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ABCA1 deficiency-mediated glomerular cholesterol accumulation exacerbates glomerular endothelial injury and dysfunction in diabetic kidney disease

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Running title: ABCA1 deficiency exacerbates diabetic kidney injury

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Abstract

Background: Hyperglycemia and dyslipidemia are two major characteristics of diabetes. In this study, the effects of glomerular cholesterol accumulation primarily due to ABCA1 deficiency on glomerular endothelial injury in diabetic kidney disease (DKD) and the possible mechanisms were investigated.

Methods: The effects of ABCA1 deficiency on glomerular lipid deposition and kidney injury were examined in a type 2 diabetic mouse model with ABCA1 deficiency in glomerular endothelial cells (DM-ABCA1-/- mice) and human renal glomerular endothelial cells (HRGECs) cultured in high glucose and high cholesterol conditions, which simulated type 2 diabetes in vitro.

Results: ABCA1 deficiency in glomerular endothelial cells exacerbated renal lipid deposition and kidney injuries in type 2 diabetic mice and manifested as increased creatinine levels, more severe proteinuria, mesangial matrix expansion and fusion of foot processes, and more pronounced renal inflammatory injury and cell death. In HRGECs cultured under high glucose and high cholesterol conditions, ABCA1 deficiency increased the deposition of cellular cholesterol, contributed to inflammation and apoptosis, damaged the endothelial glycocalyx barrier, and induced endoplasmic reticulum stress (ERS). Conversely, ABCA1 overexpression enhancing cholesterol efflux or inhibition of ERS in vitro, significantly protected against glomerular endothelial injury stimulated by high glucose and high cholesterol.

Conclusions: These findings establish a pathogenic role of ABCA1 deficiency in glomerular endothelium injury and dysfunction and imply that ABCA1 may represent a potential effective therapeutic target for early diabetic kidney disease.

Keywords: ABCA1, glomerular endothelial cell, diabetic kidney disease, cholesterol efflux, endoplasmic reticulum stress

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Introduction

Diabetes mellitus (DM) is one of the major health issues worldwide and has increased at an alarming rate in the 21st century. It casts shadows on more than one-half a billion adults worldwide [1] and has become the major cause of end-stage renal disease (ESRD) [2]. Diabetic kidney disease (DKD), a diabetes-driven kidney injury, occurs in 30%~40% of diabetic individuals [3] and can progress to renal failure despite multifactorial interventions, including lifestyle and medications. Thus, there is an urgent need to identify additional strategies and new therapeutic targets to delay the progression of DKD.

The glomerular endothelium is a highly specialized structure consisting of glomerular endothelial cells (GECs) and an endothelial surface glycocalyx layer (SGL), which is rich in gelatinous glycoprotein and separates GECs from the blood. [4] The glycocalyx layer carrying a negative charge can effectively block the filtration of negatively charged plasma proteins, especially albumin [5]. In addition, the glycocalyx layer constitutes the first layer of the glomerular filtration barrier (GFB) and is thus first affected by metabolic and hemodynamic alterations, and maintaining the integrity of its structure is essential to prevent the development of albuminuria in early diabetes [6]. Several recent studies have indicated that dysfunction of the glomerular endothelium may represent a critical deleterious hallmark in early DKD that is related to increased GFB permeability and albuminuria in DM patients [7, 8]. However, the pathogenesis of glomerular endothelial dysfunction in this disease remains obscure.

One proposed mechanism is glucolipotoxicity, in which toxic sugars and lipids accumulate, leading to endothelial cell inflammation and apoptosis [9-11]. Saturated free fatty acids (FFAs), such as palmitate, can induce endothelial cell dysfunction and death [12, 13], but the role of cholesterol accumulation in GEC injury in DKD has received little attention. ATP-binding cassette A1 (ABCA1) is a plasma membrane protein that mediates a crucial and critical step in the process of cholesterol efflux [14].

Emerging evidence has shown that ABCA1-mediated cholesterol accumulation is related to several kidney diseases, such as focal segmental glomerulosclerosis (FSGS) and DKD[15, 16]. This information led us to hypothesize that ABCA1 is very important for renal cholesterol homeostasis and that its dysfunction could aggravate renal injury. However, the underlying mechanism remains unclear.

Therefore, the aim of this study was to investigate the effects of GEC-ABCA1 deficiency-mediated cholesterol efflux dysfunction on early renal damage in type 2 diabetic mice and to explore its possible mechanisms.

Methods

Generation of diabetic endothelial ABCA1-deficient mice

Animal studies were performed according to the guidelines of and approved by the Sichuan University Animal Ethics Committee. Mice were housed at the Laboratory Animal Center of West China Hospital and provided free access to chow and water. ABCA1$^{flox/flox}$ mice on a B6.129S6-Abca1tm1Jp/J background (Jackson Laboratories)[17] were crossed with Tie2-Cre mice on a B6.Cg-Tg (Tek-cre)1Ywa/J background (Jackson Laboratories)[18] to generate GEC-specific ABCA1 knockout mice (ABCA1-/-), which were based on the Cre/loxP recombinase system. Breeding and genotyping by polymerase chain reaction (PCR) detection using genomic DNA isolated from tail tissue were conducted in accordance with standard procedures[17].

The mice were divided into the following groups (n = 6 mice for each group): (1) ABCA1$^{flox/flox}$ mice (WT group); (2) ABCA1$^{flox/flox}$, Tie2^{+} mice (ABCA1-/- group); (3) diabetic ABCA1$^{flox/flox}$ mice (DM-WT group); and (4) diabetic ABCA1$^{flox/flox}$, Tie2^{+} mice (DM-ABCA1-/- group). To induce type 2 diabetes, DM-WT and DM-ABCA1-/- mice were fed a high-fat and sucrose diet (66.7% regular diet, 10.0% lard, 20% sucrose, 3.0% cholesterol, and 0.3% sodium cholate; Chengdu Dossy Experiment Animals Co., LTD) from 8 weeks of age to the end of the experiment. In addition, DM-WT and DM-ABCA1-/- mice were injected with five low-dose streptozotocin

(STZ, 55 mg/kg/d, Sigma–Aldrich) in citrate buffer (pH 4.0) at the age of 16 weeks old, as described previously[10]. The citrate vehicle-injected WT and ABCA1-/- groups served as the respective controls. Diabetes was defined as fasting blood glucose > 16.7 mmol/L for 3 consecutive days. All mice were sacrificed at 8 weeks after diabetes induction.

Biochemical measurements in animal studies

Prior to euthanasia, random blood glucose was measured using a glucose analyzer (Roche). Blood urea nitrogen, serum creatinine, serum total cholesterol, LDL, HDL, and serum triglycerides were measured on a biochemistry autoanalyzer (BS-240VET, Mindray Co., Ltd.). Urine albumin was detected using a commercial kit (Mouse Albumin ELISA Kit Bethyl E99-134, BioVision). Urine creatinine levels were assessed in the same samples using a Creatinine Colorimetric Assay Kit (BioVision) according to the manufacturer's protocol.

Histology and immunofluorescence staining in kidney cortex sections

Paraffin sections (4 μm) of mouse kidneys stained with hematoxylin-eosin (HE), periodic acid–Schiff (PAS), and Masson's trichome stain were used for evaluation of kidney histology. Quantification of mesangial expansion was performed using Image-Pro Plus version 6.0 software as previously described[10].

Frozen or paraffin sections of mouse kidney were stained with the following primary antibodies: mouse anti-CHOP (1:250, Abcam), mouse anti-ATF6 (1:200, HuaBio), rabbit anti-ZO-1 (1:500, Thermo Fisher Scientific), mouse anti-ET-1 (1:200, Abcam), mouse anti-syndecan-1 (1:200, Abcam), mouse anti-IL-6 (1:200, HuaBio), and rabbit anti-TNF-α (1:200, HuaBio) along with the endothelial cell marker rat anti-CD31 (1:100, Santa Cruz Biotechnology).

To assess the glomerular endothelial surface glycocalyx, paraffin sections were stained with rhodamine-labeled wheat germ agglutinin lectin (WGA, VectorLabs) for 1 hour at room temperature after deparaffinization and costained with cell nuclei using 4',6-diamidino-2-phenylindole (DAPI, Sigma).

Lipid staining in kidney cortex sections

Frozen sections of renal cortex were cut into 6 μm sections for oil red O staining to evaluate lipid accumulation. The procedure was conducted as previously described[19]. GECs grown on the glass slide were fixed in 4% paraformaldehyde for 30 min at 4 °C. Then, the slides were rinsed with 60% isopropanol for 30 s and incubated with mixed Oil Red O solution (Solarbio) for 15 min at room temperature. Then, slides were rinsed again with 60% isopropanol for 10-20 s and washed with ddH_2O. Finally, the sections were stained with hematoxylin for 1 min, washed with ddH_2O again and mounted.

Filipin staining (which stains free cholesterol) was performed according to the manufacturer's protocol, as previously reported[10]. Briefly, frozen sections of kidney tissue were washed in PBS, fixed with 4% paraformaldehyde for 30 min, washed with PBS thrice, and then incubated with 125 μg/ml filipin solution for 30 min at 37 °C. After washing in PBS, sections were viewed by confocal laser scanning microscopy.

Bodipy staining (which stains neutral lipids) was conducted on 6 μm-thick fixed frozen sections of mouse kidney. The procedure was performed as previously described. [15] The sections of kidney tissue were washed in PBS, incubated with 1 mg/ml BODIPY (Sigma) solution for 30 min at 37 °C and costained with cell nuclei using 4',6-diamidino-2-phenylindole (DAPI, Sigma).

Cholesterol extraction and measurement in cell and animal studies

Cholesterol was extracted from the renal cortex according to the manufacturer's protocol as previously described [20, 21]. Cholesterol content was measured using kits purchased from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China).

HRGEC culture and transfection of shRNA in cell studies

The human renal glomerular endothelial cell (HRGEC) line was purchased from BNCC (No. BNCC339278) and subcultured for no more than passage 10. Cells were cultured in DMEM (HyClone) containing 10% FBS (GIBCO) and 1% penicillin/streptomycin under permissive conditions at 37 °C in a 5% CO_2

atmosphere.[10] Cells were starved in DMEM-1% FBS for 12 h prior to treatment and then divided into the following groups: normal glucose (NG, 5.6 mmol/L), high glucose (HG, 35 mmol/L), high glucose plus cholesterol (HC, 100 μg/ml Ac-LDL, Yiyuan Biotechnologies), high glucose plus cholesterol and shRNA-ABCA1 (HCshA1), and high glucose plus cholesterol and shRNA-Con (HCshCO) for 24 h. In the intervention study, HRGECs were pretreated for 24 h with the ER stress inhibitor TUDCA (6 mM, CSNpharm, HCshA1+TUDCA) and the LXR agonist GW3965 (1 μM, CSNpharm HC+GW3965). After the 24-h treatment, 20 μg/ml human plasma apolipoprotein A-I (Merck & Co Inc.) was added to the cells for 6 h to promote cholesterol efflux[10].

For knockdown of ABCA1, lentiviral (RFP-shRNA) plasmids for hABCA1 and control shRNA were purchased from Vigene Biosciences[22]; HRGECs grown at 37 °C were transfected with the corresponding lentivirus and 10 μg/ml polybrene at a multiplicity of infection of 10 and selected for puromycin resistance. Stably transduced HRGECs were cultured for ≥5 days prior to experiments.

Apoptosis analysis in cell and animal studies

TUNEL staining was performed using the TMR Red In Situ Cell Death detection kit (Roche Diagnostics, Switzerland) following the manufacturer's instructions[23] in a mouse model. Briefly, the frozen renal sections were washed thrice with PBS for 5 minutes each, incubated with proteinase K (20 μg/ml) for 6 min at room temperature, washed with PBS, and then treated with TUNEL reaction mixture for 45 min at 37 °C. A FITC Annexin V Apoptosis Detection Kit (BD Co Ltd) was used to examine the apoptosis rate of cultured HRGECs based on the manufacturer's protocols for the in vitro study. The number of cells labeled with Annexin V-FITC and propidium iodide was quantified using a flow cytometer (BD Biosciences).

Real-time PCR in animal studies

Total RNA was isolated from the isolated renal cortex by using an RNeasy kit (Promega Biotech Co., Ltd. Beijing) and reverse-transcribed DNase-treated RNA

using Superscript II (Vazyme Biotech). RNA concentrations were quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). Real-time PCR was performed using SYBR Green PCR Master Mix (Vazyme Biotech) in a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA)[24]. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The following primer sequences were used: mABCA1F, GGCAACAAACGAAAGCTC; mABCA1R, CTTAGGGCACAATTCCA CA; m18sF, AGGG AGAG CGGG TAAG AGA; m18sR, GGAC AGGA CTAG GCGG AACA.

Western blot analysis in cell and animal studies

Total protein was extracted from the renal cortex or HRGECs with RIPA buffer and then quantified and subjected to western blot analysis using CHOP, ATF6, ATF4, XBP1s, BIP, IL-6, TNF-α, NF-κB, cleaved caspase-3, Bax, Bcl-2, ZO-1, ET-1, syndecan-1 and glypican-1 antibodies. Finally, the specific protein bands were visualized using a hypersensitive ECL chemiluminescence solution (Beijing 4A Biotech Co., Ltd) and a Fusion FX7 System (Vilber).

Statistical analyses

The results are expressed as the mean±SEM. Two groups were compared by 2-tailed, unpaired t tests. Multiple groups were compared by ANOVA or the Kruskal–Wallis H test, as appropriate. Analyses were performed with SPSS software (version 26, IBM Corp., NY, USA), and all figures were drawn using GraphPad Prism 7.0 software. $p < 0.05$ was considered a significant difference.

Results

1. Generation of type 2 diabetic mice with ABCA1 deficiency in GEC

To investigate the role of ABCA1 in glomerular endothelial cell injury, we generated mice lacking GEC-ABCA1(ABCA1-/-) by crossing loxP-flanked ABCA1 mice ($ABCA1^{Flox/Flox}$) with mice expressing Cre recombinase under the control of the receptor tyrosine kinase Tek (Tie2) promoter/enhancer, which has been shown to

provide uniform expression in vascular endothelial cells. PCR amplification across the ABCA1 locus from tail tissue of ABCA1-/- mice and WT controls ($ABCA1^{Flox/Flox}$) indicated efficient Cre-mediated recombination (Fig. 1A, B). ABCA1 mRNA expression levels in the isolated renal cortex of ABCA1-/- mice was markedly reduced compared with that in WT mice, as was ABCA1 expression in DM-WT mice and DM-ABCA1-/- mice (Fig. 1C). The residual ABCA1 expression evident in the renal cortex from ABCA1-/- mice suggested the expression of ABCA1 in non–GECs. ABCA1 mRNA levels were also reduced in DM mice, indicating that diabetes could downregulate ABCA1 expression in renal tissue. ABCA1-/-mice had no significant differences in body weight at 8 weeks of age (Fig. 1D) and had normal fasting blood sugar (FBS) (Fig. 1E) and urine albumin creatinine ratio (UACR) (Fig. 1F).

Type 2 diabetes was induced by injections of low-dose streptozotocin (STZ) at 16 weeks of age and feeding a high-fat and sucrose diet to DM-WT and DM-ABCA1-/-mice as described previously[10]. Citrate vehicle-injected WT and ABCA1-/- mice served as the respective controls. Type 2 diabetes induction resulted in significantly higher levels of random blood glucose, HbA1c, LDL-C and UACR and lower levels of body weight, kidney weight/body weight and serum insulin in both DM-WT and DM-ABCA1-/-mice compared with the respective nondiabetic control mice (Fig. 2 and Supplemental Table 1). In addition, DM-WT mice had higher levels of total cholesterol and HDL-C than WT mice, whereas these levels appeared to be decreased in DM-ABCA1-/- mice mainly due to ABCA1 knockout in GECs. Furthermore, serum creatinine levels were also significantly increased in DM-ABCA1-/- mice compared with DM-WT mice, and the blood urea nitrogen level was higher in DM-ABCA1-/- mice than in WT mice. However, we did not find a difference in serum triglyceride levels among the four mouse groups. The above results suggested that the mouse model of type 2 diabetic kidney disease was successfully constructed.

2. ABCA1 deficiency increased albuminuria and aggravated mesangial matrix

accumulation and tubular injury in early DKD

We previously reported that ABCA1 expression was significantly decreased in the kidneys of diabetic apoE-/- mice compared with nondiabetic apoE-/- mice[10]. In addition, the reduction of ABCA1 expression in kidneys occurs in DM without renal injury[25]. Therefore, we next examined whether ABCA1 deficiency resulted in DKD progression using a T2DM-ABCA1-/- mouse model. As anticipated, further exacerbations of all indicators were observed in DM-ABCA1-/- versus DM-WT mice. The biochemical analysis (Fig. 2 and Supplemental Table 1) showed that ABCA1 deficiency deteriorated random blood glucose, HbA1c, LDL, serum creatinine and UACR in DM mice. In addition, ABCA1-deficient mice (ABCA1-/- and DM-ABCA1-/- mice) had significantly lower plasma HDL levels than WT mice, further confirming that the ABCA1 deletion mouse model was successfully constructed. HE, PAS and Masson staining of the kidneys of the four mouse groups indicated that GEC-ABCA1 deficiency worsened glomerular basement membrane (GBM) thickening and mesangial expansion in diabetic mice (Fig. 3A, B). In addition, as shown in Figure 4, A–B, ABCA1 deficiency promoted glomerular endothelial proliferation and endoplasmic reticulum injury, GBM thickening and foot process fusions of podocytes in DM-ABCA1-/- mice. Furthermore, immunohistochemical (IHC) staining of kidney tissues showed stronger staining of NGAL, a marker of renal tubular injury, in DM-WT mice than in WT mice, and the strongest staining was noted in DM-ABCA1-/- mice among the four mouse groups (Fig. 4C-D).

3. ABCA1 deficiency exacerbated lipid accumulation and induced endothelial glycocalyx layer injury in early DKD

Lipid staining, including oil red O staining, filipin staining and BODIPY staining, and quantitative analysis of cholesterol revealed greater accumulation of lipid droplets and free cholesterol in the glomeruli of diabetic ABCA1-/-mice than in DM-WT mice and controls (Fig. 5A-C, G). The localization of CD31 and lipid staining showed that ABCA1 deficiency could aggravate the degree of lipid deposition in glomerular

endothelial cells in diabetic mice. (Fig. 5D-F)

The results of rhodamine-wheat germ agglutinin (WGA) staining revealed gradually reduced staining of glycocalyx thickness in DM-WT mice compared with controls, which was worsened by deletion of GEC-ABCA1 in DM-ABCA1-/- mice (Fig. 6A, B). Immunofluorescence and western blot also showed that the expression levels of the tight junction-associated protein ZO-1 as well as glycocalyx core proteins syndecan-1 and glypican-1 in GECs were decreased in DM-WT mice, whereas ABCA1 deficiency exacerbated the above alterations in early DKD-ABCA1-/- mice. (Fig. 6 C-H). Additionally, GEC-ABCA1 knockout also caused an increase in ET-1 expression in DM-ABCA1-/- mice compared with DM-WT mice (Fig. 6E-H).

4. ABCA1 deficiency exacerbated glomerular endothelial inflammation and apoptosis in early DKD

We also detected the levels of NK-κB, IL-6, TNF-α and apoptosis in the kidney cortex of mice. Immunofluorescence results indicated significantly increased staining of IL-6 and TNF-α, which serve as markers of inflammation. This staining overlapped with CD31 in the glomeruli of both DM-WT and DM-ABCA1-/- mice. More intense staining of IL-6 and TNF-α was detected in DM-ABCA1-/- GECs than in DM-WT GECs (Fig. 7A-D). Similarly, western blot assays and real-time PCR also indicated that increased NK-κB, TNF-α, IL-6, cleaved aspase-3 and Bax levels were observed in the renal cortex of DM-ABCA1-/- mice compared with DM-WT mice (Fig. 7E-G, Fig. 8C-D), indicating that ABCA1 deficiency may worsen the extent of inflammation and apoptosis in early diabetes-driven GECs injury. Evaluation of apoptotic cells using terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) labeling further confirmed these observations (Fig. 8A-B).

5. ABCA1 deficiency-mediated cholesterol accumulation induced ERS in early DKD and HRGECs

Previous studies indicated that cholesterol accumulation contributes to

endoplasmic reticulum stress (ERS) activation and apoptosis[26]. Therefore, we investigated the levels of ERS-related proteins in ABCA1-deficient diabetic mice. Double immunostaining results showed that many CHOP-expressing cells in glomeruli were located in free-cholesterol-rich areas in DM-ABCA1-/- mice (Fig. 9A), suggesting that ABCA1 deficiency-mediated perturbed cellular cholesterol might activate ERS. In addition, we detected significantly increased staining of activating transcription factor-6 (ATF6) and C/EBP homologous protein (CHOP), markers of ERS that overlapped with CD31 in the glomeruli of both DM-WT and DM-ABCA1-/- mice. More intense ATF6 and CHOP staining was detected in DM-ABCA1-/- GECs than in DM-WT GECs (Fig. 9B-E). Consistent with this finding, the western blot results also showed increased expression levels of ERS-related proteins, including binding protein (BIP), ATF6, X-box binding protein-1 (XBP1s), activating transcription factor-4 (ATF4) and CHOP, in the renal cortex of DM-ABCA1-/- mice compared with DM-WT mice (Fig. 9 F, G). Furthermore, the results of western blot in vitro also demonstrated that the levels of cell cholesterol and ERS-related proteins of HRGECs increased under high glucose and cholesterol conditions, and this change was aggravated by silencing ABCA1 using shRNA (Fig. 10 and Supplemental Fig. 1), suggesting that ABCA1 deficiency-mediated cholesterol accumulation may induce ERS to initiate the related injuries.

6. ABCA1 mediated the NF-κB and caspase 3 pathways through ERS in HRGECs

To explore the mechanism by which ABCA1 deficiency exacerbated GECs inflammatory injury and apoptosis under type 2 diabetic conditions, we next conducted in vitro experiments using shRNA-slicing ABCA1 (Supplemental Fig. 1), GW3965 (which induces the overexpression of ABCA1, Supplemental Fig. 2) and tauroursodeoxycholate sodium (TUDCA, an ERS inhibitor, Supplemental Fig. 3) interventions. The results indicated that silencing ABCA1 significantly upregulated ERS-related proteins, including BIP, ATF6, XBP1s, ATF4 and CHOP; increased

NK-κB, IL-6, TNF-α (Fig. 10, Fig. 11A-B) and ET-1 expression (Fig. 11 C-D); increased apoptosis levels (Fig. 12); and decreased syndecan-1, glypican-1 and ZO-1 expression (Fig. 11 C-D) of HRGECs under high glucose and cholesterol conditions. This effect was attenuated by treatment with the ERS inhibitor TUDCA. These findings indicated the involvement of ERS in ABCA1 deficiency-induced NK-κB signaling and apoptosis.

To further confirm the effects of ABCA1 on HRGECs, we examined the influence of the liver X receptor agonist GW3965, which effectively promotes ABCA1 expression, on HRGECs. The results of cholesterol quantitative analysis showed that GW3965 treatment decreased the cell cholesterol content and increased ABCA1-dependent cholesterol efflux (Supplemental Fig. 4). Western blot assays indicated that GW3965 downregulated ERS-related proteins, ameliorated inflammatory damage and apoptosis, and increased the levels of glycocalyx protein expression in HRGECs under high glucose and cholesterol stimulation (Figs. 10-12). The above results confirmed that ABCA1 deficiency mediates cholesterol deposition through excessive activation of ERS to aggravate diabetes-induced kidney injury.

Discussion

ABCA1 plays an important role in DKD

With the introduction of sodium–glucose cotransporter 2 (SGLT2) inhibitors and glucagon-like peptide 1 (GLP1) agonists in recent years, several randomized trials have indicated that the improvement of dyslipidemia is one of the common mechanisms by which SGLT2 and GLP1 exert renoprotective effects, and increasing attention has been given to the role of abnormal lipid metabolism in DKD progression[27-29]. We and others have identified that glomerular accumulation of lipids appears in DKD[10, 15], focal segmental glomerulosclerosis (FSGS)[30], and hypertensive nephropathy[31]. In addition, glomerular accumulation of lipids is significantly associated with a reduction in glomerular ABCA1 expression and renal

dysfunction[32]. Furthermore, Fornoni et al.[25] found that the change in ABCA1 expression in podocytes incubated with DKD patient sera might serve as the trigger from nonprogressive to progressive DKD. The above findings provide important clinical evidence for the role of ABCA1 in DKD progression.

ABCA1 deficiency exacerbates glomerular endothelial cell injury in DKD

We and others have demonstrated that pharmacological intervention-induced or genetic ABCA1 overexpression could ameliorate lipotoxicity-induced renal injury in models of DKD[10, 30, 33]. In addition, podocyte-specific ABCA1 deficiency could increase the susceptibility of mice to diabetes-induced injury through cardiolipin-driven mitochondrial dysfunction [25]. However, the possible role of ABCA1 in glomerular endothelial cell injury in DKD is currently unknown. Therefore, we generated a type 2 diabetic mouse model with GEC-specific deletion of ABCA1 (DM-ABCA1-/-) to investigate the possible role of ABCA1 in GECs injury in DKD. The results showed that blood glucose, HbA1c, LDL and TG levels were significantly higher in DM-ABCA1-/- mice than in their controls, whereas plasma HDL levels and ABCA1 expression in the renal cortex were extremely low, suggesting that the proposed mouse model was successfully constructed.

Our results also demonstrated that GEC-ABCA1 deficiency aggravated the renal phenotype of diabetic mice, which manifested as increased creatinine levels, more severe proteinuria, mesangial matrix expansion, fusion of foot processes and tubular injury, and more pronounced renal inflammatory injury and cell death. It should be mentioned that chow-fed ABCA1-/-mice did not exhibit significant differences from WT controls, suggesting that GEC-ABCA1 deficiency alone was not sufficient to cause renal injury but rather rendered the GECs susceptible to injury in DKD. This possibility was consistent with the findings of podocyte-ABCA1 deficiency in DKD[25], and the underlying mechanisms remain to be further established. In addition, decreased ABCA1 expression in the renal cortex was not a specific finding for STZ-T2DM mice, as similar observations were also reported in ob/ob-driven T2DM

mice and STZ-T1DM mice.

To define the effect of ABCA1 deficiency on the glomerular endothelial barrier in DM mice, we investigated the levels of core glycoproteins of the glycocalyx layer, ZO-1 and ET-1. We demonstrated that ABCA1 deficiency could aggravate glomerular endothelial damage in diabetic mice, which was associated with worsened albuminuria. ET-1 is a strong vasoconstrictor and is associated with a variety of cardiovascular and renal diseases. ET-1 is secreted by glomerular endothelial cells and acts by binding to endothelin receptors (ETRs).[34, 35] Previous studies have shown that ET-1 can activate NF-κB, MAPK and other signaling pathways, leading to mesangial matrix expansion, glomerulosclerosis, and podocyte injury[36]. However, blockade of the ET/ETR axis using atrasentan (ETR antagonist) subsequently reduces proteinuria levels, restores the endothelial glycocalyx barrier, and exerts renoprotective effects in type 2 diabetic mice [37]. Therefore, it seems possible that in a type 2 diabetic context, GEC injury caused by ABCA1 deficiency might aggravate glycocalyx, podocyte and mesangial damage through the ET-1/ETR signaling pathways. In addition, we also identified that loss of GEC-ABCA1 exacerbates inflammatory and apoptotic injuries both in vitro and in vivo. These data suggested that the inflammatory and apoptotic response aggravated by ABCA1 deficiency contributed to glomerular endothelial dysfunction and glycocalyx damage in diabetes.

To assess how ABCA1 deficiency renders GECs susceptible to worsened DKD, we examined the lipid content and ERS-related protein levels in vivo models and in vitro shABCA1-HRGECs. We observed increased cellular lipid content and increased ERS-related proteins in DM-ABCA1-/-mice compared with DM-WT mice, and a similar finding was also demonstrated in vitro in shABCA1-HRGECs compared with shCO-HRGECs. Cellular cholesterol levels are closely related to endothelial cell dysfunction. Chronic inflammation, cholesterol deposition, foam cell formation and ultimately endothelial dysfunction are typical features of atherosclerosis [38]. Studies have confirmed that cholesterol accumulation through the induction of ERS activation

results in macrophage apoptosis and is one of the important mechanisms to exacerbate atherosclerosis progression[39], and a similar evolution process may exist in GECs injury in DKD. In support of this hypothesis, we used multiple lipid staining colocalization with CD31 immunofluorescence in renal sections of DM-ABCA1-/- mice, revealing that a large amount of lipid droplets with significant levels of free cholesterol accumulated in GECs. Moreover, many CHOP-expressing cells in glomeruli are located in free-cholesterol-rich areas. Consistent with these observations, ABCA1 deficiency-mediated perturbation of cellular cholesterol induced ERS in vivo. More importantly, silencing ABCA1 aggravated ERS induced by high glucose and cholesterol stimulation, whereas inflammation, apoptosis and glycocalyx damage were ameliorated by intervention with TUDCA or GW3965 in vitro. The above results confirmed that ABCA1 deficiency mediated cholesterol deposition through excessive activation of ERS to aggravate diabetes-induced kidney injury.

ESR and ABCA1 in DKD

The endoplasmic reticulum is an important organelle for cholesterol metabolism and has become an important target for "cholesterol toxicity"[40]. ERS is closely related to several diseases associated with disturbed cholesterol metabolism, such as atherosclerosis, obesity, T2DM and DKD[41, 42]. ER stress mutually promotes the inflammatory response of metabolic diseases, forming a vicious cycle, and can also lead to apoptosis through multiple pathways, ultimately accelerating disease progression. ERS mainly activates downstream effector molecules through three signaling pathways, including PERK-eIF2α-ATF4-CHOP, IRE1α-XBP1s and ATF6[43]. Studies have confirmed that ERS initiates the inflammatory response and induces apoptosis by triggering the CHOP pathway, the caspase pathway, and the c-JNK pathway via several signaling pathways, such as NF-κB and JNK/AP1[44, 45]. The present study indicated that ERS mediated by ABCA1 deficiency could induce NF-κB. CHOP and caspase-3 signaling to accelerate inflammation and cell death in GECs of DKD.

Clinical translation for ABCA1 agonists

Although ABCA1 has been shown to be very important in DKD progression, there are currently no clinically available direct agonists for ABCA1. Substantial research efforts to develop ABCA1 agonists are ongoing. A recently published study[46] demonstrated that novel compounds targeting OSBPL7 (oxysterol binding protein 7) increase ABCA1-mediated cholesterol efflux, preserving renal function in Alport and adriamycin-induced nephropathy models. Wang et al.[47] found that erythrodiol, an olive oil constituent, specifically increases the half-life of ABCA1 in macrophages to enhance cholesterol efflux. Furthermore, previous studies also indicated that multiple pharmacological agents (GLP-1R agonists, Tangshen formula and visfatin, etc.) treatment to upregulate ABCA1 is sufficient to attenuate kidney injury in diabetic mice [10, 48, 49]. Similarly, agonists of liver X-receptors (LXR), such as GW3965, DMHCA, and T091317[50, 51], can also significantly increase ABCA1 expression and promote cholesterol efflux. In diabetic mice, GW3965 reduced proteinuria, renal inflammation, and cholesterol deposition by increasing ABCA1 expression [52]. We also demonstrated that GW3965 improved ABCA1-dependent cholesterol efflux, reduced cellular cholesterol content, and ameliorated lipotoxicity-induced GECs injury in vitro. These data further validate our previously published findings [10] that ABCA1 mightrepresent a therapeutic target for the improvement of DKD prognosis.

The strengths of this study are as follows. First, to our knowledge, this was the first study to investigate the effects of ABCA1 deficiency on glomerular endothelial injury in diabetes. Second, we generated a type 2 diabetic mouse model with ABCA1 deficiency in glomerular endothelial cells (DM-ABCA1-/-mice) to investigate the specific molecular changes occurring in glomerular endothelial cells. The limitations of this study are also worth mentioning. First, we could not exclude the effects of insulin on the phenomena observed in diabetic mice (especially on apoptosis).

Because there are currently no commercially available direct agonists for ABCA1 and

its molecular weight is too large, technical difficulties are experienced when increasing its expression directly by lentivirus. Therefore, the agonists (GW3965 in this study) of their transcription factors (such as LXR) are used to indirectly achieve their overexpression effect, which may interfere to some extent with the outcome.

In summary, ABCA1 deficiency contributed to glomerular endothelial injury and dysfunction in early DKD through the inflammation and apoptosis induced by ERS. The potential mechanism was associated with ABCA1 deficiency-mediated glomerular cholesterol accumulation in GECs, which finally resulted in functional and tissue damage to the glomerular endothelium, increased creatinine levels, more severe proteinuria and DKD progression (Fig. 13). These findings suggest that ABCA1 may represent a potential effective therapeutic target for early DKD progression, and promoting endogenous homeostasis of cholesterol seems to represent an effective therapeutic paradigm to combat DKD.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

FL and JLZ designed and conducted the study. YCW, RZ, and JZ participated in the data collection and analysis. The manuscript was prepared by JLZ and YCW. All authors participated in discussions about the manuscript and approved the final version.

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Figure legend.

Fig. 1 Generation of mice lacking ABCA1 in GECs (ABCA1–/–). (A) Schematic representation of recombination between loxP sites and ABCA1 alleles. loxP sites were indicated as white triangles, and PCR primer positions were indicated by arrows. **(B)** The agarose gel electropherograms showed the results of PCR amplification using primers from genomic DNA isolated from tail tissue of 2 weeks old wild type ($ABCA1^{+/+}$), $ABCA1^{Flox/Flox}$ and ABCA1-/- mice. **(C)** ABCA1 mRNA expression in renal cortex from four group mice (n= 3 per group). **(D)** Average body weight of WT and ABCA1-/- mice (n = 6). **(E)** Average fast blood sugar of WT and ABCA1-/- mice (n = 6). **(F)** Average urinary albumin creatinine ratio (UACR) of WT and ABCA1-/- mice (n = 6). *$p < 0.05$ (two-tailed Student's t-test), n.d. not detected, NS: no significance.

Fig.2 Type 2 diabetes induction resulted in significant hyperglycaemia, dyslipidemia, and albuminuria in early T2DKD. (A) Body weight**, (B)** the ratio of kidney weight to body weight**, (C)** random blood glucose levels, **(D)** glycated hemoglobin, **(E)** total serum cholesterol, **(F)** serum LDL, **(G)** serum HDL, **(H)** serum triglyceride, **(I)** blood urea nitrogen, **(J)** serum creatinine, **(H)** the urinary albumin/creatinine ratio in mice were measured. (Mean ± SEM, n=6). *p<0.05 and **p<0.01 vs. WT group; #p<0.05 and ##p<0.01 vs. DM-WT group.

Fig.3 Histological analysis of glomerular injury in mice. (A) Hematoxylin-eosin (HE) staining, periodic acid-Schiff (PAS) staining and MASSON staining of kidney sections showed glomerular or cortical interstitial morphological changes**. (B)**The bar graph showed quantitative

analysis of the PAS-positive area, and MASSON-positive area. Results are expressed as mean

± SEM (n = 6); ***p<0.001 vs. WT group; ###p<0.001 vs. DM-WT group.

Fig.4 Histological analysis of glomerular injury in mice. (A) The ultramicrostructure of glomerular filtration barrier was observed by transmission electron microscopy (EM). Black arrow showed the fusion of podocytic process in ABCA1 deficiency diabetic glomeruli. Red arrows showed the normal structure of the endoplasmic reticulum in GECs of WT and ABCA1-/- mice. Yellow arrow indicated the swollen and shortened change of endoplasmic reticulum in glomerular endothelial cells of DM-WT mice. Green arrow indicated significant glomerular endothelial cell proliferation and chromatin margination of nuclei, suggesting that ABCA1 deficiency aggravated endothelial cell injury in DM-ABCA1-/- mice. (**B**) The ultramicrostructure of podocytic process was detected by scanning EM. White arrows showed the fusion of podocytic process in diabetic glomeruli. Scale Bar=1μm. **(C-D)** Immunohistochemistry analysis of NGAL expression in kidneys from WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice. (Scale bar = 50μm, ***p<0.001, n.d. not detected. n =3 mice for each group).

Fig.5 Increased lipid accumulation in diabetic mice. (A) Oil Red O staining **(B)** Bodipy staining **(C)** Filipin cholesterol staining in renal tissues from WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice. The double immunofluorescence (IF) staining of endothelial cell marker CD31 with **(D)** Oil Red O **(E)** Bodipy and **(F)** Filipin staining in DM-ABCA1-/- mice. Representative images are shown. Scale bars = 10 μm. **(G)** The cholesterol content in renal cortex from WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice.

Fig.6 ABCA1 deficiency exacerbated endothelial glycocalyx injury of diabetic mice. (A-B) Glomerular endothelial glycocalyx layer was determined by staining with WGA in WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice, and semiquantitative analysis of the WGA intensity per glomerular area. **(C-D)** Representative images of syndecan-1 with CD31 immunofluorescence in glomeruli of WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice, and

semiquantitative analysis of the syndecan-1intensity per CD31 positive area. **(E-F)** Representative images of tight junction protein ZO-1 with endothelin ET-1 immunofluorescence in glomeruli of WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice, and semiquantitative analysis of the ET-1 intensity per ZO-1 positive area. (**G-H**) Western blot analyses of ET-1, ZO-1, syndecan-1 and glypican-1 expression in renal cortex from WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice. Scale bar=10μm, n=3 mice for each group. Results are expressed as mean ± SEM (n = 6); *p<0.05 and **p<0.01 vs. WT group; #p<0.05 and ##p<0.01 vs. DM-WT group.

Fig.7 TNF-α and IL-6 expressions in the glomeruli of mice. Representative images of **(A-B)** TNF-α and **(C-D)** IL-6 with CD31 immunofluorescence in glomeruli of WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice, and semiquantitative analysis of the TNF-α intensity per CD31positive area and IL-6 intensity per CD31positive area (scale bar = 10 μm, n=3 mice for each group). **(E-F)** Western blot analyses of NF-κB, IL-6 and TNF-α expression in renal cortex from WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice. (**G**) The mRNA expressions of NF-κB, IL-6 and TNF-α in renal cortex from WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice. Results are expressed as mean ± SEM (n = 6); *p<0.05 and **p<0.01 vs. WT group; #p<0.05 and ##p<0.01 vs. DM-WT group.

Fig.8 GEC-ABCA1 deficiency worsen the levels of cell apoptosis in glomeruli of diabetic mice. Determination of apoptosis in glomeruli by TUNEL assay. (**A**) Representative fluorescent images of CD31 and TUNEL-positive cells in mouse glomeruli. White arrows showed TUNEL+ cells in DM-ABCA1-/- glomeruli. Scale Bar=10μm. (**B**) Quantification of TUNEL+ cells per glomerular cross section. Results are Mean ± SEM of at least 20 glomeruli evaluated per group (n=3 mice per group, ***p<0.001 vs. WT group; ###p<0.001 vs. DM-WT group). **(C)** The proteins expression of cleaved Caspase-3, Bax and Bcl-2 of renal cortex form WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice were assessed by western blot . **(D)**The densitometry analyses western blots are shown. Values are expressed as means

±SEM. *P <0.05, ***P <0.001, n = 3.

Fig. 9 ABCA1 deficiency-mediated cholesterol accumulation in glomeruli induced ERS activation of diabetic mice. (A) Representative images of filipin (which labeled free-cholesterol) with CHOP immunofluorescence in glomeruli of DM-ABCA1-/- mice. (**B, D**) Representative images of ATF6 and CHOP with CD31 immunofluorescence in glomeruli of WT, ABCA1-/-, DM-WT and DM-ABCA1-/- mice. (**C, E**) The semiquantitative analysis of the ATF6 intensity per CD31 positive area and CHOP intensity per CD31 positive area. **(F)** The expression of ERS-related proteins including BIP, ATF6, XBP1s, ATF4 and CHOP were assessed by western blot. (**G**)The densitometry analyses western blots are shown. Values are expressed as means ±SEM. *P <0.05, **P <0.01, ***P <0.001, n = 3.

Fig. 10 ABCA1 deficiency induced ERS activation of HRGECs under high glucose and cholesterol. (A) The expression of ERS related proteins including BIP, ATF6, XBP1s, ATF4 and CHOP were assessed by western blot. (**B**)The densitometry analyses western blots are shown. Values are expressed as means ±SEM. *P <0.05, **P <0.01, ***P <0.001, n = 3.

Fig.11 ABCA1 deficiency exacerbated HRGECs injury. (A) The proteins expression of NK-κB, IL-6 and TNF-α were assessed by western blot. (**B**)The densitometry analyses western blots are shown. **(C)** The proteins expression of ZO-1, ET-1, syndecan-1 and glypican-1 were assessed by western blot. (**D**)The densitometry analyses western blots are shown. Values are expressed as means ±SEM. *P <0.05, **P <0.01, ***P <0.001, n = 3.

Fig.12 Inhibition of ERS and overexpression of ABCA1 ameliorates HRGECs injury. (A) The proteins expression of cleaved Caspase-3, Bax and Bcl-2 were assessed by western blot. **(B)**The densitometry analyses western blots are shown. (**C**) Flow cytometry was conducted to analyze HRGECs apoptosis after labeling with Annexin V or propidium iodide. (**D**) Quantification analyses of apoptotic cells in

different groups. Values are expressed as means ±SEM. *P <0.05, **P <0.01, ***P <0.001, n = 3.

Fig.13 Schematic diagram showing that ABCA1 deficiency contributed to glomerular endothelial injury in early diabetic kidney disease.

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Graphical abstract

Highlights

1. Diabetic kidney disease (DKD)-the diabetes-driven kidney injury, has become the leading cause of end-stage renal disease. We and others have demonstrated ABCA1-mediated cholesterol accumulation was related with kidney injury and disease progression in diabetic patients. It led us to hypothesize that ABCA1 is very important for renal cholesterol homeostasis and its dysfunction could aggravate the renal injury.
2. Previous glomerular endothelial cells studies based on the total diabetic mouse kidneys have provided important insights on diabetic injury. However, those work provided limited information on specific molecular changes occurring in glomerular endothelial cells. In this study, we generated a type 2 diabetic mouse model with ABCA1 deficiency in glomerular endothelial cells (DM-ABCA1-/-mice) to investigate the role of ABCA1 in glomerular endothelial cells injury for the first time.
3. The results indicated that ABCA1 deficiency contributed to inflammatory injury and apoptosis of glomerular endothelial cells through endoplasmic reticulum stress. These findings suggest ABCA1 maybe a potential effective therapeutic target for early DKD progression, and promoting endogenous homeostasis of cholesterol seems to be an effective therapeutic paradigm to combat DKD.

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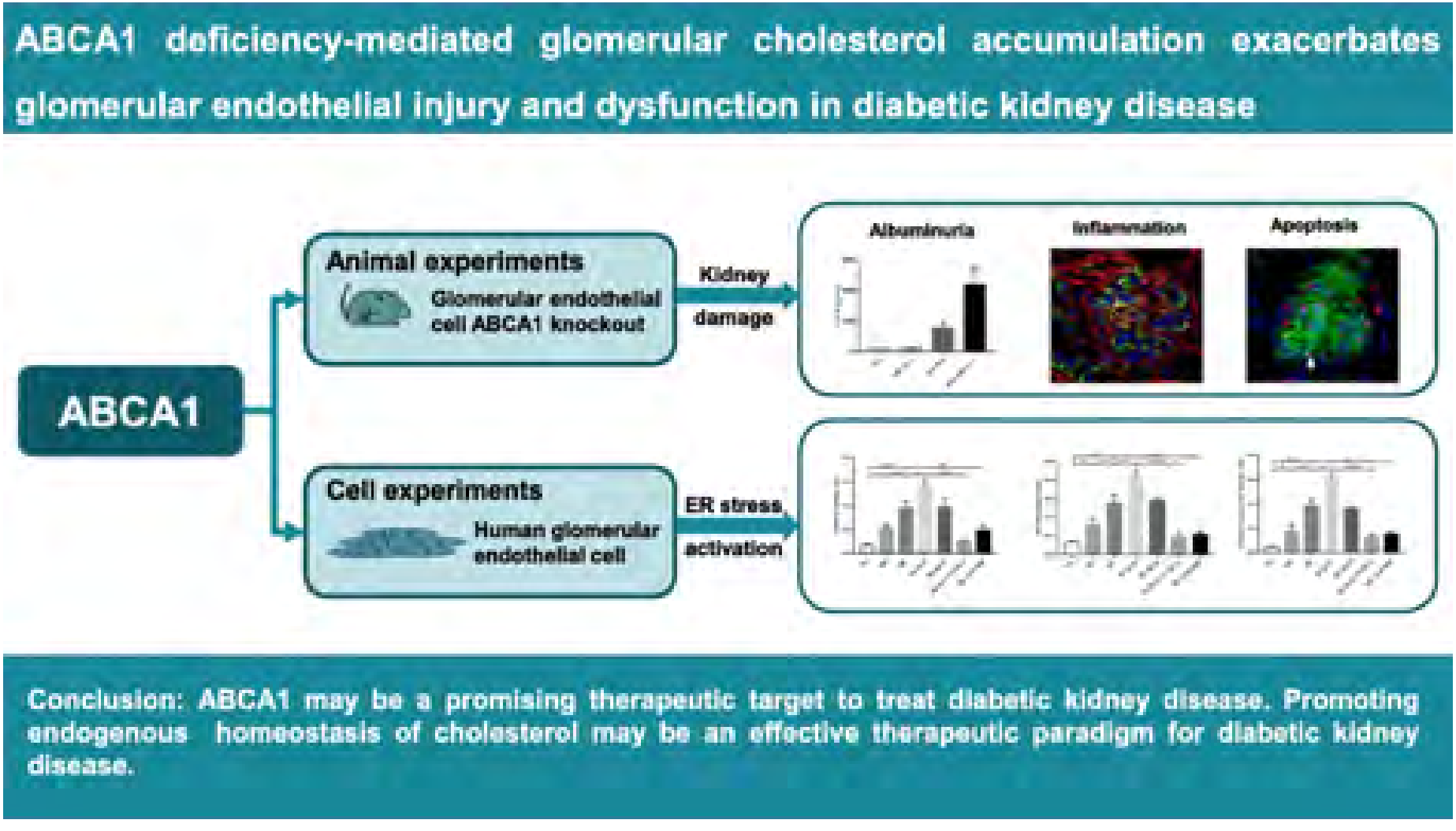
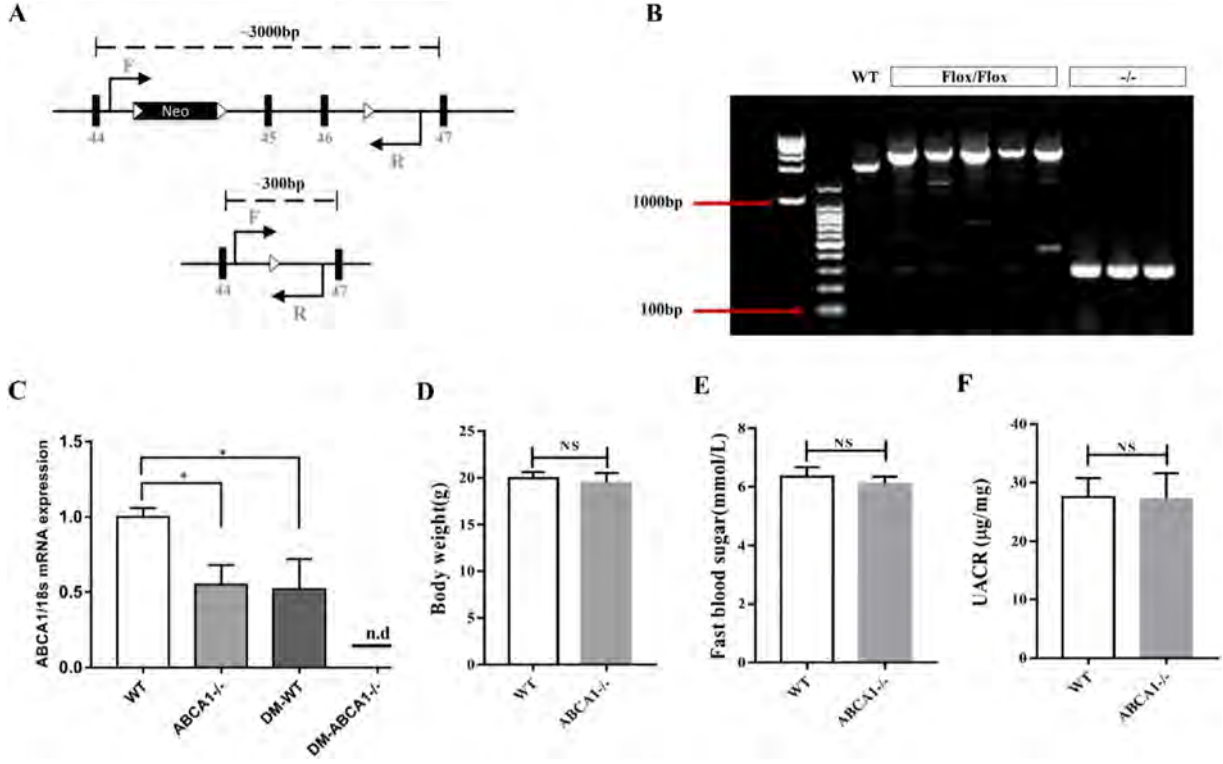


Graphics Abstract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WT
ABCA1-/-
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20
15
10
5
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Fast blood sugar(mmol/L)
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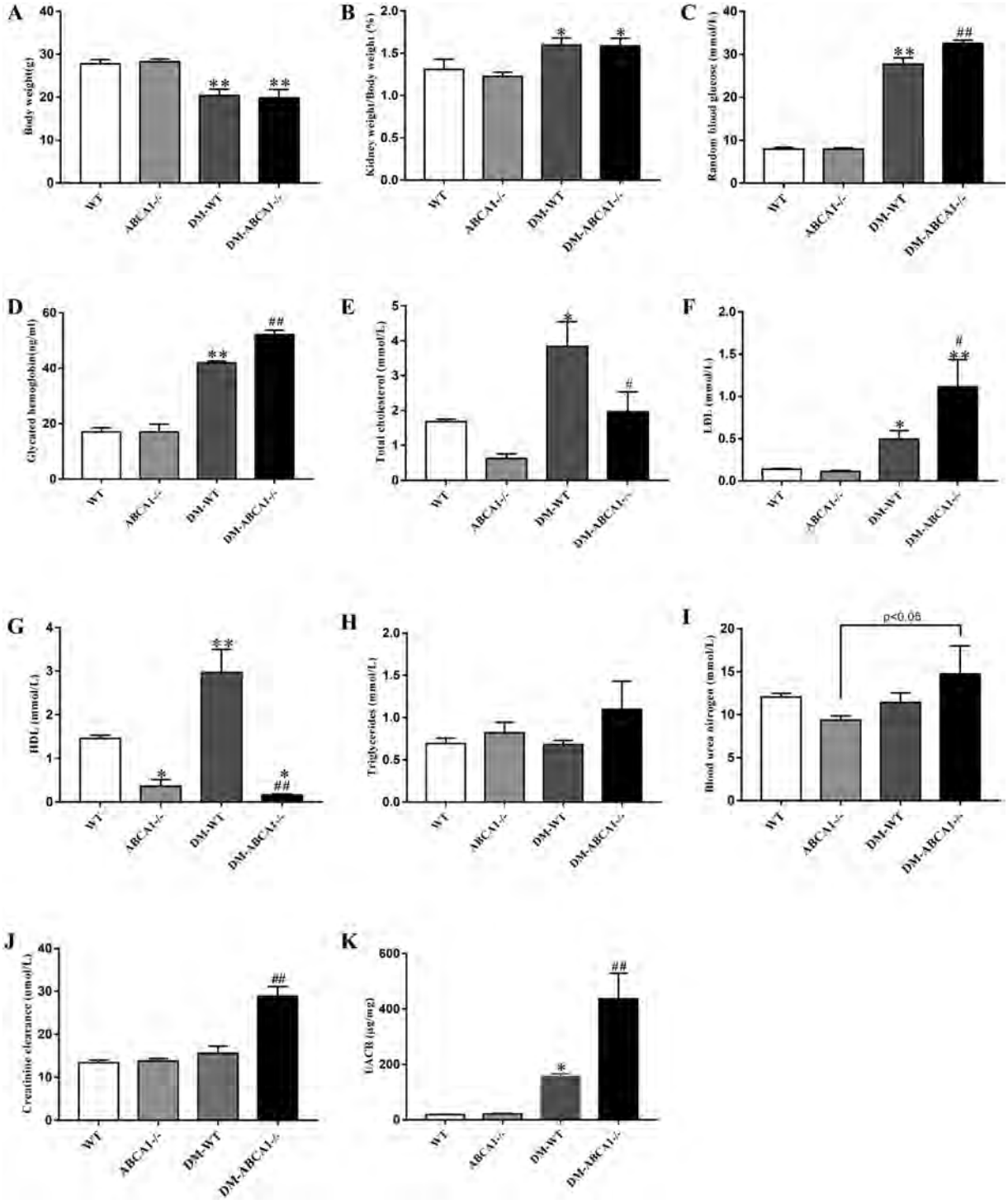
Figure 1


A
Body weight(g)
B
Kidney weight/Body weight (%)
C
Random blood glucose (mmol/L)
D
Glycated hemoglobin(ng/ml)
E
Total cholesterol (mmol/L)
F
LDL (mmol/L)
G
HDL (mmol/L)
H
Triglycerides (mmol/L)
I
Blood urea nitrogen (mmol/L)
p<0.05
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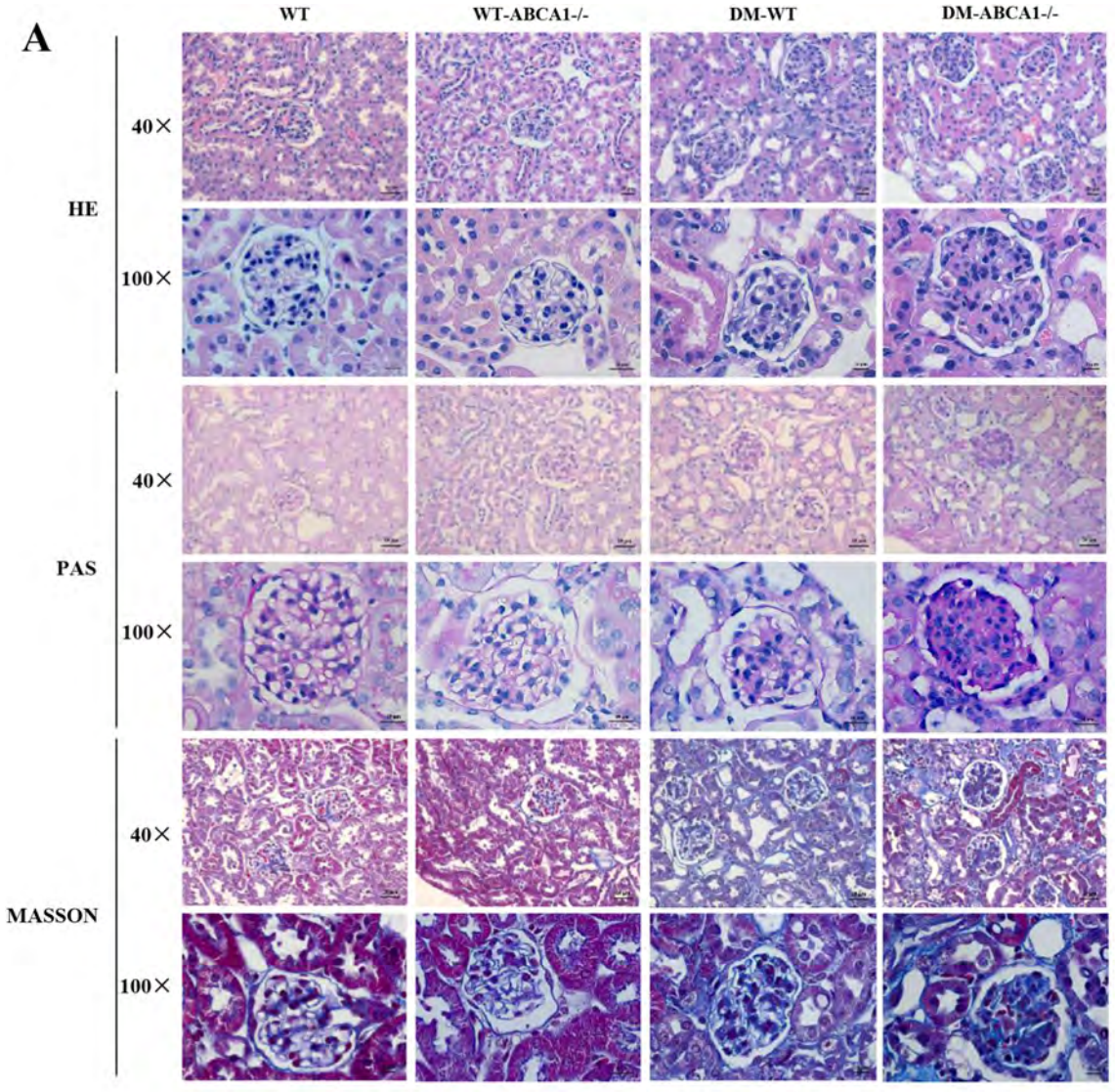
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 2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DM-WT
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0.2
0.4
0.6
***
###
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 3

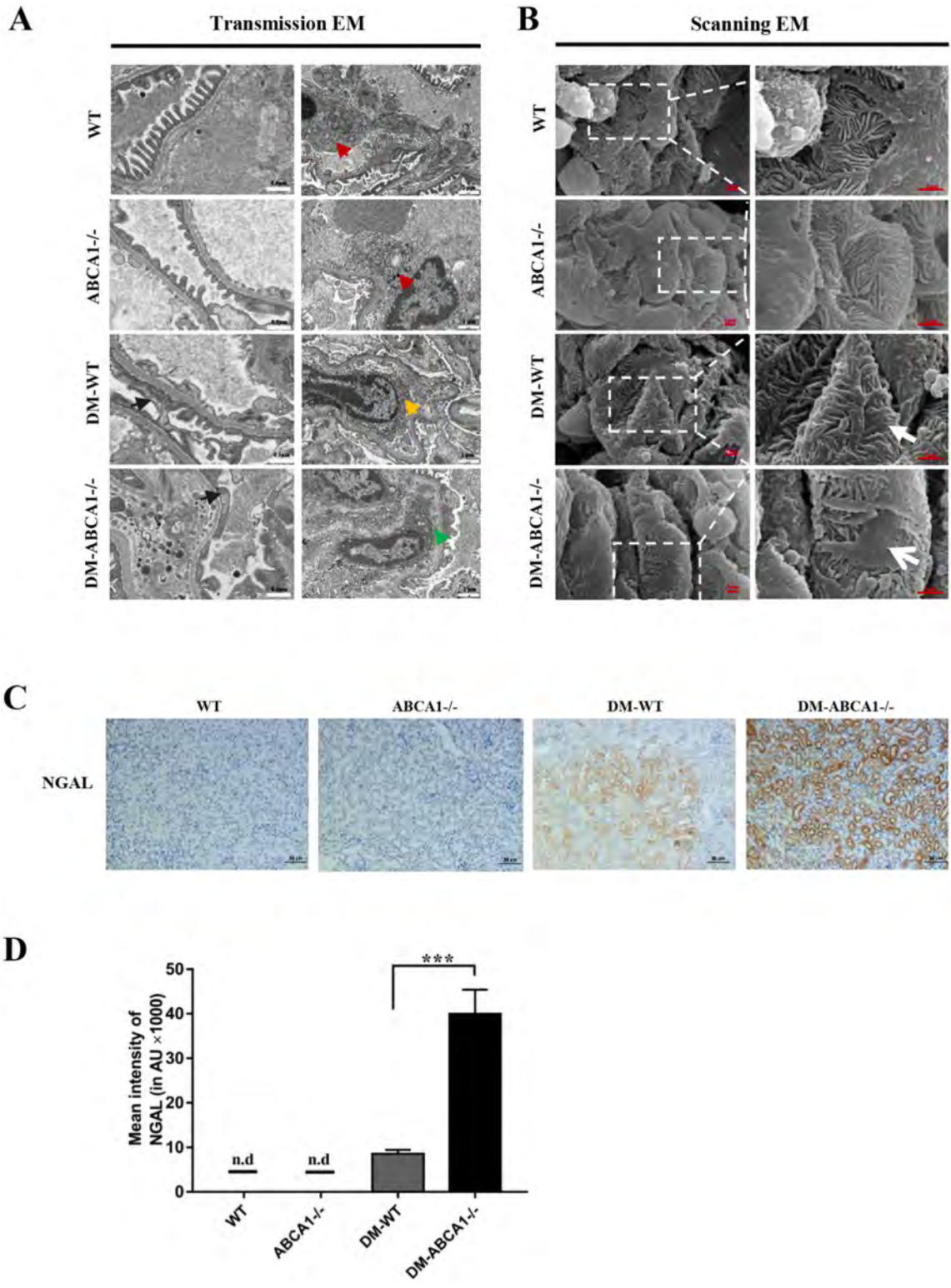
A
Transmission EM
B
Scanning EM
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
C
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
NGAL
D
Mean intensity of NGAL (in AU ×1000)
50
40
30
20
10
0
***
n.d
n.d
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 4

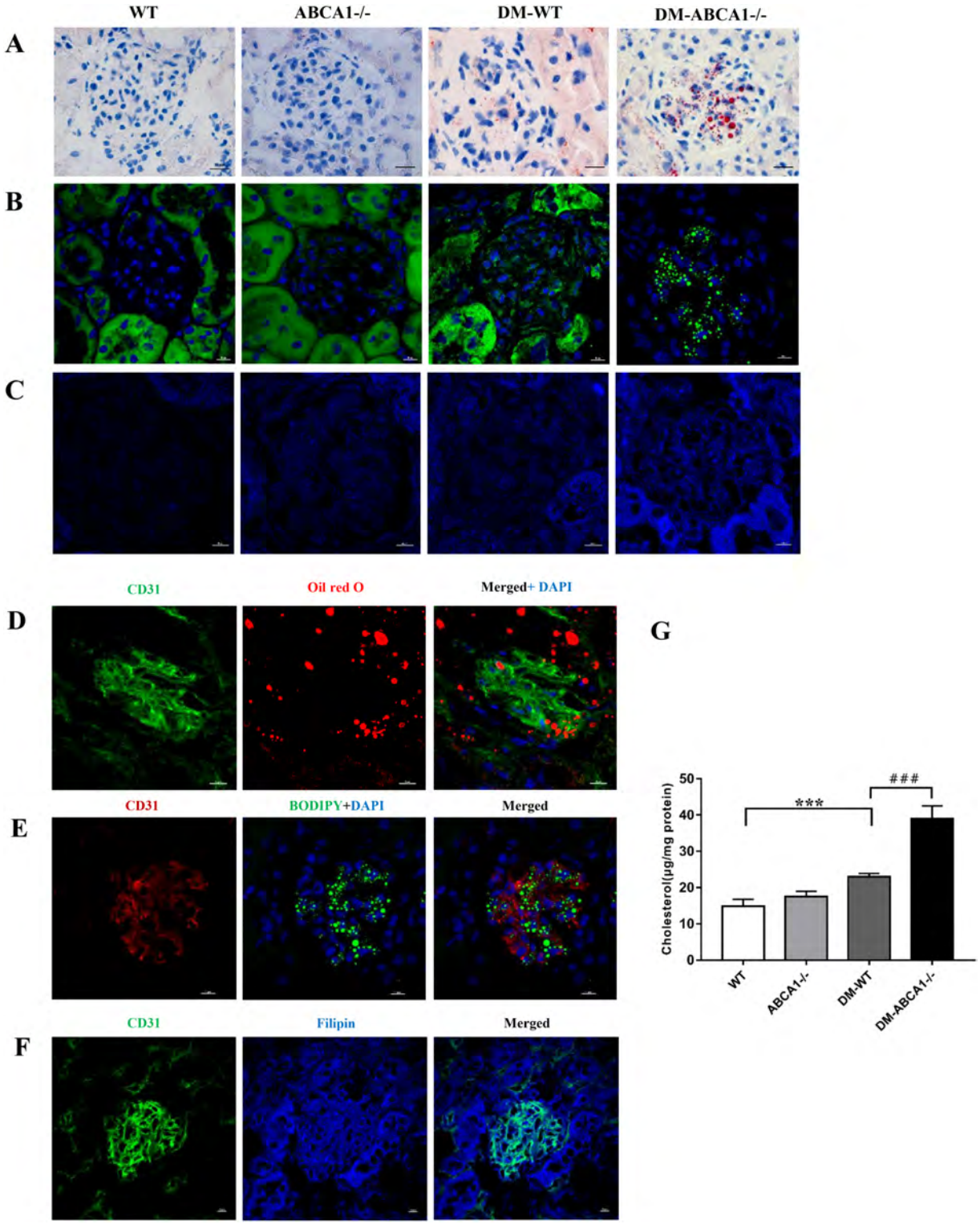
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
A
B
C
CD31
Oil red O
Merged+ DAPI
D
CD31
BODIPY+DAPI
Merged
E
CD31
Filipin
Merged
F
G
Cholesterol(μg/mg protein)
50
40
30
20
10
0
***
###
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 5

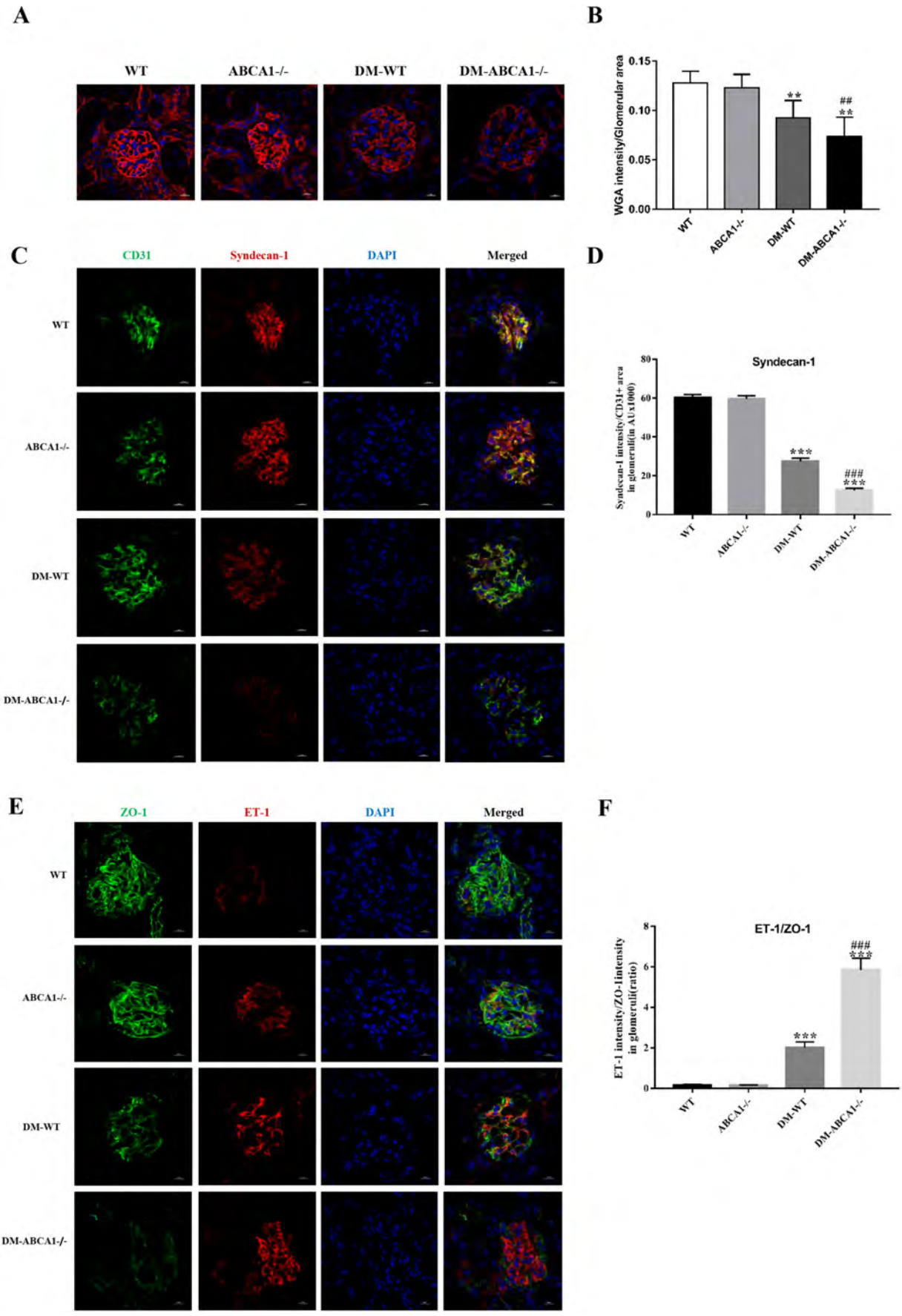
A
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
B
WGA intensity/Glomerular area
0.15
0.10
0.05
0.00
**
##
**
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
C
CD31
Syndecan-1
DAPI
Merged
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
D
Syndecan-1
Syndecan-1 intensity/CD31+ area in glomeruli(in AU x1000)
80
60
40
20
0
***
###
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
E
ZO-1
ET-1
DAPI
Merged
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
F
ET-1/ZO-1
ET-1 intensity/ZO-1intensity in glomeruli(ratio)
8
6
4
2
0
***
###
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 6af

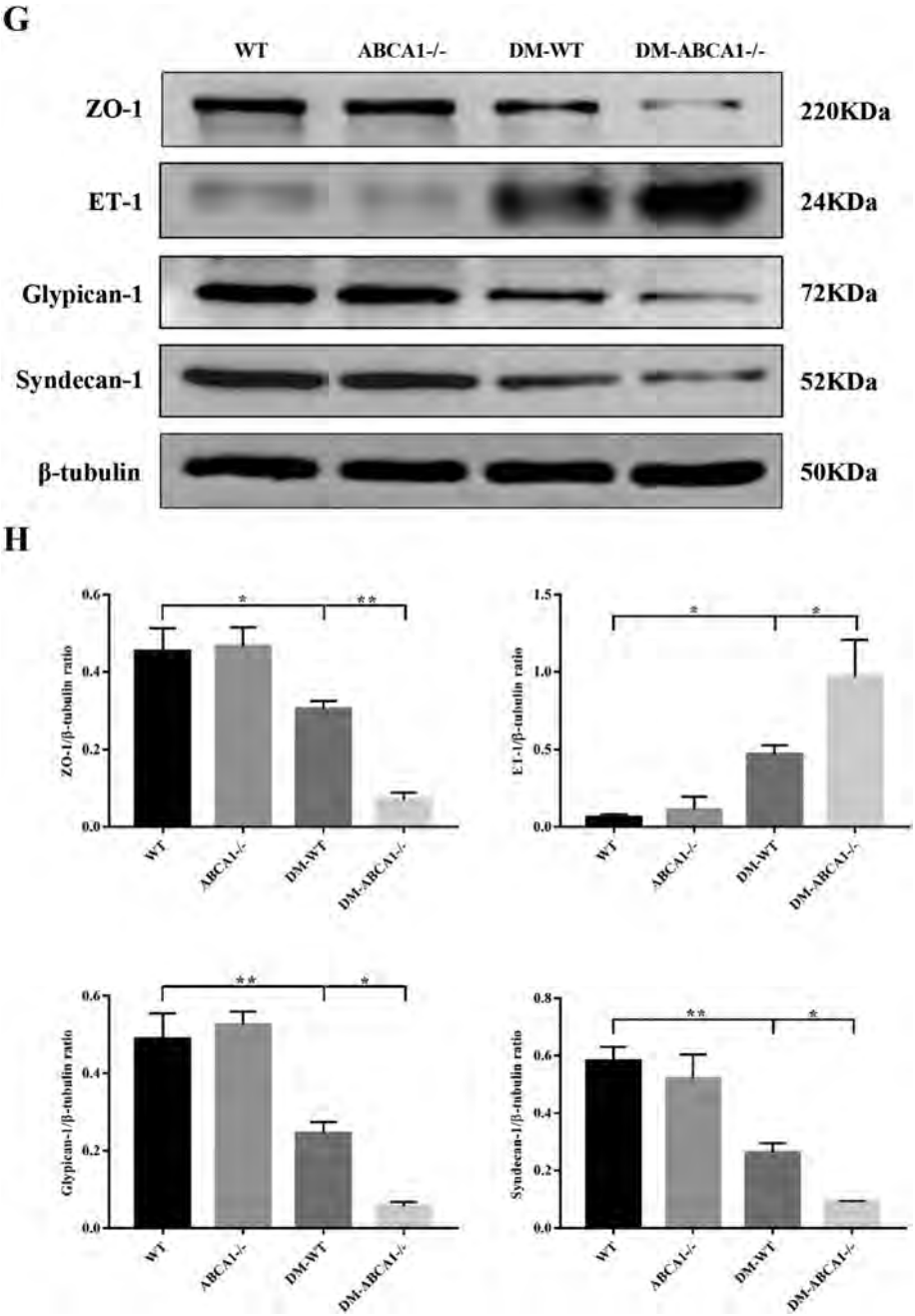
G
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
ZO-1
220KDa
ET-1
24KDa
Glypican-1
72KDa
Syndecan-1
52KDa
β-tubulin
50KDa
H
ZO-1/β-tubulin ratio
ET-1/β-tubulin ratio
Glypican-1/β-tubulin ratio
Syndecan-1/β-tubulin ratio
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 6gh

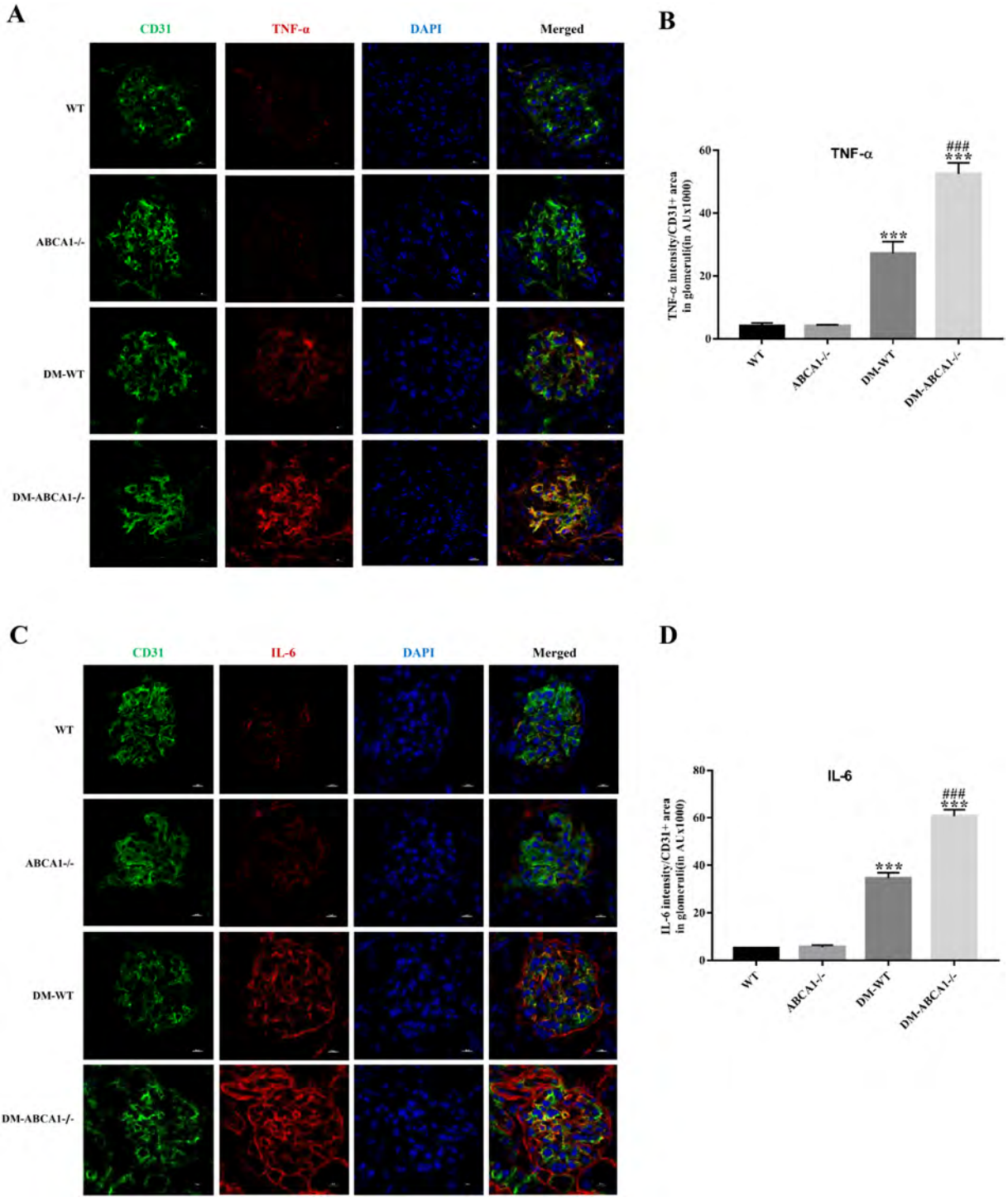
A
CD31
TNF-α
DAPI
Merged
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
B
TNF-α
TNF-α intensity/CD31+ area in glomeruli(in AUx1000)
60
40
20
0
###
***
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
C
CD31
IL-6
DAPI
Merged
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
D
IL-6
IL-6 intensity/CD31+ area in glomeruli(in AUx1000)
80
60
40
20
0
###
***
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 7ad

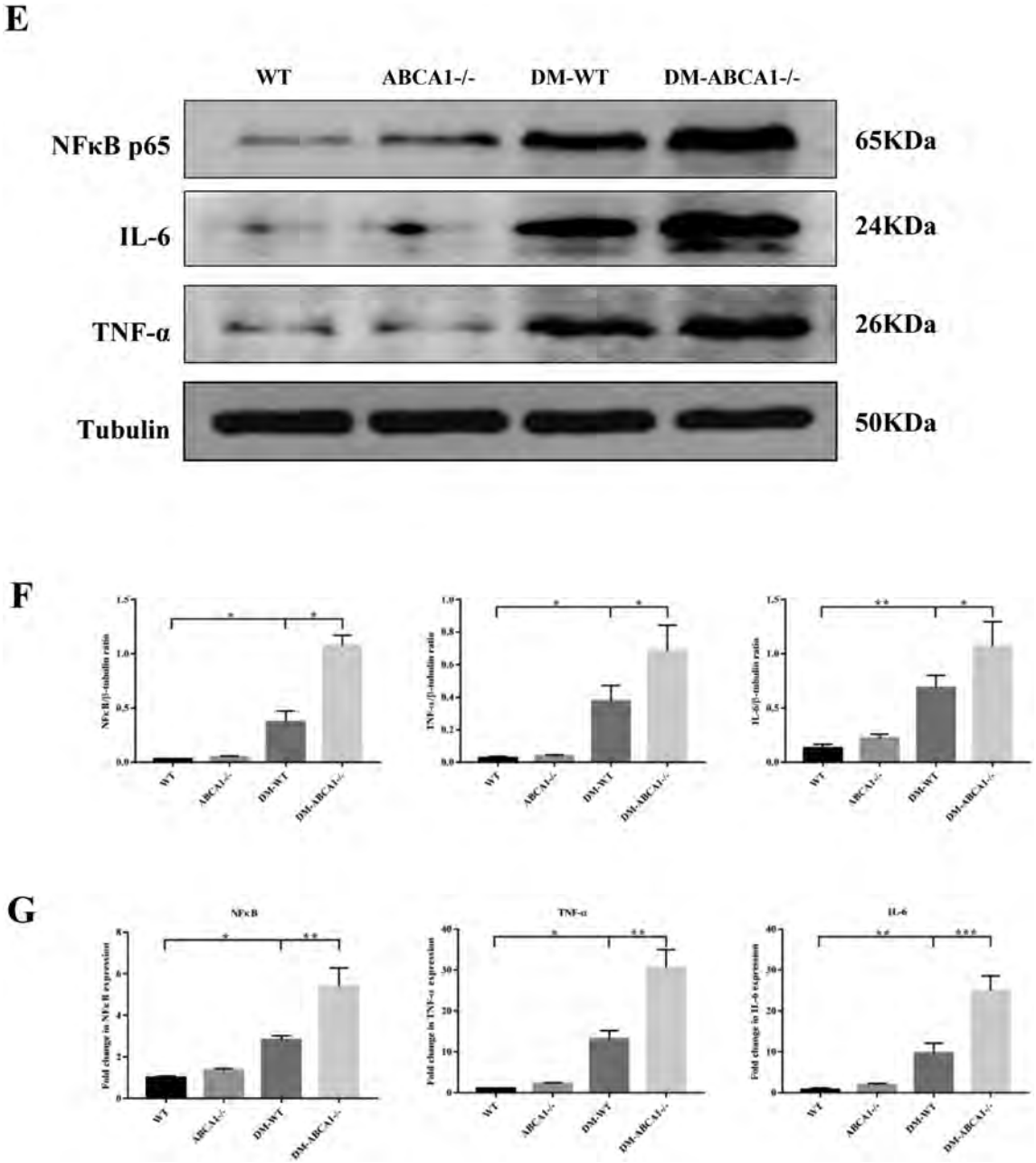
E
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
NFκB p65
65KDa
IL-6
24KDa
TNF-α
26KDa
Tubulin
50KDa
F
NFκB/β-tubulin ratio
TNF-α/β-tubulin ratio
IL-6/β-tubulin ratio
G
NFκB
TNF-α
IL-6
Fold change in NFκB expression
Fold change in TNF-α expression
Fold change in IL-6 expression

Figure 7eg

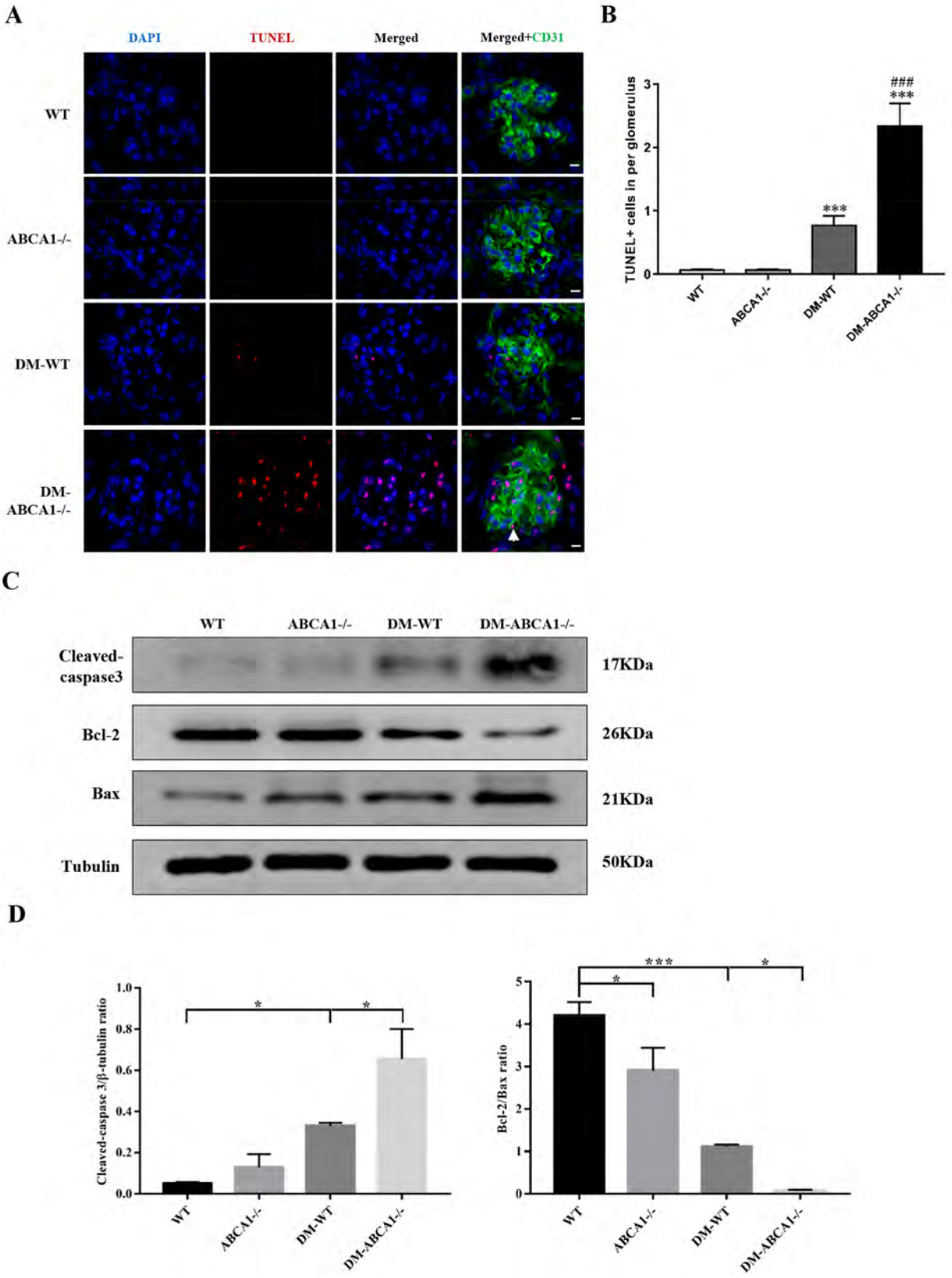
A
DAPI
TUNEL
Merged
Merged+CD31
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
B
TUNEL+ cells in per glomerulus
###
***
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
C
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
Cleaved-caspase3
17KDa
Bcl-2
26KDa
Bax
21KDa
Tubulin
50KDa
D
Cleaved-caspase 3/β-tubulin ratio
Bcl-2/Bax ratio
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 8

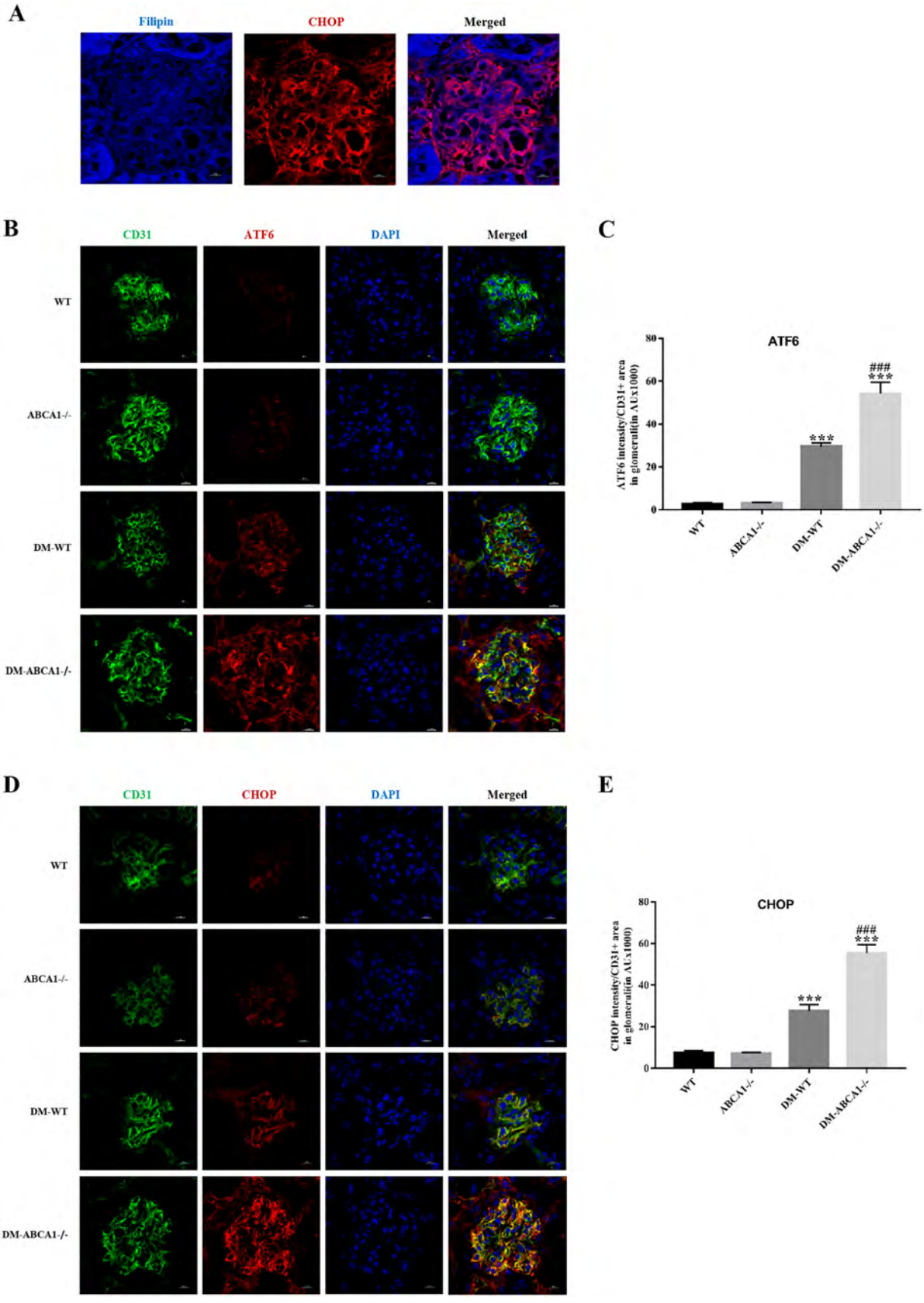
A
Filipin
CHOP
Merged
B
CD31
ATF6
DAPI
Merged
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
C
ATF6
ATF6 intensity/CD31+ area in glomerulii(in AUx1000)
80
60
40
20
0
###
***
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
D
CD31
CHOP
DAPI
Merged
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
E
CHOP
CHOP intensity/CD31+ area in glomerulii(in AUx1000)
80
60
40
20
0
###
***
***
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 9ae

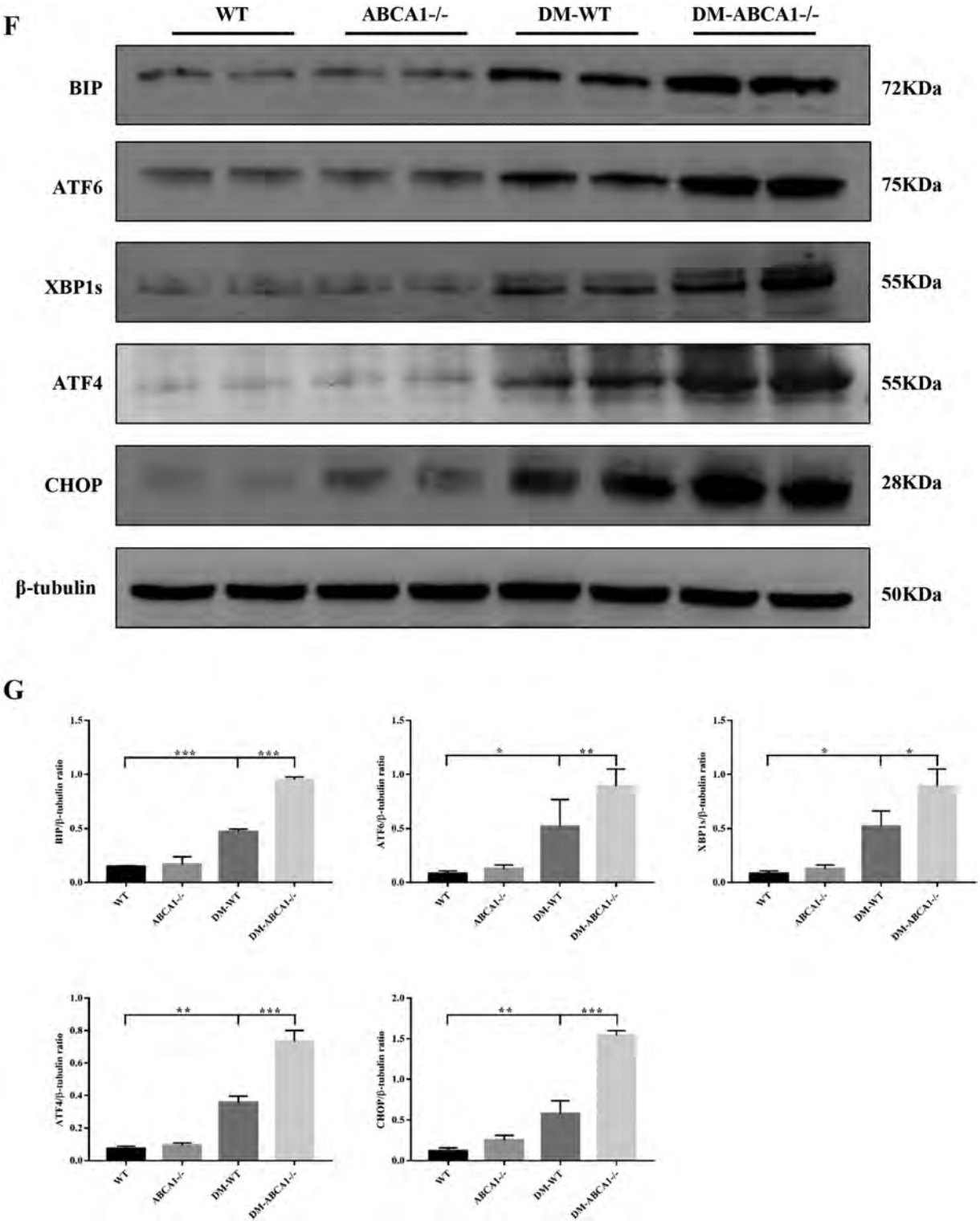
F
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-
BIP
72KDa
ATF6
75KDa
XBP1s
55KDa
ATF4
55KDa
CHOP
28KDa
β-tubulin
50KDa
G
BIP/β-tubulin ratio
ATF6/β-tubulin ratio
XBP1s/β-tubulin ratio
ATF4/β-tubulin ratio
CHOP/β-tubulin ratio
WT
ABCA1-/-
DM-WT
DM-ABCA1-/-

Figure 9fg

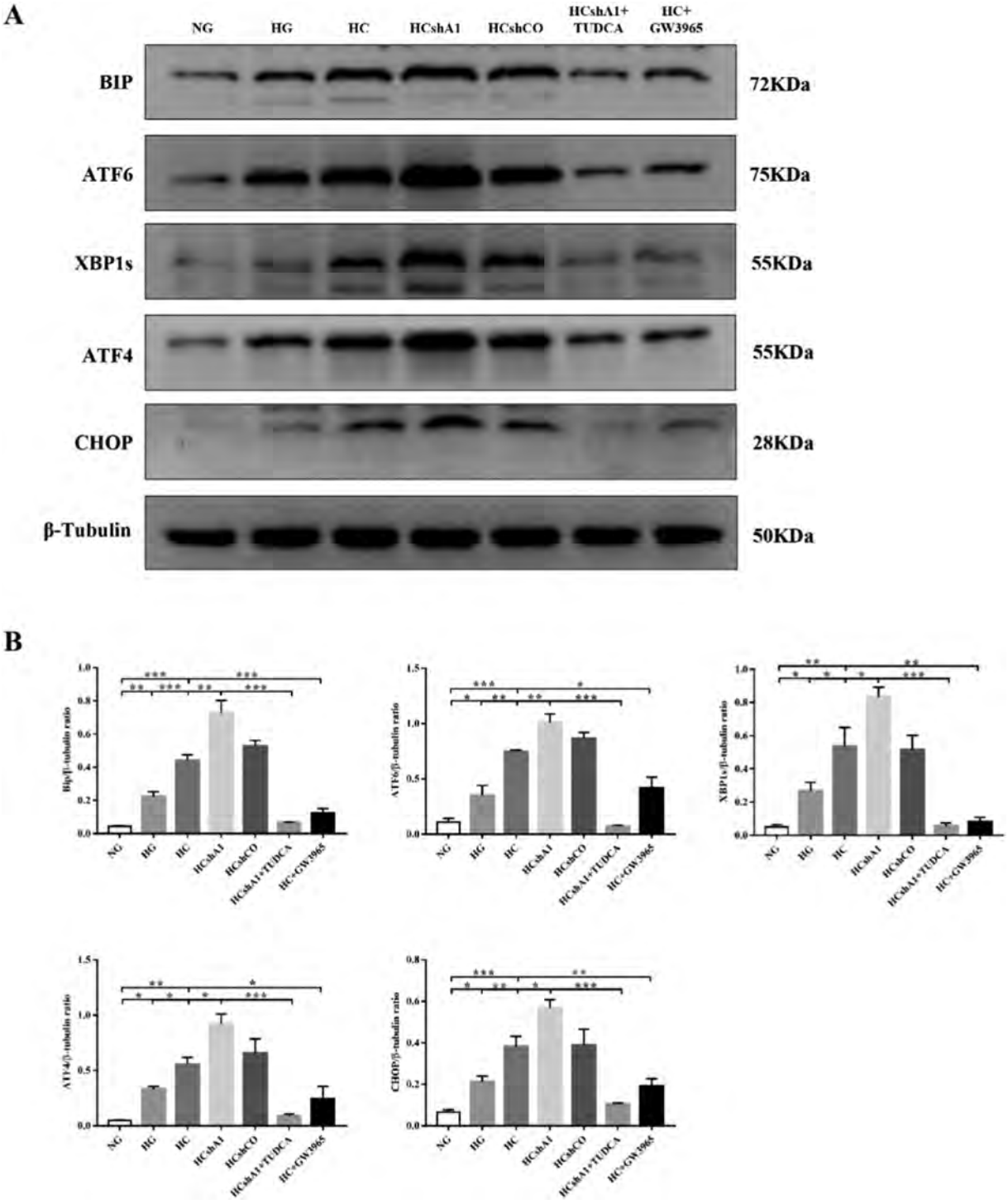
A
NG
HG
HC
HCshA1
HCshCO
HCshA1+ TUDCA
HC+ GW3965
BIP
72KDa
ATF6
75KDa
XBP1s
55KDa
ATF4
55KDa
CHOP
28KDa
β-Tubulin
50KDa
B
Bip/β-tubulin ratio
ATF6/β-tubulin ratio
XBP1s/β-tubulin ratio
ATF4/β-tubulin ratio
CHOP/β-tubulin ratio
NG
HG
HC
HCshA1
HCshCO
HCshA1+TUDCA
HC+GW3965

Figure 10

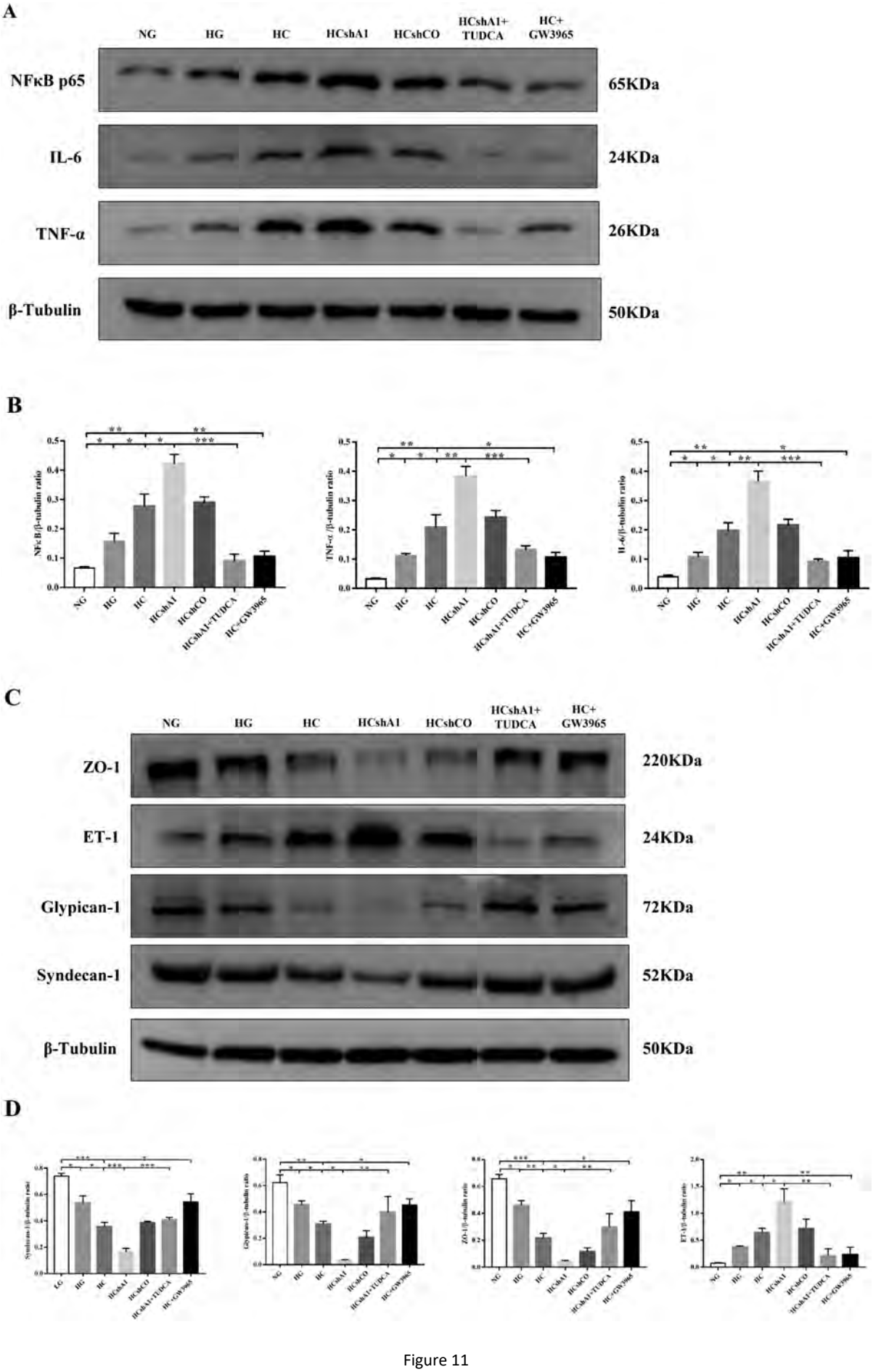
A
NG
HG
HC
HCshA1
HCshCO
HCshA1+ TUDCA
HC+ GW3965
NFκB p65
65KDa
IL-6
24KDa
TNF-α
26KDa
β-Tubulin
50KDa
B
NFκB/β-tubulin ratio
TNF-α /β-tubulin ratio
IL-6/β-tubulin ratio
C
ZO-1
220KDa
ET-1
24KDa
Glypican-1
72KDa
Syndecan-1
52KDa
β-Tubulin
50KDa
D

Figure 11

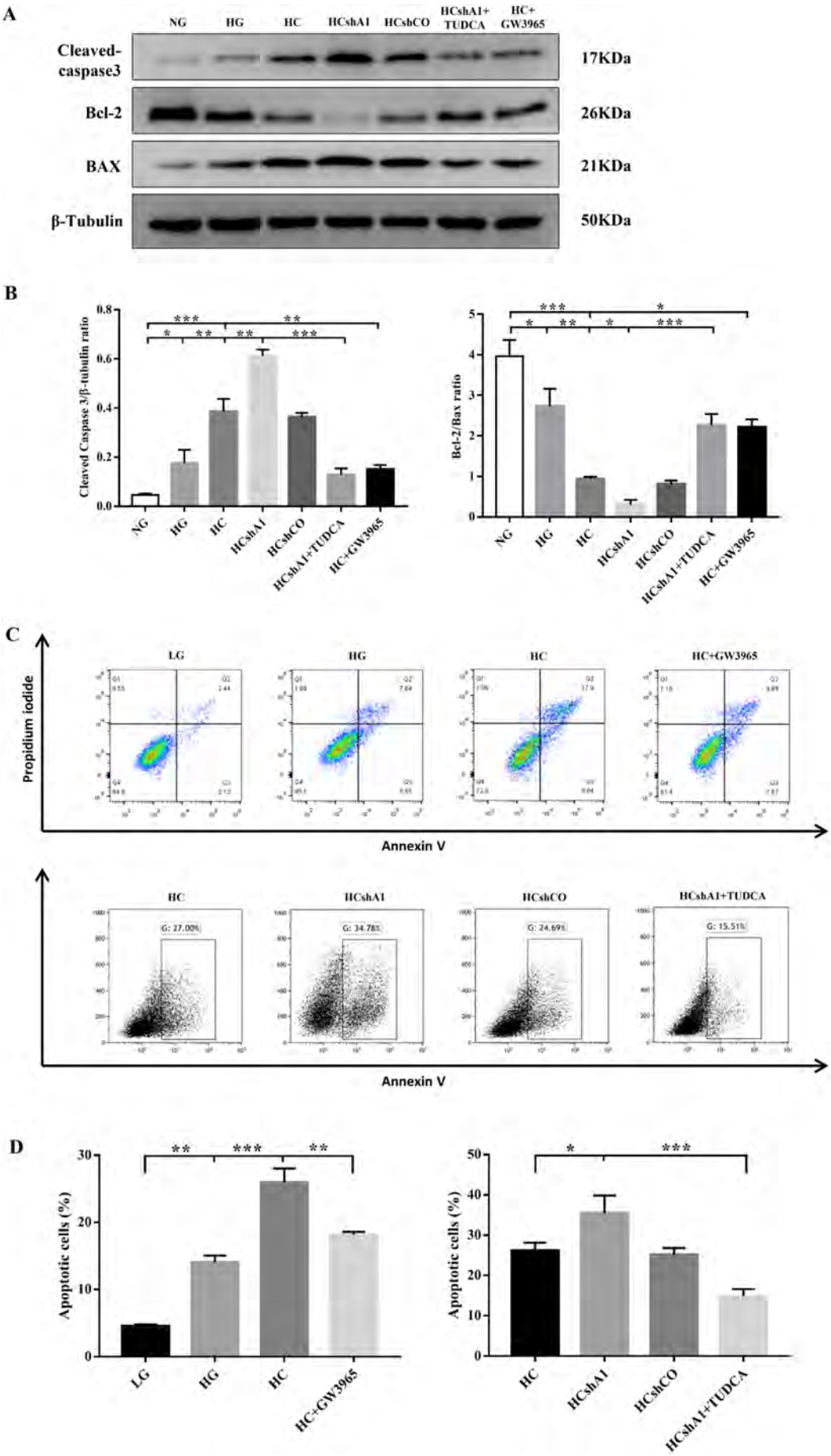
A
NG
HG
HC
HCshA1
HCshCO
HCshA1+ TUDCA
HC+ GW3965
Cleaved-caspase3
17KDa
Bcl-2
26KDa
BAX
21KDa
β-Tubulin
50KDa
B
Cleaved Caspase 3/β-tubulin ratio
Bcl-2/Bax ratio
NG
HG
HC
HCshA1
HCshCO
HCshA1+TUDCA
HC+GW3965
C
LG
HG
HC
HC+GW3965
Propidium iodide
Annexin V
HC
HCshA1
HCshCO
HCshA1+TUDCA
G: 27.00%
G: 34.78%
G: 24.69%
G: 15.51%
Annexin V
D
Apoptotic cells (%)
LG
HG
HC
HC+GW3965
Apoptotic cells (%)
HC
HCshA1
HCshCO
HCshA1+TUDCA

Figure 12

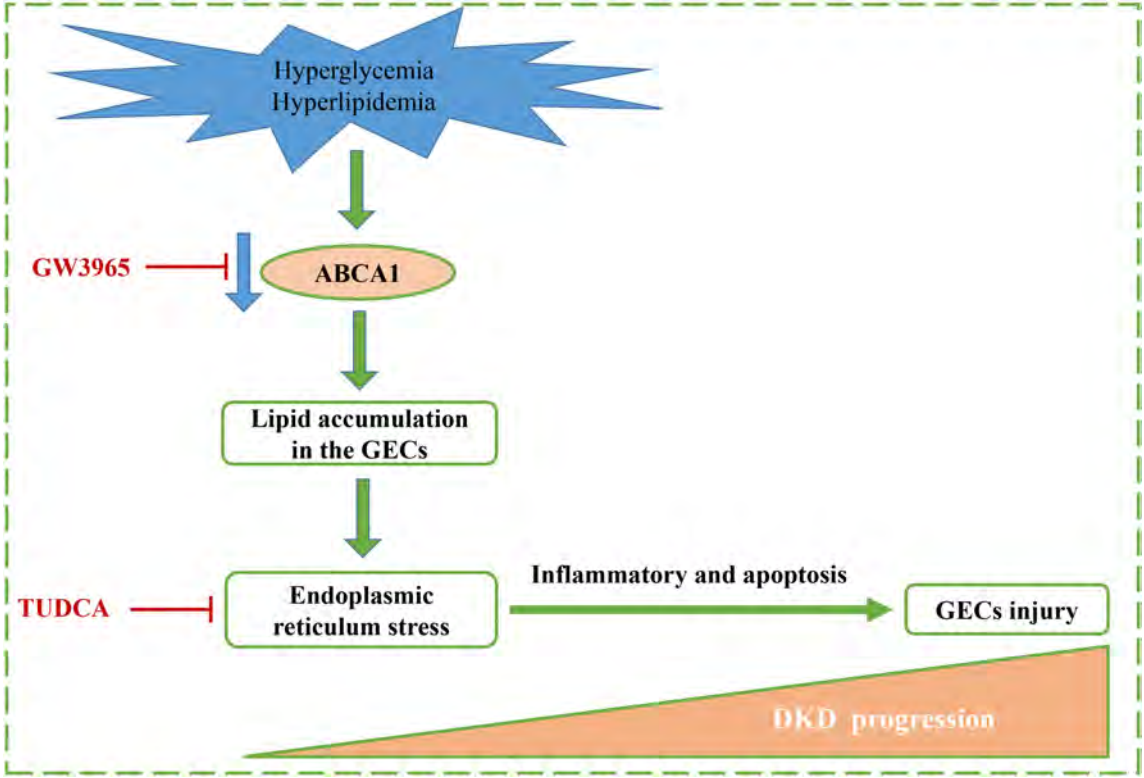
Hyperglycemia
Hyperlipidemia
GW3965
ABCA1
Lipid accumulation in the GECs
TUDCA
Endoplasmic reticulum stress
Inflammatory and apoptosis
GECs injury
DKD progression

Figure 13