

# The Zinc-finger factor *Insm1* (IA-1) is essential for the development of pancreatic $\beta$ 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  $\beta$  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  $\alpha$ ,  $\beta$ ,  $\delta$ , 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  $\beta$  cells is fundamental for energy homeostasis of the body, and dysfunction or loss of  $\beta$  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  $\beta$  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

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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  $\beta$ - and  $\delta$ -cell fate, and suppresses the emergence of  $\alpha$  cells. *Nkx2.2* and *Nkx6.1* are both essential for  $\beta$ -cell differentiation: *Nkx2.2* activity is required to initiate  $\beta$ -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*, *Hnf1 $\alpha$* , *Hnf1 $\beta$* , *Hnf4 $\alpha$* , or *NeuroD1* are also required for differentiation and maintenance of  $\beta$  cells (Edlund 2002; Murtaugh and Melton 2003; Wilson et al. 2003). Heterozygous mutations of *Pdx1*, *Hnf1 $\alpha$* , *Hnf1 $\beta$* , *Hnf4 $\alpha$* , or *NeuroD1* in humans cause maturity-onset diabetes of the young (MODY), and further genes that control  $\beta$ -cell development might correspond to susceptibility genes that contribute to the occurrence of diabetes (Bell and Polonsky 2001; Shih and Stoffel 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 endocrine, 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* (*Insm1*, *IA-1*) gene encodes a DNA-binding protein with five Zinc-finger domains and is conserved in evolution (Goto et al. 1992). The human *Insm1* 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 expres-

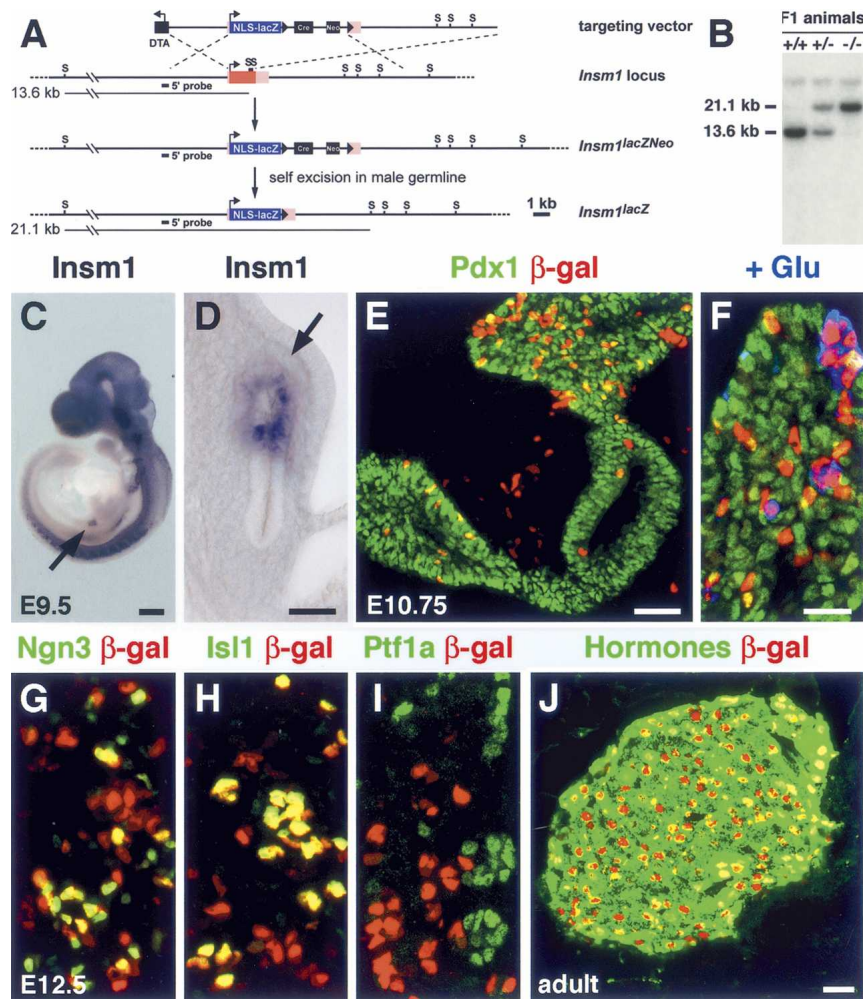
sion of *Insm1* is controlled by transcription factors of the bHLH family, and that *Insm1* acts as a transcriptional repressor (Breslin et al. 2002, 2003). The homologous genes in *Drosophila* and *Caenorhabditis elegans*—*nerfin-1* and *egl-46*, respectively—play essential roles in neuronal development (Wu et al. 2001; Kuzin et al. 2005). The developmental or physiological roles of *Insm1* in vertebrates and the genes controlled by the factor have not been determined. To characterize the function of *Insm1*, we have introduced a targeted mutation in mice. Analysis of the mutant mice demonstrates that, in *Insm1* mutant mice, only few insulin<sup>+</sup>  $\beta$  cells are produced. Despite the fact that  $\alpha$  cells are generated eventually in the correct proportion in *Insm1* 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 *Insm1* mutant mice, as reflected in the downregulation of genes that control hormone processing and secretion. Furthermore, the differentiation of endocrine cells of the intestine is impaired in *Insm1* mutant mice. Our analysis indicates that, in the mutant pancreas and intestine, endocrine precursor cells are generated but are blocked in their differentiation. *Insm1* thus plays a key role in differentiation of endocrine cells that derive from the endoderm.

## Results

### *Insm1* is expressed in the endocrine cells of the pancreas

*Insm1* 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 *Insm1* initiation codon and *Insm1* 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 *Insm1<sup>lacZ</sup>* allele, exogenous sequences (*lacZ* and a single *loxP* site) stay behind to replace the *Insm1* coding sequences, but noncoding *Insm1* sequences remain intact. Homozygous *Insm1<sup>lacZ</sup>* mutant embryos generated by heterozygous matings were normal in size and well developed until E12.5. After E12.5, however, *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* embryos were recovered at frequencies that were lower than the expected Mendelian ratios and, at birth, *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* mice were apparently unable to breathe and died (for additional details see Materials and Methods).

*Insm1* expression was previously reported in insulin-expressing pancreatic cell lines (Zhu et al. 2002). In situ hybridization analysis demonstrated *Insm1* expression in the dorsal pancreatic primordium already at E9.5, and



**Figure 1.** Generation of the *Insm1<sup>lacZ</sup>* 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 (S) restriction sites are depicted. Black lines indicate the predicted fragment sizes obtained after StuI 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 StuI-digested genomic DNA from wild-type, *Insm1<sup>lacZ</sup>/+*, and *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* 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) Immunohistological analysis of the developing pancreas of *Insm1<sup>lacZ</sup>/+* 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) Immunohistological analysis of the dorsal pancreas of *Insm1<sup>lacZ</sup>/+* 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)

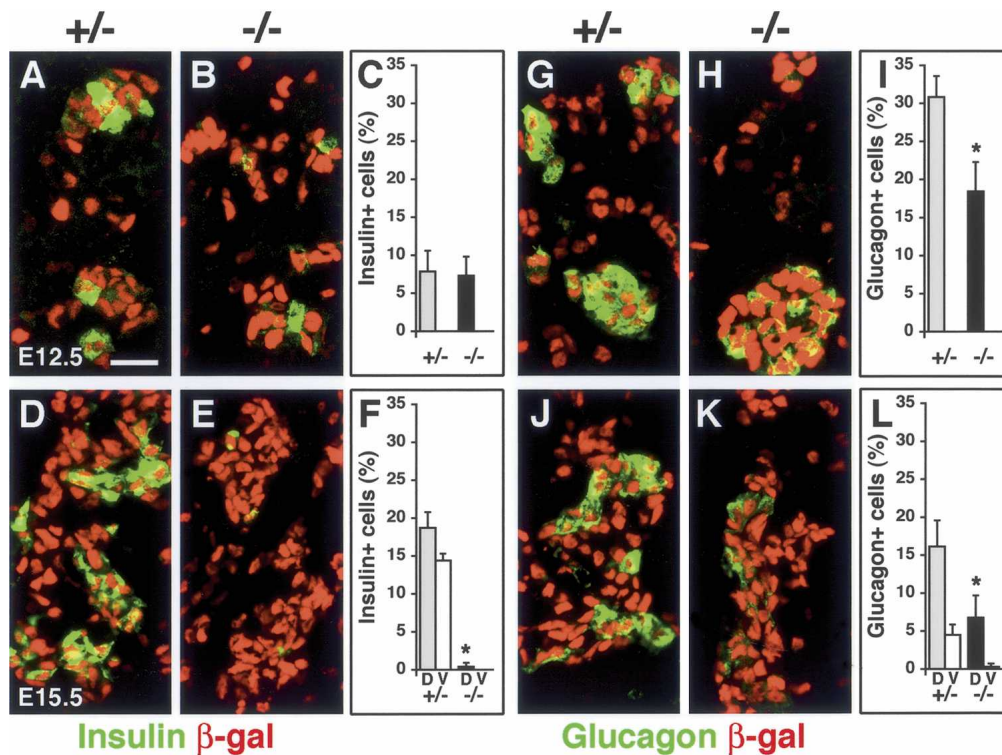
Immunohistological analysis of the pancreas of adult *Insm1<sup>lacZ</sup>/+* mice using antibodies against β-galactosidase (red) and a mixture of antibodies directed against insulin, glucagon, PP, somatostatin, and ghrelin (green). Bars: C, 400 μm; D, 50 μm; E,F,I, 20 μm.

in the dorsal and ventral primordium at E10.5 (Fig. 1C,D; Supplementary Fig. 1). In addition, widespread *Insm1* expression was observed in the developing central and peripheral nervous system. Heterozygous *Insm1<sup>lacZ</sup>* 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<sup>+</sup> cells also coexpressed glucagon (Fig. 1E,F). Many β-galactosidase<sup>+</sup> cells were also Ngn3<sup>+</sup>, Isl1<sup>+</sup>, or NeuroD1<sup>+</sup>, 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, 3O). At E18.5, β-galactosidase<sup>+</sup> cells that coexpressed insulin, glucagon, somatostatin, PP, and ghrelin were present in the pancreas of *Insm1<sup>lacZ</sup>/+* mice (see below). *Insm1* transcripts are also present in adult pancreatic islets. Immunohistological analysis of adult *Insm1<sup>lacZ</sup>/+* mice in-

dicated 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. 1J; for further details on *Insm1* expression, see Supplementary Fig. 1). The majority of β-galactosidase<sup>+</sup> 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<sup>lacZ</sup>* 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

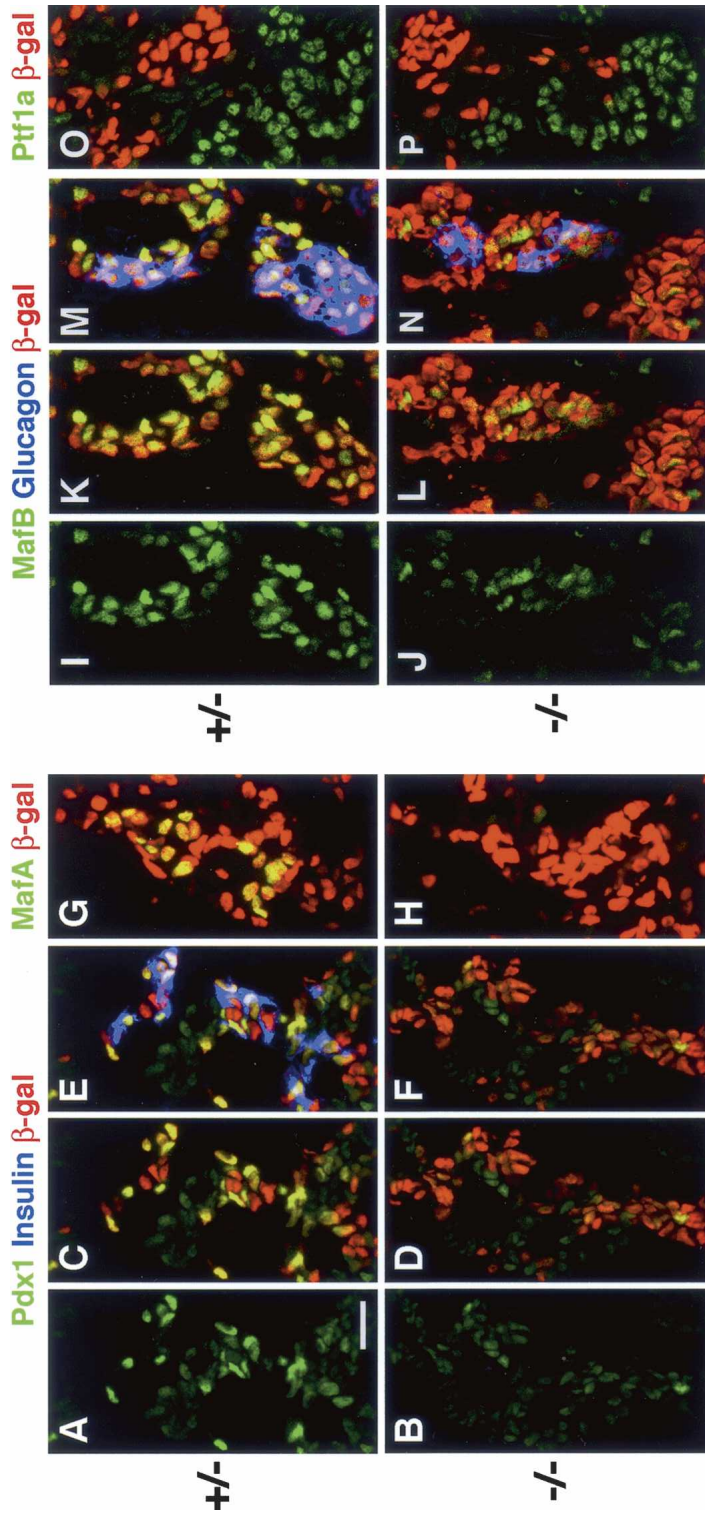


**Figure 2.** Impaired differentiation of  $\alpha$  and  $\beta$  cells in the pancreas of *Insm1* mutant mice. Immunohistological analysis of the developing dorsal pancreas of *Insm1<sup>lacZ/+</sup>* and *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice at E12.5 (A–C, G–I) and E15.5 (D–F, J–L); the genotypes are indicated by +/- and -/-. The antibodies used were directed against  $\beta$ -galactosidase (red) and insulin (green) (A, B, D, E), and  $\beta$ -galactosidase (red) and glucagon (green) (G, H, J, K). (C, I) Proportions of  $\beta$ -galactosidase<sup>+</sup> cells that express insulin (C) and glucagon (I) in the dorsal pancreas in *Insm1<sup>lacZ/+</sup>* (gray column) and in *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* (black column) mice at E12.5. (F, L) Proportions of  $\beta$ -galactosidase<sup>+</sup> cells that express insulin (F) or glucagon (L) in the dorsal (D) and ventral (V) pancreas of *Insm1<sup>lacZ/+</sup>* and *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice at E15.5. Asterisks indicate *p*-values of <0.01. Bar, 20  $\mu$ m.

$\beta$ -galactosidase<sup>+</sup> cells in the pancreas were similar, and a comparable proportion of  $\beta$ -galactosidase<sup>+</sup> cells coexpressed NeuroD1 or Isl1 in heterozygous and homozygous *Insm1<sup>lacZ</sup>* 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<sup>+</sup> cells appear in the dorsal pancreas and coexpress  $\beta$ -galactosidase in *Insm1<sup>lacZ/+</sup>* mice, and we detected similar numbers of insulin<sup>+</sup> cells in the dorsal pancreas of heterozygous and homozygous *Insm1<sup>lacZ</sup>* mutant mice at E12.5 (Fig. 2A–C). Many  $\beta$  cells arise during the secondary transition, a wave of differentiation of endocrine cells that starts around E13. By E15.5, insulin<sup>+</sup> cells are abundant in the dorsal and ventral pancreas of *Insm1<sup>lacZ/+</sup>* mice, but we observed only rare insulin<sup>+</sup> cells in the dorsal and ventral pancreas of *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice (Fig. 2D–F). This indicates that  $\beta$ -cell neogenesis during the secondary transition does not occur. Glucagon<sup>+</sup> cells differentiate first in the dorsal pancreas, and glucagon<sup>+</sup> cells can be observed in the dorsal pancreas of *Insm1<sup>lacZ/+</sup>* and *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice at E12.5. The number of glucagon<sup>+</sup> cells was reduced at this stage in *Insm1<sup>lacZ/</sup>*

*Insm1<sup>lacZ</sup>* mice, and the mutant  $\alpha$  cells contained lower levels of the hormone than the  $\alpha$  cells of control mice (Fig. 2G–I). At E15.5, numbers of glucagon<sup>+</sup> cells were reduced in the dorsal and ventral pancreas of *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice, and the mutant  $\alpha$  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  $\beta$  cells (Ahlgren et al. 1998; Sander et al. 2000; Holland et al. 2002; Fujitani et al. 2006). At E15.5, differentiating  $\beta$  cells express high levels of *Pdx1* protein; *Pdx1<sup>high</sup>* cells coexpress  $\beta$ -galactosidase in *Insm1<sup>lacZ/+</sup>* animals. The number of *Pdx1<sup>high</sup>* cells was markedly reduced in the pancreas of *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mutants. 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 *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mutants (Fig. 3A–F). *MafA* marks developing  $\beta$  cells (Kataoka et al. 2002; Olbrot et al. 2002), and *MafA* protein was absent in *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* 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 *Insm1<sup>lacZ/+</sup>* and *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice at E15.5. The following antibodies were used: (A–F) Pdx1 (green),  $\beta$ -galactosidase (red), and insulin (blue). (G,H) MafA (green) and  $\beta$ -galactosidase (red). (I–N) MafB (green),  $\beta$ -galactosidase (red), and glucagon (blue). (O,P) Ptf1a (green) and  $\beta$ -galactosidase (red). Bar, 20  $\mu$ m.

trol and in *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* mice, Nkx6.1 was down-regulated (Supplementary Fig. 3). We conclude, therefore, that the expression of Pdx1, Nkx6.1, and MafA is altered in *Insm1* mutant mice, indicating that the loss of *Insm1* affects the transcriptional network that controls  $\beta$ -cell differentiation. We further assessed the development of the  $\alpha$ -cell lineage in the *Insm1<sup>lacZ</sup>* mutant mice. MafB is expressed in developing  $\alpha$  cells of control mice (Artner et al. 2006) and was markedly reduced in the *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* 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 *Insm1* mutant mice. In contrast, the exocrine lineage, as assessed by histology and immunohistological analysis of Ptf1a and amylase expression, appeared unchanged in *Insm1* mutant mice (Figs. 3O,P, 4A,B; Supplementary Fig. 4). We conclude, therefore, that the *Insm1* mutation causes major deficits in the differentiation of pancreatic endocrine cells, but does not impair the exocrine lineage.

Histological analysis at E18.5 demonstrated the presence of islets of Langerhans in the pancreas of heterozygous and homozygous *Insm1<sup>lacZ</sup>* mutant mice. The islets in the *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* mice, however, displayed an increased density of nuclei compared with control animals (Fig. 4A,B). We also counted the overall number of  $\beta$ -galactosidase<sup>+</sup> endocrine cells in the pancreas, and observed a 26% reduction at E18.5 in the mutant mice and no significant differences at E15.5 (599  $\pm$  48 cells per section and 444  $\pm$  47 cells per section in heterozygous and homozygous *Insm1<sup>lacZ</sup>* mice at E18.5, respectively; *p*-value: 0.0079, *n* = 3 pancreata; 355  $\pm$  82 cells per section and 281  $\pm$  69 cells per section in heterozygous and homozygous *Insm1<sup>lacZ</sup>* mice at 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<sup>+</sup> cells in the pancreas at E18.5 were more abundant in homozygous than in heterozygous *Insm1<sup>lacZ</sup>* mutant mice (Supplementary Fig. 4). We counted the proportion of  $\beta$ -galactosidase<sup>+</sup> cells that coexpressed insulin and observed a 92% reduction in the number of  $\beta$  cells in *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* mice at E18.5 (Fig. 4C–E). The proportion of  $\beta$ -galactosidase<sup>+</sup> cells that coexpressed somatostatin was also reduced in *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* mice (Fig. 4F). The proportion of glucagon<sup>+</sup> cells was similar in *Insm1<sup>lacZ</sup>/+* and *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* 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<sup>+</sup> cells observed at earlier stages. Thus, despite the fact that glucagon<sup>+</sup> cells are produced in the correct proportion, their differentiation appears to be impaired. The proportions of  $\beta$ -galactosidase<sup>+</sup> cells that coexpressed PP or ghrelin were elevated and reduced, respectively, and the propor-

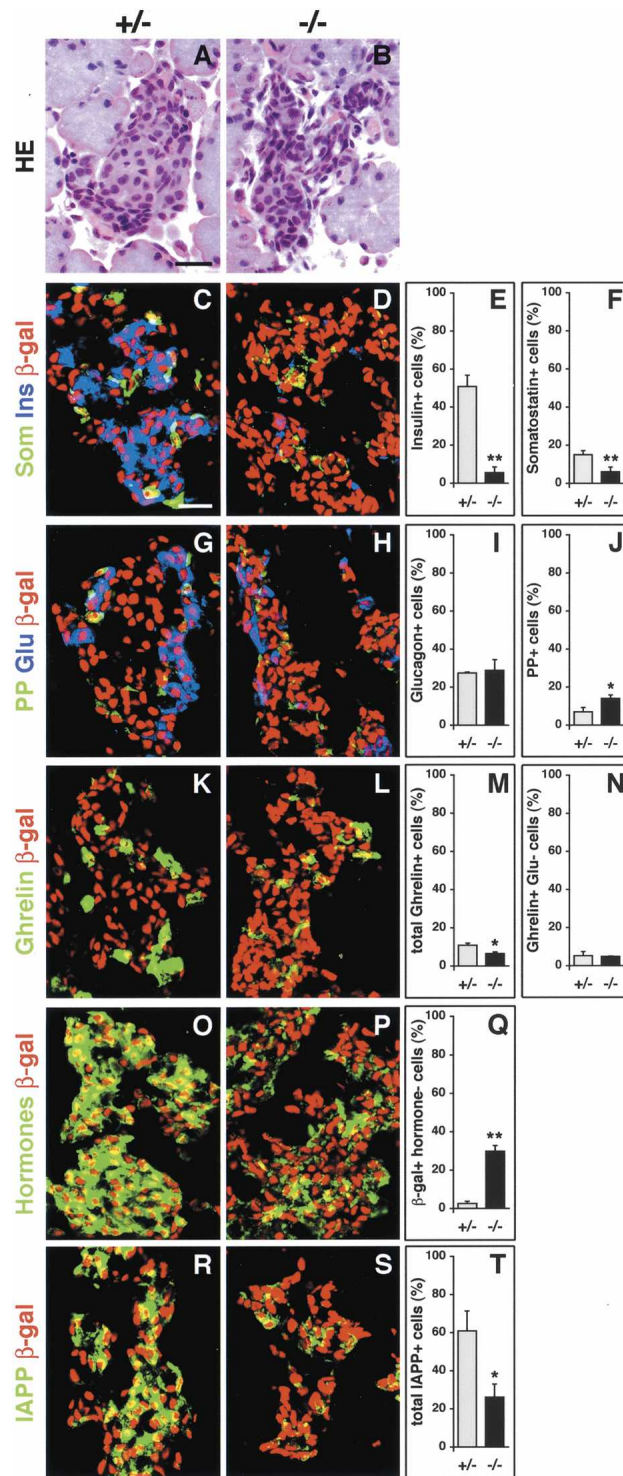
**Table 1.** Comparison of gene expression in the pancreas of heterozygous and homozygous *Insm1<sup>lacZ</sup>* mutant mice

Symbol	Gene name	Fold change	Change <i>p</i> -value
<u>Hormones</u>			
<b>Ins1</b>	Insulin I	-13.1	**
<b>Ins2</b>	Insulin II	-13.5	**
<b>Gcg</b>	Glucagon	-4.1	**
<b>Ppy</b>	PP	p, nc	ns
<b>Ghrl</b>	Ghrelin	p, nc	ns
<b>Sst</b>	Somatostatin	-2.5	**
Npy	Neuropeptide Y	-8.3	**
<u>Glucose metabolism</u>			
<b>Slc2a2</b>	Glut2	-2.9	**
G6pc2	Glucose-6-phosphatase, islet specific	-19.4	**
<u>Hormone processing and secretion</u>			
<b>Pcsk1</b>	Prohormone convertase 1/3	-2.6	**
<b>Pcsk2</b>	Prohormone convertase 2	-2.8	**
<b>Chga</b>	Chromogranin A	-24.9	**
Chgb	Chromogranin B	-40.0	**
Nnat	Neuronatin	-7.2	**
Resp18	Regulated endocrine-specific protein 18	-11.8	**
Rph3al	Rabphilin 3A-like	-2.1	**
Scg2	Secretogranin II	-33.3	**
Scg3	Secretogranin III	-16.6	**
Scgn	Secretagogin	-13.6	**
Sgne1	Secretogranin V	-9.3	**
Snx5	Sorting Nexin 5	-2.5	**
Syt14	Synaptotagmin-like 4	-4.4	**
<u>Transcription factors</u>			
<b>Nkx2-2</b>	Nkx2.2	p, nc	ns
<b>Nkx6-1</b>	Nkx6.1	-1.6	*
<b>Pax4</b>	Pax4	p, nc	ns
<b>Pax6</b>	Pax6	-2.4	**
<b>Ipf1</b>	Pdx1	-1.3	*
<b>MafB</b>	MafB	-1.9	**
<b>Arx</b>	Arx	a	—
<u>Adhesion</u>			
<b>Cdh2</b>	Ncad	p, nc	ns
<u>Others</u>			
<b>Iapp</b>	Islet amyloid polypeptide	-2.1	**
Aplp1	Amyloid $\beta$ precursor-like protein 1	-3.1	**
Gch1	GTP cyclohydrolase	-2.7	**
Gja9	Connexin 36	-6.6	**
Gng4	GTP-binding protein $\gamma$ 4 subunit	-4.9	**
Sez6l2	Seizure-related 6 homolog-like 2	-26.5	**
Slc30a8	Solute carrier family 30 member 8	-5.3	**

Systematic analysis of gene expression in control and *Insm1<sup>-/-</sup>* 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 E15.5 and 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  $\leq$ 0.05 and  $\leq$ 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<sup>+</sup>/glucagon<sup>-</sup> cells was unchanged (Fig. 4J–N). It should be noted that the numbers of  $\gamma$ , PP, or  $\epsilon$  cells remained small in the islets of *Insm1* mutant mice, indicating that transdifferentiation of the mutant  $\beta$  cells into one of these endocrine cell types did not occur.

In *Insm1*<sup>lacZ/+</sup> mice, the majority of  $\beta$ -galactosidase<sup>+</sup> islet cells express one of the pancreatic hormones at



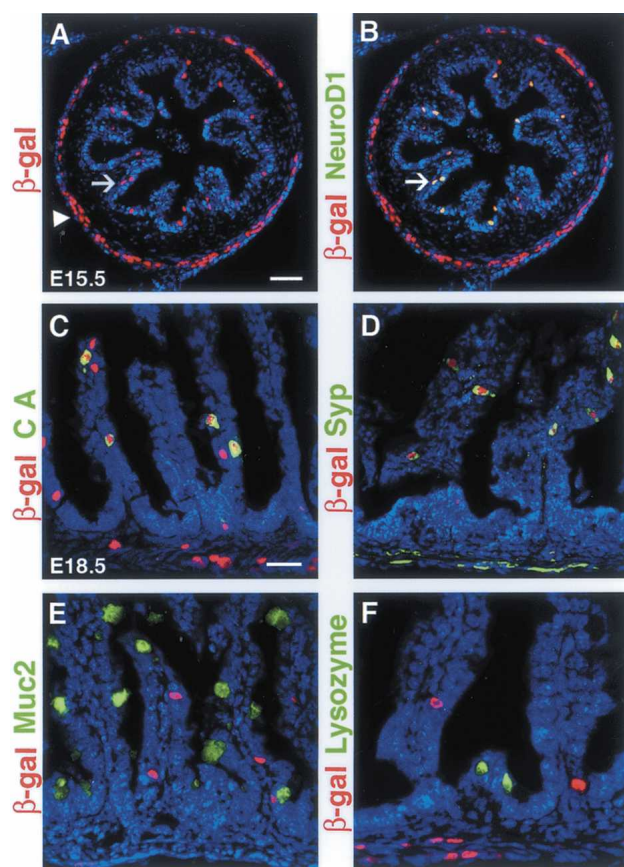
E18.5. This was, however, not observable in *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> mice, where many  $\beta$ -galactosidase<sup>+</sup> cells were present that expressed neither insulin, glucagon, somatostatin, PP, nor ghrelin (Fig. 4O–Q). Thus, other hormone-producing cell types do not form at the expense of  $\beta$  cells in *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> mice, indicating that  $\beta$  cells arrest their differentiation. We investigated if other proteins typically present in endocrine cells were expressed correctly in *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> mice. The glucose transporter 2 (Glut2) and the islet amyloid polypeptide (IAPP) are expressed in  $\beta$  cells of control mice at E18.5. Cells that expressed Glut2 were rare in *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> 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<sup>+</sup> cells, and found them down-regulated. Thus, mutant  $\alpha$  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 *Insm1*<sup>lacZ/+</sup> and *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> mice at E18.5; the genotypes are indicated by +/- and -/-. (A,B) Hematoxylin/eosin (HE) staining of semi-thin sections of the pancreas. Islets are present in *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> mice but display a changed nuclear density. Immunohistological analysis of the pancreas using antibodies directed against  $\beta$ -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- $\beta$ -galactosidase (red) antibodies simultaneously. Note that many  $\beta$ -galactosidase<sup>+</sup> cells exist in the pancreas of *Insm1*<sup>lacZ/+</sup>/*Insm1*<sup>lacZ</sup> embryos that do not express any of these five hormones. (R,S) Immunohistological analysis of the pancreas using antibodies against  $\beta$ -galactosidase (shown in red) and IAPP (green). The proportions of the  $\beta$ -galactosidase<sup>+</sup> cells that express insulin (E), somatostatin (F), glucagon (I), PP (J), 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  $\mu$ m.

doctrine cells is impaired in *Insm1* mutant mice, as reflected in a reduced expression of many genes involved in hormone secretion.

#### *Insm1* is essential for the differentiation of endocrine cells in the intestine

*Insm1* expression can also be detected in the intestine. In *Insm1<sup>lacZ/+</sup>* animals,  $\beta$ -galactosidase<sup>+</sup> cells are scattered in the intestinal epithelium and many of these coexpress NeuroD1 at E15.5 (arrows in Fig. 5A,B) or at E18.5 (Supplementary Fig. 5). Thus, these cells in the epithelium correspond to developing entero-endocrine cells. Additional  $\beta$ -galactosidase<sup>+</sup> cells are observed in a ring of

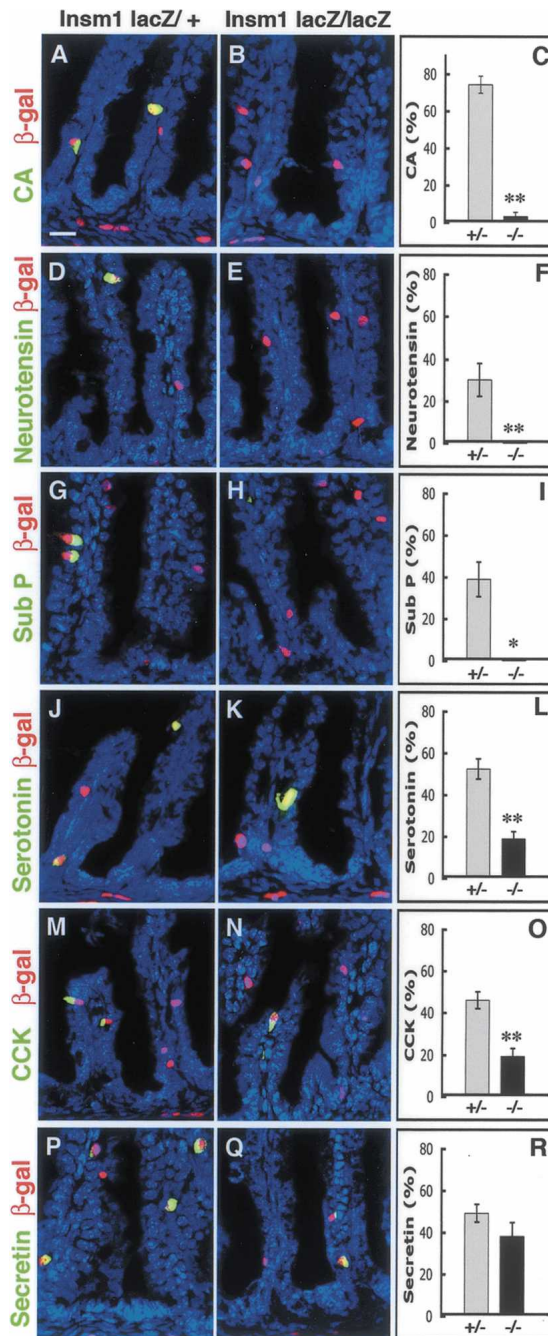


**Figure 5.** *Insm1* expression in the developing intestine. Immunohistological analysis of the developing intestine of *Insm1<sup>lacZ/+</sup>* mice at E15.5 (A,B) and E18.5 (C–F). (A)  $\beta$ -Galactosidase (red) was observed in the intestinal epithelium (arrow) and the enteric nervous system (arrowhead) of *Insm1<sup>lacZ/+</sup>* mice. (B)  $\beta$ -Galactosidase (red) is coexpressed with NeuroD1 (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) (C) or synaptophysin (Syp, green) (D) coexpressed  $\beta$ -galactosidase. In contrast, Mucin2 (Muc2, green) (E) or lysozyme (green) (F) proteins were not observed in the  $\beta$ -galactosidase<sup>+</sup> (red) cells. Sections were counterstained with TOTO-3 (blue). Bars: A, 50  $\mu$ m; C, 20  $\mu$ m.

cells located in the enteric mesenchyme (arrowhead in Fig. 5A); the distribution of these indicates that they correspond to the developing enteric nervous system. Chromogranin A identifies the majority of entero-endocrine cells (Rindi et al. 2004), and many  $\beta$ -galactosidase<sup>+</sup> cells in the intestinal epithelium of *Insm1<sup>lacZ/+</sup>* animals coexpressed chromogranin A (Fig. 5C). Similarly, synaptophysin that marks entero-endocrine cells (Rindi et al. 2004; Sancho et al. 2004) is coexpressed with  $\beta$ -galactosidase in the intestinal epithelium (Fig. 5D). Mucin 2 or lysozyme, which mark paneth and goblet cells, respectively (Sancho et al. 2004), were however not coexpressed with  $\beta$ -galactosidase (Fig. 5E,F). Expression of *Insm1* persists in the adult intestine (Supplementary Fig. 1). We conclude that *Insm1* 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 *Insm1<sup>lacZ</sup>* allele. We observed no obvious difference in the overall number of  $\beta$ -galactosidase<sup>+</sup> cells in the intestinal epithelium of *Insm1<sup>lacZ/+</sup>* and *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice at E15.5 or E18.5 (Fig. 6). At E15.5 or E18.5, the proportion of  $\beta$ -galactosidase<sup>+</sup> cells in the intestinal epithelium that coexpressed NeuroD1 was similar in heterozygous and homozygous *Insm1<sup>lacZ</sup>* mutant mice; expression of *Ngn3*, as assessed by in situ hybridization, was not affected (Supplementary Fig. 5). This indicates that endocrine precursor cells are formed correctly in *Insm1<sup>lacZ</sup>* 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<sup>+</sup> cells were absent in the intestine of *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* 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  $\beta$ -galactosidase<sup>+</sup> cells in the epithelium of the small intestine in *Insm1<sup>lacZ/+</sup>* mice at E18.5. However, neurotensin<sup>+</sup> cells were not observed in *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* animals (Fig. 6D–F). Similarly, substance P is expressed by 40% of  $\beta$ -galactosidase<sup>+</sup> cells in the intestinal epithelium of *Insm1<sup>lacZ/+</sup>* animals, and was not present in the *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice. The proportions of cells that coexpressed serotonin and  $\beta$ -galactosidase, or CCK and  $\beta$ -galactosidase, were reduced in the *Insm1<sup>lacZ/Insm1<sup>lacZ</sup></sup>* mice (Fig. 6J–O), and fewer PYY<sup>+</sup> cells were observed (data not shown). In contrast, the proportion of  $\beta$ -galactosidase<sup>+</sup> cells that express secretin was similar in heterozygous and homozygous *Insm1<sup>lacZ</sup>* 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 *Insm1* is essential for the expression of secretory vesicle proteins (chromogranin A, synaptophysin) in entero-endocrine cells. In addition, *Insm1* is required for the differentiation of particular subtypes of intestinal endocrine cells, which are either absent (substance P<sup>+</sup> or neurotensin<sup>+</sup> cells) or formed in reduced numbers (serotonin<sup>+</sup>, CCK<sup>+</sup>, or PYY<sup>+</sup> cells) in *Insm1* mutant mice.





**Figure 6.** *Insm1* is essential for the differentiation of enteroendocrine cells. Immunohistological analysis of the intestine of *Insm1<sup>lacZ/+</sup>* and *Insm1<sup>lacZ/lacZ</sup>* mice at E18.5, using antibodies directed against chromogranin A (A,B), neurotensin (D,E), substance P (G,H), serotonin (J,K), CCK (M,N), and secretin (P,Q) (all shown in green); in addition, antibodies against  $\beta$ -galactosidase (shown in red) were used. The proportion of  $\beta$ -galactosidase<sup>+</sup> cells that express chromogranin A (C), neurotensin (F), substance P (I), serotonin (L), CCK (O), and secretin (R) are displayed. Sections were counterstained with TOTO-3 (blue). Single and double asterisks indicate *p*-values of <0.005 and <0.0001, respectively. Bar, 20  $\mu$ m.

## 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,  $\beta$  cells are severely impaired in their differentiation.  $\alpha$  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<sup>+</sup> 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  $\alpha$ - or  $\beta$ -cell fate, whereas *Nkx2.2* and *Nkx6.1* control the differentiation of pancreatic  $\beta$  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  $\beta$ -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  $\beta$ -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  $\beta$ -cell lineage. In *Insm1* mutant mice, many  $\beta$ -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  $\beta$  cells, whereas in *Nkx6.1* mutants,  $\beta$ -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  $\beta$ -cell differentiation.

In the *Insm1<sup>lacZ</sup>* 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  $\alpha$ ,  $\beta$ , PP,  $\delta$ , and  $\epsilon$  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  $\beta$  cells was most severely affected. In particular, we noted a massive reduction in the number of insulin<sup>+</sup> cells in *Insm1* mutant mice, and many proteins present in  $\beta$  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  $\beta$  cells. The mutant  $\beta$  cells retained, however, the expression of  $\beta$ -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 *Insm1<sup>lacZ</sup>* mutant mice. Lineage analysis has indicated that  $\beta$ ,  $\delta$ , 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  $\beta$  cells require *Insm1* for differentiation; we also observed significant changes in the development of the  $\alpha$ -cell lineage. Analysis of the numbers of glucagon<sup>+</sup> 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  $\alpha$  cells might be distinct. Nevertheless, the expression levels of glucagon in the mutant  $\alpha$  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  $\alpha$  and  $\beta$  cells were markedly down-regulated in *Insm1* mutant mice. Thus, despite the fact that  $\alpha$  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  $\alpha$  and  $\beta$  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 aberrantly 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 *Insm1<sup>lacZ</sup>* allele, we demonstrate here that *Insm1* is expressed in developing intestinal endocrine cells. The extensive coexpression of  $\beta$ -galactosidase and *NeuroD1* at E15.5 indicates that *Insm1* is expressed mainly in post-mitotic 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).  $\beta$ -Galactosidase is coexpressed with chromogranin A and synaptophysin in heterozygous *Insm1<sup>lacZ</sup>* animals, and the proportion of coexpressing cells increases as development proceeds. This indicates that  $\beta$ -galactosidase expression initiates prior to the differentiation of entero-endocrine cells and persists in the differentiating cells. In homozygous *Insm1<sup>lacZ</sup>* mutant mice,  $\beta$ -galactosidase<sup>+</sup> 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 $\beta$ cells

Impaired function or loss of pancreatic  $\beta$  cells cause diabetes, a prevalent human disease throughout the world. We demonstrate here that  $\beta$ -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  $\beta$  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  $\beta$ -cell differentiation, is also essential for the maintenance and the correct function of  $\beta$  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<sup>+</sup> and glucagon<sup>+</sup> 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 (Osoegawa 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 *Insm1<sup>lacZ</sup>* gene by mating chimeras to C57BL/6 females. The mutant strain was subsequently expanded by mating *Insm1<sup>lacZ</sup>* males to C57BL/6 females. Routine genotyping was performed by PCR and, occa-

sionally, 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<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* 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 *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* genotype, indicating that the majority of *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* embryos died during the second half of gestation on the mixed 129/Ola and C57BL/6 genetic background. Surviving *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* mice on the 129/Ola and C57BL/6 background were apparently well developed and normal in size at E18.5. The *Insm1<sup>lacZ</sup>* 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 *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* 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 *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* and *Insm1<sup>lacZ</sup>/+* mice on the mixed 129/Ola and C57BL/6 background, and observed very few  $\beta$  cells and a comparable number of  $\alpha$  cells at E18.5 (see Supplementary Fig. 4). Regardless of the genetic background, homozygous *Insm1<sup>lacZ</sup>* 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- $\mu$ m cryosections, using the following antibodies: goat anti- $\beta$ -galactosidase (1:1000; Biogenesis), rabbit anti- $\beta$ -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; Biogenesis), 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 Planck Institute for Biophysical Chemistry, Göttingen, Germany), guinea pig anti-MafA (1:20,000), rabbit anti-Ptfla (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-CCK (1:250; Biotrend), rabbit anti-PYY (1:6000; Biotrend), 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-neurogenin3 (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-prohormone convertase 1/3 (1:100; Chemicon), rabbit anti-prohormone convertase 2 (1:100; Chemicon), goat anti-Glut2 (1:1000; Santa Cruz Biotechnology), rabbit anti-N-Cadherin (1:100; Santa Cruz Biotechnology), rabbit anti-amylase (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  $\beta$ -galactosidase<sup>+</sup> cells that express a particular pancreatic hormone, we used images taken from sections that were stained for  $\beta$ -galactosidase as well as for one of the hormones (insulin, glucagon, somatostatin, PP, or ghrelin). Cells that expressed  $\beta$ -galactosidase and a particular hormone were counted on at least three sections obtained from three to four independent heterozygous and homozygous *Insm1<sup>lacZ</sup>* mice. To determine the proportion of  $\beta$ -galactosidase<sup>+</sup> cells that coexpress each of the hormones, at least 300  $\beta$ -galactosidase<sup>+</sup> cells were analyzed at E15.5 and E18.5, and at least 100  $\beta$ -galactosidase<sup>+</sup> cells were analyzed at E12.5. The overall number of  $\beta$ -galactosidase<sup>+</sup> cells was essentially determined as described (Collombat et al. 2005). The absolute numbers of  $\beta$ -galactosidase<sup>+</sup> cells and the proportion of hormone<sup>+</sup> 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  $\alpha$  and  $\beta$  cells—shows a pronounced reduction of  $\beta$  cells and similar numbers of  $\alpha$  cells when heterozygous and homozygous *Insm1<sup>lacZ</sup>* mice were compared.

In the intestine, cells that coexpress  $\beta$ -galactosidase and various markers of endocrine cells were counted from sections obtained from three to four independent heterozygous and homozygous *Insm1<sup>lacZ</sup>* mice. To determine the proportion of  $\beta$ -galactosidase<sup>+</sup> cells that coexpress hormones/proteins, at least 100  $\beta$ -galactosidase<sup>+</sup> 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 *Insm1<sup>lacZ</sup>/Insm1<sup>lacZ</sup>* 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-moderated *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  $\leq 0.05$ .

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