Developmental Biology 408 (2015) 90–98

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Insm1 controls the differentiation of pulmonary neuroendocrine cells by repressing *Hes1*

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ARTICLE INFO

Article history:
Received 17 July 2015
Received in revised form
6 September 2015
Accepted 6 October 2015
Available online 9 October 2015

ABSTRACT

Epithelial progenitor cells of the lung generate all cell types of the mature airway epithelium, among them the neuroendocrine cells. The balance between formation of pulmonary neuroendocrine and non-neuroendocrine cells is controlled by Notch signaling. The Notch target gene *Hes1* is expressed by non-neuroendocrine and absent in neuroendocrine cells. The transcription factor *Ascl1* is expressed in a complementary pattern and provides key regulatory information that specifies the neuroendocrine cell fate. The molecular events that occur after the induction of the neuroendocrine differentiation program have received little attention. Here we show that *Insm1* is expressed in pulmonary neuroendocrine cells, and that *Insm1* expression is not initiated in the lung of *Ascl1* mutant mice. We use mouse genetics to show that pulmonary neuroendocrine cells depend on *Insm1* for their differentiation. Mutation of *Insm1* blocks terminal differentiation, upregulates Hes1 protein in neuroendocrine cells and interferes with maintenance of *Ascl1* expression. We show that Insm1 binds to the *Hes1* promoter and represses *Hes1*, and we propose that the Insm1-dependent *Hes1* repression is required for neuroendocrine development. Our work demonstrates that Insm1 is a key factor regulating differentiation of pulmonary neuroendocrine cells.

1. Introduction

The terminal buds of the developing lung contain a population of multipotent epithelial progenitor cells that gives rise to the diverse cell types found in the mature airway epithelium (Desai et al., 2014; Hogan et al., 2014; Morrisey and Hogan, 2010; Ten Have-Opbroek, 1991). Among the cell types generated are neuroendocrine cells and the more abundant non-neuroendocrine cells like Clara and ciliated cells. During development of the lung epithelium, the first cell fate decision made is the one between neuroendocrine and non-neuroendocrine cells, and neuroendocrine cells are the first differentiated cell type detected in the epithelium (Morimoto et al., 2012; Ten Have-Opbroek, 1991). Neuroendocrine cells were reported to retain developmental plasticity and generate Clara and ciliated cells upon injury (Song et al., 2012).

Ascl1 is expressed in precursors and differentiated neuroendocrine cells of the lung epithelium. *Ascl1* acts as master regulator in formation of these cells and neuroendocrine cells are not generated when *Ascl1* is mutated (Borges et al., 1997). Notch activity controls the choice between neuroendocrine and non-neuroendocrine fates and represses formation of neuroendocrine cells in the lung epithelium (Morimoto et al., 2012; Ito et al., 2000). The Notch target gene *Hes1* is expressed in non-neuroendocrine cells and thus in a complementary pattern to *Ascl1*. In the absence of *Hes1*, *Ascl1* is upregulated, neuroendocrine cells appear too early in development and their number is markedly increased (Ito et al., 2000). It is unclear how the terminal differentiation of lung neuroendocrine cells is controlled and, in particular, transcription factors which act downstream of *Ascl1* have not been identified.

Several tumor types arise in lung tissues. The most common and a highly aggressive form is small-cell lung cancer. Ascl1, CGRP and Insm1 are among the characteristic markers of small-cell lung cancer cells (Augustyn et al., 2014; Kelley et al., 1994; Lan et al., 1993). Pulmonary neuroendocrine cells express many of these genes under normal physiological conditions, providing evidence that these tumors originate from neuroendocrine cells. A second type of tumor, non-small cell lung cancer, expresses markers typical for the non-neuroendocrine lung epithelium like Hes1 and CC10 (Yuan et al., 2015; Nasgashio et al., 2011; Sutherland and Berns, 2010). The availability of lung cancer cell lines can provide a model for the analyses of transcriptional networks that control pulmonary neuroendocrine and non-neuroendocrine cell identity.

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http://dx.doi.org/10.1016/j.ydbio.2015.10.009

Insm1 encodes a zinc finger protein that is widely expressed in developing and adult endocrine cells. *Insm1* was first detected in an insulinoma cDNA library, and later observed in many neuroendocrine tumor types (Lan and Breslin, 2009; Goto et al., 1992). *Insm1* controls terminal differentiation of many endocrine cell types, for instance endocrine cells of the pancreas, intestine, pituitary and adrenal medulla, and *Insm1* is essential to maintain pancreatic β-cells in a mature state (Gierl et al., 2006; Wildner et al., 2008; Welcker et al., 2013; Osipovich et al., 2014; Jia et al., 2015). Here we show that *Insm1* is expressed in developing pulmonary neuroendocrine cells and is essential for their differentiation. In *Insm1* mutant mice, the neuroendocrine fate is induced as judged by expression of *Ascl1*, but the terminal differentiation of neuroendocrine cells is blocked. In addition, Hes1 is expressed ectopically in mutant neuroendocrine cells, and *Ascl1* expression is not maintained at late developmental stages. Hes1 is known to repress *Ascl1* in pulmonary cells (Chen et al., 1997; Ito et al., 2000). We provide evidence that Insm1 directly binds to regulatory sequences in the *Hes1* gene and represses *Hes1*. Our data demonstrate that *Insm1* is a crucial component of the regulatory network that controls the differentiation of pulmonary neuroendocrine cells.

2. Materials and methods

2.1. Genotyping, immunohistochemistry, X-gal staining and in situ hybridization

The primers used for the genotyping of the *Insm1*^{lacZ} allele have been described (Gierl et al., 2006). For immunohistochemistry, the tissue was fixed (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4), cryoprotected in 25% sucrose in PBS, embedded, and cryosections were cut at 12 μm thickness. The following primary antibodies were used: guinea pig anti-Insm1 (Jia et al., 2015), rabbit/chicken anti-β-galactosidase (1:2,500; 55976, MP/1:1000; ab9361, Abcam), rabbit anti-CGRP (1:4,000; C8198, Sigma), guinea pig/rabbit anti-Pgp9.5 (1:2,000; Ab5898/1:500; Ab1761, Millipore), goat anti-Hes1 (1:50; sc-13844, Santa Cruz), goat anti-CC10 (1:50; sc-9772, Santa Cruz), mouse anti-SSEA1 (1:100; MAB2155, R&D). Secondary antibodies conjugated to Cy2, Cy3 or Cy5 were used (1:500; Jackson ImmunoResearch). For quantifications of the analyses of E18.5 animals, 4 lungs each were dissected from control and mutant mice and the entire lung was sectioned. Every 10th section was stained using anti-Pgp9.5 and anti-CGRP antibodies, and proximal and distal airway epithelia were inspected on 20–24 sections/animal (5–6 slides). Neither single nor clustered Pgp9.5 and CGRP-positive cells were detected. In addition, histological analyses were performed on lungs from E13.5 and E15.5 mice (4 mice from each genotype). For the analysis of E13.5 lungs, 8 section (2 slides) were analyzed from each animal; for analysis of E15.5 lungs, 12–16 sections/animal (3–4 slides) were inspected. Fluorescence was imaged on a Zeiss LSM 700 confocal microscope and images were processed using Adobe Photoshop software. β-Galactosidase activity was assessed by X-gal staining as described (Lobe et al., 1999). For *in situ* hybridization, tissue was directly embedded into OCT compound. We used protocols described before for generation of digoxigenin-labeled riboprobes and hybridization (Wildner et al., 2008).

2.2. ChIP-PCR

Chromatin immunoprecipitation was done essentially as described (Jia et al., 2015). For each ChIP-PCR experiment, lungs of 12–15 embryos were used, and each ChIP-PCR experiment was performed three times using anti-Insm1 and IgG control antibodies. Briefly, embryonic lungs were cut into 1–3 mm^3 pieces, and the tissue was cross-linked in 1% formaldehyde (15 min, room temperature). Nuclei were isolated after lysis of cells and chromatin was sheared by sonication. Sonication was optimized to obtain 150–500 bp long DNA fragments. Antibodies against Insm1 were incubated with BSA blocked Protein A Dynabeads (Invitrogen), beads were washed and incubated overnight with chromatin fragments at 4 °C. To remove unspecifically bound chromatin, beads were washed seven times in washing buffer (1% NP40, 0.5 M LiCl, 1 mM EDTA, 0.7% Na-Deoxycholate, 50 mM Hepes-KOH pH 7.5), and chromatin was eluted in elution buffer at 65 °C (50 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS). After de-crosslinking, RNase *A* and proteinase *K* digestion, DNA was purified by phenol–chloroform extraction and used for PCR analysis. The PCR primers used for ChIP-PCR are shown in Supplementary material Table 2. The fragments tested here had previously been identified to bind Insm1 in SJ pancreatic β-cells using ChIP-seq (Jia et al., 2015). The anti-Insm1 antibody used for ChIP-PCR was described (Welcker et al., 2013) and its specificity was tested by immunohistology comparing control and *Insm1* mutant tissue, and by western blotting using control and mutant tissue (Jia et al., 2015).

2.3. Luciferase assay and RT-PCR analyses

DNA fragments (average size 400–800 bp) were cloned upstream of a minimal promoter driving the firefly luciferase gene (pGL4.23[luc2/minP] vector; Promega). HEK293 cells were transfected with the firefly luciferase plasmid containing putative enhancer sequences using Lipofectamine 2000 (Life technologies); as internal control, a Renilla luciferase plasmid (pRL-TK Renilla; Promega) was co-transfected. Cell lysates were prepared 24 h after transfection, and luciferase activity was determined using the Dual-Luciferase® Reporter Assay kit (Promega). For each sample, firefly luciferase values were normalized to Renilla luciferase values. We display relative luciferase activity as fold change compared to empty pGL4.23 vector. Primers used for amplification of fragments are listed in Supplementary material Table 2.

Cells were transfected with Insm1 expression plasmids as described (Welcker et al., 2013). Cells were lysed and total RNA was isolated using Trizol reagent (Invitrogen). RT-PCR analysis after first-strand cDNA synthesis was performed using a CFX96 RT-PCR system (Bio-Rad) and ABsolute QPCR SYBR Green Mix (Thermo Fisher Scientific). Primers used are listed in Supplementary material Table 2.

2.4. Western blot analyses

Proteins were extracted from transfected cells using RIPA buffer. After centrifugation, supernatants were run on 12% PAGE gels and the separated proteins were transferred to PVDF membranes (Millipore). The Insm1 antibody was used for western blot analysis (1:10,000). Secondary antibodies coupled to horseradish peroxidase (Dianova) were used, and blots were developed on a Chemismart 3000 (Vilber).

3. Results

3.1. Insm1 is essential for the differentiation of pulmonary neuroendocrine cells

During lung development, Insm1 protein appears around E13.5 in scattered epithelial cells (Fig. 1A). Pgp9.5 and CGRP mark neuroendocrine cells and, in normal development, these proteins begin to be expressed at E14.5 and E16.5, respectively (Fig. 1A). Cells co-expressing Pgp9.5 and Insm1 appeared at E14.5, and cells

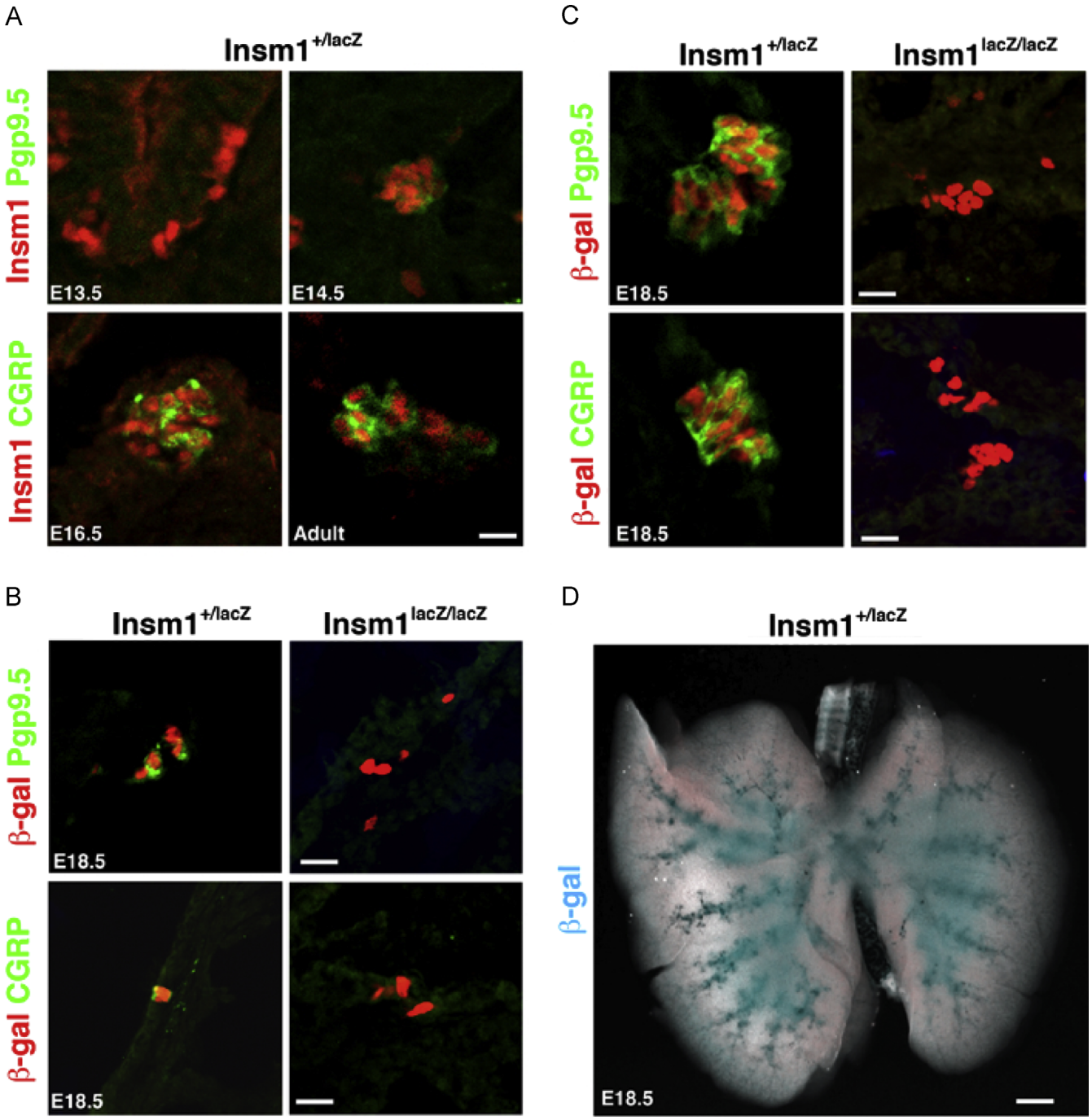

Fig. 1. Insm1 is expressed in pulmonary neuroendocrine cells during development and in the adult. (A) Immunohistological analysis of Insm1 protein (red) in the developing (E13.5–E16.5) and adult lung epithelium. Insm1 is co-expressed with Pgp9.5 and CGRP (green). (B and C) Immunohistological analysis of β-gal (red) and Pgp9.5 (green) or CGRP (green) in neuroendocrine cells of the distal (B) and proximal (C) lung epithelium of control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice at E18.5. (D) X-gal staining of the lung of heterozygous *Insm1*$^{+/lacZ}$ mice (E18.5). Scale bars: 15 μm (A, B, C); 500 μm (D).

co-expressing CGRP and Insm1 at E16.5 (Fig. 1A). Pulmonary neuroendocrine cells are present as single cells or in clusters called neuroendocrine bodies, and both expressed Insm1, but expression of Pgp9.5 and CGRP was lower in single Insm1$^+$ than clustered Insm1$^+$ cells (Fig. 1B and C). Furthermore, Insm1 continues to be co-expressed with CGRP in pulmonary neuroendocrine cells of the adult (Fig. 1A). *Insm1*$^{+/lacZ}$ mice are heterozygous and carry one *Insm1* wildtype and one null allele in which *Insm1* coding sequences are replaced by *lacZ* cDNA (Gierl et al., 2006). *LacZ* encodes β-galactosidase, and β-galactosidase$^+$ cells are found in the proximal and distal lung epithelium, as assessed by X-gal staining of the lung from *Insm1*$^{+/lacZ}$ mice at E18.5 (Fig. 1D). We conclude that Insm1$^+$ or β-galactosidase$^+$ cells in the lung co-express Pgp9.5 or CGRP, demonstrating that they correspond to pulmonary endocrine cells.

To determine the function of *Insm1* in the lung, we analyzed *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice. Cells that expressed Pgp9.5 or CGRP in the lung of E18.5 *Insm1* mutant mice were not observed in the proximal or distal epithelium (Fig. 1B and C). Nevertheless, β-galactosidase was present in some of the cells of the lung epithelium of *Insm1* mutant animals. Expression of β-galactosidase provided the first evidence that the neuroendocrine cell fate was correctly initiated in *Insm1* mutants. However, their differentiation was impaired, as demonstrated by the absence of Pgp9.5 or CGRP

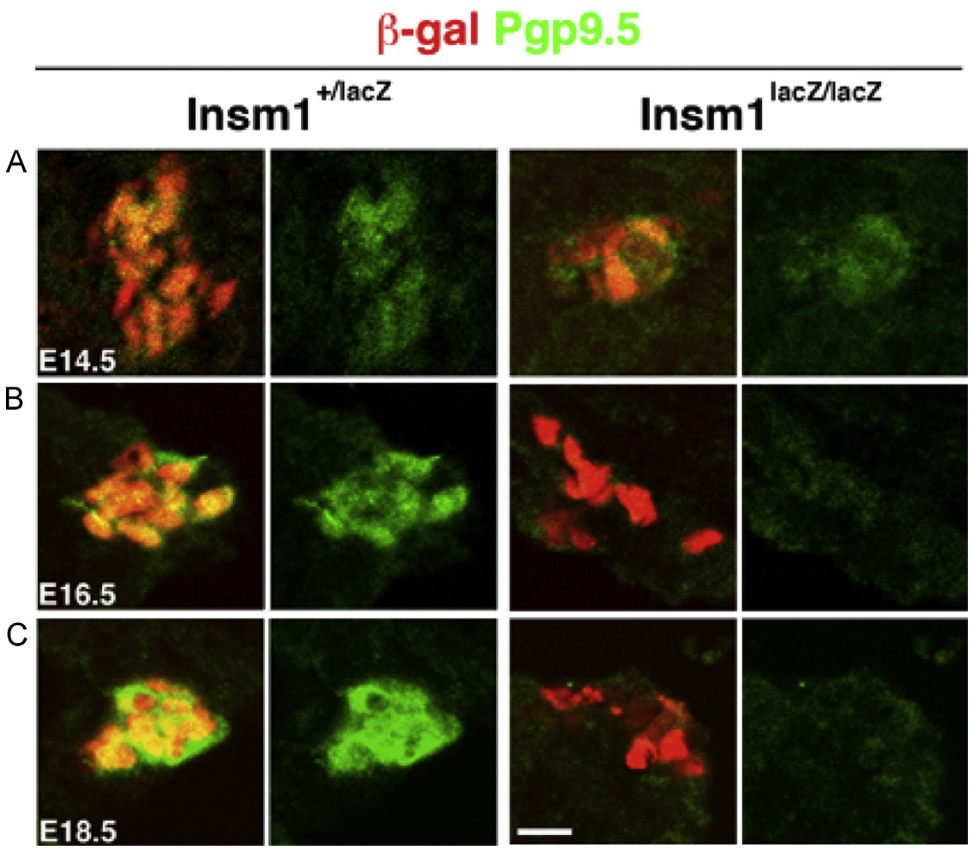

Fig. 2. Mutation of *Insm1* blocks neuroendocrine cell differentiation. (A-C) Immunohistological analysis of lung tissue from control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice at the indicated developmental stages using antibodies directed against Pgp9.5 (green) and β-gal (red). Note that Pgp9.5 expression is initiated but not maintained in the β-gal-positive neuroendocrine cells. Scale bar: 10 μm.

at E18.5 in 100 β-galactosidase$^+$ cells in the proximal lung epithelium, and in 115 β-galactosidase$^+$ clusters in the distal lung epithelium ($n=4$). Thus, the differentiation deficit was fully penetrant (Fig. 1B and C). Despite the fact that clustered β-galactosidase$^+$ cells were present, the average number of cells in the clusters was smaller in mutants than in control mice (15.1 ± 1.6 and 9.4 ± 1.8 β-galactosidase$^+$ cells per cluster in the lungs of *Insm1*$^{lacZ/+}$ and *Insm1*$^{lacZ/lacZ}$ animals, respectively; $P < 0.01$ in 2-tailed unpaired Student's *t*-test).

We further investigated the specification and differentiation of pulmonary neuroendocrine cells at different developmental stages. Pgp9.5 protein begins to be expressed at E14.5 in control mice, and we observed reduced levels of Pgp9.5 at this stage in *Insm1* mutant lung epithelium (Fig. 2A). However, at subsequent stages (E16.5 and E18.5), Pgp9.5 was no longer detectable (Fig. 2B and C).

3.2. *Hes1 is ectopically expressed in pulmonary neuroendocrine cells of Insm1 mutant mice*

Hes1 regulates the balance between neuroendocrine and non-neuroendocrine cells in the airway epithelium and is broadly expressed in non-neuroendocrine cells (Ito et al., 2000; Shan et al., 2007). In accordance, we detected Hes1 protein in pulmonary epithelial cells by immunohistology. β-Galactosidase$^+$ neuroendocrine cells were always Hes1 negative in the lung of heterozygous *Insm1*$^{+/lacZ}$ mice. In contrast, the majority of β-galactosidase-positive neuroendocrine cells co-expressed Hes1 in *Insm1* mutant mice at E15.5 and E18.5 (Fig. 3A–C).

Hes1 is known to suppress *Ascl1*, the transcription factor that functions as a master regulator of neuroendocrine development (Chen et al., 1997). We next analyzed *Ascl1* expression and observed that *Ascl1* was expressed in a comparable pattern at E13.5 in the lung of control and *Insm1* mutant embryos (Fig. 3D). *Ascl1* expression levels were reduced at E15.5, and expression was no longer detectable at E18.5 (Fig. 3E and F). *Ascl1* downregulation and Hes1 upregulation coincide thus in the lung of *Insm1* mutant mice.

Dll1 is a direct target of Ascl1 and expressed in neuroendocrine cells of pulmonary epithelia (Ito et al., 2000). We also analyzed *Dll1* expression and observed a marked downregulation of *Dll1* mRNA in the lung epithelium of *Insm1* mutant mice at E18.5 (Fig. 3G). Thus, neuroendocrine cells in the lung are correctly specified, as assessed by *Ascl1* and β-galactosidase expression in *Insm1*$^{lacZ/lacZ}$ mice, but their differentiation is impaired.

The expression of Hes1 in β-galactosidase$^+$ cells, as well as the loss of *Ascl1* expression in the lung might indicate that neuroendocrine cells in the *Insm1* mutant mice have assumed a different fate, for instance the fate of CC10$^+$ Clara cells or SSEA$^+$ epithelial progenitor cells (Morimoto et al., 2012). However, co-expression of β-galactosidase with CC10 or SSEA was not observed (Fig. 4A and B). Thus, despite the fact that neuroendocrine cells ectopically express *Hes1* and lose *Ascl1* expression in *Insm1* mutants, they do not trans-differentiate but retain a memory of having been specified as neuroendocrine cells.

Next we asked whether *Insm1* is expressed in the lung of *Ascl1* mutant mice. We detected no Insm1 protein in *Ascl1*$^{-/-}$ mice, indicating that *Insm1* requires *Ascl1* for the initiation of its expression (Fig. 4C).

3.3. *Insm1 binds to sequences in the Hes1 gene and represses Hes1*

Insm1 can bind to DNA directly and recruit other histone

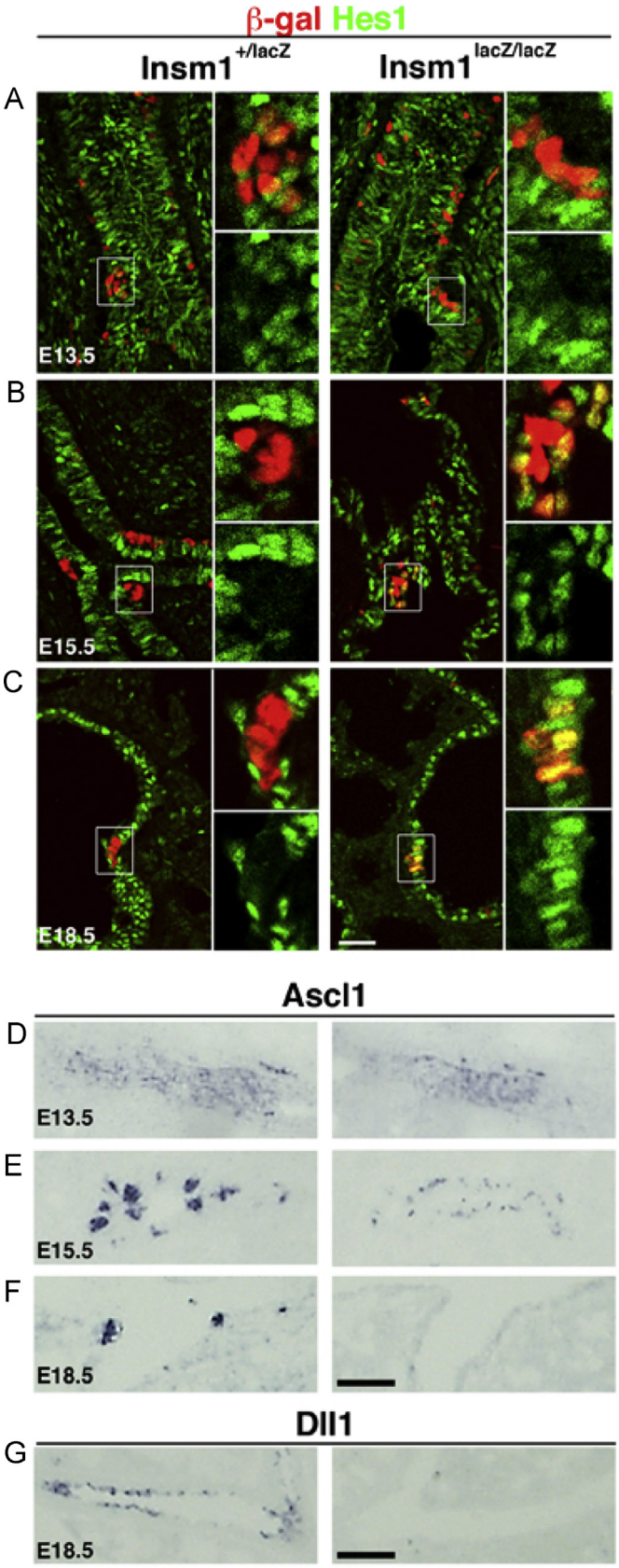

Fig. 3. Ectopic expression of Hes1 in pulmonary neuroendocrine cells of *Insm1* mutant mice. (A-C) Expression of β-gal (red) and Hes1 (green) in the lung epithelium of control and *Insm1* mutant mice analyzed by immunohistology at the indicated developmental stages. High magnifications of the indicated areas are shown twice. The insert at the top shows β-gal and Hes1 co-staining; the insert at the bottom only shows only Hes1. (D–F) *In situ* hybridization analysis of *Ascl1* expression in the lung of control and *Insm1* mutant mice at the indicated stages. (G) *In situ* hybridization analysis of *Dll1* expression in the lung of control and *Insm1* mutant mice at E18.5. Scale bars: 30 μm (A–C); 150 μm (D–G).

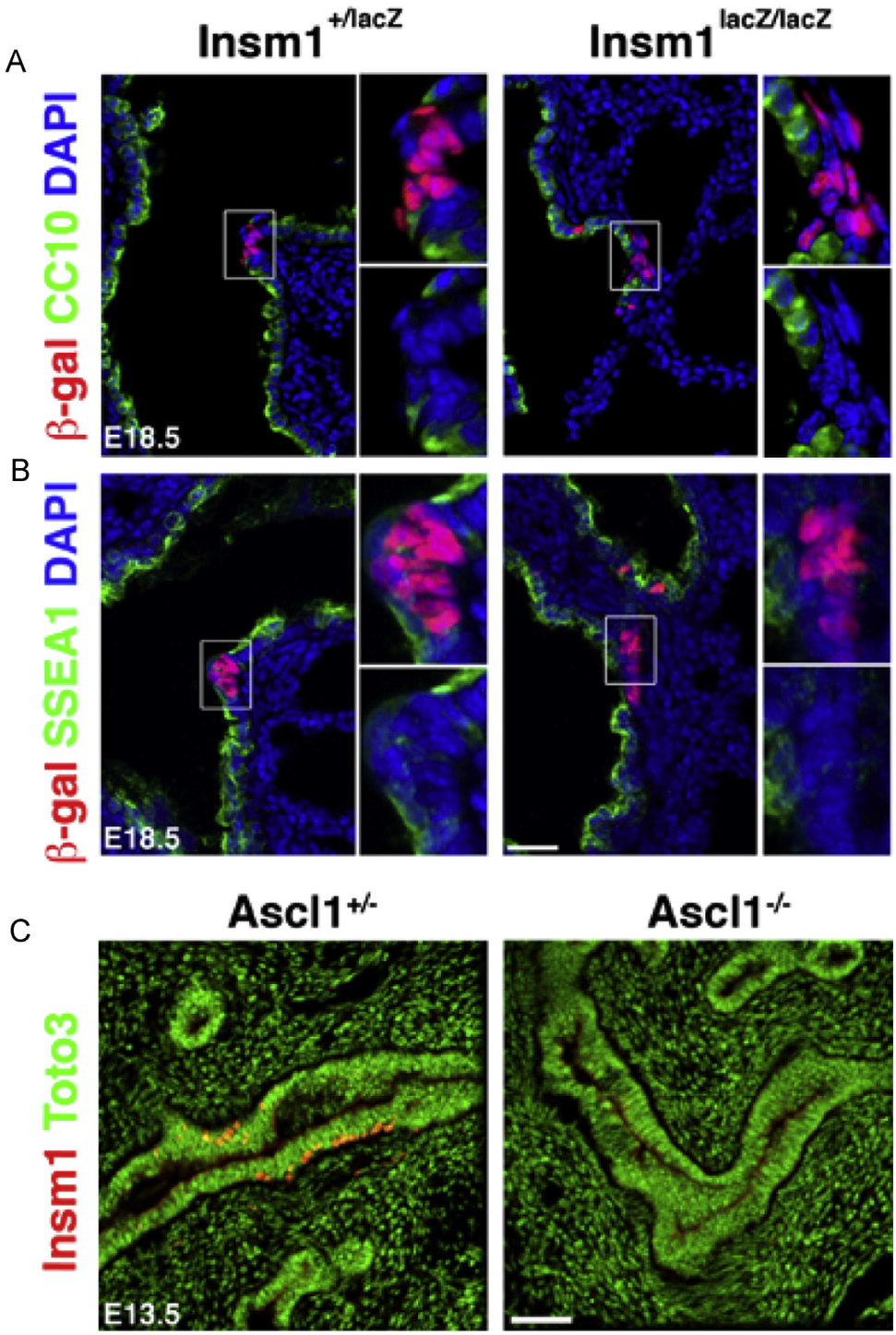

Fig. 4. Pulmonary neuroendocrine cells do not transdifferentiate in *Insm1* mutant mice. (A) Immunohistological analysis of β-gal (red) and CC10 (green) in the lung of control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice at E18.5. DAPI (blue) was used as a counterstain. High magnifications of the indicated areas are shown twice. The insert at the top shows β-gal, CC10 and DAPI co-staining; the insert at the bottom shows only CC10 and DAPI co-staining. (B) Immunohistological analysis of β-gal (red) and SSEA1 (green) in the lung of control (*Insm1*$^{+/lacZ}$) and *Insm1* mutant (*Insm1*$^{lacZ/lacZ}$) mice at E18.5. DAPI (blue) was used as a counterstain. High magnifications of the indicated areas are shown twice. The inset at the top shows β-gal, SSEA1 and DAPI co-staining; the inset at the bottom shows only SSEA1 and DAPI co-staining. (C) Immunohistological analysis of Insm1 (red) in lung epithelium of control and *Ascl1* mutant mice at E13.5. Toto3 (green) was used as a nuclear counterstain. Scale bars: 30 μm (A and B); 50 μm (C).

modifying enzymes, but is also recruited indirectly to chromatin by other transcription factors (Breslin et al., 2002; Welcker et al., 2013; Jia et al., 2015). We previously noted that Insm1 binds to *Hes1* sequences in pancreatic β-cells (Jia et al., 2015). We therefore investigated whether Insm1 binds to these sites in pulmonary neuroendocrine cells (Fig. 5A and Supplementary material Table 1). Chromatin was isolated from whole lung tissue obtained from E15.5 embryos, and immunoprecipitated using anti-Insm1 antibodies. The precipitated chromatin was analyzed for the presence of enriched sequences by PCR. We observed a strong enrichment of three sequences associated with the *Hes1* locus. These sequences located to the promoter (P2), exon four (Ex) and a 5′ upstream region (P1), and they were enriched 7- to 9-fold compared to an experiment in which a non-specific IgG was used

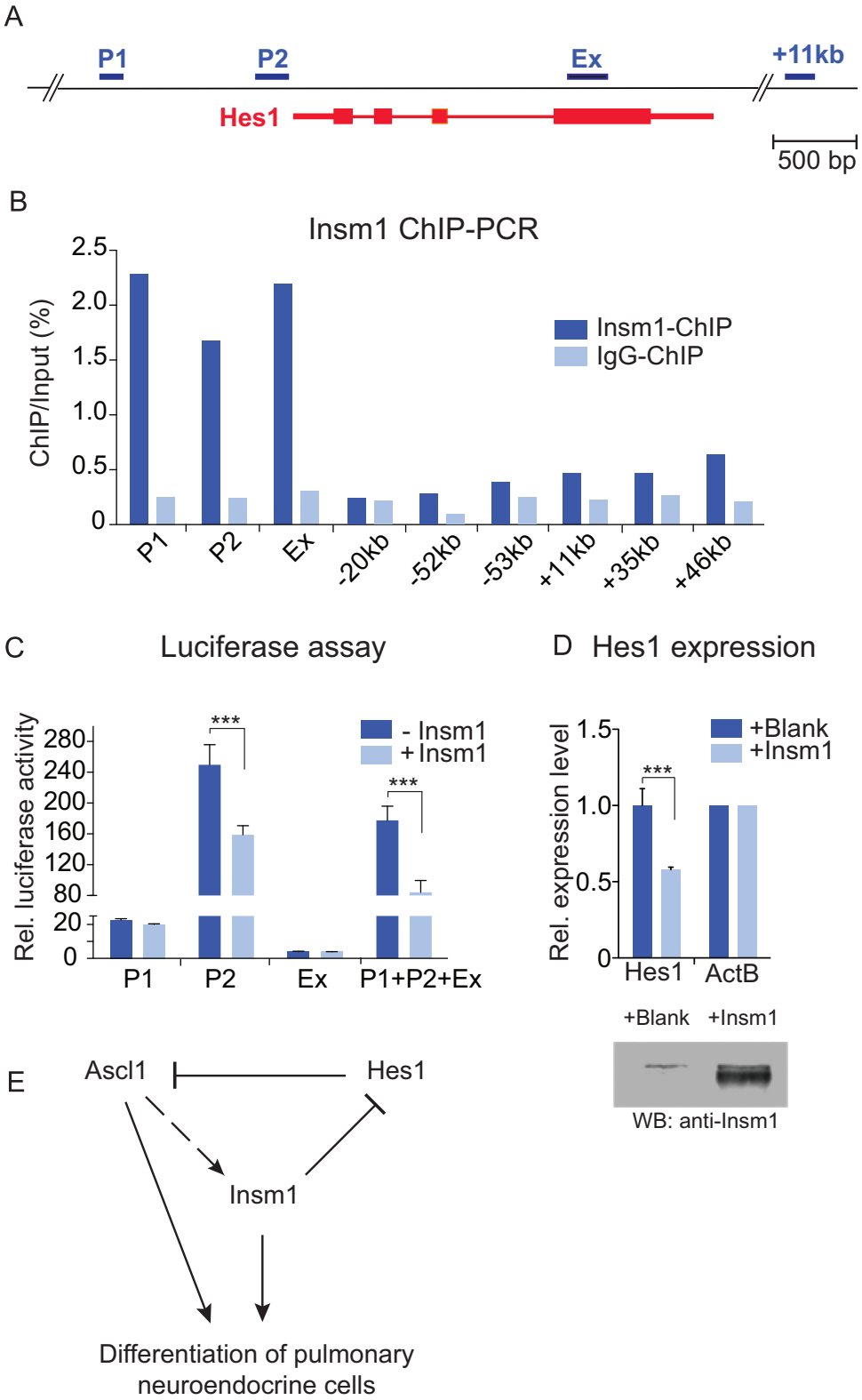

Fig. 5. Insm1 binds to the *Hes1* locus in pulmonary neuroendocrine cells and regulates *Hes1* expression. (A) Schematic diagram of the *Hes1* gene and of Insm1 binding sites in the *Hes1* locus previously identified by ChIP-seq experiments in endocrine β-cells of the pancreas (P1, P2, Ex). (B) DNA enrichment by ChIP using anti-Insm1 and IgG control antibodies. DNA in the immunoprecipitated chromatin was analyzed by quantitative-PCR. (C) Luciferase reporter assay for *cis*-regulatory activity of DNA fragments associated with Insm1 binding in the *Hes1* locus in the presence and absence of co-transfected *Insm1* cDNA. (D) RT-PCR analysis of expression of the endogenous *Hes1* gene in LCLC97 cells with or without ectopic Insm1 expression (top). Little or no Insm1 was detected in cells transfected with a control plasmid; Insm1 protein was detected after transfection of *Insm1* cDNA (bottom). (E) Schematic display of the proposed transcriptional network that controls differentiation of pulmonary neuroendocrine cells. *Ascl1* controls *Insm1* expression by direct or indirect mechanisms indicated by a broken arrow. Insm1 represses *Hes1*, and Hes1 is a known transcriptional repressor of *Ascl1*. In the absence of *Insm1*, *Hes1* is upregulated which interferes with the maintenance of *Ascl1*. In the presence of *Insm1*, *Hes1* is downregulated and *Ascl1* expression is maintained which promotes differentiation of neuroendocrine cells in lung.

(Fig. 5B). No enrichment was observed for other tested sites of the Hes1 gene that, in chromatin from pancreatic β-cells, were found to bind Insm1 (Fig. 5B). In addition, a randomly selected sequence located 11 kb downstream of the *Hes1* locus was also not enriched (Fig. 5B).

To test the sequences that bound Insm1 for transcriptional regulatory activity, we used a luciferase reporter system. The fragments were inserted into a luciferase vector containing a minimal promoter (pGL4.23), and tested in HEK293 cells. The presence of the P2 fragment from the *Hes1* promoter stimulated transcriptional activity by a factor of 250. The 5′ upstream fragment (P1) and exon fragment (Ex) also increased expression 20- and 5-fold, respectively, and the presence of all three fragments stimulated transcription 200-fold (Fig. 5C). Thus, sequences bound by Insm1 can have regulatory transcriptional activity.

We next tested whether co-transfection of *Insm1* affects the transcriptional activity of the regulatory sequences identified in the *Hes1* gene. After co-transfection of *Insm1*, we observed a significantly decreased expression when the promoter fragment (P2) was used to drive luciferase expression. Transcriptional activities of plasmids containing the exon sequence (Ex) or P1 were similar in the absence or presence of Insm1. When *Insm1* was co-transfected with a vector containing all three fragments (P1, P2 and Ex), luciferase activity decreased (Fig. 5C). In summary, Insm1 binds to several sequences in the *Hes1* gene. Among these, the promoter fragment strongly increases transcriptional activity in a heterologous system, and Insm1 represses the transcription of reporter sequences driven by this regulatory sequence.

Next we used a non-small cell lung cancer cell line, LCLC97 cells, that expresses *Hes1* but no or little *Insm1* (Fig. 5D, bottom). We tested whether the presence of Insm1 changes the expression of the endogenous *Hes1* gene by transfecting *Insm1* cDNA. The presence of Insm1 downregulated expression of the *Hes1* gene significantly, but did not affect expression of the β-*actin (Actb)* gene (Fig. 5D). Together, the data indicate that Insm1 binds the *Hes1* promoter in neuroendocrine cells of the lung, and that Insm1 can repress *Hes1* expression.

4. Discussion

Insm1 is expressed in developing and mature endocrine cell types, and is detected in neuroendocrine tumors (Lan and Breslin, 2009). Previous studies identified an essential role of *Insm1* in development of endocrine cells in the pancreas, intestine, pituitary and adrenal gland (Gierl et al., 2006; Wildner et al., 2008; Welcker et al., 2013; Osipovich et al., 2014). Here we show that *Insm1* acts as a key regulator for differentiation of pulmonary neuroendocrine cells. Mutation of *Insm1* did not affect the initial specification of these cells, but blocked their terminal differentiation. The differentiation deficit was associated with upregulated expression of *Hes1*. Notch signaling plays important roles in the specification of non-neuroendocrine cell types in the lung and needs to be repressed during development of pulmonary neuroendocrine cells (Morimoto et al., 2012). Our data indicate that Insm1 participates in the regulation of the Notch signaling network by repressing *Hes1*, an important effector of Notch signaling, and hereby allows normal neuroendocrine differentiation of lung epithelial cells.

Mechanisms responsible for cell fate specification in the pulmonary epithelium have received considerable attention, but factors regulating terminal differentiation have not been identified. We show here that after establishment of the neuroendocrine cell fate, *Insm1* needs to be present for the execution of the differentiation program. In particular, *Insm1* is essential for the repression of *Hes1* in developing neuroendocrine cells. Using ChIP experiments on lung tissue, we show that Insm1 directly binds to sequences in the *Hes1* locus. Among these, sequences close to the *Hes1* promoter had the highest transcriptional regulatory activity. Insm1 downregulated the transcriptional activity of *Hes1* promoter sequences, but not of other tested fragments. The *Hes1* promoter contains in addition to the Insm1 binding site four consensus recognition sequences of Hes1 (N-box sequences), and these sequences bind Hes1 in gel shift experiments and have regulatory function (Takebayashi et al., 1994). The overlapping binding regions might indicate a regulatory interaction between Insm1 and Hes1 proteins. Such regulatory interactions between Insm1 and another bHLH protein, Neurod1, were previously characterized in pancreatic β-cells. In particular, Insm1 can directly bind Neurod1 and frequently co-occupies chromatin together with Neurod1 and the forkhead factor Foxa2. Combinatorial Insm1/Neurod1/Foxa2 binding sites represent high affinity sites and identify regulatory sequences that maintain the mature gene expression program in β-cells (Jia et al., 2015). Regulatory interactions between Insm1 and Neurod1 might not be important during the early differentiation of pulmonary neuroendocrine cells, since Neurod1 expression is low or absent in the prenatal lung (Ito et al., 2000). It might become operative postnatally when Neurod1 is expressed in pulmonary neuroendocrine cells (Neptune et al., 2008).

Insm1 contains a SNAG motive at its N-terminus, and we showed previously that histone-modifying factors (Kdm1a, Hdac1/2 and Rcor1-3) and other proteins implicated in transcriptional regulation (Hmg20a/b and Gse1) are recruited to Insm1 via the SNAG domain (Welcker et al., 2013). Thus, Insm1 can recruit histone-modifying proteins to chromatin, and thus might repress *Hes1* expression in pulmonary neuroendocrine cells by epigenetic mechanisms.

Insm1 controls development of endocrine and neuronal cells, and similarities of the transcriptional hierarchies that regulate *Insm1* are emerging. For instance, *Insm1* expression depends on *Ascl1* in pulmonary neuroendocrine cells, sympathetic neurons and serotonergic and noradrenergic neurons of the hindbrain *in vivo* (Wildner et al., 2008; Jacob et al., 2009 and data presented here). *Ascl1* might control initiation of expression of *Insm1* directly or indirectly in pulmonary neuroendocrine cells, but it interesting to note that *Insm1* is a direct Ascl1 target gene in the developing brain (Castro et al., 2006; Jacob et al., 2009). In pancreatic endocrine cells, the initiation of Insm1 expression depends on *Ngn3* encoding a distinct bHLH factor (Mellitzer et al., 2006). Thus, different bHLH factors act upstream to initiate *Insm1* expression in various cell types.

We show here that Insm1 expression is not initiated in *Ascl1* mutants. In *Insm1* mutants, *Ascl1* expression is correctly initiated but expression is profoundly downregulated at late stages of fetal development, and this does not affect the activity of the *Insm1* promoter as assessed by the expression of *lacZ*/β-galactosidase. Therefore, the roles of Ascl1 in initiation and maintenance of *Insm1* expression differ. Ascl1 is known to act as pioneer factor during neuronal differentiation (Vierbuchen et al., 2010; Raposo et al., 2015). Pioneer factors bind sequences in closed chromatin and open it, making the DNA accessible to other transcription factors that then take over the regulation of gene expression. Such a mechanism might be operative during development of neuroendocrine cells of the lung and make expression of β-galactosidase independent of Ascl1 at late stages of differentiation.

We show here that loss of *Insm1* results in increased *Hes1* expression in pulmonary neuroendocrine cells. Upregulated *Hes1* expression was recently also detected in *Insm1* mutant endocrine pituitary cells (Welcker et al., 2013). In addition, Insm1 binding sites identified here in the *Hes1* locus were chosen for a ChIP-PCR experiment because these were previously identified as Insm1 binding sites in pancreatic β-cells (Jia et al., 2015). We observed

that a subset, but not all sites showed enrichment in ChIP-PCR experiment using chromatin of pulmonary neuroendocrine cells. This indicates that common Insm1 target genes exist in distinct endocrine cell types, but that few of the regulatory sequences are shared in the different endocrine cell types.

Acknowledgments

We thank Weihua Tao and Bettina Brandt for technical assistance, Dominique Bröhl and Thomas Müller for critically reading the manuscript, Petra Stallerow and Claudia Päseler for expert animal husbandry support. This work was supported by the SFB 665 and by Helmholtz Program on Disorders of the Nervous System.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.10.009.

References

Augustyn, A., Borromeo, M., Wang, T., Fujimoto, J., Shao, C., Dospoy, P.D., Lee, V., Tan, C., Sullivan, J.P., Larsen, J.E., et al., 2014. ASCL1 is a lineage oncogene providing therapeutic targets for high-grade neuroendocrine lung cancers. Proc. Natl. Acad. Sci. USA 111, 14788–14793.

Borges, M., Linnoila, R.I., van de Velde, H.J., Chen, H., Nelkin, B.D., Mabry, M., Baylin, S.B., Ball, D.W., 1997. An achaete-scute homologue essential for neuroendocrine differentiation in the lung. Nature 386, 852–855.

Breslin, M.B., Zhu, M., Notkins, A.L., 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.

Castro, D.S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I.J., Parras, C., Hunt, C., Critchley, J.A., Nguyen, L., Gossler, A., Gottgens, B., et al., 2006. Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Dev. Cell 11, 831–844.

Chen, H., Thiagalingam, A., Chopra, H., Borges, M.W., Feder, J.N., Nelkin, B.D., Baylin, S.B., Ball, D.W., 1997. Conservation of the Drosophila lateral inhibition pathway in human lung cancer: a hairy-related protein (HES-1) directly represses achaete-scute homolog-1 expression. Proc. Natl. Acad. Sci. USA 94, 5355–5360.

Desai, T.J., Brownfield, D.G., Krasnow, M.A., 2014. Alveolar progenitor and stem cells in lung development, renewal and cancer. Nature 507, 190–194.

Gierl, M.S., Karoulias, N., Wende, H., Strehle, M., Birchmeier, C., 2006. The zinc-finger factor Insm1 (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., 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.

Hogan, B.L., Barkauskas, C.E., Chapman, H.A., Epstein, J.A., Jain, R., Hsia, C.C., Niklason, L., Calle, E., Le, A., Randell, S.H., et al., 2014. Repair and regeneration of the respiratory system: complexity, plasticity, and mechanisms of lung stem cell function. Cell Stem Cell 15, 123–138.

Ito, T., Udaka, N., Yazawa, T., Okudela, K., Hayashi, H., Sudo, T., Guillemot, F., Kageyama, R., Kitamura, H., 2000. Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. Development 127, 3913–3921.

Jacob, J., Storm, R., Castro, D.S., Milton, C., Pla, P., Guillemot, F., Birchmeier, C., Briscoe, J., 2009. Insm1 (IA-1) is an essential component of the regulatory network that specifies monoaminergic neuronal phenotypes in the vertebrate hindbrain. Development 136, 2477–2485.

Jia, S., Ivanov, A., Blasevic, D., Muller, T., Purfurst, B., Sun, W., Chen, W., Poy, M.N., Rajewsky, N., Birchmeier, C., 2015. Insm1 cooperates with Neurod1 and Foxa2 to maintain mature pancreatic beta-cell function. EMBO J. 34, 1417–1433.

Kelley, M.J., Snider, R.H., Becker, K.L., Johnson, B.E., et al., 1994. Small cell lung carcinoma cell lines express mRNA for calcitonin and alpha- and beta-calcitonin gene related peptides. Cancer Lett. 81, 19–25.

Lan, M.S., Breslin, M.B., 2009. Structure, expression, and biological function of INSM1 transcription factor in neuroendocrine differentiation. FASEB J. 23, 2024–2033.

Lan, M.S., Russell, E.K., Lu, J., Johnson, B.E., Notkins, A.L., 1993. IA-1, a new marker for neuroendocrine differentiation in human lung cancer cell lines. Cancer Res. 53, 4169–4171.

Lobe, C.G., Koop, K.E., Kreppner, W., Lomeli, H., Gertsenstein, M., Nagy, A., 1999. Z/AP, a double reporter for cre-mediated recombination. Dev. Biol. 208, 281–292.

Mellitzer, G., Bonne, S., Luco, R.F., Van De Casteele, M., Lenne-Samuel, N., Collombat, P., Mansouri, A., Lee, J., Lan, M., Pipeleers, D., et al., 2006. IA1 is NGN3-dependent and essential for differentiation of the endocrine pancreas. EMBO J. 25, 1344–1352.

Morimoto, M., Nishinakamura, R., Saga, Y., Kopan, R., 2012. Different assemblies of Notch receptors coordinate the distribution of the major bronchial Clara, ciliated and neuroendocrine cells. Development 139, 4365–4373.

Morrisey, E.E., Hogan, B.L., 2010. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev. Cell. 18, 8–23.

Nasgashio, R., Sato, Y., Matsumoto, T., Kageyama, T., Hattori, M., Iyoda, A., Satoh, Y., Ryuge, S., Masuda, N., Jiang, S.X., et al., 2011. The balance between the expressions of hASH1 and HES1 differs between large cell neuroendocrine carcinoma and small cell carcinoma of the lung. Lung Cancer 74, 405–410.

Neptune, E.R., Podowski, M., Calvi, C., Cho, J.H., Garcia, J.G., Tuder, R., Linnoila, R.I., Tsai, M.J., Dietz, H.C., 2008. Targeted disruption of NeuroD, a proneural basic helix-loop-helix factor, impairs distal lung formation and neuroendocrine morphology in the neonatal lung. J Biol Chem. 283, 21160–21169.

Osipovich, A.B., Long, Q., Manduchi, E., Gangula, R., Hipkens, S.B., Schneider, J., Okubo, T., Stoeckert Jr., C.J., Takada, S., Magnuson, M.A., 2014. Insm1 promotes endocrine cell differentiation by modulating the expression of a network of genes that includes Neurog3 and Ripply3. Development 141, 2939–2949.

Raposo, A.A., Vasconcelos, F.F., Drechsel, D., Marie, C., Johnston, C., Dolle, D., Bithell, A., Gillotin, S., van den Berg, D.L., Ettwiller, L., et al., 2015. Ascl1 coordinately regulates gene expression and the chromatin landscape during neurogenesis. Cell Rep. 10, 1544–1556.

Shan, L., Aster, J.C., Sklar, J., Sunday, M.E., 2007. Notch-1 regulates pulmonary neuroendocrine cell differentiation in cell lines and in transgenic mice. Am. J. Physiol. Lung Cell Mol. Physiol. 292, L500–L509.

Song, H., Yao, E., Lin, C., Gacayan, R., Chen, M.H., Chuang, P.T., 2012. Functional characterization of pulmonary neuroendocrine cells in lung development, injury, and tumorigenesis. Proc. Natl. Acad. Sci. USA 109, 17531–17536.

Sutherland, K.D., Berns, A., 2010. Cell of origin of lung cancer. Mol. Oncol. 4, 397–403.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S., Kageyama, R., 1994. Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. J. Biol. Chem. 269, 5150–5156.

Ten Have-Opbroek, A.A., 1991. Lung development in the mouse embryo. Exp. Lung Res. 17, 111–130.

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Sudhof, T.C., Wernig, M., 2010. Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463, 1035–1041.

Welcker, J.E., Hernandez-Miranda, L.R., Paul, F.E., Jia, S., Ivanov, A., Selbach, M., Birchmeier, C., 2013. Insm1 controls development of pituitary endocrine cells and requires a SNAG domain for function and for recruitment of histone-modifying factors. Development 140, 4947–4958.

Wildner, H., Gierl, M.S., Strehle, M., Pla, P., 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.

Yuan, X., Wu, H., Xu, H., Han, N., Chu, Q., Yu, S., Chen, Y., Wu, K., 2015. Meta-analysis reveals the correlation of Notch signaling with non-small cell lung cancer progression and prognosis. Sci. Rep. 5, 10338.