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TRANSCRIPTIONAL REGULATION OF NEURAL DEVELOPMENT AND DEGENERATION

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Transcriptional regulation of neural development and degeneration

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Thursday 29th of March, 2018 at 09:30

To mom, who built my first laboratory.

To grandma, whose garden I destroyed with my experiments.

ABSTRACT

The architecture and functional interactions of the cerebral cortex are fascinating in their complexity of billions of neurons and glial cells connected in intricate circuitries and spatial regions. During development, transcription factors and chromatin modifiers work together to coordinate gene expression programs that drive the formation of the cerebral cortex. Notably, neurological aberrations can arise when perturbation occurs in these tightly regulated programs. Although our understanding of corticogenesis has advanced with the identification of master regulators of neural development, we only have rudimentary knowledge of the molecular mechanisms these factors use to execute the developmental programs.

The aim of this thesis is to investigate the transcriptional mechanisms of gene regulation by key transcription factors involved in telencephalic development and disease, using primarily human neural progenitors as an *in vitro* model of development.

Study I reports a novel mechanism through an interaction between the co-repressor NCOR and the transcription factor FOXP2. Genome-wide mapping of common binding sites of NCOR/FOXP2 in human iPS-derived neural progenitors included two putative regulatory elements in the proximity of the SLITRK gene cluster. Chromosome conformation capture (3C) confirmed the interaction between the SLITRK cluster gene promoters and the regulatory elements where FOXP2/NCOR binds, which proposes a possible role for this regulatory mechanism in accurate development and possibly evolution of vocal and motor skills. Study II demonstrates that the transcription factor PAX6 can function as a repressor and recruit the histone demethylase KDM5C to repress a subset of genes involved in Notch signaling, which is critical for several neuronal functions, proposing that neurodevelopmental aberrations by PAX6 and/or KDM5C mutations maybe be associated with defects in Notch signaling. Study III explores the gene regulation effects on pluripotency mediated by different handling techniques of human embryonic stem cells and human induced pluripotent stem cells, and shows that reversible gene expression changes indeed occur during prolonged culture in enzymatic conditions. Study IV reveals that a single nucleotide polymorphism (SNP) in the promoter of the *HTT* gene, responsible for Huntington's disease, disrupts the NF-κB binding and transcriptional regulation of the *HTT* gene, indicating that silencing of the *HTT* gene is a promising therapeutic strategy in Huntington's disease.

Taken together, the work of this thesis strengthens the hypothesis that the interplay between transcription factors and chromatin structure is critical for maintaining neurological fitness.

LIST OF SCIENTIFIC PAPERS

I. **Giulia Gaudenzi**, Nina Heldring, Bianca Migliori, Hannah Bruce, Olga Dethlefsen, Saiful Islam, Raju Tomer, Sten Linnarsson, Kristen Jepsen, Michael G. Rosenfeld and Ola Hermanson

NCOR and FOXP2 regulate expression of the SLITRK family via distinct and distant regulatory elements.
Manuscript

II. **Giulia Gaudenzi**, Olga Dethlefsen, Julian Walfridsson, and Ola Hermanson

Pax6 and KDM5C co-occupy a subset of developmentally critical genes including Notch signaling regulators in neural progenitors.
Submitted Manuscript

III. Frida Holm, Hero Nikdin, Kristín Rós Kjartansdóttir, **Giulia Gaudenzi**, Kaj Fried, Pontus Aspenström, Ola Hermanson, and Rosita Bergström-Tengzelius

Passaging techniques and ROCK inhibitor exert reversible effects on morphology and pluripotency marker gene expression of human embryonic stem cell lines
Stem Cells and Development. 2013, 22:1883-1892.

IV. Kristina Bečanović, Anne Nørremølle, Scott J Neal, Chris Kay, Jennifer A Collins, David Arenillas, Tobias Lilja, **Giulia Gaudenzi**, Shiana Manoharan1, Crystal N Doty, Jessalyn Beck, Nayana Lahiri, Elodie Portales-Casamar, Simon C Warby, Colúm Connolly, Rebecca A G DeSouza, REGISTRY Investigators of the European Huntington's Disease Network, Sarah J Tabrizi, Ola Hermanson, Douglas R Langbehn, Michael R Hayden, Wyeth W Wasserman & Blair R Leavitt

A SNP in the HTT promoter alters NF-kB binding and is a bidirectional genetic modifier of Huntington disease.
Nature Neuroscience. 2015, 18:807-816.

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LIST OF ABBREVIATIONS

3C	Chromosome conformation capture
ASD	Autism spectrum disorder
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic protein
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FOXP2	Forkhead box protein P2
GFAP	Glial fibrillary acidic protein
HAR	Human accelerated regions
HAT	Histone acetyl transferase
HD	Huntington disease
HDAC	Histone deacetylase
HDM	Histone demethylase
hESC	Human embryonic stem cell
hiPSC	Human Induced Pluripotent Stem Cell
HMT	Histone methyltransferase
HTT	Huntingtin gene
IPC	Intermediate progenitor cell
iROCK	Rho-associated kinase inhibitor
JARID	Jumonji- and AT-rich interaction domain *(*ARID)-domain
KO	Knock out
NCOR	Nuclear receptor co-repressor
NE	Neuroepithelial cell
NESC	Neuroepithelial-like stem cell
NSC	Neural stem cell
PAX6	Paired box protein 6

PRC2	Polycomb repressive complex 2
RGC	Radial glial cell
SFRP2	Secreted Frizzled Related Protein 2
SHH	Sonic hedgehog
SLITRK	Slit- and Trk-like
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SNP	Single-nucleotide polymorphism
SVZ	Subventricular zone
TF	Transcription factor
TFBS	Transcription factor binding site
TSS	Transcruption start site
VPA	Valproic acid
VZ	Ventricular zone
WNT	Wingless-INT
WT	Wild type

1 INTRODUCTION

Syntactical-grammatical language, symbolic thought, self-reflection, long-term planning ability, autobiographical memory, the theory of mind, and the capacity to create art, are distinctively human aspects of cognition and behavior (Sousa et al., 2017). The computational center of these higher cognitive functions is the cerebral cortex, which is arguably the most complex structure in the human brain. Deciphering the architecture and functional interactions of billions of different cell types connected in intricate circuitries and spatial regions in the cerebral cortex, is a fascinating yet challenging central question of neurobiology.

1.1 EVOLUTIONARY COMPLEXITY

When considering brain complexity, at first several descriptive efforts using comparative neuroanatomy have focused on creating a *scala naturae* of brains, and even post-Darwin, the goal was merely the reconstruction of phylogenies (Northcutt, 2001). However, it was not until the 1980s when applying an evo-devo approach to embryology and the extensive studies of several species' embryonic brains such as rodents (mostly *Mus musculus*), humans (*Homo sapiens)* and non-human primates (mostly *Macaca mulatta*) that several scientists began to focus not on explaining species differences, but on finding similarities (Striedter, 2011) and uncovering developmental principles that elucidated how the human neocortex has developed at the cellular and molecular level (Swanson, 2000) (Rakic, 2009). Although simplicity may out-survive complexity, as "brainlessness" itself is an adaptive and persisting trait throughout the history of life (e.g. prokaryotes, viruses, or simple multicellular organisms), the adaptive advantage conferred by an organized nervous system is a general feature of the evolutionary process (Konner, 2010). Connecting and mapping DNA changes in the human brain during health and disease to uniquely human neuromolecular mechanisms through which these genetic differences are expressed, is paving the way to understanding the evolution of our species' high cognitive ability, e.g. like the capacity to elaborate complex language (Franchini and Pollard, 2015a).

1.2 CORTICOGENESIS

Studying complex traits as well as diseases requires a full understanding of corticogenesis. The cerebral cortex is a cellular sheet composed of pyramidal neurons (excitatory glutamatergic) and interneurons (inhibitory GABAergic), which are organized in a horizontal fashion, and intersected by vertical radial columns, sharing extrinsic connectivity. These characteristics are common among all mammalian species (Rakic, 2009).

At first, neurons are generated via a process called neurogenesis which is followed by a precise sequence of events, such as organized neuronal migration, differentiation, dendrite and axon development, synaptogenesis and the formation of circuitry. These events are interlinked with non-neuronal processes such as the generation of astrocytes and oligodendrocytes (gliogenesis), which is followed by myelination, angiogenesis and the establishment of the blood-brain barrier (Rakic, 1988) (Götz and Huttner, 2005) (Lois and Alvarez-Buylla, 1993) (Noctor et al., 2004).

1.2.1 Proliferation and neurogenesis

During development, the most rostral region of the early embryonic mammalian neural tube give rise to the cerebral cortex, which at this stage consist of a monolayer of neuroepithelial (NE) cells lying the ventricles throughout the neuraxis. NE cells are neural stem cells (NSCs) that can generate both neurons and glia. As neurogenesis begins, NE cells are transformed in radial glial cells (RGCs), which are highly related to the former by exhibiting apical-basal cell polarity but also possess glial markers (Götz and Huttner, 2005) such as BLBPs, vimentin and the paired box gene 6 (Pax6) (**Figure 1**). RGCs at the ventricular zone (VZ) undergo symmetric cell division to expand the progenitor pools, as well as asymmetric cell division to generate an RGC plus a daughter cell, e.g. a neuronal precursor (Noctor et al., 2004) or an intermediate progenitor cell (IPC). IPCs divide at the subventricular zone (SVZ) and they all express the transcription factor Tbr2. Studies of mice lacking Tbr2 (Sessa et al., 2008) and of familial mutation in humans (Baala et al., 2007) have demonstrated that IPCs contribute to cells in all cortical layers and that they generate most cortical excitatory neurons. In addition to the aforementioned progenitors, in lissencephalic cortices (like of the mouse), other types of progenitors have been found, although less abundant. These additional progenitors are classified in apical intermediate progenitors, subapical and basal radial progenitors (bRGCs). The latter have been found in higher abundance in gyrencephalics

cortices such as those of humans and monkeys which have thicker SVZ, especially at later stages of neurogenesis.

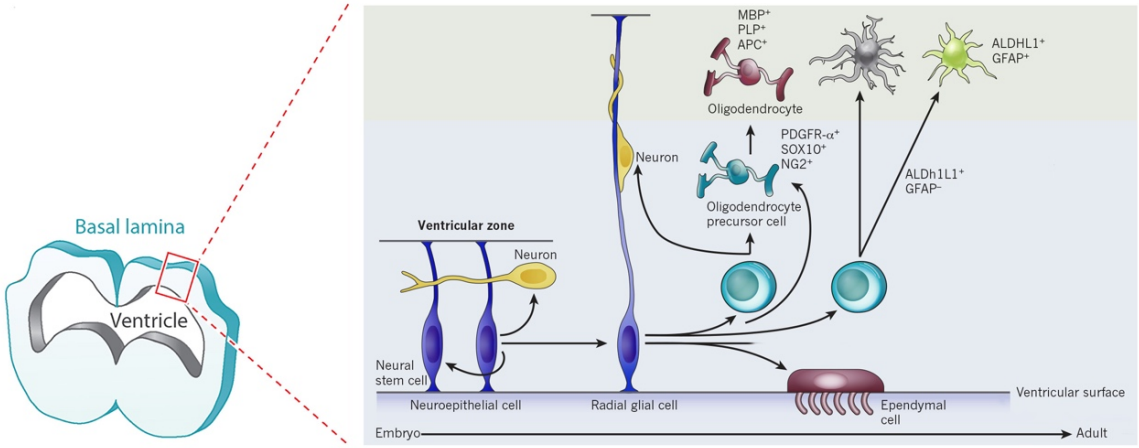

Figure 1. *Snapshots of neurogenesis and gliogenesis. Modified from Rowitch and Kriegstein, 2010.*

1.2.2 Migration and differentiation

The newborn neural pool of excitatory neurons or interneurons is derived from the migration of RGCs from VZ or SVZ to the uppermost cortical surface. This process is named "radial migration" (Rakic, 1972), and refers to the fashion in which cortical layers are formed. Radial glial fibers act as efficient train tracks by providing a perpendicular scaffold between the ventricular and pial surface of the cortex. Once neural progenitors finish migrating radially, they detach and begin to differentiate. At this stage, the "layerization" of the cortex begins in an inside-out fashion: early-born neurons migrating from the cortical plate will contribute to the deeper neocortical layers (VI and V), whereas late-born neurons will migrate and then form the additional superficial layers (IV and then II, III) (Rakic, 1972).
The neural pool of newborn inhibitory GABAergic interneurons distinctly originates from the ganglionic eminences of the ventral forebrain. Once they have exited the cell cycle they migrate non-radially into the cortical plate (Pleasure et al., 2000).

1.2.3 Patterning by molecular cues

Extracellular signals from the altering environment of the developing brain, together with a combination of intrinsic temporal programs, drive the changes of differentiation during corticogenesis (Okano and Temple, 2009).

The extracellular milieu is characterized by the biophysical factors, such as the oxygen and extracellular matrix, the expression of signalling molecules and of the generation of morphogens at patterning regions at the margins of the neocortical primordium, which coordinately drive early patterning and the necortex arealization. As described in **Figure 2**, in the early patterning of the development of the mouse forebrain, the principal signalling molecules are the FGFs (Fibroblast Growth Factor), secreted rostromedially by the commissural plate; WNTs and BMPs (Bone Morphogenetic Proteins), secreted caudomedially from the cortical hem and SFRP2 (Secreted Frizzled Related Protein 2) and EGF (Epidermal Growth Factor) that are secreted laterally from the anti hem. Altogether, these diffusible elements are responsible for reading/interpreting temporal and spatial cues as well as for inducing transcription factors gene expression in VZ progenitors in a graded manner. Transcription factors then execute the intrinsic programs to control the position and relative size of the cortical areas (Greig et al., 2013).

BMPs mediate the expansion of the VZ, and together with transcription factors critical for forebrain regionalization such as Emx1/2, Pax6, Foxg1 and Tlx1, they promote neural progenitor proliferation and suppress neuronal differentiation. In addition, cell signaling pathways such as Notch, a cell surface receptor, transduces signals that affect neural progenitor maintenance and neuronal migration, while the morphogene Sonic Hedgehog (SHH) regulates neural progenitor proliferation (O'Leary et al., 2007).

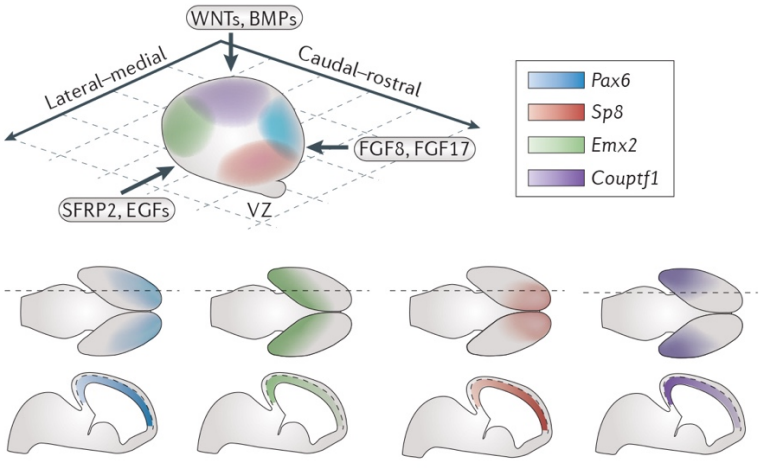

Figure 2. *When gradients of extracellular signalling molecules in the telencephalon are induced, trans-acting factors such as transcription factors binding to specific genomic sequences mediates this spatio- temporal developmental regulation by thereby executing a series of gene expression programs (Spitz and Duboule, 2008). Here are shown the expression patterns Emx2 and Pax6 Sp8 and Couptf1, respective to the morphogens gradients. Modified from Greig et al., 2013.*

Importantly, transcription factors are the effectors of the environmental cues provided by morphogenic gradients. The highly controlled regulation/coordination of transcription factors is needed throughout brain development, from establishment of neural identity to terminally differentiated neurons. In fact, several studies of lineage tracing and fate-mapping of RG and IPCs (reviewed in Sun and Hevner, 2014), followed by gain- and loss-of-function experiments using *in utero* electroporation, have elucidated that manipulation of the transcription factors gradient is enough to change the position and size of cortical areas but not the neuronal identity. In addition, postmitotic controls are also essential to specify the identities of newly born neurons' subtypes (Molyneaux et al., 2007)

The last 20 years of an information-intense generation of computational data complementing traditional wet-lab (from proteomics to cell biology) studies of transgenic mice, combined with recent advances in spatially-resolved transcriptomics at the single-cell level, are making it possible to create a spatially accurate map of the developing transcriptional cascade governing corticogenesis (**Figure 3**) as well as building a molecular-logical code of the TF network e.g. by using Boolean network modelling (Greig et al., 2013).

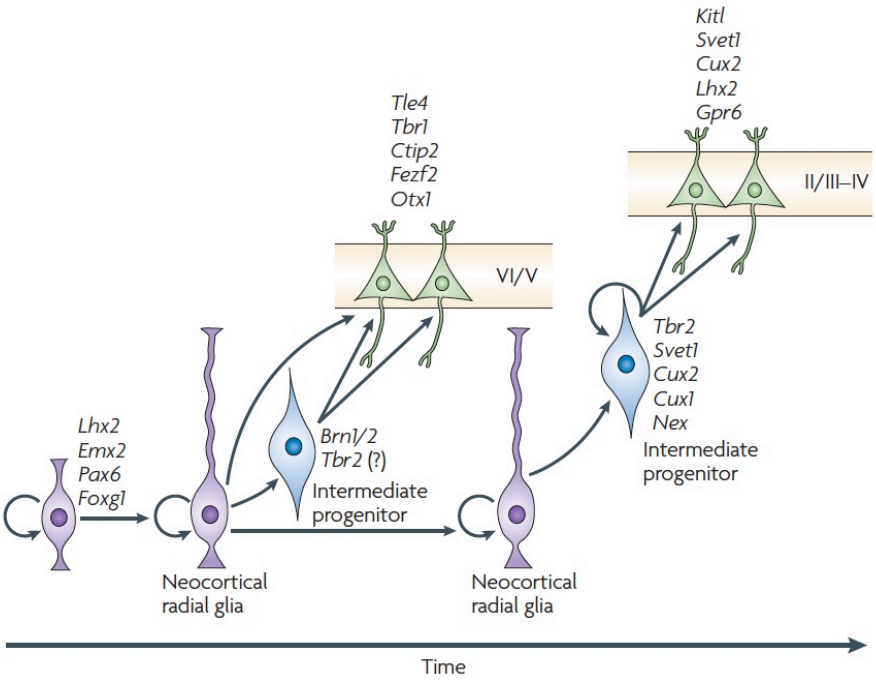

Figure 3. *Examples of layer-specific genes include, among many others: Cux2 and Lhx2, markers of layers II/III to IV; Brn2, a marker of layer II/III and V; Rorβ, a marker of layer IV; markers of layer V; and Foxp2, a marker of layer VI (not in the picture). Modified from Molyneaux et al., 2007.*

1.3 TRANSCRIPTIONAL CONTROL OF NEUROGENESIS

Now that cell-intrinsic programs and extracellular signals regulating fate restriction of neural stem cells have been generally discussed, it is apt to examine the actual mechanism of transcriptional control during gene expression in the developing cortex. The expression of genes during cortex development can be controlled at different levels, such as during initiation, elongation, at mRNA processing and stability, transport to cytoplasm and lastly during translation. Since this process begins with transcription, this initial stage becomes one of the most crucial.

The route of gene regulation, whether during activation or repression, is orchestrated by sequence-specific DNA-binding proteins encoded in the genome, such as transcription factors (*trans*-acting factors) acting together with proximal and distal coding or non-coding DNA sequences e.g. promoters and enhancers, in the genome (*cis*-acting elements). Transcription factors can bind in multiple regions and genes, making this combinatorial regulation between *cis*- and *trans* elements one of the most crucial aspects of differential gene expression (Carey et al., 2009).

A few questions immediately arise: How do transcription factors execute activation and/or repression during the elegant transcription factor cascade that specifies mammalian corticogenesis? Which other proteins are involved in such regulatory mechanisms? And finally, what happens when some of these mechanisms are failing?

To tackle these fundamental questions, it is crucial to understand the 3D architecture of gene regulation and to overview the key aspects of chromatin organization through which activation and repression take place in model systems of neural development.

1.3.1 Gene expression regulation

In all eukaryotes, the chromosomal DNA is packed into chromatin, which can be classified into heterochromatin (dense and compact with limited transcription) and euchromatin (decondensed and transcriptionally active) (Trojer and Reinberg, 2007). The fundamental unit of chromatin is the nucleosome, which consists of an octamer of histones (H2A, H2B, H3, H4) that have several isoforms playing specific roles during gene regulation. Every one of the four histones contain lysis-rich amino terminal tails, which are subject to numerous

post-translational modifications, control chromatin behavior and are necessary for regulation during transcriptional activation and silencing (**Figure 4**).

Transcription of protein-coding genes in mammals is carried out by the DNA-dependent RNA polymerase II (RNA Pol II). A typical mammalian gene is comprised of several regions that control the transcriptional activity, including a core promoter, a proximal promoter, enhancers and insulators. Transcription begins at the transcription start site (TSS), and for transcription to occur, general transcription factors and regulators are typically bind TATA-boxes (or other core promoter elements) and recruit a large complex of proteins and ultimately the RNA pol II. Transcription factors bound at distal regions could also control expression, often through the mediator complex. The regulatory elements within promoters and enhancers cooperatively regulate the expression patterns, as the core promoters alone often drive only low levels of basal transcription (Lenhard et al., 2012).

Gene expression is also affected by the chromatin structure beyond the genomic sequence itself through modification of the histone tails (Tessarz and Kouzarides, 2014). There are a large number of histone post-translational modifications that can be categorized into "small chemical groups" such as methylation, acetylation and phosphorylation as well as into the much "larger peptides" including ubiquitination, SUMOylation, citrullination, and ADP-ribosylation. In the literature these modifications are broadly regarded under the umbrella of "epigenetics", but it is worth noting that this word is often misused in light of recent advancement in the field (Ptashne, 2007) (Lappalainen and Greally, 2017) (Greally, 2018). Across the mammalian genome, histone modification influences the state of the chromatin, demarcates functional elements (promoters, gene bodies, enhancers, insulators) and fine-tunes the stability of repressive domains, which makes it fundamental for long-term repression/activation of developmental genes.

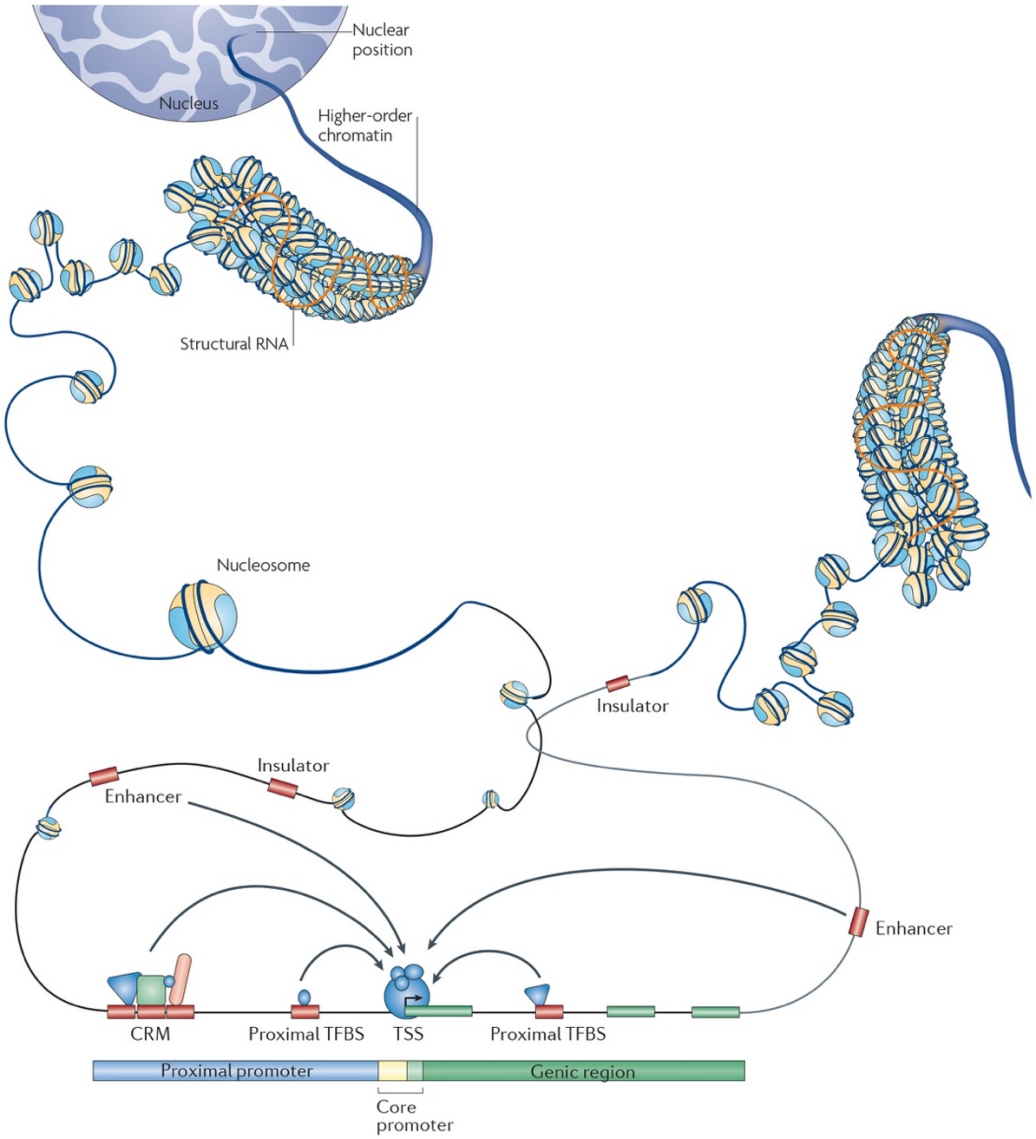

***Figure 4**. The structure of eukaryotic chromatin structure can be viewed as organized layers: from DNA sequence, to nucleosomes, to histone modifications and variants and, finally, to chromatin higher-order structures. To recruit RNA polymerase II (RNAPII) and to activate gene transcription, sequence specific transcription factors bind to transcription factor binding sites (TFBSs), alone or in clusters of cis-regulatory modules (CRMs). Modified from Lenhard et al., 2012*

1.3.2 Regulatory elements: Enhancers

Enhancers are short, *cis*-acting DNA sequences of 50-1500 bp that can be found many kilobases upstream of a gene, downstream or in introns. Enhancers associate with proximal promoters to form active chromatin hubs. Genes can contain multiple enhancers, which makes it an advantage for combinatorial expression among cell types or in response to different signals (Carey et al., 2009). Additionally, mammalian transcription is episodic, consisting of a series of discontinuous bursts, and recent evidence from drosophila studies proposes that enhancers regulate bursting frequency, making them a key parameter of gene control during development (Fukaya et al., 2016).

Genome-wide studies combined with chromatin conformation capture (3C), "Hi-C", and mechanistic approaches, help to elucidate high-resolution neuroanatomical annotations of enhancer activities and their associated histone modifications signature in the developing cortex (Nord et al., 2013) (Visel et al., 2009). Interestingly, Visel and colleagues have postulated that different cohorts of enhancers are active at different stages of brain development (**Figure 5**). Between E11.5 and E13.5, the majority of the enhancers revealed analogous patterns of activity, yet by E14.5, enhancers activity decreased and/or became limited to a smaller area in mouse forebrain development, suggesting that gene expression at later stages of the developmental processes is driven by other enhancers. Indeed, during early forebrain development, enhancers are enriched for annotation terms such as *neural precursor cell proliferation* and *axonogenesis*; later in forebrain development instead are enriched with biological processes for *synaptic transmission* and *cognition,* as well as phenotypes comprising of *abnormal learning, memory, conditioning* and *neurodegeneration* (Visel et al., 2009).

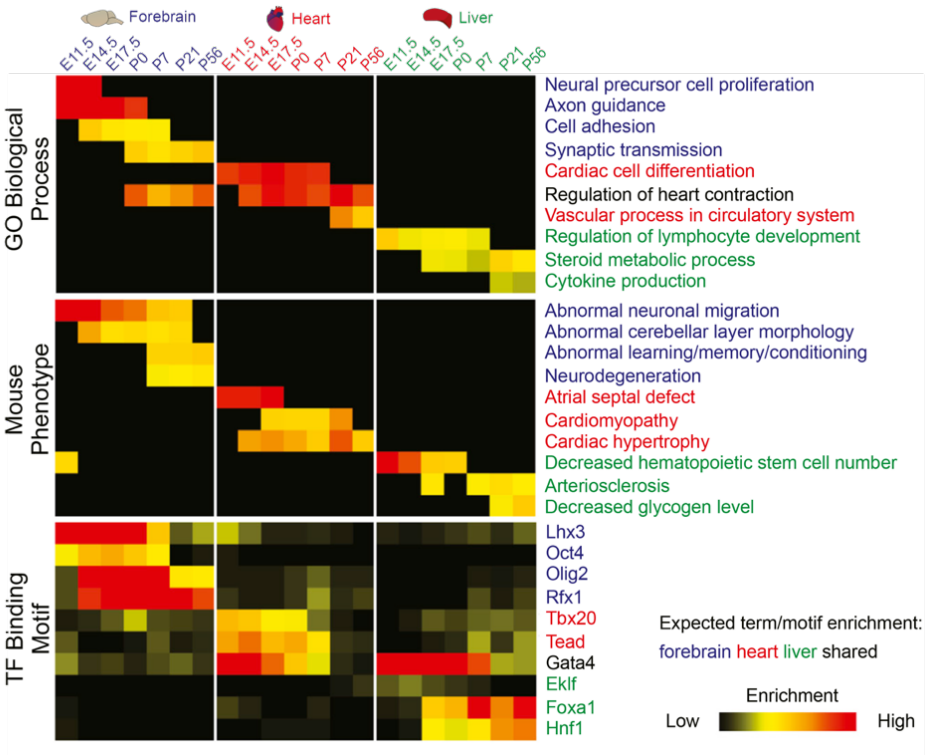

Figure 5*. Heatmaps displaying enhancer enrichment-GO biological functions across different tissues (forebrain, heart and liver) and timepoints (E11.5 to P56). Modified from Visel et al., 2009.*

Recent reports have also identified "developmental enhancers" which exhibit tissue-specific regulatory activity. Fascinatingly, several of these are 'human accelerated regions' (HARs), regions that are conserved during evolution but have dramatically mutated in humans and

have shown to directly being involved in changes that effected brain structure and activity (Capra et al., 2013) (Franchini and Pollard, 2015a) (Franchini and Pollard, 2015b) (Franchini and Pollard, 2017).

1.3.3 Chromatin modifications during neural differentiation

Early evidence of the importance of enzymes' mediation of post-translational histone modifications resulted from experiments in *Saccharomyces cerevisiae* during the late 1980's. Significant technical advancements in the field, including the use of chromatin immunoprecipitation (ChIP) to study protein binding to particular DNA sequences *in vivo*, lead to the overall understanding that these fundamental molecular mechanisms were evolutionary conserved, from yeast to flies and humans. During cortex development, several classes of enzymes were then discovered to be capable of maintaining NSC self-renewal and of orchestrating developmental progression towards a specific neural lineage (**Figure 6**).

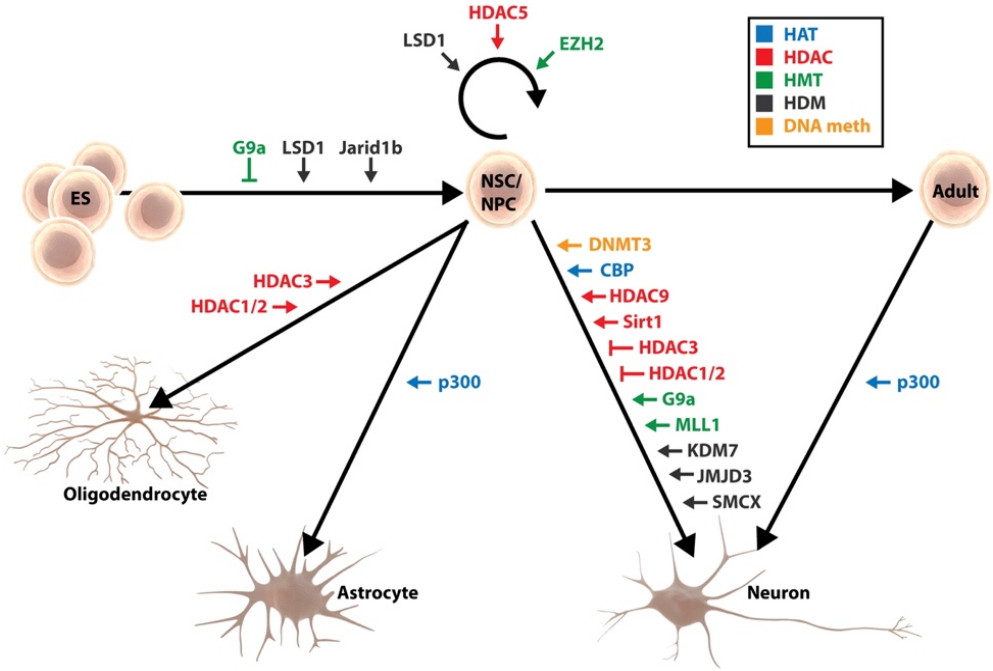

***Figure 6**. Histone-modifying enzymes involved in neurodevelopment and differentiation, color-coded for their mechanism of action. Modified from Lilja et al., 2013.*

1.3.3.1 Histone acetyl transferases (HATs) and histone deacetylases (HDACs)

The enzymatic activities of HATs and HDACs are intricately linked with transcription and these enzymes are recruited to modify histone tails at enhancers, promoters and within the gene body during transcription (early hypothesis: Allfrey et al., 1964) (Bannister and

Kouzarides, 2011). DNA-binding activators recruit HATs to acetylate nucleosomal histones, which are known to contribute to transcriptional activation, while repressors recruit HDACs to deacetylate histones, and therefore repress transcription (Strahl and Allis, 2000).

From the group of HATs, p300 is needed for astrocyte development and axonal regeneration, and CBP is required for neural development (reviewed in Lilja et al., 2013). In addition, CBP/p300 has been shown to be an accurate predictor of *in vivo* enhancer activity in the developing mouse (Visel et al., 2009).

The class of HDACs contains numerous enzymes falling into three groups: types I, II (with related mechanism of acetylation) and III (Sirt-2 related enzymes that require co-factors). During glial differentiation, HDACs 1, 2 and 3 (Castelo-Branco et al., 2014) are reported to be involved, while HDAC3 has been shown to modulate H4K8 acetylation levels and reduce long-term memory formation in hippocampal neurons (reviewed in Lilja et al., 2013).

Many of the HDACs are found in large multi-subunit complexes. Nuclear hormone corepressors, exemplified by the nuclear receptor corepressor (NCoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT), recruit HDAC 3. By repressing GFAP transcription, NCoR/NCoR1 represses astrocyte and oligodendrocyte differentiation in neural stem cells (NSCs), while NCoR2/SMRT represses neuronal and astrocytic differentiation by binding to the retinoic acid (RA) receptor and its target genes in the absence of retinoic acid (Hermanson et al., 2002) (Perissi et al., 2010).

1.3.3.2 Histone methyl transferases (HMT) and histone demethylases (HDMs)

Methylation is a histone modification that has multiple layers of complexity, and it 1) can occur either on lysines (referred to as "K") or arginines; 2) can be either positive or negative towards transcriptional expression and; 3) there can be multiple methylation states for each residue, such as mono- (me1), di- (me2) or tri- (me3). Such combinational potential allows for precise regulation of sequentially and timed events, during the transcription (Zhang and Reinberg, 2001) (Bannister et al., 2002). Methylation of H3 on K4, K36, K79 is usually associated with genes that are active, while methylation of H3 on K9, K27 and H4K20 is usually associated with genes that are repressed. Genome-wide studies of H3K27me in ES cells differentiating towards neural progenitors have found that H3K27me plays an important and flexible role in many promoters, which highlights the necessity for enzymes that add and remove H3K27me. The enzymes responsible are histone methyl transferases (HMT) such as

Mll1 or Ezh2. Studies on mice lacking Mll1 show impaired postnatal neurogenesis, and found to be associated with Dlx2, which had diminished expression (Lim et al., 2009). Another HMT with a key role in the development cortex is Ezh2, which is a HMT associated with the Polycomb Repressor Complex 2 (PRC2) and is found to indirectly promote astrocytic differentiation (Hirabayashi et al., 2009).

Enzymes that can de-methylate histones also play a key role in stem cells differentiation, such as LSD1 which is crucial to embryonic stem cell differentiation but not to self-renewal (Sun et al., 2010). Other demethylases that possess a distinct catalytic structure from LSD1 include the JmjC-domain family. A member of this family of demethylases is Jarid1c/SMCX/KDM5C, which specifically demethylates H3K4me3 to a mono- or di-methylation. Iwase and colleagues, using zebrafish and primary mammalian neurons models, demonstrated the fundamental role of SMCX in neuronal survival and dendritic development (Iwase et al., 2007) (Iwase et al., 2016) .

Taken together, enzymatic events of chromatin structure control have been shown to be implicated in the whole process of neural development, from proliferation at the stem cells state, at the neural/gliogenic switch and for neuronal survival and neurite outgrowth.

Regrettably, aberrations of the enzymatic activities described in this paragraph, have been linked to neural developmental disorders and neurological conditions. There should therefore be continuous emphasis on attempting to understand these enzyme function and roles in neurobiology (Iwase et al., 2017).

1.4 NEURODEVELOPMENTAL DISORDERS AND DEGENERATION

Neurodevelopmental disorders are complex and diverse conditions, both for pathophysiology and possible treatments. Increasing evidence supports the claim that transcriptional and higher-order chromatin abnormalities play a strong role in the development of neurological and psychiatric diseases (Mitrousis et al., 2015). Several studies in humans have suggested that, even in post-mitotic neural cells, the chromatin landscape and its regulation remain plastic, and that in addition disordered chromatin functions have been identified in several neurodevelopmental syndromes of early childhood and in a subset of adult-onset hereditary neurodegenerative syndromes (Jakovcevski and Akbarian, 2012). At least a dozen neurological spectrum syndromes have thus far been linked to single-gene mutation encoding

for histone modifying enzymes, their associated proteins or DNA methyltransferases. Deficits in language and motor skills, obesity, autistic features and epilepsy are common features in this category of mutations (Deriziotis and Fisher, 2017).

Chromatin-related diseases can be categorized into two classes: those with *trans* effects, include the loss or dysfunction of chromatin-associated factors (e.g. chromatin effector mutations) which can in turn alter chromatin structure and gene expression at certain genomic regions, while those with *cis* effects, represent mutations in non-coding regulatory sequences (e.g. promoters). These mutations, which may include the expansion of DNA repeats, can lead to chromatin alterations which affect the gene expression (Zoghbi and Beaudet, 2016).

An example of a *trans*-effect mutation, is the postnatal neurodevelopmental disorder Rett syndrome (RTT) which is initiated by mutations in the gene encoding for the transcriptional repressor involved in chromatin remodeling and the modulation of RNA splicing, MeCP2 (methyl-CpG binding protein 2). Another well-studied genetic disorder that affects chromatin structure in *trans* is the Rubinstein-Taybi syndrome, which is characterized by mental retardation, congenital heart defects and increased risk of tumor formation (Amir et al., 1999). The cause of Rubinstein-Taybi syndrome is a decreased HAT activity due to mutation in *CREBBP* and *EP300* genes, which encode for CBP and P300 proteins respectively (Petrif et al., 1995). In mice models, those with haploinsuffiency of CBP mutations show impaired learning and memory, altered synaptic plasticity and abnormal chromatin acetylation (Goodman and Smolik, 2000).

An example of a *cis*-effect mutation is the Fragile X syndrome, which is one of the most common causes of inherited mental retardation. The protein encoded by the FMR1 gene is the Fragile X mental retardation protein (FMRP), which is an RNA binding protein that is proposed to tightly regulate local translation in neurons by inhibiting translation pre-synaptically. The disease's etiology has been proposed to stem from an epigenetic silencing of the locus and consequential loss of the FMRP protein, which results in increasing protein translation at synapses (Christopher et al., 2017).

HDACs play an important role in brain development mechanisms that lead to Autism Spectrum Disorder (ASD. The early generation epileptic drug Valproic Acid (VPA) in an HDAC inhibitor. A 10-year population study of children born in Denmark, has proven that the maternal use of VPA during gestation is associated with ASD in the offspring

(Christensen et al., 2013). Similarly, studies on rat uterine exposure of VPA (Schneider and Przewłocki, 2005) have identified significant increases of acetylation at the promoter of the transcription factor Pax6 and subsequent *Pax6* gene up-regulation, resulting in autism-like behavior (Kim et al., 2014).

Iwase and colleagues uncovered another important link between histone modifications and X-linked mental retardation through SMCX/JARID1C/KDM5C, and they demonstrated its role in neuronal survival and dendritic development as well as its link to the demethylase activity (Iwase et al., 2007).

Intriguingly, whole-genome sequencing studies have revealed that molecular networks known to be involved in speech and language impairment are found to intersect to molecular networks involved in ASD, epilepsy, and other rare neurodevelopmental disorders. (Deriziotis and Fisher, 2017).

Recent studies have also revealed the vital importance of chromatin modifiers during neurodegeneration, elucidating that higher order chromatin folding affects gene transcription in post-mitotic neurons.

Interestingly, in Huntington disease (HD), a neurodegenerative disease of the striatum and cortex characterized by cognitive dysfunction, psychiatric symptoms and choreic movements, reducing the levels SMCX/JARID1C/KDM5C in primary neurons reversed down-regulation of key neuronal genes caused by mutant Huntingtin expression. In addition, SMCX was neuroprotective in a *Drosophila* HD model (Vashishtha et al., 2013). The RE1-silencing transcription factor (REST) was also shown to be neuroprotective and aberrantly associated with protein aggregates during the course of neurodegenerative diseases (Lu et al., 2014) while independently LSD1 might be affected in the aging brain and in human dementia (Hwang et al., 2017). Taken together, these findings both from neurological spectrum disorders of both development and degeneration, highlight the importance of understanding the molecular mechanism governing these diseases for future therapeutic interventions.

2 AIMS

General aim

The overall aim of the work presented in this thesis is to investigate the transcriptional mechanisms of gene regulation by key transcription factors involved in telencephalic formation and function, using human neural progenitor as *in vitro* model of development.

Specific aims listed according to each study:

I. To investigate the functional role for the co-repressor NCOR and its interaction of recruitment with the transcription factor FOXP2 in regulation of a subset of genes involved in cortical development.

II. To describe the PAX6 transcription factor mechanism of repression via recruitment of the histone demethylase KDM5C.

III. To characterize the histone landscape of promoters involved in the maintainance of pluripotency in long-term *in vitro* culturing of human embryonic stem cells and human induced pluripotent stem cells, under different passaging conditions.

IV. To investigate the *in vitro* correlation between the binding of the transcription factor NF-κB to the *HTT* promoter and its mechanistic importance in Huntington's disease (HD).

3 METHODS

This section presents the essential material and methods that have been used across the four studies included in this thesis. For a more exhaustive description, please refer to the individual publication.

3.1 MODELLING HUMAN CORTICAL DEVELOPMENT *IN VITRO*

The recent technical advances in generating *in vitro* models of human cortical development have been fundamental to progressing the understanding of the complex human neurobiology in health and recapitulate disease models. Prior to the discovery of human induced pluripotent stem cells (hiPS) by Yamanaka and colleagues, the only available source of embryonic neural progenitor was via differentiation of human embryonic stem cells (hESC) toward the neural lineage or via the primary culture of neural progenitors from aborted human fetuses donated for research (**Figure 7**). This restricted accessibility of the material due to both issues of rarity and bioethical limitation made the use of human neural progenitor cells for extensive biomedical research inadequate and less standardized.

In studies I and II we made extensive use of "long-term self-renewing neuroepithelial-like stem cells" (NESCs) established and generated in the Falk laboratory to use as a robust and reliable model system to study the properties of these cells in culture. Briefly, human iPSC lines were produced by standard retroviral transduction with OCT3/4, KLF4, SOX2 and c-MYC. Post-transduction, the individual clones are picked and transferred to separate monolayer cultures, where the "neuralization" took place according to previously described protocols (Falk et al., 2012) (Koch et al., 2009) (Shahsavani et al., 2017). The established NESCs lines are positive for neural precursor markers for Nestin and SOX2, and they express SOX1 and PAX6, but the lines are negative for differentiated neuronal and glial markers such as Tubb3 and GFAP.

Overall, the NESC system is a robust 2D-culture model, for several reasons: It is easy to handle in a well-equipped stem cell laboratory with basic good manufacturing practices (GMP) conditions, its wide use among different labs has proven that there is limited

batch-to-batch variation and it results in a stable genotype and phenotype even when subjected to freeze-thawing cycles or long-term culturing.

However, like every model, the NESC system has its limitations to take into considerations. The 2D culture system is profoundly lacks the *in vivo* three-dimensional conditions, e.g. the self-organization of the cortex, individual cell-to-cell contacts, the extracellular milieu and the intrinsic signalling that occur during corticogenesis, as previously described.

It is important to be aware of these limitation, and therefore it is always recommended to make use of several models, and to reproducing the findings using additional complementary systems for cultural development. This includes: *in vivo* studies in animal models, meta-analyses of data generated in different laboratories and finally transitioning to 3D culture systems, such as brain organoids, under defined and standardized conditions.

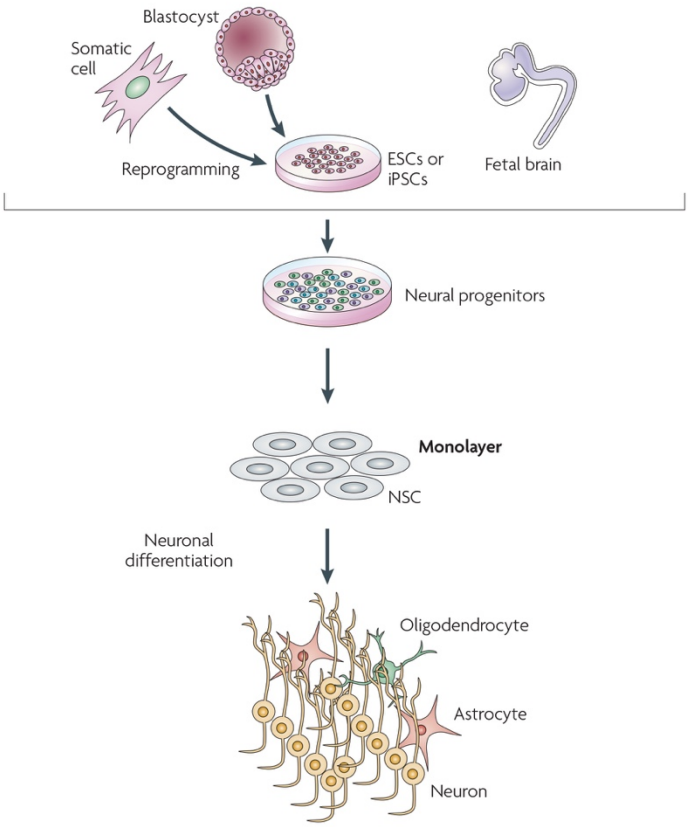

***Figure 7**. Neural Stem Cells (NSCs) sources. Modified from Conti and Cattaneo, 2010.*

3.2 GENOMIC METHODS FOR PREDICTING INTERACTIONS

3.2.1 ChIP-seq

One of the first experimental strategies to execute when studying functional regulation of a control region by DNA-TF binding and/or histone modification or chromatin protein is the well-established Chromatin Immuno-Precipitation assay (ChIP) coupled with new generation sequencing (ChIP-seq). Commercially available kits have become a reliable standard for reproducibility among this assay. However, it is usually recommended to trouble shoot the experimental conditions according to the starting material (e.g. whether cell lines or tissues). Briefly, DNA/protein are cross-linked, the extracted chromatin is fragmented by sonication and immunoprecipitated with the antibody of choice, the DNA is extracted and reverse cross-linked to be fragmented and analyzed through massively parallel DNA sequencing.

The obtained results (reads) are processed through a standardized quality control pipeline that uses samtools/1.3 to sort and index .BAM files and NGSUtils/0.5.9 to calculate simple reads statistics, NGSUtils/0.5.9 to remove duplicated reads and the blacklisted genomic regions with artificially high signals that can lead to false positive results and phantompeakqualtools/1.1 to calculate the standard cross-correlation metrics and plots after removal of the duplicated reads and blacklisted genomic regions. DeepTools/1.1.2 is used to obtain ChIP-seq cumulative enrichment (fingerprint) and to assess overall similarity between libraries, spearman- and pearson-based clustering heatmaps in bin mode. MACS/2.1.0 is used to call peaks, using 2.45e9 as an estimated effective genome size and default settings. DiffBind_2.2.12 R Bioconductor package is used to prepare a list of all peaks present across libraries and to calculate fractions of reads in peaks (FRiP). ChIPpeakAnno_3.8.9 is used for down-stream analysis of peaks, i.e. annotations and visualizations binding site distribution relative to features and for obtaining enriched pathways.

ChIP-seq also has its limitation regarding the quality of the primary reagents (e.g. antibody affinity or cross-reactivity), as the ChIP alone cannot really provide evidence that the protein of interest carries out an important function at the location of its binding, and finally standardized pipeline for bioinformatics analyses and quality control are necessary to compare data across experiments.

ChIP-seq was used in Studies I and II, and ChIP-qPCR in Studies I, II, III, and IV.

3.2.2 Chromosome conformation capture (3C)

3C was developed by the Nancy Kleckner laboratory as a general assay to study the frequency of interaction between different genomic loci (Dekker et al., 2002). In Study I, 3C was used to characterize NCOR and FOXP2 putative regulatory elements. As described briefly in **Figure 8**, the technique begins with the crosslinking of the protein complexes to DNA, followed by a digestion of the chromatin using restriction enzymes. ATP and DNA ligase enzymes are then added to the diluted solution to ligate the loose ends of the DNA that are in close proximity. Interactions are then monitored via qPCR using primers specific to the region of interest (**Figure 9A**).

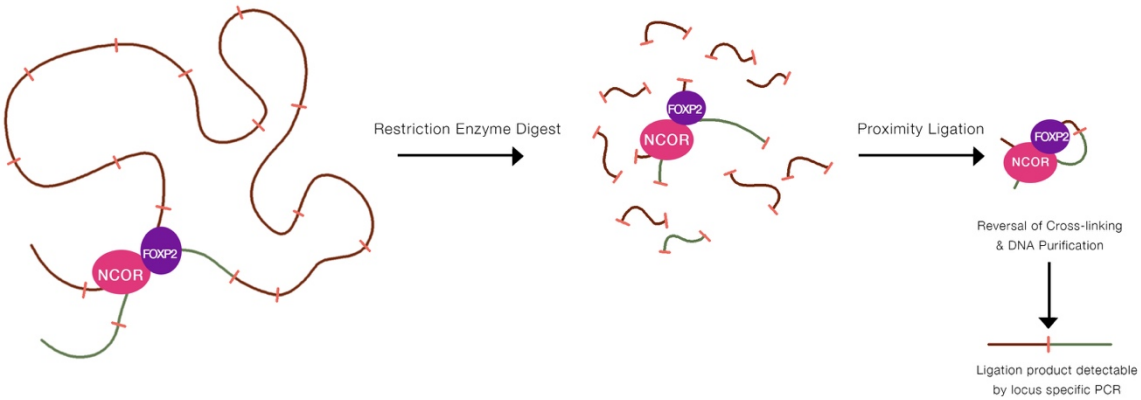

***Figure 8**. Schematic representation of 3C technique.*

To study the putative regulatory region identified in Study I, 3C in human NESC was established. **Figure 9A** shows a schematic view of the SLITRK gene cluster on Chromosome 13. The cut sites for HindIII, the restriction enzyme employed in the assay, are shown as perpendicular lines. Forward primers were designed so that they flanked a restriction enzyme cut site in a fashion so that they tiled the region of interest. Reverse qPCR primers and probes (shown in yellow) were designed to be located at a HindIII cut site in proximity to the putative regulatory elements 1 and 2 (shown in red). To calculate the ligation event efficiency, control qPCR primers are designed, as exemplified in **Figure 9B**. Amplification of the "total DNA background" is calculated via the product of qPCR primers A+B, which should always occur as no restriction enzyme HindIII cut site lies between the two. Primers A+C allow the percent of digestion efficiency to be determined, as the HindIII site interrupts the amplification if the restriction has occurred as expected.

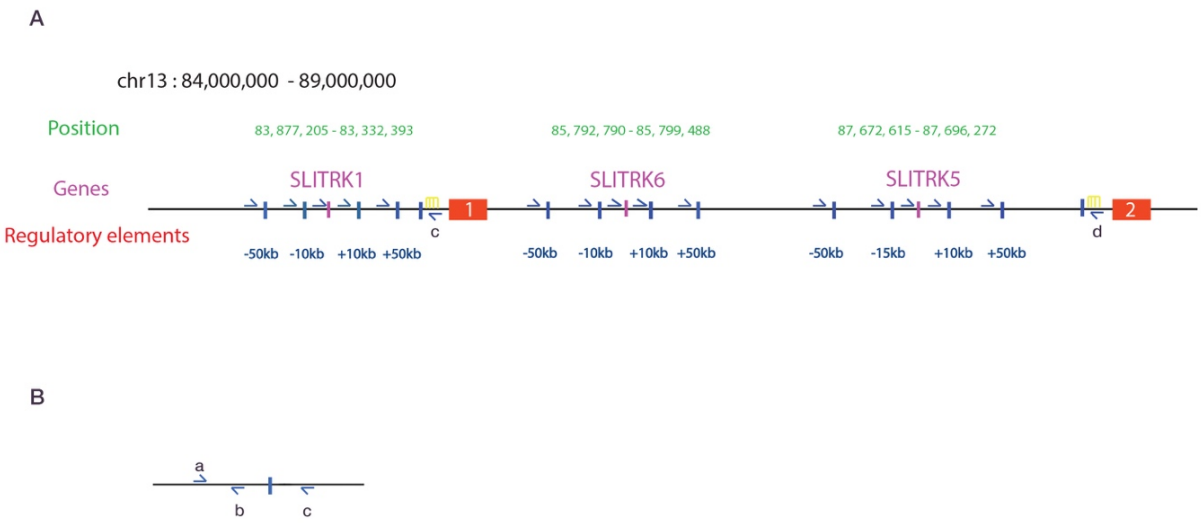

***Figure 9**. **A)** SLITRKs gene cluster and primer design for the respective regions. **B)** Control primer design.*

Since its first application in yeast, the 3C technique has been adapted to several mammalian cells and newer sequencing methods have developed it further in 4C, 5C, Hi-C and so on (methods reviewed in Shlyueva et al., 2014).

4 RESULTS AND DISCUSSION

4.1.1 STUDY I

NCOR and FOXP2 regulate expression of the SLITRK family via distinct and distant regulatory elements

The transcription factor FOXP2 has been implicated to carry out crucial neurogenetic mechanisms facilitating human spoken language. The gene was first discovered during a search for an underlying reason for developmental verbal dysplaxia; a speech and language impairment found in humans (Lai et al., 2003). FoxP2 is expressed in multiple regions within the developing brain including the cortical plate, basal ganglia, thalamus, cerebral cortex and cerebellum.

Along with the closely related transcription factor FoxP1, FoxP2 has been suggested to play essential roles in the regulation of growth and differentiation during organogenesis (Wijchers et al., 2006). A functional interaction between FoxP1 and the corepressor SMRT was discovered to be required for cardiac growth (Jepsen et al., 2008). SMRT is important for proper brain development and in particular for the control of the differentiation progress of cortical progenitors (Jepsen et al., 2007) (Castelo-Branco et al., 2014). As FoxP1 and FoxP2 proteins have an especially high sequence homology, it was hypothesized that interactions between corepressors and FoxP proteins might be a general mechanism in the development of target organs, and that FoxP2-mediated regulation of gene expression in brain development would require interactions with corepressors, such as SMRT and/or the closely related NCOR.

A genetic screen in human NESC (*N.B.* in the manuscript referred to as hNPs) for chromatin regions binding FOXP2, NCOR and SMRT established using ChIP-seq. Favorable control of the quality of the FOXP2 ChIP-seq was confirmed by the presence of peaks in the well-established FOXP2 target gene *CNTNAP2*, which is from the neurexin family that is expressed in the developing human cortex. Neurexins are a family of neuronal cell surface proteins with proposed roles in cell adhesion and intercellular signaling and are suggested to affect the glia-neuronal signaling. The phenotype of individuals with neurexin gene deletions is variable but includes autism spectrum disorders, mental retardation as well as language delays (Vernes et al., 2008).

ChIP-seq results identified 1326 overlapping regions between FOXP2 and NCOR and 1795 overlapping regions between FOXP2 and SMRT. Analyses of the peaks revealed significant enrichment of FOXP2 binding in two regions in proximity to SLITRK family regulatory regions. SLITRK genes are of interests as several of its members have been associated with neuropsychiatric disorders. To further investigate the nature of the two regions regarding putative regulatory function, a profiling of the regions for chromatin marks was conducted together with 3C experiments and indicated that FOXP2-NCOR binding sites represent active regulatory elements and that the expression of SLITRK genes is regulated by NCOR. Intriguingly, one of the regions has been identified as a "human accelerated region" by three different reports, further supporting the importance of these regulatory elements as key mechanisms in proper human development and speech.

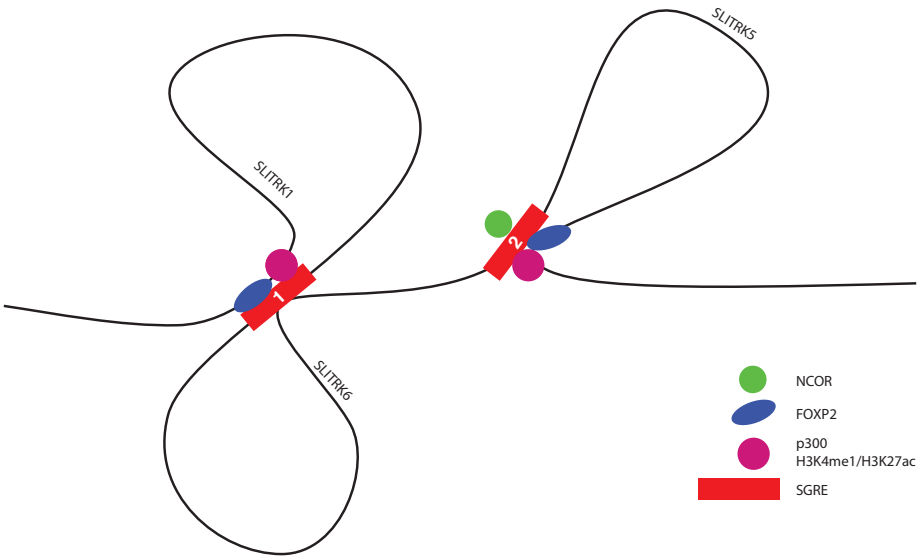

***Figure 10**. Simplified model of interaction.*

In conclusion, the findings outlined in this manuscript support the presence of a novel mechanism for NCOR, in which interaction with FOXP2 regulates the expression of SLITRK genes via two distinct and distant elements. Since FOXP2 entails implications in development and evolution of speech, this regulatory model exemplified in **Figure 10**, is of a great interest for further investigations of neurodevelopmental disorders connected with speech impairment. Collaborators have access to mice harboring conventional and conditional gene deletions of SMRT and NCoR in excitatory projection neurons in the cortex, and studies to visualize morphological impairments are currently on-going.

4.1.2 STUDY II

Pax6 and KDM5C co-occupy a subset of developmentally critical genes including Notch signaling regulators in neural progenitors

Pax6 is an evolutionary conserved transcription factor, found in all animals with bilateral symmetry and is a master regulator of a plethora of functions, such as embryonic and forebrain development as well as pancreatic, pituitary and ocular genesis (reviewed in Ypsilanti and Rubenstein, 2016).

It has been noted that genetic ablation of Pax6 can result in both down- and up-regulation of gene expression in the developing CNS, suggesting that Pax6 can act as both a transcriptional activator and repressor. When acting as a transcriptional activator, Pax6 has been associated with H3K4me3 methyl transferases; however further reports show that Pax6 preferentially binds to methylated DNA (Bartke et al., 2010), and show that when Pax6 is ablated, the expression of a subset of genes has been shown to be increased.

Using the inducible/integrated GAL reported system, we show that Pax6 can indeed act as a direct transcriptional repressor and that the Pax6-mediated repression is primarily associated with demethylation of trimethylated lysine 4 on histone H3 (H3K4me3).

H3K4me3 is a positive mark for transcription and is strongly associated with the recruitment of the transcriptional initiator complex to the promoter, and a demethylation of H3K4me3 is considered to have a robust repressive effect. A known H3K4me3 demethylase expressed at significant levels in neural stem cells and progenitors is SMCX/Jarid1C/KDM5C (Jung et al., 2005). Jumonji domain-proteins similar to KDM5C interact with sequence-specific transcription factors, but less is known regarding such KDM5C-recruiting factors.

Two separate published ChIP-seq data sets for Pax6, KDM5C and H3K4me3, generated in the developing mouse forebrain and in neural progenitor cells, were analyzed and compared for co-occupancy. Results show that Pax6 and KDM5C overlapped with H3K4me3-negative regions. Several of those regions were associated with Notch signaling, including Dll1, Dll4, and Hes1, when analyzing for Reactome pathway terms. Considering the fundamental role of Notch signaling in neural development, we suggest that this regulatory mechanism may play a role in pathologies associated with aberrant Pax6 and/or KDM5C activity.

RNA knockdown of PAX6 and KDM5C in human NESCs results in a change of gene expression levels of DLL1 and DLL4 but not HES1. These results can be explained by the dual role of PAX6 as activator and repressor or by the chromatin landscape at the binding site, e.g. at *HES1* there multiple H3K4me3 peaks. One could also argue that the method of choice for mRNA silencing (siRNA) should be complemented to more efficient genome editing technique (e.g. CRISPR KO system) for generating a PAX6 Knock-Out line in NESC. This was indeed attempted in this study, but NESC single cells of PAX6 KO clonal line did not survive, which is why the approach was discarded.

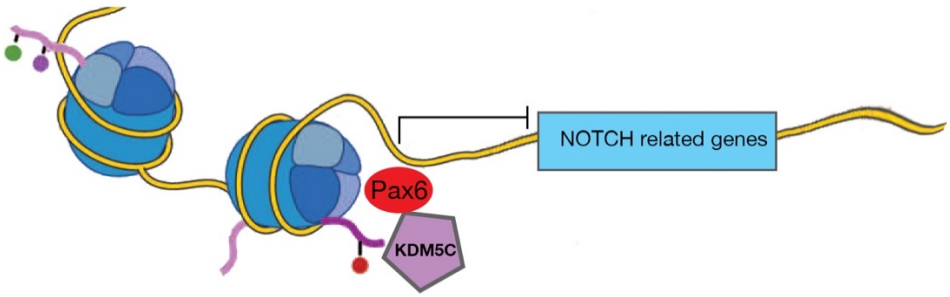

Figure 11. *Model*

Thakurela and colleagues have previously shown that Pax6 directly targets the promoter of Notch signaling components in ES cells (Thakurela et al., 2016). Here we expand these results by adding another level of tuning and propose that Pax6 directly recruits KDM5C to repress a subset of genes involved in Notch signaling and function in NESC cells. Notch signaling is critical for many events during brain development, such as proliferation, differentiation, migration, morphology, survival and plasticity. Study II suggests that neurodevelopmental aberrations by PAX6 and/or KDM5C mutations maybe be associated with defects in Notch signaling (**Figure 11**).

4.1.3 STUDY III

Passaging techniques and ROCK inhibitor exert reversible effects on morphology and pluripotency marker gene expression of human embryonic stem cell lines

The use of human embryonic stem cells (hESC) and human induced pluripotent stem cells (hIPS) is an exciting area of research regarding the potential insights into the basic biology of pluripotency and regarding their promising clinical potential they hold as cell replacement in degenerative disease. Since their discovery, significant progress has been made in characterizing the *in vitro* behavior of hESC and hIPS.

The aim of Study III was to investigate the gene expression profiles and histone modifications associated with pluripotency marker genes in, at the time, three common ways to passage hESC and hiPS cells, i.e. using mechanical splitting techniques by surgical scalpel and enzymatical splitting with or without a ROCK inhibitor. ROCK-dependent signaling pathways play a significant role in many physiological functions, such as cell proliferation, adhesion, migration and inflammation. When applied to human embryonic stem cells, the ROCK inhibitor has been shown to decrease disassociation-induced apoptosis, increase cloning efficiency and facilitate subcloning after gene transfer (Watanabe et al., 2007) . One report found that the morphology was relatively unchanged and that pluripotency markers to have maintained expression after ROCK inhibitor administration to hESCs (Gauthaman et al., 2010).

However, when the three ways of passaging hESC and hIPS were investigated, a decreased in pluripotency markers was observed in the enzymatic passaged cells. These differences in the gene expression profile however correlated poorly with the analysis of histone modifications in the promoters of the same genes. To evaluate whether these effects on the morphology and gene expression were permanent, cells passaged enzymatically were then passaged mechanically instead and re-analyzed, with the results showing that the earlier decreased levels in gene expression of the most commonly used pluripotency markers had reverted to initial levels.

In conclusion, it is important to review this work using an “historical” approach. Since this study was conducted and published, several advancements were made in the field of pluripotency. It is particularly relevant to consider the following points: Early hESC line derivation used a highly empirical culture system (e.g. feeder cells, fetal calf serum) which

impacted on the pluripotency state of the cells. The original settings of derivation of hESC lines were In Vitro Fertilization (IVF) hospital research units and were often conducted by embryologists who relied heavily on morphological cues to assess their cell behavior. In addition, hESC lines derivation is rare, which implies that the cell lines obtained are maintained in culture for long period of time. In addition, before the iPS revolution, the knowledge of reprogramming and transdifferentiation was in its early days, and still lacked the technological support of accessible single cells techniques and genome-wide high throughput methods. Some of the important questions in the field are now beginning to be understood, such as: Are mouse ES cells the same as hESC (Guo et al., 2016)? And How is the gene expression profile of mouse and human preimplantation embryos? (Deng et al., 2014) (Petropoulos et al., 2016).

With that said, the important observation from this study is, how reversible was the effect on pluripotency genes when the culture methods were overturned. A possible speculation, could be inferred regarding the plasticity of the chromatin landscape (Cheloufi and Hochedlinger, 2017) of pluripotency cells and how perturbation (e.g. a rough enzymatic splitting) can indeed affect the cellular state, but that does not consolidate these changes into a new cell identity that would require stable modification of chromatin structures.

4.1.4 STUDY IV

A SNP in the HTT promoter alters NF-κB binding and is a bidirectional genetic modifier of Huntington disease

Huntington's disease (HD) is a neurodegenerative disorder characterized by progressive cognitive, motor and emotional impairments initiated by a single genetic mutation in the HTT gene, which is responsible for a trinucleotide "CAG" repeat expansion and disease age onset. Although the genetics of HD have been known for over 20 years, no treatment for HD is yet available.

Findings by Bečanović and colleagues at the University of British Columbia, have proven through *in vitro* reporter assays, in silico screening and site direct mutagenesis experiments that the HTT promoter transcriptional activity is reduced of 50% by a cis-regulatory SNP in the NF-κB binding site. NF-κB is a transcription factor involved in inflammation and the regulation of cytokine production and has been previously been shown to be connected with HD (Träger et al., 2014). *In vivo* ChIP analyses in WT mouse striatum, prefrontal cortex and

cerebellum as well as *in vitro* ChIP analyses from Lymphoblastoid (LCLs) cell lines from HD patients and rat striatal ST14A cell line stimulated with TNFα, demonstrated that the mouse HTT promoter indeed contains the NF-κB binding site. The effect on HTT mRNA expression was therefore investigated upon transfection with siRNA against NFκB1 and p65 in HEK293 cells. NFκB1 and p65 encode for the p50/p65 heterodimer complex and indeed targeting their precursor mRNA indeed resulted in decreased HTT mRNA expression. The same decrease was seen in p53 mRNA levels, an established NF-κB target. SiRNA knockdown efficiency was on average 82% for NF-κB1 and 70% for p65. To further validate the findings, a cohort of HD patients in Denmark and Canada were screened for the disease modulatory effect of a single nucleotide polymorphisms (SNP). Findings were striking, as indeed the cis-regulatory variant was proven to be a "bidirectional modifier" and to modulate the HTT promoter region and the disease onset in the two independent cohorts of patients. When genotyping was conducted in the familial HD cases in both cohorts, it was revealed that when the SNP was present in the WT allele, the onset of the disease was extra-early (circa 9 years), whereas when present in the HD allele the disease onset was delayed (**Figure 12**).

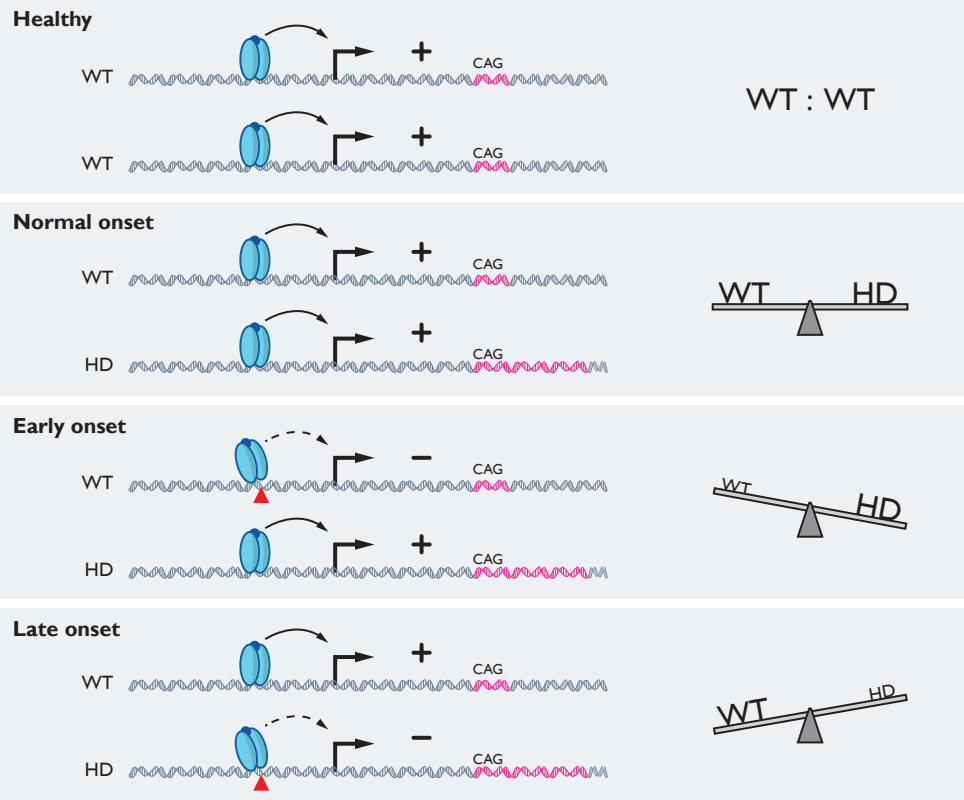

***Figure 12**. Model. The SNP is located in the identified NF-κB TFBS in the HTT promoter immediately proximal to the HTT gene. NF-κB binding is impaired and transcriptional activity of the HTT gene and HTT protein levels are reduced. When the variant is present on the wild-type allele, there are reduced wild-type HTT protein levels and earlier AO, while on the HD disease allele, there are lower mutant HTT (mHTT) protein levels and delayed AO in HD patients.*

These results are significant in different ways: Firstly, professional genetic counselling for patients and their family that falls under the studied carrier genotypes could be tailored, and relevant prognosis could, therefore, modulate therapeutic approaches; secondly the allele-specific targeting tactics for HTT mRNA may have greater therapeutic efficacy and finally, other cohorts of patients should be screened to improve knowledge of cis- and trans regulatory elements in HD.

5 CONCLUSION

In this thesis, novel transcriptional mechanisms of neurodevelopment and neurodegeneration have been described. In addition, common underlying threads highlighted in the interplay between the transcription factors and chromatin structure have been shown.

Study I reports a novel mechanism through an interaction between the co-repressor NCOR and the transcription factor FOXP2 and proposes a possible role for this regulatory mechanism in accurate development and possibly evolution of vocal and motor skills. To further investigate this interaction, several analyses could further take place 1) analyses of vocalization/behavior on the post-natal brain of conditional KO mice 2) genetic screening for mutations in the enhancer regions of fibroblast lines or hIPS cells derived from individuals with speech impairments 3) in-depth characterization of the implication of SGRE1/2 as possible HAR. Study II demonstrates that the transcription factor PAX6 can function as a repressor and recruit the histone demethylase KDM5C to repress a subset of genes involved in Notch signaling, proposing that neurodevelopmental aberrations by PAX6 and/or KDM5C mutations maybe be associated with defects in Notch signaling. To overall improve upon these results, the next-generation of standardized (in terms of cell cultures and handling), 3D *in vitro* models of the developing human cortex, are promising tools to continue exploring the complexity of the human brain in health and disease (Di Lullo and Kriegstein, 2017) . In addition, technological advancement such as genome editing techniques (e.g. CRISPR/Cas9) should be integrated in these works and should gradually replace siRNA silencing, when possible. In addition, new techniques to manipulate chromatin-looping and 3D chromatin structures are emerging and could contribute to the transcriptional regulation tool box available for mammalian cells. Single-cell technologies to create topographical gene expression mapping with the respective chromatin snapshots should also be considered and implemented in these kinds of studies. Study IV reveals a mechanism by which a single nucleotide polymorphism (SNP) in the promoter of the HTT gene, disrupts the NF-κB binding and transcriptional regulation of the HTT gene. These findings show that silencing of the HTT gene could be a promising therapeutic strategy in Huntington's disease. To improve upon these results, continue identification of *cis*- and *trans* regulatory elements could provide insights for new therapeutic targets in Huntington's disease, and additional molecular tool such as CRISPR/Cas9 could further elucidate the role of the SNP in HD.

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