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MICROENVIRONMENTAL REGULATION OF NEURAL STEM CELL DIFFERENTIATION

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Microenvironmental Regulation of Neural Stem cell Differentiation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The central nervous system consists of complex networks of neurons and other specialized cells that process information from within and outside the organism to create an output. The development of this complex machinery requires spatio-temporal regulation of neural stem cell (NSC) differentiation into the right type of cells at the right time. Deregulation of these developmental stages results in embryonic malformations or neurodevelopmental disorders. During development, the NSCs are instructed by their intrinsic gene regulatory network as well as the microenvironment which includes signaling molecules secreted by other cells, rigidity of the extracellular matrix, and cell-cell contact. The role of the metabolic signals in NSC differentiation is however less clear.

The main aim of this thesis was to elucidate how microenvironmental metabolic cues are integrated into differentiation of embryonic neural stem cells focusing on developing spinal cord and cortex.

Since vascularization is not complete during development cells are dependent on the oxygen and nutrients that diffuse through the tissue just like secreted signaling molecules. As a result each cell is exposed to varying levels of oxygen and glucose depending on their distance from the developing blood vessels and this leads to changes in the reduction-oxidation (redox) state of the cell. C-terminal binding protein (CtBP) is an evolutionarily conserved transcriptional co-repressor that can sense the redox changes and links the microenvironmental metabolic signals to the transcriptional regulation of cells. We found that the developing chick spinal cord displays an oxygen gradient throughout its dorsoventral axis, and CtBP is required to integrate the oxygen levels with local growth factor concentrations to regulate neurogenesis in the roof plate. Manipulation of the oxygen levels or downregulation of CtBP leads to misdifferentiation of neurons which demonstrates that the oxygen level is a required component of extracellular regulation of NSC differentiation. We further investigated the biochemical regulation of CtBP by metabolic changes. We showed that the acetylation and dimer detection of CtBP is regulated in inverse manner by oxygen levels in proliferative NSC. These post-translational regulation mechanisms may affect the transcriptional repressor activity of CtBP.

The mammalian cortex contains six highly specialized layers that consist of several subtypes of neurons that form connections between the layers, different parts of the cortex and subcortical structures. These connections are important for information processing and input to output computation. Perturbations in this network lead to neurodevelopmental disorders such as autism and schizophrenia. We found that NSC in the developing rodent cortex change their energy supply mechanisms as they differentiate, and manipulation of extracellular metabolic cues influences the gene expression of layer-specific neuronal markers. As we showed in the chick spinal cord, CtBP is the bridge between metabolism and differentiation also in the cortex. When CtBP is downregulated in developing mouse cortex the NSC pool is deregulated and cortical neuronal differentiation and migration is perturbed.

Altogether these results demonstrate that metabolic cues in the neural stem cell microenvironment regulate neuronal differentiation in the developing spinal cord and cortex and that the metabolic changes are translated into cellular behavior via transcriptional co-repressor CtBP. Thus, controlled regulation of energy supply mechanisms is a required part of nervous system development.

LIST OF SCIENTIFIC PAPERS

I. Dias JM, Ilkhanizadeh S, **Karaca E**, Duckworth JK, Lundin V, Rosenfeld MG, Ericson J, Hermanson O, and Teixeira AI (2014) CtBPs sense microenvironmental oxygen levels to regulate neural stem cell state. Cell Rep 8, 665-670.

II. **Karaca E**, Lewicki J, Hermanson O (2014) Oxygen-dependent acetylation and dimerization of the corepressor CtBP2 in neural stem cells. Exp Cell Res (*in press*)

III. **Karaca E**, Lewicki J, Guérout N, Barnabé-Heider F, Hermanson O. Metabolism regulates cortical neurogenesis via oligomerization of the corepressor CtBP2. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

Blbp	Brain lipid binding protein
BMP	Bone morphogenic protein
BP	Basal Progenitors
CP	Cortical plate
CtBP	C-terminal binding protein
CtIP2	(COUP-TF)-interacting proteins 1 and 2
Cux1/2	Cut-like homeobox-1/2
DCX	Doublecortin
DV	Dorsoventral
FoxP2	Forkhead box protein P2
GFAP	Glial fibrillary acidic protein
HIF	Hypoxia inducible factor
IZ	Intermediate zone
Map2	Microtubule associated protein-2
NSC	Neural stem cells
Pax6	Paired box-6
PLA	Proximity ligation assay
RGC	Radial glia cell
ROS	Reactive oxygen species
Satb2	Special AT-rich sequence-binding protein 2
Shh	Sonic hedgehog
Sox5	SRY-box-5
SVZ	Subventricular zone
Tbr2	T-box brain protein 2
TGF-β	Transforming growth factor beta
Tubb3	β-tubulin-III
VZ	Ventricular zone

1 INTRODUCTION

1.1 DEVELOPMENT OF SPINAL CORD AND THE CEREBRAL CORTEX

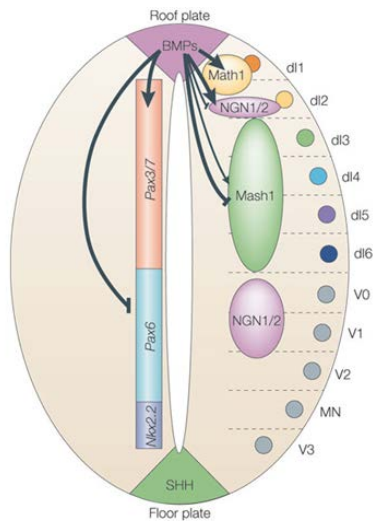### 1.1.1 Neural tube development

The nervous system development initiates at the embryonic midline ectoderm. The neural plate folds into neural tube during neurulation, followed by the patterning of the neural tube which determines areas that will give rise to specific types of neurons and glial cells in dorsoventral (DV) and anterior-posterior axis.

The neural tube DV axis patterning requires Sonic hedgehog (Shh) and bone morphogenic protein (BMP) signaling from ventral and dorsal organizing centers floor plate and roof plate respectively. Shh and BMP are gradient forming morphogens although BMP diffusion range is more limited compared to Shh. The neural progenitors at each coordinate along the DV axis are exposed to specific concentrations of Shh and BMP. Binding of these signaling molecules to their receptors leads to activation of their downstream transcriptional factors that regulate neuronal differentiation.

Figure 1. Dorsoventral patterning of developing neural tube (Liu and Niswander, 2005)

Secreted from the floor plate, Shh binds to its receptor Patched and releases its inhibition of another membrane protein Smoothened. As a result of Smoothened activation downstream Gli family zinc finger transcription factors bind to DNA and regulate transcription of their target genes. The Shh gradient leads to the differentiation of ventral interneurons or motoneurons from the progenitors in the ventral neural tube

Counterbalancing the Shh gradient, BMPs on the dorsal neural tube are secreted proteins of the transforming growth factor-β (TGF-β) superfamily. Upon binding of BMPs to their receptors, receptor-activated Smad factors (Smad1/5/8) are phosphorylated and

form a complex with Smad4 which eventually regulate transcription. Several BMPs are expressed in the roof plate such as BMP1, 2, 4 and 7 however only BMP2 and 4 were shown to diffuse through the tissue to form a gradient (Hu et al., 2004). BMP signaling in dorsal neural tube leads to activation of pro-neuronal basic helix-loop-helix (bHLH) transcription factors and differentiation of interneurons (Liu and Niswander, 2005).

1.1.2 Cortical development

The mammalian cortex consists of excitatory glutamatergic pyramidal neurons and inhibitory GABAergic interneurons. The glutamatergic neurons are produced in the ventricular zone and migrate radially whereas the GABAergic neurons originate at the ganglionic eminence and migrate tangentially. At the beginning of cortical development, in the psedostratified neuroepithelium that lines the lateral ventricles, neuroepithelial cells with polarized cell body and attachments to both apical and basal surfaces divide symmetrically to increase the stem cell pool. As the thickness of the epithelial layer increases the neuroepithelial cells keep extending their processes as they start demonstrating glial phenotype differentiating into radial glia cells (RGC) with neural stem cell markers Nestin, Pax6 and lectin RCA expression. RGCs are heterogeneous and they can also express astrocyte-specific glucose transporter GLAST or brain lipid-binding protein (BLBP). The cell bodies of RGCs remain at the germinal zones while their processes towards the basal surface guide neurons during migration (Okano and Temple, 2009).

The RGCs can undergo symmetric cell division to produce either two proliferating daughter cells or two neural precursors. Or, they divide asymmetrically to produce one stem cell and one neural precursor. RGCs also give rise to Tbr2-positive basal progenitors (BP) which lose their attachment to apical and basal surfaces and reside in the SVZ. The BPs have limited self-renewal ability and undergo one more symmetric cell division to produce two neurons or two progenitors (Götz and Huttner, 2005).

At the beginning of cortical development most cell divisions are symmetric increasing the stem cell pool. The amount of asymmetric cell divisions increases at later stages. During symmetrical cell division the division axis is vertical to the ventricle

distributing the cell body contents equally between the two daughter cells. However, as a result of asymmetric cell division which can have either horizontal (rare) or vertical division axis the daughter cell with self-renewing ability inherits the apical part of the cell soma contents and the other daughter cell which differentiates into neuron gets the basal part of the soma. As RGCs undergo cell division the cell body moves between the apical and basal borders of SVZ exposing the cells to changing environmental cues (Paridaen and Huttner, 2014).

The temporal distribution of symmetric and asymmetric cell divisions correlates with the length of the cell cycle in germinal zones (VZ and SVZ). When RGCs are undergoing stem cell pool expansion via symmetric cell divisions the cell cycle length is shorter compared to neurogenic asymmetric divisions. The reason for long cell cycle is mainly the lengthening of the G1 phase which exposes differentiating cells to extracellular cues for a longer time allowing for the integration of microenvironmental factors to intrinsic differentiation program (Dehay and Kennedy, 2007).

Following neurogenic cell divisions of RGCs and BPs neurons migrate radially into the cortical plate to eventually form six layers of mostly glutamatergic neurons followed by gliogenesis with differentiation of astrocytes and oligodendrocytes. Differentiation of the neuronal subtypes of the cortical layers as well as the glial cells is regulated by the integration of extrinsic and intrinsic factors. Cells will only respond to extracellular cues if they are competent which may depend on the expression of receptors, or epigenetic regulation of promoter activation of target genes.

1.1.3 Spatial and temporal regulation of cortical layer formation

The formation of six cortical layers is subject to spatial and temporal regulation. First, the RGCs give rise to the preplate neurons which later splits into two to form marginal zone and the subplate. The cortical plate is formed with the migration of early neurons and formation of the cortical layers in an inside-out fashion: early born neurons form the deep layers (V-VI) and late born neurons migrate past those and constitute the upper cortical layers (II-IV). The neural subtypes in each layer have marker expression of transcriptional regulators and they form specific projections to cortical or subcortical regions.

According to lineage tracing analysis of neurons, at early stages of cortical neurogenesis the neural stem cells and progenitors have the potential to differentiate into all subtypes of neurons of layers II-VI. However, as development progresses the NSC become more restricted in their potential cell-fates (Shen et al., 2006). The heterochronic transplantation studies showed that when early born progenitors were transplanted in older embryonic cortices they could differentiate into upper layer neurons. However, late born neural progenitors failed to differentiate into deep layer neurons when transplanted in young embryos (Desai and McConnell, 2000).

The temporal regulation of layer specification requires genetic cross inhibition of layer determinants. Sox5, Fezf2 and CtIP2 transcriptional regulators are responsible for the differentiation of neurons that constitute deep layers and project to subcortical regions. During deep layer neuronal specification Fezf2, which is expressed in VZ progenitors and young deep layer neurons, represses chromatin remodeling protein Satb2 which later leads to differentiation of upper layer neurons via suppressing layer V marker CtIP2 in the post-mitotic neurons. A mammalian ortholog of a temporal-identity factor which determines the fate of early born neurons in *Drosophila melanogaster*, Ikaros, has been demonstrated to have a similar role in the mammalian cortex. Overexpression of Ikaros led to increase in the deep layer neurons and less upper layer neurons were generated (Alsiö et al., 2013; Kohwi and Doe, 2013; Okano and Temple, 2009).

There are different opinions about the source of deep and upper layer neurons. Initially, the studies on VZ genes *Pax6* and *Otx1*, and the SVZ genes *Cux1*, *Cux2* and *Svet1* demonstrated that RGCs gave rise to deep layers V and VI whereas SVZ progenitors formed the upper layers II-IV. Other studies showed the expression of *Cux2* in RGCs before the formation of SVZ starting from E10.5. *In utero* fate-mapping demonstrated that these Cux2 expressing progenitors were fate-restricted to upper layer neurons from the beginning not depending on the birthdate or location (Franco et al., 2012). In another model, labeling of individual RGCs with GFP using *in utero* electroporation could show that both deep and upper layer excitatory neurons could be generated from the progeny of individual RGCs (Kohwi and Doe, 2013).

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At the end of neurogenesis at E17 gliogenesis is initiated to give rise to astrocytes and oligodendrocytes. This neuron-to-glia switch is regulated by intrinsic factors such as pro-neural bHLH protein neurogenins that inhibit gliogenesis and Sox9 that is required for the production of glial cells, and extrinsic factors such as the interleukin - 6 (IL-6) family cytokines cardiotrophin-1 (CT-1) (Barnabé-Heider et al., 2005), leukemia inhibitory factor (LIF) and ciliary neurotrphic factor (CNTF). During neurogenesis *Gfap* promoter is highly methylated preventing early neurons from differentiating into astrocytes even in the presence of IL-6 cytokines. At the time of neuron-glia switch COUP-TFI/II are expressed transiently in order to remove chromatin silencing factors enabling cells to respond to CT-1 secreted from differentiated neurons and initiate astrogenesis. Similar epigenetic regulations may play a role determining the competence of progenitors to respond to extracellular cues (Kohwi and Doe, 2013).
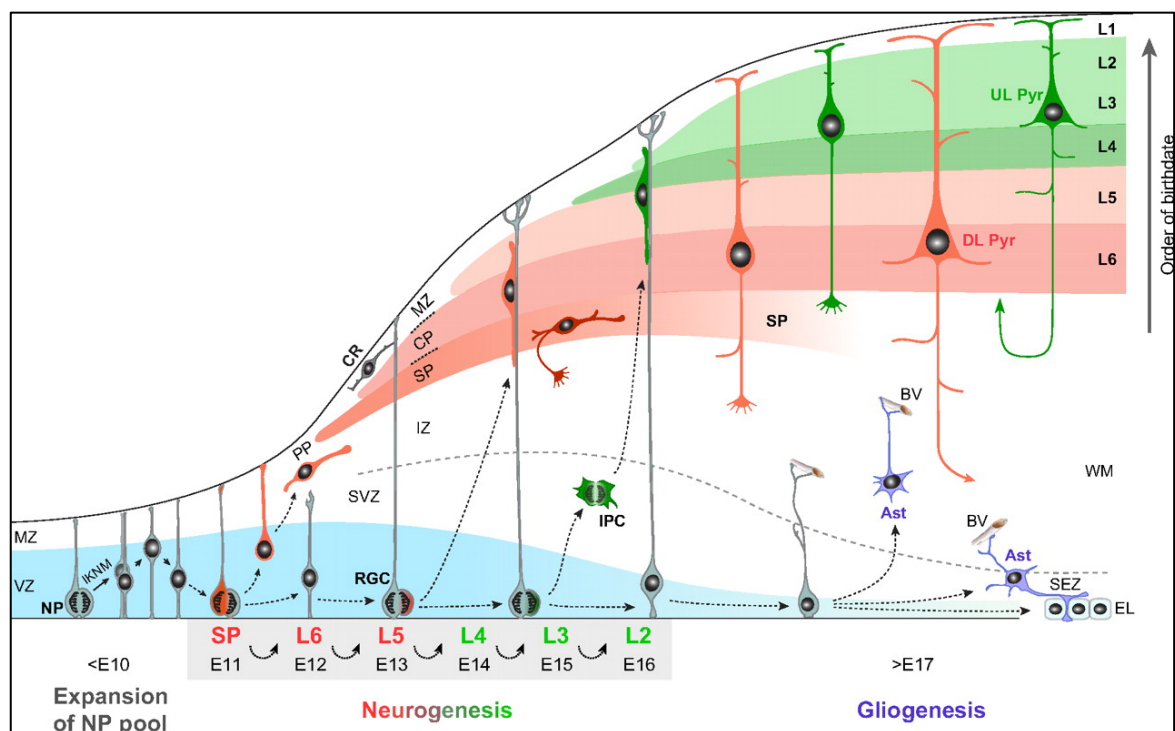

Figure 2. Stages of cortical development *in vivo* (Kwan et al., 2012)

1.1.4 Modeling cortical development *in vitro*

The temporal regulation of cortical development with the production of neurons first followed by astrocytic differentiation as well as the differentiation of layer specific subtypes of neurons can be recapitulated *in vitro*. Primary cultures of embryonic mouse cortices starting as early as E10.5 were shown to give rise to preplate as well as cortical plate neurons in the absence of extracellular factors (Shen et al., 2006). The post-mitotic neurons then start secreting IL-6 family cytokine CT-1 which leads to astrogenesis following neuronal differentiation (Barnabé-Heider et al., 2005). Furthermore, when seeded in low density in the absence of serum or growth factors embryonic stem cells give rise to neural stem cells by default *in vitro* differentiating first into Nestin positive neural stem cells, then TuJ1 and Map2 positive neurons and eventually astrocytes (Fig 3) (Gaspard et al., 2009; Gaspard et al., 2008; Smukler et al., 2006).

In this thesis, E15.5 rat cortical neural stem cells were used as an *in vitro* model (Hermanson et al., 2002; Johe et al., 1996). These cells are kept proliferative and undifferentiated with FGF2 treatment. Upon addition of various growth factors they can be differentiated into neurons, astrocytes and oligodendrocytes. Differentiation outcome of rat NSC is density dependent (Andersson et al., 2011). To mimic *in vivo* cortical neurogenesis, rat NSC are seeded at high density and differentiated spontaneously in the absence of FGF2.

1.2 METABOLISM

The stem cell niche is described as the microenvironmental factors that contribute to the preservation, differentiation and migration of stem cells such as the rigidity of the extracellular matrix, how secreted molecules from the other cells diffuse through the tissue, cell-cell contact and distance from the blood vessels which provide oxygen and glucose.

Highly proliferative cells have increased requirement for extracellular metabolic input and production of building blocks for the cells. In order to adapt to changes on the
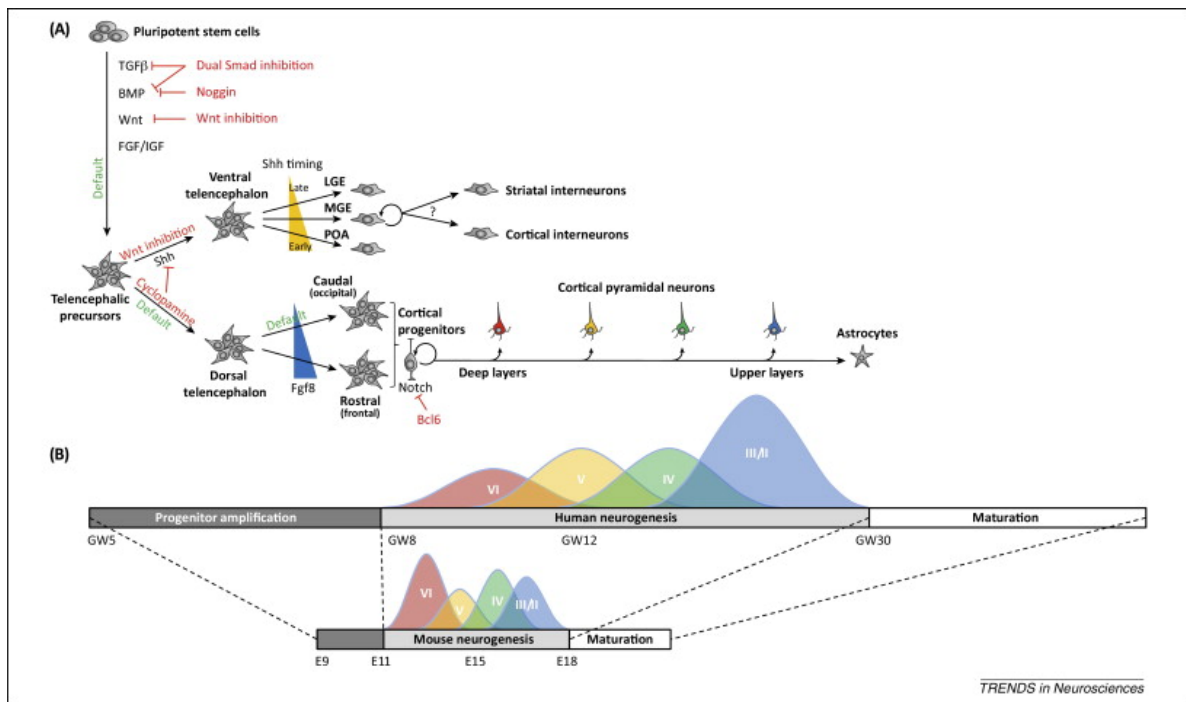
Figure 3. *In vitro* modeling of mammalian cortical development (van den Ameele et al., 2014)

availability of oxygen, glucose and other substrates for oxidation cells can shift their metabolic pathways. Besides, these metabolic changes can also contribute to the transcriptional regulation of the cells and influence cellular behavior such as proliferation, differentiation and maintenance of stem cell pool.

The metabolic cues in the stem cell microenvironment are particularly important for proper embryonic development and regeneration of adult tissues. The stem cell niche should protect cells from reactive oxygen species (ROS) induced DNA damage, preserve stem cells' potency preventing them from going into senescence or premature differentiation and assist cells during differentiation and migration.

Here I will mainly focus on how oxygen, glucose and ROS levels can regulate neural stem cells and discuss the crosstalk between neurogenesis and angiogenesis during development.

1.2.1 Oxygen

Although in *in vitro* cell culture studies ambient oxygen level (21%) is taken as normoxia under physiological conditions the oxygen content depends largely on the extent of vascularization, the oxygenation of blood vessels and the oxygen diffusion rate into the tissue. As a result an oxygen gradient is created exposing cells that reside close to blood vessels with higher oxygen level than those that are distant. In adult tissues oxygen is predicted to be in 2-9% range (Simon and Keith, 2008) whereas during embryogenesis tissues can be exposed to lower oxygen tension due to incomplete vascularization.

Physiological oxygen levels are predicted to be 0.5-8% in mouse brain (Erecińska and Silver, 2001) and 3-4% in the human brain (Dings et al., 1998). It has been found that most stem cell niches are hypoxic since under low oxygen levels cells accumulate less DNA damage that occurs with exposure to reactive oxygen species. Although 3-5% oxygen was found to be increasing stemness of cells and reducing differentiation, further decrease in oxygen levels to 1% reduced proliferation while maintaining pluripotency in stem cells.

The cellular hypoxic response is mainly initiated by the hypoxia-inducible factor (HIF) family. Hif1 is a heteromeric protein that consists of Hif1-α which senses oxygen and Hif-β (ARNT) that is constitutively expressed. Under high oxygen levels Hif1-α is subjected to cytoplasmic translocation and proteasomal degradation. When cells are exposed to low oxygen levels (<9%) (Rafalski et al., 2012). Hif1-α is stabilized, dimerizes with Hif1-β, localizes to nucleus and regulates transcriptional activation of genes involved in angiogenesis and glycolytic pathways (Bruick, 2003).

Hif1-α is involved with other pathways important for development such as the Notch, Wnt and BMP. Wnt and Notch pathways are important to keep the stem cells at undifferentiated state whereas BMP pathway promotes differentiation. Hypoxia was shown to suppress differentiation of neural stem cells in hippocampus via Wnt pathway (Mazumdar et al., 2010). Hif1-α also interacts with intracellular domain of Notch to activate transcription of Notch targets such as Hes-1 which represses transcription of pro-neural bHLH genes to inhibit neuronal differentiation (Gustafsson et al., 2005). On the other hand, the activation of Smad1/5/8 of BMP pathway was

inhibited by hypoxia which prevented the differentiation of neural stem cells (Pistollato et al., 2007).

1.2.2 Glycolysis and Reactive Oxygen Species

Cells adapt to low oxygen levels by switching to glycolysis for the production of ATP. Since amount of net ATP obtained from glycolysis is much lower compared to aerobic respiration (2 vs 36 ATP per glucose molecule) there is a greater flux through the glycolytic pathway to sustain cellular energy needs and the glucose consumption is higher.

However, as shown in cancer cells, stem cells can also have upregulation of glycolysis even in the presence of oxygen. Named Warburg effect or aerobic glycolysis, this shift in metabolism is considered to be important to accumulate building blocks for the proliferating cells (Dang, 2013).

So far, it has been shown that NSC have higher glycolysis rate compared to differentiated neurons. Cortical NSC were found to be more susceptible to glycolysis inhibition *in vitro* (Candelario et al., 2013) and cerebellar NSCs were found to use aerobic glycolysis for energy production *in vivo* which was found to be important for their neuronal differentiation (Gershon et al., 2013).

Another possible molecular signal that can regulate NSC is reactive oxygen species (ROS) that are produced with exposure to oxygen. Most research on ROS focuses on the context of DNA damage and cytotoxicity. However, low amount of ROS that is non-cytotoxic can also be involved in regulation of cell signaling promoting proliferation and cell survival (Ray et al., 2012). As an example, proliferative cortical NSC were shown to have high levels of endogenous ROS which regulated their self-renewal and neurogenesis (Le Belle et al., 2011).
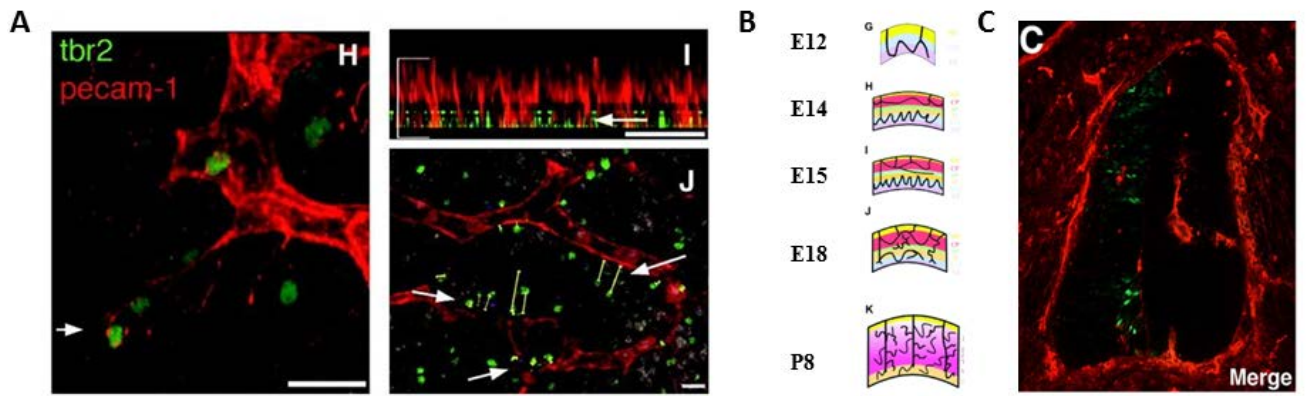
Figure 4. A) Localization of Tbr2 positive basal progenitors near blood vessels (pecam-1) (Javaherian and Kriegstein, 2009) **B)** Sketches of vascularization during cortical development (Stubbs et al., 2009) **C)** Blood vessels in avian developing spinal cord (James et al., 2009; Stubbs et al., 2009)

1.2.3 Neural stem cells and angiogenesis

In developing mouse cortex blood vessels run parallel to the basal surface and the lateral ventricles in the SVZ, creating branches radially as development progresses. In recent years, NSC have been linked to angiogenesis in the cortex (Fig 4). Proliferative cells positive for Ki67 as well as Tbr-2 positive basal progenitors were shown to accumulate around blood vessels in embryonic cortex (Javaherian and Kriegstein, 2009; Stubbs et al., 2009). Similarly, in adult cortex proliferative neural stem cells were found in close proximity to the blood vessels running through SVZ (Shen et al., 2008). The proximity of NSC to blood vessels may underline the importance of oxygen and glucose availability for NSC self-renewal and proper differentiation. In addition to these results, radial glia cells were demonstrated to support vascularization in developing cortex (Ma et al., 2013).

1.3   C-TERMINAL BINDING PROTEIN (CTBP)

C-terminal binding proteins (CtBP) are metabolism sensing transcriptional co-repressors which have been implicated in cancer progression due to their involvement in promotion of cell survival, proliferation and migration and epithelial to mesenchymal transition (EMT) (Chinnadurai, 2009).

CtBP was discovered in a yeast-two hybrid screen for identification of co-repressors that interact with oncogene E1A. CtBP are evolutionarily conserved and have two homologs in vertebrates CtBP1 and 2. According to its crystal structure CtBP1 has a C-terminal which lacks a defined structure and most of the post-translational modifications target this domain (Nardini et al., 2006). In the center of the protein is a dimerization domain for self-oligomerization and PXDLS sequence to interact with transcription factors and other epigenetic regulators with the same motif. CtBP can also bind to NAD(H) enabling it to sense cellular metabolism. NADH has been shown to bind to CtBP with higher affinity than NAD+ and increase the dimerization of CtBP (Kumar et al., 2002) (Fig 5).

Oligomerization of CtBP is important for its repressor activity. The crystal structure suggested that dimerization or higher order oligomerization is possible and the biochemical studies over the years demonstrated that NADH-induced CtBP dimerization increased the transcriptional repression of its targets. CtBP has been shown in the same complex with epigenetic regulators such as histone deacetylases (HDAC1,2), co-repsressor complex CoREST and demethylase LSD1 that target transcriptional activation marker H3K4 (Kuppuswamy et al., 2008). CtBP is also recruited by the CSL complex to repress transcription of Notch targets (Nagel et al., 2005; Oswald et al., 2005). A suggested model for how dimerization can facilitate repression is that of one monomer of CtBP binding to PXDLS DNA binding transcription factors and recruiting epigenetic factors with the other.

Comparison of CtBP1 aminoacid sequence with CtBP2 showed the two homologs are highly similar except for N-terminal of CtBP2 which contains a nuclear localization signal that requires acetylation by p300 (Bergman et al., 2006; Kim et al., 2005; Zhao et al., 2006).

CtBP1/2 have been demonstrated to be important for embryonic development due to the phenotypes of their null-mutants. CtBP1 null mutants were viable and could reach adulthood however they were smaller in size they died early. CtBP2 null mutants on the other hand, led to embryonic lethality at E10.5 with neurodevelopmental defects. The double mutant had the most severe phenotype with embryonic lethality before head fold stage (Hildebrand and Soriano, 2002).

CtBP2 was found to be upregulated in embryonic stem cells as opposed to committed progenitors and interact with stem cell marker Oct4 (van den Berg et al., 2010). Besides, CtBP1 interacts with Ikaros (in *D. Melanogaster*) which has been recently shown to regulate temporal identity of neurons in developing mouse cortex (Alsiö et al., 2013). Despite these recent findings, it is not clear what role CtBP play in neural stem cells or stem cells in general.
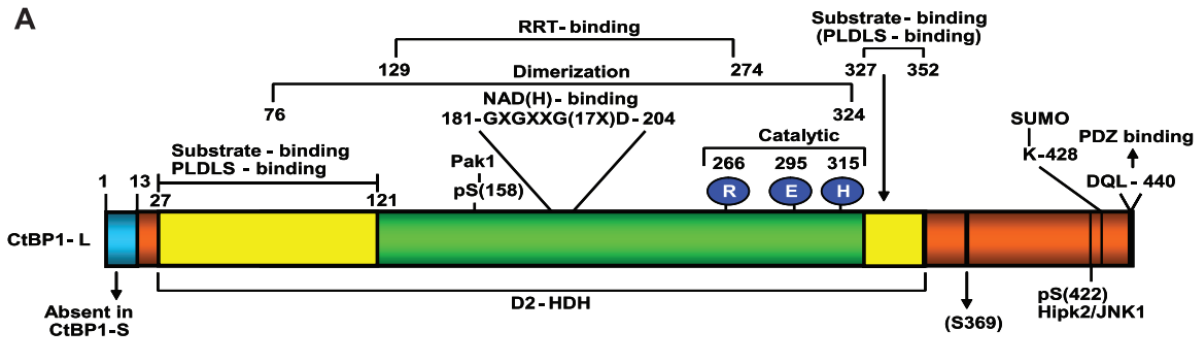

Figure 5. CtBP structure (Kuppuswamy et al., 2008)

2 AIMS

The main aim of this thesis was to elucidate the role of microenvironmental metabolic cues in neural stem cell differentiation in developing spinal cord and cerebral cortex.

Specific aims of the constituent papers are to investigate:

I.   The role of oxygen in neural stem cell differentiation in chick spinal cord.

II. How metabolic signals can influence post-translational modification and oligomerization of redox sensor transcriptional co-repressor CtBP2.

III. Role of metabolic cues in rodent cortical neurogenesis.

3 MATERIALS AND METHODS

For the detailed description of materials and methods please refer to Papers I-II-III. In this section I would like to give additional information relating to proximity ligation assay and the quantification of immunohistochemistry results.

3.1 PROXIMITY LIGATION ASSAY (PLA)

In this thesis proximity ligation assay (PLA) has been used quite often to investigate dimerization and post-translational modifications of CtBP1/2 as well as their interactions with the other proteins. PLA has advantages over more traditional biochemical techniques such as immunoprecipitation since it allows us to visualize the endogenous protein levels, interaction and modifications in a quantitative manner -and most importantly for this thesis- at single cell level (Fig 6) (Söderberg et al., 2006).
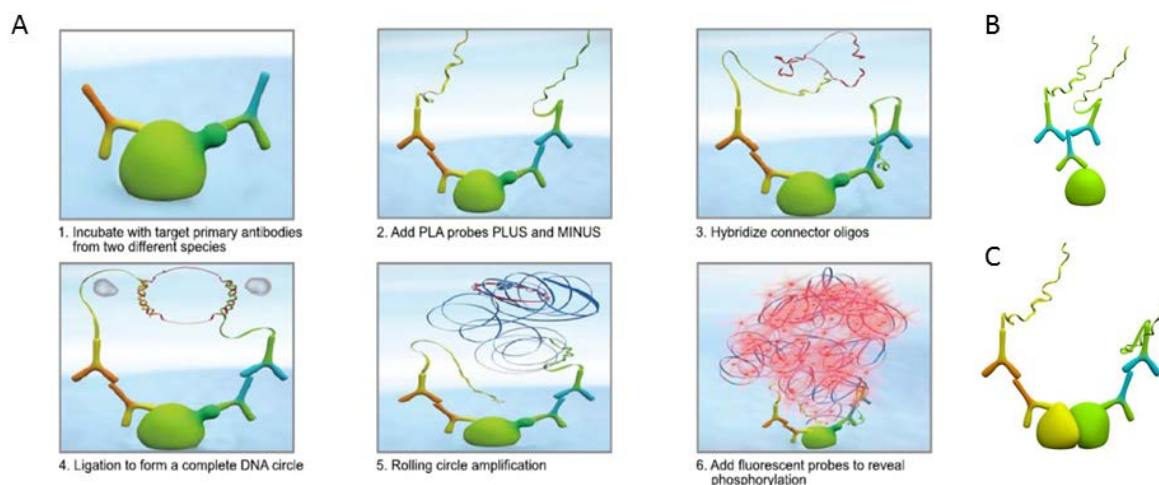

Figure 6. A) Proximity ligation assay protocol for detection of protein modifications. **B)** PLA can also be used for detection of total protein levels called single PLA or **C)** protein-protein interactions and dimerization. (olink.com)

3.2 QUANTIFICATION OF IMMUNOHISTOCHEMISTRY RESULTS

For the analysis of *in utero* electroporation results, we quantified strips of electroporated regions from 3-5 different sections from each brain. The total number of cells was 300-500. (Fig 7)
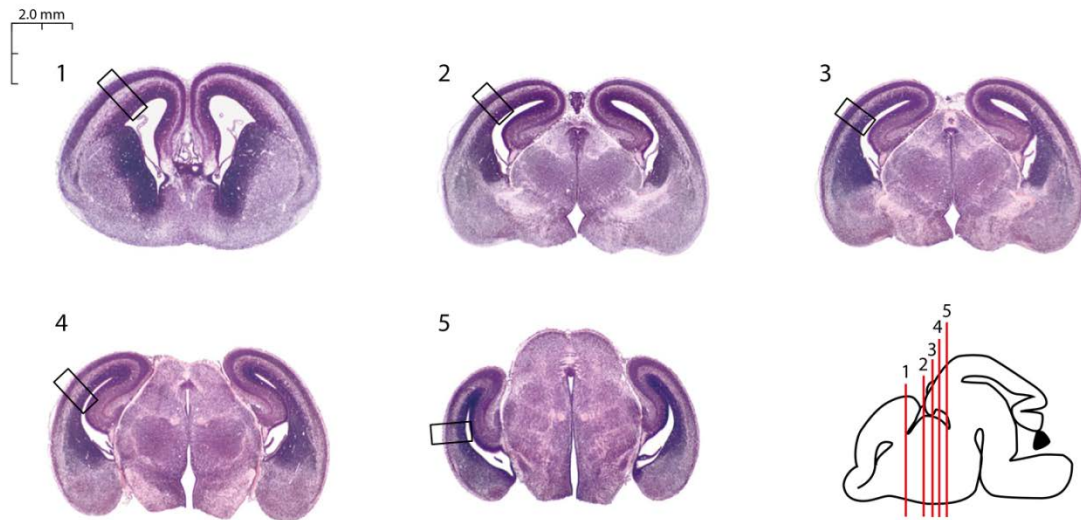

Figure 7. Approximate locations on E18 brain sections used for quantifications of immunohistochemistry images following *in utero* electroporations (Schambra, U. 2008. Prenatal mouse bran atlas)

4 RESULTS AND DISCUSSION

4.1 PAPER I

During vertebrate neural tube development, opposing dorsoventral gradients of BMP and Shh morphogens secreted from roof plate and floor plate respectively, lead to differential integration of these signaling pathways by cells at various coordinates resulting in different cell fates. Although microenvironmental oxygen levels have been shown to regulate the maintenance of stem cells, the role it plays during neuronal differentiation in the spinal cord is not clear.

In this paper, we aimed at elucidating the mechanisms integrating information from BMP signaling and microenvironmental oxygen levels and their resultant effect on neuronal differentiation. For *in vivo* studies we used the chick spinal cord as a model and as *in vitro* counterpart we employed E15.5 rat cortical neural stem cells. In order to determine *in vivo* oxygen levels in developing chick spinal cord, chick embryos were injected with hypoxia marker EF5, which is only stable when oxygen level is lower than 1%, and subsequently performed immunostaining with ELK3 antibody. We found that the oxygen distribution was dynamic in Hamburger Hamilton (HH) 13 and HH17 stages with high oxygen levels (low EF5 immunoreactivity) in the roof plate (RP) and floor plate (FP) and low oxygen levels (high EF5 immunoreactivity) in between, in the middle of the spinal cord. Incubation of chick embryos in 1% oxygen led to increased EF5 stabilization both in the RP and FP, whereas reduced oxygen levels did not lead to a change in the expression of the FP marker Lmx1B. Interestingly, the Notch pathway target Hes1, a transcriptional repressor, was downregulated in RP under hypoxia treatment whereas it remained unchanged in FP. This result suggested that Hes1 is regulated by a combination of BMP signaling and oxygen levels. Previously, it was shown that TGF-β signaling pathway works together with Notch signaling to increase Hes1 expression (Blokzijl et al., 2003) as well as Hif1-α under hypoxic conditions (Gustafsson et al., 2005). However, similar to our results, combination of BMP signaling with hypoxia led to neuronal differentiation in neural crest cells (Morrison et al., 2000). Since Hes1 represses pro-neuronal bHLH transcription factors Mash1 and Math1, we studied the effect of BMP4 treatment under normoxia and hypoxia in NSCs and found that hypoxia led to an increase in the

expression of the neuronal marker Tubb3, whereas under normoxia, BMP4 treatment resulted in increased levels of GFAP and SMA.

Next, we investigated how BMP and oxygen levels are translated into transcriptional regulation of Hes1 and neuronal markers. We hypothesized that evolutionarily conserved NAD(H)-binding transcription co-repressor C-terminal Protein 1 and 2 (CtBP) could be candidates for this role. Indeed, the exogenous expression of CtBP1 protein tagged with a strong viral transactivator domain VP16 as well as RNA knockdown of CtBP1 by siRNA led to an increase in staining of TuJ1 both in NSCs treated with BMP4 as well as the roof plate in the chick spinal cord, a non-neurogenic region. These results suggest that CtBP1 represses neuronal differentiation. To investigate the underlying mechanism, we performed chromatin immunoprecipitation (ChIP) experiments which showed increased CtBP1 binding on Hes1 promoter under hypoxic conditions. This result provides a mechanism for the downregulation of Hes1 expression in the RP under hypoxia and BMP signaling. We further demonstrated that CtBP1 protein binds to Hes1 and these transcriptional regulators could be co-precipitated at promoter regions of the proneural bHLH gene Math1 using ChIP. In addition, RNA knockdown of CtBP proteins by siRNA resulted in an increase in Math1 expression. Proximity ligation assays (PLA) did not reveal any significant changes in CtBP1-Hes1 interaction under different oxygen levels. Our results demonstrate that the transcriptional co-repressor CtBP1 integrates high BMP and high oxygen levels to repress neuronal differentiation in RP of chick spinal cord and in NSCs via its interaction with Notch target Hes1 (Fig 8).
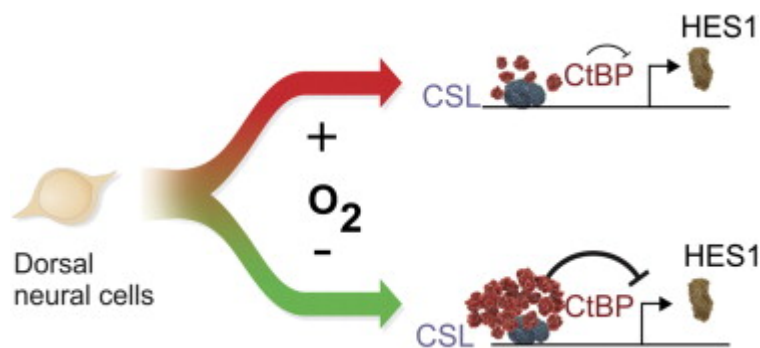

Figure 8. Summary of paper II **(Dias et al., 2014)**

4.2 PAPER-II

The main purpose of this study was to investigate whether CtBP2 protein interacts with another NAD+-binding transcriptional regulator, Sirt1. Since CtBP2 is acetylated by histone deacetylase p300 (Zhao et al., 2006) which has been shown to counteract deacetylase activity of Sirt1 in many proteins such as p53, and since Sirt1 and CtBP2 null mutant phenotypes have similarities including embryonic lethality with neurodevelopmental defects (Hildebrand and Soriano, 2002), we hypothesized that CtBP2 and Sirt1 could be linked functionally, and further that CtBP2 could be a deacetylase target for Sirt1.

Using proximity ligation assay (PLA), we first could demonstrate that endogenous CtBP2 and Sirt1 interact mainly in the nucleus in proliferating NSCs. Next, we asked if different metabolic cues leading to changes in NADH/NAD+ ratio affected the acetylation level of CtBP2. According to PLA results, the total acetylation level of CtBP2 was increased approximately 50% under hypoxia as well as 2-DG treatments, while the CtBP2-Sirt1 interaction however remained unchanged.

To study if Sirt1 regulation was involved in the increased CtBP2 acetylation, we treated NSCs with the Sirt1 inhibitor Ex527 in combination with normoxia and hypoxia. Ex527 treatment did not lead to any further increase in CtBP2 total acetylation in either condition. PLA results demonstrated that the CtBP2-Sirt1 interaction also remained unchanged with Ex527 treatment under normoxia or hypoxia. Sirt1 RNA knockdown led to an approximately 30% decrease in CtBP2 total acetylation levels, but this difference was not statistically significant. In order to confirm that the CtBP2 acetylation results were not influenced from regulation of total protein levels, we performed single PLA and immunoblotting experiments which showed that the total CtBP2 and Sirt1 levels did not change in response to metabolic treatments and Ex527.

Since the results were inconclusive on whether Sirt1 was the deacetylase of CtBP2, we wanted to confirm the suitability of PLA as an approach to study protein acetylation by investigating a well-established Sirt1 target, namely p53. Genotoxic H2O2 treatment for 30 min and 1hr resulted in an upregulation of total p53 acetylation in proliferative NSCs. However, Ex527 did not result in further increase in total p53

acetylation. This may be due to detection of total acetylation levels instead of acetylation of Sirt1 targeted lysines. Interestingly, the effect of H2O2 treatment on total CtBP2 acetylation was the opposite of p53 with approximately 45% decrease. This may indicate that p53 and CtBP2 acetylation maybe regulated by different mechanisms. Notably, PLA signal for single total CtBP2 did not change with H2O2.

Next, we investigated if acetylation of CtBP2 correlated with its dimerization. We employed PLA using mouse and rabbit anti-CtBP2 antibodies directed against overlapping regions on the C-terminal domain to detect CtBP2 homodimerization. Hypoxia led to a small (approx. 25%) but reproducible decrease in PLA detection of CtBP2 homodimers whereas 2-DG had no effect. Sirt1 RNA knockdown also failed to influence CtBP2 homodimerization. The decrease in CtBP2 homodimerization PLA while single PLA remains unchanged may indicate either a shift towards monomers or higher order oligomerization such as tetramers. Based on literature a shift towards tetramers is more likely (Madison et al., 2013).

In conclusion, this study showed that CtBP2 total acetylation levels and homodimerization are regulated by metabolic cues in proliferating NSCs. Although CtBP2 and protein deacetylase Sirt1 interacted in the nucleus of NSCs, we could not conclude whether CtBP2 is a substrate for Sirt1 deacetylase activity.

4.3 PAPER-III

To pursue a deeper investigation of the implications of the results from the chick spinal cord that CtBP links oxygen and BMP levels to neuronal differentiation, we next investigated the role of the other mammalian homolog of CtBP, CtBP2, in the developing cerebral cortex. First we studied the expression of CtBP2 in the cortex and found that CtBP2 is highly expressed at the subventricular zone (SVZ) and the cortical plate (CP) according to immunostaining of E18 mouse cortex.

In order to study cortical development *in vitro*, we studied spontaneously differentiated cortical NSCs derived from E15.5 rat cortices at high density after removal of FGF2. Upon neuronal differentiation, total CtBP1/2 protein levels did not change, while the average PLA signal representing CtBP2 homodimerization per cell was significantly reduced. This reduction in homodimerization was not equal in all

TuJ1-positive cells suggesting that these neurons may have different metabolic state influencing CtBP2 oligomerization. Next, changes in cellular metabolism during NSC differentiation into neurons were investigated. We found that the NADH/NAD+ ratio increased when proliferative NSCs were differentiated for two and four days. In parallel, the high endogenous ROS levels found in proliferative NSCs were downregulated as the cells differentiated. At day two, approximately half of the cells displayed low endogenous ROS whereas the other half with high ROS levels corresponded to cells positive for the early neuronal marker doublecortin (DCX). In order to identify cortical regions with different oxygen tensions *in vivo,* pregnant mice were injected with Pimonidazole (Pimo) which is stable in cells under 1.3 % (Raleigh et al., 1998) or lower oxygen levels, and subsequently performed immunostaining on E17 embryo cortices with a Pimo antibody. Interestingly, the Pimo positive cells constituted a layer running parallel to and overlapping with the marker CtIP2, demarking Layer V. These results demonstrated that neuronal differentiation is associated with cellular metabolism changes in NSCs.

Next, we investigated how external metabolic cues can regulate cell fate decisions in the cortex. NSCs were spontaneously differentiated under normoxia (21%), hypoxia (1%), 2-DG (glycolysis inhibitor), and H2O2 (1uM) treatments. We then subsequently studied the gene expression and protein levels of cortical layer specific markers. We found that upper layer markers Cux1 and Cux2 were upregulated by 2-DG treatment whereas Satb2 was regulated in an opposite manner. Hypoxia led to an increase in the deep layer marker Sox5, whereas Foxp2 expression was decreased, and the layer V marker CtIP2 was increased both with hypoxia and 2-DG treatments. Intriguingly, RNA knockdown of CtBP2 in NSCs either abolished or reversed the effect of metabolic changes on layer markers. To investigate the effect of CtBP2 knockdown *in vivo* we performed *in utero* electroporation of E15 mouse embryos. At E18, we found that lack of CtBP2 led to accumulation of cells in VZ/SVZ and IZ, decreased proliferation in the germinal zones, decreased number of cells in the cortical plate, irregular radial glia morphology and thinner cortical plate. Since proliferative progenitors were shown to be in close proximity to blood vessels (Javaherian and Kriegstein, 2009) and hence high oxygen levels in SVZ, the decrease we observe in

proliferative progenitors may indicate that these cells cannot sense oxygen levels in the absence of CtBP2 and leave their niche prematurely.

In summary, these results show that cortical neural stem cell differentiation is regulated by metabolic changes via CtBP2 oligomerization. Furthermore, CtBP2 is required for maintenance of neural progenitors and proper cortical development *in vivo*.

4.4 CONCLUSION AND FUTURE PERSPECTIVES

This thesis underlines the importance of microenvironmental metabolic signals in neural stem cell differentiation in developing spinal cord and cerebral cortex and presents redox sensor transcriptional co-repressors CtBP1/2 as the bridge between metabolism and cell fate decision making.

To improve upon these results, investigation of blood vessel formations in parallel to hypoxia markers and CtBP oligomerization at early stages of cortical development can help elucidate underlying mechanisms of metabolic regulation of neurogenesis. Several questions remain: Since RGCs were shown to support angiogenesis, does the CtBP2 knockdown affect vascularization in the cortex? Does the localization of progenitors with respect to blood vessels change in absence of CtBP2? Does metabolism or CtBP2 play a role in regulation of temporal identity in the cortex?

5 ACKNOWLEDGEMENTS

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