ALKBH1-demethylated DNA N^6-methyladenine modification triggers vascular calcification via osteogenic reprogramming in chronic kidney disease

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Conflicts of interest: The authors have declared that no conflict of interest exists.

Supplementary Materials

Supplemental Table 1. Plasma biochemical parameters and bodyweight of adenine-diet-induced CKD mice with ALKBH1 deficiency.

Parameters	Control	CKD	CKD + sh-Scr	CKD + sh-ALKBH1
Body weight (g)	28.58±0.9213	21.49±1.36*	20.63±0.9	21.26±2.285
Fast blood glucose (mg/dL)	134.4±10.65	138.3±14	131.5±12.3	136.7±11.94
Cholesterol (mg/dL)	130.69±26.64	140.61±23.81	135.51±31.91	142.09±31.3
Triglyceride (mg/dL)	66.56±10.64	64.44±10.55	69.20±11.53	61.63±9.73
Calcium (mmol/L)	2.042±0.3229	2.002±0.3451	1.919±0.34	1.943±0.2816
Phosphorus (mmol/L)	1.82±0.34	3.47±1.31*	3.45±1.46	3.22±1.79
ALP (IU)	112.5±30.23	169.2±19.21*	178.2±9.814	130.5±19.2#
suPAR (ng/mL)	0.98±0.35	11.61±3.06*	10.29±3.96	8.93±3.44
Creatinine (μmol/L)	12.90±2.67	41.4±9.25*	41.87±8.31	42.27±7.36
BUN (mg/dL)	10.39±2.55	37.25±8.5*	35.71±7.26	36.63±9.48

Mice tail veins were inoculated with AAV encoding Scrambled (Scr shRNA) or *Alkbh1* shRNA at four weeks after the adenine diet and then fed for four weeks. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. Values are means ± SD (n=11-12 per group). *$P<0.05$ vs. Control. #$P<0.05$ vs. CKD+sh-Scr.

Supplemental Table 2. Plasma biochemical parameters and bodyweight of adenine-diet-induced CKD mice with ALKBH1 overexpression.

Parameters	Control	CKD	CKD + AAV-Vector	CKD + AAV-ALKBH1
Body weight (g)	29.64±1.306	22.39±1.789*	22.9±1.194	23.37±2.15
Fast blood glucose (mg/dL)	142.3±12.54	138.7±12.94	136.6±10.62	143.1±12.75
Cholesterol (mg/dL)	134.69±37.69	143.85±32.81	137.90±28.02	141.46±36.59
Triglyceride (mg/dL)	63.14±13.37	61.25±7.07	63.38±9.90	62.20±11.34
Calcium (mmol/L)	2.045±0.4514	1.973±0.3258	1.895±0.3746	1.944±0.3414
Phosphorus (mmol/L)	1.97±0.28	3.59±1.69*	3.73±1.71	3.54±1.87
ALP (IU)	100.3±24.62	173.7±37.93*	170.3±34.57	208.3±45.2#
suPAR (ng/mL)	0.98±0.31	10.82±3.53*	11.95±3.21	12.70±4.26
Creatinine (μmol/L)	11.86±3.12	42.15±7.59*	46.78±7.23	44.72±5.42
BUN (mg/dL)	10.63±1.94	38.29±11.95*	35.47±11.01	35.82±10.32

Mice tail veins were inoculated with AAV vector or AAV-ALKBH1 at four weeks after the adenine diet and then fed for four weeks. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. Values are means ± SD (n=11-12 per group). *$P<0.05$ vs. Control. #$P<0.05$ vs. CKD+AAV-Vector.

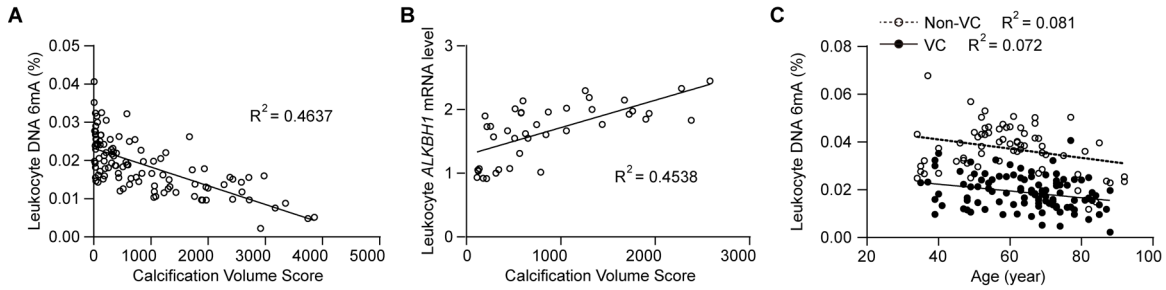
Supplemental Table 3. Plasma biochemical parameters and bodyweight of 5/6-nephrectomy-induced CKD mice with ALKBH1 deficiency or overexpression.

Parameters	Sham	5/6Nx	5/6Nx + sh-Scr	5/6Nx + sh-ALKBH1	5/6Nx + AAV-Vector	5/6Nx + AAV-ALKBH1
Body weight (g)	31.32±4.42	21.45±1.95*	21.41±1.75	21.29±1.43	22.08±2.33	21.71±1.46
Fast blood glucose (mg/dL)	139.9±12.29	135.8±12.29	133.4±9.44	139.8±7.60	132.3±8.88	138.4±12.82
Cholesterol (mg/dL)	136.86±23.16	143.80±31.29	141.04±24.08	148.12±31.73	146.95±23.74	144.05±18.19
Triglyceride (mg/dL)	68.53±9.39	67.90±9.26	65.27±8.04	65.29±14.32	60.70±12.33	64.92±6.86
Calcium (mM)	1.94±0.21	2.06±0.15	2.02±0.2	1.91±0.23	2.14±0.16	1.96±0.16
Phosphorus (mM)	1.94±0.20	3.31±0.63*	3.69±0.82	3.48±0.74	3.65±1.41	3.70±1.15
ALP (IU)	92.62±20.97	160.79±21.72*	156.98±20.92	111.65±19.35#	159.56±23.42	203.79±17.09†
suPAR (ng/mL)	1.10±0.31	12.73±2.62*	10.55±3.15	8.22±3.60	11.02±3.97	13.43±3.22
Creatinine (μM)	11.84±2.95	40.57±10.32*	38.95±10.33	38.53±6.66	43.16±7.59	40.07±8.08
BUN (mg/dL)	9.75±1.38	36.45±7.20*	35.96±8.70	35.74±6.32	36.52±7.97	37.31±7.69

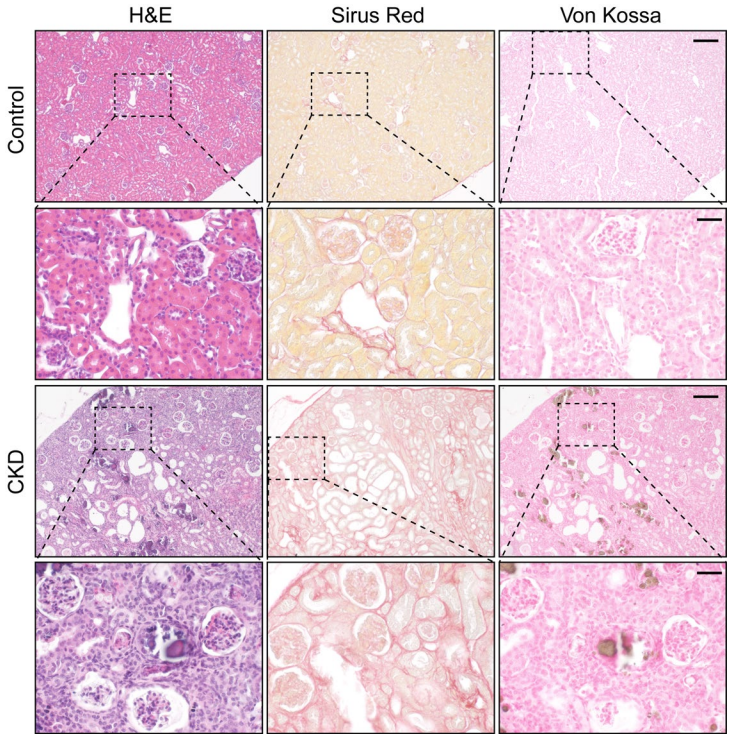
Mice tail veins were inoculated with AAV encoding Scrambled (Scr shRNA) or *Alkbh1* shRNA, or AAV-vector or AAV-ALKBH1 at the 4th week after the sham or nephrectomy operation, and mice were sacrified at the 8th week. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. Values are means ± SD (n=5-8 per group). *$P<0.05$ vs. sham. #$P<0.05$ vs. 5/6Nx + sh-Scr. †$P<0.05$ vs. 5/6Nx + AAV-Vector.

Supplemental Table 4. The primer sequences.

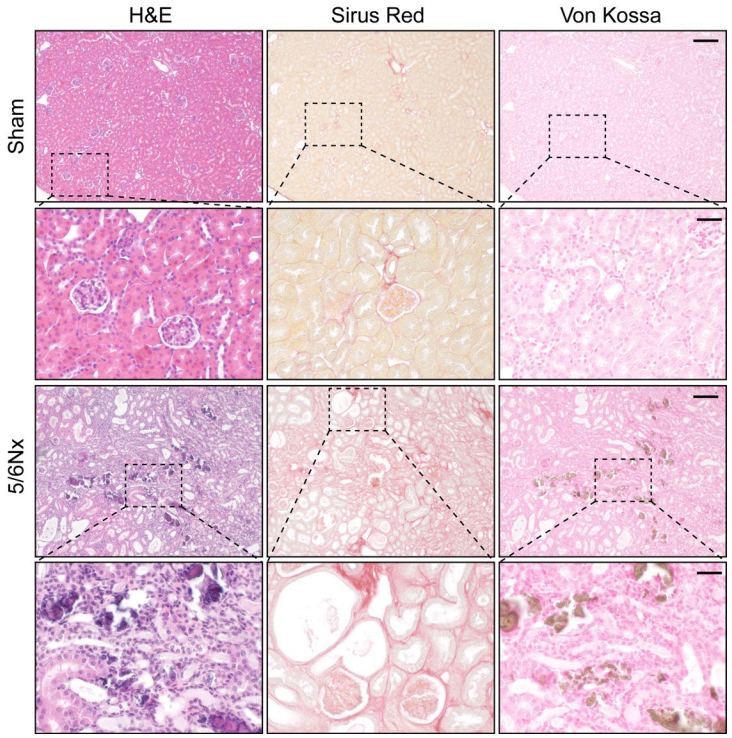
Species	Gene	Sequence (forward/reverse)
qRT-PCR		
Human	*BMP2*	5' – GGCATCCTCTCCACAAAAGA – 3'
		5' – ACGTCTGAACAATGGCATGA – 3'
	ALKBH1	5' – AGAAGCGACTAAACGGAGACC – 3'
		5' – GGGAAAGGTGTGTAATGATCTGC – 3'
	N6AMT1	5' – GCAGGGGAGAACTTCGCTAC – 3'
		5' – CAGCGCGTTCAAAAGCAGAAA – 3'
	GAPDH	5' – GGAGTCAACGGATTTGGT – 3'
		5' – GTGATGGGATTTCCATTGAT – 3'
Mouse	*Bmp2*	5' – TGAGGATTAGCAGGTCTTTG – 3'
		5' – CACAACCATGTCCTGATAA – 3'
	N6amt1	5' – AGCCGCATGTACCTTGGAAA – 3'
		5' – TACCTCTTCAGGCGGAGTCA – 3'
	Alkbh1	5' – AAGCGAAGACCCCGAAGTTTA – 3'
		5' – CAGTGGCGACTTGCTCTGA – 3'
	Gapdh	5' – ATTGTCAGCAATGCATCCTG – 3'
		5' – ATGGACTGTGGTCATGAGCC – 3'
***BMP2* ChIP assay**		
Human	ChIP-1	5' – CCGAGTCTTGTCCACACACAA – 3'
		5' – ATGCATCAGAGGGTACAGACAA – 3'
	ChIP-2	5' – CTAGTCCTTTCTCCAGTGGCTT – 3'
		5' – TGGAGGGCCAGTGAAGTCAA– 3'
	ChIP-3	5' – CTGGTCTGGCTTTGGTGTCA – 3'
		5' – CAAGGACTGTGTTTGGCCTG – 3'
***BMP2* promoter luciferase constructs**		
Human	-3319 bp~ -22 bp	5' – ACGGTACCCAGTCATCCATGACAGAACCAGG– 3'
		5' – TGCTCGAGCTTTTAAAGGGGACGCCGCCT– 3'
	-2882 bp~ -22 bp	5' – ACGGTACCGCCACAGGTTTCCATGGAATGAC – 3'
		5' – TGCTCGAGCTTTTAAAGGGGACGCCGCCT – 3'
	-2300 bp~ -22 bp	5' – ACGGTACCGGCAAACAGACACTCTCCCAAAG – 3'
		5' – TGCTCGAGCTTTTAAAGGGGACGCCGCCT – 3'
	Oct4 Del	5' – GATTGTGTGTGGACAAGACTCGG – 3'
		5' – TTTCCACTATCTCCTGAGAAACTGCC – 3'



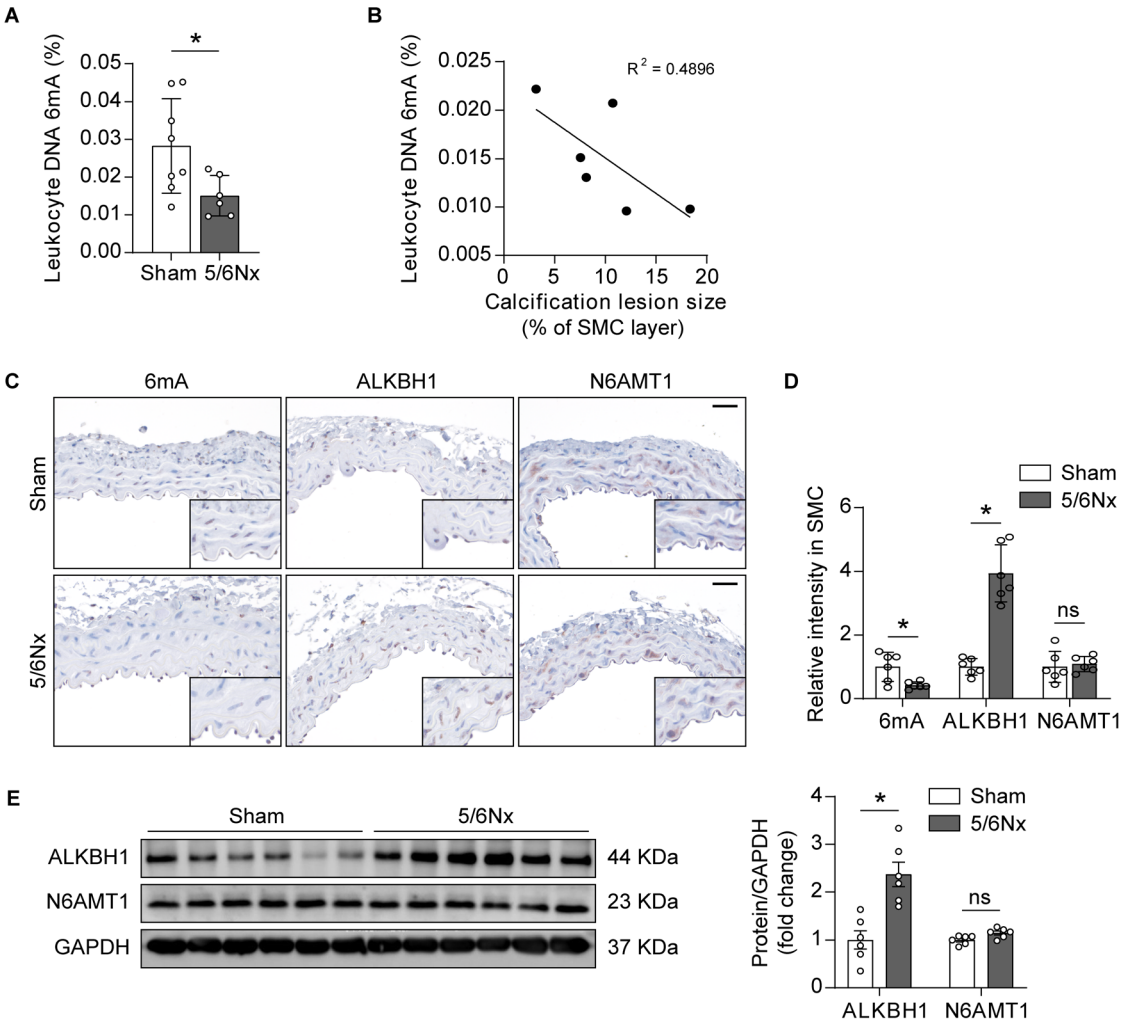
Supplemental Figure 1. Correlation between ALKBH1 and vascular calcification during CKD. (**A**) Scatter dot plot of correlation between leukocyte DNA 6mA level and calcification Volume score from CKD patients with aortic arch calcification (n = 106). (**B**) Scatter dot plot of correlation between leukocyte ALKBH1 mRNA expression level and calcification Volume score from CKD patients with aortic arch calcification (n = 40). (**C**) Reduced DNA 6mA level negatively correlated to age in CKD patients without (Non-VC, n = 67) and with aortic arch calcification (VC, n = 106). Statistical significance was assessed using Pearson's correlation coefficient analysis for correlations.



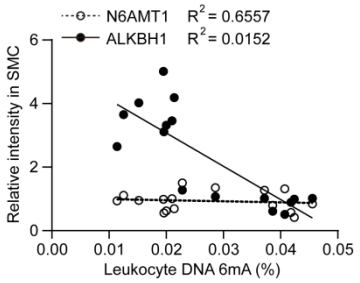
Supplemental Figure 2. Histology assessment of the kidney of adenine-diet-induced mice CKD model. H&E, Masson's trichrome and von Kossa staining of the kidney sections of adenine-diet-induced mice CKD model. Scale bars: 200 μm and 50 μm (enlarged).



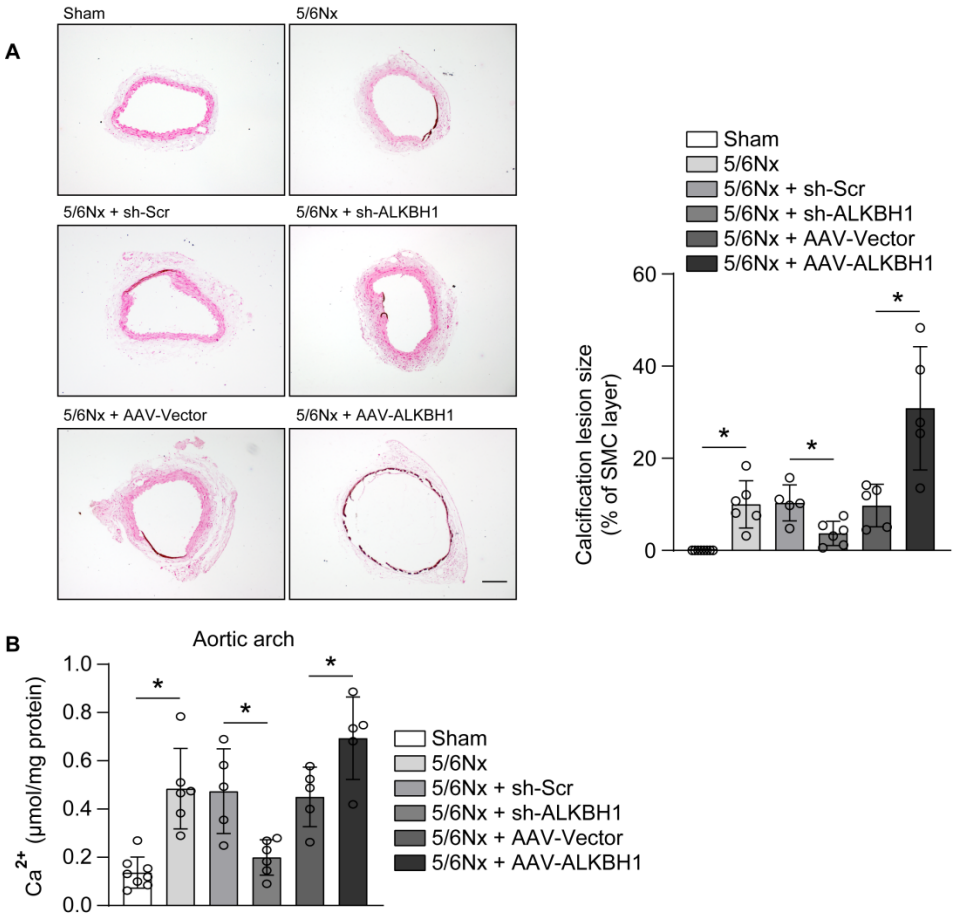
Supplemental Figure 3. Histology assessment of the remnant kidney of 5/6-nephrectomy-induced mice CKD model. H&E, Masson's trichrome and von Kossa staining of the remnant kidney sections of 5/6-nephrectomy-induced mice CKD model. Scale bars: 200 μm and 50 μm (enlarged). 5/6Nx, 5/6 nephrectomy.



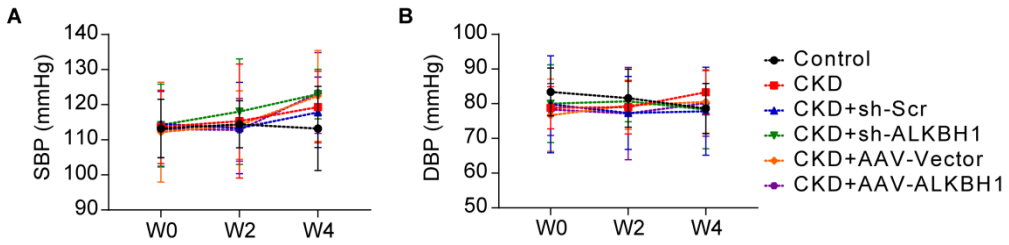
Supplemental Figure 4. ALKBH1-demethylated DNA 6mA modification is reduced in CKD mice induced by 5/6 nephrectomy. (**A**) Leukocyte DNA 6mA level in mice challenged with sham operation (n = 8) or 5/6 nephrectomy (n = 6). Leukocytes were isolated from peripheral blood. (**B**) Scatter dot plot of correlation between mice leukocyte DNA 6mA level and percentage of calcification lesion size in aortic smooth muscle layer from mice with 5/6 nephrectomy (n = 6). (**C** and **D**) Representative immunohistochemistry pictures (**C**) and quantification (**D**) of ALKBH1, N6AMT1, and 6mA in aortic smooth muscle layer of mice subjected to sham operation or 5/6 nephrectomy (n = 6 for Sham; n = 6 for 5/6Nx). Scale bars: 50 μm. (**E**) Western blot analysis of ALKBH1 and N6AMT1 expression in mice aortic arch (n = 6 per group). Statistical significance was assessed using two-tailed *t*-tests (**A**, **D** and **E**) and Pearson's correlation coefficient analysis (**B**). All values are presented as means ± SD, ns: no significance, **p* < 0.05.



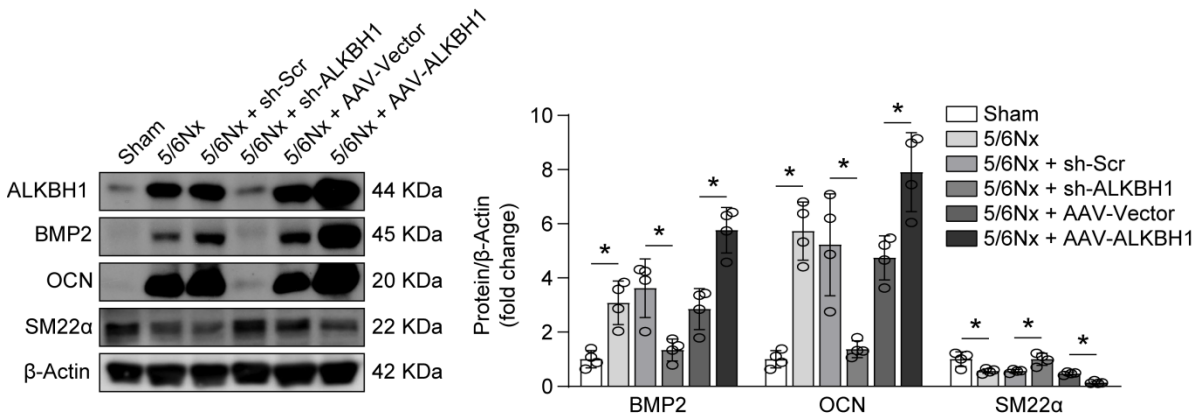
Supplemental Figure 5. ALKBH1 expression in the smooth muscle layer is inversely correlated to reduced leukocyte 6mA level. Pearson's correlation coefficient analysis for leukocyte DNA 6mA level correlated with ALKBH1 and N6AMT1 level assessed by IHC staining in the smooth muscle layer of mice aortas, respectively (n = 10-12 per group).



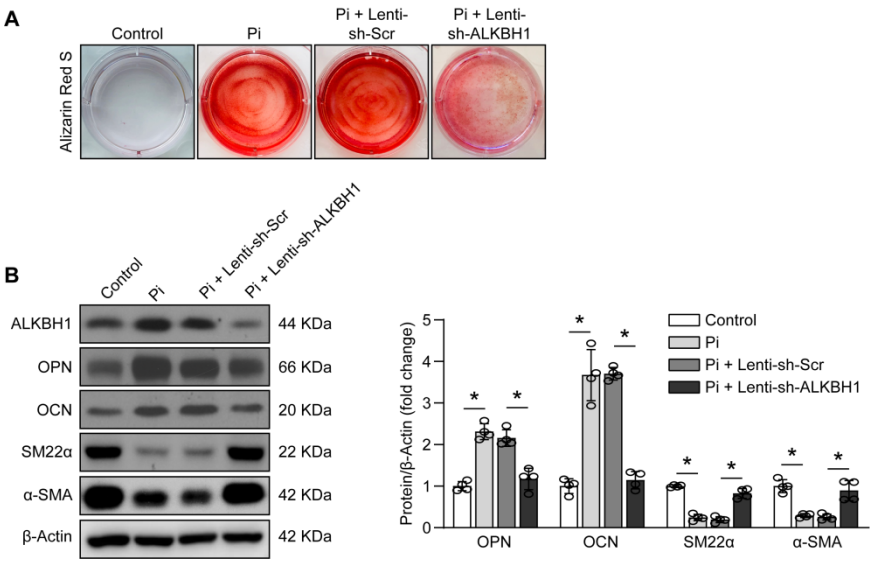
Supplemental Figure 6. ALKBH1 is essential for the regulation of CKD-induced vascular calcification in 5/6-nephrectomy model. (**A** and **B**) Von Kossa staining (**A**) and calcium content quantification of aortic arch (**B**) performed in different experimental groups for detecting mineralization (n = 5-8 per group). Scale bar: 100 μm. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. All values are presented as means ± SD, ns: no significance, *$p < 0.05$.



Supplemental Figure 7. Blood pressure of CKD mice with ALKBH1 deficiency or overexpression. (**A**) Systolic blood pressure (SBP) in adenine-diet-induced CKD mice before (W0), 2 (W2) and 4 (W4) weeks after indicated virus injection (n = 5-6 per group). (**B**) Diastolic blood pressure (DBP) in adenine-diet-induced CKD mice before (W0), 2 (W2) and 4 (W4) weeks after indicated virus injection (n = 5-6 per group). Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. All values are presented as means ± SD.



Supplemental Figure 8. BMP2 is modulated by ALKBH1 in 5/6-nephrectomy model. Western blot analysis of ALKBH1, BMP2, OCN and SM22α expression in arteries from mice with indicated treatments (n = 4 per group). Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. All values are presented as means ± SD, ns: no significance, *$p < 0.05$.



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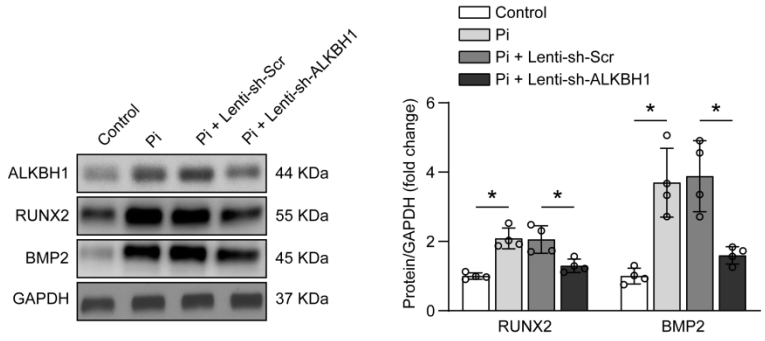
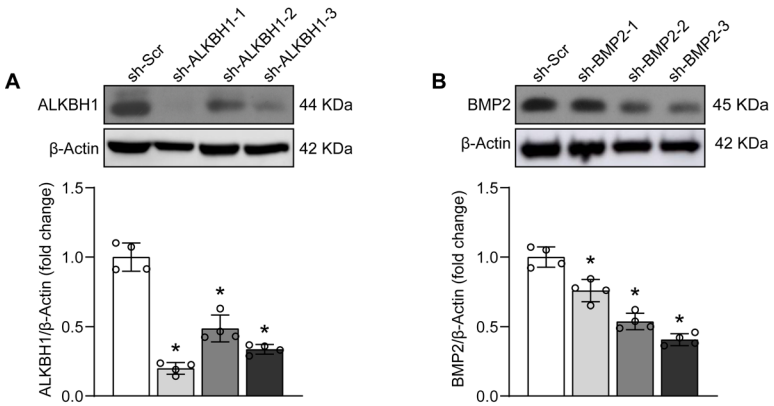
107 **Supplemental Figure 9. ALKBH1 deficiency suppresses HASMCs osteogenic reprogramming**
108 **and calcification.** (**A**) Representative photomicrographs of Alizarin red staining in HASMCs pre-
109 transfected with scrambled shRNA lentiviruses or *ALKBH1* shRNA lentiviruses and exposed in
110 osteogenic medium for another 14 days. (**B**) Western blot analysis of osteogenic phenotype marker
111 (OPN and OCN) and contractile phenotype marker (SM22α and α-SMA) expression in HASMCs with
112 ALKBH1 depletion (n = 4 per group). Statistical significance was assessed using one-way ANOVA
113 followed by Dunnett's test. All values are presented as means ± SD, **p* < 0.05.



Supplemental Figure 10. BMP2 is downregulated by ALKBH1 depletion in HASMCs. Western blot analysis showing the downregulation of BMP2 and RUNX2 in calcified HASMCs pre-transfected with scrambled shRNA lentiviruses or *ALKBH1* shRNA lentiviruses (n = 4). Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. All values are presented as means ± S.D, **p* < 0.05.



Supplemental Figure 11. The efficiency of ALKBH1 and BMP2 shRNA. (**A**) Western blot analysis showing the efficiency of three different lines of AAV sh-ALKBH1. Mice primary VSMCs were infected with AAV encoding three different lines of *Alkbh1* shRNA for 48 hours and then cultured in an osteogenic medium for 14 days (n = 4). (**B**) Western blot analysis showing the efficiency of three different lines of AAV sh-BMP2 (n = 4). Statistical significance was assessed using one-way ANOVA followed by Dunnett's test. All values are presented as means ± S.D, **p* < 0.05 vs. sh-Scr.