INTRODUCTION

Cyclin G2 (CCNG2) is an atypical cyclin that inhibits cell cycle progression and is often dysregulated in human cancers. Cyclin G2 in the occurrence and development of diabetic nephropathy (DN), one of the most severe diabetic complications, has not been fully identified. In this study, we investigated the function and regulatory mechanism of cyclin G2 in DN. In vivo studies revealed that a deficiency of cyclin G2 significantly increased albuminuria and promoted tubulointerstitial fibrosis in established DN. Cyclin G2 regulated the expression of fibrosis-related proteins via the canonical Wnt signalling pathway in renal tubular epithelial cells. Moreover, the binding of cyclin G2 to Dapper1 (Dpr1/DACT1), a protein involved in Wnt signalling, decreased the phosphorylation of Dpr1 at Ser762 by casein kinase 1 (CK1) and suppressed the Wnt signalling pathway. These findings reveal that cyclin G2 can protect against renal injury and fibrosis associated with DN and, thus, is a new target for the prevention and treatment of diabetic complications.

KEYWORDS

Cyclin G2, Dapper1, diabetic nephropathy, tubulointerstitial fibrosis, Wnt signalling
The pathological features of DN include renal glomerular hypertrophy, thickening of the glomerular and tubular basement membrane, extracellular matrix accumulation, and eventual tubular interstitial fibrosis and glomerulosclerosis. The clinical outcome of DN involves progressive, irreversible renal dysfunction and ultimately renal failure. The pathogenesis of DN is not fully understood, and effective approaches for the treatment and prevention of DN are lacking. Therefore, studies that address the molecular pathogenesis of DN are vital. Previously, we reported that cyclin G2 attenuated glomerulosclerosis in DN through the Wnt pathway. However, it has recently been demonstrated that the development of tubulointerstitial lesions is more closely correlated with a progressive decline in renal function, compared with glomerular lesions, and the function of cyclin G2 in tubulointerstitial fibrosis is not fully identified.

The canonical Wnt signalling pathway is an evolutionarily conserved, developmental signalling system that plays an important role in organ development and tissue homeostasis. The Wnt signalling pathway is activated in various kidney diseases such as obstructive nephropathy, diabetic nephropathy and polycystic kidney disease. The downstream proteins associated with Wnt signalling are up-regulated in the kidneys of patients with diabetes and in diabetic mouse models. Similarly, high-glucose induction activates the Wnt signalling pathway in glomerular podocytes and mesangial cells and causes excessive apoptosis of intrinsic renal cells. Suppression of Wnt signalling improves proteinuria and tubulointerstitial fibrosis in patients with type 1 diabetes. As a central mediator of Wnt signalling, Dishevelled (Dvl) plays an important role in both β-catenin–mediated canonical and β-catenin–independent noncanonical Wnt signalling. Dvl-1, originally identified as a Dvl-interacting protein, has been shown to mediate Dvl degradation and thus inhibit both Dvl-mediated canonical and noncanonical Wnt signalling.

In this study, we investigated the involvement of cyclin G2 in pathological processes associated with DN, both in vitro and in vivo, using several molecular biology techniques. We found that cyclin G2 attenuates the development of tubulointerstitial fibrosis in DN by regulating Wnt signalling via interaction with Dpr1. Our results highlight a novel role for cyclin G2 as a mediator of metabolic disease progression.

2 | MATERIALS AND METHODS

2.1 | Mouse model of streptozotocin-induced diabetes

Whole-body cyclin G2 knockout C57BL/6 mice (Ccn2−/−) were generated at the Shanghai Model Organisms Center (China). Male wild-type (WT; C57BL/6, n = 25) and Ccn2−/− mice (n = 27) were housed in cages, fed standard chow and maintained on a 12-hour light-dark cycle. Diabetes was induced as described previously. Briefly, 8-week-old mice received five consecutive intravenous injections of streptozotocin (STZ) (50 mg/kg; Sigma-Aldrich; V900890) in citrate buffer (pH 4.6) or citrate buffer only. A blood glucose level >11.2 mmol/L was confirmed 3 days after STZ administration at three different time-points. Mice with blood glucose levels less than 11.2 mmol/L at 2 weeks after the final injection of STZ were excluded from the experiment. Metabolic cages were used for collection of urine over a 24-hour period. Urinary albumin levels were measured using the QuantiChrom BCG Albumin Assay Kit (BioAssay Systems; DIAG-250); urinary creatinine levels were measured using the Parameter Creatinine Assay (R&D Systems; KGE005). Mice were killed 16 weeks after STZ injection, and an age-matched WT (n = 6) or Ccn2−/− mouse (n = 6) was also killed at the same time (Figure S1).

2.2 | Morphological studies

Renal tissue specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 4-μm thickness were examined by haematoxylin-eosin (HE) staining and Masson’s trichrome staining (Solarbio life sciences; G1120; G1340) as well as immunohistochemistry assays. The percentage fibrosis area was determined from 15 fields of Masson’s trichrome-stained specimens viewed at 200× magnification. Lesions were quantified using Image-Pro Plus software. The fibrotic area was digitized and subjected to colour-threshold analysis. Scores from ten non-overlapping fields per kidney were averaged to obtain the final percentage fibrosis area.

For immunohistochemistry, sections were deparaffinized, rehydrated and autoclaved for 10 minutes in citrate buffer for antigen retrieval. Nonspecific binding was blocked by incubation with 10% goat or rabbit serum for 30 minutes. The samples were incubated with anti-β-catenin (Sigma-Aldrich; C2206), anti-collagen IV (Abcam; ab6586) or anti-N-cadherin (Cell Signaling Technology; 13116) primary antibodies at 4°C overnight. After washing in PBS, the sections were incubated with an appropriate secondary antibody and detected using the Ultrasensitive S-P Kit (streptavidin-peroxidase; Sigma-Aldrich; S2438).

2.3 | Cell culture, transfection and treatment

Cells from a human renal tubular epithelial cell line (HK-2) were purchased from the ATCC and cultured with DMEM containing 10% foetal bovine serum (Gibco, Life Technologies). Cells were infected with lentivirus particles harbouring CCNG2 or control GFP lentivirus (GeneChem) and divided into high-glucose (HG; 30 mmol/L d-glucose) and low-glucose (LG; 5.5 mmol/L d-glucose and 24.5 mmol/L l-glucose) treatment groups (Figure S2). Cells were starved by incubation in DMEM containing 1% serum for 24 hours, and then,
the media was replaced with DMEM containing 10% serum and the indicated concentrations of glucose. Cells were infected with lentivirus particles harbouring CCNG2 and exposed to the Wnt signalling activator CHIR99021 (1 mmol/L; R&D Systems; 4423) or an equivalent volume of DMSO in DMEM (negative control) for 72 hours.

The eukaryotic expression vectors pCMV-3 × FLAG-G2, pEGFP-C1, pCMV-Tag5A-Dpr1 and PPGU6/GFP/Neo-shDACT1 were prepared. HK-2 cells were cultured to 80% confluence, then transfected with one or more of the following recombinant expression vectors: p3 × FLAG-CMV-BAP (Sigma-Aldrich), pCMV-3 × FLAG-G2, pEGFP-N3, PPGU6/GFP/Neo (BD Biosciences), pEGFP-C1, PPGU6/GFP/Neo-shDACT1, pCMV-Tag5A (Huayueyang Bio), pCMV-Tag5A-Dpr1 or pCMV-Tag5A-Dpr1 S762A. Transfections were performed using Lipofectamine 2000 (Life Technologies; 11668500) according to the manufacturer’s instructions.

2.4 | Immunoprecipitation and Western blot analysis

Total cellular protein (3 mg) from HK-2 cells were incubated with an anti-Dpr1 antibody (Abcam; ab51260) and Protein G Plus Agarose (Santa Cruz Biotechnology; sc-500778) at 4°C overnight. After washing, Ser/Thr-phosphorylated proteins were isolated using a phosphorylation purification kit according to the manufacturer’s instructions (Qiagen, Life Technologies; 37145). The purified protein concentration was adjusted to 0.1 mg/mL; a 30-µL aliquot was used for Western blotting. Western blotting was performed using an antiphosphoserine/threonine antibody (Sigma-Aldrich; P3430).

For Western blotting, protein concentrations were determined using BCA (Life Technologies; 23227). The anti-E-cadherin (#3195), anti-N-cadherin (#13116), anti-cyclin D1 (#2978), anti–β-tubulin (#86298), anti–phospho-β-catenin (Ser33/37/Thr41) (#9561) and anti-Dvl2 (#3224) antibodies were from Cell Signaling Technology. The anti-collagen IV (ab6586) antibody was from Abcam. The anti–β-catenin (Ser33/37/Thr41) (#9561) and anti–phospho-GSK3β (Ser9) (#9272) antibodies were from Cell Signaling Technology. The anti-collagen IV antibody was from Abcam. The anti–β-catenin (C2206) and anti-cyclin G2 (Hpa034684) antibodies were from Sigma-Aldrich, and the anti-MMP7 (10374-2-AP) antibody was from Proteintech. All experiments were performed in triplicate.

2.5 | In situ proximity ligation assay

For in situ proximity ligation assays (PLA) (Duolink; Sigma-Aldrich; DUO92103), oligonucleotide-conjugated ‘PLA probe’ antibodies were directed against the primary antibodies for Dpr1, cyclin G2 or CK1. The annealing of the PLA probes occurred when Dpr1, cyclin G2 or CK1 was in close proximity, which initiated the amplification of repeat sequences recognized by the fluorescently labelled oligonucleotide probe. Briefly, HK-2 cells were seeded on glass-bottom cell culture dishes (Nest Biotechnology) and stimulated as described.39,40 The cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 minutes at room temperature. The cells were permeabilized with 0.1% Triton X-100 for 20 minutes at room temperature. The primary antibodies used in this study were anti-Dpr1 (Abcam; ab51260; Santa Cruz Biotechnology; sc-745999), anti-cyclin G2 (Sigma-Aldrich; Hpa034684) and anti-CK1 (Santa Cruz Biotechnology; sc-6478). The PLA probes were anti-goat (minus) and anti-rabbit (plus). Duolink in situ Detection Reagent Red was used for detection. Imaging was performed using a confocal fluorescence microscope. Images were analysed with Image-Pro Plus software. At least 50 cells were analysed in each experiment.

2.6 | Immunofluorescence

Cells were fixed, permeabilized and then blocked with bovine serum albumin (BSA) for 30 minutes. The cells were incubated with primary antibody at 4°C overnight and then incubated with secondary antibody for 45 minutes at room temperature. DAPI was used to stain the nuclei. Imaging was performed using an inverted fluorescence microscope.

2.7 | Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using a Student’s t test for comparisons between two groups or using a one-way ANOVA for analyses between three or more groups. Statistical significance was defined as P < .05.

3 | RESULTS

3.1 | Cyclin G2 expression is abnormally decreased in kidneys of DN mice and high glucose–induced renal tubular epithelial cells

Cyclin G2 expression was evaluated by Western blotting using the renal tissue homogenate of normal mice and those with STZ-induced DN at 16 weeks after the onset of diabetes. The protein expression of cyclin G2 was significantly decreased in the renal tissue homogenate of DN mice (Figure 1A,B). Immunohistochemical staining showed that the protein expression of cyclin G2 was down-regulated in renal tissues from STZ-induced DN mice (Figure 1C). We further examined cyclin G2 protein expression in HK-2 cells cultured with low- and high-glucose concentrations. The protein expression level of cyclin G2 was significantly decreased in HK-2 cells cultured with high glucose (Figure 1D,E).

3.2 | Cyclin G2 deficiency increases albuminuria in DN mice

To evaluate whether cyclin G2 protects against renal dysfunction developed in diabetes, we assessed blood glucose, 24-hour urinary
albumin, urinary creatinine, body weight and kidney weight in our animal model. Blood glucose, renal weight ratio, urinary albumin and urinary albumin/creatinine ratio (UACR) were unchanged in \( \text{Ccng2}^{-/-} \) mice as compared to WT control mice in the absence of STZ treatment. However, the 24-hour urinary albumin levels and the UACR were significantly increased in the \( \text{Ccng2}^{-/-} \) mice following the induction of DN by STZ as compared with DN WT mice. The renal weight ratio was also higher in the DN \( \text{Ccng2}^{-/-} \) group, but blood glucose levels were not significantly increased (Figure 2A-E). Therefore, a lack of cyclin G2 may cause an increase in proteinuria in DN mice.

3.3 | Cyclin G2 deficiency increases the severity of renal injury and tubulointerstitial fibrosis in DN mice

Pathological alterations in the renal tissues of DN mice were determined by HE staining (Figure 3A). Masson’s trichrome staining was used to evaluate the collagen fibres in the renal cortex. Cyclin G2 deletion in mice resulted in pathological changes, including renal tubular atrophy, tubular dilation and interstitial fibrosis. In addition, the largest areas of fibrosis were observed in the renal tissues of DN \( \text{Ccng2}^{-/-} \) mice (Figure 3B,C).

To evaluate the contribution of cyclin G2 on fibrosis-related proteins in vivo, we determined the protein levels of N-cadherin and collagen IV in the renal tissues from \( \text{Ccng2}^{-/-} \) and WT mice following the induction of DN. Western blotting and immunohistochemistry revealed that DN \( \text{Ccng2}^{-/-} \) mice had larger increases in N-cadherin and collagen IV levels as compared to DN WT mice (Figure 3D-F). Hence, cyclin G2 might attenuate tubulointerstitial fibrosis and relieve renal injury in this animal model of diabetes by decreasing the changes in fibrosis-related proteins induced during the development of DN.

In parallel, we evaluated whether cyclin G2 could abrogate tubulointerstitial fibrosis in vitro by overexpressing cyclin G2 in HK-2 cells using lentiviral particles (Figure 3G,H) and measuring N-cadherin, E-cadherin and collagen IV protein levels under conditions of high glucose. At normal cyclin G2 levels, N-cadherin and collagen IV were up-regulated in high-glucose–induced HK-2 cells (Figure S3).

3.4 | Cyclin G2 down-regulates the expression of tubular fibrosis-related proteins by regulating canonical Wnt signalling

Other researchers have noted that activation of Wnt signalling induces renal injury and fibrosis. Therefore, we investigated whether cyclin G2 could regulate the progression of tubulointerstitial fibrosis through Wnt signalling. Specifically, we assessed the effect of cyclin G2 on the protein expression of Wnt signalling factors in vitro and in vivo. As expected, ectopic expression of cyclin G2 inhibited the expression of \( \beta \)-catenin (ie the key regulator of the Wnt signalling pathway), \( \beta \)-GSK3\( \beta \) and its targets (cyclin D1 and MMP7); meanwhile \( \beta \)-catenin and GSK3\( \beta \) were up-regulated in high glucose–induced HK-2 cells (Figure 4A,B). In addition, the levels of \( \beta \)-catenin, cyclin D1 and MMP7 in DN \( \text{Ccng2}^{-/-} \) mice were substantially higher than those of DN WT mice (Figure 4C-E). These results
suggested that cyclin G2 negatively regulates the Wnt pathway in high glucose–induced HK-2 cells as well as in mouse kidney.

To discover the mechanisms by which cyclin G2 influences tubulointerstitial fibrosis, we activated the Wnt signalling pathway using the GSK3β inhibitor CHIR99021. Before CHIR99021 addition, cyclin G2 overexpression inhibited both the expression of proteins associated with Wnt signalling and tubular interstitial fibrosis. After the addition of CHIR99021, the inhibition of Wnt signalling factor expression was abolished. Moreover, down-regulation of N-cadherin and collagen IV and up-regulation of E-cadherin were diminished (Figure 4F,G). These data indicate that cyclin G2 can regulate the expression of tubular interstitial fibrosis-related proteins via Wnt signalling in HK-2 cells.

### 3.5 Cyclin G2 interacts with Dpr1 and decreases CK1-mediated phosphorylation of Dpr1

To explore the mechanisms by which cyclin G2 regulates Wnt signalling, we conducted yeast two-hybrid experiments and screened cyclin G2-conjugated proteins, which identified Dpr1, a Dvl binding antagonist protein. Coimmunoprecipitation and Duolink in situ
A deficiency in cyclin G2 contributes to renal injury and fibrosis in DN mice. A, HE staining identified pathological renal alterations in WT mice, Ccng2−/− mice, STZ-induced DN WT mice and STZ-induced DN Ccng2−/− mice (original magnification, 100×, 200×, n = 6). B, C, Masson’s trichrome staining identified an interstitial fibrosis area (original magnification, 100×, 200×, n = 6). D, Western blot and (E) densitometry analyses of N-cadherin and collagen IV in the renal cortex in WT mice, Ccng2−/− mice, STZ-induced DN WT mice and STZ-induced DN Ccng2−/− mice (n = 6). F, Representative immunohistochemistry of the expression of N-cadherin and collagen IV in WT mice, Ccng2−/− mice, STZ-induced DN WT mice and STZ-induced DN Ccng2−/− mice (original magnification, 200×, n = 6). G, H, Western blotting and densitometry for E-cadherin, N-cadherin and collagen IV in HK-2 cells (n = 3). The values are expressed as the mean ± SD; *P < .05; **P < .01; n.s., not significant. Three independent experiments were performed.

3.6 Cyclin G2 decreased the phosphorylation of Dpr1 Ser762 site

Using KinasePhos and Scansite software, we predicted the effects of cyclin G2 on the phosphorylation of Dpr1 by CK1. We presumed that the binding of cyclin G2 could affect Dpr1 phosphorylation by CK1. Using a Duolink in situ PLA, we detected a reduction in the binding between Dpr1 and CK1 after overexpression of cyclin G2 in HK-2 cells (Figure 6A). In contrast, overexpression of CK1 up-regulated the phosphorylation level of Dpr1. However, co-overexpression of CK1 and cyclin G2 decreased the phosphorylation level of Dpr1 (Figure 6B). These findings reveal that cyclin G2 interacts with Dpr1 and inhibits its phosphorylation by CK1, thus negatively regulating the Wnt signalling pathway.

4 DISCUSSION

The pathogenesis of DN is a complex process involving multiple factors and many molecular mechanisms. Metabolic factors and activation of some signalling pathways are thought to be the driving force behind renal cell injury and DN. Cyclin G2 is an unconventional cyclin that can function as a positive regulator of adipocyte differentiation through the transcriptional activity of PPARγ. Recently, we reported that cyclin G2 expression plays an important role in controlling glioma progression by regulating proliferation and the Warburg effect, which play pivotal roles in the development of DN. These results suggest that cyclin G2 likely plays additional roles in cell signalling and metabolic processes. Our previous research has demonstrated that cyclin G2 has a role in inhibiting glomerulosclerosis in DN, but increasing evidence showed that the degree of renal function injury is more closely related to tubulointerstitial fibrosis. In this study, we found low expression of cyclin G2 in the kidneys of STZ-induced diabetic mice and high glucose–induced HK-2 cells (Figure 1), suggesting that cyclin G2 could be involved in modulating tubulointerstitial fibrosis in DN; the mechanism by which this may occur requires further exploration.

Recent reports consistently point to cyclin G2 as having cell cycle inhibitory functions. Cells with cyclin G2 deficiency are more likely to evolve into malignant tumours. Our results have shown that cyclin G2 is down-regulated in DN mice. Taken together, these results led us to consider the possibility of cyclin G2 deficiency contributes to renal cell injury. In our study, cyclin G2 deficiency increased the severity renal injury and tubulointerstitial fibrosis in DN mice compared with STZ-induced DN WT mice. In addition, collagen fibres were markedly increased and tubulointerstitial extracellular matrix accumulated in DN Ccng2−/− mice. Furthermore, we found that fibrosis-related proteins were also elevated and other pathophysiological changes were evident (Figure 3). These results suggest a protective function for cyclin G2 in diabetic kidney complications. However, nondiabetic Ccng2−/− mice exhibited significant changes in the extent of pathologic renal injury and fibrosis as compared with WT mice, yet, did not exhibit a significant increase in urinary albumin or the UACR (Figures 2 and 3). These results demonstrate that nondiabetic cyclin G2 deficiency causes tubulointerstitial fibrosis and pathological changes, but is probably not sufficient to cause

proximity ligation assays (PLA) were used to confirm that Dpr1 interacted with cyclin G2 in HK-2 cells (Figure 5A,B). Silencing of Dpr1 induced the expression of Dvl2, β-catenin and cyclin D1. In contrast, cyclin G2 inhibited the expression of these three proteins. However, the inhibition of Dvl2, β-catenin and cyclin D1 expression by cyclin G2 was blocked after Dpr1 silencing (Figure 5C,D). These results indicate that cyclin G2 suppresses Dvl2 expression and up-regulates GSK3β activity by binding to Dpr1 and suppressing the Wnt signalling pathway.

Other authors have reported that the phosphorylation level of Dpr1 determines whether Wnt signalling is inhibited or activated. Therefore, we investigated whether cyclin G2 affected the phosphorylation level of Dpr1. Because no reference site or antibody for Dpr1 phosphorylation had been described previously, we determined the level of Dpr1 phosphorylation using an anti-phosphoserine/threonine antibody after coimmunoprecipitation with an anti-Dpr1 antibody. Overexpression of cyclin G2 in HK-2 cells reduced the level of phosphorylated Dpr1 (Figure 5E).

It was previously shown that Dpr1 is unable to bind to Dvl2 and inhibit Wnt signalling following its phosphorylation by CK1. We presumed that the binding of cyclin G2 could affect Dpr1 phosphorylation by CK1. Using a Duolink in situ PLA, we detected a reduction in the binding between Dpr1 and CK1 after overexpression of cyclin G2 in HK-2 cells (Figure 6A). In contrast, overexpression of CK1 up-regulated the phosphorylation level of Dpr1. However, co-overexpression of CK1 and cyclin G2 decreased the phosphorylation level of Dpr1 (Figure 6B). These findings reveal that cyclin G2 interacts with Dpr1 and inhibits its phosphorylation by CK1, thus negatively regulating the Wnt signalling pathway.
albuminuria. Together, these data further suggest that cyclin G2 functions as an inhibitory factor for the progression of renal injury and fibrosis.

Recently, emerging evidence has suggested that insulin may be able to attenuate the activation of Wnt signalling via lowering blood levels in renal tissues of diabetic animal models. Meanwhile, high glucose activated Wnt signalling in renal proximal tubular epithelial cells, whereas inhibition of Wnt by an anti-LRP6 antibody ameliorated tubulointerstitial fibrosis. Previously, we reported that cyclin G2 can suppress Wnt/β-catenin signalling and inhibit

**FIGURE 4** Cyclin G2 inhibits the expression of proteins associated with tubulointerstitial fibrosis via canonical Wnt signalling. A, B, Western blotting and densitometry for β-catenin, GSK3β, cyclin D1 and MMP7 in CCNG2-overexpressing HK-2 cells exposed to 30 mmol/L D-glucose (HG) or 5.5 mmol/L D-glucose + 24.5 mmol/L L-glucose (LG; control group) for 72 h (n = 3). C, D, Western blotting and densitometry results of β-catenin, cyclin D1 and MMP7 in the renal cortex of WT mice, Ccng2−/− mice, STZ-induced DN WT mice, STZ-induced DN Ccng2−/− mice (n = 6). E, Representative immunohistochemistry of the expression of β-catenin in WT mice, Ccng2−/− mice, STZ-induced DN WT mice and STZ-induced DN Ccng2−/− mice (original magnification, 200×, n = 6). F, G, Western blotting and densitometry of β-catenin, p-β-catenin, GSK3β, p-GSK3β, cyclin D1, MMP7, E-cadherin, FN, N-cadherin and collagen IV in cyclin G2-overexpressing HK-2 cells (LV-CCNG2) treated with 5 μmol/L CHIR99021 or DMSO (control group) for 72 h (n = 3). Values are expressed as the mean ± SD; *P < .05; **P < .01; n.s., not significant. Three independent experiments were performed.
gastric cancer cell growth and migration. Furthermore, cyclin G2 also inhibits the epithelial-to-mesenchymal transition by disrupting Wnt/β-catenin signalling in epithelial ovarian cancer. In our study, cyclin G2 attenuated Wnt/β-catenin signalling induced by high-glucose treatment of HK-2 cells. In contrast, cyclin G2 deficiency activated Wnt/β-catenin signalling in our mouse model; this effect was enhanced in mice with STZ-induced DN. Further experiments demonstrated that the GSK3β inhibitor, CHIR99021, interfered with the inhibitory effects of cyclin G2 on the Wnt signalling pathway as well as on expression of fibrosis-related proteins (Figure 4). These data provide strong evidence that cyclin G2 acts as a suppressor of Wnt signalling in DN. Binding between Dpr1 and cyclin G2 resulted in Dvl2 degradation via polyubiquitination, which increased the activity of GSK3β, promoted association of β-catenin with degradation complexes, and interfered with nuclear entry. Finally, we report here that silencing of Dpr1 interrupted the inhibitory effect of cyclin G2 on Dvl2, β-catenin and cyclin D1; this indicates that cyclin G2 suppressed Dvl2 expression by binding to Dpr1 and subsequently inhibiting the expression of fibrosis-related proteins by negatively regulating the Wnt signalling pathway (Figure 5).

CK1 has been shown to phosphorylate Dpr1 and impede the interaction of Dpr1 with cyclin G2, which results in the activation of Wnt signalling. Thus, we demonstrated that cyclin G2 overexpression could influence the binding of CK1 and Dpr1, and inhibit CK1-mediated phosphorylation of Dpr1. Dpr1 has two known domains: a leucine zipper (LZ) domain at the N-terminus and the PDZ-B domain at the C-terminus. Despite affecting the ability of Dpr1 to inhibit the Wnt signalling pathway, the LZ-domain is unnecessary for the binding of Dpr1 to Dvl; however, mutation or absence of the PDZ-B domain does interfere with the binding of Dpr1 and Dvl. After truncating the Dpr1 protein, we identified an interaction between cyclin G2 and the amino acid domain at residues 1-619 and 670-836 of the Dpr1 C-terminal domain (Figure S4). Prediction analysis showed that Dpr1 Ser762 was a candidate site for CK1-mediated phosphorylation of Dpr1 (Figure S4). We constructed a Dpr1 Ser762-mutant vector and found that phosphorylation of Dpr1 and the expression of β-catenin and cyclin D1 were decreased after cotransfection of the cyclin G2 expression vector and the wild-type Dpr1 expression vector, while the expression of phosphorylated β-catenin was increased (Figure 6). These findings have preliminarily identified a new mechanism by which cyclin G2 regulates CK1-mediated Dpr1 phosphorylation at Ser762 in the C-terminal PDZ-B domain.

In conclusion, our study reveals that cyclin G2 negative regulation of Wnt signalling through Dpr1 is part of a novel mechanism by which tubulointerstitial fibrosis is suppressed in DN. We also demonstrated that the interaction between cyclin G2 and Dpr1 decreased CK1-mediated phosphorylation of Dpr1 at Ser762, resulting in the degradation of Dvl2 and the inhibition of β-catenin expression.
Our findings delineate a previously unidentified function for cyclin G2, as a protective factor against the pathologic progression of tubulointerstitial fibrosis, and suggest that the interaction between cyclin G2 and the Wnt signalling pathway renders a new therapeutic target for tubulointerstitial fibrosis in DN.

**ACKNOWLEDGEMENTS**

This work was supported by the Foundation of Liaoning Educational Committee (Nos. LZDK201703, JC2019031 and 2019-BS-285) and National Natural Science Foundation of China (Nos. 81601874 and 81571440).
CONFLICT OF INTEREST
The authors declare that there are no competing interests associated with the manuscript.

AUTHOR CONTRIBUTIONS
Yang Luo and Qi Liu designed research; Xuesha Xing and Manni sun analysed data; Sen Li, Xiaoyu Hou, Danning Wang and Chenyang Zhao performed animal and cell experiments; Chenyang Zhao wrote the paper; Shusen Wang and Jinlan Gao contributed new reagents or analytic tools.

DATA AVAILABILITY STATEMENT
All data generated or analysed during this study are included in this published article (and its additional files).

ORCID
Yang Luo https://orcid.org/0000-0003-1349-0443

REFERENCES
1. Horne MC, Donaldson KL, Goolsby GL, et al. Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J Biol Chem. 1997;272:12650-12661.
2. Zimmermann M, Arachchige-Don AS, Donaldson MS, Dallapiazza RF, Cowan CE, Horne MC. Elevated cyclin G2 expression intersects with DNA damage checkpoint signaling and is required for a potent G2/M checkpoint arrest response to doxorubicin. J Biol. 2012; 287:22838-22853.
3. Kim Y, Shintani S, Kohno Y, Zhang R, Wong DT. Cyclin G2 dysregulation in human oral cancer. Cancer. 2004;64:8980-8986.
4. Hasegawa S, Nagano H, Konno M, et al. Cyclin G2: A novel independent prognostic marker in pancreatic cancer. Oncology Letters. 2015;10:2986-2990.
5. Yin G, Zhou H, Xue Y, Yao B, Zhao W. MicroRNA-340 promotes the tumor growth of human gastric cancer by inhibiting cyclin G2. Oncol Rep. 2016;36:1111-1118.
6. Zimmermann M, Arachchige-Don APS, Donaldson MS, Patriarchi T, Horne MC. Cyclin G2 promotes cell cycle arrest in breast cancer cells responding to fulvestrant and metformin and correlates with patient survival. Cell Cycle. 2016;15(23):3278-3295.
7. Pivovarova O, von Loeffelholz C, Ilkavets I, et al. Modulation of insulin degrading enzyme activity and liver cell proliferation. Cell Cycle. 2015;14:2293-2300.
8. Zimmermann M, Arachchige-Don APS, Donaldson MS, Patriarchi T, Horne MC. Cyclin G2 promotes cell cycle arrest in breast cancer cells responding to fulvestrant and metformin and correlates with patient survival. Cell Cycle. 2016;15:3278-3295.
9. Li S, Gao J, Zhaung X, et al. Cyclin G2 inhibits the warburg effect and tumour progression by suppressing LDHA phosphorylation in glioma. Int J Biol Sci. 2019;15:544-555.
10. Sos KM, Kayampilly P, Byun J, et al. Tissue-specific metabolic reprogramming drives nutrient flux in diabetic complications. JCI Insight. 2016;1:e86976.
11. Zhang G, Darshi M, Sharma K. The warburg effect in diabetic kidney disease. Semin Nephrol. 2018;38:111-120.
12. Moresco RN, Sangoi MB, De Carvalho JAM, Tatsch E, Bochi GV. Diabetic nephropathy: traditional to proteomic markers. Clin Chim Acta. 2013;421:17-30.
13. Gao C, Huang W, Kanasaki K, Xu Y. The role of ubiquitination and sumoylation in diabetic nephropathy. Biomed Res Int. 2014;2014:1-11.
14. Yang L, Wu L, Fan YI, Ma J. Vitamin D receptor gene polymorphisms in association with diabetic nephropathy: a systematic review and meta-analysis. BMC Med Genet. 2017;18:95.
15. Mader JR, Resch ZT, McLean GR, et al. Mice deficient in PAPP-A show resistance to the development of diabetic nephropathy. J Endocrinol. 2013;219:51-58.
16. Xiao L, Wang M, Yang S, Liu F, Sun L. A glimpse of the pathogenetic mechanisms of Wnt/beta-catenin signaling in diabetic nephropathy. Biomed Res Int. 2013;2013:1-7.
17. Zhao C, Gao J, Li S, et al. Cyclin G2 suppresses glomerulosclerosis by regulating canonical Wnt signalling. Biomed Res Int. 2018;2018:6938482.
18. Guo Y, Xiao L, Sun L, Liu F. Wnt/beta-catenin signaling: a promising new target for fibrosis diseases. Physiol Res. 2012;61:337-346.
19. He J, Xu Y, Koya D, Kanasaki K. Role of the endothelial-to-mesenchymal transition in renal fibrosis of chronic kidney disease. Clin Exp Nephrol. 2013;17:488-497.
20. He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y. Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol. 2009;20:765-776.
21. Kavanagh DH, Savage DA, Patterson CC, et al. Haplotype association analysis of genes within the WNT signalling pathways in diabetic nephropathy. BMC Nephrol. 2013;14:126.
22. Ina K, Kitamura H, Tatsukawa S, Takayama T, Fujikura Y, Shimada T. Transformation of interstitial fibroblasts and tubulointerstitial fibrosis in diabetic nephropathy. Med Electron Microsc. 2002;35:87-95.
23. Dickinson ME, McMahon AP. The role of Wnt genes in vertebrate development. Curr Opin Genet Dev. 1992;2:562-566.
24. Nusse R. The Wnt gene family in tumorigenesis and in normal development. J Steroid Biochem Mol Biol. 1992;43:9-12.
25. Bejsovec A. Wnt signalling shows its versatility. Curr Biol. 1999;9:R684-R687.
26. Marotti N, Corrado A, Neve A, Cantatore FP. Systemic effects of Wnt signalling. J Cell Physiol. 2013;228:1428-1432.
27. Dai C, Stolz DB, Kiss LP, Monga SP, Holzman LB, Liu Y. Wnt/beta-catenin signaling promotes podocyte dysfunction and albuminuria. J Am Soc Nephrol. 2009;20:1997-2008.
28. Kawakami T, Ren S, Duffield JS. Wnt signalling in kidney diseases: dual roles in renal injury and repair. J Pathol. 2013;229:221-231.
29. Lin CL, Wang JY, Huang YT, Kuo YH, Surendran K, Wang FS. Wnt/beta-catenin signaling modulates survival of high glucose-stressed mesangial cells. J Am Soc Nephrol. 2006;17:2812-2820.
30. Zhou T, He X, Cheng R, et al. Implication of dysregulation of the canonical wingless-type MMTV integration site (WNT) pathway in diabetic nephropathy. Diabetologia. 2012;55:255-266.
31. Clevers H, Nusse R. Wnt/beta-catenin signalling and disease. Cell. 2012;149:1192-1205.
32. Cheyette BN, Waxman JS, Miller JR, et al. Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation. Developmental Cell. 2002;2:449-461.
33. Chen H, Liu L, Ma B, et al. Protein kinase A-mediated 14-3-3 association impedes human Dapper1 to promote dishevelled degradation. J Biol Chem. 2011;286:14870-14880.
34. Wen J, Chiang YJ, Gao C, et al. Loss of Dact1 disrupts planar cell polarity signalling by altering dishevelled activity and leads to posterior malformation in mice. J Biol Chem. 2010;285:11023-11030.
35. Zhang L, Gao X, Wen J, Ning Y, Chen Y-G. Dapper1 antagonizes Wnt signalling by promoting dishevelled degradation. J Biol Chem. 2006;281:8607-8612.
36. Yung S, Chau MKM, Zhang Q, Zhang CZ, Chan TM. Sulodexide decreases albuminuria and regulates matrix protein accumulation in C57BL/6 mice with streptozotocin-induced type I diabetic nephropathy. PLoS ONE. 2013;8:e54501.
37. Zhou T, He X, Cheng R, et al. Implication of dysregulation of the canonical wingless-type MMTV integration site (WNT) pathway in diabetic nephropathy. *Diabetologia*. 2012;55:255-266.

38. Cheng R, Ding L, He X, Takahashi Y, Ma JX. Interaction of PPARalpha with the canonic Wnt pathway in the regulation of renal fibrosis. *Diabetes*. 2016;65:3730-3743.

39. Brobel A, Kämmerer F, Tag C, Steger K, Gattenlöchner S, Wimmer M. PTPIP51—A New RelA-tionship with the NFκB Signaling Pathway. *Biomolecules*. 2015;5:485-504.

40. Konishi A, Izumi T, Shimizu S. TRF2 protein interacts with core histones to stabilize chromosome ends. *J Biol Chem*. 2016;291:20798-20810.

41. Chen L, Chen DQ, Wang M, et al. Role of RAS/Wnt/beta-catenin axis activation in the pathogenesis of podocyte injury and tubulo-interstitial nephropathy. *Chem Biol Interact*. 2015;212:52-63.

42. Li Z, Zhou L, Wang Y, et al. (Pro)renin Receptor Is an Amplifier of Wnt/beta-Catenin Signaling in Kidney Injury and Fibrosis. *J Am Soc Nephrol*. 2016;27:2339-2349.

43. Bock AS, Leigh ND, Bryda EC. Effect of Gsk3 inhibitor CHIR99021 on aneuploidy levels in rat embryonic stem cells. *Vitro Cell Dev Biol Anim*. 2014;50:572-579.

44. Lam AQ, Freedman BS, Morizane R, Lerou PH, Valerius MT, Bonventre JV. Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers. *J Am Soc Nephrol*. 2014;25:1211-1225.

45. Gao J, Zhao C, Liu QI, et al. Cyclin G2 suppresses Wnt/beta-catenin signaling and inhibits gastric cancer cell growth and migration through Dapper1. *J Exp Clin Cancer Res*. 2018;37:217.

46. Teran E, Branscomb AD, Seeling JM. Dpr Acts as a molecular switch, inhibiting Wnt signaling when unphosphorylated, but promoting Wnt signaling when phosphorylated by casein kinase Idelta/epsilon. *PLoS ONE*. 2009;4:e55022.

47. Bielecki P, Jensen V, Schulze W, et al. Cross talk between the response regulators PhoB and TctD allows for the integration of diverse environmental signals in *Pseudomonas aeruginosa*. *Nucl Acids Res*. 2015;43:6413-6425.

48. Li X, Zhong LI, Wang Z, et al. Phosphorylation of IRS4 by CK1α/β promotes its degradation by CHIP through the ubiquitin/lysosome pathway. *Theranostics*. 2018;8:3643-3653.

49. Grayson PC, Eddy S, Taroni JN, et al. Metabolic pathways and immunometabolism in rare kidney diseases. *Ann Rheum Dis*. 2018;77:1226-1233.

50. Brosius FC, Khoury CC, Buller CL, Chen S. Abnormalities in signaling pathways in diabetic nephropathy. *Expert Rev Endocrinol Metab*. 2010;5:51-64.

51. Aguilar V, Annicotte JS, Escote X, Vendrell J, Langin D, Fajas L. Cyclin G2 regulates adipogenesis through PPAR gamma coactivation. *Endocrinology*. 2010;151:5247-5254.

52. Bernaudo S, Khazai S, Honarpavar E, Kopteva A, Peng C. Epidermal growth factor promotes cyclin G2 degradation via calpain-mediated proteolysis in gynaecological cancer cells. *PLoS ONE*. 2017;12:e0179906.

53. Bernaudo S, Salem M, Qi X, et al. Cyclin G2 inhibits epithelial-to-mesenchymal transition by disrupting Wnt/beta-catenin signaling. *Oncogene*. 2016;35:4816-4827.

54. Zhou T, He X, Cheng R, et al. Implication of dysregulation of the canonical wingless-type MMTV integration site (WNT) pathway in diabetic nephropathy. *Diabetologia*. 2012;55:255-266.

55. Cheyette BN, Waxman JS, Miller JR, et al. Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation. *Dev Cell*. 2002;2:449-461.

56. Gloy J, Hikasa H, Sokol SY. Frodo interacts with Dishevelled to transduce Wnt signals. *Nature Cell Biology*. 2002;4:351-357.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhao C, Gao J, Li S, et al. Cyclin G2 regulates canonical Wnt signalling via interaction with Dapper1 to attenuate tubulointerstitial fibrosis in diabetic nephropathy. *J Cell Mol Med*. 2020;24:2749-2760. [https://doi.org/10.1111/jcmm.14946]