AMERICAN UNIVERSITY OF BEIRUT

CHARACTERIZATION OF THE MOLECULAR MECHANISMS BY WHICH THE RB/E2F PATHWAY REGULATES ADULT NEUROGENESIS IN THE OLFACTORY BULB

by
NOUR NABIL HALABY

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
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AMERICAN UNIVERSITY OF BEIRUT

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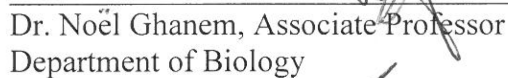

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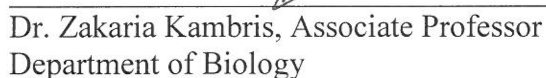

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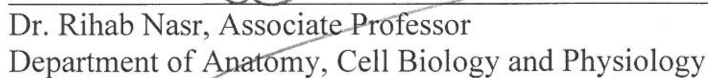

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Ever since my childhood, I’ve been enthralled with science and this fascination played a major role in studying Biology. During my bachelors, I was amazed by how systematic our human body is, and how a single organ was at the center of all body functions, the brain. Intrigued by the complexity of a fundamental part of our body, I pursued my masters in Biology with a focus on Neuroscience. Cajal once said, "The brain is a world consisting of a number of unexplored continents and great stretches of unknown territory”, and thus I am more than ready to plunge myself, along with high caliber scientists, into exploring this attractive unknown territory.

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I still remember my happy days at the 303 lab. It is because of you my colleagues, the NG lab team! Thank you for being a wonderful family, for helping me, answering my questions and teaching me. I want to thank first the soon-to-be Dr. Carine Jaafar (the mother of the lab) for being my “co-PI”. I never met someone as patient and helpful as you are. Someone that is capable of explaining difficult ideas in an easy way as you did. You are simply the best and you give a beautiful image of women in science! Second, I want to thank the encyclopedia and google of the lab, future Dr. Saad Omais. When I first came to the lab, they told me that this guy has an answer to any scientific question you may ask, and this was true. Up till now, I still can’t understand from where you get all the information you have! You are the real meaning of a scientist. Moreover, I want to thank the newest lab member Anthony Bejjani for explaining to me many things that I couldn’t understand without his help, for being my gym mate and a great friend (even though you get on my nerves sometimes). Last but not least, I would like to thank Sawsan Al Lafi,

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// AN ABSTRACT OF THE THESIS OF

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Title: Characterization of the molecular mechanisms by which the Rb/E2F pathway regulates adult neurogenesis in the olfactory bulb

Adult neurogenesis is an ongoing developmental process that is persistent in two major sites in the mammalian brain throughout life: the adult subventricular zone (aSVZ) linked to the olfactory bulb (OB) and the subgranular zone in the dentate gyrus of the hippocampus. Adult neural stem cells (aNSCs) are relatively quiescent populations that give rise to distinct neuronal subtypes throughout life, yet, at a very low rate and restricted differentiation potential. The Retinoblastoma protein (Rb) is a key cell cycle protein that controls distinct aspects of neurogenesis during development. We have recently shown that targeted loss of Rb in adult neural stem and progenitor cells (NSPCs) leads to a specific increase in progenitor proliferation in the aSVZ but does not affect aNSCs self-renewal nor neuronal migration or terminal differentiation. These findings were also replicated in culture using neurosphere assays derived from Rb-/- versus Rb+/- aSVZ tissues. However, Rb is required for the long-term survival of newborn adult OB neurons. Yet, the molecular mechanisms mediating these Rb functions in the adult brain are still unknown.

Previous studies have shown that during brain development: 1) Rb regulates neuronal survival through its interaction with E2F1 and/or E2F3 depending on the cell type, 2) each of p107 and E2F3 mediate neural progenitor responsiveness to growth factors specifically through the direct control of the gene encoding the fibroblast growth factor 2 (FGF2) ligand, 3) p130 primarily maintains neuronal survival and inhibits cell cycle re-entry in mature neurons. Moreover, loss of E2F1 impairs adult neurogenesis while p107 negatively regulates self-renewal of aNSCs found in the SVZ.

In this study, we investigated whether FGF2, E2F1 and/or E2F3 could be mediating the control of adult neurogenesis by Rb, typically progenitor proliferation control. We also examined potential compensatory role(s) played by two Rb family members, p107 and p130, during neuronal differentiation and/or survival in the absence of Rb. To do this, we assessed and quantified the changes in gene expression levels of these Rb target genes in young Rb conditional Knock-out mice. Our data indicate that the Rb/E2F pathway and the two other Rb pocket proteins play conserved roles in the control of adult neurogenesis at least partially.

Uncovering and manipulating the molecular mechanisms controlling adult neurogenesis will eventually contribute to enhance neuronal regeneration and help in the treatment of brain damage following injury or cases of neurodegenerative diseases or as a result of normal aging.
Keywords: Rb, E2F1, E2F3, FgF2, p107, p103, adult neurogenesis, olfactory bulb

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ABBREVIATIONS

aSVZ: adult subventricular zone

BrdU: bromodeoxyuridine

CCE: cell-cycle re-entry

cdk: cyclin-dependent kinase

ChIP: Chromatin immunoprecipitation

CNS: central nervous system

DCX: doublecortin

ddH2O: double distilled water

DF: double floxed

DG: dendate gyrus

EPL: external plexiform layer

Fgf2: fibroblast growth factor 2

Flox: floxed

GC: granule cell

GCL: granule cell layer

gDNA: genomic DNA

GF: growth factor

GL: glomerular layer

HB: hybridization buffer

Hi: hippocampus

INP: intermediate neural progenitor

IPL: internal plexiform layer

KO: knock-out

LMD: laser microdissection

LV: lateral ventricle

MC: mitral cell

MCL: mitral cell layer

Neo1: neogenin

NeuN: neuronal nuclear marker

NPS: neural progenitor cell

NSC: neural stem cell

NSPCs: neural stem and progenitor cells

OB: olfactory bulb

OE: olfactory epithelium

ONL: olfactory nerve layer

OSN: olfactory sensory neuron

PBS: phosphate buffer solution

PCR: polymerase chain reaction

PFA: paraformaldehyde

PGC: periglomerular cell

PSA-NCAM: polysialylated neural cell adhesion molecule

QNP: quiescent radial-glia-like type I neural progenitor cell

Rb: retinoblastoma

RMS: rostral migratory stream

RT-qPCR: real-time PCR

SEL: subependymal layer

SEM: standard error of the mean

SGZ: subgranular zone

ssDNA: single strand DNA

SVZ: subventricular zone

TAM: Tamoxifen

TC: tufted cell

YFP: yellow fluorescent protein

CHAPTER I

INTRODUCTION

A. Adult Neurogenesis

1. Definition and Historical Perspective

Neurogenesis or "birth of neurons" is defined as the developmental process of engendering functional and mature neurons in the brain from precursor cells (Bond et al., 2015; Ming and Song, 2011). It was conventionally considered to occur strictly during embryonic and perinatal phases in mammals (Ming and Song, 2005; Cajal, S. R., and May, R. M., 1991). However, Altman's revolutionary studies decades ago challenged this dogma and gave the first anatomical proof for the existence of newly born cells in the postnatal rat hippocampus (Altman and Das, 1965). Later, new neurons' integration in the adult central nervous system (CNS) was presented in songbirds (Paton and Nottebohm, 1984). Then, multipotent neural stem cells (NSCs) were isolated and derived from two sites in the adult mammalian brain (refer to next section; Richards et al., 1992; Reynolds and Weiss, 1992), and ongoing adult neurogenesis was validated in nearly all mammalian species studied, including humans albeit its persistence into mid-to-late adulthood is still debatable in humans (Boldrini et al., 2018; Sorrells et al., 2018; Bonfanti, L., and Peretto, P. 2011; Eriksson et al., 1998). The adult neurogenesis field started to grow fast, mainly after the introduction of the thymidine analog bromodeoxyuridine (BrdU) that marks cells in the S-phase and is widely used as a lineage tracer (Kuhn et al., 1996).

In spite of the fact that neurogenesis was initially defined as a developmental process, the new discovery that it continues throughout mammals' life (with clear spatio-temporal dissimilarities and cell fate constraints within species) required a reconsideration of the adult brain's regenerative ability and plasticity (Bonfanti and Peretto, 2011). In fact, adult neurogenesis is a dynamic process and can be controlled negatively by aging and stress, as well as positively, by learning, environmental enrichment and physical activity (Farioli-Vecchioli et al., 2015). Consequently, considerable research is under way attempting to uncover if neurogenesis potentially contribute to neuro-regeneration and restoration in the adult brain along with its modulatory involvement in normal brain functioning (Omais et al., 2018). Given the latest improvements and novel technologies available at hand, studies of adult neurogenesis will continue to highly progress in the upcoming years. The major challenge is to pinpoint the cellular and molecular mechanisms controlling various aspects of this intriguing developmental program including progenitor proliferation, neuroblast differentiation and integration of newborn neurons as well as their contribution to function and behavior (Ming and Song, 2011; Farioli-Vecchioli et al., 2015).

2. The Neurogenic Niches

The neurogenic niches represent permissive microenvironments that host actively dividing stem and progenitor cells and control their growth and development via a variety of refined extrinsic and intrinsic signals in vivo (Ming and Song, 2011; Bartesaghi, S., and Salomoni, P., 2012). The niches also encompass different cellular components including

ependymal cells, microglia, astrocytes, endothelial cells and vascular cells, which effectively synchronize adult neural stem and progenitor cells (NSPCs) development from proliferation to differentiation (Ming and Song, 2011).

Even though most of the stem/progenitor cells e.g. radial glia are depleted at the end of the development to produce glia and neurons, a minimal amount of quiescent neural stem cells (NSCs) is set aside during mid-development, only to be re-activated during adulthood (Fuentealba et al., 2015; Furutachi et al., 2015; Bartesaghi, S., and Salomoni, P., 2012). Moreover, functional adult neurogenesis is spatially limited under regular circumstances to two anatomically distinct neurogenic brain regions: the subgranular zone (SGZ) of the hippocampal (Hi) sub-region called the dentate gyrus (DG), where new excitatory dentate granule cells (GC) are generated; and the adult subventricular zone (aSVZ) or subependymal layer (SEL) which is a germinal area situated within the walls of the lateral ventricles (LV) under a thin layer of ependymal cells and where new inhibitory neurons are produced and migrate through the rostral migratory stream (RMS) until they reach and settle inside the olfactory bulb (OB) (Figure 1) (Gage, F. H. 2000; Givre, S. 2003; Farioli-Vecchioli et al., 2015; Alvarez Buylla, A., and Kirn, J. R., 1997).

Despite the fact that these two neurogenic zones have a comparable structural organization and a tight connection with vasculature (Bartesaghi, S., and Salomoni, P., 2012), they display clear differences (Ming and Song, 2011). Actually, the SGZ is very rich in diverse nerve terminals and exposed to dynamic circuit activity dependent control through many neurotransmitters. Conversely, the SVZ is not present within a condensed neuronal network and is physically separated from the OB where incorporation of newborn neurons happens (Ming and Song, 2011).

Latest studies started to uncover the plastic and dynamic nature of adult neurogenic niches. Through feedback, new neurons are capable of modulating NSPCs' behavior (Ming and Song, 2011).

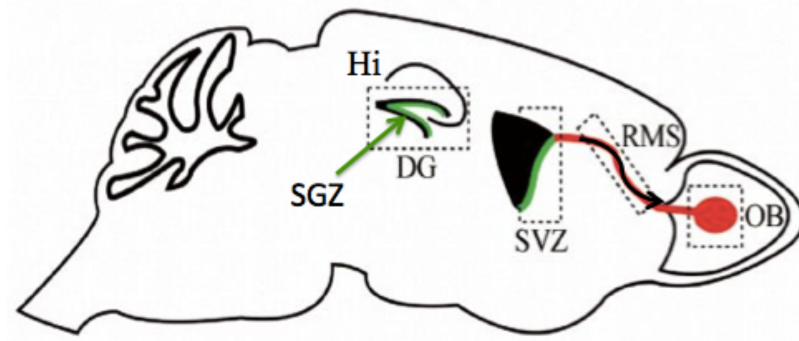

Figure 1: Sagittal section of the adult mouse brain. The two neurogenic niches (in green), where adult neurogenesis persists throughout life, are shown: the aSVZ lining the LV and linked to the OB through the RMS (in red), and the SGZ of the DG inside the Hi (as modified from Braun, S. M., and Jessberger, S. 2013).

3. *The Adult Subventricular Zone and the Olfactory Bulb*

The generation of new inhibitory neurons in the adult olfactory bulb (OB) neurogenic niche begins in the aSVZ bordering the lateral ventricles where quiescent stem cells (also known as radial glia-like type B1 cells) undergo symmetric divisions and become proliferating intermediate neural progenitors (INPs), (also known as transient-amplifying type C cells), that in turn give rise to neuroblasts or type A cells (Obernier et al., 2018; Ming and Song, 2011). These continuously produced neuronal progenitors exit the cell cycle and migrate rostrally in the form of cell aggregates along astrocytic fibers named

“chains” from their site of origin inside the SVZ to the OB. This migratory path is formally called rostral migratory stream (RMS) (Farioli-Vecchioli et al., 2015; Breton-Provencher et al., 2012). In this highly delimited route, and between 2–7 days from their birth, neuroblasts travel in close proximity to blood vessels inside a tube formed by astrocytes, while undergoing their final round(s) of cell division (Whitman, M. C., and Greer, C. A. 2009; Lledo, P., and Saghatelyan, A., 2005; Lois et al., 1996). Following their rostral migration, and once in the core of the OB, immature neurons disconnect from the RMS and spread radially where they differentiate into distinct types of interneurons and incorporate in the pre-existing neural networks (Breton-Provencher et al., 2012; Ming and Song, 2011). As such, within 2–3 weeks, young neurons finish their differentiation as they settle in the granule cell layer (GCL) and the glomerular layer (GL) of the OB until they finally acquire electrophysiological characteristics of mature neurons (Breton-Provencher et al., 2012; Lledo et al., 2006) (Figure 2 and Figure 3A).

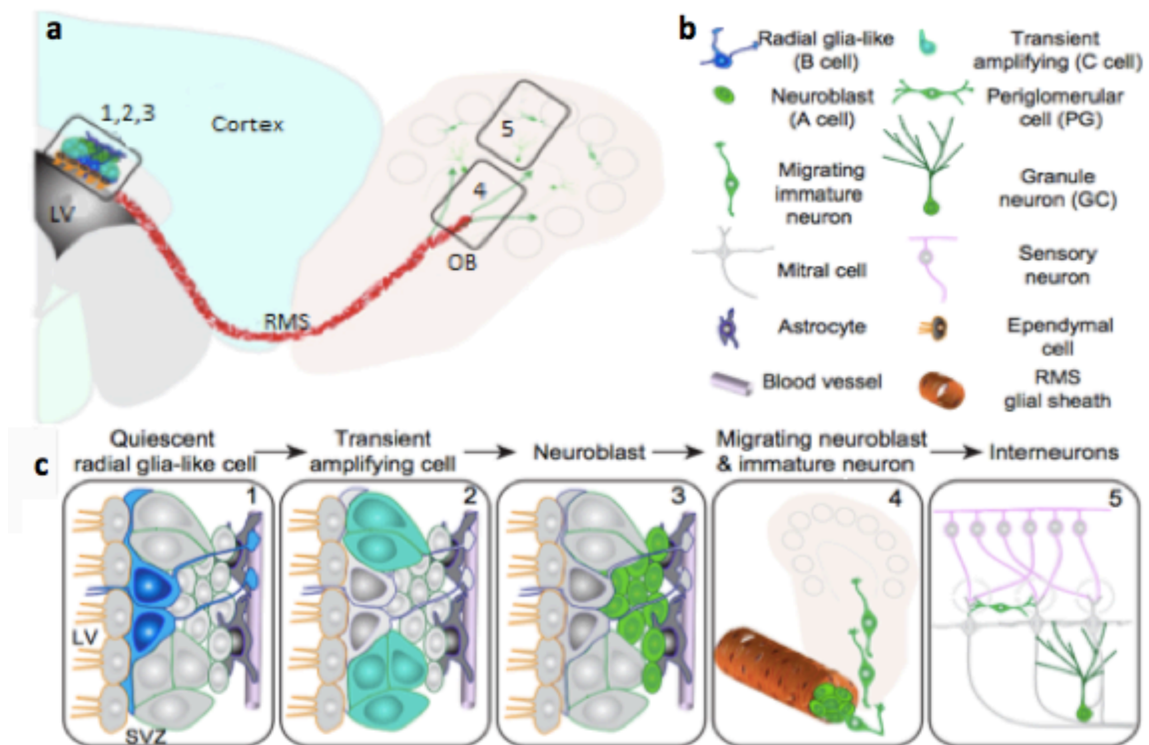

Figure 2: Adult Neurogenesis in the aSVZ and olfactory bulb. Quiescent radial glia-like cells (type B cells or number 1) divide and give rise to transient amplifying cells (type C cells or 2) that next produce neuroblasts (type A cells or 3). These young neurons migrate through the RMS (4) until they reach the core of the OB, where they disconnect from the RMS, spread radially and differentiate into mature PG and GC interneurons (5). (a) Schematic representation of the SVZ/OB niche. (b) Distinct types of cells found in the aSVZ/OB. (c) NSPCs lineage in the SVZ/OB (as modified from Ming and Song, 2011).

Newly born neurons that are produced during adulthood acquire mature morphology, establish functional synaptic connections with the principal cells of the OB and are triggered by odor stimuli (Panzanelli P et al., 2009; Whitman and Greer, 2007; Carlen M et al., 2002). Of the great number of cells reaching the OB, the majority (97%) gives rise to GABAergic granule cells (GC) that lack axons and form dendro-dendritic

synapses with tufted cells (TCs) and/or mitral cells (MCs), which are the principal projection neurons inside the OB. Only a minority (3%) becomes GABAergic periglomerular cells (PGC) (Breton-Provencher et al., 2012; Ming and Song, 2011) (Figures 2 and 3B). One study also showed the generation of a very minute number of glutamatergic juxtaglomerular neurons (Brill et al., 2009).

As previously described, newborn cells are integrated in the pre-existing system of the OB where they build new synaptic connections with the principal neurons. Accordingly, how are the OB old (perinatal) neurons and newborn ones structured? In fact, the OB is characterized by its laminar organization in 6 distinct layers based on the conventional classification of the existing neurons: olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) and granule cell layer (GCL) (Figure 3B). In mammals, odors are detected by olfactory sensory neurons (OSNs) of the olfactory epithelium (OE) that send stimuli through the ONL (axonal projections), which in turn transmit them to the primary dendrites of the principal projection neurons, mitral cells (MCs) and tufted cells (TCs). The somas of MCs are found in the MCL, whereas those of the TCs are scattered throughout the EPL. Thus, these projection neurons are under the permanent inhibitory regulation of the newborn GABAergic interneurons residing in the GC and PGC layers. The GCs are largely found in the GL and their axons extend to the IPL, while PGCs are present in the GCL (Lim, D. A. and Alvarez-Buylla, A. 2016; Nagayama et al., 2014; Gheusi et al., 2013; Breton-Provencher et al., 2012) (Figure 3B).

The perpetual arrival of new cells provides the OB with a considerable pool of flexible neurons that can adapt to the operational demands of the neuronal network that is

present. In fact, newborn neurons are implicated in short-term odor memory and recognition of perceptually similar odors, thus they adjust and regulate the bulbar network's functioning to the continuously fluctuating sensory environment (Lazarini, F., and Lledo, P. 2011). However, long-lasting neurons produced during embryonic and early postnatal period are needed for central olfactory functions as the typical odor perception. Consequently, neurons born during adulthood differ from mature neurons born around birth by their morphology, physiology, level of plasticity and excitability in addition of having distinct targets (Omais et al., 2018; Hardy and Saghatelyan, 2017; Valley et al., 2013; Breton-Provencher et al., 2012; Breton-Provencher et al., 2009). Moreover, odor stimulation and learning cause variations in the morphology and amount of newborn neurons that integrate and survive, hence can modulate neuronal turnover (Watt et al., 2004).

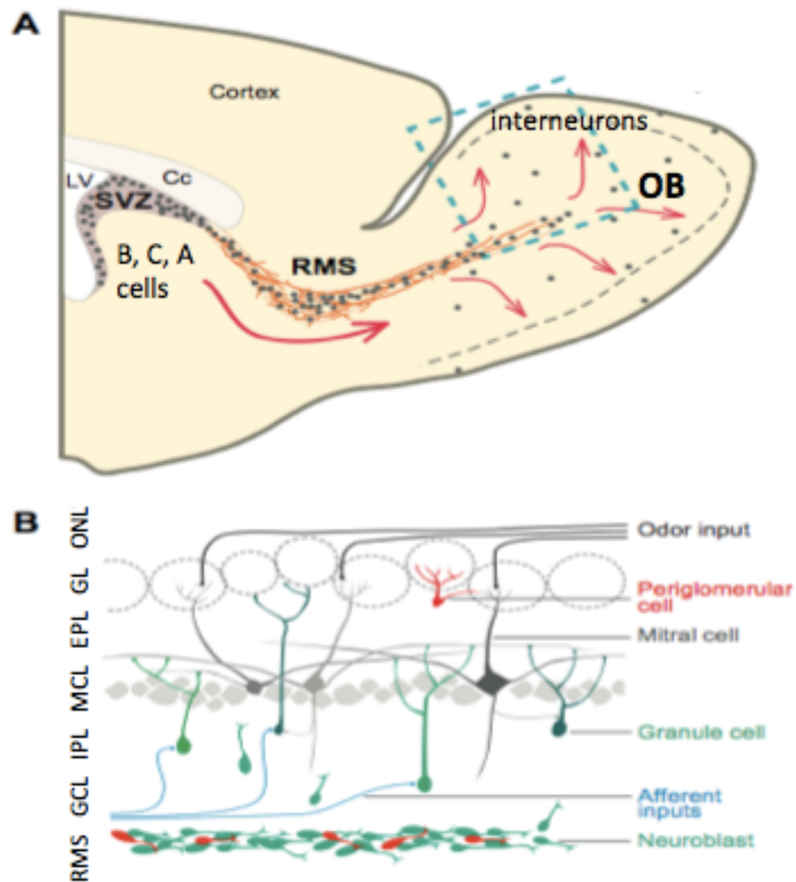

Figure 3: Newborn interneurons or inhibitory neurons are continuously arriving to the OB. (A) Adult neurogenesis in the aSVZ and OB showing the different cell types (B, C, A cells) and the RMS connecting them. (B) Closer representation of the boxed area in A showing the OB laminar organization into 6 layers: GCL, IPL, MCL, EPL, GL and ONL. Cc: corpus callosum (as modified from Breton-Provencher et al., 2012).

4. *The Subgranular Zone and the Hippocampus*

In the DG of the adult Hi, the generation of new neurons starts from quiescent radial-glia like stem cells that give rise to proliferating intermediate progenitors, which in turn produce neuroblasts. These young neurons migrate towards the inner granule cell layer

where they differentiate into dentate granule neurons. In few days, these adult neurons outspread their dendrites to the molecular layer and prolong their axons throughout the hilus toward the CA3. Finally, and based on a stereotypic process, adult-born neurons establish their synaptic incorporation in the pre-existing system (Boldrini et al., 2018; Ming and Song, 2011; Tashiro et al., 2006) (Figure 4).

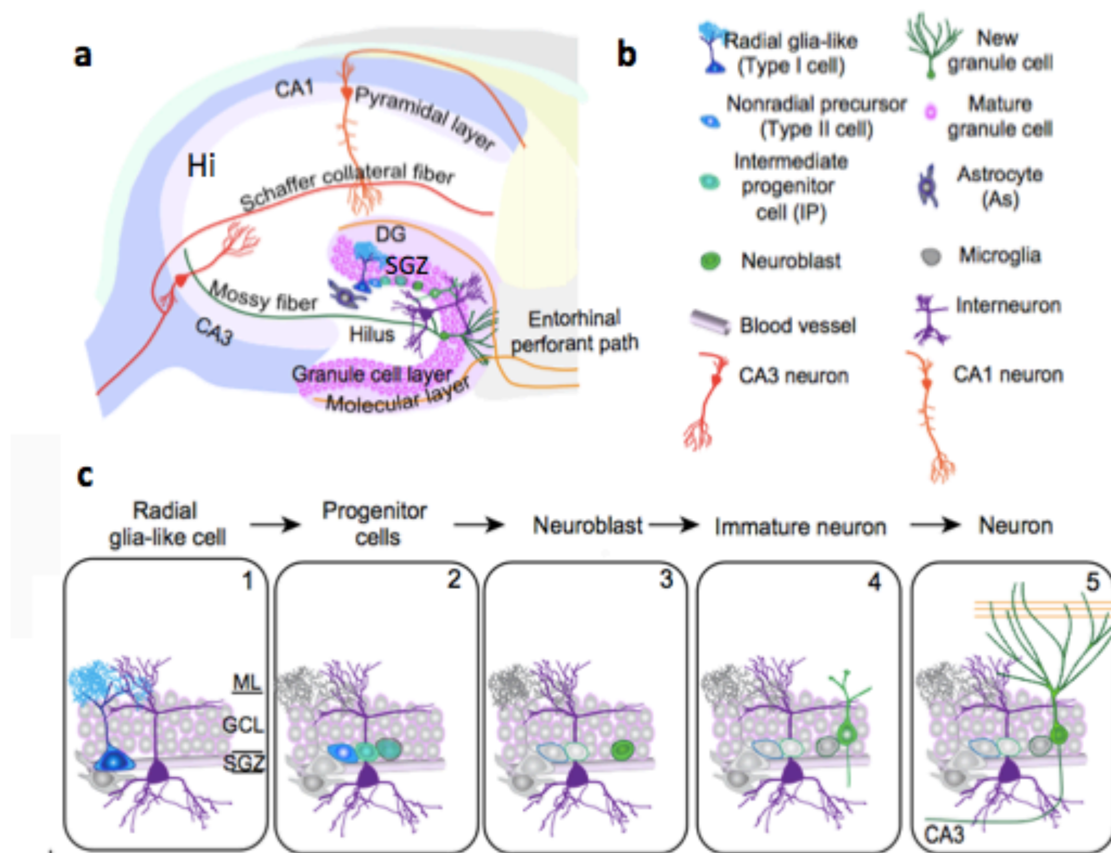

Figure 4: Adult Neurogenesis in the Dentate Gyrus of the Hippocampus. The five stages of adult neurogenesis are summarized as follows: first, the activation of quiescent radial glia-like cells in the SGZ; second, the proliferation of intermediate progenitors; third, the production of neuroblasts; fourth, the incorporation of young neurons in the pre-existing

system; and fifth, the maturation of these immature neurons into mature dentate granule cells (as modified Ming and Song, 2011)

B. Properties of the different types of Adult Neural Cells found in the aSVZ and OB

In both the embryonic and adult brain, it is well known that cell lineages are hierarchically organized with cells of the highest plasticity at the top and fully differentiated cells at the bottommost. Accordingly, stem cells are considered as the top layer followed by constrained neural progenitors and, at the end, specialized cell types: oligodendrocytes, astrocytes, and neurons (Bonaguidi et al., 2016).

1. Adult Neural Stem Cells (aNSCs)

Stem cells are generally defined as the cells having the ability for both, unlimited self-renewal through division, and continuous generation of specialized types of cells through differentiation (Ming and Song, 2011). Adult neural stem cells (aNSCs) found in the adult SVZ also known as quiescent radial glia-like type B1 cells are regionally specified and thus, heterogeneous cells are giving rise to distinct types of GABAergic and dopaminergic neurons in the OB (Bonaguidi et al., 2016; Bartesaghi, S., and Salomoni, P., 2012). These aNSCs can alternate between two different states: the first is a quiescent state where aNSCs slowly divide symmetrically to self-renew and maintain their pool throughout life, and (Bartesaghi, S., and Salomoni, P., 2012) the second is an actively dividing state (consuming symmetrical divisions) whereby they give rise to rapidly-dividing progenitor cells that undergo several rounds of division before they differentiate into functional neurons and oligodendrocytes during adulthood, hence having a limited self-renewal capacity (Obernier et al., 2018; Bonaguidi et al., 2016).

Interestingly, aNSCs are characterized by the expression of specific markers such as glial fibrillary acid protein (GFAP, a marker of mature astrocytes), brain lipid-binding protein (BLBP), and glutamate-aspartate transporter (GLAST) (Bonaguidi et al., 2016; Encinas et al., 2011). They also express markers shared with progenitors such as Nestin and Sex determining region Y-box 2 (Sox2). Moreover, they have a characteristic radial branch, which connects to the blood vessels in the SVZ (Fuentealba et al. 2012).

2. Adult Intermediate Neural Progenitor cells (INPs)

Intermediate neural progenitors (INPs) also known as proliferating transient-amplifying cells or type C cells are the result of aNSCs' asymmetric divisions (Bartesaghi, S., and Salomoni, P., 2012; Encinas et al., 2011). Each type of these neural progenitor cells (NPCs) becomes committed to a particular lineage under normal physiological circumstances; therefore, gives rise to a specific subtype of inhibitory neurons (Bonaguidi et al., 2016). Progenitors are the most abundant cell population within the aSVZ, and undergo several rounds of cell divisions (Obernier et al., 2018; Ponti et al. 2013; Encinas et al. 2011).

aSVZ progenitor cells express nestin, Mash1, Dlx2, Ki-67 among other genes and highly incorporate BrdU and have little tangential processes (Ponti et al. 2013; Encinas et al., 2011; Doetsch et al. 2002). Furthermore, intermediate progenitors are considered transient amplifying precursors as they shortly express doublecortin (DCX), a marker of committed young/immature neurons (Bonaguidi et al., 2016).

NSPCs proliferation in the aSVZ is under a firm control by extrinsic factors such as

growth factors (GF) and morphogens as well as intrinsic factors such as transcription factors and epigenetic mechanisms (Omais et al., 2018; Sun et al., 2011; Imayoshi et al., 2010; Ma et al., 2010; Zhao et al., 2008). Noteworthy, although not well acknowledged, is the influence of environmental cues and physical activity and learning on neurogenesis in the aSVZ together with the effect of pathological disorders for example Alzheimer's disease (Winner, B., & Winkler, J. 2015; Gallarda, B., & Lledo, P. 2012; Winner, B., Kohl, Z., & Gage, F. H. 2011)

3. *Adult Neuroblasts*

Even though adult neurogenesis in the aSVZ generates a minute amount of astroglia in the corpus callosum and RMS (Sohn et al., 2015) in addition to myelinated and non-myelinated oligodendrocytes (Menn et al., 2006), the majority of progenitors commit to a neuronal fate by differentiating into primitive neurons. These young and immature neurons are called migrating neuroblasts or type A cells. They migrate along the RMS and give rise to inhibitory GCs and PGCs (Whitman et al., 2009).

When NPCs differentiate into migrating neuroblasts, they stop to express GFAP and Sox2, however, they turn on the expression of polysialylated neural cell adhesion molecule (PSA-NCAM) as well as DCX (Boldrini et al., 2018).

4. *Adult-born Neurons*

In adult rodents, approximately 20,000–30,000 immature neurons reach the OB every day from which 50% are removed by natural turnover (Breton-Provencher, V., and Saghatelyan, A., 2012). The surviving cells finish their differentiation and achieve their

final maturational stage by giving rise to GCs mainly as well as PGCs that are both inhibitory GABAergic interneurons. These fully mature neurons will live up to 12 months in mice and 19 months in rats approximately, and will be replaced by a new round of fresh newborn neurons later on (Breton-Provencher et al., 2012).

Mature neurons are post-mitotic cells, which means that, under normal physiological conditions, they are unable to proliferate because they are inhibited from re-entering the cell cycle (Bartesaghi, S., and Salomoni, P., 2012). Despite this, it is believed that neuronal cell-cycle re-entry (CCE) can be triggered by numerous assaults such as the buildup of DNA damage and/or oxidative stress and/or with age (Sedelnikova et al., 2004). In this context, the roles played by key cell-cycle proteins such as Rb, p130 and cdk5 are essential to maintain the arrested state of neurons at the G0 phase (Omais et al. 2018; Herrup and Yang, 2007).

It should be noted that adult-born OB neurons have distinctive roles. They display several electrophysiological and morphological characteristics allowing them to apply quick, time-restricted and effective alterations in the neuronal system in response to the continuously fluctuating environment. The excessive production of dendritic spines, the increase in sodium excitability and currents, in addition to further, yet undiscovered aspects of mature adult neurons, distinguish this type of cells from their embryonic (and neontatal) counterparts and imply that they mediate particular functions in encoding and discriminating between new odors (Breton-Provencher et al., 2012).

Newborn neurons, which are the last outcome of the differentiation cascade, are

characterized by the expression of several maturation markers including PSA-NCAM, neuronal nuclear marker (NeuN), calbindin and/or calretinin (Boldrini et al., 2018; Encinas et al., 2011).

C. The Retinoblastoma Protein, Rb

1. Definition

The retinoblastoma gene (Rb) is one chief tumor suppressor gene that controls the cell cycle at the G1-S phase checkpoint. Its name originates from a rare childhood cancer that occurs in the retina upon its loss during development. In fact, the ocular tumor retinoblastoma is predisposed by germline mutations in the Rb gene at the chromosomal location 13q14. Upon loss of the second Rb allele, susceptible individuals develop retinal tumors (Dyer, M., and Bremmer, R. 2005; Macpherson, D., 2004; Marino, S., 2003). Almost all retinoblastoma cancers are characterized by the absence of functional Rb. Moreover, it is often inactivated in many human cancers such as osteosarcoma, lung carcinoma and brain tumors (Vélez-Cruz, R.,2016; Bartesaghi, S., and Salomoni, P., 2012).

Furthermore, the Rb protein carries CNS-specific roles that are independent from its standard function as a tumor suppressor protein. In fact, Rb regulates various cellular activities in the nervous system apart from cell proliferation. It is implicated in the laminar organization of the cortex, neuronal migration and differentiation as well as survival and maintenance of a post-mitotic state in mature neurons (refer to next sections for detail). Therefore, Rb protein has a key role in the regulation of neuronal progenitor populations and the generation of neurons during neurogenesis (Mcclellan et al., 2009).

2. Role of Rb in Cell Cycle Regulation

The cell cycle is the mechanism by which a mother cell gives rise to two daughter cells by division. This process consists of a sequence of events beginning from the quiescent state (G0), followed by the first gap/growth phase (G1 phase) where the cell prepares itself for DNA synthesis/replication (or S phase). After DNA duplication, the cell enters the second gap phase (G2) in order to continue its growth, until it finally reaches the mitosis phase (M) (Farioli-Vecchioli et al., 2015). Cytokinesis or C phase follows the M phase and leads to the separation of the two daughter cells. This process is preciously orchestrated by the coordinated activity of cell cycle molecules that control stem cells' quiescence and expansion as well as neural progenitor differentiation (Mateus-Pinheiro et al., 2013; Vandenbosch et al., 2011). Moreover, the number of neurons produced depends on three factors. First, the mode of division, a symmetric division of NSC gives rise to two neurons at the expense of the mother cell, whereas an asymmetric one preserves the original pool by engendering one committed/differentiated cell and one mother cell. Second, the amount of cells that differentiates by exiting the cycle and going into quiescence: in fact, it is unquestionable that cell cycle's inhibition is enough to cause differentiation of NSCs. Third, the cell cycle length, which can be shortened by accelerating the rate of division, leading this way to a larger number of neurons. Cell cycle's dysregulation is one of the principal reasons causing severe fluctuations in adult neurogenesis homeostasis (Farioli-Vecchioli et al., 2015).

Appropriate progression throughout the cell cycle is supervised by checkpoints that anticipate potential defects in DNA duplication and/or chromosomal segregation. The

activation of checkpoints stops the cell cycle by modulating the levels of cyclins and cyclin-dependent kinases complexes (cyclin/cdk) that are the key executers of cell cycle progression. Hence, these checkpoints are essential for the fine-tuning of the balance between NSCs expansion and differentiation (Bartesaghi, S., and Salomoni, P., 2012). Numerous studies have discovered that Rb is a master regulator of the G1/S checkpoint that governs the cell's entry into the S phase. Actually, Rb synchronizes neural precursors proliferation, assesses the timing of cell cycle withdrawal and regulates neuroblasts' terminal mitosis in the central and peripheral nervous systems as well as the retina (Vélez-Cruz, R., 2016; Dick, F. A., and Rubin, S. M. 2013; Mcclellan et al., 2009). Accordingly, what is the mechanism by which Rb regulates cell cycle progression through the G1/S restriction point? Rb gene encodes a 110-kDa nuclear phosphoprotein capable of binding and inhibiting E2F transcription factors by engaging chromatin modifiers to the E2F target genes' promoters, consequently repressing the E2F-mediated gene transcription by blocking S-phase entry. Progression through the G1/S checkpoint is dependent on the phosphorylation of Rb. Therefore, Rb's phosphorylation by Cdks results in the release of Rb from the E2F factors, thus allowing the transcriptional activity to occur leading to cell cycle progression (Figure 6) (Vélez-Cruz, R.,2016; Bartesaghi, S.,and Salomoni, P.,2012; Degregori, J., and Johnson, D. G. 2006).

Besides its function in the control of G1-to-S-phase transition, Rb regulates different cellular processes. Rb mediates the timing and accuracy of DNA replication, regulates appropriate segregation of chromosomes during mitosis, facilitates the condensation of chromosomes and controls the correct orientation and condensation of centromeres on duplicated chromosomes. Moreover, it regulates the cells' genomic stability

as well as the global expression of the genes by cooperating with chromatin remodeling enzymes (Sage et al., 2012; Bartesaghi, S., and Salomoni, P., 2012; Manning, A. L., & Dyson, N. J., 2012). Because Rb interacts with many different proteins and since it has various functions in addition to its established role in cell cycle control, the Rb protein has been defined as the multifunctional chromatin-associated protein (Dyson 2016).

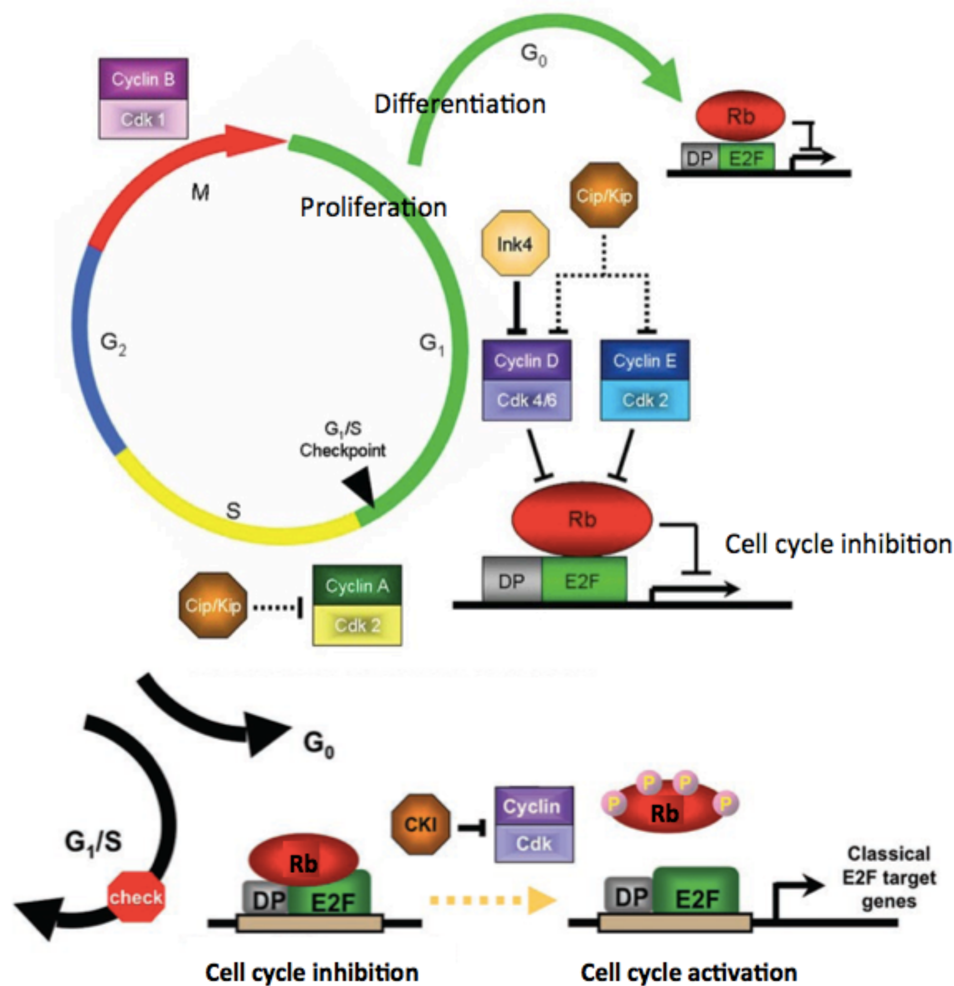

Figure 5: Schematic diagram of the cell cycle with emphasis on the regulation of G1-S phase transition by Rb/E2F. The Rb protein binds to the E2F transcription factors to repress cell cycle progression through the G1-S checkpoint. Once phosphorylated by Cylcin/Cdk complexes, Rb detaches from the E2F transcription factors allowing cell cycle progression (as modified from Mcclellan, K. A., and Slack, R. S. 2006; Mcclellan, K. A., and Slack, R. S. 2007).

3. Role of Rb during Embryonic Neurogenesis

The Rb protein is vital for embryonic development, since Rb knockout embryos display intense defects in the neural tube and placenta as well as skeletal muscle deficiencies, leading to their death by embryonic day 15 (E15) (Clarke et al., 1992). Conditional Rb inactivation and deletion in NSPCs in the telencephalon cause ectopic cell proliferation and an increase in apoptosis in progenitor cells present in the developing SVZ and dorsal cortex (Ghanem et al. 2012, Sage et al., 2012; Bartesaghi, S., and Salomoni, P., 2012; Ferguson et al. 2002, Clarke et al., 1992). Thus, Rb is fundamental for proper cell cycle exit in NSPCs and the maintenance of post-mitotic state in mature neurons (Sage et al., 2003). A block in neuronal differentiation and migration also accompanies these aberrations observed in Rb conditional knock-out mice as was shown in the ventral telencephalon (SVZ and OB) where loss of Rb leads to, E2f-mediated suppression of key differentiation genes' expression such as Dlx gene expression (Ghanem et al., 2012;). Furthermore, Rb was shown to control newborn interneurons' migration in the embryonic cortex in a cell-autonomous manner and through specific interaction with E2F3, implying a role for Rb beyond cell cycle regulation (Bartesaghi, S., and Salomoni, P., 2012; McClellan et al. 2007; Ferguson et al., 2005). A recent study by Vandenbosch indicated that the deletion of Rb in the hippocampal dendate gyrus during development provokes an immense ectopic proliferation and a delay in cell cycle exit of immature neurons especially at late developmental stages, without any effect on NSCs. However, this phenotype was partly compensated by an increase in cell death (Vandenbosch et al., 2016).

4. Role of Rb during Adult Neurogenesis

Previous findings have established the different roles of Rb during embryonic neurogenesis that go beyond its canonical function of controlling the cell cycle. These functions may be applicable to adult neurogenesis since recent studies imply that quiescent aNSCs originate from embryonic stem cell populations as described earlier. Until recently, little was known about Rb's function in aNSPCs, however, the use of Tamoxifen-inducible Cre transgenic mouse lines (e.g. Nestin-CreERT2) crossed with Rb floxed/floxed lines helped overcome embryonic lethality (due to loss of Rb) and study the role of Rb in the adult brain. In a recent study performed in our laboratory, Naser et al. revealed conserved but also distinct roles for Rb during adult neurogenesis in the SVZ and OB compared with embryonic development. First, as seen during development, loss of Rb is associated with enhanced progenitor proliferation in the aSVZ and RMS but without any obvious effect on aNSCs' self-renewal. Therefore, Rb appears to exclusively regulate the pool of proliferating progenitors in the aSVZ and RMS, without influencing the properties of aNSCs' with respect to cell division rate. Second, and unlike development where Rb conditional loss triggers serious differentiation and migration defects, Rb-null neuroblasts exit properly the cell cycle, migrate to the OB and differentiate into mature adult GABAergic neurons. These findings were also true in vitro. Therefore, the Rb seems to be dispensable for the rostral migration and terminal differentiation of newborn neurons in the adult brain (Naser et al. 2016). However, the enhanced SVZ/OB neurogenesis observed after loss of Rb reached a peak at one month and was only transient and followed by gradual loss of all Rb-null newborn neurons three months later due to increased apoptosis. This highlights the necessity of Rb for the long-term survival of adult OB neurons (Naser et

al. 2016). Additionally, the Rb regulates adult neurogenesis in the hippocampal dendate gyrus similarly to the embryonic neurogenesis. In fact, defects in Rb cause ectopic proliferation of young neurons and severely affect their survival. Therefore, the Rb has an imperative function in the production and the survival of dendate gyrus cells in the embryonic as well as the adult brain (Vandenbosch et al., 2016).

The above findings suggest that transient manipulation of the tumor suppressor protein Rb e.g. targeted Rb knock-down may help improve the regenerative capacity inside the adult brain and in aNSPCs cultures by enhancing the generation of mature and functional neurons without affecting the self-renewal or differentiation potentials of aNSCs. In fact, Rb knockdown results in an enhancement of neuron plasticity, improvement of outgrowth after axotomy and thus helping in the recovery after nerve injuries by facilitating axonal regeneration (Zochodne et al., 2014). Future experimentations are necessary for better understanding of the molecular mechanisms by which Rb controls aNPCs proliferation and neuronal commitment as well as long-term survival. This can ultimately increase our ability to regulate adult neurogenesis and thus, improve the regeneration capacity inside the brain (Zochodne et al., 2014).

D. The Rb Family of Pocket Proteins

1. Definition

The latest studies by Naser et al. and Vandenbosch et al. uncovered critical Rb functions during adult neurogenesis, particularly in the control of progenitor proliferation and long-term survival of newborn neurons (Naser et al. 2016 and Vandenbosch et al.

2016). In fact, Rb is part of a greater network of regulatory effectors, comprising two other Rb family members of pocket proteins, p107 (also known as retinoblastoma-like 1) and p130 (also known as retinoblastoma-like 2) (Bartesaghi, S., and Salomoni, P., 2012). These three closely related mammalian pocket proteins share a sequence homology in their pocket domain, which regulates their interactions with transcription factors such as E2Fs. This structural similarity leads to some functional redundancy/compensation between the pocket proteins at least partially (Bartesaghi, S., and Salomoni, P., 2012). In fact, even though the three members bear different binding properties/affinities for diverse transcription factors, overexpression experiments revealed functional resemblances in the control of cell cycle and development (Cobrinik, D. 2005; Trimarchi, J. M., and Lees, J. A. 2002). For example, all three members of pocket proteins can trigger an arrest at the G1 phase by repressing the E2F-mediated gene transcription when overexpressed. Furthermore, all three members are phosphorylated by Cdks (Bartesaghi, S., and Salomoni, P., 2012; Classon, M., and Harlow, E. 2002). However, mice lacking functional p107 or p130 survive to adulthood without any overt phenotypes, and this is in contrast with Rb-germline KO mice that die during mid-gestation and Rb-conditional KO mice in the telencephalon that die at birth (Clarke et al. 1992, Ferguson et al. 2002, Bartesaghi, S., and Salomoni, P., 2012). Besides the above redundant roles played by the Rb family of pocket proteins, studies have shown that each pocket protein regulates specific aspect(s) of neurogenesis including specific cell population(s) such as the control of stem versus progenitor cell proliferation as well as regulating cell cycle exit and/or terminal differentiation and survival (maintenance of post-mitotic state) (refer to next sections Sage et al., 2012). Therefore, future work must focus on the distinctive roles of Rb, p107 and p130 in the context of adult neurogenesis in order

to define their imperative functions in controlling the fate and size of neurogenic niches (Fong, B. C., and Slack, R. S., 2017).

2. *p107*

Studies have shown that p107 plays unique functions in the control of neural stem cell quiescence and commitment in the embryonic and adult brain, and this is dissimilar from the role of Rb in the regulation of adult progenitor cells (Vanderluit et al., 2004). Neural stem cells lacking functional p107 are characterized by an increased capability for self-renewal and thus, display an expanded pool of stem/progenitor cells. Accordingly, unlike Rb, p107 controls cell division and commitment in uncommitted aNSCs in the aSVZ but not progenitors, and acts in dual fashion. First, it suppresses the self-renewal capacity of aNSCs through the direct inhibition of the Notch1-Hes1 pathway (Vanderluit et al., 2004). Second, it is required for the neuronal commitment and differentiation (Figure 7) (Vanderluit et al., 2007). Moreover, p107 does not appear to have any direct role in the survival of post-mitotic neurons even though there is an increase in apoptosis in the aSVZ of p107-deficient mice. This is probably a secondary effect due to p107 loss and to counterbalance the increase in NSCs self-renewal and subsequent progenitor proliferation (Vanderluit et al., 2004). In summary, p107 expression, is limited to the slowly-cycling undifferentiated neural stem cells in the aSVZ, and is down regulated in committed progenitors as these cells progress towards a neuronal lineage before exiting the cell cycle and completing their differentiation.

3. p130

The involvement of p130 in adult neurogenesis is still the least studied among the Rb family members of pocket proteins and very little is known about its role. During development, when NPCs finish their terminal differentiation, the expression level of Rb decreases significantly whereas p130 expression becomes predominant (Vanderluit et al., 2007). Studies indicated that p130 is greatly expressed in post-mitotic cells and implicated in the control of long-term cortical neurons' survival via E2F-mediated inhibition of pro-apoptotic genes (Liu et al., 2005; Liu, D. X., and Greene, L. A. 2001).

In summary, the three Rb family members display different functions in neurogenesis. Throughout the progression from early precursors to mature neurons, all three proteins work sequentially to control distinct stages and cell types during this process. At the beginning, in the NSCs compartment, p107 negatively regulates self-renewal, which eventually controls the size of neural precursor population, and induces commitment to a neuronal fate. Afterwards, the Rb level upsurges in order to control progenitor proliferation as well as terminal mitosis of newly differentiated cells. Once immature neurons become post-mitotic, p130 takes over to ensure proper cell survival and inhibit cell cycle re-entry (Liu et al., 2005; Liu, D. X., and Greene, L. A. 2001).

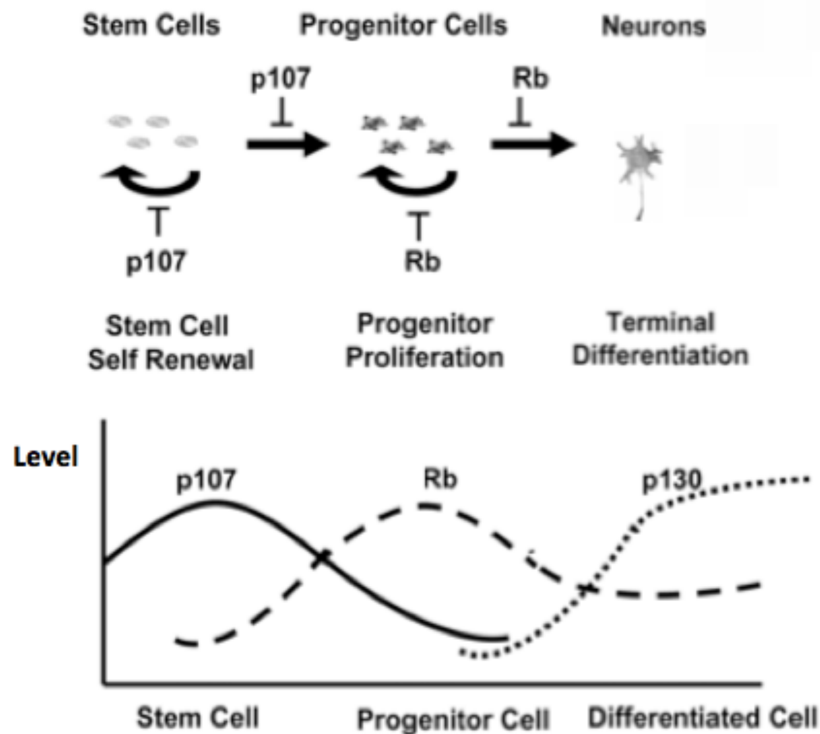

Figure 6: The Rb family members of pocket proteins play temporally different functions during neural lineage development. Initially, p107 inhibits NSCs' self-renewal, and induces commitment to a neuronal fate. Subsequently, Rb regulates progenitor proliferation as well as terminal mitosis. Finally, p130 functions to control long-term neuronal survival and repress cell cycle re-entry post-mitotic neurons (as modified from Vanderluit et al., 2004).

E. The E2F Family of Transcription Factors

1. Definition

E2Fs are transcription factors that control the expression of a broad spectrum of genes that are essential for cell proliferation (progression through the cell cycle), as well as the regulation of various physiological activities (Mcclellan et al., 2009; Lees et al., 1993). The E2F family of transcription factors includes 8 known E2F proteins (E2F1-8) in addition to two E2F3 isoforms (E2F3a and E2F3b), some of which play an essential role in

controlling the progression of cell cycle as well as various aspects of neurogenesis during development (Liao, Y., & Du, W., 2018; Julian et al., 2013). The E2F proteins are broadly divided into 2 groups. The first group comprises 5 transcription factors (E2F1-5) that can interact/bind with the Rb family members of pocket proteins (Rb, p107 and p130) in addition to proteins from the polycomb group, and are activated by binding to their dimerization partners (DP1 and DP2 proteins). The second group forms homodimers (E2F6-8), and does not bind or interact with the Rb family members (Calzone et al., 2008). Based on function, the E2F transcription factors are subdivided further into three subgroups. The first subgroup comprises the activators of transcription that can bind to the Rb (E2F1, E2F2 and E2F3a), the second subgroup includes the repressors of transcription that can bind to p107 or p130 (and to some extent Rb) (E2F3b, E2F4, E2F5), and the third subgroup, which is made of Rb-independent transcriptional repressors, which do not bind to any of the Rb family members in order to repress transcription (E2F6, E2F7a, E2F7b and E2F8) (Figure 8) (Chong et al., 2009; Calzone et al., 2008).

E2F transcription factors are implicated in the regulation of many different cellular properties during nervous system development including cell proliferation and cell death (Cooperkuhn et al., 2002). For example, E2F4 controls the ventral telencephalon's development via a genetic interaction with the Sonic hedgehog pathway (Shh), whereas both E2F1 and E2F3 mediate the proliferation of neural precursors in the SVZ and have redundant roles in this context (Ruzhynsky et al., 2007; Mcclellan et al., 2007; Cooperkuhn et al., 2002). Nonetheless, it is not known if this function targets NSCs and/or NPCs proliferation. Furthermore, many important developmental pathways and cell fate genes are

known to be E2f-regulated targets mediating E2f-dependent fate decisions in neural precursor cells. Examples of such targets include neuronal differentiation and migration genes such as Dlx1 and Dlx2, the neural specification gene Neogenin (Ngn1), the pluripotency and self-renewal factor Sox2, the fibroblast growth factor 2 (Fgf2), and Shh and Notch/Hes1 signaling pathways (Pakenham et al., 2015). During development, E2F1 and E2F3 start to be expressed in the cortex at embryonic day E11.5 and are both physiologically relevant Rb-interacting partners. Therefore similar interactions may possibly regulate NSPCs proliferation during adult neurogenesis as well, even though there is no direct validation yet (Omais et al., 2018; Mcclellan et al., 2007). Conclusively, understanding the complete regulatory function of the cell cycle machinery in the brain imposes an assessment of the E2f target genes' repertoire in neural precursors (Pakenham et al., 2015).

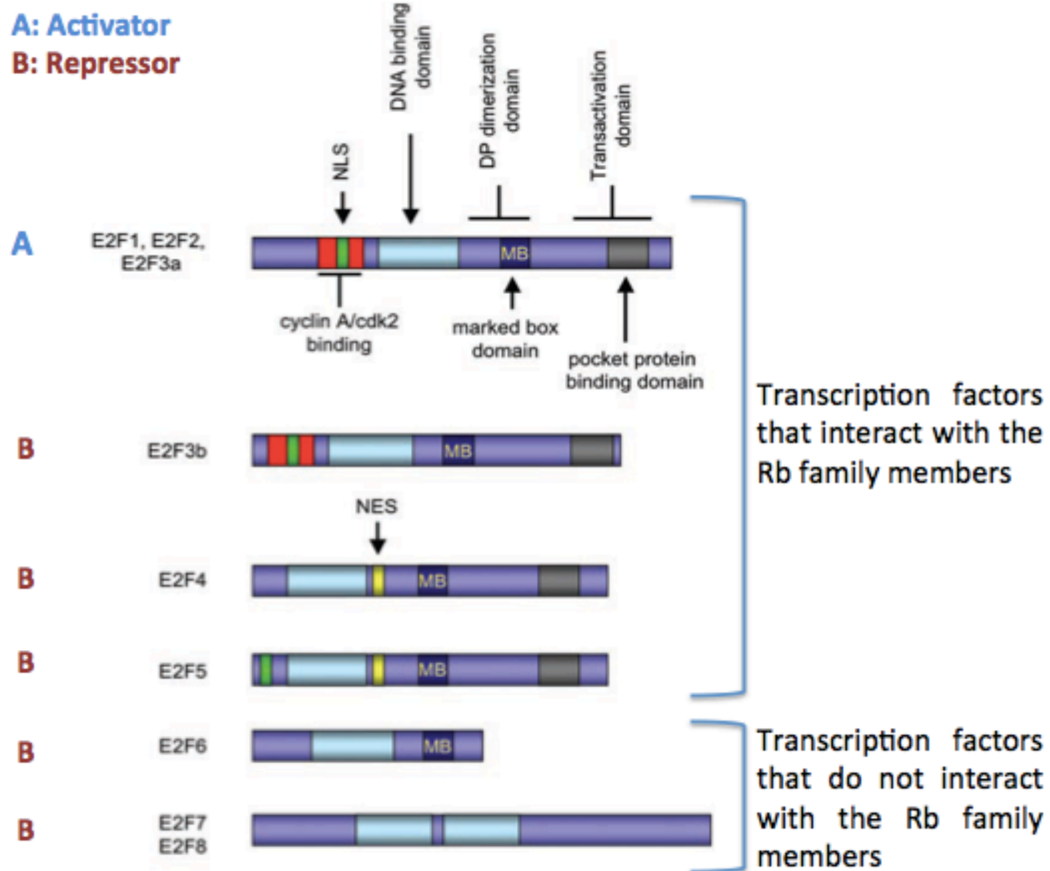

Figure 7: The E2F family of transcription factors (E2F1-E2F8). E2f1, E2F2 and E2F3b are able to bind to Rb and act as activators. E2F3a, E2F4 and E2F5 can bind to the Rb family members of pocket proteins but act as repressors. E2F6, E2F7 and E2F8 cannot bind to the Rb family members and function as independent repressors. The different domains found in the E2F proteins are illustrated in distinct colors (as modified from Mcclellan, K. A., and Slack, R. S. 2007).

2. E2F1

Of all the E2F family members, E2F1 is the most significantly expressed in the developing brain, especially in regions of NSPCs activity. E2F1 expression is cell cycle controlled, with a high expression level at G1/S. Mice with a targeted E2F1 deletion show

normal anatomy and behavior compared to wild-type mice, possibly because of the functional compensation by other E2Fs (Mcclellan et al., 2007; Cooperkuhn et al., 2002). In fact, E2F1 has an important function in the generation of granule cells in the brain by promoting cell proliferation; hence, its deficiency decreases the total number of neurons produced by impairing NPCs proliferation in the proliferative sites of the lateral ventricle, rather than altering brain's structures (Pakenham et al., 2015; Mcclellan et al., 2007; Cooperkuhn et al., 2002).

Furthermore, similar to Rb, E2F1 has also many roles independent of its transcriptional activity (Malewicz and Perlmann 2014; Biswas et al. 2014; Velez-Cruz and Johnson 2012). Actually, E2F1 is phosphorylated upon DNA damage and recruited to the site of damage through this phospho-specific interaction independently of the E2F1 transcription activation domain or DNA binding domain. Consequently, E2F1-deficient cells present genome instability and are not able to repair DNA damage because of impairment in the recruitment of DNA repair proteins to the damaged sites (Vélez-Cruz, R., 2016; Guo et al. 2010).

Besides its well-established function in cell cycle control, the best-characterized role of E2F1 is its capability to determine cell fate through the regulation of apoptotic programs (Thangue, N. B., 2003; Sears, R. C., and Nevins, J. R., 2002; Ginsberg, D., 2002). Therefore, many studies refer to the function of E2F1 as a tumor suppressor gene rather than an oncogene (Tsai et al., 1998; Yamasaki et al., 1996; Field et al., 1996). Actually, mice with E2F1 deficiency (E2F1-/-) show apoptotic defects and higher frequency of developing tumors (Yamasaki et al., 1996; Field et al., 1996). In addition, the

apoptotic level detected in Rb deficient mice can be suppressed by E2F1 deletion (Tsai et al., 1998). The mode of action of the E2F1-induced apoptosis is mediated via different pathways, some of which are p53-dependent while others are p53-independent. In fact, E2F1-induced cell death is essentially linked to the E2F-mediated upregulation of numerous pro-apoptotic genes encoding caspases, BH3-only proteins, Apaf1, p73 and p53 pro-apoptotic cofactors such as ASPP1 and ASPP2 (Korotayev, K., and Ginsberg, D., 2008). Consequently, E2F1 works as a tumor surveillance factor that detects abnormal proliferation and guards the organisms from tumor development through distinct apoptotic mechanisms (Pützer, B. M., 2007).

3. *E2F3*

Previous studies have shown that E2f3 is one of the most expressed E2f members in neural precursor cells (Julian et al., 2013; Callaghan et al., 1999). Moreover, its expression is upregulated in Rb deficient neural precursor cells, implying that it is an essential regulator of neural development and under the tight control of Rb (Julian et al., 2013). In fact, in addition to its prominent role in cell proliferation, E2F3 directly regulates target differentiation and migration genes such as those involved in axon guidance and actin dynamics (Pakenham et al., 2015). Accordingly, it was shown to be necessary for appropriate neuronal migration in the cortex (Mcclellan et al., 2007). Moreover, only 25% of mice lacking functional E2F3 survive postnatally but exhibit deficiencies in their cortical functioning as well as the long-term neurogenesis (Pakenham et al., 2015; Mcclellan et al., 2007).

Understanding the mode of function of the E2f3 and how it controls the cell cycle is

attributed to the presence of two functionally distinct isoforms with opposing roles (Julian et al., 2013). In fact, the E2F3 locus generates two transcripts: the full-length E2F3a transcript and the N-terminal-truncated E2F3b transcript transcribed from an intronic promoter (Mcclellan et al., 2007). Thus, both transcripts share the transactivation-, DNA binding- and Rb-binding domains, but differ by their N termini (Julian et al., 2013). Similar to E2F1 and E2F2, E2F3b works as a transcription activator and its expression is cell cycle regulated. Its level of expression oscillates throughout the cell cycle with a peak during the transition from G1 phase to S phase (similar to E2F1). Consistent with this function in the progression through the G1-S phase, E2F3b's ectopic expression in quiescent neural cells results in the activation of E2F target genes, thus driving cells to enter the S phase. Conversely to the function of E2F3b in the control of cell cycle, E2F3a participates with the Rb in the maintenance of quiescence in growth-hindered G0 cells; therefore, it is considered a transcription repressor. Together with the E2F4 and E2F5 genes, E2f3a is expressed throughout the cell cycle (Saavedra et al., 2002). Accordingly, the absence of E2f3a and E2f3b causes contrasting defects in the maintenance and differentiation of the progenitor pool (Pakenham et al., 2015). Julian et al. further revealed that the pocket protein p107 precisely interacts with E2f3a leading to the formation of transcriptional repressor complex whereas E2F3b generates a transcriptional activator complex by engaging RNA polymerase II. This interplay between the two complexes during neurogenesis helps fine-tune the equilibrium between neural precursors' pool differentiation and expansion through the direct transcriptional control of Sox2 in the developing and adult brain. In fact, Sox2's level of expression firmly affects neurogenesis; elevated levels of Sox2 as induced by E2F3b isoform activates proliferation of neural

precursors at the expense of differentiation into mature neurons, whereas low levels of Sox2 as mediated by E2f3a-p107 complex result in the opposite response (Julien et al. 2013).

F. FgF2

Embryonic and adult neurogenesis are regulated by different transcription factors, signaling molecules as well as mitogens and growth factors (Omais et al., 2018; Lim, D. A. and Alvarez-Buylla, A. 2016; Götz et al., 2016; Urbán, N., and Guillemot, F. 2014). Fibroblast growth factors (FgFs) constitute a group of proteins characterized by a conserved core of 140 amino acids and their ability to bind heparin or heparin sulfate proteoglycans. From the 22 FgF family members, the multi-functional growth factor FgF2 is known to be the basic FgF (Woodbury, M. E., and Ikezu, T. 2013). While the FgF family plays several roles in the CNS, the leading growth factor controlling precursor proliferation and survival is FgF2 (Rowland et al., 2011; Mcclellan et al., 2009; Martens et al., 2000; Tropepe et al., 1999; Reynolds, B., and Weiss, S. 1992). In fact, FgF2 is a well-known neurogenic factor regulating NSCs proliferation, differentiation and survival both in the embryonic and adult mouse CNS. Following Fgf2 deletion in mice, the number of proliferating NPCs considerably decreases causing a 45% decline in cortical neurons number at the end of neurogenesis (Mcclellan et al., 2009; Raballo et al., 2000). Conversely, FgF2's ventricular microinjection leads to an upsurge in the number of cortical neurons and an enlargement of the NPCs pool (Mcclellan et al., 2009). During development, FgF2's elevated level starts to be detected from neurulation onwards and its embryonic expression is spatially and temporally controlled (Woodbury, M. E., and Ikezu,

T. 2013). Interestingly, Mcclellan et al showed that the FgF2 is abnormally upregulated in the embryonic brains of mice with deficient Rb or p107, which explains the increased cell proliferation observed in these deficient mice. Moreover, E2F3 and p107 were shown to directly control the gene encoding FgF2 at the transcriptional level. Chromatin immunoprecipitation assays revealed that p107 and E2F3 occupy E2F consensus sites on the FgF2 promoter. Accordingly, E2F3 acts in concert with p107 in a physiologically appropriate context to functionally regulate FgF2's responsiveness in NPCs. In fact, p107 functions in the developing brain by repressing the E2F-mediated transcription activation at the FgF2 promoter. The identification of new machineries by which the Rb/E2F pathway regulates proliferation by mediating growth factor responsiveness permit to uncover the mode of genes' regulation outside of the traditional cell cycle mechanism (Mcclellan et al., 2009).

During adult neurogenesis, FgF2 is implicated in CNS neurogenesis; therefore, it is detected in the two neurogenic niches (aSVZ and SGZ) where it is able to direct NSCs to a particular fate (Woodbury, M. E., and Ikezu, T. 2013; Raballo et al., 2000). Conclusively, the trophic system regulated by the FgF2 generates an essential micro-environmental niche that endorses neurogenesis in the aCNS (Belluardo et al., 2009). Due to its potential significance in adult neurogenesis, as well as its importance for neuron-glia interaction and synaptic formation, the manipulation of FgF2's ligands and receptors will be a milestone in discovering treatments that stimulate neurogenesis as a therapy for neurodegenerative diseases, like Parkinson's and Alzheimer's diseases, brain injuries and multiple sclerosis (Woodbury, M. E., and Ikezu, T. 2013; Belluardo et al., 2009).

G. The Rb/E2F Pathway

The Rb/E2F pathway has long been known for its essential function in the control of various aspects of the cell cycle (Andrusiak et al., 2010). In fact, together with E2F transcription factors, the Rb family members of pocket proteins regulate crucial functions in the brain (Mcclellan et al., 2009). The established Rb/E2f pathway controlling the cell cycle is one of the principal effectors of fate decision in different cell types, including the nervous system. As previously mentioned, when Rb is phosphorylated by Cdks, it gets inactivated and detaches from the E2F transcription factors that become activated. These transcription factors are capable of regulating distinct fate choices in neural precursor cells including self-renewal/maintenance of stem cell pool, neuronal proliferation, differentiation and cell death. Such control occurs by binding directly to the promoters of numerous cell fate-associated genes whose roles are closely related to entry into the S phase and the progression through cell cycle in neural precursors (Pakenham et al., 2015; Saavedra et al., 2002). Consequently, a direct and clear function of the Rb/E2f pathway in the control of cell fate-associated genes is well established, however the extent of this interaction is still undetermined. In fact, most of the studies have concentrated on single Rb or E2f transcription factor knockouts, and since E2fs display a widespread redundancy in their genomic binding sites and roles, it is expected that many more genes targeted by E2Fs and important for cell fate determination are actually present. Therefore, the identification of these Rb/E2f targets and effectors will give fundamental understanding of these developmental mechanisms (Pakenham et al., 2015).

The Rb/E2F pathway has a fundamental function in the regulation of NSCs

proliferation. For example, Rb cooperates with E2F1 and E2F3 to regulate cell proliferation, the timing of cell cycle exit as well as cell survival (Mcclellan et al., 2009; Mcclellan et al., 2007). Using compound mutant mice for Rb/E2F1 or Rb/E2F3, Mcclellan et al showed that both transcription factors are functionally significant regulators of NPCs' proliferation as well as laminar patterning and cell cycle exit (Mcclellan, K. A., and Slack, R. S. 2007, Mcclellan et al., 2007). Saavedra et al also stated that loss of one of these two transcription factors is able to stop the ectopic proliferation and cell death seen in the Rb-null embryos in the CNS (Mcclellan, K. A., and Slack, R. S. 2007; Saavedra et al., 2002).

In fact, many studies have also implicated the Rb/E2F pathway in different roles beyond the well-known functions in the control of cell proliferation (Pakenham et al., 2015; Ghanem et al., 2012; Andrusiak et al., 2010). An Rb-specific deletion targeted to the telencephalon leads to abnormal NPCs differentiation in the SVZ as well as migration defects through the RMS and inside the OB (Ghanem et al. 2012). These defects were associated, at least partially, with the fact that the Rb/E2F pathway is needed to coordinate the transition between cell proliferation and differentiation by directly activating the level of expression of Dlx genes, particularly the Dlx2 transcription factor (Ghanem et al., 2012). Also, Rb was shown to regulate neuronal differentiation and migration in the telencephalon, independently of its role in cell cycle control and by interacting exclusively to E2F3 (Andrusiak et al., 2010; Mcclellan et al., 2009; Mcclellan, K. A., and Slack, R. S. 2007).

H. Rationale, Aims and Hypothesis

As stated above, studies have uncovered important molecular mechanisms by which the Rb/E2f pathway controls neurogenesis during development including cellular functions that extend beyond cell cycle control. In addition, key target genes mediating various Rb/E2f functions in brain development were identified and have contributed to our knowledge of how nervous system development proceeds. However, little is known about the target genes and mechanisms operating downstream of Rb in the adult brain despite recent evidence highlighting a central role for this pathway in the regulation of adult neurogenesis.

In addition, the generation of a high number of functional adult-born neurons from aNSCs is still a major obstacle facing regenerative medicine and crucial for neuronal replacement following injury or neurodegeneration. Considering the important contribution of adult neurogenesis to specific olfactory tasks, we hypothesized that at least some of the known Rb target genes during development including FGF2, E2F1 and E2F3 may play conserved role(s) in the control of cell proliferation in the adult brain. Moreover, the other two members of the Rb family of pocket proteins, p107 and p130, may compensate for the loss of Rb in the control of NSCs self-renewal and terminal differentiation, respectively.

1. Aim 1:

Given the above, we will examine whether the control of progenitor proliferation in the aSVZ by Rb is mediated by FGF2, E2F3 and/or E2F1 by assessing their levels of expression (transcript and protein levels) in the absence of Rb both in vivo and in vitro compared with controls. To do this, we will perform in situ hybridization on brain sections

using anti-sense RNA riboprobes, and, real-time PCR and western blot analyses on cDNA and protein lysates derived from dissected SVZ tissues and neurospheres in culture, respectively.

2. ***Aim 2:***

We will determine whether p107 and/or p130 is/are compensating for the loss of Rb in the control of neuronal proliferation, differentiation and/or survival. We will assess the level of expression of these two pocket proteins using similar approaches as those described under aim 1.

I. Significance of the study

Given that adult neurogenesis holds promising therapeutic benefits for regenerative medicine, neuroscientists have focused on uncovering the molecular mechanisms and pathways by which neuronal regeneration can be enhanced and targeted successfully to sites of injury while optimizing neuronal differentiation and survival inside the brain and even in culture. This study will help uncover some of the molecular mechanisms by which Rb and its E2F partners regulate adult neurogenesis at the molecular level since these proteins are major regulators of cell division and survival both in the embryonic and adult brains. Finally, the projected outcomes of this study will uncover how we can take advantage of the presence of enhanced neurogenesis e.g. as induced by a transient loss of Rb to boost the regenerative capacity and rescue death/loss of adult neurons inside the brain. In fact, brain plasticity associated with neuronal regeneration is such an important property that, if manipulated properly, may help in the treatment of brain damage following injury or cases of neurodegenerative diseases or as a result of normal aging.

CHAPTER 2

MATERIALS AND METHODS

A. Generation of adult Rb conditional KO mice in the adult brain

All animal experiments and procedures were performed according to the standard protocols as approved by the "Institutional Animal Care and Use Committee" (IACUC) at the American University of Beirut.

We generated an inducible conditional Rb deletion in aNSPCs in 2 month-old mice using NestinCreERT2-YFP mice obtained from the laboratory of Dr. Amelia Eisch at the University of Texas Southwestern Medical Center, USA (Lagace et al., 2007) and $Rb^{flox/flox}$ mice (Marino et al. 2000) In order to engender $NestinCre^{+/-}$, $YFP^{+/-}$, $Rb^{flox/+}$ mice (Rb heterozygous control mice) and $NestinCre^{+/-}$, $YFP^{+/-}$, $Rb^{flox/flox}$ mice (Rb mutant mice), $Rb^{flox/flox}$ mice were mated with NestinCreERT2-YFP/YFP mice. Nestin is a type VI intermediate filament protein that is specifically expressed in NSCs and their progeny. The Cre recombinase gene is fused with a mutated estrogen receptor ERT2 and under the control of the Nestin promoter and regulatory elements as described previously (Battiste et al. 2007). Two LoxP sites border the exon 19 of the Rb floxed allele. A stop codon located between the yellow fluorescent protein (YFP) gene and the ubiquitous Rosa26 promoter, is also flanked by 2 LoxP sites. Tamoxifen (TAM), an estrogen receptor antagonist, was administered to mice and bind to the mutated estrogen receptor of the Cre cassette. This activates Cre translocation into the nucleus in Nestin-positive cells and induces

recombination and excision of floxed sequences, thus removing one or both Rb allele(s) and activating the expression of YFP in recombined cells.

B. Tamoxifen administration

On daily basis and before each treatment, TAM (15mg/ml) is prepared from powder (Sigma T5648-5G) and dissolved in 10% absolute ethanol (Sigma Aldrich 65533) and sunflower seed oil (Sigma S5007). By oral gavage, fresh TAM is administered to the mice for 4-5 successive days according to body weight (180 mg of TAM per 1 Kg mouse weight) (Lagace et al., 2007).

C. Mice genotyping

3 weeks following their birth, mice are weaned and earpieces are taken for genotyping purposes. DNA is extracted using phenol-chloroform-isoamyl extraction procedure. Animal screening and genotyping for Rb, Cre and YFP is carried by PCR. The primers (BioRad) used are the following:

- Rb flox primers for the genomic DNA: Rb-18 forward 5' GGCGTGTGCCATCAATG 3' and Rb-19 reverse 5' AACTCAAGGGAGACCTG 3', and the amplicons sizes are 680 bp for the wild-type allele and 320 bp for the recombined allele.
- Rb flox primers for the mRNA transcript: Rb-exon17 forward 5' TTTGTCCTTCCGTGGATTCT 3' and Rb-exon20 reverse 5' GATGTGCTCTAGCTCTGGGTG 3', and the amplicons sizes are 502 bp for the wild-type allele and 359 bp for the recombined allele.

- Nestin-Cre primers: Cre-T2A forward 5' ATTTGCCTGCATTACCGGTC 3' and Cre-T2B reverse 5' ATCAACGTTTTCTTTTCG G 3', giving rise to a single band of 350 bp if the Cre is present.
- ROSA26YFP primers: forward wild-type 5'AAAGTCGCTCTGAGTTGTTAT 3', forward mutant 5' GCGAAGAGTTTGTCCTCAACC 3', and reverse 5' GGAGCG GGAGAAATGGATATG 3', giving a band of 560 bp for the wild-type allele and a band of 310 bp for the mutant allele.

D. RNA extraction and cDNA synthesis

RNA was extracted from dissected tissues and sorted green fluorescent cells derived from neurospheres in culture using the RNeasy Plus Mini Kit (Qiagen 74134). For RNA extraction from SVZ tissues, adequate volumes of RLT buffer and B-mercaptoethanol are added according to tissue mass, then the tissue is homogenized using a syringe and the supernatant recovered by centrifugation. For RNA extraction from sorted cells, RLT buffer and B-mercaptoethanol are added according to the number of cells and then homogenized using a syringe without centrifugation. The lysates obtained are transferred to the gDNA Eliminator spin column placed in a 2 ml collection tube. After centrifugation, 1 volume of 70% cold ethanol is added to the flow-through and then the mixture is transferred to the RNeasy spin column placed in a 2 ml collection tube. After centrifugation, the flow-through is discarded and RW1 buffer is added to the RNeasy spin column. After centrifugation, RPE buffer is added twice to the RNeasy spin column and the flow-through is discarded. Finally, 30 µl of RNase free water is added to the RNeasy spin column and pure RNA is eluted. It is then stored in aliquots at -80°C or directly used for cDNA synthesis.

In order to synthesize cDNA, reverse transcription is performed on the purified RNA using QuantiTect Reverse Transcription Kit (Qiagen 205311), after quantifying the RNA. The first step consists of removing any trace of genomic DNA by adding gDNA wipeout buffer to a total volume of 14ul. The mixture is incubated for exactly 2 mins at 42°C and then directly placed on ice. The second step consists of adding 4 µl of RT buffer, 1 µl of RT primer mix and 1 µl of RTase (to the +RT reaction) or 1 µl of RNase free water (to the –RT reaction). The mix is incubated at 42°C for 25 mins and then at 95°C for 3mins. Finally, the cDNA obtained is quantified using single strand DNA (ssDNA) option in the nanodrop and stored in aliquots at -80°C or 4°C for immediate use.

E. Real-Time PCR (RT-qPCR)

As a first step and before proceeding to the RT-qPCR, a standard curve for each gene is generated in order to know the exact efficiency of each set of primers used. To do so, a serial dilution of the cDNA ranging from 1:1 up to 1:100 is performed. 2 µl from each concentration of the cDNA is used, together with 10 µl of SYBR green (iTaq™ Universal SYBR® Green Supermix 1725121), 6 µl of RNase free water, and 1 µl of each primer (5mM) to obtain a total volume of 20 µl. RT-qPCR is carried using 1/10 or 1/20 dilution of cDNA depending on gene expression and duplicates of each sample. Three independent RT-qPCR experiments were carried to confirm the results obtained for each gene along with at least n=3 samples per genotype The 18S ribosomal gene and GAPDH were used as reference genes to normalize gene expression.

The RT-qPCR primers (BioRad) used are the following:

- FgF2 primers: FgF2 forward RT 5' GGCTGCTGGCTTCTAAGTGTG 3' and FgF2 reverse RT 5' TAATACGACTCACTATAGGGCAGTATGGCCTTCTGTCCAGGTCC 3' used with a 60°C annealing temperature.
- E2F1 primers: E2F1 forward RT 5' CCTCATGCCAGGAGACATCCTCTG 3' and E2F1 reverse RT 5' GGCAATACTGCTTCTTGCTCCAGG 3' used with a 59°C annealing temperature.
- E2F3 primers: E2F3 forward RT 5' AAGCCCACTTCCAAAGACTTGGCT 3' and E2F3 reverse RT 5' TCTTGGAGCAGGGGAGGCAG 3' used with a 61°C annealing temperature.
- p107 primers: p107 forward RT 5' CCGAAGCCCTGGATGACTT 3' and p107 reverse RT 5' GCATGCCAGCCAGTGTATAACTT 3' used with a 61°C annealing temperature.
- p130 primers: p130 forward RT 5' GGACCGCTGAAGGAAACTATGT 3' and p130 reverse RT 5' CTCCCCCACTTCTTCATCTTGTTAAAA 3' used with a 61°C annealing temperature.
- 18S primers: 18S forward RT 5' GTAACCCTTGAACCCCATT 3' and 18S reverse RT 5' CCATCCAATCGGTAGTAGCG 3' used with a 59-62°C annealing temperature.
- GAPDH primers: GAPDH forward RT 5' GGTGAAGGTCGGTGTGAACG 3' and GAPDH reverse RT 5' CTCGCTCCTGGAAGATGGTG 3' used with a 59-62°C annealing temperature.

F. Brain tissue preparation and sectioning

Mice were euthanized with Xylazine (0.25µl/g) and Ketamine (1.5µl/g), followed by a cardiac perfusion using 20-25ml of 1x cold phosphate buffer solution (PBS), and then subjected to 15-20ml of 4% cold paraformaldehyde (PFA) (ACROS). Afterwards, brains

were dissected and post-fixed in 4% PFA overnight. On the following day, brains were washed with 1x PBS and cryoprotected with 20% sucrose in 1x PBS for 2 days and next with 30% sucrose for 5 days. At the end, Tissue-Tek O.C.T. (SAKURA 4583) was used for brains' embedding and isopentane (-35°C) (Sigma Aldrich M32631) on dry ice using for brains' freezing. Using a cryostat (Leica, CM1850), brains were cut into 8-10μm thick sagittal sections and then mounted on SuperFrost adhesion slides (Fisher scientific and Thermo Scientific) and stored at -80°C.

G. Immunohistochemistry

Frozen tissue sections are removed from -80°C, air-dried and warmed at room temperature for at least 30 mins. After washing in 1xPBS, the sections are blocked for 1-2 hours in the blocking solution freshly prepared from 100% donkey serum, 10% Triton X, 10% BSA and 1x PBS. Afterward, slides are incubated with primary antibody overnight at room temperature. The following day, slides are washed 3 times in 1x PBS for 5-10 mins each, then incubated with fluorescent secondary antibodies at room temperature for 2 hours. After 3 washes in 1xPBS for 5-10 minutes each, slides are mounted using 1xPBS/Glycerol (3:1), and analyzed with a fluorescent microscope (upright Leica microscope DM6B).

The primary antibodies used are chicken anti-YFP (1:1000) (ab13970) and mouse anti-E2F1 (1:200) (sc-251).

The secondary antibodies used are donkey anti-chicken 488 (Jackson Immunoresearch) and donkey anti-mouse 594 (Jackson Immunoresearch).

Slides were also counterstained with Hoechst (1:100) to stain nuclei.

H. Probe synthesis and In situ hybridization

Before riboprobe synthesis, cDNA is amplified by PCR using specific primers for each gene and purified with the illustra GFX PCR DNA and Gel Band Purification Kit (GE healthcare 28903470). Riboprobe synthesis was carried by adding in the following order: 1 μg of DNA and RNase free water up to 15 μl in volume, 6 μl of 5x buffer, 2 μl of (100mM) cofactor DDT, 1 μl of RNase out Ribolock, 3 μl of 10x Digoxigenin-labeled dNTP mix and finally 2 μl of polymerase T7. The reaction is incubated at 37°C for 2-3h. Subsequently, the anti-sense RNA is precipitated using 2.5 μl of (4 M) LiCl and 75 μl of 100% cold ethanol after a 30min incubation at -80°C. This is followed by washes in 70% cold ethanol. After drying well, the pellet is re-suspended in 50 μl of RNase free water and 1 μl of RNase inhibitor. The probe is stored in aliquots at -80C. The extended primers (with T7 or T3 polymerase promoter, BioRad) used are the following:

- FgF2 primers: FgF2 forward T3 5' AATTAACCCTCACTAAAGGGTCTTCCTGCGCATCCATCCCG 3' and FgF2 reverse T7 5' TAATACGACTCACTATAGGG/CAGTATGGCCTTCTGTCCAGGTCC 3' used with a 60°C annealing temperature.
- E2F1 primers: E2F1 forward T3 5' AATTAACCCTCACTAAAGGGCCTCATGCCAGGAGACATCCTCTG 3' and E2F1 reverse T7 5' TAATACGACTCACTATAGGGACACACTGTACAACATCCTTCCCA 3' used with a 59°C annealing temperature.
- E2F3 primers: E2F3 forward T3 5'

AATTAACCCTCACTAAAGGGAAGCCCACTTCCAAAGACTTGGCT 3' and
E2F3 reverse T7 5'
TAATACGACTCACTATAGGGTCCATTCCGTGGTAGCAGACTCAC 3' used
with a 61°C annealing temperature.

In situ hybridization is performed according to a 3-days-protocol. During the first day, frozen sections are first pre-warmed for at least 30 mins at room temperature while warming the hybridization buffer (HB) as well as an incubation chamber containing wet Whatman paper (50% Formamide) at 65°C. Then, the probe is added to the hot HB and warmed again with vortexing every 5 minutes. After 30 mins, the slides are placed in the chamber, and 200 µl of the HB-probe mix is added to each slide. Finally, coverslips are placed gently on the slides and incubated at 65°C overnight. The next day, fresh wash buffer is prepared from 20x SSC, 50% deionized formamide, 10% Tween-20 and double distilled water (ddH2O), and pre-warmed at 65°C. After 30 mins, the coverslips are removed by holding the slides vertically (without exerting excessive force) and slides are washed twice with the hot wash buffer. Then, 3 consecutive cold washes are carried in 1x MABT buffer. Slides are transferred to a humidified box and incubated with the blocking solution (prepared from 100% sheep serum, 10% blocking reagent, 5x MABT and RNase free water) at room temperature. After 2 hours, anti-Digoxigenin antibody is diluted in blocking solution (1:1500) and added to the sections at room temperature overnight. The following day, sections are washed 4-5 times for 20 mins each in 1x MABT. Then they are incubated for 15-30mins with pre-staining buffer prepared from 5N NaCl, 1M Tris (pH=9), 1M MgCl2, 10% Tween-20 and ddH2O. Finally, slides are incubated in staining solution containing 10% PVA, NBT and BCIP in addition to the pre-staining buffer for hours or

days at room temperature in the dark until the desired staining is obtained. Subsequently, slides are washed in 1x PBS and staining is stopped using Tris (10mM) and EDTA (1mM) at pH=8 for 15 mins. At the end, slides are washed with ddH2O and mounted using a mix of PBS/Glycerol (1:1).

I. Protein lysate preparation and Western Blot

Protein lysastes are extracted from sorted cells derived from neurospheres in culture or dissected SVZ tissues by first, homogenization in 1x cold PBS with a syringe, then addition of RIPA-PI lysis buffer on ice for 45 mins, followed by centrifugation at full speed for 30 mins. Protein lysates are recovered in the supernatant and quantified. 10% acrylamide running gel is prepared by adding ddH2O, 2.5 M of Tris (pH=8.8), 10% SDS, 10% APS and 10% TEMED to 30% acrylamide. Approximately 25 µg of protein lysates per sample are run on gel, then transferred to a nitrocellulose membrane. The membrane is incubated for 30 mins in blocking buffer prepared from skimmed milk (Regilait), Tween 20 and 10% TBS (pH=7.5), then placed in primary antibody overnight at 4°C (prepared in 5% BSA). The following day, the membrane is washed 3 times with 1xTBST and incubated with the secondary antibody (prepared in 5% milk) for 1 hour at room temperature. The signal is detected by chemiluminescence by adding luminol Clarity ECL Substrate to the membranes and the signal's intensity is detected using ChemiDoc^{TM}MP.

Laminin gene is used as loading control.

The primary antibodies used are rabbit anti-p130 (1:200) (sc-317), rabbit anti-p107 (1:200) (sc-318) and mouse anti-Rb (1:500) (BD pharmigen; 554-136)

The secondary antibody used is HRP goat anti-rabbit (1:5000) (Jackson Immunoresearch) and goat anti-mouse (1:5000) (Jackson Immunoresearch).

CHAPTER 3

RESULTS

A. Generation of adult Rb conditional KO mice and proof of Cre recombination

Rb floxed mice having exon 19 of the Rb gene flanked between 2 loxP sites were crossed with NestinCreERT2 YFP mice in order to generate NestinCreERT2-$Rb^{flox/+}$ heterozygous mice having one normal Rb allele and one floxed allele, serving as Rb controls (referred to as Rb+/- after Cre recombination), as well as mice with 2 floxed alleles or NestinCreERT2-$Rb^{flox/flox}$ or Rb mutants (Rb-/-). Genotyping of Rb mice was done by PCR using gDNA extracted from earpieces taken from weaned mice and Rb specific primers (refer to material and methods for detail). In order to induce Rb deletion in the adult brain, Tamoxifen (TAM) was administered to 2 month-old mice (Figure 8). Upon TAM treatment, Cre nuclear translocation is activated in NSPCs expressing Nestin, an intermediate filament protein that specifically labels these stem cells and their progeny. Inside the nucleus, the Cre enzyme excises the Rb floxed allele(s) as well as the stop codon (flanked by loxP sites and located between the promoter and YFP gene), thus activating YFP expression. Accordingly, fully recombined cells will theoretically be YFP-positive and $Rb^{-/-}$ or $Rb^{+/-}$(Figure 8). The proof of recombination was confirmed in 3 different methods: 1) PCR analysis performed on gDNA extracted from sorted green fluorescent neurospheres (Figures 9; Naser et al. 2016). 2) Western blot analysis performed on protein lysates extracted from sorted green fluorescent neurospheres (Figures 10; Naser et al.

2016). 3) PCR analysis performed on cDNA extracted from sorted green fluorescent neurospheres (Figures 12).

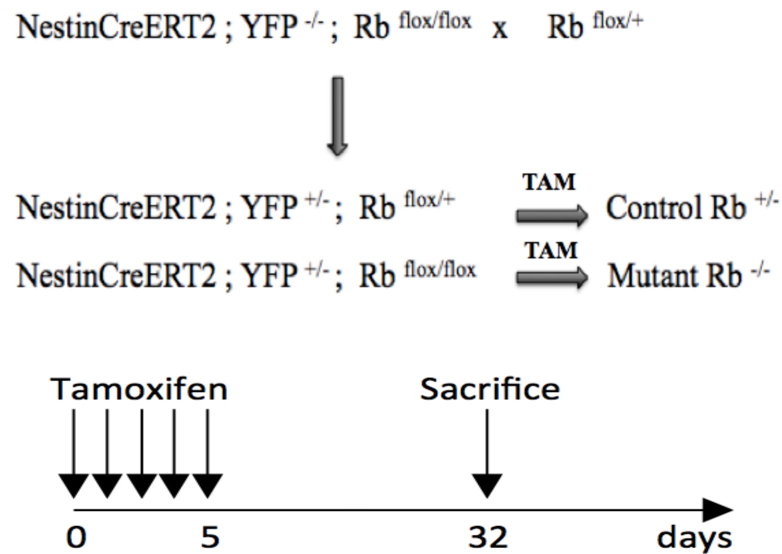

Figure 8: Schematic representation of the TAM treatment performed in 2 month-old mice. Fresh TAM was administered to mice by oral gavage for 4-5 consecutive days. 4 weeks later, mice were sacrificed and their brains were dissected, treated and sectioned using a cryostat for histological analyses (refer to material and methods for experimental detail).

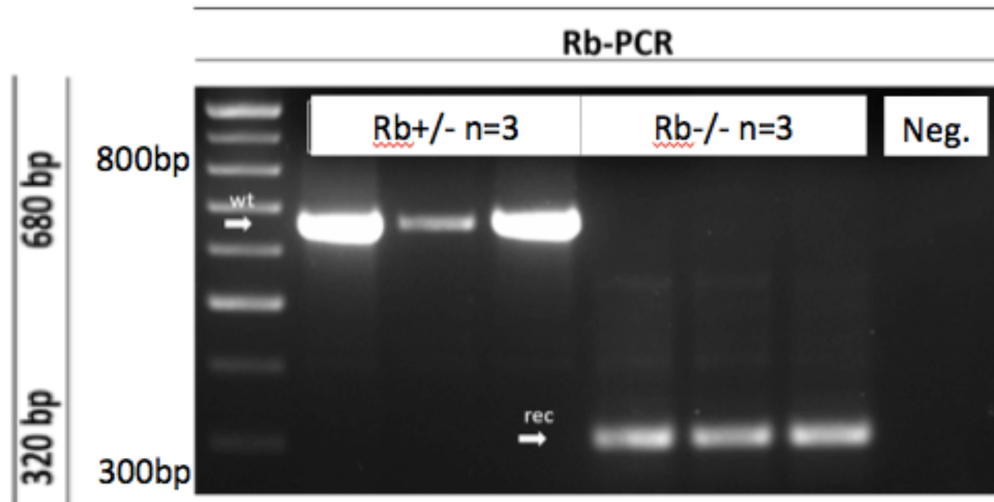

Figure 9: Proof of recombination performed by PCR using gDNA extracted from sorted green fluorescent neurospheres in culture and Rb flox primers for the genomic DNA. The ladder used is a 100bp ladder. The first 3 lanes (samples 1, 2 and 3) show a band of 680bp characteristic of the wild-type Rb allele. Lanes 4, 5 and 6 represent samples with 320 bp band characteristic of the recombined Rb allele. Sample 7 is a negative control (water; without gDNA).

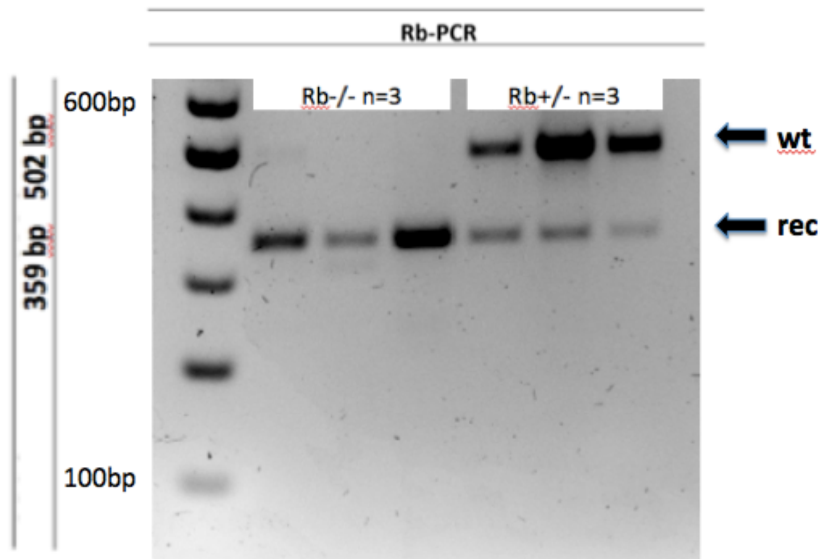

Figure 10: Proof of recombination performed by PCR using cDNA extracted from sorted green fluorescent neurospheres in culture and Rb flox primers for the mRNA transcript. The ladder used is a 100bp ladder. The first 3 lanes (samples 1, 2 and 3) show a band of 359 bp band characteristic of the recombined Rb allele. Lanes 4, 5 and 6 represent samples with a band of 502 bp characteristic of the wild-type Rb allele.

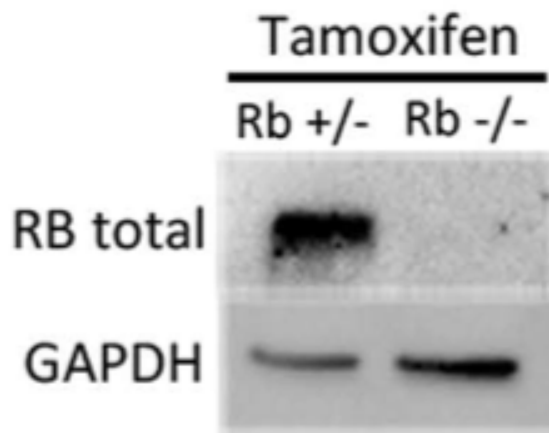

Figure 11: Proof of Cre recombination by Western Blot analysis performed on protein lysates from green fluorescent neurospheres that are derived from ($Rb^{+/-}$and $Rb^{-/-}$) cultures. The housekeeping gene GAPDH was used as a positive control (As modified from Naser et al. 2016)

B. Increased levels of E2F1, E2F3 and FgF2 transcripts' expressions in-vivo in the absence of Rb

Previous studies showed that the Rb/E2F pathway is an essential regulator of embryonic neurogenesis in the developing brain. In fact, Rb cooperates with E2F1 and E2F3 to control NPCs' proliferation, the timing of cell cycle exit and cell survival during development (Vélez-Cruz, R., 2016; Bartesaghi, S., and Salomoni, P., 2012; Mcclellan et al., 2009; Mcclellan et al., 2007; Degregori, J., and Johnson, D. G. 2006). Saavedra et al also stated that loss of one of these two transcription factors is able to stop the ectopic proliferation and cell death seen in the Rb-null embryos in the CNS (Mcclellan, K. A., and Slack, R. S. 2007; Saavedra et al., 2002). More recently, our laboratory identified a fundamental role of Rb in controlling progenitor proliferation in the aSVZ without disturbing the division rate (self-renewal) of aNSCs, which is consistent with its function during development (Naser et al. 2016). Moreover, the neurogenic growth factor FgF2 was to shown to regulate NSC/NPCs proliferation in the embryonic brain, where Fgf2 deletion leads to a reduced number of proliferating cells, thus a considerable decline in cortical neurons generated during neurogenesis in mice (Raballo et al., 2000). Interestingly, Mcclellan et al., 2009 showed that Fgf2 is under the direct transcriptional control of p107/E2F pathway (and possibly Rb/E2F), which regulates the responsiveness of NSCs to this factor during development (Mcclellan et al., 2009). All of the above led us to investigate whether the Rb controls progenitor proliferation during adult neurogenesis by cooperating with the E2F1 and/or E2F3 and whether Fgf2 could be a potential Rb target gene mediating such function as seen during embryonic neurogenesis.

Given that loss of Rb leads to specific increase in aNPCs' proliferation, we anticipated upregulation in the expression(s) of E2F1, E2F3 and/or Fgf2 in this context. In order to investigate this, we performed in situ hybridization using anti-sense RNA labeled probes targeted against the mRNA transcripts of all three genes. This was done on sagittal brain sections of mice sacrificed 28 days post-Rb deletion. Our in-vivo results revealed increased mRNA expressions of FgF2, E2F1 and E2F3 in the aSVZ and the RMS upon loss of Rb (Figure 12). This increase was detected in the dorsal, medial and ventral compartments of the aSVZ and consistent along the rostro-caudal axis (n=3 animals per genotype).

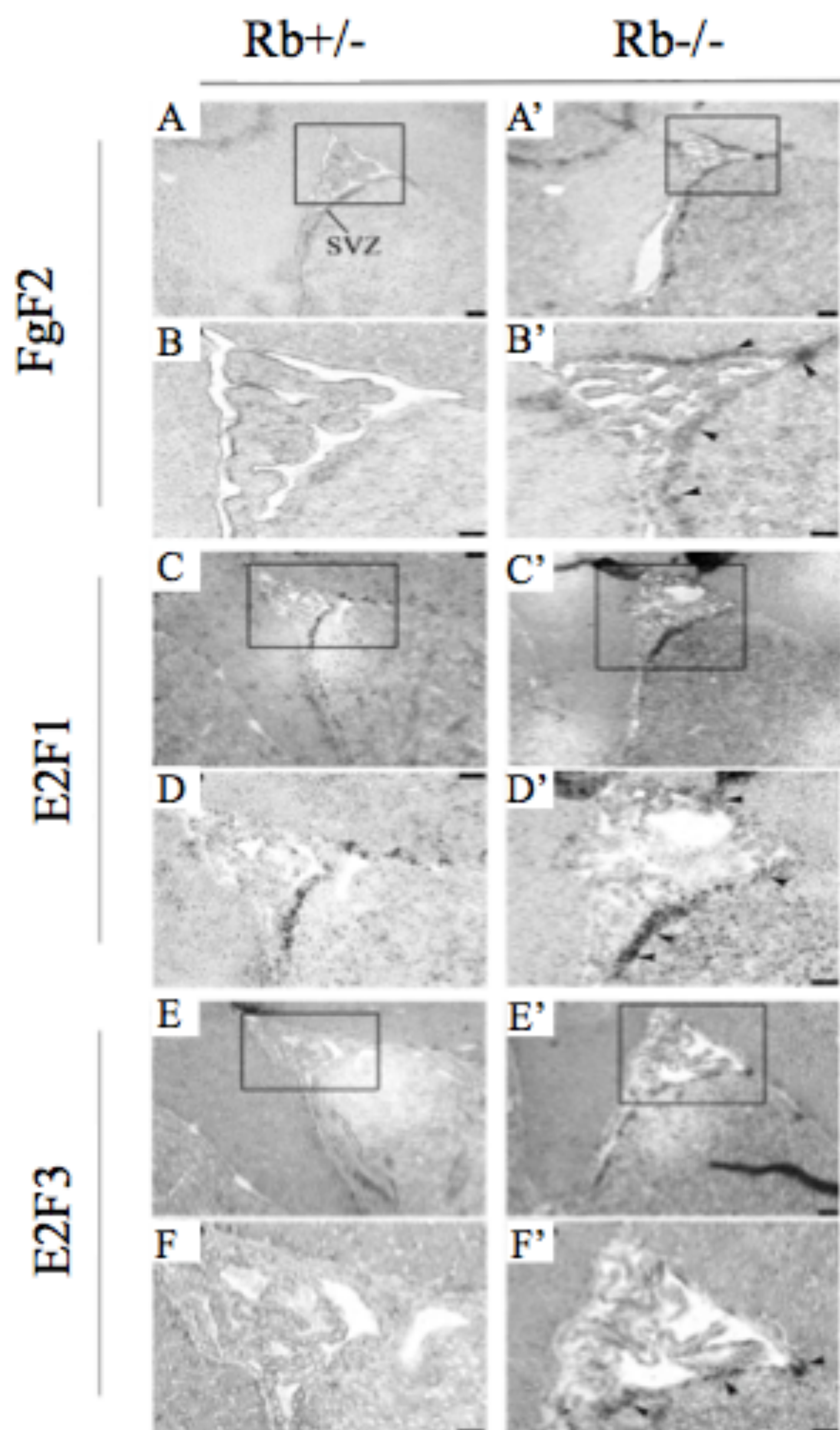
Rb+/-
Rb-/-
FgF2
E2F1
E2F3
A
A'
SVZ
B
B'
C
C'
D
D'
E
E'
F
F'

Figure 12: Increased transcript expressions of FgF2, E2F1 and E2F3 during adult neurogenesis in the aSVZ in the absence of Rb. (A-F') In situ hybridization was performed on sagittal adult brain sections using riboprobes for FgF2 (A-B'), E2F1 (C-D') and E2F3 (E-F') at 28 days post TAM treatment. In the absence of Rb ($Rb^{-/-}$ mice), note the increased transcript expressions of FgF2, E2F1 and E2F3 in the aSVZ (arrowheads in B', D' and F'). (A', B', C', D', E' and F') are higher magnifications of the boxed areas shown in (A, B, C, D, E, and F).

Next, we tried to confirm our in situ results by conducting Real-time PCR (RT-qPCR) analysis using cDNA derived from dissected aSVZ tissues from 2 month-old Rb+/- and Rb-/- mice. SVZ tissues surrounding the whole lateral ventricles were manually dissected 28 days post-TAM treatment, and total RNA extraction followed by cDNA synthesis was carried (as described in material and methods for detail). Our results showed either no or a slight increase in the levels of E2F1/3 transcription factors or Rb pocket proteins, p107 and p130, however, this increase was not statistically significant with some variability among distinct samples (Figure 13 presents one example of transcription factor and one example of Rb family member). Despite proper optimization and several repetitions, these results were consistent and this is likely due to the fact that the Rb-recombinant cells (|Rb-null cells) were outnumbered by a large number of wild type cells (Rb+/+) in the 'roughly dissected' SVZ tissues, which are masking or diluting any existing changes in gene expression levels.

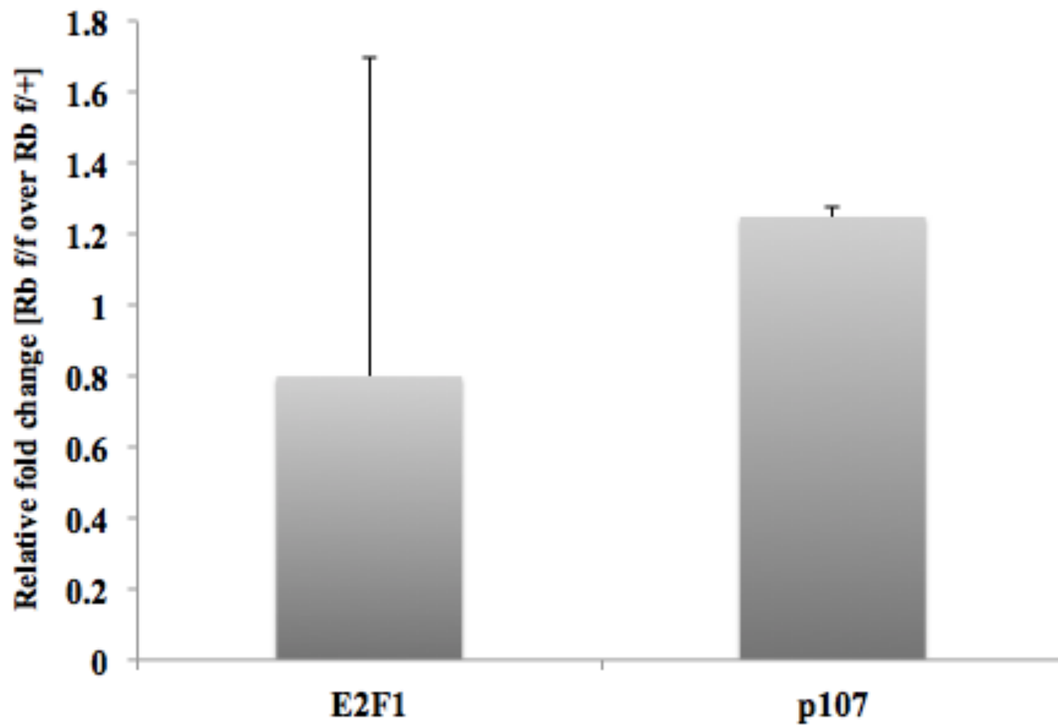

Figure 13: Graph showing no or slight increase but not significant in the transcript levels of E2F1 and p107 as quantified in-vivo by RT-qPCR using cDNA extracted from dissected SVZ tissues. The relative fold change in mRNA expression ($Rb^{-/-}$ over $Rb^{+/-)}$ was 0.8 and 1,25 for E2F1 and p107, respectively (n=5 controls and n=4 mutants). Error bars represent the standard error of the mean (SEM).

C. Increased mRNA expression levels of FgF2, E2F1 and E2F3 in-vitro in the absence of Rb

In order to confirm the upregulation in Fgf2, E2F1 and E2F3 mRNA levels in the absence of Rb, we performed RT-qPCR on cDNA extracted from sorted green neurospheres derived from Rb+/- and Rb-/- aSVZ tissues in culture. Briefly, 2 month-old mice were TAM-treated and 7 days later, primary cultures were grown from dissected aSVZ tissues from both genotypes and passaged after 7 days. On passage 2, all neurospheres derived from each sample were collected and dissociated into single cells; then, green recombinant cells were sorted by flow cytometry (FACS) (Figures 14 and 15). Subsequently, RNA extraction and cDNA synthesis was performed on the sorted green cells. Our results showed that, in the absence of Rb, the transcript levels of FgF2 and E2F1 are significantly upregulated in Rb-null neurospheres derived from aSVZ tissues versus those from Rb+/- tissues in-vitro. This increase is consistent with the in-vivo results that we obtained by in situ hybridization. Of note, the increase in E2F3 mRNA level was not significant (Figure 16), which might be due to the presence of two E2F3 isoforms, E2F3a and E2F3b that are acting in opposing manner to regulate neurogenesis as previously demonstrated (Julian et al. 2013 and refer to discussion)

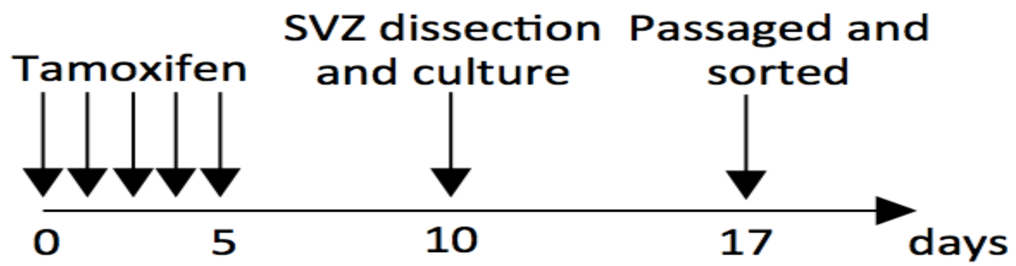

Figure 14: Schematic representation of aSVZ tissues derived cultures. Mice were sacrificed 5 days following TAM treatment and aSVZ tissues were dissected, dissociated and plated to generate primary cultures. Neurospheres were collected and passaged every 7 days. On passage 2 (day 17 after TAM treatment) green recombinant cells were sorted from pooled and dissociated spheres by FACS.

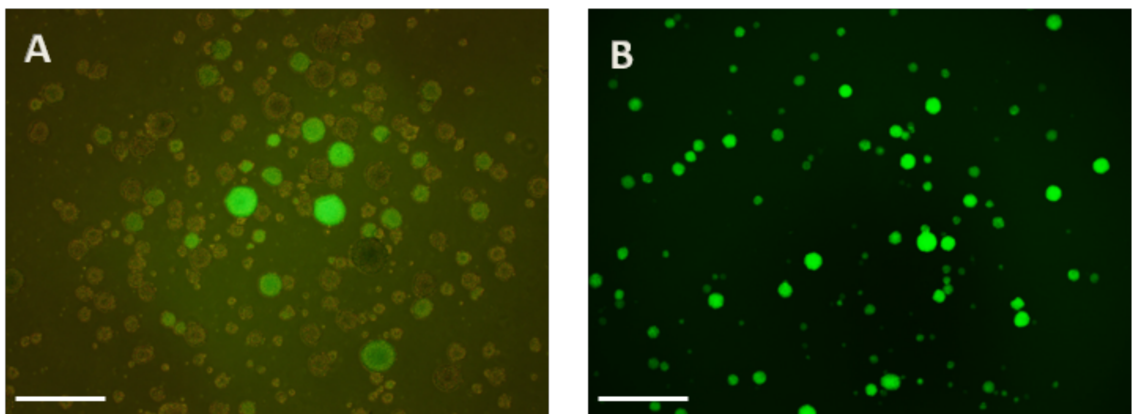

Figure 15: Fluorescent pictures of aSVZ neurospheres in culture before FACS sorting (A; showing a mix of green and non-recombinat spheres) and after FACS sorting (B; with green spheres only).

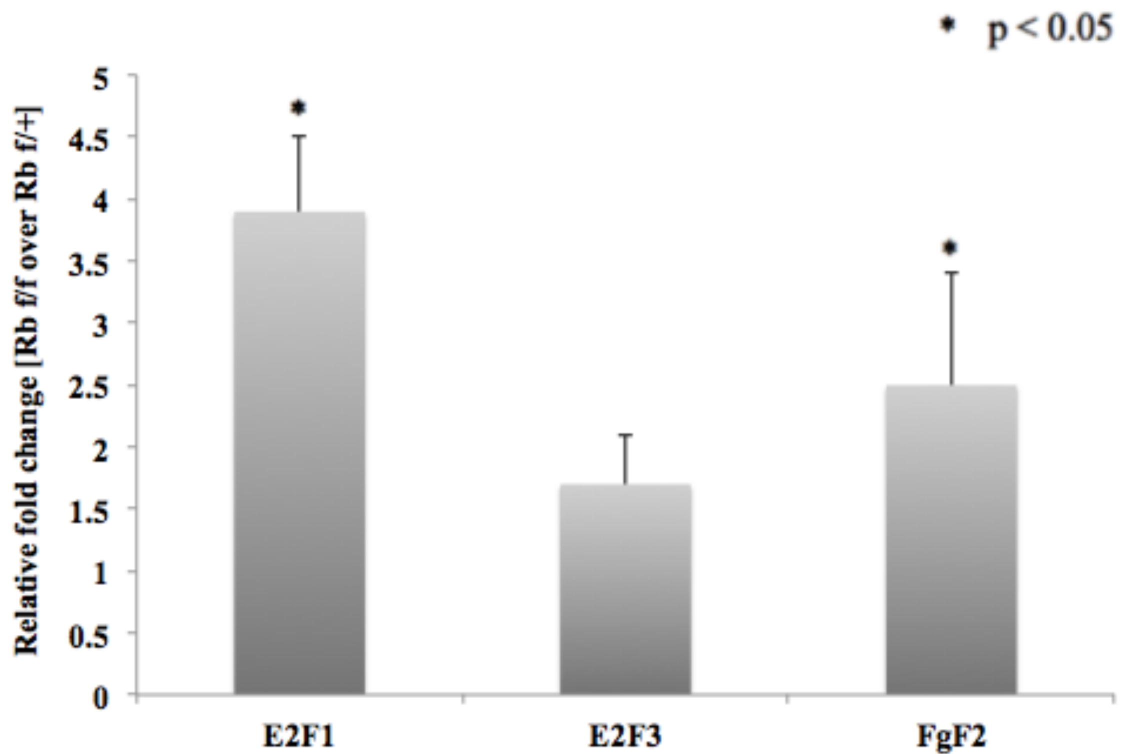

Figure 16: Graph showing significant increase in the mRNA levels of two Rb target genes, E2F1 and Fgf2, in culture in the absence of Rb. RT-qPCR performed on cDNA derived from Rb+/- and Rb-/- sorted green neurospheres in culture showed 4 folds, 2.5 folds and 1,5 folds increase in E2F1, FgF2 and E2F3 transcript levels in the absence of Rb, respectively. (n=4 control and n=3 mutants). Error bars represent the standard error of the mean (SEM).

D. Increased mRNA levels of expression of p107 and p130 in-vitro in the absence of Rb

As previously explained, Rb is one member of a family of pocket proteins including two other members, p107 and p130, which are all involved in the regulation of distinct stages of NSCs/NPCs development including self-renewal, cell proliferation, terminal differentiation and survival. They also share sequence homology in their pocket domain that regulates their interactions with other transcription factors and leads to a particular functional similarity between them (Bartesaghi, S., and Salomoni, P., 2012). Even though the three members bear different binding properties to diverse transcription factors, overexpression experiments revealed some level of functional redundancy in the control of cell cycle and neural development (Cobrinik, D. 2005; Trimarchi, J. M., and Lees, J. A. 2002). This prompted us to investigate whether p107 and p130 can compensate for the loss of Rb during adult neurogenesis owing to the fact that neuronal differentiation and short-term survival were not dramatically affected by the loss of Rb (Naser et al. 2016). Hence, we assessed their levels of expression in the absence of Rb by RT-qPCR using cDNA derived from neurospheres in culture as described above (Figure 14 and 15). Our in-vitro results showed a significant increase of 4.3 folds in the cDNA level of p107, and a highly significant increase in the level of p130 that reached 16 folds in the absence of Rb (Figure 17), suggesting that both pocket proteins may be compensating for Rb-related functions.

Given all of the above results, ongoing experiments are aimed at confirming the increase in p107, p130, E2F1 and E2F3 gene expression at the protein level by Western Blot analyses. This will be done on protein lysates extracted from Rb+/- and Rb-/- neurospheres derived from aSVZ tissues in culture.

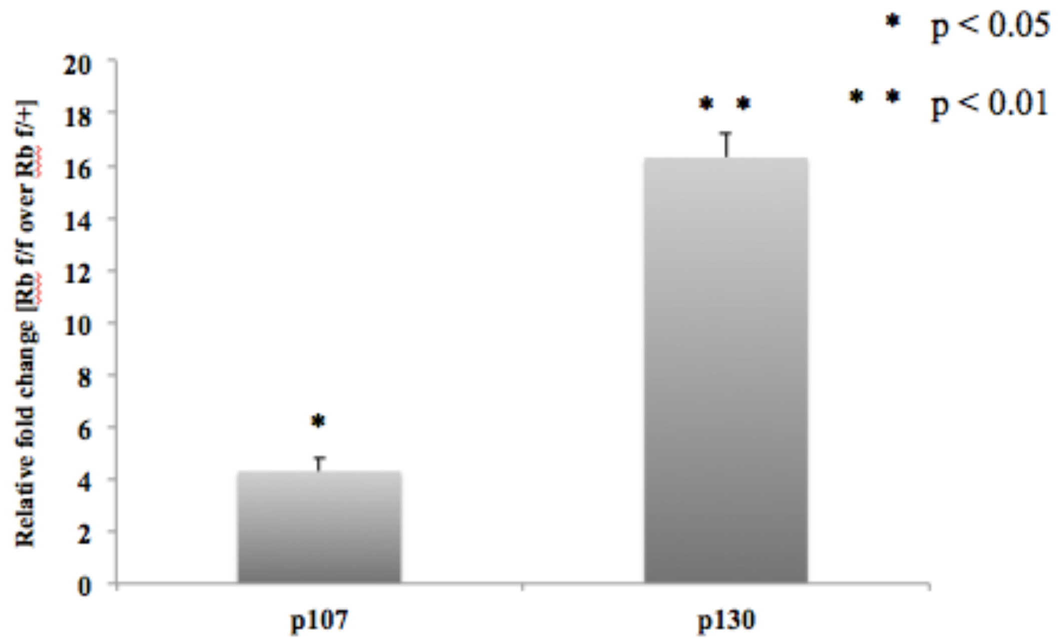

Figure 17: Graph showing significant increase in transcript levels of two pocket proteins, p107 and p130, as quantified by RT-qPCR using cDNA derived from Rb+/- versus Rb-/- neurospheres in culture. The relative fold change (ratio of Rb-/- over Rb+/-) is 4.3 and 16 folds increase in p107 and p130 mRNA expressions, respectively (n=4 controls and n=3 mutants) Error bars represent the standard error of the mean (SEM).

CHAPTER 4

DISCUSSION

In this study, we have examined the role of Rb/E2f pathway in the control of adult neurogenesis in the SVZ/OB. For this purpose, we applied different experimental methods to assess the gene expression levels of potential Rb target genes. We demonstrated thereafter that the Rb/E2F pathway plays conserved functions in the control of embryonic as well as adult neurogenesis via common downstream effectors such as FgF2, E2F1 and E2F3 (Swiss, V. A., and Casaccia, P., 2009). Our data also suggest that p107 and p130, the two Rb-related pocket proteins, may be compensating for the absence of Rb in the control of adult neurogenesis at least partially. Several findings strongly support this conclusion.

First, we found that the two transcription factors E2F1 and E2F3 as well as the growth factor FgF2 are likely to be mediating the control of cell proliferation by Rb during adult neurogenesis given that, in the absence of Rb, their mRNA levels of expression are significantly upregulated in the adult brain as shown by in situ hybridization (Figure 12) and by RT-qPCR on cDNA derived from neurospheres in culture (Figure 16). In fact, previous studies showed that Rb is vital for the control of E2F1/E2F3 transcriptional activities as well as the onset of terminal mitosis in NSPCs, two tightly regulated processes during neurogenesis. For instance, Callaghen et., al demonstrated that upon Rb deletion, this regulation is brutally disturbed in vitro and in vivo, indicating that E2F1/E2F3 are key regulatory targets for Rb in the embryonic nervous system (Callaghan et al., 1999). Furthermore, E2F1 or E2F3 deficiency in the CNS corrected the ectopic proliferation

observed in Rb deficient cells (Callaghan et al., 1999). Mcclellan et al., also reported that E2F1 and E2F3 are each functionally relevant targets in Rb-mediated control of NSPCs proliferation during development as both Rb E2F1 double knock-out (DKO) and Rb E2F3 DKO cells were able to rescue the proliferation defects (Mcclellan et al., 2007). Similarly, FgF2 was found to be abnormally upregulated in the brains of Rb-deficient and p107-deficient mice, separately, and studies performed on E2F3 and p107 mutant mice indicated that both proteins functionally collaborate to mediate FgF2 responsiveness in NPCs (Mcclellan et al., 2009). Therefore, our results with respect to the upregulation in gene expression of E2F1, E2F3 and FgF2 and its correlation with the control of proliferation by Rb during adult neurogenesis are consistent with the above studies performed during embryonic neurogenesis. Additionally, the interaction between Rb and E2F1/E2F3 or FGF2 is likely to be direct as seen during development, however, formal evidence is still lacking (Mcclellan et al., 2009; Mcclellan et., al 2007). Accordingly, chromatin immunoprecipitation (ChIP) can be applied to establish whether Rb is directly recruited to the promoter region of FGF2 as shown for p107 and E2F3 during development (Mcclellan et al., 2009). Subsequently, it would also be interesting to examine whether Rb negatively regulate FGF2 transcription by recruiting chromatin/nucleosome remodeling complexes in vivo such as histone deacetylases and/or HDAC hSWI/SNF complex as previously shown for the regulation of E2F and cyclins A and E by Rb. On the other hand, it is worth exploring the possibility that Rb may be controlling proliferation via other/unknown targets genes. One can systematically identify such targets by performing microarray analysis or RNA sequencing experiments on material extracted from sorted/recombined NSPCs in the aSVZ or cultured neurospheres. Of note, our in-vitro results showed a significant increase

by 4 folds and 2.5 folds in the transcript levels of E2F1 and FgF2 in the absence of Rb, respectively (Figure 16). However, the increase in the level of E2F3 was not statistically significant (1.5 folds; $p < 0.05$). This might be due to the presence of two E2F3 isoforms, E2F3a and E2F3b, which have been recently found to act in an opposing fashion. Actually, with respect to proliferation control, E2F3a was shown to form a transcriptional activator complex while E2F3b functions as part of a transcriptional repressor complex, and both isoforms act through the regulation of Sox2 gene expression (Pakenham et al., 2015; Julian et al., 2013; Saavedra et al., 2002). In fact, when E2f3b is deleted, E2F3a becomes the dominant isoform, and functions together with the p107 to repress Sox2 expression, thus inhibiting NSPCs' self-renewal and inducing differentiation. Contrariwise, following E2f3a deletion, the dominant isoform E2F3b activates Sox2 transcription by engaging RNA Polymerase II to the Sox2 promoter, therefore triggering NSCs self-renewal and expansion of NPCs pool at the expense of differentiation (Julian et al., 2013). Given that the E2F primers that we used recognize both isoforms, it is very difficult to discriminate and accurately assess at this point a potential change in gene expression in either isoforms. In order to overcome this limitation, it will be essential to design and use different set of primers that are specific for E2F3a and E2F3b in future experiments.

Second, our study is the first to show that both p107 and p130 may compensate for specific function(s) carried by Rb in the aSVZ/OB during adult neurogenesis given that, in the absence of Rb, their mRNA levels of expression are significantly upregulated as shown by RT-qPCR on cDNA derived from neurospheres in culture (Figure 17). We tried to confirm these results in vivo by performing RT-qPCR on cDNA extracted from dissected SVZ tissues. We detected slight increases in p107 and p130 transcript levels but this change

did not reach significance and showed high fluctuations. We believe that this might be due to the heterogeneity of the dissected tissues where a large number of cells may not be recombined (not green) or belong to other cell types e.g. astrocytes, in addition to the presence of a small but significant percentage of incompletely recombined cells (green but heterozygous for Rb or wild type cells), all of which may therefore, be masking and/or diluting the gene expression level of our target genes. To overcome this limitation, numerous techniques are available to isolate a specific cell type among others that are present in a complex tissue; the most reliable and accurate one is the Laser Microdissection (LMD). The cells of interest can be recognized from their morphology or expression of particular antigens or even better by the expression of fluorescent proteins e.g. YFP such as in our case. However, this in vivo method relies on fixing fluorescence in brain cells and separating them by LMD, followed by an improved RNA extraction that guarantees a precise isolation of an enough quantity and high purity RNA (Khodosevich et al., 2007). Morevoer, a recent study challenged the old established belief that it is practically impossible to separate/sort different cells from frozen and fixed neural tissues: Martin et., al developed an optimized method to successfully isolate specific types of cells from frozen/fixed tissue samples by separating cells into a suspension, then fluorescently tagging and sorting them (Martin et al., 2017).

Given that a change in transcript level may not necessarily correspond to a change in protein expression, ongoing work in the laboratory is presently aiming to assess p107 and p130 expressions at the protein level by Western Blot using protein lysates that are extracted from neurospheres in culture. Cell culture is underway in order to generate a high number of sorted/recombined cells from neurospheres for this purpose.

Owing to the existing structural and functional similarities between all three pocket proteins, many studies focused on the compensatory roles played by Rb, p107 and p130 in different types of tissues during development (Cheffer et al., 2013). Our data suggest that the adult brain does not seem to be an exception in this regard. In fact, Callaghan et al., showed that upon Rb deletion, NSPCs upregulate p107's protein level of expression in-vitro implying that p107 might substitute for the role of Rb in E2F-mediated transcriptional regulation (Callaghan et al., 1999). Also, Berman et al., demonstrated that pRb and p107 function together to regulate cell proliferation in different embryonic tissues including the CNS. Hence, by comparing Rb and p107 DKOs with Rb single KO, they showed the presence of an even more significant increase in ectopic proliferation as well as apoptosis in CNS in the former line, suggesting that p107 may compensate at least partially for the loss of Rb and limit - ectopic proliferation and cell death in Rb deficient CNS (Berman et al., 2009). Alternatively, the two pockets proteins could be acting via distinct mechanisms to control cell proliferation (e.g. in stem cell vs progenitor cells) and their roles could be additive. As a result, the two explanations are not mutually exclusive but it is very difficult to tease them apart in this context. The fact, that no major cell cycle exit or migration defects are observed in the adult brain in the absence of Rb, implies that the Rb family members display common and compensatory roles in regulating these functions. As for the ectopic progenitor proliferation in Rb-null NPCs, it is likely due to E2F deregulation as shown here, however, it is still not clear whether p107 can compensate for Rb in this context. In order to better investigate functional redundancy between pocket proteins and their distinct roles in E2F gene regulation, future studies should aim to generate compound

double and triple mutant animals such as NestinCreERT2 $Rb^{f/f} p107^{f/f}$, $Rb^{f/f} p130^{f/f}$, $Rb^{f/f}$ $E2F1^{f/f}$ and $Rb^{f/f}p107^{f/f}p130^{f/f}$.

As a matter of fact, we have generated in collaboration with Dr Ruth Slack's laboratory at the University of Ottawa Triple Knock-Out (TKO) mice carrying the following genotype: $p107^{-/-}$, $Rb^{f/f}$, $p130^{f/f}$. Our preliminary analyses revealed that, 4 weeks post Tamoxifen treatment, these animals show excessive ectopic proliferation in migrating neuroblasts and inside the OB accompanied with severe cell cycle exit defects as well as massive cell death starting in the RMS and reaching a peak inside the OB. In addition, the newborn neuroblasts aberrantly migrate to the cortex and the striatum rather than reaching their normal destination in the OB while those that reach the OB fail to detach from the RMS and migrate radially. Consistently, examining the TKO-aSVZ at 8 weeks post-Tam showed extremely enlarged lateral ventricles due to excessive cell death compared to triple heterozygous controls (unpublished data, Bejjani A and Ghanem N). Altogether, these results emphasize the presence of functional redundancy in the activities carried by all three pocket proteins at least in specific aspects such as the control of cell cycle exit and neuroblast migration.

In addition, it should be mentioned that previous studies showed that the functions of the three Rb family members are species-specific. For example, a whole genome study (Zhang et al., 2012) aimed at finding the genetic factors that trigger the Retinoblastoma cancer in humans, showed that biallelic mutations in the Rb gene are sufficient to cause tumorigenesis. In contrast, unlike in humans, loss of Rb protein alone in mice is not sufficient to cause retinoblastoma. In fact, p107 upregulation in murine retinal cells counterbalances the effects of Rb loss, thus preventing CCE and protecting against tumor

development. Accordingly, Rb-p107 and Rb-p130 DKO mice develop retinoblastoma tumors whereas in the triple KO mice ($Rb^{-/-}$ $p107^{-/-}$ $p130^{-/-}$), these tumors rapidly develop to reach to a metastatic state (Ajioka, I. 2014; Zhang et al., 2012). Consequently, since the adult neurogenesis field is mostly studied in animal models e.g. rodents, and despite the presence of few studies carried in humans, the adult neurogenesis field in humans still needs more in depth investigation in order to solve existing controversies and debates revolving around the presence as well as the temporal and functional extent of adult neurogenesis in the aSVZ and SGZ (Boldrini et al., 2018; Sorrells et al., 2018; Wang et al., 2011; Curtis et al., 2007; Sanai et al., 2004). Moreover, it is crucial to develop new tools and imaging techniques as well as identify reliable markers to study the distinct cell types found in post-mortem human tissues (Wang et al., 2011; Knoth et al., 2010).

In addition to the ongoing studies mentioned above, we are currently examining changes in gene expression of target genes following combined loss of Rb and p53, another tumor suppressor gene that was found to critically control aNSCs self-renewal and neuronal differentiation in the adult brain (Meletis et al. 2005 and Gil-Perotin et al. 2006). Add to this, the fact that both the Rb and p53 pathways show crosstalk at distinct levels during development and may display synergistic or complementary roles in the control of AN. Our RT-qPCR preliminary data (not shown here) show a significant and comparable increase in the levels of FgF2 and other pockets proteins in Rb-p53 conditional DKO in culture.

In conclusion, the detection of ongoing neurogenesis in the adult mammalian brain has challenged the old dogma denying neuronal regeneration inside the brain and proposed a novel perspective on the plastic nature of the CNS throughout life. Given the fast and continuous technical development achieved thus far and the use of sophisticated genetic

models that permit the targeting of particular subtypes of NSPCs or neuroblasts populations at different developmental stages, the field of adult neurogenesis is poised to make chief breakthroughs by focusing on the molecular mechanisms that govern NSCs and neuronal development as well as integration into the preexisting system. Moreover, it is intriguing to learn that adult neurogenesis recapitulates and relies on many common developmental processes that are at action during embryonic neurogenesis. Thus, the progress in this field is without a doubt further facilitated and guided by our understanding of such basic developmental events such as the ones highlighted in this study with respect to the role of the Rb/E2f pathway. Future comparative studies of adult and embryonic neurogenesis will surely continue to be rewarding. Additionally, a broader range of mutual neuronal and oncogenic proteins and pathways may be present, and their manipulation in a temporary and local fashion can help improve neurological outcomes (Zochodne et al., 2014). However, due to the absence of applicable regeneration of neurons after injury or neurodegenerative disorders in the adult mammalian brain (Kim et al., 2006), much more effort is required to explore and clinically benefit from the plasticity of the CNS. These studies will not merely aid in the development of the regenerative medicine, but will correspondingly disclose principles of stem cell biology in addition to new understandings of the olfactory circuitry and hippocampal functions as well as novel approaches for treating neurological and psychiatric disorders. Finally, as expressed by Ming and Song, combinatorial approach including studies carried at the molecular, cellular, circuitry and behavioral levels, will push the adult neurogenesis field to make another huge leap forward by explaining how it contributes to memory, learning, olfaction, mood control and many other concepts that are not recognized yet (Ming and Song, 2011).

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