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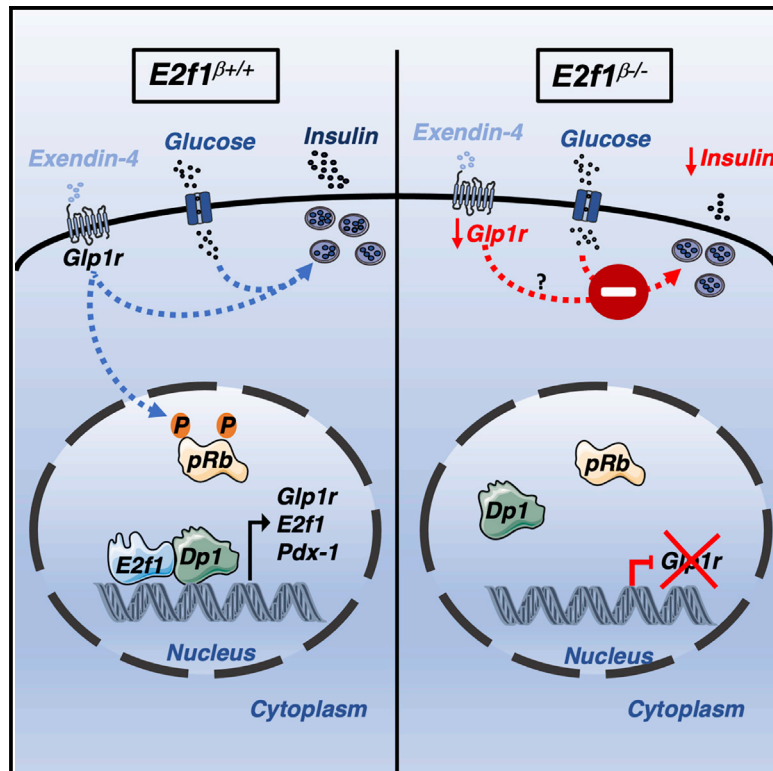
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The transcription factor E2F1 controls the GLP-1 receptor pathway in pancreatic β cells

Graphical abstract



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In brief

The glucagon-like peptide-1 (GLP-1) is an enterohormone that binds the GLP-1 receptor (GLP1R) and modulates insulin secretion. Bourrouh et al. show that β cell-specific genetic ablation of *E2f1* impairs glucose homeostasis associated with decreased *GLP1R* expression, highlighting molecular crosstalk between E2f1 and Glp-1 signaling pathways in pancreatic islets.

Highlights

- Deletion of *E2f1* in β cells impairs glucose homeostasis and insulin secretion
- E2F1 regulates Glp-1 receptor gene expression in β cells
- The GLP-1R agonist exendin-4 increases E2F1/pRB transcriptional activity
- Crosstalk between E2F1 and GLP1R signaling pathways modulates β cell function



Report

The transcription factor E2F1 controls the GLP-1 receptor pathway in pancreatic β cells

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SUMMARY

The glucagon-like peptide 1 (Glp-1) has emerged as a hormone with broad pharmacological potential in type 2 diabetes (T2D) treatment, notably by improving β cell functions. The cell-cycle regulator and transcription factor E2f1 is involved in glucose homeostasis by modulating β cell mass and function. Here, we report that β cell-specific genetic ablation of *E2f1* (*E2f1*^{B-/-}) impairs glucose homeostasis associated with decreased expression of the Glp-1 receptor (*Glp1r*) in *E2f1*^{B-/-} pancreatic islets. Pharmacological inhibition of E2F1 transcriptional activity in nondiabetic human islets decreases *GLP1R* levels and blunts the incretin effect of GLP1R agonist exendin-4 (ex-4) on insulin secretion. Overexpressing *E2f1* in pancreatic β cells increases *Glp1r* expression associated with enhanced insulin secretion mediated by ex-4. Interestingly, ex-4 induces retinoblastoma protein (pRb) phosphorylation and E2f1 transcriptional activity. Our findings reveal critical roles for E2f1 in β cell function and suggest molecular crosstalk between the E2F1/pRb and GLP1R signaling pathways.

INTRODUCTION

Glucose homeostasis is finely tuned by the coordinated action of the glucagon and insulin that are produced by pancreatic α and β cells, respectively. Dysregulation of this homeostatic process leads to type 2 diabetes (T2D), which is characterized by impaired function and mass of pancreatic β cells (Weir and Bonner-Weir, 2004). Since lifestyle modifications are usually ineffective to cure T2D, pharmacological treatments are necessary to restore normoglycemia. Targeting the glucagon-like peptide-1 (Glp-1) pathway has demonstrated promising efficacy to treat T2D (Drucker, 2018). Glp-1 is an enterohormone produced by intestinal L cells (Holst et al., 1987) and pancreatic α cells (Chambers et al., 2017) that bind the Glp-1 receptor (GLP1R), a G protein-coupled receptor (GPCR) expressed by several cell types, including pancreatic β cells (Thorens, 1992). The activation of this GPCR by natural Glp-1 or synthetic Glp-1 analogs, such as exendin-4 (ex-4) or liraglutide (Nauck et al., 2021) favors glucose-stimulated insulin secretion (GSIS) by pancreatic β cells (Holz et al., 1993; Kreymann et al., 1987), inhibits glucagon secretion by pancreatic α cells (de Heer et al., 2008), but also in-

creases β cell proliferation (Cornu et al., 2010; Friedrichsen et al., 2006; Liu and Habener, 2008), and prevents pancreatic β cells from apoptosis (Buteau et al., 2004; Li et al., 2003b; Quoyer et al., 2010).

The E2F1-pRb-CDK4 pathway plays a major role in controlling β cell mass and function (Annicotte et al., 2009; Fajas et al., 2004; Iglesias et al., 2004; Kim and Rane, 2011; Li et al., 2003a). The E2F1 transcriptional activity is finely regulated through its association with the dimerization partner-1 (DP-1) protein, members of the retinoblastoma protein family such as pRb (Mendoza and Grossniklaus, 2015), and cyclin and cyclin-dependent kinase (CDK) complexes, such as CDK4/cyclinD1, that phosphorylate pRb (Kato et al., 1993). This leads to pRb release from E2F1, promoting E2F1 transcriptional activity, cell-cycle progression, and CDK inhibitors, namely p16^{INK4A}, that bind to CDK and avoid CDK4/cyclin D complex formation, and subsequently repress pRb phosphorylation and E2F1 transcriptional activity (Schulze et al., 1994). Although these mechanisms mainly control the progression of the cell cycle (Poppy Roworth et al., 2015), recent studies have reported important roles for the E2F1-pRb-CDK4 pathway beyond the sole regulation of cell



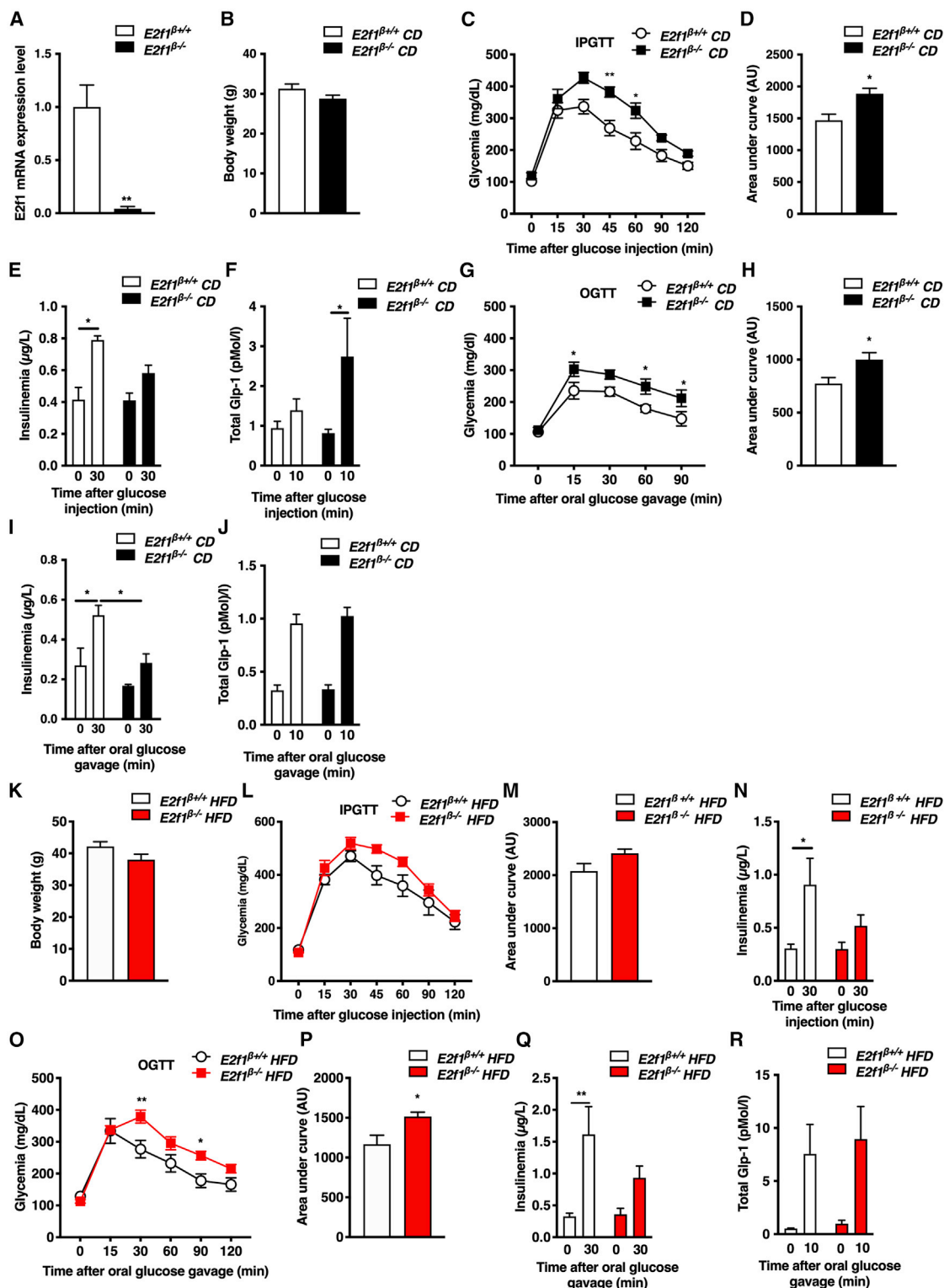


Figure 1. β cell-specific loss of $E2f1$ expression impairs glucose tolerance

(A) $E2f1$ mRNA expression levels from $E2f1^{\beta+/+}$ and $E2f1^{\beta-/-}$ pancreatic islets ($n = 4$).

(B) Body weight of 16-week-old $E2f1^{\beta+/+}$ and $E2f1^{\beta-/-}$ mice fed with chow diet (CD, $n = 12$ –15).

(C and D) IPGTT in $E2f1^{\beta+/+}$ and $E2f1^{\beta-/-}$ mice fed with CD ($n = 5$ –7) and the corresponding area under curve (AUC, D).

(E) Insulin plasma levels during IPGTT measured 0 and 15 min after glucose injection in CD-fed $E2f1^{\beta+/+}$ and $E2f1^{\beta-/-}$ mice ($n = 5$ –7).

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proliferation (Denechaud et al., 2017; Kahoul et al., 2020). Indeed, several reports have demonstrated the role of the E2F1 pathway in the control of metabolic functions in non-proliferative cells, including adipocytes (Chen et al., 2020; Fajas et al., 2002), hepatocytes (Denechaud et al., 2016; Giralt et al., 2018; Lai et al., 2017), muscle and brown adipose tissue (Blanchet et al., 2011, 2012), and pancreatic β cells (Annicotte et al., 2009; Fajas et al., 2004; Grouwels et al., 2010; Kim and Rane, 2011). Interestingly, germline-deficient *E2f1* mice are resistant to diet-induced obesity (Fajas et al., 2002) and have enhanced energy metabolism in muscle (Blanchet et al., 2011), as observed in global *Glp1r* knockout (KO) mice (Ayala et al., 2010).

While E2F1 and Glp-1 pathways are both involved in the control of β cell function and mass, whether they were physiologically connected remains unknown. Here, we demonstrate a molecular crosstalk between the GLP1R and E2F1/pRb pathways in pancreatic β cells. We show that E2F1 modulates the Glp-1 signaling pathway through the transcriptional control of *Glp1r* expression by pancreatic β cells. Importantly, we found that a treatment of human islets with the pan E2F inhibitor HLM006474 decreases *E2F1* expression, as observed in T2D human islets (Lupi et al., 2008), as well as *GLP1R* expression.

RESULTS

Loss of β cell-specific *E2f1* expression impairs glucose tolerance under a chow diet and during metabolic stress

To evaluate the role of *E2f1* in the control of glucose homeostasis, we generated pancreatic β cell-specific *E2f1*-deficient mice, called hereafter *E2f1* ^{β -/-}. The recombination efficiency at the *E2f1* locus was confirmed in pancreatic islets isolated from *E2f1* ^{β -/-} mice compared to *E2f1* ^{β +/+} control littermates (Figure 1A). *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice displayed comparable body weight (Figure 1B), fasting glycemia, pancreatic islet morphology, islet number, islet density, the percentage of endocrine cells per pancreatic section and insulin-positive cells per islet, and the insulin content (Figures S1A–S1G, respectively). Intraperitoneal glucose tolerance tests (IPGTTs) in 3-month-old *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice fed with chow diet (CD) revealed that glucose clearance was impaired in *E2f1* ^{β -/-} mice (Figures 1C and 1D), associated with defects in secreting insulin in response to glucose (Figure 1E). Plasma Glp-1 levels were not affected in control mice before and 10 min after the intraperitoneal (i.p.) injection of glucose (Figure 1F). Intriguingly, circulating Glp-1 levels were increased by the deletion of *E2f1* in β cells after glucose injection (Figure 1F). Similarly, 4-month-old *E2f1* ^{β -/-} mice demonstrated impaired glucose tolerance when chal-

lenged with an oral bolus of glucose compared to age-matched *E2f1* ^{β +/+} mice (Figures 1G and 1H), associated with a significant alteration of insulin secretion only upon glucose stimulation but not in fasting conditions (Figure 1I). Strikingly, although insulin levels were dampened in *E2f1* ^{β -/-} mice upon glucose stimulation, glucose-induced plasma Glp-1 levels during an oral GTT (OGTT) were not affected by the deletion of *E2f1* in β cells (Figure 1J).

Metabolic stress, such as high-fat diet (HFD) feeding, profoundly affects β cell function and mass. Using public datasets, we monitored *E2f1* transcript levels in pancreatic islets from mice fed CD or HFD (Gao et al., 2021). *E2f1* transcripts were significantly increased in pancreatic islets isolated from mice after 12 weeks of HFD compared to age-matched CD-fed controls (Figure S1H). We next evaluated whether such metabolic dyshomeostasis promoted by β cell-selective *E2f1* knockdown could be exacerbated upon an HFD. Accordingly, we challenged *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice with an HFD for 16 weeks. Despite body weight (Figures 1K and S1I), glucose tolerance during IPGTT (Figures 1L and 1M), and insulin sensitivity (Figure S1J) being similar between both genotypes, insulin secretion was impaired in *E2f1* ^{β -/-} mice as compared to controls fed an HFD (Figure 1N). Again, HFD-fed *E2f1* ^{β -/-} mice demonstrated impaired glucose tolerance when challenged with an oral bolus of glucose compared to *E2f1* ^{β +/+} mice (Figures 1O and 1P). Although insulinemia was similar between controls and *E2f1* ^{β -/-} mice in fasting conditions, insulin secretion was dampened 30 min after oral glucose intake in *E2f1*-deficient mice (Figure 1Q), whereas circulating Glp-1 levels before and 10 min after an oral glucose bolus were not significantly different between control and *E2f1* ^{β -/-} mice either when fed an HFD (Figure 1R). Our data demonstrate that the loss of *E2f1* expression within β cells impairs glucose homeostasis and insulin secretion upon physiologic conditions and during metabolic stress.

Overexpression of human E2F1 in murine β cells has a limited impact on glucose homeostasis and insulin secretion

We next investigated whether *E2F1* overexpression in pancreatic β cells of wild-type animals could mirror the phenotype observed in *E2f1* ^{β -/-} (i.e., increased insulin secretion in response to glucose). Therefore, we generated a mouse model that overexpresses the human form of *E2F1* (*hE2F1*) specifically in β cells (*E2f1* ^{β over}). *hE2F1* overexpression was confirmed at the mRNA levels (Figure 2A). The β cell-specific expression of *hE2F1* proteins was confirmed using co-immunostaining with anti-insulin and anti-hemagglutinin (HA) antibodies, an HA tag being fused

(F) Blood Glp-1 levels before and 10 min after an i.p. glucose injection (n = 5).

(G and H) OGTT in *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice fed with CD (G) (n = 5–7) and the corresponding AUC (H).

(I) Insulin plasma levels during OGTT measured 0 and 30 min after glucose gavage in CD-fed *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice (n = 5–7).

(J) Blood Glp-1 levels before and 10 min after glucose gavage of *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice (n = 5).

(K) Body weight of *E2f1* ^{β -/-} and *E2f1* ^{β +/+} mice fed an HFD for 16 weeks (n = 7).

(L and M) IPGTT in *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice fed with HFD (L) (n = 7) and the corresponding AUC (M).

(N) Insulin plasma levels during IPGTT in HFD-fed *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice (n = 7).

(O and P) OGTT (O, n = 7) and its respective AUC (P, n = 7).

(Q) Insulin plasma levels during OGTT in HFD-fed *E2f1* ^{β -/-} and *E2f1* ^{β +/+} mice (n = 6).

(R) Total Glp-1 levels before and 10 min after an oral bolus of glucose in *E2f1* ^{β +/+} and *E2f1* ^{β -/-} mice after 20 weeks of HFD (n = 7). All of the values are expressed as means \pm SEMs. *p < 0.05, **p < 0.01.

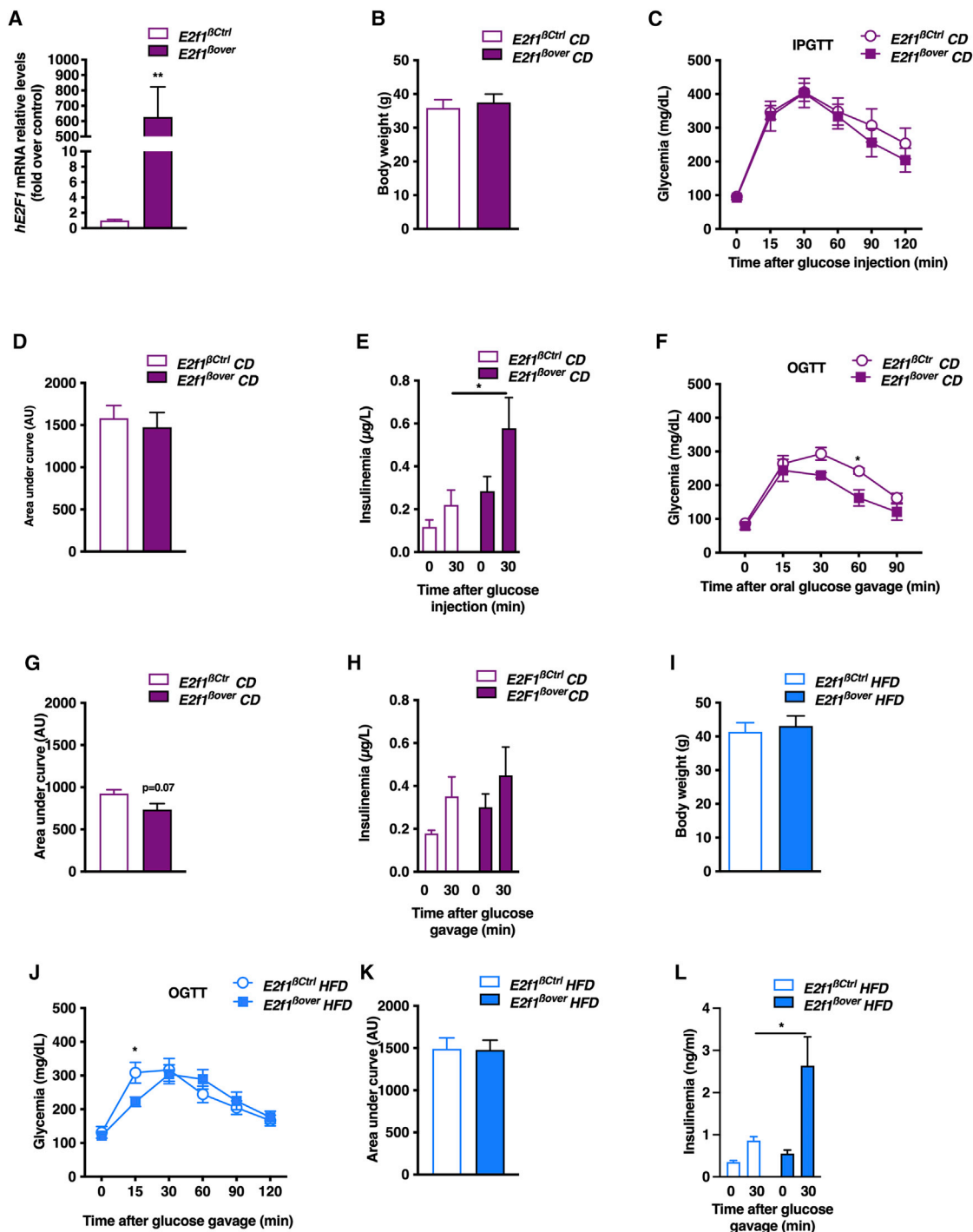


Figure 2. The overexpression of E2F1 in pancreatic β cells slightly increases insulin secretion but does not improve glucose homeostasis

(A) mRNA expression of hE2F1 in $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ pancreatic islets (n = 6).
 (B) Body weight of CD-fed $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ mice (n = 10).
 (C) IPGTT in $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ mice (n = 11–15).
 (D) AUC of IPGTT from $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ mice.
 (E) Insulin plasma levels during IPGTT before (0 min) and 30 min following glucose injection (n = 11–15).
 (F) Blood glucose levels during OGTT of $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ mice under CD (n = 5).
 (G) AUC of OGTT from $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ mice (n = 5).
 (H) Insulin plasma levels during OGTT measured 0 and 30 min after glucose gavage in $E2f1^{\beta ctrl}$ and $E2f1^{\beta over}$ mice under CD (n = 5).

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with the *hE2F1* transgene (Scheijen et al., 2003) (Figure S2A). Overexpressing human E2F1 did not affect mouse *E2f1* transcript levels (Figure S2B). No particular phenotype was observed in these mice when fed CD, including islet morphology (Figures S2C and S2D), insulin content (Figure S2E), body weight (Figure 2B), fasting glycemia (Figure S2F), insulin sensitivity (Figure S2G), and i.p. glucose tolerance (Figures 2C and 2D). Intriguingly, the insulin levels were increased in *E2f1^{βover}* mice in response to *in vivo* glucose injection when compared to littermate controls (*E2f1^{βctrl}*, Figure 2E). Finally, *E2f1^{βover}* mice demonstrated a weak but not significant ($p = 0.07$) improvement in glucose tolerance when challenged with an oral bolus of glucose (Figures 2F and 2G), associated with comparable insulin levels in basal conditions and after glucose gavage in controls and *E2f1^{βover}* mice (Figure 2H).

We next sought to evaluate the effect of *hE2F1* overexpression in pancreatic β cells on glucose homeostasis when challenged with HFD for 16 weeks. The body weight and the weight gain of *E2f1^{βover}* mice remained comparable to control mice (Figures 2I and S2H, respectively). The overexpression of *hE2F1* in pancreatic β cells did not alter the insulin sensitivity (Figure S2I) or the glucose tolerance after an i.p. injection of glucose (Figures S2J and S2K). OGTT revealed similar glucose tolerance in *E2f1^{βover}* mice after glucose gavage (Figures 2J and 2K). Interestingly, *E2f1^{βover}* mice secreted more insulin 30 min after an oral glucose load compared to control mice (Figure 2L). Our data demonstrate that *E2f1^{βover}* mice exhibit limited improvement of glucose homeostasis and insulin secretion upon physiologic conditions and during metabolic stress.

GSIS potentiation by the Glp-1 agonist exendin-4 is impaired in the absence of E2f1

To evaluate whether Glp-1 agonists modulate E2f1-dependent insulin secretion in a cell-autonomous manner, we performed static GSIS experiments in the *E2f1* knockdown mouse Min6 β cell line and their controls. Strikingly, a 55% decrease in *E2f1* mRNA levels was sufficient to blunt GSIS (Figures 3A and 3B). While ex-4 potentiated glucose effect on insulin secretion in control Min6 cells, that was not the case following *E2f1* knockdown (Figure 3B). To corroborate these findings, we performed a similar GSIS experiment using pancreatic islets isolated from *E2f1^{β+/+}* and *E2f1^{β-/-}* mice. Whereas *E2f1^{β+/+}* pancreatic islets responded well to glucose, with an outcome potentiated in the presence of ex-4, *E2f1^{β-/-}* pancreatic islets failed to exhibit both enhanced response to glucose and potentiation by ex-4 (Figure 3C). The treatment of non-diabetic human islets with the E2F pan inhibitor HLM006474 (Rosales-Hurtado et al., 2019) provoked a marked decrease in insulin secretion in response to ex-4 treatment, compared to DMSO-treated human islets (Figure 3D). Interestingly, GSIS assays on pancreatic islets isolated from control and *E2f1^{βover}* mice showed that the overexpression of *E2F1* increased the potentiation impact of ex-4 on

GSIS (Figure 3E). To further demonstrate a specific loss of ex-4 capacity to potentiate insulin secretion in the absence of *E2f1*, we treated pancreatic islets with forskolin to evaluate whether activating the glp-1 pathway downstream of its receptor is functional in *E2f1^{β+/+}* and *E2f1^{β-/-}* pancreatic islets. We observed that treating pancreatic islets with forskolin potentiated more efficiently GSIS in *E2f1^{β-/-}* pancreatic islets than in controls (Figure 3F). These data suggest that E2f1, both in mouse and human pancreatic islets, affects ex-4 effects on GSIS, a defect that can be rescued by forskolin treatment.

E2f1 controls Glp1r expression in pancreatic β cells

As *E2f1* deficiency did not affect Glp-1 circulating levels during OGTT (Figures 1J and 1R) but inhibits insulin secretion in response to glucose stimulation and incretins, we hypothesized that E2f1 modulates Glp-1 signaling in β cells rather than Glp-1 production. Since Glp-1 agonists modulate insulin secretion through the Glp1r (Muller et al., 2019), we analyzed *Glp1r* expression in our different mouse and human models of *E2f1* modulation. Knocking down *E2f1* in Min6 cells resulted in a significant decrease in *Glp1r* mRNA levels (approximately 65% of decrease, $p = 0.0022$; Figure 3G). We confirmed these results in *E2f1^{β-/-}* pancreatic islets (Figure 3H) and human islets treated with the E2F inhibitor HLM006474 (Figure 3I). Interestingly, this E2F inhibitor decreased not only *GLP1R* expression in islets from nondiabetic humans but also *E2F1* mRNA levels (Figure 3I). Notably, we observed a mirrored rise in *Glp1r* mRNA levels in *E2f1^{βover}* isolated islets (Figure 3J). *In silico* analysis revealed the presence of E2f1 binding sites in the promoter region of the *Glp1r* gene (Figures 3K, S3A, and S3B). To further demonstrate the ability of the E2f1-dependent pathway to control *Glp1r* expression, we performed co-transfection experiments in Min6 cells using a plasmid construct combining the mouse *Glp1r* promoter DNA sequence and the luciferase reporter gene. To activate or repress E2F1 pathways, we used expression vectors encoding E2F1 and its dimerization partner DP-1, as well as pRb, the bona fide repressor of E2F1 (Mendoza and Grossniklaus, 2015). Our data demonstrate that the activation of the E2F pathway increased *Glp1r*-dependent luciferase activity, while pRb repressed the increased luciferase activity mediated by E2F1-DP-1 (Figure 3L). Finally, we performed chromatin immunoprecipitation (ChIP)-qPCR experiments to determine whether E2F1 binds directly to the *Glp1r* promoter at the chromatin level. In the absence of reliable antibodies to ChIP E2F1, Min6 cells were transfected with plasmid containing the cytomegalovirus promoter (pCMV) or pCMV-hE2F1-FLAG to immunoprecipitate E2F1 using a FLAG antibody. Enrichment at the *Glp1r* promoter was observed when cells were transfected with pCMV-hE2F1-FLAG, demonstrating that E2F1 directly binds to the *Glp1r* promoter (Figure 3M). Our data highlight that E2F1 modulates *Glp1r* expression both in mouse and human islets, likely through the control of its promoter region. Strikingly, when E2F1-DP-1

(I) Body weight of *E2f1^{βctrl}* and *E2f1^{βover}* mice after 16 weeks of HFD ($n = 5$).

(J) OGTT in *E2f1^{βctrl}* and *E2f1^{βover}* mice after 16 weeks of HFD ($n = 5$).

(K) AUC of OGTT from *E2f1^{βctrl}* and *E2f1^{βover}* mice ($n = 5$).

(L) Insulin plasma levels during OGTT.

All of the values are expressed as means \pm SEMs. * $p < 0.05$, ** $p < 0.01$.

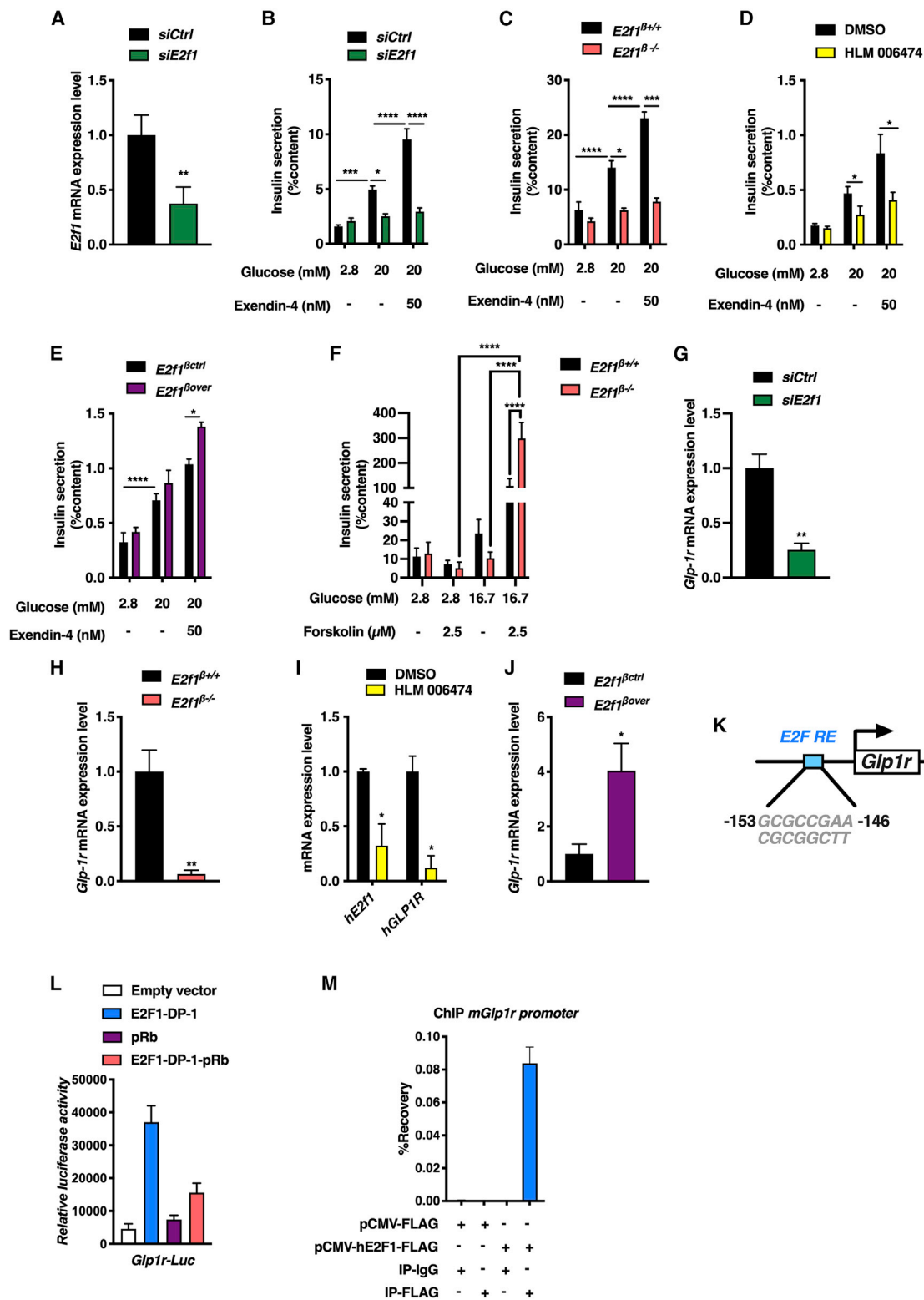


Figure 3. *E2f1* modulates Glp-1-mediated insulin secretion associated to *Glp1r* expression

(A) *E2f1* mRNA expression levels from control (siCtrl) or *E2f1* silencing (si*E2f1*) in Min6 cells (n = 5).

(B) GSIS measured in siCtrl or si*E2f1* Min6 cells treated as indicated (n = 7).

(C) GSIS in *E2f1*^{β+/+} and *E2f1*^{β-/-} pancreatic islets (n = 3–9).

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are co-transfected with pRb, the activity of the *Glp1r* promoter is dampened (Figure 3L), showing that the activity of the E2F1-DP-1 complex on the *Glp1r* promoter is regulated by pRb.

Glp-1 increases the phosphorylation of pRb through the activation of the CDK signaling pathway in mouse pancreatic islets

Since the molecular mechanisms of serine and threonine kinase activation controlled by the Glp-1 pathway are not fully understood, a kinome analysis using the Pamgene technology was performed on pancreatic islets isolated from C57Bl6/J mice treated with 20 mM glucose compared to 20 mM glucose plus 50 nM ex-4 for 30 min (Figure 4A). Following these treatments, an increase in 43 phosphorylation sites of 41 peptides was observed when pancreatic islets were treated with glucose-ex-4 compared to glucose alone (Figure 4B; Table S3). It is noteworthy that treatment of mouse islets with 20 mM glucose plus 50 nM ex-4 increased Creb phosphorylation at serine 133 (Creb^{S133}, Figure 4B, log2 fold change = 0.45, p = 0.003), as previously shown (Jhala et al., 2003). Strikingly, the co-treatment of mouse pancreatic islets with 20 mM glucose plus 50 nM ex-4 for 30 min increased the phosphorylation level of the pRb on serine 807/811 (pRb^{S807/811}; log2 fold change = 0.63, p = 0.043), the bona fide repressor of E2F1 transcriptional activity. The ex-4-mediated induction of pRb^{S807/811} phosphorylation was further confirmed in Min6 β cells (Figures S4A–S4C). Integrating the phosphorylation data using Bionavigator software revealed the specific activation of kinases upon ex-4 treatment, including several CDKs (Figure 4C). Finally, Ingenuity Pathway Analysis further demonstrated that several canonical pathways were controlled by ex-4 treatment, such as the opioid, AMP kinase (AMPK) or protein kinase A (PKA) signaling pathways (Figure 4D). These results demonstrate that the activation of the Glp-1 pathway by its agonist ex-4 in mouse pancreatic islets activates several kinases, including CDKs, leading to the phosphorylation of pRb.

Ex-4 increases E2F1 transcriptional activity

These data led us to postulate that GLP1R activation through an ex-4 treatment could increase E2F1 transcriptional activity, since the *E2f1* target gene expression is strongly controlled by the phosphorylation status of pRb (Mendoza and Grossniklaus, 2015). To test this hypothesis, we performed luciferase assays using a plasmid containing E2F-responsive elements (E2F-RE) cloned in front of the thymidine kinase (Tk) promoter and the luciferase gene (E2F-RE-Tk-Luc). Upon treatment with 2.8 mM glucose,

co-transfection with the E2F1 and DP-1 expression vectors induced luciferase activity compared to control conditions, validating increased E2F1 transcriptional activity in the presence of E2F1 and DP-1 (Figure 5A). The treatment of Min6 cells with 20 mM glucose further increased E2f1 transcriptional activity when compared to 2.8 mM glucose treatment (Figure 5A). Interestingly, this effect was even more potentiated with a 50-nM ex-4 treatment (Figure 5A), suggesting that ex-4 enhanced the E2f1 transcriptional activity on the promoter of its target genes. However, the co-transfection with a pRb expression vector nearly abolished E2f1 transcriptional activity (Figure 5A), thus demonstrating, as expected, that pRb inhibits E2f1 transcriptional activity. However, when Min6 cells were treated with 50 nM of ex-4, the pRb repressive effect was partially alleviated, thus allowing a weak transcriptional activity of the E2F1-DP1 heterodimer on the E2F-RE-Tk promoter (Figure 5A). These data suggest the loss of the pRb repressive activity following the treatment with the GLP1R agonist (Figure 5A). Since ex-4 increased E2f1 transcriptional activity, we next wondered whether the expression of genes involved in β cell mass and/or function was modulated upon ex-4 treatment. Since PDX-1, a master regulator of β cell identity that controls *Insulin* gene expression (Fujitani, 2017), is regulated by ex-4 treatment (Kodama et al., 2005; Wang et al., 2001), we checked whether short-term treatment with 50 nM of ex-4 (1–4 h) modulated its expression in Min6 cells or mouse pancreatic islets in which *E2f1* expression level is downregulated. Our results showed that treatment with 50 nM of ex-4 increased the mRNA levels of *E2f1* (Figure 5B) and *Pdx1* (Figure 5C) in Min6 cells transfected with a non-targeting, control small interfering RNA (siRNA). Knocking down *E2f1* in Min6 cells completely blocked the ex-4 mediated increase in *E2f1* (Figure 5B) and *Pdx-1* (Figure 5C) mRNA levels. These results were also observed when C57Bl6J pancreatic islets were treated with glucose and ex-4 (Figures 5D and 5E). Interestingly, treating *E2f1* ^{β -/-} pancreatic islets with ex-4 failed to increase *E2f1*, confirming the reproducibility and robustness of our model, but most interestingly failed to increase *Pdx1* expression levels (Figures 5F and 5G, respectively). Altogether, these results suggest that ex-4 modulates pRb phosphorylation and E2F1 transcriptional activity, which in turn may modulate insulin secretion through the upregulation of E2F1-dependent genes such as *E2f1* itself or *Pdx1* mRNA levels.

DISCUSSION

Following previous studies demonstrating that the E2F1-pRb pathway controls pancreatic β cell mass (Cai et al., 2013,

(D) GSIS experiments on human isolated islets treated for 48 h with 0.1% DMSO or 10 μ M HLM006474 (n = 3).

(E) GSIS in *E2f1* ^{β ctrl} and *E2f1* ^{β over} isolated islets (n = 3–4).

(F) GSIS in *E2f1* ^{β +/+} and *E2f1* ^{β -/-} pancreatic islets exposed to 2.8 mM glucose, 16.7 mM glucose, treated or not with 2.5 μ M forskolin (n = 8–10).

(G) *Glp1r* mRNA expression levels in *siCtrl* or *siE2f1* Min6 cells (n = 6).

(H) *Glp1r* mRNA expression levels in *E2f1* ^{β +/+} and *E2f1* ^{β -/-} isolated islets (n = 4).

(I) E2F1 and GLP1R mRNA expression levels in human islets treated for 48 h with 0.1% DMSO or 10 μ M HLM006474 (n = 3).

(J) *Glp1r* mRNA expression levels in *E2f1* ^{β ctrl} and *E2f1* ^{β over} isolated islets (n = 4–5).

(K) Sequence of the E2F1 responsive element (E2F RE) in the mouse *Glp1r* promoter region.

(L) Transient transfection experiment in Min6 cells with a mGlp1r-promoter-luciferase construct (Glp1r-Luc vector) and pcDNA3 vector (empty vector, negative control), E2F1-DP-1, pRb, E2F1-DP-1-pRb.

(M) ChIP-qPCR demonstrating E2F1 binding to the mouse *Glp1r* promoter from Min6 cells transfected with pCMV or pCMV-hE2F1-FLAG plasmids (n = 2).

All of the values are expressed as means \pm SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

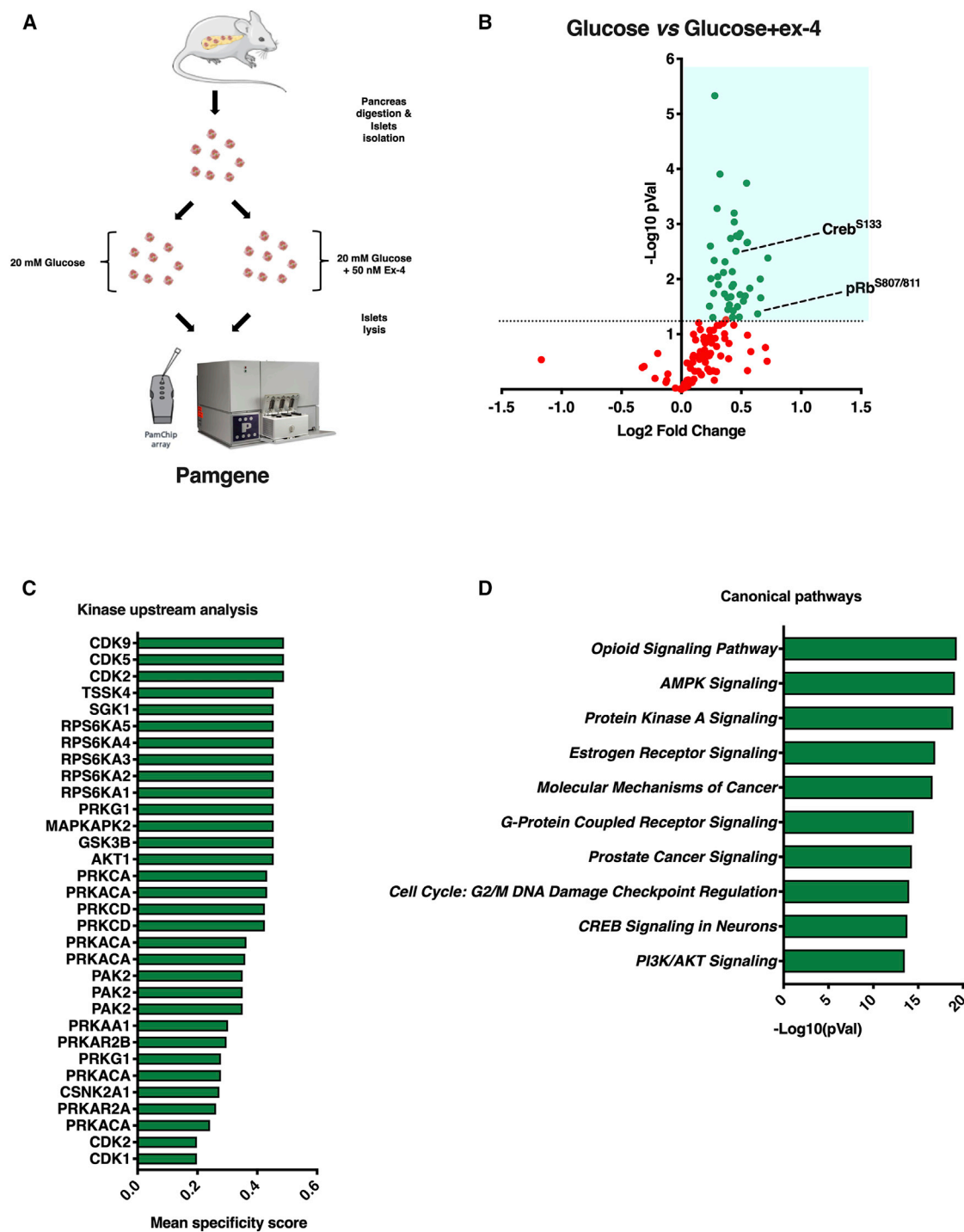


Figure 4. Short-term Glp1r activation induces pRb phosphorylation in pancreatic islets

(A) Schematic representation of the kinome profiling strategy following ex-4 treatment of mouse pancreatic islets.

(B) Volcano plot exhibiting differential peptide phosphorylation in mouse pancreatic isolated islets treated with 20 mM glucose (n = 3) or 20 mM glucose + 50 nM ex-4 (n = 3) for 30 min.

(C) Analysis of upstream kinases involved in differential peptide phosphorylation.

(D) Canonical pathways controlled by ex-4 treatment.

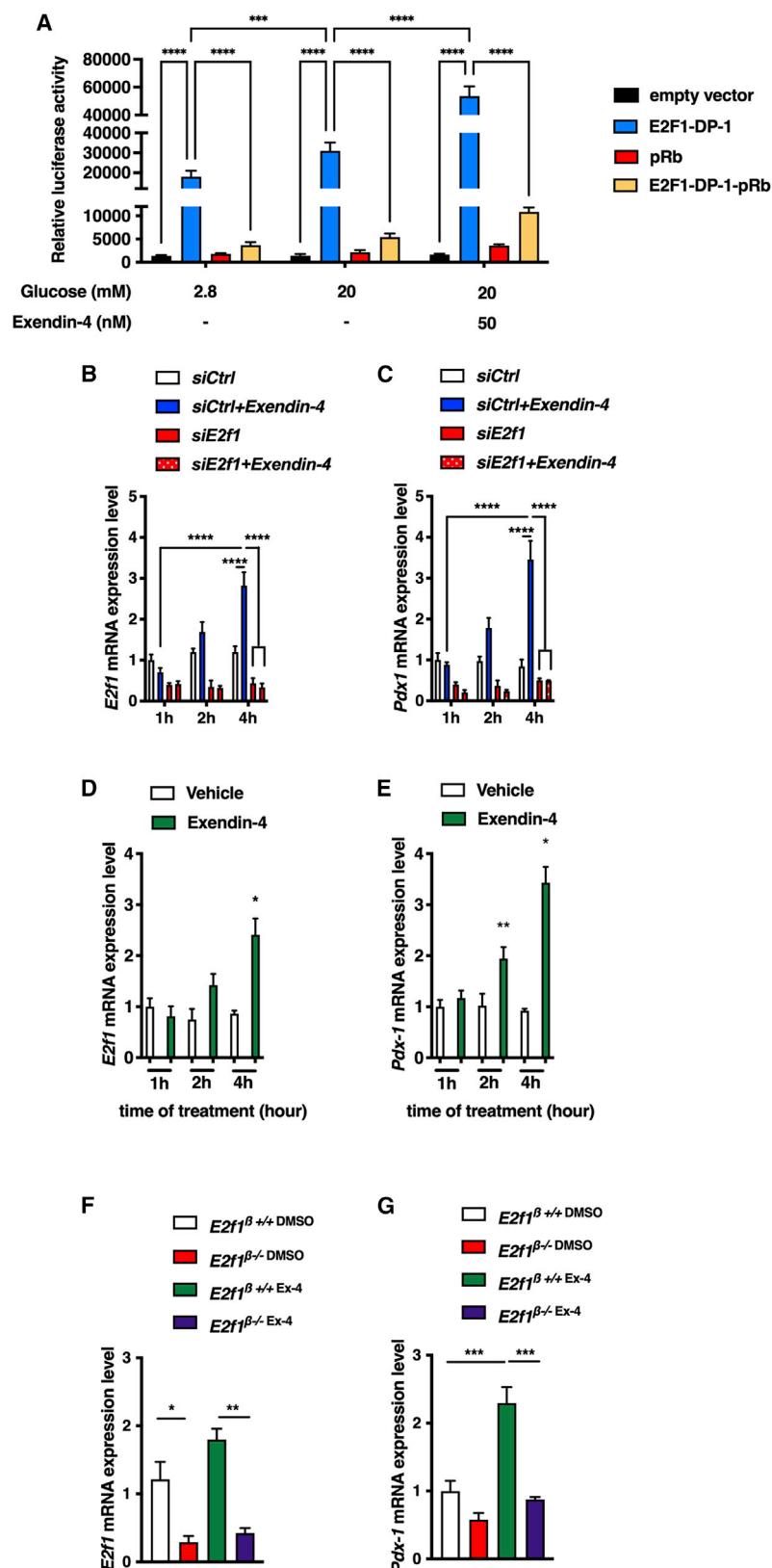


Figure 5. Ex-4 modulates E2f1 transcriptional activity

(A) Min6 cells transiently co-transfected with an E2F-RE-Tk-luciferase construct (E2F-RE-Tk-Luc vector), an empty pcDNA3 vector (negative control), or E2F1-DP-1, or pRb or E2F1-DP-1-pRb, and treated for 30 min with 2.8 mM glucose, 20 mM glucose, or 20 mM glucose + 50 nM ex-4 (n = 3).

(B) E2f1 mRNA expression from siCtrl or siE2f1 Min6 cells treated with 20 mM glucose or 20 mM glucose and 50 nM ex-4 (ex-4) during 1, 2, or 4 h (n = 3).

(C) Pdx1 mRNA expression from siCtrl or siE2f1-Min6 cells treated as in (B) (n = 3).

(D) E2f1 mRNA expression from 12-week-old C57Bl6J pancreatic islets treated with 20 mM glucose (vehicle) or 20 mM glucose and 50 nM ex-4 (ex-4) (n = 3).

(E) Pdx1 mRNA expression from pancreatic islets as in (D) (n = 3).

(F) E2f1 mRNA expression in E2f1 $\beta^{-/-}$ and E2f1 $\beta^{+/+}$ pancreatic islets treated for 4 h with 20 mM glucose (DMSO) or 20 mM glucose and 50 nM ex-4 (ex-4) (n = 3–4).

(G) mRNA expression of Pdx1 in E2f1 $\beta^{-/-}$ and E2f1 $\beta^{+/+}$ pancreatic islets treated as in (F) (n = 3–4).

All of the values are expressed as means \pm SEMs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

2014; Fajas et al., 2004; Grouwels et al., 2010) and function (Annicotte et al., 2009; Boni-Schnetzler et al., 2018), we identify here a molecular link between this transcription factor and the drug-gable Glp-1 signaling.

In the last 2 decades, a particular emphasis has been placed on the development of specific GLP1R agonists to treat T2D (Hinnen, 2017). Indeed, GLP1R agonists improve glycemia in patients with T2D by increasing insulin synthesis and secretion and by inducing β cell proliferation (Baggio and Drucker, 2007; Campbell and Drucker, 2013). Although targeting the GLP1R signaling pathway is a promising therapeutic strategy, it has been reported that the incretin effects decrease in humans with the onset of obesity and T2D, thus urging us to uncover the underlying mechanisms involved in the loss of GLP1R agonist sensitivity in patients with T2D (Ahrén, 2013; Knop et al., 2007). Several studies suggest that part of this deleterious mechanism could rely on the downregulation of the *Glp1r* expression in different tissues involved in metabolic homeostasis, including pancreatic β cells (Pan et al., 2009; Xu et al., 2007). Other studies have demonstrated that hyperglycemia not only downregulates both *Glp-1* et *Gip* receptors expression in pancreatic islets but it also impairs the Glp-1 signaling pathway through the loss of *Glp1r* at the β cell cytoplasmic membrane (Rajan et al., 2015). Preserving *Glp1r* expression in metabolic and non-metabolic tissues appears to be crucial to maintain glucose homeostasis. In addition, the identification of the signaling pathways that regulate *Glp1r* expression could represent potential targets toward T2D treatment. For instance, the Pax6 (Gosmain et al., 2012) or the farnesoid X receptor (Kong et al., 2021) directly modulate the *Glp1r* gene expression at the chromatin level; therefore, targeting these pathways may be promising for the improvement of glucose homeostasis and insulin secretion in a metabolic stress context. We also observe that overexpressing *E2F1* in pancreatic β cells increases *Glp1r* expression, suggesting that targeting this pathway would also represent an alternative strategy, with caution regarding the proliferative functions displayed by the E2F1/pRb pathway. Moreover, since *E2F1* expression is decreased in T2D islets (Lupi et al., 2008), our study suggests a putative association between the loss of *GLP1R* expression in patients with T2D and the loss of *E2F1* expression. Although single-cell RNA sequencing analyses are required to unequivocally demonstrate concomitantly decreased levels of *E2F1* and *GLP1R* within the same cell, we can speculate that the long-term loss of efficacy of Glp1r agonist treatment to restore glucose homeostasis in patients with T2D may be related, at least partially, to impaired E2F1-dependent modulation of *GLP1R* expression.

Following pioneer observations that the E2F1-CDK4-pRb pathway is regulated by glucose and insulin in pancreatic islets (Annicotte et al., 2009), we show here that the treatment of pancreatic islets with a Glp1r agonist modulates the E2F1 pathway through the phosphorylation of pRb and the activation of the E2F1 transcriptional activity. Our data are in line with previous studies showing that the treatment of Ins1 cells with ex-4 decreased both mRNA and protein levels of pRb and increased E2f1 protein levels (Cai et al., 2014). It is well established that ex-4 and Glp1r agonists increase cell-cycle progression through the increased expression of cyclins A2 and D1, stimulating

pancreatic β cell proliferation (Kim et al., 2006; Song et al., 2008). Since the E2F1-pRb-CDK4 pathway positively modulates the expression of *Ccna2* and *Ccnd1* (Stanelle et al., 2002), our data suggest that the cell-cycle progression of pancreatic β cell through the induction of *Ccna2* and *Ccnd1* with ex-4 treatment could be associated with an increase in E2f1 expression and/or activity. Moreover, in human islets, the treatment with ex-4 also increases the expression of several cyclins, leading to increased pancreatic β cell proliferation (Dai et al., 2017). Recent data obtained from hypophosphorylated pRb knockin mice demonstrated that pRb phosphorylation may affect glucose homeostasis at the β cell level and mediates β cell proliferation upon ex-4 treatment (Jiang et al., 2022). Although islet number and β cell morphology were preserved in *E2f1* ^{β -/-} in basal conditions, further investigations are warranted to unequivocally demonstrate a functional link between E2f1 and β cell proliferation upon ex-4 treatment during metabolic stress or β cell regeneration.

In this study, through the use of β cellspecific *E2f1* KO mice, we further confirm a cell-autonomous function for *E2f1* in controlling insulin secretion. More important is the fact that in *E2f1* ^{β -/-} mice, E2f1 controls insulin secretion and, contrary to global *E2f1* KO mice (Fajas et al., 2004; Iglesias et al., 2004; Li et al., 2003a), without modifying β cell mass.

In conclusion, our study reveals a crosstalk between E2f1 and Glp-1 signaling pathways in both mice and human pancreatic islets. It opens the possibility of understanding the differential effects of Glp-1 in the control of β cell mass and function and their relevant molecular mechanisms. How this crosstalk modulates β cell proliferation, β cell dedifferentiation, or loss of identity; which downstream signaling molecules are involved; and how these mechanisms are actionable in T2D pathophysiology remain open questions that require further investigation.

Limitations of the study

A limitation of this study is the observation that *E2f1* ^{β -/-} mice display glucose intolerance, pointing to a more general dysfunction of the *E2f1* ^{β -/-} pancreatic islets to secrete insulin in response to glucose and, *de facto*, to Glp1-R agonists. In addition, although we demonstrate that ex-4 increases pRb phosphorylation and E2F1 activity, we do not provide evidence that this mechanistic link between the E2f1/pRb/Glp-1 directly modulates insulin secretion and contributes to the phenotype we observe in *E2f1* ^{β -/-} mice. Future work is needed to precisely assess how lack of glucose responsiveness in the absence of *E2f1* influences the Glp-1 pathway and contributes per se to glucose homeostasis impairments.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.111170>.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-phospho-Rb ^{S807/811}	Cell signaling	CS#9308; RRID: AB_331472
anti-Rb 4H1	Cell signaling	CS#9309; RRID: AB_823629
Anti-tubulin	Sigma-Aldrich	T5168; RRID: AB_477579
Anti-glucagon	Sigma-Aldrich	G2654; RRID: AB_259852
Anti-HA	Sigma-Aldrich	H3663; RRID: AB_262051
Anti-insulin	Agilent technologies	A0564; RRID: AB_10013624
Anti-insulin	Agilent technologies	IR00261-2; RRID: AB_2800361
Anti-mouse alexa fluor 488 donkey	Thermo Scientific	A21202; RRID: AB_141607
Anti-rabbit alexa fluor 488 donkey	Thermo Scientific	A21206; RRID: AB_2535792
Anti-guinea pig alexa fluor 594 goat	Thermo Scientific	A11076; RRID: AB_2534120
Bacterial and virus strains		
DH5 α Chemically Competent Cells	Thermo Scientific	18265017
Biological samples		
Human islets	ECIT Islet for Basic Research program	https://ecit.dri-sanraffaele.org
Chemicals, peptides, and recombinant proteins		
Glucose 45%	Sigma-Aldrich	G8769
Insulin	Sigma-Aldrich	I9278
HEPES	Sigma-Aldrich	83264
Histopaque 1119	Sigma-Aldrich	11191
RPMI 1640	Fisher scientific	12027599
Exendin-4	Sigma-Aldrich	E7144
HLM006474	Sigma-Aldrich	SML1260
DMEM high glucose glutamax	Fisher scientific	12077549
β -mercaptoethanol	Sigma-Aldrich	M6250
Fatty-acid free BSA	Sigma-Aldrich	A7030
Dharmafect1	Horizon discovery	T-2001-03
Lipofectamine 2000	Fisher scientific	10696153
Protease Inhibitor cocktail	Sigma-Aldrich	05892791001
Fetal bovine serum	Fisher scientific	10270106
Penicillin-streptomycin	Fisher scientific	15-140-122
Phosphatase inhibitors	Fisher scientific	10668304
Type V collagenase	Sigma-Aldrich	C9263
SuperSignal West Dura	Fisher scientific	10220294
Trizol reagent	Fisher scientific	15596-018
FastStart SYBR Green master mix	Sigma-Aldrich	4913914001
Pierce ECL Western blotting substrate	Fisher scientific	A38555
RIPA Lysis Buffer	Fisher scientific	89901
M-PER protein extraction reagent	Fisher scientific	78501
Dipeptide A (DPP4 inhibitor)	Sigma-Aldrich	I9759
Critical commercial assays		
Mouse insulin ELISA kit	Mercodia	10-1247-01
Total GLP-1 NL-ELISA kit	Mercodia	10-278-01
Mini Elute PCR purification kit	Qiagen	28004
RNeasy Plus Microkit	Qiagen	74034

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
RNA-seq data from pancreatic islets isolated from HFD-fed C57Bl6N mice	Gao et al., 2021	GSE153222
Experimental models: Cell lines		
Min6 cells	AddexBio	C0018008
Experimental models: Organisms/strains		
C57Bl6/J homozygous <i>E2f1</i> floxed mice (<i>E2f1^{fl/fl}</i>)	This study	N/A
Rip-Cre Mouse - Tg(Ins2-cre)23Herr	Laboratory of Pr Pedro Herrera, University of Geneva, Switzerland	RRID: MGI:6195021
RIPcre ^{+/+} / <i>E2f1^{fl/fl}</i>	This study	N/A
RIPcre ^{Tg/+} / <i>E2f1^{fl/fl}</i>	This study	N/A
Rosa-26-loxP-LacZ-loxP-h <i>E2F1</i> mice (<i>RFC^{Tg/+}</i>)	Laboratory of Dr Ulrike Ziebold (MDC, Berlin, Germany)	N/A
RIPcre ^{+/+} / <i>RFC^{Tg/+}</i> mice	This study	N/A
RIPcre ^{Tg/+} / <i>RFC^{Tg/+}</i> mice	This study	N/A
Oligonucleotides		
Primers for RT-qPCR and ChIP-qPCR, see Table S1	This study	N/A
Recombinant DNA		
pCMV10	This study	N/A
pCMV10-h <i>E2F1</i> -Flag	This study	N/A
pCMV-E2F1	Fajas et al., 2002	N/A
pCMV-DP-1	Fajas et al., 2002	N/A
pECE-pRB	Fajas et al., 2000	N/A
pE2fRE-Tk-Luc	Laboratory of Pr L. Fajas, CIG, Lausanne, Switzerland	N/A
pCMV-Glp1r-Luc	E-zyvec	This study
si <i>E2f1</i>	Dharmacon	#13555
siControl	Dharmacon	D-001810-01-20
Software and algorithms		
GraphPad Prism 9.0 software	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Image J	Schneider et al., 2012	https://imagej.nih.gov/ij/index.html
Ingenuity Pathway Analysis	Qiagen	https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/
LASAGNA-Search tool	Lee and Huang, 2013	N/A
Other		
High-fat diet	Research Diet	D12492i
AccuCheck Performa	Roche Diagnostics	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and request for reagents and resources should be directed to and will be fulfilled by the lead contact, Dr Jean Sébastien Annicotte (jean-sebastien.annicotte@inserm.fr).

Materials availability

All unique/stable reagents generated in this study are available from the [lead contact](#) with a completed Materials Transfer Agreement.

Data and code availability

- This paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the [key resources table](#). All original data reported in this paper will be shared by the [lead contact](#) upon request.
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice were maintained according to European Union guidelines for the use of laboratory animals. *In vivo* experiments were performed in compliance with the French ethical guidelines for studies on experimental animals (animal house agreement no. 59-350294, Authorization for Animal Experimentation, project approval by our local ethical committee no. APAFIS#2915-201511300923025v4). All experiments were performed with male mice. Mice were housed under a 12-hr light/dark cycle and given a regular chow. For high-fat diet (HFD) studies, 6-week-old mice were placed on a HFD (60% of calories from fat) for 16 to 20 weeks. IPGTT and OGTT was performed after 16h of fasting and glucose (2g/kg) was administered by intraperitoneal injection or oral gavage respectively. For mice under HFD, IPGTT and OGTT was performed after 16h of fasting and glucose (1.5g/kg) was administered by intraperitoneal injection or oral gavage respectively. Glycemia was measured using the AccuCheck Performa. Circulating insulin levels were measured using the insulin ELISA kit. Circulating GLP-1 levels were measured using Total GLP-1 NL-ELISA kit. C57Bl6/J homozygous *E2f1* floxed (*E2f1^{fllox/fllox}*) mice and heterozygous rat insulin 2 promoter (RIP)-Cre mice (RIPcre^{Tg/+}) were previously described (Denechaud et al., 2016; Herrera et al., 1998) and then further intercrossed to generate RIPcre^{+/+}/*E2f1^{fllox/fllox}* (*E2f1^{β+/+}*) and RIPcre^{Tg/+}/*E2f1^{fllox/fllox}* (*E2f1^{β-/-}*) mice. A PCR genotyping strategy was subsequently used to identify *E2f1^{β+/+}* and *E2f1^{β-/-}* littermates. C57Bl6/J mice harboring the Rosa-26-loxP-LacZ-loxP-h*E2F1* conditional expression cassette (RFC mice) were obtained from Dr Ulrike Ziebold (MDC, Berlin, Germany; (Scheijen et al., 2003)). Heterozygous RFC/+ mice were crossed with RIPcre^{Tg/+} mice to obtain RIPcre^{Tg/+}/RFC^{Tg/+} (*E2f1^{βover}*) and control RIPcre^{+/+}/RFC^{Tg/+} (*E2f1^{βCtrl}*) mice.

METHOD DETAILS

Pancreatic islets studies

For mouse islets studies, pancreas was digested by type V collagenase (1.5mg/mL) for 10 minutes at 37°C as previously described (Annicotte et al., 2009; Rabhi et al., 2016). After pancreas digestion and separation of pancreatic islets in a polysucrose density gradient medium, islets were purified by handpicking under a microscope and were cultured during 16 hours before experiments. For GSIS experiments, approximately 30 islets were exposed to 2.8 mM glucose, 16.7 mM glucose, 20 mM glucose, 20 mM glucose and 50 nM exendin-4 or 16.7 mM glucose and 2.5 μM forskolin in Krebs-Ringer buffer supplemented with HEPES and 0.5% fatty-acid free BSA. Insulin released in the medium was measured using the mouse insulin ELISA kit. Insulin content was measured after lysis in a buffer containing 75% of ethanol and 1.5% HCl using mouse insulin ELISA kit. Data are expressed as a ratio of total insulin content. Human pancreatic tissue was harvested from human, non-diabetic, adult donors. Isolation and pancreatic islet culture were performed as previously described (Kerr-Conte et al., 2010). Human islets were treated for 48 hours with the pan-E2F inhibitor HLM006474 at 10 μM (Rosales-Hurtado et al., 2019) and data were expressed as a ratio of total insulin content. For mRNA quantification, human islets were isolated as described above and snap-frozen for further processing.

Cell culture, transfections, and siRNA knock-down

Min6 cells were cultured in DMEM with 15% fetal bovine serum, 100 mg/mL penicillin-streptomycin and 55 mM β-mercaptoethanol. Cells were transfected with non-targeting siRNA mouse negative controls (siCont) and si*E2f1* (#13555, SMARTpool, Dharmacon) using Dharmafect1 and experiments were performed 48 hours later. Transient transfection experiments were performed in Min6 cells using Lipofectamine 2000 following the manufacturer's instructions. Luciferase assays were performed 48 hours post-transfection and normalized to β-galactosidase activity as previously described (Annicotte et al., 2009). For pharmacological treatments, Min6 cells and pancreatic islets were treated with HLM006474 (Rosales-Hurtado et al., 2019) at 10 μM for 48h. Exendin-4 was used at 50 nM at different time as indicated.

Protein extracts and western blot experiments

Western blot was performed as previously described (Rabhi et al., 2018). Min6 cells were washed twice with cold PBS 1X and lysed with cell lysis buffer (50 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40) supplemented with Protease Inhibitor cocktail and phosphatase inhibitors. Immunoblotting experiments were performed using 40 μg of total proteins and loaded on Precast SDS gel (Biorad). After electromigration, proteins were transferred on nitrocellulose membrane during 1h at 110V that were further incubated in TBS-Tween 0.05% (TBS-T) supplemented with 5% of milk. Membranes were incubated 16 h at 4°C with primary antibodies as indicated in blocking buffer supplemented with 3% of bovine serum albumin or milk. After washing, membranes were incubated 1 h with the secondary antibody conjugated with horseradish peroxidase. The revelation of luminescent bands was performed using Pierce ECL Western blotting substrate or SuperSignal West Dura Extended duration substrate with Chemidoc Xrs+ (Biorad).

In silico analysis of the mouse *Glp1r* promoter region

The DNA sequence of the promoter region of the *Glp1r* gene was obtained from Ensembl (<https://www.ensembl.org/index.html>). Motif search was performed using LASAGNA-Search tool (Lee and Huang, 2013).

Chromatin immunoprecipitation

Min6 cells were transfected with the empty vector pCMV10 or pCMV10-hE2F1-Flag. 48 hours after transfection, DNA-protein complexes from Min6 cells were formaldehyde-crosslinked to DNA at a final concentration of 1% for 10 minutes. The reaction was stopped by adding glycine at a concentration of 0.125M during 5 minutes. After cell lysis and sonication with the Bioruptor Pico (Diagenode, ref B01060010) for 8 minutes, proteins were immunoprecipitated with either the non-specific Ig G or anti-Flag 2 antibodies. After washing, protein-DNA complexes were decrosslinked by heating the samples for 16 hours at 65°C. DNA was purified using Mini Elute PCR purification kit and qPCR were performed using promoter-specific primers.

RNA extraction, measurements, and profiling

Total RNA was extracted from Min6 cells using trizol reagent. For mouse and human islets, total RNA was extracted with the RNeasy Plus Microkit following manufacturer's instructions. mRNA expression levels were measured after reverse transcription by quantitative real-time PCR (qRT-PCR) with FastStart SYBR Green master mix using a LC480 instrument (Roche). qRT-PCR were normalized to cyclophilin mRNA levels. The results are expressed as the relative mRNA level of a specific gene expression using the formula $2^{-\Delta\Delta C_t}$.

Kinome profiling

Serine-threonine kinase (STK) microarrays were purchased from PamGene International BV. Each array contained 140 phosphorylatable peptides as well as 4 control peptides. Sample incubation, detection, and analysis were performed in a PamStation 12 according to the manufacturer's instructions. The experiments were performed on mouse pancreatic islets as previously described (Rabhi et al., 2018).

Immunohistochemistry, immunofluorescence, and morphometry

Immunofluorescence and immunohistochemistry were performed exactly as described previously (Rabhi et al., 2016). Pancreatic tissues were fixed in 10% formalin, embedded in paraffin and sectioned at 5 μ m. For immunofluorescence microscopy analyses, after antigen retrieval using citrate buffer, 5- μ m formalin-fixed paraffin embedded (FFPE) pancreatic sections were incubated with the indicated antibodies. Immunofluorescence staining was revealed by using a fluorescein-isothiocyanate-conjugated anti-mouse (for Glucagon and HA), fluorescein-isothiocyanate-conjugated anti-rabbit (for pRb^{S807/811}), anti-guinea pig (for Insulin) secondary antibodies. Nuclei were stained with Hoechst. Morphometrical parameters (percentage of endocrine cells and insulin positive cells, islet number, and density) were determined as previously described (Courty et al., 2019; Rabhi et al., 2016).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as mean \pm s.e.m. Statistical analyses were performed using a two-tailed unpaired Student's t-test, one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test or two-way ANOVA with Tukey's *post hoc* tests comparing all groups to each other, using GraphPad Prism 9.0 software. Differences were considered statistically significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).