Zinc inhibits phosphate-induced vascular calcification via TNFAIP3-mediated suppression of NF-kB

Jakob Voelkl[1], Rashad Tuffaha[2], Trang T. D. Luong[1], Daniel Zickler[3], Jaber Masyout[1], Martina Feger[2], Nicolas Verheyen[4], Florian Blaschke[1], Makoto Kuro-o[5], Andreas Tomaschitz[4,6], Stefan Pilz[7], Andreas Pasch[8], Kai-Uwe Eckardt[3], Juergen E. Scherberich[9], Florian Lang[2], Burkert Pieske[1,10,11], Ioana Alesutan[1,10]

[1]Department of Internal Medicine and Cardiology, Charité – Universitätsmedizin Berlin, Berlin, Germany; [2]Department of Physiology I, Eberhard-Karls University, Tübingen, Germany; [3]Department of Nephrology and Medical Intensive Care, Charité – Universitätsmedizin Berlin, Berlin, Germany; [4]Department of Cardiology, Medical University of Graz, Graz, Austria; [5]Center for Molecular Medicine, Jichi Medical University, Japan; [6] Division of Internal Medicine, Specialist Clinic of Rehabilitation Bad Gleichenberg, Bad Gleichenberg, Austria; [7]Department of Internal Medicine, Division of Endocrinology and Diabetology, Medical University of Graz, Graz, Austria; [8]Calciscon AG, Aarbergstrasse 5, 2560 Nidau-Biel, Switzerland; [9]Department of Nephrology and Clinical Immunology, Klinikum München-Harlaching, Teaching Hospital of the Ludwig-Maximilians-Universität, München, Germany; [10]Berlin Institute of Health (BIH), Berlin, Germany; [11]Department of Internal Medicine and Cardiology, German Heart Center Berlin (DHZB), Berlin, Germany.

Supplemental Material

Supplemental materials and methods

Cell culture of HAoSMCs

Primary human aortic smooth muscle cells (HAoSMCs; Thermo Fisher Scientific) were routinely cultured in Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (1:1, Thermo Fisher Scientific) containing 10% FBS (Thermo Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific). HAoSMCs were grown to confluence and used in all experiments from passages 4 to 10 (n indicates number of independent experiments performed at different passages of the cells). At least 4 different batches of HAoSMCs were used during the course of this study and each experiment was performed in at least 2 different batches of HAoSMCs depending on the availability of the cells. HAoSMCs were treated for the indicated periods with 2 mM β-glycerophosphate (Sigma Aldrich), 5 µg/ml hydroxyapatite nanoparticles (<200 nm particle size, Sigma Aldrich), the indicated concentrations of $ZnSO_4$ (Sigma Aldrich), 10 µM BAY11-7082 (stock in DMSO, Sigma Aldrich), 10 µM BMS-345541 (stock in DMSO, Sigma Aldrich) and 10 µM parthenolide (stock in DMSO, Sigma Aldrich). Treatment for 11 days with 10 mM β-glycerophosphate and 1.5 mM $CaCl_2$ (Sigma-Aldrich) was used as calcification media for the calcium deposition analysis and Alizarin Red staining [1]. For silencing experiments, HAoSMCs were transfected with 10 nM A20 siRNA (ID no. s14260, Thermo Fisher Scientific), 10 nM GPR39 siRNA (ID no. s6074, Thermo Fisher Scientific) or with 10 nM negative control siRNA (ID no. 4390843, Thermo Fisher Scientific) using the siPORT amine transfection reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The cells were used 48 hours after silencing. Silencing efficiency was verified by quantitative RT-PCR and Western Blotting.

Animal experiments

All animal experiments were conducted according to the recommendations of the Guide for Care and Use of Laboratory Animals of the National Institutes of Health as well as the German law for the welfare of animals and were approved by local authorities (PY715, G019716).

Klotho wild-type (WT) and klotho-hypomorphic (*kl/kl*) mice [2] had access to drinking water (distilled water) with or without addition of 7.18 g/l $ZnSO_4$ (Sigma-Aldrich) and standard rodent chow ad libitum. Lifelong treatment with $ZnSO_4$ was maintained in this study. At the age of 9 weeks, blood was collected by retroorbital puncture under isoflurane inhalative anaesthesia. Mice were immediately sacrificed by cervical dislocation and aortic tissues were collected and snap-frozen for further experiments.

C57Bl/6 mice were fed with calcium reduced diet (C1031, Altromin) and were treated with $ZnSO_4$ (7.18 g/l in distilled water, Sigma-Aldrich) or control drinking water. The mice were injected subcutaneously with vehicle or 400000 IU/kg BW of cholecalciferol (Sigma-Aldrich) for three days [3]. After six days, blood was collected by retroorbital puncture. Mice were sacrificed by cervical dislocation under inhalative isoflurane anaesthesia and aortic tissues were rapidly collected and snap-frozen.

Subtotal nephrectomy was performed in female DBA mice in a two-step procedure [3]. First, surgical subtotal nephrectomy of the right kidney was conducted, followed by left renal nephrectomy. After recovery from the surgical procedure, diet was switched to a phosphate-rich diet (0.6% Calcium, 0.9% phosphorous, Altromin) with or without the addition of $ZnSO_4$ to the drinking water (distilled water). Control mice without nephrectomy received standard rodent diet. Pulse propagation velocity (PPV) was determined by ultrasound measurements (VEVO 3100, MX-700 transducer) in the abdominal aorta using EKV-image acquisition. Analysis was performed with the Vevo-Vasc software (Visualsonics, Fujifilm). After 8 weeks, mice were sacrificed and tissues were collected and snap-frozen for further experiments.

The plasma concentrations of BUN, phosphate and calcium were measured by a photometric method (FUJI FDC 3500i/4000i, Sysmex), plasma zinc concentrations by using QuantiChrom Zinc assay kit (BioAssay Systems) and plasma concentrations of FGF23 C-term by using an ELISA kit (Immutopics) according to the manufacturer's instructions.

Calcium content

Aortic arch and kidney calcifications were quantified by incubation of the tissues in 0.6 M HCl overnight at 37°C. Calcium content in the supernatant was determined by using QuantiChrom Calcium assay kit (BioAssay Systems) according to the manufacturer's protocol. Tissues were lysed with 0.1 M NaOH/ 0.1% SDS and total protein concentration was measured by the Bradford assay (Bio-Rad Laboratories). HAoSMCs were decalcified in 0.6 M HCl for 24 hours at 4°C. Calcium content was determined by using QuantiChrom Calcium assay kit (BioAssay Systems). HAoSMCs were lysed with 0.1 M NaOH/0.1% SDS. Calcium content was normalized to total protein concentration measured by the Bradford assay (Bio-Rad Laboratories).

Alizarin Red staining

To visualize calcification, aortas were stained with Alizarin Red (0.0016% in 0.5% KOH). Images are representative for four mice per group. The calcified areas are shown as red staining [3]. VSMCs were fixed with 4% paraformaldehyde and stained with 2% Alizarin Red (pH4.5). The calcified areas are shown as red staining.

Von Kossa staining

Paraformaldehyde-fixed thoracic aortic tissues were cryoprotected in 30% sucrose, frozen in mounting medium (Tissue-Tek, Sakura Finetek), and sectioned at a thickness of 8 µm on coated slides. Sections were stained for calcification by using the Von Kossa staining

kit (Abcam) according to the manufacturer's protocol. The calcified areas are shown as grey/black staining.

Alkaline phosphatase (ALP) activity assay

ALP activity in HAoSMCs was determined by using the ALP colorimetric assay kit (Abcam) according to the manufacturer's protocol. ALP activity was normalized to total protein concentration measured by the Bradford assay (Bio-Rad Laboratories).

Luciferase assay

HAoSMCs were transfected with 1 μg DNA mixture of NF-kB-responsive luciferase construct and a constitutively expressing *Renilla* construct (40:1 ratio, Qiagen) using X-tremeGENE HP DNA transfection reagent (Roche Applied Science) for 48 hours. After the incubation period, cells lysed with Passive Lysis Buffer (Promega) and assayed for transcriptional activity using Dual-Luciferase Reporter Assay (Promega) and a luminometer (Victor 2 plate reader, Perkin Elmer) according to the manufacturer's protocol. All results are expressed as the ratio of NF-kB Firefly-Luciferase to *Renilla*-Luciferase (relative light units) normalized to Neg.si silenced or control treated HAoSMCs.

Quantitative RT-PCR

Total RNA was isolated from HAoSMCs and mouse aortic tissue by using Trifast Reagent (Peqlab) according to the manufacturer's instructions. Reverse transcription of 2 μg RNA was performed using oligo$(dT)_{12\text{-}18}$ primers (Thermo Fisher Scientific) and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Quantitative RT-PCR was performed with the iCycler iQ^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories) and iQ^{TM} Sybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions.

The following human primers were used (5'→3' orientation):

ACTA2 fw: AAAAGACAGCTACGTGGGTGA;

ACTA2 rev: GCCATGTTCTATCGGGTACTTC;

ALPL fw: GGGACTGGTACTCAGACAACG;

ALPL rev: GTAGGCGATGTCCTTACAGCC;

CBFA1 fw: GCCTTCCACTCTCAGTAAGAAGA;

CBFA1 rev: GCCTGGGGTCTGAAAAAGGG;

GAPDH fw: GAGTCAACGGATTTGGTCGT;

GAPDH rev: GACAAGCTTCCCGTTCTCAG;

GPR39 fw: TCTTCGTGATGGGCCTTCTG;

GPR39 rev: ACCTCCTTCTGCAAGTATCCTTT;

MSX2 fw: TGCAGAGCGTGCAGAGTTC;

MSX2 rev: GGCAGCATAGGTTTTGCAGC;

TNFAIP3 fw: TCAACTGGTGTCGAGAAGTCC;

TNFAIP3 rev: CAAGTCTGTGTCCTGAACGC.

The following mouse primers were used (5'→3' orientation):

Alpl fw: TTGTGCCAGAGAAAGAGAGAGA;

Alpl rev: GTTTCAGGGCATTTTTCAAGGT;

Cbfa1 fw: AGAGTCAGATTACAGATCCCAGG;

Cbfa1 rev: AGGAGGGGTAAGACTGGTCATA;

Gapdh fw: AGGTCGGTGTGAACGGATTTG;

Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;

Msx2 fw: TTCACCACATCCCAGCTTCTA;

Msx2 rev: TTGCAGTCTTTTCGCCTTAGC;

Tnfaip3 fw: ACTGGAATGACGAATGGGACA;

Tnfaip3 rev: CAGGGAATTGTACTGAAGTCCAC.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate and relative mRNA fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as internal reference.

Western blot analysis

Murine aortic tissue and HAoSMCs were lysed with ice-cold IP lysis buffer (Thermo Fisher Scientific) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). After centrifugation at 10000 rpm for 5 min, the proteins were boiled in Roti-Load1 Buffer (Carl Roth GmbH) at 100ºC for 10 min. Equal amounts of proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with primary antibodies: mouse anti-A20 HRP-conjugated (diluted 1:1000, sc-166692, Santa Cruz Biotechnology), goat anti-Alpl (diluted 1:200, sc-23430, Santa Cruz Biotechnology), rabbit anti-phospho-IkBα (Ser^{32}) (diluted 1:1000, #2859, Cell Signaling), rabbit anti-IkBα (diluted 1:1000, #4814, Cell Signaling), rabbit anti-NF-kB p65 (diluted 1:1000, #8242, Cell Signaling) or rabbit anti-GAPDH antibody (diluted 1:5000, #2118, Cell Signaling) and then with secondary anti-goat HRP-conjugated (diluted 1:5000, Santa Cruz Biotechnology) or anti-rabbit HRP-conjugated antibody (diluted 1:1000, Cell Signaling) for 1 hour at RT. For loading controls, the membranes were stripped in stripping buffer (Thermo Fisher Scientific) at RT for 10 min. Antibody binding was detected with ECL detection reagent (Thermo Fisher Scientific). Bands were quantified by using ImageJ software and the results are shown as the ratio of total protein to GAPDH and phosphorylated to total protein to GAPDH normalized to the control groups.

Immunocytochemistry and confocal microscopy

HAoSMCs cultured onto four-well chamber slides (BD Biostatus) were fixed with ice-cold 100% methanol for 10 minutes at RT. Slides were incubated with 5% normal goat serum in PBS/ 0.1% Triton-X100 for 1 hour at RT. Cells were incubated overnight at 4°C with primary rabbit polyclonal anti-NF-kB p65 antibody (diluted 1:50, sc-372, Santa Cruz Biotechnology) and then with goat anti-rabbit Alexa488-conjugated antibody (diluted 1:1000, Thermo Fisher Scientific) for 1 hour at RT. Nuclei were stained using DRAQ5 dye (diluted 1:1000, Biostatus) for 10 min at RT. The slides were mounted with Prolong Gold antifade reagent (Thermo Fisher Scientific). Images were collected with a confocal imaging system (A1Rsi+, Nikon Instruments) using a 60x (Oil), 1.4NA objective. Confocal images are representative for three independent experiments. Negative controls were carried out simultaneously with all experiments by omitting incubation with primary antibody.

Calcium phosphate precipitation assay

Calcium phosphate mineral phase formation assay was performed using a homogeneous system containing 10 mM $CaCl_2$ (Sigma-Aldrich) and 10 mM sodium phosphate buffer (pH7.4, Sigma Aldrich) in 500 mM HEPES buffer (pH7.4, Sigma Aldrich) in the presence of the indicated concentrations of $ZnSO_4$ (0 – 100 µM; Sigma Aldrich). After incubation for 10 minutes at RT, the samples were centrifuged at 1890g for 30 seconds and the obtained pellet was dissolved in 150 mM HCl. Calcium levels were determined colorimetric by using QuantiChrom Calcium assay kit (BioAssay Systems) according to the manufacturer's protocol.

Hydroxyapatite dissociation assay

Hydroxyapatite dissociation assay was performed using a homogeneous system containing 2 mM hydroxyapatite nanoparticles (<200 nm particle size, Sigma Aldrich) in 500 mM HEPES buffer (pH7.4, Sigma Aldrich) in the presence of the indicated concentrations of

$ZnSO_4$ (0 – 100µM; Sigma Aldrich). After overnight incubation at 37°C on a shaker (100 rpm), the samples were centrifuged at 1890g for 30 seconds and the obtained pellet was dissolved in 150 mM HCl. The calcium levels in the supernatant and the pellet, respectively, were determined colorimetric by using QuantiChrom Calcium assay kit (BioAssay Systems) according to the manufacturer's protocol.

Human samples

Ethics approval was obtained and all participants proved informed consent, adhering to the declaration of Helsinki 1964 and Istanbul 2013. For the first study cohort, blood was collected at the Charite Berlin, Germany from patients with known CKD, dialysis patients before the start of dialysis and from healthy volunteers as controls. All patients and volunteers gave informed consent. Patients were selected due to their medical history of CKD. Blood chemistry was obtained from routine laboratory measurements. Serum was obtained by immediate centrifugation and stored at -80°C. HAoSMCs were serum starved for 24 hours prior to treatment for 24 hours with 15 % uremic serum from hemodialysis patients (uremic serum, US) or control serum from matched healthy individuals (normal serum, NS). For validation, serum from a second cohort of CKD patients was analysed. This cohort was described in detail previously [4].

Serum calcification propensity was analysed in Berlin by determining the one-half maximal transition time (T_{50}) of in-vitro transformation from primary to secondary calciprotein particles as described by Pasch et al. [5] by using a Nephelostar Plus nephelometer (BMG Labtech, Ortenberg, Germany). Where indicated, $ZnCl_2$ or $ZnSO_4$ were added to the serum samples. Plasma zinc concentrations were measured by using QuantiChrom Zinc assay kit (BioAssay Systems).

Statistics

Data are shown as scatter dot plots and arithmetic means ± SEM. N indicates the number of independent experiments performed at different passages of the cells or the number of mice examined, respectively. Statistical analysis was performed by using SPSS and JMP software. Normality was tested with Shapiro-Wilk test. Non-normal datasets were transformed (log, reciprocal or sqrt) prior to statistical testing to provide normality according to Shapiro-Wilk test. Statistical testing was performed by one-way Anova followed by Tukey-test for homoscedastic data or Games-Howell test for heteroscedastic data. Non-normal data were tested by the Steel-Dwass method. Two groups were compared by unpaired two-tailed t-test. For correlations, Spearman correlation test was used. $P<0.05$ was considered statistically significant.

Supplemental References

1. Villa-Bellosta, R, Millan, A, Sorribas, V: Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. *American journal of physiology Cell physiology,* 300**:** C210-220, 2011.
2. Voelkl, J, Alesutan, I, Leibrock, CB, Quintanilla-Martinez, L, Kuhn, V, Feger, M, Mia, S, Ahmed, MS, Rosenblatt, KP, Kuro, OM, Lang, F: Spironolactone ameliorates PIT1-dependent vascular osteoinduction in klotho-hypomorphic mice. *The Journal of clinical investigation,* 123**:** 812-822, 2013.
3. Alesutan, I, Feger, M, Tuffaha, R, Castor, T, Musculus, K, Buehling, SS, Heine, CL, Kuro, OM, Pieske, B, Schmidt, K, Tomaschitz, A, Maerz, W, Pilz, S, Meinitzer, A, Voelkl, J, Lang, F: Augmentation of phosphate-induced osteo-/chondrogenic transformation of vascular smooth muscle cells by homoarginine. *Cardiovascular research,* 110**:** 408-418, 2016.
4. Scherberich, JE, Gruber, R, Nockher, WA, Christensen, EI, Schmitt, H, Herbst, V, Block, M, Kaden, J, Schlumberger, W: Serum uromodulin-a marker of kidney function and renal parenchymal integrity. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*, 2017.
5. Pasch, A, Farese, S, Graber, S, Wald, J, Richtering, W, Floege, J, Jahnen-Dechent, W: Nanoparticle-based test measures overall propensity for calcification in serum. *Journal of the American Society of Nephrology : JASN,* 23**:** 1744-1752, 2012.

Supplemental Tables

Suppl. Table 1. Effect of $ZnSO_4$ supplementation in *kl/kl* mice. Arithmetic means ± SEM of body weight and plasma calcium, phosphate and FGF23 (C-term) concentrations in klotho-hypomorphic mice (*kl/kl*) and corresponding wild-type mice (WT) following treatment without or with $ZnSO_4$. *(p<0.05), ***(p<0.001) statistically significant vs. control treated WT mice. †††(p<0.001) statistically significant vs. *kl/kl* mice.

	WT	*kl/kl*	WT^{ZnSO4}	kl/kl^{ZnSO4}	
Bodyweight [g]	19.8 ± 0.9	8.7 ± 0.3 ***	22.5 ± 1.1	17.7 ± 1.1 †††	n=10
Calcium [mg/dl]	9.2 ± 0.2	10.8 ± 0.3 ***	9.4 ± 0.2	10.2 ± 0.2 *	n=10
Phosphate [mg/dl]	7.2 ± 0.5	12.6 ± 0.4 ***	7.9 ± 0.4	11.5 ± 0.5 ***	n=10
FGF23 C-term [pg/ml]	619 ± 136	294063 ± 18429 ***	419 ± 44	117000 ± 51541	n=6

Suppl. Table 2. Effect of $ZnSO_4$ supplementation during cholecalciferol overload. Arithmetic means ± SEM of plasma calcium, phosphate and CaxPi product concentrations in mice receiving vehicle (CTR) or high-dosed cholecalciferol (vD) without or with additional treatment with $ZnSO_4$. **(p<0.01), ***(p<0.001) statistically significant vs. control mice; †††(p<0.001) statistically significant vs. vD treated mice.

	CTR	vD	vD + $ZnSO_4$	
Calcium [mg/dl]	8.9 ± 0.2	19.8 ± 1.2 ***	14.4 ± 0.9 **, †††	n=6-7
Phosphate [mg/dl]	8.0 ± 0.3	7.1 ± 0.3	8.8 ± 0.9	n=6-7
CaxPi product	70.8 ± 3.9	138.4 ± 6.0 ***	127.9 ± 19.1	n=6-7

Suppl. Table 3. Effect of $ZnSO_4$ supplementation during subtotal nephrectomy. Arithmetic means ± SEM of plasma BUN before (BUN start) and at the end (BUN final) of zinc treatment, calcium and phosphate levels as well as aortic pulse propagation velocity (PPV) and heart rate during PPV examinations in DBA mice with or without subtotal nephrectomy (Nx) or additional treatment with $ZnSO_4$. **(p<0.01), ***(p<0.001) statistically significant vs. control mice; †††(p<0.001) statistically significant between Nx and Nx+$ZnSO_4$ mice.

	CTR	$ZnSO_4$	Nx	Nx + $ZnSO_4$	
BUN start [mg/dl]	25.4 ± 2.1	23.9 ± 1.7	45.5 ± 2.3 ***	48.5 ± 2.0 ***	n=6-8
BUN end [mg/dl]	28.2 ± 3.9	21.4 ± 0.8	70.2 ± 3.7 ***	47.6 ± 3.9 ** †††	n=6-8
Calcium [mg/dl]	10.7 ± 0.1	10.5 ± 0.2	11.3 ± 0.2	10.9 ± 0.2	n=6-8
Phosphate [mg/dl]	7.8 ± 0.3	7.5 ± 0.4	9.8 ± 0.7	9.1 ± 0.5	n=6-8
PPV [m/s]	2.02 ± 0.12	2.22 ± 0.29	3.63 ± 0.40 *	2.28 ± 0.15 †	n=6-8
Heart rate [bpm]	447 ± 25	429 ± 30	434 ± 19	468 ± 21	n=6-8

Suppl. Table 4. Descriptive characteristics of study cohort 1. Table describing patient numbers, gender and arithmetic means ± SEM of age, serum zinc levels, creatinine levels and serum calcification propensity measured as calciprotein particle maturation time (T_{50}) in healthy volunteers (CTR), patients with known CKD (CKD) or dialysis patients (Dialysis). Descriptive characteristics of number of patients with known cardiovascular disease (CVD, defined as coronary heart disease, peripheral artery disease or stroke), diabetes and treatment with vitamin D or derivates, calcium-based phosphate binder or non-calcium based 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 patients.

	CTR	CKD	Dialysis
N	21	16	20
Female [n]	7	11	8
Age [years]	34.7 ± 1.4	74.1 ± 2.6 ***	61.6 ± 2.8 ***
Creatinine [mg/dl]	0.93 ± 0.04	1.79 ± 0.12 ***	7.18 ± 0.64 ***
Zinc [µM]	18.3 ± 1.3	14.8 ± 2.5	13.2 ± 0.8 **
T_{50} [min]	224.6 ± 18.8	167.6 ± 16.3	121.4 ± 12.8 ***
CVD [n]	-	10	9
Diabetes [n]	-	5	4
Non-calcium phosphate binder [n]	-	0	6
Calcium based phosphate binder [n]	-	0	9
Vitamin D or derivates [n]	-	3	15

Suppl. Table 5. Serum calcification propensity in hemodialysis patients. Arithmetic means ± SEM of serum calcification propensity measured as calciprotein particle maturation time (T_{50}) in respective human healthy volunteers and hemodialysis patients before dialysis described in Suppl. Table 4 and the measurements performed in the presence of 15 µM $ZnSO_4$ or 15 µM $ZnCl_2$. *($p<0.05$), ***($p<0.001$) statistically significant vs. control patients.

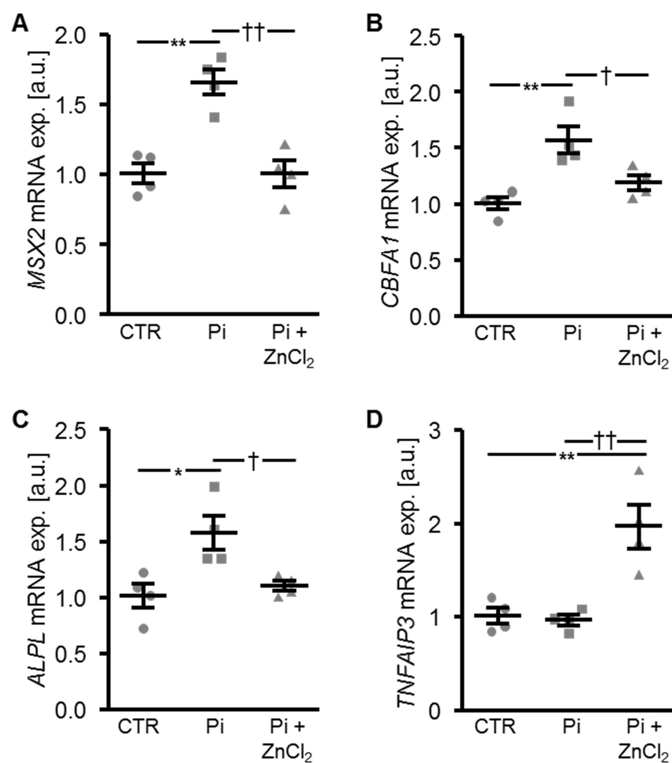
	T_{50} [min]	T_{50} [min] ($ZnSO_4$)	T_{50} [min] ($ZnCl_2$)	n
CTR	226.22±19.65	207.75±19.51	222.20±16.80	20
Dialysis	121.35±12.75***	132.91±17.06*	119.30±11.46***	20

Suppl. Table 6. Descriptive characteristics of CKD patients study cohort 2. Table describing patient numbers, gender and arithmetic means ± SEM of age, serum zinc levels, creatinine levels and serum calcification propensity measured as calciprotein particle maturation time (T_{50}) in human CKD patients according to estimated glomerular filtration rate (MDRD). Descriptive characteristics of number of patients with dialysis, known cardiovascular disease (CVD, defined as coronary heart disease, peripheral artery disease or stroke), diabetes and treatment with vitamin D or derivates, calcium-based phosphate binder or non-calcium based phosphate binder according to the patient medical history. *(p<0.05), ***(p<0.001) statistically significant vs. eGFR>60 patients.

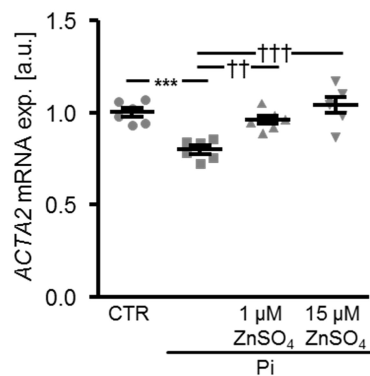
	eGFR >60	eGFR 30-60	eGFR 15-30	eGFR<15
N	30	45	17	46
Female [n]	13	15	3	20
Age [years]	56 ± 3	65 ± 2 *	67 ± 4	65 ± 2
Creatinine [mg/dl]	0.92 ± 0.03	1.35 ± 0.03 ***	2.76 ± 0.14 ***	7.56 ± 0.48 ***
Zinc [μM]	21.1 ± 3.6	12.7 ± 1.9 *	9.4 ± 2.6 *	10.4 ± 1.9 ***
T_{50} [min]	135.1 ± 6.6	118.0 ± 5.6	104.9 ± 6.3 *	96.3 ± 3.4 ***
Dialysis [n]	0	0	0	28
CVD [n]	0	6	10	19
Diabetes [n]	7	7	5	12
Non-calcium phosphate binder [n]	0	0	0	22
Calcium based phosphate binder [n]	0	0	2	15
Vitamin D or derivates [n]	21	24	17	44

Supplemental Figures

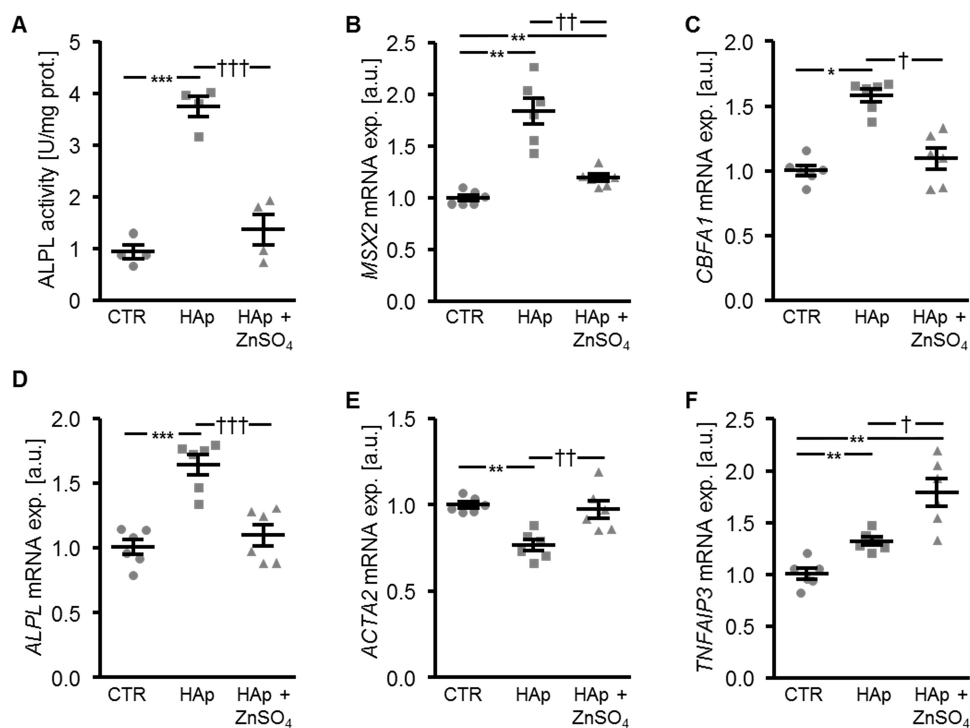
Suppl. Fig. 1. $ZnCl_2$ blunts the phosphate-induced osteogenic markers mRNA expression and up-regulates *TNFAIP3* expression in HAoSMCs. Scatter dot plots and arithmetic means ± SEM (n=4; arbitrary units, a.u.) of *MSX2* (**A**), *CBFA1* (**B**), *ALPL* (**C**) and *TNFAIP3* (**D**) relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with β-glycerophosphate (Pi) without or with additional treatment with 15 µM $ZnCl_2$. *(p<0.05), **(p<0.01) statistically significant vs. control treated HAoSMCs; †(p<0.05), ††(p<0.01) statistically significant vs. HAoSMCs treated with Pi alone.



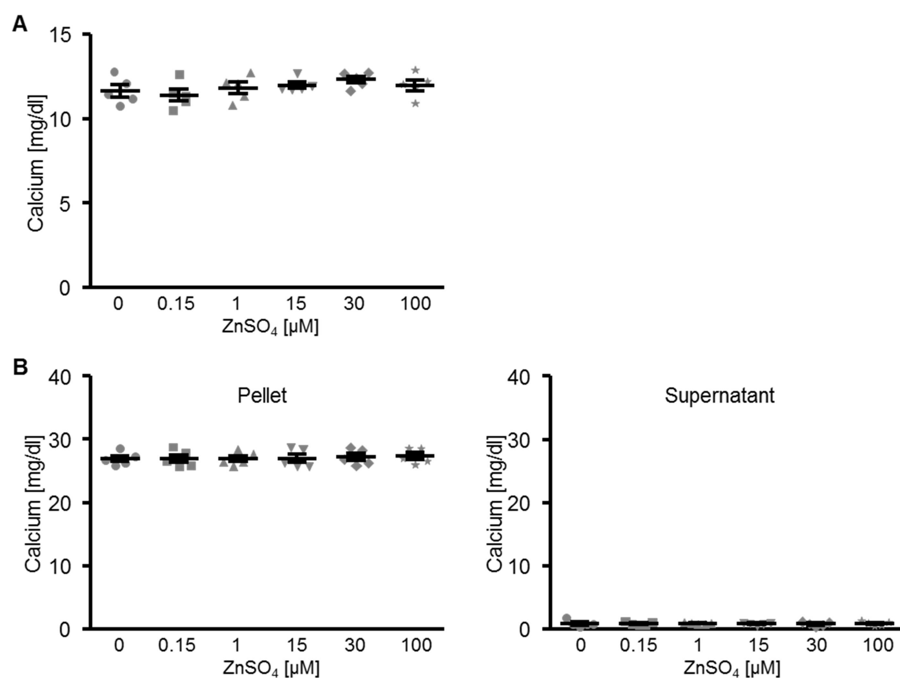
Suppl. Fig. 2. $ZnSO_4$ blunts the phosphate-induced down-regulation of *ACTA2* expression in HAoSMCs. Scatter dot plots and arithmetic means ± SEM (n=6; arbitrary units, a.u.) of *ACTA2* relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with β-glycerophosphate (Pi) without or with additional treatment with 1 μM or 15 μM $ZnSO_4$. ***(p<0.001) statistically significant vs. control treated HAoSMCs; ††(p<0.01), †††(p<0.001) statistically significant vs. HAoSMCs treated with Pi alone.



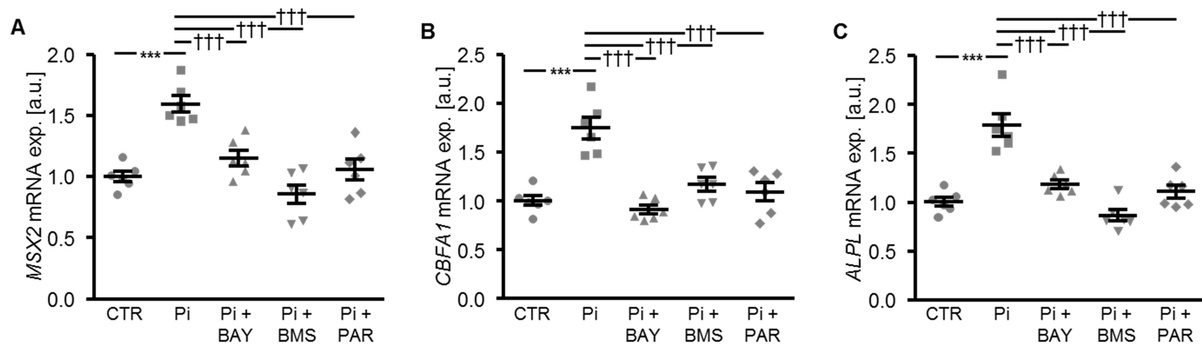
Suppl. Fig. 3. $ZnSO_4$ inhibits hydroxyapatite-induced osteoinductive signaling and up-regulates *TNFAIP3* expression in HAoSMCs. A. Scatter dot plots and arithmetic means ± SEM (n=4, U/mg protein) of alkaline phosphatase activity in HAoSMCs following treatment for 7 days with control or with hydroxyapatite nanoparticles (HAp) without or with additional treatment with 15 µM $ZnSO_4$. **B-F**. Scatter dot plots and arithmetic means ± SEM (n=6; arbitrary units, a.u.) of *MSX2* (**B**), *CBFA1* (**C**), *ALPL* (**D**), *ACTA2* (**E**) and *TNFAIP3* (**F**) relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with hydroxyapatite nanoparticles (HAp) without or with additional treatment with 15 µM $ZnSO_4$. *(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. HAoSMCs treated with HAp alone.



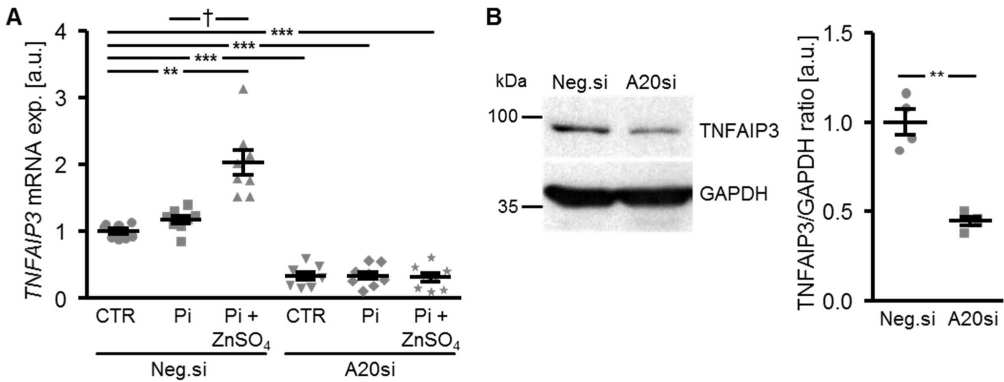
Suppl. Fig. 4. $ZnSO_4$ does not affect calcium-phosphate precipitation. A. Scatter dot plots and arithmetic means ± SEM (n=5; mg/dl) of calcium levels in the precipitated pellet following calcium phosphate precipitation in the presence of the indicated concentrations of $ZnSO_4$ (0–100 µM). **B.** Scatter dot plots and arithmetic means ± SEM (n=5; mg/dl) of calcium levels in the pellet and supernatant, respectively, following hydroxyapatite nanoparticle dissociation in the presence of the indicated concentrations of $ZnSO_4$ (0–100 µM).



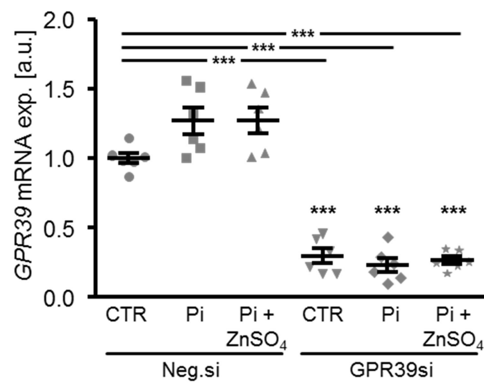
Suppl. Fig. 5. NF-kB inhibition blunts the phosphate-induced osteogenic markers mRNA expression in HAoSMCs. Scatter dot plots and arithmetic means ± SEM (n=6; arbitrary units, a.u.) of *MSX2* (**A**), *CBFA1* (**B**) and *ALPL* (**C**) relative mRNA expression in HAoSMCs following treatment for 24 hours with control or with β-glycerophosphate (Pi) without or with additional treatment with 10 µM BAY11-7082 (BAY), 10 µM BMS-345541 (BMS) or 10 µM parthenolide (PAR). ***(p<0.001) statistically significant vs. control treated HAoSMCs; †††(p<0.001) statistically significant vs. HAoSMCs treated with Pi alone.



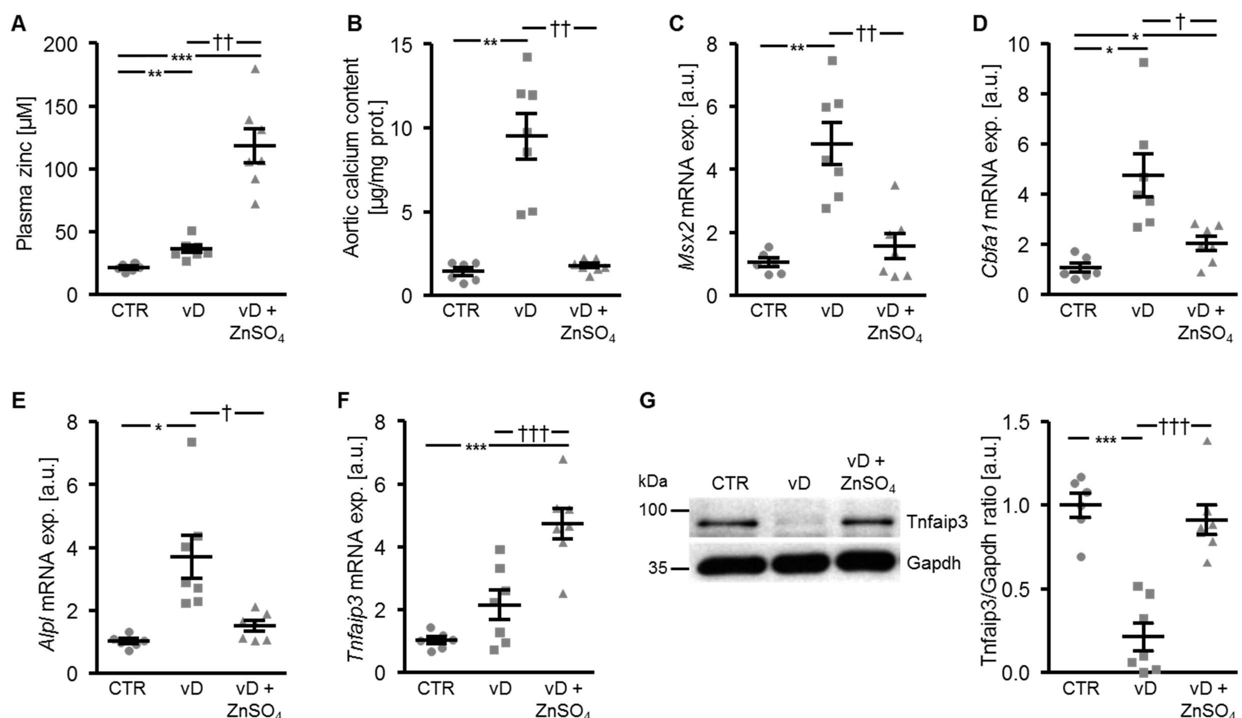
Suppl. Fig. 6. A20 silencing efficiency in HAoSMCs. A. Scatter dot plots and arithmetic means ± SEM (n=8; arbitrary units, a.u.) of *TNFAIP3* relative mRNA expression in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or A20 siRNA (A20si) and treatment for 24 hours with control or with β-glycerophosphate (Pi) without or with additional treatment with 15 μM $ZnSO_4$. **B**. Representative original Western blots and scatter dot plots and arithmetic means ± SEM (n=4; a.u.) of normalized TNFAIP3/GAPDH protein ratio in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or A20 siRNA (A20si). **(p<0.01), ***(p<0.001) statistically significant vs. Neg.si silenced HAoSMCs; †(p<0.05) statistically significant vs Neg.si silenced and Pi treated HAoSMCs.



Suppl. Fig. 7. ZnR/GPR39 silencing efficiency in HAoSMCs. Scatter dot plots and arithmetic means ± SEM (n=6; arbitrary units, a.u.) of *GPR39* relative mRNA expression in HAoSMCs following silencing for 48 hours with negative control siRNA (Neg.si) or GPR39 siRNA (GPR39si) and treatment for 24 hours with control or with β-glycerophosphate (Pi) without or with additional treatment with 15 μM $ZnSO_4$. ***(p<0.001) statistically significant vs. Neg.si silenced HAoSMCs.



Suppl. Fig. 8. $ZnSO_4$ supplementation ameliorates vascular calcification and osteoinductive signaling during cholecalciferol overload-induced calcification. A. Scatter dot plots and arithmetic means ± SEM (n=6-7, µM) of plasma zinc levels in mice receiving vehicle (CTR) or high-dosed cholecalciferol (vD) without or with additional treatment with $ZnSO_4$. **B.** Scatter dot plots and arithmetic means ± SEM (n=6-7, µg/mg protein) of calcium content in the aortic arch of mice receiving vehicle (CTR) or high-dosed cholecalciferol (vD) without or with additional treatment with $ZnSO_4$. **C-F.** Scatter dot plots and arithmetic means ± SEM (n=6-7; arbitrary units, a.u.) of *Msx2* (**C**), *Cbfa1* (**D**), *Alpl* (**E**) and *Tnfaip3* (**F**) relative mRNA expression in aortic tissue of mice receiving vehicle (CTR) or high-dosed cholecalciferol (vD) without or with additional treatment with $ZnSO_4$. **G.** Representative original Western blots and scatter dot plots and arithmetic means ± SEM (n=6-7; a.u.) of normalized Tnfaip3/Gapdh protein ratio in aortic tissue of mice receiving vehicle (CTR) or high-dosed cholecalciferol (vD) without or with additional treatment with $ZnSO_4$. *(p<0.05), **(p<0.01), ***(p<0.001) statistically significant vs. control mice; †(p<0.05), ††(p<0.01), †††(p<0.001) statistically significant vs. vD treated mice.



Suppl. Fig. 9. $ZnSO_4$ supplementation ameliorates kidney calcification in renal failure. Scatter dot plots and arithmetic means ± SEM (n=5-6, µg/mg protein) of calcium content in the kidney tissue of DBA mice without (CTR) or with subtotal nephrectomy (Nx) and treatment without or with $ZnSO_4$. *(p<0.05), ***(p<0.001) statistically significant vs. control mice; †(p<0.05), statistically significant vs. Nx mice.