### Title

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# Protein tyrosine phosphatase 1B deficiency in podocytes mitigates hyperglycemia-induced renal injury

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## Abstract

**Objective—**Diabetic nephropathy is one of the most devastating complications of diabetes, and growing evidence implicates podocyte dysfunction in disease pathogenesis. The objective of this study was to investigate the contribution of protein tyrosine phosphatase 1B (PTP1B) in podocytes to hyperglycemia-induced renal injury.

**Methods—**To determine the *in vivo* function of PTP1B in podocytes we generated mice with podocyte-specific PTP1B disruption (hereafter termed pod-PTP1B KO). Kidney functions were determined in control and pod-PTP1B KO mice under normoglycemia and high-fat diet (HFD)- and streptozotocin (STZ)-induced hyperglycemia.

**Results—**PTP1B expression increased in murine kidneys following HFD and STZ challenges.

Under normoglycemia control and pod-PTP1B KO mice exhibited comparable renal functions. However, podocyte PTP1B disruption attenuated hyperglycemia-induced albuminuria and renal injury and preserved glucose control. Also, podocyte PTP1B disruption was accompanied with improved renal insulin signaling and enhanced autophagy with decreased inflammation and fibrosis. Moreover, the beneficial effects of podocyte PTP1B disruption *in vivo* were recapitulated

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**Author Contributions**

YI, MFH, AB and FGH designed research; YI, MFH, AB, SK, AM conducted the research and collected data; YI, MFH and FGH analyzed data; and YI and FGH wrote the manuscript. FGH is the guarantor of this work. All authors approved the final version of the manuscript.

in E11 murine podocytes with lentiviral-mediated PTP1B knockdown. Reconstitution of PTP1B in knockdown podocytes reversed the enhanced insulin signaling and autophagy suggesting that they were likely a consequence of PTP1B deficiency. Further, pharmacological attenuation of autophagy in PTP1B knockdown podocytes mitigated the protective effects of PTP1B deficiency.

**Conclusions—**These findings demonstrate that podocyte PTP1B deficiency attenuates hyperglycemia-induced renal damage and suggest that PTP1B may present a therapeutic target in renal injury.

### Keywords

Diabetic nephropathy; podocytes; renal injury; hyperglycemia; protein tyrosine phosphatase 1B

## Introduction

The increasing prevalence of diabetes mellitus has become a global health issue, and it is estimated that people with diabetes worldwide will increase to 592 million by 2035 [1]. Diabetic nephropathy (DN) is one of the most devastating vascular complications of diabetes and the leading cause of end-stage renal disease [2]. The disease is associated with renal inflammation and decline in glomerular filtration barrier (GFB) [3, 4]. GFB is composed of podocytes and glomerular endothelial cells [5]. The podocyte is of particular importance in maintaining GFB integrity in humans [6, 7], and growing evidence suggest that podocyte dysfunction contributes to the pathogenesis of DN [8]. Therefore, elucidating the molecular mechanisms underlying podocyte dysfunction is of paramount importance for understanding DN pathogenesis and developing therapeutic modalities.

Protein tyrosine phosphorylation and dephosphorylation are key signaling mechanisms that are controlled by the actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs) [9]. PTPs are implicated in normal renal function and repair after injury. Indeed, Src homology 2 domain-containing phosphatase 2 (Shp2) deficiency in podocytes attenuates acute renal injury [10, 11]. Also, Shp1 is upregulated in podocytes under hyperglycemia leading to decreased tyrosine phosphorylation of nephrin, a key podocyte protein that regulates the actin cytoskeleton [12]. Protein tyrosine phosphatase 1B (PTP1B, encoded by *PTPN1*) is a widely expressed phosphatase that is an established metabolic regulator *in vivo* and a therapeutic target for obesity and type 2 diabetes [13, 14]. Whole-body PTP1B knockout (KO) mice exhibit improved insulin sensitivity, enhanced glucose tolerance and resistance to a high-fat diet (HFD)-induced obesity [15, 16]. Tissue-specific PTP1B disruption demonstrates distinct metabolic actions of this enzyme [17–24].

Studies *in vitro* and rodent models establish a role for PTP1B in renal function. PTP1B podocyte deficiency or pharmacological inhibition attenuate complement-mediated glomerular injury [25] and protect against proteinuria [26]. Moreover, several PTP1B substrates are implicated in podocyte function. Nephrin is a significant contributor to filtration barrier integrity and is a PTP1B substrate [27]. Also, insulin receptor (IR) is a *bona fide* PTP1B substrate and mice with podocyte IR deficiency develop albuminuria and histological features that resemble DN [28]. In the current study, we determined the effects

of podocyte-specific PTP1B disruption on renal functions under normoglycemia and HFD- and STZ-induced hyperglycemia then investigated the underlying molecular mechanism.

## Methods

### Mouse studies

PTP1B floxed (*PTPN1*$^{fl/fl}$) mice on the C57Bl/6J background were generated and described previously [19]. Mice expressing Cre recombinase under the control of podocin promoter on the C57Bl/6J background were purchased from Jackson Laboratories. PTP1B floxed mice were bred to podocin-Cre mice to generate mice with podocyte-specific PTP1B disruption as described [25, 26]. Genotyping for the *PTPN1* floxed allele and Cre was performed by polymerase chain reaction using DNA extracted from tails. Mice were maintained on a 12-hour light-dark cycle in a temperature-controlled facility, with free access to water and standard laboratory diet (CHD; Purina laboratory chow, # 5001). For streptozotocin (STZ)-induced hyperglycemia, 8–12 week old pod-PTP1B KO (*PTPN1*$^{fl/fl}$ Pod-cre$^{+}$) and control (*PTPN1*$^{fl/fl}$) male mice were injected once intraperitoneally with STZ (160μg/g body weight in 100mM sodium citrate buffer; Sigma-Aldrich) as described [29, 30]. For HFD-induced hyperglycemia, 8 weeks old pod-PTP1B KO and control male mice were fed HFD (60% kcal from fat, # D12492, Research Diets). For the nephrotoxic nephritis model, 10–12 week old control and pod-PTP1B KO male mice were injected retro-orbitally with sheep anti-rat glomerular serum (Probetex, 5μl/g body weight) or saline. Urine samples were collected at 24 and 47h post injection, and blood samples were collected at sacrifice (48h). Metabolic parameters were determined in urine and serum of fed and fasted mice. Fed measurements were performed between 7–9 am and fasted measurements were determined using mice fasted for 12–14 hours. Glucose, albumin, creatinine, and blood urea nitrogen (BUN) concentrations were measured using corresponding kits (Sigma; GAGO20, MAK124, MAK080, MAK006) according to manufacturer's instructions. Mice were sacrificed at 13 and 25 weeks after STZ and HFD challenges, respectively. Kidneys were harvested and processed for biochemical, histological and ultrastructural analyses. For insulin signaling studies male mice (10 weeks old) were injected intraperitoneally with a bolus of insulin (10mU/g body weight) after an overnight fast and sacrificed 10 minutes after injection as described [31]. Mouse studies were conducted following federal guidelines and were approved by the Institutional Animal Care and Use Committee at the University of California Davis.

### Histology and electron microscopy

For immunohistochemistry, 4μm kidney sections from control and pod-PTP1B KO mice were fixed (4% paraformaldehyde), embedded in paraffin and deparaffinized in xylene. Sections were stained using Synaptopodin (Santa Cruz Biotechnology), PTP1B (R & D systems), pAKT (S473) and pERK (Y202/T204) (Cell Signaling Technology), Collagen III and pNephrin (Y1176/Y1193) (Abcam) antibodies overnight at 4°C. Detection was performed with appropriate Alexa Fluor secondary antibodies (Thermo Fisher Scientific) and visualized using Olympus BX51 microscope and Olympus FV1000 Laser Scanning Confocal microscope. Transmission electron microscopy was performed on cortical kidney tissue. Kidneys were cut on ice, fixed with 2.5% glutaraldehyde dissolved in 0.1M sodium

cacodylate (pH:7.4) at 4°C overnight then washed in the same buffer. Tissue fragments were post-fixed in 1% cacodylate-buffered $OsO_4$ for 2h, dehydrated, and embedded in Epon. Blocks were sectioned in an Ultracut Reicher ultramicrotome to obtain semi-thick (0.5–1μm width) and ultra-thin (40–60nm width) sections. Semi-thick sections were mounted on glass slides and stained with 1% toluidine blue in a 1% borax aqueous solution for 2 min to evaluate the general structure. Ultra-thin sections were mounted on nickel grids and stained with aqueous 4% uranyl acetate and modified Sato's lead citrate. High magnification pictures of glomerular basement membrane (GBM), originally at 30,000X, were used to obtain the average thickness of the GBM and podocyte foot processes (PFP) width. For this purpose, we obtained 8–10 pictures per glomeruli from about 3–4 glomeruli per resin block showing unaltered morphology. Glomeruli displaying sclerotic symptoms were discarded. GBM was considered as the space between the external plasma membrane of a capillary vessel and the perpendicular-fronted plasma membrane of a podocyte process, and GBM thickness was measured every 0.5μm along and perpendicularly to the GBM. We considered PFP width as the portion of PFP plasma membrane in contact with the GBM, and all the visible PFP were scored in the same pictures used for measuring GBM. Measures of GBM and PFP width were performed using ImageJ software (N.I.H.).

## Cell culture and glomeruli isolation

E11 murine kidney podocyte cell line was purchased from Cell Lines Service (Eppelheim, Germany). Cells were cultured in RPMI medium supplemented with 2mM L-glutamine and 10% fetal bovine serum (FBS) at 33°C and induced to differentiate by culture at 37°C for two weeks. E11 cells with PTP1B knockdown (KD) and reconstitution were generated as we previously described [32, 33]. Briefly, PTP1B was silenced by testing five different hairpin shRNAs (Open Biosystems). Lentiviruses were obtained by co-transfection of vectors in HEK293FT cells using Lipofectamine 2000 following manufacturer's guidelines. Lentivirus-infected E11 cells were selected using puromycin (2μg/ml), and drug-resistant pools were propagated. PTP1B knockdown (KD) podocytes were reconstituted (KD-R) by transfection of pWZL human PTP1B, and stable cell lines were generated by hygromycin (400μg/ml) selection. Differentiated E11 podocytes were cultured in normal glucose (5.6mM) or high glucose (25mM) RPMI medium supplemented with 2mM L-glutamine and 10% FBS for 72h. For insulin signaling studies, differentiated E11 podocytes were cultured under normal, and high glucose then starved overnight using RPMI medium with 2mM L-glutamine and stimulated with insulin for 10 and 20 min. In some experiments, PTP1B knockdown E11 podocytes were treated with autophagy inhibitor N2, N4-dibenzylquinazoline-2,4-diamine (DBeQ: 15μM) for 16h under normal glucose [34]. Also, glomeruli were isolated from control and pod-PTP1B KO mice using established protocols with minor modifications as we described [11]. Glomeruli were lysed in radio-immunoprecipitation assay (RIPA) buffer (20mM Tris-HCl (pH:7.4), 150mM NaCl, 0.1% SDS, 5mM EDTA, 20mM NaF, 10mM $Na_4P_2O_7$, 1% Triton X-100, 1% sodium deoxycholate and proteases inhibitors) [35].

## Biochemical analyses

Tissues were ground in liquid nitrogen and lysed using RIPA buffer. Lysates were centrifuged at 13,000rpm for 10min and protein concentrations determined using

bicinchoninic acid protein assay kit (Pierce Chemical). Proteins were resolved by SDS-PAGE then transferred to PVDF membranes. Immunoblotting of lysates was performed with antibodies for mouse PTP1B (R & D Systems), human PTP1B (FG6; EMD Millipore), phosphotyrosine (4G10; EMD Millipore), pIKKα/β (S178/180), IKKα/β, pIκBα (S32), IκBα, pNF-κBp65 (S536), NF-κBp65, pAKT (S473), Beclin, Atg5, Atg7 (all from Cell Signaling Technology), LC3A/B (Bio-Rad), AKT, pSmad2 (S465), Smad2, TGFβRII, IR, phosphotyrosine (PY99) and Tubulin (all from Santa Cruz Biotechnology). Proteins were visualized using enhanced chemiluminescence (HyGLO; Denville Scientific), and pixel intensities of immuno-reactive bands were quantified using FluorChem 8900 (Alpha Innotech). Also, frozen kidneys were homogenized by mortar and pestle to generate fine powder. After TRIzol (Invitrogen) extraction, total RNA was converted to cDNA using high-capacity cDNA Synthesis Kit (Applied Biosystems). *Ptpn1* mRNA was quantitated by real-time PCR using SsoAdvanced Universal SYBR Green Supermix (iCycler, Bio-Rad). Relative gene expression was normalized to Tata-box binding protein (*Tbp*) and calculated using the $2^{-\Delta\Delta C_T}$ method. Primers used were: *Ptpn1*, forward 5′-TTTCAAAGTCCGAGAGTCAGGG and reverse 5′-AGCCAGACAGAAGGTTCCAGA; *Il1b*, forward 5′-AGCTTCAGGCAGGCAGTATC and reverse 5′-AAGGTCCACGGGAAAGACAC; *Il6*, forward 5′-ACAACCACGGCCTTCCCTACTT and reverse 5′-CACGATTTCCCAGAGAACATGTG; *Tnfa*, forward 5′-GACGTGGAACTGGCAGAAGAG and reverse 5′-TGCCACAAGCAGGAATGAGA; *Tbp*, forward 5′-TTGGCTAGGTTTCTGCGGTC and reverse 5′-GCCCTGAGCATAAGGTGGAA.

### Statistical analyses

Data were expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS program (IBM, Armonk) or GraphPad Prism 6.01 (Graph Pad Software Inc.). Post-hoc analysis was performed using Bonferroni honestly significant difference test. Comparisons between frequency distributions were assessed by using two-sample Kolmogorov-Smirnov test. Differences were considered significant at $p \leq 0.05$ and highly significant at $p \leq 0.01$ or $p \leq 0.001$.

## Results

### Generation of mice with podocyte-specific PTP1B disruption

Studies in rodent models of podocyte injury report marked upregulation of PTP1B in the glomeruli, notably in podocytes [26]. In this study, we investigated the effects of hyperglycemia on renal PTP1B expression. Wild-type male mice were fed standard laboratory diet or rendered hyperglycemic by STZ and HFD challenges as detailed in methods. Renal PTP1B expression increased at the transcript and protein levels under STZ- and HFD-induced hyperglycemia compared with normoglycemia (Fig. 1A). Similarly, PTP1B expression increased in differentiated E11 murine podocytes cultured in high glucose compared with normal glucose (Fig. 1B). As a control, E11 podocytes with PTP1B knockdown (KD, see later) were used revealing a significant reduction in PTP1B expression and demonstrating the specificity of antibodies. Together, these findings establish upregulation of PTP1B in podocytes under high glucose and suggest that PTP1B may

impact hyperglycemia-induced renal injury. To investigate the potential contribution of PTP1B in podocytes to renal injury *in vivo* we generated mice with podocyte-specific PTP1B disruption as detailed in methods. Pod-PTP1B KO mice appeared healthy and with no gross defects in the kidneys consistent with previous reports [25, 26]. Podocyte-specific PTP1B disruption was confirmed using biochemical and immuno-histochemical approaches. The recombined PTP1B allele was detected only in kidneys of pod-PTP1B KO mice (Fig. 1C). Also, immunoblots revealed PTP1B ablation in isolated glomeruli from knockout mice compared with controls (Fig. 1D). In contrast, PTP1B expression was comparable between knockout and control mice in other tissues suggesting specificity of deletion. Moreover, co-immunostaining of PTP1B and Synaptopodin (podocyte marker) in kidney sections revealed ablation of the former in podocytes of knockout mice (Fig. 1E). Together, these data demonstrate specific and efficient PTP1B disruption in podocytes and establish pod-PTP1B KO mice as a suitable model to investigate the potential contribution of this enzyme to hyperglycemia-induced renal injury.

## Podocyte-specific PTP1B disruption protects renal functions under hyperglycemia

Podocyte PTP1B deficiency attenuates complement-mediated glomerular injury and protects against proteinuria [25, 26]. Herein, we investigated the effects of podocyte PTP1B disruption on hyperglycemia-induced renal injury. Body weights of control and pod-PTP1B KO mice were similar on standard laboratory diet while high-fat feeding led to a comparable increase in body weights of both genotypes (Fig. 2A). Also, high-fat feeding increased fed and fasted serum glucose concentrations, but these were significantly less in pod-PTP1B KO mice compared with controls demonstrating better glucose control. Serum creatinine was comparable between control and pod-PTP1B KO mice at normoglycemia and hyperglycemia. A monitor for renal injury is albuminuria which is an early and sensitive marker of kidney damage [36, 37]. High fat feeding increased urine albumin/creatinine (ACR), but that was significantly less in pod-PTP1B KO mice compared with controls indicative of preserved renal function. Similarly, renal function and glucose control were also preserved under STZ-induced hyperglycemia as evidenced by lower fed and fasted serum glucose and ACR in pod-PTP1B KO mice compared with controls (Fig. 2B). Also, podocyte PTP1B deficiency attenuated renal injury in anti-glomerular basement membrane (GBM) serum-induced nephritis model as evidenced by lower ACR and blood urea nitrogen in pod-PTP1B KO mice compared with controls (Sup Fig. 1). Collectively, these findings demonstrate that podocyte PTP1B disruption protects renal functions under STZ- and HFD-induced hyperglycemia and identify podocytes as contributors to the beneficial effects of PTP1B deficiency.

## Podocyte PTP1B disruption attenuates hyperglycemia-induced renal injury

Diabetic kidney disease is characterized by the accumulation of extracellular matrix in the glomerular and tubulointerstitial compartments, resulting in progressive fibrosis and decline in renal function [38]. The effects of PTP1B podocyte deficiency were first examined with a light microscope using toluidine blue staining of semi-thick sections. No obvious differences were noted basally in toluidine blue-stained paraffin sections. However, hyperglycemia caused severe damage to the kidneys of control mice but to a lesser extent in pod-PTP1B KO mice as evidenced by the distorted architecture of the glomeruli and tubules in

hyperglycemic control mice (black arrows, Sup Fig. 2A). These alterations were confirmed by electron microscopy (arrows in Fig. 3A), which also revealed significant alterations in the morphology of podocytes and GBM under hyperglycemia (Fig. 3B). Swollen podocytes with large cytoplasmic vacuoles and effaced foot processes (black arrow in Fig. 3B) were observed in STZ-treated control mice. On the other hand, pod-PTP1B KO mice exhibited mild focal foot process effacement indicative of lower STZ-induced renal damage. GBM thickness and PFP width were determined as quantitative estimates of the ultrastructural preservation of the filtration barrier. STZ treatment led to an increase of GBM thickness both in controls and pod-PTP1B KO mice with the latter exhibiting significantly less increase consistent with protection against hyperglycemia-induced renal damage (Fig. 3C). Furthermore, whereas PFP width was slightly higher in pod-PTP1B KO than in control mice under basal conditions, an increase of PFP width upon STZ treatment was more in controls than pod-PTP1B KO, in such a way that under hyperglycemic conditions PFP width was greater in the controls (Fig. 3D). Frequency distribution analysis of PFP width revealed significant alteration caused by STZ in both genotypes, with no significant differences between control and pod-PTP1B KO under basal conditions, but a statistically significant difference between genotypes under hyperglycemia (Sup Fig. 2B). Altogether, these observations demonstrate that PTP1B disruption protects podocyte structure and foot processes against hyperglycemia-induced damage.

## PTP1B disruption enhances podocyte insulin signaling and autophagy

Insulin signaling to the glomerular podocytes is a significant regulator of kidney function as evidenced by podocyte-specific IR-deficient mice which develop albuminuria and histological features that resemble DN [28]. Also, IR is an established PTP1B substrate, and PTP1B attenuates insulin signaling to a significant part, by dephosphorylating IR [15, 17, 39, 40]. Accordingly, the effects of podocyte PTP1B disruption on renal insulin signaling were determined in control and pod-PTP1B KO mice. Immunoblotting of kidney lysates demonstrated insulin-induced AKT and ERK phosphorylation in control and pod-PTP1B KO mice, but those were significantly increased in pod-PTP1B KO mice indicating enhanced insulin signaling (Fig. 4A). Similarly, immunostaining of kidney sections revealed increased insulin-induced AKT and ERK phosphorylation in glomeruli of knockout mice compared with controls (Fig. 4B). In line with enhanced insulin signaling, the hyperglycemia-induced inflammatory response was attenuated in pod-PTP1B KO mice as evidenced by decreased hyperglycemia-induced IKKα, IκBα and NF-κB activation in pod-PTP1B KO mice compared with controls (Fig. 4C and Sup Fig. 3A). Consistent with this observation, hyperglycemia-induced increase in interleukin-1b (Il-1b), interleukin-6 (Il-6) and tumor necrosis factor alpha (Tnfa) renal mRNA was significantly lower in pod-PTP1B KO mice compared with controls (Fig. 4D).

Autophagy regulates several aspects of normal and diabetic kidney function and is proposed as a protective mechanism against podocyte injury [41, 42]. The effects of podocyte PTP1B deficiency on autophagy were investigated by determining the expression of proteins that are involved in the formation and maturation of autophagosomes namely Beclin, microtubule-associated protein 1A/1B-light chain 3 (LC3A/B) and autophagy-related genes (Atg) [43, 44]. Under hyperglycemia, pod-PTP1B KO mice exhibited enhanced autophagy compared

with controls as evidenced by increased Beclin1, LC3-II, and Atg5 and Atg7 expression (Fig. 5A, Sup Fig. 3B). Consistent with enhanced autophagy, pod-PTP1B KO mice exhibited lower hyperglycemia-induced fibrosis compared with controls as evidenced by decreased renal transforming growth factor beta receptor II (TGFβRII) expression and Smad2 phosphorylation (Fig. 5B, Sup Fig. 3B). Fibrosis was further evaluated by immunostaining for collagen III in kidney sections. STZ and HFD increased collagen III immunostaining in control and pod-PTP1B KO mice but that was less in the latter consistent with decreased fibrosis (Fig. 5C). In total, these findings demonstrate that podocyte PTP1B disruption is associated with increased insulin signaling and enhanced autophagy with a decrease in hyperglycemia-induced inflammation and fibrosis.

### PTP1B deficiency in cultured podocytes recapitulates the beneficial effects of its disruption *in vivo*

The renal response to hyperglycemia is the integrated outcome of several cell types that include podocytes. To further delineate the molecular mechanism underlying PTP1B function in podocytes we generated E11 murine podocytes with knockdown (KD) and reconstituted (KD-R) expression of PTP1B as detailed in methods (Sup Fig. 4A). Differentiated podocytes were cultured under normal and high glucose, and alterations in insulin signaling determined. Under normal glucose (NG) culture, PTP1B knockdown podocytes exhibited enhanced insulin-stimulated IR, AKT and ERK phosphorylation compared with KD-reconstituted podocytes (Fig. 6A). High glucose (HG) culture induced significant attenuation in insulin-stimulated IR, AKT and ERK phosphorylation but to a lower extent in knockdown compared with reconstituted podocytes. Moreover, E11 podocyte cell lines were used to determine the effects of PTP1B deficiency on high glucose-induced alterations in inflammation, autophagy, and fibrosis. Consistent with observations *in vivo*, high glucose culture increased inflammation (IKKα, IκBα, and NF-κB phosphorylation), autophagy (Beclin, LC3-II, Atg5 and Atg7 expression) and fibrosis (TGFβRII expression and Smad2 phosphorylation) (Fig. 6B, C). Notably, PTP1B deficiency attenuated the deleterious effects of high glucose culture, and knockdown podocytes exhibited decreased inflammation and fibrosis and increased autophagy compared with KD-reconstituted podocytes. To decipher the contribution of autophagy to the beneficial effects of PTP1B deficiency, pharmacological modulation of autophagy was performed in PTP1B knockdown podocytes as detailed in methods. Importantly, attenuating autophagy mitigated the protective effects of PTP1B deficiency on fibrosis as evidenced by TGFβRII expression and Smad2 phosphorylation (Fig. 6D). Altogether, these data are in keeping with *in vivo* findings and are consistent with cell-autonomous effects that are due to PTP1B deficiency.

## Discussion

Diabetic nephropathy is one of the most devastating complications of diabetes and podocyte dysfunction contributes to disease pathogenesis. It is, therefore, important to decipher the intricate molecular mechanisms underlying podocyte dysfunction to aid in developing effective therapeutic interventions. Herein, we investigated the potential contribution of PTP1B in podocytes to hyperglycemia-induced renal injury. We provide evidence of increased PTP1B expression in podocytes under high glucose. Also, podocyte-specific

PTP1B disruption attenuated hyperglycemia-induced renal injury and preserved glucose control. The beneficial effects of podocyte PTP1B disruption were associated with, and likely caused by, improved insulin signaling and enhanced autophagy. Further, PTP1B knockdown in E11 podocytes recapitulated the beneficial effects of its deficiency *in vivo*, whereas PTP1B reconstitution reversed these effects indicating that they were a consequence of PTP1B deficiency. Altogether, these findings identify PTP1B in podocytes as a contributor to renal function under hyperglycemia.

Phosphotyrosine-based signaling, mediated by the opposing actions of kinases and phosphatases, contributes to podocyte function and repair [45, 46]. Accordingly, alterations in PTPs expression and activity will likely modulate podocyte function. In this regard, PTP1B expression increased in murine kidneys upon HFD- and STZ-induced hyperglycemia and E11 podocytes cultured in high glucose. Increased PTP1B expression likely correlates with elevated enzymatic activity, but several elements regulate PTP1B activity including post-translational modifications [24, 47] so it remains to be determined if PTP1B upregulation translates to increased activity. Nevertheless, the current findings are in line with PTP1B upregulation in three rodent models of podocyte injury (puromycin amino-nucleoside nephrosis, adriamycin-induced nephrosis, and lipopolysaccharide-induced proteinuria) [26]. Kumagai *et al.*, propose that PTP1B upregulation in podocytes promotes a migratory response leading to foot process effacement and proteinuria. Indeed, podocyte-specific PTP1B overexpressing mice develop spontaneous proteinuria and foot process effacement [26]. Moreover, PTP1B expression in podocytes under hyperglycemia is comparable to other PTPs. The Src homology 2 domain-containing phosphatases Shp1 and Shp2 are upregulated in podocytes under hyperglycemia [12, 48] as well as puromycin- and lipopolysaccharide-induced injury, respectively [10, 11].

Genetic and biochemical approaches demonstrated that podocyte PTP1B deficiency partially protected against the effects of high glucose *in vivo* and culture. We provided biochemical and immuno-histochemical data demonstrating specific and efficient deletion of PTP1B in podocytes, and are consistent with the validity of this approach to achieve podocyte-specific disruption [25, 26, 28]. A limitation of this study is the use of high-dose STZ to cause pancreatic β-cells death and induce hyperglycemia since STZ may also have renal toxic effects [49]. Another limitation is that mice were on the C57BL/6 genetic background which is relatively resistant to renal injury in experimental models of DN [50]. Nevertheless, podocyte PTP1B disruption yielded significant renal protective effects against STZ-induced hyperglycemia. Also, the beneficial effects of podocyte PTP1B disruption were observed under HFD-induced hyperglycemia (where hyperinsulinemia precedes hyperglycemia) demonstrating that they were not unique to a challenge. Moreover, the beneficial effects of podocyte PTP1B deficiency were confirmed in a nephrotoxic nephritis model of renal injury. Of note, the effects of podocyte PTP1B disruption *in vivo* were recapitulated in E11 podocytes with PTP1B knockdown consistent with being cell autonomous, whereas PTP1B reconstitution reversed these effects suggesting that they were likely a consequence of its deficiency. The current *in vivo* findings are in keeping with the reported effects of podocyte PTP1B disruption which attenuates complement-mediated glomerular injury and protects against podocyte injury [25, 26]. Herein, we established that podocyte PTP1B disruption mitigated hyperglycemia-induced albuminuria and renal injury and preserved glucose

control. Diabetic albuminuria in humans is associated with characteristic histopathologic features, including glomerular hypertrophy and thickening of the GBM [51]. Consistent with decreased albuminuria in pod-PTP1B KO mice electron microscopy revealed lower hyperglycemia-induced renal damage as evidenced by the protection against the occurrence of lesion nodules in glomeruli and decreased GBM thickness and PFP width. Also, podocyte PTP1B disruption was associated with better glucose control. A pressing challenge is to delineate the impact of podocyte PTP1B deficiency on systemic glucose homeostasis, and we postulate that several factors may be implicated. 1) Enhanced insulin signaling in pod-PTP1B KO mice is likely a significant contributor given the role of insulin signaling in podocytes [28, 52]. 2) Podocyte PTP1B deficiency may impact other renal cells to enhance glucose clearance. In support of this scenario, biochemical alterations were detected in total kidney lysates of pod-PTP1B KO mice, and podocytes constitute a minor portion. Of note, a therapy for type 2 diabetes is inhibition of sugar glucose transporter 2 to lower plasma glucose levels by reducing reabsorption of filtered glucose and enhancing glucose clearance [53, 54]. 3) Also, podocyte PTP1B deficiency may impact other renal cells to decrease renal gluconeogenesis. Kidneys are contributors to glucose homeostasis via gluconeogenesis in healthy individuals [55] and significantly more in diabetic patients [56]. 4) We cannot rule out effects of podocyte PTP1B deficiency on other tissue(s) that modulate glucose homeostasis such as the liver.

Podocyte PTP1B deficiency *in vivo* was associated with enhanced insulin signaling as evidenced by increased renal insulin-stimulated ERK and AKT phosphorylation. A limitation is the use of total kidney lysates which encompass many cell types. However, immuno-histochemical evaluations of kidney sections and studies in E11 cells implicate podocytes. The IR is a physiologically-relevant PTP1B substrate, and it remains to be established if this is the case in podocytes, but the increased insulin-induced IR phosphorylation in PTP1B knockdown podocytes supports this notion. Also, PTP1B may impact insulin signaling by targeting another substrate(s). Podocyte PTP1B deficiency may increase activation of insulin growth factor 1 receptor (IGF-1R). Indeed, IGF-1R is a PTP1B substrate [57], and IGF-1R activation is protective in podocytes [58]. Insulin signaling to podocytes is a significant regulator of renal function, and podocytes respond to insulin stimulation with an increase in PI3K and MAPK signaling [52]. Podocyte IR-deficient mice develop albuminuria due to podocyte damage and exhibit features that are characteristic of human DN [28]. These changes occur in the absence of hyperglycemia underscoring the impact of insulin signaling on podocyte function. Therefore, it is reasonable to stipulate that the renal protective effects of podocyte PTP1B disruption under hyperglycemia are due, at least partly, to the increased insulin signaling. Concomitant with enhanced insulin signaling, podocyte PTP1B deficiency attenuated high glucose-induced inflammation as evidenced by decreased NF-κB signaling *in vivo* and culture. Previous studies implicate PTP1B in the control of inflammatory processes but suggest it may be context (stimulus and/or cell type)-dependent with PTP1B deficiency exacerbating inflammation in certain conditions [23, 59–61] while attenuating it in others [62].

Podocyte PTP1B deficiency *in vivo* and culture was associated with enhanced autophagy. Autophagy is implicated in normal and diabetic kidney function and is a protective mechanism against podocyte injury [41, 42]. The role of autophagy in DN may depend on

the disease stage, but we postulate that enhanced autophagy in PTP1B-deficient podocytes is likely a contributor to its renal protective effects under hyperglycemia. The molecular mechanism underlying regulation of autophagy in podocytes by PTP1B requires additional investigation but likely includes several effectors such as AMP-activated protein kinase (AMPK) which is a positive regulator of autophagy [63, 64]. Indeed, PTP1B-deficient adipose tissue [65] and adipocytes [66] exhibit increased AMPK phosphorylation and activity. Moreover, PTP1B deficiency confers cardio-protective effects against HFD through activation of AMPK and autophagy [67]. Consistent with enhanced autophagy, PTP1B deficient podocytes exhibited less hyperglycemia-induced fibrosis. Importantly, pharmacological attenuation of autophagy in PTP1B knockdown podocytes mitigated the protective effects of PTP1B deficiency on fibrosis highlighting the contribution of autophagy.

The current study provided insights into the molecular mechanisms that are associated with podocyte PTP1B deficiency but does not identify new PTP1B substrate(s). Nephrin is a PTP1B substrate in podocytes [27] and may mediate PTP1B actions in certain disease models [26, 68] but apparently not in others such as albuminuria in anti-GBM nephritis [25]. Herein, we observed alterations in nephrin phosphorylation *in vivo* and culture that are consistent with nephrin contributing to PTP1B action in podocytes under hyperglycemia (Sup Fig. S4). However, we cannot rule out that additional PTP1B substrate(s) may contribute to podocyte function under hyperglycemia. Indeed, preliminary studies using substrate-trapping and mass spectroscopy approaches in podocytes support this notion (Ito and Haj, unpublished). Accordingly, we postulate that PTP1B impacts podocyte function through engaging a multitude of substrates. In summary, the current findings demonstrate beneficial effects of podocyte PTP1B deficiency under hyperglycemia and suggest that PTP1B and its substrates may serve as therapeutic targets in renal injury.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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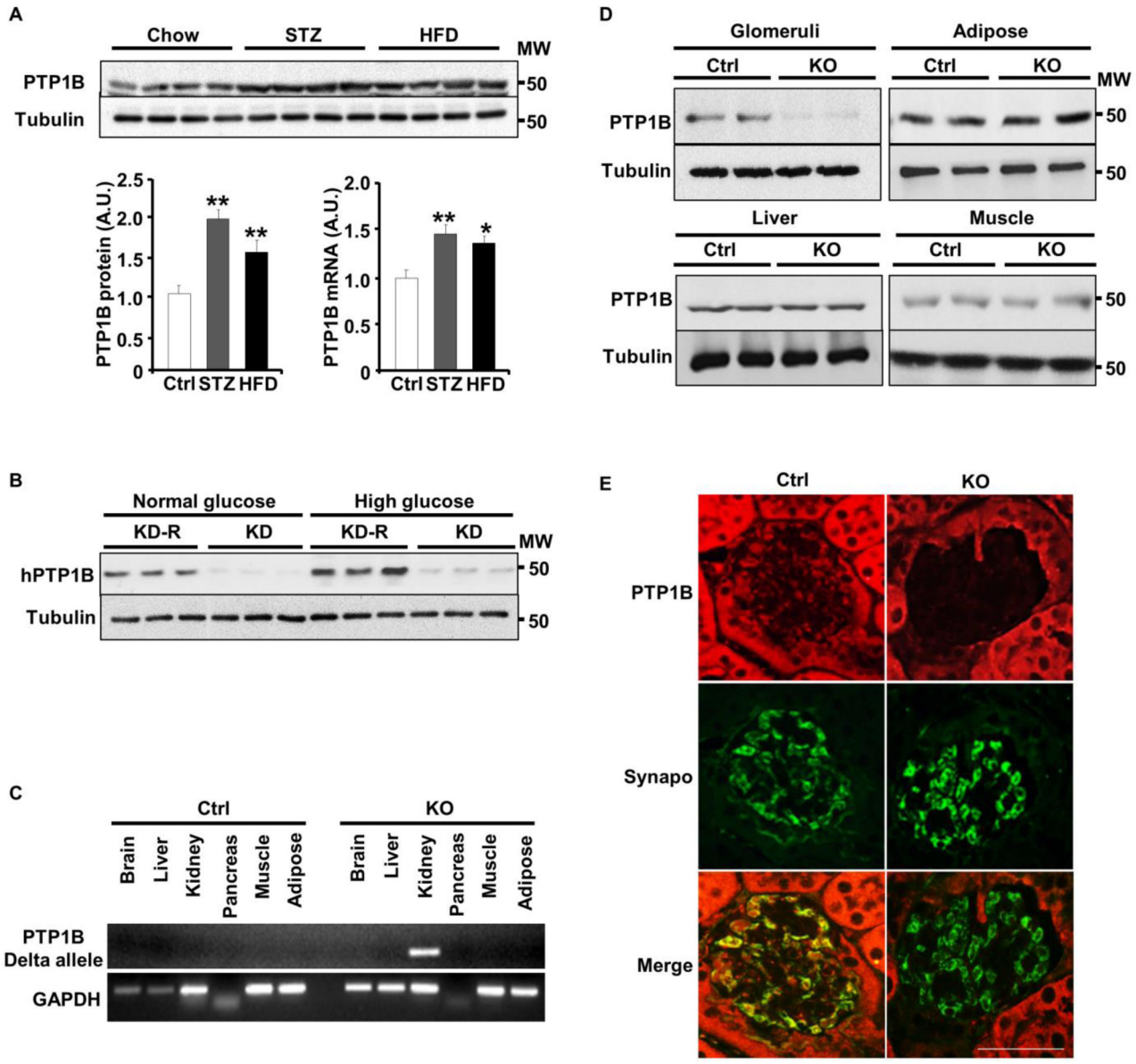


**Figure 1. Podocyte-specific PTP1B disruption**

**A**) Increased renal PTP1B expression under STZ- and HFD-induced hyperglycemia. Immunoblots of PTP1B expression in kidney lysates of wild-type male mice fed standard laboratory diet (Chow), HFD (for 25 weeks) or challenged with STZ (160μg/g body weight). Tubulin was used as a loading control, and each lane represents tissue from a separate animal. Bar graphs indicate PTP1B protein (left) and mRNA (right) in kidney lysates from control (Ctrl; chow fed, n=4), STZ-treated (n=4) and HFD-fed (n=4) mice and presented as means ± SEM. *$p \leq 0.05$, **$p \leq 0.01$ indicate a significant difference between control versus STZ-treated and HFD-fed mice. **B**) Immunoblots of human PTP1B (FG6 antibodies) in differentiated E11 podocytes with knockdown (KD) and reconstituted (KD-R) expression of hPTP1B cultured under normal and high glucose. Tubulin was used as a loading control. **C**) DNA was extracted from different tissues of control (Ctrl), and pod-PTP1B knockout (KO)

mice and the delta allele was detected by PCR with GAPDH as a control. Recombination was detected only in the kidney of knockout mice. **D**) Immunoblots of PTP1B expression in lysates of isolated glomeruli, epididymal fat, liver and muscle of Ctrl and KO mice (n=2). Representative immunoblots are shown. **E**) Immunostaining of PTP1B (red) and synaptopodin (green) in kidney paraffin sections of Ctrl and KO mice. Scale bar: 50μm. AU: arbitrary unit.

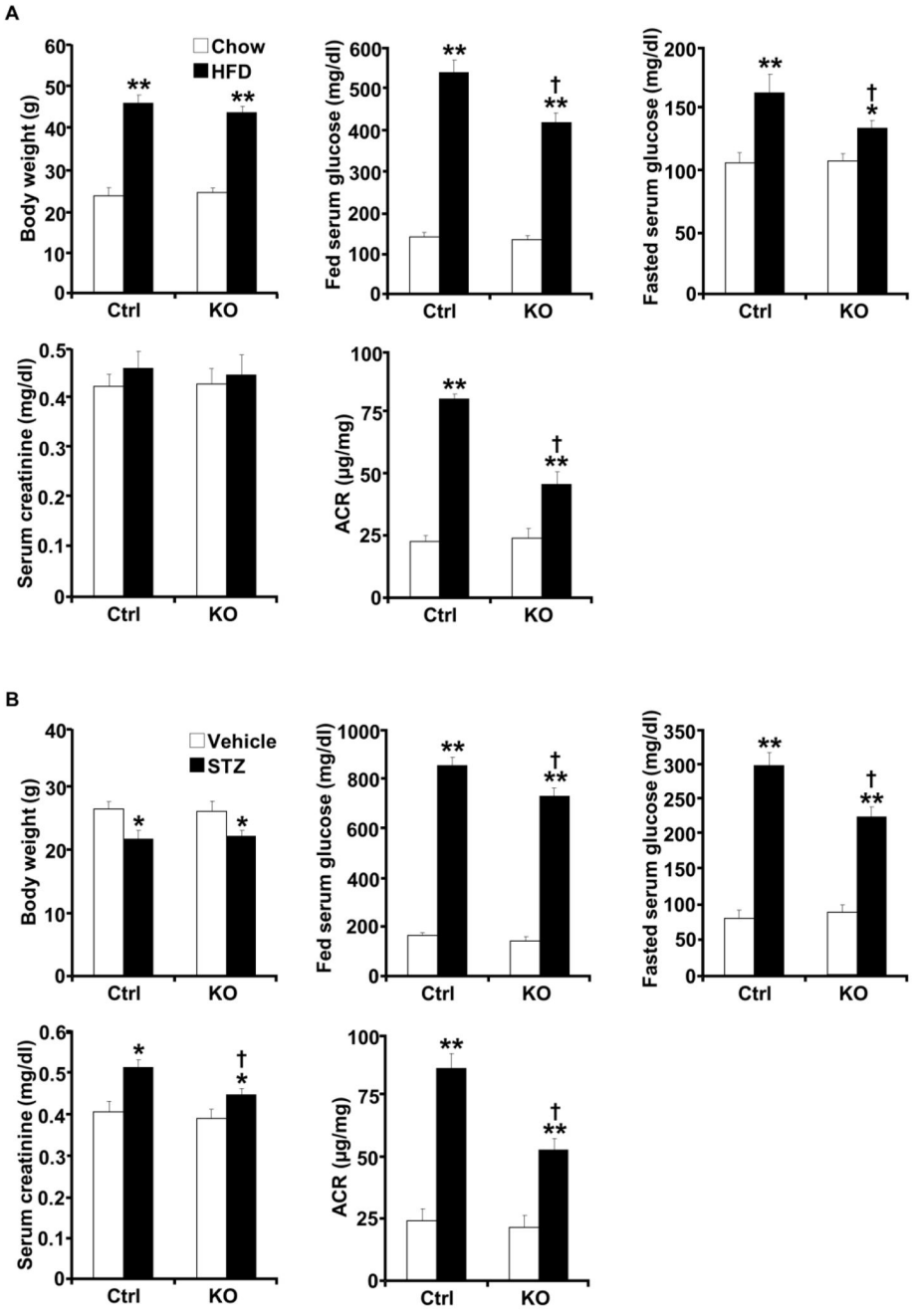


**Figure 2. Podocyte PTP1B disruption preserves renal function under HFD- and STZ-induced hyperglycemia**

**A**) Body weights, fed and fasted serum glucose, serum creatinine, and urinary albumin to creatinine ratio (ACR) of control (Ctrl, n=7) and pod-PTP1B KO (KO, n=9) mice fed chow and HFD. *$p \leq 0.05$; **$p \leq 0.01$ indicate a significant difference between chow and HFD fed mice, and †$p \leq 0.05$ indicates a significant difference between Ctrl and KO. **B**) Body weights, fed and fasted serum glucose, serum creatinine and ACR of Ctrl (n=10) and KO (n=8) mice with saline and STZ treatments. *$p \leq 0.05$; **$p \leq 0.01$ indicate a significant difference between

saline and STZ treatments, and †$p$<0.05 indicates a significant difference between Ctrl and KO.

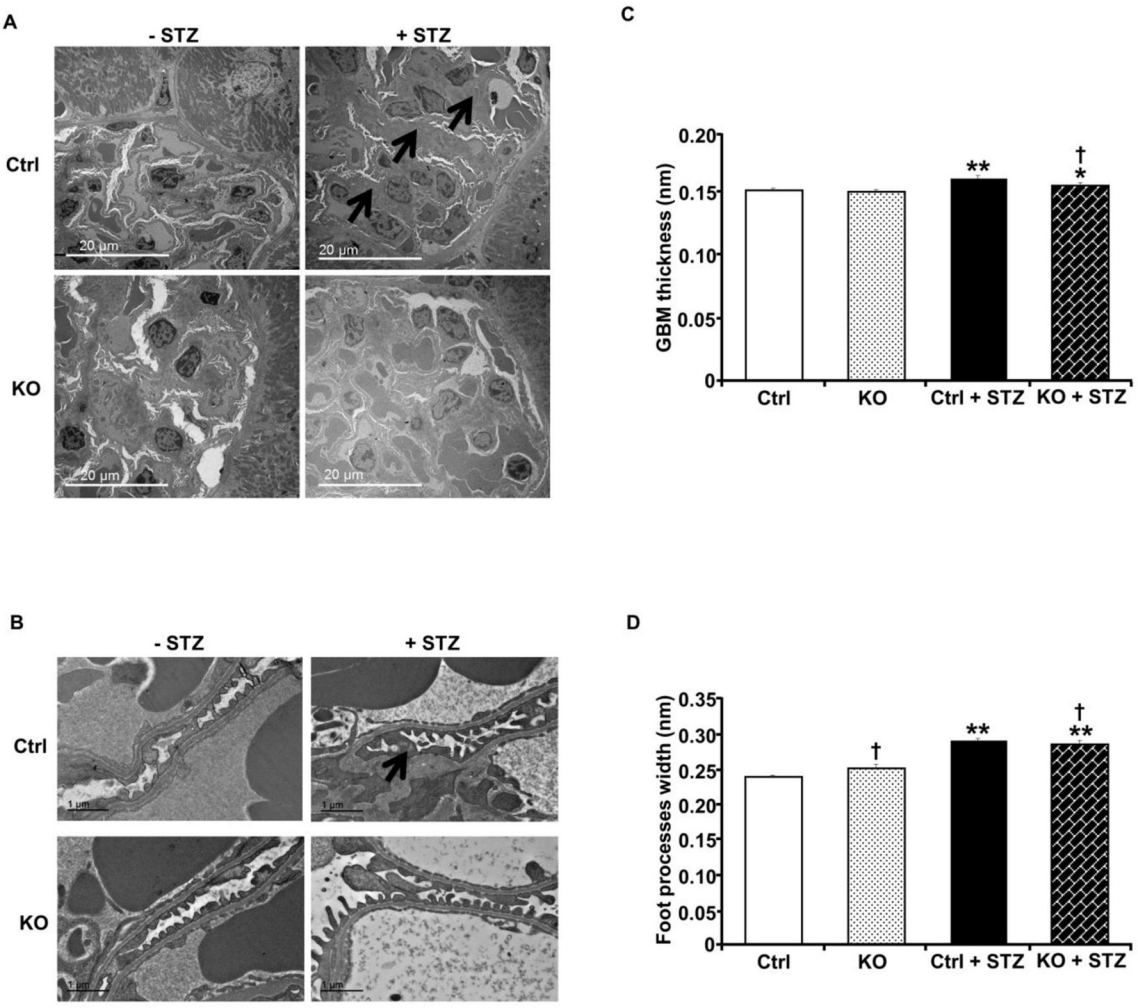


**Figure 3. Podocyte PTP1B deficiency attenuates hyperglycemia-induced glomerulosclerosis**

**A**) TEM of podocytes from control (Ctrl) and pod-PTP1B KO (KO) mice without (−) and with (+) STZ at 13 weeks post injection. Arrows denote Kimmelstiel-Wilson-like nodules in glomeruli from control mice under hyperglycemia. Scale bar: 20μm. **B**) High-magnification micrographs depicting GBM and PFP from Ctrl and KO mice without (−) and with (+) STZ. Black arrows indicate swollen podocytes with large cytoplasmic vacuoles and effaced foot processes. Scale bar: 1μm. Quantitation of GBM thickness (**C**) and PFP width (**D**) in Ctrl (n=3) and KO (n=3) mice without and with STZ challenge. In **C** and **D**, *$p \leq 0.05$; **$p \leq 0.01$ indicate a significant difference between without and with STZ treatments, and †$p \leq 0.05$ between Ctrl and KO.

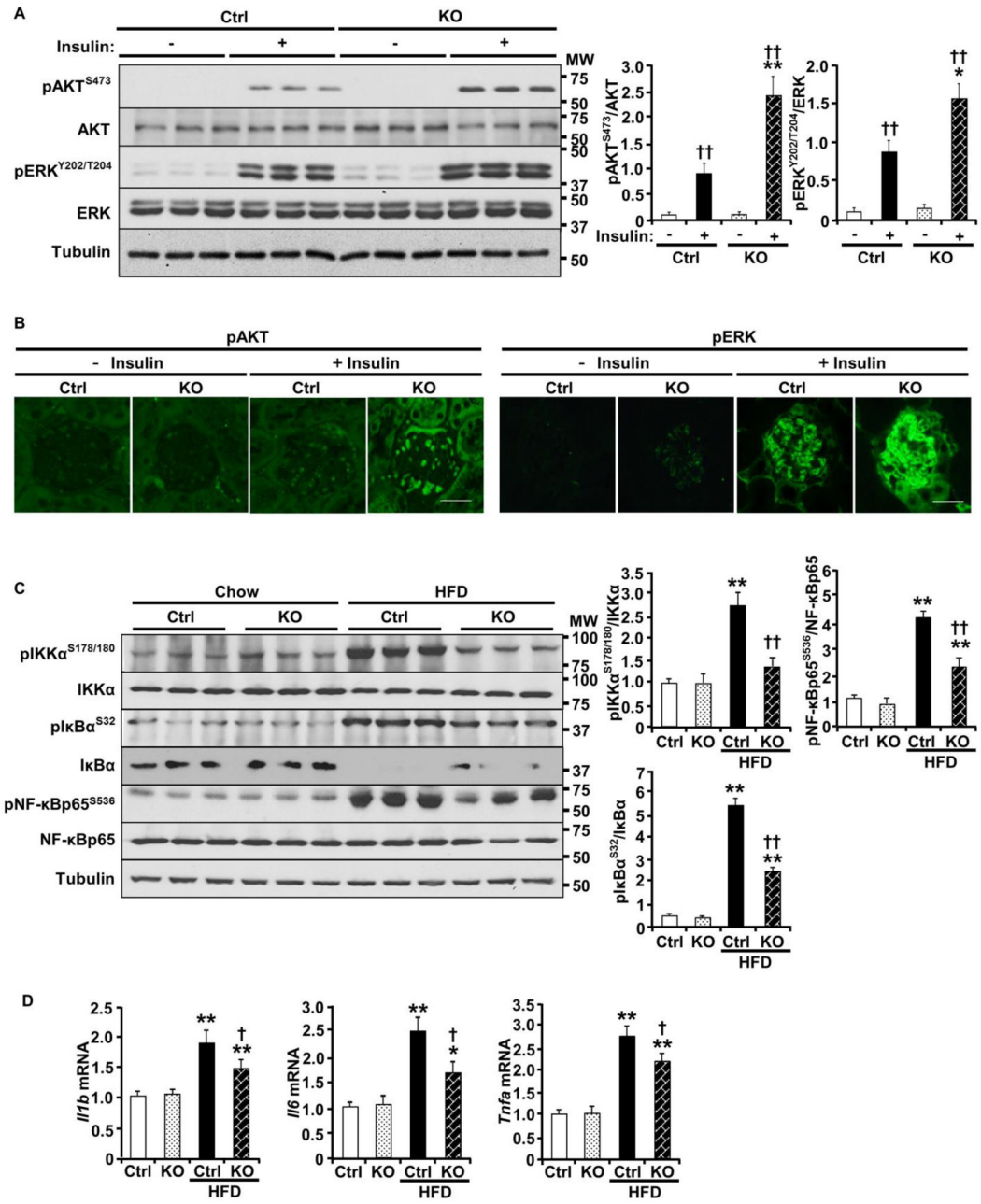


**Figure 4. Increased insulin signaling in mice with podocyte PTP1B disruption**

**A**) Immunoblots of pAKT (S473), AKT, pERK (Y202/T204), ERK and Tubulin in kidney lysates from Ctrl (n=3) and KO (n=3) mice without (−) and with (+) insulin (10mU/g body weight) at 10 minutes after injection. Each lane represents tissue from a different animal. Bar charts represent pAKT and pERK normalized to expression of the respective protein. *$p \leq 0.05$; **$p \leq 0.01$ indicate a significant difference between Ctrl and KO, and ††$p \leq 0.01$ indicates a significant difference between mice without (−) and with (+) insulin injection. **B**) Immunostaining of pAKT (S473) and pERK (Y202/T204) in kidney sections from Ctrl and KO mice without and with insulin injection. Scale bar: 50μm. **C**) Immunoblots of NFκB signaling proteins in kidney lysates from Ctrl (n=3) and KO (n=3) mice fed standard

laboratory diet (chow) and HFD. Bar charts represent normalized data for pIKKα (S178/180)/IKKα, pIKBα (S32)/IKBα and pNF-κBp65 (S536)/NF-κBp65 as means ± SEM. **D**) Renal mRNA of inflammatory cytokines *Il1b*, *Il6* and *Tnfa* in mice fed chow (n=60 and HFD (n=6). In **C** and **D**, *$p \leq 0.05$; **$p \leq 0.01$ indicate a significant difference between chow and HFD fed mice, and †$p < 0.05$; ††$p < 0.01$ between Ctrl and KO. The Y-axes of bar charts are in arbitrary unit.

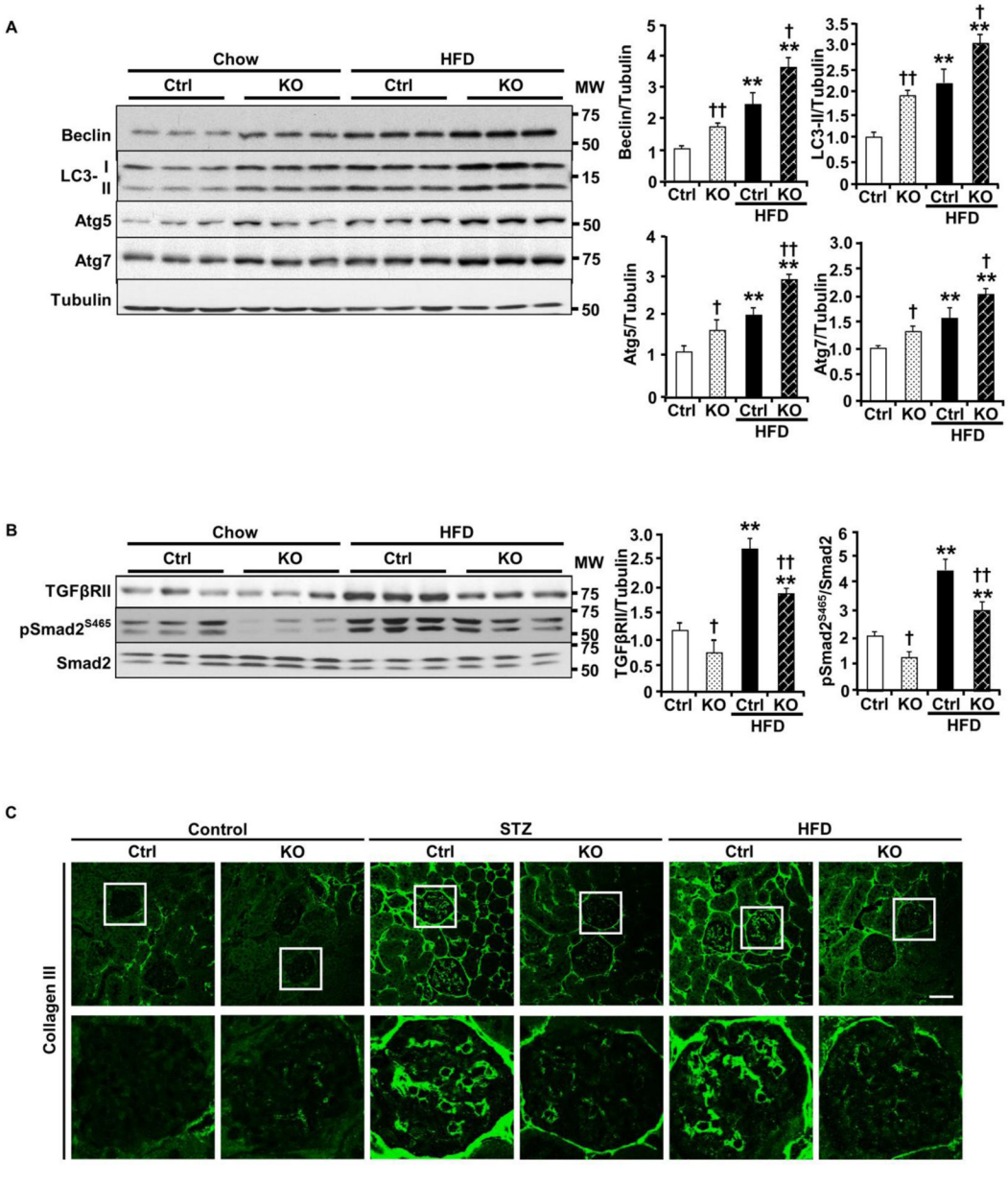


**Figure 5. Enhanced autophagy and attenuated fibrosis in mice with podocyte PTP1B disruption**

**A**) Immunoblots of Beclin, LC3-I/II, Atg5, Atg7 and Tubulin in kidney lysates from Ctrl (n=3) and KO (n=3) mice fed standard laboratory diet (chow) and HFD. Each lane represents tissue from a different animal. Bar charts represent Beclin, LC3-II, Atg5 and Atg7 normalized to Tubulin as means ± SEM. **B**) Immunoblots of TGFβRII, pSmad2 (S465) and Smad2 in kidney lysates from Ctrl (n=3) and KO (n=3) mice fed chow and HFD. Bar charts represent TGFβRII normalized to Tubulin and pSamd2/Smad2 as means ± SEM. In **A** and **B** $^{**}p \leq 0.01$ indicates a significant difference between chow and HFD fed mice, and $\dagger p \leq 0.05$; $\dagger\dagger p \leq 0.01$ between Ctrl and KO. **C**) Immunostaining of Collagen III in kidney sections from Ctrl and KO mice with Chow, STZ and HFD treatments. Lower panels are enlarged images

highlighted by white boxes in the upper panels. Scale bar: 50 μm. The Y-axes of bar charts are in arbitrary unit.

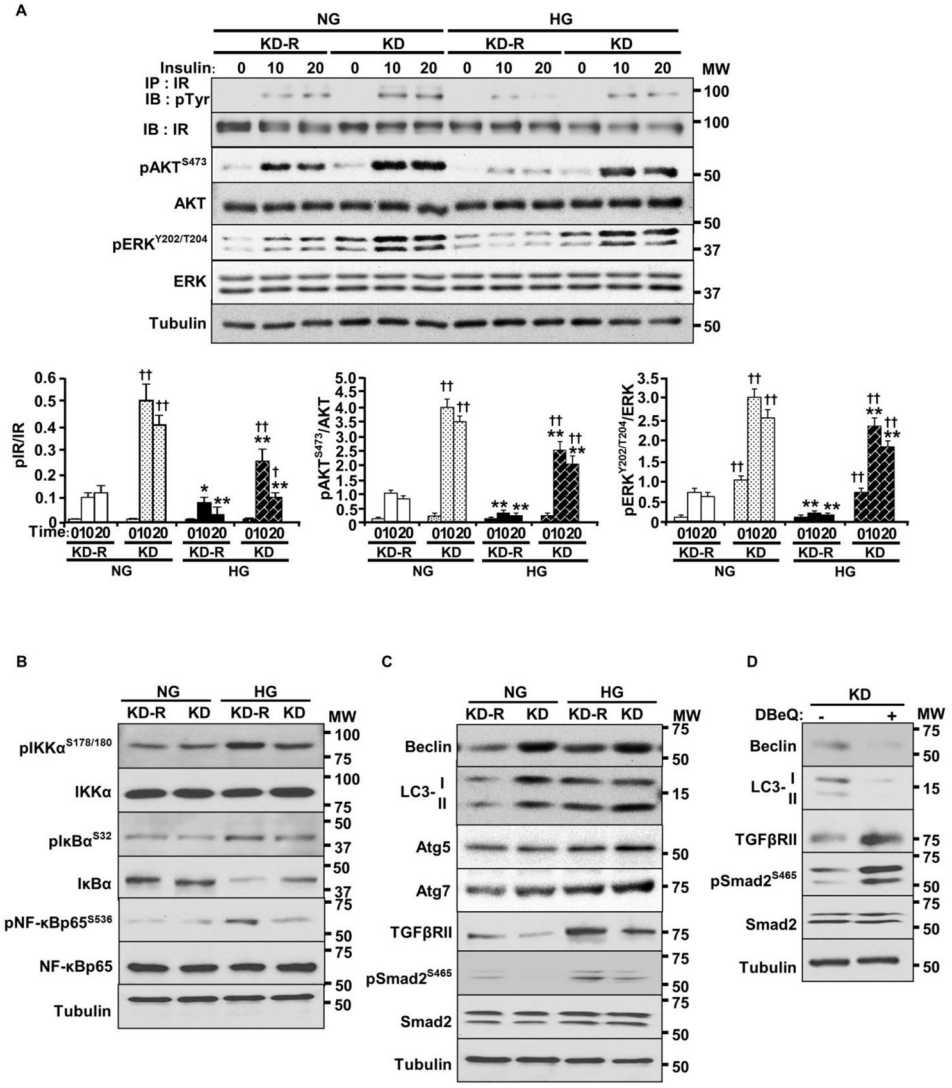


**Figure 6. Signaling alterations in E11 podocytes with PTP1B knockdown and reconstitution under normal and high glucose culture**

**A**) Differentiated E11 podocytes with PTP1B knockdown (KD) and reconstitution (KD-R) were cultured under normal glucose (NG) and high glucose (HG), starved overnight then stimulated with insulin (10nM) for 10 and 20 minutes. IR was immunoprecipitated then immunoblotted with phosphotyrosine (Ptyr; 4G10 and PY99) and IR. Lysates were immunoblotted for pAKT (S473), AKT, pERK (Y202/T204), ERK and Tubulin as a loading control. Bar charts represent normalized data for pIR/IR, pAKT/AKT, and pERK/ERK as means ± SEM. *$p \leq 0.05$; **$p \leq 0.01$ indicate a significant difference between NG and HG treatments, and †$p \leq 0.05$; ††$p \leq 0.01$ between KD-R and KD cells. **B**) Immunoblots of pIKKα, IKKα, pIκBα, IκBα, pNF-κB and NF-κB in differentiated KD and KD-R podocytes cultured in NG and HG for 72h. **C**) Immunoblots of Beclin, LC3-1/II, Atg5, Atg7, TGFβRII, pSmad2 (S465) and Smad2 in differentiated KD and KD-R podocytes cultured in NG and HG for 72h. **D**) PTP1B-KD podocytes were cultured in NG for 72h

treated with autophagy pharmacological inhibitor (DBeQ: 15μM) for 16h, then immunoblotted for Beclin, LC3, TGFβRII, pSmad2 and Smad2.