ONLINE SUPPLEMENT

Materials and methods

Cell culture

Conditionally immortalized human podocytes were kindly provided by Dr. Moin A. Saleem (Academic Renal Unit, Southmead Hospital, Bristol, UK).Podocytes were cultured at 33 °C in medium that consisted of RPMI 1640 medium (HyClone, USA) with 10% fetal bovine serum (BI, Israel), 100 μg /mL streptomycin, 100 U/mL penicillin G, and 1×insulin-transferrin- selenium (ITS) (Invitrogen, USA) for proliferation. To differentiate, the cells were cultured at 37°C for 10-14 days with ITS-free medium. The differentiated cells were stimulated for 24 h with normal glucose (NG) (5 mM), HG (30 mM) or hypertonic solution (5 mM glucose combined with 25 mM mannitol). All of the experimental results were confirmed in at least three independent cultures of podocytes.

Transfection

The transfection of the Sirt6 plasmid (Addgene) was conducted using the X-tremeGENE HP DNA Transfection Reagent (Roche) in terms of the manufacturer's instructions. A density of 2×10^5 cells was first seeded in each well of a six-well plate and then transfected with complexes containing 2 μg of WT Sirt6 plasmid or Sirt6 H133Y mutant plasmids or a negative control with pcDNA3.1 and 2 μl of the X-tremeGENE transfection reagent. Then, the cells were incubated under normal condition for 48 h at 37°C.

Western immunoblotting

Total protein from the glomeruli and podocytes was extracted with RIPA buffer (Beyotime, China) mixed with a protease inhibitor cocktail (Sigma-Aldrich, USA). The extractions were then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatants were mixed with loading buffer prior to being boiled at 100°C for 5 min. Equal amounts of protein samples were separated through SDS-PAGE and then transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with milk for 1-2 h before incubation with a primary antibody (Sirt6 rabbit monoclonal antibody, 1:1000, Abcam; Sirt1 rabbit polyclonal antibody, 1:1000, Immunoway ; p-AMPKα1/2 (Thr172) rabbit polyclonal antibody, 1:100, Santa Cruz Biotechnology; AMPKα1/2（H-300）rabbit polyclonal antibody, 1:100, Santa Cruz Biotechnology; Notch1 rabbit monoclonal antibody, 1: 1,000,Cell Signaling Technology ; Notch4 goat polyclonal antibody, 1:200, Santa Cruz Biotechnology ; PGC-1α rabbit polyclonal antibody, 1:1000, Novus Biologicals ; H3K9ac rabbit monoclonal antibody, 1:1000, Abcam; H3K56ac rabbit polyclonal antibody, 1:1000, Epigentek; α-Tubulin rabbit polyclonal antibody, 1:1000, Proteintech) overnight at 4°C. An Alexa Fluor 680/790-labeled goat anti-rabbit/goat anti-mouse IgG antibody (1:10,000, LI-COR Biosciences, USA) was used as the secondary antibody, and the blots were visualized using a LI-COR Odyssey Infrared Imaging System.

Real-time PCR

Total RNA was extracted from mouse kidney using TRIzol reagent (MRC, USA), and spectrophotometry was used to measure the concentration of the collected RNA. Next, cDNA was synthesized by a reverse transcription kit (TaKaRa, Japan). A real-time fluorescence-based quantitative PCR machine (Illumina Eco, USA) was used to determine the mRNA expression levels. GAPDH was used as control for normalization. The sequences of the primers used are listed in Table 1.

Table 1. Primers used in real-time PCR.

Gene	Forward	Reverse
TFAM	**CTCCTAATCTTTACTGTTGC**	**CTCTAAGCCTCCTCAATACAA**
NRF1	**CCTTTGGAGAATGTGGTGCG**	**CCCCGACCTGTGGAATACTTG**
GAPDH	**TCAAGAAGGTGGTGAAGCAGG**	**TCAAAGGTGGAGGAGTGGGT**

Podocyte staining for mitochondrial superoxide

Human podocytes were cultured on cell-climbing films at 37°C. After culturing with different stimuli, mitochondrial superoxide was measured by MitoSox Red staining according to the manufacturer's instructions (MitoSox Red Mitochondrial Superoxide Indicator, Yeasen, China).The images were recorded with a confocal microscope (Olympus, Japan).

Detection of ROS

The intracellular ROS level was measured using the dichlorofluorescein diacetate (DCFH-DA) fluorescent probe (Beyotime, China). Cells were reacted with moderate DCFH-DA (10 μM) at 37.0 °C for 30 min. Then cells were washed with PBS three times. Subsequently, the fluorescence intensity was measured by flow cytometry at an excitation/emission wavelength of 488/520 nm.

Supplementary Figure 1. Effects of Sirt1 on Sirt6 expression in podocytes treated with HG.

The cultured podocytes were treated with the Sirt1 inhibitor EX-527(10uM, 24h) and then cells were stimulated with HG (30mM, 24h). Western blots analysis and quantitative data showing Sirt6 expression in cultured podocytes under HG conditions in the presence or absence of EX-527 (n=4). $^{*}P< 0.05$ compared with podocytes with HG treatment, $^{\#}P< 0.05$ compared with podocyte in normal glucose.

Supplementary Figure 2. Effects of AMPK on Notch signaling in cultured podocytes.

The cultured podocytes were transfected with scrambled or Sirt6 siRNA. After transfection for 24h, cells were treated with the AMPK activator AICAR (1mM, 24h) and then exposed to HG (30mM, 24h). Western blots analysis and quantitative data showing Notch1, Notch4 ,p-AMPK and Sirt6 expression in cultured podocytes transfected with Sirt6 siRNA in the presence or absence of AICAR (n=4). $^{*}P< 0.05$ compared with podocytes transfected with scrambled siRNA, $^{\#}P< 0.05$ compared with podocytes transfected with Sirt6 siRNA.

Supplementary Figure 3. Effects of Sirt6 on mediators of renal mitochondrial biogenesis in podocytes stimulated by HG.

(A) Representative Western blots of glomerular PGC-1α expression in the different groups and quantitation of these results (n=6). $^{*}P< 0.05$ relative to control. (B) NRF-1 and TFAM mRNA levels in glomerular from each group (n=6). $^{*}P< 0.05$ relative to control. CTL=control, DN=diabetic nephropathy. (C) The cultured podocytes were transfected with pcDNA3.1, or pcDNA3.1-SIRT6 and then stimulated with HG (30mM, 24h). Untreated and untransfected podocytes were regarded as normal cells. Western blots analysis and quantitative data showing the effects of Sirt6 on the protein level of PGC-1α in podocytes with HG treatment (n=4). $^{*}P< 0.05$ compared with normal cells, $^{\#}P< 0.05$ compared with podocytes under high glucose. (D)Summarized data showing the effect of Sirt6 on the mRNA levels of NRF-1 and TFAM in podocytes treated with HG (n=4). $^{*}P< 0.05$ compared with normal cells.

Supplementary Figure 4. Effects of Sirt6 on H3K9ac and H3K56ac expression in podocytes under HG conditions.

The cultured podocytes were transfected with pcDNA3.1, pcDNA3.1 SIRT6 or pcDNA3.1 SIRT6-H133Y and then stimulated with HG (30mM, 24h). Untreated and untransfected podocytes were regarded as normal cells. Western blots analysis and quantitative data showing the effects of Sirt6 on the protein level of H3K9ac and H3K56ac in podocytes under HG treatment (n=4). $^{*}P< 0.05$ compared with normal cells. $^{\#}P< 0.05$ compared with podocytes stimulated with HG.

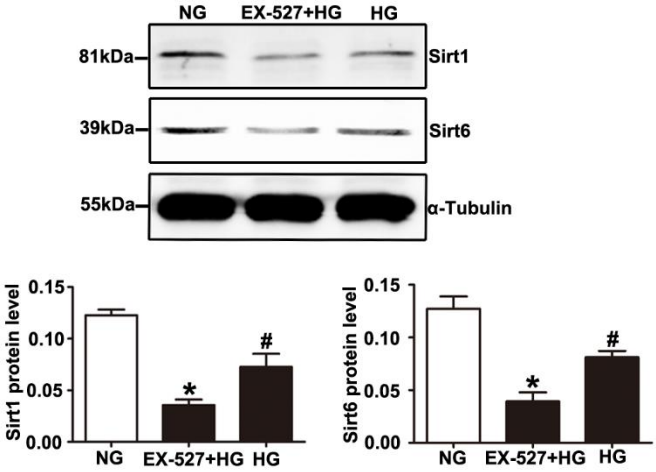
Supplementary Figure 5. Sirt6 expression and mitochondrial superoxide production in podocytes incubated in medium without glucose.

(A) Western blots analysis and quantitative data showing Sirt6 expression in podocytes in each group (n=4).$^{*}P< 0.05$ compared with cells cultured in medium with 5mM glucose. (B) Mitochondrial superoxide production in podocytes were determined by MitoSox Red fluorescence staining in each group (original magnification, ×1 000) and quantitation of these results (n=4). $^{*}P< 0.05$ compared with cells cultured in medium with 5mM glucose.

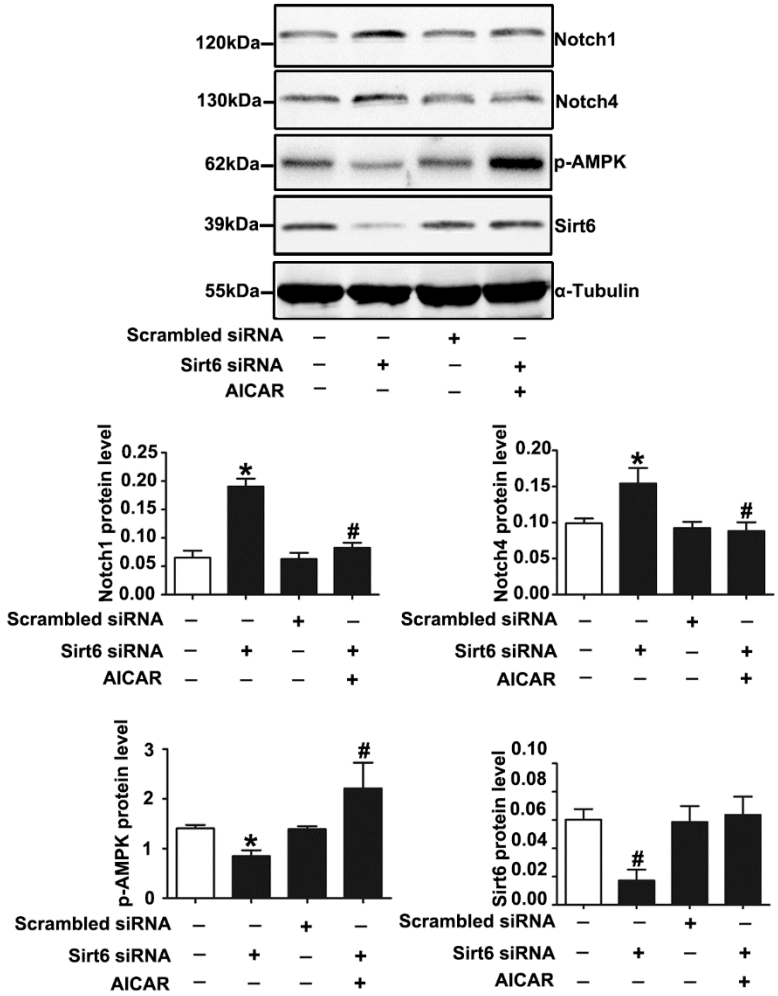
Supplementary Figure 6. Effects of Sirt6 on HG-induced ROS production in podocytes.

The cultured podocytes were transfected with pcDNA3.1, or pcDNA3.1-SIRT6 and then stimulated with HG (30mM, 24h). Untreated and untransfected podocytes were regarded as normal cells. Flow cytometry analysis of ROS production in podocytes in different groups and quantitation of these results (n=4). $^{*}P< 0.05$ compared with normal cells. $^{\#}P< 0.05$ compared with podocytes stimulated with HG.

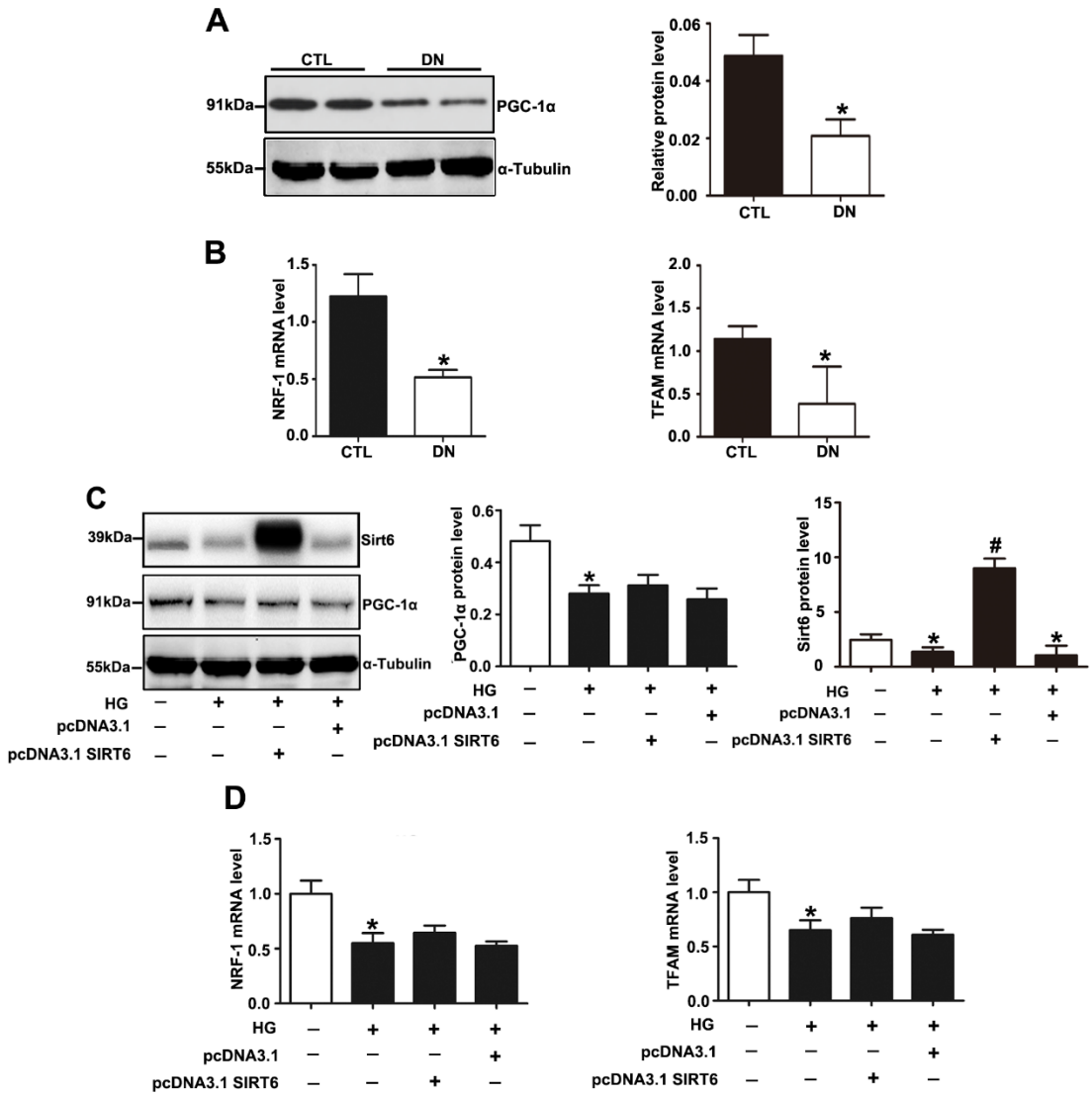
Supplementary Figure 1.



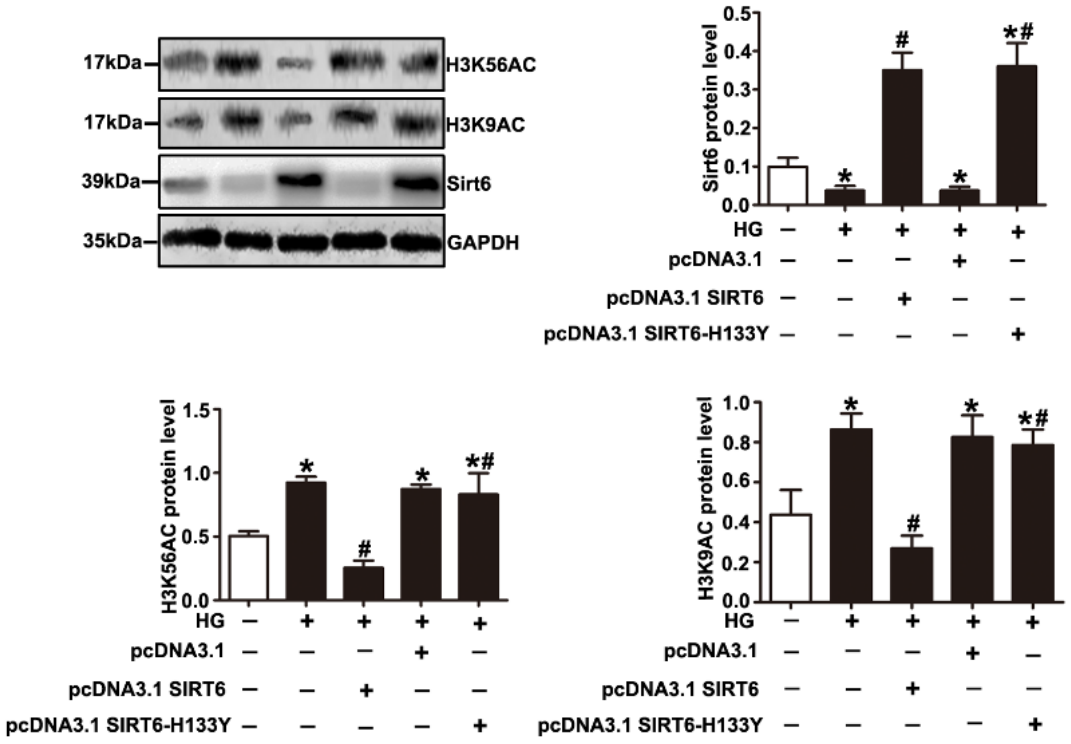
Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.



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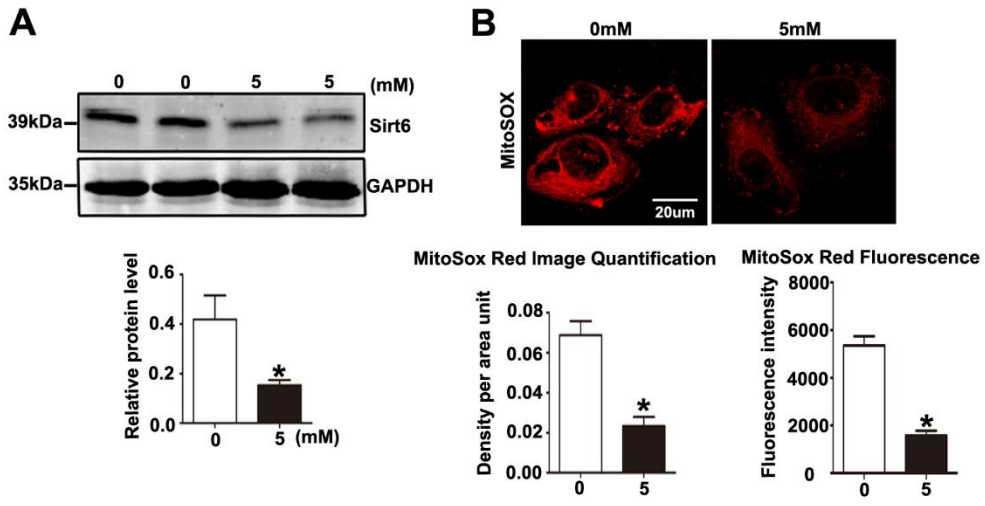

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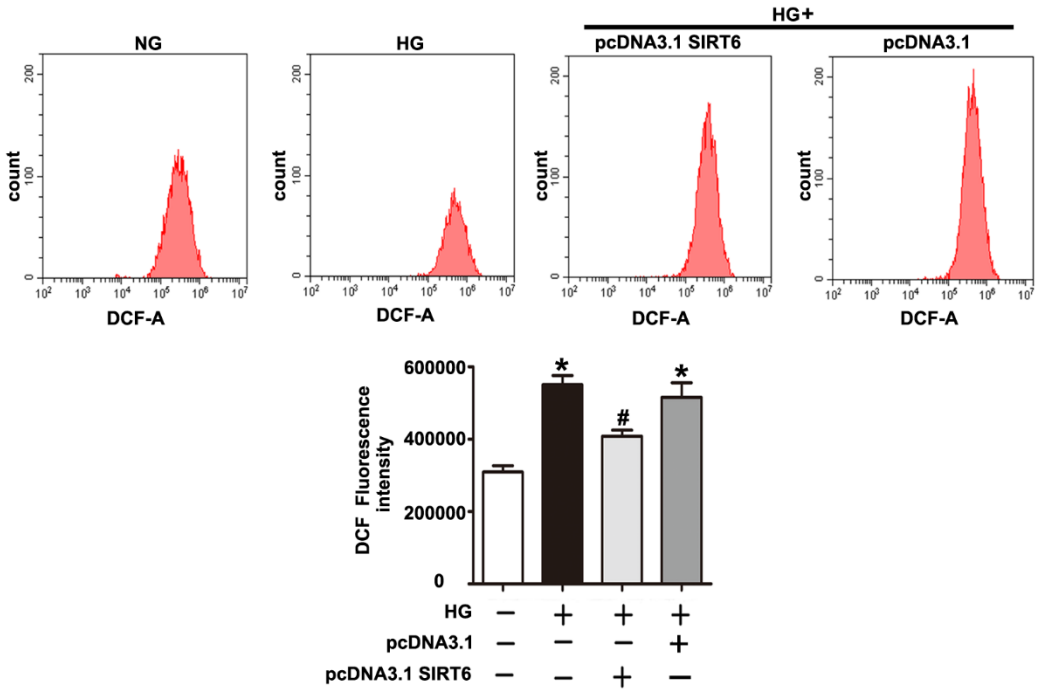
NG
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HG+
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