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To cite this version:

HAL Id: hal-01837159
https://hal.umontpellier.fr/hal-01837159
Submitted on 26 May 2020

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A Specific ChREBP and PPARα Cross-Talk Is Required for the Glucose-Mediated FGF21 Response

Highlights
- Fgf21 is a unique hepatic gene inducible by both catabolic and anabolic signals
- The ChREBP-mediated induction of Fgf21 in hepatocytes requires PPARα
- Loss of PPARα impairs Fgf21 promoter accessibility at the ChoRE
- PPARα is required for the control of sucrose preference in vivo

Authors
Alison Iroz, Alexandra Montagner, Fadila Benhamed, ..., Renaud Dentin, Hervé Guillou, Catherine Postic

Correspondence
renaud.dentin@inserm.fr (R.D.), herve.guillou@inra.fr (H.G.), catherine.postic@inserm.fr (C.P.)

In Brief
FGF21 is a hepatokine with beneficial metabolic effects, including control of sucrose preference. Iroz et al. demonstrate that Fgf21 is a unique hepatic gene inducible by both fasting and glucose signals and that the transcription factors PPARα and ChREBP both regulate the endocrine control of sugar intake by hepatic FGF21.

Data and Software Availability
GSE92502

Iroz et al., 2017, Cell Reports 21, 403–416
October 10, 2017 © 2017 The Authors.
https://doi.org/10.1016/j.celrep.2017.09.065
A Specific ChREBP and PPARα Cross-Talk Is Required for the Glucose-Mediated FGF21 Response

Alison Iroz, Alexandra Montagner, Fadila Benhamed, Francoise Levasseur, Arnaud Polizzi, Eloie Anthony, Marion Regnier, Edwin Fouche, Celine Lukowicz, Michele Cauzac, Emilie Tournier, Marcio Do-Cruzeiro, Martine Daujat-Chavaniou, Sabine Gerbal-Chalouin, Veronique Fauveau, Solenne Marmier, Anne-Francoise Burnol, Sandra Guilmeau, Yannick Lippi, Jean Girard, Walter Wahl, Renaud Dentin, Herve Guillou, and Catherine Postic

1INSERM U1016, Institut Cochin, Paris 75014, France
2CNRS UMR 8104, Paris 75014, France
3University of Paris Descartes, Sorbonne Paris Cite, Paris 75005, France
4Toxalim, Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse 31027, France
5INSERM, U1183, Institute for Regenerative Medicine and Biotherapy, Montpellier, France
6Universite de Montpellier, UMR 1183, Montpellier, France
7CHU Montpellier, Institute for Regenerative Medicine and Biotherapy, Montpellier, France
8Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232, Singapore
9Center for Integrative Genomics, University of Lausanne, Genopode Building, Lausanne 1015, Switzerland
10These authors contributed equally
11Senior author
12Lead Contact
*Correspondence: renaud.dentin@inserm.fr (R.D.), herve.guillou@inra.fr (H.G.), catherine.postic@inserm.fr (C.P.)

https://doi.org/10.1016/j.celrep.2017.09.065

SUMMARY

While the physiological benefits of the fibroblast growth factor 21 (FGF21) hepatokine are documented in response to fasting, little information is available on Fgf21 regulation in a glucose-overload context. We report that peroxisome-proliferator-activated receptor α (PPARα), a nuclear receptor of the fasting response, is required with the carbohydrate-sensitive transcription factor carbohydrate-responsive element-binding protein (ChREBP) to balance FGF21 glucose response. Microarray analysis indicated that only a few hepatic genes respond to fasting and glucose similarly to Fgf21. Glucose-challenged Chrebpx−/− mice exhibit a marked reduction in FGF21 production, a decrease that was rescued by re-expression of an active ChREBP isoform in the liver of Chrebpx−/− mice. Unexpectedly, carbohydrate challenge of hepatic Pparα knockout mice also demonstrated a PPARα-dependent glucose response for Fgf21 that was associated with an increased sucrose preference. This blunted response was due to decreased Fgf21 promoter accessibility and diminished ChREBP binding onto Fgf21 carbohydrate-responsive element (ChoRE) in hepatocytes lacking PPARα. Our study reports that PPARα is required for the ChREBP-induced glucose response of FGF21.

INTRODUCTION

The liver is central for the regulation of energy homeostasis, controlling several biochemical pathways important for metabolism of lipids, carbohydrates, proteins, bile synthesis, and detoxification of drugs and toxins. The liver also controls endocrine responses through the production of hepatokines. These proteins secreted by the hepatocytes act as hormones, and several hepatokines are considered promising leads for metabolic therapy development (Iroz et al., 2015). Among them, fibroblast growth factor 21 (FGF21) has emerged as an interesting target (Kharitonenkov and Adams, 2013). Originally targeted for its glucose-lowering properties in rodents and primates (Berglund et al., 2009; Fisher et al., 2011; Kharitonenkov et al., 2007), FGF21 is also able to improve insulin sensitivity and lipid homeostasis and induce weight loss (Coskun et al., 2008; Markan et al., 2014).

FGF21 is a direct target of the nuclear receptor peroxisome-proliferator-activated receptor α (PPARα) in response to fasting (Badman et al., 2007; Lundäsen et al., 2007). Activated by free fatty acids derived from lipolysis (Jaeger et al., 2015; Montagner et al., 2016), PPARα is essential to liver health, as its deletion promotes the development of non-alcoholic fatty liver disease (NAFLD) and hypercholesterolemia during aging (Montagner et al., 2016). The beneficial role of PPARα in response to dyslipidemia is thought to be mediated, at least in part, through FGF21 (Ong et al., 2012). Indeed, anti-diabetic therapies and PPARα agonists (fenofibrate or Wy-14653) significantly induce liver-derived FGF21 in both mouse and human plasma (Christodoulides et al., 2009; Gålman et al., 2008; Lundäsen et al., 2007).

Hepatocyte PPARα plays an essential role during fasting, which triggers transcriptional regulation for the maintenance of glycemia and ketogenesis through fatty acid catabolism for use as an alternative energy source (Goldstein and Hager, 2015). In agreement, PPARα-deficient mice exhibit impaired fatty acid oxidation and ketogenesis that promotes hepatic steatosis during fasting (Kersten et al., 1999; Kroetz et al., 1998; Leone et al., 1999; Montagner et al., 2016).
Recent work reported that FGF21 is also activated in response to glucose and fructose in rodents and humans (Herman et al., 2012; Iizuka et al., 2009; Sánchez et al., 2009; Uebanono et al., 2011). Enriched in liver, the transcription factor carbohydrate-responsive element-binding protein (ChREBP) mediates the response to dietary carbohydrates (Abdul-Wahed et al., 2017). A physiological role for the ChREBP-FGF21 axis was revealed in experiments showing that in response to sugar consumption, ChREBP-enhanced FGF21 secretion from the liver blocked sugar-seeking behavior in mice and primates by targeting the paraventricular nucleus of the hypothalamus (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). 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 GRAcE by glucose promotes ChREBP transcriptional activity and binding to the carbohydrate-responsive element (ChoRE) of its target genes, including L-pyruvate kinase (Lpk), a rate-limiting enzyme in glycolysis, fatty acid synthase (Fas), and steroyl CoA desaturase (Scd1), key enzymes of de novo lipogenesis (Kawaguchi et al., 2001). Another isoform of Chreb, Chrebβ, originating from an alternative promoter, was identified in adipose tissue and liver (Herman et al., 2012). This alternative splicing results in a constitutively active ChREBP isoform lacking the LID, a domain associated with inhibition of ChREBP activity (Herman et al., 2012).

Understanding the regulation of FGF21 is currently a research focal point. Through the use of Chreb knockout mice, we report here that ChREBP is required for the expression and secretion of hepatic FGF21 in response to carbohydrate intake. Unexpectedly, studies in hepatocyte-specific Pparβ knockout mice reveal a physiological role for PPARβ in the context of glucose challenge, as ChREBP is unable to induce Fgf21 in the absence of hepatic PPARβ. Altogether, our results suggest that FGF21’s glucose-mediated response is dependent on both ChREBP and PPARβ.

RESULTS

FGF21 Is Induced by Both Fasting and Glucose Challenge

To characterize gene expression during fasting and a glucose challenge, a microarray analysis was conducted using liver samples from wild-type mice (Figure 1). Genes sensitive to glucose or fasting that were markedly different from the fed group were incorporated into a heatmap (Figure 1A). 67 genes were significantly induced by glucose in comparison to fed conditions (cluster 1), and 675 genes were significantly upregulated between fed and fasted groups (cluster 6). Gene ontology analysis revealed that pathways identified as specifically impacted by glucose and not by fasting are involved in pyruvate and insulin-sensitive metabolism (Figure 1B). Gene ontology revealed that pathways specifically sensitive to fasting, but not to glucose, are involved in PPAR signaling (Figure 1C). Interestingly, among the top genes upregulated by glucose and fasting (Figure 1A), only 3 genes (Fgf21, Fut1, and Atp5f2) were significantly upregulated (log fold change [FC] > 1; p ≤ 0.01) as compared to fed conditions (Figure 1D). When the stringency of the selection was increased to a log FC > 2 (Figure 1E), Fgf21 was left to be the sole gene upregulated by both fasting and glucose challenge (log FC = 3.9 and log FC = 3.7, respectively (p ≤ 0.01) (Figure 1E). qPCR analysis confirmed that Fgf21 expression was significantly upregulated by fasting and glucose compared to fed conditions (Figure 1F). The glucose effect was validated through analysis of Chrebp, Chrebβ, and Lpk gene expression, while the effect of fasting was assessed by measuring the expression of two typical PPARβ targets, Cyp4a10 and Vnn1 (Figure 1F).

ChREBP Is Necessary for Glucose-Mediated Expression and Secretion of Hepatic FGF21

To address the importance of ChREBP in the Fgf21 glucose response, experiments were first performed in mouse hepatocytes from wild-type mice (Figure 2). We observed that Fgf21 expression was induced by elevated glucose concentrations and paralleled with Chreb, Chrebβ, and target gene expression (Figure 2A). ChREBP recruitment onto the Fgf21 and Lpk promoters significantly increased under high glucose concentrations (25 mM) (Figure 2B). This stimulatory effect of glucose was specific and not linked to an osmotic shock, since Fgf21 expression was not induced in response to mannitol (Figure S2). To determine whether ChREBP is mandatory for upregulation of Fgf21 in response to glucose, experiments were completed in hepatocytes lacking ChREBP (Chrebβ−/−) (Figures 2C–2E). Mice lacking exons 9–15 of the Chreb gene were generated through homologous recombination (Figure S3). The absence of the ChREBP protein (α isoform, 94 kDa) was validated in Chrebβ−/− hepatocytes by western blot analysis (Figure 2C). Under these conditions, the Chrebβ protein (72 kDa) could not be detected (data not shown). Similarly to Chreb, Chrebβ, and the ChREBP and ChREBPβ target genes Lpk and Scd1, Fgf21 robustly responded to 25 mM glucose stimulation in wild-type hepatocytes, but this response was blunted in Chrebβ−/− hepatocytes (Figure 2D). In addition, no increase in Fgf21 in culture medium was detected when Chrebβ−/− hepatocytes were cultured in 25 mM glucose (Figure 2E). Of note, basal Fgf21 production (5 mM glucose) was also significantly decreased in culture medium from Chrebβ−/− hepatocytes (Figure 2E). These findings indicate that ChREBP is mandatory for the glucose-mediated expression and secretion of FGF21 by hepatocytes.

Liver-Specific ChREBP Expression Rescues FGF21 Plasma Concentrations in ChREBP Knockout Mice

We next performed glucose challenge experiments in vivo. 10- to 12-week-old Chrebβ+/− and Chrebβ−/− male mice were given 24-hr access to a bottle of glucose-free water (fed) or a bottle containing 20% glucose (glucose challenge) (Figure 3). A significant elevation in blood glucose was observed in glucose-challenged Chrebβ−/− mice compared to Chrebβ+/− mice under the same conditions (Figure 3A). A trend toward higher insulin concentrations was observed in Chrebβ−/− mice compared to controls (under fed and glucose conditions), but this difference did not reach statistical significance (Figure 3B). Glucose challenge raised ChREBP protein content in liver of Chrebβ+/− mice (Figure 3C). A significant stimulation in hepatic triglyceride (TG) concentrations was also observed in glucose-challenged Chrebβ−/− mice, but not Chrebβ−/− mice (Figure 3D).
Figure 1. **FGF21 Is Highly Induced by Both Fasting and Glucose Challenge**

Wild-type C57BL/6J 10-week-old male mice were fed ad libitum, fasted for 24 hr, or fed for 24 hr a standard diet with addition of 20% glucose in drinking water (glucose challenge). Mice were killed at ZT14 (14 hr after the start of light period in the animal housing unit). Transcriptomic analysis was performed on livers (n = 6 per condition) using gene expression microarrays.

(A) Heatmap of differentially expressed probes (false discovery rate [FDR] < 5%).

(B and C) KEGG categories corresponding to functions impacted by glucose (B) and fasting (C).

(D) Venn diagram presenting the overlap between glucose- and fasting-induced gene expression (log FC > 1; adjusted p < 0.01).

(E) Venn diagram presenting the overlap between glucose- and fasting-induced gene expression (log FC > 2; adjusted p < 0.01).

(F) Gene expression determined by qPCR. Data are expressed as means ± SEM, n = 6 individual mice per group. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test. *p < 0.05, **p < 0.01, ***p < 0.001.
A significant increase in Chrebp, Chrebpβ, Lpk, and Scd1 mRNA levels was observed in liver of glucose-challenged Chrebp+/+ mice compared to fed mice from the same genotype (Figure 3E). Fgf21 mRNA and plasmatic FGF21 protein concentrations markedly increased in glucose-challenged Chrebp+/+ mice. While a residual (nonsignificant) glucose effect was observed in Chrebp-/- mice, this response was significantly reduced compared to Chrebp+/+ mice (Figures 3F and 3G), despite elevated blood glucose levels (Figure 3A). These results show that ChREBP is required for the in vivo glucose-mediated induction of Fgf21.

We next addressed whether liver-specific re-expression of Chrebp in a context of global ChREBP deficiency could rescue FGF21 production. Chrebp+/+ and Chrebp-/- adult mice were injected with an adenovirus vehicle containing the GFP protein or a truncated isoform of ChREBP lacking the LID domain (Li et al., 2006) corresponding to a constitutively active ChREBP isoform (ChREBP<sup>CA</sup>). Mice received a 20% glucose solution for 24 hr before sacrifice (Figures 3H–3K). Blood glucose concentrations were higher in GFP-ChREBP-/- mice than in GFP-ChREBP+/+ mice and were rescued to basal values when ChREBP<sup>CA</sup> was injected into Chrebp-/- mice (Figure 3H). Western blot analysis confirmed the absence of native ChREBP protein (94 kDa) but the presence of ChREBP<sup>CA</sup> (72 kDa) in Chrebp-/- mice injected with ChREBP<sup>CA</sup> (Figure 3I). Importantly, ChREBP<sup>CA</sup> rescued the circulating level of FGF21 (Figure 3J). This correlates with the effect of ChREBP<sup>CA</sup> on the hepatic expression of Chrebp, Chrebpβ, and their targets, Lpk, Scd1, and Fgf21 mRNA (Figure 3K). Altogether, we report that ChREBP<sup>CA</sup> administration rescued ChREBP activity in the liver of Chrebp-/- mice and was sufficient to restore FGF21 gene expression and production.
Glucose Stimulation or ChREBP Overexpression Is Not Efficient to Induce FGF21 Expression or Secretion in the Absence of Hepatic PPARα

Since the Fgf21 promoter contains overlapping peroxisome proliferator response element (PPRE) and ChRE units (−88 to −54 bp) (Girer et al., 2016; Uebanso et al., 2011), we investigated whether PPARα could impact the glucose response of Fgf21 mediated by Chrebp. Primary hepatocytes from liver-specific Pparα knockout mice (Pparα<sup>hep</sup>−/−) and their littermates, Pparα<sup>hep</sup>+/+ mice (Montagner et al., 2016), were stimulated by glucose in a dose-dependent manner (Figure 4). A similar glucose-mediated induction of Chrebp, Chrebpβ, Lpk, and Scd1 mRNA was observed in hepatocytes from Pparα<sup>hep</sup>−/− and Pparα<sup>hep</sup>+/+ mice (Figure 4A). Although a residual glucose effect was observed for Fgf21 expression in Pparα<sup>hep</sup>−/− mice hepatocytes, this response was reduced compared to Pparα<sup>hep</sup>+/+ mice (Figure 4B). Importantly, FGF21 production in response to glucose was reduced by 60% in culture medium from Pparα<sup>hep</sup>−/− hepatocytes compared to controls (Figure 4C). This profile was specific to Fgf21, since the expression of other typical PPARα targets (Cyp4a10, Cyp4a14, or Vnn1) was not induced by glucose (Figure S4). We
Figure 4. *Fgf21* Is Not Efficiently Induced by Glucose or by ChREBP Overexpression in the Absence of Liver PPARα

(A–C) Primary hepatocytes derived from adult male *Pparα*<sup>hep+/+</sup> or *Pparα*<sup>hep−/−</sup> mice were incubated 1 day after plating for 24 hr with medium containing 5, 10, 15, 20, 25, or 30 mM glucose. Relative *Chrebp*, *Chrebpβ*, *Lpk*, and *Scd1* gene expression (A) and *Fgf21* gene expression (B) was determined by qPCR. (C) ELISA quantification of FGF21 protein (ng/ml) in medium collected at end of glucose stimulation. Figures are presented as means ± SEM from 4 independent cultures completed in duplicate. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001; nd, not detectable).

(D–F) Primary hepatocytes derived from adult male mice *Pparα*<sup>hep+/+</sup> or *Pparα*<sup>hep−/−</sup> were incubated 6 hr after plating with 3 x 10<sup>9</sup> pfu GFP or ChREBP<sup>CA</sup> adenovirus at a glucose concentration of 5 mM for 24 hr. Relative *Chrebp*, *Chrebpβ*, *Lpk*, and *Scd1* expression (D) and of *Fgf21* expression (E) was determined by qPCR. (F) ELISA quantification of FGF21 protein (in ng/ml) in medium collected at end of adenoviral treatment. Data are presented as means ± SEM from 4 independent cultures completed in duplicate. Significance is based on two-way ANOVA followed by a Bonferroni post hoc test (*p < 0.05; **p < 0.01; ***p < 0.001).
next addressed whether Fgf21 could be rescued by Chrebpb overexpression in the context of Pparα deficiency. Hepatocytes from Pparα<sup>hep+/+</sup> and Pparα<sup>hep</sup>−/− mice were infected with the constitutive active form of ChREBP (ChREBP<sub>Ca</sub>) for 24 hr. The expression of Chrebpb and Chrebpb<sup>Ca</sup> was significantly increased in response to ChREBP<sub>Ca</sub> in both Pparα<sup>hep+/+</sup> and Pparα<sup>hep</sup>−/− hepatocytes, and as a result, Lpk and Scd1 mRNA expression was stimulated compared to GFP conditions (Figure 4D). However, while Chrebpb overexpression led to a 50-fold increase in Fgf21 expression in Pparα<sup>hep+/+</sup> hepatocytes, it failed to significantly induce Fgf21 expression in Pparα<sup>hep</sup>−/− hepatocytes (Figure 4E). FGF21 measured in the medium of cell culture confirmed the Fgf21 mRNA expression profile (Figure 4F). These results suggest that neither glucose nor ChREBP overexpression is efficient in inducing Fgf21 gene expression or protein production in the absence of PPARα in hepatocytes.

FGF21 Synergistically Responds to Glucose and a Pharmacological PPARα Activator in Both Mouse and Human Hepatocytes

To determine whether ChREBP and PPARα act in synergy to regulate Fgf21 gene expression, mouse and human hepatocytes were stimulated for 24 hr with low (5 mM) or high glucose concentrations (25 mM) in the presence of the PPARα agonist Wy-14643 (Figure S5). Expression of Chrebpb and Chrebpb<sup>Ca</sup> mRNA confirmed a positive glucose response in both mouse and human hepatocytes (Figures S5A and S5B). Activation of PPARα by Wy-14643 was validated by a significant upregulation in Acox1 expression, a PPARα target gene (Figures S5C and S5D). Fgf21 mRNA levels were drastically increased when hepatocytes (mouse and human) were incubated in the combined presence of high glucose (25 mM) and Wy-14643 (Figures S5E and S5F). Altogether, these results show that ChREBP and PPARα can act synergistically to induce Fgf21 in mouse and in human liver cells.

Hepatocyte PPARα Is Required for the Effect of Glucose on FGF21 In Vivo

To investigate whether PPARα is involved in the glucose-mediated induction of Fgf21 in vivo, glucose challenge experiments were performed in 10- to 12-week-old Pparα<sup>hep+/+</sup> and Pparα<sup>hep</sup>−/− mice. Three nutritional conditions were assigned to both genotypes: (1) mice fasted 24 hr with free access to water (fasted), (2) mice fed ad libitum with access to standard chow diet and free access to water (fed), and (3) mice fed ad libitum with access to standard chow diet and a 20% glucose solution in water (glucose challenge) (Figure 5). Chrebpb, Chrebpb<sup>Ca</sup>, and their target genes (Lpk and Scd1) were induced in a similar manner in the liver of Pparα<sup>hep+/+</sup> and Pparα<sup>hep</sup>−/− mice when challenged by glucose (Figure 5A). In contrast, the glucose-mediated induction of Fgf21 (compared to the fed state) was significantly reduced in the absence of PPARα (Figure 5A). FGF21 in the circulation matched hepatic mRNA levels, revealing an upregulation of FGF21 in the plasma of Pparα<sup>hep+/+</sup> that was significantly reduced in glucose-challenged Pparα<sup>hep</sup>−/− mice (Figure 5B). As expected, loss of PPARα markedly impacted the fasting response of FGF21 (Figure 5A-B). Because the Fgf21 promoter contains overlapping PPRE and ChoRE units (Figure 5C), we investigated whether lack of PPARα could impair ChREBP binding in response to glucose in vivo. When primers amplifying both ChoRE and PPRE were used (Figure 5C), a significant enrichment in PPARα binding within the proximal region of the Fgf21 promoter in liver of Pparα<sup>hep</sup>−/− mice was observed (Figure 5D). While PPARα binding was elevated under fasting conditions, a significant PPARα recruitment onto the proximal region of the Fgf21 promoter was detected under both fed and glucose-challenge conditions (Figure 5D). Surprisingly, in liver of glucose challenged Pparα<sup>hep</sup>−/− mice, recruitment of ChREBP on the Fgf21 promoter ChoRE was significantly reduced (Figure 5D). Interestingly, a significant decrease in RNA polymerase II (Pol II) recruitment and histone H3 lysine 9 acetylation (H3K9ac), a histone mark related to transcriptional activation, was observed in parallel in the liver of these mice (Figure 5D). In contrast, ChREBP recruitment on the Lpk ChoRE was similar in glucose-challenged Pparα<sup>hep</sup>−/− and Pparα<sup>hep</sup>−/− mice (Figure 5E). The presence of Pol II on the Lpk promoter (Figure 5E) was significantly elevated in response to glucose in liver of mice from both genotypes and correlated with Lpk mRNA expression (Figure 5A). Altogether, our results show that the absence of PPARα impairs the binding of ChREBP to its ChoRE and the subsequent glucose-mediated induction of Fgf21. Surprisingly, significant ChREBP recruitment was also detected on the Fgf21 ChoRE under fasting conditions (Figure 5D). Further analysis will be required to determine whether ChREBP interferes with the fasting-mediated induction of Fgf21 expression in the liver, as suggested in a recent report showing that liver-specific Chrebpb knockout mice have decreased Fgf21 expression compared to controls (Jois et al., 2017).

Fgf21 Promoter Accessibility Is Reduced in the Absence of PPARα

To provide insights into the mechanisms by which PPARα affects ChREBP binding onto the Fgf21 promoter in response to glucose, a series of experiments were conducted in vitro (Figure 6). First, we performed formaldehyde-assisted isolation of regulatory elements (FAIRE)-qPCR analysis (Simon et al., 2016) to determine whether ChREBP accessibility to the Fgf21 promoter could be altered in the absence of PPARα (Figure 6A). We observed that while Fgf21 ChoRE promoter accessibility tendency to increase in response to high glucose concentrations in Pparα<sup>hep+/+</sup> hepatocytes, it failed to increase in Pparα<sup>hep</sup>−/− hepatocytes (Figure 6A). To get insight into the molecular mechanisms involved, we hypothesized that PPARα could affect ChoRE accessibility through epigenetic processes and tested whether a pan-histone deacetylase (pan-HDAC) inhibitor (Imai et al., 2016) could increase Fgf21 promoter accessibility. Treatment of hepatocytes with a HDAC inhibitor further enhanced the difference observed in Fgf21 accessibility between Pparα<sup>hep+/+</sup> and Pparα<sup>hep</sup>−/− hepatocytes (Figure 6A), which correlated with a potentiated effect of glucose on Fgf21 expression (Figure 6B). While no significant change in Fgf21 promoter accessibility was observed in Pparα<sup>hep</sup>−/− hepatocytes treated with the HDAC inhibitor (Figure 6A), a modest but significant effect was observed for Fgf21 mRNA levels measured under these conditions. However, treatment failed to fully rescue Fgf21 gene expression to control levels (Figure 6B). These data
suggest that Fgf21 promoter accessibility at the ChoRE is altered in the absence of PPARα by a mechanism that may partly rely on histone acetylation. To determine whether the potentiating effect of the HDAC inhibitor on glucose-induced Fgf21 expression was due to enhanced ChREBP activity, experiments were performed in Chrebp+/+ and Chrebp−/− hepatocytes (Figure 6C). Similar to what was observed in Pparαhep+/+ hepatocytes (Figure 6B), the effect of glucose (25 mM) on Fgf21 expression was increased when Chrebp−/− hepatocytes were treated with the HDAC inhibitor (Figure 6C). Chrebp, Chrebpβ, and Lpk followed a similar trend, with a significant effect observed for Chrebp (Figure 6C). Importantly, the potentiated effect of the HDAC inhibitor on Fgf21 expression was lost in Chrebp−/− hepatocytes (Figure 6C), indicating a dependence on ChREBP activity. Altogether, our results suggest that PPARα is essential to allow ChREBP to gain access to the promoter of Fgf21. Moreover, our data show that the use of HDAC inhibitors enhances the ChREBP-dependent effect of glucose on Fgf21 but cannot fully rescue the effect of PPARα deficiency. 

Sucrose Intake Is Increased in Mice Lacking PPARα in the Liver
Finally, we determined whether the decrease in circulating FGF21 observed in glucose-challenged Pparαhep−/− mice (Figure 5B) paralleled with an increase in sucrose preference, since it was recently demonstrated that FGF21’s action on the paraventricular nucleus of the hypothalamus blocks sugar-seeking behavior and sugar intake in mice (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). First, we observed
that there was no change in body weight or food or water intake in Pparαhep−/− compared to Pparαhep+/+ mice fed with standard chow (Figures 7A–7C). Sucrose preference was then evaluated by giving Pparαhep+/+ and Pparαhep+/+ littermates matched for age and body weight free choice between a bottle containing a 10% sucrose solution and water (Figure 7D). Consumption, which was measured daily for 3 days, demonstrated that Pparαhep−/− mice consumed 30% more sucrose solution than Pparαhep+/+ mice, while the volume of water drunk remained similar between genotypes (Figure 7E).

DISCUSSION

The regulation of FGF21 in the liver is complex due to the paradoxical regulation of this key hepatokine by fasting and glucose signals. In the current study, we uncovered a cross-talk between ChREBP and PPARα for the induction of hepatic FGF21 in response to a glucose challenge. The main finding of our study is that hepatic PPARα is necessary for the glucose-mediated induction of Fgf21 by ChREBP.
Key to β-oxidation and ketogenesis, FGF21 mobilizes energy in the liver for peripheral use, protecting against dyslipidemia and hepatosteatosis (Inagaki et al., 2007; Potthoff et al., 2009). First described as a fasting hormone regulated by PPARα, transcriptional regulation of Fgf21 in the context of excessive blood glucose has only recently been explored (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016), despite early identification of the glucose-sensing region, ChoRE, on the Fgf21 promoter in both mouse (−74 to −52 bp) and human (−380 to −366 bp) (Iizuka et al., 2009). Our experiments confirm that FGF21 is significantly expressed and released by cultured mouse and human hepatocytes in response to glucose upon ChREBP binding to the ChoRE (Iizuka et al., 2009; Uebanso et al., 2011). Our study demonstrates that deletion of ChREBP blunts transcription and secretion of hepatic FGF21 in response to glucose. Importantly, hepatic rescue of ChREBP in global ChREBP knockout mice is sufficient to restore Fgf21 mRNA in the liver and protein in circulation, demonstrating the specificity of hepatic ChREBP activity on the induction of FGF21 in response to a glucose challenge. Interestingly, ChREBP rescue in the liver of Chrebp−/− mice significantly normalized blood glucose concentrations to control levels. This suggests that hepatic ChREBP activity and not peripheral ChREBP is essential for glucose-homeostasis maintenance. This result is consistent with a previous study in which hepatic ChREBP overexpression improved impaired glucose tolerance of high-fat-diet-challenged mice (Benhamed et al., 2012) and is in agreement with a recent study reporting that liver Chrebp knockout mice exhibit impaired insulin sensitivity and glucose intolerance (Jois et al., 2017). In this study, while hepatic Chrebp deletion protected against hepatic steatosis, it also resulted in gene expression changes in white and brown adipose tissues, suggesting inter-organ communication. The contribution of ChREBP to whole-body energy balance may therefore rely on its regulation of lipid species and/or hepatokine production that could contribute to inter-tissue coordination of energy homeostasis.

Activated in response to free fatty acids liberated from adipocytes during fasting (Montagner et al., 2016), a role for hepatocyte PPARα in the response to glucose had not yet been identified. Our study reveals that the synergic induction of Fgf21 by glucose and the PPARα agonist Wy-14643 occurs in both mouse and human hepatocytes. We hypothesize that this cross-talk is specific to Fgf21, as we found no effect of glucose...
on other typical PPARα target genes. This Fgf21 specificity may be due to the proximity of ChREBP- and PPARα-binding sites on the Fgf21 proximal promoter, as both a PPRE and a ChoRE region coexist (−88 to −54 bp) (Srirer et al., 2016). Microarray analysis comparing gene regulation during fasting and glucose challenge highlights a small subset of genes that are upregulated in both conditions. Of those genes Fgf21 is the most significantly induced. Therefore, FGF21 is a unique hepatic hormone showing dual regulation by fasting and carbohydrate signaling. Few publications have proposed a dialog between ChREBP and PPARα for the coordination of energy metabolism. Some examples of cross-talk and regulatory mechanisms have been described for lipid metabolism in brown adipose (Iizuka et al., 2013) and pancreatic β cell function (Boergesen et al., 2011). In the liver, it was also described that the PPARα co-factor PPAR γ coactivator-1α (PGC-1α) can act as a co-activator of ChREBP in response to glucose. PGC-1α is known to activate genes responsible for fatty acid oxidation and hepatic gluconeogenesis during fasting (Vega et al., 2000; Yoon et al., 2001), and it was also reported to upregulate de novo lipogenic genes during glucose stimulation in a ChREBP-dependent manner (Chambers et al., 2013). Altogether, these studies strongly suggest a direct and/or indirect interaction between ChREBP and the PPAR nuclear receptor family.

Here, we reveal a ChREBP-PPARα dialog in hepatocytes required for the induction of Fgf21 by glucose. Indeed, despite normal hepatic ChREBP activity toward glycolytic and lipogenic genes, we report that in the absence of PPARα, ChREBP binding to the Fgf21 ChoRE is significantly reduced, suggesting that hepatocyte PPARα is necessary for the ChREBP-mediated induction of Fgf21 in response to glucose. Our study also reports that Fgf21 promoter accessibility is reduced in the absence of PPARα. Deficient recruitment of ChREBP as well as Pol II was observed at the proximal Fgf21 promoter locus under elevated glucose conditions in the liver of mice lacking hepatocyte PPARα. The fact that epigenetic marks (i.e., H3K9ac) of active transcription were reduced at the Fgf21 promoter when PPARα was lacking in liver, under both fasting and glucose conditions, also supports the hypothesis of reduced promoter accessibility. Indeed, even under conditions of ChREBP overexpression, Fgf21 expression was not efficiently induced in the context of PPARα deficiency. Further experiments will be needed to determine whether PPARα acts as a “pioneer” transcription factor for Fgf21 transcription. Among several described functions, pioneer factors can trigger the opening and/or organization of the local chromatin, in turn allowing the binding of other transcription factors, histone-modification enzymes, chromatin modifiers, and/or nucleosome remodelers (Zaret and Carroll, 2011). Of note, an HDAC inhibitor potentiated a ChREBP-dependent effect of glucose on Fgf21 expression in primary hepatocytes. The HDAC inhibitor strategy in glucose-challenged PPARα-deficient hepatocytes only led to a modest restoration of Fgf21 expression. While this approach was not sufficient to fully restore accessibility to the Fgf21 promoter, it does suggest that Fgf21 promoter accessibility at the ChoRE is altered in the absence of PPARα by a mechanism that may partly rely on histone acetylation. We employed FAIRE-qPCR analysis to determine Fgf21 promoter accessibility in response to glucose. A more sensitive approach, the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) (Buenrostro et al., 2013) may have allowed us to determine whether Fgf21 promoter accessibility is indeed modified by an HDAC inhibitor strategy in Pparα−/− hepatocytes. While other molecular mechanisms are clearly involved, the implication of specific HDACs should be further investigated, since it was recently shown that PPARα prevents the recruitment of HDAC3 to the Fgf21 promoter in hepatocytes when concentrations of β-hydroxybutyrate, a key product of β-oxidation, are elevated (Rando et al., 2016).

At the physiological level, we observed that Pparα<sup>hep+/−</sup> mice consumed more sucrose (+30%) than Pparα<sup>hep+/+</sup> mice. Two studies have recently unraveled the mechanistic link between sucrose-derived FGF21 and nutrient preference (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). FGF21 production in response to carbohydrates was shown to markedly reduce sweet taste preference and suppress consumption of simple sugars by acting on specific regions of the brain (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). In these studies, hepatic FGF21 production in response to carbohydrate intake was attributed to ChREBP activity. The fact that PPARα is also involved in this liver-to-brain axis following simple sugar consumption opens a new molecular path of regulation of macronutrient preference/intake and expands liver PPARα function from a fasting regulator to a modulator affecting the physiological sugar response.

In conclusion, we identify a transcriptional node in the control of hepatic FGF21 in response to glucose. The glucose sensor ChREBP requires PPARα for the induction of FGF21 in response to dietary sugar. This is a unique collaboration between transcription factors that are activated in response to distinct nutritional conditions (high glucose for ChREBP and fasting for PPARα). These data imply that drug targeting of PPARα may exert part of its beneficial effects on metabolic homeostasis by supporting the ChREBP-induced loop controlling sweet preference via FGF21.

**EXPERIMENTAL PROCEDURES**

**Generation of ChREBP Knockout Mice**

Mice lacking exons 9–15 of the Chrebp gene were generated through homologous recombination. Correspondence regarding Chrebp<sup>−/−</sup> mice should be addressed to R.D. (renaud.dentin@inserm.fr) and C.P. (catherine.postic@inserm.fr). Experimental details are provided in Supplemental Experimental Procedures.

**Animals**

10- to 12-week-old adult male C57BL/6J, Chrebp<sup>+/−</sup>, Chrebp<sup>−/−</sup>, Pparα<sup>hep+/+</sup>, and Pparα<sup>hep+/−</sup> mice (Montagner et al., 2018) were used for all in vivo experiments. For hepatocyte cultures, male and female mice were used as described in the figure legends. Procedures were carried out according to the French guidelines for the care and use of experimental animals (animal authorization agreement number CEEA34.AFB/CP.082.12, Paris Descartes Ethical Committee). Mice were maintained in a 12-hr light/dark cycle with water and a standard diet (65% carbohydrate, 11% fat, and 24% protein) unless otherwise specified. Nutritional challenges details are described in Supplemental Experimental Procedures.

**Injection of Adenovirus In Vivo**

Adenovirus coding GFP and ChREBP<sup>GAA</sup> (ChREBP isoform deleted of the LID domain) (Li et al., 2008) produced by Laboratoire de thérapie génique (Nantes, France; requests to R.D. at renaud.dentin@inserm.fr) were delivered through
penis vein injection (3 x 10^6 [pfu]/mouse) to adult mice. Four days later, nutritional protocols were applied.

**Primary Cultures of Human and Mouse Hepatocytes**

Human hepatocytes were prepared from lobectomy segments resected from adult patients under the approval of the National Ethics Committee as described previously (Pichard et al., 2006; Marmier et al., 2015). Mouse hepatocytes were isolated as described previously (Dentin et al., 2004). Experimental details regarding culture conditions are provided in the figure legends and Supplemental Experimental Procedures.

**ChiP Analysis**

In vivo chromatin immunoprecipitation (ChiP) assays from mouse livers were performed by Active Motif. Briefly, genomic DNA regions of interest were isolated using antibodies against H3K9Ac (Active Motif), RNA Pol II (Active Motif), ChREBP (Novus), and PPARγ (Santa Cruz Biotechnology). 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). Specific enrichment was expressed as percentage of input. Further details are provided in Supplemental Experimental Procedures.

In vitro ChiP assays from cultured hepatocytes were performed as described previously (Marmier et al., 2015). Briefly, genomic DNA regions of interest were isolated using antibodies against ChREBP (Novus) and immunoglobulin G (IgG) (Cell Signaling). DNA fragments were quantified by qPCR using primers described in Table S1. Results are expressed as fold enrichment. Further details are provided in Supplemental Experimental Procedures.

**FAIRE-qPCR**

FAIRE-qPCR was performed in Pparα<sup>hep+/–</sup> and Pparα<sup>hep</sup>/–/– hepatocytes using a protocol previously described (Simon et al., 2012). Briefly, cells were treated with 1% formaldehyde at room temperature for 5 min to form DNA-protein crosslinks, and the crosslinking was stopped by addition of glycine to a final concentration of 125 mM. FAIRE was analyzed by qPCR on genomic DNA using the ChRoRE Fgf21 primers (Figure 5C) and calculated using relative FGF21 signals. FGF21 primers were calculated using the comparative Ct method, such that a geometric test was applied and the differentially expressed probes using 1% (Promega). For qPCR analysis, total RNA samples (2 μg) were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers for SYBR green assays are presented in Tables S2 and S3. Primers used to measure Chrebp expression were designed to detect the Chrebp<sup>a</sup> and Chrebp<sup>p</sup> isoforms. A specific primer to detect only the j isoform was also used. Primers for Vnn1, Cyp4a10, and Cyp4a14 were previously described (Montagnier et al., 2016). 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 or 18S ribosomal (18S) for human samples and analyzed with LinRegPCR.22.

Transcriptomic profiles were obtained using Agilent SurePrint G3 Mouse V2 GE 8x60K (Design 074809) following the manufacturer’s instructions. Data were analyzed with R (http://www.r-project.org) using Bioconductor packages (http://www.bioconductor.org, v 2.12; Gentleman et al., 2004) as described in GEO: GSE26728. A model was fitted using the limma lmFit function (Wittenher and Smyth, 2004). Correction for multiple testing was applied using a false discovery rate (Benjamini and Hochberg, 1995). Probes with adjusted p ≤ 0.05 were considered differentially expressed (n = 6). Hierarchical clustering was applied and the differentially expressed probes using 1 – Pearson correlation coefficient as distance and Ward’s criterion for agglomeration. The resulting dendrogram were illustrated as a heatmap. The enrichment of gene ontology (GO) biological processes was evaluated using a conditional hyper-geometric test (GOstats package; Falcon and Gentleman, 2007).

**Western Blotting Analysis**

Proteins from hepatocytes and liver tissue were extracted from whole-cell lysates. Proteins were subjected to 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Rabbit polyclonal ChREBP (1:1,000, Novus Biologicals) and L-PK (1:1,500, a gift from Dr. Axel Kahn) antibodies were used. Protein β-actin (1:5,000) (Cell Signaling Technology) was used to normalize data.

**Analytical Analysis**

Blood glucose was measured from total blood using an Accu-Check glucometer (Roche). Liver triglycerides were measured with a colorimetric diagnostic kit (Triglycerides FS, Diasys). Serum insulin concentrations were determined using a rat insulin ELISA assay kit (Crystal Chem) with a mouse insulin standard. The mouse ELISA kit (Millipore) was used to measure FGFI in cell culture medium (30 μL of medium was used) and mouse plasma (20 μL).

**Statistical Analysis**

Data represent at least three independent experiments, are reported as means ± SEM, and were analyzed with analysis of variance 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 more than two groups followed by Bonferroni post hoc test. Statistical significance was defined as p < 0.05.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the microarray data reported in this paper is GEO: GSE92502.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.09.065.

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We would like to thank Dr. B. Staels and Dr. P. Lefebvre (Inserm U1011, Lille), Dr. N. Venteclef (Inserm UMR_S1138, Paris), and Dr. D. Langin (Inserm UMR 1048, Toulouse) for scientific discussion. We also thank Dr. A. Kayali and Dr. T. Issad (Institut Cochin, Inserm U1016) for critical reading of the manuscript. We thank L. Francese, S. Topcu (Institut Cochin, Inserm U1016), C. Naylies (GeT-TRix, INRA ToxAim), and G.Michel for providing excellent technical assistance. We thank the animal facility staff (EZOP, INRA ToxAlim) for their excellent work. H. Guillou’s lab (TIM, ToxAlim) is supported by grants from ChroME Network (Marie Curie Skłodowska Action H2020-MSCA-ITN-2015-675610), the Foundation for the Medical Research (FRM) (DEQ20150331744), and the European Foundation for the Study of Diabetes (EFSD) Novonordisk. H. Guillou’s and C. Postic’s labs are also supported by grants from the National Agency for Research (ANR) (ANR-12BSV1-0025-ObeLiP and ANR-15-CE14-0026-Hepatokind). R. Dentin’s lab (U1016-Institut Cochin) is supported by the European Research
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