Thesis for doctoral degree (Ph.D.) 2009

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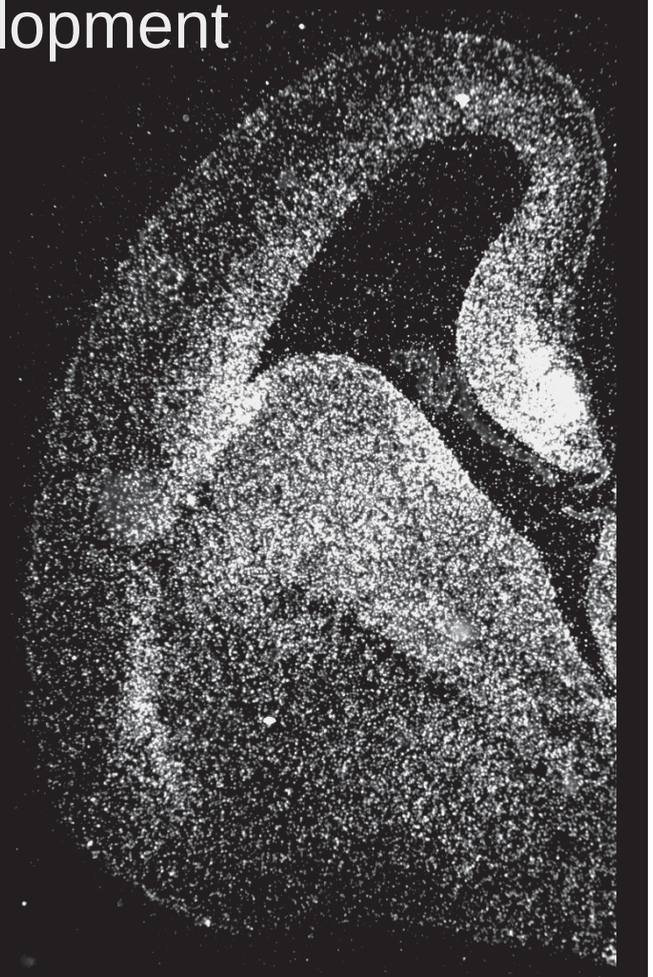
On Molecular Mechanisms of Neural Differentiation and Forebrain Development



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On Molecular Mechanisms of Neural Differentiation and Forebrain Development

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Stockholm 2009

Published by Karolinska Institutet. Printed by Larserics Digital Print AB

ISBN 978-91-7409-585-2

Abstract

Telencephalic development is an extraordinary complex process where neural structures of highly diverse cell compositions emerge as the result of multiple developmental strategies imposing tight regulation, balance and timing of gene expression. Embryonic telencephalic neural stem cells (NSCs) can form the major cell types found in the telencephalon, and are thus an excellent model for studying transcriptional control of lineage choice mechanisms in CNS development.

In paper I, we show that a member of the BTB/POZ family, Zbtb20, is required for proper repression of genes involved in interneuron and oligodendrocyte differentiation as well as proliferation and cell cycle exit in NSCs. Zbtb20 was expressed in the subpallium in a Dlx and Mash1 overlapping fashion and interacted with the co-regulator SMRT. siRNA-mediated knockdown of Zbtb20 in NSCs modulated expression of key regulatory genes in control of differentiation and proliferation, including Dlx1/2/5 and p57kip2, thus affecting the outcome of cell fate acquisition in favor of oligodendrocytic differentiation. Zbtb20 protein was accumulated in regulatory regions of the *Dlx1/2* bi-gene cluster along with a T3-dependent accumulation to the MBP M1 promoter. Furthermore, the zebrafish homolog to Zbtb20 appeared to be necessary for proper gastrulation and formation of neuroectoderm *in vivo* possibly due to precautious expression of p57kip2, analogous to the NSCs phenotype.

In paper II, we suggest a novel role for the cyclin-dependent kinase inhibitor p57Kip2 as a context-dependent repressor of neurogenic transcription factors and telencephalic neuronal differentiation. p57Kip2 interacted with pro-neuronal basic helix-loop-helix factors such as Mash1. Increased levels of p57Kip2 inhibited Mash1 transcriptional activity in a cyclin-dependent kinase (CDK) independent manner and acted as a direct repressor in transcriptional assays. Proliferating telencephalic neural progenitors co-expressed basal levels of Mash1 and p57Kip2, and endogenous p57Kip2 accumulated transiently in the nuclei of NSCs during early stages of astrocyte differentiation, independent of cell-cycle exit and at times when Mash1 expression was still prominent. p57Kip2 repressed neuronal differentiation, but exerted little or no effect astroglial differentiation of NSCs.

In paper III we propose that Zbtb45 is a novel regulator of glial differentiation. Zbtb45 mRNA was ubiquitously expressed in the developing CNS in mouse embryos but Zbtb45 mRNA knockdown in NSCs resulted in a rapid decrease in the expression of oligodendrocyte characteristic genes and significantly increased numbers of astrocytes. Zbtb45 mRNA knockdown in oligodendrocyte precursors completely inhibited oligodendrocyte differentiation upon mitogen withdrawal as assessed by a down-regulation of the expression of markers for oligodendrocytic differentiation such as CNPase, MBP and Sox10, whereas CD44 expression was found to be increased in all experiments.

In paper IV we propose that the previously uncharacterized protein CXXC5 is acting as a BMP4–induced inhibitor of Wnt signaling in neural stem cells. CXXC5 expression overlapped with BMP4 adjacent to Wnt3a expression in the dorsal regions of the telencephalon. CXXC5 showed partial homology with Idax, shown to interact with the Wnt-signaling intermediate Dishevelled (Dvl). Indeed CXXC5 and Dvl co-localized in NSCs and interacted *in vitro*. CXXC5 over-expression attenuated Wnt signaling. Interestingly, BMP4-induced decrease in Axin2 levels was abolished by siRNA-mediated knockdown of CXXC5.

List of publications

I. **Södersten E, Lewicka M, Joseph B, Andersson-Lendahl M, Mitchelmore C and Hermanson O.** Zbtb20/HOF regulates Dlx expression and neural stem cell differentiation. *Manuscript*

II. **Joseph B[*], Andersson ER[*], Vlachos P, Södersten E, Liu L, Texeira AI and Hermanson O.** p57Kip2 is a repressor of Mash1 activity and neuronal differentiation in neural stem cells. *Cell Death Differ*. doi: 10.1038/cdd.2009.72. 2009

III. **Södersten E and Hermanson O.** The novel BTB/POZ zinc finger factor Zbtb45 is essential for proper glial differentiation of embryonic neural stem cells and oligodendrocyte progenitors. *Manuscript*

IV. **Anderson T, Södersten E, Duckworth JK, Cascante A, Fritz N, Sacchetti P, Cervenka I, Bryja V and Hermanson O.** CXXC5 is a novel BMP4-regulated modulator of Wnt-signaling in neural stem cells. *J. Biol. Chem.*, 284, 3672-3681. 2009.

[*] Equal contribution

CONTENTS

ABBREVIATIONS

AEP	Anterior Entopeduncular Area
A-P	Anterior-Posterior
bHLH	basic Helix-Loop-Helix
BMP	Bone Morphogenic Protein
BTB	Broad complex, Tramtrack and Bric à brac
CDK	Cyclin Dependen Kinase
CGE	Caudal Ganglionic Eminence
CKI	Cyclin dependen Kinase Inhibitor
Clr	Calretinin
CNS	Central Nervous System
CT	Cardiotrophin
D-V	Dorsal-Ventral
E	Embryonic
FGF	Fibroblast Growth Factor
FRET	Förster Resonance Energy Transfer
G1	Gap-phase 1
GFAP	Glial Fibrillary Acidic Protein
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylases
ICD	Intra Cellular Domain
IPC	Intermediate Progenitor Cell
LGE	Lateral Ganglionic Eminences
LIF	Leukemia Induced Factor
MBP	Myelin Binding Protein
MGE	Medial Ganglionic Eminences
NSCs	Neural Stem Cells
NuRD	Nucleosomal Remodeling Deacetylase
OLP	Oligodendrocyte Progenitor
PcG	Polycomb group
PML	Promyelocytic Leukemia Protein
PNS	Peripheral Nervous System
Pv	Parvalbumin
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RG	Radial Glia
RMS	Rostral Migratory Stream
S	Synthesis phase
Shh	Sonic hedgehog
Sm	Somatostatin
SMA	Smooth Muscle Actin
SMRT	Silencing Mediator of Retinoic Acid and Thyroid hormone receptor
SOP	Sensory Organ Progenitor
SUMO	Small Ubiquitin-like Modifier
SVZ	Sub Venticular Zone
T3	3,5,3'-triiodothyronine, thyroid hormone
TGFß	Transforming Growth Factor ß
TR	Thyroid hormone Receptor
trxG	trithorax Group
TSA	Trichostatin A
VPA	Valproic Acid
VZ	Ventricular Zone

PREFACE

During development, the genomic content of a single cell is manifested in a self-orchestrated process, ultimately resulting in a self-reflective complexity. Please read the previous sentence again.

1 TELENCEPHALIC DEVELOPMENT

The telencephalon is the seat of higher cognition, consciousness, emotions and motor coordination in humans. It comprises the layered structures of the cerebral cortex, hippocampus and the olfactory bulb, along with the basal ganglia and amygdala. Construction of the telencephalon is an extraordinary complex process where neural structures of highly diverse cell compositions emerge as the result of multiple developmental strategies imposing tight regulation, balance and timing of gene expression.

1.1 Regional patterning in the developing telencephalon

Despite the vast cellular diversity and regional complexity of the adult telencephalon, the basic framework emerges as result of the same molecular patterning mechanisms that operate generally in the developing CNS and the rest of the embryo: In the developing spinal cord, the neural tube is polarized along its dorsal-ventral (D-V) axis and distinct progenitor domains are defined by patterned expression of transcription factor genes that are established by the coordinated signaling of bone morphogenic proteins (BMPs) and Wnts from the dorsal midline and sonic hedgehog (Shh) from the ventral midline respectively [1]. In this way, the progenitor populations retain their positional identity by graded local exposure to morphogenic proteins, that in turn yield distinct transcriptional outputs [2]. These pathways intersect so that BMPs act to limit the ventralizing activity of Shh [3]. Similar signaling of morphogenic molecules occurs during anterior-posterior (A-P) patterning as well. For example fibroblast growth factor 8 (FGF8), which is expressed in the midbrain primordium, controls A-P patterning of the midbrain [4,5]. Many of the fundamental mechanisms involved in the patterning of the spinal cord have been shown to be applicable on telencephalic development as well. Signals from four identified patterning centers set up the basic framework for generating cell diversity and regionalization of the developing telencephalon (**Fig 1a**). First, a rostral patterning center is a source of FGF-proteins, including FGF8 and FGF17 [6,7,8] that gives positional identity along the A-P axis of the developing telencephalon [9]. If a new FGF8 source is introduced at the posterior pole of the cortical primordium, the posterior cortex is induced to take on a more anterior fate. As result of additional ectopic posterior FGF8, the two opposing A-P signaling sources induce formation of apparently mirror-image partial area duplicates [10]. Second, the caudal-dorsal edge of each cerebral cortical hemisphere, the cortical hem, is a source of BMP and Wnt proteins [11,12,13,14]. Without a canonical Wnt signal from the hem, no hippocampus will be formed [15]. Furthermore, when telencephalic BMP activity is partially blocked, choroid plexus formation from the medial neuroepithelium is impaired [16]. Conversely, continuous activation of BMP signaling transforms the entire cortical primordium into choroid plexus epithelium [17]. Third, and analogous with spinal cord development, expression of Shh in the ventral telencephalon has been implicated in D-V patterning [13,18,19,20,21], in concert with the transcription factor Gli3, FGF8 and possibly Wnts. Finally, a lateral center termed the anti-hem interface secretes FGFs and Wnts.

1.2 Neuronal differentiation

At around E10.5 in the mouse embryo, the telencephalon develops from a morphologically uniform ventricle in the caudal part of the telencephalic vesicles. The developing telencephalon can be divided into two main subdivisions – the dorsal pallium (cortex) and the ventral subpallium (basal ganglia).

1.2.1 Dorsal neuronal differentiation

The pallium develops from a densely packed layer of pseudostratified neruroepithelial progenitor cells that are positive for Sox1, RC2 and the progenitor marker Nestin, with single cells touching the ventricle and pia. Then, the first round of mitosis generates the first post-mitotic neurons, called the preplate neurons. The preplate is thought to function primarily as a framework for further development of the cortex, organizing its laminar structure and some of its connections. The preplate is subsequently split into a superficial layer (marginal zone or Cajal-Retzius layer) and a transient underlying layer (subplate) [22,23] by inward radial migration of new cortical neurons that forms a middle layer termed cortical plate. Thus, the neurons of cortical plate grows in an "inside-out" order, from the deep layer 6, which contains the earliest-born cortical plate neurons, to the superficial layer 2, which contains the latest-born neurons [24]. Interestingly, this ontogenetic inside-out process is repeated later on in the developing hippocampal formation of the mammalian cerebral cortex (with the exception of the dentate gyrus) [25]. Soon after the onset of neurogenesis, at E12-13 in mouse, these neuroepithelial progenitors produce elongated radial glial cells (RG) that span from the ventricular zone to the pial surface [26]. The elongated processes of the RGs was initially proposed to serve as a guide for newborn neurons migrating from the ventricular side of the neuroepithelium to the pial surface [27]. This property was later shown to be particularly important at later stages of development when neurons have to migrate long distances [28]. Fate mapping studies have shown that the RG cells are progenitors and that they give rise to most glutamatergic pyramidal-projection neurons in the cortex [29,30]. However, RG cells are diverse and sometimes indistinguishable from neuroepithelial progenitor cells. Most RGs express the transcription factor Pax6 (paired box 6), RC2 (a highly disputed RG antigen [31]) and Nestin, whereas some sub-populations express a variety of other markers including brain lipid binding protein (BLBP) and glutamate–aspartate transporter (GLAST). Moreover, RGs also exhibit functional differences in Notch signal transduction and differentiation potential [32,33,34]. A particular feature of individual RGs is that in synchrony with cell-cycle progression, the RG cell nuclei migrate from apical (ventricular) to basal (in the G1 phase) and back (in the G2 phase), a process referred to as interkinetic nuclear migration [35,36]. This behavior is believed to regulate the exposure of the nuclei of the RGs to neurogenic versus proliferative signals such as Notch signaling. The RG divide asymmetrically at the ventricular surface with vertical cleavage planes to produce (self-renew) a radial glial cell and either a neuron (direct neurogenesis) or an intermediate progenitor cell (IPC, indirect neurogenesis) [37]. The IPCs are multipolar cells that express the T-box transcription factor Tbr2, and migrate away from the ventricular surface, expressing the bHLH transcription factor NeuroD to produce a secondary germinal zone, the subventricular zone (SVZ), where they undergo symmetrical, generally horizontal

divisions [38] to produce two neuronal daughters expressing Tbr1 [39]. The sequential transcription factor cascade Pax6 > Tbr2 > NeuroD > Tbr1 have been extensively studied in over-expression and knockout experiments in mice and have been suggested to make up the master regulatory components in the transcriptional network in control of glutamatergic neurogenesis [40,41].

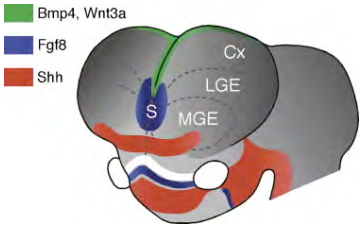

Fig 1a *Patterning centers in the developing telencephalon. Modified from [42].*

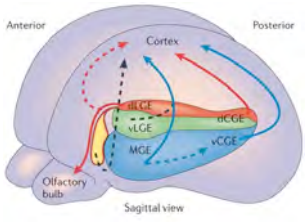

Fig 1b *Sources of GABAergic neurons and their migratory routes. Modified from [43].*

1.2.2 Ventral neuronal differentiation

The subpallium develops into two main regions, a medial domain that is known as the medial ganglionic eminence (MGE), and two posterior and lateral regions that are designated the lateral ganglionic eminence (LGE) and the caudal ganglionic eminence (CGE). In contrast to the dorsal telencephalon, each of these regions are sources of GABAergic inhibitory neurons that populate the basal ganglia and the associated limbic structures, including the amygdala and the nucleus accumbens [44]. Interestingly, the ganglionic eminences (GE) are also the major source of the GABAergic neurons in the dorsal telencephalon [45,46,47]. These putative cortical GABAergic interneurons are generated from around E12 and migrate tangentially through precise pathways towards the cortex [48] (**Fig 1b**).

GABAergic neurons are highly diverse and based on morphological, immunohistochemical and electrophysiological criteria, can be subdivided into at least 15 different subclasses that frequently include those that express the calcium-binding proteins parvalbumin (Pv) or calretinin (Clr) or the peptide somatostatin (Sm) and their subtypes [49,50]. Specification of neural progenitors in the GE appears to depend on a gene regulatory network: Members of the Dlx family of homeobox transcription factors are expressed throughout the subpallial SVZ and have been shown to be critical for interneuron specification. Mice containing compound *Dlx1/Dlx2* mutation die at birth and have a severe reduction in the tangential migration of interneurons from the GE to the cortex, resulting in a massive reduction of GABAergic cells in the cortex [51]. This may be dependent on misexpression of the direct Dlx target *Arx* (a homeodomain protein encoding gene), which in turn have been shown to regulate migration of GABAergic neurons to the cortex. Mutations in the human *ARX* gene have been found in several human neuropathological conditions

[52,53,54]. The expression of *Dlx*-genes is tightly controlled, presumably by auto-regulation along with regulation by the broadly expressed proneural bHLH factor Mash1 [55]. Furthermore, data indicate that differential activity of distinct cis-regulatory elements of the *Dlx1/2* bi-gene cluster direct GABAergic subtype specification [56].

The nature of progenitors in the subpallium has been under some dispute. Interneurons that express Pv, Clr or Sm represent largely non-overlapping populations with different origins in the GE. Some groups report that RG cells give rise to neurons in all regions of the CNS including the subpallium [57] whereas others claim that there are regional differences in RG fate and that the RGs are less neurogenic in the GE and only give rise to a few subtypes of GABAergic neurons [58]. In contrast to the pallium, the subpallial SVZ is relatively large and lack expression of Pax6, whereas expression of Dlx-genes and Mash1 is absent from the developing cortex, opening up for alternative mechanisms of progenitor production.

1.3 The neurogenic to gliogenic switch

A fundamental feature of neural development in vertebrates is that different cell types are generated in a precise sequence. First neurons, in mice generated from embryonic day 12 (E12) to E18, followed by astrocytes around E16 whereas mature oligodendrocytes are first seen postnatally [59]. Retroviral lineage tracing studies have shown *in vivo* that single neural precursors contribute to both the neuronal and glial lineages but that the cell types generated from these precursors change in favor for the glial lineage depending on the time-point [60,61]. Cortical progenitors are thus multipotent, however changing their pattern of differentiation in a temporally regulated fashion. An astonishing feature of this timed genesis is that it can be preserved in culture: clones of E10-E12 cortical stem cells generate only neurons the first days *in vitro*, followed by the sequential genesis of astrocytes and oligodendrocytes [62]. Even during the neurogenic phase, these clones generate neurons of temporally distinct subtypes in the same precise order: First the neurons of the cortical plate in the deep layers 6 and 5, followed by those destined for the more superficial layers 4, 3 and 2 [62,63]. However, embryonic cortical precursors differentiate into neurons when cultured on embryonic cultural slices, but astrocytes when cultured on postnatal cortical slices. Thus, the sequential generation of different neuronal subtypes and the subsequent neurogenic to gliogenic switch must depend on the interplay between some intrinsic timing mechanism and environmental signals [64,65].

1.3.1 Astrocytic differentiation

A major extrinsic timing mechanism for regulating the onset of gliogenesis involves a feedback loop, where neurons excrete a cytokine, cardiotrophin (CT-1) that in turn acts instructively on neural progenitors to generate astrocytes. Ablation of neuron-derived CT-1 completely blocks the neurogenic to gliogenic transition in cultured cortical progenitors. Furthermore, mice lacking CT-1 expression exhibit a 50-70% deficit in the level of cortical astrogenesis [66]. Thus, as neurogenesis proceeds, levels of CT-1 will gradually increase, and the increasing numbers of neurons will inhibit

further neuronal production. CT-1 along with leukemia induced factor (LIF) and ciliary neurotrophic factor (CNTF) signals through the co-receptors LIFRß and gp130, which in turn activate the JAK-STAT pathway [67]. Inhibition of either the co-receptors or STAT3 is sufficient to abolish astrocytic differentiation in neural precursors [66,68,69]. STAT3 is a transcription factor that directly binds to regulatory sequences of two astrocyte-specific genes, namely *gfap* (glial fibrillary acidic protein, an intermediate filament protein) and *s100ß* (an astrocyte-specific calcium binding protein). STAT3 interact with two closely related histone acetyl transferases, p300 and CBP (p300/CBP), that act as co-activators in part by acetylating histone tails, thus causing the chromatin to adopt a more accessible conformation [70].

Notch signaling has also been proposed to promote gliogenesis in a JAK-STAT dependent manner: During the gliogenic period, the notch intracellular domain (NICD) and its partner CSL forms a repressive complex together with the repressive cofactor N-CoR in *gfap*-regulatory regions. When the JAK-STAT pathway becomes activated by a gliogenic cytokine (e.g. CT-1, LIF or CNTF), N-CoR is translocated to the cytoplasm and the gfap-gene becomes de-repressed [71], in a mechanism probably involving Akt-kinase. Indeed, mice lacking the *n-cor* gene exhibit robust premature astrogenesis by E15.5 in the developing telencephalon. The CSL complex further inhibits neurogenesis by activating *hes*-genes that in turn repress proneural genes such as *mash1* and *ngn2* [72].

In another model, BMP2-signaling activates the downstream transcription factor Smad1 [73], and in the presence of a gliogenic cytokine Smad1 forms a transcriptional complex with the STAT3:p300/CBP complex that transactivates gliogenic genes [74]. However, Ngn2, a proneural bHLH factor has also been shown to act through the p300/CBP complex in activating neuronal genes (such as *tubb1*). BMP2 on the other hand induces the expression of Id1 (inhibitor of differentiation or inhibitor of DNA binding) that antagonize Ngn2 by inhibiting its function. In this way, under the neurogenic period when Ngn2 levels are high, a ngn1:p300/CBP:Smad complex may be present that inhibits BMP2-induced gliogenesis by sequestering away the p300/CBP from STAT3 [75].

Finally, the neurogenic to gliogenic switch may in part be caused by an intrinsic epigenetic timing mechanism since cortical precursors at E10 respond poorly to gliogenic cytokines compared to E14 precursors [76]. Genome-wide methylation profiling of early and late precursors have shown that astrocyte genes such as *gfap* and *s100ß* becomes demethylated prior to the onset of astrocytic differentiation [77,78], along with genes in the gp130-JAK-STAT pathway. Furthermore, conditional deletion of a DNA methyltransferase, *Dnmt1* results in DNA hypomethylation and precautions astrocyte differentiation in neural precursors [79]. Dnmt1 acts to methylate cytosine residues in CpG dinucleotides and thereby interfering with binding of transcriptional regulators to their targets [80] or by creating a binding site for members of a transcriptional repressor family, the methyl-CpG binding proteins (MBDs), that recognize methylated CpG sites [81]. One such MBD, namely MeCP2 have been shown to prevent the conversion of neurons to astrocytes by binding to highly methylated regions in transcription initiation sites of astrocytic genes [82].

1.3.2 Oligodendrocytic differentation

Similar to neurons and astrocytes, oligodendrocytes arise from multipotent precursor cells of the telencephalon. Typically, oligodendrocyte precursors (OLPs) are generated at defined times and in restricted regions of the ventricular zone in the developing CNS and have to migrate from their birthplace to the future white and gray matter. [83,84,85]. OLPs remain proliferative until they reach their final destination where they exit the cell cycle. During terminal differentiation, substantial amounts of myelin proteins are produced (such as myelin binding protein, MBP, proteolipid protein, PLP or myelin-associated glycoprotein, MAG) that become integrated in the myelin sheets with which oligodendrocytes wrap neuronal axons. In the telencephalon, the VZ of the MGE and anterior entopeduncular area (AEP), LGE/CGE as well as the cortex are believed to be the major sources of OLPs in a sequential manner [86]. The first round of OLP originates from the ventral-most part of the MGE/AEP around E12.5 and migrates laterally and dorsally to the cortex where they are first end up at E16. After E18, the contribution of ventrally derived OLPs starts to decrease in the cortex and a second wave emerges that appears to have a more local origin. Remarkably, lineage tracing experiments have shown that the first MGE/AEP-derived OLPs disappear after birth in favor for more recently generated cells. These findings also suggest that OLPs generated in different locations are functionally equivalent [86]. Whether this reflect some selective advantage of more recently generated OLP that leads to competitive elimination of ventrally derived cells or the existence of a continual turnover of oligodendrocytes is to date unknown. A turnover model is likely though since OLP production in the adult is restricted to the LGE/CGE [87].

OLP specification has been extensively studied in the mouse spinal cord, where a ventral source of Shh induces the patterning of an area (termed pMN area) that initially produces motor neurons before switching to OLP production. In response to Shh, the neuroepithelial cells of the pMN domain express Pax6 as well as the closely related homeodomain proteins Nkx6.1 and Nkx6.2 which in turn control the expression of the bHLH transcription factor Olig2 [88,89,90,91]. This region is further specified by mutual repression of Olig2 and the Nk homeodomain protein Nkx2.2. In the spinal cord, the neurogenic to gliogenic switch is believed to in part be dependent of Ngn1 and Ngn2 because their combination with Olig2 suppresses glial fate and favors the generation of motor neurons [92]. Similar, the ventral telencephalon is in part specified by a source of Shh, and Olig2 is broadly expressed at the onset of OLP production. A homolog to Nkx2.2, Nkx2.1 is also present in the MGE, but its role in OLP specification remains to date unknown. The bHLH protein Mash1 has been shown to be required for the specification of and early OLP population in the subpallium, and is co-expressed with Olig2 [93,94]. Mash1 on one hand regulate the expression of Dlx1/2 that in turn have been proposed to promote neurogenesis in favor of OLP specification, but can also counteract their antigliogenic activity together with Olig2 [51].

Analogous to the dorsal telencephalon, a neurogenic versus OLP switch is likely to take place in the ventral telencephalon because of the delayed onset of OLP production relative to neurogenesis. Although the nature of ventral progenitors may be unclear, the above mentioned transcription factors are likely to take part in such event. Notch signaling is clearly involved, since deletion of the Notch ligands Delta A

and Delta B in zebrafish results in impaired OLP formation [95,96], whereas mice lacking the Notch affector CSL have opposite results [96]. In addition, there is evidence that BMPs also inhibit differentiation of OLPs by inducing expression Id proteins that in turn can bind to and sequester the function of Olig2 [97].

After specification, OLPs must undergo terminal differentiation to acquire functionality. In the mouse spinal cord, Sox10 is the earliest known transcription factor to be specifically turned on in the oligodendrocyte lineage after specification [98]. In Sox10-deficient mice progenitors develop, but terminal differentiation is disrupted [99]. Thyroid hormone has also shown to strongly influence the decision of OLPs in tissue culture to switch from a proliferative to a differentiating mode [100]. However, data on mouse mutants, including those in which all TRα and TRβ isoforms have been jointly deleted, have failed so far to substantiate such an essential direct and cell-intrinsic role in vivo [101,102].

1.4 Cell cycle control

During telencephalic development a precise balance between proliferation and differentiation must be maintained. If too many neural progenitor cells would exit cell cycle during the early stages of development, there might be a proportional increase in early born cell types at the expense of later born cell types. On the other hand, lack of proliferative control means cancer. Although the molecular mechanisms underlying proliferative versus differentiative divisions are largely unknown, converging evidence suggests that the mode of division is correlated to cell-cycle components and, more specifically, to G1-phase regulation. Mouse cortical precursors treated with differentiation-promoting factors show an increase in the duration of the G1 phase. Conversely, treatment with mitogenic factors decreases G1 length [103]. At the single-cell level, time-lapse video microscopy studies show that the G1 phase is long in differentiative divisions and short in proliferative divisions. Differentiation results in G0 entry. Several factors are in control of G1 to S-phase progression, but the core proteins constitute of holoenzymes composed of regulatory (cyclin) and catalytic (CDK) subunits. A key target for CDKs (such as cyclin-D and cyclin-E) is the tumor suppressor protein pRb (retinoblastoma protein) which they phosphorylate during G1-phase [104]. pRb phosphorylation in turn results in the repression of E2F (family of transcriptional activators) activated genes, whose activity is required for entry into S phase [105].

G1 to S phase transitions is believed to in part be under control of cyclin-dependent kinase inhibitors (CKIs) such as the Cip/Kip (p21Cip1, p27Kip1 and p57Kip2) and Ink4 families (p16Ink4a and Bmi-1) [106]. CKIs mainly inhibit S phase entry by physical interaction with CDKs. In particular, *in vivo* and *in vitro* studies have proposed that p27Kip1 have to function for normal brain development. p27Kip1 is weakly expressed in germinal zones of the developing telencephalon but is present in areas with post-mitotic neurons in the cortical plate dorsally as well as in the subpallium [107]. Mice lacking p27Kip1 display an overall increased brain size [108] and resemble to some extent pRb mutants.

Control of cell cycle progression is tightly linked to differentiation: Clonal analyses of OLPs isolated from the developing optic nerve suggest that a cell-intrinsic program is

required to determine when the precursor cells stop dividing and differentiate terminally [100]. p27Kip1 have been suggested to take part in such a timing property, since protein levels of p27Kip1 progressively increases in the precursors as they proliferate and is high when the cells stop dividing and differentiate in the presence of thyroid hormone [109]. p57Kip2 is also involved in the generation of oligodendrocytes but appears to be more functionally dynamic. Expression of p57Kip2 has been shown to be directly regulated by Notch signaling in zebrafish and is required for OLP specification [110]. Another report suggests that p57Kip2 *in vitro* takes part in a timer mechanism similar to p27Kip1, in control of the number of OLP cell divisions before terminal differentiation [111]. However, shRNA-mediated knockdown of p57Kip2 *in vitro* leads to increased terminal differentiation of OLPs and myelinating Schwann cells of the peripheral nervous system [112,113]. Analysis of p57Kip2/BrdU/TuJ1 triple immunostaining in BrdU injected E14.5 mice indicate that p57Kip2 expression occurs as neuronal precursors initiate differentiation [114]. In differentiating post-mitotic midbrain dopamine cells, the expression of p57Kip2 is dependent on an orphan nuclear receptor, Nurr1 that is essential for dopamine neuron development. Mice lacking p57Kip2 display impaired maturation of dopamine neurons. Surprisingly, p57Kip2-promoted maturation is not dependent on CDK inhibition but through direct interaction with Nurr1 [115]. Furthermore, Wnt-1 stimulation of cultured ventral midbrain precursors *in vitro* leads to down-regulation of p57Kip2 expression and increased numbers of DA neurons [116]. In paper II we propose that p57Kip2 is a repressor of Mash1 activity and a context-dependent repressor of neuronal differentiation, possibly to allow proper glial differentiation [117].

Finally, p21Cip1 is involved in cell cycle regulation in ways similar to the other CKIs [118] but have also been implicated in p53-dependent cell cycle arrest in response to DNA-damage [119]. p21Cip1 deficient mice do not display the over-proliferating phenotypes of p27 and p57 mutants, but fail to undergo DNA-damage-induced cell-cycle arrest [120]. Over-expression of p21Cip1 results in G1-, G2-, or S-phase arrest upon exposure to DNA-damaging agents [121]. Interestingly, expression of p21Cip1 appears to be under control of several proteins of the BTB/POZ family [122,123,124,125,126] although a common regulatory mechanism for these proteins has not been elucidated. While Zbtb5 is proposed to sequester away p53 from the p21Cip1 promoter Zbtb27/Bcl-6 and Zbtb17/Miz-1 jointly regulate p21Cip1 in a p53-independent mechanism.

1.5 Epigenetic mechanisms

Beyond transcription factors and signaling pathways, the dynamic change of gene expression profiles is controlled at large by gene accessibility: Multipotent neural progenitors are maintained in an undifferentiated state in part due to epigenetic silencing of lineage-specific genes. Even though differentiation usually is initiated in response to extracellular cues, the outcome of differentiation is much dependent on epigenetic mechanisms. Given that each signaling pathway is used repeatedly during the development of an organism [127], cells must have different epigenetic settings to interpret signaling cues differently. So far, several modes of epigenetic modulation have been identified to be involved in maintenance of self-renewal and neural fate determination.

In chapter **1.3.1** I briefly discussed the contribution of DNA methylation on the timing of gliogenesis. DNA methylation is a major epigenetic mechanism that acts on global chromatin. Actually, most CpG dinucleotides in the genome on average are methylated [128], but any type of cell has its own methylation pattern so that a unique sub-set of the genome may be expressed. During differentiation, this methylation pattern change; during the neurogenic period it is necessary to inhibit astroglial marker genes by DNA methylation.

Both global and local chromatin structure is greatly influenced by histone amino-terminal tail modifications. Among the well-known covalent modifications of histones, histone acetylation directly modifies chromatin structure by decreasing the interaction of the positively charged histone tails with the negatively charged phosphate backbone of DNA, resulting in relaxation of the higher order of chromatin. In this way, DNA becomes transiently accessible for transcription [129]. Histone acetylation is conducted by histone acetyltransferases (HATs), such as GNATs, MYSTs or p300/CBP [130] and was briefly discussed in the context of astrocytic differentiation in chapter **1.3.1**.

Histone deacetylases (HDACs) catalyze the reverse reaction; in the deacetylated state, histones package the DNA into condensed chromatin, which in turn prevent access of transcriptional activators to their target sites, thus resulting in transcriptional repression. Several different HDACs exists as components of multimeric repressor complexes, such as Sin3, nucleosome remodeling deacetylase (NuRD), co-repressor of RE1-silencing transcription factor (CoREST) and nuclear corepressor (N-CoR/SMRT) [131,132]. Other proteins of these complexes, including hormone nuclear receptors such as thyroid hormone receptors [133] and developmental transcription factors such as Hes5 [133], or proteins of the BTB/POZ family [134], can lead to the recruitment of HDACs to cis-regulatory elements to promotors of specific genes. Treatment with pharmacological inhibitors of HDAC activity, such as valproic acid (VPA) leads to neuronal differentiation of NSCs [135], and many HDAC-associated co-factors have been shown to be involved in telencephalic development. CoREST and Sin3 modulate the expression of late neuronal genes in embryonic stem cells and NSCs [136], whereas the Zbtb20 (paper I) interacting co-repressor SMRT-deficient mice displays premature, presumably GABAergic neuronal differentiation [137]. The picture gets more complicated though, since various HDACs and HDAC-containing complexes have differential spatial and temporal expression during development and may thus have different functions. Also, administration of another HDAC-inhibitor, trichostatin A (TSA) to OLPs inhibits terminal differentiation into mature oligodendrocytes [138].

Histone methylation is more complicated than histone acetylation because it generates diverse effects on gene transcription depending on the sites and the numbers of metylation on the histone tails. For example, low levels of acetylation and high levels of methylated H3K9, H3K27 and H4K20 are associated with a silent heterochromatic state, whereas high levels of acetylation and trimethylated H3K4, H3K36, and H3K79 are associated with transcriptionally active euchromatin [139]. However, recent data suggest that the extent of impact of histone lysine methylation on gene transcription depends largely upon the number of methylated histones. The mono-methylations of H3K4, H3K27, H3K9, H4K20, H3K79 and H2BK5 are all linked to gene activation,

whereas trimethylations of H3K27, H3K9 and H3K79 are linked to repression [140,141]. To make things even more complicated, many promotors of preferentially highly conserved genes display a "bivalent" chromatin structure, in which active (acetylation of H3K9 and tri-methylation of H3K4) and repressive (tri-methylated H3K27) chromatin marks are juxtaposed [142]. In this way, developmentally important genes in stem cells and progenitors are believed to be kept "poised" in a repressed but rapidly activatable state. During differentiation, erasing H3K27me3 activates bivalent lineage-specific genes. Histone methylation is facilitated by a set of general transcriptional repressors of the Polycomb group (PcG) proteins that contain members specifically binding to tri-methylated H3K27. PcG group proteins also function as histone methyltransferases that accordingly facilitate high levels of tri-methylated H3K27. These proteins are counter-acted by another set of proteins, the trithorax group (trxG) family, that in turn exhibit H3K4 methyltransferase activity, but also facilitate re-modeling of nucleosomes in favor of transcription. Very little is known to date regarding the impact of histone methylations during telencephalic development. The PcG-member Bmi-1 have been shown to be required for post-natal NSC self-renewal [143,144] and recently the trxG member Mll1 (mixed-lineage leukemia 1) was proposed to activate the Zbtb20-regulated (paper I) *Dlx2* gene, also post-natally. Mll-deficient mice displays bivalently marked chromatin at the *Dlx2* locus, resulting in transcriptional repression [145]. Finally, due to the high thermodynamic stability of the N-CH_3 bond, histone methylation was until recently regarded as irreversible and the consensus in the epigenetic field was that the only way to revert histone methylation was by histone exchange or by cleavage of the methylated histone tail [146]. To date, several histone demethylases have been identified and are often found associated with large gene regulatory complexes from where they exert demethylation of specific histon methylation marks. Interestingly, in SMRT deficient mice, JMJD3, a histone demethylase specifically targeting tri-methylated H3K27, is up-regulated in a mechanism involving de-repression of the unliganded RA-receptor. Furthermore, during RA-induced neuronal differentiation, JMJD3 was found recruited to the *Dlx5* promoter [137,147].

2 PROTEINS OF THE BTB/POZ FAMILY

Proteins of the BTB/POZ family are characterized by the presence in their structures of a N-terminal BTB-domain (Broad complex, Tramtrack and Bric à brac) and several C_2H_2-type (also called *Krüppel*-type due to the fact that it resembles the *Drosophila* segmentation protein *Krüppel*) C-terminal zinc fingers. Several proteins of the BTB/POZ family have been associated with transcriptional repression, although some members have also been implicated in transcriptional activation [148,149,150]. Apparently, BTB/POZ proteins are able to directly repress transcription in *in vitro* transcriptional assays [151], but it is not known how this property is executed since neither conserved transcriptional repressor nor activation domains have been identified within the BTB/POZ protein family [152]. A more likely mechanism for transcriptional repression by the BTB/POZ family is through their well-documented ability to recruit transcriptional co-repressors, including N-CoR, SMRT, BCoR, Co-REST and CtBP [134,153]. These co-repressors are in turn part of large, multi-subunit complexes that can contain various chromatin-modifying enzymes such as methyl-

DNA binding proteins and HDACs [132]. Since the composition of the C_2H_2-type zinc fingers vary from protein to protein, BTB/POZ proteins are likely to function as site-specific docking bays that recruit general chromatin-modifying complexes to their target genes. In this way, the composition of the multi-subunit complex that is brought via the BTB domain to the target sequence will determine whether a BTB/POZ protein will act as an activator or a repressor in a particular context.

The ancient BTB-domain most likely evolved in eukaryotes, as it can be found in fungi, plants and metazoans, but not in archaebacteria or in bacteria [154]. BTB-domains attached to C_2H_2-type zinc fingers is however an unique property of metazoans, with only two proteins to be found in *C. elegans* but 15 in *Drosophila*, 45 in mice and 43 in humans. The primary structure of the BTB-domain can be very diverse but the tertiary structure appears to remain conserved overall and have been resolved by X-ray crystallography, displaying a cluster of five alpha-helices capped at one end by a short, three-stranded beta-sheet. The BTB-domain forms obligate dimer-dimer interactions when crystallized and it have been shown that dimerization is necessary for transcriptional repression [155]. Dimerization of BTB-domains creates a charged pocket that is crucial for facilitating interactions with co-repressors (**Fig 2e**). Mutations of the BTB domain that neutralize key charged pocket residues does not disrupt the actual dimerization, but abrogates the ability of BTB/POZ proteins to interact with co-repressors and HDACs [156]. Because of the conserved tertiary structure, BTB/POZ proteins can also form heterodimers with proteins of the same family. Dimerization often increases the binding affinity for a molecule to a specific DNA-motif. However, dimerizations of BTB/POZ proteins have also been shown to facilitate loop formations of DNA and can thereby remodel the three-dimensional structure of local chromatin [157,158] and in some cases even bridge different chromosomes and bring about regulatory activity *in trans* [159]. Besides direct DNA-binding by zinc-fingers, proteins of the BTB/POZ family can also be recruited to chromatin through the dimerization ability of their BTB-domains, by forming a dimer with another BTB/POZ protein that is already attached to DNA. In this way, some BTB/POZ proteins can formally function as co-repressors. This is evident in the mechanism in which Zbtb27/Bcl-6 and Zbtb17/Miz-1 jointly regulate p21Cip1 expression (as discussed earlier) [123]. Furthermore, truncated mutants where the zinc finger part is missing in the *Drosophila* BTB/POZ protein Tramtrack have been shown to be functional *in vivo* [160]. Several proteins of the BTB/POZ family have previously been implicated in cancer, development and stem cell biology, and I will now highlight a few of its more well-studied members.

2.1 Tramtrack

The *Drosophila tramtrack* (*ttk*) gene encodes two different BTB/POZ proteins, ttk69 and ttk88, that were initially isolated due to its genetic interaction with the *fushi tarazu* gene [161] that in turn is essential for proper development of the *Drosophila* body plan. Later, ttk69 was found to be involved in fate decision events by directing asymmetric cell divisions in progenitors of the *Drosophila* external sensory (es) organ. The es organ is composed by one neuron and three different support cells (**Fig 2a**). A single sensory organ progenitor (SOP) divides to give rise to two secondary precursor cells, pIIa and pIIb (**Fig 2b**). The IIa cell then divides to form two outer support cells, a bristle (hair) cell and a socket cell, whereas the pIIb precursor divides

to form a glial cell and a tertiary precursor cell pIIIb. Next the pIIIb cell divides to generate a neuron and a sheath cell. Finally, the glial cell undergoes apoptosis [162]. In *ttk*69 loss of function mutants (**Fig 2c**), only pIIb precursors are formed in the first asymmetric cell division of the SOP, giving rise to supernumerary neurons and sheath cells, whereas over-expression leads to duplicated bristles and socket cells (**Fig 2d**) [163]. The first asymmetric division of the SOP is directed by lateral inhibition by Notch signaling [164]. Specific activation of Notch occurs in pIIa but is inhibited in the pIIb daughter by the asymmetrically inherited intracellular protein Numb. Intriguingly, ttk69 mRNA levels are equal in both the pIIa and pIIb cells. Asymmetric distribution of ttk69 protein in the pIIa progenitor is instead achieved by the RNA-binding protein musashi (msi) that bind to the 3´-UTR of ttk69 mRNA. In the *Drosophila* CNS, ttk69 acts as a determinant in a process that somewhat resembles some impacts of mammalian CKIs during telencephalic development: Lineage tracing studies have shown that the individual progenitor cells in each segment of the lateral *Drosophila* CNS are stem cells transiently expressing *Drosophila* neural stem cell markers *asense* and *deadpan* [165,166]. A neurogenic to gliogenic switch appears to be present since these progenitors do not adopt a glial fate until a glial-determining pathway is activated by the *glial cells missing* (*gcm*) gene [167]. Over-expression of ttk69 in this system leads to suppressed neuronal differentiation and reduced glial numbers. This is because ttk69 simultaneously repress the expression of *asense* and *deadpan* while regulating G1 length in glial progenitors by repressing the S phase entry CDK *Cyclin E* [168]. Regulation of *Cyclin E* have also been shown to be crucial for S phase entry in the SOP model, again under control of ttk69 and interestingly, the CKI homolog *dacapo* [162].

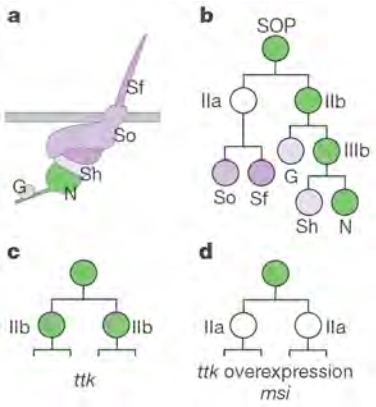

Fig2a-d *Drosophila sensory organ. Lineages of the sensory organ progenitor. Outcomes of tk69 loss- and gain of function. Modified from [169].*

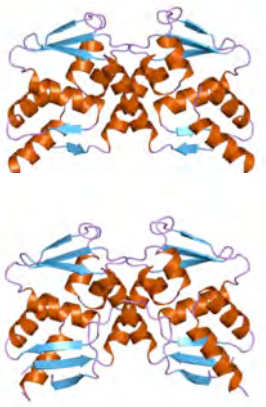

Fig 2e *Above: Crystal structure of dimerized BCL-6 BTB domains. Below: Crystal structure of dimerized BCL-6 BTB domains complexed with SMRT.*

2.2 BTB/POZ proteins in telencephalic development

At least 18 out of 43 BTB/POZ proteins encoded for in the mouse genome are expressed during brain development: Zbtb5, Pokemon/Zbtb7A, ThPOK/Zbtb7B, Zbtb16/PLZF, Zbtb17/Miz-1, Zbtb18/RP58, Zbtb20/HOF, Zbtb21, Zbtb23/GZF1, Zbtb24/Bif-1, Zbtb27/Bcl-6, Zbtb29/HIC-1, Zbtb33/Kaiso, Zbtb36, Zbtb45, MAZR, Zfp131 and Nac-1 [126,170,171,172,173,174,175,176,177]. Several of these factors are oncogenes and a few have been implicated fate choice mechanisms. In healthy animals, Zbtb27/Bcl-6 is regarded as a master regulator of B cell differentiation because it represses active germinal center B cell differentiation into terminally differentiated B cells by regulating p21Cip1 in cooperation with Zbtb17/Miz-1 [123,178]. Zbtb27/Bcl-6 was however originally identified as a gene frequently involved in chromosome 3 and 14 translocation events in non-Hodgkin's lymphomas (NHL) [179]. This pathogenesis is in part dependent on direct repression of the key tumor suppressor p53 by Zbtb27/Bcl-6 [180]. In acute promyelocytic leukemia (APL), Zbtb16/PLZF is found to fuse with the retinoic acid receptor α (RAR-α) in another chromosomal translocation event [181]. Normally, un-liganded RARs recruit co-repressors such as N-CoR and SMRT that inhibit retinoid signaling, but when retinoic acid (RA) is present, RAR-α releases these co-repressors and activates transcription of retinoid-responsive genes. The mutant PLZF–RAR-α fusion protein fails to dissociate the co-repressor interactions which in turn leads to inhibited RA-signaling [182,183]. Despite the impact of BTB/POZ proteins in cell fate decisions, oncogenesis and cell cycle regulation along with their well-documented ability to interact with co-factors and chromatin remodeling complexes, and given the profound role of Tramtrack in *Drosophila* PNS and CSN development - extremely little is known about the function of the 18 different BTB/POZ proteins present in the developing mammalian brain. Some efforts have been made however, and I will present the results from those experiments in chapter 4.

3 AIMS OF THE THESIS

1. Investigate a putative function of Zbtb20 in neural development.

2. Investigate novel mechanisms involving p57Kip2 in differentiation and proliferation of telencephalic stem cells.

3. Investigate a putative function of Zbtb45 in neural development.

4. Further the understanding of BMP4-signaling in neural differentiation by investigating a putative function of the BMP4 regulated factor CXXC5.

4 RESULTS AND DISCUSSION

4.1 Zbtb20/HOF regulates Dlx expression and neural stem cell differentiation.

FGF2-expanded E14.5 rat embryonic neural stem cells (NSCs) have proven to be an excellent model system to study transcriptional regulation and fate-choice mechanisms during telencephalic development. Although dissected out from the dorsal neocortex, FGF2 expansion acts to deplete differential expression of regional identity markers such as Pax6, Olig2 and GAD65, presumably due to induction of endogenous Shh [184]. NSCs can thus serve as a model system to study molecular mechanisms in control of both dorsal and ventral fates [185]. NSCs have the capability to form the major cell types found in the telencephalon, including pyramidal- and interneurons, astrocytes and oligodendrocytes as well as smooth muscle cells [184]. Upon mitogen withdrawal, NSCs mainly form neurons and astrocytes, but can also be brought to differentiate into the oligodendrocytic lineage by thyroid hormone (T3) treatment or specifically into astrocytes by treatment with interleukin-6 related compounds such as cliliary neurotrophic factor (CNTF) (**Fig 3**).

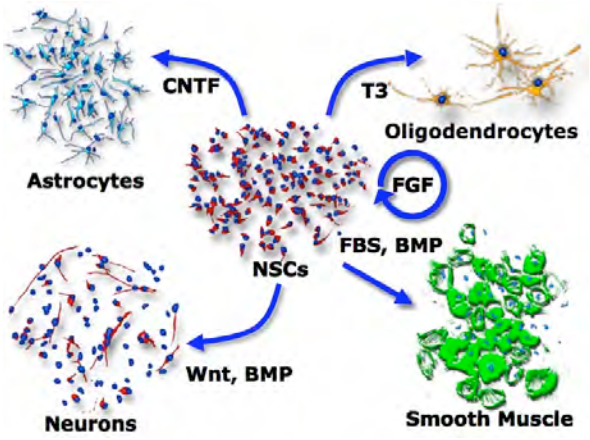

Fig 3 *Embryonic telencephalic neural stem cells self-renew when stimulated with FGF2, but can be brought to differentiate into astrocytes, oligodendrocytes, neurons and smooth muscle cells when treated with different stimuli. The picture was originally published in [186].*

In search for a putative mammalian ortholog to the *Drosophila* BTB/POZ protein Tramtrack, we set off by performing a simple *in silico* screen using the Basic Local Alignment Search Tool (BLAST) and the genomic databases provided by the NCBI [187]. We reasoned that blasting the ttk69 amino acid sequence would be more fruitful than comparing introns since evolutionary pressure acts on protein function and not DNA composition. A second rule we had for this screen was that a putative ttk69 ortholog, if important for basic fate choices, should be equally conserved in chick, zebrafish, mouse and human. A list of candidates appeared, and out of an initial PCR-screen seven of these genes turned out to be expressed in NSCs. Fortunately, the

gene that showed the highest homology in our screen, Zbtb20, was already cloned and partly characterized by a Danish group led by C. Mitchelmore and N.A. Jensen [188]. The Danes had established the expression of Zbtb20 from embryonic day (E) 16.5 to post-natal stages in mouse. Zbtb20 protein was predominantly present in newly born pyramidal neurons in areas CA3 and CA1 of the developing mouse hippocampus, at rostro-caudal levels of the post-natal sub-ventricular zone and later on in GFAP-positive cells populating the rostral migratory stream (RMS) along with white matter oligodendroglia and reactive astrocytes. Later, a competing group showed that transgenic mice over-expressing Zbtb20 under the dorsal forebrain-specific D6 promoter-enhancer [189] displayed delayed radial migration of immature cortical neurons and ectopic formation of a CA-like pyramidal cell layer in the subiculum and posterior retrosplenial cortex [173]. Then, another group interested in the function of Zbtb20 in liver concluded that Zbtb20 null mice exhibited a stark phenotype characterized by postnatal growth retardation, metabolic dysfunction, and lethality [190].

To elucidate a putative role for Zbtb20 in fate choice mechanisms in the telencephalon we performed *in situ* hybridizations in mice embryos at E10.5, E12.5 and E14.5. These time-points were chosen to cover the onset of neurogenesis as well as the time-window when neurogenesis and gliogenesis occur simultaneously. At E10.5 and E12.5 Zbtb20 mRNA was predominantly abundant in the VZ and SVZ of the CGE, MGE and LGE along with expression in the hippocampal anlagen. Later on, at E14.5 Zbtb20 expression was found in the developing hippocampus and also in the dorsal VZ and the VZ and SVZ of the GE and the corticostriatal boundary (CSB). We found this expression pattern intriguing since it was overlapping the expression of Mash1, Olig2 and Dlx1/2; genes that have been implicated in fate choice mechanisms in progenitors of the ventral telencephalon (**Fig 4**) [51]. Western blot from nuclear extracts and immunocytochemistry further showed that the Zbtb20 protein was present in NSCs. These simple analyses also provided some information about the Zbtb20 protein. First, the western blot showed that two isoforms of Zbtb20 (Zbtb20-S and Zbtb20-L) were present in the nuclei of NSCs. Interestingly, additional bands were also present but the molecular weight of these bands did not correspond to the expected size of homo-dimerized Zbtb20 nor to heterodimerizations of the both isoforms. These modifications should be covalently attached to the Zbtb20 isoforms due to their presence despite the denaturating conditions in the western blot procedure. One possibility is that the modified Zbtb20 bands are the result of SUMO (small ubiquitin-like modifier) modifications: Both tramtrack and PLZF can be SUMOylated on multiple sites [191,192]. Along with several other proteins of the BTB/POZ family, Zbtb20 displayed a punctuate, speckled pattern in the nuclei of NSCs [193,194,195]. Several compartments of the cell nucleus can appear in this form, such as splicing speckle domains, nuclear gems, Cajal bodies and PML bodies (promyelocytic leukemia protein bodies). With the exception of PML bodies, all other described punctuate nuclear bodies are absent of chromatin or DNA. Given that PLZF, a protein highly similar to Zbtb20, binds to and co-localizes with PML it would be interesting to test if Zbtb20 can interact with PML as well [196]. Most, but not all proteins of the BTB/POZ family can bind to transcriptional co-repressors that in turn are part of larger mutimeric gene regulatory complexes. Because of a few reported distinctions [197], we performed a GST-pulldown assay where *in vitro* transcribed Zbtb20-L, in line with the majority of BTB/POZ proteins indeed interacted with the co-repressor SMRT.

Although Zbtb20 previously was reported to affect hippocampal development, the intriguing expression pattern during earlier developmental stages suggested that Zbtb20 might be involved in the generation of some ventrally derived cell type, such as GABAergic neurons or oligodendrocytes. NSCs spontaneously differentiate into neurons and astrocytes upon mitogen withdrawal along with a small percentage of oligodendrocytes. We therefore used siRNA-mediated knockdown of Zbtb20 mRNA levels in NSCs and analyzed its effect on the expression of a set of genetic markers 1, 3 and 7 days post siRNA delivery after mitogen withdrawal (-FGF2) or stimulation with thyroid hormone (T3) that is a known inducer of oligodendrocytic differentiation [184]. The siRNA was designed to target both the isoforms of Zbtb20 mRNA. Based on the data generated from this approach we could conclude three major findings that are not mutually exclusive.

Zbtb20 knockdown resulted in up-regulation of a set of markers for GABAergic differentiation such as Dlx1, Dlx2, Dlx5 and Gad67 relative to control after 1, 3 and 7 DIV in both conditions. Interestingly, Dlx2, Dlx5 and Gad67 have previously been shown to be repressed by SMRT in NSCs and *in vivo* in SMRT deficient mice [137], and this result was thus in line with the Zbtb20-SMRT interaction. T3-treatment alone led to down-regulation of *Dlx* gene expression. This effect was however overruled in combination with Zbtb20 knockdown. A western blot on extract from siZbtb20 nucleofected NSCs that were differentiated in FGF2 or T3 condition for 1 DIV suggested Dlx2-regulation by Zbtb20 on the protein level. Notably, we did not detect any change in mRNA levels at any time point of Meis1 or Mash1, both known regulators of *Dlx* gene expression [55,198]. To investigate whether Zbtb20 could regulate *Dlx* gene expression directly, the presence of Zbtb20 protein on *Dlx1/2* regulatory regions was examined by chromatin immunoprecipitation (ChIP) experiments in NSCs (FGF2 or T3-stimulated). Zbtb20 was found to accumulate at the URE2 distal cis-regulatory element of the *Dlx1/2* bi-gene cluster, and was also present at the *Dlx1* promoter and the *Dlx1* and *Dlx2* intergenic regulatory regions I12a and I12b to a lesser extent. This accumulation was not significantly altered in T3-stimulated NSCs.

Given the presence of Zbtb20 on URE2 in NSCs, we hypothesized that over-expression of an URE2 containing construct in NSCs would cause a sequestering effect on endogenous Zbtb20-binding and therefore possibly mimic the effect of siRNA-mediated knockdown on Dlx gene expression. In this highly experimental setup, we decided to use a construct containing the I12a intergenic regulatory region as control, since it has been shown not to be active in neural development [199]. In line with our data, T3-treatment caused a deregulation of Dlx1 expression after 1 DIV compared to the FGF2-condition, while surprisingly, URE2 over-expression led to a down-regulation of Dlx in the same magnitude as T3-treatment, whereas over-expression of I12a had no significant effect. This result suggests that the URE2 region may not only modulate but also be required for Dlx1 expression in NSCs, and that Zbtb20 may not directly repress Dlx, but could act as a molecular docking bay for other regulatory factors.

To investigate a putative requirement of functional Zbtb20 for proper differentiation of NSCs, we used siRNA-mediated mRNA knockdown of Zbtb20 again and analyzed the outcome of differentiation in mitogen withdrawn or T3-stimulated cells after 7

DIV by immunocytochemistry. Dlx have previously been reported to inhibit oligodendroglial fate acquisition of ventral progenitors *in vivo* [51] and we thus expected a similar phenotype in the progeny of NSCs lacking Zbtb20. However upon knockdown, the numbers of oligodendrocyte procursors (RIP-antigen) and mature oligodendrocytes (MBP), were markedly increased in the FGF2 withdrawal condition, and appeared to be morphologically more mature compared to control, whereas no significant difference in oligodendrocyte numbers were detected in T3-stimulated cells. In parallel, the numbers of astocytes (GFAP) where reduced in both conditions while neuronal numbers (TuJ1) were left unchanged. To confirm the apparent effect of Zbtb20 knockdown on cell fate acquisitions of NSCs, we analyzed the expression of mRNA of Tubb3 (Tuj1), GFAP, CNPase (RIP), and MBP after 1, 3 and 7DIV. The levels of CNPase, MBP and Tubb3 were up-regulated in cells lacking Zbtb20 compared to control. However, we did not detect differences in the expression of CNPase or MBP in FGF2 withdrawal conditions corresponding to the numbers of the repective cell types seen from the immunocytochemical analysis. Unexpectedly, levels of MBP mRNA were greatly increased in T3-stimulated Zbtb20 lacking cells compared to T3-stimulated control. We therefore hypothesized that the MBP gene was under control of Zbtb20 in a thyroid hormone-dependent fashion. In order to examine this hypothesis, we performed ChIP analysis in NSCs to investigate the presence of Zbtb20 protein on the MBP regulatory regions. Indeed, Zbtb20 was accumulated at the MBP M1 promoter, and this occupancy was greatly reduced in T3-stimulated NSCs compared to FGF2 expanded cells. Furthermore, Zbtb20 expression appeared to be T3-regulated as Zbtb20 mRNA levels were gradually increased in T3-stimulated NSCs compared to FGF2 control. We therefore concluded that a thyroid hormone-dependent mechanism regulated Zbtb20 mRNA expression and protein localization in NSCs, and that Zbtb20 may take part in thyroid hormone transcriptional control of MBP gene expression.

Many proteins of the BTB/POZ family have been implicated in control of proliferation and several have been shown to regulate the expression of CKIs, specifically p21Cip1 (see chapter **1.4** and chapter **2**). To evaluate the effect on cell proliferation by Zbtb20 knockdown in NSCs, we assessed the distribution of cells in different phases of cell cycle by FACS analysis in FGF2 withdrawal condition. NSCs lacking Zbtb20 displayed a larger proportion of cells in G1 phase than control. We further analyzed the expression of CKI mRNA by qRT-PCR. Interestingly, levels of p57Kip2 were specifically increased upon Zbtb20 knockdown whereas levels of p21Cip1 and p27Kip1 remained unchanged. T3 stimulation alone caused a down-regulation of p57Kip2 but Zbtb20 knockdown overruled this effect. Furthermore Zbtb20 knockdown had no significant effect on cell death as assessed by the proportion of pyknotic nuclei.

Finally, we decided to investigate Zbtb20 knockdown effects *in vivo*. The zebrafish antisense morpholino system is advantageous for analyzing knockdown of genes when the loss of function phenotype is stark. Indeed, high morpholino concentrations yielded a poor survival rate compared to control animals. However, using lower morpholino concentrations, embryos were alive at 48 hours post fertilization (hpf), although displaying developmental abnormalities. The most striking morphological difference observed was an overall smaller body size and an obvious absence of neural structures. Analysis of pooled whole-embryo tissue by RT-qPCR displayed an almost complete absence of the neuroectodermal marker Sox1, along with more

moderate, but significant deregulation of the mesodermal markers Brachyury and MyoD and the endodermal marker Sox17. A significant increase in the expression of Goosecoid, another marker for mesoderm, was also detected, suggesting that the effect of Zbtb20 knockdown was not exclusively due to a general inhibition of germ layer formation. We further analysed the expression of zebrafish CKI homologues. Strikingly, the only transcript detected in our analysis pre-gastrulation at 4 hpf was p57Kip2 mRNA in the Zbtb20 knockdown. This was followed by a significant up regulation of p21Cip1 just after gastrulation at 9 hpf. We could therefore conclude that Zbtb20 was required for proper repression of p57Kip2 pre-gastrulation and p21Cip1 post gastrulation and overall neuroectoderm formation *in vivo.*

Taken together, our analyses of Zbtb20 loss of function in NSCs and zebrafish demonstrate that Zbtb20 may be important for differentiation as well as proliferation. These findings are not unrelated, since differentiation and proliferation are tightly connected, as discussed in chapter **1.4**. In paper II, we further show that the Zbtb20 regulated CKI p57Kip2 can regulate differentiation by binding to and regulate the activity of Mash1, that in turn have been shown to control expression of the *Dlx1/2* genes. Although Zbtb20 have been shown to be able to mediate direct repression [151], given the interaction with SMRT and the finding of Zbtb20 in URE2, a distal (12 kb) cis-regulatory element in the 5′-flanking region of *Dlx1/2* it is not unlikely that regulation of a higher order chromatin structure is required for bringing Zbtb20 in proximity to the promoter regions of *Dlx1/2*. Alternatively, a higher order chromatin structure mediated by Zbtb20 may be required to bring an activator to the Dlx1/2 promoter: Sequestering away what ever present at URE2 may be sufficient to repress Dlx1 expression in NSCs. It has been shown that Dlx2 itself provides regulation of *Dlx1/2* via URE2 [56]. It is thus possible that Zbtb20 regulate *Dlx1/2* expression as a part of a Dlx auto-regulatory mechanism in cooperation with Dlx2. Recently, the chromatin remodeling factor Mll, a member of the trxG proteins, was shown to be essential for *Dlx* gene expression during neurogenesis in a Mash1 independent fashion [145]. trxG proteins may thus me good candidates for interactions with Zbtb20. Finally, Zbtb20 expression was up-regulated by thyroid hormone in NSCs, while Dlx levels were down-regulated in these conditions. We also noted that the expression of MBP was regulated by T3 and that T3-stimulation altered the presence of Zbtb20 on the *MBP* M1 promoter. It is thus possible that Zbtb20 is directly involved in a T3-dependent gene regulation: N-CoR, another co-repressor that is highly similar to SMRT, is known to interact with proteins of the BTB/POZ family as well as thyroid hormone receptors (**Fig 5**) [133].

4.2 p57Kip2 is a repressor of Mash1 activity and neuronal differentiation in neural stem cells.

As stated earlier in this thesis, differentiation and proliferation are two tightly linked events that are very much dependent on cell cycle regulatory mechanisms. The cell cycle exit of NSCs is not merely inhibition of proliferation, but also involves a lineage choice. The Cip/Kip family of cell cycle inhibitors (p21Cip1, p27Kip1 and p57Kip2, CKI) have mainly been characterized by their ability to bind and inhibit cyclin-dependent kinases (CDK) thereby inhibiting S phase entry and regulating length of the G1 phase [200]. However, there are accumulating evidence that CKIs also modulate other cellular pathways that go beyond control of cell division that are

independently of CDK interaction. For example, over-expression experiments where the CDK interaction sites on p27Kip1 have been removed shows that specifically the N-teminal part of the protein promotes differentiation of neural progenitors in cultured cortical slices by inducing the expression of Ngn2, whereas over-expression of the C-terminal part affects radial migration [201]. p57Kip2 has also been shown to play a direct role in regulating the activity of transcription factors implicated in neuronal differentiation. For example, p57Kip2 interacts directly with the nuclear receptor Nurr1 and promote maturation of dopaminergic neurons in postmitotic precursors [115]. The take home message in paper II is that p57Kip2 selectively and physically can interact with and regulate the function of several bHLH proteins, thereby exerting a context-dependent inhibition of neuronal differentiation. The bHLH proteins neurogenin1 (Ngn1), Ngn2 and Mash1 are expressed in complementary patterns in the dorsal and ventral parts of the developing telencephalon, contributing to the generation of glutamatergic and GABAergic neurons, respectively [202]. Previous studies have suggested that p57Kip2 is involved in the differentiation of skeletal muscle and can interact with the myogenic bHLH factor MyoD [203]. We show that HA-tagged p57Kip2 robustly co-immunoprecipitated with FLAG or Myc-tagged bHLH factors Mash1, NeuroD and Nex/Math2 when expressed in HEK293 cells. In contrast, Ngn1/2/3 or Hes1 failed to co-immunoprecipitate with p57Kip2 under the same conditions. Over-expressed HA-tagged p57Kip2 pulled down endogenous Mash1 in the neuroblastoma cell line SH-SY5Y. Furthermore, a subset of Mash1 positive progenitors in the ventralmost parts of the developing telencephalon co-expressed nuclear p57Kip2 and accordingly, chromatin immunoprecipitation experiments in NSCs showed that p57Kip2 accumulated at defined Mash1 response elements in regulatory regions of the Dlx1 gene, an important regulator of telencephalic progenitor differentiation. MyoD is a repressor of several genes required for myogenic differentiation and *in vitro* studies have shown that p57Kip2 interaction leads to transactivation of those MyoD-repressed genes. In our study we came to a similar conclusion: co-transfection experiments with Mash1 and a TK-luciferase reporter construct attached to an E-box led to transcriptional activation of the construct, whereas addition of p57Kip2 to the cells resulted in repressed reporter activity in a dose-dependent manner. Consequently, p57Kip2 binding seems to counteract the gene-regulatory ability of these bHLH proteins. This interaction was independent of functional CDK interaction ability, since a mutated version of p57Kip2 with non-functional CDK binding sites also was able to repress Mash1-activation. Interestingly, p57Kip2 appears to have repressive effect on gene expression on its own: p57Kip2 fused to a DNA binding protein domain significantly repressed the activity of a GAL4 reporter construct.

Given that Mash1 and p57Kip2 are co-expressed *in vivo* and that p57Kip2 abrogates the activating ability of Mash1, what would be the biological relevance to these observations? Interleukin-6 related compounds such as ciliary neurotrophic factor (CNTF, see chapter **1.3.1**) are well known to induce a rapid and efficient differentiation of NSCs into astrocytes. Time course experiments revealed that p57Kip2 transiently accumulated in the nuclei in differentiating CNTF-stimulated NSCs, whereas proliferating FGF2 stimulated NSCs expressed basal levels in both the cytoplasm and nucleus. In further support for a cell cycle exit independent function of p57Kip2, this accumulation did not correlate to an increased cell cycle exit. Finally, over-expression of p57Kip2 as well as p57CKmut that does not interact with CDKs resulted in impaired neuronal differentiation of NSCs when allowed to differentiate

after FGF2 withdrawal after 120h, whereas astrocytic differentiation appeared unaffected, since CNTF-treated cells did not display any significant difference in the number of GFAP positive cells. Accordingly, NSCs transfected with antisense constructs for p57Kip2 displayed increased expression of neuronal-specific antigens and a simultaneous decrease in GFAP positive cells. These results suggested that the CNTF-induced accumulation of p57Kip2 in the nuclei of NSCs, although transient, indeed might have resulted in the inhibition of some neurogenic activator, presumable Ngn1/2 or Mash1 and that this inhibition may be required to suppress neuronal genes during glial differentiation in a CDK independent manner.

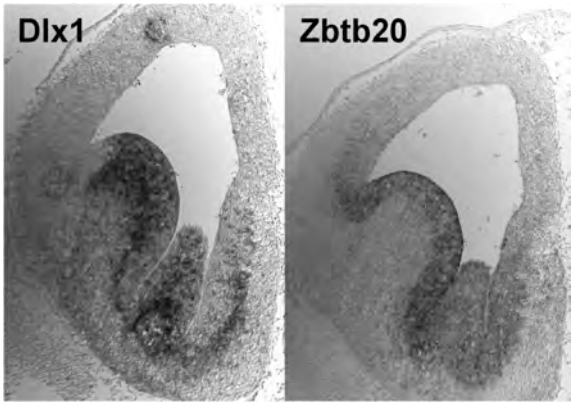

Fig 4 *Expression of Dlx1 and Zbtb20 in E13.5 mouse brain. Coronal sections.*

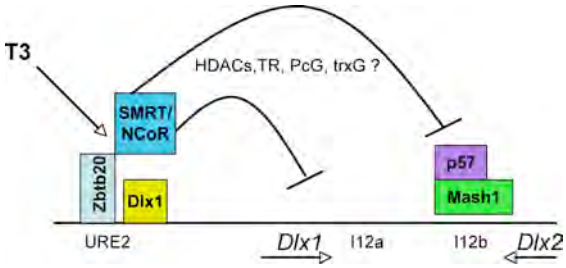

Fig 5 *Possible mechanism of Dlx-regulation in NSCs: T3 induces Zbtb20-mediated repression. In absence of Zbtb20, Dlx-expression is derepressed, perhaps in part due to p57Kip2 interaction with Mash1. Repression of Dlx by Zbtb20 is mediated by co-repressor complexes.*

4.3 The novel BTB/POZ zinc finger factor Zbtb45 is essential for proper glial differentiation of embryonic neural stem cells and oligodendrocyte progenitors.

Encouraged by the apparent importance of Zbtb20 in neural differentiation, we decided to investigate its previously uncharacterized sibling protein Zbtb45 in the same context. An initial phylogenetic analysis showed that the Zbtb45 gene was conserved in mouse, rat and human, encoding a putative 520–residue transcript that was predicted to contain a N-terminal BTB-domain and typical zinc fingers of the C2H2 type, a composition of protein domains that define the members of the BTB/POZ family. Sequence alignment further confirmed that the N-terminal part of Zbtb45 shared typical features of the BTB/POZ domain, including components of a hydrophobic core and a charged pocket [204]. Interestingly, the human ZBTB45 gene is located on chromosome 19q13, a region that have been linked to beneficial outcomes when lost in oligodendrogliomas and gliomas [205]. Quantitative RT-PCR analysis of cDNA from NSCs suggested that Zbtb45 mRNA was present in these cells so we started off by characterizing the Zbtb45 expression during mouse telencephalic development by *in situ* hybridization. To our surprise, the Zbtb45 mRNA appeared to be ubiquitously expressed at E10.5, E12.5 and E14.5, with staining density following cell density. This pattern was achieved using three different DIG-antisense RNA probes along with two synthetically generated ^{35}S-labeled RNA probes and further analysis of Zbtb45 mRNA expression in various tissues dissected from E15.5 rat embryos (olfactory bulb, thalamus, cortex, ganglionic eminences, heart and forelimb) by qPCR confirmed the results from *in situ* hybridization. Ubiquitous expression appears to be an uncommon feature amongst proteins of the BTB/POZ family, with the exception of HIC-1 that is ubiquitously expressed in normal tissue but under-expressed in different tumor cells where it is hypermethylated [206].

Interestingly, knockdown of Zbtb45 in NSCs resulted in a significant gradual relative increase in the expression of the astrocyte marker GFAP mRNA and the astrocyte-associated protein CD44 mRNA upon FGF2 withdrawal or stimulation with thyroid hormone, as compared to NSCs nucleofected with control siRNA. Accordingly, an assessment of the number of GFAP-positive cells by immunocytochemistry confirmed that the relative abundance of astrocytes had increased. Notably, the increase in astrocytic differentiation was accompanied with a relative down-regulation of the mRNA levels of oligodendrocyte related genes such as the OLP marker CNPase as well as the later marker MBP. However, we did not detect any significant decrease in the abundance of MBP expressing cells by immunocytochemistry one week post Zbtb45 knockdown. In line with the decrease of oligodendrocyte marker genes, Olig2 expression was slightly down regulated followed by decreased Sox10 expression compared to control. We also observed a temporal but moderate up-regulation of Hes5, which previously have been linked to inhibition of myelin gene expression, downstream the Notch pathway [207]. Furthermore there was no increase in levels of BMP4 or BMP7 mRNA, signaling factors known to promote astrocytic and inhibit oligodendrocytic differentiation of telencephalic NSCs [184]. As discussed in chapter **1.3**, increased astrocytic differentiation is often the result of provoked mechanisms underlying the neurogenic to gliogenic switch during telencephalic development and is therefore associated with a decrease in neuronal differentiation. Nevertheless, we could not detect any significant change in neuronal marker mRNA

levels, such as ß-tubulin III or REST, or increased number of neurons as a result of Zbtb45 knockdown in NSCs.

Although proteins of the BTB/POZ family are known to interact with co-repressors and chromatin remodeling complexes, as discussed in chapter **2**, the only interacting protein of ZBTB45 (human) reported so far is MED31, a sub-unit of the mediator complex, as identified in a yeast two-hybrid assay [208]. Med31 mRNA levels where detected in NSCs at a similar range as TBP (TATA-binding protein) and protein expression was confirmed by immunocytochemistry. We therefore employed the same assays described above for Zbtb45 knockdown, but surprisingly, we did not find any significant difference in NSC survival or differentiation after Med31 knockdown compared to control, despite a 90% knockdown efficiency.

To further investigate a potential shift in glial fate proportions induced by Zbtb45 knockdown in NSCs, we turned to the bi-potent oligodendrocyte precursor cell line CG-4 (central glia 4). CG-4 cells spontaneusly differentiate into oligodendrocytes upon mitogen withdrawal (FGF2 and PDGF), but can also form astrocytes when grown in 10% FBS [209]. In line with our data from the NSCs, an inhibitory effect of Zbtb45 knockdown was strongly confirmed by immunocytochemistry. siZbtb45 transfected cultures displayed a striking reduction in mature oligodendrocytes in contrast to the fully differentiated control cells. The differentiating CG-4 cells did not express GFAP mRNA at any time point after mitogen withdrawal but interestingly, and in further support of the results from the NSC analysis, the expression of the astrocyte-associated marker CD44 was increased.

Finally, the effects of Zbtb45 knockdown in NSCs and CG-4 cells appeared to be independent of the expression of cell cycle inhibitors and general aspects of proliferation: No significant differences in BrdU incorporation in NSCs or CG-4 cells was seen after Zbtb45 knockdown versus control cultures in any of the conditions tested nor could we detect changes in the expression of members of the Cip/Kip family explaining the magnitude in which Zbtb45 knockdown affected glial differentiation.

How can an ubiquitously expressed factor have specific effects on glial differentiation? So far, no common mechanism for BTB/POZ gene regulation has been pinpointed. However, the ubiquitous expression of Zbtb45 in the developing telencephalon suggests a more general function than specifically regulating glial differentiation of NSCs or glia progenitors. On the contrary, it gives further support to view BTB/POZ proteins as part of the transcriptional machinery acting as molecular docking bays for co-factors and other transcription factors, rather than being the end-point in a given signaling pathway or acting as a transcriptional repressor. We can speculate then that the effect of Zbtb45 knockdown is the result of impaired recruitment of some other key regulatory element. Thus finding an interaction partner for Zbtb45 will be crucial for understanding the function of Zbtb45 in telencephalic development. Given the impact of Zbtb45 on glial differentiation in NSCs and CG-4 cells, the location of the human ZBTB45 gene on chromosome 19q13 is puzzling and suggests that Zbtb45 may be involved in tumorigenesis. If so, the charged pocket of the BTB-domain could be a potential target for therapies. The BTB-motif has been shown to be a possible target for inhibitory peptides that can disrupt the interaction with co-repressors [210].

4.4 CXXC5 is a novel BMP4-regulated modulator of Wnt-signaling in neural stem cells.

The bone morphogenic proteins (BMPs) are classified as members of the transforming factor ß (TGFß) superfamily and have profound roles in a vast majority of developmental and cellular processes, such as differentiation, proliferation, and migration [211]. BMP-signaling exert cross-regulation with other signaling pathways, including Notch, FGF and Wnt signaling during telencephalic development [212]. As briefly discussed in chapter **1.1** and **1.3.1**, the caudal-dorsal edge of each cerebral cortical hemisphere, the cortical hem, is a source of BMP and Wnt proteins [11,12,13,14]. Without a canonical WNT signal from the hem, no hippocampus will be formed [15]. Furthermore, when telencephalic BMP activity is partially blocked, choroid plexus formation from the medial neuroepithelium is impaired [16]. Conversely, continuous activation of BMP signaling transforms the entire cortical primordium into choroid plexus epithelium [17]. In line with this, NSCs can be brought to differentiate into smooth muscle cells as measured by smooth muscle α-actin (SMA) in response to BMP4-stimulation, when the cells are grown at low local density [213], presumable corresponding to choroid plexus formation. In an attempt to increase the understanding of the molecular mechanisms underlying the responses of NSCs to BMP-signaling, we pursued gene expression profiling using microarrays to identify putative direct and novel targets for BMP4 in NSCs. NSCs where treated with BMP4 or FGF2 for 3h and the gene expression profile was subsequently investigated. One significantly BMP4-regulated gene that appeared in the screen was the previously uncharacterized factor CXXC5 that displayed a repeatable and rapid (40 min) 15-fold up-regulation. We concluded that CXXC5 was a direct target of the BMP4 stimulation since inhibition of *de novo* protein synthesis by cyclohexamide, an inhibitor of translation, not significantly affected the BMP4-mediated up-regulation of CXXC5. Expression patterns are always informative, so we performed *in situ* hybridizations in mouse E10.5, E12.5 and E14.5 to elucidate the putative context of CXXC5 *in vivo*. At E10.5 CXXC5 expression was restricted to the medial neuroepithelium of the dorsal pallium, overlapping or in close proximity to BMP4 and Wnt3a expression. Interestingly, CXXC5 and BMP4 remained co-expressed in the developing choroid plexus at E12.5 whereas Wnt3a expression was detected in the adjacent hippocampal anlagen. At 14.5 however, only low levels of CXXC5 expression were detected whereas BMP4 mRNA remained highly present in the developing choroid plexus. At E14.5 a marked expression of the Wnt3a gene was detected just lateral to the BMP4 expression. The expression pattern of CXXC5 thus suggested that this factor could play a role in telencephalic development and strengthened the association between CXXC5 and high BMP4 signaling activity.

The overlapping expression patterns of CXXC5 and BMP4 and the adjacent Wnt3a expression during telencephalic developing was intriguing, since several reports have described cross-talk between the BMP and WNT signaling pathways [212,214]. Furthermore, CXXC5 was predicted to be a paralog of another CXXC domain containing factor [215,216], CXXC4 (Idax) that in turn was identified in a screen for proteins interacting with the PDZ domain of the intracellular signaling factor Dishevelled (Dvl) [217]. Wnt3a signals through the canonical WNT signaling pathway - a series of events that occurs when a Wnt protein binds to cell-surface receptors of the Frizzled family. This causes the receptors to activate Dishevelled family proteins and ultimately resulting in a change in the amount of β-catenin that

reaches the nucleus. In the absence of a Wnt signal, β-catenin is constitutively phosphorylated by GSK3-kinase resulting in its degradation. The β-catenin-GSK3-kinase complex is in turn facilitated by a scaffold protein, Axin. When Wnt is present, Dishevelled proteins inhibits the scaffolding activity of Axin which leads to inhibited phosphorylation of β-catenin. CXXC4-Idax is believed to function as an intracellular protection molecule against active Wnt signaling because of its ability to directly bind to Dvl at the binding site of Axin [217]. Indeed, GFP-tagged CXXC5 constructs with Myc-tagged full-length Dvl interacted and co-immunoprecipitated when expressed in COS7 and HEK-293, demonstrating a close functional similarity between CXXC5 and CXXC4-Idax. This interaction was further confirmed for endogenous Dvl3 and GFP-CXXC5 in another co-immunoprecipitation experiment, by overlapping sub-cellular localization of GFP-CXXC5 and Myc-Dvl in COS7 cells and finally by Förster (Fluorescent) Resonance Energy Transfer (FRET) measurements [218,219] in NSCs revealing close proximity of GFP-CXXC5 and Alexa555-IgG coupled to a Dvl2 antibody.

Did the BMP4-induced increase in CXXC5 expression levels and the interaction between CXXC5 and Dvl have any functional importance? To elucidate this question we next aimed for investigating the effects of CXXC5 over-expression on the response to Wnt signaling in NSCs. Axin2 gene expression is directly regulated by the Wnt signaling mediator ß-catenin and is commonly used as a measurement for canonical Wnt signaling activity [220]. BMP4-treatment of NSCs resulted in significantly reduced levels of Axin2 mRNA expression within 6h. Accordingly, treatment with Wnt3a led to an up-regulation of Axin2 levels but over-expression of CXXC5 markedly attenuated this increase. Over-expression of CXXC5 also led to a decrease in Axin2-levels in FGF2-stimulated control NSCs. These results all suggested that high levels of CXXC5 interfered with the response to Wnt3a stimulation in NSCs. To see whether the effect of CXXC5 on Wnt signaling was selective for Axin2 expression in NSCs or a general effect on canonical Wnt signaling, we next investigated the effects of CXXC5 expression on Wnt3a mediated activation of a TOPflash reporter in HEK-293 cells, a commonly used assay for studies of canonical Wnt signaling [217]. Control experiments confirmed that reporter activity was robustly activated by over-expression of the canonical Wnt signaling mediator ß-catenin. Transfections of increasing amounts of CXXC5 significantly inhibited the TOPflash reporter activity induced by recombinant Wnt3a. In addition, a decrease in Wnt3a-induced activated (de-phosphorylated) ß-catenin was observed in CXXC5-transfected cells, although no significant changes in the total protein levels of ß-catenin or Dvl could be detected. Finally we investigated the effect of siRNA-mediated knockdown of CXXC5 in NSCs. Interestingly, introduction of siRNA against CXXC5 24h before BMP4 stimulation resulted in an almost complete abolishment of the BMP4-mediated reduction of Axin2 levels and Wnt3a stimulation of CXXC5-reduced cells facilitated an increase in Axin2 compared to control. We could thus conclude that CXXC5 is a mediator of BMP4-mediated modulation of Wnt-signaling in NSCs and possible that the functional role of CXXC5 in telencephalic development may be to contribute to the establishment of the demarcation between BMP4 and Wnt3a signaling regions during the development of hippocampal and choroid plexus formations.

ACKNOWLEDGEMENTS

My 6 years at the Karolinska Institute have been fantastic in many ways, but also very hard sometimes. A lot of people around me have been irreplaceable for me at work as well as in the outside real world. Thank you so much for all the support, advice and friendship. Without you, this thesis would not have been possible:

Liv

Mom and Dad, Gunnar & Anna

My fantastic friends:
Arvid, NC, Erik & Anders, Aiva, Annika, Grunkan, Robban, Ecke, Maggan & Jessika, Sara, Berra, Jana, Schneider, Jonas, Mattias, Katja, London-Emma, Richard, Charlotte, Calle H, Magsch, Oskar P, Jon, Knut, Anders S, Tor H, Tor S, Tomilla, Sebastian, Micke, Fredrik & Maiken, Marcus & Sarah, Farbror Henrik, Pär, Oskar E, Måns, Martin R, Anders B, Sofia, Dan, Erik F, Erik Z, Kristoffer, Mats, Ubbe + everyone that I forgot to include! Thank you all for all the fun we have had during these years, the adventures, parties, discussions, nerdyness..

And my fantastic collegues:
Thank you all past and present members of the Hermanson lab (with no particular order): Josh, Karolina, Shirin, Tobias, Amilcar, Gonçalo, Ana T, Anna C, Therese, Anna, Anna, Ana, Emma, Lining, Anna, Ana, Alex, Luisa, Mariana, Pontus, Stephanie, Michalina, Susanne & Adrian. Thanks for enduring my moodiness and what I considered being music day in and out. Thanks for great company, support and scientific discussions!

And all my other equally fantastic collegues and co-workers throughout the years: Magnus, Pinelopi, Ulrika N, Vanessa, Anna H, Anna B, Kylie, Richard, Kalle, Karin, Abdul, Peter, Jorrit, Sindhulakshmi, Nicholas, Seth, Peter, Olle, Micke, Maurice, Joanna, Mattias, Jenny, Sanya, Jesper, Mimmi, Emil, Karin, Niklas, Ulrika M, Banafsheh, Sara, Kirsty, Elisabeth, Nick, Maria, Idha, Paola, Fanie, Gustavo & Fernanda, José, Tony, Nina, Julianna, Jorge, Fredrik, Johan, Marcus, Stina, Qiaolin...

Special thanks to Jonas Muhr, Jonas Frisén, Christian Broberger, Urban Lendahl, Björn Vennström, Johan Ericson, Andras Simon, Filip Färnebo, Sandra Ceccatelli, Ernest Arenas, Monika Andersson-Lendahl, Bertrand Joseph, Patrik Ernfors, Per Uhlen, Agneta Richter-Dahlfors, Thomas Perlmann, Lars Björklund and all you other hotshots. Several of my key experiments were made in collaboration or based on direct advice from you or on reagents found in your freezers.

I would also like to acknowledge the administrative personnel on CMB and the Neuro department. Thank you very much.

And finally, thank you Ola Hermanson for accepting me as a PhD student in your lab. It has been heaven and it has been hell, but I wouldn't change this time for the world.

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