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MicroRNA Expression is Required for Pancreatic Islet Cell Genesis in the Mouse

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Running Title: Regulation of β-cell genesis by miRNA

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Abstract:

Objective: The generation of distinct cell types during the development of the pancreas depends on sequential changes in gene expression. We tested the hypothesis that microRNAs (miRNAs), which limit gene expression through post-transcriptional silencing, modulate the gene expression cascades involved in pancreas development.

Research Design and Methods: MiRNAs were cloned and sequenced from developing pancreas and expression of a subset of these genes was tested using LNA *in situ* analyses. To assess the overall contribution of miRNAs to pancreatic development, Dicer1, an enzyme required for miRNA processing, was conditionally deleted from the developing pancreas.

Results: Sequencing of small RNAs identified over 125 miRNAs, including 18 novel sequences, with distinct expression domains within the developing pancreas. To test the developmental contribution of these miRNAs, we conditionally deleted the miRNA processing enzyme *Dicer1* early in pancreas development. Dicer null animals displayed gross defects in all pancreatic lineages, although the endocrine cells, and especially the insulin-producing β-cells, were most dramatically reduced. The endocrine defect was associated with an increase in the notch-signaling target Hes1 and a reduction in the formation of endocrine cell progenitors expressing the Hes1 target gene neurogenin3.

Conclusions: The expression of a unique profile of miRNAs is required during pancreas development and is necessary for β-cell formation.

Pancreatic organogenesis begins at e9.5 in the mouse embryo with the budding of the dorsal anlagen from the prospective gut endoderm (1). Pancreatic duodenal homeobox-1 (Pdx1) appears in the same area one day earlier, and is expressed in all pancreas progenitors (2; 3). As the pancreatic program continues, the expression of bHLH factor neurogenin3 initiates the differentiation of the endocrine cells. Neurogenin3 expression peaks coincidently with the "secondary transition" – a time of rapid endocrine cell generation that occurs between e13.5 and e14.5 (4; 5). The cell-specific factors *Onecut1*, *Tcf1, Tcf2* and *Sox9* as well as the Notch signaling pathway regulate *Neurog3* expression during development (5-9).

Many tissues of the developing organism express miRNAs, which are small (~20 nt) RNAs that mediate post-transcriptional gene silencing by interacting with the RNA-induced silencing complex (RISC) and binding to the 3' untranslated region of their cognate mRNA targets (10; 11). Here we hypothesize that miRNAs are expressed in the developing pancreas, that they are necessary for the normal development of pancreatic β-cells and that they may be involved in regulating genes important for normal pancreas morphogenesis. Ultimately, an intimate understanding of how miRNAs govern pancreas development may be necessary for stem cell based therapies for all forms of *diabetes mellitus*.

Research Design and Methods:

Animals

Mice were housed on a 12-hr light-dark cycle in a controlled climate according to the University of California, San Francisco regulations. Timed matings were carried out with embryonic day 0.5 being set as midday of the day of discovery of a vaginal plug. Control mice in this study were double heterozygous for the *Dicer1* (12) and *Pdx1*-Cre (3) alleles. CD-1 mice were obtained from Charles River Laboratories (Wilmington MA, USA).

Cloning of Small RNAs from the Developing Mouse Pancreas

118 embryos from 10 CD-1 mice were removed from pregnant mothers at e14.5 and embryonic pancreata was dissected and isolated from the spleen and other surrounding tissue. RNA was then isolated using Trizol and the manufacturer's protocols (Invitrogen, Carlsbad CA, USA) with the exception that the RNA pellet was not washed with 70% ethanol following isopropanol precipitation. We obtained 189 μg of RNA from 118 pancreata. MiRNA cloning was carried out as previously described by Lau et al (13); briefly, linkers were then ligated onto the 3' and 5'-ends of size fractionated RNA, RNA was reverse-transcribed and amplified using PCR. The PCR products were restriction digested with BanI, concatermized with T4 DNA Ligase, and gel-purified fragments ranging from 600-800bp were TOPO cloned (Invitrogen). Plasmid DNA was isolated from 100 colonies and sequenced using M13 primers. Sequences were deconvolved using a Java applet that is available on request. Sequences were then aligned against the miRNAome (http://microrna.sanger.ac.uk/sequences/) and unknown sequences were aligned against genome. Regions of high sequence homology were then folded using RNAshapes (14) and those that

folded into hairpin loops were considered miRNAs.

Immunofluorescence analysis

Embryonic tissue was harvested and immediately fixed at 4°C with 4% paraformaldehyde. Tissues were then dehydrated through 50, 70, 95, and 100% ethanol and then through xylene. Tissues were then impregnated with paraffin (Paraplast; Fisher Life Sciences) at 60 °C under vacuum, and then embedded in cassettes. Tissue was then cut in 5 μM sections and adhered to glass slides (Superfrost Plus; Fisher). For immunoanalyses, tissue was first deparafinized with xylene and the rehydrated through a reverse ethanol series to ddH_20. Antigen retrieval was then carried out by microwave in a citrate buffer (Biogenex, San Ramon CA, USA). Tissues were then blocked at room temperature with 5% goat serum and goat anti-mouse IgG (1:30, MP Biomedicals/Cappel, Solon OH, USA) in PBS. Incubations with the primary antibodies were performed overnight at 4°C using standard techniques in PBS containing 1% goat serum with the following primary antisera: 1:2000 guinea pig anti-pdx-1 (15), 1:3000 guinea pig anti-glucagon (Linco, St Charles, MO), 1:4000 mouse anti-glucagon (Sigma-Aldrich, St Louis MO, USA), 1:3000 guinea pig anti-insulin (Linco), 1:2000 guinea pig anti-neurogenin3 (15), 1:200 hamster anti-mucin (NeoMarkers, Fremont, CA), 1:200 mouse anti-synaptophysin (Biogenex), 1:1000 rabbit anti-amylase (Sigma) 1:1000 rabbit anti-Hes1 (a gift from T Sudo, Toray Scienfic, Japan (16)) 1:150 rabbit anti-activated Caspase-3 (Cell Signalling, Danvers MA, USA) 1:200 rabbit anti-*MKi67* (Novocastra, Newcastle upon Tyne, UK) or 1:100 rabbit anti-Dicer (Santa Cruz Biotechnology) . Secondary antibodies were obtained from Jackson Immunoresearch (West Grove PA, USA) and used at 1:200 (FITC) to 1:400 (Cy3) dilutions and coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA). Slides were imaged on a Leica TCS SL Confocal microscope using sequential scanning of dyes. Morphometric analyses were done by serially sectioning the entire pancreas followed by staining and counting every 12th section for e18.5 embryos or every 8th section for e15.5 embryos. DAPI stained cell nuclei were counted using the cell counting application and cytoplasmic staining was quantified using the integrated metamorphic analysis: both in the Metamorph suite of programs (version 7, Molecular Devices; Union City, CA). Statistical analyses were done using Prism (version 4, Graphpad Software; San Diego, CA) and either T-tests or non-parametric Mann-Whitney U tests were used. P values of < 0.05 were considered statistically significant.

Locked Nucleic Acid In Situ Hybridization

Embryos (e14.5) were harvested and fixed overnight at 4°C in 4% paraformaldehyde. Following fixation, embryos were briefly washed with PBS and transferred to 30% sucrose in DEPC-treated PBS and kept for an additional 24 hours at 4°C. Embryos were then mounted in Tissue-Tek O.C.T (Sakura Finetek, Torrance CA, USA) and frozen on dry ice. Embryos were sectioned with a cryostat into 10 μm sections, adhered to slides (Superfrost, Fisher Scientific) and slides were kept at -80°C until use. Slides were removed from freezer and dried for 1 hour at room temperature. Sections were then re-fixed for 10 minutes at room temperature in 4% paraformaldehyde and then washed with RNAse-free PBS.

Slides were then incubated for 2 minutes with 1 μg/ml proteinase K (Roche Applied Science, Indianapolis IN, USA) in 50 mM Tris pH 7.5 containing 5 mM EDTA. Slides were then washed again in PBS and acetylated (1.2% triethanolamine, 0.018N HCl, 0.25% acetic anhydride) for 10 minutes at room temperature. Slides were then washed again before prehybridization in 50% formamide, 5x SSC, 0.1% Tween-20, 9.2 mM citric acid, 50 μg/ml heparin, 500 μg/ml yeast tRNA for 2 hours at 60°C. Antisense locked nucleic acid (LNA) probes were ordered from Exiqon (Vedbaek, Denmark) and were subsequently labeled using the DIG Oligonucleotide 3'-End Labeling kit (Roche Applied Science) and the manufacturer's protocol. Labeled LNAs were then diluted to 50 μl with RNAse-free water and separated from unincorporated label using Microspin G-25 columns (Amersham Biosciences, Piscataway NJ, USA). 1 pmol of DIG-labeled LNA was then diluted into 250 μl of hybridization buffer, briefly heated to 80°C and the iced. Labeled probes were then applied to the slides and allowed to hybridize overnight at 60°C. Slides were then washed for 3 hours at 60°C in 0.2x SSC and then incubated with alkaline phosphatase conjugated sheep anti-DIG antibody (Roche Applied Sciences) in 100 mM Tris, pH 7.5, 150 mM NaCl with 1% Goat Serum (Invitrogen) overnight at 4°C. Slides were then washed extensively in the aforementioned buffer and then equibrated with 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl2 prior to alkaline phosphatase reaction in the same buffer with 1:50 dilution of NBT/BCIP Stock (Roche Applied Sciences) for 1-3 days. For *Pdx1* staining, sections were then washed with PBS, endogenous peroxidases were quenched with a 15 min incubation in methanol containing 0.6 % hydrogen peroxide. Slides were then incubated with 1:3000 dilution of guinea pig anti-Pdx1 overnight at 4°C. Slides were the washed and incubated with 1:200 dilution of biotinylated goat anti-guinea pig secondary antibody (Vector Labs, Burlingame CA, USA). Slides were then incubated with ABC solution and then reacted with diaminobenzidine (VECTASTAIN, Vector Labs). Sections were dehydrated, coverslips mounted and imaged on a Zeiss Axio Imager.A1 equipped with an AxioCam MrC5 and using Zeiss AxioVision software (Thornwood NY, USA).

Quantitative PCR

Embryonic pancreas were harvested at e12.5 and immediately placed in 0.5ml of Trizol (Invitrogen) and RNA was extracted using the manufacturer's protocols. 250ng of total RNA was subjected to reverse transcription using random hexamers, Superscript III (Invitrogen) and the manufacturer's suggested reaction conditions. Complementary DNA was then amplified in quadruplicate using Taqman with an intron-spanning primer-probe set for *Hes1* (Forward, AAG GCA GAC ATT CTG GAA ATG AC; Reverse, CGC GGT ATT TCC CCA ACA C; Probe, AGA TGA CCG CCG CGC TCA GC). *Hes1* expression was normalized to Taqman quantified mouse *glucuronidase* expression in the individual pancreatic cDNA samples (17).

Results:

MicroRNA are expressed in the developing pancreas*:*

To determine their expression profile and identify novel miRNAs, small RNAs were cloned and sequenced from the pancreata of e14.5 mouse embryos

using standard miRNA cloning and sequencing protocols (13). In total, 107 known mouse miRNAs were cloned: 5 of which are evolutionarily conserved but had not previously been found in the mouse (Table 1). Unknown sequences were then aligned with the genome and the surrounding, conserved genomic regions were folded into hairpins (Supplemental Table 1 available at http://diabetes.diabetesjournals.org).
Using this approach, 18 novel mouse miRNAs were discovered, including some that lie in potential multicystronic miRNA transcripts (Supplemental Table 1). In addition, significant differences in a number of miRNA sequences were observed from those annotated in miRBase (18). For example, multiple copies of mmu-miR-720 and mmu-miR-411 differed significantly from the sequences in miRBase (Table 1). Differences identified in the 5' end of several miRNA are of particular significance, because the majority of miRNA target prediction algorithms rely on Watson-Crick base pairing of nucleotides 2 through 7 from the 5'-end of the miRNA; thus, 1 or 2 base pair differences at the 5' end could completely change the target profile of any miRNA (19; 20).

Many of the miRNAs that were cloned here have not been previously detected at high levels in other tissues; therefore, we hypothesized that they might play specific roles in pancreas development. To assess the expression domain of these miRNAs during pancreas development, *in situ* hybridization with highly-specific locked-nucleic acid (LNA) probes was carried out for a few of these highly expressed miRNAs (Figure 1) (21). We first tested mmu-miR-375 because it was overrepresented in our cloning experiment (Table 1) and has previously been demonstrated to be expressed in, and important for, pancreatic β-cell function. *In situ* analyses showed localization of miR-375 in the pancreatic ductal epithelium (*i.e.* the progenitor cells from which the endocrine and exocrine cells differentiate) at e14.5 (Figures 1A&B). Furthermore**,** Figure 1B demonstrates co-localization of miR-375 with Pdx1 in the e14.5 pancreas; thus, in addition to regulating insulin secretion in mature β-cells (22), miR-375 expressed in the progenitor cells may also be important during normal development. We also performed *in situ* analyses for three other overrepresented miRNAs. Both miR-503 and miR-541 were expressed in a similar pattern to miR-375 in progenitor cells in e14.5 pancreas, while miR-214 was expressed throughout the gut (Figures 1C-1H).

MicroRNA expression is necessary for pancreas organogenesis

To determine the importance of miRNAs in pancreatic development, the second RNAse III domain of *Dicer1* (3; 12) was conditionally ablated in all pancreatic cells starting at e9.5 using *Pdx1*-Cre-mediated deletion (3). Biogenesis of miRNAs requires the sequential activity of two ds-RNA-specific endonucleases, Drosha and Dicer1, that act in the nucleus and cytosol respectively (23). In mammals, removal of the pre-miRNA hairpin loop by a single Dicer homolog is a prerequisite for interaction of the mature sequence with RISC. Therefore, loss of the RNAse III domain of *Dicer1* blocks the formation of miRNAs (12; 24).

The pancreas-specific *Dicer1* knockout mice survived until birth but failed to grow and died by P3 (Figures 2A&B). Pancreata from e18.5 null mice had a dramatic reduction in the ventral

pancreas, as well as a reduction in the overall epithelial contribution to the dorsal pancreas (Figure 2D). The remaining tissue in the *Dicer1* null pancreata was comprised of clumps of disorganized epithelium interspersed with large vacuous areas and dilated ducts filled with impactions of debris (Figures 2F&H).

Early pancreatic miRNA expression is necessary for normal pancreatic cell differentiation

Cell-type specific antisera were utilized to determine the effects of Dicer1 loss on specific pancreatic lineages. Co-staining of the e18.5 pancreas for synaptophysin, an endocrine marker, and the exocrine enzyme amylase revealed reductions in both the number of stained cells and the intensity of staining for both markers (Figures 3A&B). Immunoreactivity for the duct marker mucin1 remained unchanged in the knockout animal at this stage, but the debris filling the dilated ducts stained strongly for both mucin and amylase (arrow heads; Figures 3B&J). Staining for specific hormones demonstrated a significant loss (94%; Figure 3K) of insulin-producing β–cells in the knockout animals and 79%, 95%, and 86% losses of alpha, delta and PP cells respectively (Figure 3K), but no compensatory increase in ghrelin producing ε-cells (Figures 3C-J) (25). The differences in the reductions of the various endocrine cell types were significant, as demonstrated by significant changes in the ratios of distinct endocrine cell types in the *Pdx-Cre Dicer1*$^{flox/flox}$ relative to their wildtype littermates (Figures 3L). Pdx1 immunoreactivity, which is restricted predominantly to β–cells at this stage, was also decreased within e18.5 knockout pancreases (*cf.* Figures 3E&F).

To distinguish between a role in β-cell genesis and in maintenance or proliferation of mature beta-cells, we also specifically removed *Dicer1* from differentiated β-cells by crossing the *Dicer1*^{flox} mice with mice carrying a *Cre* allele driven by the rat *insulin 2* (RIP2) promoter (26) (Figure 4). β-cell-specific Dicer1 knockout animals survived and their islets cells appeared morphologically normal at 8 months of age (*c.f.* Figures 4A&B). In addition, to exclude the possibility that Dicer1 loss specifically impacts the maintenance or proliferation of mature β-cells in the embryo, β-cell counting was performed also at e18.5. Control and RIP2-Cre Dicer$^{flox/flox}$ animals at this developmental stage contained equal numbers of insulin-immunoreactive β-cells (Figure 4C). Finally we also conditionally ablated *Dicer1* using a Neurogenin3-Cre transgene, which causes recombination in all endocrine cells. These animals also exhibit normal islet-cell hormone staining at e18.5 (data not shown).

To identify the origins of the Pdx1-mediated Dicer deletion phenotypes that were observed at e18.5, earlier time points were examined. At e12.5 staining for Pdx1 and glucagon - markers of undifferentiated pancreatic epithelial progenitors and the earliest differentiated cells in the pancreas respectively - was not different between the mutant and wild-type pancreata (Figures 5A&B). However, by e15.5 knockout embryos lacked Dicer1 immunoreactivity in the Pdx1 expressing cells (Supplemental Figure 1) and had significant reductions in pancreatic size and ductal branching (Figures 5C-H). At this stage in development, Pdx1 antisera weakly stains the majority of cells lining the ducts and strongly stains the nascent β-cells, and neurogenin3 expression marks the

committed endocrine progenitors. In the knockout animals, a decrease in the number of cells staining brightly for Pdx1 (Figure 5D) and a significant, 89%, reduction in neurogenin3 expressing cells was observed (Figures 5D,F,H&I), indicating a defect in the endocrine differentiation program. Ductal Pdx1 and mucin1 staining was not grossly altered in the *Dicer1* null animals at e15.5 (Figures 4D,H).

MicroRNAs regulate the notch pathway and prevent programmed cell death during pancreatic development

Notch signaling inhibits the expression of neurogenin3 and thereby constrains endocrine differentiation in the developing pancreas in a manner analogous to lateral inhibition in the developing nervous system(5; 27; 28). The notch target gene *Hes1* is expressed in the majority of early pancreatic epithelial cells and strongly represses *Neurog3* gene transcription within those cells(8; 16). We hypothesized that specific miRNAs may activate endocrine differentiation by inhibiting components of the Notch signaling pathway: thereby relieving the constraint on neurogenin3 expression. We tested this hypothesis by assessing *Hes1* expression using Taqman and found a modest increase in *Hes1* mRNA (35%) at e12.5 (Figure 6K). Because Hes1 is expressed in the pancreatic mesenchyme as well as the Pdx1-expressing epithelium, and because Hes1 may be a target for translational regulation, we also stained e12.5 pancreas for Hes1 and observed an increase in Hes1 expression specifically within the Pdx1 expression domain in *Dicer1* deficient pancreas (Figure 6).

In addition to the loss of neurogenin3, starting at e15.5 there was an overall decrease in cells derived from the pancreatic epithelium in the knockout animals (Figure 2 and data not shown). Since miRNAs can regulate apoptosis during organ development (12; 29; 30), rates of programmed cell death and cell proliferation were assessed in the pancreatic specific *Dicer1* knockout animal, using specific antisera for activated Caspase 3 and Ki-67 (*MKi67*) respectively. A clearly significant increase (4.6-fold) was observed in total apoptotic cells at e15.5 in pancreases from knockout animals, and this increase appeared to be limited to the epithelial cells, as apoptosis was unchanged in the surrounding mesenchymal cells, which still expressed Dicer (Figures 6H,I&J). Cell proliferation was unchanged (Supplemental Figure 2; counting data not shown).

Discussion:

Here we demonstrated that the developing pancreas expresses a unique complement of miRNAs and that Dicer1, the enzyme that generates these miRNAs, is essential for normal pancreatic development. Many of the miRNAs that were highly expressed in the developing pancreas have not been previously cloned in high numbers or from specific tissues (*e.g.* miR-503 & miR-541). High expression in the developing pancreas could suggest that these miRNAs play important roles in the developmental program; although, to date we have not identified any targets of these genes with known roles in pancreas development. In contrast, the novel miRNAs identified in this study all had fairly low expression levels as gauged by the number of clones obtained. Despite their low overall abundance, these miRNAs could still play important roles at specific target genes or in cell types at low abundance in the e14.5 pancreas.

Although exocrine and duct cells were also affected, loss of miRNA processing uniquely impaired the development of the endocrine lineage, especially the β- and δ-cells. Two lines of evidence suggest that miRNAs are important for β-cell genesis rather than β-cell maintenance. First, a dramatic reduction in the number of neurogenin3 positive endocrine progenitor cells from which all β-cells are derived was observed at e15.5. Consistent with a reduction in islet cell progenitors, glucagon, somatostatin and pancreatic polypeptide expressing endocrine cells were also reduced, although ghrelin expressing cells were not. The relatively less severe α-cell phenotype may be solely a timing issue, as Pdx1-mediated loss of Dicer1 may not be complete until after the first glucagon positive cells differentiate, and Pdx1 null animals do have glucagon positive cells (31). Alternatively, miRNAs may act downstream of neurogenin3 to influence cell fate decisions, and in the absence of miRNAs the few remaining neurogenin3-expressing progenitors may preferentially differentiate into α- and ε-cells.

Secondly, when *Dicer1* was ablated using the *RIP2-Cre (*or the *Neurog3-Cre)* transgene (Figure 4) we saw little effect on pancreatic islet cell morphology and no apparent effect on β-cell development (Figure 4C) or β-cell maintenance. A previous study demonstrated that miR-375 normally constrains β-cell insulin secretion in a mytrophin dependent fashion (22). Our data here focused on the role of miRNAs in the formation of β-cells are not at odds with these previous data on islet function. We have shown that islet and β-cell development remains unaffected in the *RIP2-Cre Dicer1*$^{flox/flox}$ mice, but we have not directly tested the glucose-stimulated insulin secretory response of these islets. It is possible that there could be a functional defect in the islets of these animals, although given the proposed inhibitory role of miR-375 on insulin secretion such defects might not result in glucose intolerance or overt diabetes. Furthermore, glucose metabolism defects might be confounded by the expression of the *RIP2-Cre* transgene in cells in the hypothalamus that also impact glucose metabolism(26).

The large number of miRNAs expressed in the developing pancreas, and the large number of potential targets for each of these complicate the task of identifying the miRNAs that normally maintain neurogenin3 expression and endocrine cell generation, but the increase in the neurogenin3 inhibitor Hes1 suggests that Hes1 or upstream genes in the notch signaling pathway could be miRNA targets in pancreatic progenitor cells. Previous studies have implicated Hes1 protein as a target of miR-23a (32; 33), and miR-23a was expressed in the developing pancreas (Table1) (Although, this paper was withdrawn because the authors mistakenly used the wrong gene sequence for their analyses, the observation that miR-23a can influence the expression of Hes1 remains a possibility from this publication). In addition, it seems likely that miRNA may target other notch pathway components upstream of Hes1, yielding an increase in Hes1 expression in the absence of Dicer1.

In summary, these data demonstrate for the first time that a unique set of miRNAs emerge during pancreas development and regulate pathways important for normal ductal, exocrine and endocrine development. Exploration of the roles of individual miRNA will provide a more complete understanding of pancreatic development and the differentiation of the distinct cell types,

especially β–cells, and help in developing methods for generating these cells for therapeutic uses.

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References:

1. Pictet RL, Rutter WJ: Development of the embryonic pancreas. In *Handbook of physiology* Steiner DF, Freinkel N, Eds. Washington DC, American Physiological Society, 1972, p. 25-66

2. Jonsson J, Carlsson L, Edlund T, Edlund H: Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* 371:606-609, 1994

3. Gu G, Dubauskaite J, Melton DA: Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* 129:2447-2457, 2002

4. Gradwohl G, Dierich A, LeMeur M, Guillemot F: neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* 97:1607-1611, 2000

5. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund H: Notch signalling controls pancreatic cell differentiation. *Nature* 400:877-881, 1999

6. Jacquemin P, Durviaux SM, Jensen J, Godfraind C, Gradwohl G, Guillemot F, Madsen OD, Carmeliet P, Dewerchin M, Collen D, Rousseau GG, Lemaigre FP: Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. *Mol Cell Biol* 20:4445-4454, 2000

7. Boj SF, Parrizas M, Maestro MA, Ferrer J: A transcription factor regulatory circuit in differentiated pancreatic cells. *Proc Natl Acad Sci U S A* 98:14481-14486, 2001

8. Lee JC, Smith SB, Watada H, Lin J, Scheel D, Wang J, Mirmira RG, German MS: Regulation of the pancreatic pro-endocrine gene neurogenin3. *Diabetes* 50:928-936, 2001

9. Lynn FC, Smith SB, Wilson ME, Yang KY, Nekrep N, German MS: Sox9 coordinates a transcriptional network in pancreatic progenitor cells. *Proc Natl Acad Sci U S A* 104:10500-10505, 2007

10. Ambros V: The functions of animal microRNAs. *Nature* 431:350-355, 2004

11. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297, 2004

12. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ: The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci U S A* 102:10898-10903, 2005

13. Lau NC, Lim LP, Weinstein EG, Bartel DP: An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* 294:858-862, 2001

14. Steffen P, Voss B, Rehmsmeier M, Reeder J, Giegerich R: RNAshapes: an integrated RNA analysis package based on abstract shapes. *Bioinformatics* 22:500-503, 2006

15. Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS: Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* 127:3533-3542, 2000

16. Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD: Control of endodermal endocrine development by Hes-1. *Nat Genet* 24:36-44, 2000

17. Gasa R, Mrejen C, Leachman N, Otten M, Barnes M, Wang J, Chakrabarti S, Mirmira R, German M: Proendocrine genes coordinate the pancreatic islet differentiation program in vitro. *Proc Natl Acad Sci U S A* 101:13245-13250, 2004

18. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ: miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 34:D140-144, 2006
19. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: Prediction of mammalian microRNA targets. *Cell* 115:787-798, 2003
20. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N: Combinatorial microRNA target predictions. *Nat Genet* 37:495-500, 2005
21. Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH: In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* 3:27-29, 2006
22. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M: A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* 432:226-230, 2004
23. Kim VN: MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 6:376-385, 2005
24. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ: Dicer is essential for mouse development. *Nat Genet* 35:215-217, 2003
25. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* 101:2924-2929, 2004
26. Gannon M, Shiota C, Postic C, Wright CV, Magnuson M: Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. *Genesis* 26:139-142, 2000
27. Murtaugh LC, Stanger BZ, Kwan KM, Melton DA: Notch signaling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci U S A* 100:14920-14925, 2003
28. Hald J, Hjorth JP, German MS, Madsen OD, Serup P, Jensen J: Activated Notch1 prevents differentiation of pancreatic acinar cells and attenuate endocrine development. *Dev Biol* 260:426-437, 2003
29. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K: Aberrant T cell differentiation in the absence of Dicer. *J Exp Med* 202:261-269, 2005
30. Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'Connor E, Godwin J, Wilson CB, Brockdorff N, Fisher AG, Smale ST, Merkenschlager M: T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med* 201:1367-1373, 2005
31. Ahlgren U, Jonsson J, Edlund H: The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* 122:1409-1416, 1996
32. Kawasaki H, Taira K: Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. *Nature* 423:838-842, 2003
33. Kawasaki H, Taira K: Retraction: Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. *Nature* 426:100, 2003

Table Legend

Table 1: The miRNA profile of the developing mouse pancreas.
Total RNA (185 μg) was extracted from 118 e14.5 mouse pancreata. Small RNA was isolated, cloned and analyzed as described. Out of 1012 sequences obtained, 835 were miRNAs. The notes column reflects differences from annotated miRNA sequences.

Table 1: The miRNA profile of the developing mouse pancreas.

miRNA name	e14.5 cloning sequence	x cloned	Notes
mmu-Let-7a	TGAGGTAGTAGGTTGTATAGTT	63	
mmu-Let-7b	TGAGGTAGTAGGTTGTGTG	3	
mmu-Let-7c	TGAGGTAGTAGGTTGTATGG	19	
mmu-Let-7d	GAGGTAGTAGGTTGCATAGT	75	
mmu-Let-7e	TGAGGTAGGAGGTTGTATAGT	8	
mmu-Let-7i	TGAGGTAGTAGTTTGTGCTG	1	
mmu-miR-7	TGGAAGACTAGTGATTTTGTTG	11	
mmu-miR-10a	TACCCTGTAGATCCGAATTTGT	7	
mmu-miR-15b	TAGCAGCACATCATGGTTTACA	7	
mmu-miR-16	TAGCAGCACGTAAATATTGGCG	13	
mmu-miR-17-3p	ACTGCAGTGAGGGCACTTGTAG	3	
mmu-miR-17-5p	CAAAGTGCTTACAGTGCAGGCTAG	6	
mmu-miR-18	TAAGGTGCATCTAGTGCAGATA	4	
mmu-miR-19b	TGTGCAAATCCATGCAAAACTGA	7	
mmu-miR-20a	TAAAGTGCTTATAGTGCAAGTAG	4	
mmu-miR-21	TAGCTTATCAGACTGATGTTGA	2	
mmu-miR-23a	ATCACATTGCCAGGGATTTCCAA	1	
mmu-miR-23b	ATCACATTGCCAGGGATTACC	2	
mmu-miR-24	TGGCTCAGTTCAGCAGGAACAG	6	
mmu-miR-25	CATTGCACTTGTCTCGGTCTGA	5	
mmu-miR-26b	TTCAAGTAATTCAGGATAGGTT	19	
mmu-miR-27a	TTCACAGTGGCTAAGTTCCGC	3	
mmu-miR-29b	TAGCACCATTTGAAATCAGTGTT	2	
mmu-miR-30a-3p	CTTTCAGTCGGATGTTTGCAGC	4	
mmu-miR-30a-5p	TGTAAACATCCTCGACTGGAAG	2	
mmu-miR-30b	TGTAAACATCCTACACTCAGCT	1	
mmu-miR-30c	TGTAAACATCCTACACTCTCAGCT	2	
mmu-miR-31	AGGCAAGATGCTGGCATAGCTGT	1	
mmu-miR-92-1	TATTGCACTTGTCCCGGCCTGT	4	
mmu-miR-93	CAAAGTGCTGTTCGTGCAGGTAG	2	
mmu-miR-99b	CACCCGTAGAACCGACCTTGCG	8	
mmu-miR-100	AACCCGTAGATCCGAACTTGTG	3	
mmu-miR-101a	TACAGTACTGTGATAACTGAAG	1	
mmu-miR-101b	TACAGTACTGTGATAGCTGAAG	1	
mmu-miR-106b	TAAAGTGCTGACAGTGCAGATA	3	
mmu-miR-107	AGCAGCATTGTACAGGGCTATCA	3	
mmu-miR-122a	TGGAGTGTGACAATGGTGTTTGT	5	
mmu-miR-125a	TCCCTGAGACCCTTTAACCTGT	5	
mmu-miR-125b	TCCCTGAGACCCTAACTTGTGAA	23	
mmu-miR-126-3p	TCGTACCGTGAGTAATAATGCG	35	
mmu-miR-126-5p	CATTATTACTTTTGGTACGCG	9	
mmu-miR-127	TCGGATCCGTCTGAGCTTGGC	7	
mmu-miR-130a	CAGTGCAATGTTAAAAGGGCAT	14	
mmu-miR-134	TGTGACTGGTTGACCAGAGGGG	5	
mmu-miR-136	ACTCCATTTGTTTTGATGATGGA	25	
mmu-miR-138	AGCTGGTGTTGTGAATCAGGCCG	1	

mmu-miR-140	CAGTGGTTTTACCCTATGGTAG	4	
mmu-miR-143	TGAGATGAAGCACTGTAGCTCA	13	
mmu-miR-154	TAGGTTATCCGTGTTGCCTTCG	2	
mmu-miR-181a	ACATTCAACGCTGTCGGTGAGT	2	
mmu-miR-181b-2	AACATTCATTGCTGTCGGTGGGTTT	4	
mmu-miR-191	CAACGGAATCCCAAAAGCAGCT	9	
mmu-miR-199a	CCCAGTGTTCAGACTACCTGTTC	11	
mmu-miR-199a*	TACAGTAGTCTGCACATTGGTTAA	13	
mmu-miR-200a	TAACACTGTCTGGTAACGATGTT	7	
mmu-miR-200b	TAATACTGCCTGGTAATGATGAC	3	
mmu-miR-200c	TAATACTGCCGGGTAATGATGGA	12	
mmu-miR-203	GTGAAATGTTTAGGACCACTAG	3	5' G
mmu-miR-210	CTGTGCGTGTGACAGCGGCTGA	2	
mmu-miR-214	ACAGCAGGCACAGACAGGCAG	32	
mmu-miR-218	TTGTGCTTGATCTAACCATGT	4	
mmu-miR-222	AGCTACATCTGGCTACTGGGTCTC	2	
mmu-miR-297	ATGTATGTGTGCATGTGCATGT	1	
mmu-miR-298	GGCAGAGGAGGGCTGTTCTTCCCT	4	3' CT
mmu-miR-299	TGGTTTACCGTCCCACATACAT	1	
mmu-miR-299*	TATGTGGGACGGTAAACCGCT	1	
mmu-miR-301	CAGTGCAATAGTATTGTCAAAGCAT	7	5' AT
mmu-miR-322	AAAACATGAAGCGCTGCAACA	4	5' A
mmu-miR-324-3p	CCACTGCCCCAGGTGCTGCTGG	2	
mmu-miR-324-5p	CGCATCCCCTAGGGCATTGGTG	3	
mmu-miR-329	AACACACCCAGCTAACCTTTTT	1	
mmu-miR-331	GCCCCTGGGCCTATCCTAGAA	4	
mmu-miR-335	TCAAGAGCAATAACGAAAAATGT	14	
mmu-miR-342	TCTCACACAGAAATCGCACCCGT	7	
mmu-miR-345	TGCTGACCCCTAGTCCAGTGC	1	
mmu-miR-351	TCCCTGAGGAGCCCTTTGAGCCT	3	
mmu-miR-369-3p	AATAATACATGGTTGATCTT	7	
mmu-miR-369-5p	AGATCGACCGTGTTATATTCGC	2	
mmu-miR-370	GCCTGCTGGGGTGGAACCTGGTT	3	
mmu-miR-375	TTTGTTCGTTCGGCTCGCGTGA	48	
mmu-miR-376a	ATCGTAGAGGAAAATCCACGT	12	
mmu-miR-376c	AACATAGAGGAAATTTCACG	12	
mmu-miR-379	TGGTAGACTATGGAACGTAGG	14	
mmu-miR-382	GAAGTTGTTCGTGGTGGATTCG	4	
mmu-miR-409	AATGTTGCTCGGTGAACCCCTT	3	5' G-
mmu-miR-410	AATATAACACAGATGGCCTGTT	3	
mmu-miR-411	TATGTAACACGGTCCACTAAC	17	
mmu-miR-422b	CTGGACTTGGAGTCAGAAGGCC	2	
mmu-miR-423	AGCTCGGTCTGAGGCCCCTCAGT	1	3' CAGT
mmu-miR-424	CAGCAGCAATTCATGTTTTGGA	4	
mmu-miR-429	TAATACTGTCTGGTAATGCCGT	1	
mmu-miR-431	TGTCTTGCAGGCCGTCATGCA	5	3' GG-
mmu-miR-451	AAACCGTTACCATTACTGAGTTTAGT	3	3' TAGT
mmu-miR-466	ATACATACACGCACACATAGA	1	
mmu-miR-484	TCAGGCTCAGTCCCCTCCCGA	2	3' T

mmu-miR-485-5p	AGAGGCTGGCCGTGATGAATTC	1	
mmu-miR-503	TAGCAGCGGGAACAGTACTGCAG	19	3' AG
mmu-miR-501*	AATGCACCCGGGCAAGGATTTGG	1	3' G
mmu-miR-539	GGAGAAATTATCCTTGGTGTGT	4	
mmu-miR-541	AAGGGATTCTGATGTTGGTCACACT	18	3' CT
mmu-miR-652	AATGGCGCCACTAGGGTTGTGCA	2	
mmu-miR-708	CAACTAGACTGTGAGCTTCTAGT	2	3' GT
mmu-miR-720	TCAGATCTCGCTGGGGCCTCCA	2	
novel mouse (hsa-miR-409-5p)	AGGTTACCCGAGCAACTTTGCATCT	1	
novel mouse (rno-miR-382*)	ATCATTCACGGACAACACTTT	2	
novel mouse (hsa-miR-421)	ATCAACAGACATTAATTGGGCGC	2	
novel mouse (xtr-miR-125b)	CGGGAATACTTCGAAATGT	2	
novel mouse (hsa miR-154*)	AATCATACACGGTTGACCTATT	2	
btc 325	TGAGAGGCAGAGAGCGAGACTTT	1	
14.5 486	GAGGGTTGGGTGGAGGCTCTCCG	1	
14.5 438	GCACTGAGATGGGAGTGGTGTAA	1	
14.5 307	TGACACCTGCCACCCAGCCCAAGT	1	
14.5 388	GATGAGAGCGCGGTACTGATGGC	1	
14.5 2 427	GCACTGAGATGGGAGTGGTGT	1	
14.5 2 425	CTCACCTGGAGCATGTTTTC	2	
14.5 2 380	GCCCTAAGGTGAATTTTTTGGG	1	
14.5 2 27	GGTGGTGCAGGCAGGAGAGCCA	1	
14.5 2 250	TATCGTCCTTCTGCCCAGCGAT	1	
14.5 2 222	TGCCCCCTCCAGGAAGCCTTCT	1	
14.5 2 192	AAGACGGGAGAAGAGAAGGGAG	2	
14.5 2 155	ACGGAGAGTCTTTGTCACTCAGT	1	
14.5 2 135	ACTAGATTGTGAGCTGCTGGAGT	1	
14.5 454	CACAGCTCCCATCTCAGAACAA	1	
14.5 142	AAGGTTACTTGTTAGTTCAGGA	2	
14.5 99	TCAGTAACAAAGATTCATCCTTGA	1	
14.5 91	TGCAGTCCACGGGCATATACAC	1	
		835	
	total sequences	1012	
	miRNA cloning efficiency	82.50	

Figure Legends:

Figure 1: *In situ* hybridization for developmentally expressed miRNAs. Embryonic pancreas was harvested at e14.5, fixed, embedded and sectioned. Tissues were then subjected to *in situ* hybridization with DIG-labeled LNA probes. MiR-375 (A,B), miR-503 (C,D) and miR-541 (E,F) were specifically enriched within the ductal structures within the developing pancreas. In contrast, the intronic miR-214 was observed fairly uniformly across the gut and pancreatic epithelium (G,H). Panels A,B were Pdx-1 immunostained following *in situ* analyses and intense Pdx1-immunoreactivity (brown DAB staining) was observed in a subset of miR-375 positive cells lining the ducts (arrowheads). Scale bars = 50 μm

Figure 2: The gross phenotype of the *Pdx-Cre Dicer1*$^{flox/flox}$ mouse. Panels A & B show the runting of the mutant (*) animal and pancreas respectively at day P3. *Pdx-Cre Dicer1*$^{flox/flox}$ mice (B,D,F,H) and their littermates (A,C,E,G) were harvested at e18.5 and pancreas, stomach, attached spleen and duodenum were removed (C, D). Tissues were then paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E) (E-H). Dramatic reductions in both ventral (arrow head) and dorsal (arrow) pancreas can be observed in the *Pdx-Cre Dicer1*$^{flox/flox}$ animals (*cf.* C & D). In addition, H&E staining shows large vacuous spaces within the pancreas with general disorganization of the pancreatic morphology: including dilated ducts (arrow; F), a decrease in exocrine tissue (arrowhead; F), a decrease in eosin staining, and an increase in the centroacinar spaces (*cf.* E,F&G,H). Scale Bars (E-H) = 50 μm.

Figure 3: Immunohistochemical characterization of the *Dicer1*$^{flox/flox}$ mouse. Pancreata from the *Pdx-Cre Dicer1*$^{flox/flox}$ mice displayed reductions in both synaptophysin (red) and amylase (green) immunoreactivity (*cf.* A&B). Pancreatic ducts were dilated in the mutant animals and filled with large amylase and mucin immunoreactive impactions (B&J; arrowheads). Endocrine differentiation was disrupted in the *Pdx-Cre Dicer1*$^{flox/flox}$ pancreas with a near complete loss of insulin (green; C,D&K) producing β-cells, a decrease in glucagon-producing α-cells (red; C,D&K), a loss of PP cells (red; E,F&K) and Pdx1 positive cells (green; E&F), and a near complete loss of somatostatin-producing δ-cells (red; G,H&K). There was no change in ghrelin (green; I&J) positive ε-cells observed in the mutant pancreas, and mucin (red; I&J) staining indicated the presence of a large number of secreting duct cells (I&J). Cells expressing distinct islet hormones were counted as described in the Methods section of the text and expressed as percent of total pancreatic cells (K) and ratios of islet cell types (L). Data are expressed as mean ± SEM; asterisks indicate statistically significant differences between wildtype and *Pdx-Cre Dicer1*$^{flox/flox}$ littermates (*, $P<0.05$; **, $P<0.005$; $n=4$). Scale Bars = 50 μm.

Figure 4: *RIP2*-Cre *Dicer1*$^{flox/flox}$ mice show normal islet insulin (green: A&B) and glucagon (red; A&B) staining at 8 months of age, and no difference in the number of insulin positive cells at e18.5 relative to wildtype (C). Data are expressed as mean ± SEM ($n=3$).

Figure 5: Immunohistochemical analysis of markers of pancreas development of the *Pdx-Cre Dicer1*$^{flox/flox}$ mice. At e12.5 there were no discernable differences in Pdx1 (green; A&B) or glucagon (red; A&B) immunostaining between wildtype (A) and *Pdx-Cre Dicer1*$^{flox/flox}$ (B) mice. However, at e15.5 there was a dramatic loss of the endocrine pancreas progenitor marker, neurogenin3 immunostaining (green; C-I), a decrease in Pdx1 immunostaining (red; C&D), no significant decrease in glucagon immunostaining (red; E,F&I) and no dramatic change in mucin staining (red; G&H). Additionally, there was a decrease in the total pancreatic size at this stage (data not shown). Cells expressing distinct neurogenin3 and glucagon were counted as outlined in the Methods section of the text and expressed as percent of total pancreatic cells (I). Data are expressed as mean ± SEM where asterisks (*) indicate statistically significant differences between wildtype and *Pdx-Cre Dicer1*$^{flox/flox}$ littermates ($P<0.05$, $n=4$). Scale Bars = 50 μm.

Figure 6: Hairy enhancer of split 1 (Hes1) and activated caspase-3 immunostaining in the *Dicer1*-null pancreas. In an effort to define pathways by which miRNAs regulate pancreas development staining for Hes1 was carried out. At e12.5 an upregulation of Hes1 (red; B,E&K) was observed in the pancreatic bud from the *Pdx-Cre Dicer1*$^{flox/flox}$ animals when compared to the double heterozygous (wildtype; A,B,C&K) controls; while, as previously described, there was no difference in Pdx1 staining (green; *cf.* A&D). In an effort to determine why there was an overall decrease in pancreatic mass, e15.5 pancreas was stained for activated caspase-3 (red; G,H,I&J). There was an increase in activated caspase-3 immunoreactivity in the ductal cords (Pdx1 positive; green) of the developing pancreas of the *Pdx-Cre Dicer1*$^{flox/flox}$ mice (H,I&J). Data are expressed as mean ± SEM where asterisks (*) indicate statistically significant differences between wildtype and *Pdx-Cre Dicer1*$^{flox/flox}$ littermates ($P<0.05$; J, $n=4$; K, $n=8$). Scale Bars = 50 μm.

Figure 1

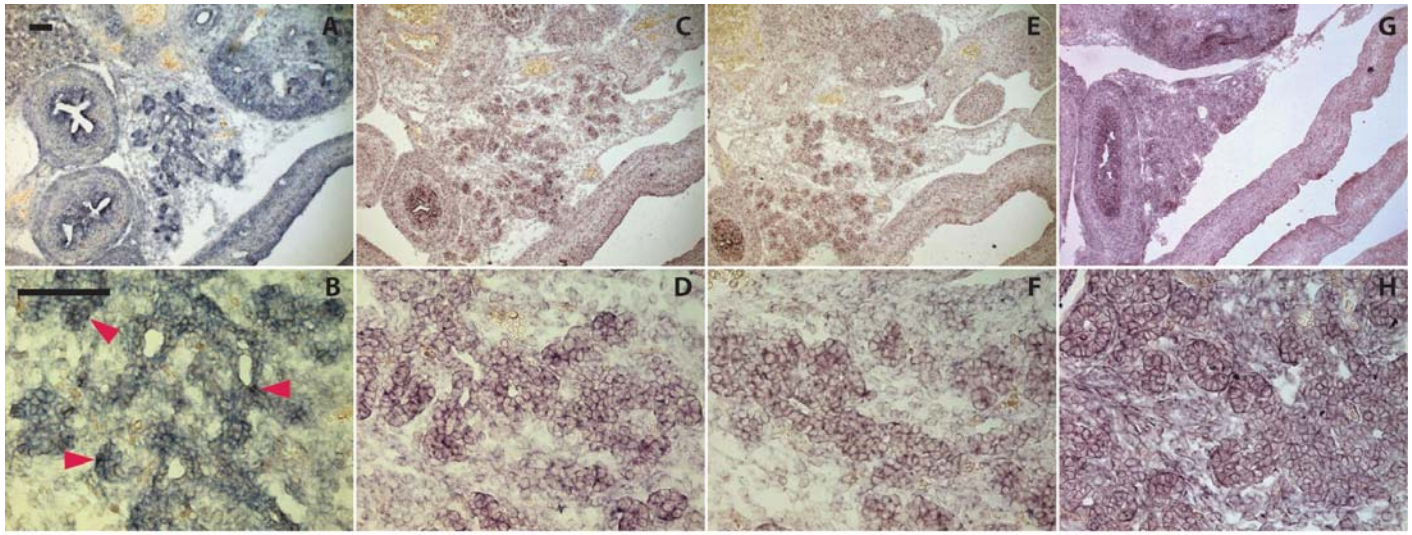


Figure 2

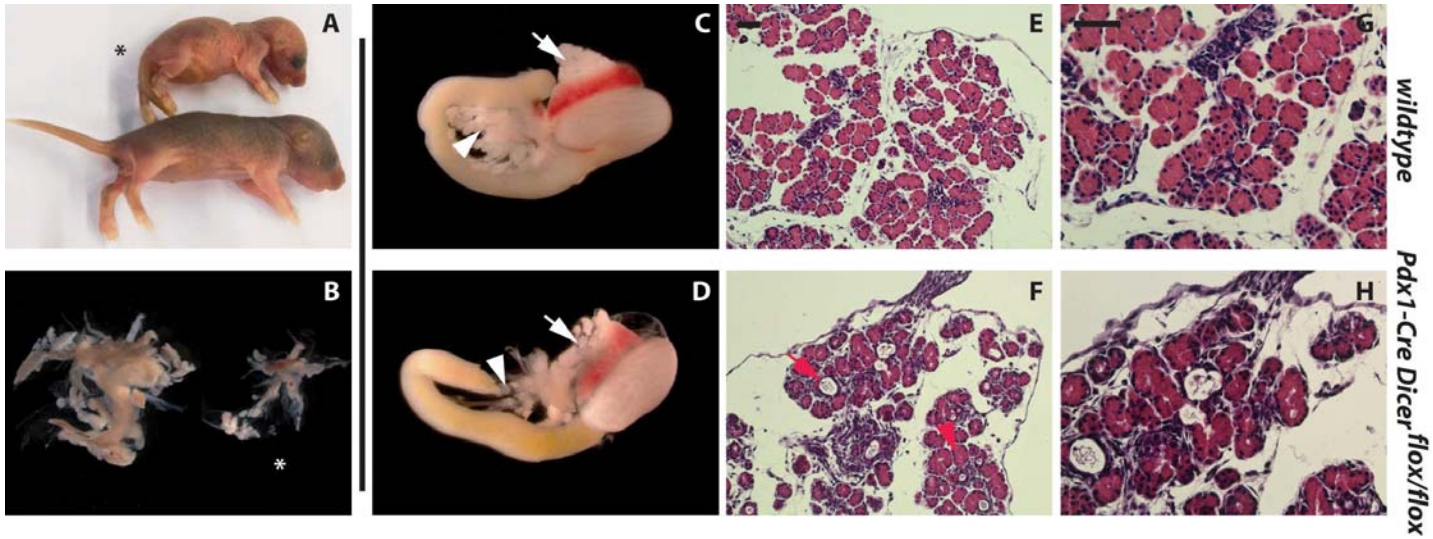


Figure 3

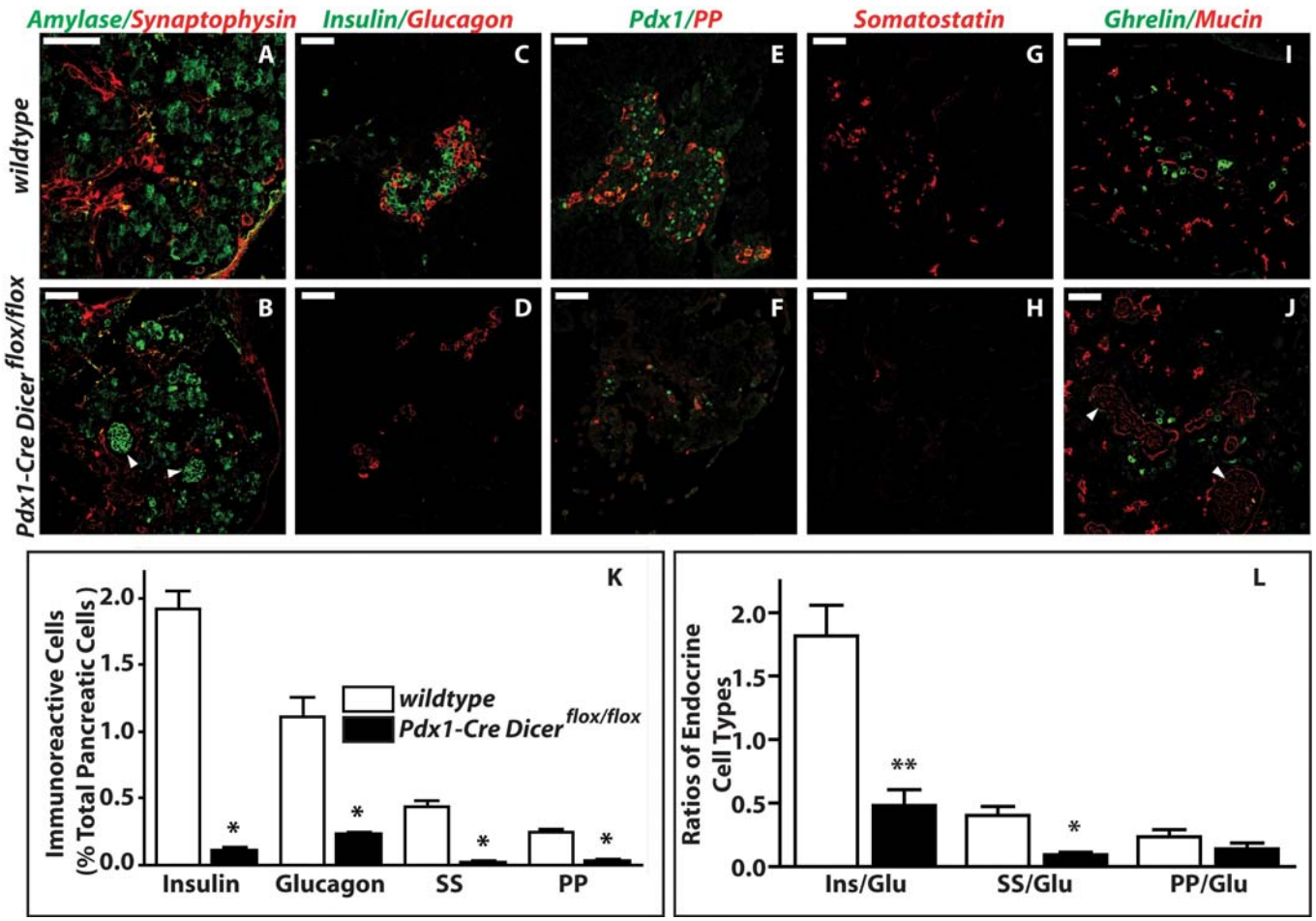

Amylase/Synaptophysin
Insulin/Glucagon
Pdx1/PP
Somatostatin
Ghrelin/Mucin
wildtype
Pdx1-Cre Dicer flox/flox
A
B
C
D
E
F
G
H
I
J
K
Immunoreactive Cells (% Total Pancreatic Cells)
2.0
1.5
1.0
0.5
0.0
wildtype
Pdx1-Cre Dicer flox/flox
*
Insulin
Glucagon
SS
PP
L
Ratios of Endocrine Cell Types
**
Ins/Glu
SS/Glu
PP/Glu

Figure 4

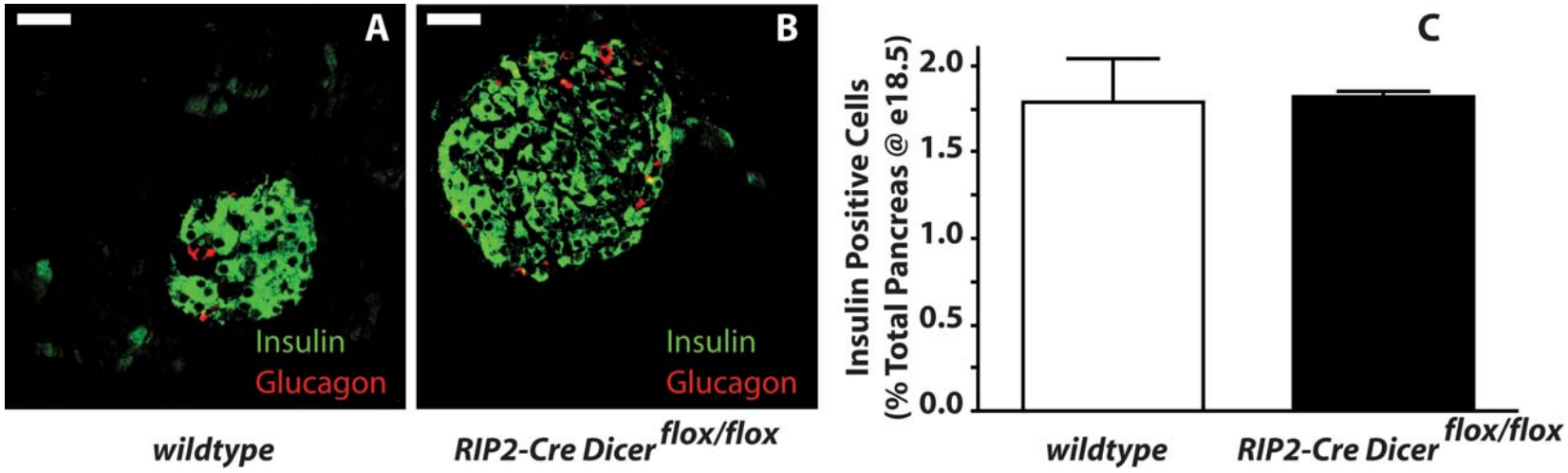

A
Insulin
Glucagon
wildtype
B
Insulin
Glucagon
RIP2-Cre Dicer flox/flox
C
Insulin Positive Cells
(% Total Pancreas @ e18.5)
2.0
1.5
1.0
0.5
0.0
wildtype
RIP2-Cre Dicer flox/flox

Figure 5

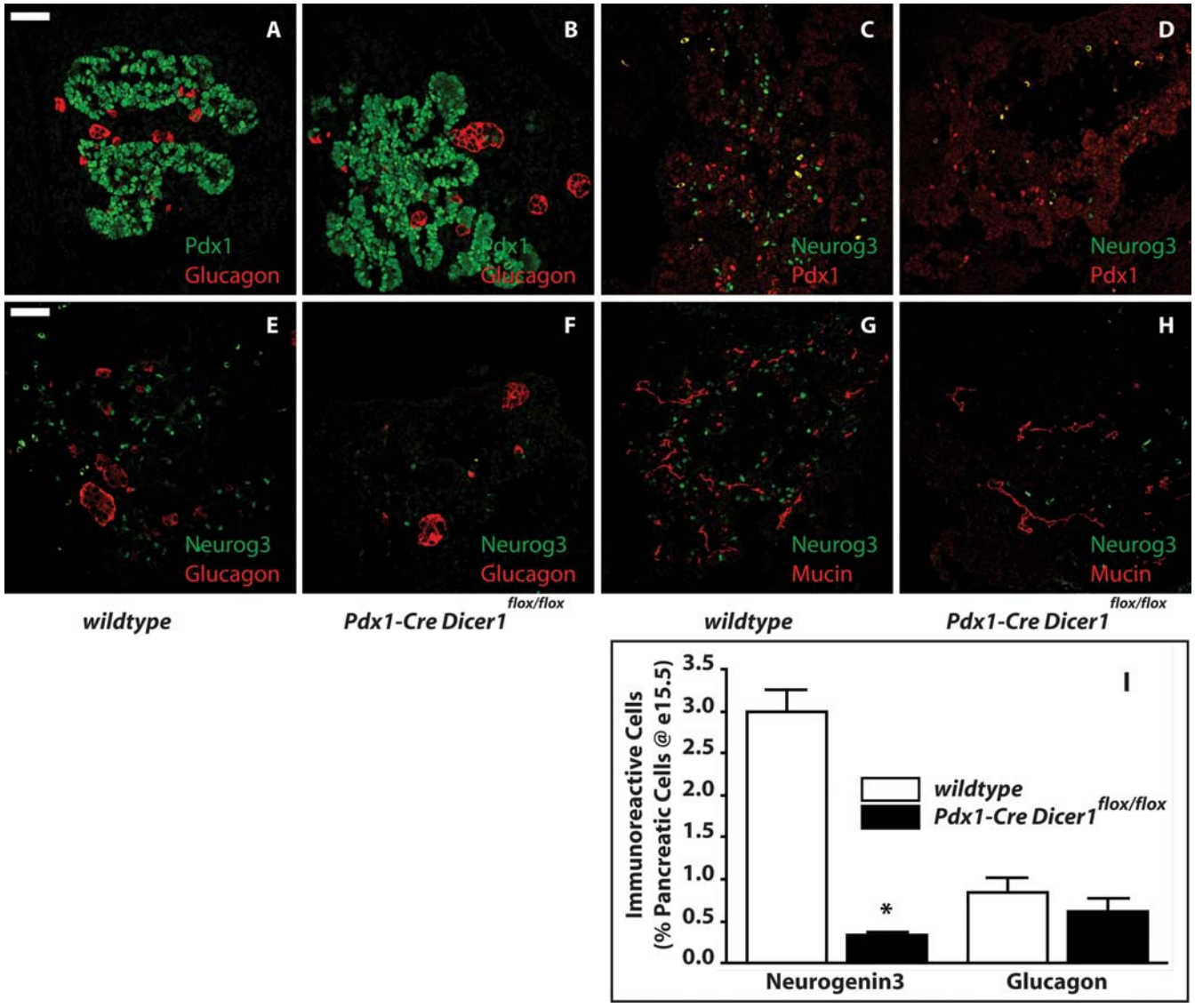
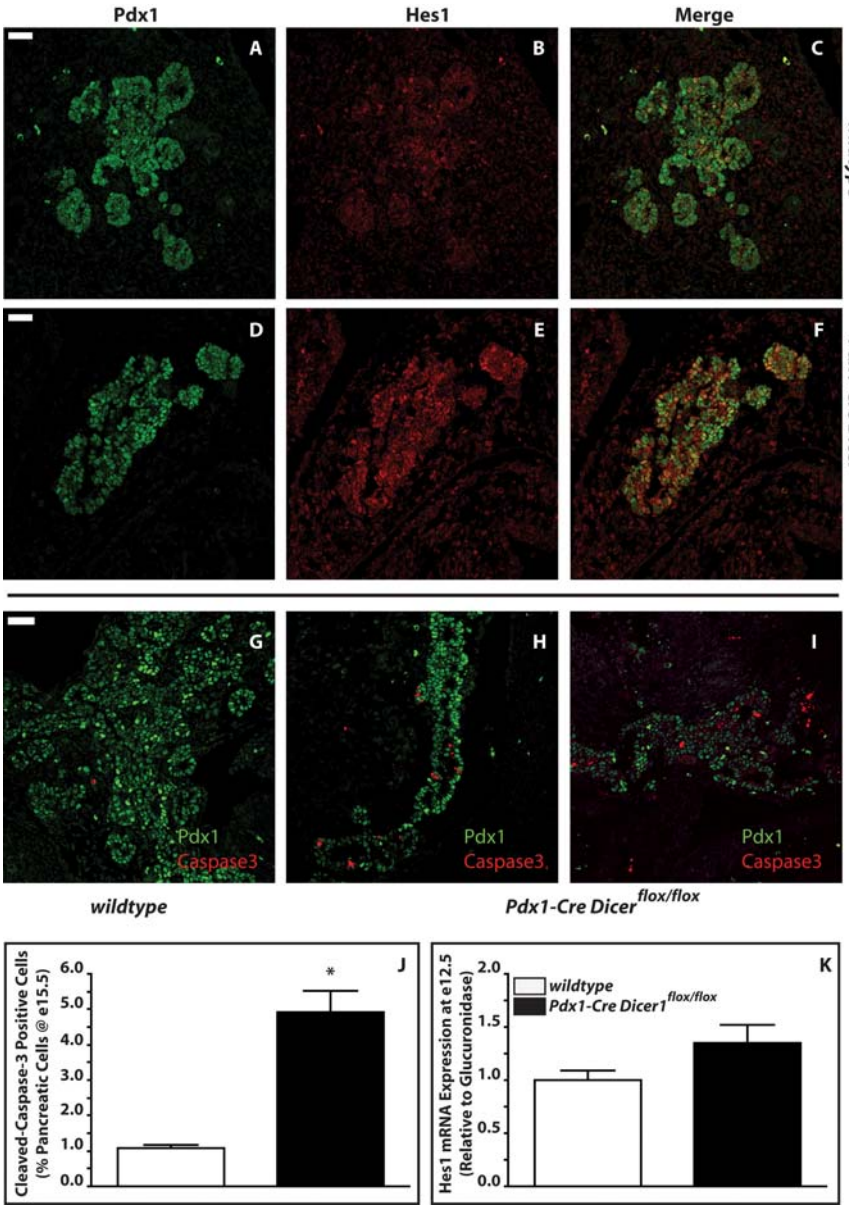

A
Pdx1
Glucagon
B
Pdx1
Glucagon
C
Neurog3
Pdx1
D
Neurog3
Pdx1
E
Neurog3
Glucagon
F
Neurog3
Glucagon
G
Neurog3
Mucin
H
Neurog3
Mucin
wildtype
Pdx1-Cre Dicer1flox/flox
wildtype
Pdx1-Cre Dicer1flox/flox
I
Immunoreactive Cells
(% Pancreatic Cells @ e15.5)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
wildtype
Pdx1-Cre Dicer1flox/flox
*
Neurogenin3
Glucagon

Figure 6


Pdx1
Hes1
Merge
A
B
C
wildtype
D
E
F
Pdx1-Cre Dicer flox/flox
G
H
I
Pdx1
Caspase3
wildtype
Pdx1-Cre Dicer flox/flox
J
Cleaved-Caspase-3 Positive Cells (% Pancreatic Cells @ e15.5)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
K
Hes1 mRNA Expression at e12.5 (Relative to Glucuronidase)
2.0
1.5
1.0
0.5
0.0
wildtype
Pdx1-Cre Dicer1 flox/flox