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Age-dependent pancreatic gene regulation reveals mechanisms governing human β -cell function

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Data Accession Numbers

The data presented in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002), under the accession number GEO SuperSeries GSE79469.

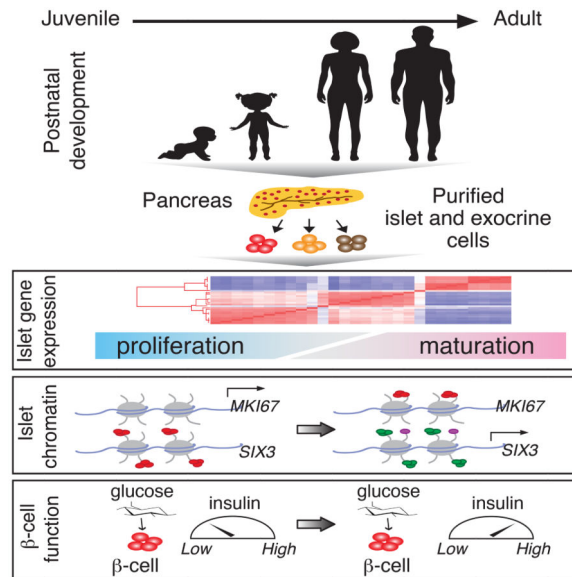
AUTHOR CONTRIBUTIONS

H.E.A., H.Y.C., and S.K.K. conceived and supervised the project, H.E.A., L.L., E.A.T., Y.R., R.C.S., C.D., X.G., H.P. and P.W. performed the experiments, M.S.S. supervised sequencing, M.G. and R.S. shared materials, R.B. and J.W. isolated human islets, J.T. and K.Q. performed statistical analysis, H.E.A., J.T., C.D., A.C.P., S.K.K. analyzed the data and wrote the manuscript.

SUMMARY

Intensive efforts are focused on identifying regulators of human pancreatic islet cell growth and maturation to accelerate development of therapies for diabetes. After birth, islet cell growth and function are dynamically regulated; however establishing these age-dependent changes in humans has been challenging. Here we describe a multimodal strategy for isolating pancreatic endocrine and exocrine cells from children and adults to identify age-dependent gene expression and chromatin changes on a genomic scale. These profiles revealed distinct proliferative and functional states of islet α -cells or β -cells, and histone modifications underlying age-dependent gene expression changes. Expression of *SIX2* and *SIX3*, transcription factors without prior known functions in the pancreas and linked to fasting hyperglycemia risk, increased with age specifically in human islet β -cells. *SIX2* and *SIX3* were sufficient to enhance insulin content or secretion in immature β -cells. Our work provides a unique resource to study human-specific regulators of islet cell maturation and function.

Graphical abstract



INTRODUCTION

The pancreas is a vital compound organ with an exocrine component comprised of acinar and duct cells that aid with digestion of nutrients, and an endocrine component which has hormone producing cells organized into structures called Islet of Langerhans, that regulate metabolism of glucose and other macromolecules (Arda et al., 2013; Benitez et al., 2012). Pancreatic islet β -cells are the sole source of insulin in the body; loss or impairment of pancreatic islet β -cells is a common cause of diabetes mellitus. Thus, the goals of human β -cell replacement therapies for type 1 diabetes (T1DM) or β -cell functional restoration in type 2 diabetes (T2DM) motivate global efforts to identify factors that regulate and stimulate β -cell insulin production and secretion.

After birth, pancreatic islet cells proliferate, expand and mature (Chen et al., 2011; Goodyer et al., 2012; Gregg et al., 2012; Meier et al., 2008), providing a physiological setting to study the genetic mechanisms underlying these crucial changes. Postnatal β -cell proliferation drives the expansion of β -cell mass, a feature that may influence T2DM risk (Butler et al., 2007). Although several cell cycle genes promoting or inhibiting human β -cell proliferation have been identified, the genetic programs controlling postnatal β -cell expansion remain poorly understood (Chen et al., 2011; Fiaschi-Taesch et al., 2009; Goodyer et al., 2012).

Glucose-stimulated insulin secretion is a hallmark feature of functional β -cells that may also change after birth (reviewed in Benitez et al., 2012). Prior work in rodents showed that, compared to adult islets, neonatal islets have blunted insulin secretion in response to glucose or other secretagogues (Aguayo-Mazzucato et al., 2011; Avrahami et al., 2015). Likewise, studies with human fetal β -cells demonstrated that glucose-stimulated insulin secretion was impaired (Otonkoski et al., 1988; Rorsman et al., 1989). While molecular analysis suggested involvement of different mechanisms including expression levels of specific transcription factors, Glucokinase and K_{ATP} channel activity (Jermendy et al., 2011; Rorsman et al., 1989; Taniguchi et al., 2000), systematic analysis of gene expression on a genomic scale during human islet cell maturation has not been reported. Moreover, assessment of functions like insulin secretion has not been systematically compared in islets from children and adults.

Regulation of gene expression is governed by several levels of molecular control, including chromatin structure and histone modifications. Studies by the ENCODE consortium and others have shown that chromatin modifications can modulate the activity or silencing of gene transcripts (The ENCODE Project, 2012). Elucidation of genome-wide distribution of histone marks can provide mechanistic insights into the regulation of gene expression including the pancreas (Bramswig et al., 2013; Pasquali et al., 2014; Zhou et al., 2013). However, the delineation of chromatin states in the context of human postnatal islet development from purified cells has not been achieved.

Here we describe a systems approach to identify age-dependent gene expression programs in human islet cells that includes (1) procurement of pancreatic tissue from children and adults, (2) developing robust and reliable cell purification methods, (3) generation of comprehensive transcriptome and histone modification maps, and (4) systematic assays of islet physiology and function. Our work generated datasets identifying several hundred genes whose expression changed with age in human islet cells, including regulators of human postnatal islet development and function.

RESULTS

Purification of pancreatic cells from juvenile and adult donors

To measure pancreatic gene expression changes with age in humans, we established an infrastructure involving a network of collaborators, which includes organ procurement organizations and islet isolation centers to obtain and isolate islets from human donors in a timely manner, including donors of young age. For this study, we targeted a rare set of juvenile donors under 9 years of age, in whom islet β -cells are expanding, permitting studies of human β -cell proliferation and function. In addition, we procured both juvenile and adult

human pancreata with uniform, strict criteria, crucial for optimizing the quality and reliability of gene expression analysis (see Experimental Procedures).

Pancreatic cells were initially separated by fluorescence activated cell sorting (FACS) into endocrine (HPi2) and exocrine (HPx1) populations (Dorrell et al., 2008, 2011) (Figures 1A–B and S1A). Unlike sorting of adult islet α -cells, however, juvenile α -cells were not separated from β -cells by existing FACS strategies using the HPA2 antibody (Bramswig et al., 2013; Dorrell et al., 2008) (Figure S1B). Prior work suggested that cluster of differentiation antigen 26 (CD26, also known as DPP4) is expressed in human α -cells (Dorrell et al., 2011), and FACS analysis revealed that juvenile or adult HPi2^{POS} islet cells from multiple donors can be successfully separated into α -cell-enriched and β -cell-enriched fractions using anti-CD26 antibody (Figures 1B and S1A). In addition, we purified CD133^{POS} duct cells and HPx1^{POS} acinar cells (Dorrell et al., 2008; Sugiyama et al., 2007) (Figures 1B and S1A). We used quantitative RT-PCR (qPCR; Figure S1C) to confirm enrichment or depletion of expected marker genes for β -cells (*INS*), α -cells (*GCG*), δ -cells (*SST*), acinar (*CPA1*) and duct cells (*KRT19*). We also performed intracellular sorting with GLUCAGON- and INSULIN-specific antibodies (Hrvatín et al., 2014) to isolate juvenile α - and β -cells (Figure 1B), and found that the molecular signatures of GCG^{POS} and INS^{POS} cells matched those of cells purified with surface antigens (Figure 1D, also see below). Thus, our FACS strategies generated highly enriched endocrine and exocrine pancreatic cell subsets from children and adult donors.

High-depth transcriptome maps of human juvenile and adult pancreatic cells

To obtain comprehensive gene expression profiles, we performed high-throughput RNA sequencing (RNA-Seq), yielding 28 expression libraries (Table S1). An average of approximately 130 million paired-end sequences per sample uniquely mapped to the human genome, with a total of >3.5 billion sequence reads (Table S1). Consistent with our initial qPCR assessment (Figure S1C), RNA-Seq transcript counts and abundance matched the sorted cell type (Figure 1C). Pearson Correlation Analysis (de Hoon et al., 2004), which measures overall similarity between samples, followed by unsupervised hierarchical clustering of these 28 RNA-Seq samples showed high correlation within each cell type. This included appropriate clustering of GCG^{POS}-cells with HPi2^{POS} HPA2^{POS} CD26^{POS} α -cells and INS^{POS}-cells with HPi2^{POS} HPA2^{NEG} CD26^{NEG} β -cells, as well as distinct clustering of acinar cells and primary pancreatic duct cells (Figure 1D). A separate correlation analysis demonstrated that our RNA-Seq data were well-matched to recent transcriptome profiling of adult human acinar and β -cells (Morán et al., 2012) (Figure S1D). Thus, our cell purification strategy generated high-quality, age-dependent gene expression profiles of human pancreatic endocrine and exocrine cells.

Age-dependent changes of cell growth, fate and function in humans are determined by intrinsic and extrinsic factors that vary greatly between individuals. Thus, as expected, we found the dispersion (a statistical measure to estimate variance; Anders and Huber, 2010) of transcript counts by age to be high. To gain additional statistical power, we combined α - and β -cell data sets into ‘juvenile’ (< 9 years) or ‘adult’ (> 28 years) age groups. Using the DE-Seq algorithm (Anders and Huber, 2010) we identified more than 500 genes whose

expression changed significantly in α - or β -cells with age (fold change > 1.5, FDR 0.2, Table S2). We computed the abundance of age-dependent transcripts in α - and β -cells for each age category and represented this as heat maps (Figures 2A and 2B). We observed a subset of genes increased in juveniles (Figure 2A) or adults (Figure 2B) that were enriched in either α - or β -cells. However, the majority of genes differentially expressed with age were shared between α - and β -cells, consistent with the view that genetic programs common to both cells regulate postnatal development (Bramswig et al., 2013). For instance, we found that *CDKN2A* (*p16*) and *CDKN2B* (*p15*), cyclin dependent kinase inhibitors with functions in cellular senescence and organismal aging (López-Otín et al., 2013), were increased in both adult α - and β -cells. Thus, our bioinformatics analysis successfully identified age-dependent gene expression changes in human islet cells.

Age-dependent gene expression in human islet cells indicates distinct functional states

Gene ontology (GO) analysis suggested molecular functions of the enriched gene sets in α - and β -cells. Genes with greater expression in juvenile α -cells and β -cells were enriched for terms related to cell cycle, proliferation and nucleotide biosynthesis, consistent with a neonatal proliferative state (Figure 2C). For example, we found genes including *MKI67*, *BUB1B*, *NUSAPI* and *CDK9*, which encode products known to regulate mitosis (M phase) and cell cycle progression. In addition, *HSPA1* and *HSPH1* are involved in the unfolded protein responses, and were also found to be significantly more abundant in juvenile islets. Consistent with these findings, immunohistology showed that proliferation marker Ki67 (encoded by *MKI67*) was increased both in juvenile α -cells and β -cells, compared to those cells in adult islets (Figures 2D and 2E).

By contrast, genes with transcripts increased in adulthood were enriched for GO terms like hormone activity, and regulation of hormone levels, consistent with physiological functions of mature endocrine islet cells (Figure 2F). When we applied the same analysis to pancreatic acinar and duct cell samples, unexpectedly, we found fewer than 20 genes whose expression significantly changed with age, and there was no enrichment for a specific GO term (Table S3, Figure S2A). Thus, our findings reveal specific gene expression programs that control endocrine cell function as humans age. Prior work in rodents has shown that insulin secretion changes with age (Aguayo-Mazzucato et al., 2011; Blum et al., 2012; Rorsman et al., 1989; Avrahami et al., 2015); however, systematic studies of human islet insulin secretion with advancing age have not been previously reported. We used in vitro perfusion to measure insulin secretion by juvenile and adult human islets used for gene expression studies (see Experimental Procedures). Compared to equivalent numbers of juvenile islets, adult human islets had higher insulin secretion when exposed to basal glucose (5.6 mM, Figures 2G–H, S2B) and high glucose (16.7 mM, Figures 2G and S2C). By contrast, total insulin content was indistinguishable in equivalent numbers of juvenile or adult islets (Figure 2I), consistent with our observation that insulin mRNA did not change detectably with age (Table S2). Together, these studies demonstrate functional differences in islet proliferation and insulin secretion between children and adults, consistent with our analysis of age-dependent gene expression in endocrine cells.

Analysis of histone marks reveals age-dependent chromatin signatures underlying gene expression changes in human islet cells

To elucidate mechanisms underlying age-dependent gene expression changes in human islets, we generated genome-wide occupancy maps of histone marks using ChIP-Seq from purified juvenile or adult pancreatic cells (Table S4). We studied the abundance of three well-defined histone modifications: H3K4me3 and H3K27ac, modifications that are known to correlate with active transcription, and H3K27me3 known to be present at inactive or silenced genomic loci (Heintzman et al., 2009; The ENCODE Project, 2012).

To evaluate the chromatin dynamics involving age-dependent gene expression in human islets, we focused on the promoter regions of genes we identified as differentially expressed with age in human α - or β -cells (Table S2). We calculated the mean ChIP-Seq signal of the three histone marks 2 kb upstream or downstream of the transcription start site (TSS) of age-dependent genes. For the 209 genes whose expression is increased in adult α - or β -cells (adult upregulated, Table S2), the ChIP-Seq signal from adult islet samples showed a significant enrichment of H3K27ac and H3K4me3 marks (Figures 3A–B, 3D–E, and S3) when compared to 356 genes whose expression is higher in juvenile samples (juvenile upregulated, Table S2). However, we did not detect substantial changes in levels of H3K27me3, a repressive modification, at the TSS of all age-dependent genes in adult samples (Figure 3F, J). Surprisingly, the ChIP-Seq results from juvenile samples did not show a similar pattern: H3K27ac and H3K4me3 levels were not detectably increased at juvenile-upregulated genes (Figures 3G–I, and S3). Rather, in juvenile islet samples we observed relative absence of the repressive H3K27me3 mark at juvenile-upregulated genes and significant enrichment of the repressive H3K27me3 mark at genes whose expression increases later in adult α - or β -cells (Figures 3C, I, J). As a further control, we randomly selected from our RNA-Seq datasets a subset of genes that are expressed in both α - or β -cells (normalized read counts >100) whose expression does not change with age (fold change < 0.5) and examined the histone ChIP-Seq signal at their TSS (Figure S3, red lines). We found that in juvenile samples the control gene set has substantially higher H3K4me3, H3K27ac and low H3K27me3 signal, correlating with their gene expression status. Thus, our genome-wide histone maps revealed evidence for distinct modes of histone-mediated regulation of age-dependent genes in juvenile and adult human islet cells (Figure 3J).

Regulators of age-dependent islet gene expression programs and their linkage to diabetes

Transcription factors (TFs) are crucial regulators of islet development and disease (Conrad et al., 2014). For instance, mutations in 9 of 15 genes that have been linked with maturity-onset diabetes of the young (Sellick et al., 2004) (MODY) or pancreatic agenesis (Lango Allen et al., 2012) encode TFs, like *PDX1*, *HNF1 β* , *NEUROD1*, *PTF1A* and *GATA6*. Comparison of age-regulated genes found here with a human TF compendium (Weirauch et al., 2014) revealed 25 TFs, most of which have not been yet linked to diabetes or studied in the context of islet development and function (Table S2). We analyzed the mean expression levels of each TF in juvenile or adult α - or β -cells, and identified several TFs whose expression increased with age specifically in β -cells, such as *ONECUT2*, *MAFA*, *TSHZ3*, *SIX2* and *SIX3* (Figure 4A). Prior work in rodents has shown that Mafa and Mafb regulate β -cell maturation by controlling genes that function in insulin synthesis, secretion and glucose-

sensing (Aguayo-Mazzucato et al., 2011; Artner et al., 2007, 2010). Our analysis reveals age-dependent expression of *MAFA* specifically in human β -cells; by contrast, *MAFB* is expressed both in α - and β -cells at levels that do not detectably vary with age (Figure 4A).

In addition to mRNAs encoding proteins, we identified over 50 non-coding RNAs whose expression changed with age in α - and/or β -cells (Table S2). In six cases, the lncRNA is adjacent to a protein-coding gene whose expression also changes with age. For example, the long non-coding RNA *lnc-PCSK1-1* neighbors *PCSK1* which encodes a proprotein convertase essential for proinsulin processing (Figure 4B). While *PCSK1* mRNA increased with age in β -cells, *lnc-PCSK1-1* was most abundant and increased with age in α -cells. A single nucleotide polymorphism (SNP, rs13179048) was previously associated with *PCSK1* in a genome-wide association study (GWAS) (Manning et al., 2012) examining fasting glucose levels and we found that this SNP is substantially closer to *lnc-PCSK1-1* (6 kb) in the genome than to *PCSK1* (160 kb). Thus, our findings suggest that *lnc-PCSK1-1* deserves consideration in evaluating the role of this locus to disease risk.

GWAS reports have identified potential causal genetic variants associated with diabetes or related metabolic traits, such as fasting glucose impairment or altered fasting insulin levels. We compared genes in reported GWAS loci linked to these traits to genes whose expression changed with age in α -cells and β -cells; our analysis revealed significant enrichment of genes increased in adult β -cells or α -cells associated with risk for pre-diabetes phenotypes or diabetes (Figure 4C). Notably, our analysis revealed the TFs *SIX2* and *SIX3*, which are encoded at loci previously linked by GWAS to impaired fasting glucose (Kim et al., 2011), but whose roles in β -cells has not been reported. Thus, we next analyzed *SIX2* and *SIX3* function in human β -cells.

***Sine Oculis* homeobox (*SIX*) family transcription factors are human β -cell regulators**

Based on their increased expression in adult β -cells, we postulated that *SIX2* and *SIX3* could regulate key age-dependent features of β -cells, such as insulin production or secretion. *SIX2* and *SIX3* belong to the *Sine Oculis* family of homeodomain TFs and have functions in kidney, forebrain and eye development (Kumar, 2009). We developed immunohistology methods to detect *SIX2* and *SIX3* protein (Figure S4A), and demonstrated that *SIX2* and *SIX3* localized to the nucleus of adult β -cells (Figures 5A–B, identified by co-expression of *INS* and *PDX1*). By contrast, we did not detect *SIX2* or *SIX3* in juvenile β -cells (Figures 5A–B), consistent with our RNA-Seq results. To test the function of *SIX2* and *SIX3*, initially we used a human β -cell line, EndoC- β H1, derived from human fetal pancreas by simian virus 40 large T-Antigen transformation (Ravassard et al., 2011). EndoC- β H1 cells proliferate, have modest insulin content and secretion, and do not express detectable *SIX2* or *SIX3* mRNA (Figure S4B), features characteristic of immature β -cells. Using a lentivirus system, we generated EndoC- β H1 cells that stably express *SIX2*, *SIX3*, or GFP alone (Figure S4A). Compared to controls, *SIX3*-producing cells had increased insulin content (Figure S4C). Upon glucose challenge, both *SIX2*- and *SIX3*-producing cells secreted significantly more insulin than controls (Figure S4D). We assessed the expression of *PDX1*, *NKX6.1* and *MAFA* mRNA but found no significant increase of these by *SIX2*- and *SIX3*-producing cells (Figure S4E). These findings indicate that *SIX2* and *SIX3* are β -cell specific

factors sufficient to enhance insulin production and secretion, possibly in parallel to other TFs known to regulate β -cell functional maturation.

Recent studies showed that conditional deletion of T-Antigen reduced proliferation in the EndoC- β H2 cell line (Scharfmann et al., 2014), a change accompanied by enhanced insulin production and glucose-stimulated insulin secretion (GSIS). Using specific siRNAs, we knocked down T-Antigen efficiently in EndoC- β H1 cells (hereafter EndoC- β H1^{TKD}), and observed markedly reduced Ki67 production (Experimental Procedures; see also Figures S4F–G), enhanced insulin production and glucose-stimulated insulin secretion (Figure 5C–D). Thus, EndoC- β H1^{TKD} cells permitted assessment of SIX2 or SIX3 effects in non-proliferating β -cells after T-Antigen knock down. We found insulin content and secretion were not detectably altered by SIX2 expression in EndoC- β H1^{TKD} cells (Figure 5C). By contrast, SIX3 expression increased insulin content and secretion of EndoC- β H1^{TKD} cells (Figure 5C–D).

To gain further insights into β -cell gene regulation by SIX2 and SIX3, we performed RNA-Seq experiments in EndoC- β H1^{TKD} cells stably expressing these factors. Comparison of EndoC- β H1^{TKD} cells expressing SIX3 to control cells expressing GFP revealed over 200 differentially expressed genes (\log_2 fold change > 0.5, FDR 0.2, Table S5). A significant number of these overlapped with human islet age-dependent genes ($P < 0.001$, Chi-square test with Yates' correction, Figure S5A), with enrichment of GO terms such as cell-cell signaling, growth factor and calcium binding (Figure S5B). Similar analysis of SIX2^{POS} EndoC- β H1^{TKD} cells revealed a much smaller set of genes, and there was no significant overlap with the islet age-dependent genes. This suggests that the gene expression differences we observe in SIX3^{POS} EndoC- β H1^{TKD} cells likely reflect SIX3 activity.

SIX3 functional studies in primary juvenile human islets

To assess the function of SIX3 in primary human islets, we developed lentiviral methods for expressing transgenes in human primary islet cells (Figure 5E, see Experimental Procedures). Pancreatic islets can be dispersed to single cell suspensions for efficient viral transduction; these cells spontaneously re-aggregate into 'pseudo-islet' clusters (Hopcroft et al., 1985; Zaldumbide et al., 2013). Using this approach we detected marker gene production (GFP, Figure 5F) in 70–80% of islet cells after dispersion, lentiviral transduction and re-aggregation. Insulin secretion stimulated by a step-increase of glucose or glucose + IBMX was robust in transgenic GFP^{POS} pseudo-islets (Figure 5G), demonstrating that fundamental mechanisms regulating insulin secretion remained intact in pseudo-islets.

To test the prediction that SIX3 expression could enhance insulin secretion in juvenile islet cells, we obtained islets from juvenile donors and generated pseudo-islets that were transduced with lentivirus carrying either a control GFP or SIX3 expression construct. Using qPCR we found that juvenile pseudo-islets transduced with GFP virus, as expected, did not have detectable *SIX3* mRNA, whereas juvenile pseudo-islets transduced with SIX3 virus produced *SIX3* mRNA at levels comparable to adult pseudo-islets transduced with GFP virus (Figure 5H). We assessed insulin secretion by juvenile pseudo-islets and found that compared to GFP controls, SIX3 mis-expression significantly enhanced insulin release at basal glucose levels (2.8 mM), while stimulated secretion levels remained similar or

improved (Figure 5I). These findings are consistent with our perfusion studies of primary islets from children and adults (Figure 2D). Taken together, our gain-of-function experiments using either EndoC- β H1 cells or primary juvenile human islets provide evidence that expression of *SIX2* or *SIX3* were sufficient to enhance cardinal functions of human β -cells.

DISCUSSION

Here we present the transcription and chromatin landscape of age-dependent human pancreatic cell maturation, based on organ procurement and flow cytometry methods to isolate primary cells from adult and rare child donors. Our study generated a unique gene expression atlas and maps of major histone modifications during postnatal development and maturation of human pancreatic endocrine cells. Thus, our study provides several resources that should impact understanding of islet genetics and signaling relevant to metabolism, diabetes and regenerative biology.

Prior studies have described the purification of pancreatic cells using either cell surface antibodies, non-specific zinc-chelating dye, or cell fixation and intracellular labeling (Blodgett et al., 2015; Dorrell et al., 2011; Hrvatin et al., 2014; Morán et al., 2012). However, none of these studies enriched for cells obtained from juvenile islets. Here, we used surface marker-based purification of islet cell subtypes from juvenile as well as adult donors, with appropriate enrichment of cells in each isolated fraction. Our method complements existing protocols for islet cell sorting and will be particularly useful for future applications that require live, native cells, such as open chromatin analysis (Buenrostro et al., 2013) or derivation of cell lines (Scharfmann et al., 2014).

Our data identified several hundred genes whose expression changes with age in human islet cells, most without known functions in the pancreas. We showed that the age-dependent gene expression programs are dynamic in endocrine α -cells and β -cells, and do not represent a general feature of aging cells. Genes with age-dependent expression cluster in classes with different functions, showing that postnatal development and maturation of islet endocrine cells are highly dynamic and involve multiple distinct molecular and signaling pathways. Our observation that genes like *SIX2* and *SIX3* may regulate hallmark features of β -cells such as insulin content or secretion, but are not expressed for at least several years after birth, suggests that the duration of human β -cell postnatal development may be longer than previously considered (McKnight et al. 2011; Arda et al., 2013). Our studies suggest that insulin secretion increases with age in human β -cells. However, definitive evidence establishing increased β -cell insulin secretion with advancing age, and when this occurs in postnatal life, will require data from a larger set of human donors than achieved here.

Our comparative analysis of histone modifications from human juvenile and adult islet cells provides insights about the chromatin dynamics of age-dependent genes. Chromatin marks associated with active gene expression, like H3K4me3 and H3K27ac, are significantly enriched in adult samples for adult-upregulated genes, but not in juvenile samples for juvenile-upregulated genes. Conversely, the H3K27me3 repressor mark is enriched at the adult-upregulated genes in juvenile samples but not in adult samples. These findings suggest

that the Polycomb repressor complex (PRC2) establishes and maintains the H3K27me3 modification in juvenile islet cells to suppress expression of adult-specific genes. Thus, PRC2 might regulate the maturation of human β -cell function observed in our studies. During adulthood, the abundance of the repressive H3K27me3 mark at specific genes is reduced and the expression of adult-upregulated genes coincides with increased levels of H3K4me3 and H3K27ac at those loci. Trithorax Group (TrxG) proteins regulate H3K4me3 methylation in β -cells (Zhou et al., 2013); thus our findings further suggest that the TrxG controls age-dependent gene expression and maturation in human α - and β -cells.

One aspect of our analysis included TFs, a class of genes prior studies have shown are crucial regulators of pancreas and islet development (reviewed in Benitez et al 2012; Arda et al 2013). Comparatively less is known about TF regulation of postnatal human islet cell growth and maturation. Our analysis revealed age-dependent expression of more than a score of TFs, and some factors, like *MAFA*, *SIX2* and *SIX3*, showed age-dependent induction specifically in β -cells. *SIX2* and *SIX3* were sufficient to enhance cardinal features of human β -cells like insulin content or secretion. Our gain-of-function studies also suggest that *SIX2* and *SIX3* may have distinct roles and are not functionally redundant, consistent with findings in other settings (reviewed by Kumar, 2009).

From GWAS studies of T1DM, T2DM and prediabetes, over 100 risk loci have emerged, but the function of candidate risk genes identified through these efforts has been elusive (Rutter, 2014). Unexpectedly we show here that genes whose expression in islet cells is age-dependent, like *SIX2*, *SIX3*, *BACE2* and *DLK1*, are significantly enriched in gene sets linked by GWAS to T1DM and T2DM risk. *DLK1* encodes a transmembrane protein containing Epidermal Growth Factor motifs with possible roles in Notch and MAP Kinase signaling (Wang et al., 2015). Similar to *SIX2*, *SIX3* and *BMP5* mRNA, *DLK1* mRNA was highly enriched in β -cells and levels were increased in adult islets. In transgenic mice, mis-expression of *Dlk1* in β -cells increased insulin production, β -cell mass, and insulin secretion (Wang et al., 2015). Findings and resources generated in this study, including stable *SIX3*-expressing human β -cell lines, should advance further studies to delineate the molecular mechanisms linking *SIX3* and *DLK1* functions to human β -cells. Expression of the mouse homologues *Six2*, *Six3* and *Dlk1* was not detected in adult mouse β -cells (Benitez et al., 2014; Benner et al., 2014; Kalousova et al., 2010; Ku et al., 2012; Martens et al., 2014), highlighting the importance of the human-specific approaches reported here.

Generating replacement islet β -cells from renewable sources such as embryonic stem cells, or non-islet cells, including exocrine duct cells is an intensive area of research (Lee et al., 2013; Pagliuca et al., 2014; Reznica et al., 2014; Russ et al., 2015). These approaches have been largely guided by findings from non-human experimental systems, especially mouse pancreas developmental biology. While recent progress toward this goal has been made, progeny from these produce insulin at levels far below those measured in native β -cells, and fail to recapitulate regulated insulin secretion characterized in islet β -cells. Our work provides a crucial genetic framework describing innate human-specific gene expression changes that control functional maturation of human islet cells. We speculate that integration of findings from this study into future β -cell engineering approaches could enhance

development of insulin-producing cells from stem cell sources, including improvements in the quality and levels of insulin production and regulated insulin secretion.

EXPERIMENTAL PROCEDURES

Human Pancreas and Islet Procurement

All studies involving human pancreas or islets were conducted in accordance with Stanford University Institutional Review Board guidelines. De-identified human pancreata, islets or acinar tissues were obtained from healthy, non-diabetic organ donors with BMI<30, less than 15 hours of cold ischemia time, and deceased due to acute trauma or anoxia. Organs and islets were procured through Integrated Islet Distribution Network (IIDP), National Diabetes Research Institute (NDRI), and International Institute for the Advancement of Medicine (IIAM). For FACS, RNA-Seq and ChIP-Seq studies, islets from eight adult donors (ages 28, 33, 40, 42, 48, 54, 66 years) and seven juvenile donors (ages 0.5, 0.8, 1.4, 1.5, 5, 5, 6 years) were used (also see Tables S1 and S4). For perfusion assays, islets from 16 adult donors (ages 19, 20, 20, 23, 29, 38, 39, 40, 43, 43, 43, 48, 50, 51, 60, 60 years) and nine juvenile donors (ages 0.5, 1.2, 1.6, 3, 5, 5, 6, 9 years) were used. For total insulin content and corresponding basal secretion, islets from ten adult donors (ages 20, 20, 24, 28, 31, 37, 51, 53, 55, 65 years) and three juvenile donors (ages 4, 6, 9 years) were used. For immunohistologic analysis of proliferation, pancreata from four adult (ages 27, 30, 31, 37 years) and four juvenile donors (ages 1, 2, 3, 4 years) were used. For pseudo-islet studies islets from 0.8-, 2-, 42-year-old donors were used.

Islet Perfusion Assays, Insulin Content Analysis

For each assay 60 size-matched islets (approximately 100 islet equivalents [IEQ]) from juvenile (n=9) or adult (n=16) donors were perfused in an islet perfusion apparatus using two secretagogues. Insulin secretion normalized to IEQ using the method described (Dai et al., 2012; Kayton et al., 2015). To eliminate confounding effects that may stem from islet viability or fitness, only islet samples that matched a defined 'normal profile' were considered for this study (Kayton et al., 2015). Basal insulin was defined by the insulin concentration of the last fraction before introduction of 16.7 mM glucose. For insulin content analysis, 60 adult or juvenile islets of 180 μ m diameter were processed (100 IEQs) as detailed in Kayton et al., 2015. Insulin was quantified using a human insulin ELISA kit following the manufacturer's protocol (10-1113-10, Merckodia, Inc).

Pseudo-islet generation and GSIS Assays

Juvenile islets were dispersed into monolayer cell suspension using enzymatic digestion (Accumax, Invitrogen). For each experimental group, approximately 1×10^6 cells were transduced with lentivirus corresponding to 1×10^9 viral copies in 1 mL as determined by the Lenti-X qPCR titration kit (Clontech, 631235). Transduced islet cells were seeded in ultra-low attachment 96-well plates (Corning) and cultured at 37°C with 5% CO₂ in medium containing 10% fetal bovine serum (Hyclone), RPMI 1640 (Gibco), 2.25 g/dL glucose, 1% penicillin/streptomycin (v/v, Gibco). After 24 hours, the islet cells were pooled into 6-well ultra-low attachment plates and maintained under the same culture conditions for seven days. At the day of GSIS, 30–40 pseudo-islets were handpicked and pre-incubated in KRBB

without glucose for one hour, which was repeated three times, for a total of three hours. After the initial equilibration, stimulated insulin secretion was performed by static incubation of pseudo-islets for one hour in KRBB containing 2.8 mM (basal) followed by 16.7 mM (high) glucose, and 16.7 mM (high) glucose +IBMX. Pseudo-islets were then lysed in acid-ethanol solution to extract the total insulin content. Human insulin from supernatants and pseudo-islet lysates were quantified using a human insulin ELISA kit (Merckodia Inc., Sweden).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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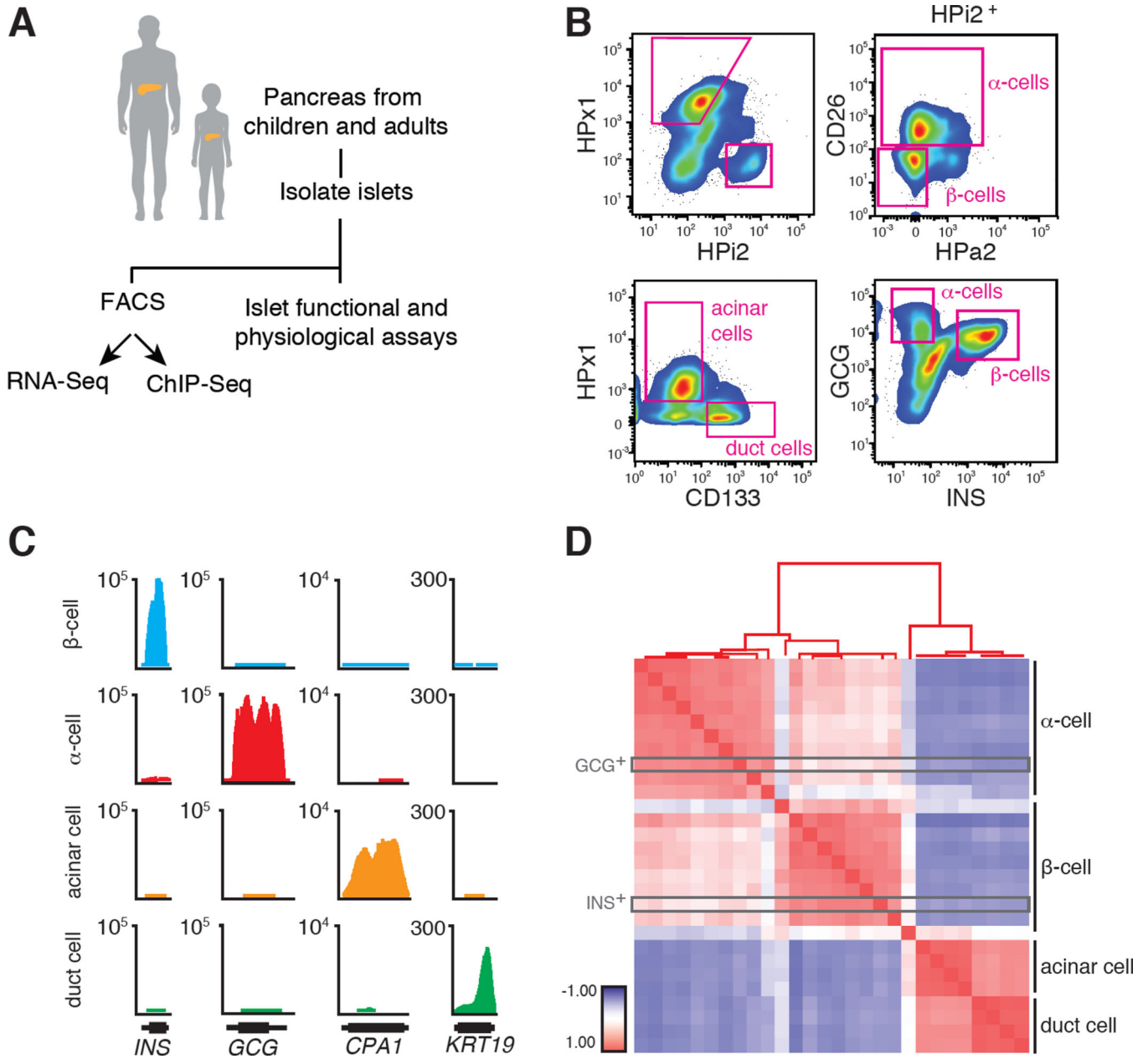


Figure 1. Cell purification approach to isolate native juvenile and adult islet cell subpopulations
 (A) Schematic summary of this study design. (B) FACS plots showing the distribution of distinct pancreatic cell populations. (C) RNA-Seq tracks representing the enrichment of transcript reads in *GCG*, *INS*, *CPA1* and *KRT19* loci. Gene model (black, 5' to 3') excludes introns. (D) Pearson Correlation Coefficient matrix of all RNA-Seq samples used in this study. +1 indicates perfect correlation (red), 0 indicates no correlation (white), -1 indicates anti-correlation (blue). The positions of intracellular sorted juvenile samples are boxed in grey.

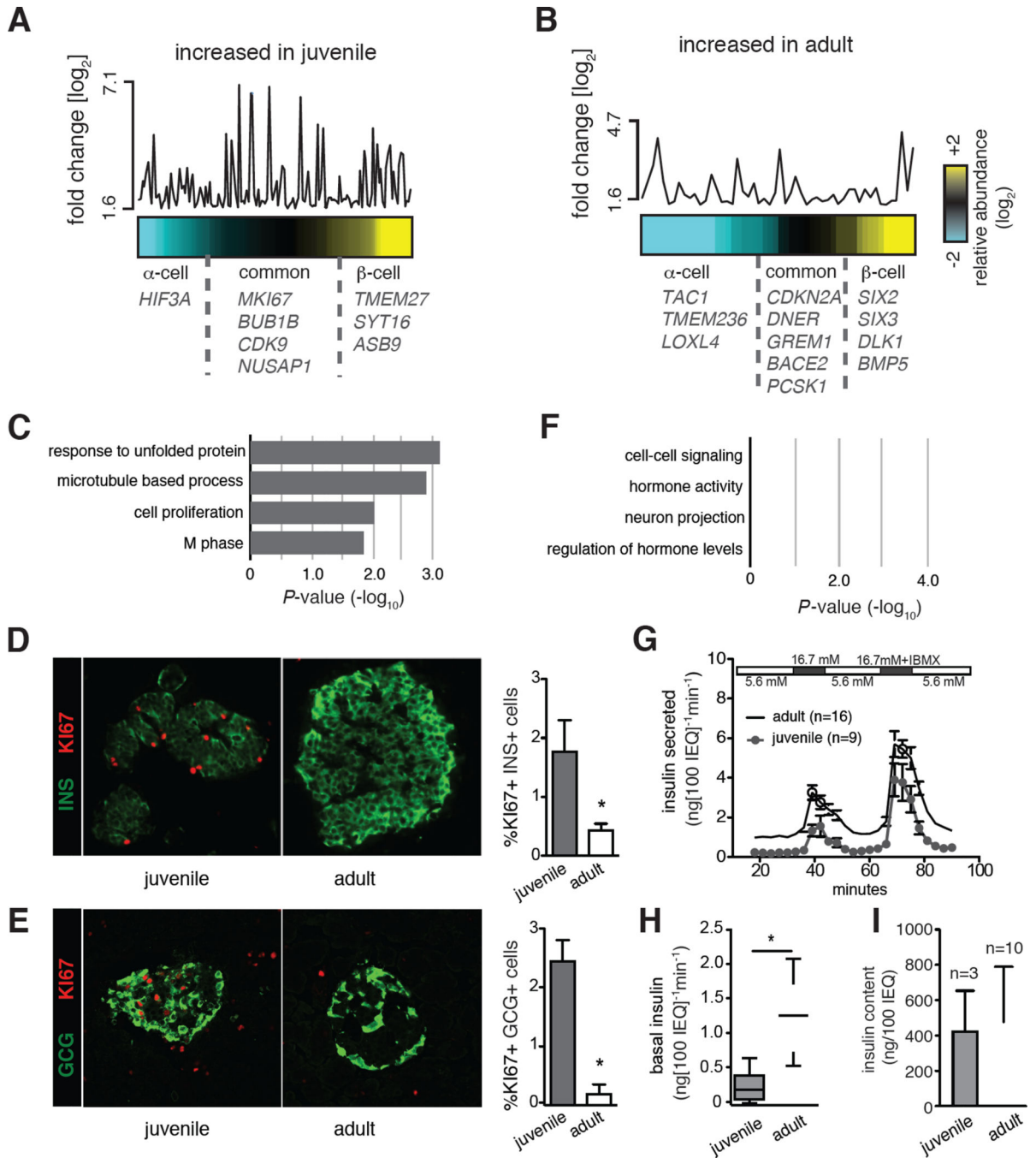


Figure 2. Age-dependent gene expression and functional changes in human islet cells

Heat maps representing the relative abundance of age-dependent genes increased either in juvenile (A) or adult (B) samples. The y-axis shows the fold change of expression between different age groups. Each point corresponds to an age-dependent gene and its position is aligned with the column position in the heat map. Age-dependent genes more abundant in β -cells are colored yellow, in α -cells colored cyan, equal abundance is represented in black. Names of select genes are indicated on the plots. For the full list, see Table S2. (c) GO Term enrichment analysis of genes increased in juvenile (C) or adult (F) samples. Top scoring

biological process terms are graphed. Immunostaining for KI67 and (D) Insulin (INS) or (E) Glucagon (GCG) in juvenile (2-year-old) and adult (31-year-old) pancreatic sections. Quantifications on the right (* t-test $P < 0.05$). Error bars indicate S.D. (G) Dynamic GSIS results of perfused human juvenile (n=9) and adult (n=16) islets, IEQ: islet equivalent. (H) Box plots show secreted insulin levels of juvenile (n=9) and adult (n=16) islets in the last fraction exposed to 5.6 mM glucose (basal) before a step increase to 16.7 mM glucose. (I) Insulin content of equivalent numbers of juvenile (n=3) and adult islets (n=10). Also see Experimental Procedures (* t-test $P < 0.05$; Error bars indicate S.E.).

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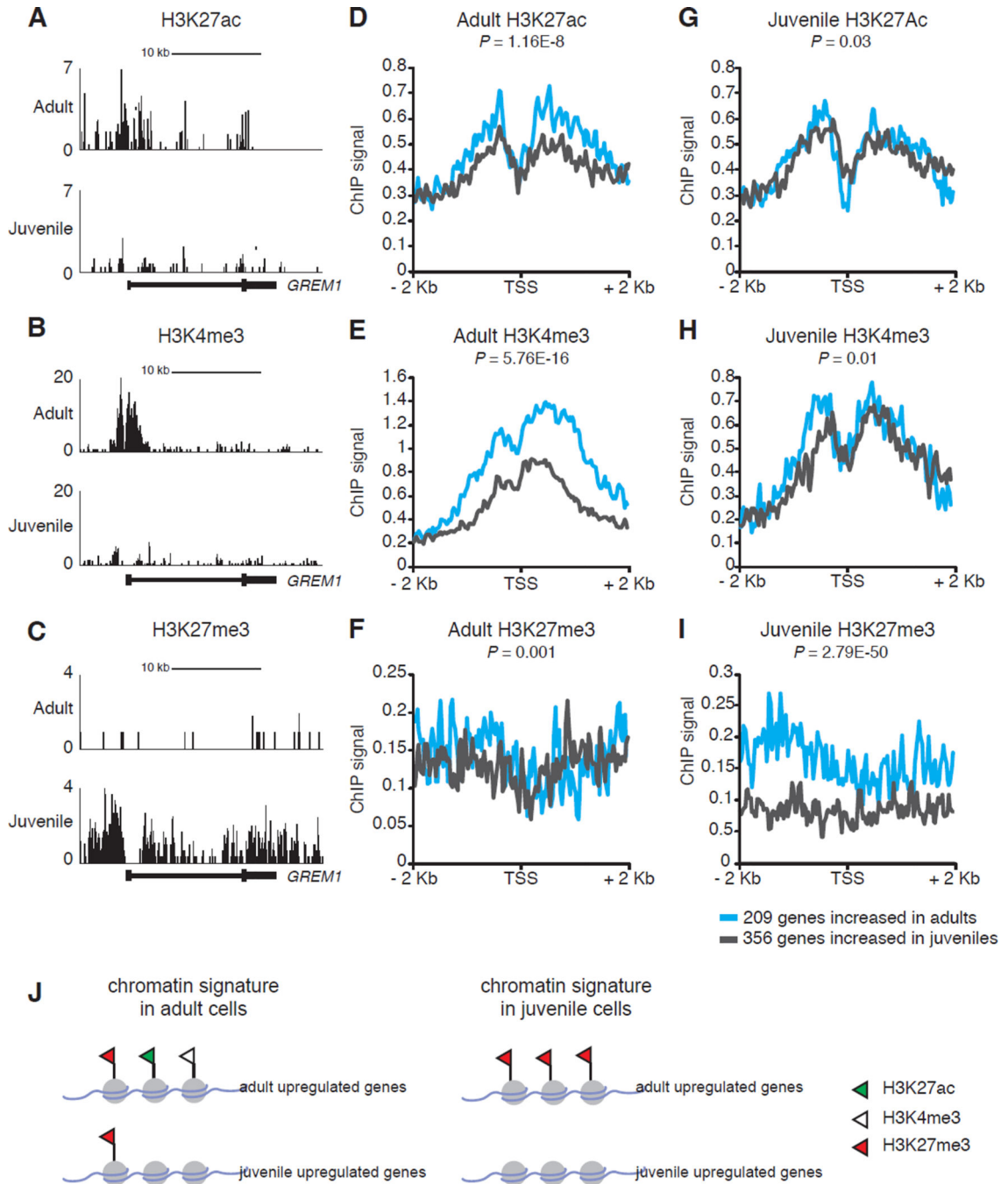


Figure 3. Histone ChIP-seq maps reveal distinct modes of chromatin modifications associated with age-dependent genes in human islets

UCSC browser tracks showing (A) H3K27ac, (B) H3K4me3, (C) H3K27me3 normalized ChIP-Seq signal at the *GREM1* locus. (D–I) Plots represent mean aggregate signal 2 kb upstream or downstream around the transcriptional start site (TSS) of age-dependent genes that are increased in adult (cyan lines, total of 209 genes) or juvenile samples (grey lines, total of 356 genes). For a complete list of genes see Table S2. Histone ChIP-Seq signals obtained from (D–F) 48-year-old adult donor, (G) 0.8-year-old and (H–I) 0.5-year-old juvenile donors. Wilcoxon rank sum test was used to calculate the P values. (J) Schematic

depicting histone modifications found at genes expressed in an age-dependent manner in juvenile and adult islet samples.

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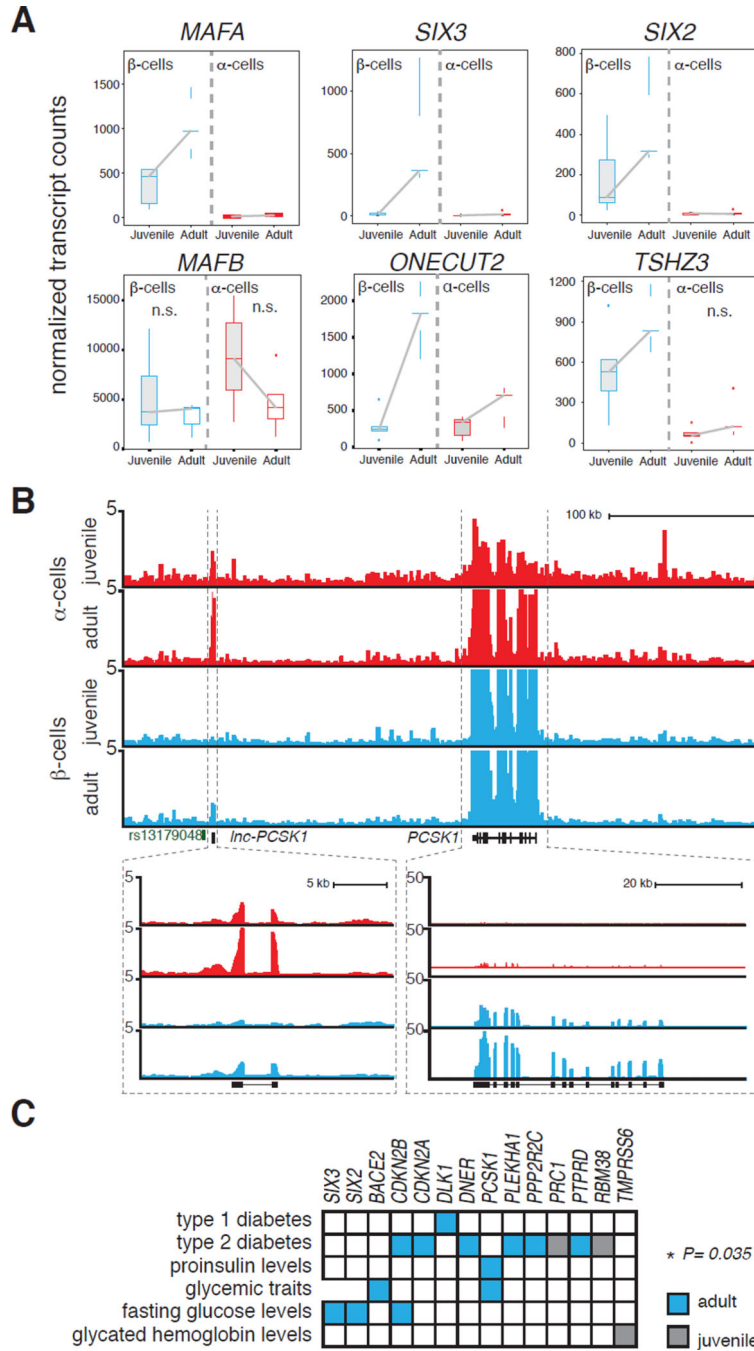


Figure 4. Age-dependent islet genes are enriched for genes linked to diabetes and related metabolic traits

(A) Box plots displaying normalized transcript counts of TFs whose expression changes significantly with age (except for *MAFB*) in human α - or β -cells, (n=5 juvenile, n=5 adult). (B) RNA-Seq profiles of α - and β -cells from juvenile and adult samples highlighting the *PCSK1* and *IncPCSK1-1* locus. (C) Matrix indicating the overlap of age-dependent genes and GWAS genes linked to diabetes and associated metabolic traits (* Chi-square test with Yates' correction).

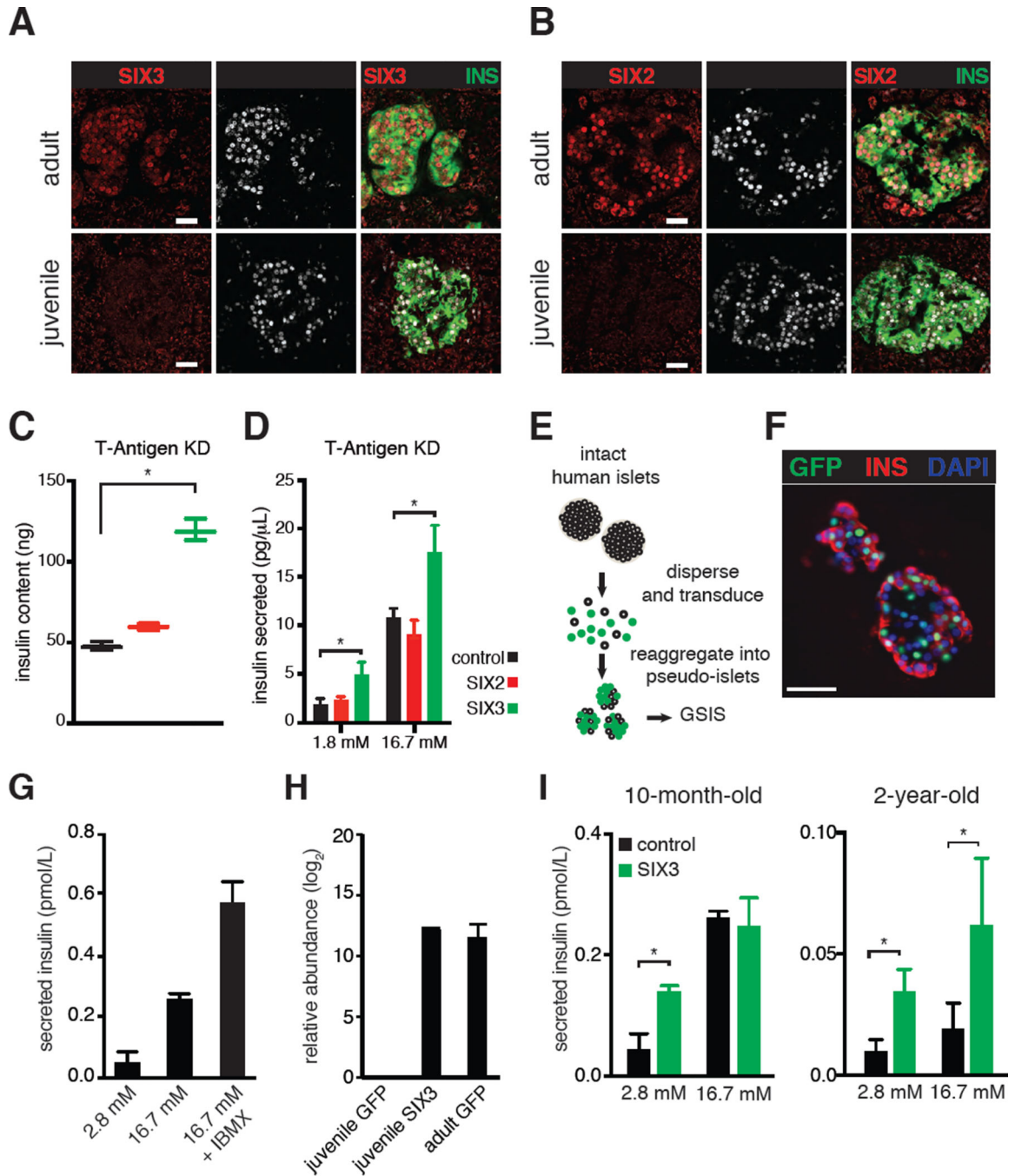


Figure 5. SIX3 and SIX2 increase with age specifically in human β -cells and enhance β -cell maturation

SIX3 (A) and SIX2 (B) immunostaining of adult (31-year-old) and juvenile (4-year-old) human pancreas sections, scale bar: 100 μ m. Insulin content (C) and secreted insulin levels (D) of EndoC- β H1 cells expressing *GFP*, *SIX2* or *SIX3* without T-Antigen (T-Antigen KD). Asterisks indicate statistically significant results reproduced in multiple experimental replicates ($P < 0.05$, two-way ANOVA followed by Fisher's Least Significant Difference test). Bars indicate S.D. (E) Schematic detailing pseudo-islet techniques. Human islet cells are enzymatically dispersed, and transduced with lentivirus which co-expresses a GFP

transgene. 5–7 days after dispersion, islet cells spontaneously re-aggregate into pseudo-islet clusters can be assayed using glucose-stimulated insulin secretion (GSIS) in vitro. (F) Representative immunohistologic images of human pseudo-islets stained with Insulin, GFP or DAPI, scale bar: 50 μm . (G) Bar graphs show secreted insulin levels of cultured human pseudo-islets exposed to glucose or glucose +IBMX. Secreted insulin is normalized to total insulin content. (H) Bar graphs indicate relative abundance of *SIX3* transcript detected in pseudo-islets after transduction with GFP or *SIX3* lentiviral vectors. (I) Graphs show secreted insulin levels from cultured pseudo-islets obtained from juvenile donors, expressing GFP alone (control) or *SIX3*. Secreted insulin is normalized to total insulin content (* $P < 0.05$, t -test). Error bars indicate S.D.