Materials and Methods

Experimental protocol: All experiments were performed using mouse strains on the FVB/NJ background. In previous studies, we characterized the phenotype of both the CD TG model [1] and FVB/NJ Akita mice [2]. Briefly, FVB/NJ Akita mice develop albuminuria and early histopathologic features of DN in humans by 20 weeks of age [2]. CD TG mice express the enzyme CD under the control of the tet operator sequence and a minimal CMV promoter [3]. This strategy permits targeting CD to renal or extrarenal tissues using available Tet-On mice (reverse tetracycline transactivator or rtTA) or Tet-Off mice (tTA) [3]. For the experiments, both transgenes were bred onto Akita mice and then Akita "double" TG mice were created by crossing male Akita TG mice with female wild type TG mice; thus, offspring from this mating did not develop in the diabetic milieu. For the experiments, we used only male offspring because female Akita mice develop only mild hyperglycemia as well as modest functional and histologic features of diabetic kidney disease [4,5].

After creating Akita "double" TG mice, Akita CTLs (Akita "single" TG mice and Akita wild type mice) and CTLs ("single" TG mice and wild type mice), male animals were treated with doxycycline in 2% sucrose water (to enhance palatability) after weaning. At 4 weeks of age, mice were treated with 500 mg/kg 5-FC for 5 days as previously described [1]. This time period corresponds to the onset of hyperglycemia in FVB/NJ Akita mice [2]. Blood glucose measurements, 24 hour urine collections and body weights were obtained at 12, 16 and 20 weeks of age and systolic blood pressure (SBP) was measured at 12 and 20 weeks of age. After the last urine collection, mice were sacrificed and blood, kidneys and hearts were removed. Kidney and hearts were weighed and kidney tissue was saved in both formalin and glutaraldehyde. The experiments conformed to the Guide for the Care and Use of Laboratory Animals [6].

BP measurements: Systolic BP was measured using a computerized tail-cuff system (Hatteras Instruments, Cary, NC, USA) in conscious mice as previously described [7]. This technique has previously been shown to correlate closely with intra-arterial measurements [8].

Histopathology: Light microscopic sections were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) as well as Masson trichrome and then evaluated be a pathologist (A.F.B.) blinded to genotype. Mesangial expansion was graded on a semi-quantitative scale of 0-3 (0-normal, 1-mild, 2-moderate, 3-severe) as previously described [2]. Quantitation of kidney collagen was performed using a Sirius Red/Fast Green Collagen Staining Kit from Chondrex (Redmond, WA) according to the manufacturers directions. Briefly, deparaffinized tissue sections are stained with Sirius Red/Fast Green and the dye extracted from the tissue sections according to the manufacturers directions. Optical density values were obtained at 540nm and 605nm and used to calculate the total collagenous and non-collagenous protein content, respectively.

Albuminuria: Urine was collected for 24 hours in metabolic cages specifically designed for collection of urine in mice (Hatteras Instruments, Cary, NC). Urinary albumin concentrations were measured using a kit from AssayPro (St. Charles, MO) and urine creatinine levels were

measure using a kit from Exocell (Philadelphia, PA). Urinary albumin excretion was expressed both as the albumin excretion rate per 24 hours and the albumin/creatinine ratio.

Serum creatinine and glucose measurements: Serum creatinine levels were measured using a kit from Crystal Chemistry (Downers Grove, IL). The kit utilizes an enzymatic method to measure creatinine levels and is suitable for use with mouse serum. To measure blood and serum glucose levels, we used the AlphaTRAK testing system (Abbott Laboratories, Chicago, IL). For measurements that were greater than the upper limit of the testing system, samples were diluted 1:1 with phosphate buffered saline and re-tested.

Transmission electron microscopy (TEM) and GBM thickness: Electron microscopy was performed as previously described [7]. Analysis at the ultrastructural level was performed in a qualitative fashion and areas of interest selected in semithin sections for preparation of ultrathin sections for examination by a pathologist (A.F.B.) blinded to genotype. GBM width was measured as previously described [2].

Quantitation of podocyte number, podocyte density and glomerular volume: The number of podocytes per glomerular profile was measured as previously described [1,2]. Briefly, podocyte nuclei were stained with a WT1 antibody (rhodamine), the glomerular tuft was stained with a synaptopodin antibody (fluorescein) and nuclei were countered stained with DAPI. Podocyte nuclei were then quantitated by counting nuclei that both co-localized with DAPI and were associated with synaptopodin staining. Using this technique, nuclear WT1 staining is consistently associated with synaptopodin stained cells. Data were expressed as the average number of podocyte nuclei per glomerular profile as previously described [1]. Podocyte density [Nv(P/Glom)] was calculated using the Weibel-Gomez method [9] and confocal microscopy to obtain optical dissectors. The mean glomerular volume (VGlom) was obtained from the harmonic mean of the glomerular surface area as previously described [2,7]. An estimate of the total number of podocytes per glomerulus [N(P,Glom)] was obtained by multiplying Nv(P/Glom) by the glomerular volume Vglom [9].

Statistical analysis: Data are presented as the mean ± standard error of the mean (SEM) and statistical analyses were performed using the Prism computer program (GraphPad Software, Inc.). For comparison of continuous variables, a test of normality was performed (Kolmogorov–Smirnov test) prior to assessing statistical significance using the following statistical methods: 1. T-test for variables passing the normality test, or 2.Mann-Whitney test for variables that were not normally distributed. For comparisons between more than two groups, statistical analysis included either: 1. A one way Analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons post-test for normally distributed variables, or 2. A Kruskal-Wallis test followed by a Dunn multiple comparisons post test for variables that were not normally distributed. For non-continuous variables data was analyzed using a Chi-square analysis.

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Supplementary Figure Legends

Supplementary Figure 1. Urine output, heart weight and kidney weight in Akita mice. (A) Urine output was significantly increased in Akita CD mice and Akita CTLs compared to CTL animals. (B) Heart-to-body weight was increased in Akita CTLs compared to the CTLs at 20 weeks of age. Heart-to-body weight was also tended to be increased in 20-week old Akita CD compared to the CTLs but these differences did not reach statistical significance. (C) Kidney-to-body weight ratio was significantly increased in both groups of Akita mice compared to CTLs. Eight Akita CTLs, 9 CTLs and 5-6 Akita CD mice were studied (one mouse died during the study). *P<0.05 †P<0.01 vs. age-matched CTLs.