Supporting Information

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SI Materials and Methods

Mice. $TM^{P/P}$, APC^{high}, and $p66^{Shc}$ knockout mice were described (1–3). In the current study, littermates, which have been backcrossed for at least 10 generations on a C57BL/6 background, were used. All animal experiments were conducted by following standards and procedures approved by the local Animal Care and Use Committee (Regierungspräsidium Karlsruhe and Landesverwaltungsamt Halle, Germany).

Reagents. The following antibodies were used in the current study: rabbit polyclonal antibody to synaptopodin (H-140), rabbit polyclonal antibodies to mouse PAR1 (H111), and mouse PAR3 (H103), and rabbit polyclonal antibody to Wilms' tumor-1 (C-19) from Santa Cruz; rabbit polyclonal antibody to β-actin and GCN5 from Cell Signaling; mouse monoclonal antibody to $p66^{Shc}$ from Invitrogen; rabbit polyclonal anti-nitrotyrosine and anti-Acetyl-H3 from Millipore; mouse monoclonal anti-8-hydroxydeoxyguanosine (anti-8-OH-dG) from Trevigen; and HRP- or FITC-conjugated secondary antibodies to mouse and rabbit IgG from Abcam.

The following reagents were obtained from Sigma-Aldrich: Streptozotocin, 1% collagen, HOECHST 33258, and TRIzol reagent (Invitrogen).

Other reagents used in the current study were as follows: RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas); shRNA for PAR-1 and PAR-3 (Openbiosystems); Hifect transfection reagent (Lonza); mouse PAR agonist peptides (final concentration, 20 μM; PAR1-AP: SFFLRN-OH; PAR2-AP: SLIGRL-OH; PAR3-AP: SFNGGP-OH) and control peptide (FSLLRN-NH2, from Bachem); mouse albumin ELISA quantification kit (Bethyl Laboratories); DMEM, Trypsin-EDTA, FBS, and Hepes (PAA Laboratories); IFN-γ (Cell Sciences); Accu-Chek system and EpiQuick Acetyl-Histone H3 ChIP Kit (Epigentek); EpiTect Methylation-Specific-PCR Kit (Qiagen), protease inhibitor mixture (Roche Diagnostics); BCA reagent (Perbio Science); Vectashield mounting medium and DAB (Vector Laboratories); PVDF membrane (Millipore); and enhanced chemiluminescence (ECL) reagent (Amersham).

Long-Term (26 wk) Diabetic Nephropathy Model. In nonuninephrectomized mice, diabetes was induced by i.p. administration of 60 mg/kg streptozotocin (STZ) freshly dissolved in 0.05 M sterile sodium citrate at pH 4.5 for 5 d successively in 8-wk-old mice, as described (2). Mice were considered diabetic if blood glucose levels were above 300 mg/dL 16–25 d after the last STZ injection. Blood glucose levels were determined from blood samples taken from the tail vein by using ACCU-CHEK glucose strips. In the first 3 wk after the onset of diabetes, blood glucose values were measured three times per week, and after 3 wk, they were measured once per week. Mice displaying blood glucose levels above 500 mg/dL received 1–2 U of insulin (Lantus) to avoid excessive and potentially lethal hyperglycemia. Blood and tissue samples were obtained after 26 wk of persistent hyperglycemia in diabetic mice. Age-matched littermates served as controls.

Determination of Albuminuria. The day before tissue preparation, individual mice were placed in metabolic cages and 12-h urine samples were collected. Urine albumin was determined by using a mouse albumin ELISA kit according to the manufacturer's instructions, and urine creatinine was determined by using a commercially available assay of a modified version of the Jaffe method (X-Pand automated platform; Siemens) (4).

Generation of Stable Knockdown Cell Lines Using shRNA. Stable knockdown mouse podocyte cells were generated by shRNAs cloned into pLKO.1 vectors, as described (5). This vector contains a puromycin-resistant gene for positive colony selection. To generate stable cell clones, podocytes were grown in six-well plates and transfected with shRNA-containing plasmids according to the manufacturer's instructions. Stably transfected cell lines were selected in the presence of 0.4 μg/mL puromycin. Efficient knockdown of target genes was confirmed in stably transfected cell lines by Western blot. Podocyte cell lines with stable knockdown were maintained in complete podocyte medium with 0.2 mg/mL puromycin, and experiments were performed after 14 d of differentiation, as described above. Cells transfected with empty vectors served as controls.

Subcellular Fractionation. For determination of $p66^{Shc}$ translocation into mitochondria, cytosolic, and mitochondrial fractions were isolated as described (2). Briefly, cells were washed in PBS and scraped into isotonic buffer (200 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes–NaOH at pH 7.40, 1 mM DTT, and protease inhibitors) and homogenized with a Potter-Elvehjem homogenizer. Nuclei and unbroken cells were removed by centrifugation at 500 × g for 10 min at 4 °C. The supernatant was centrifuged at 100,000 × g for 60 min, and the resulting supernatant was taken as the cytosolic fraction. The pellet was washed in isotonic buffer (15,000 × g, for 5 min at 4 °C), resuspended in extraction buffer (20 mM Hepes at pH 7.40, 250 mM NaCl, 1% NP–40, 1 mM DTT, 2 mM EDTA, 2 mM sodium orthovanadate, and protease inhibitors) and centrifuged at 20,000 × g for 5 min to remove debris. Samples were subsequently used for immunoblotting.

Immunoblotting. Proteins were isolated and immunoblotting was performed as described (5). In brief, cell lysates were prepared in RIPA buffer (50 mM Tris at pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM NaF supplemented with protease inhibitor mixture). Lysates were centrifuged (10,000 × g for 10 min at 4 °C), and insoluble debris was discarded. The protein concentration in supernatants was quantified by using BCA reagent. Equal amounts of protein were electrophoretically separated on 10% (vol/vol) or 12.5% (vol/vol) SDS polyacrylamide gels, transferred to PVDF membranes, and probed with the desired primary antibodies overnight at 4 °C. Membranes were then washed with TBST and incubated with anti-mouse IgG (1:3,000) or anti-rabbit IgG (1:3,000) horseradish peroxidase-conjugated antibodies, as indicated. Blots were developed with the enhanced chemiluminescence system. To compare and quantify levels of proteins, the density of each band was measured by using ImageJ software. Equal loading was confirmed by Western blot with an actin antibody.

Cell Culture. Conditionally immortalized mouse podocytes were cultured as described (5). In brief, podocytes were grown on collagen type I at 33 °C in the presence of IFN-γ (10 U/mL) to enhance expression of the thermosensitive T antigen. Under these conditions, the cells proliferate and are undifferentiated. To induce differentiation, podocytes were grown at 37 °C in the absence of IFN-γ for 14 d. Experiments were performed 14 d after the induction of differentiation. Differentiation was confirmed by examining the expression of synaptopodin and Wilms' tumor-1 protein. All in vitro experiments were performed in the presence of hirudin (1 mg/mL). Primary mouse glomerular en-

dothelial cells were obtained from Cell Biologics and cultured at 37 °C in a humidified 5% CO_2 incubator in M1168 mouse endothelial cell medium with growth factors, as provided by the manufacturer. For coculture of podocytes and endothelial cells, a modified Boyden chamber system was used, as described (6). Briefly, cell culture inserts (6.5 mm, Corning) were placed in each well of a 24-well plate (Corning-Costa), thus creating transwell chambers. Murine podocytes were plated in the cell culture inserts and differentiated for 14 d. Primary glomerular endothelial cells (GENCs) were grown on the bottom of each well. SVEC4-10 were cultured as described (7). HAEC and HPMVEC were cultured according to the manufacturer's instructions (Promocell).

RT-PCR. RNA was isolated by using TRIzol reagent according to the manufacturer's instructions. cDNA was generated by using 1 μg of total RNA after treatment with DNase (5 U/5 μg of RNA) followed by reverse transcription using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). PCR primers used for analyses of $p66^{Shc}$ expression are as follows: forward, 5′-ACTACCCTGTGTTCCTTCTTTC-3′; reverse, 5′-TCGGTGGATTCCTGAGATACTGT-3′. PCR products were separated on a 1.5% (wt/vol) agarose gel and visualized by ethidium bromide staining. Expression was normalized to β-actin. Reactions lacking reverse transcriptase served as negative controls.

Mitochondrial Membrane Potential. Mitochondrial membrane potential (MMP) was determined by the polarity sensor fluorescent dye JC-1 (Biomol). Analysis was performed according to the manufacturer's instructions. In brief, 1×10^3 mouse podocytes per well were grown in 96-well plates. After experimental treatment, 100 μL of JC-1 working solution (10 μM) was added to the wells and the cells were incubated at 37 °C in a 5% CO_2 incubator for 15 min. The JC-1 working solution was removed, and the cells were washed (3×) in HBSS and analyzed with a fluorescence plate reader (BioTek Instruments; FLx800). The extent of mitochondrial depolarization was assessed by measuring the ratio of red JC-1 aggregates (polarized mitochondria) at Ex/Em = 490/590 nm to green monomeric JC-1 (depolarized mitochondria) at Ex/Em = 490/525.

Histology and Immunohistochemistry. After sacrifice, the mice were perfused with ice-cold PBS and then with 4% buffered paraformaldehyde. Tissues were further fixed in 4% buffered paraformaldehyde for 2 d, embedded in paraffin, and processed for sectioning. Extracellular matrix deposition in the glomeruli was assessed by performing periodic acid-Schiff (PAS) staining followed by calculation of the fractional mesangial area (FMA), as recommended by current Diabetes Complications Consortium protocols. Briefly, a 5-μm-thick section was stained with periodic acid-Schiff (PAS) reagent. At least 30 different superficial glomeruli were randomly chosen for analysis. For every investigated glomerulus, the total glomerular area and glomerular tuft area were determined by tracing the outline of the Bowman's capsule and the tuft, respectively, by using ImageJ software. FMA was calculated as the percentage of area positive for PAS in the glomerular tuft area. For analysis of immunohistochemical nitrotyrosine or $p66^{Shc}$ images, the background-corrected integrated optical density (IOD) was determined as described (7). Control images obtained after incubation with a nonspecific primary antibody were used for background correction. Image Pro Plus software (version 6.0) and ImageJ software were used for image analysis. All histological analyses were performed by two independent blinded investigators. For immunohistochemistry and immunofluorescence, images were captured with an Olympus Bx43-Microscope. For confocal images, a Zeiss LSM 4 Pascal microscope was used.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation (ChIP) analysis was performed as described (8). ChIP was performed on cell extracts by using the Epiquik ChIP assay kit for acetyl-H3 by following the manufacturer's instructions. Briefly, cells were exposed to 1% formaldehyde in cell culture medium on a rocking platform (10 min at room temperature) to crosslink proteins. To quench the cross-linking reaction, cells were washed in 125 mM glycine in PBS. After centrifugation, the pellet was resuspended in lysis buffer CP3 containing protease inhibitors and incubated on ice for 10 min. DNA was sheared by sonication (5- to 15-s cycles of 1 pulse per second at 20%). After centrifugation (8,000 × g, 10 min, 4 °C), the supernatant was diluted with buffer CP4, and an aliquot was removed as "input" control and processed on an agarose gel to check for equal DNA shearing. Proteins from the remaining solution were precipitated by using rabbit polyclonal anti-acetyl H3 IgG antibody. After incubation (4 °C overnight), supernatants were removed and the precipitates were washed six times with buffer CP1. DNA was released by using proteinase K in buffer CP5 (15 min at 65 °C) and then captured, washed, and eluted by using spin columns. Purified DNA was stored at −20 °C until use. Recovered DNA was analyzed by Q-PCR (LightCycler 1.5; Roche) using the following primers for the mouse $p66^{Shc}$ promoter region: forward, 5′-TTT GTA TTG GGC TGG GAG AG-3′; reverse, 5′-TTG AGA GCA CAT CCT TGC AC-3′.

Mitochondrial ROS Production. ROS production was assessed as described (9) and colocalized with mitochondria by using MitoTracker. Briefly, cells grown on collagen-coated coverslips were loaded with 30 μM of the ROS-sensing dye dihydrorhodamine 123 (DHR 123; Invitrogen) and/or 100 nM MitoTracker Red CMXRos (Invitrogen) in PBS at 37 °C. After washing with PBS, green (DHR 123) or red (MitoTracker Red) fluorescence was detected at room temperature with an Olympus Ax70 microscope and a digital color camera (ColorView II) equipped with CY3/FITC filters. Images were captured by using Cell F Imaging Software (Olympus). Colocalization was evaluated by using an ImageJ plug-in for automated quantification and visualization of colocalized fluorescent signals, as described (10). Results are presented as the mean correlation index (Icorr) ± SEM. The Icorr indicates the automatically computed fraction of positively correlated pixels in the image.

Methylation-Specific PCR. Methylation-specific PCR was performed as described (11). Briefly, genomic DNA was isolated by using the QIAamp DNA Mini Kit (Qiagen) and treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research) by following the manufacturer's instructions. PCR amplification was performed with the EpiTect MSP Kit (Qiagen) by using primer pairs designed to specifically detect either methylated or unmethylated CpG sites in the mouse $p66^{Shc}$ promoter. For ratio analyses, amplicons against methylated and unmethylated CpGs were visualized on a 2% (wt/vol) agarose gel. Universal methylated mouse DNA (Millipore) was used as a positive control. The sequences of the primers used were as follows: methylated forward, 5′- TTT TTT TGG TTT GTT TAC GTC - 3′; methylated reverse, 5′- GAC GCG AAA AAA AAA TAA AA - 3′; unmethylated forward, 5′- TGT TTT TTT TGG TTT GTT TAT GTT - 3′; and unmethylated reverse, 5′- CCA ACA CAA AAA AAA AAT AAA AA - 3′.

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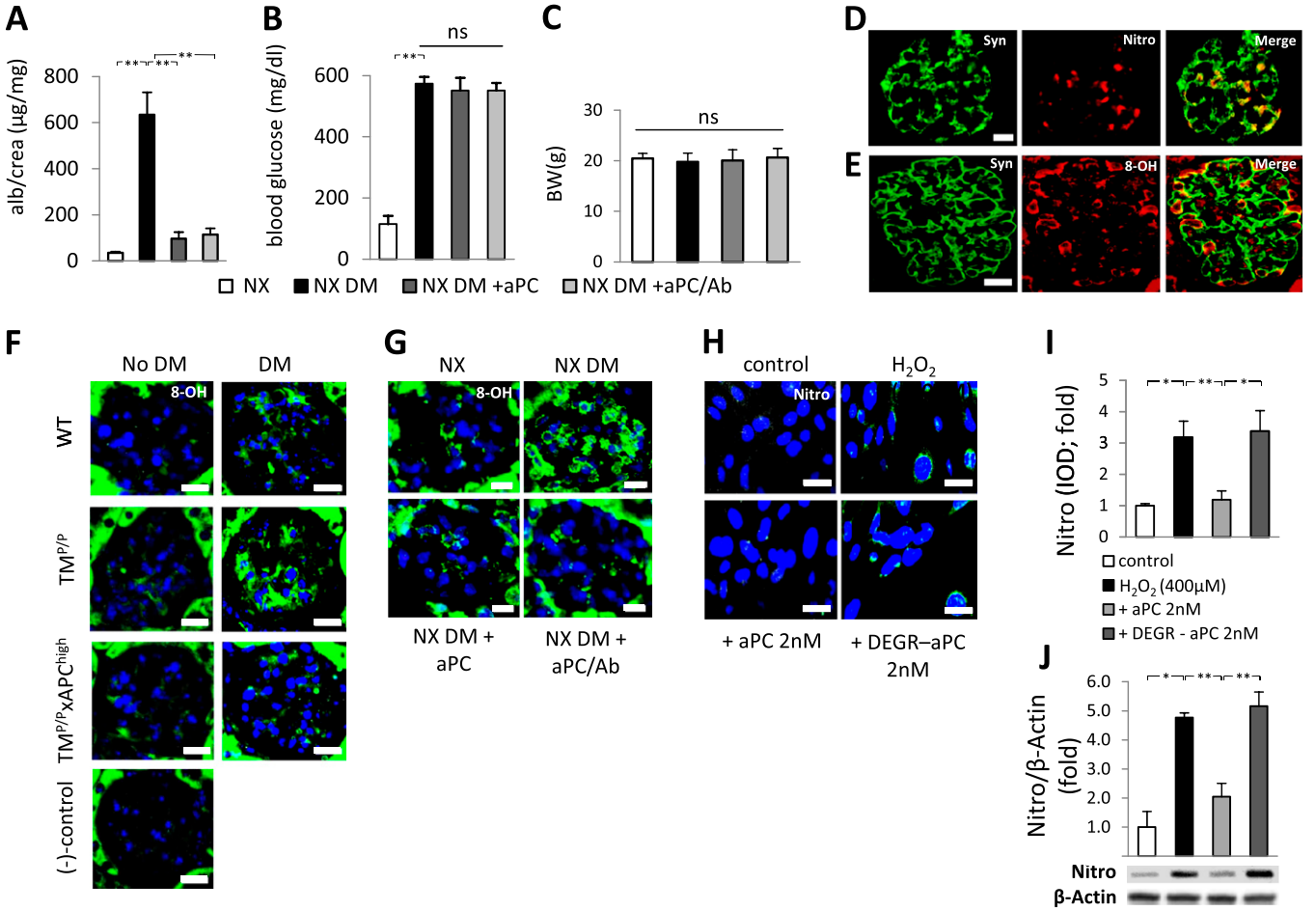

Fig. S1. Markers of oxidative stress are regulated by aPC in glomeruli and mouse podocytes. (*A–C*) Administration of aPC (NX DM+aPC) and anticoagulant function-blocked aPC (NX DM+aPC/Ab) in uninephrectomized diabetic wild-type mice (NX DM) decreases diabetes-induced albuminuria (*A*) despite comparable blood glucose levels and body weight (BW) between the diabetic groups (*B* and *C*); ns, not significant. (*D* and *E*) Immunofluorescence images showing co-localization of nitrotyrosine (red; *D*) or 8-OH-dG (red; *E*) and the podocyte marker synaptopodin (green) in diabetic $TM^{P/P}$ mice. Conventional fluorescence microscopy was performed on paraffin-embedded sections. Nitro, nitrotyrosine; p66, $p66^{Shc}$; Syn, synaptopodin. (*F* and *G*) Increased staining intensity of the oxidative stress marker 8-OH-dG (green) is evident in the glomeruli of diabetic (DM) $TM^{P/P}$ mice (*F*) and uninephrectomized wild-type diabetic mice (NX DM; *G*). Staining intensity for 8-OH-dG is reduced in mice with concomitantly increased plasma levels of aPC ($TM^{P/P}$ x APC^{high}; *F*) or with the exogenous application of aPC (NX DM+aPC) and aPC-HAPC1573 (NX DM+aPC/Ab; *G*). No staining of 8-OH-dG is observed in the glomeruli of diabetic $TM^{P/P}$ mice by using a nonspecific primary antibody [(-)-control; *F*]. Hoechst 33258 nuclear counterstain is shown by blue staining. (*H–J*) Immunocytochemical staining of nitrotyrosine (Nitro, green) and Hoechst 33258 (nuclear counter stain, blue; *H*) and representative immunoblot of nitrotyrosine (*J*) in murine podocytes in vitro shows increased nitrotyrosine accumulation after H_2O_2 (400 µM) treatment, which is prevented by aPC but not by active-site blocked aPC (DEGR-aPC). Bar graphs summarizing results as integrated optical density (IOD; *I*) or densitometric ratio of nitrotyrosine to β-actin (*J*) are shown. Data are presented as the mean ± SEM from three independent experiments. $*P < 0.05$; $**P < 0.005$. (Scale bars: *D–G*, 20 µm; *H*, 10 µm.)

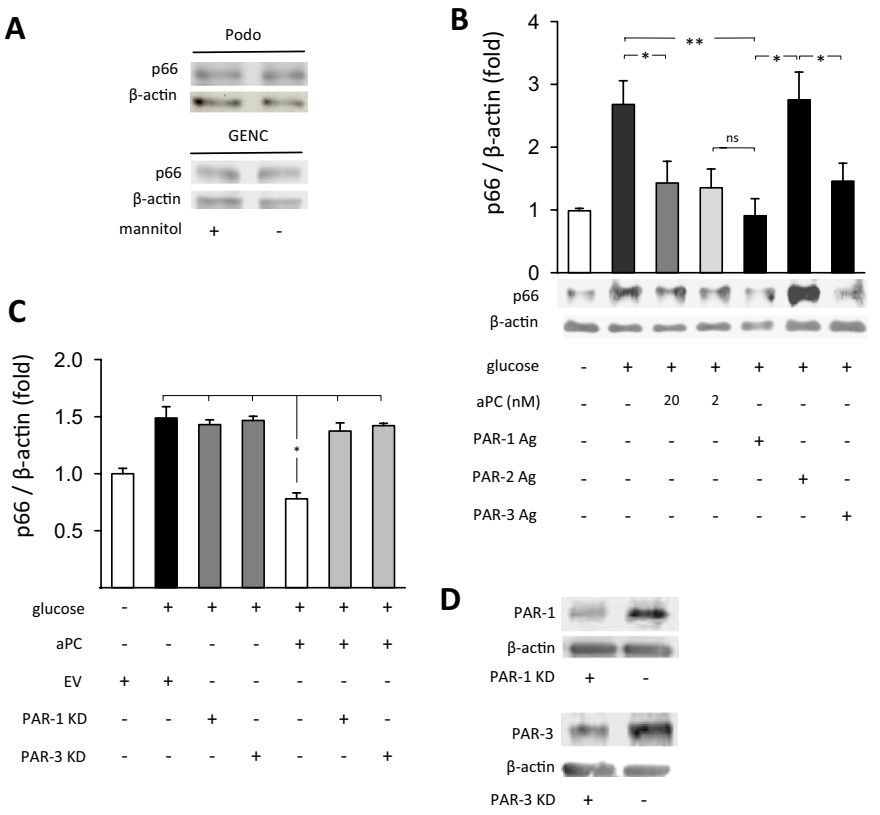

Fig. S2. PAR-1 and PAR-3 are required for aPC-dependent suppression of glucose induced p66^{Shc}. (*A*) Osmotic controls for glucose treatment in cell culture. Mannitol at 25 mM has no effect on p66^{Shc} protein levels in mouse podocytes (Podo) and mouse glomerular endothelial cells (GENC). (*B*) Immunoblot images demonstrating that the PAR-1 and PAR-3 peptide agonists are sufficient to inhibit glucose-induced p66^{Shc}, whereas the PAR-2 agonist has no effect on p66^{Shc}. Ag, agonist. Representative immunoblots of three independent experiments and a bar graph summarizing the results are shown. (*C*) Bar graph summarizing the results of at least three independent experiments with PAR-1 and PAR-3 knockdown (KD) podocytes with the indicated treatments (25 mM glucose ± aPC). (*D*) Immunoblot images of knock-down control experiments confirming the suppression of PAR-1 and PAR-3 with the PAR-1 and PAR-3 knockdown constructs (PAR-1/PAR-3-KD). Data are presented as the mean ± SEM. p66, p66^{Shc}; $*P < 0.05$; $**P < 0.005$.

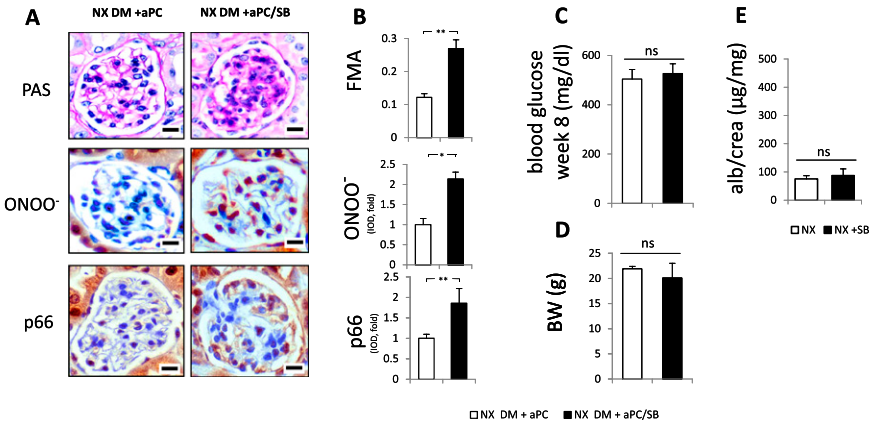

Fig. S3. The hyperacetylating agent sodium butyrate abolishes the nephroprotective effects of aPC despite unchanged blood glucose and body weight. (*A* and *B*) Representative histological images of uninephrectomized diabetic wild-type mice treated with aPC without (NX DM+aPC) or with sodium butyrate (NX DM+aPC/SB) showing PAS, nitrotyrosine (ONOO$^-$) and p66^{Shc}-stained glomeruli of paraffin-embedded tissues (*A*), and bar graphs summarizing the results as fractional mesangial area (FMA) or mean integrated optical density (IOD; *B*). (C–*E*) SB-treated diabetic mice show unaltered blood glucose (*C*) and body weight (BW; *D*). Sodium butyrate treatment has no effect on albuminuria in nondiabetic control mice (*E*). Data are presented as the mean ± SEM of at least six mice per group and analyses of ≥30 glomeruli per mouse (*C*). $*P < 0.05$; $**P < 0.005$; ns, not significant. (Scale bars: 20 µm.)

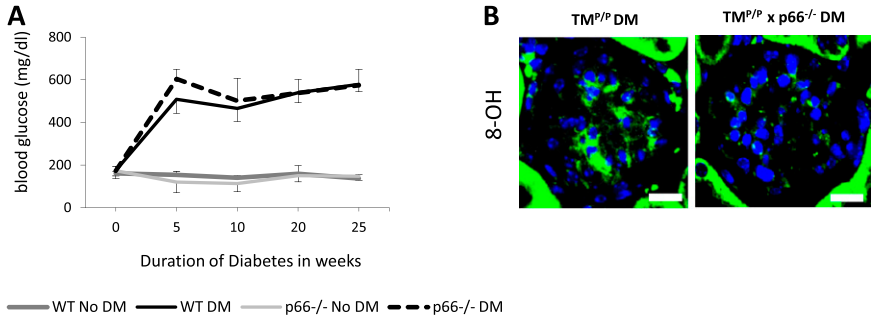

Fig. S4. p66^{shc} deficiency corrects for increased 8-OH-dG accumulation in the glomeruli of diabetic TM$^{P/P}$ mice. (*A*) No difference in blood glucose levels was observed between wild-type and p66$^{shc-/-}$ mice, when comparing either diabetic or nondiabetic mice. (*B*) Representative immunofluorescence images of diabetic TM$^{P/P}$ (TM$^{P/P}$ DM) mice and diabetic TM$^{P/P}$ x p66$^{Shc-/-}$ (TM$^{P/P}$ x p66$^{-/-}$ DM) mice showing reduced 8-OH-dG accumulation in the glomeruli of diabetic TM$^{P/P}$ x p66$^{Shc-/-}$ mice compared with diabetic TM$^{P/P}$ mice. Conventional immunofluorescence microscopy was performed on paraffin-embedded tissues. 8-OH-dG, green; Hoechst 33258 nuclear counter stain, blue. At least six mice per group and ≥30 glomeruli per mouse were analyzed (*B*). (Scale bars: 20 μm.)