Supplemental Information

Adventitial MSC-like cells are progenitors of vascular smooth muscle cells and drive vascular calcification in chronic kidney disease

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Inventory of Supplemental Information

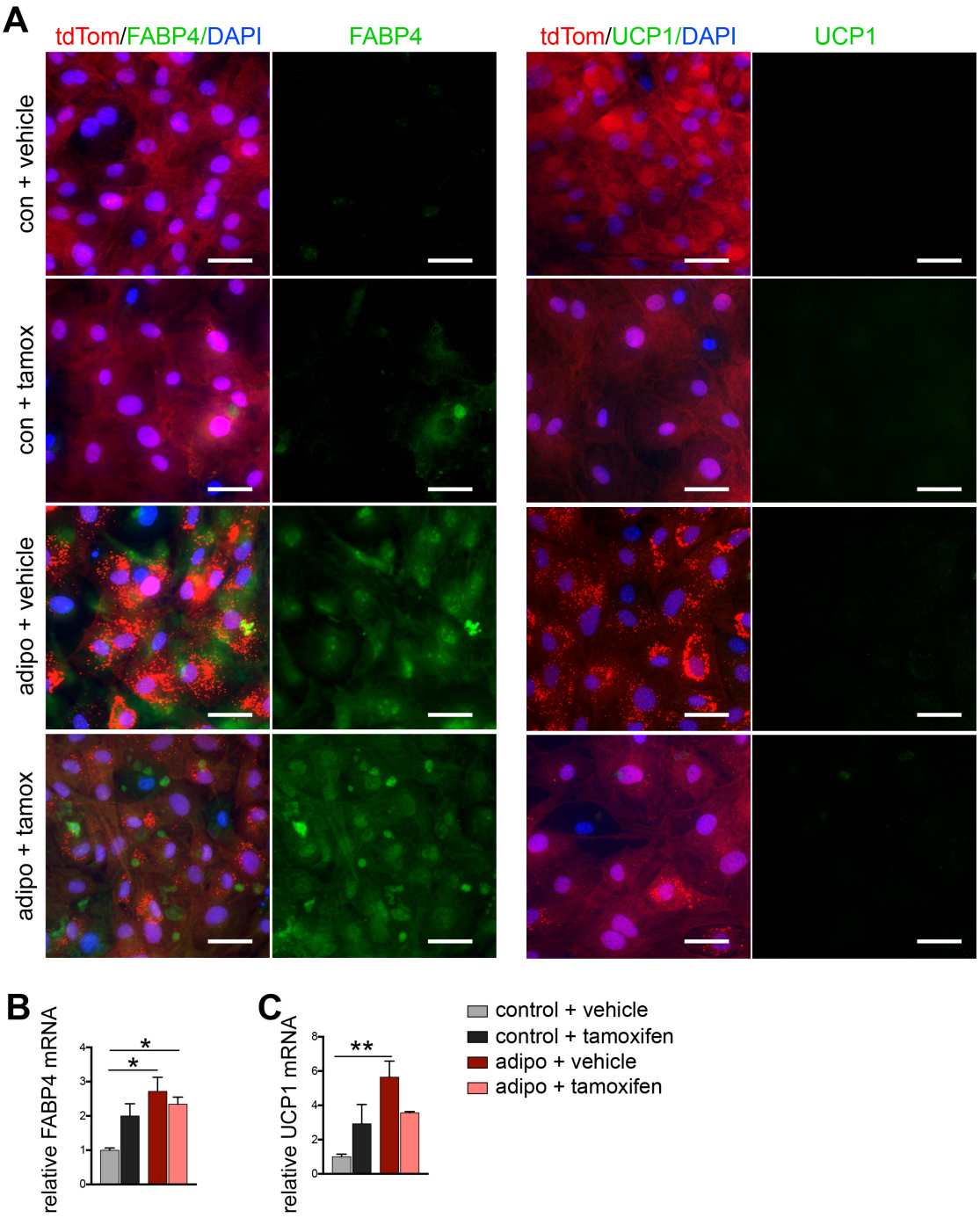

Figure S1: Tamoxifen does not significantly affect adipogenic differentiation of Gli1$^+$ cells

(A) Representative picture of Gli1$^+$ cells cultured in adipogenic differentiation medium or standard MSC medium with addition of 5-hydroxytamoxifen in ethanol or vehicle (ethanol) for 21 days. Staining was performed for fatty acid binding protein 4 (FABP4) as marker of adipogenic differentiation and uncopling protein 1 (UCP1) as a marker for brown adipose tissue. Scale bars 50µm

(B) Quantitative realtime PCR for FABP4 and UCP1 indicating no signficicant effect of tamoxifen treatment. * $p<0.05$; ** $p<0.01$ by one way ANOVA with posthoc Tukey

See also figure 1

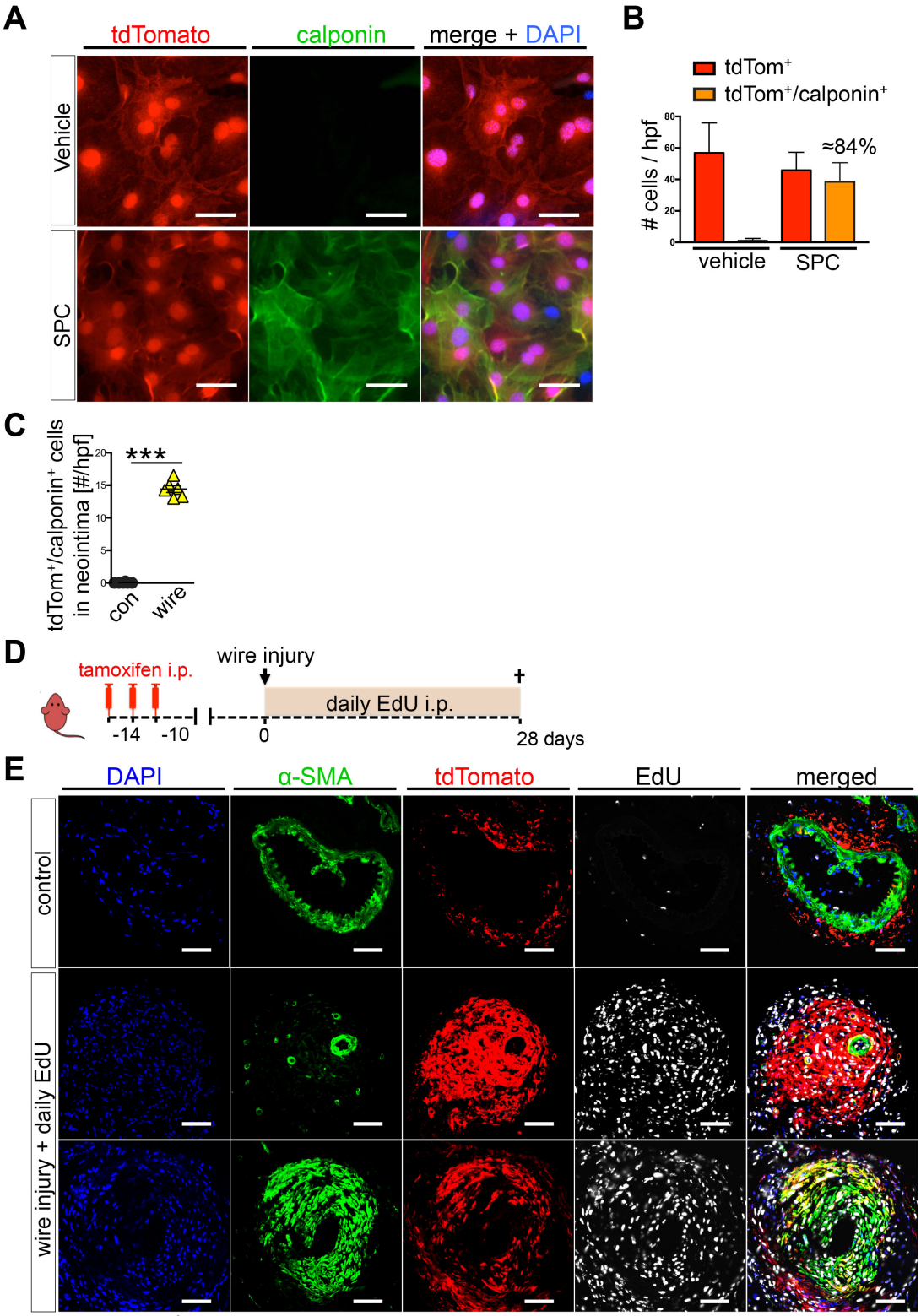

Figure S2: Gli1$^+$ cells are vSMC progenitors in vitro and in vivo

(A-B) Representative images and quantification of calponin stained adventitial Gli1$^+$ cells cultured with Sphingosylphosphorylcholine (2μM in ethanol) versus vehicle (ethanol). Scale bars 50μm, hpf-high power fields 400x

(C) Quantification of calponin positive tdTomato$^+$ cells at 4 weeks following wire injury to the femoral artery (Figure 2 E). ***p<0.001 by t-test

(D-E) To elucidate the relative contribution of Gli1$^+$ cells to newly formed vSMCs we performed an wire injury experiment on bigenic Gli1CreER;tdTomato mice (n=6 males, 8 week old) with daily injections of 5-ethynyl-2-deoxyuridine (EdU) to label all proliferating cells.

Represenative images of contralateral non-injured femoral artery (control) and injured femoral arteries stained for alpha smooth muscle actin (α-SMA) and EdU.
See also Figure 2

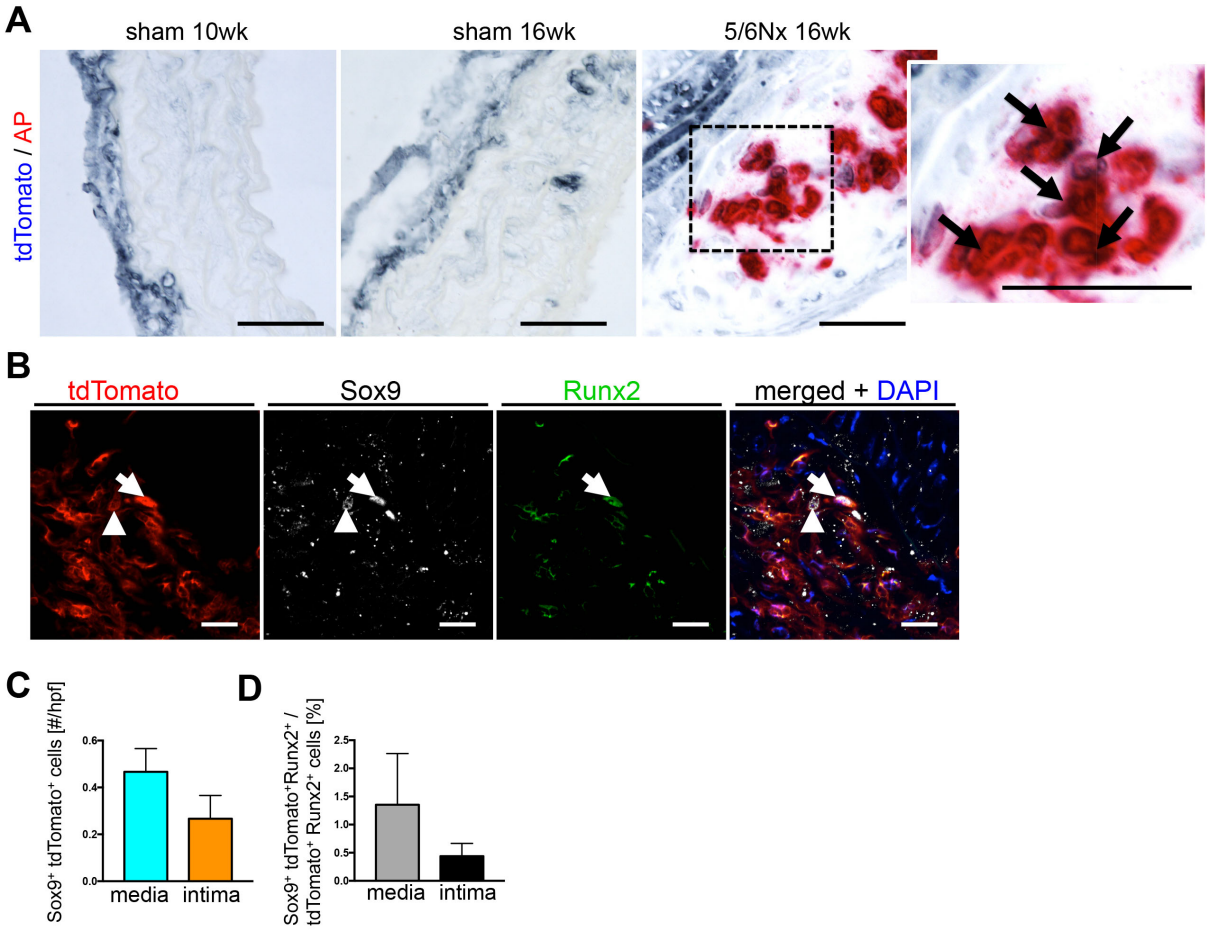

Figure S3: Gli1^{+} cells become ALP expressing osteoblast like cells during vascular calcification, while chondrogenic differentiation is a rare event.
(A) Representative images of aortic arches from Gli1CreER^{t2};tdTomato;ApoEKO mice immunostained for tdTomato (blue) and costained for alkaline phosphatase activity (red). Scale bars 50µm
(B-D) Representative images and quantification of tdTomato^{+} cells coexpressing Runx2 and Sox9 in aortic arches of Gli1CreERt2;tdTomato;ApoEKO mice at 16 weeks after subtotal nephrectomy. Scale bars 25µm
See also Figure 5

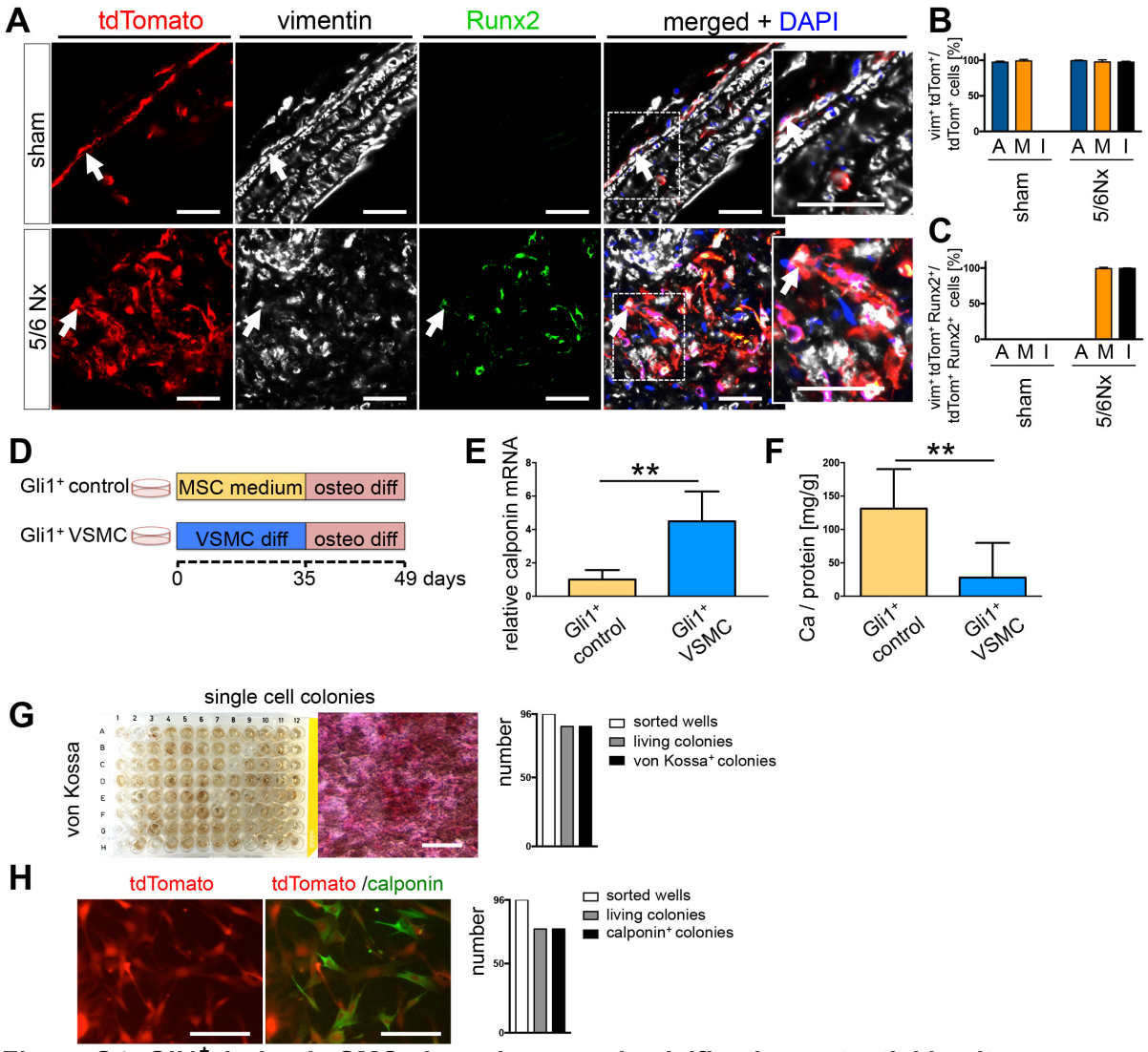

Figure S4: Gli1$^+$ derived vSMC show decreased calcification potential in vitro

(A-C) Representative images and quantification of tdTomato$^+$ cells co-stained for Runx2 and vimentin at 16 weeks. (arrows, tdTomato$^+$ cells expressing vimentin, scale bars 25µm)

(D) To elucidate whether Gli1$^+$ progenitors and Gli1$^+$cell derived vSMC differ in their calcification potential adventitial Gli1$^+$ cells were differentiated towards the vSMC lineage or kept in standard medium for 35 days followed by osteogenic differentiation for 14 days.

(E-F) Relative mRNA expression of SM-calponin and calcium quantification in the cell pellets. **p<0.01 by t-test

(G-H) Representative images and quantification of Gli1$^+$ single cell colonies differentiated towards osteoblasts or vSMC.

See also Figure 6

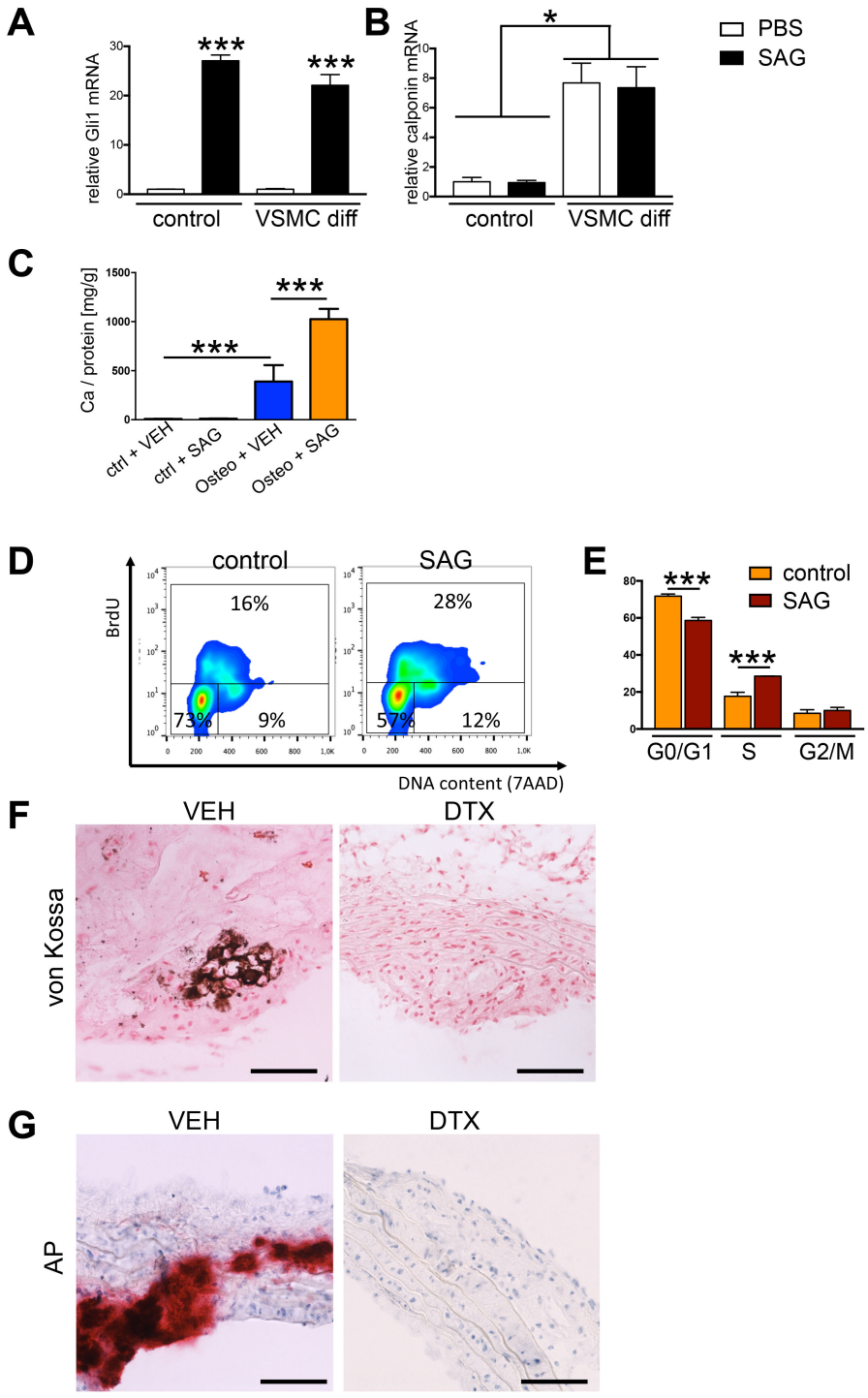

Figure S5: Smoothened activation increases proliferation and enhances calcification of Gli1$^+$ cells

(A-B) Relative mRNA expression of Gli1 and calponin from Gli1$^+$ cells differentiated towards the vSMC lineage versus control-medium (control) with addition of smoothened agonist (SAG) or vehicle (PBS) .*p<0.5, ***p<0.001 by one way ANOVA with posthoc Tukey.

(C) Calcium quantification of Gli1$^+$ cells cultured in osteogenic differentiation medium with addition of smoothened agonist (SAG) or phosphate buffered saline (VEH).

(D-E) Representative flow cytometric cell-cycle plots and quantification of Gli1$^+$ cells after treatment with smoothened agonist (SAG) versus vehicle (control). ***p<0.001 by t-test

(F-G) Representative images of von Kossa staining and staining for alkaline phosphatase (AP) activity in the aortic wall of Gli1CreERt2;iDTR;ApoEKO mice where Gli1 cells were ablated (DTX) and the control group (VEH). See also Figure 7

Methods

Animals

All mouse experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Harvard University and Washington University. Gli1CreER^{t2} (i.e. Gli1$^{tm3(re/ERT2)Alj/}$J, JAX Stock #007913), Rosa26tdTomato (i.e. B6-Cg-Gt(ROSA)26Sort$^{tm(CAG-tdTomato)Hze}$/J JAX Stock # 007909) iDTR mice (i.e. C57BL/6-Gt(ROSA)26Sor$^{tm1(HBEGF)Awai}$/J, JAX Stock # 007900) and ApoEKO mice (ApoEtm1Unc, JAX Stock # 002052) were purchased from Jackson Laboratories (Bar Harbor, ME). Offspring were genotyped by PCR according to the protocol from the Jackson laboratory. For lineage tracing studies 6-7 week old mice received 3x 10 mg tamoxifen in corn oil / 3% ethanol (Sigma) via oral gavage 10days before surgery. Subtotal nephrectomy surgery (5/6Nx) was performed in a two step method as described previously (Aikawa et al., 2009) with subtotal nephrectomy of the left kidney followed by right uninephrectomy one week later.

Mice received a high fat diet (21% fat and 0.21% cholesterol) that was obtained from Research Diets (D12079B, New Brunswick, NJ). In the sham group kidneys were exposed by flank incision. Sham mice received standard mouse chow. Transluminal arterial injury was induced surgically as previously described, (Sata et al., 2000). Briefly, a straight spring wire (0.38mm, C-SF-15-15, Cook) was inserted into the femoral artery under microscopic observation. The wire was moved 10 times in and out of the femoral artery. Mice were sacrificed at day 28 after surgery. For all surgical procedures mice were anesthetized with 100 mg/kg bodyweight ketamine and 10mg/kg bodyweight xylazine and 3mg/kg acepromazine (intraperitoneally) and received buprenorphine (0.1mg/kg bodyweight subcutaneously) to achieve analgesia. BUN was measured using the Infinity Urea assay (Thermo Fisher Scientific) according to the manufacturer instructions. To assess proliferation of Gli1^{+} cells following wire injury mice were injected daily with 5-ethynyl-2-deoxyuridine (EdU) (SantaCruz Biotech) at 100mg/kg bodyweight in PBS subcutaneously.

Macroscopic Fluorescence Reflection Imaging

Calcification was monitored by ex vivo molecular imaging as described previously (Aikawa et al., 2007). A bisphosphonate-derivatized near-infrared fluorescent imaging agent (Osteosense 680 EX, PerkinElmer, Boston, MA, 2nmol/100µl PBS / 25g) was intravenously injected via tail vein into the mice 24 hours before imaging. After mice were euthanized, aorta was perfused with saline, dissected and imaged to map the macroscopic NIR fluorescent signals elaborated by Osteosense 680nm (excitation/emission: 668/687) and tdTomato (excitation/emission 554/581) using fluorescent reflection imaging (Image Station 4000MM, Eastman Kodak Co., New Haven, CT). Quantification was performed using MathLab. A pixel intensity above 4500 was considered as positive signal. Data are given as Osteosense positive area related to total area.

Cell specific ablation experiments

For all ablation experiments, bigenic Gli1CreER^{t2}; iDTR mice received tamoxifen (3x 10mg per oral gavage) and were injected with diphtheria toxin dissolved in PBS (List Biological Laboratories) at a dose of 50ng/g bodyweight intraperitoneally 10 days after the last tamoxifen dose as indicated (3 injections every other day). Subtotal nephrectomy of the left kidney was performed 3 days after the last DTX dose and right uninephrectomy was performed one week later. Mice were sacrificed 16 weeks after the last surgery. All mice received a high fat diet (Research Diets #D12079B, New Brunswick) starting at the subtotal nephrectomy surgery.

Tissue Preparation and Histology

Mice were anesthetized with isoflurane (Baxter) and subsequently perfused via the left ventricle with 4°C PBS for 1 minute.

The aortic arch was carefully dissected under 30x magnification. For immunofluorescence and histology studies, kidneys were fixed in 4% paraformaldehyde on ice for 1 hour, then incubated in 30% sucrose in PBS at 4°C overnight. OCT-embedded (Sakura Finetek) tissues were cryosectioned into 7 µm sections and mounted on Superfrost slides (Fisher Scientific). Sections were washed in 1X PBS, blocked in 10% normal goat serum (Vector Labs) and incubated with primary antibodies specific for alpha SMA (1:200, Sigma, Cat No. A2547), alpha SMA-FITC (1:200, Sigma, # F3777), CD31 (1:100, ebioscience, #14-0311), calponin (1:200, milipore, #04-589), PDGFRß (1:200, ebioscience, #14-1402), Sca1 (1:200, abcam, #ab51317) CD34 (1:200, Abcam, #ab116043) Runx2 (1:100, Abcam, ab114133), CD68 (1:100, AbD Serotec, #CA1957A488), Shh (1:500, abcam #ab53281), Gli1 (1:100, Novus biological, NB-600-600), Tpm4 (1:500 millipore,#AB5449) nmMHCIIb (1:200, biolegend #909901) vimentin (1:500, Abcam #ab92547), UCP1 (1:100, abcam #ab10983, FABP4 (1:100, RnD Systems # AF1443), Sox9 (1:100, Chemicon, #AB5545). Secondary antibodies were FITC-, or Cy5-conjugated (Jackson ImmunoResearch). Sections were then stained with DAPI (4´,6´-´diamidino-2-phenylindole) and mounted in Prolong Gold (Life Technologies). Immunohistochemistry for tdTomato and costaining for alkaline phosphatase activity was performed using the Red Alkaline Phosphatase Substrate kit (Vector Labs) and the primary antibody against DsRed (1:50, Clontech #632496), and a biotinylated secondary antibody (Jackson Immuno). Antigen retrieval was achieved by pressure cooker treatment and antigen unmasking solution (Vector). Staining was achieved using Avidin/Biotin Blocking kit, the ABC kit and the TMB Peroxidase (HRP) Substrate Kit (all Vector laboratories) according to the manufacturer instructions. Cell numbers and co-staining was evaluated in 400x magnification at the confocal microcope (Nikon C1 eclipse, Nikon, Melville, NY) by an experienced investigator using X, Y and Z axes of 6 random high power fields (400x) of the aortic arch.

For histological analyses tissues were stained according to routine histology protocols. Plaque size of the aortic arch lesser curvature was determined in two hematoxylin eosin stained sections per animal in a blinded manner using the Eclispe 80i microscope with with Elements 3.20 software (Nikon).

Fluorescence activated cell sorting

For flow cytometric analysis or fluorescence activated cell sorting (FACS) the aortic arch including carotid and subclavian arteries was dissected from bigenic Gli1CreERt2;tdTomato mice 10 days after injection of tamoxifen (3x10mg p.o.) at 30x magnification. The arches were placed in FACS Buffer (PBS, 10%FBS, 2% Penicillin Streptomycin, Life Technologies). After thoroughly mincing the tissue using a sterile scalpel (Feather), the tissue was placed in gentleMACS C Tubes (Miltenyi Biotec) containing 1.5ml DMEM (Life Technologies) with 0.1mg/ml Liberase TL (Roche). The tissue was then dissociated using the D program of the gentleMacs dissociator (Miltenyi Biotec) followed by 30 min incubation at 37°C. Following washing steps with FACS buffer and centrifugation (1500 rpm 5 min) the solution was filtered twice trough a 40µm cell strainer (BD Biosciences) and transferred to 5ml Polystyrene Round-Bottom FACS tubes (BD Biosciences). FACS sorting was performed using the FACSAria II cell sorter (BD Biosciences). Data were analyzed by using Flow Jo software (Version 9.6.2, Tree Star Inc).

Cell Culture Experiments

$Gli1^+$ cells were grown in alpha MEM (GlutaMAX, Life Technologies) containing 20% MSC qualified FBS (Life Technologies), 2% Penicillin Streptomycin (Life Technologies), 1ng/ml murine basic fibroblast growth factor (Thermo Fisher Scientific) and 5ng/ml murine epidermal growth factor (Peprotech). For osteogenic or adipogenic differentiation flow-purified cells were plated in a 48 well, at a 60-70% or 90-100% confluence, the alpha MEM medium was exchanged with osteogenic or adipogenic differentiation medium, respectively

(R&D Systems). After 21 days of cultivation, cells were stained according to routine protocols using Oilred O (Sigma). To achieve co-staining for alkaline phosphatase and von Kossa staining, cells were washed once with PBS, fixed with 10% neutral formalin buffer (VWR) for 10min, incubated for 15 min with distilled water followed by an incubation in an Naphthol-AS MX-PO4 (Sigma), Dimethylformamide (Fisher), Tris-HCL buffer with Red Violet LB salt (Sigma) for 45 min. After washing with distilled water cells were stained with 2.5% silver nitrate (Sigma) for 30 minutes. For chondrogenic differentiation 2-10x10^4 cells were resuspended in 1ml chondrogenic differentiation medium (RnD Systems) in a 15ml conical tube (BD Biosciences) and centrifuged at 1500rpm for 5 min. The cell-pellets were cultivated upright in the 15 ml conical for 21 days. For detection of chondrogenic differentiation the cell-pellet was fixed in 4% paraformaldehyde. OCT-embedded (Sakura Finetek) cell-pellets were cryosectioned into 4 µm sections and mounted on Superfrost slides. After PBS washing the sections were stained with Alcian blue (Santa Cruz Biotechnology) and counterstained with nuclear fast red (Sigma).

For smooth muscle cell differentiation cells were cultivated in alpha MEM (GlutaMAX, Life Technologies) containing 20% MSC qualified FBS (Life Technologies), 2% Penicillin Streptomycin (Life Technologies), 10ng/ml transforming growth factor beta (Peprotech) and 5ng/ml platelet derived growth factor BB (Peprotech). The control cells were cultivated in the standard medium as mentioned above.

For single cell differentiation experiments Gli1$^+$ cells were sorted from dissected adventita as described above. Living tdTomato$^+$ clones were identified by fluorescence microscopy. Cells were differentiated towards osteoblasts (21 days) and vSMC lineage (35 days) as described above. To asses whether Sphingosylphosphorylcholine (SPC) induces vSMC differentiation of Gli1$^+$ cells were treated with 2µM D-erythro-SPC (Biotrend) in ethanol. Controls were treated with the same concentration of DMSO.

To test the effect of smoothened activation on Gli1 cells osteogenic or vSMC differentiation cells were treated with smoothened agonist (SantaCruz Biotech) at 1µM in PBS versus vehicle (PBS). Medium was changed daily. In order to test the effect of tamoxifen on Gli1 cell differentiation, adventitial Gli1$^+$ cells were cultured in regular MSC medium (control) or adipogenic differentiation medium with addition of 4- hydroxytamoxifen (Sigma) in ethanol at 5µM versus ethanol alone.

For cell-cycle analysis, cells were grown in 6-well plates until 50%confluency, serum starved in 0.5% FBS for 4 hours to synchronize the cell cycle, and then cultured for 48 hours in 10% FBS medium, together with 1µM SAG or PBS (vehicle). Before fixation,cells were incubated for 75 minutes in 10 µM BrdU and stained according to the BD APC BrdU Flow Kit protocol (catalog 552598; BD Biosciences).Cells were analyzed by flow cytometry (FACSCanto II; BDBiosciences) within 1 hour of staining, and data were analyzed using FlowJo software, version 7.5

Calcium Determination

Calcium was extracted from cells overnight with HCL (0.6mol/L) and quantified with the use of cresolphthalein complexone chemistry for a colorimetric assay at 578nm (Randox Laboratories). The calcium content was normalized to the protein content to allow for comparison. The protein content was measured colorimetrically at 562nm (BC-Assay, Uptima).

Human arteries

We examined femoral arteries obtained from consented clinical autopsies of 12 dialysis patients (8 men, age 68 ± 15 years, dialysis vintage 6 ±2 years) and 10 age-matched non-CKD patients (6men, age 66 ± 10 years) between April 2009 and April 2011 at the Department of Pathology of the RWTH Aachen University. The study was approved by the ethical committee of the RWTH Aachen University (ethical votes EK 180/14 and EK239/11) and carried out according to the declaration of Helsinki.

Real Time PCR Experiments

Tissue was harvested and immediately snap frozen in liquid nitrogen. RNA from was extracted according to the manufacturer instructions using the RNeasy Mini Kit (Qiagen) and 600 ng of total RNA was reverse transcribed with iScript (BioRad). During the RNA extraction DNA was removed by a DNAse digestion step (Life Technologies). Quantitative polymerase chain reactions were carried out with iQ-SYBR Green supermix (BioRad) and the BioRad CFX96 Real Time System with the C1000 Touch Thermal Cycler. Cycling conditions were 95°C for 3 minutes then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by one cycle of 95°C for 10 seconds. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Data was analyzed using the $2^{-\Delta\Delta ct}$ method.

Primer pairs used for PCR

Gene	Sequence
GAPDH	Fw 5´-AGGTCGGTGTGAACGGATTTG -3´
	Rv 5`-TGTAGACCATGTAGTTGAGGTCA -3´
iDTR (HB-EGF)	Fw 5´-GGAGCACGGGAAAAGAAAG-3´
	Rv5´-GAGCCCGGAGCTCCTTCACA-3
Runx2	Fw5´- AGACTGCAAGAAGGCTCTGG -3´
	Rv5´- CACTGACTCGGTTGGTCTCG -3´
Gli1	Fw 5´- ATCACCTGTTGGGGATGCTGGAT-3´
	Rv 5`- CGTGAATAGGACTTCCGACAG -3´
Calponin	Fw5´- GCAGTGGACACACGCATTTT -3´
	Rv5´- AACAACTGGCCCCAAGACTC -3´
UCP1	Fw5´- AGTACCCAAGCGTACCAAGC -3´
	Rv5´- CCCTTTGAAAAAGGCCGTCG -3´
FABP4	Fw5´- CGATGAAATCACCGCAGACG-3´
	Rv5´- CCAGCTTGTCACCATCTCGT -3´

Single cell realtime PCR Experiments

Bigenic Gli1CreER;tdTomato mice were injected with tamoxifen (3x10mg) at 8 weeks of age and sacrificed 10 days after the last tamoxifen dose. The adventitia was carefully dissected and prepared for FACS as mentioned above. Single cell sort and reverse transcription-specific target amplification (RT-STA) was performed as described in the Real-Time PCR protocol from Fluidigm. Briefly, $Gli1^{+}$ single cells were sorted into two 96 well qPCR plate (Biorad) containing 5.1µl of 2x Cells Cirect reaction mix (CellsDirect One-Step qRT-PCR Kit, Invitrogen, #11753-100) with Superase-In (Ambion, PN AM2694; for one 96 well plate 580µl of the 2x Cells Cirect reaction mix was mixed with 11.6µl SUPERase-In). Plates were sealed with adhesive film (Biorad) before and immediately after the sort. In a DNA-free hood the following TaqMan Gene Expression Assays were pooled and diluted with DNA Supsension buffer (TEKnova, #PNT0221) for reverse transcription-specific target amplification (RT-STA): CD140a (assay ID: Mm00440701_m1), CD140b (Mm00440701_m1), CD44 (Mm01277161_m1), CD73 (Mm00501910_m1), NG2 (Mm00507257_m1), CD29 (Mm01253230_m1), Tie2 (Mm00443243_m1), CD90 (Mm00493681_m1), Sox2 (Mm03053810_s1), Nestin (Mm00450205_m1), Sox1 (Mm00486299_s1), Sox10 (Mm00569909_m1), CD105 (Mm00468252_m1), Sca1 (Mm00726565_s1), CD14 (Mm00438094_g1), smoothelin (Mm00449973_m1), calponin (Mm00487032_m1), SM22a

(Mm00487032_m1), nmMHCIIb (Mm00805131_m1), TPM4 (Mm01245298_m1), vimentin (Mm01333430_m1), c-kit (Mm00445212_m1), CD34 (Mm00519283_m1) , VGFR2 (Mm01222421_m1), MSX1 (Mm00440330_m1), MSX2 (Mm00442992_m1), GAPDH (Mm99999915_g1), Gli1 (Mm00494654_m1), Ptc1 (Mm00436026_m1), cyclin d2 (Mm00438070_m1), N-myc (Mm00476449_m1), IGF2 (Mm00439564_m1), and GILZ (Mm00726417_s1) (all Thermofisher). Super Script Taq Mix from the CellsDirect One-Step qRT-PCR Kit, (Invitrogen, #11753-100) was added and RT-STA was performed on a 96 well thermocycler (Biorad) according to the fluidigm protocol mentioned above. The cDNA was diluted 1:5 in DNA Suspension buffer. Quantitative Taqman gene expression for GAPDH was carried out with the two worted 96 well PCR plates and cDNA from 96 clones with high GAPHD expression was selected for single cell qPCR analysis. Single cell qPCR was performed using a 96.96 Dynamic Array IFC (Fluidigm) on the Biomark system (Fluidigm) by the Gene Technology Access Center (GTAC) of the Washington University (St Louis, MO).
Unsupervised hierarchical clustering was performed in the open source R package Fluidigm® SINGuLAR™ analysis toolkit version 3.5.2 freely available athttps://www.fluidigm.com/software . Data from Fluidigm BioMark System were imported into the SINGuLAR package and expression values for the replicated genes were averaged.Missing data is converted to a Log2Ex value of -1. Missing data is given a value of the mean expression for the gene of that sample group if sample group information is provided. Thus, all values whose Log2Ex is negative will be converted to the average value of that gene. Low quality data was discarded by manually providing a limit of detection (LOD) value of 35. Co-profiled genes are clustered together using the Pearson method, and samples are clustered together using the Euclidean method. In each case, the complete linkage method is then used to find similar clusters.

Western Blot

Tissue was snap frozen in liquid nitrogen immediately after mice were euthanized and stored at -80°C. Kidney tissue samples were homogenized in lysis buffer containing 10mM HEPES, pH 7.4, 0.32M sucrose, 2mM EDTA, 1mM DTT, 1mM PMSF and 1 protease inhibitor tablet per 10ml of lysis buffer (Roche Cat. No. 11836153001). Protein from $Gli1^{+}$ cultured cells was isolated after PBS washing using a cell scratcher with the same buffer. The samples were sonicated and protein concentration was determined by the Bradford Assay using Bio-Rad Protein Assay Dye (Biorad). 10-20µg of protein from lysates was loaded on a 10% polyacrylamide gel and separated by SDS electrophoresis. Proteins were transferred to a Immobilion membrane (Millipore) blocked in 5% milk in PBST, probed overnight at 4°C with the primary antibodies: mouse anti-αSMA at 1:4000 (Sigma #A2547), rabbit anti calponin (Milipore, #04-589), rabbit anti smoothelin (Santa Cruz #sc28562) and rabbit anti-GAPDH at 1:4000 (Bethyl Laboratories, #A300-641A). Following incubation with primary antibody blots were washed probed with respective horseradish-peroxidase conjugated secondary antibodies at 1:4000 (Dako) for 1 hour at room temperature and then visualized using the Western Lightning ECL kit from PerkinElmer (#NEL100001EA).

Statistical Analysis

Data are presented as mean±SEM . Comparison of two groups was performed using unpaired t-test. Paired t-test was used for comparsion of repeated measured in the same group. For multiple group comparison analysis of variance with posthoc Tukey correction was applied. Statistical analyses were performed using GraphPad Prism 5.0c (GraphPad Software Inc., San Diego, CA). A p value of less than 0.05 was considered significant.

Single cell qPCR standard output

Standard output of single cell qPCR, related to figure 6

Single cell qCPR heatmap output

Heatmap output of single cell qPCR for analysis with the singulair software package from Fluidigm, related to figure 6

Supplementary References

Aikawa, E., Aikawa, M., Libby, P., Figueiredo, J.L., Rusanescu, G., Iwamoto, Y., Fukuda, D., Kohler, R.H., Shi, G.P., Jaffer, F.A.*, et al.* (2009). Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease. Circulation *119*, 1785-1794.

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