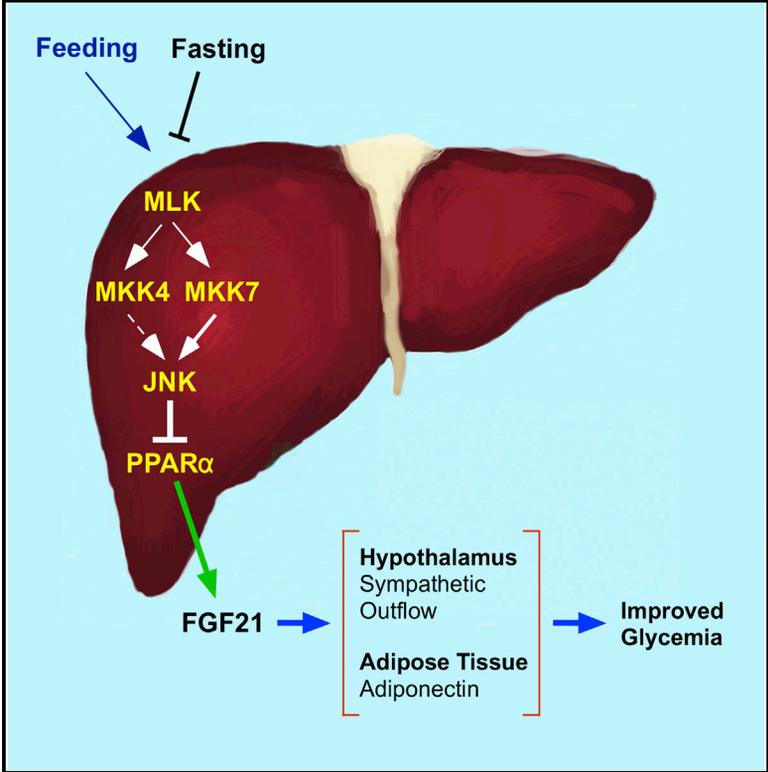


## Fibroblast Growth Factor 21 Mediates Glycemic Regulation by Hepatic JNK

### Graphical Abstract



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### In Brief

Vernia et al. examine the role of circulating FGF21 in the response to JNK signaling. Hepatic JNK deficiency promotes expression of FGF21 and improves glycemia. Liver-specific ablation of the *Fgf21* gene prevents this improvement of glycemia. These data argue that FGF21 mediates metabolic actions of hepatic JNK.

### Highlights

- Hepatic JNK activity regulates FGF21 expression during fasting/feeding cycles
- Hepatic JNK suppresses circulating FGF21 and promotes metabolic syndrome
- Hepatocyte FGF21 is required for the effects of hepatic JNK on metabolic syndrome

# Fibroblast Growth Factor 21 Mediates Glycemic Regulation by Hepatic JNK

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## SUMMARY

The cJun NH<sub>2</sub>-terminal kinase (JNK)-signaling pathway is implicated in metabolic syndrome, including dysregulated blood glucose concentration and insulin resistance. Fibroblast growth factor 21 (FGF21) is a target of the hepatic JNK-signaling pathway and may contribute to the regulation of glycemia. To test the role of FGF21, we established mice with selective ablation of the *Fgf21* gene in hepatocytes. FGF21 deficiency in the liver caused marked loss of FGF21 protein circulating in the blood. Moreover, the protective effects of hepatic JNK deficiency to suppress metabolic syndrome in high-fat diet-fed mice were not observed in mice with hepatocyte-specific FGF21 deficiency, including reduced blood glucose concentration and reduced intolerance to glucose and insulin. Furthermore, we show that JNK contributes to the regulation of hepatic FGF21 expression during fasting/feeding cycles. These data demonstrate that the hepatokine FGF21 is a key mediator of JNK-regulated metabolic syndrome.

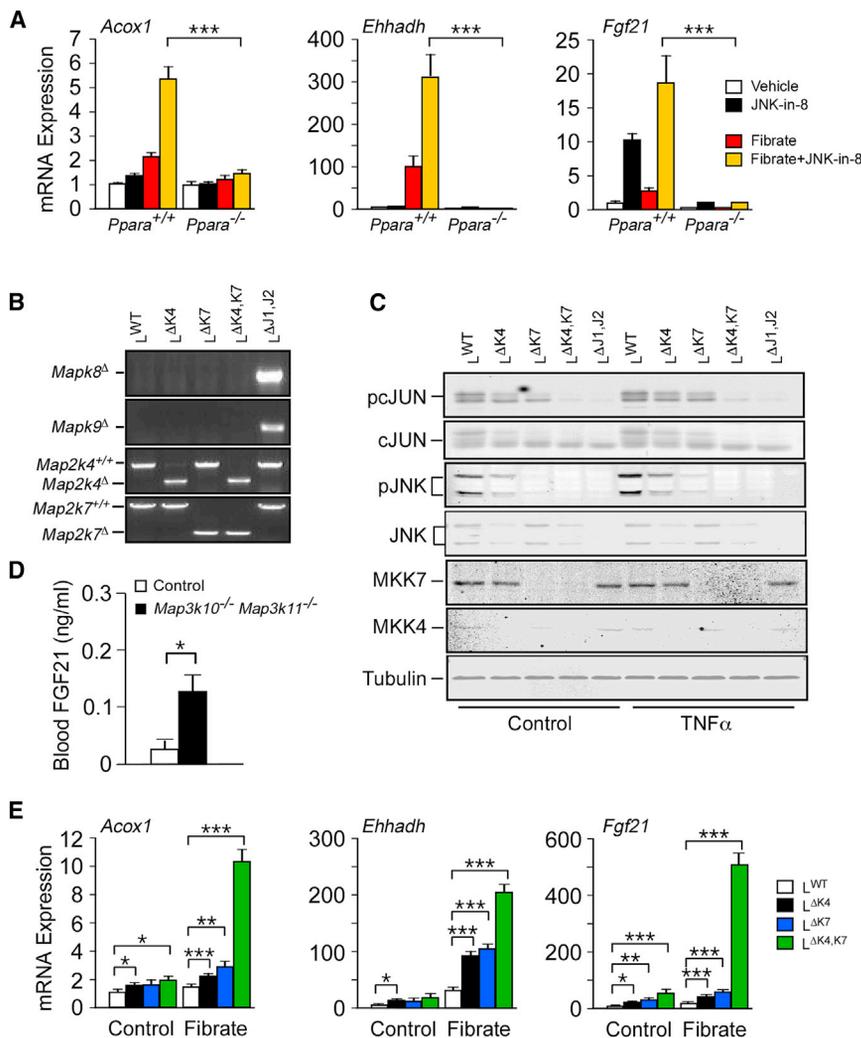
## INTRODUCTION

The cJun NH<sub>2</sub>-terminal kinase (JNK)-signaling pathway is activated by metabolic stress and contributes to the development of metabolic syndrome in response to the consumption of a high-fat diet (HFD), including obesity, hyperglycemia, and insulin resistance (Sabio and Davis, 2010). JNK plays different roles in multiple tissues to cause these phenotypes. Thus, JNK in the hypothalamus and pituitary gland suppress energy expenditure to cause obesity (Belgardt et al., 2010; Sabio et al., 2010a; Vernia et al., 2013), and JNK in macrophages promotes chronic inflammation to cause insulin resistance (Han et al., 2013). JNK in peripheral tissues also contributes to the development of insulin resistance, including adipose tissue, liver, and muscle (Sabio et al., 2008, 2010b; Sabio and Davis, 2010). Interestingly, hepatic JNK deficiency causes systemic protection against insulin resistance in HFD-fed mice (Vernia et al., 2014). The mech-

anism of systemic protection may be caused by JNK-mediated repression of the peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ )/fibroblast growth factor 21 (FGF21)-signaling axis (Vernia et al., 2014).

FGF21 circulates in the blood and is a potent regulator of metabolism (Owen et al., 2015). The effects of FGF21 are mediated by binding to FGF receptors (FGFR1c, 2c, or 3c) together with the obligate co-receptor  $\beta$ Klotho (Owen et al., 2015). Clinical trials using FGF21 analogs demonstrate improved dyslipidemia in humans (Gaich et al., 2013; Dong et al., 2015). Murine studies demonstrate that the liver is a target of the metabolic actions of FGF21 by regulating fatty acid oxidation, ketogenesis, gluconeogenesis, and lipogenesis (Potthoff et al., 2012). Indeed, FGF21 deficiency is associated with increased hepatic steatosis, fibrosis, and inflammation (Fisher et al., 2014; Tanaka et al., 2015). Direct effects of FGF21 on the liver may contribute to these hepatic responses, but recent studies indicate that FGF21 primarily mediates these effects by indirect mechanisms that initially target other tissues, including the CNS and adipose tissue (Owen et al., 2015). The major hepatic action of FGF21 mediated by adipose tissue is the PPAR $\gamma$ -induced expression of the adipokine adiponectin (Holland et al., 2013; Lin et al., 2013). FGF21 also acts on the hypothalamus-pituitary axis to regulate adrenal glucocorticoid secretion during starvation-induced hepatic gluconeogenesis (Liang et al., 2014). Moreover, FGF21 acts on the hypothalamus within the suprachiasmatic nucleus (Owen et al., 2014) and the paraventricular nucleus (Douris et al., 2015) to increase sympathetic outflow to peripheral tissues, including the liver. This sympathetic activity also targets brown fat and beige/brite cells in sub-cutaneous white fat depots (Douris et al., 2015), and it may contribute to the blood glucose-lowering actions of FGF21 by causing increased UCP1-dependent energy expenditure (Kwon et al., 2015).

The purpose of the study reported here was to test the role of FGF21 in the metabolic response to hepatic JNK activation. We show that hepatic JNK deficiency in HFD-fed mice causes reduced hyperglycemia and improved tolerance to glucose and insulin. These effects of JNK deficiency were not detected in mice with hepatocyte-specific ablation of the *Fgf21* gene. Collectively, these data demonstrate that FGF21 is required for glycemic regulation by hepatic JNK.



**Figure 1. Inhibition of the Hepatic JNK-Signaling Pathway Promotes FGF21 Expression**

(A) Primary hepatocytes were treated without or with JNK inhibitor (JNK-in-8) or the PPAR $\alpha$  agonist WY14043 (Fibrate). The expression of the PPAR $\alpha$  target genes *Acox1*, *Ehhadh*, and *Fgf21* mRNA was examined by measurement of mRNA using qPCR (mean  $\pm$  SEM; n = 6). PPAR $\alpha$  deficiency suppressed gene expression, including in cells treated with fibrate plus JNK-in-8 (\*\*p < 0.001).

(B and C) Primary hepatocytes were prepared from control mice and mice with liver-specific deficiency of MKK4, MKK7, or JNK1/2. Genomic DNA was examined by PCR to detect gene ablations (B). Primary hepatocytes treated without or with TNF- $\alpha$  were examined by immunoblot analysis (C).

(D) The blood concentration of FGF21 in wild-type and MLK2 plus MLK3-deficient (*Map3k10*<sup>-/-</sup>*Map3k11*<sup>-/-</sup>) mice fed ad libitum was measured by ELISA (mean  $\pm$  SEM; n = 6; \*p < 0.05).

(E) Expression of *Acox1*, *Ehhadh*, and *Fgf21* mRNA by primary hepatocytes prepared from control mice and mice with liver-specific deficiency of MKK4 or MKK7 was examined by qPCR (mean  $\pm$  SEM; n = 6; \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001).

including low-protein and high-carbohydrate diets (Iizuka et al., 2009; Chartoumpakis et al., 2011; De Sousa-Coelho et al., 2012; Li et al., 2014).

To obtain independent evidence for repression of *Fgf21* expression by the JNK-signaling pathway, we examined the effect of defects in the upstream regulatory mechanism that activates JNK. It is established that JNK activation is mediated

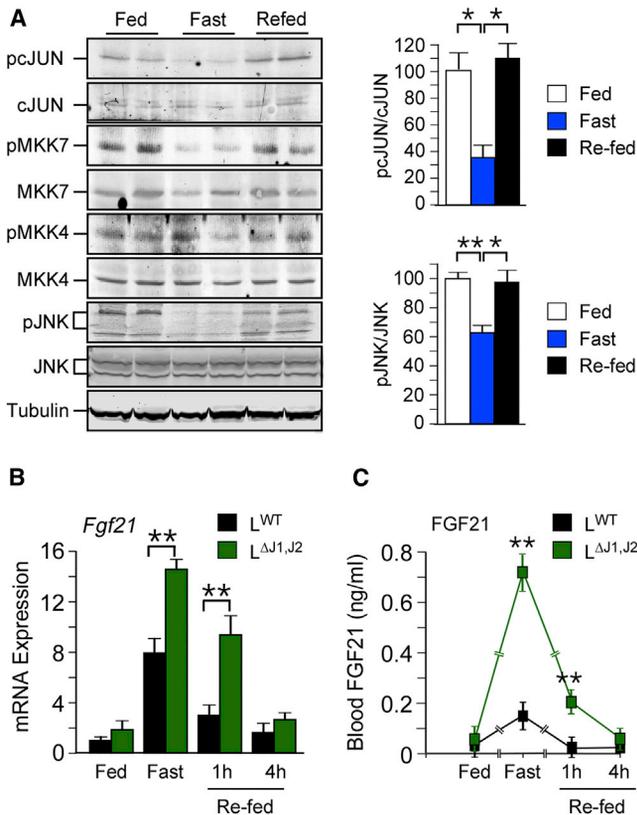
by the protein kinases MKK4 and MKK7 (Tournier et al., 2001). Disruption of the *Map2k4* gene or the *Map2k7* gene in hepatocytes partially suppressed JNK signaling, indicated by reduced phosphorylation of JNK and cJun (Figures 1B and 1C). In contrast, compound ablation of *Map2k4* plus *Map2k7* (like compound ablation of *Mapk8* plus *Mapk9*) prevented JNK signaling in hepatocytes (Figure 1C). Analysis of *Fgf21* mRNA expression demonstrated that both MKK4 deficiency and MKK7 deficiency caused increased *Fgf21* expression, but compound MKK4 plus MKK7 deficiency strongly increased *Fgf21* expression (Figure 1E). Similarly, compound deficiency of the mixed-lineage protein kinases MLK2 plus MLK3, which function as activators of MKK4 and MKK7 in response to metabolic stress (Kant et al., 2013), causes increased expression of FGF21 circulating in the blood (Figure 1D). Together, these data demonstrate that the JNK-signaling pathway acts to suppress FGF21 expression.

To test the role of JNK signaling during physiological regulation of FGF21 expression, we investigated the effect of fasting and re-feeding mice on hepatic FGF21 expression and JNK activity. It is established that FGF21 expression is strongly induced by starvation and that re-feeding rapidly represses FGF21

## RESULTS AND DISCUSSION

### JNK Signaling Suppresses *Fgf21* Gene Expression

Studies of mice with compound ablation of the *Mapk8* plus *Mapk9* genes (also known as *Jnk1* and *Jnk2*) in the liver demonstrate increased expression of PPAR $\alpha$  target genes, including *Fgf21* (Vernia et al., 2014). The JNK-signaling pathway can therefore repress PPAR $\alpha$  signaling. To obtain additional evidence to support this conclusion, we examined the effect of a specific small molecule inhibitor of JNK protein kinase activity (JNK-in-8; Zhang et al., 2012) on PPAR $\alpha$ -dependent gene expression, including *Acox1*, *Ehhadh*, and *Fgf21*. We found that treatment of wild-type hepatocytes, but not *Ppara*<sup>-/-</sup> hepatocytes, with JNK-in-8 caused increased PPAR $\alpha$ -dependent gene expression in response to the PPAR $\alpha$  agonist WY14043 (Figure 1A). These data support the conclusion that JNK signaling represses PPAR $\alpha$  target gene expression, including *Fgf21*. This PPAR $\alpha$  pathway is important for starvation-induced FGF21 expression (Badman et al., 2007; Inagaki et al., 2007), while additional pathways (e.g., ATF4, ChREBP, GCN2, NRF2, and SIRT1) play key roles in FGF21 expression caused by other dietary stresses,



**Figure 2. Hepatic JNK Regulates FGF21 Levels in Response to Fasting**

(A) Mice were fed a CD ad libitum, fasted overnight, or fasted overnight and then re-fed a CD (1 hr). Liver extracts were prepared and examined by immunoblot analysis (left). The amounts of phospho-JNK and phospho-cJUN (right) were quantitated (mean  $\pm$  SEM; n = 3; \*p < 0.05 and \*\*p < 0.01). (B and C) The amount of hepatic *Fgf21* mRNA (B) was measured by qPCR (mean  $\pm$  SEM; n = 6–10). The blood concentration of FGF21 (C) was measured by ELISA (mean  $\pm$  SEM; n = 10–12). Statistically significant differences between L<sup>WT</sup> and L<sup>ΔJ1, J2</sup> mice are indicated (\*\*p < 0.01).

expression (Potthoff et al., 2012). Previous studies have demonstrated that hepatic JNK is activated by over-nutrition when mice are fed a HFD (Sabio and Davis, 2010). However, it was not known whether hepatic JNK is acutely regulated by fasting and re-feeding. We therefore examined JNK activation in mice fed a chow diet (CD) ad libitum, mice fed a CD and then starved overnight, and mice that were starved overnight and subsequently re-fed a CD (1 hr). This analysis demonstrated the presence of activated hepatic JNK in fed mice, a reduction in JNK activation in starved mice, and rapid re-activation of JNK in re-fed mice (Figure 2A). The hepatic JNK pathway is therefore acutely regulated by fasting/feeding cycles. These changes in JNK pathway activation appear to reflect the activation state of MKK7 rather than MKK4 (Figure 2A). Interestingly, JNK activation and FGF21 expression are anti-correlated; feeding-induced activation of JNK is associated with downregulation of FGF21 expression (Figures 2B and 2C). These data indicate that JNK-mediated repression may contribute to the physiological regulation of FGF21 during cycles of fasting and feeding.

### Conditional Ablation of the *Fgf21* Gene in Mice

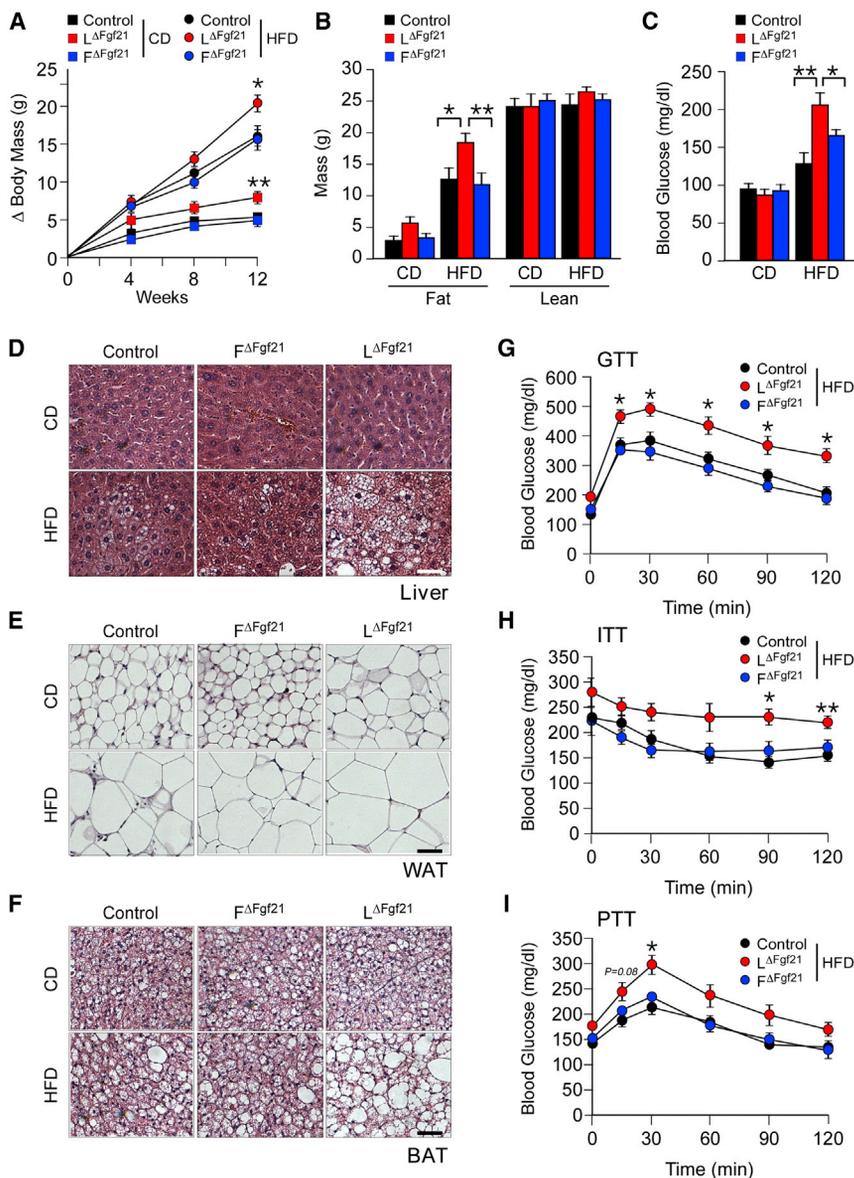
We established *Fgf21*<sup>Loxp/Loxp</sup> mice to examine the effect of tissue-specific ablation of the *Fgf21* gene (Figures S1A and S1B). To test the role of hepatic FGF21 expression, we obtained L<sup>ΔFgf21</sup> mice (*Alb-cre*<sup>+</sup> *Fgf21*<sup>Loxp/Loxp</sup>) with *Fgf21* gene ablation selectively in hepatocytes. Analysis of L<sup>ΔFgf21</sup> mice demonstrated no hepatic expression of *Fgf21* mRNA or FGF21 protein circulating in blood (Figures S1C–S1E). In contrast, F<sup>ΔFgf21</sup> mice (*Adipoq-cre*<sup>+</sup> *Fgf21*<sup>Loxp/Loxp</sup> mice) with *Fgf21* gene ablation selectively in adipose tissue demonstrated the presence of FGF21 protein in blood, despite the complete loss of *Fgf21* mRNA expression in adipose tissue (Figures S1C–S1E). These observations confirm that the liver is the major source of FGF21 circulating in blood (Markan et al., 2014).

### Hepatic FGF21 Deficiency Promotes Metabolic Syndrome

We examined the effect of feeding a HFD to control mice and mice with FGF21 deficiency in liver (L<sup>ΔFgf21</sup>) or adipose tissue (F<sup>ΔFgf21</sup>). Measurement of body mass demonstrated increased weight gain by L<sup>ΔFgf21</sup> mice, but not F<sup>ΔFgf21</sup> mice, compared with control mice (Figures 3A and S2A). Analysis of total body composition by <sup>1</sup>H-MRS demonstrated that the increased body mass was caused by accumulated fat mass rather than increased lean mass (Figure 3B). Examination of organ mass at necropsy indicated that the majority of the increased weight gain was associated with increased mass of the liver, white adipose tissue, and brown adipose tissue (Figure S2B). Sections prepared from the liver demonstrated that L<sup>ΔFgf21</sup> mice exhibited increased hepatic steatosis compared with F<sup>ΔFgf21</sup> mice and control mice (Figure 3D). Similarly, analysis of sections prepared from brown and white adipose tissues demonstrated increased adipocyte hypertrophy in L<sup>ΔFgf21</sup> mice compared with F<sup>ΔFgf21</sup> mice and control mice (Figures 3E and 3F). The increased obesity of L<sup>ΔFgf21</sup> mice was associated with increased HFD-induced hyperinsulinemia and circulating concentrations of the adipokines leptin and resistin (Figures S2C–S2E). This effect of hepatic FGF21 deficiency to promote obesity is similar to observations reported for whole-body FGF21 knockout mice (Badman et al., 2009; Hotta et al., 2009).

The phenotype of L<sup>ΔFgf21</sup> mice suggests that hepatic FGF21 deficiency may promote metabolic syndrome. We therefore examined glycemic regulation in L<sup>ΔFgf21</sup> mice, F<sup>ΔFgf21</sup> mice, and control mice. No significant differences in blood glucose concentration were detected in CD-fed mice. Similarly, studies of glucose, insulin, and pyruvate tolerance of CD-fed mice demonstrated no differences among control mice, L<sup>ΔFgf21</sup> mice, and F<sup>ΔFgf21</sup> mice (Figures S3A–S3C). In contrast, the blood glucose concentration was increased in HFD-fed L<sup>ΔFgf21</sup> mice compared with HFD-fed F<sup>ΔFgf21</sup> mice and HFD-fed control mice (Figure 3C). Furthermore, HFD-fed L<sup>ΔFgf21</sup> mice were significantly more glucose intolerant (Figure 3G), more insulin resistant (Figure 3H), and more pyruvate intolerant (Figure 3I) than HFD-fed control mice or F<sup>ΔFgf21</sup> mice.

Collectively, these data demonstrate that hepatocyte-specific FGF21 deficiency dramatically suppresses the amount of FGF21 that circulates in the blood. Moreover, hepatocyte-specific



**Figure 3. FGF21 Deficiency in Liver Increases HFD-Induced Obesity**

(A) The total body mass gain of CD-fed and HFD-fed mice was examined (mean ± SEM; n = 6–10; \*p < 0.05 and \*\*p < 0.01). FGF21 deficiency in liver or fat was studied by comparing control mice (*Fgf21<sup>LoxP/LoxP</sup>*) with liver-specific FGF21-deficient mice (*L<sup>ΔFgf21</sup>*) and adipose tissue-specific FGF21-deficient mice (*F<sup>ΔFgf21</sup>*).

(B) The fat and lean mass of CD-fed and HFD-fed (12 weeks) mice were measured by <sup>3</sup>H-MRS analysis (mean ± SEM; n = 6–10; \*p < 0.05 and \*\*p < 0.01).

(C) The blood glucose concentration in CD-fed and HFD-fed (12 weeks) mice starved overnight was examined (mean ± SEM; n = 6–10; \*p < 0.05 and \*\*p < 0.01).

(D–F) Sections of liver (D), epididymal white adipose tissue (WAT, E), and interscapular brown adipose tissue (BAT, F) from CD-fed and HFD-fed (12 weeks) mice were stained with H&E. Scale bar, 100 μm.

(G–I) Glucose tolerance test (GTT, G), insulin tolerance test (ITT, H), and pyruvate tolerance test (PTT, I) using HFD-fed (12 weeks) mice were performed (mean ± SEM; n = 6–10; \*p < 0.05 and \*\*p < 0.01).

See also Figures S1, S2, and S3.

FGF21 deficiency promotes obesity and hallmarks of metabolic syndrome in HFD-fed mice, including increased hyperglycemia and hyperinsulinemia together with intolerance to glucose, pyruvate, and insulin.

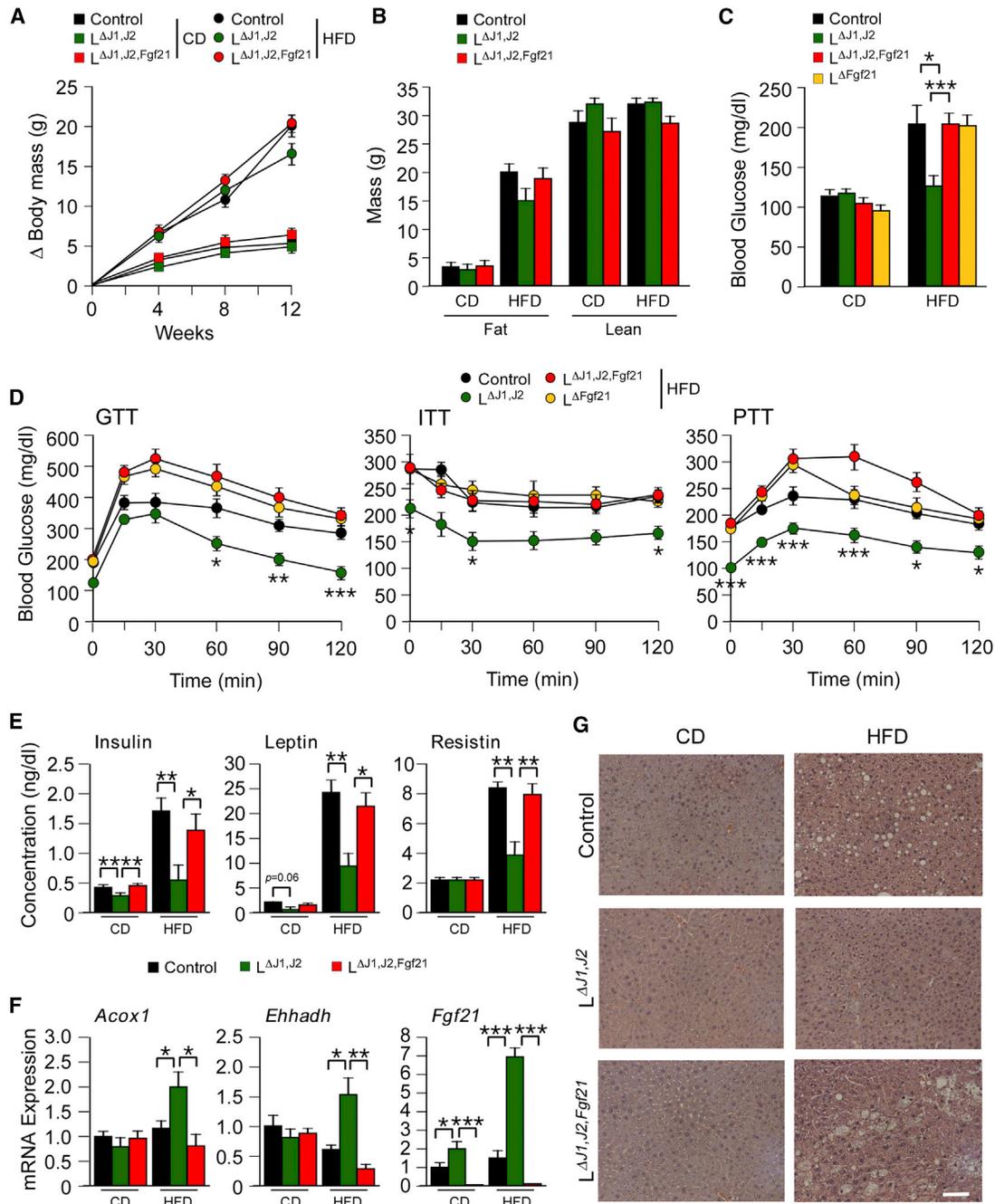
### Fgf21 Gene Ablation Suppresses the Metabolic Effects of Hepatic JNK Deficiency

Hepatic JNK deficiency causes increased expression of PPAR $\alpha$  target genes in the liver, including *Fgf21* (Vernia et al., 2014). To test the role of FGF21, we examined the effect of compound deficiency of JNK plus FGF21 in hepatocytes by comparing *L<sup>ΔJ1,J2</sup>* mice (*Alb-cre<sup>+</sup> Mapk8<sup>LoxP/LoxP</sup> Mapk9<sup>LoxP/LoxP</sup>*) with *L<sup>ΔJ1,J2,Fgf21</sup>* mice (*Alb-cre<sup>+</sup> Mapk8<sup>LoxP/LoxP</sup> Mapk9<sup>LoxP/LoxP</sup> Fgf21<sup>LoxP/LoxP</sup>*). No significant differences in total body mass, fat mass, or lean mass among *L<sup>ΔJ1,J2</sup>* mice, *L<sup>ΔJ1,J2,Fgf21</sup>* mice, and control mice were detected (Figures 4A and 4B). However, the HFD-induced

hyperglycemia in control mice that was suppressed by JNK deficiency (compare control and *L<sup>ΔJ1,J2</sup>* mice; Figure 4C) was not detected in FGF21-deficient mice (compare *L<sup>ΔFgf21</sup>* and *L<sup>ΔJ1,J2,Fgf21</sup>* mice; Figure 4C). These data indicate that FGF21 is essential for the effect of hepatic JNK deficiency to suppress HFD-induced hyperglycemia.

To examine the requirement of FGF21 for glycemic regulation, we performed glucose, insulin, and pyruvate tolerance tests. Studies of CD-fed mice demonstrated no significant differences between groups (Figures S3D–S3F), but JNK deficiency in HFD-fed mice caused improved performance in each of these tests (Figure 4D). This effect of JNK deficiency was not detected in FGF21-deficient mice (compare HFD-fed *L<sup>ΔFgf21</sup>* and HFD-fed *L<sup>ΔJ1,J2,Fgf21</sup>* mice; Figure 4D). Moreover, hepatic JNK deficiency did not suppress HFD-induced hyperinsulinemia or circulating concentrations of the adipokines leptin and resistin in liver-specific FGF21-deficient mice (Figure 4E). Together, these data confirm that FGF21 is required for improved glycemic regulation caused by hepatic JNK deficiency.

Interestingly, the effect of hepatic JNK deficiency to increase the expression of the PPAR $\alpha$  target genes *Acox1* and *Ehhadh* in HFD-fed mice was suppressed in hepatic FGF21-deficient mice (Figure 4F). This observation suggests that FGF21 plays a key role in the promotion of fatty acid oxidation caused by hepatic JNK deficiency, consistent with the established physiological



**Figure 4. FGF21 Is Critically Required for the Metabolic Actions of Hepatic JNK**

(A) The total body mass gain of CD-fed and HFD-fed mice was examined. The effect of FGF21 deficiency in liver was examined by comparing control mice (*Mapk9*<sup>LoxP/LoxP</sup> *Mapk9*<sup>LoxP/LoxP</sup>) and mice with liver-specific deficiency of JNK ( $L^{\Delta J1,J2}$ ) or JNK plus FGF21 ( $L^{\Delta J1,J2,Fgf21}$ ). No statistically significant differences were detected between groups (mean  $\pm$  SEM; n = 8–10).

(B) The fat and lean mass of CD-fed and HFD-fed (12 weeks) mice were measured by <sup>1</sup>H-MRS analysis (mean  $\pm$  SEM; n = 8–10).

(C) Blood glucose concentration was measured in overnight-fasted CD-fed and HFD-fed (12 weeks) mice (mean  $\pm$  SEM; n = 6–10; \*p < 0.05 and \*\*\*p < 0.001).

(D) GTT, ITT, and PTT using HFD-fed (12 weeks) mice were performed (mean  $\pm$  SEM; n = 6–10; \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001).

(E) The blood concentrations of insulin, leptin, and resistin in overnight-fasted CD-fed and HFD-fed (12 weeks) mice were measured (mean  $\pm$  SEM; n = 6–10; \*p < 0.05 and \*\*p < 0.01).

(F) The expression of *Acox1*, *Ehhadh*, and *Fgf21* mRNA in the liver of CD-fed and HFD-fed (12 weeks) mice were measured by qPCR (mean  $\pm$  SEM; n = 6; \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001).

(G) Sections of liver from CD-fed and HFD-fed (12 weeks) control,  $L^{\Delta J1,J2}$ , and  $L^{\Delta J1,J2,Fgf21}$  mice were stained with H&E. Scale bar, 100  $\mu$ m.

See also Figures S3 and S4.

role of FGF21 (Badman et al., 2007). Indeed, the reduction in HFD-induced hepatic steatosis caused by JNK deficiency was not detected in the liver of mice with hepatic FGF21 deficiency (Figure 4G). These data highlight the crucial role of FGF21 in hepatic JNK signal transduction.

One pathway that mediates the hepatic actions of FGF21 is represented by the adipokine adiponectin (Holland et al., 2013; Lin et al., 2013). We therefore examined the expression of adiponectin in control,  $L^{\Delta Fgf21}$ , and  $F^{\Delta Fgf21}$  mice; these data demonstrated that hepatic FGF21 deficiency, but not adipocyte FGF21 deficiency, caused reduced expression of *Adipoq* mRNA in epididymal white adipose tissue (Figure S4A). Moreover, studies of control,  $L^{\Delta J1, J2}$ , and  $L^{\Delta J1, J2, Fgf21}$  mice indicated that hepatic JNK deficiency caused increased adiponectin expression that was suppressed in mice with compound hepatic deficiency of JNK plus FGF21 (Figure S4B). Together, these data demonstrate that the increased circulating concentration of FGF21 caused by hepatic JNK deficiency promotes adipose tissue expression of adiponectin. The FGF21-promoted adiponectin expression, together with FGF21-promoted sympathetic stimulation of brown and beige/brite adipose tissue, may contribute to the improved hepatic function of mice with hepatic JNK deficiency.

In summary, the requirement of FGF21 for the metabolic actions of hepatic JNK provides a mechanism for metabolic regulation by the JNK-signaling pathway in the liver. FGF21 plays a major role in the response to starvation, including increased hepatic gluconeogenesis, ketogenesis, and fatty acid oxidation (Potthoff et al., 2012). Feeding terminates this response by activation of the hepatic JNK-signaling pathway and repression of FGF21 expression. JNK activation, therefore, provides a regulatory mechanism that contributes to the termination of the starvation response. Conversely, starvation-induced inhibition of the JNK pathway contributes to de-repression of FGF21 expression and promotes adaptive responses to starvation. These data establish a key role played by FGF21 in the metabolic response to hepatic JNK activation caused by cycles of fasting and re-feeding.

The interplay between hepatic JNK and FGF21 contributes to the development of metabolic syndrome in response to diet-induced obesity (Vernia et al., 2014). Indeed, the protective effects of hepatic JNK deficiency on insulin resistance were not observed in mice with hepatic FGF21 deficiency (Figure 4). This finding is similar to a report of autophagy-deficient mice that exhibit improved metabolic syndrome that also was dependent on increased FGF21 expression (Kim et al., 2013). Similarly, FGF21 improves hyperglycemia in mice with hepatic deficiency of the insulin receptor (Emanuelli et al., 2014). FGF21 also can provide protection against some forms of drug-induced hepatotoxicity (Ye et al., 2014). Together, these data demonstrate that FGF21 can play strong protective roles in models of metabolic and cellular dysfunction.

## EXPERIMENTAL PROCEDURES

### Blood Analysis

Blood glucose was measured with an Ascensia Breeze 2 glucometer (Bayer). FGF21 was measured using the Rat/Mouse Fibroblast Growth Factor 21

ELISA kit (EZRMFGF21-26K, Millipore). Adipokines and insulin in plasma were measured by multiplexed ELISA using a Luminex 200 machine (Millipore).

### Glucose and Insulin Tolerance Tests

Glucose, insulin, and pyruvate tolerance tests were performed by intraperitoneal injection of mice with glucose (1 g/kg), insulin (0.5 U/kg), or pyruvate (1 g/kg) using methods described previously (Sabio et al., 2008).

### RNA Analysis

Tissue isolated from mice starved overnight was used to prepare total RNA with the RNeasy mini kit (QIAGEN). Total RNA (500 ng) was converted into cDNA using the high-capacity cDNA reverse transcription kit (Life Technologies). The diluted cDNA was used for real-time qPCR analysis using a Quantstudio PCR machine (Life Technologies). TaqMan assays (Life Technologies) were used to quantify *Acox1* (Mm01246831\_m1), *Adipoq* (Mm00456425-m1), and *Ehhadh* (Mm00619685\_m1). *Fgf21* expression was quantified using the primers FGF21F67 (5'-AGATGGAGCTCTCTATGGATCG-3') and FGF21R67 (5'-GGGC TTCAGACTGGTACACAT-3') with universal probe 67 (Universal Probe Library, Roche). The relative mRNA expression was normalized by measurement of the amount of 18S RNA in each sample using Taqman assays (catalog number 4308329, Life Technologies).

### Immunoblot Analysis

Liver extracts were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM  $\beta$ -glycerophosphate, 1  $\mu$ M sodium orthovanadate, 1  $\mu$ M PMSF, and 10  $\mu$ g/ml leupeptin plus aprotinin). Extracts (30–50  $\mu$ g protein) were examined by immunoblot analysis by probing with antibodies to pSer<sup>63</sup>-cJUN, pJNK, pMKK4, MKK4, pMKK7, and MKK7 (Cell Signaling Technology); JNK1/2 (BD Pharmingen); cJUN and GAPDH (Santa Cruz Biotechnology); and  $\beta$ -Tubulin (Covance). Immunocomplexes were detected by fluorescence using anti-mouse and anti-rabbit secondary IRDye antibodies (LI-COR Biosciences) and quantitated using the LI-COR Imaging system.

### Analysis of Tissue Sections

Histology was performed using tissue fixed in 10% formalin (24 hr), dehydrated, and embedded in paraffin. Sections (7  $\mu$ m) were cut and stained using H&E (American Master Tech Scientific). Sections isolated from five mice per group were examined by a board-certified pathologist who was blinded with respect to the identity of each group of mice.

### Statistical Analysis

Differences between groups were examined for statistical significance using the Student's test or ANOVA with the Fisher's test.

### Institutional Review Board Approval

The Institutional Animal Care and Use Committee of the University of Massachusetts Medical School approved all studies using animals.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.02.026>.

## AUTHOR CONTRIBUTIONS

S.V. and R.J.D. designed the study. S.V., J.C.-K., and T.B. performed experiments. S.V., C.T., and R.J.D. analyzed data and wrote the paper.

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