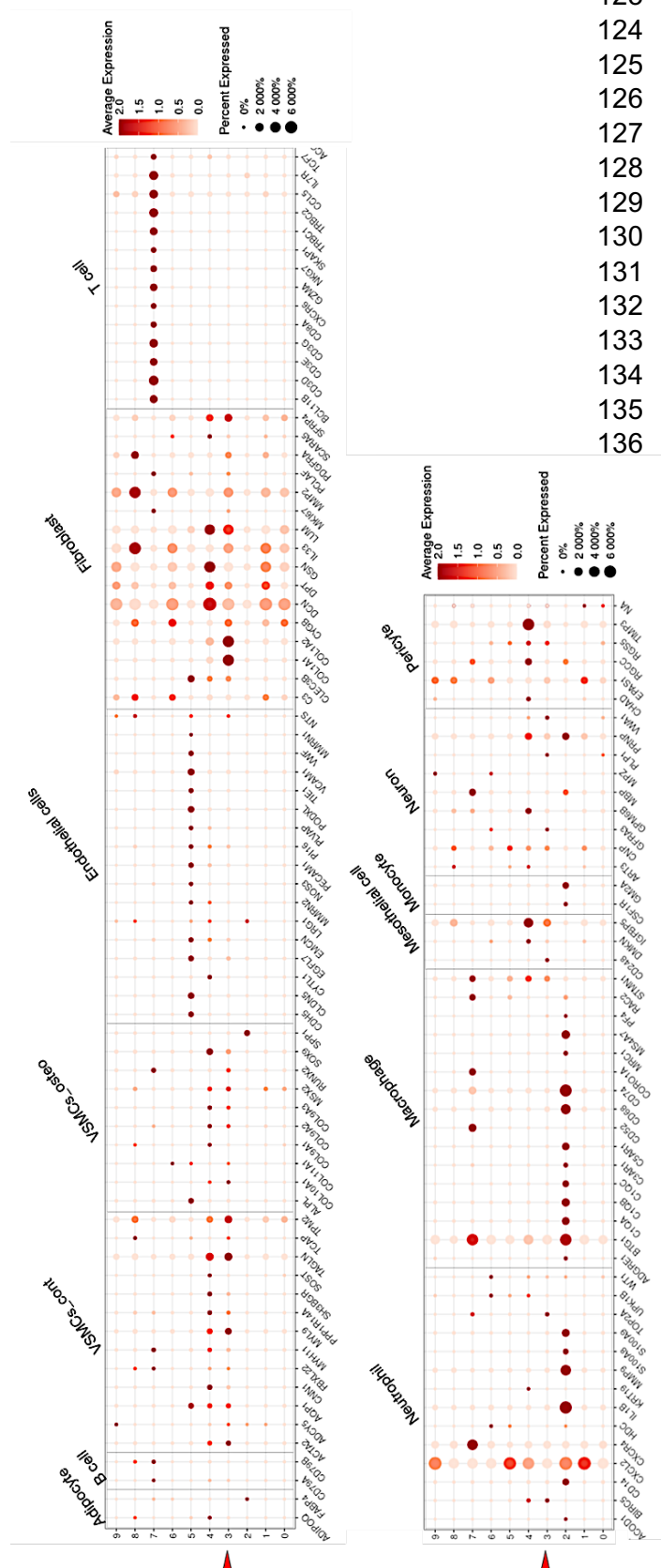
Uniform Manifold Approximation and Projection (UMAP) plots displaying CALD1-positive cells co-expressing PECAM1 or CD68 in AS valves (upper panel) and Control valves (lower panel), with corresponding heatmaps showing co-expression intensities (colour threshold set to 0.5).

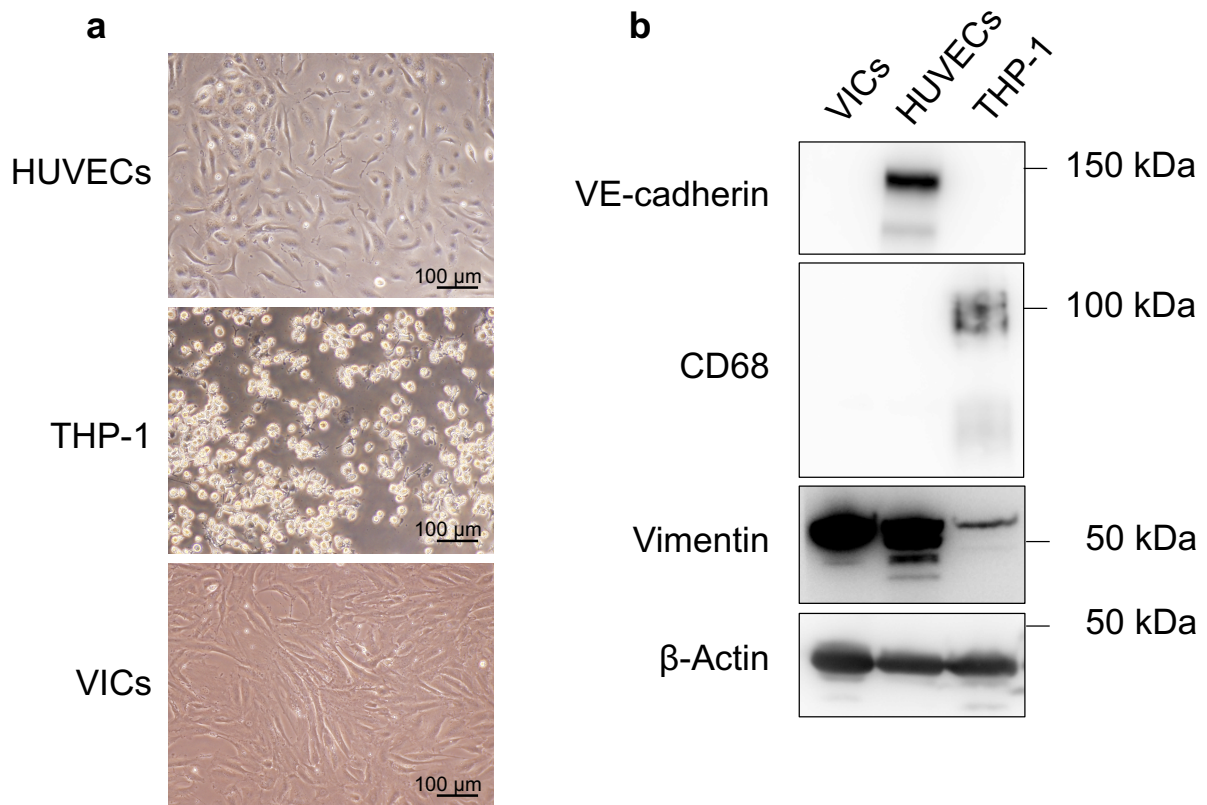


Supplementary Fig. S2. Tissue distribution of CD31 and CD68 in aortic valve leaflets.

a. Immunohistochemical and immunofluorescent staining for CD31 in normal and AS valves. Left panels: DAB staining with anti-CD31 antibody (clone 89C2, Cell Signaling Technology, Catalogue no. #3528) in normal and AS valve leaflets. In normal valves, CD31-positive endothelial cells were detected only on the leaflet surface, whereas in AS valves, CD31-positive cells were also observed in newly formed blood vessels within the interstitium. Some of these neovessels were surrounded by CALD1-positive perivascular cells. Immunofluorescence staining was performed using anti-CD31 rabbit polyclonal antibody (Catalogue no. ab28364, Abcam) and anti-CALD1 antibody (Catalogue no. #66693-1-Ig, Proteintech).

b. Tissue localization of CD68-positive macrophages in AS valves. CD68-positive immunofluorescence signals (red) were not detected in CALD1-positive VICs. Detailed staining protocols are described in the Materials and Methods section of the main text.

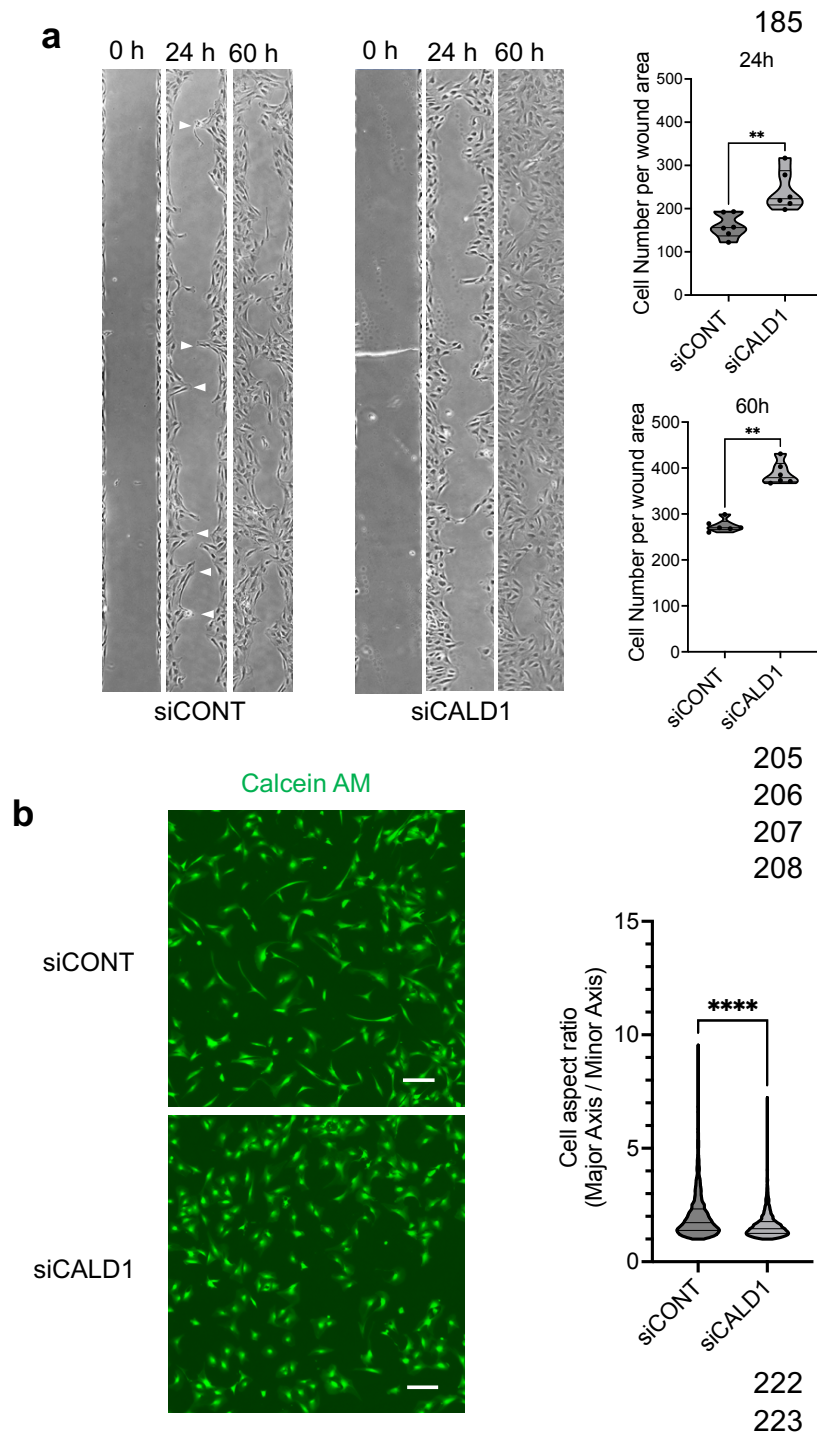




Supplementary Fig. S4. Characterization of pooled VICs used in this study.

a. Representative images of HUVECs, THP-1 cells, and pooled VICs. HUVECs (passage 3) were cultured in EGM-2 medium (Lonza). THP-1 cells were stimulated with 100 ng/mL phorbol ester for 72 hours to induce macrophage differentiation. Pooled VICs were used at passage 3. Images were acquired using an Olympus FSX100 microscope.

b. Western blot analysis of VE-cadherin (R&D Systems, Catalogue no. AF938, 1:400), CD68 (Cell Signaling Technology, MultiMab® Rabbit mAb mix, Catalogue no. 86985, 1:400), vimentin (Cell Signaling Technology, Clone D21H3 XP®, Catalogue no. 5741, 1:1000), and β -actin (Sigma, Clone AC-15, Catalogue no. A5441, 1:4000). Detailed western blotting procedures are described in the Materials and Methods section of the main text. VE-cadherin (endothelial marker) and CD68 (macrophage marker) were detected in lysates from HUVECs and THP-1 cells, respectively. Vimentin (mesenchymal marker) was predominantly expressed in VICs and HUVECs. The uncropped original blots are shown in Supplementary Fig. 12.

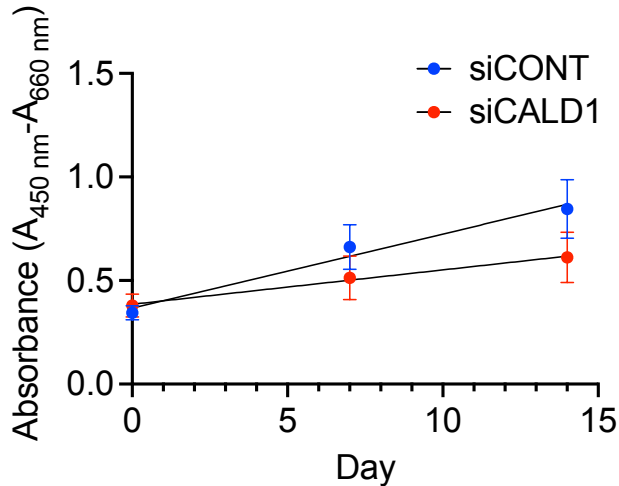


Supplementary Fig. S5. Cell migration activity and cellular morphology of control valvular interstitial cells (VICs) and CALD1-depleted VICs.

a. Control (CONT) siRNA or caldesmon-1 (CALD1) siRNA were transfected into the VICs, followed by culturing for 24 h. siCONT-transfected or siCALD1-transfected VICs were seeded in a 35-mm high Culture-Insert 3 Well in μ -Dish (IBIDI GmbH, Martinsried, Germany, Catalogue no. 80366), according to the manufacturer's instructions and allowed to adhere and grow to confluence in Dulbecco's Modified Eagle Medium

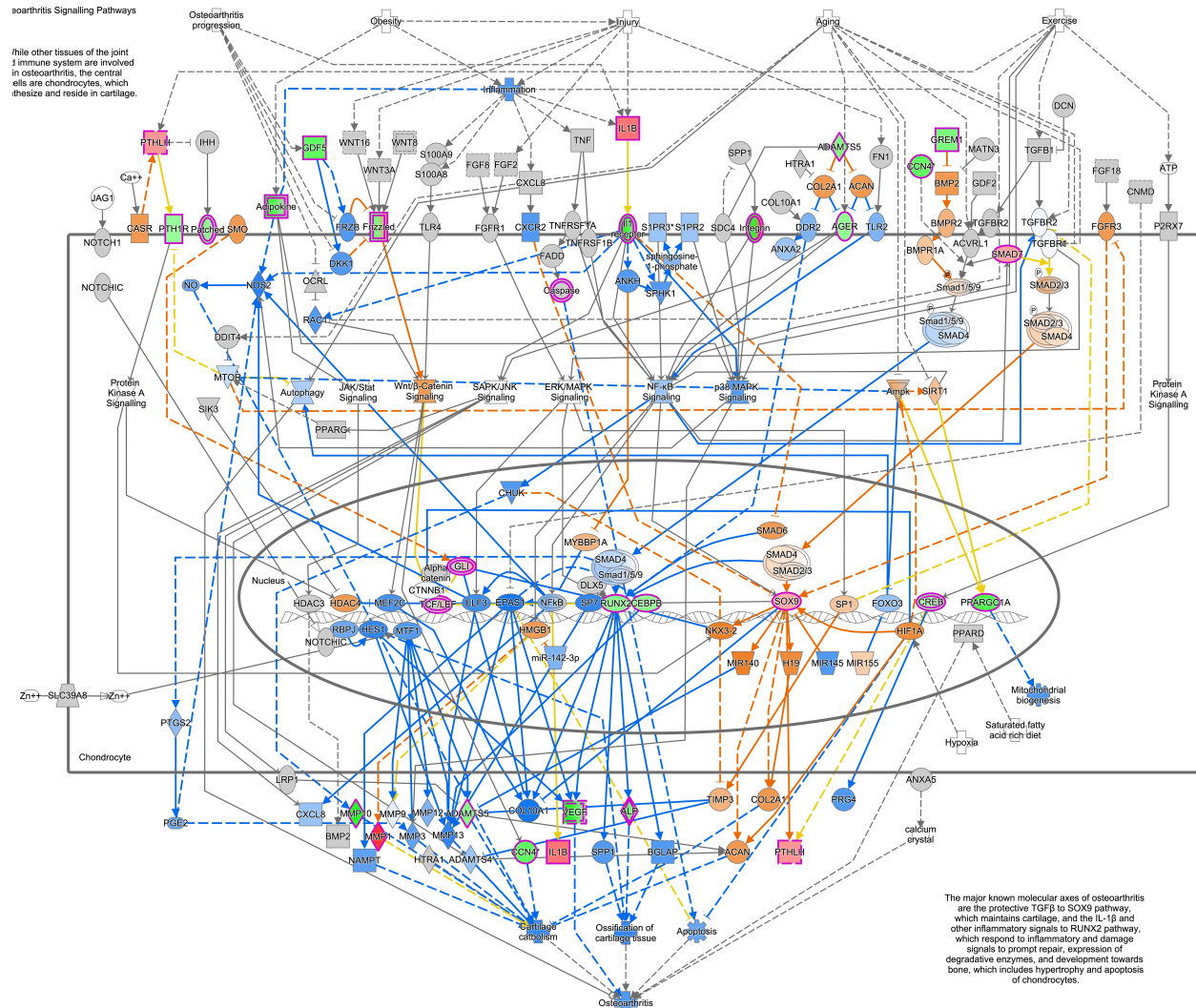
(DMEM). At 100% confluence, the insert was removed, as per the protocol provided by
ibid, initiating cell migration into the cell-free area. BZ-X800 (Keyence, Osaka, Japan)
was used to capture the migrated cells at 0, 24, and 60 h post-insert removal. Cell
numbers in the wound area were counted at each time point to evaluate the effects of
CALD1 knockdown on VIC migration. Statistical analysis was performed using a Mann–
Whitney *U* test. ***P* < 0.01.

b. Cellular morphology of VICs transfected with control siRNA or CALD1 siRNA. Cells
were stained with Calcein AM (Dojin Chemical, Kumamoto, Japan) for 30 min. Scale
bars = 200 μ m. Quantification of cell morphology and calculation of the aspect ratio
(major axis/minor axis) were performed using Keyence Macro Cell Count software. Data
are presented as mean $\pm$ SD. *****P* < 0.0001 by Mann–Whitney *U* test. The aspect ratio
of VICs was significantly reduced by CALD1 knockdown.

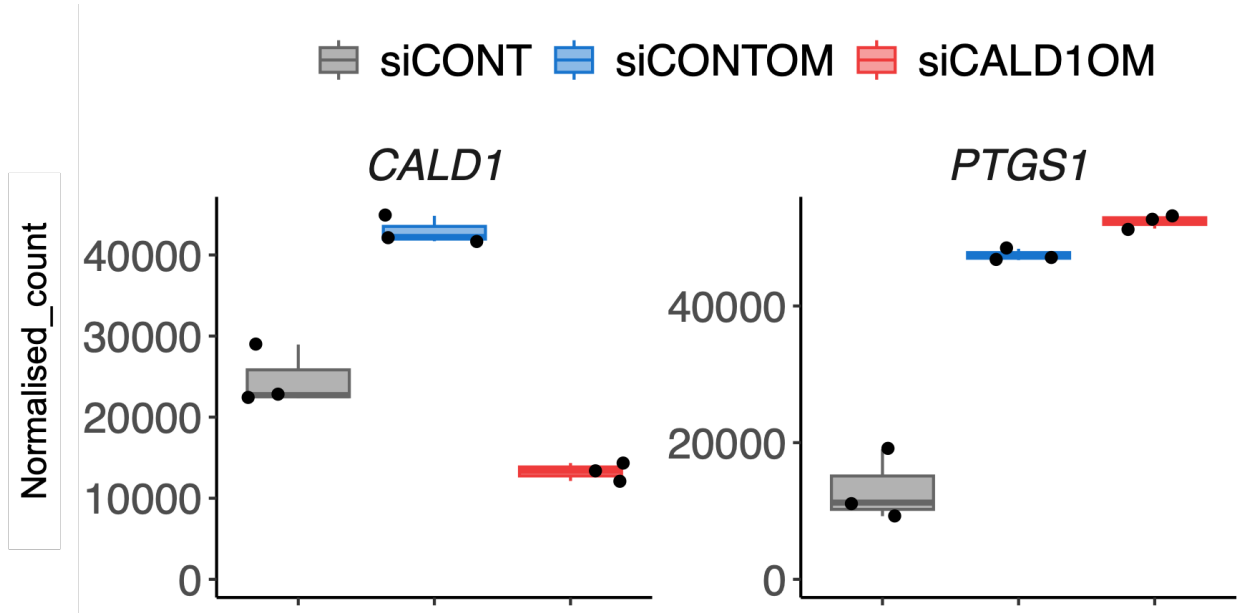


Supplementary Fig. S6. Cell growth assay of control and CALD1-depleted VICs.

WST-1 assay (CELLPRO-RO, Roche, Basel, Switzerland) was performed for VICs treated with siCONT or siCALD1 at days 0, 7, and 14 following cell plating (n=32, biological replicates). The absorbance was measured at 450 nm with a reference wavelength of 660 nm ($A_{450\text{nm}} - A_{660\text{nm}}$). Data are shown as mean $\pm$ SD. Significant differences were observed on days 7 ($P < 0.0001$) and 14 ($P < 0.0001$) by the Mann–Whitney U test.

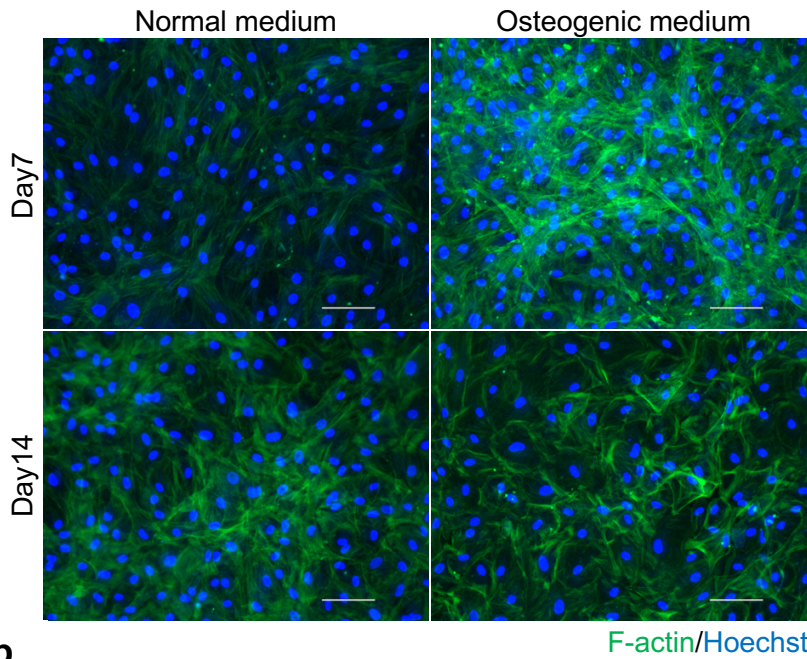


Supplementary Fig. S7. A detailed map of signalling pathways involved in osteoarthritis, with a focus on osteogenic and inflammatory cascades. Genes predicted to be downregulated in CALD1-depleted VICs are marked in blue, while predicted upregulated genes are in orange. Genes measured as upregulated are labelled in red, and those downregulated are labelled in green.

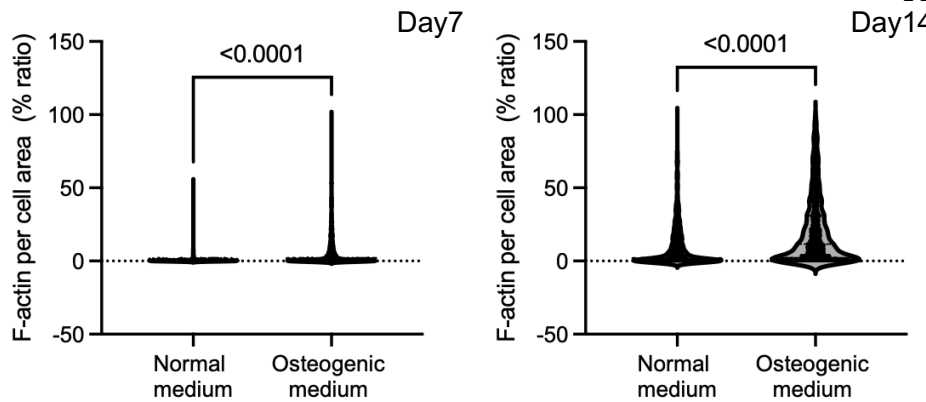


Supplementary Fig. S8. Expression levels of PTGS1 in control siRNA- or CALD1 siRNA-transfected VICs cultured in normal or osteogenic medium. Differential gene expression analysis was performed using the RNAseq Chef pipeline, as described in the main text. Although PTGS1 expression was increased by osteogenic medium, this upregulation was not attenuated by CALD1 knockdown.

a



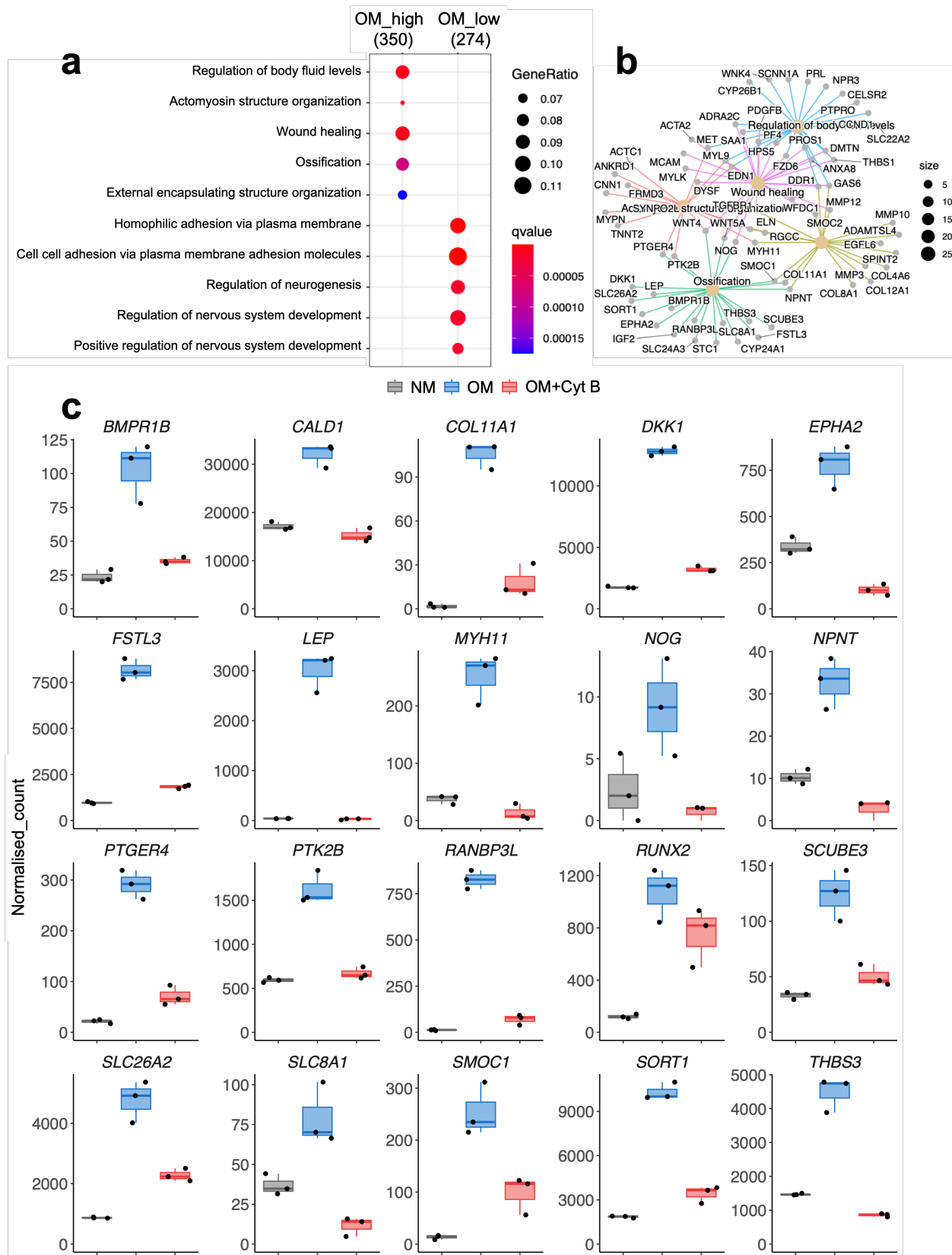
b



Supplementary Fig. S9. Actin dynamics in siCONT- or siCALD1-transfected VICs cultured in normal or osteogenic medium.

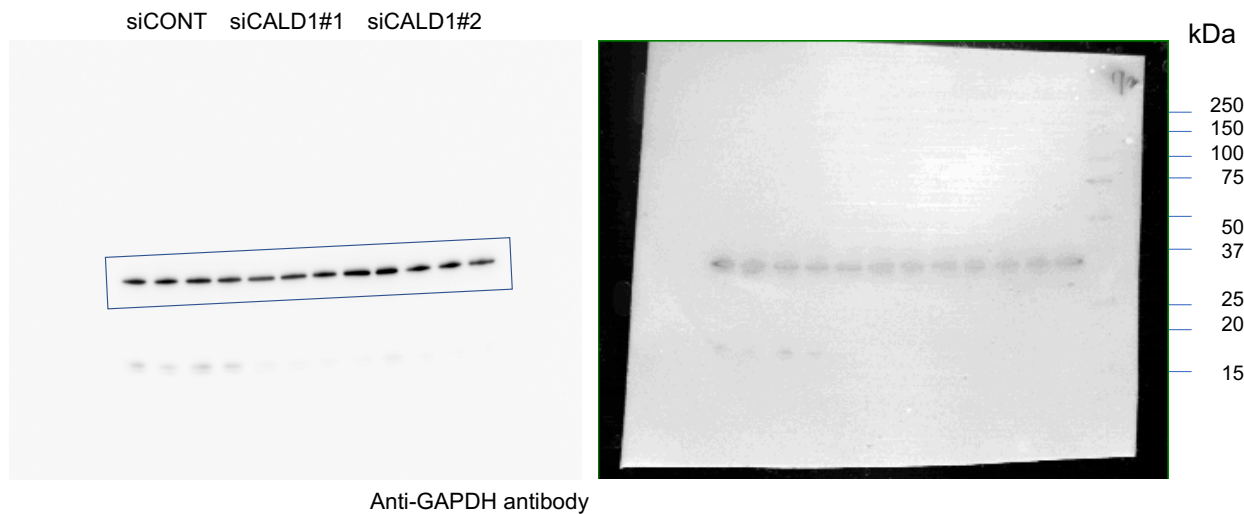
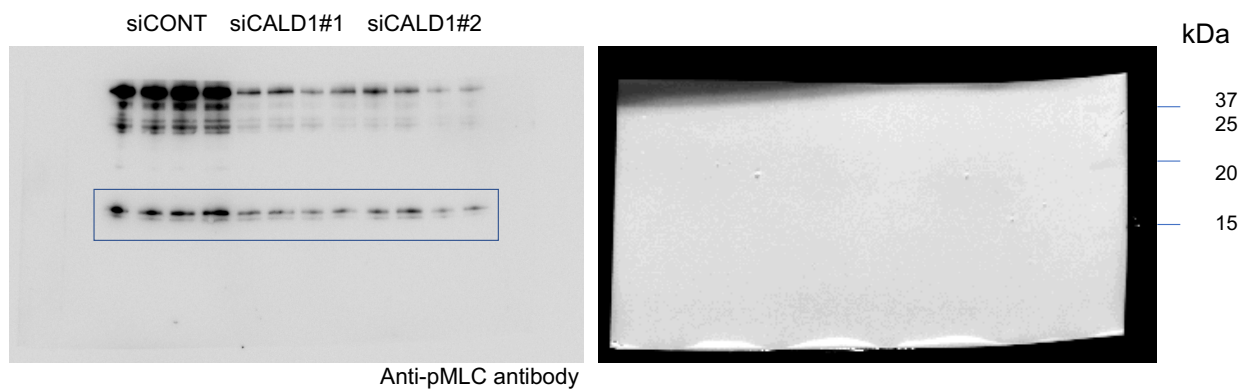
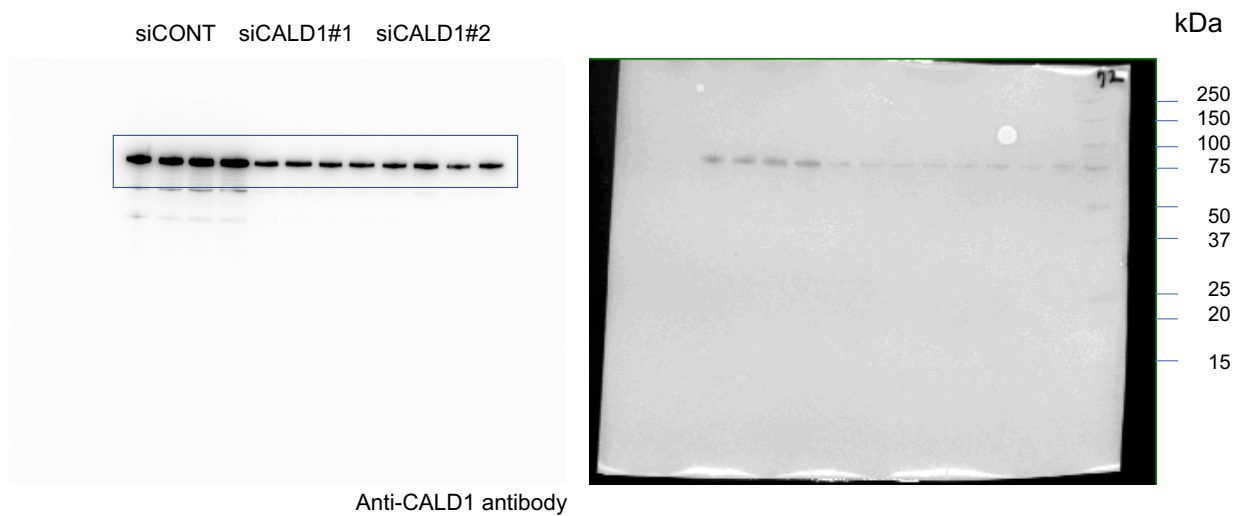
a. VICs were cultured in control or osteogenic medium for 7 or 14 days, fixed with 4% paraformaldehyde (PFA) for 30 min at 25 °C, and permeabilized with PBS containing 0.1% Triton X-100. Cells were stained with Alexa Fluor™ 488-conjugated phalloidin (Thermo Fisher Scientific) for 60 min and counterstained with Hoechst 33342. Fluorescent images were acquired using a Keyence BZ-X800 microscope. Scale bars, 100 µm.

b. Quantification of F-actin fluorescence per cell was performed using Keyence Macro Cell Count software. Statistical analysis was performed using the Mann–Whitney U test. Actin polymerisation was significantly increased in cells cultured in osteogenic medium at both day 7 and day 14.

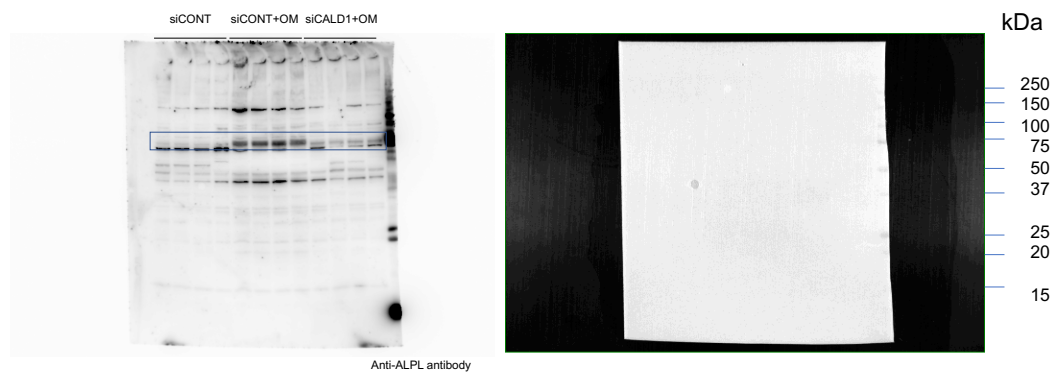
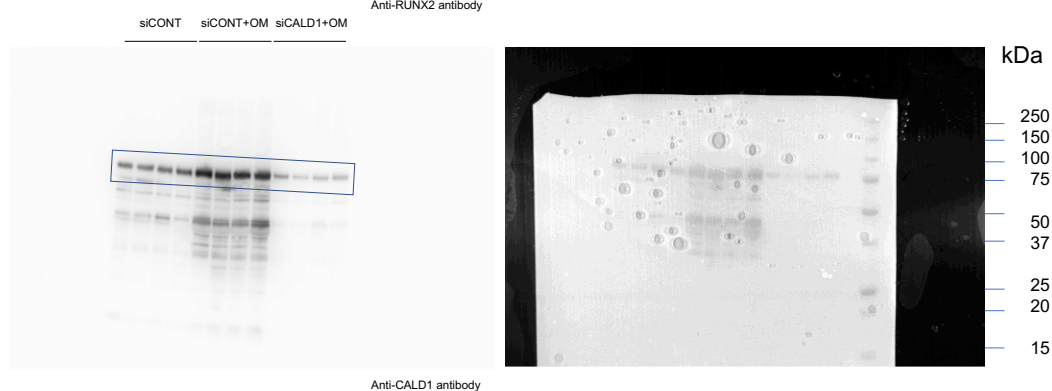


**Supplementary Fig. S10. Bioinformatic analysis of RNA-seq data from Cyt B–
treated osteogenic VICs.**

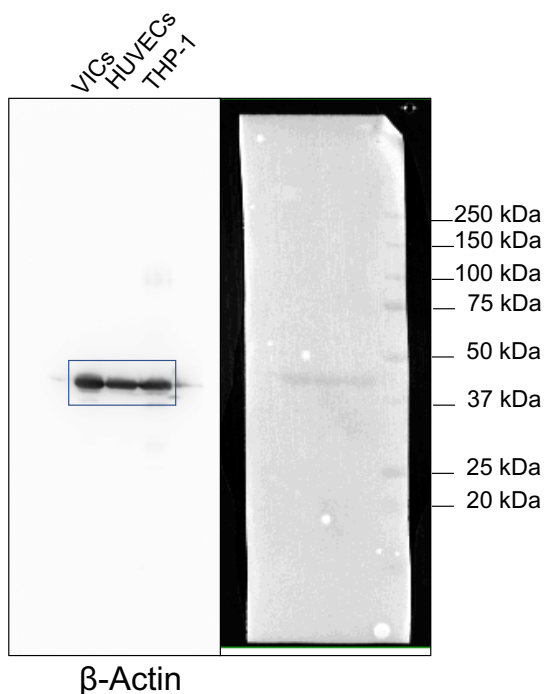
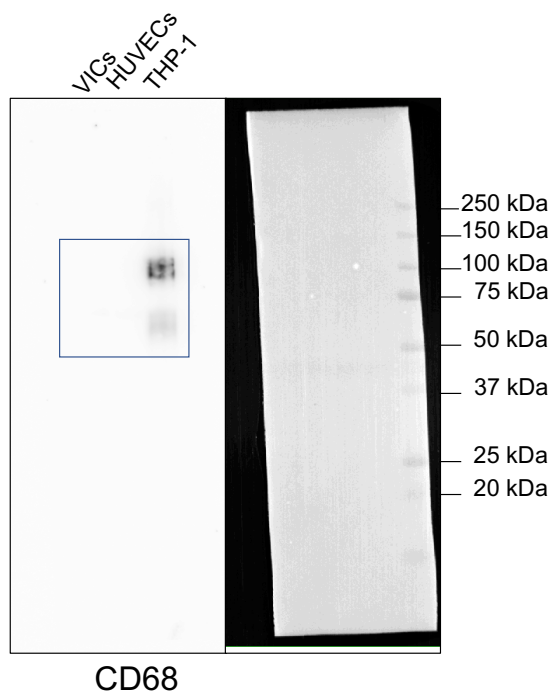
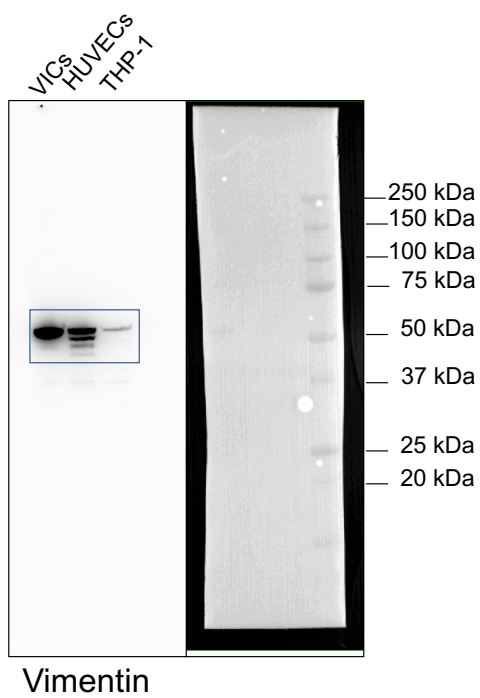
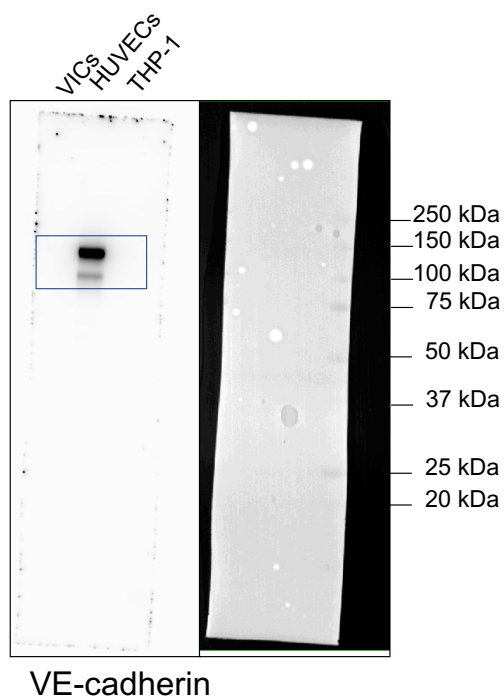
- a. Pathway analysis based on gene expression profiles of VICs cultured in normal medium (NM) or osteogenic medium (OM) with 1.0 μ M Cyt B.
 - b. Genes contributing to activated pathways in OM-treated VICs.
 - c. Box plots showing expression levels of ossification-related genes in VICs cultured in NM or OM with 1.0 μ M Cyt B.
- Differential gene expression analysis was performed using the RNA-seq Chef pipeline, as described in the main text.



Supplementary Fig. S11. Uncropped western blotting data (for Fig. 3b)

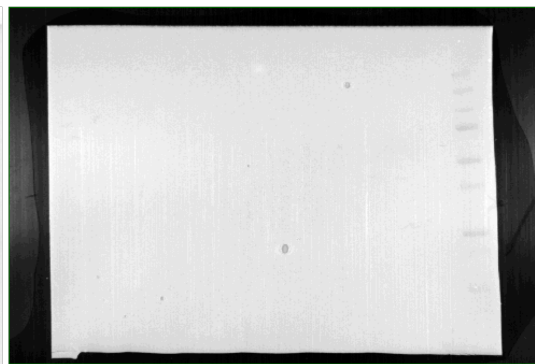
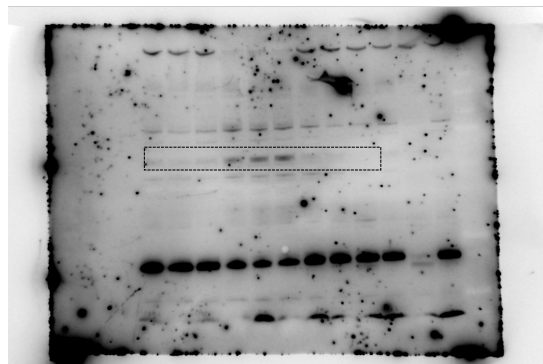


Supplementary Fig. S12. Uncropped western blotting data (for-Fig. 5b)



Supplementary Fig. S13. Uncropped western blotting data (for Supplementary Fig. S4b).

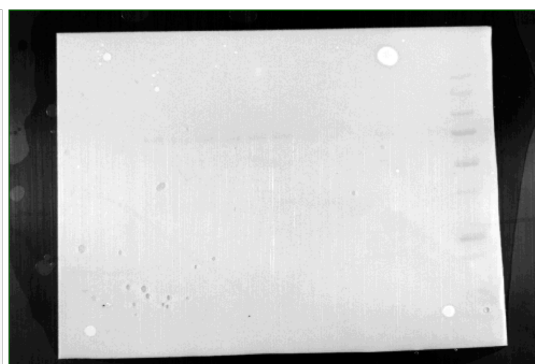
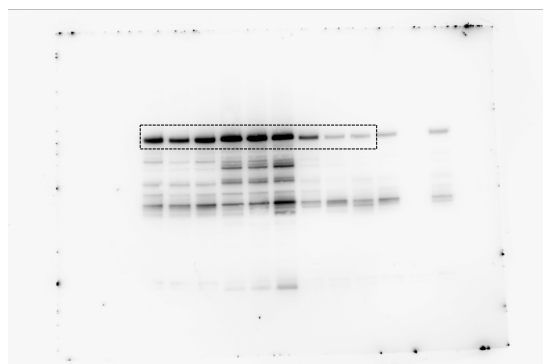
NM OM OM+Cyt B
(n=3) (n=3) (n=3)



kDa

250
150
100
75
50
37
25
20
15

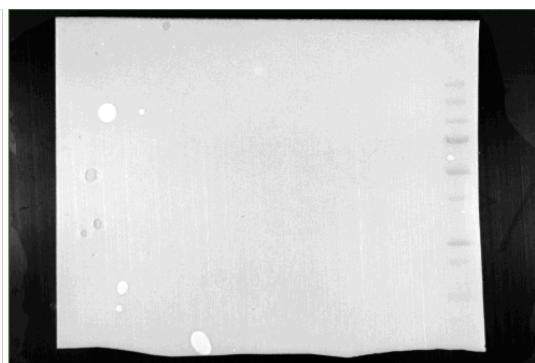
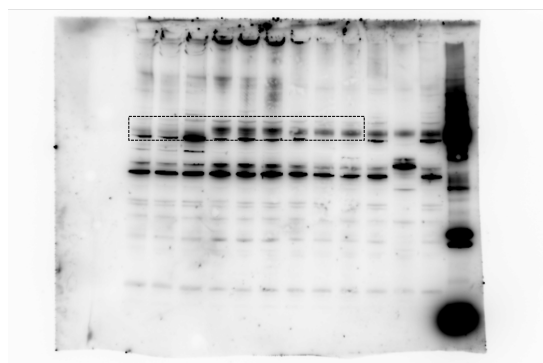
Anti-RUNX2 antibody



kDa

250
150
100
75
50
37
25
20
15

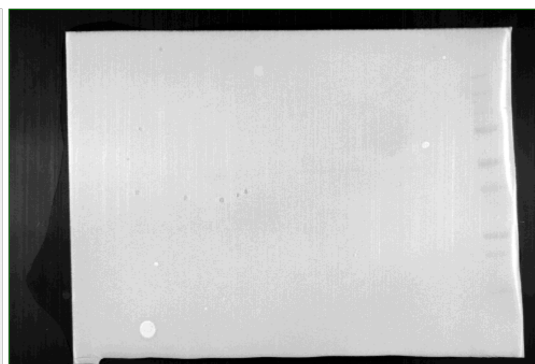
Anti-CALD1 antibody



kDa

250
150
100
75
50
37
25
20
15

Anti-ALPL antibody



kDa

250
150
100
75
50
37
25
20
15

Anti-Tubulin antibody

402
403
404
405

Supplementary Fig. S14. Uncropped western blotting data (for Supplementary Fig. S3b).