RESEARCH

Txnip deficiency promotes β-cell proliferation in the HFD-induced obesity mouse model

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Abstract

Elucidating the mechanisms of regulation of β-cell proliferation is key to understanding the pathogenesis of diabetes mellitus. *Txnip* is a tumor suppressor that is upregulated in diabetes and plays an important role in the regulation of insulin sensitivity; however, its potential effect on pancreatic β-cell proliferation remains unclear. Here, we evaluated the role of *Txnip* in pancreatic β-cell compensatory proliferation by subjecting WT and *Txnip* knockout (KO) mice to a high-fat diet (HFD). Our results demonstrate that *Txnip* deficiency improves glucose tolerance and increases insulin sensitivity in HFD-induced obesity. The antidiabetogenic effect of *Txnip* deficiency was accompanied by increased β-cell proliferation and enhanced β-cell mass expansion. Furthermore, *Txnip* deficiency modulated the expression of a set of transcription factors with key roles in β-cell proliferation and cell cycle regulation. *Txnip* KO in HFD mice also led to activated levels of p-PI3K, p-AKT, p-mTOR and p-GSK3β, suggesting that *Txnip* may act via PI3K/AKT signaling to suppress β-cell proliferation. Thus, our work provides a theoretical basis for *Txnip* as a new therapeutic target for the treatment of diabetes mellitus.

Key Words

- *Txnip*
- pancreatic β cell
- proliferation
- PI3K/AKT signaling pathway

Endocrine Connections (2022) **11**, **e210641**

Introduction

Diabetes mellitus (DM) is a progressive and chronic metabolic disease with multiple etiologies. DM is characterized by defective glucose metabolism due to low levels or an absolute lack of insulin, which seriously threatens human physical and mental health. Diabetes is correlated with the occurrence and development of many diseases, including exacerbated effects of coronavirus (COVID-19) infection (1). Compared with non-diabetic individuals, diabetic patients require more medical interventions and have a higher rate of severe pneumonia, multiple organ injury and mortality (2, 3). As a result, DM has become a major threat to human health worldwide. In the pathogenesis of diabetes, for either type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), a deficiency in the functional pancreatic β-cell mass and decreased numbers of β cells cause insufficiency of insulin secretion, which constitutes the key mechanism of DM (4). Pancreatic β-cell replacement and regeneration therapies provide promising approaches to the treatment of diabetes. Therefore, a key goal in the treatment of diabetes is to promote the proliferation of β cells and expand the functional β-cell mass. However, the mechanism driving β-cell proliferation as an adaptive process to insulin deficiency remains unclear.

In healthy individuals, the number of β cells is maintained by neogenesis of endocrine progenitor cells, reprogramming of non-β cells or replication of pre-existing β cells (5, 6, 7). However, in adults, mature β cells rarely replicate and proliferate (8). β-cell neogenesis predominantly takes place during embryogenesis and neonatal life, whereas β-cell proliferation rates decrease dramatically with age (9). Nevertheless, in response to

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increased physiological and pathological metabolic demands in adults, such as insulin resistance, obesity, pregnancy or pancreatic injury, β cells may undergo compensatory proliferation that is critical for increasing insulin demands and maintaining glucose homeostasis (10, 11, 12, 13, 14). Previous studies have demonstrated that a high-calorie Western diet or a high-fat diet (HFD) stimulates mild β-cell proliferation and expansion of β-cell mass in humans and rodents (15, 16). However, the molecular mechanisms underlying this progression remain unclear. Understanding the mechanisms behind β-cell regeneration is crucial for identifying novel targets to prevent or revert disease. Numerous studies have demonstrated that pancreatic transcription factors, including *Pdx1*, *Mafa* and *Foxo1*, play vital roles in β-cell proliferation, development, differentiation, maturation and functional maintenance (8, 17, 18, 19). Insulin, glucose, growth factors, hormones and nutrients stimulate multiple pathways that promote β-cell replication, at least in part through the activation of pro-proliferative/pro-survival protein kinases, such as PI3K and AKT. The PI3K/AKT signaling pathway plays a critical role in regulating β-cell mass and function (20, 21). The function of AKT in stimulating β-cell replication may occur via the activation of cell cycle regulators, such as *Cyclin D2*, *Cdk4* and *p27Kip1* (22, 23, 24). In addition, the pancreatic transcription factor *Foxo1* is a downstream target of AKT, and *Pdx1* localization and protein levels are tightly regulated throughout the cell cycle (25, 26). Thus, the precise regulatory mechanisms of HFD-induced β-cell proliferation are likely to be multifaceted.

Thioredoxin-interacting protein (*Txnip*; also known as thioredoxin-binding protein-2/*TBP-2*) is a member of the α-arrestin family that was first identified as vitamin-D3-upregulated protein-1 (*VDUP1*) in the human promyelocytic leukemia cell line (HL-60) (27, 28). *Txnip* is a multifunctional protein that is ubiquitously expressed and is localized in the nucleus, mitochondria and cytoplasm. It is associated with the development of diabetes, cardiovascular disease, CNS diseases, cancer and other diseases (29, 30, 31). Recently, *Txnip* has been the focus of investigation due to its unique characteristic of specifically binding and regulating Trx activity (32). *Txnip* is closely associated with glucose and lipid metabolism and is a major regulator of glucose homeostasis (33, 34). Consistently, several studies have demonstrated that *Txnip* expression is increased in DM and that it plays an important role in the regulation of insulin sensitivity (35, 36, 37). *Txnip*-deficient HcB-19 (*HcB*) mice have been shown to have a three-fold increase in insulin levels (37), increased pancreatic β-cell mass and enhanced insulin secretion and sensitivity (36, 38). However, the associated mechanisms for *Txnip* in DM, including its potential role in β-cell proliferation, have not been fully characterized.

As a tumor suppressor, *Txnip* has been shown to be involved in cellular growth, with functions in suppressing cellular proliferation and arresting cell cycle progression (39, 40). Numerous studies have demonstrated that *Txnip* is downregulated in various cancers and human cancer cells, such as breast cancer, stomach cancer, lung cancer, colon cancer, cervical cancer, gastrointestinal cancer, prostate cancer, cutaneous T-cell lymphoma and adult T-cell leukemia (31, 41, 42, 43, 44, 45, 46). There is also increasing evidence that *Txnip* can inhibit the proliferation of tumor cells. Therefore, we hypothesized that *Txnip* may also modulate the proliferation of pancreatic β cells.

In this study, to uncover the mechanism of *Txnip* in regulating insulin sensitivity in DM, we explored the relationship between *Txnip* and pancreatic β-cell proliferation in HFD-induced mice. Our results provide insight into the mechanism of *Txnip* in regulating DM and suggest that it may serve as a novel target for clinical treatment.

Materials and methods

Animals and ethics

Experimental studies were performed on male mice on a C57BL/6J background. *Txnip* knockout mice (KO, catalog number: ENSMUST00000074519.12) were originally generated by GemPharmatech Co., Ltd (Nanjing, China) using CRISPR/Cas9 technology. In brief, Cas9 mRNA and gRNA (sequence, S1: 5'-ACACGGTGTGCTCCTAGCG-3'; S2: 5'-CAACCAATCAGCGAGGCCGC-3'; S3: 5'-GGTCTCCGGCTTCAAG-3'; S4: 5'-CTATGCCACAGTGCGGGCAC-3') generated by *in vitro* transcription were injected into fertilized eggs of C57BL/6J mice via the microinjection technique. Then, Cas9 protein binds to the target site under the guidance of gRNA, resulting in DNA double-strand break, thus realizing the deletion of base sequence at the target site. *Txnip* KO homozygous mice and WT littermate mice were used. All mice were bred, reared and housed in our specific pathogen-free facility at the Shanxi Medical University. The mice were maintained under standardized conditions on a fixed 12 h light: 12 h darkness cycle with free access to food and water. When the mice were 4 weeks old, they were randomly divided into four groups: (1) WT group; (2) KO group; (3) WT-HFD group; (4) KO-HFD group. They were

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fed normal chow diet (NC) or high-fat diet (HFD) starting at age 4 weeks, with continuous feeding for 12 weeks. HFD was composed of 60% fat, 20% carbohydrate and 20% protein and was prepared by Beijing Vital River Laboratory Animal Technology Co., Ltd (D12492, Beijing, China). The mice were weighed weekly until the end of the experiment, at which time they were anesthetized with isoflurane and sacrificed by cervical dislocation.

All animal procedures were approved by and performed in accordance with the Ethical Committee of Shanxi Medical University with the approval of the Institutional Animal Care and Use Committee. Concerted efforts were made to minimize the number of animals used and their suffering.

Bio-Plex assays

Mice were anesthetized with isoflurane, and the blood was collected from the retroorbital venous plexus using a microcapillary tube. Blood samples were collected and allowed to clot at room temperature for 30–45 min. Then, the samples were centrifuged at 1000 ***g*** for 15 min at 4°C, and the supernatant was transferred to a clean centrifuge tube. Centrifugation was repeated at 10,000 ***g*** for 10 min to completely remove platelets and other precipitates. Serum samples were then diluted 1:4 and stored at −80°C until assay.

For Mouse Bio-Plex Pro Assays, 25 μL of serum per sample was analyzed. The concentration levels of insulin, glucagon, glucagon-like peptide-1 (GLP1) and Leptin were measured using the Mouse Diabetes Panel 8-Plex Assay (catalog no. 171-F7001M, BIO-RAD) according to the manufacturer's instructions.

Oral glucose tolerance test and insulin tolerance test

To perform the oral glucose tolerance test (OGTT), mice were fasted overnight for 16 h. Fasting blood glucose was measured from tail vein blood using the OneTouch Ultra glucose meter (Life Scan, Milpitas, USA). All mice were administered glucose (2 g/kg body weight) by oral gavage, and the blood glucose was measured at 15, 30, 60, 90 and 120 min after administration. For the insulin tolerance test (ITT), mice were fasted for 6 h. The fasting blood glucose was measured, and then, the mice were injected intraperitoneally with 0.075 U/mL human insulin in filter-sterilized PBS at 0.1 mL per 10 g body weight. Blood glucose levels were measured at 15, 30, 60, 90 and 120 min after injection.

β-cell mass and β-cell proliferation analysis

For morphometric analysis of the β-cell mass, the pancreas was removed, and fat and lymph nodes were cleared in saline solution. The whole body and the pancreas were weighed, and then, the pancreas was fixed in 4% paraformaldehyde for 48 h, embedded in paraffin and sectioned at a thickness of 5 μm. Seven to 11 sections of each pancreas were prepared approximately 200 μm apart. Then, the sections were immunostained for insulin (1:6400, CST, 3014s) and scanned at 40× using a Nano Zoomer-SQ Digital slide scanner (Hamamatsu, Japan). Images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). The β-cell mass was determined by calculating the insulin-positive percent area of each pancreas and multiplying the pancreas weight. Five to six mice per group were analyzed.

β-cell proliferation was assessed by immunostaining for *insulin* (1:100, Abcam, ab7842) and either *Ki67* (1:200, Abcam, ab15580) or *Pcna* (1:2400, CST, 2586S). Two to three slides per pancreas were used to calculate the average percentage of *insulin*/*Ki67* or *insulin*/*Pcna* double-positive cells. At least 4000 cells were counted per animal. Images were captured using an Olympus FV3000 laser scanning confocal microscope (Olympus), and cells were counted using Image-Pro Plus 6.0 software. The percentage of proliferating β cells was calculated by dividing the total number of *insulin*/*Ki67* or *insulin*/*Pcna* double-positive cells by the total number of insulin-positive cells.

Islet isolation

Islets were isolated by collagenase v (Sigma, 1 mg/mL) perfusion through the common hepatic bile duct into the pancreas. Then, the pancreas was dissected and digested for 15 min in a 37°C shaking water bath. Digestion was halted with 10% serum HBSS. The samples were then centrifuged at 1200 ***g*** for 15 min, and the supernatant was discarded. The islets were washed three times, observed under a light microscope and cultured in RPMI medium 1640 containing 10% fetal bovine serum and antibiotics.

RT-qPCR

Total RNA was extracted from islets using the RNAiso Plus (code no.9109) (Takara). The integrity of the RNA was verified by agarose gel electrophoresis, and the absorbance was determined by NanoDrop (NanoDrop Technologies, Wilmington, USA). The cDNA was synthesized using a PrimeScript™RT reagent Kit with gDNA Eraser (code no.

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RR047A) (Takara) according to the manufacturer's protocol. The mRNA expression was quantified by RT-qPCR with TB Green®Premix Ex Taq™II (Takara) using a LightCycler® 96 System (Roche), with the data shown as the $2^{-\Delta\Delta Ct}$ value. All mRNA expression levels are normalized to *β-actin*. The primer sequences are listed in Table 1.

Table 1 Primer sequences.

Gene	Primer sequences
β-actin	F: 5′CACTATTGGCAACGAGCGGTTCCG3′ R: 5′ACGGATGTCAACGTCACACT3′
Txnip	F: 5′CAAGGGTCTCAGCAGTGCAAAC3′ R: 5′AAGCTCGAAGCCGAACTTGTACTC3′
Ki67	F: 5′GTGTCAAACAAACTTGAATCTGTGG3′, R: 5′TCTGCAGATGCATCAAACTTGG3′
Pcna	F: 5′GAGAGCTTGGCAATGGGAACA3′, R: 5′CAGAGCAAACGTTAGGTGAACAGG3′;
Pdx1	F: 5′GCCCGGGTGTAGGCAGTAC3′ R: 5′CAGTGGGCAGGAGGTGCTTA3′
Mafa	F: 5′GGAGGTCATCCGACTGAAACA3′ R: 5′GCACCTCTCGCTCTCCAGAAT3′
Mafb	F: 5′TGAGCTAGAGGGAGGAAGGA3′ R: 5′CCGGGTTTCTCTAACTCTGC3′
Foxo1	F: 5′GAGAAGAGGCTCACCCTGTC3′ R: 5′ACAGATTGTGGCGAATTGAA3′
Foxm1	F: 5′CACTTGGATTGAGGACCACTT3′ R: 5′GTCGTTTCTGCTGTGATTCC3′
Nkx6.1	F: 5′AGAGAGCACGCTTGGCCTATTC3′ R: 5′GTCGTCAGAGTTCGGGTCCAG3′
Ins2	F: 5′TCAACATGGCCCTGTGGAT3′ R: 5′AAAGGTGCTGCTTGAAAAAGC3′
Glut2	F: 5′CTCGTGGCGCTGATGCT3′ R: 5′CTGGTTGAATAGTAAAATATCCCATTGA3′
Ccna2	F: 5′TTGGCTGCACCAACAGTAAATCA3′ R: 5′ACGGGTCAGCATCTATCAAACTCA3′
Ccnb1	F: 5′CATGCTGGACTACGACATGGTG3′ R: 5′ACATTCTTAGCCAGGTGCTGCATA3′
Ccnd1	F: 5′GCGTACCCTGACACCAATCTC3′ R: 5′CTCCTCTTCGCACTTCTGCTC3′
Ccnd2	F: 5′GGCTAAGACAGGGTGGCTTTCA3′ R: 5′GATTGCTACCTCCAGTTCCCACA3′
Ccne1	F: 5′TGATCGTTACATGGCATCACAA3′ R: 5′GCGCCATCTGTAACATAAGCAA3′
Cdk2	F: 5′GGACTAGCAAGAGCCTTTGG3′ R: 5′AAGAATTTCAGGTGCTCGGT3′
Cdk4	F: 5′ACATGTGGAGCGTTGGCTGTA3′ R: 5′CAACTGGTCGGCTTCAGAGTTTC3′
Cdkn2a(p16)	F: 5′CGTGAACATGTTGTTGAGGCTAGAG3′ R: 5′ATCTGCACCGTAGTTGAGCAGAAG3′
Cdkn1a(p21)	F: 5′AGTCTCATGGTGTGGTGGAA3′ R: 5′GACATCACCAGGATTGGACA3′
Cdkn1b(p27)	F: 5′AGTGTCCAGGGATGAGGAAG3′ R: 5′CTTCTGTTCTGTTGGCCCTT3′
Cdkn1c(p57)	F: 5′AATCAGCCAGCCTTCGAC3′ R: 5′ATCACTGGGAAGGTATCGCT3′
Rb	F: 5′CCCAACACATGCTTTGAACTGA3′ R: 5′TGGGTTGTACTGTACTAGGGTCCTC3′

Western blot analysis

Total proteins were extracted from islets, and protein levels were measured by the BCA method. Equal quantities of protein were electrophoresed using a TGX Stain-Free FastCast Acrylamide Kit, 10% (BIO-RAD) and then transferred onto PVDF membranes (Millipore). The membranes were incubated in TBST buffer containing 5% BSA (Boster Biological Technology Co., Ltd, Wuhan, China) to block non-specific binding at room temperature and then were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were washed in TBST buffer and then were incubated with secondary antibodies for 1 h at room temperature. Finally, the proteins bands were visualized using ECL Plus and the ChemiDocTM Imaging System (BIO-RAD). The band intensities were analyzed with ImageJ software (US National Institutes of Health). Protein levels were normalized to the β-actin protein, and phosphorylated forms were normalized to phosphorylation-independent levels of the same protein. Antibodies used in this study are listed in Table 2.

Statistical analysis

Statistical analysis was performed using SPSS20.0 software (IBM). Data are expressed as the mean ± s.e.m. The Kolmogorov–Smirnov test was used to verify data normality. The independent samples *t* test was applied for comparisons between two groups, while one-way ANOVA was applied for multi-groups. *P* value <0.05 was considered statistically significant.

Table 2 Antibodies used for Western blot analysis.

Antibody	Catalog no.	Company	Dilution ratio
TXNIP	ab188865	Abcam	1:2000
Cyclin D2	ab230883	Abcam	1:2000
CDK4	ab137675	Abcam	1:3000
p27	ab92741	Abcam	1:5000
p-PI3K	17366s	CST	1:1000
PI3K	4292s	CST	1:1000
p-AKT	9271s	CST	1:1000
AKT	9272s	CST	1:1000
p-mTOR	5536s	CST	1:1000
mTOR	2983s	CST	1:1000
p-GSK3β	5558s	CST	1:1000
GSK3β	9315s	CST	1:1000
β-actin	AP0060	Bioworld	1:10,000

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Results

Txnip deficiency and HFD feeding increase the pancreatic β-cell mass

To ensure that *Txnip* KO mice were eligible, *Txnip* protein expression level and mRNA expression level were measured. The results show that *Txnip* was almost undetectable of both protein and mRNA levels in *Txnip* KO mice (Fig. 1A and B). The proliferation ability of adult pancreatic β cells is normally very low; however, previous studies have demonstrated that high-fat feeding can induce pancreatic β cell compensatory proliferation (15). Thus, to induce mouse pancreatic β-cell proliferation, we fed mice NC or HFD. To evaluate the role of *Txnip* in the response to HFD, we treated both WT and KO mice. The mice were weighed weekly, and after 12 weeks, we observed that HFD resulted in substantially increased weight gain in the WT-HFD mice, whereas the weight gain of the KO-HFD mice was not significantly increased, thus supporting a protective effect of *Txnip* deficiency in preventing weight gain during HFD (Fig. 1C). Next, we measured the ratio of the pancreas to body weight. As shown in Fig. 1D, the ratios of the WT and KO groups were not significantly different, while the ratio of the KO-HFD group was significantly greater than the ratio of the WT-HFD group of mice. To verify these findings, we performed immunohistochemical staining to visualize the pancreatic islet morphology. The islet number and size were increased in both WT-HFD and KO-HFD mice (Fig. 1E). Furthermore, the β-cell mass was significantly increased in both WT-HFD and KO-HFD mice when compared to their control groups fed with normal chow, and the β-cell mass in the KO-HFD group was significantly increased compared with WT-HFD group mice (Fig. 1F). Collectively, these results indicate that *Txnip* KO mice fed on HFD have increased pancreatic β-cell mass in the absence of significant weight gain, which is indicative of productive pancreatic β-cell compensatory proliferation.

Txnip deficiency improves glucose tolerance and insulin sensitivity in HFD-induced obesity

To verify that *Txnip* KO improves glucose metabolism and insulin sensitivity in the HFD mouse model, we measured the serum insulin and fasting blood glucose levels of each

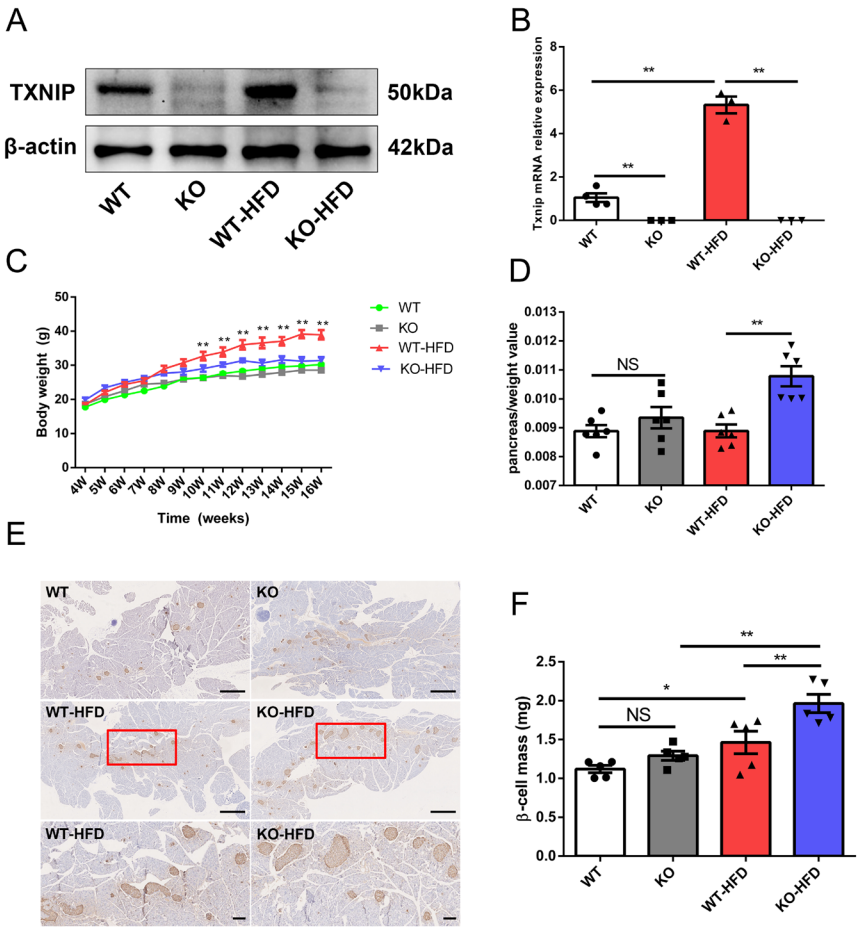

Figure 1
Txnip deficiency and HFD accelerate the accumulation of β-cell mass. (A) The protein expression levels of TXNIP ($n = 3$). (B) The mRNA expression levels of *Txnip* ($n = 3$). (C) WT and *Txnip* knockout (KO) mice were fed normal chow diet (NC) or high-fat diet (HFD) starting at 4 weeks of age and continuing for 12 weeks. The mice were weighed weekly. **Significant difference in weight gain for WT-HFD vs KO-HFD mice. No significant differences were observed for the WT and KO groups of mice ($n = 10$). (D) The ratios of pancreas/body weight after 12 weeks. **Significant difference for KO-HFD vs WT-HFD mice. No significant differences were observed between the other three groups ($n = 6$). (E) Immunohistochemical staining of pancreatic tissue for insulin. The islet numbers and sizes were increased in KO-HFD mice. Scale bars, 1 mm (upper) and 0.5 mm (lower). (F) The β-cell mass in the four groups of mice. The β-cell mass was significantly greater in KO-HFD vs WT-HFD mice, while there were no significant differences between the WT and KO groups ($n = 5$). NS, not significant, $^*P < 0.05$, $^{**}P < 0.01$.

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group of mice. For both WT and KO mice, the serum insulin levels of HFD mice were significantly higher than those of NC mice. Furthermore, the insulin level of the KO-HFD group was greater than the insulin level of the WT-HFD group mice, whereas there was no significant difference between the insulin levels of the WT and KO groups of mice that were fed with NC (Fig. 2A). Furthermore, the fasting blood glucose levels of HFD mice were higher than those of NC mice, and the level of fasting blood glucose in the KO-HFD group was lower than that in the WT-HFD group (Fig. 2B). Double immunofluorescence staining of insulin/glucagon demonstrated that the pancreatic α cells were evenly distributed around islets in the WT and KO groups of mice, while the number of pancreatic α cells was higher in the WT-HFD and KO-HFD groups, and fewer cells were infiltrated into the middle of the islets (Fig. 2C).

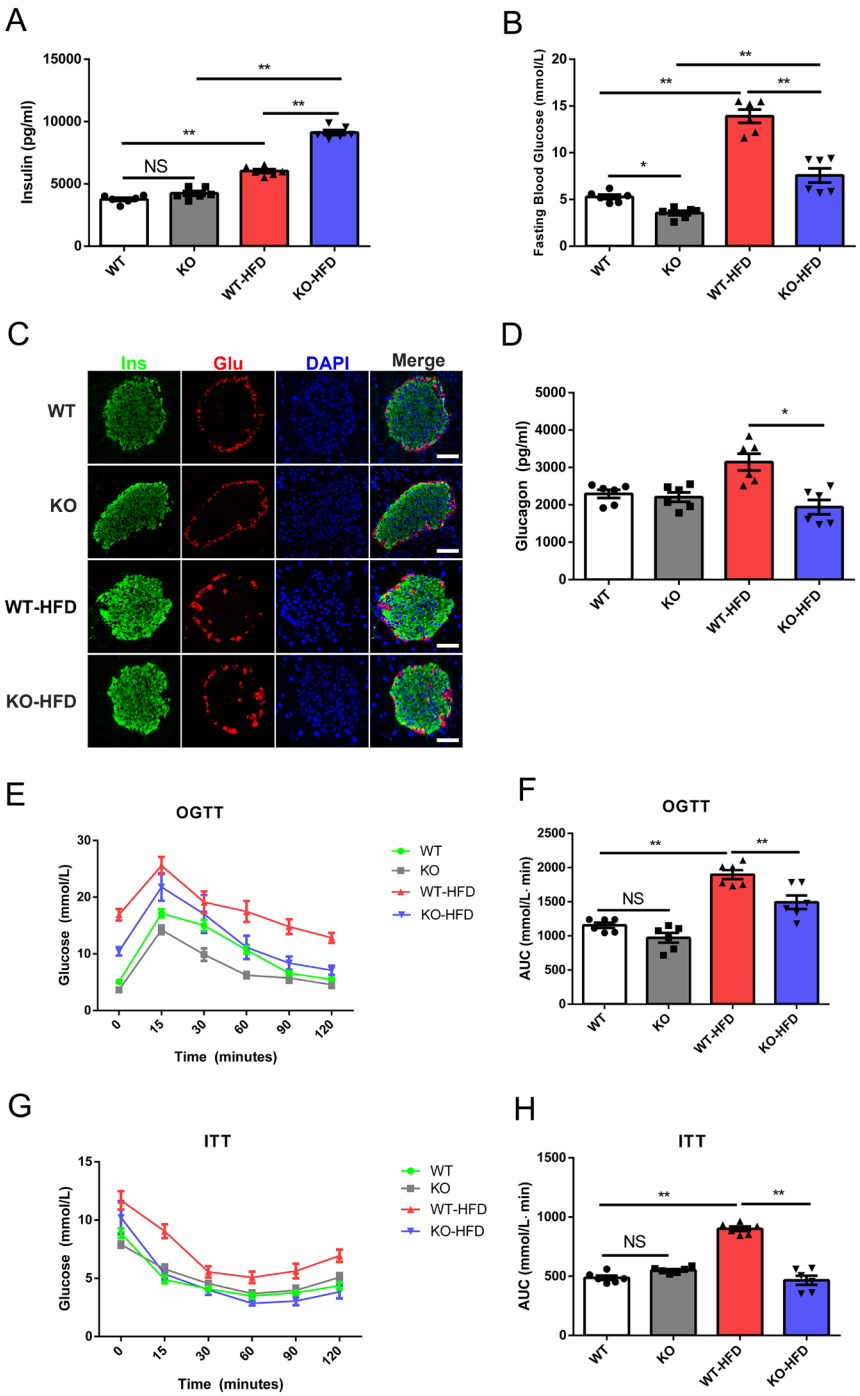

Figure 2
Txnip knockout increases insulin secretion, decreases fasting blood glucose and improves glucose tolerance and insulin sensitivity. (A) Bio-Plex assays showing serum insulin levels in four groups of mice. The serum insulin levels were significantly higher in HFD mice. Additionally, the serum insulin levels were higher in KO-HFD vs WT-HFD mice (n = 6). (B) Fasting blood glucose levels. The levels were significantly higher in HFD mouse groups. Additionally, the fasting blood glucose levels were lower in *Txnip* knockout vs WT mouse groups (n = 6). (C) Double immunofluorescence staining of insulin/glucagon. The number of pancreatic α cells was higher in HFD mice, but there was no apparent difference between *Txnip* knockout and WT mice. Scale bar, 50 μm. (D) The glucagon levels were lower in KO-HFD vs WT-HFD mice (n = 6). (E) Oral glucose tolerance test (OGTT) showing changes in blood glucose levels. (F) The area under the curve (AUC) was calculated for the OGTT (n = 6). (G) Insulin tolerance test (ITT) showing changes in blood glucose levels. (H) The AUC of ITT is shown (n = 6). NS, not significant, $^*P < 0.05$, $^{**}P < 0.01$.

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Furthermore, the glucagon levels were lower in the KO-HFD group compared to the WT-HFD group, though there was no observable difference between the WT and KO groups of mice (Fig. 2D). These results confirm that *Txnip* KO protects mice from the effects of HFD in inducing diabetes.

To further investigate β-cell mass functionality, we performed the OGTT and ITT before sacrificing the mice. The blood glucose levels in the OGTT were higher in HFD mice than in NC mice, while the post-load glucose levels were significantly lower in the *Txnip* KO groups than in their control groups mice (Fig. 2E and F). The results indicate that the impaired glucose tolerance in mice on HFD is improved by deletion of *Txnip*. Similarly, the blood glucose levels from the ITT were significantly lower in KO-HFD mice than in WT-HFD group mice, though there were no differences between the WT and KO groups of mice (Fig. 2G and H). Collectively, these results indicate that HFD impairs insulin tolerance and that deletion of *Txnip* can improve β-cell mass functionality and insulin sensitivity.

Txnip deficiency promotes β-cell proliferation in HFD-induced mice

To measure the effect of *Txnip* deficiency on β-cell proliferation, we performed double immunofluorescence staining of *insulin* and proliferation marker (*Ki67*) or *insulin* and proliferating cell nuclear antigen (*Pcna*). *Ki67*/*insulin* double-positive cells were barely detectable in NC-fed WT and KO mice; however, significantly more β-cell proliferation was observed in the HFD groups of mice (1.7- to 2.3-fold more $Ki67^{+}Ins^{+}$ cells). In addition, the percentages of $Ki67^{+}Ins^{+}$ cells were almost 1.4-fold greater in KO-HFD mice compared with WT-HFD mice, whereas there was no significant difference in the β-cell proliferation between WT and KO groups of mice (Fig. 3A and B). Similar results were obtained with *Pcna*. The percentage of $Pcna^{+}Ins^{+}$ cells in the HFD groups was 1.9- and 2.6-fold greater than in the NC-fed groups. Furthermore, the percentage of $Pcna^{+}Ins^{+}$ cells was almost 1.8-fold greater in KO-HFD mice than in WT-HFD mice (Fig. 3D and E). These results indicate that *Txnip* KO can promote β-cell proliferation in HFD-treated mice.

To further confirm these differences, we evaluated the relative expression of *Ki67* and *Pcna* mRNA levels. The results suggest that the mRNA levels of *Ki67* and *Pcna* were significantly greater in the KO-HFD group compared with WT-HFD group mice. However, there were no differences between the WT and KO groups of mice (Fig. 3C and F). Thus, these results suggest that *Txnip* deficiency increases the β-cell proliferation caused by HFD.

Txnip deficiency modulates transcription factor expression in HFD mice

A variety of pancreatic transcription factors, including *Pdx1* and *Mafa*, play crucial roles in the pancreas and function in the maintenance of mature β cells. To identify pancreatic transcription factors that are modulated in mice, we performed real-time RT-PCR. Our results show that the mRNA levels of *Pdx1*, a marker of mature β cells, were upregulated in the KO-HFD group compared with the WT-HFD group of mice, whereas there was no significant difference between the WT and KO groups of mice that were fed with NC (Fig. 4A). Similarly, the mRNA levels of *Mafa*, a β-cell-specific transcription factor that functions as a potent activator of insulin gene transcription (Fig. 4B), as well as *Mafb*, *Foxm1* and *Ins2* (Fig. 4C, E and G), were significantly more highly expressed in KO-HFD mice as compared with WT-HFD mice. By contrast, the mRNA levels of *Foxo1*, a key transcription factor in insulin signaling, were decreased in the KO-HFD group of mice (Fig. 4D), and the expression of *Nkx6.1* or *Glut2* mRNA was not significantly different (Fig. 4F and H). These results suggest that *Txnip* modulates a set of transcription factors with known roles in regulating gene expression in β cells.

Txnip deficiency accelerates cell cycle progression to promote β-cell replication

To further dissect the molecular events underlying the enhanced β-cell proliferation in mice, we evaluated the levels of *cyclins* and *Cdks* that are known to be modulated during cell cycle progression in mammals. This includes *cyclinD2*, a key regulator of the cell cycle in adult β-cell growth; *Cdk4*, which forms a complex with *cyclinD2* and is known to have a critical role in the regulation of β-cell mass; and *p27Kip1*, an inhibitor of *Cdk* activity that is also known to regulate β-cell mass. Western blot results showed that the protein levels of cyclin D2 and CDK4 were upregulated, and the protein levels of *p27* were downregulated in the KO-HFD group compared with the WT-HFD group. Yet, there was no significant change between the WT and KO groups of mice that were fed with NC (Fig. 5A, B, C and D), which is consistent with the increased proliferation of β cells in *Txnip*-deficient mice. Furthermore, real-time quantitative PCR results were consistent with the Western blot results (Fig. 5E, F and G), suggesting that the expression of these proteins is regulated at the level of the mRNA. To further investigate the role of cell cycle regulators, we performed real-time quantitative PCR of *Rb*, *cyclins* and cyclin-dependent kinase inhibitors (*CDKIs*),

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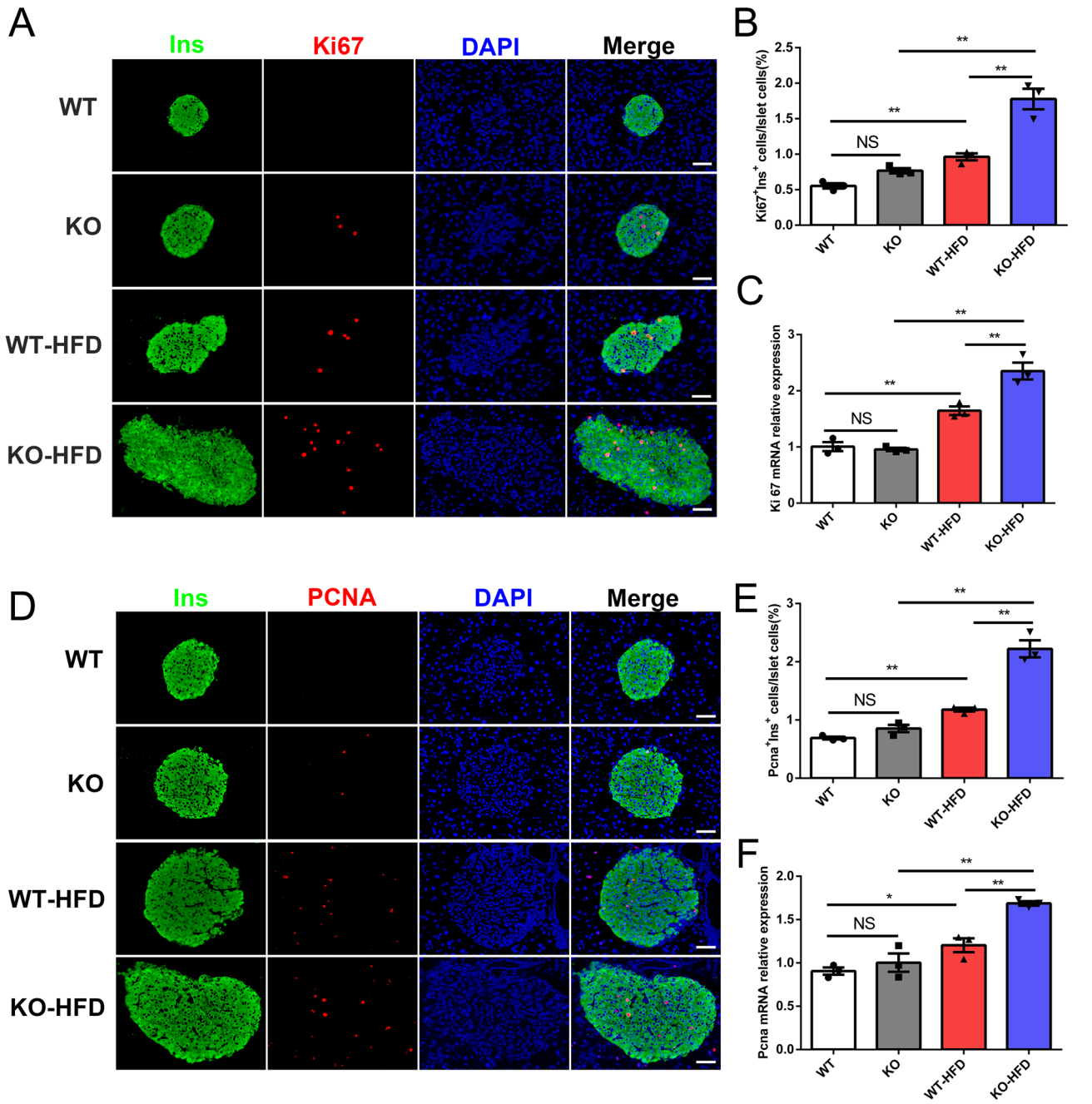

Figure 3

Txnip deficiency and HFD feeding lead to increased β-cell accumulation. (A) Double immunofluorescence staining of *insulin* and *Ki67*. Scale bar, 50 μm. (B) The percentage of *Ki67*$^{+}$*Ins*$^{+}$ cells. The *Ki67* positive cells number was increased by HFD and was significantly greater in KO-HFD vs WT-HFD mice. NS, no significant difference in WT vs KO mice(n = 3). (C) The mRNA expression levels of *Ki67* were significantly higher in KO-HFD vs WT-HFD mice. NS, no significant difference in WT vs KO mice (n = 3). (D) Double immunofluorescence staining of *insulin* and *Pcna*. The *Pcna*-positive cell number was increased by HFD and was significantly greater in KO-HFD vs WT-HFD mice. NS, no significant difference in WT vs KO mice. Scale bar, 50 μm. (E) The percentage of *Pcna*$^{+}$*Ins*$^{+}$ cells (n = 3). (F) The mRNA expression levels of *Pcna* were significantly higher in KO-HFD vs WT-HFD mice. NS, no significant difference in WT vs KO mice (n = 3). NS, not significant, *P < 0.05, **P < 0.01.

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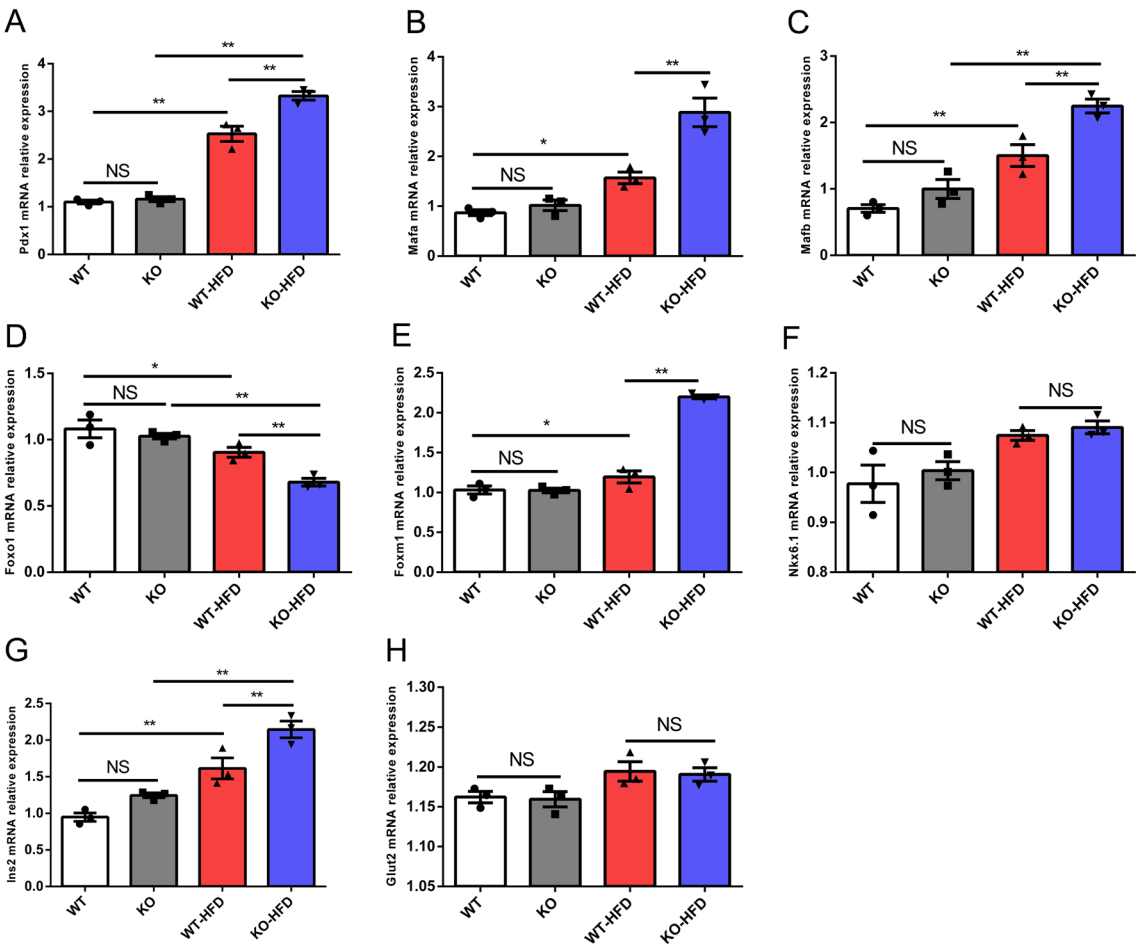

Figure 4

Txnip deficiency promotes changes in transcription factor mRNA levels in HFD-induced mice. (A, B, C, D, E, F, G and H) The mRNA expression levels of *Pdx1*, *Mafa*, *Mafb*, *Foxo1*, *Foxm1*, *Nkx6.1*, *Ins2* and *Glut2* were detected by RT-qPCR. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

each of which are key regulators of cell cycle progression. The mRNA expression levels of *Rb*, *Cdkn2a* and *Cdkn1c* were significantly decreased and *Ccna2*, *Ccnb1*, *Ccnb2* and *Cdk2* were significantly increased in the KO-HFD group. There was no significant change between the WT and KO groups. However, the mRNA expression levels of *Ccnd1*, *Ccne1* and *Cdkn1a* were not affected by *Txnip* knockdown (Fig. 5H). Collectively, the above results suggest that *Txnip* KO may promote β-cell replication by accelerating cell cycle progression via transcriptional regulation of specific cell cycle regulators in HFD-induced mice.

Txnip may regulate β-cell proliferation via the PI3K/AKT pathway

We next sought to explore the possible molecular mechanism by which *Txnip* deficiency may promote β-cell proliferation. A previous study demonstrated that the PI3K/AKT signaling pathway has the potential to regulate β-cell proliferation (22). Thus, we hypothesized that *Txnip* might regulate β-cell proliferation in the mouse DM model via the PI3K/AKT pathway. Western blot assays demonstrated that the phosphorylation of PI3K and AKT was increased significantly after *Txnip* KO in HFD fed mice (Fig. 6A, B and C). Furthermore, phosphorylation of the pivotal downstream mediators of the PI3K/AKT pathway, p-mTOR and p-GSK3β, were upregulated significantly in KO-HFD mice (Fig. 6D and E). Thus, these results suggest that the deletion of *Txnip* may promote β-cell proliferation via the activation of the PI3K/AKT pathway.

Discussion

Diabetes is a disease characterized by an absolute or relative deficiency of β cells that underlies both T1DM and T2DM.

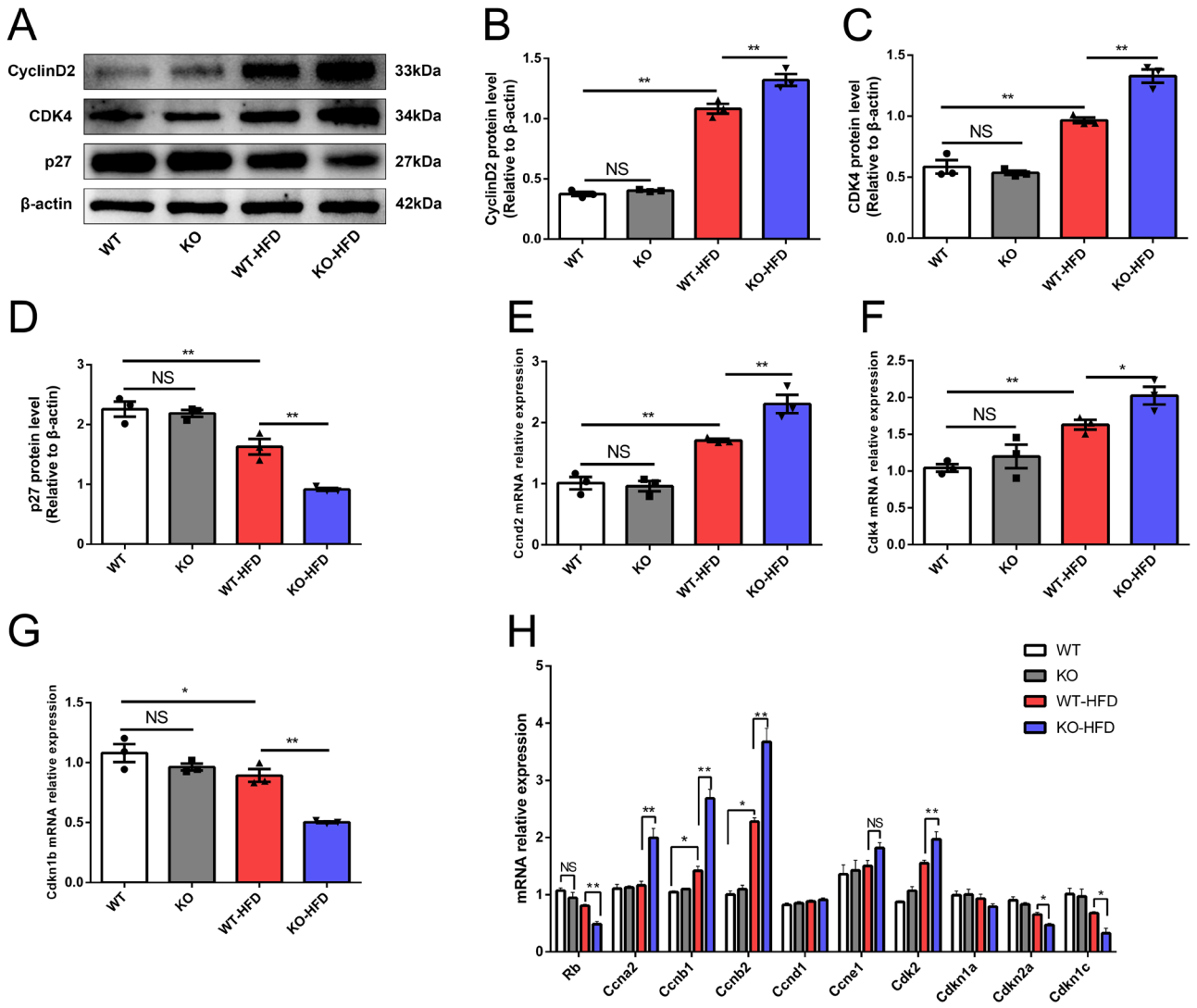

Figure 5

Txnip deficiency promotes β-cell replication by regulating the levels of cell cycle regulatory proteins. (A, B, C and D) The protein expression levels of cyclin D2, CDK4 and p27 were analyzed by Western blotting. (E, F and G) The mRNA expression levels of *Ccnd2*, *Cdk4* and *Cdkn1b* were detected by RT-qPCR. (H) The mRNA expression levels of additional cell cycle regulators were detected by RT-qPCR. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

Loss of the functional β-cell mass is a key process leading to DM and understanding the molecular mechanisms behind β-cell failure is critical for preventing or reverting disease (47). Furthermore, replenishing the lost β-cell mass is a strategy that can alleviate some of the burdens of the disease. In this study, our results suggest that *Txnip* deficiency can improve glucose tolerance and insulin sensitivity by increasing β-cell mass. We found that *Txnip* deficiency can modulate the expression of transcription factors and cell cycle regulators and may activate PI3K/AKT signaling pathways to promote β-cell proliferation in HFD-induced obesity. Thus, our findings provide a molecular mechanism by which *Txnip* deficiency may provide a therapeutic target for DM.

The prevalence of obesity is increasing rapidly worldwide and is associated with peripheral insulin resistance and β-cell proliferation (16, 48). A commonly used model of obesity is HFD feeding in mice, which has been shown to promote β-cell proliferation and β-cell mass expansion (15, 49). In this study, we used HFD mice as a model for evaluating the mechanisms of β-cell compensatory proliferation, including the potential role of *Txnip*. After feeding for 12 weeks, the weight was increased in both WT-HFD and KO-HFD mice when compared to their control mice. Interestingly, the weight was significantly increased in WT-HFD mice compared to the other groups, while the weight of KO-HFD mice was not significantly

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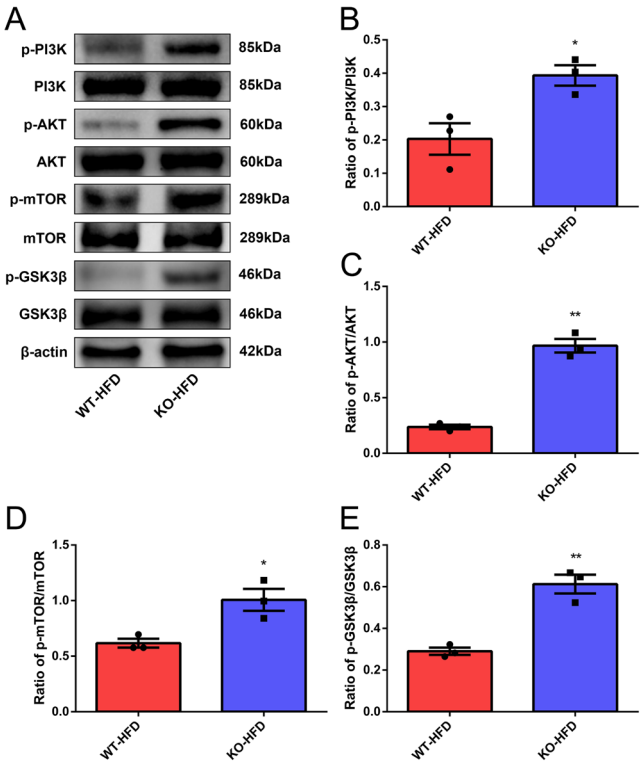

Figure 6

Txnip deficiency may activate the PI3K/AKT signaling pathway. (A) Western blot analysis was performed to determine PI3K/AKT signaling pathway-related protein expression and phosphorylation in WT-HFD vs KO-HFD mice. Representative images are shown. (B) The phosphorylation ratio of PI3K. (C) The phosphorylation ratio of AKT. (D) The phosphorylation ratio of mTOR. (E) The phosphorylation ratio of GSK3β. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$.

increased. This suggests a mechanistic role of *Txnip* as a regulator of glucose and lipid metabolism and is consistent with previous studies demonstrating that *Txnip*-deficient (*Hcb-19*) mice have increased β-cell mass expansion and that this effect is more apparent when animals are stressed by multiple low-dose STZ administration or obesity (38, 50). Our data also show that *Txnip* KO mice have a greater pancreas to bodyweight ratio and significantly increased β-cell mass in KO-HFD mice. In contrast, these parameters were largely unchanged between WT and KO mice fed NC diet.

Aberrant glucagon production correlates with diabetes. Research has shown that blockade of glucagon signaling lowers glycemia in mouse models while enhancing the formation of functional β-cell mass (51). In our study, plasma glucagon levels significantly decreased in KO-HFD mice compared with WT-HFD group. This change may be related to the regulation of β cells by glucagon, while it also may be due to the conversion of α cells into β cells. However, the specific mechanism underlying this process still needs to be further investigated. Thus, our results suggest that HFD-induced obesity enhances β-cell mass expansion in *Txnip* KO mice. These findings are consistent with those of a previous study (38), though our results provide additional understanding of the ability of *Txnip* deficiency to improve glucose tolerance and insulin sensitivity in HFD-induced mice. Our results are also consistent with previous reports demonstrating that *Txnip* plays an important role in the regulation of insulin sensitivity (36, 52), thus providing a mechanism for *Txnip* in a DM model. We also measured the GLP1 and Leptin levels and found that the level of GLP1 was increased in KO-HFD mice, while the Leptin level was decreased in KO-HFD mice (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article). Given that GLP1 has the ability to enhance β-cell proliferation and stimulate β-cell mass expansion in rodents and humans (53, 54), it is possible that *Txnip* may function in part by regulating the *leptin/GLP-1* balance. Leptin is additional negative regulator of β-cell mass expansion (55) and may be worth evaluating in the future to further enhance our understanding of the mechanism of *Txnip* deficiency in promoting β-cell mass expansion in HFD-induced obesity.

Many studies have demonstrated that *Txnip* comprises a major tumor suppressor that inhibits the proliferation of tumor cells (31, 40, 43, 44, 45, 46), which motivated us to speculate that *Txnip* may promote diabetes by inhibiting β-cell proliferation. To explore the relationship between *Txnip* and pancreatic β-cell proliferation, we performed immunofluorescence staining of proliferative antigens, such as *Ki67* and *Pcna*. Our data show that in *Txnip* KO mice, $Ki67^{+}Ins^{+}$ and $Pcna^{+}Ins^{+}$ cells were significantly increased after 12 weeks of HFD. Thus, our results suggest that *Txnip* deficiency can promote pancreatic β-cell proliferation in HFD-induced obesity.

Numerous studies have identified a variety of pancreatic transcription factors that play crucial roles in β-cell regeneration and maintenance of mature β-cell function (8, 17, 18). To understand factors that regulate β-cell proliferation, we used real-time RT-PCR to examine the mRNA levels of specific transcription factors, such as *Pdx1*, *Mafa*, *Foxo1* and *Nkx6.1*. *Pdx1*, which is a marker of β cells, is expressed in mature β cells and is required for β-cell differentiation and proliferation. *Mafa* also is expressed exclusively in β cells and functions as a potent activator of insulin gene transcription (17, 18, 19). Recently, *Pdx1* has been shown to contribute to β-cell mass expansion and proliferation induced by the AKT pathway (56).

Foxo1 is a key cell cycle entry factor that inhibits cell proliferation through the transcriptional regulation of cell cycle inhibitors and activators (57). Interestingly, *Foxo1* is mutually exclusive with *Pdx1* and promotes *Pdx1* nuclear exclusion to counteract *Pdx1* function in β cells (58, 59). *Foxm1*, on the other hand, promotes progression through the cell cycle by regulating genes important for the G1/S and G2/M transitions, as well as genes required for karyokinesis and cytokinesis (60, 61). Our results show that these pancreatic transcription factors are differentially and coordinately regulated at the level of mRNA in β cells, indicating that *Txnip* may inhibit β-cell proliferation by regulating pancreatic transcription factors, though the individual roles of these factors are still unclear and need further study.

Adult pancreatic β-cell regeneration mainly results from the proliferation of existing β cells (5). Thus, cell cycle molecules are likely to play a pivotal role in the proliferation of β cells. We have shown that TXNIP modulates the expression of a variety of genes involved in the cell cycle, including increases in the expression of *Ccnd2* and *Cdk4* and reduction in the expression of *p27*, at both the protein and mRNA levels. This is consistent with reports demonstrating that cyclin D2/CDK4 complex formation is essential for β-cell proliferation (62); and that, conversely, increased accumulation of *p27* can inhibit β-cell proliferation (24, 63). Our data also show that *Txnip* deficiency leads to the activation of the PI3K/AKT pathway. This included the activation of mTOR as a downstream target of the PI3K/AKT pathway and increased phosphorylation of GSK3β. Previous work has shown that mTOR is an important regulator of the β-cell mass and β-cell proliferation in the pancreas. Furthermore, there is evidence that cyclin D2 stability acts downstream of mTOR signaling in pancreatic β cells (64). mTOR has been shown to regulate β-cell proliferation via cell cycle progression by modulating cyclin D2 and CDK4/cyclin D activity (64, 65). Interestingly, cyclin D2 has also been reported to act downstream of GSK3β (22). GSK3β inhibition promotes β-cell proliferation by regulating cell cycle activators and inhibitors, such as p27, cyclinD1, cyclinD2, CDK4 and CDK2 (66). Therefore, *Txnip* deficiency is likely to promote β-cell proliferation in HFD-induced obesity via PI3K/AKT signaling. However, the specific regulatory mechanism needs further investigation. We also demonstrated that the mRNA level of retinoblastoma protein (*Rb*) was reduced in KO-HFD mice, which is consistent with the possibility that *Txnip* deficiency may promote G1 to S transition to accelerate cell cycle progression and cell proliferation (67), though additional studies will be needed to verify this possibility. We examined the mRNA levels of other key cell cycle regulators that may influence cell cycle progression, including *Ccna2*, *Ccnb1*, *Ccnb2*, *Ccnd1*, *Ccne1*, *Cdk2*, *Cdkn1a*, *Cdkn2a* and *Cdkn1c*, most of which showed significant differences in KO-HFD vs WT-HFD mice. Thus, further studies may clarify the roles of these regulators in *Txnip*-mediated β-cell proliferation.

In conclusion, our results demonstrate that *Txnip* plays an important role in the regulation of β-cell mass expansion and β-cell proliferation. *Txnip* deficiency promotes β-cell proliferation in HFD-induced obesity, which may be mediated via activation of the PI3K/AKT signaling pathway. Our work thus provides a theoretical basis for *Txnip* as a new therapeutic target for the treatment of DM.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-21-0641.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Applied Basic Research Project of Natural Science Foundation of Shanxi Province (grant no. 20210302124377 and 201901D111192).

Acknowledgement

The authors thank LetPub (www.letpub.com) for linguistic assistance and pre-submission expert review.

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Received in final form 10 March 2022
Accepted 16 March 2022
Accepted Manuscript published online 16 March 2022