The Zinc-finger factor Insm1 (IA-1) is essential for the development of pancreatic β cells and intestinal endocrine cells

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The pancreatic and intestinal primordia contain epithelial progenitor cells that generate many cell types. During development, specific programs of gene expression restrict the developmental potential of such progenitors and promote their differentiation. The Insm1 (insulinoma-associated 1, IA-1) gene encodes a Zinc-finger factor that was discovered in an insulinoma cDNA library. We show that pancreatic and intestinal endocrine cells express Insm1 and require Insm1 for their development. In the pancreas of Insm1 mutant mice, endocrine precursors are formed, but only few insulin-positive β cells are generated. Instead, endocrine precursor cells accumulate that express none of the pancreatic hormones. A similar change is observed in the development of intestine, where endocrine precursor cells are formed but do not differentiate correctly. A hallmark of endocrine cell differentiation is the accumulation of proteins that participate in secretion and vesicle transport, and we find many of the corresponding genes to be down-regulated in Insm1 mutant mice. Insm1 thus controls a gene expression program that comprises hormones and proteins of the secretory machinery. Our genetic analysis has revealed a key role of Insm1 in differentiation of pancreatic and intestinal endocrine cells.

Keywords: Insm1; development; endocrine; intestine; mouse; pancreas

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Endocrine cells in the pancreas and intestine play key roles in nutritional homeostasis through the regulated synthesis and secretion of hormones and growth factors. The islets of Langerhans contain the four principal endocrine cell types of the pancreas, the α, β, δ, and PP cells, which produce the hormones glucagon, insulin, somatostatin, and pancreatic polypeptide (PP), respectively (Edlund 2002; Murtaugh and Melton 2003; Wilson et al. 2003). More recently, a fifth endocrine cell type was identified that expresses ghrelin (Prado et al. 2004). The role of β cells is fundamental for energy homeostasis of the body, and dysfunction or loss of β cells causes diabetes. Endocrine cells are also scattered in the epithelium of the intestine and stomach. These endocrine cells are heterogeneous, and they can be distinguished on the basis of ultrastructure or content of hormone(s) such as substance P, secretin, cholecystokinin (CCK), neurotensin, somatostatin, serotonin and peptide YY (PYY) (Rindi et al. 2004). Interestingly, some basic similarities exist in the molecular mechanisms that control the development of endocrine cells in the intestine and pancreas.

Pancreas development initiates morphologically with the formation of epithelial buds that protrude dorsally and ventrally from the foregut [at embryonic day 9.5 (E9.5) in mice] and later fuse to form the definitive pancreas. These primordia contain progenitor cells that are capable of generating all pancreatic cell types, the endocrine, exocrine, and duct cells (Gu et al. 2003). Specific programs of gene expression restrict the developmental potential of these progenitors and promote their differentiation into the distinct cell types. Significant efforts have been made to identify the mechanisms that control the specification and differentiation of pancreatic endocrine cells, particularly β cells. Such knowledge may provide a basis for the generation of insulin-producing cells from endodermal or embryonic stem (ES) cells (Edlund 2002; Murtaugh and Melton 2003; Wilson et al. 2003). Mutations in genes encoding components of the Notch signaling pathway lead to a premature and exclusive differentiation of endocrine cells, and Notch signaling is, thus, essential to maintain the progenitor pool (Apelqvist et al. 1999; Jensen et al. 2000). The basic helix-loop-helix (bHLH) transcription factors Ngn3 and NeuroD1
are essential factors that control the endocrine fate. Mutation of Ngn3 precludes the development of all endocrine cells in the pancreas, mutation of NeuroD1 reduces their number, and the ectopic expression of Ngn3 or NeuroD1 under the control of the Pdx1 promoter allows premature and ectopic differentiation of islet cells that mainly produce glucagon [Naya et al. 1997; Gradwohl et al. 2000; Schwitzgebel et al. 2000]. Thus, these genes determine an endocrine fate in the pancreas, but the allocation and differentiation of the different cellular subtypes appear to be controlled by other genes. Pax4 promotes the specification of the β- and δ-cell fate, and suppresses the emergence of α cells. Nkx2.2 and Nkx6.1 are both essential for β-cell differentiation: Nkx2.2 activity is required to initiate β-cell differentiation, while Nkx6.1 acts downstream from Nkx2.2 [Sosa-Pineda et al. 1997; Sussel et al. 1998; Sander et al. 2000; Wang et al. 2004; Collombat et al. 2005]. In addition to their roles in the early development of the pancreas, Pdx1 [IPF1], Isl1, Hnf1α, Hnf1β, Hnf4α, or NeuroD1 are also required for differentiation and maintenance of β cells [Edlund 2002; Murtaugh and Melton 2003; Wilson et al. 2003]. Heterozygous mutations of Pdx1, Hnf1α, Hnf1β, Hnf4α, or NeuroD1 in humans cause maturity-onset diabetes of the young (MODY), and further genes that control β-cell development might correspond to susceptibility genes that contribute to the occurrence of diabetes [Bell and Polonsky 2001; Shih and Stoffers 2002; Habener et al. 2005].

The intestinal epithelium contains epithelial progenitors that generate endocrine cells, other secretory cells like goblet and paneth cells, as well as enterocytes. Notch signaling suppresses the development of intestinal endocrine cells, and mutations in genes that encode components of the Notch signaling cascade lead to the appearance of supernumerary entero-endocrine and other secretory cells [Jensen et al. 2000; Fre et al. 2005; van Es et al. 2005]. The bHLH factor Math1 is an important regulator of entero-endocrine development, and mutation of Math1 precludes the differentiation of enteroendocrine, goblet, and paneth cells [Yang et al. 2001]. Gfi1 controls the allocation of subtypes of secretory cells; in Gfi1 mutant mice, supernumerary endocrine cells are formed at the expense of paneth and goblet cells [Shroyer et al. 2005]. Furthermore, Ngn3 is required for the development of all endocrine cell types of the intestine, whereas NeuroD1, Pax4, and Pax6 are necessary for differentiation of subtypes of endocrine cells [Naya et al. 1997; Larsson et al. 1998; Jenny et al. 2002; Lee et al. 2002].

The Insulinoma-associated 1 (Ins1, IA-1) gene encodes a DNA-binding protein with five Zinc-finger domains and is conserved in evolution [Goto et al. 1992]. The human Ins1 gene was first identified in a subtraction library from an insulinoma [Goto et al. 1992] and was subsequently found to be expressed in a large number of insulinomas and other neuroendocrine tumors, as well as in the developing pancreas and nervous system [Goto et al. 1992; Zhu et al. 2002; Breslin et al. 2003]. Available biochemical evidence indicates that expression of Ins1 is controlled by transcription factors of the bHLH family, and that Ins1 acts as a transcriptional repressor [Breslin et al. 2002, 2003]. The homologous genes in Drosophila and Caenorhabditis elegans—nerfin-1 and egl-46, respectively—play essential roles in neuronal development [Wu et al. 2001; Kuzin et al. 2005]. The developmental or physiological roles of Ins1 in vertebrates and the genes controlled by the factor have not been determined. To characterize the function of Ins1, we have introduced a targeted mutation in mice. Analysis of the mutant mice demonstrates that, in Ins1 mutant mice, only few insulin-β cells are produced. Despite the fact that α cells are generated eventually in the correct proportion in Ins1 mutant mice, their differentiation appears to be delayed. In addition, we provide evidence that the differentiation program of pancreatic endocrine cells is not correctly established in Ins1 mutant mice, as reflected in the down-regulation of genes that control hormone processing and secretion. Furthermore, the differentiation of endocrine cells of the intestine is impaired in Ins1 mutant mice. Our analysis indicates that, in the mutant pancreas and intestine, endocrine precursor cells are generated but are blocked in their differentiation. Ins1 thus plays a key role in differentiation of endocrine cells that derive from the endoderm.

**Results**

Ins1 is expressed in the endocrine cells of the pancreas

Ins1 is a single exon gene that is located on mouse chromosome 2. We introduced a targeted mutation into the gene by homologous recombination in ES cells. In the targeting vector, a lacZ cassette was fused in-frame to the Ins1 initiation codon and Ins1 coding sequences were deleted. In addition, the vector contained a neomycin resistance gene (self-excision neo cassette) [Bunting et al. 1999] and a sequence encoding fragment A of diphtheria toxin (DTA) for positive and negative selection in ES cells [Fig. 1A; see also Materials and Methods]. Targeted ES cells were selected, used to generate chimeras, and transmission of the mutant allele through the male germline excised the neo cassette [Fig. 1B]. In the resulting Ins1lacZ allele, exogenous sequences [lacZ and a single loxP site] stay behind to replace the Ins1 coding sequences, but noncoding Ins1 sequences remain intact. Homozygous Ins1lacZ mutant embryos generated by heterozygous matings were normal in size and well developed until E12.5. After E12.5, however, Ins1lacZ/Ins1lacZ embryos were recovered at frequencies that were lower than the expected Mendelian ratios and, at birth, Ins1lacZ/Ins1lacZ mice were apparently unable to breathe and died (for additional details see Materials and Methods).

Ins1 expression was previously reported in insulin-expressing pancreatic cell lines [Zhu et al. 2002]. In situ hybridization analysis demonstrated Ins1 expression in the dorsal pancreatic primordium already at E9.5, and
Insm1 in endocrine development

Figure 1. Generation of the Insm1^lacZ mouse strain and Insm1 expression in the pancreas. (A) Schematic representation of the targeting vector, the wild-type Insm1 locus, and the mutated Insm1 allele, before and after removal of the self-excision neomycin (cre, neo) cassette. Coding (red) and noncoding (pink) Insm1 sequences, NLS-lacZ (blue), DTA, the self-excision neomycin cassette, loxP (arrowhead), and StuI restriction sites are depicted. Black lines indicate the predicted fragment sizes obtained after Stul digestion of genomic DNA. A black bar shows the 5’ sequence used as a probe for Southern analyses shown in B. (B) Southern blot analyses of Stul-digested genomic DNA from wild-type, Insm1^lacZ/+ and Insm1^lacZ/lacZ mice. (C,D) In situ hybridization of E9.5 mouse embryos using an Insm1-specific probe. Insm1 is expressed in the dorsal pancreatic bud (arrows) and the developing central and peripheral nervous system. (E,F) Immunohistochemical analysis of the developing pancreas of Insm1^lacZ/+ embryos at E10.75 using antibodies against β-galactosidase (red) and Pdx1 (green) [E], and β-galactosidase (red), Pdx1 [green], and glucagon [blue] [F]. (G–I) Immunohistochemical analysis of the dorsal pancreas of Insm1^lacZ/+ embryos at E12.5 using antibodies against β-galactosidase (red), and Ngn3 [green] [G], Isl1 [green] [H], and Ptf1a [green] [I]. Coexpression of β-galactosidase with proteins that mark endocrine cells [Isl1, Ngn3, glucagon] is observed; in contrast, Ptf1a is present in exocrine cells and is not coexpressed with β-galactosidase. (J)

Immunohistological analysis of the pancreas of adult Insm1^lacZ/+ mice using antibodies against β-galactosidase (red) and a mixture of antibodies directed against insulin, glucagon, PP, somatostatin, and ghrelin (green). Bars: C, 400 µm; D, 50 µm; E,F,J, 20 µm.

In the dorsal and ventral primordium at E10.5 [Fig. 1C,D, Supplementary Fig. 1], Insm1 expression was observed in the developing central and peripheral nervous system. Heterozygous Insm1^lacZ embryos were used for a detailed analysis of Insm1 expression in the developing pancreas. The epithelium of the dorsal and ventral pancreas is marked by Pdx1, and scattered cells that expressed β-galactosidase were present in the epithelium at E10.75; β-galactosidase+ cells also coexpressed glucagon [Fig. 1E,F]. Many β-galactosidase+ cells were also Ngn3+, Isl1+, or NeuroD1+, and coexpression of β-galactosidase and insulin or glucagon was also detectable at E12.5 [Figs. 1G,H, 2]. This indicates that developing endocrine cells express β-galactosidase. Ptf1a marks the exocrine lineage of the pancreas at E12.5 (Krapp et al. 1996), and was not coexpressed with β-galactosidase at E12.5 or subsequent stages [Figs. 1I, 30]. At E18.5, β-galactosidase+ cells that coexpressed insulin, glucagon, somatostatin, PP, and ghrelin were present in the pancreas of Insm1^lacZ/+ mice [see below]. Insm1 transcripts are also present in adult pancreatic islets. Immunohistological analysis of adult Insm1^lacZ/+ mice indicated that the pancreatic hormones insulin, glucagon, somatostatin, PP, and ghrelin were coexpressed with β-galactosidase, indicating that all endocrine cell types maintain Insm1 expression [Fig. 1], for further details on Insm1 expression, see Supplementary Fig. 1. The majority of β-galactosidase+ cells express a pancreatic hormone at E18.5 (97.6%) and in the adult (99.5%). Thus, Insm1 expression marks endocrine cells in the developing and adult pancreas.

Insm1 is essential for the differentiation of pancreatic endocrine cells

We compared mice heterozygous and homozygous for the Insm1^lacZ allele to assess the function of Insm1 in the development of endocrine cells of the pancreas. In situ hybridization with Pdx1 as a probe demonstrated that the epithelia of the dorsal and ventral pancreatic buds were similar in size in control and mutant mice at E12.5. We observed no change in the levels of Pdx1 protein or in the hybridization intensity at this stage [Supplementary Fig. 2]. At E12.5 or E15.5, numbers of
β-galactosidase+ cells in the pancreas were similar, and a comparable proportion of β-galactosidase+ cells coexpressed NeuroD1 or Isl1 in heterozygous and homozygous Insm1lacZ/− mutant mice (Supplementary Figs. 2, 3). This indicates that early pancreas development and the generation of the early endocrine precursor cells occurred correctly. However, we found profound changes in the subsequent differentiation of endocrine cells in the mutant mice. The first insulin+ cells appear in the dorsal pancreas and coexpress β-galactosidase in Insm1lacZ/+ mice, and we detected similar numbers of insulin+ cells in the dorsal pancreas of heterozygous and homozygous Insm1lacZ/+ mice at E12.5 (Fig. 2A–C). Many β cells arise during the secondary transition, a wave of differentiation of endocrine cells that starts around E13. By E15.5, insulin+ cells are abundant in the dorsal and ventral pancreas of Insm1lacZ/+ mice, but we observed only rare insulin+ cells in the dorsal and ventral pancreas of Insm1lacZ/lacZ mice (Fig. 2D–F). This indicates that β-cell neogenesis during the secondary transition does not occur. Glucagon+ cells differentiate first in the dorsal pancreas, and glucagon+ cells can be observed in the dorsal pancreas of Insm1lacZ/+ and Insm1lacZ/lacZ mice at E12.5. The number of glucagon+ cells was reduced at this stage in Insm1lacZ/lacZ mice, and the mutant α cells contained lower levels of the hormone than the α cells of control mice (Fig. 2G–I). At E15.5, numbers of glucagon+ cells were reduced in the dorsal and ventral pancreas of Insm1lacZ/+ and Insm1lacZ/lacZ mice, and the mutant α cells contained reduced glucagon levels (Fig. 2J–L).

To further assess developing endocrine cells in the Insm1 mutant mice, we analyzed the expression of transcription factors known to control development and maintenance of pancreatic endocrine cells. Pdx1 is known to be essential for the differentiation of β cells (Ahlgren et al. 1998; Sander et al. 2000; Holland et al. 2002; Fujitani et al. 2006). At E15.5, differentiating β cells express high levels of Pdx1 protein; Pdx1high cells coexpress β-galactosidase in Insm1lacZ/+ animals. The number of Pdx1high cells was markedly reduced in the pancreas of Insm1lacZ/lacZ animals. The number of Pdx1high cells was markedly reduced in the pancreas of Insm1lacZ/lacZ mice. In addition, Pdx1 is expressed at low levels in many cells of the pancreatic epithelium in control mice, and this was not changed in the Insm1lacZ/lacZ mutants (Fig. 3A–F). MafA marks developing β cells (Kataoka et al. 2002; Olobrot et al. 2002), and MafA protein was absent in Insm1lacZ/lacZ mice (Fig. 3G, H). In addition, the expression of Nkx2.2, Isl1, and Nkx6.1 was analyzed. Whereas Nkx2.2 and Isl1 expression was similar in con-
Figure 3. Changed expression of transcription factors in the pancreas of Insm1 mutant mice. Immunohistological analysis of transcription factors in the developing dorsal pancreas of Insm1lacZ/+ and Insm1lacZ/lacZ mice at E15.5. The following antibodies were used: (A–F) Pdx1 (green), β-galactosidase (red), and insulin (blue). (G,H) MafA (green) and β-galactosidase (red). (I–N) MafB (green), β-galactosidase (red), and glucagon (blue). (O,P) Ptf1a (green) and β-galactosidase (red). Bar, 20 µm.
control and in \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} mice, Nkx6.1 was down-regulated (Supplementary Fig. 3). We conclude, therefore, that the expression of Pdx1, Nkx6.1, and MafA is altered in \textit{Insm1} mutant mice, indicating that the loss of \textit{Insm1} affects the transcriptional network that controls \(\beta\)-cell differentiation. We further assessed the development of the \(\alpha\)-cell lineage in the \textit{Insm1}^{lacZ} mutant mice. MafB is expressed in developing \(\alpha\) cells of control mice (Artner et al. 2006) and was markedly reduced in the \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} mice (Fig. 3I–N). Arx and Pax6 are expressed in \(\alpha\) cells and are important determinants in the development of this lineage (St-Onge et al. 1997; Collombat et al. 2003). Arx and Pax6 expression was also reduced (Table 1; Supplementary Fig. 3). Thus, the transcriptional network that controls \(\alpha\)-cell differentiation is also changed in the pancreas of \textit{Insm1} mutant mice. In contrast, the exocrine lineage, as assessed by histology and immunohistological analysis of Ptf1a and amylase expression, appeared unchanged in \textit{Insm1} mutant mice (Figs. 3O–P, 4A,B, Supplementary Fig. 4). We conclude, therefore, that the \textit{Insm1} mutation causes major deficits in the differentiation of pancreatic endocrine cells, but does not impair the exocrine lineage.

Histological analysis at \textit{E18.5} demonstrated the presence of islets of Langerhans in the pancreas of heterozygous and homozygous \textit{Insm1}^{lacZ} mutant mice. The islets in the \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} mice, however, displayed an increased density of nuclei compared with control animals [Fig. 4A,B]. We also counted the overall number of \(\beta\)-galactosidase\(^{+}\) endocrine cells in the pancreas, and observed a 26\% reduction at \textit{E18.5} in the mutant mice and no significant differences at \textit{E15.5} (599 ± 48 cells per section and 444 ± 47 cells per section in heterozygous and homozygous \textit{Insm1}^{lacZ} mice at \textit{E18.5}, respectively; \(p\)-value: 0.0079, \(n = 3\) pancreata; 355 ± 82 cells per section and 281 ± 69 cells per section in heterozygous and homozygous \textit{Insm1}^{lacZ} mice at \textit{E15.5}, respectively; \(p\)-value: 0.11, \(n = 4\) pancreata). Apoptosis at late developmental stages appears to account for the reduced cell number, since TUNEL\(^{+}\) cells in the pancreas at \textit{E18.5} were more abundant in homozygous than in heterozygous \textit{Insm1}^{lacZ} mutant mice (Supplementary Fig. 4). We counted the proportion of \(\beta\)-galactosidase\(^{+}\) cells that coexpressed insulin and observed a 92\% reduction in the number of \(\beta\) cells in \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} mice at \textit{E18.5} (Fig. 4C–E). The proportion of \(\beta\)-galactosidase\(^{+}\) cells that coexpressed somatostatin was also reduced in \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} mice (Fig. 4F). The proportion of glucagon\(^{+}\) cells was similar in \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} and \textit{Insm1}^{lacZ}/\textit{Insm1}^{lacZ} mice (Fig. 4G–I). However, mutant \(\alpha\) cells contained less glucagon protein as assessed by the intensity of the immunohistological staining, and glucagon mRNA was down-regulated [Table 1]. A delayed accumulation of glucagon protein might thus account for the reduction in the number of glucagon\(^{+}\) cells observed at earlier stages. Thus, despite the fact that glucagon\(^{+}\) cells are produced in the correct proportion, their differentiation appears to be impaired. The proportions of \(\beta\)-galactosidase\(^{+}\) cells that coexpressed PP or ghrelin were elevated and reduced, respectively, and the propor-

| Symbol | Gene name | Fold change | \(p\)-value |
|--------|-----------|-------------|-------------|
| Hormones | | | |
| Ins1 | Insulin I | -13.1 | ** |
| Ins2 | Insulin II | -13.5 | ** |
| Ggc | Glucagon | -4.1 | ** |
| Ppy | PP | p, nc | ns |
| Ghrl | Ghrelin | p, nc | ns |
| Sst | Somatostatin | -2.5 | ** |
| Npy | Neuropeptide Y | -8.3 | ** |
| Glucose metabolism | Slec2a2 | Glut2 | -2.9 | ** |
| G6pc2 | Glucose-6-phosphatase, islet specific | -19.4 | ** |
| Hormone processing and secretion | | | |
| Pesk1 | Prohormone convertase 1/3 | -2.6 | ** |
| Pesk2 | Prohormone convertase 2 | -2.8 | ** |
| Chga | Chromogranin A | -24.9 | ** |
| Chgb | Chromogranin B | -40.0 | ** |
| Nnat | Neuronatin | -7.2 | ** |
| Resp18 | Regulated endocrine-specific protein 18 | -11.8 | ** |
| Rph3a1 | Rabphilin 3A-like | -2.1 | ** |
| Sgc2 | Secretogranin II | -33.3 | ** |
| Sgc3 | Secretogranin III | -16.6 | ** |
| Sgcn | Secretogamin | -13.6 | ** |
| Sgnl | Secretogranin V | -9.3 | ** |
| Snx5 | Sorting Nexin 5 | -2.5 | ** |
| Syl14 | Synaptotagmin-like 4 | -4.4 | ** |
| Transcription factors | Nkx2-2 | Nkx2.2 | p, nc | ns |
| Nkx6-1 | Nkx6.1 | -1.6 | * |
| Pax4 | Pax4 | p, nc | ns |
| Pax6 | Pax6 | -2.4 | ** |
| Ipf1 | Pdx1 | -1.3 | * |
| MafB | MafB | -1.9 | ** |
| Arx | Arx | a | — |
| Adhesion | Cbd2 | Ncad | p, nc | ns |
| Others | Iapp | Islet amyloid polypeptide | -2.1 | ** |
| Aplp1 | Amyloid \(\beta\) precursor-like protein 1 | -3.1 | ** |
| Gch1 | GTP cyclohydrolase | -2.7 | ** |
| Gat9 | Connexin 36 | -6.6 | ** |
| Gng4 | GTP-binding protein \(\gamma\) subunit | -4.9 | ** |
| Sez6l2 | Seizure-related 6 homolog-like 2 | -25.6 | ** |
| Scl30a8 | Solute carrier family 30 member 8 | -5.3 | ** |

Systematic analysis of gene expression in control and \textit{Insm1}^{lacZ} mice using Affymetrix oligonucleotide microarrays. The average signal fold change and the change \(p\)-value as calculated by Affymetrix MAS 5.0 software are shown. Genes were selected based on a consistent and significant differential expression at \textit{E15.5} and \textit{E18.5}. We also display data for those genes that were analyzed in this study by immunohistological analysis, in situ hybridization, or RT–PCR. Genes whose expression was analyzed by microarray analysis and by additional techniques are marked in bold. Genes whose expression was changed with a \(p\)-value ≤0.05 and ≤0.001 are marked by single or double asterisks, respectively. No probe sets exist for MafA on the MOE 430 2.0 GeneChip. \([p]\) Present; \([a]\) absent; \([nc]\) not changed; \([ns]\) not significant.
tion of ghrelin+/glucagon− cells was unchanged [Fig. 4J–N]. It should be noted that the numbers of γ, PP, or ε cells remained small in the islets of Insm1 mutant mice, indicating that transdifferentiation of the mutant β cells into one of these endocrine cell types did not occur.

In Insm1lacZ/+ mice, the majority of β-galactosidase+ islet cells express one of the pancreatic hormones at E18.5. This was, however, not observable in Insm1lacZ/+ Insm1lacZ mice, where many β-galactosidase+ cells were present that expressed neither insulin, glucagon, somatostatin, PP, nor ghrelin [Fig. 4O–Q]. Thus, other hormone-producing cell types do not form at the expense of β cells in Insm1lacZ/+Insm1lacZ mice, indicating that β cells arrest their differentiation. We investigated if other proteins typically present in endocrine cells were expressed correctly in Insm1lacZ/+Insm1lacZ mice. The glucose transporter 2 (Glut2) and the islet amyloid polypeptide (IAPP) are expressed in β cells of control mice at E18.5. Cells that expressed Glut2 were rare in Insm1lacZ/+Insm1lacZ mice, but many cells remained that expressed low levels of IAPP [Fig. 4R–T; Supplementary Fig. 4]. Furthermore, the prohormone convertase 1/3 as well as chromogranin A, which are expressed broadly in the pancreatic endocrine cell types, were significantly down-regulated in the mutant mice. We analyzed the expression of these differentiation markers, specifically in glucagon+ cells, and found them down-regulated. Thus, mutant α cells expressed not only glucagon at lower levels, but also other differentiation markers like the prohormone convertase 2 and chromogranin A. In addition, changes in gene expression were also analyzed systematically using microarray analysis (Table 1). This indicated changes in transcript levels of pancreatic hormones, prohormone convertases, IAPP, and chromogranin A in the Insm1 mutant mice, and thus verified the reduced levels of the corresponding proteins observed by immunohistological analysis. Interestingly, this systematic analysis revealed an additional set of significantly down-regulated transcripts, whose protein products function in vesicle transport and secretion—for instance, chromogranin B, secretogranin II, III, and V; sorting nexin 5; and synaptogamin-like protein 4. We conclude, therefore, that terminal differentiation of pancreatic en-

Figure 4. Islet histology and hormone expression in the pancreas of Insm1 mutant mice. Histological and immunohistological analyses of the developing pancreas of Insm1lacZ/+ and Insm1lacZ/Insm1lacZ mice at E18.5. The genotypes are indicated by +/− and −/−, respectively. (A,B) Hematoxylin/eosin (HE) staining of semi-thin sections of the pancreas. Islets are present in Insm1lacZ/Insm1lacZ mice but display a changed nuclear density. Immunohistological analysis of the pancreas using antibodies directed against β-galactosidase [shown in red]. In addition, antibodies directed against insulin [blue] and somatostatin [green] (C,D), glucagon [blue] and PP [green] (G,H), and ghrelin [green] (K,L) were used. (O,P) Immunohistology using rabbit anti-insulin, rabbit anti-glucagon, rabbit anti-somatostatin, rabbit anti-PP, and rabbit anti-ghrelin [green], and goat anti-β-galactosidase [red] antibodies simultaneously. Note that many β-galactosidase− cells exist in the pancreas of Insm1lacZ/+Insm1lacZ embryos that do not express any of these five hormones. (R,S) Immunohistological analysis of the pancreas using antibodies against β-galactosidase [shown in red] and IAPP [green]. The proportions of the β-galactosidase− cells that express insulin [E], somatostatin [F], glucagon [G], PP [I], ghrelin [M], ghrelin but not glucagon [N], any of the five hormones [Q], or IAPP [T] are displayed. Single and double asterisks indicate p-values of <0.01 and <0.001, respectively. Bars, 20 µm.
Endocrine cells are observed in a ring of mutant mice, but further analysis showed expression in the developing intestine. Immunohistochemistry of animals co-expressed serotonin and -galactosidase in the intestinal epithelium of mutant mice, and was not expressed in developing intestine of animals (Fig. 6A-C). The proportion of -galactosidase cells in the intestinal epithelium that coexpressed -galactosidase was similar in heterozygous and homozygous mutant mice, expression of -galactosidase persists in the adult intestine [Supplementary Fig. 1]. We conclude that -galactosidase is expressed in developing endocrine cells of the intestine.

We compared the development of endocrine cells in the intestine of mice heterozygous and homozygous for the -galactosidase allele. We observed no obvious difference in the overall number of -galactosidase cells in the intestinal epithelium of mutant and mutant mice at E15.5 or E18.5 (Fig. 6). At E15.5 or E18.5, the proportion of -galactosidase cells in the intestinal epithelium that coexpressed -galactosidase was similar in heterozygous and homozygous mutant mice, expression of -galactosidase was not affected [Supplementary Fig. 5]. This indicates that endocrine precursor cells are formed correctly in mutant mice, but further analysis showed that their differentiation was altered. For instance, the number of cells that expressed chromogranin A was severely reduced and synaptophysin
cells were absent in the intestine of mutant and mutant animals (Fig. 6A-C; data not shown). Subtypes of entero-endocrine cells express a particular hormone, and subtypes that coexpress more than one hormone exist. Neurotensin is expressed by 30% of -galactosidase cells in the epithelium of the small intestine in mutant mice at E15.5 or E18.5. However, neurotensin
cells were not observed in mutant and mutant animals (Fig. 6D-F). Similarly, substance P is expressed by 40% of -galactosidase cells in the intestinal epithelium of mutant animals, and was not present in the mutant mice. The proportions of cells that coexpressed serotonin and -galactosidase, or CCK and -galactosidase, were reduced in mutant mice. Fewer PYY+ cells were observed [data not shown]. In contrast, the proportion of -galactosidase cells that express secretin was similar in heterozygous and homozygous mutant mice [Fig. 6P-R]. Alcian blue and periodic acid-Schiff stainings indicated that paneth and goblet cells were produced correctly [Supplementary Fig. 5]. We conclude, therefore, that -galactosidase is essential for the differentiation of particular subtypes of intestinal endocrine cells, which are either absent [substance P+ or neurotensin+ cells] or formed in reduced numbers [serotonin+, CCK+, or PYY+ cells] in -galactosidase mutant mice.

**Figure 5.** -galactosidase expression in the developing intestine. Immunohistochemical analysis of the developing intestine of -galactosidase mice at E15.5 (A,B) and E18.5 (C-F). (A) -Galactosidase (red) was observed in the intestinal epithelium (arrow) and the enteric nervous system (arrowhead) of -galactosidase mice. (B) -Galactosidase (red) is coexpressed with -galactosidase (green) in entero-endocrine cells located in the intestinal epithelium (arrows), but not in the enteric nervous system (arrowhead); coexpressing cells appear yellow, an overlap of the red and green fluorescence. In the intestinal epithelium, all cells that contained chromogranin A (CA, green) or synaptophysin (Syp, green) (D) coexpressed -galactosidase. In contrast, Mucin2 (Muc2, green) (E) or lysozyme (green) (F) proteins were not observed in the -galactosidase (red) cells. Sections were counterstained with TOTO-3 (blue). Bars: A, 50 μm; C, 20 μm.
Discussion

Insm1, a gene encoding a Zn-finger transcription factor, is expressed in endocrine cells of the pancreas and intestine. Our genetic analysis demonstrates the essential function of Insm1 in the differentiation of pancreatic and intestinal endocrine cells. We show that, in the pancreas, β-cells are severely impaired in their differentiation. α-Cells eventually form in a correct proportion, but their appearance is delayed and they express low levels of glucagon. Furthermore, intestinal endocrine cells do not develop correctly in Insm1 mutant mice. We observed a 25-fold reduction in the numbers of chromogranin A+ cells, and particular subtypes of intestinal endocrine cells are absent or reduced in number. Notch signals and the transcription factors Ngn3, NeuroD1, Pax4, and Pax6 have previously been shown to control the development of endocrine cells in both, the pancreas and intestine, demonstrating that an overlapping set of genes directs the developmental program of these endodermal-derived endocrine lineages (Schonhoff et al. 2004; Habener et al. 2005). The phenotypes that we observed indicate that Insm1 determines particular aspects of the differentiation program of endocrine cells and acts downstream from Notch and Ngn3, which promote an endocrine fate.

The function of Insm1 in development of the endocrine pancreas

The mature pancreas contains endocrine, exocrine, and ductal cells, which develop from a single progenitor cell type (Gu et al. 2003). A number of genes have been identified that specifically control the development of the endocrine lineage. Among those, Ngn3 promotes an endocrine fate, Pax4 and Arx determine whether an endocrine progenitor cell adopts an α- or β-cell fate, whereas Nkx2.2 and Nkx6.1 control the differentiation of pancreatic β-cells (Sussel et al. 1998; Gradwohl et al. 2000; Sander et al. 2000; Schwitzgebel et al. 2000; Prado et al. 2004). In addition to its early function in the development of the pancreas, Pdx1 is also essential for β-cell differentiation (Jonsson et al. 1994; Stoffers et al. 1997; Fujitani et al. 2006). Here we identified a novel factor, Insm1, to be essential for the development of the pancreatic β-cell lineage. The Insm1 gene was previously shown to be expressed in insulinomas, other endocrine tumors, and the pancreas (Goto et al. 1992; Zhu et al. 2002), but the function of this Zn-finger factor had not been assessed. Our genetic analysis using Insm1 mutant mice demonstrates that Insm1 is indispensable for the correct differentiation of the β-cell lineage. In Insm1 mutant mice, many β-cell precursors appear to be maintained, but these do not express any of the pancreatic hormones. This phenotype is not identical to the one observed in Nkx2.2, Pax4, and Nkx6.1 mutant mice: In Nkx2.2 or Pax4 mutant mice, ghrelin-expressing cells replace β-cells, whereas in Nkx6.1 mutants, β-cell neogenesis is defective, but undifferentiated endocrine cells do not accumulate (Sander et al. 2000; Prado et al. 2004). Immunohistological and microarray analyses reveal a
down-regulation of Nkx6.1 and Pdx1 in the pancreas of Insm1 mutant mice, indicating that Insm1 affects the transcriptional network that determines β-cell differentiation.

In the Insm1lacZ mutant allele, Insm1 coding sequences were replaced by lacZ. This allowed us to follow cells that express the allele in heterozygous and homozygous mutant mice. Our analysis indicates that Insm1 is expressed in all endocrine cell types of developing and adult pancreatic islets, i.e., in α, β, PP, δ, and ε cells. In Insm1 mutant mice, endocrine precursor cells were generated, as assessed by the expression of Isl1, NeuroD1, and Ngn3, but their differentiation was impaired. The development of δ cells was most severely affected. In particular, we noted a massive reduction in the number of insulin− cells in Insm1 mutant mice, and many proteins present in δ cells of control mice (Glut2, IAPP, prohormone convertase 1/3, chromogranin A) were strongly down-regulated. Furthermore, we did not observe an up-regulation of genes that would be indicative of a transdifferentiation of cells in the mutant pancreas and, in particular, other pancreatic endocrine cell types did not replace the mutant β cells. The mutant β cells retained, however, the expression of β-galactosidase and some expressed also IAPP at low levels, indicating that they keep the character of pancreatic endocrine cells, and we propose that they are arrested in their terminal differentiation. In addition, we observed changes in the proportions of endocrine cells that express somatostatin or PP in Insm1lacZ mutant mice. Lineage analysis has indicated that β, δ, and PP cells derive from a common lineage (Herrera et al. 1994; Herrera 2000). Our data indicate, therefore, that the molecular networks that ensure that these endocrine cell types are formed in correct proportion are also affected by the Insm1 mutation.

Not only β cells require Insm1 for differentiation; we also observed significant changes in the development of the α-cell lineage. Analysis of the numbers of glucagon+ cells indicated that they were initially reduced [E12.5, E15.5], but eventually these cells were present in correct proportion [E18.5], indicating that the effects of Insm1 on early and late α cells might be distinct. Nevertheless, the expression levels of glucagon in the mutant α cells remained low. Furthermore, other proteins [prohormone convertases 1/3 and 2, chromogranin A] that are expressed in many pancreatic endocrine cell types and are present in α and β cells were markedly down-regulated in Insm1 mutant mice. Thus, despite the fact that α cells form in a correct proportion, their terminal differentiation is impaired. By microarray analysis, we found additional genes that function in hormone maturation, secretion, and vesicle transport to be down-regulated in the pancreas of Insm1 mutant mice, indicating that Insm1 controls a genetic program that allows pancreatic hormone production and secretion. It is possible that the expression of some of these genes is altered not only in α and β cells, but also in the other subtypes of pancreatic endocrine cells. Available biochemical evidence indicates that Insm1 acts as a transcriptional repressor. Members of the snail family of Zn-finger transcription factors are related to Insm1, display a similar overall domain structure—i.e., a “SNAG” domain and five Zn-fingers—and are known to act as transcriptional repressors (Grimes et al. 1996). However, in our microarray analysis, genes that were significantly and consistently up-regulated at E15.5 and E18.5 were not observed in the mutant mice, raising the possibility that Insm1 might also function as a transcriptional activator. It is noteworthy that immunohistological analysis had revealed abnormally high levels of N-cadherin in pancreatic islets of Insm1 mutant mice (Supplementary Fig. 4), but microarray analysis indicated no significant change in the level of the corresponding transcript. Changes in the turnover of N-cadherin protein might be responsible for this and could contribute to the altered islet morphology in Insm1 mutant mice.

The function of Insm1 in the development of intestinal endocrine cells

Our analysis of Insm1 mutant mice demonstrates that this factor is essential not only for development of pancreatic endocrine cells, but also for the differentiation of endocrine cells of the intestine. The intestinal epithelium contains secretory cells (endocrine, paneth, and goblet cells) and absorptive enterocytes, which all derive from multipotent epithelial stem cells (Cohn et al. 1991). This stem cell does not appear to produce directly intestinal endocrine cells but generates a series of intermediary cells that become further restricted in their developmental potential. Available evidence indicates that a committed progenitor exists that gives rise to all secretory cell types (Yang et al. 2001). This progenitor produces cells that generate either the paneth/goblet or the endocrine lineage (Shroyer et al. 2005). The endocrine precursor expresses Ngn3 and requires Ngn3 for its formation, whereas NeuroD1 functions downstream from Ngn3 to coordinate cell cycle exit and terminal differentiation (Mutoh et al. 1998; Jenny et al. 2002). Using the Insm1lacZ allele, we demonstrate here that Insm1 is expressed in developing intestinal endocrine cells. The extensive coexpression of β-galactosidase and NeuroD1 at E15.5 indicates that Insm1 is expressed mainly in postmitotic cells destined to form the entero-endocrine lineage. Differentiated entero-endocrine cell types express chromogranin A and synaptophysin (Rindi et al. 2004; Sancho et al. 2004). β-Galactosidase is coexpressed with chromogranin A and synaptophysin in heterozygous Insm1lacZ animals, and the proportion of coexpressing cells increases as development proceeds. This indicates that β-galactosidase expression initiates prior to the differentiation of entero-endocrine cells and persists in the differentiating cells. In homozygous Insm1lacZ mutant mice, β-galactosidase+ cells form in the intestinal epithelium and coexpress NeuroD1, demonstrating that entero-endocrine precursor cells are generated. However, the Insm1 mutation affected their further differentiation; i.e., the expression of hormones and of secretory vesicle proteins like chromogranin A and synaptophysin. Interestingly, chromogranin A expression was down-
regulated in intestinal and pancreatic endocrine cells of Insm1 mutant mice, indicating that similarities in the gene expression program controlled by Insm1 might exist in these two organs. We conclude that Insm1 controls endocrine differentiation in the intestine and pancreas and acts downstream from Notch and Ngn3 that specify the endocrine fate in both organs.

**Insm1 and pancreatic β cells**

Impaired function or loss of pancreatic β cells cause diabetes, a prevalent human disease throughout the world. We demonstrate here that β-cell differentiation is arrested in Insm1 mutant mice. Insm1 mutant mice appear unable to breathe and die at birth, indicating that other, as-yet-uncharacterized phenotypes are present in these mice. The postnatal lethality has precluded the analysis of the consequences of the Insm1 mutation on nutritional homeostasis. Many genes that contribute to the occurrence of diabetes encode transcription factors that play key roles in development of pancreatic β cells (Bell and Polonsky 2001; Shih and Stoffel 2002, Habener et al. 2005). Further studies are required to assess whether Insm1, beyond its role in β-cell differentiation, is also essential for the maintenance and the correct function of β cells.

While this manuscript was under review, a study on Insm1 expression and function appeared in press (Melitzer et al. 2006). This paper demonstrates that Insm1 is not expressed in Ngn3 mutant mice, and provides evidence that Insm1 is a direct target gene regulated by Ngn3. Furthermore, after application of Insm1 morpholinos to cultured pancreatic tissue, the authors report a reduction in numbers of developing insulin+ and glucagon+ cells, confirming, thus, our data obtained in genetically modified mice.

**Materials and methods**

**Generation of Insm1-null mice**

The 129/Sv mouse PAC clone RPCIP711L1640Q2 [Resource Center/Primary Database, http://www.rzpd.de] containing Insm1 was isolated from the RPCI-21 library (Osogawa et al. 2000). A 14-kb DNA fragment containing the Insm1 gene was isolated by gap repair (Lee et al. 2001). Homologous recombination in bacteria [Lee et al. 2001] was used to fuse an NLS-lacZ cassette to the ATG of Insm1, to introduce the self-excision neo cassette (Bunting et al. 1999), and to delete the coding sequence of Insm1. In addition, the MC1-diphtheria toxin A ([DTA] cassette) was placed at the 5′ end of the vector and was used for negative selection. E14.1 ES cells [129/Ola] were electroporated, and colonies that had incorporated the targeting vector into their genome were selected by G418 and analyzed for homologous recombination by Southern blot analysis using 5′ and 3′ sequences that lie outside of the targeting vector, as well as lacZ sequences as probes. We injected blastocysts and identified chimeras that transmitted the mutant Insm1lacZ+ gene by mating chimeras to C57BL/6 females. The mutant strain was subsequently expanded by mating Insm1lacZ males to C57BL/6 females. Routine genotyping was performed by PCR and, occasionally, genotypes were verified by Southern blot hybridization. Lines deriving from two independently targeted ES cells were established. One line was used for the characterization of Insm1 function, and the other for verification of the phenotype.

Embryos derived from heterozygous matings were isolated at different stages of development. Insm1−/−/Insm1lacZ+ embryos were observed at the expected Mendelian frequency at E12.5 and earlier stages, but were recovered at lower ratios subsequently. At E18.5, only four out of 76 mice (5.2%) generated by heterozygous matings had an Insm1lacZ+/Insm1lacZ genotype, indicating that the majority of Insm1lacZ−/Insm1lacZ embryos died during the second half of gestation on the mixed 129/Ola and C57BL/6 genetic background. Surviving Insm1lacZ−/Insm1lacZ males on the 129/Ola and C57BL/6 background were apparently well developed and normal in size at E18.5. The Insm1lacZ− allele was then crossed for two to three generations onto the CD1 out-bred strain. On such a mixed genetic background, the lethality was reduced, and 60 out of 451 (13%) mice generated by heterozygous matings had an Insm1lacZ−/Insm1lacZ genotype at E18.5. Again, the surviving mice were apparently well developed and normal in size. We used animals of a mix of the CD1, 129/Ola, and C57BL/6 background to generate the homozygous mice used for the analysis presented here. In addition, we also compared the pancreas of Insm1lacZ+/Insm1lacZ and Insm1lacZ−/− mice on the mixed 129/Ola and C57BL/6 background, and observed very few β cells and a comparable number of α cells at E18.5 (see Supplementary Fig. 4). Regardless of the genetic background, homozygous Insm1lacZ− mice remained cyanotic—i.e., appeared to be unable to breathe—and died shortly after birth.

**In situ hybridization, histology, and immunohistology**

In situ hybridization and histological analysis of mouse embryos were performed as previously described (Britsch et al. 1998). A 510-base-pair (bp) fragment located in the 3′ untranslated region (UTR) of the mouse Insm1 gene was used to generate the RNA probe. For immunohistological analysis, tissue was dissected and fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) for 2 h. Intestinal tissues were flushed gently with PBS prior to fixation. Antibody staining was performed on 10- to 12-µm cryosections, using the following antibodies: goat anti-β-galactosidase [1:1000, Biogenes]; rabbit anti-β-galactosidase [1:10,000, ICN Biochemical]; rabbit anti-glucagon [1:1000, ImmunoStar]; guinea pig anti-glucagon [1:500, Linco]; rabbit anti-insulin [1:1000, ImmunoStar]; guinea pig anti-insulin [1:1000, Biogenes]; rabbit anti-PP [1:500, Chemicon]; rabbit anti-somatostatin [1:500, DakoCytomation]; rabbit anti-Pdx1 [1:500, Chemicon]; goat anti-NeuroD1 [1:500, Santa Cruz]; guinea pig anti-Isl1 [1:20,000, a gift from Tom Jessell, Columbia University, New York]; guinea pig anti-NKX6.1 [1:500, a gift from Maike Sander, University of California at Irvine, Irvine, CA]; rabbit anti-Arx [1:500, a gift from Ahmed Mansouri, Max Plank Institute for Biophysical Chemistry, Göttingen, Germany]; guinea pig anti-MafA [1:20,000]; rabbit anti-Ptf1a [1:500, a gift from Helena Edlund, University of Umea, Umea, Sweden]; rabbit anti-secretin [1:50, Abcam]; rabbit anti-neurotensin [1:3000, Sigma]; rabbit anti-lysozyme [1:500, DakoCytomation]; rabbit anti-CKC [1:250, Bioretent]; rabbit anti-PYY [1:6000, Bioretent]; rabbit anti-chromogranin A [1:1000, ImmunoStar]; rabbit anti-synaptophysin [Zymed]; rabbit anti-serotonin [1:5000, Sigma]; rabbit anti-substance P [1:1000, Zymed]; rabbit anti-mucin 2 [1:5000, Santa Cruz Biotechnology]; rabbit anti-neurogenin1 [1:2000, a gift from Michael German, University of California at San Francisco, San Francisco, CA]; guinea pig anti-MafB [1:20,000], rabbit anti-ghrelin [1:2000, a gift from Cathrine Tomasetto, Institut National
de la Santé et de la Recherche Médicale [INSERM], Strasbourg, France), goat anti-ghrelin [1:1000, Santa Cruz Biotechnology], rabbit anti-iAPP [1:1000, Progen], guinea pig anti-Nkx2.2 [1:4000], a gift from Beatriz Sosa-Pineda, St. Jude’s Children’s Hospital, Memphis, TN), rabbit anti-proliferating cell nuclear antigen (PCNA [1:100; Chemicon], rabbit anti-proliferating cell nuclear antigen (PCNA [1:100; Chemicon], guinea pig anti-Nkx2.2 [1:4000], a gift from Beatriz Sosa-Pineda, St. Jude’s Children’s Hospital, Memphis, TN), rabbit anti-proliferating cell nuclear antigen (PCNA [1:100; Chemicon], goat anti-Insulin [1:1000, Santa Cruz Biotechnology], rabbit anti-N-Cadherin [1:100, Santa Cruz Biotechnology], rabbit anti-amylose [1:100; Santa Cruz Biotechnology], and secondary antibodies conjugated with Cy2, Cy3, or Cy5 [Jackson Immunoresearch]. Intestinal sections were counterstained with the nuclear dye TOTO-3 [1:10,000; Molecular Probes]. Cell death was determined by TUNEL staining using an Apop-Tag fluorescein in situ apoptosis detection kit (Intergen). Fluorescence was imaged on a Zeiss LSM 5 Pascal confocal microscope, and images were processed in Adobe Photoshop.

**Cell counts**

To determine the proportion of β-galactosidase+ cells that express a particular pancreatic hormone, we used images taken from sections that were stained for β-galactosidase as well as for one of the hormones (insulin, glucagon, somatostatin, PP, or ghrelin). Cells that expressed β-galactosidase and a particular hormone were counted on at least three sections obtained from three to four independent heterozygous and homozygous Ins11lacZ mice. To determine the proportion of β-galactosidase+ cells that coexpress each of the hormones, at least 300 β-galactosidase+ cells were analyzed at E15.5 and E18.5, and at least 100 β-galactosidase+ cells were analyzed at E12.5. The overall number of β-galactosidase+ cells was essentially determined as described (Collombat et al. 2005). The absolute numbers of β-galactosidase+ cells and the proportion of hormone+ cells can be used to calculate the absolute numbers of the cells that express a particular hormone. A comparison of the calculated absolute numbers—for instance, of α and β cells—shows a pronounced reduction of β cells and similar numbers of α cells when heterozygous and homozygous Ins11lacZ mice were compared.

In the intestine, cells that coexpress β-galactosidase and various markers of endocrine cells were counted from sections obtained from three to four independent heterozygous and homozygous Ins11lacZ mice. To determine the proportion of β-galactosidase+ cells that coexpress hormones/proteins, at least 100 β-galactosidase+ cells were analyzed. To determine the significance of the observed differences, a Student’s t-test for a one-tailed distribution and a two-sample unequal variance was applied.

**Microarray analysis**

Whole pancreata were collected in RNAlater (Ambion) from E15.5 and E18.5 wild-type and Ins11lacZ/Ins11lacZ embryos and homogenized in Trizol (Invitrogen). RNA extraction, probe synthesis, and hybridization to Affymetrix MOE430 2.0 microarrays (Affymetrix) were performed according to the manufacturer’s protocol. Further data processing and identification of differentially expressed genes were carried out in the R environment for statistical computing (R Development Core Team 2005) using the Bioconductor base installation (Gentleman et al. 2004) and packages affyPLM, gcrma, and limma. Briefly, array quality was assessed with affyPLM, and data were normalized with gcrma. Probe sets with low variance of expression across all arrays were filtered out, and differentially expressed genes were identified using the empirical Bayes-modernized t-test implemented in the limma package. P-values associated with the t-statistics were adjusted using a false-discovery-rate approach to compensate for multiple testing. Genes were considered differentially expressed if the difference of their expression level had a p-value of ≤0.05.

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