From the Department of Cell and Molecular Biology and the
Ludwig Institute for Cancer Research
Karolinska Institutet, Stockholm, Sweden

TRANSCRIPTIONAL REGULATION OF NEURONAL DIFFERENTIATION IN THE DEVELOPING CNS

Vilma Rraklli

Karolinska Institutet

Stockholm 2017

Published by Karolinska Institutet.
Printed by E- Print AB 2017

ISBN 978-91-7676-593-7

Transcriptional Regulation of Neuronal Differentiation in the Developing CNS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Vilma Rraklli

Principal Supervisor:
Associate Professor Johan Holmberg
Karolinska Institutet
Department of Cell and Molecular Biology
and Ludwig Institute for Cancer Research

Co-supervisor(s):
Post Doctoral Fellow Erik Sodersten
Karolinska Institutet
Department of Cell and Molecular Biology
and Ludwig Institute for Cancer Research

Application Specialist, Ulrika Nyman
Roche Diagnostic Scandinavia

Opponent:
Professor Carol Schuurmans
University of Toronto
Department of Biochemistry

Examination Board:
Associate Professor Eva Hedlund
Karolinska Institutet
Department of Neuroscience

Associate Professor Margareta Wilhelm
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Professor Karin Forsberg Nilsson
Uppsala Universitetet
Department of Immunology, Genetics and Pathology

The public defense of this thesis will be held in the CMB lecture hall (Berzelius väg 21) on Friday 24th of February 2017 at 9.30 AM.

To my family

ABSTRACT

The central nervous system (CNS) is responsible for our intellectual and cognitive functions and it comprises the brain and spinal cord. Generation of the CNS occurs during embryonic development from the neural tube that initially consist of a pool of immature progenitors that will give rise to all the neurons in the brain and spinal cord. CNS development is a highly coordinated process and any defect has a high risk of generating malformations and/or sensory, motor and cognitive impairments.

The large number and variety of neurons that form the CNS mirrors the complexity and multitude of functions of the system itself. Despite that they are all generated from the same pool of immature progenitors, neurons greatly differ from each other in morphology, function and in gene expression. During development, generation of newborn neurons requires immature progenitor cells to undergo sequential fate restriction from a pluripotent stem cell to neural progenitor and finally to a differentiated neuron. The journey from a progenitor cell to a mature neuron with specific functions occurs in different developmental stages that involve interpretation of environmental cues, cell cycle exit, downregulation of progenitor markers, migration, expression of neuronal genes and repression of genes of other lineages. During these processes, the morphological metamorphosis of a cell is matched by changes in gene expression. Consequently, neuronal differentiation of a cell leads to a final epigenetic and transcriptional landscape quite distinct from the one of the cell of origin.

During neuronal differentiation transcriptional regulation plays fundamental role in each step of the process from neural fate determination to neuronal specification. At a molecular level, neuronal differentiation is coordinated by transcription factors involved in all steps, such as cell cycle exit, loss of progenitor properties, restriction of other lineages, migration and acquisition of neuronal features. Despite the progress made in the field, a lot remains to be clarified about regulation of gene expression and regulation of transcriptional activity. The papers presented in this thesis aim to shed some light regarding the role of specific transcriptional factors at different stages of neuronal differentiation.

Paper I investigates the role of chromatin remodeler CHD5 during neurogenesis, focusing on two specific aspects of terminal neuronal differentiation: induction of neuronal features and repression of other lineages determinants. Our data, *in vitro* and *in vivo*, suggest that CHD5 has a dual role during neuronal differentiation: it facilitates the activation of neuronal genes and it synergizes with Polycomb group proteins to facilitate repression of alternative lineages determinant.

Paper II focuses on the role of ZAC1 transcription factor during neurogenesis and the importance of controlling its expression levels. Our data shows that elevated levels of

ZAC1 transcription factor promote cell cycle exit, block neuronal specification and induce non-neuronal lineage determinants.

Paper III investigates how changes in the surrounding environment, such as heat shock induced stress, affect transcriptional regulation, through the NOTCH pathway. Our *in vitro* and *in vivo* data show that stress induces sumoylation of NOTCH and its accumulation in the nucleus which results in repression of Notch target gene (*Hes1*, *Hes5*).

LIST OF SCIENTIFIC PAPERS

I. Chris M Egan, Ulrika Nyman, Julie Skotte, Gundula Streubel, Siobhan Turner, David J. O´Connell, **Vilma Rraklli**, Micheal J. Dolan, Naomi Chadderton, Klaus Hansen, Gwyneth Jane Farrar, Kristian Helin, Johan Holmberg and Adrien P. Bracken. CHD5 Is Required for Neurogenesis and Has a Dual Role in Facilitating Gene Expression and Polycomb Gene Repression. *Developmental Cell* (2013) 26, 223-226

II. **Vilma Rraklli**, Erik Sodersten, Ulrika Nyman, Daniel W. Hagey, Johan Holmberg Elevated levels of ZAC1 disrupt neurogenesis and promote rapid in vivo reprogramming. *Stem Cell Research* (2016) 16, 1-9

III. Christian A.M. Antila, **Vilma Rraklli**, Henri A. Blomster, Kathe M. Dahlstrom, Tiina A. Salminen, Johan Holmberg, Lea Sistonen, Cecilia Sahlgren.. Stress-inducible sumoylation of NOTCH1 represses its target gene expression. Manuscript submitted

PAPERS NOT INCLUDED IN THE THESIS

IV. Sjöqvist M, Antfolk D, Ferraris S, **Rraklli V**, Haga C, Antila C, Mutvei A, Imanishi SY, Holmberg J, Jin S, Eriksson JE, Lendahl U, Sahlgren C. PKCζ regulates Notch receptor routing and activity in a Notch signaling-dependent manner. Cell Research (2014) 4: 433-50.

V. Shen X, Burguillos MA, Osman AM, Frijhoff J, Carrillo-Jiménez A, Kanatani S, Augsten M, Saidi D, Rodhe J, Kavanagh E, Rongvaux A, **Rraklli V,** Nyman U, Holmberg J, Östman A, Flavell RA, Barragan A, Venero JL, Blomgren K, Joseph B. Glioma- induced inhibition of caspase-3 in microglia promotes a tumor supportive phenotype. Nat. Immunology (2016) 11:1282-1290.

CONTENTS

1. INTRODUCTION

1.1 Neural Development
- 1.1.1 Early nervous system and Neural Tube formation
- 1.1.2 Spinal cord development
- 1.1.3 Cortical development

1.2 Neuronal Differentiation in the developing CNS
- 1.2.1 Neuronal differentiation in spinal cord
- 1.2.2 Notch signaling
- 1.2.3 bHLH proneural genes
- 1.2.4 Neuronal diversity and differentiation in cerebral cortex

2. AIMS

3. RESULTS

3.1 Paper I
3.2 Paper II
3.3 Paper III

4. DISCUSSION

5. ACKNOWLEDGMENTS

6. REFERENCES

LIST OF ABBREVIATIONS

BMP	Bone Morphogenetic Protein
bHLH	basic Helix-Loop-Helix
CC	Corpus Callosum
CDK	Cyclin Dependent Kinase
CIP/KIP	CDK Interacting Protein/Kinase Interacting Protein
CKI	Cyclin Dependent Kinases Inhibitor
CNS	Central Nervous System
CPN	Callosal Projection Neuron
CThPN	Corticothalamic Projection Neuron
ESC	Embryonic Stem Cell
FGF	Fibroblast Growth Factor
H3K4me3	Histone 3 Lysine 4 Tri Methylated
H3K27me3	Histone 3 Lysine 27 Tri Methylated
NE	Neuroepithelium
NGN2	Neurogenin 2
NICD	Notch Intracellular Domain
NPC	Neural Progenitor Cell
PRC2	Polycomb Repressive Complex 2
RA	Retinoic Acid
RG	Radial Glia
SHH	Sonic Hedgehog
SP	Subplate
SUMO	Small Ubiquitine-like Modifiers
SVZ	Subventricular Zone
TGFß	Transforming Growth Factor Beta
VZ	Ventricular Zone

1. INTRODUCTION

The central nervous system (CNS), beyond being the seat of our highest intellectual functions and responsible for our cognitive ones, is one of the most complex organs. Despite the progress made the last centuries in the field of neural development, a lot remains to be discovered about its generation and the establishment of neural circuits during development.

The first description of the brain dates back to about 4000 years ago in ancient Egypt: the Edwin Smith surgical papyrus, named after the person who discovered it and dated to the 17th century BC It contains not only symptoms, diagnosis and prognosis of patients with head trauma but also a description of what is now known as the brain.
The complexity of the CNS, its vital role for life and its extension throughout the organism, might explain why the major discoveries about the brain have been achieved only in the last century. Advances in technology and methodology are setting the stage for new discoveries and a deeper understanding of the CNS.

The nervous system is responsible for coordinating actions, voluntary and involuntary movements by transmitting signals from and to all parts of the body (Kandel et al. 2012) hence, malfunction in the nervous system can lead to disease if not death of the animal.

In vertebrates, the nervous system is divided in peripheral nervous system and CNS. The CNS is a bilateral and symmetrical structure composed by the brain and spinal cord. In mammals, the brain comprises seven major structures: the medulla oblongata, pons, cerebellum, midbrain, diencephalon and cerebrum (Kandel et al. 2012).

The human brain has about 100 billion neurons (Herculano-Houzel 2009) of several different classes capable to communicate and interact in functional neural circuits.Specific tasks of the brain such as sensation, movement and cognition, are controlled by distinct regions (40-50 in the human brain) that differ in lamination, connectivity and neurochemistry (Caviness 1975; Job and Tan 2003; Sur and Rubenstein 2005)

While the brain is responsible for the cognitive functions and processing of information, the vertebrate spinal cord transmits sensory information from the periphery to the brain and then sends back the processed information to pertinent parts of the body. The spinal cord is also responsible for a number of sensory-motor computations among which those needed for proper control of movement. It has been observed that a de-cerebrated cat is still capable of coordinated movements when suspended above a treadmill even though control from the forebrain has been lost (Brown, 1911; Gifford et al. 2013).

Neurons and most of neural circuits are generated before birth during neural development and defects during these processes, result in malformations and numerous sensory, motor

and cognitive impairments. Hence, in order to understand the complexity of the CNS and possible malfunctions therein, the knowledge of neural development is of vital importance.

1.1 Neural development

The formation of the nervous system is initiated early during development and it produces the most complex structure in the embryo. It is a highly organised process that requires coordination of many events among which proliferation, cell cycle exit, migration and differentiation. During each step of development, cells receive signals, via morphogens or cell-cell communication, interpret the message and respond by changes in gene expression. Errors during development can lead to severe defects or even be lethal for the organism therefore, these processes need to be tightly controlled.

1.1.1 Early nervous system and neural tube formation

Immediately after the formation of the primitive streak, the structure that determines the bilateral symmetry of the embryo, cells start migrating and through the process of gastrulation generates the three germ layers: endoderm, mesoderm and ectoderm, which will form all the tissue and organs of the embryo (Reh et al 2005). The CNS is derived from cells within the ectoderm. Early nervous system development starts with neurulation, the process of neural tube formation (Figure 1, Jessell 2000).

During neurulation, cells keep proliferating and migrating along the primitive streak forming the neural folds and between them the neural groove. The neural folds start elevating and the groove deepens gradually (Figure 1a). Once the neural folds come closer to each other, they meet in the middle line converting the groove into a closed tube (Figure 1c), the neural tube, which will form the nervous system (Jessell 2000).

Initially, the neural tube consists of a pool of immature cells that can differentiate into all the cells of the future brain and spinal cord (Gifford et al. 2013). As the neural tube develops, its most rostral part will give rise to the telencephalon (Hebert 2005) (future cerebral cortex and basal ganglia) and its most caudal part to the spinal cord (Gifford et al. 2013). The process that leads equally potential cells of the neural tube to diverse fates is regulated during early development mostly via morphogen gradients that are released from the neighboring tissues, mainly notochord, floor plate, roof plate and presomitic mesoderm along three orthogonal axes: mediolateral, dorsoventral and rostrocaudal (Gifford et al. 2013; Carpenter et al. 2013). The effectors responsible for the acquisition of different neuronal identities are distinct transcription factors that are induced according to gradient concentrations of morphogens. (Wilson and Maden 2005).

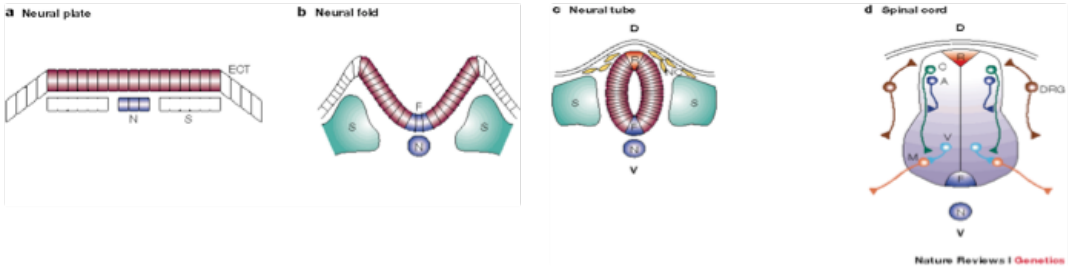

Figure 1. Neural tube formation and Spinal cord development. At the neural plate stage (a) cells proliferate and migrate toward the ventral midline to form the neural folds (b). As the neural folds come closer, they meet to form the neural tube (c). In the developing spinal cord (d), commissural (C) and association (A) neurons differentiate in the dorsal half of the spinal cord, and motor neurons (M) and ventral interneurons (V) develop in the ventral half of the neural tube. Adapted **from Jessell 2000**

1.1. 2. Spinal cord Development

In the developing spinal cord, rostrocaudal orientation is determined by fibroblast growth factor (FGF) signaling, released from the primitive node and presomitic mesoderm, and retinoic acid (RA), released from somites in the paraxial mesoderm (Gifford et al. 2013; Carpenter et al. 2013). In response to the FGF and RA signaling, cells of the developing spinal cord will express transcription factors encoded by the *Hox* gene family which contain a turn-helix-turn DNA binding domain and function as transcription regulators by activating genes responsible for the division of the spinal cord into cervical, thoracic, lumbar and and sacral domain along the rostrocaudal axis (Figure 2 left) (Carpenter et al. 2002, Dasen and Jessel 2009). In fact, it is thought that specific combinatorial signals from paraxial mesoderm define regionalisation along the rostrocaudal axis of the neural tube: cervical -high RA, brachial - low RA - low FGF, thoracic - high FGF, lumbar – high FGF – high Gdf11 (Carpenter et al 2103; Wilson and Maden 2005; Dasen and Jessell 2009).

A transverse section of the spinal cord reveals a topological organization. Neurons responsible for receiving and communicating sensory information from peripheral neurons of the dorsal root ganglia are located in the dorsal half of the spinal cord, while neurons responsible for the processing of motor output reside in the ventral part of the spinal cord. This dorsoventral organisation of the spinal cord is mirrored during its early development; the dorsal part is populated with interneuron progenitors whereas the ventral contains also motorneuron progenitors (Figure 1d) (Gifford et al 2013).

Mediolaterally, within the most inner layer of the developing spinal cord, the ventricular zone hosts many progenitor domains with specific transcriptional signature which will give rise to definite neuronal and glial lineages. While these progenitors stop proliferating, exit cell cycle, migrate to the mantle layer (outer layer of the developing spinal cord), and finally mature, expression of an ulterior transcriptional program is needed for proper differentiation (Jessell 2000, Shirasaki and Pfaff 2002, Briscoe and Ericson 2001).

According to the specific transcriptional signature, 11 progenitor domains can be found in the ventricular zone of the developing spinal cord: 6 interneuron progenitor domains (dp1-6) located in the dorsal part and 5 progenitor domains in the ventral one (p0-3, pMN). These progenitors will then generate the corresponding neuron population: dI1-dI6 interneurons will reside dorsally while V0-V3 interneurons and motor neurons (MN) will reside in the ventral half of the spinal cord. (Jessell 2000, Shirasaki and Pfaff 2002).

Expression of the transcriptional factors that determine the distinct progenitor domains is regulated by signals secreted from the ventral floor plate (Sonic Hedgehog) and the dorsal roof plate (WNT and BMP) (Wilson and Maden 2005). Expression and secretion of SHH from the notochord induces expression of the morphogen in the floor plate which results in different concentration of the morphogen along the dorsal ventral axis of the developing spinal cord: high in the ventral half and low in the dorsal one (Jessell 2000) (Figure 2 Right). In the dorsal region of the spinal cord BMP, TGFß secreted from the overlying ectoderm and WNT are responsible for defining not only dorsal progenitors identity but also the ventral ones. Even though morphogen gradient along the developing spinal cord have been thoroughly studied in the last decades, it is not completely understood how they interact with each other. It has been shown that the combination of exposure to specific morphogens at different concentration induces a certain set of transcription factors that determine the identity of definite progenitor domains (Gifford et al. 2013, Wilson and Maden 2005). For example, ventrally, high concentrations of SHH correspond to low concentrations of Class I transcription factors (*Pax6*, *Dbx1*, *Dbx2*) and induced expression of Class II transcription factors (*Nkx2.2*, *Nkx6.1*, *Nkx6.*). The expression of specific transcription factors in each cell along the neural tube will determine its belonging to a certain progenitor domain therefore, its fate (Figure 2 Right).

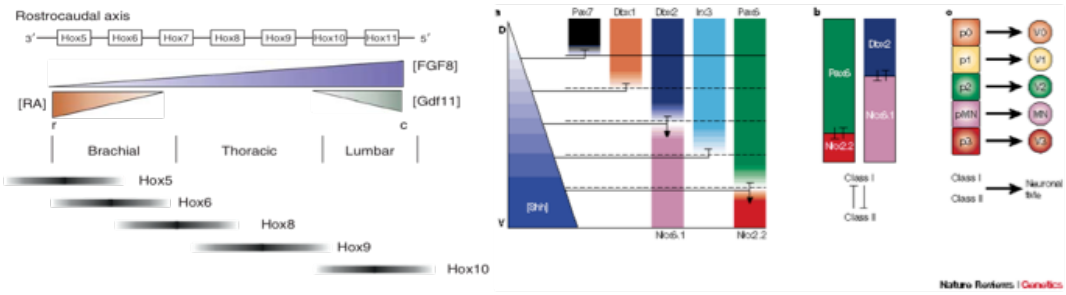

Figure 2. Patterning along the rostrocaudal (left) and dorsoventral (right) axes of the neural tube. Left: *Hox* genes at one end of the cluster are expressed in the rostral part (r) while genes at the other end are expressed in the caudal part (c) where there are higher levels of FGF. Rostrally *Hox* genes are regulated by graded RA signaling while caudally *they* are regulated by graded GDF11. (Figure from Dasen and Jessell 2009) **Right**. Motor neurons and ventral interneurons are generated along the dorsoventral (d-v) axis according to the graded concentration of sonic hedgehog (SHH) which induces expression of specific transcription factors in progenitor cells. Class I factors are induced by Shh while Class II transcription factors are repressed. The identity of progenitor domains is also influenced by selective cross- repressive interactions between Class I and Class II transcription factor (Briscoe et al., 2000). Figure from Jessell 2000.

1.1.3. Cortical Development

During early embryonic development, the caudal part of the neural tube gives rise to the future spinal cord and the most rostral part, generates the telencephalon. The cortex, also known as pallium develops from the ventricular zone of the dorsocaudal part of the telencephalic vescicle (Sur and Rubenstein 2005).

The neocortex, which is generated from the dorsal pallium, is the largest part of the cerebral cortex and is of vital importance for the cognitive functions. Different areas of the neocortex are responsible for different tasks, e.g. rostral regions of the neocortex regulate motor and executive function while caudal regions process somatosensory, auditory and visual inputs (Sur and Rubenstein2005).

Until 1990, two hypotheses were trying to explain how regionalization of the brain was generated: the protocortex (O´Leary 1989) and protomap (Rakic 1988) hypotheses. While the protocortex hypothesis claimed that thalamic afferent axons induce cortical areal identity via activity dependent mechanisms, the protomap one states that the information responsible for the identity of distinct cortical areas resides within the cortical progenitor zone (Sur and Rubinstein 2005). Further research conducted in the field suggested that the protomap hypothesis better describes the early steps of neocortical patterning (Sansom and Livesey 2009). Nowadays, it is well accepted that secreted proteins from patterning centers generate positional information: *Shh* is expressed in the ventral telencephalon, *Bmp* and *Wnt* families are expressed along the dorsal midline whereas *Fgf8* is expressed at the rostral margin of the telencephalon (Figure 3) (Sur and Rubenstein 2005). Similarly to the spinal cord, the graded signals coordinate regionalization of the cortex by inducing graded expression of distinct transcription factors such as *Foxg1 (BF1*), *COUPTF1*, *Emx2*, *Lef1*, *Lhx2* that control proliferation, neurogenesis, migration, connectivity and cell death/ survival (Sur and Rubenstein 2005, Sansom and Livesey 2009). For instance, Fukuchi-Shimogori and Grove in 2001 demonstrated that FGF8, secreted from the rostral midline, acts in a concentration dependent manner to induce rostrocaudal positional identity in neocortical stem cells via changes in gene expression (Fukuchi-Shimogori and Groove 2001; Sansom and Livesey 2009). More specifically, the data suggests that high concentrations of FGF determine the rostral most motor cortex while low concentrations of FGF specify somatosensory and visual cortex (Sansom and Livesey 2009). Moreover, *Fgf8* increases *Foxg1* expression and reduces *Emx2* and *COUPTF1* transcription factor expression that are necessary for regionalization of the cortex (Bishop et al 2002; Sansom and Livesey 2009). For instance, Bishop et al. in 2002 showed that *Emx2* induces caudomedial area identities while *Pax6* induces rostrolateral ones. In 2007, Armentano et al. demonstrated that *COUPTF1* is crucial for promoting a caudal identity in the neocortex. In mice where *COUPTF1* was depleted in the cortex, there was an increase in cells with a motor cortex transcriptional signature whereas the somatosensory and visual areas were significantly smaller. Transcription factors induced by gradient signals in turn induce

expression of distinct region and layer-specific transcription factors that determines the fate of the future neurons.

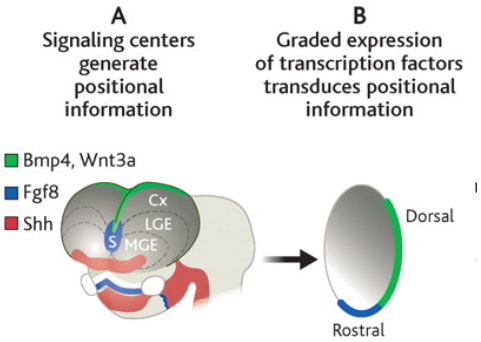

Figure 3. During development regionalization of cortical areas in the neocortex is determined by secreted proteins form patterning center which in turn induce expression of transcription factors specific to the area. Adapted **from Sur and Rubenstein 2005**

1. 2. Neuronal Differentiation in the developing CNS

The human brain is estimated to contain roughly 100 billion neurons (Herculano-Houzel 2009). The variety of the building blocks of the CNS mirrors the multitude of functions of the system itself. For instance, according to their position, function, even "birthdate" they can greatly differ from each other in morphology, connectivity, neurochemistry etc., despite the fact they all generate from the same pool of immature progenitors. The journey from a progenitor cell to a mature neuron with specific functions has different stages that involve cell cycle exit, downregulation of progenitor markers, migration, expression of neuronal genes and repression of genes of other lineages. During these processes, the morphological metamorphosis of a cell is matched by changes in gene expression. Consequently, neuronal differentiation of a cell leads to a final epigenetic and transcriptional landscape quite distinct from the one of the cell of origin.

1.2.1. Neuronal Differentiation in the developing spinal cord

In the developing spinal cord, proliferative progenitor cells reside in the ventricular zone surrounding the central canal and their proliferation expands the progenitor pool (Matise and Sharma 2013). As the cell cycle progresses, they extend long processes from the pial (outside) part of the spinal cord to the apical (close to the central canal) part along which they translocate their nuclei during what is known as interkinetic nuclear migration (Matise and Sharma 2013). Once a progenitor cell has exited cell cycle, it migrates towards the mantle zone, upregulates expression of neuronal genes and thus terminally differentiates into a neuron.

In the developing spinal cord, cell cycle exit is accompanied by upregulation of cyclin-dipendent kinase inhibitors CKI proteins, inhibitors of G1 cyclins, in particular two CDK (cyclin dependent kinase) interacting protein/kinase interacting protein (CIP/KIP) class CKIs, P27 and P57. Despite the fact that both factors can be induced by proneural basic-helix-loop-helix (bHLH) factors such as *Ngn2*, they are not necessary for cell cycle exit (Gui et al. 2007). It has also been suggested that cell cycle exit in the ventral spinal cord is triggered by the diminishing concentrations of G1 cyclins which is due to reduced concentrations of dorsal WNT signaling. In fact, lower levels of WNT in ventral regions result in a decrease of cell proliferation (Megason and McMahon 2002).

Self-renewing progenitors in the ventricular zone express SOXB1 transcription factors (SOX1-SOX3) and when cells exit cell cycle the levels of SOX1, SOX2, and SOX3 are downregulated. SOXB1 transcription factors belong to the SOX family, an HMG-box transcription factors known to be among the first regulators of neurogenesis and they can act as both transcriptional activators and repressors. It has been shown that these transcription factors when overexpressed in the chick developing spinal cord, block neuronal differentiation and maintain progenitor cells in an undifferentiated state by maintaining the expression of progenitor properties and inhibiting the capacity of bHLH proteins to promote cell cycle exit and consequently neuronal differentiation (Bylund et al. 2003, Holmberg et al. 2008).

1.2.2. Notch signaling

One signaling strategy in the developing spinal cord, also well studied in other tissues and many organisms and adopted for maintenance of uncommitted progenitor pool, is the NOTCH pathway (Andersson et al. 2011). The *Notch* genes (*Notch1–4* in mammals) encode membrane-bound receptors whose cognate ligands include the DELTA (DLL11, DLL3, DLL4) and JAGGED (JAG1, JAG2) families of membrane-bound proteins (Lai et al. 2013).

The NOTCH pathway is activated by cell-cell communication with a NOTCH-ligand expressing cell. In the proliferative cells of the VZ of the spinal cord three Notch receptors have been found to be expressed: JAGGED 1, JAGGED 2, and DLL1. Once a Notch receptor has been activated by contact with an adjacent Notch positive cell, the intracellular domain of the Notch receptor is cleaved releasing the NOTCH intracellular domain (NICD) that is then transported to the nucleus. Once in the nucleus, NICD binds to the transcription factor CSL (CBF1/Suppressor of Hairless/Lag-1) which leads to an allosteric change, consequent activation of CSL and recruitment of the transcriptional coactivator protein Mastermind-like (MAML). The formation of the ternary complex NICD-CSL-MAML is necessary for activating expression of NOTCH target genes (Louvi and Artavanis-Tsakonas, 2006, Kopan and Ilagan 2009, Wang et al 2015).

Among the target genes of activated NOTCH are the bHLH transcription factors *Hes1* and *Hes5* that act as DNA binding repressors and counteract the expression of proneural genes (Louvi and Artavanis-Tsakonas 2006, Ohtsuka et al 1999). It has also been suggested that HES transcription factors antagonise neurogenesis by forming non-functional pairs with proneural proteins or E-proteins (Fischer and Gessler, 2007; Sasai et al., 1992, Holmberg et al 2008).

One role of NOTCH in the developing CNS is to control the balance of undifferentiated and differentiated neural cells via regulation of proneural bHLH proteins and E-proteins (ubiquitously expressed bHLH proteins) expression levels. It has been suggested that E – proteins fact as obligatory heterodimerizing partner factors of proneural proteins preventing this way neuronal differentiation (Holmberg et al. 2008).
During early development of CNS, Notch mediated lateral inhibition is initiated by bHLH proneural genes: bHLHs upregulate expression of NOTCH ligands that in turn activate NOTCH signaling in the neighbouring cell (Huang et al. 2014; Skeath and Carroll 1994; Johnson and Glasgow 2009) which will block neurogenesis via *Hes1* and *Hes5* as described previously and keep the neighboring cells in a progenitor state (Figure 4).

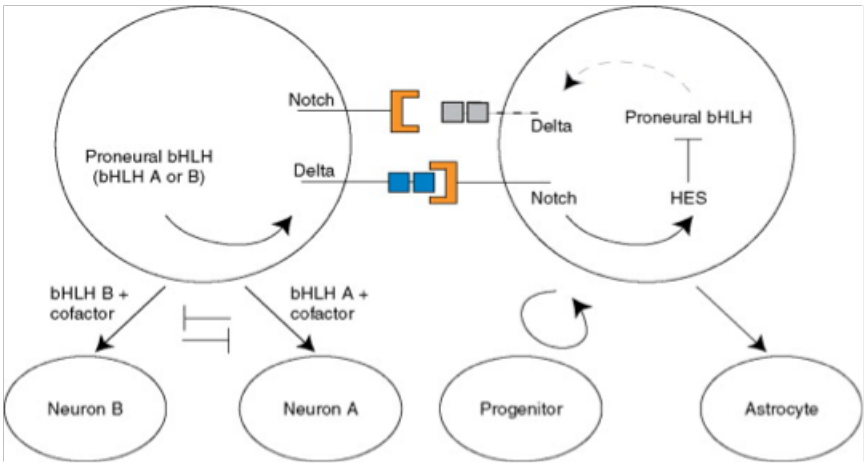

Figure 4. Interaction between Notch signaling and bHLH transcription factors. High levels of bHLH proneural protein in one cell enhances the expression of Delta ligand that in turn activates Notch signaling in the neighboring cell. Activation of Notch signaling induces expression of bHLH transcription factors Hes1and Hes5 which in turn represses the proneural bHLH transcription factors in that cell. High levels of Notch and low levels of proneural bHLH transcription factors will maintain the progenitor state whereas low levels of Notch and high levels of proneural bHLH transcription factors will induce neuronal differentiation. **From Johnson and Glasgow 2009**

1.2.3 bHLH proneural genes

bHLH proneural genes encode for a family of transcription factors that are involved in the regulation of different developmental processes comprising cellular differentiation and lineage commitment not only in the nervous system but also in other organs such as heart and muscle. During the entire development of the nervous system their role is crucial from neural fate determination to neuronal subtype specification (Johnson and Glasgow 2009). The ubiquity of these transcription factors and the ability to regulate different processes is partly due to the structure of the proteins they encode for. Their basic domain is responsible for contacting the major groove of the DNA while the HLH (Helix-Loop-Helix) domain composed by two helices divided by a loop mediates protein hetero or homodimerization (Bertrand et al. 2002; Powell and Jarman 2008, Huang et al. 2014). The 60 amino acid bHLH (basic-Helix-Loop-Helix) motif binds to a core hexa-nucleotide e-box, CANNTG (Johnson and Glasgow 2009).

The bHLH family, highly conserved through evolution, was first discovered in Drosophila melanogaster responsible for promoting a neural identity, rather than an epidermal one, onto naïve ectodermal cells (Skeath and Carrol 1994, Huang et al 2014). This family included members of the achete-scute complex (achete (*ac*), scute (*sc)*, lethal of scute (*lsc*) and asense (*as*)), *ato*, *amos* and *cato* (Bertrand et al 2002). In vertebrates, *ato* genes belong to three different families: neurogenin genes (*Neurog1*, *Neurog2*, *Neurog3*) neurogenic differentiation genes (*NeuroD1, NeuroD2, Neurod4/Math3/Atoh3, Neurod6/ Math2/Atoh2, Atoh1/Math1, Atoh7/Math5*) and *Olig* genes (*Olig1, Olig2, Olig3*) (Huang et al. 2014).

According to their dimerization capabilities, DNA binding specificities and expression pattern, the bHLH transcription factors have been classified in two groups: class A and class B. Broadly expressed and often called E-proteins, class A comprises all class I bHLH transcription factors including HEB (TCF12, MGI), E2-2 (TCF4, MGI), and the two splice variants of E2A (TCFE2A, MGI), E12 and E47 in vertebrates. E-proteins form homodimers as well heterodimers with class B proteins. Class B proteins comprises from class II to class VII, which vary in protein motifs and activity, can act both as repressors and activators of downstream target genes resulting in both inhibition or activation neuronal differentiation.

Class II includes *Mash1 (Ascl1, MGI), Math1 (Atoh1, MGI), Math5 (Atoh7, MGI), Ngn1, 2, 3 (Neurog1, 2, 3, MGI)*, and *Neurod1* which are known to form heterodimers with E-proteins, bind DNA at e-box elements and promote neurogenesis, regulation and activation of neuronal specification. It has been shown that class II factors such as *AsclI, AtohI, Neurog1* and *Neurog2* promote neuronal differentiation while inhibiting gliogenesis (Bertrand et al 2002). Also, introduction of *Ascl1*, *Neurog1/2*, *Neurod4* or *Atoh1* in chick spinal cord resulted in excess neurons (Lee et al 2005, Nakada et al 2004, Lai et al 2013) while forced expression of *Neurog1* in cortical progenitor cultures promoted neurogenesis

(Sun et al 2001, Lai et al 2013). Moreover, misexpression of *Ascl1* and *Neurog1* induced neuronal differentiation in P19 embryonal carcinoma cells by inducing cell cycle exit and neuronal specific genes (Farah et al 2000, Lai et al 2013). Forced cell cycle exit and consequent neuronal differentiation was observed also after electroporation of *Mash1, Math1, Ngn1* and *Ngn2* in the chick neural tube (Johnsons 2009). In fact, it is well accepted that upregulation of proneural genes results in cell cycle exit and neuronal differentiation (Bertand et al. 2002, Lai et al. 2013).

Class VI bHLH transcription factors include HES proteins (homologs to Drosophila hairy and enhancer-of-split) and are known inhibitors of neurogenesis by binding to N-boxes with recognition site CACNAG located in the upstream regulatory regions of proneural genes inhibiting this way their function (Huang at el 2014, Lai et al 2013)
As described previously, another functional characteristic of proneural genes is their ability to transactivate and initiate NOTCH signaling. For instance, class II and class VI transcription factors participate in a regulatory circuit based on cell-cell communication via the NOTCH-DELTA signaling pathway that serves to limit the number of differentiating cells and maintain the progenitor population (Johnson and Glasgow 2009)
Also, it has been shown that genes encoding for NOTCH ligands *Dll1* and *Dll3* are direct targets of *Ascl1* in the telencephalon and neural tube together with other genes involved in cell cycle exit and differentiation (Castro et al. 2006).

The omnipresence of bHLH transcription factors during the entire process of neurogenesis and given the complexity and sophistication of the process itself requires a great number of such factors that are activated at different stages of neurogenesis and development in general. For instance, some proneural bHLH proteins are crucial for neural fate determination while others are needed for neuronal differentiation.

1.2.4. Neuronal Diversity and Differentiation in the developing cortex

The human cerebral cortex makes up two thirds of the neuronal mass and contains about three quarters of all our synapses. The cerebral cortex is organised in cytoarchitectonic areas with distinct cellular, biochemical, connectional and physiological features that were found to serve specific functions such as language, facial recognition or spatial orientation (Rakic 1988, Parnavelas 2000). These specific areas and the interactions among them are established during development of the cerebral cortex. In 1988, Pasko Rakic formulated a currently well accepted theory describing cortical development and neuronal differentiation, the radial unit hypothesis: it postulated that areas of the cortex develop in cortical columns or "radial units" which originate form a neural stem cell (known as radial glial) layer called the ventricular zone. Neurons generated from a specific proliferative radial unit migrate along the radial glial guides through the intermediate zone until they reach the subplate. Moreover, formation of specific cytoarchitectonic areas depends on

spatial distribution while position within specific layers and neuronal phenotype depend on their time of origin, (Rakic1988).

In spite of functional differences among different areas in which the cerebral cortex is subdivided, it displays a general organisation of neurons into six histologically distinct layers defined as supragranular (layer I/II-III), granular (layer IV), and intragranular layers (layers V-VI). Layers contain different type of neurons endowing them with diverse morphological characteristics that vary in an area- specific manner. (Lodati and Arlotta 2015, Greig et al. 2013).

There are two main classes of cortical neurons: interneurons responsible for local connections and projection neurons whose axons extent to distant intracortical, subcortical and subcerebral targets. Interneurons, together with Cajal- Retzius neurons, are GABA (gamma aminobytiric acid) inhibitory neurons and are generated from progenitors located in the ventral telencephalon and cortical hem respectively, that migrate long distances in order to reach their final destination in the neocortex (Molyenaux et al. 2007, Wonders and Anderson 2006). Projection neurons characterized by a pyramidal morphology and responsible for transmitting information between different areas of the neocortex and other regions of the brain, are excitatory glutamatergic neurons that are generated from progenitors situated in the ventricular zone of the dorsal lateral wall of the telencephalon.

With interneurons making up for 20-30% of the neuronal population in the cortex (Arlotta and Lodato2015), glutamatergic pyramidal neurons are the most represented type of neurons and based on their hodology, they are classified into three basic classes of cortical projection neurons. Associative projection neurons extend axonal projections within a single hemisphere, commissural projection neurons within the cortex to the opposite hemisphere through the corpus callosum or the anterior commissure whereas corticofugal projection neurons, such as subcerebral and corticothalamic projection neurons reach targets outside the cortex (Molyenaux et al. 2007, Greig et al. 213). Layers of the cerebral cortex are occupied by a variety of distinct neurons: for instance, while layer I is mostly composed of Cajal–Retzius neurons, layer II/III is populated by different classes of commissural neurons such as callosal projection neurons (CPN). CPNs reside also in layer V together with subcerebral projection neurons that connect the cortex with other areas of the brain (brain stem, spinal cord and superior colliculus). Layer VI is the result of different classes of corticothalamic projection neurons (CThPN) projecting to distinct thalamic nuclei and CPNs that connect through the corpus callosum (CC). Specific cortical projection neurons express a distinct gene signature: for example, *SatB2* is expressed in CPNs of layer II/III, and V; *FezF2* is expressed in *CThPN* of layer VI while *Cux1* is expressed in CPNs of layer II/IIII (Lodato and Arlotta 2015).

All neurons forming the mammalian cortex are generated in a determined window of time that is species specific: for example, in humans cortical neurogenesis lasts from gestation week 5 to 20 (Bystron et al. 2008) whereas in mice it lasts from embryonic day E11 to E19

(Lodato and Arlotta 2015). During early development, the undifferentiated epithelial cells of the telencephalic wall proliferate via symmetric divisions in order to expand the progenitor pool and start differentiation into radial glia (around E10.5 in mice) that will result in the formation of the ventricular zone (Haubensak et al. 2004) (Figure 5). Radial glia divides asymmetrically to self-renew while also giving rise to outer radial glia and intermediate progenitor that will constitute the subventricular zone (SVZ) and act as transit amplifying cells to increase neuronal production (Noctor et al. 2004). The first neurons to be generated are the Cajal-Retzius ones at E10.5 in mice and they migrate from the ventricular surface to form the preplate. The next wave of neurons migrates into the preplate and divides it into preplate and subplate while establishing the cortical plate around E12.5 in mice. From now on, neurons will be generated in an inside-out manner: early born neurons will reside in deeper layers (layer VI then layer V) whereas late born neurons will migrate past them, using radial glia as a scaffold, to populate the upper layers (first layer IV, then layer II/III) (Figure 5). At the end of neurogenesis, around E17.5 in mice, the radial scaffold begins to be dismantled and neural progenitors switch to gliogenesis giving rise to subependymal zone astrocytes and to a layer of ependymal cells (Kwan et al. 2012, Molyenaux et al. 2007, Greig et al. 2013)

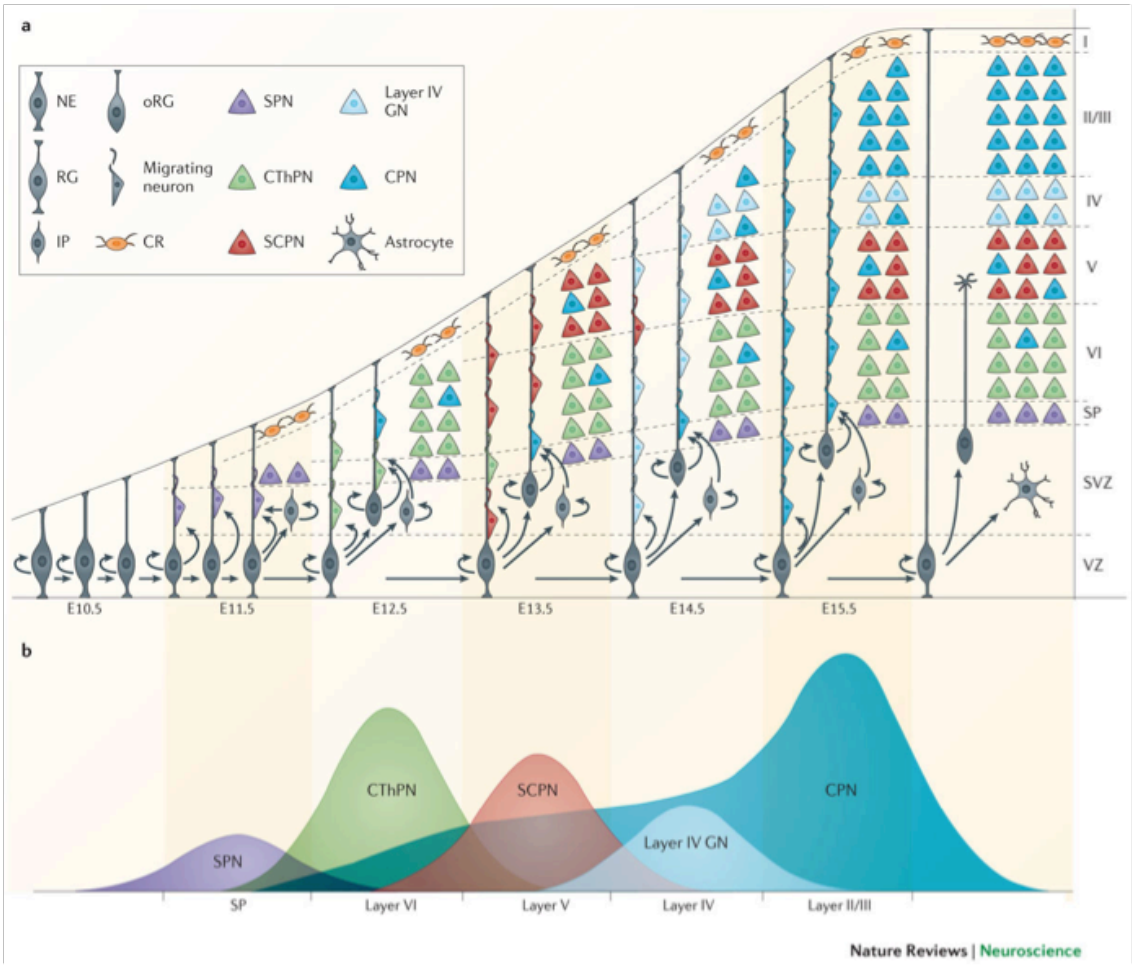

Figure 5. Schematic of inside-out manner of generation of projection neurons form diverse progenitor types in the VZ/SVZ during development of the mouse brain. From **Greig et al 2013**

The diversity of neuronal subtypes, their controlled organization and, the capability to interpret spatial and temporary clues in order to give rise to specific neurons, is mirrored by the unique transcriptional signature in each step of neuronal differentiation from progenitors to postmitotic neurons.
Initially, the transition from neuroepithelium (NE) to radial glia (RG) at the onset of neurogenesis in the dorsal telencephalon corresponds to activation of NOTCH signaling and expression of NOTCH ligand delta like (*Dll1)*. This activation, as mentioned earlier, is followed by upregulation of Notch downstream genes *Hes1* and *Hes5* (Martynoga 2012, Hatakeyama et al. 2004). Induction of *Dll*1 is accompanied by the appearance of proneural proteins *Ngn2* and *Ascl1*, known transcriptional regulators of neurogenesis. It has been suggested that in order for Ngn2, at first characterized by oscillatory expression, to induce neuronal differentiation it has to be stably expressed. Progenitors exposed to stable levels of NGN2 have more time to respond to its proneural effect and undergo neuronal differentiation (Huang et al 2014). In fact, it has been observed that an increase in number of progenitors undergoing neuronal differentiation coincides with G1 lengthening (Shimojo, Ohtsuka, Kageyama 2008, Calegari, Haubensak, Haffner and Huttner 2005, Huang et al 2014). Moreover, in 2006 Castro et al. showed that NGN2 activates downstream transcription factors necessary for neuronal differentiation such as *Neurod1, Neurod4, Tbr2, Rnd2, Dll1* and *Dll3*. Similarly to *Ngn2, Ascl1*, because of its ability to transactivate Notch signaling and initiate Notch mediated lateral inhibition, keeps progenitors in a proliferative state when its expression is oscillating whereas it promotes differentiation when it is stably expressed (Imayoshi et al 2013, Huang et al 2014).

During the generation of early born neurons, which will give rise to deep layers neurons, specific transcription factors, such as SOX5, have been found to be expressed or enriched in particular layers or subtype of cortical projections. SOX5 is expressed in post mitotic SP and layer VI projection neurons and it has been shown that it regulates migration, molecular differentiation and axonal projection of deep layer neurons (Kwan et al. 2008, Lai et al. 2008). Moreover, it has been shown that SOX5 downregulates *Fezf2* (fez family zinc finger 2) and *Ctip2* that are specific to layer V neurons (Kwan et al 2008, Lai et al 2008).
Another transcription factor involved in the regulation of early born neurons is TBR1 which is expressed in corticothalamic projection neurons and similarly to SOX5, it downregulates *Fezf2* and regulates laminar positioning, molecular differentiation and axonal path finding (Hevner et al., 2001; Han et al., 2011; McKenna et al., 2011).

Laminar position and differentiation of late-born neurons, which will occupy upper layers of the cortex, are similar to the early born ones but they are regulated by different transcription factors. One important transcription factor that controls multiple aspects of upper layer neurons development is special AT rich sequence binding protein SATB2 (Alcamo et al. 2008) and is present in corticocortical projection neurons of layer II-V, starting at E13.5 in mice. SATB2 is also a positive regulator of layer specific markers of

corticocortical projection neurons such as *Cux2* and cadherin10 (*cdh10*) (Alcamo et al 2008).
Other well studied regulators of upper layer neurons migration and identity are POU3F2 (*Brn2*) and POU3F3 (*Brn1*), two domain transcription factors that are expressed at layers II-V starting at E14.5. In fact, double deletion of these transcription factors reduces not only expression of some upper layer markers but also proliferation in the VZ and SVZ form E14.5 affecting in this way, the generation of late born neurons (McEvilly et al. 2002, Sugitani et al. 2002).

Neuronal differentiation in the developing cortex is a highly coordinated process where transcriptional regulation plays a vital role during each step and every aspect of it. In spite of the great knowledge acquired in the past years regarding the molecular mechanisms behind transcriptional regulation, many aspects remain to be elucidated.

2. AIMS

During neuronal differentiation in the developing CNS, transcriptional regulation plays fundamental role in each step of the process from neural fate determination to neuronal specification. Control of gene regulation by transcription factors has been extensively studied in all cardinal stages of neuronal differentiation such as cell cycle exit, loss of progenitor properties, restriction of other lineages, migration and acquisition of neuronal features. However, despite the progress made in the field, due to the abundance of transcription factors, their ubiquity, their multiple functions and short timing of distinct differentiation stages, a lot remains to be clarified about regulation of gene expression and regulation of transcription factors themselves. The papers presented in this thesis aim to shed some light on the role of specific transcriptional factors at different stages of neuronal differentiation. Specifically, we explore the following aspects:

Paper I focuses around two specific aspects of terminal neuronal differentiation: induction of neuronal features and repression of other lineages determinants. Particularly, we investigate the role of chromatin remodeler CHD5 during neurogenesis and the mechanisms that cause aberration of neuronal differentiation upon CHD5 downregulation.

Paper II investigates the importance of controlling expression levels of transcription factors during neurogenesis. We aim to understand how elevated levels of the Zac1 transcription factor block neuronal specification and induce non-neuronal lineage determinants.

Paper III aims to deconstruct interpretation of environmental cues and consequent regulation of gene expression during neurogenesis. More precisely, the authors investigate the mechanisms responsible for altering gene expression in stem cells during development upon changes in the cellular environment, such as heat shock induced stress and how it leads to Notch sumoylation and repression of its target genes: the bHLH transcription factors *Hes1* and *Hes5*.

3. RESULTS

3.1 Paper I: CHD5 is required for neurogenesis and has a dual role in facilitating gene expression and polycomb gene repression

During development, generation of new cortical neurons from neural stem cells requires a loss of progenitor properties, acquisition of neuronal features (Molyenaux et al. 2007) and restriction of possible alternative lineages. Even though acquisition of neuronal properties via transcriptional regulation has been extensively studied, molecular mechanism responsible for repression of alternative lineages remain still unclear.

It has been shown that during lineage specification, chromatin regulators, seen as facilitators of such process rather than "main protagonists" (Holmberg and Perlman 2012), are involved in gene control regulation by "writing" and "reading" posttranslational modifications (Kouzadires 2007, Schubeler 2009). "Writer" proteins include Polycomb Repressive complex 2 that is associated with gene repression by mediating trimethylation of histone 3 at lysine 27 (H3K27me3) (Margueron and Reinberg 2011) whereas Trithorax group proteins are associated with gene activation by mediating deposition of H3K4me3 (Schuetengruber et al. 2011). Chromatin "reader" proteins have a conserved structural domain such as chromodomains, plant homeodomains (PHDs) and Tudor domains (Yap and Zhou 2010) responsible for interaction with histone N-terminal tails that allows them to bind or read posttranslationally modified histone proteins (Taverna et al. 2007).

The chromatin remodeler CHD5 belongs to the CHD1-9 family of reader proteins characterised by the presence of two N-terminal chromodomains and a helicase-like motif associated with nucleosome remodelling (Clapier and Cairns 2009). The reader proteins belonging to the subgroup CHD3-5 have also double PHD domains that have been shown, for CHD4 and CHD5 to bind unmodified H3K4 (Musselman et al. 2009; Paul et al. 2013).
CHD5 gene is located, together with other 23 genes, at 1p36 a region commonly lost in high-risk neuroblastoma (Brodeur 2003; Okawa et al. 2008). Even though CHD5 is expressed in the adult CNS (Garcia et al. 2010; Potts et al. 2011), very little is known about its role during development.

Several members of the CHD family have been suggested to be key players during development (Ho and Crabtree 2010) and *CHD5* has been shown to be expressed in neuronal tissue (Garcia et. al. 2010). Thus, we wanted to determine whether CHD5 played a role during neurogenesis. And, if so, how it affects neuronal differentiation.

We first established via qRT-PCR, western blot and immunohistochemistry that CHD5 is expressed in neural tissue during development and, it is upregulated during terminal differentiation of late stage neuronal progenitors. CHD5 expression pattern suggested that CHD5 is a neuron specific chromatin remodeler with roles in both CNS and peripheral

tissues of neural origin. In agreement with what observed in adult tissue, we found that CHD5 is expressed in the developing spinal cord and cortex as cells downregulated progenitor properties, exited cell cycle and acquired neuronal identity.

To determine the role of CHD5 during cortical development we performed *in vivo* knockdown *via in utero* electroporation at E14.5. CHD5 knockdown resulted in failure of cells to exit the proliferative zone (VZ/SVZ and intermediate zone) while a slight but significant upregulation of proliferative markers (KI67) was observed. Failure to move out of the proliferative zone was accompanied by failure to reach upper layers and consequently terminally differentiate into neurons. *In vivo* experiments showed that cells where CHD5 had been depleted are not able to downregulate progenitor properties and, consequently, to terminally differentiate.

We next performed knockdown of CHD5 into an *in vitro* model of neurogenesis in order to be able to perform qRT-PCR and gene expression array that would allow us to better understand the mechanisms by which CHD5 facilitates neuronal differentiation. Our data showed that inhibition of neuronal differentiation due to CHD5 reduced levels was accompanied by failure to activate several neuronal genes such as *Tubb3, NeuN*, and *Ncam*. Moreover, upon CHD5 depletion, polycomb target genes were upregulated.

Peptide pull down-assays performed in nuclear lysates generated from mouse cortices and human SH-SY5Y neuroblastoma cell line showed that CHD5 binds to H3K27me3 via its tandem chromodomains. In addition, genome wide ChIP-seq analysis showed that CHD5 binds a large cohort of genes and is required to activate neuronal genes during neuronal differentiation while. Also, CHD5 chromodomains bind to H3K27me3 while localization of CHD5 on a cohort of Polycomb target genes is necessary to maintain their repressed state.

Our *in vitro* combined with the *in vivo* data supports a dual role for CHD5 where it both facilitates the activation of neuronal genes and synergizes with Polycomb group proteins to facilitate the repression of genes encoding regulators of alternative lineages.
Our study suggests that, in addition to cell cycle exit, downregulation of progenitor features and upregulation of neuronal traits ,there is also a need to restrict expression of factors determining non-neuronal lineages.

3.2 Paper II: Elevated levels of ZAC1 disrupt neurogenesis and promote rapid *in vivo* reprogramming.

During neurogenesis, timing of cell cycle exit is crucial for proper neuronal differentiation and generation of new neurons requires strict control of gene expression during each step of the process: from proliferation of a progenitor until migration and maturation of the newly born neuron. In paper II we focused on the role of the transcription factor ZAC1 during cortical development. ZAC1, already shown to induce apoptosis and cell cycle

arrest (Spengler et al. 1997) belongs to an imprinted gene network (IGN) that is involved in embryonic growth (Varrault et al. 2006). Zac1 is expressed in the germinal zone (Valente et al. 2005) and recently, it has been shown that ZAC1 is necessary in the developing cortex for proper neuronal migration (Adnani et al. 2015). Despite this, a lot remains to be elucidated regarding the role of ZAC1 during neurogenesis.

To elucidate its role during neurogenesis, we first established, via immunohistochemistry in chick spinal cord and mouse cortex, that ZAC1 is expressed in proliferating progenitors both in the forebrain and spinal cord during CNS development whereas its expression is lost upon cell cycle exit and in postmitotic neurons. *In vivo* overexpression of *Zac1* in cortex and spinal cord via, respectively, *in utero* and *in ovo* electroporation led to premature cell cycle exit and, differently to what observed upon overexpression of proneural genes, there was no precocious differentiations. On the contrary, further immunostaining with post mitotic markers showed that neuronal differentiation was obstructed.

To elucidate the mechanisms that led to precocious cell cycle exit and obstructed neuronal differentiation we performed genome wide analysis *via* RNA-Seq in FACS sorted cells from mouse cortices electroporated with *Zac1*. Cell cycle exit observed initially was mirrored also in the genome wide analysis by reduced expression of positive regulators of cell cycle such as *MycN, Ccnf* and *Cxl12*. Moreover, RNA-seq showed that, while expression levels of early proneural bHLH factors such as *Neurog1* and *Neurog2* were unaltered, their downstream targets such as *Neurod1*, *Neurod6*, *Dlx2* and *Rnd3* together with pan-neuronal genes such as *Map2* and *Tubb3* showed decreased expression. In *Zac1* overexpressing cells, we also observed an increased expression of genes belonging to IGN such as *Igf2, H19, Dlk1, Gnas* and *Igf2as*. Interestingly, GO analysis of the sequencing data showed an enrichment of mesodermal and muscle associated genes in cells overexpressing *Zac1* and a significant increase of determinants of early mesoderm/ endoderm formation such as *Tbx6* (Takemoto et al. 2011) myogenic fate determination such as (*Myod1* and *Myog*) together with *Desmin* and *Myoz1* known structural proteins of muscle fibers. This data, was supported by further RT- qPCR performed in mouse neuronal Neuro2a and human neuronal SHSY-5Y cell lines electroporated with *Zac1*.

Other genes that resulted enriched from the RNA sequencing analysis upon *Zac1* overexpression were pluripotency associated genes such as *FoxH1*, an inducer of mesenchymal to epithelial transition and *Glis1* both inducers of iPSC formation (Takahashi et al. 2014). The newly acquired "pluripotency" of *Zac1* overexpressing cells was confirmed in *Zac1* electroporated cortices by staining for AP activity, a common marker for increased "stemness" together with augmented levels of *Alpl* observed in the RNA sequencing data suggesting that elevated levels of *Zac1* cause a loss of neuronal identity *via* induction of pluripotency and non-neuronal genes.

Further analysis on electroporated cortices together with luciferase activity assay demonstrated that *Zac1* triggers precocious cell cycle exit via activation *Cdkn1c* (P57). However, when a rescue experiment (electroporation of both *Zac1* and shRNA for *Cdkn1C*) was performed, we observed that while cell cycle is restored thus dependent from P57 activation, induction of non neuronal factors such as DESMIN was still there suggesting the latest is an event triggered solely by *Zac1* levels and it is not related to precocious cell cycle exit.

In addition, ChiP-Seq analysis for SOX2 performed on E11.5 mice cortices, showed that *Zac1* is bound by SOX2 in a conserved region containing the TTTGT motif. Moreover, data analysis from *in utero* electroporations of *Sox2* exhibited decreased levels of *Zac1* transcript whereas data from electroporations with shRNA against *Sox1-3* showed increased levels of *Zac1* transcript thus, suggesting that *Zac1* is negatively regulated by SOXB1 transcription factors.

3.3 Paper III: Stress-Inducible sumoylation of NOTCH1 represses its target gene expression.

One crucial aspect of neurogenesis and proper neuronal differentiation is the capability of a progenitor cell to process environmental information and adapt by effectuating changes in gene expression. An extensively studied signaling pathway during neurogenesis is the one mediated by NOTCH. During development, many processes require oscillating and dynamic expression of Notch genes (Kobayashi and Kageyama 2014). Activation of NOTCH signaling, which is strictly dose-dependent (Anderson et al. 2011, Anderson and Lendhal 2014) results in induction of NOTCH target genes which in turn need to be fine-tuned for cells in order to meet changes in their physiological environment (Main et al. 2010, Gustafsson et al. 2005, Sahlgren et al. 2008). Despite the abundance of literature regarding NOTCH signaling during neurogenesis, the mechanisms behind its diversity and the dynamic regulation of NOTCH target genes are not fully understood.

Notch signaling is activated by direct contact with a cell expressing a Notch ligand. An activated notch receptor results in release and translocation of the NOTCH intracellular domain (NICD) to the nucleus where it activates specific target genes by forming a complex with other transcription factors (Pezeron et al. 2014). Once NICD translocates to the nucleus, it forms a transcriptional complex with the DNA binding protein RBPJ and coactivators of the mastermind like family (MAML) that in turn interacts with P300, a crucial step for transcriptional activation (Wang et al. 2014).

Regulation of NOTCH transcriptional activity can be regulated by interaction with other signaling pathways and post-transcriptional modifications (PTM) (Andersson and Lendhal 2014). A PTM shown to affect NOTCH activity is modification by small ubiquitin–like modifiers (SUMO), also known as sumoylation, which leads to degradation of the modified

protein. It has been reported that inhibition of sumoylation increases NOTCH target genes (Licciardello et al. 2014). However, it is not known how sumoylation affects modification and regulation of NICD1. In mammals, four different isoforms of SUMO (SUMO 1-4) have been reported (Betterman et al. 2012). They are involved in cell fate determination and various differentiation processes during development (Lomeli and Vazquez 2011). Many sumoylated lysines are also targets of other PTMs, such as ubiquitination and acetylation suggesting there is a cross talk between SUMO and other PTMs (Hendriks et al 2014). SUMO binds to its substrates via E1 activating enzyme, an E2 ubiquitin enzyme 9 (Ubc9) and, in most cases, E3 ligases (Watts 2013). Desumoylating proteins rapidly reverse sumoylation and, for sumoylation to mediate a specific function, usually several cycles of conjugation/deconjugation are needed (Wilkinson & Henley 2010; Bossis & Melchior 2006).

It has been reported that class II histone deacetylases (HDACs), including HDAC4, function as SUMO E3 ligases (Sando et al. 2012) and they have been shown to regulate gene expression during stem cell differentiation (Zhang et al 2014). Acetylation and deacetylation affect chromatin landscape and regulate gene expression (Abend and Kehat 2015): in fact, it has been shown that histone deacetylases have a crucial role during differentiation (Clocchiatti et al 2011). HDACs are divided into 4 classes according to their function and DNA sequence similarity: class I (HDACs 1,2,3,8), class II (HDACs 4, 5, 6, 7, 9, 10), sirtuin class III and class IV (HDAC 11) (Foti et al. 2013).

The aim of this study is to understand the molecular mechanisms behind the regulation of Notch target genes upon sumoylation, in response to environmental stimuli.
To understand how sumoylation influences Notch activity, we first assessed, via previously developed approaches (Bloomster et al. 2009; Bloomser et al. 2010), that NOTCH1 is a putative substrate of SUMO2 and that sumoylation of NOTCH1 may occur within a sequence of the RAM domain containing 4 lysine residues (K1774, K1780, K1781, K1782). Immunoprecipitation of whole cells extract of HeLa cells transfected with *Notch1* and SUMO1 showed sumoylation of NICD.

To assess the effect of stress, immunoprecipitation performed on heat shocked treated HeLa cells showed that heat shock increases modification of NOTCH1 by SUMO1 and SUMO3. To establish which endogenous isoform of SUMO was capable of modifying NOTCH1, immunoblot with antibodies against SUMO1 and SUMO2/3 was performed on immunoprecipitated NOTCH1 from HeLa cells extract transfected with NICD1. Only endogenous SUMO2/3 was able to modify NOTCH1. Immunofluorescence analysis COS7 cells transfected with *Notch1* and/or SUMO showed that they interact in the nucleus and that this interaction increases NICD nuclear levels. Moreover, stronger nuclear localization was observed when heat shock was combined with overexpression of NOTCH and SUMO. To determine the effects of NOTCH accumulation due to stress induced sumoylation we analyzed NOTCH1 activity by using a 12XCSL luciferase reporter gene in cells that were transfected with SUMO+NICD1 and cells transfected with SUMO and a tetra SUMO

mutant of NICD, where all four conserved lysines in the RAM domain were mutated to arginine so it cannot be sumoylated. Upon heat stress, the accumulation of TSM in the nucleus was weaker that the wild type and interestingly, TSM diplayed higher NOTCH activity than the wild type NICD upon luciferase assay with 12XCSL. To verify whether the repressive effect of sumoylation applies also to target gene expression, RT-qPCR analysis was performed for classical NOTCH1 target genes. RT-qPCR analysis showed that *Hes1*, *Hey1* and *Hey2* decreased upon heat treatment when NICD1 was overexpressed but *Hey1* and *Hey2* increased when TSM was overxpressed instead.

To determine if proper sumoylation is needed to activate downstream target genes and negatively regulate neurogenesis *in vivo*, *in ovo* electroporation was performed with vectors encoding either for the wild type NICD or TSM lacking the domains necessary for sumoylation. When electroporated, both constructs inhibited upregulation of the neuronal marker TUJ1 and induced expression of NOTCH1 target genes such as *Hes5*. Upon heat induced stress however, there was a reduction of *Hes5* RNA levels in neural tubes electroporated with wild type NICD whereas in embryos electroporated there was still a robust increase of *Hes5* suggesting that sumoylation of NOTCH1 is needed for activation of it downstream target genes.

To unravel the mechanisms beyond suppressed NOTCH1 activity upon sumoylation we focused on HDAC4, a known negative regulator of transcription.
Downregulation of HDAC4 with small interfering RNAs (siRNA) increased the stressed induced Notch1 activity and even more in the presence of SUMO. Moreover, the interaction between HDAC4 and NICD was increased in the presence of SUMO and it was more increased upon heat shock. Whereas binding of TSM to HDAC4 was reduced suggesting sumoylation of NICD enhanced binding to HDAC4. Analysis of Notch target genes expression upon overexpression and siRNA medated knock down of HDAC4 in the presence of heat shock, showed that silencing of HDAC4 activated Notch target genes whereas overexpression of HDAC4 had an opposite effect. Taken together, our data showed that HDAC4 has a repressive role in the regulation of NOTCH target genes upon NICD sumoylation due to heat-induced stress.

4. DISCUSSION

During development stem cells undergo sequential fate restriction from a pluripotent cell to neural progenitor and finally to a differentiated neuron. Throughout this process the ability of a cell to generate cell types is reduced during each developmental stage (Hirabayashi and Gotoh 2010). Reduced potential is accompanied by dramatic changes in gene expression, which are mostly coordinated by transcriptional activity. For instance, whereas in pluripotent stem cells global transcriptional activity is hyperactive, in NPCs its levels drop to 50% (Hirabayashi and Gotoh 2010). The change in transcription regulation is mirrored by the changes in chromatin compaction suggesting that the chromatin is in a loosened state and more readily accessible to transcription in ESCs than NPCs (Meshorer et al. 2006).

Neurogenesis begins with neural induction: stem cells undergo fate restriction from a pluripotent state to being able of producing only neural lineages. During this stage, the ability to sense stimuli from the surrounding environment and consequently respond by changing gene expression is crucial. In fact, expression of proneural genes during neural induction is determined not only by intrinsic factors but also spatial and temporal cues. An extensively described pathway, vital during neurogenesis is the one mediated by Notch signaling. In paper III we show that cues form the surrounding environment have a great influence on neurogenesis and consequently on neuronal differentiation. Indeed, we showed that stress induces sumoylation and accumulation of NICD in the nucleus. This accumulation results in interaction of NICD with HDAC4 that in turn suppresses the expression of NOTCH target genes suggesting that environmental cues such as stress, by changing transcriptional activity, have the potential to influence neurogenesis.

Once neural fate has been established in the progenitor population, NPCs (neural progenitors) will undergo further fate restriction in order to produce first, neuronal cell types during the neurogenic phase and, later, glial cell types during the gliogenic phase (Hirabayashi and Gotoh 2005). Moreover, during the neurogenic phase, different types of neurons, which will be located in different layers of the neocortex, are produced. Generation of new neurons is the result of further fate restriction mirrored by changes in gene expression that in turn is coordinated by specific transcriptional signatures. Gradual fate restriction of a progenitor cell to a more differentiated state, occurs by expression of neuronal genes and downregulation of other lineages determinants. The elevated number of changes in gene expression and the short window of time during which they have to occur require a strict and precise control of transcriptional activity. In paper I we show that, beside upregulation of neuronal genes, repression of other lineage determinants is as important for neuronal differentiation to occur. Our data suggest that CHD5 chromatin remodeler is essential for neurogenesis because not only it facilitates the activation of neuronal genes but also interacts with Polycomb group (PcG) proteins to mediate the repression of genes regulating of alternative lineages.

PcG proteins play a crucial role in the repression of key developmental genes via trimethylation at histone H3 lysine 27 (H3K27me3) (Margueron and Reinberg 2011; Hirabayashi and Gotoh 2010). Many developmental genes are bivalent (also known as poised state) during early development i.e. they have both a repressive mark (such as H3K27me3) and active mark (such as H3K4me3). It has been shown that neural genes such as *Ngns*, *Pax6* (paired box gene 6), *Sox1, Nkx2.2* (NK2 transcription factor related, locus 2) and *Ascl1* are bivalent in ESCs and they become active, i.e. lose their repressive mark but keep the active one, at the onset of neural specification (Hirabayashi and Gotoh 2010; Mikkelsen et al. 2007).

Similarly to the concept of poised state, the seesaw model proposes that pluripotency of stem cells is defined by transcriptional competition of lineage specifying transcription factors. According to this model, many individual transcription factors, responsible for differentiation into various lineages, constantly attempt to specify stem cell differentiation to their own lineage of interest.This constant tug-of-war, balances out the effects of single transcription factors and serves to maintain the cell in a pluripotent state (Loh and Lim 2011). Loss or downregulation of individual transcription factors will result in differentiation of the stem cell towards lineages of the other pluripotency factors. Hence, activation and repression of specific transcription factors is essential during development but is not the only modality of transcriptional regulation. For instance, fine-tuning of expression levels of certain transcription factors is a determining aspect of neuronal differentiation. In paper II we show that elevated *in vivo* levels of the imprinted transcription factor *Zac1* inhibit neuronal differentiation and induce upregulation of mesodermal/myogenic lineage determinants accompanied by expression of genes known to regulate pluripotency. The study shows that SOXB1 transcription factors, despite being in the same NPC population, negatively regulate *Zac1* suggesting that its expression levels need to be controlled in order to avoid aberrant induction of non neuronal genes and premature cell cycle exit.

Transcription factor control of gene expression is crucial for development. Thanks to intense research efforts the last decades, our understanding of this process have been much improved. Despite this, a lot remains to be elucidated regarding their mode of activity, regulation, interaction between each other and how non-autonomous stimuli exerts control over their mode of action.

5. ACKNOWLEDGMENTS

The past years have been challenging in many ways but when I look back I am overwhelmed with gratitude. So, thank you all, colleagues and friends, for your support during this time.

To my main supervisor, **Johan Holmberg,** thank you for having me in your group and for being a peerless support during all these years from day one. Your passion for research and curiosity for scientific questions have been a source of inspiration for me. Under your supervision, I have acquired not only technical skills and comprehension of the field but also learned how to address my scientific questions. Your enthusiasm and your constant presence/interest on the development of different projects have been fundamental for progression and completion of my work. Your supervision has provided me with motivation to carry out projects and at the same time taught me how to be independent in my work. A lot of work has been performed during these years and it has always been fun thanks to you. I truly think this thesis and my work would have not been possible without you. For this, I am sincerely grateful.

Many thanks to my co-supervisor **Erik** for his help during these years. You have found the time to teach me even when you didn't have time for your own experiments. You're a great co-supervisor and it has been a pleasure to work with you!

I am grateful to my co-supervisor **Ulrika** for teaching me during the first years of my PhD. Thank you for finding time for me even though we are not in the same lab anymore.

I would like to thank all the members of the Holmberg group (past and present) **Erik**, **Yao**, **Ulrika**, **Isabelle**, **Konstantinos**, **Eva** for creating a great work environment. It has been fun to work with you not only for the cozy atmosphere in the lab and office but also because when I needed help I knew I could rely on you.

I am grateful to **Susanne Schlisio** for her insightful suggestions during these years. It has been wonderful to share the lab space and office with the Schlisio group: **Stuart, Shuije**, **Olga**, **Veronica**, **Karin**. It has not only been a prosperous "cohabitation" when it comes to lab meeting, exchange of scientific ideas and solution to technical issues but also very fun in terms of atmosphere and social activities.

I would like to thank **Charlotta** …if it wasn't for you, my permanence in Sweden would have lasted only for the first six months ;)

Many thanks to all the people working at **LICR**. I felt like I was part of a big family. I am very grateful for all your help and support during these years, from borrowing reagents

to help with protocols. Lunches, fika, coffees and chats have made these years very enjoyable.

I would like to thank my co-authors and collaborators: it has been a great opportunity to work and discuss science with you.

To my friends, I would have never thought I could find such warmth in northern Europe. I am truly grateful for your time, your ears, for sharing your thoughts with me and, in general, for your support.
Cecile, you´re the first person I met when I arrived in Stockholm and since then mamma Cecilia has always been looking out for me. I really appreciate your honesty and your friendship all these years. **Karina**, I think you're the second person I met … the rest is history! Thank you for your thoughtful advices these years and for being there for me when I needed it. **Giulia Z**, you´re a unique combination of humor and deep analytical thoughts…said Kant! I am immensely happy we met…since then it has been a continuous (killer) laugh (even in "tragi-comic" situation). Grazie di esistere! **Mustafa**, I always enjoy spending time with you and I am very grateful for your friendship. In particular, I would like to thank you for making me believe that the iphone could perform incredible tasks ;). **Susie**, thank you for all your hugs…they have been a great support these years. **Konstantinos**, thank you for bringing me coffee when you see that I desperately need it …it has been a blast having you in the lab (and not just for the coffees)! **Sanja**, I´m really happy we met and even though we don't meet as often as we used to, it always feels like time hasn't passed. **Haythem**, I always enjoyed spending time with you and specially our talks (miss you!). **Andrea** I always had a good time hanging out with you either for a run or for a beer (or both in the same order). **Steffi** your energy is contagious and is always fun to be with you. I miss both you and Andrea. **Ale** is always fun to hang out with you specially because of your overwhelming positivism ;). Many thanks to **Tanya**, **Giulia G**., **Andreas, Sophie**, **Eva, Aileen, Aga** for your friendship, good company and the good times.

Mami, Babi dhe Andi: Ju falenderoj per te gjitha sakrificat qe keni bere gjate gjith keto vite. Pa ndihmen e juaj, nuk do isha ku jam tani. Duke ndjekur shembullin e juaj, kam gjetur vullnetin per pune, kurajon per te ndjekur idet e mia, durim dhe force per ti realizuar. Per gjitha keto arsye, ju falenderoj me gjithe zemer.

6. REFERENCES

- Abend, A. & Kehat I., 2015. Histone deacetylases as therapeutic targets - From cancer to cardiac disease. *Pharmacology & therapeutics*, 147C, pp.55–62.
- Adnani L., Langevin M.L., Gautier E, Dixit R., Parsons K., Li S., Kaushik G., Wilkinson G., Wilson R., Childs S., Ngyen M.D., Journot L., Dehay C., Schuurmans C. 2015. *Zac1* Regulates the Differentiation and Migration of Neocortical Neurons via *Pac1*. *The Journal of Neuroscience,* 35(39): 13430 –13447.
- Alcamo, E. A., Chirivella, L, Dautzenberg, M, Dobreva, G, Fariñas, I, Grosschedl, R. and McConnell, S. K. (2008). Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron* 57.
- Andersson, E.R., Sandberg, R. & Lendahl, U., 2011. Notch signaling: simplicity in design, versatility in function. *Development (Cambridge, England)*, 138(17), pp. 3593–612.
- Andersson, E.R. & Lendahl, U., 2014. Therapeutic modulation of Notch signalling-are we there yet? *Nature reviews. Drug discovery*, 13(5), pp.357–78.
- Armentano M., Chou S.J., Tomassy G.S., Leingartner A., O'Leary D.D., Studer M. 2007. COUP-TFI regulates the balance of cortical patterning between frontal/ motor and sensory areas. *Nat Neurosci* 10: 1277–1286.
- Bertrand N., Castro D.S., and Guillemot F. (2002) Proneural genes and the specification of neural cell types. *Nature Reviews Neuroscience* 3: 517–530.
- Bettermann K., Benesch M., Weis S., Haybaeck M. 2012. SUMOylation in carcinogenesis. *Cancer letters*, 316(2), pp.113–25.
- Bishop K.M., Rubenstein J.L.R., O'Leary D.D.M. 2002. Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. *J Neurosci* 22: 7627–7638.
- Blomster H.A., Imanishi S.A., Siimes J., Kastu J., Morrice N.A., Eriksson J.E., Sistonen L. 2010. In vivo identification of sumoylation sites by a signature tag and cysteine-targeted affinity purification. *The Journal of biological chemistry*, 285(25), pp.19324–9.
- Blomster H.A., Hietakangas V., Wu J., Kouvonen P., Hautaniemi S., Sistonen L. 2009. Novel proteomics strategy brings insight into the prevalence of SUMO-2 target sites. *Molecular & cellular proteomics : MCP*, 8(6), pp.1382–90.
- Bossis, G. & Melchior, F., 2006. Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Molecular cell*, 21(3), pp.349–57.
- Briscoe E. and Ericsson E. 2001. Specification of neuronal fates in the ventral neural tube. *Current Opinion in Neurobiology* 2001, 11:43–49.
- Brodeur GM 2003. Neuroblastoma: biological insights into a clinical enigma. *Nature Reviews* 3, 203-216.
- Brown, T.G., 1911. The intrinsic factors in the act of progression in of the mammal. *Proceedings of the Royal Society of London* 84, 308–309.
- Bylund, M., Andersson, E., Novitch, B.G., Muhr, J., 2003. Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nature Neuroscience* 6, 1162–1168
- Bystron I, Blakemore C, Rakic P. 2008. Development of the human cerebral cortex: Boulder committee revisited. *Nature Review Neuroscience* 9(2): 110-22.
- Calegari F, Haubensak W, Haffner C, & Huttner, WB. 2005. Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. *The Journal of Neuroscience*, 25(28), 6533–6538.

- Calpier CR and Cairns BR 2009. The biology of chromatin remodeling complexes. *Annu. Rev. Biochem*. 78:273–304.
- Carpenter E. 2002 Hox genes and spinal cord development. *Dev Neurosci* 2002; 24:24–34.
- Castro, D.S., Skowronska-Krawczyk, D., Armant, O., et al., 2006. Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. Developmental Cell 11, 831–844
- Caviness VS. Jr. 1975 Architectonic map of neocortex of the normal mouse. *J Comp Neurol* 164(2): 247-63.
- Clocchiatti, A., Florean, C. & Brancolini, C., 2011. Class IIa HDACs: from important roles in differentiation to possible implications in tumourigenesis. *Journal of cellular and molecular medicine*, 15(9), pp.1833–46.
- Farah M.H., Olson J.M., Sucic H.B., Hume R.I., Tapscott S.J., Turner D.L., 2000. Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* 127, 693–702.
- Fischer, A. and Gessler, M. (2007). Delta-Notch-and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic Acids Res.* 35, 4583-4596.
- Foti S.B., Chou A., Moll A.D., Roskams A.J. 2013. HDAC inhibitors dysregulate neural stem cell activity in the postnatal mouse brain. *International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience*, 31(6), pp.434–47.
- Fukuchi-Shimigori and Groove 2001. Neocortex patterning by the secreted signaling molecule FGF8. *Science* 294(5544): 1071-4.
- Garcia I., Mayol G., Rodriguez E., Sunol M., Gershon T.R., Rios J., Cheung N.K., Kieran M.W., George R.E., Perez-Atayde A.R. et al 2010. Expression of the neuron-specific protein CHD5 is an indipendent marker of outcome in neuroblastoma. *Molecular Cancer* 9:277.
- Gifford W.D., Hayashi M., Sternfeld M., Tsai J., Alaynick W.A., Pfaff S.L. 2013. Chapter 7.Spinal cord Patterning. From Patterning and Cell Type Specification in the Developing CNS and PNS. *Comprehensive Developmental Neuroscience*, Volume 1.
- Greig LC, Woodworth MB, Galazzo MJ, Padmanabhan H, Macklis JD. 2013 Molecular Logic of neocortical Projection neuron specification, development and diversity. *Nature Review Neuroscience*. 14(11): 755-69.
- Gui, H., Li, S., Matise, M.P., 2007. A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord. *Developmental Biology* 301, 14–26.
- Gustafsson M.V., Zheng X., Pereira T., Gradin K., Jin S., Lundkvist J., Ruas J.L., Poellinger L., Lenhal U., Bondesson M. 2005. Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Developmental cell*, 9(5), pp.617–28.
- Han, W., Kwan, K. Y., Shim, S., Lam, M. M., Shin, Y., Xu, X., Zhu, Y., Li, M. and Sestan, N. (2011). TBR1 directly represses Fezf2 to control the laminar origin and development of the corticospinal tract. *Proc. Natl. Acad. Sci. USA* 108, 3041-3046.
- Hatakeyama J, Bessho Y, Katoh K, Ookawara S, Fujioka M, Guillemont F, Kageyama R. 2004. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentation. *Development*. 131. 5539-5550.
- Haubnesak W, Attardo A, Denk W and Huttner WB. 2004. Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc. Natl. Acad. Sci USA* 101. 3196-3201.

- Hebert JM. 2005. Unraveling the molecular pathways that regulate early telencephalon development. Chapter 2 Brain Development. *Current Topics in developmental biology vol. 69*.
- Hendriks I.A., D´souza R.C., Yang B., Verlaan-de Vries M., Mann M., Vertegaal A.C. 2014. Uncovering global SUMOylation signaling networks in a site-specific manner. *Nature Structural & Molecular Biology*, 21(10), pp.927–936.
- Herculano-Houzel 2009. The human brain in numbers: a linearly scaled up primate brain. *Frontiers in Human Neuroscience*.
- Hevner, R. F., Shi, L., Justice, N., Hsueh, Y., Sheng, M., Smiga, S., Bulfone, A., Goffinet, A. M., Campagnoni, A. T. and Rubenstein, J. L. (2001). Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* 29, 353-366.
- Hirabayashi Y. and Gotoh Y 2010. Epigenetic control of neural precusor cell fate during development. *Nature Reviews Neuroscience* 11 377-388.
- Hirabayashi Y and Gotoh Y. 2005. Stage dependent fate determination of neural precursor cells in mouse forebrain. *Neuroscience Research* 51, 331-336
- Ho L. and Crabtree G. 2010 Chromatin remodeling during development. *Nature* 463, 474-484.
- Holmberg J., Hansson E., Malewicz M., Sandberg M., Perlmann T., Lendahl U., Muhr J. 2008. SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation. *Development* 135, 1843-1851.
- Holmberg J and Perlmann T 2008. Maintaining differentiated cellular identity. *Nature Reviews Genetics* 13, 429-439.
- Huang C., Chan J.A., Schuurmans C. 2014 Proneural bHLH Genes in Development and Disease. *Current Topics in Developmental Biology*, Volume 110.
- Imayoshi, I., Isomura, A., Harima, Y., Kawaguchi, K., Kori, H., Miyachi, H., et al. (2013). Oscillatory control of factors determining multipotency and fate in mouse neural progenitors. *Science* 342(6163), 1203–1208.
- Jan, Y. N., and Jan, L. Y. (1994). Genetic control of cell fate specification in Drosophila peripheral nervous system. *Annu. Rev. Genet*. 28, 373–393.
- Jessell TM 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nature Reviews Genetics* 1, 20-29
- Job C. and Tan S. 2003 Constructing the mammalian neocortex: the role of intrinsic factors. *Developmental Biology* 257 (2003) 221–232.
- Johnson and Glasgow 2009. Helix–Loop–Helix (bHLH) Proteins: Proneural.
- Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspeth AJ. 2012 *Principles of neural science*. Fifth edition.
- Kobayashi and Kageyama 2014. Expression Dynamics and Functions of Hes Factors in Development and Diseases. *Current Topics in Developmental Biology*, Volume 10.
- Kopan, R. & Ilagan, M.X.G., 2009. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, 137(2), pp.216–33.
- Kouzarides T. 2007 Chromatin Modifications and their function. Cell *128*, 693–705
- Kwan KW, Sestan N and Anton ES. 2012. Transcriptional co-regulation of neuronal migration and laminar identity in the neocortex. *Development* 139, 1535-1546
- Kwan, K. Y., Lam, M. M., Krsnik, Z., Kawasawa, Y. I., Lefebvre, V. and Sestan, N. (2008). SOX5 postmitotically regulates migration, postmigratory differentiation, and projections of subplate and deep-layer neocortical neurons. *Proc. Natl. Acad. Sci. USA* 105, 16021-16026.
- Lai H.C., Meredith D.M., Johnson J.E. Chapter 18 bHLH Factors in Neurogenesis and Neuronal Subtype Specification. Patterning and Cell Type Specification in the

Developing CNS and PNS. *Comprehensive Developmental Neuroscience*, Volume 1.

- Lai, T., Jabaudon, D., Molyneaux, B. J., Azim, E., Arlotta, P., Menezes, J. R. and Macklis, J. D. (2008). SOX5 controls the sequential generation of distinct corticofugal neuron subtypes. *Neuron* 57, 232-247.
- Le Dreau G and Marti E. 2012 Dorsal–Ventral Patterning of the Neural Tube: A Tale of Three Signals. *Developmental Biology*.
- Lee, S.K., Lee, B., Ruiz, E.C., Pfaff, S.L., 2005. Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells. *Genes & Development* 19, 282–294.
- Licciardello M.P., Mullner M.K., Durnberger G., Kerzendorfer C., Boidol B., Trefzer C., Sdelci S., Berg T., Penz T., Schuster M., Bock C., Kralovics R., Superti-Furga G., Colinge J., Nijman S.M., Kubicek S. 2014. NOTCH1 activation in breast cancer confers sensitivity to inhibition of SUMOylation. *Oncogene*.
- Lodato S. and Arlotta P. Generating neuronal diversity in the mammalian cerebral cortex. *The Annual Review of Cell and Developmental Biology* 2015; 31:699-700.
- Loh K.M and Lim B. 2011. A precarious balance: Pluripotency factors as lineage specifiers. *Cell Stem Cell* 8, 363-369
- Lomelí, H. & Vázquez, M., 2011. Emerging roles of the SUMO pathway in development. *Cellular and molecular life sciences : CMLS*, 68(24), pp.4045–64.
- Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci*. 7, 93-102.
- Main H., Lee K.L., Yang H., Haapa-Paananen S., Edgren H., Jin S., Sahlgren C., Kallioniemi O., Poellimger L., Lim B., Lendhal U. 2010. Interactions between Notch- and hypoxia-induced transcriptomes in embryonic stem cells. *Experimental cell research*, 316(9), pp.1610–24.
- Margueron R and Reinberg D 2009. The polycomb complex PRC2 and its mark in life. *Nature Revies* 469, 343-349
- Martynoga B, Drescsel D and Guillemont F. 2012. Molecular Control of Neurogenesis: a view from the mammalian cerebral cortex. *Cold Spring Harbor Perspective Biology.*
- Matise and Sharma 2013. Chapter 21. The Specification and Generation of Neurons in the Ventral Spinal Cord. Patterning and Cell Type Specification in the Developing CNS and PNS. *Comprehensive Developmental Neuroscience*, Volume 1.
- McEvilly, R. J., de Diaz, M. O., Schonemann, M. D., Hooshmand, F. and Rosenfeld, M. G. (2002). Transcriptional regulation of cortical neuron migration by POU domain factors. *Science* 295,
- McKenna, W. L., Betancourt, J., Larkin, K. A., Abrams, B., Guo, C., Rubenstein, J. L. and Chen, B. (2011). Tbr1 and Fezf2 regulate alternate corticofugal neuronal identities during neocortical development. *J. Neurosci.* 31, 549-564.
- Megason, S.G., McMahon, A.P., 2002. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* 129, 2087–2098.
- Meshorer E. et al 2006. Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev. Cell*. 10 105-116.
- Mikkelsen T.S., Ku M., Jaffe D.B., Issac B., Lieberman E., Giannoukos G., Alvarez P., Brockman W., Kim T.K., Koche R.P., Lee W., Mendenhall E., O´Donovan A., Presser A., Russ C., Xie X., Meissner A., Wernig M., Jaenisch R., Nusbaum C., Lander E.S., Bernstein B.E. 2007. Genome-wide maps of chromatin state in pluripotent and lineage commitment cells. *Nature* 448, 553-560.
- Molyenaux BJ, Arlotta P, Menez JRL, Macklis JD 2007. Neuronal sybtype specification in the cerebral cortex. *Nature Reviews Neuroscience*. Vol 8, 427-437.

- Musselman C.A., Mansfield R.E., Garske A.L., Davrazou F., kwan A.H., Oliver S.S., O´Leary H., Denu J.M., Mackay J.P. and Kutateladze T.G. 2009. Binding of the CHD4 PHD2 finger to histone H3 is modulated by covalent modifications. *Biochem. J*. 423, 179–187.
- Nakada, Y., Hunsaker, T.L., Henke, R.M., Johnson, J.E., 2004. Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification. *Development 131*, 1319–1330.
- Noctor SC, Martinez-Cerdeno V, Ivic L and Kriegstein AR. 2004. Cortical Neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nature Neuroscience* 7. 136-144
- Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J*. 18, 2196-2207.
- Okawa E.R., Gotoh T., Manne J., Igarashi J., Fijuta T., Silverman K.A., Xhao H., Mosse Y.P., White P.S. and Brodeur G.M. Expression and sequence analysis of candidates for the 1p36.31 tumor suppressor gene deleted in neuroblastomas. *Oncogene* 27, 803-810.
- O'Leary D., 1989. Do cortical areas emerge from a protocortex? *Trends Neurosci*. 12, 400
- Parnavelas J.G. 2000 The origin and migration of cortical neurons: new vistas. *Trends in Neuroscience* Vol 23, 126-131; 2000.
- Paul S., Kuo A., Schalch T., Vogel H., Joshua-Tor L., McCombie W.R., Gozani O., Hammell M. and Mills A.A. 2013. Chd5 requires PHD-mediated histone 3 binding for tumor suppression. *Cell Reports 3*, 92–102.
- Pézeron G., Millen K., Boukhatmi H., Bray S. et al., 2014. Notch directly regulates the cell morphogenesis genes Reck, talin and trio in adult muscle progenitors. *Journal of cell science*, 127(21), pp.4634–44.
- Potts R.C., Zhang P., Wurster A.L., Precht P., Mughal M.R., Wood W.H., Zhang Y., Becker K.G., Mattson M.P. and Pazin M.J. 2011. CHD5, a brain-specific paralog of Mi2 chromatin remodeling enzymes, regulates expression of neuronal genes. *PloS One* Vol.6.
- Powell, L. M., & Jarman, A. P. (2008). Context dependence of proneural bHLH proteins. *Current Opinion in Genetics & Development*, 18(5), 411–417.
- Rakic P. 1988 Specification of Cerebral Cortical Areas. Science 241, 170.
- Sahlgren C., Gustafsson M.V., Jin S., Poellinger L., Lendahl U. 2008. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proceedings of the National Academy of Sciences*, 105(17), pp.6392–6397.
- Sando R. 3rd., Gounko N., Pieratus S., Liao L., Yates J 3rd, Maximov A. et al., 2012. HDAC4 governs a transcriptional program essential for synaptic plasticity and memory. *Cell*, 151(4), pp.821–34.
- Sanes D.H., Reh T.A., Harris W.A. 2005 Chapter1. *Development of the nervous system*. Elsevier Science.
- Sansom and Livesey 2009. Gradients in the Brain: The Control of the Development of Form and Function in the Cerebral Cortex. *Cold Spring Harb Perspect Biol* 1:a002519
- Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev*. 6, 2620-2634.
- Schubeler D. 2009 Methylation matters. *Nature* 462, 296-297
- Schuettengruber B., Martinez A.M., Iovino N. and Cavalli G. 2011 Trithorax group proteins: switching genes on and keeping them active. *Nature Reviews, Molecular Cell Biology* 12, 799-814.

- Shimojo H, Ohtsuka T, & Kageyama, R. 2008. Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron*, 58(1), 52–64.
- Shirasaki R. and Pfaff S.L. 2002. Transcriptional Codes and the control of neuronal identity. *Annu. Rev. Neurosci*. 2002. 25:251–81.
- Skeath, J. B., & Carroll, S. B. (1994). The achaete-scute complex: Generation of cellular pattern and fate within the Drosophila nervous system. *The FASEB Journal*, 8(10), 714–721.
- Sugitani, Y., Nakai, S., Minowa, O., Nishi, M., Jishage, K., Kawano, H., Mori, K., Ogawa, M. and Noda, T. (2002). Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. *Genes Dev.* 16, 1760-1765.
- Sun, Y., Nadal-Vicens, M., Misono, S., et al., 2001. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* 104, 365–376.
- Sur and Rubenstein 2005. Patterning and Plasticity of the Cerebral Cortex. *Science* Vol310.
- Takahashi K. et al 2013. Induction of pluripotency in human somatic cells via a transient state resembling primitive streak-like mesendoderm. *Nature communications* 5:3678.
- Takemoto T. et al 2011. Tbx6-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. *Nature* 470, 394-399.
- Taverna et al. 2007. How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nature Structural & Molecular Biology* 14, 1025-1044.
- Valente T., Junyent F., Auladell C. 2005. Zac1 Is Expressed in Progenitor/Stem Cells of the Neuroectoderm and Mesoderm During Embryogenesis: Differential Phenotype of the Zac1-Expressing Cells During Development. *Developmental Dynamics* 233:667–679.
- Van der Loos H. and Woosley T.A. 1973. Somatosensory cortex: Structural alterations following early injury to sense organs. *Science*, 179: 395–398.
- Varrault A., Gueydan C., Dalalbre A., Bellman A., Houssami S., Aknin C., Severac D., Chotard L., Kahli M., Le Digarcher A., Pavlidis P., Journot L. 2006. Zac1 Regulates an Imprinted Gene Network Critically Involved in the Control of Embryonic Growth. *Developmental Cell* 11, 711–722.
- Wang, H., Zang C., Liu XS., Aster J.C. 2015. The role of notch receptors in transcriptional regulation. *Journal of cellular physiology*, 230(5), pp.982–8.
- Watts, F.Z., 2013. Starting and stopping SUMOylation. What regulates the regulator? *Chromosoma*, 122(6), pp.451–63.
- Wilkinson, K.A. & Henley, J.M., 2010. Mechanisms, regulation and consequences of protein SUMOylation. *The Biochemical journal*, 428(2), pp.133–45.
- Wilson L. and Maden M. 2005. The mechanisms of dorsoventral patterning in the vertebrate neural tube. *Developmental Biology* 282, 1– 13.
- Wonders C.P. and Anderson S.A.. 2006. The origin and specification of cortical interneurons. *Nature Review Neuroscience*. 7, 687-696.
- Yap and Zhou 2010. Keeping it in the family: diverse histone recognition by conserved structural folds. *Critical Reviews in Biochemistry and Molecular Biology* 45(6): 488–505.
- Zhang L.X., DeNicola M., Qin X., D J., Ma J., Tina Zhao Y., Zhuamg S., Liu PY., Wei L., Qin G., Tang Y., Zhao T.C. 2014. Specific inhibition of HDAC4 in cardiac progenitor cells enhances myocardial repairs. *American journal of physiology. Cell physiology*, 307(4), pp.C358–72.