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ARTICLE

Glucose regulation of a cell cycle gene module is selectively lost in mouse pancreatic islets during ageing

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Abstract
Aims/hypothesis Transcriptional networks in beta cells are modulated by extracellular signals such as glucose, thereby ensuring beta cell adaptation to systemic insulin demands. Ageing is a main risk factor for type 2 diabetes and has been associated with perturbed expression of genes essential for beta cell function. We aimed to uncover glucose-dependent gene modules in mouse pancreatic islets and investigate how this regulation is affected by ageing.
Methods Global gene expression was assessed in pancreatic islets from young and aged wild-type and *Cdkn2a* (*Ink4a/Arf*)-deficient mice exposed to different glucose concentrations. Gene modules were identified by gene ontology and gene set enrichment analysis.
Results Gene expression profiling revealed that variations in glucose levels have a widespread and highly dynamic impact on the islet transcriptome. Stimulatory glucose levels induced the expression of highly beta cell-selective genes and repressed the expression of ubiquitous genes involved in stress and antiproliferative responses, and in organelle biogenesis. Interestingly, a module comprising cell cycle genes was significantly induced between non-stimulatory and stimulatory glucose concentrations. Unexpectedly, glucose regulation of gene expression was broadly maintained in islets from old mice. However, glucose induction of mitotic genes was selectively lost in aged islets and was not even restored in the absence of the cell cycle inhibitors $p16^{INK4a}$ and $p19^{ARF}$, which have been implicated in the restricted proliferative capacity of beta cells with advanced age.
Conclusions/interpretation Glucose-dependent transcriptional networks in islets are globally conserved during ageing, with the exception of the ability of stimulatory glucose levels to induce a cell cycle gene module.

Keywords Ageing · Beta cell · *Cdkn2a* · Cell cycle · Gene modules · Gene regulation · Glucose · $p16^{INK4a}$ · $p19^{ARF}$ · Pancreatic islet · Transcriptional networks

Abbreviations

BMI-1	Bmi1 polycomb ring finger oncogene
EZH2	Enhancer of zeste homolog 2 (*Drosophila*)
FDR	False detection rate
G3	3 mmol/l glucose
G5	5.5 mmol/l glucose
G11	11 mmol/l glucose
G16	16 mmol/l glucose
GSEA	Gene Set Enrichment Analysis
NRF1	Nuclear respiratory factor 1

Introduction

In recent years, knowledge of the tightly regulated transcriptional networks that control pancreatic beta cell fate and

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specialised functions has increased [1, 2]. The identity of beta cells is defined during pancreatic development by epigenetic mechanisms that ensure the appropriate activation and repression of genes in beta cells [3–5]. Nevertheless, the beta cell transcriptome is highly dynamic and influenced by environmental and metabolic signals that allow beta cells to adapt to systemic insulin demands [1, 2, 6].

Glucose and nutrient metabolism is central for stimulus–secretion coupling in beta cells. Besides its role as an insulin secretagogue, glucose mediates long-term adaptive responses in beta cells, including cell proliferation, survival and function [6–9]. Beta cell mass is tightly regulated and beta cell growth matches changes in systemic insulin demand, which increases during common physiological and pathological states such as insulin resistance, obesity and pregnancy [10, 11]. It has long been known that glucose: (1) is a potent beta cell mitogen in mouse, rat and humans [7, 8, 12, 13]; and (2) plays a dominant role in beta cell compensation of insulin resistance [10]. Glucose stimulates insulin secretion and proliferation in beta cells through signals derived from glycolytic metabolism, which allows homeostatic control of beta cell mass by metabolic demand [14]. Beta cell stimulation by glucose is also known to suppress apoptosis, which may also contribute to the glucose-induced increase in beta cell mass [9].

Despite these observations, the signalling and molecular networks linking glucose to beta cell mass and function remain unresolved. Glucose stimulates several pathways in beta cells, including insulin secretion. Via an autocrine loop, insulin may in turn mediate the effects of glucose on beta cell function, growth and survival [15, 16]. Global genomic studies have shown that glucose metabolism provides major signals for beta cell gene regulation, which may be instrumental for the long-term effects of glucose [6, 17–22].

Ageing is a major risk factor for the development of type 2 diabetes [23], but neither the underlying mechanisms behind the increased susceptibility to diabetes in the elderly, nor the contribution of beta cells to this process are clearly understood. Several studies in pancreatic islets have uncovered age-dependent changes in the expression of genes and proteins, including key transcription factors for beta cell function (e.g. HNF-4α and PDX-1) and the Fas ligand [24–27]. Moreover, basal and adaptive beta cell proliferation and regeneration are severely restricted with advanced age [11, 24, 28–31]. This has been associated in human and mouse beta cells with a progressive increase in expression of the cell cycle inhibitors $p16^{INK4a}$ and $p19^{ARF}$, both encoded by *Cdkn2a* [11, 29, 32, 33].

Despite this body of evidence showing age-related transcriptional and functional changes in beta cells, it is not known how regulation of the beta cell transcriptome by a central stimulus for beta cells such as glucose is globally affected during ageing. Here, we aimed to uncover the main gene modules that are regulated by glucose in pancreatic islets from young and old mice. We show that in islets glucose regulates a wide range of genes affecting a variety of functional categories, with regulation occurring in a dose-dependent manner. This response was broadly maintained during ageing, with the exception of the ability of glucose to induce a cell cycle gene module in aged islets.

Methods

Biological samples and experimental design Mouse pancreatic islets were isolated from wild-type and *Cdkn2a*$^{-/-}$ [34] C57Bl/6J male mice by collagenase digestion and a Histopaque gradient (Sigma-Aldrich, St Louis, MO, USA) [35]. Islets were allowed to recover overnight at 37°C and 5% CO_2 in RPMI containing 11 mmol/l glucose, supplemented with 10% FCS (vol./vol.) and penicillin/streptomycin. Islets were then cultured for 2 days at different glucose concentrations, unless otherwise indicated. Primary cultures of mouse cortical neurons and astrocytes were obtained as described previously [36]. Protocols were approved by the Animal Ethics Committee of the University of Barcelona and the Principles of Laboratory Animal Care were followed.

Gene expression analysis Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using SuperScript (Invitrogen). Quantitative PCR of at least three different biological replicates was performed using SYBR Green (Invitrogen) or Taqman assays (Applied Biosystems, Foster City, CA, USA) in a 7900HT Fast Real-Time PCR system (Applied Biosystems). Primer sequences are listed in electronic supplementary material (ESM) Table 1. Expression levels were normalised to the expression of *Hprt1*.

Global gene expression profiling mRNA from cultured and freshly isolated pancreatic islets from 5-week-old and 13-month-old mice, and from mouse cortical neurons and astrocytes, was amplified through two cycles of cDNA synthesis. Labelled cRNA from biological duplicates was hybridised to Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Expression data were normalised with a robust multi-array average (RMA). The LIMMA software package available from Bioconductor (www.bioconductor.org) was used for statistical analysis to identify differences in gene expression using a multiple test-adjusted p value (false detection rate [FDR]) of $p<0.05$, as previously described [37]. Data have been deposited in Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), accession numbers GSE42591 and GSE42607.

Determination of tissue-specificity factor A tissue-specificity factor for a given gene in each tissue was

calculated as the $\log_2$-ratio between the expression level of that gene in a particular tissue and the median of its expression levels across a panel of tissues. The gene expression profiles used for this analysis were generated in this study or have been published elsewhere [4, 37, 38]. To generate gene cluster representations, expression levels of each gene were normalised across all the tissues analysed and then clustered on the basis of their similarity according to the Euclidian distance using Cluster3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software). Clusters were represented using Java TreeView1.1.6r2 (http://jtreeview.sourceforge.net).

Functional category analysis The DAVID Functional Annotation Tool (http://david.abcc.ncifcrf.gov, accessed 1 August 2012) and Gitools (www.gitools.org, accessed 1 August 2012) [39] were used to identify enriched functional categories in differentially expressed genes. Gene Set Enrichment Analysis (GSEA) software (www.broad.mit.edu/GSEA accessed 1 June 2012) [40, 41] was used to determine the enrichment of gene sets across the expression data generated.

Immunofluorescence and morphometric analysis Immunofluorescence in paraffin-embedded pancreases from at least three different mice was performed as described elsewhere [35], using guinea pig anti-insulin (1:2,500) and mouse anti-glucagon (1:1,000) (Dako, Glostrup, Denmark), and Cy2- and Cy3-labelled secondary antibodies (1:200; Jackson ImmunoResearch, Newmarket, UK). Hoechst 33258 (Sigma-Aldrich) was used as a nuclear marker. Each pancreas was analysed at three different levels. Images were taken with an epifluorescence microscope (DMR HC; Leica Microsystems, Wetzlar, Germany) and analysed using Image J software (NIH, Bethesda, MD, USA). Ki67-positive beta cells were determined in intact islets using mouse anti-Ki67 (1:20; BD Biosciences, Franklin Lakes, NJ, USA), guinea pig anti-insulin (1:500; Dako) and DAPI (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (ESM Fig. 1). Approximately 50 islets from three different biological samples were analysed for each age and condition.

Western blot Protein lysates from three different biological samples were immunoblotted with mouse anti-cyclin D1 (1:2,000; Cell Signaling, Beverly, MA, USA), mouse anti-cyclin D2 (1:200; Abcam, Cambridge, UK) and rabbit anti-actin (1:1,000; Sigma-Aldrich) antibodies.

Statistical analysis Data are expressed as mean±SEM and statistical significance was determined by Student's *t* test. A value of $p<0.05$ was considered statistically significant.

Results

Glucose regulation of the mouse islet transcriptome Several genome-wide analyses have studied the effects of glucose on beta cell lines, rat beta cells and rat pancreatic islets [6, 17–22], but a systematic analysis of the regulation of the mouse islet transcriptome at different glucose concentrations was still missing. To decipher the transcriptional response to glucose in mouse pancreatic islets, we profiled mRNA from islets isolated from 5-week-old mice and cultured for 2 days at two non-stimulatory glucose concentrations (3 mmol/l glucose [G3] and 5.5 mmol/l glucose [G5]) and two stimulatory concentrations (11 mmol/l glucose [G11] and 16 mmol/l glucose [G16]). As rodent islets are usually maintained ex vivo at stimulatory glucose concentrations, which result in better survival outcomes [9, 22], the expression levels at G11 were taken as reference values. Using a 5% FDR, we found 201 downregulated genes (54 downregulated by more than twofold) and 383 upregulated genes (78 upregulated by more than twofold) in islets cultured at G5 (Fig. 1a,b, ESM Table 2). A much more widespread perturbation of gene expression was observed at G3, with 2,140 genes downregulated (310 by more than twofold) and 2,981 upregulated (566 by more than twofold); these numbers represent 9.9% and 13.8% of all islet genes, respectively. Conversely, there were no significant differences between G11 and G16, even though the number of genes differentially expressed in G5 vs G16 was higher than in G5 vs G11. Expression levels of the mesenchymal marker vimentin did not differ between the different glucose concentrations (ESM Fig. 2), thus excluding the possibility that the global transcriptional changes are due to changes in islet levels of mesenchymal cells such as fibroblasts.

Remarkably, in freshly isolated islets from mice with free access to food, the expression of most glucose-dependent genes matched that found in islets cultured at stimulatory glucose levels (Fig. 1a). Thus, less than 11% and 2% of genes downregulated and upregulated, respectively, at G3 and G5 (compared with G11) showed similar expression levels in freshly isolated islets (Fig. 1b). This is exemplified by: (1) the glucose-induced genes *Mafa* and *Pclo* (Piccolo) (Fig. 1c,d), which are involved in regulation of the insulin gene and other key genes for beta cells [42], as well as in insulin secretion [43], respectively; and (2) other markers of beta cell differentiation (ESM Fig. 2). Confirming previous studies in rat pancreatic islets and beta cell lines [20, 22], we found that the expression of several stress genes, including *Ddit3* (*Chop*) and *Trib3* (Fig. 1e,f), was highly increased at G3 and G5, thus illustrating how stimulatory glucose concentrations repress the expression of such genes in islets. Remarkably, a missense polymorphism in human *TRIB3* that results in greater protein stability has been linked to increased risk of type 2 diabetes and to impaired insulin

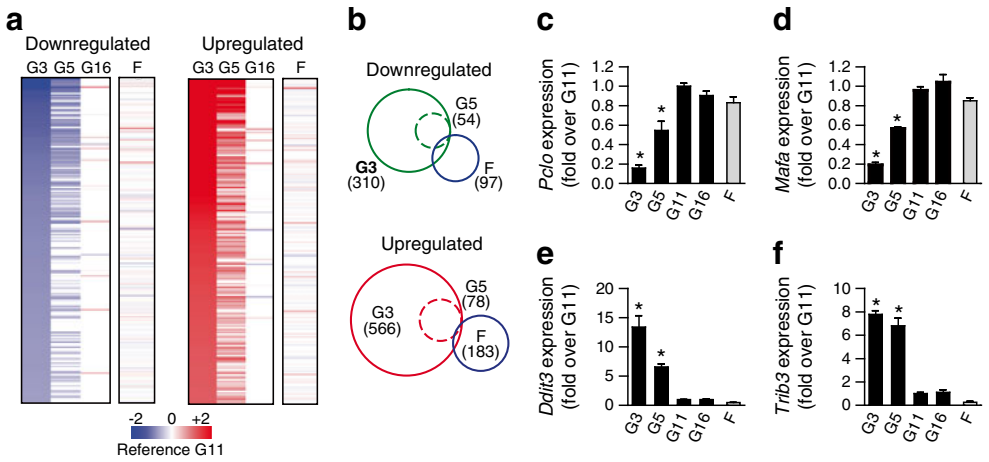

Fig. 1 Glucose is a potent regulator of the islet transcriptome. (**a**) Heat maps depicting changes in gene expression (log_2-transformed) in islets from 5-week-old mice cultured for 2 days at G3, G5 and G16, as well as in freshly isolated islets (F), relative to the expression in islets cultured at G11. The data are sorted in the G3 column by decreasing fold change absolute values. (**b**) Venn diagrams showing the overlap of genes in which expression differed more than twofold in islets cultured as above (**a**). (**c**) Quantitative RT-PCR determination of mRNA levels of *Pclo*, (**d**) *Mafa*, (**e**) *Ddit3* and (**f**) *Trib3* in islets cultured at the indicated glucose concentrations and in freshly isolated islets (F). The results show that, for glucose-induced (*Pclo*, *Mafa*) and glucose-repressed (*Ddit3* [*Chop*], *Trib3*) genes, expression in islets cultured at stimulatory conditions (G11, G16) and in freshly isolated islets is more similar than in islets cultured at lower glucose concentrations (G3, G5). Data were normalised against *Hprt1* and are shown relative to levels at G11, which were set arbitrarily to 1 (n=3 per group). Error bars indicate SEM; *p<0.05

exocytosis and beta cell proliferation [44]. Other stress genes such as *Nupr1* (*p8*), *Nrip2*, *Areg* and *Hspa1b* followed a similar regulatory pattern, as did a GSEA-identified set of genes regulated by C/EBP homologous protein (CHOP), which includes *Trib3*, *Stbd1*, *Odz4* (also known as *Tenm4*), *Tcea1*, *Ptrh2* and *Chka* (ESM Table 2). Taken together, these results indicate that glucose-triggered metabolic and signalling pathways play a central role in regulating the beta cell transcriptome.

To explore the kinetics of the glucose-regulated transcriptional programme, we next profiled the islet transcriptome after short periods of incubation at different glucose concentrations. The expression of about 95% of genes differentially expressed at G3 after a 2-day incubation period was significantly affected after just 10 h of incubation at the same concentration (Fig. 2a–e). Moreover, the expression of 34% and 27% of the genes upregulated and downregulated at G3, respectively, was partially or totally restored to normal levels after 4 h of incubation at stimulatory glucose (G11) (Fig. 2a–e. ESM Table 3). These results point to a highly dynamic regulation of gene expression by glucose in mouse islets.

Glucose induces beta cell-selective and neuronal programmes in islets We next assessed to what extent glucose-dependent genes are ubiquitous or in contrast exhibit a selective tissue expression pattern. To this end, we compared the expression profiles of a panel of different tissues and cell types, and a tissue-specificity score was calculated for a given gene in each tissue. This analysis revealed that glucose-induced genes are highly expressed in islets compared with other tissues (Fig. 3a). Remarkably, glucose-induced genes exhibited similar expression levels in islets and FACS-sorted beta cells. Conversely, genes upregulated at low glucose were expressed ubiquitously (Fig. 3b), pointing to the induction of common signalling and transcriptional networks triggered by stress conditions.

Although many glucose-induced genes were found to be unique or highly specific to islets and beta cells (Fig. 3a,c), the expression of about 30% of such genes was shared with brain regions and neurons, but not with astrocytes (Fig. 3c). Examples include genes encoding piccolo, neurotrophic tyrosine kinase, receptor, type 2 (the receptor for the neurotrophin BDNF) and the prohormone VGF, which has been shown to enhance beta cell survival and function [45] (Figs 1c and 3d,e). Collectively, these results indicate that glucose stimulation is required to maintain the expression of beta cell-specific genes as well as genes shared with neurons.

Non-stimulatory glucose concentrations activate gene modules related to organelle biogenesis A major aim of this study was to dissect the glucose-dependent transcriptional programme into gene modules. Ontology analysis of genes upregulated at G3 and G5 revealed the enrichment of categories such as ribosomes, mitochondria and unfolded protein binding (Fig. 4a). This is clearly illustrated by GSEA, which uncovered a strong enrichment of genes related to ribosomes, mitochondrial ribosomes and oxidative phosphorylation (Fig. 4b). We next inspected the proximal

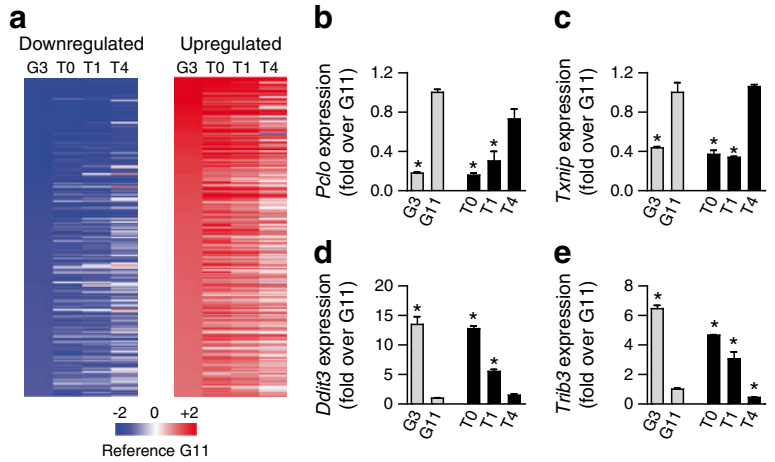

Fig. 2 Glucose regulation of gene expression in pancreatic islets is highly dynamic. (**a**) Expression profiling data sets of mouse pancreatic islets cultured at 3 mmol/l glucose for 10 h (T0) and then challenged with 11 mmol/l glucose for 1 h (T1) and 4 h (T4). The transcriptional profiles of islets cultured at G3 for 2 days are also represented. The heat maps depict the changes in gene expression (log_2-transformed) relative to islets incubated for 2 days at G11. Upregulated and downregulated genes are ordered by decreasing fold change absolute values at G3. (**b**) Quantitative RT-PCR determination of mRNA levels of glucose-induced genes *Pclo* and (**c**) *Txnip*, and (**d**) glucose repressed genes *Ddit3* and (**f**) *Trib3* at time points T0, T1 and T4, compared with expression at G3 and G11. Data were normalised against *Hprt1* and are shown relative to levels at G11, which were set arbitrarily to 1 (n=3 per group). Error bars indicate SEM; *p<0.05

promoters of glucose-regulated genes in order to detect the enrichment of transcription factor binding sites that could point to the mechanisms involved in the response to low glucose. Analysis of the promoters of genes upregulated at

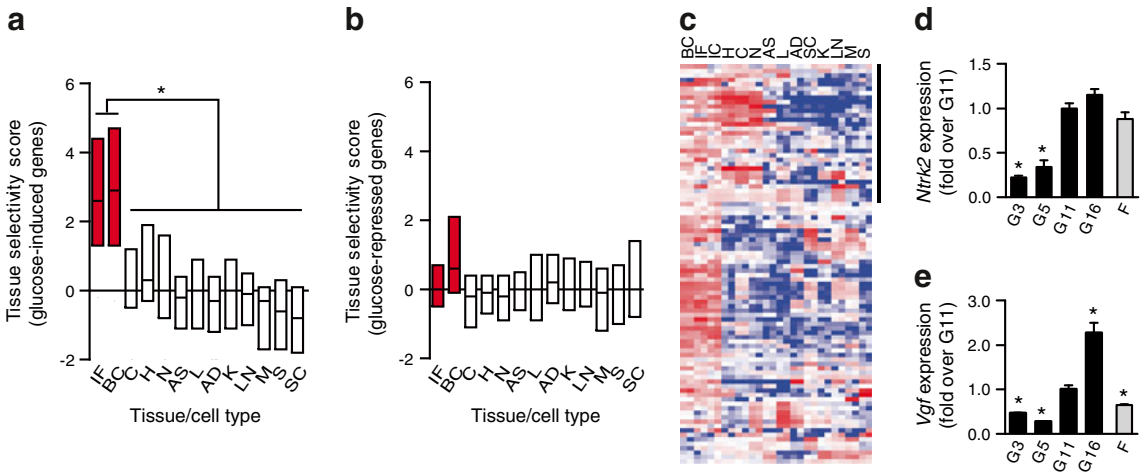

Fig. 3 Glucose induces the expression of beta cell-selective genes and neuronal genes in pancreatic islets. Tissue-selectivity scores for glucose-induced (**a**) and glucose-repressed (**b**) genes in a given tissue according to their expression across a panel of tissues and cell types (see Methods). All genes whose expression levels in pancreatic islets changed more than twofold between G5 and G11 were included in the analysis. Values are plotted in boxplots, where the bottom and top of the box represent the 25th and 75th percentile. The inside line indicates the median and the ends of the whiskers, the 10th and 90th percentiles. A one-way ANOVA using Tukey's multiple comparison test was performed for comparison of different tissues and cell types, *p<0.05. Red boxplots denote values for freshly isolated islets (IF) and FACS-sorted beta cells (BC). C, brain cortex; H, hypothalamus; N, neurons; AS, astrocytes; L, liver; AD, adipose tissue; K, kidney; LN, lung; M, muscle; S, spleen; SC, embryonic stem cells. (**c**) Expression profiling data sets across a panel of tissues and cell types for a module of glucose-induced genes (G11 vs G5). Log_2-transformed probe signal values are shown relative to the average signal level of each probe set across all samples. Red and blue represent higher-than-average and lower-than-average signal levels, respectively. The results show that glucose-induced genes are highly selective for islets and beta cells. The black bar highlights a cluster of genes that are expressed in islets and neuronal tissues. The expression profiling data sets used in this analysis were from mouse freshly isolated (IF) and cultured (IC) islets and tissues as above (**a**,**b**). (**d**) Quantitative RT-PCR determination of islet mRNA levels of representative neuronal genes *Ntrk2* (neurotrophic tyrosine kinase, receptor, type 2) and (**e**) *Vgf* (VGF nerve growth factor inducible), both of which are induced by glucose. Islets were cultured at the indicated glucose concentrations or freshly isolated (F). Data were normalised against *Hprt1* and are shown relative to levels at G11, which were set arbitrarily to 1 (n=3 per group). Error bars indicate SEM; *p<0.05

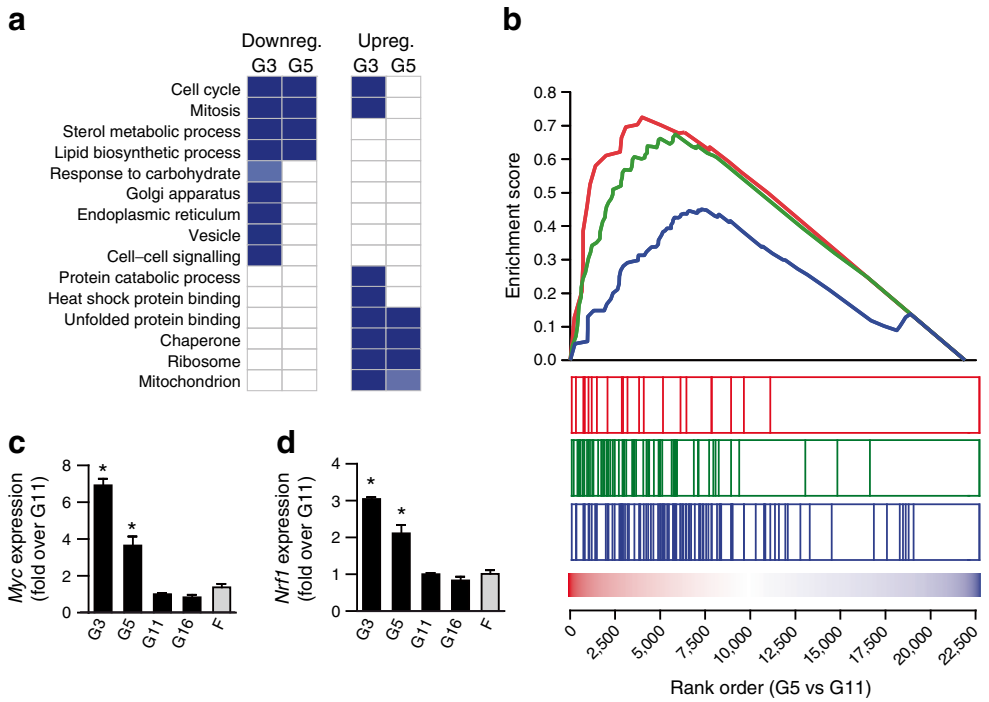

Fig. 4 Mitochondrial, ribosomic and related transcription factor genes are induced in islets cultured at non-stimulatory glucose concentrations. (**a**) Gene ontology analysis of downregulated and upregulated genes in islets cultured at G3 and G5, taking G11 as a reference. Light blue, $p<0.05$; dark blue, $p<0.01$. (**b**) GSEA of genes related to mitochondria and ribosomes across genes ranked according to their different levels of expression in pancreatic islets cultured at G5 vs G11. Vertical lines beneath the graphs depict rank positions of each gene in the colour-coded gene sets. The results show that the modules related to mitochondrial ribosome (red line) ($p=0.002$), structural constituent of ribosome (green line) ($p<0.001$) and oxidative phosphorylation (blue line) ($p=0.006$) were significantly enriched within upregulated genes. (**c**) Quantitative RT-PCR determination of mRNA levels of two glucose-repressed genes, *Myc* and (**d**) *Nrf1*, which encode transcription factors that promote mitochondrial biogenesis. Islets were cultured at the indicated glucose concentrations or freshly isolated (F). Data were normalised against *Hprt1* and are shown relative to levels at G11, which were set arbitrarily to 1 ($n=3$ per group). Error bars indicate SEM; *$p<0.05$

G3 showed that the binding sites for nuclear respiratory factor 1 (NRF1), which have a central role in the regulation of mitochondrial biogenesis, were enriched (ESM Table 4). Myelocytomatosis oncogene (Myc) has been shown to act together with NRF1 to activate mitochondrial genes and regulating mitochondrial biogenesis [46, 47], establishing a direct link between these two transcription factors. Accordingly, both *Nrf1* and *Myc* were induced at G3 and G5 in the same concentration-dependent manner as the mitochondrial genes (Fig. 4c,d), further supporting a coordinated action of both transcription factors on the induction of such genes at non-stimulatory glucose concentrations.

Glucose regulates a cell cycle gene module in pancreatic islets Ontology analysis revealed that the categories enriched among genes downregulated at G3 were related to lipid biosynthesis, the endoplasmic reticulum, the Golgi apparatus and vesicles. Notably, a cell cycle gene module was enriched among genes downregulated at G3 and G5 (Fig. 4a). This category was also enriched in genes upregulated at G3, due to the induction of genes encoding growth suppressors such as *Ccng1* (cyclin G1).

The majority of glucose-induced cell cycle genes are involved in late stages of the cell cycle, namely the progression from G_2 to M and the mitotic phase, which have been shown to be transcriptionally regulated during the cell cycle [48] (Fig. 5a–d). Consistently, the percentage of Ki67-positive beta cells was threefold higher in islets cultured at G11 than in those cultured at G3 (see below). Moreover, glucose also induced the expression of genes involved in the G_1 phase, such as *Ccnd1* and *Ccnd2* (Fig. 5e,f), although only cyclin D2 protein levels were increased by glucose (see below). Importantly, the maximal induction of cell cycle genes was found between G5 and G11. This suggests that mild, but sustained increases in glucose concentrations within this range of concentrations, as occurs in the early stages of diabetes, may induce a proliferative response in beta cells.

Glucose induction of mitotic genes is selectively lost in aged islets Given that ageing has been related to transcriptional and functional changes in beta cells, we next evaluated how the transcriptional response to glucose is affected in aged islets. Interestingly, islets from young and old mice

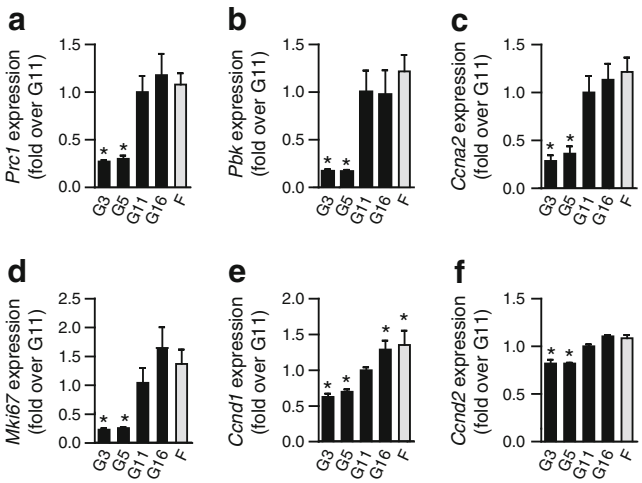

Fig. 5 A cell cycle gene module is regulated by glucose. (**a**–**f**) mRNA levels of glucose-induced cell cycle genes at G3, G5, G11 and G16, and in freshly isolated islets (F) were determined by quantitative RT-PCR. (**a**) *Prc1*, (**b**) *Pbk* and (**c**) *Ccna2* are representative mitotic genes, which have been shown to be transcriptionally regulated during cell cycle progression [48]. (**d**) *Mki67* encodes a protein increased throughout the cell cycle, while (**e**) the *Ccnd1* and (**f**) *Ccnd2* products are involved in G_1 phase, and are activated by mitogenic stimuli. The maximal induction was found between G5 and G11. Data were normalised against *Hprt1* and are shown relative to levels at G11, which were set arbitrarily to 1 (n=4 per group). Error bars indicate SEM; *p<0.05

exhibited a similar insulin secretion response to glucose (ESM Fig. 3), indicating that aged beta cells can sense glucose and secrete insulin in close correlation to glucose levels. We next sought to determine how ageing affects the ability of glucose to modulate gene expression. Thus, we analysed the transcriptional profiles of old islets (13-month-old mice) cultured under different glucose concentrations. Strikingly, glucose-regulated genes and gene modules were broadly maintained in old islets (Fig. 6a, ESM Fig. 4). Moreover, glucose regulation exhibited the same concentration-dependent pattern, as shown for representative glucose-induced and glucose-repressed genes (Fig. 6b,c).

Despite this highly conserved transcriptional response to glucose during ageing, the gene modules related to cell cycle and mitosis were not enriched among genes downregulated at G3 and G5 (ESM Fig. 4). Indeed, these were the only glucose-regulated categories that behaved differently in young and old islets. This selective lack of response to glucose was observed for all genes involved in the latter stages of the cell cycle (Fig. 6d–j). Importantly, the levels of these genes at non-stimulatory glucose concentrations also progressively declined with age. Similar results were obtained when analysing nuclear protein expression of the proliferation marker Ki67 (Fig. 6k, ESM Fig. 1). In striking contrast, *Ccnd1* and *Ccnd2* maintained high levels of expression in old islets and were still able to be stimulated by glucose (Fig. 6d,i,j). As found in young islets, glucose induced a threefold increase in protein levels of Cyclin D2 (Fig. 6l). Moreover, genes comprised in the cell cycle gene module, but exhibiting growth inhibitory functions were similarly induced at G3 (ESM Fig. 4). Taken together, these findings show that although glucose sensing and glucose-induced transcriptional networks are very similar in young and old islets, the induction of mitotic genes is selectively lost in aged islets.

Interestingly, a detailed inspection of the gene modules associated in a strain- and age-dependent manner with obesity-induced diabetes susceptibility [49] revealed that the mitotic genes induced by glucose only in young islets were in a cell cycle gene module linked to diabetes susceptibility. Consistent with our results, *Ccnd1* and *Ccnd2* were not included in such a diabetes-associated gene module.

Glucose induction of mitotic genes is not restored in aged $Cdkn2a^{-/-}$ islets *Cdkn2a* encodes two cell cycle inhibitors, $p16^{INK4a}$ and $p19^{ARF}$, whose expression increases with age in human and mouse islets, and has been suggested to be involved in the reduced proliferative capacity of old beta cells [29, 32, 33]. We thus sought to determine whether the induction of mitotic genes by glucose may be restored in old beta cells in the absence of these inhibitors. To this purpose, the expression of cell cycle genes was analysed in islets from 6-month-old $Cdkn2a^{-/-}$ mice exposed to different glucose concentrations. Although p16 has been previously shown to be highly expressed in almost all beta cells in 6-month-old mice [11] and previous studies have reported increased Ki67-positive beta cells in $Cdkn2a^{-/-}$ mice [29], control and $Cdkn2a^{-/-}$ mice exhibited a similar islet morphology, total beta cell area and number of islets (Fig. 7a–c). Strikingly, glucose-dependent mitotic genes were not induced by stimulatory glucose concentrations in 6-month-old $Cdkn2a^{-/-}$ islets (Fig. 7d–i). These results indicate that the absence of the cell cycle inhibitors, p16 and p19, is not sufficient to restore the induction of mitotic genes by a mitogenic stimulus such as glucose in aged beta cells.

Discussion

Glucose is a fundamental signal in the regulation of gene expression of pancreatic islet beta cells [6, 17–22]. Here we dissected the mouse islet transcriptional response to glucose into gene modules and analysed how this response is affected during ageing. Gene ontology analysis revealed that gene modules related to cell cycle and mitosis were significantly enriched among genes induced by glucose in young islets. Remarkably, the maximal induction of this module occurs between G5 and G11. Thus, glucose can induce the cell cycle gene module within the range of concentrations that are found in prediabetic settings, in which basal blood

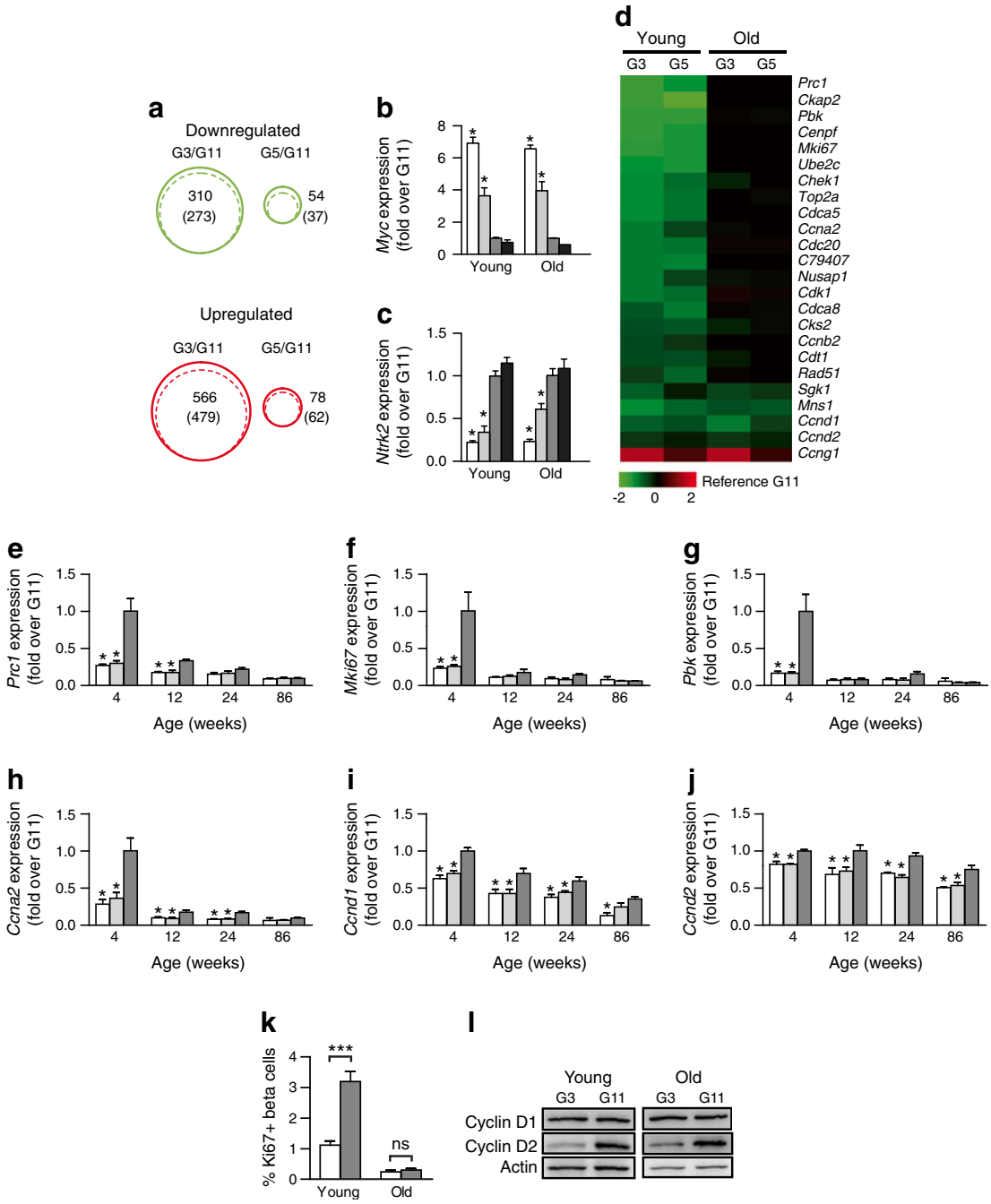

Fig. 6 Age-dependent differences in glucose regulation of gene expression in pancreatic islets. (**a**) Diagrams showing the genes downregulated and upregulated more than twofold in islets from 5-week-old (young, continuous line) mice cultured at G3 and G5 glucose, taking the value at G11 as a reference. The circles with dotted lines represent genes conserving a significant variation in 13-month-old mice (old). Numbers indicate the number of genes (in parenthesis, data from old mice). (**b**) Quantitative RT-PCR gene expression of representative glucose-repressed (*Myc*) and (**c**) glucose-induced (*Ntrk2*) genes in young and old islets cultured at different glucose concentrations (G3, white bars; G5, light grey bars; G11, dark grey bars; G16, black bars). The same pattern of glucose regulation was observed in both age groups. (**d**) Heat map illustrating the changes in expression of cell cycle genes ($\log_2$-transformed) at G3 and G5 compared with G11 in islets from young and old mice. *C79407*, also known as *Mis18bp1*. (**e**) Quantitative RT-PCR determination of expression of glucose-induced cell cycle genes *Prc1*, (**f**) *Mki67*, (**g**) *Pbk*, (**h**) *Ccna2*, (**i**) *Ccnd1* and (**j**) *Ccnd2* in pancreatic islets from mice at ages indicated and cultured at G3 (white bars), G5 (light grey bars) and G11 (dark grey bars). Glucose regulation of mitotic genes, but not of *Ccnd1* and *Ccnd2* was progressively abrogated during ageing. (**k**) Percentage of Ki67-positive beta cells in pancreatic islets from 4-week-old (young) and 86-week-old (old) mice cultured for 2 days at G3 and G11. (**l**) Representative western blots of the cell cycle markers cyclin D1 and cyclin D2 in pancreatic islets as above (**k**). Actin was used as a loading control. Gene expression data were normalised against *Hprt1* and are shown relative to levels in islets cultured at G11, which were set arbitrarily to 1 (n=4 per group). Error bars indicate SEM; *p<0.05 and ***p<0.001

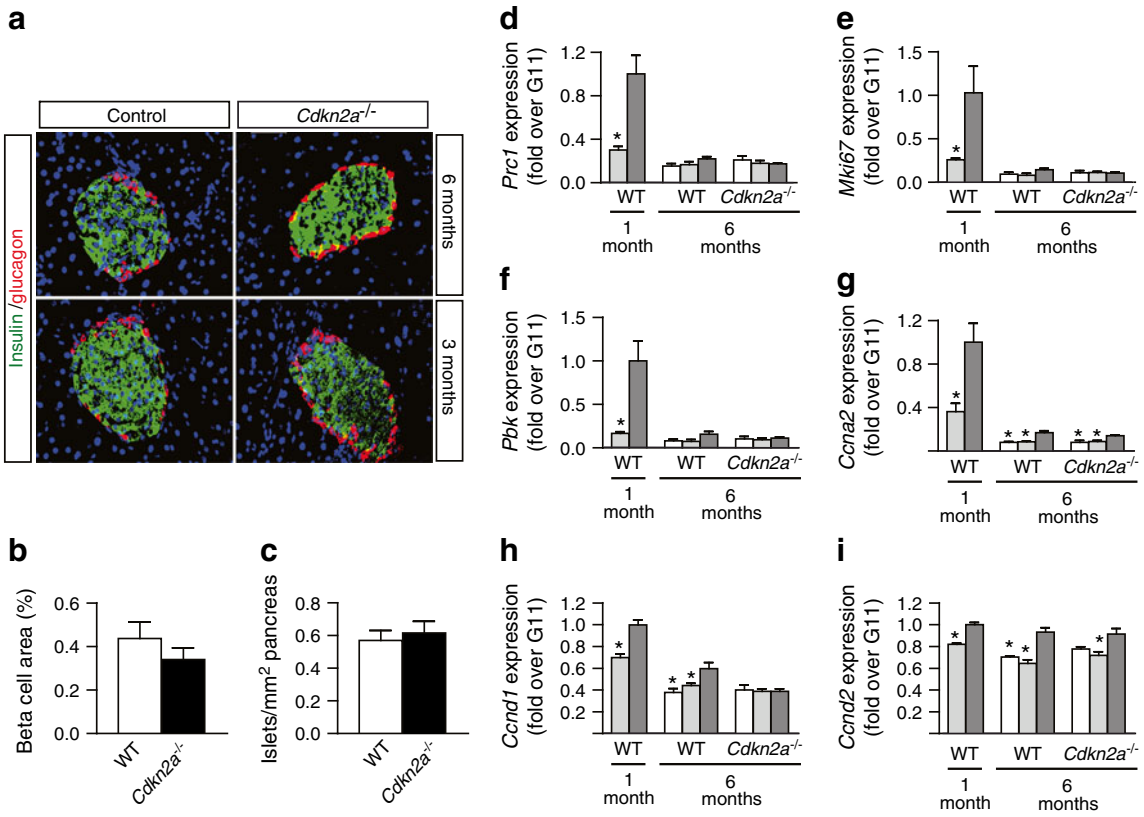

Fig. 7 Glucose induction of mitotic genes is not restored in islets from aged *Cdkn2a*$^{-/-}$ mice. (**a**) Immunostaining of insulin (green) and glucagon (red) in pancreases from 3-month-old and 6-month-old wild-type (control) and *Cdkn2a*$^{-/-}$ mice. (**b**) Beta cell area in 6-month-old wild-type (WT) and *Cdkn2a*$^{-/-}$ mice, calculated as percentage of the insulin-positive area relative to total pancreatic area. (**c**) Islet density represented as number of islets per mm^2 of pancreas. (**d**) Quantitative RT-PCR determination of mRNA levels of cell cycle genes *Prc1*, (**e**) *Mki67*, (**f**) *Pbk*, (**g**) *Ccna2*, (**h**) *Ccnd1* and (**i**) *Ccnd2* in pancreatic islets from 1- and 6-month-old wild-type control mice and 6-month-old *Cdkn2a*$^{-/-}$ mice cultured at G3 (white bars), G5 (light grey bars) and G11 (dark grey bars). Data were normalised against *Hprt1* and are shown relative to levels in islets cultured at G11, which were set arbitrarily to 1 (n=4 per group). Error bars indicate SEM; *p<0.05

glucose levels are slightly increased. In parallel, and as previously described [6, 20, 22], glucose also represses genes that have deleterious and antiproliferative effects on beta cells, such as *Ddit3* (*Chop*) and *Trib3*, with repression occurring in a similar dose-dependent manner. Our findings thus support the notion that (1) the induction of a cell cycle gene module and (2) the repression of genes that are deleterious for beta cells may contribute to the glucose-mediated increase in beta cell mass, enabling adaptation to increased systemic insulin demands.

Ageing has been associated with type 2 diabetes and beta cell dysfunction. Gene-specific and genome-wide analyses have uncovered changes in the expression of key genes for beta cells during ageing [24–27]. Unexpectedly, our gene expression profiling of islets cultured at different glucose concentrations revealed that the transcriptional response to glucose is highly similar in young and old islets. However, the ability of glucose to induce a mitotic gene module was selectively lost in aged islets. Studies performed in mice from obesity-induced diabetes-resistant and diabetes-susceptible strains revealed an age-dependent link between a cell cycle regulatory module in islets and diabetes susceptibility [49]. Strikingly, the mitotic genes that are induced by glucose in young, but not aged islets are included in such a module. This, together with the fact that glucose has been shown to play a major role in beta cell compensation in insulin resistance [10], suggests that the inability of glucose to activate mitotic genes in aged beta cells may be central to the onset of diabetes in a setting of insulin resistance.

The other gene modules regulated by glucose were maintained during ageing, as well as the glucose-induced insulin secretory response. Thus, stimulatory glucose concentrations activated islet- and beta cell-selective genes, as well as a cluster of genes shared with neurons, and repressed ubiquitous genes involved in stress responses, nutrient sensing and organelle biogenesis, a mechanism occurring in a similar way in young and old islets. These findings show that glucose signalling overall ensures an appropriate transcriptional programme in mouse pancreatic islets during ageing; they also highlight the fact that the inability of

glucose to induce mitotic genes in close correlation to glucose levels is the main defect observed in the transcriptional response to glucose in aged islets.

The proliferative capacity of beta cells has been shown to decline with age, although there is some disagreement about the extent of this process. Thus, it has recently been reported that a significant compensatory proliferation of beta cells is retained in old mice after partial ablation of beta cells or administration of a glucokinase activator [50]. In contrast, a complete loss of the mitogenic response of beta cells in response to a glucagon-like peptide-1 analogue or a high-fat diet, or after streptozotocin administration, has been described in old islets [11]. Our findings show that the mitogenic response to stimulatory glucose concentrations is also lost in aged islets. The fact that the overall glucose response is not perturbed rules out the possibility that this loss is due to global defects in glucose signalling. Indeed, old islets retain the same glucose regulation of cyclin D1 and D2, which are mainly involved in the G_1 phase of the cell cycle. This could be in part required for cyclin D-controlled functions other than regulation of the cell cycle to take place [51]. Remarkably, glucose repressed to a similar extent and in a dose-dependent manner the expression of genes with antiproliferative and apoptotic effects in young and old beta cells, such as *Trib3* and *Ddit3* (*Chop*). Thus, abrogation of the induction by glucose of the cell cycle gene module cannot be attributed to defective glucose repression of such genes.

Age-dependent increased levels of the cell cycle inhibitors $p16^{INK4a}$ and $p19^{ARF}$, which are both encoded by *Cdkn2a*, have been related to the decreased capacity of human and mouse beta cells to proliferate and regenerate with advanced age [29, 32, 33]. This age-dependent induction of *Cdkn2a* is correlated to a progressive decrease of the epigenetic regulators enhancer of zeste homologue 2 (*Drosophila*) (EZH2) and Bmi1 polycomb ring finger oncogene (BMI-1), which maintain repressive epigenetic marks at the *Cdkn2a* promoter [32, 33]. Accordingly, beta cells lacking EZH2 and BMI-1 showed a significant increase in $p16^{INK4a}$ and reduced beta cell proliferation [32, 33]. Interestingly, in EZH2-deficient beta cells, there was no change in mRNA levels of other cell cycle inhibitors [33], suggesting that EZH2 has a specific effect on the *Cdkn2a* locus. Moreover, islet-specific expression of $p16^{INK4a}$, but not of other cyclin-dependent kinase inhibitors increases markedly with ageing [29]. Despite all this evidence pointing to a critical role of *Cdkn2a* in restricting beta cell proliferation, the induction of mitotic genes by glucose was not restored in islets from aged $Cdkn2a^{-/-}$ mice. These unexpected results indicate that, in addition to a potential role for *Cdkn2a*, other mechanisms play a pivotal role in the reduced proliferative capacity of old beta cells in response to mitogenic cues such as stimulatory glucose concentrations. In line with our findings, it has recently been shown that increased gene dosage of *Ink/Arf* does not alter islet number and beta cell area during physiological ageing in mice [52], somehow contradicting a previous study in $Cdkn2a^{-/-}$ mice that reported increased Ki67-positive beta cells and enhanced islet proliferation after streptozotocin-induced beta cell ablation [29]. This discrepancy between data from a severe model of beta cell ablation and data from glucose stimulation may reflect differences between the effects of acute damage and a physiological stimulus.

In conclusion, we have uncovered a cell cycle gene module whose glucose induction in mouse islets is progressively abrogated during ageing. In contrast, the global glucose-dependent transcriptional response to glucose is broadly maintained in old islets, indicating that the signalling and transcriptional networks regulated by glucose are globally conserved during ageing. Further research is warranted to decipher the mechanisms of the age-dependent selective loss of the induction of mitotic genes by glucose, and thus to possibly identify targets for promoting beta cell adaptive proliferative responses during the early stages of diabetes in old organisms.

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Contribution statement AMA designed and performed the study, analysed and interpreted data, and wrote the manuscript. CC performed the study, analysed and interpreted data, and critically revised the manuscript. AG analysed and interpreted data and critically revised the manuscript. AN contributed to the interpretation of data and discussion, and critically revised the manuscript. JMS designed and performed the study, supervised the project, analysed and interpreted data, and wrote the manuscript. All authors approved the final version of this manuscript.

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