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Insm1 (IA-1) is a crucial component of the transcriptional network that controls differentiation of the sympatho-adrenal lineage

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Insm1 (*IA-1*) encodes a Zn-finger factor that is expressed in the developing nervous system. We demonstrate here that the development of the sympatho-adrenal lineage is severely impaired in *Insm1* mutant mice. Differentiation of sympatho-adrenal precursors, as assessed by the expression of neuronal subtype-specific genes such as *Th* and *Dbh*, is delayed in a pronounced manner, which is accompanied by a reduced proliferation. Sympathetic neurons eventually overcome the differentiation blockade and mature correctly, but sympathetic ganglia remain small. By contrast, terminal differentiation of adrenal chromaffin cells does not occur. The transcription factors Mash1 (Ascl1), Phox2a, Gata3 and Hand2 (previously dHand) control the differentiation of sympatho-adrenal precursor cells, and their deregulated expression in *Insm1* mutant mice demonstrates that Insm1 acts in the transcriptional network that controls differentiation of this lineage. Pronounced similarities between *Mash1* and *Insm1* phenotypes are apparent, which suggests that *Insm1* might mediate aspects of *Mash1* function in the subtype-specific differentiation of sympatho-adrenal precursors. Noradrenaline is the major catecholamine produced by developing sympatho-adrenal cells and is required for fetal survival. We demonstrate that the fetal lethality of *Insm1* mutant mice is caused by catecholamine deficiency, which highlights the importance of Insm1 in the development of the sympatho-adrenal lineage.

KEY WORDS: Sympatho-adrenal lineage, Chromaffin cells, Sympathetic neurons, Zn-finger transcription factor, Endocrine differentiation, Mash1 (Ascl1)

INTRODUCTION

Neural crest cells constitute a transient population of stem cells that generate many different cell types. Considerable progress has been made in elucidating the molecular mechanisms that control the migration, specification and differentiation of neural crest-derived cells. In particular, sympatho-adrenal precursors are generated from neural crest cells and give rise to mature neurons of secondary sympathetic ganglia, to chromaffin cells of the adrenal medulla and to the extra-adrenal chromaffin tissue (Anderson, 1993; Goridis and Rohrer, 2002; Huber, 2006; Unsicker et al., 2005). Sympatho-adrenal precursor cells, sympathetic neurons and chromaffin cells express a common set of genes essential for differentiation and catecholamine biosynthesis (Howard, 2005). The differentiation of these cells requires exogenous signals as well as an endogenous network of transcription factors.

Migration of neural crest cells to the dorsal aorta depends on neuregulin 1 and the Erbb2/Erbb3 receptors (Britsch et al., 1998). Upon arrival in the mesenchyme lateral of the dorsal aorta, BMP signals induce the sympatho-adrenal differentiation of neural crest cells, which results in the formation of the primary sympathetic ganglion chain (Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996). Initiation of sympatho-adrenal differentiation can be assessed by the expression of *Phox2b* and *Mash1* (*Ascl1* – Mouse Genome Informatics), which encode homeobox and basic helix-loop-helix (bHLH) transcription factors, respectively. During further maturation of sympatho-adrenal precursor cells, Phox2a, the bHLH factor Hand2, the Zn-finger factors Gata2/3 and pan-neuronal proteins appear in sympatho-adrenal precursors. Finally, enzymes that are characteristic for noradrenergic neurons and that are required for catecholamine synthesis, such as tyrosine hydroxylase (Th) and dopamine-β-hydroxylase (Dbh), are produced (Goridis and Rohrer, 2002). In *Phox2b* mutant mice, Mash1 expression is correctly initiated, but none of the other genes expressed in differentiating sympatho-adrenal cells appears (Pattyn et al., 1999). In *Mash1* mutant mice, *Phox2b* expression is initiated correctly, but pan-neuronal and neuronal subtype-specific genes are expressed delayed and the sympathetic ganglia remain small (Guillemot et al., 1993; Pattyn et al., 2006). In *Gata3* mutant mice, the sympatho-adrenal differentiation is correctly initiated, as assessed by the expression of Phox2a/b, Mash1 and pan-neuronal genes. However, Gata2 and Th expression is severely downregulated, the size of sympathetic ganglia is reduced, and Phox2b expression is not correctly maintained (Lim et al., 2000; Moriguchi et al., 2006; Tsarovina et al., 2004). Mutations of *Phox2b*, *Mash1* and *Gata3* also interfere with the terminal differentiation of chromaffin cells in the adrenal gland (Huber et al., 2002; Huber et al., 2005; Moriguchi et al., 2006). Various lines of evidence indicate that a simple hierarchical model cannot account for all aspects of the functions of Mash1, Phox2a/b, Hand2 and Gata2/3. Instead, these factors crossregulate each other, forming a transcriptional network that coordinately regulates differentiation of the sympatho-adrenal lineage (Goridis and Rohrer, 2002).

The insulinoma-associated 1 (*Insm1*, *IA-1*) gene encodes a DNA-binding protein with five zinc-finger domains that is conserved in evolution (Goto et al., 1992). *Insm1* is expressed in the developing central and peripheral nervous system, in a large number of endocrine tumors, and in endocrine cells of the developing pancreas and intestine (Gierl et al., 2006; Goto et al., 1992; Mellitzer et al.,

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2006). *Insm1* is required for differentiation of endocrine cells in the pancreas and intestine, and its mutation affects the execution of a gene expression program that comprises hormones and secretory proteins (Gierl et al., 2006). Available evidence indicates that expression of *Insm1* can be controlled by transcription factors of the bHLH family such as Mash1, Ngn1 or Ngn3 (Breslin et al., 2003; Castro et al., 2006; Mellitzer et al., 2006). We investigate here the function of *Insm1* in the peripheral nervous system, and demonstrate that *Insm1* is a crucial component of the transcriptional network that coordinates the differentiation of sympatho-adrenal cells. Our data indicate that *Insm1* genetically acts downstream of *Mash1* and *Phox2b*, and that in addition *Insm1* represses *Mash1*. Furthermore, we show that the fetal lethality of *Insm1* mutant mice is caused by insufficient catecholamine synthesis, highlighting the importance of *Insm1* in development of the sympatho-adrenal lineage.

MATERIALS AND METHODS

Mouse strains and determination of noradrenaline levels

The generation and genotyping of $Insm1^{lacZ}$, $Mash1^{GFP}$, and $Phox2b^{lacZ}$ mutant mice were described (Gierl et al., 2006; Pattyn et al., 1999; Wildner et al., 2006). We observed a pronounced fetal lethality of $Insm1^{lacZ}/Insm1^{lacZ}$ mice on a 129/Ola/C57/BL6 genetic background. In order to rescue this fetal lethality pharmacologically, pregnant $Insm1^{lacZ}/+$ dams that had been mated to $Insm1^{lacZ}/+$ males received L-DOPA (1 mg/ml; 0.25% ascorbic acid) in their drinking water (Thomas et al., 1995). Rescued animals died shortly after birth. The $Insm1^{lacZ}$ allele was also crossed for two to three generations onto the CD1 strain, and the fetal lethality was less pronounced on this outbred genetic background (Gierl et al., 2006). However, changes in development of the sympatho-adrenal lineage were comparable on both genetic backgrounds.

Noradrenaline levels were analyzed essentially as described (Britsch et al., 1998; Thomas et al., 1995). In short, $Insm1^{lacZ}/+$ and $Insm1^{lacZ}/Insm1^{lacZ}$ mice at E12.5 were homogenized in 0.1 M perchloric acid, and the protein concentrations in the homogenates were determined (Bio-Rad, Hercules, CA, USA). Catecholamines were purified over alumina columns, and noradrenaline levels were determined by HPLC chromatography (Haema Institute for Laboratory Medicine, Berlin, Germany).

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

For in situ hybridization, embryonic or adult tissues were embedded either in OCT compound or paraffin and sectioned. Hybridization was performed with DIG-labeled riboprobes. Fragments amplified from cDNA were used to generate the RNA probes for Insm1, Chromogranin A and B; other probes were generated from plasmids obtained from various laboratories. Detection of β-galactosidase activity by X-gal staining was performed as described previously (Lobe et al., 1999).

Immunohistochemistry was performed on 12 μm cryosections of mouse embryos fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH7.4. The following primary antibodies were used: goat anti-β-galactosidase (1:1000; AbD Serotec, Oxford, UK), rabbit anti-β-galactosidase (1:1000; ICN Biochemical, Eschwege, Germany), mouse anti-Mash1 (1:500 BD Biosciences, San Jose, CA, USA), rabbit anti-Th (1:200; Pel-Freez, Rogers, AR, USA), rabbit anti-Phox2a, rabbit anti-Phox2b (both 1:1000; Christo Goridis and Jean-Francois Brunet, Ecole Normale Superieure, Paris, France); rabbit anti-Npy (1:8000; Sigma, St Louis, MO, USA); rabbit anti-p75 (1:200; Promega, Madison, WI, USA), rabbit and guinea-pig anti-Tlx3 (Muller et al., 2005); mouse anti-Tuj1 (1:1000, Covance, Berkeley, CA, USA); rabbit anti-Pnmt (1:1000; ImmunoStar, Hudson, WI, USA) and secondary antibodies conjugated with Cy2, Cy3, or Cy5 (1:500; Jackson ImmunoResearch, West Grove, PA, USA). Cell death was determined by TUNEL staining using an Apop-Tag fluorescein in situ apoptosis detection kit (Millipore, Billerica, MA, USA).

For BrdU labeling, BrdU (75 μg/g body weight; Sigma) was injected intraperitoneally, and embryos were isolated at the indicated times. Sections were treated with primary antibodies that specifically detect various cell types, and subsequently labeled with anti-BrdU antibodies. Incorporated BrdU was detected with either mouse (1:200; Sigma) or rat anti-BrdU antibodies (1:200; AbD Serotec, Oxford, UK). Fluorescence was imaged on a Zeiss LSM 5 Pascal confocal microscope and images were processed using Adobe Photoshop software.

Cell counts

Cells in the anlage of the sympathetic nervous system were stained with antibodies against β-galactosidase, BrdU, Phox2a, Phox2b, Th, Tuj1 or Mash1, and the numbers of double-positive cells were counted on at least three sections obtained from three or four heterozygous and homozygous $Insm1^{lacZ}$ mice. TUNEL+ cells were counted in the adrenal medulla on sections obtained from three heterozygous and three homozygous $Insm1^{lacZ}$ mice at E18.5; at least three sections per animal were counted. The numbers of β-galactosidase+ cells in the adrenal gland were determined by counting β-galactosidase+ cells on cryosections of adrenal glands that were obtained from three heterozygous and three homozygous $Insm1^{lacZ}$ mice at E14.5, E16.5 and E18.5. The entire adrenal gland was sectioned, and every second (E14.5) or forth section (E16.5 and E18.5) was counted. To assess the statistical significance, a Student's *t*-test for a two-tailed distribution and a two-sample unequal variance was applied.

Microarray analysis

Adrenal glands were dissected from E18.5 wild-type and $Insm1^{lacZ}/Insm1^{lacZ}$ embryos and homogenized in Trizol (Invitrogen, Carlsbad, CA, USA). RNA extraction, probe synthesis and hybridization to Affymetrix MOE430 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) were performed according to the manufacturer's protocol. Data processing and identification of differentially expressed genes was carried out as described (Gierl et al., 2006). Genes were considered differentially expressed if the difference of their expression level had a *P*-value of 0.05.

RESULTS

Noradrenaline deficiency causes the fetal lethality of *Insm1* mutant mice

We have reported previously that the majority of $Insm1^{lacZ}/Insm1^{lacZ}$ mice died during the second half of gestation. On a mixed 129/Ola/C57/BL6 genetic background, 23.8%, 22.5% and 6.5% of the offspring of heterozygous matings had an $Insm1^{lacZ}/Insm1^{lacZ}$ genotype at E10.5, E12.5 and P0, respectively (see also Gierl et al., 2006). Noradrenaline is the major catecholamine produced by developing sympatho-adrenal cells and is required for fetal survival (Britsch et al., 1998; Lim et al., 2000; Pattyn et al., 2000; Thomas et al., 1995; Zhou et al., 1995). We found that noradrenaline concentrations in total extracts of $Insm1^{lacZ}/Insm1^{lacZ}$ embryos were significantly lower than in control mice (2.5±1.0 and 0.5±0.2 ng noradrenaline/mg protein in $Insm1^{lacZ}/+$ and $Insm1^{lacZ}/Insm1^{lacZ}$ mice at E12.5, respectively, n=5). The precursor of noradrenaline synthesis is tyrosine, which is converted into L-DOPA by Th. Decarboxylation of L-DOPA by aromatic amino acid decarboxylase produces dopamine, which is converted by Dbh into noradrenaline. We observed that *Th* and *Dbh* transcripts were markedly downregulated in the primary sympathetic ganglion chain of $Insm1^{lacZ}/Insm1^{lacZ}$ mice, indicating that diminished Th and Dbh synthesis were responsible for the low noradrenaline levels (see also below). Alterations in sympathetic activity in *Phox2b*, *Gata3*, *Th* or *Dbh* mutant mice have been directly linked to fetal lethality apparently caused by heart failure. Fetal lethality can be rescued by feeding catecholamine intermediates or noradrenaline receptor agonists to pregnant dams (Lim et al., 2000; Pattyn et al., 2000; Thomas et al., 1995; Zhou et al., 1995). We fed the catecholamine intermediate L-DOPA to pregnant $Insm1^{lacZ}/+$ females that had been crossed to $Insm1^{lacZ}/+$ males. Analysis of their offspring demonstrated that homozygous *Insm1* mutant mice could be recovered at normal Mendelian ratios at birth, i.e. we observed 24.6% of the offspring to display an $Insm1^{lacZ}/Insm1^{lacZ}$ genotype at

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P0. We conclude that a catecholamine deficiency causes the intrauterine death of *Insm1* mutant mice. Control of rescued mutant mice were comparable in overall size and appearance at birth, but the *Insm1*^{lacZ} mutant mice were unable to breathe and died postnatally.

Insm1 is expressed widely in the differentiating peripheral and central nervous system (Breslin et al., 2003; Gierl et al., 2006). Here, we used mice carrying one copy of an *Insm1*^{lacZ} mutant allele to characterize *Insm1* expression, and analyzed the appearance of the *lacZ* gene product, β-galactosidase, in the sympatho-adrenal lineage. We did not detect expression of β-galactosidase in migrating neural crest cells that were visualized by anti-p75 antibodies (Fig. 1A). The first β-galactosidase+ cells in the peripheral nervous system appeared around E9.5 and were detected in the anlage of the primary sympathetic ganglion chain lateral of the dorsal aorta on interlimb levels, and in differentiating neurons of the condensing dorsal root ganglia (Fig. 1A; see also Fig. S1 in the supplementary material). At E10.5, the entire primary sympathetic ganglion chain expressed β-galactosidase, as assessed by X-gal staining (Fig. 1B). Immunohistological analysis showed that β-galactosidase+ cells co-expressed Phox2b, indicating that sympatho-adrenal precursor cells express *Insm1* (Fig. 1C). In addition, some but not all β-galactosidase+ cells co-expressed Mash1 (Fig. 1D); the transient nature of the expression of Mash1 might account for this. *Insm1* expression in sympathetic ganglia was observable at E18.5 (data not shown). β-Galactosidase expression or *Insm1* transcripts were also detected in chromaffin cells of the adrenal gland (Fig. 1E and data not shown). Expression in the adrenal gland was detected at E13.5, and persisted in the adult adrenal gland.

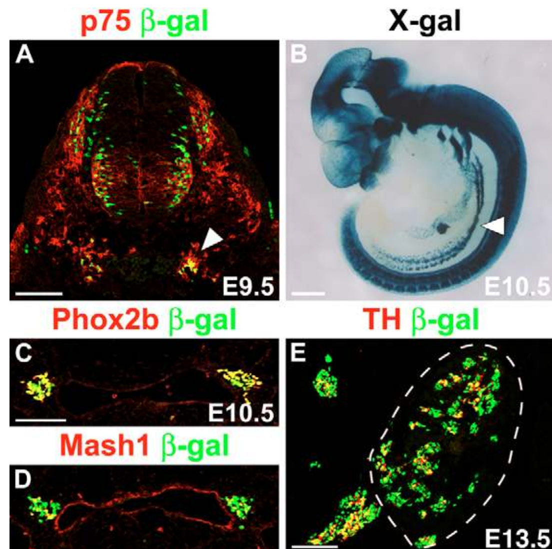

Fig. 1. Expression of Insm1 in the developing peripheral nervous system. To analyze Insm1 expression, we took advantage of the *Insm1*^{lacZ} allele in which *lacZ* sequences replace the *Insm1*-coding sequence. *Insm1*^{lacZ}/+ animals were analyzed at the indicated developmental stages, using antibodies directed against the *lacZ* gene product β-galactosidase (A,C-E) or X-gal staining (B). (**A**) Immunohistochemical analysis using anti-β-galactosidase (green) and anti-p75 (red) antibodies demonstrates expression of β-galactosidase in the primary sympathetic ganglion chain located lateral of the dorsal aorta (arrowhead), in the spinal cord and in condensing dorsal root ganglia at E9.5. (**B**) At E10.5, X-gal staining is detected in the entire primary sympathetic ganglion chain (arrowhead), as well as in sensory ganglia and in the central nervous system. (**C**,**D**) Immunohistochemical analyses of the primary sympathetic ganglion chain using antibodies directed against β-galactosidase (green), Phox2b (red in C) and Mash1 (red in D) indicate that the majority of β-galactosidase+ cells lateral of the dorsal aorta co-express Phox2b, and some β-galactosidase+ cells also express Mash1. (**E**) Immunohistochemical analysis of the adrenal gland at E13.5 using anti-β-galactosidase (green) and anti-Th (red) antibodies demonstrates *Insm1* expression in chromaffin cells of the adrenal medulla. Scale bars: 100 μm in A,C,E; 500 μm in B.

Insm1 is required for the differentiation of sympatho-adrenal precursor cells

We next investigated the formation and differentiation of the primary sympathetic ganglion chain of *Insm1* mutant mice. Neural crest cells that initiate sympatho-adrenal differentiation were identified by in situ hybridization using *Phox2b* as a probe, and we

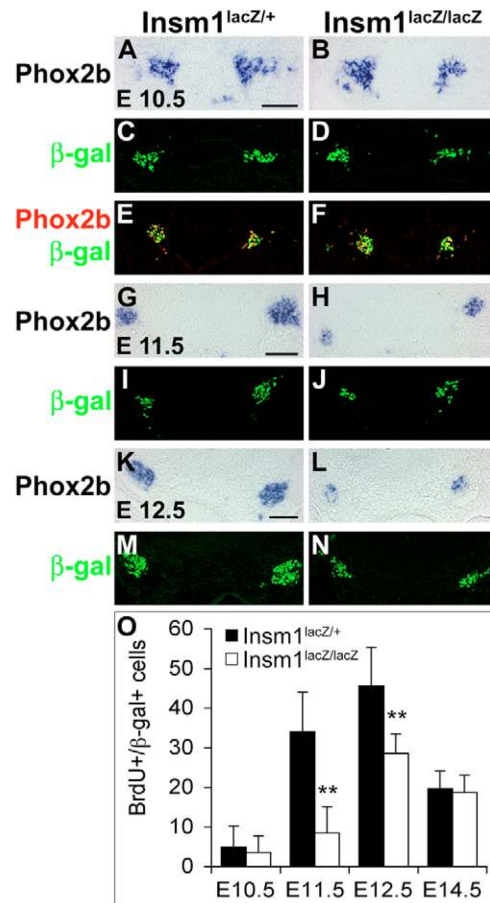

Fig. 2. Reduced proliferation of sympatho-adrenal precursor cells causes a smaller size of primary sympathetic ganglia in *Insm1* mutant mice. Phox2b and β-galactosidase expression was analyzed in the sympathetic ganglion chain at E10.5 (**A-F**), E11.5 (**G-J**) and E12.5 (**K-N**) of *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice by in situ hybridization or immunohistochemical analyses. (A,B,G,H,K,L) In situ hybridization using a Phox2b-specific probe. (C-F,I,J,M,N) Immunohistochemistry using antibodies against (C-F,I,J,M,N) β-galactosidase (green) and (E,F) Phox2b (red). The number of cells in the primary sympathetic ganglion chain is reduced in the mutant at E11.5 and E12.5. (**O**) Cell proliferation, as determined by BrdU injections at the indicated time points in the primary sympathetic ganglion chain of *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice. Shown is the proportion (%) of β-galactosidase+ cells that had incorporated BrdU 2 hours after the injection. Double asterisks indicate $P<0.01$. Scale bars: 100 μm in A,G,K.

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observed Phox2b+ sympatho-adrenal precursors lateral of the dorsal aorta in heterozygous and homozygous *Insm1*^{lacZ} mice (Fig. 2). At E10.5, the size of the primary ganglion chain was comparable when analyzed by Phox2b in situ hybridization, by immunohistochemistry using anti-β-galactosidase antibodies or by X-gal staining (Fig. 2A-F and data not shown). We observed comparable proportions of β-galactosidase+ cells that expressed Phox2b in heterozygous and homozygous *Insm1*^{lacZ} mice at E10.5 (81.2±4.7% and 79.9±5.0% in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice, respectively, n=3; see also Fig. 3A,B), indicating that the differentiation of sympatho-adrenal progenitor cells is correctly initiated in *Insm1* mutant mice. However, at subsequent developmental stages we noted a pronounced reduction in the size of the primary sympathetic ganglion chain in *Insm1*^{lacZ}/*Insm1*^{lacZ} mice, regardless whether *Phox2b* in situ hybridization, β-galactosidase immunohistochemistry or X-gal staining were used (Fig. 2G-N). Sympatho-adrenal precursors possess proliferative capacities (Rohrer and Thoenen, 1987). We compared the proliferation in control and *Insm1* mutant mice using BrdU injections, and determined the proportions of β-galactosidase+ sympatho-adrenal precursor cells that incorporated BrdU. In control mice, we observed considerable proliferation rates of sympatho-adrenal precursor cells at E11.5 and E12.5, which were reduced in *Insm1*^{lacZ}/*Insm1*^{lacZ} mice (Fig. 2O). At E14.5, proliferation rates were comparable in control and *Insm1* mutant mice (Fig. 2D). Apoptosis in cells lateral of the dorsal aorta, as assessed by TUNEL staining, was not augmented at E10.5 and E12.5 (data not shown). We therefore conclude that a reduced proliferation of sympatho-adrenal precursor cells accounts for the reduced size of the primary sympathetic ganglion chain in homozygous *Insm1* mutant mice.

We also assessed the expression of genes that appear after Phox2b in the differentiating sympatho-adrenal lineage. We observed that few sympatho-adrenal precursor cells expressed *Phox2a*, *Hand2*, *Gata3*, *Ret*, *Th* and *Dbh* in *Insm1* mutant mice at E10.5 (Fig. 3A-N). Thus, at E10.5, the time point at which the analysis of Phox2b or β-galactosidase expression did not reveal a change in the size of the primary ganglion chain of *Insm1* mutant mice, many differentiation markers were expressed in only few sympatho-adrenal precursor cells. However, the expression of these genes recovered at later stages. We quantified the proportion of β-galactosidase+ sympatho-adrenal precursor cells that co-expressed Th at various developmental stages. In control mice at E10.0, 60% of the β-galactosidase+ cells expressed Th, but only 10% co-expressed Th in the *Insm1*^{lacZ}/*Insm1*^{lacZ} mutant animals (Fig. 3Q-S). At E14.5, the proportions of co-expressing cells were similar in control and mutant mice (Fig. 3S). We also observed a delay in the differentiation of the sympatho-adrenal precursor cells when we analyzed Phox2a expression in *Insm1* mutant mice (Fig. 3T-V). At E10.5, 59% of β-galactosidase+ cells co-expressed Phox2a in control mice, but only 13% in the *Insm1*^{lacZ}/*Insm1*^{lacZ} mutant mice. By contrast, by E12.5 similar proportions of β-galactosidase+ cells co-expressed Phox2a in heterozygous and homozygous mutant mice (Fig. 3V). By

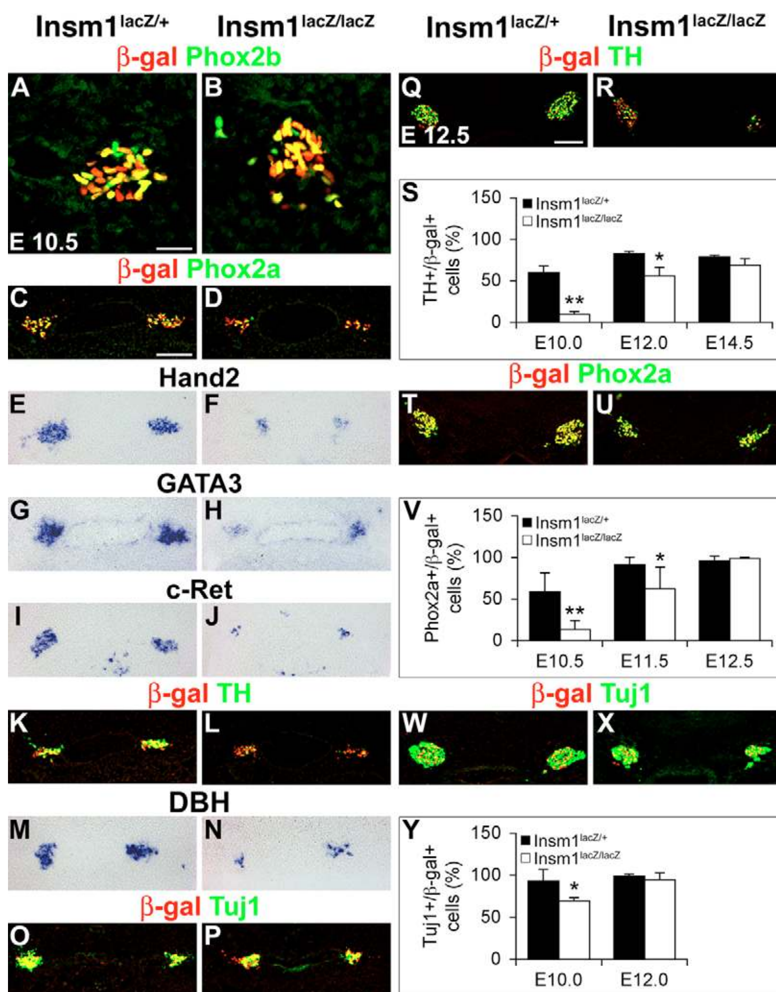

Fig. 3. Delayed differentiation of sympatho-adrenal precursor cells in *Insm1* mutant mice. The differentiation of the sympatho-adrenal precursor cells in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice was assessed at the indicated stages using immunohistochemical analysis or in situ hybridization. (A-P) Analysis of E10.5 embryos. (**A-D**) Immunohistochemistry using antibodies against β-galactosidase (red), (A,B) Phox2b (green) and (C,D) Phox2a (green). (**E-J**) In situ hybridization using probes specific for (E,F) Hand2, (G,H) Gata3 and (I,J) Ret. (**K**,**L**) Immunohistochemistry using antibodies against β-galactosidase (red) and Th (green). (**M**,**N**) In situ hybridization using a Dbh-specific probe. (**O**,**P**) Immunohistochemistry using antibodies against β-galactosidase (red) and Tuj1 (green). There is a pronounced reduction in the number of sympatho-adrenal precursor cells that express Phox2a, Hand2, Gata3, Ret, Th or Dbh at E10.5 in *Insm1*^{lacZ}/*Insm1*^{lacZ} mice. (**Q**,**R**) Analysis of E12.5 embryos by immunohistochemistry using antibodies against β-galactosidase (red) and Th (green). (**S**) Quantification of β-galactosidase+ sympatho-adrenal precursor cells that co-express Th in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice at various developmental stages. (**T**,**U**) Analysis of E12.5 embryos by immunohistochemistry using antibodies against β-galactosidase (red) and Phox2a (green). (**V**) Quantification of the β-galactosidase+ cells in sympathetic ganglia that co-express Phox2a at various developmental stages in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice. (**W**,**X**) Analysis of E12.5 embryos by immunohistochemistry using antibodies against β-galactosidase (red) and Tuj1 (green). (**Y**) Quantification of β-galactosidase+ sympatho-adrenal precursor cells that co-express Tuj1 in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice at various developmental stages. Single or double asterisks in S,V,Y indicate P<0.05 and P<0.01, respectively. Scale bars: 50 μm in A; 100 μm in C,Q.

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comparison, expression of the pan-neuronal antigen, neuronal class III β-Tubulin (Tuj1), was delayed only mildly in *Insm1*^{lacZ}/*Insm1*^{lacZ} mice (Fig. 3W-Y). We conclude that proliferation and differentiation of sympatho-adrenal precursor cells are affected by the *Insm1* mutation.

Mash1 expression appears early during differentiation of the sympathetic ganglion chain. In situ hybridization demonstrated a markedly upregulated expression of *Mash1* at E10.5 in the primary sympathetic ganglion chain of *Insm1* mutant mice (Fig. 4A,B). At this stage, the *Mash1* target gene delta-like 1 (*Dll1*) was also markedly upregulated (Fig. 4C,D). The expression of genes controlled by Notch signaling, Hes1, Hes5, Hes1r or Nrarp, were not affected (not shown). Upregulated expression of *Mash1* was less pronounced at E12.5 (Fig. 4I,J). Mash1 protein in the primary ganglion chain was also assessed by immunohistochemistry. In heterozygous *Insm1* mutant mice, the proportion of β-galactosidase+ cells that co-expressed Mash1 was 48% at E10.5, but in homozygous *Insm1* mutant mice, the proportion of co-expressing cells was 85% (Fig. 4E,F). Phox2a and Mash1 were, however, not co-expressed in sympatho-adrenal precursor cells of heterozygous or homozygous *Insm1* mutant mice (Fig. 4G,H).

Sympatho-adrenal precursor cells migrate from the primary ganglion chain to generate secondary sympathetic ganglia. The reduction in the overall size of the primary sympathetic ganglion chain in *Insm1* mutant mice at E12.5 was accompanied by a reduced size of secondary sympathetic ganglia at E14.5 and E18.5. Thus, for instance the superior cervical and stellate ganglia were smaller in *Insm1*^{lacZ}/*Insm1*^{lacZ} than in *Insm1*^{lacZ}/+ mice when assessed by whole-mount staining using X-gal (Fig. 5A,B). Similarly, immunohistochemical analyses of control and mutant stellate ganglia demonstrated a reduction in size when antibodies directed against Tuj1, Th or Phox2a were used (Fig. 5C-H). However, similar proportions of neurons expressed NPY, Tlx3 and Ret in the secondary sympathetic ganglia of control and mutant mice at E18.5 (Fig. 5C-H and data not shown), indicating that maturation of sympathetic neurons had occurred.

Differentiation of chromaffin cells in Insm1 mutant mice

Sympatho-adrenal precursor cells move to the anlage of the adrenal gland around E12.5, and by E14.5 their derivatives, the chromaffin cells, can be detected in great numbers in the adrenal medulla (Britsch et al., 1998; Huber et al., 2002). Chromaffin cells that express β-galactosidase were present in comparable numbers in the adrenal medulla of heterozygous and homozygous *Insm1*^{lacZ} mutant mice at E14.5, but these cells appeared more dispersed in heterozygous mice (Fig. 6A,B; for a quantification

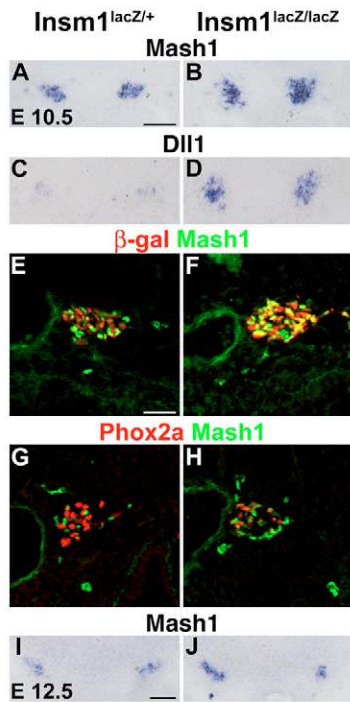

Fig. 4. *Mash1* and *Dll1* are upregulated in sympatho-adrenal precursor cells of *Insm1* mutant mice. Analysis of Mash1 expression by in situ hybridization and immunohistochemistry at E10.5 (**A**,**B**,**E-H**) and E12.5 (**I**,**J**), and of Dll1 expression at E10.5 (**C**,**D**) by in situ hybridization of *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice. In situ hybridization using a probe specific for Mash1 (A,B,I,J) and Dll1 (C,D). Immunohistochemistry using antibodies against (E,F) β-galactosidase (red) and Mash1 (green); (G,H) Phox2a (red) and Mash1 (green). Scale bars: 100 μm in A,I; 50 μm in E.

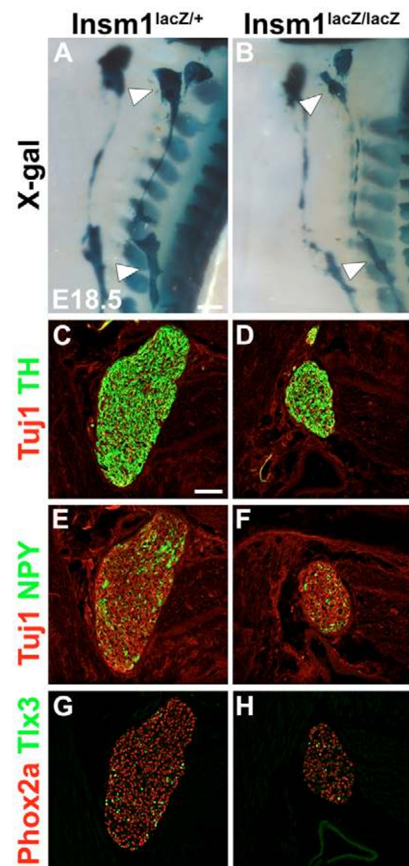

Fig. 5. Reduced size of secondary sympathetic ganglia in *Insm1* mutant mice. Secondary sympathetic ganglia of *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice (E18.5) were analyzed by (**A**,**B**) X-gal staining and by (**C-H**) immunohistochemistry. Antibodies used were directed against: (C,D) Tuj1 (red) and Th (green), (E,F) Tuj1 (red) and NPY (green); (G,H) Phox2a (red) and Tlx3 (green). Arrowheads in A and B indicate superior cervical and stellate ganglia. Scale bars: 500 μm in A; 100 μm in C.

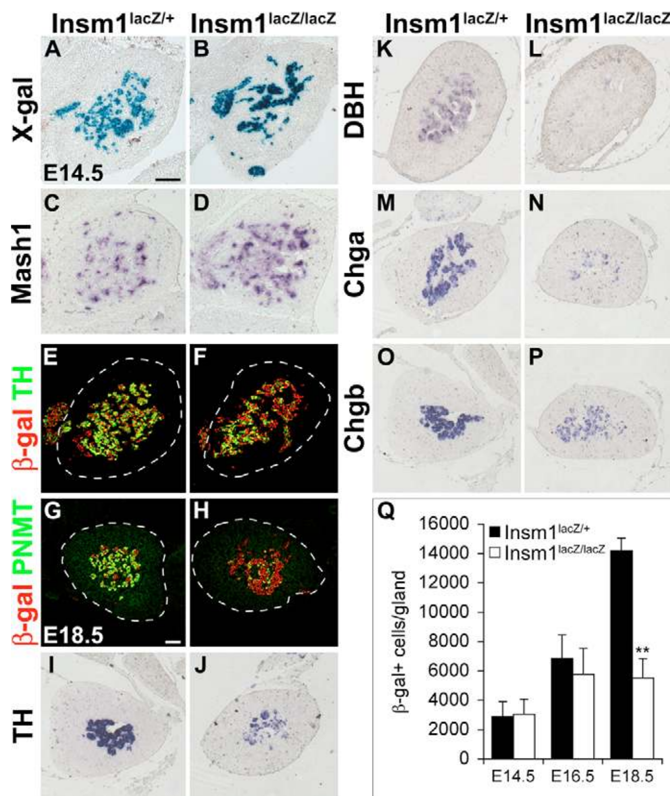

Fig. 6. Changed differentiation of chromaffin cells in *Insm1* mutant mice. Adrenal glands of *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice (E14.5) were analyzed by (**A**,**B**) X-gal staining, (**C**,**D**) in situ hybridization using a Mash1-specific probe and (**E**,**F**) immunohistochemistry using antibodies against β-galactosidase (red) and Th (green). Adrenal glands of *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice (E18.5) were analyzed by (**G**,**H**) immunohistochemistry using antibodies against β-galactosidase (red) and PNMT (green), and by in situ hybridization using probes specific for (**I**,**J**) Th, (**K**,**L**) Dbh, (**M**,**N**) chromogranin A and (**O**,**P**) chromogranin B. (**Q**) Quantification of total numbers of β-galactosidase+ cells in adrenal glands at various developmental stages in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice. Double asterisks indicate a $P<0.01$. Scale bars: 100 μm (A,G).

see Q). At E18.5, the numbers of β-galactosidase+ cells were reduced by 61% in homozygous *Insm1* mutants (Fig. 6Q). Analysis of cell proliferation using BrdU injection demonstrated comparable proliferation rates of β-galactosidase+ cells in the medulla at E14.5 and E16.5 (24.1±8.3% and 20.7±6.3% at E14.5 in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice, respectively, n=3; 20.6±3.0% and 19.8±4.1% at E16.5 in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice, respectively, n=3). However, TUNEL staining indicated a marked increase in apoptosis at E18.5 (0.2±0.1 and 2.0±0.8 TUNEL+ cells/mm^2 of the adrenal medulla in *Insm1*^{lacZ}/+ and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice, respectively, n=3). Thus, enhanced cell death accounts for the reduction in chromaffin cell numbers.

We analyzed various genes expressed in differentiating chromaffin cells. *Mash1* expression was increased in the adrenal gland of the *Insm1* mutant mice at E14.5 and E18.5 (Fig. 6C,D and Table 1). By contrast, the expression of genes that encode enzymes responsible for catecholamine biosynthesis like Th, Dbh and the adrenalin-synthesizing enzyme phenylethanolamine N-methyltransferase (Pnmt) was downregulated (Fig. 6E-L and Table 1). To assess changes in gene expression of chromaffin cells systematically, we isolated RNA of adrenal glands from *Insm1*^{lacZ}/*Insm1*^{lacZ} and *Insm1*^{lacZ}/+ animals, and compared transcripts by microarray analysis. This also demonstrated that *Th*, *Dbh* and *Pnmt* expression was reduced significantly in the mutants, and revealed additional pronounced changes in gene expression. In particular, genes typically expressed in mature endocrine cells, such as chromogranin A and B, were strongly downregulated (Table 1 and Fig. 6M-P). The 2.5-fold reduction in chromaffin cell numbers cannot account for these pronounced changes in transcript levels. In addition, a small number of genes, among them *Mash1* and *Nf68* (*Nefl* – Mouse Genome Informatics), were significantly upregulated (Table 1). By contrast, changes in the transcript level of *Phox2a*/*b*, *Gata2*/*3* and *Hand2* were small (fold changes of transcript levels between 1.8 and 3.4; see Table 1). The remaining chromaffin cells appeared to express similar transcript levels of *Phox2a*/*b*, *Gata2*/*3* and *Hand2* when analyzed by in situ hybridization (data not shown). Thus, the 2.5-fold reduction in the number of chromaffin cells contributes significantly to the observed downregulation of *Phox2a*/*b*, *Gata2*/*3* and *Hand2* transcript levels, and might even account for it (compare Fig. 6Q and Table 1). *Dll1* was not significantly expressed in the adrenal gland of control and mutant mice. We conclude that the terminal differentiation of chromaffin cells is impaired in *Insm1* mutant mice, and that gene products essential for catecholamine production and secretion are not correctly expressed.

Epistasis between Insm1, Phox2b and Mash1

Sympatho-adrenal neurons require *Phox2b* for their differentiation (Pattyn et al., 1999). We analyzed *Insm1* expression in *Phox2b* mutant mice at E10.5 and E12.5, and detected no *Insm1* expression lateral of the dorsal aorta (Fig. 7A-D and data not shown). This demonstrates that *Insm1* expression is indeed restricted to the neuronal population in the developing sympathetic nervous system, and indicates that *Phox2b* is required to initiate *Insm1* expression. In the primary sympathetic ganglion chain of *Mash1* mutant mice, *Phox2b* expression is correctly initiated, but other markers appear delayed (Pattyn et al., 2006). We observed no *Insm1* expression in *Mash1* mutant mice at E10.5 in sympatho-adrenal precursors, and at E12.5 expression was downregulated in a pronounced manner (Fig. 7E,F). Thus, *Mash1* is also required for correct *Insm1* expression during development of sympatho-adrenal precursors (see Fig. 7G for a summary).

DISCUSSION

Insm1 encodes a Zn-finger factor that is expressed widely in the developing peripheral and central nervous system. In the central nervous system, for example in the spinal cord, neurons express *Insm1* for only a short period during their differentiation (Gierl et al., 2006). Our analyses indicate that Insm1 is dispensable for generic neurogenesis and neuronal specification in spinal cord (H.W., M.S.G. and Thomas Müller, unpublished). By contrast, neural crest-derived sympatho-adrenal precursors express *Insm1* for prolonged periods, and require *Insm1* for their differentiation. In particular, differentiation of sympatho-adrenal precursors was delayed in *Insm1* mutant mice, and we found that many subtype-specific genes were expressed behind schedule. Sympatho-adrenal precursors subsequently escaped the differentiation block in *Insm1* mutant mice, and generated sympathetic neurons that matured correctly. However, secondary sympathetic ganglia in *Insm1* mutant mice remained small, owing to a decreased proliferation of the precursors. Sympatho-adrenal precursors also generate chromaffin cells in the

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Table 1. Comparison of gene expression in the adrenal gland of heterozygous and homozygous *Insm1*^{lacZ} mutant mice

Gene symbol	Gene name	FC	*P*
Hormone processing and secretion			
Akr1c18	Aldo-keto reductase family 1, member C18	–39.7	0.001
*Pnmt**	Phenylethanolamine-N-methyltransferase	–28.1	0.001
*Th**	Tyrosine hydroxylase	–6.7	0.001
*Dbh**	Dopamine beta hydroxylase	–6.9	0.001
*Chga**	Chromogranin A	–13.3	0.001
*Chgb**	Chromogranin B	–8.7	0.001
Scg2	Secretogranin II	–4.0	0.001
Scg3	Secretogranin III	–4.1	0.001
Sgne1	Secretogranin V	–4.8	0.001
Slc18a1	Vesicular monoamine Transporter (VMAT1)	–4.2	0.001
Slc7a8	Cationic amino acid transporter (LAT2)	–4.1	0.001
Transcription factors			
Gata2	GATA binding protein 2	–3.4	0.001
*Gata3**	GATA binding protein 3	–3.4	0.001
*Phox2a**	Paired-like homeobox 2a	–2.9	0.001
*Phox2b**	Paired-like homeobox 2b	–1.9	0.001
*Hand2**	Hand2	–1.8	0.001
*Mash1**	Achaete-scute complex homolog-like 1 (Ascl1)	2.6	0.001
Ebf1	Early B-cell factor 1	2.1	0.001
Other factors			
Resp18	Regulated endocrine-specific protein 18	–10.5	0.001
Disp2	Dispatched homolog 2	–9.2	0.001
Sez6l2	Seizure related 6 homolog like 2	–4.2	0.001
Igf1	Insulin-like growth factor 1	4.5	0.001
Dkk2	Dickkopf homolog 2 (*Xenopus laevis*)	4.4	0.001
*Nefl**	Neurofilament 68	2.1	0.001

Systematic analysis of gene expression in control and *Insm1*^{lacZ}/*Insm1*^{lacZ} mice using Affymetrix oligonucleotide microarrays. The average signal fold change is shown. We selected the following genes for display: (1) genes that are downregulated at least fourfold; (2) genes of known function during chromaffin cell development; (3) genes that were upregulated at least twofold.
*Also analyzed by immunohistochemistry or by in situ hybridization.
FC, fold change.

adrenal medulla. We observed a marked change in terminal differentiation of chromaffin cells, reduced expression of genes whose protein products control catecholamine synthesis and secretion, and low catecholamine levels in *Insm1* mutant mice. Catecholamines are essential for fetal survival and for heart function during development (Lim et al., 2000; Pattyn et al., 2000; Thomas et al., 1995; Zhou et al., 1995). We were able to rescue the fetal lethality of *Insm1* mutant embryos by the administration of catecholamine intermediates. Thus, deficits in catecholamine synthesis are responsible for the reduced viability of *Insm1* mutant mice during gestation.

Insm1 and the transcriptional network that regulates sympatho-adrenal differentiation

The transcription factors Phox2a/b, Mash1, Gata2/3 and Hand2 control sympatho-adrenal differentiation, and form a transcriptional network that regulates their own expression as well as the expression of generic and subtype-specific neuronal genes (Goridis and Rohrer, 2002). Changes in the differentiation of sympatho-adrenal precursors cells were reported for *Phox2b*, *Mash1* and *Gata3* mutant mice (Guillemot et al., 1993; Lim et al., 2000; Moriguchi et al., 2006; Pattyn et al., 2006; Pattyn et al., 1999; Tsarovina et al., 2004). We show here that several factors of the transcriptional network

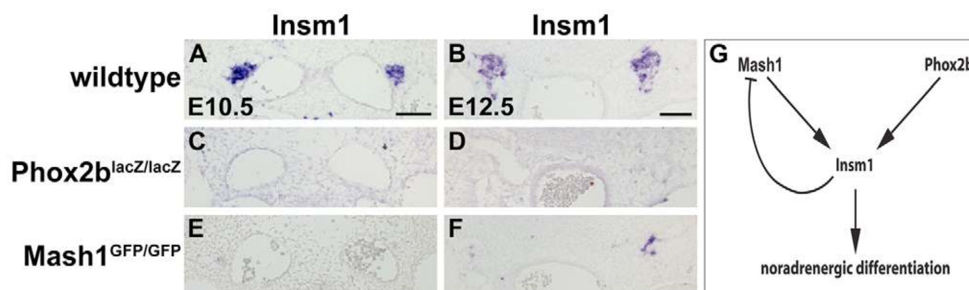

Fig. 7. Epistasis between *Insm1*, *Phox2b* and *Mash1* in the differentiation of sympatho-adrenal precursor cells. Analysis of the primary sympathetic ganglion chain in wild-type (**A**,**B**), *Phox2b* (**C**,**D**) and *Mash1* (**E**,**F**) mutant mice at E10.5 (A,C,E) and E12.5 (B,D,F) by in situ hybridization using a probe specific for Insm1. (**G**) Schematic drawing of the epistatic relationship between *Mash1*, *Phox2b* and *Insm1*. Scale bars: 100 μm in A,B.

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(Phox2a, Gata3, Mash1) and subtype-specific genes (*Th*, *Dbh*) are not correctly expressed during the differentiation of sympatho-adrenal precursor cells in *Insm1* mutant mice. Conversely, Insm1 expression is not correctly initiated in sympatho-adrenal precursors of *Phox2b* and *Mash1* mutant mice. *Insm1* was recently predicted to be a direct Mash1 target (Castro et al., 2006). Our data are in accordance with a function of Mash1 in the control of Insm1 expression, and demonstrate that Insm1 acts downstream of Phox2b and Mash1 during the development of sympatho-adrenal precursor cells.

Similarities in the phenotypes of *Mash1* and *Insm1* mutant mice are apparent. For example, in both mutant strains sympatho-adrenal differentiation is correctly initiated, as assessed by Phox2b expression. *Phox2a*, *Gata3*, *Hand2* and *Th* transcripts appear behind schedule in all sympatho-adrenal precursor cells of *Mash1* mutant mice (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 2006). These genes are also expressed delayed in *Insm1* mutant mice, but this delay is apparent in the majority, and not all sympatho-adrenal precursors. Finally, in *Mash1* mutant mice a block in the differentiation of enteric neurons is present in the esophagus but not in other parts of the gastrointestinal tract (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 1999). Similarly, differentiation of esophageal neurons is severely impaired in *Insm1* mutant mice, as assessed by the absence Phox2a expression in the esophagus at E10.5 and E18.5; Phox2a is, however, expressed in enteric neurons located in more posterior parts of the enteric nervous system (H.W., M.S.G. and C.B., unpublished). The similarities in phenotypes observed in *Mash1* and *Insm1* mutant mice suggest that Insm1 mediates aspects of Mash1 functions in the differentiation of catecholaminergic neurons. Mash1 controls generic and subtype-specific aspects of neuronal differentiation of catecholaminergic neurons. Expression of pan-neuronal markers is only mildly affected in Insm1 mutant mice, indicating that Insm1 exerts its role primarily in the control of subtype-specific neuronal differentiation.

In addition, our experiments revealed an upregulated expression of Mash1 and its direct target gene delta-like 1 (*Dll1*) in sympatho-adrenal precursor cells of *Insm1* mutant mice. During normal differentiation, Mash1 is expressed transiently in sympatho-adrenal precursor cells. The de-repression of *Mash1* might interfere with differentiation of sympatho-adrenal precursors of *Insm1* mutant mice. It should be noted that we did not observe upregulated expression of other Notch target genes such as *Hes1*, *Hes5*, *Hes1r* and *Nrarp*, indicating that upregulated Notch signaling is not responsible for the delayed differentiation. It has previously been noted that the *Mash1* promoter is de-repressed in *Mash1* mutant mice, but Mash1 does not directly mediate this negative regulation (Meredith and Johnson, 2000). De-repression of *Mash1* was also observed in chromaffin cells of *Gata3* mutant mice (Moriguchi et al., 2006), indicating that the Zn-finger factors Gata3 and Insm1 participate in the regulatory feedback loop that controls *Mash1*.

Mash1 and Phox2b are the first transcription factors that appear upon initiation of differentiation of sympatho-adrenal precursors (Tsarovina et al., 2004). Hand2, Phox2a and Gata3 act genetically downstream of Mash1 and Phox2b, but mis-expression of Hand2 and Phox2a induces Mash1 and Phox2b, and Gata3 is required to maintain correct Phox2b expression (Howard et al., 1999; Howard et al., 2000; Lucas et al., 2006; Moriguchi et al., 2006; Stanke et al., 1999; Stanke et al., 2004; Tsarovina et al., 2004). These transcription factors seem thus to collaborate during specification of the sympatho-adrenal lineage, and despite their sequential appearance during development, they form a regulatory network rather than a linear cascade. We report here changes in the expression of several of these transcription factors in *Insm1* mutant mice, which demonstrates that Insm1 is an essential component of the transcriptional network that controls differentiation of sympatho-adrenal precursor cells.

Timing of differentiation and execution of the differentiation program

Sympatho-adrenal precursors migrate in order to form secondary sympathetic ganglia, as well as adrenal and extra-adrenal chromaffin cells (Huber, 2006; Unsicker et al., 2005). Mature sympathetic neurons and chromaffin cells share characteristics, like the expression of Th and Dbh, but they also display distinct features. Sympathetic neurons extend axons and maintain typical neuronal markers such as neurofilament 68 (Nefl), whereas neuronal markers are downregulated in chromaffin cells. The presence of secretory granules and the expression of Pnmt are typical for chromaffin cells and are further properties that distinguish the two cell types. We report here a delayed differentiation of sympatho-adrenal precursor cells in *Insm1* mutant mice. Remaining precursor cells of *Insm1* mutant mice eventually escape the block, and undergo sympatho-adrenal differentiation. During development of sympatho-adrenal precursor cells, Insm1 is thus crucial for the correct timing of differentiation.

Sympatho-adrenal precursors of *Insm1* mutant mice subsequently form sympathetic neurons, albeit at reduced numbers. In marked contrast, the further development of chromaffin cells was significantly altered, and enzymes of catecholamine biosynthesis (Th, Dbh, Pnmt) and components of secretory granules (chromogranin A/B) were markedly downregulated, whereas neurofilament (NF68) expression was increased. This was accompanied by an altered morphology of the adrenal medulla, and by increased apoptosis of chromaffin cells. Insm1 is therefore required for the correct execution of the differentiation program of chromaffin cells. Upregulated expression of Mash1 and neurofilament 68 indicate that chromaffin cells retain the character of immature sympatho-adrenal precursors in *Insm1* mutant mice. This represents a further similarity in the phenotypes of *Mash1* and *Insm1* mutant mice [compare Huber et al. (Huber et al., 2002) with this study]. It has previously been proposed that two distinct types of sympatho-adrenal precursors exist in the primary ganglion chain, one population destined to form sympathetic neurons and a second destined to form chromaffin cells (Huber et al., 2002). Such a hypothesis is supported by the fact that the number of sympatho-adrenal cells in the primary ganglion chain is markedly reduced in *Insm1* mutant mice, which affects the numbers of sympathetic neurons but not the numbers of chromaffin precursors that arrive in the adrenal gland.

Insm1 is also essential for terminal differentiation of endocrine cells of the pancreas and intestine (Gierl et al., 2006; Mellitzer et al., 2006). Genetic evidence indicates that Insm1 expression depends on two bHLH transcription factors Ngn3 and Mash1 in the pancreas and sympatho-adrenal cells, respectively, indicating that similar molecular mechanisms function upstream of Insm1 in these distinct organs. Chromaffin cells and endocrine cells of the pancreas and intestine produce different hormones, but they share endocrine characteristics such as the expression of granins. Chromogranin A/B are two genes whose expression is markedly downregulated in chromaffin cells, as well as in endocrine cells of the pancreas and intestine in *Insm1* mutant mice. The identification of direct target genes will reveal how Insm1 participates in the execution of a gene expression program that controls endocrine features. During adrenergic differentiation of neurons, Insm1 appears to mediate

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aspects of Mash1 functions in subtype-specific differentiation. Alternatively, Insm1 and Mash1 might co-operate to control expression of subtype-specific genes in developing peripheral neurons.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/3/473/DC1

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