The function of the zinc finger transcription factor Insm1 in neuronal progenitors

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Madeleine Julie Larrosa
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1st Reviewer: Prof. Dr. Holger Gerhardt

2nd Reviewer: Prof. Dr. Stephan Sigrist

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This doctoral thesis is dedicated to my mother Juliette Ban, who offered unconditional love and endless devotion, and taught me to work hard for the things I aspire to achieve.

I also dedicate this work to Mohammed Marine, who has been a constant source of support and encouragement, and without whom I would not have completed this doctoral thesis.

In loving memory of my friend Lorelei Arbeille, who taught me to never give up through her incredible strength and perseverance.

Statement of contribution

I confirm that the work presented in this doctoral thesis is my own and that all information derived from other sources is indicated.

Madlen Sohn, technician in the group of Prof. Dr. Wei Chen at the MDC, performed the sequencing of the immunoprecipitated chromatin and the transcriptome for the ChIP-seq and RNA-seq experiments, respectively. Dr. Mahmoud Ibrahim and Dr. Scott Lacadie, bioinformaticians in the group of Prof. Dr. Uwe Ohler at the MDC, analyzed the ChIP-seq data and Dr. Scott Lacadie analyzed the RNA-seq data.
In the group of Prof. Dr. Carmen Birchmeier at the MDC, Xun Li generated the *Insm1* mutant P19 cell lines and Dr. Kira Balueva performed *in situ* hybridizations on the mouse subventricular zone during her doctoral studies.

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1. Introduction

The mature central nervous system is composed of a vast diversity of neurons that vary in anatomical, chemical and electrophysiological properties. These neurons are organized in functional circuits that control various functions from elementary reflexes (i.e. spinal cord circuits) to high cognitive brain function such as thinking or memory (i.e. forebrain circuits). Studies in the last century have shown that both neuron diversity and circuit formation depend on a set of developmental processes defined as neurogenesis. There exist a number of common rules that occur in diverse parts of the central nervous system.

First, neuroectodermal pluripotent cells differentiate from the neural plate and become the embryonic neural stem cells, also known as radial glial cells, which symmetrically divide in order to increase the progenitor pool. Second, radial glial cells asymmetrically divide to generate more committed cell types, including specialized neuronal progenitors, in various combinations according to developmental stages and/ or central nervous regions in which they reside (see below). Third, neuronal progenitors express genes that initiate and drive neuronal differentiation. Fourth, cells undertake an irreversible decision to leave the cell cycle and fully acquire the neuronal identity. Last, neurons mature and acquire a particular phenotype by activating specific transcriptional programs that lead to formation of functioning neurons. Neurogenesis is not restricted to embryonic development, as it continues in postnatal life at well-characterized and restricted brain areas in various species including the human.

In the following sections of my thesis introduction, I will describe the neurogenic stages mentioned above with a particular emphasis on the function of transcription factors in such processes. In the last part of my introduction, I will focus on a repressive transcription factor named Insm1, which has been shown to participate in neurogenesis but whose particular function has not yet been studied.

1.1 Neurogenesis and neuronal differentiation

1.1.1 Embryonic neurogenesis

Upon the formation of the three germinal embryonic layers, the ectoderm differentiates into the epidermis and the neural plate. This process occurs by embryonic day E7.5 in mouse. By embryonic day E8.5 in mouse, the neural plate invaginates to form the neural tube from which the whole central nervous system emerges. The neural tube is composed of a single layer of pluripotent neuroepithelial cells that symmetrically divide to increase their number and serve as the earliest neural stem cells (Alvarez-Buylla *et al*., 2001). These symmetric divisions are directed by the organization of centrosomes that run perpendicular to the apical surface. Neuroepithelial cells are characterized for undergoing interkinetic nuclear migration between the basal and the apical surface of the neural tube, as the S-phase and M-phases of the cell cycle occur in the basal and apical surfaces, respectively (Haubensak *et al*., 2004; Miyata *et al*., 2004; Noctor *et al*., 2004).

Because the cell cycle is not synchronized among neuroepithelial cells, the overall appearance of this epithelial layer is pseudostratified. By embryonic day E10 in mouse, the thickness of the neural tube increases and neuroepithelial cells modify their morphology by elongating apical and basal processes, which attach their somas to both surfaces of the neural tube. In addition, these cells initiate the expression of certain glial proteins, such as Nestin, Vimentin or Glial Fibrillary Acidic Protein (GFAP), and are known as radial glial cells. Similar to neuroepithelial cells, radial glial cells symmetrically divide to increase their number. They also possess the capacity to self-renew through their symmetric divisions. A subset of radial glial cells starts asymmetric divisions, a consequence of a change in the positioning of the poles of the mitotic spindle and the division plane.

These asymmetric divisions result in the generation of two distinct cell types: i) one retains the self-renewal capacity and ii) the other differentiates into a more committed fate. The committed fates that differentiated cells can adopt are either the terminal differentiation of a neuron or the commitment to become an intermediate progenitor (Malatesta *et al*., 2003; Götz and Huttner, 2005).

The generation of neurons and intermediate progenitors laminates the neural tube into three zones: i) a ventricular zone located in the apical surface of the neural tube which contains radial glial cells; ii) a subventricular zone, located at the basal region of the ventricular zone, which contains the intermediate progenitors also known as basal progenitors; and iii) a marginal zone, basal to the subventricular zone, which contains terminal differentiated neurons (Haubensak *et al*., 2004; Miyata *et al*., 2004; Noctor *et al*., 2004).

1.1.2 Postnatal neurogenesis

Neurogenesis in postnatal life is restricted to two regions of the brain: the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone of the lateral ventricles (Kempermann *et al*., 1997). Neural stem cells are present in these two regions (Doetsch, 2003; Kriegstein and Alvarez-Buylla, 2009). These adult neural stem cells are mostly quiescent/ dormant and only occasionally divide to produce new neurons, which integrate into the pre-existing neural circuits (Seri *et al*., 2001; Lagace *et al*. 2007; Imayoshi *et al*., 2008; Bonaguidi *et al*., 2011; Encinas *et al.*, 2011; Pilz *et al.*, 2018).

The subgranular zone of the mouse hippocampus generates neuroblasts that migrate into the granular cell layer. The subventricular zone of the mouse brain gives rise to neuroblasts that migrate along the rostral migratory stream and differentiate into interneurons of the olfactory bulb (Lois and Alvarez-Buylla, 1993; Luskin, 1993), reviewed in (Alvarez-Buylla and Garcia-Verdugo, 2002; Lledo *et al*., 2006).

1.1.3 The adult subventricular zone

The subventricular zone is the largest neurogenic zone of the adult mammalian brain. It lies along the lateral walls of the lateral ventricles (Doetsch and Alvarez-Buylla, 1996) and corresponds to an important reservoir of progenitor cells in the adult brain.

Radial glial cells produce neural stem cells and ependymal cells in the postnatal subventricular zone (Merkle *et al*., 2004; Spassky *et al*., 2005; Ventura and Goldman, 2007; Merkle *et al*., 2007). Postnatally, the subventricular zone becomes thicker and its size decreases later in postnatal life (Marshall and Goldman, 2002). The cellular architecture of the subventricular zone is described below.

1.1.3.1 Ependymal cells

Ependymal cells form an epithelial layer that separates the lateral ventricles and the adult subventricular zone where neural stem cells reside (Spassky *et al*., 2005). There are two types of ependymal cells in the adult brain: i) multi-ciliated cells and ii) cells possessing only two cilia and a complex basal body. Ependymal cells form “pinwheel structures” around stem cells (Mirzadeh *et al*., 2008).

1.1.3.2 Neural stem cells

Neural stem cells generate transit amplifying progenitors that give rise to glial and neuronal lineages. Several thousand neural stem cells are distributed along the lateral ventricle walls (Mirzadeh *et al.,* 2008; Ponti *et al.*, 2013).
Neural stem cells have many characteristics of brain astrocytes and express astroglial proteins, such as Glial Fibrillary Acidic Protein (GFAP) and Nestin (Doetsch, 1999; Liu *et al*., 2006; Colak *et al*., 2008; Nomura *et al*., 2010). Quiescent and activated stem cells express Tlx (Shi *et al*., 2004; Li *et al*., 2012) and activated stem cells express Ascl1 (Kim *et a*l., 2011) and EGF receptor (Pastrana *et al*., 2009). However, none of those proteins are expressed exclusively by stem cells, making them challenging to identify *in vivo*.

There are two types of neural stem cells, i) B1 neural stem cells, adjacent to ependymal cells, that extend an apical process between ependymal cells to directly contact the ventricle, and ii) B2 neural stem cells, that are multipolar and dispersed in the subventricular zone, but do not contact the ventricle (Doetsch *et al*., 1997; Mirzadeh *et al*., 2008). Type B1 cells can be either quiescent or activated stem cells (Codega *et al*., 2014), which proliferate and give rise to transit amplifying progenitors. These cells can also give rise to glial lineages, including oligodendrocytes and nonneurogenic astrocytes (Menn *et al*., 2006).

1.3.3.3 Transit amplifying progenitors

Transit amplifying progenitors are generated by neural stem cells and proliferate extensively (Shen *et al*., 2008). They express Ki67, Sox2 (Ferri *et al*., 2004) and proneural transcription factors like Ascl1 (Kohwi *et al*., 2005; Kim *et al*., 2007; Brill *et al*., 2008; Kim *et al*., 2011). Ascl1 depleted postnatal mice present impaired subventricular zone neurogenesis and oligodendrogliogenesis. Ascl1 is therefore essential for the specification of both neuronal and glial lineages (Parras *et al*., 2004).

Transit amplifying progenitors give rise to neuroblasts (Doetsch *et al*., 1997). The expression of doublecortin (DCX) distinguishes transit amplifying progenitor cells from neuroblasts (Doetsch *et al.,* 1997). *In vivo* analysis of cell proliferation by mitotic marker incorporation indicates that transit amplifying progenitor cells divide symmetrically approximately three times before giving rise to neuroblasts (Ponti *et al.,* 2013).

1.1.3.4 Neuroblasts

Early neuroblasts form chains that keep proliferating during migration (Menezes *et al*., 1995; Doetsch and Alvarez-Buylla, 1996; Jankovski and Sotelo, 1996; Lois *et al*., 1996; Peretto *et al*., 1997; Wichterle *et al*., 1997). In fact, neuroblatsts divide at least twice while migrating to the olfactory bulb (Ponti *et al*., 2013).

Neuroblasts migrate along a path called the rostral migratory stream (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Petreanu and Alvarez-Buylla, 2002; Carleton *et al*., 2003). They are committed to specific neuronal fate before reaching the olfactory bulb (Hack *et al*., 2005; Kohwi *et al*., 2005).

Neuroblasts are surrounded by neural stem cells and transit amplifying progenitors (Lois *et al*., 1996; Doetsch *et al*., 1997) and express proteins such as Tubb3 and Doublecortin (DCX).

1.1.3.5 Neurons

Progenitor cells of the subventricular zone continuously give rise to new neurons that migrate to the olfactory bulb (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994).
In the olfactory bulb, newly generated neurons differentiate into multiple neuronal types operating in the olfactory bulb circuits (Luskin, 1993; Brill *et al.*, 2009). These neurons are derived from neural stem cells, according to their position in the subventricular zone (Merkle *et al*., 2014).

In embryonic life, the subventricular zone produces the principal projection neurons (mitral and tufted cells) of the olfactory bulb as well as glutamatergic periglomerular cells (Hinds, 1968). In postnatal life, most GABAergic interneurons of the olfactory bulb are generated (Altman and Das, 1966; Petreanu and Alvarez-Buylla, 2002). They arise from the subventricular zone and migrate along the rostral migratory stream into the deep granular cell layer (Luskin, 1993; Lemasson *et al*., 2005). Granule cells from the adult subventricular zone reach the olfactory bulb where they mature (Lois and Alvarez-Buylla, 1994; Petreanu and Alvarez-Buylla, 2002) and make synaptic connections with projection neurons in the olfactory bulb (Greer, 1987; Imamura *et al*., 2006).

The adult subventricular zone also gives rise to periglomerular neurons of the olfactory bulb (Luskin, 1993; Petreanu and Alvarez-Buylla, 2002). Those include GABAergic neurons expressing tyrosine hydroxylase (TH) (Kosaka *et al*., 1995), calretinin or calbindin (Kosaka *et al*., 1998). All of these subtypes are generated during adult neurogenesis (Whitman and Greer, 2007) from specific locations in the subventricular zone and from the rostral migratory stream (Hack *et al*., 2005; Ventura and Goldman, 2007; Brill *et al*., 2009; Ihrie *et al*., 2011). GABAergic periglomerular cells are local circuit interneurons that synapse on olfactory sensory neurons and on projection neurons of the olfactory bulb and regulate the function of projection neurons (Kasowski *et al*., 1999; De Saint Jan and Westbrook, 2005).

1.1.3.6 Oligodendrocytes and astrocytes

Most oligodendrocytes are generated during embryogenesis and in the early postnatal period. In adult life, neural stem cells generate some oligodendrocytes (Jackson *et al*., 2006; Menn *et al*., 2006), which migrate radially from the subventricular zone to the corpus callosum and fiber tracks of the striatum. The adult born oligodendrocytes do not migrate to the cortex or olfactory bulb (Menn *et al*., 2006).

Astrocytes that are generated early postnatally in the subventricular zone migrate radially into the white matter, striatum and cortex but do not join the rostral migratory stream (Marshall *et al*., 2005). In contrast, the adult subventricular zone generates no migrating astrocytes under normal conditions.

1.1.3.7 Microglia

Recent studies have shown that microglial cells are also an essential component of the subventricular zone, where they contact with neural stem cells, neuroblasts and transit amplifying progenitor cells (Gonzalez-Perez *et al.,* 2012: Solano Fonseca *et al.,* 2016).
Neural progenitor cells and microglia communicate through different junctions and this communication is important for maintaining a precise control of neurogenesis (Matarredona *et al*., 2018).

1.2 Ascl1, a proneural bHLH transcription factor

Proneural genes encode basic helix-loop-helix (bHLH) transcription factors whose function is essential for neurogenesis. They can specifically bind DNA sequences containing an E-box motif (Bertrand *et al*., 2002). Proneural genes were identified in *Drosophila* and include the achaete-scute complex (achaete, scute, lethal of scute and asense) and atonal related genes (atonal, amos, and cato) (Bertrand *et al*., 2002).

Different types of proneural genes have been described in Vertebrates and are expressed in different regions of the developing nervous system, such as *Achaete-scute homolog1 (Ascl1)* (Guillemot and Joyner, 1993). bHLH transcription factors like *Neurogenin* and *NeuroD* are homologues of the atonal gene in *Drosophila* (Bertrand *et al*., 2002). During neural development, these genes are differentially expressed in distinct neuronal progenitor cells. For example, *Neurogenin2* is expressed in the dorsal telencephalon, which generates glutamatergic neurons (Fode *et al*., 1998), while *Ascl1* is predominantly expressed in the ventral telencephalon, which produces GABAergic neurons (Letinic *et al*., 2002). Therefore, distinct proneural genes are involved in the generation of specific neuronal types (Wilson and Rubenstein, 2000).

Proneural transcription factors are both required and sufficient to induce neuronal differentiation (Bertrand *et al*., 2002; Wilkinson *et al*., 2013; Vasconcelos and Castro, 2014). Consequently, the genetic ablation of proneural genes in mice results in neural developmental defects, which are often associated to impaired neurogenesis, whereas the overexpression of proneural genes induces the neuronal differentiation program (Guillemot *et al*., 1993; Casarosa *et a*l., 1999; Berninger *et al*., 2007; Geoffroy *et al*., 2009).

In addition to its crucial role in neural development, Ascl1 has been used in protocols to reprogram somatic cells, including fibroblasts and astrocytes, into neurons (Berninger *et al*., 2007; Vierbuchen *et al*., 2010; Karow *et al*., 2012; Chanda *et al*., 2014). Ascl1 plays a critical role in the activation of quiescent neural stem cells and generation of neuroblasts in the mouse brain. It is expressed at low levels in subsets of activated neural stem cells and at high levels in transit amplifying cells (Pastrana *et al*. 2009; Kim *et al*. 2011; Andersen *et al*. 2014).

Previous work has shown that Ascl1 regulates and is regulated by the Notch signaling pathway (Guillemot *et al.*, 1993; Bertrand *et al.*, 2002). In fact, Ascl1 induces the transcription of the Notch ligand *Dll1* (Castro *et al.*, 2006) in neuronal progenitor cells. This results in the repression of Notch signaling activity in differentiating progenitors, which is needed for the induction of neuronal differentiation (described below).

1.3 The function of the Notch signaling pathway in the maintenance of neuronal progenitor cells

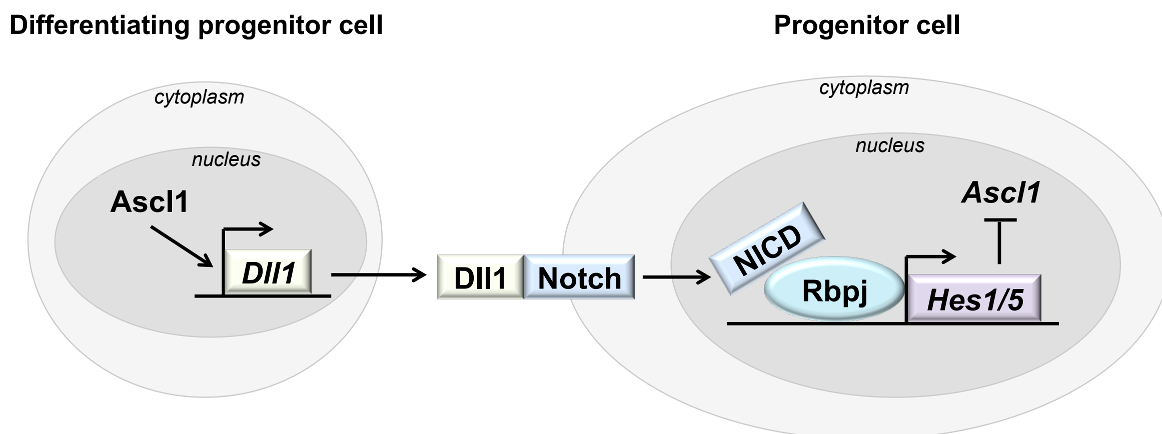
Notch signaling is a key mediator of neural stem cell maintenance in the embryonic and postnatal brain (Ables *et al.*, 2011; Pierfelice *et al.*, 2011). The maintenance of neural stem cells and neuronal progenitor cells in embryonic and postnatal life requires the activation of Notch receptors by their ligands Delta and Jagged (Chambers *et al.*, 2001; Shimojo *et al.*, 2008). Inhibition of Notch or Rbpj as well as depletion of Hes1 in adult neural stem cells results in their loss and impaired neurogenesis (Ables *et al*. 2010; Ehm *et al*. 2010; Imayoshi *et al*. 2010; Andersen *et al*. 2014).

Notch target genes of the Hes family maintain neural stem cells undifferentiated by suppressing proneural genes like *Ascl1* (Louvi and Artavanis-Tsakonas, 2006). *Hes1* and *Hes5* are expressed in neural stem cells (Aguirre *et al.*, 2010; Imayoshi *et al.*, 2010).
In the adult subventricular zone, *Notch1* is expressed in transit amplifying progenitors and in early neuroblasts (Givogri *et al.*, 2006; Basak *et al.*, 2012). Notch3 is mostly found in stem cells, whereas Notch2 is present in both cell types. The two canonical Notch ligands Dll1 and Jagged1 were found expressed in the adult subventricular zone (Stump *et al.*, 2002; Nyfeler *et al.*, 2005; Givogri *et al.*, 2006; Aguirre *et al.*, 2010).

In order to prevent differentiation of all neuronal progenitors at the same time, the activity of bHLH transcription factors is tightly controlled. Lateral inhibition is crucial to regulate the differential expression of bHLH factors (Henrique *et al.*, 1995). In fact, the activity of bHLH factors induces the expression of the transmembrane ligand Delta, which is able to bind to Notch receptors expressed in neighbouring cells. Interaction between Notch receptor and Delta ligand leads to the intracellular cleavage of Notch by gamma-secretase into NICD, Notch intracellular domain, the active form of Notch receptor.

NICD is then translocated into the nucleus, where it forms a complex with the DNA-binding transcriptional regulator Rbpj and further coactivators (Honjo, 1996; Ohtsuka *et al*., 1999; Selkoe and Kopan, 2003).

Direct targets of the NICD/Rbpj complex include the transcriptional effectors Hes1 and Hes5, known repressors of proneural genes like *Ascl1* (Louvi and Artavanis-Tsakonas, 2006; Kageyama *et al*., 2008). Consequently, *Notch* expressing cells remain neuronal progenitors whereas *Delta* expressing cells differentiate into neurons (Henrique *et al*., 1995).



Schematic view of Notch signaling lateral inhibition. In the differentiating progenitor cell, Ascl1 drives the expression of *Dll1* ligand. In the neighboring cell, Dll1 induces cleavage of the transmembrane Notch receptor into NICD. NICD replaces transcriptional corepressors with activators such as RBPJ to enable the transcription of *Hes* genes. Hes effectors will inhibit *Ascl1* expression, driving this cell to remain a progenitor.

1.4 The zinc-finger transcription factor Insm1

1.4.1 Structural features

The Insulinoma-associated 1 *Insm1* (previously known as *IA-1*) is an intronless gene located on mouse chromosome 2 and on human chromosome 20. It was first cloned from an insulinoma-glucagonoma library (Goto *et al*., 1992). It encodes a protein of 510 amino acids that contains an amino-terminal sequence (amino acid 1-250) composed of a great percentage of proline, glycine and alanine residues. The carboxyl-terminal sequence (amino acid 251-510) contains the DNA-binding domain, which is characterized by five Cys2-His2 type zinc finger motifs. These motifs span a conserved length of 45/46 amino acids (De Caestecker *et al*., 2000; Zilfou *et al*., 2001). Cys2His2 (C2H2) type zinc-fingers are DNA-binding motifs that can also function as protein-protein and RNA-protein interaction domains (Klug and Schwabe, 1995; Brayer and Segal, 2008).

The Insm1 protein contains a nuclear localization signal right before the third zinc finger and is localized to the nucleus (Xie *et al*., 2002).
Sequence comparisons identified homologs of Insm1 in Vertebrates and Invertebrates: EGL-46 in *C. elegans*, insm1a and insm1b in *Danio rerio,* Nerfin1/2 in *Drosophila melanogaster* and insm1 in *Xenopus laevis*.

The Vertebrate Insm1 protein possesses a SNAG domain (Grimes *et al*., 1996; Parlier *et al*., 2008). The SNAG domain is known to bind to co-repressors that modify chromatin structure such as Lsd1, Hdac1/2 and Rcor1-3 (Saleque *et al*., 2007; Lin *et al*., 2010; Laurent *et al*., 2012; Welcker *et al*., 2013). The presence of a SNAG domain in Insm1 supports its transcriptional repressor function.

1.4.2 Panneurogenic expression of *Insm1*

The transcription factor Insm1 has a panneurogenic expression in the embryonic and adult nervous system. In fact, Insm1 is expressed transiently in neuronally committed progenitor cells and in early neurons (Farkas *et al*., 2008) and its expression in correlation with neurogenesis is maintained in postnatal and adult life (Alvarez-Buylla and Garcia-Verdugo, 2002; Ninkovic and Götz, 2007).

Moreover, the panneurogenic expression of *Insm1* is conserved in evolution. In fact, Insm1 ortholog *Nerfin-1* in *Drosophila* is expressed in neurogenic delaminating neuroblasts and ganglion mother cells, but not in postmitotic neurons (Kuzin *et al*., 2005).

In neurogenic regions, Insm1 is often co-expressed with proneural factors such as Ascl1 and Ngn2, which induce *Insm1* expression (Castro *et al*., 2006; Jacob *et al*., 2009).
Interestingly, *Insm1* mutants and *Ascl1* mutant embryos show similar proneural defects, which suggests that Insm1 operates in the same cascade as Ascl1 in neuronal differentiation (Parras *et al*., 2004; Pattyn *et al*., 2006; Farkas *et al*., 2008; Wildner *et al*., 2008).

1.4.3 Function of Insm1 in endocrine and neuronal lineages

Endocrine cell types in the pancreas, intestine, pituitary and adrenal medulla express *Insm1*, which plays an essential role for their development (Gierl *et al*., 2006; Wildner *et al*., 2008; Welcker *et al*., 2013; Osipovich *et al*., 2014; Jia *et al*., 2015). In the sympathetic nervous system, differentiation of several neuronal subtypes is impaired in *Insm1* mutants (Wildner *et al*., 2008; Jacob *et al*., 2009).

In the nervous system, analysis of *Insm1* mutant mice indicates that Insm1 is important for the transition from transit amplifying progenitors to differentiated neurons (Farkas *et al.*, 2008; Masserdotti *et al.*, 2015). In postnatal life, it was shown that Insm1 regulates postnatal neurogenesis and controls the exit of transit amplifying progenitors stage (Balueva, 2013). Nevertheless, little is known on how Insm1 regulates this transition and the neurogenic transcriptional program directed by Insm1 has not been characterized.

Insm1 binds to several proteins that regulate chromatin structure in nervous system as well as neuroendocrine cells. The SNAG domain of Insm1 is essential to recruit the chromatin-modifying enzymes Lsd1, Hdac1/2 and the corepressors Rcor1-3 (Welcker *et al*., 2013; Monaghan *et al.*, 2017). The multiprotein complexes Rcor, Lsd1 and Hdac repress transcription by removing histone modifications associated with transcriptional activation. Therefore, Insm1 acts as a repressor to allow differentiation via epigenetic regulation of gene expression.

1.5 Aim of the study

The Insulinoma-associated 1 Insm1 zinc finger transcription factor is highly expressed in the embryonic nervous system and its expression is maintained in the neurogenic areas of the adult brain. Insm1 is a direct target of Ascl1 and has been shown to be important for neuronal differentiation (Wildner *et al.*, 2008; Farkas *et al.*, 2008; Masserdotti *et al.*, 2015; Monaghan *et al.*, 2017).

However, the understanding of Insm1 molecular network has remained limited. In this study, I have sought to undertake a genomic approach to decipher Insm1 molecular mechanisms that control the progression of neuronal progenitor cells into neurons.

To this end, I generated datasets of Insm1 expression profiling and its genomic binding profile to unravel the complex molecular program regulated by Insm1 for the correct establishment of neuronal differentiation through both genetic and epigenetic mechanisms.

2. Material and Methods

2.1 Abbreviations

4% PFA: 4% paraformaldehyde

Bp: base pair

BSA: bovine serum albumin

cDNA: complementary DNA

DAPI: 4'-6-Diamin-2-2phenylindol

DMSO: dimethylsulfoxide

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide acid

E: embryonic day

EDTA: ethylene diamine tetraacetic acid

EGTA: ethylene glycol tetraacetic acid

F: forward primer

FACS: Fluorescent Activated Cell Sorter

GFP: green fluorescent protein

IF: immunofluorescence

Ig: immunoglobulin

IP: immunoprecipitation

Kb: kilobase

mRNA: messenger ribonucleic acid

Neo: Neomycin resistance gene

PBS: phosphate buffered saline

PBST: PBS containing 0.1% Triton X-100

PCR: polymerase chain reaction

PCR: polymerase chain reaction

PFA: paraformaldehyde

QPCR: quantitative real-time polymerase chain reaction

R: reverse primer

RNA: ribonucleic acid

RNase: ribonuclease

Rpm: revolutions per minute

RT-PCR: reverse transcription polymerase chain reaction

RT: room temperature

SDS: sodium dodecyl sulfate

SVZ: subventricular zone

Tris: 2-Amino-2-(hydroxymethyl)-propan-1,3-diol

Tween-20: polyoxyethylene (20) sorbitan monolaurate

V: voltage

2.2 Material

2.2.1 Chemicals, kits and enzymes

Chemicals and kits for molecular biology were purchased from the following companies: Clontech (Heidelberg, Germany), Gibco BRL (Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), Molecular Probes (Eugene, USA), Qiagen Sciences (Maryland, USA), Roth (Karlsruhe, Germany), Roche (Mannheim, Germany), Sigma (Steinheim, Germany). Enzymes were obtained from New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were produced by Eurofins genomics (Ebersberg, Germany) unless otherwise stated.

2.2.2 DNA-oligonucleotides

Oligonucleotides were synthetized by Eurofins Genomics (Ebersberg).

2.2.2.1 Primers for genotyping

Gene	Condition	Sequence (5’ 3’)	Product size
Insm1^{Flox}	(F)	AGGGCGATCGAAAACGTGCTAT	Mutant 567 bp /
Insm1^{Flox}	(R)	AATGGCCCCAAATATGTCAGGAG	Wild type 364 bp
Brn4^{Cre}	Mutant (F)	GGTTCCCCCGCACCATAGATGT	Mutant 412 bp /
Brn4^{Cre}	Mutant (R)	AGCCCGGACCGACGATGAA	Wild type -

2.2.2.2 Primers for quantitative real-time polymerase chain reaction (qPCR)

Gene	Primer	Sequence (5’ 3’)
Ascl1	(F)	TCTCCGGTCTCGTCCTACTCCT
Ascl1	(R)	ATCCTGCTTCCAAAGTCCATTCCC
Dll1	(F)	GATACACACAGCAAACGTGACACC
Dll1	(R)	TTCCATCTTACACCTCAGTCGCTA
Gapdh	(F)	AGGTCGGTGTGAACGGATTTG
Gapdh	(R)	TGTAGACCATGTAGTTGAGGTCA
Hes1	(F)	GAAGCGCCGACGAGACCGAATCAA
Hes1	(R)	CAGGGCGTGCGCGTCAAAATAACC
Hes5	(F)	GCTCCGCTCGCTAATCGCCTCCAG
Hes5	(R)	GTCCCGACGCATCTTCTCCACCAC
Insm1	(F)	TCTTCTCGCTGTGACTCC
Insm1	(R)	TGAACACAGCGAATACTAGCAA
Notch1	(F)	TCAATGCCGTGGATGACCTAG
Notch1	(R)	GAAGAACGGAGCAACAAGG
Tbp	(F)	CCCCACAACTCTTCCATTCT
Tbp	(R)	GCAGGAGTGATAGGGGTCAT
Tubb3	(F)	ATTCCCTGGTCAGCTCAATG
Tubb3	(R)	TTCTTGGCATCGAACATCTG

2.2.2.3 Primers for chromatin immunoprecipitation quantitative real-time polymerase chain reaction (ChIP-qPCR)

Primers were designed 3 kb upstream and downstream of the identified peaks. Negative control (NC) primers were designed 10 kb either upstream or downstream of the identified peaks.

Gene	Primer	Sequence (5’ 3’)
Ascl1	(F)	AGCGTCTCCACCTTGCTC
Ascl1	(R)	GCTCAACTTCAGCGGCTT
Ascl1 NC	(F)	ATTAAATGTCTGAGGTGCTT
Ascl1 NC	(R)	CAAAACTCTTCTCTTGCTCT
Dll1 M	(F)	GTGTCCGGTCCCACT
Dll1 M	(R)	TCGCACAATAACAGACTGCC
Dll1 N	(F)	TGTATTGGGGAAATCAAGCA
Dll1 N	(R)	CAGGCTCCTATAGAATCACT
Dll1 NC	(F)	CAATAAAGAATGCACTCACC
Dll1 NC	(R)	CCATCTTAGAACAATGCTCA
Insm1	(F)	GGCTTCACCCTTGTTCGAT
Insm1	(R)	CTTTGTTCGGCACATTAGCAT
Insm1 NC	(F)	AGGTTCTTAAACTCTACCCC
Insm1 NC	(R)	GTAGCCTGATTTTACCAAGGA
Hes5	(F)	CGGGGCTCTCAGCATCA
Hes5	(R)	CGCACGCTAAATTGCCT
Hes5 NC	(F)	GTGCCTTGGAGTCGAAT
Hes5 NC	(R)	AAGAGACTTGTCAAGGCAT
Notch1 A	(F)	ACAGCATTAATCGCCTCC
Notch1 A	(R)	AGACACTAATGAATGCACAC
Notch1 B	(F)	TGAATAAGAAGCACAGCGAGA
Notch1 B	(R)	CGCCTGATTTATTGGCCAT
Notch1 NC	(F)	ACATGGTATGCTTGGGTGGA
Notch1 NC	(R)	TGGACACTTCTTGGATCGCT

NC: negative control

2.2.3 Vectors

Name	Source
pCMV-Insm1	Insm1 mammalian expression vector
pCMV-Ascl1-IRES-GFP	Ascl1-GFP bicistronic expression vector
3XFLAGNICD1	Addgene
pCMV-Hes1	Hes1 mammalian expression vector
pCMV-Hes5	Hes5 mammalian expression vector

2.2.4 Antibodies

Antibody	Host	Source	Dilution
Ascl1	mouse IgG	BD Pharmingen	ChIP: 5 µg IF: 1:100
GFP	rat IgG	Nacalai Tesque	IF: 1:2000
Insm1	guinea pig IgG	Jochen Welcker, MDC	ChIP: 5 µg IF: 1:10000
NICD	rabbit IgG	Abcam	IF: 1:200
Tubb3	mouse IgG	Chemicon International	IF: 1:500
Tubb3	rabbit IgG	Abcam	IF: 5 µg/ml
H3K27ac	rabbit IgG	Diagenode	ChIP: 5 µg
K3K24me3	rabbit IgG	EMD Millipore	ChIP: 5 µg

Secondary antibodies coupled with Cy2, Cy3, Cy5 or DL649 and DL488 fluorescent compounds were purchased from Dianova (Hamburg) and dissolved in 50% glycerol to a final concentration of 0.5 mg/ml. The concentration used for secondary antibodies was of 1 µg/ml.

2.2.5 Cell lines

P19 cells were purchased from ATCC-LGC Standards (Wesel, Germany).

2.2.6 Mouse strains

Wildtype mice: C57Bl/6J-inbred and CD1-outbred mouse strains.

Insm1^{Flox}: (Dinko Blazevic, MDC): Insm1 conditional allele was obtained by introduction of two *loxP* sites that flanked the *Insm1* gene into the *Insm1* locus. Embryonic stem clones containing the *loxP*-flanked *Insm1* locus (*Insm1^{Flox}*) were selected to generate chimeric mice and the mutant mouse line was established through germ-line transmission. The vector contained a self-excision neomycin resistance cassette downstream of the first *loxP* site at the 5´ end of *Insm1* locus. The neomycin cassette was removed by crossing *Insm1 $^{Flox-neo/+}$* mice with *Flp* deleter mice.

Brn4^{Cre}: *Tg(Pou3f4-cre)32Cren*; or *bcre-32 (Bcre32 Tg(Pou3f4-cre)32Cren* (Kyung Ahn, University of Pennsylvania Medical School, USA): The *Brn4^{Cre}* transgenic construct was obtained using a 5.6 kb SalI-BamHI promoter region of the *Brn4* gene that was fused to *Cre* recombinase gene and *polyA* sequence (Ahn *et al*., 2001). *Bcre32 Tg(Pou3f4-cre)32Cren* transgene restrains *Cre* expression to progenitor cells of the central nervous system from E9 in mouse (Zechner *et al*., 2003).

2.3 Methods

Standard protocols from (Sambrook, 2001) were used for procedures such as DNA restriction, ligation, bacterial culture and other cloning techniques. Other procedures were carried out according to kit manufacturers' instructions. Specific techniques are described below.

2.3.1 Nucleic acids methods

2.3.1.1 Isolation of genomic DNA from mouse tissue for genotyping

To genotype postnatal mice, DNA was isolated from tail biopsies. Tissue was lysed at 55°C in 50 µl of lysis buffer (200 mM NaCl, 100 mM Tris-HCl; pH 8.5, 5 mM EDTA, 0.2% SDS) containing 1 mg/ml of proteinase K for 2 hours. Proteinase K was inactivated by incubation at 95°C for 10 min. 300 µl of H_2O was added to the samples and 2 µl of each sample were used for each PCR reaction. The PCR conditions for genotyping were the following: 1 cycle of 94ºC for 2 minutes, followed by 35 cycles as follows: 94ºC for 1 minute; 60ºC for 1 minute; 72ºC for 2 minutes; and a final extension at 72ºC for 5 minutes. PCR products were detected by 1-2% agarose gel electrophoresis.

2.3.1.2 RNA extraction and cDNA preparation from mouse subventricular zone and differentiated P19 cells

Total RNA was isolated from microdissected mouse subventricular zone or differentiated P19 cells using TRIzol (Ambion) and the RNeasy Cleanup kit (Qiagen), according to the manufacturers' instructions. Microdissected tissue or differentiated P19 cells pellets were homogenized in 400 µl of TRIzol and the volume was then adjusted with TRIzol to 1600 µl. Samples were incubated for 5 minutes at RT, before addition of 320 µl of chloroform. After 15 minutes of centrifugation at 10,000 g at 4°C (Eppendorf 5417R centrifuge), 800 µl of the upper phase were collected and transferred to new Eppendorf tubes. 660 µl of isopropanol were then added and the samples were incubated at RT for 10 minutes. After centrifugation for 10 minutes at 12,000 g at 4°C, RNA pellets were washed with 1.5 ml of 75% ethanol, centrifuged for 5 minutes at 12,000 g and air-dried for 10 minutes. Total RNA was dissolved

in 100 µl of RNAse-free H_2O. Total RNA was purified with RNeasy Cleanup kit 74204 (Qiagen) and eluted with 14 µl of H_2O. RNA concentration was measured with NanoDrop (Thermo Scientific). cDNA was generated with 1 µg of RNA using SuperScript^{TM} kit (Invitrogene) as described in the manufacturer's instructions.

2.3.1.3 Quantitative real-time PCR (qPCR) on mouse subventricular zone and differentiated P19 cells

Primers for qPCR were designed by Eurofins Genomics (see 2.2.2.2). QPCR reactions were performed using SYBR Green reagent (Sigma) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Scientific). PCR conditions were 94ºC for 2 minutes, followed by 40 three-step cycles of 94ºC for 15 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds. *Tbp* was used as an endogenous housekeeping gene. Each primer set amplified a single PCR product of predicted size as determined by melt-curve analysis following PCR and by agarose gel electrophoresis (see below), and had approximately equal amplification efficiencies when validated using a serial dilution of representative cDNA. Each qPCR was performed in duplicates and relative quantification was determined according to the delta-delta C(t) method (Livak and Schmittgen, 2001).

2.3.1.4 Agarose gel electrophoresis

1% or 2% agarose TAE gel was used in resolving DNA samples. 1% or 2% agarose was made up with 1x TAE buffer (0.4 M Tris, 0.2 M sodium acetate, 20 mM EDTA; pH 8.3) in an Erlenmeyer flask, boiled, and left to cool down before adding ethidium bromide (Sigma) to a final concentration of 0.2 µg/ml. The solution was poured in a gel tray containing a toothed comb, and left to solidify. DNA samples were mixed with 10x loading buffer prior to loading into wells. 1 kb or 100 bp DNA ladders (Gibco) were run for size comparison.

2.3.2 Chromatin immunoprecipitation

2.3.2.1 Chromatin collection and cross-linking

P19 cells were differentiated with retinoic acid (see 2.3.3.2) in 10 mm cell culture dishes. $1x10^6$ cells were used per reaction. The cells were harvested at day 5 of neuronal differentiation in a 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cells were washed with 10 ml of PBS and centrifuged at 1000 rpm for 5 minutes. PBS was discarded and 1% formaldehyde fixative solution was added to the cells. The cells were left on a rocking platform at 100 rpm for 10 minutes at RT. To stop the fixation, 1 ml of 1.25 M Glycine solution was added to the cells that were then centrifuged at 1000 rpm for 5 minutes.

2.3.2.2 Cell lysis and chromatin extraction

The fixative solution was removed and 6 ml of lysis buffer 1 (50 mM Hepes-KOH; pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X100, 1X protease inhibitor) were added. Cells rotated at 20 rpm for 20 minutes at 4ºC and were collected by centrifugation at 1000 rpm for 10 minutes. Supernatant was discarded. 6 ml of lysis buffer 2 (10 mM Tris-HCl; pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1X protease inhibitor) were added and cells rotated at 20 rpm RT for 10 minutes. Cells were collected by centrifugation at 1000 rpm for 10 minutes. Supernatant was discarded. 150 µl of lysis buffer 3 (10 mM Tris-HCl; pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% SDS, 1X protease inhibitor) were added. Cells were left on ice and pipetted up and down for 10 minutes in order to break the nuclei. 600 µl of buffer 4 (20 mM Tris-HCl; pH 8.0, 2 mM EDTA, 1X protease inhibitor) and 750 µl of buffer 5 (20 mM Tris-HCl; pH 8.0, 2 mM EDTA, 2% Triton X100, 300 mM NaCl, 1X protease inhibitor) were added to the cells.

2.3.2.3 Chromatin shearing

Cells were transferred to a new 15 ml falcon tube and sonicated at 4ºC in a Bioruptor® sonication device (Diagenode) at medium energy for 30 cycles of 30 seconds on/off each. After sonication, cells were centrifuged at 12000 rpm for 10 minutes at 4ºC. The supernatant

was transferred to a new Eppendorf tube. Input conditions were stored at -80ºC until decross-link step.

2.3.2.4 Preparation of magnetic beads

20 µl of magnetic Dynabeads were used per chromatin sample. The beads were transferred to a new Eppendorf tube and collected using a magnetic rack. 1 ml of 0.5% BSA blocking solution was then added to the beads and the supernatant was removed using a magnetic rack. The beads were then resuspended in 2.5 X volume of blocking solution. 5 µg of the antibody of interest were added to the beads which were left rotating at 20 rpm over night at 4°C.

2.3.2.5 Immunoprecipitation assay

2.3.2.5.1 Binding of sheared chromatin to Dynabeads

Magnetic beads bound to antibodies were collected using a magnetic rack, supernatant was discarded and beads were washed 3 times in 1 ml of blocking solution. 700 µl of sheared chromatin were added to the beads that had been previously split in the appropriate number of Eppendorf tubes. 100 µl of blocking solution were added to each tube. The tubes were left rotating at 20 rpm over night at 4°C.

2.3.2.5.2 Washes of sheared chromatin bound to Dynabeads

Beads were collected using a magnetic rack and washed the following way:

-1 ml of sonication buffer (20 mM Tris-HCl; pH 8.0, 2 mM EDTA, 1% Triton X100, 0.1% SDS, 150 mM NaCl, 1X protease inhibitor) at 20 rpm for 4 minutes at RT.

-1 ml of sonication buffer + 500 mM NaCl (20 mM Tris-HCl; pH 8.0, 2 mM EDTA, 1% Triton X100, 0.1% SDS, 150 mM NaCl, 1X protease inhibitor, 500 mM NaCl) at 20 rpm for 4 minutes at RT.

-1 ml 250 mM LiCl buffer (10 mM Tris-HCl; pH 8.0, 2 mM EDTA, 250 mM LiCl, 1% NP40, 1X protease inhibitor) at 20 rpm for 4 minutes at RT.

-1 ml of TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA; pH 8.0, 50 mM NaCl) at 20 rpm for 4 minutes at RT.

2.3.2.5.3 Elution and decross-link of chromatin

Dynabeads were collected on a magnetic rack and transferred to new Eppendorf tubes. 1 ml of TE buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA; pH 8.0, 50 mM NaCl) was added to each tube. The tubes were then centrifuged at 1000 rpm for 3 minutes at 4°C. Supernatant was discarded.
200 µl of elution buffer (50 mM Tris-HCl; pH 8.0, 10 mM EDTA; pH 8.0, 1% SDS) pre-warmed at 65°C were added to the tubes that then incubated in a thermoshaker at 800 rpm for 30 minutes at 65°C.
The supernatant was collected and transferred to a new Eppendorf tube for each sample. Input samples were thawed and their NaCl and SDS concentrations were adjusted to 110 mM and 1%, respectively. Beads incubated in a thermoshaker at 800 rpm at 65°C over night to decross-link the chromatin.

2.3.2.6 DNA purification and extraction

RNA was digested by adding 2.5 µl of 20 mg/ml RNAseA to the chromatin which incubated at 37°C for 1 hour. Chromatin was then treated with 5 µl of 20 mg/ml proteinase K at 55°C for 2 hours to remove all proteins.
DNA was extracted in phenol-chloroform and chloroform and precipitated with ethanol. The chromatin was resuspended in 20 µl of DNAse free water and used for the experiment of interest.

2.3.3 *In vitro* cell culture experiments

2.3.3.1 Cell culture of P19 cells

P19 cells are a cell line that comes from a murine embryo-derived teratocarcinoma, which was obtained after transplantation of a mouse embryo at E7.5 into the testis of an adult male mouse (Rossant and McBurney, 1982). They are pluripotent cells that can generate all three germ layers following treatment with certain reagents (McBurney *et al.*, 1982). For instance, treatment with retinoic acid induces neuronal differentiation (described below) (Jones-Villeneuve *et al.*, 1982; Monzo *et al.*, 2012). Cells were grown in a standard growth medium

(89% DMEM, 10% FBS and 1% Pen/Strep) and maintained at 37°C and 5% CO_2 in an incubator.

2.3.3.2 Neuronal differentiation of P19 cells

P19 cells were harvested using PBS and 0.05% trypsin. Subsequently, cells were resuspended in differentiation medium (94% DMEM, 5% FBS, 1% Pen/Strep and 1 µM retinoic acid). Cells were grown in bacteriological Petri dishes to allow cell aggregates to form. Aggregates were incubated for 4 days in differentiation medium. At day 4 of differentiation, aggregates were dissociated using 0.05% trypsin and 50 mM of DNAseI. Dissociated cells were then collected by centrifugation, resuspended in differentiation medium and counted using a hemocytometer. Last, dissociated cells were seeded on 18 mm poly-D-lysine coated coverslips at a $1x10^5$ cell density and grown until day 5 of differentiation.

2.3.3.3 Transfection of *Insm1* mutant P19 cell lines with an *Insm1* expression vector

The pCMV-Insm1 expression vector was generated by subcloning the coding sequence of *Insm1* into a CMV (cytomegalovirus) vector expressing a neomycine resistance gene. Wildtype and *Insm1* mutant cells were transfected with the *Insm1* expression vector using lipofectamine 2000. Transfected cells were selected by adding 200 mM neomycine into the growth medium.

2.3.3.4 FAC-sorting of P19 cells transfected with an *Ascl1-IRES-GFP* expression vector

P19 cells were transfected with a vector expressing *Ascl1-IRES-GFP* that had been generated previously by members of the group of Prof. Dr. Carmen Birchmeier, by subcloning the coding sequence of *Ascl1* into a pCDNA3.1 vector expressing *IRES-GFP* under a CMV promoter. Ascl1 could drive neuronal differentiation in combination with different expression vectors (*NICD* and/ or *Insm1* expression CMV vector). Transfected P19 cells were FAC-sorted for GFP at day 1 post-transfection. Sorted P19 cells were then plated on 18 mm poly-D-lysine coated coverslips at a $1x10^5$ cell density and fixed in 4% PFA at day 2 post-transfection.

2.3.3.5 Immunohistochemical staining of P19 cells

Cells were fixed with 4% paraformaldehyde made in PBS for 15 minutes, and then washed three times with PBS. Cells were fixed at various timepoints after plating and processed for immunofluorescence.

Immunofluorescence: fixed cells were washed in PBS and incubated with blocking solution (0.1% Triton X 100, 5% horse serum made in PBS). After, cells were incubated overnight in blocking solution containing specific primary antibodies of interest. Subsequently, cells were washed with PBS, incubated with DAPI and fluorescence conjugated secondary antibodies for 3 hours and mounted with the immunofluorescence medium Immunomount.

2.3.3.6 Cell counts

Images were acquired at 40X by using a Zeiss inverted confocal and optimized for brightness and contrast using the Adobe Photoshop CS5 program. For cell quantification, 3-5 fields (40X) were randomly chosen for each condition, and all cells included in the field were quantified.

2.3.4 Statistics

P-values were calculated by student's t-test when comparing two groups and by ANOVA when comparing more than two groups. Statistical significance was calculated by using the Prism 5 for mac OS x software and set to a p-value of <0.05. For all graphs, the mean and the standard error of the mean are displayed. Error bars reflect standard error of the mean.

3. Results

3.1 Expression of Insm1 in differentiating P19 cells

3.1.1 P19 cells, an *in vitro* system to model neuronal differentiation

The Insulinoma-associated 1 (Insm1) zinc-finger transcription factor is expressed panneurogenically during development, i.e. in all differentiating central and peripheral neurons (Farkas *et al.*, 2008). Analysis of *Insm1* mutant mice indicates that Insm1 might control the transition from transit amplifying progenitors to differentiated neurons (Wildner *et al.*, 2008; Farkas *et al.*, 2008; Jacob *et al.*, 2009; Masserdotti *et al.*, 2015: Monaghan *et al.*, 2017). Nevertheless, little is known on how Insm1 regulates this transition. Moreover, the neurogenic transcriptional program directed by Insm1 has not been systematically characterized. Ascl1 is a proneural factor expressed in transit amplifying progenitors but not in differentiated neurons. Ascl1 acts upstream of Insm1 (Castro *et al.*, 2006) and regulates Notch signaling, which is required for the maintenance of neuronal progenitors and its downregulation is necessary for terminal neuronal differentiation (Imayoshi & Kageyama, 2014).

In order to study the function of Insm1 in neuronal progenitors, I used a pluripotent murine embryonic carcinoma cell line called P19 cells. These cells can differentiate into neurons upon retinoic acid treatment. A schematic view of P19 cells differentiation protocol and the morphology of P19 cells during neuronal differentiation are shown in Figure 1.

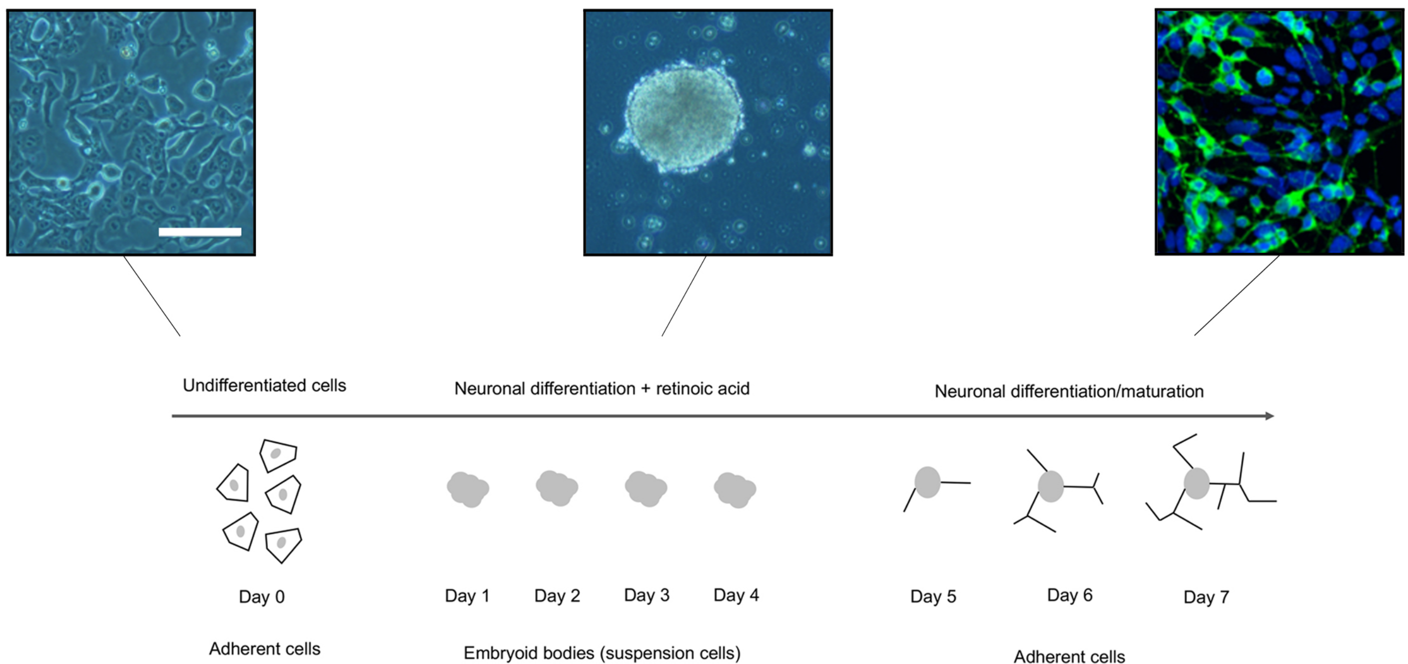


Figure 1. P19 cells, an *in vitro* system to model neuronal differentiation. Bottom, schematic view of the P19 cell neuronal differentiation: at day 1, cells are treated with retinoic acid and resuspended in non-adherent culture dishes to form embryoid bodies. At day 4, embryoid bodies are dissociated and plated on adherent cell culture dishes. At day 5, neuronal progenitors become post-mitotic and undergo terminal differentiation that is completed at day 8. Top, photographs of P19 cells during neuronal differentiation at day 0 and day 3. At day 5, immunochemistry shows developing neurons that express Tubb3 (green). Cell nuclei were counterstained with DAPI (blue). Scale bar: 40 µm.

3.1.2 Expression of *Insm1*, *Ascl1* and *Tubb3* in differentiating P19 cells

The bHLH transcription factor Ascl1 is an important regulator of embryonic neurogenesis. It has been shown that Ascl1 controls the expression of *Insm1* (Breslin *et al.*, 2003; Castro *et al.*, 2006). Besides, many phenotypes observed in the nervous system of *Insm1* mutant mouse embryos were reminiscent of the proneural defects found in *Ascl1* mutants (Parras *et al.*, 2004; Farkas *et al.*, 2008; Wildner *et al.*, 2008). This suggests that Insm1 mediates aspects of Ascl1 function.

To assess the expression of *Insm1* and *Ascl1* during neuronal differentiation in P19 cells, I performed quantitative PCR (qPCR) from day 0 to day 7. I also analyzed the expression of *Tubb3*, a neuronal marker.
I observed that *Insm1* and *Ascl1* expression is low between day 0 and day 4 of neuronal differentiation until it peaks at day 5 and day 6, respectively. The expression is then downregulated at day 7 (Figure 2A). *Tubb3* expression is upregulated from day 5 of neuronal differentiation and keeps increasing, as progenitor cells differentiate into neurons (Figure 2B).

I characterized the expression of Insm1, Ascl1 and Tubb3 by immunochemistry in P19 cells at day 5 of neuronal differentiation. At this timepoint, about 50% of the cultured cells co-expressed Insm1 and Ascl1, and were negative for the neuronal marker Tubb3 (Figure 2C). The remainder of the cells corresponded to differentiated neurons that were Insm1-/Ascl1-/Tubb3+ (Figure 2D).

The large proportion of Insm1+/Ascl1+ progenitor cells observed at day 5 of differentiation makes P19 cells a suitable *in vitro* system for genome-wide studies.

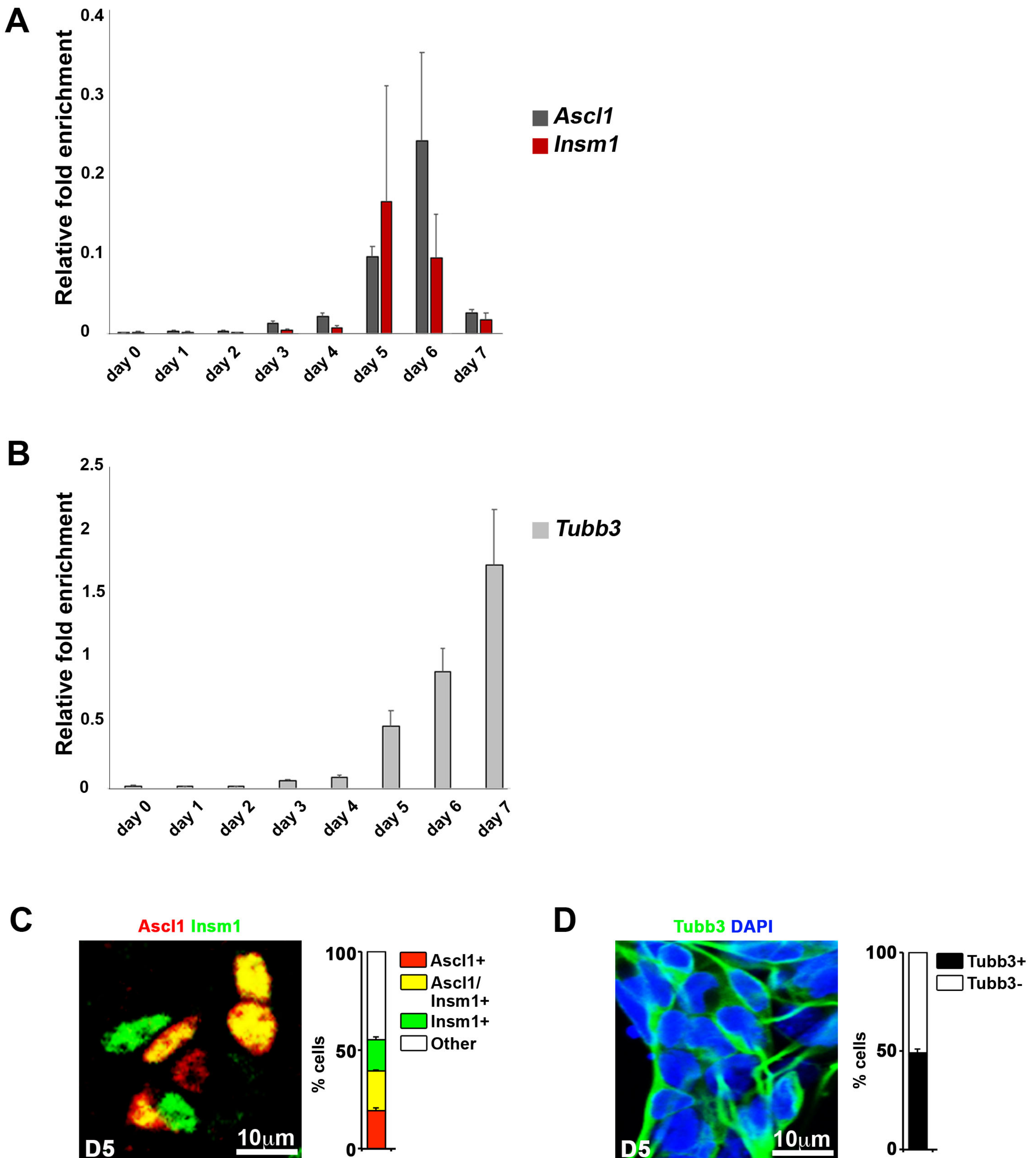


Figure 2. Expression of *Insm1*, *Ascl1* and *Tubb3* in differentiating P19 cells. (A, B) Quantification of *Insm1*, *Ascl1* and *Tubb3* expression in differentiating P19 cells (relative to *Tbp* expression). (C) Left, immunochemistry analysis of P19 cells at day 5 of neuronal differentiation stained with Ascl1 (red) and Insm1 (green) antibodies. Right, quantification of Ascl1+/Insm1+ progenitors at day 5 of differentiation. (D) Left, immunochemistry analysis of P19 cells at day 5 of neuronal differentiation stained with Tubb3 (green) antibodies. Cell nuclei were counterstained with DAPI (blue). Right, quantification of Tubb3+ neurons at day 5 of differentiation.

3.2 Genome-wide identification of Insm1 and Ascl1 binding sites in neuronal progenitor cells

3.2.1 Chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) for Insm1 and Ascl1: co-occupation of sites in the chromatin of differentiating P19 cells

The number of neuronal progenitors is scarce in the developing and postnatal brain, which makes the study of a transcription factor function laborious and expensive. In particular, chromatin-immunoprecipitation analysis of transcription factors requires around 10^7 cells.

To assess the function of Insm1 in neuronal progenitors, I used P19 cells at day 5 of neuronal differentiation to perform chromatin-immunoprecipitation followed by deep sequencing (ChIP-seq) for Insm1 and Ascl1, as a large proportion of Insm1+/Ascl1+ progenitor cells can be observed at this stage (this *in vitro* system is described in 3.1.1 and 3.1.2).
The chromatin of P19 cells at day 5 of differentiation was extracted and sonicated into fragments of 300 base pairs, which were immunoprecipitated with the antibody of interest. Insm1 and Ascl1 binding sites were identified from two independent biological replicates using specific antibodies that recognize the endogenous Insm1 and Ascl1 proteins, respectively. The efficiency of Insm1 and Ascl1 antibodies in chromatin-immunoprecipitation studies has been previously tested in the group of Prof. Dr. Carmen Birchmeier (Jia *et al.*, 2015). The sequencing of the immunoprecipitated chromatin was generated by Madlen Sohn in the group of Prof. Dr. Wei Chen at the MDC, and the sequencing analyses were performed by Dr. Mahmoud Ibrahim and Dr. Scott Lacadie, bioinformaticians in the group of Prof. Dr. Uwe Ohler at the MDC.

The ChIP-seq analyses identified 8261 and 7725 reproducible binding sites for Insm1 and Ascl1, respectively, of which 4265 sites overlapped (Figure 3). This indicates that Insm1 and Ascl1 have many target genes in common, which suggests a transcriptional cooperation of both factors.

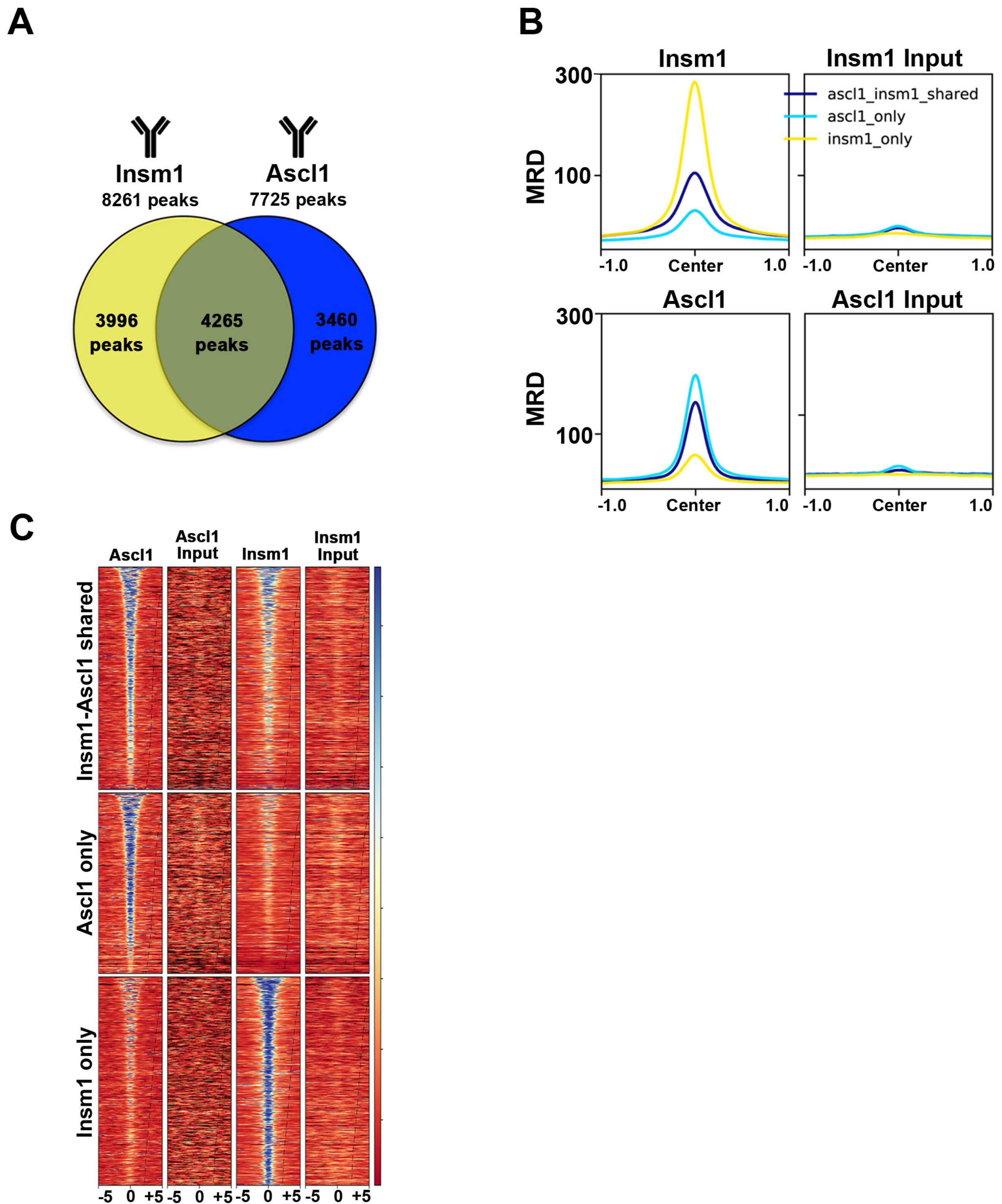


Figure 3. Insm1 and Ascl1 have many target genes in common. (A) Venn diagram illustrating the overlap of Insm1 and Ascl1 binding sites. (B) Insm1 (yellow), Ascl1 (light blue) and Insm1/Ascl1 (dark blue) mean read densities (MRD). MRD center at the binding sites +/- 1 kb distances. Input MRD were used as controls. (C) Heatmaps showing binding sites coverage of Insm1 only, Ascl1 only or Insm1 and Ascl1. Color code indicates read density +/- 5 kb around the binding sites. Input tracks used as controls are also provided.

3.2.2 Co-occupation by Insm1 and Ascl1 is prominent in genes of the Notch signaling pathway

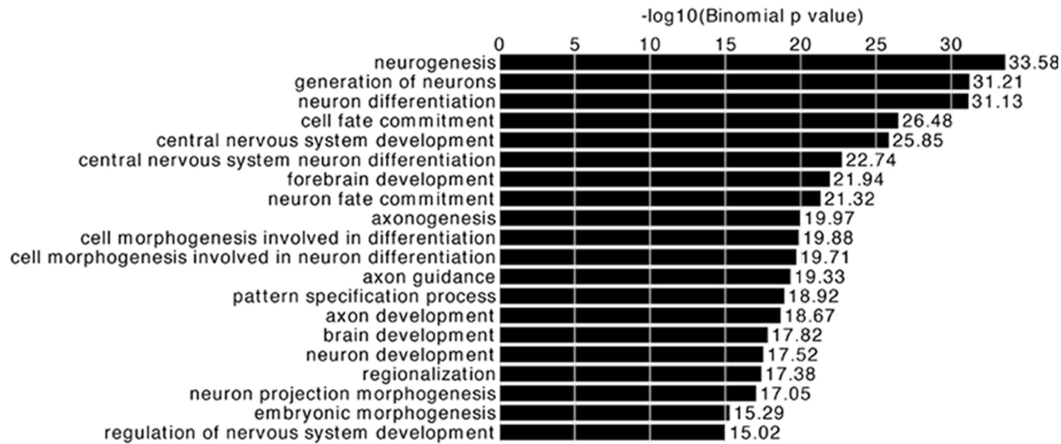
PANTHER analysis was used to determine the gene ontology of the binding sites of Insm1 only and Ascl1 only. This showed that genes bound by Insm1 and Ascl1 were involved in biological processes relevant to development of the nervous system, such as neurogenesis, axon development and brain development (Figure 4A, B).

As described in 3.2.1, the ChIP-seq revealed that Insm1 and Ascl1 have many target genes in common, which suggests a transcriptional co-operation of both factors. Interestingly, the pathway analysis of the sites co-occupied by both Insm1 and Ascl1 revealed enrichment for genes of the Notch signaling pathway (Figure 4C).
Additionally, ChIP-seq tracks showed that Insm1 and Ascl1 shared similar binding to intronic and intergenic loci of Notch signaling genes, such as *Notch1*, *Dll1* and *Hes5* (Figure 5).

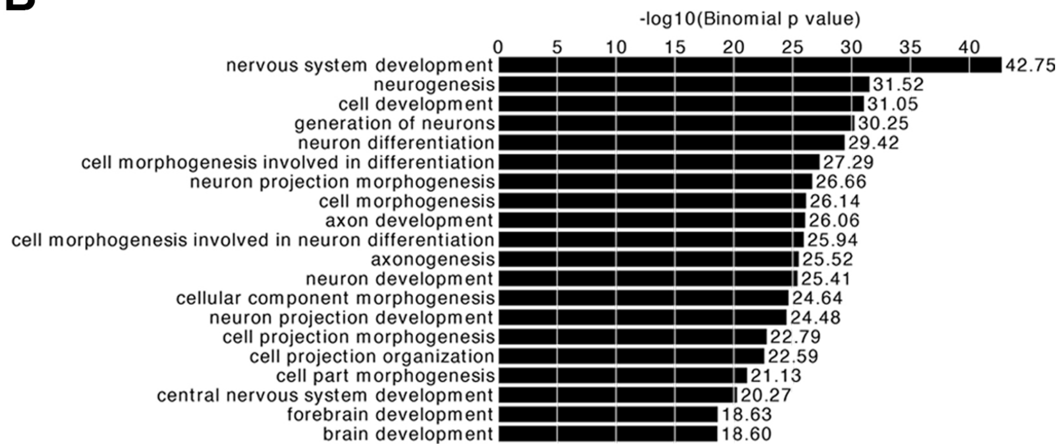
The Notch signaling pathway is known to regulate postnatal neurogenesis and is required for the long-term maintenance of adult neural stem cells (Imayoshi *et al*., 2010) and it has been shown that Ascl1 can modulate Notch signaling by directly regulating the expression of *Dll1* (Castro *et al.*, 2006).

Therefore, enrichment for Insm1 and Ascl1 at genes of the Notch signaling pathway, as well as the similar occupancy at these genes loci, suggest that Insm1 might cooperate with Ascl1 to regulate Notch signaling genes in neuronal progenitors.

A



B



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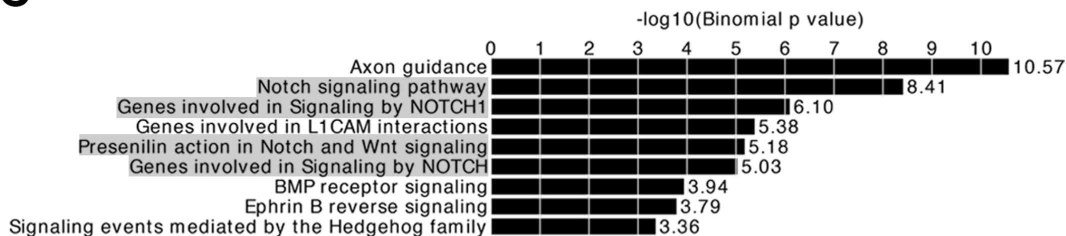


Figure 4. Gene ontology category and pathway analysis of the target genes bound by Insm1 and Ascl1 in P19 cells at day 5 of differentiation. (A) Gene ontology analysis for all Insm1 binding genes and (B) all Ascl1 binding genes. (C) Pathway analysis for both Insm1 and Ascl1 binding sites. Notch signaling related enrichment is highlighted in grey.

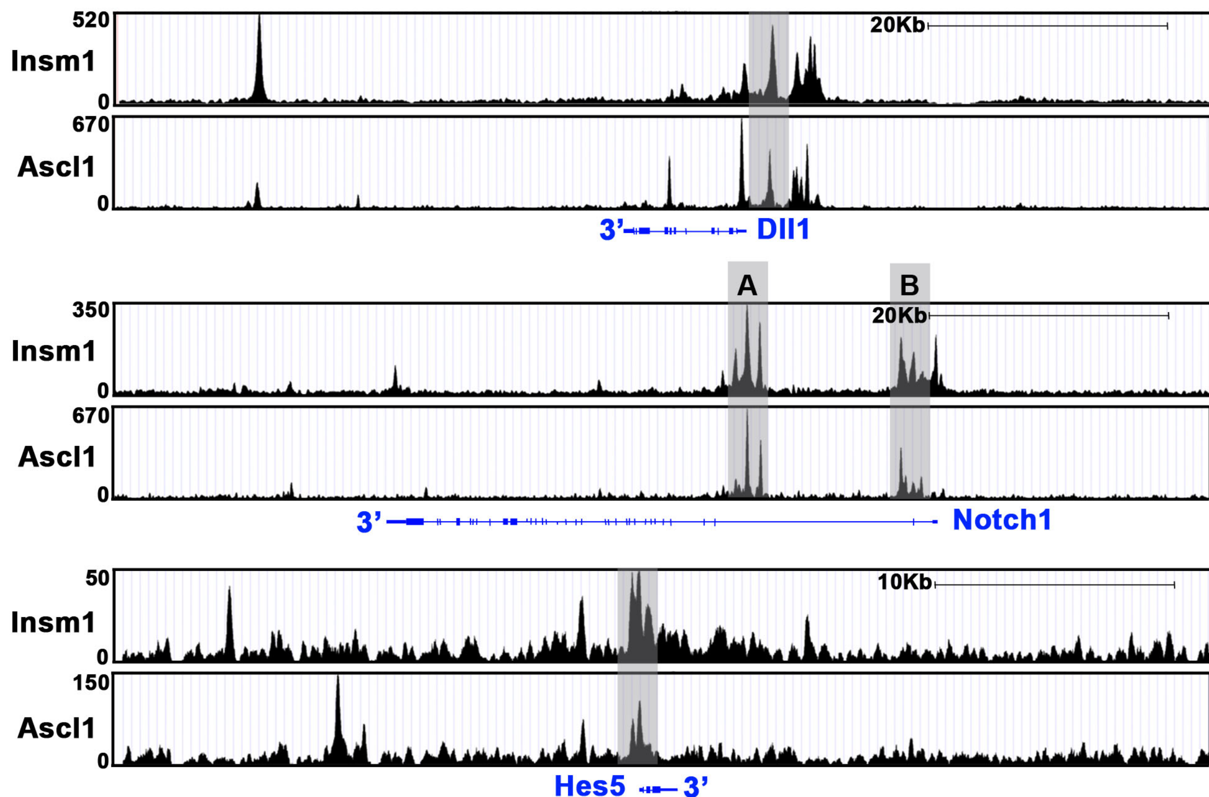


Figure 5. ChIP-seq tracks of Insm1 and Ascl1 show similar occupancy on *Notch1*, *Dll1* and *Hes5* loci. Top tracks: Insm1 binding sites, bottom tracks: Ascl1 binding sites. Regions highlighted in grey indicate Insm1 and Ascl1 similar loci occupancy.

3.2.3 Notch signaling genes occupied by Insm1 in P19 cells correspond to bona fide Insm1 occupied sites in the chromatin of neuronal progenitors *in vivo*

To verify Insm1 binding sites identified in P19 cells *in vivo*, I used progenitor cells of the mouse subventricular zone to perform chromatin-immunoprecipitation combined with quantitative PCR (ChIP-qPCR). For this, I prepared chromatin from micro-dissections of the subventricular zone of P10-12 wildtype mice. I chose this stage, as progenitor cells are more abundant than in the adult (Maslov *et al.*, 2004). Three wildtype animals were used and pooled for chromatin extraction. The chromatin was then sonicated into fragments of 300 base pairs, which were immunoprecipitated with Insm1 antibody.

The efficiency of the Insm1 antibody in chromatin-immunoprecipitation studies has been previously tested in the group of Prof. Dr. Carmen Birchmeier (Jia *et al.*, 2015).

The enrichment for Insm1 at different Notch signaling gene loci was assessed by quantitative PCR. I analyzed *Notch1*, *Dll1* and *Hes5* loci that were identified in P19 cells (Figure 5, selected regions highlighted in grey were used for ChIP-qPCR validation of Insm1 binding sites *in vivo*).

ChIP-qPCR showed enrichment for Insm1 at *Notch1*, *Dll1* and *Hes5* loci *in vivo* (Figure 6). The identified binding sites in P19 cells represent bona fide sites occupied by Insm1 in the chromatin of neuronal progenitors *in vivo*.

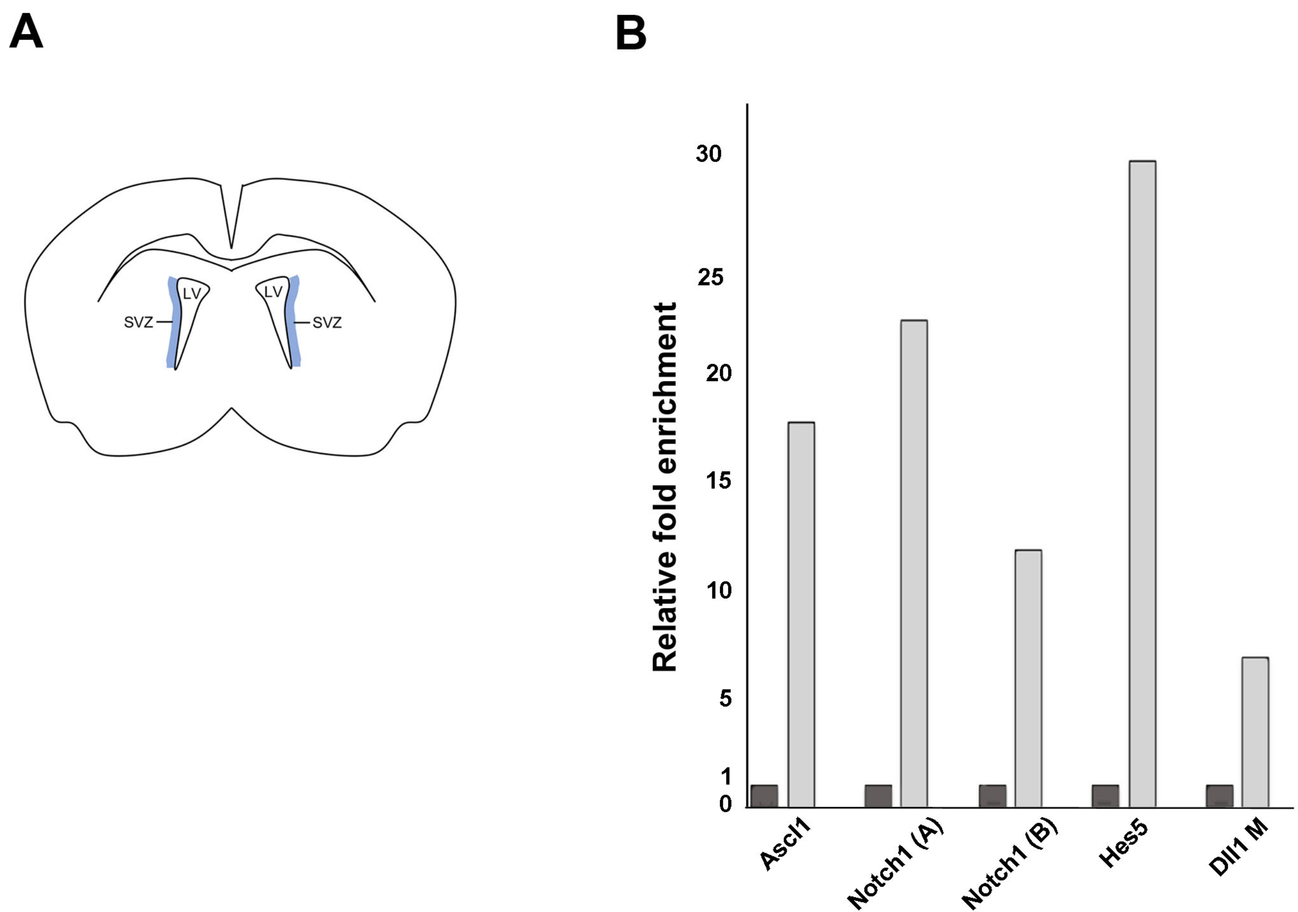


Figure 6. Validation of Insm1 binding sites *in vivo*. (A) Scheme of the mouse subventrivular zone. The SVZ (in blue) was micro-dissected and isolated from P10-P12 mouse brains. LV: lateral ventricle: SVZ: subventricular zone. (B) ChIP-qPCR quantification of Insm1 occupancy on *Notch1*, *Dll1* and *Hes5* loci in progenitor cells of the mouse subventricular zone. Enrichment was normalized to negative control areas located +/- 2 kb from the center of the binding site.

3.3 Insm1 is required for neuronal progenitor cell differentiation and the regulation of Notch signaling gene expression

3.3.1 Deregulated genes of the Notch signaling pathway in *Insm1* mutant P19 cells

Previous results indicate that Insm1 cooperates with Ascl1 to regulate Notch signaling genes in neuronal progenitors. To test whether Insm1 is important for the transcription of *Notch1*, *Dll1* and *Hes5*, I first assessed the expression of these genes in *Insm1* mutant P19 cells.

To this end, I used P19 cells carrying *Insm1* mutant loci that were previously generated by Xun Li, a former Master student in the laboratory of Prof. Dr. Carmen Birchmeier.
Two homozygous mutant cell clones (here called $Insm1^{M1}$ and $Insm1^{M2}$) were obtained using CRISPR/Cas9 and independently derived from two distinct guide RNAs that targeted PAM sequences close to the transcriptional start site of *Insm1*. The mutations resulted in a depletion of Insm1 protein in both cell lines, as confirmed by western blot analyses.
A schematic view of the CRISPR/Cas9 methodology used to induce *Insm1* mutant cell lines is shown in Figure 7.

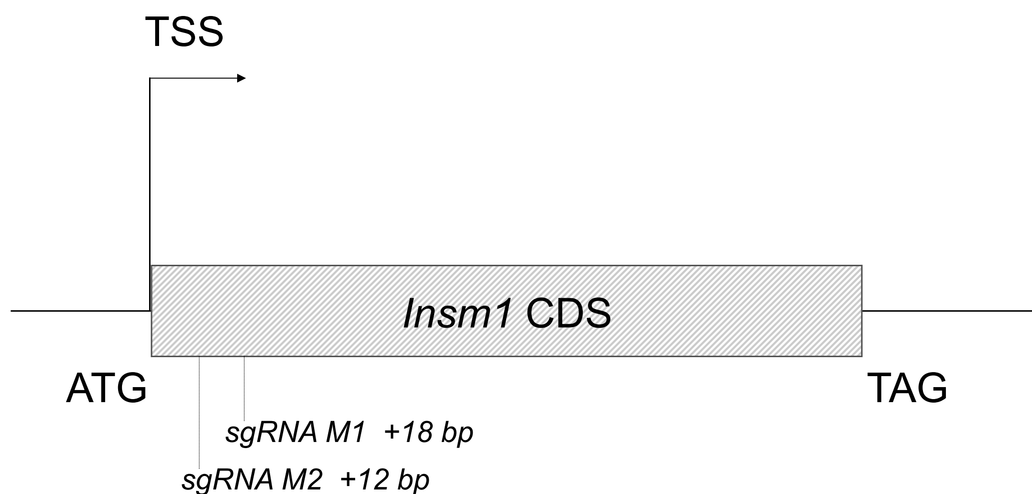


Figure 7. Schematic view of CRISPR/Cas9 derived *Insm1* mutant P19 cell lines. Two mutant cell clones (here called $Insm1^{M1}$ and $Insm1^{M2}$) were obtained using CRISPR/Cas9 and independently derived from two distinct single guide RNAs (sgRNA) that targeted PAM sequences close to the transcriptional start site of *Insm1*. TSS: transcription start site; CDS: coding sequence; ATG: start codon; TAG: stop codon.

I assessed the expression of *Notch1*, *Dll1* and *Hes5* in *Insm1* mutant cells compared to wildtype cells. To this end, I differentiated the *Insm1* mutant cell lines with retinoic acid and performed quantitative PCR (qPCR) at day 5 of neuronal differentiation. This showed upregulation of *Notch1*, *Dll1* and *Hes5* in both *Insm1* mutant cell lines (Figure 8).

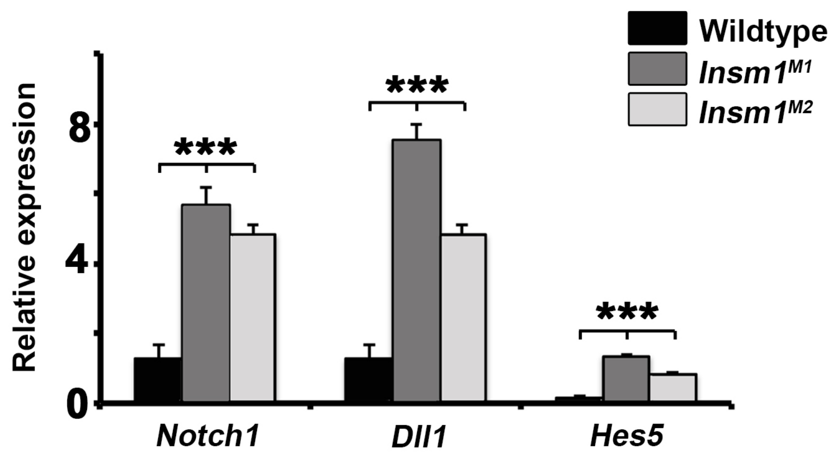


Figure 8. *Insm1* mutation in P19 cells results in the upregulation of Notch signaling genes expression. Quantification of *Notch1*, *Dll1* and *Hes5* expression in *Insm1* mutant cells (*Insm1*^{M1} and *Insm1*^{M2}) at day 5 of neuronal differentiation (relative to *Gapdh* expression).

I next assessed whether Insm1 directly regulates *Notch1*, *Dll1* and *Hes5* gene expression.
To evaluate this, I transfected the *Insm1* mutant cell lines with a vector expressing wildtype *Insm1* coding sequence under a CMV promoter and generated two distinct clonal lines of "rescued" cells (here called *Insm1*^{rescued1} and *Insm1*^{rescued2} for *Insm1*^{M1} and *Insm1*^{M2}, respectively).

I differentiated *Insm1* rescued cell lines with retinoic acid and the following were assessed:
i) Notch signaling gene expression and ii) neuronal progenitor cell production.

To test whether Insm1 directly regulates the expression of Notch signaling genes, I assessed the expression of *Notch1*, *Dll1* and *Hes5* in *Insm1* rescued cells compared to wildtype cells. To this end, I differentiated the *Insm1* rescued cell lines with retinoic acid and performed quantitative PCR at day 5 of neuronal differentiation. *Notch1*, *Dll1* and *Hes5* were downregulated upon re-introduction of *Insm1* in the *Insm1* mutant cell lines (Figure 9).

The re-introduction of *Insm1* in *Insm1* mutant cells restored the repression of Notch signaling gene expression, as observed by the Notch signaling gene expression levels of *Insm1* rescued cells comparable to those of wildtype cells.

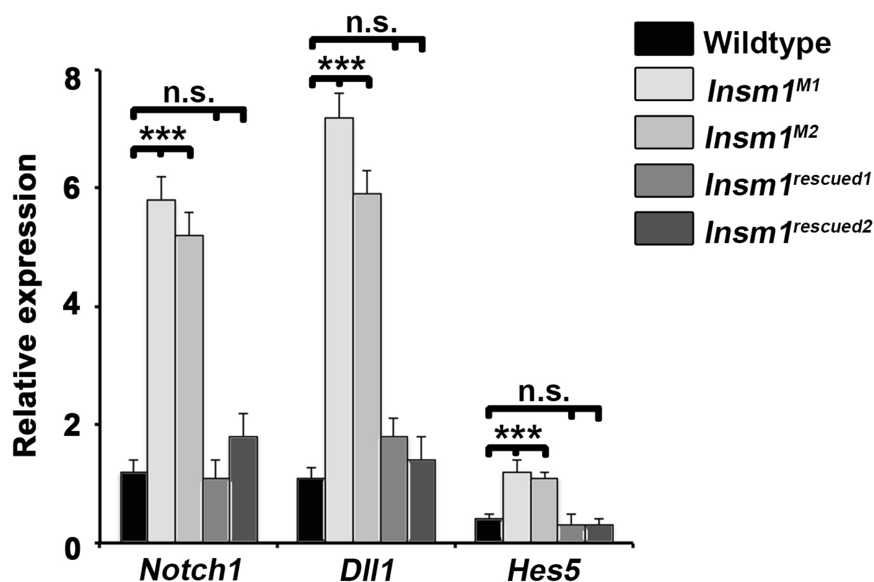


Figure 9. The re-introduction of *Insm1* in *Insm1* mutant cells restored Notch signaling gene expression. Quantification of *Notch1*, *Dll1* and *Hes5* expression in *Insm1* mutant cells (*Insm1*^{M1} and *Insm1*^{M2}) and rescued *Insm1* mutant cells (*Insm1*^{rescued1} and *Insm1*^{rescued2}) at day 5 of neuronal differentiation (relative to *Gapdh* expression). The re-introduction of *Insm1* in the mutant cells restores the expression of Notch signaling genes.

To characterize the expression of Insm1, Ascl1 and Tubb3 in *Insm1* mutant cells, I differentiated the *Insm1* mutant and *Insm1* rescued cell lines with retinoic acid and performed immunochemistry at day 5 of neuronal differentiation.

At this timepoint, *Insm1* mutant cells showed increased numbers of Ascl1+ progenitor cells and reduced numbers of Tubb3+ neurons compared to wildtype cells, whereas quantifications showed similar numbers of Ascl1+ and Tubb3+ cells when *Insm1* rescued and wildtype P19 cells were compared (Figure 10). *Insm1* mutation in P19 cells results in increased numbers of neuronal progenitor cells at the expense of differentiated neurons and this can be restored by the re-introduction of *Insm1* in *Insm1* mutant cells.

Taken together, these results indicate that Insm1 is required for the direct regulation of Notch signaling gene expression and neuronal progenitor cell differentiation.

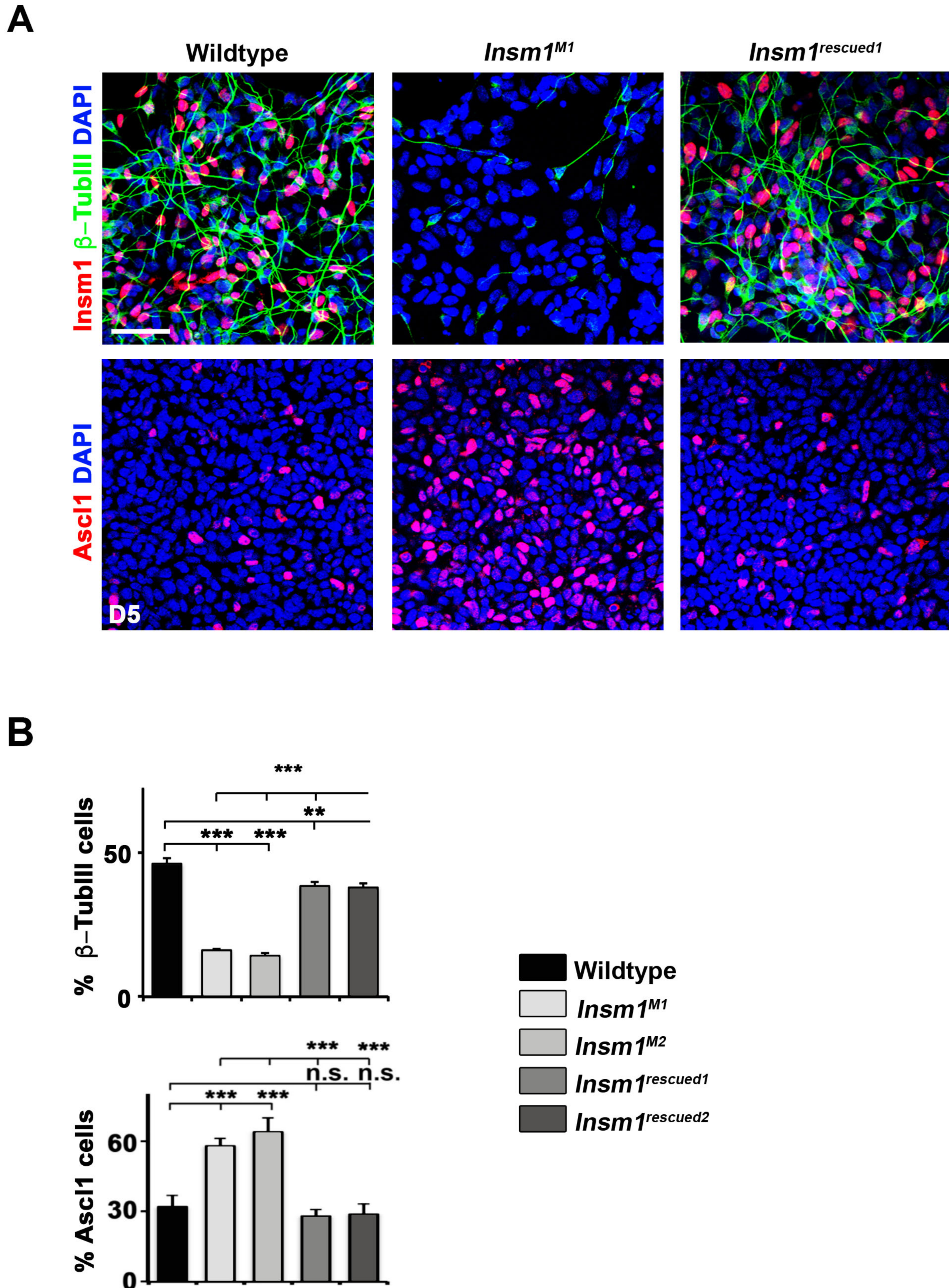


Figure 10. *Insm1* mutation in P19 cells results in increased numbers of neuronal progenitor cells at the expense of differentiated neurons. (A) Top, histological analysis of 5-day differentiated P19 cells stained with antibodies against Insm1 (red) and Tubb3 (green). Bottom, histological analysis of 5-day differentiated P19 cells stained with antibodies against Ascl1 (red). DAPI (blue) was used to counterstain. (B) Top, quantification of Tubb3+ neurons at day 5 of differentiation. Bottom, quantification of Ascl1+ progenitor cells at day 5 of differentiation. Scale bar: 40 µm.

3.3.2 Deregulated genes of the Notch signaling pathway in conditional *Insm1* mutant mice

The results presented in paragraph 3.3.1 indicate that Insm1 is required for the direct regulation of Notch signaling gene expression and neuronal progenitor cell differentiation.
To investigate the function of Insm1 *in vivo*, I analyzed mice in which *Insm1* is conditionally mutated in progenitor cells of the subventricular zone, a region of the mouse forebrain that expresses *Insm1* and actively undergoes neurogenesis in development and postnatal life (Kempermann *et al*., 1997). I used the Cre recombinase driven by *Brn4* gene ($Brn4^{Cre}$ line) and $Insm1^{Flox}$ alleles. The resulting control ($Brn4^{Cre/+};Insm1^{+/Flox}$, here called *control*) and *Insm1* mutant ($Brn4^{Cre/+};Insm1^{Flox/Flox}$, here called *coInsm1*) were used for the following experiment.

To evaluate the deregulation of Notch signaling genes *in vivo*, I performed RNA-sequencing (RNA-seq) in mouse neuronal progenitors. I micro-dissected the subventricular zone of P10-12 *control* and *coInsm1* mice and isolated the RNA. I used 5 animals per condition. The transcriptome was sequenced by Madlen Sohn in the group of Prof. Dr. Wei Chen at the MDC. The sequencing analyses were performed by Dr. Scott Lacadie, a bioinformatician in the group of Prof. Dr. Uwe Ohler at the MDC.
Analyses of the RNA-seq data showed an upregulation of genes of the Notch signaling pathway in the subventricular zone of *coInsm1* mice. Among these genes were *Dll1* and *Notch1* (Figure 11A). The phenotype observed *in vivo* resembles the one that is apparent in cell culture.

Dr. Kira Balueva, a former PhD student in the group of Prof. Dr. Carmen Birchmeier, performed *in situ* hybridization of *control* and *coInsm1* mice subventricular zone during her doctoral studies (Balueva, 2013). This revealed the enhanced expression of *Dll1* and *Notch1* in the neuronal progenitors of the mouse subventricular zone (Figure 11B).

These results show that Notch signaling pathway genes are deregulated in the mouse subventricular zone in the absence of Insm1.

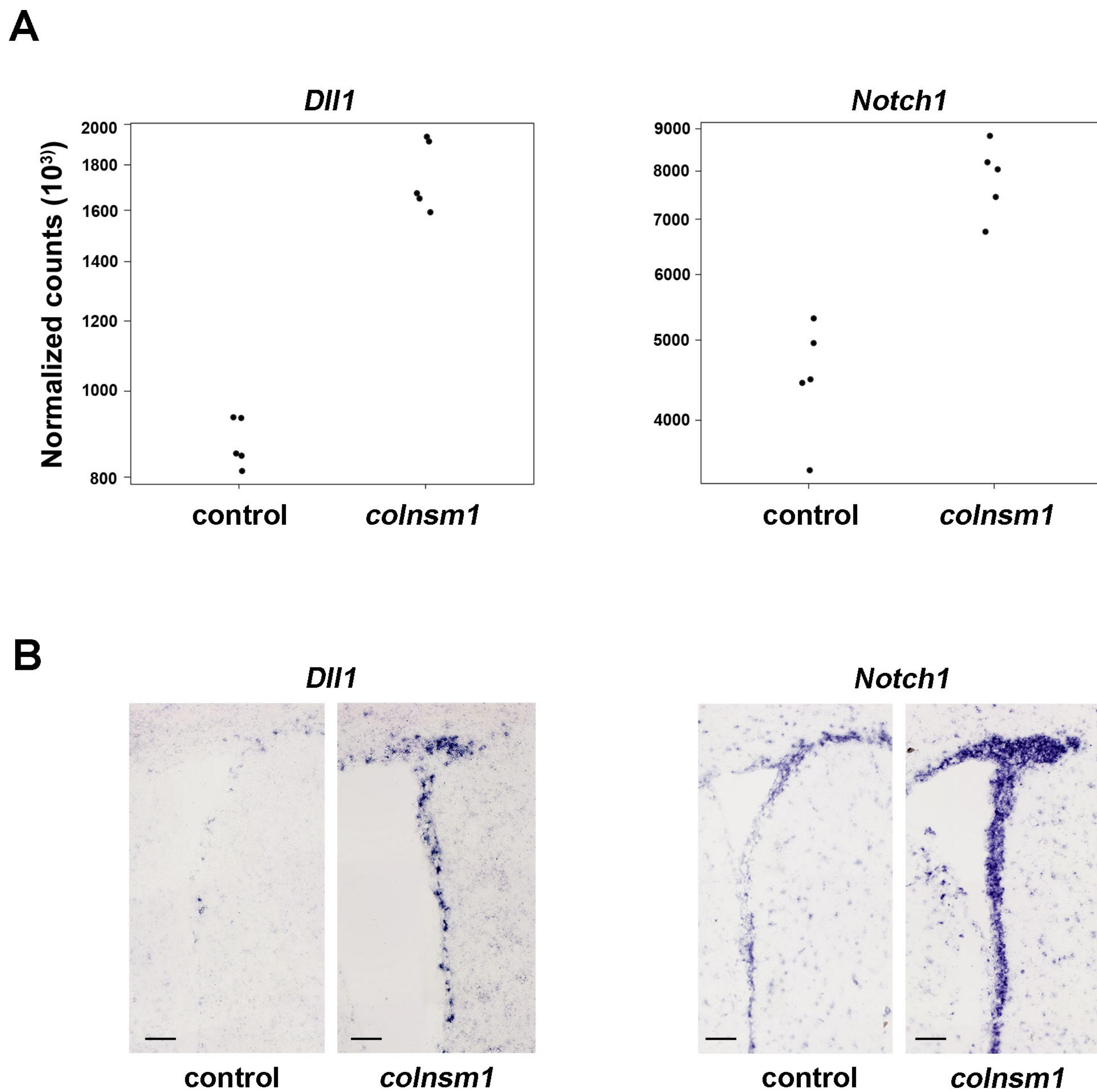


Figure 11. Genes of the Notch signaling pathway are upregulated in *coInsm1* mice. (A) Transcript read counts for *Dll1* and *Notch1* in the subventricular zone of *control* and *coInsm1* P10-P12 mice. RNA was extracted from the subventricular zone. Each dot represents an animal. (B) *In situ* hybridizations for *Dll1* and *Notch1* in the subventricular zone of P10 *control* and *coInsm1* mice (performed by Kira Balueva, 2013). Scale bar: 100 µm.

3.3.3 Deregulated neuronal progenitor genes and non-neuronal genes in conditional *Insm1* mutant mice

Further analyses of the transcriptome data revealed that genes known to be expressed in transit amplifying progenitors and activated neural stem cells, i.e. *Nestin* and *Ascl1,* were also upregulated in *Insm1* mutant mice (Table 1). It is thus tempting to speculate that Insm1 represses a transcriptional program specific to transit amplifying progenitors.
Additionally, the transcriptome data identified an upregulation of genes typically expressed in non-neuronal tissue, e.g. skeletal muscle genes like *Myl9* and *Egln3* (Table 2).

The ectopic expression of inappropriate fates indicates that Insm1 might not only repress the transcriptional program of transit amplifying progenitors but also non-neuronal fates to allow neuronal differentiation.

Gene symbol	Gene name	log2 fold change	*P*-value
Progenitor state genes			
*Draxin**	dorsal repulsive axon guidance protein	1,289209338	8,04E-38
*Fgfr2**	fibroblast growth factor receptor 2	1,106169359	5,20E-35
Nes	nestin	0,757454802	1,04E-12
Sox2	SRY-Box 2	0,61698009	4,74E-38
*Ascl1**	achaete-scute homolog 1	0,542734003	1,29E-07

Table 1. Neuronal progenitor genes are upregulated in *coInsm1* mice. Systematic analysis of neuronal progenitor gene expression in the subventricular zone of *control* and *coInsm1* P10-P12 mice. The average signal fold change is shown. *Deregulated gene expression was also analyzed by *in situ* hybridization (Kira Balueva).

Gene symbol	Gene name	log2 fold change	*P*-value
Non-neuronal genes			
Myl2	myosin light chain 2	2,685222345	9,31E-05
Vnn1	vanin 1	1,85460893	1,47E-48
Flnc	filamin C	1,476712295	2,59E-09
Egln3	egl nine-like 3	1,454499819	1,62E-59
Tnnt3	troponin T3	1,258261245	2,85E-06
Myl9	myosin light chain 9	1,047643433	5,64539E-06
Myl4	myosin light chain 4	0,937475879	0,000271833
Myo7b	myosin VIIb	0,83016768	4,34E-05

Table 2. Non-neuronal genes are upregulated in *coInsm1* mice. Systematic analysis of non-neuronal gene expression in the subventricular zone of *control* and *coInsm1* P10-P12 mice. The average signal fold change is shown.

3.3.4 Transcriptional activation of *Notch1*, *Dll1* and *Hes5* in *coInsm1* mice

I have previously shown that Notch signaling genes were deregulated in *Insm1* mutant mice. I next asked whether the genomic regions bound by Insm1 on *Notch1*, *Dll1* and *Hes5* loci corresponded to regulatory elements.

To this end, I performed chromatin-immunoprecipitation combined with quantitative PCR (ChIP-qPCR) using H3K27ac and H3K27me3 antibodies on chromatin prepared from micro-dissections of the subventricular zone of P10-P12 *control* and *coInsm1* mutant mice. Three animals per condition were used and pooled for chromatin extraction. H3K27ac and H3K27me3 are chromatin modifications that mark active and repressed enhancers, respectively (Creyghton *et al.*, 2010; Rada-Iglesias *et al.*, 2011; Shlyueva *et al.*, 2014). The chromatin was sonicated into fragments of 300 base pairs, which were immunoprecipitated with either H3K27ac or H3K27me3 antibody.

Both chromatin marks were enriched at the tested chromatin sites in different patterns.
In *control* mice, higher enrichments for the repressed chromatin mark H3K27me3 were detected at the tested Notch signaling gene loci. However, in *coInsm1* mutant mice chromatin, higher and lower enrichments for H3K27ac and H3K27me3 respectively were detected at these same loci (Figure 12). The tested genomic regions are transcriptionally activated in the absence of Insm1.

The data indicates that genomic regions bound by Insm1 on *Notch1*, *Dll1* and *Hes5* loci correspond to regulatory elements that possess dynamic histone modifications.

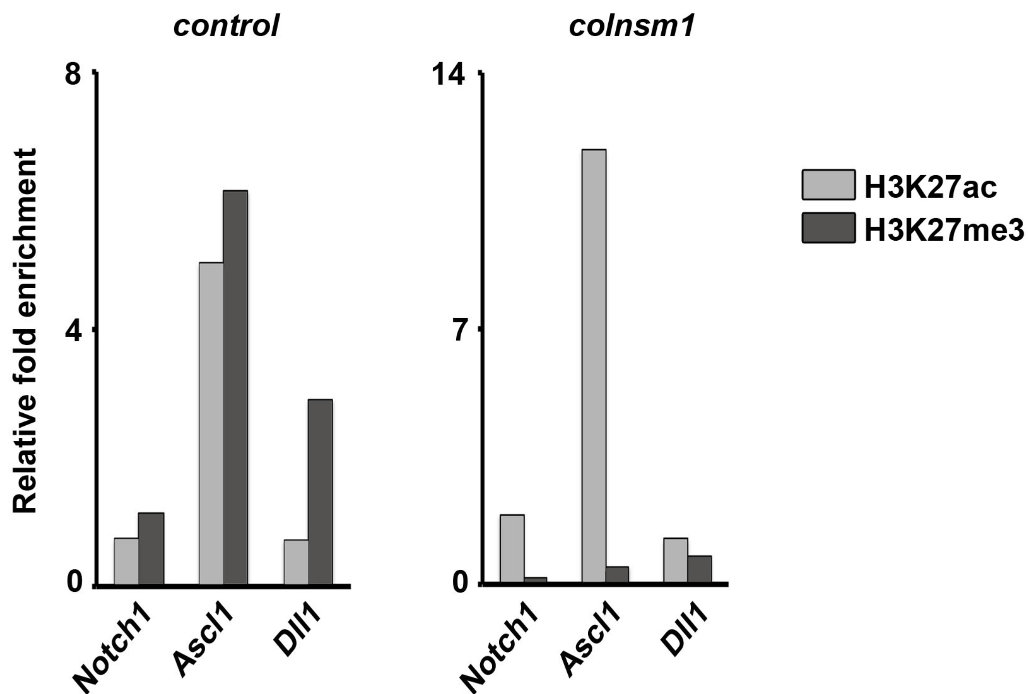


Figure 12. Genomic regions bound by Insm1 on *Notch1*, *Dll1* and *Hes5* loci correspond to regulatory elements. ChIP-qPCR for H3K27ac and H3K27me3 chromatin marks on *Notch1*, *Ascl1* and *Dll1* loci bound by Insm1 *in vivo*. Chromatin was isolated from the subventricular zone of P10-P12 *control* (left) and *coInsm1* mutant mice (right).

3.4 Insm1 represses Notch signaling by counteracting NICD

3.4.1 Insm1 supersedes NICD activity to promote neurogenesis

The regulation of Notch pathway by Insm1 could occur at multiple levels. I tested whether Insm1 directly competes with NICD, the active form of the Notch receptor, to regulate neuronal differentiation.

To this end, I performed Ascl1-mediated neuronal reprogramming of P19 cells by transfecting them with a bicistronic vector encoding *Ascl1-IRES-GFP* only or with different combinations of vectors and FAC-sorted cells expressing GFP at day 1 post-transfection. The sorted cells were then plated on 18-mm poly-D-lysine coated coverslips and analyzed at day 2 post-transfection.

The transfection of *Ascl1-IRES-GFP* alone reprogrammed >85% of GFP+ cells into Tubb3+ neurons. Co-transfection of *Ascl1-IRES-GFP* and a *NICD* encoding vector inhibited neuronal differentiation in all GFP+/NICD+ cells. However, additional expression of *Insm1* was sufficient to inhibit NICD effect and restore neuronal differentiation. Indeed, >78% GFP+/NICD+ cells also co-expressed Tubb3 in presence of *Insm1* (Figure 13).

This indicates that Insm1 counteracts NICD activity to promote neuronal differentiation in neuronal progenitor cells.

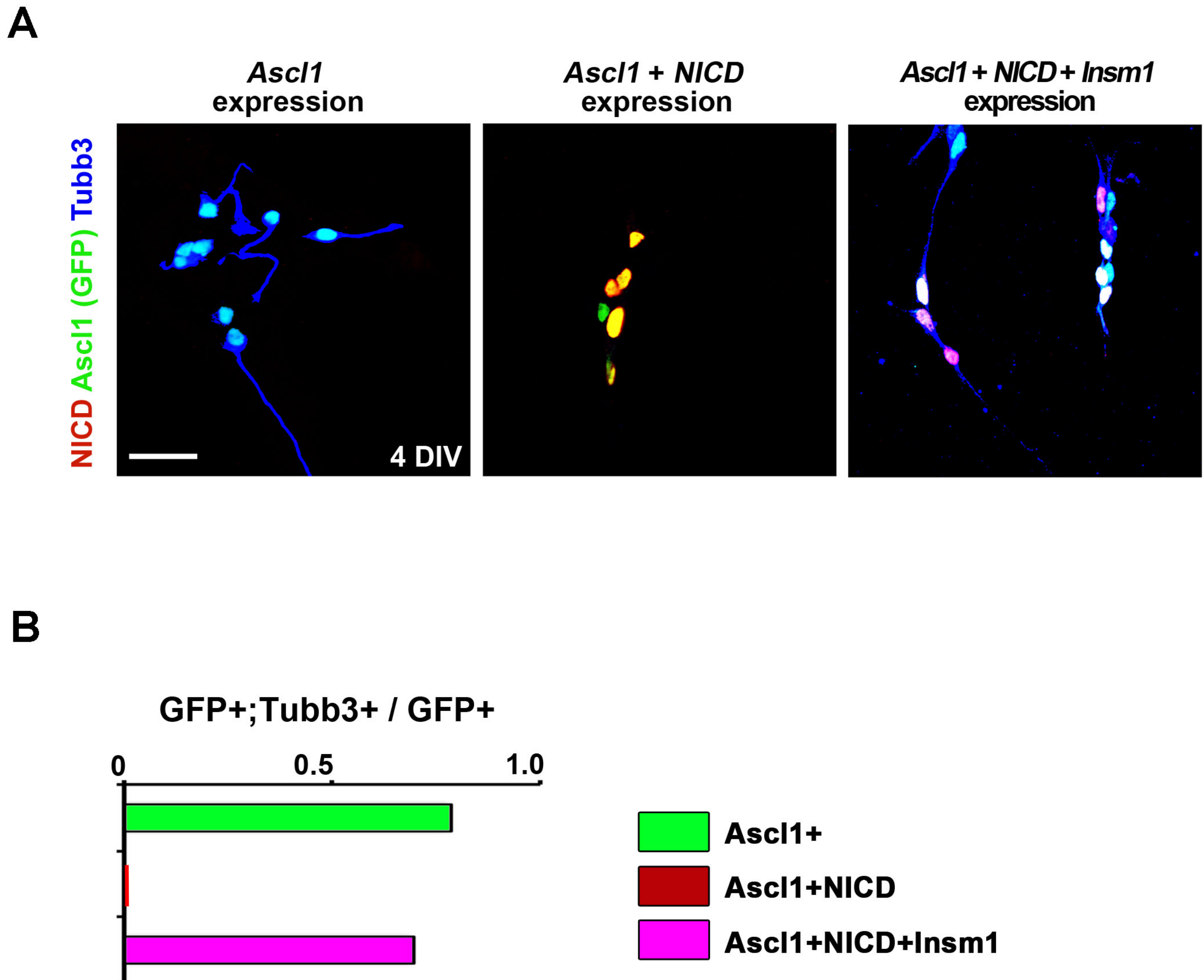


Figure 13. Insm1 counteracts NICD to promote neurogenesis. (A) histological analysis of Ascl1-mediated neuronal reprogrammed wildtype P19 cells transfected with different combinations of expression vectors. Cells were stained with antibodies against GFP (Ascl1, green), NICD (red) and Tubb3 (blue). (B) quantification of Tubb3+ differentiated neurons 2 days after transfection. Scale bar: 40 µm.

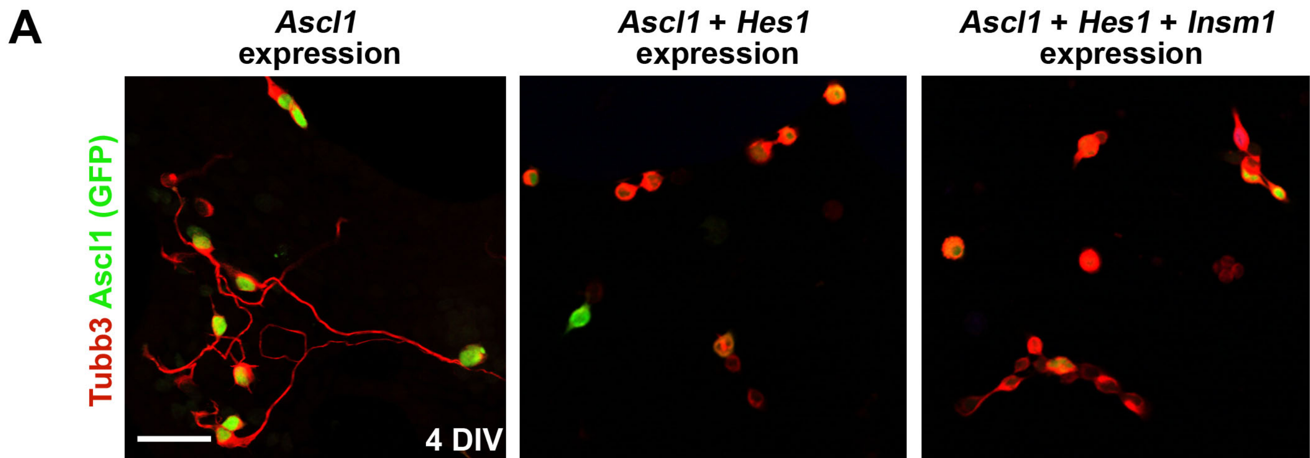
3.4.2 Hes1 and Hes5 alter neurite elongation in neuronal progenitors

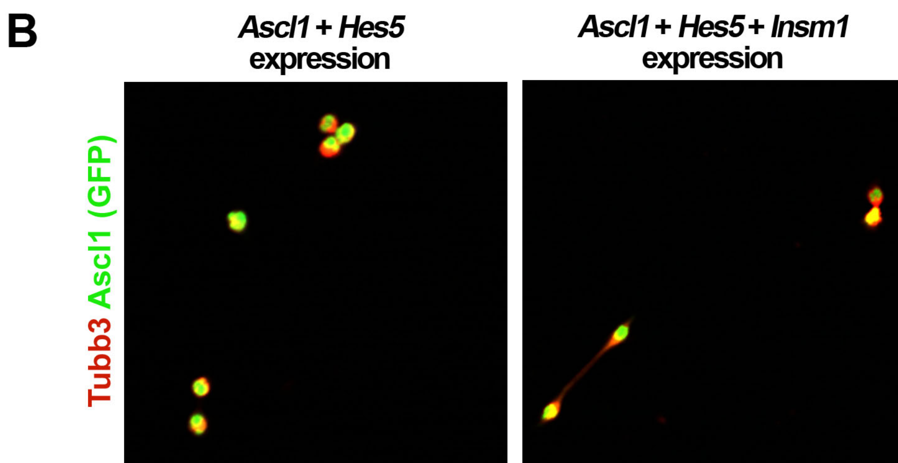
To assess the competitivity of Insm1 with the Notch signaling effectors Hes1 and Hes5, I performed the same P19 cells reprogramming experiment. I transfected P19 cells with an *Ascl1-IRES-GFP* bicistronic vector only or with different combinations of vectors and FAC-sorted cells expressing GFP at day 1 post-transfection. The sorted cells were then plated on 18-mm poly-D-lysine coated coverslips and analyzed at day 2 post-transfection.

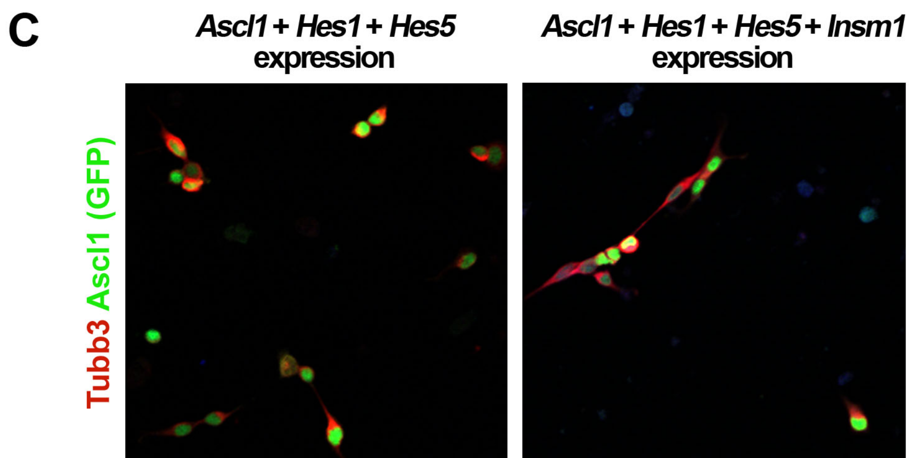
The transfection of *Ascl1-IRES-GFP* alone reprogrammed >97% of GFP+ cells into Tubb3+ neurons (Figure 14A, D). Co-transfection of *Ascl1-IRES-GFP* and a *Hes1* encoding vector (Figure 14A) or a *Hes5* encoding vector (Figure 14B) did not prevent neuronal differentiation in GFP+ cells. In fact, >94% of GFP+ cells also expressed Tubb3+ (fig. 14D). However, no neurite elongation was observed compared to *Ascl1* only transfected cells.
When *Insm1* was expressed additionally to *Ascl1-IRES-GFP* and *Hes1* or *Hes5*, >97% GFP+ cells also co-expressed Tubb3 (Figure 15A, B, D). Nevertheless, the Tubb3+ cells presented a different morphology with a few elongated neurites, compared to *Hes1* or *Hes5* only transfected cells that presented no neurite elongations.

I then co-transfected P19 cells with *Ascl1-IRES-GFP* and both *Hes1* and *Hes5* encoding vectors. I observed that >69% of GFP+ cells expressed Tubb3+. The GFP+/Tubb3+ cells did not possess neurite elongations (Figure 14C, D).
However, additional co-expression of *Insm1* partially restored neurite elongations (Figure 14C).

Taken together, these data show that unlike NICD, neither Hes1 nor Hes5 are sufficient to prevent neuronal differentiation induced by Ascl1, as shown by the numbers of Tubb3+ cells. However, upon *Hes1* and/ or *Hes5* overexpression, cells present no neurite elongations compared to *Ascl1* only transfected cells. Interestingly, neurite elongations could be observed when *Insm1* was also co-expressed. I conclude that overexpression of Notch signaling effectors Hes1 and Hes5 can alter neurite elongation in neuronal progenitors and that Insm1 is able to counteract this activity.


A
Ascl1
expression
Ascl1 + Hes1
expression
Ascl1 + Hes1 + Insm1
expression
Tubb3 Ascl1 (GFP)
4 DIV


B
Ascl1 + Hes5
expression
Ascl1 + Hes5 + Insm1
expression
Tubb3 Ascl1 (GFP)


C
Ascl1 + Hes1 + Hes5
expression
Ascl1 + Hes1 + Hes5 + Insm1
expression
Tubb3 Ascl1 (GFP)

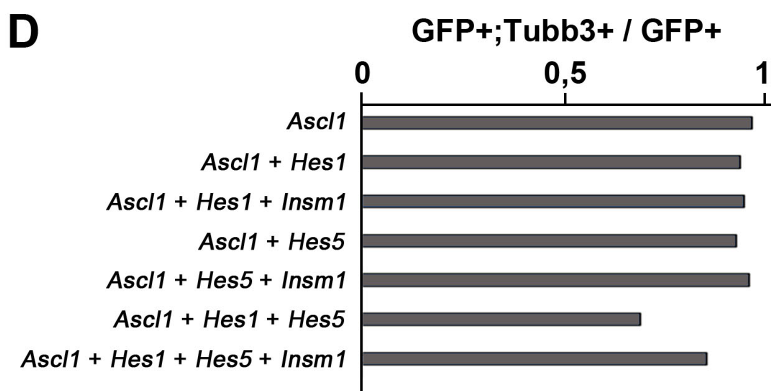

D
GFP+;Tubb3+ / GFP+
0
0,5
1
Ascl1
Ascl1 + Hes1
Ascl1 + Hes1 + Insm1
Ascl1 + Hes5
Ascl1 + Hes5 + Insm1
Ascl1 + Hes1 + Hes5
Ascl1 + Hes1 + Hes5 + Insm1

Figure 14. Hes1 and Hes5 effectors alter neurite elongation in neuronal progenitors. (A, B, C) Histological analysis of Ascl1-mediated neuronal reprogrammed wildtype P19 cells transfected with different combinations of vectors. Cells were stained with antibodies against GFP (Ascl1, green) and Tubb3 (red). (D) Quantification of Tubb3+ differentiated neurons 2 days after transfection. Scale bar: 40 μm.

4. Discussion

4.1 Insm1 and Ascl1 cooperate to regulate a common transcriptional program

4.1.1 Co-expression of Insm1 and Ascl1 in neuronal progenitors

The transcription factor Insm1 has a panneurogenic expression in the embryonic and adult nervous system. In fact, Insm1 is expressed transiently in neuronally committed progenitor cells and in early neurons (Farkas *et al*., 2008) and its expression in correlation with neurogenesis is maintained in postnatal and adult life (Alvarez-Buylla and Garcia-Verdugo, 2002; Ninkovic and Götz, 2007). In neurogenic regions, Insm1 is often co-expressed with proneural factors such as Ascl1, which induces *Insm1* expression (Castro *et al*., 2006; Jacob *et al*., 2009).

In order to study Insm1 function, I used the *in vitro* P19 cell system allowing me to generate neuronal progenitors and neurons. I noted that 50% of the cultured cells co-expressed Insm1 and Ascl1 at day 5 of differentiation (3.1.2). These differentiating cells corresponded to neuronal progenitors, while the rest of the cells had differentiated into Tubb3+ neurons.
At that same timepoint in the *Insm1* mutant P19 cell lines $Insm1^{M1}$ and $Insm1^{M2}$, I observed increased numbers of neuronal progenitors expressing Ascl1 and decreased numbers of Tubb3+ neurons, indicating that neuronal differentiation was impaired. This was associated with the upregulation of Notch signaling pathway gene expression (3.3.1).
Furthermore, Ascl1 is expressed in transit amplifying progenitors and is known to cooperate with Notch signaling to promote the expression of proliferation genes in progenitor cells and thereby their expansion (Castro *et al*., 2011).

In the absence of Insm1, the upregulation of *Ascl1* and Notch signaling genes might directly lead to increased numbers of neuronal progenitors and delayed neuronal differentiation.

4.1.2 Co-occupation of binding sites by Insm1 and Ascl1 in the chromatin of neuronal progenitors *in vitro*

It is known that Ascl1 induces the expression of *Insm1* and interestingly, previous studies showed similarities in the nervous system of *Insm1* and *Ascl1* mutants, which both present proneural defects (Parras *et al*., 2004; Farkas *et al*., 2008; Wildner *et al*., 2008).
This supports the idea that Insm1 is part of the neuronal differentiation pathway initiated by Ascl1 and that both transcription factors regulate a common transcriptional program.

In order to study the role of Insm1 in neuronal progenitors and unveil the transcriptional cascade in which it operates, I performed chromatin-immunoprecipitation followed by deep sequencing (ChIP-seq) on P19 cells at day 5 of differentiation for both Insm1 and Ascl1 transcription factors. This identified 8261 and 7725 reproducible binding sites for Insm1 and Ascl1 respectively, of which 4265 sites overlapped. Insm1 and Ascl1 therefore have many target genes in common, which supports the idea of a transcriptional cooperation of both factors.

Interestingly, Insm1 has not been shown to directly bind its sites on DNA (Jia *et al*., 2011); it hence requires cofactors to bind DNA or might be recruited by other transcription factors.
I performed co-immunoprecipitation experiments for Insm1 and Ascl1 in P19 cells at day 5 of differentiation but results were not conclusive regarding the direct interaction of both factors. However, ChIP-seq experiment for both Insm1 and Ascl1 on 5 day-differentiated P19 cells (3.2.1) allowed us to examine Insm1 binding sites by *de novo* motif analyses. This revealed enrichment for two E-box motifs, known binding motifs of bHLH transcription factors, and one zinc-finger motif, binding motif of zinc-finger transcription factors. Among the motifs was a strong preference for the E-box ACAGCTG, which is a known Ascl1 binding motif (Castro *et al*., 2011). This suggests that Ascl1 recruits Insm1 to chromatin at its binding sites.

In line with this hypothesis, it is known that Ascl1 is a pioneer transcription factor (Wapinski *et al*., 2013). In fact, Ascl1 is able to directly bind silent chromatin in order to initiate a transcriptional program by recruiting other transcription factors unable to bind silent chromatin themselves.

Altogether, the findings indicate that Ascl1 might recruit Insm1 to DNA with the help of co-factors in order to initiate the activation or repression of their common target genes.

4.2 Upregulation of Notch signaling pathway and delayed neuronal differentiation in *Insm1* mutants

Analyses of the gene ontology terms of the sites co-occupied by Insm1 and Ascl1 revealed a strong enrichment for genes of the Notch signaling pathway. Additionally, ChIP-seq tracks showed that both transcription factors shared similar binding to intronic and intergenic loci of Notch signaling genes, which supports a common regulation of these genes (3.2.3).

The initiation of neuronal differentiation requires the repression of the transcriptional program typical of neural stem cells and neuronal progenitor cells. The downregulation of the Notch signaling pathway is needed to allow neuronal differentiation (Kageyama *et al*., 2008; Imayoshi and Kageyama, 2014). According to the principle of lateral inhibition, the upregulation of Dll1 in transit amplifying progenitors leads to the induction of Notch signaling pathway in neighboring cells, which start expressing Hes effectors and inhibit *Ascl1* expression in these cells, which therefore remain neuronal progenitors (Louvi and Artavanis-Tsakonas, 2006; Kageyama *et al*., 2008). Dll1 is a direct target of Ascl1 (Castro *et al*., 2006) and has been found upregulated in neuronal tissue of *Insm1* mutants (Castro *et al*., 2006; Farkas *et al*., 2008; Wildner *et al*., 2008; Balueva, 2013; Monaghan *et al*., 2017).

I observed an upregulation of the expression of Notch signaling pathway genes (e.g. *Dll1*, *Notch1* and *Hes5*) in the *Insm1* mutant cell lines $Insm1^{M1}$ and $Insm1^{M2}$ (3.3.1), as well as in the subventricular zone of *coInsm1* mutant mice, as detected by RNA-seq and *in situ* hybridization (3.3.2). This was associated with decreased numbers of differentiated neurons *in vitro* (3.3.1), which recapitulates the delayed neuronal differentiation of transit amplifying progenitors previously observed in the subventricular zone of *coInsm1* mutant mice (Balueva, 2013).

I propose that the upregulation of *Dll1* in *Insm1* mutants activate Notch signaling pathway in neuronal progenitors, which results in delayed neuronal differentiation.

Previous work showed that upon Notch receptor activation, transit amplifying progenitor cells are unable to induce the expression of *Hes* effectors (Mizutani *et al*., 2007; Kawaguchi *et al*., 2008; Nelson *et al*., 2013). This implies the inhibition the Notch signaling pathway through cell-autonomous mechanisms, such as Insm1 that regulates the expression of Notch signaling components.

Interestingly, I observed a downregulation of Notch signaling gene expression, as well as a rescue of neuronal differentiation upon expression of *Insm1* in the *Insm1* mutant cell lines (3.3.1). In line with Insm1 function in regulating Notch signaling, the Insm1 homolog Nerfin-1 in *Drosophila melanogaster* has been recently shown to repress Notch signaling in order to prevent the dedifferentiation of medulla neurons (Xu *et al*., 2017).

This data supports cell-autonomous function of Insm1 in repressing Notch signaling components to allow neuronal differentiation in neuronal progenitors.

4.3 Possible mechanism of action of Insm1 in regulating the Notch signaling pathway

4.3.1 Insm1 transcriptionally represses genes of the Notch signaling pathway by binding to loci bearing active marks in neuronal progenitor cells

I previously showed that Insm1 directly binds and represses genes of the Notch signaling pathway in neuronal progenitors. However, the regulation of Notch signaling by Insm1 could occur at multiple levels in the Notch pathway.

To gain insight, I assessed chromatin modifications at the identified Notch signaling loci bound by Insm1 (3.2.4). I performed chromatin-immunoprecipitation combined with quantitative PCR (ChIP-qPCR) using H3K27ac and H3K27me3 antibodies on neuronal progenitors from postnatal *control* and *coInsm1* mutant mice. H3K27ac and H3K27me3 are chromatin modifications that mark active and repressed regulatory regions, respectively (Creyghton *et al*., 2010; Rada-Iglesias *et al*., 2011; Shlyueva *et al*., 2014).

Results indicate that the tested Notch signaling gene loci are transcriptionally activated in the absence of Insm1, as shown by higher enrichments for the active mark H3K27ac in *coInsm1* mutant mice (3.3.4).

The chromatin marks dynamics at the Notch signaling sites bound by Insm1 indicate that these loci correspond to regulatory regions. Insm1 therefore binds to loci bearing active marks in a repressive manner, which is in line with its repressor function. Most importantly, de-repression of these regulatory regions in *coInsm1* mutant mice results in the upregulation of *Notch1*, *Dll1* and *Hes5* in neuronal progenitors of the subventricular zone.

Previous studies have shown that Insm1 can recruit histone modifying factors, such as the histone demethylase Lsd1, the histone deacetylases Hdac1/2 and Rcor1/2, the co-repressors of the REST silencing complex (Saleque *et al*., 2007; Lin *et al*., 2010; Welcker *et al*., 2013; Monaghan *et al*., 2017). Furthermore, Insm1 epigenetic function is important for differentiation of endocrine cells in the pituitary gland (Welcker *et al*., 2013) and for balancing progenitor proliferation and neuronal differentiation during brain development (Monaghan *et al*., 2017). This supports the idea that Insm1 functions as a transcriptional repressor in neuronal progenitors to insure neuronal differentiation.

I showed that Insm1 directly binds and represses regulatory regions of *Notch1*, *Dll1* and *Hes5*, and this repression is important for exiting the transit amplifying progenitor state and progressing into terminal neuronal differentiation.

4.3.2 Insm1 promotes neuronal differentiation by counteracting NICD activity

After characterizing Insm1 transcriptional repressive function on Notch signaling pathway genes, I assessed whether Insm1 was able to counteract the inhibitory activity of Notch signaling in neuronal differentiation.

To this end, I transfected P19 cells with different combinations of expression vectors and observed that Ascl1 expression could induce the generation of Tubb3+ neurons (3.4.1). However, co-expression with the active version of Notch receptor *NICD* was sufficient to inhibit neuronal differentiation *in vitro*. Interestingly, co-expression with *Insm1*, *Ascl1* and *NICD* rescued the inhibition of neurogenesis by NICD, as revealed by the number of generated Tubb3+ neurons. These results suggest that Insm1 alone enhances the neurogenic function of Ascl1 by counteracting the inhibitory activity of NICD to allow neuronal differentiation.

Notably, the expression vectors used for transfection contained the coding sequences of *Ascl1*, *NICD* and *Insm1* with no regulatory elements. I therefore conclude that Insm1 counteracts NICD activity by operating on its target genes.

It is known that the expression of *Hes1* and *Hes5* effectors is induced by NICD (Kageyama *et al.*, 2007). I therefore assessed the effect of Insm1 on *Hes1* and *Hes5* expression.
To do so, I performed the same reprogramming experiment of P19 cells and transfected the cells with the same combinations of vectors, along with vectors expressing *Hes1* or *Hes5* coding sequences (3.4.2). I observed that upon expression of *Hes1* and/ or *Hes5* with *Ascl1*, neuronal differentiation was not inhibited. However, these cells presented no neurite elongations. Interestingly, rescue of neurite elongations could be observed when *Insm1* was co-expressed with *Ascl1* and/ or *Hes1*/*Hes5*.

It was shown that Notch signaling is involved in the control of neurite elongation, which can be inhibited via Rbpj-dependent mechanisms (Sestan *et al.*, 1999; Levy *et al.*, 2002; Ables *et al.*, 2011). Consequently, Notch signaling effectors Hes1 and Hes5 interfere with the correct generation of neurons, as they are required for neurite elongation, but they do not inhibit neuronal differentiation like NICD.

Altogether, I conclude that Insm1 promotes neuronal differentiation by counteracting NICD activity and its downstream Hes effectors in a cell-autonomous manner.

4.4 Upregulation of genes involved in neural stem cell and progenitor cell maintenance in *Insm1* mutants

The upregulation of Notch signaling components in neuronal progenitors of *coInsm1* mutant mice was accompanied by the upregulation of further genes involved in neural stem cell and progenitor cell maintenance (3.3.3).
In fact, analysis of the transcriptome data revealed the upregulation of genes known to operate in transit amplifying progenitors and activated neural stem cells, such as *Nestin* and *Fgfr2* (Frinchi *et al.*, 2008; Azim *et al.*, 2012).

Insm1 is known to regulate neurogenesis in postnatal and embryonic life where it is important for the exit from transit amplifying progenitor stage (Farkas *et al*., 2008; Balueva, 2013). I have shown that *Insm1* mutant cells remain in a progenitor stage, as shown by the increase of *Ascl1* expressing cells and the upregulation of Notch signaling.

These results support Insm1 function as a repressor of a transcriptional program specific to transit amplifying progenitors to allow neuronal differentiation.

4.5 Insm1 suppresses non-neuronal lineages to safeguard the neuronal fate

In neuronal progenitors, Insm1 promotes neurogenesis by repressing transcriptional programs that operate in progenitor cells, including Notch signaling pathway.
Remarkably, analysis of the transcriptome data identified an upregulation of genes typically expressed in non-neuronal tissue, e.g. skeletal muscle genes (3.3.3). Activation of transcriptional programs characteristic of different germ layers at such late developmental stages is not usual. The ectopic expression of inappropriate fates in *coInsm1* mutant mice implies that Insm1 might also repress other lineage fates to allow neuronal differentiation.

Previous work has shown that upon Insm1 depletion in endocrine cells of the pituitary, genes typically expressed in skeletal muscle were also found upregulated, indicating that Insm1 represses an inappropriate fate (Welcker *et al.*, 2010). Moreover, recent studies revealed that the zinc-finger transcription factor Myt1l directly represses many non-neuronal lineages in reprogramming fibroblasts in order to safeguard the neuronal identity (Mall *et al*., 2017). Among the expression programs repressed by Myt1l are Notch signaling genes and skeletal muscle genes. Another zinc-finger transcription factor named Myt1 was found to promote neurogenesis downstream of Ascl1 by directly repressing *Hes1* expression and therefore suppressing Notch signaling in differentiating progenitors (Vasconcelos *et al*., 2016).

It is therefore tempting to speculate that Insm1 represses different non-neuronal lineages to maintain the neuronal identity in differentiating neuronal progenitors. Remarkably, Insm1 might be a member of the lineage transcriptional repressors possessing the emergent function of safeguarding the neuronal fate.

5. Summary

The Insulinoma-associated 1 (Insm1) zinc finger transcription factor is highly expressed in the embryonic nervous system and its expression is maintained in the neurogenic areas of the adult brain. Insm1 is a direct target of Ascl1 and has been shown to be important for neuronal differentiation (Wildner *et al.*, 2008; Farkas *et al.*, 2008; Masserdotti *et al.*, 2015; Monaghan *et al*., 2017). However, the understanding of Insm1 molecular network has remained limited.

Here, I sought to undertake a genomic approach to decipher Insm1-dependent molecular mechanisms controlling the progression of neuronal progenitor cells. To this end, I generated datasets of Insm1-dependent expression profiling and its genomic binding profile. Interestingly, Insm1 occupies genomic regions located nearby genes of the Notch signaling pathway. *In vivo* and *in vitro* studies showed that mutation of *Insm1* in neuronal progenitor cells results in upregulation of Notch signaling components, such as *Notch1*, *Dll1* and *Hes1*. *In vitro* analyses illustrate that Insm1 directly represses the expression of these genes.

I propose that Insm1 represses the progenitor program downstream of Ascl1 in neurogenesis. Importantly, I show that Insm1 exerts its functions by directly controlling the expression of neuronal target genes and repressing non-neuronal genes. Altogether, this genomic analysis reveals the complex molecular program regulated by Insm1 for the correct establishment of neuronal differentiation through both genetic and epigenetic mechanisms.

5. Zusammenfassung

Der Zinkfinger-Transkriptionsfaktor Insm1 (Insulinoma-associated 1) ist im embryonalen Nervensystem hoch exprimiert. Die Expression wird außerdem in den neurogenen Bereichen des adulten Gehirns aufrechterhalten.
Insm1 ist ein direktes Zielgen von Ascl1 und hat sich als wichtig in der neuronalen Differenzierung erwiesen (Wildner *et al.*, 2008; Farkas *et al.*, 2008; Masserdotti *et al.*, 2015; Monaghan *et al.*, 2017). Dennoch ist das molekulare Netzwerk von Insm1 noch unzureichend beschrieben.
In der vorliegenden Arbeit habe ich versucht den Insm1-abhängigen molekularen Mechanismus zu charakterisieren, welcher das Entwicklungsprogramm neuronaler Vorläuferzellen steuert. Hierfür habe ich auf einen auf Genomanalyse basierenden Ansatz zurückgegriffen und Daten zur Insm1-abhängigen Genexpression sowie dem entsprechenden genomischen Bindungsprofil generiert.
Interessanterweise besetzt Insm1 genomische Regionen, welche sich in der Nähe von Genen des Notch-Signalweges befinden. *In-vivo*- und *in-vitro*-Analysen zeigten, dass eine Mutation von Insm1 in neuronalen Vorläuferzellen zu einer Hochregulierung der Komponenten des Notch-Signalweges führt, beispielsweise bei *Notch1*, *Dll1* und *Hes1*. *In-vitro*-Analysen zeigten ebenfalls, dass Insm1 repressiv auf die Expression dieser Gene wirkt. Ich postuliere daher, dass Insm1 das Entwicklungsprogramm der neuronalen Vorläuferzellen negativ beeinflusst. Dies betrifft den Teil des Programms, welches Ascl1 nachgeschaltet ist. Darüber hinaus stellte ich fest, dass Insm1 die Expression neuronaler Zielgene direkt kontrolliert und nicht-neuronale Gene unterdrückt.
Die vorliegende genomische Analyse zeigt somit das komplexe molekulare Programm, mit dessen Hilfe Insm1 die Etablierung neuronaler Differenzierungsprozesse durch genetische und epigenetische Mechanismen reguliert.

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Publications

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Declaration of independence

I declare that the work in this dissertation titled "The Function of Insm1 in Neuronal Progenitors" has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree at this or any other institution.
The work presented is entirely my own, except where states otherwise by reference or acknowledgment.

Berlin, May 5th 2020
Madeleine Larrosa