Supplementary materials

HDAC9-mediated epithelial cell cycle arrest in G2/M contributes to kidney fibrosis in male mice

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Running Title: HDAC9 deficiency attenuated kidney fibrosis

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Table S1. Clinical characteristics in the normal human control subjects or subjects with chronic kidney disease.

Normal group (n = 6)	
Age (years)	50.17±4.97
Gender (male, n, %)	2 (33.33%)
SCr (μmol/l)	64.33±4.72
BUN (mmol/l)	4.92±0.56
eGFR (ml/min/1.73 m^2)	75.14±4.28
Subjects with renal fibrosis (n = 17)	
Age (years)	50.47±2.72
Gender (male, n, %)	13 (76.47%)
SCr (μmol/l)	158.06±10.15 ($p < 0.0001$)#
BUN (mmol/l)	11.50±1.78 ($p = 0.0214$)*
eGFR (ml/min/1.73 m^2)	33.45±2.23 ($p < 0.0001$)*
Pathological diagnosis	N (%)
Focal segmental glomerulosclerosis	9 (52.94%)
IgA nephropathy	5 (29.41%)
Diabetic nephropathy	3 (17.65%)

SCr, serum creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; # Mann-Whitney rank sum test with two-tailed *p*-value; * Unpaired t-test with two-tailed *p*-value; SEM, Standard error of mean; Data are expressed as mean ± SEM.

Table S2. Physical and biochemical parameters of mice with *HDAC9* deficiency.

Variables		*HDAC9*$^{+/+}$	*HDAC9*$^{-/-}$
Body weight (g)		26.25 ±0.94	26.37±0.59
kidney weight (g)		0.26±0.01	0.26±0.02
Heart rate (beat/min)		490.56±14.40	496.89±17.36
Blood pressure (mmHg)	Systolic	112.11±2.98	110.84±2.77
	Diastolic	72.28±3.13	71.45±2.75
Scr (μmol/l)		9.65±0.64	9.08±0.73
BUN (mmol/l)		8.74±0.41	8.98±0.49

n = 6 mice per group. Data are expressed as mean ± SEM

Table S3. Physical and biochemical parameters of mice with *HDAC9* deficiency in tubules.

Variables		*Cre*$^{-}$*/HDAC9*$^{fl/fl}$	*Cre*$^{+}$*/HDAC9*$^{fl/fl}$
Body weight (g)		26.05 ±0.76	26.22±0.81
kidney weight (g)		0.25±0.02	0.26±0.01
Heart rate (beat/min)		487.00±20.08	490.78±17.48
Blood pressure (mmHg)	Systolic	111.39±4.29	112.44±2.86
	Diastolic	72.00±2.99	72.39±2.23
Scr (μmol/l)		9.32±0.52	8.95±0.67
BUN (mmol/l)		9.01±0.51	8.83±0.73

n = 6 mice per group. Data are expressed as mean ± SEM

Table S4. Primer pairs of target genes used for PCR in this study.

Gene	Species	Forward (5' to 3')		Reverse (5' to 3')	
Global *HDAC9* knockout	Mouse	P1	CCCCAAATTACCTCGCACCTTA	P2	CCTCCCGGATTCCACTTGTT
		P3	CGTGGGCCGTCTCTGTCTTG	P4	CCCTGGTGCCTCTTACTTTCTCG
HDAC9$^{fl/fl}$	Mouse	AAGCAGCACGAGAATTTGAC		CTAAGGAAGAACTACATTGCCCTAT	
Cdh16-Cre	Mouse	P1	GCAGATCTGGCTCTCCAAAG	P2	AGGCAAATTTTGGTGTACGG
		P3	CAAATGTTGCTTGTCTGGTG	P4	GTCAGTCGAGTGCACAGTTT
HDAC9	Mouse	AGTGCGAGACACAGATGCTC		TACATGAACGTGGCTGGAGG	
p21	Mouse	TTGTCGCTGTCTTGCACTCT		TAGAAATCTGTCAGGCTGGTCT	
β-actin	Mouse	GGCTGTATTCCCCTCCATCG		CCAGTTGGTAACAATGCCATGT	
HDAC9	Human	CTCATGCCGAGCAAATGGTT		GGAACACCTTGCCTAAGCGT	
CTGF	Human	TTAGCGTGCTCACTGACCTG		GCCACAAGCTGTCCAGTCTA	
β-actin	Human	CATGTACGTTGCTATCCAGGC		CTCCTTAATGTCACGCACGAT	
α-SMA	Rat	CCTCATGCCATCATGCGTCT		CTCACGCTCAGCAGTAGTCA	
β-actin	Rat	CTCTGTGTGGATTGGTGGCT		CGCAGCTCAGTAACAGTCCG	
IL-6	Mouse	CTTCTTGGGACTGATGCTGGT		CTCTGTGAAGTCTCCTCTCCG	
TNFα	Mouse	CAGGCGGTGCCTATGTCTC		CGATCACCCCGAAGTTCAGTAG	
IL-1β	Mouse	CTGCAGCTGGAGAGTGTGG		GGGGAACTCTGCAGACTCAA	
IL-1β	Human	ATGATGGCTTATTACAGTGGCAA		GTCGGAGATTCGTAGCTGGA	
IL-6	Human	ACTCACCTCTTCAGAACGAATTG		CCATCTTTGGAAGGTTCAGGTTG	
TNFa	Human	CCTCTCTCTAATCAGCCCTCTG		GAGGACCTGGGAGTAGATGAG	

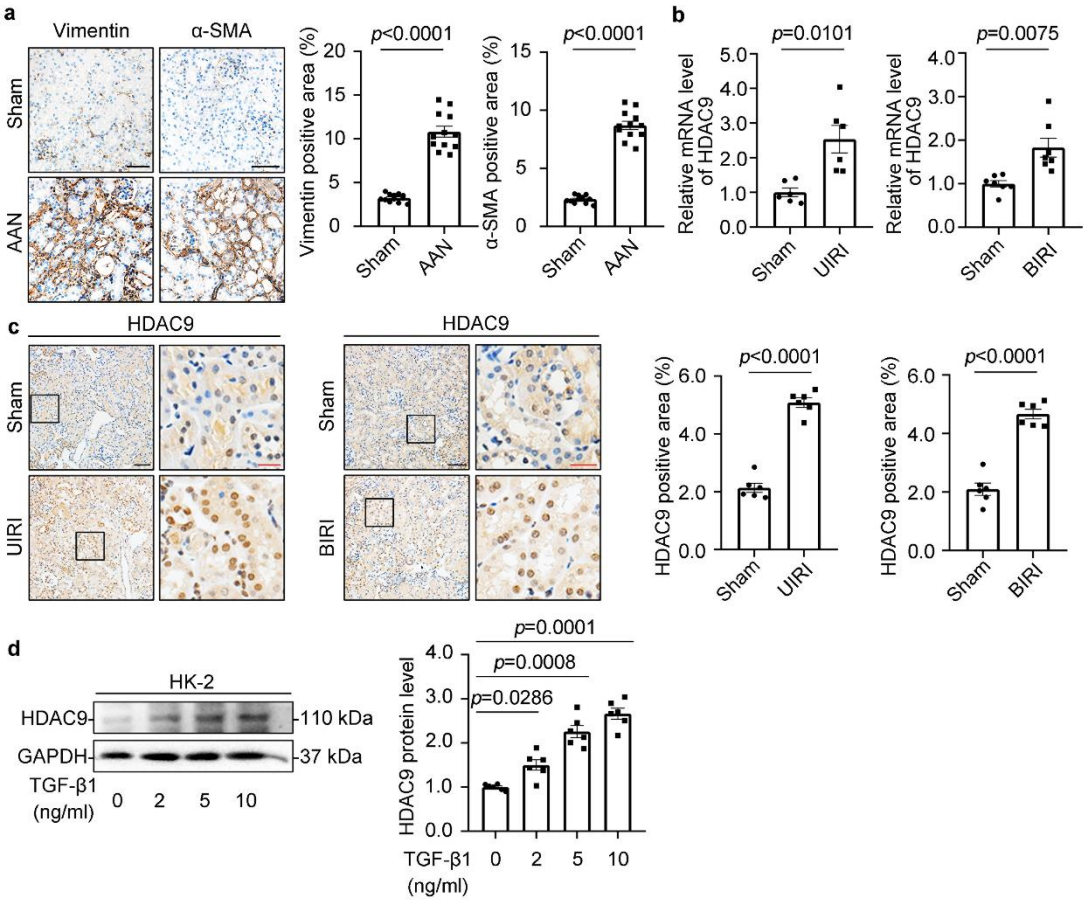
Table S5. Antibodies used in this study.

Primary antibodies	Source	Provider	Catalog	Application
HDAC9	Rabbit	ORIGENE	TA324378	WB (1:500), IHC (1:50), IF (1:50)
Fibronectin	Rabbit	ProteinTech Group	15613-1-AP	WB (1:1000)
Vimentin	Rabbit	Abcam	ab92547	IHC (1:100), IF (1:100)
Vimentin	Rabbit	Cell Signaling Technology	5741	WB (1:1000)
Alpha-smooth muscle	Rabbit	Abcam	ab124964	WB (1:2000), IHC (1:100), IF (1:100)
PCNA	Rabbit	ProteinTech Group	10205-2-AP	WB (1:1000), IHC (1:50), IF (1:100)
Collagen I	Rabbit	Affinity	AF7001	WB (1:1000)
Collagen I	Rabbit	Cell Signaling Technology	72026	IHC (1:50), IF (1:50)
Collagen IV	Rabbit	Abcam	ab236640	IHC (1:50)
Cyclin B1	Mouse	Cell Signaling Technology	4135	IHC (1:50), IF (1:50)
Cyclin B1	Rabbit	ProteinTech Group	55004-1-AP	WB (1:1000)
Cyclin D1	Rabbit	Abcam	ab16663	WB (1:1000)
Ki-67	Rabbit	Abcam	ab15580	IF (1:50)
Histone H3 (phospho S10)	Mouse	Abcam	ab14955	IF (1:50)
PDGF Receptor β	Rabbit	Cell Signaling Technology	3169	IF (1:50)
Alpha-smooth muscle	Mouse	ProteinTech Group	67735-1-lg	IF (1:100)
TGF beta 1	Rabbit	Abcam	ab215715	IHC (1:50)
TGF beta 1	Rabbit	Abcam	ab179695	WB (1:2000)
STAT1 (phospho S727)	Rabbit	Abcam	ab109461	WB (1:1000), IHC (1:50)
Acetylated-Lysine Antibody	Rabbit	Cell Signaling Technology	9441	WB (1:1000)
STAT1	Mouse	Cell Signaling Technology	9176	IP (10μg), WB (1:1000)
STAT1	Rabbit	Abcam	ab234400	WB (1:1000)

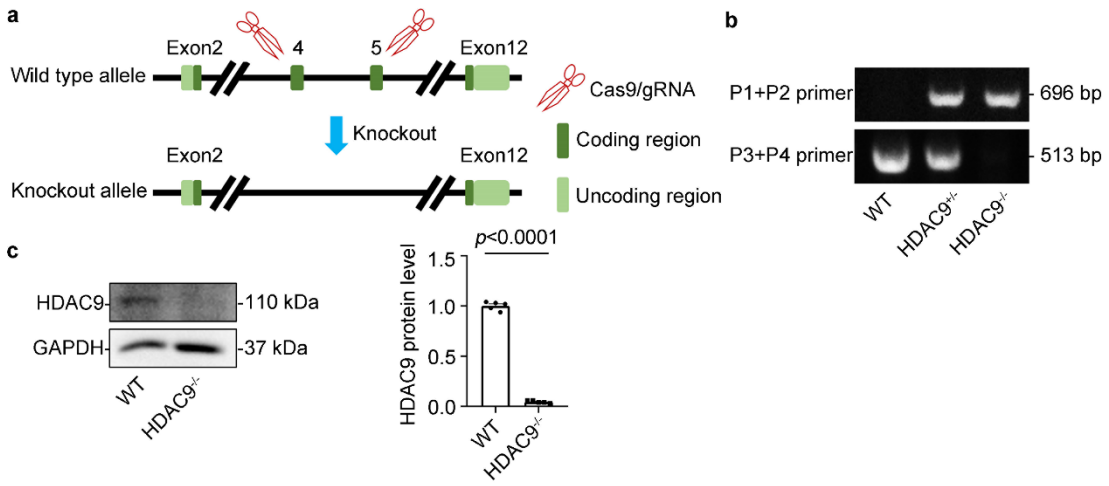
STAT1	Mouse	Abcam	ab155933	IP (10μg), WB (1:1000)
p21	Rabbit	Abcam	ab109199	WB (1:1000), IF (1:50)
STAT1 (phospho S727)	Rabbit	Cell Signaling Technology	9177	IF (1:50)
HDAC3	Rabbit	ABclonal	A2139	WB (1:1000)
HDAC7	Rabbit	Abcam	ab166911	WB (1:1000)
HDAC8	Rabbit	ABclonal	A5829	WB (1:1000)
F4/80	Rabbit	Cell Signaling Technology	70076	IHC (1:50)
DNMT3a	Rabbit	Cell Signaling Technology	3598	WB (1:1000)
STAT2	Rabbit	ABclonal	A3588	WB (1:1000)
STAT3	Rabbit	ABclonal	A1192	WB (1:1000)
STAT4	Rabbit	ABclonal	A4523	WB (1:1000)
STAT5	Rabbit	ABclonal	A5029	WB (1:1000)
STAT6	Rabbit	ABclonal	A19120	WB (1:1000)
STAT2 (phospho Y690)	Rabbit	Abcam	ab191601	WB (1:1000)
STAT3 (phospho Tyr705)	Rabbit	Cell Signaling Technology	9145	WB (1:1000)
STAT4 (phospho Y690)	Mouse	Santa Cruz Biotechnolog	sc-28296	WB (1:500)
STAT5 (phospho Tyr694)	Rabbit	Cell Signaling Technology	4322	WB (1:500)
STAT6 (phospho Y641)	Rabbit	ABclonal	AP0456	WB (1:500)
Mouse IgG1 Isotype Control	Mouse	Cell Signaling Technology	5415	IP (10μg)
Mouse Anti-rabbit IgG	Mouse	Cell Signaling Technology	5127	WB (1:2000)
Goat Anti-Rabbit IgG (H+L) HRP	Goat	Abways Technology	AB0101	WB (1:10000)
Goat Anti-Mouse IgG (H+L) HRP	Goat	Abways Technology	AB0102	WB (1:10000)
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594)	Goat	Abcam	ab150080	IF (1:200)
Goat Anti-Mouse IgG H&L (Alexa Fluor® 594)	Goat	Abcam	ab150116	IF (1:200)
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Goat	Abcam	ab150077	IF (1:200)

GAPDH	Rabbit	Abways Technology	AB0037	WB (1:10000)
β-actin	Rabbit	Abways Technology	AB0035	WB (1:10000)
AQP1	Mouse	Abcam	ab9566	IF (1:100)
THP	Mouse	Santa Cruz Biotechnolog	sc-271022	IF (1:100)

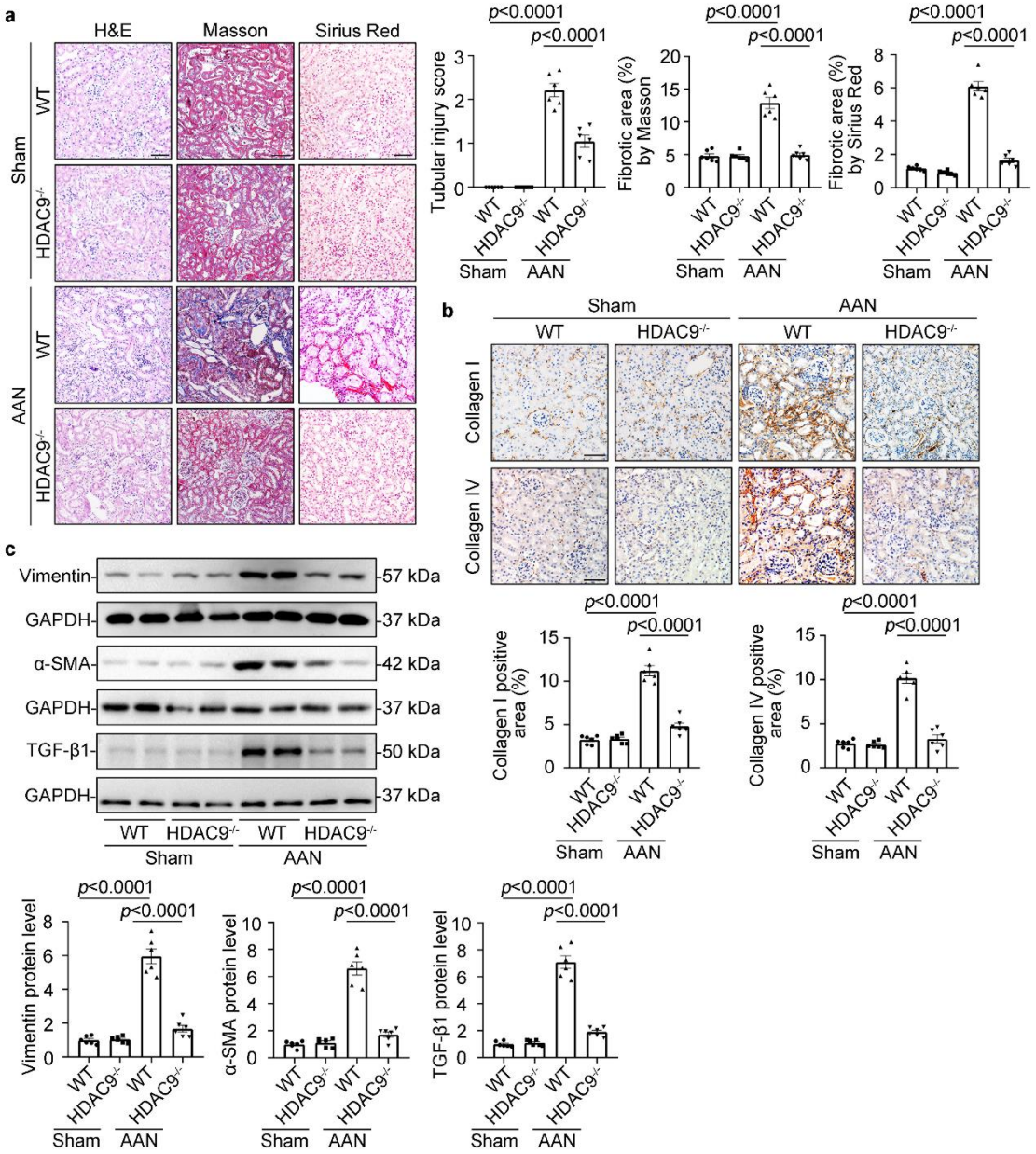
Supplementary figures



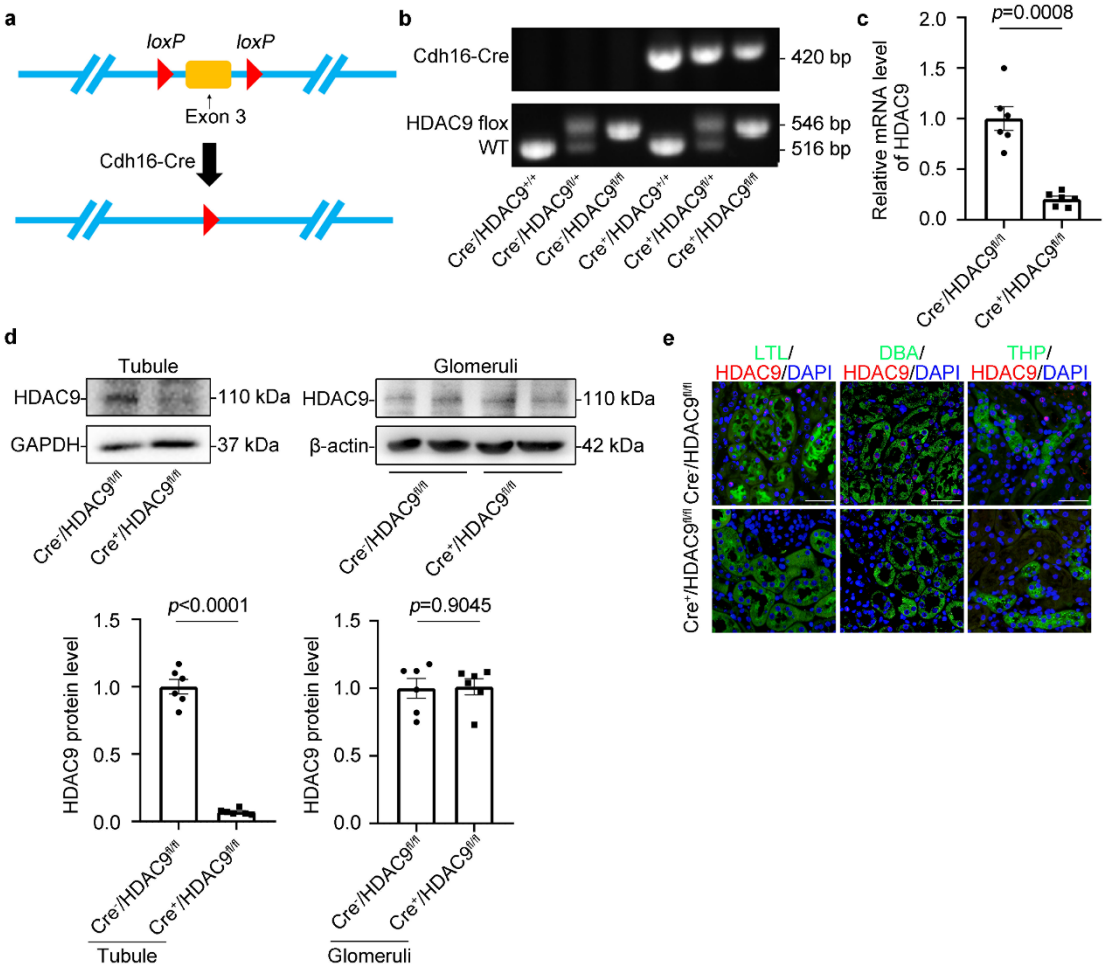
Supplementary Figure S1. HDAC9 was significantly increased in tubular epithelial cells. a. Photomicrographs and quantifications of Vimentin and α-SMA in kidney from AAN mice. Scale bar: black = 50 μm. (n = 12 mice per group). **b.** Relative mRNA level of *HDAC9* in the cortex of kidney from UIRI (n = 6 mice per group) and BIRI (n = 7 mice per group) mice. **c.** Photomicrographs and quantifications showing the expression of HDAC9 in the cortex of kidney from UIRI (n = 6 mice per group) and BIRI (n = 6 mice per group) mice. Bar: black = 50 μm; red = 20 μm. **d**. Representative Western blot gel documents and summarized data showing the relative protein levels of HDAC9 in human tubule epithelial cells (HK-2) with TGF-β1 treatment for 48 h. (n = 6 biologically independent experiments). Data are expressed as mean ± SEM (a-d). Two-tailed Student's unpaired t test analysis (a-c). One-way ANOVA followed by Tukey's post-test (d). Source data are provided as a Source Data file.



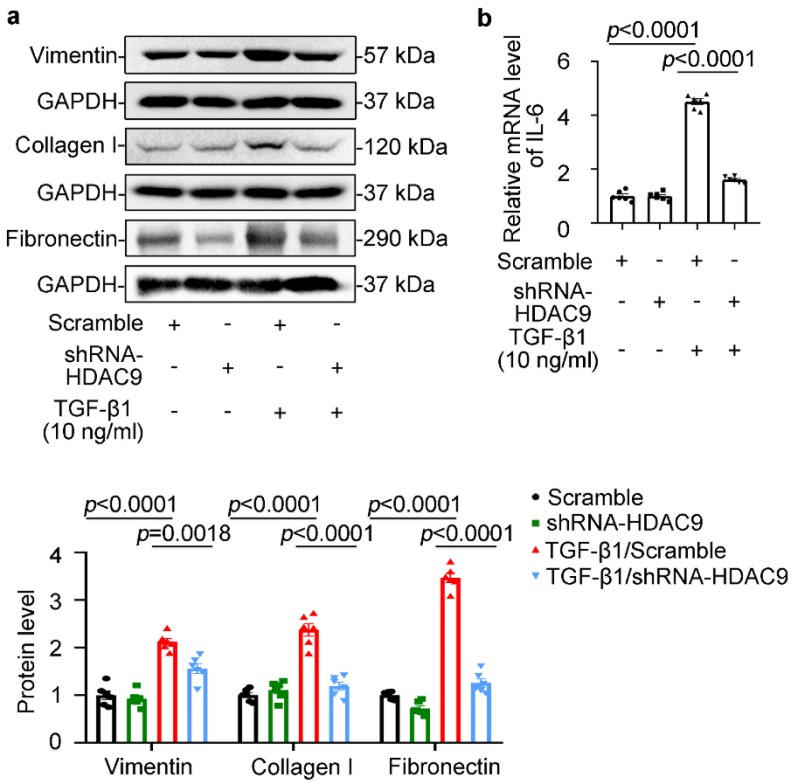
Supplementary Figure S2. Establishment of *HDAC9* knockout (*HDAC9*$^{-/-}$) mice. a. Scheme for *HDAC9* knockout mice. **b**. Genotyping was confirmed by tail preparation and PCR at 2 weeks of age. **b** was repeated five times independently with similar results. **c**. Representative Western blot gel documents and summarized data showing the relative protein levels of HDAC9 in the cortex of kidney from *HDAC9*$^{-/-}$ mice. (n = 5 mice per group). Data are expressed as mean ± SEM (c). Two-tailed Student's unpaired t test analysis (c). Source data are provided as a Source Data file.



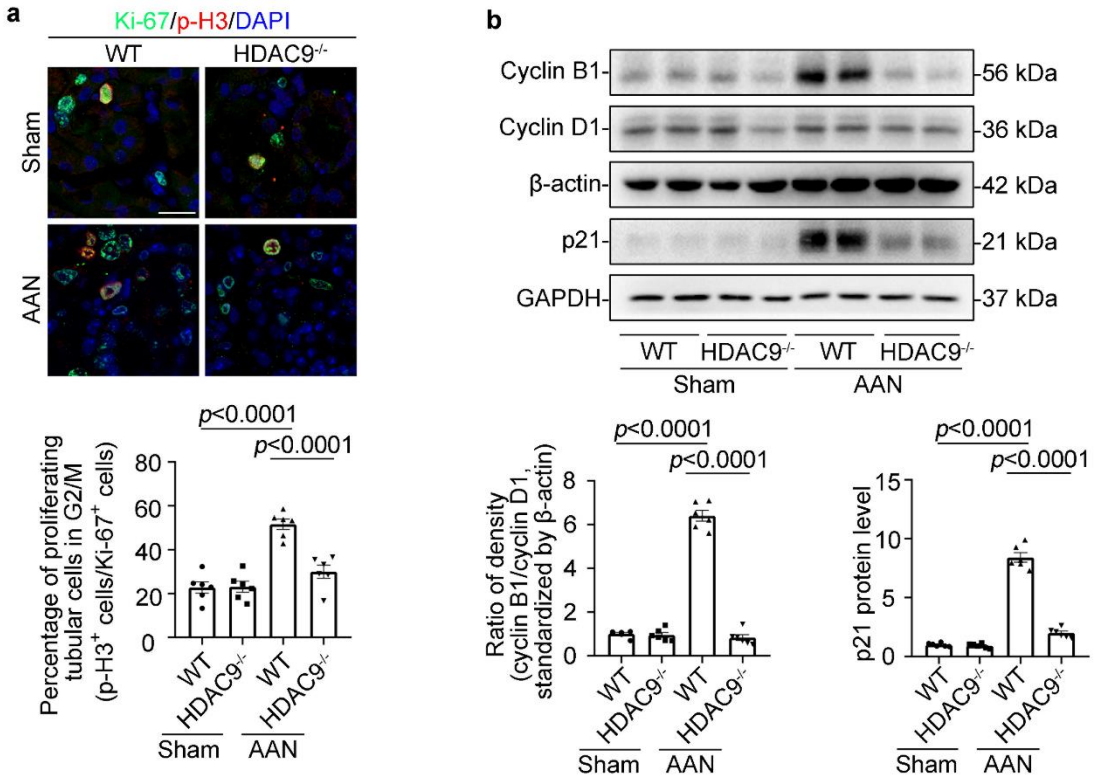
Supplementary Figure S3. *HDAC9* deficiency attenuated kidney fibrosis. a. H&E staining, Masson´s trichrome staining and Sirius Red staining were performed to assess the kidney injury and fibrosis in different groups. Scale bar: black = 50 μm. (n = 6 mice per group). **b.** Photomicrographs and quantifications showing the expression of collagen I and collagen IV in kidney from different groups of mice. Scale bar: black = 50 μm. (n = 6 mice per group). **c.** Representative Western blot gel documents and summarized data showing the relative protein levels of Vimentin, α-SMA and TGF-β1 in the cortex of kidney from different groups of mice. (n = 6 mice per group). Data are expressed as mean ± SEM (a-c). Two-way ANOVA followed by Tukey's post-test (a-c). Source data are provided as a Source Data file.



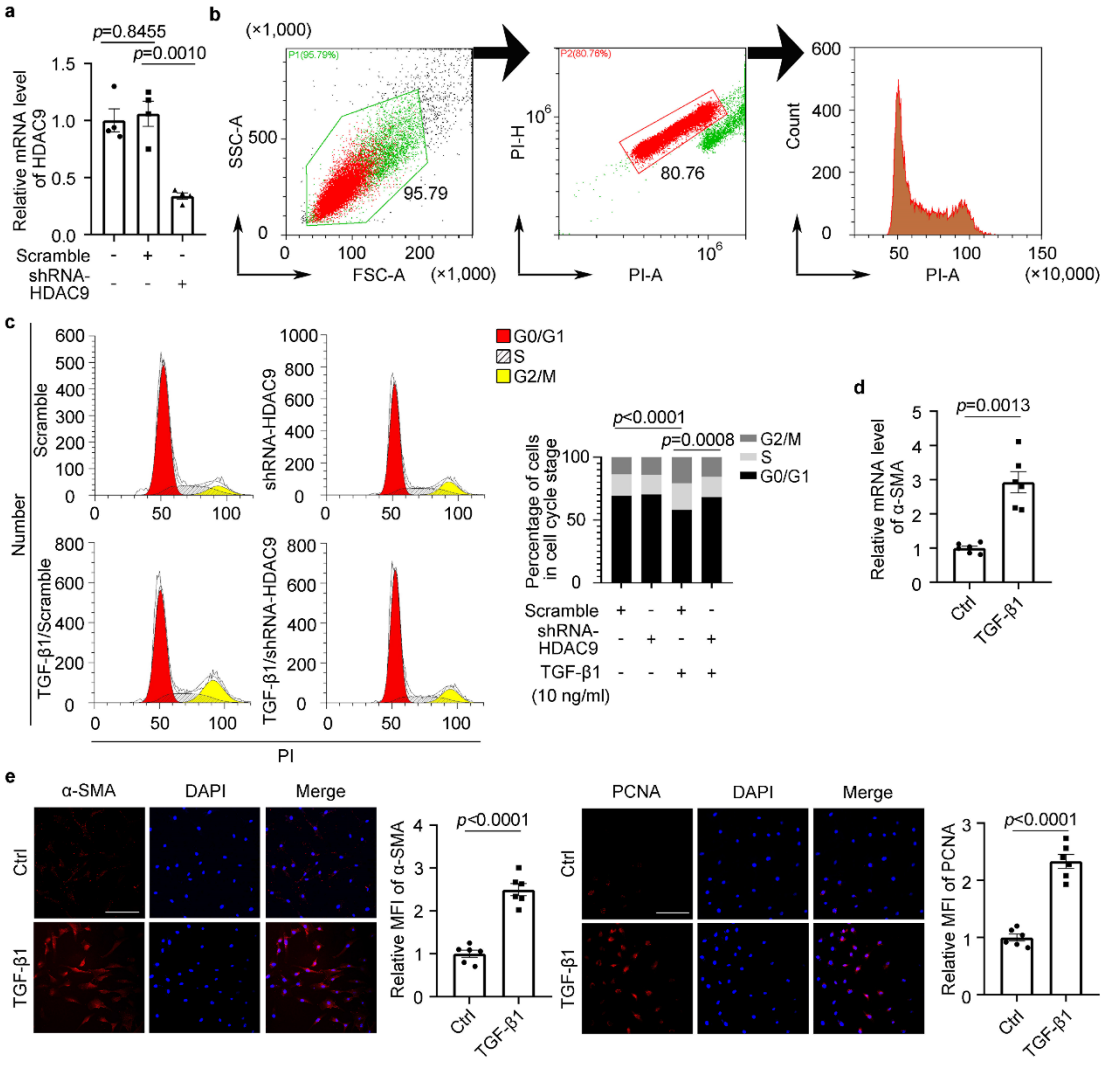
Supplementary Figure S4. Establishment of tubule-specific *HDAC9* knockout (*Cre*$^{+}$/*HDAC9*$^{fl/fl}$) mice. a. Generation of conditional knockout mice in which *HDAC9* is specifically ablated renal tubular cells by using *Cre–LoxP* recombination system. Exon 3 is deleted upon Cdh16-Cre–mediated recombination. **b**. Genotyping was confirmed by tail preparation and PCR at 2 weeks of age. This experiment was repeated five times independently with similar results. **c.** Relative mRNA level of *HDAC9* in the cortex of kidney from *Cre*$^{+}$/*HDAC9*$^{fl/fl}$ mice. (n = 6 mice per group) **d**. Representative Western blot gel documents and summarized data showing the relative protein levels of HDAC9 in isolated tubule or glomeruli from *Cre*$^{+}$/*HDAC9*$^{fl/fl}$ mice. (n = 6 mice per group). **e**. Photomicrographs showed that HDAC9 was deleted in renal tubules, including proximal tubule. Lotus tetragonolobus lectin (LTL) was used as a proximal tubular marker. Tamm-Horsfall glycoprotein (THP) was used as a marker for ascending loop of Henle; dolichos biflorus agglutinin (DBA) was used as a marker for collecting duct. Scale bar: white = 20 μm. This experiment was repeated three times independently with similar results. Data are expressed as mean ± SEM (c and d). Two-tailed Student's unpaired t test analysis (c-d). Source data are provided as a Source Data file.



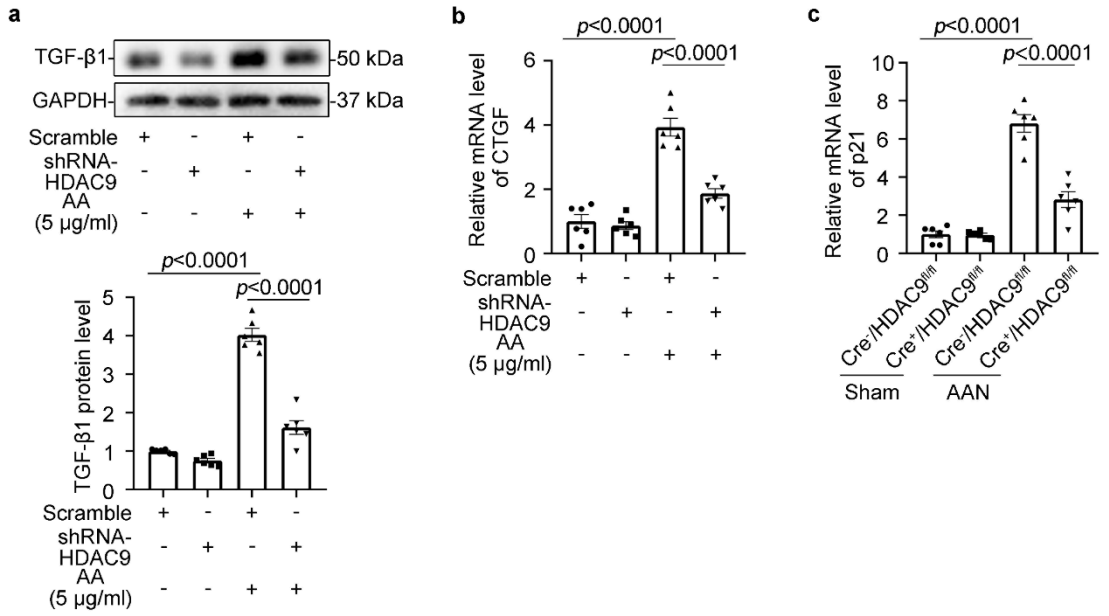
Supplementary Figure S5. *HDAC9* knockdown reduced the expression of markers associated with fibrosis and proinflammatory mediators in HK-2 with TGF-β1 treatment. a. Representative Western blot gel documents and summarized data showing the relative protein levels of Vimentin, Collagen I and Fibronectin in HK-2 with TGF-β1 treatments. (n = 6 biologically independent experiments). **b and c.** Relative mRNA level of *IL-6* and *TNFα* in HK-2 with different treatments. (n = 6 biologically independent experiments). Data are expressed as mean ± SEM (a-c). Two-way ANOVA followed by Tukey's post-test (a-c). Source data are provided as a Source Data file.



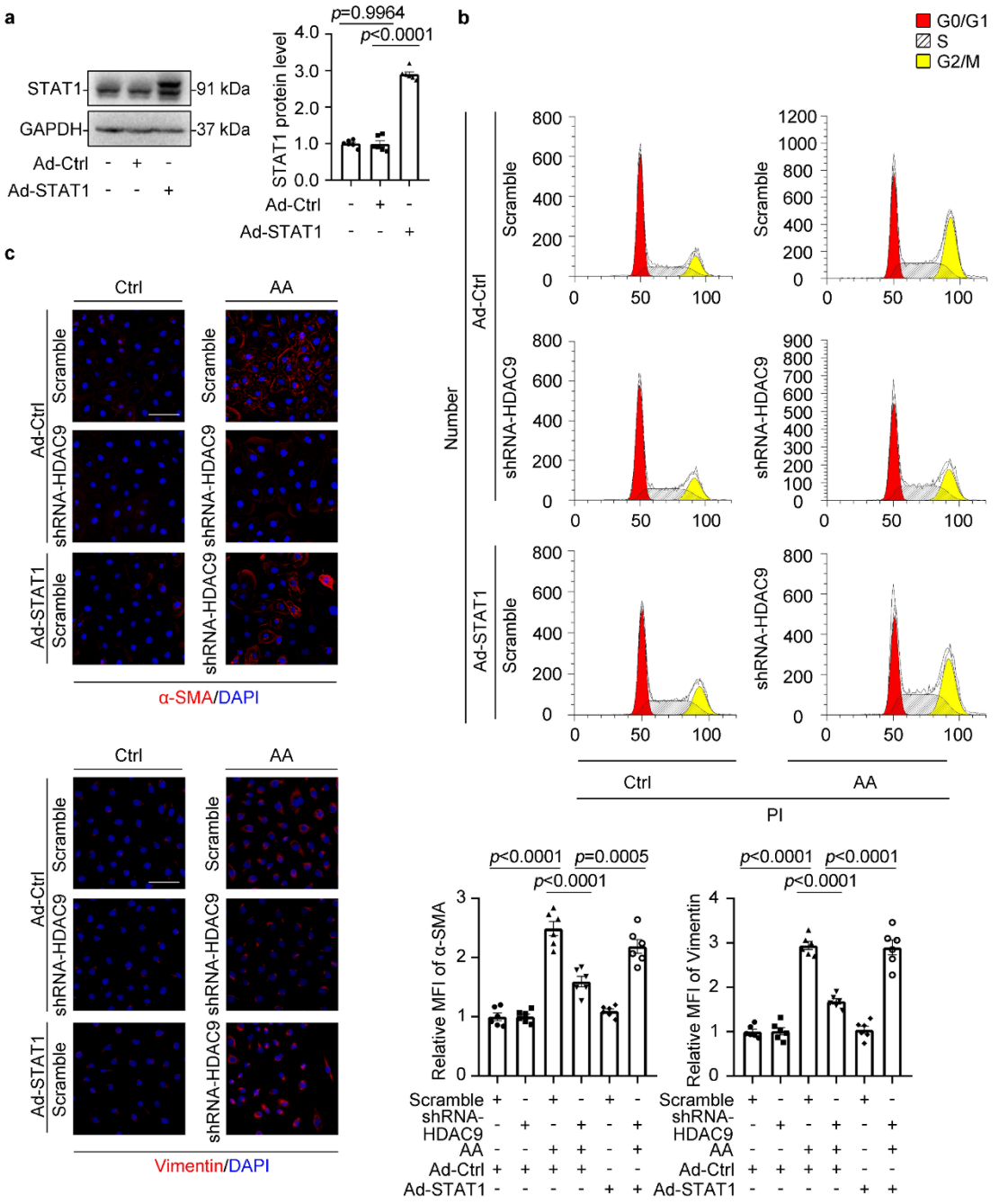
Supplementary Figure S6. *HDAC9* deficiency inhibited tubular epithelial cell G2/M arrest. a. Representative photomicrographs of coimmunostaining with antibodies to Ki-67 (anti–Ki-67) and p-H3 (anti–p-H3) on kidneys and the percentage of Ki-67$^+$ p-H3$^+$ cells among total Ki-67$^+$ tubular cells in different groups. Scale bar: white = 10 μm. (n = 6 mice per group). **b.** Representative Western blot gel documents and summarized data showing the ratio of cyclin B1 to cyclin D1 densities standardized to β-actin and the relative protein levels of p21 in the cortex of kidney from different groups of mice. (n = 6 mice per group). Data are expressed as mean ± SEM (a and b). Two-way ANOVA followed by Tukey's post-test (a and b). Source data are provided as a Source Data file.



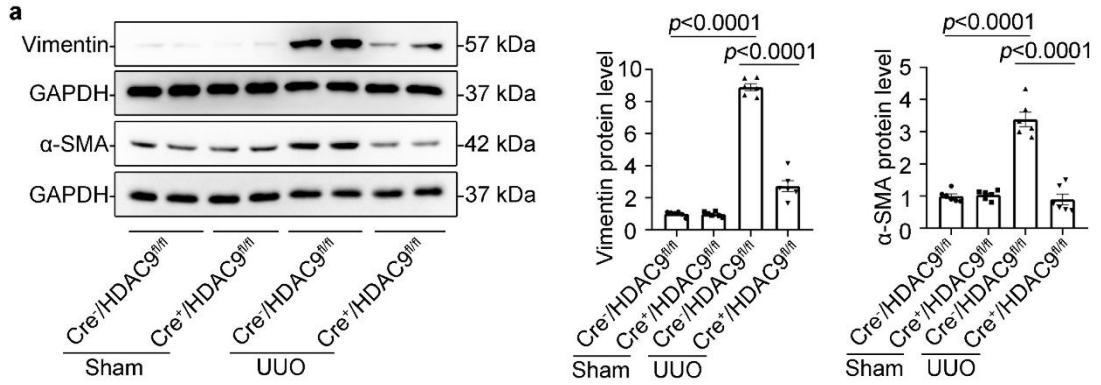
Supplementary Figure S7. HDAC9 contributed to epithelial cell cycle arrest in G2/M and activation of fibroblasts. a. The relative mRNA level of *HDAC9* in HK-2 with *HDAC9*-lentivirus transfection. (n = 4 biologically independent experiments). **b**. Representative gating strategy for analyzing the G2/M cell cycle arrest. **c.** Cell cycle analysis by flow cytometry for HK-2 in different groups. (n = 6 biologically independent experiments). Red: G0/G1; yellow: G2/M. **d**. Relative mRNA level of *α-SMA* in NRK-49F with different treatments. (n = 6 biologically independent experiments). **e.** Photomicrographs and quantifications showing the expression of α-SMA (red) and PCNA (red) in NRK-49F with TGF-β1 (5 ng/ml) treatment for 24h. Scale bar: white = 100 μm. (n = 6 biologically independent experiments). Data are expressed as mean ± SEM (a, c, d, e). Two-way ANOVA followed by Tukey's post-test (a and c). Two-tailed Student's unpaired t test analysis (d and e). Source data are provided as a Source Data file.



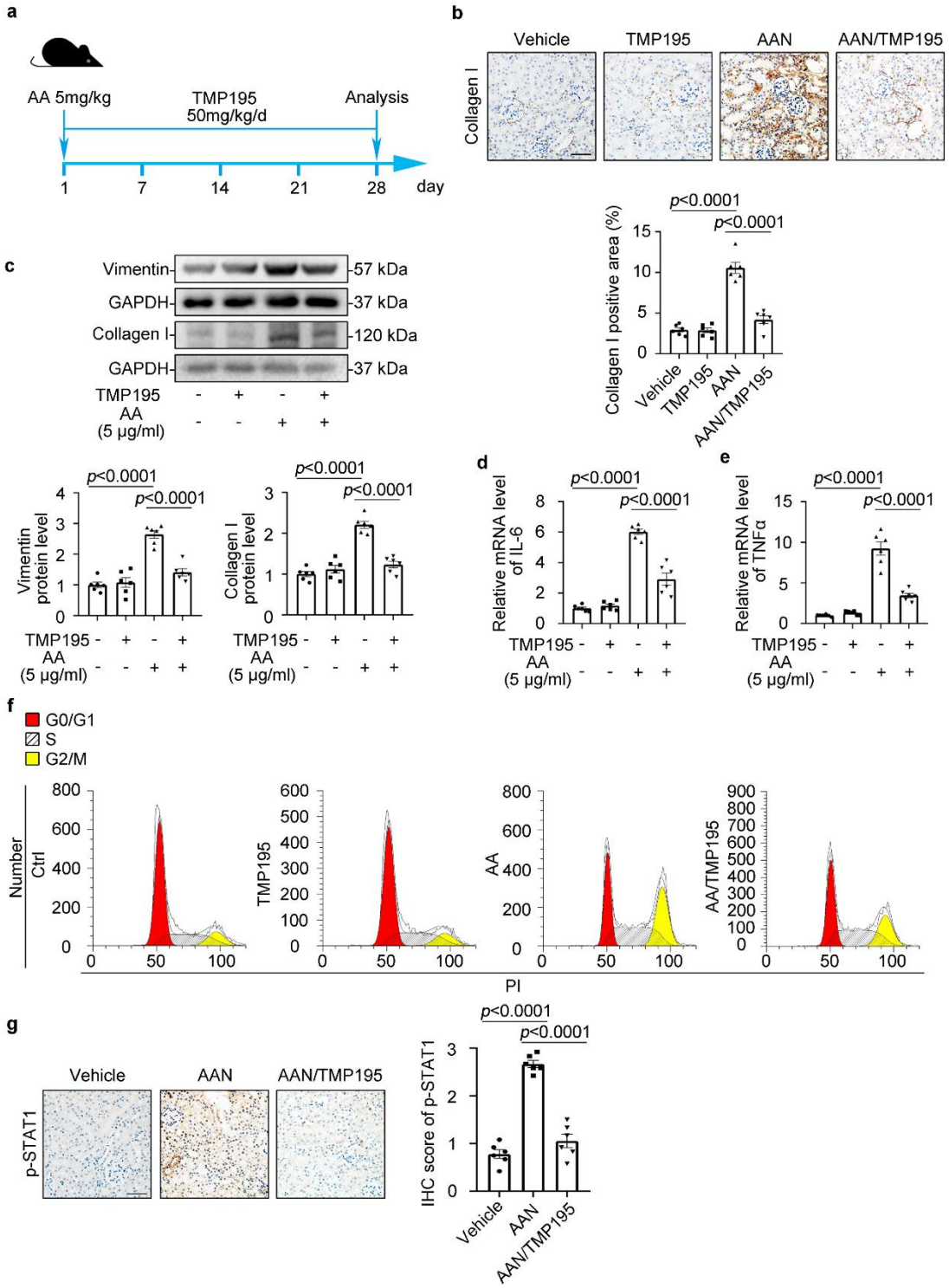
Supplementary Figure S8. *HDAC9* upregulated the expression of TGF-β1, CTGF and p21. a. Representative Western blot gel documents and summarized data showing the relative protein levels of TGF-β1 in HK-2 with AA treatment. (n = 6 biologically independent experiments). **b.** Relative mRNA level of *CTGF* in HK-2 with different treatments. (n = 6 biologically independent experiments). **c.** Relative mRNA level of *p21* in isolated tubules from different groups of mice. (n = 6 mice per group). Data are expressed as mean ± SEM (a-c). Two-way ANOVA followed by Tukey's post-test (a-c). Source data are provided as a Source Data file.



Supplementary Figure S9. STAT1 was involved in HDAC9-mediated fibrotic effect. a. Representative Western blot gel documents and summarized data showing the relative protein level of STAT1 in HK-2 with STAT1-adenovirus transfection. (n = 6 biologically independent experiments). **b**. Cell cycle analysis by flow cytometry for HK-2 in different groups. Red: G0/G1; yellow: G2/M. **c**. Photomicrographs and quantifications showing the relative protein levels of α-SMA, and Vimentin in HK-2 with different treatments. Scale bar: white = 50 μm. (n = 6 biologically independent experiments). Data are expressed as mean ± SEM (a and c). Two-way ANOVA followed by Tukey's post-test (a and c). Source data are provided as a Source Data file.

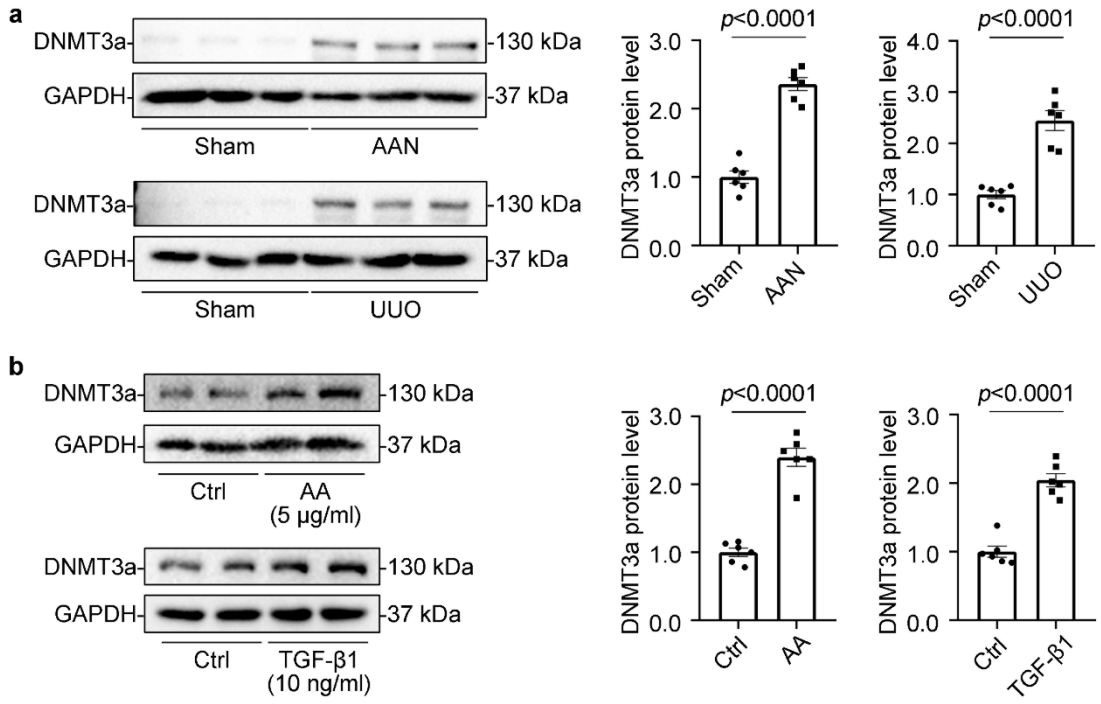


Supplementary Figure S10. Tubule-specific deletion of *HDAC9* reduced the expression of Vimentin and α-SMA. a. Representative Western blot gel documents and summarized data showing the relative protein levels of Vimentin and α-SMA in cortex of kidney from different groups of mice. (n = 6 mice per group). Data are expressed as mean ± SEM (a). Two-way ANOVA followed by Tukey's post-test (a). Source data are provided as a Source Data file.



Supplementary Figure S11. TMP195 attenuated kidney fibrosis and inhibited G2/M phase arrest in TECs. a. A schematic diagram showing TMP195 treatment in AAN mice. **b.** Photomicrographs and quantifications showing the expression of collagen I in kidney from different groups of mice. Scale bar: black = 50 μm. (n = 6 mice per group). **c.** Representative Western blot gel documents and summarized data showing the relative protein levels of Vimentin and Collagen I in HK-2 with AA treatments. (n = 6 biologically independent experiments). **d and e.** Relative mRNA

level of *IL-6* and *TNFα* in HK-2 with different treatments. (n = 6 biologically independent experiments). **f**. Cell cycle analysis by flow cytometry for HK-2 in different groups. **g.** Photomicrographs and quantifications showing the protein levels of p-STAT1 in kidney from different groups of mice. Scale bar: black = 50 μm. (n = 6 mice per group). Data are expressed as mean ± SEM (b, c, d, e and g). Two-way ANOVA followed by Tukey's post-test (b-e and g). Source data are provided as a Source Data file.



Supplementary Figure S12. DNMT3a was increased in fibrotic kidneys and HK-2 with AA or TGF-β1 treatment. a. Representative Western blot gel documents and summarized data showing the relative protein levels of DNMT3a in the cortex of kidney from AAN or UUO mice. (n = 6 mice per group). **b.** Representative Western blot gel documents and summarized data showing the relative protein levels of DNMT3a in HK-2 with different treatments. (n = 6 biologically independent experiments). Data are expressed as mean ± SEM (a and b). Two-tailed Student's unpaired t test analysis (a and b). Source data are provided as a Source Data file.