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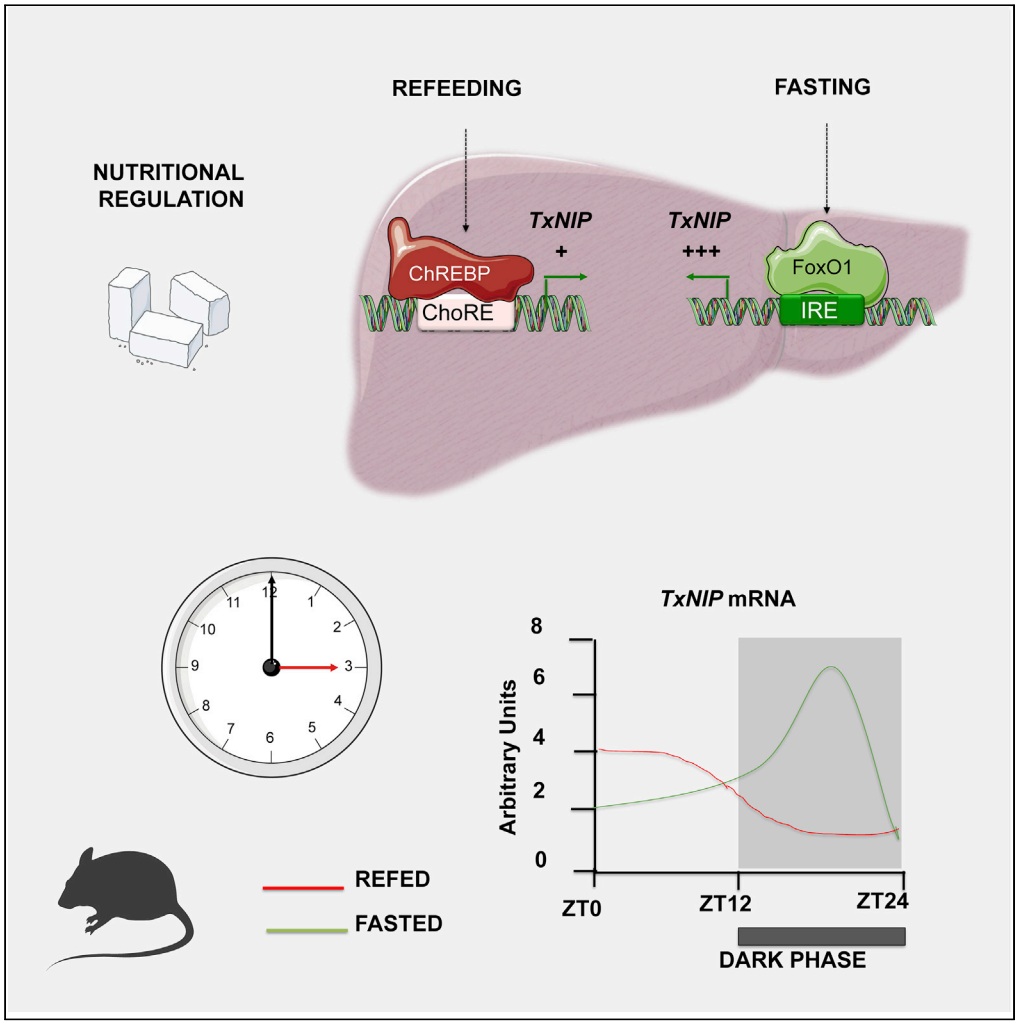

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HIGHLIGHTS

TxNIP is considered as a potential candidate drug target for type 2 diabetes

We provide better understanding of *Txnip* regulation and function in liver

Hepatic *Txnip* is up-regulated by both ChREBP and FoxO1 transcription factors

We suggest a role for TxNIP in the physiological adaptation to nutrient restriction

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Dual regulation of TxNIP by ChREBP and FoxO1 in liver

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SUMMARY

TxNIP (Thioredoxin-interacting protein) is considered as a potential drug target for type 2 diabetes. Although TxNIP expression is correlated with hyperglycemia and glucotoxicity in pancreatic β cells, its regulation in liver cells has been less investigated. In the current study, we aim at providing a better understanding of *Txnip* regulation in hepatocytes in response to physiological stimuli and in the context of hyperglycemia in *db/db* mice. We focused on regulatory pathways governed by ChREBP (Carbohydrate Responsive Element Binding Protein) and FoxO1 (Forkhead box protein O1), transcription factors that play central roles in mediating the effects of glucose and fasting on gene expression, respectively. Studies using genetically modified mice reveal that hepatic TxNIP is up-regulated by both ChREBP and FoxO1 in liver cells and that its expression strongly correlates with fasting, suggesting a major role for this protein in the physiological adaptation to nutrient restriction.

INTRODUCTION

Thioredoxin-interacting protein (TxNIP/VDUP1/TBP-2) originally discovered as a vitamin D3-inducible gene (Chen and DeLuca, 1994) has gained interest for being involved in glucose homeostasis, carcinogenesis, angiogenesis, or inflammation (Alhawiti et al., 2017; Yoshihara 2020). Structurally designated as part of the α-arrestin family, TxNIP contains two amino-terminal SH3-binding domains, whereas the carboxyl terminus contains two PPxY motifs and three SH3 domains (Patwari et al., 2009). TxNIP binds the anti-oxidant protein, thioredoxin and inhibits its disulfide reductase activity *in vitro*. In this context, TxNIP has been described as a possible link between cellular redox state and metabolism. Importantly, owing to its diverse array of functions in glucose and lipid metabolism in several cell types, TxNIP has been considered as a novel candidate drug target for type 2 diabetes (Thielen and Shalev, 2018). Interestingly, a study recently identified a novel anti-diabetic small molecule SRI-37330 that inhibits TxNIP expression and signaling in mouse and human islets (Thielen et al., 2020).

TxNIP expression and function have been extensively studied in pancreatic β cells (Shalev, 2014). In this cell type, *Txnip* is one of the most highly up-regulated genes in response to hyperglycemia (Cha-Molstad et al., 2009). As part of a negative-feedback loop, TxNIP was shown to inhibit glucose uptake and promote caspase-3 cleavage, contributing to glucose-dependent β cell death (Saxena et al., 2010). TxNIP also regulates pro-inflammatory gene expression through inflammasome activation via binding to NLRP3 (NOD-like receptor family pyrin domain containing 3) (Zhou et al., 2010). Altogether, TxNIP has emerged as an important factor in pancreatic β cell biology and tight regulation of TxNIP levels appears necessary for β cell survival. The mechanisms driving TxNIP expression in pancreatic β cells are complex and involve crosstalk between several transcription factors, including the glucose-sensitive transcription factor Carbohydrate Responsive Element Binding Protein (ChREBP) and the Forkhead boxO1 transcription factor (FoxO1). The *Txnip* promoter contains two carbohydrate response elements (ChoRE) for binding of ChREBP (Minn et al., 2005). FoxO1 was reported to up-regulate *Txnip* expression in neurons and endothelial cells (Li et al., 2009), whereas it is reported to significantly decrease *Txnip* expression in pancreatic β cells. Mechanistically, FoxO1 was reported to inhibit *Txnip* expression by reducing the glucose-induced binding of ChREBP on the *Txnip* promoter in β cells, suggesting that FoxO1 may antagonize ChREBP for binding to the *Txnip* promoter in the context of insulin-secreting cells (Kibbe et al., 2013).

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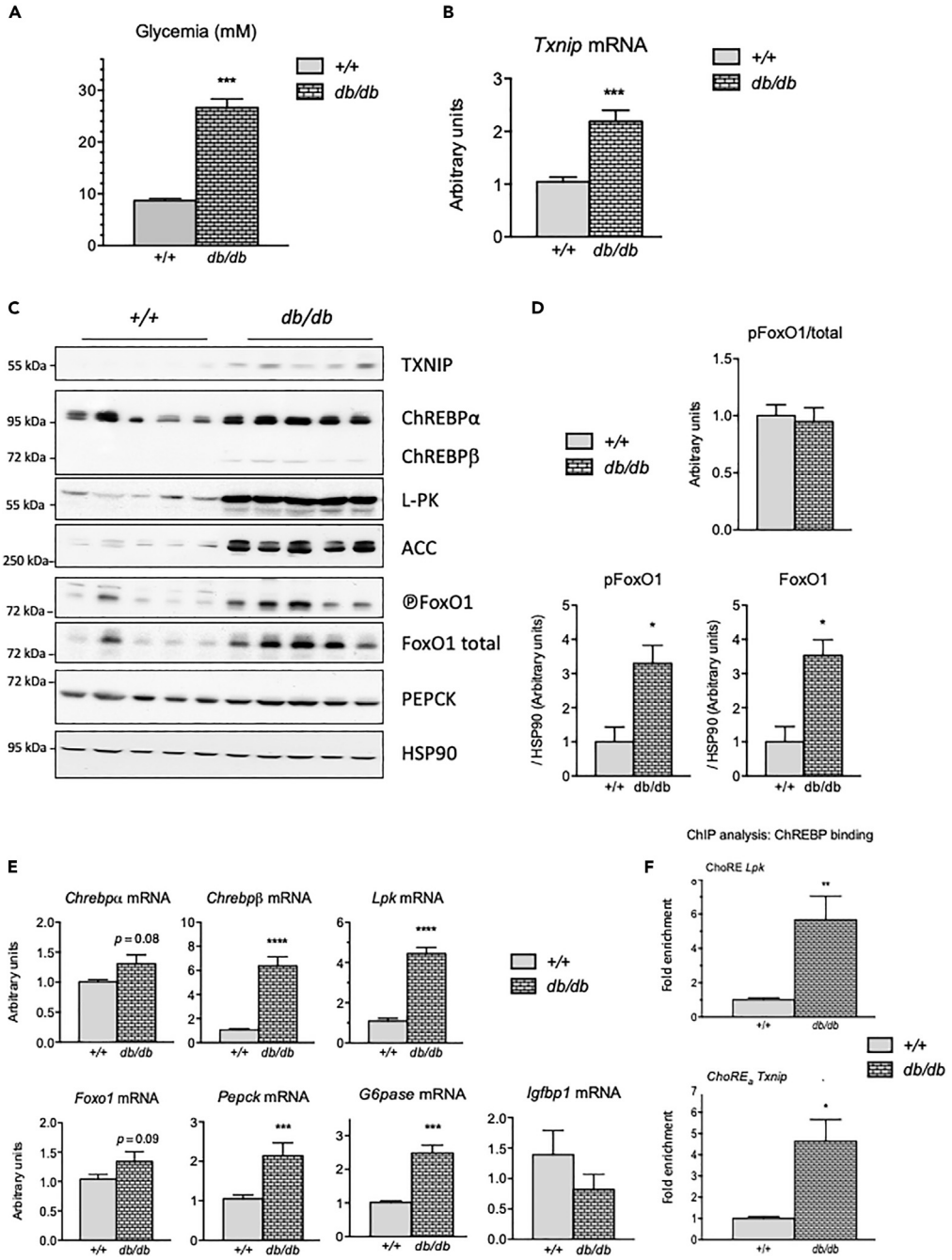

Figure 1. TxNIP expression is increased in the liver of *db/db* mice

Twelve-week-old C57BL/6J (+/+) and *db/db* male mice were fed ad libitum. Figures are presented as means ± SEM from 8 to 12 individual mice. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test. *p < 0.5, **p < 0.01, ***p < 0.001; ****p < 0.0005 when compared with (+/+) mice.

(A) Blood glucose (mM) recovered at the time of harvest from tail snip.

(B) Relative *Txnip* gene expression determined by qPCR.

(C) Western blot analysis of protein extracted from whole-liver lysate. HSP90 was used as loading control. Five representative samples are shown.

Figure 1. *Continued*
(D) Quantification of the ratio of phosphorylated FoxO1 corrected to total FoxO1 protein, of the ratio of phosphorylated FoxO1 corrected to HSP90, and of total FoxO1 corrected to HSP90.
(E) Relative *Chrebpα*, *Chrebpβ*, *Lpk*, *Foxo1*, *Pepck*, *G6pase, and Igfbp1* gene expression determined by qPCR.
(F) ChIP analysis followed by qPCR of whole mouse liver tissue. Immunoprecipitation experiments conducted with ChREBP antibodies. The DNA regions of the *Lpk* and *Txnip* promoters were amplified using primers indicated in Table S2.

The function and the mechanisms regulating *Txnip* expression are less well described in the liver. Key information, however, was gained from the analysis of *Txnip*-null mice. *Txnip*-null mice are hypoglycemic, hypoinsulinemic, and exhibit blunted glucose production following a glucagon challenge, a phenotype consistent with a defect in hepatic glucose metabolism (Chutkow et al., 2008). *In vitro* analysis confirmed that glucose release from isolated *Txnip*-null hepatocytes was lower than that from wild-type hepatocytes, supporting an intrinsic defect in hepatocyte glucose production. Although hepatocyte-specific gene deletion of *Txnip* did not alter glucose clearance compared with controls, *Txnip* expression in the liver is required for maintaining normal fasting glycemia and glucose production (Chutkow et al., 2008).

In this context, we sought to provide a better understanding of *Txnip* regulation in hepatocytes in response to physiological stimuli and in the context of diabetes. Owing to their previously reported implication, we focused on pathways governed by ChREBP and FoxO1. Studies using genetically modified mouse models of ChREBP and/or FoxO1 expression reveal that, in contrast to pancreatic β cells, hepatic TxNIP is stimulated by both ChREBP and FoxO1 and is strongly associated with the physiological response to fasting in liver.

RESULTS

TxNIP expression is increased in liver of *db/db* mice

We first measured TxNIP expression in the liver of fed *db/db* mice (Figure 1). We chose *db/db* mice for their significant hyperglycemia and confirmed that their blood glucose concentrations were elevated compared with controls under our experimental conditions (Figure 1A). As previously reported (Jo et al., 2013), *Txnip* mRNA levels and TxNIP protein content were significantly increased in the liver of fed *db/db* mice compared with +/+ mice (Figures 1B and 1C). As *Txnip* was previously reported to be a direct target of ChREBP and/or FOXO1 depending on the cell type studied, we examined the expression and activity of these transcription factors. The ChREBP protein contains a low glucose inhibitory domain (LID) and a glucose responsive activation conserved element (GRACE) located in its N terminus (Li et al., 2006). Activation of the GRACE domain by glucose promotes ChREBP transcriptional activity and binding to the ChoRE element of its target genes. Another isoform of *Chrebp*, *Chrebpβ*, originating from an alternative first exon promoter, was identified in the adipose tissue and in liver (Herman et al., 2012). This alternative splicing results in a constitutively active and potent ChREBP isoform lacking the inhibitory LID domain (Herman et al., 2012). We observed that ChREBP protein content (both α and β isoforms) was elevated in the liver of *db/db* mice and paralleled a marked induction of LPK (L-pyruvate kinase) and ACC (acetyl coA carboxylase) protein levels (Figure 1C), two known ChREBP hepatic targets (Abdul-Wahed et al., 2017). A modest increase in *Chrebpα* mRNA levels was observed in the liver of *db/db* mice, whereas 6-fold induction in *Chrebpβ* expression was measured along with a 5-fold increase in *Lpk* mRNA levels (Figure 1E). Chromatin immunoprecipitation (ChIP) analysis revealed a significant increase in ChREBP binding on the ChoRE of the *Lpk* promoter in the liver of *db/db* mice compared with controls (+/+) (Figure 1F). Interestingly, increased binding of ChREBP to the ChoRE of the *Txnip* promoter was also observed (Figure 1F), suggesting direct control of *Txnip* expression by ChREBP in the liver of *db/db* mice.

Interestingly, FoxO1 protein content was also elevated in the liver of *db/db* mice (Figure 1C). Of note, the level of phospho-FoxO1 was also increased, but in proportion to changes in total FoxO1 level (Figure 1D), indicating that levels of both phospho- (inactive) and nonphospho- (active) FoxO1 are likely increased in *db/db* liver. In contrast, the level of FoxO1 mRNA was not significantly altered in *db/db* mice, suggesting that differences in FoxO1 protein level are due to post-transcriptional mechanisms. A significant increase in the expression of several FoxO1 target genes (*Pepck* and *G6pase*) is consistent with increased FoxO1 activity in the liver of *db/db* mice (Figure 1E). Although we did not succeed in performing FoxO1 ChIP assays, our results, nevertheless, indicate that hepatic TxNIP content is increased in the liver of *db/db* mice and parallels with enhanced ChREBP and FoxO1 activity.

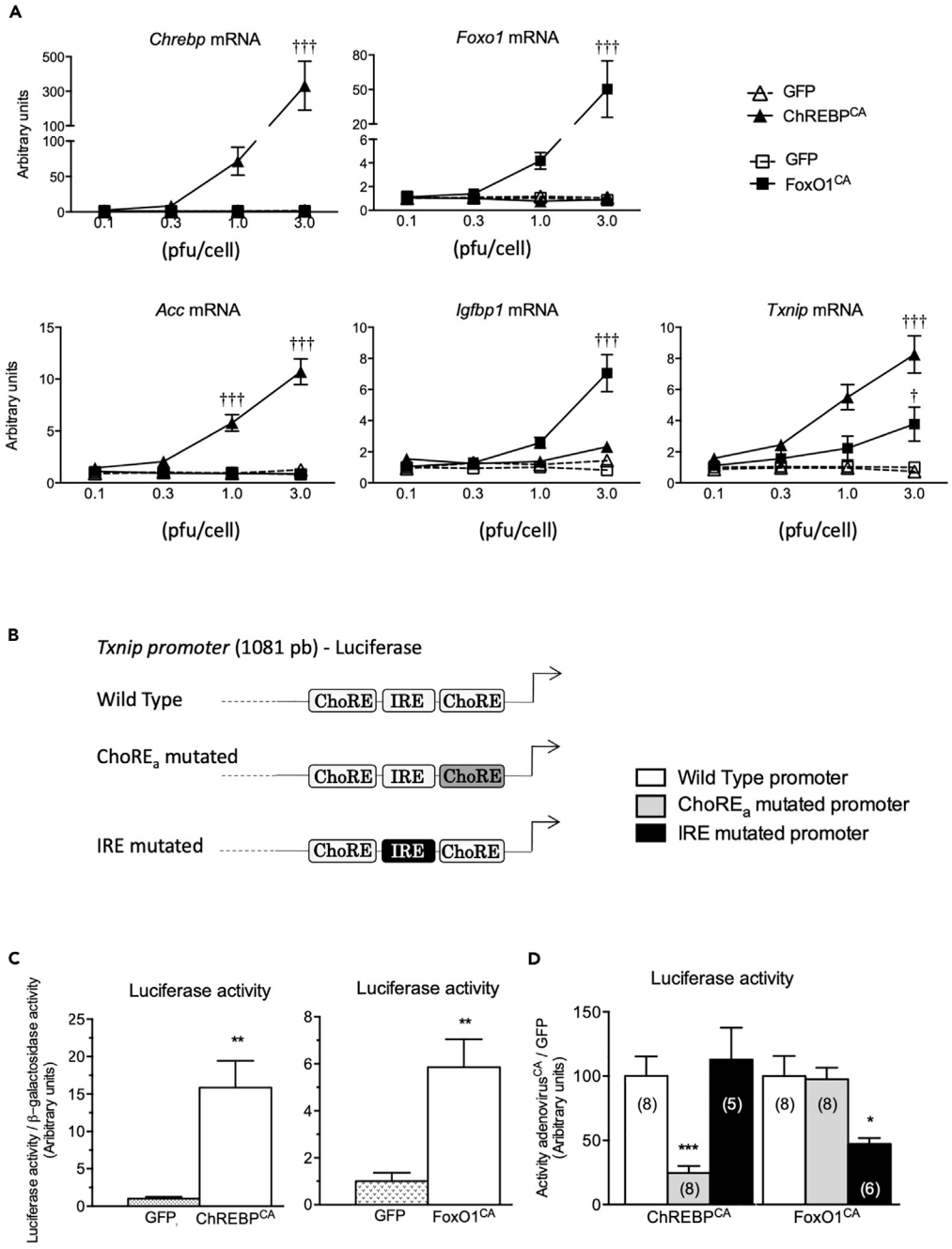

Figure 2. Effect of ChREBP and FoxO1 overexpression on *Txnip* expression and promoter activity in mouse hepatocytes

Primary hepatocytes derived from adult male mice were incubated under low glucose concentration (5 mM) with specific adenovirus as indicated (from 0.1 to 3.0 PFU/cell) for 24 h.

(A) Relative *Chrebp*, *Foxo1*, *Acc*, *Igfbp1*, and *Txnip* gene expression determined by qPCR. Figures are presented as means ± SEM from 6 to 8 independent cultures. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test †p < 0.05, ††p < 0.01, †††p < 0.001 when compared with GFP conditions.

(B) Schematic representation of the wild and mutated *Txnip* promoters (1,081 bp; the two ChREBP-binding sites [ChoRE] and the FoxO1-binding site [IRE] are indicated). Primers used for site-directed mutagenesis are indicated in Table S1.

(C) Luciferase activity of the wild-type *Txnip* promoter in primary hepatocytes in response to either ChREBP^{CA} (3 PFU/cell) or FoxO1^{CA} (3 PFU/cell). Figures are presented as means ± SEM from 6 to 8 independent cultures. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test. **p < 0.01 when compared with GFP conditions.

Figure 2. *Continued*

(D) Luciferase activity of the wild-type, ChoREa-mutated, or IRE-mutated *Txnip* promoter in primary hepatocytes in response to either ChREBP^{CA} (3 PFU/cell) or FoxO1^{CA} (3 PFU/cell). Figures are presented as means ± SEM from 5 to 8 independent cultures. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test *p < 0.05, ***p < 0.001 when compared with wild-type promoter activity.

Direct effects of FoxO1 and ChREBP on *Txnip* expression in mouse hepatocytes

To investigate direct effects of ChREBP and/or FoxO1 on *Txnip* expression in liver cells, overexpression studies of ChREBP or FoxO1 were conducted using adenovirus strategies *in vitro*. Primary hepatocytes were transfected with increasing concentrations (from 0.1 to 3.0 plaque-forming unit [PFU]/cell) of an adenovirus expressing a constitutively active isoform of ChREBP (ChREBP^{CA}) lacking the low glucose inhibitory domain (LID) (Li et al., 2006), a constitutively active form of FoxO1 (FoxO1^{CA}) (Zhang et al., 2006), or green fluorescent protein (GFP) as a control. As shown in Figure 2A ChREBP overexpression (at 3 PFU/cell) increased mRNA levels of *Acc*, a known ChREBP target gene, but not of *Igfbp1*, which is regulated by FoxO1. Conversely, FoxO1 overexpression led to a significant increase in the expression of its target gene, *Igfbp1*, but not of *Acc* (Figure 2A), demonstrating gene-specific effects of ChREBP and FoxO1 in hepatocytes. Interestingly, both ChREBP and FoxO1 were able to induce *Txnip* expression. An 8-fold increase in response to ChREBP^{CA} (3 PFU/cell) and a 4-fold stimulation in response to FoxO1^{CA} (3 PFU/cell) were observed (Figure 2A), suggesting that ChREBP and FoxO1 are able to stimulate *Txnip* expression in hepatocytes in a cell-autonomous fashion.

Next, *Txnip* promoter activity was measured by performing luciferase reporter gene assays (Figures 2C and 2D), using reporter gene constructs containing the wild-type *Txnip* promoter sequence or constructs in which ChREBP and/or FoxO1-binding sites are mutated (Figures 2B and Table S1). ChREBP^{CA} stimulated by 15-fold wild-type *Txnip* promoter activity (Figure 2C), whereas a 6-fold effect was observed in response to FoxO1^{CA} (Figure 2C). The stimulatory effect of ChREBP^{CA} was significantly reduced when one ChREBP-binding site (ChoREa) was mutated but remained unchanged when the FoxO1-binding site (IRE) was mutated (Figure 2D). Similarly, the stimulatory effect of FoxO1^{CA} was partially lost when the IRE was mutated on the *Txnip* promoter but remained unchanged when the ChoREa was mutated (Figure 2D). Together, these results indicate that *Txnip* expression is under the dual stimulatory control of ChREBP and FoxO1 operating through distinct *cis*-acting elements within the *Txnip* promoter in hepatocytes.

Correlation between TxNIP expression and blood glucose concentrations

To characterize the physiological regulation of TxNIP in liver, its expression was measured in liver of fasted, freely fed, and refed mice (Figure 3). Interestingly, whereas TxNIP was described as a highly up-regulated gene in response to glucose (Cha-Molstad et al., 2009), TxNIP expression (both mRNA and protein levels) was lower in the liver of refed than in fasted mice (Figures 3A and 3B). To better understand the regulation of hepatic TxNIP expression depending on the nutritional status, we established correlations between the relative amount of *Txnip* expression as a function of glycemia. We plotted *Txnip* expression depending on blood glucose concentrations in 32 C57BL/6J mice and calculated the Spearman's correlation coefficient (R) (Figure 3C). Interestingly, we observed positive correlations between *Txnip* mRNA levels and low glucose concentrations below 6 mM (Figure 3D, n = 11), and also with elevated glucose concentrations above 13 mM (Figure 3F, n = 5). No positive correlation was found when glucose concentrations were in the normal range, between 6 and 13 mM (Figure 3E, n = 15). Taken together, our results suggest that hepatic *Txnip* expression correlates with glycemia under both low- and high-glucose conditions.

Regulation of TxNIP during fasting and refeeding

To further study the physiological regulation of TxNIP, we next focused on the fasting/refeeding transition, characterized by marked differences in blood glucose concentrations (Figure 4A) and in the ChREBP and FoxO1 activities (Figures 4C and 4D). As expected, the expression of ChREBPα and of its target genes *Chrebpβ* and *Lpk* were significantly induced in the liver of refed mice (Figures 4C and 4D). The refed state was also characterized by an increase in the ratio of phosphorylated FoxO1 to total FoxO1 (Figure 4E) and by decreased PEPCK expression (Figures 4C and 4D), consistent with a reduction in FoxO1 activity. We confirmed that TxNIP expression (both mRNA and protein levels) was lower in the liver of refed than of fasted mice (Figures 4B and 4D). To determine whether ChREBP or FoxO1 was recruited onto the *Txnip* promoter under these nutritional conditions, ChIP analysis was performed in the liver of fasted and refed mice (Figure 4F). Under fasted conditions, a significant enrichment of FoxO1 binding on the IRE of the

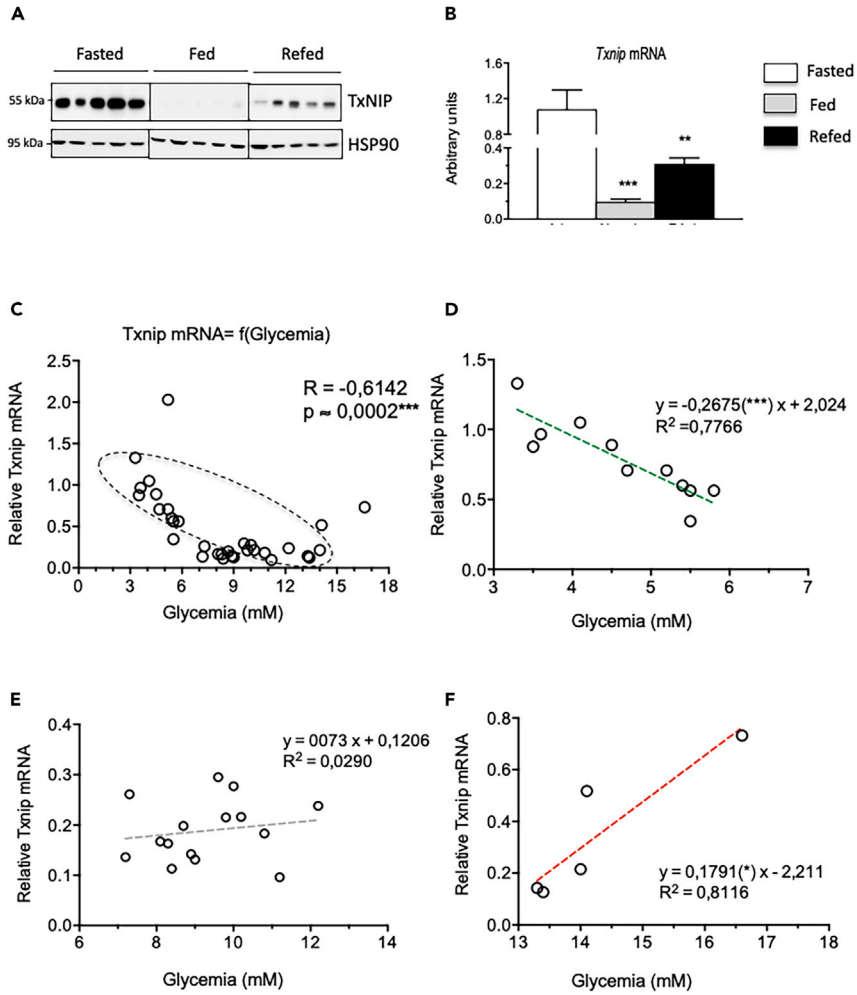

Figure 3. Correlation between *Txnip* expression and blood glucose concentrations

(A and B) Twelve-week-old C57BL/6J (+/+) male mice were studied at the fed, fasted, and refed state. (A) Western blot analysis of protein extracted from whole-liver lysate. HSP90 was used as loading control. Five representative samples are shown. (B) Relative *Txnip* gene expression determined by qPCR. Figure is presented as means ± SEM from 8 to 12 individual mice. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test. **p < 0.01, ***p < 0.001 when compared with the fasted state.

(C–F) Representation of the relative amounts of *Txnip* mRNA levels as a function of glycemia. (C) Correlation using a set of C57BL/6J male mice (n = 32 points) measured at either the fasted, fed, and refed state. R, Spearman's correlation coefficient, ***p < 0.001 (coefficient other than zero). Dashed oval, point cloud approximation. (D–F) Linear regressions for blood glucose concentrations below 6 mM (D, n = 11), between 6 and 13 mM (E, n = 15) and above 13 mM (F, n = 5). R2, coefficient R squared (adequacy of the points to the right); y = ax + b, equation of the regression line with a slope.

Pepck promoter was observed. Although it did not reach significance, FoxO1 binding on the IRE of the *Txnip* promoter was also enriched compared with refed conditions (Figure 4F). As expected, under refed conditions, a marked increase in ChREBP binding was observed on the *Lpk* ChoRE. Interestingly, a significant enrichment in ChREBP binding was also observed on the ChoRE of the *Txnip* promoter (Figure 4F). Altogether, these results support the concept that FoxO1 and ChREBP contribute to the regulation of *Txnip* expression in the liver under fasting (FoxO1 active) and refed (ChREBP active) conditions.

Glucose-mediated induction of *Txnip* requires ChREBP

To address the direct contribution of ChREBP to *Txnip* expression in the refed state, ChREBP was silenced in the liver of mice before fast-refeeding using a validated short hairpin RNA (shRNA) strategy (Dentin et al., 2004). We confirmed that ChREBP protein (Figure 5A) and mRNA (Figure 5B) levels were significantly

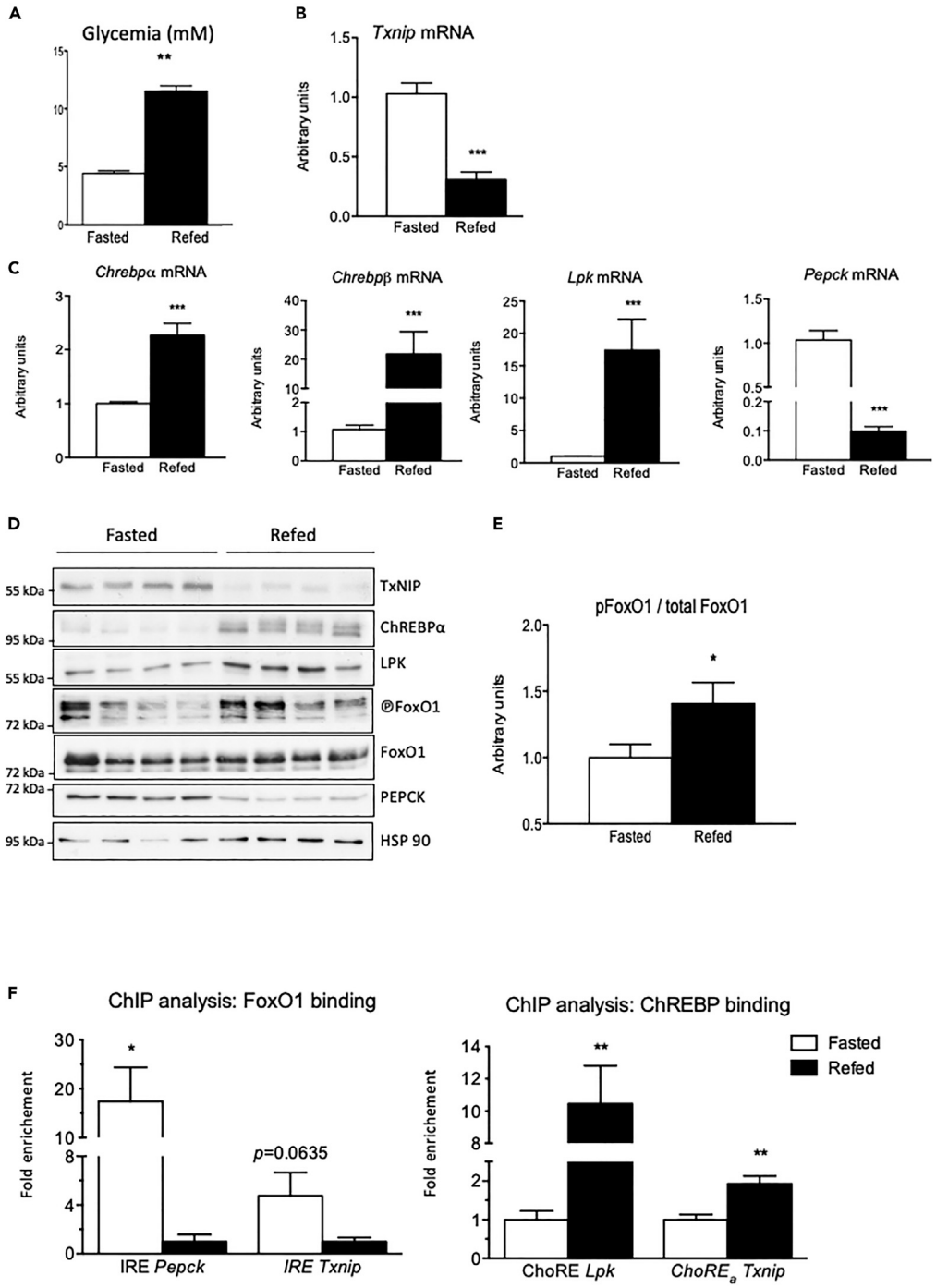

Figure 4. Differential regulation of TxNIP during fasting and refeeding. Adult C57BL/6J male mice were studied under fasting (24 h fast) or refed (a 18-h refeeding period) at ZT12

Data are expressed as means ± SEM, n = 6 to 8 individual mice/group. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test *p < 0.05, **p < 0.01, ***p < 0.001 when compared with fasted conditions.

(A) Blood glucose (mM) recovered at the time of harvest from tail snip.

(B) Relative *Txnip* gene expression determined by qPCR.

(C) Relative *Chrebpα*, *Chrebpβ*, *Lpk*, and *Pepck*, gene expression determined by qPCR.

(D) Western blot analysis of protein extracted from whole-liver lysate. HSP90 was used as loading control. Four representative samples are shown.

(E) Quantification of the ratio of phosphorylated FoxO1 corrected to total FoxO1 protein is provided.

Figure 4. *Continued*

(F) ChIP analysis followed by qPCR of whole mouse liver tissue. Immunoprecipitation experiments conducted with FoxO1 and ChREBP antibodies. The DNA regions of the *Pecpk*, *Lpk*, and *Txnip* promoters were amplified using primers indicated in Table S1.

Data are expressed as means ± SEM, n = 6 to 8 individual mice/group. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test *p < 0.05, **p < 0.01, ***p < 0.001 when compared with fasted conditions.

decreased in the liver of refed ShChREBP mice compared with refed ShControl. *Chrebp* silencing led to 80% to 90% reduction in *Lpk* and *Acc* mRNA levels (Figure 5B). A 50% decrease in *Txnip* mRNA (Figure 5B) and protein (Figure 5A) levels was also was observed in the liver of refed ShChREBP mice, suggesting that ChREBP does contribute, at least partially, to *Txnip* expression under refeeding conditions.

Because ChREBP silencing was only partial using the shRNA strategy (Figure 5A), we also examined *Txnip* regulation in hepatocytes lacking both ChREBP isoforms (*Chrebp*$^{-/-}$) (Iroz et al., 2017) (Figure 5C). We observed that *Txnip* expression was robustly increased in response to 25 mM glucose in wild-type hepatocytes (*Chrepb+/+*) and was comparable to the increase in *Chrebpβ*, *Lpk,* and *Acc* expression (Figure 5C). The stimulatory effect of glucose was totally prevented for *Lpk* and *Acc* in *Chrebp*$^{-/-}$ hepatocytes, and the induction of *Txnip* also was significantly reduced, although not completely disrupted, in response to 25 mM in these hepatocytes (Figure 5C). Together, these results confirm that ChREBP-independent mechanisms contribute to the glucose-mediated induction of *Txnip* in mouse hepatocytes.

Daily rhythms of *Txnip* correlate with the one of FoxO1

To gain further insight into the physiological regulation of *Txnip* in liver, we measured its pattern of expression in the liver during daily rhythms under control, fasted, or refed conditions (Figure 6). Variations in blood glucose concentrations (Figure 6A) and in hepatic *Reverbα* (a well-known circadian-regulated gene) (Figure 6B) were measured to validate the experimental conditions used. Hepatic mRNA levels of *Chrebpα*, *Chrebpβ*, *Lpk*, *Txnip*, *Pepck, and Foxo1* were measured at ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT24 by qPCR (Figure 6C). The expression of *Chrebpα*, *Chrebpβ,* and *Lpk* increased in the dark phase in fed mice, when feeding largely occurs, but decreased in the dark under fasting conditions, indicating that diurnal changes in the expression of these genes coincide with changes in food intake. In contrast, the expression of *Txnip* underwent a different pattern with a major peak of induction at ZT14 in the dark phase under fasting conditions. Interestingly, the profile of *Txnip* paralleled the one of *Foxo1* and of its target gene *Pepck* (Figure 6C), suggesting that food intake is necessary to prevent the induction and activation of *FoxO1*, *Pepck,* and *Txnip* expression during the transition from light to dark phases. These data support the concept that *Txnip* expression correlates with the expression of *Foxo1* and *Pepck* in the transition to the fasting state in a physiological context.

FoxO1 stimulates the expression of TxNIP in liver

To further characterize the contribution of FoxO1 to the regulation of *Txnip* in liver, we performed a series of experiments using mice with genetic modifications of the FoxO proteins in liver (Figure 7). First, *Txnip* expression was measured in the fasting state in the liver of mice with a liver-specific deficiency in the three FoxO proteins (FoxO1, FoxO3, and FoxO4) (LFoxO^{TKO} mice) (Zhang et al., 2016). *Txnip* expression was significantly decreased along with the expression of *Igfbp1*, a well-known target of FoxO proteins, whereas the expression of *Chrebpα*, *Chrebpβ*, and *Lpk* was only modestly affected (Figure 7A). To address the specific role of FoxO1 on *Txnip* expression when insulin signaling is decreased, we measured the expression of *Txnip* in the liver of refed liver-specific insulin receptor knockout (LIR^{KO}) and IR/FoxO1 double knockout (LIRFoxO1^{KO}) mice (O-Sullivan et al., 2015) (Figure 7B). In the liver of LIR^{KO} mice, where FoxO1 activity is enhanced (O-Sullivan et al., 2015), the expressions of *Txnip* as well as other FoxO1 targets (*Igfbp1 and Pepck*) were significantly increased and reversed when FoxO1, the predominant FoxO protein expressed in the liver (Zhang et al., 2016), was knocked out in this model (i.e., LIRFoxO1^{KO} mice) (Figure 7B). These results confirm that endogenous FoxO1 promotes *Txnip* expression in the liver when insulin signaling is disrupted.

We also analyzed the expression of *Txnip* in the liver of transgenic mice that selectively express a constitutively active form of FoxO1 in the liver (FoxO1^{TGN}) (Zhang et al., 2006). *Txnip* expression was markedly increased in the liver of FoxO1^{TGN} mice and paralleled with changes in the expression of PEPCK at both protein and RNA levels (Figures 7C and 7D). In contrast, ChREBP activity was found to be reduced in the

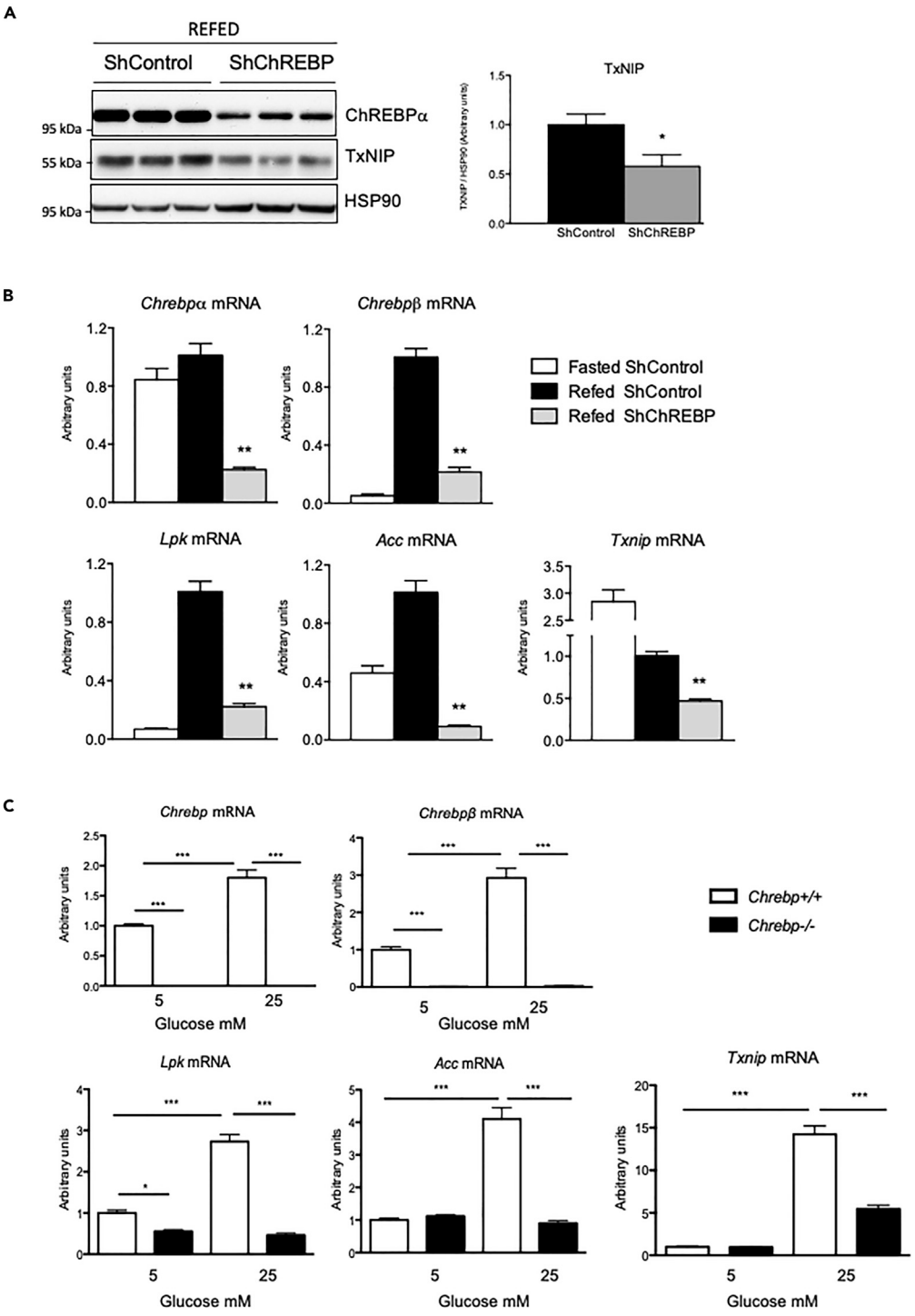

Figure 5. The glucose-dependent induction of *Txnip* requires ChREBP

C57Bl/6J male mice were injected intravenously with a single dose of 5 × 10^9 PFU of shCTRL or shChREBP adenovirus at Day1. Seven days later, mice were challenged to nutritional manipulations as indicated (fasted or refed).

(A) Western blot analysis of protein extracted from whole-liver lysate. HSP90 was used as loading control. Three representative samples are shown. Quantification of the ratio of TxNIP protein content corrected to HSP90 is shown.

*$p < 0.05$ when compared with ShControl conditions.

Figure 5. *Continued*

(B) Relative gene expression of *Chrebpα*, *Chrebpβ*, *Lpk*, *Acc*, and *Txnip* gene was determined by qPCR. Figures are presented as means ± SEM from 6 individual mice. Significance is based on two-way ANOVA followed by Bonferroni post test, *p < 0.05, **p < 0.01, when compared with refed ShControl conditions.

(C) Primary hepatocytes from *Chrebp*$^{-/-}$ and *Chrebp*$^{+/+}$ littermates were stimulated 1 day after platting for 24 h with cell culture medium containing 5 or 25 mM glucose. qPCR analysis of *Chrebp*, *Chrebpβ*, *Lpk*, *Acc*, and *Txnip*. Figures are presented as means ± SEM from 3 independent cultures done in triplicates. Significance is based on two-way ANOVA followed by Bonferroni post test, *p < 0.05, ***p < 0.0 01.

liver of these mice. Indeed, we observed that the expression of *Chrebpβ* and *Lpk* was decreased in the liver of FoxO1^{TGN} mice. We hypothesized that this decrease could be due to reduced ChREBP O-GlcNAcylation (ChREBPα OGlcNAc) (Figure 7C). O-GlcNAcylation is a post-translational modification dependent on glucose metabolism that stimulates ChREBP transcriptional activity in the liver (Guinez et al., 2011). Interestingly, it was previously reported that ChREBPα OGlcNAcylation is reduced in response to FoxO1 in the liver (Ido-Kitamura et al., 2012), presumably due to the suppression of glucokinase expression and glucose utilization by FoxO1 (Zhang et al., 2006). Together these results indicate that FoxO1 is sufficient to promote *Txnip* expression in the liver, including under conditions where ChREBP expression and activity are reduced.

Txnip silencing in liver reduces hyperglycemia in *db/db* mice

We next evaluated the contribution of TxNIP to the hyperglycemic phenotype of *db/db* mice using a TxNIP shRNA strategy (Figure 8). TxNIP protein content was decreased by 50% in the liver of *db/db* mice treated with the ShTxnip adenovirus (Figure 8A). This decrease was associated with a significant improvement in the pyruvate tolerance test (PTT), which reflects production of glucose from exogenous pyruvate (Figure 8B). Blood glucose concentrations in *db/db* ShTxnip mice were significantly reduced compared with *db/db* ShControl under both fasted and fed states (Figure 8C). No change in body weight was observed upon TxNIP silencing in *db/db* mice (Figure 8D), indicating that effects of shTxnip on glucose levels and pyruvate tolerance were not due to modification in body weight. *Txnip* knockdown was associated with a significant decrease in *Pepck*, *G6pase*, and *Pgc1α* expression (Figure 8E).

To determine whether TxNIP silencing also could affect glucose homeostasis in a physiological context, TxNIP was knocked down in the liver of C57Bl/6J mice (Figure S1A). We observed that the PTT was improved in ShTxNIP-treated mice compared with mice injected with a ShControl (Figure S1B). Blood glucose concentrations also were significantly decreased at the time of sacrifice (Figure S1C). qPCR analysis confirmed that *Txnip* mRNA levels were significantly decreased in the liver of ShTxNIP mice in parallel with reduced expression of gluconeogenic genes, including *Pepck* and *G6pase* and the co-activator *Pgc1α* (Figure S1D). Altogether, these results support a role for TxNIP in regulating hepatic glucose production and gluconeogenic gene expression under hyperglycemic and physiological conditions.

DISCUSSION

In the current study, we sought to provide a better understanding of the mechanisms involved in the regulation of TxNIP in the liver under physiological and hyperglycemic conditions. In recent years, TxNIP has emerged as a key regulator of glucose and lipid metabolism and has been shown to influence metabolic regulation via multiple actions including insulin release from pancreatic β cells, glucose production by the liver, and glucose uptake in peripheral tissues including muscle and adipose tissue (Alhawiti et al., 2017). In addition, genetic and epigenetic variations in TxNIP are associated with chronic metabolic disorders including diabetes and hypertension (van Greevenbroek et al., 2007; Ferreira et al., 2012). Interestingly, anti-diabetic agents like insulin, metformin, glucagon-like peptide-1 (GLP-1) agonists, and resveratrol have been reported to inhibit TxNIP expression, which may contribute to their therapeutic efficacy in the treatment of diabetes (Chai et al., 2012; Bedarida et al., 2016; Nivet-Antoine et al., 2010; Shao et al., 2010). Interestingly, after a screen of more than 300,000 molecules, Thielen and co-workers recently identified a compound that downregulates TxNIP in mouse and human pancreatic islets. When given orally to mice, this small molecule (SRI-37330) lowered serum levels of glucagon, prevented fatty liver, and inhibited glucose production by the liver (Thielen et al., 2020). In this context, TxNIP continues to generate significant interest as a potential therapeutic target for the management of diabetes and other metabolic disorders (Yoshihara, 2020). We report in the present study that TxNIP is dually regulated in the liver by the transcription factors ChREBP and FoxO1 under specific nutritional conditions.

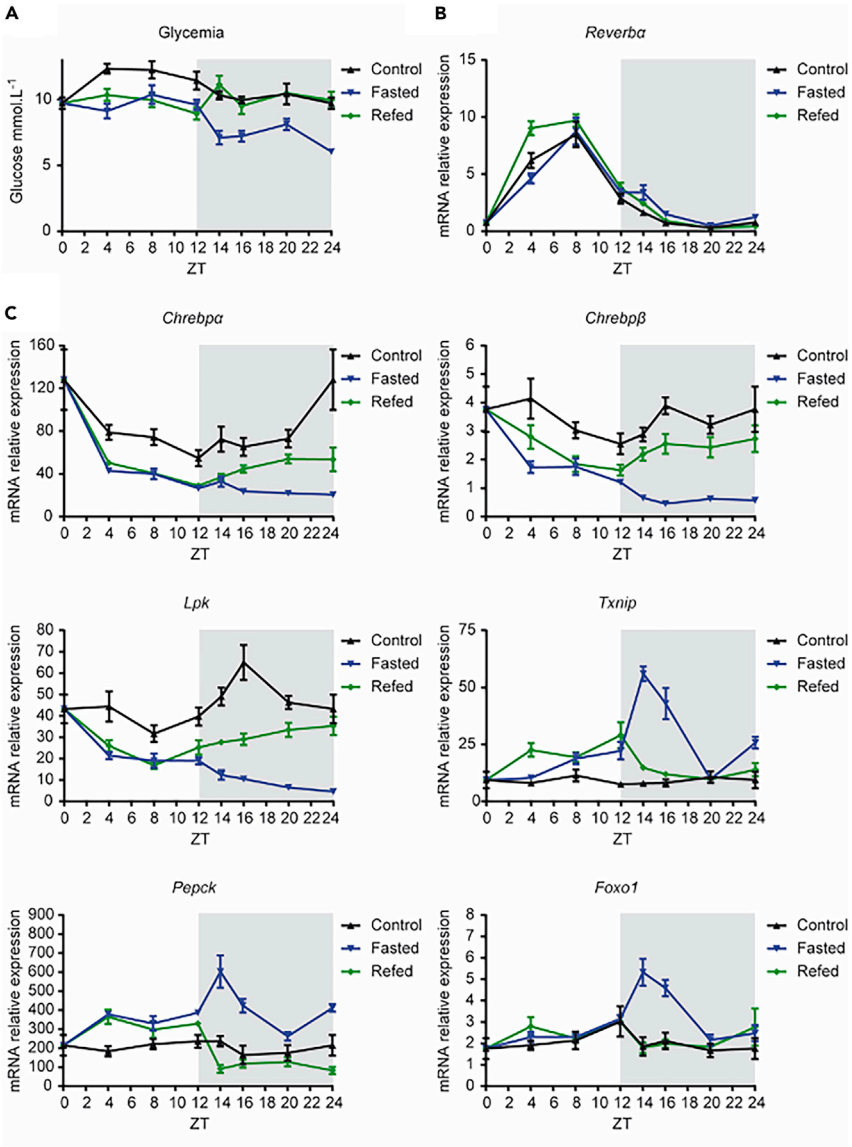

Figure 6. Daily rhythms of hepatic gene expression under control, fasted, and refed conditions

Adult C57BL/6J male mice were studied under control conditions (fed at libitum), fasted, or refed at ZT12. Mice were killed by cervical dislocation at several time points in a pairwise manner: ZT0, ZT4, ZT8, ZT12 ZT14, ZT16, ZT20, and ZT24 as indicated. Figures are presented as means ± SEM from 10 individual mice per time point.

(A) Blood glucose (mmol/L) recovered at time of harvest from tail snip.

(B) qPCR analysis of *Reverbα*.

(C) qPCR analysis of *Chrebpα*, *Chrebpβ*, *Lpk*, *Txnip*, *Pepck*, and *Foxo1*.

TxNIP can be induced by glucose in a ChREBP-dependent fashion in a variety of cell types and the TxNIP promoter contains two ChREBP response elements (Cha-Molstad et al., 2009). *Txnip* is one of the most highly up-regulated genes in response to glucose in pancreatic β cells (Cha-Molstad et al., 2009) where its induction by glucose was previously reported to be ChREBP dependent (Minn et al., 2005). Our study shows that *Txnip* expression also is significantly up-regulated by high glucose concentrations in primary hepatocytes and that the glucose-sensitive transcription factor ChREBP contributes to glucose-mediated induction of *Txnip* in hepatocytes. Although the results demonstrate that ChREBP does contribute to the induction of TxNIP in refed conditions, our data also suggest that other molecular mechanisms may be involved. Indeed, a significant (although reduced) glucose effect was maintained on *Txnip* in *Chrebp*$^{-/-}$

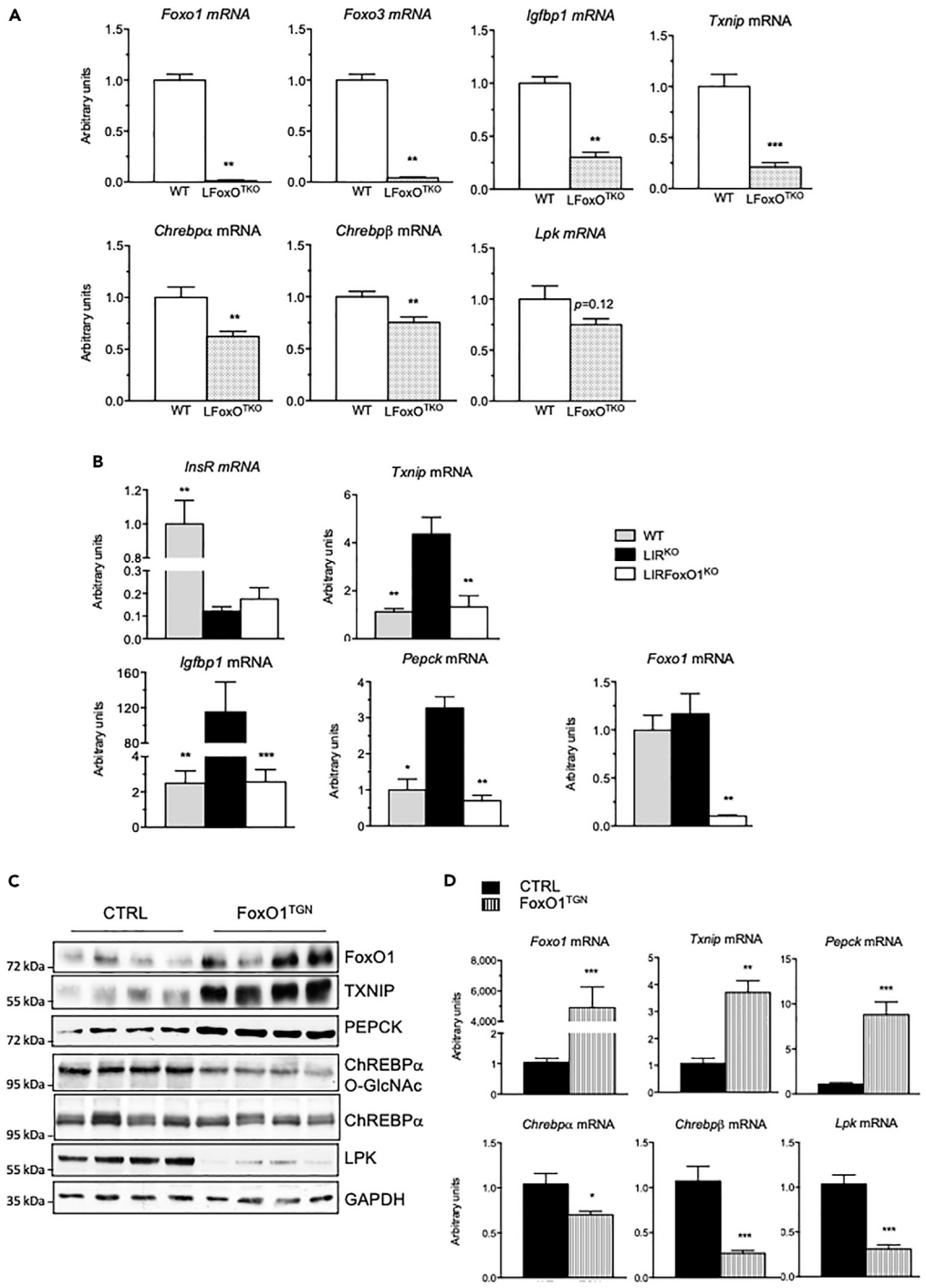

Figure 7. FoxO1 stimulates the expression of TXNIP in liver

(A and B) (A) Wild-type (WT) and LFoxO^{TKO} mice were fasted for 24 h qPCR analysis of *Foxo1*, *Foxo3*, *Igfbp1*, *Txnip*, *Chrebpα*, *Chrebpβ*, and *Lpk*. Data are expressed as means ± SEM, n = 6 to 8 individual mice/group. Significance is based on two-way ANOVA followed by Bonferroni post hoc test, **p < 0.01, ***p < 0.001 when compared with WT mice. (B) Wild-type (WT), LIR^{KO}, and LIRFoxO1^{KO} mice were studied at the fed state. qPCR analysis of *InsR*, *Txnip*, *Igfbp1*, *Pecpk*, and *Foxo1*. Data are expressed as means ± SEM, n = 6 to 8 individual mice/group. Significance is based on two-way ANOVA followed by Bonferroni post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001 when compared with LIR^{KO} mice.

(C and D) Control (CTRL) and transgenic mice overexpressing a constitutive active form of FoxO1 (FoxO1^{TGN}) were studied at the fed state. (C) Western blot analysis of protein extracted from whole liver lysate. GAPDH was used as loading

Figure 7. *Continued*
control. Four representative samples are shown. (D) qPCR analysis of *Foxo1*, *Txnip*, *Pecpk Chrebpα*, *Chrebpβ*, and *Lpk*. Data are expressed as means ± SEM, n = 6 individual mice/group. Significance is based on two-way ANOVA followed by Bonferroni post hoc test, Significance is based on two-way ANOVA followed by a Bonferroni post hoc test *p < 0.05, **p < 0.01, ***p < 0.001.

hepatocytes challenged with high glucose concentrations. One possible candidate for mediating this effect could be MondoA, the paralog of ChREBP (Richards et al., 2017), which was shown to be important for *Txnip* expression in skeletal muscles (Ahn et al., 2016). Interestingly, we also reported that glucose still induces *Txnip* expression in islets from *Chrebp*$^{-/-}$ mice and that this effect does in fact require MondoA in human β cells (Richards et al., 2018). While MondoA functions were predominantly reported in skeletal muscle and proliferative cells (Wilde and Ayer, 2015), a role for MondoA in hepatocytes should not be excluded. In fact, the contribution of MondoA to liver metabolism was previously evidenced by its direct control of the glycogen-targeting protein (PTG) in response to glucose (Petrie et al., 2013). The potential contribution of MondoA to the *Chrebp*$^{-/-}$phenotype remains to be determined.

Surprisingly, unlike other ChREBP targets, our study reveals that liver *Txnip* expression is higher under fasting than under refed conditions. Interestingly, correlation studies established that *Txnip* mRNA levels correlate with both low glucose concentrations (below 6 mM) and elevated glucose concentrations (above 13 mM). In the liver of hyperglycemic *db/db* mice, we confirmed that *Txnip* expression is elevated and that ChREBP binding is enriched on the ChoRE of the *Txnip* promoter. In this mouse model, we cannot exclude that elevated *Txnip* expression also could result from enhanced glucocorticoid signaling or other factors. Indeed, several studies have suggested that chronic corticosterone treatment may upregulate *Txnip* by targeting the glucocorticoid receptor (Bharti et al., 2018; Reich et al., 2012). Glucocorticoids have been shown to regulate TxNIP in neuronal cells (Bharti et al., 2018) and often function cooperatively to regulate the expression of FoxO1 target genes in the liver, including *Pepck*, *G6pase*, and *IGFBP-1* (Goswami et al., 1994). Interestingly, our study suggests that FoxO1 activity may be enhanced in the liver of *db/db* mice, where FoxO1 is known to play a significant role in promoting hyperglycemia (Zhang et al., 2012). Although we were not able to address the direct contribution of FoxO1 to the expression of *Txnip* in the liver of *db/db* mice due to unresolved technical problems, we were able to demonstrate that FoxO1 plays a key role in promoting *Txnip* expression in the liver, based on complementary approaches *in vitro* and *in vivo*.

To date, the role of FoxO1 on *Txnip* expression has been rather complex. FoxO1 was reported to stimulate *Txnip* expression in neurons (Al-Mubarak et al., 2009) and glucose-treated endothelial cells (Li et al., 2009), and to down-regulate its expression in liver tumor cells (de Candia et al., 2008). A study also reported that the expression of FoxO1 and TxNIP is inversely correlated in alcohol-induced hepatitis. However, this study did not examine the direct effect of FoxO1 on TxNIP expression in liver cells in this pathophysiological context (Heo et al., 2019). Here, we report that FoxO proteins contribute to the regulation of *Txnip* in the liver under a variety of physiological conditions. Using liver-specific knockout mice, we demonstrate that *Txnip* expression is strongly induced during the fasting state in a FoxO-dependent manner, and that FoxO1, the major FoxO protein expressed in the liver (Zhang et al., 2016), plays a crucial role in promoting *Txnip* expression when insulin signaling is disrupted in the liver of LIR-KO mice. Studies in transgenic mice expressing a constitutively active form of FoxO1 in the liver also demonstrated that FoxO1 promotes the expression of *Txnip* in the liver, and studies with adenoviral vectors demonstrated that FoxO1 stimulates hepatic *Txnip* expression in primary hepatocytes in a cell-autonomous fashion. Reporter gene studies and ChIP analysis showed that this effect of FoxO1 is mediated through the *cis*-acting FoxO1 target site located in the Txnip promoter, supporting the concept that TxNIP is a direct downstream target of FoxO1 in the liver (Zhang et al., 2012). Together, these data provide strong support for the concept that FoxO1 promotes TxNIP expression in the liver under conditions where insulin levels are low (fasting) or insulin signaling is impaired.

Crosstalk between FoxO1 and ChREBP was previously reported in pancreatic β cells (Kibbe et al., 2013). In these cells, FoxO1 was reported to inhibit the ability of ChREBP to stimulate *Txnip* expression, and it was suggested that FoxO1 might exert this effect by binding to a FoxO target site overlapping a nearby ChoRE in the *Txnip* promoter, thereby displacing ChREBP from its ChoRE (Kibbe et al., 2013). In addition, FoxO1 has been reported to suppress the expression of glucokinase in pancreatic β cells (Buteau et al., 2007) similar to the liver, and might therefore limit ChREBP activation to Txnip promoter by an indirect mechanism. In the present study, we found that FoxO1 and ChREBP are able to both stimulate the expression of *Txnip* in hepatocytes. Although

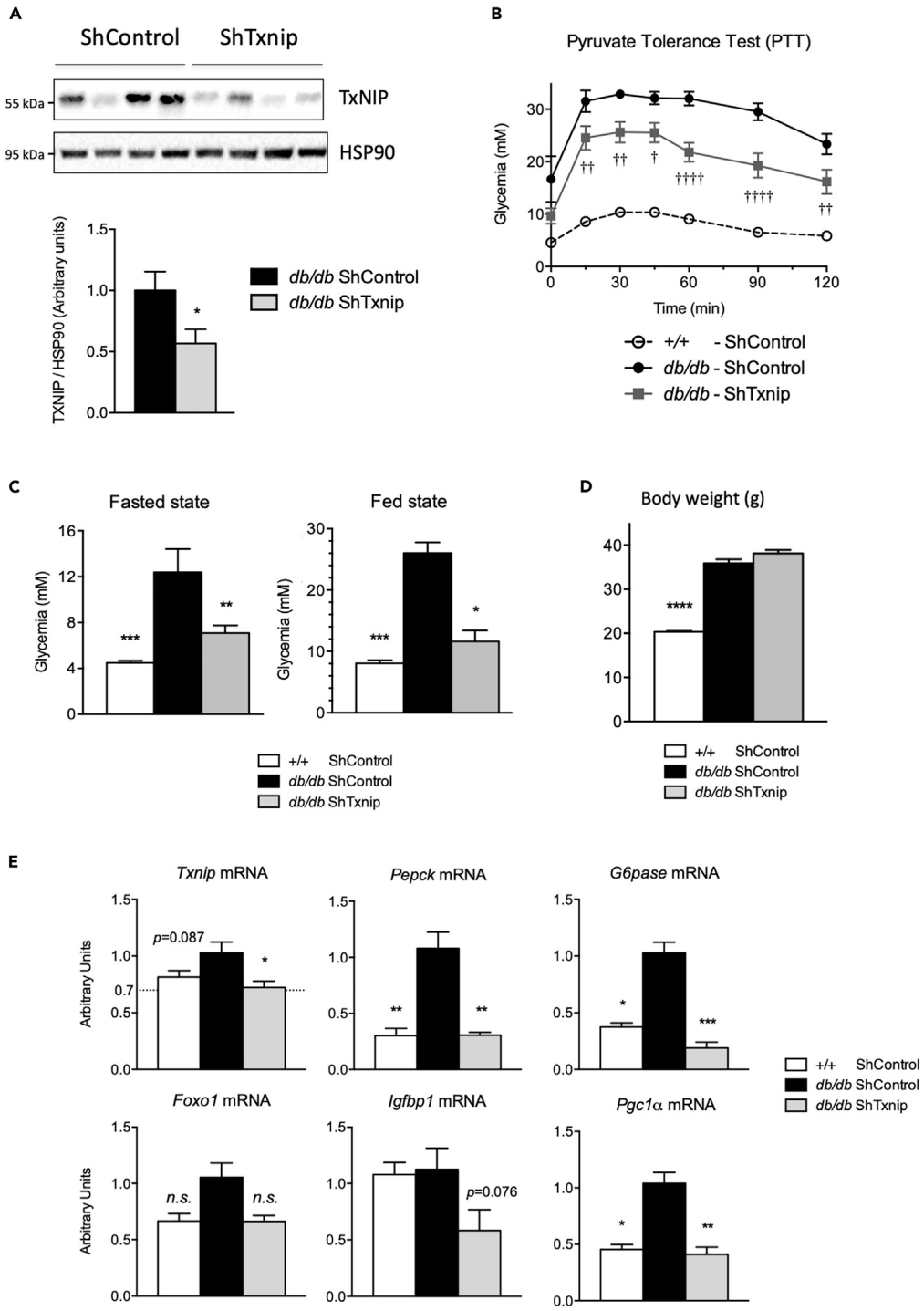

Figure 8. *Txnip* silencing in liver reduces hyperglycemia in *db/db* mice

Adult C57BL/6J (+/+) and *db/db* male mice were injected intravenously with a single dose of 5×10^9 PFU of ShControl or ShTxnip adenovirus (GeneCust) at Day1. Seven days later, mice were challenged to a pyruvate tolerance test (PTT) or to nutritional manipulations as indicated (a 24-h fast [Fasted] or analyzed at the fed state [Fed]). Data are expressed as means ± SEM, n = 6 to 8 individual mice/group. Significance is based on two-way ANOVA followed by Bonferroni post hoc test, (A) Western blot analysis of protein extracted from whole-liver lysate. HSP90 was used as loading control. Four

Figure 8. *Continued*
representative samples are shown. Quantification of the ratio of TxNIP protein to HSP90 is shown. *p<0.05 when compared to *db/db* ShControl. (B) Pyruvate tolerance test (PTT) †p < 0.05, ††p < 0.01, †††p < 0.001, ††††p < 0.0005 when compared with *db/db* ShControl.. (C) Blood glucose concentrations under fasted and fed state. (D) Body weight at the time of the sacrifice. (E) qPCR analysis of *Txnip*, *Pecpk G6Pase*, *Foxo1*, and *PGC1α*. *p<0.05, **p<0.01, ***p<0.001 when compared to *db/db* ShControl.

this apparent discrepancy might reflect tissue-specific differences in FoxO1 and ChREBP function, it is important to note that FoxO1 and ChREBP activity are regulated very differently under physiological conditions in the liver. FoxO1 activity is inhibited by insulin due to its phosphorylation by Akt, so that it is most active when insulin levels are low (i.e., in fasting) or insulin signaling is disrupted; conversely, FoxO1 activity is suppressed when insulin levels are high and Akt is activated (i.e., in the fed state). Conversely, ChREBP is activated in the postprandial state, where glucose utilization is increased, and its activity is suppressed when glucose utilization is limited in the liver (e.g., fasting). Based on these observations, we suggest that FoxO1 and ChREBP act coordinately to maintain and promote the expression of TxNIP under both fasting (FoxO1 active, ChREBP inactive) and fed (ChREBP active, FoxO1 inactive) conditions.

It is important to note that other factors also may contribute to the coordinated regulation of TxNIP in the response to fasting and feeding and during daily rhythms, such as PPARα, Indeed, while a degenerate sequence rather than the consensus AGGTCA-N-AGGTCA sequence was identified on the *Txnip* promoter (AGGACA-G-AGGGGG) (Tzeng et al., 2015), functional evidence reveals that the expression of *Txnip* is significantly reduced in the liver of fasted hepatocyte-specific *Pparα* deficient mice (Régnier et al., 2018). Interestingly, a novel PPARα-dependent regulatory axis involving Gm15441, a long non-coding RNA, was recently identified (Brocker et al., 2020). In this study, Gm15441 was shown to be transcribed in a liver-specific, PPARα-dependent manner and protect against metabolic stress by suppressing the expression of TxNIP and thereby suppressing TxNIP-mediated NLRP3 inflammasome activation.

Like its regulation, the physiological functions of the TXNIP protein also appear to be complex when considered in a tissue-specific manner. Several studies, including the current one, demonstrate that TxNIP plays a critical role in the regulation of hepatic glucose production. HcB-19 mice, which contain a nonsense mutation of the *Txnip* gene, and *Txnip* knockout mice have an inherent defect in maintaining blood glucose levels through glucose production, as well as other defects in lipid and glucose metabolism (Hui et al., 2004). Moreover, liver-specific *Txnip* knockout mice suffer from fasting hypoglycemia and elicit a diminished response to glucagon (Chutkow et al., 2008). Hepatocytes isolated from TxNIP-deficient animals produce significantly less glucose when compared with hepatocytes obtained from their wild-type littermates. In the current study, we report that acute suppression of TxNIP in the liver of hyperglycemic *db/db* mice leads to normalization of blood glucose concentrations under both fasted and fed states and is associated with normalized expression of key genes that promote hepatic production, including *Foxo1* and FoxO1-regulated genes. Thus, in addition to its expression being positively regulated by FoxO1, *Txnip* also appears to support the expression and function of FoxO1 and its downstream targets on glucose production by hepatocytes, suggesting that these factors may function in a feedforward fashion. The induction of *Txnip* also may provide other protective functions during prolonged fasting. Interestingly, it was reported that the expression of *Txnip* is induced to a great extent in torpid animals (both fasting-induced and natural torpor) (Hand et al., 2013). The fact that *Txnip* expression is increased in both the hypothalamus and peripheral tissues (including liver) of torpid mice suggests that it may play an essential role in adapting to major shifts in energy supply in hypometabolic states. In agreement with this concept, mice lacking *Txnip* die during a prolonged fast (Oka et al., 2006).

In summary, we report *Txnip* to be up-regulated in the liver by two master nutritional regulators, ChREBP and FoxO1, in response to glucose/refeeding and fasting, respectively. *Txnip* expression is inducible by glucose through a ChREBP-dependent manner, whereas *Txnip* expression is greater and rapidly switched on during fasting in the liver in a FoxO-dependent manner, suggesting a major role for this protein in the liver in the physiological adaptation to nutrient restriction.

Limitations of the study

In our study, we have provided a molecular basis to explain the dual regulation of TxNIP in response to fasting and refeeding in liver, via the transcription factors FoxO1 and ChREBP, respectively. However, we have not

clearly determined which of these two factors contributes to enhanced TxNIP expression in the liver of hyperglycemic *db/db* mice. In addition, although we report that TxNIP silencing reduces blood glucose concentrations and improves PTTs in C57Bl/6J and *db/db* mice, the mechanism(s) involved was not elucidated.

Resource availability

Lead contact

Further information, requests, and inquiries should be directed to and will be fulfilled by the lead contact, Catherine Postic (catherine.postic@inserm.fr).

Materials availability

All tables and figures are included in the text and supplemental information.

Data and code availability

The published article includes all data generated or analyzed during this study.

METHODS

All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102218.

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AUTHOR CONTRIBUTIONS

B.N., F.B., I.O.-S., W.Z., G.F., A.M., A.P., and S.M. designed experiments, performed experiments, and analyzed the data. F.B., A.-F.B., S.G., T.I., H.G., and C.B. contributed to the critical review of the manuscript. C.P. and T.U. analyzed the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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Supplemental information

Dual regulation of TxNIP

by ChREBP and FoxO1 in liver

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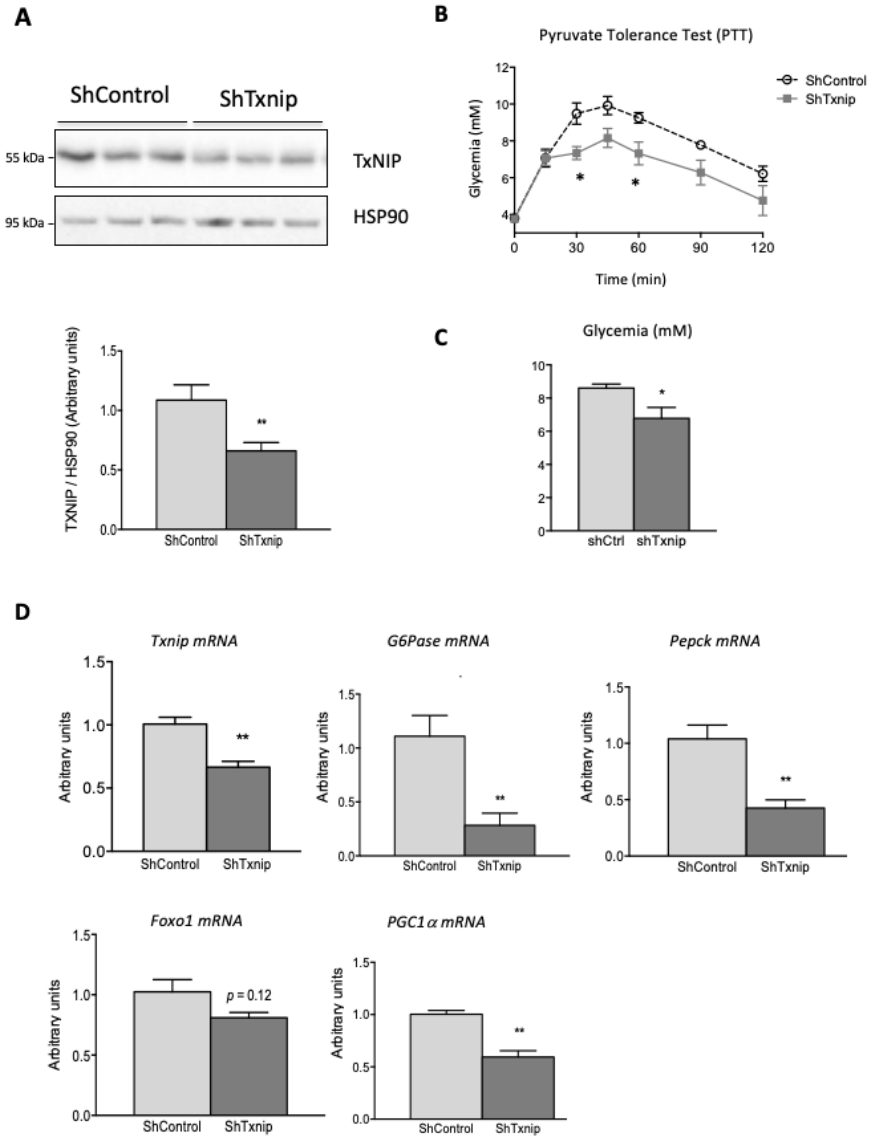
Figure S1. *Txnip* silencing in liver reduces blood glucose concentrations in C57BL/6J mice. **Related to Figure** 8.



Adult C57BL/6J male mice were injected intravenously with a single dose of 5.10^9 pfu of ShControl or ShTxnip adenovirus (GeneCust) at Day1. Seven days later, mice were challenged to a pyruvate tolerance test (PTT). Data are expressed as means ± SEM, n=6 to 8 individual mice/group. Significance is based on two-way ANOVA followed by Bonferroni post hoc test, *p<0.05, **p<0.01, when compared to ShControl (**A**) Pyruvate tolerance test (PTT) (**C**). Blood glucose concentrations at sacrifice. (**D**). qPCR analysis of *Txnip*, *G6Pase, Pepck, Foxo and PGC1α.*

Table S1. Sequences of PCR primers used for mutagenesis. Related to Figure 2.

	Forward Primer	Reverse Primer
ChoRE$_a$ WT	CTG TGC ACG AGG GCT GCA CGA GCC TCC	GGA GGC TCG TGC AGC CCT CGT GCA CAG
ChoRE$_a$ mutated	CTG TGC ACC ATG GCT GGA CGA GCC TCC	GGA GGC TCG TCC AGC CAT GGT GCA CAG

IRE WT	AGG CCT GGT AAA CAA GGG CCA AGT A	TAC TTG GCC CTT GTT TAC CAG GCC T
IRE partial mutant 1	AGG CCT AGT AAC CAA GGG CCA AGT A	TAC TTG GCC CTT GGT TAC TAG GCC T
IRE partial muant 2	AGG TCT AGC AAC CAA GGG CCA AGT A	TAC TTG GCC CTT GGT TGC TAG ACC T
IRE mutated	AGG TCT AGC AAC CAA TGG CCA AGT A	TAC TTG GCC ATT GGT TGC TAG ACC T

The consensus sequences for recognition and binding of the transcription factors ChREBP and FoxO1 are underlined. The sequences of the endogenous promoter (murine-WT) are indicated for comparison. Mutations are indicated in red. The 'IRE partial mutant 1' mutant serves as a template for obtaining the 'IRE mutated' (last lane). Abbreviations: ChoREa, Carbohydrate Response Element proximal to the *Txnip* promoter; IRE, Insulin Response Element; WT, unmutated wild type promoter.

Table S2. Sequences of the primers used for qPCR and ChIP-qPCR. Related to Figures 1, 3, 4, 5, 6, 7, 8 and S1.

	Forward	Reverse
Acc	GAG GTG GCT AAG AGG AGG CTC T	CAG CAC CGA GAC TGA ACT GTA AGG
Chrebp total	ATG ACC CCT CAC TCA GGG AAT A	GAT CCA AGG GTC CAG AGC AG
Chrebp α	CGA CAC TCA CCC ACC TCT TC	TTG TTC AGC CGG ATC TTG TC
Chrebp β	TCT GCA GAT CGC GTG GAG	CTT GTC CCG GCA TAG CAA C
Foxo1	TGT TAC TTA GCT CTC TCC CCT CG	AGA CGA GCA GTG GCT CAA T
Foxo1 (TGN)	GGA TGG TGA AGA GCG TGC CC	CGC TCT TGC CTC CCT CTG GA
Foxo3	CTCTCAGGCTCCTCACTGTA	ATGAGTTCACTACGGATGAT
G6Pase	TTA CCA GCC TCC TGT CGG	GAC ACA ACT GAA GCC GGT TAG
Lpk	CTT GCT CTA CCG TGA GCC TC	ACC ACA ATC ACC AGA TCA CC
Txnip	GAC TAG AGA GCC CCA CCA CC	GGA CGC ACG GAT CCA CCT CA
Igfbp1	CCT GCC AAC GAG AAC TCT AT	AGG GAT TTT CTT TCC ACT CC
Pepck	TGG CTA CGT CCC TAA GGA A	GGT CCT CCA GAT ACT TGT CGA
Cyclophilin	ATG GCA CTG GTG GCA AGT CC	TTG CCA TTC CTG GAC CCA AA
Tbp	CCC CAC AAC TCT TCC ATT CT	GCA GGA GTG ATA GGG GTC AT
Chore *Lpk*	GTC CCA CAC TTT GGA AGC AT	CCC CAA CAC TGA TTC TAC CC
Chore *Txnip*	AAG GGC CAA GTA GCC AAT GGG	GTG CTG GCC TGG AGG
IRE *Pepck*	TAC AGA CAT TAT CTA GAA GTC TCA	CAA GGG CAG GCC TAG CCG AGA
IRE *Txnip*	AAC AAC AAC CAT TTT CCC CGC TAG	ATA GCC GCC TGG CTT GGC GCT

Table S3. Antibodies and dilutions used for Western Blot analysis. Related to Figures 1, 3, 4, 5, 7, 8 and S1.

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Protein	Size (kDa)	Dilution (solution)	Secondary	Reference
ACC	265	1/5000 (BSA 5%)	Rabbit	Cell Signaling (#3662)
ChREBP	93	1/3000 (BSA 5%)	Rabbit	Nouvs (Nb400-135)
FoxO1	70	1/1000 (BSA 5%)	Rabbit	Cell Signaling (#2880)
GAPDH	36	1/2000 (lait 5%)	Rabbit	Santa Cruz (sc-25778)
HSP90	85	1/1000 (lait 5%)	Rabbit	Cell Signaling (#4874)
L-PK	62	1/300 (BSA 2%)	Rabbit	Dr A. Kahn (Cochin)
PEPCK	69	1/2000 (lait 5%)	Rabbit	Santa Cruz (sc-32879)
℗FoxO1	70	1/800 (BSA 5%)	Rabbit	Cell Signaling (#9461)
TxNIP	44	1/2000 (lait 5%)	Mice	MBL (K0205-3)

Transparent Methods

Animals

Ten to twelve week-old adult male C57BL/6J, *db/db*, *Chrebp*$^{+/+}$ and *Chrebp*$^{-/-}$ (Iroz et al., 2017), transgenic mice overexpressing a constitutively active form of FoxO1 in liver (FoxO1^{TGN}) (Zhang et al., 2006), liver specific triple FoxO1, 3, 4 knockout (LFoxO^{TKO}) (Zhang et al., 2016), liver specific insulin receptor knockout (LIR^{KO}) and liver specific insulin receptor and FoxO1 double knockout (LIRFox01^{KO}) (O-Sullivan et al., 2015) mice were used for *in vitro* and *in vivo* experiments as indicated. Procedures were carried out according to the French guidelines for the care and use of experimental animals (Animal authorization agreement n° CEEA34.AFB/CP.082.12, Paris Descartes Ethical Committee). Mice were maintained in a 12-hour light/dark cycle with water and standard diet (65% carbohydrate, 11% fat, and 24% protein) unless specified.

Nutritional and circadian challenges

Mice were studied in the fasted, fed or refed state. ZT stands for Zeitgeber time: ZT0 is defined as the time when the lights are turned on and ZT12 as the time when lights are turned off (7 pm). The fed group was fed ad libitum. The fasted group was fasted from ZT0 until ZT12. The refed group was fasted from ZT0 to ZT12 (included) and refed from ZT12 to ZT24. For circadian rhythms experiments, mice were killed by cervical dislocation at several time points in a pair-wise manner: ZT0, ZT4, ZT8, ZT12, ZT14, ZT16, ZT20, ZT24 as indicated. Liver was removed, snap-frozen in liquid nitrogen and stored at −80 °C until use.

Primary cultures of mouse hepatocytes

Mouse hepatocytes were isolated as described (Dentin et al., 2004). Briefly, hepatocytes were isolated from the livers of fed male mice by a modification of the collagenase method (Berry and Friend, 1969). Briefly, livers from mice were perfused with Hank's balanced salt solution (HBSS, KCl, 5.4 mm; KH_2PO_4, 0.45 mm; NaCl, 138 mm; $NaHCO_3$, 4.2 mm; Na_2HPO_4, 0.34 mm; glucose, 5.5 mm; HEPES, 1 m; EGTA, 50 mm; $CaCl_2$, 50 mm; pH 7.4). Livers were washed at a rate of 5 ml/min using the portal vein before collagenase (0.025%) was added. Cell viability was assessed by the trypan blue exclusion test and was always higher than 60%. Hepatocytes were seeded (in 60-mm Petri dishes at a density of 2×10^6 cells for RNA extraction or 6-well plates at a density of 4×10^5 cells per well for luciferase assays) in medium M199 with Earle salts (Invitrogen), supplemented with 10 μg/ml of streptomycin, 100 units/ml of

penicillin, 2.4 mm of glutamine, 0.1% (w/v) bovine serum albumin, 2% (v/v) Ultrocer G (Invitrogen), 100 nm dexamethasone (Soludecadron, Merck Sharp), and 100 nm insulin (Actrapid, Novo-Nordisk). After cell attachment (6 h), the medium was replaced by fresh M199 medium for 24 h. For adenoviral infections, hepatocytes from C57BL/6J male mice were incubated under low glucose concentration (5 mM) with specific adenovirus (from 0.1 to 3 pfu/cell) for 24 h. For glucose stimulation experiments, hepatocytes from *Chrebp*$^{+/+}$ and *Chrebp*$^{-/-}$ mice (Iroz et al., 2017) were incubated in the presence of low (5mM) or high glucose concentrations (25 mM) for 24 hours.

Adenoviral injection *in vivo*

To silence TxNIP expression, adult C57BL/6J (+/+) and *db/db* male mice were injected intravenously with a single dose of $5.0x10^9$ pfu of ShControl or ShTxnip adenovirus (GeneCust) at Day1. At Day 4, fasting blood glucose was measured. At Day 7, mice were challenged with a pyruvate tolerance test (PTT). At Day 8, fed blood glucose concentrations were measured. Mice were sacrificed under fasting conditions. To silence ChREBP, adult C57BL/6J male mice were injected with a single dose of $5.0x10^9$ pfu of ShControl or ShChREBP adenovirus (GeneCust) at Day1. Seven days later, mice were challenged to nutritional manipulations as indicated (Fasted or Refed) before sacrifice.

Pyruvate tolerance test

Intraperitoneal pyruvate tolerance test (PTT, 2g/kg body weight) was performed in overnight fasted awake adult C57BL/6J (+/+) and *db/db* mice 7 days post adenovirus injection.

Mutagenesis and Luciferase assays

A TxNIP promoter-luciferase reporter plasmid (pGL3B-1081) was obtained from Addgene (cat #18758). Hepatocytes from C57BL/6J male mice were plated in 6-well plates (4×10^5 cells per well) and transfected with TxNIP luciferase reporter constructs (0.2 µg DNA per well) including either Wild type or mutated on the ChoRE (ChoREa mutated) or on the IRE (IRE mutated) (Yu et al., 2009) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Mutants were generated using oligonucleotides described in Table S1. The luciferase assay was conducted using the dual luciferase substrate system (E1501; Promega, Madison, WI), and the result was normalized with the internal control Beta galactosidase. Each experiment was performed in triplicate and repeated 5 to 8 times as indicated.

ChIP analysis

In vivo ChiP assays from mouse livers were performed as described (Marmier et al., 2015). Briefly, genomic DNA regions of interest were isolated using antibodies against ChREBP (Novus) or Fox01 (Cell Signaling) or non-immune IgG as a control (Cell Signaling). QPCR reactions were carried out in triplicate using SYBR Green Supermix (Bio-Rad) on a CFX Connect™ Real Time PCR system. Positive and negative control sites were tested for each factor as well as the sites of interest. The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using input DNA (pooled unprecipitated genomic DNA from each sample). DNA fragments were quantified by qPCR, using primers described in Table S2. Results are expressed as fold enrichment.

Gene expression analysis

Total cellular RNA was extracted using the SV total RNA isolation system (Promega). For qPCR analysis, total RNA samples (2 µg) were reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers for SYBR Green assays are presented in Table S2. Amplifications were performed on an ABI Prism 7300 Real Time PCR System (Applied Biosystems). qPCR data were normalized by TATA-box binding protein (TBP) mRNA levels and analyzed with LinRegPCR.22.

Western blotting analysis

Proteins from liver lysates were subjected to 10% SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes. Antibodies and dilutions used are indicated in Table S3.

Wheat germ agglutinin purification

For ChREBP immunoprecipitation, cells were lysed on IPH buffer (20 mmol/L Tris/HCl, 150 mmol/L NaCl, 0.5% NP-40 [v/v], and protease inhibitors) as described (Guinez et al., 2011). Briefly, proteins were incubated with 2 µg of anti-ChREBP antibody (Novus) and placed at 4°C overnight. Bound proteins were recovered after addition of 30 µl of Sepharose-labeled protein G (Sigma) for 1 h at 4°C. Beads were gently centrifuged for 1 min and washed four times for 5 min each. For wheat germ agglutinin ([WGA] a GlcNAc-binding lectin) precipitation, 1 mg of proteins was incubated with 30 µl of WGA agarose beads (Sigma). Then, proteins were eluted from the beads in a Laemmli buffer and separated by SDS-PAGE.

Biochemical analysis

Blood glucose was measured in total blood using an Accu-Check glucometer (Roche).

Statistical Analysis

Data representing at least three independent experiments are reported as means ± S.E.M, and were analyzed using Prism 5.0, GraphPad) software. A student's T-test was used when comparing two groups (followed by Mann-Whitney post hoc test) or two-way ANOVA when comparing three or more groups followed by a Bonferroni post hoc test. Statistical significance was defined as $p<0.05$.

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