Fyn directly phosphorylates Tgm2 and is involved in the development of diabetic kidney disease by modulating autophagy and p53 expression

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ABSTRACT

Diabetic kidney disease (DKD) is the leading cause of end-stage renal disease (ESRD). Autophagy was shown to regulate DKD. Fyn functions as a muscle mass regulator by suppressing autophagy through STAT3 but it is still not known whether Fyn regulates autophagy in kidneys, especially in renal proximal tubular epithelial cells (RPTCs). In this study, we aimed to examine the role of Fyn on autophagy in RPTCs *in vivo* and *in vitro*. We demonstrated Fyn-dependent STAT3 phosphorylation by IL6 *in vitro*. Autophagy decreased via both IL6-dependent and Fyn-dependent mechanisms. Phospho-proteomic analysis revealed that Fyn phosphorylated transglutaminase 2 (Tgm2) at Y369. Moreover, we demonstrated that Fyn-dependent phosphorylation of Tgm2 regulated autophagy in RPTCs. Tgm2 forms a complex with p53 and p62 to degrade p53 in autophagosome in cancer cells. Interestingly, we found that p53 expression was decreased upon autophagy in Tgm2-knockdown RPTCs. In high-fat diet fed mice, we found increased Tgm2 expression and phosphorylation and decreased autophagy accompanied with increased p53 expression in RPTCs. Finally, We confirmed that Fyn regulates autophagy to mediate p53 expression using streptozocin-induced hyperglycemia mice model. Taken together, these data provide a molecular basis for the role of Fyn-Tgm2-p53 axis in the development of DKD.

One of the major complications of diabetes is diabetic nephropathy (DN), the leading cause of end-stage renal disease (ESRD) (1). Glomerular disorders due to hyperglycemia have been

considered as the cause of DN, but in recent years, renal tubule disorders due to aging or arteriosclerotic lesions have also been shown as the cause (2,3). Therefore, DN is also called diabetic kidney disease (DKD) since the disorder may not be limited to nephrons. Moreover, autophagy is attracting attention as a target for DKD (2). While autophagy is a system of bulk degradation, it has the aspect of maintaining intracellular quality in the basal state. In glomeruli and tubular cells in DN, autophagy deteriorates cell quality and causes cell death (2).

Although autophagy is originally described as an evolutionarily conserved cellular recycling program, recent studies have demonstrated the role of autophagy in metabolic functions (4-10). Deregulation of these functions is also associated with inflammatory processes, suggesting that inflammatory molecules modulate autophagy (11). In line with this, prolonged exposure of tissues and organs to high concentrations of inflammatory mediators inhibits autophagy, resulting in severe cell damage (11-13). Molecular mechanisms underlining the crosstalk between autophagy, energy metabolism, and immune function have been under scrutiny.

Fyn is one member of the Src family of non-receptor tyrosine kinases and a regulator of T cell signaling in immune response (14). Previous studies examining the role of Fyn in insulin action and energy expenditure *in vivo* relied on Fyn-null mice, indicating that the role of Fyn in energy metabolism and immune function could be mediated by autophagy (15,16). Indeed, recent molecular analyses revealed a novel Fyn signaling pathway in skeletal muscles through a Fyn-dependent activation of STAT3 with reduced Vps34/Vps15/beclin1 expression to modulate its participation in autophagy, indicating that Fyn functions as part of the nutrient-sensing system to regulate skeletal muscle autophagy and muscle degeneration (17).

Although how beclin1 complex regulates autophagy is not well-known, beclin1 crosslinking by transglutaminase 2 (Tgm2) is one of the mechanisms of autophagy regulation, resulting in decreased beclin1 activity to decrease autophagy (18). More recently, it has been reported that

Tgm2 binds to p62, an autophagy-specific substrate, and p53, a tumor suppressor, to regulate autophagic activity and the expression of p53 in tumor cells (19). Although p53 is an exacerbating factor for DKD, there are few reports on its regulation (20-22).

In this study, we aimed to examine the role of Fyn and Tgm2-p62-p53 complex on autophagy in proximal tubular cells *in vivo* and *in vitro*.

RESEARCH DESIGN AND METHODS

Antibodies and reagents

Rabbit polyclonal antibodies against Fyn, STAT3, and phospho$^{-(Y705)-}$STAT3, as well as mouse monoclonal antibodies against Tgase2, p53, and AQP-1, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody against p62 was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Rabbit polyclonal antibody against LC3 was obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal against anti-GAPDH antibody was purchased from MBL International (Woburn, MA, USA). Anti-Phosphotyrosine, clone 4G10 antibody was purchased from Millipore (Billerica, MA, USA). Leupeptin Hemisulfate, IL-1β, and IL6 were obtained from ThermoFisher Scientific (Pittsburgh, PA, USA) and IL18 from MBL. All other reagents were purchased from Merck (Burlington, MA, USA).

cDNA constructs

pcDNA3 and pcDNA3-Fyn-CA were constructed or obtained as previously explained (16). The IMAGE clone 3256943 (GenBank: BC016492) for mouse TG2 was obtained from imaGenes (Berlin, Germany) and PCR was performed with a pair of oligonucleotides as follows: 5'-CACCATGGACTACAAGGACGATGACGACAAG-ATGGCAGAGGAGCTG-3' and 5'-TTAGGCCGGGCCGATGATAA -3'. PCR product was separated on a 2% agarose gel, and the

specific single band was extracted using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands). The purified PCR product was cloned into the pcDNA3.1D/V5-His-TOPO using the pcDNA3.1 Directional TOPO Expression Kit (ThermoFisher). pcDNA3.1-Flag-Tgm2-Y369 was obtained using the QuickChange 2-XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with the following pair of oligonucleotides: 5'-GAAGAGCGAAGGGACATTCTGTTGTGGCCCA-3' and 5'-TGGGCCACAACAGAATGTCCCTTCGCTCTTC-3'. pcDNA3.1-Flag-Tgm2-Y369/617F was obtained using the QuickChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) with the following pairs of oligonucleotides: 5'-GAAGAGCGAAGGGACATTCTGTTGTGGCCCA-3' and 5'-ACCCACTTTCCGATCCCCTGTTTGACTGCATCT-3'; 5'-TGGGCCACAACAGAATGTCCCTTCGCTCTTC-3' and 5'-AGATGCAGTCAAACAGGGGATCGGAAAGTGGGT-3'.

Cell culture

The human renal proxima l tubular epithelial cell line HK-2 was obtained from ATCC (Manassas, VA, USA). The cells were cultured in a 100 cm^2 dish and grown at 37°C in an atmosphere of 5% CO_2 in Keratinocyte-SFM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with bovine pituitary extract (0.05 mg/mL) and epithelial growth factor (5 ng/mL; Thermo Fisher Scientific). Cells were sub-cultured when they reached 70–80% confluency. Human renal proximal tubule epithelial cells (PTECs) obtained from ATCC were cultured in a 100 cm^2 dish and grown at 37°C in an atmosphere of 5% CO_2 in REBM Basal Medium and REGM SingleQuots supplements as described previously (Lonza. Muenchensteinerstrasse, Basel, Switzerland).

Transfection

After seeding 2×10^5 cells onto 12-well plates in Dulbecco's modified Eagle medium (DMEM) low glucose for 12 h, cells were transfected with either 2 μg of myc-DDK-Tgm2 wild-type (WT) or

Tgm2-YF with 2 μl of X-tremeGENE HP DNA transfection reagent (Roche Diagnostics) using OptiMEM (Thermo Fisher Scientific) according to the manufacturer's protocol. After 12 h, OptiMEM was changed to DMEM low glucose medium followed by harvesting transfected cells after 48 h. For siRNA-mediated knockdown of Fyn and Tgm2, cells were transfected with siGENOME siRNA SMART pool using DharmafectDuo (Dharmacon, Thermo Scientific, USA) according to the manufacturer protocol. Briefly, 16 h after seeding 1.0×10^5 cells onto 12-well plates, cells were transfected with either 0.2 μM of non-target siRNA or Fyn/Tgm2 siRNA with DharmafectDuo followed by harvesting to perform subsequent experiments after 48 h.

Western blotting analysis

Proteins were extracted using a lysis buffer containing a protease inhibitor as described previously (19) and quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes, which were blocked with 5% fatty acid-free powdered milk for 2 h. The membranes were incubated with the following primary antibodies overnight at 4°C: Anti-p STAT3 (1:500), anti-STAT3 (1:1,000), anti-Fyn (1:1,000), anti-p62 (1:1,000), anti-LC3 (1:1,000), anti-Tgm2 (1:1,000), anti-4G10 (1:1,000), anti-p53 (1:1,000), and anti-GAPDH (1:1,000). Thereafter, they were incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min. The membranes were extensively washed in tris-buffered saline with Tween 20, and antigen-antibody complexes were visualized via chemiluminescence using an ECL kit (Pierce).

Immunoprecipitation

Cells were lysed or tissues from mouse models were homogenized using a beads-based homogenizer, beads crusher (μT-12, Taitec, Saitama, Japan), with Zirconia beads with the

following setting: 2500 rpm, 90 s in NP40 lysis buffer. Collected samples were centrifuged at maximum speed (15000 rpm) for 10 min at 4°C. The supernatants were collected and protein concentration was determined using the BCA method. Ten milligrams of lysate was mixed with 30 μl of anti-Tgm2 antibody while gently rocking for 2 h at 4°C followed by incubation with 70 μl of protein A/G PLUS-Agarose (Santa Cruz) for 1 h at 4°C. Samples were washed thrice in washing buffer and boiled and separated via 10% SDS-PAGE followed by an overnight transfer. Western blotting analysis was performed with the indicated antibodies.

***In vitro* Tgm2 phosphorylation assay**

Human GST-Tgm2 fusion protein and Flag-Fyn-CA were purchased from Abnova (Taipei, Taiwan) and BPS Bioscience (San Diego, CA, USA), respectively. GST-Tgm2 (100 ng) was incubated with the active recombinant FynT kinase (1.8 U) in presence of ATP and kinase buffer (Cell Signaling) for 1 h at 30°C. Samples were separated on 8% SDS-polyacrylamide gels and immunoblotting was performed with an anti-4G10 antibody.

Measurement of autophagy flux in HK-2/ PTECs

After 48 h, cells were cultured in a 100 cm^2 dish followed by stimulation with 2 M NH_4Cl and 10 mM Leupeptin for 2 h. Cells were then harvested to perform western blotting with the anti-LC3 antibody.

Animals

pp59fyn null mice (FynKO) were provided by RIKEN BRC, with the support of the National BioResource Project of Ministry of Education, Culture, Sports, Science and Technology, Japan. Heterozygous FynKO mice were bred to get FynKO and their controls. C57BL/6J were purchased from Charles River (Wilmington, MA, USA). All mice were housed in a facility with a 12/12 h light/dark cycle and fed a standard chow diet (Research Diets, New Brunswick, NJ) containing 67% (Kcal) carbohydrates, 19% protein, and 4% fat *ad libitum*. All studies were approved by and

performed in compliance with the guidelines of the institutional animal care and use committee of Gunma University. For diet-induced obesity model mice, C57BL/6J, were fed a high-fat, low-carbohydrate diet (containing 5.2 kcal/g, with 60% of the calories from fat, 20% from protein, and 20% from carbohydrate; diet D12492 from Research Diets, New Brunswick, NJ) from 4 weeks. For streptozocin (STZ) induced diabetes models, the animals (8-12 weeks old) were injected STZ (50 mg/kg) dissolved in citrate buffer (pH 4.5) or the same amount of citrate buffer for controls intraperitoneally. The injection was administered twice every 5 days consecutively. After 4 and 8 weeks, blood samples were collected from the tails, to perform a blood sugar test. The mice were fed a standard chow diet for 2 months, and the blood sugar test were repeated. The mice were sacrificed via cerebral dislocation after fasting for 16 h and the kidneys were collected. This protocol has been adopted by the Animal Models of Diabetic Complications Consortium.

Immunofluorescence

Frozen tissue slides were blocked in 1% BSA at room temperature for 30 min followed by incubation with the diluted primary antibody (1:50 for Fyn, Tgm2, p62, AQP-1 and p53) in 1% BSA at room temperature for 60 min. Secondary antibodies used were as follows: Alexa Fluor 488 anti-goat antibody, Alexa Fluor 546 anti-rabbit antibody (ThermoFisher Scientific). Sections were washed thrice with PBS, mounted with Prolong Gold anti-fade reagent with DAPI (ThermoFisher Scientific), and imaged using a confocal fluorescence microscope, BZ-X710 (KEYENCE, Osaka, Japan). Settings (Iris (pinhole), laser intensity, gain and offset) were fixed for all samples. p62 signal was quantified using the Image J software (National Institutes of Health). Images of 15 representative cells were processed and results represent three independent experiments

RNA isolation and real-time qPCR

Frozen kidney samples were placed into QIAzol Lysis Reagent (Qiagen), and homogenized using beads crusher (μT-12, Taitec) followed by total RNA isolation from tissues using the RNeasy Plus

Mini Kit (Qiagen) according to the manufacturer's protocol. Total RNA (1 mg) was reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (ThermoFisher Scientific) followed by real-time quantitative PCR performed on an Applied Biosystems 7500HT system (Applied Biosystems, Branchburg, NJ, USA) with EagleTaq Master Mix (Roche Lifescience). *Fyn, Tgm2,* and *p53* mRNAs were quantified using TaqMan RT-PCR (ThermoFisher Scientific) and gene expression levels were internally normalized against those of *Hrpt1* (mouse) and analyzed using a standard curve method. Each experiment was performed in triplicate and independently repeated three times.

Statistics

Results are expressed as the mean ± standard error of the mean (SEM). Differences between animals and/or treatments were tested for statistical significance using the Student's unpaired t-test.

RESULTS

IL-6 regulates autophagy through Fyn in HK-2 cells

We first examined whether inflammatory cytokines could regulate STAT3 activity in HK-2 cells. We incubated inflammatory cytokines for 30 min. Moreover, we used TGF beta and IL-1 and IL-6 as pro-inflammatory cytokines (23) and IL18 as an anti-inflammatory cytokine (24). STAT3 phosphorylation was observed in HK-2 cells only upon IL-6 administration (Figure 1A). Next, to investigate whether STAT3 phosphorylation induced by IL-6 was dependent on Fyn, we administered cytokines to HK-2 cells via non-target or Fyn (siFyn) siRNA transfection and observed STAT3 phosphorylation. siRNA-mediated Fyn knockdown was quite efficient (~95%) (Figure 1B). STAT3 phosphorylation was observed in both HK-2 cells transfected with non-target siRNA or siFyn, but it was significantly less in HK-2 cells transfected with siFyn than those

transfected with non-target siRNA (Figure 1B). To further examine if Fyn-dependent STAT3 phosphorylation regulates autophagy, we first knocked down Fyn and observed p62 expression. p62 expression dramatically decreased in HK-2 cells transfected with siFyn, denoting Fyn-dependent autophagy in renal tubular cells (Figure 1C and D). Moreover, autophagy flux was assessed by comparing LC3-II expression in HK-2 cells with and without ammonium chloride/Leupeptin (N/L) treatment (25,26). The increase in LC3-II expression was observed upon N/L treatment in HK-2 cells expressing non-target siRNA, indicating the basal autophagic flow (Figure 1E and F). However, the increase in LC3-II expression was observed upon N/L treatment in HK-2 cells expressing siFyn, confirming Fyn-dependent autophagy. Finally, we performed an autophagic flow assay in HK-2 cells transfected with siFyn and incubated with IL-6, as well as N/L. The increase in LC3-II expression was observed upon N/L and IL-6 treatment in HK-2 cells expressing non-target siRNA, indicating the basal autophagic flow (Figure 1G and H). However, few changes in LC3-II expression were observed in the cells treated with IL-6, demonstrating that IL-6 negatively regulated autophagy in HK-2 cells. In contrast, the increase in LC3-II expression was still observed upon N/L treatment in HK-2 cells expressing siFyn, which was also observed in the cells treated with IL-6, demonstrating that Fyn knockdown rescued the IL-6-induced autophagic flow decay in HK-2 cells (Figure 1G and H).

Tgm2 Y369 is phosphorylated by Fyn *in vivo* and *in vitro*

We next examined whether Fyn phosphorylates Tgm2 and regulates beclin 1 crosslinking. Exogenous Flag-Tgm2 was phosphorylated by overexpressed constitutively active Fyn but not by the mock (Figure 2A). To examine whether Tgm2 is a direct substrate target of Fyn, we performed *in vitro* kinase assay, demonstrating that purified GST-Tgm2 fusion protein was phosphorylated by purified recombinant Fyn kinase *in vitro* (Figure 2B). To identify the Tgm2 tyrosine sites

phosphorylated by Fyn, we inspected our previous phosphotyrosine proteome screening and identified Y369 and Y617 as the putative sites (Supplementary Figure 1A and B). Therefore, we generated double point mutants where Y369 and Y617 were substituted with a phenylalanine residue. Co-expression of Fyn-CA with WT and double mutant (D-YF) Tgm2 demonstrated equal expression levels for all Tgm2 mutants (Supplementary Figure 1C). The phosphorylation of the D-YF mutant decreased. Moreover, to examine whether phosphorylation happens at only one site, we generated single-point mutants, demonstrating that when Y369 was substituted by phenylalanine, tyrosin phosphorylation by Fyn-CA was almost diminished, indicating that Y369 was the only phosphorylation site (Figure 2C).

Tgm2 Y369 phosphorylation regulates autophagic activity in renal tubular cells

To further examine if Fyn-dependent Tgm2 phosphorylation regulates autophagy, we first knocked down Tgm2 and observed p62 expression. p62 expression was dramatically decreased in HK-2 cells transfected with siTgm2, indicating Tgm2-dependent autophagy in renal tubular cells (Figure 3A and B). Moreover, autophagy flux was assessed by comparing the expression of LC3-II in HK-2 cells transfected with siTgm2 with and without N/L treatment. The increase in LC3-II expression was observed upon N/L treatment in HK-2 cells expressing non-target siRNA, indicating basal autophagic flow (Figure 3C and D). The increase in LC3-II expression was also observed upon N/L treatment in HK-2 cells expressing siTgm2, confirming Tgm2-dependent autophagy. This result was further confirmed by overexpressing Tgm2-WT in HK-2 cells, demonstrating no change in LC3-II expression upon N/L treatment (Figure 3E and F). Furthermore, we performed an autophagic flow assay in HK-2 cells transfected with Tgm2-WT and Tgm2-Y369F. No change in LC3-II expression was observed upon N/L treatment in HK-2 cells expressing Tgm2-WT, indicating basal autophagic flow inhibition by Tgm2 (Figure 3E and F). A significant increase in

LC3-II expression was observed in the cells transfected with Tgm2-Y369F, demonstrating that non-phosphorylated Tgm2 had a dominant-negative effect and rescued autophagic flow in HK-2 cells (Figure 3G and H).

Tgm2 knockdown regulates p53 through autophagy

We transfected HK-2 cells with Tgm2-WT and Fyn-CA followed by western blotting with Nε-(ϒ-L-glutamyl)-L-lysine isopeptide (18). There was no evidence of beclin1 crosslinking in HK-2 cells transfected with either mock or Fyn-CA construct (Supplementary Figure 2A). To further confirm these data, we transfected HK-2 cells with Tgm2-WT and Tgm2-Y369F and performed western blotting under normal and denatured conditions, which showed no evidence of beclin1 crosslinking (Supplementary Figure 2B). Therefore, we suspected another mechanism involving p53 (19). Indeed, autophagy inhibition by N/L increased p53 expression, indicating p53 degradation through autophagy in PTECs (Figure 4A and B). Interestingly, p53 expression decreased in Tgm2-knockdown PTECs (Figure 4C and D). Finally, p53 expression was assessed in PTECs transfected with siTgm2 with and without N/L treatment. p53 expression increased upon N/L treatment, clarifying p53 as an autophagy substrate in PTECs (Figure 4E and F). Importantly, this increase in p53 expression was initiated upon siTgm2 treatment, demonstrating that Tgm2-regulated autophagy-mediated p53 expression.

Higher Tgm2 phosphorylation and autophagy inhibition is observed in the high-fat diet (HFD) mice model

We next examined the expression of Fyn and Tgm2 in the kidneys of insulin-resistant mice. WT mice were fed with an HFD for 13 weeks (27). Fyn expression did not change significantly, but Tgm2 expression significantly increased in the kidneys of HFD mice (Figure 5A and B). While Fyn expression was not changed, Fyn activity and Tgm2 phosphorylation changed in HFD mice. To explore this regulation, we examined Tgm2 phosphorylation in HFD mice. As shown in Figure

5C, HFD induced Tgm2 expression. Moreover, we performed immunoprecipitation to observe the phosphorylation of Tgm2, demonstrating that HFD dramatically induced endogenous Tgm2 phosphorylation *in vivo*.

We then examined if HFD affected autophagy *in vivo,* specifically in renal tubular cells of HFD mice. To explore autophagy in renal tubular cells, we performed immunofluorescence using a p62 antibody with AQP1 followed by the quantification of the p62 puncta (Figure 5D, E and Supplementary Figure 3), demonstrating that autophagy was inhibited in renal tubular cells in HFD mice. We also examined p53 expression in HFD mice, revealing that p53 expression increased while *p53* mRNA expression did not change (Figure 5F, G and H). Taken together, Tgm2 phosphorylation by Fyn was promoted in HFD mice, leading to autophagy inhibition to regulate p53 expression.

FynKO rescues STZ-induced DKD by inducing autophagy-mediated p53 degradation

We observed Tgm2 phosphorylation in HFD mice; however, we did not know whether this phosphorylation was Fyn-dependent or DKD-causing. To further explore this, we utilized FynKO mice with a slight modification (28), taking advantage of STZ and inducing a diabetic model instead. Two weeks after STZ injection, we observed hyperglycemia in both WT and FynKO mice (data not shown). To explore autophagy in renal tubular cells, we performed immunofluorescence using a p62 antibody with AQP1 followed by quantifying the p62 puncta in both mice. The p62 signal intensity increased significantly in kidney renal tubular cells of STZ-induced WT diabetic mice, indicating a lack of autophagy in renal tubular cells (Figure 6A and B). However, no change was observed in the renal tubular cells of STZ-induced diabetic FynKO mice, indicating that autophagy was rescued (Figure 6A and B). Finally, we examined whether this autophagy regulation changes p53 expression to induce DKD. p53 expression increased in the kidneys of

STZ-induced WT hyperglycemic mice but not in the kidneys of FynKO mice, indicating that Fyn regulated autophagy and p53 expression (Figure 6C and D).

DISCUSSION

The data presented in this study provide a new beclin-1- and hyperglycemia-independent autophagy mechanism in DKD. We identified Fyn as a novel autophagy regulator in proximal tubular cells, which is mediated through IL-6, one of the pro-inflammatory cytokines induced under diabetic conditions.

DKD is one of the chronic kidney diseases caused by hyperglycemia, induced by many factors such as accumulation of advanced glycation products (AGEs), free radicals, and activation of protein kinase C (29). More recently, a variety of studies established the new idea that autophagy also has important roles in the pathogenesis of chronic kidney disease (2,30). However, the role of autophagy in DKD remains elusive (30).

Proximal tubular cells are responsible for the reabsorption of nutrients and consume a large amount of energy (31). Therefore, they are susceptible to the metabolic state, have higher autophagic activity than the cells in other parts of the kidneys, and their dysfunction may be closely related to the progression of DKD (31). Hyperglycemia reportedly inhibits the expression of beclin-1, resulting in the inhibition of autophagosome membrane formation (32), but the underlying mechanisms of how beclin1 modulates autophagy in proximal tubular cells under diabetic conditions are still elusive (31).

Chronic inflammation is in charge of pathogenesis and the causative reason for the progression of kidney disease in patients with diabetes (11,33). Among the pro-inflammatory cytokines, IL6 signaling contributes to the progression of DKD, which is supported by both *in vitro* and *in vivo* studies (29,33,34). To identify Fyn substrates in autophagy, we previously performed a

phosphotyrosine proteome screening comparing skeletal muscles from Fyn-null mice with those from the HSA-Fyn transgenic mice and identified STAT3 as a predominant substrate (17). We also identified AMPK as a substrate for Fyn and an autophagy regulator (35). In this regard, we also recently identified TNFα as a Fyn-mediated AMPK regulator to modulate autophagy in HEK293T cells (35). More importantly, while we demonstrated that Fyn enhances STAT3 phosphorylation and alters the expression of Vps34, as well as the assembly of the Vps34/Vps15/Beclin1/Atg14 complex, the mechanism of how it regulates autophagy is still unknown (17).

Tgm2 is a member of a family of Ca^{2+}-dependent transamidases, catalyzing the isopeptide bond between glutamate and lysine residues, resulting in a covalent crosslink (36). To date, discrete roles of Tgm2 have been identified (37-40). A recent study showed that the role of extracellular Tgm2 is induced in glucose-stimulated cell lines and that Y369 is the potential phosphorylation site (41). However, the role of Tgm2 in renal tubular cells under diabetic conditions was unclear and the significance of tyrosine phosphorylation or the responsible tyrosine kinase is still unknown (41). Here, we identified Tgm2 as a novel autophagy regulator in proximal tubular cells. We determined Fyn as the kinase that phosphorylates Tgm2 and demonstrated that Fyn phosphorylated Tgm2 at Y369 and this phosphorylation determined the autophagic activity in proximal tubular cells.

It was reported that beclin1 is crosslinked by Tgm2 (18). Therefore, we first examined if beclin1 crosslinking by Tgm2 also occurs in HK-2 cells. However, this was not the case. A more recent study showed that Tgm2 forms a complex with p53 and p62, known autophagy regulators/substrates, to degrade p53 in autophagosomes in cancer cells (19). p53, as a well-known tumor suppressor, is a key component of the cellular response to stress (42). Indeed, p53 knockdown in renal proximal tubules protects against ischemic-reperfusion injury. Importantly,

p53 knockdown in other renal tubular segments is ineffective (43). In the present study, we found that autophagy regulation by Tgm2 mediated p53 expression, implicating the role of Tgm2-regulated autophagy. p53 is degraded via ubiquitination in other tissues (44). Therefore, this is the first evidence that p53 is degraded via autophagy in renal proximal tubular cells. Moreover, given that p53 activation in tubular cells plays a critical role in AKI pathogenesis and maladaptive kidney repair after AKI (26), p53 regulation by Tgm2 could be a novel and potential strategy for the treatment of this disorder.

Finally, we utilized STZ-induced hyperglycemia and HFD mice models to confirm the underlying mechanisms of Tgm2 phosphorylation by Fyn to regulate autophagy and p53 expression in DKD. HFD inhibits autophagy through lysosomal dysfunction (45). These mechanisms have been observed in the kidneys of patients with obesity (46). Factors like lipotoxicity (46) or oxidative stress (47) regulate autophagy but few studies have demonstrated the impaired autophagy signaling in the renal tubular cells under these circumstances. Moreover, several recent studies have shown the role of p53 in metabolic tissues (48-50), but only a few confirmed the underlying mechanism of how its expression is controlled via degradation. Here, we confirmed that p53 is degraded via autophagy through a Tgm2-dependent axis in HFD mice in a Fyn-dependent manner.

We provide a molecular basis for the role of the Fyn-Tgm2-p53 axis in the development of DKD. Tissue-specific or time-dependent deletion mice models are necessary to confirm these mechanisms; however, this regulatory mechanism is not mediated by blood glucose levels and may lead to new therapeutic targets for DKD regardless of the control of diabetes. Moreover, as this is the first study showing a tyrosine kinase mediating autophagy and DKD, and there are already many Fyn inhibitors commercially available, this mechanism could contribute to the treatment of DKD.

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REFERENCES

1. Tuomilehto J, Lindström J, Eriksson JG, Valle TT, Hämäläinen H, Ilanne-Parikka P, Keinänen-Kiukaanniemi S, Laakso M, Louheranta A, Rastas M, Salminen V, Uusitupa M, Finnish Diabetes Prevention Study Group. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 2001;344(18):1343–50.

2. Tang C, Livingston MJ, Liu Z, Dong Z. Autophagy in kidney homeostasis and disease. Nat Rev Nephrol 2020;16(9):489–508.

3. O'Sullivan ED, Hughes J, Ferenbach DA. Renal aging: causes and consequences. J Am Soc Nephrol 2017;28(2):407–20.

4. Kim KH, Lee MS. Autophagy - a key player in cellular and body metabolism. Nat Rev Endocrinol 2014;10(6):322–37.

5. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ. Autophagy regulates lipid metabolism. Nature 2009;458(7242):1131–5.

6. Auranen M, Villanova M, Muntoni F, Fardeau M, Scherer SW, Kalino H, Minassian BA. X-linked vacuolar myopathies: two separate loci and refined genetic mapping. Ann Neurol 2000;47(5):666–9.

7. Raben N, Schreiner C, Baum R, Takikita S, Xu S, Xie T, Myerowitz R, Komatsu M, Van der Meulen JH, Nagaraju K, Ralston E, Plotz PH. Suppression of autophagy permits successful enzyme replacement therapy in a lysosomal storage disorder - murine Pompe disease. Autophagy 2010;6(8):1078–89.

8. Singh R, Xiang Y, Wang Y, Baikati K, Cuervo AM, Luu YK, Tang Y, Pessin JE, Schwartz GJ, Czaja MJ. Autophagy regulates adipose mass and differentiation in mice. J Clin Invest 2009;119(11):3329–39.

9. Ebato C, Uchida T, Arakawa M, Komatsu M, Ueno T, Komiya K, Azuma K, Hirose T, Tanaka K, Kominami E, Kawamori R, Fujitani Y, Watada H. Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet. Cell Metab 2008;8(4):325–32.

10. Jung HS, Chung KW, Won Kim J, Kim J, Komatsu M, Tanaka K, Nguyen YH, Kang TM, Yoon KH, Kim JW, Jeong YT, Han MS, Lee MK, Kim KW, Shin J, Lee MS. Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. Cell Metab 2008;8(4):318–24.

11. Yao RQ, Ren C, Xia ZF, Yao YM. Organelle-specific autophagy in inflammatory diseases: a potential therapeutic target underlying the quality control of multiple organelles. Autophagy 2021;17(2):385–401.

12. Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM. Autophagy and inflammation in chronic respiratory disease. Autophagy 2018;14(2):221–32.

13. Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. J Hepatol 2013;59(3):583–94.

14. Comba A, Dunn PJ, Argento AE, Kadiyala P, Ventosa M, Patel P, Zamler DB, Núñez FJ, Zhao L, Castro MG, Lowenstein PR. Fyn tyrosine kinase, a downstream target of receptor tyrosine kinases, modulates antiglioma immune responses. Neuro Oncol 2020;22(6):806–18.

15. Bastie CC, Zong H, Xu J, Busa B, Judex S, Kurland IJ, Pessin JE. Integrative metabolic regulation of peripheral tissue fatty acid oxidation by the SRC kinase family member Fyn. Cell Metab 2007;5(5):371–81.

16. Yamada E, Pessin JE, Kurland IJ, Schwartz GJ, Bastie CC. Fyn-dependent regulation of energy expenditure and body weight is mediated by tyrosine phosphorylation of LKB1. Cell Metab 2010;11(2):113–24.

17. Yamada E, Bastie CC, Koga H, Wang Y, Cuervo AM, Pessin JE. Mouse skeletal muscle fiber-type-specific macroautophagy and muscle wasting are regulated by a Fyn/STAT3/Vps34 signaling pathway. Cell Rep 2012;1(5):557–69.

18. Luciani A, Villella VR, Esposito S, Brunetti-Pierri N, Medina D, Settembre C, Gavina M, Pulze L, Giardino I, Pettoello-Mantovani M, D'Apolito M, Guido S, Masliah E, Spencer B, Quaratino S, Raia V, Ballabio A, Maiuri L. Defective CFTR induces aggresome formation and lung inflammation in cystic fibrosis through ROS-mediated autophagy inhibition. Nat Cell Biol 2010;12(9):863–75.

19. Kang JH, Lee JS, Hong D, Lee SH, Kim N, Lee WK, Sung TW, Gong YD, Kim SY. Renal cell carcinoma escapes death by p53 depletion through transglutaminase 2-chaperoned autophagy. Cell Death Dis 2016;7(3):e2163.

20. Ma Z, Li L, Livingston MJ, Zhang D, Mi Q, Zhang M, Ding HF, Huo Y, Mei C, Dong Z. p53/microRNA-214/ULK1 axis impairs renal tubular autophagy in diabetic kidney disease. J Clin Invest 2020;130(9):5011–26.

21. Wang Y, Zheng ZJ, Jia YJ, Yang YL, Xue YM. Role of p53/miR-155-5p/sirt1 loop in renal tubular injury of diabetic kidney disease. J Transl Med 2018;16(1):146.

22. Hong Q, Zhang L, Das B, Li Z, Liu B, Cai G, Chen X, Chuang PY, He JC, Lee K. Increased podocyte Sirtuin-1 function attenuates diabetic kidney injury. Kidney Int 2018;93(6):1330–43.

23. Amdur RL, Feldman HI, Gupta J, Yang W, Kanetsky P, Shlipak M, Rahman M, Lash JP, Townsend RR, Ojo A, Roy-Chaudhury A, Go AS, Joffe M, He J, Balakrishnan VS, Kimmel PL,

Kusek JW, Raj DS, CRIC Study Investigators. Inflammation and progression of CKD: the CRIC study. Clin J Am Soc Nephrol 2016;11(9):1546–56.

24. Araki S, Haneda M, Koya D, Sugimoto T, Isshiki K, Chin-Kanasaki M, Uzu T, Kashiwagi A. Predictive impact of elevated serum level of IL-18 for early renal dysfunction in type 2 diabetes: an observational follow-up study. Diabetologia 2007;50(4):867–73.

25. Mauro-Lizcano M, Esteban-Martínez L, Seco E, Serrano-Puebla A, Garcia-Ledo L, Figueiredo-Pereira C, Vieira HL, Boya P. New method to assess mitophagy flux by flow cytometry. Autophagy 2015;11(5):833–43.

27. Rossmeisl M, Rim JS, Koza RA, Kozak LP. Variation in type 2 diabetes - related traits in mouse strains susceptible to diet-induced obesity. Diabetes 2003;52(8):1958–66.

28. Lee TW, Kwon H, Zong H, Yamada E, Vatish M, Pessin JE, Bastie CC. Fyn deficiency promotes a preferential increase in subcutaneous adipose tissue mass and decreased visceral adipose tissue inflammation. Diabetes 2013;62(5):1537–46.

29. Kawanami D, Matoba K, Utsunomiya K. Signaling pathways in diabetic nephropathy. Histol Histopathol 2016;31(10):1059–67.

30. Lin TA, Wu VC, Wang CY. Autophagy in chronic kidney diseases. Cells 2019;8(1):61.

31. Isaka Y, Kimura T, Takabatake Y. The protective role of autophagy against aging and acute ischemic injury in kidney proximal tubular cells. Autophagy 2011;7(9):1085–7.

32. Guan ZF, Zhou XL, Zhang XM, Zhang Y, Wang YM, Guo QL, Ji G, Wu GF, Wang NN, Yang H, Yu ZY, Zhou HG, Guo JC, Liu YC. Beclin-1- mediated autophagy may be involved in the elderly cognitive and affective disorders in streptozotocin-induced diabetic mice. Transl Neurodegener 2016;5:22.

33. Furuya F, Ishii T, Kitamura K. Chronic inflammation and progression of diabetic kidney disease. Contrib Nephrol 2019;198:33–9.

34. Donate-Correa J, Martín-Núñez E, Muros-de-Fuentes M, Mora-Fernández C, Navarro-González JF. Inflammatory cytokines in diabetic nephropathy. J Diabetes Res 2015;2015:948417.

35. Yamada E, Okada S, Bastie CC, Vatish M, Nakajima Y, Shibusawa R, Ozawa A, Pessin JE, Yamada M. Fyn phosphorylates AMPK to inhibit AMPK activity and AMP-dependent activation of autophagy. Oncotarget 2016;7(46):74612–29.

36. Tatsukawa H, Hitomi K. Role of transglutaminase 2 in cell death, survival, and fibrosis. Cells 2021;10(7):1842.

37. Olsen KC, Sapinoro RE, Kottmann RM, Kulkarni AA, Iismaa SE, Johnson GV, Thatcher TH, Phipps RP, Sime PJ. Transglutaminase 2 and its role in pulmonary fibrosis. Am J Respir Crit Care Med 2011;184(6):699–707.

38. Lai TS, Greenberg CS. TGM2 and implications for human disease: role of alternative splicing. Front Biosci (Landmark Ed) 2013;18:504–19.

39. Eligini S, Fiorelli S, Tremoli E, Colli S. Inhibition of transglutaminase 2 reduces efferocytosis in human macrophages: role of CD14 and SR-AI receptors. Nutr Metab Cardiovasc Dis 2016;26(10):922–30.

40. Yeo SY, Itahana Y, Guo AK, Han R, Iwamoto K, Nguyen HT, Bao Y, Kleiber K, Wu YJ, Bay BH, Voorhoeve M, Itahana K. Transglutaminase 2 contributes to a TP53-induced autophagy program to prevent oncogenic transformation. eLife 2016;5:e07101.

41. Furini G, Burhan I, Huang L, Savoca MP, Atobatele A, Johnson T, Verderio EAM. Insights into the heparan sulphate-dependent externalisation of transglutaminase-2 (TG2) in glucose-stimulated proximal-like tubular epithelial cells. Anal Biochem 2020;603:113628.

42. Sionov RV, Haupt Y. The cellular response to p53: the decision between life and death. Oncogene 1999;18(45):6145–57.

43. Hao J, Wei Q, Mei S, Li L, Su Y, Mei C, Dong Z. Induction of microRNA-17-5p by p53 protects against renal ischemia-reperfusion injury by targeting death receptor 6. Kidney Int 2017;91(1):106–18.

44. Kang JH, Lee SH, Lee JS, Oh SJ, Ha JS, Choi HJ, Kim SY. Inhibition of transglutaminase 2 but not of MDM2 has a significant therapeutic effect on renal cell carcinoma. Cells 2020;9(6):1475.

26. Zhang D, Liu Y, Wei Q, Huo Y, Liu K, Liu F, Dong Z. Tubular p53 regulates multiple genes to mediate AKI. J Am Soc Nephrol 2014;25(10):2278–89.

45. Yamamoto T, Takabatake Y, Takahashi A, Kimura T, Namba T, Matsuda J, Minami S, Kaimori JY, Matsui I, Matsusaka T, Niimura F, Yanagita M, Isaka Y. High-fat diet-induced lysosomal dysfunction and impaired autophagic flux contribute to lipotoxicity in the kidney. J Am Soc Nephrol 2017;28(5):1534–51.

46. Engin AB. What is lipotoxicity? Adv Exp Med Biol 2017;960:197–220.

47. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. Cell Death Differ 2015;22(3):377–88.

48. Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, Nojima A, Nabetani A, Oike Y, Matsubara H, Ishikawa F, Komuro I. A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat Med 2009;15(9):1082–7.

49. Krstic J, Galhuber M, Schulz TJ, Schupp M, Prokesch A. p53 as a dichotomous regulator of liver disease: the dose makes the medicine. Int J Mol Sci 2018;19(3):921.

50. Quiñones M, Al-Massadi O, Folgueira C, Bremser S, Gallego R, Torres-Leal L, Haddad-Tóvolli R, García-Caceres C, Hernandez-Bautista R, Lam BYH, Beiroa D, Sanchez-Rebordelo E, Senra A, Malagon JA, Valerio P, Fondevila MF, Fernø J, Malagon MM, Contreras R, Pfluger P, Brüning JC, Yeo G, Tschöp M, Diéguez C, López M, Claret M, Kloppenburg P, Sabio G,

Nogueiras R. p53 in AgRP neurons is required for protection against diet-induced obesity via JNK1. Nat Commun 2018;9(1):3432.

FIGURE LEGENDS

Figure 1: The inflammatory cytokine, IL-6 regulates autophagy through the non-receptor tyrosine kinase Fyn in HK-2 cells. **(A)** 2 h after serum starvation, HK-2 cells were incubated with indicated compounds for 30 min (TGF-β 2 ng/mL, IL-1β 10 ng/mL, IL-6 20 ng/mL, IL-18 10 ng/mL). Western blotting was performed with the indicated antibodies. These are representative images from three independent experiments. **(B)** After HK-2 cells were transfected with either non-target or Fyn siRNA for 48 h, cells were incubated with 20 ng/mL IL-6 for 30 min. Western blotting was performed with the indicated antibodies. These are representative images of three independent experiments. **(C)** HK-2 cells transfected with either non-target or Fyn siRNA for 48 h were harvested, followed by western blotting with the indicated antibodies. These are representative images of three independent experiments. **(D)** p62 expression was normalized to GAPDH. **(E)** After HK-2 cells were transfected with either non-target or Fyn siRNA for 48 h, cells were incubated with NH_4Cl and Leupeptin for 2 h. Western blotting was performed with the indicated antibodies. These are representative images of four independent experiments. **(F)** Signal quantifications of expression levels of LC3-II normalized to GAPDH. **(G)** After HK-2 cells were transfected with either non-target or Fyn siRNA for 48 h, cells were incubated with NH_4Cl and Leupeptin for 2 h followed by 20 ng/mL IL-6 for 30 min. Immunoblots were performed with the indicated antibodies. These are representative images of

four independent experiments. **(H)** LC3-II expression was normalized to GAPDH. Data are shown as the mean ± SD, *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Figure 2: Tgm2 Y369 is phosphorylated by Fyn both *in vivo* and *in vitro*. **(A)** 48 h after HEK-293T were co-transfected with Flag-Tgm2 and Fyn-CA, immunoprecipitation was performed using the Flag antibody followed by western blotting. Cell lysates were immunoprecipitated with the Flag monoclonal antibody and immunoblotted with the indicated antibodies. These are representative images of three independent experiments. **(B)** Purified Human GST-Tgm2 and either mock or Flag-Fyn-CA were incubated with src kinase buffer supplemented with ATP at 30°C for 30 min. Western blotting was performed with the indicated antibodies. **(C)** 48 h after HEK-293T were co-transfected with Fyn-CA and Tgm2-WT or Tgm-Y369F, immunoprecipitation was performed using the Flag antibody followed by western blotting with the indicated antibodies. These are representative images of three independent experiments.

Figure 3: Phosphorylation of Tgm2 Y369 by Fyn regulates autophagic activity in renal tubular cells. **(A)** After HK-2 cells were transfected with either non-target or Tgm2 siRNA for 48 h, cells were harvested followed by western blotting with the indicated antibodies. These are representative images of three independent experiments. **(B)** p62 expression normalized to GAPDH. **(C)** After HK-2 cells were transfected with either non-target or Fyn siRNA for 48 h, cells were incubated with NH_4Cl and Leupeptin for 2 h. Western blotting was performed with the indicated antibodies. These are representative images of four independent experiments. **(D)** LC3-II expression was normalized to GAPDH. **(E)** After HK-2 cells were transfected with either mock or Tgm2 constructs for 48 h, cells were incubated with NH_4Cl and Leupeptin for 2 h.

Western blotting was performed with the indicated antibodies. These are representative images of four independent experiments. **(F)** LC3-II expression was normalized to GAPDH. **(G)** After HK-2 cells were transfected with either Tgm2-WT or Tgm2-Y369F for 48 h, cells were incubated with NH_4Cl and Leupeptin for 2 h. Western blotting was performed with the indicated antibodies. These are representative images of four independent experiments. **(H)** LC3-II expression was normalized to GAPDH. Data are shown as the mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 4: siRNA-mediated Tgm2 knockdown regulates p53 expression through autophagy. **(A)** Representative images of three independent experiments showing PTECs transfected with either non-target or Tgm2 siRNA for 48 h. Cells were harvested followed by western blotting with the indicated antibodies. **(B)** p53 expression levels normalized to GAPDH. **(C)** PTECs transfected with either non-target or Tgm2 siRNA for 48 h and incubated with NH_4Cl and Leupeptin for 2 h. Western blotting was performed with the indicated antibodies. Representative images of four independent experiments are shown. **(D)** p53 expression levels normalized to GAPDH. **(E)** After PTECs were transfected with either non-target or Tgm2 siRNA for 48 h, they were incubated with NH_4Cl and Leupeptin for 2 h. Western blotting was performed with the indicated antibodies. These are representative images of four independent experiments. **(F)** p53 expression levels normalized to GAPDH. Data are shown as the mean ± SD, $^{*}p < 0.05$, $^{\#\#}p < 0.01$, $^{***}p < 0.001$.

Figure 5: Fyn and Tgm2 are expressed in the renal proximal tubular epithelial cells *in vivo* and expression and phosphorylation of Tgm2 is upregulated in the kidneys of the high-fat diet (HFD) mice model. **(A)** Kidneys of either normal chow-fed (CD) or HFD mice were

removed followed by western blotting with the indicated antibodies. These are representative images of three independent experiments. **(B)** Fyn and Tgm2 expressions were normalized to GAPDH. **(C)** Tissue lysates from the kidneys of either CD or HFD mice were removed followed by immunoprecipitation with an anti-Tgm2 antibody and western blotting was performed with the indicated antibodies. These are representative images of three independent experiments. **(D)** 13 wks after C57BL/6J fed high fat diet mice were fasted for 16 hrs. Immunofluorescence was performed with the indicated antibodies (p62; green, AQP-1 (red) and DAPI (blue)). These are representative images of three independent experiments. **(E)** Quantification of p62 puncta in each cell. Data are representative of five experiments. **(F)** Kidneys of either C57BL/6J fed HFD for 13 weeks were fasted and removed followed by immunoblots with the indicated antibodies. These are representative images of three independent experiments. **(G)** p53 expression was normalized to GAPDH. **(H)** *p53* mRNA espression normalized to *Hrpt1* in the kidneys of HFD mice determined using qRT-PCR (n = 5 independent experiments). Data are shown as the mean ± SD, $^{***}p < 0.001$.

Figure 6: FynKO rescues STZ-induced DKD by inducing autophagy-mediated p53 degradation. **(A)** Representative images of DKD or WT mouse (66 weeks, 16 h fasting) kidneys showing p62 (Green), AQP1 (Red), and DAPI (Blue). **(B)** Quantification of p62 puncta in each cell described in (A). **(C)** Western blotting results showing kidney tissues of STZ-induced DKD Fyn KO or WT mice with the indicated antibodies. These are representative images of three independent experiments. **(D)** p53 expression was normalized to GAPDH. Data are shown as the mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$.