

E2F1 promotes hepatic gluconeogenesis and contributes to hyperglycemia during diabetes



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ABSTRACT

Objective: Aberrant hepatic glucose production contributes to the development of hyperglycemia and is a hallmark of type 2 diabetes. In a recent study, we showed that the transcription factor E2F1, a component of the cell cycle machinery, contributes to hepatic steatosis through the transcriptional regulation of key lipogenic enzymes. Here, we investigate if E2F1 contributes to hyperglycemia by regulating hepatic gluconeogenesis.

Methods: We use different genetic models to investigate if E2F1 regulates gluconeogenesis in primary hepatocytes and *in vivo*. We study the impact of depleting E2F1 or inhibiting E2F1 activity in diabetic mouse models to evaluate if this transcription factor contributes to hyperglycemia during insulin resistance. We analyze E2F1 mRNA levels in the livers of human diabetic patients to assess the relevance of E2F1 in human pathophysiology.

Results: Lack of E2F1 impaired gluconeogenesis in primary hepatocytes. Conversely, E2F1 overexpression increased glucose production in hepatocytes and in mice. Several genetic models showed that the canonical CDK4-RB1-E2F1 pathway is directly involved in this regulation. E2F1 mRNA levels were increased in the livers from human diabetic patients and correlated with the expression of the gluconeogenic enzyme Pck1. Genetic inactivation or pharmacological inhibition of E2F1 improved glucose homeostasis in diabetic mouse models.

Conclusions: Our study unveils that the transcription factor E2F1 contributes to mammalian glucose homeostasis by directly controlling hepatic gluconeogenesis. Together with our previous finding that E2F1 promotes hepatic steatosis, the data presented here show that E2F1 contributes to both hyperlipidemia and hyperglycemia in diabetes, suggesting that specifically targeting E2F1 in the liver could be an interesting strategy for therapies against type 2 diabetes.

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Keywords Gluconeogenesis; E2F1; Liver metabolism; Hyperglycemia; Diabetes; Cell cycle regulators

1. INTRODUCTION

Sustaining blood glucose levels within a relatively narrow range is essential for maintaining global homeostasis. This status is achieved through a complex balance between glucose production and utilization by different tissues. These processes are dynamically regulated by hormonal and nutritional signals [1]. Hepatic glucose production, which comprises glycogenolysis and gluconeogenesis (*de novo* production of glucose from non-carbohydrate substrates), is critical for adaptation to fasting conditions and represents up to 80% of total endogenous glucose production [2]. Hepatic gluconeogenesis is largely controlled by substrate flux and by transcriptional regulation of the key rate-

limiting enzymes phosphoenolpyruvate carboxykinase 1 (PCK1) and glucose-6-phosphatase (G6PC), which catalyze the first committed steps and the terminal steps of gluconeogenesis, respectively. Several transcription factors and coactivators, control the expression of these gluconeogenic enzymes in response to hormones, especially insulin and glucagon [1]. Aberrant regulation of hepatic glucose production is a major contributor to the hyperglycemia observed in type 2 diabetes and the insulin-resistant state. Indeed, clinical suppression of hepatic glucose production to lower glycemia is one of the main targets of diabetes treatment [2]. In normal subjects, hepatic gluconeogenesis and lipogenesis are mutually exclusive events. In contrast, during diabetes, the liver exhibits abnormally high levels of gluconeogenesis

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Abbreviations: PCK1, Phosphoenolpyruvate carboxykinase 1; G6PC, glucose-6-phosphatase G6PC; PTT, Pyruvate tolerance test; CDK4, cyclin-cyclin-dependent kinase 4; PKA, protein kinase A; cAMP, Cyclic adenosine monophosphate; PPAR γ C1A, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; FOXO1, Forkhead box protein O1; CREB, cAMP response element-binding protein; HFD, high fat diet; FAO, fatty acid oxidation

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as well as high levels of cholesterol and triglyceride synthesis, resulting in both dyslipidemia and hyperglycemia. This highlights the apparent paradox of selective insulin resistance in the liver [3]. Although some explanations have been proposed, this phenomenon is not yet fully understood [4,5].

E2F transcription factors are the downstream effectors of distinct signaling cascades that regulate the expression of genes involved in cellular homeostasis. In proliferating cells, E2F target genes include effectors of DNA replication, mitosis, DNA repair, and apoptosis [6]. E2Fs exist either as heterodimers associated with dimerization partner (DP) proteins or within larger complexes, including members of the retinoblastoma family of proteins (pRBs). In general, the association of E2Fs with pRB family members induces the repression of their target genes. When phosphorylated by active cyclin–cyclin-dependent kinase (CDK) complexes, pRBs are released, enabling E2Fs to drive transcriptional activation [6].

We have previously demonstrated that E2F1, the first member of the E2F family to be described and studied extensively, has important metabolic functions beyond the control of the cell cycle in non-proliferating cells [7–9]. Studies of the retinoblastoma protein RB1 further support a major role of E2F1 in metabolism [10]. In recent studies, we showed that E2F1 is essential for controlling liver metabolism, regulating cholesterol uptake and promoting lipid synthesis through transcriptional regulation of key lipogenic enzymes [11,12]. We also highlighted that widely used animal models of non alcoholic fatty liver disease (NAFLD) had increased levels of *E2f1* expression and activity in the liver [12]. Here, we demonstrate that E2F1 contributes to mammalian glucose homeostasis through the control of hepatic glucose production. Importantly, our results indicate that E2F1 is critical for inducing hyperglycemia in type 2 diabetes. This suggests that reducing E2F1 activity could protect against obesity-induced hyperglycemia.

2. MATERIALS AND METHODS

Animal experiments. C57BL/6J mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). *E2f1*^{−/−} (B6; 129S4-*E2f1*^{tm1Meg/J}) mice and *db/+* mice (Janvier Labs) were crossed to obtain *db/db*; *E2f1*^{+/-} mice. *E2F1*^{tg/+} mice harboring the Rosa26-loxP-LacZ-LoxP-E2F1 conditional expression cassette were obtained from Ulrike Ziebold (MDC, Berlin, Germany) [13]. *E2F1*^{tg/+} mice were crossed with *Alb-cre*⁺ mice to obtain *E2F1*^{tg/+} *Alb-Cre*⁺ mice and their control littermates *E2F1*^{tg/+} *Alb-Cre*[−] mice. *E2f1*^{fl/fl} mice (Taconic Biosciences, NY, USA) were crossed with *Alb-cre*⁺ mice to obtain *E2f1*^{fl/fl} *Alb-Cre*⁺ (*E2f1*-LKO) and their control littermates *E2f1*^{fl/fl} *Alb-Cre*[−] mice. Mice were housed under a 12-hour light/dark cycle and sacrificed as indicated. For high fat diet (HFD) experiments, mice were fed during 12 or 16 weeks, as indicated, with a HFD (2127, Kliba Nafag or TD06414, Envigo). For pharmacological inhibition of E2F, during the last 10 days of the HFD, mice were gavaged daily with 40 mg/kg of E2F inhibitor HLM006474 (324461, Merck-Millipore) or vehicle. For Pyruvate tolerance test (PTT), mice were fasted overnight and injected with pyruvate (1 g/kg). For insulin tolerance test (ITT), mice were fasted for 6 h and injected with insulin 0.75 U/kg (Actrapid, Novonordisk). Blood glucose was determined using a glucometer and glucose strips (Accu-Chek Aviva, Roche). Serum levels of lactate dehydrogenase, ALT and AST were measured by the Mouse Metabolic Facility of the University of Lausanne. All animal experiments were performed according to Swiss animal welfare laws and were approved by the Canton of Vaud SCAV (authorization VD2627 and VD3046).

Cell culture. HepG2 cells were obtained from the American Type Culture Collection (ATCC). *E2F1*^{−/−} HepG2 cells were described here [11]. Primary hepatocytes were isolated as previously described [14]. Primary hepatocytes were isolated from C57BL/6J mice, *E2f1*^{−/−} mice (B6; 129S4-*E2f1*^{tm1Meg/J}), *E2F1*^{tg/+} crossed with *Abl-Cre*⁺ mice, *Rb1*^{fl/fl} mice (B6,129-*Rb1*^{tm3Tyj/J}) crossed with *Abl-Cre*⁺ mice, *Cdk4*^{−/−} mice, and *Cdk4*^{R24C/R24C} mice [15] and their respective control mice. C57BL/6J hepatocytes were not used as controls for mutant hepatocytes. Mouse hepatocytes were isolated, cultured, and infected with adenoviruses Ad-GFP and Ad-E2F1, as previously described [12,14]. For mRNA expression experiments, primary hepatocytes were treated for 3 h with 10 μM Forskolin (Cat. F6886, Sigma-Aldrich) or vehicle (DMSO). For glucose production experiments, hepatocytes were rinsed twice with PBS and cultured in media without glucose, glutamine, and phenol red (Cat. A14430, Life Technologies) supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, and forskolin (10 μM). After 6 h, media was collected for glucose quantification (Cat. GAGO20, Sigma-Aldrich) and proteins were quantified for normalization.

Real-time quantitative PCR analysis. Total mRNA was extracted from 20 to 30 mg of mouse liver or cultured cells using an RNeasy kit (Cat. 74106, Qiagen) according to the manufacturer's protocol. One microgram of the RNA was subsequently reverse-transcribed and quantified via real-time quantitative PCR using an ABI 7900HT instrument. Gene expressions were determined using the delta Ct method or the standard curve method normalized housekeeping gene ribosomal protein S9 (RS9) levels. The complete list of primers is presented in Supplemental Table 2.

Seahorse analyses. Mitochondrial function was determined with an XF-24 extracellular flux analyzer (Seahorse Bioscience). Oxygen consumption Rate (OCR) was measured in control and mutant hepatocytes that were seeded in an XF 24-well cell culture microplate 16 h before the experiment. OCR was expressed as pmol of O₂ per minute and was normalized by protein content. Just before the experiment the cells were washed, and the hepatocyte culture medium was replaced with KHB containing 2.5 mM Glucose and 1.5 mM of carnitine. After measuring baseline OCR as an indication of basal respiration, OCR was measured after an acute injection of 300 μM of palmitate coupled to BSA.

ChIP experiment. ChIP was performed as previously described [16]. The sheared DNA was immunoprecipitated by an E2F1 antibody (Cat. 3742, Cell Signaling Technology) coupled to magnetic beads. The immunoprecipitated chromatin was washed, reverse cross-linked at 65°C overnight and purified using minielute columns (Cat. 28006, Qiagen). Finally, E2F1 DNA binding was quantified via real-time quantitative PCR using an ABI 7900HT instrument.

Immunoblotting. Total protein extract and immunoblot analysis were performed as previously described [14]. The following antibodies were used: E2F1 (Cat. 3742, Cell Signaling Technology), Ser⁷⁸⁰ phosphorylated RB (Cat. 8180, Cell Signaling Technology), RB (Cat. C-15 Santa Cruz Biotechnology), CREB (Cat. 9197, Cell Signaling Technology), Ser¹³³ phosphorylated CREB (Cat. 9198, Cell Signaling Technology), and beta-ACTIN (Cat. A2066, Sigma-Aldrich).

Human samples. All obese patients included in this study are from the ABOS study (Atlas Biologique de l'Obésité Sévère). This study also included a group of lean and normoglycemic control patients that had surgery for benign and non-inflammatory pathologies. Clinical data were collected at Centre Hospitalier Régional Universitaire de Lille. The protocol concerning the use of biopsies from patients follows French regulations and was approved by the Institutional Ethical Committee of the University of Lille and the Centre Hospitalier Régional Universitaire de Lille (ClinGov NCT 01129297). All patients gave written and

informed concern. The obese, type 2 diabetic patients displayed high Hba1c and high HOMA-IR. Metabolic parameters of the two groups are presented in [Supplemental Table 1](#). Liver samples were collected during surgery, within the first 15 min of the procedure, weighed and snap-frozen in liquid nitrogen. Total RNA and protein were extracted from 10 mg of liver samples using the All in One Purification Kit (NorGen Biotek Corp). First-strand cDNA synthesis was performed using 500 ng total RNA as a template and Superscript II reverse transcriptase (Life Technologies) primed with 50 pmol of random hexamers (New England Biolabs). Quantitative real-time PCR was performed using the Bio-Rad MyiQ Single-Color Real-Time PCR Detection System and the Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories). Gene expression was normalized to two housekeeping genes Beta-actin (*Actb*) and ribosomal protein S9 (RS9).

Statistical analysis. All data are expressed as the mean \pm SEM except human data. Statistical significance was assessed with unpaired t-test using Prism 7 software (GraphPad Software, La Jolla, CA USA). The correlation analysis was performed using Pearson correlation test. Differences were considered statistically significant at $P < 0.05$.

3. RESULTS

3.1. Lack of E2F1 blunts hepatic gluconeogenesis in hepatocytes

We have recently shown that the cell cycle regulator E2F1 promotes hepatic lipogenesis during insulin resistance. We wondered if E2F1 could participate in glucose production in the liver, a metabolic pathway that is abnormally active in type 2 diabetes. For this purpose, we analyzed the gluconeogenic capacity of primary hepatocytes lacking E2F1. Mouse primary hepatocytes obtained from *E2f1*^{-/-} mice and their wild type littermates were treated with forskolin, a protein kinase A (PKA) activator that mimics glucagon action in the liver by increasing cAMP levels, to activate gluconeogenesis. *Pck1* and *G6pc* mRNA expression levels were significantly reduced in *E2f1*^{-/-} hepatocytes after forskolin stimulation ([Figure 1A](#)). This led to a decrease in glucose production in *E2f1*-deficient primary hepatocytes ([Figure 1B](#)). No differences between the two genotypes were observed in basal conditions, when gluconeogenic gene expression is very low (data not shown). Similarly, depleting E2F1 in HepG2 cells, a human hepatoma cell line, using CRISPR/Cas9 technology resulted in a significant decrease in gluconeogenic gene expression ([Figure 1C,D](#)).

Gluconeogenesis is an energy-consuming process that requires intact mitochondrial function. In fasting conditions, fatty acids produced by the lipolysis in white adipose tissue are oxidized in the liver to sustain gluconeogenesis [1]. We have previously shown that E2F1 regulates oxidative metabolism [8]. This prompted us to investigate if the decrease in gluconeogenesis observed in *E2f1*^{-/-} hepatocytes could be due to a defect in mitochondrial oxidation of fatty acids. Seahorse analyses showed that *E2f1*-deficient primary hepatocytes presented increased fatty acid oxidation ([Figure 1E,F](#)), which indicated that the decrease in glucose production observed in *E2f1*^{-/-} hepatocytes was not caused by impaired mitochondrial function.

The above findings showed that lack of E2F1 in hepatocytes impairs glucose production due to a decrease in gluconeogenic gene expression.

3.2. Overexpression of E2F1 induces gluconeogenesis in primary hepatocytes and in liver

To further examine whether E2F1 may promote gluconeogenesis, we transduced mouse primary hepatocytes with an adenovirus overexpressing human E2F1 (ad-E2F1). E2F1 overexpression markedly

increased *Pck1* mRNA levels, which led to a concomitant increment in glucose production ([Figure 2A,B](#)). In contrast to what was observed in *E2f1*^{-/-} hepatocytes, E2F1 overexpression had no major effect on *G6pc* mRNA levels ([Figure 2A](#)). The effect of E2F1 overexpression on gluconeogenesis was completely abrogated in the presence of the PKA inhibitor H89 ([Figure 2A,B](#)). These results suggested that E2F1 promotes gluconeogenesis in a PKA-dependent manner.

To investigate if E2F1 could promote hepatic gluconeogenesis *in vivo*, we generated a liver-specific E2F1 overexpressing mouse model by crossing transgenic mice harboring the Rosa26-loxP-LacZ-LoxP-E2F1 conditional expression cassette (*E2F1*^{tg/+} mice) [13] with *Alb-Cre* mice. Under Cre recombinase activity, these mice express human E2F1 under the control of the Rosa promoter, which results in a mild increase in hepatic E2F1 protein levels ([Suppl. Fig. 1A](#)). When challenged with a pyruvate tolerance test, which reflects hepatic gluconeogenesis, hepatic glucose production increased to a much greater extent in *E2F1*^{tg/+} *Alb-Cre*⁺ mice compared to their control littermates ([Figure 2C](#)). Remarkably, this increase was not due to a defect in insulin sensitivity ([Suppl. Fig. 1B](#)). Similar to what we observed in primary hepatocytes infected with ad-E2F1 ([Figure 2A](#)), transgenic mice overexpressing E2F1 in the liver presented higher hepatic *Pck1* mRNA levels, but no changes in *G6pc* mRNA ([Figure 2D](#)). In addition, primary hepatocytes isolated from *E2F1*^{tg/+} *Alb-Cre*⁺ mice presented the same pattern of gluconeogenic gene expression, and increased glucose production ([Figure 2E,F](#)). No differences were observed in terms of fatty acid oxidation ([Suppl. Fig. 1C-D](#)). Altogether, these results showed that increasing E2F1 levels in the liver is sufficient to promote hepatic gluconeogenesis *in vivo*.

We next performed chromatin immunoprecipitation (ChIP) to examine whether E2F1 is recruited to the promoters of gluconeogenic genes. Immunoprecipitation of endogenous E2F1 from the livers of mice led to an enrichment of the proximal region of the *Pck1* promoter ([Figure 2G](#)). No significant binding of E2F1 was observed in the promoter of *G6pc*. Notably, the enrichment in the *Pck1* promoter was higher during fasting conditions, whereas no differences due the nutritional status were observed in the binding of E2F1 to its own promoter. Moreover, promoter analysis revealed the presence of putative E2F response elements in the mouse and human PCK1 promoters ([Suppl. Fig. 2A](#)). These results suggested that E2F1 directly activates the expression of PCK1. Lack of E2F1 caused a marked decrease of *G6pc* expression ([Figure 1](#)), but we were not able to detect a significant binding of E2F1 to its promoter ([Figure 2G](#)). This prompted us to investigate if E2F1 could also regulate gluconeogenesis by affecting the PKA pathway. In the cAMP/PKA pathway, cAMP elevation in response to glucagon leads to activation of serine kinase PKA, which phosphorylates the transcription factor CREB on Serine¹³³. Phosphorylated CREB promotes gluconeogenesis through the induction of *Pck1* and *G6pc* transcription [1]. CREB phosphorylation was examined in *E2f1*^{-/-} primary hepatocytes after forskolin stimulation. The phosphorylation signal was significantly reduced in *E2f1*^{-/-} hepatocytes indicating a decrease in PKA activity ([Suppl. Fig. 2B](#)). On the contrary, E2F1 adenoviral transduction in hepatocytes led to exacerbated CREB Serine¹³³ phosphorylation at ([Suppl. Fig. 2C](#)). To assess if E2F1 also enhanced PKA signaling *in vivo*, we evaluated the phosphorylation status of CREB in the livers of fasted *E2F1*^{tg/+} *Alb-Cre*⁺ mice. No differences were observed in the phosphorylation of CREB in mice overexpressing E2F1 in the liver ([Suppl. Fig. 1E](#)).

The above results suggested that E2F1 promotes gluconeogenesis in hepatocytes directly by regulating PCK1 expression and indirectly by modulating the activity of the PKA pathway. Nevertheless, *in vivo* the major effect of E2F1 is probably direct PCK1 regulation.

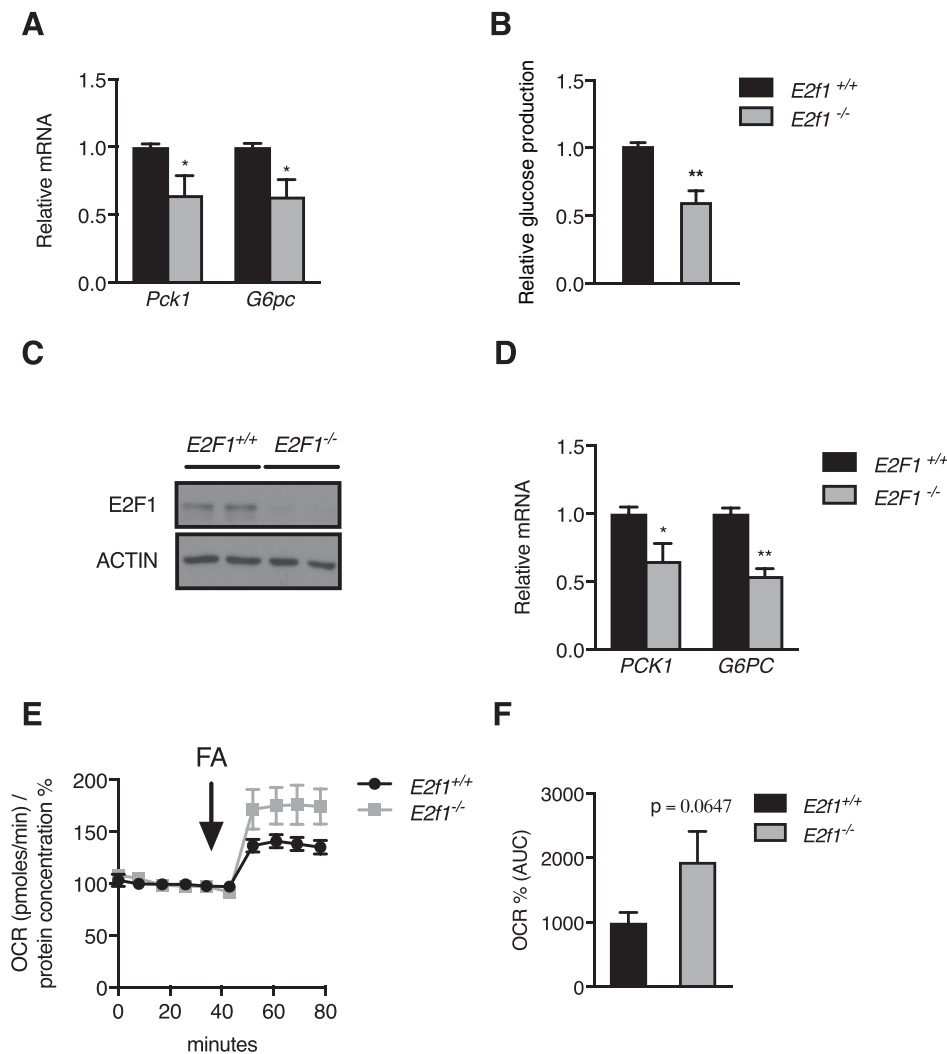


Figure 1: E2F1 deficiency reduces gluconeogenesis in hepatocytes. (A) Relative expression levels of *Pck1* and *G6pc* mRNA in primary hepatocytes from *E2f1*^{+/+} and *E2f1*^{-/-} mice treated for 3 hours with 10 μ M forskolin (Fsk). (B) Glucose production by *E2f1*^{+/+} and *E2f1*^{-/-} primary hepatocytes treated for 6 hours with 10 μ M Fsk (n = 3). (C) E2F1 protein levels in *E2F1*^{+/+} and *E2F1*^{-/-} HepG2 cells. (D) Relative expression levels of *PCK1* and *G6PC* mRNA in *E2F1*^{+/+} and *E2F1*^{-/-} HepG2 cells incubated overnight in a serum-free medium and treated for 3 hours with forskolin (10 μ M) (n = 3). (E) Fatty acid oxidation in *E2f1*^{+/+} and *E2f1*^{-/-} primary hepatocytes. (F) Quantification of the area under curve of the palmitate induced OCR. Data represent the mean \pm SEM. *P < 0.05, **P < 0.01, (2-tailed, unpaired t test).

3.3. The CDK4/RB1 pathway participates with E2F1 in the control of gluconeogenesis

Given that E2F1 modulates gluconeogenesis in a PKA-dependent manner (Figure 2A,B), we wanted to determine whether CDK4 and RB1, upstream regulators of E2F1, participated in the control of gluconeogenesis. CDK4, when associated with the regulatory D-type cyclins, promotes E2F1 transcriptional activity by phosphorylating RB1 and therefore releasing E2F1 from its repression [6]. We have previously shown that CDK4 can be activated in response to insulin, both in adipocytes [17] and in hepatocytes [12]. We investigated next, whether the gluconeogenic hormone glucagon could modulate the CDK4/RB1 pathway in hepatocytes. We observed an increase in RB1 Ser⁷⁸⁰ phosphorylation in response to glucagon stimulation in primary hepatocytes (Figure 3A). The same observation was confirmed in HepG2 cells in response to forskolin stimulation (Figure 3B), which suggests a PKA-dependent activation of CDK4. This dual phosphorylation of RB1 both by insulin and by glucagon in primary hepatocytes was also reproduced in HepG2 cells (Suppl. Fig. 3A-B). These results suggested

that in response to PKA activation, RB1 is released from E2F1 in order to promote the expression of gluconeogenic genes. Consistently, *Rb1*^{-/-} hepatocytes showed higher expression levels of *Pck1* and *G6Pc* mRNAs and a concomitant increase in glucose production (Figure 3C,D), which was the reverse phenotype of hepatocytes lacking E2F1 (Figure 1A and B).

Furthermore, primary hepatocytes isolated from *Cdk4*^{-/-} mice (Figure 3E,F) or with depleted *Cdk4* expression by shRNA (Suppl. Fig. 3C-D) showed decreased gluconeogenic gene expression and glucose production. In a complementary approach, we characterized the gluconeogenic capacity of primary hepatocytes isolated from *Cdk4*^{R24C/R24C} mice, which express a mutant CDK4 protein that is not sensitive to INK4 inhibitors and that is consequently more active [15]. In sharp contrast with hepatocytes lacking CDK4 activity, *Cdk4*^{R24C/R24C} hepatocytes had an increased gluconeogenic profile, namely higher *Pck1* and *G6pc* gene expression and increased glucose production (Figure 3G,H). This is in agreement with the observation that p16 (INK4a) deficiency enhances hepatic gluconeogenesis [18].

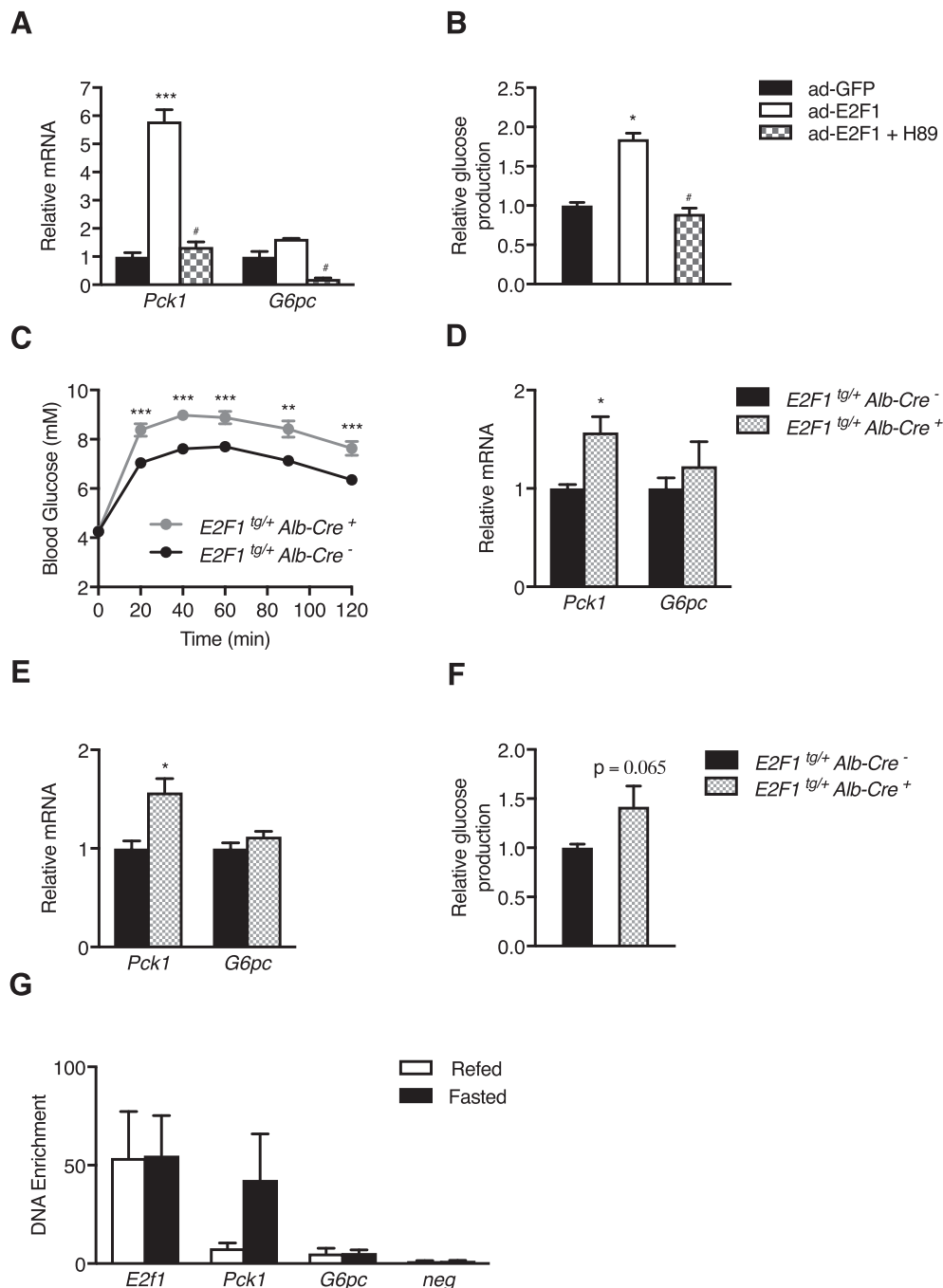


Figure 2: Overexpression of E2F1 induces gluconeogenesis in hepatocytes and in mice. (A) Relative expression levels of *Pck1* and *G6pc* mRNA and (B) glucose production in primary hepatocytes infected with ad-GFP or ad-E2F1 for 48 hours and treated with forskolin (10 μ M) in the presence or absence of the PKA inhibitor H89 (5 μ M). (C) Pyruvate tolerance test (n = 6–11) and (D) relative liver expression of *Pck1* and *G6pc* mRNA of *E2F1*^{tg/+} *Alb-Cre*⁺ and control mice (n = 5). (E) Relative mRNA expression levels of *Pck1* and *G6pc* and (F) glucose production in primary hepatocytes from *E2F1*^{tg/+} *Alb-Cre*⁻ and *E2F1*^{tg/+} *Alb-Cre*⁺ mice after forskolin (10 μ M) treatment. (G) E2F1 ChIP-qPCR on livers of mice fasted for 24 hours or refed overnight after a 24-hour fasting (n = 4–6). Data represent the mean \pm SEM. *P < 0.05, ***P < 0.005 compared to control, # P < 0.05 compared to ad-E2F1 (2-tailed, unpaired t test).

Crucially, *Cdk4*^{R24C/R24C} hepatocytes lacking *E2f1* presented normalized gluconeogenic gene expression levels and decreased glucose production (Figure 3G,H), which revealed that CDK4 promoted gluconeogenesis in hepatocytes in an E2F1-dependent manner. The above findings obtained in different genetic models that present an alteration of the CDK4-RB1 pathway support the notion that E2F1 promotes gluconeogenesis in hepatocytes.

3.4. E2F1 deficiency reduces gluconeogenesis in mouse models of diabetes

Aberrant increased hepatic gluconeogenesis is the main contributor to hyperglycemia during diabetes [2]. Mutants for the leptin receptor, *db/db* mice have hyperphagia, obesity, and type 2 diabetes [19]. *db/db* mice presented increased E2F1 mRNA and protein levels (Figure 4B and Suppl. Fig 4A). In order to determine whether deletion

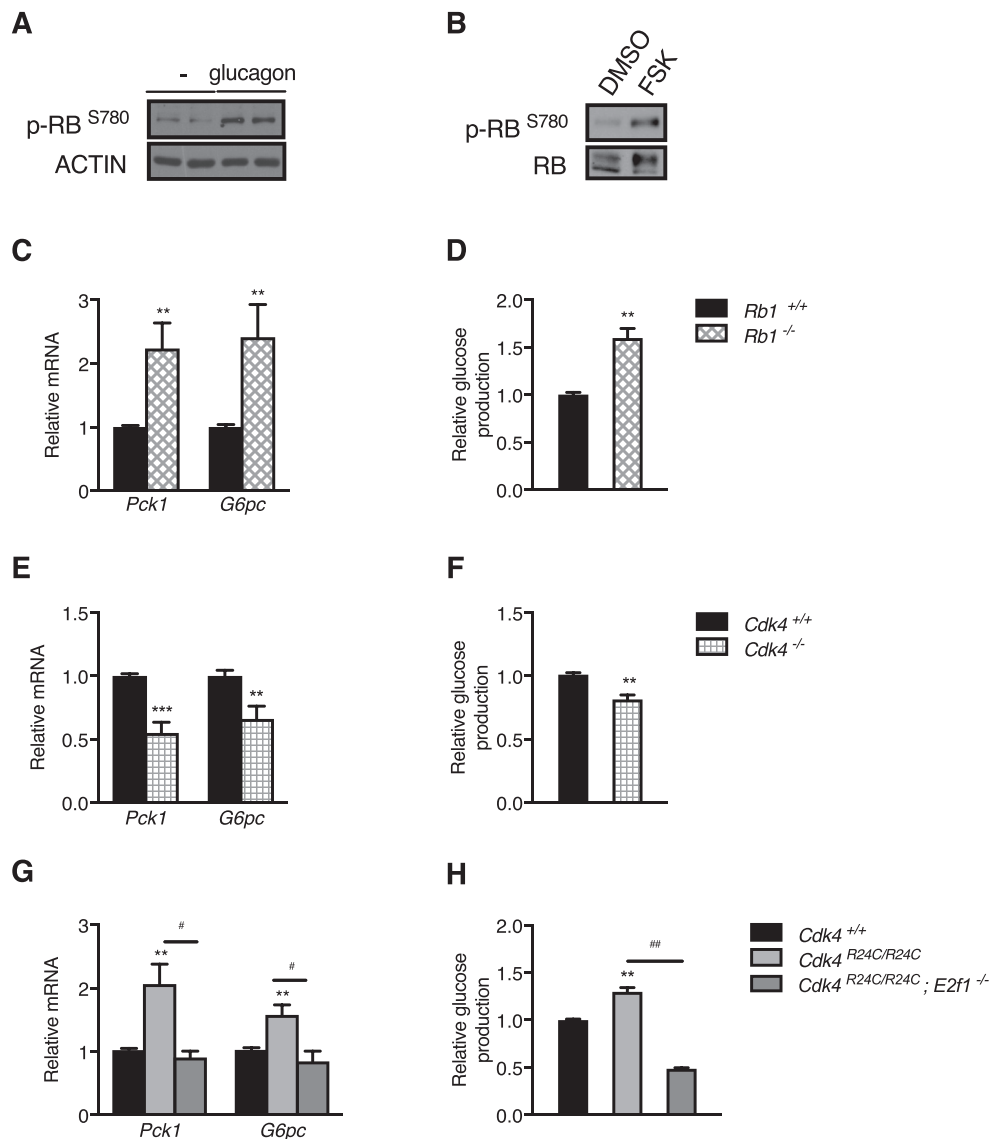


Figure 3: The CDK4/RB1 pathway participates with E2F1 in the control of gluconeogenesis. Ser⁷⁸⁰ phosphorylation of RB1 in (A) primary hepatocytes treated for 6 hours with glucagon (100 nM) and (B) HepG2 cells treated for 2 hours with 10 μ M forskolin (n = 3). (C, E and G) Relative *Pck1* and *G6pc* mRNA levels and (D, F and H) glucose production in primary hepatocytes of the indicated genotypes (n = 3) treated with Fsk for 3 or 6 hours, respectively. Data represent the mean \pm SEM. **P < 0.01, ***P < 0.005 compared to their controls. #P < 0.05, ###P < 0.01 compared to *Cdk4*^{R24C/R24C} hepatocytes (2-tailed, unpaired t test).

of *E2f1* could decrease hyperglycemia in diabetic mouse models, we generated *db/db* mice invalidated for one allele of *E2f1*: *db/db E2f1*^{+/-}. *E2f1* homozygous mutants could not be used in this study because *db/db E2f1*^{-/-} mice cannot produce sufficient insulin due to defective beta cell function [7, 12]. *db/db E2f1*^{+/-} mice developed obesity and became hyperinsulinemic like their *db/db E2f1*^{+/+} littermates (Suppl. Fig 4B,C) but presented *E2f1* levels in the liver comparable to non-diabetic mice (Figure 4B). Remarkably, partial deletion of *E2f1* in the *db/db* background was sufficient to significantly decrease fasting blood glucose levels (Figure 4A). This was associated with an almost complete normalization of *Pck1* mRNA levels and a significant decrease of *G6pc* gene expression (Figure 4B).

Next, in order to assess if the amelioration of hyperglycemia in *db/db* mice lacking one copy of *E2f1* was due to decreased *E2f1* activity in

the liver, we generated liver-specific *E2f1*^{-/-} mice (*E2f1* LKO) and fed them with a high fat diet (HFD). HFD-induced obesity is considered to be a more accurate representation of human type 2 diabetes onset [19]. After 12 weeks on this diet, *E2f1* LKO mice showed no differences in body weight compared to their wild-type littermates (Suppl. Fig. 4D). However, *E2f1* LKO mice presented decreased fasting hyperglycemia (Figure 4C). After sacrifice, liver gene expression was analyzed and *E2f1* LKO mice showed a marked decrease in gluconeogenic gene expression compared to their control littermates (Figure 4D). Similarly to what we had observed in the livers of *E2f1*^{tg/+ Alb-Cre} mice, the changes in hepatic gluconeogenic gene expression were not a consequence of differences in PKA activity (Suppl. Fig 4F).

These results demonstrate that *E2f1* contributes to hyperglycemia during obesity by regulating hepatic gluconeogenesis.

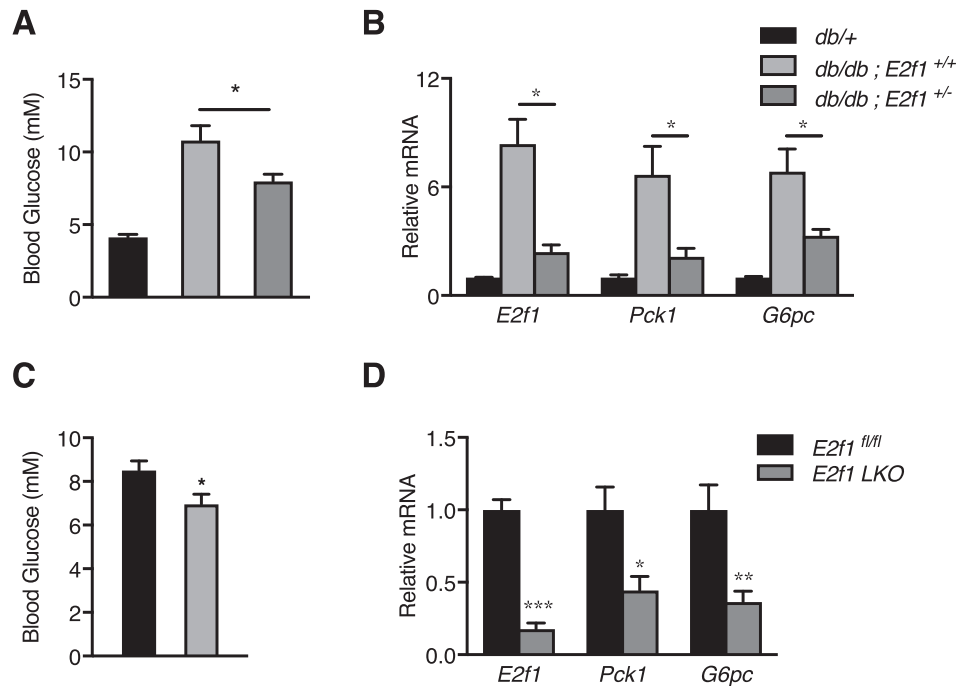


Figure 4: E2F1 deficiency reduces hyperglycemia in diabetic mouse models. (A) Fasting glycemia and (B) relative liver mRNA expression levels of the indicated genes in *db/+*, *E2f1^{+/+}*, *db/db E2f1^{+/+}* and *db/db E2f1^{+/-}* mice (n = 5–10). (C) Fasting glycemia and (D) relative liver mRNA expression levels of the indicated genes in *E2f1* LKO and control mice fed for 12 weeks on high fat diet (n = 6). Data represent the mean \pm SEM. *P < 0.05, **P < 0.01, ***P < 0.005 (2-tailed, unpaired t test).

3.5. E2F1 mRNA expression is increased in the livers of diabetic patients and correlates with PCK1 levels

Next, we sought to examine whether hepatic E2F1 could also contribute to hyperglycemia in humans. For this purpose, we quantified E2F1 mRNA levels in liver biopsies from a cohort of 38 human patients (the metabolic profiles of these patients are presented in Suppl. Table 1). E2F1 mRNA levels were significantly increased in the livers of obese diabetic patients compared to lean patients (Figure 5A). Moreover, analyzing liver gene expression in the whole cohort, we found a significant correlation between E2F1- and PCK1 mRNA levels (Figure 5B).

3.6. Pharmacological inhibition of E2F1 reduces hyperglycemia in diabetic mice

Given that E2F1 contributes to hyperglycemia during insulin resistance (Figure 4), we investigated whether targeting E2F1 activity could be used to treat hyperglycemia in diabetic mice. First, mouse primary hepatocytes were treated overnight with the E2F chemical inhibitor HLM006474. This led to a decrease in glucose production that correlated with impaired expression of gluconeogenic genes (Figure 6 A,B). Next, wild-type mice fed with a HFD during 16 weeks were treated for ten days with the same compound. Strikingly, treatment with the E2F chemical inhibitor decreased hyperglycemia and completely normalized gluconeogenic gene expression in the liver of this diabetic mouse model (Figure 6 C,D). Noteworthy, the treatment did not have an impact on the body weight of the mice or in the circulating levels of lactate dehydrogenase and of the transaminases ALT and AST, which suggested lack of hepatic toxicity (Suppl. Fig.5 A-C).

4. DISCUSSION

We report here that E2F1 is a major component of the transcriptional response to induce hepatic gluconeogenesis and that this transcription

factor plays a critical role in the pathophysiology of type 2 diabetes by actively contributing to hyperglycemia in diabetic mouse models. Importantly, we show that E2F1 gene expression is increased in human liver biopsies from diabetic patients and that it correlates with the levels of PCK1, which is implicated in the development of hyperglycemia in mice [20] and probably also in humans [21]. Furthermore, we identify that this regulation involves the canonical regulators of E2F1, RB1 and CDK4.

The impact of modulating E2F1 activity on gluconeogenesis was only evident in primary hepatocytes in conditions that mimic the liver during fasting or in mice during insulin resistance. This suggests that the PKA pathway somehow promotes E2F1 action during gluconeogenesis. In this regard, we show that glucagon treatment leads to retinoblastoma protein phosphorylation in hepatocytes (Figure 3). These findings are

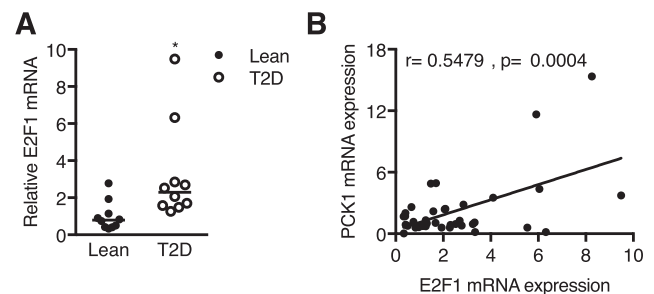


Figure 5: E2F1 expression is increased in diabetic patients and correlates with PCK1 levels. (A) Relative expression of E2F1 mRNA in livers of lean subjects and obese type 2 diabetic patients (n = 10). Data represent the median with individual plots. * P < 0.05 (2-tailed, unpaired t test). (B) Correlation between hepatic E2F1 and PEPCK1 mRNA levels in a cohort of human patients (n = 38). A Pearson correlation analysis was performed.

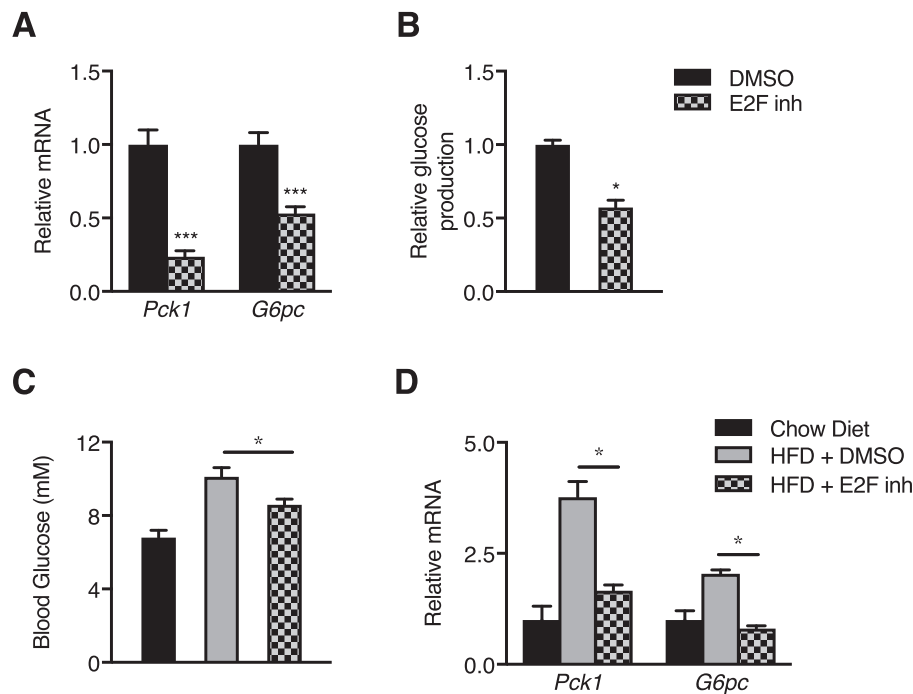


Figure 6: Pharmacological inhibition of E2F1 reduces hyperglycemia in diabetic mice. (A) Relative expression levels of *Pck1* and *G6pc* and (B) glucose production in primary hepatocytes incubated overnight with DMSO or a E2F inhibitor (HLM006474, 25 μ M) followed by forskolin treatment (10 μ M) during 3 hours ($n = 4$). (C) Fasting glycemia and (D) relative liver mRNA expression levels of the indicated genes of mice fed during 16 weeks with a high-fat diet and treated 10 days with HLM006474 (40 mg/kg/day) or vehicle ($n = 5-8$). Data represent the mean \pm SEM. * $P < 0.05$, *** $P < 0.005$ (2-tailed, unpaired t test).

consistent with previous studies in other tissues that have described a PKA-dependent activation of CDK4 in thyroid cells [22] and increased RB1 Ser⁷⁸⁰ phosphorylation in muscle in response to the administration of isoproterenol, a β -3 adrenergic agonist that also promotes PKA activity [8]. Moreover, glucagon has been reported to induce, in a PKA-dependent manner, chromatin epigenetic changes that are required for the full induction of gluconeogenic gene expression [23]. It is also possible that the action of E2F1 on gluconeogenesis depends on the interaction with other transcription factors such as CREB, FOXO1, or PPARGC1A, that are activated by PKA, and typically dysregulated during diabetes [2]. In this regard, it has been shown that E2F1 promotes FOXO1 expression in neurons, which could also be a mechanism of gluconeogenic regulation in the liver [24].

The dual phosphorylation of RB1 in hepatocytes both by insulin and by glucagon seems paradoxical given that in physiological conditions, depending on the nutritional status, only one signal or the other is active. This dual activation might not accurately reflect the physiological situation *in vivo*. We could presume that basal E2F1 activity is regulated during the feeding-fasting cycle by other additional mechanisms than the CDK4-RB axis.

Recently, Lee et al. reported that under normal conditions, in the fed state, CDK4 phosphorylates and activates the acetyltransferase GCN5, which, in turn, results in the inhibition of PPARGC1A activity and the decreased expression of its gluconeogenic target genes [25]. This is at odds with our observations in *Cdk4*^{-/-} hepatocytes (Figure 3E,F and Suppl. Fig. 3 C-D). Nevertheless, both Lee et al. and our group reported the hyperphosphorylation of the retinoblastoma protein in the livers of insulin-resistant mice [12,25]. In addition, hepatic E2F1 expression is increased during diabetes in both mice and humans (Figure 4A,

Suppl. Fig. 4A, and Figure 5A). This indicates that in the diabetic state there is a pathological hyperactivation of the CDK4-RB1-E2F1 pathway in the liver that contributes to hyperglycemia. This conclusion is supported by the fact that specifically depleting E2F1 in the liver reduces gluconeogenesis and decreases hyperglycemia in a diabetic mouse model (Figure 4 C,D). One possible explanation for the increased hepatic E2F1 activity during diabetes is the increased chronic inflammation and reticulum stress observed during this pathological state [26]. Interestingly, increased E2F1 expression and retinoblastoma protein hyperphosphorylation has also been observed in the white adipose tissue from obese rodents and humans [27–29]. This suggests that E2F1 activity may also contribute to the pathophysiology of insulin resistance in other tissues than the liver.

In conclusion, the results presented here, along with our previous finding that E2F1 contributes to hepatic steatosis, demonstrate that this transcription factor critically contributes both to hyperlipidemia and hyperglycemia during insulin resistance. This suggests that an interesting therapeutic window exists to simultaneously target these two key metabolic pathways that are abnormally increased in type 2 diabetes.

AUTHOR CONTRIBUTIONS

AG, PDD, and LF conceptualized the study and designed the experiments. AG, PDD, ICLM, BD, EB, and JSA performed the experiments. CB and FP performed the human study. AG, PDD, and LF wrote the manuscript. LF is the guarantor of the study and has full access to 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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CONFLICT OF INTEREST

The authors of this study have no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.molmet.2018.02.011>.

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