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RESEARCH ARTICLE

Insm1 controls development of pituitary endocrine cells and requires a SNAG domain for function and for recruitment of histone-modifying factors

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ABSTRACT

The *Insm1* gene encodes a zinc finger factor expressed in many endocrine organs. We show here that *Insm1* is required for differentiation of all endocrine cells in the pituitary. Thus, in *Insm1* mutant mice, hormones characteristic of the different pituitary cell types (thyroid-stimulating hormone, follicle-stimulating hormone, melanocyte-stimulating hormone, adrenocorticotrope hormone, growth hormone and prolactin) are absent or produced at markedly reduced levels. This differentiation deficit is accompanied by upregulated expression of components of the Notch signaling pathway, and by prolonged expression of progenitor markers, such as Sox2. Furthermore, skeletal muscle-specific genes are ectopically expressed in endocrine cells, indicating that *Insm1* participates in the repression of an inappropriate gene expression program. Because *Insm1* is also essential for differentiation of endocrine cells in the pancreas, intestine and adrenal gland, it is emerging as a transcription factor that acts in a pan-endocrine manner. The Insm1 factor contains a SNAG domain at its N-terminus, and we show here that the SNAG domain recruits histone-modifying factors (Kdm1a, Hdac1/2 and Rcor1-3) and other proteins implicated in transcriptional regulation (Hmg20a/b and Gse1). Deletion of sequences encoding the SNAG domain in mice disrupted differentiation of pituitary endocrine cells, and resulted in an upregulated expression of components of the Notch signaling pathway and ectopic expression of skeletal muscle-specific genes. Our work demonstrates that Insm1 acts in the epigenetic and transcriptional network that controls differentiation of endocrine cells in the anterior pituitary gland, and that it requires the SNAG domain to exert this function *in vivo*.

KEY WORDS: Hormone, Differentiation, Kdm1a, Mouse

INTRODUCTION

The pituitary gland is a central neuroendocrine organ that relays information from the central nervous system to peripheral targets by secreting hormones and other factors. The anterior pituitary gland contains six different endocrine cell types that control physiological processes, such as growth, stress response, metabolic status, reproduction and lactation (Cushman and Camper, 2001). During embryogenesis, transcription factors and signaling events control the development of these hormone-secreting cell types (reviewed by Zhu et al., 2005; Kelberman et al., 2009). The discovery of these regulatory cascades facilitated genetic analyses of patients with hormone deficiencies. All hormone-secreting cell types of the pituitary gland derive from Rathke's pouch. Rathke's pouch is an invagination of the oral ectoderm. Inductive interactions between surrounding tissues and Rathke's pouch control the expression of transcription factors, such as Lhx3, Lhx4, Hesx1, Prop1 and Pitx1, that specify fates of pituitary cells (reviewed by Ericson et al., 1998; Rosenfeld et al., 2000). Differentiation of the six endocrine cell types (thyrotropes, corticotropes, melanotropes, somatotropes, lactotropes, gonadotropes) depends on transcription factors, such as Pit1 (Pou1f1), Math3 (NeuroD4), Nr5a1 (Sf1), Tbx19 and NeuroD1 (Camper et al., 1990; Shinoda et al., 1995; Pulichino et al., 2003; Lamolet et al., 2004; Zhu et al., 2006). Sox2 is expressed in Rathke's pouch and in endocrine progenitor cells during development and in the adult, but is downregulated upon differentiation and hormone expression (Fauquier et al., 2008; Jayakody et al., 2012).

The *Insm1* gene encodes a zinc finger transcription factor that is expressed in various endocrine cell types as well as in tumors that derive thereof (Goto et al., 1992; Breslin et al., 2002; Gierl et al., 2006; Mellitzer et al., 2006; Wildner et al., 2008). Insm1 contains five zinc finger domains of the C2H2 type, a motif frequently associated with transcription factors (Razin et al., 2012). At the very N-terminus, Insm1 contains a SNAG domain (Grimes et al., 1996), which was first identified in Snail/Slug and Gfi1 and Gfi1b zinc finger transcription factors and recruits Rcor1, the histone deacetylases Hdac1 and Hdac2 and the histone demethylase Kdm1a (Saleque et al., 2007; Lin et al., 2010). Rcor1 acts as a co-repressor and was first identified together with the histone deacetylases Hdac1 and Hdac2 in the REST silencing complex (Andrés et al., 1999). Together, these proteins modulate gene expression by chromatin modification and provide key epigenetic information. Zinc finger factors related to Insm1 exist in *Drosophila* (Nerfin-1) and *Caenorhabditis elegans* (EGL-46), where they function in neuronal differentiation, but the invertebrate homologs do not contain SNAG domains (Wu et al., 2001; Kuzin et al., 2005). In accordance with a potentially repressive function, Insm1 is known to bind Hdac1 and Hdac3 (Liu et al., 2006), but whether the Insm1 SNAG domain is responsible for this interaction has not been assessed.

Genetic analysis in mice demonstrated that *Insm1* encodes a key factor in development of endocrine cells of pancreas, intestine and adrenal gland. *Insm1* is required for differentiation but not for the

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specification of these endocrine cell types (Gierl et al., 2006; Wildner et al., 2008). Insm1 is expressed also in endocrine cells of the pituitary (our unpublished observations) and pituitary tumor cells (Goto et al., 1992), but a potential function in the pituitary has not been assessed. Here, we show that Insm1 expression in the pituitary is initiated when endocrine cells differentiate, and persists during further development and in the adult. Mutation of *Insm1* leads to the formation of an anterior pituitary gland of normal overall morphology, but this pituitary is devoid of the entire repertoire of differentiated endocrine cell types expressing the appropriate hormones. In the mutant endocrine cells, skeletal muscle-specific genes are ectopically expressed, indicating that Insm1 suppresses an inappropriate differentiation program. We also define the role of the SNAG domain in the differentiation of pituitary endocrine cells. We used biochemistry to show that Insm1, via its SNAG domain, interacts with a number of histone-modifying factors (i.e. Kdm1a, Hdac1/2 and Rcor1-3) and with factors implicated in transcriptional regulation (Hmg20a/b and Gse1). Furthermore, deletion of the SNAG domain *in vivo* disrupts the differentiation of endocrine cells in the pituitary. Our work demonstrates that Insm1 acts in the epigenetic and transcriptional network that controls differentiation of endocrine cells in the anterior pituitary gland, and requires the SNAG domain to exert its role in differentiation and in suppression of inappropriate gene expression programs.

RESULTS

Insm1 is expressed in differentiating endocrine cells of the anterior pituitary gland

We analyzed expression of Insm1 during organogenesis of the pituitary gland by immunohistological analysis. The first Insm1$^+$ cells appear at embryonic day (E) 11.5 and locate to the most ventral part of the pituitary anlage (Fig. 1A). This domain is known to contain proliferating progenitors that initiate differentiation into the early thyrotropes of the pituitary (Pope et al., 2006; Bilodeau et al., 2009). Counterstaining with DAPI demonstrated nuclear localization of the Insm1 protein (Fig. 1A, inset). At E13.5, Insm1 expression has expanded (Fig. 1B), and is detected below the progenitor zone known to contain proliferating progenitors (c.f. Bilodeau et al., 2009). By E17.5, Insm1 is present in the entire anterior pituitary (Fig. 1C,D). This indicates that Insm1 expression accompanies the spreading of endocrine cell differentiation. Sox2 and Sox9 are expressed in endocrine progenitors of the pituitary (Fauquier et al., 2008; Jayakody et al., 2012), and we detected few cells that co-express Insm1 and Sox2, or Insm1 and Sox9 at E17.5 (Fig. 1C,C′). Instead, the majority of Insm1$^+$ cells co-expressed hormones, as assessed by immunohistology using a mix of antibodies directed against pituitary hormones (Fig. 1D,D′). Further analysis indicated that Insm1 was co-expressed with growth hormone (GH), prolactin (Prl), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), adrenocorticotrope hormone (ACTH), as well as melanocyte-stimulating hormone (MSH) (Fig. 1E-J). Thus, Insm1 is present in all differentiated endocrine cell types, and its expression persists into adulthood (supplementary material Fig. S1A). In accordance with this, we observed only very rarely Insm1$^+$ cells that expressed Ki67 at E17.5, indicating that most Insm1$^+$ cells have exited the cell cycle (Fig. 1K,K′). Insm1 was not co-expressed with markers for fibroblasts (ER/TR7), folliculostellate cells (S100) or endothelia (PCAM), and is thus restricted to the endocrine lineage of the pituitary (Fig. 1L-N). This pattern indicates that Insm1 expression initiates in pituitary progenitors when differentiation of endocrine cells begins. Insm1 continues to be expressed in differentiated endocrine cells during development and in adulthood.

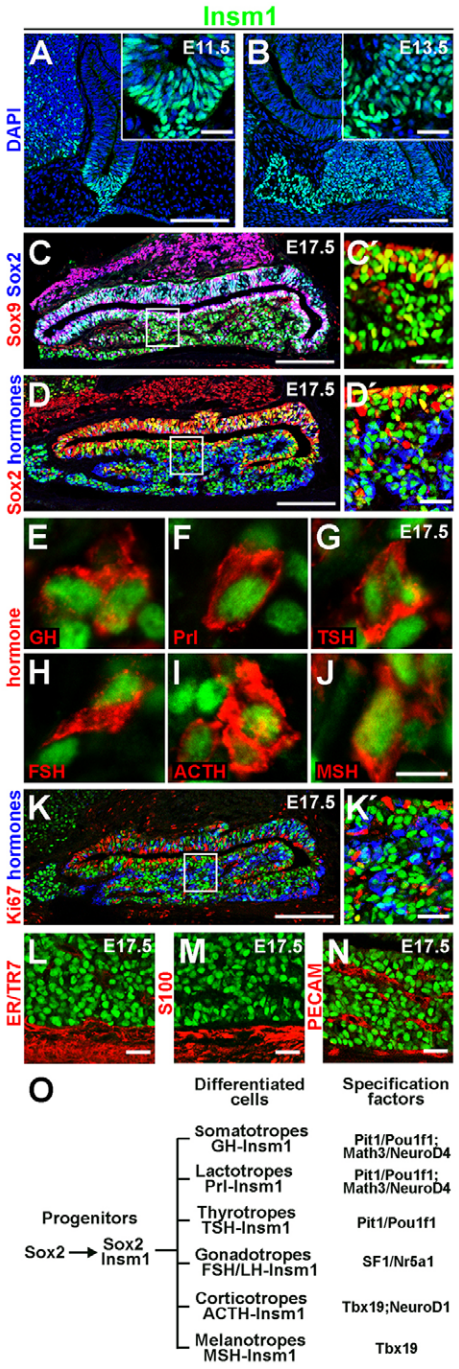

Fig. 1. Expression of Insm1 in the developing mouse pituitary. (A-N) Insm1 expression was analyzed by immunohistochemistry in the developing pituitary at E11.5 (A), E13.5 (B) and E17.5 (C-N). Insm1 expression starts around E11.5 in a few cells of the ventral domain of Rathke's pouch and subsequently expands, encompassing many cells in the developing anterior lobe of the pituitary (A,B). At E17.5, Insm1 is present in the entire anterior pituitary (C). At this stage, a few Insm1$^+$ cells co-express the progenitor markers Sox9 and Sox2 (C,C′), but the majority of Insm1$^+$ cells co-express hormones (D,D′). Insm1 is present in all endocrine cell types and is co-expressed with GH, Prl, TSH, FSH, ACTH and MSH (E-J). Insm1 was very rarely co-expressed with Ki67, indicating that Insm1$^+$ cells are postmitotic (K,K′). Insm1 is not expressed in fibroblast (L), folliculostellate (M) or endothelia (N) cells. (O) The developmental progression of pituitary cell types is summarized; expression of Sox2, Insm1 and other transcription factors that contribute to the differentiation of the distinct cell types is indicated. Scale bars: 100 μm (A-D,K); 10 μm (E-J); 20 μm (insets in A,B; C′,D′,K′,L-N).

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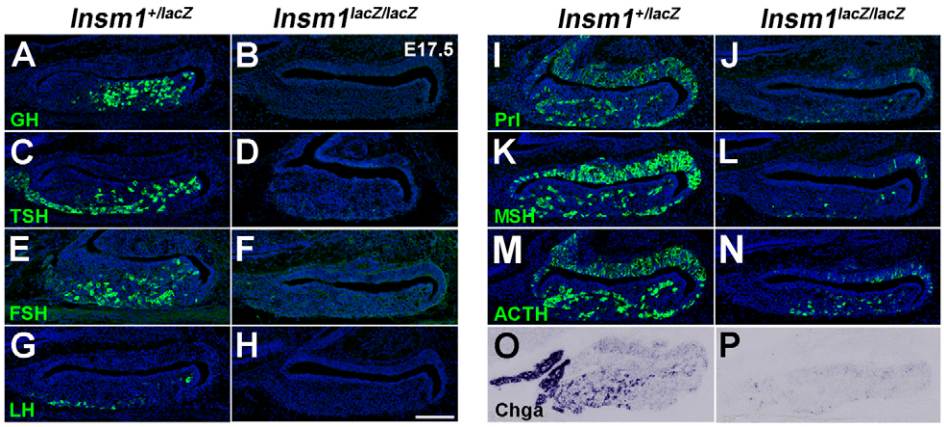

Fig. 2. Mutation of *Insm1* disrupts differentiation of endocrine cells in the pituitary. (A-N) Immunohistochemical analysis of pituitary hormone expression in control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice at E17.5. The expression of GH (A,B), TSH (C,D), FSH (E,F), LH (G,H), Prl (I,J), MSH (K,L) and ACTH (M,N) was markedly downregulated in *Insm1* mutant pituitaries. It should be noted that *Prl* mRNA is not detectable in the intermediate lobe; the signal detected in this domain by antibodies is thus caused by cross-reaction. (O,P) In addition, the expression of chromogranin A was downregulated as assessed by *in situ* hybridization. Scale bar: 100 µm.

Furthermore, Insm1 is expressed in all endocrine cell types of the pituitary (see Fig. 1O for a summary of the different endocrine cell types and their expression of Insm1).

Insm1 is required for the differentiation of endocrine cells in the anterior pituitary gland

Next, we used mouse genetics to define the role of *Insm1* in the development of endocrine cells, using a previously generated null allele in which the *Insm1* coding sequence is replaced by *lacZ* (Gierl et al., 2006). As assessed by immunohistochemistry, GH was produced at E17.5 in heterozygous *Insm1*^{lacZ} animals, which served as controls, but was not detectable in homozygous *Insm1*^{lacZ} mice (Fig. 2A,B; we refer subsequently to heterozygous and homozygous *Insm1*^{lacZ} mice also as control and *Insm1*^{lacZ} mutants). *In situ* hybridization demonstrated a strong reduction in *Gh* transcripts in *Insm1*^{lacZ} mutants (supplementary material Fig. S1B,C). Thus, somatotropes require *Insm1* for their differentiation. Similarly, TSH, FSH and LH proteins were present in the pituitary of control animals, but were not detectable in *Insm1*^{lacZ} mutants (Fig. 2C-H), and the corresponding mRNAs, as well as the mRNA encoding the common α-subunit of the three hormones, Cga, were strongly downregulated (supplementary material Fig. S1D-K). Thus, thyrotropes and gonadotropes also require *Insm1* for differentiation. Lactotropes express Prl, which was markedly downregulated in *Insm1*^{lacZ} mutants compared with control animals (Fig. 2I,J). Finally, melanotropes and corticotropes produce MSH and ACTH, respectively, which are generated from a common precursor protein, pro-opiomelanocortin (POMC). Antibodies that detect mature MSH and ACTH demonstrated a strong reduction of these hormones in pituitaries of homozygous *Insm1*^{lacZ} mutants (Fig. 2K-N), whereas *Pomc* mRNA was mildly downregulated (supplementary material Fig. S1L,M). A further hallmark of endocrine cell differentiation is the production of granin proteins, which are associated with secretory vesicles. Chromogranin A (*Chga*) transcripts were present at reduced levels in the pituitary of homozygous *Insm1*^{lacZ} mutants compared with control mice (Fig. 2O,P). We conclude that none of the endocrine cell types of the pituitary differentiates correctly in *Insm1*^{lacZ} mutants, which is reflected by a lack or marked downregulation of hormones and proteins associated with secretory vesicles. The deficit in differentiation of endocrine cells was accompanied by a marked increase in the number of cells expressing Sox2 and Sox9 (Fig. 3A-D′). The supernumerary Sox2^{+} and Sox9^{+} cells co-expressed β-gal in *Insm1*^{lacZ} mutants, and we noted that β-gal^{+} nuclei in the anterior lobe of the pituitary were packed more densely in mutant than in control mice (Fig. 3A-D′). Thus, the disrupted differentiation of endocrine cells is accompanied by an increase in the number of endocrine progenitor cells. We did not detect obvious changes in the distribution or morphology of other cells types of the pituitary (fibroblasts, folliculostellate cells, endothelia) in *Insm1*^{lacZ} mutant mice (Fig. 3B,B′,F,F′).

We next investigated whether the disrupted differentiation affected proliferation and survival of endocrine cells in the pituitary. Cell proliferation was assessed by BrdU incorporation, which revealed no significant changes when control and *Insm1*^{lacZ} mutants were compared (supplementary material Fig. S2A-C). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining did not detect apoptosis in the pituitary at E13.5 or E15.5 (supplementary material Fig. S2D-G). The overall shape of pituitary glands of control and homozygous mutant mice were similar at E17.5 (Fig. 3G,H). Nissl-stained consecutive sections of the pituitary were used to estimate the size of the pituitary at E17.5, which detected a small decrease (~10%) in mutant mice (Fig. 3I-K). Thus, the pituitary size is reduced mildly in *Insm1*^{lacZ} mutants, apparently owing to a denser packing of cells. We conclude that the mutation of *Insm1* interferes in a striking manner with endocrine differentiation but not cell cycle exit in the pituitary.

Insm1 controls the expression of a pan-endocrine differentiation program and is required to repress myogenic genes in the pituitary

To assess global changes in gene expression, we performed gene profiling of control and *Insm1*^{lacZ} mutant pituitary glands using Illumina microarrays. This confirmed the marked downregulation of genes encoding pituitary hormones, and demonstrated that many other genes that function in hormone production and secretion were also downregulated, e.g. proprotein convertases and various granins, such as secretogranin II/III (Table 1). Interestingly, comparison with previous experiments that analyzed genes deregulated in the adrenal gland and pancreas of *Insm1*^{lacZ} mutants (Gierl et al., 2006; Wildner et al., 2008) detected a set of genes that are deregulated in all three endocrine organs (Table 2; supplementary material Fig. S5). Thus, *Insm1* functions in many endocrine cell types and its mutation affects the expression of an overlapping gene set in different endocrine organs.

Remarkably, further analysis of the microarray data showed that a number of genes of the Notch pathway were upregulated in the pituitary of *Insm1*^{lacZ} mutant mice (Table 1). Among these were *Dll1* and the Notch target genes *Hey1*, *Hes1* and *Hes5*. We confirmed the enhanced expression of *Dll1* and *Hey1* using *in situ* hybridization and qPCR (Fig. 4A-F,K). *Notch2* is expressed during pituitary development (Raetzman et al., 2004), and *in situ* hybridization demonstrated a pronounced upregulation of *Notch2* expression in

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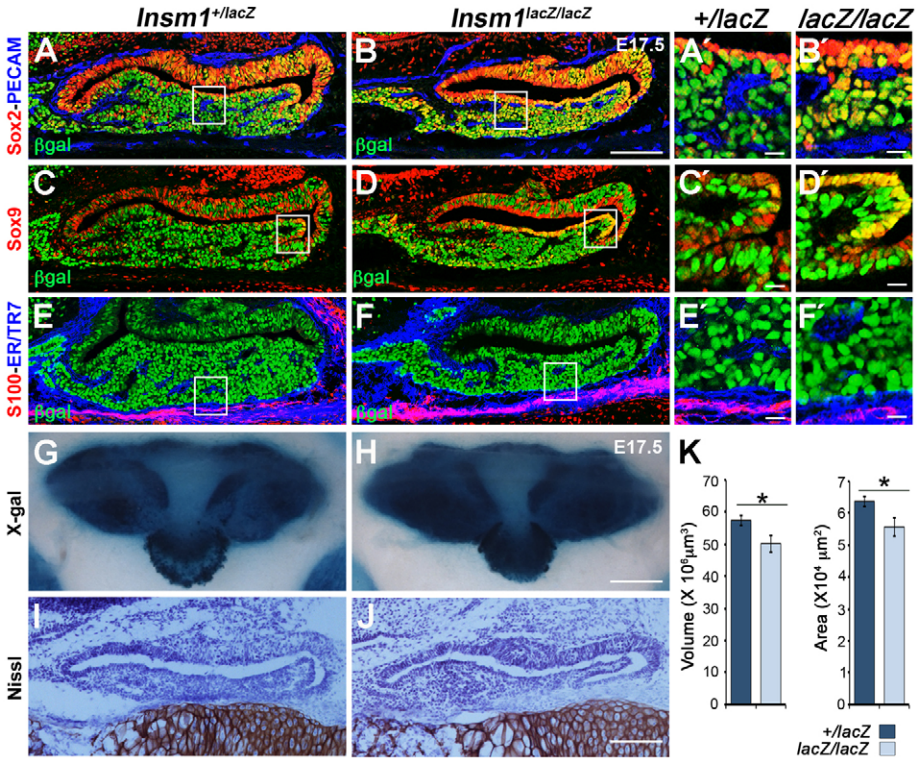

Fig. 3. Mutation of *Insm1* increases the number of endocrine progenitor cells without altering the overall morphology of the pituitary gland. (A-F′) Immunohistochemical analysis of endocrine progenitor cells in the pituitary of control (*Insm1*$^{+/lacZ}$) and mutant (*Insm1*$^{lacZ/lacZ}$) mice at E17.5. Increased numbers of double-positive cells for β-gal and Sox2 (A-B′) or Sox9 (C-D′) were observed in *Insm1*$^{lacZ/lacZ}$ embryos compared with control littermates. No apparent alteration of endothelial (A-B′), fibroblast or folliculostellate (E-F′) cells was found in *Insm1* mutant mice. (G,H) Whole-mount X-gal staining revealed no obvious changes in the overall shape of pituitary gland of homozygous mutant mice compared with control littermates. (I-K) Estimation of the pituitary gland volume and area detected in consecutive sections stained with Nissl from *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/lacZ}$ mice at E17.5. **P*<0.05. Scale bars: 100 µm (A-F,I,J); 20 µm (A′-F′); 500 µm (G,H).

the *Insm1*^{lacZ} mutant pituitary at E15.5; at E17.5, altered *Notch2* expression was detected by qPCR but not by *in situ* or microarray experiments.

In addition, the array analysis identified a marked upregulation of a number of genes typically expressed in skeletal muscle, e.g. mRNAs encoding myosin light polypeptide 1 (Myl1), alpha cardiac muscle 1 actin (Actc1) expressed in skeletal and cardiac muscle, and musculin (Msc), a myogenic transcription factor (Table 1). Upregulated expression of *Myl1* and *Actc1* mRNAs in a subpopulation of cells was confirmed by *in situ* hybridization and qPCR (Fig. 4G-K). We conclude from these data that *Insm1* is required for repression of components of the Notch signaling pathway and of skeletal muscle-specific genes in endocrine cells of the pituitary.

The development of different endocrine cell types depends on a number of transcription factors. In particular, somatotrope, lactotrope and thyrotrope lineages depend on Pit1 for differentiation (Camper et al., 1990), and Pit1 expression was indeed not detected at E14.5 and was markedly downregulated at E17.5 in *Insm1*^{lacZ} mutants (Fig. 5A-D). Math3 controls differentiation of somatotropes and lactotropes, and Nr5a1 the differentiation of gonadotropes (Shinoda et al., 1995; Zhu et al., 2006). *Math3* and *Nr5a1* transcripts were markedly downregulated in *Insm1*^{lacZ} mutant mice (Fig. 5E-H). Tbx19 and NeuroD1 mark differentiating corticotropes (Tbx19, NeuroD1) and melanotropes (Tbx19) and control their differentiation as well as POMC expression (Lamolet et al., 2001; Pulichino et al., 2003; Lamolet et al., 2004); expression of *Tbx19* and *NeuroD1* was downregulated in mutant mice (Fig. 5I-L). In conclusion, transcription factors that direct the differentiation of endocrine cell types of the pituitary are not correctly expressed in *Insm1*^{lacZ} mutant mice. We also assessed the early specification of endocrine cells by analyzing transcription factors, such as Hesx1, Pitx1, Pitx2, Lhx3 and Prop1, which are known to be essential for pituitary development (Sheng et al., 1996; Dattani et al., 1998; Wu et al., 1998; Szeto et al., 1999; Suh et al., 2002). No obvious change in the expression of the corresponding transcripts was detected at E12.5 (supplementary material Fig. S3A-P). *Prop1* expression was compared at various stages, and was unchanged at E12.5 and mildly upregulated at E14.5 and E15.5 (supplementary material Fig. S3I-P). We conclude that in the absence of *Insm1*, endocrine progenitor cells of the pituitary are correctly specified, but their differentiation is disrupted.

Insm1 interacts with Kdm1a, Rcor1 and Hdac1/2 via its SNAG domain

To discover Insm1-interacting proteins, we combined stable isotope labeling by amino acids in cell culture (SILAC) and affinity purification, an approach that can identify protein-protein interactions with very high confidence (Selbach and Mann, 2006; Paul et al., 2011). This technology relies on the quantification of proteins that co-immunoprecipitate with Insm1. The proteome of AtT-20 cells, which is a mouse corticotrope tumor cell line, was labeled with amino acids carrying either heavy or light stable isotopes (‘heavy’ and ‘light’ cells). ‘Heavy’ and ‘light’ cells were transduced with retroviruses encoding non-tagged Insm1 (Insm1^{wt}) and FLAG-tagged Insm1 (Insm1^{FLAG}), respectively. Immunoprecipitates obtained from the ‘heavy’ and ‘light’ cells using anti-FLAG antibodies were combined and subjected to mass spectrometry-based quantification (see Fig. 6A for an outline of the experiment). As expected, mass spectrometry found Insm1 among the proteins most abundant for the ‘light’-label state when anti-FLAG immunoprecipitates from ‘heavy’-labeled Insm1^{wt} and ‘light’-labeled Insm1^{FLAG} cells were compared. In addition, Kdm1a, Rcor1-3, Hdac1/2, Gse1 and Hmg20a/b were identified and quantified with similar protein ratios (Table 3). Swapping isotope labels resulted in reciprocal abundance ratios for Insm1 and detected co-precipitated proteins, supporting specificity of the interaction. Western blot analysis was used to confirm these interactions, using

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Table 1. Comparison of gene expression in the pituitary of control and Insm1 mutant mice

Gene symbol	Gene name	Fold change	*P*-value
Hormones			
*Gh**	growth hormone	−85.74	2.1E–09
*Prl**	prolactin	−2.75	1.9E–04
*Tshb**	thyroid-stimulating hormone, β-subunit	−17.95	4.2E–13
*Lhb**	luteinizing hormone, β-subunit	−3.94	1.3E–03
*Cga**	glycoprotein hormones,α-subunit	−7.30	3.6E–09
*Pomc**	pro-opiomelanocortin	–	–
Processing and secretion of hormones			
*Chga**	chromogranin A	−4.80	5.9E–09
*Chgb**	chromogranin B	−17.58	1.8E–12
*Pcsk1n**	proprotein convertase subtilisin/kexin type 1	−5.14	1.6E–08
*Pcsk2**	proprotein convertase subtilisin/kexin type 2	−5.95	5.3E–07
Scg2	secretogranin II	−5.25	9.7E–10
*Scg3**	secretogranin III	−6.01	1.9E–09
Scgn	secretagogin	−2.39	6.5E–08
Notch signaling pathway			
*Dll1**	delta-like 1	1.88	1E–06
*Hey1**	hairy/enhancer-of-split related with YRPW motif 1	2.2	3.7E–06
*Hes1**	hairy and enhancer of split 1	1.42	6.9E–04
*Hes5**	hairy and enhancer of split 5	1.2	3.2E–04
Muscle-specific genes			
*Myl1**	myosin, light polypeptide 1	18.09	2.05E–06
*Actc1**	actin, alpha, cardiac muscle 1	8.02	8E–07
Sln	sarcolipin	7.26	6.2E–07
*Msc**	musculin	5.43	4.6E–07
Thsd7b	thrombospondin, type I, domain containing 7B	3.58	1.7E–07
Myh8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	2.86	3.9E–06
Tnnt1	troponin T1, skeletal, slow	3.24	3.7E–07
Mustn1	musculoskeletal, embryonic nuclear protein 1	2.69	4.5E–08
Mylpf	myosin light chain, phosphorylatable, fast skeletal muscle	2.80	9.5E–06

Systematic analysis of gene expression in $Insm1^{+/lacZ}$ and $Insm1^{lacZ/lacZ}$ mice using Illumina oligonucleotide microarrays. The average signal fold change is shown. We selected the following genes for display: (1) genes encoding hormones and proteins that participate in hormone processing and secretion; (2) components of the Notch signaling pathway; (3) muscle-specific genes.

*Deregulated expression was also analyzed by immunohistochemistry, *in situ* hybridization or qPCR.

protein extracts from AtT-20 cells and from a second pituitary cell line, the rat somatotrope line GH3. In these experiments, Kdm1a, Rcor1 and Hdac1/2 were co-precipitated with endogenous Insm1 (Fig. 6B).

We next tested biochemically whether the SNAG domain of Insm1 is essential for the interaction with Kdm1a, Rcor1 and Hdac1/2. For this, cDNAs encoding FLAG-tagged Insm1 with intact SNAG domain ($Insm1^{FLAG}$) and a truncated FLAG-tagged variant that lacks seven highly conserved amino acids at the N-terminus were constructed ($Insm1\Delta SNAG^{FLAG}$); the deletion of these seven amino acids disrupts the SNAG domain of Insm1 (Fig. 6C). $Insm1^{FLAG}$ and $Insm1\Delta SNAG^{FLAG}$ cDNAs were expressed in AtT-20 cells and immunoprecipitated with anti-FLAG and anti-Insm1 antibodies (Fig. 6D). Kdm1a, Rcor1 and Hdac1/2 were no longer co-immunoprecipitated with $Insm1\Delta SNAG^{FLAG}$, but were present in $Insm1^{FLAG}$ precipitates (Fig. 6D). Similarly, Kdm1a, Rcor1 and Hdac1/2 antibodies co-immunoprecipitated full-length $Insm1^{FLAG}$, but not $Insm1\Delta SNAG^{FLAG}$ (Fig. 6E). Thus, Kdm1a, Rcor1 and Hdac1/2 are recruited to Insm1 via its N-terminal SNAG domain.

The SNAG domain of Insm1 is essential for Insm1 functions *in vivo*

To assess the function of the Insm1 SNAG domain in the developing pituitary, we generated a mouse strain in which wild-type *Insm1* was replaced by a sequence encoding *Insm1ΔSNAG* by the use of homologous recombination in embryonic stem cells and blastocyst injections (supplementary material Fig. S4). We compared Insm1 protein in pituitary extracts from mice with the genotypes $Insm1^{\Delta SNAG/+}$ (also called control) and $Insm1^{\Delta SNAG/lacZ}$ (also called $Insm1^{\Delta SNAG}$ mutants), using anti-Insm1 and anti-FLAG antibodies. This demonstrated that the Insm1ΔSNAG protein was present and stable, and it was detected even at mildly increased amounts in the pituitaries of $Insm1^{\Delta SNAG}$ mutants compared with control mice (Fig. 7A). To test whether this is caused by transcriptional autoregulation, we quantified *lacZ* transcripts by qPCR and found upregulated *lacZ* expression in $Insm1^{\Delta SNAG/lacZ}$ compared with $Insm1^{lacZ/+}$ pituitaries mice (Fig. 7B). The heterozygous $Insm1^{\Delta SNAG}$ animals that were used as controls were viable and fertile and displayed no overt phenotype.

We next compared pituitary differentiation in control and $Insm1^{\Delta SNAG}$ mutant mice. None of the endocrine cell types of the pituitary differentiated correctly in $Insm1^{\Delta SNAG}$ mutants. In particular, we did not detect GH, TSH and FSH proteins in the pituitary (Fig. 7C-H). Furthermore, LH, Prl, MSH and ACTH were absent or present at reduced levels (Fig. 7I-P). The expression of pan-endocrine genes, such as proprotein convertases and granins, were compared using qPCR and were downregulated to similar extents in the pituitary of $Insm1^{lacZ}$ and $Insm1^{\Delta SNAG}$ mutants (Fig. 7U). By contrast, muscle-specific genes, such as *Myl1*, *Actc1* and *Msc* were upregulated to a similar extent, as assessed by *in situ* hybridization and/or qPCR (Fig. 7Q-T,V). qPCR analysis also demonstrated that *Dll1*, *Notch2* and the Notch target genes *Hey1*, *Hes1* and *Hes5* were upregulated to a similar extent in the pituitary of $Insm1^{\Delta SNAG}$ and $Insm1^{lacZ}$ mutants (Fig. 7W). Overall, our data show that the SNAG domain of Insm1 is essential for its function during the differentiation of pituitary endocrine cell types.

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Table 2. A common set of genes are deregulated in the pituitary, adrenal gland and pancreas of *Insm1* mutant mice

Gene symbol	Gene name	Fold change in pituitary gland	Fold change in adrenal gland	Fold change in pancreas
Resp18	regulated endocrine-specific protein 18	−14.6***	−7.0**	−4.2*
Chgb	chromogranin B	−17.8***	−6.8**	−43.0**
Scg3	secretogranin III	−6.0***	−2.0*	−10.8**
Pcsk1n	proprotein convertase subtilisin/kexin type 1 inhibitor	−5.1***	−1.9*	−2.4*
Scg2	secretogranin II	−5.2***	−3.3*	−44.0**
Chga	chromogranin A	−4.8***	−9.8**	−26.2**
Gng4	guanine nucleotide binding protein (G protein)	−3.3**	−1.7*	−2.9*
Sez6l2	seizure related 6 homolog like 2	−3.2**	−2.5*	−4.0**
Aplp1	amyloid beta (A4) precursor-like protein 1	−2.5**	−1.8*	−2.4*
Slc35d3	solute carrier family 35, member D3	−1.8**	−2.8*	−1.4*
Snx5	sorting nexin 5a	−1.7**	−1.7*	−2.2*
Cplx2	complexin 2	−1.7*	−1.8*	−1.8*
Efcab1	EF hand calcium binding domain 1	−1.5*	−1.5*	−2.0*
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	−1.4*	−2.2*	−2.0*
Trp53inp2	transformation related protein 53 inducible nuclear protein 2	−1.3*	−2.1*	−1.9*
Ccrn4l	CCR4 carbon catabolite repression 4-like (*S. cerevisiae*)	−1.3*	−1.3*	−2.0*
Pclo	piccolo (presynaptic cytomatrix protein)	−1.2*	−1.6*	−1.7*
Hbs1l	Hbs1-like (S. cerevisiae)	−1.2*	−1.3*	−2.0*
Cyp51	cytochrome P450, family 51	−1.1*	−1.7*	−1.7*
Btg3	B-cell translocation gene 3	−1.1*	−1.3*	−1.4*
Efcab14 (4732418C07Rik)	EF-hand calcium binding domain 14	−1.1*	−1.4*	−1.4*
Gmpr2	guanosine monophosphate reductase 2	1.1*	1.7*	2.0*
Tcea3	transcription elongation factor A (SII)	1.2*	1.3*	2.1*
Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase	1.3*	2.2*	1.8*
Meis1	Meis homeobox 1	1.5**	1.4*	3.1*
Bcl6	B-cell leukemia/lymphoma 6 (Bcl6)	1.6**	1.2*	1.6*

Systematic analysis of gene expression in pituitary glands, adrenal glands and pancreata of $Insm1^{+/lacZ}$ and $Insm1^{lacZ/lacZ}$ mice using Illumina or Affymetrix microarrays. The average signal fold change is shown. We selected the following genes for display: (1) genes that are deregulated in all three endocrine organs; (2) genes that display a fold change >1.4 in at least one of these endocrine organs; (3) genes that are consistently up- or downregulated in all three endocrine organs.

*P<0.05; **P<0.01; ***P<0.001.

The fact that mutant endocrine cells continue to express the truncated Insm1ΔSNAG protein allowed us to use anti-Insm1 antibodies to analyze and quantify cells co-expressing (wild-type or truncated) Insm1, Sox2 or Sox9 protein in control and $Insm1^{\Delta SNAG}$ mutants. First, we quantified overall numbers of $Sox2^+$, $Sox9^+$ and $Insm1^+$ cells in the anterior lobe of the pituitary (supplementary material Fig. S6). This demonstrated a significant increase in the number of $Sox2^+$ and $Sox9^+$, but not $Insm1^+$ cells. Furthermore, the proportion of $Sox2^+$ or $Sox9^+$ cells co-expressing Insm1 was increased in $Insm1^{\Delta SNAG}$ mutants compared with control mice (Fig. 8A-F), providing further evidence that the mutant cells remain in a progenitor state for a prolonged period. Interestingly, a combination of *in situ* hybridization and immunohistology demonstrated that a subpopulation of cells expressing the truncated Insm1 protein co-express *Myl1* (Fig. 8G,H). Thus, Insm1 acts cell-autonomously when repressing the expression of muscle-specific genes in the pituitary.

DISCUSSION

We show here that all endocrine cell types of the pituitary express Insm1 and depend on Insm1 for differentiation. Early specification of endocrine cells proceeded correctly, whereas transcription factors that control differentiation of endocrine cells were not correctly expressed in *Insm1* mutants. Furthermore, the endocrine differentiation program was disrupted in *Insm1* mutants, and hormones, proprotein convertases and granins were not expressed or expressed at markedly reduced levels. In addition, we noted prolonged expression of progenitor markers (Sox2/9), as well as ectopic expression of Notch signaling components and of genes typical for the muscle lineage in the *Insm1* mutant pituitary (summarized in Fig. 8I). Our previous work has demonstrated a severe disruption of the differentiation of endocrine cells in pancreas, intestine and adrenal medulla (Gierl et al., 2006; Wildner et al., 2008). A comparison of the deregulated genes in the pancreas, adrenal and pituitary glands detects a small set of overlapping genes (Table 2). Insm1 is thus emerging as a pan-endocrine differentiation factor.

The SNAG domain of Insm1

The SNAG domain consists of fewer than ten amino acids located at the immediate N-terminus of the Insm1 protein. We use here mouse genetics to show that this domain is essential for Insm1 function. The SNAG domain is found in other transcription factors, such as Gfi1 and Snail1 (Snai1), two well characterized zinc finger factors that act as transcriptional regulators (Hock and Orkin, 2006; Moreno-Bueno et al., 2008; Möröy and Khandanpour, 2011). We show in biochemical experiments that the SNAG domain of Insm1 recruits a battery of chromatin modifying enzymes, such as Kdm1a, Rcor1-3 and Hdac1/2, as well as additional factors, such as Gse1 and Hmg20a/b.

Snail1 is a repressor of E-cadherin (cadherin 1) gene expression and controls epithelial-mesenchymal transition of cells, for instance in tumorigenesis (Cano et al., 2000). The SNAG domain of Snail1 also recruits Kdm1a, Rcor1/2 and Hdac1/2; it resembles the tail of histone H3, and the active site of Kdm1a binds the histone H3 tail and the SNAG domain (Lin et al., 2010). To the Snail1-Kdm1a complex, Rcor is recruited, and the formation of the ternary complex stabilizes Snail1 (Lin et al., 2010). By contrast, the steady state levels of Insm1ΔSNAG are not markedly lowered, indicating that Insm1 stability does not depend on the formation of the ternary

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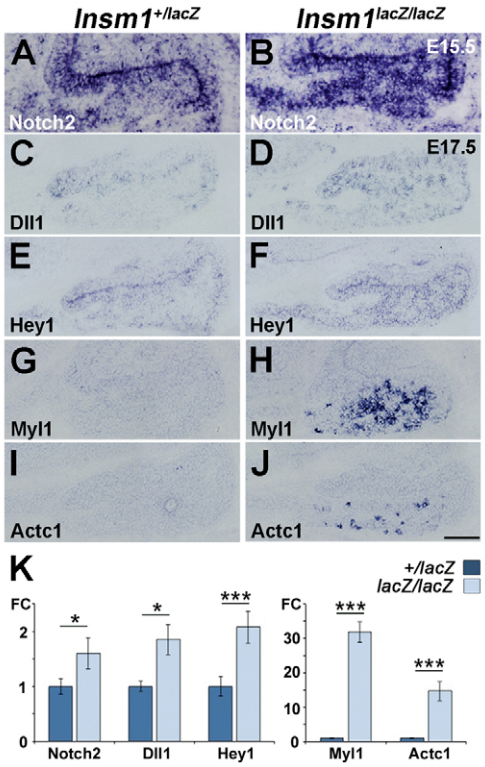

Fig. 4. Enhanced expression of *Notch2*, *Dll1* and *Hey1* and de-repression of skeletal muscle genes in the pituitary of *Insm1* mutants. (A-J) Analysis of *Notch2* (A,B), *Dll1* (C,D), *Hey1* (E,F), myosin light polypeptide 1 (*Myl1*; G,H) and alpha cardiac muscle 1 actin (*Actc1*; I,J) expression by *in situ* hybridization in control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice at E15.5 (A,B) and E17.5 (C-J). (K) Upregulated expression of these genes was verified by qPCR. *P<0.05; ***P<0.001. Scale bar: 100 µm.

Insm1-Rcor-Kdm1a complex. The transcription factor Gfi1 is essential for hematopoietic differentiation, and a single point mutation in the SNAG domain of Gfi1 interferes with Gfi1 function in hematopoietic differentiation as well as in recruitment of Kdm1a, Rcor1/2 and Hdac1/2 (Saleque et al., 2007). Thus, the function of the SNAG domains of Insm1, Snail1 and Gfi1 are conserved, and they act as hub to recruit chromatin-modifying factors. The recruitment of these modifying factors is essential for the function of these zinc finger transcription factors.

Transcriptional deregulation in the developing pituitary of *Insm1* mutant mice

Our analysis of pituitary development in *Insm1*^{lacZ} mutant mice indicates that the differentiation of the different endocrine cell types is disrupted and that neither the hormones nor pan-endocrine-specific genes, such as proprotein convertases or granins, are expressed correctly. However, morphogenesis of the pituitary remains intact and, for instance, endocrine progenitors leave the proliferative zone in the *Insm1* mutant pituitary. This requires an epithelial-mesenchymal transition, which thus occurs in the absence of Insm1. The first changes that we detect during development of endocrine cells in the pituitary of *Insm1*^{lacZ} mutants are associated with the failure to initiate expression of transcription factors that control the lineage-specific differentiation (e.g. Pit1, Sp1, Math3). Insm1 might thus participate in the activation of such genes or indirectly preclude the expression of such factors. We also observed upregulated expression of the genes encoding Notch2, the Notch ligand Dll1 and of target genes of the Notch signaling pathway, such as *Hey1*, *Hes1* and *Hes5*. In normal development, the Notch2 and Notch3 receptors and Hes1 are expressed in progenitor cells, but are excluded from differentiating endocrine cells (Raetzman et al., 2004). A tight control of Notch signaling is required for pituitary development, and sustained Notch activation in progenitor cells by expression of Notch1/2 intracellular domains disrupts and dramatically delays endocrine differentiation (Raetzman et al., 2006; Zhu et al., 2006). Furthermore, the maintenance of progenitors and formation of melanotropes depend on the Notch target genes *Hes1* and *Hes5* (Kita et al., 2007; Raetzman et al., 2007). Upregulated expression of Notch signaling components is associated with a prolonged expression of Sox2/9, indicating that the *Insm1* mutant endocrine cells remain in a progenitor stage. The *Insm1*^{lacZ} mutation arrests differentiation of pituitary endocrine cells in fetal development, but the lethal phenotype of the mutation precludes analysis during postnatal stages. Thus, it is possible that by upregulating Notch signaling, the *Insm1*^{lacZ} mutation merely delays differentiation, as described for the Notch2 gain-of-function mutation (Raetzman et al., 2006). Upregulation of the Notch signaling pathway, particularly of Dll1 and Hes1, was also noted in

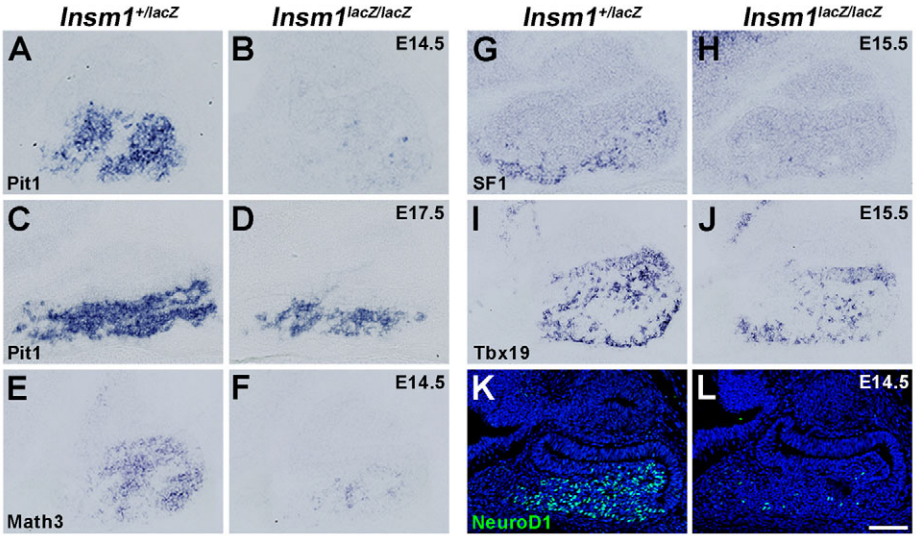

Fig. 5. Transcription factors that regulate differentiation of endocrine cells are deregulated in the pituitary of *Insm1* mutant mice. (A-J) Analysis of *Pit1* (A-D), *Math3* (E,F), *SF1* (*Nr5a1*) (G,H) and *Tbx19* (I,J) expression by *in situ* hybridization in control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice. (K,L) Immunohistological analysis of NeuroD1 in control and *Insm1* mutant mice. The developmental stages analyzed are indicated. Scale bar: 100 µm.

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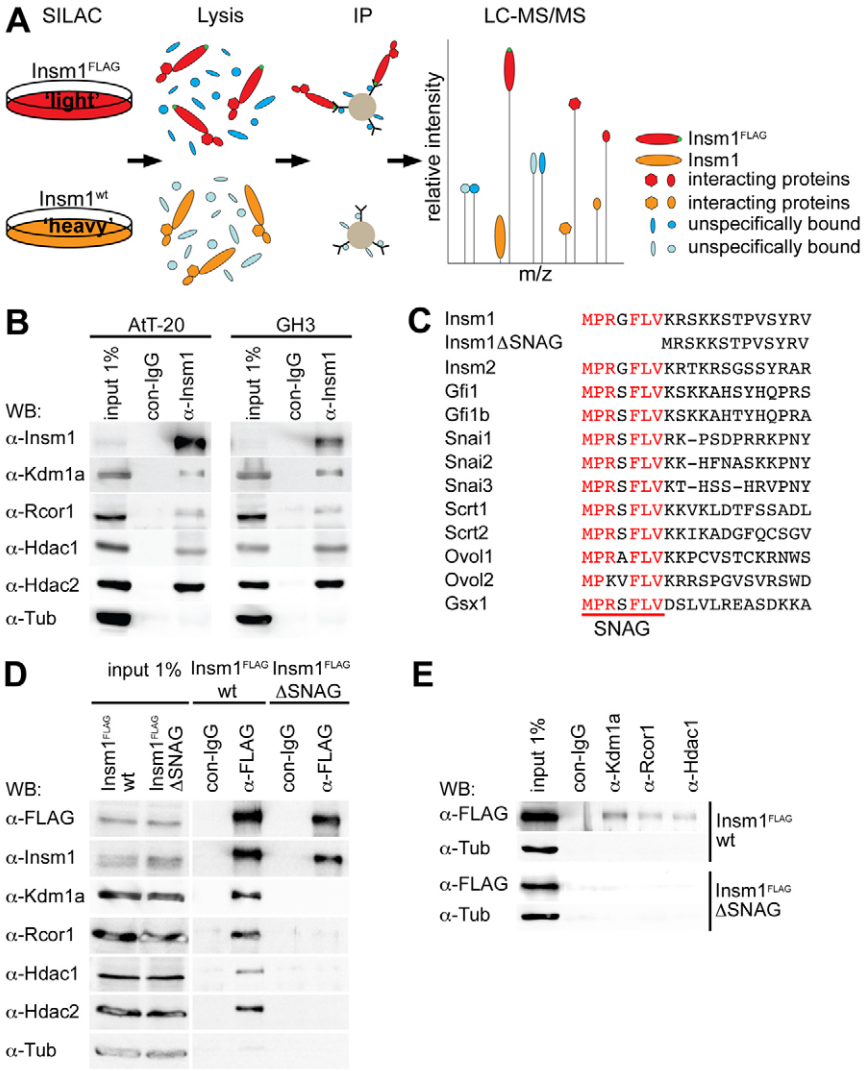

Fig. 6. Insm1 recruits chromatin-modifying factors and factors implicated in transcriptional regulation via its SNAG domain. (A) Schematic outline of the SILAC experiment (stable isotope labeling by non-radioactive amino acids in cell culture combined with mass spectrometry) used to identify proteins that interact with Insm1. Cells were grown in the presence of amino acids labeled with heavy or light non-radioactive isotopes, and transduced with retroviruses encoding non-tagged Insm1 ($Insm1^{wt}$) and FLAG-tagged Insm1 ($Insm1^{FLAG}$), respectively. Immunoprecipitates from 'heavy' or 'light' cells were pooled and analyzed by mass spectrometry. (B) Verification of Insm1 interaction partners in AtT-20 and GH3 cell lines that derive from mouse corticotropes and rat somatotropes, respectively. Proteins that interact with Insm1 were co-immunoprecipitated and identified by western blotting. (C) Comparison of N-terminal sequences of various transcription factors containing a SNAG domain; the sequence of Insm1ΔSNAG is also shown. (D) Insm1 interaction partners require the SNAG domain for binding to Insm1, as assessed by immunoprecipitation of FLAG-tagged Insm1 and Insm1ΔSNAG and western blotting. (E) Immunoprecipitations of Kdm1a, Rcor1 and Hdac1 co-immunoprecipitate Insm1 but not Insm1ΔSNAG.

our previous analysis on the development of the sympathetic nervous system in *Insm1*^{lacZ} mutant mice (Wildner et al., 2008). The fact that we observe upregulated *Dll1* and *Hes1* expression in several tissues might indicate that activation of Notch is a direct and primary event in the deregulation of gene expression caused by the *Insm1*^{lacZ} mutation.

Deletion of sequences encoding the SNAG domain of Insm1 (*Insm1*$^{\Delta SNAG}$) and the null mutation of Insm1 (*Insm1*^{lacZ}) have indistinguishable effects on the development of endocrine cells of the pituitary, indicating that the recruitment of proteins by the SNAG domain is essential for Insm1 function. Remarkably, conditional mutation of *Kdm1a* (also known as *Lsd1*) in the entire anlage of the

Table 3. Screen for Insm1-interacting proteins using SILAC technology

Protein symbol	Protein name	Ratio H/L normalized	Quant. events	Ratio H/L normalized 'label swap'	Quant. events 'label swap'
Insm1	insulinoma-associated 1	0.02	51	9.04	63
Kdm1a	lysine (K)-specific demethylase 1A	0.34	2	n.d.	n.d.
Rcor1	REST corepressor 1	0.13	16	5.47	6
Rcor2	REST corepressor 2	0.27	4	5.58	2
Rcor3	REST corepressor 3	0.30	2	n.d.	n.d.
Hdac1	histone deacetylase 1	0.04	1	6.13	2
Hdac2	histone deacetylase 2	0.05	5	5.44	4
Gse1	genetic suppressor element 1	0.06	27	7.25	8
Hmg20a	high mobility group 20A	0.05	6	9.37	4
Hmg20b	high mobility group 20B	0.14	10	10.46	4

AtT-20 cells were transfected with Insm1 and FLAG-tagged Insm1 after labeling with 'heavy' and 'light' amino acids, respectively; proteins in immunoprecipitates obtained by the use of anti-Flag antibodies were identified using mass spectrometry. Shown are the gene symbol and name, the ratio of the abundance of heavy and light labeled peptides of a particular protein identified by mass spectrometry (ratio H/L normalized) and the number of quantification events/protein (quant. events). In a second experiment, 'heavy' and 'light' amino acids label was swapped (label swap).
n.d., not detected.

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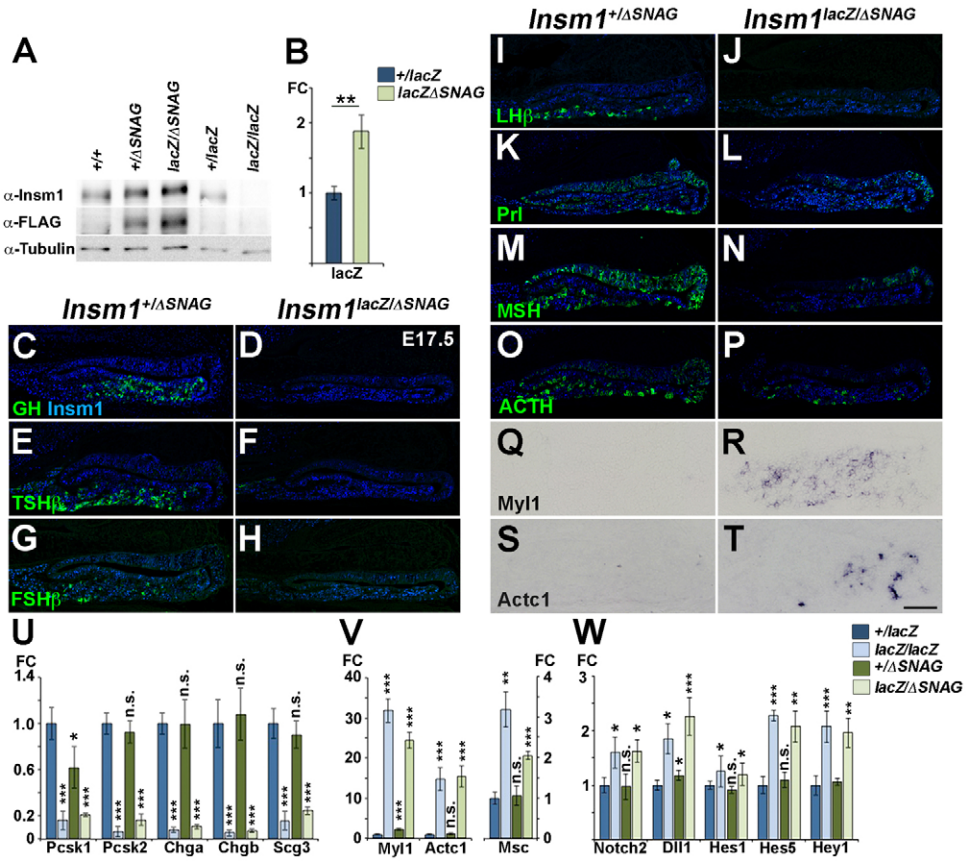

Fig. 7. The SNAG domain is required for Insm1 function. (A) Expression of Insm1 and Insm1ΔSNAG protein in the pituitary of wild-type, *Insm1*$^{+/\Delta SNAG}$, *Insm1*$^{lacZ/\Delta SNAG}$, *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/lacZ}$ mice was assessed by western blotting using antibodies against Insm1 and FLAG. Note that the allele encoding Insm1ΔSNAG contains a FLAG-tag (supplementary material Fig. S4). (B) qPCR of *lacZ* transcripts expressed in the pituitary of *Insm1*$^{+/lacZ}$ and *Insm1*$^{lacZ/\Delta SNAG}$ mice. (C-P) Analysis of hormone expression in the pituitary of control and *Insm1*$^{lacZ/\Delta SNAG}$ mice at E17.5 using antibodies directed against GH (C,D), TSH (E,F), FSH (G,H), LH (I,J), Prl (K,L), MSH (M,N) and ACTH (O,P). Note that all hormones are produced at much reduced levels in *Insm1*$^{\Delta SNAG}$ mutant mice. (Q-T) Analysis of myosin light polypeptide 1 (*Myl1*; Q,R) and alpha cardiac muscle 1 actin (*Actc1*; S,T) expression by *in situ* hybridization in the pituitary of control and *Insm1*$^{\Delta SNAG}$ mutant mice. (U-W) qPCR analysis of proprotein convertases and granins (U), various muscle-specific genes (V) and components of the Notch signaling pathway (W). Compared are pituitaries of control (*Insm1*$^{+/\Delta SNAG}$ and *Insm1*$^{+/lacZ}$), *Insm1*$^{lacZ/lacZ}$ and *Insm1*$^{lacZ/\Delta SNAG}$ mice at E17.5. **P*<0.05; ***P*<0.01; ****P*<0.001; n.s., not significant. Scale bar: 100 μm.

pituitary results in stunningly similar deficits and, like the *Insm1* mutation, blocks differentiation of all endocrine cell types of the pituitary (Wang et al., 2007). Furthermore, the expression of Prop1 but not Pit1 is correctly initiated, and Hey1 is upregulated in *Kdm1a* and *Insm1* mutant pituitaries (this work) (Wang et al., 2007). Kdm1a acts as a histone demethylase that demethylates both Lys-4 (H3K4me) and Lys-9 (H3K9me) of histone H3 and acts thus in a context-dependent manner as co-activator or co-repressor (Lee et al., 2005; Metzger et al., 2005). It is possible that Insm1 also takes over such dual roles in the pituitary. In support for a function as a repressor, we observed many genes that were upregulated, among them Dll1 and Notch target genes. Interestingly, among the top 20 upregulated genes, nine are strongly expressed in muscle tissue in normal development. Muscle and pituitary derive from mesoderm and ectoderm, respectively. Activation of particular gene expression programs that are characteristic of different germ layers at such late developmental stages is unusual. It should be noted that this ectopic expression in the pituitary did not reach levels comparable to those observed in mature skeletal muscle, and the changed expression does therefore not represent a complete fate shift. The data indicate that Insm1 participates in the repression of inappropriate differentiation programs. We conclude that Insm1 recruits chromatin-modifying factors and thus directs differentiation of all endocrine cell types of the pituitary.

MATERIALS AND METHODS

Mouse strains

Generation and genotyping of *Insm1*^{lacZ} mutant mice were carried out as described (Gierl et al., 2006). The Insm1ΔSNAG targeting vector was generated by homologous recombination in bacteria (Lee et al., 2001); in particular, the *Insm1* coding sequence was replaced by an Insm1ΔSNAG sequence that lacks the seven N-terminal amino acids (Fig. 6C; supplementary material Fig. S4) and contains a C-terminal 3xFLAG-tag (Sigma-Aldrich) sequence. The targeting vector contained a self-excision *neo* cassette (Bunting et al., 1999). R1/E embryonic stem cells 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. Chimeric mice obtained after blastocyst injection were mated to C57BL/6 mice for transmission of the Insm1ΔSNAG allele. Routine genotyping was performed by PCR using the following primers: Insm1ΔSNAG-fw CCCAACCAGTGCGTCGCCCTT and Insm1ΔSNAG-rv AGCAGCGCCCGGTCACTGTCC.

In situ hybridization, X-Gal staining, BrdU labeling and immunohistochemistry

For *in situ* hybridization, tissue was embedded into OCT compound directly after dissection. Digoxygenin-labeled riboprobes, which were used for hybridization, were generated by *in vitro* transcription. β-Galactosidase activity was assessed by X-Gal staining as described previously (Lobe et al., 1999). For immunohistochemistry, tissue was fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and cryoprotected in 25% sucrose in PBS prior to embedding, and cryosections were cut at 14 μm thickness. The following primary antibodies were used: rabbit/guinea pig anti-Insm1 (1:10,000; antibodies were raised against an antigen produced in *Escherichia coli* and the plasmid used for production of the antigen was kindly provided by G. Gradwohl, IGBMC Illkirch, France), rabbit anti-ACTH (1:2000; AFP15610278912, NHPP, Torrance, USA), chicken anti-β-galactosidase (1:5000; Ab9361, Abcam), rat anti-BrdU antibodies (1:200; AbD Serotec), rat-anti ER/TR7 (1:500; Ab51829, Abcam), rabbit anti-FSH (1:2000; AFP7798-1289, NHPP), rabbit anti-GH (1:1000; Ab940, Merck-Millipore), rat anti-Ki67 (1:500; M724901-8, Dako Cytomation), rabbit anti-LH (1:2000; AFPC697071P, NHPP), rabbit anti-MSH (1:1000; Peninsula Laboratories LLC), goat anti-NeuroD1 (1:500; sc-1084, Santa Cruz), rat anti-PECAM (1:300; 553930, BD Pharmingen), rabbit anti-Prl (1:2000; AFP131078rb, NHPP), rabbit anti-S100 (1:1000;

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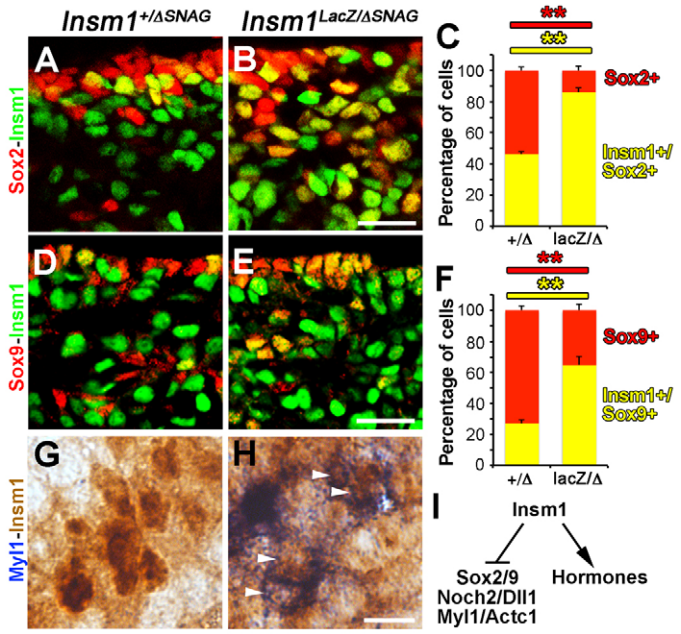

Fig. 8. Mutant endocrine cells in *Insm1*$^{lacZ/\Delta SNAG}$ mice maintain Sox2/9 expression and ectopically express the muscle-specific gene *Myl1*. (A-F) Immunohistological analysis of co-expressed Sox2/Sox9 and wild-type/truncated Insm1 protein in control (*Insm1*$^{+/\Delta SNAG}$) and mutant *Insm1*$^{lacZ/\Delta SNAG}$ mice (A,B,D,E); quantification of co-expressing cells in the anterior lobe of the pituitary (C,F). (G,H) Analysis of co-expression of *Myl1* and wild-type/truncated Insm1 in control (*Insm1*$^{+/\Delta SNAG}$) and mutant *Insm1*$^{lacZ/\Delta SNAG}$ mice; arrowheads indicate that a subpopulation of Insm1^{+} cells co-expresses *Myl1* in the mutant pituitary. (I) Summary of Insm1 functions in gene expression in endocrine cells of the pituitary. Insm1 suppresses the expression of progenitor markers (Sox2/9), various components of the Notch pathway (Notch2, Dll1) and muscle-specific genes (*Myl1*, *Actc1*) in developing endocrine cells of the pituitary, and drives differentiation and hormone gene expression. ***P*<0.01. Scale bars: 25 µm (B,E); 12.5 µm (H).

Z0311, Dako), goat anti-Sox2 (1:500; GT15098, Immune Systems), rabbit anti-Sox9 (1:2500; Ab5535, Merck-Millipore) and rabbit anti-TSH (1:1000; Ab976, Merck-Millipore). Secondary antibodies conjugated to Cy2, Cy3 or Cy5 were used at a dilution of 1:500 (Jackson ImmunoResearch). All quantifications from histological sections were performed on at least three sections of three or more animals/genotype. Unpaired Student's two-tailed *t*-tests were performed using Graphpad Prism 5 to determine significance. Average and s.e.m. are displayed. For visualization of Myl1 and Insm1ΔSNAG co-expression, *in situ* hybridization was combined with immunohistochemistry. TUNEL was performed by the use of a kit (57110, Merck-Millipore) according to the manufacturer's instructions. DAPI was used as a nuclear counterstain. Fluorescence was imaged on a Zeiss LSM 700 confocal microscope and images were processed using Adobe Photoshop software.

Morphometric analysis

For the estimation of the volume and area of the pituitary gland, 14-µm-thick sagittal sections were collected and Nissl stained. A total of 75 sections of the pituitary gland were obtained per animal (three animals/genotype). The area of every third section was measured using imaging analysis software (ImageJ; NIH, version 1.34n). Estimation of the total volume of the pituitary gland was obtained by application of Cavalieri's method (West, 2012).

qRT-PCR and microarrays

E17.5 pituitary glands were dissected and total RNA was isolated using Trizol reagent (Invitrogen). qRT-PCR analysis after first-strand cDNA synthesis was performed using a CFX96 RT-PCR system (Bio-Rad), ABsolute QPCR SYBR Green Mix (Thermo Fisher Scientific) and the primers are listed in supplementary material Table S1. Total RNA from pituitary glands was labeled and hybridized to MouseRef-8 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

Microarray data analysis

All microarray datasets were analyzed using R Bioconductor package (Gentleman et al., 2004). To compare samples, we applied log2 transformation and normalized using the 'quantile' method, and adjusted *P*-values were obtained using the Benjamini-Hochberg FDR method (cutoff $P<0.05$ for all cell types). Expression data of control and *Insm1* mutant pituitary glands have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through accession number GSE46139.

Cell culture, biochemical experiments, mass spectrometry and data analysis

AtT-20 and GH3 cells were grown in high-glucose DMEM (Gibco/Invitrogen) containing 10% fetal calf serum (FCS; Sigma-Aldrich). For SILAC experiments, AtT-20 cells were grown in SILAC DMEM (PAA Laboratories, Velizy-Villacoublay, France), 10% dialyzed FCS (SAFC), 4 mM glutamine and amino acids labeled either with 'light' [^{12}C;^{14}N]arginine/lysine (Sigma-Aldrich) or 'heavy' [^{13}C;^{15}N]arginine/lysine (Sigma-Aldrich) isotopes for four passages.

For immunoprecipitation, cells were washed with PBS, harvested in lysis buffer containing 50 mM Tris pH 7.4, 140 mM NaCl, 1% Triton X-100, complete protease inhibitors (Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). After pre-clearing, supernatants were incubated with antibodies conjugated and covalently coupled to Dynabeads ProteinA/G for 3 hours at 4°C. Precipitates of the corresponding 'heavy' and 'light' pull-downs were combined and washed three times with lysis buffer. The last wash was performed with 5 mM Tris pH 7.4, 140 mM NaCl. Bound proteins were eluted with 3×100 µl 100 mM glycine pH 2.5 and subsequently precipitated by adding 1 µl GlycoBlue (Ambion/Life Technologies), 80 µl 2.5 M sodium acetate pH 5.0 and 1500 µl ethanol. Sedimentation was carried out at 20,000 ***g*** for 30 minutes. Protein pellets were dissolved in 6 M urea/2 M thiourea/10 mM HEPES (pH 8.0), reduced with 1 mM dithiothreitol, alkylated with 5.5 mM iodoacetamid and subsequently digested by lysyl-endopeptidase (Lys-C, Wako Chemicals) and sequencing grade modified trypsin (Promega) in 50 mM ammonium bicarbonate following standard procedures (Shevchenko et al., 2007). Peptides were desalted by stop and go extraction (STAGE) tips containing C18 Empore disks (3M) (Rappsilber et al., 2003). Peptide mixtures were analyzed by C18 reversed phase nanoflow HPLC on an Eksigent nanoLC-1D Plus system (Eksigent Technologies) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Mass spectrometers were operated in the data-dependent mode with a TOP5 method (Olsen et al., 2005). Raw data files were processed using the MaxQuant software platform with standard settings (Cox and Mann, 2008; Cox et al., 2009). Peak lists were searched against an in-house curated mouse International Protein Index (IPI) human protein database using MASCOT (Matrix Science, Boston, USA) and a database containing common contaminants. Proteins with one unique peptide were considered identified, and peptide and protein false discovery rates were restricted to 0.01.

For western blot analysis, immunoprecipitated proteins were eluted from beads by boiling in 1 × Laemmli buffer for 5 minutes and then subjected to SDS-PAGE. Proteins were transferred to PVDF membranes. The following antibodies were used for western blot analysis: rabbit anti-Kdm1a (1:1000, #2139, Cell Signaling Technology), goat anti-CoREST (1:200, sc-23448, Santa Cruz), rabbit anti-Hdac1 (1:1000, #2062, Cell Signaling Technology), rabbit anti-Hdac2 (1:2000, ab32117, Merck-Millipore), rabbit/guinea pig anti-Insm1 (1:6000), mouse anti-tubulin (1:10,000, T9026, Sigma-Aldrich), mouse anti-Flag (1:1000, F1804, Sigma-Aldrich). Secondary antibodies coupled to horseradish peroxidase (Dianova, Hamburg, Germany) were used, and blots were developed on a Chemi-smart 3000 (Vilber,

Eberhardzell, Germany). Wild-type and modified Insm1 cDNAs [Insm1^{FLAG} (Insm1 containing 3xFLAG-tag, Sigma-Aldrich), Insm1ΔSNAG^{FLAG} (Insm1ΔSNAG containing 3xFLAG-tag, Sigma-Aldrich)] were expressed in AtT-20 cells using a MMULV-based system developed by the Nolan lab (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html).

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Competing interests
The authors declare no competing financial interests.

Author contributions
J.E.W. performed the majority of the experiments; L.R.H.-M., F.E.P. and S.J. performed experiments; J.E.W., L.R.H.-M., M.S. and C.B. designed experiments; A.I. analyzed microarray data; and J.E.W. and C.B. wrote the paper.

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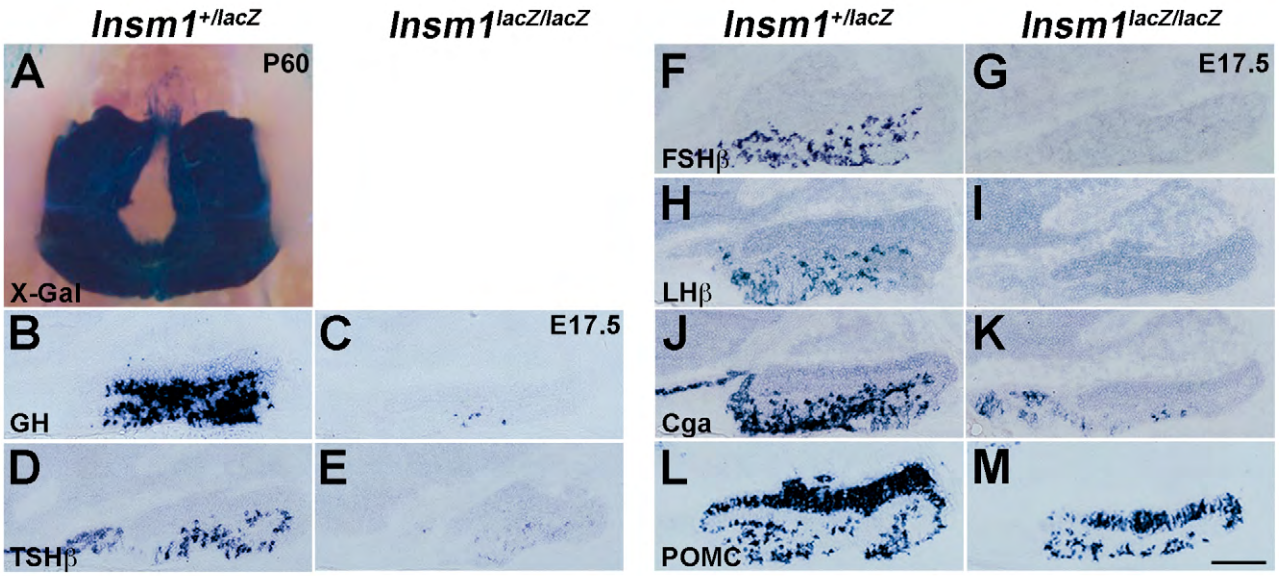

Fig. S1. Insm1 expression in the adult pituitary gland and reduced transcription of genes encoding pituitary hormones. Whole mount X-Gal staining of a P60 *Insm1*$^{+/lacZ}$ pituitary gland demonstrates transcriptional activity of the *Insm1* locus in the adult (A). Transcript levels of hormone coding genes were assessed by *in situ* hybridization in control and mutant (*Insm1*$^{lacZ/lacZ}$) mice at E17.5 (B-M). GH (B,C), TSHb (D,E), FSHb (F,G) and LHb (H,I) transcripts were abundant in control pituitaries and absent in pituitaries of *Insm1*$^{lacZ/lacZ}$ mice, and Cga (J,K) and POMC (L,M) transcripts were present at reduced levels in mutants. Scale bar**:** 100 µm.

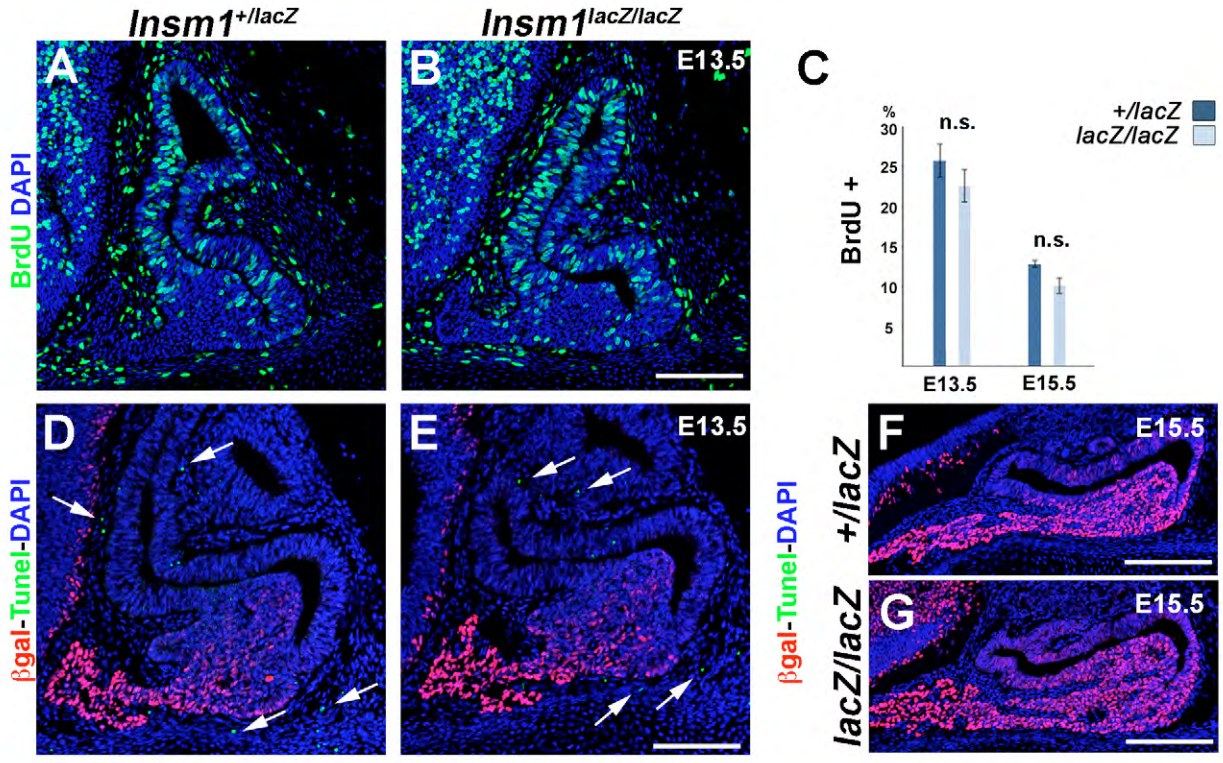

Fig. S2. The *Insm1* mutation does not promote proliferation or cell death. Proliferation was assessed at E13.5 and E15.5 by BrdU incorporation (A-C) in control (*Insm1*$^{+/lacZ}$) and mutant (*Insm1*$^{lacZ/lacZ}$) mice. Nuclei were counterstained with DAPI, and the percentage of BrdU+ cells was determined (C). Apoptosis was assessed by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay at E13.5 (D,E) and E15.5 (F,G). In control and mutant mice at E13.5 or E15.5, a few apoptotic cells (arrows) were observed in parenchymal and other tissues adjacent to the pituitary gland, but not within the gland (D-G). Scale bars**:** 100 µm.

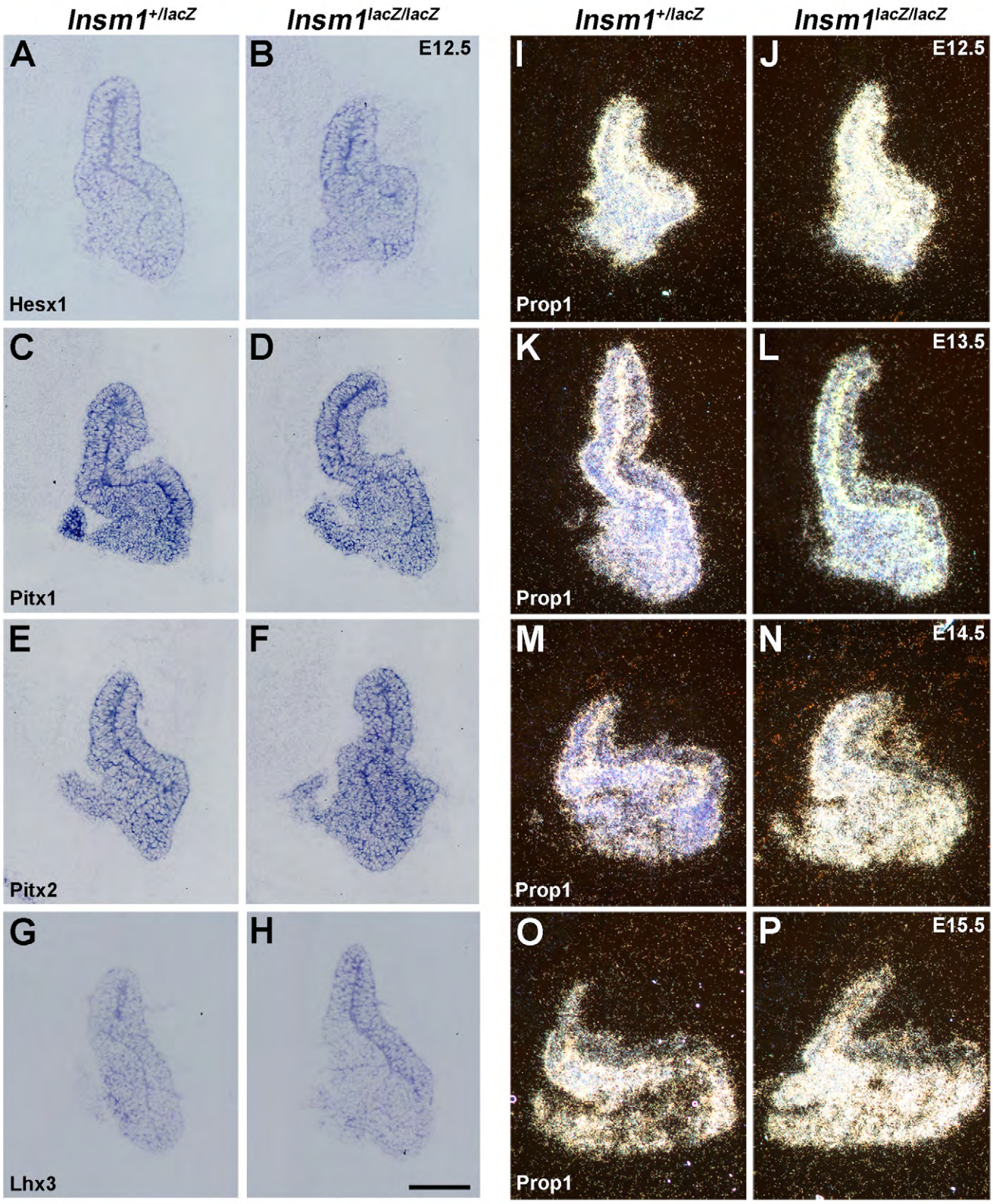

Fig. S3. Pituitary endocrine cells are specified correctly in *Insm1* mutant mice. The expression of transcription factors expressed during specification of pituitary endocrine cells was assessed by *in situ* hybridization. The transcripts of Hesx1 (A,B), Pitx1 (C,D), Pitx2 (E,F), Lhx3 (G,H), and Prop1 (I-P) were detected at comparable levels in control (*Insm1*$^{+/lacZ}$) and mutant (*Insm1*$^{lacZ/lacZ}$) mice at E12 or E13.5. At E14.5 and E15.5, expression of Prop1 was mildly enhanced in the anterior lobe. Scale bar: 100 μm.

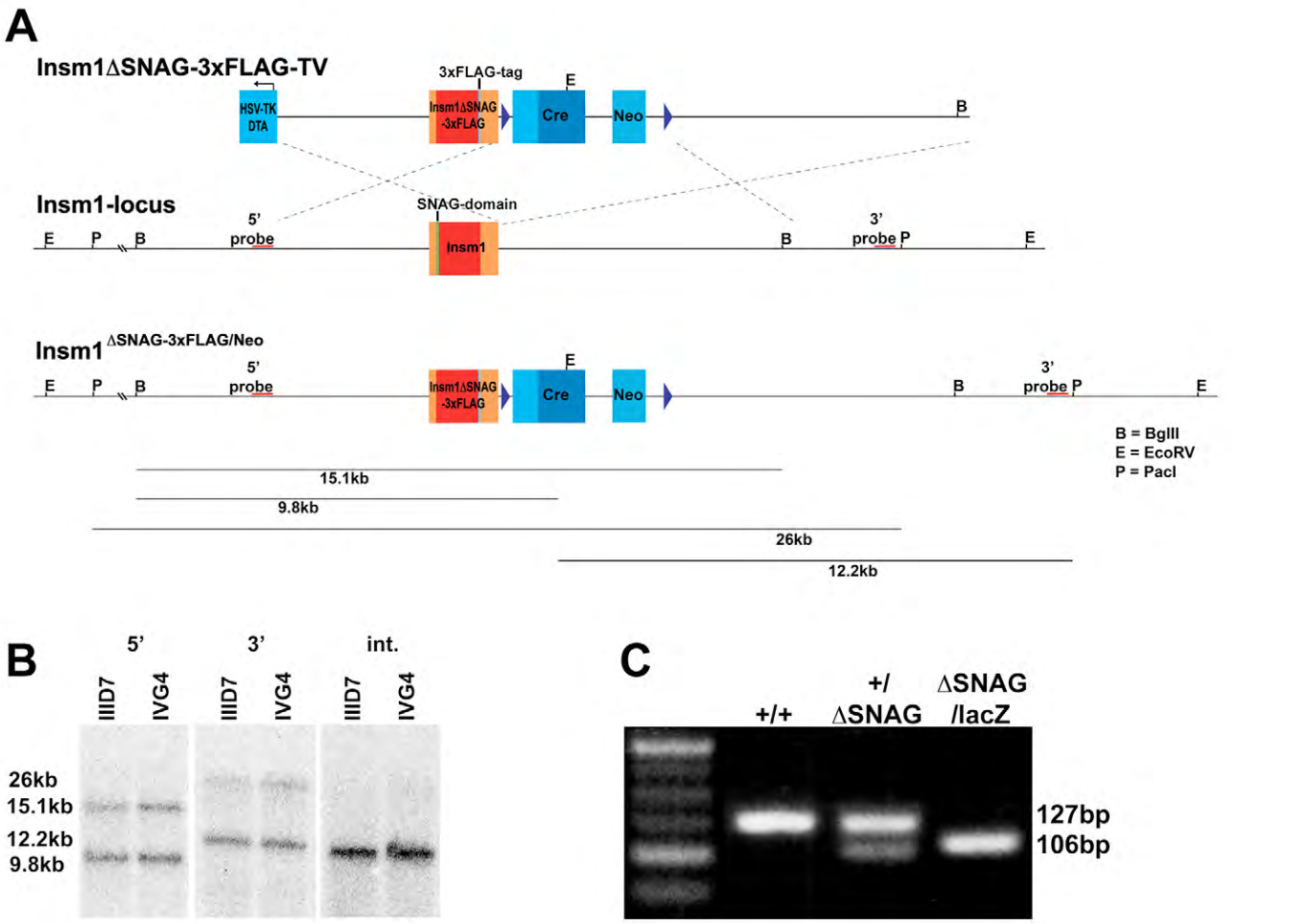

Fig. S4. Generation of *Insm1*$^{\Delta SNAG}$ mutant mice. Schematic representation of the targeting vector, the wild-type *Insm1* locus, and the mutated *Insm1*$^{\Delta SNAG}$ allele (A). Coding (red) and non-coding (orange) sequences, the SNAG domain (green), the N-terminal 3xFLAG-tag (grey), DTA, the self-excision neomycin cassette, loxP (arrowhead), Southern blot probes (red bars) and *Bgl*II, EcoRV and PacI restriction sites are depicted. Black lines indicate the predicted fragment sizes obtained after digestion of genomic DNA. G418 resistant clones were analyzed by Southern blotting (B). Hybridization with a probe located 5′ of the targeting vector detected 15.1 kb and 9.8 kb DNA fragments corresponding to *Insm1* wildtype and *Insm1*$^{\Delta SNAG}$ alleles, respectively; the DNA from ES cells was digested with *Bgl*II/EcoRV. Hybridization with a probe located 3′ of the targeting vector detected 26 kb and 12.2 kb fragments of *Insm1* wildtype and *Insm1*$^{\Delta SNAG}$ alleles; the DNA was digested by PacI/EcoRV. A probe containing sequences present in the targeting vector was used to ensure the absence of random integration events. (C) *Insm1* wildtype and *Insm1*$^{\Delta SNAG}$ alleles are distinguishable by PCR. PCR amplification of *Insm1* wildtype and *Insm1*$^{\Delta SNAG}$ alleles produce a 127 bp and 106 bp fragment, respectively.

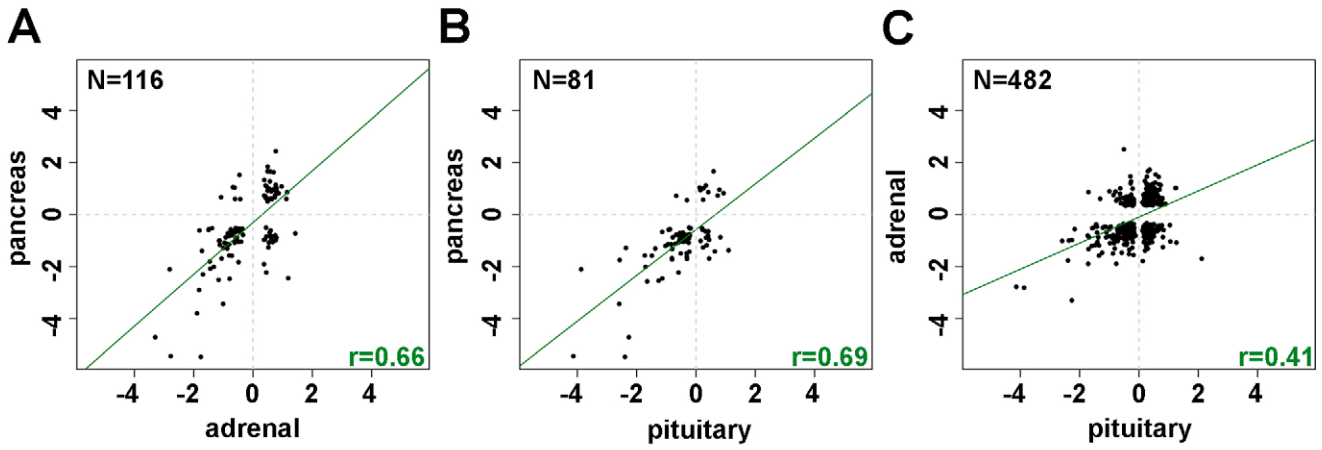

Fig. S5. Comparison of gene expression changes in three different endocrine cell types of Insm1 mutant mice. Cross comparison of expression changes (log2 values are given) in three different (adrenergic, pancreatic and pituitary) endocrine organs of *Insm1* mutant versus control mice. Green: linear regression line and Pearson's correlation coefficients. Each scatter plot represents genes that are deregulated in both organs (FC more than 1.7). (A) Comparison of deregulated genes in the adrenal gland and pancreas (116 genes); (B) Comparison of deregulated genes in the pituitary and pancreas (81 genes); (C) Comparison of deregulated genes in the pituitary and adrenal gland (482 genes).

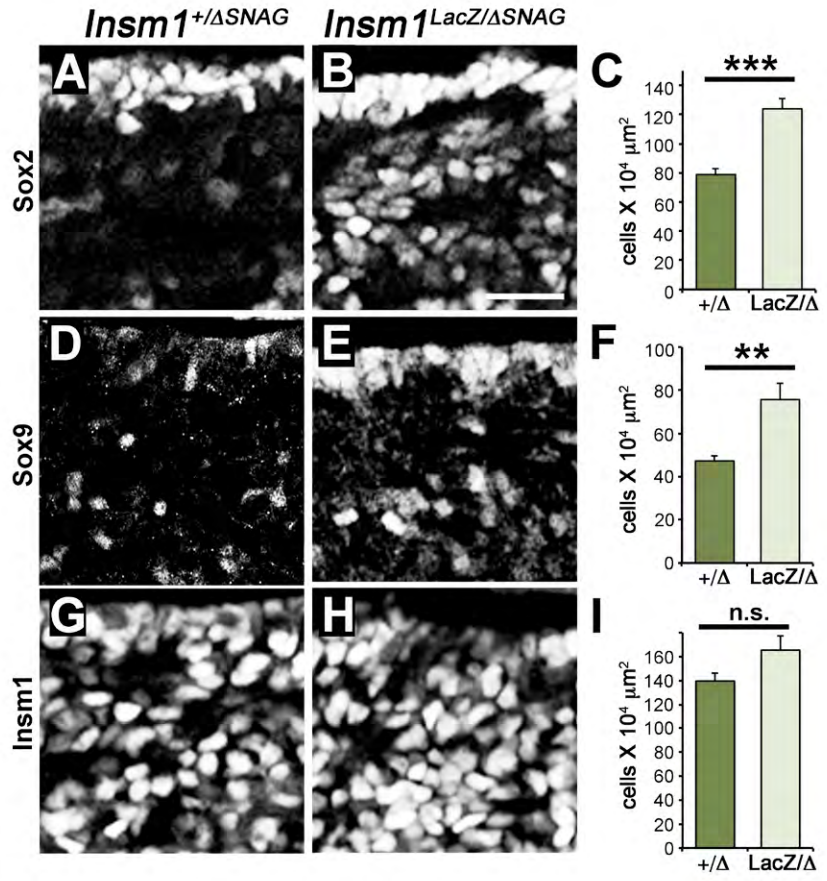

Fig. S6. Mutation of *Insm1* results in an increased number of cells that retain progenitor characteristics. Immunohistochemical analysis of endocrine progenitor cells using antibodies directed against Sox2 (A,B), Sox9 (D,E) and Insm1 (G,H) in the pituitary of Insm1 control (*Insm1*$^{+/\Delta SNAG}$) and mutant (*Insm1*$^{lacZ/\Delta SNAG}$) mice at E17.5. Quantification of numbers of Sox2+ (C), Sox9+ (F) and Insm1+ cells (I) in the anterior lobe of the pituitary; whereas the numbers of Sox2+ and Sox9+ cells were increased, the number of Insm1+ cells was not significantly changed. Scale bars: 25 µm. $^{**}P<0.01$

Table S1. Primers used for qRT-PCR

Name	Primer Sequence 5' to 3'
Myl1-fw	CACATCATGTCTGTCTAAACGG
Myl1-rv	CTGGTGTTGACAGTTAGCCAT
Actc1-fw	AGGCCCATCCATTGTCCA
Actc1-rv	CAAGAAGCACAATACGGTCA
Msc-fw	GCTTTGTGGAACTTCCGCTT
Msc-rv	AGGGCAAACCACACTTGTCT
Chga-fw	ACACTTCTGCAGGGCAGC
Chga-rv	AGTTATTGCAGTTGTGCCCC
Chgb-fw	ATTCACCCACAGGCAGAAAG
Chgb-rv	ACAAGTCACGCTAGTCACATGG
Pcsk1-fw	CCATGCTGCGACTCCT
Pcsk1-rv	TGATTGTTTTGAAAGTGCATT
Pcsk2-fw	ACCTTTGGCATCAGTATTAACACC
Pcsk2-rv	CATCAGACTCAGGGGCATCA
Scg3-fw	TGTCTCGGCATGCTAGACAC
Scg3-rv	GACGTGGGTTTATTTCCGTG
Notch2-fw	GCTATAAGTGCCTCTGCGAT
Notch2-rv	AGGCACACTCATCTATATTCACC
Dll1-fw	GATACACACAGCAAACGTGACACC
Dll1-rv	TTCCATCTTACACCTCAGTCGCTA
Hes1-fw	CAGACATTCTGGAAATGACTGTGAA
Hes1-rv	CGCGGTATTTCCCCAACAC
Hes5-fw	GCTCCGCTCGCTAATCGCCTCCAG
Hes5-rv	GTCCCGACGCATCTTCTCCACCAC
Hey1-fw	GCCGACGAGACCGAATCAATAACA
Hey1-rv	TCCCGAAACCCCAAACTCCGATAG