

# Ubiquitin-Specific Protease 2 Regulates Hepatic Gluconeogenesis and Diurnal Glucose Metabolism Through 11 $\beta$ -Hydroxysteroid Dehydrogenase 1

Matthew M. Molusky, Siming Li, Di Ma, Lei Yu, and Jiandie D. Lin

Hepatic gluconeogenesis is important for maintaining steady blood glucose levels during starvation and through light/dark cycles. The regulatory network that transduces hormonal and circadian signals serves to integrate these physiological cues and adjust glucose synthesis and secretion by the liver. In this study, we identified ubiquitin-specific protease 2 (USP2) as an inducible regulator of hepatic gluconeogenesis that responds to nutritional status and clock. Adenoviral-mediated expression of USP2 in the liver promotes hepatic glucose production and exacerbates glucose intolerance in diet-induced obese mice. In contrast, *in vivo* RNA interference (RNAi) knockdown of this factor improves systemic glycemic control. USP2 is a target gene of peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), a coactivator that integrates clock and energy metabolism, and is required for maintaining diurnal glucose homeostasis during restricted feeding. At the mechanistic level, USP2 regulates hepatic glucose metabolism through its induction of 11 $\beta$ -hydroxysteroid dehydrogenase 1 (HSD1) and glucocorticoid signaling in the liver. Pharmacological inhibition and liver-specific RNAi knockdown of HSD1 significantly impair the stimulation of hepatic gluconeogenesis by USP2. Together, these studies delineate a novel pathway that links hormonal and circadian signals to gluconeogenesis and glucose homeostasis. *Diabetes* 61:1025–1035, 2012

**H**epatic gluconeogenesis is important for maintaining blood glucose homeostasis in mammals during prolonged fasting. Glucocorticoids and glucagon are major physiological hormones that stimulate the expression of gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), whereas insulin suppresses the action of these counterregulatory hormones in the liver (1–3). Hepatic gluconeogenesis is abnormally elevated in both type 1 and type 2 diabetes, resulting in excess glucose secretion that exacerbates hyperglycemia in diabetes (4,5). Recent studies have demonstrated that the circadian clock exerts profound influences on energy metabolism and is required for maintaining energy and nutrient homeostasis (6). Mice with defective liver clock develop hypoglycemia following starvation and at certain time points during the day because of impaired hepatic gluconeogenesis

(7,8). As such, hormonal and circadian signals likely converge on key regulatory nodes to coordinate hepatic gluconeogenesis and glucose secretion.

Glucocorticoids are steroid hormones secreted by the adrenal gland that stimulate gluconeogenic genes through binding to glucocorticoid receptor (GR) in the liver. In humans, glucocorticoids circulate as protein-bound inactive precursor (cortisone) that can be converted into active hormone (cortisol) in tissues by 11 $\beta$ -hydroxysteroid dehydrogenase 1 (HSD11 $\beta$ 1 or HSD1), an endoplasmic reticulum membrane protein (9,10). Similarly, this enzyme catalyzes the conversion of inactive dehydrocorticosterone to active corticosterone in rodents. Excess activity of glucocorticoid signaling has been implicated in the development of glucose intolerance in patients with Cushing's Syndrome and also contributes to the pathogenesis of metabolic syndrome (11–14). Tissue-specific activation of glucocorticoid signaling by transgenic expression of HSD1 leads to the development of key features of metabolic syndrome, including central obesity, glucose intolerance, and hypertension (15,16). On the contrary, HSD1 inhibitors improve glycemic control in rodents as well as in humans, in part through attenuation of hepatic gluconeogenesis and glucose output (17,18).

Reversible protein ubiquitination and deubiquitination modulate the biochemical functions of target proteins. The latter is carried out by deubiquitinating enzymes, which remove ubiquitin or ubiquitin-like protein from their substrates (19,20). Ubiquitin-specific proteases (USPs) constitute a major family of deubiquitinases that is emerging as a versatile regulator of diverse biological processes, including cell cycle regulation, signaling, transcriptional regulation, and mitochondrial dynamics (21–25). Whether USP members are nutritionally regulated and participate in the regulation of glucose metabolism remains unexplored. In this study, we profiled USP expression in the liver under different nutritional conditions and identified USP2 as a fasting-inducible and clock-regulated deubiquitinase. USP2 stimulates hepatic gluconeogenesis and is required for maintaining normal glucose homeostasis. In addition, USP2 regulates systemic glucose tolerance in diet-induced obesity through induction of HSD1 expression and glucocorticoid signaling in the liver.

## RESEARCH DESIGN AND METHODS

**Cultured primary hepatocytes.** Primary hepatocytes were isolated from C57BL/6J mice using collagenase type II (Invitrogen, Carlsbad, CA), as previously described (26). Hepatocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine growth serum and antibiotics at 37°C and 5% CO<sub>2</sub>. Cells were switched to DMEM supplemented with 0.1% BSA for 16–24 h before treatments with hydrocortisone (1  $\mu$ M), glucagon (40 nmol/L) or insulin (100 nmol/L) for 6 h. For adenoviral transduction, recombinant adenoviruses were generated using AdEasy adenoviral vector (Stratagene, Santa Clara, CA) as previously described (27). Hepatocytes were transduced for 48 h at similar moiety of infection before RNA isolation and gene expression analysis.

From the Life Sciences Institute, University of Michigan Medical Center, Ann Arbor, Michigan; and the Department of Cell and Developmental Biology, University of Michigan Medical Center, Ann Arbor, Michigan.

Corresponding author: Jiandie D. Lin, [jdlin@umich.edu](mailto:jdlin@umich.edu).

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See accompanying commentary, p. 993.

**Gene expression analysis.** Total hepatocyte RNA was isolated using Trizol (Invitrogen), reverse transcribed using MMLV reverse transcriptase, and analyzed by quantitative PCR (qPCR) using the Sybr Green method. The primers used for qPCR analysis are listed in Supplementary Fig. 5 or described in previous studies (27,28).

**In vivo studies.** C57BL/6J mice were kept on a 12:12 light-dark cycle with food and water freely available. For fasting/refeeding studies, mice were provided food ad libitum, fasted for 20 h, or re-fed for 18 h following 20 h of fasting. Tissues were harvested at the same time and frozen immediately for gene expression analysis. For in vivo adenoviral transduction, chow or high-fat diet (HFD)-fed male mice were injected via tail vein with purified adenoviruses at approximately 0.15 optical density per mouse. Metabolic studies and gene expression analysis were performed 5–7 days after tail vein injection. For carbenoxolone (CBX) treatments, HFD-fed transduced mice were subcutaneously injected with either saline or CBX (40 mg/kg by body weight) once daily for 3 consecutive days between 11 A.M. and 12 P.M. *Bmal1* flox/flox mice were obtained from The Jackson Laboratory. Liver-specific *Bmal1* knockout mice were generated by breeding the flox/flox mice with albumin-Cre transgenic mice.

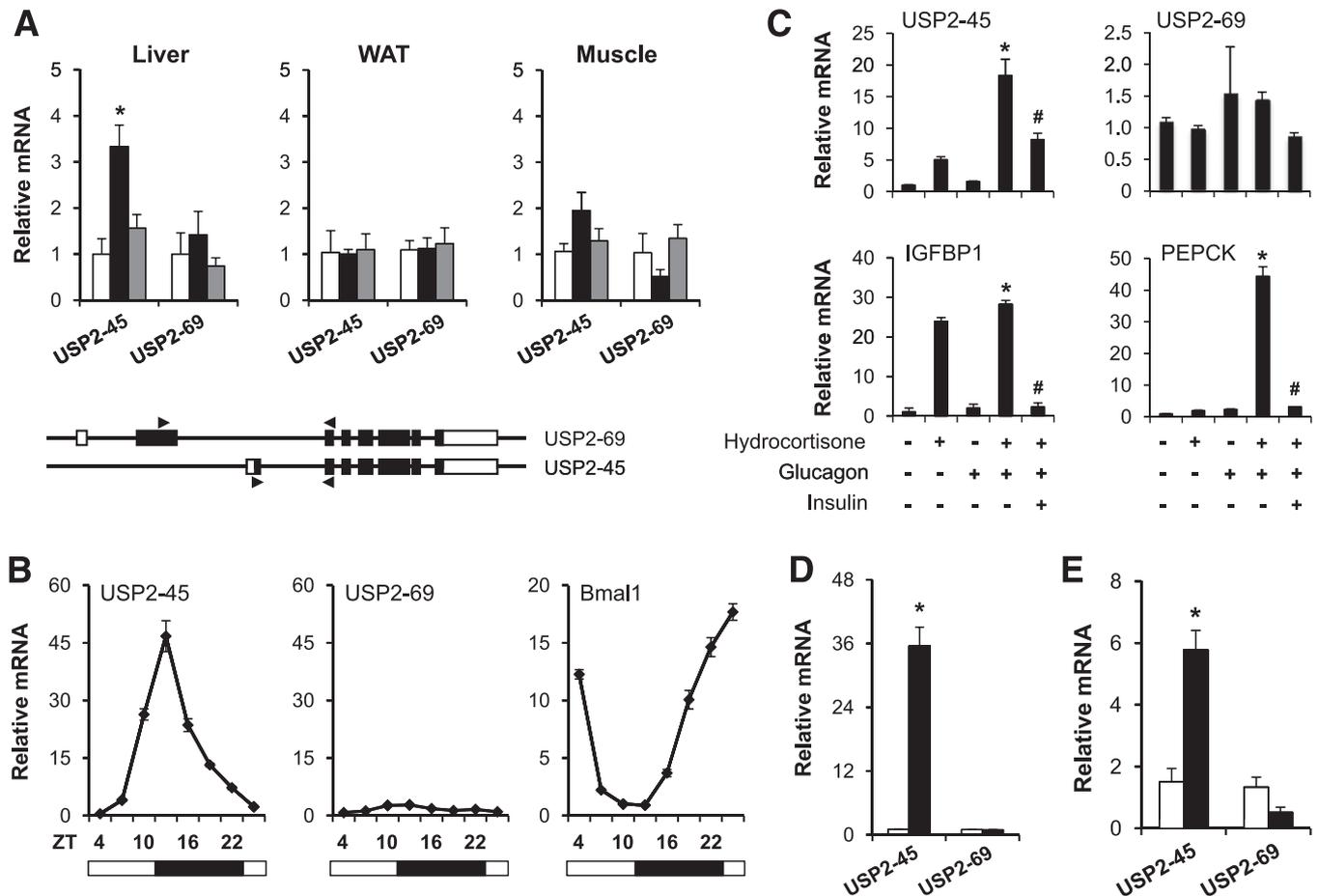
**Pyruvate, insulin, and glucose tolerance tests.** For insulin tolerance tests (ITTs), transduced HFD-fed (10–12 weeks old) mice were placed in clean cages without food for 4–5 h and injected intraperitoneally with an insulin solution at 0.8 units/kg of body weight. For glucose tolerance tests (GTTs), transduced HFD-fed mice were fasted overnight and injected intraperitoneally with glucose (2 g/kg, in 0.9% NaCl). For the pyruvate tolerance test (PTT), transduced chow-fed mice were fasted for 16 h and injected intraperitoneally with 2 g/kg of body weight of sodium pyruvate. Blood glucose levels were

measured before insulin injection and at 20, 40, 60, and 120 min after injection. Liver glycogen levels were measured as previously described (29).

**Histology.** Liver tissue from transduced mice was fixed in 4% paraformaldehyde and processed for paraffin embedding. Paraffin-embedded liver sections were stained for glycogen using Periodic Acid Schiff's Stain Kit (Polysciences, Inc., Warrington, PA).

**RESULTS**

**Nutritional and circadian regulation of USP2 expression in the liver.** The physiological role of USPs in glucose and lipid metabolism remains largely unexplored. To identify nutritionally regulated deubiquitinases, we profiled mRNA expression of 47 USP family members in mouse liver under ad libitum, fasted, and re-fed conditions. We found that USP2 mRNA expression is significantly induced by starvation and decreased following overnight refeeding (Fig. 1A), whereas other USP members are modestly affected. The USP2 gene has been annotated to generate two isoforms (USP2-45 and USP2-69) through alternative transcriptional initiation sites (30). To determine whether nutritional regulation of USP2 is isoform-specific, we performed qPCR analyses using primers specific for individual isoforms. As shown in Fig. 1A, mRNA expression of USP2-45 is



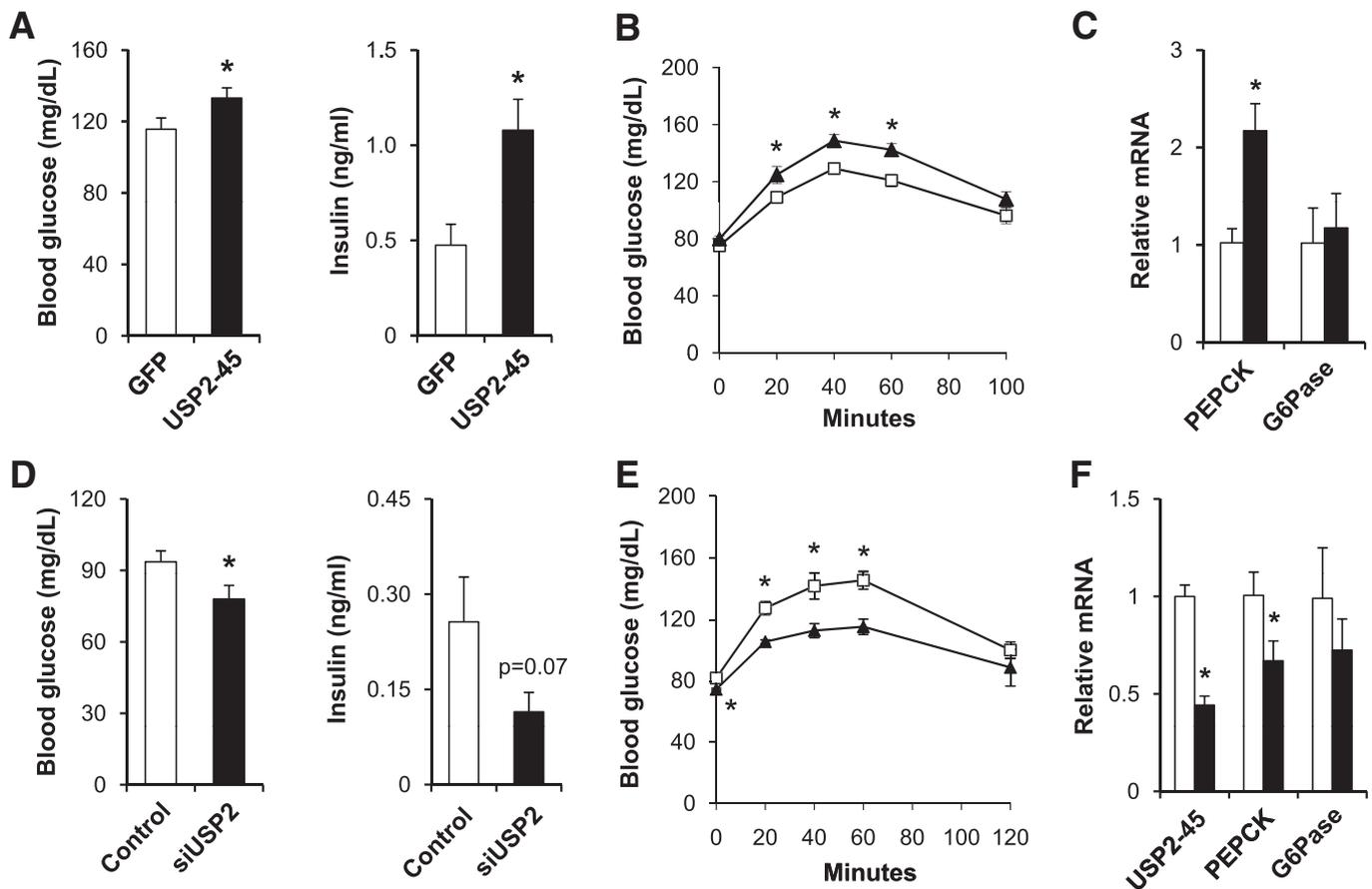
**FIG. 1.** Nutritional and circadian regulation of USP2 isoforms. **A:** Realtime qPCR analysis of USP2-45 and USP2-69 mRNA in liver, white adipose tissue (WAT), and skeletal muscle from fed (open), fasted (filled), and re-fed (gray) mice. Shown below is a schematic of the USP2 gene locus and the qPCR primers (arrowheads) used to detect USP2-45 and USP2-69 isoforms. Data are means  $\pm$  SEM ( $n = 4$ ). \* $P < 0.05$ . **B:** qPCR analysis of liver gene expression at different time points. ZT0 indicates the onset of light phase. **C:** qPCR analysis of total RNA from hepatocytes in triplicate wells treated with hydrocortisone, glucagon and insulin. Data are means  $\pm$  SD of samples from one representative experiment. \* $P < 0.01$ , hydrocortisone plus glucagon vs. control. # $P < 0.01$  insulin vs. no insulin. **D–E:** Expression of USP2-45 and USP2-69 in cultured primary hepatocytes (**D**) or livers from mice (**E**) transduced with GFP (open,  $n = 4$ ) or PGC-1 $\alpha$  (filled,  $n = 5$ ) adenovirus. Data are means  $\pm$  SEM. \* $P < 0.01$ , GFP vs. PGC-1 $\alpha$ .

induced by approximately 3.2-fold in the liver during fasting. In contrast, mRNA levels of USP2-69 remain similar under these feeding conditions. The induction of USP2-45 by starvation appears to be liver-specific, as mRNA levels for both isoforms remain largely unchanged in white adipose tissue and skeletal muscle. Although USP2 expression was found to be rhythmic in previous microarray studies (31,32), whether this diurnal regulation is unique for a specific isoform is unknown. Using isoform-specific primers, we found that USP2-45, but not USP2-69, exhibits robust diurnal regulation (Fig. 1B). USP2-45 mRNA levels increase toward the end of light phase and reach their peak at the onset of dark phase (ZT12). The peak of USP2-45 mRNA expression is antiphase to *Bmal1*, a core component of the molecular clock.

To determine the nature of starvation signals that regulate USP2-45 expression, we treated cultured mouse primary hepocytes with glucagon, hydrocortisone, and endogenous glucocorticoid in rodents, and insulin alone or in combination. As expected, the combination of glucagon and hydrocortisone strongly induces the expression of PEPCK and insulin-like growth factor binding protein-1, known glucocorticoid targets in the liver (Fig. 1C). The expression of USP2-45, but not UCP2-69, is robustly induced by hydrocortisone and further augmented in the presence of glucagon. Similar to PEPCK and insulin-like growth factor

binding protein-1, the induction of USP2-45 by hydrocortisone and glucagon is strongly suppressed by insulin treatments. Previous studies have implicated PGC-1 $\alpha$ , a transcriptional coactivator, in coordinating several aspects of hepatic fasting response, including hepatic gluconeogenesis, heme biosynthesis, and fatty acid  $\beta$ -oxidation (33,34). We next examined whether USP2 gene expression is regulated by PGC-1 $\alpha$  in primary hepatocytes transduced with green fluorescent protein (GFP) or PGC-1 $\alpha$  adenoviruses. Compared with control, PGC-1 $\alpha$  induces mRNA expression of USP2-45, but not USP2-69, by approximately 35-fold (Fig. 1D). Similar induction of USP2-45 by PGC-1 $\alpha$  was also observed in the livers from mice transduced with PGC-1 $\alpha$  adenovirus (Fig. 1E). These results indicate that USP2-45 is regulated by nutritional and circadian signals and is a target of hepatic glucocorticoid signaling.

**USP2-45 stimulates hepatic gluconeogenesis and glucose output.** De novo glucose synthesis via gluconeogenesis is essential for maintaining blood glucose levels during starvation in mammals. To determine whether USP2-45 regulates hepatic glucose production, we transduced chow-fed mice via tail vein injection of recombinant adenoviruses expressing GFP (control) or Flag/HA-tagged USP2-45. Compared with control, adenoviral-mediated expression of USP2-45 in the liver results in elevated plasma glucose and insulin concentrations (Fig. 2A). We next performed



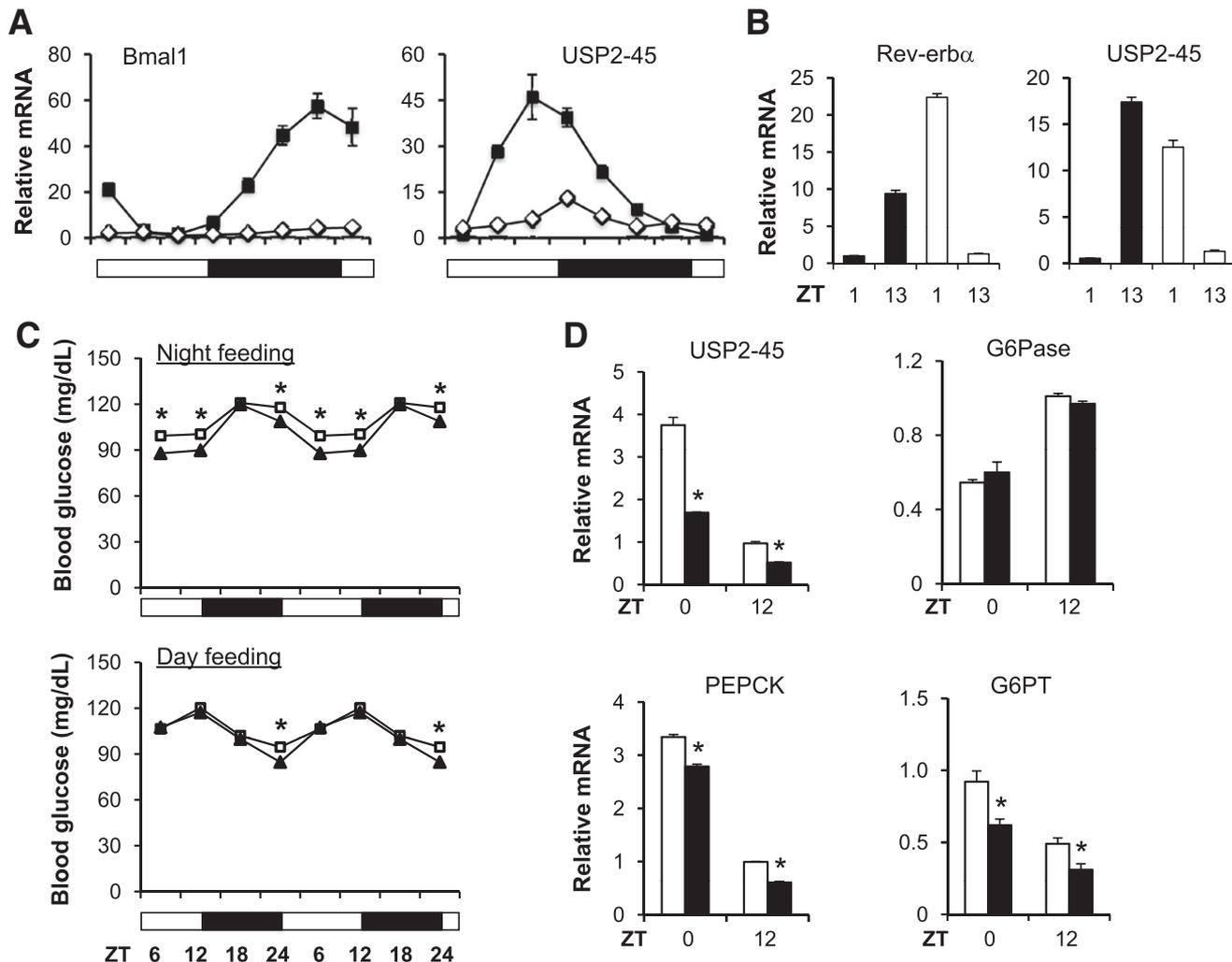
**FIG. 2.** USP2-45 regulates hepatic gluconeogenesis. **A:** Plasma glucose and insulin concentrations in transduced mice measured under fed condition (ZT5). **B:** PTT in mice transduced with GFP (open squares,  $n = 7$ ) or USP2-45 (filled triangles,  $n = 7$ ) adenovirus. **C:** qPCR analysis of PEPCK and G6Pase gene expression in livers from mice transduced with GFP (open) or USP2-45 (filled) adenovirus. Data in A-C are means  $\pm$  SEM. \* $P < 0.05$ , GFP vs. USP2-45. **D:** Blood glucose and insulin levels in mice transduced with control or siUSP2 adenovirus following overnight fast (ZT5). **E:** PTT in mice transduced with control (open squares,  $n = 6$ ) or siUSP2 (filled triangles,  $n = 7$ ) adenovirus. **F:** qPCR analysis of liver gene expression from mice transduced with control (open) or siUSP2 (filled) adenovirus. Data in D-F are means  $\pm$  SEM. \* $P < 0.05$ , control vs. siUSP2.

PTT to directly assess hepatic gluconeogenic function in transduced mice. We injected a single bolus of pyruvate, a gluconeogenic substrate, and measured blood glucose levels at different time points. Mice transduced with USP2-45 adenovirus have significantly higher blood glucose levels following pyruvate injection (Fig. 2B), suggesting that hepatic gluconeogenic activity is augmented by USP2-45. Consistently, we found that mRNA expression of PEPCK is increased by USP2-45 (Fig. 2C). The expression of G6Pase remains unchanged.

We next examined whether USP2 is required for maintaining normal blood glucose levels during starvation. We generated recombinant adenoviruses that express short hairpin RNA directed toward USP2 (Supplementary Fig. 1) and transduced C57BL/6J mice with control or small interfering USP2 (siUSP2) adenoviruses. Recombinant adenoviruses efficiently and nearly exclusively transduce hepatocytes when delivered via tail vein (35). Measurements of fasting blood glucose indicate that mice with

hepatic knockdown of USP2 have significantly lower glucose levels (Fig. 2D). In addition, circulating insulin concentration is also reduced in the knockdown group, although the difference only reaches borderline statistical significance ( $P = 0.07$ ). Consistently, we found that mice transduced with siUSP2 adenovirus have impaired ability to convert pyruvate into glucose during PTT (Fig. 2E), suggesting that hepatic gluconeogenesis and glucose output are impaired by RNAi knockdown of USP2 in the liver. Gene expression analysis revealed that PEPCK mRNA expression is lower in mice transduced with siUSP2 adenovirus (Fig. 2F). Interesting, RNAi knockdown of USP2 has modest effects on the induction of hepatic gluconeogenesis by PGC-1 $\alpha$  (Supplementary Fig. 2). We conclude from these studies that USP2-45 regulates hepatic gluconeogenesis and is required for plasma glucose homeostasis.

**Diurnal regulation of glucose homeostasis by USP2.** In mammals, the biological clock regulates diverse behavioral and physiological rhythms, notably pathways of nutrient



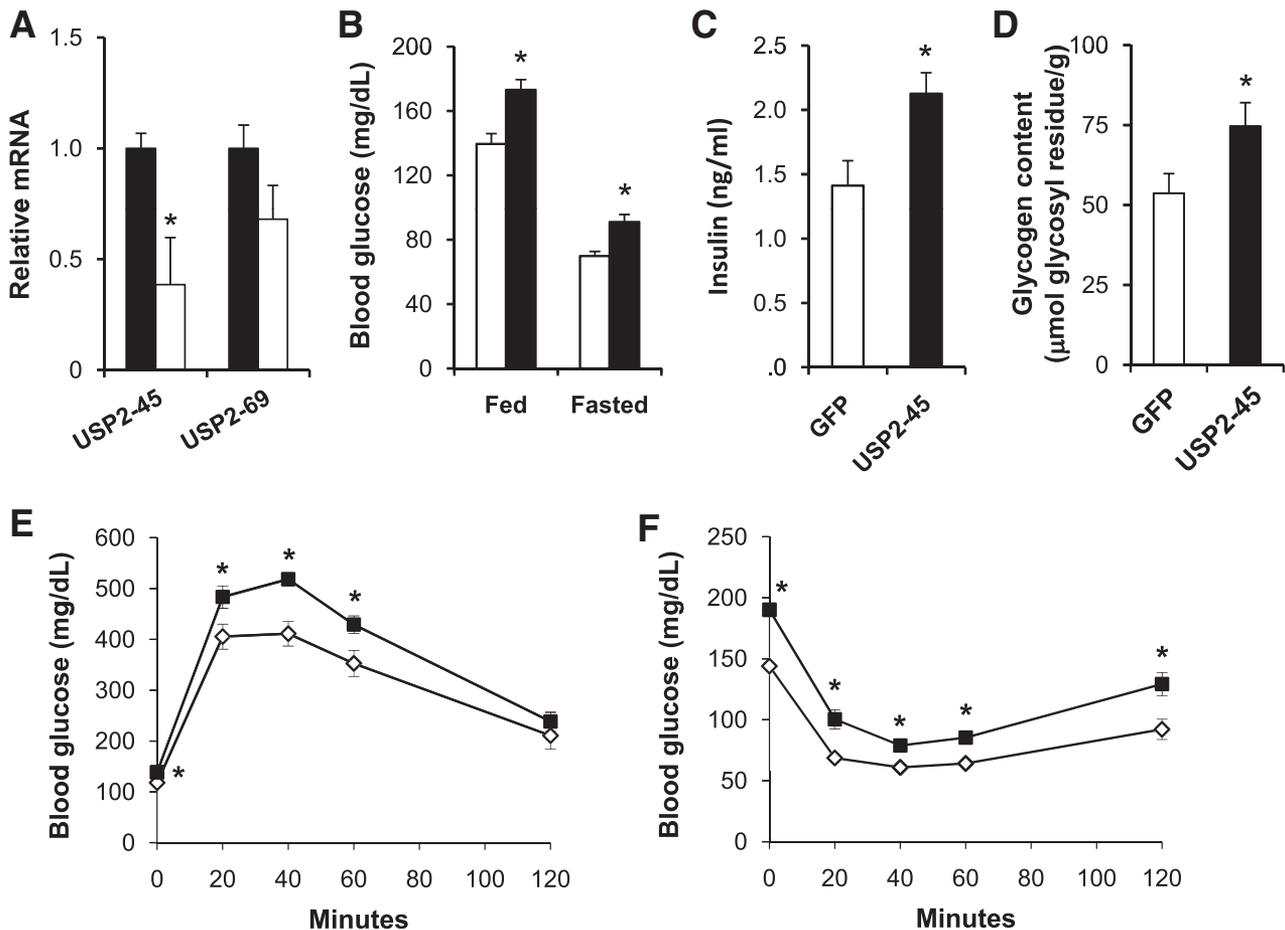
**FIG. 3.** USP2-45 is downstream of clock and regulates circadian glucose metabolism. **A:** qPCR analysis of liver gene expression in *Bmal1* flox/flox (filled squares) and liver-specific *Bmal1* null mice (open diamonds). Data are means  $\pm$  SD using pooled liver RNA ( $n = 3-5$ ). **B:** Regulation of liver *Rev-erb $\alpha$*  and USP2-45 expression by restricted feeding. Livers were harvested at ZT1 and ZT13 from mice maintained on night feeding (filled,  $n = 4$ ) or after switching to day feeding for 4 days (open,  $n = 4$ ). **C:** Blood glucose levels in mice transduced with control (open squares,  $n = 11$ ) or siUSP2 (filled triangles,  $n = 12$ ) adenovirus with feeding restricted to nighttime (upper panel) or 3 days after a switch to daytime feeding (lower panel). Data are double plotted means  $\pm$  SEM. **D:** qPCR analysis of liver gene expression from restricted daytime fed mice transduced with control (open) or siUSP2 (filled) harvested at ZT0 or ZT12. Data are means  $\pm$  SD using pooled RNA assayed in triplicate. \* $P < 0.05$ , control vs. siUSP2.

and energy metabolism. The clock oscillator, which is composed of multiple positive and negative transcriptional feedback loops, exerts its effects on tissue physiology in part through output pathways. We postulated that USP2–45 may serve as a downstream target of clock and examined whether a functional liver clock is required for the rhythmic expression of USP2–45. Recent studies have demonstrated that liver-specific deletion of *Bmal1* abolishes clock function in this tissue without perturbing locomotor activity and feeding behavior (7). As expected, rhythmic expression of several clock genes, including *Period 2* (*Per2*) and *Rev-erba*, is severely perturbed in the liver deficient in *Bmal1* (data not shown). Hepatic USP2–45 mRNA expression exhibits a strong diurnal rhythm in flox/flox control mice. In contrast, this cyclic expression is nearly abolished in *Bmal1*-deficient mouse livers (Fig. 3A). These results strongly suggest that USP2–45 is a cell-autonomous target of clock in the liver.

Restricted feeding has been demonstrated to play a dominant role in setting the phase of clock and metabolic gene expression in peripheral tissues (28,36). As such, switching mice from night feeding to day feeding results in phase resetting of clock gene expression (Fig. 3B). Although USP2–45 mRNA expression peaks at zeitgeber

time (ZT; ZT0 is the onset of light phase) 13 (ZT13) under night feeding condition, this diurnal pattern is reversed in the liver following 4 days of day feeding, suggesting that USP2–45 expression is tightly linked to the phase of liver clock. To explore the role of USP2–45 in circadian glucose regulation, we transduced C57BL/6J mice with control or siUSP2 adenoviruses and measured plasma glucose levels under restricted feeding conditions. We observed significantly lower plasma glucose levels at ZT6, ZT12, and ZT24, but not ZT18, when mice were fed exclusively during dark phase (Fig. 3C). Following a switch to daytime feeding, siUSP2 transduced mice have significantly lower glucose levels at ZT24, suggesting that hepatic USP2–45 is required for diurnal regulation of glucose homeostasis in response to restricted feeding. Gene expression analysis at ZT0 and ZT12 reveals that mRNA levels of USP2, PEPCK, and glucose-6-phosphate transporter (G6PT), but not G6Pase, are reduced in response to RNAi knockdown of USP2 (Fig. 3D). We conclude from these studies that USP2–45 is a target of circadian signaling in the liver and participates in diurnal regulation of glucose metabolism.

**Hepatic overexpression of USP2 exacerbates glucose intolerance in diet-induced obese mice.** Hepatic gluconeogenesis and glucose secretion are elevated in diabetes



**FIG. 4.** Hepatic USP2–45 overexpression promotes glucose intolerance in HFD-fed mice. **A:** qPCR analysis of hepatic USP2 gene expression in mice fed chow (filled,  $n = 3$ ) or HFD (open,  $n = 3$ ). Data are means  $\pm$  SEM. \* $P < 0.05$ , chow vs. HFD. **B:** Plasma glucose concentrations in HFD-fed mice transduced with GFP (open,  $n = 7$ ) or USP2–45 (filled,  $n = 7$ ) adenovirus. **C–D:** Plasma insulin concentrations (**C**) and liver glycogen (**D**) in ad libitum transduced mice. **E:** GTT in HFD-fed mice transduced with GFP (open diamonds,  $n = 8$ ) or USP2–45 (filled squares,  $n = 8$ ) adenovirus. **F:** ITT in transduced mice. Data in **B–F** are means  $\pm$  SEM. \* $P < 0.05$ , GFP vs. USP2–45.

and exacerbate hyperglycemia in diabetic states. We next examined whether USP2 expression is altered in the liver of HFD-fed obese mice. Compared with lean control, mRNA expression of USP2-45 is significantly decreased in the liver of diet-induced obese mice (Fig. 4A). Chronic high-fat feeding results in obesity and hyperinsulinemia. Given that USP2-45 expression is suppressed by insulin in hepatocytes, our results suggest that insulin may inhibit hepatic USP2-45 expression in obese mice, potentially serving as an adaptive mechanism to restrain hepatic gluconeogenesis when blood glucose is readily available and insulin levels are high.

We next examined whether liver-specific overexpression of USP2 exacerbates glucose intolerance in the insulin resistant state. We transduced HFD-fed mice with recombinant adenoviruses expressing GFP or USP2-45. Compared with control, blood glucose concentrations are significantly elevated in mice transduced with USP2-45 adenovirus under fed and fasted conditions (Fig. 4B). Fasting blood insulin concentrations are also significantly higher in mice transduced with USP2 adenovirus (Fig. 4C). To rule out the possibility that enhanced glycogenolysis may contribute to elevated blood glucose in response to USP2-45, we measured liver glycogen content in transduced mice.

Compared with GFP, USP2-45 significantly increases liver glycogen content (Fig. 4D and Supplementary Fig. 3). Because gluconeogenesis is important for the indirect pathway of hepatic glycogen synthesis (37), these results suggest that USP2-45 augments gluconeogenic flux that leads to increased glucose production and glycogen storage. GTTs indicate that adenoviral-mediated overexpression of USP2-45 further impairs glucose tolerance in transduced mice (Fig. 4E). Consistently, blood glucose levels remain significantly elevated at all time points in an ITT (Fig. 4F). The rate of glucose lowering in response to insulin injection, however, appears to be similar in these two groups of mice (Supplementary Fig. 3)

Analysis of hepatic gene expression indicates that PEPCK mRNA expression is significantly induced by USP2-45 (Fig. 5A). Unlike chow-fed mice, the mRNA levels of G6Pase and G6PT are also increased in response to USP2-45 overexpression in diet-induced obese mice. Excess glucocorticoid signaling is responsible for the development of diabetes in patients with Cushing's Syndrome and has also been implicated in the pathogenesis of metabolic syndrome (11-14). In humans, cortisone is released by the adrenal gland and converted to active cortisol locally in tissues by

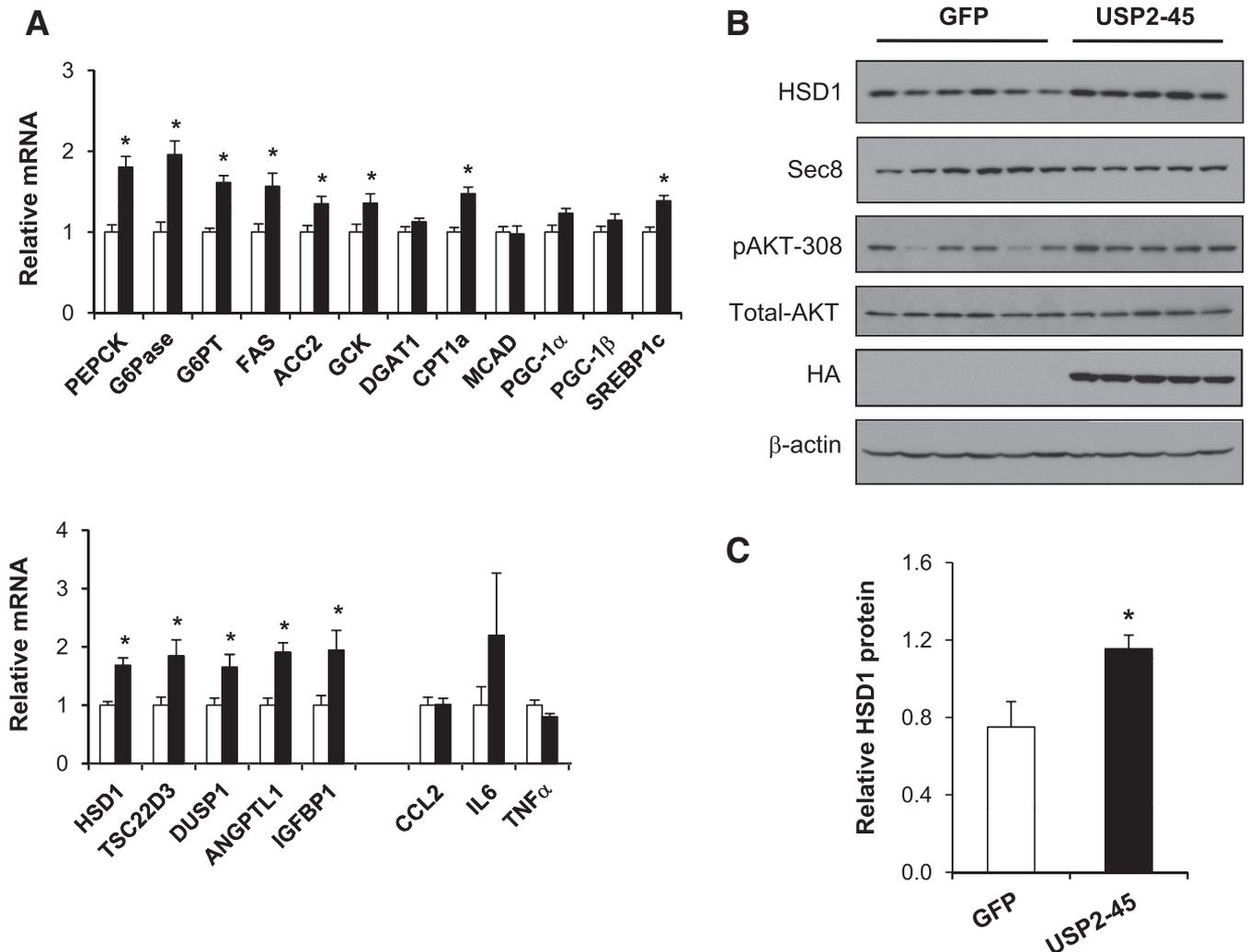


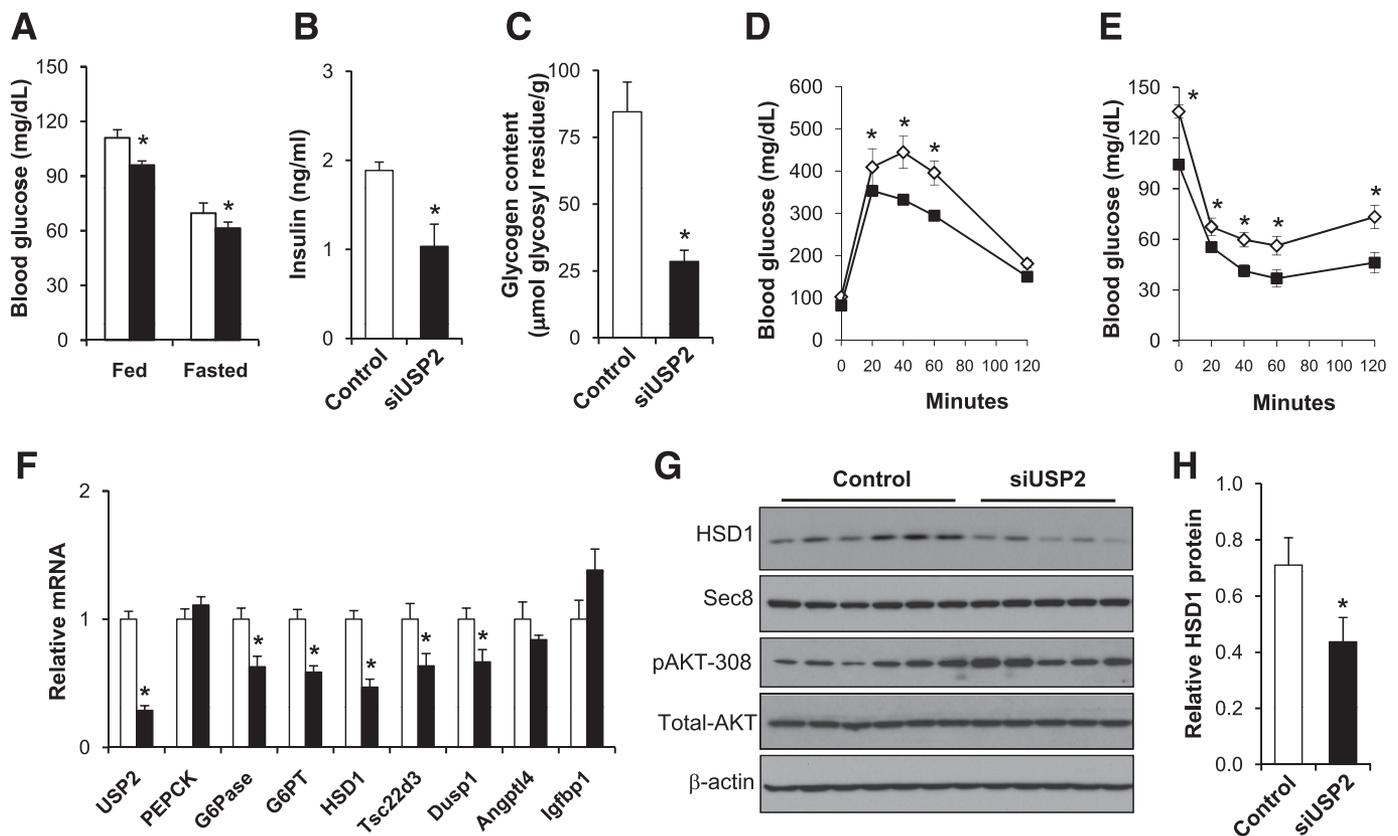
FIG. 5. Hepatic gene expression analyses. A: qPCR analysis of liver gene expression in mice transduced with GFP (open) or USP2-45 (filled) adenovirus. Data are means ± SEM. \*P < 0.05, GFP vs. USP2-45. B: Immunoblotting analysis of total liver lysates from transduced mice as indicated. C: Quantitation of HSD1 protein expression as normalized to β-actin.

HSD1. Because a major physiological target of glucocorticoid signaling in the liver is gluconeogenesis, we postulated that USP2-45 may augment hepatic glucocorticoid signaling. In support of this, we found that mRNA levels for several known glucocorticoid target genes, including *IGFBP1*, *TSC22D3*, *DUSP1*, and *ANGPTL4* (38), are also induced by USP2-45. Interestingly, both mRNA and protein levels of HSD1 are significantly increased in response to hepatic overexpression of USP2-45 (Fig. 5B). In contrast, the expression of Sec8, another endoplasmic reticulum membrane protein, and GR remains similar. Although mRNA levels of *CCL2*, *IL6*, and *TNF $\alpha$* , genes involved in inflammatory response, remain similar between two groups, the expression of several lipogenic genes, including fatty acid synthase, acetyl-CoA carboxylase 2 (*ACC2*), and glucokinase (*GCK*), appears to be enhanced by USP2-45 (Fig. 5A). These results are consistent with elevated plasma insulin concentrations in mice transduced with USP2-45 adenovirus. In fact, the expression of *SREBP1c*, an insulin-responsive regulator of lipogenesis, is also induced by USP2-45. *AKT* phosphorylation is modestly affected by USP2-45. Together, these results demonstrate that USP2-45 augments glucocorticoid signaling in the liver and exacerbates glucose intolerance in the insulin resistant state.

**Hepatic USP2 knockdown improves glucose homeostasis in obese mice.** The expression of USP2 is stimulated by starvation and reaches peak levels at the onset of dark phase (Fig. 1). Both of these conditions are characterized by

active glucocorticoid signaling in the liver. As such, it is possible that USP2 plays an important role in modulating local glucocorticoid activation through its regulation of HSD1. We next examined whether RNAi knockdown of USP2 suppresses the gluconeogenic pathway and ameliorates glucose intolerance in HFD-fed mice. We transduced high-fat fed mice with control or USP2 RNAi adenoviruses for 5–7 days. Compared with control, blood glucose levels are significantly lower in mice transduced with siUSP2 adenovirus when measured under ad libitum and fasted conditions (Fig. 6A). In addition, fasting insulin concentrations are lower in the knockdown group (Fig. 6B). Liver glycogen content is also reduced in mice transduced with siUSP2 adenovirus, as revealed by quantitative assays and periodic acid Schiff's staining (Fig. 6C and Supplementary Fig. 4). We next performed GTT and ITT in transduced mice to assess whole body glucose tolerance and insulin sensitivity. As shown in Fig. 6D, blood glucose levels following an intraperitoneally glucose bolus are significantly lower in mice transduced with siUSP2 adenovirus. Similarly, USP2 knockdown results in lower blood glucose levels following insulin injection (Fig. 6E and Supplementary Fig. 4). These results demonstrate that depletion of USP2 in the liver improves glucose homeostasis in an insulin resistant state.

Gene expression analysis reveals that RNAi knockdown of USP2 results in approximately 65% reduction in USP2 mRNA levels in the liver (Fig. 6F). The expression of *G6Pase* and *G6PT* are decreased in response to USP2 knockdown.



**FIG. 6.** RNAi knockdown of USP2 in the liver ameliorates glucose intolerance in HFD-fed mice. **A:** Plasma glucose concentrations in HFD mice transduced with control (open,  $n = 7$ ) or siUSP2 (filled,  $n = 7$ ) adenovirus. **B:** Plasma insulin levels in transduced mice following overnight fasting. **C:** Liver glycogen content in transduced mice. **D** and **E:** GTT (**D**) and ITT (**E**) in mice transduced with control (open diamonds) or siUSP2 (filled squares) adenovirus. **F:** qPCR analysis of liver gene expression in HFD-fed mice transduced with control or siUSP2 adenovirus. Data are means  $\pm$  SEM.  $*P < 0.05$ , control vs. siUSP2. **G:** Western blot analysis of total liver lysates from transduced mice. **H:** Quantitation of HSD1 protein expression as normalized to  $\beta$ -actin.

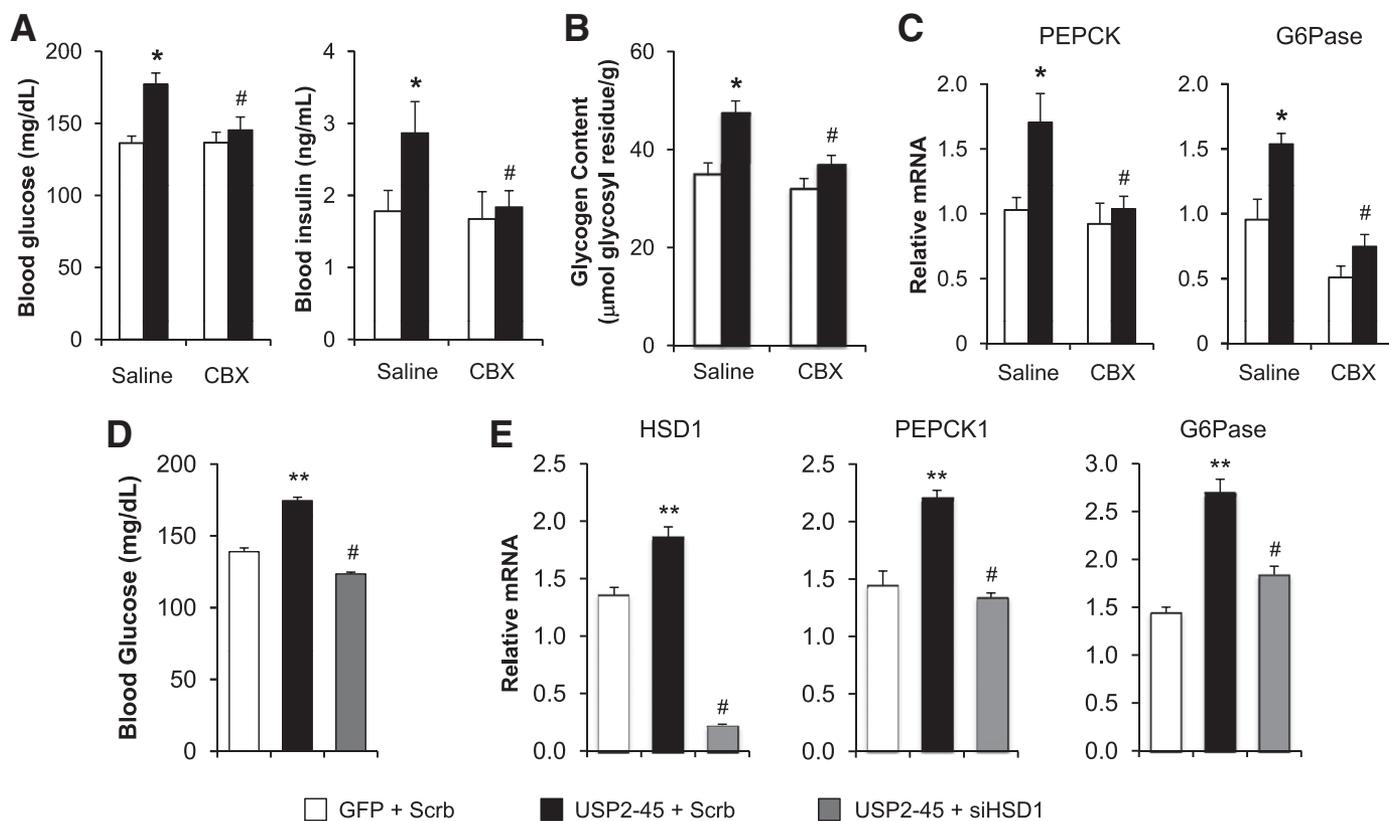
Surprisingly, PEPCK expression is only modestly affected, possibly as a result of lower insulin levels in mice transduced with siUSP2 adenovirus. Compared with control, HSD1 mRNA and protein levels are decreased in mice transduced with siUSP2 adenovirus (Fig. 6G–H). In addition, hepatic expression of TSC22D3 and DUSP1 are decreased following USP2 knockdown. We conclude from these studies that hepatic USP2 is required for maintaining normal levels of HSD1 expression and glucocorticoid action in the liver.

**HSD1 inhibitor blocks the stimulatory effects of USP2 on hepatic gluconeogenesis.** To directly assess the significance of HSD1 in mediating the effects of USP2–45 on glucose metabolism, we transduced HFD fed mice with GFP or USP2–45 adenoviruses followed by subcutaneous injection of saline or CBX (40 mg/kg), an inhibitor of HSD1. Chronic CBX treatments have been previously demonstrated to improve glycemic control in rodents and humans (18,39,40). As expected, adenoviral-mediated expression of USP2–45 significantly elevates circulating glucose and insulin concentrations (Fig. 7A). While daily injections of CBX for three days have modest effects on blood glucose levels in control mice, these treatments nearly abolished the hyperglycemic effects of USP2–45. Plasma insulin levels are also decreased by CBX in mice with hepatic overexpression of USP2–45. Further, the augmentation of liver glycogen accumulation in response to USP2–45 is significantly blocked by CBX treatments (Fig. 7B). Analysis of hepatic gene expression indicates that the induction of PEPCK and G6Pase

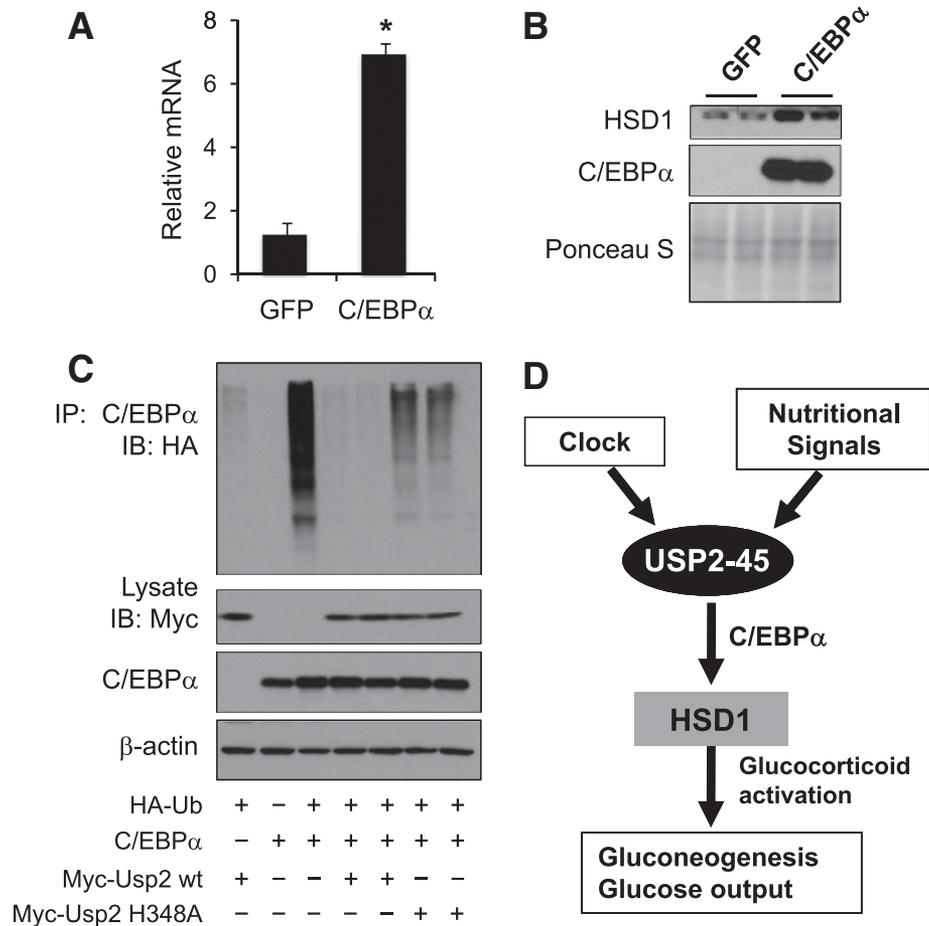
by USP2–45 is significantly diminished by HSD1 inhibition (Fig. 7C).

To rule out the possibility that CBX treatment may alter plasma glucose levels through nonhepatic mechanisms, we constructed a recombinant adenovirus expressing short hairpin RNA targeting HSD1. We transduced HFD-fed mice with control (GFP) or USP2–45 in the presence or absence of small interfering HSD1 (siHSD1) adenovirus. Compared with control, hepatic expression of USP2–45 results in elevated blood glucose levels (Fig. 7D). This hyperglycemic effect of USP2–45 is significantly dampened when HSD1 is reduced by RNAi knockdown. Consistently, the induction of PEPCK and G6Pase gene expression by USP2–45 is also decreased in mice transduced with siHSD1 adenovirus (Fig. 7E). Together, these results strongly suggest that the activation of glucocorticoid signaling through HSD1 is critical for mediating the metabolic effects of USP2–45 on hepatic gluconeogenesis and glucose production.

Previous studies have demonstrated that transcription factor C/EBP $\alpha$  increases HSD1 promoter activity (41). To determine whether C/EBP $\alpha$  regulates endogenous HSD1 gene expression, we transduced hepatocytes with GFP or C/EBP $\alpha$  adenovirus. Gene expression analyses indicate that C/EBP $\alpha$  robustly induces both mRNA and protein expression of HSD1 in transduced hepatocytes (Fig. 8A and B). To explore whether C/EBP $\alpha$  may serve as a substrate for USP2–45, we transiently transfected 293 cells with C/EBP $\alpha$  and hemagglutinin-tagged ubiquitin plasmids in the presence or absence of USP2–45. As shown in Fig. 8C, C/EBP $\alpha$



**FIG. 7. HSD1 inhibition blocks the effects of USP2–45 on glucose metabolism.** A and B: Plasma glucose and insulin concentrations (A) and liver glycogen content (B) in mice transduced with GFP (open,  $n = 6$ ) or USP2–45 (filled,  $n = 6$ ) followed by treatments with vehicle or CBX for 3 days. Data are means  $\pm$  SEM. \* $P < 0.05$  USP2–45 vs. GFP; # $P < 0.05$  CBX vs. saline. C: qPCR analysis of liver gene expression. Data represents mean  $\pm$  SD using pooled RNA samples assayed in triplicate. D–E: Plasma glucose (D) and liver gene expression (E) in mice transduced with indicated combinations of adenoviruses. Data are means  $\pm$  SEM. \*\* $P < 0.05$  USP2–45+Scrub vs. GFP+Scrub; # $P < 0.05$  USP2–45+siHSD1 vs. USP2–45+Scrub. Samples were collected at ZT11–12.



**FIG. 8.** C/EBP $\alpha$  induces HSD1 expression and is a substrate of USP2-45. **A** and **B**: Induction of HSD1 mRNA (**A**) and protein (**B**) expression by C/EBP $\alpha$  in primary hepatocytes. \* $P < 0.01$ , C/EBP $\alpha$  vs. GFP. **C**: Deubiquitination of C/EBP $\alpha$  by USP2-45 in transiently transfected 293 cells. **D**: Model depicting the role of USP2-45 in mediating circadian and nutritional regulation of hepatic gluconeogenesis and glucose secretion.

is heavily ubiquitinated in the presence of MG132, a proteasome inhibitor. The level of C/EBP $\alpha$  ubiquitination is significantly reduced when USP2-45 is coexpressed in the cell. In contrast, the levels of ubiquitinated C/EBP $\alpha$  remain high in cells cotransfected with a catalytic dead mutant of USP2-45 (42). To our surprise, USP2-45-mediated deubiquitination appears to exert modest effects on C/EBP $\alpha$  protein stability (data not shown). These data suggest that USP2-45 may regulate HSD1 gene expression through deubiquitinating C/EBP $\alpha$  and potentially modulating its transcriptional activity.

## DISCUSSION

The mechanisms that integrate these hormonal and circadian signals in the regulation of gluconeogenesis are incompletely understood. In this study, we identified USP2 as a nutrient-inducible and clock-regulated deubiquitinase that regulates hepatic gluconeogenesis and glucose homeostasis through modulating glucocorticoid signaling in the liver (Fig. 8D). In vivo gain- and loss-of-function studies demonstrate that USP2 is sufficient and necessary for maintaining normal gluconeogenic activity in the liver. Tail vein injection of a recombinant adenovirus expressing USP2-45 leads to elevated blood glucose and insulin concentrations. Direct assessment of hepatic gluconeogenesis using pyruvate as a substrate indicates that USP2-45 significantly

increases hepatic glucose production. In contrast, depletion of endogenous USP2 by in vivo RNAi knockdown impairs gluconeogenic gene expression and results in the development of hypoglycemia during fasting. USP2 deficiency in the liver also perturbs normal diurnal glucose rhythms under restricted feeding conditions. In HFD-fed mice, adenoviral-mediated expression of USP2 exacerbates glucose intolerance and insulin resistance, as illustrated by elevated blood glucose and insulin levels. In this case, hepatic USP2-45 overexpression impairs the ability of transduced mice to clear glucose from circulation during GTT. Though it is possible that impaired response to insulin is local in nature, i.e., suppression of gluconeogenesis in the liver, we cannot rule out the possibility that other tissues, such as skeletal muscle and white adipose tissue, may also develop insulin resistance secondary to hepatic insulin resistance. Accordingly, RNAi knockdown of USP2 ameliorates glucose intolerance in diet-induced obese mice. As such, USP2-45 appears to serve as a "rheostat" in the liver that adjusts hepatic gluconeogenesis and glucose output. Pharmacological modulation of USP2-45 activity is likely to have significant impact on glucose homeostasis and glycemic control in diabetes.

Perhaps the most intriguing aspect of USP2 regulation of hepatic gluconeogenesis is its role in modulating HSD1 expression. Hepatic overexpression of USP2 increases HSD1 mRNA and protein levels, leading to the induction of gluconeogenic genes as well as several other glucocorticoid

targets, whereas RNAi knockdown of USP2 significantly lowers HSD1 gene expression in the liver. Importantly, chemical inhibition of HSD1 activity by CBX abolished the ability of USP2-45 to activate gluconeogenic genes and raise blood glucose levels, suggesting that HSD1 induction is required for mediating the effects of USP2 on glucose metabolism. Previous studies have demonstrated that HSD1 is required for the generation of active glucocorticoid from inactive precursor in tissues (43). Mice lacking HSD1 fail to properly activate PEPCK and G6Pase expression in the liver in response to starvation and have improved glucose tolerance (44). In addition, blood glucose levels are lower in HSD1 null mice following high-fat feeding, suggesting that HSD1 plays an important role in physiological activation of hepatic gluconeogenesis and contributes to the development of hyperglycemia in obesity. Our data indicate that USP2-45 is itself a glucocorticoid target that also plays an important role modulating glucocorticoid signaling in the liver. This potential feedforward regulatory loop may serve to amplify glucocorticoid action following prolonged starvation and toward the onset of dark phase.

USPs modulate cellular signaling through deubiquitinating substrate proteins. Several substrates have been identified for USP2, including fatty acid synthase, epithelial Na<sup>+</sup> channel, MDM2, and MDMx (42,45–47). Although the exact substrates of USP2-45 that mediate its regulation of hepatic gluconeogenesis remain unknown, C/EBP $\alpha$  provides a plausible link. C/EBP $\alpha$  is deubiquitinated by USP2-45 and regulates the expression of endogenous HSD1 in hepatocytes. These results are consistent with previous findings that C/EBP $\alpha$  activates HSD1 promoter activity (41). Surprisingly, deubiquitination of C/EBP $\alpha$  by USP2-45 does not appear to significantly alter the stability of C/EBP $\alpha$ . As ubiquitination of transcription factors may affect their transcriptional functions (20), it is possible that USP2-45-mediated deubiquitination of C/EBP $\alpha$  may augment its transcriptional activity, resulting in the induction of HSD1 gene expression. Future studies are needed to elucidate the exact molecular mechanisms involved.

The expression of USP2-45 is induced by starvation and rises toward the end of the light phase. This regulation of USP2 is unique for the USP2-45 isoform, since mRNA levels of USP2-69 remain similar under these conditions. In cultured hepatocytes, hydrocortisone potently stimulates, whereas insulin represses, USP2-45 gene expression. It is likely that the inhibition of USP2-45 expression by insulin may serve as an adaptive mechanism to limit hepatic glucose production under physiological conditions when blood glucose is abundant and insulin levels are high. Previous studies have demonstrated that PGC-1 $\alpha$  coordinates several aspects of hepatic fasting response, including the activation of gluconeogenesis, heme biosynthesis, and fatty acid  $\beta$ -oxidation (48–50). In addition, PGC-1 $\alpha$  itself is rhythmically expressed in the liver and directly regulates core clock genes, including Bmal1 and Rev-erb $\alpha$  (28). Given that PGC-1 $\alpha$  strongly induces USP2-45 expression, it is possible that USP2-45 is a component of the hepatic metabolic response orchestrated by PGC-1 $\alpha$  during fasting and throughout light/dark cycles. Although the molecular details that regulate the transcriptional regulation of USP2-45 expression remain to be explored, one potential scenario is that USP2-45 may be a direct target of GR, which itself is a transcriptional partner for PGC-1 $\alpha$ . Alternatively, PGC-1 $\alpha$  may regulate USP2-45 gene expression indirectly through its modulation of clock pathway.

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M.M.M. and J.D.L. designed and performed experiments, analyzed data, and wrote and edited the manuscript. S.L. and D.M. designed and performed experiments, analyzed data, and reviewed the manuscript. L.Y. performed studies. J.D.L. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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