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Mechanisms Regulating Specification and Maintenance of Pancreatic Cell Fate

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Mechanisms Regulating Specification and Maintenance of Pancreatic Cell Fate

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in Molecular Biology

by

Murtaza Kanji

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ABSTRACT OF THE DISSERTATION

Mechanisms Regulating Specification and Maintenance of Pancreatic Cell Fate

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Murtaza Kanji

Doctor of Philosophy in Molecular Biology

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Professor Anil Bhushan, Chair

An important aim of Type I diabetes therapeutic strategies is the replenishment of functional beta cells. Directing the efficient differentiation of stem cell sources *in vitro* into functional insulin secreting beta cells is the preferred approach, however, existing experimental protocols have not been able to successfully yield functionally responsive insulin positive cells. One obstacle in this approach can be attributed to gaps in the understanding of mechanisms that regulate pancreatic beta cell development *in vivo*. This research work seeks to better understand the mechanisms by which important genetic and epigenetic regulators govern pancreatic cell fate specification and maintenance.

Our data reveals that microRNAs (miRNAs) - post-transcriptional regulators of gene expression - play an important role in the functional maintenance and survival of newly specified pancreatic beta cells. In the absence of the miRNA-processing enzyme Dicer1, newly specified beta cells lose insulin expression and acquire a neuronal transcriptional profile. The deregulation of neuronal genes is associated with a loss of binding of the neuronal transcriptional repressor REST to its neuronal target genes. This suggests that miRNAs play an important role in modulating critical regulators of cell fate during pancreatic islet cell development.

Interestingly, an investigation into a role for REST during early pancreatic development revealed that activation of REST target genes in pancreatic progenitors resulted in the epithelium adopting a progenitor and duct-like state with defects in endocrine and acinar cell specification. This data argues for a previously unknown role for REST in modulating pancreatic organogenesis.

In addition to discovering novel transcriptional regulators of pancreatic development, our data provides insights into a critical role for a DNA methyltransferase - DNMT1 - in coordinating pancreatic organogenesis. Abrogation of DNMT1 expression in pancreatic progenitors resulted in p53-dependant apoptosis of the progenitors that was subsequently rescued upon introduction of p53 haploinsufficiency to the DNMT1 deficient progenitors.

This body of work adds to the overall understanding of normal embryonic pancreas development and may guide the creation of efficient beta cell differentiation protocols allowing for in *vitro* differentiated beta cells to possibly become an important source for replenishing beta cells in diabetic patients.

The dissertation of Murtaza Shabbir Kanji is approved.

Karen Lyons

Karen Reue

Senta K. Georgia

Martin G. Martin

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2013

To my parents, Shabbir and Zainab Kanji

And my wife Ismat

I am forever indebted

TABLE OF CONTENTS

LIST OF FIGURES

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Chapter 2 is a published manuscript in the journal "Diabetes" titled "**Dicer1 is required to repress neuronal fate during endocrine cell maturation**". Murtaza S. Kanji, Martin G. Martin and Anil Bhushan authored the manuscript.

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PUBLICATIONS AND PRESENTATIONS

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Kanji MS, Bhushan A. Dicer1 is required to repress neuronal fate during endocrine cell

maturation, UCLA annual MBI Research Retreat, Oct 2010 (**Poster Presentation**)
Kanji MS, Bhushan A. "Dicer1 is required to repress neuronal fate during endocrine cell maturation." Western Region of Islet Study Group Conference, April 2011 (**Oral Presentation**)

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Chapter 1

Introduction

The increasing incidence of diagnosed diabetes nationally (1,2) along with the substantial burden placed on the economy and healthcare system (3) has created an urgent push on scientific researchers and medical practitioners to come up with new strategies to effectively cure this disease in addition to developing temporary treatments that prevent the symptoms and complications associated with the disease.

The two most common forms of diabetes – Type 1 and Type 2 diabetes – both result from an insufficient mass of functional insulin-producing pancreatic beta cells. Type 1 diabetes, often referred to as juvenile diabetes, is a form of diabetes most commonly diagnosed in young children and is characterized by an autoimmune destruction (4) of pancreatic beta cells resulting in insulin deficiency and hyperglycemia. This results in an absolute insulin deficiency. The autoimmune attack usually results from exposure to environmental triggers in genetically susceptible individuals (5). In contrast, Type 2 diabetes is generally associated with obesity or older age; however, there has been an increasing prevalence of Type 2 diabetes and prediabetes in children and adolescents in recent times (6). Type 2 diabetes results from insulin resistance of insulin responsive tissues such as liver and peripheral skeletal tissues coupled with the inability of the existing pancreatic beta cell mass to expand to meet the metabolic demand due to beta cell dysfunction (7). Although pancreatic beta cell dysfunction has an underlying genetic basis, the peripheral insulin resistance is more commonly associated with environmental factors in addition to genetic makeup (8).

Current therapeutic strategies for treatment of diabetes include subcutaneous insulin injections for Type 1 diabetes (4) and less commonly, islet transplantation from cadaveric organ donors, has been used to provide temporary remission for patients (9). The islet transplantation procedure however is limited in scope due to the scarcity of islet donors and the need for chronic immunosuppression in patients transplanted with the islets making it an unfeasible mass-market treatment approach. For Type 2 diabetes, disease management through lifestyle modifications (10) is often encouraged along with the use of oral medications such as incretin-based therapies that stimulate insulin secretion or increased peripheral tissue sensitivity to insulin (11). While there is evidence that medications such as GLP-1 receptor agonists can successfully restore normal glycaemia (11), serious concerns have been raised about the long-term use of such medications and their potential to promote acute pancreatitis and eventually pancreatic cancer (12).

The non-curative nature of current diabetes therapies along with the safety concerns that have been raised for some of the oral medications (12) has resulted in a renewed effort to seek novel approaches in generating insulin producing beta cells for replacement therapies from autologous sources. The obvious advantage of using an individual patients' own stem cell sources or adult cell types to generate beta cells would be to avoid the use of immunosuppressive drug therapies needed to prevent the rejection of islet cell transplants. Additionally it would address the problem of extremely high demand that hospitals are facing for islet donor material.

Current research is focused on generating beta cells from alternate sources such as stem cells as well as looking into the potential of related cell types to transdifferentiate into beta cells. The pancreatic acinar cells and endocrine cells derive from common progenitors and the plasticity of acinar cells has allowed them to be manipulated and transdifferentiated into beta cells-like cells

(13,14) through the expression of a cocktail of transcription factors. Additionally, several attempts have been made at differentiating pancreatic beta cells in vitro from stem cells sources (15,16). However these efforts have met with limited success, as the insulin-expressing cells generated cannot fully recapitulate the insulin sensitivity of endogenous beta cells and are thus not functional beta cells (14).

The successful programing of progenitor cell populations or reprogramming of non beta cell fates into functionally mature beta cells requires a complete understanding of the mechanisms that regulate endogenous formation of functional pancreatic beta cells. Whereas a lot of progress has been achieved in the understanding of various signaling pathways and transcriptional cascades that guide the specification of beta cells (17), there is still a lot to be learned about how the various genetic and epigenetic modulators govern pancreas development during embryogenesis. These *in vivo* discoveries can then be translated into creating more effective protocols for the *in vitro* generation of insulin producing beta cells.

Pancreas Development

The pancreas consists of endocrine and exocrine cell compartments that arise from a common progenitor population (18). The exocrine compartment consists of acinar cells that produce digestive enzymes that are secreted into the small intestine through a branched network of ductal cells (19). The hormone producing endocrine cells are organized into clusters called islets of Langerhans and generally account for 1-2% of the pancreatic cell mass. Within a typical mouse islet, the insulin producing beta cells form at the core whereas glucagon producing alpha cells are located at the periphery, along with a small number of cells that produce other hormones like

somatostatin, pancreatic polypeptide and ghrelin. The majority of cells within an islet are beta cells.

During embryogenesis, the pancreas arises as two distinct evaginations along the dorsal and ventral surfaces of the posterior foregut endoderm (20). The early patterning and specification of the pancreatic endoderm is governed by signaling molecules from adjacent tissues (20) with the epithelial buds undergoing branching morphogenesis. Each of these epithelial buds consists of pancreatic progenitors expressing the marker pancreatic duodenal homeobox 1 (Pdx1) that subsequently give rise to the endocrine, acinar and ductal cell lineages of the mature pancreas. The early multipotent progenitor population also co-expresses Ptf1a, carboxypeptidase A (CPA) and the Sry/HMG box transcription factor Sox9 (21,22). The expression of Pdx1 is critical for normal pancreatic development to proceed as loss of Pdx1 results in pancreatic agenesis (18, 23-25) with subsequent development of diabetes in neonatal life (26). As pancreatic lineage specification continues, Pdx1 expression is downregulated in the endocrine and exocrine precursors before strong expression is restored exclusively in the newly differentiated beta cell population with low levels of Pdx1 expression maintained in the other pancreatic epithelial cells (27-29). Sox9 is also a critical factor required for maintenance and proliferation of the multipotent pancreatic progenitors and subsequently gets downregulated in lineage-committed progenitors and the differentiated acinar and endocrine cell populations (30). In contrast, the ductal cell population and a subset of the centroacinar cells continue to express Sox9.

Between embryonic days 8.5-11.5, the progenitors proliferate to increase the size of progenitor pool. This expansion is mediated by Notch and FGF signaling which prevents the progenitors from undergoing premature differentiation (31-34). The differentiation of the endocrine and exocrine cell lineages from the multipotent progenitor population also involves Notch signaling.

The Notch target Hes1, which is activated in progenitor cells prevents the downstream activation of Neurogenin 3 (Ngn3), a basic helix-loop-helix (bHLH) transcription factor whose expression is essential for the progenitors to adopt an endocrine cell differentiation program (35-37). Though Notch signaling is thought to prevent the differentiation program, recent findings seem to indicate that the Notch pathway plays an important role in activating the expression of Sox9, which in turn induces the downstream activation of Ngn3 allowing for endocrine differentiation to proceed (38). The activation of Ngn3 in the endocrine progenitors is transient and induces a downstream transcriptional cascade resulting in the specification of post-mitotic endocrine cells (39-40). While the majority of the endocrine cells are specified from Ngn3-positive endocrine progenitors between e12.5 and e15.5 during a period termed as the 'secondary transition', a small fraction of hormone expressing endocrine cells, mostly alpha cells, have been observed as early as e9.5. These primary transition endocrine cells are also present in Pdx1, Ptf1a and Ngn3 mutant pancreata demonstrating their unique origin and have yet to be subject to intensive characterization (41).

As with endocrine specification, the specification of the exocrine lineage involves Notch and FGF and EGF signaling (42, 43). The activation of these signaling pathways primarily functions to promote the period of progenitor cell expansion thereby allowing for an appropriate balance to be achieved between progenitor cell proliferation and differentiation resulting in high number of endocrine and exocrine cells being specified. In contrast to the requirement for transient Ngn3 activation for endocrine cell specification, Ptf1a is the bHLH lineage-specifying transcription factor that interacts with Notch signaling and is required both for the initiation of the transcriptional cascade regulating exocrine specification as well as its maintenance (44). The differentiated exocrine cells – acinar cells, ductal epithelial cells and the centroacinar cells are

each characterized by a different set of markers. The most interesting of these cell types are the centroacinar cells, which have been thought to possibly function as multipotent progenitor cells in the adult pancreas according to several studies (30,44). The capacity of these cells to rapidly proliferate in response to casts them as a potentially appealing stem cell source that could be utilized for the regeneration of beta cells.

The majority of beta cells during the prenatal period are accounted for by differentiation from progenitors and are mitotically quiescent (45). In contrast, self-duplication of the beta cells rather than neogenesis is the predominant mechanism through which the beta cell mass is expanded during the post-natal period (46,47). The neonatal period is also characterized by functional beta cell maturation during which the newly formed beta cells become capable of appropriately regulating glucose stimulated insulin secretion. A different set of transcription factors are activated in each of the islet cell types assisting in the maintenance of these mature cells. For example, the repression of MafB (48) and activation of NeuroD1 (49) is a critical step for post-natal maturation. Along with repression of the low Km hexokinase – HKII (50), these changes prevent the beta cells from exhibiting impaired insulin secretion.

Epigenetic modulation of differentiation and maintenance of pancreatic cell types

Over the years, a great deal of progress has been achieved in elucidating the transcriptional regulators and signaling pathways that govern pancreas development during embryogenesis. This information has in turn been used to inform the creation of in *vitro* protocols for the differentiation of embryonic stem cells or induced pluripotent stem cells into glucose-responsive, insulin-secreting beta cells (51). Given the plastic nature of the different pancreatic cell types

there are also efforts to reprogram these cell types into beta cells (13). In almost all cases however, these protocols have been unable to generate functionally mature beta cells. Recent research efforts have focused on looking toward epigenetic mechanisms that could potentially be regulating endocrine cell differentiation and maintenance.

Broadly defined, epigenetics refers to mechanisms that modulate gene expression patterns or cellular phenotype without altering the underlying DNA sequence. Epigenetic modifications of the genome do not involve any changes of the nucleotide sequence and are deemed to be heritable. DNA methylation, histone modifications and non-coding RNAs (ncRNAs) are the three main classes of epigenetic modifications that may affect the cellular phenotype [52]. Understanding the epigenetic network that regulates transcription factors and the signaling pathways during pancreatic organogenesis is as yet in its infancy, with significant promise being attributed to deciphering the changing epigenetic landscape as endocrine pancreas development proceeds and using this information to design more efficient stem cell differentiation protocols for therapeutic purposes.

Role of miRNAs in pancreas development and maintenance

Given that the protein-coding gene sequences account for approximately 2% of the genome, an important question is raised regarding the significance of the remainder of the genome. Recent studies have discovered that the vast majority of the genome codes for regulatory non-protein-coding RNAs (ncRNAs) such as microRNAs (miRNAs), small interfering RNAs (siRNAs) and long ncRNAs amongst others (53,54). MiRNAs are non-protein-coding small RNAs about 22 nucleotides in length that act by negatively regulating gene expression at the post-transcriptional

level (55) and have been implicated as important regulators of animal development (56). RNA Polymerase II transcribes them as long primary transcripts called primary miRNA transcripts (pri-miRNAs) and they undergo a series of processing steps that require the RNase III enzymes Drosha and Dicer1 before becoming functionally mature (57,58). The double-stranded mature miRNAs thereafter get incorporated into an RNA-induced silencing complex (RISC) and exert their silencing effects on target mRNAs that contain sequences complementary to the mature miRNA at the three prime untranslated regions (3' UTR). The silencing effect is mediated either through the repression of the translation process or through mRNA degradation (54,59) of the miRNA targets. It is important to note that several miRNAs can target a specific mRNA and conversely one mRNA can be the target of a combination of miRNAs giving rise to a vast array of miRNA-mRNA interactions that could be potentially regulating various biological processes. Adding to this layer of complexity is the potential of miRNAs to activate certain gene targets in addition to their traditional view as negative regulators of gene expression (60). The biology of miRNA regulation of gene expression in pancreatic islet specification and maturation is not completely understood. Several miRNAs have been proposed to regulate beta cell genes during development (reviewed in 61) and computational algorithms have identified various miRNAs that target pancreatic transcription factors involved in the specification process. Additionally, conditional deletion of Dicer1 - the critical enzyme required for miRNA biogenesis - in Pdx1 progenitor cells resulted in an almost total loss of all differentiated pancreatic lineages due to the Notch signaling target Hes1 failing to downregulate (62). MiRNAs also play important roles in maintaining the function of mature pancreatic beta cells. For example, miR-375 is a critical regulator of glucose homeostasis (63). Furthermore, inactivation of the miRNA pathway in adult

beta cells resulted in the de-repression of transcriptional repressors of insulin expression leading to hyperglycemia (64).

Most of the studies to date on miRNA regulation of pancreatic transcription factors have only been shown *in vitro* and it remains to be seen what role these regulators play during embryonic pancreatic organogenesis. One objective of this work therefore seeks to understand the role of miRNAs *in vivo* during the specification and maintenance of pancreatic beta cells. This information will add significantly to the current understanding of mechanisms involved in the transition of pancreatic progenitor cells into functional beta cells.

REST in pancreas organogenesis

The changing transcriptional landscape during pancreatic development is partly regulated by stage and tissue-specific miRNAs raising an important question as to what regulatory networks modulate the expression and function of these miRNAs during organogenesis. Evidence from neurogenesis suggests that a large number of neuron specific miRNAs are targets of the neuronal transcriptional repressor REST (RE-1-silencing transcription factor), and REST itself is a target of different miRNAs (65,66), indicating that this complex REST-miRNAs interplay is important in maintaining the tissue-specific gene expression profiles necessary for nervous system development to proceed and a similar layer of regulatory control could be present during pancreas specification. During neuronal development, REST mediates the silencing of neural target genes in neural stem cells (NSCs) thereby preventing these progenitors from undergoing differentiation (67). It also plays an important role in maintaining the transcriptional integrity of non-neuronal cells by silencing the expression of terminally differentiated neuronal genes (68).

The REST protein structure contains a DNA binding domain that binds specifically to target RE-1 binding regions in the regulatory regions of its target genes, as well as two repressor domains. The regulatory domains function to recruit several epigenetic regulators that render a repressive chromatin environment at the promoter regions thereby silencing active transcription of the target (68,69). REST binding sites have been discovered at the promoter regions of several key pancreatic beta cell transcription factors such as Pdx1, NeuroD and Pax4 (70). Additionally, *in vivo* genome-wide mapping of REST binding sites by ChIPSeq analysis on fibroblast cells identified the islet-cell development transcription factors NEUROD1, HNF4α, HNF6, Hes1 and Ngn3 as REST targets (71) implying a role for REST in islet cell development. In contrast the absence of REST expression in adult insulin-producing cell lines and the detrimental effect of conditional REST overexpression in beta cells on insulin exocytosis argues against a role for REST in mature beta cells (72,73). An important fact to consider regarding REST-mediated regulation is that the amount of available REST protein in different cell types and the binding affinity of the REST complex vary between its target genes. For example, in the ChIPSeq study (71), genes encoding endocrine cell regulators were bound relatively poorly by REST compared to terminally differentiated neuronal genes suggesting that a potentially larger amount of REST protein would be required to regulate the islet development factors compared to the neuronal genes. This regulation is therefore not an on-off function and is very much context dependent. Part of this research work seeks to delineate a role for REST in pancreatic development.

DNA methylation during pancreas specification

Pancreatic organogenesis involves a combination of signaling pathways and pancreas-specific transcriptional regulators interacting to coordinate the balance between proliferation of pancreatic progenitor cells and cell cycle exit followed by differentiation that eventually results

in the complex pancreatic organ being patterned appropriately. Various epigenetic mechanisms have been suggested to regulate transcriptional cascades during development (52), one of them being DNA methylation – an epigenetic process that involves the methylation of the Cytosine at the 5 position in CG dinucleotides and leads to gene repression in a variety of tissues (52). Previous work has shown that changing patterns of DNA methylation during embryogenesis have a profound effect on cell-fate specification (74) implying that DNA methylation could also potentially contribute towards the maintenance of pancreatic progenitor pluripotency or the initiation of lineage commitment.

Proteins known as the DNA methyltransferases carry out the establishment and maintenance of DNA methylation patterns. The *de novo* methyltransferases Dnmt3a and Dnmt3b are required for the establishment of DNA methylation patterns early during development (75). In contrast DNMT1 is regarded as a maintenance methyltransferase that functions to methylate hemi methylated DNA sequences in proliferating cells thereby propagating methylation patterns in daughter strands (76). Previous studies have offered a contrasting view on the role of Dnmt1 during development. Whereas a loss of Dnmt1 in hematopoietic progenitor cells promotes a shift toward myeloid cell differentiation (77), a similar loss of Dnmt1 in human epidermal stem cells promotes the maintenance of progenitor cell self-renewal (78).

This research work aims to understand the role of DNA methylation during pancreatic lineage specification potentially yielding novel information as to how the manipulation of the epigenome controls the balance between progenitor maintenance and lineage specification during pancreatic embryogenesis. Small molecule therapeutics could possibly be developed to target these epigenetic regulators and achieve more efficient differentiation of stem cell sources into functional beta cells.

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Chapter 2

Dicer1 is required to repress neuronal fate during endocrine cell maturation

ABSTRACT

MicroRNAs (miRNAs) are important regulators of gene expression programs in the pancreas, however little is known about the role of miRNA pathways during endocrine cell specification and maturation during neonatal life. In this study, we deleted *Dicer1*, an essential ribonuclease for active miRNAs biogenesis, specifically from NGN3+ endocrine progenitor cells. We found that deletion of *Dicer1* in endocrine progenitors did not affect the specification of hormone-expressing endocrine cells. However the islets in the mutant mice in the neonatal period exhibited morphological defects in organization, loss of hormone expression and the mutant mice subsequently developed diabetes. Dicer1-deficient β-cells lost insulin expression while maintaining the expression of β-cell transcription factors such as Pdx1 and Nkx6.1 early in the postnatal period. Surprisingly transcriptional profiling showed that that the Dicer1-deficient endocrine cells expressed neuronal genes prior to the onset of diabetes. The derepression of neuronal genes was associated with a loss in binding of the neuronal transcriptional repressor REST to its targets in Dicer1-deficient β-cells. These studies suggest that miRNAs play a critical role in suppressing neuronal genes during the maturation of endocrine cells.

INTRODUCTION

A potential therapeutic approach to replenish the pancreatic β-cell mass in diabetic patients involves the transplantation of functional, glucose responsive β-cells differentiated from human pluripotent stem cells (HPSCs). Several attempts have been made at differentiating β-cells *in vitro* from stem cells with limited success (1-2) as the insulin-expressing cells generated lack the characteristic hallmarks of functionally mature β-cells such as the ability to regulate glucose-stimulated-insulin-secretion (GSIS). Although many transcription factors and signaling pathways underlying the stepwise cell fate acquisition during β-cell development are known (3-6), a complete understanding of the molecular basis of β-cell specification and functional maturation is lacking.

Of significant interest is the role of microRNAs (miRNAs) in regulating the pancreatic developmental program. miRNAs are non-protein-coding small RNAs (~19-25 nucleotides) that negatively regulate gene expression at the post-transcriptional level (7) and have been implicated as important regulators of animal development (8). Newly transcribed miRNAs undergo a series of processing steps that require the RNase III enzymes Drosha and Dicer1 before becoming functional (9-10). Although several miRNAs have been proposed to regulate β-cell transcription factors during development (11), many of these computationally predicted miRNA-mRNA interactions have not been experimentally validated *in vivo*. The dysregulation of miRNAs through *Dicer1* ablation in the early embryonic Pdx-1 expressing pancreatic progenitor cells resulted in severe deficiencies in the formation of all islet cell lineages (12). More recently, it has been shown that deletion of *Dicer1* in β-cells leads to loss of insulin expression and development of diabetes in adult mice (13). Although these studies reveal key functions of miRNA dependent

pathways during early pancreatic development and in adult β-cells, they preclude analysis of the role of miRNAs during the specification of endocrine cells and their functional maturation in postnatal life. In this study, we used a mouse model where expression of Cre recombinase directed by the *Ngn3* promoter conditionally deleted floxed *Dicer1* alleles in endocrine progenitor cells. In addition, by crossing these mice onto the R26RYFP reporter line, we were able to trace the lineage of the Dicer1-deficient islet progenitor cells. Our data demonstrates that Dicer1-deficient endocrine progenitors differentiate into hormone-expressing endocrine cells but lose hormone expression subsequently during the neonatal period and develop diabetes. More surprisingly, we found that the Dicer1-deficient islet cells expressed neuronal genes supporting a model in which miRNA pathways control important transcriptional networks required for suppressing neuronal fate during the maintenance and maturation of newly specified endocrine cells.

RESEARCH DESIGN AND METHODS

Mice and physiology. Mice were maintained in a 12 h light/dark cycle under standard conditions. Studies involving mice were performed in accordance with NIH policies on the use of laboratory animals and approved by the University of California, Los Angeles Animal Research Committee. The mice used in this study are the conditional *Dicer1* $^{flox/flox}$ line (14), the *Ngn3-Cre* (15) and *R26R-YFP* (16) lines. The control mice used throughout the paper were heterozygous for the conditional *Dicer1* allele and the Ngn3-Cre transgene (NC:*Dicer1*$^{fl/+}$). All mice were maintained in the C57BL6 background. DNA extracted from tails was used for PCR based genotyping. Blood glucose levels were measured from tail vein blood using a FreeStyle glucometer (Abbot Diabetes Care) and pancreatic insulin content was measured using a mouse

insulin ELISA kit (Mercodia) after acid ethanol (0.18 mol/l HCl in 70% ethanol) extraction according to the protocol recommended by the Animal Models of Diabetes Complications Consortium (http://www.amdcc.org/).

Histology and immunohistochemistry. Pancreatic tissue was processed for immunohistochemical analyses as previously described (17). Briefly, the pancreas was dissected and fixed in 4% formaldehyde for 2h before being embedded in paraffin. 5-µm-thick sections were deparaffinized, rehydrated, subjected to antigen retrieval using Antigen Unmasking Buffer (Vector Labs) and permeabilized in 0.4% Triton X-100/TBS. Tissues were subsequently blocked with 3% IgG-free BSA (Jackson ImmunoResearch Laboratories). Incubation with primary antibodies was performed overnight at 4°C in blocking solution at the following dilutions: 1:200 guinea pig anti-insulin (Dako), 1:500 rabbit anti-glucagon (Immunostar), 1:200 rabbit anti-amylase (Sigma-Aldrich), 1:100 mouse anti-Pdx1 (DHSB), 1:200 rabbit anti-Pdx1 (Chemicon), 1:250 chicken anti-GFP (Aves Labs Inc.), 1:500 rabbit anti-MafA (18), 1:100 rabbit anti-MafB (Bethyl), 1:1000 rabbit anti-Glut2 (Millipore), 1:250 rabbit anti-Th (Millipore), 1:50 mouse anti-Nkx2.2 (BCBC), 1:50 mouse anti-Nkx6.1 and 1:50 mouse anti-Ki67 (BD Pharmingen). Secondary antibodies (Jackson ImmunoResearch Laboratory) conjugated to FITC (1:200 dilution) or Cy3 (1:1000 dilution) were diluted in blocking buffer. *In situ* cell death detection assay (TUNEL) was performed according to manufacturer's instructions (Roche). Slides were mounted with Vectashield with DAPI (Vector Labs) and images were obtained using Openlab software (Perkins Elmer) and a Leica DM6000 microscope.

β-cell mass analysis. β-Cell mass was calculated as previously described (19) by analyzing

pancreata from four mice for each age and genotype.

Islet isolation and FACS sorting. Islet isolation from mice was performed using a Liberase enzyme blend and purified by centrifugation in a Histopaque gradient as described previously (17) with slight modifications. Instead of Liberase perfusion through common bile duct, neonatal pancreata were randomly injected with Liberase blend. To obtain purified pancreatic and endocrine progenitor cells, pancreatic buds from E12.5 Pdx1-YFP and E14.5 Ngn3-GFP (20) embryos were dissected and dissociated into a single cell suspension using non-enzymatic cell dissociation buffer (Sigma-Aldrich). GFP+ cells were sorted by FACS (FACSaria BD biosciences). A similar process was followed to obtain β cells from islets isolated from 2-week-old MIP-GFP (21) mice.

RNA isolation, RT-PCR and real-time qPCR. Total RNA from islets, dissected pancreata and Ngn3-GFP and MIP-GFP cells was isolated using an RNeasy RNA extraction Kit (Qiagen). Single stranded cDNA was prepared using Superscript III Reverse Transcriptase (Invitrogen) with oligodT priming. Real-time qPCRs were performed using the 7900HT Fast Real-time PCR system (Applied Biosystems). The expression level of each transcript was normalized to the housekeeping gene *Cyclophilin*. Data and standard deviations shown were measured from at least three independent biological replicate experiments. All RT-PCR and real-time qPCR primer sequences used are available upon request.

ChIP assays and ChIP-qPCR. To obtain purified β-cells from P7 control and NC:*Dicer1*$^{fl/fl}$ mice for ChIP analyses, dissected pancreata were dissociated into a single cell suspension. The

pancreatic cells were immunostained for insulin, following fixation and permeabilization with BD Cytofix reagent (BD PharMingen), using guinea pig anti-insulin antibody, followed by incubation with a Cy3-conjugated secondary antibody, and sorted by FACS (FACSaria BD Bioscience). Pancreatic cells processed without primary antibody were used as a negative control for FACS. ChIP experiments on purified β cells was then performed using the micro-ChIP protocol previously described (22). The sequence-specific primers used to amplify the region around the RE1 sites on the Stmn2, Stmn3, Th and Syn1 locus are available upon request. qPCR analysis was performed using the 7900HT Fast Real-time PCR system (Applied Biosystems). The data shown is using independent biological triplicates and ChIP-qPCR signals are reported as percentage of input.

Microarray gene expression analysis

Two pairs of independent pancreata from control and mutant NC:*Dicer1*$^{fl/fl}$ mice were dissected out at postnatal day 7 (P7) and total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen) as per manufacturer's protocol. Total RNA quality was assessed using an Agilent 2100 Bioanalyzer and an RNA Integrity Number (RIN) generated using 2100 Expert Software (Agilent Technologies). All RNA samples used had a RIN greater than 7. 1 μg of total RNA was processed, labeled and hybridized to the mouse 1.0ST GeneChip Microarray (Affymetrix) according to manufacturer's recommendations by the UCLA DNA Microarray Core Facility.

The CEL files (raw expression measurement data) generated from array image analysis were imported into Partek Genomics Suite Software (23), RMA normalized and converted to $\log_2$ values. The software was further used to perform analysis of variance (ANOVA) and identify

statistically significant differentially expressed genes (P-values) between control and NC:*Dicer1*$^{fl/fl}$ pancreata by applying a false discovery threshold of 5% to the *p* values.

Statistical analyses.

All data are expressed as means ± SEM. Statistical significance was determined by an unpaired Student's t test and a P value <0.05 was used to reject the null hypothesis.

RESULTS

Dicer1-null endocrine precursor cells specify appropriately but display loss of hormone expression during neonatal phase and subsequently develop diabetes

The catalytic role of *Dicer1* is central to the functional maturation of miRNAs from their precursors (9). To investigate *in vivo* the role of miRNAs during islet cell specification from *Neurogenin3* (*Ngn3*) expressing endocrine precursor cells, the *Dicer1* conditional allele (*Dicer1*$^{fl/fl}$) (14) was deleted specifically from endocrine precursor cells using *Ngn3-Cre* (NC)-mediated excision (15) thereby preventing the generation of active miRNAs in these cells. Additionally, an R26R-YFP reporter line (16) was crossed onto the Ngn3-Cre:*Dicer1*$^{fl/fl}$ (NC:*Dicer1*$^{fl/fl}$) mice, to heritably label any cells undergoing recombination and deletion of Dicer1. Co-immunostaining of NC:*Dicer1*$^{fl/fl}$ pancreatic sections for Insulin /YFP (Figure 1A-F) and Glucagon/YFP (data not shown) showed a high degree of overlap of these markers in the mutant islets, therefore confirming that a high percentage of specified islet cells were derived from endocrine precursor cells that had undergone cre-mediated recombination. Real-time qPCR analysis revealed that the *Dicer1* transcript was reduced by 80% in the mutant NC:*Dicer1*$^{fl/fl}$

islets at 1 week (Figure 1G) further confirming the deletion of *Dicer1* from a majority of islet cells.

To determine whether loss of *Dicer1* affected the specification of islet cells, pancreatic tissue isolated from control (heterozygous for the *Dicer1* conditional allele - NC:*Dicer1*$^{fl/+}$) and mutant NC:*Dicer1*$^{fl/fl}$ mice were co-immunostained for insulin and glucagon at different stages of neonatal development. Immunostaining of pancreas from embryonic day 18.5 (e18.5) NC:*Dicer1*$^{fl/fl}$ mice for insulin and glucagon revealed that α and β-cells specified appropriately in the pancreas and no morphological differences in islet architecture were apparent (Figure 1H, K). However, pancreas from one week old NC:*Dicer1*$^{fl/fl}$ mice exhibited altered islet organization with many instances of α-cells prevalent within the core of the islet rather than at the periphery, an observation also confirmed by Pdx1 and glucagon staining at the same stage (Figure 1I,L and Supplementary Figure S1A, B). Pancreas from two weeks old NC:*Dicer1*$^{fl/fl}$ mice displayed severe defects in morphology and the expression of insulin and glucagon was severely diminished (Figure 1J, M). Quantification at 1 day after birth revealed similar of β and α cell mass (Figure 1P, Q). A similar comparison showed a dramatic reduction in endocrine cell mass in the mutant NC:*Dicer1*$^{fl/fl}$ mice at 2 weeks after birth. Consistent with the reduction in β cell mass by 2 weeks (Figure 1P), an almost total loss of pancreatic insulin content was observed in the mutant NC:*Dicer1*$^{fl/fl}$ animals (shown at three weeks Figure 1O). However, only a very modest decrease in insulin content was noticeable in the mutant NC:*Dicer1*$^{fl/fl}$ animals at 1 week. The mutant NC:*Dicer1*$^{fl/fl}$ animals displayed an inability to metabolize glucose and developed hyperglycemia and frank diabetes within two weeks of birth (Figure 1 N) consistent with a nearly total loss of β cells by that age.. Taken together, our results suggest that *Dicer1* in the endocrine progenitors was not required for specification of endocrine cells during

embryogenesis but was required postnatally to maintain the expression of hormones and the maintenance of endocrine cell mass. These observations therefore underscore the key role miRNAs play during the neonatal period when endocrine cells became functionally mature and capable of maintaining blood glucose levels.

Endocrine cells from *Dicer1-null* mice maintain expression of characteristic transcription factors despite losing hormone expression

Next, we asked whether the loss of hormone expression in mutant NC:*Dicer1*$^{fl/fl}$ islet cells was due to any potential effect on key islet transcriptional regulators upon ablation of miRNAs . We tested this hypothesis by assessing the expression of characteristic β-cell transcription factors Pdx1, Nkx6.1 and MafA. Immunostaining of pancreata from two-week-old control mice showed the expected co-expression of all these transcription factors with insulin (Figure 2A-C and data not shown). In contrast, while the pancreata from mutant NC:*Dicer1*$^{fl/fl}$ littermates showed normal expression levels of Pdx1, Nkx6.1 and MafA in the islets, only a few of these cells were also positive for insulin with the REST devoid of insulin expression. (Figure 2D-F). Similarly, two-week-old mutant NC:*Dicer1*$^{fl/fl}$ pancreatic sections showed a number of cells that expressed MafB but not glucagon (Supplementary Figure S1C-D). Furthermore, real-time qPCR analyses of a set of key β-cell genes between control and mutant NC:*Dicer1*$^{fl/fl}$ pancreata revealed a significant reduction in the expression of *Insulin1* and *Insulin2* mRNA, whereas no differences in the expression of transcription factors markers was observed (Supplementary Figure S1E-F and S2A) . These results suggested thatendocrine hormone expression was lost although the

transcriptional regulatory genes that characterize endocrine cells was intact in the absence of *Dicer*1.

Deletion of *Dicer-1* upregulates neuronal genes in islet cells

To investigate the molecular changes in endocrine cells of mutant NC:*Dicer1*$^{fl/fl}$ animals that could be responsible for the loss of hormone expression in endocrine cells, we performed genome-wide transcription profiling using microarrays on 2 independent pairs of control and mutant NC:*Dicer1*$^{fl/fl}$ pancreata isolated at postnatal day 7 (P7). The microarray was performed at this stage, which precedes any apparent physiological changes. Transcription profiling analysis revealed 162 differentially expressed genes with at least 2-fold change at a false discovery rate of 5 % of which 145 genes were upregulated and 17 were downregulated. A surprisingly disproportionate number of neuronal genes were up-regulated in the NC:*Dicer1*$^{fl/fl}$ pancreata, whereas the expression of transcription factors and other characteristic islet cell markers did not exceed the 2-fold change threshold (Figure 3A-C). The upregulation of these neuronal genes in NC:*Dicer1*$^{fl/fl}$ pancreata was further validated by performing real-time qPCR analysis from the microarray samples (Figure 3E) as well as on a triplicate of samples independent of those used for the microarray analysis (Supplementary Figure 2A). Gene ontology (GO) analysis for biological processes associated with the dysregulated genes revealed a significant enrichment of genes involved in nervous system development, synaptic transmission, transmission of nerve impulse and neurogenesis (Figure 3D). The upregulated genes included pan-neuronal markers such as Scg10, Stmn3, the neurofilament markers Nefl and Nefm as well as molecular markers of noradrenergic neurons such as the neurotransmitter-

synthesizing enzymes tyrosine hydroxylase (Th) and dopamine beta hydroxylase (Dbh), the vesicular transport molecule – vesicular monoamine transporter 2 (Vmat2) and the plasma membrane transporter - norepinephrine transporter (Net). In fact, many components of the noradrenergic program were upregulated. A number of these neuronal genes are expressed to some extent early during endocrine pancreas development but their expression declines significantly by 3-4 weeks of age (24). For example, real-time qPCR analysis indicated that the expression of NPY, which is normally downregulated upon maturation was expressed at high levels in the mutant NC:*Dicer1*$^{fl/fl}$ pancreata. To test whether the increase in transcript levels of the neuronal genes was associated with a corresponding increase in protein levels, we immunostained one week control and NC:*Dicer1*$^{fl/fl}$ pancreatic sections for insulin and tyrosine hydroxylase (Th). Very few Th and insulin double-positive cells were observed in control sections (Figure 4A-C). In contrast, the mutant NC:*Dicer1*$^{fl/fl}$ sections displayed a larger percentage of cells that co-expressed insulin and Th (Figure 4D-F). Thus, the upregulation of mRNA of neuronal genes due to the absence of *Dicer1* also results in a concomitant increase at the protein level. To assess whether the upregulation of Th was limited to β-cells, we co-stained Th with Pdx1. All Th-positive cells in the NC:*Dicer1*$^{fl/fl}$ islets also co-expressed Pdx1 (Figure 4H-L, M) suggesting that in the absence of *Dicer1*, β-cells upregulated the neuronal marker Th. In order to verify the endocrine lineage of these Th-positive cells, we performed immunostaining for the lineage trace marker YFP and Th in the mutant NC:*Dicer1*$^{fl/fl}$pancreata. All of the Th-positive cells were also positive for YFP staining (Figure 4G,J) therefore confirming that the Th-positive cells were derived from endocrine precursor cells that had undergone recombination and lost Dicer1 expression.

We also examined Th expression in the mutant NC:*Dicer1*$^{fl/fl}$ islets at 2 weeks. Consistent with observations in islets from 1-week-old control mice, co-staining of Th with insulin (Figure 4N-S) revealed that control islets from 2-week-old mice also typically displayed only a few Th positive cells that also stained for insulin (Figure 4N-P). In contrast, mutant NC:*Dicer1*$^{fl/fl}$ islets displayed a massive upregulation of Th staining (Figure 4Q-S). While a few insulin and Th co-positive islet cells were evident, a majority of the Th positive cells did not show any insulin staining suggesting that neuronal gene upregulation persists in mutant islet cells that have already downregulated endocrine hormones. Taken together, our results suggest that miRNAs likely play an important role in suppressing a neuronal gene program during the maturation phase of endocrine islet cells.

Loss of REST-mediated repression of neuronal targets in *Dicer1*-null β-cells

We hypothesized that the acquisition of a neuronal transcriptional profile by the mutant NC:*Dicer1*$^{fl/fl}$ islet cells could possibly be attributed to the dysregulation of transcriptional programs dedicated towards the maintenance of islet cell identity. To investigate transcription factors regulating neuronal gene repression in islet cells, we employed the MetaCore Interactome characterization tool **(**GeneGo Inc. MI, http://www.genego.com) to identify the top transcription factors that exhibited significantly large numbers of interactions with the dysregulated genes in the mutant NC:*Dicer1*$^{fl/fl}$ islet cells. The interactome analysis revealed that 16% of the dysregulated genes were targets of the neuronal transcriptional repressor REST (RE1-Silencing Transcription Factor (REST) also known as NRSF) (Figure 5A). REST is a repressor of neuronal genes that binds in a sequence-specific manner to the RE1 elements of its target genes and

mediates transcriptional repression (25-26). The upregulation of REST target genes in NC:*Dicer1*$^{fl/fl}$ islet cells suggested an important role for REST during islet cell development and maturation. We first investigated whether REST was expressed at different stages of islet cell development. Real-time qPCR analysis of REST expression showed that *REST* transcripts were present in FACS-purified Ngn3-positive endocrine precursor cells at e14.5 and in β-cells at post-natal day 14 (P14) at levels comparable to those expressed in e14.5 brain tissue and adult lung tissue where REST is known to be highly expressed (27; 28) (Figure 5B). Though microarray and real-time qPCR analysis (data not shown and Figure 5D) did not reveal any changes in REST mRNA levels between control and NC:*Dicer1*$^{fl/fl}$ islets, we hypothesized that changes in miRNAs could potentially be regulating molecular events which may alter recruitment of REST protein and/or members of the REST repressive complex . To analyze whether REST binding to the RE1 sites of its target genes was lost in the mutant NC:*Dicer1*$^{fl/fl}$ β-cells, chromatin immunoprecipitation (ChIP) analysis on a subset of the upregulated REST target genes showed that REST was bound to the RE1 sites of all four genes examined in FACS-purified control β-cells at P7. In contrast, REST binding to its target genes was significantly reduced in the mutant NC:*Dicer1*$^{fl/fl}$ β-cells (Figure 5C) and a concomitant increase in mRNA expression of these genes was observed in the mutant NC:*Dicer1*$^{fl/fl}$ pancreata (Figure 3E). This suggests that miRNAs play an important role in regulating the recruitment of the REST repressive complex, which in turn represses the transcription of its target neuronal genes. In addition to REST repression of the neuronal genes, we also identified other potential candidate transcription factors using the interactome analysis, including the neuronal homeobox transcription factors Phox2a and Phox2b – which are important regulators of the noradrenergic phenotype in vertebrates (29-31). Real-time qPCR analysis indicated that both transcripts were significantly upregulated in the

mutant NC:*Dicer1*$^{fl/fl}$ pancreata (Figure 5D). Taken together, our results reveal an important role of miRNAs in regulating key transcriptional programs required for maintaining islet cell identity during neonatal development.

***Dicer1*-null islet cells die by early adulthood**

To determine the ultimate fate of the *Dicer1*-null islet cells that had acquired an altered transcriptome, we immunostained the islets of control and NC:*Dicer1*$^{fl/fl}$ pancreatic sections for the islet hormone markers at six weeks. By six weeks, most insulin expression (Figure 6A-B, Supplementary Figure S3A-B) as well as other hormone expression in the islet was lost (data not shown). To determine whether the hormone negative Dicer1-null islet cells survived into adulthood, immunostaining of control and NC:*Dicer1*$^{fl/fl}$ pancreatic sections with the lineage trace marker YFP revealed that by six weeks, very few YFP-positive cells remained in the mutant NC:*Dicer1*$^{fl/fl}$ pancreatic sections compared to the littermates (Figure 6C-D). Since the genetic ablation of *Dicer1* is associated with apoptosis during development (14, 32-33), we assessed the apoptosis rate in control and mutant NC:*Dicer1*$^{fl/fl}$ islets using TUNEL immunostaining. A significant increase (11.7 fold) in apoptosis was observed in mutant NC:*Dicer1*$^{fl/fl}$ β-cells at P12 (Figure 6E-G) as well as other islet cells (data not shown) therefore suggesting that *Dicer1* was ultimately responsible for the survival of mature islet cells.

DISCUSSION

Our study illustrates an essential role of Dicer1-dependent miRNA pathways in the suppression of a subset of neuronal markers, and the maintenance of endocrine hormone expression during the maturation of newly specified islet cells in neonatal development. Surprisingly, we found that deletion of the miRNA processing enzyme Dicer1 in islet progenitors cells (NC:*Dicer1*$^{fl/fl}$ mice) did not affect the specification of endocrine cells. However, these newly specified endocrine cells acquired an altered transcriptome in which neuronal genes were upregulated and endocrine hormone expression was lost, resulting in failure to regulate blood glucose and onset of diabetes by 2 weeks of age. One possible explanation for this is that the loss of miRNAs early in endocrine development may alter later developmental pathways involved in the maturation of endocrine cells.

Both neuronal and β-cells share the expression of a large number of proteins and many similarities exist in terms of the transcription factors involved in their differentiation programs (34). The neuronal genes could be direct targets of miRNAs, such that *Dicer1* ablation would result in their derepression in the mutant NC:*Dicer1*$^{fl/fl}$ islet cells. Alternatively, it is a more likely scenario where transcriptional regulators of the neuronal markers that are direct or indirect targets of a combination of miRNAs could be deregulated in the NC:*Dicer1*$^{fl/fl}$ islet cells, resulting in the derepression of a neuronal transcripts. The decreased binding of the neuronal transcriptional repressor REST to target neuronal genes, and increased levels of the neuronal transcriptional activators Phox2a and Phox2b in mutant NC:*Dicer1*$^{fl/fl}$ β-cells suggests that these miRNA-controlled transcriptional regulators are likely part of a broader transcriptional program that actively suppresses neuronal differentiation genes to maintain islet cell identity.

In vivo genome-wide mapping of REST binding sites by ChIPSeq analysis has previously identified the critical islet-cell development transcription factors NEUROD1, HNF4α, HNF6, Hes1 and Ngn3 as targets of REST (35). Interestingly, genes encoding these endocrine cell regulators were bound relatively poorly by REST compared to terminally differentiated neuronal genes such as Stmn2, Stmn3 and the neurofilament genes which bound with greater affinity. This suggests that REST might not play a major role during islet cell differentiation during embryogenesis, but may instead control terminally differentiated neuronal genes, that might be involved in maturation of endocrine cells. *Dicer1* deletion results in deregulation of REST recruitment resulting in the derepression of its target neuronal genes. The eventual loss of islet cells is not surprising given that the loss of Dicer1 expression is associated with massive cell death in a range of different tissues during development (14). Our results are supported by a recent study on the deletion of *Dicer1* in β-cells using the transgenic RIP-Cre line which showed that neonatal pancreas contained ultrastucturally normal beta cells, although these mice developed progressive hyperglycaemia and full-blown diabetes mellitus in adulthood (36). The derepression of neuronal genes in β-cells however was not explored.

Given the similarities in the neuronal and islet cell differentiation programs, our studies demonstrate a novel role of miRNA pathways in maintaining islet cell identity through the repression of neuronal markers while maintaining the expression of endocrine hormones. Identifying the individual miRNAs involved in the maturation and maintenance of newly specified islet cells, particularly β-cells will aid in the development of therapeutic strategies to generate functionally mature replacement β-cells. These results also have important implications for engineering β-cells from ES cells.

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No potential conflicts of inteREST relevant to this article were reported.

M.K. designed and performed research, analyzed data and wrote the manuscript. M.G.M. designed research and reviewed and edited the manuscript. A.B. designed research, analyzed data and wrote the manuscript. Dr. Anil Bhushan is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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Figure 1-1

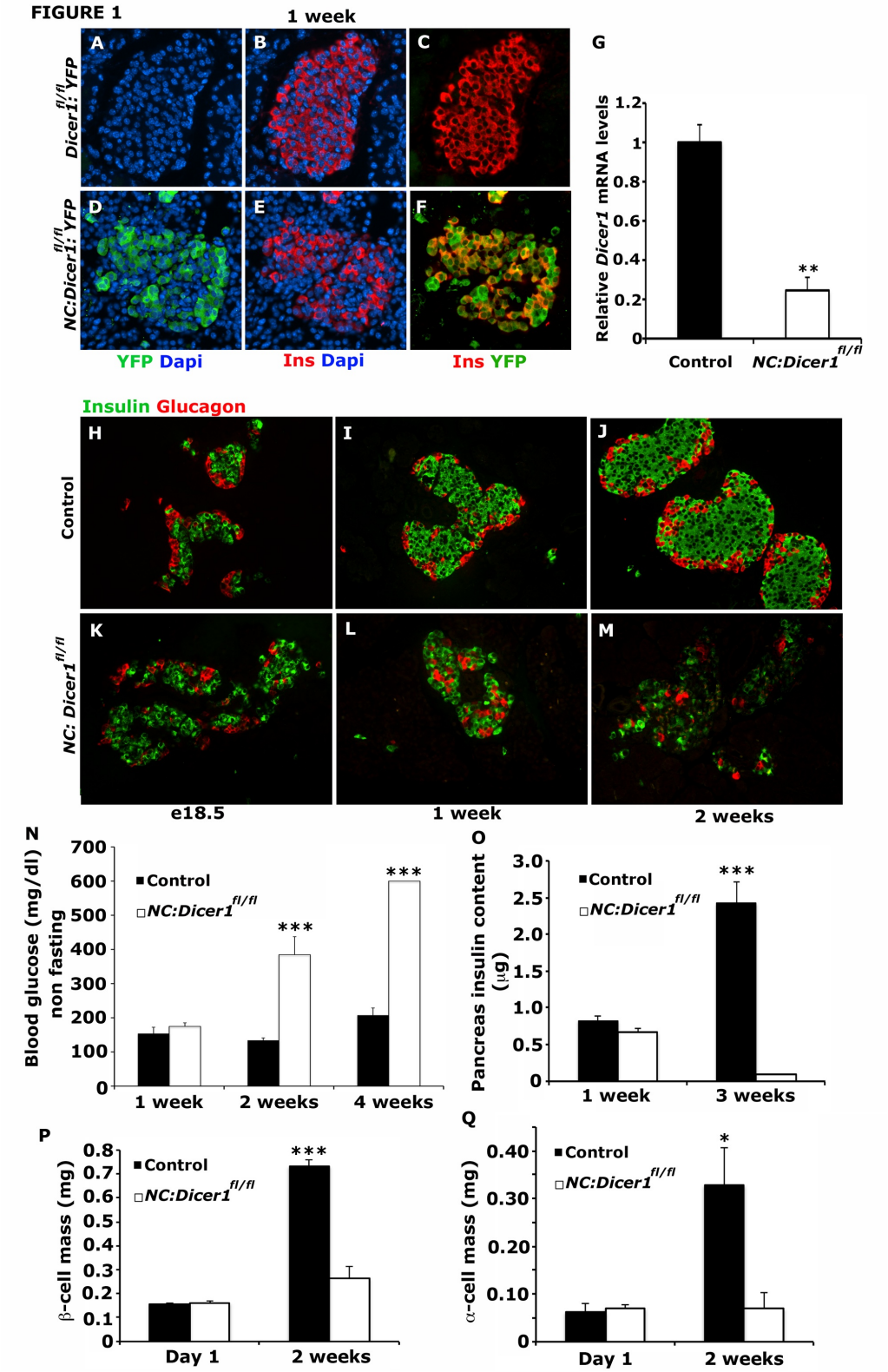


FIGURE 1
1 week
A
B
C
D
E
F
Dicer1: YFP fl/fl
NC:Dicer1: YFP fl/fl
YFP Dapi
Ins Dapi
Ins YFP
G
Relative Dicer1 mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
**
Control
NC:Dicer1 fl/fl
Insulin Glucagon
H
I
J
K
L
M
Control
NC: Dicer1 fl/fl
e18.5
1 week
2 weeks
N
Blood glucose (mg/dl) non fasting
700
600
500
400
300
200
100
0
Control
NC:Dicer1 fl/fl
***
***
1 week
2 weeks
4 weeks
O
Pancreas insulin content (µg)
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
NC:Dicer1 fl/fl
***
1 week
3 weeks
P
β-cell mass (mg)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
NC:Dicer1 fl/fl
***
Day 1
2 weeks
Q
α-cell mass (mg)
0.40
0.30
0.20
0.10
0
Control
NC:Dicer1 fl/fl
*
Day 1
2 weeks

Figure 1-1. Mutant NC:*Dicer1*$^{fl/fl}$ islet cells specify normally but lose hormone expression and develop hyperglycemia during neonatal development. Immunostaining of 1-week-old Cre negative (A-C) and mutant NC:*Dicer1*$^{fl/fl}$ (D-F) pancreatic sections (n=3) for insulin (red, B, E), YFP that marks recombined cells (green, A, D) and DAPI to visualize the nuclei (blue). A majority of the insulin+ β-cells co-localize with YFP in the mutant islets but not in the control islets (C, F). G: Transcript levels of *Dicer1* determined by real-time qPCR using RNA isolated from islets of control and mutant NC:*Dicer1*$^{fl/fl}$ mice (n=3) at P7. Expression levels in control were set as one arbitrary unit. Representative pancreatic sections (n=3 for each age and genotype) from e18.5, 1-week-old and 2-week-old control (H-J) and mutant NC:*Dicer1*$^{fl/fl}$ (K-M) littermates were immunostained for insulin (green) and glucagon (red). N: Non-fasting blood glucose levels in neonatal control and mutant NC:*Dicer1*$^{fl/fl}$ mice at 1 week, 2 weeks and 4 weeks (n=5 for each age group). NC:*Dicer1*$^{fl/fl}$ mutant mice are hyperglycemic by 2 weeks. O: Total pancreatic insulin content in control and NC:*Dicer1*$^{fl/fl}$ mice at 1 week and 3 weeks reveals minimal pancreatic insulin content remaining in NC:*Dicer1*$^{fl/fl}$ mice at 3 weeks (n≥3). P-Q: β and α-cell mass in control and mutant NC:*Dicer1*$^{fl/fl}$ mice at P1 and 2 weeks was assessed as described in RESEARCH DESIGN AND METHODS (n = 4). The error bars represent standard error of the mean (SEM). * P<0.05, ** P<0.01, ***P<0.005. In all cases, at least two to three pancreas sections were used for each animal.

Figure 1-2

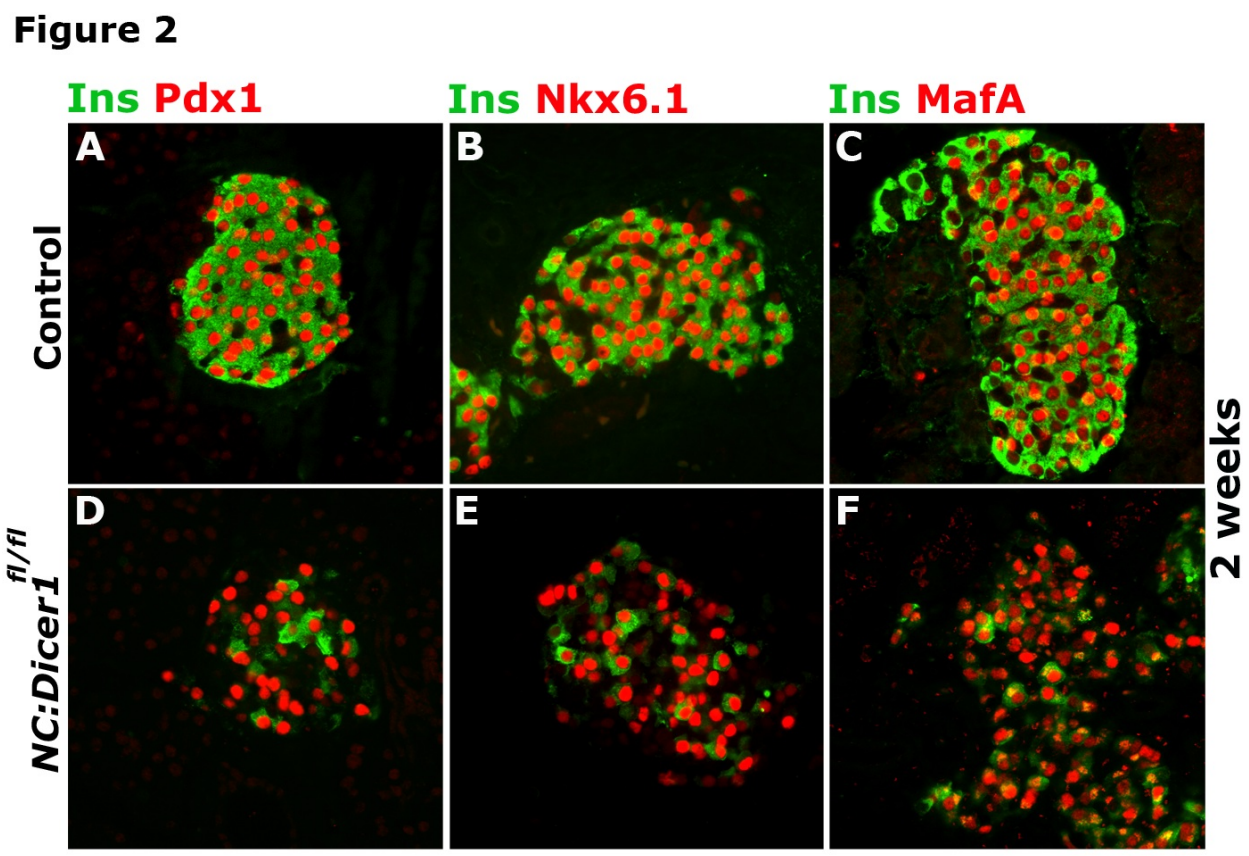


Figure 1-2. Loss of insulin expression in mutant NC:*Dicer1*$^{fl/fl}$ β-cells is not preceded by downregulation of characteristic β-cell transcription factors. Representative pancreatic sections from 2-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ mice co-immunostained for insulin (Ins; green) and Pdx1 (red, A, D), Nkx6.1 (red, B, E) and MafA (red, C, F) . Many instances of insulin-negative β-cells that still express the β-cell transcription factors are observed in the mutant sections. In control animals, β-cells always co-express insulin with the transcription factors. n ≥ 3 for each group of animals. In all cases, at least two to three pancreas sections were used for each animal.

Figure 1-3

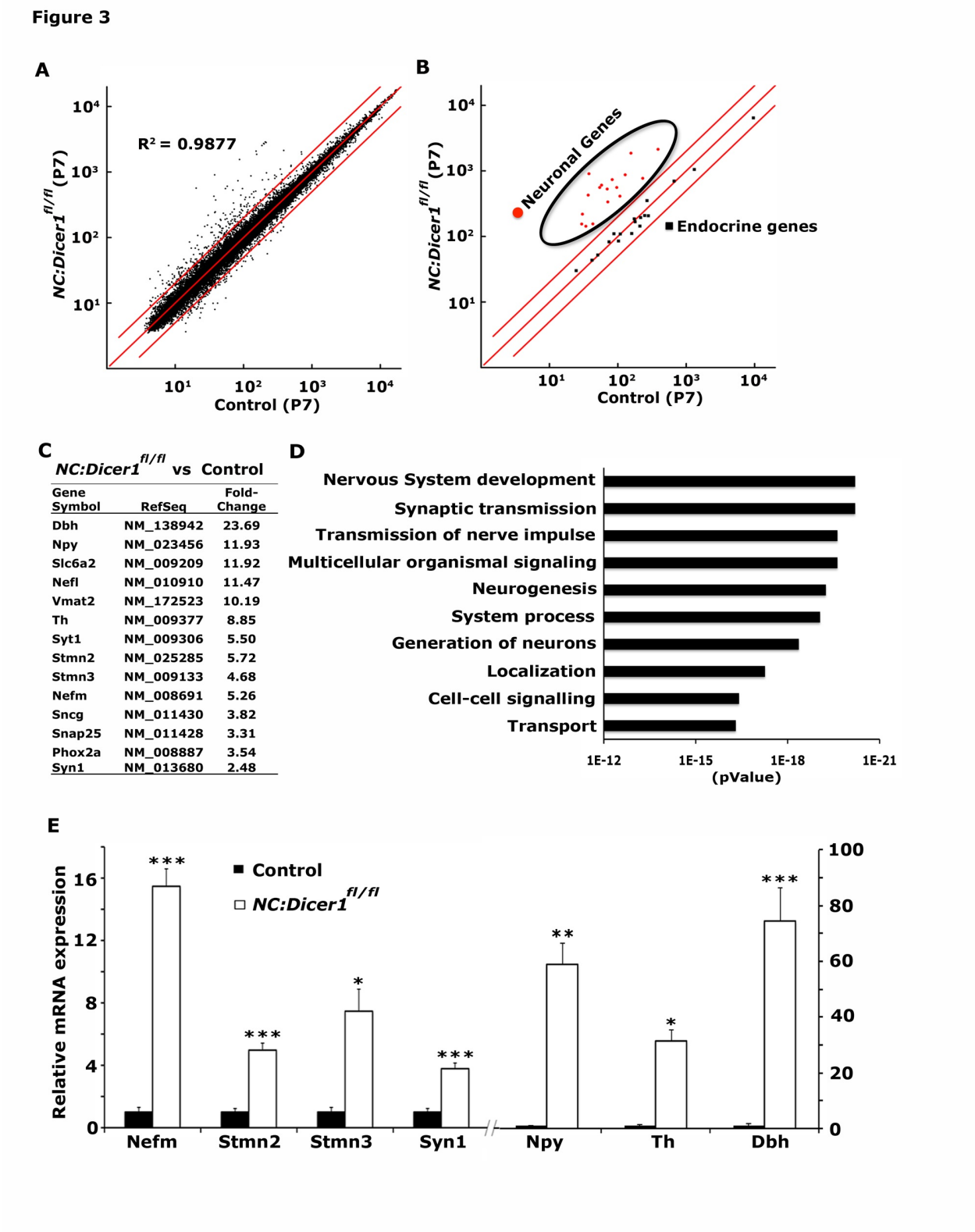


Figure 3
A
B
R² = 0.9877
NC:Dicer1fl/fl (P7)
Control (P7)
Neuronal Genes
Endocrine genes
C
NC:Dicer1fl/fl vs Control
Gene Symbol | RefSeq | Fold-Change
Dbh | NM_138942 | 23.69
Npy | NM_023456 | 11.93
Slc6a2 | NM_009209 | 11.92
Nefl | NM_010910 | 11.47
Vmat2 | NM_172523 | 10.19
Th | NM_009377 | 8.85
Syt1 | NM_009306 | 5.50
Stmn2 | NM_025285 | 5.72
Stmn3 | NM_009133 | 4.68
Nefm | NM_008691 | 5.26
Sncg | NM_011430 | 3.82
Snap25 | NM_011428 | 3.31
Phox2a | NM_008887 | 3.54
Syn1 | NM_013680 | 2.48
D
Nervous System development
Synaptic transmission
Transmission of nerve impulse
Multicellular organismal signaling
Neurogenesis
System process
Generation of neurons
Localization
Cell-cell signalling
Transport
1E-12
1E-15
1E-18
1E-21
(pValue)
E
Control
NC:Dicer1fl/fl
Relative mRNA expression
Nefm
Stmn2
Stmn3
Syn1
Npy
Th
Dbh

Figure 1-3. Unchanged endocrine gene expression but increased neuronal gene expression in mutant NC:*Dicer1*$^{fl/fl}$ islet cells at the presymptomatic stage (P7). A: Representative scatter plot of global gene expression obtained from microarray analysis of control and mutant NC:*Dicer1*$^{fl/fl}$ pancreas at P7. A high similarity (R^2) in gene expression is observed between control and mutant NC:*Dicer1*$^{fl/fl}$ samples. Red lines indicate a two-fold difference in expression. B: The expression levels of many neuronal markers as identified by the Gene Ontology database are upregulated in the mutant NC:*Dicer1*$^{fl/fl}$ samples. Key upregulated neuronal genes (red dots) are circled. The expression levels of most endocrine cell markers are unchanged (black dots). C: List of key neuronal genes (circled in B) upregulated in microarray chips of mutant NC:*Dicer1*$^{fl/fl}$ vs. control samples. D: Gene ontology (GO) analysis displaying biological processes associated with gene function of the differentially expressed genes with fold change greater than 2. The x-axis values are in logarithmic scale and correspond to raw binomial P-values. E: Real-time qPCR validation of representative upregulated neuronal gene transcripts *Nefm*, *Stmn2*, *Stmn3*, *Syn1*, *Npy*, *Th*, and *Dbh* using RNA isolated from pancreas of control and mutant NC:*Dicer1*$^{fl/fl}$ mice. Expression levels in control were set as one arbitrary unit. All data points represent means ± SEM of at least 3 biologically independent experiments. * $P<0.05$, ** $P<0.01$, ***$P<0.005$.

Figure 1-4

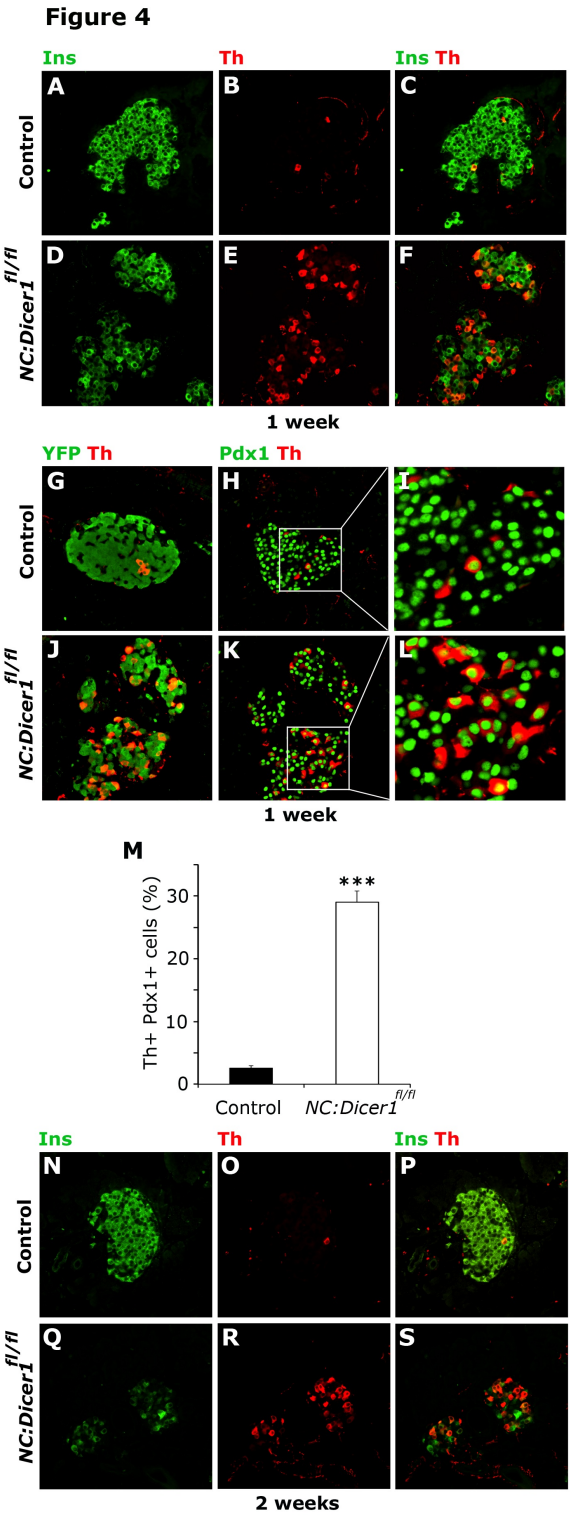


Figure 4
Ins
Th
Ins Th
A
B
C
Control
D
E
F
NC:Dicer1fl/fl
1 week
YFP Th
Pdx1 Th
G
H
I
Control
J
K
L
NC:Dicer1fl/fl
1 week
M
Th+ Pdx1+ cells (%)
30
20
10
0
***
Control
NC:Dicer1fl/fl
Ins
Th
Ins Th
N
O
P
Control
Q
R
S
NC:Dicer1fl/fl
2 weeks

Figure 1-4. Expression of tyrosine hydroxylase in mutant NC:*Dicer1*$^{fl/fl}$ pancreas. Pancreatic sections from 1-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ littermates showing immunostaining for insulin (Ins; green, A, D) , tyrosine hydroxylase (Th; red, B, E) and merged view (C, F) reveals sharp upregulation of Th expression in the mutant β-cells (n=3). Immunostaining of 1-week-old control (G, H) and NC:*Dicer1*$^{fl/fl}$ mutant (J, K) pancreatic sections for Th (red) / YFP (green) and Th (red) /Pdx1 (green) (n=3). Most Th expression is limited to Pdx1+ cells. The inset marks an area in (H,K) that is magnified and shown in the right panel (I,L). M: Quantification of Th positive cells expressed as % of insulin positive and Pdx1 positive cells in 1-week-old mice. Data points represent means ± SEM of at least 3 independent experiments. ***P<0.005. Immunostaining of 2-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ littermates for insulin(Ins; green, N, Q), tyrosine hydroxylase (Th; O, R) and merged view (P, S) indicates the presence of many insulin-negative β-cells that express tyrosine hydroxylase (n=3). In all cases, at least two to three pancreas sections were used for each animal.

Figure 1-5

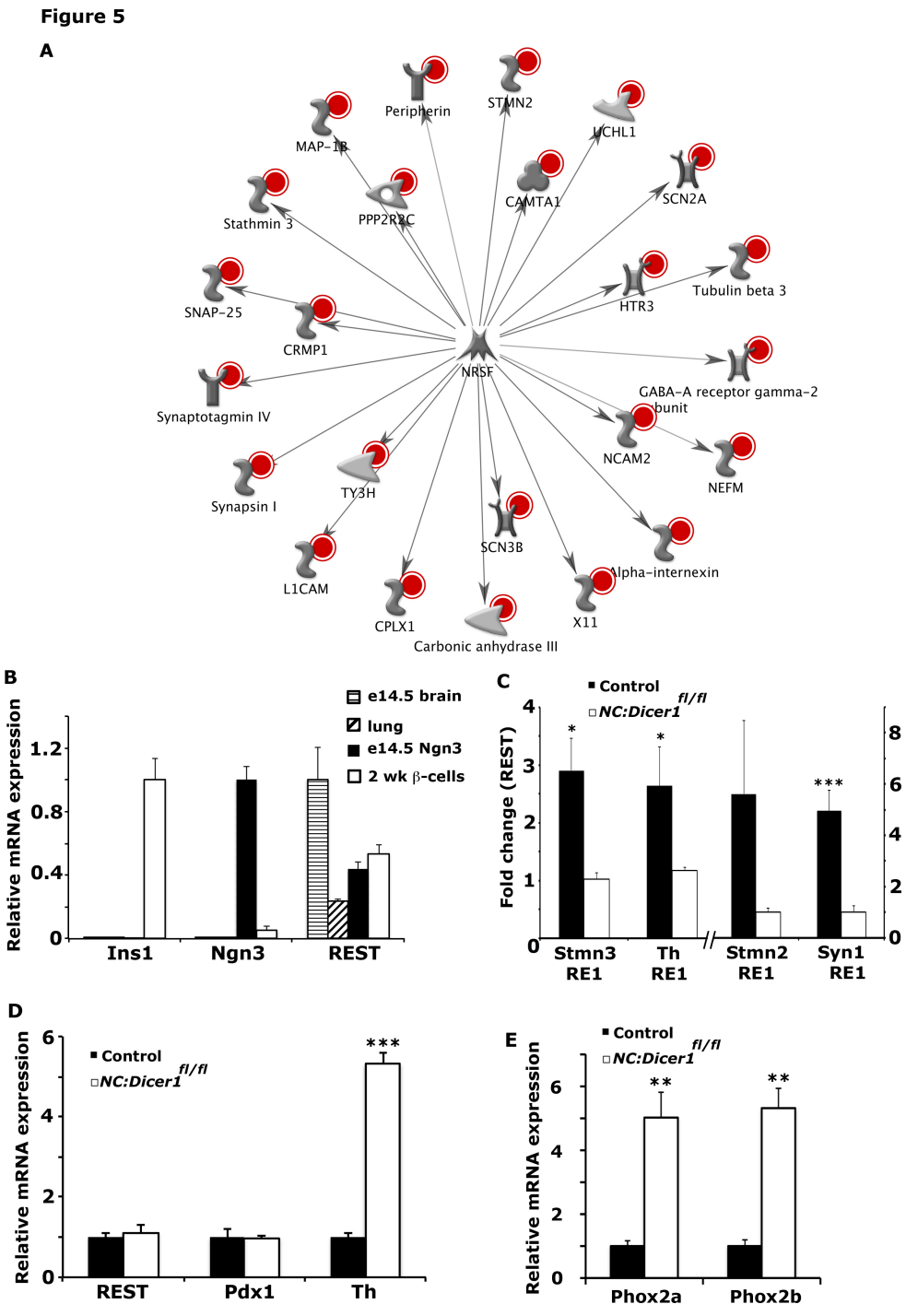


Figure 5
A
Peripherin
STMN2
MAP-1B
UCHL1
CAMTA1
SCN2A
Stathmin 3
PPP2R2C
HTR3
Tubulin beta 3
SNAP-25
CRMP1
NRSF
GABA-A receptor gamma-2 subunit
Synaptotagmin IV
NCAM2
NEFM
Synapsin I
TY3H
SCN3B
Alpha-internexin
L1CAM
CPLX1
X11
Carbonic anhydrase III
B
Relative mRNA expression
e14.5 brain
lung
e14.5 Ngn3
2 wk β-cells
Ins1
Ngn3
REST
C
Control fl/fl
NC:Dicer1
Fold change (REST)
Stmn3 RE1
Th RE1
Stmn2 RE1
Syn1 RE1
D
Relative mRNA expression
Control fl/fl
NC:Dicer1
REST
Pdx1
Th
E
Control fl/fl
NC:Dicer1
Relative mRNA expression
Phox2a
Phox2b

Figure 1-5. Loss of REST binding to neuronal targets in mutant NC:*Dicer1*$^{fl/fl}$ β-cells. A: MetaCore Interactome analysis (GeneGo Inc.) revealed that 16% of the dysregulated genes (> 2-fold change) are targets of the neuronal transcriptional repressor REST. B: Real-time qPCR analysis of *Ins1, Ngn3 and REST* mRNA transcript levels in adult lung tissue, brain tissue isolated at e14.5, FACS-purified Ngn3+ pancreatic endocrine progenitor cells at e14.5 and MIP-GFP β-cells at 2 weeks C: ChIP analysis of FACS-purified control and mutant NC:*Dicer1*$^{fl/fl}$ mice β-cells at P7. REST binding to the RE1 regions of *Stmn2*, *Stmn3*, *Th* and *Syn1* locus was reduced in mutant β-cells. ChIP data are reported as fold change of REST binding at the RE1 locus of the respective genes relative to a negative control region and represent means ± SEM of at least 3 biologically independent experiments. D: Real-time qPCR analysis of *REST, Pdx1 and Th* mRNA transcript levels in isolated islets from pancreas of control and mutant NC:*Dicer1*$^{fl/fl}$ mice at P7. E: Transcript levels of *Phox2a* and *Phox2b* as determined by real-time qPCR using RNA isolated from pancreas of control and mutant NC:*Dicer1*$^{fl/fl}$ mice at P7. Expression levels in control were set as one arbitrary unit. All data points represent means ± SEM of at least 3 biologically independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.005$.

Figure 1-6

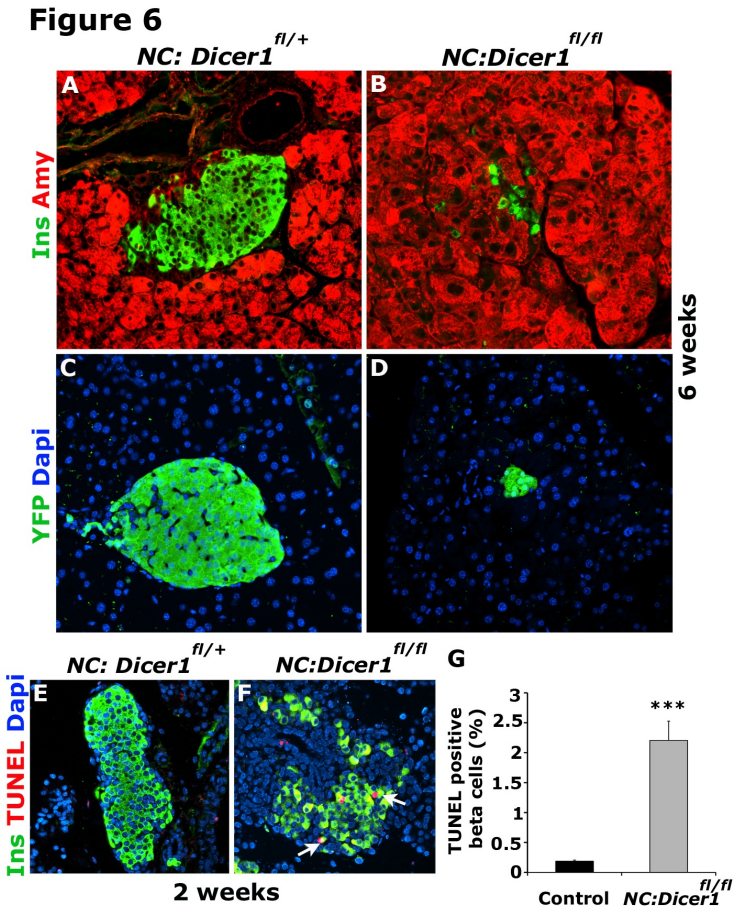
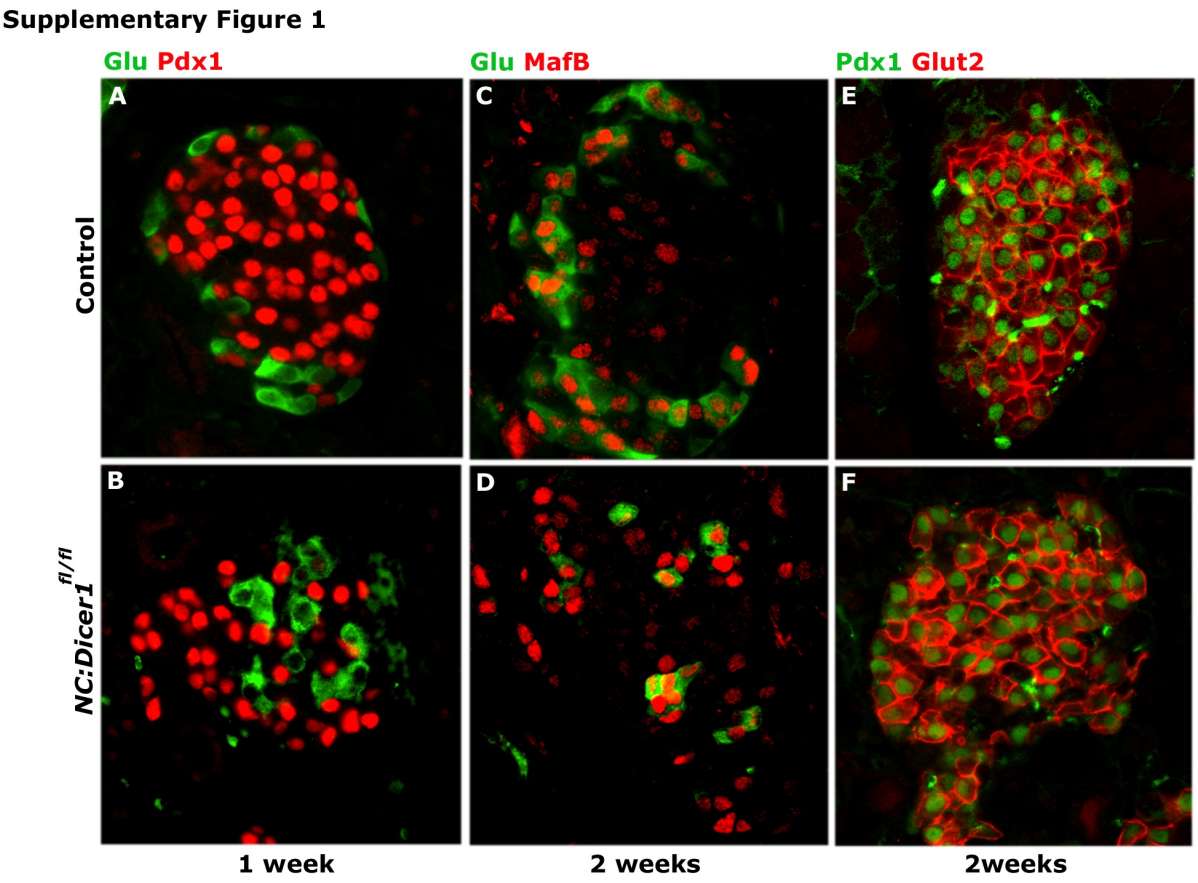


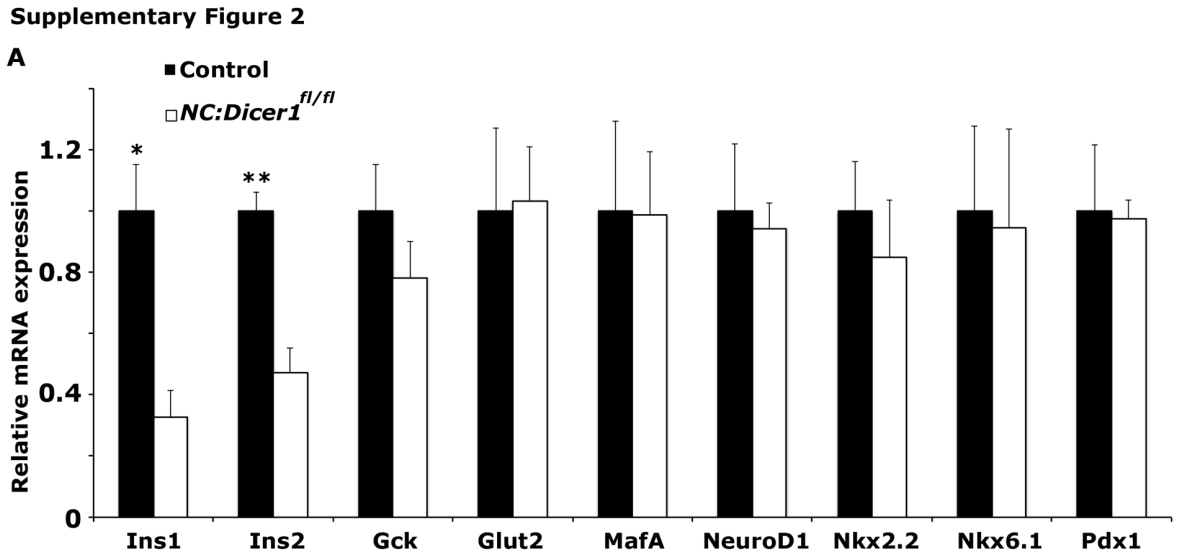
Figure 1-6. *Dicer1*-null islet cells undergo apoptosis during neonatal period. A-D: Representative pancreatic sections (n=3) from 6-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ mice co-immunostained for insulin (green, A, B) /Amylase (red A, B) and YFP (green, C, D) /DAPI (blue, C, D). E, F: Immunostaining of pancreatic sections (n=3) from 12-day-old control and mutant NC:*Dicer1*$^{fl/fl}$ mice for insulin and TUNEL reveals an increase in TUNEL+ apoptotic cells in the mutant mice. G: Quantification of TUNEL positive cells expressed as % of β-cells in 12-day-old mice. Data points represent means ± SEM of at least 3 independent experiments. ***P<0.005. In all cases, at least two to three pancreas sections were used for each animal.

Figure 1-S1



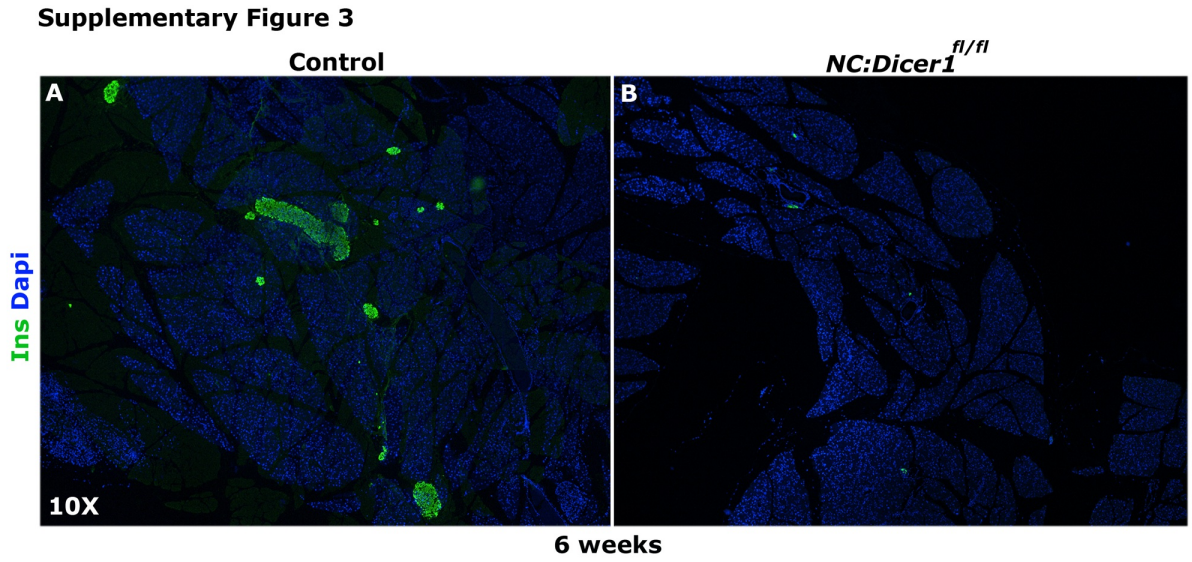
Supplementary Figure 1. Endocrine gene expression in mutant NC:*Dicer1*$^{fl/fl}$ islets A-B: Representative pancreatic section (n=3) from 1-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ mice co-immunostained for Glucagon (Glu; green) and Pdx1 (red) showing altered organization of α-cells within islet core. C-D: Immunostaining of pancreatic sections (n=3) from 2-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ mice for glucagon and MafB reveals the prevalence of MafB-positive Glucagon-negative α-cells in the mutant mice. E-F: Immunostaining of control and mutant NC:*Dicer1*$^{fl/fl}$ pancreatic sections (n=3) for Pdx1 (green) and Glut2 (red) at 2 weeks reveals no difference in Glut2 expression. In all cases, at least two to three pancreas sections were used for each animal.

Figure 1-S2



Supplementary Figure 2. : Regulation of β-cell genes in mutant NC:*Dicer1*$^{fl/fl}$. A: Real-time qPCR analysis of β-cell gene transcripts from RNA isolated from pancreas of control and mutant NC:*Dicer1*$^{fl/fl}$ mice at P7. The expression levels of Gck, Glut2, MafA, NeuroD1, Nkx2.2, Nkx6.1 and Pdx1 is unchanged whereas Ins1 and Ins2 expression is significantly decreased. Expression levels in control were set as one arbitrary unit. All data points represent means ± SEM of at least 3 biologically independent experiments. * $P<0.05$, ** $P<0.01$.

Figure 1S-3



Supplementary Figure 3. : Representative montage of pancreatic sections (n=3) from 6-week-old control and mutant NC:*Dicer1*$^{fl/fl}$ mice co-immunostained for insulin (green, A, B) and Dapi (blue A, B) showing an almost total loss of β-cells at 6 weeks in the mutant mice.

Chapter 3

Effect of activation of REST target genes in early pancreatic development

Abstract

The neuronal transcriptional repressor REST is a critical regulator of neurogenesis, raising the possibility that it could be playing an analogous role in modulating pancreatic development. We report that activation of REST target genes *in vivo* in pancreatic progenitors using a RESTVP16 transgene results in the attenuation of acinar and endocrine cell specification with the pancreatic epithelium adopting a combination of progenitor and duct-like state. This brake in lineage differentiation resulting from REST target gene activation is time-dependent. Our results suggest that the repression of REST target genes in progenitors is critical to allow the pancreatic differentiation programs to proceed normally.

Introduction

The mechanisms underlying the specification of pancreatic cell types share a lot of similarities to neuronal development, providing a unique opportunity to take key discoveries made from the study of neurogenesis and test for analogous roles during pancreatic organogenesis. The neuronal transcriptional repressor REST (RE-1-silencing transcription factor) is a critical regulator of the transcriptional cascades involved in the multistage differentiation of neurons from their progenitors raising a question as to whether (1) a similar layer of regulatory control could be present during pancreas specification.

REST mediates the silencing of neural target genes in neural stem cells (NSCs) thereby preventing these progenitors from undergoing differentiation (1). It also plays an important role in maintaining the transcriptional integrity of non-neuronal cells by silencing the expression of terminally differentiated neuronal genes (2). The REST protein structure contains a DNA binding domain that binds specifically to target RE-1 binding regions in the regulatory regions of its target genes, as well as two repressor domains. The regulatory domains function to recruit several epigenetic regulators that render a repressive chromatin environment at the promoter regions thereby silencing active transcription of the target (2) (3). REST binding sites have been discovered at the promoter regions of several key pancreatic beta cell transcription factors such as Pdx1, NeuroD and Pax4 (4). Additionally, *in vivo* genome-wide mapping of REST binding sites by ChIPSeq analysis on fibroblast cells identified the islet-cell development transcription factors NEUROD1, HNF4α, HNF6, Hes1 and Ngn3 as REST targets (5) implying a role for REST in islet cell development. Previous research has also implicated a role for REST in regulating the expression of terminally differentiated neuronal genes in newly specified beta cells

(6).

In contrast, the absence of REST expression in adult insulin-producing cell lines and the detrimental effect of conditional REST over-expression in beta cells on insulin exocytosis argues against a role for REST in mature beta cells (7) (8). An important fact to consider regarding REST-mediated regulation is that the amount of available REST protein in different cell types and the binding affinity of the REST complex vary between its target genes. For example, in the ChIPSeq study (5), genes encoding endocrine cell regulators were bound relatively poorly by REST compared to terminally differentiated neuronal genes, suggesting that a potentially larger amount of REST protein would be required to regulate the islet development factors compared to the neuronal genes. REST regulation of its targets is therefore not an on-off function and is very much context dependent.

To investigate an *in vivo* role for REST in the maintenance of pancreatic progenitors and activation of lineage specific differentiation programs, we generated a mouse model that could temporally (doxycycline inducible) activate a RESTVP16 transgene through out the pancreatic epithelium. This transgene functions to not only to counter REST repression, but also activates REST targets (9). Our studies indicate that *in vivo* expression of RESTVP16 in pancreatic progenitors resulted in the activation of REST target genes but did not affect the maintenance of the progenitor pool. We found that subsequent differentiation of endocrine and acinar lineages was repressed with the epithelium adopting a combination of progenitor and duct-like state. Subsequent activation of the transgene in the epithelium at the onset of differentiation did not affect the specification program. These data suggest a role for REST in the repression of regulators whose silencing is required for normal pancreatic organogenesis to proceed.

Results

RESTVP16 expression in early pancreatic epithelium does not affect progenitor population

The absence of REST expression in mature pancreatic beta cells and the negative influence of beta cell-specific REST overexpression on the insulin exocytosis machinery (7) (8) does not exclude a role for REST early during pancreatic lineage specification. We first determined whether REST was expressed at various stages of pancreas development. RT-PCR analysis showed that *REST* mRNA was expressed in FACS-purified Pdx1-positive progenitors cells at e12.5, Ngn3-positive endocrine precursor cells at e14.5 and in beta-cells at post-natal day 14 (P14) (Figure 1A) possibly implying a role for REST during early pancreatic development. To investigate an *in vivo* role for REST during pancreatic development, we generated a transgenic mouse that could temporally express the recombinant transcription factor RESTVP16 specifically in the pancreatic epithelium. The RESTVP16 transgene has been previously described and consists of the DNA-binding domain of REST, with the terminal repressor domains replaced with the activation domain of herpes simplex virus protein VP16. This allows for the transgene to bind to the same endogenous binding sites that REST binds to except that it activates these target genes (9; 10).

A transgenic line harboring a tetracycline responsive element (TRE) that controlled expression of the transgene encoding for RESTVP16 (*TRE-RESTVP16*, see materials and methods) was first generated. Founder mice harboring the *TRE-RESTVP16* transgene were then crossed into *Pdx1-Cre* transgenic line (11) to generate double transgenic *Pdx1-Cre: TRE-RESTVP16* mice. These double transgenic mice were further crossed with a *ROSA26R-rtTAEGFP* transgenic line (12) to generate the doxycycline inducible Pdx1:RESTVP16:EGFP triple Tg mice. The resulting mice

could conditionally express the transgene in pancreatic epithelial cells upon doxycycline administration. Additionaly, all cell lineages derived from those Pdx1 progenitors that underwent recombination would be heritably labeled by EGFP. To determine whether doxycycline administration induced expression of the RESTVP16 transgene *in vivo* in pancreatic epithelium of Tg embryos, pregnant females carrying control and RESTVP16 Tg fetuses were Doxycycline treated 8 days after discovery of vaginal plug (e8.5), coinciding with the onset of Pdx1 expression in the pancreatic progenitors (13). Doxycycline administration was maintained until the day of analysis (Figure 1B). Immunofluorescence staining for EGFP (Figure 1C-D) on control and RESTVP16 Tg pancreatic sections at e12.5 showed that a majority of the Tg pancreatic epithelium stained for EGFP illustrating a high degree of recombination efficiency. The Tg pancreas also stained for REST (Figure 1E-F) indicating that the REST-VP16 protein was being expressed within the pancreatic progenitor population. In contrast, no REST expression was observed in the control pancreas. The generation of this mouse model therefore permitted its use as a tool to investigate the effect of activation of REST target genes on pancreatic lineage specification *in vivo* .

We next examined the effect of RESTVP16 expression on the early pancreatic progenitor population. Immunofluorescence staining for the multipotent progenitor markers Pdx1, Sox9 and Nkx6.1 (14) at e12.5 showed similar expression of these markers in both the control and RESTVP16 Tg pancreatic epithelial buds (Figure 1G-L). Glucagon staining in the RESTVP16 Tg buds also appeared comparable to the control sections (Figure 1G-H) indicating that RESTVP16 expression did not affect the few hormone expressing cells that arise during the primary transition phase (15). Our data therefore suggests that induction of RESTVP16 expression at e8.5 does not affect the early pancreatic progenitor population.

RESTVP16 expression in pancreatic progenitors results in absence of differentiated acinar and endocrine cells

We next wanted to assess differentiation of the various pancreatic lineages in control and RESTVP16 Tg pancreas. We immunostained control and RESTVP16 Tg pancreatic buds for markers of differentiated endocrine cells and acinar cells at e16.5, a time point that coincides with the final stages of the secondary transition phase, during which the bulk of endocrine cell differentiation from a bipotential ductal/endocrine progenitor domain is completed (16). A striking absence of differentiated insulin expressing cells was observed in the RESTVP16 Tg pancreas at e16.5, with very few tiny clusters of glucagon expressing cells observed compared to the control buds (Figure 2A-B). Coimmunostaining of glucagon with EGFP showed that the glucagon positive clusters were EGFP negative (data not shown) suggesting that these cells had not undergone recombination, or more plausibly, these glucagon-positive cells had arisen during the primary transition phase. A similar deficiency in somatostatin and pancreatic polypeptide immunostaining was observed in the RESTVP16 Tg pancreas (data not shown) showing that differentiation of all endocrine lineages was attenuated. Antibody staining against acinar cells expressing amylase showed a massive loss of amylase expression in the RESTVP16Tg bud with minimal expression restricted at the tips of the acinar domain (Figure 2C-D). Quite surprisingly, immunostaining for DBA (*Dolichos biflorus agglutinin*) showed dramatic upregulation of this ductal cell marker throughout the epithelium of the RESTVP16 Tg bud (Figure 2E-F). Immunofluorescence staining of control and RESTVP16 Tg sections for endocrine and exocrine markers at post natal day three revealed that the Tg pancreas was atrophic with few acinar cells remaining (Supplementary Figure 1) illustrating a permanent loss of endocrine and acinar

lineages. Taken together our data suggests that RESTVP16 expression in pancreatic progenitors represses the acinar and endocrine differentiation programs and favors the adoption of a duct-like fate.

Pan-epithelial expression of Sox9 and down-regulation of Ngn3 in RESTVP16 Tg pancreas

Next, we asked whether the loss of differentiated endocrine cells in the RESTVP16 Tg bud was a consequence of changes in expression patterns of key regulators of the endocrine specification program. The transcription factor Sox9 is expressed in multipotent progenitor cells of the early pancreatic epithelium (17; 18) and plays an important role in regulating Ngn3 expression, a critical bHLH transcription factor required for the endocrine differentiation program to proceed (19) (11) (20; 21). Immunostaining of control and RESTVP16 Tg pancreatic buds for Ngn3 (green) and REST (red) at e14.5 revealed that the RESTVP16 Tg buds had significantly fewer Ngn3+ cells (Figure 3B-C). These Ngn3+ cells mostly did not co-express REST (white arrows) implying that the transgene was not active in these cells, possibly due to lack of recombination. In contrast, control pancreatic buds stained for large numbers of Ngn3+ cells, consistent with the massive formation of Ngn3+ cells that is known to occur during this stage (Figure 3A). As differentiation proceeds, the expression of Sox9 gets restricted to the bipotential duct/progenitor domain and is absent from committed endocrine and acinar progenitor and differentiated cells (17) and only remains in ductal cells during the postnatal period. Immunofluorescence staining of control and RESTVP16 Tg pancreatic buds for Sox9 (red) at e16.5 revealed that while Sox9 was getting restricted to a subset of cells of the epithelial cords in the control buds (Figure 3D), widespread pan-epithelial Sox9 expression was prevalent in the Tg buds. These Sox9+ cells in the Tg pancreas could either be uncommitted, pluripotent pancreatic progenitors or might have adopted a ductal fate. Co-staining of control and RESTVP16 Tg sections for Sox9 and the ductal

marker DBA showed that the majority of Sox9+ cells in the Tg bud also expressed DBA suggesting that these cells had adopted a ductal fate. Our results therefore suggest that the failure of Sox9 to downregulate results in an attenuation of both the endocrine and acinar differentiation program.

Activation of RESTVP16 transgene in pancreatic epithelial cells after onset of secondary transition does not affect differentiation programs

The temporal inducibility of the transgene allowed us to investigate the effect of RESTVP16 transgene activation at different stages of pancreatic organogenesis. We hypothesized that the endocrine and acinar differentiation defect observed by activating RESTVP16 in the early pancreatic epithelium might be abrogated if the onset of RESTVP16 expression was delayed. To investigate this hypothesis, we administered doxycycline treatment to pregnant females starting at e12.5 coinciding with the start of secondary transition (Figure 4A). Immunostaining of e15.5 embryos for REST (Figure 4B-C) showed that the transgene was expressed in the RESTVP16 Tg epithelium. However, staining for endocrine and exocrine markers revealed that both lineages differentiated normally (Figure 4D-E). In contrast to transgene activation at e8.5, delayed activation of RESTVP16 resulted in appropriate downregulation of Sox9, getting restricted to the bipotential epithelial cords in the Tg pancreas (Figure 4F-G). Our results therefore suggest that the activation of REST target genes might only be affecting the initiation of the endocrine/acinar differentiation program and subsequent RESTVP16 transgene expression in the pancreatic epithelium after the onset of differentiation program does not effect terminal differentiation.

Stem and multipotent progenitor cell markers persist in RESTVP16 Tg pancreas

We next wanted to examine the molecular identity that the RESTVP16 Tg pancreatic epithelial cells had adopted after failing to undergo differentiation. We performed genome-wide expression analysis on pancreatic epithelial cells FACS-sorted from control and RESTVP16 Tg embryos at e16.5. RNA-Seq analysis revealed 277 genes that were at least 2-fold down-regulated and 409 transcripts 2-fold up-regulated with a false discovery rate of 5% (Figure 5A). Not surprisingly, the MetaCore Interactome characterization tool **(**GeneGo Inc. MI, http://www.genego.com) identified 56 of the up-regulated genes as direct REST targets. REST was also identified as the transcription factor exhibiting the largest number of interactions with the dysregulated genes suggesting that the transgene was functioning *in vivo* as expected to activate REST target genes (Figure 5B). The changed gene expression signature in the Tg epithelium was further validated by performing real-time qPCR analysis on a triplicate of samples, independent of those used for the RNA-Seq analysis (Figure 6F).

Branching morphogenesis of the pancreas is initiated at e12.5 and results in the separation of a tip region that constitutes a multipotent progenitor domain and a trunk domain that consists of endocrine-ductal progenitors and their differentiated progeny (16). We categorized the control and RESTVP16 Tg RNA-Seq expression data according to the tip-trunk model. Our data revealed that transcription factors characteristic of the tip domain – Ptf1a, Cpa1, Spdef, Aldh1b1, Rbpjl- were up-regulated, whereas the key trunk transcription factors (Neurod1, Pou3f4, Isl1, Arx MafB, Ngn3, Nkx6.1) were down-regulated (Figure 6A-B). Furthermore, characteristic stem cell markers such as Lin28 and Lgr5 (22; 23) were also up-regulated in the Tg epithelial cells (Figure 6C). Subsequently, as earlier noted, a massive reduction in expression of markers characteristic of terminally differentiated acinar and endocrine cells was also observed (Figure

6B). Although immunostaining for Sox9 and the ductal marker DBA indicated the RESTVP16 Tg epithelial cells had adopted a duct-like fate (Figure 2E-F, 3E, G), a number of other ductal cell markers such as K19, Hnf1b and Car2 (24) were not up-regulated, suggesting that these cells were not mature ductal cells. Taken together, our results suggest that activation of REST target genes results in the pancreatic epithelium maintaining a progenitor like identity and failing to activate key differentiation programs. We hypothesized that the de-repression of critical regulators (REST targets) might be contributing to the maintenance of a progenitor-like state in the RESTVP16 epithelium. We analyzed the top up-regulated genes from the RNA-Seq data to identify transcriptional regulators that were direct REST targets. Amongst the highly up-regulated genes were the transcription factors Lhx3, Pax5 and Scrt1, as well as the RNA binding protein Lin28. The LIM homeodomain protein Lhx3 is particularly interesting as it acts combinatorially with other homeodomain proteins to allow specification into various neuronal cell fates (25). Immunostaining for Lhx3 in control and RESTVP16 Tg sections at e16.5 revealed a pan-epithelial Lhx3 expression in the Tg bud. In contrast, no Lhx3 expression was observed in the control bud suggesting that de-repression of Lhx3 might be contributing to the observed phenotype in the RESTVP16 Tg model.

Discussion

Our results suggest that the repression of REST target genes in the pancreatic progenitor population is important to facilitate normal lineage differentiation programs during pancreatic organogenesis. Our work also suggests that expression of the RESTVP16 transgene results in the de-repression of critical regulators that maintain the pancreatic epithelium in a progenitor state, while also promoting the acquisition of duct-like characteristics. The lack of any defects in the initiation of differentiation programs when RESTVP16 is expressed at a later time point (e12.5) in the pancreatic epithelium suggests that repression of REST targets is required before the initiation of branching morphogenesis during the secondary transition phase. It is important to note that REST exhibits differential binding to its targets (3) and therefore the transcriptional repression of REST targets is very much context dependent. Therefore, the RESTVP16 Tg model, which serves to activate REST targets, does not necessarily substitute for a REST loss-of-function (knockout) model, because loss of REST does not imply de-repression of its target. Further studies will need to be conducted with a knockout model to better inform us on the role of REST during pancreatic organogenesis. Our model however serves as an important tool in accomplishing this aim.

An important question is raised as to the mechanism through which REST target gene activation attenuates the downstream specification process. We propose several scenarios that could be responsible for observed phenotype. One possibility is that activated REST targets could be responsible for the failure of Sox9 expression to down-regulate in the pancreatic epithelium. Sox9 expression marks the multipotent progenitor domain in the early epithelial cells and its subsequent down-regulation is important for the initiation of the endocrine and acinar differentiation programs (17). Since Sox9 transcript levels remain unchanged in the Tg

epithelium, the de-regulation of a post-transcriptional mechanism could possibly be responsible for the observed pan-epithelial Sox9 protein expression. A potential candidate for such post-transcriptional modulation is the RNA-binding protein Lin28, an important developmental regulator that is normally expressed in progenitor populations. One of its functions is to block the biogenesis of the *let-7* family of microRNAs (26). Given that *Sox9* is a predicted target for *let-7c* and *let-7g*, we hypothesize that the observed up-regulation of Lin28 in the RESTVP16 Tg epithelium could possibly be responsible for loss of *Let-7* mediated repression of Sox9, thereby preventing the initiation of the differentiation programs.

The upregulation of the LIM homeodomain protein Lhx3, a direct REST target, in the RESTVP16 Tg epithelium could also be responsible for the duct-like cell fate switch observed. During neuronal development Lhx3 acts combinatorially with other homeodomain proteins, including Isl1, to dictate the specification of different neuronal subtypes (25). We propose that the changing composition of these complexes could potentially be overriding the existing differentiation programs during pancreas development. The identification of such novel regulators of pancreas development is critical in aiding the creation of efficient protocols for the conversion of stem cell sources into functional beta cells for therapeutic purposes.

Figure 2-1

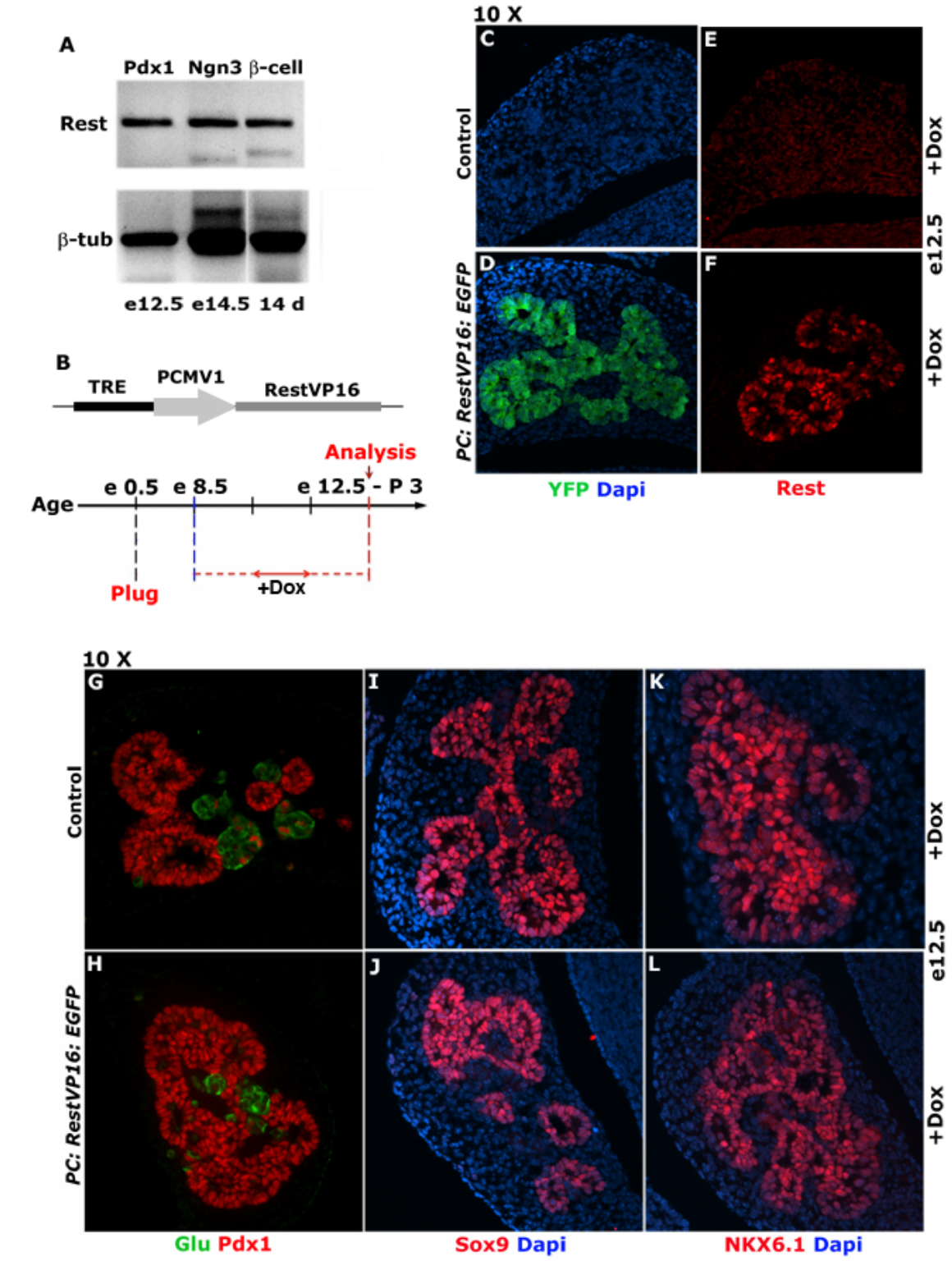


FIGURE 1
A
Pdx1 Ngn3 β-cell
Rest
β-tub
e12.5 e14.5 14 d
B
TRE
PCMV1
RestVP16
Analysis
Age
e 0.5 e 8.5 e 12.5 - P 3
Plug
+Dox
10 X
C
E
D
F
Control
PC: RestVP16: EGFP
+Dox
e12.5
+Dox
YFP Dapi
Rest
10 X
G
I
K
H
J
L
Control
PC: RestVP16: EGFP
+Dox
e12.5
+Dox
Glu Pdx1
Sox9 Dapi
NKX6.1 Dapi

Figure 2-1. RESTVP16 expression in early pancreatic epithelium does not affect progenitor population (A) RT-PCR analysis indicates the expression of *REST* mRNA in FACS-purified, Pdx1-YFP pancreatic progenitor cells at e12.5, FACS-purified Ngn3-YFP endocrine progenitor cells at e14.5 and MIP-GFP β-cells at 2 weeks. (B) Schematic of the targeting cassette containing TRE-RESTVP16. Pregnant females carrying control and *PC:RESTVP16:EGFP* transgenic fetuses were Doxycycline treated 8 days after discovery of vaginal plug (e8.5). Doxycycline treatment was maintained until day of analysis D and mutant NC:*Dicer1*$^{fl/fl}$ (C-D) Immunofluorescence staining of pancreatic sections (n=3) for YFP (Green) that marks recombined cells and DAPI to visualize the nuclei (blue) illustrates high efficiency of recombination in RESTVP16 transgenic pancreata. (E-F). Immunohistochemistry for REST (Red) demonstrates the activation of RESTVP16 transgene in majority of Tg pancreatic bud. (G-L) Immunofluorescence staining for Pdx1, Sox9 and Nkx6.1 in control and RESTVP16 Tg pancreatic buds at e12.5. No observable differences in the expression of multipotent progenitor markers between Tg and control buds. In all cases, at least two to three pancreas sections were used for each animal.

Figure 2-2

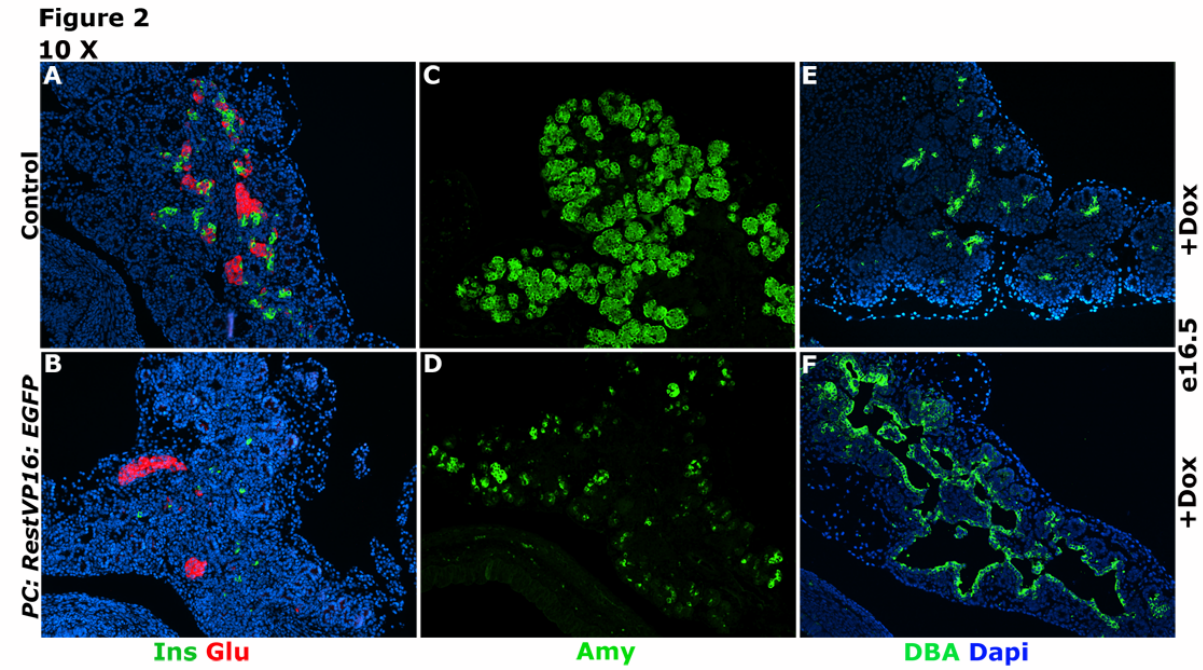


Figure 2-2: Endocrine and acinar cell differentiation severely attenuated in the RESTVP16 Tg pancreas. (A-B) Staining for Insulin (green) and Glucagon (green) illustrates the severely reduced number of insulin and glucagon cells in the RESTVP16 pancreatic bud at e16.5. Glucagon expressing cells from primary transition still remain. (C-D) Immunohistochemistry for amylase (green) illustrates few amylase expressing acinar cells at the periphery of the RESTVP16 pancreatic bud. (E-F) Immunofluorescence staining for DBA lectin (green) - a marker for ductal cells, demonstrates a shift in phenotype toward ductal fate in the RESTVP16 Tg bud. Both control and Tg buds are from embryos administered with doxycycline treatment starting e8.5.

Figure 2-3

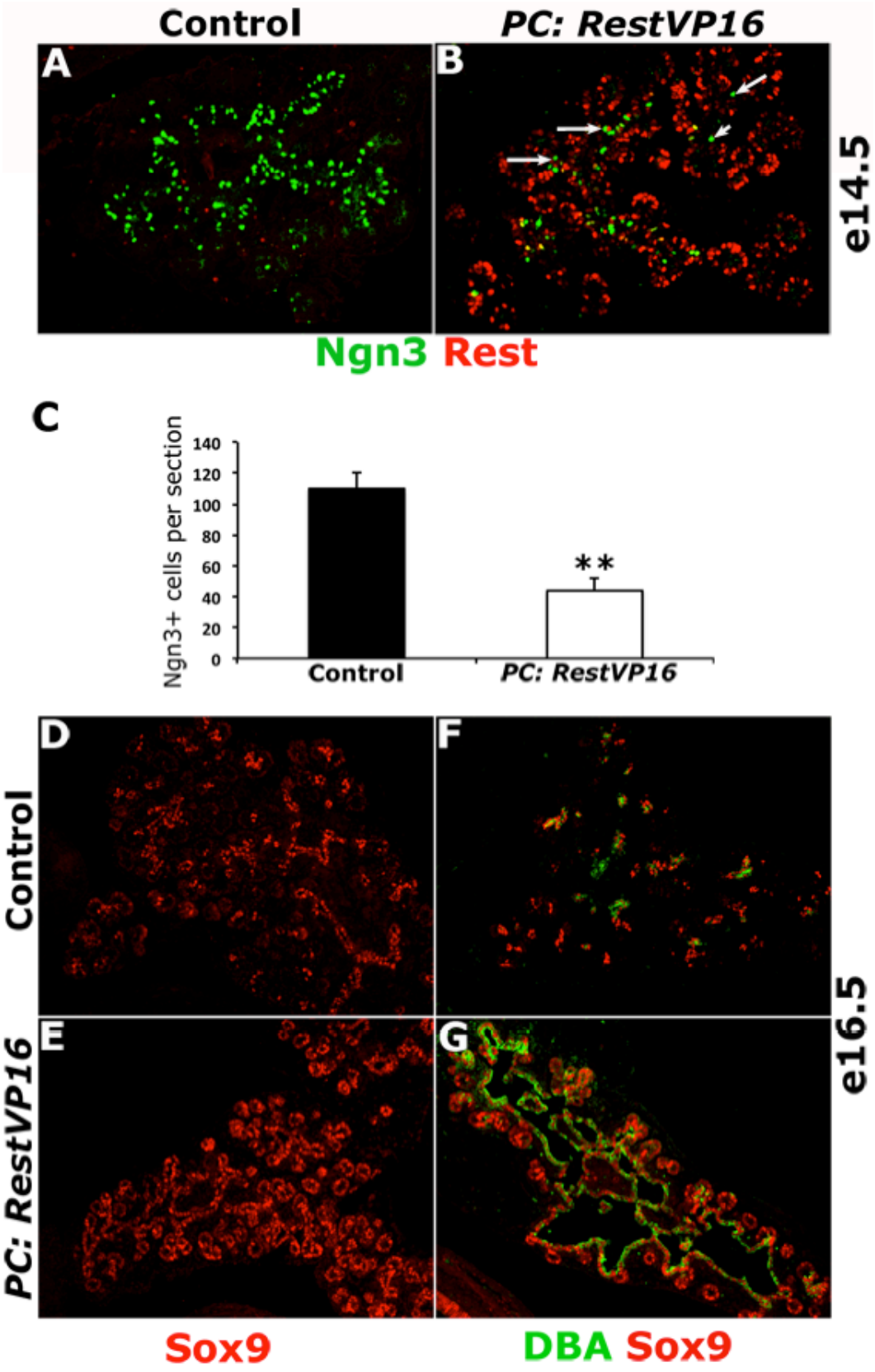

Control
PC: RestVP16
A
B
e14.5
Ngn3 Rest
C
Ngn3+ cells per section
140
120
100
80
60
40
20
0
**
Control
PC: RestVP16
D
F
Control
E
G
PC: RestVP16
e16.5
Sox9
DBA Sox9

Figure 2-3: Pan-epithelial expression of Sox9 and downregulation of Ngn3 in RESTVP16 Tg pancreas (A-B) Immunofluorescence staining for Ngn3 (green) and REST (red) illustrates a reduction in Ngn3+ cells in the RESTVP16 Tg pancreatic bud at e14.5. White arrows show Ngn3+ REST- cells indicating that these cells might have escaped recombination. (C) Quantification of Ngn3 positive cells expressed as average number of Ngn3+ per pancreatic section in e14.5 embryos. Data points represent means ± SEM of at least 3 independent embryos (between 3-5 sections per embryo). **P<0.01. (D-E) Immunohistochemistry for Sox9 (red) illustrates failure of Sox9 expression to get restricted to ductal/endocrine epithelial cords in RESTVP16 Tg pancreatic bud at e16.5. (F-G) Immunofluorescence staining for Sox9 (Red) and DBA lectin (green) shows most of RESTVP16 Tg pancreatic epithelium co-expressing these markers. Both control and Tg buds are from pregnant dam administered with doxycycline treatment starting at e8.5. In all cases, at least two to three pancreas sections were used for each animal.

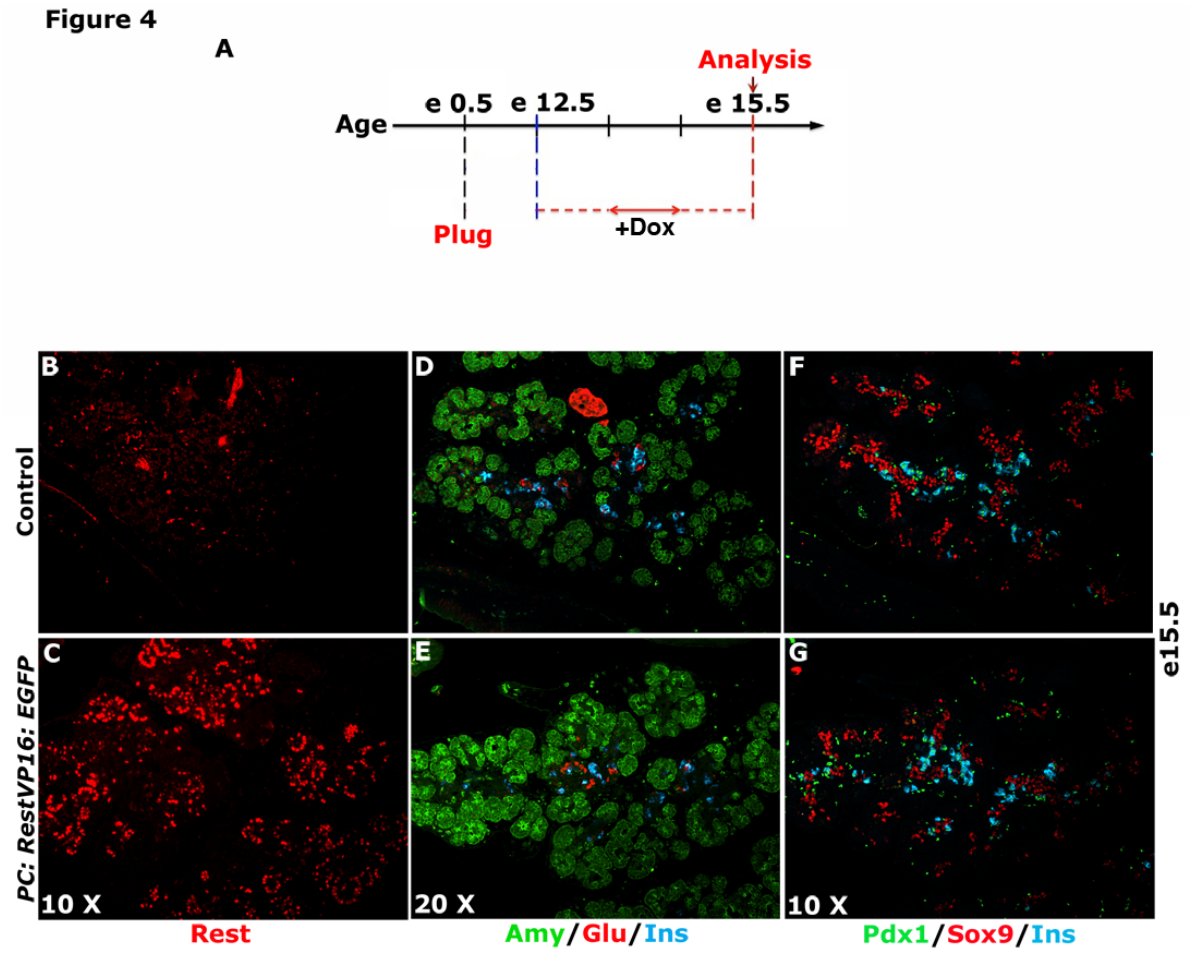


Figure 2-4. Activation of RESTVP16 transgene in pancreatic epithelial cells after onset of secondary transition does not affect organogenesis (A) Pregnant females carrying control and *PC:RESTVP16:EGFP* transgenic fetuses were administered Doxycycline twelve days after discovery of vaginal plug (e12.5). Doxycycline administration was maintained until day of analysis at e15.5. (B-C) Immunohistochemistry for REST (Red) shows activation of the RESTVP16 transgene in Tg pancreatic bud. (D-E) Immunostaining for Amylase (green) Glucagon (red) Insulin (light blue) illustrates no defects in terminal differentiation of acinar and endocrine cells in the RESTVP16 Tg bud. (F-G) Immunofluorescence staining for Pdx1 (green), insulin (blue) and Sox9 (red) illustrates localization of Sox9 to ductal compartments. Most Pdx1 positive cells co-stain with insulin in the RESTVP16 Tg bud similar to the control bud.

Figure 2-5

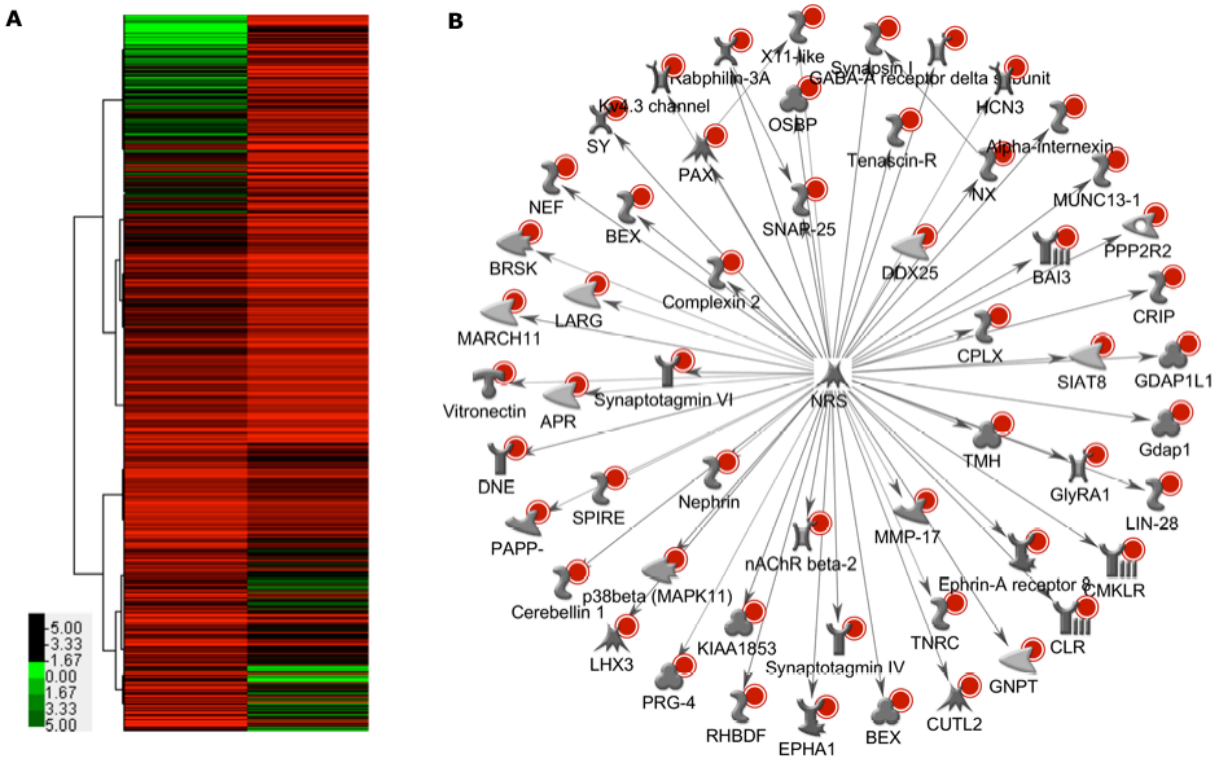
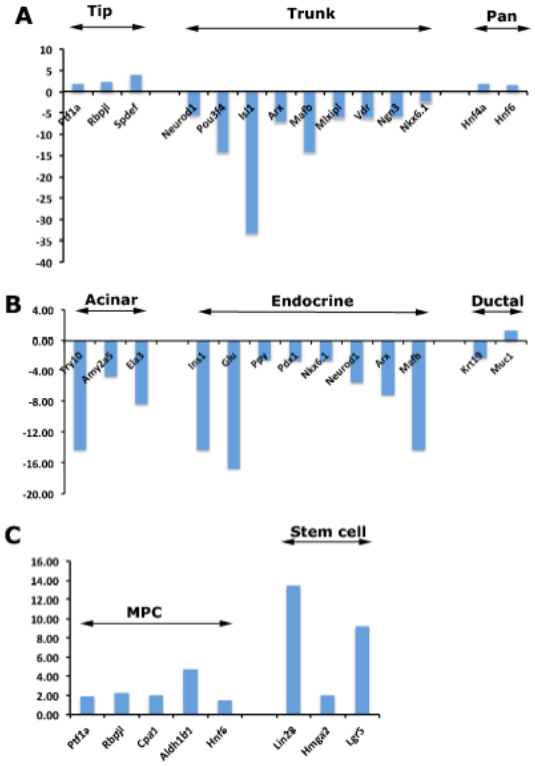


Figure 2-5: RNA-Seq analysis confirms selective upregulation of REST target genes in RESTVP16 Tg pancreatic epithelium. (A) Representative heat map illustrating global gene expression profile from RNA-Seq analysis of FACS-sorted control and RESTVP16 pancreatic epithelial cells at e16.5. (B) MetaCore interactome analysis (GeneGo Inc.) revealed that 14% (56 genes) of the upregulated genes (> 2-fold change) in Tg epithelial cells are REST targets.

Figure 2-6



D

RestVP16 Tg vs Control

Gene Symbol	Fold-change
Cacng2	560.61
Cacng3	446.62
Lhx3	277.06
Scrt1	250.63
Npas1	242.97
Pax5	216.74
Gm6537	188.78
Tmhs	148.26
Ntsr2	135.15
Lin28	13.37

E

RestVP16 Tg vs Control

Gene Symbol	Fold Change
Ghrl	-191.41
Irs4	-110.59
Gm20554	-61.56
Gip	-47.79
Gast	-39.86
Sorcs1	-37.73
Fut1	-35.85
Gpr119	-31.28
Isl1	-30.28
Pde3a	-28.25
Irx2	-27.71
Gcg	-17.87
Mafb	-14.14
Ins1	-13.52

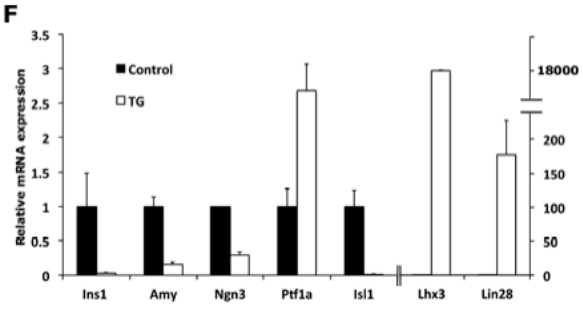


Figure 2-6: Stem and multipotent progenitor markers persist in RESTVP16 Tg pancreas (A-C): Genome-wide expression analysis showing downregulation of differentiated markers of all endocrine and acinar lineages in RESTVP16 Tg epithelium. Transcription factors characteristic of the multipotent progenitor tip domain are upregulated whereas trunk transcription factors are downregulated. Normally repressed stem cell markers are also upregulated in Tg epithelium. The expression levels of most endocrine cell markers are unchanged (black dots). C: List of top changing genes from RNA-Seq analysis indicates down regulation of endocrine hormones and their precursors. Upregulated genes include the transcription factors LHX3 and Pax5 – direct targets of REST. F: Real-time qPCR validation of representative pancreatic progenitor and differentiated gene transcripts *Ins1*, *Amy*, *Ngn3*, *Isl1*, *Ptf1a* as well as the normally repressed transcripts *Lhx3*, and *Lin28*. RNA was isolated from sorted pancreatic epithelial cells of control and RESTVP16 Tg mice. Expression levels in control were set as one arbitrary unit. All data points represent means ± SEM of at least 3 biologically independent experiments.

Figure 2-7

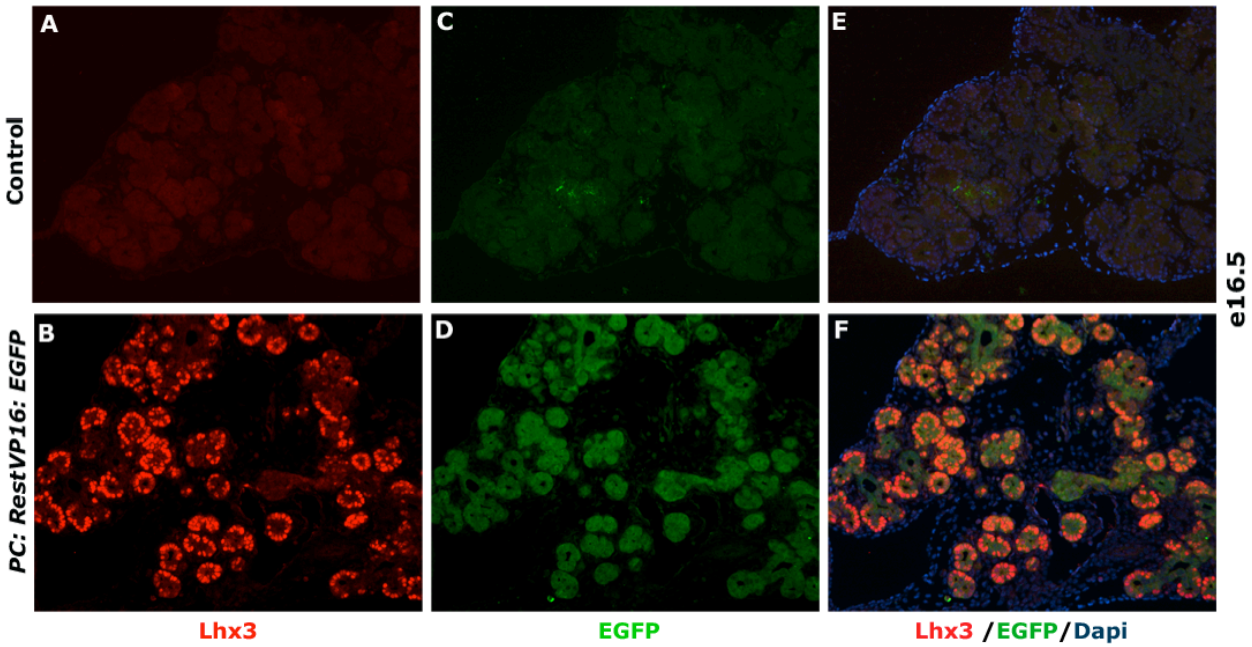


Figure 2-7: LIM homeodomain gene LHX3 upregulated in RESTVP16 Tg epithelium (A-B) Pancreatic sections (n=3) at postnatal day 3 (P3) immunostained for Lhx3 (red) illustrates the high expression of Lhx3 in the Tg epithelium at e16.5. (C-D) EGFP expression (green) is only observed in the RESTVP16 Tg pancreatic epithelium. (E-F) Costaining of control and RESTVP16 Tg epithelium for EGFP and Lhx3 illustrates that most of the Tg epithelium is Lhx3+/YFP+. A total absence of Lhx3 expression is observed in the control pancreas. Both control and Tg pancreata are from embryos administered with doxycycline treatment starting e8.5.

Figure 2-S1

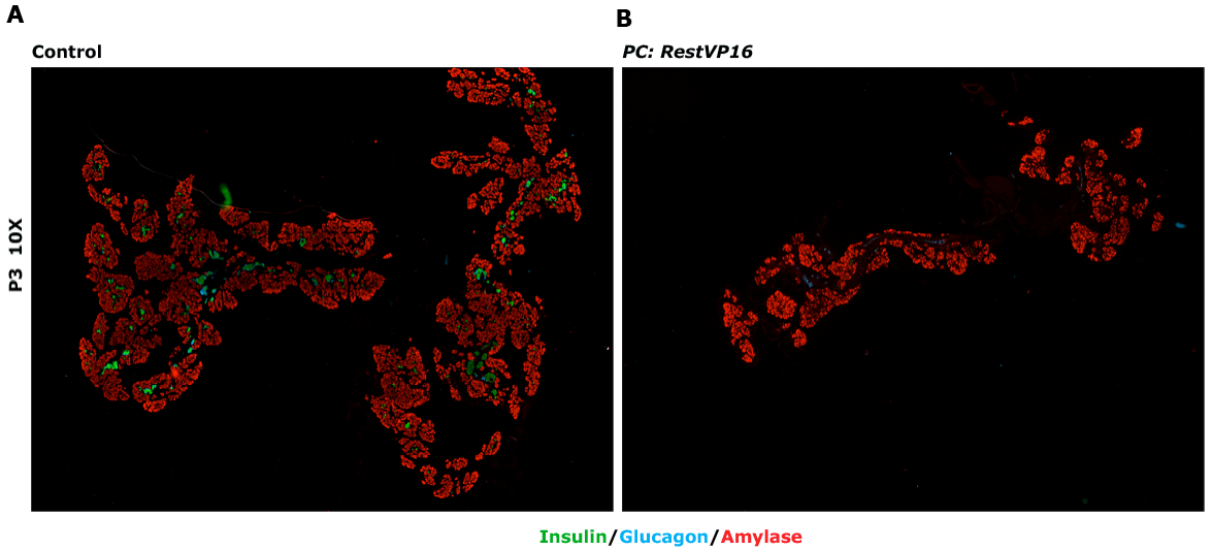


Figure 2-S1: RESTVP16 induction in pancreatic progenitors results in atrophic pancreas

(A-B) Representative montage of pancreatic sections (n=3) at postnatal day 3 (P3) immunostained for acinar (amylase, red) and endocrine markers (insulin - green, glucagon – red) illustrates the atrophic size of the RESTVP16Tg pancreas. Both control and Tg pancreata are from pups administered with doxycycline treatment starting e8.5.

RESEARCH DESIGN AND METHODS

Mouse husbandry

Mice were maintained in a 12 h light/dark cycle under standard conditions. Studies involving mice were performed in accordance with NIH policies on the use of laboratory animals and approved by the University of California, Los Angeles Animal Research Committee. The mice used in this study are the transgenic *TRE:RESTVP16* line that was developed in-house, the *Pdx1-Cre* transgenic line (11) and the *ROSA26R-rtTAEGFP* (12) line.

Generation of inducible Pdx1:RESTVP16:EGFP triple transgenic mice

The RESTVP16 vector was kindly donated by Sadhan Majumder. The expression vector had the REST DNA-binding domain fused to a VP16 activation domain. The N-and C-terminal repressor domains were deleted (9). The *TRE-RESTVP16* DNA fragment was digested from pcDNA3-NMyc-RESTVP16 plasmid and injected into F2 donor embryos of (C57BL/6 x DBA/2) and FVB/N background to generate the *TRE-RESTVP16* transgenic animals. Founder mice expressing the *TRE-RESTVP16* transgene were then crossed into *Pdx1-Cre* transgenic mice to generate double transgenic *Pdx1-Cre: TRE-RESTVP16* mice. These double transgenic mice were further crossed with *ROSA26R-rtTAEGFP* transgenic (Tg) mice to the generate the doxycycline inducible Pdx1:RESTVP16:EGFP triple Tg mice. This strategy allowed for doxycycline inducibility of the *RESTVP16* transgene in cells derived from pancreatic progenitors expressing Pdx1 (Jackson Labs). Additionaly, all cell lineages of the progenitors would be marked by EGFP expression. Transgenic mice were genotyped by PCR amplification of genomic DNA obtained from tail biopsy using the following primers: RESTVP16 forward primer *tccaaagccccatacgagac* and reverse primer *tccaaagccccatacgagac* that yielded a 397 bp product.

The control mice used throughout the paper were either double transgenic Pdx1:EGFP or RESTVP16:EGFP mice that could not express the *RESTVP16* transgene.

Doxycycline administration

Doxycycline was administrated in drinking water supplemented with 0.5% sucrose at a concentration of 2g/L. The water was stored in red bottles to shield from artificial light. Additionaly, the mice were fed with food pellets containing doxycycline (200mg/kg, Bio-Serv). Food and water supplies were replenished every 2-3 days.

Histology and immunohistochemistry

BrdU (25 μg per g of body weight) was injected intraperitoneally into pregnant dams 45min before harvesting the embryos. The embryos were considered to be 0.5 days of gestation at noon on the day of discovery of vaginal plugs. The gastrointestinal tracts along with the pancreas were dissected put from the embryos in cold PBS, fixed in 4% formaldehyde for 1 hour followed by dehydration in ethanol and stored at −20°C until processed for paraffin embedding. During embedding, the gastrointestinal tracts were oriented in paraffin such that sections were cut along the anterior–posterior axis. Five-micrometer sections were deparaffinized in toluene, rehydrated in grades of alcohol, and washed in H2O. All slides were subject to antigen retrieval protocols using Antigen Unmasking Buffer (Vector Labs). After antigen unmasking, the slides were cooled to room temperature. All slides were permeabilized in 0.4% Triton X-100/TBS for 20 min, and non-specific binding of antibodies was subsequently blocked with 3% IgG-free BSA (Jackson ImmunoResearch Laboratories).

Incubation with primary antibodies was performed overnight at 4°C in blocking solution at the following dilutions: 1:200 guinea pig anti-insulin (Dako), 1:500 rabbit anti-glucagon

(Immunostar), 1:200 rabbit anti-amylase (Sigma-Aldrich), 1:100 mouse anti-Pdx1 (DSHB), 1:200 rabbit anti-Pdx1 (Chemicon), 1:250 chicken anti-GFP (Aves Labs Inc.), 1:500 rabbit anti-Sox9 (Millipore), 1:50 mouse anti-Nkx6.1 (DSHB), 1:100 fluorescein-conjugated DBA lectin (Vector Labs) and 1:50 mouse anti-NRSF (12C11) (27). Donkey- and goat-derived secondary antibodies conjugated to FITC, Cy3 or Cy5 (Jackson ImmunoResearch Laboratory) were diluted in blocking buffer at1:500. Slides were mounted with Vectashield with DAPI (Vector Labs) and images were obtained using Openlab software (Perkins Elmer) and a Leica DM6000 microscope.

Cell sorting

To obtain purified pancreatic progenitor cells and neonatal beta cells, pancreatic buds from e12.5 Pdx1-YFP, e14.5 Ngn3-GFP and 2 week MIP-GFP transgenic mice were dissected and gently dissociated into a single cell suspension using Accumax (Innovative Cell Technologies) as previously described (28). A similar protocol was used to isolate sorted cells from control and RESTVP16 Tg fluorescent pancreatic buds at e16.5. The pancreatic buds were suspended in Accumax at 37°C for 15 minutes with occasional pipetting followed by addition of quenching media. The dissociated cell suspension was thereafter manually filtered, resuspended in FACS sorting buffer and sorted at low pressures by FACS (FACSaria BD biosciences) to an average percent purity of 95%-100%. Cells from wild-type non-fluorescent buds were used as negative control for FACS gating.

RNA isolation, RT-PCR and real-time qPCR

Total RNA from sorted cell populations was isolated using an RNAqueous-Micro Kit (Ambion) with slight protocol modification to allow for recovery of small RNAs including miRNAs. Single stranded cDNA was prepared using Superscript III Reverse Transcriptase (Invitrogen) with

oligodT priming. Real-time qPCRs were performed using the 7900HT Fast Real-time PCR system (Applied Biosystems). The expression level of each transcript was normalized to the housekeeping gene *Cyclophilin*. Data and standard deviations shown were measured from at least three independent biological replicate experiments. All RT-PCR and real-time qPCR primer sequences used are available upon request.

RNA-seq data acquisition and analysis

Three pairs of independent pancreatic buds from control and RESTVP16 Tg embryos from a doxycycline-fed dam were dissected out at e16.5 and total RNA extracted using the RNAqueous-Micro Kit (Ambion). Total RNA quality was assessed using an Agilent 2100 Bioanalyzer and an RNA Integrity Number (RIN) generated using 2100 Expert Software (Agilent Technologies). All RNA samples used had a RIN greater than 7. 10-20 ng of total RNA was processed using Ovation® RNA-Seq System V2 to generate amplified cDNA according to manufacturer's instructions. The amplified cDNA was used to generate sequencing libraries at the UCLA Clinical Microarray Core Facility and sequencing was performed on an Illumina Hi-Seq instrument.

The raw sequencing reads were assessed for their quality using the FASTX tool kit (http://hannonlab.cshl.edu/fastx_toolkit) and poor quality reads trimmed. The RNA-seq raw reads were then aligned and assigned to the mouse reference genome using the BWA program (29) (Li et al). The aligned reads were then assigned to genes according to gene annotations from GenBank. Thereafter, gene counts were generated from mapped reads as FPKM values using HTSeq software (http://www-huber.embl.de/users/anders/HTSeq). To identify differential gene expression between control and RESTVP16 sorted cells, the DESeq statistical method was employed (30) with the P- and q-values used to assess significance. Group functional annotation

of differentially expressed genes was performed using the DAVID Bioinformatics Database (31; 32)

Statistical analyses.

All data are expressed as means ± SEM. Statistical significance was determined by an unpaired Student's t test and a P value <0.05 was used to reject the null hypothesis.

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Chapter 4

DNMT1 represses *p53* to maintain progenitor cell survival during pancreatic organogenesis

Abstract

In the developing pancreas, self-renewal of progenitors and patterning of cell fates are coordinated to ensure the correct size and cellular makeup of the organ. How this coordination is achieved, however, is not clear. We report that deletion of DNA methyltransferase 1 (Dnmt1) in pancreatic progenitors results in agenesis of the pancreas due to apoptosis of progenitor cells. We show that DNMT1 is bound to the p53 regulatory region and that loss of Dnmt1 results in derepression of the p53 locus. Haploinsufficiency of p53 rescues progenitor cell survival and cellular makeup of the Dnmt1-deleted pancreas..

Introduction

The vertebrate pancreas is a complex organ made of functionally different tissue types that arise from a common progenitor cell pool during embryogenesis (Gu et al. 2003; Jensen 2004; Gittes 2009). Lineage tracing analysis indicates that a pool of pancreatic progenitor cells persists throughout organogenesis to give rise to differentiated mature cell types in a spatially and temporally regulated manner (Herrera 2000; Jensen et al. 2000a; Gu et al. 2002). The maintenance of the progenitor cell pool requires that progenitor cell self-renewal is balanced with cell cycle exit to coordinate differentiation of mature cell types of the pancreas (Georgia et al. 2006).

DNA methylation is a potential epigenetic mechanism that may be involved in maintaining the balance between self-renewal and terminal differentiation in the developing pancreas. DNA methylation may serve as a means of repressing pluripotency genes in multipotent progenitor cells or a means of repressing lineage-specific differentiation genes in multipotent progenitor cells (Reik 2007). It is not clear whether pancreatic progenitor DNA methylation patterns must be inherited during self-renewal to maintain a functional progenitor population by repressing differentiation genes or to serve as a basis for establishing lineage-specific methylation patterns during differentiation.

DNA methyltransferase 1 (Dnmt1) is the enzyme that propagates DNA methylation patterns during cell division. Previous studies on Dnmt1 have yielded interesting yet divergent data about its role in maintenance and differentiation of progenitor cell types. Dnmt1-null mouse embryonic stem cells (mESCs) undergo limited differentiation and apoptosis and are eliminated in competition assays with wild-type mESCs yet have a proliferative capacity comparable with that of wild-type mESCs when maintained in an undifferentiated state (Lei et al. 1996; Panning and

Jaenisch 1996). Dnmt1-null embryos expire from growth arrest and severe neural tube distortions by embryonic day 8.5 (E8.5), which indicates that Dnmt1-null mESCs are capable of undergoing more extensive differentiation in vivo than in vitro (Li et al. 1992; Lei et al. 1996). Loss of Dnmt1 in hematopoetic progenitor cells results in defects in self-renewal and niche retention and an inappropriate shift to myeloid progenitor cell cycling and differentiation (Trowbridge et al. 2009). Studies in human adult epidermal stem cells indicate that Dnmt1 maintains progenitor self-renewal by repressing genes that trigger cell cycle exit and premature differentiation (Sen et al. 2010). During neurogenesis, Dnmt1 restricts precocious astrogliogenesis of neural precursor cells, but deletion of Dnmt1 in telencephalic precursors results in cell death and forebrain degeneration (Fan et al. 2005; Hutnick et al. 2009). Taken together, these studies present context-dependent roles for Dnmt1 that may have differential effects in vitro or in vivo.

To investigate the in vivo role of DNMT1 in progenitor maintenance and concomitant lineage specification during organogenesis, we conditionally deleted Dnmt1 in pancreatic progenitor cells. Analysis of progenitor cell self-renewal dynamics revealed that Dnmt1-null pancreatic progenitor cells undergo G2 arrest. Our studies indicate that DNMT1 functions to repress p53. In its absence, p53 expression is derepressed, resulting in apoptosis of pancreatic progenitor cells. We show that p53 haploinsufficiency rescues progenitor cell survival and restores pancreatic organogenesis in the Dnmt1-null embryos. These data suggest that Dnmt1 is required to prevent p53-dependent apoptosis during lineage specification of pancreatic progenitor cells.

Results

Loss of Dnmt1 results in a severely atrophic pancreas

We bred mice with a floxed allele of Dnmt1 ($DNMT1^{fl/fl}$) with mice transgenic for Cre recombinase under the control of the Pdx1 promoter (Jackson-Grusby et al. 2001; Gu et al. 2002). This RESTricted Dnmt1 excision to the pancreatic epithelium ($DNMT1^{PC}$). To facilitate lineage tracing studies, we also bred in the stop-floxed-$R26R^{YFP}$ to mark all cells derived from progenitor cells that expressed the Pdx1-Cre transgene (Srinivas et al. 2001). To confirm that deletion of Dnmt1 resulted in hypomethylation of the self-renewing pancreatic progenitor pool, we used immunohistochemistry to detect 5-methyl-cytosine (5mC). Unlike the control epithelium, where 5mC uniformly stained the epithelium and surrounding mesenchyme, 5mC staining was grossly diminished in the epithelium of the $DNMT1^{PC}$ pancreas but maintained in the surrounding mesenchyme (Supplemental Fig. 1A,B). This indicated that deletion of Dnmt1 resulted in hypomethylation of the pancreatic epithelium during organogenesis. Previous reports have shown that loss of Dnmt1 leads to derepression of intracisternal A particle (IAP), a core retroviral element protein (Fan et al. 2001). Consistent with these previous studies, IAP staining was not detected in the pancreatic epithelium of control E13.5 embryos, but high levels of IAP were detected in the $DNMT1^{PC}$ pancreatic epithelium (Supplemental Fig. 1C,D). Some glucagon-positive cells in the $DNMT1^{PC}$ pancreatic epithelium were negative for IAP expression and were likely derived from cells that had escaped Cre-mediated recombination. These data indicated that Pdx1-Cre expression efficiently deleted Dnmt1 from the large majority of pancreatic progenitor cells early in development and led to hypomethylation of the pancreatic epithelial cells.

We next examined the effects of Dnmt1 deletion on pancreatic organogenesis by analyzing DNMT1^{PC} litters at birth. DNMT1^{PC} animals were born alive at expected Mendelian ratios. Examination of littermates indicated that the pancreas of pups in which one allele of Dnmt1 was deleted were grossly normal and comparable with wild-type control littermates (Fig. 1 A,B, D,E). YFP expression was absent in the control animals and homogenously distributed throughout the pancreas in heterozygous pups (Fig. 1G,H). Strikingly, gross examination of the DNMT1^{PC} pancreas revealed a severely atrophic pancreas (Fig. 1C,F). The rudimentary DNMT1^{PC} pancreas displayed little YFP expression, indicating that most of the atrophic pancreatic tissue was derived from cells that had escaped recombination (Fig. 1I). These data indicated that Dnmt1 is essential for the formation of the pancreas.

Previous studies in zebrafish indicated that loss of Dnmt1 resulted in degeneration of the acinar pancreas, but ductal and endocrine lineages were spared, leaving open the possibility that the atrophic pancreas that we observed could consist of primarily endocrine and ductal cells (Anderson et al. 2009). To determine whether the absence of Dnmt1 resulted in loss of specific cell lineages, we carried out immunohistological analysis for differentiated cell types in the DNMT1^{PC} pancreas. Antibody staining against exocrine cells expressing amylase and endocrine cells expressing insulin showed that scattered clusters of both endocrine and exocrine cells were present in the DNMT1^{PC} pancreas, but the typical rosette architecture of the acinar tissue and islet clusters of insulin cells was disrupted (Supplemental Fig. S2A–D). Cells that stained for the ductal marker mucin were scattered throughout the DNMT1^{PC} pancreas (data not shown). From this analysis, we concluded that deletion of Dnmt1 from pancreatic epithelial progenitor cells did not impact any particular lineage and instead disrupted the architecture and severely reduced the numbers of differentiated cells of all lineage of the mature pancreas.

Deletion of *Dnmt1* in pancreatic progenitors results in the absence of differentiated cells

We investigated whether a severely reduced pancreas size could result from depletion of the pancreatic progenitor pool during embryogenesis. We examined DNMT1^{PC} and control littermate pancreas by immunostaining for pancreatic progenitor markers Pdx1 and Sox9. At E13.5, the overlapping Pdx1 and Sox9 populations were comparable in the control and DNMT1^{PC} pancreatic epithelium (P = 0.2568) (Fig. 2A,B,E). In contrast, the number of cells staining for Sox9 at E15.5 was reduced by >40% in the DNMT1^{PC} pancreas compared with the control littermate pancreas (P = 0.0137) (Fig. 2C,D,E). This indicated that the progenitor pool is depleted during pancreatic organogenesis and results in a severely atrophic pancreas. The depletion of pancreatic progenitors could be due to either a defect in self-renewal or precocious differentiation. Precocious differentiation leading to depletion of the pancreatic progenitor pool has been previously described (Jensen et al. 2000b; Bhushan et al. 2001). To assess precocious differentiation, we carried out immunohistochemistry of E12.5 pancreas with antibodies against Sox9 to mark progenitor cells and glucagon to mark differentiated cells. The size of the pancreatic epithelial bud and the number of glucagon cells from E12.5 embryos immunostained with Sox9 and glucagon were comparable in DNMT1^{PC} and control littermates (Fig. 2F–G). To further assess differentiation, we compared global gene expression by microarray analysis in control and DNMT1^{PC} pancreas. There was less than a twofold difference between most transcripts in the control and DNMT1^{PC} pancreas, which suggests that the majority of the cells in the DNMT1^{PC} pancreas had a transcriptome similar to that of the control pancreas (Supplemental Fig. 3A). Gene enrichment analysis indicated that a subset of genes involved in pancreatic differentiation was specifically down-regulated. Neurogenin 3, the gene required for differentiation of all endocrine cell types, was one of the most down-regulated gene in the array

analysis, with a >12-fold decrease in expression in the DNMT1^{PC} pancreas (Schwitzgebel et al. 2000). Other transcription factors involved in endocrine and exocrine lineage specification—such as Nkx6.1, Nkx2.2, Rfx6, Rbjk-l, and Ptf1—were specifically down-regulated (Supplemental Fig. 3B). We sought to confirm the gene expression profiles by immunohistochemistry. Immunostaining of the control pancreas at E15.5 showed Ngn3^{+} cells along the embryonic duct surrounded by pancreatic progenitor cells expressing Pdx1. In striking contrast, the number of Ngn3^{+} cells in the DNMT1^{PC} pancreas was <15% that of the control littermates ($P < 0.005$) (Fig. 2H–J). These results indicated that in the absence of Dnmt1, pancreatic epithelial cells were not depleted due to precocious differentiation, and in fact, very few differentiated cells were observed in the DNMT1^{PC} pancreas.

Loss of *Dnmt1* resulted in accumulation of progenitor cells in the G2 phase

To investigate whether loss of Dnmt1 resulted in depletion of pancreatic progenitor cells due to defects in self-renewal, we analyzed embryos from pregnant dams injected with a short pulse of BrdU. Control and DNMT1^{PC} pancreas were immunostained with antibodies against Pdx1 to mark pancreatic progenitor cells and BrdU to identify cells actively in S phase of the cell cycle (Fig. 3A,B). The Pdx1^{+}BrdU^{+} cells were counted to determine the proliferation index, calculated as (Pdx1^{+}BrdU^{+})/Pdx1^{+}. There were no differences in the proliferation index between control and DNMT1^{PC} progenitor cells at E12.5 (Fig. 3C). Because a short BrdU pulse only measures the cells that are actively in S phase, we pursued additional proliferation studies to determine whether the progenitor cells were completing the cell cycle. Control and DNMT1^{PC} pancreas were immunostained with antibodies against phosphorylated histone H3, a marker for G2/M

phase, and Pdx1 (Fig. 3D,E). Cells with a speckled PHH3 pattern were categorized as G2 phase of the cell cycle (Fig. 3D,E, white arrows), and cells with more robust staining were categorized as M phase (Fig. 3D,E, orange arrows). Ratios of $Pdx1^{+}PHH3^{+}/Pdx1^{+}$ were used to calculate the proliferation index. Comparing control and $DNMT1^{PC}$ pancreas revealed that the number of M-phase progenitor cells was not significantly different, but the number of progenitor cells in G2 phase in the $DNMT1^{PC}$ pancreas was increased by 40% ($P < 0.005$) (Fig 3F). The fact that S-phase and M-phase progenitor cells did not change in the $DNMT1^{PC}$ pancreas indicated that cell cycle entry and mitosis of pancreatic epithelial cells were not perturbed by the deletion of Dnmt1. The accumulation of a subset of pancreatic epithelial cells in G2 arrest suggested that depletion of these cells could be due to apoptosis.

Deletion of *Dnmt1* results in p53-dependent apoptosis of pancreatic progenitors

We next investigated whether apoptosis of pancreatic epithelial cells could result in the atrophic phenotype of the $DNMT1^{PC}$ pancreas at birth. Pancreatic sections from E15.5 $DNMT1^{PC}$ and control littermate embryos were stained for cleaved caspase 3, a marker for apoptosis, and Pdx1. Cleaved caspase 3 was not detected in control pancreas; however, $DNMT1^{PC}$ pancreas showed a number of cells within the pancreatic epithelium that were positive for cleaved caspase 3 (Fig. 4A,B).

Several studies in fibroblast and cancer cell lines indicated that loss of Dnmt1 resulted in p53-dependent apoptosis, although the mechanism remains unclear (Jackson-Grusby et al. 2001; Chen et al. 2007; Anderson et al. 2009). To determine whether deletion of Dnmt1 in pancreatic progenitors resulted in changes in p53, we first assessed whether levels of p53 were altered in

DNMT1^{PC} pancreas. Immunohistochemical analysis using a p53 antibody revealed that p53 accumulated in the DNMT1^{PC} epithelium, while no p53 staining was observed in control littermate pancreas (Fig. 4C,D, white arrows). To confirm this accumulation of p53 in DNMT1^{PC} pancreas, we measured p53 using Western blot analysis at E12.5, prior to detectable differences in the pancreatic epithelium, and at E15.5, after the onset of p53 expression. Western blot analysis indicated that there was no difference in the accumulation of p53 at E12.5, but there was a significant sixfold increase in p53 in the DNMT1^{PC} pancreas compared with control pancreas from littermates at E15.5 ($P = 0.0098$) (Fig 4E,F). Western blot analysis indicated that the p53 downstream transcriptional target p21 accumulated in the DNMT1^{PC} pancreas (Fig. 4E). To assess whether the increase in p53 protein correlated with an increase in mRNA levels of p53, we used quantitative PCR to quantify any changes in p53 gene expression. Our analysis revealed a 20-fold increase in p53 mRNA in DNMT1^{PC} pancreas compared with control pancreas from littermates (data not shown). The increase in p53 mRNA indicated that Dnmt1 could potentially regulate p53 at a transcriptional level.

We next assessed how loss of Dnmt1 resulted in transcriptional up-regulation of p53. We hypothesized that DNMT1 could play a role in directly repressing p53 transcription and that loss of Dnmt1 would result in derepression of p53. Supporting our hypothesis, a previous study showed that DNMT1 was bound to the p53 promoter 2 kb upstream of the p53 transcription start site. Interestingly, this upstream regulatory region also contained a binding sequence for the transcriptional repressor CTCF. Binding of CTCF to this region suppressed reporter activity (Su et al. 2009). We reasoned that DNMT1 and CTCF associated at the p53 regulatory region, and binding of CTCF to the p53 regulatory region may be disrupted in the absence of Dnmt1. To test this, E14.5 pancreas from DNMT1^{PC} and control littermates were subjected to chromatin

immunoprecipitation (ChIP) analysis (Fig. 4G). In control pancreas, both DNMT1 and CTCF were bound to the p53 regulatory region, confirming that DNMT1 and CTCF binding was associated with p53 repression ($P < 0.005$) (Fig. 4H). In contrast, there was no enrichment of CTCF in $DNMT1^{PC}$ embryos ($P = 0.0268$) (Fig. 4H). Moreover, enrichment of H3K27ac within the p53 promoter region was observed in the $DNMT1^{PC}$ embryos, which is consistent with transcriptional activation ($P = 0.0423$) (Fig. 4H). These results suggested that DNMT1 directly repressed p53 transcription, and loss of Dnmt1 resulted in derepression of p53.

Haploinsufficiency of *p53* rescues pancreatic organogenesis

To investigate whether inactivation of Dnmt1 resulted in p53-mediated apoptosis of pancreatic progenitors, we crossed the p53-null animals into the $DNMT1^{PC}$ background. p53-null animals are phenotypically indistinguishable from wild-type littermates at birth, and pancreas formation was not perturbed (Jacks et al. 1994).

We first assessed whether haploinsufficiency of p53 restored pancreatic progenitor populations at E15.5. Immunohistochemical assessment of Sox9 and Pdx1 revealed no significant differences in the pancreatic epithelium between the control and $DNMT1^{PC}p53^{+/-}$ pancreas (Fig. 5A,B). To evaluate whether p53 haploinsufficiency would restore the expression of ngn3, we examined $DNMT1^{PC}p53^{+/-}$ pancreas for expression of Ngn3. Immunohistochemistry at E16.5 indicated that $Ngn3^{+}$ and $Pdx1^{+}$ cell populations persisted through organogenesis with a staining pattern similar to that of control littermates. Lineage tracing indicated that $DNMT1^{PC}p53^{+/-}$ cells expressing Ngn3 had undergone recombination, thus leading us to conclude that the expression of Ngn3 was restored by p53 haploinsufficiency (Fig. 5C,D). At birth, $DNMT1^{PC}$ and $DNMT1^{PC}p53^{+/-}$

animals were present at the expected Mendelian ratios. Gross histological examination of the neonatal pancreas revealed that while the pancreas of DNMT1^{PC} animals were severely atrophic, DNMT1^{PC}p53$^{+/-}$ littermates had a restoration of a full, albeit smaller, pancreas (Fig. 5E,F). Lineage tracing suggested that recombination was extensive, although not complete, in the DNMT1^{PC}p53$^{+/-}$ background (Fig. 5G,H). Insulin and YFP immunostaining indicated normal morphology of the endocrine tissue compartment in the control and DNMT1^{PC}p53$^{+/-}$ littermate pancreas (Supplemental Fig. 4A,B). Immunostaining for amylase and YFP indicated that exocrine cells formed morphologically normal rosette structures (Supplemental Fig. 4C,D). Mucin^{+}/YFP^{+} ductal structures were present and morphologically normal in both the control and DNMT1^{PC}p53$^{+/-}$ pancreas (Supplemental Fig. 4E,F). Taken together, these data indicated that p53 haploinsufficiency rescued the ability of Dnmt1-null pancreatic progenitor cells to differentiate into a normal mature organ during embryogenesis. The area of DNMT1^{PC}p53$^{+/-}$ islets decreased by postnatal day 15, and blood glucose levels were elevated into the diabetic range. This suggests that while haploinsufficiency of p53 may permit organogenesis in the DNMT1^{PC} pancreas, these cells are not able to support glucose homeostasis in adolescent mice (Supplemental Fig. 5A–E).

Discussion

Our results suggest that p53 repression mediated by DNMT1 is not required for self-renewal of pancreatic progenitor cells per se but is critical for progenitor cell survival during differentiation. Furthermore, p53 haploinsufficiency prevented apoptosis and rescued the survival of Dnmt1-null progenitor cells; this suggests that p53 is a primary target of DNMT1 action in pancreatic

progenitor cells. Recently, the role of Dnmt1 in epidermal progenitor cells suggested that Dnmt1 was required to retain proliferative stamina and suppress differentiation (Sen et al. 2010). Deletion of Dnmt1 in epidermal progenitors resulted in precocious differentiation and eventual tissue loss. Although the phenotype in the pancreatic deletions of Dnmt1 also was the eventual loss of tissue, no evidence of premature differentiation or defects in proliferation were observed. The mechanism by which Dnmt1 maintains progenitor populations may vary in different tissue depending on the cellular context. This suggests that at a molecular level, DNMT1 may associate with different effectors to carry out repressive functions depending on the cellular context.

We attribute the inability of the $DNMT1^{PC}$ pancreatic progenitor population to undergo differentiation to the accumulation of p53. Part of p53 accumulation may be due to the derepression of the p53 locus when DNMT1 is not present on the promoter. It is to be noted that there is no CpG methylation around the DNMT1-CTCF-binding area (Su et al. 2009; our unpublished results), suggesting that DNMT1 is working as part of a repressive complex independent of its DNA methylation activity. Methylation-independent functions of DNMT1 have been shown to repress target genes as part of complexes with E2F1 and histone deacetylases (Valdez et al. 2011; Clements et al. 2012). The inability of $DNMT1^{PC}$ cells to differentiate because of derepression of p53 is similar to the $mdm2^{-/-}$ mouse phenotype (Jones et al. 1995; Montes de Oca Luna et al. 1995). While $mdm2^{-/-}$ are embryonic-lethal at E6.5, $mdm2^{-/-}p53^{-/-}$ compound mutants are normal and viable. Analysis of compound $mdm2^{-/-}p53^{515c\text{-}515c}$, which express a mutated p53 that can mediate growth arrest but not apoptosis, revealed hematapoetic and lymphoid deficiencies because of progenitor cell cycle and differentiation defects (Liu et al. 2007). Thus, it is possible that repression of p53 serves to facilitate pancreatic progenitor cell differentiation during normal organogenesis and that the

absence of Dnmt1 results in transcriptional derepression and subsequent accumulation of p53 that initiates cell cycle arrest and apoptosis during differentiation. The ability of DNMT1^{PC}p53$^{+/-}$ pancreatic progenitor cells to persist through differentiation and form a pancreas at birth suggests that Dnmt1 is not required to maintain progenitor cell self-renewal or differentiation but is required to attenuate the role of p53 as a checkpoint during differentiation.

Materials and methods

Information on mouse strains is in the Supplemental Material. All animal experiments were performed in accordance with NIH policies on the use of laboratory animals and approved by the Animal Research Committee of the Office for the Protection of Research Subjects at University of California at Los Angeles. For immunohistochemical experiments, tissues were prepared, oriented, and stained, and images were captured as previously described (Georgia et al. 2006). Calculations of the proliferative index were done as previously described using at least three embryos from each genotype (Zhong et al. 2007). ChIP experiments with the E14.5 dorsal pancreas cells were carried out using the micro-ChIP protocol (Dahl and Collas 2008). Statistical significance was determined using Student's t test. Information on mouse strains, antibodies, and primers is in the Supplemental Material.

Acknowledgements

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JDRF, and the Helmsley Trust to A.B. S.G. and M.K. performed the experiments. A.B. and S.G. conceived and planned the experiments and interpreted data. A.B. and S.G. wrote the manuscript

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Figure 3-1

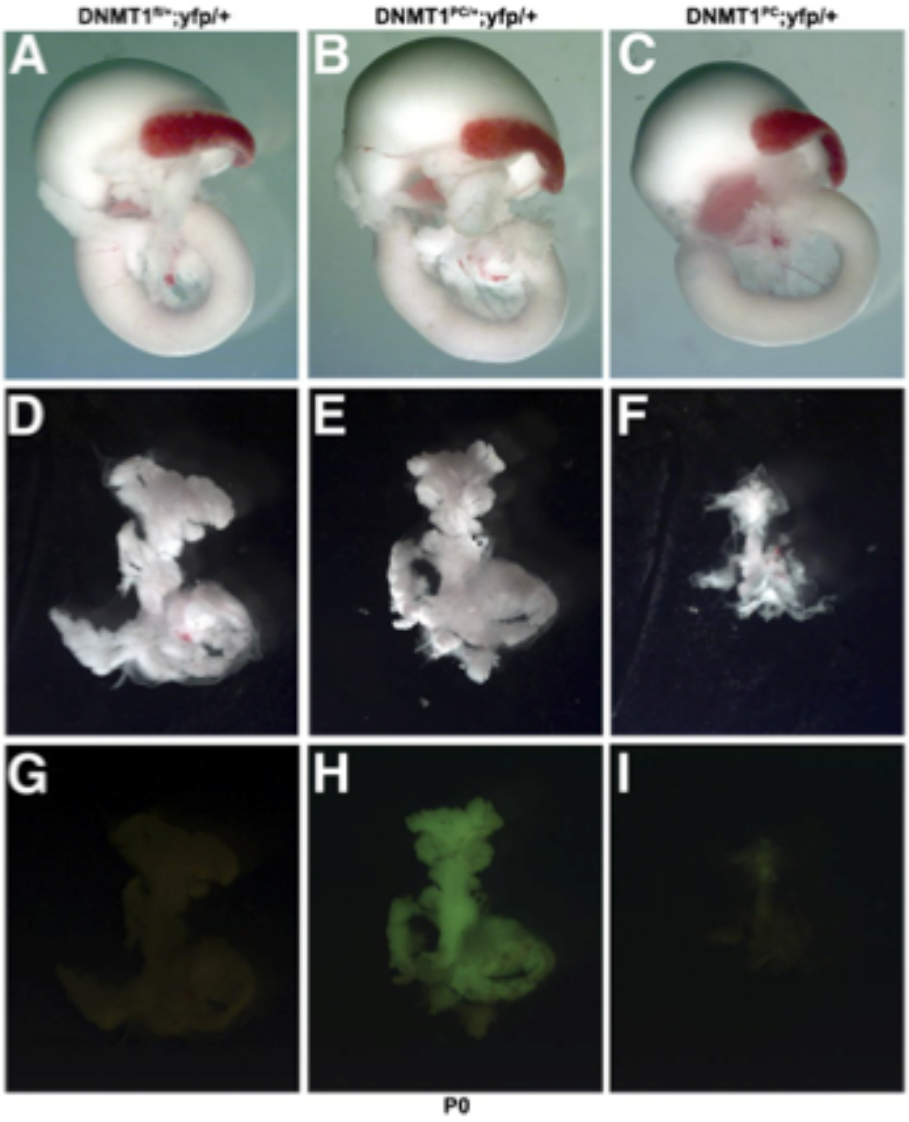


Figure 3-1: DNMT1 deletion results in an atrophic pancreas. (A–C) Gastrointestinal tract of control ($DNMT1^{fl/fl}$) (A), heterozygous ($DNMT1^{PC/+}$) (B), and conditional knockout ($DNMT1^{PC}$) (C) pancreas at postnatal day 0 (P0). (D–F) Pancreas dissected from the gastrointestinal tract illustrate the atrophic size of the $DNMT1^{PC}$ pancreas. (G–I) Lineage trace analysis indicates that there is efficient recombination in the heterozygous $DNMT1^{PC/+}$ pancreas (H), but very few recombined cells persist in the $DNMT1^{PC}$ pancreas (I)

Figure 3-2

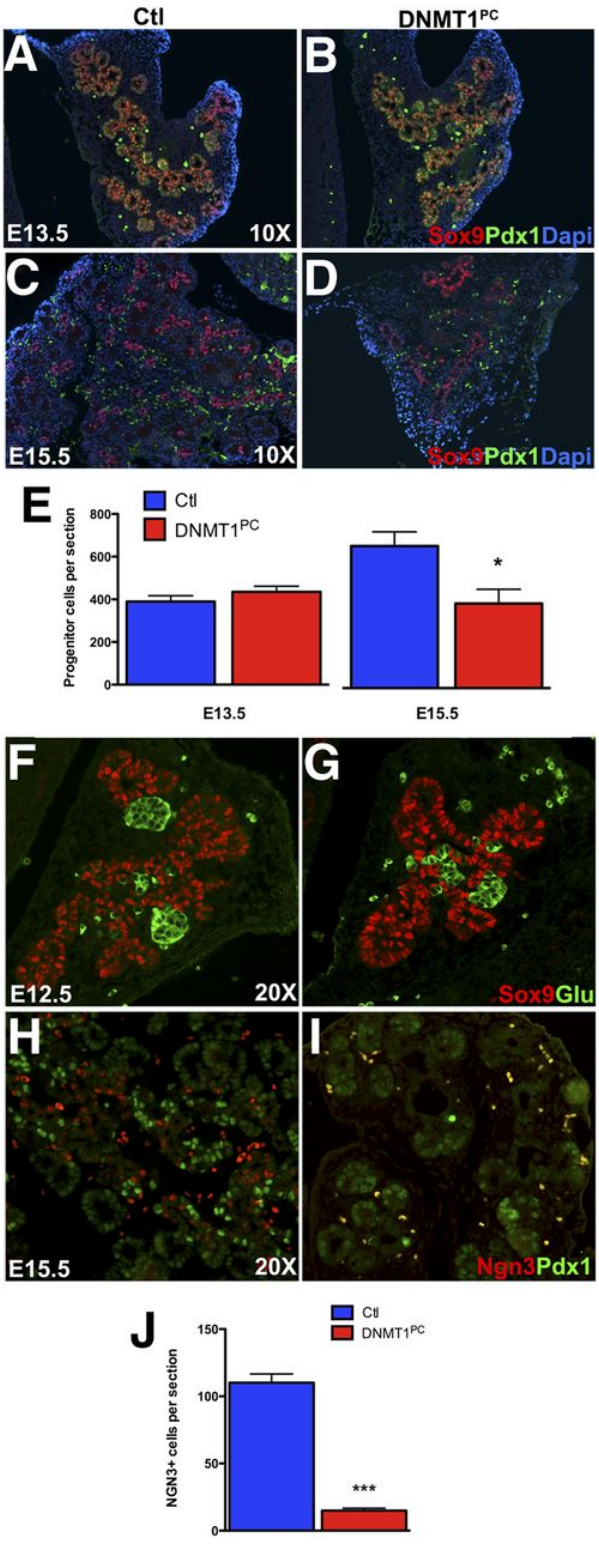

Ctl
DNMT1PC
A
B
E13.5
10X
Sox9Pdx1Dapi
C
D
E15.5
10X
Sox9Pdx1Dapi
E
Ctl
DNMT1PC
Progenitor cells per section
800
600
400
200
0
*
E13.5
E15.5
F
G
E12.5
20X
Sox9Glu
H
I
E15.5
20X
Ngn3Pdx1
J
Ctl
DNMT1PC
NGN3+ cells per section
150
100
50
0
***

Figure 3-2: DNMT1^{PC} pancreatic atrophy is the result of progenitor cell depletion. (*A*,*B*) Immunohistochemistry for Sox9 and Pdx1 at E13.5 indicates no notable difference in the size or morphology in control (*A*) and DNMT1^{PC} (*B*) pancreatic epithelium. (*C*,*D*) Immunohistochemistry of pancreatic epithelium for Sox9 and Pdx1 at E15.5 reveals a decrease in the number progenitor cells in the DNMT1^{PC} pancreatic epithelium. (*E*) Quantification of progenitor cells (E13.5) and Sox9 cells (E15.5). (*F*,*G*) Immunohistochemical comparison of the progenitor and glucagon populations at E12.5 in the control and DNMT1^{PC} suggests that precocious differentiation is not responsible for progenitor depletion. (*H*,*I*). By E15.5, DNMT1^{PC} pancreas have little expression of pre-endocrine cell marker Ngn3 (*I*) in comparison with control littermates (*H*). (*J*) Quantification of Ngn3^{+} cells at E15.5.

Figure 3-3

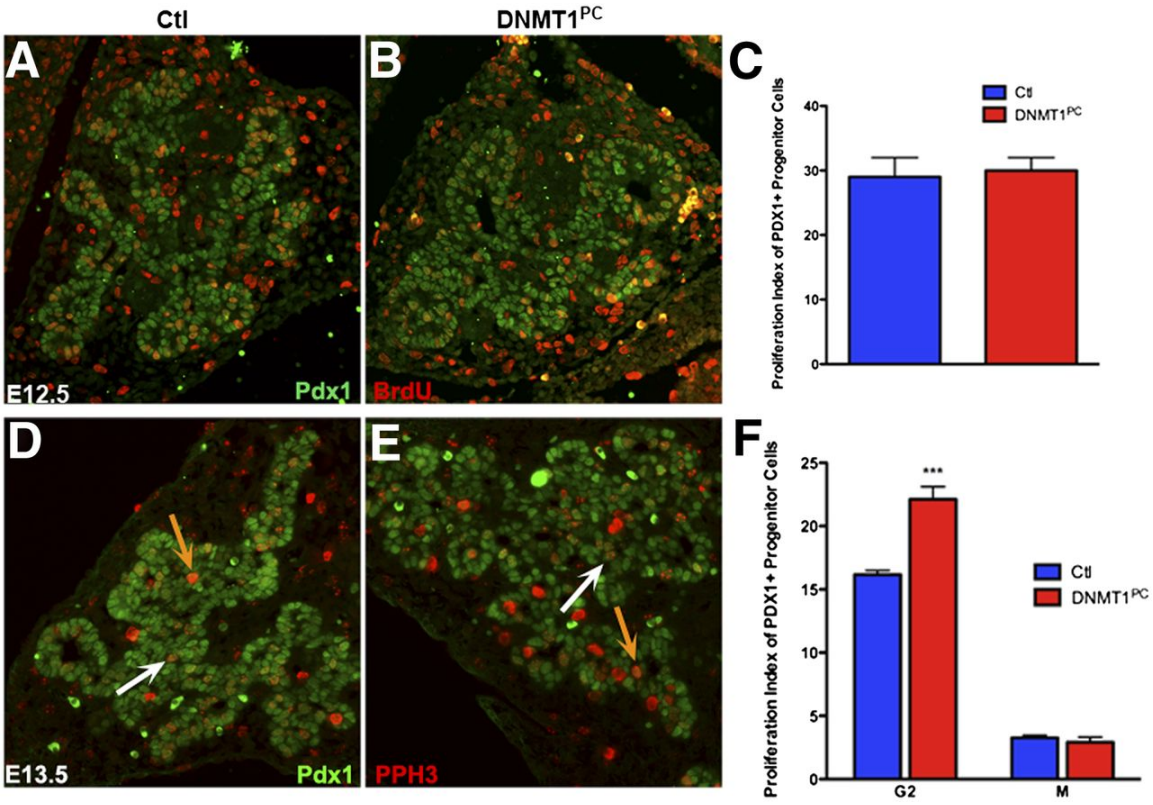


Figure 3-3: A subset of DNMT1^{PC} progenitor cells accumulates in G2 arrest. (*A–C*) E12.5 pancreatic sections immunostained for BrdU and Pdx1 to quantify progenitor proliferation. (*D–F*) Quantification of pancreatic progenitor cell proliferation at E13.5 as a percentage of phosphorylated histone H3^{+} Pdx1^{+} double cells indicates that there is an increase in the number of cells in G2 in the DNMT1^{PC} pancreas. (White arrows) G2-phase cells; (orange arrows) M-phase cells. (***) $P < 0.005$.

Figure 3-4

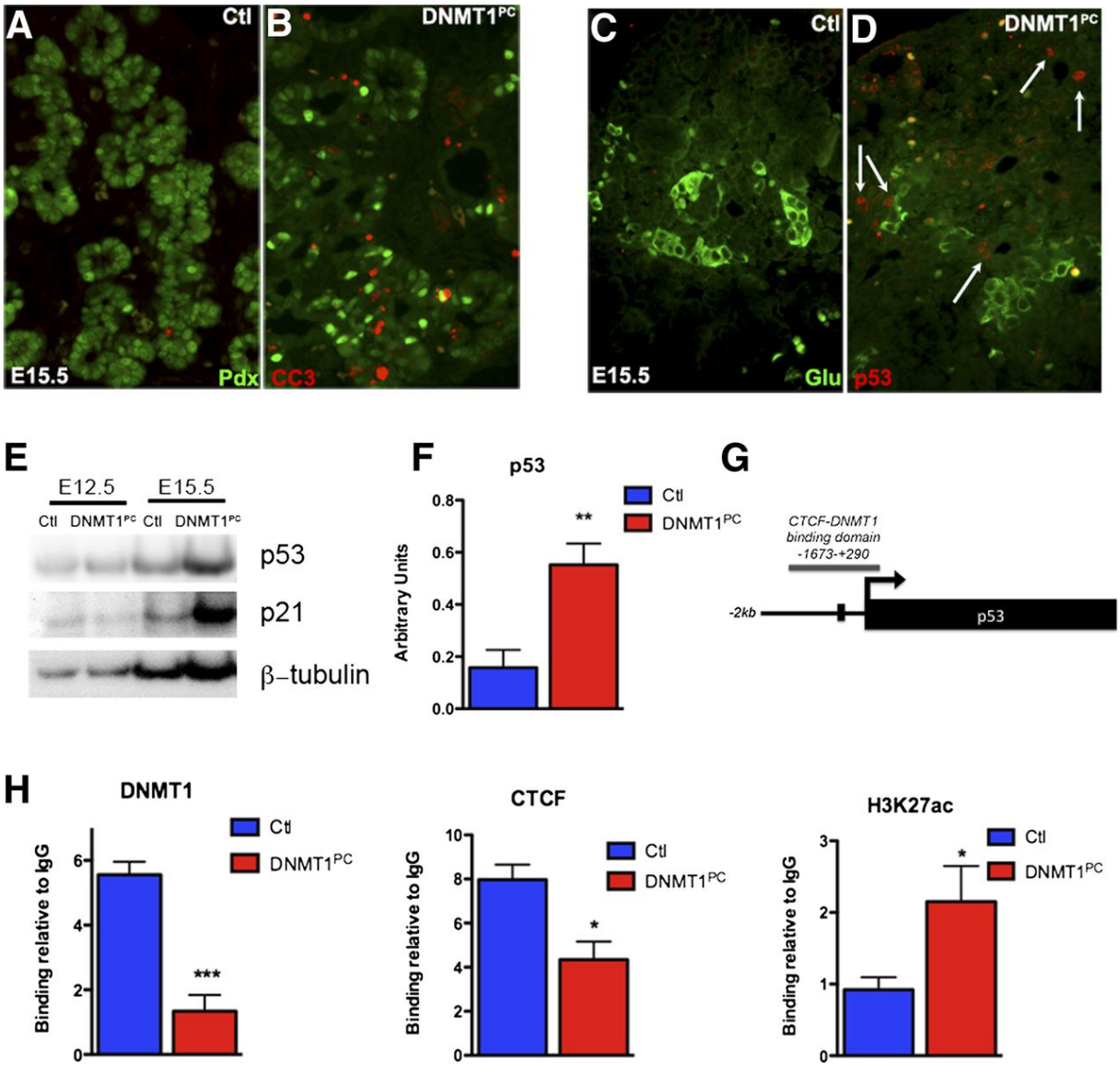

A
Ctl
E15.5
Pdx
B
DNMT1PC
CC3
C
Ctl
E15.5
Glu
D
DNMT1PC
p53
E
E12.5
E15.5
Ctl
DNMT1PC
Ctl
DNMT1PC
p53
p21
β–tubulin
F
p53
Ctl
DNMT1PC
Arbitrary Units
0.8
0.6
0.4
0.2
0.0
**
G
CTCF-DNMT1
binding domain
-1673-+290
-2kb
p53
H
DNMT1
Ctl
DNMT1PC
Binding relative to IgG
6
4
2
0
***
CTCF
Ctl
DNMT1PC
Binding relative to IgG
10
8
6
4
2
0
*
H3K27ac
Ctl
DNMT1PC
Binding relative to IgG
3
2
1
0
*

Figure 3-4: Progenitor depletion is a result of apoptosis in the DNMT1^{PC} pancreatic epithelium. (*A*,*B*) Immunohistochemistry for cleaved caspase 3 did not reveal any apoptosis in the control pancreas (*A*), but apoptosis was widespread in the DNMT1^{PC} pancreas (*B*). (*C*,*D*) The presence of apoptosis markers coincides with the appearance of p53 accumulation in the DNMT1^{PC} pancreatic epithelium (*D*, white arrows) and is absent in control littermates at E15.5 (*C*). (*E*) Western blot analysis for p53 protein and its downstream target, p21, at E12.5 and E15. (*F*) Densitometric quantification of Western blots indicates a sixfold increase in p53 protein at E15.5. (*G*) Schematic of the ChIP assay binding in the *p53* locus. (*H*) ChIP analysis indicates that DNMT1 binds to the *p53* locus in control pancreas but is lost in DNMT1^{PC} pancreas. Chromatin insulator protein CTCF is also bound to the *p53* locus in control pancreas but is lost in the DNMT1^{PC} pancreas. The activating histone mark H3K27ac is enriched in the DNMT1^{PC} pancreas.

Figure 3-5

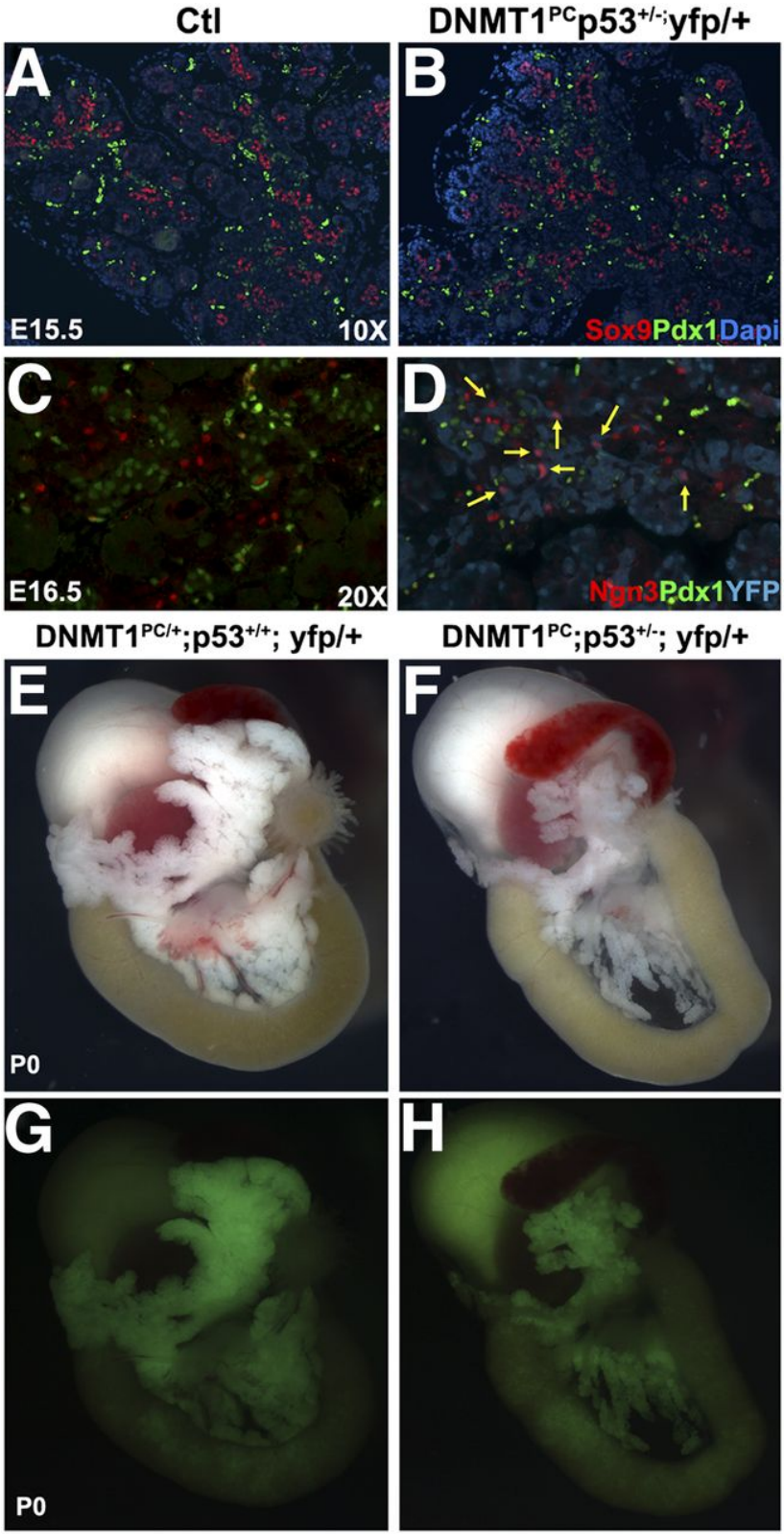

Ctl
DNMT1PCp53+/-;yfp/+
A
E15.5
10X
B
Sox9Pdx1Dapi
C
E16.5
20X
D
Ngn3Pdx1YFP
DNMT1PC/+;p53+/+; yfp/+
DNMT1PC;p53+/-; yfp/+
E
P0
F
G
P0
H

Figure 3-5: Haploinsufficiency of p53 rescues apoptosis and allows for cell survival during differentiation in DNMT1^{PC}p53$^{+/-}$ pancreas. (A,B) Immunohistochemical staining for Sox9 and Pdx indicates no significant difference in the control (A) and DNMT1^{PC}p53$^{+/-}$ (B) cell populations at E15.5. (C,D) Immunohistochemical analysis of control (C) and DNMT1^{PC}p53$^{+/-}$yfp^{+} (D) pancreas revealed a restoration of Ngn3^{+} expression in the absence of Dnmt1. Overlap with YFP indicates that these cells have arisen from cells that underwent recombination. (E,F) Gross analysis of control and DNMT1^{PC}p53$^{+/-}$yfp^{+} indicates that mutant pancreas are comparable with control littermates at P0. (G,H) Lineage tracing by YFP expression indicates that most cells in the DNMT1^{PC}p53$^{+/-}$yfp^{+} pancreas have undergone recombination.

SUPPLEMENTARY MATERIALS AND METHODS

Mice

All animal experiments were performed in accordance with NIH policies on the use of laboratory animals and approved by the Animal Research Committee of the Office for the Protection of Research Subjects at UCLA. We used Cre/loxP system to conditionally delete Dnmt1 in pancreatic progenitor cells. Dnmt1fl/fl mice with loxP sites flanking exons 4 and 5 of the Dnmt1 gene and Pdx1-Cre mice expressing Cre recombinase from Pdx1 promoter have been described previously (Gu et al. 2002). The Pdx1-Cre mice were bred into the Rosa26R-YFP background to indelibly mark all cells that were derived from pancreatic progenitor cells (Jackson Labs) (Soriano 1999; Srinivas et al. 2001). BrdU (25 μg per g of body weight) was injected intraperitoneally into pregnant dams 45min before harvesting the embryos. Isolated embryos were considered to be 0.5 days of gestation at noon of the day the plugs were detected. Embryos were dissected in cold PBS, fixed in 4% formaldehyde for 45 min to 3 h, depending on the age of the embryo, followed by dehydration in ethanol and stored at −20°C until processed for paraffin embedding.

Immunohistochemistry

The gastrointestinal tracts were oriented during the embedding process in paraffin so that sections were cut along the anterior–posterior axis. Five-micrometer sections were deparaffinized in toluene, rehydrated in grades of alcohol, and washed in H2O. All slides were subject to antigen retrieval protocols using Antigen Unmasking Buffer (Vector Labs). After antigen unmasking, the slides were cooled to room temperature. All slides were permeabilized in 0.2% Triton X-100/TBS for 20 min, and non-specific binding of antibodies blocked with 0.2%Tween-20, 3% IgG-free BSA/TBS. Primary antibodies were diluted in the blocking solution

at the following dilutions: rabbit anti-glucagon 1:500 (Immunostar); guinea pig anti-insulin 1:500 (Dako); rabbit anti-amylase (1:200, Sigma-Aldrich), hamster anti-mucin (1:200, NeoMarkers); rabbit anti-cleaved caspase-3 1:200 (Cell Signaling); mouse anti-p53 (1:250, Santa Cruz); mouse anti-BrdU (1:100, Amersham), rabbit anti-Pdx1 (1:200,Chemicon); mouse anti-Pdx1 (1:100, DHSB); rabbit

Georgia et al. Supplementary Info 8 anti-phospho-histone H3 1:200 (Upstate); mouse anti-Ngn3 (1:200, DHSB); mouse anti 5-methyl cytosine (1:200; Aviva Systems Biology AMM99021); and chicken anti-GFP (1:500; Aves Labs, Inc., 1020). Donkey- and goat-derived secondary antibodies conjugated to FITC, Cy3 or Cy5 were diluted in blocking buffer 1:500 (Jackson Laboratories). All slides were mounted with Vectashield with or without DAPI (Vector Labs). Slides were viewed using a Leica DM6000 microscope and images acquired using Openlab software (Perkins Elmer). BrdU injections, proliferative index and cell quantification BrdU (25 µg per g of body weight) was injected intraperitoneally into pregnant dams 45 min (or otherwise stated) before harvesting the embryos. Mouse anti-BrdU antibody (1:100) (Amersham/Pharmacia) and the Pdx1 antibody were diluted in the nuclease buffer provided with the BrdU antibody. For calculation of the proliferative index, the number of Pdx1-positive cells and Pdx1- BrdU or PPH3 -positive cells in the dorsal pancreatic buds were counted and the percentage of BrdU/PPH3 incorporation calculated (proliferative index). For cell quantification, four sections (3 sections apart) were analyzed in this manner, giving a total of 24 data points. Quantification of progenitor population was carried out using at least three embryos from each genotype. Statistical significance was determined using Student's t test.

RNA Isolation, Microarray Analysis, and Western Blotting Analysis

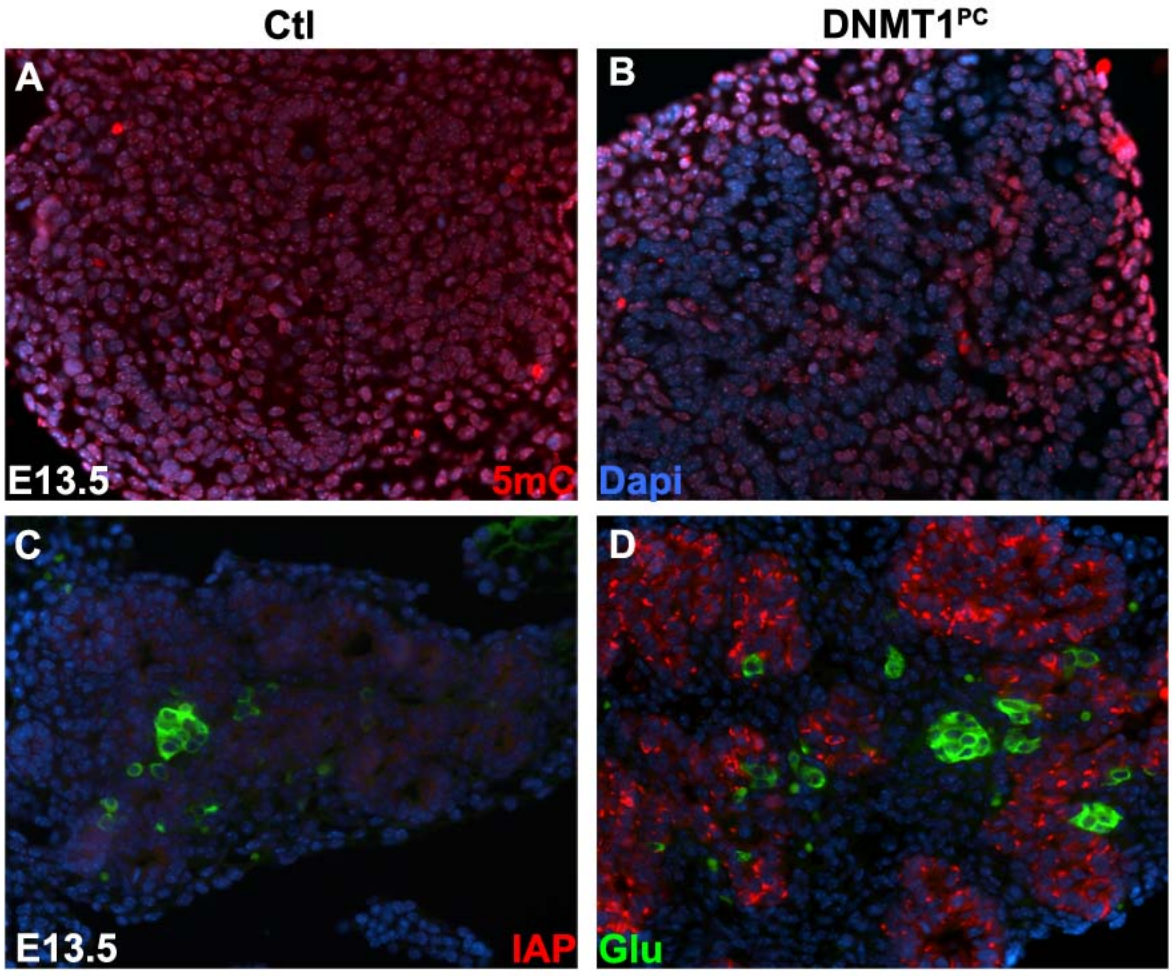
Dorsal pancreatic buds were dissected from E14.5 embryos and stored in RNA isolation buffer from the micro RNA Easy Kit (Qiagen) at -80°C until genotyping was completed. RNA was isolated using the micro RNA Easy Kit as per the manufacturer's protocol. For microarray studies, isolated RNA was processed, labeled, and hybridized to the Mouse 1.0ST GeneChip (Affymetrix) by the UCLA DNA Microarray Core Facility using standard protocols recommended by the manufacturer. Primary analysis of the microarray data, including RMA normalization, transcript expression levels, and the probability of change in transcript expression between genotypes was performed with Genomics Suite software (Partek). For western blotting studies, E15.5 dorsal pancreatic buds were stored in cell extraction buffer (Invitrogen) and stored 8rat -20°C until genotyping was completed. Western blots were performed as described previously

(Tschen et al. 2009) with the following primary antibodies: mouse anti-p53 (1:200, Santa Cruz), rabbit anti-p21 (1:500, Santa Cruz), mouse anti-beta tubulin (1:1000, Sigma Aldrich). Densitometric measurements were calculated from at least three western blots using Labworks (Media Cybernetics).

Chromatin Immunoprecipitation Primers and Antibodies

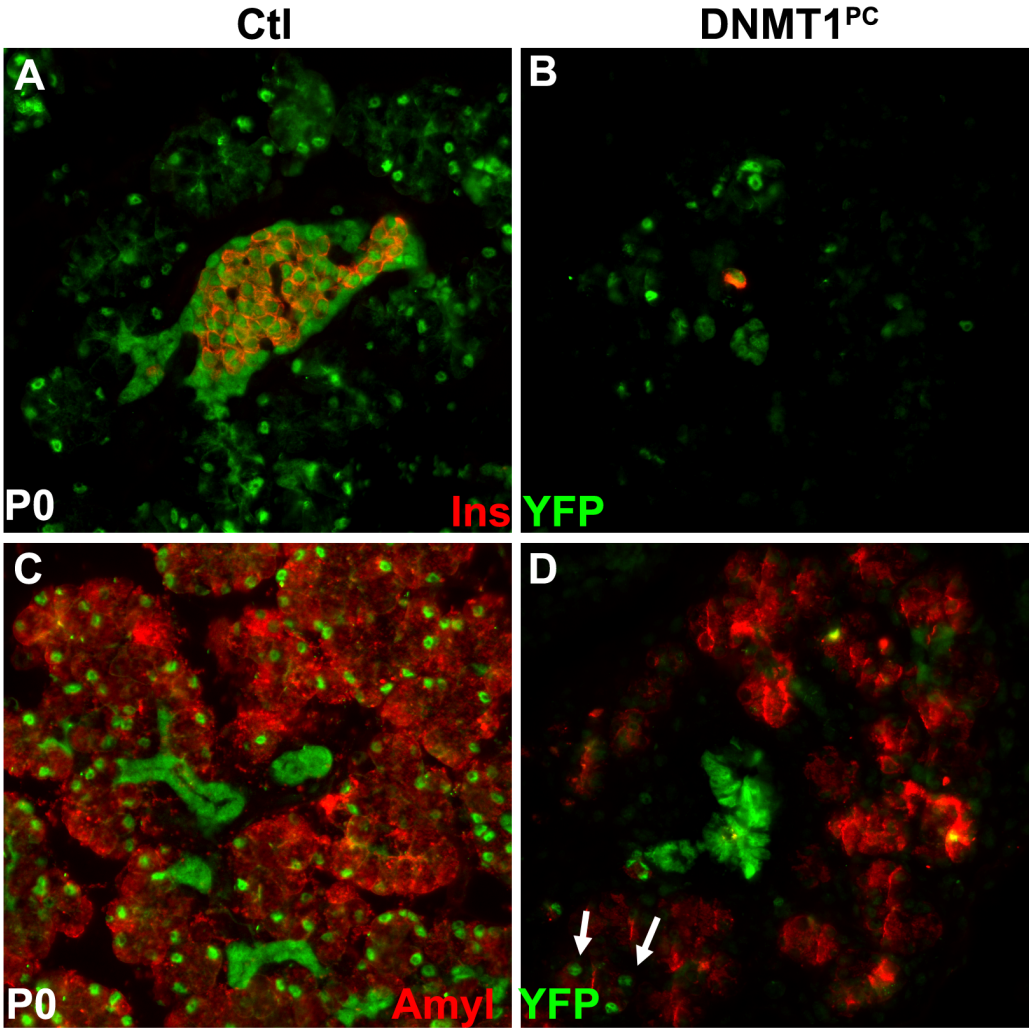
Primers for the DNMT1 and CTCF binding site on the p53 locus were designed in with reference to Su et al. 2009. The primers were For-AACACGGTGGTGCGATACCAAG and Rev-CCAACACGGGCCCCTAAGTTC. Antibodies used for chromatin immunoprecipitation were mouse anti-DNMT1 (IMG-261A), rabbit anti-CTCF (Millipore 07-729), rabbit anti-H3K27ac (Abcam, ab-4729).

Figure 3-S1



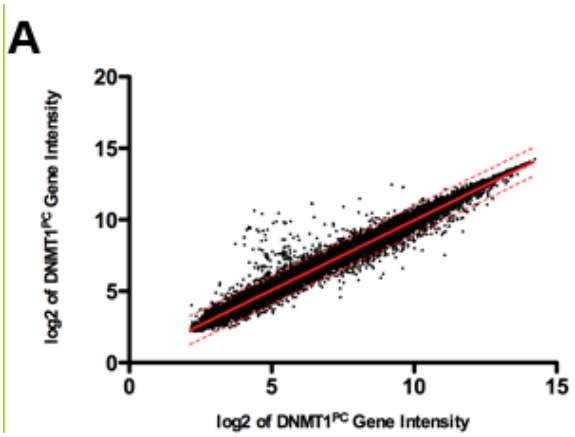
Supplementary Figure 3-S1: Cre excision is efficient in the DNMT1PC pancreatic epithelium. (A-B) 5-methylcytosine expression is homogenous in the control pancreas, but is decreased in the DNMT1PC pancreatic epithelium. DNMT1PC mesenchymal staining, which arises from cells that do not express Pdx1, is comparable to control littermates. (C-D) The expression of retroviral element IAP is repressed in control pancreas (C), but is seen throughout the epithelium of the DNMT1PC pancreas.

Figure 3-S2



Supplementary Figure 3-S2: All cell types are severely decreased in the DNMT1^{PC} pancreas. (A-B) Staining for insulin (red) and YFP (green) illustrates the high rate of recombination in control animals, and the severely reduced number of cells that underwent recombination in the DNMT1^{PC} pancreas. (C-D) Immunohistochemistry for amylase (red) and YFP (green) illustrates the majority of surviving exocrine cells present in the DNMT1^{PC} pancreas are did not undergo recombination, with a few exceptions (D, white arrows). The recombination rate in control littermates heterozygous for *Dnmt1* was high (C).

Figure 3-S3

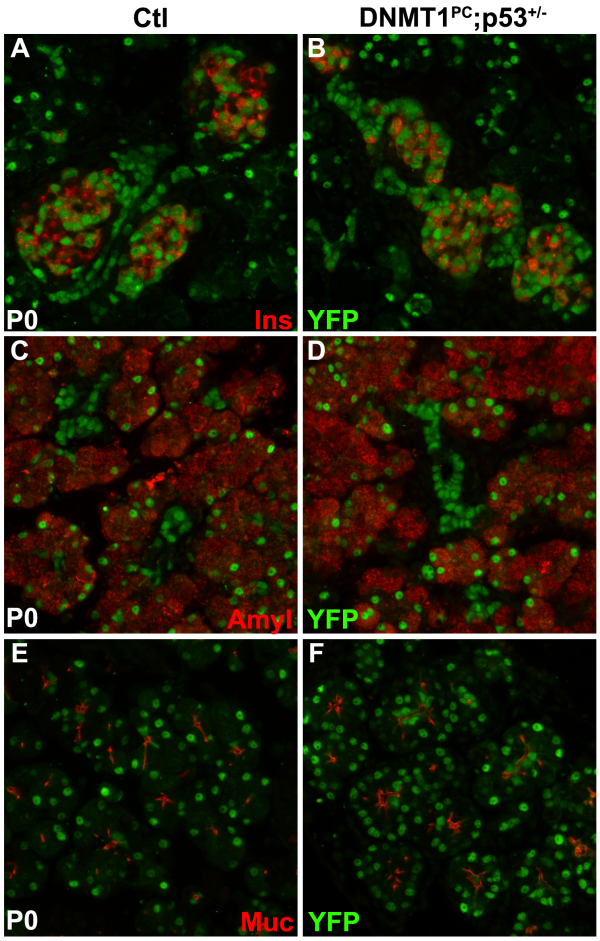


B GSEA Identifed Pancreatic Develloment Genes with > 2X Change

Gene Name	GenBank Acc. No.	Fold Change DNMT1^{PC} to Ctl
Neurogenin 3	NM_009719	-12.63
Onecut1/HNF6	BC023444	-4.23
Rbpjl	NM_009036	-3.41
Ptf1a	NM_018809	-2.96
Nkx2.2	NM_010919	-2.92
Nkx6.1	NM_144955	-2.55
Pdx1	NM_008814	-2.42
Rfx6	NM_001159389	-2.04

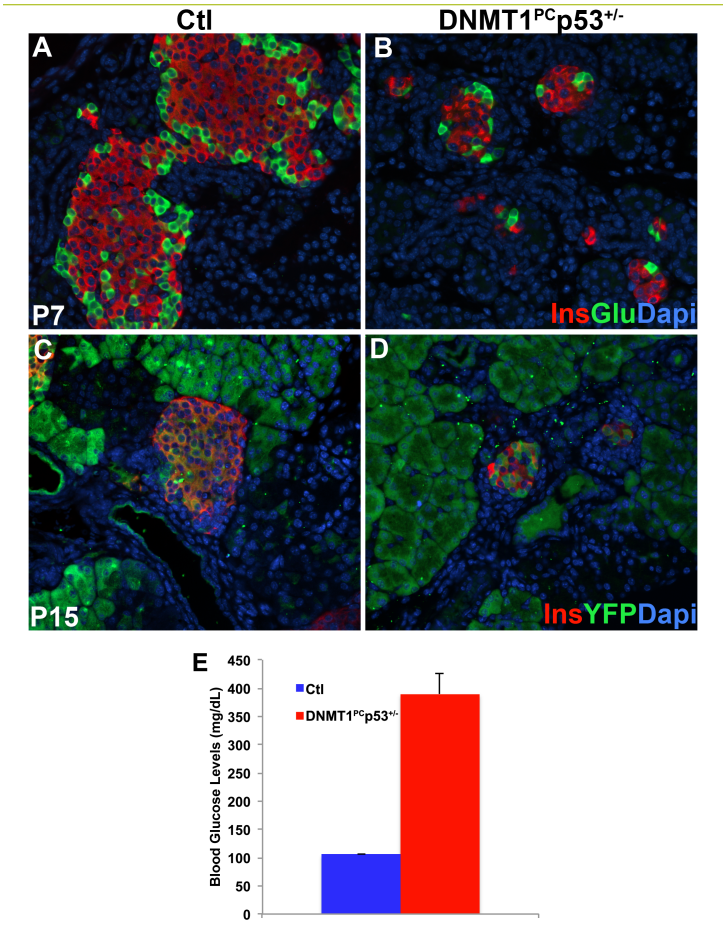
Supplementary Figure 3-S3: Changes in gene expression suggest DNMT1^{PC} cells do not complete terminal differentiation. (A) Comparison of probe intensities on expression microarrays. Dots that lie outside of the dotted lines represent a gene that has more than a two-fold change between the control and DNMT1^{PC} microarray data. (B) Gene set enrichment analysis indicates that genes that determine pancreatic endocrine and exocrine lineages are downregulated.

Figure 3-S4



Supplementary Figure 3-S4: Haploinsufficency of *p53* rescues the ability of DNMT1^{PC};p53$^{+/-}$ progenitor cells to undergo terminal differentiation. (A-B) Immunohistochemical analysis of insulin and YFP staining indicates that clusters of insulin cells derived from DNMT1^{PC};p53$^{+/-}$ progenitor cells are present at P0. (C-D) Immunohistochemical analysis of amylase expression, a marker for exocrine tissue, reveals that terminal exocrine lineage differentiation does proceed in the DNMT1^{PC};p53$^{+/-}$ pancreas. (E-F) The presence of mucin by immunohistochemistry suggests that ductal structures of the pancreas at P0 are comparably intact in the control (E) and DNMT1^{PC};p53$^{+/-}$ pancreas.

Figure 3-S5



Supplementary Figure 3-S5: DNMT1PC;p53+/- islet mass is unable to sustain glucose homeostasis during postnatal development. (A-B) Immunohistochemical analysis of insulin and glucagon staining indicates that islet clusters are diminished in the DNMT1PC;p53+/- pancreas at P7. (C-D) Immunohistochemical analysis of YFP, a marker recombination, and insulin reveals that most of the remaining insulin cells did not undergo recombination in the diminished islet at E15. (E) Analysis of nonfasting blood glucose levels reveals that DNMT1PC;p53+/- animals become diabetic prior to weaning.

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Su CH, Shann YJ, Hsu MT. 2009. p53 chromatin epigenetic domain organization and p53 transcription. *Molecular and cellular biology* **29**: 93-103.

Tschen S-I, Dhawan S, Gurlo T, Bhushan A. 2009. Age-dependent Decline in Beta Cell Proliferation restricts the Capacity of Beta Cell Regeneration in Mice. *Diabetes*: db08-1651.