An Integrated Cell Purification and Genomics Strategy Reveals Multiple Regulators of Pancreas Development

Cecil M. Benitez1, Kun Qu2, Takuya Sugiyama3, Philip T. Pauerstein1, Yinghua Liu1, Jennifer Tsai1, Xueying Gu1, Amar Ghodasara1, H. Efsun Arda1, Jiajing Zhang2, Joseph D. Dekker3, Haley O. Tucker3, Howard Y. Chang2,4,5, Seung K. Kim1,5,6*

1 Department of Developmental Biology, Stanford University School of Medicine, Stanford, California, United States of America, 2 Program in Epithelial Biology, Stanford University School of Medicine, Stanford, California, United States of America, 3 Institute for Cellular and Molecular Biology and Department of Molecular Biosciences, University of Texas at Austin, Austin, Texas, United States of America, 4 Department of Dermatology, Stanford University School of Medicine, Stanford, California, United States of America, 5 Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California, United States of America, 6 Department of Medicine (Oncology Division), Stanford University School of Medicine, Stanford, California, United States of America

Abstract

The regulatory logic underlying global transcriptional programs controlling development of visceral organs like the pancreas remains undiscovered. Here, we profiled gene expression in 12 purified populations of fetal and adult pancreatic epithelial cells representing crucial progenitor cell subsets, and their endocrine or exocrine progeny. Using probabilistic models to decode the general programs organizing gene expression, we identified co-expressed gene sets in cell subsets that revealed patterns and processes governing progenitor cell development, lineage specification, and endocrine cell maturation. Purification of Neurog3 mutant cells and module network analysis linked established regulators such as Neurog3 to unrecognized gene targets and roles in pancreas development. Iterative module network analysis nominated and prioritized transcriptional regulators, including diabetes risk genes. Functional validation of a subset of candidate regulators with corresponding mutant mice revealed that the transcription factors Etv1, Prdm16, Runx1t1 and Bcl11a are essential for pancreas development. Our integrated approach provides a unique framework for identifying regulatory genes and functional gene sets underlying pancreas development and associated diseases such as diabetes mellitus.

Introduction

The pancreas is a vital internal organ with exocrine and endocrine cells. The exocrine pancreas is composed of acinar cells that secrete digestive enzymes into a branched network of bicarbonate-secreting duct cells. Endocrine cell forms clusters called islets of Langerhans that secrete hormones such as insulin, glucagon, pancreatic polypeptide, somatostatin, and ghrelin produced, respectively, by beta cells, alpha cells, PP cells, delta cells and a transient population of epsilon cells [1]. Classical genetic approaches revealed that exocrine and endocrine cells develop from a common multipotent progenitor that expresses the transcription factors Sox9 [2] [3], Pdx1 [4], and Ptf1a [5]. Through mouse embryonic development, Sox9+ multipotent progenitors generate endocrine progenitors that express the basic helix-loop-helix (bHLH) transcription factor Neurog3 [6], which produce all pancreatic endocrine cells [7]. Although these approaches have revealed much about individual factors that regulate pancreatic development [8], we have yet to understand the regulatory logic underlying pancreas formation [9].

Genome-scale approaches to organ development can provide unbiased views of the genetic interactions regulating transient cell populations such as multipotent progenitor cells and their lineage-restricted progeny. However, to decipher the regulatory logic that culminates in successful organogenesis, gene expression in distinct, developing cell types must be acquired. Then, algorithms that analyze expression patterns across multiple cell types can be executed. Such integrated efforts have been used successfully for studies of hematopoiesis [10] [11]. We postulate that similar approaches to identify gene sets orchestrating formation and maturation of pancreas cells should advance islet beta cell-replacement efforts in diabetic patients. However, the application of such an approach to solid organs like the pancreas has proven difficult. In particular, crucial transient cell subsets like multipotent pancreatic progenitors and endocrine precursors represent a small fraction of cells in the developing pancreas – making analysis of these cells challenging. Thus, while prior studies used cell fractionation and genome-scale analysis of gene expression to advance understanding of pancreas development [12] [13] [14] [15] none was able to comprehensively assess purified progenitors.
Author Summary

Discovery of specific pancreas developmental regulators has accelerated in recent years. In contrast, the global regulatory programs controlling pancreas development are poorly understood compared to other organs or tissues like heart or blood. Decoding this regulatory logic may accelerate development of replacement organs from renewable sources like stem cells, but this goal requires identification of regulators and assessment of their functions on a global scale. To address this important challenge for pancreas biology, we combined purification of normal and mutant cells with genome-scale methods to generate and analyze expression profiles from developing pancreas cells. Our work revealed regulatory gene sets governing development of pancreas progenitor cells and their progeny. Our integrative approach nominated multiple pancreas developmental regulators, including suspected risk genes for human diabetes, which we validated by phenotyping mutant mice on a scale not previously reported. Selection of these candidate regulators was unbiased; thus it is remarkable that all were essential for pancreatic islet development. Thus, our studies provide a new heuristic resource for identifying genetic functions underlying pancreas development and diseases like diabetes mellitus.

and their endocrine or exocrine progeny from multiple developmental stages. Moreover, most previous studies were limited to pair-wise comparisons of wild-type cells in the endocrine lineage, thereby precluding powerful inferences from mutant analysis, and none prioritized or validated multiple candidate regulators by phenotyping mutant mice.

Here, we used a combination of cell sorting and transgenic cell labeling to purify and profile twelve pancreatic cell types at specific stages of development and used methods to optimize RNA quantification from relatively small numbers of cells. Our data set encompasses multipotent Sox9\(^+\) pancreatic progenitors, Neurog3\(^+\) endocrine progenitors, fetal and adult alpha cells and beta cells, and exocrine cells including fetal acinar cells and adult duct cells. Statistical comparisons demonstrate the highly reproducible quality of gene expression profiles obtained. We found that iterative probabilistic modeling, optimized with data on established pancreatic regulators, succeeded in nominating and ranking scores of novel candidate regulators and their functions. We validated a subset of these predictions using mutant Neurog3 mice and by phenotyping pancreas development in appropriate mutant mice. This comprehensive, integrated effort with discrete, operationally-defined populations of purified fetal and adult pancreatic cells provides gene expression profiling at higher resolution than previously achieved, identifies new regulators of pancreas development that are validated in vivo, and elucidates new elements of the regulatory logic underlying development of the endocrine and exocrine pancreas.

Results

Purification and gene expression profiling of fetal and adult pancreatic cells

To dissect developmental mechanisms of pancreatic development and maturation, we adopted a strategy using staged mice, FACS purification of specific cell subsets, genome-scale gene expression profiling coupled to bioinformatics analysis, and validation using mutant mice (Figure 1A). Using a combination of surface markers and transgenic reporter mice, we isolated 12 cell populations and profiled gene expression using GeneChip microarrays (Figure 1B; Methods). These included embryonic day (E) 11 cells enriched for Sox9\(^+\) multipotent pancreatic progenitors [3], E15 pancreatic ‘progenitors’ enriched for the markers Sox9 and CD24 [16] [17], E15 Neurog3\(^+\) endocrine progenitors enriched for CD133 and CD40 [16], E15 acinar cells, Glucagon\(^+\) alpha cells from postnatal day (P) 1 and 8–12 weeks, fetal and adult beta cells from E15, E17, P1, P15, and 8–12 weeks, and duct cells from 8–12 weeks. To our knowledge, comparative analysis of this range of mouse pancreatic cell types and developmental stages has not been reported.

To assess the quality and reproducibility of replicate cell isolations, RNA collection and gene expression profiles, we obtained the Pearson correlation coefficient of pair-wise-comparisons between samples and performed unsupervised hierarchical clustering. This analysis revealed tight clustering of biological replicates for each cell subset isolated (Figure 1C). We verified the expression of established pancreatic markers and developmental regulators [9] for each specific cell type profiled, using microarray (Figure S1A), or with quantitative PCR for a subset of ultra-abundant mRNAs encoding proteins like insulin and glucagon, which can saturate microarray probes (Figure S1B, S1C). Sox9\(^+\) E11 and E15 pancreatic progenitor cells were enriched for expression of expected mRNAs encoding Sox9, cMye, and Onecut1. Likewise we confirmed that adult ductal cells were enriched for expression of Muc1, Sox9, Onecut1 and Hes1 mRNA (Figure S1A). At multiple stages, purified beta cells were highly enriched for mRNAs encoding Pdx1, Insulin and Glucokinase, while alpha cells expressed expected markers, including Pax6, MafB, Arx, and Glucagon (Figure S1A). Thus, appropriate scaling of mouse collections overcame inherently low numbers of fetal pancreatic cell subsets to generate a unique, coherent set of highly reproducible gene expression data sets spanning multiple pancreatic cell lineages and developmental stages.

Pearson correlation and unsupervised hierarchical clustering analysis revealed grouping of cell types and their gene expression based on developmental stage, and exocrine or endocrine function. Undifferentiated fetal pancreatic progenitors from E11 clustered closest to E15 progenitor cells and E15 acinar cells (Figure 1C). E15 Neurog3\(^+\) endocrine progenitors clustered closely with fetal alpha cells and beta cells, forming a cluster distinct from fetal progenitor, ductal and acinar cells. Adult duct cell gene expression clustered with that of E11 and E15 pancreatic progenitors cells, instead of other adult cells, likely reflecting the postulated origins of pancreatic progenitors from primitive fetal ductal cells [18]. Unexpectedly, we did not observe clustering by endocrine cell type; rather, we observed clustering of postnatal and adult beta cells with adult alpha cells, and close clustering of fetal beta cells with neonatal alpha cells (Figure 1C).

Pair-wise differential expression analysis (Table S1) and unsupervised hierarchical clustering analysis with over 30 adult mouse tissues [19] supported this conclusion (Figure S2). As described below, this similarity likely reflects common functions of mature adult alpha cells and beta cells as nutrient-responsive cells that produce, process and secrete peptide hormones—functions distinct from those in fetal alpha and beta cells.

Identifying distinct gene sets in pancreas progenitor and exocrine development

The clustering of cell types in our Pearson correlation analysis (Figure 1C) indicated that specific cell types expressed distinct genes. To investigate this further, we used module mapping [20], which determines if a set of genes associated with or governing a specific biological function is significantly enriched or depleted.
within a sample (see Methods). As expected, module mapping revealed gene sets enriched in E11 pancreatic progenitors that remained enriched in E15 pancreatic progenitors, fetal acinar cells, and duct cells (Group I; Figure 2). This included gene sets regulating cell proliferation, cell fate commitment, branching morphogenesis and gland development; together, these functions reflect the known proliferative capacity and differentiation potential of these cell populations [21]. In addition, we observed that progenitor and duct cells shared modules with fetal endocrine cells (Group I; Figure 2), suggesting common genetic regulatory features. These commonalities are consistent with recent findings indicating a latent potential in pancreatic acinar or duct cells for conversion into endocrine cells [22], [23]. To identify gene sets enriched or uniquely expressed in E11 and E15 Sox9+ pancreatic progenitors, acinar or duct cells, we (1) obtained the gene signatures for each cell type (Figure S3; Table S2; see Methods for gene signature criteria), and (2) identified differentially expressed genes (Figure S4; Tables S2, S3, S4). This analysis revealed that E11, E15 progenitors and E15 acinar cells shared distinct but overlapping gene signatures (Figure S3A). Genes in these signatures included many transcriptional regulators, including Bcl11a, and genes involved in RNA processing and translation such as Spin2, Rpp40, and Rpl23, whose possible roles have not been previously noted in pancreas development. Thus, our analysis identified genes and gene sets that are expressed during pancreatic progenitor and exocrine cell development.

Identifying gene sets in endocrine progenitors and their fetal and adult endocrine progeny

Maturation of defining beta cell functions, such as glucose sensing and insulin secretion, increases from fetal through postnatal stages [7] [24]; however, a comprehensive analysis of beta cell gene expression from fetal to adult stages has not, to our knowledge, been reported in mice. Likewise, little is known about gene expression changes accompanying maturation of alpha cells [25]. Module mapping revealed gene sets initially expressed in Sox9+ pancreatic progenitor cells and Neurog3+ endocrine progenitors (including the terms cell proliferation and cell fate commitment) that were maintained in fetal beta and alpha cells but extinguished in adult endocrine cells (Group I; Figure 2). A second group of gene sets (with terms like cell adhesion, angiogenesis, hormone activity and eye development) was expressed after the Neurog3+ stage in alpha and beta cells. Strikingly, nearly all these modules were transiently downregulated in P15 beta cells and lost in adult alpha cells, but maintained in adult beta cells (Group II; Figure 2). These findings are consistent with prior studies showing that at early stages of development (E15, E17, and P1) immature alpha and beta cells are establishing neurovascular connections [26] [27], proliferating [28] [29], and
Figure 2. Module map analysis of differentially expressed gene sets. The module map algorithm of Genomica software was executed to identify gene-sets (representing gene ontology biological functions) that are differentially expressed between 12 cell populations representing various stages and cell types of the pancreas. Each individual block represents the average expression of statistically enriched (yellow) or depleted (teal) genes based on a log2 scale ($P < 0.05$ and FDR $< 0.05$). Cut-off values >1 or < −1, based on a log2 scale. Black blocks indicate that there was no significant enrichment or depletion of a gene-set. Because of resolution and space constraints not all gene set terms are displayed (signified with dots). Endocrine progenitor (EP).

doi:10.1371/journal.pgen.1004645.g002
Iterative Network Module (IMNA) identifies regulators of pancreas development

After obtaining genes and gene sets that were differentially expressed during pancreas development, we sought to identify the regulatory logic governing their expression. Mutations in loci encoding transcription factors constitute an important group of risk factors for diabetes mellitus and pancreatic malformations in humans [32-33]. Thus, we focused on identifying regulatory networks governed by transcription factors. To do this, we adapted the module network function in Genomica, a probabilistic algorithm that groups genes based on co-expression patterns (modules) and predicts the regulators that might control such gene co-expression [34-35]. To identify regulators, we first analyzed 1642 genes expressed in the developing pancreas that encode transcription factors (TFs) or DNA-binding factors [36-37] [38]. We optimized our parameters with a ‘training’ set of 82 established pancreatic regulators (Methods; [9]). Because Genomica is constrained to choose one group of regulators per gene set - a recognized limitation [39] - we employed an Iterative Module Network Analysis (IMNA) approach, in which we identified candidate regulators after multiple iterations (‘runs’) of the module network program. We then systematically varied the number of modules and runs (Figure 3B; Figure S5; Methods), and found that 100 iterations of 75 modules identified 99% (81/82) of established transcriptional regulators of pancreas development that included Neurog3, Arx, Gli3, Pax6, Idf1, Foxo and Myt1 (Figure 3D; Table S5). The quality of predictions did not improve with more iterations or modules (Methods). To determine the validity of our outputs, we ranked candidate regulators based on their frequency of occurrence across all iterations (Figure 3D) and performed Gene Set Enrichment Analysis on these ranked regulators (GSEA; Figure 3C; [40]). This analysis revealed that established pancreatic regulators ranked significantly higher in the list indicating that the top-ranked predictions were likely to be true regulators (Figure 3C, 3D; Table S5). One highly-ranked candidate regulator was Bcl11a, a gene previously linked by human GWAS studies to increased type 2 diabetes (T2D) risk [41-42] [43]. Of 26 loci encoding DNA-binding factors that have been linked to diabetes risk, we found that 22 (85%, \( P = 1.38 \times 10^{-15} \)) were expressed in the developing pancreas, and 21 of these were highly ranked by IMNA as possible regulators (Figure 3E, 3F).

In vivo validation of genes regulated by Neurog3

To validate predictions from IMNA, we initially focused on analyzing gene expression changes associated with the loss of Neurog3 in vivo. We purified Neurog3-null cells from the pancreata of E15 Neurog3+GFP/+GFP mutant embryos [45-46], an approach not previously reported [47-48] [49]. Of the 6367 differentially expressed, 3188 were downregulated and 3179 were upregulated by the loss of Neurog3 expression (Table S6). These included both known targets such as Pax4, Rx6, Nkx2.2, Snail2 and Ins1, as well as novel candidates like Etv1, Runx1 and Runx11 (Figure 4A). We did not detect known regulators of pancreas development among the gene set predicted to be repressed by Neurog3, an area not previously well-characterized [9]; thus, we prioritized analysis of the gene set predicted to be activated by Neurog3. Functional enrichment analysis for biological processes through DAVID (Figure 4E) predicted roles for these Neurog3 targets in processes including RNA biosynthesis and transcription, protein transport, localization and secretion, catabolic processes, cell cycle control and chromatin organization. These functional categories were corroborated independently by in vivo testing (see below). Thus, the module network algorithm readily identified both established and previously unrecognized Neurog3-dependent gene regulatory programs and target genes governing pancreatic endocrine development.

Integrative Deconstruction of Pancreas Development
substantially validated specific predictions made by IMNA about Neurog3 target genes and their Neurog3-dependent biological functions.

Functional validation of previously unrecognized pancreas developmental regulators in vivo

To assess and validate candidate transcriptional regulators nominated by IMNA, we chose to assess in vivo functions of genes (1) highly ranked by IMNA (Table S5), (2) predicted to regulate target genes or functions involved in pancreas development (Figure S7), (3) without known roles in pancreas development at the initiation of these studies, and (4) with available mutant mouse alleles [50] [51] [52] [53]. These included Pdx1, Etv1, Runx111, and Bcl11a (Table S5). During pancreas development, mRNAs encoding each of these factors were more abundant in Neurog3 endocrine progenitors than in Sox9 pancreatic progenitors or beta cells (Figure 5A, 5B, 5D, 5G). This suggested possible roles for each factor in islet development. We also analyzed Gfi, a transcriptional regulator expressed in the fetal pancreas (Figure S8E–G), which has an established role in hematopoietic development [54] [55], but not nominated by IMNA as a regulator of pancreas development.

Figure 3. Expression-based identification of pancreatic regulators. (A) Schematic of approach used to identify regulators of pancreas development, their targets, and their predicted biological functions using the module network algorithm of Genomica. To identify regulators two lists are loaded into the program: 1) a list of potential regulators and 2) normalized expression values of samples. Genes with similar expression patterns are grouped (termed a module). Regulators that are most predictive of a specific module expression pattern are learned. Output information includes a list of regulators and their potential targets. Functional enrichment analysis is used to predict the biological function of each regulator (see Methods for details). An example of module-network analysis nominating Neurog3 as a candidate regulator of endocrine development is shown along with its potential targets. (B) Optimal number of modules and iterations were determined by calculating the percentage of known regulators of pancreas development for each module and iteration combination. (C) Gene set enrichment analysis (GSEA) for 100 iterations of 75 modules yielded an enrichment score greater than >0.5 when known regulators were used. Distribution of known regulators based on their rank is displayed on the top panel. (D) Ranking of candidate regulators based on their frequency. Among the most reproducible candidates included known pancreas regulators such as Pdx1 and Neurog3 (red font) and candidate regulators validated in subsequent analysis (red font). (E) GSEA plot for the distribution of diabetes risk factors among list of predicted regulators. (F) Ranking of diabetes risk factors based on their frequency score. Validated GWAS genes include Bcl11a (red). doi:10.1371/journal.pgen.1004645.g003
Figure 4. Identifying biological functions and targets of Neurog3. (A) Venn diagram displaying the number of predicted activated targets of Neurog3 using the module network algorithm of Genomica based on a cut-off value of two-fold (orange), and the number of genes that are downregulated upon the loss of Neurog3 by a two-fold difference based on expression profiling of E15 Neurog3-null cells (yellow). Overlap of a subset of activated Neurog3 target genes is shown to the right. Validated targets are in red. Fisher’s exact test was used to calculate the P-value. (B) mRNA expression of a subset of nominated regulators (Etv1, Prdm16, Runx1t1, and Bcl11a) in Neurog3 mutant pancreata (n = 3) and control mice (n = 3) at E15. (C) Adeno-based overexpression of Neurog3 in ductal cell line (mPAC) and its effect on Runx1t1, Bcl11a, Etv1, and Prdm16 expression, (n = 3, each). (D) Immunohistochemistry showing the expression of Runx1t1 (red) in a subset of Neurog3-eGFP+ cells in heterozygous Neurog3eGFP/+ (left panel). Loss of Runx1t1 (red) in the epithelium of Neurog3-null pancreas (right panel). No change in expression of Runx1t1 (red) in mesenchymal cells in Neurog3-null pancreas. Epithelial cells are labeled with E-cadherin (white). (E) Genomica-based predicted biological functions of Neurog3 based on the target genes that were positively correlated with the expression of Neurog3. (F) Biological functions of targets based on expression profiling of Neurog3+ endocrine progenitor cells and E15 Neurog3-null cells based on a 2-fold difference. (B–C) data are represented as mean ±/SEM. (D–E) functional enrichment analysis for each set of targets genes was performed through DAVID. FDR<0.05.
doi:10.1371/journal.pgen.1004645.g004
Prdm16 encodes a transcriptional regulator and histone methyltransferase [56]. Our gene expression studies showed that Prdm16 is highly expressed in Sox9+ pancreatic progenitor cells and Neurog3+ endocrine progenitors, then maintained at lower levels in alpha cells and beta cells (Figure 5A). IMNA predicted that Prdm16 regulates expression of Arx (Figure S7A). Supporting these findings, we recently reported that homozygous null mutation of Prdm16 leads to impaired development of pancreatic islets [17]. This included inappropriately increased expression of Arx, increased alpha cell and PP cell numbers (a known outcome of Arx misexpression; [57]), and disrupted beta cell development [17]. Thus, our work supports the prediction that Prdm16 is required for pancreas development in vivo.

Bcl11a encodes a zinc-finger transcription factor involved in hematopoiesis [53] but without known roles in pancreas develop-
ment. IMNA nominated Bcl11a as a candidate regulator of pancreas development, and predicted new target genes Ins2, Glucagon and Ppy (Figure S7B). Remarkably, we observed reduced mRNA expression of each of these genes in FACSpurified endocrine cells from homozygous null mutant Bcl11a−/− mice (Figure 5C). We did not find significant changes in allocation of fetal islet cell subsets in Bcl11a mutants (Figure S8C), and lethality at P1 precluded further phenotyping. Thus, in vivo analysis confirmed a requirement for Bcl11a in endocrine development.

Etv1 (also known as Er81) encodes a transcription factor involved in neurogenesis and maturation of neural cells [50], but has no known function in pancreas development. Etv1 mRNA levels were reduced in Neurog3−/− mutant pancreata (Figure 4B), indicating that Etv1 is a direct or indirect target of Neurog3 and might have roles in islet cell development. Consistent with this possibility, we observed decreased levels of mRNAs encoding islet cell hormones, including a significant reduction of Pancreatic polypeptide mRNA in Etv1−/− mutant pancreas (Figure 5E). Likewise, morphometry of P1 null Etv1−/− pancreas revealed severely reduced islet PP cell mass (Figure 5F). Thus, in vivo analysis confirmed a requirement for Etv1 in pancreatic islet development.

Runx11l (also called Eto or Mlg8; [51]) encodes a transcription factor related to the Drosophila runt protein, and mutations in this gene have been linked to blood, lung and breast neoplasia [58] [59]. We detected Runx11l and Neurog3 co-expression in fetal pancreatic epithelial cells (Figure 4D). Runx11l mRNA was reduced in homozygous Neurog3−/− mutant pancreas (Figure 4B), and Runx11l protein was undetectable in homozygous null Neurog3−/−GFP/GFP mutant cells (Figure 4D), supporting the view that Neurog3 regulates Runx11l expression. IMNA analysis indicated Runx11l regulates Pancreatic polypeptide (Figure S7D).

Analysis of pancreas development in mice lacking Runx11l, which expire at birth, revealed increased mRNA levels of Pancreatic polypeptide and Ghrelin expression (Figure 5H). Together with findings of significant islet cell hyperplasia in Runx11l null mutants (P. Pauerstein, C.B. and S.K.K., in preparation), our analysis confirmed an essential role for Runx11l in pancreas development.

Gfi1 encodes a transcriptional regulator [60] and is expressed in fetal pancreas (Figure S8E) but was not nominated by IMNA as a regulator of pancreas development. Consistent with this prediction, we did not detect disrupted islet development or glucose regulation in mice lacking Gfi1, despite exhaustive systematic phenotyping (Figure S8F, S8G). Thus, our rigorous integration of developmental, genomic and bioinformatic approaches identified four candidate regulators of pancreas development, and mutant mouse analysis confirmed that all four were also required in vivo.

Discussion

Elucidating the regulatory interactions underlying global transcriptional programs that control development of solid organs like the pancreas has been a challenge. Classical and recent studies have advanced our understanding of the cellular origins, genetics, morphogenesis, and cell lineage relationships in the developing pancreas, and have identified features of transient pancreatic progenitors or lineage-specific endocrine progenitors [8]. However these and other fetal pancreatic cell subsets are generated in relatively small numbers, hampering prior comprehensive genomic-scale efforts to dissect pancreas development. Here we combined several powerful approaches — including cell sorting, transgenic cell labeling, genomic-scale expression profiling, bioinformatics, and targeted mutagenesis in mice — to identify elements comprising genetic regulatory hierarchies in the developing pancreas. This effort has revealed both the complexity and structural framework of transcriptional programs underlying pancreas cell differentiation and maturation, and provides a strategy for similar studies in other solid organs.

Purification of cell subsets from defined genetic mouse strains by flow cytometry ([16] [17], this study) generated highly-reproducible gene expression profiles of a dozen pancreatic cell subsets — a degree of comprehensiveness unprecedented in prior studies. This innovation permitted deconvolution of gene expression profiles into co-expressed and co-regulated genes. We found that many gene sets were re-used in multiple lineages and stages. These findings are reminiscent of the general gene regulatory circuitry identified during hematopoiesis [10]. For example, immature alpha cell and beta cells from fetal or neonatal pancreas shared common gene sets that clustered distinctly from those in mature alpha and beta cells from adult pancreas. This feature likely reflects commonalities of mature alpha and beta cells as nutrient-responsive cells that produce, process and secrete polypeptide hormones, and corroborate similarities of gene regulation observed in adult human alpha and beta cells [61]. Global similarities of gene expression in adult alpha and beta cells shown here are also consistent with recent findings that in specific experimental settings, adult alpha cells may acquire beta cell features [62] [63] or vice-versa [64]. Identification of gene sets controlling function of mature beta cells may foster progress in producing replacement beta cells from renewable stem cell sources for diabetic patients [65]. For example, gene sets regulating calcium ion transport or responsiveness were enriched in adult beta cells, consistent with studies showing that calcium-dependent signaling pathways regulate beta cell maturation in mice and humans [31] [66]. Stimulation of calcium-responsive pathways, such as calcineurin/NFATc signaling, can enhance functional maturation of beta cells [31]. Thus, our reference data sets should prove useful for advancing efforts to produce or replace beta cells in diabetes.

Compared to the endocrine cell lineage or exocrine acinar cells, little is known about the genetic programs defining pancreatic exocrine duct cells [21]. Pearson correlation identified clustering between adult ductal cells and fetal pancreatic cells, including endocrine progenitor cells. This indicated that regulatory programs maintaining adult ductal cell gene expression and fate are unexpectedly similar to those in transient oligopotent fetal cell subsets, as suggested by unsupervised clustering with 30 mouse tissues. Thus, our study provides support for strategies focused on ‘reprogramming’ duct cells into other desired fates, including insulin-producing cells [67] [23] and could accelerate use of somatic cell reprogramming for therapeutic aims.

Human genetic studies have revealed that transcription factors have major roles in the pathogenesis of pancreatic malformation, including agenesis and diabetes mellitus [32] [68]; thus, we focused here on elucidating previously unrecognized transcriptional regulators required for pancreas development. Our general strategy was to exploit co-variation of transcription factors in gene sets and the cellular states they might regulate. Iterative use of a gene expression-based probabilistic program identified known regulators with high efficiency and predicted new regulatory functions for scores of transcription factors. IMNA identified known and novel regulators of endocrine development (see below), including a subset of transcription factors previously implicated by human GWAS in type 2 diabetes risk [41,43]. We also noted that the frequency of detecting regulators of exocrine differentiation, like Mist1, was lower (Table S5). This likely reflects the lower
representation of gene sets from differentiated exocrine cell types (2/12 cell subsets purified and analyzed here) compared to endocrine cell subsets. Therefore, further studies of gene regulation in subsets of purified fetal pancreatic exocrine cells could therefore likely identify additional exocrine pancreatic regulators.

To validate and assess the biological significance of predictions based on gene expression and module analysis and to control for variables introduced by our FACS-based approach, we analyzed relevant mutant mouse strains, including Neurog3 mutants. This combined approach proved to be a powerful way to test, for example, predictions of Neurog3 target gene expression, and to functionally validate transcriptional regulators identified by IMNA. Bcl11a, Runx1t1, Prdm16 and Etv1 encode transcription factors not previously linked to roles in pancreas development when these studies began. Prior studies had revealed crucial roles for Bcl11a in regulating blood development [69] [53] and diseases [70] [71], and for Runx1t1 in midgut development [51] and neoplasias of blood, lung and breast [58] [59]. Prdm16 has well characterized functions in adipogenesis [72] [73], leukemia pathogenesis [74], and neuronal stem cells maintenance [75]. Etv1/Ere81 is an established regulator of fetal neuronal development [50] whose mis-expression leads to cancer pathogenesis in diverse tissues [76]. Independent genetic screens in our group, concurrent with studies here, identified roles for Prdm16 in regulating allocation of pancreatic islet cells in development [17].

Strikingly, after unbiased selection of these four candidate regulators, analysis of mouse strains harboring targeted mutations in Etv1, Prdm16, Bcl11a and Runx1t1 here or in recent studies from our group [17] revealed defects of pancreas development in all of the mutants. Though expressed in islet development, Gfi1, an established regulator of myeloid and enteric development [54] [77] [78], did not meet criteria of a regulator through our bioinformatic analysis. Accordingly, intensive investigation revealed no detectable phenotypes in pancreas development or glucose control in mice lacking Gfi1. Thus, our integrated approach accurately predicted essential regulators of islet development, demonstrating the robustness of our cell purification and gene expression profiling. This level of functional validation with mutant mouse phenotyping is, to our knowledge, unprecedented for integrative genomic approaches to pancreas development. Clearly, additional in-depth phenotypic studies, including in mice permitting conditional or pancreas-specific gene targeting, could prove valuable for understanding the molecular roles of these factors in pancreas development. Improved methods to isolate, purify and analyze cognate human pancreatic cell subsets [79] should enable an analogous integrative approach to identify factors regulating human pancreatic development.

Endoderm-derived epithelial cells and their progeny accomplish the vital physiological functions of the adult pancreas, and in other gastrointestinal organs. Thus, in these initial investigations we focused on deciphering the transcriptional hierarchies underlying epithelial cell development in the pancreas. However, prior studies have revealed that non-epithelial cells, including vascular endothelium, neuronal cells, and mesenchyme-derived signals control basic aspects of pancreas development [7] [80]. Thus, a complete deconstruction of pancreas development will require assessments, akin to those described here, of gene expression data from additional important cell subsets. Likewise additional data from epigenetic, genome-scale ChIP-Seq, proteomics and enhancer analyses [81] need to be integrated into the regulatory frameworks described here. The coordinated developmental, cellular, molecular and computational approaches described here should provide a paradigm for identifying genes and circuitry underlying development and postnatal maturation in other visceral organs, as well as assessment of regenerated pancreatic cell types.

Methods

Animals

All animal studies were approved by Stanford University and performed in accordance with Stanford University Animal Care and Use guidelines. Discomfort of animals was limited to that which was unavoidable in the conduct of scientifically valuable research. Analgesic, anesthetic, and tranquilizing drugs used were indicated and where appropriate to minimize discomfort and pain.

Mice harboring the Sox9-CreER T2 BAC transgene were obtained from Mutant Mouse Regional Resource Center, University of California at Davis [82]. Because of CreER T2 perdurance, Sox9-CreER T2 cells in Sox9-CreER T2 mice contain a mixture of Sox9+ and Sox9− progeny. However, the percent of Sox9− cells is low [17]. Neurog3-GFP transgenic mice were a kind gift from Drs. Guoqiang Gu and Douglas Melton [13]. Neurog3 knock-in reporter mice were a kind gift from Dr. M. Hara (University of Chicago, Chicago, II.; [83]) and maintained in a CD-1 background. Glucagon-Venus mice were a gift from Dr. Fiona Gribble [84]. Runx1t1 mutant mice were rederived from MRC Harwell (Stock number FESA:000373). Etv1 mutant mice were a gift from Dr. Thomas Jessell and provided by Dr. Julia Kaltschmidt (Memorial Sloan Kettering Institute). Bcl11a mutant mice were derived from Bcl11a floxed mice [53] by crossing with a Cre deleter strain (CMV-Cre), obtained from Jackson Laboratories (Stock number 00654). Prdm16 mutant mice were obtained from Jackson Laboratories (Stock number 013100). Gfi1 mutant mice are described in [54]. Genotyping follows published methods. Mice were mated overnight and checked for plugs. Noon on the day of vaginal plug appearance was counted as embryonic day 0.5 (E0.5).

Cell sorting strategy and flow cytometry

Pancreata were obtained at E11, E15, E17, postnatal day (P) 1, P15, and 8–12 weeks of age. To obtain E11 Sox9+ pancreatic progenitors, we dissected the dorsal pancreas from Sox9-CreER T2 reporter embryos and dissociated with TrypLE Express (Invitrogen, Carlsbad, CA) at 37°C for 5 min and triturated. TrypLE was neutralized with 10% (v/v) FBS in PBS [17]. Approximately 200 pancreata were dissected to obtain four replicates of Sox9+ pancreatic progenitors. E15 pancreatic progenitors, Neurog3+ endocrine progenitors, and acinar cells were collected using a combination of cell surface markers and transgenic cell labeling. This approach was used to collect hormone+ progenitors, Neurog3+ endocrine progenitors because GFP perdurance labels their hormone+ descendants. Briefly, the Neurog3-GFP transgenic pancreata were dissected and visually assessed for GFP expression. GFP+ pancreata were pooled and dissociated with 0.05% Trypsin EDTA at 37°C for 3 min and triturated (GFP− pancreata were collected for FACS gating purposes). Trypsin EDTA was neutralized with 10% (v/v) FBS in 10 mM EGTA, PBS [16] and the cells were treated for 15 min in a blocking solution composed of FACS buffer (2% fetal bovine serum in PBS, 10 mM EGTA was supplemented for experiments of E15 pancreas) containing 300 ng/ml rat IgG (Jackson ImmunoResearch, West Grove, PA). We used the following primary antibodies: biotin anti-CD133 (13A4, 1:100; eBioscience, San Diego, CA), Pacific Blue anti-CD24 (M1/69, 1:100; BioLegend, San Diego, CA), and PE anti-CD49f (GoH3, 1:50; R&D, Minneapolis, MN). Streptavidin-APC.
respectively. Live-dead cell exclusion was performed with 10
(1:100; Biosciences), which label erythroid cells and leukocytes,
BioLegend, San Diego, CA). To exclude blood cell contamination
their ability to couple glucose detection with insulin secretion [85].
cells from P15 and adult mice are ‘postnatal’ beta cells based on
stages E15, E17 and P1 are termed ‘fetal’ beta cells, while beta
Each replicate consisted of at least 3 male mice. Beta cells from the
intraductal ligation and digestion with 1 mg/ml collagenase [31].
pancreata. P15 and adult pancreata were dissociated by standard
u
followed by mixing, spinning and further dissociation with 1 mg/
dl dispase for 8 minutes at 37°C from approximately 230
pancreata. P15 and adult pancreata were dissociated by standard
intraductal ligation and digestion with 1 mg/ml collagenase [31].
Each replicate consisted of at least 3 male mice. Beta cells from the
stages E15, E17 and P1 are termed ‘fetal’ beta cells, while beta
cells from P15 and adult mice are ‘postnatal’ beta cells based on
their ability to couple glucose detection with insulin secretion [85].
Adult duct cells were collected using APC anti-CD133 (1:100;
BioLegend, San Diego, CA). To exclude blood cell contamination
from our sorts we used cell-surface markers Ter119 and CD45
(1:100; Biosciences), which label erythroid cells and leukocytes,
respectively. Live-dead cell exclusion was performed with 10 µg/
ml Propidium Iodide (PI; Sigma) or 10 µg/mL Aqua (L34957;
Invitrogen).
Cell sorting was performed in FACS Aria I and II machines
fitted with a 100 µm nozzle using DIVA software (BD Biosciences,
San Jose, CA). FACS data were analyzed by using FlowJo software
(Tree Star, San Carlos, CA). Cells were collected in 10% fetal
bovine serum in PBS and processed for RNA collection (for acinar
cells, 2% fetal bovine serum in PBS supplemented with 10 mM
EGTA). Cell death did not exceed 30% per sample. All cell types
were collected in at least triplicate from a minimum of 15,000 cells
per sample.

Molecular biology
Total mRNA was isolated using the Arcturus PicoPure kit
(Applied Biosystems) for all microarray samples. For quantitative
PCR analysis, whole pancreas mRNA at E18 or P1 was collected
by homogenizing each pancreas in 1.5 ml of RLT buffer and
RNA was extracted using the Qiagen RNAeasy Micro kit
(Qiagen). cDNA synthesis was performed with Ambion Retroscrip
t kit. Quantitative PCR studies were performed using an
ABI7500 system, Applied Biosystems (Foster City, CA). Replicates
were processed independently, and each cDNA was tested in
duplicate. Expression level was normalized to beta-actin. Infor
mation about primer and probe sets is available upon request.

Microarray data preprocessing, normalization and clustering
RNA quality was accessed with Agilent’s BioAnalyzer (Stanford
PAN facility). 50 ng to 100 ng of mRNA with a RNA integrity
number (RIN) score >9 were amplified with NuGen Ovation Kit
V2 (NuGEN) and fragmented and labeled with the Biotin and
fragmentation labeling kit (NuGEN) following manufacturer’s
protocol. Hybridization and image analysis processing was done
in accordance to the Stanford PAN facility, The Affymetrix Mouse
430 2.0 GeneChip was used.

For gene expression analysis, arrays were RMA normalized using
justRMA package in R. After normalization, probes with raw
expression value of 100 in all arrays were filtered out—leaving a
total of 23,093 probes. For each expressed probe, expression values
were log2-transformed, and mean-centered across all the conditions
before pair-wise Pearson correlation was performed. Unsupervised
hierarchical clustering and array clustering of pair-wise Pearson
correlation was performed using Cluster 3.0 [86]. This dendrogram
was overlaid with the Pearson correlation plot (Figure 1C).

Unsupervised hierarchical clustering analysis with Microarray
data from over 30 adult mouse tissues was downloaded from NIH
GEO, with accession number GSE1135 [19]. The data discussed
in this publication have been deposited in NCBI’s Gene
Expression Omnibus [87] and are accessible through GEO Series
accession number GSE54374 [http://www.ncbi.nlm.nih.gov/
geotquery/acc.cgi?acc=GSE54374].

Module mapping
The module mapping function of Genomica [34] was executed to
identify gene sets that are enriched or depleted in each sample. Here,
we entered gene ontology terms belonging to biological processes (BP-
GO terms) from mouse and the mean-centered normalized
expression values for all the arrays (23,093 probes). We used the
default settings of the program: P-value < 0.05, FDR correction of
0.05, and the hierarchical agglomerative (correction centered)
clustering method. Probes with an expression level > +1 (on a log2
scale) were considered upregulated, while probes with an expression
level < -1 (on a log2 scale) were considered downregulated. We
displayed the average expression of gene hits from each enriched gene
set (based on a log2 scale of mean-centered values). GO gene sets of
biological functions were downloaded from website DAVID and
imported into the Genomica Software [20] [34].

Gene signatures and pair-wise comparisons
Gene signatures for each cell type were calculated using Student
t-test comparing signals in the arrays of a particular cell-type versus
the rest of the arrays. The genes selected as ‘signature genes’
met four parameters: P-value < 0.001, FDR < 0.05, log2 fold change
> +1, and standard deviation < 0.5 of arrays in the same cell type. FDR
correction was estimated using the p.adjust package in R. Negative gene signatures were selected using a log2 fold change
< -1, P-value < 0.001, FDR < 0.05, and standard deviation < 0.5 of arrays in the same cell type. Similar
methods were applied for transcription factor signatures. For each
gene signature, we performed functional enrichment analysis of
Gene Ontology terms related to biological processes (GO-
TERM_BP_FAT) through DAVID. BP-GO terms were consid-
ered significant if FDR > 0.05. DAVID default settings were used.

To obtain differentially expressed genes between two cell types
we used the Student t-test where the obtained P-values were
adjusted for multiple testing using the p.adjust function of R with
Benjamini-Hochberg method (adjusted P<0.05). The log2 fold
change difference between each representative cell type was
measured as average of the transformed probes in the arrays of
the first cell type and subtract that from the second cell type.
Fetal endocrine cells included E15 beta cells, E17 beta cells, P1 beta
cells, and P1 alpha cells. Postnatal endocrine cells include P15
beta cells, Adult beta cells, and Adult alpha cells.

IMNA expression-based prediction of regulators, their
target genes and functions
We identified candidate regulators and their regulation
programs using the Module Networks algorithm in the Genomica
null cells. We obtained ~8000 probes that were differentially expressed with a 2-fold difference (4604 probes enriched in E15 endocrine progenitors and 4217 probes enriched in Neurog3-null cells). We compared this list to targets predicted by Genomica. This analysis yielded an 85% overlap in probes (303/358; \( P = 2.01 \times 10^{-106}; \) Figure 4A) or 87.2% overlap in genes (285/327; \( P = 1.05 \times 10^{-153} \)) when we filter out probes in each predicted module that have an expression value <1 (based on a log2 scale). The same approach was used to predict repressed targets of Neurog3. When we filter probes with an expression value \( >2 \), 67% of probes overlap (220/328, \( P = 2.60 \times 10^{-85} \); Figure S6A) or 73% genes overlap (192/263; \( P = 7.02 \times 10^{-102} \)). Fishér’s exact test was used to determine statistical significance with \( P < 0.05 \) against the total number of probes or genes. Next, we performed functional enrichment analysis on each list of Genomica predicted targets through DAVID using default settings and compared these results to targets obtained by gene expression profiling of Neurog3-null cells (FDR <0.05).

The analysis was integrated through GenomeSpace (http://www.genomespace.org/). Venn diagrams were obtained with the Venny program [89] (http://bioinfolg.cnb.es/tools/venny/index.html). Fishér’s exact analysis was performed using the following website: http://www.langsrud.com/fisher.htm

### Statistical analyses

Each variable was analyzed using the two-tailed Student’s t test. For all analyses, a \( P \) value of less than 0.05 was considered significant. Results are given as mean +/- SEM.

### Over-expression of Neurog3

A mouse duct cell line (mPAC) was infected with adenoviruses expressing the mouse Neurog3 gene and the red fluorescent protein (RFP) from separate CMV promoters. The control sample included mPAC cells that were infected with Adenoviruses expressing RFP. We verified that RFP did not have an effect on the expression of Neurog3 downstream genes in non-treated mPAC cells. Cells were cultured with the virus for 1 day and then media was changed. Cells were harvested after 3–4 days. Each experimental condition was performed in triplicate.

### Immunohistology

For measurement of endocrine-cell mass, a minimum of 12 pancreas sections spanning the entire pancreas were assessed for at least 3 different mice per genotype. The total cross-sectional area of hormone cells was summed and normalized to total pancreatic area using Image-Pro Plus analysis software (Media Cybernetics). Statistical analysis was performed using a two-tailed Student’s t-test. For staining Runx1t1 and Etv1-LacZ expression, E15 and 2-month-old mouse pancreata were dissected and fixed with 4% paraformaldehyde overnight at 4°C, and cryo-embedded. Sections were permeabilized with 1% Triton-X-100 for 1 hr before blocking with 2%BSA, 1% DMSO in PBS. We used the following primary antibodies: Goat anti-Runx1t1 (1:200, Santa Cruz, C-20); Rabbit anti-LacZ (1:500, Invitrogen), and Rat anti-E-cadherin (1:400, Invitrogen). Secondary antibodies were from Jackson ImmunoResearch and Molecular Probes. Samples were mounted with Vectashield containing DAPI (Vector Laboratories). Microscopic images were obtained using a Leica SP2 AOBS confocal laser-scanning microscope.

### Supporting Information

**Figure S1** Heat map of mRNA expression of a subset of known pancreatic markers. (A) Heat map of genes that are representative...
of each major cell type collected. High relative expression is shown in red and low relative expression in blue based on a log2 scale. (B) Insulin and Glucagon mRNA-expression analysis of sorted beta cells from adult mice. (C) Insulin and Glucagon mRNA-expression of sorted alpha cells from adult mice.

Figure S2 Hierarchical clustering of pancreatic cells with 30 adult mouse tissues. The data was normalized and clustered. The cells in this study are in red and those from [19] are in black.

Figure S3 Gene signatures across 12 sorted cell types. (A) Genes that are enriched in each cell type were termed 'positive gene-signatures' based on four parameters (P-value < 0.001, FDR < 0.05, log2 fold change > = 1, and standard deviation < 0.5 of arrays in the same cell type). Range of expression values (~5.015, 4.84). More than 75% of probes with positive values lie within the range of ±2.1 to ±2.1 based on a log2 scale. (B) Genes that are repressed in each cell type were termed 'negative gene-signatures' based on parameters (P-value < 0.001, FDR < 0.05, log2 fold change > = −1, and standard deviation < 0.5 of arrays in the same cell type). Range of values (~6.55, 3.98). >75% of probes with negative values lie within the range of −2.1 to +2.1 based on a log2 scale. (A-B) E15, E17, and P1 beta cell samples were grouped to obtain the gene signature of fetal beta cells and P15 and 8−12 week beta cells were grouped to obtain the gene signature of postnatal beta cells. SPP (Sox9 Pancreatic Progenitor), EP (Endocrine Progenitor). Scale bar based on a log2 scale. The number of genes corresponding to each gene signature are shown below each heat map. Corresponding values for each figure are shown in Table S2.

Figure S4 Pair-wise comparisons between endocrine cells. Volcano plots representing the distribution of probes against their P-value and FDR cut off of <0.05 (horizontal red line). Horizontal red lines represent an expression cut off with a log2 value of −1 and +1. X-axis represents the log2 fold change between each pair of conditions, while the Y-axis represents the −log10 value of the P-value. The annotated probes with the highest fold change difference are noted in each graph. A full list of differentially expressed genes for each condition is shown in Table S4. Color scheme of cell types as shown in Figure 1B, i.e. blue (beta cells), green (alpha cells).

Figure S5 GSEA of various module and iterations parameters used in IMNA. Gene set enrichment analysis displaying the enrichment score and distribution of known regulators of pancreas development based on their frequency. We show 25 modules at 100 iterations, 50 modules at 100 iterations, 75 modules at 120 iterations, and 100 modules at 100 iterations. The enrichment score for these parameters was worse than the enrichment score for 75 modules at 100 iterations. All statistical tests had a P-value and FDR value of <0.05.

Figure S6 Validation of repressed Neurog3 functions and targets. (A) Venn diagram showing genes that were upregulated in E15 Neurog3-null cells (yellow) and predicted repressed targets of Neurog3 based on module network analysis in Genomica (orange). Fisher’s exact test was used to calculate the P-value. (B) Functional enrichment analysis of biological functions of predicted repressed targets of Neurog3 based on the module network analysis algorithm. (C) Functional gene set analysis of genes that were enriched in Neurog3-null cells vs. E15 Neurog3+ endocrine progenitors (by 2-fold) was performed using DAVID (FDR < 0.05), similar biological terms were grouped.

Figure S7 Predicted targets and GO terms of a subset of regulators. (A-D) Predicted biological functions of Bcl11a, Runx1l1, Gfi1, and Prdm16 as determined by DAVID analysis of Genomica predicted targets for positively-correlated genes. FDR < 0.2. X-axis shows the −log(p-value) of each biological function as calculated in DAVID. A sample of the predicted targets is shown to the right. Validated targets are shown in red.

Figure S8 Phenotypic mutant analysis of nominated regulators. (A) Expression of Gfi1 expression in adult mouse pancreas using the Env1lacZ knock-in reporter mouse with Glucagon staining (red). (B) Immunostaining showing that Runx1l1 (green) is expressed in a subset of islets sells as determined by overlap with islet marker Chromogranin A (ChgA, green), epithelial cells are shown in white in E15 fetal pancreas. (C) Morphometric analysis comparing the insulin+ cell area and glucagon+ cell area in Bcl11a mutant mice compared to littermate controls (n = 3 each) at birth (P1). (D) Morphometric analysis of pancreatic polypeptide+ (PP), insulin+ (Ins), and glucagon+ (Gcg), and somatostatin+ (Sst) cell area in Etv1 mutant mice on embryonic day 18 (n = 5, each). In (C) and (D) there were no statistically significant changes in each comparison. (E) mRNA expression of Gfi1 from E15 pancreatic progenitors, E15 endocrine progenitors, E15 endocrine cells, E15 acinar cells, and E15 Neurog3-null cells. (F) Fasting glucose tolerance between 8−12 week old Gfi1 mutant mice and control littermates (n = 3, each). (G) mRNA expression analysis comparing a set of pancreatic markers between Gfi1 mutant whole pancreas and control mice at P1 (n = 2, mean ± SEM).

Table S1 Pair-wise comparison of alpha and beta cells by developmental stage. Fetal beta cells represent E15, E17, P1 beta cells while postnatal beta cells represent P15 and 8−12 week beta cells. Percentage of affymetrix probes that are differentially expressed based on a total probe number of 45101.

Table S2 Gene signatures of pancreatic cells types. Tab1: Positive signature. Tab2: Negative signature. Fetal beta cells (E15, E17, and P1 beta cells). Postnatal beta cells (P15 and 8−12 week beta cells). Parameters used to obtain gene signatures are described in methods. Corresponding Figure S3A−B.

Table S3 Pair-wise comparisons of cells of progenitors. Tab1: E11 SPP vs. E15 SPP. Tab 2: E11 SPP vs. E15 acinar cells. Tab 3: E11 SPP vs. adult duct cells. Tab 4: E11 SPP vs. E15 EP. Tab 5: E15 SPP vs. adult duct cells. Expression values represent Log2 normalized values. Only values that are differentially expressed by a 2-fold change and have an adjusted P-value of <0.05 (based on a multiple hypothesis correction) are shown. SPP (Sox9+ pancreatic progenitor), EP (endocrine progenitor).

Table S4 Pair-wise comparisons of cells of endocrine cells. Tab1: Postnatal (P15 and adult beta cells) vs. fetal (E15, E17, P1) beta cells. Tab 2: Fetal beta vs. P1 alpha cells. Tab 3: P1 alpha vs adult alpha cells. Tab 4: Postnatal beta cells vs. adult alpha cells. Tab 5: Fetal (E15, E17, and P1 beta cells and P1 alpha cells) vs. postnatal endocrine cells (P15 and adult beta cells and adult alpha cells). Tab 5: E15 EP vs E15 beta cells. Expression values represent Log2 normalized values. Only values that are differentially
expressed by a 2-fold change and have an adjusted P-value of < 0.05 (based on a multiple hypothesis correction) are shown. Corresponding volcano plots are displayed in Figure S4. EP (endocrine progenitor).

Table S5 Predicted regulators of pancreas development by IMNA. The frequency ratio was calculated based on the number of times each regulator appears after each iterative run. A total of 100 iterative runs were performed each comprising of 75 gene-network modules. Analysis was executed using the module network algorithm of Genomica. (XLS)

Table S6 Pair-wise comparison of Neurog3-wt vs. Neurog3 null cells. Differentially expressed genes were obtained by comparing the expression values of E15 Neurog3

Corresponding volcano plots are displayed in Figure S4. EP (endocrine progenitor). Differentially expressed genes were obtained by comparing the expression values of E15 Neurog3

Predicted regulators of pancreas development by IMNA. The frequency ratio was calculated based on the number of times each regulator appears after each iterative run. A total of 100 iterative runs were performed each comprising of 75 gene-network modules. Analysis was executed using the module network algorithm of Genomica. (XLS)

Author Contributions
Conceived and designed the experiments: CMB SKK. Performed the experiments: CMB KQ TS PTP YL XG AG JZ JT. Analyzed the data: CMB SKK. Contributed reagents/materials/analysis tools: JDD HOT. Wrote the paper: CMB SKK.

References
1. Prado CL, Pagh-Bernard AE, Elghazli L, Soza-Plaede B, Suelm L (2004) Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proceedings of the National Academy of Sciences of the United States of America 101: 2924–2929.
2. Lynn FG, Smith SB, Wilson ME, Yang KY, Nekrep N, et al. (2007) Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proceedings of the National Academy of Sciences of the United States of America 104: 10500–10505.
3. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, et al. (2007) SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences of the United States of America 104: 1065–1070.
4. Jonsson J, Carlsson L, Edlund T, Edlund H (1994) Insulin-promoter factor 1 is required for pancreatic development in mice. Nature 371: 600–609.
5. Krapp A, Knoller M, Ledermann B, Kurki B, Berny C, et al. (1998) The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes & development 12: 3752–3763.
6. Kopp JL, Dubois CL, Schaffer AE, Hao E, Shih HP, et al. (2011) Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. Development 138: 633–646.
7. Benitez CM, Goodyer WR, Kim SK (2012) Deconstructing pancreas developmental biology. Cold Spring Harbor perspectives in biology 4: doi: 10.1101/cshperspect.a012401.
8. Shih HP, Wang A, Sandor M (2013) Pancreas Organogenesis: From Lineage Determination to Morphogenesis. Annual review of cell and developmental biology.
9. Arda HE, Benitez CM, Kim SK (2013) Gene regulatory networks governing pancreas development. Developmental cell 25: 5–13.
10. Novershtern N, Subramanian A, Lawton LN, Mak RH, Haining WN, et al. (2011) Densely interconnected transcriptional circuits control cell states in human hematopoiesis. Cell 144: 296–309.
11. McKinney-Freeman S, Cahah P, Li H, Lacadie SA, Huang HT, et al. (2012) The transcriptional landscape of hematopoietic stem cell ontology. Cell stem cell 11: 701–714.
12. Scarce LM, Brestelli JE, McWeeney SK, Lee CS, Mazzarelli J, et al. (2002) Functional genomics of the endocrine pancreas: the pancreas clone set and PanChip, new resources for diabetes research. Diabetes 51: 1997–2004.
13. Gu G, Wells JM, Dombrowski D, Prelfer F, Aronow B, et al. (2004) Global expression analysis of gene regulatory pathways during endocrine pancreatic development. Developmental cell 25: 5–13.
14. Hoffman BG, Zavadga B, Witzache J, Ruiz de Algarra T, Beach M, et al. (2008) Identification of transcripts with enriched expression in the developing and adult pancreas. Genome biology 9: R99.
15. van Arensbergen J, Garcia-Hurtado J, Moran I, Maestro MA, Xu X, et al. (2010) Derepression of Polycistron targets during pancreatic organogenesis allows insulin-producing beta-cells to adopt a neural gene activity program. Genome Res 20: 722–732.
16. Sugiyama T, Rodriguez RT, McLean GW, Kim SK (2007) Conserved markers of fetal pancreatic epithelium permit prospective isolation of islet progenitor cells by FACS. Proceedings of the National Academy of Sciences of the United States of America 104: 175–180.
17. Sugiyama T, Benitez CM, Ghodasara A, Liu L, McLean GW, et al. (2013) Reconstituting pancreas development from purified progenitor cells reveals genes essential for islet differentiation. Proceedings of the National Academy of Sciences of the United States of America 110: 12691–12696.
18. Seymour PA, Sandor M (2013) Historical perspective: beginnings of the beta-cell current perspectives in beta-cell development. Diabetes 60: 364–376.
19. Su AI, Wilshire T, Batalov S, Lapp H, Ching KA, et al. (2004) A gene atlas of the mouse and human protein-encoding transcriptionome. Proceedings of the National Academy of Sciences of the United States of America 101: 6062–6067.
20. Segal E, Friedman N, Koller D, Regev A (2004) A module map showing conditional activity of expression modules in cancer. Nature genetics 36: 1090–1098.
21. Benitez CM, Goodyer WR, Kim SK (2012) Deconstructing pancreas developmental biology. Cold Spring Harb Perspect Biol 4.
22. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 455: 627–632.
23. Lee J, Sugiyama T, Liu Y, Wang J, Gu X, et al. (2013) Expansion and conversion of human pancreatic ductal cells into insulin-secreting endocrine cells. Elife 2: e00940.
24. Pagliuca FW, Melton DA (2013) How to make a functional beta-cell. Development 140: 2472–2483.
25. Mezza T, Kulkarni RN (2014) The regulation of pre- and post-maturational plasticity of mammalian islet cell mass. Diabetologia 57: 1291–1303.
26. Reinert RB, Cai Q, Hong YJ, Flank JL, Aamodt K, et al. (2014) Vascular endothelial growth factor coordinates islet innervation via vascular scaffolding. Development 141: 1480–1491.
27. Cleaver O, Dor Y (2012) Vascular instruction of pancreas development. Development 139: 2033–2043.
28. Georgia S, Hainaut C, Kasnoperv D, Hu J, Meyer J, et al. (2016) Cyclin D2 is essential for the compensatory beta-cell hyperplastic response to insulin resistance in rodents. Diabetes 59: 987–996.
29. Teta M, Long SY, Wartchow LM, Rankin MM, Kushner JA (2005) Very slow turnover of beta-cells in aged adult mouse. Diabetes 54: 2357–2367.
30. Gu C, Stein GH, Pan N, Goebels H, Hering H, et al. (2010) Pancreatic beta cells require NeuroD to achieve and maintain functional maturity. Cell Metab 11: 290–300.
31. Goodyer WR, Gu X, Liu Y, Bottino R, Crabtree GR, et al. (2012) Neonatal beta cell development in mice and humans is regulated by calcineurin/NFAT. Development 139: 23–34.
32. McKnight KD, Wang P, Kim SK (2010) Deconstructing pancreas development to reconstruct human islets from pluripotent stem cells. Cell Stem Cell 6: 300–308.
33. De Franco E, Shaw-Smith C, Flanagan SE, Shepherd MH, Hattersley AT, et al. (2005) Genetic variation in PPARδ affects insulin resistance in rodents. Diabetes 54: 987–996.
34. Segal E, Shapira M, Regev A, Pe’er D, Botstein D, et al. (2005) Module networks: identifying regulatory modules and their condition-specific regulators from gene expression data. Nature genetics 34: 166–176.
35. Novershtern N, Regev A, Friedman N (2011) Physical Module Networks: an integrative approach for reconstructing transcription regulation. Bioinformatics 27: 1177–1185.
36. Kanamori M, Konno H, Osato N, Kawaji J, Hayashizaki Y, et al. (2004) A genome-wide and nonredundant mouse transcription factor database. Biochem Biophys Res Commun 322: 787–793.
37. Ravasi T, Suzuki H, Caminstraci CV, Katayama S, Bajic VB, et al. (2010) An atlas of combinatorial transcriptional regulation in mouse and man. Cell 140: 744–754.
38. Zhang HM, Chen H, Liu W, Liu H, Gong J, et al. (2012) AnimalTFDB: a comprehensive animal transcription factor database. Nucleic Acids Res 40: D144–149.
39. Joshi A, De Smet R, Marchal K, Van de Peer Y, Michiels T (2009) Module networks revisited: computational assessment and prioritization of model predictions. Bioinformatics 25: 490–496.

40. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America 102: 15545–15550.

41. Imamura M, Maeda S (2011) Genetics of type 2 diabetes: the GWAS era and future perspectives [Review]. Endocr J 58: 723–739.

42. Morni AF, Vought BF, Teslovich TM, Ferreira T, Segre AV, et al. (2012) Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. Nat Genet 44: 961–969.

43. Diamant F, Lagoiu V, Barlovic A, Knospe JW, Magi R, et al. (2014) Impact of type 2 diabetes susceptibility variants on quantitative glycemic traits reveals mechanistic heterogeneity. Diabetes 63: 2158–2171.

44. Gradwohl G, Dierich A, LeMeur M, Guillemot F (2000) neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proceedings of the National Academy of Sciences of the United States of America 97: 1607–1611.

45. Lee CS, Perreault N, Brestell JE, Kaestner KH (2002) Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes & development 16: 1488–1497.

46. Wang S, Jensen YM, Seymour PA, Hsu W, Dor Y, et al. (2009) Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. Proceedings of the National Academy of Sciences of the United States of America 106: 9715–9720.

47. Juhl K, Sarkar SA, Wong R, Hutton JC (2008) Mouse pancreatic endocrine cell transcriptome defined in the embryonic Ngn3-null mouse. Diabetes 57: 2752–2761.

48. White P, May CL, Lamounier RN, Brestelli JE, Kaestner KH (2008) Defining pancreatic endocrine precursors and their descendants. Diabetes 57: 654–668.

49. Suyer J, Halle L, Raffelberger W, Buecher A, Orvain C, et al. (2010) Rfx5 is an Ngn3-dependent winged helix transcription factor required for pancreatic islet development. Development 137: 1703–1712.

50. Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2001) Gene targeting reveals a crucial role for peripherin 2 in the development of type 2 diabetes. Diabetes 50: 2221.

51. Calabi F, Pannell R, Pavloska G (2001) Gene targeting reveals a crucial role for peripherin 2 in the development of type 2 diabetes. Diabetes 50: 2221.

52. Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2001) Gene targeting reveals a crucial role for peripherin 2 in the development of type 2 diabetes. Diabetes 50: 2221.

53. Sankaran VG, Xu J, Ragsdale CW, Ippolito GC, Walkley CR, et al. (2009) Developmental and species-divergent globin switching are driven by BCL11A. Development 136: 2095–2105.

54. Karsunky H, Zeng H, Schmidt T, Zevnik B, Kluge R, et al. (2002) Inflammatory repression of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods 5: 948–960.

55. Bjork BC, Turbe-Doan A, Prysak M, Herron BJ, Beier DR (2010) Prdm16 is essential for normal lymphoid development. Nature immunology 11: 729–735.

56. Al-Hasani K, Pfister T, Courchet D, Bousso P, Ares M, et al. (2002) Inactivation of Arx in pancreatic alpha-cells triggers their neogenesis and conversion into functional beta-like cells. PLoS Genet 9: e1003954.

57. Dhawan S, Geogia SB, Tsien SL, Fan G, Bhansali A (2011) Pancreatic beta cell identity is maintained by DNA methylation-mediated repression of Arx. Developmental cell 20: 419–429.

58. Hembrok M (2012) Generating beta cells from stem cells-the story so far. Cold Spring Harbor perspectives in medicine 2: a007674.

59. Pearis H, Raghupathi R, Jessup CF, Zanin MP, Mohanasundaram D, et al. (2012) Increased expression of the glucose-responsive gene, RCAN1, causes hypoinsulinaemia, beta-cell dysfunction, and diabetes. Endocrinology 153: 5212–5221.

60. Al-Hasani K, Pfister T, Courchet D, Bousso P, Ares M, et al. (2002) Inactivation of Arx in pancreatic alpha-cells triggers their neogenesis and conversion into functional beta-like cells. PLoS Genet 9: e1003954.

61. Dhawan S, Geogia SB, Tsien SL, Fan G, Bhansali A (2011) Pancreatic beta cell identity is maintained by DNA methylation-mediated repression of Arx. Developmental cell 20: 419–429.

62. Hembrok M (2012) Generating beta cells from stem cells–the story so far. Cold Spring Harbor perspectives in medicine 2: a007674.

63. Courtney M, Gijmes E, Duvelle N, Rasoul C, Vieira A, et al. (2013) The inactivation of Arx in pancreatic alpha-cells triggers their neogenesis and conversion into functional beta-like cells. PLoS Genet 9: e1003954.

64. Dhawan S, Geogia SB, Tsien SL, Fan G, Bhansali A (2011) Pancreatic beta cell identity is maintained by DNA methylation-mediated repression of Arx. Developmental cell 20: 419–429.

65. Hembrok M (2012) Generating beta cells from stem cells-the story so far. Cold Spring Harbor perspectives in medicine 2: a007674.