The CDK4-pRB-E2F1 pathway controls insulin secretion

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Abstract

CDK4-pRB-E2F1 cell cycle regulators are robustly expressed in non-proliferating β-cells, suggesting that besides the control of β-cell number the CDK4-pRB-E2F1 pathway has a role in β-cell function. We show here that E2F1 directly regulates the expression of Kir6.2, which is a key component of the K_{ATP} channel involved in the regulation of glucose-induced insulin secretion. We demonstrate, by in tissue chromatin immunoprecipitation analysis that Kir6.2 expression is regulated at the promoter level by the CDK4-pRB-E2F1 pathway. Consistently, inhibition of CDK4, or genetic inactivation of E2F1 results in decreased expression of Kir6.2, impaired insulin secretion, and glucose intolerance in mice. Furthermore we show that rescue of Kir6.2 expression restores insulin secretion in E2f1 −/− β-cells. Finally, we demonstrate that CDK4 is activated by glucose through the insulin pathway, ultimately resulting in E2F1 activation and consequently in Kir6.2 increased expression. In summary we provide evidence that the CDK4-pRB-E2F1 regulatory pathway is involved in glucose homeostasis, defining a new link between cell proliferation and metabolism.

MESH Keywords Animals ; Blotting, Western ; COS Cells ; Cell Line ; Cercopithecus aethiops ; Chromatin Immunoprecipitation ; Cyclin-Dependent Kinase 4 ; metabolism ; E2F1 Transcription Factor ; genetics ; metabolism ; Gene Expression Profiling ; Glucose ; pharmacology ; Immunohistochemistry ; Insulin ; secretion ; Islets of Langerhans ; cytology ; drug effects ; metabolism ; Mice ; Mice, Inbred C57BL ; Mice, Knockout ; Models, Biological ; Phosphorylation ; Potassium Channels, Inwardly Rectifying ; genetics ; metabolism ; Protein Binding ; RNA, Small Interfering ; genetics ; Retinoblastoma Protein ; metabolism ; Reverse Transcriptase Polymerase Chain Reaction ; Signal Transduction ; Transfection

E2F transcription factors exist either as free E2F/DP heterodimers, or associated in larger complexes containing members of the retinoblastoma (pRB, p107, p130), and of the cyclin/cdk protein families. Association of E2Fs with the pRB family facilitates active repression through recruitment of histone deacetylases1 and lysine/arginine methyl-transferases2 . Subsequent phosphorylation of the retinoblastoma protein by the cyc/cdk complexes results in the release of the E2F transcription factors and activation of the transcription of genes required for progression through G1 into the S phase of the cell cycle. It is now accepted that E2Fs participate in cellular processes beyond the cell cycle, including apoptosis, differentiation, and development (reviewed in3). Of particular interest is the observation that E2Fs could be involved in the regulation of a metabolic network, including the control of mitochondrial function4 . We recently implicated E2Fs in the control of adipocyte differentiation5 . Furthermore, we also showed an overall reduction in pancreatic size, as the result of impaired postnatal pancreatic growth in E2f1 −/ − mice6 . Surprisingly, we observed that E2F1 was highly expressed in non-proliferating pancreatic β-cells6 , suggesting that besides its impact on β-cell number, E2F1 could also play a role in pancreatic β-cell function and insulin secretion. Pancreatic β-cell K_{ATP} channels are composed by the heteromeric association of the sulfonylurea receptor 1 (SUR1) with the K^+ ATP channel inward rectifier (KIR6.2). These channels play a critical role in the regulation of glucose-induced insulin secretion by controlling membrane polarization7 ,8 .

The first result suggesting a role of the CDK4-pRB-E2F1 pathway in pancreas physiology, independent of cell proliferation was the observation that E2F1, cyclin-dependent kinase-4 (CDK4) and pRB were expressed in almost all insulin-producing β-cells (Fig. 1a), whereas only a few of these cells (6%) were proliferating, as assessed by PCNA labeling (Fig. 1b , and Fig. S1b). Co-immunoprecipitation assays further indicated coexpression of pRB and E2F1 in these cells (Fig. S1a). Participation of CDK4-pRB-E2F1 in glucose homeostasis was revealed by intra-peritoneal glucose tolerance test (IPGTT) showing that E2f1 −/− mice are glucose intolerant (Fig. 1c , and6). Interestingly, insulin secretion in response to glucose was impaired in both E2f1 −/− mice (Fig. 1d), and E2f1 −/− isolated islets (

Fig. 1e). No differences in insulin content were observed in E2f1 −/− and +/+ cells, when normalized by DNA content (Fig. S1c). This suggested that E2F1 is critical to maintain normal blood glucose levels through the control of insulin secretion in a cell autonomous manner, rather than defects in insulin synthesis. The lack of sensitivity of E2f1 −/−, compared to + /+ mice to glibenclamide, which is an antidiabetic drug of the sulfonylurea family that stimulates insulin secretion, and therefore decreases glycemia by blocking ATP-sensitive potassium channel in pancreatic β-cells suggested that E2F1 is involved in insulin secretion through the controls of mechanisms such as plasma membrane depolarization (Fig. 1f).

We next identified E2F1 target genes that could mediate the effects of E2F1 in β-cell biology. Comparative gene expression profiling revealed that several genes known to be involved in insulin production or secretion were differentially expressed between E2f1 −/− and E2f1+/+ pancreatic islets (Fig. S2a , and supplementary table 1 and 2). Notably, mRNA expression of Kir6.2 (also referred as Kcnj11), which is subunit of the inwardly rectifying and ATP-sensitive potassium channel was strongly decreased in E2f1 −/− compared to +/+ islets, whereas expression of other genes involved in the control of insulin secretion, such as the sulfonylurea receptor Sur1, the pyruvate carboxylase (Pcx) or the uncoupling potein 2 (Ucp2), were not significantly changed (Fig. 2a). Consistent with this observation knock-down of E2F1 by siRNA decreased endogenous Kir6.2 mRNA in isolated islets and in Min6 cells (Fig. 2c–d , and Fig. S2c), which followed the expected decrease in E2F1 protein (Fig. 2d). This resulted in decreased glucose-stimulated insulin secretion in Min6 cells (Fig. S2d). Most importantly, rescue of Kir6.2 expression in E2f1 −/− isolated islets restored glucose-stimulated insulin secretion in these cells, demonstrating that Kir6.2 mediated the observed effects of E2F1 in insulin secretion (Fig. 2e , and Fig. S2e).

Computational analysis of the murine Kir6.2 promoter sequence revealed the presence of an E2F binding site located 577 base pairs upstream of the transcription start site (Fig. S3a). Transient transfection experiments indicated that the E2F1/DP-1 heterodimer could activate the Kir6.2 promoter up to 10-fold induction (Fig. 2f). This transactivation capability was abrogated when the E2F1 response element found in the Kir6.2 promoter was mutated, suggesting that E2F1 was directly involved in the control of Kir6.2 promoter activity (Fig. 2f). Binding of E2F1 on the promoter region of the Kir6.2 gene was demonstrated by chromatin immunoprecipitation (ChIP) experiments on genomic DNA isolated from whole pancreatic tissues. A fragment of the mouse Kir6.2 promoter containing the E2F binding site was amplified by PCR when different anti-E2F1 antibodies were used (Fig. 2g and Fig. S3b). Same results were observed when chromatin isolated from islets was used (Fig. 2h). No E2F1 was detected in a non-related genomic region, or in extracts from E2f1−/− pancreas, demonstrating the specificity of the binding (Fig. 2g , and Fig. S3c). Direct binding of E2F1 to this site, at the same extend that to the consensus E2F1 binding site was shown by EMSA (Fig. S3d). Furthermore, E2F1 could not bind to a mutated version of the E2F site in the Kir6.2 promoter in the same assay (Fig. S3d). These results demonstrated that E2F1 regulates Kir6.2 expression through direct promoter binding in a cell-autonomous manner. Same results were obtained when Min6 cells were used (Fig. S3e). In this cellular context, E2F1 was equally well associated to the Kir6.2 or Dhfr promoter, suggesting that the E2F1 response element found in the Kir6.2 promoter could be considered as a bona fide E2F1 target gene (Fig. S3e).

E2F1 transcriptional activity is regulated through interactions with pocket proteins and cdks. When associated to pRB, E2Fs repress transcription. Upon phosphorylation of pRB by cdks, E2F1 is released and activates transcription of its target genes (reviewed in9). Since E2F1 can regulate the expression of Kir6.2 (Fig. 2), and Kir6.2 mRNA expression is regulated by glucose (Fig. 3a , and 10) we hypothesized that a glucose load could trigger the E2F1 pathway through inactivation of pRB by phosphorylation in pancreatic β-cells. This was demonstrated by the observed increase of pancreatic CDK4 activity in response to glucose in mice, as measured by the capacity of immunoprecipitated CDK4 to phosphorylate recombinant pRB protein in vitro (Fig. 3b). Consistent with this increase in CDK4 activity, enhanced pRB phosphorylation was observed in pancreatic islets in response to intra-peritoneal glucose injection (Fig. 3c and Fig. S4). We also showed that glucose-induced phosphorylation of pRB increased E2F1 transcriptional activity in Kir6.2 promoter-reporter assays. Glucose treatment increased Kir6.2 promoter activity in the presence of the E2F1/DP-1 heterodimer, whereas no induction was observed when the E2F1 responsive element in the Kir6.2 promoter construct was mutated (Fig. 3d). Moreover, co-transfection of increasing amounts of pRB counteracted the stimulatory effects of E2F1 on the Kir6.2 promoter (Fig. 3e). Similarly, cell treatment with the CDK4 inhibitor, 2-Bromo-12,13-dihydro-5H-indolo(2,3-a)pyrrolo (3,4)carbazole (IDCX), also repressed this E2F1-mediated transactivation of the Kir6.2 promoter activity (Fig. 3e). These results were further validated by in vivo ChIP experiments. E2F1 was associated to the Kir6.2 promoter regardless of glucose treatment (Fig. 3f). In sharp contrast, pRB was only associated to this promoter region in the absence of glucose treatment, suggesting that glucose facilitated pRB release from the E2F1 complex bound to the Kir6.2 promoter (Fig. 3f). This is consistent with the known repressor effect of hypophosphorylated pRB on E2F1 activity. To further prove that E2F1 and pRB are part of the same complex re-ChIP experiments were performed. After a first ChIP using anti-E2F1 antibody, a second immunoprecipitation using an anti-pRB antibody or nonspecific IgGs was performed. Same results as in simple ChIP experiments were observed, demonstrating that pRB forms a complex with E2F1 in this promoter under these conditions (Fig. 3g). Finally, the physiological relevance of pRB phosphorylation was evaluated during glucose-stimulated insulin secretion. Transient transfection of a pRB non-phosphorylatable mutant (pRB-Δp34, 11) in Min6 cells resulted in a hypophosphorylated form of pRB upon normal and glucose-stimulated conditions, whereas the wild-type form was still sensitive to phosphorylation (Fig. 3h). Most importantly, expression of this mutant in Min6 cells resulted in decreased Kir6.2 mRNA expression (Fig. 3i) and subsequent glucose-stimulated insulin secretion (Fig. 3j). We can conclude from these results that phosphorylation of pRb is involved in glucose-stimulated insulin secretion through the

transcriptional control of Kir6.2 mRNA expression. Taken together these results suggested that glucose increases CDK4 activity, alleviates pRB-mediated repression and activates E2F1 transcriptional activity towards Kir6.2, highlighting a novel role for the CDK4-pRB-E2F1 pathway in the control of the insulin/glucose metabolic response in pancreatic islets.

In pancreatic β-cells, insulin regulates expression of genes such as insulin gene itself 12 , or glucokinase 13 in response to glucose through autocrine effects. We therefore wanted to evaluate whether CDK4-pRB-E2F1 regulation of Kir6.2 expression by glucose was direct or rather mediated by insulin autocrine effects. Concomitant treatment of glucose and diazoxide, which is a synthetic inhibitor of insulin secretion in β-cells through opening of K+ channels abrogated the positive effect of glucose on Kir6.2 mRNA expression in these cells (Fig. 4a). This transcriptional effect was mediated by E2F1 as evidenced by the absence of effect in E2f1 −/− islets (Fig. 4a). Increased Kir6.2 expression in response to glucose correlated with increased CDK4 activity in pancreas of glucose-treated mice, as demonstrated by CDK4 kinase assays following immunoprecipitation of equal amounts of CDK4 (Fig. 4b). Interestingly, blockade of insulin secretion with diazoxide, or inhibition of insulin signaling with LY290034, which is a PI3K inhibitor resulted in the abrogation of CDK4 activity in these mice (Fig. 4b). The resulting decrease in CDK4 activity upon diazoxide treatment was correlated to increased binding of the repressor pRB-E2F1 complex on the Kir6.2 promoter, as demonstrated by in vivo ChIP and Re-ChIP experiments (Fig. 4c–d). This was consistent with the observed decrease in Kir6.2 mRNA expression. Similar to diazoxide treatment, mice under fasting conditions show inhibited insulin secretion. Consistently, the pRB-E2F1 repressor complex is associated to the Kir6.2 promoter. Conversely, glucose or insulin infusion of mice resulted in pRB phosphorylation (Fig. 4c–d) and release from the E2F1 complex on the Kir6.2 promoter, leading to increased E2F1 transcriptional activity, as indicated by increased histone acetylation in this promoter region (Fig. 4c–d). Taken together these results suggested that the effect of glucose on CDK4 activity, pRB phosphorylation and E2F1 transcriptional activity are mediated through an autocrine effect of insulin on β-cells, secondary to glucose-stimulated insulin secretion.

We next investigated whether increased CDK4 activity upon glucose and insulin treatment could be the result of increased regulatory activity of D-type cyclins, which are major regulators of CDK4. We observed a 2- to 3-fold increase in binding of Cyclin D2 to CDK4 in Min6 cells treated with glucose, and most importantly with insulin, suggesting that insulin and glucose could exert their positive effects on CDK4 activity through an increase in cyclin D2 and CDK4 mRNA levels and subsequent protein-protein interaction (Fig. 4e–f). Cell cycle is tightly regulated, with D-type cyclins acting as growth factors sensors. Upon mitogenic stimulation, D-type cyclins expression and assembly into CDK complexes is increased, finally resulting in cell cycle progression. Interestingly, we observed that treatment of Min6 cells with growth factors, i.e. EGF or IGF, resulted in increased mRNA levels of CcnD2 and Cdk4, and most importantly, increased Kir6.2 (Fig. 4g–h). Altogether, these data indicate that CDK4 activity is regulated upon growth factor stimuli through increased mRNA levels of CcnD2 and CDK4, resulting in increased association of Cyclin D2 and CDK4, which finally increases mRNA Kir6.2 expression.

Finally, to unequivocally implicate the CDK4-pRB-E2F1 pathway in the control of glucose homeostasis, we evaluated the effects of pharmacological inhibition of CDK4 activity on glucose tolerance and insulin secretion in mice. Clearance of injected glucose, as evaluated by IPGTT was dramatically decreased in treated, compared to non-treated mice with IDCX, which is a specific CDK4 inhibitor (Fig. 5a). This was consistent with decreased glucose-stimulated insulin secretion in IDCX treated, compared to non-treated mice (Fig. 5b). In addition, inhibition of CDK4 activity in vivo resulted in decreased Kir6.2 mRNA and protein levels (Fig 5c–d). These results are of particular interest since they are closely related to those observed in E2f1 −/− mice and reinforce our hypothesis that the CDK4-pRB-E2F1 pathway plays a crucial role in the control of glucose homeostasis.

We have previously demonstrated the importance of cell cycle regulators, in particular E2Fs in the control of post-natal pancreatic β -cell growth and replication. Decreased proliferation and renewal rate of pancreatic β-cells could not fully explain, however the observed decrease in insulin secretion in E2f1 −/− mice. We provide now enough evidence to propose that cell cycle regulators, in particular the CDK4-pRB-E2F1 pathway are critical mediators of nutrient sensing and insulin secretion. This is supported by several findings. First , E2F1, CDK4, and pRB proteins are highly expressed in β-cells. Only few of these cells are, however proliferating despite high levels of expression of these cell cycle regulators, suggesting an additional role of these proteins in β-cells. Second , we show that E2F1 translates nutritional signaling, such as glucose to a metabolic, and not proliferative response through the transcriptional regulation of specific β-cell genes involved in insulin secretion, such as Kir6.2. Consistent with this, we found that Kir6.2 mRNA and protein were almost absent in E2f1 −/− pancreatic β-cells, and rescue of Kir6.2 expression in E2f1 −/− islets restored glucose-stimulated insulin secretion. We show that E2F1-pRB directly bind to the Kir6.2 promoter, as demonstrated by in vivo ChIP assays. Furthermore we show that the cyclin D-CDK4-pRB-E2F1 pathway is involved in the transcriptional activation of the Kir6.2 promoter. Strikingly, the phenotype of Kir6.2 −/ − mice strongly resembles E2f1 −/− mice phenotype. Both show impaired insulin secretion. Furthermore, these mice are not diabetic although impaired glucose-stimulated insulin secretion because of their increased insulin sensitivity in peripheral tissues14 ,6 ,5 ,15 . These observations underline a close link between E2F1 and KIR6.2 in metabolic function. Finally , we demonstrate that the CDK4-pRB-E2F1 pathway is regulated through an autocrine effect of insulin on β-cells, secondary to glucose-stimulated insulin secretion. We demonstrate here that upon high glucose concentration, β-cells respond by inducing insulin secretion, which subsequently induces the activation of transcription by an autocrine effect (Fig. S5). Transcriptional activation in β-cells is controlled, at least in part by insulin signaling through the PI3 kinase/AKT pathway. We show that this pathway is implicated on the CDK4-pRB-E2F1-mediated transcriptional regulation,

through increasing CDK4 kinase activity. CDK4, on its turn phosphorylates pRB, and releases E2F1-DP complex, which activate transcription of Kir6.2. Consistent with our hypothesis is the finding that CDK4 activity was increased in transgenic mice over-expressing a constitutively active form of AKT in β-cells (caAKT^{Tg}; 16).

Participation of other cell cycle regulators of the CDK4-pRB-E2F1 pathway in the metabolic response of pancreatic β-cells is likely. Similar to E2f1 −/− mice, cyclin D1 −/−, and cyclin D2 −/− mice show decreased β-cell mass, concomitantly with decreased insulin levels 17 ,18 . Conversely, mice with gene inactivation of the cell cycle inhibitors p27 19 ,20 or p16 INK4A 21 have increased islet mass. These observations raise the question of how this cell cycle regulatory pathway can regulate both cell proliferation and metabolism in β-cells. In line with this duality is the established role of insulin as an important factor for both β-cell proliferation, and β-cell metabolic function22 – 25 . Since our findings indicate that the CDK4-pRB-E2F1 pathway is regulated by insulin, we can hypothesize that both the proliferative and metabolic effects of insulin on β-cells are mediated by the increase of CDK4 activity and subsequent E2F1 transcriptional activity (Fig. 4).

In summary, we have shown that the CDK4-pRB-E2F1 pathway mediates the transcriptional response to glucose of genes implicated in insulin secretion, such as Kir6.2. These results underscore a dual role of the E2F1 pathway in the control of both cell proliferation, and metabolic response.

Methods

Materials and oligonucleotides

All chemicals, except if stated otherwise, were purchased from Sigma (St Louis, MO, USA). LY290034 and IDCX were purchased from VWR-Calbiochem (Fontenay sous Bois, France). Anti-CDK4 (C-22), anti-E2F1 (C-20 for immunofluorescence, KH-95X for ChIP), anti-KIR6.2 (D-14) and anti-PCNA (PC-10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-acetyl H4 (Lys 12), anti-pRb and anti-Phospho-Rb (ser 807/811) were from Cell Signaling (Beverly, MA, USA), anti-insulin was from Linco Research (Millipore, Billerica, MA, USA), anti-BrdU was from Dako (Glostrup, Denmark). The oligonucleotide sequences used for various experiments in this manuscript are available upon request.

Protein extracts, co-immunoprecipitation assays and immunoblot analysis

Protein extracts and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), electrotransfer and immunoblotting were performed as described 26 . Co-immunoprecipitation assays were performed as described 27 .

Immunofluorescence (IF) and immunohistochemistry (IHC)

IF and IHC were performed as described previously 6 ,27 . Briefly, after antigen retrieval, 5μ m formalin-fixed pancreatic sections were incubated with the indicated antibodies. Immunostainings were revealed using peroxydase-conjugated anti-rabbit (Jackson Immunoresearch, Cambridgeshire, UK) secondary antibodies and the DAB (for PCNA and Phospho-Rb, DAKO, Glostrup, Denmark) and NovaRed (for insulin, Vector Laboratories, Burlingame, USA) chromogens as a substrate. Sections were counterstained with haematoxylin or methyl green. Immunofluorescence stainings were revealed using a FITC-conjugated anti-rabbit (Jackson Immunoresearch), alexa-conjugated anti guinea pig, or anti-goat (for KIR6.2 staining, Jackson Immunoresearch) secondary antibody. Negative controls using rabbit or goat IgGs were performed and no staining was observed in these conditions.

Animal experiments

C57Bl/6J and E2f1 +/+ and −/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and were maintained according to European Union guidelines for use of laboratory animals. In vivo experiments were performed in compliance with the French guidelines for experimental animal studies (animal house agreement # B-34-172-27, authorization for animal experimentation # 34.324). Glucose and insulin measurements were performed as described 6 on overnight-fasted mice immediately before and at the indicated time after intra-peritoneal injections of (dose/body weight) glucose (2g/Kg) and glibenclamide (5mg/Kg). IDCX was intra-peritoneally injected at a dose of 5mg/Kg/day for 3 days.

Pancreatic islet studies

Briefly, small pieces of pancreas were digested by collagenase (1.5 mg/ml) and isolated in oxygenated-Krebs Ringer Bicarbonate Hepes buffer (KRBH) containing 0.5% FFA-free BSA. For insulin secretion assays, approximately five islets per condition were handpicked and exposed to either 2.8 or 20 mM glucose. Insulin released in the medium was measured 1 h later by RIA (Linco Research, Inc., St Charles, USA). For Kir6.2 re-expression experiments, E2f1 −/− islets were isolated as described above and cultured in DMEM medium supplemented with FBS and antibiotics in 12-well plates. Sixteen hours later, isolated islets were transfected with empty pCDNA3 or pCDNA3 encoding the mKir6.2 cDNA (a kind gift of Pr Y. Kurachi, Osaka University, Japan) using jetPEI (Polyplus Transfection, Illkirch, France) following manufacturer's instructions. Five hours after transfection, medium was replaced by fresh DMEM

medium supplemented with FBS and antibiotics and islets were subsequently cultured for 20 h. For siRNA experiments, islets were transfected with DharmaFect2 (2μ 1ml) and smart-pool E2F1 validated siRNA (100nM) following manufacturer's instructions (Dharmacon, Inc., Lafayette, USA) and cultured for 24 h in DMEM containing 2.8mM glucose and 10% FBS. Islets were then rinsed in PBS, handpicked (approximately five islets per condition, in triplicate) and pre-incubated in oxygenated-KRBH containing 0.5% FFA-free BSA for 30 minutes. After this pre-incubation step, islets transiently transfected with empty pCDNA3, pCDNA3-mKir6.2, siRNA-control or siRNA-E2F1 were subsequently incubated in oxygenated-KRBH containing 2.8 or 20 mM glucose. Insulin released in the medium was measured using an insulin ELISA kit (Mercodia, Uppsala, Sweden). Data are expressed as a ratio per DNA or total insulin content. For mRNA quantification, islets were isolated and incubated in KRBH containing 10 mM glucose for 1 h 30, subsequently handpicked and finally incubated with or without 20 mM glucose, 100 nM insulin and/or inhibitors (200μ M diazoxide) for 1 h. Islets were then processed for mRNA extraction.

RNA extraction, Affymetrix arrays, RT-PCR and Q-PCR

After isolation of E2f1 +/+ and −/− islets RNA extraction and reverse transcription were performed as described 6 ,28 . mRNA expression was measured by quantitative real-time PCR (Q-PCR) with Power SYBR Green master mix using a 7300 ABI PRISM Sequence Detector System (Applied Biosystems, Foster City, USA) according the manufacter's recommendations. Q-PCR was performed using gene-specific oligonucleotides under the following conditions: 2 minutes at 50°C and 10 minutes at 95°C and then 40 cycle of 15 seconds at 95°C and 1 mintue at 60°C. The results were normalized to endogenous 18S reference mRNA expression levels. Values are expressed as the relative mRNA level of specific gene expression as obtained using the formula $2^{-\Delta Ct}$. The oligonucleotide sequences used for various experiments in this manuscript are available upon request. All Affymetrix experiments were performed on the High Density Microarray core facility at the Institute for Research in Biotherapy of the University Hospital of Montpellier (https://195.220.112.5/http/0/irb.montp.inserm.fr/).

Site-directed mutagenesis

The pGL3-Kir6.2 -Luc (a kind gift of Dr K.H. Kaestner, University of Pennsylvania, USA) containing a mutated E2F responsive element (GCCGCC**g** G**c** GG**gcggccgc** TCGGCTCCTGA) was created using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, USA).

Cell culture, transient transfections

The Min6 and COS7 cell lines were derived from stocks routinely maintained in the laboratory. Experiments on Min6 cells were performed between passages 20 to 28. Monolayer cell cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 25 mM glucose and 10% foetal calf serum (FCS) (Invitrogen) for COS7 cells and 15% FCS and 100 mM β -mercaptoethanol for Min6 cells. Transient transfections were performed as described previously 27 using jetPEI (Polyplus Transfection) and pGL3-Kir6.2 -Luc, pGL3-muKir6.2 -Luc pCMV-E2F1 5 , pCMV-DP-1 5 pECE-pRbwt and pECE-pRbΔp34 11 . Luciferase activity measurements were normalized for β-galactosidase activity to correct for differences in transfection efficiency. Graph values represent the mean of three independent experiments. For glucose treatment experiments, cells were maintained in glucose free DMEM overnight, transfected and treated 8 h after transfection when stated with glucose for 24 h. Cells were then harvested and luciferase activity was measured.

Chromatin immunoprecipitation (ChIP) and Re-ChIP

ChIP and Re-ChIP assays were performed as described previously 27 . Briefly, proteins from Min6 cells or pancreatic tissues were formaldehyde cross-linked to DNA. After homogeneization, lysis and DNA sonication, proteins were then immunoprecipitated using purified IgGs, anti-E2F1, anti-pRb or anti-acetyl H4 antibodies. After washing, for Re-ChIP experiment, DNA-protein-complexes were incubated in 10mM dithiotreitol (DTT) at 37°C for 30 min, diluted in Re-ChIP buffer and subjected to a second immunoprecipitation. After washing, DNA-protein-complexes obtained from ChIP and Re-ChIP experiment were subsequently eluted and cross-linking was reversed by heating the samples at 65°C for 16 h. DNA was then purified using Qiagen PCR purification kit (Qiagen, Courtaboeuf, France) and PCR amplification was performed using Kir6.2, FABP4, and Dhfr promoter-specific primers.

EMSA

Electromobility shift assays were performed exactly as previously described using nuclear extracts from transfected Min6 cells with E2F1 and DP1 expression vectors 27 and radiolabelled gene-specific oligonucleotides (E2F-Dhfr, 5′ -AATTCTGCGATTTCGCGCCAAACTTGACG-3′; E2F-Kir, 5′-TCAGGAGCCGATCTGGCGCCCTCTGGCGGC-3′; E2F-mutKir, 5′ -TCAGGAGCCGAG CG GC CGCCCG CC GGCGGC-3′).

Kinase assays

Mice were fasted 16 hours, intra-peritoneally injected with glucose (2 g/Kg) and glucose (2 g/Kg) combined with diazoxide (25 mg/Kg) or LY290034 (100 mg/Kg). Mice were then sacrificed and CDK4 immunoprecipitation and kinase assays were performed exactly as previously described29 . Briefly, kinase assays were performed using immunoprecipitated CDK4 and 100 ng of recombinant Rb protein (Santa Cruz, California) as a substrate. Reactions were performed in kinase buffer (25mM Tris/HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl2, 1mM DTT, 5 mM Na4P2O7, 50 mM NaF, 1 mM vanadate and protease inhibitor cocktail (Sigma, St. Louis, Missouri)) using 40 μ M ATP and 8 μ Ci [γ-^{33} P] ATP for 30 min at 37°C. CDK6/cycD3 kinase (Upstate, Charlottesville, Virginia) was used as positive control. The reaction was stopped by boiling the samples for 5 min in the presence of denaturing sample buffer. Samples were subsequently subjected to SDS-PAGE, and the gels were then dried in a gel dryer for 1 hr at 80°C and exposed to an X-ray film.

Statistical analysis

Data are presented as means ± s.e.m Statistical analysis were performed with unpaired Student's t-test. Differences were considered statistically significant at $p < 0.05$. (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$).

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Footnotes:

Author contributions

J-S. A. and E.B. contributed equally to this work. J-S. A. and L.F designed the study. J-S. A., E.B., C.C., I.I., S.C., S.A. and J.T performed the experiments. S.D. and C.S. provided reagents and data. J-S. A. and L.F wrote the manuscript.

Supplementary information

Supplementary tables

Supplementary Information, table I . Genes with decreased expression in pancreas of E2f1 −/− mice.

Supplementary Information, table II . Genes with increased expression in pancreas of E2f1 −/− mice.

Supplementary Information, table III . Oligonuleotide sequences used in this study.

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Figure 1

Decreased secretagogue-stimulated insulin secretion in E2f1 −/− mice

a, Immunofluorescence analysis of serial pancreatic sections showing co-expression of E2F1, CDK4 and pRB (green) in β-cells, as demonstrated by insulin staining (red). Nuclei are stained with Hoechst reagent. Scale bars represent 100 μ m. **b,** The Proliferating Cell Nuclear Antigen (PCNA, black arrow) proliferation marker is detected in β-cells (insulin, pink) by immunohistochemistry. Cells were counterstained with methyl green. Scale bars represent 100 μ m. **c, d,** IPGTT measuring the levels of glucose (c) and insulin (b) at the indicated times after an intra-peritoneal injection of glucose in E2f1 +/+ and −/− mice (means ± s.e.m., n=7). **e,** Insulin secretion of E2f1 +/+ and −/− isolated islets in the presence of 2.8 mM and 20 mM glucose. Results were normalized by total insulin content. A representative result of 5 independent experiments is shown (means ± s.e.m.). **f,** Serum glucose levels were determined before and 60 minutes after intra-peritoneal injection of glibenclamide (means ± s.e.m., n=5).

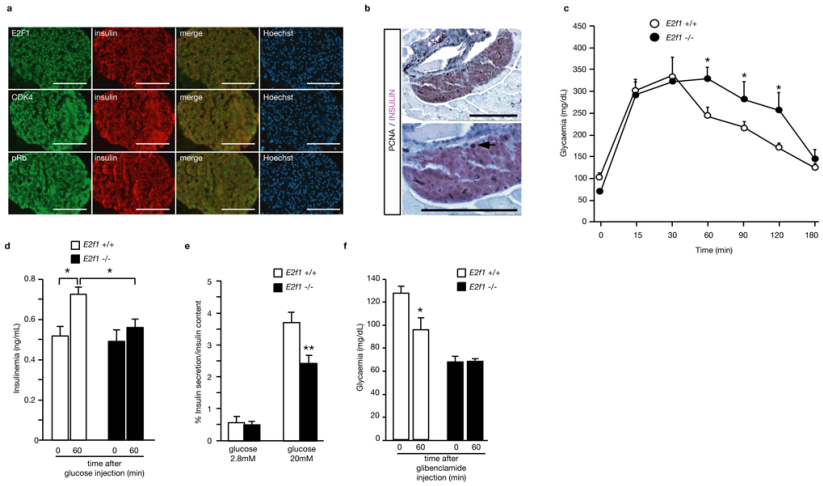

Figure 2

Kir6.2, a component of the K_{ATP} channels regulating insulin secretion, is a direct E2F1 target gene

a, Gene expression analysis in E2f1 **+/+** and **−/−** isolated islets. Quantification of the expression by Q-PCR of E2f1 and relevant genes involved in insulin secretion. Kir6.2, inwardly rectifying potassium channel; Sur1, sulfonyl urea receptor 1; Pcx, pyruvate carboxylase; Ucp2, uncoupling protein-2 (means **±** s.e.m., n=5). **b,** Western blot showing KIR6.2 expression in E2f1 **+/+** and **−/−** pancreas. **c,** Quantification of E2f1 and Kir6.2 mRNA expression in E2f1 **+/+** isolated islets transfected with siRNA-control or siRNA-E2F1. The experiment was performed in triplicate (means **±** s.e.m., n=3). **d,** Western blot showing E2F1 expression in isolated islets transiently transfected with a siRNA control or a siRNA targeting E2f1 mRNA. Actin was used as a loading control. **e,** Insulin secretion of E2f1 **+/+** and **−/−** isolated islets transfected with pCDNA3 or pCDNA3-mKir6.2 in the presence of 2.8 mM and 20 mM glucose. Results were normalized by total insulin and DNA content (means **±** s.e.m., n=3). **f,** The E2F1/DP-1 heterodimer modulates Kir6.2 promoter activity. COS7 cells were transiently co-transfected with the pGL3 (empty vector, negative control), the Kir6.2 promoter (Kir6.2 -luc) and the mutated E2F1 responsive element Kir6.2 promoter (mutKir6.2 -luc) luciferase constructs in the presence or absence of E2F1/DP-1. Results were normalized to β-galactosidase activity and are expressed as relative luciferase units (R.L.U.). **g,** ChIP demonstrating specific binding of E2F1 to the Kir6.2 promoter. Cross-linked chromatin from E2f1 **+/+** and **−/−** pancreata were incubated with two different antibodies against E2F1 or IgG. Immunoprecipitates were analyzed by PCR using specific primers for the E2F-RE present in the Kir6.2 promoter. As a control, a sample representing 10% of the total chromatin was included in the PCR (Input). **h,** ChIP demonstrating specific binding of E2F1 to the Kir6.2 promoter in isolated islets. Cross-linked chromatin from E2f1 **+/+** and **−/−** isolated islets were incubated with antibodies against E2F1 or IgG. Immunoprecipitates were analyzed as in **g.** See Supplementary Information, Fig S6 for full scans of blots in **b** and **d.**

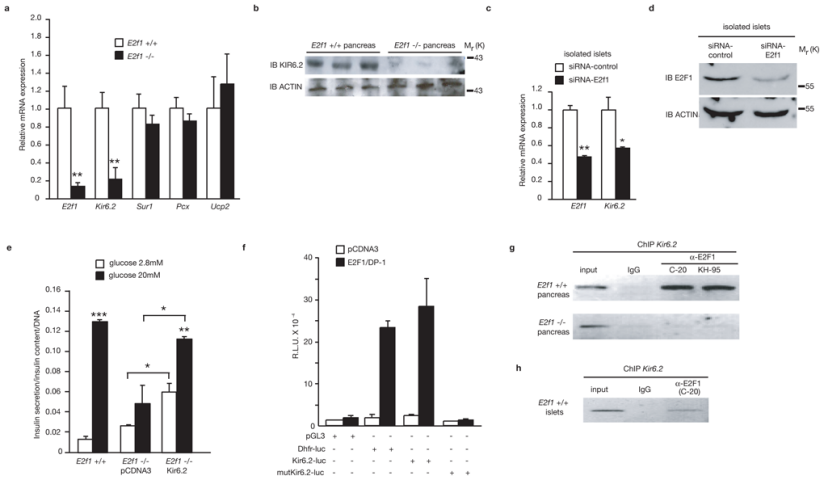

Figure 3

Glucose regulates CDK4 activity, pRB phosphorylation and E2F1 transcriptional activity both in vitro and in vivo

a, Kir6.2 mRNA levels following glucose treatment of isolated islets (means ± s.e.m, n=3). **b,** CDK4 activity in vivo. SDS-PAGE autoradiography showing phosphorylated purified pRB by immunoprecipitated CDK4 from pancreata after an intra-peritoneal injection of glucose (lane 2) or a saline solution (lane 1). As a positive control for pRb phosphorylation, purified CyclinD3/CDK4 proteins were used (lane 3). **c,** Quantification of phosphorylated pRB in pancreatic islets after in vivo injection of glucose. Mice were treated with (n=5) or without glucose (n=5), killed one hour post-injection. Pancreata were then collected, fixed in 4% PFA and subsequently processed for paraffin embedding. IHC was performed on pancreas sections using an anti-phospho pRb antibody. Total and positively-stained cells were counted. Around 50 islets per condition were counted (means ± s.e.m., n=5). **d,** Glucose treatment modulates the Kir6.2 promoter activity in the presence of E2F1/DP-1 heterodimer. Min6 cells were treated as indicated, and results are presented in fold induction. **e,** Activity generated by the Kir6.2 -Luc reporter cotransfected with the E2F1/DP-1 expression vector in the absence or presence of increasing amount of wild-type pRB and the CDK4 inhibitor IDCX (1, 5 and 10 μ M). Experiments were performed in COS7 cells in the presence of glucose. **f,** ChIP demonstrating binding of E2F1 and pRB to the Kir6.2 promoter. As a control, a sample representing 10% of the total chromatin was included in the PCR (Input). **g,** Re-ChIP assays demonstrating interaction between E2F1 and pRB on the Kir6.2 promoter. Immunoprecipitates were analyzed as in **f. h,** Western blot analysis of pRb phosphorylation status in Min6 cells upon glucose treatment transiently transfected with wild-type pRb or a phosphorylation-defective constitutive active pRB mutant construct (pRB Δp34). **i,** Quantification of Kir6.2 mRNA expression in Min6 cells transfected with wild-type pRb (pRb wt) or pRB Δp34 treated or not with 20mM glucose (means ± s.e.m., n=3). **j,** Insulin secretion of Min6 cells transfected with pRb wt or pRBΔp34 in the presence of 2.8 mM and 20 mM glucose. Results are presented as fold induction compared to 2.8 mM glucose (means ± s.e.m, n=3). See Supplementary Information, Fig S6 for full scans of blots in **b** and **h.**

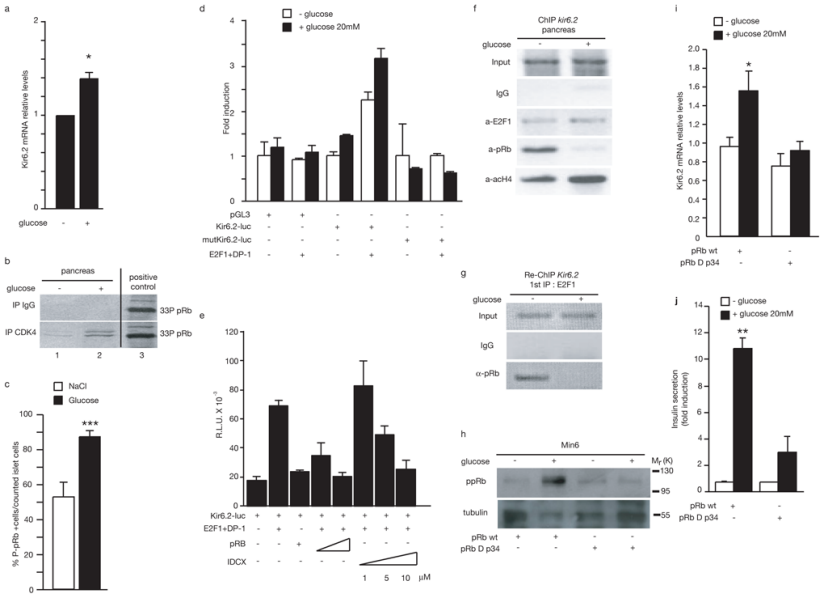

Figure 4

Insulin regulates CDK4 activity, pRB phosphorylation and E2F1 transcriptional activity in vivo through an autocrine effect

a, Kir6.2 mRNA levels in E2f1 +/+ and −/− isolated islets 1 hour after glucose, insulin, diazoxide and glucose/diazoxide treatment. The experiment was done using ~20 islets per condition in triplicate (means ± s.e.m.). **b,** CDK4 activity in E2f1 +/+ pancreata. SDS-PAGE autoradiography showing radiolabelled phosphorylated purified pRB (^{33P} pRb) by immunoprecipitated CDK4 from pancreatic tissue of mice treated 1 hour as indicated (lane 1 to 4). As a positive control for pRb phosphorylation, purified Cyclin D3/CDK6 proteins were used (lane 5). **c,** ChIP showing differential binding of E2F1 and pRb to the Kir6.2 promoter in the pancreas of mice treated as indicated. **d,** Re-ChIP assays demonstrating differential interaction between E2F1 and pRB on the Kir6.2 promoter. Chromatin was prepared from pancreatic tissues obtained after i.p. injection of a saline, a glucose, an insulin or glucose/diazoxide solution and subjected to the ChIP procedure with the antibody against E2F1 and re-immunoprecipitated using IgG or anti-pRB antibody. Immunoprecipitates were analyzed as described above. **e,** Representative immunoprecipitation (IP) assays showing interaction between CCND2 and CDK4. Immunoblot (IB) analysis revealed an increased interaction between CDK4 and CCND2 in Min6 cells after glucose and insulin treatment. **f,** Densitometry analysis of results shown in 4e, and in two independent experiments. Results are expressed as the ratio of the signal reported to the input signal (means ± s.e.m.). Images were analyzed by ImageJ software. **g,** Quantification of Cyclin D2 (CcnD2) and Cdk4 mRNA levels in Min6 cells treated or not with 5 nM EGF or 10 nM IGF (triplicate, means ± s.e.m.). **h,** Kir6.2 mRNA levels in Min6 cells treated as described (triplicate, means ± s.e.m.). See Supplementary Information, Fig S6 for full scans of blots in **b** and **e.**

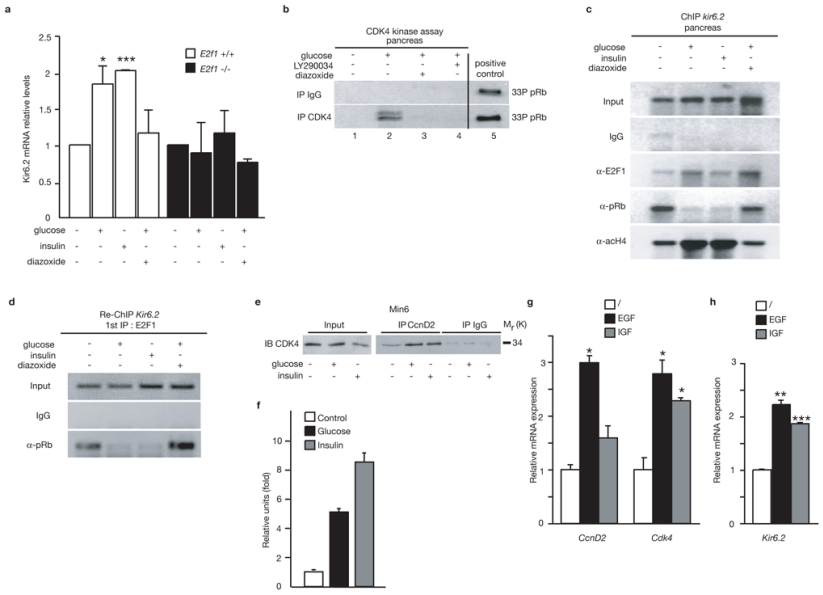

Figure 5

Glucose intolerance, decreased insulin secretion and KIR6.2 levels in C57Bl/6 treated with CDK4 inhibitor

a, b, IPGTT measuring the levels of glucose (a) and insulin (b) at the indicated times after an intra-peritoneal injection of glucose in C57/Bl6J mice treated for 3 days with vehicle or IDCX solutions (means ± s.e.m., n=4). **c,** Kir6.2, Sur1 and Pcx mRNA levels in E2f1 +/+ islets isolated from mice treated with IDCX as described in **a.** The experiment was done using ~40 islets per condition in triplicate (means ± s.e.m.). **d,** Western blot analysis of KIR6.2 protein levels in E2f1 +/+ pancreas isolated from mice treated without (control, n=4) or with IDCX (n=4) as described in **a.** A representative radiography of pancreas from 2 non-treated and treated animals is shown. Levels of induction (n-fold) are indicated. Tubulin was used as a loading control. See Supplementary Information, Fig S6 for full scans of blots in **d.**