Supplemental Information

Ptf1a and Nkx6 transcription factors function as antagonistic lineage determinants in multipotent pancreatic progenitors

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A. Supplemental Figures

Figure S1 related to Figure 1

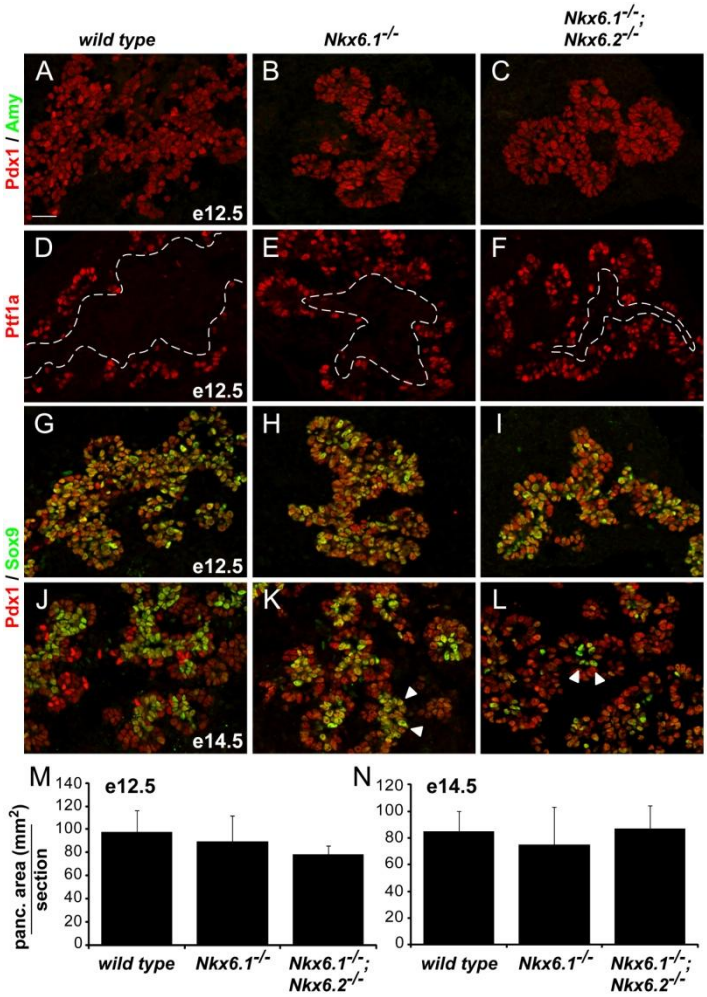

Figure S1. *Nkx6* mutant embryos maintain residual Ptf1a$^-$ progenitors. (**A-L**) Immunofluorescence staining of pancreata from *Nkx6* mutant embryos. (**A-C**) Nkx6 deficiency is not associated with premature expression of amylase at e12.5. (**D-F**) Ptf1a and Pdx1/Sox9 staining on adjacent sections at e12.5 shows a residual Ptf1a$^-$ area (dashed line) in the interior of the organ of *Nkx6.1$^{-/-}$ and Nkx6.1$^{-/-}$;Nkx6.2$^{-/-}$* embryos that expresses Sox9 and Pdx1 (**H,I**).

(**J-L**) A small area of Sox9^{+}/Pdx1^{+} “trunk” cells is also present at e14.5 (arrowheads, **K**,**L**). (**M,N**) *Nkx6.1$^{-/-}$ and Nkx6.1$^{-/-}$;Nkx6.2$^{-/-}$* embryos have normal pancreas size. Scale bar=50 µm.

Figure S2 related to Figure 2

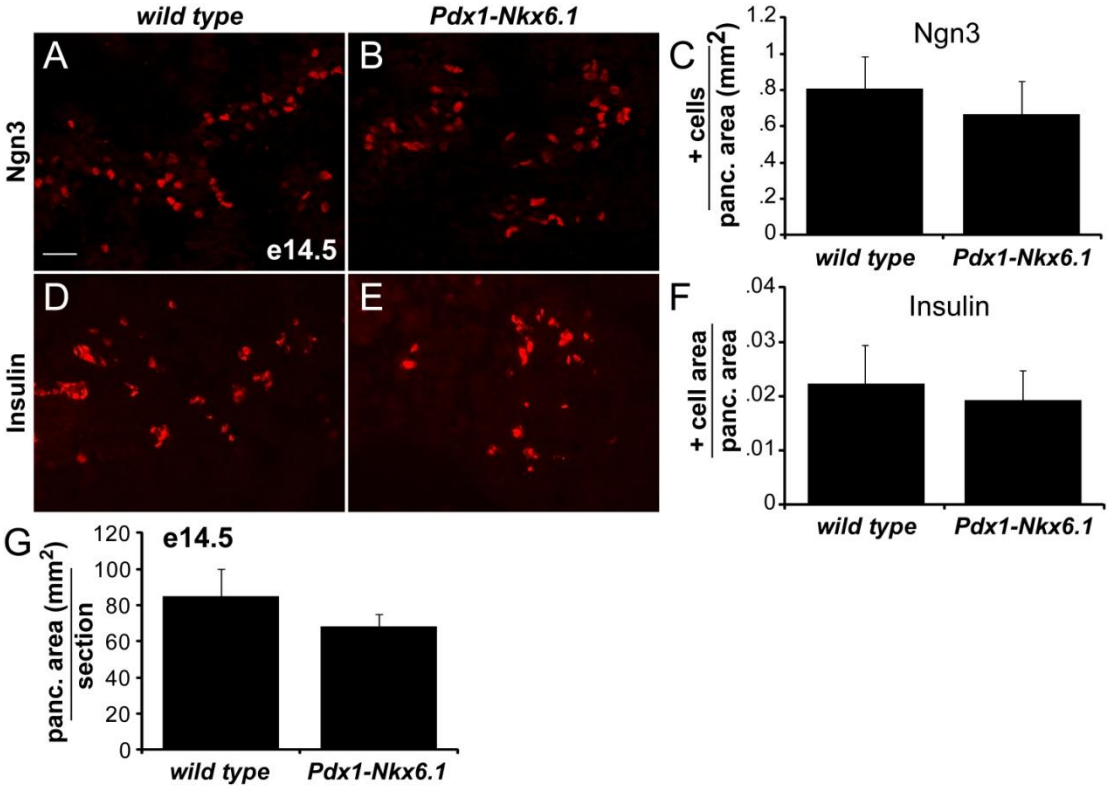

Figure S2. *Pdx1-Nkx6.1* mice do not display premature endocrine cell differentiation. (**A,B,D,E**) Immunofluorescence staining of pancreata from *Pdx1-Nkx6.1* embryos at e14.5 shows no difference in Ngn3^{+} and insulin^{+} cells compared to wild type embryos. (**C,F**) Quantification of Ngn3^{+} cells and insulin^{+} area relative to total pancreatic area. (**G**) *Pdx1-Nkx6.1* embryos have normal pancreas size. Scale bar=50 µm.

Figure S3 related to Figure 3

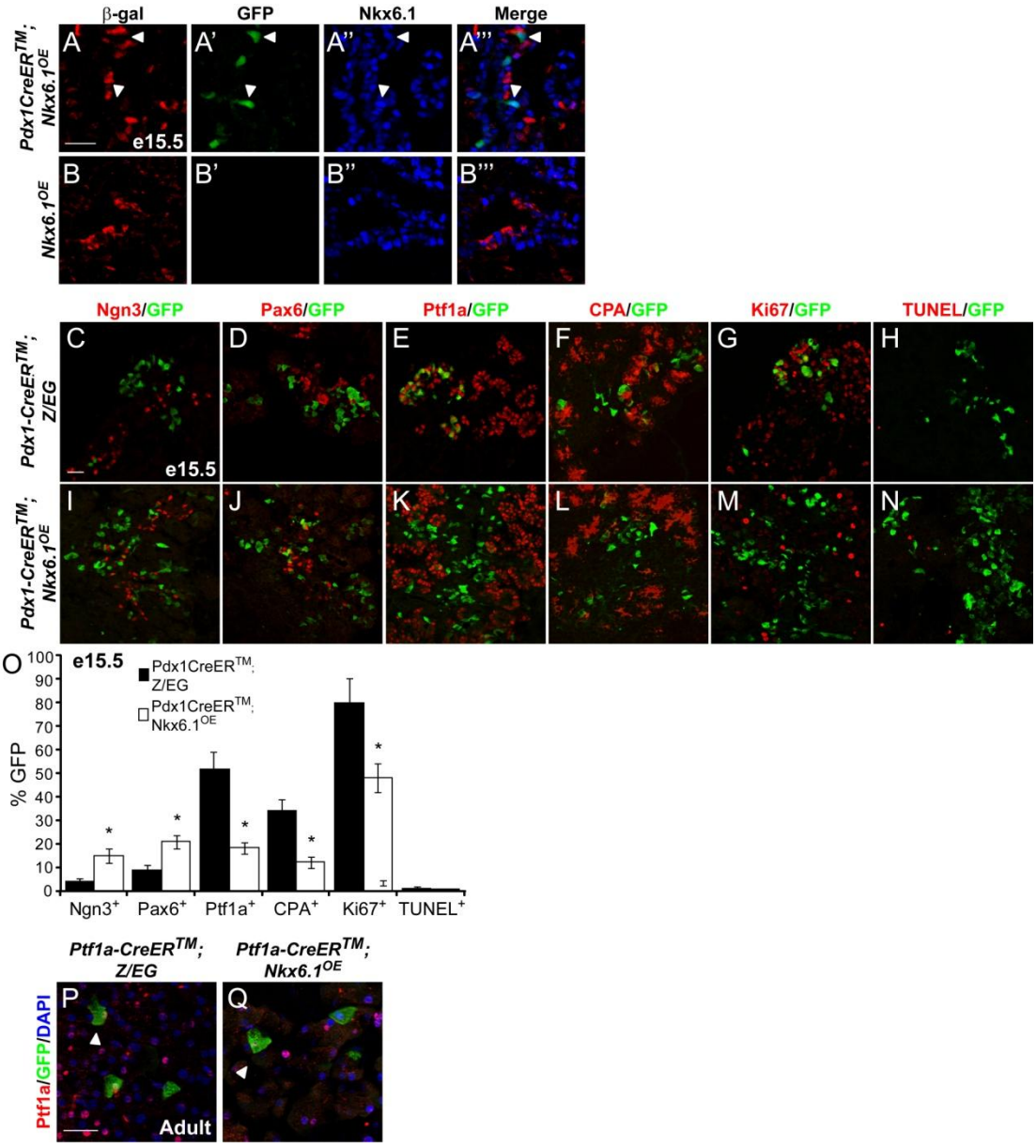

Figure S3. Heritable Nkx6.1 expression excludes Ptf1a from multipotent embryonic progenitors, but not from adult acinar cells. (**A**) Tamoxifen injection of *Pdx1-CreER*^{TM}*;Nkx6.1*^{OE} embryos at e8.5 results in Cre-mediated excision of a *Bgeo* cassette and expression of GFP and Nkx6.1. As predicted, all recombined GFP^{+} cells (arrowheads) co-express Nkx6.1, but do not

express β-galactosidase (β-gal). (**B**) In pancreata from *Nkx6.1*^{OE} embryos at e15.5, β-gal^{+} cells, but not GFP^{+} cells, are detected. Immunofluorescence staining of pancreas sections from *Pdx1-CreER*^{TM}*;Z/EG* (**C-H**) and *Pdx1-CreER*^{TM}*;Nkx6.1*^{OE} (**I-N**) embryos injected with one dose of tamoxifen at e8.5 and analyzed at e15.5. Quantification of GFP^{+}/marker^{+} cells relative to the total number of GFP^{+} cells. (**O**) In *Pdx1-CreER*^{TM}*;Nkx6.1*^{OE} embryos, a higher percentage of GFP^{+} cells express the endocrine marker Ngn3 and Pax6, while a lower percentage express the acinar marker Ptf1a and CPA compared to *Pdx1-CreER*^{TM}*;Z/EG* control embryos. The smaller number of GFP^{+} cells expressing Ki67 in *Pdx1-CreER*^{TM}*;Nkx6.1*^{OE} compared to *Pdx1-CreER*^{TM}*;Z/EG* embryos is explained by a lower proliferative capacity of endocrine cells than acinar cells. Nkx6.1 misexpression does not affect cell death. (**P,Q**) *Ptf1a-CreER*^{T2}*;Nkx6.1*^{OE} and *Ptf1a-CreER*^{T2}*;Z/EG* control mice were injected with tamoxifen 3x at 5 weeks of age. Recombined cells expressed GFP at 7 weeks of age. (**P,Q**, arrowhead) GFP^{+} cells in *Ptf1a-CreER*^{T2}*;Nkx6.1*^{OE} and *Ptf1a-CreER*^{T2}*;Z/EG* control mice express Ptf1a protein. CPA, carboxypeptidase A; Scale bar=50 μm.

Figure S4

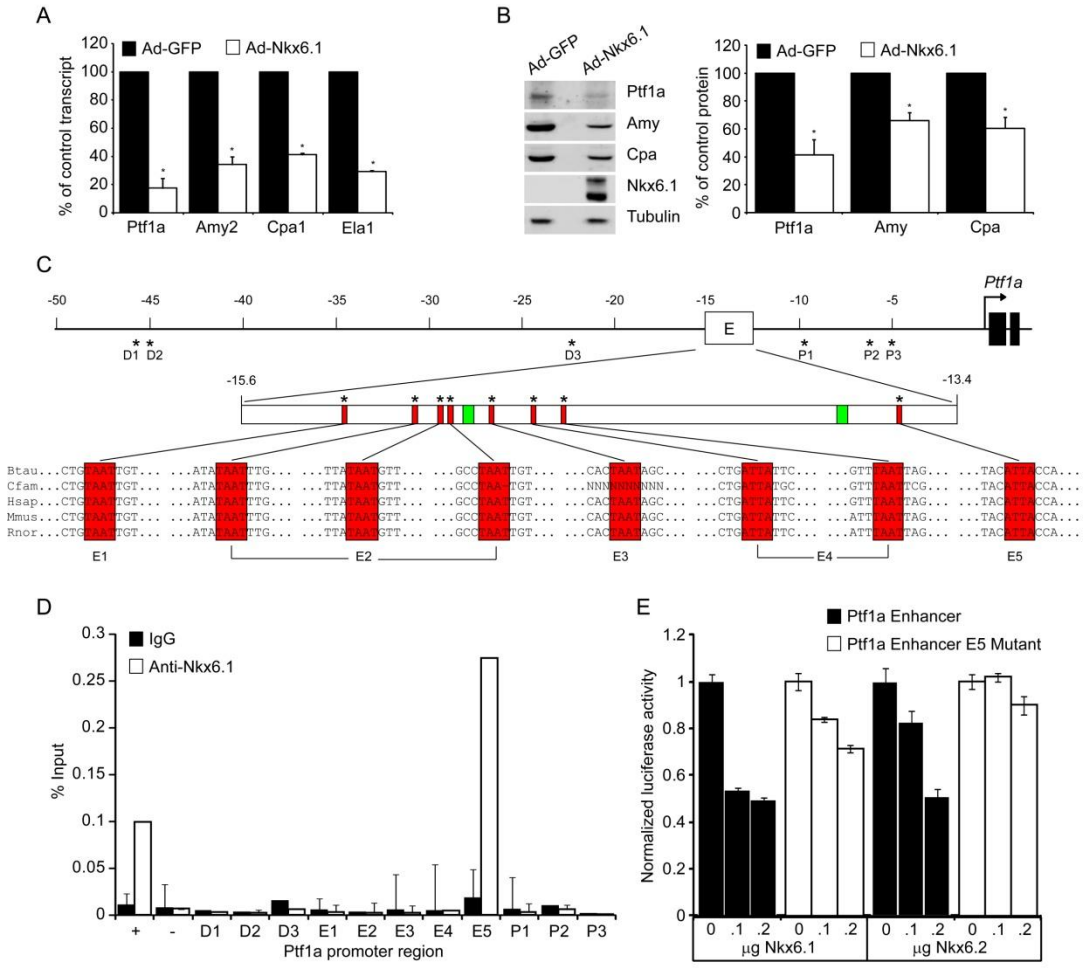

Figure S4. Nkx6.1 binds to the *Ptf1a* enhancer and represses *Ptf1a* transcription. qRT-PCR (**A**) or Western Blot (**B**) analysis for acinar cell gene products in AR42J cells infected with *AdCMV-Nkx6.1* or *AdCMV-GFP*. Amounts in *AdCMV-GFP* infected cells were set to 100%. Misexpression of Nkx6.1 represses acinar gene expression. (**C**) Schematic of 50 kb of 5' flanking region of the *Ptf1a* gene. Asterisks indicate phylogenetically-conserved binding motifs for Nkx6 factors. A *Ptf1a* enhancer (E) element contains two confirmed PTF1 binding sites (Masui et al., 2008) (green boxes) and eight putative Nkx6 binding sites (red boxes). (**D**) Nkx6.1 binds to the E5

site within this *Ptf1a* enhancer in nuclear chromatin from βTC3 cells analyzed by ChIP with antibodies against Nkx6.1 or control immunoglobulin G (IgG). Mouse glucagon promoter (Schisler et al., 2005) and intergenic primers were used as positive (+) and negative (-) controls, respectively. (**E**) Co-transfection of 266-6 cells with the 5' *Ptf1a* enhancer or the 5' *Ptf1a* enhancer with a mutation in the Nkx6.1 binding site linked to a minimal promoter driving luciferase expression together with a *CMV-Nkx6.1* or *CMV-Nkx6.2* expression construct. Promoter activity without *CMV-Nkx6.1* or *CMV-Nkx6.2* was set to 1. The 5' *Ptf1a* enhancer shows dosage-dependent repression by Nkx6.1 and Nkx6.2, with Nkx6.2 being less potent at lower concentrations. Amy, amylase; Cpa, carboxypeptidase A; Ela, elastase; Btau, Bos Taurus; Cfam, *Canis familiaris*; Hsap, *Homo sapiens*; Mmus, *Mus musculus*; Rnor, *Rattus norvegicus*.

B. Supplemental Experimental Procedures

Generation of *CAG-Bgeo,-Nkx6.1,-eGFP*, *Pdx1-Ptf1a* and *Ptf1a-CreER^{T2}* mice

CAG-Bgeo,-Nkx6.1,-eGFP mice were generated by inserting the murine *Nkx6.1* cDNA between the *Bgeo* and *IRES-eGFP* cassette in the *CAG-Bgeo,-eGFP* vector (Novak et al., 2000). The linearized construct was injected into the pronuclei of CB6F1 fertilized oocytes (UC Irvine Transgenic Mouse Facility). Using PCR for *lacZ,* eight potential founders were identified and expression verified by X-gal staining on embryonic pancreata. Three founders showed significant X-gal staining, two of which efficiently recombined the transgene and activated expression of Nkx6.1 and GFP upon expression of Cre-recombinase. To generate *Pdx1-Ptf1a* embryos, a murine *Ptf1a* coding sequence fused to FLAG-SV40polyA was cloned downstream of 4.5 kb of the mouse *Pdx1* regulatory sequences and a human *HSP68-β-globin* cassette. The linearized construct was injected into the pronuclei of CB6F1 fertilized oocytes (UC Irvine Transgenic Mouse Facility) and embryos harvested at e14.5. Out of a total of 48 embryos, 18 integrated the *Pdx1-Ptf1a* transgene into their genome. Four embryos showed significant expression of FLAG protein in Pdx1^{+} cells.

Additional mouse strains

R26^{NotchIC} mice were kindly provided by D. Melton (Murtaugh et al., 2003) and *Ptf1a-CreER*^{T2} mice by C. Wright (unpublished). *Pdx1-Cre* mice for NotchIC activation in *R26*^{NotchIC} mice have been previously described (Gu et al., 2002). For the experiments described in Suppl. Fig. 7, a mosaic substrain was used that manifested partial silencing of the *Pdx1-Cre* transgene in our mouse colony.

Morphometric analysis

At e10.5 and e12.5, every pancreas section was analyzed from a minimum of 3 embryos per genotype, while every fifth pancreas section (a total of 10-20 sections per embryo) was analyzed at e14.5 and e15.5. The number of marker$^+$ cells was determined by counting every DAPI$^+$/marker$^+$ cell in each section. The total pancreatic area per section was measured using Image Pro Plus 5.0.1 software (Media Cybernetics), which was calibrated to calculate values in µm. The number of marker$^+$ cells per section was subsequently divided by the total area of the section and expressed as marker$^+$ cells/mm^2. For lineage analysis, the number of GFP$^+$/DAPI$^+$/marker$^+$ cells was divided by the total number of GFP$^+$/DAPI$^+$ cells and multiplied by 100. The values were averaged for each genotype and displayed as ± standard error of the mean. Statistical analysis was performed using an unpaired, 2-tailed, t-test, and significance determined with a $p<0.05$.

Adenoviral infections of cells, protein quantification, DNA-protein binding, and reporter gene assays

Procedures for infection of AR42J cells with AdCMV-Nkx6.1 and AdCMV-GFP control virus, protein preparation, and Western Blot analysis have been described previously (Schisler et al., 2005; Seymour et al., 2008). Western Blots were quantified with the Odyssey detection system (LI-COR Biosciences).

Based on previously identified motifs (Jorgensen et al., 1999; Mirmira et al., 2000), a custom positional weight matrix was used to identify putative Nkx6.1 binding sites. ChIP assays were performed as described (Gerrish et al., 2001). Nkx6.1 (1:250) or rabbit IgG antisera were used for immunoprecipitations. Each ChIP assay was quantified in triplicate by qPCR.

The *Ptf1a* 5' enhancer construct and procedures for transient transfections and luciferase assays have been described previously (Masui et al., 2008). For the E5 mutation in the enhancer TAC at -13412 bp to -13414 bp from the *Ptf1a* transcription start site was changed to CTG. For each data point, values were normalized to the activity of the minimal promoter. All reporter gene analyses were performed in triplicate and data expressed as means ± SEM.

Table S1. Primers used in chromatin immunoprecipitation experiments

Primer name	Relative position to Ptf1a transcriptional initiation site	Sequence
Positive (Glucagon) forward		5'-AAG CAG ATG AGC AAA GTG AGT G-3'
Positive (Glucagon) reverse		5'-AGG CTG TTT AGC CTT GCA GAT A-3'
Negative (non-coding) forward		5'-CAC TCA GAT CCT GAG CCA CA-3'
Negative (non-coding) reverse		5'-GCT CTC TGC CTT CCA CTT TG-3'
D1 forward	-45558	5'-TCC TAG ACA TGG TCC TGG TGT C-3'
D1 reverse	-45457	5'-CCA ACC CCT ACA CCC CAT AA-3'
D2 forward	-45020	5'-TCA CTG TGC CTG TCA AGG AGA-3'
D2 reverse	-44888	5'-AGC AGC ACG GTG ACT GTG AA-3'
D3 forward	-22451	5'-GCA AGG CTG GTG CGG TGC CAA C-3'
D3 reverse	-22310	5'-GAT AGG GGG GCT GGG AGT GGC C-3'
E1 forward	-15324	5'-GCG TCC AGT CGT GCG TCC ATC-3'
E1 reverse	-15171	5'-CGG CAG AGG AAG GCA CCA GAA G-3'
E2 forward	-14952	5'-GGA AAT CGC CCG CGA CGC ACC-3'
E2 reverse	-14842	5'-GAG GGG GTG CGC TGC CCT TTC-3'
E3 forward	-14806	5'-ACA AGT GGC GAC ATT CCC ATG-3'
E3 reverse	-14670	5'-CCG GTT CTC AAT TGA ATG CG-3'
E4 forward	-14612	5'-GGC AGC CAC TGT TCA CTT TCT-3'
E4 reverse	-14465	5'-GCT CTC TGC TGT CCC AGA ACT-3'
E5 forward	-13509	5'-CCC CGG AGA CGT GCA GGA CAT AG-3'
E5 reverse	-13399	5'-CCG ACA CAA GGC GCT GGT ACC TG-3'
P1 forward	-9821	5'-TGC AGC TGT CTC TGT TGC TG-3'
P1 reverse	-9747	5'-CCT GGT GCT CAA AAT TGT CC-3'
P2 forward	-6597	5'-ACA GGA GGA AAA TAC ACT GGA AGC-3'
P2 reverse	-6457	5'-GGC TTC CAC AAT GTG TAC ATG A-3'
P3 forward	-5193	5'-CAG ACC TTG GCA TGT CAG AA-3'
P3 reverse	-5107	5'-TTC CTG CCT TGC ACT TCT CA-3'

Table S2. Primers used in qRT-PCRs

Primer name	Sequence
HPRT forward	5'-CAG GCC AGA CTT TGT TTG GAT-3'
m-HPRT reverse	5'-TTG CGC TCA TCT TAG GCT TT-3'
r-HPRT reverse	5'-GCC GCTGTC TTT TAG GCT TT-3'
m-Ptf1a forward	5'-TAA AGT GTG GAC CCC AGA GG-3'
m-Ptf1a reverse	5'-CGA TGT GAG CTG TCT CAG GA-3'
m-Amylase forward	5'-TTG CCA AGG AAT GTG AGC GAT-3'
m-Amylase reverse	5'-CCA AGG TCT TGA TGG GTT ATG AA-3'
m-Kallikrein5 forward	5'-TTT GAG GAT GAA CCC TCT GC-3'
m-Kallikrein5 reverse	5'-CAG CAT CAG GTC ATT GCT GT-3'
r-Amylase forward	5'-GGT CAT TCA TCT TGG TGG TGA-3'
r-Amylase reverse	5'-CCT TCT CCC CAG TTC TTT AAG T-3'
r-Carboxypeptidase A forward	5'-ATT CTG AGT CTG CTG CTG GAA G-3'
r-Carboxypeptidase A reverse	5'-AGT CCA ACT GCA AGT GCT CCA-3'
r-Ptf1a forward	5'-AGG TCA TCA TCT GCC ATC GAG-3'
r-Ptf1a reverse	5'-GGG TCC ACA CTT TAG CTG TAC G-3'
r-Elastase forward	5'-ATC GCC CTA TTG CGC TTG-3'
r-Elastase reverse	5'-GCC CAT TGG TTC TGG TTC TC-3'

Table S3. Antibodies used for immunofluorescence and Western blot analysis

Primary Antibodies			
Antigen	Species	Dilution	Source
Amylase	mouse	1:250	Santa Cruz
Amylase	rabbit	1:500	Sigma
β-gal	rabbit	1:500	ICN
Carboxypeptidase A	rabbit	1:1000	Biotrend
DBA	N/A	1:500	Vector Laboratories
FLAG	rabbit	1:2000	Sigma
GFP	Rat	1:2000	C. Kioussi, Oregon State University
Glucagon	Guinea Pig	1:2000	Sigma
HNF1β	Goat	1:1000	Santa Cruz
Insulin	Mouse	1:5000	Sigma
Ki67	Rabbit	1:500	Lab Vision
Ngn3	Guinea Pig	1:2000	Henseleit et al. 2005
Nkx6.1	Mouse	1:500	BCBC clone 2023
Nkx6.1	Rabbit	1:2000	P. Serup, Hagedorn Institute
Nkx6.2	Guinea Pig	1:2000	J. Ericson, Karolinska Institute
Pancreatic Polypeptide	Rabbit	1:2000	Dakocytomation
Pax6	Rabbit	1:500	Chemicon
Pdx1	Guinea Pig	1:10000	C. Wright, Vanderbilt
Ptf1a	Rabbit	1:3000	B. Breant, INSERM-Paris
Sox9	Rabbit	1:1000	Chemicon
Somatostatin	Rabbit	1:3000	Dakocytomation
Tubulin	Mouse	1:4000	DSHB
Secondary Antibodies			
Antigen	Conjugation	Dilution	Source
Guinea Pig	Alexa 488	1:2000	Invitrogen
Guinea Pig	Cy3	1:2000	Jackson ImmunoResearch
Mouse	Alexa 488	1:2000	Invitrogen
Mouse	Cy3	1:2000	Jackson ImmunoResearch
Mouse	Biotin	1:250	Vector Laboratories
Mouse	IR-800	1:10000	LI-COR
Rabbit	Cy3	1:2000	Jackson ImmunoResearch
Rabbit	IR-680	1:10000	LI-COR
Rabbit	Alexa 488	1:2000	Invitrogen
Rat	Alexa 488	1:2000	Invitrogen

C. Supplemental References

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