AMERICAN UNIVERSITY OF BEIRUT

STUDY OF THE COMBINED ROLES OF RB AND P53 IN THE CONTROL OF MURINE ADULT NEUROGENESIS *IN VITRO*

by
AFAF RAJI SALIBA

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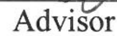

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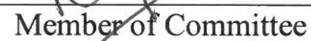

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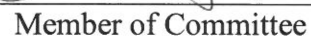

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AN ABSTRACT OF THE THESIS OF

Afaf Saliba for Master of Science

Major: Biology

Title: Study of the Combined Roles of Rb and p53 in the Control of Murine Adult Neurogenesis *in vitro*.

Adult neurogenesis (AN) is a highly dynamic process that is restricted to two main neurogenic zones in the brain, the sub-granular zone (SGZ) and the subventricular zone (SVZ). In the SVZ, activated adult neural stem and progenitor cells (aNSPCs) commit to differentiating into neuroblasts while migrating through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they finally differentiate into GABAergic interneurons. This process requires high orchestration between cell cycling and cell death. This study aims at studying the combined roles of two main cell cycle regulators, the Retinoblastoma protein (Rb) and p53, during AN in the SVZ using inducible Nestin-CreERT2-YFP-transgenic mice and Rb/p53$^{floxed/floxed}$ mice. aNSPCs are regulated by extrinsic and intrinsic signals. Previous studies found that loss of p53 induced hyper-population of aNSPCs in the SVZ whereby p53 negatively regulates self-renewal of aNSPCs (Gil-Perotin et al. 2006; Meletis et al. 2006). More recently, we have studied the role of Rb during AN in the SVZ and showed that it mainly controls proliferation in adult neural progenitor cells (not stem cells). Thus, loss of Rb led to significant increase in the pool of progenitors resulting in a transient increase in OB neurogenesis. Moreover, Rb was necessary for the long-term survival of newborn neurons in the olfactory bulb (Naser et al. 2016). Knowing that neuronal loss in the absence of Rb might be mediated by p53-dependent mechanism(s), and that both Rb and p53 pathways have major crosstalk in the control of the cell cycle, we hypothesized that manipulation of both pathways through the combined deletions of these genes may help amplify the pool of aNSPCs and their progeny, and, might compensate for neuronal cell death of Rb-null newborn neurons inside the OB. Our results showed that the effects of Rb and p53 in the control of aNSPCs proliferation are indeed additive (synergistic) in culture, and, combined loss of both genes further increase the proliferative potential of these cells compared to Rb and p53 single deletions and wild type cells. Moreover, p53 and Rb were both required for the long-term survival of newborn neurons in the adult OB; hence, p53 loss did not rescue neuronal cell death in Rb-null neurons. Our study provides insights for the in vitro manipulation of the Rb and p53 pathways in order to expand the progeny of aNSPCs in the future. A transient deletion of both genes may be a potential tool to significantly expand those cells for cell therapy and replacement in regenerative medicine e.g. following brain injury or neurodegenerative diseases.

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ABREVIATIONS

ABAM	antibiotic-antimycotic
AN	adult neurogenesis
aNSC	adult neural stem cells
aNSPC	adult neural stem and progenitor cells
aSVZ-NSPCs	adult neural stem and progenitor cells of the subventricular zone
Cdks	cyclin dependent kinases
Cdki	cyclin dependent kinases inhibitors
CNS	central nervous system
Cre	Cre-recombinase enzyme
DCX	doublecortin
DG	dentate gyrus
Dlx	*Distal-less* related mouse homologue
DNA	deoxyribonucleic acid
E	embryonic day
EGF	epidermal growth factor
ERT2	modified estrogen receptor
FGF-2	fibroblast growth factor-2
GABA	gamma-aminobutyric acid
GCL	granule cell layer
GCs	granule cells
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GL	glomerular layer

GLAST	glutamate-aspartate transporter
NCSs	Neural stem cells
NSPCs	Neural stem and progenitor cells
OB	olfactory bulb
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PFA	paraformaldehyde
Rb/pRb	retinoblastoma protein
RMS	rostral migratory stream
SGZ	subgranular zone
SVZ	subventricular zone
Ttp53/p53	tumor protein 53
VZ	ventricular zone
YFP	yellow fluorescent protein

CHAPTER I.

INTRODUCTION

A. Adult neurogenesis: Definition and brief history

1. Definition and significance of adult neurogenesis

Neurogenesis is defined as "a process of generating functional neurons from progenitor cells, including proliferation and neuronal fate specification of neural progenitors, and maturation and functional integration of neuronal progeny into neuronal circuits" (Ming & Song 2005). Adult neurogenesis (AN) validates the brain's distinctive plasticity and potential to continually add, eliminate and reshape neural connections in context-dependent manner to optimize the appropriate behavior in response to environmental stimuli (Jessberger & Gage 2014; Yau et al. 2014). It is involved in the "use it or lose it" learning and adaptation process of the brain (Shors et al. 2012). Historically, the primate's adult brain was considered having no or very limited neurogenesis and scientists associated the brain's stability with its higher complexity (Rakic 1985). Today, it is well established that active neurogenesis occurs in the subventricular zone (SVZ) and the subgranular zone (SGZ) of most mammals including humans. Apart from these conventional regions, AN is found in other regions mainly in response to excessive pathological cell death in the brain (reviewed in (Marlier et al. 2015). Accumulating studies depicted NSCs activated following ischemia in the cerebral cortex. These NSCs were found to generate, *in vitro*, neurospheres that are nestin-positive, and were detected using retroviral labeling to generate successfully-integrated GABAergic new born neurons in the cortex (Ohira et al. 2010; Nakagomi et al. 2011). Several studies provided evidence for the involvement of the hippocampal

neurogenesis in acquiring new information, specifically in spatial learning (Dupret et al. 2008), long-term retention of spatial memory (Snyder et al. 2005), trace and contextual fear conditioning (Pan et al. 2012; Seo et al. 2015) (reviewed in (Bond et al. 2015; Lieberwirth et al. 2016), in addition to its relation with several psychological disorders such as anxiety, stress and depression (Deng et al. 2010; Yau et al. 2011). In comparison, AN in the subventricular zone (SVZ) was found to be primarily needed for odor discrimination and olfaction-related-learning (Moreno et al. 2009; Arisi et al. 2012) in addition to reproduction (Larsen et al. 2008; Veyrac & Bakker 2011) and sexual behavior (Corona et al. 2016) reviewed in (Peretto & Paredes 2014; Peretto & Bonfanti 2015; Bond et al. 2015).

2. A brief history on adult neurogenesis

In 1913, neurogenesis was restricted , according to one of the founders of modern neuroscience Santiago Ramon Cajal, to embryonic development (Cajal & May 1991) despite previous suggestions made by Hamilton A. in 1901 on the presence of dividing cells within the postnatal brain (Hamilton 1901). It was not until the generation of [H3]-thymidine autoradiography method in a study on developing central nervous system histogenesis (Sidman et al. 1959) that Altman J and Das GD (1965) showed evidence of newly formed neurons in different adult brain regions including the dentate gyrus (DG) of the hippocampus in rats (Altman & Das 1965) and guinea pigs (Altman & Das 1967), and, the cerebellum in cats (Das & Altman 1971). These findings by Altman and colleagues on the existence of AN gained appraisal when evidence of long-term survival of newborn neurons was provided in 1977 (Kaplan & Hinds 1977) as well as evidence of neurogenesis in the neocortex of adult primates (Gould et al. 1999).

Since then, several studies started erupting on the functionality of these newborn neurons, for instance the role of AN in song learning in birds (Nottebohm 1989).

In the early 1990s, another major discovery in the field was done by Reynolds BA and Weiss S who identified for the first time the presence of adult neural stem cells (aNSCs) in the rodent brain and isolated them in culture (Reynolds et al. 1992; Reynolds & Weiss 1996). Which was followed by several reports of NSCs isolation from the SVZ and the hippocampus of rodents' brains (Richards et al. 1992; Palmer et al. 1999). Later on, Kukekov et al. isolated adult neural stem and progenitor cells (aNSPCs) from the subependymal zone and the hippocampus of human brain biopsies (Kukekov et al. 1999).

During the same period and after the generation of bromodeoxyuridine (BrdU), a thymidine analogue that marks cells in the S-phase (Gratzner 1982), AN was assessed and described in several mammals by BrdU incorporation including macaques and humans (Eriksson et al. 1998; Gould et al. 1999).

After the discovery of AN in the mammalian brain, the functionality and the synaptic integration of the newly formed neurons was later addressed in depth by more recent studies combining electrophysiological techniques, retroviral and genetic-based methods to study lineage tracing as well as loss- or gain- of gene function (van Praag et al. 1999; Van Praag 2008; Praag et al. 2002) reviewed in (Ming & Song 2011; Bond et al. 2015).

B. Overview on the adult neurogenic niches

1. Neurogenic niche definition

The neurogenic niche is the micro-environment that hosts, supports and regulates NSPCs development and survival. It is a source where appropriate signals and/or supporting cells induce NSCs quiescence, proliferation and/or differentiation in context-dependent manner (Reviewed in Ming & Song 2011).

2. Adult neurogenesis in the conventional neurogenic niches in rodents

The conventional neurogenic niches found in rodents including mice and rats are the SGZ and the SVZ. The SGZ is the germinal layer laying between the DG and the hilus in the hippocampus, and hosting radial and non-radial precursors that give rise to intermediate progenitors which eventually generate neuroblasts. These neuroblasts or immature neurons migrate to the inner granule cell layer (GCL) where they differentiate into dentate granule cells (DGCs). It requires few days for the newborn neurons to start forming synapses and integrate with the existing mature neuronal circuitry through dendritic extensions to the molecular layer and axonic extensions to the hilus toward the CA3 region of the hippocampus (Zhao et al. 2006; Toni et al. 2007). Importantly, these newly formed neurons were shown to display, during their development, higher synaptic plasticity and hyper excitability as compared to the already existing mature neurons in both the Hippocampus (Ge et al. 2007; Ge et al. 2008) and the OB (Nissant et al. 2009). However, once they become mature they exhibit similar electrophysiological properties with respect to their inhibitory GABA-ergic outputs and excitatory glutamatergic inputs compared to existing neurons (reviewed in Mongiat & Schinder 2011; Malvaut & Saghatelyan 2016). At the functional level, the newly formed neurons are known to integrate anatomically and participate in hippocampal memory

and information processing (Deng et al. 2010). Controversially, increased hippocampal neurogenesis, was found to contribute to forgetting through destabilizing the already existing neuronal circuitry in guinea pigs models (Akers et al. 2014).

The adult subventricular zone (aSVZ) lines the lateral ventricles. This neurogenic niche hosts a population of quiescent glial-like stem cells (type B cells) that, when activated, divide asymmetrically to generate transient-amplifying progenitor cells (type C cells) which in turn proliferate extensively to expand the pool of progenitors. Late progenitors exit the cell cycle and initiate their differentiation programs as immature neurons or neuroblasts (type A cells). Neuroblasts form converging and aligned “chains of cells” that migrate from the SVZ rostrally, thus following a route to the OB called the rostral migratory stream (RMS). This migration is a very dynamic event in the aSVZ neurogenesis. in rodents type A cells migrate rostrally through a few mm distance (up to 5 mm) to reach the OB (Lois & Alvarez-Buylla 1994; Doetsch & Alvarez-Buylla 1996). It was found to take 4 to 10 days in rodents (Luskin 1993; Lois & Alvarez-Buylla 1994; Hu & Rutishauser 1996). *In vitro* videomicrography analysis of this migration showed that type A cells migrate at a high speed of 120 um/h (Wichterle et al. 1997). After reaching the OB neuroblasts spread radially and differentiate into GABAergic subtypes of interneurons in the periglomular and GCLs (reviewed in Lim & Alvarez-Buylla 2016). A study by Brill et al. showed neurogenic generation of glutamatergic interneurons in the olfactory bulb derived from NSCs residing in the dorsal region of the SVZ (Brill et al. 2009).

A recent study showed different route for the SVZ-NSPCs. Where in response to stroke in the cortex, SVZ NSPCs migrate to the stroke region and differentiate into reactive astrocyte which promote scar formation (Faiz et al. 2015).

3. The neurogenic properties of the subgranular and the subventricular zones

The SVZ and SGZ are both characterized by a neurogenic environment enriched with endothelial cells, astrocytes, microglia, and ependymal cells in addition to the presence of NSPCs. They are also at proximity with dense vasculatures, and, the SVZ is in close interaction with the cerebrospinal fluid (CSF). These properties allow access to dense amount of signaling molecules such as pro-proliferation e.g. growth factors, hormones, etc. and other important regulatory signals in their environment (Bjornsson et al. 2015). Indeed, co-culture of aSVZ progenitors with endothelial cells led to an increase in neuronal differentiation (Leventhal et al. 1999) in addition to a dense number of proliferating cells located anatomically in proximity to vasculature (Palmer et al. 2000).

Moreover, astrocytes co-cultured with aNSPCs increased neuronal differentiation and progenitor proliferative potential (Lim & Alvarez-Buylla 1999). This could be attributed to the fact that astrocytes in the adult brain display diverse subtypes with different functions. Hence, some subtypes have stem-cell-like properties as stated earlier (Doetsch et al. 1999) while other subtypes act as a pro-neurogenic source of signals (Lim & Alvarez-Buylla 1999) or factors that promote synaptogenesis of newborn neurons (Sultan et al. 2015).

4. Unconventional neurogenic niche in rodents

Scientists sought to find regions in the adult brain other than the SGZ and the SGZ that might be hosting NSCs or at least a suitable environment for neurogenesis. Thus, several studies used *in vitro* cultures of neurospheres and BrdU incorporation to show the presence of NSPCs in several regions namely, the cortex (Seri et al. 2006), the substantia nigra (Zhao et al. 2003), the cerebellum (Lee et al. 2005) and the

hypothalamus (Pierce & Xu 2010). However, compared to the conventional niches, the proliferative capacity of NSPCs extracted from these non-conventional neurogenic niches exhibit less proliferative and differentiation capacities which can be enhanced with the additions of certain supplements such as vitamins (reviewed in Oyarce et al. 2014). Moreover, it remains unclear whether these regions are active neurogenic sites *in vivo*. Interestingly, a recent study showed that the substantia nigra is indeed a suitable neurogenic environment that can promote differentiation of transplanted mouse embryonic stem cells into neuroblasts and neurons in the murine host brain (Maya-Espinosa et al. 2015).

C. Cellular organization and structure of the adult subventricular zone niche

Along the CSF-filled lateral ventricles lie 4 main types of SVZ-cells including ependymal cells (Type-E), NSCs (type B) and their progeny (types C and A) as stated earlier (reviewed in Lim & Alvarez-Buylla 2016). The NSCs also known by type B cells share astrocytic characteristics (Doetsch et al. 1999) and express glial markers such as glial-fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST), and brain lipid-binding protein (BLBP) (Doetsch et al. 1997). In addition, they make end-feet connections with vasculatures within the SVZ and have direct contacts with ependymal cells (type E) that are lining immediately the lateral ventricles.

There are two states in which B cells can exist; quiescence or active (Codega et al. 2014). Active and not quiescent type B cells upregulate the expression of the intermediate filament protein nestin and epidermal growth factor receptor (EGFR). They were shown by time-lapse video microscopy to be able to undergo asymmetric division to self-renew and give rise to the transit-amplifying cells including Dlx2-expressing type C cells (Ortega et al. 2013). Using *in vivo* incorporation of mitotic cell markers,

type C cells were found to divide symmetrically around three times before committing into doublecortin-expressing-neuroblasts or type A cells which then undergo one or two rounds of divisions while migrating through the RMS to the OB where they differentiate into neurons (Ponti et al. 2013).

The subtypes of OB interneurons born to SVZ-type B cells was shown to be dependent on the spatial localization of these cells within the SVZ (regional distribution). Hence, deep granule neurons and periglomerular cells expressing calbindin originate from ventral NSCs (V-SVZ), whereas superficial granule cells (GCs) and periglomerular cells expressing tyrosine hydroxylase are derived from dorsal NSCs (D-SVZ). Interestingly, heterotypic grafts showed that this regionalization of NSCs is due to the cell intrinsic properties of the heterogeneous NSC populations found in the SVZ. Thus, when ventral NSCs are grafted dorsally in the SVZ they still generate calbindin-positive periglomerular cells in the OB. Therefore, the authors suggested the presence of significant differences in the epigenetic profiles of type B cells related to their physical location (Alvarez-Buylla et al. 2008).

It is clear now that there is a minimum of six subtypes of OB interneurons three of which are periglomerular cells while the other three are GCs. Recently, Merckle et. al. revealed the existence of an additional four subtypes of OB-interneurons that are derived from microdomains in the anterio-ventral regions of the SVZ and differentiate specifically in the mitral cell layer of the OB (Merkle et al. 2014).

The different sub-regions of the V-SVZ were recently classified into three domains by Lim and buylla (Lim & Alvarez-Buylla 2016) based on the SVZ structure and different arrangement of type B-1 cells:

- Apical domain I: The body of ependymal cells (type E) and apical processes of type B cells
- Intermediate domain II: The cell body of most type B cells, where they are found in contact with type C and A cells
- Basal domain III: The basal processes of B cells making “end-feet” on the blood vessels

D. Regulation of neural stem cells and neurogenesis in the adult subventricular zone

1. Extrinsic signaling pathways involved in the regulation of neurogenesis in the subventricular zone

Although different NSCs might exhibit different intrinsic properties, they are subject to and dependent on external regulation exerted by the microenvironment of the sub-region(s) where they are located. This signals-dependent neurogenesis in the SVZ was evidenced by transplantation experiments. Grafting murine SVZ-cells in another mice’s SVZ resulted in enormous amount of newborn neurons in the OB of the recipient mice (Lois & Alvarez-Buylla 1994). Contrarily, a negligible amount of neurons was generated after transplanting SVZ cells in a non-neurogenic regions of the recipient’s brain (Herrera et al. 1999). Several extrinsic signals play important roles in the regulation of AN in the SVZ including growth factors, morphogens, other autocrine/paracrine factors, neurotransmitters etc.

Growth Factors regulate NSCs properties

Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2) are two major mitogens used in cultures of SVZ-NSCs (Reynolds et al. 1992). Interestingly, EGF was found to mediate the conversion of transient-amplifying type C cells into

multipotent stem cells *in vivo* (Doetsch, Petreanu, et al. 2002). EGF and FGF and their signaling pathways interfere also in the *in vivo* control of SVZ-neurogenesis (Craig et al. 1996; Kuhn et al. 1997). Moreover, transforming growth factor-alpha (TGF-a) which is the most prevalent ligand of the EGF-Receptor (EGFR) was found to be involved in enhancing the SVZ-NSC proliferation and migration (Craig et al. 1996). In addition, both FGF-2 null mice (Zheng et al. 2004) and TGF- α null mice (Tropepe et al. 1997) displayed significant decrease in SVZ-neurogenesis and the maintenance of NSCs pool (reviewed in Bath & Lee 2010; Lim & Alvarez-Buylla 2016).

<u>Morphogens reach the SVZ through the CSF and affect neurogenesis</u>

Bone morphogenetic proteins (BMP), Sonic hedgehog (Shh) and Wnt ligands (wnts) are morphogens that can reach the SVZ through the CSF and are known to affect SVZ-neurogenesis (reviewed in Lim & Alvarez-Buylla 2016). BMPs, are secreted by adult neural progenitor cells (aNPCs) in the SVZ and were found to down regulate SVZ-neurogenesis (Lim et al. 2000). Ependymal cells secrete a BMP antagonist called noggin that antagonizes BMP signaling and increase SVZ-NSPCs proliferation (Lim et al. 2000).

In contrast to BMP signaling, studies showed that Wnt signaling (Wnt3a and Wnt5a) upregulates SVZ-NSCs proliferation and differentiation of NPCs into neurons in culture (Yu et al. 2006).

As for Shh, studies showed its involvement in regulating SVZ-neurogenesis at several levels. For instance, deletion of smoothened (Smo), a shh-signaling protein, resulted in decreased number of SVZ-NPCs marked with increase programed cell death (Machold et al. 2003). Also, Shh was found to control specifically the development of

ventral type B cells in the SVZ which express the transcription factor Gli1 in response to Shh signaling. This study emphasized on the important role of shh in the specification of the NSCs or type B cell positional identity in the SVZ (Ihrie et al. 2011). More recently, Shh signaling was found crucial for SVZ-NSCs' quiescence. And over expression of SHH exhibited dramatic increase in NSCs symmetric division which resulted in NSCs depletion (Ferent et al. 2014).

Notch and eph/ephrin signaling maintain type B cells quiescence

The aSVZ exhibit high expression of Notch1 and its major membrane-bound ligands, Jagged1 and Delta1 (Givogri et al. 2006). Canonical Notch signaling was found to maintain the SVZ-NSCs pool through negative feedback mechanisms whereby an increase in the number of type A cells, that express Jagged1 and Delta1, activates Notch1 signaling in NSCs (type B1). This activation of Notch1 signaling increases the self-renewal activity of type B cells while inhibiting their differentiation (Givogri et al. 2006). Another example of feedback mechanism controlling SVZ-neurogenesis involves the eph/ephrins membrane-bound ligands. For instance, ephrin A2 signaling by type A and C cells acting on EphA7 receptor expressed on type B cells and ependymal cells downregulates NSCs proliferation and SVZ neurogenesis (Holmberg et al. 2005). Furthermore, a recent study showed that the NSCs direct contacts with the endothelial cells in the SVZ further promotes their quiescence through Notch/jagged1 and eph/ephrin signaling (Ottone et al. 2014).

Neurotransmitters play a role in regulating SVZ-neurogenesis

Neuroblasts release GABA that acts through a negative-feedback to regulate proliferation of GFAP-expressing progenitors (Liu et al. 2005). Interestingly, this

negative effect of GABA can be counteracted with the action of diazepam-binding inhibitor (DBI) that is secreted by type B and type C cells; DBI competitively inhibits GABA binding to its receptors, thus resulting in increased SVZ-neurogenesis (Alfonso et al. 2012).

2. Intrinsic factors involved in the regulation of neurogenesis in the subventricular zone

Studies have shown that the different intrinsic properties of the NSCs and their progeny lead to distinct interpretation(s)/cellular response(s) downstream of the same extrinsic signal(s). Due to the fact that different BMP receptors are expressed by different cells along the NSPCs cell lineage, BMP signaling induces astrocytic differentiation in early neural precursor cells but promotes enhanced neuroblast survival (Lim et al. 2000).

Sox2, a member of the Sox family transcription Factors, is another key regulator of SVZ-NSC self-renewal and maintenance of SCs identity (Ferri et al. 2004). The arsenite-resistance protein 2 (Ars2) is directly required for the expression of *Sox2,* and, NSCs lose their self-renewal capacity upon Ars2 deletion and regain it upon overexpression of Sox2 (Andreu-Agullo et al. 2012).

The basic helix–loop–helix (bHLH) transcription factor Ascl1 (Mash-1) controls SVZ-neural precursors lineage fate and is mainly expressed by a subgroup of type B cells and type C cells. Loss of *Ascl1* results in defects in SVZ neurogenesis and oligodendrogenesis, thus highlighting its central role in specifying both neuronal and glial cell fates of SVZ- NPCs (Parras et al. 2004).

Finally, studies have demonstrated that different expression profiles of transcription factor(s) in the SVZ-NSPCs are linked to different interneurons

differentiation programs inside the OB. These expression profiles are also directly associated with *the* spatial cellular organization of NSCs inside the SVZ sub-regions as well as their intrinsic properties as described earlier (Reviewed in (Lim & Alvarez-Buylla 2016). For example*, Dlx1/2* expressing SVZ precursors were mapped using inducible genetic fate mapping and found to be expressed in most interneurons subtypes generated in the OB (seven subtypes)(Batista-Brito et al. 2008). Moreover, SVZ-neural progenitors expressing the *Emx1* homeobox gene and *Dlx5/6* genes give rise to a subset of calretinin-positive interneurons residing superficially in the GCL and the periglomerular layer of the OB (Kohwi et al. 2007).

In another study, the transcription factor Pax-6 (Homeobox gene *Paired box 6*) was shown to mediate cell-autonomous control of the dopaminergic-interneurons generation in the OB (Kohwi et al. 2005); hence, SVZ-type C cells deficient in Pax6 generated neuroblasts that migrated successfully to the OB but failed to differentiate into dopaminergic periglomerular interneurons.

E. *In vitro* properties of adult neural stem cells

1. The neurosphere assay

Since their discovery, the properties of aNSCs have long been studied and characterized *in vitro* including their self-renewal capacity, proliferative potential and multipotency (Reynolds et al. 1992; Richards et al. 1992; Rietze & Reynolds 2006) (reviewed in (Bond et al. 2015).

The aNSCs culture system known by the neurosphere assay, was first developed by Reynolds and Weiss (Reynolds et al. 1992) and is commonly used by stem cell biologists given its ease of use and suitability as a model for studying NSCs'

basic biology in addition to its use in stem cell therapy (reviewed by (Gil-Perotín et al. 2013). Briefly, this assay consists of seeding and growing aNSCs that are dissociated from the tissue of interest (*in vivo* dissection) in growth-factors-rich (FGF-2 and EGF) serum-free medium. aNSCs form free floating sphere-like clusters in culture referred to as neurospheres. Neurospheres are comprised of a heterogenous populations of neural precursors including a small fraction of slow-dividing NSCs, a large fraction of fast-dividing progenitors with some committed progenitors (Hulspas & Quesenberry 2000; Bez et al. 2003; Lobo et al. 2003). Neurospheres can be dissociated into single cells and manipulated in culture to perform different assays. Thus, cells can be cultured at low densities to measure the proliferative capacities of the NSPCs. In addition, seeding single cells or cells derived from a single dissociated neurosphere to generate primary and secondary neurospheres, respectively are assays used as an indication of the clonal-self-renewal capacities of NSPCs. Moreover, neurospheres can be maintained in culture for several passages; the numbers and size of the newly generated neutrospheres would indicate the amplification rate of the NSPCs. Finally, seeding single NSPCs on adherent substrate(s) in a serum-containing medium, would promote their differentiation into neurons, astrocytes, and/or oligodendrocytes (Protocol found in (Azari et al. 2010) (Reviewed in (Gil-Perotín et al. 2013).

2. Study of adult neural stem cells quiescence

Adult NSCs in the SVZ exist in either a quiescent and an active state inside the SVZ as described earlier. Using anti-mitotic drugs to eliminate fast-dividing neural precursors, aNSCs were reported to be primarily quiescent *in vivo* (Doetsch et al. 1999); their quiescence was essentially attributed for maintaining their genomic stability (Kippin et al. 2005). The neurosphere assay faces a major caveat when studying

quiescence of aNSCs (reviewed in (Gil-Perotín et al. 2013; Bond et al. 2015). First, because the quiescent NSCs (qNSCs) are normally mixed with the activated ones in a heterogeneous population and tend to be eliminated progressively with successive passages due to their slow division rates compared to the higher proliferative rates of the activated precursors. Second, it is still a major challenge to sort out specifically qNSCs from activated NSPCs extracted from the adult neurogenic niches including the aSVZ due to the lack of appropriate markers to discriminate between the two types (reviewed in (Gil-Perotín et al. 2013; Bond et al. 2015). A recent study reported prospective isolation of quiescent SVZ-NSCs using a combination of GFAP and CD133 markers *in vitro*; In fact, the authors showed that, unlike active NSCs that are positive for GFAP; CD133; EGFR and nestin, qNSCs express GFAP and CD133 only and rarely exhibit neurospheres formation (Codega et al. 2014). This observation was consistent with the dormant state of these cells as depicted *in vivo* and emphasized the fact that the neurosphere assay can only be useful to assess the *in vitro* properties of activated NSCs and their derived progenitors (Codega et al. 2014).

3. Study of the self-renewal capacity of adult neural stem cells

Using the neurosphere assay, several studies demonstrated that NSCs lines derived from embryonic (Vescovi et al. 1999) and adult neurogenic tissues can be maintained and expanded *in vitro* for a long time (minimum of ten passages depending on the age and source of NSCs) (Gritti et al. 1999; Gritti et al. 2002). The self-renewing NSCs are positively selected at each subsequent passage and the more committed neural precursors are negatively selected. This confirms their unlimited self-renewal capacity (Reynolds et al. 1992; Galli et al. 2004) (reviewed in De Filippis & Binda 2012). However, this assay is not perfect and relies on measuring self-renewal and clonal

potential by counting the number of neurospheres generated from one neurosphere over many passages (Louis et al. 2013) despite the fact that both stem cells and progenitors derived from the same sphere are capable of colony formation (Gil-Perotín et al. 2013). To overcome this limitation modified method denoted as the neural colony forming cell (NCFC) assay was introduced by which the size of the neurospheres formed is considered an indicative of the proliferative potential; thus, one would be able to differentiate between the progenies of NSCs and NPCS with larger colonies attributed to the actively self-renewing NSCs (Louis et al. 2008).

In comparison with the above *in vitro* studies, it is still controversial whether aNSCs are also capable of maintaining such a strong long-term/unlimited self-renewal potential *in vivo* (Bond et al. 2015). One study has shown that deletion of *Pten* in a subpopulation of aNSCs in the SVZ leads to persistently enhanced NSCs self-renewal without sign of exhaustion (Gregorian et al. 2009). In contrast, a recent study by Calzolari et al. showed that the progeny of individual NSCs exhibits fast clonal expansion as well as limited stem cell self-renewal and exhaustion within few weeks in the SVZ (Calzolari et al. 2015). Similarly, other studies reported depletion of the NSC pool upon specific gene manipulations *in vivo*. For instance, overexpression of Shh caused NSPCs depletion due to the dramatic increase in the symmetric division of NSCs (Ferent et al. 2014). Of note, several studies have shown that the proliferative potential of aNSCs declines with aging (Conover & Shook 2011; Kempermann 2011; Mobley et al. 2013) but this is likely due to lack of extrinsic support from the niche e.g. growth factors in addition to potential change(s) in the intrinsic properties of the NSCs overtime e.g. increased quiescence (Luo et al. 2006) .

4. Differentiation potential of adult neural stem cells

Using the neurosphere assay, adult SVZ-NSCs were found to be tri-potent and able to differentiate into neurons, astrocytes and oligodendrocytes when cultured in monolayers in the presence of serum and absence of growth factors (Reynolds et al. 1992). Similar *in vitro* differentiation assays are reliably used in other studies to investigate the properties of aNSCs following specific genetic manipulations *in vivo* (Meletis et al. 2006; Gil-Perotin et al. 2006; Naser et al. 2016) . Of note, since the differentiation potential of NSCs is triggered under 'artificial conditions' set in culture by the experimenter choice of factors and serum(s), this assay remains at best an correlative indication of the true potential of these cells *in vivo* where there is a higher level of extrinsic control and a complex spatio-temporal regulation of aNSCs development (Herrera et al. 1999; Merkle et al. 2007; Alvarez-Buylla et al. 2008; Merkle et al. 2014; Hooper et al. 2014) (Reviewed in Bond et al. 2015; Lim & Alvarez-Buylla 2016). In fact, studies have shown that aNSCs are regionalized in the SVZ and give rise to distinct interneurons in the OB depending on their location in the dorsal versus medial or ventral SVZs (Alvarez-Buylla et al. 2008; Merkle et al. 2014). Interestingly, one recent study showed some individual NSCs in the SVZ generate a neuronal lineage only (Calzolari et al. 2015).

F. Role of the Retinoblastoma protein, Rb, in adult neurogenesis

1. Role of Rb in cell cycle regulation

The tumor suppressor gene (*Rb*) encodes a 110-kDA nuclear phosphoprotein. It was first identified and defined to predispose, when mutated, to a rare retinal cancer that occurs in infants during development, the ocular tumor Retinoblastoma (Friend et al. 1986; Lee et al. 1987; Blanquet et al. 1993). Two other members of the Rb family

were later identified based on similarity in their genomic sequences: RbL1 that codes for p107 protein, and, RbL2 that codes for p130 protein. The three Retinoblastoma members, referred to as the pocket proteins family, interact through their conserved pocket A/B domain with a specific motif, L-X-C-X-E, found in certain viral oncogenes such as simian virus, human papilloma virus and adenovirus (Münger et al. 1989; Ewen et al. 1989; Dyson et al. 1989; Ewen et al. 1991; Li et al. 1993; Dyson 1998) (reviewed in Sage 2012; Henley & Dick 2012).

Despite the structural similarity among the pocket proteins, they act distinctly but synchronize their activities towards arresting the cell cycle at G1 phase. Moreover, they share the ability to interact, in their hypo-phosphorylated states, with members of the E2F transcription factors family through their pocket domain (Chow et al. 1996; Chow & Dean 1996;reviewed in Dick & Rubin 2013). Regulation of the G1/S cell cycle transition by the pocket proteins can also be mediated through E2F- independent routes; for example, p107 and p130 arrest the cell cycle progression through inhibiting cyclin/cyclin dependent kinases (cyclin/Cdks) complexes such as cyclin E/cdk2 and cyclin A/cdk2 (Sherr & Roberts 1999). In addition, Rb upregulates and promotes the stabilization of p27 protein which acts as a cyclin-dependent kinase inhibitor (cdki), thus arresting the cell at G1 phase (Park et al. 1999,reviewed in Henley & Dick 2012 and Dick & Rubin 2013). In turn, Cdks transiently phosphorylate and inactivate Rb and other pocket proteins, thus promoting cell cycle progression. For instance, cyclin D/Cdk4/6 was found to mono- phosphorylate Rb, during early G1 phase to allow the release of E2F transcription factors while at late G1 phase Rb is hyper-phosphorylated by cyclin E/Cdk2 (Narasimha et al. 2014). This phosphorylated/dephosphorylated Rb

state is highly regulated at different cell cycle checkpoints by the binding/unbinding state of Cdki to the cyclin/Cdk complexes (reviewed in Giacinti & Giordano 2006).

In addition to regulating the G1/S transition, Rb is involved in controlling DNA replication timing and mitotic chromosomal segregation (Coschi et al. 2010; Van Harn et al. 2010). Rb was also found to mediate appropriate centromeres condensation where studies correlated compromised chromosomal segregation with compromised Rb function (Manning & Dyson 2012; reviewed in Bartesaghi & Salomoni 2013).

2. Role of Rb in cell quiescence and cell death

Mice null for p107 or p130 can develop normally into adults (Lee et al. 1996; Mulligan & Jacks 1998). However, Rb-null mice die by embryonic day 15 (E15) due to severe neural tube and skeletal muscle developmental defects associated with hyper-proliferation and cell death (Jacks et al. 1992; Lee et al. 1992; Clarke et al. 1992). Rb was suggested to have a more important compensatory role in p107- and p130-null mice, thus supporting their survival (reviewed in Bartesaghi & Salomoni 2013). Hyper-proliferation in Rb-null embryos was found to be cell-autonomous (Sage et al. 2003). However, the increase in cell death was shown to be cell non-autonomous resulting from hypoxia induced by placental developmental defects (MacPherson et al. 2003). To overcome the research limitation associated with embryonic lethality upon Rb loss, it was essential to generate mice with conditional and transient loss of Rb in a tissue-and-time-specific manner (Vooijs et al. 1998; MacPherson et al. 2003). Using conditional deletions, Rb was found essential in maintaining cell quiescence in development and adulthood (Sage et al. 2003; Li & Clevers 2010) . For instance, acute Rb loss, promoted cell cycle re-entry in quiescent and senescent cells which are arrested from cell cycling

but are distinguished with high metabolic activity (Sage et al. 2003), in addition to inducing reprogramming of differentiated cells back to a pluripotent state (Kareta et al. 2015). Moreover, Rb loss increased NPCs proliferation, accompanied with an increase in cell death and differentiation defects (Ferguson et al. 2002; Ferguson et al. 2005; McClellan et al. 2007). The increase in death of the differentiating and/or differentiated NPCs lacking Rb may involve p53-dependent apoptotic pathways (Macleod et al. 1996; Morgenbesser et al. 1994;reviewed in Sage 2012; Polager & Ginsberg 2009).

3. Role of Rb in the developing central nervous system

Mice embryos with homozygous deletion of the *Rb* gene had ectopic NPCs proliferation accompanied with increase in cell death in the developing cortical regions including the ventricular zone (VZ) and subventricular zone (SVZ) (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). Conditional Rb deletion in the telencephalon also resulted in NPCs hyper-proliferation. However, most of these progenitors/neuroblasts survived and differentiated normally resulting in increased brain cellularity (Ferguson et al. 2002). In addition to the previous cell-autonomous control of NPCs proliferation, Rb was shown to regulate the newborn interneurons' migration in the embryonic cortex. Transplanted Rb-null newborn neurons exhibited tangential migration defects in the hosts' mice brain, thus validating a cell-autonomous requirement for Rb in the control on cell migration (Ferguson et al. 2005). A recent study showed that the neuronal differentiation and migration defects observed in the SVZ after telencephalic/conditional loss of Rb are associated with dramatic decrease in the expression of *Dlx1* and *Dlx2*, key genes that are required for the development of all interneurons in the brain (Ghanem et al. 2012). This again confirms the cell-autonomous control of NPCs proliferation and migration by Rb. More recently, mice

carrying the same telencephalic-specific deletion of Rb showed severe neurogenic defects in the developing olfactory system including ectopic proliferation of the NPCs, severe radial migration and axonal guidance defects leading to gradual olfactory system degeneration (Jaafar et al. 2016).

Of interest, a recent *in vitro* study sought to study the role of Rb during human CNS development. The authors differentiated wild type and RB1-knock-out human embryonic stem cells into cerebral organoids thus forming a three-dimensional culture model of the developing cortical tissue. RB loss was associated with increased cerebral organoids' volume indicating an increase in neuroblast proliferation. In addition, the authors observed increased apoptosis of NSCs, neuroblasts and neurons, thus mimicking the role of Rb in controlling neuronal survival in the rodent brain during development (Matsui et al. 2017).

4. Role of Rb in adult neurogenesis

While the role of Rb has been extensively studied in the developing brain, few studies have investigated its role and the role of other pocket proteins and their E2F target genes in controlling AN. Two key regulatory target proteins in the Rb signaling pathways, E2F1 and E2F3 were shown to be positive regulators of precursor cell proliferation in the adult brain with some redundancy in function (Cooper-Kuhn et al. 2002; McClellan et al. 2007). Consistently, mice deficient in E2F3 exhibited a decrease in the adult neural progenitor pool inside the SVZ (McClellan et al. 2007), and this finding was also true for stem cells and progenitors found in the SVZ and the hippocampus in E2F1-null mice (Cooper-Kuhn et al. 2002)

Vanderluit et al. studied the role of p107 during embryonic and AN. and reported that the expression of *p107* was upregulated in NPCs but was progressively downregulated during progression along the neurogenic lineage; p107 was shown to promote neural progenitor commitment to a neuronal fate (Vanderluit et al. 2007). Moreover, adult neural precursor cells lacking p107 had enhanced self-renewal capacity in addition to increased proliferation which was found be mediated by the Notch-Hes signaling pathway (Vanderluit et al. 2004). In a related study, p107 was found to regulate FGF2 level, thus affecting specifically NPCs responsiveness to FGF2 signaling (McClellan et al. 2009).

More recently, Rb was found to be involved in the regulation of adult DGCs neurogenesis. Rb loss in the adult hippocampus hence mimicked its functional role in CNS development and resulted in increased proliferation of immature DGCs, however, this effect was coupled to decreased differentiation and/or survival of in the population of mature DGCs (Vandenbosch et al. 2016).In comparison, Naser et al. investigated the role of Rb in the adult SVZ-OB neurogenesis and found that, similarly to its role in CNS development, Rb did not affect the self-renewal capacity of aNSCs in the SVZ, however, it specifically regulates aNPCs proliferation both *in vivo* and *in vitro.* Loss of Rb hence led to an increase in NPCs pool in the aSVZ and RMS and without affecting the rostral migration of the adult neuroblasts or their terminal maturation inside the OB. This resulted in a transient enhancement in OB neurogenesis that was offset by long-term survival defects of Rb-null newborn neurons (Naser et al. 2016).Moreover, aNPCs of the SVZ lacking Rb did not exhibit differentiation defects *in vitro* nor *in* vivo.

Taken together, the above studies on the role of Rb role in adult neurogenesis inside SGZ and SVZ have shown that, Rb displays similar functions inside the adult

neurogenic niches and is mainly needed to control aNPCs proliferation and subsequent survival of newborn adult neurons (reviewed in Fong & Slack 2017).

G. The role of p53 in adult neurogenesis

1. p53 and the control of cell cycle and apoptosis

The p53 protein is encoded by the *TP53* gene in human and *Trp53* gene in mice. It was identified in 1979 as a protein that forms an oligomer complex with the Simian Vacuolating Virus 40 T antigen (SV40 TAg) (Lane & Crawford 1979). p53 turned out to be a major tumor suppressor protein that is frequently found mutated in human cancers (Bartesaghi & Salomoni 2013). Upon DNA damage, p53 halts/stops cell cycle progression and activates repair mechanisms, or can induce cell death, thus maintaining genomic stability. As a result, it has been called the "guardian of the genome"(Lane 1992) and a "life guard with license to kill" (Kruiswijk et al. 2015).

Nuclear p53 acts as a transcription factor that targets specific DNA sequences and regulates the expression of several genes involved in cell cycle control, programmed cell death and metabolism (reviewed in Bartesaghi & Salomoni 2013).

Cellular stress activates p53 which in turn triggers G1 phase arrest and regulate transition from G2 phase to mitosis. p53 mainly performs these functions by directly targeting and activating cyclin-dependent kinases inhibitors (Cdkis). The major p53 Cdkis targets are p21 and p27; they are involved in the control of G1/S phase and G2/M phase checkpoints where they inhibit several cyclin/Cdk complexes mainly, cyclin E/Cdk2 and cyclin B/Cdc2 (Lookeren Campagne & Gill 1998; Flatt et al. 2000; Deng et al. 1995; Pietsch et al. 2008). In addition to p21 and p27, p53 also targets other cell cycle inhibitory proteins such as 14-3-3σ (sigma) protein and the Growth-arrest-and-

DNA-damage-45 protein (Gadd45). For instance, 14-3-3σ binds to and inhibits cyclin B1 and Cdc25C phosphatase from promoting cell cycle entry to mitosis (Chan et al. 1999) . On the other hand, Gadd45 sequesters Cdc2, thus inhibiting its binding to cyclin B (Jin et al. 2000). Moreover, p53 can inhibit indirectly the activity of cyclins and Cdks by downregulating the levels of phosphatases, namely Cdc25A that activates cyclin E/Cdk2, and, Cdc25C that activates cyclin B/Cdc2 (Rother et al. 2007; Clair et al. 2004). When genomic stability is irreversibly jeopardized by cellular stress, p53 induces programmed cell death or apoptosis by upregulating the expression of pro-apoptotic targets through its transcriptional activity such as Pten, Bax, Apaf-1and Puma (reviewed in Pietsch et al. 2008). In addition, p53 can translocate to the mitochondria where it promotes the activation and oligomerization of pro-apoptotic Bcl-2 family of proteins (Bax, BAK and Bid), which in turn induce mitochondrial cytochrome C release into the cytosol followed by caspase cascade activation (active-caspases) resulting in apoptosis (Leu et al. 2004; Chipuk et al. 2004).

p53 was also shown to control cell autophagy depending on its cellular distribution; hence, when in the cytoplasm, it was found to repress autophagy (Maiuri et al. 2010) whereas upon nuclear localization it promotes autophagy through the upregulation of DRAM1 (DNA damage-regulated autophagy modulator 1) (Crighton et al. 2006).

2. Role of p53 in the developing central nervous system

High p53 mRNA levels were depicted in rodents' embryos until mid-gestation. However, during organogenesis, these levels gradually decrease and reach almost no expression in differentiated tissues (Schmid et al. 1991). During CNS development in rodents, *p53* is highly expressed in proliferating NSPCs (Lookeren Campagne & Gill

1998). Although p53-null embryos undergo 'normal' development (Donehower et al. 1992) a small percentage displayed developmental defects. hence, 8% to 16% of *p53*-null females were reported to exhibit exencephaly or neural tube closure defect (Armstrong et al. 1995; Sah et al. 1995) This is in addition to other reports of neural tube defects associated with p53 deficiency such as spina bifida (Hosako et al. 2009) and retinal dysplasia (Ikeda et al. 1999). These observations validate the role of p53 in regulating neural tube closure during development, a process that requires effective orchestration between cell proliferation, cell migration and programmed cell death (Mendrysa et al. 2011). However, the low percentages of p53-null embryos with CNS-defected suggests the existence of compensatory mechanisms overcoming the loss of p53 and/or the presence of strain-specific effects (dependence on the genetic background). This compensation was proposed to be mediated by other members of the p53 family of transcription factors such as p63 and p73 which are structural and functional homologs of p53 (Dötsch et al. 2010; Fatt et al. 2014; Bartesaghi & Salomoni 2013).

p53 activity is tightly regulated by multiple regulatory pathways of which is the mostly cited group of p53 inhibitors, Mdm4 (Francoz et al. 2006) and the ubiquitin-protein ligase Mdm2 (Haupt et al. 1997). Interestingly, p53 auto-regulates its own level of activity by targeting and regulating the expression of the *Mdm2* gene (Shvarts et al. 1996). Putting this interaction in context, Mdm2 conditional loss in the developing CNS resulted in dramatic increase in apoptosis that was mediated by p53 (Francoz et al. 2006; Xiong et al. 2006). Moreover, the perinatal lethality caused by loss of *Mdm2* and *Mdm4 in* mice, was overcome by p53 deletion (Jones et al. 1995; Parant et

al. 2001; Migliorini et al. 2002), thus highlighting the importance of p53 regulation during development (reviewed in Mendrysa et al. 2011).

Finally, NSCs isolated from p53-KO mice embryos at E13.5 exhibited significant increase in their self-renewal and proliferative capacities, and, were significantly less apoptotic compared to wild type cells following exposure to radiation. These NSCs had also enhanced neuronal lineage (Armesilla-Diaz et al. 2009).

3. Role of the p53 transcription factors family in the central nervous system

p53, p63, and p73 share domain and sequence identity. They all trans-activate p53-target genes, regulate cell cycle transitions and induce apoptosis in response to cellular stress. However, p63 and p73 exist in multiple isoforms due to the presence of an alternative promoter and splicing events related to their carboxyl-terminal end. The full lengths p63 and p73 isoforms (denoted as TAp63 and TAp73) include a trans-activation domain (TAD) and thus, exhibit similar transcriptional activities to p53. However, their truncated isoforms (ΔN) lack the TAD rendering them transcriptionally inactive and therefore, function in specific contexts as inhibitors of the active family members (reviewed in Fatt et al. 2014; Dötsch et al. 2010).

Both p73 and p63 are involved in CNS development. For instance, mice deficient in all p73 isoforms displayed severe CNS developmental defects including hydrocephalus (abnormal CSF accumulation in the brain) and hippocampal dysgenesis (Yang et al. 2000). In comparison, p63 was found to be required for stem cells renewal in the olfactory epithelium, the horizontal basal cells (HBCs) and it functions in haltering HBC differentiation (Fletcher et al. 2011).

4. Role of p53 in adult neurogenesis

The role of p53 in AN has been extensively studied and p53 was shown to mainly control the properties of aNSPCs. Hence, NSPCs highly express *p53* in contrast to the differentiated neurons found in the adult SVZ-RMS-OB system that exhibit a weak p53 level (Meletis et al. 2006). A study conducted by Gil-Perotin et al. reported an increase in aSVZ-NSPCs proliferation in p53-null mice and this was observed both *in vivo* and *in vitro*. This observation resulted with enlarged cellularity along the lateral ventricles' walls and was associated with spontaneous SVZ-NSPCs cell death. The increase in cell death, was attributed to compensatory apoptotic mechanisms in the absence of p53 as assessed with Tunnel assay, of note, the authors found that SVZ-NSPCs with conditional p53 loss did not exhibit increase in cell death compared to wild type cells. Moreover, p53-null NSPCs exhibited an enhanced differentiation potential associated with bias towards the neuronal lineage (Gil-Perotin et al. 2006).

Consistent with the previous study, Meletis et al. detected increased self-renewal and proliferation of SVZ-NSPCs in p53-null male mice both *in vivo* and *in vitro.* However, they did not detect any significant change in the rate of SVZ-NSPCs cell death *in vivo* but a significant decrease *in vitro* as assessed by flow cytometry (Annexin-V assay) (Meletis et al. 2006). Using microarray analysis of the transcriptome in p53-null SVZ-NSPCs, the same study identified down regulation in p21, thus implicating a role for this p53 downstream target gene in controlling the self-renewal capacity of NPCs (Meletis et al. 2006).

Other studies have shown that p53 loss decrease the irradiation-induced-apoptosis in NPCs extracted from both the SVZ (Chow et al. 2000) and SGZ (Li et al.

2010). More recently, a study reported that this phenotype specifically affected DCX-positive neuroblast cells in the latter region (Li et al. 2016).

The roles of the downstream effectors of p53 have been also studied in the context of AN. For instance, NSCs lacking p21 exhibited cell cycle exit defects in addition to a significant increase in their number. These findings support a role for p21 in the maintenance of aNSCs' quiescence (Kippin et al. 2005). On the other hand, p27 loss did not affect the self-renewal capacity of aNSCs. However, it was involved in regulating the pool of transient-amplifying progenitors (Doetsch, Verdugo, et al. 2002).

Besides p53, aNPCs express two major p53 family members, ΔNp63 and TAp73. While p53 was reported to regulate proliferation and apoptosis of NPCs (Meletis et al. 2006; Gil-Perotin et al. 2006), ΔNp63 was found to antagonize p53-mediated apoptosis (Cancino et al. 2013) whereasTAp73 was mainly shown to promote NPCs long-term self-renewal through the transcriptional regulation of the bHLH transcription factor, Hey2which inhibits NSCs differentiation (Fujitani et al. 2010). Finally, in a recent study, Fatt et al. further validated the crucial interaction between p63 and p73 in regulating aNPCs' p53-dependent apoptosis or senescence (Fatt et al. 2014).

H. Interplay between the Rb and p53 pathways in the control of cell growth and programed cell death

The Rb-E2F and MDM2-p53 pathways are known to independently control the cell cycle via maintaining a tight balance between cell growth and apoptosis. However, given that both pathways are recurrently found defective in most human cancers, scientists have uncovered the presence of significant crosstalk between the two pathways, thus mediating and coordinating several cellular decisions in response to mitotic signals (reviewed in (Polager & Ginsberg 2009). On one hand, p53 can keep Rb

active by upregulating the expression of *p21* and other Cdkis such as p16 that prevent Rb phosphorylation. On the other hand, loss of Rb may result in the upregulation of p53 via ARF-mediated inhibition of Mdm2 by high E2F levels (Zhang et al. 1998; Pomerantz et al. 1998; Sherr & McCormick 2002). In fact, ARF (known as p19 in rodents) is activated upon signals of hyperproliferation caused by deregulated E2Fs hyper-activities in the absence of Rb. This ARF activity stabilizes p53 and result in its activation (Komori et al. 2005). The interplay between Rb and p53 is mediated in large extent by the activities of both repressor E2Fs and activator E2Fs (mainly E2F1) (reviewed in Polager & Ginsberg 2009).

1. Perplexing features of activator E2Fs

Activator E2Fs especially E2F1 have the potential to induce contradictory events such as proliferation or apoptosis in a context-dependent manner (DeGregori & Johnson 2006). For instance, E2F1 showed both positive and negative tumorigenic effects (Johnson & DeGregori 2006). It was hypothesized that this dichotomy may be due to the pro-apoptotic activity of E2F1 as compensatory mechanism in cases of deregulated proliferation or hyper-proliferation. In contrast, the increased hypoxia-p53-dependent apoptosis observed in Rb-null embryos in post-mitotic regions of the developing CNS was suppressed following deletion of E2F1 and E2F3 (Tsai et al. 1998; Ziebold et al. 2001; Saavedra et al. 2002).

2. Crosstalk between the Rb-E2Fs and Mdm2-p53 pathways

The most established regulators of E2Fs and p53 are Rb and Mdm2, respectively. Mdm2 acts as an E3-ubiquitin ligase that selectively binds to the N-terminal trans activation domain of p53, thus inhibiting its transcriptional activity and

promoting its proteosomal degradation (Francoz et al. 2006). More recently, Mdm2 was also found to inhibit p53 translation (Ofir-Rosenfeld et al. 2008).

In addition to inhibiting p53, Mdm2 can promote cell cycle and tumor progression by interacting with E2F1 or its binding partner, DP1, in order to upregulate the transcription of E2F1 target genes that are needed for the G1-S phase transition (Martin et al. 1995). Surprisingly, Mdm2 was also shown to promote the sequestering of the E2F1-DP1 heterodimer in p53-null cells (Loughran & La Thangue 2000), however, this effect comes in line with the pro-proliferative effect of Mdm2 which, in this situation, downregulates the apoptotic activity mediated by E2f1 (Loughran & La Thangue 2000).

Finally, it is noteworthy that Rb deficiency was reported to be associated with upregulated Mdm2 activity that, in turn, could eventually downregulate p53 and promote additional tumor transformation signals (Xu et al. 2009).

3. E2Fs and p53 interactions in growth control

E2Fs and p53 interact to control cell proliferation and cell cycle exit (reviewed in Polager & Ginsberg 2009). Hence, activator E2fs were reported to suppress p53 while repressor E2F complexes were found to mediate p53-dependent cell cycle arrest. In addition, some p53 target genes can repress activator E2Fs and inhibit cell proliferation.

For instance, targeted disruption of the activator E2Fs (E2F1-3) resulted in p53 activation. Mutually, p53 expression due to DNA damage was found to recruit the repressor E2F4-p130 complex which repressed E2F target genes and triggered cell cycle arrest at G1/S phase and G2/M phase (Polager & Ginsberg 2003; Taylor et al. 2001). Moreover, repressor E2Fs such as E2F7 and E2F8 that function in Rb-

independent pathways were found to suppress cell cycle progression in a p53-dependent manner. E2F7 regulates p53 activity by maintaining a physiological level of E2F1. Hence, mice lacking both E2F7 and E2F8 do not survive development due to catastrophic increase in p53-dependent apoptosis that was induced by E2F1 (Li et al. 2008). In addition, p53 was sufficient to upregulate the expression of *E2F7* in response to cellular damage. E2F7 was also found to couple the Rb and p53 pathways and promote cell cycle arrest and senescence by interacting and inhibiting E2Fs target genes promoters (Aksoy et al. 2012).

4. E2Fs and p53 interactions during apoptosis

p53 and E2F1 were suggested to act in a "feedforward loop" in promoting cell death (Polager & Ginsberg 2009). First, both E2F1 and p53 were reported to regulate the expression of several apoptotic genes including PUMA, NoxA, APAF and SIVA (Moroni et al. 2001; Han et al. 2001; Nakano & Vousden 2001; Oda 2000; Fortin et al. 2004; Moon et al. 2005). In addition, p53 can be activated by E2F1 (Tian et al. 2011). In fact, E2F1 indirectly affects p53 activation and stability, through regulating specific genes that activate p53. For instance, overexpression of E2F1 as a result of Rb loss, promotes p53 activation through upregulating the transcription of p19ARF (ARF in mice), an Mdm2 inhibitor (Hernández-Monge et al. 2016). Ectopic expression of E2F1 has been shown to activate apoptotic genes even in p53-independent manner in p53-null cells (Irwin et al. 2000) .

Taken together, , the above studies demonstrated that, in response to cellular stress and/or DNA damage, E2F1 and p53 co-upregulate the expression of the aforementioned apoptotic genes in addition to specific genes related to autophagy such as DRAM1 and SIRT1 (Wang et al. 2006; Polager et al. 2008; Nemoto et al. 2004). Of

note, E2F1 may also promote apoptosis independently of p53 by directly upregulating the expression of *p73* (Irwin et al. 2000).

RATIONALE, HYPOTHESIS and AIMS

As described earlier, adult neurogenesis in the brain is restricted to two neurogenic regions only, the OB and Hi, where few aNSCs constantly give rise to specific cell types albeit at low numbers. However, several studies have confirmed that, with age, the adult mammalian brain progressively loses its 'active' neurogenesis and this is due to the fact that aNSCs have their proliferative activity considerably decreased overtime and tend to enter a permanent state of quiescence or senescence with aging (Conover & Shook 2011; Mobley et al. 2013; Capilla-Gonzalez et al. 2015). These age-related changes in aNSCs properties and cell dynamics render the brain's regenerative capacity very limited and thus, incapable of compensating for extensive neuronal loss upon brain injury or following neurodegenerative disease. The urge to enhance neuronal regeneration to treat brain injury and neurodegenerative disorders e.g. Alzheimer's disease and Parkinson's disease pushed scientists towards a better understanding of the key factors and molecular pathways as well as the environmental conditions that control the regenerative capacity of the adult brain, hoping that by manipulating them one can enhance this important function inside the brain. For instance, physical exercise and low-caloric intake was shown to significantly increase AN (Yau et al. 2014; Hornsby et al. 2016). Moreover, the rise of advanced and optimized research methods for *in vitro* studies of NSPCs such as the neurosphere assays (Reynolds et al. 1992; Azari et al. 2010) allowed scientists to consider using *ex-vivo* expansion of these cells followed by transplantation for cell therapy and replacement (Azari et al. 2010; Iwai et al. 2015).

In addition to the above, AN is a highly regulated process that is tightly dependent on the cell cycle machinery for the proper regulation of key checkpoints; hence, aNSCs undergo cellular divisions to self-renew and/or generate committed progenitors that proliferate, then exit the cell cycle and ultimately differentiate. Hence, it is crucial to understand the genetic pathways that regulate the swing of aNSCs from quiescence to cell division or cell cycle exit before differentiation or apoptosis. Although several studied have tackled this question and provided interesting data about this process, many aspects of AN remain to be uncovered. In this context, Rb and p53 interact to regulate the cell cycle kinetics and hence, play key roles in coordinating stem cells/progenitors' self-renewal, proliferation, differentiation and programmed cell death (reviewed in Bartesaghi & Salomoni 2013). As described earlier, p53 was found to negatively regulate the self-renewal capacity of aSVZ-NSCs; its loss was accompanied with a decrease in aNSCs programmed cell death and a dramatic increase in the proliferative potential and neuronal lineage differentiation of these cells (Meletis et al. 2006). Of note, the hyper-popularity of aNSCs in the aSVZ was found to be associated with p53-independent apoptosis in p53-null mice (Gil-Perotin et al. 2006). On the other hand, our laboratory has recently studied the role of Rb during AN in the adult SVZ and SGZ, and showed that it specifically controls aNPCs proliferation in both regions (and *in vitro*) without affecting their differentiation potential. As a result, loss of Rb caused enhanced neurogenesis inside the OB and Hi, however, this was coupled with dramatic increase in apoptosis of Rb-/- newborn neurons inside the OB and Hi (Naser et al. 2016; Vandenbosch et al. 2016).

Given all the above, we have **hypothesized** that the p53 and Rb tumor suppressor pathways may synergistically interact in the regulation of adult

neurogenesis, primarily cell proliferation and survival of aNSPCs and their progeny inside the SVZ/OB. As such, the combined loss of both genes may help amplify further the pool (numbers) of aNSCs and/or aNPCs as well as their differentiation rate *in vitro*, and, may rescue the long-term survival defects of OB-newborn neurons observed in the absence of Rb. We hence plan to examine whether neuronal cell death in Rb-null mice is p53-dependent or not, knowing that, during development, Rb-null embryos had increased p53-mediated cell death in distinct CNS regions with post-mitotic neurons (Tsai et al. 1998; Ziebold et al. 2001; Saavedra et al. 2002).

To test our hypotheses, our **first aim** is to investigate the *in vitro* properties of aNSPCs isolated from the SVZ of young adult mice (2 months) carrying single p53 deletion versus Rb;p53 double deletions that will be generated using an inducible Cre-ERT2 knock-out system. Our *in vitro* experiments will be based on neurosphere assays and differentiation assays (Rietze & Reynolds 2006; Azari et al. 2010). We anticipate that combined loss of both genes will lead to a significant increase in aNSPCs proliferation and self-renewal capacities as well as enhanced neuronal differentiation potential. In our **second aim**, we will assess the survival rate of aNSPCs and their differentiated progenies in culture and *in vivo* following loss of both genes. We expect that, if the decreased survival of Rb-null newborn neurons is mediated by p53-dependent apoptosis (as seen during development), then loss of p53 will rescue this phenotype and sustain long-term survival of newborn neurons.

CHAPTER II.

MATERIALS AND METHODS

A. Generation of Adult Rb and/or p53 conditional knockout Mice

All animal procedures and experiments were approved and conducted in accordance with the guidelines of "the Institutional Animal Care and Use Committee" (IACUC) at the American University of Beirut (AUB).

Temporal and conditional deletion(s) of *Rb* and/or *p53* genes were induced in 8-week-old mice to study the single and combined role(s) of these genes in AN. The conditional knock out(s) was/were done using the Nestin-Cre-LoxP inducible system by tamoxifen administration (see section below). The Nestin-CreERT2/R26R-YFP mice was obtained through material transfer agreement from Dr. Amelia Eisch (University of Texas Southwestern Medical Center, USA) who generated this line in collaboration with Dr. Pierre Chambon at IGBMC, France (Lagace et al. 2007). The Cre recombinase gene is fused to a mutated estrogen receptor, ERT2, and expressed under the control of the Nestin gene promoter and enhancer, that are specifically active in aNSPCs. Tamoxifen (TAM),an estrogen analog, binds to the Nestin-Cre-ERT2 cassette and activates the translocation of the Cre enzyme into the cell nucleus where it recognizes and remove the sequences flanked by *LoxP* sites including a stop signal sequence upstream of the yellow fluorescent protein (YFP) sequence, exon 19 of the *Rb* gene, Rb flox/flox mice (Marino et al. 2000), and/or exons 2-10 in *p53* (p53flox/flox mice).The Cre recombination thus leads to *YFP* expression and deletion(s) of Rb/p53.

Nestin-CreERT2; Rosa26YFP/YFP transgenic mice were crossed with $Rb^{flox/flox}$; $p53^{flox/flox}$ mice to generate the following genotypes: $Rb^{flox/flox}$; $p53^{flox/flox}$; Nestin-$Cre^{+/-}$; $YFP^{+/-}$ (denoted as double floxed), and, $p53^{flox/flox}$; Nestin-$Cre^{+/-}$; $YFP^{+/-}$ (denoted as p53 floxed/floxed).

Nestin-$Cre^{+/-}$; $YFP^{+/-}$ were used as controls. After TAM treatment (Cre recombination), these three genotypes were referred to as Rb; p53 double conditional knock out mice (dc-KO), p53-cKO, and wild type (wt) mice, respectively.

B. Genotyping and polymerase chain reaction analysis

Mice earpieces were excised and used for genomic DNA extraction. Ear pieces were incubated in DNA lysis buffer overnight at 55°c. The next day, phenol chloroform (1:1 ratio) was added to the lysed tissues. After centrifuging, the aqueous layer was removed and added to isopropanol in 1:1 ratio and the resulting centrifuged pellet was subsequently washed with 70% cold ethanol. The final DNA pellet was suspended and allowed to homogenize in 10µm Tris solution. The same DNA extraction method was applied on YFP-positive cells sorted and dissociated from aSVZ-NSPCs neurospheres and from the OB.

- To screen for the Rb wt, floxed and recombined alleles we used Rb-18 forward primer *5'GGCGTGTGCCATCAATG 3'* and Rb-19 reverse primer *5'AACTCAAGGGAGACCTG 3'* that yield 490 bp band for wt allele, 580 bp band for floxed allele and 320 bp for the recombined allele. Thermo-cycling conditions for this set of primers: 35 cycles of 30 s at 94°C, 30 s at 58°C and 60 s at 72°C then 10 minutes at 72°C.

- To screen for p53 floxed versus wt alleles we used p53 floxed primers T008 *5’CACAAAAACAGGTTAAACCCA 3’* and T009 *5’AGCACATAGGAGGCAGAGAC 3’* that yield 288 bp band for the wt allele and 370 bp band for floxed alleles. Thermo-cycling conditions for this set of primers: 35 cycles of 30 s at 94°C, 30 s at 60°C and 60 s at 72°C then 10 minutes at 72°C.

- To screen for recombined p53 allele(s) we used T008 and p53-intro-rev primer *5’GAAGACAGAAAAGGGCCA 3’* that yields a 612 bp for the recombined p53 band. Thermo-cycling conditions for this set of primers: 30 cycles of 30 s at 94°C, 30 s at 58°C and 50 s at 72°C.

- To screen for the Nestin-Cre transgene we used Cre-T2A primer forward 5' *ATTTGCCTGCATTAC CGGTC* 3' and Cre-T2B primer reverse: 5' *ATCAACGTTTTCTTTTCG G* 3' that yields a 350 bp in the presence of Cre. Thermo-cycling conditions for this set of primers: 32 cycles of 30 s at 94°C, 30 s at 51°C and 60 s at 72°C, followed by 10 minutes in 72°C.

- To screen for R26R-YFP alleles we used as forward primers *5’AAAGTCGCTCTGAGTTGTTAT* 3’, and 5’ *GCGAAGAGT TTGTCCTCAACC* 3’, and reverse primer 5’ *GGAGCGGGAGAAATGGATATG* 3’ that yields a 310 bp band for the mutant YFP allele and a 560 bp for the wt allele. Thermo-cycling conditions for this set of primers: 32 cycles of 30 s at 94°C, 30 s at 50°-61°C and 60 s at 72°C followed by 10 mins at 72°C.

PCR reactions with total volume of 25 µl consisted of 2.5 µl dNTPs (10x), 2.5 µl of MgCl2 (25nM), 2.5µl of Taq PCR buffer (10x), 0.4µl primer (100µm) and 1:20 Taq polymerase.

C. Tamoxifen administration

Freshly prepared TAM was administered by oral gavage for 5 consecutive days (180 mg Tamoxifen/1 kg mouse body weight). TAM powder (Sigma T5648-5G) was dissolved in sunflower seed oil and absolute ethanol (Sigma Aldrich 65533) at a final concentration of 15 mg/ml as per standard protocol (Lagace et al. 2007).

D. Cell culture and cell sorting

Microdissection of the SVZ tissues from the adult brains of two-month-old mice was performed 5 days post-TAM administration. Mechanical and chemical digestion of the SVZ was done using razor blade to chop the tissue followed by 30-minutes incubation in digestion media [DMEM-F12 (Sigma SD8437), 1 µg/µl Papain (Sigma P3125), 0.5M EDTA (Sigma 27285)]. To recover the cells, post digestion, a quick and mild centrifugation was performed followed by a single wash in FBS solution [10% FBS (Sigma 14A173) diluted in DMEMF12] and two subsequent cell pellet washes with DMEMF12. Finally, the extracted cells were passed through a 40-µm sterile filter and cultured in 10 cm petri dishes (Corning 430167) containing stem cell media [DMEMF12, 0.02 ug/ml FGF (Feldan 1D-07-017b), 0.02 µg/ml EGF (Sigma SE1257), B27 (Unitech 17504044), 2ug/ml Heparin (Sigma SH3149) and 1% antibiotic/anti-mycotic (ABAM) (Sigma SA5955)]. 2 days post initial seeding, neurosphere colonies start to form from aNSPCs. Neurospheres dissociation and passages were performed every 6-7 days when neurospheres had a size ranging between

100 and 200 µm (Azari et al. 2010). Cell cultures were incubated in sterile conditions at 37°c and 5% CO_2. All cultures and assays were performed in specialized tissue culture rooms (TC) with TC biosafety Level 2 (BSL-2) with a minimum of n=3 cultures or replicates per genotype. Neurospheres counts and size calculations were performed using an upright fluorescent microscope (Olympus IX70).

1. Cell sorting

After neurosphere dissociation into single cells at passage 2 (P2), cells were sorted by fluorescent-activated cell sorting (FACS) using "BD-FACS-Aria-SORP cell sorter" at 40 Psi using a 100 µm nozzle. Fluorescence was detected using the blue laser (488 nm excitation laser line) with FITC filter set of 520/35nm. Gating strategy consisted of excluding debris and cell duplicates to avoid false positive during cell sorting (see figure 1) and to yield high purity YFP-positive sorted and live NSPCs. The gate of YFP-negative cells was set using a negative control sample/culture derived from SVZ-extracted NSPCs of two-month-old B6- non-transgenic wild type mice. Sorted cells were collected with stem cell media containing double the normal amounts of growth factors, and were washed twice in DMEMF12 prior to seeding at a density of 50 cell/µl of stem cell media.

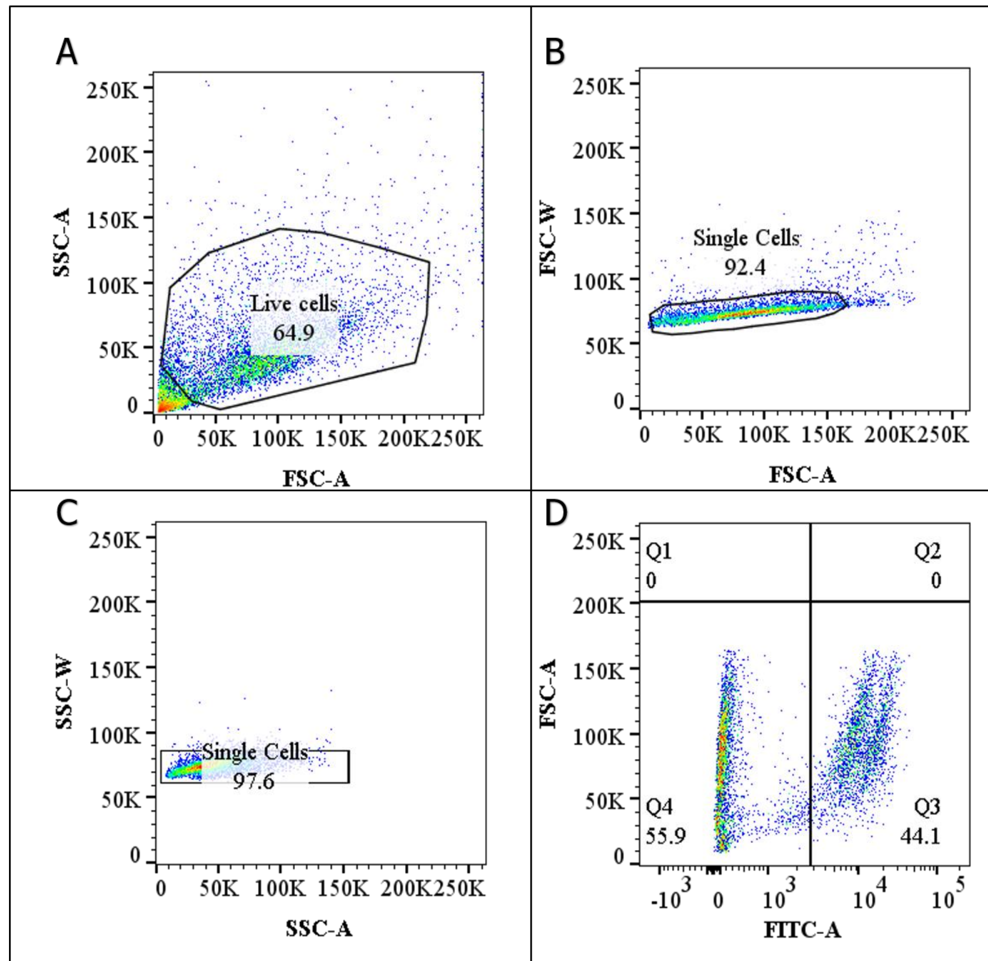

Figure 1: Gating strategy for NSPCs single cells sorting. Panel A shows gating of live cells excluding cell debris that exhibit low FSC-A and SSC-A intensities. Panel B and C show gating of singlets that do not have high width pulse intensities. Panel D shows gating of FITC-positive cells (Q3) that exhibit high FITC intensity as compared to the low FITC intensity in the group of YFP-negative cells (Q4). This analysis was performed using FlowJo software and the original cell population record was obtained from the BD-FACS-DIVA software.

2. Primary neurosphere assays

The proliferative capacity (amplification rate) of aNSPCs and the frequency of neurosphere formation was assessed by plating the sorted cells at decreasing densities (5000 cells/ml, 2500 cells/ml, 1250 cells/ml and 625 cells/ml) in 24-well plates containing 500µl stem cell media with a minimum of three replicates for each cell density and at least n=3 per genotype. neurospheres counts and size measurements were done at day 7.

3. Secondary neurosphere assay

The self-renewal potential of sorted NSPCs was assessed by measuring the frequency of secondary neurospheres formation from dissociated single neurospheres. Individual neurospheres of equal size (80µm-100µm) were plated in 96-well plate and dissociated mechanically in 200 µl stem cell media and neurospheres counts were scored 7 and 10 days later.

4. Differentiation assay

Green sorted cultures of aNSPCs were passaged and dissociated into single cells. Cells were allowed to grow in 24-well plates containing round glass cover slips coated with 15% poly-ornithine (Sigma P4957) and laminin 10µg/mL (Sigma 47743-734, corning 354232) in the presence of monolayer media [DMEMF12, 1 % ABAM, 2ug/ml Heparin, 1% N2 supplement (Gibco CSMSN200-OE) and 0.02 ug/ml FGF and EGF]. When monolayers reached 60-70% confluency (after 48-72 hours), the media was switched to differentiation media [DMEMF12, 1% N2 supplement, 1 % ABAM, 2ug/ml Heparin and 1% FBS] and cells were left in differentiation conditions for 5 or 10 days. Then, cells were incubated for 2 hours in BrdU at 0.02mg/ml (Sigma B5002-250MG) prior to fixation with 4 % paraformaldehyde (PFA) for 20 minutes at room

temperature. Gentle washes were made with cold WIFI distilled water (Irvie scientific 9309) to remove excess PFA.

5. Immunocytochemistry

After fixation, differentiated cells were in incubated for 1 hour in blocking solution (BS) [1% BSA (amresco 0332-25G), 0.3% Triton X, 5% donkey serum in 0.1M PBS]. Primary antibodies were added to cells and left overnight at room temperature. The following day, three rounds of 10-minutes gentle washes were done in cold 1xPBS; this was followed by 2-hours incubation in secondary antibodies (1:400 in BS) and Hoescht stain (1:100 in BS). Finally, cells are gently washed for 3 consecutive 10-minutes washes in cold 1xPBS. Mounting with cover slips was done using glycerol diluted in 1xPBS (1:3).

The primary antibodies used were: goat anti-GFAP (1:100; Santa Cruz SC-6170), mouse anti-Tuj1 (1:5000; hybridoma), rabbit anti-active caspase 3 (1:500, Cell Signaling 9664).

The secondary antibodies used were (Alexa Fluor, Molecular Probes, Invitrogen, and, Jackson Immunoresearch): donkey anti-goat 488, 596 and 647 Alexa Fluor, donkey anti-mouse 488 and 596 Alexa Fluor, donkey anti-rabbit 488 and 594 Alexa Fluor.

6. Imaging and cell counts

Visualization, picture acquisition and analyses were performed using the Upright Fluorescence Microscope (DFC7000 T by Leica) at AUB -Central Research Science Laboratory (CRSL) facility.

Cell counts from differentiation assays were performed from 2-4 different regions (at magnification X20) per coverslip using ImageJ software and confirmed manually on selected regions (n=2 per genotype). Statistical analysis was done using t-test and ANOVA test in excel and SPSS programs.

7. YFP-positive cells isolation and sorting from the olfactory bulb

Olfactory bulbs of 2-months-old TAM-treated-mice (p53-cKO and dc-KO) were isolated 120 days-post tamoxifen treatment. At least 8 OBs of each genotype were collectively digested to sort YFP-positive cells. OBs were mechanically and enzymatically digested using trituration and digestion medium incubation for 30 minutes same as previously described in NSPCs isolation from the SVZ. After recovering the digested tissue with 10% FBS, the cells were centrifuged for 15 minutes at 2000 rpm in in 1:5 ratio of 22 % percoll solution (GE-healthcare 17-0891-01), diluted in DMEMF12. Finally, the cell pellet was washed twice with DMEMF12 and passed through a 40-µm sterile filter and collected in 15 ml conical tube. The final cell pellet was suspended in 1xPBS and subsequently analyzed and sorted using the “BD-FACS-Aria-SORP cell sorter” using the previously described FITC filters for YFP-positive cell sorting. Sorted cells were collected in 50% FBS (diluted in DMEMF12) and the cells were washed twice with DMEMF12 and the final cell pellet was subsequently subjected to DNA extraction and PCR analysis (performed as previously described).

CHAPTER III.

RESULTS

A. Neurosphere assays and sorting of Nestin-Cre-YFP positive cells in culture

In order to study the *in vitro* properties of aNSPCs carrying deletion(s) in p53 and/or Rb, we induced Cre recombination by the administration of tamoxifen to 8-week-old mice carrying the following genotypes: $Rb^{flox/flox}$; p53 $^{flox/flox}$; $NestinCre^{+/-}$; $YFP^{+/-}$ (denoted as Rb; p53 double floxed/floxed), p53 $^{flox/flox}$; $NestinCre^{+/-}$; $YFP^{+/-}$ (denoted as p53 floxed/floxed), and, $NestinCre^{+/-}$; $YFP^{+/-}$ (used as controls). Tamoxifen was administered by oral gavage for five consecutive days and mice were sacrificed five days following the last TAM treatment (for detail on mice generation and treatment, refer to material and methods section). The SVZ tissues harboring NSPCs were micro-dissected, then mechanically and enzymatically dissociated and neurosphere assays were performed as previously described (Rietze & Reynolds 2006; Azari et al. 2010). At passage 2, neurospheres (green and non-green) were pooled and dissociated into single cells suspensions and subjected to fluorescent-activated cell sorting (FACS) to isolate live green-recombined cells or YFP+ cells only (for more experimental detail, refer to material and methods). Sorted cells were seeded and allowed to form neurospheres (figure 2) over several passages while performing the desired neurosphere assays and differentiation assays. FACS allowed the isolation of a heterogeneous population of YFP-positive cells primarily enriched in NSPCs that can generate neurospheres in growth factors-rich medium, and, can be induced to differentiate in monolayers as described below (figure 8 and see next sections).

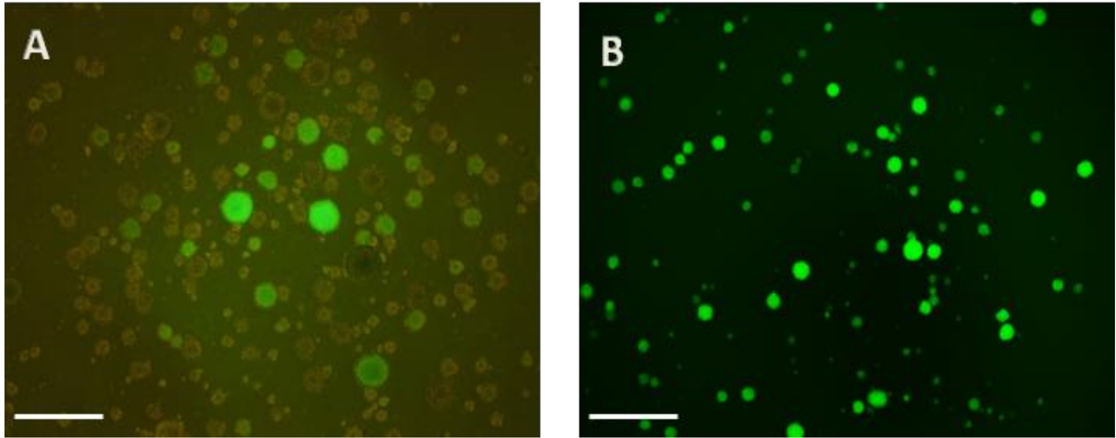

Figure 2: Bright field and fluorescent images of floating neurospheres before and after FACS. Panel A shows neurospheres suspension before cell sorting including green (YFP+) and non-green (YFP-) colonies. Panel B shows a neurosphere culture post-FACS where only green/recombined neurospheres can be detected.

B. Proof of Cre recombination in NSPCs derived from p53-cKO and Rb;p53-dc-KO mice

Following sorting, green recombinant cells were collected at distinct passages and genomic DNA was extracted from samples derived from all three genotypes (1 million cells per sample, n=3 samples per genotype) as described in the methods section. PCR analysis was performed to check the Cre recombination using specific sets of primers for p53 and Rb floxed versus recombined alleles. Successful Cre recombination was detected in p53-cKO and dc-KO mice as shown by the presence of a recombined band of 612 bp corresponding to the cleaved p53 allele, and, a 320 bp band in the case of the recombined Rb allele (figure 3), thus confirming the successful deletion of p53 and Rb genes in neurosphere cultures derived from aSVZ-NSPCs. (refer to material and methods for experimental detail).

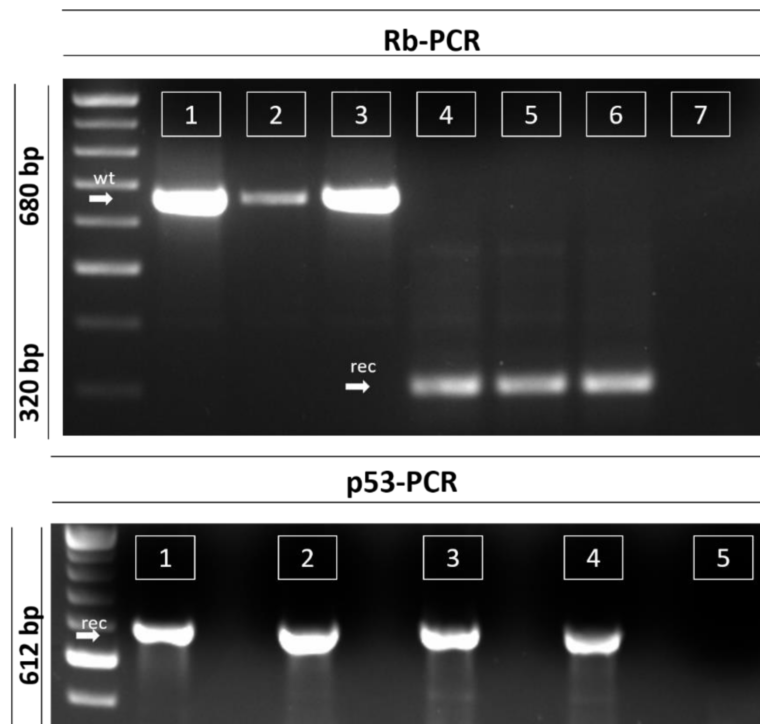

Figure 3: Proof of Cre recombination in neurosphere cultures derived from sorted aSVZ-NSPCs.
PCR analysis was performed on genomic DNA (gDNA) extracted from neurospheres derived from aSVZ-NSPCs. Top panel shows Rb-PCR performed on p53-cKO cells (samples 1, 2 and 3) and dc-KO cells (samples 4, 5 and 6) using Rb18 and Rb19 primers; the Rb wt and recombined alleles are marked by 680bp and 320bp bands, respectively. Sample 7 is a negative control (no DNA; water). Bottom panel shows a p53-PCR performed on gDNA from p53-cKO (samples 1 and 2) and dc-KO (samples 3 and 4) using the p53 T008 and p53 intron-reverse primers. The 612 bp band corresponds to the recombined p53 allele. Sample 5 is a negative control (no DNA; water).

C. p53 and Rb act synergistically to expand the pool of NSPCs in culture

1. p53; Rb double conditional knocked-out NSPCs exhibit an amplified proliferation rate in culture compared to p53-cKO and wt cells

Sorted wt, p53-cKO and dc-KO aSVZ-NSPCs were plated in stem cell medium at four decreasing dilutions in 24 well-plates with three replicates per density. The starting cell density was 5000 cells/ml and the final dilution was 625 cells/ml. Primary neurospheres were counted and categorized based on their size 7 days later. This primary neurosphere assay examines the proliferative capacity of NSPCs or their ability to form neurospheres which is an indication of the original NSPCs' population viability (Xiong et al. 2011). Our results showed that dc-KO-NSPCs exhibited a significant increase in the number of primary neurospheres obtained at all densities as compared to wild type NSPCs [r=2.03 in 5000 cells/ml (dc-KO: 184 ± 2 vs. wt: 91 ± 3, p=2.27854E-05), r=1.93 in 2500 cells/ml (dc-KO: 97 ± 5 vs. wt: 50 ± 8, p=0.003407), r=1.68 in 1250 cells/ml (dc-KO: 53 ± 3 vs. wt: 31 ±1, p=0.001942), r= 2 in 625 cell/ml (dc-KO: 32 ± 2 vs. wt: 16 ± 1, p=0.000309)]. When compared to p53-cKO cells, the dc-KO-NPSCs exhibited significant increase in the neurospheres counts at high densities only (5k and 2.5k cells/ml) [r= 1.25 in 5000 cells/ml (dc-KO: 184 ± 2 vs. p53-cKO: 147 ± 2, p=0.000227613), r= 1.24 in 2500 cells/ml (dc-KO: 97 ± 5 vs. p53-cKO: 78 ± 1, p=0.005967), r= 1.14 in 1250 cells/ml (dc-KO: 53 ± 3 vs. p53-cKO: 46 ± 4, p=0.157331), r=1.19 in 625 cell/ml (dc-KO: 32 ± 2 vs. p53-cKO: 27 ± 3, p=0.10499)]. p53-cKO NSPCs had also significant increase in neurosphere counts at all cell densities when compared to wt-NSPCs [r= 1.62 in 5000 cells/ml (p53-cKO: 147 ± 2 vs. wt: 91 ± 3, p=4.6988E-06), r=1.56 in 2500 cells/ml (p53-cKO: 78 ± 1 vs. wt: 50 ± 8, p=0.001783), r=1.48 in 1250 cells/ml (p53-cKO: 46 ± vs. wt: 31 ±1, p=0.005668),

r=1.68 in 625 cell/ml (p53-cKO: 27 ± 3 vs. wt: 16 ± 1, p=0.00289)]. Data is shown in Table 1 and figure 4.

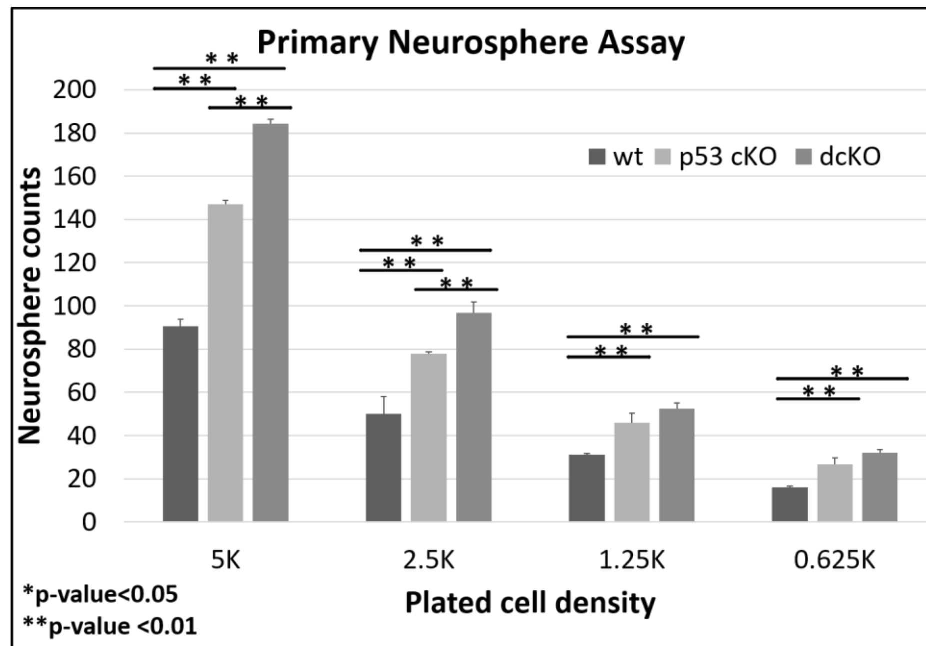

Figure 4: Quantification of the total numbers of primary neurospheres derived from aSVZ-NSPCs in culture at decreasing cell densities.
Neurospheres counts were performed on day 7 of primary neurosphere assays at different plating densities after cell sorting. Results showed significant increase in the average numbers of spheres generated in dc-KO cultures compared with both p53-cKO and wt cultures. Counts were performed in triplicates for each cell density with n=3 per genotype. Error bars represent the standard deviations from the mean (SD). T-test and ANOVA test were used to assess statistical significance; (*) and (**) represent p-values <0.05 and <0.01, respectively.

Ratio	5K	2.5K	1.25K	0.625K
dc-KO / wt	2.03**	1.93**	1.68**	2**
dc-KO / p53-cKO	1.25**	1.24**	1.14	1.19
p53-cKO / wt	1.62**	1.56**	1.48**	1.68**

Table 1: Comparative ratios of total number of primary neurospheres yielded 7 days post-seeding of Rb;p53 dc-KO-, p53-cKO- and wt-NSPCs. (*) and (**) represent p-values <0.05 and <0.01, respectively.

2. p53;Rb double conditional knocked-out NSPCs exhibit significant increase in primary neurospheres size compared with p53-cKO and wt cultures

Compared to wt and p53-cKO cultures, dc-KO cultures had a significant increase in the size of primary neurospheres generated after 7d in culture (see figure 5 for significance). Hence, 27% of dc-KO neurospheres had a diameter larger than 140 μm compared with 4.31% and 9.22% in wt and p53-cKO cultures, respectively (see table 2). Moreover, both dc-KO and p53-cKO cultures showed high frequency of medium and large size spheres (80-140 μm) compared with wt cultures. On the contrary, most of the wt neurospheres (42.6%) had a relatively smaller sphere size (less than 110 μm). These findings point to higher cell viability and content in proliferative/active NSCs in dc-KO cultures compared with the other two genotypes (Louis et al. 2008; Azari et al. 2010; Louis et al. 2013).

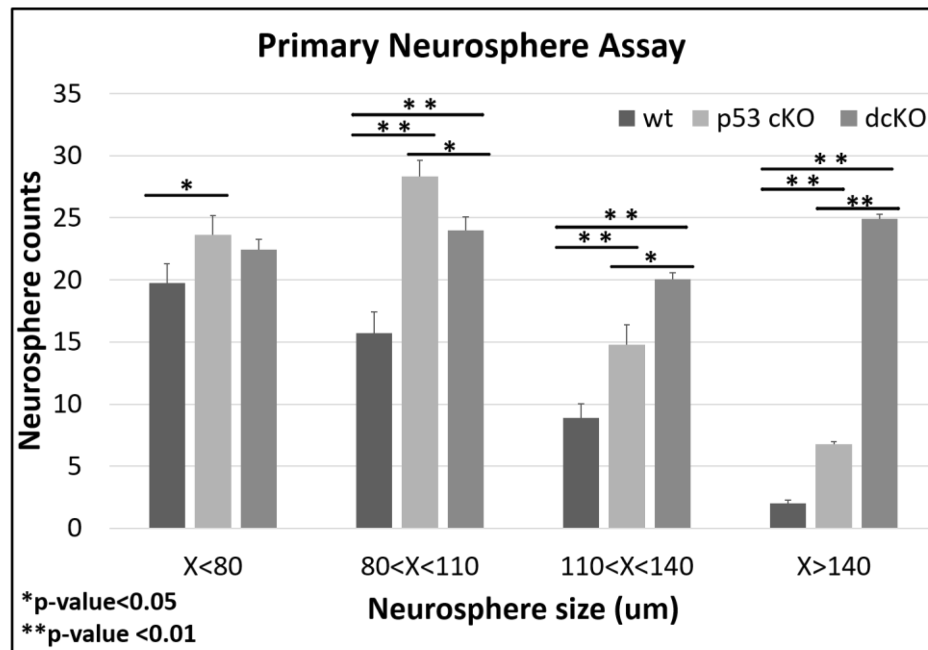

Figure 5: Primary neurospheres size measurements in Rb; p53-dc-KO, p53-cKO and wt cultures. The size of primary neurospheres was recorded on day 7after seeding. Note the high frequency of dc-KO neurospheres with size >140 µm compared with the other genotypes. Counts were performed in triplicates for each cell density and n=3 per genotype. Error bars represent the standard deviations from the mean (SD). T-test and ANOVA test indicate statistical significance with (*) and (**) representing p-value<0.05 and p-value<0.01, respectively.

Percentage	X<80	80<X<110	110<X<140	X>140
Wt	42.60	33.91	19.17	4.31
p53-cKO	32.11	38.53	20.13	9.22
dc-KO	24.53	26.26	21.93	27.27

Table 2: Percentages of primary neurospheres generated in aSZV-NSCs cultures according to size (µm). Note the high frequency of extra-large (>140 µm) neurospheres formed in dc-KO compared with other genotypes.

3. p53; Rb dc-KO NSPCs display higher amplification rate over several passages in culture

The amplification rate is a direct measurement of the proliferative capacity of NSPCs and their potential to generate viable cells with high frequency over several passages. It is calculated by dividing the number of viable cells after each passage over the number of initially seeded cells. We examined the effect of combined loss of Rb and p53 on NSPCs proliferation and long-term potential to generate viable neurospheres and detected significant enhancement in the amplification rate (AR) in p53; Rb dc-KO cultures compared to p53-cKO and wt cultures (see figure 6). Hence, after 3 consecutive passages, dc-KO cultures exhibited 2.29-fold increase in their AR compared to p53-cKO cultures (dc-KO AR=12.62 ±4.30 vs. p53-cKO AR= 5.50 ± 0.37, p=0.046135575), and, 4.69-fold increase compared to wt (dc-KO AR=12.62 ±4.30 vs. wt AR= 2.69 ± 1.03, p=0.017712139). In turn, p53-cKO cultures had a 2.05-fold increase in the AR compared to wt cultures (p53-cKO AR= 5.50 ± 0.37 vs. wt AR= 2.69 ± 1.03, p= 0.011341124). These results suggest that Rb and p53 cooperate to control the proliferative capacities of NSCs and/or NPCs, and, their effects are additive in this context.

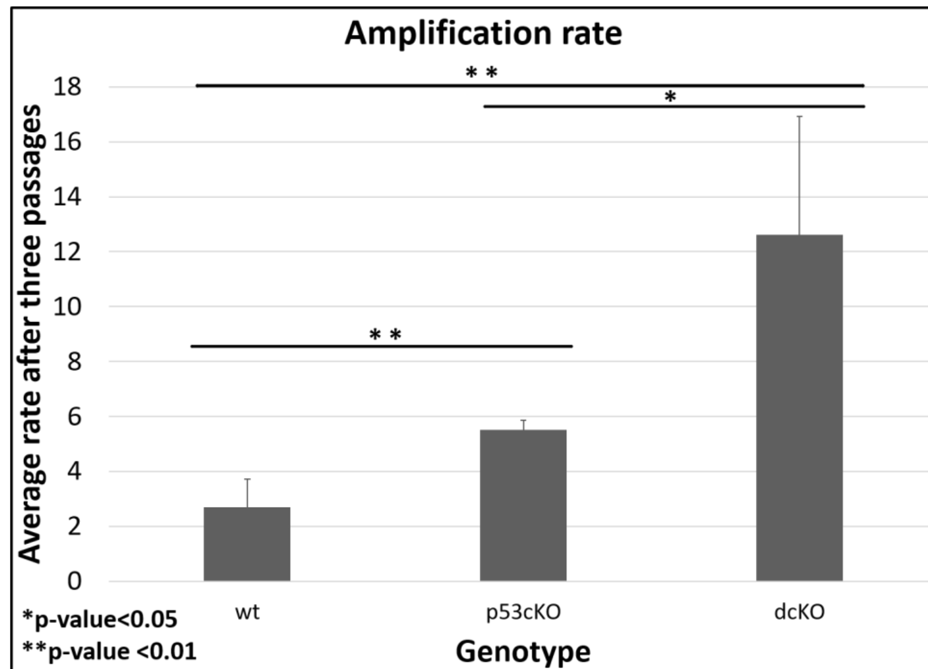

Figure 6: Higher amplification rate observed in Rb; p53dc-KO NSPCs compared to p53-cKO and wt cells in culture. The amplification rates were calculated and averaged from 3 consecutive passages (see text for detail). dc-KO cultures show significantly higher amplification rate even after prolonged numbers of passages (data not shown here). Error bars indicate the standard deviations from the mean (SD) (n=3 per genotype). T-test was used to assess statistical significance with asterisk (*) and (**) representing p-values <0.05 and <0.01, respectively.

D. p53 and not Rb controls the self-renewal capacity of NSPCs *in vitro*

To measure the self-renewal and clonal capacity of aNSPCs lacking both Rb and p53, single YFP-positive neurospheres of equal size (80-90 µm) were picked from all three cultures on day 3 from similar passages and placed in 96-well-plate (1 sphere per well, 6-10 replicates, n=3 per genotype), then mechanically dissociated by gently pipetting up and down. The generated secondary neurospheres were quantified and analyzed 10-days later. In a previous study, we have not observed a difference in the self-renewal capacity of aNSPCs lacking Rb (*in vitro* and *in vivo*) (Naser et al. 2016). However, a p53 role in negatively regulating the self-renewal capacity of aNSCs has been reported. Our results showed that significantly higher numbers of secondary neutrospheres can be derived from p53-cKO and Rb; p53-dc-KO cultures compared with wt cultures; (figure 7; (dc-KO: 64 ± 5 vs. wt: 33 ± 2, ratio=1.94, p=0.000971126, and, p53-cKO: 55 ± 1 vs. wt: 33 ± 2, ratio=1.66, p=6.89976E-05). However, no statistical significant difference was obtained when comparing dc-KO vs p53-cKO cultures (dc-KO: 64 ± 5 vs. p53-cKO: 55 ± 1, ratio=1.16, p=0.1163281). This data indicates that p53 and not Rb controls the self-renewal capacity of aNSPCs as previously shown in p53-null mice (Meletis et al. 2006; Gil-Perotin et al. 2006). Moreover, this data is consistent with our previous study where no effect for Rb on the self-renewal capacity of aNSPCs was detected both *in vivo* and *in vitro* (Rb-/- versus Rb+/-; Naser et al. 2016)

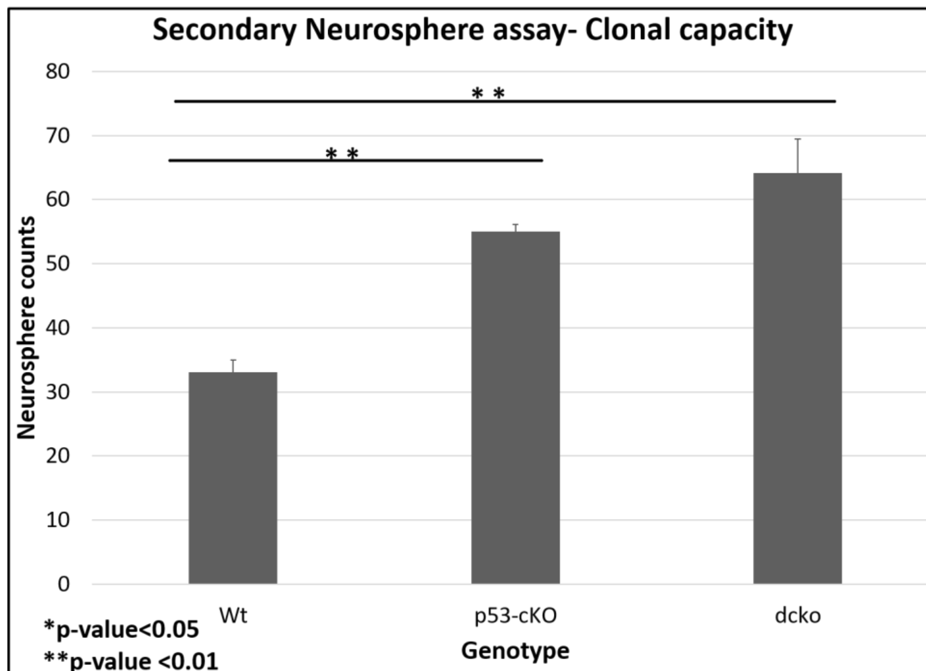

Figure 7: Quantification of the number of secondary neurospheres obtained in cultures lacking p53 and Rb; p53 compared with wt cultures. Both dc-KO and p53-cKO cultures exhibited significantly higher numbers of secondary neurospheres compared to wt cultures, suggesting that they have a higher rate of clonal capacity. Error bars indicate the standard deviations from the mean (SD). T-test assessment of statistical significance with asterisk (*) and (**) representing p-values <0.05 and <0.01, respectively.

E. Combined loss of p53 and Rb did not affect the differentiation potential of aNSPCs and was associated with increased differentiation towards a neuronal lineage

To assess the differentiation potential of aNSPCs lacking both Rb and p53, YFP-positive neurospheres were dissociated and plated on poly-ornithine and laminin-coated glass cover slips in 24-well-plates to form monolayers. When monolayers reached 60-70% confluency, the media was switched to a serum-rich differentiation media to induce cell differentiation for 5d and 10d after which cells were fixed with 4% PFA (for 20 minutes) and processed for histological analyses to assess the glial and neuronal phenotypes. Immunostaining was performed using antibodies against GFAP (marker of mature astrocytes) and Tuj-1 (marker of early differentiated/immature neurons) (n=2 per genotype and 2 replicates per sample). Our results showed that, similar to wt cells, both p53-cKO and dc-KO cells were able to differentiate into astrocytes and neurons. Interestingly, NSPCs derived from p53-cKO and dc-KO-NSPCs exhibited significant increase in their neuronal lineage upon differentiation at day 5 and 10 compared with wt cells (figure 8). On average, 300-700 cells per replicate were analyzed and the number of Tuj-1-positive cells was counted and normalized to the total number of Hoechst cells [(Graphs 5 and -:5-days-post-differentiation; Tuj-1+ cells, 42.18 % ± 0.68% in dc-KO vs. 26.63% ± 3.70% in p53-cKO (p=0.027996969) and 9.37 % ± 0.68% in wt cells (p=0.000298109), p53-cKO vs wt; p=0.02246231]. Similar results were obtained 10 days-post-differentiation [(Figure 9; Tuj-1+ cells; 54 % ± 0.46% in dc-KO cells vs. 41% ± 3.72% in p53-cKO (p=0.036961316) and 23 % ± 1.91% in wt cells (p=0.001986558), p53-cKO vs wt; p=0.026913665]. This data indicates that combined loss of p53 and Rb does not affect differentiation of NSPCs,

however, it preferentially direct it towards the neuronal lineage as previously described for p53-cKO mice (Meletis et al. 2006; Gil-Perotin et al. 2006).

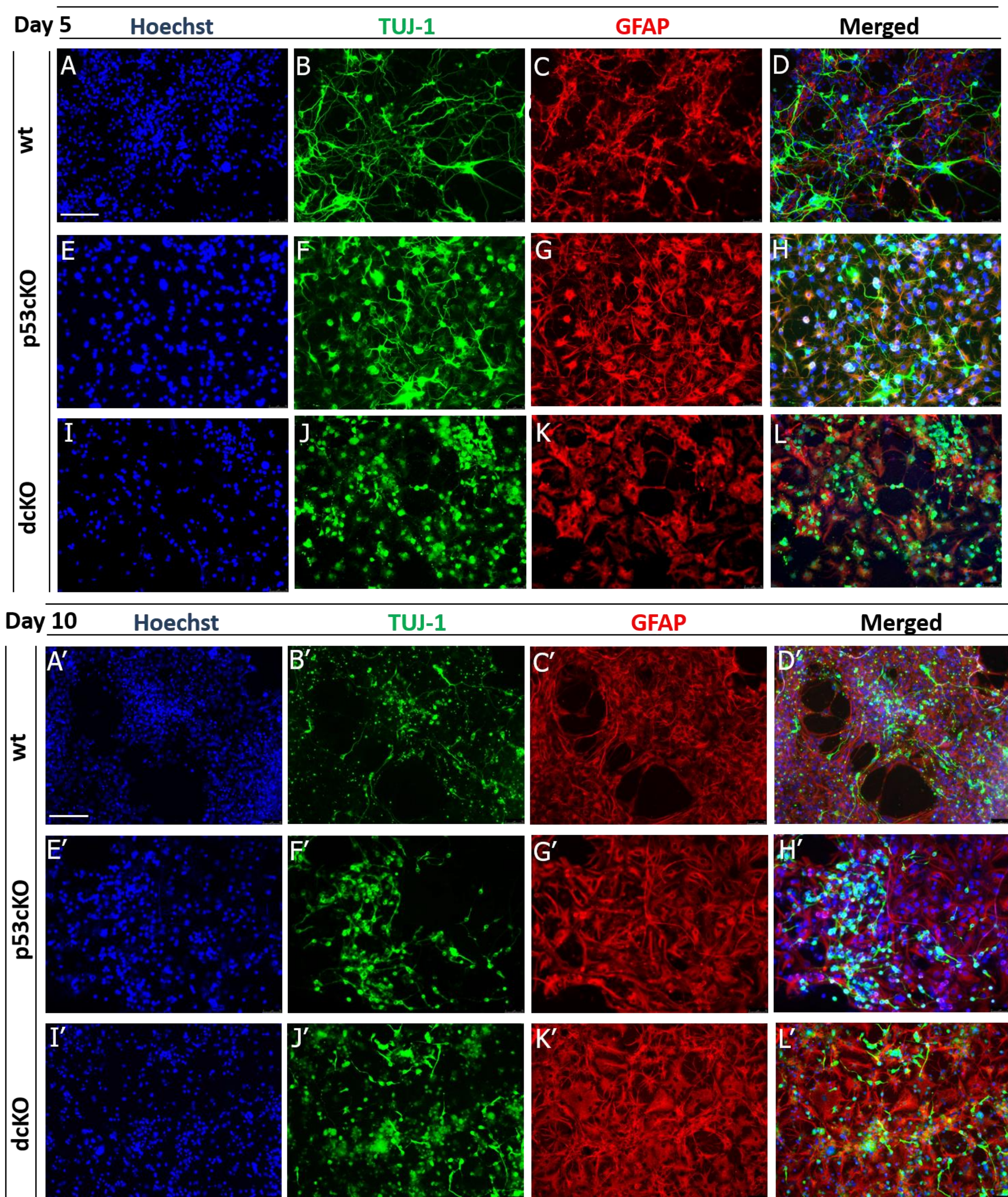

Figure 8: Rb; p53 dc-KO NSPCs display similar differentiation potential in culture compared with wt cells with preference towards differentiation into a neuronal lineage. Double immunocytochemistry staining performed against GFAP (red: astrocytes) and Tuj-1 (green: immature neurons) on differentiated aSVZ-NSPCs fixed at days 5 and 10 post-differentiation. YFP-positive dc-KO, p53-cKO and wt NSPCs exhibited similar differentiation potentials and give rise to astrocytes and neurons. (A-D, A'-D') represent wt cells, (E-H. E'-H') p53-cKO cells, and, (I-L, I'-L') dc-KO cells. Scale bar = 100 µm.

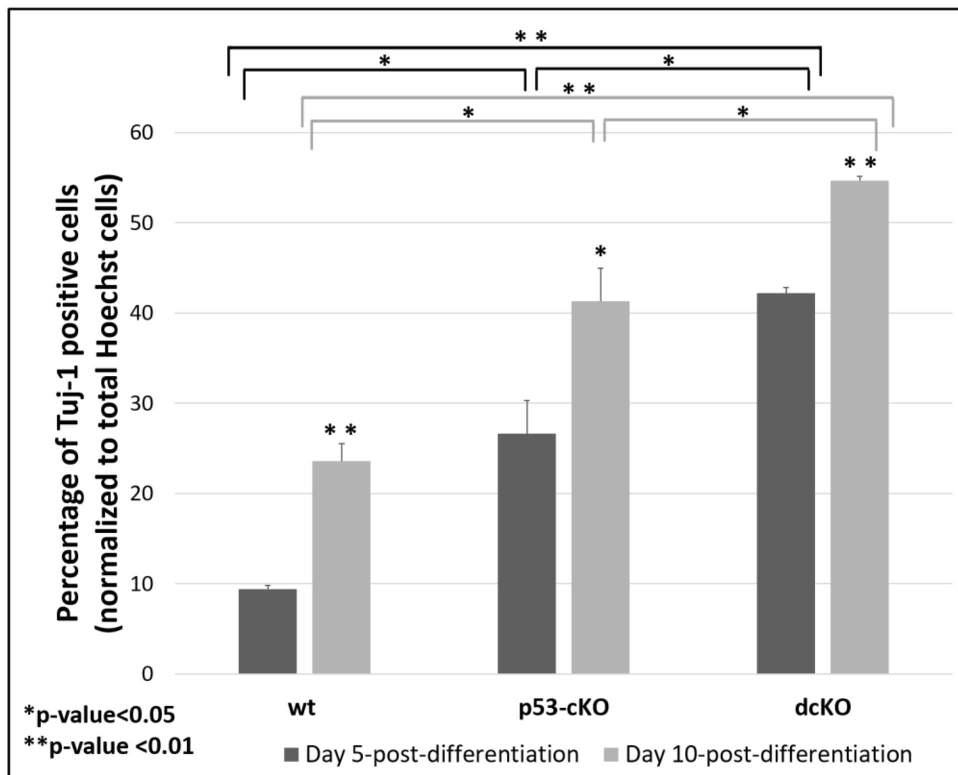

Figure 9: Quantification of the number of Tuj-1 positive immature neurons derived post-differentiation in culture and showing a significant increase in neuronal lineage in dc-KO cultures compared to p53-cKO and wt cultures at day 5 and 10. Hence, at 5d, there is 1.58 and 4.50-fold increase in neuronal lineage in dc-KO cultures compared to p53-cKO and wt cultures, respectively, and, 1.32 and 2.31-fold increase at day 10. p53-cKO cultures also had significant increase in their neuronal lineage compared to wt cultures with ratios of 2.84 and 1.75 at day 5 and 10, respectively. Error bars are the standard deviations from the mean (SD) with n=2 per genotype. T-test was used for statistical significance with asterisk (*) and (**) representing p-values <0.05 and <0.01, respectively.

F. No change in active caspase-3 level in differentiated aSVZ-NSPCs lacking both Rb and p53 compared with wt cells

To assess the survival of differentiated cells derived from aNSPCs lacking both Rb and p53 in comparison with p53-cKO and wt cells, we performed immunocytochemistry with active-caspase 3 (AC-3; apoptosis marker) after 5d and 10d of differentiation. We found that 45.59% ± 0.07% were AC-3+ in wt cultures at day 5 post-differentiation, however, there were significantly less AC-3-posistve cells in p53-cKO (33.44 % ± 2.5%, p=0.020594831) and dc-KO cells (26.22% ± 0.37, p=0.000191985) at this time-point (figure 10, figure 11). In contrast, at day 10, all 3 genotypes showed higher but similar levels of AC-3 (dc-KO: 58.92 % ± 0.54%; p53-cKO: 52.19% ± 1.07%; wt: 57.80% ± 0.21%) (figure 10, figure 11), suggesting no difference in the level of apoptosis among them. This finding in association with other findings describing a non-apoptotic effect of active caspase-3 during neuronal differentiation (Pasan Fernando et al. 2005; Oomman et al. 2006; D'Amelio et al. 2010; Solá et al. 2012) suggest that AC-3 staining may not be ideal to assess cell death in this context (refer to discussion for more explanation). Other apoptotic markers and tests should be used to accurately assess and compare survival of differentiated aNSPCs in all three genotypes.

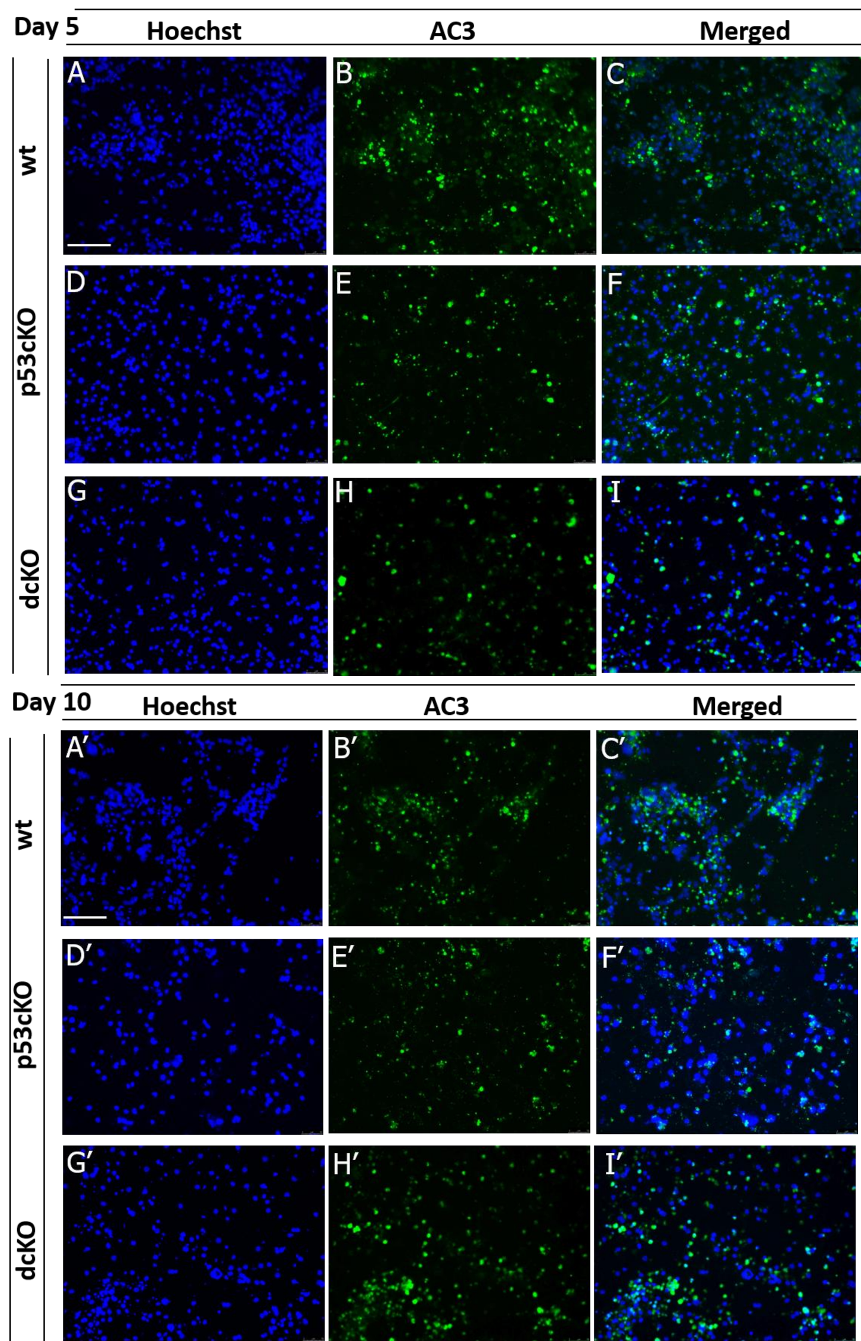

Figure 10: Active-caspase-3 level in differentiated aSVZ-NSPCs. Immunocytochemistry staining against AC-3 (green) performed on differentiated cells derived from aSVZ-NSPCs fixed at day 5 and day 10. p53-cKO and dc-KO cultures showed significantly less AC-3 positive cells compared to wt cultures at day 5, but all three genotypes had higher and similar numbers of AC-3+ cells at day 10 (see graph below). Scale bar = 100 µm.

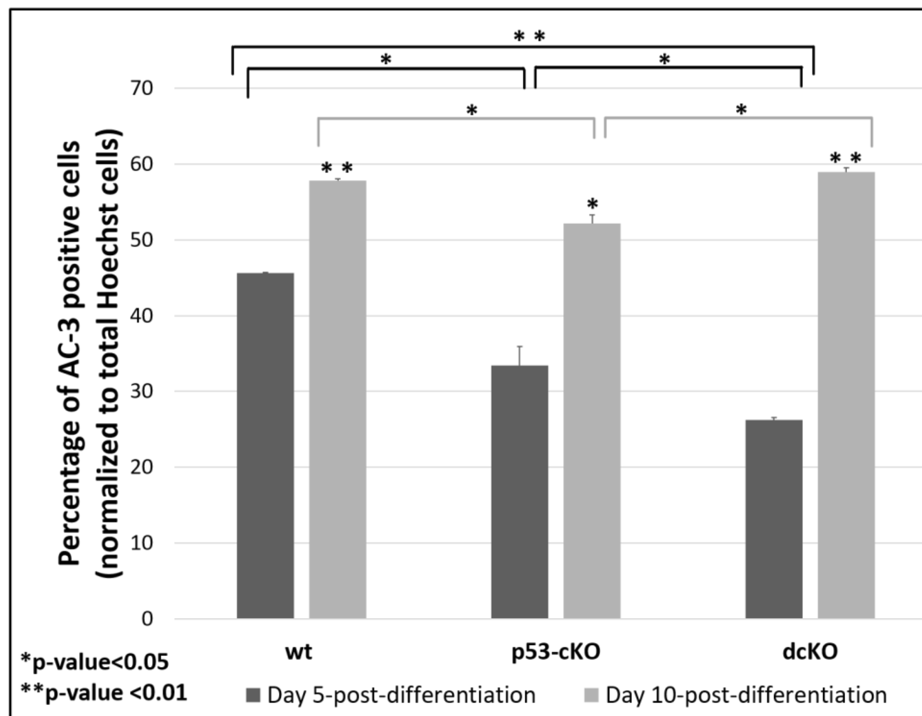

Figure 11: Quantification of AC-3 positive cells post-differentiation of aNSPCs in the absence of p53 and Rb. Error bars are the standard deviations from the mean (SD) with n=2. T-test was used for statistical significance with asterisk (*) and (**) representing p-values <0.05 and <0.01, respectively.

G. Loss of p53 does not rescue the long-term survival defects of Rb-null newborn neurons inside the OB

We recently reported that Rb-cKO newborn neurons in the olfactory bulb have long-term survival defects and are lost 120d post-Rb deletion inside the OB. Knowing that loss of Rb may result in E2Fs hyper-activity leading to ARF-mediated stabilization of p53 and thus activation of p53-dependent apoptotic pathways (Komori et al. 2005), we ought to check whether this scenario would occur in adult SVZ/OB neurogenesis. If true, loss of p53 in Rb-null mice may then rescue the survival defects described earlier. To test this, we induced single p53- and double Rb; p53 deletion(s) in the adult brain by tamoxifen treatment of 8-week-old mice for 5 consecutive days followed by a survival period of 120d. Next, we collected and pooled the OBs of treated mice in each genotype; then, we dissociated and successfully sorted the YFP-positive cells found in the OB tissues (refer methods section for more detail). We extracted gDNA from YFP-positive cells and genotyped by PCR the cells derived from each genotype to check for the presence of floxed and/or recombined p53 alleles. Unexpectedly, we found that YFP-sorted cells in both p53-cKO and dc-KO mice were not recombined (data not shown) and thus, carried p53 floxed/floxed alleles (Figure 12), implying that the aNSPCs recombined progenies in p53-cKO and dc-KO did not survive 4 months-post-treatment. This phenotype is consistent with the previously reported one in Rb-null mice (Naser et al. 2016) and indicates that loss of p53 did not rescue the long-term survival defects in Rb-null newborn neurons, suggesting that both genes are required for neuronal survival, and, cell death is mediated by p53-independent mechanisms in this context.

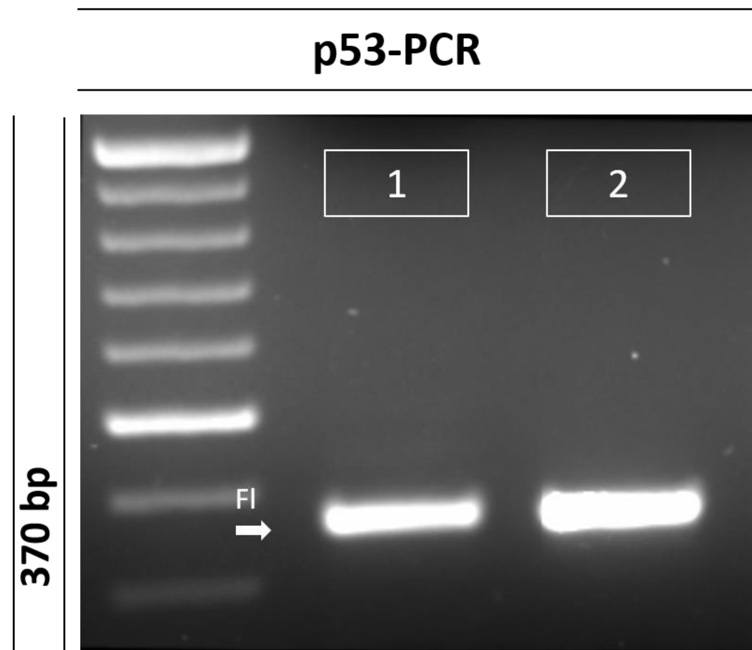

Figure 12: PCR analysis of genomic DNA extracted from YFP-positive cells sorted from the olfactory bulbs of p53-cKO (1) and dc-KO mice (2) at 120d post-tamoxifen treatment. Results showed that cells from both samples (1, 2) carried the p53 floxed/floxed alleles (378bp band) and not the recombined ones (data not shown).

CHAPTER IV.

DISCUSSION

A. Synergistic roles for Rb and p53 in the control of aNSPCs proliferative capacity and amplification rate

In this study, we have assessed how two interrelated cell cycle genes, Rb and p53, cooperate in the regulation of different features of adult neurogenesis in the SVZ niche by studying the *in vitro* properties of aSVZ-NSPCs in the absence of one or both genes. As previously mentioned, the Rb and p53 pathways interact in regulating the cell cycle kinetics primarily through the Rb/E2Fs and ARF/MDM2/p53 pathways, respectively (Komori et al. 2005). While p53 was found to control aSVZ-NSCs self-renewal capacity and proliferation (Meletis et al. 2006; Gil-Perotin et al. 2006), Rb was confined to regulating proliferation in aSVZ-NPCs without affecting NSCs properties both *in vivo* and *in vitro* (Naser et al. 2016). Our results showed that the effects of both genes with respect to the control of aSVZ-NSPCs proliferation are additive in culture; hence, compared to wt-aNSPCs and p53-cKO-NSPCs that are derived from 2-month-old mice, Rb; p53-dcKO-NSPCs gain a significant increase in their proliferation capacity. This was assessed by measuring the neurosphere formation frequency (number) and efficiency (size) in primary and secondary cultures of YFP+ aSVZ-NSPCs (sorted cells/recombined). Furthermore, compared to wt and p53-cKO cultures, dc-KO cultures displayed a strikingly higher amplification rate (2-5 fold) when calculated over 3 consecutive passages, implying that they have acquired an intensified neurosphere formation potential with capacity to survive and expand very fast over several weeks. Of note, this strong amplification rate attributed to dc-KO cells was

preserved even after cryopreservation; in fact, these cells were consistently able to propagate at very high rate up to 12 passages without any sign of exhaustion unlike in wt cultures where decreased proliferative capacity was noted overtime (data not shown).

In addition to the increase in the number of neurospheres generated after 7 days, we detected a high percentage of extra-large neurospheres in dc-KO cultures (>27.27 % of neurospheres with size >140um) which was remarkable in comparison with wt and p53-cKO cultures where less than only 9.22% and 4.31% had such size, respectively. The majority of neurospheres in p53-cKO cultures were of medium/large size (38.53% ~ 110um) whereas a considerable number of neurospheres were still of small size in wt cultures (42.6~ 80uM).

In a recently published study, we also showed that Rb-cKO cultures had significant increase (2-3 fold) in neurosphere numbers at decreasing dilutions compared with Rb+/- controls with high occurrence of large neurospheres (70-110 um), however, we did not observe any noticeable extra-large neurospheres as seen here. Taken together, these results indicate that the dramatic increase in sphere size is likely due to an effect on stem cells' proliferative capacity rather than progenitor cells, with a higher content of actively proliferating stem/progenitor cells yielding larger neurospheres (Louis et al. 2013). Moreover, it can be interpreted as a result of the capacity of neurospheres to being able to spontaneously fuse especially when they come in close contact. This adhesion property of neurospheres has indeed been reported and is mediated by the extensive presence of filopodia processes on the surface of neurospheres. The whole adhesion and fusion process was estimated to take about 38 seconds to complete as assessed by real time recording of floating neurospheres in culture, and can happen irrespective of the initial density of seeded cells (Ladiwala et al.

2012). In summary, combined loss of Rb and p53 may provide aNSPCs with cell-intrinsic property to proliferate faster and generate larger neurospheres in short time, in addition to the fact that an excessive number of neurospheres in close proximity make them more prone to adhere and fuse.

We next assessed over a period of ten days in culture the self-renewal capacity of aNSPCs by performing secondary neurospheres assays derived from single dissociated neurospheres of similar size. Our results showed that dc-KO and p53-cKO cultures yielded similar numbers of secondary neurospheres that were significantly higher compared to wt, thus confirming that p53 solely control self-renewal of NSCs (and not Rb). This finding is in line with our previous study ruling out any role for Rb in controlling self-renewal of aNSPCs (Naser et al. 2016) as well as other studies showing that p53 negatively controls the self-renewal capacity of aNSPCs in p53-null mice (Gil-Perotin et al. 2006; Meletis et al. 2006).Interestingly, the latter studies noted that the increased proliferative potential of aNSPCs was also partially due to lower cell death as shown by flow cytometric analysis of cultured NSPCs (Meletis et al. 2006).

The combined roles of Rb and p53 have been previously studied in the adult brain in the context of brain tumor formation. Hence, dual loss of both genes was associated with hyper-proliferation of NSPCs and decreased neural progenitor cell death in the adult SVZ (Sutter et al. 2010; Jacques et al. 2010). Moreover, Jacques and colleagues used adenoviral-GFAP-cre mediated deletions of Rb and p53 in NSPCs in the aSVZ (GFAP; marker of NSCs and mature astrocytes) and showed that this leads to increase neuronal lineage *in vivo*, which is consistent with our *in vitro* results (see next section). However, they also found that, nine months post-inducing the double deletions, 19.8% of dc-KO-mice exhibited phenotypes with tumorigenic lesions

resembling primitive neuroectodermal tumors (PNETs) of undifferentiated malignant cells (Jacques et al. 2010). It is still to be determined whether the Nestin-CreERT2-dc-KO mice that we have used here will show a similar phenotype *in vivo*.

B. Combined loss of Rb and p53 is associated with increased potential of differentiation into a neuronal lineage

Our *in vitro* differentiation assays demonstrated that YFP+ aNSPCs derived from p53; Rb dc-KO mice, can differentiate into astrocytes (GFAP-positive) and immature neurons (TUJ-1-positive) and do not seem to exhibit any major differentiation defects when compared to wt cultures. Notably, their differentiation potential (and the one of p53-cKO) was associated with a significantly higher tendency towards a neuronal lineage compared to wt cells. This effect seems to be also exclusively mediated by p53 given that our previous study did not identify such potential in Rb-cKO-aNSPCs compared to Rb+/--aNSPCs in culture (Naser et al. 2016). This finding is again consistent with other studies correlating the loss of p53 with increased neuronal differentiation (Gil-Perotin et al. 2006; Armesilla-Diaz et al. 2009; Liu et al. 2013) except for one study where authors failed to detect an effect on neuronal differentiation *in vitro* using p53-null cells (Meletis et al. 2006). For instance, Gil-Perotin et al. showed that differentiated cultures derived from p53-null NSCs exhibit 11-folds increase in the number of Tuj1-positive cells compared to wt counterparts (Gil-Perotin et al. 2006). In addition, this increase in neuronal production was not a result of decreased neuronal apoptosis even though the authors have observed a compensatory increase in neural progenitor cell death (by Tunnel assay *in vivo and in vitro*) in p53-null mice. Of note, the same group reported that, similar to p53-null cells, p53-cKO aSVZ-NSCs had also increased neuronal differentiation potential compared to wt, indicating that this p53

function is rather cell-autonomous and mediated by intrinsic pathway(s) unlike its role during apoptosis in neural progenitors that seem to be partially cell non-autonomous. Finally, a recent study suggested that this role of p53 in controlling neuronal lineage differentiation *in vitro* may be mediated via the BMP-Smad1 pathway (Liu et al. 2013).

C. Combined loss of Rb and p53 was not associated with any change in the level of active-caspase-3 in differentiated cultures of NSPCs

To assess neuronal survival in the absence of p53 and Rb, we examined the level of active-caspase 3 (AC-3), a commonly used marker of apoptosis, by immunostaining performed on differentiated YFP+-aNSPCs derived from wt, p53-cKO and dc-cKO mice. As a result, we found significantly less AC-3 positive cells in cKO and dc-KO cultures compared to wt cultures after 5d of differentiation, however, this was not true after 10d post-differentiation where there were significantly higher but similar numbers of AC-3+ cells in all three cultures. These results could be explained by two possible scenarios that are not mutually exclusive. First, considering that AC-3 can be used to assess neuronal apoptosis (Clark et al. 2000; Xing et al. 2009), it is rather expected to observe decreased neuronal cell death after loss of p53 especially if apoptosis is p53-dependent in this context. However, our results are not consistent between 5d and 10d post-differentiation whereby the significant rise in the numbers of AC-3+ positive cells in all three genotypes at 10d suggest the presence of other compensatory cell death mechanisms. This explanation is however unlikely to be true given that all three cultures showed similar counts of AC-3+ at 10d. Alternatively, the remarkable increase in AC-3 expression at this stage could be linked to higher cellularity and thus, lack of nutrient support for appropriate maintenance of the newly differentiated progeny. The second scenario supports a hypothesis for a distinct role of

AC-3 in non-apoptotic activities during neuronal differentiation as has been previously reported in many studies as well as during differentiation of non-neuronal cell types (P. Fernando et al. 2005; Oomman et al. 2006; reviewed in D'Amelio et al. 2010). This AC-3 activity was found to be mediated through DNA strand breaks required for neural stem cell differentiation (Larsen et al. 2010). Interestingly, a study by Fernando et al. showed a direct correlation between increasing AC-3 levels and enhanced neurogenesis *in vitro*, and this increase in AC-3 was not associated with cell death (P. Fernando et al. 2005). Furthermore, they showed the presence of delayed and altered neuronal differentiation when AC-3 activity was inhibited in neurospheres before inducing differentiation. Also, differentiated neurons had significant reduction in their neurites growth when AC-3 expression was downregulated, confirming the involvement of AC-3 activity in neuronal differentiation (P. Fernando et al. 2005). Given all the above, it is not clear at this stage whether combined loss of p53 and Rb affects neuronal survival in our cultures and therefore, ongoing experiments are aiming to assess apoptosis using different markers and assays such as Tunnel assay and Annexin-V staining.

D. Long-term survival of newborn olfactory bulb neurons requires the functions of both Rb and p53

During development, Rb-null embryos display excessive proliferation with dramatic neuronal death that is partially mediated by high levels of E2Fs e.g. E2F1, and, due to the inability of newborn neurons to exit properly the cell cycle. Consequently, cellular stress caused by this defective neuronal differentiation activated the apoptotic p53-mediated pathways (Morgenbesser et al. 1994; Macleod et al. 1996; Tsai et al. 1998; Slack et al. 1998). Owing to these observations and that we have recently reported that Rb-null newborn neurons do not survive in the olfactory bulbs few months post-Rb

deletion, we examined here whether the latter phenotype is p53-dependent and hence, can be rescued by deleting p53 in Rb-cKO-NSPCs. We collected and sorted YFP+ cells from the OBs of p53-cKO and dc-KO mice at 120d post-treatment and genotyped the cells to determine whether they carried recombined p53 alleles or not (floxed alleles). Unexpectedly, our in vivo results showed that loss of p53 alone or both Rb; p53 led to neuronal death inside the OB as manifested by the absence of recombined progeny (p53-null YFP+ cells), and this is consistent with what was previously reported in Rb-null mice, indicating that neuronal apoptosis is mediated by p53-independent pathways and/or other compensatory pathways may be involved. In fact, Rb and p53 were both implicated in maintaining cell quiescence in terminally differentiated cells (reviewed in Cheung & Rando 2013), and, inhibition of either of the two genes resulted in cell cycle re-entry in differentiated (and senescent) cells (Beauséjour et al. 2003; Pajcini et al. 2010). Post-mitotic neurons lacking Rb have been reported to re-enter the cell cycle (Lipinski et al. 2001; Andrusiak et al. 2012), and this cell cycle re-entry has been closely associated with increased apoptosis, especially that tetraploid neurons are more prone to commit apoptosis and were closely associated with degenerative diseases (Arendt et al. 2010, Andrusiak et al. 2012, reviewed in Frade & Ovejero-Benito 2015). Tetraploid neurons usually undergo apoptosis at the G1/S checkpoint (Liu & Greene 2001) by a process called “abortive cell cycle re-entry” which is mainly mediated by deregulated E2Fs activity e.g. high E2F1 levels triggering p53-independent apoptosis (Giovanni et al. 2000; O’Hare et al. 2000). Finally, future studies should aim at exploring whether such alternative cell death pathways are activated in the absence of Rb and/or p53, and, whether dc-KO immature neurons exit properly the cell cycle following ectopic proliferation and whether they are functional or not.

In conclusion, we uncovered here that Rb and p53 cooperate to control proliferation of aNSPCs in vitro and play complementary roles in this regard whereby p53 solely controls NSCs self-renewal and Rb primarily regulates NPCs proliferation. Moreover, we demonstrated that both genes are required for long-term neuronal survival in vivo. In addition to deciphering interactive molecular mechanisms involving these pathways in adult SVZ neurogenesis, our study could provide an insight for cell therapy in the long run, where potentially-speaking transient deletion(s) of Rb and/or p53 in vitro can be used to amplify and expand the pool of adult neural stem cells and progenitors. This may be an essential step to help optimize recent strategies of in vivo transplantation and cell therapy in cases of traumatic brain injury especially that studies have shown that when aNSPCs are amplified and differentiated in vitro prior to transplantation better therapeutic results are achieve (Fortin et al. 2016).

REFERENCES

Akers, K., Martinez-Canabal, a & Restivo, L., 2014. Hippocampal Neurogenesis Regulates Forgetting During Adulthood and Infancy. *Science (New York, N.Y.)cience*, 598(May), pp.598–602. Available at: http://www.sciencemag.org/content/344/6184/598.short.

Aksoy, O. et al., 2012. The atypical E2F family member E2F7 couples the p53 and RB pathways during cellular senescence. *Genes and Development*, 26(14), pp.1546–1557. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22802529 [Accessed April 3, 2017].

Alfonso, J. et al., 2012. Diazepam binding inhibitor promotes progenitor proliferation in the postnatal SVZ by reducing GABA signaling. *Cell Stem Cell*, 10(1), pp.76–87.

Altman, J. & Das, G.D., 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *Journal of Comparative Neurology*, 124(3), pp.319–335.

Altman, J. & Das, G.D., 1967. Postnatal neurogenesis in the guinea-pig. *Nature*, 214(5093), pp.1098–1101.

Alvarez-Buylla, A. et al., 2008. The heterogeneity of adult neural stem cells and the emerging complexity of their niche. In *Cold Spring Harbor Symposia on Quantitative Biology*. pp. 357–365.

Andreu-Agullo, C. et al., 2012. Ars2 maintains neural stem-cell identity through direct transcriptional activation of Sox2. *Nature*, 481(7380), pp.195–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22198669%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3261657.

Andrusiak, M.G. et al., 2012. The Retinoblastoma Protein Is Essential for Survival of Postmitotic Neurons. *Journal of Neuroscience*, 32(42). Available at: http://www.jneurosci.org/content/32/42/14809 [Accessed March 31, 2017].

Arendt, T. et al., 2010. Selective cell death of hyperploid neurons in Alzheimer's disease. *The American journal of pathology*, 177(1), pp.15–20. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2893646&tool=pmcentrez&rendertype=abstract.

Arisi, G.M. et al., 2012. The role of olfactory stimulus in adult mammalian neurogenesis. *Behavioural Brain Research*, 227(2), pp.356–362.

Armesilla-Diaz, A. et al., 2009. p53 regulates the self-renewal and differentiation of neural precursors. *Neuroscience*, 158(4), pp.1378–1389.

Armstrong, J.F. et al., 1995. High-frequency developmental abnormalities in p53-deficient mice. *Current Biology*, 5(8), pp.931–936.

Azari, H. et al., 2010. Isolation and expansion of the adult mouse neural stem cells using the neurosphere assay. *Journal of visualized experiments : JoVE*, (45), pp.8–11.

Bartesaghi, S. & Salomoni, P., 2013. Tumor suppressive pathways in the control of neurogenesis. *Cellular and molecular life sciences : CMLS*, 70(4), pp.581–97. Available at: http://link.springer.com/10.1007/s00018-012-1063-9 [Accessed September 5, 2016].

Bath, K.G. & Lee, F.S., 2010. *Neurotrophic factor control of adult SVZ neurogenesis*, NIH Public Access. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20186714 [Accessed April 5, 2017].

Batista-Brito, R. et al., 2008. The distinct temporal origins of olfactory bulb interneuron

subtypes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 28(15), pp.3966–75. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2505353&tool=pmcentrez&rendertype=abstract.

Beauséjour, C.M. et al., 2003. Reversal of human cellular senescence: Roles of the p53 and p16 pathways. *EMBO Journal*, 22(16), pp.4212–4222.

Bez, A. et al., 2003. Neurosphere and neurosphere-forming cells: Morphological and ultrastructural characterization. *Brain Research*, 993(1–2), pp.18–29.

Bjornsson, C.S. et al., 2015. It takes a village: constructing the neurogenic niche. *Developmental cell*, 32(4), pp.435–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25710530 [Accessed March 23, 2017].

Blanquet, V. et al., 1993. Identification of germline mutations in the RB1 gene by denaturant gradient gel electrophoresis and polymerase chain reaction direct sequencing. *Human Molecular Genetics*, 2(7), pp.975–979.

Bond, A.M.M.M., Ming, G.L. & Song, H., 2015. Adult Mammalian Neural Stem Cells and Neurogenesis: Five Decades Later. *Cell Stem Cell*, 17(4), pp.385–395. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26431181 [Accessed March 24, 2017].

Brill, M.S. et al., 2009. Adult generation of glutamatergic olfactory bulb interneurons. *Nature Neuroscience*, 12(12), pp.1524–1533. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19881504.

Cajal, S.R. y & May, R.M., 1991. Degeneration and Regeneration of the Nervous System. *Oxford Press*, (185), pp.799–802. Available at: http://www.oxfordscholarship.com/view/10.1093/acprof:oso/9780195065169.001.0001/acprof-9780195065169.

Calzolari, F. et al., 2015. Fast clonal expansion and limited neural stem cell self-renewal in the adult subependymal zone. *Nature neuroscience*, 18(4), pp.490–2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25730673.

Cancino, G.I. et al., 2013. p63 Regulates adult neural precursor and newly born neuron survival to control hippocampal-dependent Behavior. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(31), pp.12569–85. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23904595%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3728680.

Capilla-Gonzalez, V., Herranz-Pérez, V. & García-Verdugo, J.M., 2015. The aged brain: genesis and fate of residual progenitor cells in the subventricular zone. *Frontiers in cellular neuroscience*, 9(September), p.365. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4585225&tool=pmcentrez&rendertype=abstract.

Chan, T. a et al., 1999. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature*, 401(6753), pp.616–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10524633.

Cheung, T.H. & Rando, T.A., 2013. Molecular regulation of stem cell quiescence. *Nature reviews. Molecular cell biology*, 14(6), pp.329–40. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23698583 [Accessed November 27, 2016].

Chipuk, J.E. et al., 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*, 303(5660), pp.1010–4.

Chow, B.M., Li, Y.Q. & Wong, C.S., 2000. Radiation-induced apoptosis in the adult

central nervous system is p53-dependent. *Cell death and differentiation*, 7(8), pp.712–720.

Chow, K.N. & Dean, D.C., 1996. Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. *Molecular and Cellular Biology*, 16(9), pp.4862–4868. Available at: http://mcb.asm.org/lookup/doi/10.1128/MCB.16.9.4862 [Accessed March 29, 2017].

Chow, K.N., Starostik, P. & Dean, D.C., 1996. The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif. *Molecular and Cellular Biology*, 16(12), pp.7173–7181. Available at: http://mcb.asm.org/lookup/doi/10.1128/MCB.16.12.7173 [Accessed March 29, 2017].

Clair, S.S. et al., 2004. DNA damage-induced downregulation of Cdc25C is mediated by p53 via two independent mechanisms: One involves direct binding to the cdc25C promoter. *Molecular Cell*, 16(5), pp.725–736.

Clark, R.S. et al., 2000. Caspase-3 mediated neuronal death after traumatic brain injury in rats. *Journal of neurochemistry*, 74(2), pp.740–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10646526.

Clarke, A.R. et al., 1992. Requirement for a functional Rb-1 gene in murine development. *Nature*, 359(6393), pp.328–330. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1406937.

Codega, P. et al., 2014. Prospective Identification and Purification of Quiescent Adult Neural Stem Cells from Their In Vivo Niche. *Neuron*, 82(3), pp.545–559.

Conover, J.C. & Shook, B. a, 2011. Aging of the subventricular zone neural stem cell niche. *Aging and disease*, 2(1), pp.49–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3295044&tool=pmcentrez&rendertype=abstract.

Cooper-Kuhn, C.M. et al., 2002. Impaired adult neurogenesis in mice lacking the transcription factor E2F1. *Molecular and cellular neurosciences*, 21, pp.312–323.

Corona, R. et al., 2016. Sexual Behavior Increases Cell Proliferation in the Rostral Migratory Stream and Promotes the Differentiation of the New Cells into Neurons in the Accessory Olfactory Bulb of Female Rats. *Frontiers in neuroscience*, 10, p.48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26955325 [Accessed March 23, 2017].

Coschi, C.H. et al., 2010. Mitotic chromosome condensation mediated by the retinoblastoma protein is tumor-suppressive. *Genes and Development*, 24(13), pp.1351–1363.

Craig, C.G. et al., 1996. In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. *J Neurosci*, 16(8), pp.2649–2658. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8786441.

Crighton, D. et al., 2006. DRAM, a p53-Induced Modulator of Autophagy, Is Critical for Apoptosis. *Cell*, 126(1), pp.121–134.

D'Amelio, M., Cavallucci, V. & Cecconi, F., 2010. Neuronal caspase-3 signaling: not only cell death. *Cell death and differentiation*, 17(7), pp.1104–14. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19960023.

Das, G.D. & Altman, J., 1971. Postnatal neurogenesis in the cerebellum of the cat and tritiated thymidine autoradiography. *Brain Research*, 30(2), pp.323–330.

DeGregori, J. & Johnson, D.G., 2006. Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis. *Current molecular medicine*, 6(7), pp.739–748.

Deng, C. et al., 1995. Mice Lacking p21 CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell*, 82(4), pp.675–684.

Deng, W., Aimone, J.B. & Gage, F.H., 2010. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nature reviews. Neuroscience*, 11(5), pp.339–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20354534%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2886712.

Dick, F.A. & Rubin, S.M., 2013. Molecular mechanisms underlying RB protein function. *Nature Publishing Group*, 14. Available at: http://search.proquest.com.ezproxy.aub.edu.lb/docview/1346865188?pq-origsite=summon [Accessed March 28, 2017].

Doetsch, F., Petreanu, L., et al., 2002. EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron*, 36(6), pp.1021–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12495619 [Accessed March 25, 2017].

Doetsch, F., Verdugo, J.M.-G., et al., 2002. Lack of the cell-cycle inhibitor p27Kip1 results in selective increase of transit-amplifying cells for adult neurogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 22(6), pp.2255–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11896165.

Doetsch, F. et al., 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*, 97(6), pp.703–716.

Doetsch, F. & Alvarez-Buylla, A., 1996. Network of tangential pathways for neuronal migration in adult mammalian brain. *Proceedings of the National Academy of Sciences of the United States of America*, 93(25), pp.14895–14900. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8962152%5Cnhttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC26233/pdf/pq014895.pdf.

Doetsch, F., García-Verdugo, A. & Alvarez-Buylla, J., 1997. Cellular Composition and Three-Dimensional Organization of the Subventricular Germinal Zone in the Adult Mammalian Brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(13)(13), pp.5046–61. Available at: www.ncbi.nlm.nih.gov/pubmed/9185542.

Donehower, L. a et al., 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, 356(6366), pp.215–221.

Dötsch, V. et al., 2010. p63 and p73, the ancestors of p53. *Cold Spring Harbor perspectives in biology*, 2(9).

Dupret, D. et al., 2008. Spatial relational memory requires hippocampal adult neurogenesis. *PLoS ONE*, 3(4).

Dyson, N. et al., 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, 243(4893), pp.934–937. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.2537532 [Accessed March 29, 2017].

Dyson, N., 1998. The regulation of E2F by pRB-family proteins. *Genes & Development*, 12(15), pp.2245–2262. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.12.15.2245 [Accessed March 29,

2017].
Eriksson, P.S. et al., 1998. Neurogenesis in the adult human hippocampus. *Nat Med*, 4(11), pp.1313–1317. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9809557%5Cnhttp://www.nature.com/nm/journal/v4/n11/pdf/nm1198_1313.pdf.
Ewen, M.E. et al., 1989. An N-Terminal transformation-governing sequence of SV40 large T antigen contributes to the binding of both p110Rb and a second cellular protein, p120. *Cell*, 58(2), pp.257–267. Available at: http://linkinghub.elsevier.com/retrieve/pii/0092867489908404 [Accessed March 29, 2017].
Ewen, M.E. et al., 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell*, 66(1985), pp.1155–1164. Available at: http://linkinghub.elsevier.com/retrieve/pii/009286749190038Z [Accessed March 29, 2017].
Faiz, M. et al., 2015. Adult Neural Stem Cells from the Subventricular Zone Give Rise to Reactive Astrocytes in the Cortex after Stroke. *Cell Stem Cell*, 17(5), pp.624–634. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26456685 [Accessed April 3, 2017].
Fatt, M.P. et al., 2014. P63 and P73 Coordinate P53 Function To Determine the Balance Between Survival, Cell Death, and Senescence in Adult Neural Precursor Cells. *Cell death and differentiation*, 21(10), pp.1546–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24809925.
Ferent, J. et al., 2014. Genetic activation of hedgehog signaling unbalances the rate of neural stem cell renewal by increasing symmetric divisions. *Stem Cell Reports*, 3(2), pp.312–323.
Ferguson, K.L. et al., 2005. A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. *EMBO Journal*, 24(24), pp.4381–4391. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16308563%5Cnhttp://www.nature.com/emboj/journal/v24/n24/pdf/7600887a.pdf.
Ferguson, K.L. et al., 2002. Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. *EMBO Journal*, 21(13), pp.3337–3346.
Fernando, P., Brunette, S. & Megeney, L.A., 2005. Neural stem cell differentiation is dependent upon endogenous caspase-3 activity. *The FASEB Journal*, 19(12), pp.1671–3. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16103108 [Accessed April 20, 2017].
Fernando, P., Brunette, S. & Megeney, L. a, 2005. Neural stem cell differentiation is dependent upon endogenous caspase 3 activity. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 19(12), pp.1671–1673.
Ferri, A.L.M. et al., 2004. Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development*, 131(15), pp.3805–3819. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15240551 [Accessed April 5, 2017].
De Filippis, L. & Binda, E., 2012. Concise review: self-renewal in the central nervous system: neural stem cells from embryo to adult. *Stem cells translational medicine*,

1(4), pp.298–308. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23197809 [Accessed April 8, 2017].

Flatt, P.M. et al., 2000. p53 regulation of G(2) checkpoint is retinoblastoma protein dependent. *Molecular and cellular biology*, 20(12), pp.4210–23. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=85790&tool=pmcentrez&rendertype=abstract.

Fletcher, R.B. et al., 2011. P63 regulates olfactory stem cell self-renewal and differentiation. *Neuron*, 72(5), pp.748–759.

Fong, B.C. & Slack, R.S., 2017. RB: An essential player in adult neurogenesis. *Neurogenesis*, 4(1), p.e1270382. Available at: https://www.tandfonline.com/doi/full/10.1080/23262133.2016.1270382 [Accessed March 27, 2017].

Fortin, A. et al., 2004. The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. *Journal of Biological Chemistry*, 279(27), pp.28706–28714.

Fortin, J.M. et al., 2016. Transplantation of Defined Populations of Differentiated Human Neural Stem Cell Progeny. *Scientific reports*, 6(March), p.23579. Available at: http://www.nature.com/srep/2016/160331/srep23579/full/srep23579.html.

Frade, J.M. & Ovejero-Benito, M.C., 2015. Neuronal cell cycle: the neuron itself and its circumstances. *Cell cycle (Georgetown, Tex.)*, 14(5), pp.712–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25590687 [Accessed April 20, 2017].

Francoz, S. et al., 2006. Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 103(9), pp.3232–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1413884&tool=pmcentrez&rendertype=abstract.

Friend, S.H. et al., 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*, 323(6089), pp.643–6. Available at: http://www.nature.com/doifinder/10.1038/323643a0 [Accessed March 29, 2017].

Fujitani, M. et al., 2010. TAp73 acts via the bHLH Hey2 to promote long-term maintenance of neural precursors. *Current Biology*, 20(22), pp.2058–2065.

Galli, R. et al., 2004. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Research*, 64(19), pp.7011–7021.

Ge, S. et al., 2007. A Critical Period for Enhanced Synaptic Plasticity in Newly Generated Neurons of the Adult Brain. *Neuron*, 54(4), pp.559–566.

Ge, S. et al., 2008. Synaptic integration and plasticity of new neurons in the adult hippocampus. *The Journal of physiology*, 586(16), pp.3759–65. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2538931&tool=pmcentrez&rendertype=abstract.

Ghanem, N. et al., 2012. The Rb/E2F Pathway Modulates Neurogenesis through Direct Regulation of the Dlx1/Dlx2 Bigene Cluster. *Journal of Neuroscience*, 32(24), pp.8219–8230. Available at: http://www.jneurosci.org/content/jneuro/32/24/8219.full.pdf [Accessed March 25, 2017].

Giacinti, C. & Giordano, A., 2006. RB and cell cycle progression. *Oncogene*, 25(38), pp.5220–5227. Available at:

http://www.nature.com/doifinder/10.1038/sj.onc.1209615 [Accessed March 30, 2017].

Gil-Perotin, S. et al., 2006. Loss of p53 induces changes in the behavior of subventricular zone cells: implication for the genesis of glial tumors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(4), pp.1107–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16436596 [Accessed February 26, 2016].

Gil-Perotín, S. et al., 2013. Adult Neural Stem Cells From the Subventricular Zone: A Review of the Neurosphere Assay. *The Anatomical Record*, 296(9), pp.1435–1452. Available at: http://doi.wiley.com/10.1002/ar.22746 [Accessed March 25, 2017].

Giovanni, A. et al., 2000. E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. *Journal of Biological Chemistry*, 275(16), pp.11553–11560.

Givogri, M.I. et al., 2006. Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. *Developmental Neuroscience*, 28(1–2), pp.81–91.

Gould, E. et al., 1999. Neurogenesis in the neocortex of adult primates. *Science (New York, N.Y.)*, 286(5439), pp.548–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10521353 [Accessed April 5, 2017].

Gratzner, H.G., 1982. Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science (New York, N.Y.)*, 218(4571), pp.474–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7123245.

Gregorian, C. et al., 2009. Pten deletion in adult neural stem/progenitor cells enhances constitutive neurogenesis. *Journal of Neuroscience*, 29(6), pp.1874–1886. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19211894 [Accessed April 8, 2017].

Gritti, A. et al., 1999. Epidermal and Fibroblast Growth Factors Behave as Mitogenic Regulators for a Single Multipotent Stem Cell-Like Population from the Subventricular Region of the Adult Mouse Forebrain. *Journal of Neuroscience*, 19(9). Available at: http://www.jneurosci.org/content/19/9/3287.long [Accessed April 8, 2017].

Gritti, A. et al., 2002. Multipotent Neural Stem Cells Reside into the Rostral Extension and Olfactory Bulb of Adult Rodents. *Journal of Neuroscience*, 22(2). Available at: http://www.jneurosci.org/content/22/2/437.long [Accessed April 8, 2017].

Hamilton, A., 1901. The division of differentiated cells in the central nervous system of the white rat. *Journal of Comparative Neurology*, 11(4), pp.297–320. Available at: http://doi.wiley.com/10.1002/cne.910110403 [Accessed March 23, 2017].

Han, J. et al., 2001. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. *Proceedings of the National Academy of Sciences of the United States of America*, 98(20), pp.11318–23. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=58727&tool=pmcentrez&rendertype=abstract.

Van Harn, T. et al., 2010. Loss of Rb proteins causes genomic instability in the absence of mitogenic signaling. *Genes and Development*, 24(13), pp.1377–1388.

Haupt, Y. et al., 1997. Mdm2 promotes the rapid degradation of p53. *Nature*, 387(6630), pp.296–9.

Henley, S.A. & Dick, F.A., 2012. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell Division*, 7(1),

p.10. Available at: http://celldiv.biomedcentral.com/articles/10.1186/1747-1028-7-10 [Accessed March 29, 2017].

Hernández-Monge, J. et al., 2016. Dual function of MDM2 and MDMX toward the tumor suppressors p53 and RB. *Genes & cancer*, 7(9–10), pp.278–287. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28050229 [Accessed April 4, 2017].

Herrera, D.G., Garcia-Verdugo, J.M. & Alvarez-Buylla, A., 1999. Adult-derived neural precursors transplanted into multiple regions in the adult brain. *Annals of Neurology*, 46(6), pp.867–877.

Holmberg, J. et al., 2005. Ephrin-A2 reverse signaling negatively regulates neural progenitor proliferation and neurogenesis. *Genes and Development*, 19(4), pp.462–471.

Hooper, C.M. et al., 2014. Gene Expression Analyses of the Spatio-Temporal Relationships of Human Medulloblastoma Subgroups during Early Human Neurogenesis B. Alsina, ed. *PLoS ONE*, 9(11), p.e112909. Available at: http://dx.plos.org/10.1371/journal.pone.0112909 [Accessed April 8, 2017].

Hornsby, A.K.E. et al., 2016. Short-term calorie restriction enhances adult hippocampal neurogenesis and remote fear memory in a Ghsr-dependent manner. *Psychoneuroendocrinology*, 63, pp.198–207.

Hosako, H. et al., 2009. Gene and microRNA expression in p53-deficient day 8.5 mouse embryos. *Birth Defects Research Part A - Clinical and Molecular Teratology*, 85(6), pp.546–555.

Hu, H. & Rutishauser, U., 1996. A septum-derived chemorepulsive factor for migrating olfactory interneuron precursors. *Neuron*, 16(5), pp.933–940.

Hulspas, R. & Quesenberry, P.J., 2000. Characterization of neurosphere cell phenotypes by flow cytometry. *Cytometry*, 40(3), pp.245–250.

Ihrie, R.A. et al., 2011. Persistent Sonic Hedgehog Signaling in Adult Brain Determines Neural Stem Cell Positional Identity. *Neuron*, 71(2), pp.250–262.

Ikeda, S. et al., 1999. Severe ocular abnormalities in C57BL/6 but not in 129/Sv p53-deficient mice. *Investigative Ophthalmology and Visual Science*, 40(8), pp.1874–1878.

Irwin, M. et al., 2000. Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature*, 407(6804), pp.645–8. Available at: http://www.nature.com/doifinder/10.1038/35036614%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/11034215%5Cnhttp://www.nature.com/doifinder/10.1038/35036614%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/11034215.

Iwai, H. et al., 2015. Allogeneic Neural Stem/Progenitor Cells Derived From Embryonic Stem Cells Promote Functional Recovery After Transplantation Into Injured Spinal Cord of Nonhuman Primates. *Stem cells translational medicine*, 4(7), pp.708–19. Available at: http://stemcellstm.alphamedpress.org/content/early/2015/05/21/sctm.2014-0215.abstract [Accessed December 9, 2015].

Jaafar, C. et al., 2016. Role of Rb during Neurogenesis and Axonal Guidance in the Developing Olfactory System. *Frontiers in Molecular Neuroscience*, 9, p.81. Available at: http://journal.frontiersin.org/article/10.3389/fnmol.2016.00081 [Accessed September 13, 2016].

Jacks, T. et al., 1992. Effects of an Rb mutation in the mouse. *Nature*, 359(6393), pp.295–300. Available at: papers2://publication/doi/10.1038/359295a0%5Cnhttp://www.nature.com/doifinde

r/10.1038/359295a0.

Jessberger, S. & Gage, F.H., 2014. Adult neurogenesis: Bridging the gap between mice and humans. *Trends in Cell Biology*, 24(10), pp.558–563. Available at: http://dx.doi.org/10.1016/j.tcb.2014.07.003.

Jin, S. et al., 2000. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. *Journal of Biological Chemistry*, 275(22), pp.16602–16608.

Johnson, D.G. & DeGregori, J., 2006. Putting the Oncogenic and Tumor Suppressive Activities of E2F into Context. *Current molecular medicine*, 6(7), pp.731–738. Available at: papers2://publication/uuid/192D9559-C877-4D10-BA4B-ABE4D5FB0699.

Jones, S.N. et al., 1995. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature*, 378(6553), pp.206–208.

Kaplan, M.S. & Hinds, J.W., 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science (New York, N.Y.)*, 197(4308), pp.1092–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/887941.

Kareta, M.S. et al., 2015. Inhibition of pluripotency networks by the Rb tumor suppressor restricts reprogramming and tumorigenesis. *Cell Stem Cell*, 16(1), pp.39–50.

Kempermann, G., 2011. Seven principles in the regulation of adult neurogenesis. *The European journal of neuroscience*, 33(6), pp.1018–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21395844 [Accessed April 8, 2017].

Kippin, T.E. et al., 2005. p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes & Development*, 19(6), pp.756–767. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.1272305 [Accessed September 5, 2016].

Kohwi, M. et al., 2007. A subpopulation of olfactory bulb GABAergic interneurons is derived from Emx1- and Dlx5/6-expressing progenitors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(26), pp.6878–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17596436.

Kohwi, M. et al., 2005. Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *Journal of Neuroscience*, 25(30), pp.6997–7003. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16049175%5Cnhttp://www.jneurosci.org/content/25/30/6997.full.pdf.

Komori, H. et al., 2005. Distinct E2F-mediated transcriptional program regulates p14ARF gene expression. *The EMBO journal*, 24(21), pp.3724–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1276720&tool=pmcentrez&rendertype=abstract.

Kruiswijk, F., Labuschagne, C.F. & Vousden, K.H., 2015. p53 in survival, death and metabolic health: a lifeguard with a licence to kill. *Nature Reviews Molecular Cell Biology*, 16(7), pp.393–405. Available at: http://www.nature.com/doifinder/10.1038/nrm4007.

Kuhn, H.G. et al., 1997. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J Neurosci*, 17(15), pp.5820–5829. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9221780.

Kukekov, V.G. et al., 1999. Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. *Exp Neurol*, 156(2), pp.333–44. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10328940%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/10328940.

Lagace, D.C. et al., 2007. Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. *Journal of Neuroscience*, 27(46), pp.12623–12629. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18003841.

Lane, D.P., 1992. Cancer. p53, guardian of the genome. *Nature*, 358(6381), pp.15–16.

Lane, D.P. & Crawford, L. V, 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature*, 278, pp.261–263.

Larsen, B.D. et al., 2010. Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. *Proceedings of the National Academy of Sciences of the United States of America*, 107(9), pp.4230–5. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.0913089107%5Cnhttp://www.pnas.org/content/107/9/4230.full.

Larsen, C.M., Kokay, I.C. & Grattan, D.R., 2008. Male pheromones initiate prolactin-induced neurogenesis and advance maternal behavior in female mice. *Hormones and Behavior*, 53(4), pp.509–517.

Lee, A. et al., 2005. Isolation of neural stem cells from the postnatal cerebellum. *Nature neuroscience*, 8(6), pp.723–9. Available at: http://dx.doi.org/10.1038/nn1473.

Lee, E.Y. et al., 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature*, 359(6393), pp.288–294. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1406932.

Lee, M.H. et al., 1996. Targeted disruption of p107: functional overlap between p107 and Rb. *Genes & development*, 10(13), pp.1621–32. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8682293 [Accessed April 8, 2017].

Lee, W.H. et al., 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science*, 235(4794), pp.1394–9. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.3823889 [Accessed March 29, 2017].

Leu, J.I.-J. et al., 2004. Mitochondrial p53 activates Bak and causes disruption of a Bak–Mcl1 complex. *Nature Cell Biology*, 6(5), pp.443–450. Available at: http://www.nature.com/doifinder/10.1038/ncb1123.

Leventhal, C. et al., 1999. Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. *Molecular and cellular neurosciences*, 13(6), pp.450–64. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10383830.

Li, J. et al., 2008. Synergistic Function of E2F7 and E2F8 Is Essential for Cell Survival and Embryonic Development. *Developmental Cell*, 14(1), pp.62–75.

Li, L. & Clevers, H., 2010. Coexistence of quiescent and active adult stem cells in mammals. *Science (New York, N.Y.)*, 327(5965), pp.542–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20110496%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4105182%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4105182&tool=pmcentrez&rendertype=abstract.

Li, Y. et al., 1993. The adenovirus E1A-associated 130-kD protein is encoded by a

member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes and Development*, 7(12 A), pp.2366–2377. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.7.12a.2366 [Accessed March 29, 2017].

Li, Y.-Q. et al., 2016. P53 regulates disruption of neuronal development in the adult hippocampus after irradiation. *Cell Death Discovery*, 2, p.16072. Available at: http://www.nature.com/articles/cddiscovery201672 [Accessed April 2, 2017].

Li, Y.Q., Aubert, I. & Wong, C.S., 2010. Abrogation of Early Apoptosis Does Not Alter Late Inhibition of Hippocampal Neurogenesis After Irradiation. *International Journal of Radiation Oncology Biology Physics*, 77(4), pp.1213–1222.

Lieberwirth, C. et al., 2016. Hippocampal adult neurogenesis: Its regulation and potential role in spatial learning and memory. *Brain Research*, 1644, pp.127–140. Available at: http://www.sciencedirect.com.ezproxy.aub.edu.lb/science/article/pii/S0006899316303705?np=y&npKey=db77e8fde9acbe25d53928fec303e239b1af82ab717c59448ec08e455a3a0653 [Accessed April 5, 2017].

Lim, D.A. et al., 2000. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron*, 28(3), pp.713–726.

Lim, D.A. & Alvarez-Buylla, A., 1999. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 96(13), pp.7526–31. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=22119&tool=pmcentrez&rendertype=abstract.

Lim, D.A. & Alvarez-Buylla, A., 2016. The Adult Ventricular–Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. *Cold Spring Harbor Perspectives in Biology*, 8(5), p.a018820. Available at: http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a018820 [Accessed September 5, 2016].

Lipinski, M.M. et al., 2001. Cell-autonomous and non-cell-autonomous functions of the Rb tumor suppressor in developing central nervous system. *EMBO Journal*, 20(13), pp.3402–3413.

Liu, D.X. & Greene, L.A., 2001. Neuronal apoptosis at the G1/S cell cycle checkpoint. *Cell and tissue research*, 305(2), pp.217–228.

Liu, X. et al., 2005. Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. *Nature neuroscience*, 8(9), pp.1179–1187.

Lobo, M.V.T. et al., 2003. Cellular characterization of epidermal growth factor-expanded free-floating neurospheres. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 51(1), pp.89–103.

Lois, C. & Alvarez-Buylla, A., 1994. Long-distance neuronal migration in the adult mammalian brain. *Science*, 264(5162), pp.1145–1148. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8178174%5Cnhttp://www.jstor.org/stable/pdfplus/2885135.pdf.

Lookeren Campagne, M. Van & Gill, R., 1998. Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: Comparison with expression of the cell cycle regulators p21Waf1/Cip1, p27Kip1,

p57Kip2, p16Ink4a, cyclin G1, and the proto-oncogene bax. *The Journal of Comparative Neurology*, 397(2), pp.181–198. Available at: http://doi.wiley.com/10.1002/%2528SICI%25291096-9861%252819980727%2529397%253A2%253C181%253A%253AAID-CNE3%253E3.0.CO%253B2-X [Accessed September 9, 2016].

Loughran, O. & La Thangue, N.B., 2000. Apoptotic and growth-promoting activity of E2F modulated by MDM2. *Molecular and cellular biology*, 20(6), pp.2186–97. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=110835&tool=pmcentrez&rendertype=abstract.

Louis, S.A. et al., 2008. Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay. *Stem Cells*, 26(4), pp.988–996. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18218818%5Cnhttp://onlinelibrary.wiley.com/store/10.1634/stemcells.2007-0867/asset/260988_ftp.pdf?v=1&t=hvy6zfse&s=eaa20a57fd1b3aca4bb3025b76e658a07a76451d.

Louis, S.A., Mak, C.K.H. & Reynolds, B.A., 2013. Methods to culture, differentiate, and characterize neural stem cells from the adult and embryonic mouse central nervous system. *Methods in molecular biology (Clifton, N.J.)*, 946, pp.479–506. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23179851.

Luo, J. et al., 2006. The aging neurogenic subventricular zone. *Aging Cell*, 5(2), pp.139–152.

Luskin, M.B., 1993. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron*, 11(1), pp.173–189.

Machold, R. et al., 2003. Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. *Neuron*, 39(6), pp.937–950.

Macleod, K.F., Hu, Y. & Jacks, T., 1996. Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *The EMBO journal*, 15(22), pp.6178–88. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=452439&tool=pmcentrez&rendertype=abstract.

MacPherson, D. et al., 2003. Conditional Mutation of Rb Causes Cell Cycle Defects without Apoptosis in the Central Nervous System. *Molecular and Cellular Biology*, 23(3), pp.1044–1053. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12529408%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC140709%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/12529408%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC140709%5Cnhttp://mcb.asm.org/.

Maiuri, M.C. et al., 2010. Autophagy regulation by p53. *Current Opinion in Cell Biology*, 22(2), pp.181–185.

Malvaut, S. & Saghatelyan, A., 2016. The role of adult-born neurons in the constantly changing olfactory bulb network. *Neural Plasticity*, 2016.

Manning, A.L. & Dyson, N.J., 2012. RB: mitotic implications of a tumour suppressor. *Nature Reviews Cancer*, 12(3), pp.220–226. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22318235.

Marino, S. et al., 2000. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum.

Genes and Development, 14(8), pp.994–1004.
Marlier, Q. et al., 2015. Mechanisms and Functional Significance of Stroke-Induced Neurogenesis. *Frontiers in neuroscience*, 9, p.458. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26696816 [Accessed April 5, 2017].
Martin, K. et al., 1995. Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. *Nature*, 375(6533), pp.691–694.
Matsui, T. et al., 2017. Retinoblastoma protein controls growth, survival and neuronal migration in human cerebral organoids. *Development*, 144(6). Available at: http://dev.biologists.org/content/144/6/1025 [Accessed March 29, 2017].
Maya-Espinosa, G. et al., 2015. Mouse Embryonic Stem Cell-Derived Cells Reveal Niches that Support Neuronal Differentiation in the Adult Rat Brain. *STEM CELLS*, 33(2), pp.491–502. Available at: http://doi.wiley.com/10.1002/stem.1856 [Accessed March 23, 2017].
McClellan, K.A. et al., 2009. The p107/E2F pathway regulates fibroblast growth factor 2 responsiveness in neural precursor cells. *Molecular and Cellular Biology*, 29(17), pp.4701–4713. Available at: papers2://publication/doi/10.1128/MCB.01767-08.
McClellan, K. a et al., 2007. Unique requirement for Rb/E2F3 in neuronal migration: evidence for cell cycle-independent functions. *Molecular and cellular biology*, 27(13), pp.4825–4843.
Meletis, K. et al., 2006. p53 suppresses the self-renewal of adult neural stem cells. *Development*, 133(2), pp.363–369. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16368933.
Mendrysa, S.M., Ghassemifar, S. & Malek, R., 2011. p53 in the CNS: Perspectives on Development, Stem Cells, and Cancer. *Genes & cancer*, 2(4), pp.431–442.
Merkle, F.T. et al., 2014. Adult neural stem cells in distinct microdomains generate previously unknown interneuron types. *Nature Neuroscience*, 17(2), pp.207–214. Available at: http://www.nature.com/neuro/journal/v17/n2/pdf/nn.3610.pdf.
Merkle, F.T., Mirzadeh, Z. & Alvarez-Buylla, A., 2007. Mosaic organization of neural stem cells in the adult brain. *Science (New York, N.Y.)*, 317(5836), pp.381–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17615304.
Migliorini, D. et al., 2002. Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol Cell Biol*, 22(15), pp.5527–5538. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12101245.
Ming, G.-L. & Song, H., 2005. Adult Neurogenesis in the Mammalian Central Nervous System. Available at: https://pdfs.semanticscholar.org/65c4/54a498f57266117105f5c854c623d319e6cc.pdf [Accessed March 22, 2017].
Ming, G. li & Song, H., 2011. Review Adult Neurogenesis in the Mammalian Brain : Significant Answers and Significant Questions. *Neuron*, 70(4), pp.687–702. Available at: http://dx.doi.org/10.1016/j.neuron.2011.05.001.
Mobley, A.S. et al., 2013. Age-dependent regional changes in the rostral migratory stream. *Neurobiology of Aging*, 34(7), pp.1873–1881.
Mongiat, L.A. & Schinder, A.F., 2011. Adult neurogenesis and the plasticity of the dentate gyrus network. *European Journal of Neuroscience*, 33(6), pp.1055–1061.
Moon, N.S. et al., 2005. Drosophila E2F1 has context-specific pro- and antiapoptotic properties during development. *Developmental Cell*, 9(4), pp.463–475.
Moreno, M.M. et al., 2009. Olfactory perceptual learning requires adult neurogenesis.

Proceedings of the National Academy of Sciences of the United States of America, 106(42), pp.17980–17985.
Morgenbesser, S.D. et al., 1994. p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature*, 371(6492), pp.72–74. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8072529.
Moroni, M.C. et al., 2001. Apaf-1 is a transcriptional target for E2F and p53. *Nature cell biology*, 3(6), pp.552–558.
Mulligan, G. & Jacks, T., 1998. The retinoblastoma gene family: Cousins with overlapping interests. *Trends in Genetics*, 14(6), pp.223–229.
Münger, K. et al., 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *The EMBO journal*, 8(13), pp.4099–105. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=401588&tool=pmcentrez&rendertype=abstract.
Nakagomi, T. et al., 2011. Ischemia-Induced Neural Stem/Progenitor Cells in the Pia Mater Following Cortical Infarction. *Stem Cells and Development*, 20(12), pp.2037–2051. Available at: http://www.liebertonline.com/doi/abs/10.1089/scd.2011.0279 [Accessed April 5, 2017].
Nakano, K. & Vousden, K.H., 2001. PUMA, a novel proapoptotic gene, is induced by p53. *Molecular Cell*, 7(3), pp.683–694.
Narasimha, A.M. et al., 2014. Cyclin D activates the Rb tumor suppressor by mono-phosphorylation. *eLife*, 3, pp.6983–6993. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24876129 [Accessed March 30, 2017].
Naser, R. et al., 2016. Role of the Retinoblastoma protein, Rb, during adult neurogenesis in the olfactory bulb. *Scientific reports*, 6, p.20230. Available at: http://www.nature.com/srep/2016/160205/srep20230/full/srep20230.html [Accessed February 26, 2016].
Nemoto, S., Fergusson, M.M. & Finkel, T., 2004. Nutrient Availability Regulates SIRT1 Through a Forkhead-Dependent Pathway. *Science*, 306(5704), pp.2105–2108. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.1101731.
Nissant, A. et al., 2009. Adult neurogenesis promotes synaptic plasticity in the olfactory bulb. *Nature neuroscience*, 12(6), pp.728–730.
Nottebohm, F., 1989. From bird song to neurogenesis. *Scientific American*, 260(2), pp.74–79.
O'Hare, M.J. et al., 2000. Induction and modulation of cerebellar granule neuron death by E2F-1. *Journal of Biological Chemistry*, 275(33), pp.25358–25364.
Oda, E., 2000. Candidate Mediator of p53-Induced Apoptosis Noxa, a BH3-Only Member of the Bcl-2 Family and. *Microbiol. Rev. Int. J. Zoon. Acta Microbiol. Acad. Sci. Hung. Nature Vet. Med. Assoc. Arch. Virol. Nature Nature Nature Science J. R. Geraci et al. Science J. Virol. Virology J. Gen. Virol. J. Virol. Methods*, 56(57), pp.1053–1505. Available at: http://www.sciencemag.org/cgi/content/full/288/5468/1053%5Cnhttp://www.sciencemag.org/cgi/content/full/288/5468/1053#otherarticles%5Cnhttp://www.sciencemag.org/cgi/collection/cell_biol.
Ofir-Rosenfeld, Y. et al., 2008. Mdm2 Regulates p53 mRNA Translation through Inhibitory Interactions with Ribosomal Protein L26. *Molecular Cell*, 32(2),

pp.180–189.

Ohira, K. et al., 2010. Ischemia-induced neurogenesis of neocortical layer 1 progenitor cells. *Nature Neuroscience*, 13(2), pp.173–179. Available at: http://www.nature.com/doifinder/10.1038/nn.2473%5Cnpapers3://publication/doi/10.1038/nn.2473.

Oomman, S. et al., 2006. Bergmann glia utilize active caspase-3 for differentiation. *Brain research*, 1078(1), pp.19–34. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16700096 [Accessed April 20, 2017].

Ortega, F., Berninger, B. & Costa, M.R., 2013. Primary culture and live imaging of adult neural stem cells and their progeny. *Methods in molecular biology (Clifton, N.J.)*, 1052, pp.1–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23640252.

Ottone, C. et al., 2014. Direct cell-cell contact with the vascular niche maintains quiescent neural stem cells. *Nature cell biology*, 16(11), pp.1045–56. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4298702&tool=pmcentrez&rendertype=abstract.

Oyarce, K., Bongarzone, E.R. & Nualart, F., 2014. Unconventional Neurogenic Niches and Neurogenesis Modulation by Vitamins. *Journal of stem cell research & therapy*, 4(3), p.184. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26203401 [Accessed March 23, 2017].

Pajcini, K. V. et al., 2010. Transient inactivation of Rb and ARF yields regenerative cells from postmitotic mammalian muscle. *Cell Stem Cell*, 7(2), pp.198–213.

Palmer, T.D. et al., 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(19), pp.8487–8497.

Palmer, T.D., Willhoite, A.R. & Gage, F.H., 2000. Vascular niche for adult hippocampal neurogenesis. *Journal of Comparative Neurology*, 425(4), pp.479–494.

Pan, Y.-W. et al., 2012. Inhibition of Adult Neurogenesis by Inducible and Targeted Deletion of ERK5 Mitogen-Activated Protein Kinase Specifically in Adult Neurogenic Regions Impairs Contextual Fear Extinction and Remote Fear Memory. *Journal of Neuroscience*, 32(19), pp.6444–6455.

Parant, J. et al., 2001. Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nat Genet*, 29(1), pp.92–95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11528400.

Park, M.S. et al., 1999. p27 and Rb are on overlapping pathways suppressing tumorigenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 96(11), pp.6382–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=26890&tool=pmcentrez&rendertype=abstract.

Parras, C.M. et al., 2004. Mash1 specifies neurons and oligodendrocytes in the postnatal brain. *The EMBO journal*, 23(22), pp.4495–505. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15496983%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC526464.

Peretto, P. & Bonfanti, L., 2015. Adult neurogenesis 20 years later: physiological function vs. brain repair. *Frontiers in neuroscience*, 9, p.71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4351634&tool=pmcent

rez&rendertype=abstract [Accessed September 24, 2015].
Peretto, P. & Paredes, R.G., 2014. *Social Cues, Adult Neurogenesis, and Reproductive Behavior*, CRC Press/Taylor & Francis. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24830028 [Accessed March 23, 2017].
Pierce, A.A. & Xu, A.W., 2010. De novo neurogenesis in adult hypothalamus as a compensatory mechanism to regulate energy balance. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 30(2), pp.723–730. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3080014%7B&%7Dtool=pmcentrez%7B&%7Drendertype=abstract.
Pietsch, E.C. et al., 2008. The p53 family and programmed cell death. *Oncogene*, 27(50), pp.6507–6521.
Polager, S. & Ginsberg, D., 2003. E2F mediates sustained G2 arrest and down-regulation of Stathmin and AIM-1 expression in response to genotoxic stress. *Journal of Biological Chemistry*, 278(3), pp.1443–1449.
Polager, S. & Ginsberg, D., 2009. p53 and E2f: partners in life and death. *Nature reviews. Cancer*, 9(10), pp.738–748. Available at: http://dx.doi.org/10.1038/nrc2718.
Polager, S., Ofir, M. & Ginsberg, D., 2008. E2F1 regulates autophagy and the transcription of autophagy genes. *Oncogene*, 27(35), pp.4860–4864.
Pomerantz, J. et al., 1998. The Ink4a tumor suppressor gene product, p19(Arf), interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell*, 92(6), pp.713–723.
Ponti, G. et al., 2013. Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. *Proceedings of the National Academy of Sciences of the United States of America*, 110(11), pp.E1045-54. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3600494&tool=pmcentrez&rendertype=abstract.
Praag, H. van et al., 2002. Functional neurogenesis in the adult hippocampus. *Nature*, 415(6875), pp.1030–1034. Available at: http://search.proquest.com/docview/204514330?accountid=15618%5Cnhttp://sfx.is.cuni.cz/sfxlcl3?url_ver=Z39.88-2004&rft_val_fmt=info:ofi/fmt:kev:mtx:journal&genre=article&sid=ProQ:ProQ%3Ahealthcompleteshell&atitle=Functional+neurogenesis+in+the+adult+hippoc.
Van Praag, H., 2008. Neurogenesis and exercise: Past and future directions. *NeuroMolecular Medicine*, 10(2), pp.128–140.
van Praag, H., Kempermann, G. & Gage, F.H., 1999. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature neuroscience*, 2(3), pp.266–270.
Rakic, P., 1985. Limits of Neurogenesis in Primates. *Science*, 227(4690), pp.1054–1056.
Reynolds, B.A. et al., 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science*, 255, p.1707–10.
Reynolds, B.A. & Weiss, S., 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Developmental biology*, 175(1), pp.1–13.
Richards, L.J., Kilpatrick, T.J. & Bartlett, P.F., 1992. De novo generation of neuronal cells from the adult mouse brain. *Proceedings of the National Academy of Sciences*

of the United States of America, 89(18), pp.8591–5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=49966&tool=pmcentrez&rendertype=abstract.

Rietze, R.L. & Reynolds, B.A., 2006. Neural Stem Cell Isolation and Characterization. *Methods in Enzymology*, 419, pp.3–23.

Rother, K. et al., 2007. p53 downregulates expression of the G1/S cell cycle phosphatase Cdc25A. *Oncogene*, 26(13), pp.1949–1953.

Saavedra, H.I. et al., 2002. Specificity of E2F1, E2F2, and E2F3 in mediating phenotypes induced by loss of Rb. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research*, 13(May), pp.215–225.

Sage, J. et al., 2003. Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature*, 424(6945), pp.223–228.

Sage, J., 2012. The retinoblastoma tumor suppressor and stem cell biology. *Genes & development*, 26(13), pp.1409–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22751497%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3403009.

Sah, V.P. et al., 1995. A subset of p53-deficient embryos exhibit exencephaly. *Nature genetics*, 10(2), pp.175–180.

Schmid, P. et al., 1991. Expression of p53 during mouse embryogenesis. *Development (Cambridge, England)*, 113(3), pp.857–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1821855.

Seo, D.-O. et al., 2015. Adult Hippocampal Neurogenesis Modulates Fear Learning through Associative and Nonassociative Mechanisms. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 35(32), pp.11330–45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4532761&tool=pmcentrez&rendertype=abstract.

Seri, B. et al., 2006. Composition and organization of the SCZ: A large germinal layer containing neural stem Cells in the adult mammalian brain. *Cerebral Cortex*, 16(SUPPL. 1).

Sherr, C.J. & McCormick, F., 2002. *The RB and p53 pathways in cancer*, Available at: http://linkinghub.elsevier.com/retrieve/pii/S1535610802001022 [Accessed March 29, 2017].

Sherr, C.J. & Roberts, J.M., 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development*, 13(12), pp.1501–1512. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.13.12.1501 [Accessed March 29, 2017].

Shors, T.J. et al., 2012. Use it or lose it: How neurogenesis keeps the brain fit for learning. *Behavioural Brain Research*, 227(2), pp.450–458.

Shvarts, A. et al., 1996. MDMX: a novel p53-binding protein with some functional properties of MDM2. *The EMBO journal*, 15(19), pp.5349–57. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=452278&tool=pmcentrez&rendertype=abstract.

Sidman, R.L., Miale, I.L. & Feder, N., 1959. Cell proliferation and migration in the primitive ependymal zone; An autoradiographic study of histogenesis in the nervous system. *Experimental Neurology*, 1(4), pp.322–333.

Slack, R.S. et al., 1998. A critical temporal requirement for the retinoblastoma protein

family during neuronal determination. *Journal of Cell Biology*, 140(6), pp.1497–1509.

Snyder, J.S. et al., 2005. A role for adult neurogenesis in spatial long-term memory. *Neuroscience*, 130(4), pp.843–852.

Solá, S., Aranha, M.M. & Rodrigues, C.M.P., 2012. Driving Apoptosis-relevant Proteins Toward Neural Differentiation. *Molecular Neurobiology*, 46(2), pp.316–331. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22752662 [Accessed April 20, 2017].

Sultan, S. et al., 2015. Synaptic Integration of Adult-Born Hippocampal Neurons Is Locally Controlled by Astrocytes. *Neuron*, 88(5), pp.957–972.

Taylor, W.R. et al., 2001. p130/E2F4 binds to and represses the cdc2 promoter in response to p53. *Journal of Biological Chemistry*, 276(3), pp.1998–2006.

Tian, X. et al., 2011. E2F1 inhibits MDM2 expression in a p53-dependent manner. *Cellular Signalling*, 23(1), pp.193–200.

Toni, N. et al., 2007. Synapse formation on neurons born in the adult hippocampus. *Nature neuroscience*, 10(6), pp.727–734. Available at: http://www.nature.com/neuro/journal/v10/n6/full/nn1908.html.

Tropepe, V. et al., 1997. Transforming growth factor-alpha null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(20), pp.7850–7859.

Tsai, K.Y. et al., 1998. Mutation of E2f-1 Suppresses Apoptosis and Inappropriate S Phase Entry and Extends Survival of Rb-Deficient Mouse Embryos. *Molecular Cell*, 2(3), pp.293–304. Available at: http://linkinghub.elsevier.com/retrieve/pii/S1097276500802749 [Accessed March 29, 2017].

Vandenbosch, R. et al., 2016. RB regulates the production and the survival of newborn neurons in the embryonic and adult dentate gyrus. *Hippocampus*, 26(11), pp.1379–1392. Available at: http://www.ncbi.nlm.nih.gov/pubmed/27325572 [Accessed March 29, 2017].

Vanderluit, J.L. et al., 2004. P107 Regulates Neural Precursor Cells in the Mammalian Brain. *The Journal of cell biology*, 166(6), pp.853–63. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2172121&tool=pmcentrez&rendertype=abstract.

Vanderluit, J.L. et al., 2007. The Retinoblastoma family member p107 regulates the rate of progenitor commitment to a neuronal fate. *Journal of Cell Biology*, 178(1), pp.129–139.

Vescovi, A.L. et al., 1999. Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Experimental neurology*, 156(1), pp.71–83. Available at: http://www.sciencedirect.com/science/article/pii/S0014488698969989.

Veyrac, A. & Bakker, J., 2011. Postnatal and adult exposure to estradiol differentially influences adult neurogenesis in the main and accessory olfactory bulb of female mice. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 25, pp.1048–1057.

Vooijs, M. et al., 1998. Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. *Oncogene*, 17(1), pp.1–12. Available at:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9671308%5Cnhttp://www.nature.com/onc/journal/v17/n1/pdf/1202169a.pdf.

Wang, C. et al., 2006. Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. *Nature cell biology*, 8(9), pp.1025–1031.

Wichterle, H., García-Verdugo, J.M. & Alvarez-Buylla, A., 1997. Direct evidence for homotypic, glia-independent neuronal migration. *Neuron*, 18(5), pp.779–791.

Xing, C. et al., 2009. Role of oxidative stress and caspase 3 in CD47-mediated neuronal cell death. *Journal of Neurochemistry*, 108(2), pp.430–436.

Xiong, F. et al., 2011. Optimal time for passaging neurospheres based on primary neural stem cell cultures. *Cytotechnology*, 63(6), pp.621–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21858692 [Accessed April 6, 2017].

Xiong, S. et al., 2006. Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. *Proceedings of the National Academy of Sciences of the United States of America*, 103(Track II), pp.3226–3231.

Xu, X.L. et al., 2009. Retinoblastoma Has Properties of a Cone Precursor Tumor and Depends Upon Cone-Specific MDM2 Signaling. *Cell*, 137(6), pp.1018–1031.

Yang, A. et al., 2000. P73-Deficient Mice Have Neurological, Pheromonal and Inflammatory Defects But Lack Spontaneous Tumours. *Nature*, 404(6773), pp.99–103. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10716451.

Yau, S. et al., 2014. Physical Exercise-Induced Adult Neurogenesis: A Good Strategy to Prevent Cognitive Decline in Neurodegenerative Diseases? *BioMed Research International*, 2014, pp.1–20. Available at: http://www.hindawi.com/journals/bmri/2014/403120/ [Accessed March 22, 2017].

Yau, S.Y., Lau, B.W.M. & So, K.F., 2011. Adult hippocampal neurogenesis: A possible way how physical exercise counteracts stress. *Cell Transplantation*, 20(1), pp.99–111.

Yu, J.M. et al., 2006. Increase in proliferation and differentiation of neural progenitor cells isolated from postnatal and adult mice brain by Wnt-3a and Wnt-5a. *Molecular and Cellular Biochemistry*, 288(1–2), pp.17–28.

Zhang, Y., Xiong, Y. & Yarbrough, W.G., 1998. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, 92(6), pp.725–734.

Zhao, C. et al., 2006. Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(1), pp.3–11.

Zhao, M. et al., 2003. Evidence for neurogenesis in the adult mammalian substantia nigra. *Proceedings of the National Academy of Sciences of the United States of America*, 100(13), pp.7925–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12792021%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC164689.

Zheng, W., Nowakowski, R.S. & Vaccarino, F.M., 2004. Fibroblast growth factor 2 is required for maintaining the neural stem cell pool in the mouse brain subventricular zone. *Dev Neurosci*, 26(2–4), pp.181–196.

Ziebold, U. et al., 2001. E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. *Genes and Development*, 15(4), pp.386–391. Available at: http://www.genesdev.org/cgi/doi/10.1101/gad.858801 [Accessed March 29, 2017].