Notch1 and Notch2 in podocytes play differential roles during diabetic nephropathy development

Running title: Notch Receptors in Diabetic Podocyte

Mariya T. Sweetwyne[1], Antje Gruenwald[1], Thiruvur Niranjan[1], Ryuichi Nishinakamura[2], Lothar J. Strobl[3] and Katalin Susztak[1*]

[1]Renal Electrolyte and Hypertension Division, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

[2]Department of Kidney Development, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan

[3] Department of Gene Vectors, Helmholtz Center Munich, German Research Center for Environment and Health, Munich, Germany

[*]Corresponding author:

Katalin Susztak, MD, PhD

Renal Electrolyte and Hypertension Division

University of Pennsylvania

415 Curie Blvd

Philadelphia, PA, 19104

Tel: 215 898 2009

ksusztak@mail.med.upenn.edu

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ABSTRACT

Notch pathway activation in podocytes has been shown to play an important role in diabetic kidney disease (DKD) development, however, the receptors and ligands involved in the process have not been identified. Here we report that conditional deletion of Notch1 in podocytes using NPHS2^{cre}Notch1$^{flox/flox}$ animals resulted in marked amelioration of diabetic kidney disease. On the contrary, podocyte-specific genetic deletion of Notch2 had no effect on albuminuria and mesangial expansion. Notch1-null podocytes were protected from apoptosis and dedifferentiation *in vitro*, likely explaining the protective phenotype *in vivo*. Deletion of Notch1 in podocytes also resulted in an increase in Notch2 expression, indicating an interaction between the receptors. At the same time, transgenic overexpression of Notch2 in podocytes did not induce phenotypic changes, while constitutive expression of Notch1 caused rapid development of albuminuria and glomerulosclerosis. In summary, our studies indicate that Notch1 plays a distinct (non-redundant) role in podocytes during diabetic kidney disease development.

INTRODUCTION

To date, approximately 9% of the US population has diabetes. Diabetes is the leading cause of chronic kidney disease (CKD) and end stage renal failure. Diabetic kidney disease (DKD) is initiated in part because high-level of blood glucose damages the glomerular filtration unit, resulting in protein leakage into the urine (1). The filtration unit is comprised of capillary endothelial cells, glomerular basement membrane and specialized epithelial cells; podocytes. Once thought to be a primarily quiescent and terminally differentiated cell, research over the last decade has demonstrated that podocytes may in fact be the true culprit of diabetic kidney disease (1). Podocyte injury is characterized by pathological loss of regularity in foot branching and widening of the foot processes; these changes are termed 'foot process effacement'. Foot process effacement is the typical mechanism of injury response in podocytes and it is usually associated with a broader dedifferentiation. Severe insult leads to podocyte loss, by apoptosis or detachment (1). Reactivation of developmental pathways, including Wnt and Notch signaling, has been shown to play an important role in podocyte injury and diabetic kidney disease development, by promoting dedifferentiation and apoptosis (2; 3).

The Notch protein family is comprised of four receptors, Notch1-4 and five canonical ligands, Jagged 1and 2 (Jag1 & 2) and Delta Like Ligands (Dll) 1, 3 and 4 (4). Canonical Notch signaling is typically transcellular; the ligand(s) expressed on one cell binds to receptors on neighboring cells and initiates cleavage of the receptor. Notch cleavage results in the release of the Notch Intracellular Domain (NICD, or ICNotch), which translocates to the nucleus to become a transcriptional co-regulator. Some of the transcriptional binding partners that engage the NICD in the nucleus are common to all Notch receptors, including Mastermind-Like 1

(MAML1) and Recombination Signal Binding Protein for Immunoglobulin Kappa J (RBPJκ)(4). Despite the common use of activation and signaling partners, Notch receptor functions are often non-redundant(5).

Notch1 and Notch2 show high structural similarities and an almost overlapping expression pattern in the developing and adult mammalian kidney. Despite their intersecting expression, Notch1 and 2 are functionally distinct. Mutations of NOTCH2 in patients cause Alagille syndrome, which is associated with renal developmental abnormalities (6; 7). Similarly, genetic studies performed in mice indicated absence of podocytes and proximal tubule development in Notch2 knock-out animals(5; 8). On the other hand, mice with kidney specific deletion of Notch1 do not show renal developmental defects, highlighting that Notch1 and Notch2 play specific (non-redundant) roles during development. To understand this specificity, the Kopan group recently performed experiments swapping the intracellular and extracellular domains of Notch1 and Notch2 in the developing kidney(9). They propose, that signal strength alterations might be responsible for the functional differences between Notch1 and Notch2 during kidney development.

Expressions of Notch pathway proteins are much lower in adult mouse and human kidneys. Increased expression of both Notch1 and Notch2 has been reported in kidney samples of patients with DKD(10), focal segmental glomerulosclerosis (10; 11), HIV-associated nephropathy (12) and tubular interstitial fibrosis (13). Our studies also indicate that Notch plays a functional role in podocytes, as inducible expression of the Notch1 intracellular domain in mature podocytes causes severe albuminuria and glomerulosclerosis (2). Functional studies performed in cultured podocytes indicated that increased Notch1 expression induces apoptosis via upregulation of p53 (2). To prove that Notch signaling plays a functional role in podocytes,

we generated mice with podocyte specific deletion of Rbpjκ. Rbpjκ is a common transcriptional binding partner of all Notch isoforms. Podocyte-specific Rbpjk deletion resulted in significant (50%) reduction of proteinuria in the setting of diabetes (2). Furthermore, Lin et al. also demonstrated that inhibition of Notch signaling by blocking release of the NICD via gamma secretase inhibitors (GSI) treatment in a rat model, ameliorated albuminuria and glomerulosclerosis in the setting of diabetes (14). Collectively, these results strongly indicate that Notch signaling plays an important role in DKD development. Unfortunately, all prior studies relied on simultaneous inhibition of all Notch isoforms, thus the receptor-specific signaling architecture remains unknown. This is a critical question for potential therapeutics development as broad inhibition of Notch signaling is associated with severe systemic side effects, while receptor or ligand specific targeting show better profiles. An additional concern is that both the gamma secretase complex and Rbpjk are involved in multiple signaling cascades. A recent report even suggested that Notch2 activating antibodies could be protective in the context of nephrotic syndrome, indicating that different Notch receptors play dissimilar roles (15).

Here we examined the role of Notch1 and Notch2 in podocytes using genetic deletion and overexpression systems *in vivo* and *in vitro*. We found that podocyte specific loss of Notch1 protected from diabetic kidney disease development by ameliorating podocyte dedifferentiation and loss.

METHODS:

Antibodies and reagents

The following antibodies were used cleaved Notch1 (Val1744, Cell Signaling, rabbit): 1:100 IHC, 1:150 IF; Notch1 (D1E11 Cell Signaling) 1:1000 WB; cleaved Notch2 (Cleaved-Ala1734,

Sigma, rabbit): 1:150 IHC; cleaved Notch2 (#07-1234 Millipore, rabbit) 1:200 IF, 1:1000 WB; Snail1 (NBP1-19529, Novus Biologicals, rabbit): 1:200 IHC, 1:200 IF, 1:1000 WB; Nephrin (Fitzgerald Labs, guinea pig): 1:250 IF, Podocin (H-130, Santa Cruz Biotechnology, rabbit) 1:1000 WB; WT-1 (C-19, Santa Cruz Biotechnology, mouse) 1:150 IF; GAPDH (Clone GAPDH-71.1, Sigma, mouse) 1:5,000 WB; β-Actin (Clone AC-15, Sigma, Mouse) 1:10,000 WB; γ-Tubulin (ab11316, Abcam, mouse) 1:1,000 WB. The following secondary antibodies were used; Rhodamine conjugated goat α guinea pig (Fitzgerald Labs) and FITC conjugated immunoadsorbed donkey α rabbit (Jackson Labs). Nuclei were visualized with either Hoechst 33342 or NucBlue® Fixed Cell stain (Life Technologies).

Animals

The Notch1$^{flox/flox}$ mice were on a 129SvJ background and purchased from Jackson Laboratory (stock # 006951), the Notch2$^{flox/flox}$ mice on a C57Bl/6J background were a kind gift of Ursula Zimber-Strobl (Helmholtz German Research Center for Environmental Health - GmbH, Munich, Germany) and the Podocin Cre NPHS2^{cre} line was a kind gift of Lawrence Holzman (University of Pennsylvania). To generate mice with Notch1 or Notch2 deletion specifically in podocytes, Notch1$^{flox/flox}$ mice or (16) Notch2$^{flox/flox}$ mice were crossed with Podocin Cre mice (17). Transgenic animals were identified by genomic PCR using transgene specific primers. Nephrectomies were performed on male littermates of the NPHS2^{cre}/Notch$^{flox/flox}$ x NPHS2^{cre}Notch$^{WT/flox}$ matings at 4 weeks of age, under sterile conditions. To induce diabetes, the uninephrectomised mice were injected with streptozotocin protocol (50 mg/kg ip. daily x5, low dose protocol) as detailed at amdcc.org. Mice were sacrificed at 20 weeks of age. NICD2 overexpressing floxed mice (R26Notch2) were a kind gift of Dr. Nishunkamura, Kumamoto

University, Japan and were previously described in detail (18). R26Notch2 mice were crossed with the NPHS2^{Cre} mice to generate podocyte-specific NICD2 overexpressing animals. Animal studies were approved by Albert Einstein College of Medicine and at the University of Pennsylvania.

Phenotype analysis

Urinary albumin and creatinine were determined using Mouse Albumin specific ELISA and Creatinine Companion kits (Exocell and Bethyl Laboratories) following manufacturer's protocol. Blood glucose was measured with OneTouch Glucometer Ultra2 (LifeScan). Formalin fixed paraffin embedded kidney sections were stained with Periodic-Acid-Schiff (PAS). Slides were examined and pictures were taken with Nikon Eclipse TE300 microscope and SPOT Diagnostic CCD camera.

Immunostaining

Formalin-fixed, paraffin-embedded sections were blocked with fish skin gelatin, bovine serum albumin, secondary antibody host serum (donkey or goat) and Triton X-100. Endogenous biotin was blocked with avidin/biotin blocking kit (Vector Labs). Peroxidase-conjugated secondary antibodies were provided with the Vectastain Elite Kit and peroxidase development was achieved with ImmPACT™DAB peroxidase substrate kit (Vector Labs). For frozen section, 7 μM thick, O.C.T. Compound (Sakura) embedded cryosections were fixed in zinc buffered formalin and probed with primary antibodies as described above.

Primary glomerular harvest and podocyte culture

Glomeruli for transcript, protein and cell culture analysis were harvested using magnetic beads and for whole glomeruli immunofluorescence, they were isolated using sieving. Magnetic Dynabeads® (Life Technologies) isolation was performed as previously described (19) but with a 10 minute red blood cell lysis following quenching of the collagenase digest (RBC Lysis buffer, ZenBio; Collagenase, Worthington Biochemical Corp). Isolation by sieving first used a 30 minute 1 mg/ml Collagenase A digest of minced kidneys 37°C, followed by collagenase inhibition with 30% volume fetal bovine serum addition. Isolates were pushed and washed through successive cell strainers of 100 and then 70 μm pore size. Glomeruli were collected in the top of a 40 μm filter.

For primary podocyte culture, harvested glomeruli were placed onto culture dishes coated with 0.1 mg/ml rat tail Collagen type I as previously described (20). Culture medium from day 1-3 was RPMI 1640 containing 15% volume fetal bovine serum (FBS) (Atlanta Bio), penicillin streptomycin and amphotericin B. Day three of culture, unattached glomeruli were washed away and medium was changed to 10% FBS. Podocytes were treated on day 6 of culture after harvest. 12 hours prior to TGF-β1 stimulation, medium was replaced with RPMI containing only 0.2% FBS.

Immortalized Podocytes

Immortalized mouse podocytes were cultured as previously reported (20),(21). Briefly, differentiation was induced when cells were approximately 60% of confluence by thermoshifting to 37°C in medium without interferon-γ for 14 days. 24 hours prior to treatment, medium FBS was changed to 0.2%. Cells were treated with 1 μM GSIXX (dibenzazepine, Calbiochem) or

vehicle for 1 hour and then recombinant mouse TGF-β1 (Preprotech) or vehicle in a reverse time course as indicated.

Apoptosis Assays

Collagen coated Lumox® gas-permeable bottom plates (Sarsted) were used to culture glomeruli for all apoptosis assays. Kidneys from two mice of the same genotype were pooled for each n. After 48 hours, unattached glomeruli were washed away with Dulbecco's PBS and medium replaced with RPMI with 0.2% FBS. After 18 hours NucBlue© Live Cell Stain to label live nuclei and CellEvent® Caspase-3/7 Green (Life Technologies) to measure apoptosis, were added to each plate as per manufacturers instructions. Only cells that had migrated away from a glomerulus were quantified by summing the total number in 3-5 10x fields per plate. Each plate was then treated with 5 ng/ml of activated TGF-β1 and re-quantified 24 hours later.

qRT-PCR

RNA was isolated using the RNeasy®Mini Kit (Qiagen) per manufacturer's instructions. RNA was reverse transcribed using the cDNA Archival Kit (Life Technology), and qRT-PCR analysis was performed using SYBRGreen Master Mix, gene-specific primers and ViiA 7 System (Life Technology). The data were normalized to β-actin gene (ACTB) levels within each sample and analyzed using the $^{\Delta\Delta}$Ct method. Primer sequences as follows; Nphs2: forward –5' ccgagaggcccacgggagaa, reverse – 5' ccgctctgctccagcatccg; Nphs1: forward – 5' agcgtgagccctcactcggt, reverse – 5' ctccgccagaggagtccccc; Notch1: forward - 5' acagtgcaacccccctgtatg, reverse – 5' tctaggccatcccactcaca; Notch2: forward - 5'-ggcatgttggggaaagctac, reverse: 5'-ggacacaaagcaggggtgag; Snai1: forward – 5'

tggaaaggccttctctaggc, reverse – 5' ggagaatggcttctcaccag; Actb: forward – 5' accgtgaaaagatgacccag, reverse – 5'agcctggatggctacgtaca

Western Blotting

Protein was isolated using RIPA buffer with phosphatase and protease inhibitors. Lysis was completed by shearing through a 26 G syringe needle. Samples were denatured with 10% β-Mercaptoethanol (Sigma) in 2x Laemmli sample buffer at 95°C for 10 minutes. Primary podocytes were run on 4-15% SDS-PAGE gradient gels (Bio-Rad Laboratories). Immortalized podocytes were run on 10% SDS-PAGE gels. All gels were transferred onto PVDF, blocked in 5% nonfat milk in PBS before being probed with primary antibodies and secondary antibodies. Blots were developed with Pierce ECL Western Blotting Substrate (Thermo Scientific).

Statistics

Analysis of differences between two groups used a student's 2-tailed *t*-test assuming unequal variance. ANOVA with Bonferroni correction was used when more than 2 groups were present. Correlations and R^2 values were calculated using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software). A *P* value less than 0.05 was considered significant.

RESULTS

Mice with podocyte specific deletion of Notch1 were protected from diabetic kidney disease

To address, the role of Notch1 and Notch 2 in podocytes in health and disease we generated mice with podocyte specific deletion of Notch1, by crossing mice carrying a Notch1

floxed allele (Notch1$^{F/F}$) (16) with podocin Cre animals (NPHS2^{cre}) (17). NPHS2^{cre}Notch1$^{F/F}$ mice were born at the expected Mendelian frequency and had no notable gross renal alterations even at 20 weeks of age. Successful deletion of Notch1 was confirmed by quantitative PCR based transcript analysis of isolated glomeruli **(Fig1A)**.

Male mice underwent a uninephrectomy at 4 weeks of age and type1 diabetes was induced by low dose streptozotocin (STZ) injection at 6 weeks of age. Littermates carrying either the podocin Cre or just the Notch1 floxed allele were used as controls (CTL). Vehicle treated uninephrectomized animals served as non-diabetic controls. At 20 weeks of age, urine was collected and animals were sacrificed. The degree of hyperglycemia was similar in the Notch1 knockout and in the control animals **(Supplemental Table 1)**. As the colony was kept on a 129 SvJ background, they developed significant albuminuria following uninephrectomy and STZ-induced diabetes (mean albumin/creatinine ratio was 3,406 ug/mg). Albuminuria was significantly lower in mice with podocyte specific Notch1 deletion (448.4 ug/mg albumin/creatinine) **(Figure 1B)**. Histological changes as seen by Periodic acid–Schiff stain **(Figure 1C)** and Masson's Trichrome stain **(Supplemental Figure 1)**, including mesangial expansion were also ameliorated in podocyte specific Notch1 knock out animals**.**

Mice with podocyte specific deletion of Notch2 were not protected from diabetic kidney disease development

Next, we generated mice with podocyte specific deletion of Notch2 by intercrossing NPHS2^{cre} and Notch2$^{F/F}$ animals. Reduced expression of Notch2 was confirmed by quantitative PCR-based transcript analysis **(Figure 1D)**. Podocyte-specific Notch2 knockout animals showed

no phenotypic abnormalities, as the Podocin Cre mediated genetic deletion occurs late during development.

Diabetes was induced by low dose STZ injection following uninephrectomy (**Supplemental Table 1**). These animals were on a C57/B6J background and the degree of proteinuria was much lower than in the 129 SvJ mice carrying the Notch1 deletion **(Figure 1E)**. We observed no significant differences in proteinuria or mesangial expansion when diabetic podocyte-specific Notch2 knockout animals were compared to control diabetic animals **(Figure 1E, F).**

Lack of Notch1 prevents podocyte dedifferentiation in vivo

Nphs1 (Nephrin) is the most widely accepted marker of podocyte differentiation and is required for maintenance of the slit diaphragm (22). Loss of Nphs1 expression is an early hallmark of glomerular disease, therefore we next analyzed Nphs1 expression. In healthy animals, Nphs1 expression was similar regardless of the genotype **(Figure 2A)**. As previously reported, Nphs1 expression was markedly reduced in diabetic mice(23). In diabetic podocyte-specific Notch2 knockout animals the decrease was similar to controls, whereas Nphs1 expression was largely preserved in podocyte-specific Notch1 knockout animals **(Figure 2A)**.

To further probe the issue of dedifferentiation we analyzed the expression of Snail1 (Snai1). Snail is a known upstream regulator of podocyte differentiation by negatively regulating Nephrin expression (24). Snail1 expression, as analyzed by immunostaining was markedly increased in control diabetic glomeruli **(Figure 2B)**. In all sections we saw strong positive staining for Snail1 in the smooth muscle layer of vasculature consistent with a known expression

pattern. Double immunofluorescence staining with Nephrin indicated increased Snai1 expression in podocytes. Snail1 expression on the other hand was not changed in podocyte-specific Notch1 knock-out animals **(Figure 2B)**.

Notch1 null podocytes were protected from TGFβ1-induced dedifferentiation

Next we wanted to examine whether Notch1 directly regulated Nphs1 expression and podocyte dedifferentiation *in vitro*. We cultured primary podocytes from control and NPHS2^{cre}Notch1$^{F/F}$ mice and treated them with transforming growth factor beta 1 (TGF-β1). TGF-β1 is a growth factor, which is strongly increased in diabetes and known to play an important role in DKD development (2; 25-28). We confirmed that TGF-β1 activity was increased in our diabetic models, regardless of genotype, by staining for nuclear localization of phosphorylated Smad 2/3, an indicator of active TGF-β signaling **(Supplemental Figure 2)**. Previous studies indicated that TGF-β1 increased cleaved Notch1 levels in podocytes via upregulating Jagged1 (ligand) expression (2). We confirmed TGF-β-mediated Jagged1 expression and Notch1 cleavage in primary control podocytes (**Supplemental Figure 3D**). As expected, in the Notch1 knockout animals, Jagged1 expression was still increased following TGF-β1 treatment but cleaved Notch1 was absent (**Supplemental Figure 3D**). After 24 hours of TGF-β1 treatment, Nphs1 transcript levels were significantly reduced in control podocytes, reflecting the dedifferentiation process that took place following TGF-β1 treatment. On the other hand, Nphs1 levels were maintained in Notch1 null podocytes even after TGF-β1 stimulation **(Figure 3A)**. Next, we examined whether the effect of Notch1 was specific for Nphs1 or if other podocyte-specific differentiation markers were also regulated. Both transcript and protein

expression of Nphs2 (Podocin) were significantly reduced 24 hours following TGF-β1 treatment in control cells. In Notch1 null podocytes, however, Nphs2 transcript expression was maintained following TGF-β1 stimulation. Podocin protein levels were significantly reduced in Notch1 null cells, but not with the severity seen in the control cells, indicating a broader effect on podocyte differentiation **(Figures 3B, C)**.

Snai1 and 2 are well-known direct, transcriptional targets of Notch (29), and as we had already observed differences in Nephrin and Snai1l expression *in vivo*, we next analyzed Snai1 and 2 regulations in cultured cells. In immortalized mouse podocytes, TGF-β1 treatment resulted in an increase in Snai1 transcript levels (Figure 3D). Treatment of podocytes with the γ-secretase inhibitor XX (GSIXX) to block Notch cleavage, did not alter Snai1 expression at the early time points (Figure 3D), but reduced the expression of Snai1 at later time points (after 8 hrs). These results indicate that the later, sustained effect of TGFβ1 on Snai1 expression is Notch dependent. To determine if the sustained expression of Snai1 was indeed Notch1-dependent, we tested the response of primary podocytes from $NPHS2^{cre}Notch1^{F/F}$ and control mice to 24 hours of TGF-β1 stimulation. Similar to immortalized podocytes, TGF-β1 treatment also lead to an increase of Snai1 expression in control primary podocytes. Snai1 expression (both transcript and protein level) was significantly lower in Notch1-null primary podocytes following TGF-β1 treatment (Figures 3E, F) indicating that sustained expression of Snai1 in podocytes depends on Notch1.

Notch signaling is highly context dependent therefore; we next analyzed podocyte dedifferentiation in primary glomeruli without significant *in vitro* culturing. In addition, this method gave us the opportunity to analyze Notch2 null glomeruli, as these cells did not tolerate *in vitro* culturing. Snai1 expression was increased following TGF-β1 treatment in podocytes of glomeruli isolated from control mice **(Figure 4)**. Nuclear specific Snai1 staining in

NPHS2^{cre}Notch1$^{F/F}$ glomeruli was notably absent following TGF-β1 treatment, consistent with the primary podocyte data. In contrast, Snai1 expression in podocytes of glomeruli isolated from NPHS2^{cre}Notch2$^{F/F}$ animals was upregulated similarly to control glomeruli **(Figure 4)**. Nphs1 expression followed the pattern we previously observed in the diabetic mice and the TGF-β1-treated podocytes. Notch1-null podocytes showed protection from dedifferentiation while it appeared that Nphs1 expression in NPHS2^{Cre}Notch2$^{F/F}$ glomeruli was similar to control, indicating a smaller role for Notch2 in regulating Nphs1 expression **(Figure 4)**. In summary, these *in vitro* results suggest that Notch1, but not Notch2, plays an important role in podocyte dedifferentiation by regulating Nphs1, Nphs2 and Snai1 expressions.

Notch1 plays an important role in podocyte apoptosis

Following severe podocyte injury, podocytes are lost by detachment and apoptosis, which is considered an irreversible step leading to glomerulosclerosis (30). Therefore we next counted glomerular podocyte number by quantifying WT-1 positive podocytes in the healthy, diabetic and podocyte-specific knockout animals. As previously reported (1; 31), glomerular podocyte number was significantly lower in diabetic control animals when compared to their healthy littermates **(Figure 5A, B)**. We found no statistically significant differences in glomerular podocyte number when diabetic NPHS2^{cre}Notch2$^{F/F}$ animals were compared to diabetic control animals **(Figure 5B)**. In contrast, podocyte number was preserved in diabetic NPHS2^{cre}Notch1$^{F/F}$ mice **(Figure 5A)**. Together, these results indicated that podocyte-specific Notch1 deletion protected kidneys from diabetes-induced podocyte loss.

Next, we asked whether the protection from podocyte loss in diabetic NPHS2^{cre}Notch1$^{F/F}$ mice could be a direct effect of Notch1 on podocyte apoptosis. We tested this by quantifying Caspase 3/7 positive primary podocytes isolated from glomeruli of control, NPHS2^{cre}Notch1$^{F/F}$ and NPHS2^{cre}Notch2$^{F/F}$ mice. Caspase3/7 is a sensitive marker of apoptosis. As described earlier, podocyte apoptosis was significantly increased 24 hours following TGFβ1 stimulation (32). We found that the number of apoptotic nuclei was significantly lower in NPHS2^{cre}Notch1$^{F/F}$ podocytes when compared to control TGFβ1-treated cells (25% vs. 44%, respectively), indicating the important role of Notch1 in podocyte apoptosis. In contrast, we found that Notch2 deletion did not ameliorate podocyte apoptosis induced by TGF-β1. In fact, podocyte apoptosis was significantly increased in podocytes isolated from NPHS2^{cre}Notch2$^{F/F}$ mice when compared to control cells **(Figure 5C, D)**. These results indicate that Notch1 play an important, roles in podocyte apoptosis *in vitro* and may explain the protective effect of Notch1 inhibition in the context of diabetes.

Notch2 expression was Notch1 dependent in podocytes

To further understand functional differences between Notch1 and Notch2 receptors and the potential interplay between them, we examined whether there is a direct regulation or compensation between these two receptors. As previously described, we found that the level of cleaved Notch1 was increased following TGF-β1 treatment **(Figure 6A, B)**. Treatment of cells with the gamma secretase inhibitor (GSIXX) ameliorated the TGF-β1-induced cleaved Notch1 expression, consistent with the previously reported ligand-mediated (Jagged1) cleavage and activation of Notch1. However, unexpectedly we found that protein level of cleaved Notch2 was decreased following TGF-β1 treatment **(Figure 6A, B)**. To see if this regulation occurred at the

transcriptional level, we analyzed Notch mRNA levels in immortalized podocytes. We found that 4 hrs following TGFβ1 stimulation Notch2 transcript levels were lower **(Figure 6C)**. This effect was not limited to cultured podocytes, as Notch2 levels were also decreased in primary podocytes following TGFβ1 treatment **(Figure 6D)**. These results indicate that while Notch1 increased following TGFβ1 treatment Notch2 expression was decreased.

Next, we examined whether Notch2 expression and cleavage was directly regulated by Notch1 levels. We found that Notch2 expression was unchanged in NPHS2^{cre}Notch1$^{F/F}$ podoctyes following TGFβ1 treatment **(Figure 6D),** indicating that the decrease in Notch2 expression depends on the presence of Notch1. We also examined whether baseline (non-stimulated) Notch2 levels were regulated by Notch1. We found that Notch2 expression was slightly, but significantly (1.9-fold, p=0.01), increased in absence of Notch1 in NPHS2^{cre}Notch1$^{F/F}$ glomeruli **(Figure 6E)**. On the other hand, Notch1 did not compensate for the Notch2 loss in the NPHS2^{cre}Notch2$^{F/F}$ mice and had levels of Notch1 identical to control glomeruli **(Figure 6F).** These results indicate that the regulation of Notch2 in podocytes, both at baseline and following TGF-β1 treatment, is Notch1-dependent.

Because we do not detect increased Notch2 levels in the primary cultured Notch1 knockout podocytes, but did detect this increase in the whole glomeruli, we further probed the Notch1-dependent Notch2 podocyte regulation in isolated glomeruli. We found increased cleaved Notch1 expression in the nuclei of podocytes from control glomeruli following TGF-β1 stimulation **(Figure 7)**. Glomeruli from NPHS2^{cre}Notch2$^{F/F}$ animals occasionally showed cleaved Notch1 expression in podocytes prior to stimulation, but following TGF-β1 treatment cleaved Notch1 expression was increased to a similar degree as in control glomeruli **(Figure 7)**. Unlike cleaved Notch1, we detected a low level of cleaved Notch2 in wild-type glomeruli at baseline

both in Nphs1-positive and negative cells. Following TGFβ stimulation, the overall expression of Notch2 decreased, **(Figure 7),** results congruent with the TGFβ-induced decrease of Notch2 protein and mRNA in the immortalized mouse podocytes **(Figure 6B-D)**. There was no change in cleaved Notch2 levels in $NPHS2^{Cre}Notch1^{F/F}$ glomeruli following TGF-β1 treatment **(Figure 6D)**. These results confirm that Notch2 levels are regulated by Notch1 both at baseline and following TGF-β1 stimulation in podocytes, but that this regulation is not reciprocal.

We also examined the expression of Notch3 and 4 receptors in our system. Notch3 (42) (43) has been proposed to play role in tubulointersitial injury and nephrotoxin-induced glomerulonephritis respectively. Upregulation of Notch4 has been described in HIV-associated Nephropathy (HIVAN) (12). In our hands, Notch3 and Notch4 transcripts were not increased in immortalized podocytes following TGF-β1 stimulation **(Supplemental Figure 6A &D)**. Similarly we did not observe differences in Notch3 transcript and protein expression following TGF-β1 stimulation of primary podocytes **(Supplemental Figure 6B &C)**.

Podocyte specific expression Notch1 intracellular domain causes glomerulosclerosis, but Notch2 intracellular domain does not

As we observed marked differences in phenotype development following podocyte specific Notch1 and Notch2 deletion; we asked if increased Notch1 and Notch2 are functionally equivalent. Previously, our and the Piscione group, found that over-expression of the Notch1 intracellular domain in podocytes resulted in rapid development of albuminuria and glomerulosclerosis (2; 33). Therefore we examined whether expression of the intracellular domain of Notch2 could have similar effects in podocytes. To address this, we developed a new

mouse model by crossing the podocin Cre mouse ($NPHS2^{Cre}$) with a transgenic mouse line expressing the intracellular domain of Notch2 (R26Notch2) (18). The R26Notch2 mouse line carries a stop codon flanked by a LoxP site just prior to the inserted ICNotch2 cassette. This mouse line has been shown earlier to drive robust expression of Notch2 during development (18). We confirmed that the domain structure of the over expressed proteins (aa1749-2293 for NICD1 and aa1701-2470 for NICD2) were structurally equivalent. In this model the Cre-mediated recombination results in cell type specific deletion of the stop codon, thus initiating expression of the Notch2 intracellular domain. Robust cleaved Notch2 expression in podocytes was confirmed using an antibody specific to amino acid, alanine 1733 (the cleaved Notch2) **(Figure 8)**.

We did not observe significant structural abnormalities in mice with podocyte-specific NICD2 expression even at 20 weeks of age. This was in sharp contrast to mice with podocyte-specific Notch1 over expression **(Figure 8)**, using the Nephrin rtTA/TRE ICNotch1. We concluded from these results that the Notch2 intracellular domain might have a different effect from the Notch1 intracellular domain and while NICD1 over expression causes severe glomerulosclerosis the NICD2 over expression did not.

DISCUSSION

In this study, we show that Notch1 expression in podocytes plays a non-redundant role in diabetic kidney disease development. We used mouse models with genetic deletion of Notch1 or Notch2 specifically from mature podocytes and show that deletion of Notch1 protected mice from diabetic kidney disease, including albuminuria and glomerulosclerosis. On the contrary,

deletion of Notch2 had no effect on disease course. We show that Notch1 plays an important role in regulating Nphs1, Nphs2 and Snai1 and thereby podocyte dedifferentiation. Snai1 is a critical transcription factor for dedifferentiation and transdifferentiation in epithelial cells and is a well-known target of both Notch and TGFβ/Smad3 (24). Regulation of Snail1 in podocytes has been shown *in vitro* and in the PAN injury model *in vivo* (24). Here we show that while the initial activation of Snail1 by TGF-β/Smad3 is independent of Notch signaling, Notch1 is required for its sustained expression. This sustained Snai1 activation might play an important role in glomerular disease, as we observed differences in Snai1 expression both in animal models and patient samples with diabetic kidney disease **(Figures 2, 3, Supplemental 2).** Our studies indicated that Notch regulated Snai1 and Nphs1 expression, and the dedifferentiation program. Direct interaction between the Nephrin ortholog (Sticks-and-Stones, Hibris) and Notch has also been described in Drosophila (34). It has previously been shown that Notch regulates Nephrin levels via endocytosis (35). Further studies shall determine the contribution of each of these mechanisms to diabetic kidney disease development.

Dedifferentiation and apoptosis are two key alterations affecting podocytes during disease conditions. It is likely that early on, dedifferentiation is an important mechanism to protect cells from apoptosis, however, with severe damage podocytes eventually die or detach leading to permanent scarring of the glomerulus (36). Deletion of Notch1 was sufficient to protect podocytes from TGF-β-induced apoptosis. This is consistent with our prior observations showing that over expression of the Notch1 intracellular domain lead to p53-mediated apoptosis (2). Putting all this together indicates that Notch1 is both sufficient and necessary for TGF-β-induced apoptosis. While Notch1 deletion protected from apoptosis, the rate of apoptosis was actually higher in Notch2 null cells. These results are also consistent with recent reports by

Tanaka et al. (15) indicating that a Notch2 agonist antibody activates Akt and other pro-survival pathways in podocytes. In summary it seems that Notch1 and Notch2 play different and, in the case of apoptosis, perhaps even antagonistic roles in podocytes.

Within the field of Notch biology, there are several examples for differential functions of Notch1 and Notch2. The closest example to our study is the differential effect of Notch1 and Notch2 deletion during kidney development. While genetic deletion of Notch2 in mouse models and genetic mutation of NOTCH2 in patients cause kidney developmental abnormalities, deletion of Notch1 does not have a similar effect. Opposite affects of Notch1 and Notch2 on cancer growth and survival are noted in embryonic brain tumors (37) and astrocytic gliomas (38). A study of more than 1,000 Chinese patients with colorectal cancer found that higher Notch1 expression was predictive of lower (~25%) 5-year survival rates (39). Conversely, higher Notch2 tissue expression predicted better survival (~60%) with the highest rates found in patients who had tumors that were both Notch1 negative but Notch2 positive (39).

Notch receptors show high degree of similarities, in particular the intracellular domains of Notch1 and Notch2 share more than a 97% homology. Therefore, it is fascinating to learn about functional differences between Notch1 and Notch2 within the same tissue. The Kopan group generated chimeric proteins between the extracellular and intracellular domains of Notch1 and Notch2. Using these genetically engineered chimeric proteins, they demonstrated that during kidney development, Notch2 is able to reach the cell surface more efficiently than Notch1 due to differences in their extracellular domains (9). In this context, the same study demonstrated that the intracellular domains of Notch1 and 2 are functionally interchangeable and that only the difference in cell surface abundance and ligand affinity was enough to confer proximal tubule developmental dependence on Notch2 but not Notch1.

Furthermore, in this study we show that genetic overexpression of Notch1 or Notch2 intracellular domains are not equivalent in podocytes. Increased expression of Notch1 causes albuminuria and glomerulosclerosis, while mice with overexpression of the Notch2 intracellular domain does not show phenotypic alterations. This is surprising as even during development, overexpression of the Notch1 intracellular domain is able to compensate for Notch2 to rescue the developmental defect (9). In our studies we made sure to use proteins with comparable domain structures with the hope that they with generate comparable signal strengths. At the moment we entertain two different but non-exclusive possibilities for our observations in the podocyte NICD overexpressing mice. The first possibility is that despite our efforts, the signal strengths generated by the Notch1 and Notch2 intracellular domains are different, leading their different transcriptional outcome. The second possibility is that despite the high similarities it is possible that Notch1 and Notch2 intracellular domains have different transcriptional binding partners. Detailed future experiments will be needed to differentiate between these possibilities.

In addition to identifying differences between Notch1 and Notch2 activation we also found that Notch1 regulated Notch2 expression in podocytes. Deletion of Notch1 resulted in an increase in Notch2 expression. Such compensatory increase is usually not the rule but it has been shown previously in mesothelial cells (40) and mouse embryonic fibroblasts (41). The Nakayama group found that in fibroblasts the loss of cell cycle regulator, Fbxw7 lead to increased stabilization of the NICD1 and a decrease in the expression of NICD2. They concluded that the decrease in NICD2 is a direct result of an increase in NICD1, as NICD1 overexpression also lowered NICD2 levels. Finding in mesothelioma cells showed striking similarities to podocytes indicating a compensatory increase in Notch2 levels upon Notch1 deletion (40). These results indicates that a potentially interesting circuit in Notch regulation that

would need to be further explored, unfortunately, while *in vitro* studies have provided some interesting and important insight into this circuitry, *in vivo* studies will be important to define this mechanism.

In our view the diabetic and/or TGF-β driven podocyte injury is more likely a reflection of increased Notch1 expression as we found little evidence that Notch2 was playing a role in pathology. In the context of podocyte injury, Notch1 was harmful and Notch2 had a protective effect on apoptosis, but only Notch1 induced podocyte dedifferentiation. This differential role of Notch1 and 2 may explain why loss of the Notch1 receptor, but preservation of the Notch2 receptor, in podocytes appears to be more effective in ameliorating glomerulosclerosis and proteinuria than was podocyte-specific loss of the pan-Notch co-transcriptional regulator, RBPj (2) or pharmacological inhibition of Notch signaling by gamma secretase inhibitors. These observations are in keeping with recent pharmacological studies indicating that Notch2 activation (by agonist antibodies) has a protective effect in the doxorubicin-induced nephrosis model (15). In that study, the authors show that a Notch2 agonist led to the activation of pro-survival pathways in podocytes. On the other hand, our genetic studies do not fully support the role of Notch2, as loss of Notch2 *in vivo* did not exacerbate diabetic nephropathy development. It is possible that while the short-term effect of increased Notch2 signaling is associated with a strong survival benefit, it is lost in long-term models such as diabetes.

Our studies indicate that the timing, duration and strength of the Notch receptor and ligand expression patterns likely play important roles in negotiating the balance between physiologic healing and pathologic fibrosis. Prior studies by the Romagnani group support these conclusions (44). The co-regulation of the Notch1 and 2 receptors in the podocyte, however, suggests that global targeting canonical Notch signaling will not be effective in preventing

diabetic renal injury. Rather, our results demonstrate the need for a more directed therapeutic approach involving both the inhibition of Notch1 and possibly the maintenance of Notch2.

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Author Contributions: M.T.S, N.T., A.G. and K.S. participated in experimental design and data analysis, M.T.S, N.T. and A.G. performed experiments and collected data, M.T.S and K.S. wrote and edited the manuscript, R.N. and L.J.S. created some of the mouse models used in these studies and provided editorial insight.

FIGURE LEGENDS

Figure 1. NPHS2^{Cre}Notch1$^{F/F}$, but not NPHS2^{Cre}Notch2$^{F/F}$ mice were protected from diabetic glomerulosclerosis.

(A) Notch1 transcript levels in control and NPHS2^{Cre}Notch1$^{F/F}$ glomeruli. Error bars represent standard deviation and p-values determined by t-test.

(B) Urine albumin and creatinine ratios in non-diabetic and diabetic NPHS2^{Cre}Notch1$^{F/F}$ and control mice. Error bars represent standard deviation and p-values determined by 2-way ANOVA.

(C) Representative images of Periodic acid-Schiff stained kidney samples from control and NPHS2^{Cre}Notch1$^{F/F}$ mice in the presence or absence of diabetes at 20 weeks of age.

(D) Notch2 transcript levels in control and NPHS2^{Cre}Notch2$^{F/F}$ glomeruli.

(E) Urine albumin creatinine ratios in non-diabetic and diabetic NPHS2^{Cre}Notch2$^{F/F}$ and control mice.

(F) Representative images of Periodic acid-Schiff stained kidney samples from control and NPHS2^{Cre}Notch2$^{F/F}$ mice in the presence or absence of diabetes at 20 weeks of age.

Figure 2. Notch1 regulated Nphs1 and Snai1 expression in podocytes in diabetic animals.

(A) Representative images of Nphs1 (Nephrin) immunofluorescence (red) in non-diabetic and diabetic NPHS2^{cre}Notch1$^{F/F}$, and NPHS2^{cre}Notch2$^{F/F}$ mice. Images were obtained with identical capture parameters.

(B) Representative immunofluorescence images of Nphs1 (red), Snail1 (green) and nuclei (Hoechst 33342, blue) of kidney sections of control, NPHS2^{cre}Notch1$^{F/F}$ and NPHS2^{cre}Notch2$^{F/F}$ mice at 20 weeks of age. Insets show examples of punctate nuclear Snail1 staining in podocytes from control diabetic mouse (Top two) and NPHS2^{cre}Notch2$^{F/F}$ mouse (Bottom two). The asterix (*) indicates positive Snail1 staining in the smooth muscle layer. Images were obtained with identical capture parameters.

Figure 3. Notch1 mediated TGFβ1-induced podocyte dedifferentiation.

(A) Transcript levels of Nphs1 (Nephrin) in primary cultured podocytes obtained from control (n = 5) and $NPHS2^{cre}Notch1^{F/F}$ mice (n = 4) and treated with or without TGFβ1 for 24 hours.

(B) Transcript levels of Nphs2 (Podocin) in primary cultured podocytes obtained from control (n = 5) and $NPHS2^{cre}Notch1^{F/F}$ (n = 4) mice and treated with or without TGFβ1 for 24 hours.

(C) Representative western blots and densitometry-based quantification of Nphs2 protein levels in cultured glomeruli. Each sample was normalized to γ-Tubulin expression and expressed as a fold change between control and TGFβ1 treated samples. (control n = 6, $NPHS2^{cre}Notch1^{F/F}$ n = 6)

(D) Relative Snai1 transcript levels of immortalized mouse podocytes treated with TGFβ1 in the presence or absence of γ-secretase inhibitor XX (GSIXX) (n = 5).

(E) Relative transcript levels of Snai1 in control and Notch1 podocytes with sham or TGFβ1 treatment

(F) Representative western blots and densitometry based quantification of Snail1 protein expression in cultured primary podocytes. Each sample was normalized to GAPDH expression and expressed as a fold change between control and TGFβ1 treated samples (control n = 4, $NPHS2^{cre}Notch1^{F/F}$ n = 4).

Data information: Asterisk (*) indicates statistically significantly differences by student t-test, p-value < 0.05.

Figure 4. Podocyte Snail1 expression is regulated by Notch1.

Representative immunofluorescent images of Nephrin (red) and Snail1 (green) in glomeruli obtained from control, $NPHS2^{cre}Notch1^{F/F}$, and $NPHS2^{cre}Notch2^{F/F}$ animals - with or without 10

ng/ml TGF-β1. Nuclei were visualized using NucBlue Fixed. Insets show higher magnification of Snail1+Nephrin positive podocytes.

Figure 5. Loss of Notch1 prevented whereas loss of Notch2 exacerbates podocyte apoptosis.

(A) Quantification of WT-1 positive podocytes per glomerular cross section in control and diabetic NPHS2^{Cre}Notch1$^{F/F}$ mice. Error bars represent standard deviation and p-values determined by 2-way ANOVA.

(B) Quantification of WT-1 positive podocytes per glomerular cross section in control and diabetic NPHS2^{Cre}Notch2$^{F/F}$ mice. Error bars represent standard deviation and p-values determined by 2-way ANOVA.

(C) Mean percent of apoptotic (caspase 3/7 positive) podocytes isolated from control, NPHS2^{Cre}Notch1$^{F/F}$ or NPHS2^{Cre}Notch2$^{F/F}$ mice and quantified before (NT) or after 24 hours of TGF-β1 stimulation (TGF-β). Error bars represent standard deviation and p-values determined by student t-test.

(D) Representative images of Caspase 3/7 reporter (green nuclei) and nuclear blue (live cells) in primary podocytes.

Figure 6. Notch1 dependent regulation of Notch2 in podocytes.

(A) Representative immunoblot of cleaved Notch1 in immortalized mouse podocytes treated with TGFβ in absence or presence of GSIXX. Graph shows Densitometry based quantification of protein levels of cleaved Notch1 (n=4) normalized to the average no treatment at 0 hour and β-Actin for loading control. P-values determined by student t-test.

(B) Representative immunoblot of cleaved Notch2 in immortalized mouse podocytes treated with TGFβ in absence or presence of GSIXX. Graph shows Densitometry based quantification of protein levels of cleaved Notch2 (n=3) normalized to the average no treatment at 0 hour and β-Actin for loading control. P-values determined by student t-test.

(C) Relative transcript levels of Notch2 in immortalized mouse podocytes treated with TGFβ for 0-24 hrs.

(D) Relative Notch2 transcript levels of control and NPHS2^{cre}Notch1$^{F/F}$ primary podocytes at baseline and following TGFβ treatment. P-values determined by student t-test.

(E) Relative Notch2 transcript levels in control and NPHS2^{cre}Notch1$^{F/F}$ glomeruli. (n = 5, p-values determined by two-way ANOVA).

(F) Relative Notch1 transcript levels in control and NPHS2^{cre}Notch2$^{F/F}$ glomeruli. (n = 4, p-values determined by two-way ANOVA).

Data Information: Error bars are presented as SEM

Figure 7. Increased Notch2 expression in NPHS2^{cre}Notch1$^{F/F}$ glomeruli.

Representative immunofluorescence images of cleaved Notch1 (green) cleaved Notch2 (green) and Nephrin (red) of glomeruli isolated from wild type (WT), NPHS2^{cre}Notch1$^{F/F}$ and NPHS2^{cre}Notch2$^{F/F}$ animals following TGF-β1 or vehicle treatment for 3 hours. Negative Rabbit IgG control is shown in bottom most row.

Figure 8. Podocyte specific constitutive expression of Notch2 intracellular domain does not induce glomerulosclerosis.

(A) Representative images of kidney section of control and NPHS2^{Cre}/R26Notch2 animals, stained for cleaved Notch2 or PAS.

(B) Representative PAS stained images of kidney sections of control and NPHS2rtTA/TRE-ICNotch1 animals treated with doxycycline for 3 weeks.

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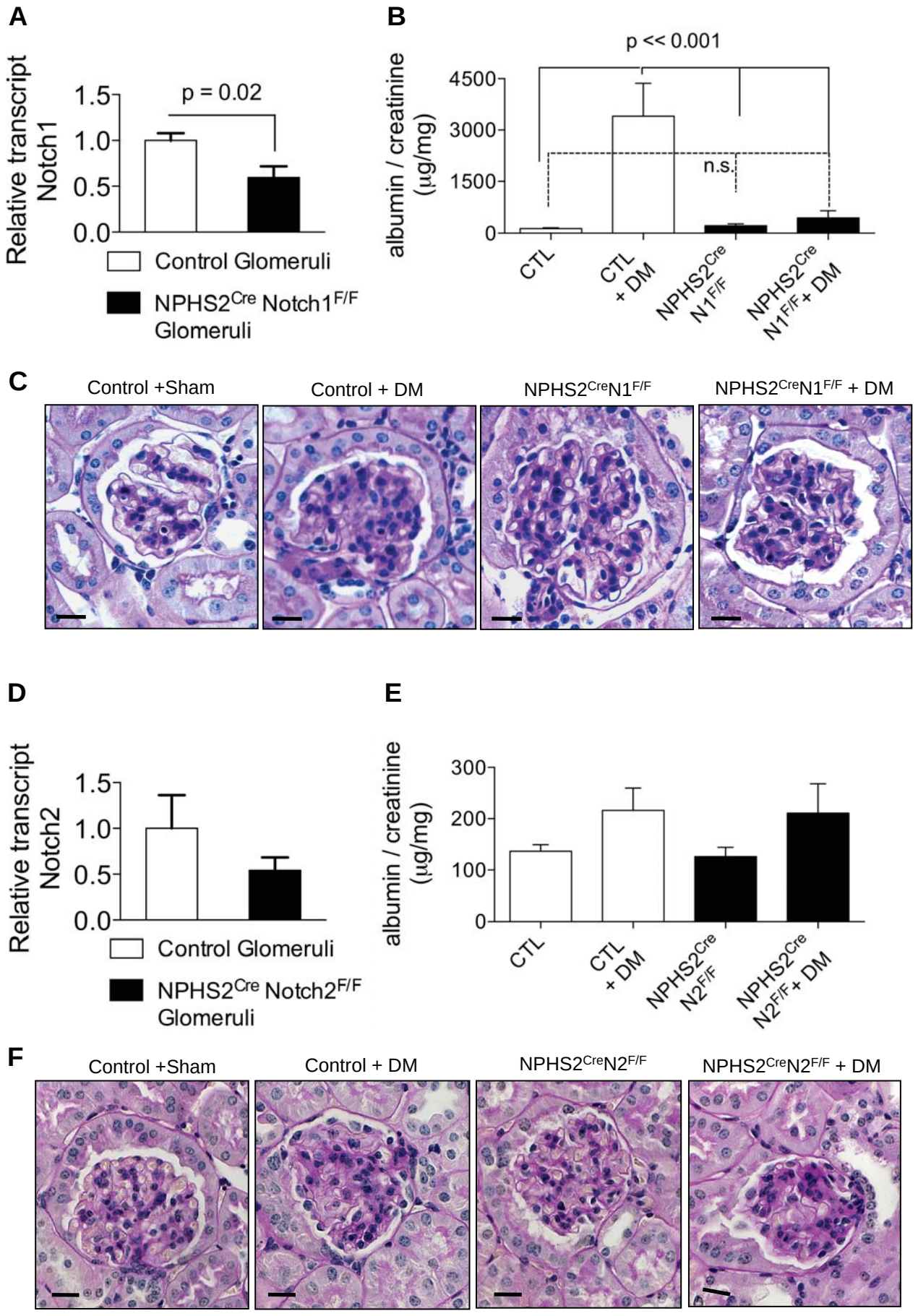
Figure -1. Sweetwyne/Susztak



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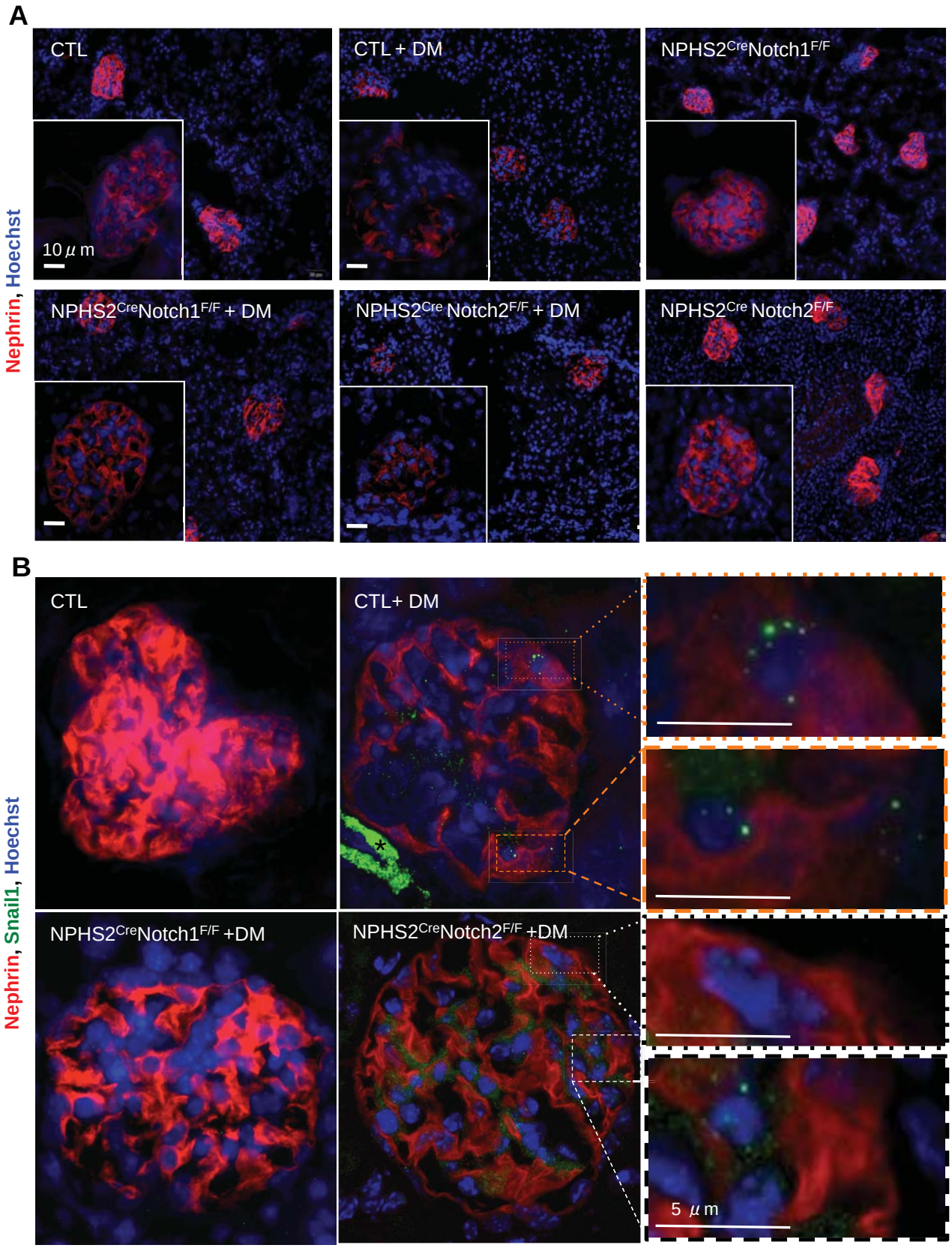

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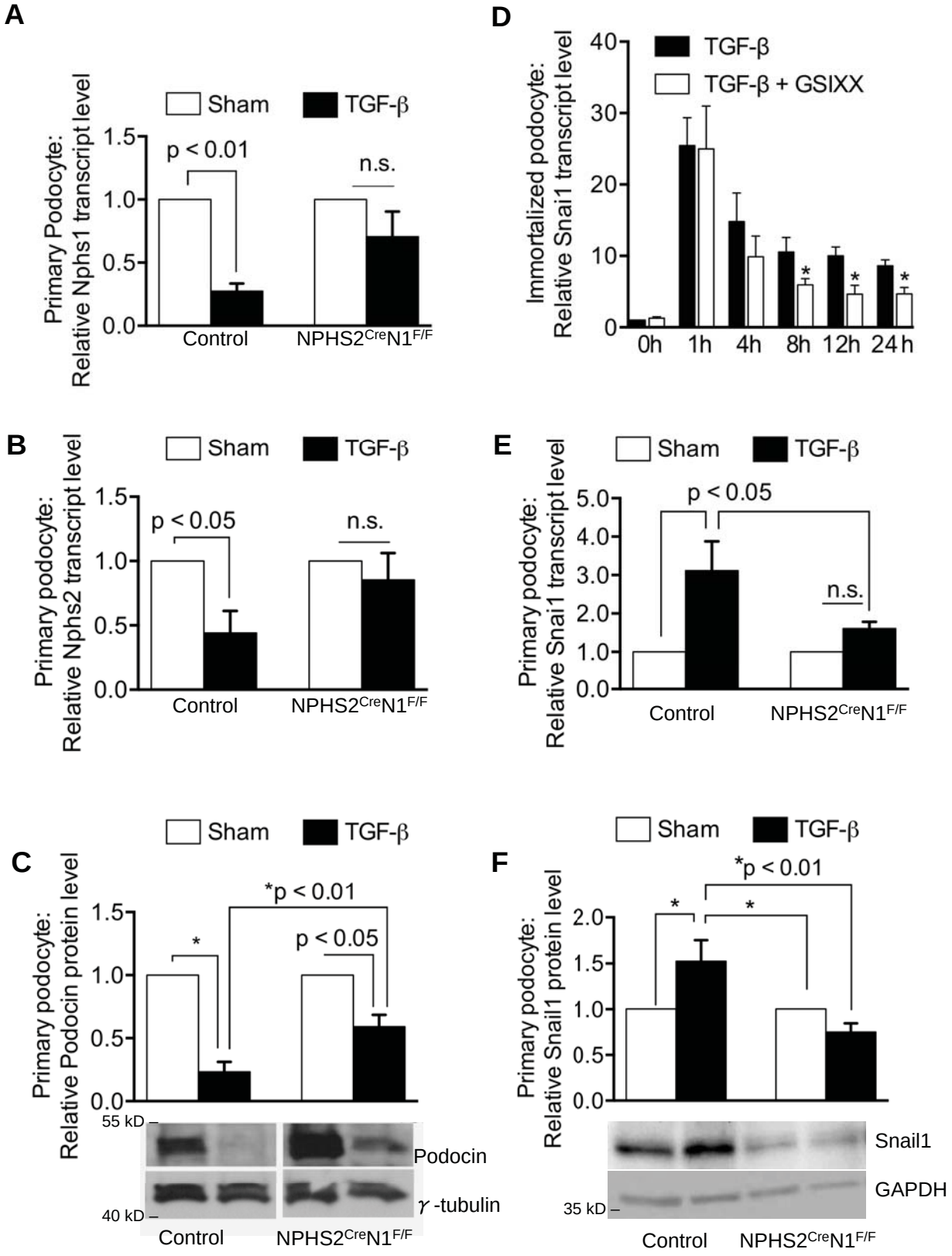

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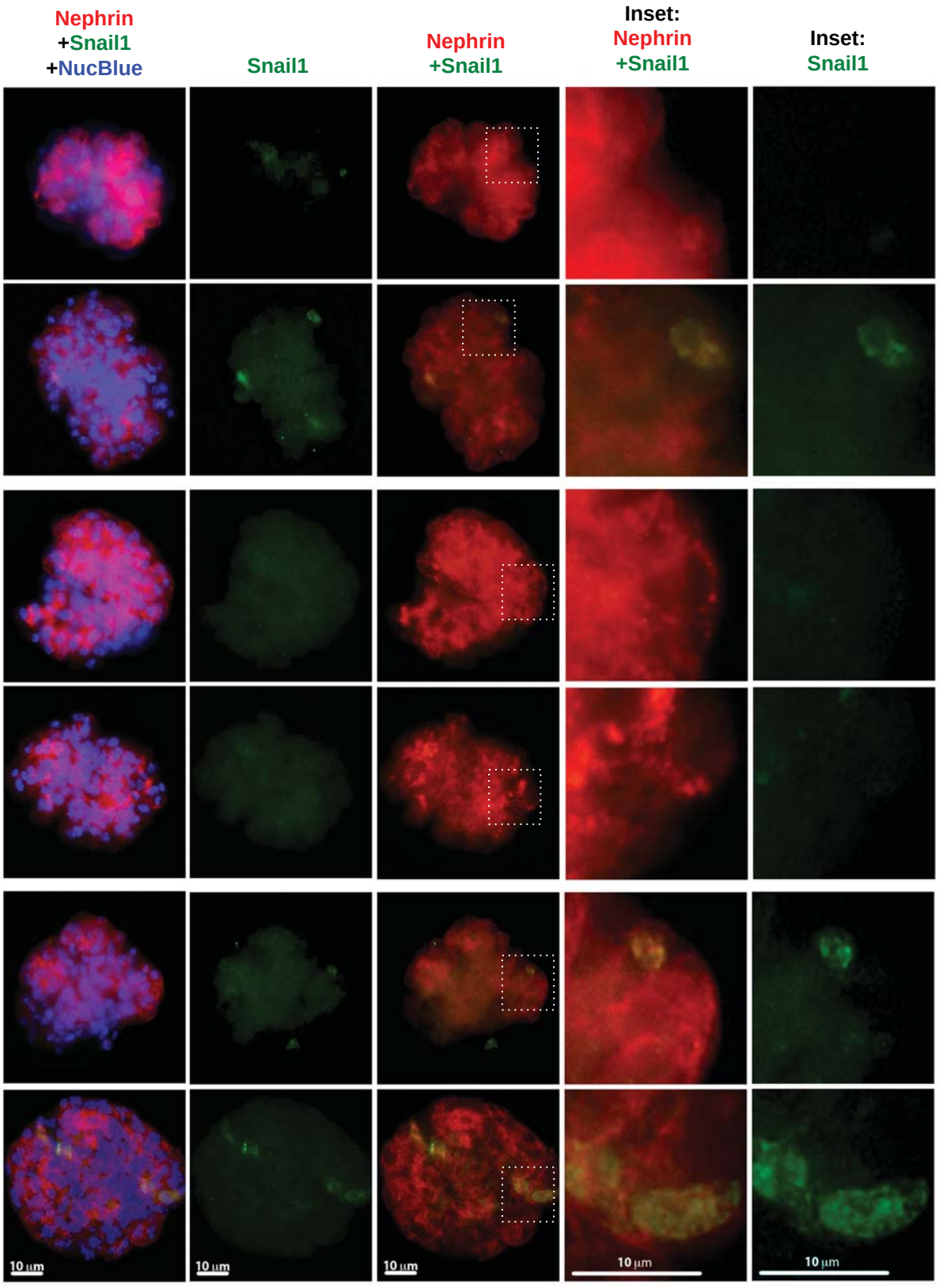

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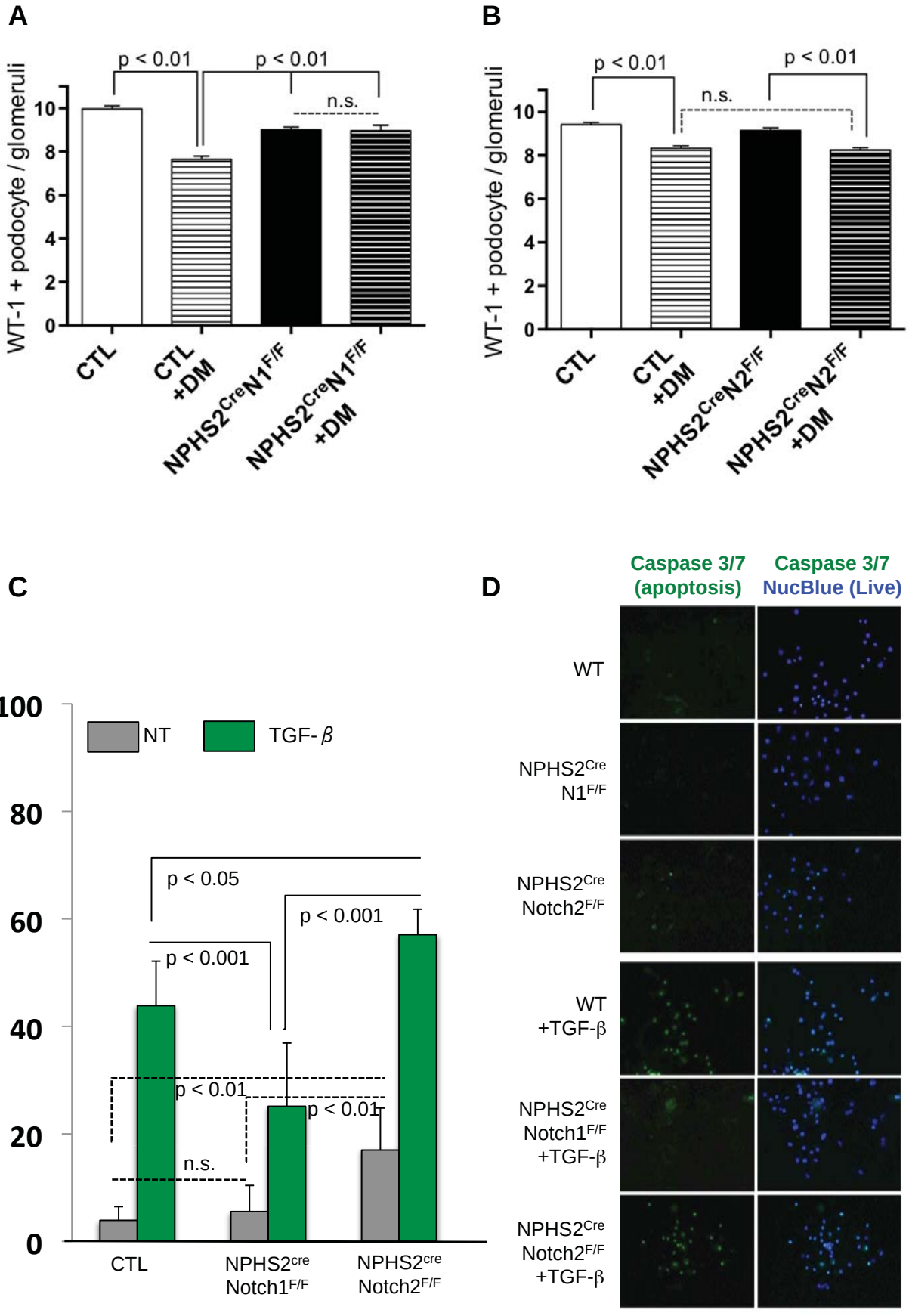

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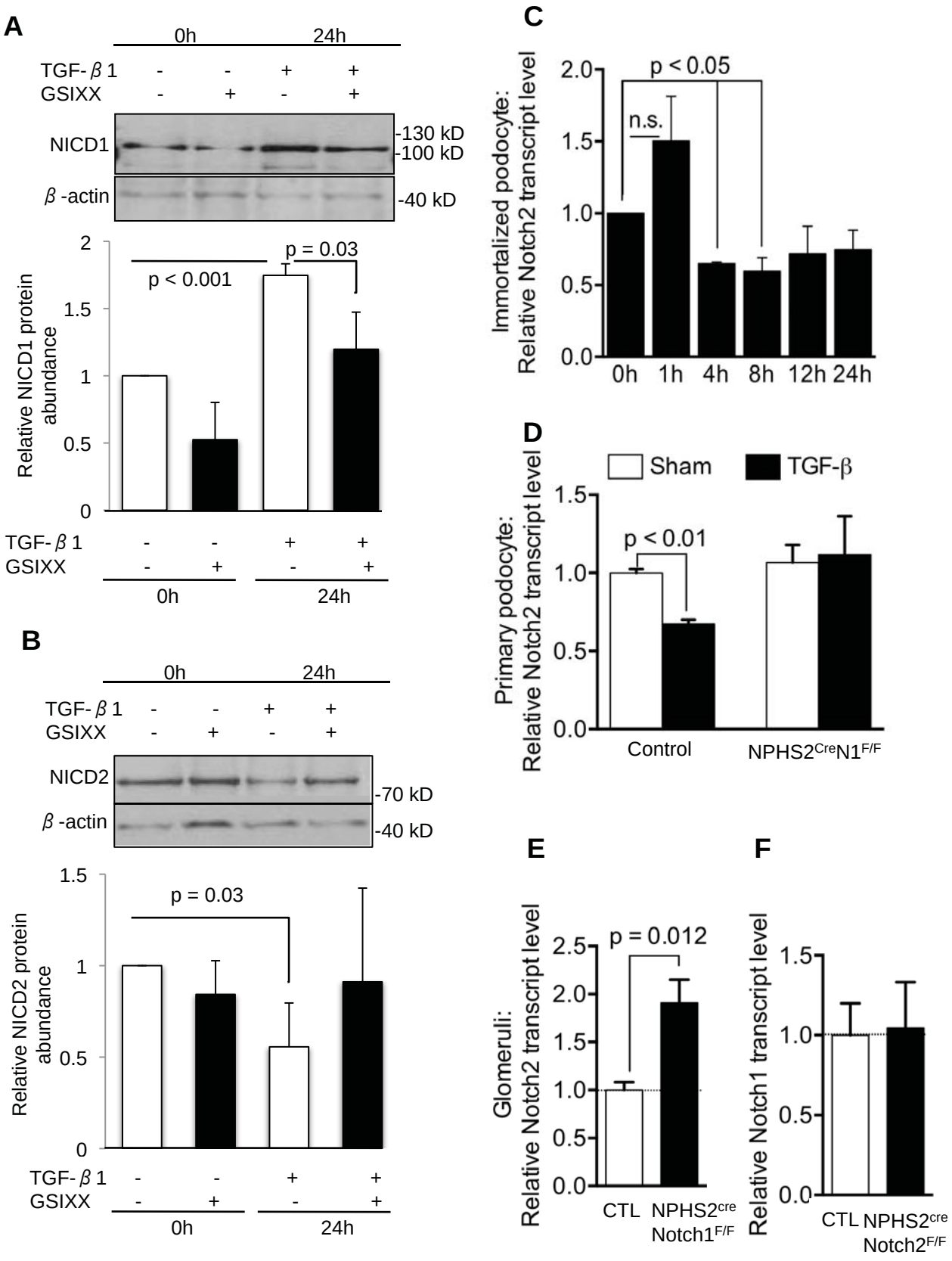

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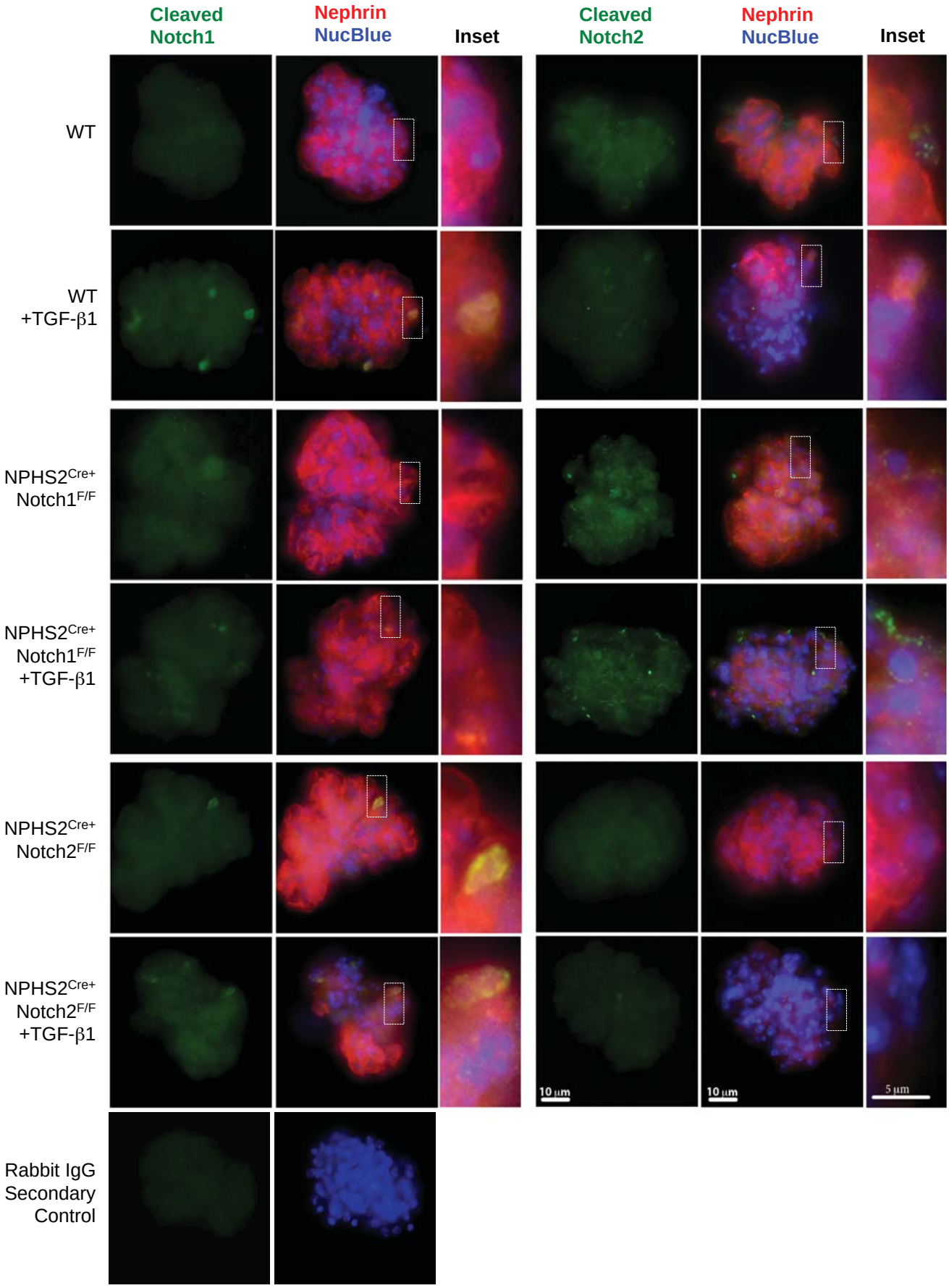

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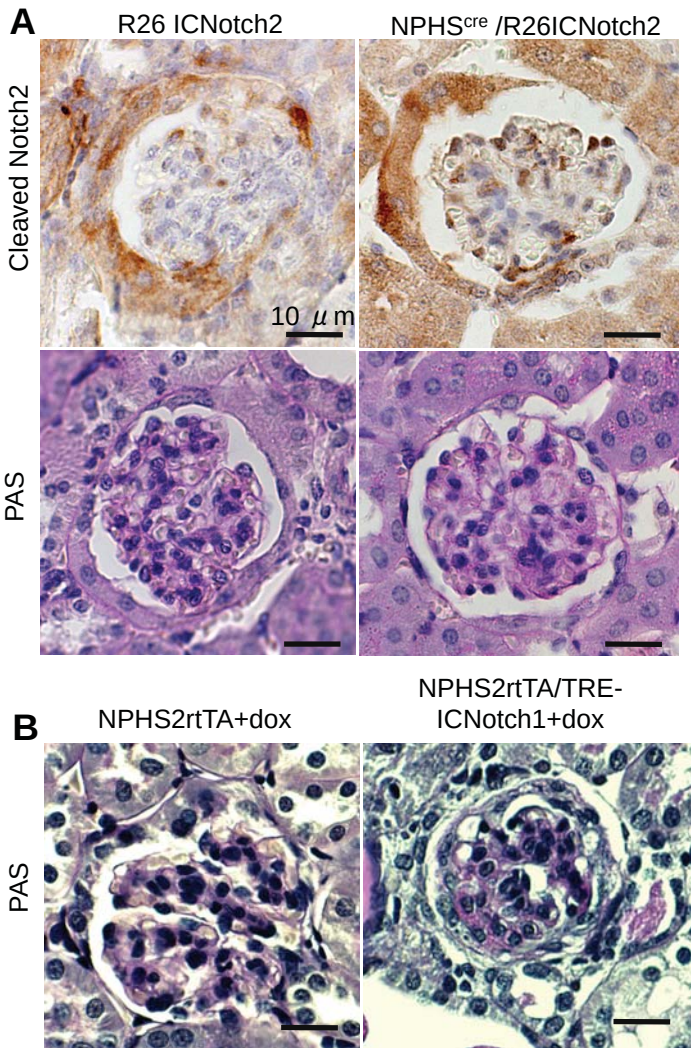
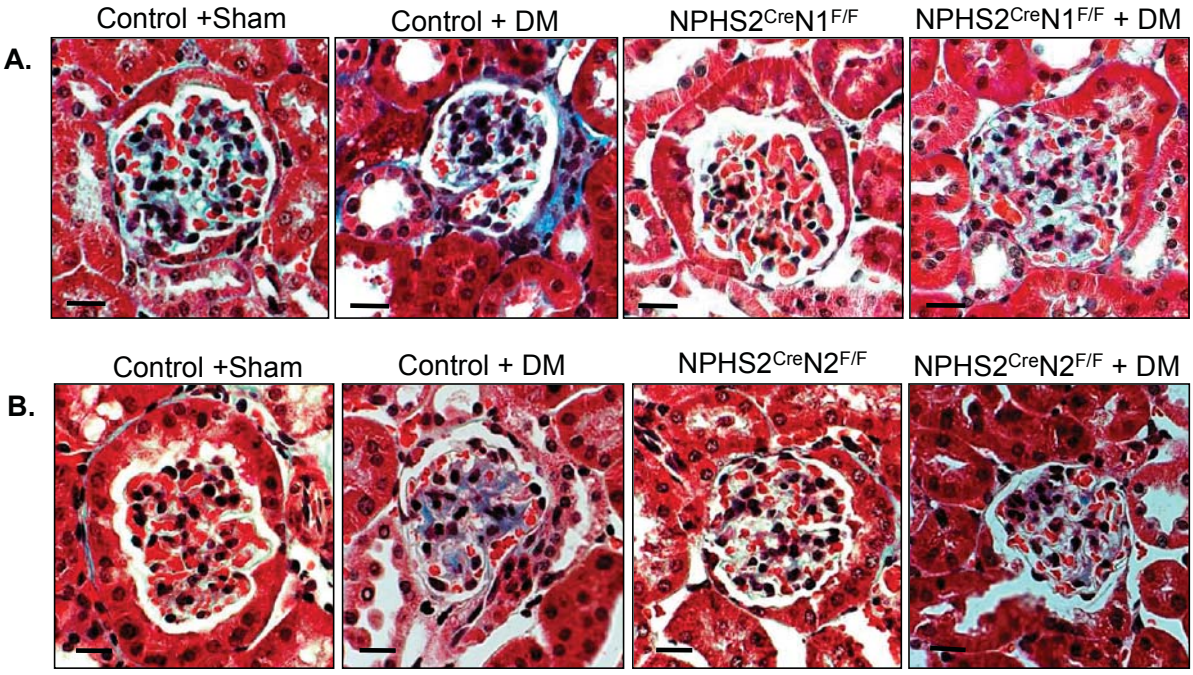


Sweetwyne/Susztak

Supporting Data Table-1. Hyperglycemia in response to streptozocin insult is not altered with podocyte loss of either Notch1 or Notch2.

	Notch1$^{F/F}$			Notch2$^{F/F}$		
	n	Body Weight (g)	Blood Glucose (mg/dl)	n	Body Weight (g)	Blood Glucose (mg/dl)
Control	9	36.1 ± 5.1	291 ± 19	14	34.9 ± 8.7	213 ± 10
NPHS2^{cre}	16	33.7 ± 5.7	296 ± 14	11	31.9 ± 4.8	188 ± 10
Control +DM	8	22.7 ± 6.9	529 ± 27	13	22.2 ± 4.8	515 ± 18
NPHS2^{cre} +DM	15	26.2 ± 3.8	530 ± 12	10	21.1 ± 4.2	502 ± 25

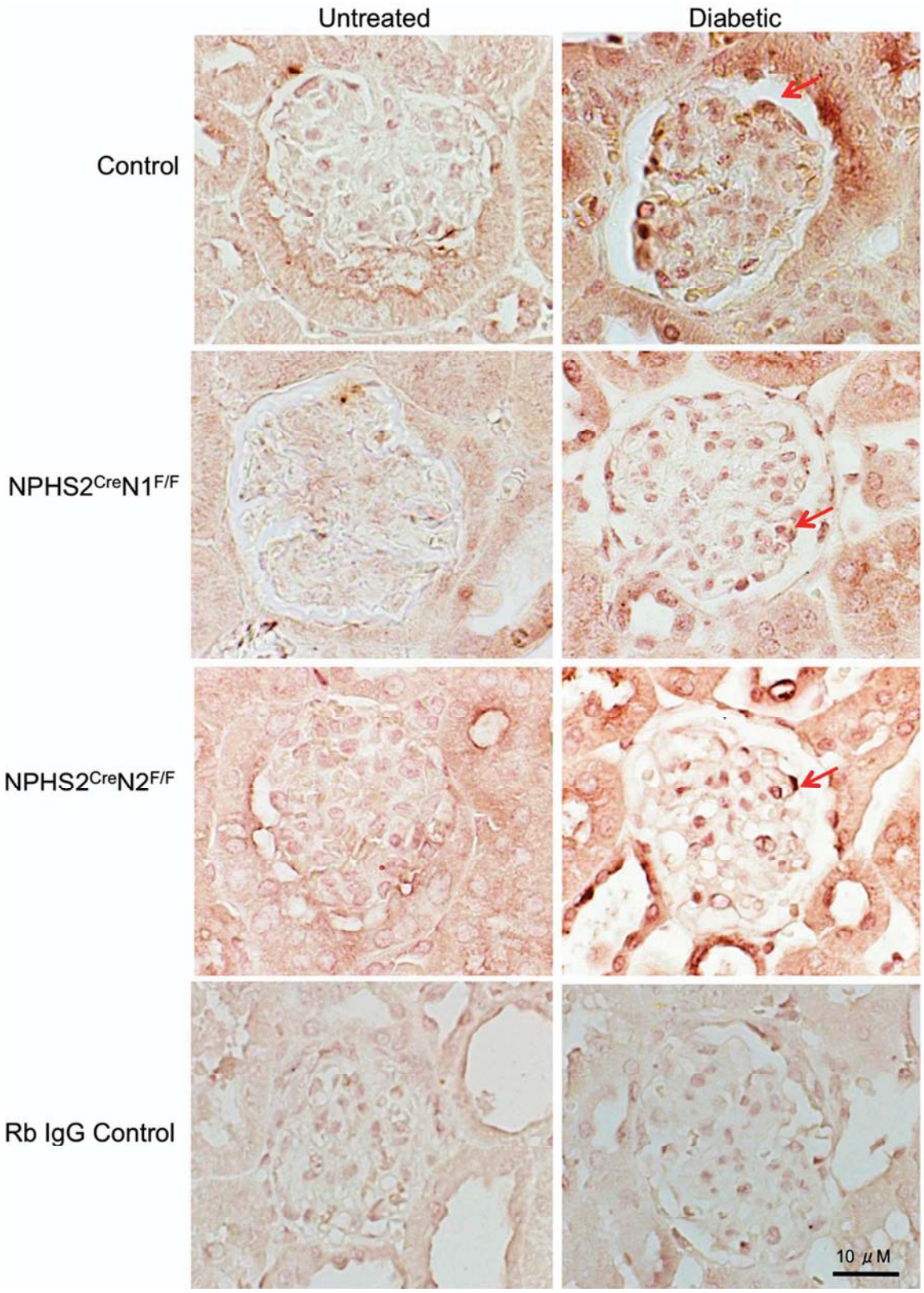
Supplemental Table 1. Serum glucose levels and body weight in podocyte specific Notch1 or Notch2 knock-out animals. Average mouse weight and non-fasting blood glucose are shown per group +/- standard deviation.



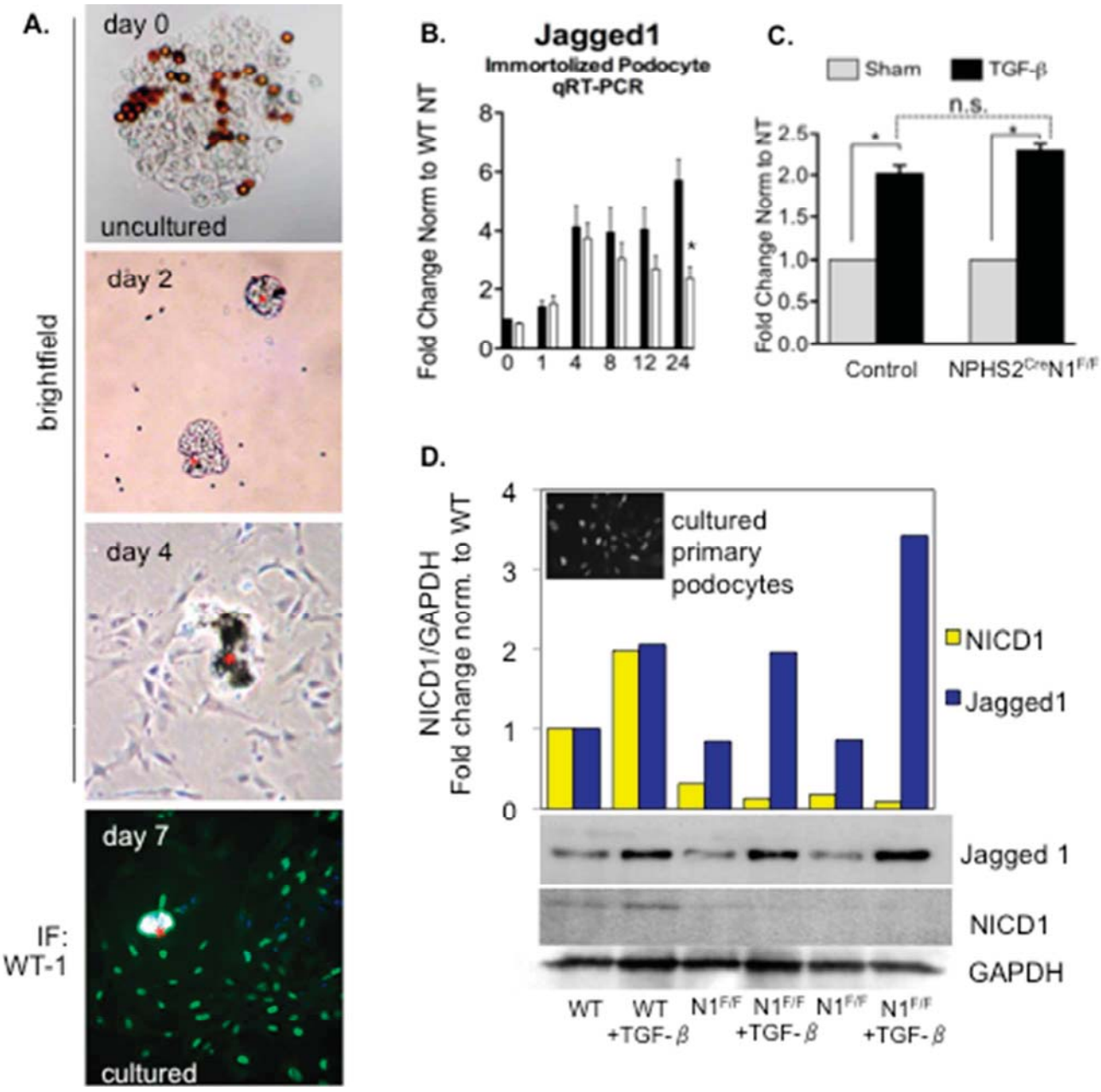
Supplemental Figure 1.

(A) Representative images of Masson's trichrome stained kidney samples from control and NPHS2^{Cre}Notch1$^{F/F}$ mice in the presence or absence of diabetes at 20 weeks of age.

(B) Representative images of Masson's trichrome stained kidney samples from control and NPHS2^{Cre}Notch2$^{F/F}$ mice in the presence or absence of diabetes at 20 weeks of age.



Supplemental Figure 2. Representative images of immunohistochemistry for phosphorylated-Smad2/3 Antibody (Ser 423/425, goat polyclonal IgG, Santa Cruz sc-11769-R) kidney samples from control, NPHS2^{Cre}Notch1$^{F/F}$ and NPHS2^{Cre}Notch2$^{F/F}$ mice in the presence or absence of diabetes at 20 weeks of age. Arrows show positive cells which are likely podocytes based on location and shape.



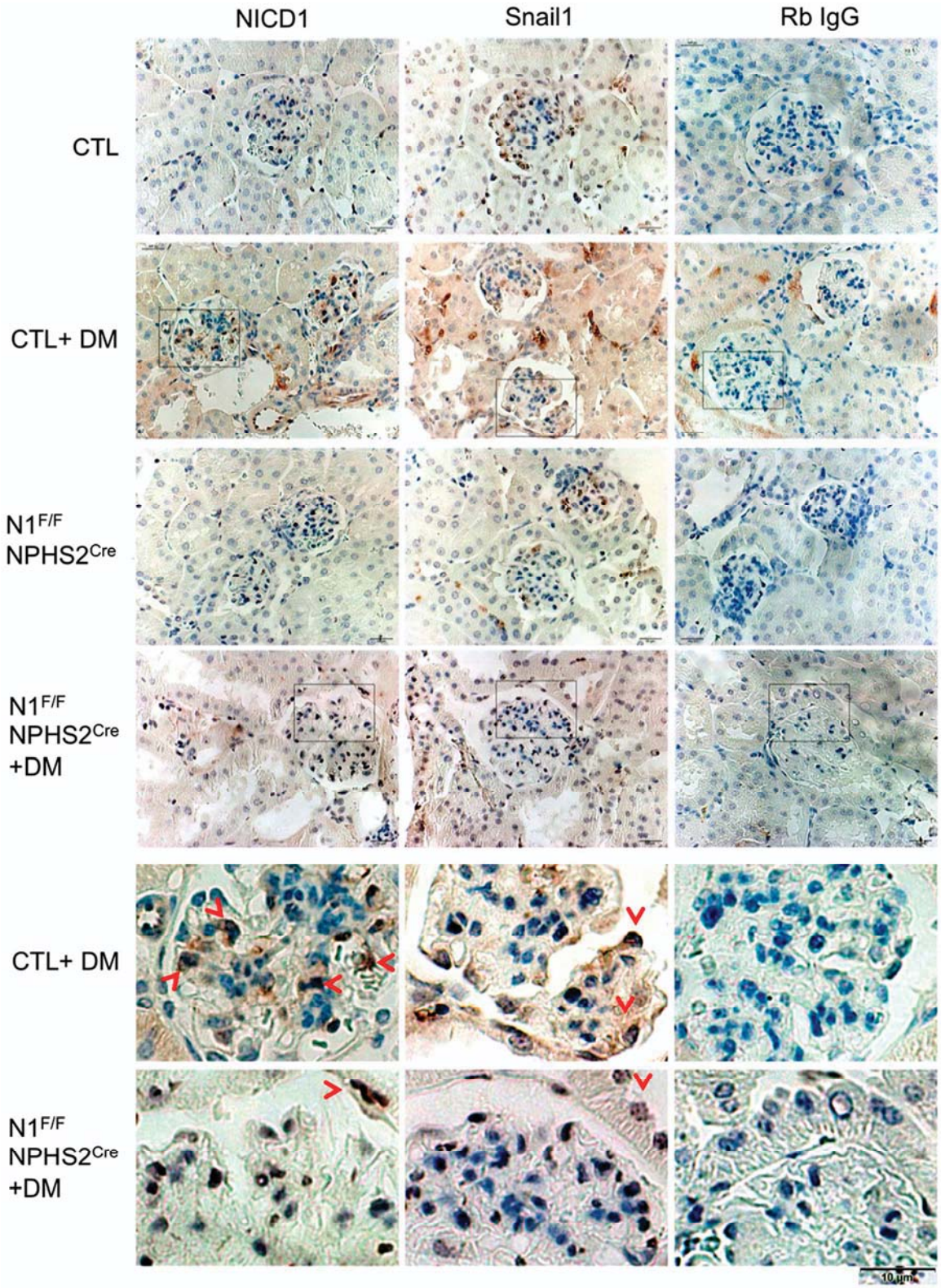
Supplemental Figure 3. TGF-β1 –induced Jagged-1 and Notch1 regulation in primary mouse podocytes.

(A) Representative images depicting stages of glomerular harvest and culture. Podocytes were identified as WT-1 positive nuclei at day 7 confirming that >85% of cultured cells were podocytes.

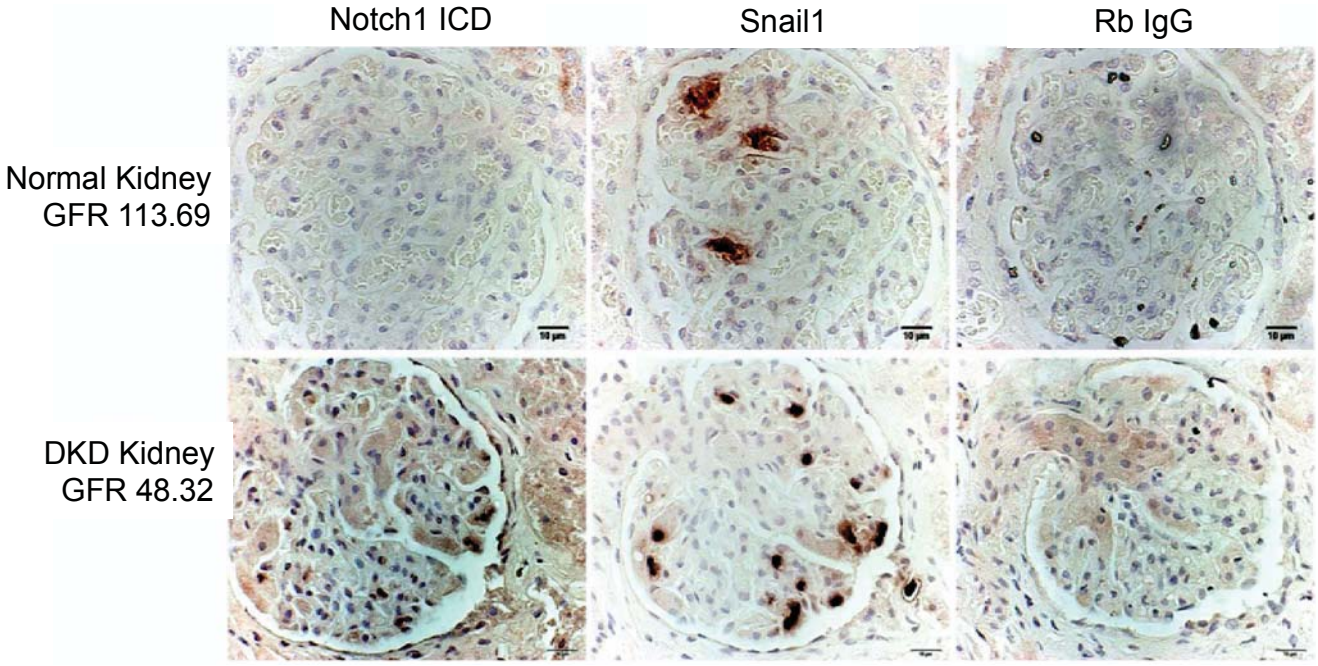
(B) Relative JAG1 transcript levels of immortalized mouse podocytes treated in time course with TGFβ1 in the presence (white bars) or absence (black bars) of γ-secretase inhibitor XX (GSIXX). * indicates p-value < 0.01.

(C) Relative transcript levels of Jagged-1 in control and Notch1 podocytes with sham or TGFβ1 treatment. n = 3, asterisk indicates p-value < 0.01, error bars SEM.

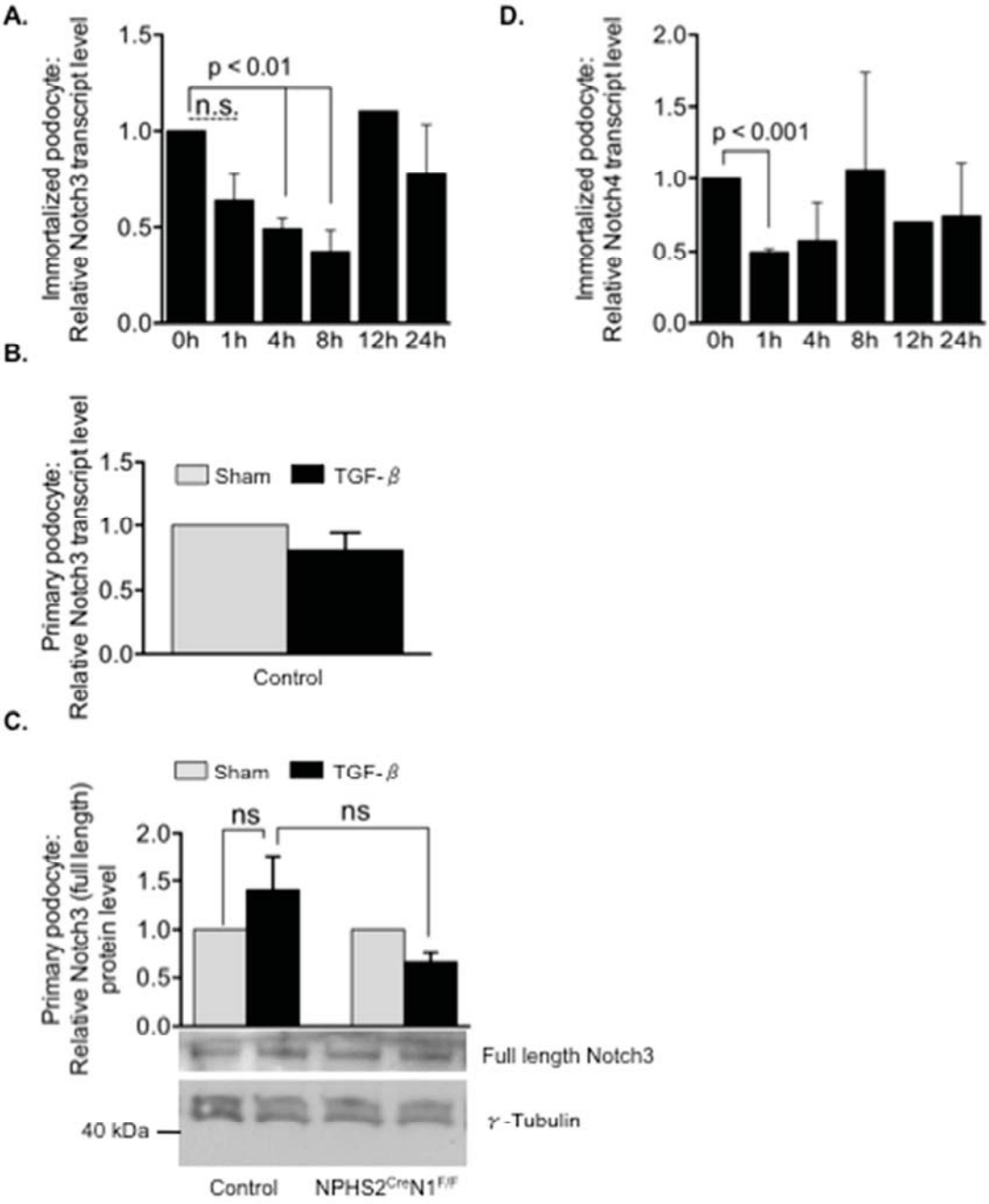
(D) Representative western blots and densitometry based quantification of Notch intracellular domain 1 (NICD1) and Jagged-1 protein expression in cultured primary podocytes.



Supplemental Figure 4. Representative immunohistochemical staining for the cleaved Notch1 (Notch1 ICD) and Snail1 on serial paraffin sections from control and $NPHS2^{Cre}Notch1^{F/F}$ mice in the presence or absence of diabetes at 20 weeks of age. Non-immune rabbit immunoglobulin (Rb IgG) at identical concentration to primary antibodies was used as a negative control Tissues are counterstained with hematoxylin. Arrows indicate positive (brown) staining.



Supplemental Figure 5. Representative immunohistochemical staining for the cleaved Notch1 (Notch1 ICD) and Snail1 on serial paraffin sections from a normal adult kidney (top row) and samples obtained from patients with diabetic kidney disease (bottom row). Non-immune rabbit immunoglobulin (Rb IgG) at identical concentration to primary antibodies was used as a negative control.



Supplemental Figure 6. Notch3 and Notch4 are not strongly regulated in podocytes by TGF-β stimulation at 24 hours.

(A) Relative NOTCH3 transcript levels of immortalized mouse podocytes treated in time course with TGFβ1. n = 3 for all except 12 hours, n = 2

(B) Relative transcript levels of NOTCH3 in control podocytes with sham or with 24 hrs of TGFβ1 treatment. n = 4

(C) Representative western blots and densitometry based quantification of full-length Notch3 protein in cultured primary podocytes with 24 hours of treatment. Notch3 molecular weight is predicted to be 230 kDa and bands ran above the highest marker of 170 kDa. n = 4

(D) Relative NOTCH4 transcript levels of immortalized mouse podocytes treated in time course with TGFβ1. n = 3 for all except 12 hours, n = 2