The role of p130/DREAM in silencing self-renewal genes in post-mitotic neurons.

Joelle Azzi

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirement for the M.Sc. degree in Cellular Molecular Medicine.

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

ABSTRACT

The recently identified DREAM complex assembles when Rb-like protein (p130, p107) recruits E2F4, DP (dimerization partner) and MuvB (multivulval complex B (Lin9, Lin37, Lin52, Lin54, and RbBp4)) during G0 and quiescence to repress cell cycle-dependent genes. DREAM assembly requires phosphorylation of the MuvB subunit Lin52 mediated by Dyrk1a, a kinase that has been linked to Down syndrome and neurodegenerative diseases. Our lab previously demonstrated an essential role for the Rb-like pocket proteins in the regulation of neural precursor population and that E2F4 is also involved in the regulation of the expression of the pluripotent gene Sox2. Here, we performed *in utero* electroporation experiments to overexpress the DREAM complex components and assess their roles during neurogenesis. Our results showed that the overexpression of DREAM components (Lin52 and p130) and Dyrk1a promotes commitment to differentiation at the expense of self-renewal. We also showed that Dyrk1a requires p130 or p107 to regulate neurogenesis. Furthermore, using harmine treatment which is an inhibitor of Dyrk1a the kinase that induces DREAM assembly, our results revealed that DREAM regulates the expression of self-renewal markers affecting the cell fate decision. Performing ChIP experiments, we detected a binding enrichment of the DREAM components on the promoters of not only classical cell cycle genes but on the self-renewal genes like Sox2 and EZH2. Taken together, our study confirmed that DREAM complex plays an important role in the cell fate determination during the regulation of neurogenesis through the control of the self-renewal genes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CDK	Cyclin dependent-kinases
ChIP	Chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
Dcx	Doublecortin
DKO	Double Knockout
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
Dp	Dimerization partner
dREAM	Drosophila RBF, E2f2, and Mip
DREAM	DP, RB-like, E2F and MuvB
DS	Down Syndrome
DYRK	Dual specificity tyrosine related kinase
E	Embryonic day
E1A	Adenovirus early region A
E2F	E2 promoter binding factor
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
Flox	Flanked by LoxP sites
FoxM1	Forkhead box M1
G1	Gap phase 1
G2	Gap phase 2
GFP	Green fluorescence protein

hr	Hour
HDAC	Histone deacetylases
IPC	Intermediate progenitor cells
KO	Knockout
MEFs	Mouse Embryonic Fibroblasts
min	minute
mL	milliliter
MMB	Myb-MuvB
MOI	Multiplicity of infection
M-phase	Mitotic phase
Muv	Multi-vulva phenotype
NE	Neuroepithelial cell
NPC	Neural Precursor Cell
NSC	Neural Stem Cell
OPC	Oligodendrocyte precursor cell
p107	Retinoblastoma like protein 1
p130	Retinoblastoma like protein 2
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
qPCR	quantitative Polymerase Chain Reaction
pRb	Retinoblastoma protein
RbBP4	Rb binding protein 4
RGC	Radial Glial Cell
RT	Room temperature
S28	Serine 28

S28A	Serine 28 to Alanine
SDS	Sodium dodecyl sulphate
Sox2	Sex determining region Y box 2
S-phase	Synthesis phase
SV40	Simian virus 40
SVZ	Sub-ventricular zone
Tbr2	T box brain gene 2
TKO	Triple knockout
VZ	Ventricular zone
WT	Wild-type
µL	Microliter
µm	Micrometer

ACKNOWLEDGMENTS

First of all, I would like to thank God without whom none of this would be possible.

To begin, I cannot thank my supervisor, Dr. Ruth Slack, enough for allowing me the opportunity to pursue my Master of Science, degree Cellular and Molecular Medicine, in her laboratory. I feel privileged for being able to work with you and join your amazing research team. Thank you, Dr. Slack for your guidance and support in helping me coordinate between my graduate studies and family. Thank you for being so kind and patient with me. Your enthusiasm for science and success is contagious, your determination and your ability to make everything look feasible pushed me more towards achieving my goals.

A sincere thank you goes to my thesis advisory committee Dr. Alexandre Blais and Dr. Jocelyn Côté for their valuable insight and advice throughout my degree. I especially like to thank Dr. Blais for giving me the opportunity to learn the ChIP technique in his lab with the help of Dr. Imane Chakroun. I also must thank Dr. Litovchick for providing our lab with the Lin52 antibodies.

I would also like to extend my thanks to all members of the Slack and Park labs, past and present, including Alysen Clark, Dr. Dave Patten, Dr. Delphie Dugal-Tessier, Edward Yakubovich, Jacob Wong, Dr. Lisa Julian, Nikita Larionov, Raghda Gemae, Dr. Ujval Anil Kumar. It has been a pleasure to work side-by-side with you. I especially like to thank Jason MacLaurin for turning the daily challenges in the lab into great experiences. Initially, I was mentored by Bensun Fong. Thank you for your support Ben and for being always there when needed. I am lucky to have been given the privilege to work with Dr.

Devon Svoboda and Dr. Mireille Khacho. To Devon, thank you for a great collaboration in the DREAM project. Thank you for your guidance, encouragement, patience and insightful suggestions regarding my project. Mireille, I am lucky to have met you. Thank you for your continued support, constructive criticisms and countless discussions often during difficult times. Your expertise helped me learn qualitative research methods. I am happy that this relationship does not end with the submission of this thesis. A sincere thank you goes to Dr. Richard Harris for reviewing my thesis.

Finally, I would like to express my gratitude to my amazing parents, sisters, and brother for their permanent support and encouragement.

To my wonderful kids: Rita, Anthony & Bryan thank you for always being a source of joy for me.

And to my amazing husband Fadi: words fail to express how grateful I am for your love and support. Thank you for everything that you do.

This work was funded through CIHR grant to RSS.

INTRODUCTION

The cerebral neocortex plays crucial roles in learning, memory, and cognition. The development of the neocortex is regulated by complex interactions between intrinsic factors and extrinsic cues in a spatial and temporal manner. The normal function of the neocortex is dependent on neurogenesis. Neurogenesis, the birth of new neurons, initiates during development and continues in the adult brain. It is the process by which multiple progenitor populations proliferate to give rise to new functional neurons (McConnell 1995; Bond, Ming, and Song 2015). Neurogenesis is described as a multistep process, beginning with the proliferation of neural precursor cells (NPCs), followed by differentiation and migration. The distinctive feature of a stem cell to self-renew and generate all neural lineage cells in the nervous system has attracted considerable attention as a potential novel therapy to cure the loss of neurons occurring in neurodegenerative diseases and aging.

Highly regulated proliferation/self-renewal of neural progenitor cells (NPCs), differentiation and migration are key steps required for the development of a healthy cortex. Disturbances affecting any of these processes contribute to the symptoms observed in several neurodevelopmental and psychological disorders, which can be grouped under disorders of proliferation, migration and cortical formation and organization (Mochida and Walsh 2004; Guerrini and Parrini 2010; Olson 2014).

At the onset of neurogenesis, NPCs undergo self-renewing symmetric division to generate a sufficient pool of cells for ongoing neurogenesis. Disturbances affecting the symmetric division results in a reduction of the progenitor pool followed by a decrease in proliferation, decrease in the neuron production leading to smaller brain size (Huttner and

Kosodo 2005; Dehay and Kennedy 2007). NPCs divide and differentiate within the ventricular and the sub-ventricular zones (VZ and SVZ respectively). Newborn neurons acquire new morphology while migrating to the cortical plate where they further differentiate (Noctor et al. 2001; Marín and Rubenstein 2003).

During the transition from multipotent proliferative stem cells to differentiated neurons, cellular and environmental factors control the expansion and the differentiation of the NSCs pool. Several factors including cell cycle regulation may contribute to cell fate determination (Shen et al. 2006; Dehay and Kennedy 2007; McClellan and Slack 2007; Vanderluit et al. 2004:107; Julian et al. 2013; Khacho et al. 2016). Previous studies, as well as studies from Slack laboratory, showed that the retinoblastoma Rb protein (pRb) negatively regulates the G0/G1 to S phase transition of the cell cycle (Sun et al. 2007; Vormer et al. 2014). The decision whether a cell continues another round of cell division or exits the cell cycle and differentiates is a critical step for tumor formation/suppression and differentiation. This decision plays an essential role during neurogenesis as this process is regulated in a spatiotemporal manner and also affects the determination of the cell fate.

1.1 Neurogenesis- An overview

The development of the mammalian cerebral cortex is a dynamic and complex process where many developmental mechanisms are involved in driving the generation of broad cellular diversity in the brain. The embryonic forebrain comprises the diencephalon and the telencephalon; the latter gives rise to the basal ganglia and the cerebral cortex, which is composed of the neocortex, archicortex and the paleocortex (Götz and Sommer 2005). Neocortex arises from multipotent neural precursor cells (NPC) and leads to the formation of a proper cortex in a timely manner (Noctor et al. 2004). Neural precursor cells may give rise to two major types of neurons: cortical projection excitatory neurons and neocortical interneurons, which migrate radially and tangentially respectively to reach the neocortex (Anderson et al. 2002). The neocortex is organized into six layers; each layer with a distinct neuronal cell type and a different pattern of connectivity. Disruption during the formation of the neocortex may result in cognitive impairments and neurological disorders (Rubenstein 2011; Manzini and Walsh 2011; Kwan, Šestan, and Anton 2012).

1.1.1 Formation of the six layers.

Following the neural tube closure during gastrulation, a single layer of neuroepithelial (NE) cells generates all neurons and glial cells within the cerebral cortex. Neuroepithelial cells exhibit a powerful capacity to both self-renew and generated different cell types. Neuroepithelial cells self-renew to expand their pool (Kriegstein and Götz 2003; Götz and Huttner 2005; Molyneaux et al. 2007) and display two processes apical and basal allowing the nucleus to undergo interkinetic nuclear migration during the phases of the cell cycle (Noctor et al. 2004; Götz and Huttner 2005).

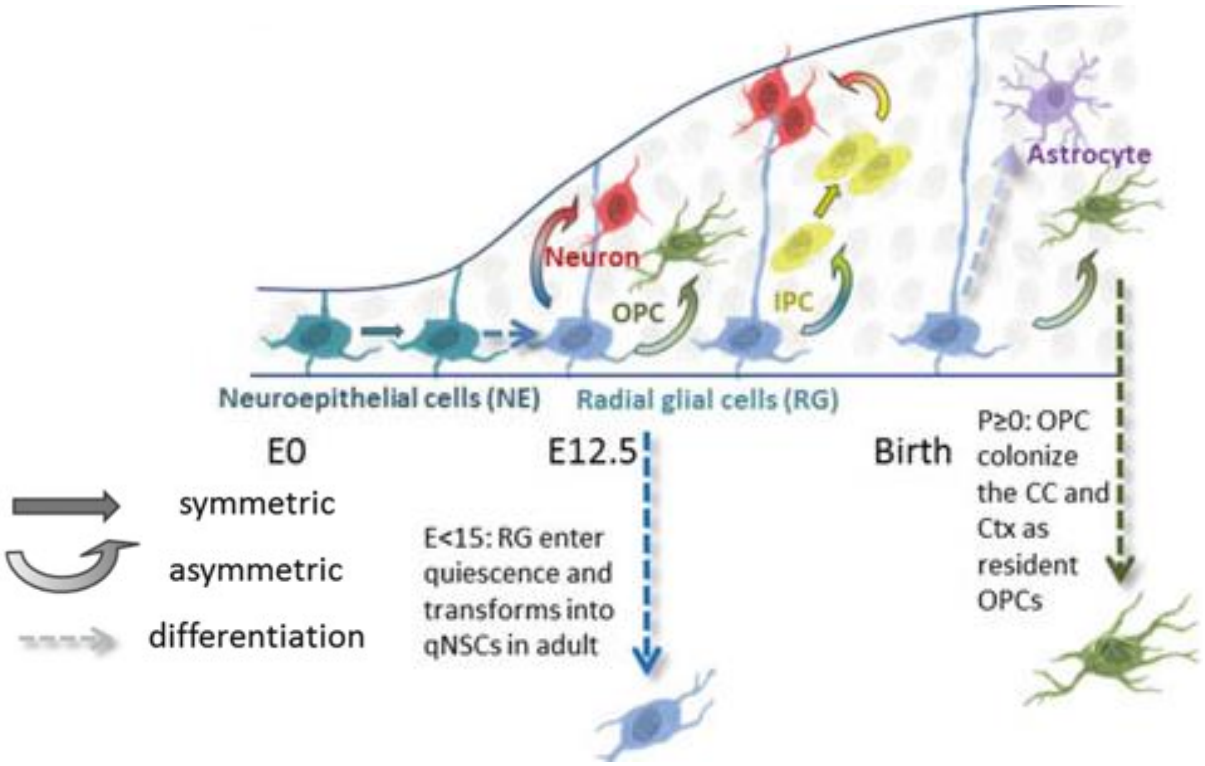


Figure 1 Cell divisions and types during mammalian embryonic neurogenesis.

Before the onset of neurogenesis, neuroepithelial (NE) cells undergo symmetric divisions to expand their pool. As neurogenesis proceeds, NE cells transform into radial glial cells (RGCs). RGCs divide either symmetrically generating two progenitors or asymmetrically to give rise to an oligodendrocyte precursor cell (OPC) and a neuron through intermediate progenitor cell (IPC).
Modified from Mathieu Daynac and Claudia K. Petritsch 2017 Regulation of Asymmetric Cell Division in Mammalian Neural Stem and Cancer Precursor Cells. Results and Problems in Cell Differentiation 61: 375–399.

At the onset of neurogenesis marked by a transition from symmetric to asymmetric division, around E11 in the mouse embryonic brain, neuroepithelial cells undergo asymmetric division and give rise to the radial glial cells (RGCs), which will reside in the ventricular zone (VZ), a specific proliferative area surrounding the ventricles. RGCs then divide asymmetrically to give rise to neurons directly or via generation of neuronal intermediate progenitors cells (IPCs) as shown in Figure 1. As cortical development proceeds, RGCs produce basal progenitors, including basal radial glial cells, transit-amplifying cells, and intermediate progenitors (Lui, Hansen, and Kriegstein 2011; Florio and Huttner 2014). Subsequently, IPCs detach from the apical membrane, migrate dorsally where a new proliferative layer named sub-ventricular zone (SVZ) forms above the VZ (Noctor et al. 2004). IPCs divide symmetrically to generate more IPCs or differentiate into more committed cells (Tamamaki et al. 2001; Noctor et al. 2004; Miyata et al. 2004; Götz and Huttner 2005; Kriegstein and Alvarez-Buylla 2009). IPCs are marked by their expression of Tbr2, which is a T-domain transcription factor (Englund et al. 2005), and divide away from the VZ at non-surface positions to generate basal progenitors. Basal progenitors undergo one to two mitotic cycles before producing postmitotic neurons that migrate in the developing cortex (Dehay and Kennedy 2007). Conversely, newborn neurons deriving from basal progenitors are marked by the expression of Doublecortin (Dcx), a protein that participates in the polymerization of the microtubules. Expression of Dcx, observed in both migrating cells and differentiated neurons, appears to be crucial for proper migration of these cells (Francis et al. 1999). Neuroepithelial cells are considered the earliest progenitors in the brain whereas RGCs are called the principal progenitor type and possess a fate-restricted feature (Haubensak

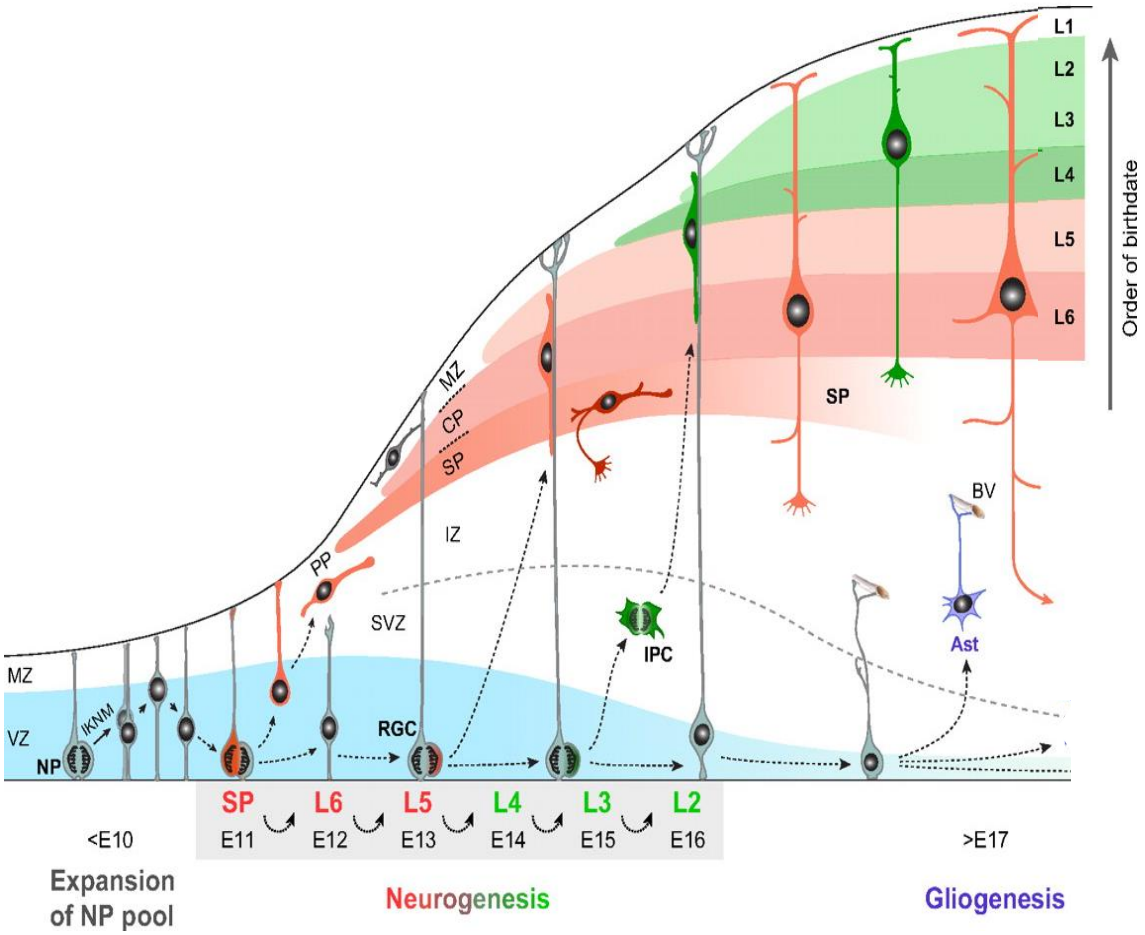


Figure 2 Formation of the six cortical layers of the neocortex.

Corticogenesis occurs in an inside-out manner. Neural progenitors (NP), present in the ventricular zone (VZ) undergo interkinetic nuclear migration (IKNM) and divide symmetrically. The earliest born neurons form the preplate (PP) above the VZ. The PP splits into marginal zone and subplate, forming the cortical plate in between. As neurogenesis progresses, neurons continue to migrate into six distinct layers, with the earliest born neurons residing in the deepest layer.
Modified from Kwan et al. 2012."Transcriptional Co-Regulation of Neuronal Migration and Laminar Identity in the Neocortex." *Development* 139, no. 9 (May 1, 2012): 1535–46. doi:10.1242/dev.069963.

et al.2004; Noctor et al. 2004). Embryonic neurogenesis continues until embryonic day (E) 17.5 in the mouse brain (Manzini and Walsh 2011). Around E18.5, the first astrocytes originating from RGCs appear, following a transition from neurogenesis to gliogenesis (Kriegstein and Alvarez-Buylla 2009).

The first cortical neurons to become postmitotic migrate toward the preplate, which is a layer of differentiated neurons above the VZ. The production of new neurons split the preplate to into the outer marginal zone and the subplate (Kriegstein and Noctor 2004). Newborn neurons radially migrate into the cortical plate along the radial glial fibers where each generation passes one another. This is performed in an "inside-out" manner resulting in a neocortex with six different and distinguishable layers: the firstborn neurons will reside in the most profound layers whereas the latest-born neurons populate the superficial layers of the cortex as shown in Figure 2 (Rakic 1972; Rakic 2009; Kwan, Šestan, and Anton 2012). Neurons residing in different layers do not share the same origin and are generated at different times (Haubensak et al. 2004; Noctor et al. 2004). Premature differentiation resulting from an early transition from proliferative zone towards differentiated layers is believed to confer susceptibility to several developmental abnormalities (Manzini and Walsh 2011).

1.1.2 Cell fate determination.

During neurogenesis, various neural cell types are generated from neural stem cells (Taverna, Götz, and Huttner 2014). The decision, of a cell to maintain its proliferative state, or exit the cell cycle to differentiate or enter into quiescence, is tightly controlled by a series of signals and pathways.

Cellular quiescence or G0 is defined as the reversible arrested cell cycle state

(Coller 2011). Traditionally quiescence was considered a passive state; however, recent studies have revealed that quiescence is an actively maintained state and that quiescent cells are transcriptionally active (Roche, Arcangioli, and Martienssen 2017; Spencer et al. 2013; Cheung and Rando 2013). Entry into quiescence is accompanied by a decrease in the expression of the genes linked to cell cycle progression (Coller 2011). Cellular quiescence has been shown to be functional in preserving the self-renewal capacity of the neural stem cells and prevent their premature differentiation. Quiescent cells may re-enter the cell cycle and subsequently differentiate into several neuronal lineages required for homeostasis or repair. Imbalance or loss of quiescence affects the progenitor populations leading to stem cell depletion.

Many factors and pathways are contributing to the determination of cell fate (Chenn and Walsh 2002; Parras et al. 2002; Molofsky, Pardal, and Morrison 2004). For this thesis, we will describe how the transcriptional and the cell cycle regulation influence the cell fate decisions.

1.1.2.1 Transcriptional regulation influences cell fate decisions.

Among several extrinsic and intrinsic factors, basic helix-loop-helix (bHLH) transcription factors have been considered as critical regulators of the cell fate decision. bHLH transcription factors described as activators such as Mash-1, Neurogenin 1 and 2 and NeuroD (Guillemot 2007) promote differentiation whereas the bHLH repressors such as notch-1and hes-1 (Kageyama et al. 2005) promote self-renewal and proliferation.

Amid the transcription factors that promote the maintenance of pluripotency, the SoxB1 high-mobility-group (HMG) protein family (Sox1-3) play key roles in the regulation of stemness and differentiation during the CNS development (Pevny and

Nicolis 2010; Sarkar and Hochedlinger 2013; Avilion et al. 2003:2). Indeed, SoxB1 factors are expressed in the neural stem and progenitor cells during the embryonic neurogenesis (Bylund et al. 2003). SoxB1 factors have been shown to be involved in maintaining the potency of the progenitor cells and preventing their premature differentiation (Sarkar and Hochedlinger 2013). Neural stem and progenitors cells are characterized by the expression of the pluripotency gene Sox2 (Zappone et al. 2000); they are capable of generating new stem cells and differentiation toward neuronal lineages (D'Amour and Gage 2003; Bani-Yaghoub et al. 2006). Overexpression of Sox2 prevented the differentiation of the progenitor cells while maintaining their uncommitted state. However, the expression of a dominant negative form of Sox2 provokes a premature exit from the cell cycle and differentiation toward neuronal fate (Bylund et al. 2003; Graham et al. 2003). Thus, an exquisite regulation of the different transcription factors is critical as the latter affects the balance between self-renewal and differentiation to generate an appropriate patterned CNS.

Extrinsic factors including multiple signaling pathways such as FGF, Notch, wnt-β-catenin, Sonic Hedgehog (Shh) and BMP (Molofsky, Pardal, and Morrison 2004; Zhang and Li 2005; Guillemot 2005) are involved in the cell fate decision as they may contribute to the maintenance of a proliferative state of the neural stem cells. For instance, FGF is essential in many aspects of the nervous system. Several reports have suggested that the FGF signaling pathway regulates through the D-type cyclins (Lobjois et al. 2004) and the Notch pathway (Rash et al. 2011) the balance between the proliferation and the differentiation of the neural stem cells. Notch signaling mainly notch-1 plays a role in the maintenance of the neural precursor population (Hitoshi et al.

2002). Activated Notch protein releases the notch intracellular domain which will translocate to the nucleus to trigger the expression of transcriptional repressor genes such as hes-1; the latter inhibits the differentiation and maintains the neural stem cell population (Bertrand, Castro, and Guillemot 2002).

1.1.2.2 Cell cycle regulation and cell fate decision.

Recently, several lines of evidence suggest that the cell cycle regulation may be implicated in cell fate determination. It has been shown that the feature of cell cycle exit is critical for the cell differentiation and commitment. These two processes are highly coordinated as they are involved in the generation of the correct number of neuronal lineage (Kintner 2002). In fact, the timing of the cell cycle exit has been linked to the neuronal lineage specification occurring during the G2 phase of the cell cycle (McConnell 1995; Guillemot 2005). Therefore, the commitment towards specific neuronal cell fate is highly linked to the different phases of the cell cycle.

Moreover, the length of the cell cycle, predominantly the length of the G1 phase, is also implicated in the cell fate regulation. Neural precursor cells exhibit an extended G1 phase and a short S-phase when committed to neuronal fate (Arai et al. 2011; Turrero García et al. 2016). The lengthening of the G1 phase occurs during the switch from proliferative to neurogenic divisions (Calegari et al. 2005). These findings highlight a prominent link between cell cycle regulation and cell fate decision.

In summary, the previous section describes the development of the central nervous system where neuronal and glial cell types are generated from a pool of neural stem cells. The development of a well-functioning brain necessitates a tight regulation of the different events including the proliferation of the NSC, cell cycle exit, differentiation,

and migration. Cell cycle regulation is considered a major mechanism of stem cell self-renewal and differentiation. The next section will describe the Rb/E2F pathway, which is a well-known regulator of the cell cycle.

1.2. Rb/E2F Pathway

1.2.1. Pocket proteins-Original discovery

The retinoblastoma susceptibility gene, which when altered or inactivated predisposes to the development of a malignant tumor of the retina, was the first tumor suppressor gene to be identified and cloned from patients with retinoblastoma (Friend et al. 1986; Lee et al. 1987; Fung et al. 1987). Mutations in the gene encoding the retinoblastoma protein (pRb) were associated with diverse human tumors including retinoblastoma, small-cell lung carcinomas and many sarcomas (Friend et al. 1987; Mendoza et al. 1988). Shortly after, pRb was discovered as a target for the DNA tumor viruses including adenovirus E1A, SV40 T antigen and the E7 proteins of human papillomavirus (Whyte et al. 1988; DeCaprio et al. 1988; Münger et al. 1989). Since that time, pRb was considered a central regulator of cell proliferation through the regulation of the cell cycle progression and a tumor suppressor (Yoshikawa 2000; Sun et al. 2007; Weinberg 1995; Dannenberg and te Riele 2006). The next section will provide a brief overview of the Rb/E2F function in cell cycle progression followed by a more detailed analysis of their functions below.

1.2.2 Rb-E2F function and regulation.

The Rb family of pocket proteins: pRb, p130 and p107 are known to interact with the E2F transcription factors, which are considered their main cellular targets

(Chellappan et al. 1991). E2F family of transcription factors consists of nine factors E2F1, E2F2, E2F 3a & 3b, E2F4, E2F5, E2F6-8. Rb represses E2F-mediated transcription (Hiebert et al. 1992) of genes required for both DNA replication such as DNA polymerase, dihydrofolate reductase, and cdc2, and progression through the cell cycle (cyclin A, cyclin E, CDC2, E2F-1, B-Myb, and p107) (Weinberg 1995; Dyson 1998; Takahashi, Rayman, and Dynlacht 2000; Trimarchi and Lees 2002).

One of the best-known functions of the Rb family of pocket proteins is its ability to regulate the G1-S phase of the cell cycle through the control of genes that drive cell division (McClellan and Slack 2006). In G0-G1 phase, hypophosphorylated pocket proteins bind and repress E2Fs (Yoshikawa 2000; Cobrinik 2005; Burkhart and Sage 2008) to form stable complexes (Figure 3). Rb family of pocket proteins may regulate gene expression through E2F in two distinct ways: by interacting and inhibiting E2F-mediated gene expression or by recruiting corepressors to promoters containing E2F sites. Examples of corepressors bound by Rb include histone deacetylase (HDAC), histones demethylases (RBP2) DNA methyltransferases (DNMT1) helicases, histone methyltransferases such as Suv39h1/2, and histone binding proteins like HP1 (Brehm et al. 1998; Magnaghi- Jaulin et al. 1998; Benevolenskaya et al. 2005; Robertson et al. 2000; Singh, Coe, and Hong 1995; Nielsen et al. 2001).

Rb function is regulated by the cyclin-dependent kinase (CDKs) complexes. In late G1, pocket protein is phosphorylated by CDKs, which causes the disruption of the Rb/E2F complex (Buchkovich, Duffy, and Harlow 1989; Cooper and Shayman 2001). Therefore Rb is released, and E2Fs may promote cell cycle progression through the activation of transcription of genes needed for cell cycle progression and S-phase

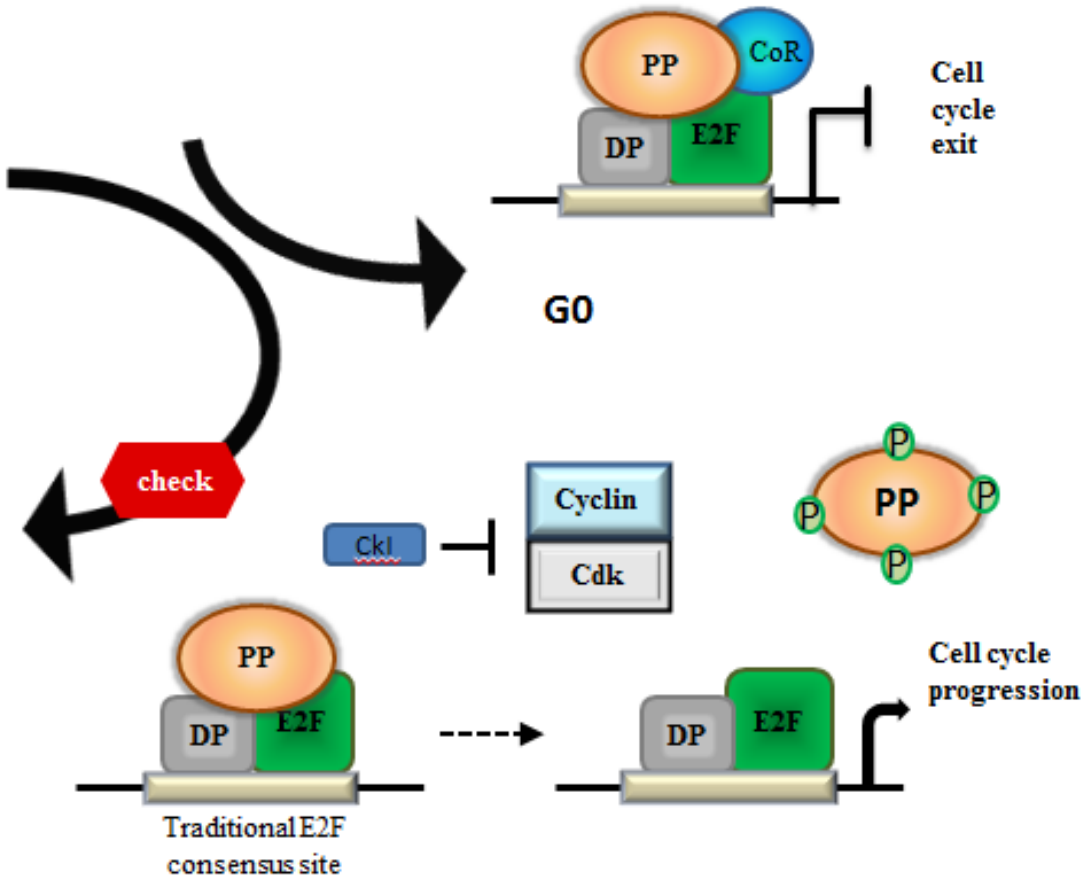


Figure 3 Pocket proteins E2Fdependent regulation during the cell cycle.

The role of Rb-E2F pathway in the control of cell proliferation.
Pocket proteins (PP) repress transcription by at least two mechanisms. PP bind to E2F family of transcription factors and block the expression of genes required for cell cycle progression. In addition, PP can actively repress transcription through formation of repressor complex on the promoters of target genes.
During G1 phase, active hypophosphorylated PP interacts with E2F and their dimerization partner (DP) at the promoter regions of target genes. As G1 progresses, Cyclins/CDK complexes phosphorylate the PP which will be released from E2Fs, allowing the transcription of pro-proliferation genes required for cell cycle progression.
During G0 or post-mitotic state, active repressor complexes are formed, PP recruit co-repressors often acting as chromatin modifiers.
Modified from McClellan & Slack, 2007 Specific in Vivo Roles for E2Fs in Differentiation and Development. Cell Cycle 6(23): 2917–2927.

transition (Chellappan et al. 1991; Dyson 1998; Harbour and Dean 2000). D-type cyclins with their associated kinases Cdk4 and Cdk6 in early G1 and Cdk2 associated with cyclins A and E in late G1 initiate the phosphorylation of Rb upon receiving mitogenic signals (Lundberg and Weinberg 1998; Ezhevsky et al. 2001). Cyclins-cyclin dependent kinases complexes are negatively modulated by Cdk inhibitor family consisting of the Ink family and the Cip/Kip family (Sherr and Roberts 1995). Inactivation of Rb can be repressed by the phosphatase activity; therefore, Rb earns back its hypophosphorylated active state (Nelson and Ludlow 1997).

1.2.3 Pocket Protein family.

As mentioned above, pRb and its close relatives p107 and p130 make up the Rb family of pocket proteins. The three members share a conserved domain by which the pocket proteins function; it is referred as "pocket" region consisting of A and B domains separated by a spacer (Classon and Dyson 2001; Dick and Rubin 2013). The pocket domains mediate the interactions with other proteins including the E2F family of transcription factors, the viral oncoproteins (Nevins 1994; Dick 2007) and a various number of cellular proteins containing a LXCXE peptide sequence which is required for their binding to the pocket proteins at the LXCXE binding site (Dahiya et al. 2000). The LXCXE binding site is not required for the interaction of the pocket protein with E2F; mutations affecting LXCXE domain prevent Rb binding to the cellular proteins such as HDAC1 and 2 whereas Rb interaction with E2F persists intact (Dahiya et al. 2000). All three members are known to bind and regulate E2F transcription factors to control the cell cycle progression (Cobrinik 2005). Loss of pRb results in a shorter G1 phase in mouse embryonic fibroblasts (MEFs) (Herrera et al. 1996), while its overexpression in

some cell types induces G1 growth arrest (Qin et al. 1992). Inactivation of all three pocket proteins in MEFs results in an unrestricted growth; cells progress through G1 at an accelerated rate and fail to arrest cell cycle (Sage et al. 2000).

Despite the fact that all the pocket proteins function in similar ways to repress transcription, they may have different non-redundant functions. This is due to both differential expression patterns of the pocket proteins during the cell cycle, and at different developmental stages and specific binding preferences to E2F. In fact, their protein levels vary during the phases of the cell cycle (Henley and Dick 2012). For example, the level of pRb is sustained throughout the cell cycle and in quiescent cells (Chicas et al. 2010), while the levels of p107 and p130 change dramatically during the different phases of the cell cycle (Shirodkar et al. 1992; Beijersbergen et al. 1995; Mayol, Garriga, and Graña 1996). pRb is expressed in dividing cells as well as differentiated cells like neurons (Callaghan et al. 1999; Ferguson and Slack 2001). p107 accumulates as cells progressed through the cell cycle and is detected in the non-differentiated cells such as the neural precursor cells residing in the ventricular zone of the developing brain (Shin et al. 1995; Vanderluit et al. 2004). In contrast, p130 is detected in G0 quiescent cells, in the differentiated cells including neurons and when the cell is forced to exit the cell cycle. The accumulation of p130-E2F complexes is linked not only to the G1/G0 transition (Cobrinik et al. 1993; Smith et al. 1996; Mayol, Garriga, and Graña 1996) but it appears to play an essential role in G0 phase (Shin et al. 1995).

Pocket proteins differ in their preference for binding to different E2Fs, which suggests that the pocket proteins may regulate some processes uniquely. pRb interacts with E2F1- 4 (Lees et al. 1993), whereas p107 and p130 preferentially form complexes

with E2Fs 4 and 5 (Chen et al. 2002; Hijmans et al. 1995). Several studies revealed that p130-E2F4 complex accumulation coincides with the G1/G0 transition (Mayol, Garriga, and Graña 1996; Takahashi, Rayman, and Dynlacht 2000; Balciunaite et al. 2005). These findings suggest a unique role for p130 in controlling the G1/G0 transition distinctly from the known pRb pathway.

1.2.4 Differential roles of Rb family in both embryonic and CNS development.

The lack of all three pocket proteins results in a more severe phenotype than the loss of a single member which indicates that the pocket proteins may be compensating each other. The absence of pRb, p107, and p130 leads to an embryonic lethality around E9-11 (Wirt et al. 2010) whereas pRb deficient embryos die at E12.5-15.5 (Jacks et al. 1992). p130 and p107 double knockout embryos exhibit bone and cartilage malformations provoking immediate postnatal death (Cobrinik et al. 1996). In contrast, p107 knockout embryos are viable while the lack of p130 causes lethality between E11-13 (Cobrinik et al. 1996; Lee et al. 1996). Embryos lacking pRb and p107 died around E11.5 (Lee et al. 1996), earlier than pRb deficient alone. These findings indicate that the pocket proteins may also have redundant functions.

p107 levels were increased in MEFs lacking either p130 or pRb, whereas pRb and p130 levels are not affected when the other members are absent (Hurford et al. 1997). p130 and p107 share the most structural similarity mainly in their pocket domain. They appear to compensate for each other and contribute to the regulation of a different set of E2F-responsive genes that are distinct from those controlled by pRb (Hurford et al. 1997). Previous work in Slack lab showed that p107 controls the expansion of the stem cell pool and promotes the commitment of neural progenitors to a neuronal fate and its

loss leads to an increase in the expression of the pluripotency factor Sox2 (Vanderluit et al. 2004; Vanderluit et al. 2007; Julian et al. 2013). Furthermore, p130, but not pRb and p107, can arrest cell cycle progression in specific cell lines such as the T98G cells (Claudio et al. 1994). These findings together suggest that the pocket proteins may complement and compensate each other in certain contexts; while in another setting they can have distinct functions and regulate different E2F target genes.

1.2.5 E2F Family of transcription factors.

Among the proteins bound and controlled by the Rb family are the E2 promoter binding factor (E2F) family of transcription factors. E2F was initially identified as the cellular factor required for the transactivation of the E2 viral promoter (Kovesdi, Reichel, and Nevins 1986). The E2F family has been studied extensively in the context of development and cell cycle (Chen, Tsai, and Leone 2009). Among the E2F factors, E2F1-5 are known for the interaction with pocket proteins (Dimova and Dyson 2005), whereas E2F6-8 function in a pocket protein independent manner (Di Stefano, Jensen, and Helin 2003; Christensen et al. 2005). E2Fs are involved in the regulation of expression and timing of numerous genes essential for various cellular processes beyond the S-phase entry, such as proliferation, differentiation, and apoptosis (Weinberg 1995; Müller et al. 2001).

E2Fs are differentially expressed during the different phases of the cell cycle to control cell proliferation. In cycling cells, E2F1-3 are detected at significant levels and bind preferentially to pRb. However, E2F4 and 5 are highly expressed in quiescent cells and are regulated primarily by p107 and p130. E2F4 and 5 may also interact with pRb when cells progress in the cell cycle (Ikeda, Jakoi, and Nevins 1996; Trimarchi and Lees

2002; Bertoli, Skotheim, and de Bruin 2013). E2F expression and activity are tightly regulated during the cell cycle progression. Furthermore, E2Fs have distinct subcellular localization: E2F1-3 are mainly nuclear whereas E2F4 and 5 change their location as they translocate from the cytoplasm to the nucleus (Verona et al. 1997; Müller et al. 1997). Indeed, E2F4 exhibits changes in the subcellular localization in a cell cycle-dependent manner. In quiescent cells, E2F4 is primarily found in the nucleus, whereas the entry into S-phase promotes its shuttle to the cytoplasm. Subcellular localization appears to modulate E2Fs activity (Lindeman et al. 1997).

1.2.6. E2Fs: activators and repressors.

E2F family members regulate the transcription of many networks of genes. E2Fs can be classified as activators or repressors: E2F1, E2F2, E2F3a function as transcriptional activators whereas E2F3b, E2F4 and 5 act as transcriptional repressors (Johnson et al. 1993; DeGregori et al. 1997; Wu et al. 2001).

E2F1, E2F2, and E2F3 form the activator class of E2Fs. The activator class is characterized by the ability to activate the transcription of target genes required for entry into S phase. Activator E2Fs associate preferentially with pRb to inhibit the expression of E2F-regulated genes. Activator E2Fs are expressed in late G1 to early S-phase in continuously cycling cells (Ikeda, Jakoi, and Nevins 1996). Traditionally, E2F1-3 were considered essential for cell proliferation as their loss induced cell cycle arrest (Wu et al. 2001). However, recent *in vivo* studies have shown that cell proliferation can occur in the absence of the E2F activators. E2F1-3 may be dispensable for cell cycle progression in some contexts (Chong et al. 2009; Chen et al. 2009; Wenzel et al. 2011). Thus, through their roles in cell cycle and apoptosis, activators E2F appear to play important roles in

both in tumorigenesis (Chen, Tsai, and Leone 2009) and cell survival.

Repressor E2Fs are known for their ability to repress E2F target gene expression. This thesis focuses on E2F4 and 5 repressors as they share similar mechanisms of repression. E2F4 and 5, in addition to E2F3b, comprise the repressor E2Fs. Although repressor E2Fs are known to function mainly in quiescent (G0) and postmitotic cells (Moberg, Starz, and Lees 1996), they may also function in proliferating cells (Hsu and Sage 2016). The major function of E2F repressor appears to be in the induction of the cell cycle exit and differentiation (Humbert et al. 2000; Gaubatz et al. 2000). E2F4 and E2F5 recruit pocket proteins to E2F-consensus sites of E2F-regulated genes. E2F4 and E2F5 may interact with all three pocket proteins, but preferentially with p130 or p107 (Dyson 1998; Cobrinik 2005). Their expression is maintained during the cell cycle; E2F4 and E2F5 can be found not only in quiescent and differentiated cells but also in stem cells (Sardet et al. 1995; Moberg, Starz, and Lees 1996; Dagnino et al. 1997; Hsu and Sage 2016). E2F4 and 5 can be found in the nucleus during G0/G1, and they are transported in the nucleus after complex formation with the pocket proteins (Müller et al. 1997; Lindeman et al. 1997). These E2Fs are weak transcriptional activators and not able to induce quiescent cells to enter the S-phase (Ikeda, Jakoi, and Nevins 1996; Moberg, Starz, and Lees 1996). E2F4 and E2F5 play essential roles during development. Mice, deficient for E2F4 or E2F5, exhibit several defects in multiple tissues and structures (Lindeman et al. 1998; Humbert et al. 2000; Rempel et al. 2000; Ruzhynsky et al. 2007). Surprisingly, MEFs from mice deficient for E2F4 and 5 can undergo cell cycle arrest following serum deprivation (Gaubatz et al. 2000; Humbert et al. 2000). Thus, E2F family of transcription factors have context-dependent roles in the regulation of cell fate

decision and cell proliferation.

1.2.7 Roles of E2F4 in diverse cellular functions.

Studies from Slack laboratory revealed that E2F4 has a cell cycle-independent role, as E2F4 can regulate sonic hedgehog (Shh) in the ventral telencephalon, which controls the self-renewal of neural precursor cells (NPCs) (Ruzhynsky et al. 2007). Besides, E2F4 may contribute to the regulation of cell fate decisions of stem cells through the control of pluripotency factors like Sox2 (Kareta et al. 2015; Li et al. 2012). Furthermore, chromatin immunoprecipitation (ChIP)-on-chip experiments showed that E2F4 is highly enriched on a set of genes associated with the cell fate decisions of NPCs such as Sox2, EZH2 and members of the Notch/Hes (Julian et al. 2016).

Different E2F complexes accumulate during the phases of the cell cycle and recruit chromatin remodeling factors to targeted genes (Moberg, Starz, and Lees 1996; Hurford et al. 1997). During quiescence G0, p130-E2F4 complexes are the most abundant among other complexes due to the upregulation of p130 and E2F4 at this stage of the cell cycle (Coller, Sang, and Roberts 2006; Takahashi, Rayman, and Dynlacht 2000; Balciunaite et al. 2005). Abundant p130-E2F4 complexes, with low levels of histone acetylation, appear to be associated with the repression of E2F target genes during quiescence or G0 (Takahashi, Rayman, and Dynlacht 2000). In contrast, p107-E2F4 complexes are formed to repress gene transcription in cycling cells (Balciunaite et al. 2005). Because of the accumulation of E2F4 or E2F5-p130 complexes in quiescent cells, together with the fact that many if not all of the E2F target genes are subject to E2F-dependent repression in quiescent cells, it has been suggested that these E2F-p130 complexes function primarily to repress the expression of these target genes and may be

implicated in the maintenance of quiescence.

The relative importance of the Rb/E2F pathway correlates with its multifaceted fundamental role in both the regulation of cell cycle progression and tumorigenesis. This pathway is involved in several cellular processes including cell proliferation, differentiation and cell death and its misregulation results in uncoordinated cell cycle progression. However, a unique role for the p130/p107 members of the Rb family has been suggested through a novel pathway. The next section discusses the formation of a multi-subunit complex called DREAM. This complex appears to be involved in the regulation of numerous cell cycle genes (Litovchick et al. 2007).

1.3. Overview of DREAM complex.

As discussed above, the Rb/E2F pathway plays fundamental roles in regulating cell cycle gene expression during the different phases of the cell cycle especially during the transition to S phase. Recently, genetic studies done in *C. elegans* and *Drosophila* identified a novel Rb related repressor complex involved in the regulation of the G0/G1 phase known as the DP, Rb-like, E2F, and MuvB (DREAM) and the Myb-MuvB (MMB) complexes (Korenjak et al. 2004). The DREAM complex is thought to be the master regulator of the cell cycle (Sadasivam and DeCaprio 2013). The DREAM complex is highly conserved from *C.elegans* to human (Korenjak et al. 2004; Schmit et al. 2007; Litovchick et al. 2007). In mammalian cells, the DREAM complex consists of dimerization partner DP, an Rb like protein (p130 or p107), E2F4 or 5 transcription factors, and five MuvB proteins homologous to products of the *C.elegans* synMuvB group of genes (Lin9, Lin37, Lin52, Rbbp4 (Lin53) and Lin54) (Litovchick et al. 2007) (Figure 4).

It was initially believed that all three pocket proteins may associate with the other components to form DREAM (Gagrica et al. 2004; Osterloh et al. 2007; Pilkinton, Sandoval, and Colamonici 2007). However, recent studies done in humans revealed that p130 and p107, but not pRb, are involved in DREAM complex assembly (Litovchick et al. 2007; Guiley et al. 2015). In fact, only p130/p107 can be recruited to bind the MuvB core due to a weak affinity with pRb. In mammalian cells, the DREAM complex binds to E2F-responsive promoters repressing the expression of cell cycle genes during G0 and G1 as shown in Figure 4 (Litovchick et al. 2007; Sadasivam and DeCaprio 2013).

A recent study suggested a role for DREAM complex during development. For instance, DREAM-deficient mice exhibited defects in bone development leading to postnatal death (Forristal et al. 2014). Upon receiving mitogenic stimulation, cyclin-dependent kinases phosphorylate the pocket proteins which dissociate from the complex enabling the MuvB core to associate with B-Myb to form Myb-MuvB complex (Korenjak et al. 2004; Litovchick et al. 2007; Sadasivam and DeCaprio 2013; Guiley et al. 2015). Therefore Myb-MuvB complex promotes gene expression and can associate to FoxM1 transcription factor (Sadasivam, Duan, and DeCaprio 2012; Sadasivam and DeCaprio 2013). During quiescence or G0, the complex formed consists of either p130 or p107, E2F4 and the MuvB core and function as a repressor of E2F-responsive genes. However, during S phase, Myb-MuvB core is assembled and contributes to the activation of genes required for the late phase of the cell cycle (Figure 4).

The MuvB core consists of five proteins: Lin54, Lin37, Lin52, Lin9 and RbBp4 (Litovchick et al. 2007) (Figure 4). Little is known about the functions of these proteins. In *C.elegans*, this set of genes controls vulval development. Increased activation of these

genes results in a multi-vulva phenotype (Muv). In mammalian DREAM, Lin52 plays a pivotal role during DREAM assembly, as it is required to interact with p130 to enable its assembly (Guiley et al. 2015). RbBp4 (Rb binding protein) is traditionally associated with histones (van den Heuvel and Dyson 2008). Lin54 binds DNA (Schmit, Cremer, and Gaubatz 2009) and appears to be involved in directing the binding of MuvB to the cell cycle genes homology regions (CHR) (Schmit, Cremer, and Gaubatz 2009:54; Müller et al. 2014), which is found in the promoters of cell cycle-regulated genes. CHR is thought to be involved in gene repression in G0 and early G1, and gene activation in G2 and M phases (Müller and Engeland 2010; Müller et al. 2012). The MuvB core may associate with p130, E2F4-DP to repress the expression of the cell cycle genes. It may also associate with BMyb and FoxM1 to coordinate mitotic gene expression during S phase and G2/M, as illustrated in Figure 4 (Osterloh et al. 2007; Sadasivam and DeCaprio 2013).

1.3.1. DREAM complex assembly and regulation

Litovchick et al. revealed that DREAM components share overlapping binding enrichment in G0-arrested cells. These components appear to regulate cell cycle genes as they are enriched close to the transcription sites of these genes (Litovchick et al. 2007). Depletion of DREAM components results in a de-repression of cell cycle genes. Thus, p130, E2F4 and MuvB core assembled as DREAM complex which acts as a repressor of cell cycle genes during quiescence.

The same group also revealed that DREAM assembly requires interaction between Lin52, member of the MuvB core, and the pocket protein p130 (Litovchick et al. 2011). Lin52 was found phosphorylated on Serine 28 when co-immunoprecipitated with

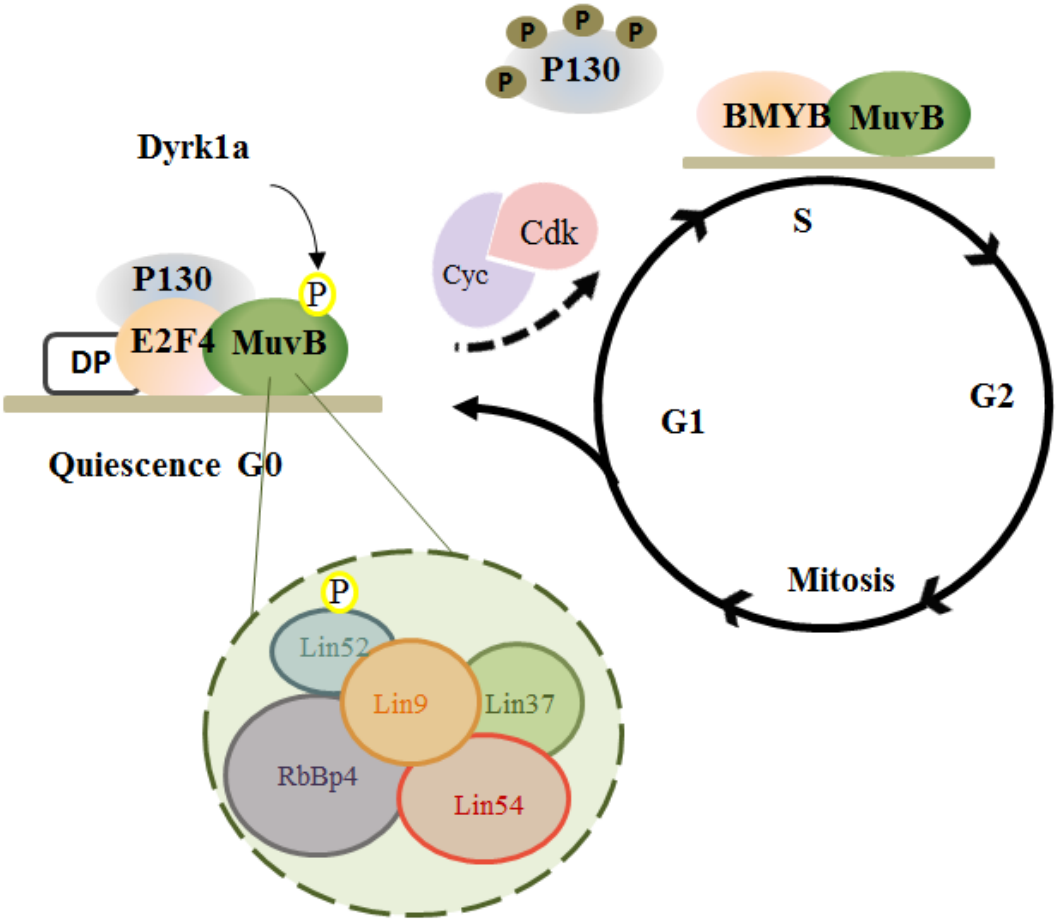


Figure 4 Possible mechanisms of transcriptional regulation through DREAM complex.

During quiescence G0 state, p130 interacts with E2F4, DP and the MuvB core in order to form DREAM complex. DREAM assembly is promoted by Dyrk1a phosphorylation of Lin52, member of the MuvB core, while disassembly is induced when cyclins/CDK complexes phosphorylate p130. In S phase, MuvB core binds to BMYB and form MMB complex involved in cell cycle progression.
Adapted from Sadasivam and DeCaprio, 2013 and Guiley et al, 2015.
The DREAM Complex: Master Coordinator of Cell Cycle Dependent Gene Expression. Nature Reviews. Cancer 13(8): 585–595.
Structural Mechanisms of DREAM Complex Assembly and Regulation. Genes & Development 29(9): 961–974.

p130. However, no interaction was detected between p130 and the mutant Lin52S28A; the Serine 28 was mutated to Alanine. A high level of the Lin52 phosphorylated form characterizes the quiescent state; an elevated level of the Lin52 phosphorylated form was detected in quiescent cells contrary to cells in G1, G2 and S phases (Litovchick et al. 2011). Therefore, phosphorylation of Lin52 on Serine 28 is considered a pivotal event to enable DREAM assembly during G0.

Litovchick and coworkers also identified the kinase involved in Lin52 phosphorylation. Dual-specificity tyrosine-(Y)-phosphorylation Regulated Kinase 1A (Dyrk1a) phosphorylates specifically Lin52 on its Serine 28 to enable DREAM assembly (Litovchick et al. 2011). Furthermore, inhibition of Dyrk1a kinase activity reduces the level of phosphorylated form of Lin52, prevents the interaction of p130 and Lin52, and prohibits DREAM assembly. Thus, DREAM disassembly through the inhibition of Dyrk1a function prevents the cells from entering the quiescence state. During cell cycle re-entry, the DREAM complex disassembles and dissociates from the promoters of genes required for the G1/S progression. DREAM complex disassembly is mediated through the cyclin-dependent kinases which will phosphorylate p130. Subsequently, p130 separates from E2F4 and the MuvB core (Guiley et al. 2015).

The MuvB core then associates with the BMyb, forms the MMB complex involved in the activation of mitotic genes. Based on the fundamental function of these complexes during the cell cycle, any imbalance between the DREAM and the MMB complex may contribute to cell cycle defects. Increased MMB activity may be associated with uncontrolled cell proliferation. In fact, high levels of BMyb are observed in tumors (Musa et al. 2017:2).

DREAM complex functions are best understood in the oncogenesis field, however little is known about its role in the mammalian brain. Failure of a cell to enter quiescence and repress mitotic genes may contribute not only to defects in proliferation but also in self-renewal and cell fate decisions. During neurogenesis, the cell cycle is highly regulated, as the precise timing may affect the cell fate decision to cycle or differentiate. Premature exit of the cell cycle or extended proliferative state confer susceptibility to several developmental disorders.

1.4. Dyrk1a regulates DREAM assembly.

Given that Lin52 phosphorylation by Dyrk1a is a crucial event for DREAM assembly (Litovchick et al. 2011), the function of Dyrk1a will be further described. Dyrk1a belongs to the DYRK family of kinase consisting of five mammalian members. Among those five kinases, Dyrk1a was extensively characterized; it is a dual specificity protein kinase that catalyzes the phosphorylation of serine and threonine residues on its substrates (Becker et al. 1998) as well as its autophosphorylation (Kentrup et al. 1996; Himpel et al. 2001).

Dyrk1a is activated through autophosphorylation on a tyrosine residue in the activation loop. Subsequently, Dyrk1a acquires an activated conformation, whereas dephosphorylation leads to a loss of its activity (Becker and Sippl 2011). Several synthetic compounds were developed to target other kinases and also appear to be efficient on Dyrk1a, such as INDY and Purvalanol A. Natural compounds derived from plants are also available and inhibit Dyrk1a activity, including epigallocatechin-gallate (EGCG) and harmine. Harmine a β-carboline alkaloid extracted from plants can inhibit Dyrk1a specifically among other kinases (Becker and Sippl 2011).

The Dyrk1a gene is located within the Down syndrome (DS) Critical region (DSCR), which is the minimal region of chromosome 21 that, when found in triplicates, generates DS phenotypes (Delabar et al. 1993; Korenberg et al. 1994; Becker and Joost 1999). Over- or under-expression of Dyrk1a levels result in phenotypes observed in DS individuals; thus Dyrk1a function is dosage dependent. Furthermore, Dyrk1a expression may be enhanced by the overexpression of E2F1 transcription factor (Maenz et al. 2008).

Among those genes located within the DSCR region, Dyrk1a is considered a strong candidate for the developmental, cognitive and neurodegenerative phenotypes exhibited in DS (Hämmerle et al. 2003; Tejedor and Hämmerle 2011; Dierssen and de Lagrán 2006). Dyrk1a was identified as the mammalian homologue of the *Drosophila* minibrain kinase involved in neurogenesis (Tejedor et al. 1995). Moreover, recent findings have associated Dyrk1a to several neurodegenerative diseases such as Alzheimer's and Pick diseases (Ferrer et al. 2005).

Dyrk1a is involved in brain development and has a tightly regulated expression pattern. Dyrk1a is expressed briefly before the transition from symmetric to asymmetric divisions and maintained at lower levels in neural progenitors. Dyrk1a level is upregulated in newborn neurons, downregulated during migration, and again expressed when the neurons differentiate (Fernández-Martínez, Zahonero, and Sánchez-Gómez 2015). These findings support that Dyrk1a function as an inhibitor of cell cycle progression. In fact, Dyrk1a is inherited by only one of the daughter cells (Hämmerle et al. 2002). Yang et al. showed that overexpression of a kinase-deficient Dyrk1a attenuated neurite outgrowth (Yang, Ahn, and Chung 2001). Yet, Dyrk1a overexpression inhibits proliferation and induces premature neuronal differentiation of neural progenitor cells

(Park, Song, and Chung 2009; Yabut, Domogauer, and D'Arcangelo 2010). Thus, a tightly regulated expression of Dyrk1a is required for normal neurogenesis.

Given that the pocket proteins p107/p130 are key components in the DREAM complex, studies from Slack lab examined their functions in depth. Unpublished data revealed that the absence of all three pocket proteins results in upregulation of Sox2, EZH2 and PCNA expression. Cortical neurons from Rbflox/flox and triple knock out (TKO) (Rbflox/flox, p107-/- and p130flox/flox) animals were cultured and infected with lentivirus expressing GFP as control and Cre to knock out the expression of Rb and p130. When Rb was knocked out, we observed an increase in the level of p130. However, when all three pocket proteins were absent, we noticed an upregulation in Sox2, EZH2 and PCNA levels (Figure 5). These findings revealed a potential involvement of p130 and p107 in the regulation of self-renewal genes.

ChIP-on-chip experiments revealed that E2F4 binds and regulates several genes involved in the regulation of cell fate decision and differentiation (Julian et al. 2016). Furthermore, preliminary ChIP experiments performed in our lab on NPCs and post-mitotic neurons have shown an expansive role of the DREAM complex as shown in Figure 6. We observed that DREAM complex members including E2F4, p130, p107, Lin9 and RbBp4 share an overlapping binding enrichment not only at the promoters sites of classical cell cycle genes known as DREAM target such as CycB2 but also at promoters sites of self-renewal genes such as Sox2 and EZH2. Therefore, the DREAM complex may be involved in the regulation of self-renewal genes. Further, direct protein immunoprecipitation using neurons was performed using specific antibodies against p130 and Lin52. This immunoprecipitation revealed an interaction between p130 and Lin52

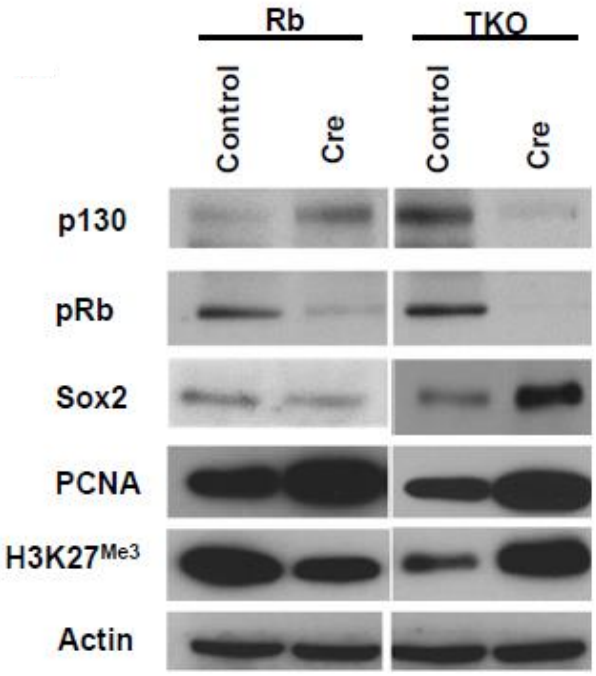


Figure 5 Absence of all pocket proteins results in upregulation of Sox2, EZH2, PCNA and H3K27^{Me3}.

Cortical neurons cultured from Rb$^{flox/flox}$ and TKO (Rb$^{flox/flox}$, p107$^{-/-}$, p130 $^{flox/flox}$) were treated with control (pWPXLD-GFP) or Cre (pWPXLD-CreGFP) expressing lentivirus. An increase in the level of Sox2 was detected in the cortical neurons derived from TKO, which is not the case in the Rb KO.
Andrusiak and Slack (Unpublished)

as shown in Figure 7. p130 interacts with Lin52, member of the MuvB core, in cortical neurons. This interaction between p130 and Lin52 is a key event in order to assemble DREAM (Litovchick et al. 2007; Guiley et al. 2015). Based on all these data together, we hypothesize that DREAM complex is not only essential for silencing cell cycle genes but also for regulating self-renewal genes which will influence the determination of cell fate.

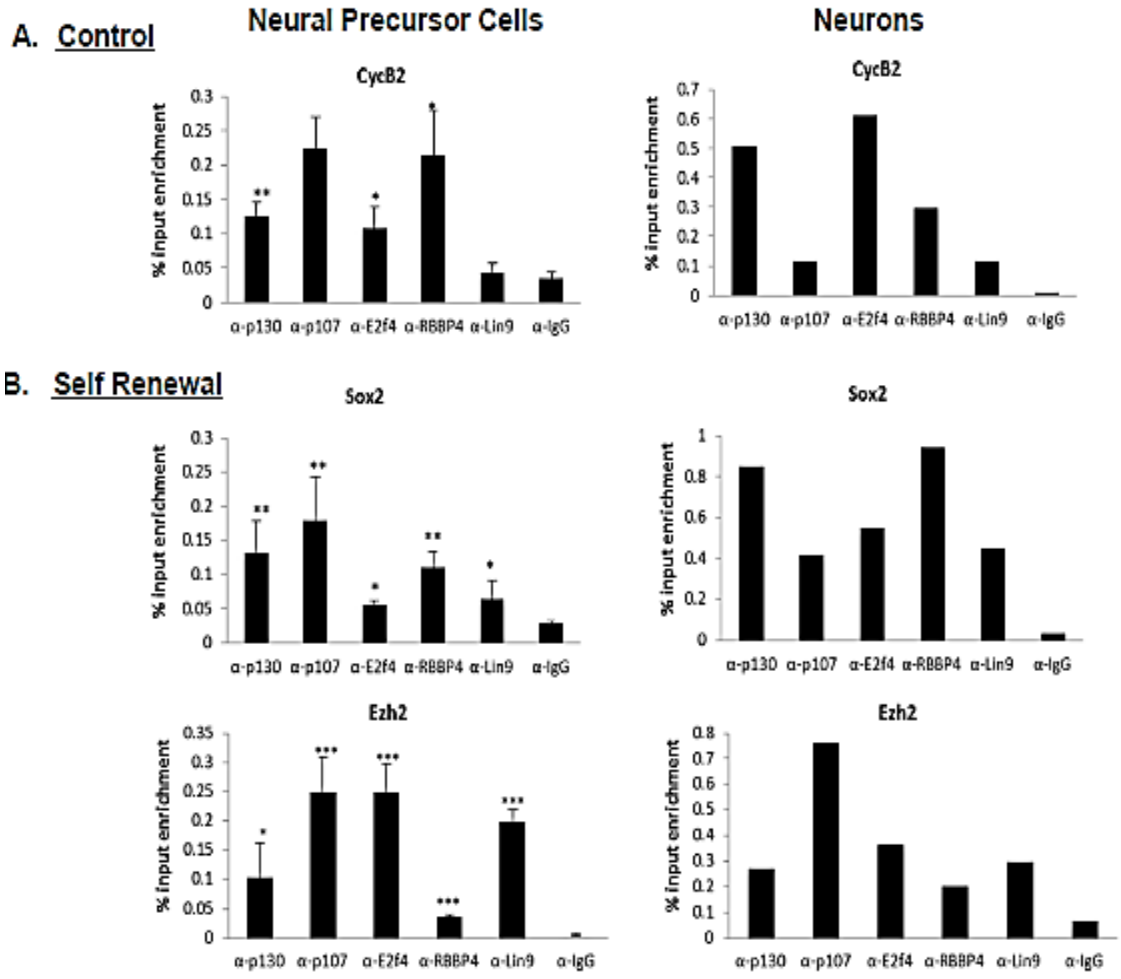


Figure 6 Chromatin Immunoprecipitation showing enrichment of DREAM components on promoters.

ChIPs were performed on neural precursor cells (n=3) and neurons (n=1).
Binding enrichment of DREAM components p130, p107, E2F4, RBBP4 and Lin9 on the promoters of known DREAM target like CycB2. **B.** Self-renewal represents binding enrichment of DREAM components on novel targets such as the self-renewal gene Sox2 and Ezh2. IgG is considered the negative control.
Julian and Slack Unpublished.

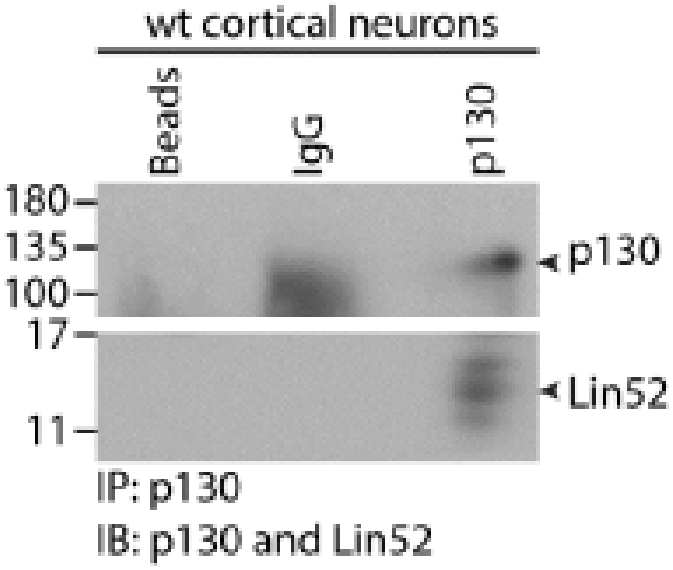


Figure 7 p130 interacts with Lin52, member of the MuvB core in cortical neurons.

Direct protein immunoprecipitation were performed using cortical neurons. Lysates from cultured neurons were incubated with p130 antibody to pull-down and isolate the p130 protein. The isolated protein was then probed with a specific Lin52 antibody. Two bands were observed in the column corresponding to p130 immunoprecipitation indicating an interaction between p130 and Lin52. P130 interacts with Lin52 in cortical neurons. Khacho and Slack Unpublished.

1.5. Rationale and Hypothesis

All of the above studies suggest a potential role for the p130/E2F4 complex in the regulation of neurogenesis. We hypothesize that the p130-mediated DREAM complex is involved not only in the repression of classical cell cycle genes but also in self-renewal genes, such as Sox2, whereby changes in the expression of self-renewal genes due to DREAM activity may lead to a change in cell fate. The proposed studies will test this hypothesis.

Hypothesis:

p130/ p107 mediated recruitment of the DREAM complex is crucial not only for silencing cell cycle genes but also in regulating self-renewal genes leading to change in cell fate decision.

To test this hypothesis, I have two specific objectives:

Objective 1: Characterize the function of DREAM components on cell fate decisions in neural precursor cells *in vivo*.

Objective 2: Molecular assessment of DREAM co-repressors in p130-mediated silencing of E2F target genes in NPCs and post-mitotic neurons and assess the impact on differentiation.

MATERIALS AND METHODS

2.1. Mice

p130/p107 double knockout (DKO) mice were originally generated by Dr. Julien Sage (Wirt et al. 2010) then outcrossed to C57B6. Mice were maintained on a mixed FVBN/C57/S129 background. p130flox/flox; p107-/- were generated by crossing a male p107-/-; p130flox/flox with a female p107+/-; p130flox/flox and genotyped according to previously published protocols (Lee et al. 1996; LeCouter et al. 1998). For embryonic time points, the time of plug identification was counted as embryonic day 0.5 (E0.5). All experiments were approved by the University of Ottawa's Animal Care ethics committee, adhering to the Guidelines of the Canadian Council on Animal Care. CD1 mice were used for *in utero* electroporation (IUE) experiments and were purchased from Charles River Laboratories, Wilmington, MA.

2.2. *In utero* electroporation

The *in utero* electroporation (IUE) experiments were performed as previously described (Svoboda et al. 2015). Pregnant mice at E13.5 were anesthetized with 2-5% isoflurane gas with 1 L/min O2 and their uterine horns were exposed. A plasmid DNA solution (2 μg/μl of vector DNA of interest co-injected with a reporter) was injected through the uterine wall into the lateral ventricle of each embryo. For better visibility, 0.1% Fast Green was mixed with the DNA solution. After plasmid injection, electroporation was performed by discharging five pulses at 30 V with 950 ms intervals using a tweezer-type electrode encapsulating the embryonic head. Embryos were placed back into the abdominal cavity, allowing the embryos to develop to the desired stage in the pregnant mice. The abdominal cut was carefully and aseptically sutured back.

Dyrk1a, Dyrk1a-K188r, Lin52, Lin52S28A cDNAse were gifts from Dr. Litovchick and Dr. DeCaprio (Litovchick et al. 2007; Litovchick et al. 2011). All cDNAse were subcloned into a pCIG2 vector that we modified from the pCIG vector (kind gift of Carol Schuurmans) which contains a (cDNA)-IRES-EGFP expression cassette expressed under the control of a CMV-enhancer and a chicken β-actin promoter (Hand et al. 2005).

2.3. Tissue fixation and cryosection

Pregnant mice were euthanized 48 hours following the IUE, with an injection of 1 mg/g Euthanyl (Supplied by Animal and Veterinary Care Services, University of Ottawa) followed by cervical dislocation. Then, embryos were dissected out and euthanized by decapitation. Heads were fixed overnight in 4% paraformaldehyde (PFA) (pH 7.4) dissolved in Phosphate Buffered Saline (PBS). Heads were then washed in 1% PBS, cryoprotected using 20% sucrose and 0.01% sodium azide in 1% PBS solution, embedded using Tissue-Tek OCT Compound (Sakura) and frozen using dry ice. Brains were cut coronally at 14μm and mounted onto Superfrost Plus slides (Fisher) then stored at -80°C for immunohistochemistry.

2.4. Immunohistochemistry

Immunohistochemistry was performed as previously described (Svoboda et al. 2015). Mouse brains at E15.5 were sliced into 14 μm coronal sections. Slides were left to dry at room temperature, then were washed once in 1% PBS to remove the OCT compound. Before antibody incubation, antigen retrieval was performed when necessary, by heating sections at 75^{o}C for 15 minutes in 0.01 M citric acid solution (pH 6) (Hevner et al. 2004). Slides were then rinsed three times with 1% PBS then incubated overnight at 4^{o}C in primary antibody diluted in 0.1% Triton, 0.1% Tween in 1% PBS solution. Slides

were then rinsed three times with 1% PBS and incubated at room temperature for 45 minutes with the appropriate secondary antibodies diluted in 0.1% Triton, 0.1% Tween in 1% PBS. Tissue sections were then treated for 5 minutes with 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Sigma) in 1% PBS followed by three washes in 1% PBS. Finally, the slides were coverslipped with Immunomount (Genetex) and imaged using Zeiss confocal microscope and Zen imaging software.

2.5. Primary culture

The cortices of E14.5 mice embryos were dissected out from CD1 embryos as described previously (Fortin et al. 2001). After removal of the meninges, cortical tissue was dissociated with trypsin for 20 minutes at 37^{o}C, treated with DNase then triturated. Cortical neurons were resuspended in pre-warmed Neurobasal media (Gibco) supplemented with 2% B27 (Gibco), 0.6mM L-Glutamine (Gibco), 1% N2 (Gibco), 1% Pen-Strep (Sigma). Cortical neurons were seeded in 0.01 mg/ml poly-D-Lysine (BD-Bioscience) coated glass coverslips for immunocytochemistry or on culture plates for Western-blotting and chromatin immunoprecipitation (ChIP) experiments. Cultures were kept for five days in a 5% CO_2 humidified incubator at 37°C.

For the harmine treatment experiment, harmine (Sigma-Aldrich 286044-1G) was dissolved in DMSO at 100mM. At the time of platting, cells were treated with DMSO and harmine at 5µM.

2.6. Immunocytochemistry

Neurons cultured on glass coverslips were fixed with 4% PFA for 20 minutes at room temperature (RT) then washed with PBS. Primary antibodies were diluted in a blocking solution (1% Triton X100 and 1% bovine serum albumin in PBS) to

	Genotyping primers
p130	p130-5 5'-GTG TTG TAA CAT TCT CGT GGG-3' p130-3 5'-GAC TGC TGG TAT TAG AAC CC-3'.
P107	p107-WT 5'-CAT GAA CAG ACT TGT CAT TCC AC-3' p107- NEO 5'-GC ACG AGA CTA GTG AGA CGT GC-3' p107-COMMON 5'-TCG CTG GCA GTC TGA GTC AG AG-3'.
	qPCR Primers
Sox2	Sox2-FOR 5'-CCC ATT TAT TCC CTG ACA GC-3' Sox2-REV 5'-CTC TTC TTT CTC CCA GCC CTA-3'.
EZH2	EZH2 FOR 5'-GCA AAT AGC TCA CCC CAT GT-3' EZH2 REV 5'-GGG ATT TTG CCA TTC AGA TG-3'.
CycA	FOR 5'–TGT AAG ATT CCC GTC GGG CCT TC-3' REV 5'-AGG CGG GAG GAG CGT AGA GCC -3'.
CycB2	FOR 5'–GGA GCC ATC ACC TAA GGA CA-3' REV 5'-CTG AAC GAC CTG GAG ACG AT-3'.
Pcna	FOR 5'–AGC TCG ATT TGC CTG TGA CT-3' REV 5'-AAT CGT ACC CGT GGT TTG AG -3'.
Desert region	FOR 5'-TCC TCC CCA TCT GTG TCA TC-3' REV 5'-GGA TCC ATC ACC ATC AAT AAC C-3'.

Table 1 Primers used for genotyping and ChIP Real-Time PCR.

permeabilize the membrane and block unspecific antibody binding. Cells were incubated with primaries for 2 hours at room temperature. After several rinses with PBS, conjugated secondary antibodies diluted in PBS were applied for 1 hour at RT. Finally, cells were incubated with DAPI for 5 minutes then mounted on microscope slides using Immunomount (Genetex) and dried overnight. Images were acquired with a 40X objective using a confocal microscope (Zeiss) and Zen imaging software (Zeiss). Forward and reverse primers used for genotyping purposes to amplify regions unique to the p130 and p107, and for amplifying the Sox2, EZH2, CycA, CycB2 and Pcna loci from ChIP products by Real-Time PCR (Table 1).

2.7. Neural precursor cultures

Neural precursors were obtained by dissection of the ventral telencephalic tissue of CD1 embryos E13.5. Neurosphere assays were performed as previously described (Vanderluit et al. 2004). Briefly, the dissected tissue was dissociated and grown in medium Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Sigma) supplemented with 2% B27 (Gibco), 1% Pen-Strep (Sigma), 12.5 ng/ml FGF, 12.5 ng/ml EGF(Sigma), 2 ng/µl heparin (Sigma). Cells were seeded in uncoated plates.

For lentivirus infections, cells were pre-incubated with the lentivirus at MOI 30 for 2hours at 37^{o}C at a high density of 400cells/µl (Khacho et al. 2016). Cells were triturated, plated and maintained in a humidified incubator at 37ºC with 5% CO_2 for five days.

2.8. Western blot analysis

Western blot analysis was performed as previously described (Khacho et al. 2016). Proteins from cortical neuronal cultures and neurosphere cultures were isolated. Whole cell protein lysates were prepared by mechanically shearing cells using a 25 gauge

syringe in a 4% sodium dodecyl sulfate (SDS) lysis buffer. Protein concentration was measured using Bradford assay (Bio-Rad) then 20µg was loaded per lane. Proteins were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (BioRad). BLUeye prestained protein ladder (FroggaBio) was used as a size reference. The membrane was blocked with Tween 20 phosphate buffered saline (T-PBS) containing 5% skim milk for 1 hour to prevent nonspecific binding.

For detection of Lin52, protein was extracted using a lysis buffer (50mM Tris-HCl, pH 7.4 150mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate and 1:10000 B-mercaptoethanol) supplemented with phosphatase and protease inhibitor cocktails (Sigma-Aldrich P8340), followed by centrifugation at 18800 g for 10 min at 40°C. The supernatant was then collected into a fresh tube. Protein concentration was measured using Bradford assay (BioRad) then 50µg was loaded per lane. Proteins were separated on a precast gel (Criterion TGX 4-20% Stain Free Precast Gel, 10 wells: #456-8094) and transferred to a nitrocellulose membrane (BioRad). The membrane was blocked with 5% skim milk (Carnation powder milk) for 1hour at room temperature. After washing the membranes with T-PBS, they were incubated overnight at 4°C with primaries antibodies. A corresponding secondary antibody was applied for 1hour at room temperature. Blots were developed by chemiluminescence according to the manufacturer's instructions (ECL; Bio-Rad). An anti-actin antibody was used as a protein loading control (Table 2).

2.9 Chromatin Immunoprecipitation (ChIP)

Primary cultured neurons were prepared from CD1 embryos at E14.5. After five days in culture, neurons were cross-linked with 11% fresh formaldehyde solution for 10 minutes, quenched with glycine for 5 minutes then washed with cold PBS. The resultant

pellet was either stored or immediately lysed. Cell lysates were sonicated using Covaris S220 (duty factor: 5%, peak power: 140 watt, burst 200, duration 20 minutes) resulting in chromatin fragments 300 to 500 bp. Sonicated samples were centrifuged for 10 min at 14K rpm to remove cellular debris. ChIP was performed as described previously (Andrusiak et al. 2011). Each immunoprecipitation (IP) was performed using 25µg of chromatin which was cleared with blocked protein A/G beads (Protein A-Sepharose 4B Conjugate-life Technologies 101041) for 3hours at 4^{o}C while rocking. After discarding the beads, a 10% input was taken and saved at -20^{o}C. Chromatin was then incubated overnight at 4^{o}C, with 2µg of antibody directed against the protein of interest (p130, E2F4, Lin9, Lin54 and RbBp4) or IgG considered negative control (Table 2). Blocked protein A/G beads were added to the immunocomplexes for 3hours at 4^{o}C followed by stringent washes. Crosslinks were reversed overnight using 1% SDS solution at 65^{o}C, followed by treatment with RNase A (Sigma-Roche 10109169001) at 37°C for 1 hour and proteinase K (Sigma 03115879001) at 65°C for 30 minutes. Phenol/chloroform (Fisher BP 1752I pH 6.7/8) was used to extract residual proteins from the DNA. Finally, the resulting DNA was resuspended in 100 µl of water then analyzed by quantitative PCR (qPCR) using the Mx3000P thermocycler and Quanta SYBR Green Fast mix with low ROX (Quanta CA101414-274) as an internal reference dye to which detection of SYBR Green fluorescence was normalized. Primers were specifically designed to amplify the promoter (Table 1). For ChIP-qPCR serial dilutions of the input genomic DNA were run alongside with each series and used to calculate the specific ChIP enrichments as a percent of input DNA. Each IP was performed on at least three independent samples.

2.10 Statistical Analysis

All statistical comparisons in this study were performed using an unpaired two-tailed t test, with differences considered significant with a p value of <0.05 (*), ** $p<0.01$, *** $p<0.001$.

	Antibody	Dilution	Source
Primary	GFP (rabbit)	1:5000	Abcam 6556
	Tbr2 (rat)	1:250	eBioscience 14-4875-82
	Sox2 (goat)	1:500	Santa Cruz sc-17320
	Dcx (guinea pig)	1:2000	EMD-Millipore AB2253
Secondary	Alexa Fluor-488	1:500	Molecular Probes
	Cy3	1:500	Jackson Immuno
	Dylight -649	1:500	Jackson Immuno
	DAPI	1:1000	Sigma
Western Blot	Dyrk1a (rabbit)	1:1000	Cell Signaling 2771S
	Sox2 (goat)	1:1000	Santa Cruz sc17-320
	Actin (mouse)	1:10 000	Sigma A-5316
	Lin52 (rabbit)	1:1000	Gift from Dr. Litovchick
	Lin52 S28A (rabbit)	1:3000	(Litovchick et al. 2011)
ChIP	p130 (rabbit)	Santa Cruz sc 317	
	E2F4 (rabbit)	Santa Cruz sc 1082	
	Lin9 (rabbit)	Abcam ab 62329	
	Lin54 (rabbit)	Bethyl A307-799A	
	RbBp4 (rabbit)	Bethyl A301-206A	
	IgG (rabbit)	Sigma-Aldrich I8140	

Table 2 Antibodies used for immunohistochemistry, western blotting and ChIP experiments.

RESULTS

Objective 1: To investigate the role of DREAM complex during neurogenesis.

DREAM, is a multiprotein complex consisting of E2F4, DP, and p130 interacting with the phosphorylated form of Lin52, a member of the MuvB core. Dyrk1a contributes to Lin52 phosphorylation on Serine 28 to enable its interaction with p130 allowing DREAM assembly (Litovchick et al. 2011). Here, we performed *in vivo* experiments in WT and p107/p130 DKO mice, to define the role of DREAM in neurogenesis.

Results:

3.1 Dyrk1a promotes neuronal differentiation of neural progenitor cells.

To examine the effect of the DREAM complex on neurogenesis *in vivo*, *in utero* electroporation (IUE) experiments were performed using WT mice (Charles River Laboratories, Wilmington, MA). Embryos were sacrificed 48 hours after the electroporation. Using immunohistochemical staining, we examined the effect of manipulating DREAM components on cell fate decision.

Neuroepithelial cells give rise to radial glial cells (RGCs) which divide asymmetrically to produce another RGC or another progenitor with an intermediate progenitor (IP). IPs undergo symmetric divisions to give rise to two neurons or amplifying divisions to generate new IPs (Götz and Huttner 2005; Pontious et al. 2008). RGCs are found in the ventricular zone described as the proliferative zone, and the IPs move toward the sub-ventricular zone whereas the neurons migrate towards the cortical plate (Noctor et al. 2004).

During quiescence or G0 phase, DREAM is involved in the repression of the majority of cell cycle genes (Litovchick et al. 2007). Different factors may control

DREAM assembly including Lin52 phosphorylation effected by Dyrk1a. First, we wanted to confirm previous studies showing that Dyrk1a overexpression inhibits proliferation and promotes neuronal differentiation (Yabut, Domogauer, and D'Arcangelo 2010).

WT embryos were *in utero* electroporated at E13.5. Expression plasmids carrying WT-Dyrk1a, point mutant Dyrk1a-K188R and a nuclear-targeted EGFP plasmid as control were injected into the lateral ventricle of the neocortex (Saito and Nakatsuji 2001). Forty-eight hours after the electroporation, GFP-expressing control cells were distributed in the ventricular, sub-ventricular, and intermediate zone and the cortical plate. However, most of the WT cells electroporated with the control plasmid were found in the intermediate zone (68%) with 7% and 21% in the ventricular and the sub-ventricular zone, respectively, and only 2.5% in the cortical plate. In contrast, cells expressing Dyrk1a exhibited an increase in the percentage of cells in the intermediate zone (84%) at the expense of the ventricular zone (1.4%) (Figure 8(A)). To investigate whether Dyrk1a function depends on its kinase activity, we used Dyrk1a-K188R which is a catalytically inactive kinase. A point mutation generates this inactive kinase: the Lysine residue replaced by Arginine preventing ATP binding to the catalytic domain (Himpel et al. 2001). When Dyrk1a-K188R is overexpressed, the majority of the electroporated cells were found in the intermediate zone (69%) with 7% and 22% in the ventricular and sub-ventricular, respectively, and 0.88% in the cortical plate (Figure 8(A)). Dyrk1a-K188R positive cells were distributed in a comparable pattern to the GFP-only expressing cells. The finding that the mutant kinase shows similar distribution as the control suggests that the kinase activity of Dyrk1a may be involved in the regulation of neurogenesis.

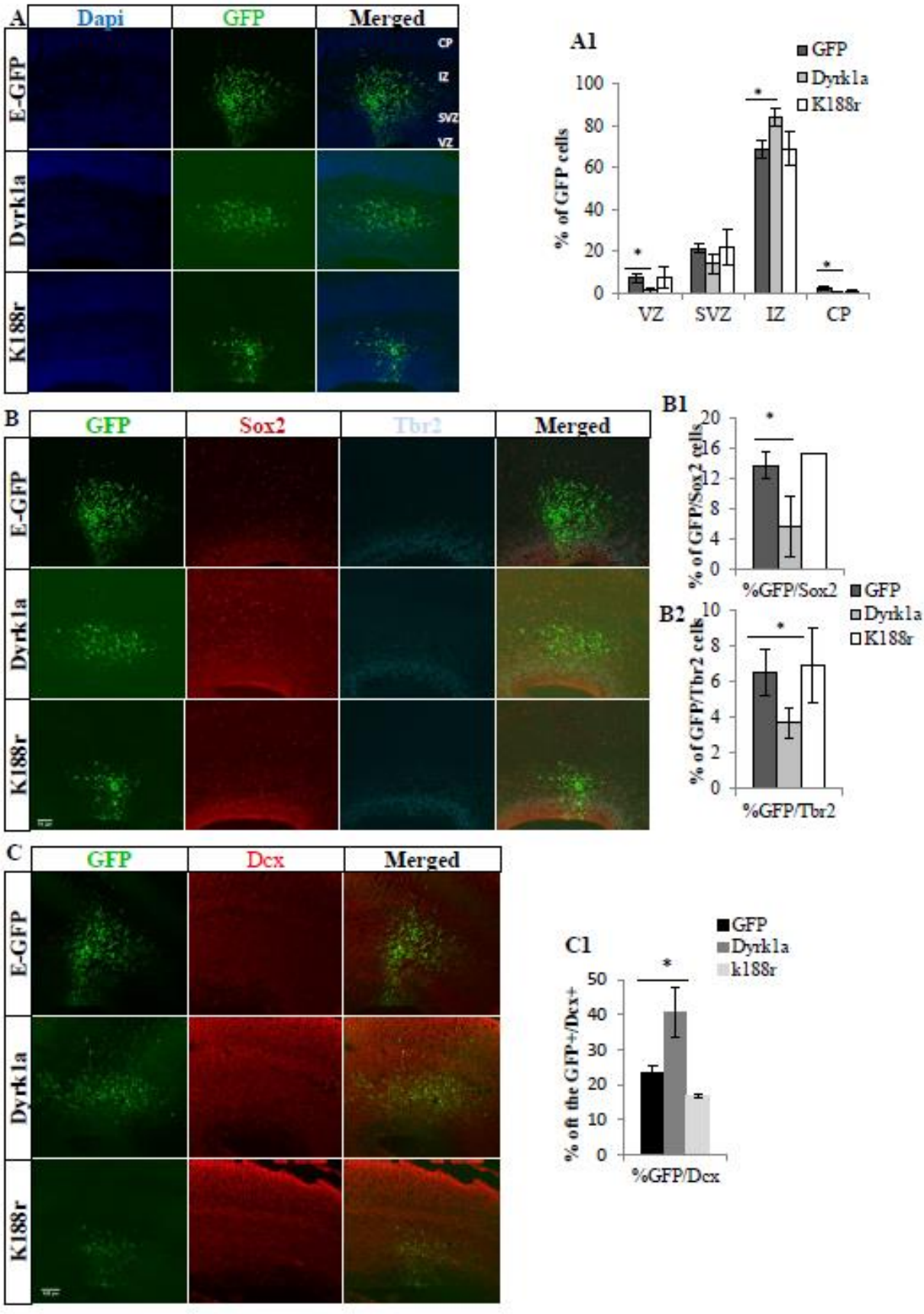

A
Dapi
GFP
Merged
E-GFP
Dyrk1a
K188r
CP
IZ
SVZ
VZ
A1
GFP
Dyrk1a
K188r
% of GFP cells
100
80
60
40
20
0
VZ
SVZ
IZ
CP
B
GFP
Sox2
Tbr2
Merged
E-GFP
Dyrk1a
K188r
B1
% of GFP/Sox2 cells
20
16
12
8
4
0
%GFP/Sox2
B2
% of GFP/Tbr2 cells
10
8
6
4
2
0
%GFP/Tbr2
C
GFP
Dcx
Merged
E-GFP
Dyrk1a
K188r
C1
GFP
Dyrk1a
k188r
% of the GFP+/Dcx+
50
40
30
20
10
0
%GFP/Dcx

Figure 8 Overexpression of Dyrk1a in WT embryos promotes commitment of neuronal fate.

Panel A, B and C Representative confocal images following 48 hours of *in-utero* electroporation of Dyrk1a, GFP and K188R, which is a mutant kinase, at E13.5 in the dorsal forebrain.

Panel A: Immunohistochemistry against GFP (electroporated cells) counterstained with DAPI to detect the expression pattern of the electroporated cells within the ventricular zone (VZ), the sub-ventricular zone (SVZ), the intermediate zone (IZ) and the cortical plate (CP). Graph A1 represents the quantification of the percentage of GFP positive cells within the VZ, SVZ, IZ and CP. The majority of electroporated cells are located in the IZ. Dyrk1a overexpression pushed the cells to change their localization; they leave the VZ and SVZ towards the IZ, which is not observed when GFP and the mutant kinase were overexpressed.

Mean± SEM, n=4, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 75μm.

Panel B: Immunohistochemistry against GFP (electroporated cells), Sox2 (apical precursors) and Tbr2 (basal progenitors). Graphs B1 and B2 represent the percentage of GFP/Sox2 and GFP/Tbr2 positive cells respectively. A decrease in the expression of Sox2 and Tbr2 was observed only following the overexpression of Dyrk1a.

Mean± SEM, n=4, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 75μm.

Panel C: Immunohistochemistry against GFP (electroporated cells) and Dcx (neuronal marker) to detect the cell identity of the electroporated cells. Graph C1 shows the percentage of GFP/Dcx positive cells. Dyrk1a overexpression leads to an increase in Dcx expression which is not observed in the mutant kinase K188R or the GFP.

Mean± SEM, n=4, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 100μm.

We then asked whether the change in the cellular localization of the cells manipulated with Dyrk1a represents a change in the cell fate. To answer this question, we quantified the Sox2+ and Tbr2+ cells marking the apical and intermediate progenitor cells, respectively, and Dcx, which is a marker for newborn neurons in the WT-Dyrk1a, the mutant, and the control. In the control, 13.6% and 6.5% were Sox2+ and Tbr2+ respectively with 25% of the manipulated cells were Dcx+ (Figure 8(B)). We detected a significant reduction in the percentage of Sox2+ (5.5%) and Tbr2+ (3.7%) cells (Figure 8(B)) with a corresponding increase in the percentage of Dcx (40%) cells only when WT-Dyrk1a was overexpressed (Figure 8(C)). In contrast, cells overexpressing Dyrk1a with a mutant kinase domain revealed similar percentages of Sox2, Tbr2, and Dcx as the control cells. These findings demonstrate that Dyrk1a through its kinase activity may regulate the cell fate decision and promote neurogenesis through its kinase activity.

3.2 Lin52 overexpression enhances neurogenesis while the mutant Lin52S28A prevents neurogenesis and promotes a proliferative state.

As discussed above, Dyrk1a phosphorylates Lin52 to promote the assembly of the DREAM complex. Phosphorylated Lin52 interacted with p130 (Figure 7), and this phosphorylation is considered a key event for the DREAM assembly. We next wanted to ask whether Lin52 overexpression will enhance neurogenesis similar to the outcome of Dyrk1a overexpression. To manipulate Lin52 expression, we used expression plasmids for wild-type (WT) Lin52, mutant Lin52 known as Lin52-S28A (point mutation of Serine 28 to Alanine preventing its phosphorylation by Dyrk1a and its interaction with p130) and a nuclear-targeted EGFP plasmid as the control.

Expression plasmids carrying WT-Lin52, Lin52-S28A and the control were

injected into the lateral ventricle of the neocortex of E13.5 WT embryos. Forty-eight hours after the electroporation, GFP-expressing control cells were found in the ventricular zone (3.5%) with 20% and 39% in the sub-ventricular and intermediate zone, respectively, and 37% in the cortical plate (Figure 9(A)). In contrast, cells expressing WT-Lin52 revealed an increase in the percentage of cells in the intermediate zone (65%) at the expense of the sub-ventricular (11.3%) zone and the cortical plate (20%). To examine whether the function of Lin52 is linked to its phosphorylation by Dyrk1a, which will enable its interaction with p130, we electroporated Lin52-S28A, as its phosphorylation by Dyrk1a is prevented due to a point mutation of the Serine 28 to Alanine. Cells expressing the phosphorylation-deficient Lin52-S28A mutation exhibited an increase in the percentage of cells in the sub-ventricular zone (37 %) at the expense of the cortical plate (21%) (Figure 9(A)). Altogether, these results suggest that overexpression of Lin52 drives apical progenitor cells out of the VZ/SVZ towards intermediate zone and that Lin52 may promote differentiation of neural stem/progenitor cells. In contrast, a more significant proportion of the mutant Lin52-S28A electroporated cells remained in the VZ, consistent with impaired differentiation.

Next, we asked whether this change in cellular localization is associated with a change in the cell fate consistent with differentiation. We quantified the Sox2+ and Tbr2+ cells marking the apical and basal progenitors, respectively, and Dcx+ marking the newborn neurons. In the control, 9.3% and 6.8% were Sox2+, Tbr2+ cells respectively with 42% Dcx+ cells. In contrast, cells expressing the WT-Lin52 revealed a decrease in the percentage of cells expressing Sox2 (4.6%) and Tbr2 (4.2%) and a corresponding increase in the Dcx+ cells (59%) (Figure 9(B)). Cells expressing the mutant Lin52-S28A

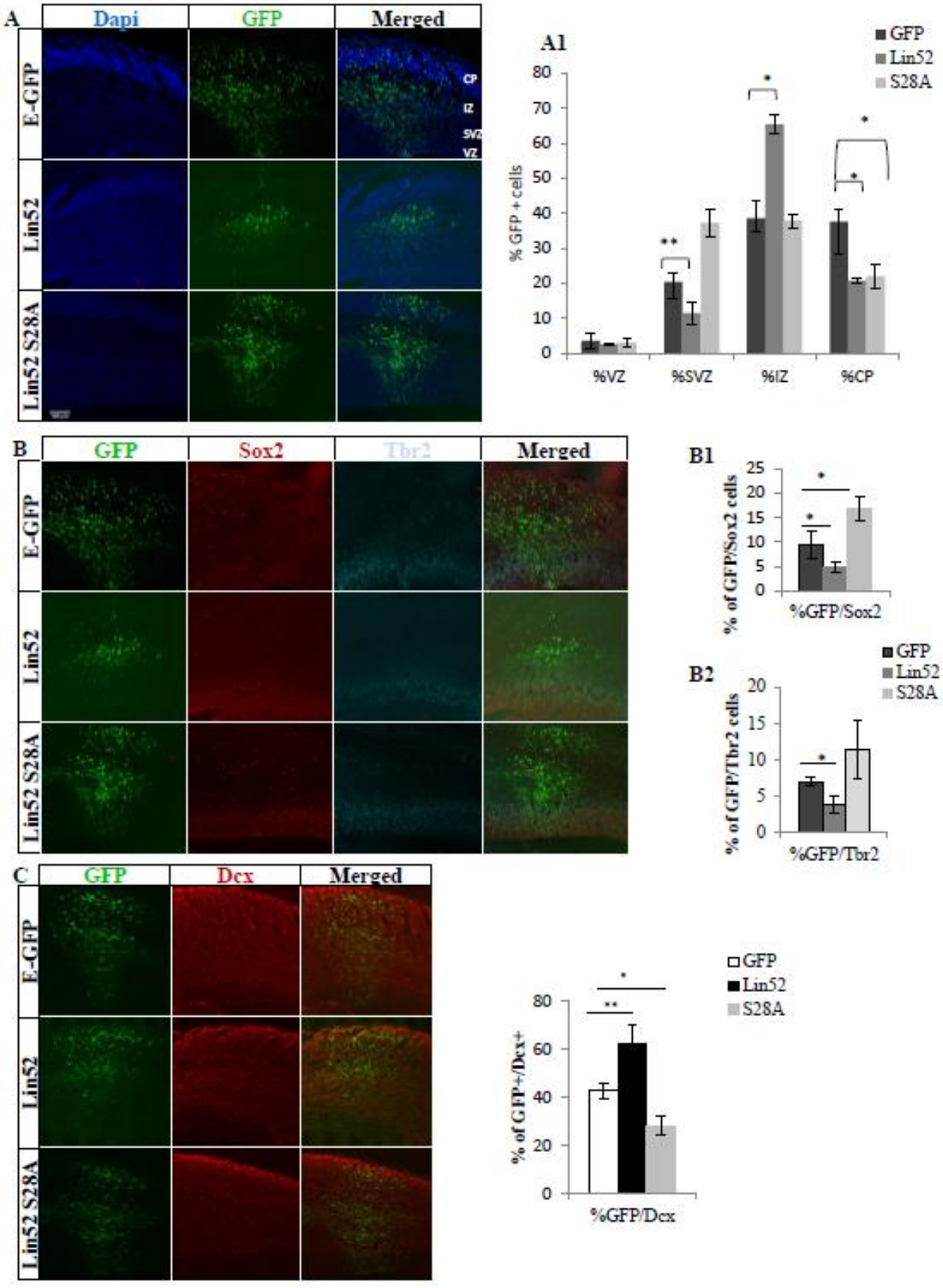

A
Dapi
GFP
Merged
E-GFP
Lin52
Lin52 S28A
CP
IZ
SVZ
VZ
A1
GFP
Lin52
S28A
% GFP + cells
%VZ
%SVZ
%IZ
%CP
B
GFP
Sox2
Tbr2
Merged
E-GFP
Lin52
Lin52 S28A
B1
% of GFP/Sox2 cells
%GFP/Sox2
B2
GFP
Lin52
S28A
% of GFP/Tbr2 cells
%GFP/Tbr2
C
GFP
Dcx
Merged
E-GFP
Lin52
Lin52 S28A
GFP
Lin52
S28A
% of GFP+/Dcx+
%GFP/Dcx

Figure 9 Overexpression of Lin52 in WT embryos promotes the acquisition of neuronal fate at the expense of the Sox2 population.

Panel A, B and C Representative confocal images following 48 hours of *in-utero* electroporation of GFP, Lin52 and Lin52 S28A, which is a Lin52 mutant at E13.5 in the dorsal forebrain.

Panel A: Immunohistochemistry against GFP and counterstained with DAPI to detect the expression pattern of the electroporated cells within the ventricular zone (VZ), the sub-ventricular zone (SVZ), the intermediate zone (IZ) and the cortical plate (CP).

(A1) Graph showing the percentage of GFP positive cells within the VZ, SVZ, IZ and CP. The majority of electroporated cells are located in the IZ. Lin52 overexpression pushed the cells to leave the VZ and SVZ towards the IZ comparable to the GFP. Conversely with the mutant Lin52, the electroporated cells maintained their localization in the VZ and SVZ.

Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 100μm.

Panel B: Immunohistochemistry against GFP, Sox2 (apical precursors) and Tbr2 (basal progenitors).Graphs (B1) and (B2) represent the quantification of the percentage of GFP/Sox2 and GFP/Tbr2 positive cells respectively. A decrease in the expression of Sox2 and Tbr2 was observed following the overexpression of Lin52. However the Sox2 expression within the cells expressing the mutant Lin52 was not only maintained but significantly increased.

Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 100μm.

Panel C: Immunohistochemistry against GFP and Dcx (neuronal marker) to detect the cell identity of the electroporated cells. Right graph shows the quantification of the percentage of GFP/Dcx positive cells. Lin52 overexpression leads to an increase in Dcx expression, which is not observed in the control. A significant decrease in Dcx expression was noted when the mutant Lin52-S28A was overexpressed.

Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 100μm.

exhibited an increase in the percentage of Sox2+ (16%) and Tbr2+ cells (10.9%) and a decrease in the percentage of the Dcx+ cells (28%) (Figure 9(C)).

Thus, Lin52 overexpression provokes a change in the cellular localization associated with a change in cell phenotype by increasing commitment to differentiation, a phenotype similar to when Dyrk1a was overexpressed. These findings showed that the overexpression of Lin52 enhances neurogenesis and decreases the proportion of uncommitted progenitor cells. Conversely, overexpression of Lin52 mutant, which impairs DREAM assembly, reduces the proportion of cells that undergo differentiation, while remaining as uncommitted stem and progenitor cells within the VZ/SVZ.

3.3 P130, member of DREAM complex, regulate neurogenesis by promoting neuronal commitment.

We next asked whether the increased expression of p130 would also enhance neurogenesis, consistent with being a component of the DREAM complex (Fig.10). Expression plasmids carrying p130 and a nuclear-targeted EGFP were injected into the ventricle of embryonic brain E13.5. Forty-eight hours after the electroporation, GFP-expressing control cells were found in the ventricular, sub-ventricular and intermediate zone. However, most of the control cells were located in the intermediate zone (72%) with 6.5% and 21% in the ventricular and sub-ventricular zone respectively. In contrast, cells expressing p130 exhibited an increase in the percentage of cells in the intermediate zone (86%) at the expense of the ventricular (1.7%) and sub-ventricular (11.7%) zones respectively (Figure 10(A)). These results suggested that the overexpression of p130 provoke a change in the cellular localization and maybe enhancing neurogenesis.

We next asked whether that change in the cellular localization corresponds to a change in the cell fate. We quantified the Sox2+ and Tbr2+ cells marking the apical and basal progenitors respectively. In the control, 12% and 4.8% of the cells were expressing Sox2 and Tbr2 respectively (Figure 10(B)). Conversely, cells manipulated with p130 revealed a decrease in the percentage of cells expressing Sox2 (8.7%) with an increase in the percentage of cells expressing Tbr2 (10.9%). Cells manipulated with p130 committed to a progenitor fate at the expense of the Sox2 population. The overexpression of p130, a component of DREAM complex, enhances neurogenesis by promoting a change in the cellular localization accompanied by the acquisition of a more differentiated fate.

In summary, overexpression of the components of the DREAM complex (p130, Lin52) and Dyrk1a, which promotes DREAM assembly, induced the expression of neuronal markers indicating the commitment to a neuronal fate. These cells had committed to a neuronal fate (Dcx expression) at the expense of the Sox2 population. DREAM complex may be regulating stemness (decrease in Sox2 and Tbr2) and promoting commitment to neuronal fate (expression of neuronal markers). However, when the phosphorylation-deficient Lin52 mutant was overexpressed, DREAM assembly was disabled causing a delay in differentiation while maintaining the cells in an undifferentiated state in a proliferative region. Therefore, DREAM promotes commitment to differentiation at the expense of self-renewal.

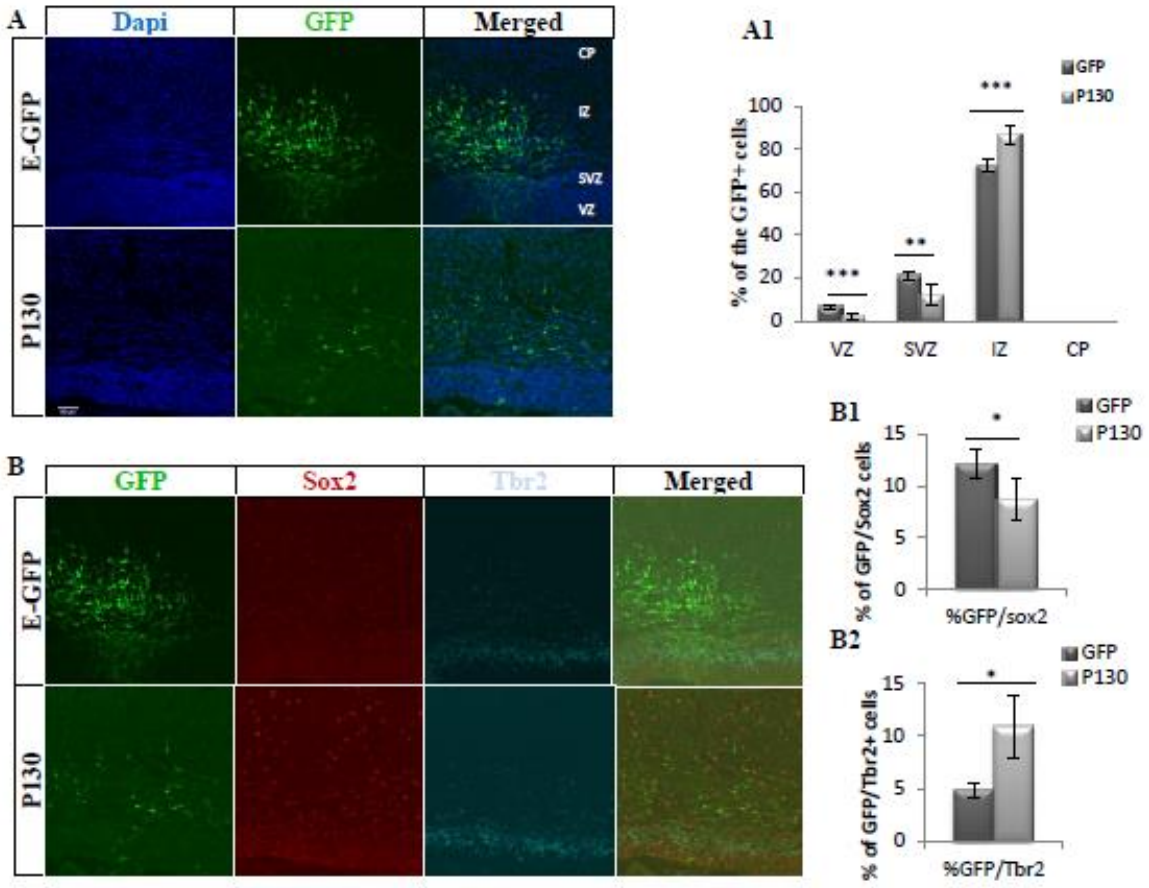


Figure 10 Overexpression of p130 in WT embryos enhances commitment.

Panel A and B Representative confocal images following 48 hours of *in-utero* electroporation of GFP and p130 at E13.5 in the dorsal forebrain.

Panel A: Immunohistochemistry against GFP (electroporated cells) counterstained with DAPI to detect the expression pattern of the electroporated cells within the ventricular zone (VZ), the sub-ventricular zone (SVZ), the intermediate zone (IZ) and the cortical plate (CP). Graph A1 represents the percentage of GFP positive cells within the VZ, SVZ, IZ and CP. The majority of electroporated cells are located in the IZ. p130 overexpression pushed the cells to change their localization; they leave the VZ and SVZ towards the IZ, which is not observed when GFP and was overexpressed. Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar =50µm.

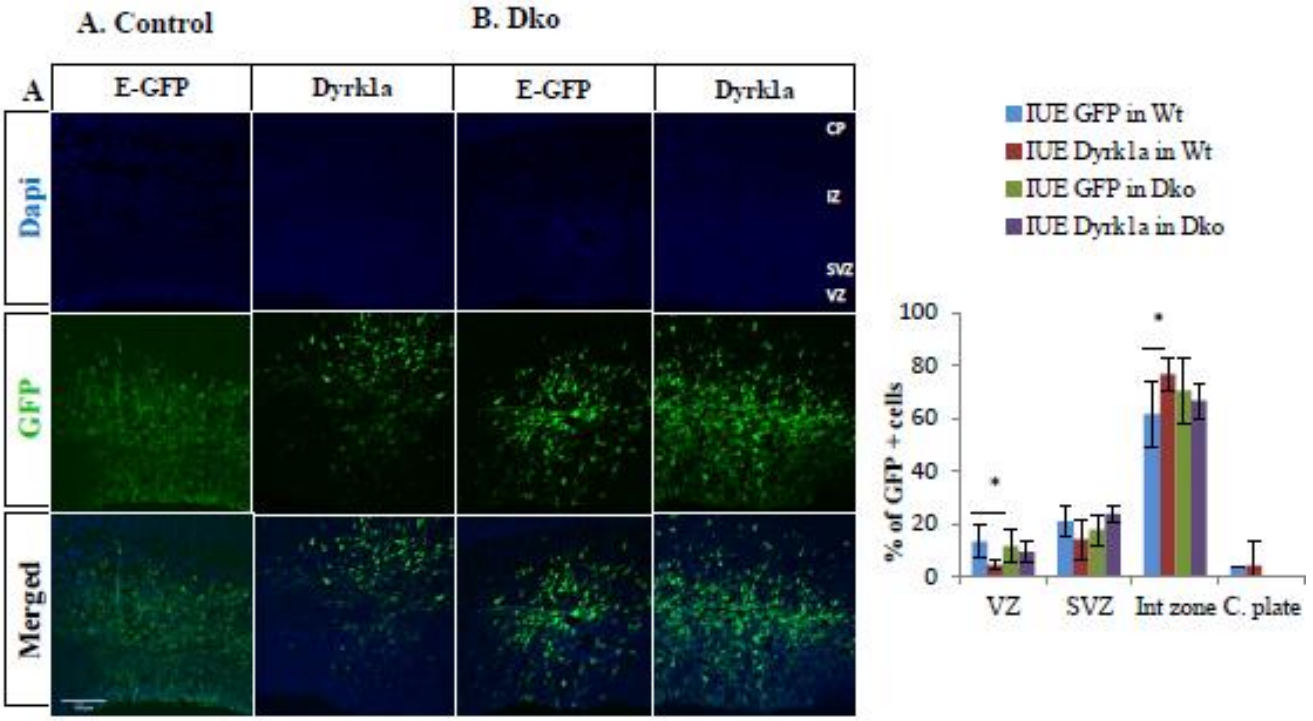
Panel B: Immunohistochemistry against GFP (electroporated cells), Sox2 (apical precursors) and Tbr2 (basal progenitors). Graphs B1 and B2 represent the percentage of Sox2+ and Tbr2+ cells respectively. Following the overexpression of p130 a decrease in the expression of Sox2 was observed accompanied with an increase in the Tbr2 expression.

Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 50µm.

3.4 Dyrk1a function on neurogenesis requires p130 or p107.

Several studies showed that p107 could interact with Dp, E2F4 and MuvB core to form DREAM complex in p130 depleted cells (Litovchick et al. 2007; Schmit et al. 2007; Pilkinton, Sandoval, and Colamonici 2007). As discussed above, pocket proteins may have redundant functions; p130 and p107 can compensate for each other. Given that DREAM complex activity is mediated through p130 as shown above, we asked whether the loss of p130 and p107 will abolish DREAM assembly and activity. To address this question, we tested whether the overexpression of Dyrk1a could increase neurogenesis in the absence of p130/p107. We used double knock-out (DKO) embryos where p107 is a germline knock-out, while p130 is a conditional knock-out. Expression plasmids carrying Dyrk1a and the GFP control were injected into the ventricle of E13.5 DKO and WT embryos considered as control.

In WT embryos, the majority of the cells electroporated with control plasmid were found in the intermediate zone (61%) with 13% and 21% in the ventricular and sub-ventricular zones, respectively, with only 3.7% located in the cortical plate (Figure 11(A)). In contrast, WT cells manipulated with Dyrk1a revealed an increase in the percentage of the cells found in the intermediate zone (76%) and the cortical plate (4.3%) at the expense of the ventricular (4.5%) and sub-ventricular (14%) zones (Figure 11). In DKO embryos, GFP-expressing control cells were found in the ventricular, sub-ventricular and intermediate zones. The majority of the cells electroporated with the control plasmid were located in the intermediate zone (70.5%) with 11.6% and 17.7% in the ventricular and sub-ventricular zones respectively. DKO cells manipulated with Dyrk1a exhibited a comparable cellular distribution to the control with 9.3%, 23.8% and


A. Control
B. Dko
A
E-GFP
Dyrk1a
E-GFP
Dyrk1a
Dapi
GFP
Merged
CP
IZ
SVZ
VZ
IUE GFP in Wt
IUE Dyrk1a in Wt
IUE GFP in Dko
IUE Dyrk1a in Dko
% of GFP + cells
100
80
60
40
20
0
*
*
VZ
SVZ
Int zone
C. plate

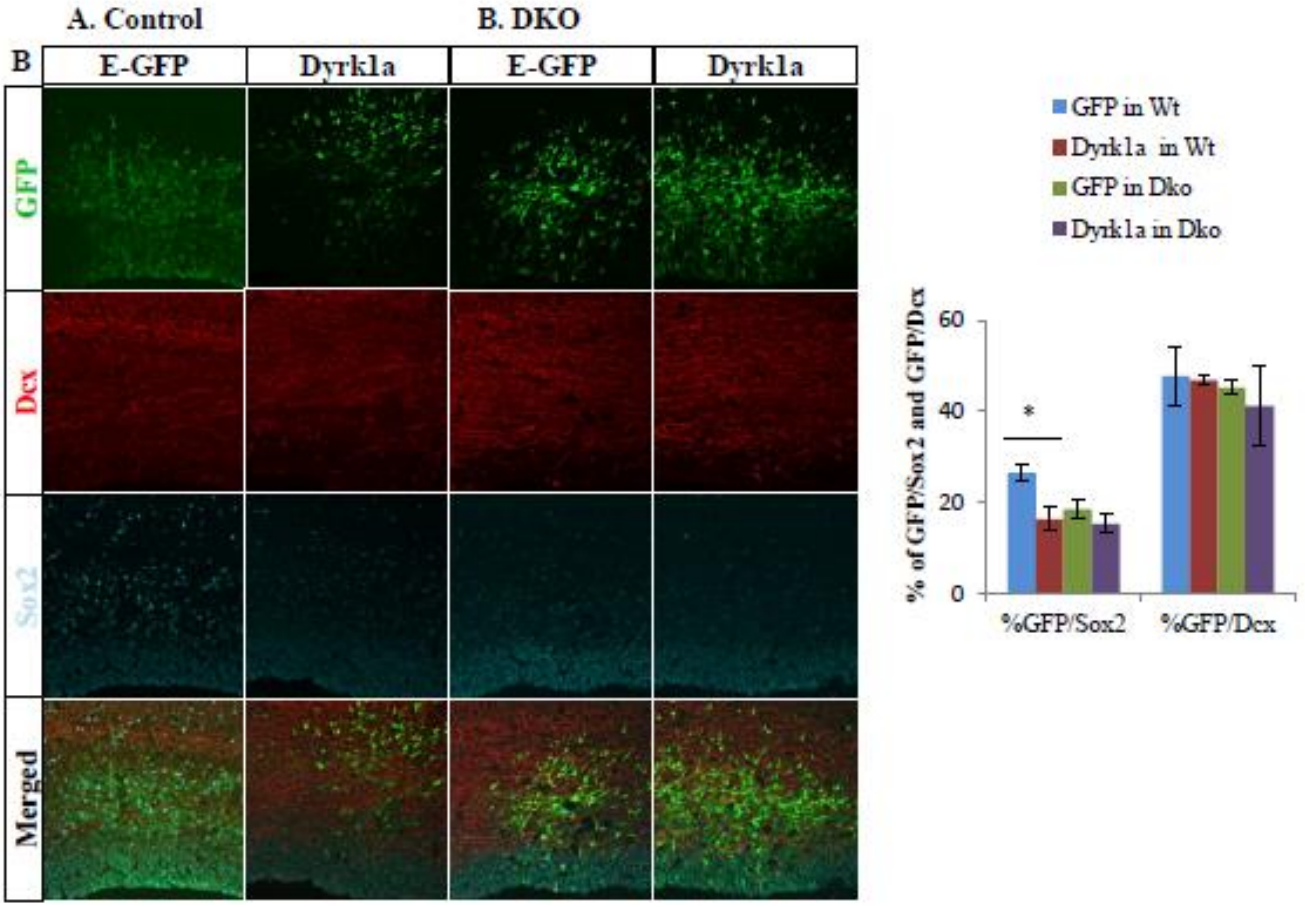

A. Control
B. DKO
B
E-GFP
Dyrk1a
E-GFP
Dyrk1a
GFP
Dcx
Sox2
Merged
GFP in Wt
Dyrk1a in Wt
GFP in Dko
Dyrk1a in Dko
% of GFP/Sox2 and GFP/Dcx
60
40
20
0
*
%GFP/Sox2
%GFP/Dcx

Figure 11 Dyrk1a function on neurogenesis requires p130/ p107.

A) Representative confocal images following *in utero* electroporation of Dyrk1a and GFP in the dorsal forebrain of WT and DKO animals, respectively. Images showing immunohistochemistry against GFP and DAPI to detect the expression pattern of the electroporated cells within the ventricular zone (VZ), the sub-ventricular zone (SVZ), the intermediate zone (IZ) and the cortical plate (CP). Graph showing the quantification of the percentage of GFP positive cells within the different layers. Dyrk1a overexpression leads to a shift in the localization of the electroporated cells in WT animals. No significant change in the expression pattern of the electroporated cells following Dyrk1a overexpression in the DKO animals. Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 100μm.

B) Representative confocal images following *in utero* electroporation of Dyrk1a and GFP in the dorsal forebrain of WT and DKO animals, respectively. Images showing immunohistochemistry against GFP, Sox2 (apical precursors) and Dcx. Graph shows the quantification of the percentage of GFP/Sox2 and GFP/Dcx positive cells. Following Dyrk1a overexpression in WT animals, the electroporated cells revealed a significant decrease in the Sox2 expression. Conversely, in DKO animals, no significant change was detected in the expression of Sox2.
Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 100μm.

66.8% found in the ventricular, sub-ventricular and the intermediate zones respectively (Figure 11(A)). This result suggests that Dyrk1a function requires p130 or p107.

We next quantified Sox2+ and Dcx+ cells marking the apical progenitors and the newborn neurons respectively. In the WT embryos, cells expressing Dyrk1a revealed a decrease in the percentage of Sox2+ cells, while no change was detected in the DKO (Figure 11(B)). Thus, Dyrk1a promoted commitment to differentiation in WT embryos associated with a decrease in the percentage of Sox2+ cells. In contrast, Dyrk1a overexpression in DKO embryos revealed no significant change in the cellular localization in comparison to the control. These cells maintained a similar cellular localization as the control. These findings suggested that Dyrk1a requires p130 or p107 in order to regulate neurogenesis.

Altogether these results demonstrate that the DREAM complex components Lin52, p130 and Dyrk1a, which is the kinase required for DREAM assembly, play important roles in the regulation of neurogenesis. Their overexpression promoted the differentiation of the stem/progenitor cells. In contrast, when the mutants Lin52 was overexpressed, most of the manipulated cells remained undifferentiated and were found in the ventricular zone.

Objective 2: Molecular assessment of DREAM co-repressors in p130-mediated silencing of E2F target genes in NPCs and post-mitotic neurons and assess the impact on differentiation.

3.5 Dysregulated DREAM assembly affects the fate decision of cells *in vitro.*

Given that Dyrk1a is the kinase responsible for Lin52 phosphorylation, we asked whether inhibition of Dyrk1a affects the level of Lin52 phosphorylation and DREAM assembly. We tested this effect using a well-known inhibitor of Dyrk1a called harmine, which shows an important specificity for Dyrk1a among other kinases (Becker and Sippl 2011; Wang et al. 2015). Neural precursor cells and postmitotic neurons from WT brains were cultured and treated with DMSO or harmine at 5μM at time of platting. After five days, western blots of protein extracts from these cells showed a decrease in phosphorylated Lin52 (Figure 12). Göckler et al. showed that harmine inhibits Dyrk1a and consequently affects neurite formation (Göckler et al. 2009). We observed a similar phenotype: neurites were shorter in the treated neurons comparable to the non-treated. These results indicate that Dyrk1a inhibition through harmine leads to a decrease in the level of Lin52 phosphorylated required for the interaction with p130 to promote DREAM assembly.

We next asked if the dysregulated DREAM assembly through harmine treatment affects the fate decision of the cells *in vitro*. To observe the harmine effect on neurons adequately, we platted the neurons on coverslips. We quantified the number of Sox2 and Dcx cells, marking stem-like and newborn neurons, respectively, and counterstained with DAPI. We observed an increase in the percentage of cells expressing Sox2 and Sox2/Dcx following the harmine treatment (Figure 13). The percentage of Dcx positive cells

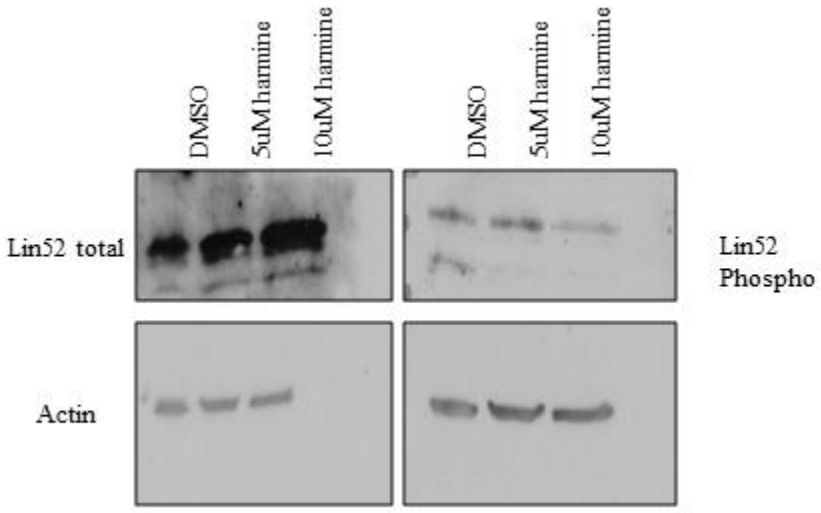


Figure 12 Decrease in the Lin52 phosphorylation level in neurons following harmine treatment.

Cultured neurons were treated with DMSO and harmine at two different concentrations 5 and 10 μM. Western blots of protein extracts from these cells 5 days after platting showed a decrease in Lin52 phosphorylated form. Lin52 total level was unchanged after the treatment. Lin52 phosphorylation level decreased following the harmine treatment.

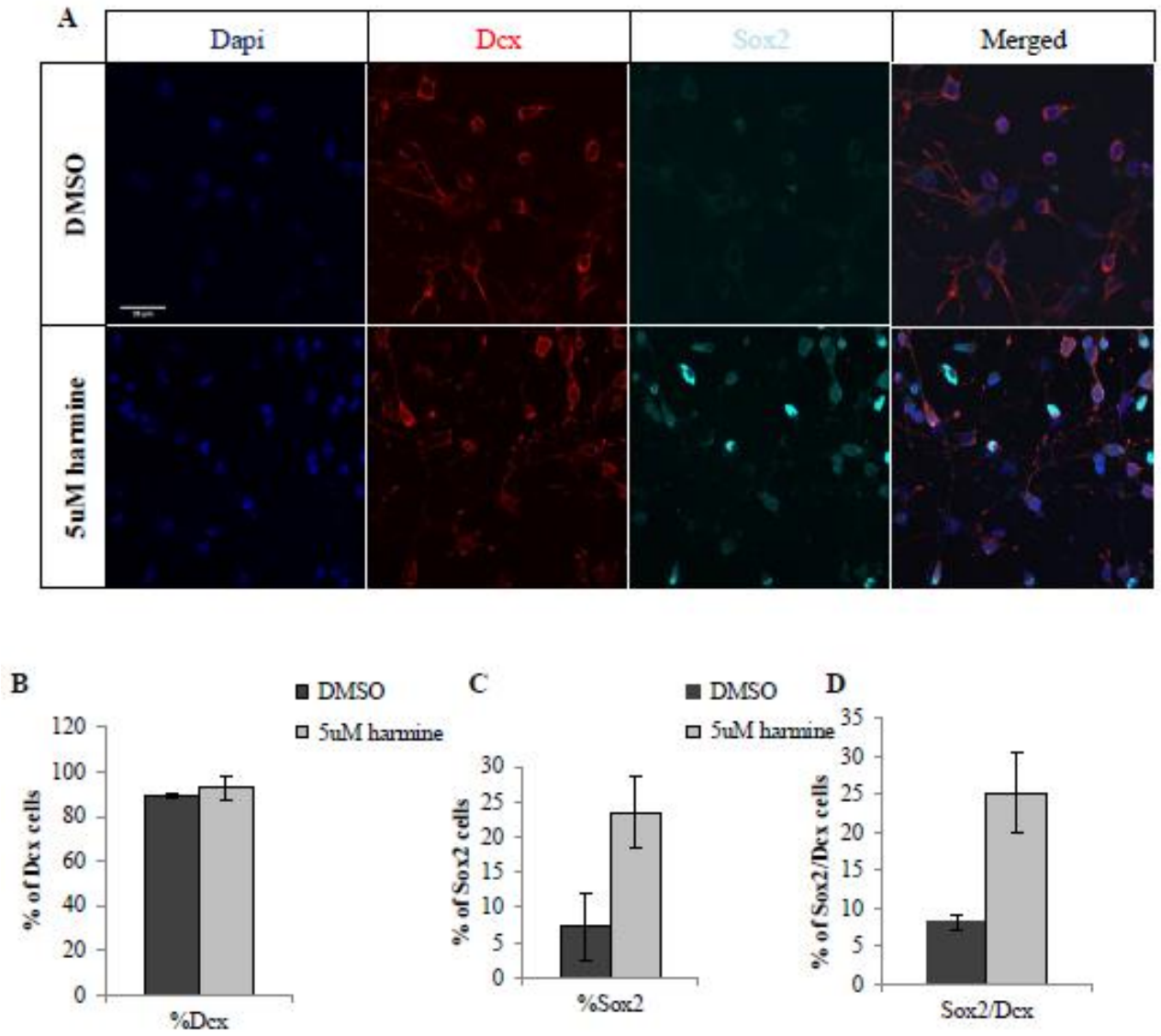


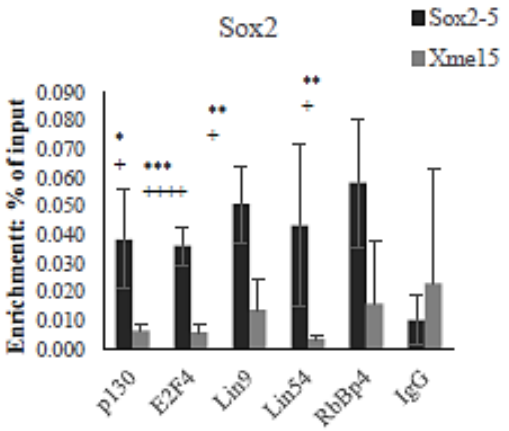
Figure 13 Cultured neurons treated with harmine reveal an increase in the level of Sox2 *in vitro*.

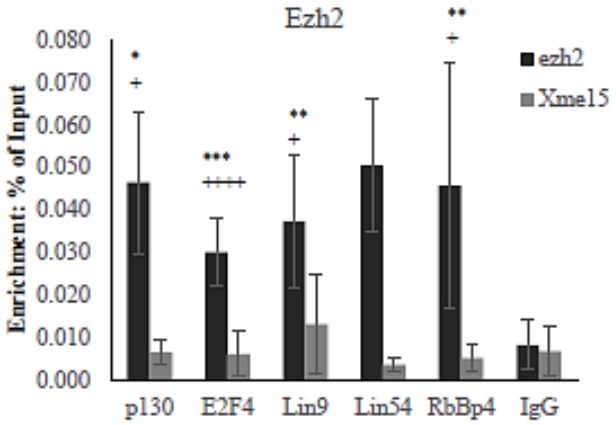
Cultured neurons were treated with DMSO and 5µM of harmine at time of platting. (A) Representative confocal images of cultured neurons on coverslips following treatment with harmine. Images show immunohistochemistry Dcx (neuronal marker) and Sox2 (apical precursor) to determine the cell type and counterstained with DAPI. (B), (C) and (D) Graphs showing the percentage of Dcx, Sox2, and Dcx/Sox2 positive cells respectively. No change in the level of expression of Dcx following the harmine treatment. Conversely, an increase in the expression of Sox2 was detected in the treated neurons. Error bars represent standard deviations of technical replicates.
Scale bar: 25 µM.

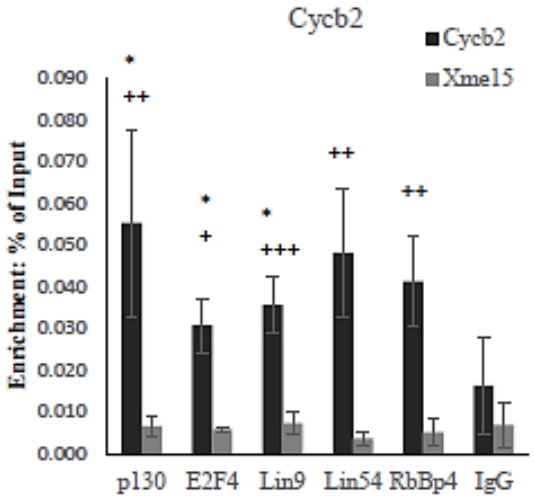
remains the same. These findings show that the expression of Sox2 marking the self-renewal capacity was maintained in neuronal cultures following harmine treatment, which dysregulates DREAM assembly. These results suggest that DREAM complex may regulate the expression of self-renewal markers, therefore, affecting the cell fate determination.

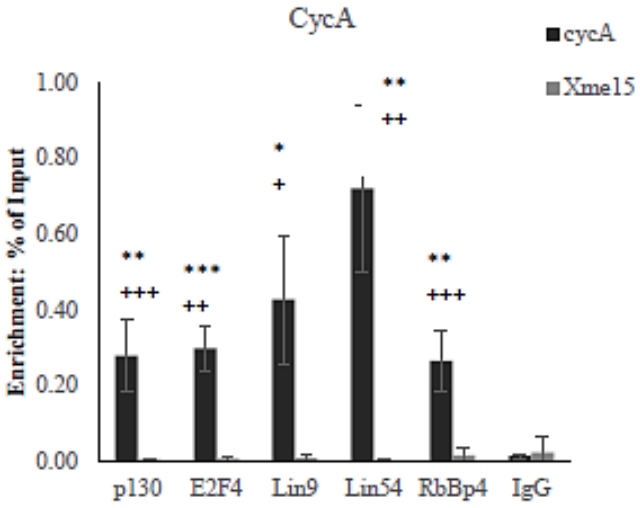
3.6 DREAM complex regulates the self-renewal capacity through the regulation of Sox2 expression: DREAM complex components bind at the promoter of Sox2 and EZH2.

Julian et al. showed that E2F4 is enriched at the promoter regions of genes involved in self-renewal such as Sox2 and EZH2 in addition to a large number of cell cycle genes (Julian et al. 2016). These results suggested a regulatory role for E2F4 on these genes in neural precursor cells. Furthermore, Litovchick et al. showed that DREAM components shared a number of target cell cycle genes (Litovchick et al. 2007). We wanted to know whether E2F4, a member of the DREAM complex, and the other components of DREAM, are involved in the regulation of expression of these genes in cortical neurons. To address this question, classical ChIP experiments were performed using chromatin from WT neurons. We detected a binding enrichment of the DREAM components at the promoters of known cell cycle target genes such as CycB2 and CyclinA (Figure 14). Our results also showed an enrichment binding of DREAM complex components at the promoter of self-renewal gene like Sox2 and EZH2. Thus, our results suggest that DREAM complex may regulate not only classical cell cycle genes but also the self-renewal genes such as Sox2 and EZH2.


Sox2
Sox2-5
Xme15
Enrichmentt: % of input
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
p130
E2F4
Lin9
Lin54
RbBp4
IgG


Ezh2
ezh2
Xme15
Enrichment: % of Input
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
p130
E2F4
Lin9
Lin54
RbBp4
IgG


Cycb2
Cycb2
Xme15
Enrichment: % of Input
0.090
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0.000
p130
E2F4
Lin9
Lin54
RbBp4
IgG


CycA
cycA
Xme15
Enrichment: % of Input
1.00
0.80
0.60
0.40
0.20
0.00
p130
E2F4
Lin9
Lin54
RbBp4
IgG

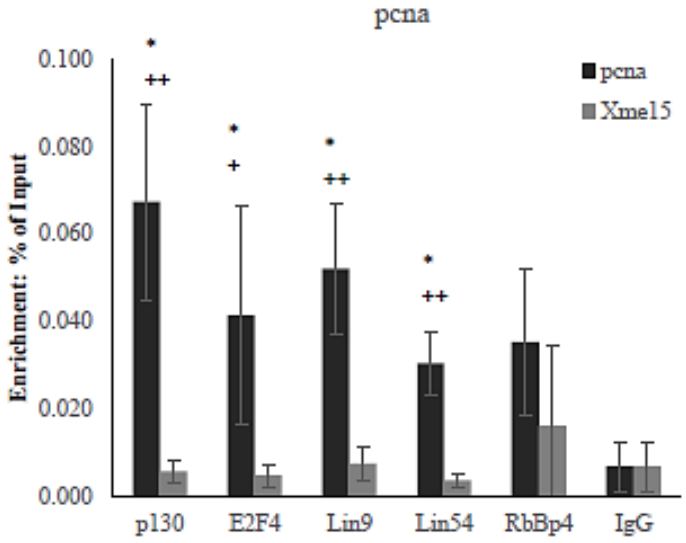

pcna
pcna
Xme15
Enrichment: % of Input
0.100
0.080
0.060
0.040
0.020
0.000
p130
E2F4
Lin9
Lin54
RbBp4
IgG

Figure 14 Chromatin Immunoprecipitations showing enrichment of DREAM components on promoters of self-renewal genes such as Sox2 and EZH2 and of cell cycle genes like PCNA, CycA and CycB2.

ChIPs assays were performed on cultured neurons from E14.5 WT embryos with antibodies against Sox2, EZH2, PCNA, CycA and Cycb2. Binding enrichment of DREAM components p130, p107, E2F4, RbBP4 and Lin9 on the promoters of DREAM targets genes like Sox2, EZH2 CycA, CycB2 and PCNA. IgG is considered the negative control. The chromosome 15 was used as a gene desert region. (n=3)

Two-tailed t-test paired with * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$ compared with the IgG**.**

Two-tailed t-test paired with + $p \leq 0.05$, ++ $p \leq 0.01$, +++ $p \leq 0.001$compared with the gene desert.

3.7 Re-expression of Sox2 rescues the outcome of the dysregulation of DREAM complex observed following Lin52 overexpression.

As shown above, DREAM complex components regulate the expression of self-renewal genes such as Sox2. We observed previously a decrease in Sox2 expression following the manipulation of Lin52 which promotes DREAM assembly. We hypothesized that if we re-express Sox2, we may be able to rescue the enhanced neurogenesis observed following Lin52 overexpression mediated through DREAM activity. To test this hypothesis, expression plasmids carrying Lin52/Sox2 simultaneously and the GFP control were injected into the lateral ventricle of the neocortex. Following the simultaneous overexpression of Lin52/Sox2, no significant changes in the cellular localization compared to the control were detected (Figure 15). In contrast, Lin52/Sox2 overexpression appears to rescue the phenotype noted previously when only Lin52 was overexpressed (Figure 9). We quantified the number of Sox2 and Dcx cells, marking the apical progenitors and the newborn neurons, respectively, to determine if Lin52/Sox2 overexpression affects the cell fate. An increase in the Sox2 expression confirmed our experiment. Cells overexpressing Lin52/Sox2 simultaneously showed a significant decrease in the percentage of the Dcx+ cells (43%) with 78.5% in the control (Figure 15(C)). Thus, Lin52/Sox2 overexpression rescues the increased neurogenesis observed when Lin52 was manipulated. Lin52/Sox2 positive cells maintained their localization within the proliferative zone (VZ and SVZ) and the expression of Sox2 self-renewal marker without gaining neuronal markers such as Dcx (Figure 15(B)). These results confirmed that DREAM complex components regulate neurogenesis through the regulation of the self-renewal genes.

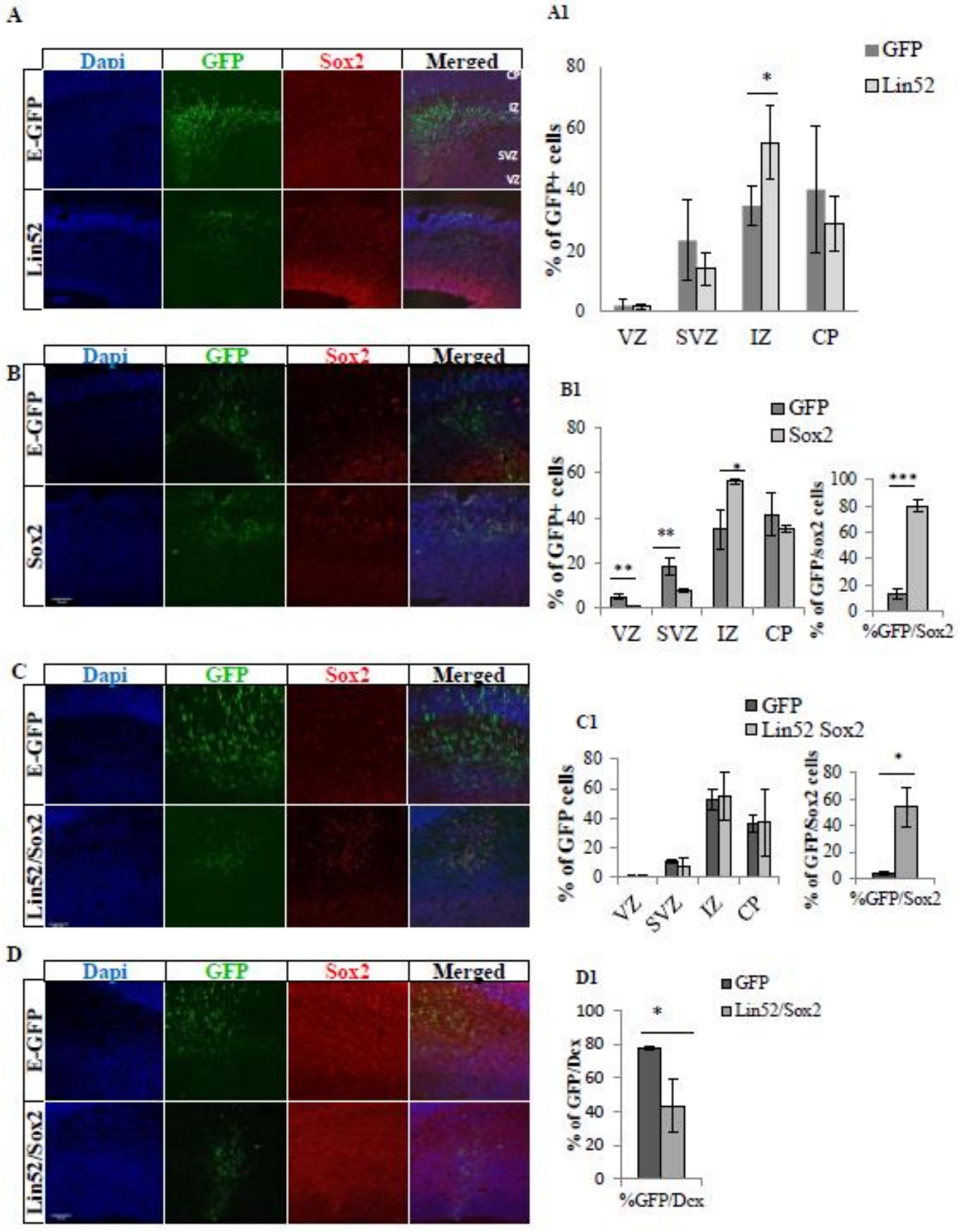

A
Dapi
GFP
Sox2
Merged
E-GFP
Lin52
CP
IZ
SVZ
VZ
A1
GFP
Lin52
% of GFP+ cells
VZ
SVZ
IZ
CP
B
Sox2
B1
% of GFP/Sox2 cells
%GFP/Sox2
C
Lin52/Sox2
C1
Lin52 Sox2
% of GFP cells
D
D1
% of GFP/Dcx
%GFP/Dcx

Figure 15 Simultaneous expression of Lin52/Sox2 prevents the cells from acquiring a neuronal fate.

Panel A: Representative confocal images following *in utero* electroporation of Lin52 and GFP in the dorsal forebrain of E13.5 WT embryos. Immunohistochemistry against GFP and Lin52 and counterstained with DAPI to detect the expression pattern of the electroporated cells within the (VZ), (SVZ), (IZ) and (CP). (A1) Graph shows the percentage of GFP+ cells within the different layers. Lin52 overexpression leads to a shift in the localization of the electroporated cells from the SVZ towards the IZ.
Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 75μm.
Panel B: Representative confocal images following *in utero* electroporation of Sox2 and GFP in the dorsal forebrain of E13.5 WT embryos. Immunohistochemistry against GFP and Sox2 and counterstained with DAPI to detect the expression pattern of the electroporated cells within the (VZ), (SVZ), (IZ) and (CP). (B1) Graph shows the percentage of GFP+ cells within the different layers (left) and the percentage of GFP/Sox2 cells (right). Sox2 overexpression leads to a shift in the localization of the electroporated cells from the VZ and SVZ towards the IZ. An increase in the percentage of GFP/Sox2 cells was detected after IUE of Sox2.
Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 75μm.
Panel C: Representative confocal images following *in utero* electroporation of Lin52/Sox2 and GFP in the dorsal forebrain of E13.5 WT embryos. Immunohistochemistry against GFP and Sox2 and counterstained with DAPI to detect the expression pattern of the electroporated cells within the (VZ), (SVZ), (IZ) and (CP). (C1) Graph shows the percentage of GFP+ cells within the different layers (left) and the percentage of GFP/Sox2 cells (right). No significant change in the expression pattern of the electroporated cells following Lin52/Sox2 IUE. An increase in the percentage of GFP/Sox2 cells was detected after IUE of Lin52/Sox2.
Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 75μm.
Panel D: Images show an immunohistochemistry against GFP and Dcx to determine the cell type and counterstained with DAPI. Graph showing the quantification of the percentage of GFP/Dcx positive cells following Lin52/Sox2 electroporation. A decrease in the percentage of GFP/Dcx cells was detected.
Mean± SEM, n=3, * p≤ 0.05, ** p≤ 0.01, ***p≤ 0.001. Scale bar = 75μm.

Altogether, these results support the findings of the in vivo experiments that suggest that DREAM complex regulate neurogenesis through the expression of the self-renewal marker Sox2. Neurons treated with harmine, which is the inhibitor of Dyrk1a kinase activity, maintained the expression of Sox2 throughout the days of the culture. This result indicates that the DREAM disassembly, caused by the harmine treatment activates the expression of Sox2. Indeed our ChIP experiments demonstrate that DREAM complex components share the binding at the promoters of novel target genes such as Sox2 and EZH2, in addition to the known classical genes. Furthermore, the simultaneous overexpression of Sox2 and Lin52 rescue the phenotype observed following the manipulation of Lin52. This indicates that Lin52 influences the expression of Sox2 through DREAM complex.

Discussion

4.1 Summary of results.

In this work, the role of DREAM complex was studied in the context of neurogenesis. We manipulated DREAM complex components and assessed their roles during neurogenesis using *in utero* electroporation technique *in vivo*. Overexpression of DREAM components including p130, Lin52, and Dyrk1a, which is the kinase required for the DREAM assembly (Litovchick et al. 2011), revealed that the DREAM components regulate the self-renewal capacity of neural stem cells and their function plays a key role to enable the neuronal differentiation. A change in the localization of the manipulated cells away from the ventricular zone was associated with a change in the cell fate. The expression of the self-renewal markers, such as Sox2, was decreased following the overexpression of the DREAM components, whereas an increase was observed in the expression of markers characterizing a more differentiated state, like Dcx. In the absence of p130/p107, which are members of the DREAM complex, no changes were noted following the overexpression of Dyrk1a. This indicates that Dyrk1a function on neurogenesis requires p130/p107 mediated DREAM complex assembly. We then assessed the DREAM complex function *in vitro* using harmine, an inhibitor for Dyrk1a kinase activity, which prevents Dyrk1a mediated DREAM complex assembly. In the harmine treated neurons, the Sox2 expression was elevated in contrast to the long term repression of Sox2 observed as neurons differentiated. Finally, our ChIP experiments revealed that DREAM components bind E2F consensus sites not only on classical cell cycle genes, but also on self-renewal genes including Sox2 and EZH2. Taken together all these findings demonstrate that DREAM functions to repress Sox2 expression to enable

neuronal differentiation. This is supported by rescue experiments, demonstrating that the re-expression of Sox2 was sufficient to rescue the phenotype observed following Lin52 overexpression in the progenitor population.

Altogether, these results support our hypothesis that the recruitment of DREAM complex plays a crucial role to regulate neurogenesis by mediating repression of not only cell cycle genes but also self-renewal genes such as Sox2. This is the first study that reveals that DREAM regulates stem cell self-renewal to enable differentiation.

4.2 Dyrk1a promotes neuronal differentiation of neural progenitor cells.

Our first goal in this study was to examine the effect of DREAM complex on neurogenesis. Dyrk1a phosphorylates Lin52 on Serine 28 (Litovchick et al. 2007); this phosphorylation is required for the DREAM complex assembly. Previous studies revealed a role for Dyrk1a in the regulation of neurogenesis (Tejedor et al. 1995; Hämmerle, Elizalde, and Tejedor 2008; Tejedor and Hämmerle 2011; Hämmerle et al. 2011; Soppa et al. 2014). Our results showed that Dyrk1a is considered a key regulator during neurogenesis, which is consistent with previous findings. Using *in utero* electroporation experiments, we showed that the overexpression of Dyrk1a enhanced neurogenesis through a change in the localization of the electroporated cells from the proliferative zone (ventricular zone) towards more differentiated zone (sub-ventricular and intermediate zone), while gaining neuronal markers such as Dcx. As predicted, the mutant Dyrk1a kinase did not affect the identity of the manipulated cells when compared to the control, indicating that Dyrk1a function relies on its kinase activity. These findings concur with a number of studies demonstrating that Dyrk1a plays a crucial role during neurogenesis. Litovchick et al. showed that Dyrk1a is a negative regulator of the cell

cycle; its overexpression in various cell lines inhibits cell proliferation through the promotion of a quiescent state (Litovchick et al. 2011). Dyrk1a controls proliferation and neuronal differentiation through the phosphorylation of the cell cycle regulator Cyclin D1 and the CDK inhibitors $p27^{kip1}$ (Yabut, Domogauer, and D'Arcangelo 2010; Hämmerle et al. 2011; Soppa et al. 2014). Dyrk1a gain-of-function prevents cell proliferation by inducing the expression of $p27^{kip1}$, acting as the main negative regulator of the cell cycle of neurons. The same group showed also that Dyrk1a loss-of-function provokes cell proliferation and death through the decrease of the expression of $p27^{kip1}$ (Hämmerle et al. 2011). Furthermore, Dyrk1a overexpression can inhibit the cell cycle progression, induce the cell cycle exit and the premature differentiation of neural progenitors through the nuclear export and degradation of the Cyclin D1 (Yabut, Domogauer, and D'Arcangelo 2010). These studies show that Dyrk1a manipulated in mid-corticogenesis regulates neural proliferation and differentiation. However, recent experiments conducted at the onset of neurogenesis revealed that modest Dyrk1a overexpression does not disturb the birth of neurons (Kurabayashi and Sanada 2013). Dyrk1a levels, whether increased or reduced, contributes to altered neurogenesis. Thus, Dyrk1a function and effect appear to depend on its dosage and the time of its manipulation. Normalization of Dyrk1a levels appears to be an effective strategy to restore Dyrk1a-associated imbalanced neurogenesis.

Interestingly, Dyrk1a gene is located within the Down syndrome (DS) critical region (Guimera et al. 1999; Hämmerle et al. 2003). Its triplicate expression contributes to the altered neurogenesis observed in the DS models (Dierssen and de Lagrán 2006). The role of Dyrk1a associated to the mental retardation in the DS models was supported by several studies using many lines of transgenic mice. The overexpression of Dyrk1a in

normal mouse was sufficient and able to provoke defects in learning and memory, and in brain structure, suggesting an essential role for Dyrk1a in mental retardation (Ahn et al. 2006).

In this thesis, we not only replicated the previous findings but we showed that Dyrk1a-mediated effects on neurogenesis function through its action on the DREAM complex, through phosphorylation of Lin52. Importantly, we also show that Dyrk1a-mediated function on neurogenesis requires the participation of p130/p107, a novel function for these pocket proteins and the DREAM complex.

4.3 Lin52 phosphorylation by Dyrk1a promotes neurogenesis.

Among many substrates phosphorylated by Dyrk1a, Lin52 is considered a key target for DREAM assembly (Litovchick et al. 2007; Guiley et al. 2015). Our results show a change in the laminar fate of the electroporated cells where Lin52 was overexpressed (Figure 9). These cells exhibited a change in their cell fate: they moved from the ventricular zone (proliferative) towards the sub-ventricular and intermediate zones (differentiated). Conversely, the phosphorylation-deficient mutant Lin52 provokes an opposite phenotype. The majority of the cells expressing the mutant Lin52 were found in the proliferative zones and maintained the expression of self-renewal markers such as Sox2. These findings support the hypothesis that Lin52 plays an essential role in the regulation of neurogenesis.

Lin52 was identified as a subunit of the *drosophila* DREAM complex (Lewis et al. 2004). Lin52 was also described as an interacting partner with the Rb family in C. *elegans* (Thomas et al. 2003). Recently, several groups showed that Lin52 is a key member of the DREAM complex in human cell lines (Litovchick et al. 2007; Schmit et

al. 2007), its role in the DREAM assembly induces the cells to enter quiescence and inhibits the expression of the majority of cell cycle genes (Litovchick et al. 2011). Lin52 ectopic expression influences the cell cycle allowing the cells to remain in G0 phase and prevents their entry into S-phase. Consistent with the negative role on the cell cycle progression and the promotion of quiescence of Lin52-mediated DREAM assembly; our results show that the overexpression of Lin52 *in vivo* provokes a decrease in the expression of the self-renewal markers such as Sox2 and the acquisition of neuronal markers. This indicates that Lin52 overexpression may function, beyond cell cycle regulation, and regulate the expression of genes that control the self-renewal-differentiation decision.

4.4 P130 promotes neurogenesis through DREAM assembly and is required for Dyrk1a-mediated enhanced neurogenesis.

We examined the effect of p130, a member of the DREAM complex, on neurogenesis using *in utero* electroporations. We observed that most of the cells manipulated with p130 were found in the intermediate zone with a decrease in the expression of Sox2, suggesting that these cells were committed toward differentiation.

Aside the other members of the pocket protein family, p130 has been shown to interact with E2F family of transcription factors to regulate the expression of genes required for cell cycle progression in G0 and in early G1 (Cobrinik 2005; Dick and Rubin 2013). The overexpression of pRb and p130 induced the differentiation of neural stem cells *in vitro* through their ability to regulate the cell fate and identity (Jori et al. 2004). Unpublished data from our lab showed that the absence of all three pocket proteins provoked an increase in the expression of Sox2 levels, whereas the absence of only pRb

did not affect the levels of Sox2 (Figure 5). The results from this thesis suggest a unique role for p130/p107 in the regulation of the cell fate and cell identity, in part through the regulation of Sox2 expression.

Recently, several studies showed that p130/p107 are members of the DREAM complex (Schmit et al. 2007; Litovchick et al. 2007). p130/p107 interact with E2F4/5 and Dp, altogether are recruited to the MuvB core through the direct interaction between p130/p107 with the phosphorylated form of Lin52 (Litovchick et al. 2007; Schmit et al. 2007; Guiley et al. 2015). Our lab performed immunoprecipitation experiments showing a direct interaction between p130 and Lin52 in neurons (Figure 7). We hypothesized that DREAM complex requires p130 to promote complex assembly to facilitate neurogenesis. To answer this question, we manipulated Dyrk1a in the absence of p130 or p107. We wanted to know if p130/p107 are required for DREAM function. Our results revealed that p130/p107 are required for Dyrk1a to enhance neurogenesis. When p130/p107 are absent, we expect the DREAM assembly is prevented.

4.5 Inhibition of Dyrk1a kinase activity results in de-repression of pluripotency gene Sox2 in post-mitotic neurons.

We wanted to examine the effect of the DREAM complex on neurogenesis *in vitro* through the inhibition of Dyrk1a. We used harmine which is extracted from plants and known for its capacity to inhibit specifically Dyrk1a among other kinases (Becker and Sippl 2011). In order to show that the harmine-mediated inhibition of Dyrk1a suppresses its kinase activity, immunoblots were performed to reveal changes in the Lin52 phosphorylation level (Figure 12). Following harmine treatment, we detected a decrease in the level of phosphorylated Lin52. In agreement with these findings,

Litovchick et al. showed that Lin52 phosphorylated form decreases following harmine treatment (Litovchick et al. 2011). The same group revealed also that unphosphorylated form of Lin52 will still able to interact with B-Myb to form the MMB complex which activates the expression of late cell cycle genes and promotes the activity of FoxM1 (Sadasivam, Duan, and DeCaprio 2012; Sadasivam and DeCaprio 2013). Following the harmine treatment Dyrk1a kinase activity is inhibited allowing us to hypothesize that the Lin52 phosphorylated form is less available to interact with p130 to enable DREAM assembly.

Harmine treatment was shown to prevent premature maturation of neuronal progenitors isolated from the Ts65Dn mouse, the mouse model for Down Syndrome where Dyrk1a is overexpressed (Mazur-Kolecka et al. 2012). Moreover, harmine and other Dyrk inhibitors have been shown to reduce the number of phosphorylated forms of the Tau protein, which is an important factor in the pathological progression of Alzheimer's disease. Thus, inhibition of Dyrk1a activity may be considered a novel therapeutic approach for several diseases where this kinase is involved (Zhong, Tao, and Yang 2015; Dakic et al. 2016; Dirice et al. 2016; Kim et al. 2016).

Furthermore, our neuronal cultures treated with harmine display shorter neurites. In accordance with this observation, Göckler et al. showed that Dyrk1a is involved in the regulation of neurite formation (Göckler et al. 2009). Altogether our *in vivo* data suggest a potential role for the components of DREAM complex including p130, Lin52 and Dyrk1a in the cell fate determination. Harmine inhibits DREAM assembly through the inhibition of Dyrk1a kinase activity. Primary neurons treated with harmine maintained the expression of the self-renewal marker Sox2 after five days from plating. Conversely,

untreated neurons downregulated the Sox2 expression as cells committed to differentiation. Our *in vitro* findings are in accordance with our *in vivo* data that suggests a role for the DREAM complex in the cell fate determination, possibly through the regulation of the Sox2 expression.

4.6 DREAM complex regulates Sox2 expression

The mechanism responsible for regulating self-renewal genes in post-mitotic and differentiated cells is not fully understood. We showed that the cells overexpressing DREAM complex components including p130, Lin52 and Dyrk1a express low levels of Sox2 and therefore we investigated whether DREAM complex influences Sox2 expression. To examine the presence of DREAM complex members on the Sox2 promoters, we performed ChIP assays using antibodies against p130, E2F4, Lin9, Lin54 and RbBp4 in WT neurons. The results showed here demonstrate the binding of the five proteins members of the DREAM complex to the Sox2 promoter. These findings suggest a novel function for DREAM complex as a repressor for the pluripotency gene Sox2 in addition to the known repression of a large number of cell cycle genes (Litovchick et al. 2007; Sadasivam and DeCaprio 2013).

As mentioned above, Sox2 is considered an intrinsic factor controlling the self-renewal capacity, the pluripotency and lineage specification (Wegner 1999; Bani-Yaghoub et al. 2006). Several regulatory elements located in the Sox2 locus such as the Sox2 promoter and several enhancers have been shown to be functional in the regulation of Sox2 expression (Uchikawa et al. 2003; Catena et al. 2004; Lengler et al. 2005). In neural precursor cells, a number of transcription factors including Pax6 and Prox1, which are required for neural development and differentiation, are involved in the regulation of

Sox2 expression (Lengler et al. 2005). These transcription factors bind the enhancer sequences of Sox2 in order to activate its expression, which suggests a positive effect of these factors on the Sox2 expression. In addition, other signalling pathways such as Akt and Stat3 and cell cycle regulators including E2F3a and E2F3b have been shown to regulate Sox2 expression (Peltier et al. 2011; Foshay and Gallicano 2008; Julian et al. 2013:2). It has been shown that E2F3a with p107 repress the expression of Sox2 and therefore affect self-renewal capacity and promote differentiation. However, E2F3b activates the Sox2 expression and promotes the self-renewal fate. Given the important functions of Sox2 for the maintenance of pluripotency and the lineage specification, diverse mechanisms cooperate together to regulate its expression. Here we suggested the DREAM complex is a novel regulator of the Sox2 expression by mediating its long-term repression to enable stem cell commitment to differentiation and neuronal maturation. To further investigate the role of DREAM complex in regulating Sox2 expression, we overexpressed Lin52 and Sox2 simultaneously to observe whether their overexpression will rescue the enhanced neurogenesis observed following the overexpression of Lin52 alone. Cells overexpressing Lin52 moved out of the ventricular zone while exhibiting a decrease in the expression of the self-renewal marker Sox2 accompanied by an increase in the expression of the newborn neurons marker Dcx (Figure 15). To confirm that Lin52 was functioning through the repression of Sox2, Lin52/Sox2 were simultaneously overexpressed, which was able to rescue the phenotype observed previously following Lin52 overexpression. This observation indicates that the re-expression of Sox2 was sufficient to prevent the premature differentiation of the Lin52 electroporated and maintained the undifferentiated state.

Taken together these findings reveal a novel and important role of DREAM complex in the regulation of the self-renewal genes in addition to the known function in the control of cell cycle genes. In addition to its known functions in silencing cell cycle genes during quiescence (Litovchick et al. 2011), we showed that DREAM plays an essential role during neurogenesis by regulating Sox2, which affects the maintenance of stem cell self-renewal. DREAM is considered an attractive therapeutic target due to the possibility of manipulating its assembly by pharmacologically targeting Dyrk1a.

Down syndrome (DS) is a genetic disorder caused by a complete or partial trisomy of the chromosome 21, and is associated with intellectual disability affecting learning and memory (Wiseman et al. 2009; Lott and Dierssen 2010). DS is also characterized by neurological abnormalities including reduced brain size and weight, impaired cell proliferation and neurogenesis (Coyle, Oster-Granite, and Gearhart 1986; Golden and Hyman 1994). These structural defects have been linked to intellectual disability, which limits the cognitive function of the individuals with DS and affect their quality of life. Several genes located on the chromosome 21 are involved in brain development, metabolism and neuronal networks. Among these genes, Dyrk1a was considered an important contributor to the brain features and cognitive deficits characterizing DS (Dowjat et al. 2007; Liu et al. 2008). These studies also suggested that the trisomy of Dyrk1a is linked to the mental retardation in subjects with DS. Consequently, Dyrk1a was considered an important therapeutic drug target for DS. A number of kinase inhibitors were developed in the past decades including harmine, EGCG, INDY and FINDY (Becker and Sippl 2011). However the administration of these molecules faced limitations due to side effects or off-target effects. Interestingly, Dyrk1a

displays a sophisticated spatio-temporal expression during development. Therefore, further studies have to be performed to determine the correct timing and the level of Dyrk1a required at specific developmental stages.

In regards to the defects in the self-renewal capacity, DS patients display a perturbation in stem and progenitor cell growth. Indeed, neurospheres derived from DS subjects exhibited a reduction in cell proliferation rate (Lu et al. 2012). This observation showing that the proliferation is altered at an early stage of the neurogenesis. We may speculate a role for Dyrk1a mediated through DREAM complex and affecting the self-renewal capacity and the proliferation rate of neural precursor population.

Various mouse models were also developed to expand our knowledge in the molecular mechanism underlying the phenotypes observed in subjects with DS (Gupta, Dhanasekaran, and Gardiner 2016). TS65Dn is the best studied mouse model for DS displaying a trisomic region of the homologue of the chromosome 21 where Dyrk1a gene is located. This model revealed an impaired production of neurons early in neurogenesis and the growth delay of the cortical wall. The growth delay was associated with the lengthening of the cell cycle in the ventricular layer (Chakrabarti, Galdzicki, and Haydar 2007). However, more recent experiments conducted at the onset of neurogenesis revealed that modest Dyrk1a overexpression does not disturb the birth of neurons (Kurabayashi and Sanada 2013).

Subjects with DS are at high risk of developing dementia and other histopathology observed in Alzheimer's disease (Lai and Williams 1989). Among the genes located on the chromosome 21, the amyloid precursor protein (APP) gene product is described as the main biomarker for Alzheimer's disease (Goldgaber et al. 1987).

However, other genes located on chromosome 21 may also be involved in the early onset of Alzheimer's disease in the DS subjects. A DS mouse model, that is independent of the amyloid precursor protein revealed the presence of hyperphosphorylated Tau protein, which is a marker for Alzheimer's disease (Shukkur et al. 2006). Moreover, Dyrk1a may be involved in the onset of Alzheimer's disease in DS patients as it may contribute to the phosphorylation of Tau allowing for its hyperphosphorylation (Ryoo et al. 2007; Liu et al. 2008), to splicing of Tau (Shi et al. 2008) and the phosphorylation of APP (Ryoo et al. 2008). Therefore, normalization of Dyrk1a levels may contribute to the improvements of various deficits observed in many diseases such as Down syndrome and Alzheimer's disease.

FUTURE DIRECTIONS

Here, we showed that DREAM complex affects the maintenance of stemness and the cell fate decision through its capacity to regulate the expression of the self-renewal marker Sox2 during development. We showed that DREAM influence the stem cell population and the cell fate decision during neurogenesis. It is also essential to investigate the role of DREAM complex during adult neurogenesis. Its function may be crucial to maintaining the quiescence of the stem cell pool as this pool is required to self-renew and give rise to newborn neurons when needed. Understanding the role of DREAM in adult neurogenesis will be important in the development of new treatments that may require the activation of the long-term maintenance of the quiescent stem cell pool.

ChIP experiments could be performed to reveal the binding of the methylation markers on the promoters of targets genes and to examine their level of activation and repression. It would also be interesting to perform the ChIP experiments following the manipulation of DREAM components *in vitro* to reveal any change in their binding to the targets genes. Besides, neurosphere assays could also be performed to examine the self-renewal capacity of the stem cells isolated from 130/p107 double knockout mice. Rescue experiments could be performed in the Down syndrome model where Dyrk1a gene is present in triplicate. We have initiated the maintenance of colonies of the Ts65Dn mouse model from Jackson's Laboratory. Given that our findings indicate that DREAM regulates the expression of Sox2, we hypothesize that the re-expression of Sox2, through the manipulation of DREAM assembly, may rescue the impairment in neurogenesis found in DS due to the overexpression of Dyrk1a.

CONCLUSION

The work presented in this thesis provides a novel pathway involved in the regulation of stemness and cell fate. We showed that DREAM complex components are involved in the regulation of neurogenesis. The overexpression of p130 and Lin52, both members of the DREAM complex, and Dyrk1a, which is the kinase required for Lin52 phosphorylation, promotes a change in the localization of the manipulated cells from proliferative zone towards more differentiated zone, associated with a decrease in Sox2 expression; the cells were committed towards differentiation. Furthermore, we showed that Dyrk1a function requires p130/p107, members of the DREAM complex. Using harmine, an inhibitor of Dyrk1a activity leading to DREAM disassembly, a decrease in the level of Lin52 phosphorylated form was associated with the maintenance of expression of the self-renewal marker Sox2. This finding supports the *in vivo* observations that the manipulation of DREAM influences the expression of Sox2. Our ChIP results revealed that DREAM components share the binding on the promoters of the self-renewal marker Sox2 and EZH2 in addition to the binding on the promoters of classical cell cycle genes.

This work revealed a novel function for the p130 mediated DREAM complex. DREAM complex components are involved in the cell fate determination during neurogenesis through the control of the expression of self-renewal marker gene Sox2.

CITATIONS

Ahn, Kyoung-Jin, Hey Kyeong Jeong, Han-Saem Choi, et al.
2006 DYRK1A BAC Transgenic Mice Show Altered Synaptic Plasticity with Learning and Memory Defects. Neurobiology of Disease 22(3): 463–472.

Bani-Yaghoub, Mahmud, Roger G. Tremblay, Joy X. Lei, et al.
2006 Role of Sox2 in the Development of the Mouse Neocortex. Developmental Biology 295(1): 52–66.

Becker, Walter, and Wolfgang Sippl
2011 Activation, Regulation, and Inhibition of DYRK1A. FEBS Journal 278(2): 246–256.

Catena, Raffaella, Cecilia Tiveron, Antonella Ronchi, et al.
2004 Conserved POU Binding DNA Sites in the Sox2 Upstream Enhancer Regulate Gene Expression in Embryonic and Neural Stem Cells. Journal of Biological Chemistry 279(40): 41846–41857.

Chakrabarti, Lina, Zygmunt Galdzicki, and Tarik F. Haydar
2007 Defects in Embryonic Neurogenesis and Initial Synapse Formation in the Forebrain of the Ts65Dn Mouse Model of Down Syndrome. Journal of Neuroscience 27(43): 11483–11495.

Cobrinik, David
2005 Pocket Proteins and Cell Cycle Control. Oncogene 24(17): 2796–2809.

Coyle, J. T., M. L. Oster-Granite, and J. D. Gearhart
1986 The Neurobiologie Consequences of down Syndrome. Brain Research Bulletin 16(6): 773–787.

Dakic, Vanja, Renata de Moraes Maciel, Hannah Drummond, et al.
2016 Harmine Stimulates Proliferation of Human Neural Progenitors. PeerJ 4. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5144684/.

Dick, Frederick A., and Seth M. Rubin
2013 Molecular Mechanisms Underlying RB Protein Function. Nature Reviews Molecular Cell Biology 14(5): 297–306.

Dierssen, M., and M. Martínez de Lagrán
2006 DYRK1A (Dual-Specificity Tyrosine-Phosphorylated and -Regulated Kinase 1A): A Gene with Dosage Effect During Development and Neurogenesis. The Scientific World Journal 6: 1911–1922.

Dirice, Ercument, Deepika Walpita, Amedeo Vetere, et al.
2016 Inhibition of DYRK1A Stimulates Human Beta-Cell Proliferation. Diabetes.

Dowjat, Wieslaw K., Tatyana Adayev, Izabela Kuchna, et al.
2007 Trisomy-Driven Overexpression of DYRK1A Kinase in the Brain of Subjects with Down Syndrome. Neuroscience Letters 413(1): 77–81.

Foshay, Kara M., and G. Ian Gallicano
2008 Regulation of Sox2 by STAT3 Initiates Commitment to the Neural Precursor Cell Fate. Stem Cells and Development 17(2): 269–278.

Göckler, Nora, Guillermo Jofre, Chrisovalantis Papadopoulos, et al.
2009 Harmine Specifically Inhibits Protein Kinase DYRK1A and Interferes with Neurite Formation. FEBS Journal 276(21): 6324–6337.

Golden, Jeffrey A., and Bradley T. Hyman
1994 Development of the Superior Temporal Neocortex Is Anomalous in Trisomy 21. Journal of Neuropathology & Experimental Neurology 53(5): 513–520.

Goldgaber, D., M. I. Lerman, O. W. McBride, U. Saffiotti, and D. C. Gajdusek
1987 Characterization and Chromosomal Localization of a cDNA Encoding Brain Amyloid of Alzheimer's Disease. Science (New York, N.Y.) 235(4791): 877–880.

Guiley, Keelan Z., Tyler J. Liban, Jessica G. Felthousen, et al.
2015 Structural Mechanisms of DREAM Complex Assembly and Regulation. Genes & Development 29(9): 961–974.

Guimera, J., C. Casas, X. Estivill, and M. Pritchard
1999 Human Minibrain Homologue (MNBH/DYRK1): Characterization, Alternative Splicing, Differential Tissue Expression, and Overexpression in Down Syndrome. Genomics 57(3): 407–418.

Gupta, Meenal, A. Ranjitha Dhanasekaran, and Katheleen J. Gardiner
2016 Mouse Models of Down Syndrome: Gene Content and Consequences. Mammalian Genome: Official Journal of the International Mammalian Genome Society 27(11–12): 538–555.

Hämmerle, B., C. Elizalde, J. Galceran, W. Becker, and F. J. Tejedor
2003 The MNB/DYRK1A Protein Kinase: Neurobiological Functions and Down Syndrome Implications. Journal of Neural Transmission. Supplementum(67): 129–137.

Hämmerle, Barbara, Carina Elizalde, and Francisco J. Tejedor
2008 The Spatio-temporal and Subcellular Expression of the Candidate Down Syndrome Gene Mnb/Dyrk1A in the Developing Mouse Brain Suggests Distinct

Sequential Roles in Neuronal Development. European Journal of Neuroscience 27(5): 1061–1074.

Hämmerle, Barbara, Edgar Ulin, Jordi Guimera, et al.
2011 Transient Expression of Mnb/Dyrk1a Couples Cell Cycle Exit and Differentiation of Neuronal Precursors by Inducing p27KIP1 Expression and Suppressing NOTCH Signaling. Development (Cambridge, England) 138(12): 2543–2554.

Jori, F. P., M. a. B. Melone, M. A. Napolitano, et al.
2004 RB and RB2/p130 Genes Demonstrate Both Specific and Overlapping Functions during the Early Steps of in Vitro Neural Differentiation of Marrow Stromal Stem Cells. Cell Death & Differentiation 12(1): 65–77.

Julian, Lisa M, Renaud Vandenbosch, Catherine A Pakenham, et al.
2013 Opposing Regulation of Sox2 by Cell-Cycle Effectors E2f3a and E2f3b in Neural Stem Cells. Cell Stem Cell 12(4): 440–452.

Kim, Hyeongki, Kyu-Sun Lee, Ae-Kyeong Kim, et al.
2016 A Chemical with Proven Clinical Safety Rescues Down-Syndrome-Related Phenotypes in through DYRK1A Inhibition. Disease Models and Mechanisms 9(8): 839–848.

Kurabayashi, Nobuhiro, and Kamon Sanada
2013 Increased Dosage of DYRK1A and DSCR1 Delays Neuronal Differentiation in Neocortical Progenitor Cells. Genes & Development 27(24): 2708–2721.

Kwan, Kenneth Y., Nenad Šestan, and E. S. Anton
2012 Transcriptional Co-Regulation of Neuronal Migration and Laminar Identity in the Neocortex. Development 139(9): 1535–1546.

Lai, Florence, and Roger S. Williams
1989 A Prospective Study of Alzheimer Disease in Down Syndrome. Archives of Neurology 46(8): 849–853.

Lengler, Johannes, Tobias Bittner, Doris Münster, Alaa El-Din A. Gawad, and Jochen Graw
2005 Agonistic and Antagonistic Action of AP2, Msx2, Pax6, Prox1 and Six3 in the Regulation of Sox2 Expression. Ophthalmic Research 37(6): 301–309.

Lewis, Peter W., Eileen L. Beall, Tracey C. Fleischer, et al.
2004 Identification of a Drosophila Myb-E2F2/RBF Transcriptional Repressor Complex. Genes & Development 18(23): 2929–2940.

Litovchick, L., L. A. Florens, S. K. Swanson, M. P. Washburn, and J. A. Decaprio
2011 DYRK1A Protein Kinase Promotes Quiescence and Senescence through DREAM Complex Assembly. Genes and Development 25(8): 801–813.

Litovchick, Larisa, Subhashini Sadasivam, Laurence Florens, et al.
2007 Evolutionarily Conserved Multisubunit RBL2/p130 and E2F4 Protein Complex Represses Human Cell Cycle-Dependent Genes in Quiescence. Molecular Cell 26(4): 539–551.

Liu, Fei, Zhihou Liang, Jerzy Wegiel, et al.
2008 Overexpression of Dyrk1A Contributes to Neurofibrillary Degeneration in Down Syndrome. The FASEB Journal 22(9): 3224–3233.

Lott, Ira T, and Mara Dierssen
2010 Cognitive Deficits and Associated Neurological Complications in Individuals with Down's Syndrome. The Lancet Neurology 9(6): 623–633.

Lu, Jie, Gewei Lian, Hui Zhou, et al.
2012 OLIG2 over-Expression Impairs Proliferation of Human Down Syndrome Neural Progenitors. Human Molecular Genetics 21(10): 2330–2340.

Manzini, M. Chiara, and Christopher A. Walsh
2011 What Disorders of Cortical Development Tell Us about the Cortex: One plus One Doesn't Always Make Two. Current Opinion in Genetics & Development 21(3): 333–339.

Mazur-Kolecka, Bozena, Adam Golabek, Elizabeth Kida, et al.
2012 Effect of DYRK1A Activity Inhibition on Development of Neuronal Progenitors Isolated from Ts65Dn Mice. Journal of Neuroscience Research 90(5): 999–1010.

Musa, Julian, Marie-Ming Aynaud, Olivier Mirabeau, Olivier Delattre, and Thomas GP Grünewald
2017 MYBL2 (B-Myb): A Central Regulator of Cell Proliferation, Cell Survival and Differentiation Involved in Tumorigenesis. Cell Death & Disease 8(6): e2895.

Peltier, Joseph, Anthony Conway, Albert J. Keung, and David V. Schaffer
2011 Akt Increases Sox2 Expression in Adult Hippocampal Neural Progenitor Cells, but Increased Sox2 Does Not Promote Proliferation. Stem Cells and Development 20(7): 1153–1161.

Rubenstein, John L.R.
2011 Development of the Cerebral Cortex: Implications for Neurodevelopmental Disorders. Journal of Child Psychology and Psychiatry, and Allied Disciplines 52(4): 339–355.

Ryoo, Soo-Ryoon, Hyun-Jeong Cho, Hye-Won Lee, et al.
2008 Dual-Specificity tyrosine(Y)-Phosphorylation Regulated Kinase 1A-Mediated Phosphorylation of Amyloid Precursor Protein: Evidence for a Functional Link between

Down Syndrome and Alzheimer's Disease. Journal of Neurochemistry 104(5): 1333–1344.

Ryoo, Soo-Ryoon, Hey Kyeong Jeong, Chinzorig Radnaabazar, et al.
2007 DYRK1A-Mediated Hyperphosphorylation of Tau. A Functional Link between Down Syndrome and Alzheimer Disease. The Journal of Biological Chemistry 282(48): 34850–34857.

Sadasivam, Subhashini, and James A. DeCaprio
2013 The DREAM Complex: Master Coordinator of Cell Cycle Dependent Gene Expression. Nature Reviews. Cancer 13(8): 585–595.

Sadasivam, Subhashini, Shenghua Duan, and James A. DeCaprio
2012 The MuvB Complex Sequentially Recruits B-Myb and FoxM1 to Promote Mitotic Gene Expression. Genes & Development 26(5): 474–489.

Schmit, Fabienne, Michael Korenjak, Mirijam Mannefeld, et al.
2007 LINC, a Human Complex That Is Related to pRB-Containing Complexes in Invertebrates Regulates the Expression of G2/M Genes. Cell Cycle 6(15): 1903–1913.

Shi, Jianhua, Tianyi Zhang, Chunlei Zhou, et al.
2008 Increased Dosage of Dyrk1A Alters Alternative Splicing Factor (ASF)-Regulated Alternative Splicing of Tau in Down Syndrome. The Journal of Biological Chemistry 283(42): 28660–28669.

Shukkur, Ebrahim Abdul, Atsushi Shimohata, Takumi Akagi, et al.
2006 Mitochondrial Dysfunction and Tau Hyperphosphorylation in Ts1Cje, a Mouse Model for Down Syndrome. Human Molecular Genetics 15(18): 2752–2762.

Soppa, Ulf, Julian Schumacher, Victoria Florencio Ortiz, et al.
2014 The Down Syndrome-Related Protein Kinase DYRK1A Phosphorylates p27Kip1 and Cyclin D1 and Induces Cell Cycle Exit and Neuronal Differentiation. Cell Cycle 13(13): 2084–2100.

Tejedor, F., X. R. Zhu, E. Kaltenbach, et al.
1995 Minibrain: A New Protein Kinase Family Involved in Postembryonic Neurogenesis in Drosophila. Neuron 14(2): 287–301.

Tejedor, Francisco J., and Barbara Hämmerle
2011 MNB/DYRK1A as a Multiple Regulator of Neuronal Development. FEBS Journal 278(2): 223–235.

Thomas, Jeffrey H, Craig J Ceol, Hillel T Schwartz, and H Robert Horvitz
2003 New Genes That Interact with Lin-35 Rb to Negatively Regulate the Let-60 Ras Pathway in Caenorhabditis Elegans. Genetics 164(1): 135–151.

Uchikawa, Masanori, Yoshiko Ishida, Tatsuya Takemoto, Yusuke Kamachi, and Hisato Kondoh
2003 Functional Analysis of Chicken Sox2 Enhancers Highlights an Array of Diverse Regulatory Elements That Are Conserved in Mammals. Developmental Cell 4(4): 509–519.

Wegner, M.
1999 From Head to Toes: The Multiple Facets of Sox Proteins. Nucleic Acids Research 27(6): 1409–1420.

Wiseman, Frances K., Kate A. Alford, Victor L.J. Tybulewicz, and Elizabeth M.C. Fisher
2009 Down Syndrome—recent Progress and Future Prospects. Human Molecular Genetics 18(R1): R75–R83.

Yabut, Odessa, Jason Domogauer, and Gabriella D'Arcangelo
2010 Dyrk1A Overexpression Inhibits Proliferation and Induces Premature Neuronal Differentiation of Neural Progenitor Cells. The Journal of Neuroscience 30(11): 4004–4014.

Zhong, Zeqi, Yuan Tao, and Hui Yang
2015 Treatment with Harmine Ameliorates Functional Impairment and Neuronal Death Following Traumatic Brain Injury. Molecular Medicine Reports. http://www.spandidos-publications.com/10.3892/mmr.2015.4437, accessed April 12, 2016.