SGK1 induces vascular smooth muscle cell calcification through NF-kB signaling

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Supplemental Material

Supplemental methods

Quantitative RT-PCR

The following human primers were used (Thermo Fisher Scientific, 5'→3' orientation):

ALPL fw: GGGACTGGTACTCAGACAACG;

ALPL rev: GTAGGCGATGTCCTTACAGCC;

ANKH fw: CCATGCTGGCATTCTCTTAAAAC;

ANKH rev: CAAGGAGAGGATCGGGATGAG;

CBFA1 fw: GCCTTCCACTCTCAGTAAGAAGA;

CBFA1 rev: GCCTGGGGTCTGAAAAAGGG;

CHUK fw: GGAGAAGTTCGGTTTAGTAGCC;

CHUK rev: TGAGGGTCCCAATTCAACATCA;

IKBKB fw: GTCTTTGCACATCATTCGTGGG;

IKBKB rev: GTGCCGAAGCTCCAGTAGTC;

GAPDH fw: GAGTCAACGGATTTGGTCGT;

GAPDH rev: GACAAGCTTCCCGTTCTCAG;

MSX2 fw: TGCAGAGCGTGCAGAGTTC;

MSX2 rev: GGCAGCATAGGTTTTGCAGC;

SGK1 fw: GCAGAAGAAGTGTTCTATGCAGT;

SGK1 rev: CCGCTCCGACATAATATGCTT;

ZFP36 fw: GACTGAGCTATGTCGGACCTT;

ZFP36 rev: GAGTTCCGTCTTGTATTTGGGG;

WNT3A fw: AGCTACCCGATCTGGTGGTC;

WNT3A rev: CAAACTCGATGTCCTCGCTAC;

WNT7A fw: CTGTGGCTGCGACAAAGAGAA;

WNT7A rev: GCCGTGGCACTTACATTCC.

The following mouse primers were used (Thermo Fisher Scientific, 5’→3’ orientation):

Alpl fw: TTGTGCCAGAGAAAGAGAGAGA;

Alpl rev: GTTTCAGGGCATTTTTCAAGGT;

Ankh fw: CCCTGATAGCCTACAGTGACT;

Ankh rev: CCCTTCGTGTTTTGCTCCC;

Cbfa1 fw: AGAGTCAGATTACAGATCCCAGG;

Cbfa1 rev: AGGAGGGGTAAGACTGGTCATA;

Col1a1 fw: ACCCGAGGTATGCTTGATCTG;

Col1a1 rev: CATTGCACGTCATCGCACAC;

Fn1 fw: GTGACACTTATGAGCGCCCTA;

Fn1 rev: CCACTTGTCGCCAATCTTGTA;

Gapdh fw: AGGTCGGTGTGAACGGATTTG;

Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;

Msx2 fw: TTCACCACATCCCAGCTTCTA;

Msx2 rev: TTGCAGTCTTTTCGCCTTAGC;

Nppa fw: GCTTCCAGGCCATATTGGAG;

Nppa rev: GGGGGCATGACCTCATCTT;

Nppb fw: GAGGTCACTCCTATCCTCTGG;

Nppb rev: GCCATTTCCTCCGACTTTTCTC;

Sgk1 fw: CTGCTCGAAGCACCCTTACC;

Sgk1 rev: TCCTGAGGATGGGACATTTTCA;

Tagln fw: CCAACAAGGGTCCATCCTACG;

Tagln rev: ATCTGGGCGGCCTACATCA;

Zfp36 fw: CCACCTCCTCTCGATACAAGA;

Zfp36 rev: GCTTGGCGAAGTTCACCCA;

Wnt3a fw: AATTTGGAGGAATGGTCTCTCGG;

Wnt3a rev: CAGCAGGTCTTCACTTCACAG;

Wnt7a fw: GGCTTCTCTTCGGTGGTAGC;

Wnt7a rev: TGAAACTGACACTCGTCCAGG.

Supplemental Tables:

Suppl. Table 1. Characteristics of human vascular sections. Arithmetic means ± SEM of age and creatinine levels as well as gender in control patients (CTR) or dialysis patients with calcifications (Dialysis). *($p<0.05$) statistically significant vs. control group (unpaired two-tailed t-test for creatinine).

	CTR	Dialysis
Age [years]	58.0 ± 4.0	62.6 ± 3.7
Female [n]	2	1
Male [n]	3	4
Creatinine [mg/dl]	1.36 ± 0.19	6.75 ± 1.22*

Suppl. Table 2. Effect of Sgk1 deficiency during vitamin D_3 overload. Arithmetic means ± SEM of plasma calcium, phosphorus, FGF23 C-term, aldosterone and cortisol concentrations as well as heart rate during PPV measurements in Sgk1-deficient mice ($sgk1^{-/-}$) or corresponding wild-type mice ($sgk1^{+/+}$) receiving vehicle or high-dosed cholecalciferol (vD). *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant vs control $sgk1^{+/+}$ mice (One-way ANOVA with Tukey-HSD post-hoc test for calcium, aldosterone or with Games-Howell post-hoc test for FGF23 C-term and Steel-Dwass method for phosphorus, cortisol).

	$sgk1^{+/+}$	$sgk1^{-/-}$	$sgk1^{+/+}$ vD	$sgk1^{-/-}$ vD	
Calcium [mg/dl]	10.0 ± 0.4	9.8 ± 0.4	20.4 ± 1.6***	19.3 ± 1.4***	n=5-7
Phosphorus [mg/dl]	7.6 ± 0.3	7.4 ± 0.7	8.3 ± 0.4	9.1 ± 0.6	n=5-7
FGF23 C-term [pg/ml]	723 ± 61	733 ± 66	136646 ± 18299**	118415 ± 13521***	n=5-7
Aldosterone [pg/ml]	211.1 ± 22.6	249.1 ± 30.3	423.5 ± 24.7**	418.7 ± 42.7**	n=5-7
Cortisol [µg/dl]	5.12 ± 1.49	3.27 ± 0.93	33.33 ± 3.50*	24.47 ± 2.33*	n=5-7
Heart rate [BPM]	405.1 ± 13.2	384.0 ± 9.3	380.8 ± 7.3	398.6 ± 11.7	n=6-9

Suppl. Table 3. Effect of Sgk1 inhibitor EMD638683 during vitamin D_3 overload. Arithmetic means ± SEM of plasma calcium, phosphorus, FGF23 C-term, aldosterone and cortisol concentrations as well as heart rate during PPV measurements in mice receiving vehicle or high-dosed cholecalciferol (vD) without or with additional treatment with SGK1 inhibitor EMD638683 (EMD). *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant vs control mice (One-way ANOVA with Tukey-HSD post-hoc test for calcium, phosphorus, FGF23 C-term, aldosterone or with Games-Howell post-hoc test for cortisol).

	CTR	vD	vD + EMD	
Calcium [mg/dl]	10.4 ± 0.4	17.9 ± 0.8***	16.2 ± 0.6***	n=7
Phosphorus [mg/dl]	6.9 ± 0.3	7.1 ± 0.4	7.3 ± 0.2	n=7
FGF23 C-term [pg/ml]	612 ± 26	113857 ± 10582***	110206 ± 17745***	n=7
Aldosterone [pg/ml]	487.3 ± 47.6	797.7 ± 79.7	801.5 ± 134.7	n=7
Cortisol [µg/dl]	7.17 ± 0.74	17.42 ± 1.51***	19.29 ± 4.03	n=7
Heart rate [BPM]	385.5 ± 12.12	379.5 ± 14.4	382.4 ± 14.1	n=10

Suppl. Table 4. Effect of Sgk1 deficiency in apoE-null mice after subtotal nephrectomy. Arithmetic means ± SEM of plasma phosphorus, total cholesterol, FGF23 C-term, aldosterone, cortisol and BUN concentrations after subtotal nephrectomy procedure (1W) and at the end of the study period (final) in Sgk1-deficient mice ($apoE^{-/-}sgk1^{-/-}$) or corresponding wild-type mice ($apoE^{-/-}sgk1^{+/+}$) under the $apoE^{-/-}$ background without or with subtotal nephrectomy (Nx). *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant vs control $apoE^{-/-}sgk1^{+/+}$ mice; †($p<0.05$) statistically significant vs respective $apoE^{-/-}sgk1^{+/+}$ mice (One-way ANOVA with Games-Howell post-hoc test for phosphorus, BUN final or with Tukey-HSD post-hoc test for cholesterol, FGF23 C-term, BUN 1W and Steel-Dwass method for aldosterone, cortisol).

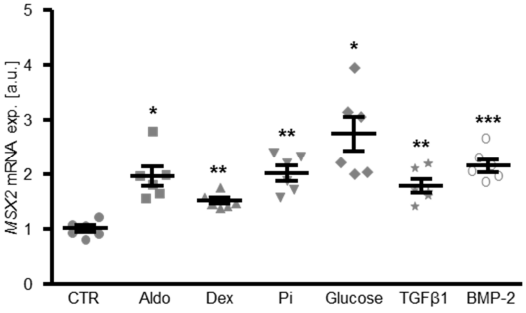
	$apoE^{-/-}$ $sgk1^{+/+}$	$apoE^{-/-}$ $sgk1^{-/-}$	$apoE^{-/-}$ $sgk1^{+/+}$ Nx	$apoE^{-/-}$ $sgk1^{-/-}$ Nx	
Phosphorus [mg/dl]	7.6 ± 0.9	7.0 ± 0.3	12.0 ± 1.2	9.9 ± 1.6	n=5-6
Cholesterol [mg/dl]	843 ± 198	548 ± 42	2425 ± 397**	1260 ± 295†	n=4-6
FGF23 C-term [pg/ml]	693 ± 69	518 ± 45	8782 ± 2563***	2871 ± 1313**	n=5-6
Aldosterone [pg/ml]	162.7 ± 12.0	167.1 ± 9.6	332.5 ± 22.6*	219.9 ± 21.9†	n=5-6
Cortisol [µg/dl]	0.67 ± 0.20	0.37 ± 0.14	31.90 ± 14.04*	1.96 ± 0.74†	n=5-6
BUN final [mg/dl]	25.9 ± 1.8	27.1 ± 2.9	75.6 ± 6.3**	52.9 ± 8.6	n=5-6
BUN start [mg/dl]	28.9 ± 2.2	23.8 ± 1.8	51.4 ± 2.6***	47.4 ± 2.4***	n=5-10

Suppl. Table 5. Characteristics of human serum. Arithmetic means ± SEM of age, creatinine levels and serum calcification propensity measured as calciprotein particle maturation time (T_{50}) in healthy volunteers (NS) or dialysis patients (US). Descriptive characteristics of gender, number of patients with known cardiovascular disease (CVD, defined as coronary heart disease, peripheral artery disease or stroke) and treatment with phosphate binder according to the patient medical history (from two control samples no creatinine values could be obtained). *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant vs. control group (unpaired two-tailed t-test for T_{50} and age, U-test for creatinine).

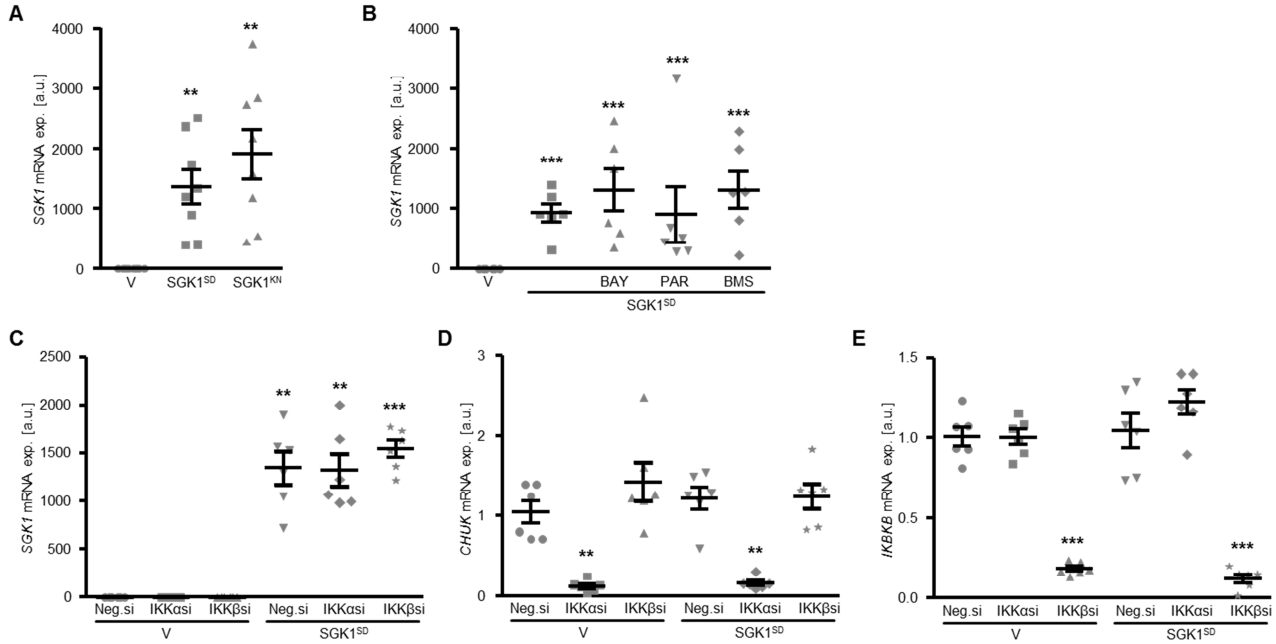
	NS	**US**
Age [years]	32.7 ± 2.7	67.6 ± 3.3 ***
Female [n]	2	2
Male [n]	5	5
Creatinine [mg/dl]	0.93 ± 0.11	7.04 ± 0.90 **
T_{50} [min]	283.6 ± 32.2	178.3 ± 29.0 *
Oral phosphate binder [n]	0	5
Known CVD [n]	0	6

Supplemental figures and figure legends:

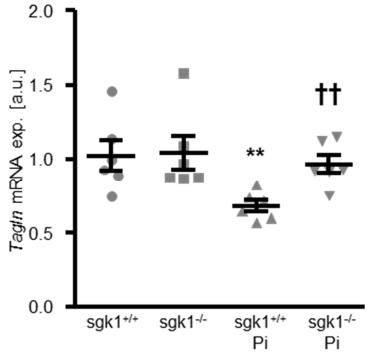
Suppl. Fig. 1. *MSX2* mRNA expression in primary human aortic smooth muscle cells. Scatter dot plots and arithmetic means ± SEM (n=6 per group; arbitrary units, a.u.) of *MSX2* relative mRNA expression in HAoSMCs following treatment with control or with aldosterone (Aldo), dexamethasone (Dex), β-glycerophosphate (Pi), glucose, human TGFβ1 protein or human BMP-2 protein. *(p<0.05), **(p<0.01), ***(p<0.001) statistically significant vs. control treated HAoSMCs (One-way ANOVA with Games-Howell post-hoc test).



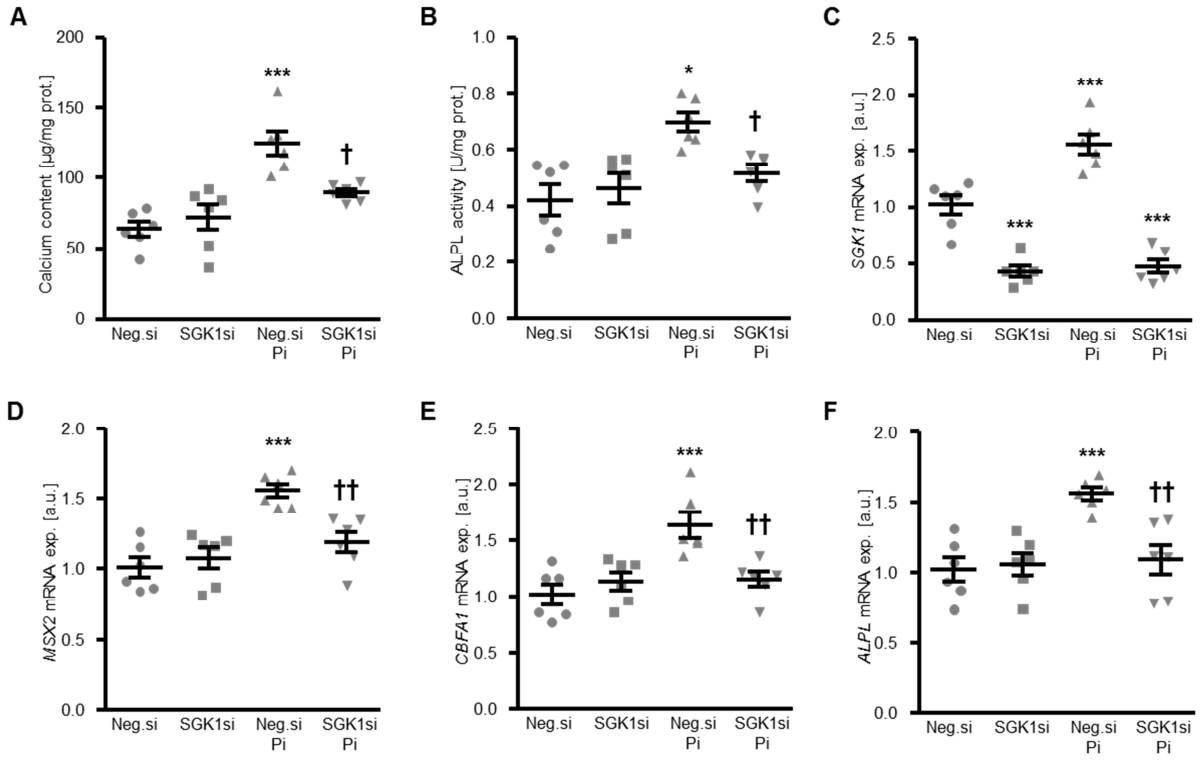
Suppl. Fig. 2. Transfection and silencing efficiency in primary human aortic smooth muscle cells. A. Scatter dot plots and arithmetic means ± SEM (n=8 per group; arbitrary units, a.u.) of *SGK1* relative mRNA expression in HAoSMCs following transfection with empty vector (V), constitutively active SGK1^{S422D} (SGK1^{SD}) or inactive SGK1^{K127N} (SGK1^{KN}). **B**. Scatter dot plots and arithmetic means ± SEM (n=6 per group; a.u.) of *SGK1* relative mRNA expression in HAoSMCs following transfection with empty vector (V) or constitutively active SGK1^{S422D} (SGK1^{SD}) and additional treatment with control, BAY11-7082 (BAY), parthenolide (PAR) or BMS-345541 (BMS). **C-E**. Scatter dot plots and arithmetic means ± SEM (n=6 per group; a.u.) of *SGK1* (**C**), *CHUK* (**D**) and *IKBKB* (**E**) relative mRNA expression in HAoSMCs following transfection with empty vector (V) or constitutively active SGK1^{S422D} (SGK1^{SD}) and additional silencing with negative control siRNA (Neg.si), IKKα siRNA (IKKαsi) or IKKβ siRNA (IKKβsi). *(p<0.05), **(p<0.01), ***(p<0.001) statistically significant vs. V transfected or V transfected and Neg.si silenced HAoSMCs, respectively (One-way ANOVA with Tukey-HSD post-hoc test for **B** or with Games-Howell post-hoc test for **C-E** and Steel-Dwass method for **A**).



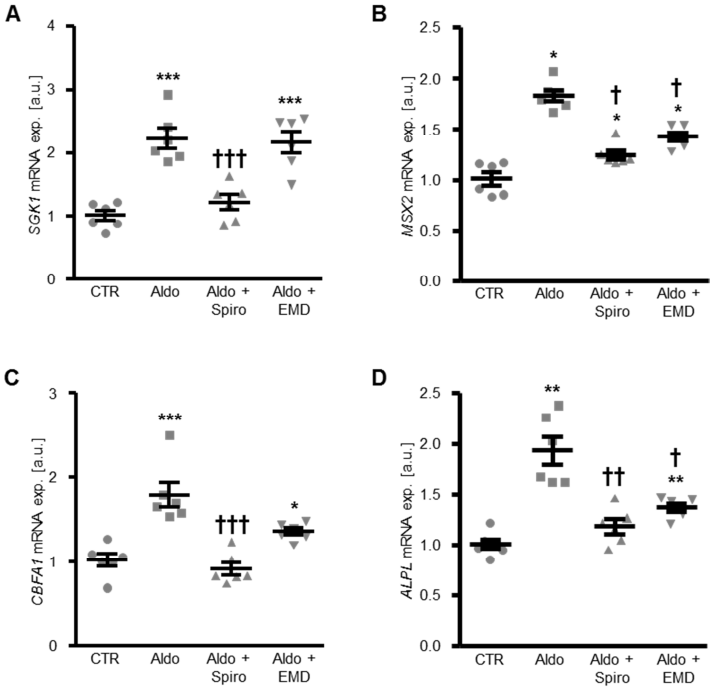
Suppl. Fig. 3. Effect of Sgk1 deficiency on *Tagln* mRNA expression in primary mouse aortic smooth muscle cells. Scatter dot plots and arithmetic means ± SEM (n=6 per group; arbitrary units; a.u.) of *Tagln* relative mRNA expression in MAoSMCs isolated from Sgk1-deficient mice ($sgk1^{-/-}$) or corresponding wild-type mice ($sgk1^{+/+}$) and treated with control or with phosphate (Pi). **($p<0.01$) statistically significant vs. control treated $sgk1^{+/+}$ MAoSMCs; ††($p<0.01$) statistically significant vs. Pi treated $sgk1^{+/+}$ MAoSMCs (One-way ANOVA with Tukey-HSD post-hoc test).



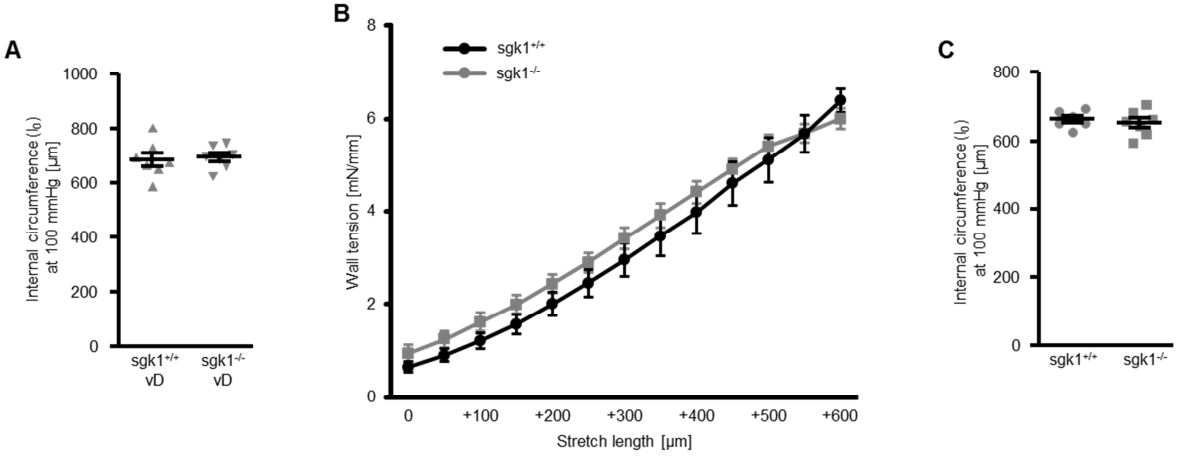
Suppl. Fig. 4. Effect of SGK1 silencing on phosphate-induced osteo-/chondrogenic transformation and calcification of primary human aortic smooth muscle cells. Scatter dot plots and arithmetic means ± SEM of calcium content (**A**, n=6 per group, µg/mg protein), alkaline phosphatase activity (**B**, n=6 per group, U/mg protein) and *SGK1* (**C**), *MSX2* (**D**), *CBFA1* (**E**) and *ALPL* (**F**) relative mRNA expression (n=6 per group; a.u.) in HAoSMCs following silencing with negative control siRNA (Neg.si) or SGK1 siRNA (SGK1si) and additional treatment with control or with phosphate (Pi). *($p<0.05$), ***($p<0.001$) statistically significant vs. Neg.si silenced HAoSMCs; †($p<0.05$), ††($p<0.01$) statistically significant vs. Neg.si silenced and Pi treated HAoSMCs (One-way ANOVA with Tukey-HSD post-hoc test for **A,C-F** and Steel-Dwass method for **B**).



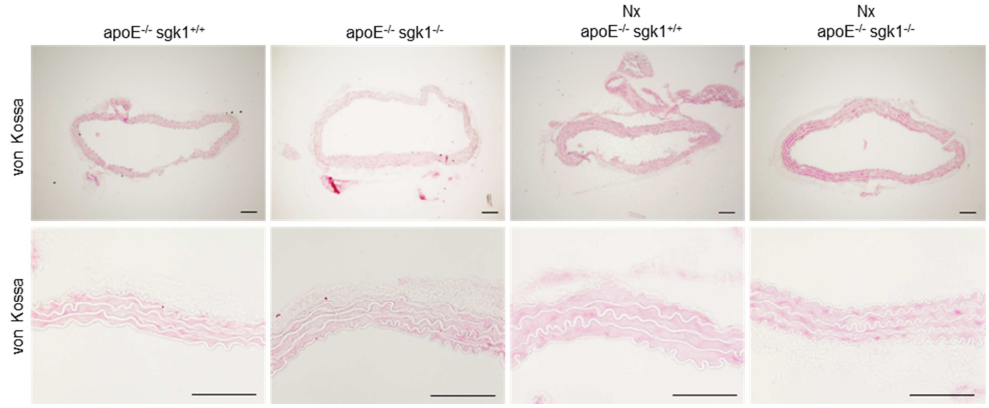
Suppl. Fig. 5. Effect of spironolactone and SGK1 inhibitor EMD638683 on aldosterone-induced osteo-/chondrogenic transdifferentiation of primary human aortic smooth muscle cells. Scatter dot plots and arithmetic means ± SEM (n=6 per group; arbitrary units, a.u.) of *SGK1* (**A**), *MSX2* (**B**), *CBFA1* (**C**) and *ALPL* (**D**) relative mRNA expression in HAoSMCs following treatment in charcoal stripped FBS media with aldosterone (Aldo) without or with additional treatment with spironolactone (Spiro) or SGK1 inhibitor EMD638683 (EMD). *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant vs. control treated HAoSMCs; †($p<0.05$), ††($p<0.01$), †††($p<0.001$) statistically significant vs. Aldo treated HAoSMCs (One-way ANOVA with Tukey-HSD post-hoc test for **A,C** or with Games-Howell post-hoc test for **D** and Steel-Dwass method for **B**).



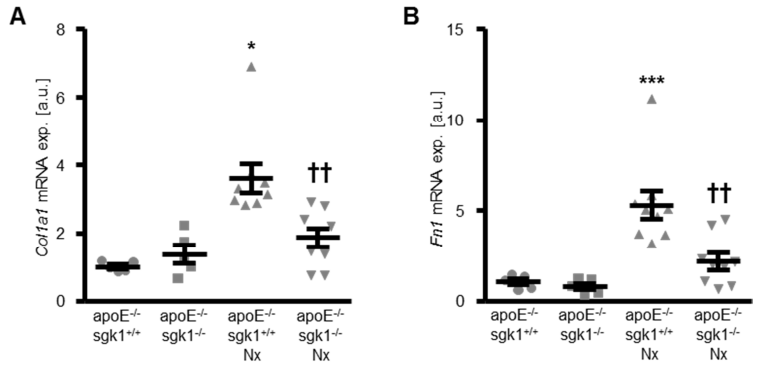
Suppl. Fig. 6. Resting length and wall tension of aortas from Sgk1-deficient mice ex-vivo. A. Resting lengths (n=7 rings, 4 mice per group; l_o) of abdominal aortas isolated from $sgk1^{+/+}$ mice or $sgk1^{-/-}$ mice receiving high-dosed cholecalciferol (vD). **B**. Summary data of wall tension (n=6-7 rings, 4 mice per group; mN/mm) during mechanical stretch (µm) of abdominal aortas isolated from $sgk1^{+/+}$ mice or $sgk1^{-/-}$ mice receiving control treatment. **C**. Resting lengths (n=6-7 rings, 4 mice per group; l_o) of abdominal aortas isolated from $sgk1^{+/+}$ mice or $sgk1^{-/-}$ mice receiving control treatment.



Suppl. Fig. 7. No visible ectopic calcifications in apoE-null mice after subtotal nephrectomy. Representative original images (n=4 per group) showing von Kossa staining (calcification – gray to black) of thoracic aorta sections from Sgk1-deficient mice (apoE$^{-/-}$sgk1$^{-/-}$) or corresponding wild-type mice (apoE$^{-/-}$sgk1$^{+/+}$) under the apoE$^{-/-}$ background without or with subtotal nephrectomy (Nx). Scale bar: 100 μm.



Suppl. Fig. 8. Sgk1 deficiency ameliorates aortic stiffness markers expression in apoE-null mice after subtotal nephrectomy. Scatter dot plots and arithmetic means ± SEM (n=5-6 per group; arbitrary units, a.u.) of *Col1a1* (**A**) and *Fn1* (**B**) relative mRNA expression in aortic tissue of Sgk1-deficient mice (apoE$^{-/-}$sgk1$^{-/-}$) or corresponding wild-type mice (apoE$^{-/-}$sgk1$^{+/+}$) under the apoE$^{-/-}$ background without or with subtotal nephrectomy (Nx). *(p<0.05), ***(p<0.001) statistically significant vs. control apoE$^{-/-}$sgk1$^{+/+}$ mice; ††(p<0.01) statistically significant vs. Nx treated apoE$^{-/-}$ sgk1$^{+/+}$ mice (Steel-Dwass method for **A** and One-way ANOVA with Tukey-HSD post-hoc test for **B**).



Suppl. Fig. 9. Sgk1 deficiency ameliorates cardiac hypertrophy markers expression in apoE-null mice after subtotal nephrectomy. Scatter dot plots and arithmetic means ± SEM (n=5-6 per group; arbitrary units, a.u.) of *Nppa* (**A**) and *Nppb* (**B**) relative mRNA expression in heart tissue of Sgk1-deficient mice (apoE$^{-/-}$sgk1$^{-/-}$) or corresponding wild-type mice (apoE$^{-/-}$sgk1$^{+/+}$) under the apoE$^{-/-}$ background without or with subtotal nephrectomy (Nx). *($p<0.05$), **($p<0.01$), ***($p<0.001$) statistically significant vs. control apoE$^{-/-}$sgk1$^{+/+}$ mice; †($p<0.05$), ††($p<0.01$) statistically significant vs. Nx treated apoE$^{-/-}$sgk1$^{+/+}$ mice (One-way ANOVA with Tukey-HSD post-hoc test for **A** or with Games-Howell post-hoc test for **B**).