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

Jochen E. Welcker¹, Luis R. Hernandez-Miranda¹, Florian E. Paul², Shiqi Jia¹, Andranik Ivanov³, Matthias Selbach² and Carmen Birchmeier¹.*

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 Ptf1 (Pouf1f1), 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...
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 (Fig. 1C, inset). By E17.5, Insm1 is present in the entire anterior pituitary (Fig. 1D,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), adrenocorticotropic 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 Sox2 and Sox9 (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).
Furthermore, Insm1 is expressed in all endocrine cell types of the pituitary (see Fig. 10 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<sup>lacZ</sup> animals, which served as controls, but was not detectable in homozygous Insm1<sup>lacZ</sup> mice (Fig. 2A,B; we refer subsequently to heterozygous and homozygous Insm1<sup>lacZ</sup> mice also as control and Insm1<sup>lacZ</sup> mutants). In situ hybridization demonstrated a strong reduction in Gh transcripts in Insm1<sup>lacZ</sup> 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<sup>lacZ</sup> 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<sup>lacZ</sup> 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<sup>lacZ</sup> mutants (Fig. 2K-N), whereas Pmc mRNAs 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<sup>lacZ</sup> mutants compared with control mice (Fig. 2O,P). We conclude that none of the endocrine cell types of the pituitary differentiate correctly in Insm1<sup>lacZ</sup> 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<sup>+</sup> and Sox9<sup>+</sup> cells co-expressed β-gal in Insm1<sup>lacZ</sup> mutants, and we noted that β-gal<sup>+</sup> 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<sup>lacZ</sup> mutant mice (Fig. 3B,B').

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<sup>lacZ</sup> 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<sup>lacZ</sup> 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<sup>lacZ</sup> 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<sup>lacZ</sup> 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<sup>lacZ</sup> 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
the Insm1lacZ 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 Insm1lacZ 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 Insm1lacZ 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 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). Propl 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 (Insm1wt) and FLAG-tagged Insm1 (Insm1 FLAG), respectively. 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 (Insm1wt) and FLAG-tagged Insm1 (Insm1FLAG), 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 Insm1wt and ‘light’-labeled Insm1FLAG cells were compared. In addition, Kdm1a, Rcor1-3, Hdac1/2, Gsp1 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...
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 |
| Cgα*        | 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 |
| Pcsk1T*     | 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        | secretogranin     | -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   |
| Slit2        | sarcolipin       | 7.26        | 6.2E-07 |
| Msc*        | musculin         | 5.43        | 4.6E-07 |
| Thsd7b       | thrombospondin, type I, domain containing 7B | 3.50     | 1.7E-07 |
| Myh8         | myosin, heavy polypeptide B, skeletal muscle, perinatal | 2.86     | 3.9E-06 |
| Tmtn1        | tropolin T1, skeletal, slow | 3.24     | 3.7E-07 |
| Mustrn1      | 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 Insm1lacZ and Insm1ΔSNAGlacZ 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.

To test biochemical 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 (Insm1wildtype) and a truncated FLAG-tagged variant that lacks seven highly conserved amino acids at the N-terminus were constructed (Insm1SNAGΔ). The deletion of these seven amino acids disrupts the SNAG domain of Insm1 (Fig. 6B). Insm1ΔSNAGΔ mutant mice 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 Insm1ΔSNAGlacZ compared with Insm1ΔSNAGΔ mutants (Fig. 7B). The heterozygous Insm1ΔSNAG animals that were used as controls were viable and fertile and displayed no overt phenotype.

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 Insm1ΔSNAGlacZ (also called control) and Insm1ΔSNAGlacZ (also called Insm1ΔSNAG 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 Insm1ΔSNAG 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 Insm1ΔSNAGlacZ compared with Insm1ΔSNAGΔ mutants (Fig. 7B). The heterozygous Insm1ΔSNAG animals that were used as controls were viable and fertile and displayed no overt phenotype.

We next compared pituitary differentiation in control and Insm1ΔSNAG mutant mice. None of the endocrine cell types of the pituitary were affected in Insm1ΔSNAG mutants. In particular, we did not detect GH, TSH and FSH proteins in the pituitary (Fig. 7C-H). Furthermore, LH and FSH proteins 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 found downregulated in Insm1ΔSNAG mutant mice (Fig. 7J). The heterozygous Insm1ΔSNAG animals that were used as controls were viable and fertile and displayed no overt phenotype.
### 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** |
| Sgc3        | secretogranin III | −6.0*** | −2.0* | −10.8** |
| Pcsk1n      | propoprotein convertase subtilisin/kexin type 1 inhibitor | −5.1*** | −1.9* | −2.4* |
| Sgc2        | 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* |
| Szx262      | seizure related 6 homolog like 2 | −3.2** | −2.5* | −4.0** |
| Appl1       | amyloid beta (A4) precursor-like protein 1 | −2.5* | −1.8* | −2.4* |
| S1c35d3     | solute carrier family 35, member D3 | −1.8* | −2.8* | −1.4* |
| Snx5        | sorting nexin 5a | −1.7* | −1.7* | −2.2* |
| Cplx2       | complex 2 | −1.7* | −1.8* | −1.8* |
| Efcab1      | EF hand calcium binding domain 1 | −1.5* | −1.5* | −2.0* |
| Tnfrs12a    | tumor necrosis factor receptor superfamily, member 12a | −1.4* | −2.2* | −2.0* |
| Tp53np2     | transformation related protein 53 inducible nuclear protein 2 | −1.3* | −2.1* | −1.9* |
| Ccm4f       | CCR4 carbon catabolite repression 4-like (S. cerevisiae) | −1.3* | −1.3* | −2.0* |
| Polo        | piccolo (presynaptic cystomatrix protein) | −1.2* | −1.6* | −1.7* |
| Hbs1-1      | 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 Insm1ΔSNAG and Insm1ΔSNAG 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 Insm1ΔSNAG 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 Insm1ΔSNAG 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 complex.
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 Insm1lacZ 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 Insm1lacZ 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 Insm1lacZ 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 Insm1lacZ 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...
our previous analysis on the development of the sympathetic nervous system in Insm1<sup>lacZ</sup> 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<sup>lacZ</sup> mutation.

Deletion of sequences encoding the SNAG domain of Insm1 (Insm1<sup>ΔSNAG</sup>) and the null mutation of Insm1 (Insm1lacZ) 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 | Quant. events |
|----------------|-------------------------------|----------------------|--------------|----------------------|--------------|
|                |                               |                      |              | 'label swap'         |              |
| Insm1          | insulinoma-associated 1       | 0.02                 | 51           |                      |              |
| Kdm1a          | lysine (K)-specific demethylase 1A | 0.34               | 2            |                      |              |
| Rcor1          | REST corepressor 1            | 0.13                 | 16           |                      |              |
| Rcor2          | REST corepressor 2            | 0.27                 | 4            |                      |              |
| Rcor3          | REST corepressor 3            | 0.30                 | 2            |                      |              |
| Hdac1          | histone deacetylase 1         | 0.04                 | 1            |                      |              |
| Hdac2          | histone deacetylase 2         | 0.05                 | 5            |                      |              |
| Gse1           | genetic suppressor element 1  | 0.06                 | 27           |                      |              |
| Hmg20a         | high mobility group 20A       | 0.05                 | 6            |                      |              |
| Hmg20b         | high mobility group 20B       | 0.14                 | 10           |                      |              |

A1T-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.
described (Gierl et al., 2006). The Insm1Δ generated by homologous recombination in bacteria (Lee et al., 2001); in 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 mutation, blocks differentiation of all endocrine cell types of the pituitary.

chromatin-modifying factors and thus directs differentiation of all differentiation programs. We conclude that Insm1 recruits that Insm1 participates in the repression of inappropriate development. Muscle and pituitary derive from mesoderm and ectoderm, respectively. Activation of particular gene expression hybridization, were generated by in situ transcription. Pituitary-gland 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 E. coli). The plasmid used for production of this antigen was kindly provided by G. Gradwohl, IGBMC Illkirch, France), rabbit anti-ACTH (1:2000; AFIP5160278912, 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; AFIP7798-1289, NHPP), rabbit anti-GH (1:1000; Ab5940, 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; 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 Insm1ASNAG allele. Routine genotyping was performed by PCR using the following primers: Insm1ASNAG-fw CCAACCATGTCGTCGCCCTT and Insm1ASNAG-rv AGACGCCCGCTCTACCTCC.

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

For in situ hybridization, tissue was embedded into OCT compound directly after dissection. Digoxigenin-labeled riboprobes, which were used for hybridization, were generated by in vitro transcription. Pituitary-gland 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 E. coli). The plasmid used for production of this antigen was kindly provided by G. Gradwohl, IGBMC Illkirch, France), rabbit anti-ACTH (1:2000; AFIP5160278912, 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; AFIP7798-1289, NHPP), rabbit anti-GH (1:1000; Ab5940, 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; MATERIALS AND METHODS

Mouse strains

Generation and genotyping of Insm1Δ mutants were carried out as described (Gierl et al., 2006). The Insm1ASNAG targeting vector was generated by homologous recombination in bacteria (Lee et al., 2001); in particular, the Insm1 coding sequence was replaced by an Insm1ASNAG...
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
ATF-20 and GH3 cells were grown in high-glucose DMEM (Gibco/Invitrogen) containing 10% fetal calf serum (FCS, Sigma-Aldrich). For SILAC experiments, ATF-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' [12C;14N]arginine/lysine (Sigma-Aldrich) or 'heavy' [13C;15N]arginine/lysine (Sigma-Aldrich) isotypes 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 C18 and 3 (Sigma-Aldrich). After pre-clearing, supernatants were incubated with complete protease inhibitors (Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) 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 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 TOPS 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 hors eradish peroxidase (Dianova, Hamburg, Germany) were used, and blots were developed on a Chemi-smart 3000 (Vilber, Paris, France).
Eberhardzell, Germany). Wild-type and modified Ins1 cDNAs [Ins1FLAG (Ins1 containing 3xFLAG-tag, Sigma-Aldrich), Ins1ASNFLAG (Ins1ASN 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).

Acknowledgements

We particularly thank Ivonne Schiffner, Bettina Brandt and Karolin Fuchs for technical assistance; Claudia Päseler and Petra Stallerow for help with the animal husbandry; Walter Birchmeier, Mathias Treier, Thomas Müller and Dominique Bröhl for critical discussions and for reading the manuscript; and Hagen Wende (all MDC, Berlin) for help with the analysis of microarray data. We are very grateful to Ronald Naumann and his team (MPI-CBG Dresden, Germany) for the gift of R1/E ES cells and blastocyst injections.

Competing interests

The authors declare no competing financial interests.

Author contributions

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

Funding

This work was funded by SFB 665 (Deutsche Forschungsgemeinschaft); the Neuroscience Program of the Helmholtz Association; and the Deutsche Bundesministerium für Bildung und Forschung. Deposited in PMC for immediate release.

Supplementary material

Supplementary material available online at http://dev.biologists.orglookup/suppl doi:10.1242/dev.097642/D1C

References

Andrés, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., Dallman, J., Ballas, N. and Mandel, G. (1999). COREST: a functional corepressor required for regulation of nuclear-specific gene expression. Proc. Natl. Acad. Sci. USA 96, 9873-9878.

Bilodeau, S., Roussel-Gervais, A. and Drouin, J. (2009). Distinct developmental roles of cell cycle inhibitors p57Kip2 and p27Kip1 distinguish pituitary progenitor cell cycle exit from cell cycle reentry of differentiated cells. Mol. Cell. Biol. 29, 1895-1908.

Breslin, M. B., Zhu, M., Notkins, A. L. and Lan, M. S. (2002). Neuroendocrine differentiation factor, IA-1, is a transcriptional repressor and contains a specific DNA-binding domain: identification of consensus IA-1 binding sequence. Nucleic Acids Res. 30, 1038-1045.

Bunting, M., Bernstein, K. E., Greer, J. M., Capecchi, M. R. and Thomas, K. R. (1999). Targeting genes for self-excision in the germ line. Gene Dev. 13, 1524-1528.

Camper, S. A., Saunders, T. L., Katz, R. W. and Reeves, R. H. (2006). The zinc-finger factor Ins1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. Genes Dev. 20, 2465-2478.

Gierl, M. S., Karoulias, N., Wende, H., Strehe, M. and Birchmeier, C. (2006). The zinc-finger factor Ins1 (IA-1) is essential for the development of pancreatic beta cells and intestinal endocrine cells. Genes Dev. 20, 2465-2478.

Goto, Y., De Silva, M. G., Toscani, A., Prabhakar, B. S., Notkins, A. L. and Lan, M. S. (1992). A novel human insulinoma-associated cDNA, IA-1, encodes a protein with "zinc-finger" DNA-binding motifs. J. Biol. Chem. 267, 15252-15257.

Grimes, H. L., Chan, T. O., Zweidler-McKay, P. A., Tong, B. and Tsichlis, P. N. (1996). The Gfi-1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawal. Mol. Cell. Biol. 16, 6863-6972.

Hock, H. and Orkin, S. H. (2006). Zinc-finger transcription factor Gfi-1: versatile regulator of lymphocytes, neutrophils and hematopoietic stem cells. Curr. Opin. Hematol. 13, 1-6.

Jayakody, S. A., Andoniadou, C. L., Gaston-Massuet, C., Signore, M., Cariboni, A., Boupoux, P. L., Teissier, P., Pevny, L. H., Dattani, M. T. and Martinez-Barbera, J. P. (2012). SOX2 regulates the hypothalamic-pituitary axis at multiple levels. J. Clin. Invest. 122, 3635-3646.

Kelmanson, D., Rizzotti, K., Lim, W., Badge, R., Robinson, I. C. and Dattani, M. T. (2009). Genetic regulation of pituitary gland development in human and mouse. Endocr. Rev. 30, 790-825.

Kita, A., Imayoshi, I., Hojo, M., Kitagawa, M., Kubukawa, H., Ohsawa, R., Ohtsuka, T., Kageyama, R. and Hashimoto, N. (2007). Hes1 and Hes5 control the progenitor pool, intermediate lobe specification, and posterior lobe formation in the pituitary gland. Mol. Endocrinol. 21, 1458-1466.

Kuzin, A., Brody, T., Moore, A. W. and Odenwald, W. F. (2005). NefR-1 is required for early axon guidance decisions in the developing Drosophila CNS. Dev. Biol. 277, 347-365.

Lamolet, B., Puilchin, A. M., Lamerone, T., Gaichier, Y., Brue, T., Enjalbert, A. and Drouin, J. (2001). A pituitary cell-restricted T box factor, Tpt, activates POMC transcription in cooperation with the homeoproteins. Cell 104, 849-860.

Lamolet, B., Puilinin, G., Chu, K., Guillot, F., Tsai, M. J. and Drouin, J. (2004). Tpt-independent function of NeuroD1(BETA2) in pituitary corticotroph differentiation. Mol. Endocrinol. 18, 995-1003.

Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swint, D. A., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targetting and subcloning of BAC DNA. Genomics 73, 56-65.

Lee, M. G., Wynder, C., Cooch, N. and Sheikhattar, R. (2005). An essential role for COREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437, 432-436.

Lin, Y., Wu, Y., Li, J., Dong, C., Ye, X., Chi, Y. I., Evers, B. M. and Zhou, B. P. (2010). The SNAG domain of Snail1 functions as a molecular hook for recruiting lysine-specific demethylase 1. EMBO J. 29, 1803-1816.

Liu, W. D., Wang, H. W., Mugira, M., Breslin, M. B. and Lan, M. S. (2006). INS1 functions as a transcriptional repressor of the NeuroD/beta2 gene through the recruitment of cyclin D1 and histone deacetylases. Biochem. J. 397, 169-177.

Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., Gertsenstein, M. and Nagy, A. (1999). ZAP, a double reporter for cre-mediated recombination. Dev. Biol. 208, 281-292.

Mellitzer, G., Bonné, S., Lucco, R. F., Van De Casteele, M., Lenne-Samuel, N., Collombat, P., Mansouri, A., Lee, J. L., Marray, M., Pielkeers, D. et al. (2006). IA-1 is NGF-dependent and essential for differentiation of the endocrine pancreas. EMBO J. 25, 1344-1352.

Metzger, E., Wissmann, M., Yin, N., Müller, J. M., Schneider, R., Peters, A. H., Günther, T., Buettner, R. and Schüle, R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437, 436-439.

Moreno-Bueno, G., Portillo, F. and Cano, A. (2008). Translational regulation of cell polarity in EMT and cancer. Oncogene 27, 6958-6969.

Moury, T. and Khandapur, C. (2011). Growth factor independence 1 (Gfi1) as a regulator of lymphocyte, neutrophils and hematopoietic stem cells. Biol. Chem. 392, 41-54.

Grotz, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. A., Court, D. L., Norlin, S., Jessell, T. M. and Edlund, T. (2008). MaxQuant enables high peptide identification rates, very local False Discovery rates and proteotypic peptide assignments. Mol. Cell. Proteomics 7, 890-907.

Rappsilber, J., Ishihama, Y. and Mann, M. (2003). Step and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75, 663-670.

Development

Development (2013) doi:10.1242/dev.097642 DEVELOPMENT
Razin, S. V., Borunova, V. V., Maksimenko, O. G. and Kantidze, O. L. (2012). Cys2His2 zinc finger protein family: classification, functions, and major members. Biokhimiya 77, 217-226.

Rosenfeld, M. G., Briata, P., Dasen, J., Gleiberman, A. S., Kioussi, C., Lin, C., O’Connell, S. M., Ryan, A., Szeto, D. P. and Treier, M. (2000). Multistep signaling and transcriptional requirements for pituitary organogenesis in vivo. Recent Prog. Horm. Res. 55, 1-13, discussion 13-14.

Saleque, S., Kim, J., Rooke, H. M. and Orkin, S. H. (2007). Epigenetic regulation of hematopoietic differentiation by Gfi-1 and Gfi-1b is mediated by the cofactors CoREST and LSD1. Mol. Cell 27, 562-572.

Selbach, M. and Mann, M. (2006). Protein interaction screening by quantitative immunoprecipitation combined with knockdown (QUICK). Nat. Methods 3, 981-983.

Sheng, H. Z., Zhadanov, A. B., Mosinger, B., Jr, Fujii, T., Bertuzzi, S., Grinberg, A., Lee, E. J., Huang, S. P., Mahon, K. A. and Westphal, H. (1996). Specification of pituitary cell lineages by the LIM homeobox gene Lhx3. Science 272, 1004-1007.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. and Mann, M. (2007). In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856-2860.

Shinoda, K., Lei, H., Yoshii, H., Nomura, M., Nagano, M., Shiba, H., Sasaki, H., Osawa, Y., Ninomiya, Y., Niwa, O. et al. (1995). Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the Pitf1 disrupted mice. Dev. Dyn. 204, 22-29.

Suh, H., Gage, P. J., Drouin, J. and Camper, S. A. (2002). Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification. Development 129, 329-337.

Szeto, D. P., Rodriguez-Esteban, C., Ryan, A. K., O’Connell, S. M., Liu, F., Kioussi, C., Gleiberman, A. S., Izpisúa-Belmonte, J. C. and Rosenfeld, M. G. (1999). Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. Genes Dev. 13, 484-494.

Wang, J., Scully, K., Zhu, X., Cai, L., Zhang, J., Prefontaine, G. G., Krones, A., Ohgi, K. A., Zhu, P., Garcia-Basssets, I. et al. (2007). Opposing LSD1 complexes function in developmental gene activation and repression programmes. Nature 446, 882-887.

West, M. J. (2012). Estimating volume in biological structures. Cold Spring Harb. Protoc. 2012, 1129-1139.

Wildner, H., Gierl, M. S., Streile, M., Pla, P. and Birchmeier, C. (2008). Insm1 (IA-1) is a crucial component of the transcriptional network that controls differentiation of the sympatho-adrenal lineage. Development 135, 473-481.

Wu, W., Cogan, J. D., Pfaffle, R. W., Dasen, J. S., Frisch, H., O’Connell, S. M., Flynn, S. E., Brown, M. R., Mullis, P. E., Parks, J. S. et al. (1998). Mutations in PROP1 cause familial combined pituitary hormone deficiency. Nat. Genet. 18, 147-149.

Wu, J., Duggan, A. and Chaffie, M. (2001). Inhibition of touch cell fate by egl-44 and egl-46 in C. elegans. Genes Dev. 15, 789-802.

Zhu, X., Lin, C. R., Prefontaine, G. G., Tollkuhn, J. and Rosenfeld, M. G. (2005). Genetic control of pituitary development and hypopituitarism. Curr. Opin. Genet. Dev. 15, 332-340.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E. H., Guillemot, F., Kageyama, R. and Rosenfeld, M. G. (2006). Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. Genes Dev. 20, 2739-2753.