AMERICAN UNIVERSITY OF BEIRUT

STUDY OF THE COMBINED ROLES OF THE RB FAMILY OF POCKET PROTEINS DURING ADULT NEUROGENESIS IN THE OLFACTORY BULB

by
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I still remember the day I received my acceptance email to AUB as being the best day of my life. Little did I know that defending my thesis 2.5 years later would top that feeling.

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AN ABSTRACT OF THE THESIS OF

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Title: The Combined Roles of the Rb Family of Pocket Proteins During Adult Neurogenesis in the Olfactory Bulb

Adult neurogenesis (AN) is a restricted and highly coordinated process that produces adult-born neurons from a preserved pool of adult neural stem cells (aNSCs). These cells are located in the adult subventricular zone (aSVZ) lining the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus inside the hippocampus. Cell-cycle proteins were shown to be critically involved in brain development, particularly the regulation of embryonic neurogenesis. One such family is the Retinoblastoma (Rb) family of pocket proteins that is comprised of Rb, p107 and p130. We have recently shown that Rb specifically controls neural progenitor cells' (NPCs) proliferation in the aSVZ without affecting stem cell fate, and is needed for long-term survival of newborn neurons inside the OB and the dentate gyrus. In contrast, p107 was previously shown to negatively regulate adult NSCs self-renewal and promote progenitor commitment to a neuronal fate. Moreover, p130 is required to maintain a post-mitotic state and survival in mature cortical neurons in culture; however, its role in the adult brain is still unknown. Hence, pocket proteins clearly play distinct functions during neurogenesis; yet, they could also carry redundant functions manifested by compensatory mechanisms that are not revealed by studies in single knock-out (KO) models. Here, we show indeed that, compared with single-KOs, loss of all three pocket proteins (triple KO) in aNSPCs leads to additive effects with respect to enhanced stem cell self-renewal and progenitor proliferation but also to unique consequences associated with ectopic neuroblast migration as well as severe and pre-mature loss of immature neurons inside the aSVZ and RMS, prior to reaching the OB. Analysis of double KO mice, confirmed the distinct roles played by p107 and Rb in the control of stem cell self-renewal and progenitor proliferation, respectively. Moreover, it highlighted the central role played by Rb in neuronal survival given that one functional Rb allele is able to rescue the phenotype observed in TKO mice. On the longer term, loss of the Rb family is associated with premature exhaustion of the aNSCs pool in the aSVZ. This study uncovered unique and redundant functions played by pocket proteins during adult neurogenesis, which may have direct implications on regenerative medicine in the adult brain.

CONTENTS

FIGURES

ABBREVIATIONS

%: percent

+: positive

µL: microliter

µm: micrometer

℃: degrees Celsius

ACC: animal care committee

AC3: active caspase 3

AN: adult neurogenesis

aNSC: adult neural stem cell

aNSPCs: adult neural stem and progenitor cells

aSVZ: adult subventricular zone

BMP: bone morphogenic protein

BrdU: 5-bromo-2’-deoxyuridine

BSA: bovine serum albumin

CB: calbindin

CC: corpus callosum

CDK: cyclin-dependent kinase

CDKi: cyclin-dependent kinase inhibitor

CR: calretinin

DCX: doublecortin

DKO: double knockout

DP: dimerization protein

E: embryonic day

EPL: external plexiform layer

EYFP: enhanced yellow fluorescent protein

f: flox

FGF2: fibroblast growth factor 2

GABA: γ-aminobutyric acid

GC: granule cell

GCL: granule cell layer

GDNF: glial-derived nerve factor

GFAP: glial fibrillary acidic protein

GL: glomerular layer

HIF1α: hypoxia inducible factor 1 subunit alpha

IP: intra-peritoneal

IPL: internal plexiform layer

LGE: lateral ganglionic eminence

LV: lateral ventricle

MCL: mitral cell layer

min: minute

mL: milliliter

mM: millimolar

NeuN: neuronal nucleus

NSCs: neural stem cells

NSPCs: neural stem and progenitor cells

OB: olfactory bulb

OSN: olfactory sensory neuron

PBS: phosphate-buffered saline

PFA: paraformaldehyde

PGC: periglomerular cell

PSA-NCAM: polysialated neuronal-cell-adhesion molecule

PTEN: phosphatase and tensin homolog

Rb: retinoblastoma

RBC: Rb C-terminal domain

RBL: retinoblastoma-like

RBN: Rb N-terminal domain

RMS: rostral migratory stream

rRMS: rostral RMS

SGZ: subgranular zone

Shh: sonic hedgehog

shRNA: short hairpin RNA

SVZ: subventricular zone

SGZ: subgranular zone

TAM: tamoxifen

TD: transactivation domain

TH: tyrosine hydroxylase

THC: triple heterozygous control

TKO: triple knockout

VZ: ventricular zone

w: week

wpt: week post-treatment

YFP: yellow fluorescent protein

CHAPTER I

INTRODUCTION

A. Adult Neurogenesis

1. Historical perspective and importance

In the mid-1960s, Joseph Altman and colleagues identified, by autoradiography, the presence of undifferentiated dividing cells in the adult brain of mammals located in the subgranular zone (SGZ) of the hippocampus (Altman & Das, 1965). Three decades later, these mammalian cells turned out to be newborn neurons and the term coined for the process of their formation in the adult brain is adult neurogenesis (AN) (Reynolds & Weiss, 1992). This discovery caused a paradigm shift and overturned a long-held dogma that "Everything may die, nothing may be regenerated" (Ramon y Cajal, 1928). In fact, it was believed that the adult brain could no longer produce new neurons since neurons are post-mitotic cells and the existence of neural stem cells (NSCs) was not reported yet. Nearly 20 years after Altman's reports, Goldman and Nottebohm demonstrated that AN contributes to the addition of new neurons into the higher vocal center of songbirds and to song learning (Goldman & Nottebohm, 1983). Since then, AN has been reported in nearly every vertebrate species studied, including canary birds, lizards, zebrafish, and goldfish (reviewed in (Chapouton, Jagasia, & Bally-Cuif, 2007)). In invertebrates such as insects, AN has been also shown to occur in the mushroom bodies of the protocerebrum and in the optic lobes, both of which are involved in the processing of sensory information, mainly visual function (Cayre et al., 1996; Fernandez-Hernandez, Rhiner, & Moreno, 2013).

The existence of AN in adult human remains a controversial issue despite several reports and related studies over the past three decades. The first report of hippocampal AN in human came in the late 1990s where 5-bromo-2'-deoxyuridine (BrdU)-treated cancer patients showed cells in their dentate gyrus that had retained the label (Eriksson et al., 1998). BrdU is a thymidine analog that gets incorporated into the DNA of dividing cells during the S-phase and is later detected using immunohistochemistry. Further immunohistochemical analysis using stage-specific markers on post-mortem tissue of humans aged between 0 and 100 years also validated the presence of hippocampal AN (Knoth et al., 2010). Moreover, Sanai et al. reported the existence of a rudimentary rostral migratory stream (RMS) in humans indicating the presence of AN in the olfactory bulbs (OBs), similar to that of the murine brain. The RMS is an intermediate structure and migratory path that connects the sub-ventricular zone (SVZ) with the OB (Sanai et al., 2011). However, human OB neurogenesis quickly declines during the first two years of infancy and therefore, is not sustained throughout life. In addition to BrdU labelling, the high level of radioactive ^{14}C in the atmosphere from nuclear bomb testing allowed a retrospective birth dating of adult-born neurons in the human brain. Using ^{14}C labelling, Spalding et al. showed that around 700 adult-born neurons are added to the hippocampus daily (Spalding et al., 2013). Moreover, humans display unique SVZ-derived AN to the striatum (Ernst et al., 2014). Research in the field of AN is especially difficult in humans due to the limited availability of tissue given that *in vivo* studies rely mostly on the availability of post-mortem tissues. On the other hand, a recent study showed that hippocampal AN in humans declines shortly after birth, reaching undetectable levels by the age of 13 (Sorrells et al., 2018). However, subsequent studies demonstrated that the human hippocampus continues to

generate new neurons throughout life, where thousands of immature neurons were detected even at 90 years old (Boldrini et al., 2018; Moreno-Jimenez et al., 2019). The conflicting results could be due to the different staining techniques used and treatment methods between the studies. The development of non-invasive and highly precise methods to image the living human brain warrants to study AN with ease and in more depth under normal conditions and diseased states.

The discovery of AN has expanded our understanding of brain plasticity and capacity of regeneration. Thus, it is important to study the cellular and molecular mechanisms by which AN is regulated in order to unlock the therapeutic potential of adult neural stem cells (aNSCs) and enhance regeneration following injury or degeneration (Omais, Jaafar, & Ghanem, 2018).

2. Neurogenic niches

The neurogenic niche is a localized anatomical microenvironment that hosts aNSCs and possesses the proper intrinsic and extrinsic conditions such as regulatory factors and cues to support stem cell survival and proliferation (Mu, Lee, & Gage, 2010). These factors include access to vasculature, cerebrospinal fluid (in the SVZ via ependymal cells), regulatory hormones, and neurotrophic factors (Shohayeb, Diab, Ahmed, & Ng, 2018). In the adult mammalian brain, two canonical neurogenic sites are present: the adult SVZ (aSVZ) and the SGZ (**Figure 1**) (Ming & Song, 2011).

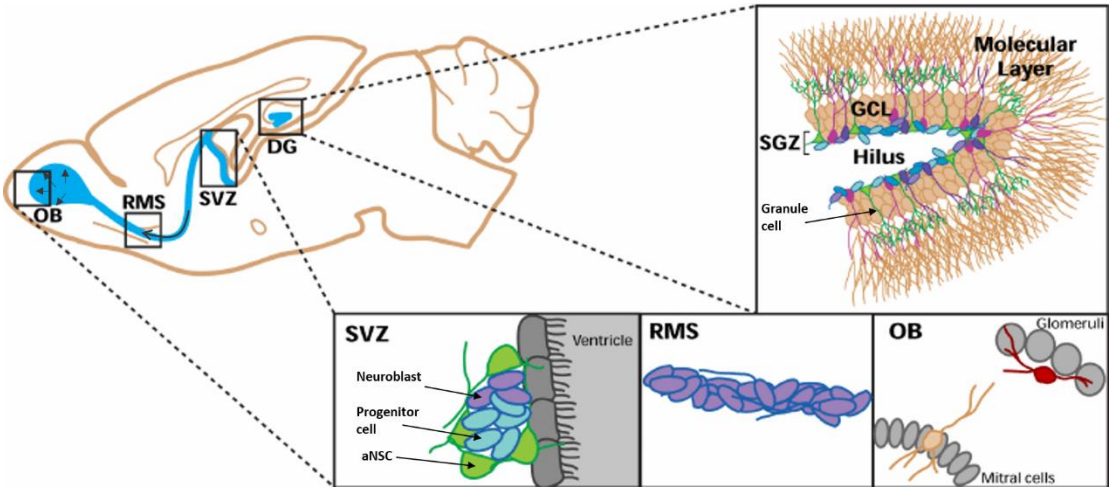


Figure 1: Neurogenic niches in the adult mouse brain. Sagittal section of an adult mouse brain showing the two neurogenic niches: the subventricular zone (SVZ) lining the lateral ventricles (LVs) and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus. The aSVZ produces neuroblasts that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into inhibitory GABAergic interneurons. aNSCs in the SGZ produce progenitors that slightly migrate apically and differentiate into glutamatergic granule cells in the granule cell layer (GCL) of the DG and extend their dendrites into the molecular layer. GCL=granule cell layer. As modified from (Johnson, Ables, & Eisch, 2009)

a. <u>SGZ</u>

The dentate gyrus of the hippocampus is made up of three layers: the outermost molecular layer, the middle granule cell layer (GCL), and the innermost polymorphic layer. aNSCs can be found in the SGZ below the GCL where they are known as type-1 radial glial-like cells due to their expression of the glial fibrillary acidic protein (GFAP). In addition, they express Nestin and the stem cell marker *Sox2* (Faigle & Song, 2013). Upon activation, aNSCs in the hippocampus divide primarily by asymmetric divisions giving rise to one stem cell and one progenitor cell, or type-2 cell. Eventually, they undergo consuming symmetric divisions and generate progenitor cells (Pilz et al., 2018). Type-2 cells lose their cell extensions and downregulate GFAP expression, and then divide a few times before becoming type-3 cells, or neuroblasts. Type-3 cells

express doublecortin (DCX) and polysialated neuronal-cell-adhesion molecule (PSA-NCAM). These cells migrate locally within the dentate gyrus into the GCL (Faigle & Song, 2013). Upon maturation, granule cells of the dentate gyrus become glutamatergic neurons and express the mature neuronal nucleus (NeuN) marker. Unlike granule cells of the OB which lack axons, these cells extend an axon and synapse with the pyramidal cells in the CA3 domain of the hippocampus and a dendrite into the outer molecular layer. Despite them being glutamatergic (glutamate isthe main excitatory neurotransmitter in the brain), the activation of granule cells in the hippocampus leads to the inhibition of the local circuit (Lepousez, Nissant, & Lledo, 2015). Moreover, adult born cells in the dentate gyrus play a role in pattern separation (Bakker, Kirwan, Miller, & Stark, 2008).

b. Adult SVZ

AN in the aSVZ is similar to that in the SGZ in that quiescent GFAP-expressing aNSCs give rise to transit-amplifying progenitors, which divide several times before committing to a specific lineage (Faigle & Song, 2013). Unlike hippocampal AN, neuroblasts leave the aSVZ and migrate rostrally through a tubular RMS made of astrocytes extensions into one of the OBs (Lim & Alvarez-Buylla, 2016; Ming & Song, 2005; Whitman & Greer, 2009). There, they migrate radially within the OB into either the GCL (97%) or the glomerular layer (GL; 3%) where they primarily mature into γ-aminobutyric acid (GABA)ergic inhibitory interneurons (**Figures 2 and 3**) [(described in detail in part B (Lim & Alvarez-Buylla, 2016; Lledo, Saghatelyan, & Lemasson, 2004)].

AN is a highly dynamic process that is affected by both intrinsic and extrinsic factors. Intrinsically, aNSCs and their progeny are regulated by a plethora of cell cycle proteins and transcription factors that form complex regulatory networks to ensure proper lineage progression (Omais et al., 2018). A number of epigenetic mechanisms also regulate AN, including microRNAs (miRNAs), other non-coding RNAs, and DNA methylases and demethylases (Gonzales-Roybal & Lim, 2013; Ming & Song, 2011; Park et al., 2014; Ramos et al., 2015). Extrinsic factors include morphogens such as bone morphogenic protein (BMP) and growth factors such as glial-derived nerve factor (GDNF) (Bond et al., 2014; Shohayeb et al., 2018). Moreover, environmental cues, physical exercise, and sensory enrichment were shown to play a role in modulating AN (Farioli-Vecchioli & Tirone, 2015). In comparison to the well-known role of adult born neurons in the dentate gyrus, the contribution of adult born granule cells to olfactory function is the subject of ongoing research. The latter cells modulate the function of existing neurons in their local circuit; they are required to improve discrimination between overlapping stimuli and contribute to tough discrimination tasks such as between similar odors, and minimize the generalization of contextual information (Sahay et al., 2011).

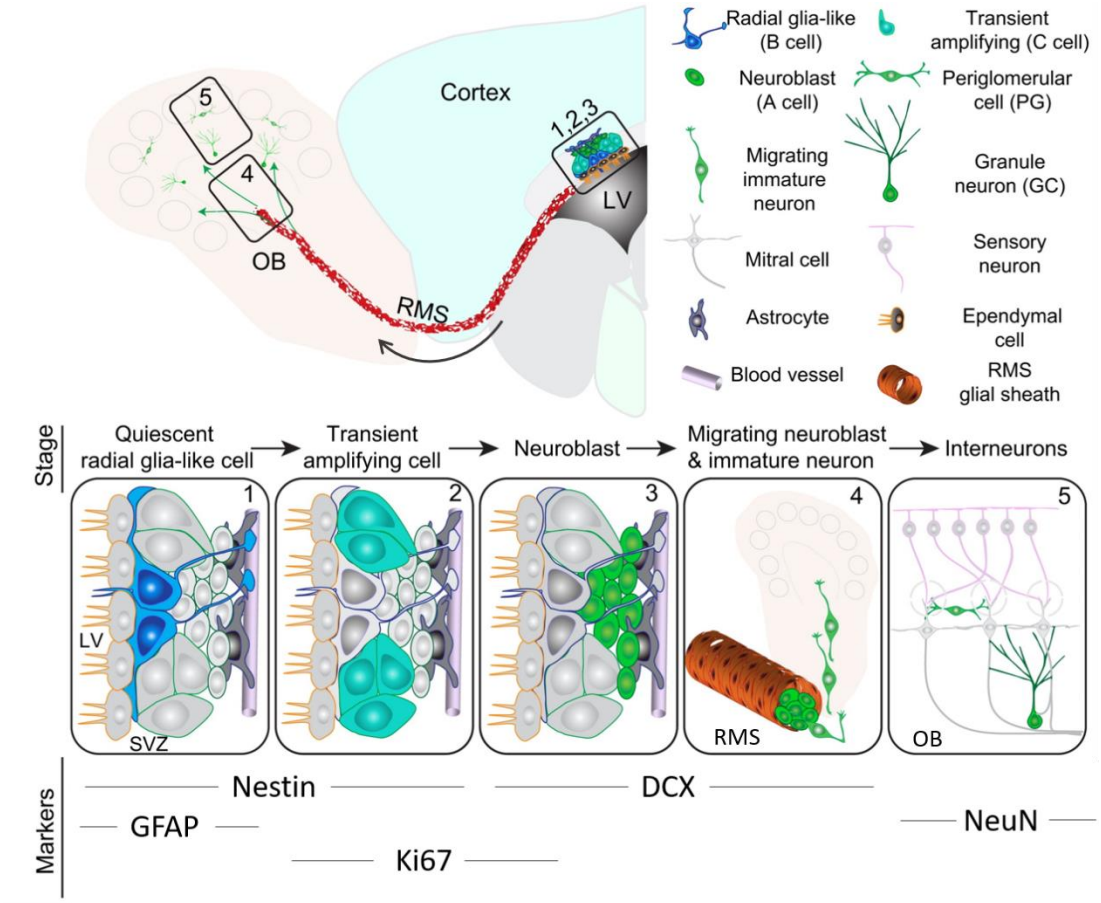


Figure 2: Adult neurogenesis in the aSVZ. GFAP-expressing quiescent radial glial-like aNSCs line the lateral ventricles (LV) with their extensions contacting the LV and the vasculature. These slowly dividing cells give rise to transient amplifying cells, which divide 3-4 times before expressing DCX and committing primarily to a neuronal fate. Neuroblasts then leave the aSVZ and migrate in a tubular rostral migratory stream (RMS) to the OB where they differentiate into inhibitory GABAergic interneurons. Mature adult born neurons express NeuN, in addition to other subtype-specific markers such as calbindin or calretinin. as modified form (Ming & Song, 2011).

c. Other neurogenic niches

Despite the restriction of aNSCs to the two canonical sites mentioned above, AN has been reported in several other brain regions. Historically, Altman reported cell proliferation in the cerebral cortex in rats (Altman, 1963). This observation was later confirmed by incorporation of ^{3}H-thymidine, a radioactive thymidine analog, in the cortex of rats (Kaplan, 1981). Since the discovery of AN in the aSVZ/OB system, it

remains controversial whether the aSVZ contributes to adult born neurons in the cerebral cortex under normal physiological conditions. The difficulty in proving cortical AN in healthy states could be due to an intrinsically low number of adult born neurons that migrate to the cortex making it difficult to detect their presence. Also, this could be due to the lack of a pro-survival environment for adult born neurons in the cerebral cortex, leading to the failure of integration into the local circuit and, therefore, cell death. However, AN into the cortex has been widely recognized to be damage-dependent, with several studies showing increased migration of adult born neurons into the cortex following stroke and traumatic brain injury (Ohira et al., 2010; Ohira, Takeuchi, Shoji, & Miyakawa, 2013; Sundholm-Peters, Yang, Goings, Walker, & Szele, 2005; Urrea et al., 2007). Moreover, Menn et al. showed that the adult aSVZ continuously produces oligodendrocytes that migrate to the corpus callosum (Menn et al., 2006). This migration increased following a demyelinating injury such as autoimmune encephalomyelitis (Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002). Although striatal AN in rodents was not reported under normal conditions, it was shown to occur following ischemic injury (Dayer, Cleaver, Abouantoun, & Cameron, 2005; Yang, You, & Levison, 2008). In contrast, aSVZ-derived AN in the striatum was reported in the human brain (Ernst et al., 2014).

3. Laminar organization of the OB

The processing of olfactory information is carried out by the olfactory epithelium, the OBs, and the associated olfactory cortices. The OE, which lines the nasal cavity, is composed of bipolar olfactory sensory neurons (OSNs) whose dendrites express a single type of olfactory receptors. The axons of the OSNs project through the

cribriform plate of the ethmoid bone to form the olfactory nerve (cranial nerve I). Each bundle of axons carrying olfactory information from the same type of OSNs converge and synapse with one or two glomeruli in the glomerular (or periglomerular) layer (GL) of the OB. Thus, glomeruli are functionally specific for a particular type of odorant molecule (Purves, 2012).

The OB has a laminar organization comprised of three cell nuclear layers separated by two plexiform layers (**Figure 3**). The axons of the OSNs synapse with the dendrites of the mitral and tufted cells. Around 25 dendrites are extended into each glomerulus, suggesting a more specific encoding of olfactory information. The cell bodies of the mitral cells form the mitral cell layer (MCL) that is separated from the GL by the external plexiform layer (EPL). The cell bodies of the tufted cells occupy the EPL. The axons of the mitral and tufted cells project out of the OB and form the lateral olfactory tract. These cells are the primary projection neurons of the OB and transduce olfactory information into the piriform cortex (Purves, 2012).

The largest layer of the OB is the GCL that is separated from the MCL by the internal plexiform layer (IPL). The GCL is composed mainly of inhibitory GABAergic interneurons known as granule cells. These interneurons are unique in that they lack axons but form two-way dendro-dendritic synapses with mitral and tufted cells (Lledo et al., 2004). They display GABAergic signaling that mediates local inhibition of the glutamatergic projection neurons. They also receive glutamatergic input from projection neurons along their dendro-dendritic synapses (Lledo et al., 2004). Functionally speaking, granule cells are responsible for the synchronization in the firing of mitral cells through lateral inhibition (Treloar, Miller, Ray, & Greer, 2010). The second group of interneurons in the OB are periglomerular cells found in the GL. Similar to granule

cells, periglomerular cells are GABAergic including a subpopulation of tyrosine hydroxylase (TH) positive dopaminergic interneurons (Whitman & Greer, 2009). These cells regulate olfactory information by forming synapses with the dendrites of mitral and tufted cells, and sometimes with axons of OSNs directly (Whitman & Greer, 2009). In addition, both granule and periglomerular cells receive cholinergic, noradrenergic, and serotonergic inputs from higher cortical areas which modulates odor codes and olfactory information based on certain behavioral demands (Lledo et al., 2004). The olfactory cortex includes the anterior olfactory nucleus (AON), the olfactory tubercle, the piriform cortex, and the entorhinal cortex (reviewed in (Wilson, Kadohisa, & Fletcher, 2006)). These regions are innervated by mitral and/or tufted cell axons via the lateral olfactory tract (LOT).

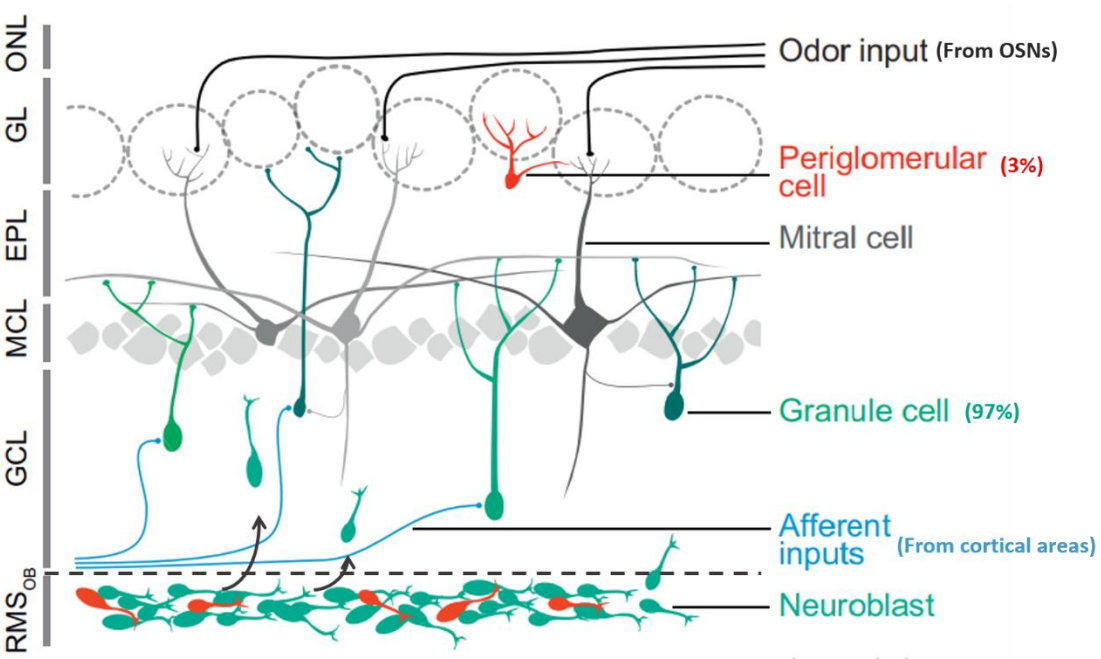


Figure 3: Laminar organization of the olfactory bulb. Olfactory sensory neurons (OSNs) coming from the olfactory epithelium through the olfactory nerve layer (ONL) synapse with dendrites of mitral and tufted cells in the glomeruli of the glomerular layer (GL) (tufted cells are not shown). Mitral and tufted cells constitute the primary projection neurons of the OB. Mitral cells occupy the mitral cell layer (MCL) whereas tufted cells are located mostly in the EPL. Granule cells and periglomerular cells are

mostly GABAergic interneurons that continuously inhibit the activity of the primary projection neurons. These former cells are continuously turned over through the process of AN. Granule cells occupy the GCL (about 97% of adult born interneurons), whereas periglomerular cells are found in the GL (about 3% of adult born interneurons). The rostral migratory stream (RMS) delivers neuroblasts from the aSVZ to the OB where they undergo radial migration and differentiate into adult born interneurons. Granule cells also receive afferent inputs from distant cortical regions that modulate their activity. As modified from (Breton-Provencher, Lemasson, Peralta, & Saghatelyan, 2009).

4. Functional significance of OB neurogenesis

Given that several subtypes of interneurons are generated from the aSVZ, potentially more complex sensory integration can be processed in the OB than in the hippocampus (Sahay et al., 2011). Interestingly, the OB is connected to the dentate gyrus via the amygdala and entorhinal cortex. This allows for the emotional association during the processing of olfactory information and potentially forms a feedback system by which AN in the OB may affect that of the hippocampus and vice versa (Kageyama, Imayoshi, & Sakamoto, 2012).

Adult born neurons in the OB are characterized by having a lower threshold for synaptic plasticity than the pre-existing ones (Nissant, Bardy, Katagiri, Murray, & Lledo, 2009). This property of adult born neurons is important for learning new odors and the discrimination between similar ones (Magavi, Mitchell, Szentirmai, Carter, & Macklis, 2005). Moreover, long-term potentiation, a form of strengthening functionally active synapses, is required for conditioning behaviors, and in the case of the OB, for aversive olfactory conditioning (W. Zhang, Tan, Atwood, & Martin Wojtowicz, 2010).

Previous studies have shown that AN is necessary for proper OB functioning. Although AN is not required for basic olfactory processing, the impairment of OB neurogenesis diminishes associative olfactory functions e.g. tough olfactory

discrimination tasks and olfactory-dependent behaviors (Breton-Provencher et al., 2009; Gheusi et al., 2000).

B. Types of cells in the aSVZ/OB system

1. aNSCs (Type B1 cells)

The lateral ventricles (LVs) in mammals are lined with thousands of aNSCs or type B1 cells that continuously generate newborn neurons, astrocytes, and oligodendrocytes throughout life (Lim & Alvarez-Buylla, 2016; Menn et al., 2006; Sohn et al., 2015). B1 cells are also referred to as glial-like stem cells due to their expression of several glial markers such as GFAP. Upon activation, aNSCs and their progenitors upregulate the expression of Nestin, an intermediate filament protein, which has been adapted in many transgenic models to study AN (**Figure 2**) (Lim & Alvarez-Buylla, 2016). The use of Nestin-driven recombination models such as Nestin-Cre allows for targeting stem cells and their progeny in the adult brain (Enikolopov, Overstreet-Wadiche, & Ge, 2015). B1 cells display a glial morphology and retain characteristics of their epithelial origin. They are characterized by an apical extension that contacts the ventricle and cerebrospinal fluid, and a basal process that contacts the brain vasculature (Mirzadeh, Merkle, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2008). B1 cells have a slower cell cycle compared to embryonic NSCs; thus dividing, on average, once every few days to weeks (Gotz, Nakafuku, & Petrik, 2016).

Moreover, B1 cells are a spatially heterogeneous population and display regional specification. Different subtypes of aNSCs generate distinct interneurons based on their location along the aSVZ (Merkle et al., 2014; Merkle, Mirzadeh, & Alvarez-Buylla, 2007). aNSCs located in the dorsal regions of the aSVZ produce TH+ periglomerular

cells (PGCs) and superficial granule cells, whereas the ventral regions generate Calbindin (CB)+ PGCs and granule cells (Merkle et al., 2007). Calretinin (CR)+ PGCs and granule cells are produced from anterior regions of the aSVZ (Merkle et al., 2007). This regional specification appears to be largely cell-intrinsic given that grafting and *ex vivo* analyses have shown that these cells retain their positional information even when extracted from the aSVZ (Lim & Alvarez-Buylla, 2016).

Several studies have investigated the cellular origin of aNSCs (Fuentealba et al., 2015; Furutachi et al., 2015). For instance, Furutachi et al. showed that between embryonic days E13.5 to E15.5, a subset of neural progenitor cells slows down their cell cycle and become quiescent. This is mediated by $p57^{Kip2}$, a cyclin-dependent kinase inhibitor (CDKi). This subpopulation remains largely quiescent until birth where it constitutes the pool of the future aNSCs (Furutachi et al., 2015). These studies showed that aNSCs are not residual cells that are left over from embryonic development but rather a specific subpopulation that is specified early on during development. Interestingly, Obernier et al. demonstrated using clonal barcoding studies that pre-B1 cells become regionally specified as early as E11.5 (Obernier et al., 2018). The exact mechanisms of regional specification are not yet known; however, it is likely to involve the action of epigenetics mechanisms and the combinatorial expression of different transcription factors.

Given the limited number of aNSCs available, it is essential to have several regulatory mechanisms that limit/control stem cell activation in order to sustain AN throughout life. Previous studies have assumed that B1 cells divide asymmetrically, generating another B1 cell and a progenitor cell (Bonaguidi et al., 2011). However, little evidence was presented to show the mode of division of B1 cells. Obernier et al.

demonstrated that B1 cells undergo symmetric divisions to either self-renew or produce two progenitors. Symmetric self-renewal of B1 cells maintains the pool of stem cells throughout life. However, only around 20% of B1 cells undergo symmetric self-renewal, whereas symmetric divisions that produce progenitor cells consume the majority. This leads to the age-dependent depletion in the numbers of aNSCs and decline in AN with time (Obernier et al., 2018). Of note, aNSCs in the aSVZ can remain quiescent for up to 4-5 months after undergoing a self-renewing division before being consumed (Obernier et al., 2018).

2. Transient-amplifying progenitors (Type C cells)

Following a consuming division, aNSCs give rise to transient-amplifying progenitor cells, or type C cells (**Figure 2**). These hyper-proliferative intermediate cells lose their radial extensions and divide several times before exiting the cell cycle, thus increasing the number of progenies generated from each aNSC. Type C cells divide an average of three times before committing to their respective lineages (Ponti et al., 2013). These cells are characterized by the expression of key transcription factors such as *Ascl1* and *Dlx2* (Lim & Alvarez-Buylla, 2016). Type C cells were recently shown to divide around once per day for 4 days before committing and becoming neuroblasts (Obernier et al., 2018). Moreover, they express the proliferation marker Ki67, which expression peaks during the S/G2/M phases of the cell cycle, allowing for the identification of these cells. Progenitor cells, much like B1 cells, are under the regulation of several intrinsic and extrinsic factors, mainly cell-cycle proteins (as discussed below) (Omais et al., 2018).

3. Neuroblasts (Type A cells)

After dividing few times, type C cells upregulate the expression of the microtubule-associated protein DCX and PSA-NCAM (Francis et al., 1999; Lim & Alvarez-Buylla, 2016). Henceforth, they become type A cells, or immature neuroblasts (**Figure 2**). DCX is essential for the stabilization of microtubules by forming links between them and actin filaments (Ayanlaja et al., 2017). Moreover, DCX is required for proper neuronal migration during development, and its deletion leads to severe migration defects and developmental disorders such as epilepsy and double cortex syndrome (Sossey-Alaoui et al., 1998). In studies performed on early post-natal mice, DCX was shown to be involved in determining the fate of progenitors, the maintenance of the morphology of type A cells, and their proper migration along the RMS (Belvindrah, Nissant, & Lledo, 2011; Koizumi et al., 2006).

Neuroblasts soon leave the aSVZ and migrate along the RMS to the OB in chains inside tubes formed from GFAP+ astrocytes and blood vessels (Lois, Garcia-Verdugo, & Alvarez-Buylla, 1996; Whitman & Greer, 2009). These tubes converge together forming the RMS that direct neuroblasts' migration into the OB. During their migration, type A cells may finally divide once or twice before existing the cell cycle and reaching the OB (Ponti et al., 2013). This migration takes about 2-7 days (Petreanu & Alvarez-Buylla, 2002). Although aNSCs can give rise to astrocytes and oligodendrocytes, most progenitors commit to a neuronal fate by becoming neuroblasts destined to differentiate into adult born OB neurons (Menn et al., 2006; Sohn et al., 2015).

4. Adult-born neurons

Upon reaching the OB, neuroblasts detach from the RMS and migrate radially into the two layers of the OB; mainly the GCL (around 97% of adult born neurons) and GL (around 3%) (**Figures 2 and 3**) (Breton-Provencher & Saghatelyan, 2012). During their maturation, neuroblasts downregulate the expression of the immature marker DCX and express mature neuronal markers such as NeuN. As mentioned earlier, adult born neurons lack axons and form dendro-dendritic synapses with mitral and tufted cells, where they mediate local inhibition in the olfactory circuit (Lledo et al., 2004).

Adult-born neurons take around 15 to 30 days before fully maturing. They can be divided into 5 classes based on the degree of their maturation (Petreanu & Alvarez-Buylla, 2002). Class 1 cells represent the immature neuroblasts entering the OB. Class 2 cells correspond to the radially migrating neuroblasts, whereas Class 3 neurons have begun extending their dendrites into the EPL. At this stage, these neurons begin receiving synaptic input from distant cortical regions. Upon forming functional dendro-dendritic synapses with mitral and tufted cells, these neurons are classified as Class 4 neurons, before becoming mature Class 5 cells characterized by full spine density and dendritic branching. The period of integration of adult-born neurons into the local circuit is significant because nearly half of incoming cells are eliminated (Petreanu & Alvarez-Buylla, 2002). This highlights the activity-dependent survival of adult-born neurons and the importance of sensory input (Gheusi, Lepousez, & Lledo, 2013).

C. The Retinoblastoma protein, pRb, and the control of adult neurogenesis

1. The Rb family of pocket proteins

The Retinoblastoma gene (Rb) was first discovered to be mutated in children carrying a rare form of cancer that develops in the retina called retinoblastoma. The presence of at least one functional Rb allele protects from the development of this form of cancer in humans (Huang et al., 1988). Thus, Rb is the first tumor suppressor gene to be discovered. Later, many other genes were shown to have tumor suppressive properties, such as p53 and phosphatase and tensin homolog (PTEN).

Rb belongs to a family of three proteins that consists of Rb itself and the Rb-like (RBL) proteins, p107 (RBL1) and p130 (RBL2). In mouse models, the inactivation of Rb alone is not sufficient to induce Retinoblastoma due to the functional compensation by p107 (Chen et al., 2004; J. Zhang et al., 2004). However, Rb inactivation alone is able to induce osteosarcomas and thyroid and pituitary tumors in mice (Cobrinik et al., 1996).

Although they play distinct or complementary roles, the Rb family members also share some structural similarities and can compensate for each other, thus displaying partial functional redundancy in specific contexts. The full-length mouse Rb is a 105 KDa protein that is structurally organized into three main domains, the Rb N-terminal domain (RBN), the pocket protein domain, and the Rb C-terminal domain (RBC), in addition to flexible inter-domain linkers (**Figure 4A**) (Dick & Rubin, 2013). The pocket protein domain, which gives this family its name, is divided into two subdomains: A and B. The B subdomain contains a conserved LXCXE binding cleft that is required for many of the functions of the Rb family members. Moreover, Rb binds to the transactivation domain (TD) of E2Fs through this pocket domain (pocket-E2F^{TD}) (C.

Lee, Chang, Lee, & Cho, 2002). The RBC is an intrinsically disordered domain that binds to the E2F/Dimerization Protein (DP) complex to induce a conformational change adopting a strand-turn-helix arrangement. This domain also interacts with other cell-cycle proteins such as the cyclin-dependent kinases (CDK) 2, 4, and 6 (Rubin, Gall, Zheng, & Pavletich, 2005).

The Rb family members, primarily Rb, regulate the cell cycle at the G1/S transition via their interaction with the E2F family of transcription factors (**Figure 4B**). The E2F family consists of 8 members: E2F1-8. E2F1-3a are considered transcriptional activators, since they activate gene expression, whereas E2F3b-8 are transcriptional repressors (Attwooll, Lazzerini Denchi, & Helin, 2004). Once expressed, the E2F members form heterodimers with DPs, and subsequently bind to DNA to modulate gene expression (Swiss & Casaccia, 2010). The Rb family proteins perform most of their functions by interacting with E2F1-5 (Chellappan, Hiebert, Mudryj, Horowitz, & Nevins, 1991). The inactivation of Rb is mediated by its hyperphosphorylation. Active Rb exists in a hypophosphorylated form that is bound to E2Fs. Thus, Rb inhibits cell cycle progression through the direct inhibition of E2F-mediated transcription of S-phase genes (Dick & Rubin, 2013). At the G1-to-S transition, Rb is hyperphosphorylated by several CDKs complexes which are activated by type D cyclins. CDKs are in turn regulated by the activity of CDKis which can be divided into two families: the Cip/Kip family and the Ink family. The Cip/Kip family is composed of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, whereas the Ink family is composed of $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, and $p19^{Ink4d}$. Different CDKs are inhibited by different CDKis. The Ink family inhibits Cdk4/6 at the G1/S transition, whereas the Cip/Kip family acts on all CDKs at every phase of the cell cycle (Frade & Ovejero-Benito, 2015).

2. Overview of the role of cell-cycle proteins in the control of neurogenesis

The development of adult neural stem and progenitor cells (aNSPCs) involves a tight regulation of the cell cycle, in order to ensure the correct balance between stem cell quiescence, maintenance, and consumption, in addition to progenitor proliferation. These cell cycle properties are directly regulated by cell cycle proteins including the Rb family, the E2F family, CDKs and CDKis (**Figure 4B**). In addition, several cell cycle proteins were shown to be involved in the regulation of cellular properties beyond cell cycle control, such as neuronal commitment, migration, differentiation, and survival (Herrup, 2013). Some of these non-cycling roles are mediated through the canonical pathways by which these proteins act, while other functions are carried through non-canonical pathways (as described in the next sections). Of note, some cell-cycle proteins including the Rb family remain indispensable for the long-term survival and proper functioning of mature neurons long after they exit the cell cycle. Given their spatiotemporal distribution, these proteins play tissue-specific roles (e.g. in SGZ versus SVZ) but are also subjected to temporal regulation (e.g. in embryonic neurogenesis versus AN). Moreover, they appear to function in a cell-type-specific manner, thus exerting their effects on specific cell types in the lineage during neurogenesis (**Figure 4C**) [reviewed in (Omais et al., 2018)].

3. Role of Rb in the control of neurogenesis

a. Role of Rb during embryonic development

Several studies have examined the role of Rb during embryonic neurogenesis. Early studies showed that a whole-embryo knockout of Rb exhibits lethality at

embryonic day 15 (E15.5) due to severe hematological and placental defects (Clarke et al., 1992; Jacks et al., 1992; E. Y. Lee et al., 1992). In comparison, the use of conditional brain-specific deletion of Rb (telencephalic-specific Cre recombination model) leads to the ectopic proliferation of neuroblasts in the cortex, along with lethality shortly after birth from respiratory difficulties associated with death of neurons in the pons and medulla (Ferguson et al., 2002). In addition, conditional deletion of Rb during embryonic development leads to severe migration and differentiation defects of specific GABAergic neurons born in the lateral ganglionic eminence (LGE) (Ghanem et al., 2012). In specific, the loss of Rb during development results in transcriptional repression of *Dlx1/Dlx2* genes, which are essential for the migration and differentiation of GABAergic interneurons in the brain. This repression is mediated by the upregulation of the repressor E2F7 which interacts with E2F-binding sites in the *Dlx* enhancers (Ghanem et al., 2012). Moreover, Rb regulates tangential migration of neural precursors in a cell-autonomous manner by inhibiting the activity of E2F3, which induces the expression of neogenin. High level of the latter protein was shown to impede migration in the absence of Rb *in vivo* (Andrusiak et al., 2011; McClellan et al., 2007).

In addition, Rb is essential for the survival of Reelin-expressing Cajal-Retzius neurons in the developing cortex given that its loss causes a reduction in the number of these neurons and a subsequent defect in radial neuronal migration during development (Ferguson et al., 2005). Our laboratory has also shown that Rb is essential for the regulation of progenitor cell proliferation, migration, differentiation, and survival of OSNs in the developing olfactory epithelium as well as the proper establishment of axonal connections between OSNs and mitral cells in the OB (Jaafar et al., 2016).

Finally, loss of Rb in the developing dentate gyrus leads to cell cycle deregulation manifested by an increased and ectopic progenitors' proliferation with no apparent effect on NSCs. This phenotype is linked to a delayed cell-cycle exit of newborn dentate granule cells, indicating a requirement of Rb in regulating the timing of cell-cycle exit in the developing dentate gyrus (Vandenbosch et al., 2016).

b. Role of Rb in adult neurogenesis

The use of germline or conditional deletion of Rb (and other cell-cycle proteins) may not be an effective method when studying the role of these proteins in AN given the birth lethality associated with loss of Rb. In addition, using such models makes it difficult to discern whether the observed phenotype is due to the specific loss of the protein in the adult brain or a secondary phenotype that was carried forward from embryonic development. To overcome these limitations, recent studies relied on inducible conditional knockout models (Cre-loxP systems) to target and delete the gene(s) of interest specifically at adult stage(s) (Dhaliwal & Lagace, 2011). Nuclear translocation of the Cre enzyme is controlled by tamoxifen (TAM) administration. Alternatively, other studies used stereotactic injection of lentiviruses expressing the *Cre* gene or a gene that codes for an shRNA to induce specific gene deletion or knock down (Enikolopov et al., 2015). As a result, the phenotypes observed in such models can be attributed to the specific function(s) of that protein in the adult brain.

i. Role of Rb in the aSVZ

Our laboratory has recently characterized the role of Rb in the regulation of AN in the aSVZ (Naser et al., 2016). Using an inducible Nestin-CreER^{T2} recombination

model, Rb deletion was induced in NSPCs and their progeny in both neurogenic niches. This study demonstrated that Rb specifically controls adult progenitor proliferation (and not stem cells) without affecting neuronal migration or terminal differentiation inside the OB. Hence, in the absence of Rb, there is a significant expansion of the progenitor pool that peaks at around 28 days post-TAM treatment in Rb mutant mice but is normalized to control levels between days 60 and 120 after TAM treatment (Naser et al., 2016). Interestingly, unlike the phenotype observed with the conditional deletion of Rb during development, Rb mutant mice did not show any major defects in rostral migration or terminal differentiation; neuroblasts were able to differentiate into mature GABAergic interneurons in both GL and GCL (Ghanem et al., 2012; Naser et al., 2016). However, the above increase in AN is only transient and is followed by the loss of Rb-null adult-born neurons in the OB by apoptosis starting around 60 days post-TAM. This study is the first to highlight a requirement for Rb as a negative regulator of progenitor proliferation in the aSVZ and critically needed for the long-term survival of newborn adult neurons in the OB (Naser et al., 2016). The control of adult progenitor proliferation by Rb in this context is likely to be mediated by E2F1, E2F3 and fibroblast growth factor 2 (FGF2) given that the levels of expression of all three transcripts are significantly increased *in vivo* after the deletion of Rb (unpublished data, Halaby N and Ghanem N). Moreover, p107 and p130 may be compensating for the loss of Rb in neuroblast migration and differentiation given that their expression levels were also increased in aNSPCs derived from the aSVZ in culture (unpublished data, Halaby N and Ghanem N).

ii. Role of Rb in the SGZ

Similar to its role during embryonic neurogenesis in the SGZ, Rb negatively regulates proliferation of late progenitors in the adult dentate gyrus and is required for the survival of immature neurons (Vandenbosch et al., 2016). The conditional deletion of Rb using a Nestin-CreER^{T2} in aNSPCs in the hippocampus did not affect NSCs' quiescence but significantly increased the numbers of late progenitors and immature neuroblasts and dramatically compromised the survival of newborn/mature dentate granule cells, indicating a conserved role for Rb in regulating AN in both the SGZ and the aSVZ (Naser et al., 2016; Vandenbosch et al., 2016). The role of Rb in the survival of adult-born granule neurons is partially mediated by an E2F1-dependent mechanism (Vandenbosch et al., 2016).

4. Role of p107 in the control of embryonic and adult neurogenesis

While Rb specifically controls progenitor proliferation and survival of post-mitotic neurons, p107 negatively regulates stem cell self-renewal and is needed for the commitment of progenitors to a neuronal fate in the adult brain (Vanderluit et al., 2004; Vanderluit et al., 2007). Mice having a germ-line knockout of p107 display a higher number of neural precursors and elevated levels of Notch signaling members such as *Hes1*, *Delta-like 1*, and *Notch1* inside the ventricular zone (VZ) (Vanderluit et al., 2004; Vanderluit et al., 2007). In fact, Notch signaling pathway is known to promote the maintenance of the neural precursor pool by promoting stem cell self-renewal (Ishibashi et al., 1994). Notably, the enhanced precursor pool was not associated with enhanced neurogenesis in the OB due to increased progenitor apoptosis around the LV (a balanced effect). Thus, p107 negatively regulates the self-renewal of stem cells during

embryonic development and this role is found to be conserved in the adult brain through the direct transcriptional repression of the *Hes-1* gene (Vanderluit et al., 2007). In addition, through the repression of Notch signaling, p107 was shown to promote cell-cycle exit of neural progenitors and commitment to a neuronal fate, as loss of p107 causes a decrease in the number of mature cortical neurons (Vanderluit et al., 2007).

Finally, p107 was shown to control the transcriptional repression of FGF2 through the inhibition of E2F3, thus regulating the pool of FGF2-responsive precursors in the developing brain (McClellan et al., 2009).

5. Role of p130 in the control of embryonic and adult neurogenesis

p130 is the least studied of the Rb family members, especially in the context of AN where its role is virtually unexplored. However, one study has shown that p130 is needed for the long-term survival of post-mitotic cortical neurons in culture. This role is mediated by the suppression of pro-apoptotic genes through the recruitment of chromatin modifiers such as Suv39H1 and HDAC1 via E2F4, which interacts with p130 in post-mitotic neurons (Liu, Nath, Chellappan, & Greene, 2005).

In conclusion, all the above studies demonstrate that the Rb family of pocket proteins carry unique and non-redundant functions in the control of embryonic and AN. In addition to their classical roles in cell cycle control, these proteins also have secondary, “non-cyclic” functions such as those directly implicated in the control of neuronal migration, differentiation, and survival.

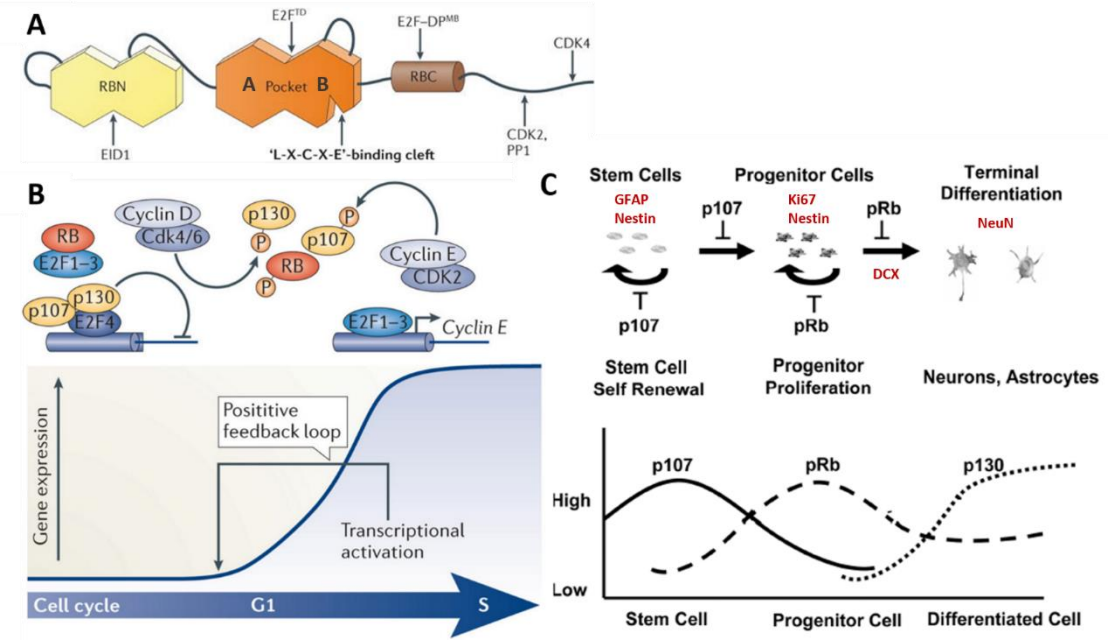


Figure 4: Regulation of AN by the Retinoblastoma (Rb) family. (A) The full-length Rb protein is comprised of an Rb N-terminal domain (RBN), a middle pocket protein domain that interacts with E2Fs including two subdomains, A and B, with the conserved LXCXE binding cleft, and an intrinsically disordered Rb C-terminal domain (RBC) which also interacts with E2Fs. **(B)** The Rb family is comprised of Rb itself, p107, and p130, all of which regulate cell cycle progression at the G1/S transition by interacting with the E2F family of transcription factors. Following CDK4/6-dependant Rb hyperphosphorylation, Rb detaches from E2F1-3s, thus allowing the transcriptional activation of cyclin E and other S-phase genes. Rb interacts with E2F1-3, whereas p107 and p130 primarily interact with E2F4-5. **(C)** In the context of AN, p107 is involved in the negative regulation of stem cell self-renewal through the inhibition of E2F3. Rb negatively regulates progenitor proliferation and is required for long-term survival of mature neurons. p130 is highly expressed in mature neurons where it may be involved in maintaining a post-mitotic state and helping in long-term survival. As modified from (Bertoli, Skotheim, & de Bruin, 2013; Dick & Rubin, 2013; Vanderluit et al., 2004).

D. The Rb/E2F Pathway

1. Overview of the E2F family

As described earlier, the E2F family of transcription factors consists of 8 members: E2F1-3a are transcriptional activators while E2F3b-8 are transcriptional repressors (Attwooll et al., 2004). Once expressed, the E2F members hetero-dimerize with a class of proteins known as dimerization proteins (DPs) and subsequently bind to DNA to modulate gene expression (Swiss & Casaccia, 2010). Pocket proteins perform

most of their cellular functions by interacting with E2F1-5 (Chellappan et al., 1991). By binding primarily to E2F1/2/3 through its pocket domain and RBC, Rb inhibits the activity of these E2Fs (Dick & Rubin, 2013). p107 interacts with E2F3a in a similar manner as that of Rb as well as with E2F4 and 5 (Dick & Rubin, 2013; Julian et al., 2013). On the other hand, p130 binds to the E2F4-DP and E2F5-DP complexes as well as other protein complexes such as MuvB to form the DREAM complex (Korenjak et al., 2004; Litovchick et al., 2007). DREAM represses genes involved in the cell-cycle and promotes quiescence and senescence (Hurford, Cobrinik, Lee, & Dyson, 1997; Moberg, Starz, & Lees, 1996). E2F6-8 are unconventional repressors in that they act in an Rb-independent manner (**Figure 5**). The functions of the different complexes in the context of AN are described below.

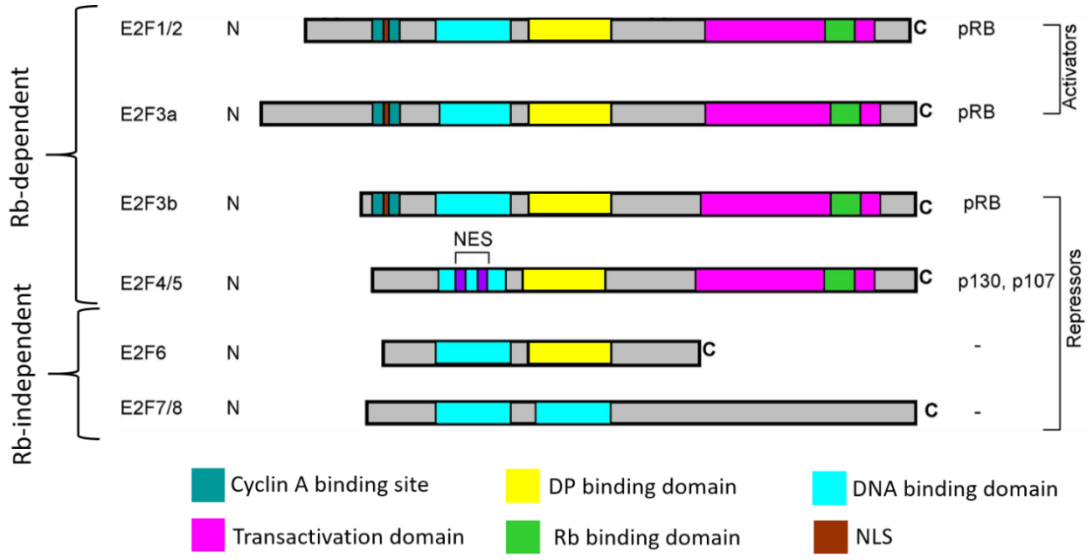


Figure 5: The E2F family of transcription factors. The E2F family consists of 8 members (E2F1-8). These are divided into transcriptional activators (E2F1,2,3a) and transcriptional repressors (E2F3b,4-8). E2F1-5 interact with pocket proteins whereas E2F6-8 act independently of Rb. The main domains of a typical E2F protein are: a DNA binding domain which allows binding of E2Fs to the gene promoters, a transactivation domain which includes an Rb binding domain that interacts with the pocket protein domain, and a DP binding domain by which E2Fs heterodimerize with DP and interact with the RBC domain of the Rb protein. As modified from (Wu, Zheng, & Yu, 2009)

2. Roles of E2Fs in regulating embryonic and adult neurogenesis

Several studies have addressed the roles of specific E2Fs in regulating neural stem and progenitor cells during development and/or in the adult brain. To begin with, E2F1-null mice exhibit significant impairment in AN in both the aSVZ and SGZ. The numbers of proliferating aNSPCs is nearly reduced by half in both neurogenic sites in $E2F1^{-/-}$ brains which results in significant decrease in the number of adult-born neurons in the OB and dentate gyrus (Cooper-Kuhn et al., 2002). Moreover, E2F1 was shown to be involved in the differentiation of oligodendrocyte progenitor cells through the transcriptional activation of cell-cycle proteins and chromatin-associated proteins (Magri et al., 2014).

The *E2F3* gene produces two protein isoforms, E2F3a and E2F3b, by alternative splicing, which act in opposing manner to regulate NSCs self-renewal by modulating the expression of the pro-neural gene *Sox2*. By interacting with p107, E2F3a directly represses *Sox2* expression, hence favoring neurogenesis over precursor self-renewal, whereas E2F3b activates *Sox2* expression and promotes precursor proliferation (Julian et al., 2013). These opposing roles of E2F3 isoforms act as a mechanism to fine-tune the balance between progenitor proliferation and differentiation (Julian et al., 2013). During development, the Rb/E2F3 complex is involved in regulating neuronal migration through the repression of neogenin expression, thus demonstrating a cell-cycle independent role for E2Fs (McClellan et al., 2007).

E2F4 is required for the proper development of the ventral telencephalon since E2F4-null mice display loss of the medial and lateral ganglionic eminences, as well as impaired precursor self-renewal. These defects are due to the disruption in Sonic

Hedgehog (Shh) signaling, which is essential for proper patterning of the ventral telencephalon (Ruzhynsky et al., 2007).

E2F7 and E2F8 are essential for embryonic development given that E2F7-8 double mutant embryos die at E11.5 and display massive p53-dependant apoptosis and dilation of blood vessels (Li et al., 2008). E2F7 can compensate for the loss of Rb in the inhibition of other E2Fs and is a key regulator of cellular senescence (Aksoy et al., 2012). Moreover, genome-wide analysis revealed that the hypoxia inducible factor 1 subunit alpha (HIF1α) forms a complex with E2F7 that leads to the transcriptional repression of neuropilin-1, a chemo-repulsive molecule. The inhibition of E2F7 leads to elevated levels of neuropilin-1 and deregulation in the axonal guidance of developing motor neurons in zebrafish *in vivo* (de Bruin et al., 2016). Whether this role of E2F7 is conserved in mammals requires further investigation. The unique role(s) of E2F8 are not fully understood, but E2F8 was shown to modulate cellular activity by repressing other E2Fs synergistically with E2F7 (Christensen et al., 2005; Li et al., 2008). The roles of the remaining E2Fs (E2F2 and E2F5) have not been characterized in the context of neurogenesis.

E. Rationale, Hypothesis, and Significance of the Study

Previous studies have explored the roles of the Rb family of proteins during AN by focusing mainly on the use of single gene knockouts (Naser et al., 2016; Vandenbosch et al., 2016; Vanderluit et al., 2004). Although these studies have shown that different pocket proteins act on distinct cell-types and thus carry distinct functions, it is likely that the presence of structural similarity between them may lead to partial functional compensation, which may in turn mask phenotypic consequences associated

with knocking out one protein at a time. Moreover, functional redundancy may reduce the extent of the observed phenotypes in single knock-out models. Thus, in order to better understand the functional interplay between all three pocket proteins, it is critical to examine their combined role(s) using double and triple knockout mouse models in comparison with single knock-outs. In fact, previous studies have utilized double and triple knockout models for the Rb family to study embryonic neurogenesis in specific brain regions and dissect the distinct versus redundant roles played by these proteins. Results showed that both Rb and p130 are required to prevent cell-cycle re-entry of retinal horizontal interneurons (Ajioka et al., 2007). Also, $Rb^{-/-}$; $p130^{-/-}$ mice do not develop retinoblastomas due to the ectopic upregulation of p107 which can compensate for their dual loss. However, the additional loss of one p107 allele leads to the development of metastatic retinoblastomas in $Rb^{-/-}$; $p107^{-/+}$; $p130^{-/-}$ (Ajioka et al., 2007). Interestingly, one intact Rb allele is sufficient for proper cell cycle exit in developing cortical excitatory neurons, since neurons lacking all three pocket proteins fail to exit the cell cycle before differentiating and remain in a proliferative state while activating the double-strand break repair pathway (Oshikawa, Okada, Nakajima, & Ajioka, 2013). In addition, Svoboda et al. showed that Rb is required for radial migration in developing cortical neurons and proper cortical lamination. This defect was enhanced in $p107^{-/-}$; $Rb^{-/-}$ mice, but was absent in single $p107^{-/-}$ brains, indicating a compensatory function of p107 in radial migration (Svoboda, Paquin, Park, & Slack, 2013). Based on the above studies, we have **hypothesized** that the Rb family members may carry compensatory, additive and/or synergistic functions during the regulation of AN in the aSVZ/OB system. In order to assess these interactions and dissect the functional relationships between distinct Rb family members, we studied the phenotypes displayed

by mouse models carrying double (DKO) and triple deletions (TKO) in these pocket proteins in the adult brain in comparison with triple heterozygote controls and single knock-outs. Using these models, the **aims** of the study are to assess **1)** self-renewal and proliferation of stem cells and progenitors in the aSVZ and RMS, **2)** the migration and differentiation of adult neuroblasts, **3)** the integration and survival of adult-born neurons inside the OB, in the absence of one or more pocket protein(s). The phenotypic analyses were carried in detail in DKO and TKO in comparison with triple heterozygote controls (THC), single Rb-KO and single p107-KO (as described by our earlier studies).

A better understanding of the various roles played by each pocket protein during cell cycle control and beyond will allow for manipulating the development of aNSPCs and their progeny, which is critical in regenerative medicine following brain injury or neurodegenerative diseases.

CHAPTER 2

MATERIALS AND METHODS

A. Generation of double and triple knock-out mice

The Animal Care Committee (ACC) at the University of Ottawa approved all animal procedures. Animal breeding and mating as well as sacrifice and brain dissection were carried at the laboratory of Dr. Ruth Slack at the University of Ottawa. The NestinCreER^{T2} transgenic FVB/NJ mouse line sued in this study is obtained from the laboratory of Dr. Suzanne Baker (Chow, Zhang, & Baker, 2008). The use of Nestin as a promoter allows for the conditional deletion of genes in aNSPCs. To generate double and triple Rb family knockout, NestinCreER^{T2} mice were crossed with Rb$^{flox/flox}$; p107$^{-/-}$; p130$^{flox/flox}$ mice bred on a mixed 129Sv/J:C57BL/6 background and obtained from the laboratory of Dr. Julien Sage (Wirt et al., 2010). Moreover, these mice were also mated with ROSA26-EYFP mice to serve as a reporter. *ROSA26* is a ubiquitously expressed promoter that is linked to the enhanced yellow fluorescent protein (EYFP) gene but separated from it by a stop codon flanked by 2 loxP sites (Srinivas et al., 2001). Upon TAM-induced Cre activation in aNSPCS, Cre translocates into the nucleus and recognizes the loxP sites and excises the DNA sequences between them. This leads to the generation of truncated Rb and/or p130 proteins, thus achieving DKO or TKO mouse models. In addition, the Cre-mediated excision of the stop codon in the EYFP cassette leads to yellow fluorescent protein (YFP) expression in aNSPCs.

B. Genotyping

Mice were weaned approximately 1 month after birth and small earpieces were collected for genotyping. DNA extraction was performed using a classic Phenol-Chloroform-Isoamyl alcohol (Sigma, 77617) extraction method. Screening was done by PCR using the following primers:

- Nestin-Cre primers: forward 5' GAACCTGATGGACATGTTCAGG 3' and reverse 5' AGTGCGTTCGAACGCTAGAGCCTGT 3'.
- ROSA26-YFP: forward wt 5'AAAGTCGCTCTGAGTTGTTAT 3', forward mutant 5' GCGAAGAGTTTGTCCTCAACC 3', and reverse 5' GGAGCG GGAGAAATGGATATG 3'.
- Rb primers: forward 5' CTCATGGACTAGGTTAAGTTGTGG 3'and 5'GCATTTAATTGTCCCCTAATCC3'.
- p107 primers: forward 5' CATGAACAGACTTGTCATTCCAC 3' and reverse-Neo 5'GCACGAGACTAGTGAGACGTGC3' and reverse-common 5'TCGCTGGCAGTCAGAG3'.
- p130 primers: forward 5' GTGTTGTAACATTCTCGTGGG 3' and reverse 5'GACTGCTGGTATTAGAACCC 3'

C. Tamoxifen treatment

Mice belonging to the triple knockout (TKO) and triple heterozygous control (THC) groups were given a solution of 50 mg/mL of TAM (Sigma T5648) by oral gavage for 4 consecutive days based on their body weight (150 mg/kg). Mice belonging to the double knockout (DKO) group received TAM via intra-peritoneal (IP) injections

for 4 days (180 mg/kg). Animals were sacrificed in two groups after two survival periods; 4 weeks and 8 weeks post-treatment.

D. Tissue preparation and cryo-sectioning

Mice were anesthesized with a solution of 1.5 µL/g of ketamine and 0.25 µL/g of Xylazine before cardiac perfusion. Mice were perfused with around 20mL of cold 1x phosphate-buffered saline (PBS) then with the same amount of cold 4% paraformaldehyde (PFA; Himedia RM3660-500G) freshly prepared. Brains were then dissected and kept overnight in 4% PFA to ensure complete fixation. Brains were then dehydrated in a 20% sucrose solution prepared in 1x PBS for 2 days followed by 30% sucrose solution for 4 consecutive days at least. Brains were shipped from the University of Ottawa to AUB in 30% sucrose. Brains were cut in half across the mid-sagittal line and frozen in a cold isopentane solution on dry ice at -35℃ for 30 seconds each. They were then embedded in tissue freezing medium (Leica, 14020108926) and sectioned using a Leica cryostat at -25℃ (Leica, CM1850). 8 µm sagittal sections were mounted on SuperFrost slides (Thermo Scientific, J1800AMNZ) and stored at -80℃.

E. Immunohistochemistry

Frozen sectioned (-80℃) were removed and warm up at room temperature for at least 30 minutes. Slides were washed with 1xPBS for 7 mins to dissolve embedding material, then blocked for 1-2 hours in a blocking solution comprised of 1% Bovine Serum Albumin (BSA, Sigma A2153), 5% donkey serum (Abcam ab7475), 0.3% Triton-X (Amresco, M143), and 0.1M PBS. For Ki67 staining, slides were treated for 10 mins in a 10mM sodium citrate antigen retrieval solution (Fisher BioReagents

BP327) at 95℃ then washed for 7 mins in 1x PBS. The slides were then incubated in primary antibody solution prepared in blocking solution at room temperature overnight. The next day, slides were washed 3 times in 1x PBS solution to remove excess primary antibody, and then incubated with a secondary antibody solution prepared in blocking solution without BSA for 1 hour. The slides were washed again 3 times with 1x PBS and mounted using a (3:1) PBS:Glycerol solution. The following primary antibodies were used: chicken anti-GFP (1:1000; Abcam ab13970), rabbit anti-Ki67 (1:400; Cell Marque 275R-15), goat anti-DCX (1:500; Santa Cruz sc-8066), rabbit anti-AC3 (1:500; Cell signaling D175), mouse anti-NeuN (1:300; Millipore MAB377), chicken anti-Nestin (1:100; Abcam ab134017), goat anti-GFAP (1:50; Santa Cruz sc-6170). The following secondary antibodies were used: donkey anti-chicken 488 (1:400; Jackson 703-545-155), donkey anti-rabbit Cy3 (1:200; Jackson 711-165-152), donkey anti-goat Cy5 (1:200; Jackson 705-605-147), donkey anti-mouse 647 (1:200; Jackson 715-605-150), donkey anti-chicken Cy3 (1:200; Jackson 703-165-155), donkey anti-rabbit 488 (1:200; Jackson 711-545-152). Hoechst dye (1:1000) was added to all slides with the secondary antibodies.

F. Imaging and data analysis

Images were taken on a Leica DM6B upright fluorescent microscope. Cell counts were made for every 4th section, for a total of 3 sections per brain per genotype. All genotypes were counted in triplicates (3 brains/genotype) except for 4w-DKO brains which were counted in duplicates. For OB counts, two representative areas of 299168.975 μm^2 were taken from the GCL and one from the GL. All cell counts and

image processing were done manually using the ImageJ software. Cell counts were statistically analyzed using independent sample t-tests on Microsoft Excel.

CHAPTER 3

RESULTS

A. The Rb family of pocket proteins negatively regulates cell proliferation in the aSVZ

We have previously shown that the loss of Rb in aNSPCs in the aSVZ leads to a significant increase in progenitor proliferation without affecting stem cell self-renewal (Naser et al., 2016). To investigate the combined roles of all pocket proteins in the control of AN, we examined the phenotypes of young adult mice carrying, in combination, double or triple deletions in these proteins. To do so, p107-null mice carrying Rb- and p130-double floxed alleles ($p107^{-/-}$; $Rb^{f/f}$; $p130^{f/f}$) are mated with Nestin-$CreER^{T2}$;Rosa26-YFP mice. The Nestin-$CreER^{T2}$ system allows for the conditional deletion of the target gene(s) specifically in activated Nestin-postive aNSPCs. Upon TAM treatment, the Cre recombinase translocates to the nucleus and Cre-mediated excision of the Rb and p130 floxed alleles takes place, in addition to the removal of a stop codon leading to YFP expression as a reporter gene. This treatment leads to the generation of triple knockout ($p107^{-/-}$; $Rb^{-/-}$; $p130^{-/-}$) mice, referred to as (TKO) as well as triple heterozygous mice ($p107^{+/-}$; $Rb^{+/-}$; $p130^{+/-}$) or (THC) that will serve as controls. Moreover, double knockout mice for p107 and p130 carrying one functional Rb allele and designated as ($p107^{-/-}$; $Rb^{+/-}$; $p130^{-/-}$) or (DKO) are generated in order to assess for distinct versus compensatory functions among these proteins. 2-month-old adult mice were treated and sacrificed at either 4 weeks (4wpt) or 8 weeks post-TAM treatment (8wpt). These timepoints were chosen owing to the fact that the process of AN from the SVZ to the OB takes until full maturation of adult-born neurons

takes about a month (4 weeks) (Obernier et al., 2018; Petreanu & Alvarez-Buylla, 2002). The 8-week timepoint represents a longer timeframe to study the effects of the loss of the Rb family. Of note, TAM treatment was performed over 5 days by IP in DKO mice and, in a separate experiment, by oral gavage in TKO and THC mice. Following treatment, adult brains are dissected, cryo-protected and sectioned as described in detail under material and methods. Animal breeding and mating as well as TAM treatments and brains dissection were carried at the laboratory of our collaborator, Dr Ruth Slack, at Ottawa University. For phenotypic analyses, double and triple immunostaining was performed on frozen brain sections and phenotypic assessment done on rostral, medial and caudal levels. However, the results shown and described here in detail correspond to medial level given the consistency of the phenotype along the rostro-caudal axis.

To assess cell proliferation, we performed a double immunostaining using antibodies against the proliferation marker Ki67 in combination with YFP. At medial level and compared with THC, DKO and TKO mice show significant increase in proliferating cells (YFP+;Ki67+) with the highest number scored with the latter group. In fact, loss of all three pocket proteins leads to the formation of ventricular heterotopia, as the aSVZ is largely thicker in TKO brains compared to THC brains (**Figure 6A-A'', B, B''and F-F''**). Hence, compared with THC, TKO mice display 5.56-fold increase in the numbers of YFP-positive (319 ± 133 in THC versus 1775 ± 429 in TKO) and 21.25-fold increase in (YFP;Ki67) double positive cells (8 ± 3 in THC versus 170 ± 23 in TKO) at 4 weeks post-treatment (wpt) (**Figure 6B-C'', L**). This increase in cell proliferation is consistent at 8wpt, where we found in TKO mice 2.99-fold increase in YFP-positive cells (245 ± 33 in THC compared to 734 ± 282in TKO) and 7.28-fold

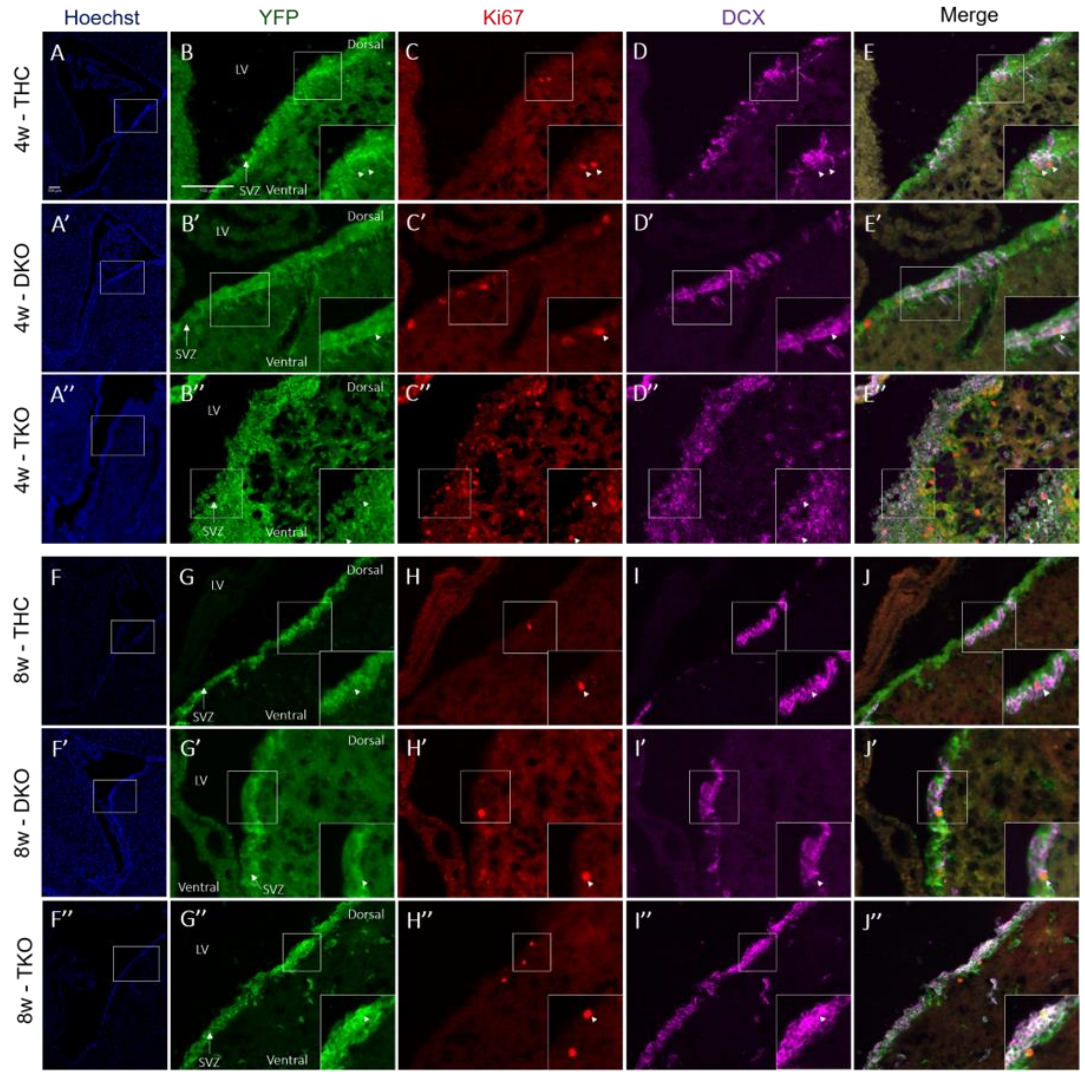
increase in (YFP;Ki67) double positive cells (7 ± 1 in THC compared to 51 ± 16 in TKO) (**Figure 6G-H'', K**). Of note, we detected a noticeable decline in the absolute numbers of both YFP+ and (YFP+Ki67+) cell populations between 4 wpt and 8 wpt, which is probably due to premature exhaustion of aNSPCs pool (**Figure 6K**). To assess whether this increase in cell proliferation is proportional or not, we calculated the proliferation index which is the ratio of the (YFP+Ki67+) double positive population over the total YFP-positive population. This ratio is significantly higher in TKO compared with THC at 4 wpt (0.02 in THC versus 0.1 TKO) but not at 8 wpt (0.03 in THC versus 0.08 in TKO), indicating the presence of an extended proliferation period affecting one or more cell type(s) along the neural lineage at 4 wpt (**Figure 6M**).

Similarly, in DKO compared to THC brains, we observed 3.25-fold increase in the number of dividing progenitors (YFP+;Ki67+) (8 ± 3 in THC versus 26 ± 10 in DKO) and a significantly higher proliferative index (0.02 in THC versus 0.11 in DKO brains) at 4 wpt (**Figure 6C, C', K, M**) but not at 8 wpt (**Figure 6J, J', K, M**). This increase in progenitor proliferation in DKO brains is likely due to an indirect effect carried forward from the loss of p107 in stem cells, which was previously shown to negatively regulate stem cells' self-renewal (Vanderluit et al., 2004).

Next, to assess whether this enhanced proliferation affects late progenitors/immature neuroblasts, we quantified the number of neuroblasts that are still dividing and corresponding to the (YFP+Ki67+DCX+) triple positive cell population. We found a significantly greater number of proliferating neuroblasts in TKO compared to THC at 4 wpt (4 ± 2 in THC versus 81 ± 24 in TKO). However, the ratio of this population over the total number of neuroblasts (YFP/Ki67/DCX over YFP/DCX) was higher but the increase was not statistically significant (0.04 in THC versus 0.07 in

TKO) (**Figure 6E-E'', K, M**). These results are consistent at 8 wpt (**Figure 6J-J'', K, M**), indicating proportional expansion and delayed cell cycle exit in this population in the absence of all pocket proteins. On the other hand, we detected a lower number of (YFP+;Ki67+DCX+) triple positive cells in DKO compared with TKO, yet the ratio of this population in the former group is still larger than the one seen in THC (0.04 in THC versus 0.17 in DKO) (**Figure 6E, E', K, M**).

Altogether, the above results show that the Rb family of pocket proteins, typically p107 and Rb, negatively regulates cell proliferation. Compared with p107 and Rb single KOs, the combined loss of all three proteins causes further expansion in the overall progenitor population including dividing neuroblasts, which is probably due to, at least, an additive effect.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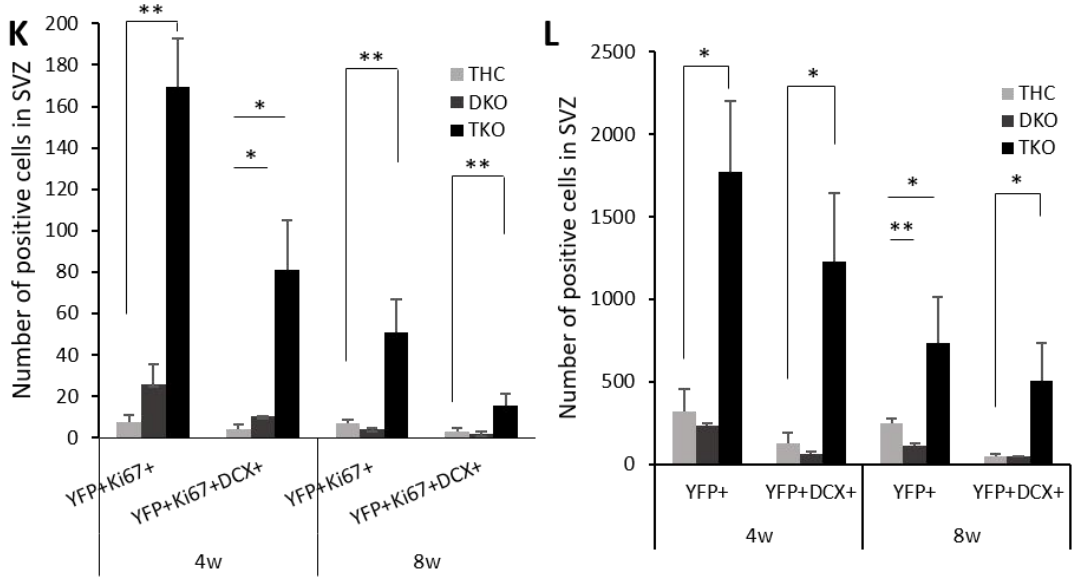
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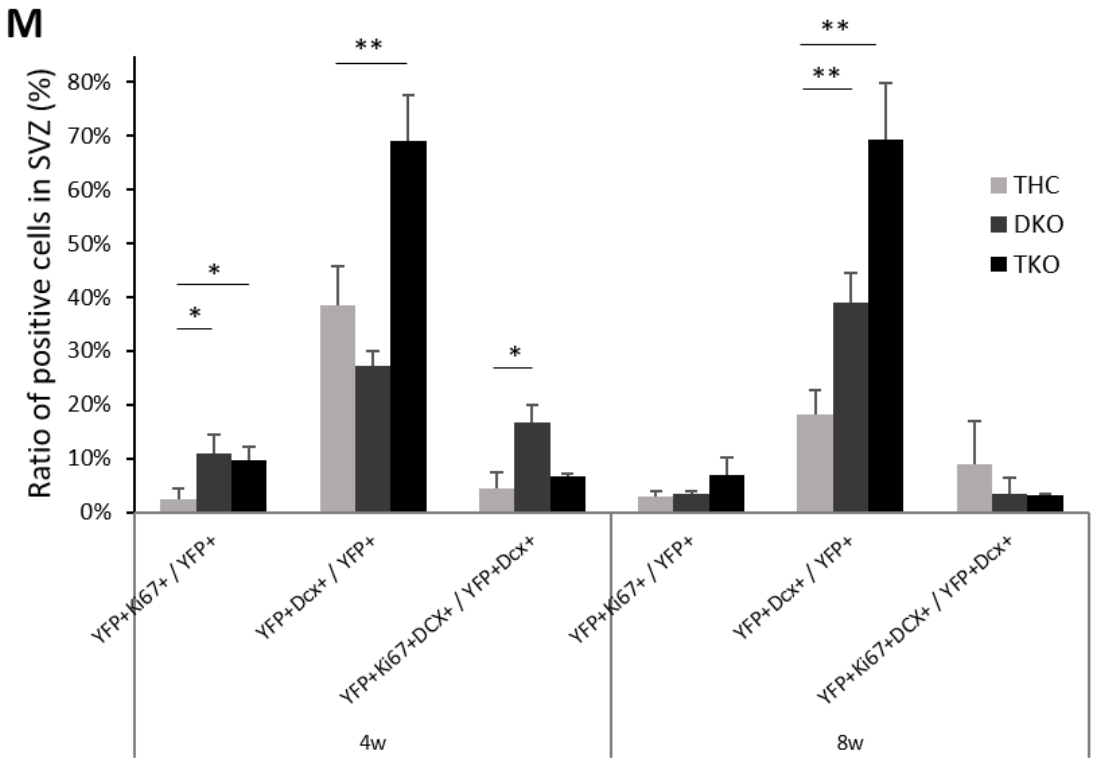
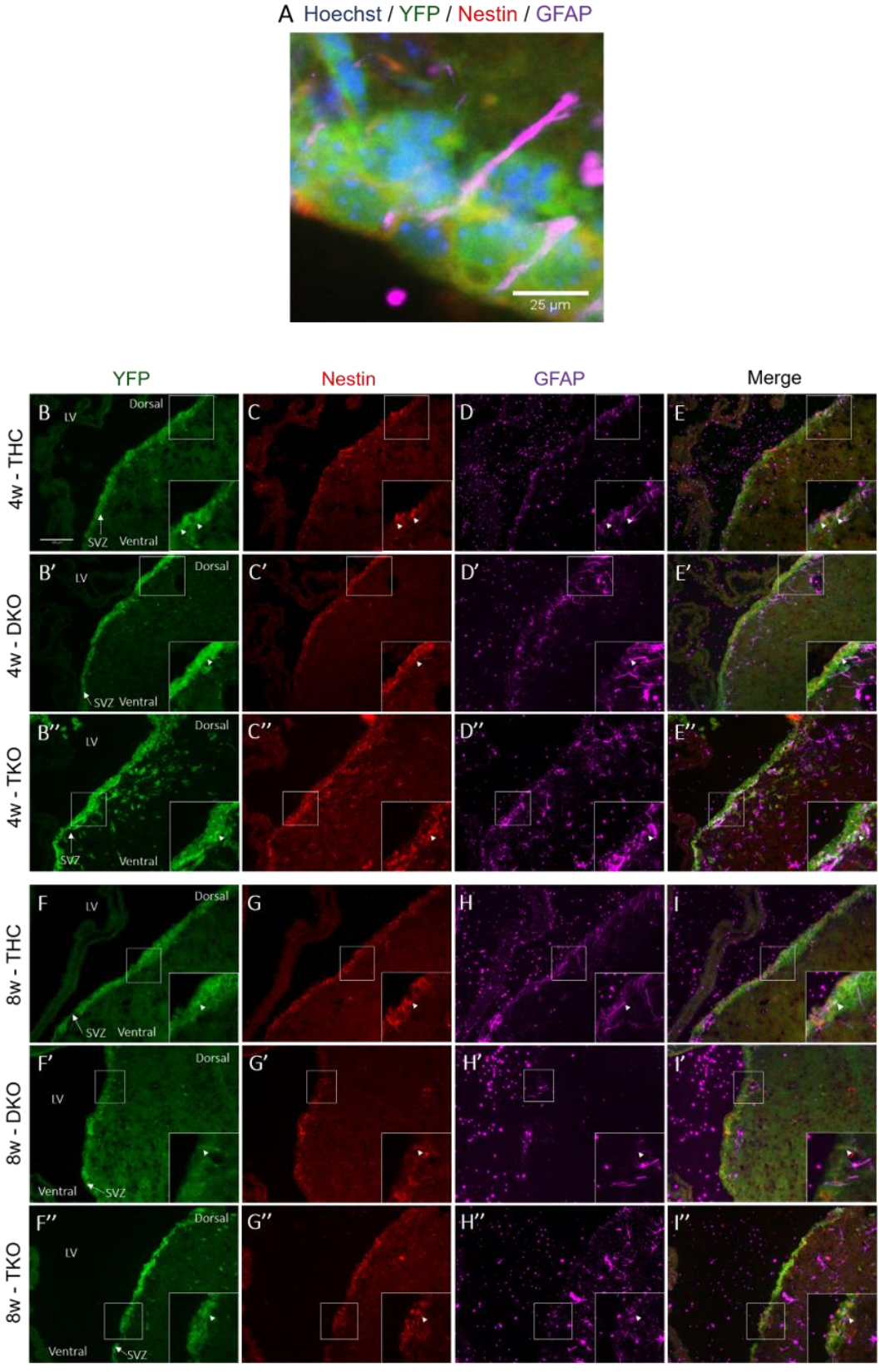


Figure 6: Increased cell proliferation and neuronal commitment in the aSVZ in the absence of pocket proteins. (A-A'', F-F'') Hoechst staining performed on mouse sagittal brain sections at medial level in the aSVZ. **(B-E'', G-J'')** Triple immunostaining of mouse sagittal sections for YFP, Ki67, and DCX at 4 wpt in THC **(B-E)**, DKO **(B'-E')** and TKO **(B''-E'')**, as well as at 8 wpt in THC **(G-J)**, DKO **(G'-J')** and TKO **(G''-J'')**. Insets in **(B-E'', G-J'')** show higher magnifications pictures of the indicated regions in the aSVZ. Arrowheads in **(B-E'', G-J'')** show triple positive (YFP+Ki67+DCX+) cells in the aSVZ. **(K, L)** Graphs showing cell counts of the populations of YFP+ cells co-stained with different markers in the aSVZ at 4wpt and 8wpt in THC, DKO, and TKO brains. **(M)** Ratios of the different cell populations quantified in the aSVZ. Error bars indicate the standard deviation from n=3 animals per genotype at 4 wpt and 8 wpt with the exception of n=2 DKO at 4 wpt. Asterisks represent statistically significant results obtained from independent sample t-tests: (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar = 100 µm. LV = lateral ventricle, SVZ = subventricular zone.

B. p107 is the main pocket protein that negatively control stem cells' self-renewal in the aSVZ

To determine whether the increase in the number of proliferating (Ki67+) cells is due to an expanded pool of stem cells and not only progenitor cells, we co-

immunostained sagittal brain sections for YFP, GFAP and Nestin. GFAP is a marker of mature astrocytes but also labels type B1 cells, which are radial glial-like stem cells (**Figure 7A)**. Once activated, stem cells upregulate the expression of Nestin, which is a type VI intermediate filament protein that is also expressed in all derived progenitor cells. Since Cre-ER^{T2} is under the control of the Nestin promoter and enhancer, only activated aNSCs and their progeny will be recombined and thus, express YFP. Our results showed a significant increase in the size of aNSPC population which is represented by (YFP+Nestin+) double positive cells in TKO brains compared with THC brains at 4 wpt (119 ± 35 in THC versus 373 ± 19 in TKO brains) (**Figure 7B-E'', J**). A similar increase in this population was observed in DKO compared with THC at 4 wpt, although not significant (119 ± 35 in THC versus 178 ± 17 in DKO brains) (**Figure 7J**). Moreover, the number of stem cells represented by (YFP+GFAP+Nestin+) triple positive population was significantly increased by 8.75 fold and 2.7 fold in TKO and DKO compared to THC brains at 4 wpt, respectively (33 ± 8 in THC versus 289 ± 7 in TKO and 89 ± 24 in DKO brains) (**Figure 7J**). Accordingly, the proliferative ratios of the number of (YFP+GFAP+Nestin+) triple positive cells over the total number of (YFP+Nestin+) double positive cells was significantly higher in TKO and DKO brains compared to THC brains at 4wpt (0.28 in THC versus 0.78 in TKO and 0.5 in DKO brains) (**Figure 7K**). In contrast, at 8 wpt, the number of stem cells was quite decreased in TKO compared with THC and the respective ratios of the stem cell populations in the two genotypes are normalized, indicating a fast exhaustion of the stem cells' pool. (**Figure 7F-I'', 7J and 7K**). The above results indicate that p107 is the primary regulator of adult NSCs self-renewal in the aSVZ as was previously shown (Vanderluit et al., 2004).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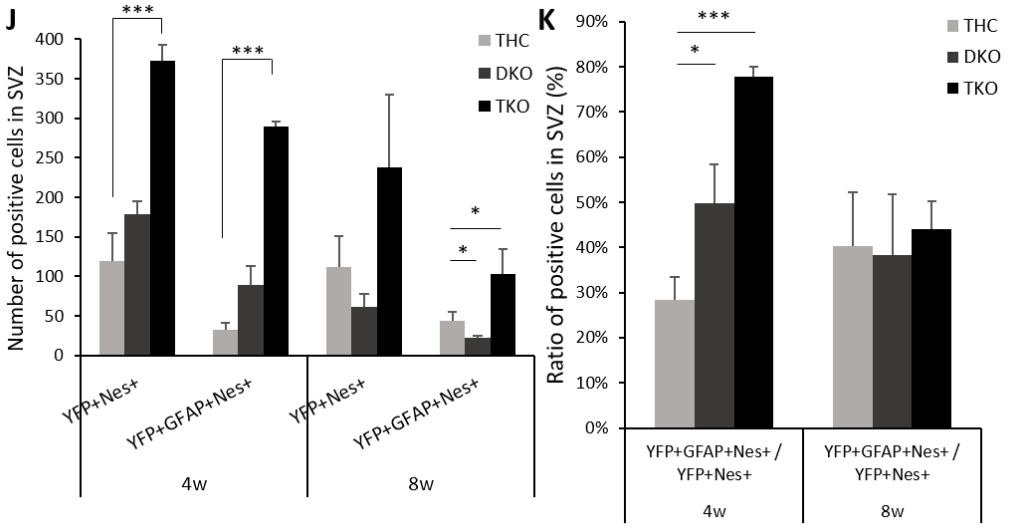


Figure 7: p107 but not Rb or p130 negatively controls stem cells' self-renewal in the aSVZ. (A) Panel is showing two aNSCs inside the aSVZ: each cell body extends GFAP+ processes that contact both the ventricle and the vasculature at the apical and basal levels, respectively. The nuclei are shown by Hoechst staining in blue. **(B-I'')** Triple immunostaining of sagittal sections for YFP, Nestin, and GFAP at 4 wpt in THC **(B-E)**, DKO **(B'-E')** and TKO **(B''-E'')** as well as at 8 wpt in THC **(F-I)**, DKO **(F'-I')** and TKO **(F''-I'')**. Insets in **(B-I'')** show higher magnifications images of the indicated regions in the aSVZ. Arrowheads in **(B-I'')** show triple positive (YFP+Nestin+GFAP+) cells in the aSVZ. **(J)** Graph showing the cell counts of different populations of YFP+ cells co-stained with Nestin (NSPCs) or both Nestin and GFAP (NSCs) in all three genotypes at 4 wpt and 8 wpt **(K)** Ratios of (YFP+GFAP+Nes+/YFP+Nes+) or (aNSCs/aNSPCs) in the aSVZ. Error bars indicate the standard deviation from n=3 animals per genotype except for n=2 for 4wpt DKO brains. Asterisks represent statistically significant results obtained from independent sample t-tests: (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar **(B-I'')** = 100 μm. Scale bar **(A)** = 25 μm, Legend as in Figure 1.

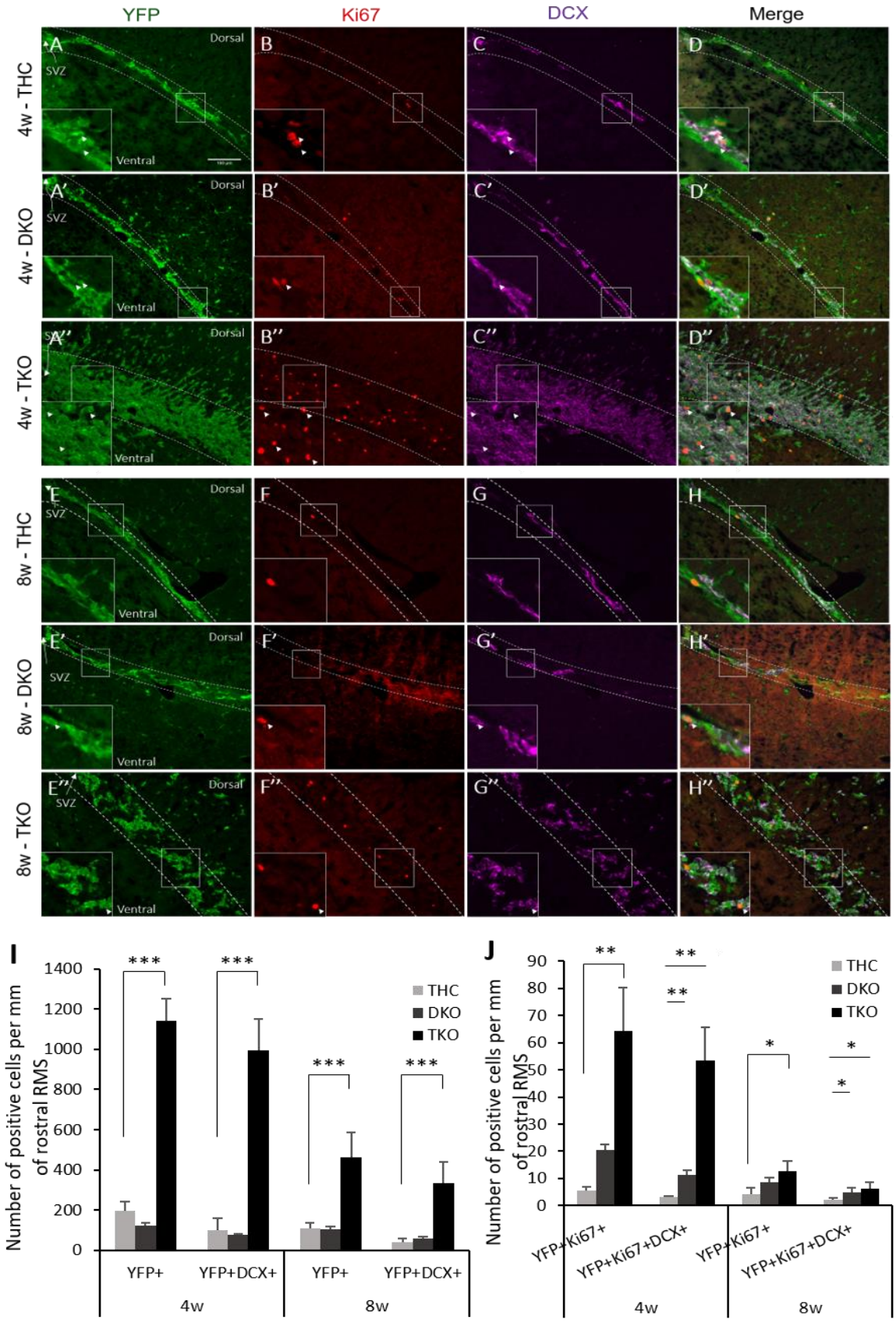
C. Enhanced production of migratory neuroblasts along the RMS and ectopic cell migration in the absence of pocket proteins

Late neuronal progenitors or immature neuroblasts undergo terminal rounds of cell division as they initiate their rostral migration to the OB. We examined the combined roles of the Rb family of proteins during neuronal commitment and migration and quantified the number of YFP+ neuroblasts found in the aSVZ and the rostral RMS. Our results showed highly significant 9.97 fold increase in the number of

(YFP+DCX+) cells in TKO brains compared to THC brains in the aSVZ at 4 wpt (123 ± 66 cells in THC brains versus 1227 ± 418 cells in TKO brains) (**Figure 6D, D'', 6L**) and 11.54 fold increase at 8 wpt (**Figure 6I, I'', 6L**). We did not detect a significant change in the absolute size of this population in DKO brains compared to THC brains at either time-points (**Figure 6D, D', I, I' and L**). We estimated the proportion of recombined neuroblasts among the total YFP population by calculating the ratio of (YFP+DCX+/total YFP) and found it to be around 40% in THC brains versus 70% in TKO at 4 wpt, and, 20% versus 70% at 8 wpt, respectively (**Figure 6M**). Similarly, a greater number of recombined neuroblasts was detected along the rostral RMS in TKO compared with THC brains, but not in DKO brains (**Figure 8C-C'', G-G'')**. At 4 wpt, this number averaged at 99 ± 59 cells in THC brains compared to 996 ± 155 cells in TKO brains (10-fold increase; **Figure 8C-C'', I**), and 38 ± 21 cells in THC brains versus 334 ± 106 cells in TKO brains at 8 wpt (8.78-fold increase; **Figure 8G-G'', I**). In addition, there is a clear increase in the proportion of neuroblasts among the total YFP population found in the rostral RMS in TKO compared to THC brains (50% in THC versus 90% in TKO brains at 4 wpt and 30% in THC brains versus 70% in TKO brains at 8 wpt) (**Figure 8K**). A comparable increase in the proportion of neuroblasts is seen in DKO brains compared with THC at 8 wpt (30% in THC brains versus 60% in DKO brains), but not at 4 wpt (**Figure 8K**). Like in the aSVZ, the number of dividing neuroblasts (YFP+;Ki67+;DCX+) in the RMS was significantly greatly in both TKO and DKO brains compared to THC mice at 4 wpt (3 ± 1 in THC, 54 ± 12 in TKO, 11 ± 2 in DKO) and 8 wpt (2 ± 1 in THC, 6 ± 2 in TKO, 5 ± 2 in DKO) (**Figure 8J, compare with 6K**). The above results indicate the presence of enhanced production of immature neurons or neuroblasts inside the RMS in the absence of all three pocket

proteins, which is consistent with the increased cell proliferation detected earlier in the aSVZ.

The enhanced production and migration of DCX+ neuroblasts caused a sharp increase in the thickness of the rostral RMS, typically in TKO compared with DKO and THC primarily at 4 wpt (**Figure 8A-H''; dashed lines**). Like in the THC, the majority of YFP-positive neuroblasts successfully migrate along the RMS and reach the OB in DKO and TKO brains. However, there is ectopic migration of a large number of YFP+ cells outside the RMS in DKO but mainly in TKO brains. These migrating cells radially invade the anterior and posterior cerebral cortex (**Figure 9A-C'**), the striatum (**Figure 9A''-C''**), and the corpus callosum (**Figure 9D-D''**), and, appear to originate from both the aSVZ and RMS. These results indicate that all three pockets proteins are required for proper rostral migration of neuroblasts and can compensate for each other loss either partially or fully when one or two protein(s) are lost but not all.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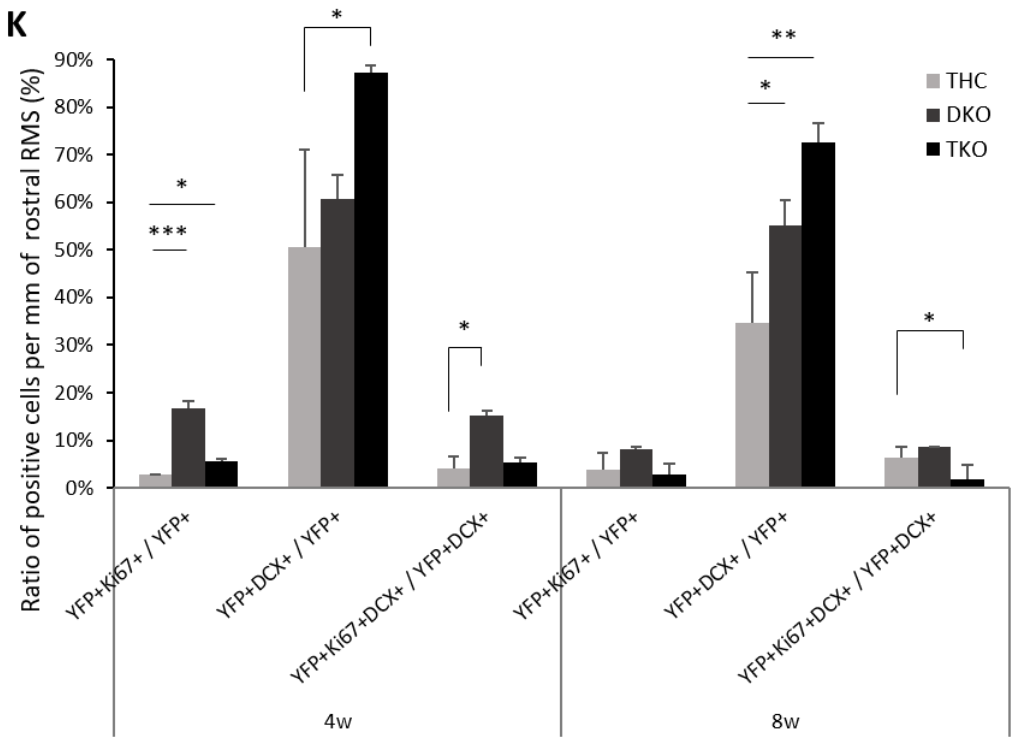


Figure 8: Enhanced neuroblast migration along the rostral RMS to the OB in the absence of pocket proteins. (A-H'') Triple immunostaining for YFP, Ki67, and DCX performed on sagittal sections at 4w in THC **(A-D)**, DKO **(A'-D')** and TKO **(A''-D''),** and, at 8w in THC **(E-H)**, DKO **(E'-H')** and TKO. The migratory route of DCX-positive neuroblasts along the rostral portion of the RMS is delimited by dashed lines in E-H''. Insets in **(A-H'')** show higher magnifications images of the indicated regions in the RMS. Arrowheads in **(A-H'')** show triple positive (YFP+Ki67+DCX+) cells. **(I, J)** Graphs showing quantification of the populations of YFP+ cells co-stained with different markers in the RMS at 4wpt and 8wpt in all three genotypes. **(K)** Graph showing the ratios of the different cell populations found in the RMS. Error bars indicate the standard deviation from n=3 mice for THC, TKO, and 8w DKO, and, n=2 from 4w DKO brains. Asterisks represent statistically significant results obtained from independent sample t-tests: (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar = 100 μm,

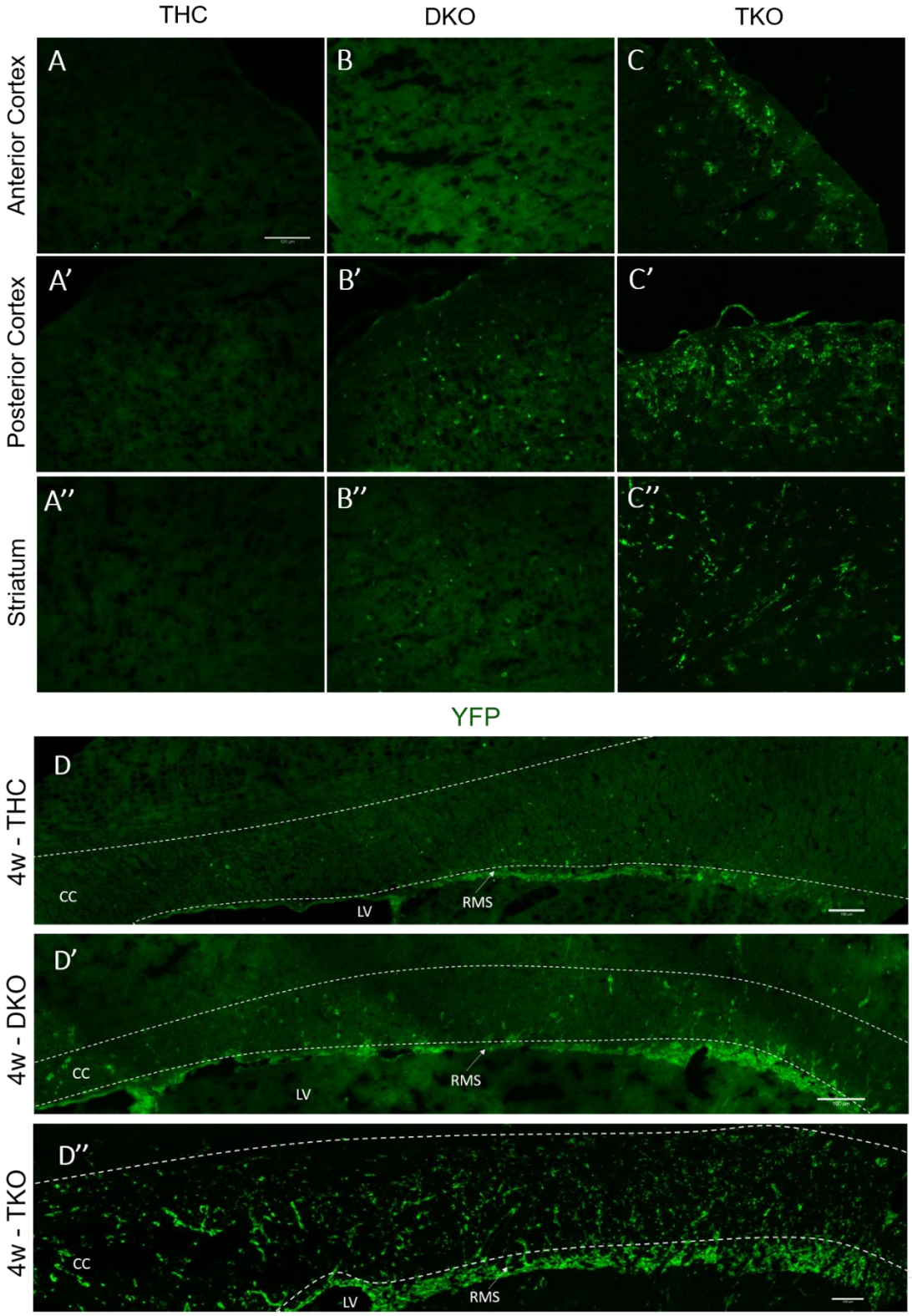


Figure 9: Ectopic migration of YFP-positive cells into different brain regions in (p107, p130) DKO and (p107, Rb, p130) TKO compared with THC. (A-D'')
Immunostaining with YFP on sagittal brain sections at 4w in THC (**A'-A'', D**), DKO (**B-B'', D'**) and TKO (**C-C'', D''**) showing ectopic migration of YFP positive cells into

the anterior cortex **(A-C)**, posterior cortex **(A'-C')**, striatum **(A''-C'')**, and the corpus callosum **(D-D''; as delimited by dashed lines)** in DKO and TKO brains but not in THC brains. cc = corpus callosum, LV = lateral ventricle, RMS = rostral migratory stream Scale bar = 100 μm

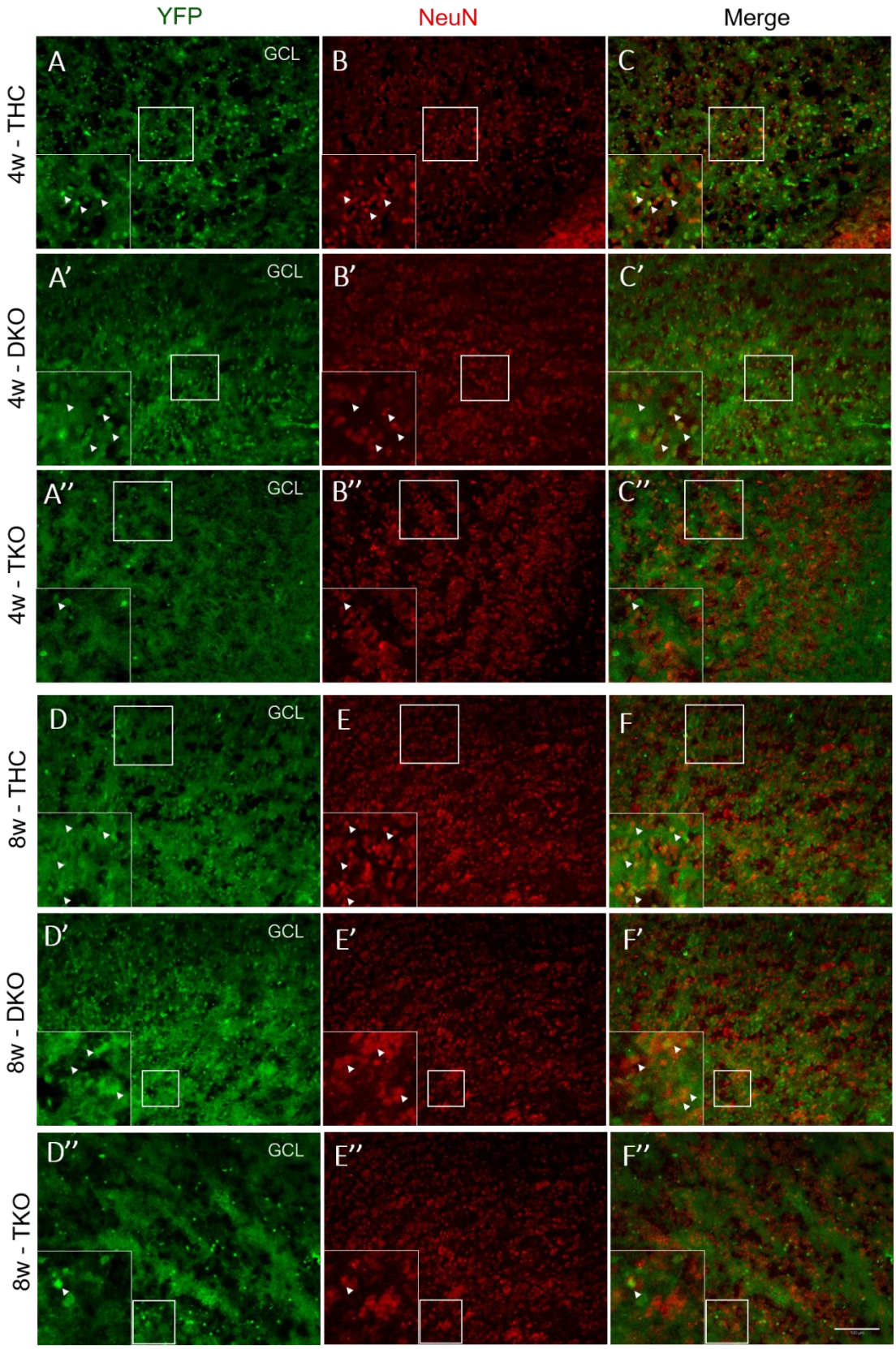
D. Loss of all three pocket proteins leads to dramatic reduction in adult neurogenesis inside the OB

A previous study has implicated p130 in the survival of developing cortical neurons in culture (Liu et al., 2005). We also showed that the Rb protein is indispensable for the long-term survival of adult-born neurons in the OB (Naser et al., 2016). To investigate the combined roles of the Rb family in the survival of adult-born neurons in the aSVZ/OB, we quantified and compared the number of YFP positive cells found in the OB layers, the GCL and the GL, in all three genotypes. Cell counts were performed on three consecutive OB sections taken at medial level from each brain, then averaged and normalized to the surface area from two representative regions inside the GCL and from one region in the GL. Results showed dramatic reduction in the number of YFP-positive cells detected in the GCL in TKO brains compared to THC brains at 4 wpt (668 ± 201 cells per mm^2 of GCL in THC versus 126 ± 36 cells in TKO) (**Figure 10A-C'', G**) as well as at 8 wpt (710 ± 38 cells per mm^2 of GCL in THC versus 97 ± 17 cells per mm^2 in TKO) (**Figure 10D-F'', G)**. However, in DKO brains, a single Rb allele was able to restore the average number of YFP+ cells inside the GCL compared with THC at 4wpt (668 ± 201 cells per mm^2 of GCL in THC versus 580 ± 211 cells in DKO brains) (**Figure 10A-C'', G**), and at 8wpt (**Figure 10D-F'', G;** 710 ± 38 cells per mm^2 of GCL in THC versus 698 ± 9 cells in DKO brains). Similarly, we detected a decrease in the number of YFP positive cells in the GL in TKO brains compared to those in THC brains at 4 wpt (565 ± 129 cells per mm^2 in THC versus 167 ± 79 cells per

mm^2 in TKO brains) (**Figure 11A-C'', G**). However, we did not detect any significant change in the number of YFP positive cells in DKO brains compared to THC brains at this time-point (565 ± 129 cells per mm^2 in THC versus 503 ± 23 cells per mm^2 in DKO brains) (**Figure 11A-C'', G**). These results are consistent with those seen in the GL at 8 wpt (**Figure 11D-F'', G;** 596 ± 120 cells per mm^2 in THC, 83 ± 20 cells per mm^2 in TKO brains, and 594 ± 100 cells per mm^2 in DKO).

To assess whether the YFP+ cells found in the OB layers are mature adult-born neurons, we co-stained for YFP and NeuN and quantified the numbers of double positive cells in all three genotypes. We observed that the majority of YFP-positive cells in the GCL co-express the mature neuronal marker NeuN in THC and DKO brains indicating that these cells are indeed newborn neurons (**Figures 10G;** 540 ± 157 cells per mm^2 in THC and 474 ± 176 cells per mm^2 in DKO brains 4 wpt). However, only about 20% of YFP+ cells in TKO brains co-express NeuN (**Figures 10G;** 540 ± 157 cells per mm^2 in THC compared to 26 ± 5 cells per mm^2 in TKO brains at 4 wpt). Similar results are obtained in the GCL at 8wpt (**Figures 10G;** 577 ± 24 cells per mm^2 in THC, 29 ± 6 cells per mm^2 in TKO brains, and 582 ± 2 cells per mm^2 in DKO). The cell counts of (YFP+NeuN+) cells in the GL are consistent with those obtained in the GCL at both time-points (**Figures 11G;** 457 ± 99 cells per mm^2 in THC, 35 ± 16 cells per mm^2 in TKO brains, and 410 ± 14 cells per mm^2 in DKO at 4 wpt, and, 484 ± 94 cells per mm^2 in THC, 25 ± 6 cells per mm^2 in TKO brains, and 331 ± 95 cells per mm^2 in DKO at 8 wpt). Taken together, the above results indicate that AN inside the OB is drastically reduced in the absence of pocket proteins, and this is likely due to terminal differentiation defect and/or massive cell death of neuroblasts. Importantly, a single Rb

allele can rescue the decline in the counts of adult-born neurons observed in TKO brains.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C
A'
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A''
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D
E
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GCL

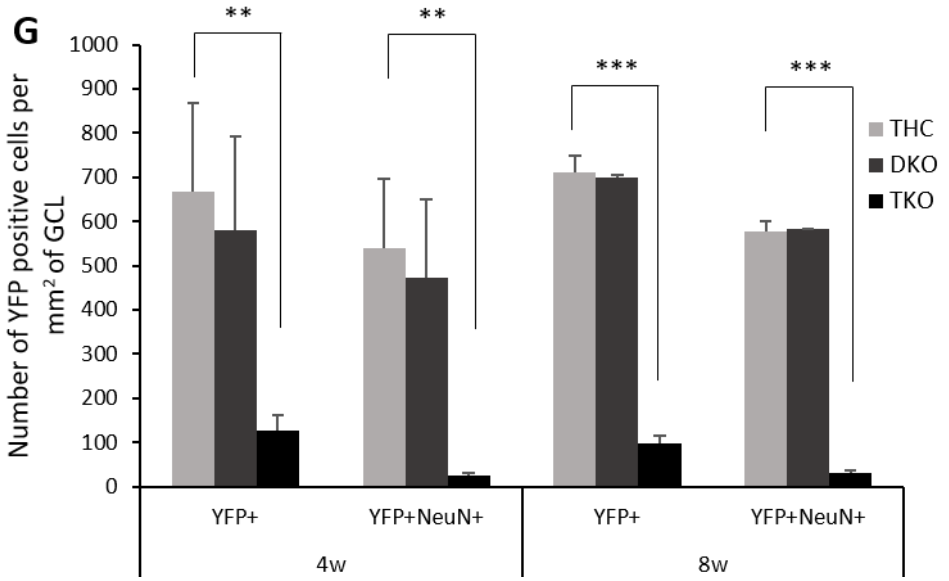
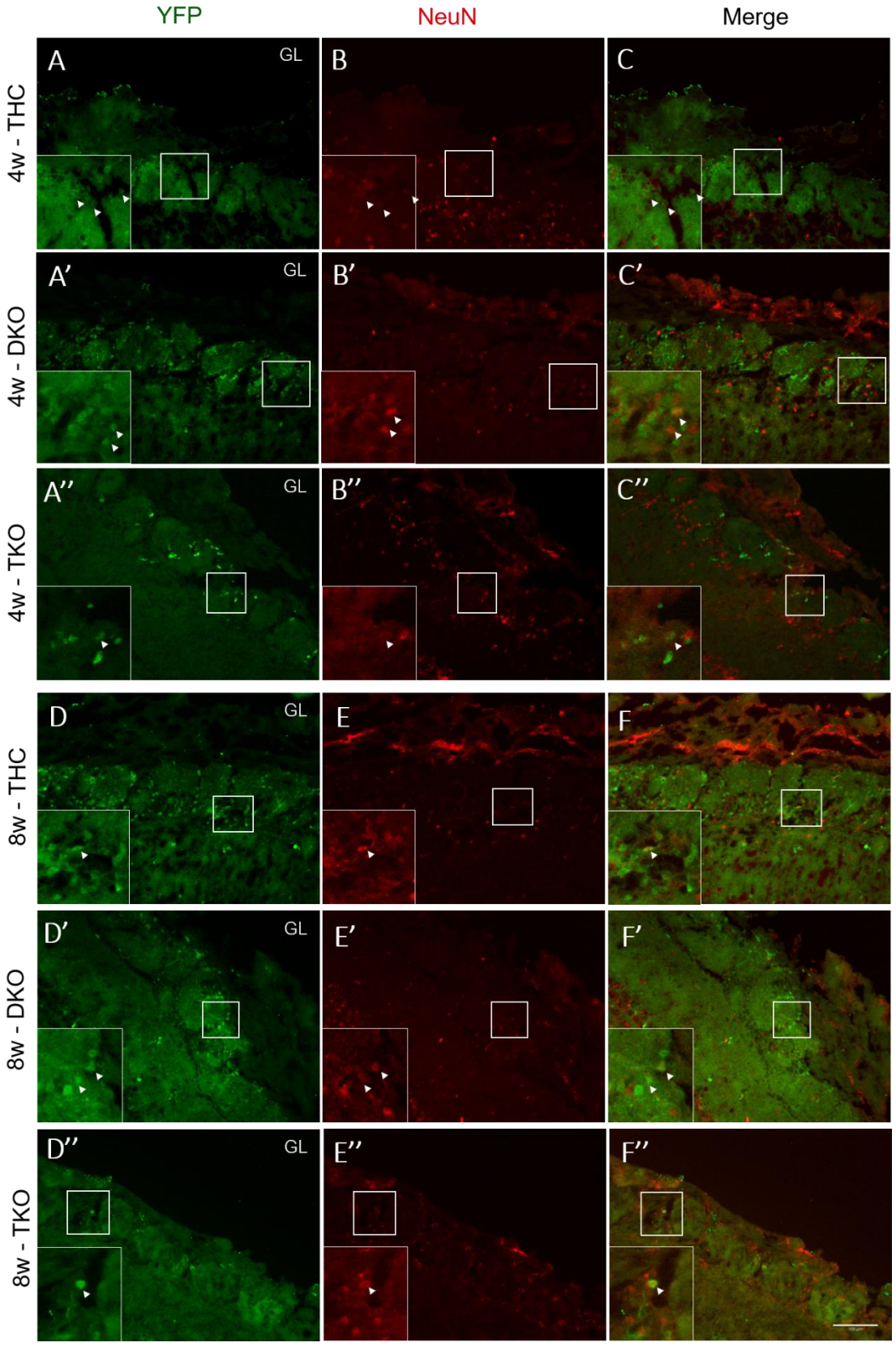


Figure 10: Dramatic reduction in the number of newborn neurons in the GCL inside of the OB in TKO brains. (A-F'') Double immunostaining for YFP and NeuN on sagittal sections at 4w in THC **(A-C)**, DKO **(A'-C')**, and TKO **(A''-C'')** as well as at 8w in THC **(D-F)**, DKO **(D'-F') and** TKO **(D''-F'')**. Insets in **(A-F'')** show higher magnifications images of the indicated regions in the GCL. Arrowheads in **(A-F'')** show double positive (YFP+NeuN+) cells inside the GCL. **(G)** Graph showing cell counts of YFP+ and YFP+NeuN+ cells in the GCL in all three genotypes. Error bars indicate the standard deviation from n=3 brains for THC, TKO, and 8wpt-DKO, and n=2 from 4wpt DKO brains. Asterisks represent statistically significant results obtained from independent sample t-tests: (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar = 100 μm, GCL = granule cell layer


YFP
NeuN
Merge
4w - THC
4w - DKO
4w - TKO
8w - THC
8w - DKO
8w - TKO
A
B
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A'
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GL

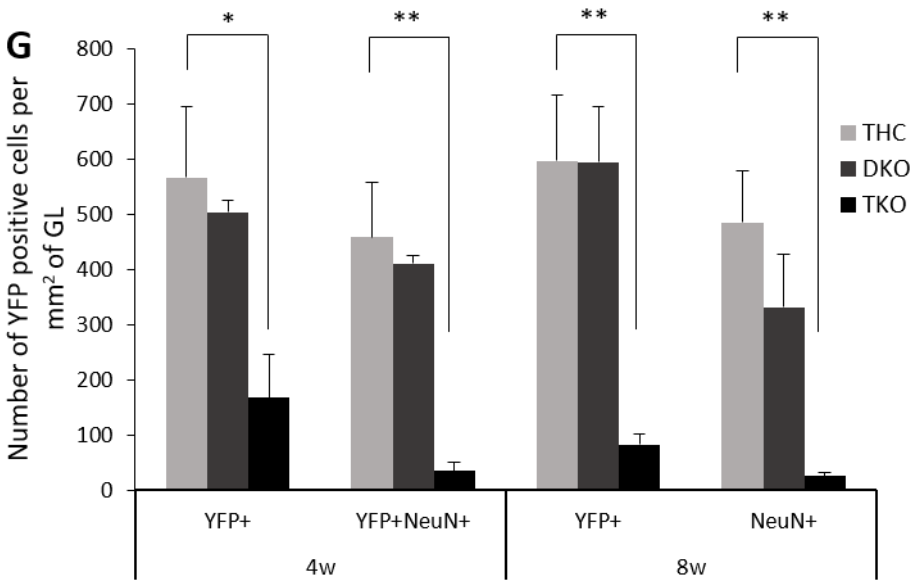


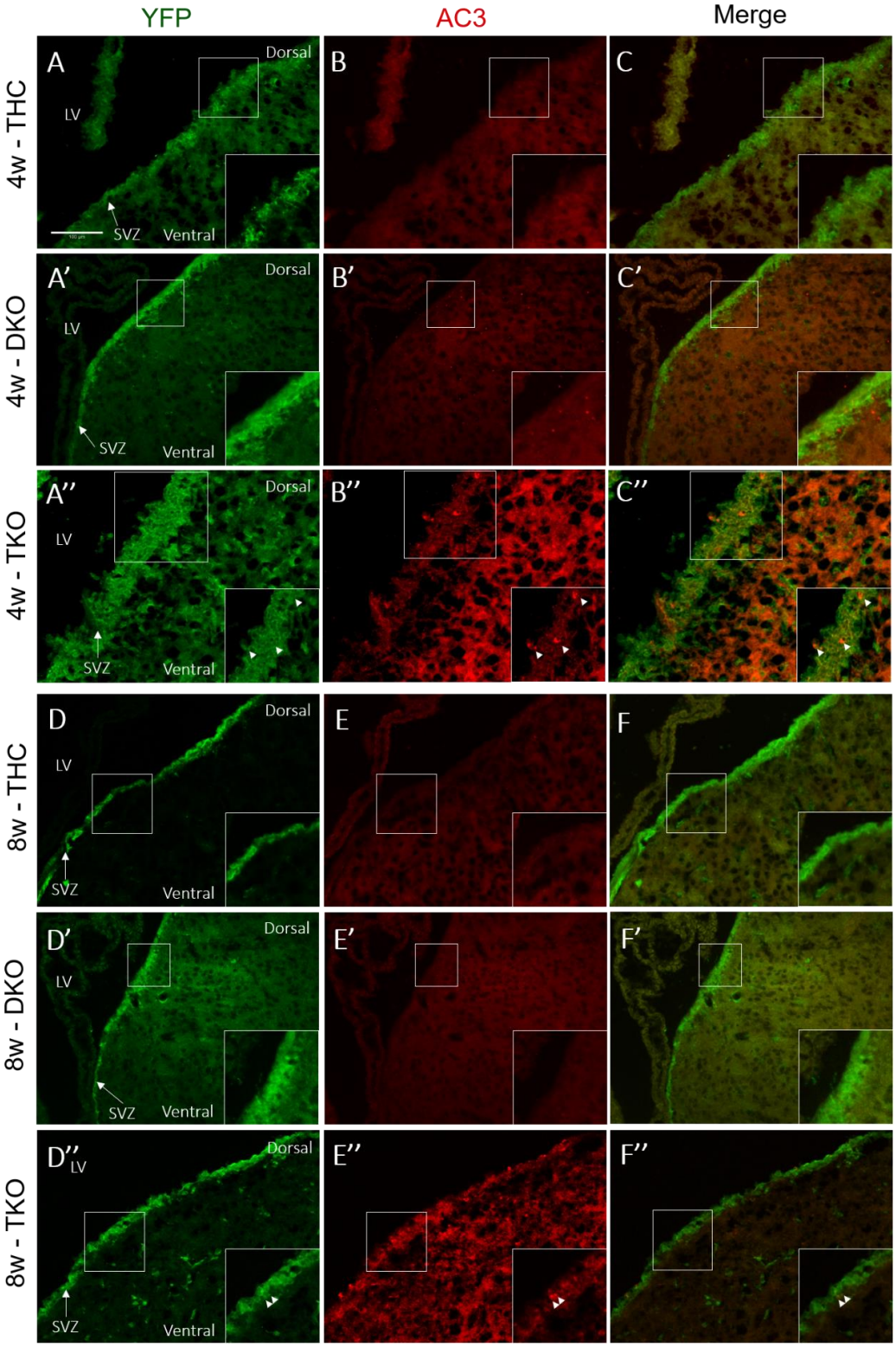
Figure 11: Dramatic reduction in the number of newborn neurons in the GL inside of the OB in TKO brains. (A-F'') Double immunostaining for YFP and NeuN on sagittal sections at 4wpt in THC **(A-C)**, DKO **(A'-C')** and TKO **(A''-C'')** as well as at 8wpt in THC **(D-F)**, DKO **(D'-F')** and TKO **(D''-F'')** showing a reduced number of YFP+ cells in the GL in TKO brains compared with DKO and THC. Insets in **(A-F'')** show higher magnifications images of the indicated regions in the GL. Arrowheads in **(A-F'')** show double positive (YFP+NeuN+) cells in the GL. **(G)** Graph showing cell counts of YFP+ and YFP+NeuN+ cells in the GL in all three genotypes. Error bars indicate the standard deviation from n=3 brains for THC, TKO, and 8w-DKO and n=2 from 4w-DKO brains. Asterisks represent statistically significant results obtained from independent sample t-tests: (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar = 100 μm, GL = glomerular layer

E. Massive apoptosis of DCX+ neuroblasts in the aSVZ and RMS in the absence of the Rb family of pocket proteins

The dramatic decline in AN observed in TKO brains could be due to survival defects in neuroblasts inside the aSVZ and/or RMS. To test this, we examined the stage at which neuroblasts are dying by co-staining for active caspase-3 (AC3), an apoptotic marker, in combination with YFP and DCX. Our results showed indeed the presence of massive cell death inside the aSVZ in TKO brains compared to THC and DKO as indicated by the number of (YFP+AC3+) double positive cells found in the region (0 cells in THC brains versus 36 ± 6 cells in TKO brains at 4 wpt; 0 cells in THC brains

versus 11 ± 2 cells in TKO brains at 8 wpt) (**Figure 12A-C'', J**). Importantly, the majority of these apoptotic cells (97%) co-labeled with DCX in the aSVZ in TKO brains (**Figure 12G, G', J**) while no apoptotic cells were detected in DKO brains at either time-points (**Figure 12A-F'', J**). The cell death phenotype was also found inside the rostral RMS with respect to the detection of (YFP+AC3+DCX+) triple positive cells in TKO but none in THC or DKO at 4wpt (0 cells per mm of RMS in THC and DKO brains versus 24 ± 5 cell per mm of RMS in TKO brains) (**Figure 13A-C'', G, G' and I**), and at 8 wpt, albeit at lower numbers (0 cells per mm of RMS in THC and DKO brains versus 5 ± 2 cells per mm of RMS in TKO brains; **Figures 13D-F'', H, H' and I**). The AC3-positive cells represent approximately 1.5-2% of all YFP-positive cells detected in both regions in TKO brains (**Figures 12K and 13J**). Of note, we did not identify any AC3-positive cells inside the OB suggesting that the vast majority of neuroblasts die before entering it (data not shown).

Taken together, these results demonstrate that the drastic decline in adult OB neurogenesis in TKO brains compared with THC and DKO is due to the massive cell death of neuroblasts that is abnormally occurring in the aSVZ and the RMS.


YFP
AC3
Merge
4w - THC
4w - DKO
4w - TKO
8w - THC
8w - DKO
8w - TKO
A
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C
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LV
SVZ
Dorsal
Ventral

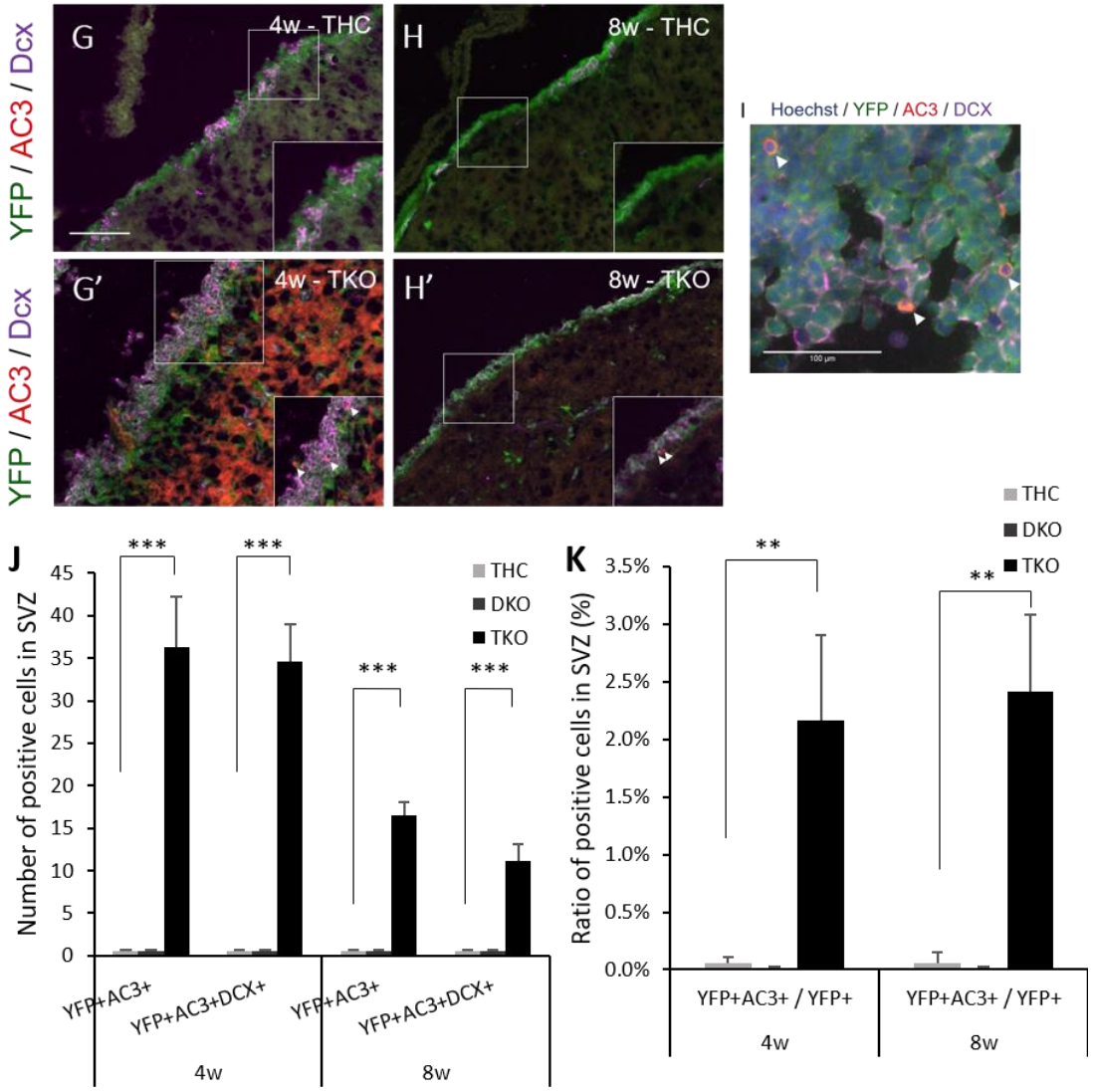
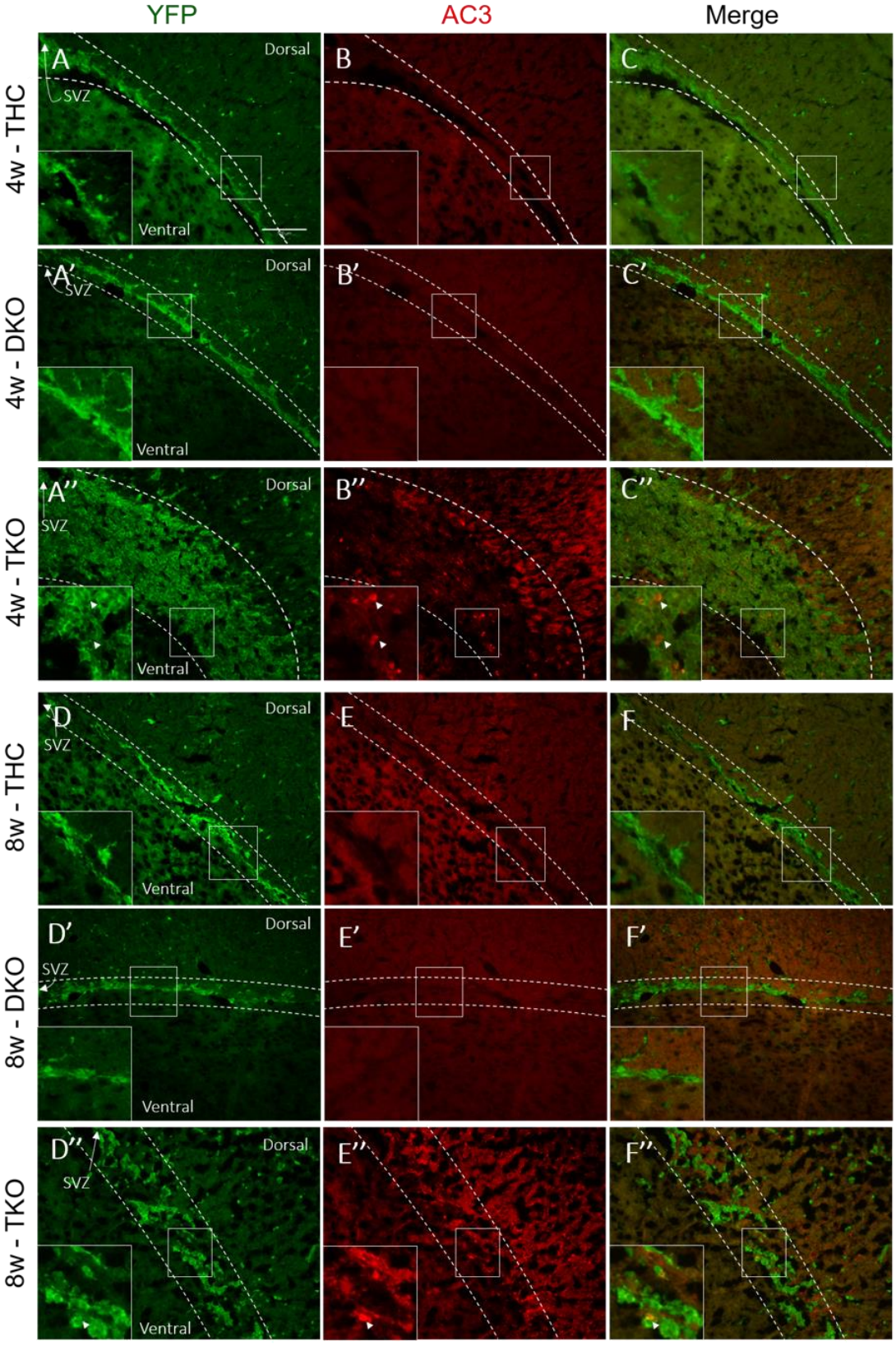


Figure 12: Massive apoptotic cell death of neuroblasts in the aSVZ in the absence of pocket proteins (A-F'') Double immunostaining for YFP and AC3 performed at 4wpt in THC **(A-C)**, DKO **(A'-C')**, and TKO **(A''-C'')**, as well as at 8wpt in THC **(D-F)**, DKO **(D'-F')**, and TKO **(D''-F'')** showing AC3+ cells in the aSVZ. Insets in **(A-F'')** show higher magnifications of the indicated regions in the aSVZ. Arrowheads in **(A-F'')** show double positive (YFP+AC3+) cells in thea SVZ. **(G-H')** Triple immunostaining for YFP, AC3 and DCX showing cell death of neuroblasts in the SVZ of TKO brains compared to THC brains. **(I)** Panel showing three (YFP+AC3+DCX+) triple positive cells (arrowheads) in the aSVZ in TKO at 4wpt. The nuclei are shown by Hoechst staining. **(J)** Graph showing the cell counts of different populations of YFP+ co-stained with AC3 and DCX in all three genotypes at 4 wpt and 8 wpt. **(K)** Ratios of YFP+AC3+YFP+ cells in the aSVZ. Error bars indicate the standard deviation from n=3 brains for THC, TKO, and 8w DKO and n=2 from 4w DKO brains. Asterisks represent statistically significant results obtained from independent sample t-tests such that (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar = 100 µm. LV = lateral ventricle, SVZ = subventricular zone.


YFP
AC3
Merge
4w - THC
4w - DKO
4w - TKO
8w - THC
8w - DKO
8w - TKO
A
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SVZ

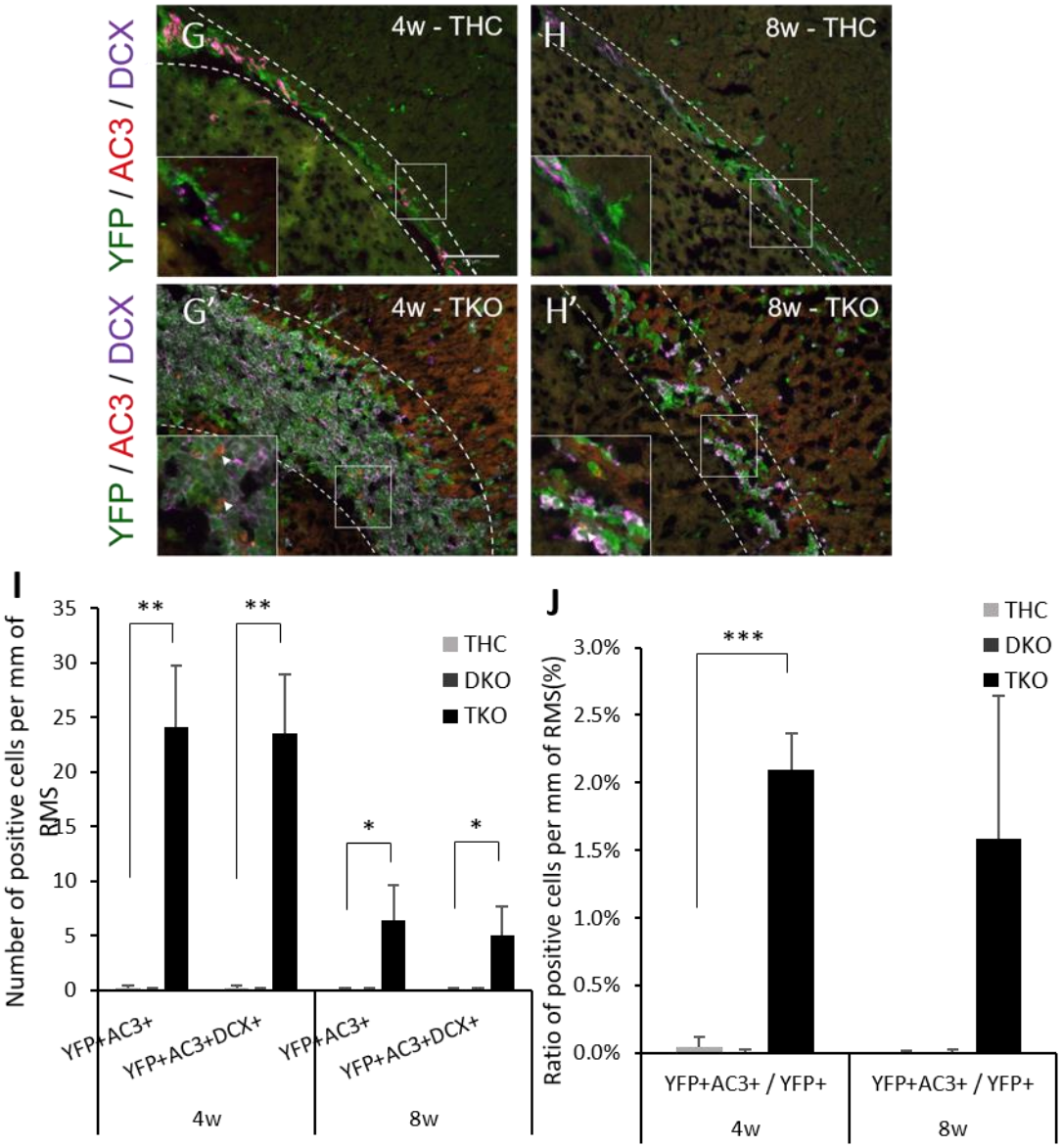

Figure 13: Massive apoptotic cell death of neuroblasts in the RMS in the absence of pocket proteins. (A-F'') Double immunostaining for YFP and AC3 performed on sagittal sections at 4w in THC **(A-C)**, DKO **(A'-C')**, and TKO **(A''-C'')**, as well as at 8w in THC **(D-F)**, DKO **(D'-F')**, and TKO **(D''-F'')** showing cell death along the rostral portion of the RMS delimited by two dashed lines. Insets in **(A-F'')** show higher magnifications of the indicated regions in the RMS. Arrowheads in **(A-F'')** show double positive (YFP+AC3+) cells in the RMS. **(G-H')** Co-immunostaining for YFP, AC3 and DCX showing cell death of neuroblasts in the RMS of TKO brains compared to THC brains. **(I)** Graph showing the cell counts of different populations of YFP+ co-stained with AC3 and DCX in all three genotypes at 4 wpt and 8 wpt. **(J)** Ratios of YFP+AC3+YFP+ cells in the RMS. Error bars indicate the standard deviation from n=3 brains for THC, TKO, and 8w DKO, and n=2 from 4w DKO brains. Asterisks represent statistically significant results obtained from independent sample t-tests such that (*) $p<0.05$, (**) $p<0.01$, (***), $p<0.001$. Scale bar = 100 µm, SVZ = subventricular zone.

CHAPTER 4

DISCUSSION

In this study, we assessed the combined roles of the Rb family of pocket proteins in regulating AN in the aSVZ/OB system. To accomplish this, we induced a double deletion of p107 and p130 (DKO with $Rb^{+/-}$) and a triple deletion of all three proteins (TKO), then examined the resulting phenotypes at different stages of AN and in cell populations along the neural lineage starting in the aSVZ, RMS, and the OB in comparison with THC. We found that the loss of the Rb family leads to significant increase in stem cell self-renewal and progenitor proliferation in the aSVZ, enhanced and ectopic neuroblast migration in the RMS and other brain regions, and severely compromised terminal differentiation and loss of newborn neurons in the OB. By comparing our data from DKO and TKO with previous data obtained from single KOs of p107 and Rb, we concluded the presence of distinct as well as synergistic functions among all three pocket proteins with potential compensatory roles in specific function(s), at least partially, during the control of AN.

A. The Rb family negatively regulates proliferation of aNSPCs in the aSVZ

Previous studies performed on single KO models of p107 and Rb have shown that the different Rb family members act at different stages during AN to control the aNSPC lineage. Our laboratory has previously shown that Rb negatively regulates progenitor proliferation in the aSVZ but does not affect stem cell self-renewal (Naser et al., 2016). In contrast, Vanderluit et al. demonstrated that p107 negatively regulates stem cell self-renewal and does not seem to have a direct control on progenitor

proliferation (Vanderluit et al., 2004). To assess the presence of possible functional redundancy and/or compensation between pocket proteins, we used a Nestin-CreER^{T2}-YFP transgenic model and Rb;p130 floxed mice in combination with p107-null mice to induce double and triple deletions of these genes.

We then examined the resulting phenotypes in the aSVZ/OB four weeks (4wpt) or eight weeks (8wpt) post-TAM treatment in comparison with THC mice. Of note, the TAM treatment was administered to DKO mice by IP injections while TKO and THC received the treatment by oral gavage separately. It is well known that compared with IP injection, oral gavage is more effective in driving Cre recombination due to its better/systematic absorption which minimizes dose fluctuation among different mice and treatment groups. Therefore, and in order to minimize errors related to the technical approach used in our study, we primarily relied on relative numbers and ratios to assess and compare the size of specific cell populations at different developmental stages rather than absolute numbers. As expected, the frequency of recombination was lower in DKO compared with THC as indicated by slightly lower numbers of YFP expressing cells in the adult SVZ in DKO brains compared with THC brains. Yet, the ratio of (YFP+;Ki67+) cells over the total YFP population was significantly higher in the former group. On the other hand, in TKO brains, we found that loss of all pocket proteins leads to a massive increase in the numbers of YFP-positive cells and (YFP;Ki67) double positive cells in the adult SVZ compared with THC brains (**Figure 1**). This is due to enhanced cell proliferation and illustrated by the vast expansion of the neurogenic aSVZ region (heterotopia) in TKO compared with THC. Importantly, DKO brains, which carry a functional Rb allele, do not exhibit the latter phenotype although they have significant increase in cell proliferation compared with THC.

Accordingly, the proliferation index (YFP+;Ki67+/ total YFP) in the aSVZ is significantly higher in both TKO and DKO compared with TKO at 4 wpt. In Rb-single KO adult brains, we reported earlier a 2-3 fold increase in (YFP;Ki67) double positive cells compared with $Rb^{+/-}$ brains at 28 days post-TAM (Naser et al., 2016). In comparison, we detect here at the same time-point a 5.56-fold increase in the number of dividing cells in TKO; this is due to an additive and possibly synergistic effect following the combined loss of p107, and Rb, in stem cells and progenitor cells, respectively.

Although p107 specifically controls stem cells self-renewal as previously shown, it could potentially compensate for the loss of Rb in directly regulating progenitor proliferation in the aSVZ given that another study in the laboratory found that its transcript expression is upregulated by 4-fold in Rb-null aNSPCs neurospheres derived from the aSVZ (Halaby N., Ghanem N., unpublished data). E2F1 and E2F3 mRNA levels are also upregulated in the absence of Rb *in vitro* and *in vivo* (Halaby N., Ghanem N., unpublished data). If true, the observed compensatory role of p107 would be partial at best and unlikely to be mediated by E2F1 or 2 given that the structural differences between p107 and Rb proteins greatly reduce its binding affinity to these two E2Fs (Liban, Thwaites, Dick, & Rubin, 2016). Direct evidence for the existence of such a role by p107 is still lacking. Moreover, it was shown that p107 can associate with E2f3a (but not E2f3b) in transcriptionally repressing *Sox2* in adult progenitors as seen with neural precursor cells during development (Julian et al., 2013). This scenario would be consistent with a previous study showing that combined loss of Rb and E2F1 or Rb and E2F3 can rescue ectopic progenitor proliferation during development (McClellan et al., 2007).

As a matter of fact, in other contexts, p107 was shown to compensate for the loss of Rb (reviewed in (Wirt & Sage, 2010)). For instance, mice lacking Rb and p107 exhibit disrupted cerebellar architecture that was associated with migration and differentiation defects of granule cell precursors, followed by their apoptosis following maturation at the dorsal part of the mid-to-hind brain junction (Marino, Hoogervoorst, Brandner, & Berns, 2003). Moreover, p107 protects from the formation of squamous cell carcinoma in the epidermis and from the development of adenomas or adenocarcinomas in the lung epithelium if Rb is lost. The combined loss of Rb and p107 leads to the formation of these cancers (Lara et al., 2008; Simpson, Mason-Richie, Gettler, & Wikenheiser-Brokamp, 2009). p107 also compensates for Rb in differentiating cortical neurons where TKO mice show a much higher proliferation index compared to the single knockouts (Oshikawa et al., 2013). Hence, a single allele of p107 can restore the ectopic proliferation of cortical progenitors during development (Oshikawa et al., 2013). Furthermore, Ajioka et al. showed that $Rb^{-/-}$; $p130^{-/-}$ retinal horizontal interneurons are protected from the development of retinoblastomas due to the upregulation of p107 which is playing a compensatory role (Ajioka et al., 2007). In addition, p107 was upregulated in mouse embryonic retinal progenitors when Rb was deleted, and vice versa, showing a reciprocal compensation between Rb and p107 in the developing retina (Donovan, Schweers, Martins, Johnson, & Dyer, 2006).

When comparing the phenotypes observed at 4wpt and 8wpt, we report similar trends in terms of increase in the numbers and ratios of dividing cells in TKO compared to THC brains. However, the absolute cell counts e.g. number of progenitors is lower at 8wpt compared with those detected at 4wpt in TKO, which is primarily due to increased

cell death in the aSVZ and RMS and could be indicative of a premature exhaustion of the stem cell pool overtime.

B. Control of neuronal commitment by the Rb family of proteins

With respect to neuronal commitment, our results indicate that the enhanced proliferation described above is directly associated with expansion in the population of neuroblasts in the absence of pocket proteins. In fact, the ratio of DCX-positive cells or neuroblasts (YFP;DCX/ total YFP) is largely higher in TKO brains versus THC brains at 4 wpt.

Upon quantifying the number of late progenitors/neuroblasts (YFP+;Ki67+;DCX+), we see a similar increase in the number of progenitors in TKO brains compared to THC brains that was not observed in DKO brains 4 wpt, indicating the expansion of the progenitor pool in the absence of the Rb family. This is also consistent with previous studies on both Rb and p107 single knockout mice, where the deletion of either gene leads to an increase in cell proliferation (Naser et al., 2016; Vanderluit et al., 2004). The expanded population in 4-week DKO brains suggests that the effect is at the level of stem cell division.

C. p107 plays a distinct role in negatively regulating stem cell division in the aSVZ

When assessing the combined roles of pocket proteins in the control of stem cells self-renewal, we found that both DKO and TKO brains show an expanded pool of aNSCs compared to THC brains at 4wpt as indicated by the larger size of the activated population of aNSCs (YFP+;Nestin+;GFAP+) found in the former two genotypes. Hence, similar to our findings with respect to the size of aNSPCs population, the ratios

of aNSCs compared to the total aNSPCs are significantly elevated in DKO and TKO brains compared to THC with no significant difference between TKO and DKO (just a slight decrease in DKO versus TKO). These findings clearly indicate that p107 (and not Rb nor p130) plays a distinct role in negatively regulating stem cell division in the adult brain, and this is consistent with previous study showing expansion in stem cells pool in p107-null mice (Vanderluit et al., 2004). Moreover, these results indicate that the increase in the number of progenitor cells observed in DKO brains results indirectly from the expanded pool of activated stem cells in the aSVZ rather than from a direct control of progenitor proliferation by p107. Previous studies have shown that p107 negatively regulates stem cell division through the transcriptional repression of several genes in the Notch pathway and $p107^{-/-}$ mice exhibit enhanced activation of this signaling pathway which was shown to promote NSC division at the expense of quiescence (Chapouton et al., 2010; Vanderluit et al., 2004). The slight reduction in the size of the stem cell pool in DKO brains compared to TKO brains at 4 wpt indicates that Rb could partially compensate for the loss of p107 in regulating the size of the stem cell pool. To validate the potential compensatory role of Rb in this context, future studies should compare p107-single knockout with Rb/p107 DKO brains where the pocket proteins are deleted specifically in the stem cell pool, for example by using a GFAP-Cre transgenic model. p130 does not seem to play a major role in the regulation of adult stem cell development; however, analysis of the phenotype in p130 single KO will provide a definitive answer.

aNSCs in the aSVZ can either undergo consuming divisions and produce two progenitors (around 70% of the time), or self-renewing divisions and produce two stem cells (around 30% of the time) (Obernier et al., 2018). This disproportionate rate of

division favoring the formation of progenitors leads to the age-dependent decline observed in AN (Obernier et al., 2018). We detected a similar but relatively faster decline in the size of the stem cells population at 8wpt in TKO and DKO brains compared with 4 wpt. This was evidenced by the remarkable decrease in the ratios of stem cells over the total population aNSPCs in DKO and TKO compared with THC levels between the two time-points. Given the relatively short time elapsed between the two time-points, this data suggests the existence of premature exhaustion of the stem cell pool in the absence of pocket proteins. Alternatively, the decline in activated aNSCs in the 8-week timepoint could possibly be due to latency of stem cells as a result of chromatin remodeling in the absence of Rb. The Rb family is known to recruit and interact with several chromatin-modifying enzymes (Macaluso, Montanari, & Giordano, 2006). Thus, future studies are needed to asses the integrity and the change in the epigenome in 4-week versus 8-week aNSCs in the absence of the Rb family.

D. p107 plays a distinct role in negatively regulating neuronal commitment in the aSVZ

When assessing the combined roles of pocket proteins in the control of stem cells self-renewal, we found that both DKO and TKO brains show an expanded pool of aNSCs compared to THC brains at 4wpt as indicated by the larger size of the activated population of aNSCs (YFP+;Nestin+;GFAP+) found in the former two genotypes. Hence, similar to our findings with respect to the size of aNSPCs population, the ratios of aNSCs compared to the total aNSPCs are significantly elevated in DKO and TKO brains compared to THC with no significant difference between TKO and DKO (just a slight decrease in DKO versus TKO). These findings clearly indicate that p107 (and not

Rb nor p130) plays a distinct role in negatively regulating stem cell division in the adult brain, and this is consistent with previous study showing expansion in stem cells pool in p107-null mice (Vanderluit et al., 2004). Moreover, these results indicate that the increase in the number of progenitor cells observed in DKO brains results indirectly from the expanded pool of activated stem cells in the aSVZ rather than from a direct control of progenitor proliferation by p107. Previous studies have shown that p107 negatively regulates stem cell division through the transcriptional repression of several genes in the Notch pathway and $p107^{-/-}$ mice exhibit enhanced activation of this signaling pathway which was shown to promote NSC division at the expense of quiescence (Chapouton et al., 2010; Vanderluit et al., 2004). The slight reduction in the size of the stem cell pool in DKO brains compared to TKO brains at 4 wpt indicates that Rb could partially compensate for the loss of p107 in regulating the size of the stem cell pool. To validate the potential compensatory role of Rb in this context, future studies should compare p107-single knockout with Rb/p107 DKO brains where the pocket proteins are deleted specifically in the stem cell pool, for example by using a GFAP-Cre transgenic model. p130 does not seem to play a major role in the regulation of adult stem cell development; however, analysis of the phenotype in p130 single KO will provide a definitive answer.

aNSCs in the aSVZ can either undergo consuming divisions and produce two progenitors (around 70% of the time), or self-renewing divisions and produce two stem cells (around 30% of the time) (Obernier et al., 2018). This disproportionate rate of division favoring the formation of progenitors leads to the age-dependent decline observed in AN (Obernier et al., 2018). We detected a similar but relatively faster decline in the size of the stem cells population at 8wpt in TKO and DKO brains

compared with 4 wpt. This was evidenced by the remarkable decrease in the ratios of stem cells over the total population aNSPCs in DKO and TKO compared with THC levels between the two time-points. Given the relatively short time elapsed between the two time-points, this data suggests the existence of premature exhaustion of the stem cell pool in the absence of pocket proteins.

E. Pockets proteins are required for proper neuroblast migration along the adult RMS and show functional redundancy in this context

To assess neuronal commitment, we quantified the number of DCX+ cells that label late/committed progenitors and neuroblasts in the rostral portion of the RMS (rRMS). We found that the numbers of DCX positive cells significantly increased in both the aSVZ and rRMS in TKO brains compared to THC brains at 4wpt and 8 wpt. This is also reflected by the higher ratios of (YFP+; DCX+/total YFP) in the rRMS in TKO versus THC brains. In contrast, the proportion of neuroblasts that are still proliferating compared to the total number of neuroblasts (YFP;Ki67;DCX / YFP; DCX) or the total number of YFP positive cells (YFP; Ki67; DCX cells/YFP) was surprisingly similar between TKO and THC brains at both time-points. This suggests that late progenitors/neuroblasts successfully commit to a neuronal lineage and exit properly the cell cycle despite the loss of all three-pocket proteins. This finding is not consistent with a previously described requirement for p107 in promoting neuronal commitment during development and highlights differential role(s) played by pocket proteins in the embryonic versus adult brain (Vanderluit et al., 2007). Consistent with the decline in the absolute numbers of aSVZ progenitors at 8wpt in TKO, we observed a similar and sharp decrease in the numbers of YFP+ neuroblasts in the rRMS in TKO

and DKO brains compared to THC at this time-point. This was again caused by massive apoptosis in neuroblasts inside the rRMS in addition to premature exhaustion of the stem cell pool in the absence of the Rb family.

In term of rostral migration, we found that a large number of neuroblasts successfully migrate along the RMS and reach the entrance of the OB as manifested by a largely thicker RMS in TKO brains compared to THC brains at 4wpt and 8wpt. However, we also detected the presence of several ectopically migrating cells towards different brain regions in TKO and DKO brains compared to THC brains. However, ectopic migration in TKO brains is much more severe than that in DKO brains. These cells are spread in the cerebral cortex and the striatum and the largest population is found inside the corpus callosum. This is likely due to close proximity of the corpus callosum as it is situated right above the LV and is lining the RMS. Moreover, it is the only other brain region where aSVZ-derived progenitors can migrate to other than the OB, albeit at low level (Menn et al., 2006). In fact, the aSVZ is known to continuously generate new oligodendrocytes in the corpus callosum; in addition, AN in this region is enhanced in de-myelinating diseases (Menn et al., 2006; Nait-Oumesmar et al., 1999; Picard-Riera et al., 2002). Similarly, migration to cortical and striatal regions is enhanced following injury (Arvidsson, Collin, Kirik, Kokaia, & Lindvall, 2002; Parent, 2003; Parent, Vexler, Gong, Derugin, & Ferriero, 2002). The above results indicate that loss of pocket proteins leads to ectopic migration of neuroblasts outside the RMS, and/or alternatively, to a possible change in lineage fate of aSVZ progenitors favoring the production of more oligodendrocytes (CC) and/or astrocytes (striatum). The latter possibility is worthy of further investigation in the future as it may have direct implications on how to manipulate cell fate in the aSVZ for regenerative purpose in case

of neurodegenerative diseases. Of note, single Rb-KOs did not display ectopic migration in the adult brain (Naser et al. 2016). Moreover, a single Rb allele significantly reduces the number of ectopically migrating YFP positive cells as detected in DKO versus TKO brains, suggesting the presence of compensatory mechanisms in the regulation of neuronal migration among pocket proteins.

Compared with AN, a role for Rb in neuronal migration is well characterized during embryonic neurogenesis. Rb was shown to promote tangential migration of cortical neurons by associating exclusively with E2F3 to inhibit the expression of neogenin during development (Andrusiak et al., 2011; McClellan et al., 2007). Moreover, Rb is required for the survival of Reelin+ Cajal-Retzius cells, which guide the radial migration of cortical neurons in the developing cortex (Ferguson et al., 2005). In addition, it plays a similar role in the migration of OSNs in the olfactory epithelium (Jaafar et al., 2016). The above data and studies indicate that pocket proteins play conserved role(s) in the control of neuronal migration in the embryonic and the adult brain and, may have potential compensatory mechanisms among each other.

F. The Rb family is required for the survival of adult-born GABA-ergic interneurons in the OB

We assessed survival of adult-born neurons inside the OB and found a drastic decline in the number of YFP positive cells in the GCL and GL in TKO mice compared to THC mice at both time-points examined. This underlines a severe survival defect and/or terminal differentiation affecting newborn neurons in the absence of the Rb family. Interestingly, one allele of Rb in DKO brains was sufficient to rescue this phenotype and restore the correct number of YFP cells to control levels, indicating that

Rb is the main pocket protein that is required for survival of adult-born GABA-ergic interneurons. Further investigation of the reason behind this drastic decline in newborn neurons in TKO brains revealed the presence of massive cell death or apoptosis in YFP;DCX double positive neuroblasts starting in the aSVZ and RMS in TKO at both time-points, but almost none in DKO or THC. Moreover, we failed to detect any (AC3; NeuN) double positive cells inside the OB in TKO, indicating that neuroblast likely died before terminally differentiating or entering the OB. Given that only a small fraction of the residual YFP+ cells found in the OBs in TKO co-labeled with NeuN, we conclude that these cells are probably incompletely recombined. On the other hand, it would be interesting to investigate whether p130 plays a role in terminal differentiation inside the OB and whether it could partially compensate for the loss of Rb in maintaining survival at least in the short-term. Future studies should aim at studying this role as well as the combined role of Rb and p130 DKO in this context. In fact, loss of Rb in mature cortical neurons during development leads to ectopic activation of cell-cycle proteins such as Ki67, DNA damage and apoptosis (Andrusiak, Vandenbosch, Park, & Slack, 2012). However, apoptosis was not caused by the upregulation of E2F-induced pro-apoptotic genes such as Apaf1 and Puma (Andrusiak et al., 2012) but by E2F-dependent chromatin remodeling (Andrusiak, Vandenbosch, Dick, Park, & Slack, 2013) Similarly, p130 was shown necessary to maintain a post-mitotic state in mature cortical neurons in culture (Liu et al., 2005). It should be noted that Rb knockout (or DKO or TKO of pocket proteins) in progenitors versus mature neurons could lead to different consequences and thus, could involve distinct regulatory mechanisms as previously shown in cortical excitatory progenitors following TKO of pocket proteins (Oshikawa et al., 2013).

Since the discovery of aNSCs, the field of AN has been evolving at a fast pace, along with the technology needed to visualize and manipulate these stem cells. Despite the progress in this field, brain regeneration and the clinical relevance of aNSCs remain limited and hindered by our understanding, or lack thereof, of the complex molecular mechanisms that underlie these functions. Thus, more studies are needed to understand such mechanisms, especially those that promote survival and integration of adult-born neurons into pre-existing or damaged neuronal circuits. The Rb family and its role in this context represent a good example of how intrinsic factors can have such an important impact on stem and progenitor cell properties and behavior at all stages in development. Finally, AN re-defined our perception of brain plasticity and challenged our viewpoint of the brain as a post-mitotic organ and gave hope for potential intervention in regenerative medicine.

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