Supplemental Research Design and Methods

Tissue Preparation, Immunohistochemistry and Microscopy – Antibodies were used at the following dilutions: rabbit anti-Smoothened (1:500) a kind gift from P.-T. Chuang, guinea pig anti-Insulin (1:250) Linco, rabbit anti-Glucagon (1:500) Linco, rabbit anti-Pdx1 (1:500) and rabbit anti-Neurogenin3 (1:500) kind gifts from M.S. German, mouse anti-Nkx6.1 (1:100) Hybridoma Bank, rabbit anti-Amylase (1:500) Sigma, armenian hamster anti-Mucin (1:200) Neomarkers, rabbit anti-Somatostatin (1:250) DAKO, guinea pig anti-Pancreatic Polypeptide (1:250) Linco, rabbit anti-Phospho-Histone H3 (1:200) Upstate, rabbit anti-cleaved Caspase3 (1:100) Cell Signaling.

Morphometric and Statistical Analysis – Morphometric analysis of tissues was completed by sectioning through whole samples and collecting all sections containing pancreatic tissue. Each section was stained with the desired markers and individually analyzed for pancreatic area or numbers of cells. All embryonic analyses were normalized to control littermates before comparative analysis. For e12.5 tissues, all sections containing pancreas tissue were manually analyzed for tissue area. Relative ratios of proliferating or apoptotic cells was calculated by first determining the percent of proliferating/apoptotic cells in the pancreas (the number of proliferative/apoptotic-positive pancreatic cells by the total number of Pdx1-positive cells). Next, mutant samples were compared to control littermate samples for relative ratios before comparative analysis. For e15.5 and P0 tissues, one fifth of total tissue was analyzed. Endocrine areas were determined by adding total insulin-positive areas with total glucagon-positive areas. Calculation of relative ratios of proliferating insulin-positive cells was performed similarly to the calculation for proliferating cells at e12.5. Since total pancreatic area had normalized by e15.5, absolute numbers of Ngn3-positive cells or Ngn3/Nkx6.1-positive cells were assessed and compared to control littermates before comparative analysis. Insulin secretion assays were performed in five technical replicates on three independent experimental replicates. Data from these experiments were pooled and analyses for statistical significance. Similarly, calculation of β-cell mass was performed by using four experimental replicates as previously described (1). Statistical analyses for all analyses were conducted using the Student t-test, except in glucose and insulin tolerance tests which were assessed by two-way ANOVA. Error bars represent standard deviation.

Patched-LacZ Stains – Tissue harvested at various embryonic dates were fixed in 0.2% glutaraldehyde prepared in 1X phosphate buffered solution (PBS) for 30 to 120 mins, depending on the size and age of tissue. Pre-fixed tissues were washed several times in 1X PBS, and then stained in X-gal staining solution (0.00435M Ferrocyanide, 0.005M Ferricyanide, 0.02% NP-40, 0.02M $MgCl_2$, 0.50mg/ml X-gal in 1X PBS) at room temperature overnight. After staining for β-gal activity, issues were post-fixed in 4% paraformaldehyde for 2 hours, washed in 1X PBS, processed for paraffin embedding, and cut into 5 μm sections. Sections were deparaffinized and rehydrated, counter-stained with nuclear fast red and imaged on a Zeiss brightfield microscope.

RNA Isolation, SybrGreen and Taqman real time quantitative PCR – Soluble Wnt and BMP ligands targeted for gene expression analysis were determined from previous reports (2-4). Target gene sequences for insulin secretion machinery and some islet genes were based on previously confirmed primers (4-7). All data was normalized

relative to *Cyclophilin* and *β-glucuronidase (GUS)* showing similar results, but presented relative to *Cyclophilin*.

Total Body Weight – Mice were weighed after overnight fast on an OHAUS CL-2000 portable scale with readability to 1g.

Fasting Serum Insulin Levels – Mice were fasted overnight for not more than 16 hours before serum collection. Protease inhibitor cocktail (Roche #11-873-580-001) was added to collected samples, and samples were assayed using Alpco Ultrasensitive Mouse Insulin ELISA kit.

REFERENCES:

1. Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M: The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* 113:11-23, 2003

2. Heller RS, Dichmann DS, Jensen J, Miller C, Wong G, Madsen OD, Serup P: Expression patterns of Wnts, Frizzleds, sFRPs, and misexpression in transgenic mice suggesting a role for Wnts in pancreas and foregut pattern formation. *Dev Dyn* 225:260-270, 2002

3. Dichmann DS, Miller CP, Jensen J, Scott Heller R, Serup P: Expression and misexpression of members of the FGF and TGFbeta families of growth factors in the developing mouse pancreas. *Dev Dyn* 226:663-674, 2003

4. Goulley J, Dahl U, Baeza N, Mishina Y, Edlund H: BMP4-BMPR1A signaling in beta cells is required for and augments glucose-stimulated insulin secretion. *Cell Metab* 5:207-219, 2007

5. Kawahira H, Ma NH, Tzanakakis ES, McMahon AP, Chuang P-T, Hebrok M: Combined activities of Hedgehog signaling inhibitors regulate pancreas development. *Development, in press*, 2003

6. Cano DA, Murcia NS, Pazour GJ, Hebrok M: Orpk mouse model of polycystic kidney disease reveals essential role of primary cilia in pancreatic tissue organization. *Development* 131:3457-3467, 2004

7. Pasca di Magliano M, Sekine S, Ermilov A, Ferris J, Dlugosz AA, Hebrok M: Hedgehog/Ras interactions regulate early stages of pancreatic cancer. *Genes Dev* 20:3161-3173, 2006

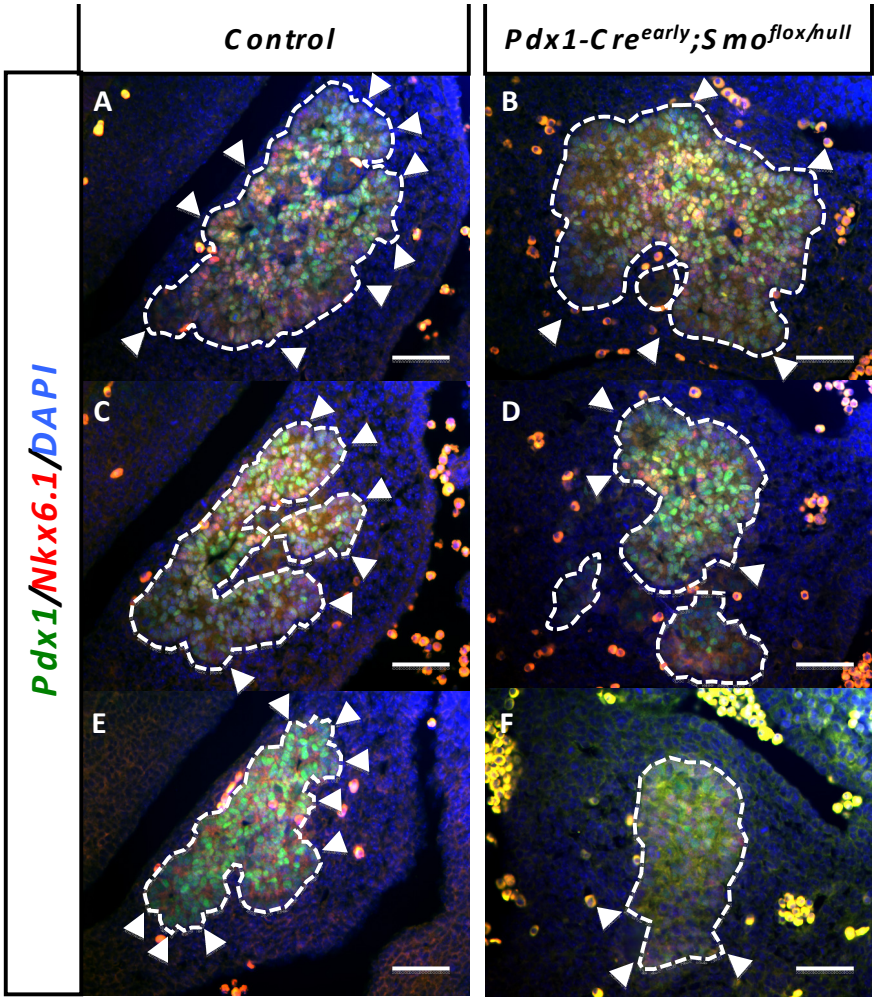
Supplemental Table 1 – Primer sequences used for quantitative PCR.

Gene	Forward	Reverse
Cyclophilin	TCACAGAATTATTCCAGGATTCATG	TGCCGCCAGTGCCATT
GUS	ACGGGATTGTGGTCATCGA	TCGTTGCCAAAACTCTGAGGTA
Smoothened	AATTGGCCTGGTGCTTATTGTG	TGGCTGCCTTCTCACTCAGAA
Patched	CCCTAACAAAAATTCAACCAAACCT	GCATATACTTCCTGGATAAACCTTGAC
Gli1	GCCACACAAGTGCACGTTTG	AAGGTGCGTCTTGAGGTTTTCA
Ihh	CACGTGCATTGCTCTGTCAA	AGGAAAGCAGCCACCTGTCTT
Shh	CAAAGCTCACATCCACTGTTCTG	GAAACAGCCGCCGGATTT
BMP4	GGACTTCGAGGCGACACTTC	TTGCTAGGCTGCGGACG
BMP5	TTCTTCAAGGCAAGCGAGGT	TATTGCGGTTTTGATTTTTCCG
BMP7	CAGGGAGTCGGACCTCTTCTT	AACACCAACCAGCCCTCCTC
ID2	CCGCTGACCACCCTGAAC	GCTCAGAAGGGAATTCAGATGC
Wnt2	AACACCCTGGACAGAGATCACA	CGTAAACAAAGGCCGATTCC
Wnt2b	AGGCTGCAGGTTCCCTAGGT	ACCCATCTGTTCCTTTAGAAGTCTTG
Wnt5a	TGCAGCACAGTGGACAATACTTCT	CGTACGTGAAGGCCGTCTCT
Wnt5b	CAGTGCAGAGACCGGAGATGT	GGGAAAGCCCAGGAAGTTG
Wnt7	TCGGGAAGGAGCTCAAAGTG	CCGCAGCGATAATCGCATA
Wnt11	AAACAGGATCCCAAGCCAATAAA	GGAGGCACGTAGAGCCTGTCT
sFRP1	CCGAGATGCTCAAATGTGACA	TCCGTGGTATTGGGCGG
sFRP2	ACGGAGATCCTTCTGTGGTTTC	GTCAAGACACACTACACAAAGCGTTT
Axin2	GCCAATGGCCAAGTGTCTCT	GCGTCATCTCCTTGGGCA
Lef1	TCATATGATTCCCGGTCCTC	CCTTCTGCCAAGAATCTGGT
Tcf1	GCTGCCATCAACCAGATCCT	AGTTCATAGTACTTGGCCTGCTCTTC
Arnt *	CTCCCACTGCCTCATCTGGTA	GTCCAGTCTCAGGAGGAAAGTTG
Calpain-10 *	TGCACCCCATTGGTTTCC	GGAACACGCGTCCTGGTT
GIPR *	TCAAAGCTGAGGACTCGACAGA	TGGAGCGAGCCAGCCTTA
GLP-1R *	AGAACTCTCCTTCACTTCCTTCCA	TCCCAGCATTTCCGAAACTC
Glucagon *	CCGCCGTGCCCAAGA	CATCATGACGTTTGGCAATGTT
Glucokinase *	GAGTGCTCAGGATGTTAAGGATCTG	GCTTTTGAGACCCGTTTTGTG
Glut2 *	GGGGTTGGTGCCATCAAC	CACAAGCAGCACAGAGACAGC
Insulin *	AGCAAGCAGGTCATTGTTTCAA	AAGCCTGGGTGGGTTTGG
Pdx1	AAATCCACCAAAGCTCACGC	GGGTTCCGCTGTGTAAGCA
Kir6.2 *	GGACCTCCGAAAGAGCATGA	GCGCACCACCTGCATGT
Nkx6.1 *	TCAGGTCAAGGTCTGGTTCCA	CGGTCTCCGAGTCCTGCTT
PC1/3 *	AGGCAGCTGGCGTGTTTG	GAAGCTGGTTCCGCTTGGA
Rab27a *	GGAGGCCCGGGAACTTG	TCTCAATCGCGTGGCTTATG
Rab3d *	GGTCTACCGACATGACAAGAGGAT	GCGATAGTAGGCCGTGGTGAT
SNAP25 *	AATCGCCAGATCGACAGGAT	GGCTTCATCAATTCTGGTTTTGT
SUR1 *	CCTCCAGAAGGTGGTGATGAC	TCTGCACTCAGGATGGTGTGTAC
UCP2 *	CAGGTCACTGTGCCCTTACCA	AGGCATGAACCCCTTGTAGAAG
FoxO1 *	GCGGGCTGGAAGAATTCA	GATTGAGCATCCACCAAGAACTC
NeuroD1	TCCGGTGCCGCTGC	GCGAATGGCTATCGAAAGACA
Fgf10	Mm00433275_m1 (Applied Biosystems)	
Neurogenin3	Mm00437606_s1 (Applied Biosystems)	

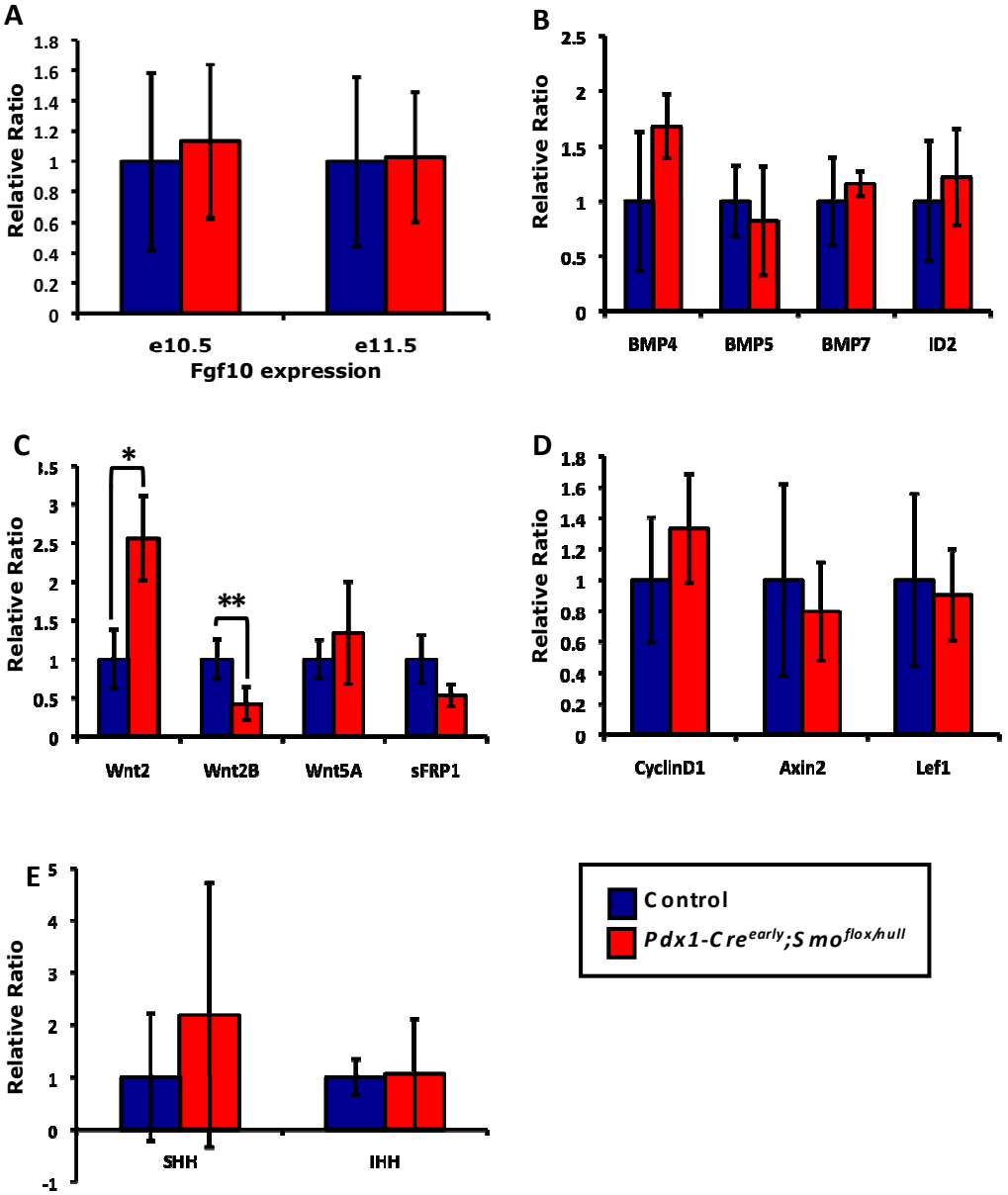
* - sequences as previously published by Goulley et al (2007) Cell Metabolism.

Appendix References

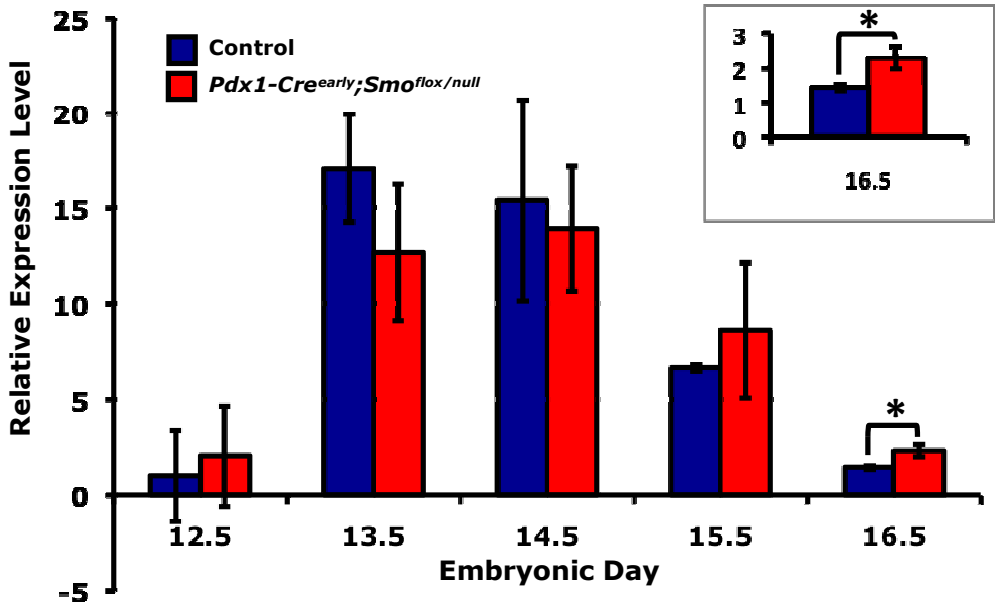
Supplemental Figure 1 – *Pdx1-Cre*^{early}*;Smo*$^{flox/null}$ mice have reduced epithelial area and branching. Sequential 5µm sections stained with Pdx1(green), Nkx6.1(red) and DAPI(blue) in e12.5 pancreata show disrupted pancreatic branching in control (**A, C, E**) versus *Pdx1-Cre*^{early}*;Smo*$^{flox/null}$ (**B, D, F**) mice. (Arrowheads indicate branching tips). Scale bars in **A-F** are equal to 100µm.



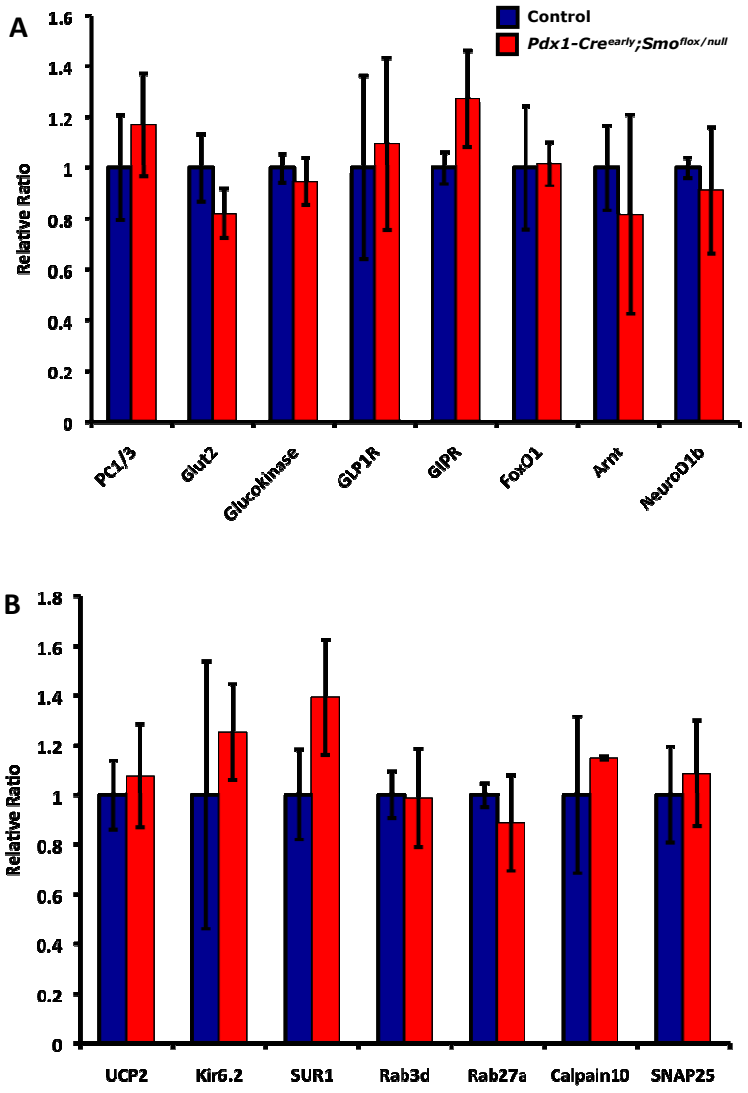
Supplemental Figure 2 – Fgf10, BMP, and Wnt signaling pathways are not altered in early *Pdx1-Cre^{early};Smo$^{flox/null}$* pancreata. (**A**) Taqman expression of Fgf10 show no significant changes in mesenchyme intact e10.5 and e11.5 pancreata. (**B**) Sybr green real time PCR showed that expression of BMP ligands and soluble BMP inhibitor ID2 in e10.5 pancreata was unchanged. While expression Wnt2 and Wnt2B suggested some significant changes at e10.5 (**C**), expression of Wnt target genes expression (**D**) indicated that the pathway was not altered. Expression of other Wnt ligands including Wnt5B, Wnt7, and Wnt11 did not show any significant changes (data not shown). (**E**) Finally, expression of Hh ligands was also unaffected. (N=4 for all samples at e10.5 and e11.5; only e10.5 data shown but e11.5 data had similar negative results, **P*<0.02, ***P*<0.05.) P-values were determined by student T-test.



Supplemental Figure 3 – Temporal expression pattern of *Neurogenin3* in *Pdx1-Cre^{early};Smo$^{flox/null}$* mice. Taqman real time PCR expression pattern for *Neurogenin3* in pancreas tissues isolated from e12.5 through e16.5 showed a trend in shifted peak expression from e13.5, in control mice, to e14.5, in *Pdx1-Cre^{early};Smo$^{flox/null}$* mice. Control mice in blue and *Pdx1-Cre^{early};Smo$^{flox/null}$* mice in red. Inset is magnified graph of neurogenin3 expression at e16.5 timepoint. (N=4 for all time points, **P*<0.05.) P-values were determined by student T-test.



Supplemental Figure 4 – Expression of genes regulating islet function and insulin secretion machinery is not affected. Sybr Green real time PCR of adult islets isolated from *Pdx1-Cre*^{early}*;Smo*$^{flox/null}$ mice. (**A**) Expression of genes involved in islet function is not altered. (**B**) Expression of genes involved in insulin secretion machinery is not altered. (N=4)



Supplemental Figure 5 – *Pdx1-Cre*^{early}*;Smo*$^{flox/null}$ mice have normal insulin serum levels and body weight. Adult *Control* and *Pdx1-Cre*^{early}*;Smo*$^{flox/null}$ mice show comparable (**A**) average body, (N=5) and (**B**) fasting insulin serum levels, (N=7).