MATERIALS AND METHODS

Antibodies and agents

Antibodies against acetyl-H3 (9649S) and E-cadherin (14472S) were purchased from Cell Signaling Technology (MA, USA). Antibodies against collagen I (ab34710), α-SMA (ab5694), and GPX4 (ab125066) were obtained from Abcam (MA, USA). Antibodies for Ngal (sc-515876), HDAC4 (sc-46672), and GAPDH (sc-137179) were purchased from Santa Cruz Biotechnology (CA, USA). The antibody for KIM-1 (AF1817) was obtained from R&D Systems (MN, USA), and the antibody for vimentin (V5255) was purchased from Sigma-Aldrich (MO, USA). Antibodies for p-H3 (NB21-1091) and fibronectin (NBP1-91258) were from Novus Biologicals (CO, USA). Antibodies against Foxo3a (A0102), phospho-Foxo3a (Ser253; AP0684), ACSL4 (A20414), and Sirt3 (A7307) were purchased from ABclonal (Wuhan, China). Secondary antibodies (SA00001-1, SA00001-2, SA00001-4) were obtained from Proteintech Group (Shanghai, China). The acetyl-lysine antibody (PTM-105RM) was purchased from PTM Biolabs (Hangzhou, China). Tasquinimod was purchased from Selleckchem (Houston, TX, USA). CMC-Na was purchased from Sigma-Aldrich (MO, USA). Malondialdehyde (MDA) assay kit, glutathione (GSH) assay kit, creatinine (Cr) assay kit, and blood urea nitrogen (BUN) assay kit were all obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and generation of renal tubule–specific HDAC4 knockout mice

C57BL/6 male mice were purchased from Shanghai JISJIE Experimental Animal Co., LTD. (Shanghai, China). HDAC4fl/fl and Cdh16-Cre mice (Cdh16-CreER$^{+/-}$) were purchased from Shanghai Nanfang Model Biotechnology Co., LTD. (Shanghai, China). HDAC4 knockout mice were generated by breeding HDAC4fl/fl with Cdh16-Cre $^{\pm}$ mice to obtain Cdh16-Cre^{+}:

HDAC4fl/fl mice (HDAC4-KO) and Cdh16-Cre^{-}: HDAC4fl/fl mice (HDAC4-WT). All animals were housed under standard conditions (12-hour light/dark cycle) with ad libitum access to food and water at the Animal Experimental Center of Tongji University. Mice were used at 8 weeks of age, weighing 20–25 g. All animal experiments were reviewed and approved by the Animal Care and Use Committee of Tongji University.

Ischemia-reperfusion (IR)-induced AKI and CKD models

AKI was induced by unilateral ischemia-reperfusion. Briefly, the right renal artery and ureter were ligated, the right kidney was removed, and the left renal artery was clamped for 23 minutes at 37 °C. In the control group, only the right kidney was removed while the left kidney remained intact. To establish the IR-CKD model, the left renal artery was clamped for 45 minutes at 37 °C while preserving the contralateral kidney. In the corresponding sham group, the right renal artery was exposed but not clamped. At least six mice were included per group. For experiments with HDAC4 knockout mice in the IR-AKI model, animals were randomly divided into four groups: (i) Cdh16-Cre^{-}: HDAC4fl/fl-Sham group; (ii) Cdh16-Cre^{+}: HDAC4fl/fl-Sham group; (iii) Cdh16-Cre^{-}: HDAC4fl/fl-IR group; (iv) Cdh16-Cre^{+}: HDAC4fl/fl-IR group. For experiments with HDAC4 knockout mice in IR-CKD model, HDAC4 knockout mice were also divided into the same four groups as described above.

For in vivo tasquinimod experiments in AKI, C57BL/6 mice were randomly assigned to four groups: (i) sham-operated group; (ii) tasquinimod-treated sham group; (iii) IR group; and (iv) IR + tasquinimod-treated group. Tasquinimod was dissolved in 5% CMC-Na and administered by gavage at 25 mg/kg/day, starting immediately after reperfusion. Mice receiving only 5% CMC-Na served as vehicle controls. Surgical procedures in knockout mice were identical to those in

tasquinimod-treated C57BL/6 mice. All AKI model mice were euthanized with phenobarbital (100 mg/kg) at 48 hours post-surgery, and kidneys and blood were collected for analysis.

For in vivo tasquinimod experiments in IR-CKD, mice were also randomly assigned to four groups: (i) sham-operated group; (ii) tasquinimod-treated sham group; (iii) IR group; and (iv) IR + tasquinimod-treated group. Tasquinimod was administered by gavage at 25 mg/kg/day starting 48 hours after reperfusion and continued daily until day 28; mice receiving only 5% CMC-Na served as vehicle controls. HDAC4 knockout mice were divided into the same four groups as described above, with identical surgical procedures. All CKD model mice were euthanized on day 28, and kidneys and blood were collected for further analysis.

Folic acid-induced AKI and CKD

To establish folic acid–induced AKI and CKD models, mice received a single intraperitoneal injection of folic acid at a dose of 250 mg/kg, dissolved in 0.3 M $NaHCO_3$. Control mice were injected with an equivalent volume of 0.3 M $NaHCO_3$ alone. To assess the impact of AKI on progression to CKD, kidney samples were collected 48 hours after folic acid injection for AKI evaluation and at 28 days for CKD assessment. Each experimental group included at least six mice.

Cell culture and treatment

Mouse renal proximal tubular epithelial cell lines (mTECs) (Dr. Jeffrey B., Kopp of the National Institutes of Health) were cultured in DMEM with F12 containing 10% fetalbovine serum (FBS) and 0.5% penicillin in an atmosphere of 5% CO2at 37°C. To determine the effect of HDAC4 inhibition on TGF-β1-induced pEMT, siRNA transfection was performed at mTECs densities of 70%. 24 hours after transfection

mTECs were incubated with TGF-β1 (5 ng/ml) for 48 hours.

Transfection of siRNA

mTECs were seeded in medium containing 10% FBS and cultured to reach 60%–70% confluence, after which the medium was replaced with serum-free OPTI-MEM. HDAC4-specific siRNA (75 pmol) was transfected into each well using the GenePharm Transfection Reagent, following the manufacturer's instructions. A scrambled siRNA (75 pmol) was used as a negative control to account for off-target effects. Twenty-four hours after transfection, cells were treated with TGF-β1 (5 ng/ml) for 48 hours and then harvested for further analysis.

Nuclear and cytoplasmic extraction

The nuclear and cytoplasmic fractions of treated mTECs were collected using an extraction kit (Vazyme, CHN) according to the manufacturer's instructions. Proteins obtained by extraction can be used for Western Blot. Specific protein markers GAPDH and lamin B1 were used to detect the purity of non-nuclear fraction and nuclear fraction.

Renal Function Analysis

Serum was prepared by centrifuging whole blood at 1,500 rpm for 15 minutes at 4 °C. Serum creatinine (Scr) levels were measured using a creatinine detection kit, and serum blood urea nitrogen (BUN) levels were determined using a BUN assay kit, following the manufacturers' instructions.

Determination of Glutathione (GSH) and malondialdehyde (MDA)

GSH and MDA levels in mouse kidney tissues were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. Tissue samples were centrifuged at 8,000 × *g* at 4 °C, and the resulting supernatant

was used for the assays. GSH levels were determined by measuring absorbance at 420 nm using a microplate reader. For MDA measurement, the supernatant was mixed with the MDA reaction reagent, boiled in a water bath for 1.5 hours, and then the absorbance was measured at 532 nm using a microplate reader.

Western Blot Analysis

Kidney tissue samples were homogenized in RIPA buffer containing phosphatase and protease inhibitors to extract total protein. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat milk for 1 hour at room temperature, incubated with the appropriate primary antibodies overnight at 4 °C, and then incubated with the corresponding secondary antibodies for 1 hour at room temperature. Protein bands were visualized and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Pathological Assessment

Kidney tissues were fixed in formalin, embedded in paraffin, and sectioned at a thickness of approximately 3–5 μm. Periodic acid–Schiff (PAS) staining and Masson's trichrome staining were performed according to standard protocols to evaluate renal injury and fibrosis, respectively. The severity of tubular injury and the extent of renal fibrosis were assessed by light microscopy in at least 10 randomly selected fields per kidney section. Tubular injury was scored as previously described: 0, none; 1, <10%; 2, 10–25%; 3, 26–50%; 4, 51–75%; and 5, ≥75%[1].

Immunofluorescence

Immunofluorescence staining of kidney tissues was performed as described in our previous

study[2]. Briefly, kidney sections were incubated with primary antibodies against Ngal (sc-515876), α-SMA (ab5694), HDAC4 (sc-46672), p-H3 (NB21-1091), Acsl4 (A20414), Foxo3a (A0102), phospho-Foxo3a (Ser253; AP0684), and GPX4 (ab125066), as well as Lotus tetragonolobus lectin (LTL)–fluorescein (#L32480, Thermo Fisher). After primary incubation, sections were incubated with appropriate fluorescence-labeled secondary antibodies. Images were acquired randomly using an Olympus BX53 microscope at ×200 magnification, and fluorescence intensity was quantified with ImageJ software (NIH, USA).

For cell staining, mTECs with different treatments were cultured on coverslips, fixed with 4% paraformaldehyde, and blocked at room temperature for 1 hour in blocking buffer (5% sheep serum in PBS). Cells were then incubated overnight at 4 °C with the primary antibody, washed three times with PBS, and incubated with the appropriate fluorescence-labeled secondary antibody for 1 hour at 37 °C. Nuclei were counterstained with DAPI. Immunofluorescence images were acquired using a ZEISS digital fluorescence microscope.

Co-immunoprecipitation (Co-IP) Assay

Total protein was extracted from mTECs treated with or without TGF-β1 stimulation for 48 hours using cell lysis buffer. To assess the binding of HDAC4 to Foxo3a and the acetylation level of cytoplasmic Foxo3a, co-immunoprecipitation was performed. After 24 hours of HDAC4 transfection or control treatment, mTECs were stimulated with TGF-β1 for 48 hours, and cytoplasmic proteins were collected. The interaction between Foxo3a and acetyl-lysine (ace-K) was examined by immunoprecipitation using Protein A/G Magnetic Beads (HY-K0202) following the manufacturer's protocol. Beads were washed five times with PBST (0.05% Tween-20), and immunoprecipitated complexes were eluted with 1× SDS loading buffer by heating in a metal bath at 100 °C.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed using the SimpleChIP Plus Enzymatic Chromatin IP Kit (Magnetic Beads) (#9005, Cell Signaling Technology) following the manufacturer's instructions with minor modifications. Briefly, minced kidney tissues were cross-linked in phosphate-buffered saline (PBS) containing 1.5% formaldehyde and 1× protease inhibitor cocktail (PIC) at room temperature for 20 minutes. Chromatin was enzymatically digested with Micrococcal Nuclease and further sonicated to yield soluble chromatin fragments of 200–500 base pairs. A 2% aliquot of chromatin was saved as input control. Immunoprecipitations were carried out overnight at 4 °C using antibodies against HDAC4 (sc-46672, Santa Cruz), Foxo3a (A0102, ABclonal), Histone H3 (#4620, Cell Signaling Technology), or IgG (#2729, Cell Signaling Technology) as a negative control. Protein-DNA complexes were captured with ChIP-Grade Protein G Magnetic Beads (#9006), eluted, and reverse cross-linked. After protease K treatment, DNA was purified using DNA purification columns and analyzed by PCR with primers targeting the GPX4 promoter region:

Forward primer: GGGCTCTTTAAGGGGATGAC

Reverse primer: GTTATCCGCGAGGTTGCCTG

Quantitative PCR data were normalized to 1% input chromatin DNA and expressed as fold enrichment relative to IgG controls. Input control represents non-immunoprecipitated total chromatin DNA.

Statistical Analyses

Data are presented as mean ± the standard error of the mean (SEM). Intergroup comparisons were performed using one-way ANOVA. Statistical significance was indicated in the figures, with $p < 0.05$ considered statistically significant.

References

[1] K. Yamada, T. Miwa, J.N. Liu, M. Nangaku, and W.C. Song. Critical protection from renal ischemia reperfusion injury by CD55 and CD59. Journal of Immunology 172, 3869-3875 (2004)

[2] M. Pang, J. Kothapally, H. Mao, E. Tolbert, M. Ponnusamy, Y.E. Chin, et al. Inhibition of histone deacetylase activity attenuates renal fibroblast activation and interstitial fibrosis in obstructive nephropathy. Am J Physiol Renal Physiol 297, F996-F1005 (2009)

Figure S1

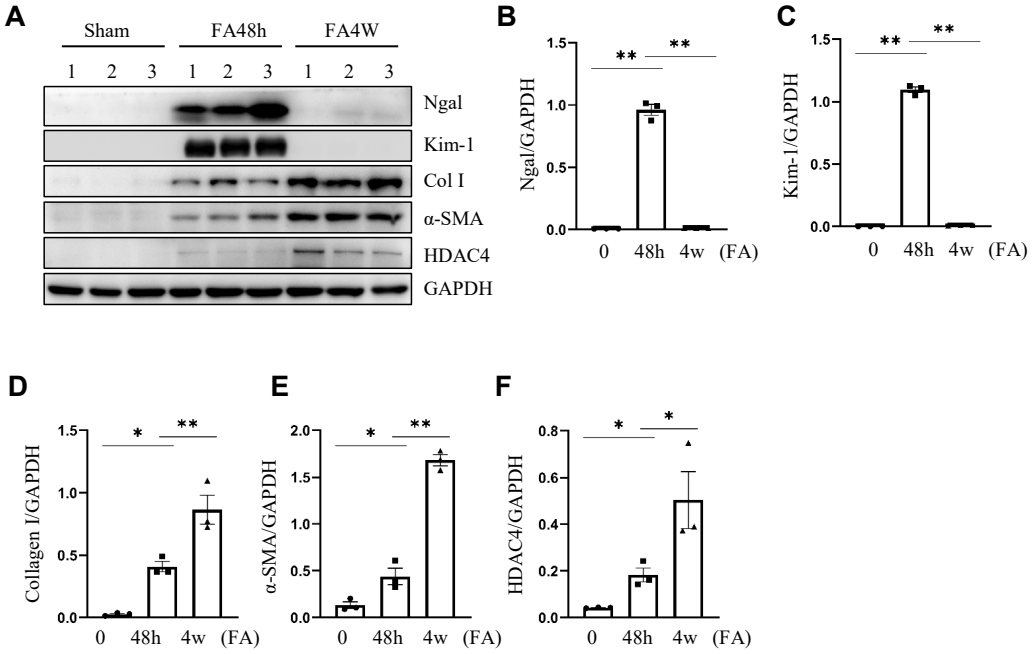

Figure S1. The kidney tissues collected at 48 h and 4weeks after sham and FA injury in C57/black mice. (A) Western blot was performed to detect the protein levels of Ngal, Kim-1, collagen I, α-SMA, HDAC4 and GAPDH. Expression levels of Ngal (B), Kim-1 (C), collagen I (D), α-SMA (E) and HDAC4 (F) were quantified by densitometry and normalized using GAPDH. Values are presented as mean ± SEM of three samples. *p < 0.05; **p < 0.01.

Figure S2

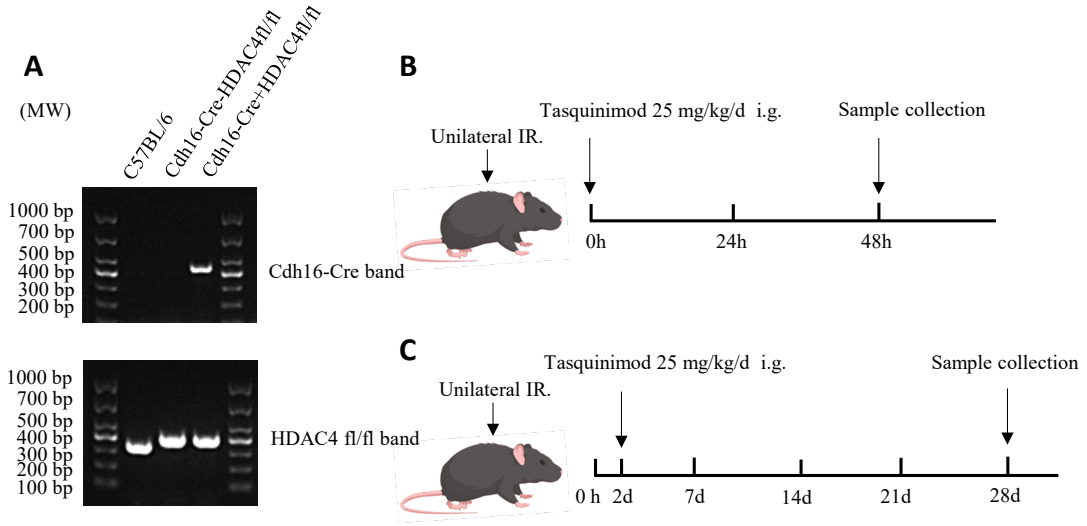

Figure S2. (A) Generation of conditional knockout mice in which HDAC4 was specifically ablated in tubular epithelial cells by using Cre-LoxP recombination system. Genotyping was confirmed by tail preparation and PCR at 2 weeks of age.

(2) Schematic of the experimental design for delayed treatment with tasquinimod. To investigate the therapeutic effect of tasquinimod on the IR mouse model for 48 hours, tasquinimod was administered intragastrically at a dose of 25mg/kg immediately after the operation, and was given continuously daily. The animals were euthanized at 48 hours to collect kidney samples. (3) Schematic of the experimental design for delayed treatment with tasquinimod. In a mouse model of renal fibrosis induced by 4 weeks of IR, tasquinimod 25 mg/kg was given by gavage 48 hours after IR and continued to be administered daily until day 28. Kidney samples were collected on the 28th day.

Figure S3

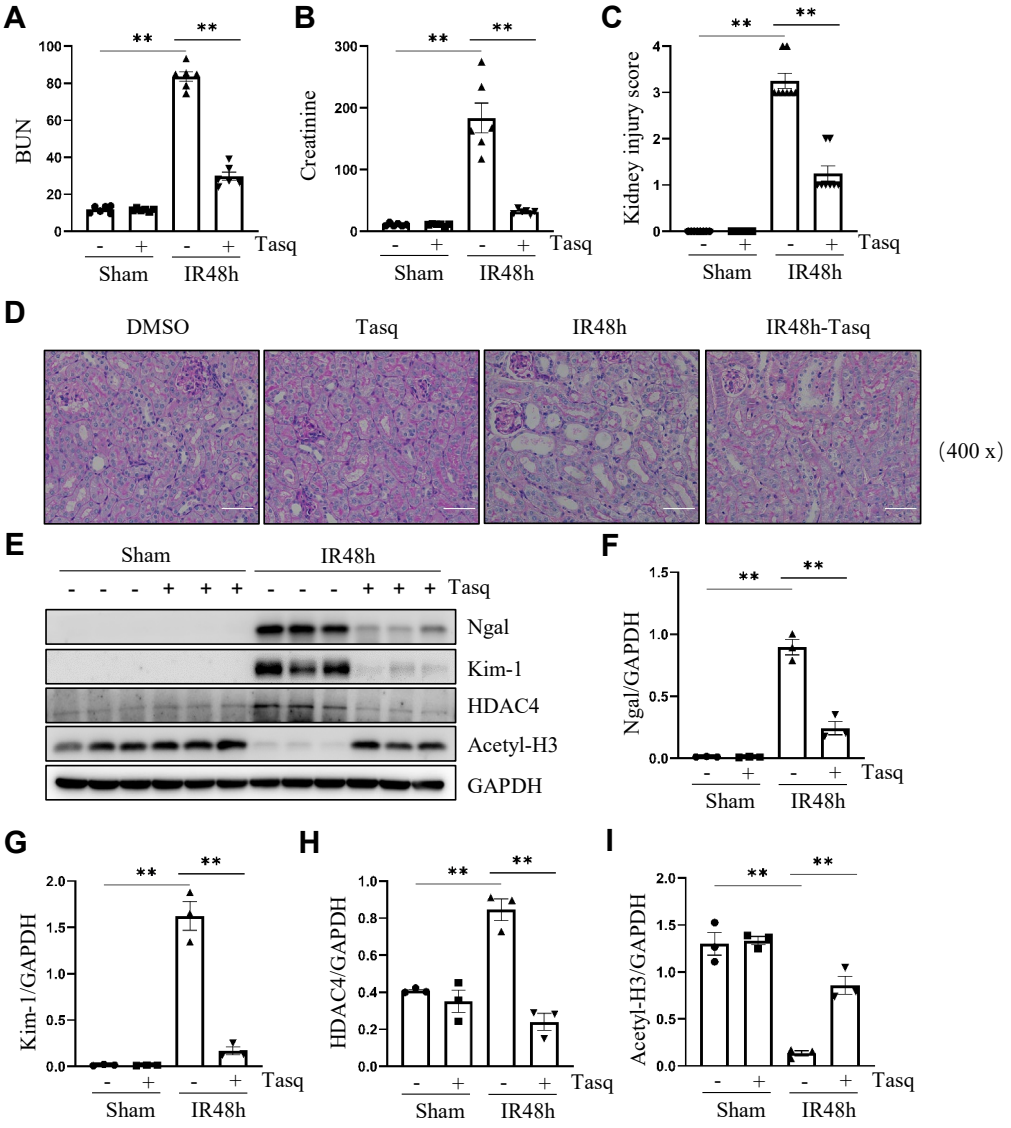

Figure S3. Pharmacologic inhibition of HDAC4 attenuates IR-induced AKI. (A, B) After various treatments as indicated, blood urea nitrogen (BUN) and serum creatinine levels were measured. (C, D) The kidneys injured by IR. Periodic acid-Schiff (PAS) staining revealed kidney structure damage in mice from each group. Data are presented as mean± SEM (n=6 per group). Scale bar = 50μm. Tubule injury scores of renal tissue were assessed in each group. (E)The kidney tissue lysates were subjected to immunoblot analysis with antibodies against Ngal, Kim-1, HDAC4, acetyl-histone 3, and GAPDH. Expression levels of Ngal(F), Kim-1(G), HDAC4(H)and acetyl-histone 3 (I) were quantified by densitometry and normalized using GAPDH. Values are presented as mean ± SEM of three samples. *$p < 0.05$; **$p < 0.01$.

Figure S4

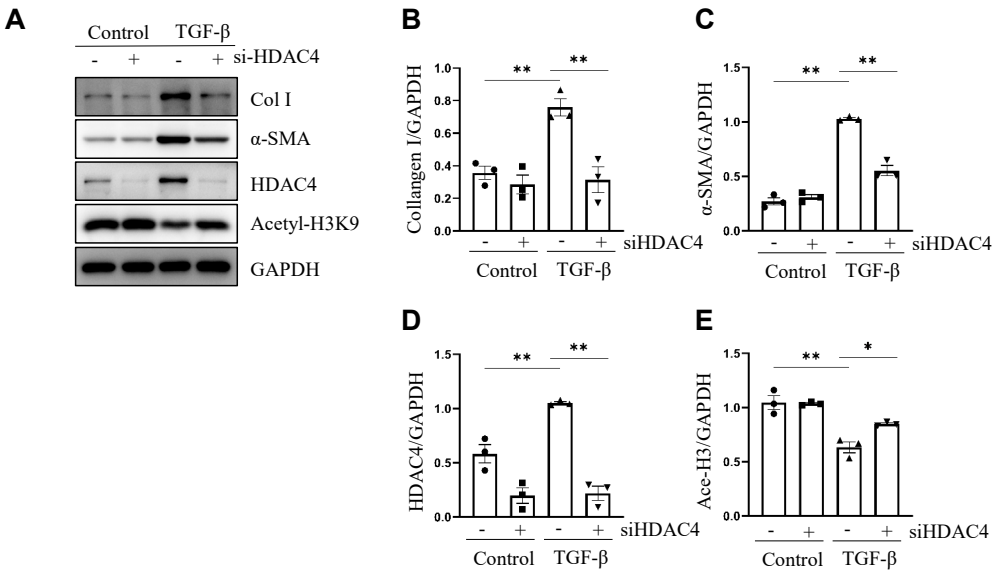

Figure S4. (A) Cell lysates transfected with HDAC4 siRNA and then stimulated with TGF-β1 were subjected to immunoblotting analysis with antibodies against collagen I, α-SMA, HDAC4, acetyl-histone 3 and GAPDH. Expression levels of collagen I (B), α-SMA (C), HDAC4 (D), acetyl-histone 3 (E) were quantified by densitometry and normalized with GAPDH. Values are presented as mean ± SEM of three samples. *$p < 0.05$; **$p < 0.01$.

Figure S5

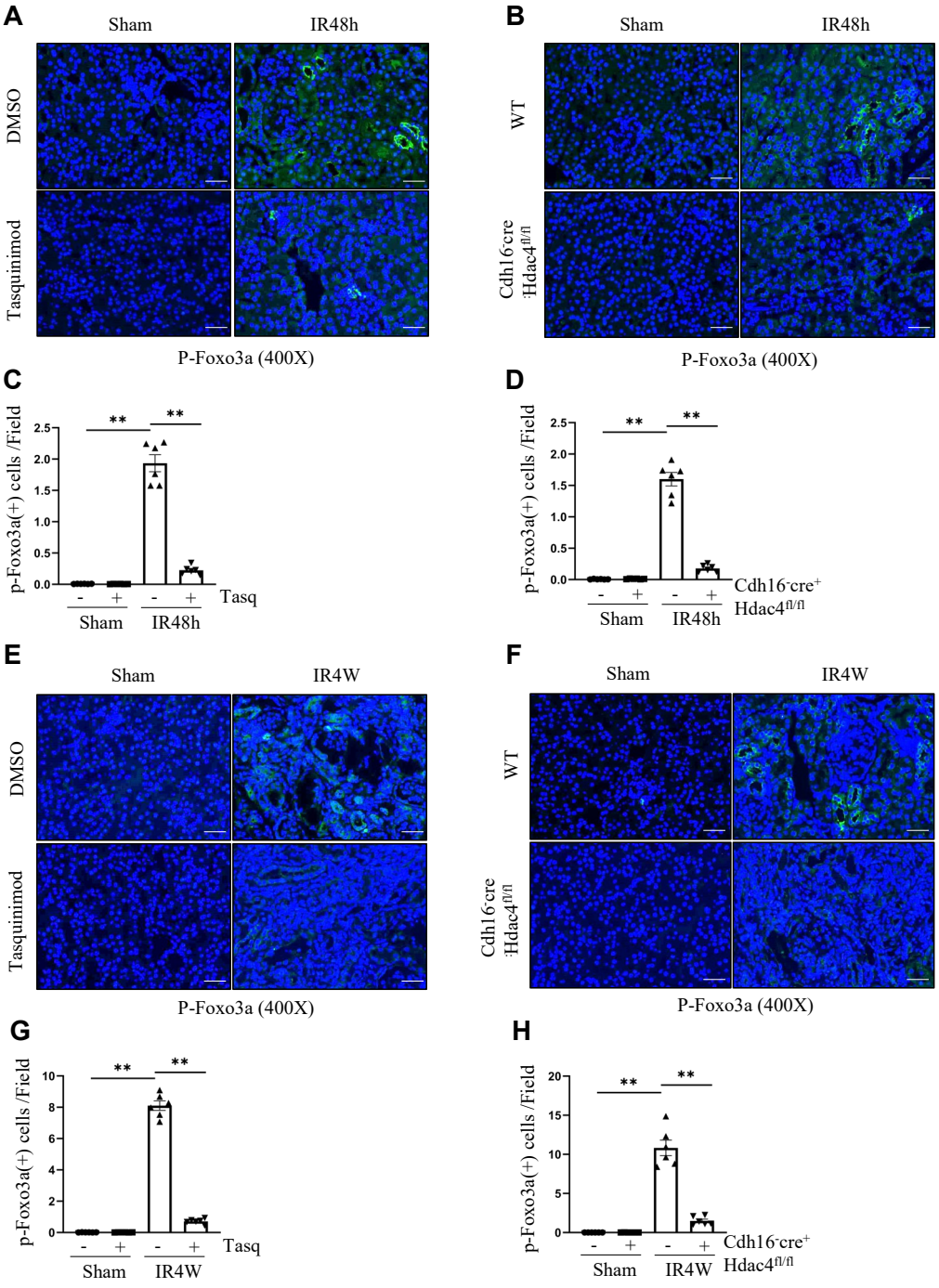

Figure S5. (A) The kidney tissues collected at 48 h after sham and IR injury with or without tasquinimod administration in C57/black mice. Micrographs of p-Foxo3a immunofluorescence staining, magnification ×400, Scale bar = 50 μm. (B) Kidney tissues were collected from the injury areas of HDAC4-WT and HDAC4-KO mice at 48 hours after sham operation and ischemia-reperfusion injury. Micrographs of p-Foxo3a immunofluorescence staining, magnification ×400, Scale bar = 50 μm. (C, D)

The percentage of p-Foxo3a (+) areas was counted in 6 high-power fields. (E) The kidney tissues collected at 4 weeks after sham and IR injury with or without tasquinimod administration in C57/black mice. Micrographs of p-Foxo3a immunofluorescence staining, magnification ×400, Scale bar = 50 μm. (F) Kidney tissues were collected from the injury areas of HDAC4-WT and HDAC4-KO mice at 4 weeks after sham operation and ischemia-reperfusion injury. Micrographs of p-Foxo3a immunofluorescence staining, magnification ×400, Scale bar = 50 μm. (G, H) The percentage of p-Foxo3a (+) areas was counted in 6 high-power fields. Data are presented as mean ± SEM (n = 6 per group). *$P < 0.05$; **$P < 0.01$.

Figure S6

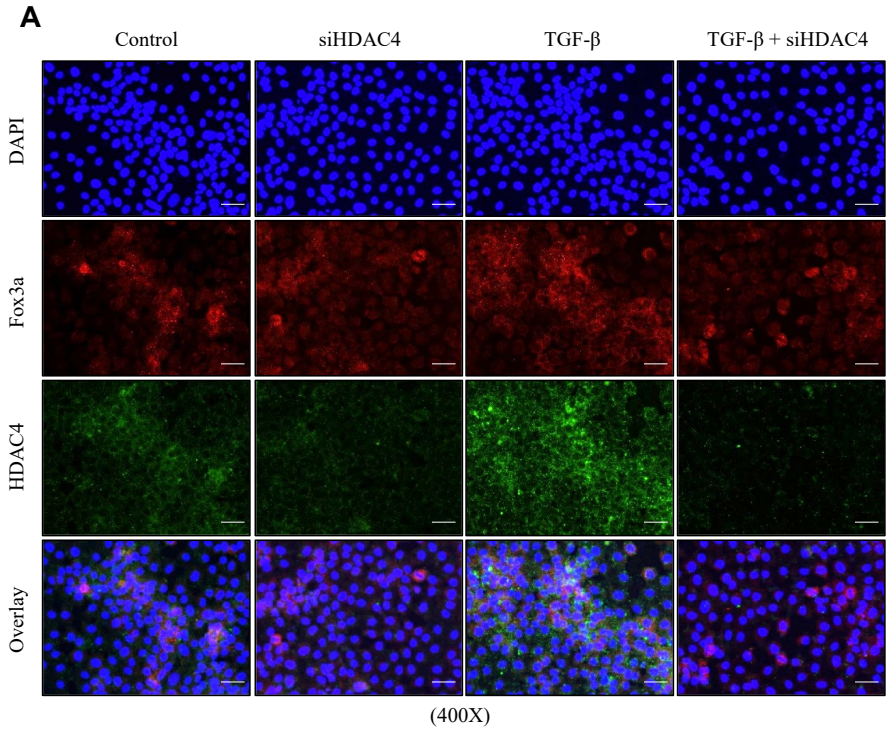

Figure S6. Murine renal tubular epithelial cells were transfected with con-siRNA or siHDAC4 siRNA for 24 h, and then exposed to TGF-β1 for 48 h. Co-immunofluorescence staining of Foxo3a and HDAC4 in mTECs to assess the co-localization of HDAC4 and Foxo3a.