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THE ROLE OF THE RETINOBLASTOMA PROTEIN (PRB) IN THE DEVELOPMENT OF THE OLFACTORY SYSTEM

by
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AN ABSTRACT OF THE THESIS OF

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TITLE: The role of the retinoblastoma protein (pRb) in the development of the olfactory system

The Retinoblastoma, pRb, is a tumor suppressor gene that plays important roles in brain development primarily by controlling cell division at the G1-S phase checkpoint. In addition, loss of Rb causes neuronal differentiation and migration defects in the developing brain. We investigated here the role of Rb in the development of the olfactory system (OS) which is comprised of the olfactory epithelium (OE) and the olfactory bulb (OB). "Neuron formation" or neurogenesis and "synapse creation" or synaptogenesis in the OS are regulated by reciprocal interactions between the OE and the OB during development. We analyzed layer organization inside the OS and studied the development of the olfactory sensory neurons (OSN) and olfactory nerve layer (ONL) in the absence of Rb. To do this, we performed a conditional Rb's deletion in the telencephalon and OS by crossing Foxg1-Cre mice and $Rb^{floxed/floxed}$ mice. Then, we analyzed the OS phenotype in Rb-null mice and control littermates between E12.5 and birth using used cresyl-eosin staining, immunohistochemistry and *in situ* hybridization. We assessed neurogenesis and synaptogenesis in the OS and found that, starting E15.5, loss of Rb leads to: 1) enhanced neurogenesis manifested by increased progenitor proliferation of immature OSNs and thereafter, increased thickness of the mutant OE, 2) aberrant radial migration causing ectopic localization of immature OSNs in the intermediate zone of OE and coupled to terminal maturation defects in OSNs, 3) increased apoptosis in both the OB and OE with gradual degeneration of the latter around birth, and finally, 4) axonal guidance defects affecting the ONL and leading to a loss of connectivity between the OB and OE during late development. Our data demonstrates that Rb is required for normal development and morphogenesis of the OS and emphasizes a novel role for this cell cycle protein in the establishment of appropriate neuronal connections between different brain regions.

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LIST OF ABBREVIATIONS

%	percent
+	positive
AC-3	active caspase 3
A-P	anterior-posterior
aNSC	adult Neural Stem Cell
bHLH	basic helix-loop-helix transcription factor
BL	basal lamina
BrdU	5-bromo-2'-deoxyuridine
cAMP	cyclic Adenosine Mono Phosphate
Cdks	cyclin dependent kinases
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CR	calretinin
Cre	Cre recombinase enzyme
Ct	control
d	day
DIG	digoxigenin
Dlx	*Distal-less* related mouse homologue
DNA	deoxyribonucleic acid
D-V	Dorsal-Ventral
E	embryonic day

E2F	E2f transcription factor
EPL	external plexiform layer
GABA	gamma-aminobutyric acid
GAP 43	Growth Associated Protein 43
GBCs	globose basal cells
GCL	granule cell layer
GCs	granule cells
GL	glomerular layer
GnRH+	Gonadotropin-releasing Hormone-positive cells
HBCs	horizontal basal cells
hr	hour
INP	Intermediate Neuronal Precursor
IPL	internal plexiform layer
Kg	kilogram
KO	knock out
LGE	lateral ganglionic eminence
LOT	lateral olfactory tract
LP	lamina propria
Mash 1	Mammalian Achaete-Scute Homolog 1
MCL	mitral cell layer
MCs	mitral cells
mg	milligram
min	minute

ml	milliliter
mm^2	millimeter square
M/T cells	mitral and tufted cells
Mut	mutant
NC	nasal cavity
Ngn	Neurogenin1
Np	nasal pit
Nrp2	Neuropilin2
Nrp1	Neuropilin
OB	olfactory bulb
OE	olfactory epithelium
ON	olfactory nerve
ONL	outermost olfactory nerve layer
OR	Olfactory Receptor
OS	Olfactory system
Ops	Olfactory placodes
OMP	olfactory marker protein
ºC	Degrees Celsius
OSN	olfactory sensory neurons
OSN_i:	immature OSN
OSN_m	mature OSN
P	post-natal day
P	placode

$p27^{kip1}$ /p27	tumor protein 27
P5	Postnatal 5
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGCs	periglomerular cells
pOB	prospective olfactory bulb
PONl	prospective Olfactory Nerve Layer
pRb	retinoblastoma protein
PSA-NCAM	polysialylated neural cell adhesion molecule
Rb	retinoblastoma
RBL1	retinoblastoma-like 1
RBL2	retinoblastoma-like 2
RMS	rostral migratory stream
SCLC	small cell lung cancer
Sema3A	Semaphorin3A
Sema3F	Semaphorin3F
SVZ	subventricular zone
SUS	sustentacular cells
T	telencephalon
Tuj1	βIII-tubulin
V	ventricle
VZ	ventricular zone

μl	microliter

CHAPTER I

INTRODUCTION

A. The Olfactory System:

A.1. Development of the Olfactory System

The Olfactory System (OS) is involved in the integration and processing of olfactory sensory information along with other components of the Central Nervous System (CNS). The main olfactory system is comprised of the Olfactory Bulb (OB) and Olfactory Epithelium (OE) (reviewed by Mori et al. 1999; Firestein 2001). The OB and the OE emerge from spatially segregated regions, and undergo concurrent, yet independent developmental programs during early formation (López-Mascaraque and de Castro 2002). Hence, the OE emerges from specialized regions of the cranial non-neural ectoderm, while the OB grows from the germinal zone of the neural tube (reviewed in López-Mascaraque and de Castro 2002). At later stages in development, the two developmental programs become interrelated as axons from olfactory cells in the OE innervate the nascent OB (Derer et al. 1977).

A.2. Formation of the Olfactory Epithelium

The OE is a pseudo-stratified epithelium that lines part of the nasal cavity. Its structure and function are vastly conserved among mammals (Nibu et al. 1999; Rawson and Gomez 2002). The mouse OE has a particularly high regenerative capacity; the whole neuronal population is regenerated every 4-6 weeks. Moreover, the mouse OE serves as an ideal

model for investigating the properties of neural stem cells and progenitor cells at the molecular and behavioral levels *in vivo* because of its simple cellular composition, made up of a single type of neuron, and due to the fact that continuous neurogenesis is maintained in it throughout life (Graziadei and Graziadei 1979; Schwob 2002; Beites et al. 2005).

OE development starts at Embryonic Day 9.5 (E9.5) and grows from the Olfactory Placodes (OPs) located in the rostro-lateral head region. Two distinct phases of OE neurogenesis are clearly defined: (i) Early Neurogenesis, and (ii) Established Neurogenesis (Beites et al. 2005).

A.2.a. Early Neurogenesis

At E10, the olfactory nasal pits form by invagination of the OPs towards the forebrain, indicating the commencement of "early neurogenesis". This process, also known as "primary neurogenesis", is characterized by continuous proliferation of OP/OE cells, some of which become neurons localized in a scattered pattern in the OP (Cau et al. 1997; Cau, Casarosa, and Guillemot 2002; Ikeda et al. 2007). At this stage of development, the OE is not spatially segregated yet and consists of a single layer of neuroepithelial cells that have their cell bodies spread across the whole OP/OE region (Ikeda et al. 2007) (**Figure 1**).

Two distinct classes of neurons are generated during primary neurogenesis: (i) the pioneer neurons and (ii) the early-differentiated neurons (Ikeda et al. 2007). Pioneer neurons are migratory cells that exit the OP/OE and reside in a region between OP/OE and the forebrain. At later developmental stages, these OE-derived neurons aggregate with the 'migratory mass', described in Section A.4 (Schwanzel-Fukuda et al. 1992). The other class includes the early-differentiated neurons which were found to express βIII-tubulin, an early

differentiation marker, however, their cell fate remain poorly characterized till date (Ikeda et al. 2010, Heron et al. 2013) (**Figure 1**).

A.2.b. Established Neurogenesis

OP/OE cells continue to proliferate during OE development and they become multipotent cells differentiating into distinct cell types, such as ensheathing cells, sustentacular cells, neurons and cells of the vomeronasal organ. Established neurogenesis starts at E12.5. During this phase, basal and apical progenitors are identified. A subpopulation of the OE cells become progenitors that express, the bHLH (basic helix-loop-helix) transcription factor, Mash 1 (Mammalian Achaete-Scute Homolog 1). In turn, Mash1+ cells give rise to Ngn1+ (Neurogenin1+) transit amplifying progenitors that are Intermediate Neuronal Precursors (INPs). INPs divide and generate neuroblasts that express the differentiation gene Neuro D. These neuroblasts differentiate into mature Olfactory Sensory Neurons (OSNs) (Cau et al. 1997; Cau, Casarosa, and Guillemot 2002; Calof et al. 2002; Manglapus, Youngentob, and Schwob 2004). Another subpopulation of the OE cells becomes apical progenitors which are mainly responsible for generating Sustentacular cells (**Figure 1**). At E13.5, the embryonic OE starts to organize into a mature layered structure atop a defined basal lamina and arranged in three compartments: the apical, the middle and the basal compartment (Beites et al. 2005; Ikeda et al. 2007; Ikeda et al. 2010, Heron et al. 2013) (**Figure 2B**).

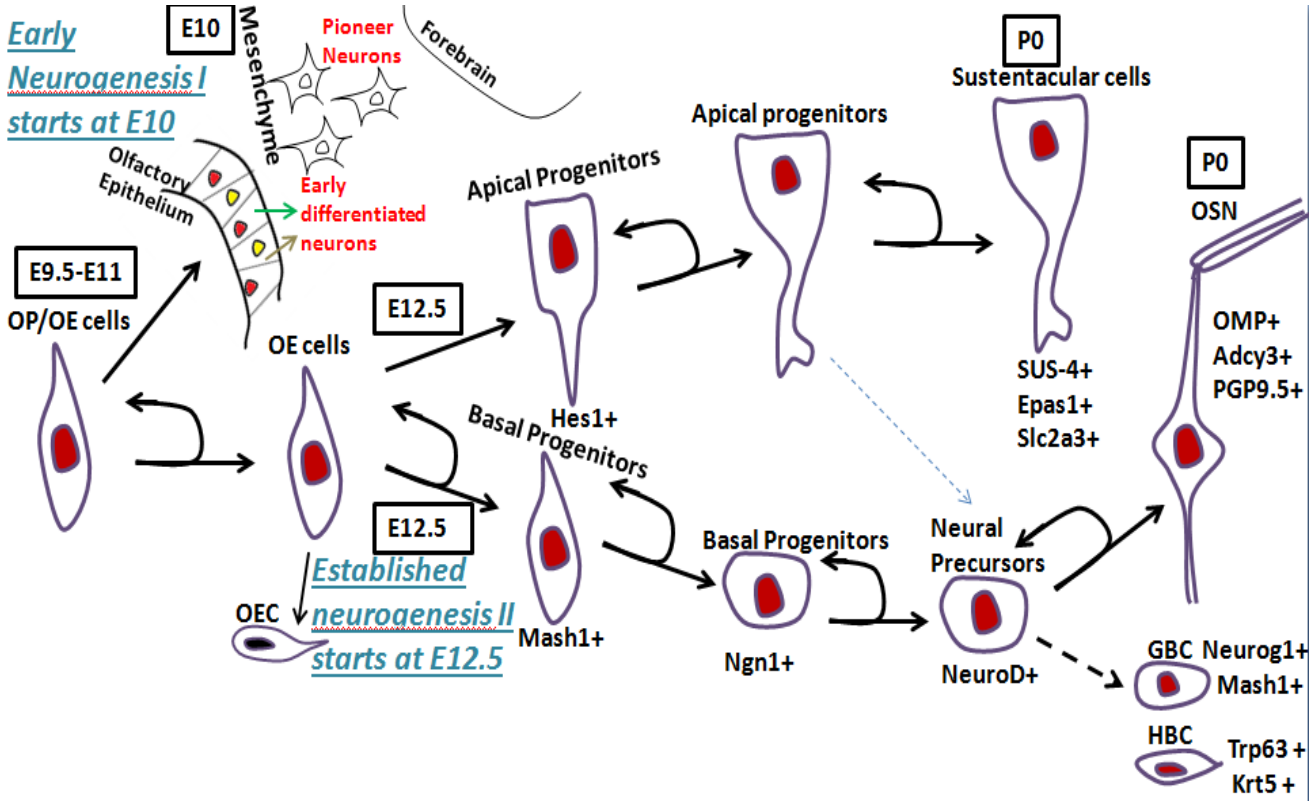


Figure 1: Early neurogenesis and established neurogenesis during OE development. (**I**) Starting at E9.5, OP/OE cells undergo proliferation. At E10, early neurogenesis begins whereby some of the OP/OE cells differentiate into neurons (early differentiated cells) and pioneer neurons exit the placode to reside in the mesenchyme between OP/OE and forebrain. (**II**) Established neurogenesis starts at E12.5: multipotent OE cells continue to proliferate and differentiate into different cell types. During this phase, basal and apical progenitors are identified: hence, a subpopulation of OE cells becomes basal progenitors that express Mash1 and reside in the basal compartment of the OE. Mash1-positive progenitors give rise Neurogenin (Ngn) positive progenitors which divide and generate NeuroD-positive neuroblasts. These cells differentiate into OMP- positive OSNs. It is still controversial whether NeuroD positive progenitors directly become GBCs and HBCs in the postnatal OE. A second subpopulation of OE cells differentiates into apical progenitors that occupy apical regions in the developing OE. These self-renewing progenitors give rise to glial-like sustentacular cells (as modified from Ikeda et al. 2010, Heron et al. 2013)

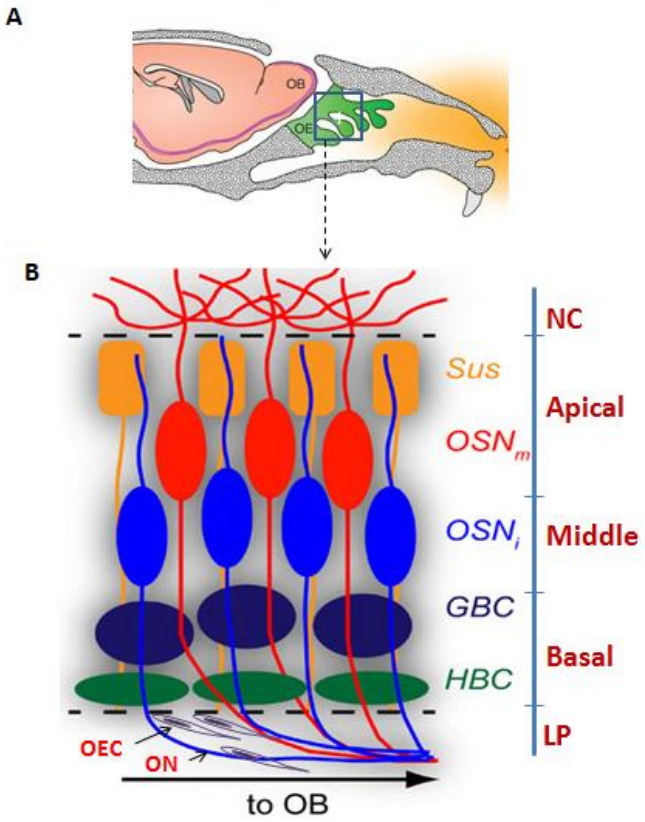


Figure 2: Structure of the Olfactory Epithelium **A**. Schematic drawing representing sagittal view of the Olfactory System **B**. Schematic diagram representing cellular organization of the OE. The glial olfactory ensheathing cells (OECs) are located in the lamina propria (LP) outside the OE. The GBCs and HBCs occupy the basal region of the OE: the HBCs are attached to the basal lamina (BL) and lie below the GBCs. The immature OSNs (OSN_i) reside in the middle region of the OE and mature OSNs (OSN_m) are bipolar neurons that reside in more apical regions of the OE. They project their axons to the LP forming an olfactory nerve (ON) while they extend their dendrites to the nasal cavity (NC). The Sustentacular cells (SUS) occupy the apical region of the OE and provide structural and functional support to OSNs (modified from Brann and Firestein 2014)

A.2.c. Basal Stem Cells: GBCs and HBCs

The OE basal compartment is close to the Lamina Propria (LP) which lies beneath the epithelium and is occupied by two distinct types of basal stem cells: the Globose Basal Cells (GBCs) and Horizontal Basal Cells (HBCs). HBCs are adjacent to the basal lamina and lie immediately below the GBCs layer (Beites et al. 2005) (**Figure 2B**). The round

shaped GBCs are actively proliferating cells and contain the major multipotent stem cells and their derived progenitors that give rise to the OSN lineage and other types of cells during OE development (Calof, Lander, and Chikaraishi 1991; Caggiano, Kauer, and Hunter 1994; DeHamer et al. 1994; Huard et al. 1998; Calof et al. 2002; Kawauchi et al. 2004). On the other hand, the flat shaped HBCs cells are largely quiescent and divide less frequently during development. It was thought that stem cells that give rise to OSNs belong only to the GBC population (Caggiano, Kauer, and Hunter 1994; Huard et al. 1998) (**Figure 2B**); however, recent fate-mapping studies showed that HBCs serve as an important source of OSNs not only during development but also in the adult OE. Furthermore, HBCs were shown to regenerate both neuronal and non-neuronal cells during normal turnover, as well as, after traumatic injury (Carter, MacDonald, and Roskams 2004; Leung, Coulombe, and Reed 2007; Iwai et al. 2008).

A.2.d. Sustentacular Cells

Early proliferative progenitors characterized by elongated nuclei densely pack the apical compartment of the developing OE (Murdoch and Roskams 2007). Some of these progenitors give rise to apical SUS-4 positive Sustentacular cells (**Figure 1** and **2B**), which are glial-like cells that structurally and functionally support OSNs and are distinct from the axon-ensheathing cells of the LP (Goldstein and Schwob 1996). The remaining progenitors produce more progenitors, some of which migrate to the basal region, where they undergo a transition from proliferative to terminally dividing cells that generate OSNs (Murdoch and Roskams 2007; Kawauchi et al. 2004; Rosenbaum, Duggan, and García-Añoveros 2011).

A.2.e. Immature and Mature Olfactory Sensory Neurons (OSNs)

OSNs can be subdivided into immature and mature populations (Farbman and Margolis 1980; Verhaagen et al. 1989). Immature OSNs express βIII-tubulin and GAP-43 (Growth Associated Factor- 43) and migrate from the basal compartment towards more apical positions of the OE as they mature (Miragall and Mendoza 1982). Mature OSNs are characterized by the expression of the OMP and their cell bodies occupy the middle compartment of the OE (Nibu et al. 1999; Rawson 1999) (**Figure 2B**). They constitute the major population of neurons that are involved in olfactory sensation (odor detection) and processing of the sensory information to the OB (Nibu et al. 1999).

A.3. Formation of the Nasal Cavities

The frontonasal epithelial thickenings that form the olfactory primordia in mice are first identified at E9; however, they are not differentiated at this early age yet. By E10, the placodal epithelium thickens considerably and starts to invaginate forming a bowl-like structured olfactory pit (**Figure 3; panel B**) (LaMantia et al. 2000). Twelve hours later, at E10.5, the thickened nasal pits invaginate further defining the olfactory epithelium (**Figure 3; panel C**). By E11.5, the pits deepen further and become gradually more elaborate leading to the formation of the nasal and vomeronasal cavities (**Figure 3; panels D and E**). This process is accompanied by the translocation of the developing nasal structures from a lateral to a medial position in the developing head (**Figure 3; panels F and G**). Finally, the fusion of the pits and cartilage condensation take place at the facial midline leading to the formation of a single nose with two nares (reviewed by Carstens 2002).

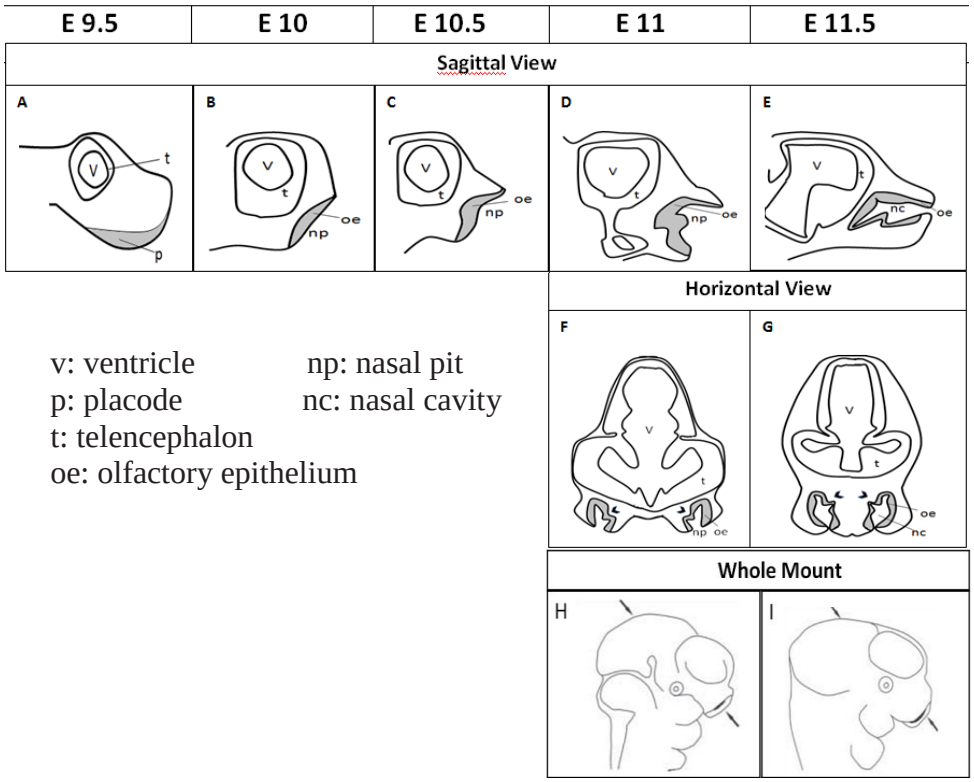


Figure 3: Development of the OE and the nasal cavity between E9.5 and E11.5. At E9.5, the olfactory placode appears as a thin cellular layer (panel A; shaded region). By E10, a cup-like structured nasal pit is formed upon invagination of the placode (panel B). At E10.5, further invagination deepens the developing pits (panel C). At E11, a second invagination within the nasal pits (arrowheads in panels F and G) lead to formation of the vomeronasal cavities (panel D). At E11.5, the nasal and vomeronasal pits deepen and become more elaborate (panel E). The developing nasal structures translocate from lateral to medial positions (horizontal view; panels F and G). The arrows in the whole mount diagrams indicate the plane of section in the horizontal diagrams (Whole Mounts; panels H and I) (as modified from Treloar et al. 2010).

A.4. Formation of the Olfactory Nerve Layer

One characteristic feature that makes OSNs unique among all other sensory transduction neurons such as glial cells is that they do not follow an established migratory pathway (Doucette 1984; Balmer and LaMantia 2005). In order to initiate formation of the olfactory nerve, the "migratory mass", must pioneer a pathway from the periphery to the telencephalon, *de novo*. Extrinsic cues underlying formation of a permissive pathway for

projecting OSN axons include guidance cues released from the adjacent frontonasal mesenchyme, in addition to, chemotrophic cues secreted from the telencephalon (Whitesides and LaMantia 1996; Balmer and LaMantia 2004).

Mature OSNs project their axons through the OE and across the underlying basal lamina, then enter the frontonasal mesenchyme and navigate towards the ventromedial region of the telencephalon where the OB will develop. These OSN axons are associated with a heterogeneous population of migratory cells forming an accumulation known as the "migratory mass" (LaMantia et al. 2000). The "migratory mass" is mainly composed of Olfactory Ensheathing Cells (OECs) (Valverde, Santacana, and Heredia 1992), in addition to, other cells which include: (i) Olfactory Receptor (OR) protein-expressing pioneer neurons that are derived from the OE (Conzelmann et al. 2002), and, (ii) Gonadotropin-releasing Hormone-positive (GnRH+) cells, which migrate to diencephalic brain regions (Schwanzel-Fukuda and Pfaff 1989; Valverde, Heredia, and Santacana 1993; De Carlos, López-Mascaraque, and Valverde 1995).

A.4.a. Role of Olfactory Ensheathing Cells (OECs)

OECs are glial cells that reside in the LP and express molecules that promote adhesion and guidance such as Integrin-alpha 6 and Laminin (Chuah and West 2002; Whitley et al. 2005). Their main function is to enwrap OSN axons and guide their projection from the OE to the OB (Raisman 1985). OECs were shown to exit the placode before OSN axons and serve as their guideposts as they migrate slightly ahead of OSN axons en route to the OB. Moreover, several studies have implicated OECs in guiding regenerating axons back towards their

final targets and this occurred not only during normal neuronal turnover, but also after injury or damage (Graziadei and Graziadei 1979; Doucette 1984; Murthy et al. 2014).

A.4.b. Stages of Olfactory Nerve Development

The growing "migratory mass" does not establish contact with the rostral telencephalic surface until E11.5 (**Figure 4; panel A**). At E12, OSN axons penetrate the rostral-most tip of the telencephalon through small fenestrations in the basement membrane of the forebrain (Marin-Padilla and Amieva 1989; Treloar, Nurcombe, and Key 1996; Gong and Shipley 1996; Balmer and LaMantia 2004). Moreover, OSN axons along with the associated migratory mass fasciculate into the prospective Olfactory Nerve Layer (pONL) and a small subset of axons extend deeper into the ventricular zone (VZ) of the telencephalon (Pellier and Astic 1994) (**Figure 4; arrow head, panel B**). At E12.5, these deep VZ-axons are no longer observed. OSN axons remain in the nerve layer surrounding the nascent OB until glomerulogenesis begins at E15 (Treloar, Purcell, and Greer 1999) (**Figure 4; panel C**). This waiting period (from E12.5 till E15) has been hypothesized to be essential for axon sorting (described in Section A.6.b) prior to the establishment of proper topographic map in the OB. As OSN axons grow deeper into the OB they form axodendritic synapses with second-order neurons, the mitral cells, located in the glomeruli of the OB. Continuous growth of the nerve layer occur as more axons extend to the OB (Treloar, Purcell, and Greer 1999) (**Figure 4; panel D**).

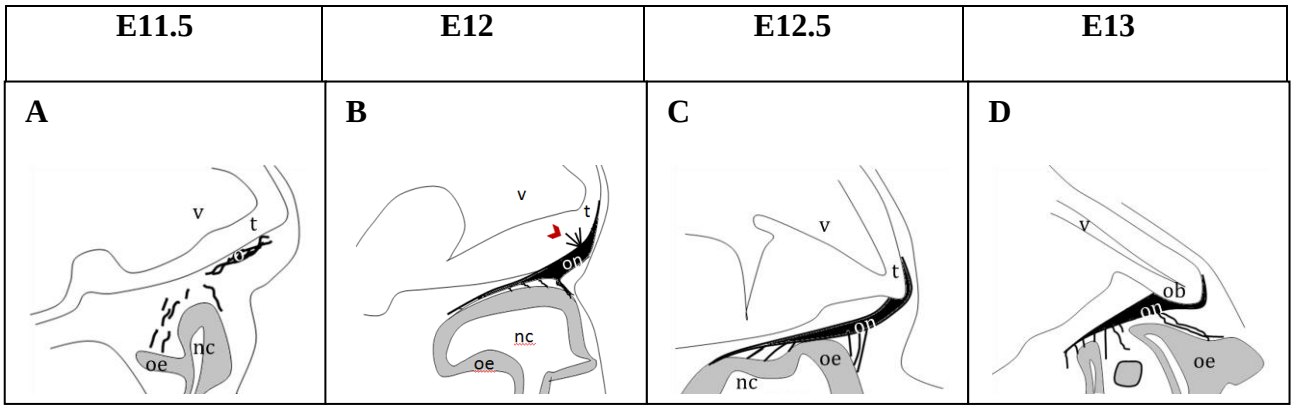


Figure 4: Development of the olfactory nerve between E11.5 and E13. At E11.5, OSN axons grow towards the telencephalon (panel A). Later at E12, axons penetrate the basement membrane and form a nerve layer around the prospective OB. Few OSN axons are seen to penetrate deeper into the telencephalon at this age (red arrowhead; panel B). At E12.5, the olfactory bulb becomes macroscopically observed. OSN axons fasciculate to form a distinct ONL surrounding the nascent OB (panel C). Continuous growth of the nerve layer occurs as more axons extend to the OB (panel D). v: ventricle, nc: nasal cavity, on: olfactory nerve, t: telencephalon, oe: olfactory epithelium. (as modified from Treloar 2010)

A.5. Development of the Olfactory Bulb

During early embryogenesis, the telencephalon is predetermined into distinct areas that give rise to different adult brain regions including the cerebral cortex, the basal ganglia, the basal forebrain and the olfactory bulb. Rakic (1988) et al. was the first to propose such a model, which became widely supported by later studies (Grove and Tole 1999; Hack et al. 2004; Mathis and Nicolas 2006; Danjo et al. 2011). The OB grows from a pre-specified rostral telencephalic region and appears as a macroscopically distinct evagination of the rostral telencephalon starting at E12.5 (Sugisaki et al. 1996) (**Figure 4;panel C**). OB development ends at birth although maturation of OB GABAergic neurons is completed around the third post-natal week (P21) in mice and is therefore experience-dependent (Kelsch, Lin, and Lois 2008).

Patterning of the telencephalon was shown to be established by the strict spatio-temporal expression of an array of signaling molecules and transcription factors (Mallamaci and Stoykova 2006). Although induction of OB growth does not require any direct input from the OE (Jiménez et al. 2000), Long et al. (2003) provided evidence for the central role of OSN axons in layer formation and neuron organization inside the developing OB. Thus, loss of OE input resulted in random packing of Reelin-positive mitral cell bodies within the OB, in addition to, cell non-autonomous OB lamination defects (Long et al. 2003). Furthermore, *in vitro* studies have demonstrated that OE regulates mitral cell dendritic growth via secretion of ligands of the TGF-β family (Slotnick 2004; Tran et al. 2008).

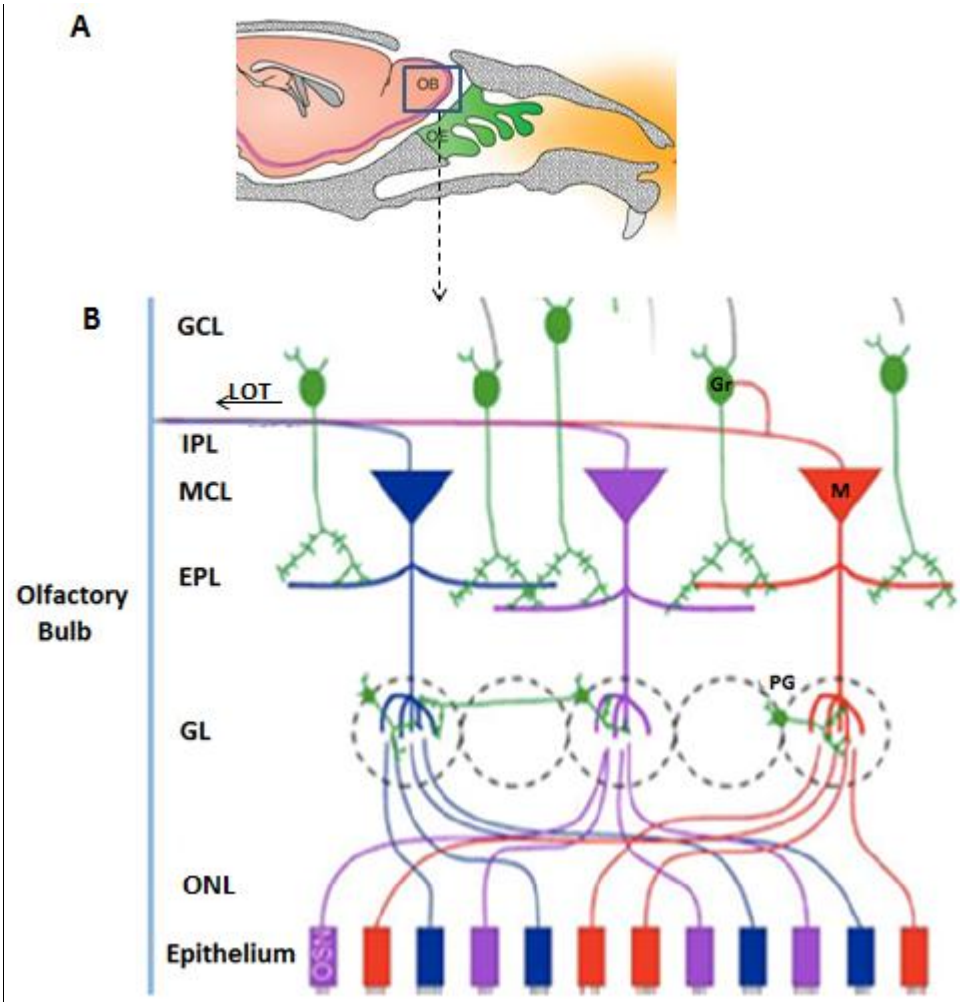


Figure 5: Structure of the Olfactory Bulb **A.** Schematic drawing representing sagittal view of the OS B. Schematic representation showing laminar organization of the OB. The outermost ONL is formed of OSN axons. The glomerular layer (GL) and granule cell layer (GCL) are comprised of distinct subtypes of GABAergic and dopaminergic neurons. The mitral cell layer (MCL) is made of mitral (M) and tufted cells sandwiched between the internal plexiform layer (IPL) and external plexiform layer (EPL). Axons of OSNs establish specific connections with dendrites of mitral and tufted cells in the GL as well as with periglomerular (PG) interneurons. Axons of mitral and tufted cells extend through the lateral olfactory tract (LOT) to the olfactory cortex. (as modified from Au et al. 2002).

The OB is a highly organized and laminated structure that integrates sensory odor information and relays it to the olfactory cortex, also known as the piriform cortex, for further processing (Shepherd 2007). It consists of six distinct layers that are anatomically characterized based on their cellular composition: (i) the outermost ONL; (ii) the

Glomerular Layer (GL) is occupied by glomeruli which are neuropils containing the OSN axons and the apical dendrites of mitral and tufted cells as well as periglomerular interneurons; (iii) the External Plexiform Layer (EPL) is made up of the lateral dendrites of mitral and tufted cells; (iv) the Mitral Cell Layer (MCL) is composed of cell bodies of mitral cells; (v) the Internal Plexiform Layer (IPL) is made up of granule cell dendrites; (vi) the Granule Cell Layer (GCL) consists of granule cells that are primarily GABAergic interneurons, the cells of highest abundance in the OB and connected to the axons of mitral cells (Parrish-Aungst et al. 2007) (**Figure 5B**).

A.5.a. Development of OB Projection Neurons

The principal excitatory projection neurons in the OB are the mitral and tufted cells (M/T cells) (Cajal 1894). These cells migrate radially from their site of origin in the ventricular zone (VZ) towards their final destination in the MCL of the OB (Hinds 1968). Blanchart et al. (2006) showed that establishment of mature morphology as well as functionality of these M/T cells is dependent on a spatio-temporal cross talk between their dendrites and the OSN axons. In this study, three developmental phases of mitral and tufted cells (M/T cells) were classified: Phase I (E11-E13) starts after genesis of M/T cells at E11. Newborn cells initially have their somas radially oriented towards the border of the prospective OB (pOB). Later at E12, a tangential orientation is adopted and these cells become parallel to the pOB surface. Phase II (E14-E16) is characterized by the arrival of OSN axons that stimulate a final radial reorientation of M/T cells. Immature M/T cells undergo elaborate apical dendritic growth towards the EPL and their axons are seen to extend in the opposite direction towards the piriform cortex through the Lateral Olfactory Tract (LOT). OSN

axons are observed to remain stationary within the ONL until E15 after which they start to fasciculate into "protoglomeruli", the site where glomerulogenesis commences later during postnatal development. During phase III (E17-adulthood), M/T cells adopt a mature morphology after which they have undergone extensive refinement and pruning. Mature M/T cells have a single dendrite extended to a glomerulus and several secondary horizontally projecting dendrites in the EPL (Blanchart, De Carlos, and López-Mascaraque 2006).

A.5.b. Development of OB Interneurons

Most OB interneurons are produced between E18 and P5 (Hinds 1968). However, recent birth dating studies showed that the initial population of interneurons is generated from LGE precursors as early as E12.5 (Tucker, Polleux, and LaMantia 2006). These early generated cells migrate selectively into the rudimentary OB pioneering a pathway that defines the rostral migratory stream (RMS) (Tucker, Polleux, and LaMantia 2006). Most OB interneurons differentiate into distinct mature subtypes by postnatal day 21 (P21) (Kelsch, Lin, and Lois 2008). Continuous neurogenesis of interneurons is maintained during adult postnatal life in mice albeit at a lower rate. LGE-derived adult Neural Stem Cells (aNSCs) are found in the SVZ lining the lateral ventricle where they continue to generate neuroblasts in the adult brain. Neuroblasts migrate along chains of astrocytes through the RMS to the OB, where they differentiate into mature granule and periglomerular interneurons (Alvarez-Buylla et al. 2008; Doetsch, García-Verdugo, and Alvarez-Buylla 1999; Gil-Perotin et al. 2009; Taupin 2006; Whitman and Greer 2009; Ming and Song 2011).

A.6. Establishment of the Glomerular Topographic Map in the Olfactory System

The formation of accurate and specific stereotypic connections between OSNs in the periphery and second-order neurons located within the CNS is critical for precise internal representations and olfactory perception of the external environment. In 1991, Axel and Buck discovered around 1,000 different Odorant Receptors (ORs) which are expressed by OSNs and detect various odorants (Buck and Axel 1991). Serizawa and Miyamichi (2004) established the "One neuron- One receptor rule" by which, each OSN expresses a single functional OR out of the 1,000 ORs. Moreover, Mombaerts et al. (1996) showed that OSNs expressing similar ORs project their axons to two specific stereotypically positioned glomeruli in the OB, thus applying "One glomerulus-One receptor rule". Hence, the pattern of the activated glomerular map in the OB serves as a topographic representation of odor information detected by OSNs in the OE (Mori et al. 2006; Johnson and Leon 2007; Matsumoto et al. 2010). During development of the OS, the glomerular map is structured by the combination of two processes: Projection of OSN axons along the Dorsal-Ventral (D-V) axis is an OR-independent process while projection of the same axons along the anterior-posterior (A-P) axis is OR-dependent (Sakano 2010).

A.6.a. Establishment of the Dorso-Ventral Arrangement of OSNs

The projection of OSN axons to a specific glomerular location along the D-V axis is correlated with the anatomical position of the corresponding OSN cell bodies in the OE (Astic, Saucier, and Holley 1987). This spatial relationship between neuronal cell somas and the target sites of their corresponding axons is also conserved in other systems in the brain such as the visual system (McLaughlin and O'Leary 2005; Luo and Flanagan 2007).

Establishment of an accurate topographic glomerular map along the D-V axis requires spatio-temporal regulation by two repulsive signaling systems: the receptor Robo2 and its ligand Slit1 on one hand and the receptor Neuropilin2 (Nrp2) and its ligand Semaphorin3F (Sema3F) on the other hand (Cloutier et al. 2002; Walz, Rodriguez, and Mombaerts 2002; Cho et al. 2007).

A.6.a.1. Robo2/Slit1 signaling

Cho et al. (2012) reported that Robo2/Slit1 repulsive signaling contribute to accurate axonal innervations along the D-V axis. OSNs in the dorsal zone of the OE mature before those in the ventral zone (Sullivan et al. 1995). Thus, repulsive signaling between Robo2 and Slit1 is required to limit the first wave of OSN projection to the dorsal domain of the OB (Miyasaka et al. 2005; Cho et al. 2007; Takeuchi et al. 2010). Early arriving Robo2+ dorsal-zone axons strictly innervate the anterodorsal domain of the OB through repulsive signals by Slit 1 expressed in the septum and the ventral domain of the OB, hence, the first glomerular structure develops in the anterodorsal region of the OB (Cho, Kam, and Cloutier 2012).

A.6.a.2. Nrp2/Sema3f signaling

Nrp2 and its repulsive ligand Sema3F are expressed in a graded manner by OSN axons (Takeuchi et al. 2010). Nrp2+ axons are late arriving-ventral zone axons and restricted to the ventral region of the OB through repulsive interaction with Sema3F, which is secreted in the dorsal region of the OB by early-arriving-dorsal zone axons. As a result, OSNs project their axons sequentially from the dorsomedial to the venterolateral zone of the OE, which contributes to the establishment of topographic map order in the OB along the

dorsoventral axis (Takeuchi et al. 2010). In summary, the sequential arrival of dorsal zone OSN axons followed by the ventral zone ones along with the graded and complementary expression of Nrp2/ Sema3F, and Robo2/Slit1 repulsive interaction altogether play important role in establishing map order in the OB.

A.6.b. Instructive Role of Olfactory Receptors in Establishment of the Anterior-Posterior Arrangement of OSNs

Anterior-Posterior (A-P) projection depends on the expressed OR species (Imai, Suzuki, and Sakano 2006). ORs controls A-P targeting at the transcriptional level by regulating the expression of the second messenger, cAMP (cyclic Adenosine Mono Phosphate). Unique cAMP levels determined by the OR species thus regulate expression of repulsive signaling cues such as the receptor Nrp1 and its ligand Sema3A. Axons of OSNs that generate elevated levels of cAMP innervate the posterior domain of the OB, whereas those that generate low levels innervate the anterior domain (Imai, Suzuki, and Sakano 2006). Similarly, Nrp1 levels in OSN axon termini show a gradient expression with low levels found in the anterior OB and high levels in the posterior domain. Any change in Nrp1 expression in OSNs therefore results in glomerular shifts either posteriorly or anteriorly (Imai et al. 2009). Recent studies have shown that the topographic order of the glomerular map is determined by sorted axon bundles, before they reach the OB. In fact, Nrp1 and Sema3A are expressed in a complementary manner in OSNs: axons arranged in the central zones of the axon bundle have low Nrp1 and high Sema3A levels whereas axons sorted to outer-lateral zones of the bundle display opposite levels of expression with high Nrp1 and low sema3A levels (Imai et al. 2009). Therefore, formation of the topographic map order

and glomerular segregation along the anteroposterior axis in the OB is regulated by pre-target sorting within projecting axon bundles.

B. The Retinoblastoma Protein (pRb)

B.1. Overview

The retinoblastoma gene, Rb, is the first known tumor suppressor gene to be identified in humans (Huang et al. 1988). It was initially cloned from infants that have "Retinoblastoma", a rare eye cancer that arises in the retina (Knudson 1971). Since its discovery, the Rb protein has been found dysfunctional in many pediatric and adult human cancers (Dunn et al. 1988). Retinoblastoma exists in two forms: heritable and inheritable. The heritable form, which appears at early age can result either in a unilateral tumor (affects a single eye; 60% of total cases) or a bilateral tumor (affects both eyes; only heritable). This form is caused by biallelic Rb inactivation; one mutation is constitutional and the other hits somatic cells in the retina. Constitutional mutation predisposes patients to other cancers in the future such as brain, skin and lung cancer. The other form is inheritable, and results only in unilateral tumors. Majority of inheritable retinoblastomas arise from biallelic mutations (*de novo*) both hitting one somatic cell in the retina (Richter et al. 2003; Dimaras et al. 2012).
In mouse models, Rb inactivation has been reported to induce thyroid and pituitary tumors, as well as, induction of osteosarcomas, but not retinoblastomas (Cobrinik et al. 1996). Development of retinoblastomas requires loss of Rb associated with co-absence of one of its family members (p107 or p130) (Robanus-Maandag et al. 1998; Chen et al. 2004). Cooperation of Rb's loss with inactivation of other tumor suppressors was further shown to

cause other types of cancer. For instance, loss of Rb and p53 in cerebellar neural stem cells can cause initiation of medulloblastoma (Sutter et al. 2010). Moreover, Rb's loss was shown not only to be involved in the initiation of the wide variety of cancers as mentioned above but also is essential for cancer progression and metastasis at later stages in some cancers such as prostate cancer (Sharma et al. 2010).

B.2. Rb and Cell Cycle Regulation

Rb regulates cell cycle progression at the G1-S phase restriction point through interaction with the E2f family of transcription factors (Bandara et al. 1991; Cobrinik et al. 1993; Sherr 1993; Hamel et al. 1992).The E2f family consists of eight members; five of them can bind to Rb (E2f 1-5) (Maiti et al. 2005). E2f(1-3) are transcriptional activators, while E2f (4,5) have repressive roles (McClellan and Slack 2007). In normal non-growth inducing conditions, Rb, in its hypophosphorylated form is active and, stays bound to E2f (primarily E2fs 1-3) thus repressing its function and blocking transcription (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Ludlow et al. 1990). At the transition from G1 to S phase, D-type Cyclin-Dependent Kinases (CDKs) phosphorylate Rb, rendering it inactive (Cobrinik et al.1992). Released E2f binds to DNA resulting in transactivation of gene expression that promotes entry to the S-phase (Frolov et al. 2004; Dimova and Dyson 2005). CDK activity is itself under the control of two cell cycle CDK- inhibitors: p16 and p21, which belong to the INK4 and CIP/KIP families, respectively (Bunz et al. 1998; Passalaris et al. 1999; Taylor et al. 1999).

B.3. Family protein members: p107 and p130

The Rb protein family constitutes of three members: pRb (less commonly known as p105), p103 (or Rb2) and p107 (or Rb1*) (*Hannon et al. 1993; Li et al. 1993; Zhu et al. *1993).* Rb proteins share sequence homology in their pocket domains by which they bind E2f transcription factors (Ewen et al. 1991; Hannon, Demetrick, and Beach 1993; Li et al. 1*993;* Trimarchi and Lees 2002*)*. However, they were shown to differ in their binding affinity to E2fs. For example, Rb interact with E2fs 1-5, however, p107 and p130 form complexes only with E2f4 and E2f5 (Lees et al. 1993; Hijmans et al. 1995; Moberg, Starz, and Lees 1996; Dyson 1998; Trimarchi and Lees 2002*;* Kong et al. 2007). In vivo studies showed that these different complexes contribute to timed regulation of S-phase entry. Rb proteins operate in a sequential manner during the cell cycle: p130 associates with E2fs 4 and 5 in G0, p107 binds E2f4 in G1, however, Rb complexes with E2f4 in the S phase (Shirodkar et al. 1992; Cobrinik et al. 1996; Dyson 1998; Dimova and Dyson 2005). Rb also interacts with E2f1-3 to control entry into S-phase as previously stated. E2f6-8s are Rb-independent repressors (de Bruin et al. 2003; Logan et al. 2004; Logan et al. 2005; Maiti et al. 2005; Christensen et al. 2005). The Rb family members were shown to exhibit different functions in general, however, studies in Rb mutant mice demonstrated also overlapping and partially redundant roles among them in specific contexts.

C. Roles of Rb During Brain Development

Neurogenesis or neuronal development is a highly regulated process by which neural stem cells give rise to progenitor cells which, after a period of proliferation, commit to a specific neuronal lineage then exit the cell cycle, and differentiate into mature neurons. Strict cell

cycle regulation is critical during this developmental process (Ferguson and Slack 2001). Rb plays a central role in the control of cell proliferation at the G1-S phase transition during neurogenesis. Thus, for a long time, Rb has been observed as a cell cycle regulator 'only'. However, recent studies have shown that Rb's role goes beyond cell cycle control thus affecting neuronal migration, differentiation and survival in the brain (Callaghan et al. 1999; McClellan et al. 2007; Ruzhynsky et al. 2007; Ferguson and Slack 2001; Ferguson et al. 2005; Ghanem et al. 2012; McClellan and Slack 2007).

C.1. Rb's Role in Cellular proliferation

Rb-deficiency in the developing brain was studied using Rb mutant mice (full knock-out) and was initially reported to result in hyper-proliferation and extensive apoptotic cell death in dividing progenitors (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). While ectopic proliferation was found to be cell autonomous, widespread apoptosis was shown to be due to decrease in oxygen levels from defective placental development (Ferguson et al. 2002; MacPherson et al. 2003; Wu et al. 2003). Ferguson et al. (2002) used telencephalon-specific Rb conditional mutant mice to assess the role of Rb in cortical neurogenesis. Rb mutant mice survived till birth and exhibited enhanced neurogenesis manifested by excessive and ectopic proliferation of committed neuroblasts that were able to survive as well as cortical lamination defects. Similarly, *c*onditional Rb deletion in the retina caused ectopic division of differentiating precursors (Chen et al. 2004). While widespread cellular survival in the forebrain was shown not to be affected upon Rb's loss, studies showed that Rb was required for survival of specific cell types such as ganglion and photoreceptor cells in the retina (Chen et al. 2004). Moreover, conditional Rb deletion in cerebellar vermis

precursor cells caused increased proliferation and apoptotic cell death affecting specifically granule cell precursors (Marino et al. 2003).

C.2. Rb's Role in Migration

The first evidence for Rb's regulatory role in neuronal migration came from a study by Ferguson et al. (2005) who showed that absence of Rb resulted in aberrant tangential migration of cortical interneurons from their site of origin in the lateral ganglionic eminences in the ventral telencephalon towards their final destination in the dorsal cortex. In absence of Rb, GABAergic interneurons were properly specified during early neurogenesis; however, by mid-neurogenesis specific subtypes exhibited severe migration defects that were shown to be cell-autonomous, thereby implicating a role for Rb in migration (Ferguson et al. 2005). Direct evidence for Rb's role in neuronal migration came from subsequent studies that demonstrated that this function was mediated by its specific interaction with E2F3 (McClellan and Slack 2007) and through its regulation of neogenin, a gene involved in neuronal migration and axonal guidance (Andrusiak et al. 2011). More recently, a study by Ghanem et al. (2012) showed that Rb deficient neuroblasts exhibited defective radial and tangential migration in the telencephalon and the olfactory bulb during late development (Ghanem et al. 2012).

C.3. Rb's Role in Differentiation in the Brain and other tissues

Rb was shown to play a central role in differentiation in several cell types and tissues both *in vivo* and *in vitro*. In the retina, Rb's loss resulted in defective differentiation of cholinergic neurons (Chen et al. 2007) and this regulation by Rb was shown to be: 1)

exclusively mediated through E2f3 and 2) independent from its role in cell cycle control (Novitch et al. 1996; Chen et al. 2007). Rb was also shown to be essential for normal cellular differentiation in skeletal muscles: hence, Rb's loss was observed to cause inhibition of myoblasts differentiation (Novitch et al. 1996; Zacksenhaus et al. 1996; Huh et al. 2004; Hosoyama et al. 2011). Moreover, during adipogenesis, Rb was demonstrated to act as a molecular switch that promotes white adipocyte formation by blocking pathways that lead to differentiation of brown adipocytes (Fajas et al. 2002; Hansen et al. 2004; Scimè et al. 2005; Calo et al. 2010). In contrast, differentiation and function of hair cells was enhanced upon conditional deletion of Rb in mice (Mantela et al. 2005; Sage et al. 2005). In addition, *in vitro* studies showed that Rb controls quiescence in skin stem cells and satellite cells which are involved in muscle repair post-injury. In satellite cells, Rb's loss was shown to cause expansion of myoblast pool (Hosoyama et al. 2011) and inhibition of differentiation (Huh et al. 2004). Moreover, conditional deletion of Rb in stratified epithelia of the skin resulted in hyperkeratosis in addition to high perturbations in terminal epidermal differentiation. Nevertheless, the severity of these defects increased with coabsence of p107, indicating a partial compensatory role of this pocket protein in absence of Rb (Ruiz et al. 2004).

During brain development, the Rb-E2F pathway plays an important role in the control of the onset of differentiation in the telencephalon via the direct regulation of *Dlx1* and *2* transcriptional activities. In absence of Rb, increased expression levels of the repressor, E2f-7, caused downregulation of *Dlx*1/2 gene expression consequently blocking terminal differentiation (Ghanem et al. 2012). Taken together, these studies demonstrate a tissue-specific requirement of Rb during cellular differentiation.

C.4. Rb's Role in Development of the Olfactory System

The only report describing a role for Rb in the development of OS came from a recent study by Ghanem et al. (2012) that revealed a requirement of Rb for proper differentiation, migration and survival of specific neuronal subtypes in the OB during late development. In fact, telencephalic-specific Rb deletion resulted in gradual reduction and loss of GABAergic interneuron subtypes in the OB (Ghanem et al. 2012). The study showed that while neuronal progenitors were produced and specified normally in the LGE in absence of Rb, they exhibited defective terminal differentiation as well as rostral and radial migration defects in the OB. The study highlighted an indispensable requirement of Rb during late development in the OB, however, the role of Rb in the development and neurogenesis of OE and the interaction between OE and OB during development is still unknown.

CHAPTER 2

MATERIALS AND METHODS

A. Mice and tissue preparation

Foxg1-Cre male mice (He´bert and McConnell 2000) were mated with $Rb^{floxed/floxed}$ females: the Rb floxed allele has exon 19 flanked by two LoxP sites (Cre-specific sites) (Marino et al., 2000). $Rb^{floxed/floxed}$ females were bred on a mixed genetic background. All animals were maintained according to protocols approved by IUCAUC. Pregnant females at five embryonic time-points -Embryonic days- E12.5, E14.5, E15.5, E17.5 and E18.5 were given a single BrDU injection intraperitonealy 2h prior to sacrifice. When staging of embryos, E0.5 corresponds to the day vaginal plug was observed. Mice were euthanized with a Xyl/Ketamin solution of 1.5µl/g ketamine and 0.25µl/g Xylasine then sacrificed by cervical dislocation. Embryonic pups were collected in 1x PBS and decapitated. Their heads were fixed in 4% PFA (paraformaldehyde) and left at 4°C (3 h for E12.5 embryos, 6-8 h for E14.5 and E15.5, overnight for E17.5 and E18.5). Following fixation, the samples were washed several times in 1xPBS and cryoprotected using a sucrose gradient in 1x PBS as follows: 12% sucrose for 1 day, 18% for 1 day then 22% sucrose for 3 days. Finally, heads were embedded in OCT medium (Tissue-Tek Surgipath FSC 22 3801480), snap frozen in cold isopentane (Sigma Aldrich M32631) on dry ice. Samples were sectioned using a cryostat onto superfrost slides (company Fisher scientific and Thermo Scientific). Sagittal sections of 10µm thickness were obtained and stored at -80°C for later use.

Bromodeoxyuridine (BrdU) injection

10 mg/ml BrdU (Sigma B5002-250MG) solution was prepared using 0.9% NaCl and 0.007N NaOH and BrdU was administered to mice according to body weight (50mg/kg). For BrdU staining, tissue sections were treated as follows: 45s of incubation in acetone for dehydration followed by 1xPBS wash for 10 minutes, then incubation in 1N HCl for 20 minutes at 37°C to denature the DNA, and finally, neutralization in 0.1M sodium borate (pH=8.5) (Fisher scientific S-249) for 10min followed by wash in 1xPBS. Slides were then processed for immunohistochemistry as described in the section below.

B. Genotyping

Mice used for line maintenance were weaned 20-25 days after birth. Small earpieces were taken from adult mice and small tail pieces from embryos for genotyping. DNA extraction was performed using Phenol-chloroform-isoamyl alcohol mixture (SIGMA 77617) according to phenol -chloroform extraction protocol. Screening was done using the following primers (BioRad): *Rb flox primers*: forward 5' GGC GTG TGC CAT CAA TG 3' and reverse 5' AAC TCA AGG GAG ACC TG 3', *Cre primers:* forward 5'- TGA CCA GAG TCA TCC TTA GCG – 3' and 5'- AAT GCT TCT GTC CGT TTG CC – 3'. Rb heterozygous (Bf-1 Cre +/- Rb flox/+) embryos and Rb wild-type (Bf-1 Cre +/- Rb +/+) embryos showed the same phenotype and thus were both used as controls while Rb mutant mice carried the Bf-1 Cre +/- Rb flox/flox genotype.

C. Immunohistochemistry

Slides were air dried for at least 30 minutes after being removed from -80 °C then blocked for 1-2 h in a blocking solution (1% BSA (amresco 0332-25G), 0.3% Triton X, 5% normal

goat or donkey serum and 0.1M PBS). 1-2 hrs later, slides were incubated with primary antibody(ies) overnight at room temperature. The next day, sections were washed 3 times with 1xPBS for 10 minutes each, then incubated with fluorescent secondary antibodies diluted in blocking solution for 2h at room temperature. The following primary antibodies were used: mouse anti-BrdU (1:50; BD Pharmagon), rat anti-BrdU (1:150; Accurate), rabbit anti-OMP (1:300; Abcam), rabbit anti-tuj1 (1:5000; Covance), mouse anti-Bax-3 (1:300; Abcam), mouse anti-AC-3 (1:500; Cell Signaling). The following secondary antibodies (Molecular Probes, Invitrogen, and, Jackson Immunoresearch) were used: donkey anti-mouse 596 Alexa Fluor (red), donkey anti-rabbit 596 Alexa Fluor (red), goat anti-rat 596 Alexa Fluor (red), donkey anti-rat 596 Alexa Fluor (red), donkey anti-rabbit 488 Alex Fluor (green). Finally, slides were washed in 1x PBS for 3x5minutes and counterstained with Hoechst stain (to label nuclei), then mounted in (3:1) PBS/Glycerol solution.

D. Imaging and cell counting

All fluorescent and bight-field images were captured using an upright UV microscope (Zeiss Axiovert 200) with a digital camera and Axiovision software. Emission wavelengths of the secondary antibodies, Alexa 350, 488 and 596, were detected using the blue, green and red filters, respectively.

For cell counts, representative images were taken from medial regions of the OE. BrdU-positive cells and AC-positive cells were counted in the whole OE using Image J software. OE surface area was measured using the Image J software. Double positive cells were

visualized on overlapping images using Adobe Photoshop CS5. For statistical analysis, independent t-test was applied using SPSS and all results were obtained in triplicates (n=3 for each genotype).

E. *In situ* hybridization

In situ hybridization was performed as described in (Wallace and Raff, 1999). Anti-sense riboprobes were synthesized using digoxigenin (DIG)-labeled UTP. Hybridization was performed overnight at 65°C. Signal detection was performed using an AP-conjugated anti-DIG antibody (1:2000) (Roche) followed by incubation in NBT/BCIP solutions at room temperature. Mouse anti-sense riboprobes for Slit1 (obtained from R.Slack's Lab), Robo 2 (obtained from Tessier-Lavigne's Lab), Robo1 (obtained from Tessier-Lavigne's Lab), Slit 2 (obtained from R.Slack's Lab) and Sema 3F (obtained from J. Verhaagen's Lab) cDNA were prepared from plasmids.

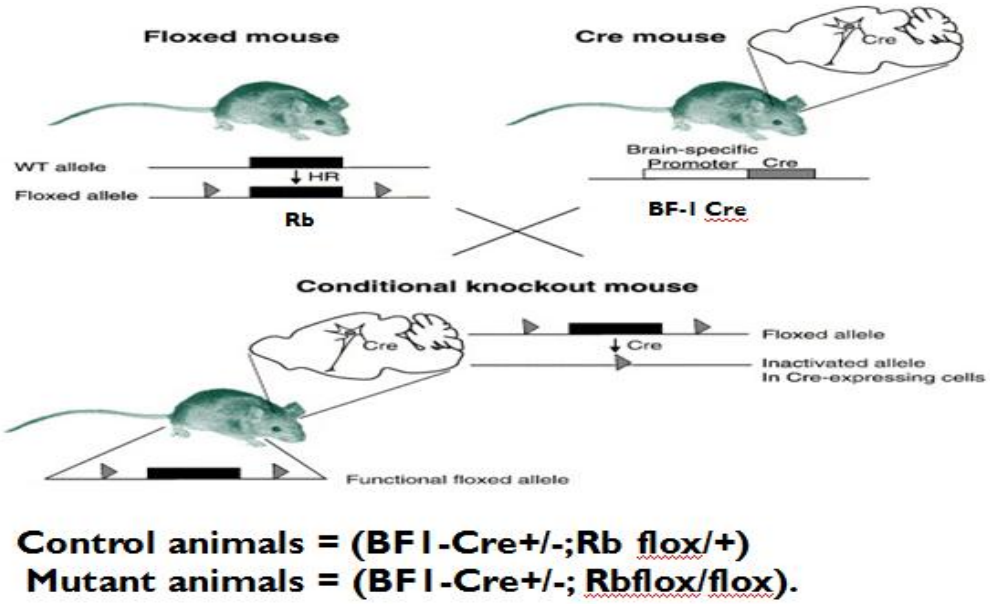
CHAPTER III

RESULTS

A. Abnormal development of the olfactory system in Rb conditional mutant mice

Rb full knockouts yield widespread neurological and hematological defects in addition to early embryonic lethality (Clarke et al.,1992; Jacks et al., 1992; Lee et al., 1992, 1994). To establish role of Rb in OS development, we used conditional Rb knock outs where Rb was specifically deleted in the telencephalon and the OS. Mice in which the Cre recombinase gene was knocked into the Foxg1locus were used. Foxg1, also known as brain factor-1 (BF-1), is a member of the Forkhead transcription factor family expressed specifically in the telencephalon and the OS (Herbert and McConell 2000). Cre-mediated excision of the floxed Rb allele would take place when Foxg-1 Cre or BF-1 Cre mice are crossed with Rb flox/flox mice (Marino et al., 2000) (**Figure 6A**). This occurs in neural progenitors starting at E9. Using the model system described above, we were able to examine the effect of Rb's loss on the development of the OS at different embryonic time points until birth (**Figure 6B**).

A



B

Figure 6: A. Schematic diagram showing the model system used to induce a conditional Rb's deletion in the telencephalon and OS by crossing BF-1 Cre mice and Rb $^{floxed/floxed}$ mice. B. Pregnant females were sacrificed at distinct embryonic time-points between E12.5 and birth. Embryos were collected and brains sectioned for histological analyses.

We compared the morphology of the OS in Rb mutant mice and their control littermates by using cresyl-eosin staining. Our analysis showed that loss of Rb affects the development of the olfactory nerve layer (ONL) starting at E15.5 until birth **(Figure 7)**. While OSN axons appeared to grow from the neuroepithelium and fasciculate in a well organized manner forming a single nerve layer surrounding the OB in control mice, these axons projected randomly and formed circle-like structures **(Figure 7B'; arrow)** at the base of the OB in Rb conditional mutant mice at E15.5 **(Figure 7A-B')**. Failure of these axons to normally connect with the OB suggests the presence of a serious axon guidance defect and therefore, lack of reciprocal interaction between OB and OE needed for the proper development of the

OS at this age. As a result, we observed a degeneration of OE structure in Rb mutant mice at P0: many neuroepithelial cells detached and were found inside the nasal cavity enclosed by the OE **(Figure 7D'; asterisk)**. These results indicate the requirement of Rb for appropriate morphogenesis of the OE and normal formation of the ONL.

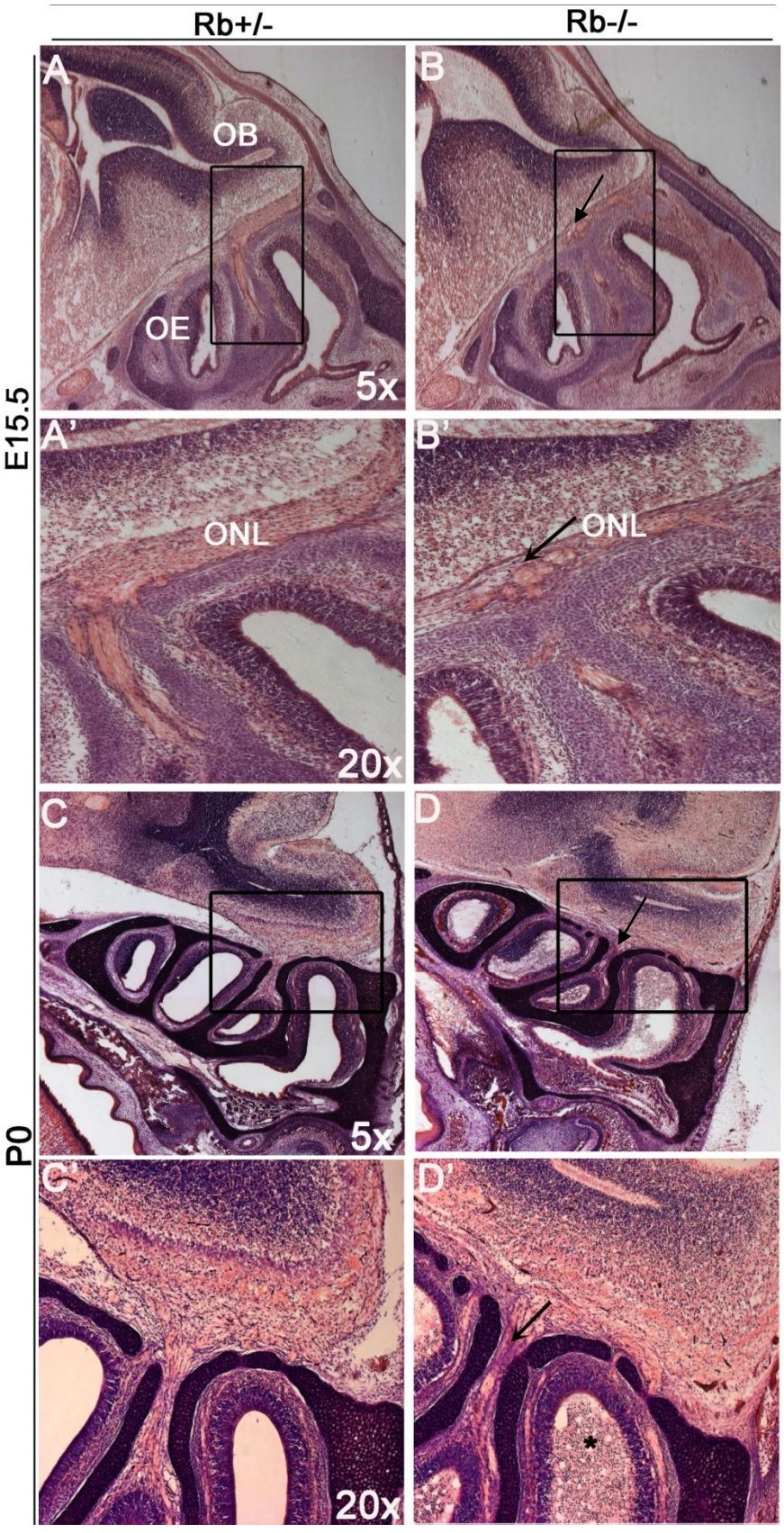

Rb+/-
Rb-/-
E15.5
P0
A
OB
OE
5x
B
A'
ONL
20x
B'
ONL
C
5x
D
C'
20x
D'

Figure 7: Abnormal development of the OS in Rb mutant mice (A-D') Cresyl-eosin stained sagittal sections of the OE at E15.5 (A-B') and P0 (C-D'). (A', B', C', D') are higher magnification images of the regions shown in black boxes in (A, B, C, D) respectively. Arrows in (B' and D') show disorganization of the ONL as OSNs fail to make proper connections with the OB. The asterisk in (D') shows degeneration of OE and the presence of detached cells inside the nasal cavity in Rb mutant mice at P0

B. Lamination defects and loss of mitral cells in the Rb mutant OB

Owing to the morphological abnormalities observed in the ONL and the OE, and since neurogenesis and synaptogenesis in the OS are regulated by reciprocal interactions between the OE and the OB during development, we investigated whether Rb deficiency was affecting OB development. Although development of the telencephalon and genesis of the OE starts around E10, the earliest time-point we analyzed was E12.5 because the OSN axons first achieve contact with the prospective OB (pOB) at this age. Our analysis showed no detectable morphological defect inside the OB between E12.5 and 14.5 (data not shown). However, starting at E15.5 until birth, we detected a gradual reduction in the size of the OB in Rb mutant mice compared with controls **(Figure 8)**, and, the existence of severe lamination defects inside the bulb as revealed by immunostaining against PSA-NCAM (Polysialylated-Neural Cell Adhesion Molecule), a marker of young migrating neurons and GABA (γ-Aminobutyric acid), a marker of inhibitory neurons or interneurons. Hence, we observed a marked layer disorganization affecting several OB layers and leading to the absence of clear borders primarily between the IPL, MCL, and EPL **(Figure 9)**. Furthermore, GABAergic neurons which are the major type of neurons normally occupying the GCL were seen ectopically scattered in the presumptive IPL and MCL **(Figure 9 G,G').** Further examination of this phenotype revealed gradual loss of mitral cells during late development **(Figure 10 A'-D')**. Thus, staining against Reelin which labels the proximal

dendrites of mitral cells did not reveal any defects in the MCL between E12.5 and E15.5 (data not shown). However, gradual loss of mitral cells was seen between E17.5 and birth **(Figure 10 B' and D').** Of note, immunostaining against MAP2 (Microtubule Associated Protein 2), a late differentiation marker, revealed impaired radial migration in the OB of Rb mutant animals as shown by the abnormal expression of MAP2 in the subependymal layer (SEL) and the deep GCL in mutant OB as opposed to being expressed in the outer most layers such as the GL in Rb control animals. Altogether, these data suggest that Rb plays a critical role in the morphogenesis of the OB during late development.

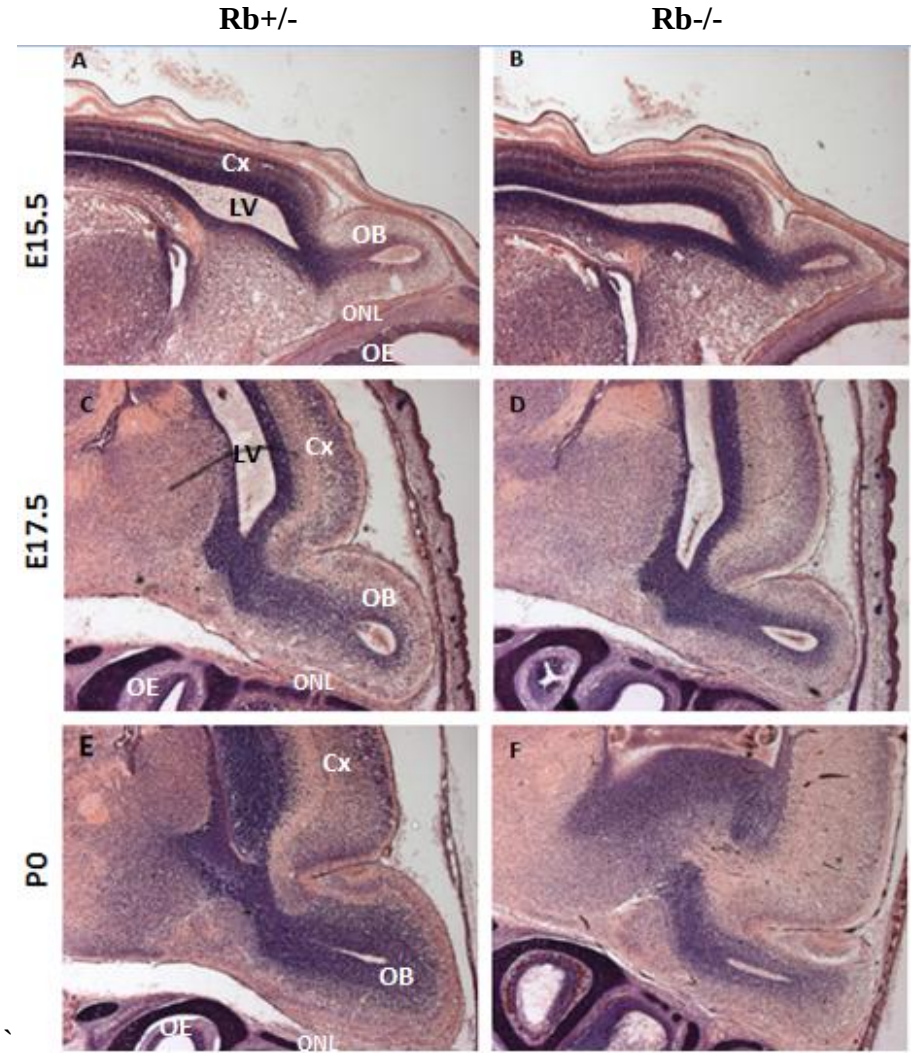


Figure 8: Loss of Rb leads to reduced OB size. Cresyl-eosin staining performed on sagittal sections of Rb control and mutant brains at E15.5 (A and B), E17.5 (C and D) and P0 (E and F). Decrease in OB size was observed in Rb mutant brains (panels B, D and F) compared with Rb controls (A, C and E) starting at E15.5.

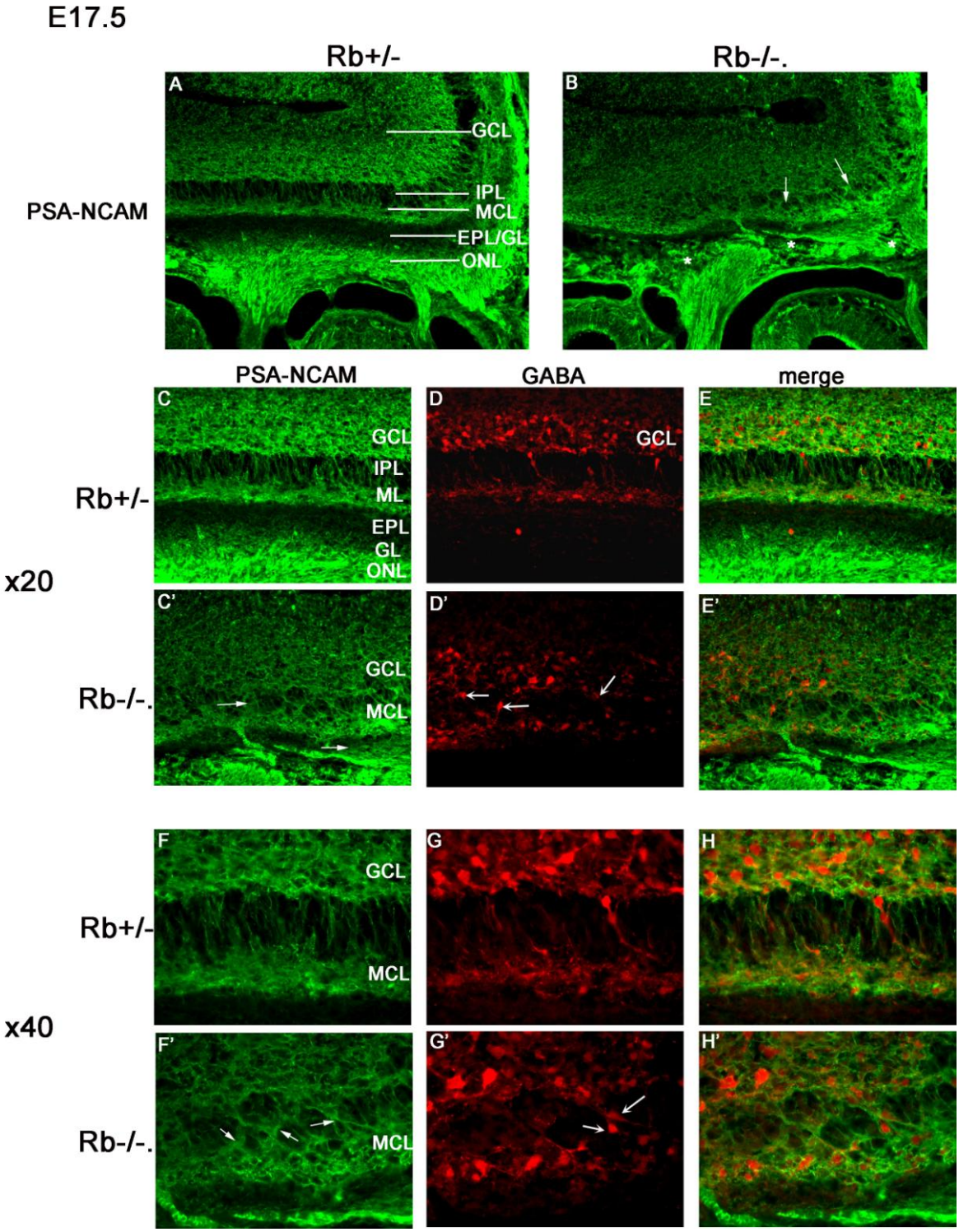


Figure 9: Lamination defects in the OB of Rb mutant mice during late development. Immunostaining against PSA-NCAM (polysialic-neuronal cell adhesion molecule) performed on sagittal sections (A, B) shows general disorganization in the structure of the

OB in Rb mutant embryos compared with controls (arrows). Asterisks point to the observed defects in the ONL as highlighted earlier in Figure 6.
(C-H') Double staining against PSA-NCAM and GABA (gamma-amino-butyric acid) showing GABAergic interneurons that were well localized in the GCL in Rb control mice but appeared scattered between the GCL, IPL and MCL in the Rb mutant bulb with loss of clear boundaries between these layers. F-H' show higher magnification images of C-E'.

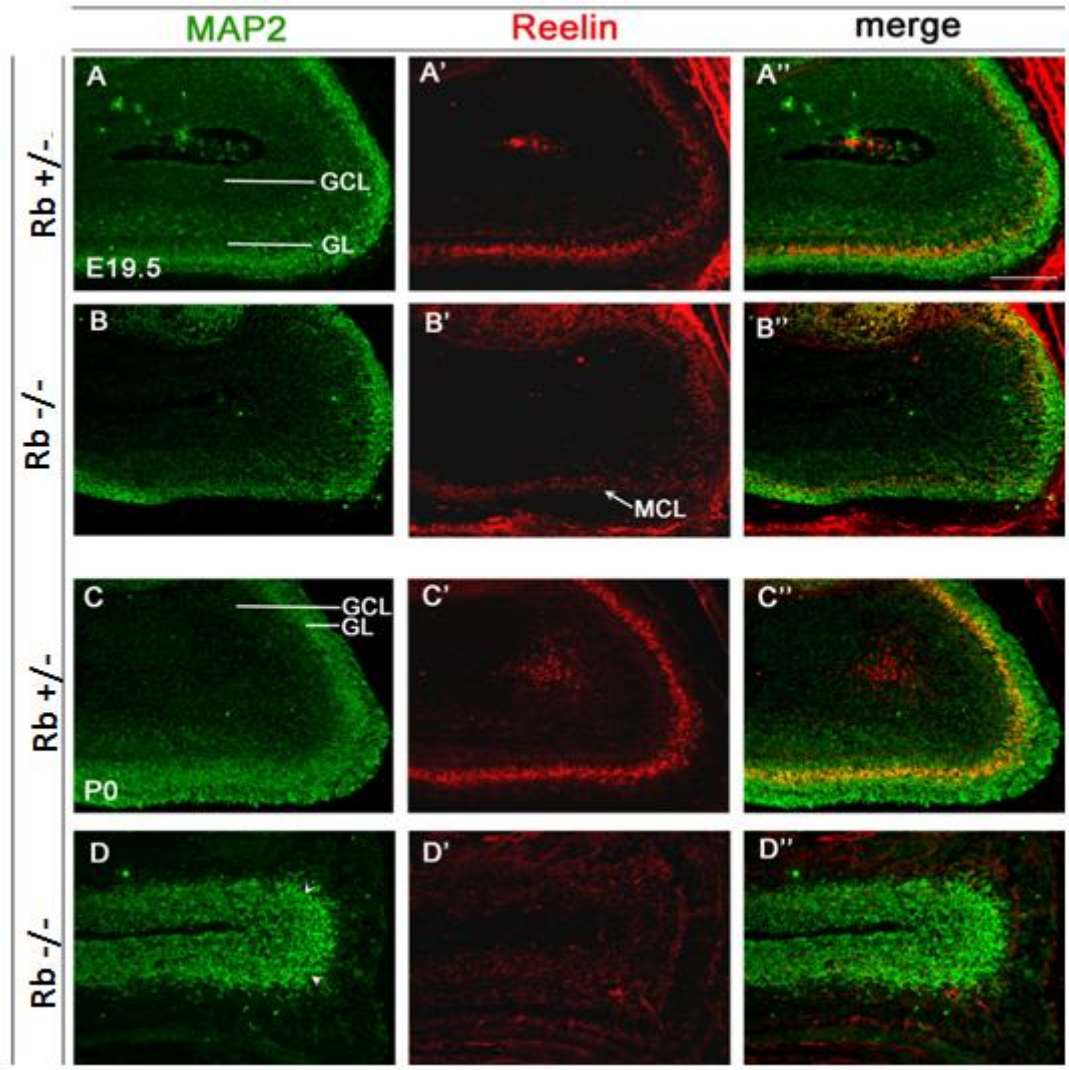


Figure 10: Loss of mitral cells and radial migration defects in the OB in Rb mutant mice. Immunostaining against Reelin, which stains the proximal dendrites of mitral cells, was performed on sagittal sections at E19.5 (A-B'') and P0 (C-D''). A gradual degeneration of the MCL was observed and could be indirectly associated with the defective development of ONL illustrated in figure 7. Immunostaining against MAP2 (Microtubule-Associated Protein 2), a late differentiation marker, revealed abnormal MAP2 expression in the GCL in

Rb mutant mice (arrowheads in D) compared with controls at P0. MAP2 is normally expressed in the outer most layers of the OB including the GL (C, C'') at this age suggesting the presence of a radial migration defect in the Rb mutant OB.

C. Impaired neurogenesis in the Rb mutant OE during late development

Ectopic proliferation and abnormal migration in the dorsal cortex (Ferguson et al. 2002) and the SVZ (Ghanem et al. 2012) were previously shown to be direct consequences for the telencephalon-specific deletion of Rb. To investigate whether Rb plays a similar role in the developing OE, Rb mutant mice and control littermates were assayed for BrdU incorporation by immunohistochemistry. Results showed that, in the absence of Rb,mice exhibited increased proliferation and extensive BrdU incorporation, mostly in the intermediate zone (IZ) of the OE **(Fig. 11C', H' ; arrows)**. Thus, 1.38-fold and 2.46-fold increase in BrdU-positive cells was detected in Rb mutant mice as compared to control littermates at E15.5 and E18.5, respectively (**Fig. 11 C,C', H, H'**; **Graph 1**). We next examined whether the ectopically proliferating Rb-/- cells are neuroblasts by co-staining with BrdU and Tuj-1, the early neuronal marker β-III tubulin. Single and double-labelled cells were quantified in the OE: BrdU/Tuj1 double positive cells showed a 2.07-fold and 2.35-fold increase in Rb mutant mice vs littermate controls at E15.5 and E18.5, respectively (**Fig. 11D-E' and I-J'; Graph 1**). These results indicate that the ectopically dividing cells had committed to a neuronal fate and initiated differentiation in the developing Rb-deficient brain. In addition, consistent with the enhanced neurogenesis detected above, quantification of the average surface area of the OE revealed a significant increase in the OE surface along the medio-lateral axis between E15.5 and P0 (**Graph 2**). Altogether, this data suggest

that Rb regulates the rate of proliferation in the developing OE and loss of Rb results in enhanced neurogenesis during late development.

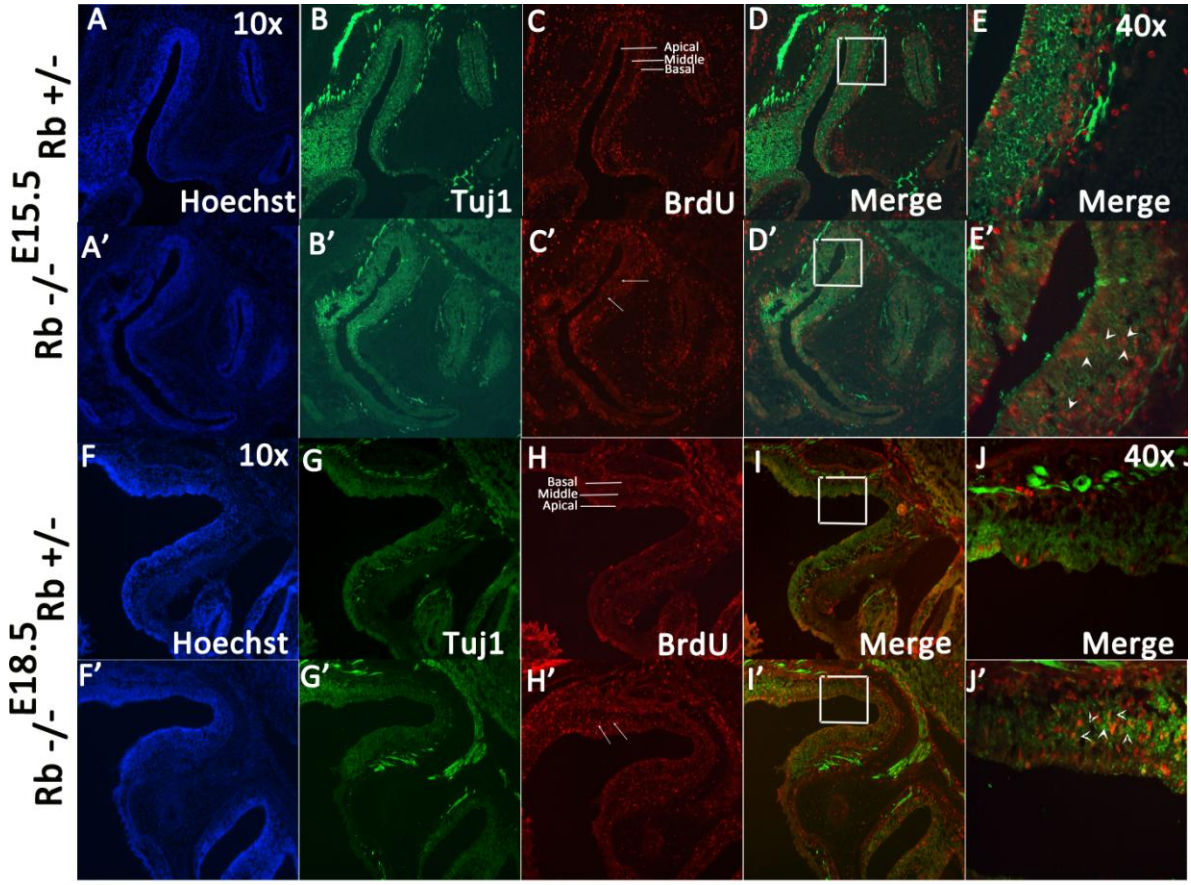
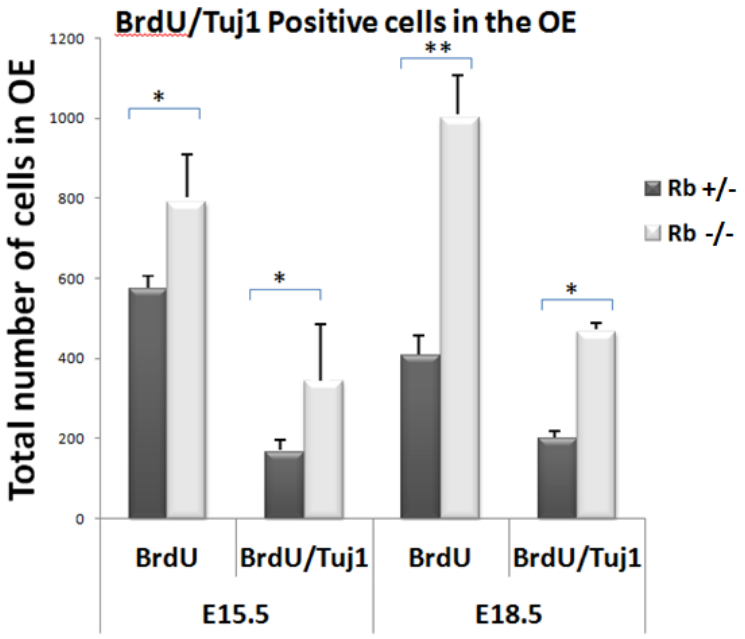
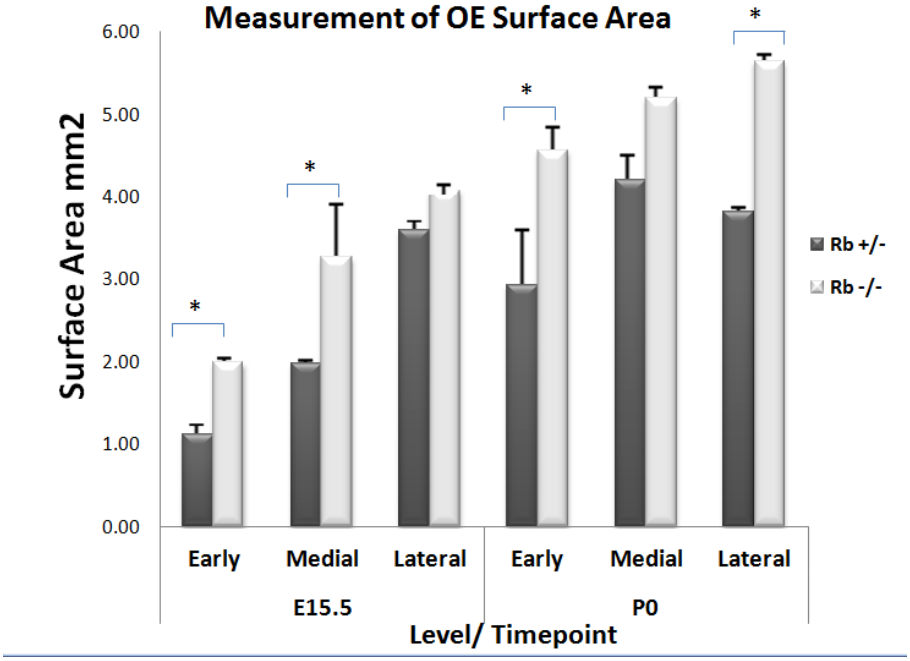


Figure 11: Rb deletion resulted in enhanced and ectopic proliferation of neuroblasts in the developing OE. (A-E') Pregnant females were injected with BrdU at E15.5 and embryos were removed 2h later. Double immunstaining was performed on sagittal sections using anti-BrdU (red) and anti-Tuj1 (green) in Rb+/- (A-E) and Rb-/- (A'-E') embryos. E and E' are high magnification pictures (40x) of the regions in the white boxes in D and D' respectively. Note the increase in the number of BrdU-Positive cells in the OE of Rb-/- mice, mostly in the intermediate zone (C'; arrows) compared with Rb+/- mice. Most of BrdU-positive cells were co-stained with Tuj-1 (E'; arrowheads) indicating that they have committed to a neuronal fate. (F-J') Same experiment as above. BrdU injection was done at E18.5. J and J' are high magnification pictures (40x) of the regions shown in white boxes in I and I' respectively. Note the increase in the number of BrdU-positive cells in the OE of

Rb-/- mice, mostly in the intermediate zone (H'; arrows) compared with Rb+/- (H). Counts were performed in the whole OE of 4 consecutive sections taken from medial level. Quantifications of these results are shown in graph1.



Graph 2: Quantification of BrdU-positive and BrdU/Tuj1-double positive cells in the whole OE. Rb-/-mice showed 1.38-fold and 2.07-fold increase in the number of BrdU-positive and BrdU/tuj1 double positive cells compared to control mice at E15.5, respectively. About 2.46-fold and 2.35-fold increase was also detected in the number of BrdU-positive and BrdU/tuj1 double positive cells in the absence of Rb at E18.5, respectively. Error bars represent SD of measurements from n=3 and asterisks indicate a statistical significant difference between control and mutant mice using t-test, (*) represents $p<0.05$ and (**) represents $p<0.01$



Graph 2: Conditional deletion of Rb leads to an increase in the surface area of the developing OE. The average surface areas of OE was measured and compared between Rb-/- and littermate controls at three different levels along the medio-lateral axis at E15.5 and P0. Consistent with the increased proliferation detected earlier in the OE, the average surface area of OE -was found increased by 20-40%depending on the sagittal level in Rb-/- mice compared to control littermates at E15.5 and P0. Error bars represent SD of measurements from n=3 and asterisks indicate a statistical significant difference between control and mutant mice using t-test, (*) represents $p<0.05$.

Radial migration defect and decreased neuronal survival in the OE in the absence of Rb

Ghanem et al. 2012 showed that Rb is required for rostral migration of neuroblasts from SVZ to the OB and radial migration in the dorsal cortex during late development (Ghanem et al. 2012). We investigated whether OSN migration in the OE is perturbed in the absence of Rb. **(Fig.12 A, A').** In fact, during normal development, immature neuroblasts undergo their final rounds of cell division close to the basal layer, then migrate radially to the apical surface in the OE as they mature into OSNs. In Rb mutant embryos, Tuj1-positive neuroblasts were seen to accumulate and cluster close to the basal region of the OE and

inside the intermediate zone suggesting the presence of a radial migration defect **(Fig. 12 A-A'; arrows)**.

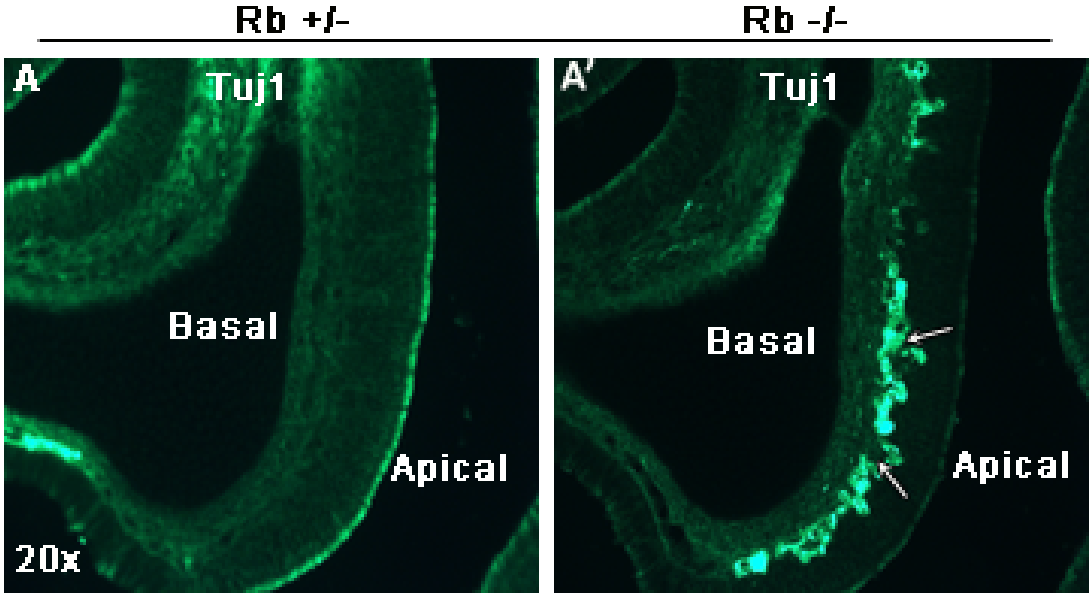


Figure 12: Radial migration defect in OE of Rb mutant mice (A-A') Immunostaining was performed on sagittal sections using antibodies for Tuj-1at E17.5. In Rb control embryos, OSNs migrated radially from the basal layer to the apical surface in the OE as they matured and thus, lined the apical surface of the OE (A). In contrast, OSNs accumulated in the intermediate zone between the apical and basal regions of the OE in Rb-/- embryos due to a radial migration defect (A'; arrows).

The OB hypoplasia and the deterioration of the neuroepithelium observed earlier in Rb mutant mice made us investigate whether loss of Rb affects cell survival in the OS. Therefore, we assessed and compared apoptotic cell death in the OS of Rb control and mutant animals at different stages of development by labeling with Active Caspase-3 (AC-3), an apoptotic marker. We detected a massive increase in apoptotic cell death inside the OB and OE between E14.5 and birth (**Fig. 13 and Graph 3, 4**). Moreover, apoptosis was more pronounced in the basal region of OE that harbours self-renewing basal stem cells and immature neuroblasts as shown by the significant increase in the number of AC-3/Tuj1 double positive cells found in Rb mutant mice at E14.5 (data not shown) and at E18.5 compared with controls (**Fig. 14 H', J' and Graph 4**). As immature OSNs begin to reach into OB glomeruli around E17, they undergo terminal differentiation and up-regulate the expression of the Olfactory Marker Protein (OMP) (Farbman and Margolis, 1980; Miragall and Monti Graziadei, 1982; Verhaagen et al., 1989). To determine the effect of Rb's deletion on the survival of mature OSNs, we co-labeled against OMP and Bax-3 (apoptotic cell death marker) at E18.5. Our results showed also an increase in OMP/Bax-3 double-positive cells at this age **Fig. 14 (F', I')** suggesting that survival of both neuroblasts and mature OSNs is compromised in the absence of Rb

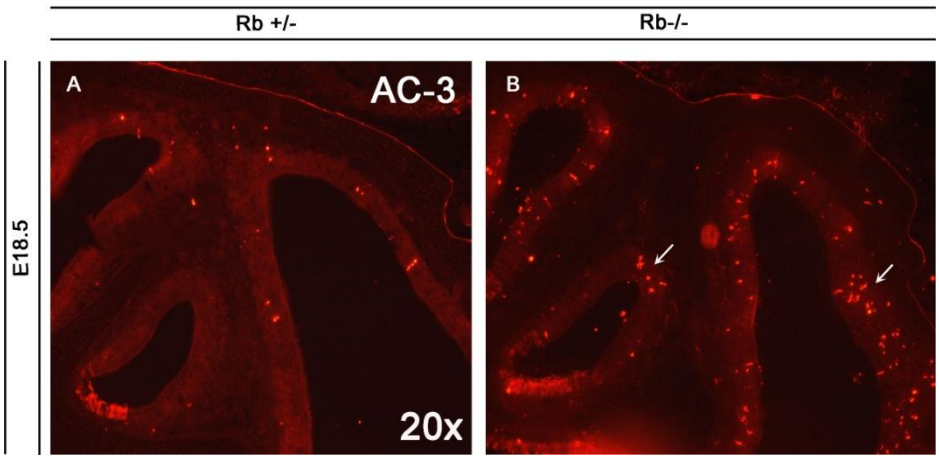
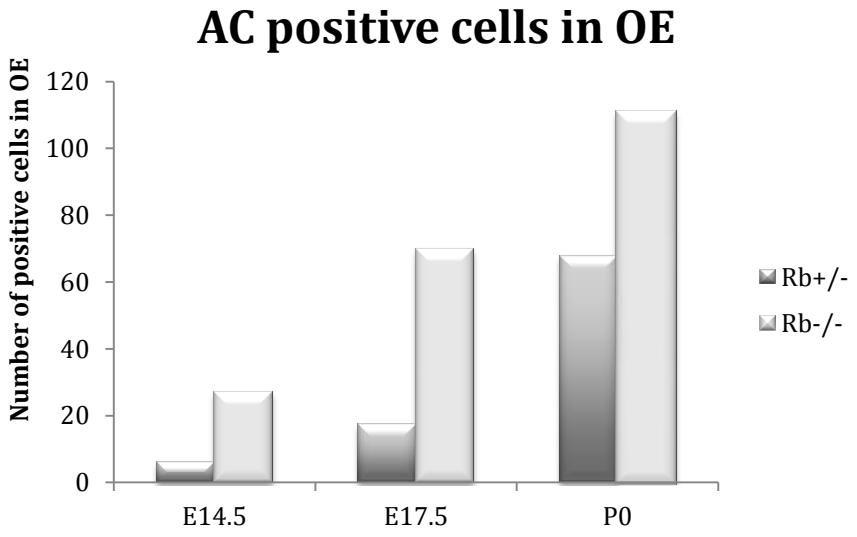
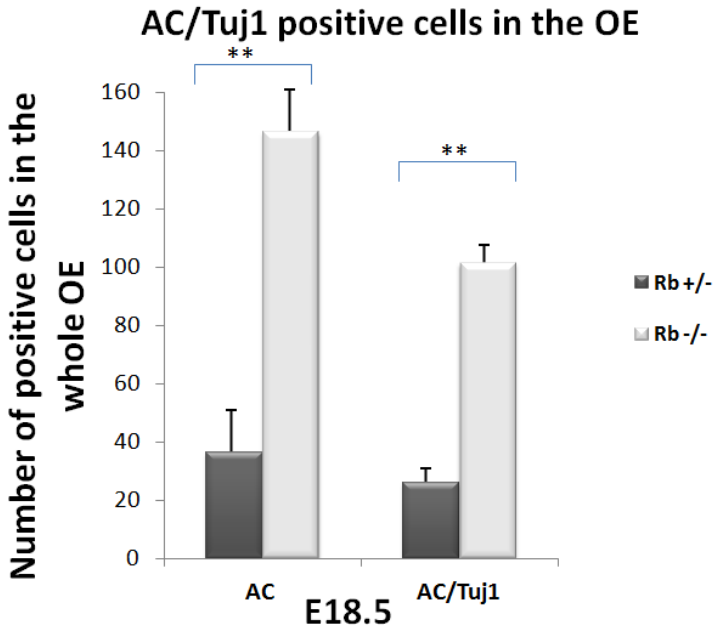


Figure 13: Increase in apoptotic cell death in Rb-/- mutant OE during development. Immunostaining against AC-3 showed increase in number of positive cells in Rb -/- mice as compared to Rb+/- mice at E18.5 (A,B)



Graph 3: Quantification of AC-3 positive cells at E14.5, E17.5 and P0. Counts were performed on the whole OE of 4 consecutive sections taken from medial levels of the brain. Results showed a 4.3-fold, 3.65 -fold and 1.73-fold increase in the number of AC-3 positive cells in Rb-/- compared to control mice at E14.5, E17.5 and P0, respectively.



Graph 4: Quantification of AC-3/Tuj-1 double positive cells. Counts were performed on the whole OE of 4 consecutive sections taken from medial levels inthe brain. Results showed a 4-fold increase in AC-3 and AC-3/tuj1 in Rb-/- compared to Rb+/- respectively, at E18.5.

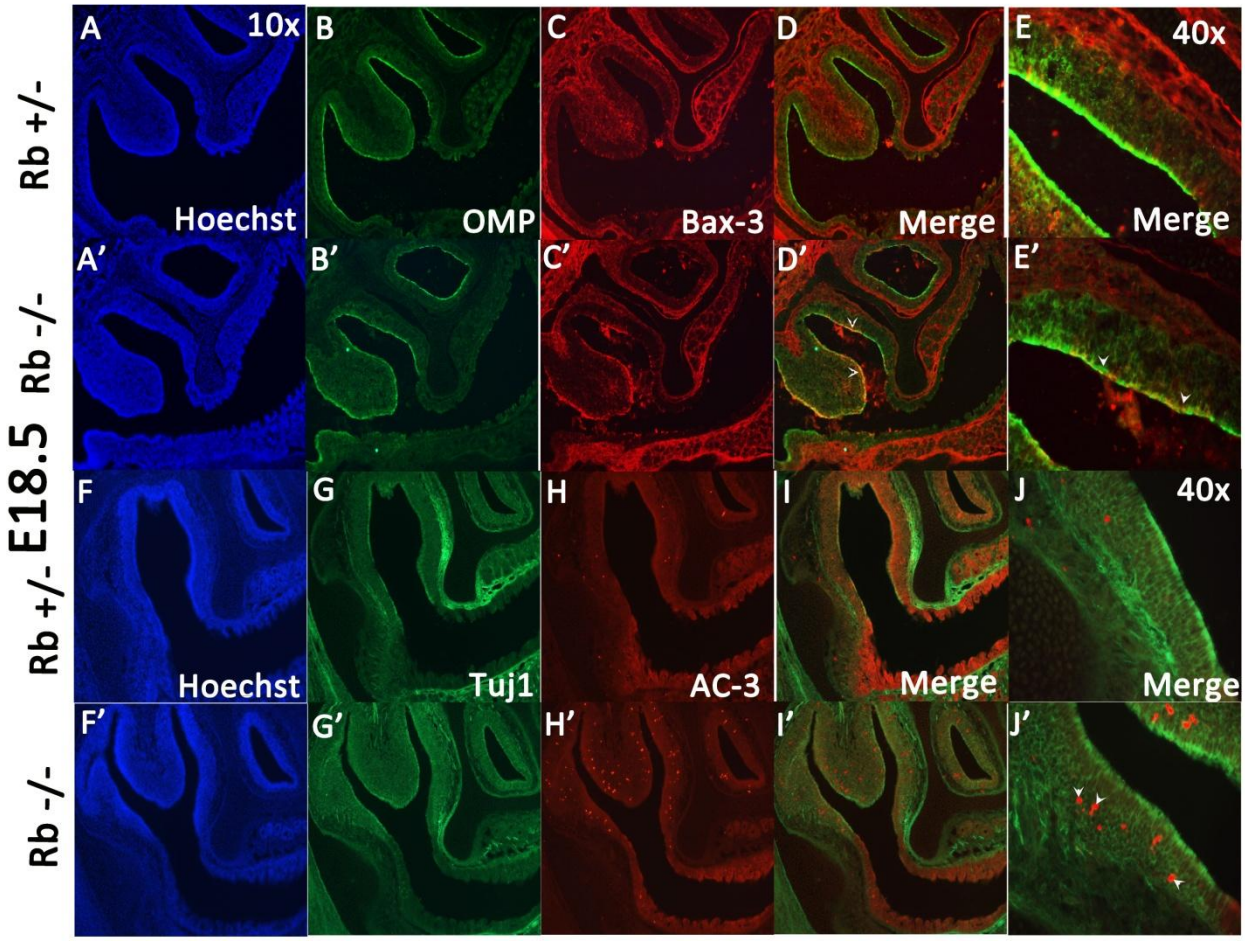


Figure 14: Increased cell death of neuroblasts and mature OSNs in the Rb mutant OE. (A-E') Immunostaining against Olfactory Marker Protein (OMP; late differentiation marker) and Bax-3 (pro-apoptotic marker), and, (F-J') Tuj1 and AC-3 (C-J') was performed at E18.5. Results showed increase in cell death of both Tuj1-positive cells and OMP-positive cells in Rb mutant mice compared with controls at E18.5.

D. Deregulated expression of key signalling molecules in Rb mutant mice

The presence of migration defects in the OE and axonal guidance defects in the ONL during late development led us to investigate whether this phenotype could be due to cell-to-cell signaling defects that might affect the expression of key signaling molecules including Slit1, Slit 2, Sema 3F and Robo 2. These molecules are known to act as guidance cues regulating the interactions between the developing ONL and OB (Cho et al., 2007; Cho et al., 2011, Cho et al. 2011; Nguyen-Ba-Charvet et al., 2008; Takeuchi et al., 2010). To do this, we performed *in situ* hybridization with sense and anti-RNA probes targeted against the mRNAs of these molecules at two critical stages in development, E14.5 and E18.5. As a result, we detected a clear increase in Robo-2 expression in the OE in Rb mutant mice at mid-gestation E14.5 compared with controls and this difference was maintained later in development at E18.5 **(Fig. 15 A-D').** We further checked whether the expression of slit 1 which is Robo2 ligand, is affected. We found that Slit 1 expression was too weak to be assessed at E14.5 but was markedly decreased at E17.5 (data not shown) and E18.5 **(Fig. 15 E-H').** This suggests that the increase in Robo-2 expression detected earlier might be to compensate for Slit-1 downregulation. In addition, we detected a significant increase in the expression of Sema3F in the mutant OE compared with controls **(Fig. 15 I-J').** Finally, we did not detect any expression of Robo1 and Slit-2 in the OE at E14.5 and E17.5 (data not shown) and this was consistent with previous studies (Cho et al. 2007; Nguyen-Ba-Charvet et al., 2008). Altogether, these results point to the involvement of Rb in the regulation of gene expression of key guidance molecules that control axonal development in the OE during late embryogenesis.

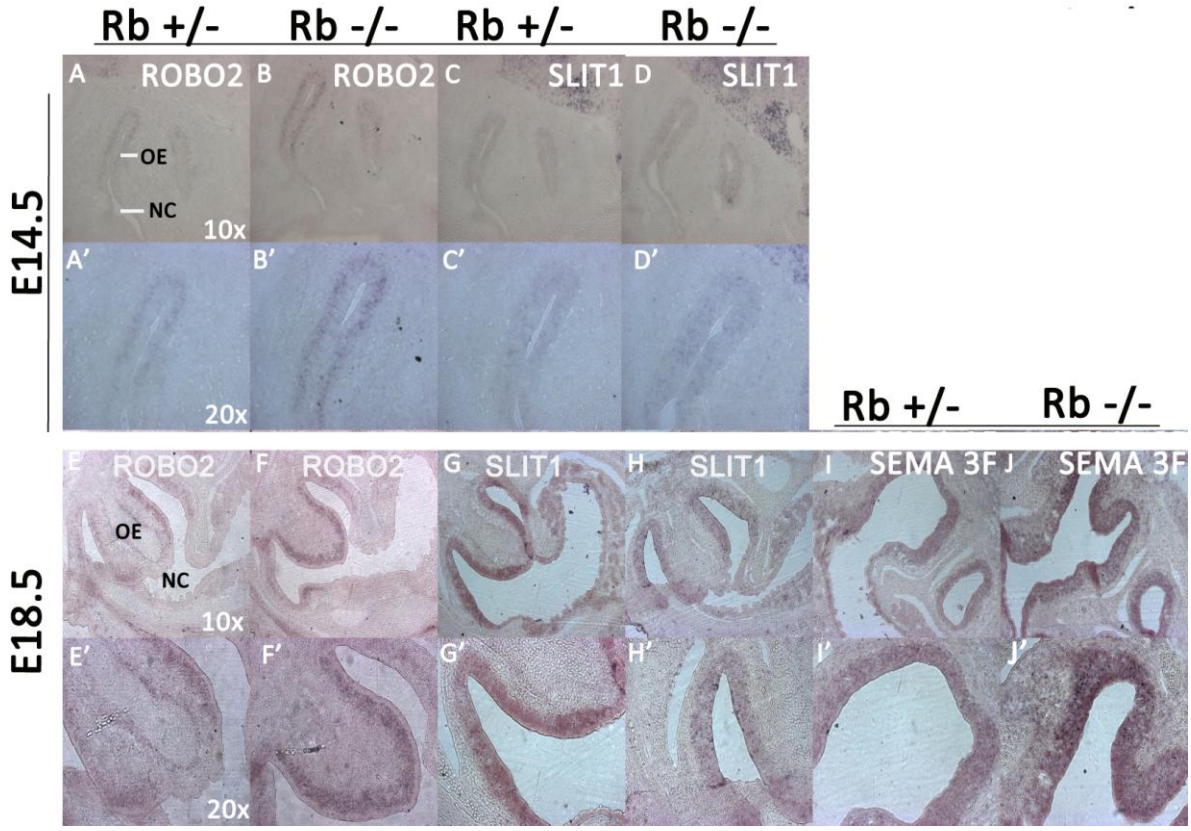


Figure 15: Axon guidance defects in the OS of Rb null mice. mRNA expression of three key guidance molecules, Robo 2 (A, A',B, B', F,F' E, E'),slit 1 (C, C', D, D', G,G', H,H') and sema 3F (I-J') was assessed by *in situ hybridization* in the OE. (A'-J') are high magnification images of regions shown in (A-J) respectively. Note the increased expression of Robo 2 in the OE at E14.5 (B') and prominently at E17.5 (F'), while slit1 expression was decreased in Rb-/- mice compared to Rb+/- (H'). Moreover, Sema 3F expression increased in Rb mutants (J') as compared to control littermates (I') suggesting the presence of migration guidance defects in the absence of Rb.

CHAPTER IV

DISCUSSION

Brain plasticity and continuous neurogenesis in the brain throughout life (adult neurogenesis) have been topics of high interest in the last decades. Thorough investigations have been carried out in an attempt to identify the molecular components/pathways including key cell cycle genes and transcription factors that control neurogenesis during development, and, in adult brain regions such as in the olfactory system and the hippocampus (McKay, 1997; Fung et al., 1997; Kuhn et al. 1997; Tropepe et al. 1997; Weiler et al. 1997; Casaccia-Bonnefil et al. 1999; Miyazawa et al. 2000). Studies of genetic mouse models that lack individual cell cycle genes e.g. Rb knock-out mice have revealed that these cell cycle regulators control not only cell proliferation and apoptosis but diverse processes beyond their cell cycle regulatory role such as neuronal differentiation and migration in the developing and adult brain (reviewed in McClellan and Slack 2006). Here, we reported a novel role for Rb in the development and morphogenesis of the primary olfactory structures, OE, ONL, and OB, and, the control of neurogenesis of OSNs. Hence, we have demonstrated that Rb is required for the control of cell proliferation and radial migration of immature OSNs in the OE as well as OSNs' survival and terminal maturation. Moreover, our study has revealed a critical requirement of Rb in the establishment of proper axonal connections between the two main components of the olfactory system during development, the OE and the OB. We have also emphasized Rb's role in laminar patterning and neuronal survival primarily of mitral cells inside the OB.

A. Abnormal development of the ONL and lamination defects in the developing OB in Rb conditional mutant mice

Using Rb conditional KO mice, we have performed a time-course study that includes analyses of different time-points corresponding to critical stages of development and established a time-dependency of Rb's role during development and morphogenesis of the olfactory system. Thus, we have linked the appearance of developmental defect(s) due to Rb's loss with specific stage(s) of OS development. Herein, we demonstrated that the OSN axons in the OE of Rb mutant mice fail to innervate the OB during mid-gestation -E15.5- (**Fig. 7B'**) and many mature OSNs undergo thereafter apoptosis later in development -E17.5- (**Fig.14 and Graph 3**). In parallel, loss of Rb resulted in reduced OB size and altered lamination as well as degeneration of the mitral cell layer in the OB after E17 (Fig. **10 B' and D'**). Our data suggests that these defects are correlated and could be, at least in part, mutually related in a 'cause-and-effect' manner specifically during later stages of development. This is the time when proper OS function depends on the establishment of proper connections between axons of OSNs on one hand and dendrites of MC inside the GL on the other hand (Nagayama et al., 2004; Shepherd et al., 2004; Hayar et al., 2004; Gire et al., 2012). Moreover, consolidation of these OE-OB connections is only completed after birth when olfactory function is triggered (reviewed in Mombaerts, 2006; Blanchart and López-Mascaraque, 2011). Hence, failure to connect and/or re-enforce the connection(s) may result in nerve cell degeneration on both sides as seen with mitral cells (MC) and GABAergic neurons in the OB (**Fig. 2** and Ghanem et al. 2012), and, OSNs in the OE (**Fig. 7 D'**). In fact, we first detected axonal guidance defects in the ONL at E15.5 in Rb mutant mice despite the fact that the MCL appeared normal at this age (**Fig. 9 and data**

not shown). However, around E17, the MCL started to degenerate and this was coincident with the time when the coalescence of OSN axons into protoglomerular structures begins (Treloar et al. 1999; Blanchart et al. 2006). While the axonal guidance and migration defects are likely associated with a cell-autonomous function of Rb (see next sections), the OB defects could be due in part to non cell-autonomous effects that result from the absence of OE input to OB during late development. This suggests that OSN axons probably play a key role in cellular organization and morphogenesis of MCL and GL. As a matter of fact, previous studies have suggested that the OP and/or the OE may have a central role in inducing OB development and neurogenesis (Graziadei and Monti-Graziadei, 1992; LaMantia et al., 1993, 2000; Gong and Shipley, 1995) although the arrival of pioneer OSN axons from the OE at E12 postdates the genesis of mitral cells, which begins on E10.5–11 (Hinds 1968a). In contrast, other studies have showed that the OB still forms despite lack of OSN innervation (Lopez-Mascaraque et al., 1998; Jimenez et al., 2000; Anchan et al., 1997; Long et al. 2003). For example, Pax6*sey/sey* (small eye) mutant mice lack olfactory axons but reveal features of a non-evaginated OB-like structure (Lopez-Mascaraque et al., 1998; Jimenez et al., 2000). Yet, the failure of complete OB evagination in Pax6*sey/sey* mutants may be contributed to a mechanism secondary to PAX6 function in the OE and the telencephalon as noted by the authors of these studies (Anchan et al., 1997). Moreover, OB growth is initiated in *Dlx5*-deficient mice despite the lack of an ONL. However, this study underlined the presence of lamination defects inside OB at E18.5 suggesting that OSN axons are still required for OB laminar properties (Long et al. 2003). The discrepancies among the studies described above may be due to the fact that the interaction(s) between OB-OE is/are differentially regulated in a complex spatio-temporal manner during

development and may be associated with cell/tissue autonomous and/or non-autonomous properties. Therefore, the olfactory input to the telencephalon might indeed be essential for normal OB development and neurogenesis at least at late developmental stages as concluded from our study. Finally, *in vitro* studies have showed that OB formation was induced via secretion of ligands of the TGF-β family using OE explants (Slotnick 2004; Tran et al. 2008), yet, the exact nature of such signals warrants further *in vivo* investigations.

B. Rb regulates proliferation and radial migration of immature OSNs as well as their terminal maturation in a cell-autonomous fashion

We examined the role of Rb in the control of cell proliferation in the OE and found that loss of Rb resulted in enhanced and 'ectopic' proliferation of immature OSNs. These neuroblasts were abnormally proliferating at high rate in the intermediate zone of the OE as indicated by BrdU incorporation (**Fig. 11)** suggesting the presence of a radial migration defect. This observation is in contrast with the phenotype of Rb control littermates where almost all BrdU-positive cells accumulated near the most basal and/or the most apical regions of the OE where basal and apical progenitors are found, respectively. The increased cell proliferation is a direct and expected consequence for the loss of Rb's tumor suppressor function during cell division. In fact, Rb-null neuroblasts were previously shown to proliferate ectopically and initiate their differentiation program without exiting the cell cycle in the developing cortical plate in the same mutant mice (Ferguson et al. 2002). However, their cell fate was uncertain and they either underwent apoptosis or failed to complete their differentiation program (Ferguson et al. 2002, Ghanem et al. 2012).

Moreover, Rb-null neuroblasts displayed abnormal rostral migration along the rostral migratory stream and radial migration in the dorsal cortex and inside the OB (Ghanem et al. 2012). These findings are consistent with our results and indicate that Rb plays a redundant role in the control of cell cycle and neuronal differentiation/migration in different brain regions. In addition, there is strong evidence that Rb's control of neuronal differentiation and migration is primarily linked to a cell-autonomous function for Rb as demonstrated by Ghanem et al. 2012 who showed that the Rb/E2F pathway directly regulates the expression of *Dlx1/Dlx2* genes, two key regulators of neuronal differentiation in the brain. Similarly, other studies have shown that Rb's control of cell migration is independent of the environment using transplantation experiments (Ferguson et al. 2005) and is specifically mediated by Rb's interaction with E2F3 (McClellan et al.2007) and Rb's control of neogenin expression in the telencephalon *in vivo* (Andrusiak et al. 2011).

Besides its role during neurogenesis, our study revealed also a requirement for Rb in the survival of OSNs during OE development. Rb's loss led to increased apoptosis of both immature OSNs starting at E14.5 till birth (**Fig. 14 H', Graph 3 and Graph 4**) and mature OSNs at E18.5 **(Fig. 13).** While loss of immature OSNs may be a direct and expected consequence in Rb mutant mice as described above for other brain regions, we believe that loss of mature OSNs is a secondary and late effect due to Rb's phenotype in the OB. In fact, the gradual attrition of the OE that peaked at P0 (**Fig 7 D'**) is likely to result from failure of OSN axons to connect with their targets in the OB or mitral cells few days before birth. Although direct evidence is still lacking, several observations support this conclusion: 1) the Cre expression in the OE is restricted to Foxg1 progeny comprised of neuronal progenitors and not mature neurons. 2) Moreover, Cre was not expressed in all OE

progenitors as seen in our Rb mutant mice (data not shown). This could explain the incomplete penetrance of the Rb's phenotype described in the OE (e.g. radial migration defect) as well as the programmed death of mature OSNs that may not be Rb-null cells in the first place. We have generated Rb floxed/floxed; YFP/YFP homozygous animals carrying the YFP reporter gene inside a cassette along a stop signal that is flanked by *loxP* sites. We are presently mating these mice with BF-1Cre animals: hence, all Rb-null cells will be labeled/traced with YFP and this will allow us to confirm the nature and identity of Rb-null cells by performing triple labeling experiments e.g. GFP-BrdU-Tuj1 and GFP-BrdU-OMP. These ongoing experiments will help confirm our previous findings as well as provide the genetic tool needed to perform future studies based on lineage tracing and the identification of specific pathways that are Rb-dependent.

C. Axonal guidance defects associated with deregulated expression of key signalling molecules in Rb mutant mice

There is a tight correlation between the position an OSN occupies in the OE and its target glomerulus within the OB, thus creating a well-defined spatial organization of both OSNs and glomeruli. For instance, OSNs positioned in the dorso-medial domain of the OE innervate the dorsal aspect of the OB while OSNs located in the ventro-medial domain of the OE project ventrally to the OB (Astic and Saucier, 1986, Miyamichi et al., 2005). Previous studies have demonstrated that two pairs of axon guidance molecules, Robo2/Slit1, and, Nrp2/Sema3F, play key roles in dorso-ventral targeting of OSN axons inside the OB (Cho et al., 2007; Nguyen-Ba-Charvet et al., 2008; Takeuchi et al., 2010;

Cho et al., 2011; Cho et al., 2012). Moreover, it is well established that interactions between the distinct pairs of axon guidance molecules are involved in the development of connectivity in the OS. Owing to the profound axonal de-fasciculation observed in the ONL of Rb mutant mice, we investigated the effect of Rb's loss on the expression of key signaling molecules such as Robo1, Robo2, Slit1, Slit2 and Sema3f. We found that Robo1 was not expressed inside the OE (data not shown) and this was consistent with a previous report showing that this receptor is not produced by OSNs, but rather by OECs (Nguyen-Ba-Charvet et al., 2008). Nevertheless, it would be interesting to determine in the future the effect of Rb's loss on Robo1 expression given that Robo1+ OECs might also function in regulating axon guidance by associating with OSN axons and repelling Slit ligands expressed in the ventral OB. In addition, we did not detect any expression of Slit-2 in the OSNs between E14.5 and E17.5 (data not shown) as shown in a previous report (Cho et al. 2007). Contrastingly, our analysis of Rb conditional mice showed a dramatic increase in Robo 2 expression in the dorso-medial regions of the OE and this increase was opposed by a decrease in its ligand's expression, Slit-1, in the same regions. This finding is particularly intriguing because previous studies have revealed that Robo-2 expression was specifically detected in OSNs across the OE in a graded manner with high levels of Robo-2 in OSNs located dorsomedially and low levels in OSNs in the ventrolateral domain of the OE (Cho et al.,2007). The opposed effect of Rb's loss on Robo2 and slit1 expressions in late development raises the possibility that the upregulation of the receptor, Robo 2, is a compensatory feedback mechanism to compensate for the downregulation in Slit 1. This type of feedback was also observed in other systems such as the μ-opiate receptor and its

ligand β-endorphin in the human epidermis where elevated levels of the ligand were compensated for by downregulation of the μ-opiate receptor (Bigliardi-Qi et al., 1999). The expression of another set of signaling molecules that regulate to a large extent D-V projection, the receptor Nrp2 and its repulsive ligand Sema 3F, was also found affected in the absence of Rb. Sema 3F expression level was drastically increased in the dorso-medial region of the OE in Rb mutant mice compared to control littermates. Ongoing experiments are focused on examining Nrp2 expression level and relating it to the altered expression of Sema 3F in Rb mutant mice. In conclusion, the deregulated expressions of several key guidance molecules in OSNs may be directly associated with the perturbations observed in the glomerular map and thus may be largely contributing to the defective phenotype seen in the mutant OB.

Taken altogether, the results of this study have advanced our knowledge of brain development particularly the formation of the olfactory system. From a broader perspective, our findings will contribute to a better understanding of nerve regeneration and brain plasticity, two highly relevant processes in the brain for many reasons: 1) the ability to regenerate nerve cells e.g. OSN regeneration and reconstitution of ONL is essential for normal brain function and in cases of disease or injury because these cells are directly exposed to the external environment and thus, are continuously prone to infection, damage and/or normal wear-out (reviewed in Schwob 2002), 2) continuous neurogenesis in the neuroepithelium has been proposed to be the second major mechanism that explain plasticity of the OB after adult neurogenesis in the SVZ (reviewed in Huart et al., 2013) , 3) deciphering the molecular mechanisms that control nerve regeneration in distinct adult

brain regions such as OB and hippocampus may provide important cues that will surely contribute to the development of intervention methods for regenerative medicine in the future.

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