The Role of the Retinoblastoma Protein in Dentate Gyrus Development

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Abstract

New neurons continue to be added to the dentate gyrus (DG) throughout adulthood and enhancing neurogenesis in this region holds therapeutic potential. However, the molecular mechanisms underlying DG neurogenesis remain elusive. Since developmental and adult neurogenesis often share the same signaling pathways, understanding how the DG develops is crucial to understanding adult neurogenesis. This study aims to determine the role of the retinoblastoma (Rb) protein in DG development and to determine if modulation of this pathway holds potential for enhancing neurogenesis in an adult system. A FoxG1 driven Cre is used to delete Rb in the developing forebrain and the resulting effects are analyzed in *in vitro* and *in vivo* mouse models. We show that Rb deletion enhances DG neurogenesis by specifically increasing proliferation of immature neurons. Overall this study suggests that Rb pathway modulation could hold potential for enhancing neurogenesis in the adult.

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ABAM	Antibiotic Antimycotic
AC3	Activated caspase3
Apaf1	Apoptotic protease activating factor 1
BLBP	Brain lipid binding protein
BrdU	5-bromo-2'-deoxyuridine
CA	Cornu Ammonis
Cdk	Cyclin-dependent kinase
CH	Cortical hem
CP	Choriod Plexus
Cre	Cre recombinase
Cyc	Cyclin
Cxcr4	C-X-C chemokine receptor type 4
DA	Dentate anlage
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DG	Dentate Gyrus
DGM	Dentate gyrus migratory stream
DMEM-F12	Dulbecco's Modified Eagle Medium: Ham's Nutrient Mixture F-12
DP	DRTF1 (Database of Rice Transcription Factor)-Polypeptide
E	Embryonic day
E2F	E2 promoter binding factor
EGF	Epidermal growth factor

Emx2	Empty spiracles homeobox 2
FBS	Fetal bovine serum
FI	Fimbria
FGF	Fibroblast growth factor-2
Flox	Flanked by LoxP sites
FoxG1	Forkhead box protein G1, Brain Factor-1
GFAP	Glial fibrillary acidic protein
HBSS	Hank's Buffered Salt Solution
HP	Hippocampal plate
HDAC	Histone deacetylase
Hes1	Hairy and enhancer of split-1
Hes5	Hairy and enhancer of split-5
IB	Intermediate band
INP	Intermediate neuronal progenitor
IZ	Intermediate zone
Ki67	Antigen Ki-67
Lef/Tcf	Lymphoid enhancer factor/ T-cell factor
Lef1	Lymphoid enhancer factor-1
LGE	Lateral ganglionic eminence
Lhx2	LIM homeobox 2
Lrp6	Low density lipoprotein receptor-related protein 6
Mash1	Mammalian Achaete-Scute Homolog 1
MGE	Medial ganglionic eminence

mM	Millimolar
NE	Neuroepithelium
NES	Nuclear export signal
NeuroD1	Neurogenic differentiation 1
NLS	Nuclear localization signal
NS	Not significant
OCT	Optimal cutting temperature
P	Postnatal day
p53	Tumor protein 53
p107	Retinoblastoma-like protein 1
p130	Retinoblastoma-like protein 2
Pax6	Paired box gene 6
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
Prox1	Prospero homeobox protein 1
Rb	Retinoblastoma protein
Sdf1	Stromal cell-derived factor-1
SEM	Standard error of the mean
Sox2	Sex determining region Y-box 2
SVZ	Subventricular zone
SWI/SNF	Switch/sucrose nonfermentable chromatin remodeling complex
Tbr2	T-box brain gene 2
Tuj1	Neuronal specific β-3-Tubulin

μm	Micrometer
VZ	Ventricular zone
Wnt	Wingless
Zbtb20	Zinc finger and BTB domain-containing protein 2

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Introduction

Since the 1950's the hippocampus, a medial cortical brain structure, has been studied extensively for its role in spatial learning and memory consolidation. The case of patient HM, whose hippocampal formation was removed in an effort to eliminate seizures and concurrently resulted in loss of memory consolidation (Corkin, 1968; Corkin, 1984; Corkin, 2002), was the first example of the importance of the human hippocampus. Since this time, many studies have lead to an increased understanding of the role of the hippocampus in episodic memory formation and spatial learning (Vargha-Khadem et al., 1997; Eichenbaum, 2001; For review see: Burgess et al., 2002).

In the past, it was believed that the neurons one is born with, are the same as those one dies with, however over the past few decades, the discovery of adult neurogenesis within dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles has proven this dogma incorrect (Altman, 1962; Reynolds & Weiss, 1992; Luskin et al., 1993; Doetsch et al., 1997). The presence of adult neurogenesis within the human DG (Eriksson et al., 1998) has thrown this structure into the spotlight as researchers strive to determine not only the endogenous role of neurogenesis, but also to understand the molecular mechanisms underlying this process in order to manipulate it for therapeutic use. Interestingly, the presence of continued neurogenesis with the SVZ of adult humans is still highly controversial. Recent evidence suggests that neurogenesis does not take place within this structure of the adult human brain (Bergmann et al., 2012), making an understanding of neurogenesis in the DG even more imperative.

Though not certain, it is believed that adult neurogenesis is required for the consolidation of new memories and during adult life, neurogenesis in the hippocampus

can increase in response to various stimuli such as environmental enrichment, injury, and exercise (Kitamura et al., 2009; Sahay et al., 2011; Snyder et al., 2011, van Praag et al., 2005; Olson et al., 2006; Parent, 2003). The presence of continued DG neurogenesis opens the door to various therapeutic possibilities, if this neurogenesis could be modulated in the desired way (Hallbergson et al., 2003). However, the mechanisms underlying the regulation of adult neurogenesis are still being determined. In order to more fully understand the regulation of adult neurogenesis, a clear understanding of hippocampal neurogenesis during development is required, as the molecular pathways essential to adult stem cell maintenance and proliferation are often similar to those required during development.

Currently, little is known about how neurogenesis in the developing DG is regulated on a molecular level. In an effort to augment neurogenesis, we turn to the retinoblastoma (Rb) protein, a cell cycle regulatory protein shown to be involved in neurogenesis during the development of the cerebral cortex. During cortical development, the Rb protein has been shown to be important in the regulation of proliferation (Ferguson et al., 2002). Thus, it is possible that through modification of the Rb pathway, endogenous neurogenesis in the developing DG could be enhanced, possibly suggesting a mechanism to enhance neurogenesis in an adult setting.

The Hippocampus: An Overview

The hippocampus is composed of two intertwined structures, Cornu Ammonis, also known as Ammon's Horn or the CA regions 1-3, and the DG. The hippocampal circuitry is composed of complex input and output circuits; the primary inputs, known as the perforant pathway enter the DG from the entorhinal cortex, impulses are processed

through two regions consisting of pyramidal neurons, known as CA1 and CA3, and the primary outputs of the CA regions innervate the deep layers of the entorhinal cortex. This forms a looped circuitry known as the trisynaptic pathway (For Review see: Witter et al., 2007).

During embryonic development, the hippocampus is derived from neuroepithelium above the cortical organizer region known as the cortical hem (Fig. 1). Hippocampal development starts around E10 in the mouse, with the DG developing in a unique fashion relative to Ammon's Horn and the cortex. As it is likely that similar pathways are involved in regulating neurogenesis during development as well as in the adult, a clear understanding of embryonic DG neurogenesis is critical.

Development of Ammon's Horn

Ammon's horn development occurs prior to the DG and can be divided into three main phases temporally; the specification of the Ammonic neuroepithelium, neurogenesis of the CA cells, and the final migration of CA cells to the correct location.

Specification of the Ammonic Neuroepithelium

The actual specification of the Ammonic neuroepithelium, the region of neuroepithelium giving right to the CA neurons, remains unclear. Lineage studies indicate that CA progenitors are not hippocampal specific, but can give rise to various types of pyramidal neurons if provided with the correct environmental cues (Grove et al., 1992). Further research suggests that hippocampal specification could be due to interactions between the CA cells and signals derived from the hippocampal poles (Grove et al., 1998). Many pathways have been implicated in the specification of the hippocampal field, with one of the most important being canonical Wnt signaling.

It has been postulated that Wnt ligands secreted from the cortical hem, which is located at the ventral hippocampal pole, work to specify the region for the hippocampus (Fig. 1). Deletion of the Wnt ligand, or downstream components of the Wnt pathway, results in complete loss or significant reduction in the size of hippocampal structures (Lee et el., 2000). However, this data could suggest that Wnt signaling is required for the maintenance or initial expansion of the hippocampal progenitor pool, not necessarily the specification of the hippocampal region itself (Lee et al., 2000; Machon et al., 2003). In addition, the importance of factors secreted from the cortical hem in hippocampal specification has been debated. Tole & Grove (2001) cultured hippocampal explants with the cortical hem removed, and found little effect on the patterning of the hippocampus, suggesting that the cortical hem is dispensable for hippocampal development. Another factor recently implicated in the specification of the Ammonic neuroepithelium is the BTB-zinc finger gene, Zbtb20. This gene is essential for the specification of the CA1 region, suggesting that multiple factors are required for the correct patterning of the hippocampal neuroepithelium (Xie et al., 2010; Nielsen et al., 2007; Nielsen et al., 2010) (Fig. 1).

CA Neurogenesis and Initial Migration

Following the specification of the hippocampal field, CA neurogenesis begins. The development of the structures composing Ammon's Horn can be likened in many ways to that of the cortex. Both structures are formed in the classic "inside-out" pattern; the cells lining the ventricle are proliferative, giving rise to cells which migrate to the correct location such that those born earliest give rise to the inner-most layers. Also similar zones of activity are classified in the cortex and Ammon's horn during

development (VZ, SVZ, intermediate zone/intermediate band, cortical plate/hippocampal plate, respectively) (Altman & Bayer, 1990) (Fig. 2). However, the layered structure of the hippocampus must be patterned not only in a deep to superficial plane, but also from CA1 through to the DG, generating some stark differences between cortical and hippocampal development. This fact, in combination with the relatively late developmental start, results in newly born cells "waiting" in the intermediate band for an extended period of time (Altman & Bayer 1990).

The cells giving rise to CA3 are born in peak numbers at E13-14 (Angevine, 1965) and move into the intermediate band, where they halt migration (Altman and Bayer, 1990; Fig. 3). Around the time CA3 neurons being their sojourn, CA1 neurogenesis peaks (E15) and these cells also move into the intermediate band then halt migration (Altman and Bayer, 1990; Nakahira & Yuasa, 2005) (Fig. 3). The duration of the stay in the intermediate band was shown to be approximately 2-3 days during mouse development (Nakahira &Yuasa, 2005). Altman and Bayer (1990) postulated that this delayed stay could be the result of the patterning of the hippocampus itself (Fig. 3), which results in the need to generate cells before the correct cues to position them are in place. It was hypothesized by Altman & Bayer (1990) that the extended stay in the intermediate band allowed for the formation of two structures critical to hippocampus development. First, the halt in migration allowed for the formation of scaffolding required to move CA1 neurons into the correct place and, second, allowed for the initiation of DG development, which is thought to be important to CA3 formation (Altman & Bayer 1990; Fig. 3). Though an intriguing hypothesis, research into the cause of this migratory delay is lacking.

Migration of Cells into Pyramidal Cell Layer

Following their delayed stay in the intermediate band, CA neurons migrate in a radial fashion toward the future site of pyramidal layer formation (Altman & Bayer 1990) eventually coming to settle in the correct location prior to birth (Angevine, 1965). Interestingly, though CA1 develops first, the cells that give rise to CA3 are actually born first (Altman & Bayer, 1990, Angevine, 1965) (Fig. 3).

The regulation of cell migration into the hippocampus is not completely understood, however it is well documented that Reelin, a chemotactic cue secreted by Cajal Retzius cells, is crucial to the correct morphology of the hippocampus.

Many classic studies in mice with mutations in the Reelin gene (Reeler mice), have documented significant defects in the lamination of the CA region in addition to those seen in the cortex (Stanfield & Cowan, 1979; For review see: Tissir et al., 2003). Reeler mice have an inverted developmental pattern, such that the hippocampal layers develop in an outside-in fashion (Stanfield & Cowan, 1979). Research suggests that Reelin may function in the correct formation of a radial glial scaffold so it is hard to determine if Reelin signaling plays a cell-autonomous role in the migration of CA neurons, or if the defects present are caused by scaffolding defects (Weiss et al., 2003).

At this time, though the gross morphology of Ammon's Horn development has been fairly well defined an understanding of the signaling pathways involved in CA neurogenesis and cell migration is still lacking.

Development of the DG

The development of the DG is unique in that it develops in a different fashion from the surrounding structures, the cortex and Ammon's Horn. In contrast to these

structures, the DG develops in an outside-in pattern, such that the earliest born neurons (ie, the oldest cells) reside postnatally in the outermost layers of the DG (Angevine, 1965; Altman and Bayer, 1990). In addition, DG development begins relatively late during brain development, starting around E14 in the mouse and continuing into postnatal life, with rapid proliferation dropping off around P10 (Angevine, 1965; Li & Pleasure, 2007; Li & Pleasure, 2005). For the sake of clarity, two broad areas of dentate gyrus development will be discussed, the morphological development and neurogenesis during embryonic development. Though these processes will be discussed separately, during development they occur concurrently.

Morphological Development of the DG

The development of the DG takes place across three structurally defined regions, each known as a matrix, and can be loosely divided into three main temporal phases of development (Fig. 4).

The primary matrix, located in the VZ, consists of the dentate neuroepithelium, and is where the initial DG progenitors begin to divide. The second matrix is considered to be the migratory stream of progenitors and differentiated cells between the primary matrix and the third matrix; the third matrix, the hook shaped area classically associated with the DG, is the location of a secondary proliferative zone during development and is the only part of the DG remaining in the adult hippocampus (Fig. 4).

The temporal divisions of DG development are as follows; first, the dentate neuroepithelium must be specified. Second, a radial glial scaffold is formed to aid in the migration of DG precursors and young granule neurons from the first to the 3rd matrix. Third, cells migrate into the third matrix and form a secondary proliferative zone in the

presumptive hilus. Following birth, the third matrix is condensed and organized into the classically layered DG structure, however this phase will only be briefly described.

Specification of the DG Neuroepithelium

The initial stage of development is the specification of the DG neuroepithelium located at the medial edge of the developing cortex, just ventral to the Ammonic neuroepithelium (Altman and Bayer, 1990). Though the defined start of DG development is not until E14, the specification of the DG neuroepithelium occurs around E12.5 (Tole & Grove, 2001), and is not fully understood. There has been research to suggest that the specification of the DG neuroepithelium is the result of "nested" transcription factor expression, regulated by the cortical hem (Fig. 1). For example, studies suggest that Emx2 and more recently, Lhx2, expression is important to the specification of the DG, (Pellegrini et al., 1996; Mangale et al., 2008) (Fig. 1). In a study by Pellegrini et al., (1996), the importance of Emx2 specifically in DG development is highlighted. Mice carrying homozygous mutations in the Emx2 allele fail to develop a DG, but do develop reduced CA structures. The authors went on to postulate that the absence of the DG is the result of a reduced dentate neuroepithelium, however it is not clear if this is the result of a patterning defect, or a result of a proliferative defect. There is also evidence to suggest that Lhx2 is involved in patterning the cortical hem, as loss of Lhx2 results in cortical hem expansion, at the expense of the hippocampal neuroepithelium (Mangale et al., 2008).

In addition to nested transcriptional factor expression, traditional developmental pathways are crucial to the early stages of DG development, with two of the most notable being Wnt signaling and Notch signaling.

Many studies have shown the importance of canonical Wnt signaling in DG development. During canonical Wnt pathway activation, Wnt binds to its' receptor, Frizzled. Receptor-ligand binding allows beta-catenin to be stabilized and translocated to the nucleus where it interacts with the Lef/Tcf family of transcription factors, leading to transcription of downstream genes (For review see: MacDonald et al., 2009). The cortical hem is known to secrete various Wnt family members and is thought to play a role in the initial specification and patterning of the dorsal cortex, including the DG (Grove et al., 1998) (Fig. 1). Wnt3a is thought to be crucial to the proper specification of the hippocampus as well as DG development. Wnt3a mutant progenitors "stall" in their proliferative ability and thus animals with mutations in Wnt3a fail to develop any hippocampal structures (Lee et al., 2000, Zhou et al., 2004 for review see Li & Pleasure, 2005). In addition, a more region specific requirement of Wnt signaling in the DG has been shown using Lef1 mutants (Galceran et al., 2000). In Lef1 mutant mice, while the CA structures formed somewhat abnormally, there was a complete absence of a DG. These results were confirmed in a later study using mutants for both Lef1 and LRP6, a Wnt co-receptor (Zhou et al., 2004), implying that variants of the Wnt signaling pathway could be affecting different regions of the hippocampus during development, with Lef1 being crucial to the development of the DG specifically (Galceran et al., 2000) (Fig. 1).

Another classic developmental pathway implicated in the early dentate neurogenic niche is Notch signaling. Pleasure et al., (2000) show that Notch effectors Hes1 and Hes5 (Ohtsuka et al., 1999 & 2001) are highly expressed in the dentate neuroepithelium prior to cell migration. Given the traditional role of Notch signaling in lateral inhibition (For review see: Beatus & Lendahl, 1998), as well as the known anti-

neurogenic roles of Hes1 and Hes5 (Ohtsuka et al., 1999 & 2001), it was postulated that this expression could be maintaining the progenitor cell population (Pleasure et al., 2000).

In recent years it has become clear that multiple intricate pathways play a key role initiating DG development, however further work into the cellular mechanisms regulating DG specification and neurogenesis are needed. Understanding the pathways involved in generating the embryonic neurogenic niche could shed light on the regulation of neurogenesis in the adult system.

Formation of the Radial Glial Scaffold

Following neuroepithelial specification, the formation of a radial glial scaffold begins. Canonical Wnt signaling and Reelin secretion from Cajal-Retzius cells have both been implicated in DG scaffold formation (Zhou et al., 2004; Weiss et al., 2003).

Mutations in components of the Wnt pathway have been shown to result in malformation of the glial scaffold required for cell migration into the 3rd matrix of the DG (Zhou et al., 2004). In 2004, it was shown that Lef1 and LRP6 mutants display disorganization of radial glial fibers and migratory defects (Zhou et al., 2004). Furthermore, it was shown that animals with mutations in beta-catenin itself display similar defects to those seen in other Wnt signaling mutants (Machon et al., 2003).

In addition to Wnt signaling, Reelin expression is also imperative to radial glial scaffold formation. It has been well documented that mice deficient in Reelin signaling display radial glial abnormalities in addition to disorganized migration and a lack of condensation of the DG (Weiss et al., 2003; Review: Förster et al., 2006). More recently, it has been suggested that the Reelin and Notch signaling pathways form a

network, leading to the correct differentiation of the radial glial cells forming the scaffold (Sibbe et al., 2009), however a direct interaction has not yet been shown.

Migration of DG Cells and formation of Secondary Proliferative Zone

Once radial glial scaffold formation is underway, the migration of DG progenitors and young DG neurons begins. During embryonic development, the migration of DG cells into the third matrix occurs in such a way that the oldest cells reside in the dorsal blade of the DG and the youngest cells form the ventral blade (Li & Pleasure, 2004). This migration is regulated primarily through the chemoattractant signaling of Cxcr4/Sdf1, though due to the radial glial defects seen in Reelin signaling mutants, a cell autonomous role for Reelin in DG cell migration has not been ruled out. Bagri et al. (2002) showed that Sdf1, secreted from the meningeal cells (Lu et al., 2002), acts as a chemoattractant to direct DG cells from the primary, through the secondary and into the tertiary matrix (Fig. 4). This study went on to show that DG cells express Cxcr4, the Sdf1 receptor, and that Cxcr4 mutants have DG malformations. Recently, it was suggested by Li et al., (2009) that Sfd1/Cxcr4 signaling is imperative not only to the formation of the DG itself, but also to the formation of the secondary proliferative zone in the hilus anlage, as in the absence of this signaling pathway, this proliferative zone fails to form. The cells that migrate into the third matrix to form this secondary proliferative zone rapidly expand the number of DG granule cells (Li et al., 2009). Rapid proliferation continues in this secondary region until P10, at which time reorganization of the DG moves these proliferative cells into the subgranular zone (SGZ) of the DG where they will continue to cycle at low levels through out adulthood (Berger et al., 2007; Li et al., 2009).

Neurogenesis in the Embryonic Dentate Gyrus

The study of embryonic DG neurogenesis is made difficult by a few factors. First, the cell lineage during DG development is not as well defined as in adult neurogenesis, however most markers used to study cortical development or adult neurogenesis appear to correlate well with DG development. Second, the migratory stream during the development of the DG is a mixture of stem-like cells, intermediate neuronal progenitors (INPs) and young granule neurons, making it difficult to define cell type based on location (For review see: Pleasure et al., 2000). Due to this, various markers have been used to identify the different populations of cells during embryonic neurogenesis and the roles and interactions of these markers are starting to become clearer. Though there are various markers used for each population, only the ones used in the present study will be discussed in detail (Fig. 5).

During development of the DG, the stem-like cell population is believed to be radial glial like and is commonly labeled with Sox2, Pax6, Mash1, brain lipid binding protein (BLBP), or low levels of Prox1 (Englund et al., 2005;Pleasure et al., 2000; Pevny et al., 2010; Galichet et al., 2008; Fig.5). For the purpose of this study, Sox2, the stem cell pluripotency factor (Ellis et al., 2004), was used as a marker of the neural stem-like cells present in the dentate neuroepithelium at E14.5, and BLBP was used to visualize the radial glial scaffold at later stages. The presence of true "stem cells" within the DG of the developing hippocampus beyond E14.5 is debatable (Clarke and van der Kooy, 2011). To circumvent this issue, analysis of the stem-like cell population was temporally limited to E14.5, a time when stem-like cells are believed to be present (Clarke and van der kooy 2011).

The intermediate neuronal progenitor (INP) population has been defined by expression of the T-box transcription factor, Tbr2, primarily in the neocortex (Englund et al., 2005) as well as in the adult hippocampus (Kempermann et al., 2004). Recent studies have confirmed that INPs in the developing DG also express Tbr2 (Li et al., 2009; Hodge et al., 2012) and it has been shown that Tbr2 expression is required during DG development for cells to mature (Fig. 5). Hodge et al., (2012) went on to provide evidence that Tbr2 could be involved in negatively regulating Sox2, suggesting a feedback loop between the stem and INP cell populations to ensure the maintenance of progenitor cell pools.

The final population discussed is the immature granule neuron population. These cells are first characterized by the expression of NeuroD1, then as the cells begin to mature, high levels of Prox1 (Fig. 5). NeuroD1, a basic-helix-loop-helix transcription factor, is expressed throughout Ammon's horn and the DG in a transient fashion such that late stage progenitors will increase NeuroD1 expression as cell cycle exit begins, and NeuroD1 expression is lost once the newborn neurons begin to mature (Miyata et al., 1999; Liu et al., 2000). Deletion of NeuroD1 expression leads to an almost complete absence of DG structures, potentially due to a defect in initiation of cell cycle exit, a theory that fits well with the temporal expression of NeuroD1 (Miyata et al., 1999; Liu et al., 2000). Interestingly, NeuroD1 is also important to the correct differentiation of granule cells in the cerebellum, possibly suggesting a differentiation role specific to granule cells (Miyata et al., 1999).

The expression of Prox1 in the development of the DG is ubiquitous, with very low expression levels indicating DG progenitors and high expression indicating newly

born granule neurons (Galichet et al., 2008) (Fig. 5). Prox1 expression is maintained at high levels in DG granule neurons throughout life (Galichet et al., 2008; Lavado et al., 2010; Pleasure et al., 2000). The expression of Prox1 is crucial to granule cell maturation during development and has also been shown to regulate Notch inhibition of neurogenesis (Lavado et al., 2010; Kaltezioti et al., 2010).

Though not much is known about the regulation of Prox1 or NeuroD1 during development, recent work in the adult brain suggests that Wnt signaling may control expression of both of these transcription factors (Karalay et al., 2011; Kuwabara et al., 2009).

An increased understanding of factors that control embryonic neurogenesis in the DG could clarify the mechanism of DG neuron production in the adult brain. Cell cycle regulation is crucial to the formation of the correct number and types of cells in the cortex (For review see: Dehay et al., 2007), and is likely imperative to correct DG development as well.

Rb and Other Pocket Protein Family Members

The first tumor suppressor protein indentified, Rb is a nuclear phosphoprotein involved in cell cycle regulation (Reviewed in Harbour & Dean, 2000). This protein is critical to normal development, as Rb deletion results in embryonic lethality between E13.5 and E15.5 (Lee et al., 1992).

The "pocket proteins" are a family of cell cycle regulatory proteins named for their common E2F binding pocket. This family consists of the retinoblastoma protein (Rb), p107 and p130. Through inhibition of the various E2Fs, these pocket proteins form

a multifaceted regulatory system to control cell cycle progression at the G1/S phase checkpoint (Fig. 6) (For review see: Harbour & Dean, 2000; Cobrinik, 2005).

Rb is known as a cell cycle exit protein as it prevents the G1-S phase transition, primarily through inhibition of the activator E2Fs; the E2F family is discussed below (Reviewed in Harbour & Dean, 2000; Ferguson & Slack, 2001; Cobrinik, 2005). Active Rb is hypophosphorylated and can either bind directly to E2F or bind to the promoter on the gene of interest as a complex with E2F, both of which inhibit E2F mediated transcription (Weintraub et al., 1995; Reviewed in Hallstrom et al., 2009). When cells receive a signal to divide, a CDK-cyclin complex is required for Rb inactivation. Lundberg et al. (1998) found that both the cyclinD-CDK4/6 complex and the cyclinE-CDK2 complex are required sequentially for phosphorylation and inactivation of Rb. Phosphorylation results in release of E2F inhibition and the transcription of downstream genes. Interestingly, one of these cell cycle progression genes was found to be cyclin E itself (Ohtani et al., 1995), suggesting a feedback loop in which Rb inhibition can lead to a further, more enhanced Rb inhibitory effect, thus further driving cell cycle progression and adding another level of complexity to Rb cell cycle regulation.

In addition to directly inhibiting E2F family members, Rb has also been implicated in altering gene expression through the recruitment of chromatin remodeling complexes (Brehm et al., 1998; Luo et al., 1998; Zhang et al., 2000; For review see: Macaluso et al., 2006). Zhang et al. (2000) suggest that through the recruitment of an HDAC complex, Rb is able to regulate the transcription of both cyclinA and cyclinE. They go on to show that through two Rb-SWI/SNF complexes, Rb maintains the correct expression timing of cyclinA and cyclinE during the cell cycle, suggesting a role of Rb

beyond a simple checkpoint protein (Zhang et al., 2000). Though not certain, there is also data to suggest that the Rb mediated recruitment of chromatin remodeling complexes could play a key role in the long term repression of cell cycle genes following cellular differentiation (Narita et al., 2003).

The Rb protein is crucial to proper development as mouse embryo's lacking functional Rb die prenatally due to a placental defect (Lee et al., 1992; Jacks et al., 1992). In a study by de Bruin et al. (2003), Rb deleted mice provided with a non-mutated umbilical cord were viable until soon after birth. Mice with deletions in Rb generally have ectopic proliferation, marked defects in differentiation and increased apoptosis in various cell types (Lee et al., 1992, de Bruin et al., 2003, Mayhew et al., 2005). However, it was recently shown by Wirt et al., (2010) that cell types from specific lineages, namely the neural and muscular lineage, are able to exit the cell cycle in the absence of all pocket proteins. These data suggest that Rb plays a complex role in cell cycle regulation and that non-Rb related pathways are critical in cell cycle arrest in G0/G1 (Wirt et al., 2010).

Though less studied than Rb, the other pocket proteins, p107 and p130 also have important roles in the maintenance of cell cycle stability and can provide partial compensation in the absence of Rb. These two pocket proteins are expressed at different times in the cell cycle than Rb and tend to have a higher affinity for the repressor E2Fs. It has been shown that p107 tends to be expressed most highly in proliferating cells (Shin et al., 1995; Kiess et al., 1995) and p130 most highly in quiescent cells (Cobrinik et al., 1993; Shin et al., 1995; Smith et al., 1996). Germ line deletion of p107 has not been found to be lethal, however p130 deletion is embryonic lethal specifically on a BalbC

background (LeCouter et al., 1998; Lee et al., 1996; Cobrinik et al., 1996). Interestingly, double-deletion of both p107 and p130 results in postnatal lethality (Cobrinik et al., 1996).

It should be noted that p107 and p130 have the ability to partially compensate for each other as well as Rb, as triple knockout animals have a much more severe proliferative defect that Rb knockouts alone (Wirt et al., 2010). Many studies have been performed trying to elucidate the roles of the pocket proteins in various systems, with the common theme being a tissue specific role; pocket proteins in neurogenesis will be discussed below.

E2F Proteins

The E2F family of transcription factors are the main binding partners of Rb. Classically, in cells that are not cycling, Rb is bound to E2Fs, inhibiting them. When the cell cycle is to proceed through to S phase, Rb is inactivated and the E2Fs become free to activate transcription of their target genes (Fig. 5), many of which consist of proteins required for cell cycle progression, and DNA synthesis (Polager et al., 2002; Dyson, 1998; Ren et al., 2002; Ferguson and Slack, 2001; Wu et al., 2001). Though accepted, the above scenario has recently been found to be an oversimplification. The role of E2F is complicated by the fact that there are known to be at least nine different E2Fs, all of which behave differently and recent work in an *in vivo* model suggests that E2Fs could play differing roles based on the presence or absence of Rb. Understanding these complex proteins is imperative to a clear understanding of the role of Rb.

The E2F family of transcription factors is composed of E2Fs 1 through 8 (Fig. 7). These proteins can be roughly divided into the categories of activator E2Fs (E2F1, E2F2,

E2F3a), repressor E2Fs (E2F3b, E2F4, E2F5, E2F6) and the atypical E2Fs (E2F7 and E2F8) (Fig. 5). Rb has been found to interact primarily with the activator E2Fs as well as the repressors, E2F3b and E2F4 (Lees et al., 1993; Moberg et al., 1996; For Review see: Trimarchi and Lees, 2002). E2F6 and the atypical E2Fs do not require pocket protein binding for function, and E2F5, though able to bind Rb, binds preferentially to p107 and p130 (For review see: Trimarchi and Lees, 2002). For the purpose of this study only the activator E2Fs and the repressors, E2F3b and E2F4, will be discussed.

The activator E2Fs, are present primarily in actively dividing cells. It was traditionally thought that activator E2Fs simply promote cell cycle progression and Rb functions to control this cell cycle progression response. The activator E2Fs are responsible for many of the proliferative defects associated with E2F deregulation. Ectopic activator E2F expression has been shown to induce cell cycle re-entry in various models of quiescent cells (Qin et al., 1994; Asano et al., 1996), and research indicates that the ectopic proliferation present in Rb deleted tissues could be rescued with concurrent E2F deletion (Ziebold et al., 2001; Saavedra et al., 2002; Parisi et al., 2007). However, new data suggests that the relationship between Rb, activator E2Fs and cell cycle progression may be more complex then initially thought. Recently, downstream E2F targets not linked to cell cycle progression have been identified (For Review see: Bracken et al., 2004). Activator E2Fs have now been shown to regulate genes involved in differentiation, migration, and apoptosis, indicating that the role of Rb-E2F is not straightforward (Hou et al., 2000; Muller et al., 2001; Macleod et al., 1996; Guo et al., 2001; Ferguson et al., 2005). In addition, *in vivo* research in mammalian models indicates that the role of activator E2Fs may change based on pocket protein binding.

Much of the data surrounding Rb dependent regulation of the activator E2Fs, was generated primarily in lower invertebrates or shown through *in vitro* assays (Asano et al., 1996), however recent *in vivo* work in the mouse suggests that activator E2Fs can actually "switch" to repressors in the presence of Rb (Chong et al., 2009a; Chen et al., 2009; Wenzel et al., 2001). Chen et al. (2009) found that contrary to the traditional belief, activator E2Fs were not required for normal proliferation but instead were important to postnatal survival of mouse retinal progenitors. A paper by Wenzel et al. (2011), supports this finding by showing that activator E2Fs are not required for proliferation in the developing mouse lens and suggest that activator E2Fs play a critical repressive role during normal development and differentiation. They go on to suggest that E2Fs only act to drive cell cycle progression under special circumstances, such as the loss of Rb function (Wenzel et al., 2011). These data suggest a complex system in which activator E2Fs can actually "switch" into a repressive role in the presence of Rb, however further study to understand this phenomenon is ongoing.

The traditional repressor E2Fs are involved primarily in the repression of E2F target genes and are generally present at highest levels in quiescent cells (For review see: Trimarchi and Lees, 2002). E2F3b is known to bind Rb, while E2F4 binds the different pocket proteins based on the phase of the cell cycle, with the highest affinity appearing to be for p107 or p130 (Moberg et al., 1996; Chong et al., 2009b). Though our understanding of the E2F family of transcription factors is beginning to clarify, further research is needed to really understand the interactions between all these family members.

Overall, though much work has been done, the role of E2Fs is continuously evolving. For this study, a classical model of Rb-E2F has been assumed, however, it is important to keep in mind that this is a complex family of transcription factors, with intricate networks regulating downstream target genes.

Rb/E2F Pathway in Neural Stem Cell regulation/Cortical development

Rb has previously been implicated in the regulation of neurogenesis. It was shown by Lee et al., (1992) that mice with deletion of the Rb gene die prenatally and the brains of these mice display ectopic proliferation in addition to widespread apoptosis. The presence of apoptosis in the Rb deleted brain lead to the concept that Rb may be directly linked to apoptotic regulation (Slack et al., 1995).

However, in a study by de Bruin et al. (2003), it was suggested that the increased apoptotic death seen in Rb mutants could be due to a placental defect as Rb deleted mice provided with a non-mutated umbilical cord were viable until birth and lacked the apoptotic activity normally seen in Rb knockout mice (Ferguson et al., 2002; MacPherson et al., 2003). Though this is an intriguing concept, there is currently more evidence to support a role of Rb inactivation initiating a p53 or Apaf1 dependent apoptotic pathway (Qin et al., 1994; Macleod et al., 1996; Morgenbesser et al., 1994; Guo et al., 2001; Hershko et al., 2005; Reviewed in Polager & Ginsberg, 2009). In 2005, p53 was found to be a downstream target of E2F1 and E2F1 was also found to transcribe various p53 cofactors, directing cell fate toward apoptotic cell death as opposed to growth arrest (Hersko et al., 2005; Chen et al., 2005). In addition, Rb deficiency has also been found to activate Apaf1, which is important for apoptotic cell death in the CNS (Guo et al., 2001).

Deletion of Rb in neural precursor cells of the telencephalon was shown to cause delayed differentiation as well as induction of p107, E2F1 and E2F3 (Callaghan et al, 1999), however no difference in neuronal specification was detected (Ferguson et al., 2002). The up regulation of E2F1 and E2F3 was accompanied by increases in their target genes, CyclinA, CyclinE and Cdk2, indicating that the mechanism of enhanced proliferation in the absence of Rb is likely be dependent on activator E2Fs (Callaghan et al., 1999). Further study showed that Rb/E2F3 double deletion rescued the proliferation phenotype in neural precursor cells (McClellan et al., 2007).

In addition to its regulatory role in proliferation, Rb has also been shown to be important in the tangential migration of interneurons in the cortex (Ferguson et al., 2005). Though there was a decrease in Cajal-Retzius cells in the Rb deleted cortex, reciprocal transplantation experiments were able to show that the migration defects present in the interneuron population were cell autonomous (Ferguson et al., 2005).

The Rb family member, p107, has been implicated in the regulation of neural precursor cells (Vanderluit et al., 2004; Vanderluit et al., 2007). It was found that p107 deleted mice had increased levels of neuronal precursors *in vitro* and that p107 was able to regulate the number of stem cells *in vivo* (Vanderluit et al., 2004). However, though it was found that p107 deletion resulted in increased numbers of neural stem cells, this did not translate into increased numbers of neurons (Vanderluit et al., 2004). Instead, p107 deficient animals exhibited a defect in neuronal commitment. The same group went on to determine that p107 regulated cell commitment to neuronal fate in developing cortical neurons (Vanderluit et al., 2007).

Finally, a study by Ferguson et al. (2002) showed that deletion of Rb specifically in the telencephalon lead to increased cortical neurogenesis. In this study, not only was proliferation increased, but the ectopically dividing cells were able to survive and differentiate, a phenotype not seen in the p107 knockouts.

Overall, the current data regarding Rb deficiency and neural precursors suggest that pocket proteins are imperative to various aspects of cortical development. In the absence of Rb, cell numbers in the cortex are increased, suggesting a similar phenotype is possible in the developing DG.

Hypothesis and Rational of the Present Study

The above studies suggest a critical role for pocket proteins in the development of the cortex, and indicate that the Rb/E2F pathway may be a crucial regulator of neurogenesis. Currently, nothing is known about the role of Rb during the development of the DG, however studies have suggested Prox1 could be a downstream E2F target (Wenzel et al., 2011), indicating a potentially unique role for Rb in DG development. In addition, Rb could be involved in many areas of DG development, including neurogenesis and migration. Therefore, I propose that Rb signaling plays an important role in regulating embryonic DG neurogenesis and hypothesize that loss of Rb will enhance neurogenesis. The results of this study are important to determine the potential for Rb pathway modulation as a novel strategy by which to enhance adult neurogenesis, in addition to increasing the understanding of DG development.

We will address this hypothesis through the use of an *in vivo* mouse model in which Rb is deleted in neural precursor cells of the telencephalon at approximately E10. There are 3 objectives: 1) to determine if Rb deletion can expand the granule cell

population. 2) To determine if Rb loss alters the generation and/or the survival of neurons. 3) To determine if neurons generated in the absence of Rb migrate to the correct location. The results of this study will shed light on the role of Rb in DG development as well as address the potential for Rb modulation to enhance adult neurogenesis.

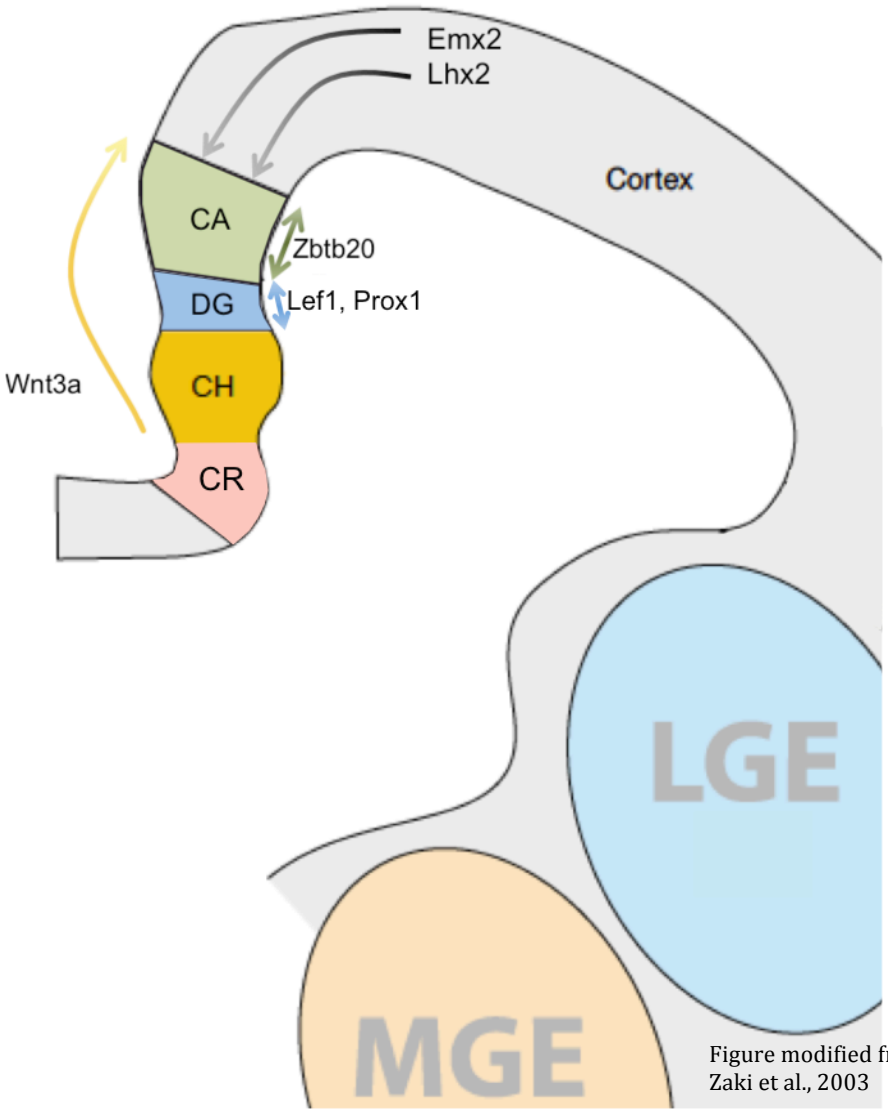


Figure 1: Factors involved in the specification of the hippocampus.
The above figure depicts a simplified version of the location and approximate expression of various signaling and transcription factors important to the definition of the hippocampal field at E13 in the mouse. Wnt3a, secreted from the cortical hem, is important to specification the hippocampal field, Lhx2 and Emx2 expression defines the boundary of the cortex. Zbtb20 is specific to just the Ammonic neuroepithelium and Lef1 and Prox1 are crucial to the definition of the DG neuroepithelium. Deletion of any of these factors is detrimental and can result in the expansion of one region at the expense of another. CR, choroid plexus; CH, cortical hem; DG, dentate gyrus neuroepithelium; CA, ammonic neuroepithelium; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence.

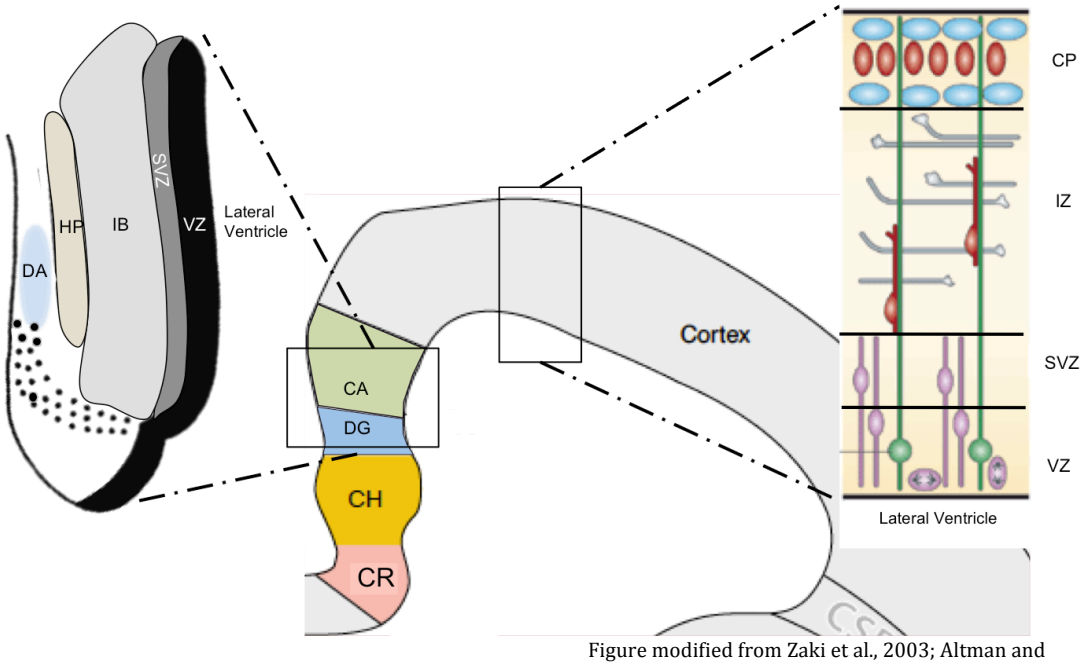


Figure modified from Zaki et al., 2003; Altman and Bayer 1990 and Nadarajah and Parnavelas, 2002

Figure 2: Comparison of Cortical and CA development.
The above figure depicts a simplified version of the parallels between cortical and CA development. Both the developing cortex and hippocampus have a ventricular zone (VZ), subventricular zone (SVZ), intermediate zone or band (IZ or IB) and cortical plate or hippocampal plate (CP or HP). In both cases, cells are generated in the proliferative VZ and SVZ and then migrate away from the ventricle. The dentate anlage (DA) is located just medial to the hippocampal plate and is the site of future dentate gyrus formation. CR, choroid plexus; CH, cortical hem; DG, dentate gyrus neuroepithelium; CA, ammonic neuroepithelium; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate.

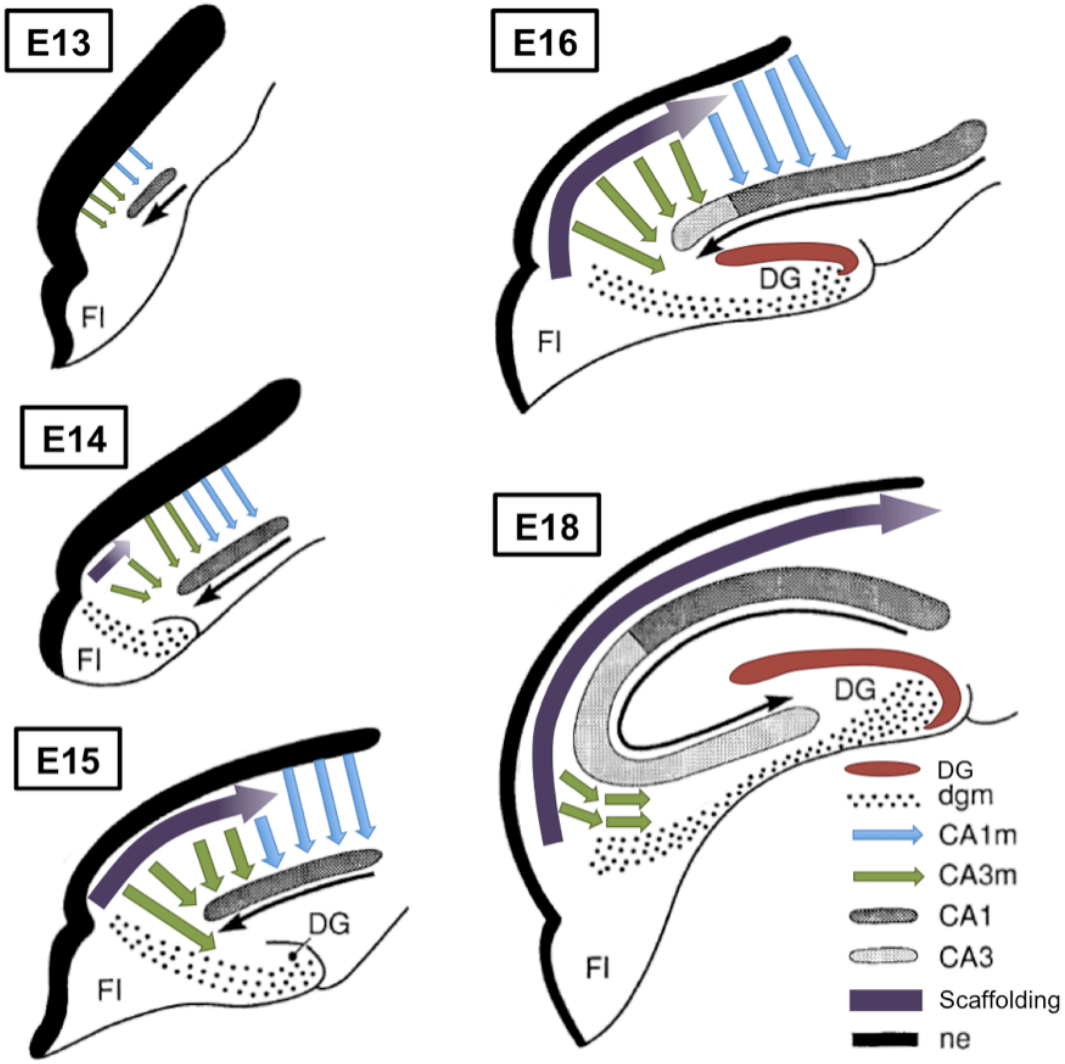


Figure modified from Altman and Bayer, 1990

Figure 3: Morphological development of the CA region of the hippocampus. The gross development of the CA structures is outlined above from E13 until E18 in the mouse. A solid black arrow shows the direction of growth of Ammons Horn. During development, the cells giving rise to CA3 are born first, but are delayed in the intermediate band. Ultimately CA1 is formed before CA3. Scaffolding to support CA1 migration is formed starting at the fimbria (FI) and progresses toward the subiculum (not shown). DG, dentate gyrus; dgm, dentate gyrus migratory stream; CA1m, migratory path of CA1 neurons; CA3m, migratory path of CA3 neurons; ne, neuroepithelium.

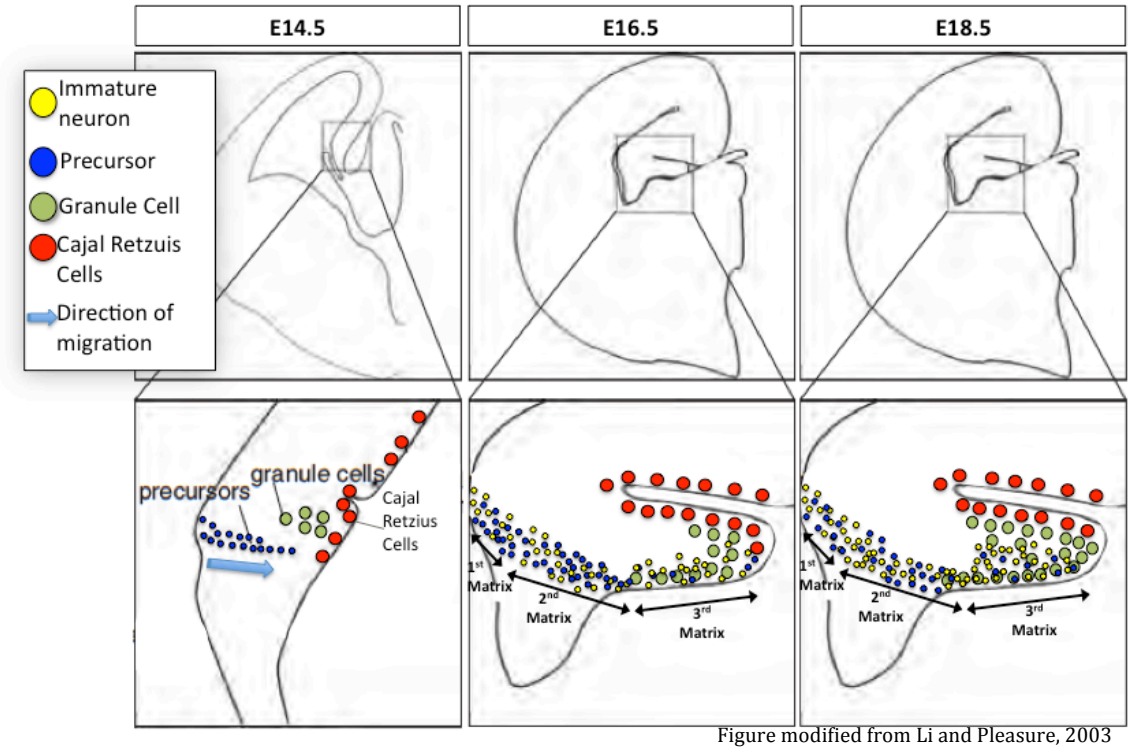


Figure modified from Li and Pleasure, 2003

Figure 4: Overview of the development of the DG.
The development of the DG begins at E14.5 with the generation of DG precursors along the ventricle. Cajal Retzius cells along the medial surface secrete Reelin, which is required for the formation of the radial glial scaffold. As development progresses, glial scaffolding increases and a migratory stream consisting of precursors and immature neurons forms, moving along the glial scaffold into the dentate anlage. Immature neurons in the anlage begin to express Prox1 at high levels and the characteristic hooked shape of the DG becomes visible. The regions of DG migration are loosely divided into three matrices. The first matrix consists of the cells coming off the ventricle. The second matrix is the migratory stream and the third matrix is the hook shaped structure traditionally associated with the DG. Once development is complete, only the third matrix of the DG remains.

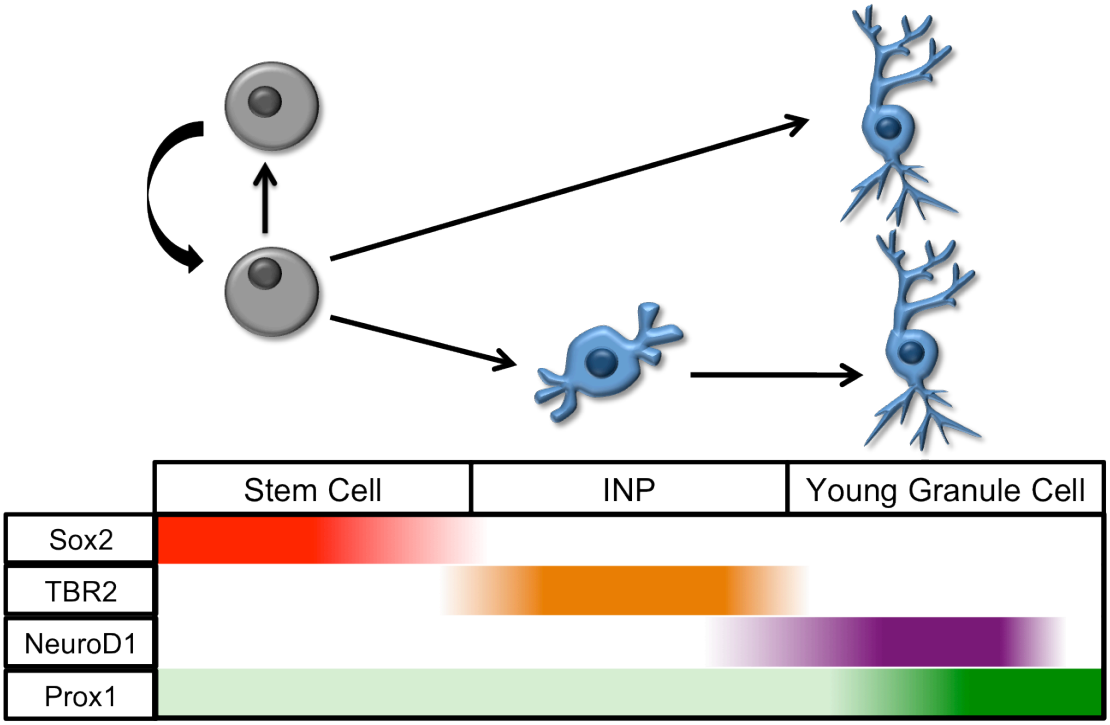


Figure 5: Expression of markers used in this study.
A brief cell lineage is shown above. Expression of the markers used in this study are shown by coloured gradients. Sox2 expression is high in the stem-like cell population and TBR2 expression is high in the intermediate neuronal progenitor (INP) cells. NeuroD1 expression begins with the differentiation of granule neurons however decreases upon complete differentiation. Prox1 is expressed at low levels in all the cells of the DG, however expression is strongly increased during differentiation into granule neurons and remains high through out mature life.

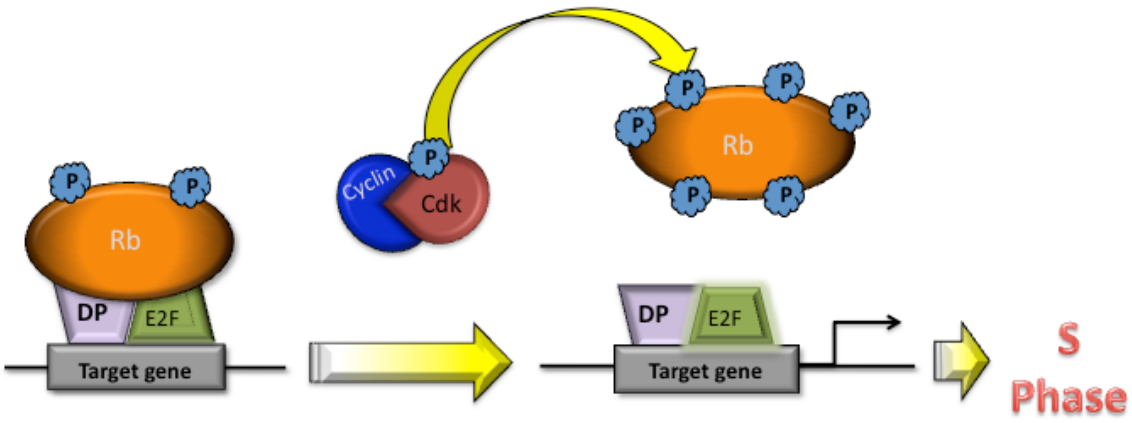


Figure 6: Rb/E2F Pathway.
The Rb protein functions as a G1 exit protein. During the G1 phase, Rb is initially hypophosphorylated (P), and works to repress activator E2F function. When the cell reaches the G1 checkpoint to move through into S phase, if the cell cycle is to progress, Rb is hyperphosphorylated (P) by a Cyclin/Cdk complex and in inactivated. Inactivation of Rb releases the activator E2Fs, allowing transcription of their target genes, usually genes involved in cell cycle progression.

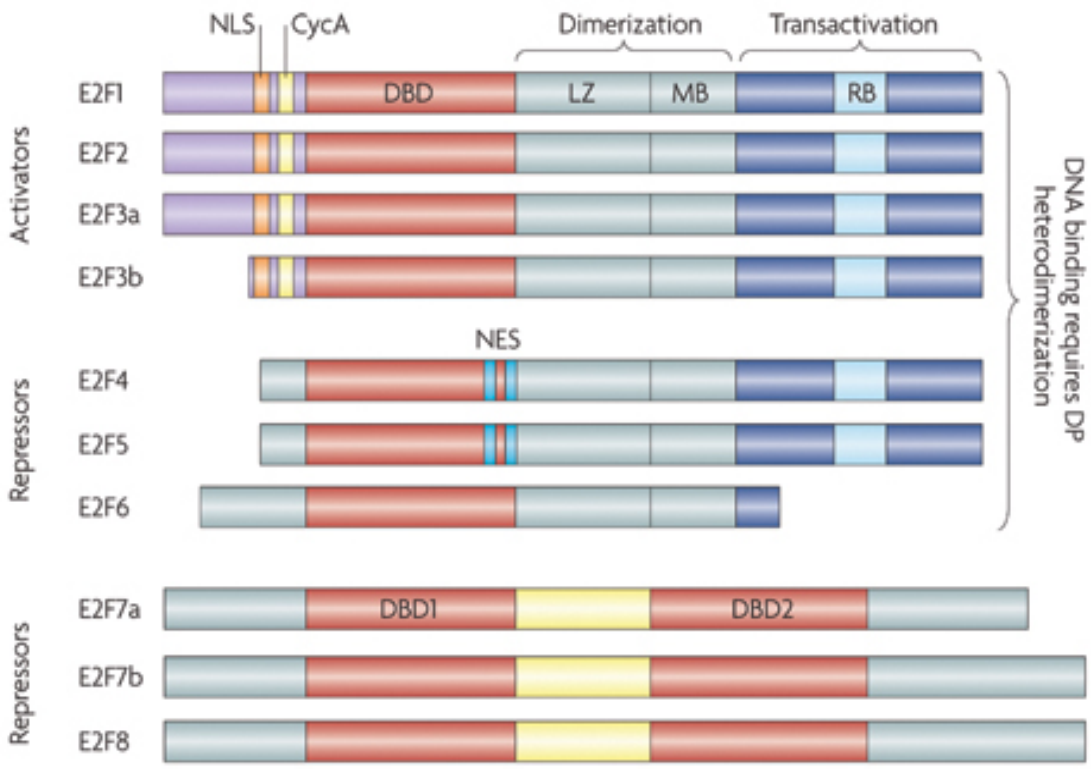


Figure modified from Chen et al., 2009b

Figure 7: E2F family of transcription factors.
The E2F family of transcription factors is divided into three main categories, activators, repressors, and "atypical" repressors that do not bind pocket proteins. All E2Fs have a DNA binding sequence (DBD) allowing activation or repression of specific target genes. Only E2Fs 1-5 are known to interact with Rb, and the repressor E2Fs, E2F4 and E2F5 are known to interact primarily with pocket proteins other then Rb. The activator E2Fs are traditionally associated with cell cycle progression, as many of their target genes are involved in this process. NLS, nuclear localization signal; CycA, cyclinA binding site; NES, nuclear export signal.

Material and Methods

Mice

The Rb floxed mice, generated by Anton Burns (Marino et al., 2000), and FoxG1 Cre mice, which have Cre knocked into the FoxG1 (Brain factor 1) locus, generated by Suzanne McConnel (Hebert and McConnell, 2000) were all maintained on an FVBN background. Animals used in experiments were generated as previously described in Ferguson et al., 2002. Briefly, females carrying homozygous floxed Rb alleles (Rb Flox/Flox) were crossed with males that were double heterozygous for FoxG1 Cre and the floxed Rb allele (FoxG1 Cre/+, Rb Flox/+). This allowed the generation of Rb deficient (Rb Flox/Flox; FoxG1Cre/+) and double heterozygous control animals (Rb Flox/+; FoxG1Cre/+) in the same litter.

Mice were genotyped using the Sigma Extract-N-Amp kit and the following primers: Rb flox: 5'- GGC GTG TGC CAT CAA TG -3' and 5' – AAC TCA AGG GAG ACC TG -3'; FoxG1Cre: 5'- TGACCAGAGTCATCCTTAGCG – 3' and 5'- AATGCTTCTGTCCGTTTGCC – 3'.

The time of plug identification was considered to be day 0.5 for all time points. All experiments were approved by the University of Ottawa's Animal Care and Ethics committee and adhere to the guidelines put forth by the Canadian Council on Animal Care.

Tissue Preparation and Immunohistochemistry

Pregnant female mice were euthanized by injection of 19.5mg of Euthanyl (Supplied by: Animal and Veterinary Care Services, The University of Ottawa) followed by cervical dislocation. E14.5, E16.5 or E18.5 embryos were dissected, euthanized by decapitation and heads were fixed over night in 4% paraformaldehyde (PFA) in 1x

phosphate buffered saline (PBS), pH of 7.4. Heads were infiltrated with 20% sucrose, embedded in Tissue-Tek OCT compound (Sakura), frozen, and sectioned at 14μm, coronal, onto Superfrost Plus slides (Fisher).

Prior to immunostaining, slides were briefly dried at room temperature, and then washed once in PBS. Antigen retrieval was performed, when necessary, by 30-minute incubation at 95-98^{o}C in Dako Antigen Retrieval solution (Dako). Slides were then allowed to cool for 15 minutes, rinsed once with PBS and incubated overnight at 4^{o}C in primary antibody diluted in 0.1% Triton/0.1% Tween/PBS (Table 1). Slides were rinsed three times in PBS and the appropriately conjugated secondary antibody was applied, 1:500 in 0.1% Triton/0.1% Tween/PBS (Table 1). Slides were incubated at room temperature for 45 minutes, then the secondary antibody solution was removed and slides were treated for 5 minutes with 1mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma), diluted 1:1000 in PBS followed by two washes in PBS. Slides were coverslipped with Immunomount (Genetex) and imaged on a Zeiss confocal microscope using Zen imaging software.

Cresyl Violet Staining

Cresyl violet staining procedures were adapted from Sirkin (1983). Briefly, frozen sections were allowed to dry completely at room temperature prior to staining. Once dry, slides were post-fixed for 10 minutes in 10% formalin followed by 2 washes with sterile water. Sections were then dehydrated and rehydrated using an alcohol gradient then incubated for 7-8 minutes in 0.25% Cresyl violet in 200mM acetate buffer. Once stained, slides were rinsed in water, dehydrated with alcohol and cleared with xylene. Slides were then coverslipped, using Permount (Fisher) mounting medium.

Cell Quantification and Statistical Analysis

For manual cell quantification, counts were performed using confocal images on four adjacent sections from each brain that had been leveled rostra-caudally based on cresyl violet staining, and values are expressed as per section or per population. For estimates of total cell number within the DG, a modified serial counting technique was used (Mandyam et al., 2007). Every sixth section through out the DG was quantified, with the DG defined as the area containing Prox1+ cells. The total population of interest within each section was imaged and then counted automatically using ImageJ software (Galichet et al., 2008) if the total population was greater then 300 cells per section, otherwise cells were counted manually. For automated counting, images were loaded into the software then colour/brightness thresholds were set and remained constant throughout the experiment (Galichet et al., 2008; Table 2). Results of these counts were summed and multiplied by 6 to generate an estimate of the total population within the DG (Mandyam et al., 2007). Results of the serial counting technique were able to validate counts performed on adjacent sections. Quantification of cultured cells was performed manually.

Statistical analysis was performed using Microsoft Excel or Prism software. Unpaired, two tailed Student's T-tests or one-way ANOVA tests were performed for all results with a minimum 95% confidence threshold. Unless otherwise stated, all data is presented as the arithmetic mean, plus or minus the standard error of the mean (+/- SEM) and analysis was performed using a minimum of 3 separate animals, an "n" value of 3.

Progenitor Cultures

DG dissection and progenitor culture techniques were adapted from Babu et al., (2011) as well as Clarke & van der Kooy (2011). Breifly, E14.5 pregnant female mice were euthanized and embryo's were removed then placed in ice cold sterile Hank's Buffered Salt Solution (HBSS). Brains were removed and the dentate neuroepithelium was dissected out of the medial lip of the cortex and placed in cold hibernation medium overnight, to allow the embryo's to be genotyped (Table 3). Embryos were genotyped as previously described and tissue from one animal was triturated and plated into 10ml of stem cell medium (Table 3). Cells were allowed to grow as spheres for 5 days, at which time they were trypsinized for 5 minutes at 30^{o}C with TrypLE, a more stable and gentle alternative to trypsin, then triturated. Cells were counted using the Countess automatic cell counter and plated at 50,000 cells per well in a 24 well plate coated with poly-L-ornithine and 10μg/mL laminin. Cells were plated in monolayer medium (Table 3) and were allowed to grow as a proliferative monolayer for approximately 3 days, at which time the medium was changed for differentiation medium (Table 3). All cells were allowed to differentiate for 7 days with BrdU added to the wells, at previously defined intervals, to a final concentration of 10μg/mL throughout differentiation. A 50% medium change was performed every second day for the 7 days of differentiation, after which time cells were fixed for immunocytochemistry. Neurons in culture were identified through Tuj1 staining, a marker of neuronal specific beta-tubulin.

For a neurosphere assay, dissection and genotyping were performed as described above but prior to plating; cells were counted using the Countess automated counter and 6 wells per animal were plated at a density of 10 cells/μL in a 24 well plate. Neurosphere

assays were left to grow for approximately 7 days, at which time the number of spheres per well was quantified.

Immunocytochemistry

Cells were fixed for 20 minutes at room temperature with 4% PFA in PBS. Following fixation, cells were washed 3 times, for 5 minutes each with PBS. To denature DNA, cells were treated for with 2N HCl at 37^{o}C for 30 minutes, and then neutralized with 0.1M borate buffer for 15 minutes at room temperature. Cells were rinsed once with PBS then permeabilized for 10 minutes in 1% Triton/PBS rinsed again with PBS and blocked for 30 minutes in 1% donkey serum/PBS. Primary antibodies were diluted in 1% donkey serum/PBS and incubated over night at 4^{o}C (Table 1). Cells were washed three times, 5 minutes each with PBS. Appropriately conjugated secondary antibodies were diluted 1:500 in 1% donkey serum/PBS and left at room temperature for 45 minutes (Table 1). Secondary antibody was removed and cells were treated with 1mg/ml DAPI, diluted 1:1000 in sterile H_2O, for 5 minutes, then washed twice with H_2O and imaged using a fluorescent microscope with Axiovision software.

Table 1: Antibodies used for Immunostaining

Antibodies used for immunostaining are listed. The dilution of each antibody as well as the source of the antibody is shown. Various secondary antibodies were used depending on the correct primary conjugate, however the fluorophores used, the dilution and their sources are listed. The species in which the antibody was generated is stated (in brackets).

Antibody	Dilution	Source
Primary		
Prox1 (rabbit)	1:1000	Millipore
NeuroD1 (goat)	1:500	Santa Cruz
Ki67 (mouse)	1:100	BD Pharmigen
Tbr2 (rabbit)	1:1000	Abcam
Sox2 (goat)	1:500	Millipore
AC3 (rabbit)	1:250	Cell Signalling
BLBP (rabbit)	1:1000	Millipore
Reelin (mouse)	1:500	Calbiochem
Caleretinin (rabbit)	1:500	Swant
Tuj1 (mouse)	1:1000	Convance
GFAP (rabbit)	1:1000	Dako
Secondary		
Alexa Fluor-488 (donkey)	1:500	Jackson Immuno
Cy3 (donkey)	1:500	Jackson Immuno
Dylight-649 (donkey)	1:500	Jackson Immuno

Table 2: Parameters for Automated Counting of Serial Sections

The ImageJ parameters used for automated counting are listed. Positive staining within an image was based on colour threshold (for co-labeling), brightness, size and shape. Once set, the software automatically identified and quantified positive staining within an image.

Stain	Colour Threshold	Brightness	Pixel Size	Circularity
Prox1/NeuroD1	blue-green 102/155	89/255	20-infinite	0-1
Ki67/NeuroD1	red-blue 184/241	71/255	20-infinite	0-1
Ki67+	0/64	N/A	20-infinite	0-1

Table 3: Cell Culture Medium

The components of the mediums used for cell culture are listed below along with their working concentrations and source.

Component	Final Concentration	Source
Stem Cell Medium		
DMEM:F12	N/A	Gibco
B27	2%	
ABAM	1%	Sigma
Heparin	2ug/mL	Sigma
EGF	0.02ug/mL	Sigma
FGF	0.02ug/mL	Sigma
Monolayer Medium		
DMEM:F12	N/A	Gibco
N2	1%	
ABAM	1%	Sigma
Heparin	2ug/mL	Sigma
EGF	0.02ug/mL	Sigma
FGF	0.02ug/mL	Sigma
Differentiation Medium		
DMEM:F12	N/A	Gibco
N2	1%	
ABAM	1%	Sigma
Heparin	2ug/mL	Sigma
FBS	1%	
Hibernation Medium		
H20	N/A	Giboo
Potassium Chloride	2.23mg/mL	Sigma
Glucose	0.9mg/mL	Sigma
Magnesium Chloride Hexahydrate	0.048mg/mL	Sigma
Sodium Phosphate Monohydrate	1.5mg/mL	Sigma
Sodium Monohydrogen Phosphate Heptahydrate	1.34mg/mL	Sigma
Lactic Acid	0.18%	Sigma
Sorbitol	25.5mg/mL	Sigma

Results

Aim 1: Gross Morphology and Neurogenesis in the DG

Previous work has shown that loss of Rb in neural tissues can increase cell production and cause overgrowth of structures (Ferguson et al., 2002). In order to determine the effect of Rb deletion on the gross morphology of the hippocampus and DG, cresyl violet staining was performed at E14.5, when DG development begins, at E16.5, a mid point in embryonic DG development, and E18.5, the latest developmental stage studied as our mouse model is lethal at P0.

Staining at E14.5 did not show any gross morphological changes; the neuroepithelial structures appeared similar between Rb deficient brains and littermate controls (Fig. 8). Midway through embryonic development, at E16.5, the first changes in morphology became apparent. The dorsal blade of the DG was visible in control animals, however was absent in Rb null brains (Fig. 8). In addition, cell overgrowth became apparent throughout Ammon's Horn in the absence of Rb. At the latest time point studied, E18.5, an increase in the cellularity of the CA regions as well as the DG was present in the Rb deleted hippocampus when compared with controls (Fig. 8). There appeared to be a slight change in the angle of the dorsal blade in the Rb null brain when compared to littermate controls; nonetheless, the dorsal blade at E18.5 is identifiable in the Rb null brain, unlike at E16.5.

The apparent overgrowth of the DG revealed by cresyl violet staining made me question whether the expanded population could be young granule neurons. To determine this, I double labeled sections with Prox1, a marker of DG cells, and NeuroD1, a marker of young neurons. Co-labeling with these two markers would therefore reveal the young granule neurons in the DG. This double stain was not used prior to E16.5, as

few granule cells are present in the DG before this time. At E16.5, a 1.8-fold increase in the number of Prox1+/NeuroD1+ cells was present in the Rb deleted DG, despite the dorsal blade not being discernable (Student's T-test, $p = 0.08$; Fig. 9). This suggests that in the absence of Rb, regardless of changes in morphology, there is an increase in neurogenesis. To determine if an increase in the number of young neurons was still present at later stages, the same double staining was performed at E18.5 and a significant 1.5-fold increase in the number of Prox1+/NeuroD1+ cells was revealed in the Rb deleted brain when compared with controls (Student's T-test, $p = 0.0001$; Fig. 10, D). This suggests, that Rb plays a central role in regulating the size of the granule cell population during DG development. However, the DG is a large formation that changes in shape and cell number throughout the rostro-caudal plane, making it difficult to know the true nature of a defect from the small area analyzed with the previous quantification. In addition, our initial morphological study revealed significant changes in DG structure in the absence of Rb, making comparisons between Rb deleted and control animals even more difficult. In order to address this, a second, more rigorous, approach for quantifying newborn neurons was used to validate the initial findings. I used a serial sectioning approach, where every sixth section throughout the DG was stained with Prox1/NeuroD1 and double-labeled cells were then quantified. Using this approach the total number of young granule neurons in the DG was estimated for each embryo. Whole DG estimates revealed a 1.5-fold increase in the total number of young granule neurons in the Rb deleted brain when compared with control animals, validating the previous individual section counts (Student's T-test, $p = 0.008$, Fig. 10, E).

During the course of the Prox1+/NeuroD1+ analysis, it was observed that the DG of Rb mutant animals contained a number of Prox1 expressing cells that did not express NeuroD1 (Prox1+/NeuroD1-) This was a surprising finding, as at this stage of development, Prox1+ cells normally also express NeuroD1 and this population was not present in control animals. Though NeuroD1 expression is transient, this factor is expressed at high levels until the granule cells mature, which usually occurs postnatally. Quantification confirmed that there was a significant 9-fold increase in the number of Prox1+/NeuroD1- cells per section (Student's T-test, $p = 0.0009$; Fig. 11) and that close to 95% of this population also co-labeled with the proliferation marker, Ki67 (Fig. 12). Further observation revealed that these cells resided almost completely within the inner layer of the dorsal blade at E18.5, correlating to some of the oldest neurons in the DG at this time, and are present as early as E16.5 (Fig. 11 & Fig. 12). The presence of this cell population could suggest that these cells are unable to maintain a quiescent state in the absence of Rb or perhaps this population of cells is unable to correctly exit the cell cycle at all. The presence of this population also suggests that Rb is important to the correct expression of differentiation markers in granule cells, however further study into this population is needed.

In summary, we determined that in the absence of Rb, defects in gross morphology become apparent at E16.5 and continued to E18.5. Cresyl violet staining at E14.5 indicated no defect in morphology, however more work is needed to determine if the stem-like cells present at this stage are affected by Rb deletion. Further analysis revealed a 1.8-fold increase in the size of the young granule neuron population at E16.5 and a 1.5-fold increase at E18.5, suggesting that though neurogenesis was enhanced, this

affect could be decreasing with time. In addition, a population of cells that are Prox1+ and NeuroD1- were present in the Rb deleted brain, and the majority of these cells expressed the cell cycle marker, Ki67. Overall these data suggest that, beginning at mid embryonic development, Rb becomes crucial to regulating the size of the granule cell population.

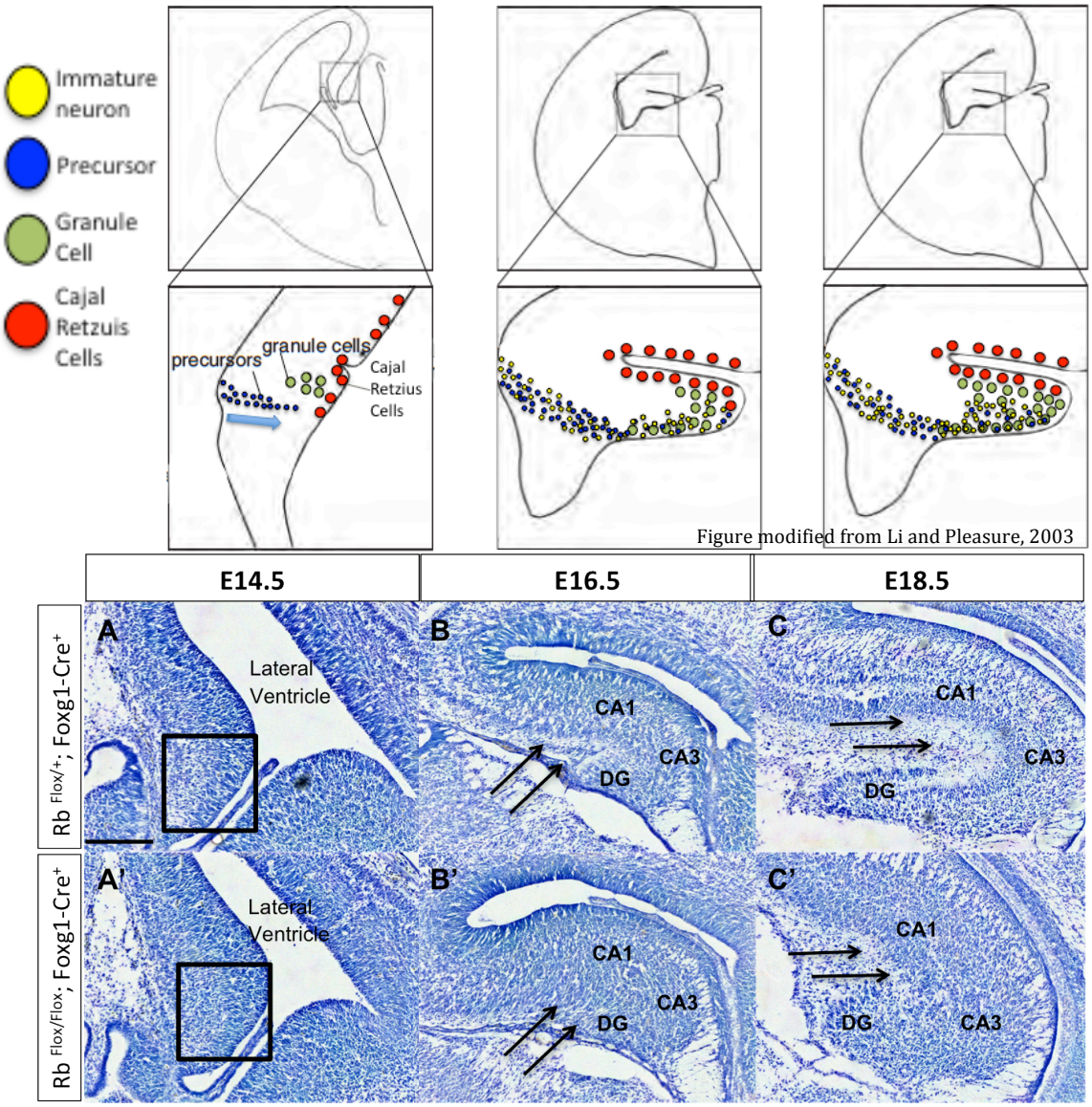


Figure 8: Gross Morphological Defect becomes apparent at E16.5 in the Rb Deleted Hippocampus.

Cresyl violet staining shows the Rb deleted (A'-C') and littermate control hippocampus at E14.5, E16.5 and E18.5. A schematic of DG development is shown above each time point for reference. Tissue was frozen and sectioned coronally. Sections were stained, leveled rostro-caudally, and imaged on a light microscope using a 10x objective and Axiovision software. (A-C) Cresyl violet staining shows littermate control embryos at the beginning of DG development, E14.5, mid embryonic DG development, E16.5 and late embryonic development, E18.5. As can be seen, an overgrowth of cells is present by E16.5 (B, B', arrows) in the Rb deleted DG and continues at E18.5 (C, C', arrows). Square boxes outline the dentate neuroepithelium at E14.5 (A, A'). The regions of Ammon's Horns are denoted by CA1 and CA3 while the dentate gyrus is denoted DG. Scale bar = 100μm

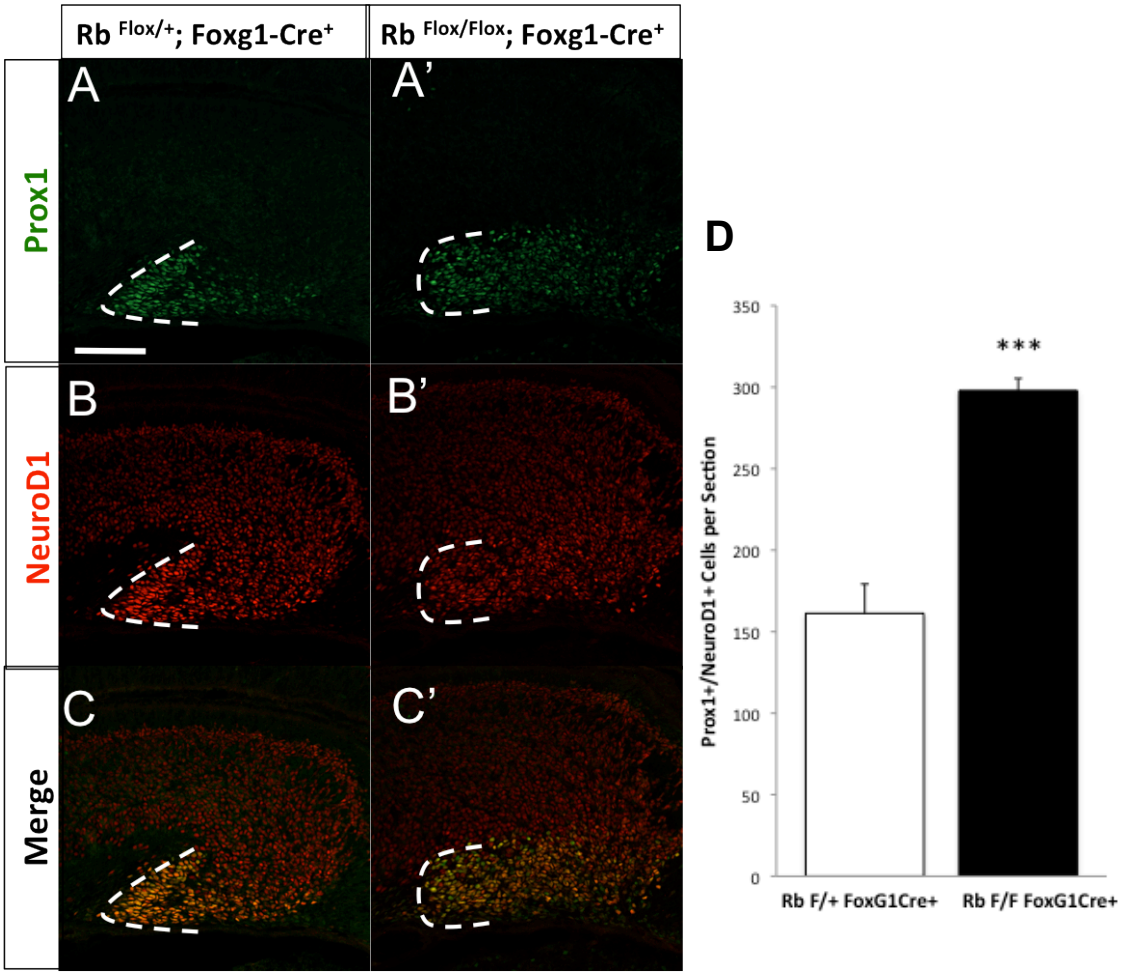


Figure 9: Increased young granule cell population at E16.5 cells in the Rb deleted DG.

Double labeling with Prox1 and NeuroD1 of Rb null and littermate controls reveals an increase in neurogenesis in the Rb deleted DG. E16.5 brains were frozen, sectioned in the coronal plane and imaged on a Zeiss confocal microscope with the 20x objective. Images were acquired with Zen software and processed using ImageJ software. The number of Prox1+/NeuroD1+ cells is increased 84% in the Rb deleted hippocampus (A'-C') when compared with littermate controls (A-C), despite the Rb null DG lacking a dorsal blade (arrows). (D) Quantification of the number of Prox1+/NeuroD1+ cells per section indicates a significant increase in neurogenesis (Mean, +/- SEM, Student's T-test, n=3, p = 0.008, Microsfot Excel sofware). Scale bar = 100μm.

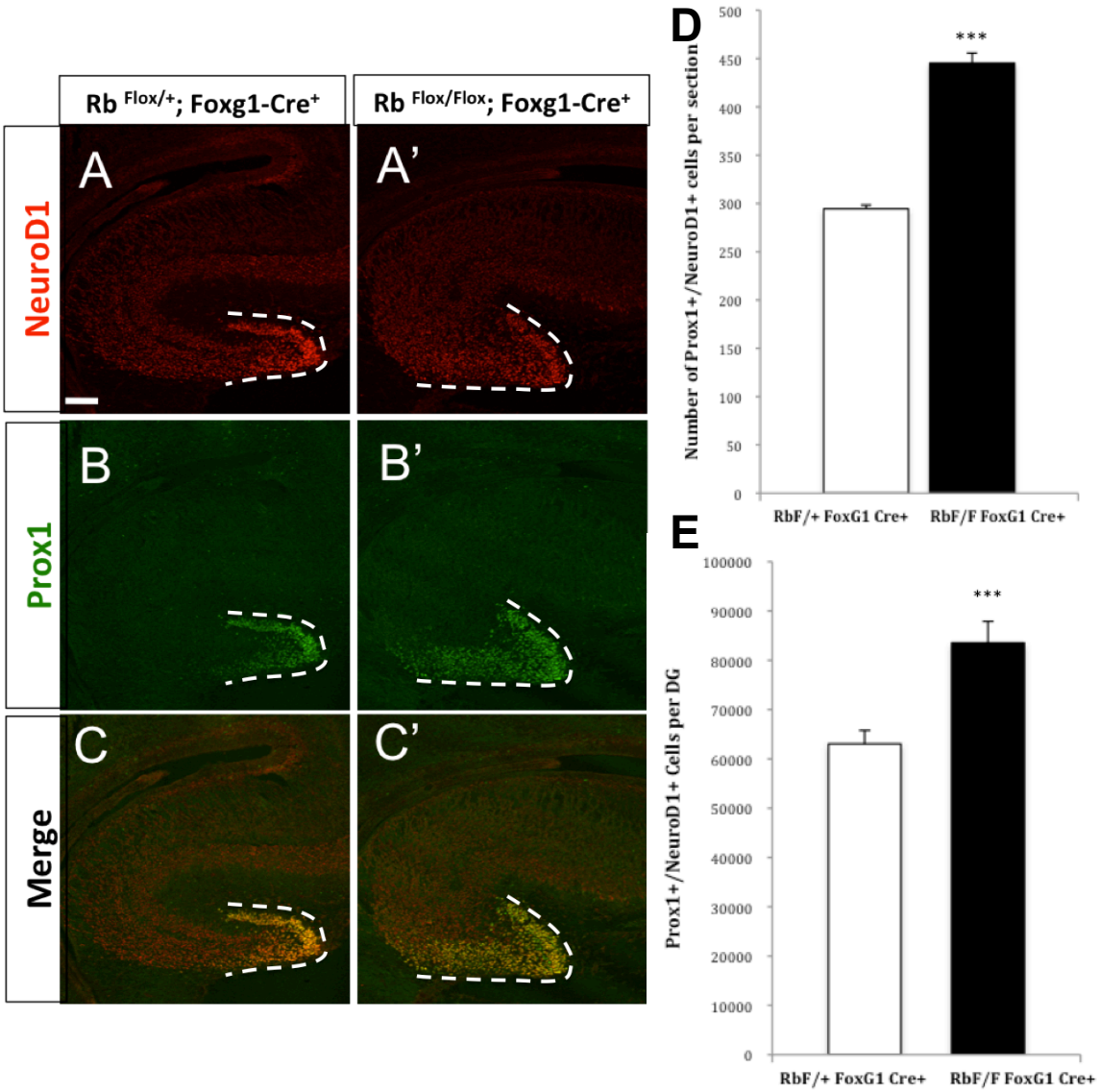


Figure 10: Increased young granule neuron population in the Rb deleted DG at E18.5.

Analysis of E18.5 Rb deleted (A'-C') and littermate control brains (A-C) using immunohistochemistry against Prox1 and NeuroD1 reveals more Prox1+/NeuroD1+ cells are present in the Rb deleted brain. Brains were frozen and sectioned coronally. A Zeiss confocal microscope with 10x objective and Zen imaging software was used for image acquisition. (D) Manual quantification of the Prox1/NeuroD1 population per section reveals a significant 51% increase in the Prox1+/NeuroD1+ population in the absence of Rb (Mean, +/- SEM, Students T-test, n=3, p = 0.0001) (E) Counting every sixth section rostro-caudally throughout the DG, using ImageJ software, indicates a significant 51% increase in the Prox1+/NeuroD1+ throughout the DG, validating the previous counting technique (Student's T-test, n=3 for Rb deleted, n=4 for controls, p = 0.008). Scale bar = 100μm.

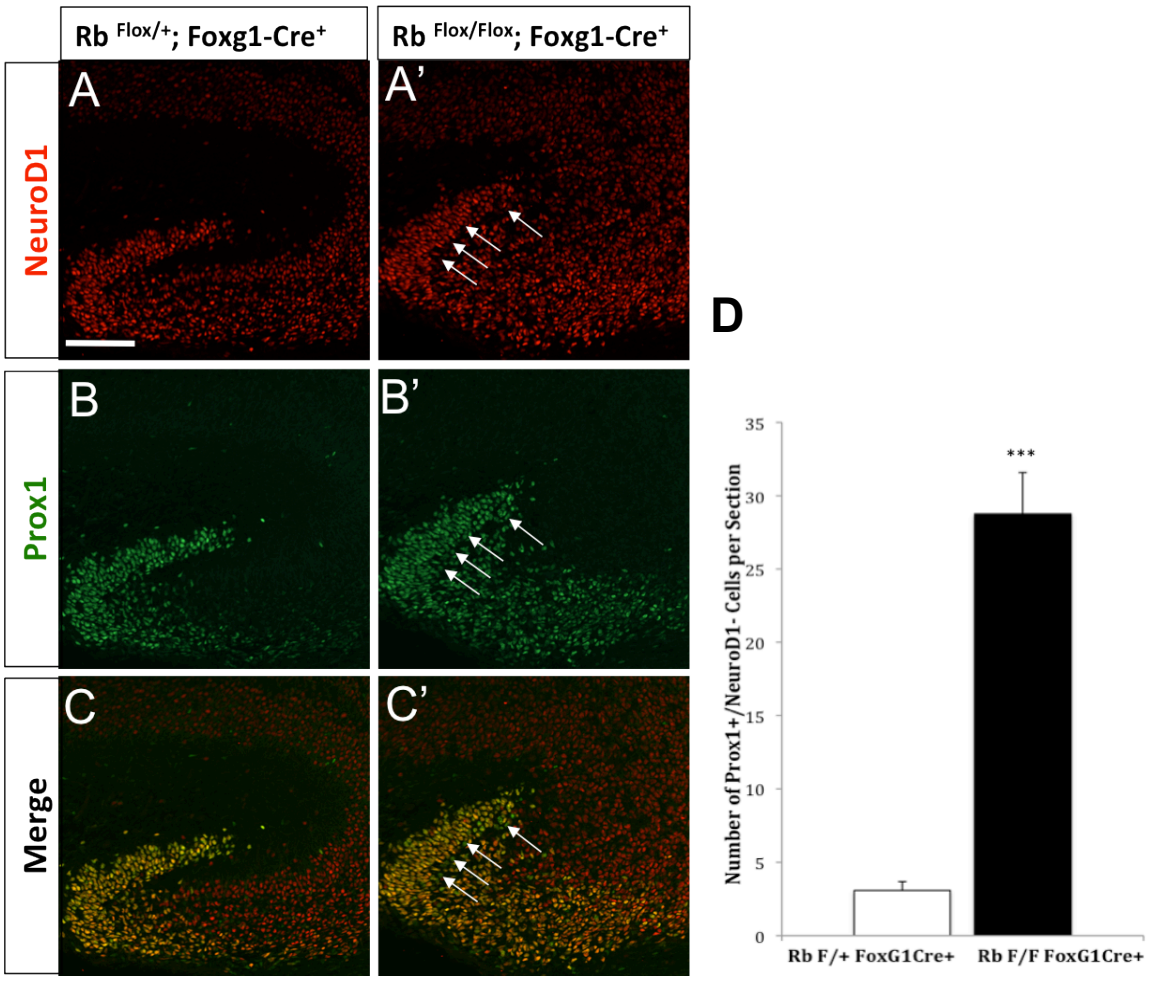
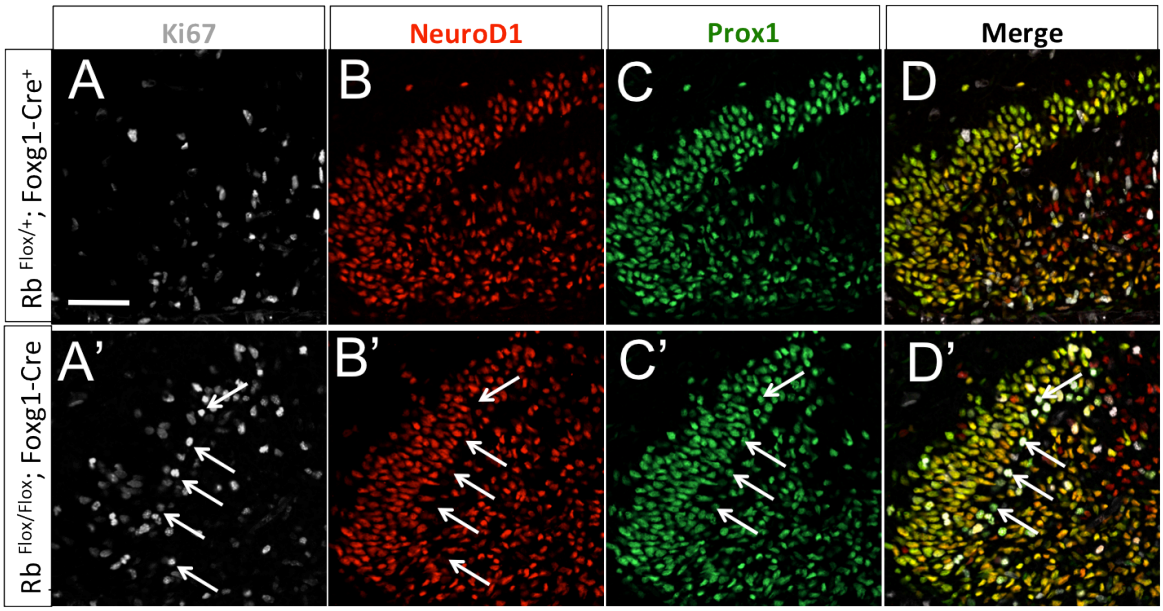


Figure 11: Presence of unique cell population in the Rb deleted DG.
Brains from E18.5 pups were isolated, tissue frozen then sectioned coronally. Sections were leveled rostro-caudally and immunostained for Prox1 and NeuroD1, then imaged with a 20x objective on a Zeiss confocal microscope using Zen image acquisition software. Closer analysis of the E18.5 Rb deleted hippocampus revealed the presence of a Prox1+/NeuroD1- population located within the inner edge of the dorsal blade (A'-C', arrows). This population is not common in the littermate control hippocampus (A-C). (D) Manual quantification indicates a significant 9-fold increase in this population of cells in the Rb deleted hippocampus when compared with littermate controls (Mean, +/- SEM, Student's T-test, n=3, p = 0.0009). Scale bar = 100μm, see figure 12 for increased magnification.



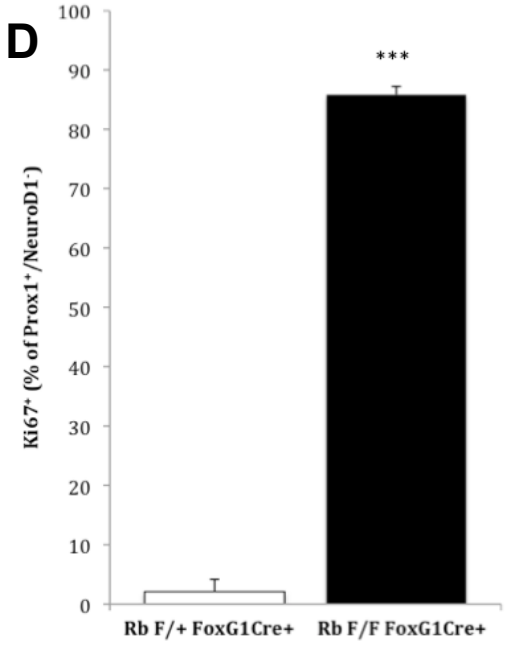


Figure 12: Prox1+/NeuroD1- Cells are Ki67+.
Further analysis of the Prox1+/NeuroD1- population uncovered that more of these cells are expressing the cell cycle marker Ki67 in the Rb deleted brain then in the control brain. Triple immunohistochemistry was used on frozen coronal sections to label Ki67, Prox1 and NeuroD1 in the E18.5 Rb null (A'-D') and littermate control (A-D) DG. A Zeiss confocal microscope with 40x oil objective and Zen software was used for image acquisition. (E) Manual quantification shows a significant increase in the number of Prox1+/NeuroD1-/Ki67+ cells (arrows) in the Rb deleted dentate gyrus (Mean +/- SEM, Student's T-test, n=3, $p < 0.0001$). Scale bar = 50μm.

Aim 2: Proliferation and Cell death in the DG

Our lab has previously shown that Rb deletion results in increased proliferation as well as decreased survival (Ferguson et al., 2002; Callaghan et al., 1999; Slack et al., 1995). Having established that Rb deficiency expands the granule cell population, I next set out to determine the proliferative state of these cells and if there was increased apoptosis. To address these questions, Ki67 staining was used to determine if there was an increase in the number of cells expressing cell cycle markers and to determine if there was an increases in apoptotic cell death in the DG, activated caspase3 staining was used at E14.5, E16.5 and E18.5.

First, to analyze the number of cycling cells in the developing DG, Ki67 staining was used and revealed a 2.5 fold increase in the percentage of Ki67+ cells in the E16.5 Rb null brain when compared with littermate controls (Students T-test, $p = 0.001$; Fig. 13). The percentage of Ki67+ cells in the E18.5 DG of Rb deleted brains was increased 2.2 fold when compared to littermate controls (Student's T-test, $p = 0.001$; Fig. 14, D). In order to validate these findings, the total number of Ki67+ cells in the DG at E18.5 was estimated using the previously described serial counting technique and a significant 3-fold increase at E18.5 was present in the Rb deleted brain when compared to controls (Students T-test, $p = 0.0009$; Fig. 14, E). In addition, it was observed that the increase in Ki67 expression was most apparent in the dorsal blade of the DG as well as in the presumptive hilar region. Though the presence of Ki67 does not decisively show that cells are actually proliferating as opposed to ectopically expressing a marker of the cell cycle, previous results in Rb deficient brains indicates that Rb deletion results in increased proliferation (Ferguson et al., 2002). Based on previous data, the presence of

increased Ki67 expression and the increased number of granule neurons, I conclude that Rb deficiency results in increased proliferation in the developing DG. Having established that an increase in proliferation occurred in the DG of the Rb deleted brain; I next determined which population, the stem-like cells, intermediate neuronal progenitors (INP) or young neurons, this increased proliferation could be attributed to. To do this, Sox2, the pluripotency factor, was used to label stem-like cells present in the DG at E14.5. This stain was used only at E14.5 as the presence of stem-like cells in the DG neuroepithelium past E14.5 is debatable (Clarke and van der Kooy, 2011).

Sox2/Ki67 double labeling was used to determine the proportion of stem-like cells expressing the cell cycle marker, Ki67. This double labeling revealed approximately 40% of the Sox2+ population to be expressing Ki67 in both the Rb null and control brain (Students T-test, $p = 0.38$; Fig. 15), suggesting that Rb deletion does not effect proliferation in the stem-like cell population.

Since expression of Ki67 in stem-like cell population appeared to be unaffected by Rb deletion, we next questioned if Rb deficiency could affect the rapidly proliferating INP population. To analyze this group of cells, Tbr2, a marker of INPs, and Ki67 co-labeling was performed at E16.5 and E18.5. This double stain was not used at E14.5, as very few INPs are present that early during normal DG development. Quantification at E16.5 indicated a 1.2-fold increase in the number of Tbr2+/Ki67+ cells, however this increase was not significant (Student's T-test, $p = 0.12$; Fig. 16). Interestingly, at E18.5, there was a significant 1.2-fold decrease in the percent of cycling Tbr2+ cells in the Rb null DG (Student's T-test, $p = 0.03$; Fig. 17). This result was surprising, as Rb deletion has not previously been documented to decrease expression of cell cycle markers within

the neuronal progenitor population. Nonetheless, it was determined that the increased Ki67 expression could not be attributed to the INP cells, therefore the young neuronal population of the DG was analyzed.

NeuroD1, a marker transiently expressed in young DG neurons, and Ki67 double labeling was performed to analyze whether the proliferative state of immature neurons in the DG was affected in the absence of Rb (Fig. 18). Staining was performed at E18.5 as this time point was the latest possible with the mouse model used, therefore would reveal the most newborn neurons. NeuroD1/Ki67 double staining revealed a significant 19-fold increase in the proportion of cycling NeuroD1 cells per DG section (Student's T-test, $p = 0.04$; Fig. 18, D). Due to the morphological differences present between the Rb null and control brains, a serial sectioning technique was used to estimate the total number of NeuroD1 cells expressing Ki67 in the Rb deleted and control brain. This analysis revealed that the total number of NeuroD1+/Ki67+ cells in the DG of Rb null mice increased 19-fold over the number in littermate controls (Students T-test, $p = 0.003$; Fig. 18, E). This result indicates that in the absence of Rb, increased proliferation is present in the young neuron population, however is it not known if this apparent increase in proliferation is due to delayed cell cycle exit or cell cycle reentry, both of which have been documented in the absence of Rb (Qin et al., 1994; Callaghan et al., 1999).

The results indicate that Rb deletion results in enhanced proliferation of young neurons while having little effect on the stem-like cell and actually decreasing proliferation in the INP cells. However these results did not determine if these were cell autonomous consequences of Rb deletion, nor whether increased expression of cell cycle markers in the young neuronal population was due to delayed cell cycle exit, or cell cycle

reentry. To address these issues, we turned to an *in vitro* model. First, we used an *in vitro* assay of DG neural precursor cells to determine if there were changes in the number of precursors in the Rb null brain. Cells isolated from the dentate neuroepithelium at E14.5 were assayed for the number of colony forming cells. Stem-like and precursor cells are capable of forming free-floating colonies *in vitro*, therefore by plating equal numbers of cells, the number of colonies formed can be used as a readout of the number of precursor or stem-like cells in a given region, thus allowing us to determine if this population was expanded in the absence of Rb. In accordance with our *in vivo* results, neurosphere assay results indicated no change in the number of colony forming cells in the absence of Rb (Fig. 19). In addition, differentiation assays with BrdU incorporation were conducted to determine if the enhanced proliferation observed in immature neurons was due to delayed cell cycle exit. Cells isolated from the E14.5 DG neuroepithelium were plated as a proliferative monolayer and then differentiated in the presence of BrdU. One issue with differentiating DG neuroepithelial cells is the low ratio of neurons generated (Clarke and van der Kooy, 2011). To circumvent this issue, all cells were differentiated for a total of 7 days with BrdU added to dishes as various times throughout differentiation. All cultures were then stained for Tuj1, a marker of neuronal specific tubulin, BrdU and GFAP, an astrocyte marker. The number of BrdU+ cells present in the Tuj population was used to assess cell cycle exit. In the Rb deleted cultures, a trending increase in the number of Tuj1+/BrdU+ cells were present in cultures exposed to BrdU at later time points of differentiation than those in littermate control cultures, implying a possible delay in cell cycle exit, which could cause prolonged expression of the cell cycle marker, Ki67 (Fig. 20). Previous findings from our lab indicate that Rb is essential for

timely cell cycle exit in the developing cortex (Callaghan et al., 1998), which supports the findings of this study. Taken together, the *in vitro* results suggest that Rb is not imperative to regulating precursor and stem-like cell proliferation or population size. Instead, the data suggest that Rb works to regulate proliferation of newborn DG neurons and could possibly act through facilitating cell cycle exit. Though the data show a tendency for a delayed cell cycle exit in the absence of Rb, further work is needed to confirm this result. In addition, this does not rule out the possibility that Rb deletion could also affect the cells ability to maintain quiescence and thus result in a re-entry of the cell cycle later on; further study is needed into this possibility.

As it has been previously documented that loss of Rb can increase cell death in addition to proliferation (Slack et al., 1995; Qin et al., 1994; Hou et al., 2000), I next analyzed cell death within the DG using active caspase3 (AC3) staining as a marker of apoptosis. AC3 staining was used at E14.5, E16.5 and E18.5 in an effort to determine if cell death was increasing with time. To begin, AC3 staining at E14.5 indicated no significant change in cell death (Student's T-test, $p > 0.05$; Fig. 21). Staining with AC3 at E16.5 resulted in a 24-fold increase in cell death in the absence of Rb (Students T-test, $p < 0.001$; Fig. 22). Further analysis at E18.5 indicated a non-significant 24-fold increase in cell death in the DG (Student's T-test, $p = 0.08$; Fig. 23, D). As done previously, estimates of whole brain cell numbers were used to get a more accurate readout of cell death. Serial sectioning and counting revealed a highly significant 8-fold increase in AC3 staining at E18.5 (Student's T-test, $p = 0.0001$; Fig. 23, E). These results indicate that Rb deletion is negatively impacting the survival of DG cells. However, it was reasoned that an increase in cell death could simply be caused by an increase in total cell

number. To address this, we analyzed the fold changes in total cell number as well as fold change in cell death. Further study revealed that cell death had increased by 8.3-fold while cell number increased by only 1.3-fold, suggesting that the increase in cell death was not cause solely by an increase in cell number (Fig. 24). It has been shown previously that Rb deficiency negatively impacts survival of neural tissues (Slack et al., 1995), as appears to be the case in my model, though further study is needed to determine if this increase in cell death is intrinsic or cause by other external factors.

Overall, these results indicate that, while it has no apparent role in early stages of DG development, Rb could be involved in regulating the rate of cell cycle exit of immature neurons. In addition, in the Rb deficient DG, increased proliferation is present within the young neuron population, leading to enhanced neurogenesis. My findings also indicate that Rb is involved in sustaining the survival of young DG neurons, however more *in vitro* work would be advantageous to determine if this is a cell-autonomous or non cell-autonomous event.

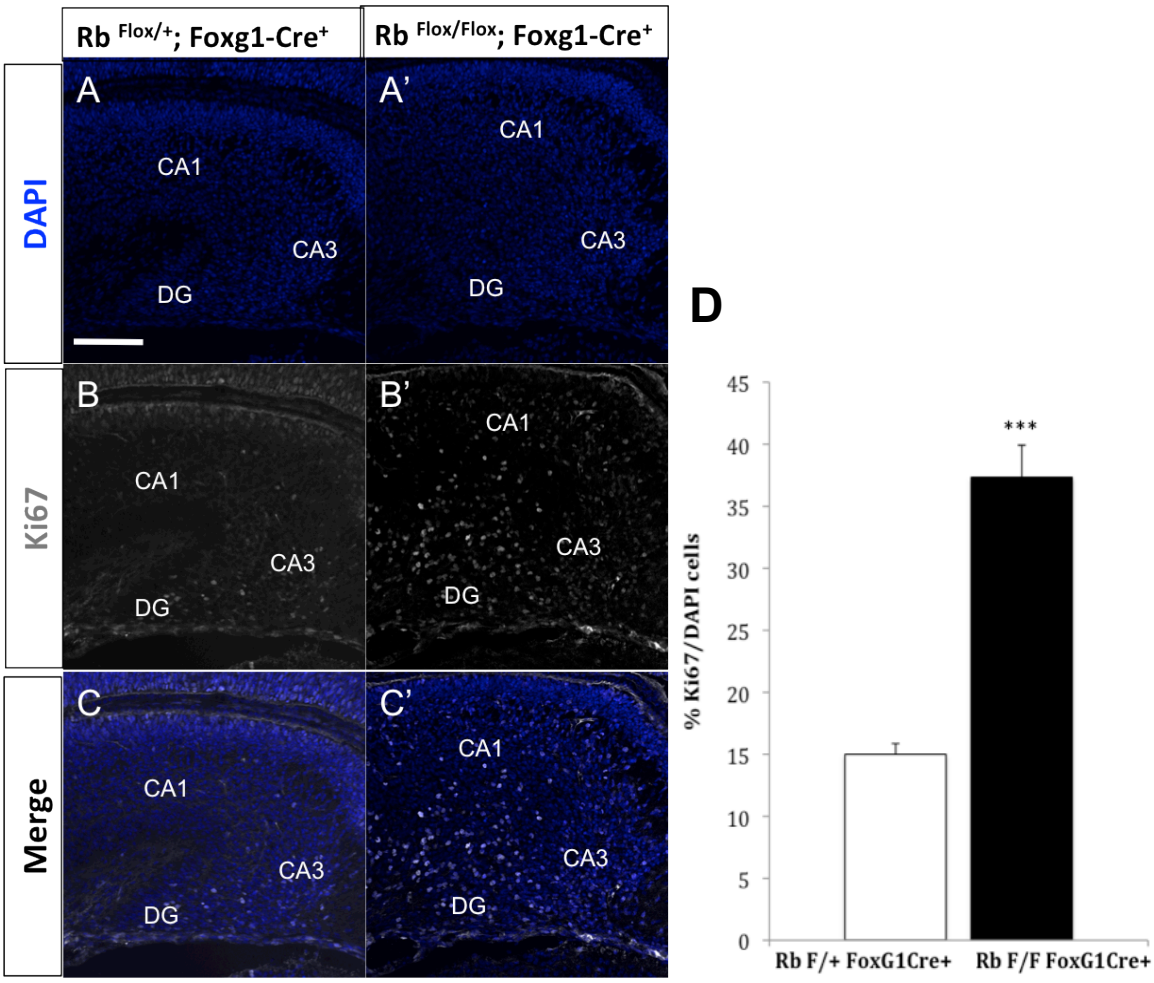


Figure 13: Proliferation is enhanced in the absence of Rb in the E16.5 Dentate Gyrus.

Ki67 immunostaining in the control (A-C) and Rb null (A'-C') hippocampus reveals an increase in Ki67+ cells in the absence of Rb. Prior to coronal sectioning, E16.5 brains were infiltrated with sucrose and frozen. Sections were leveled rostro-caudally and immunostained for Ki67. Staining was visualized using a Zeiss confocal microscope with a 20x objective and images were acquired with Zen imaging software. (D) Manual quantification of the percent of Ki67+ cells within the third matrix of the DG indicates a significant 2.5 fold increase in proliferation in the absence of Rb (Mean +/- SEM, Student's T-test, n=3, p = 0.001). The regions of the hippocampus are given as CA1 and CA3 for Ammon's horn. DG denotes dentate gyrus. Scale bar = 100μm.

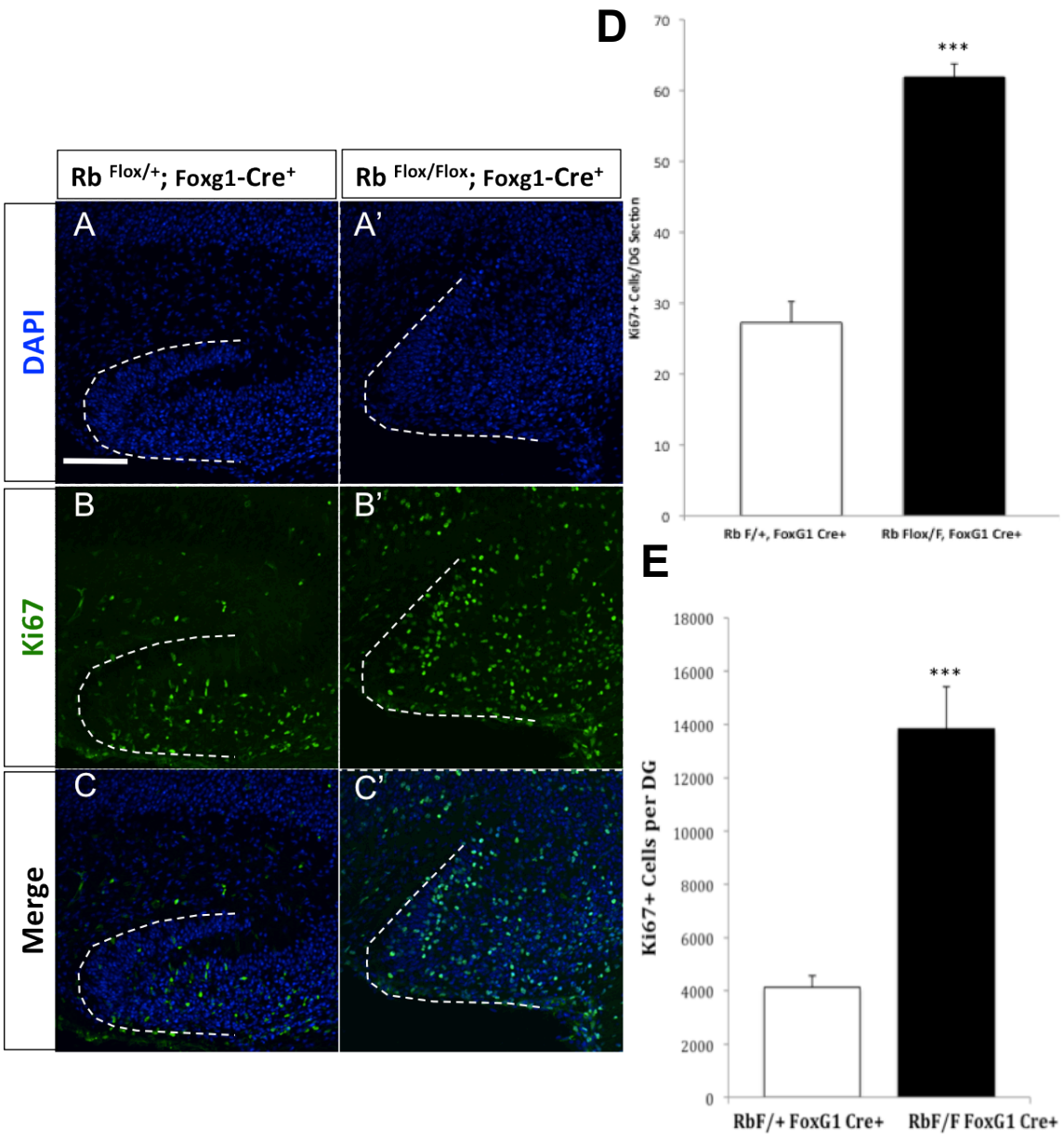


Figure 14: Increased proliferation is maintained in the E18.5 Dentate Gyrus. Immunostaining on frozen, coronal, sections for Ki67 at E18.5 indicates increased numbers of Ki67+ cells in the absence of Rb (A'-C') when compared with littermate controls (A-C). (D) Manual quantification of Ki67+ cells per DG section at E18.5 indicates a 2.2-fold increase in Ki67+ cells (Mean +/- SEM, Student's T-test, p = 0.001) (E) Quantification of every sixth sectioning throughout the shows a significant 3 fold increase in the number of Ki67+ per DG in the absence of Rb (Mean +/- SEM, Student's T-test, n=3 for Rb deleted, n=4 for controls, p = 0.0009). Images were acquired using a Ziess confocal microscope with a 20x objective and Zen imaging software. Dashed lines outline the third matrix of the DG, scale bar = 100μm.

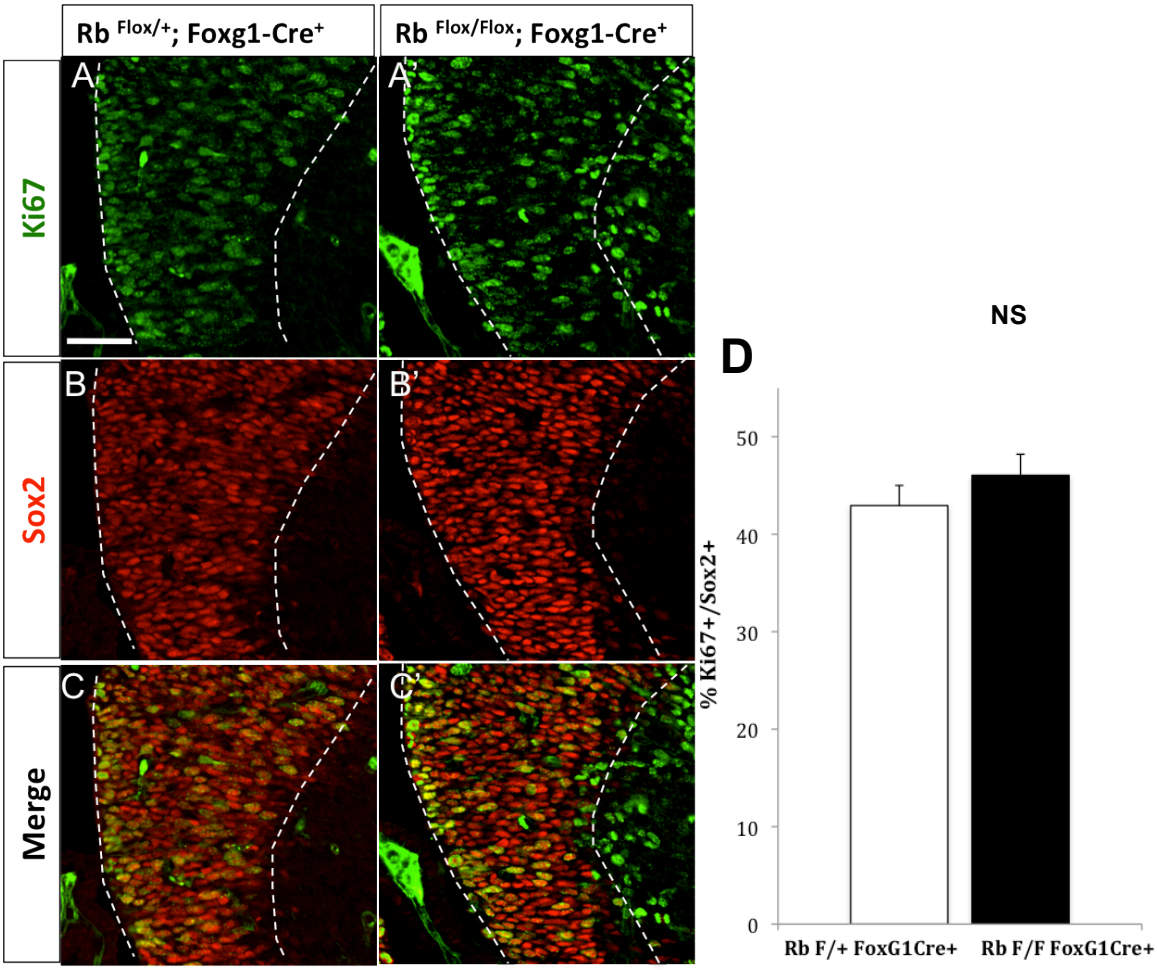


Figure 15: No change in expression of Ki67 within the stem-like cells in the E14.5 dentate neuroepithelium.

Co-labeling of Sox2 and Ki67 at E14.5 in the dentate neuroepithelium indicated no change in the proliferative state of stem cells of the control (A-C) or Rb deleted (A'-C') hippocampus. Frozen sections were immunostained with Sox2 and Ki67 then visualized using a Zeiss confocal microscope, 20x objective and Zen imaging software. (D) Manual quantification of the Ki67+/Sox2+ population indicates no significant change in the expression of cell cycle markers within the Sox2 population in the absence of Rb (Mean +/- SEM, Students T-test, p = 0.38). Dashed line outlines the dentate neuroepithelium; the lateral ventricle is to the left of the image. All images were taken with a 40x oil objective, the scale bar = 50μm.

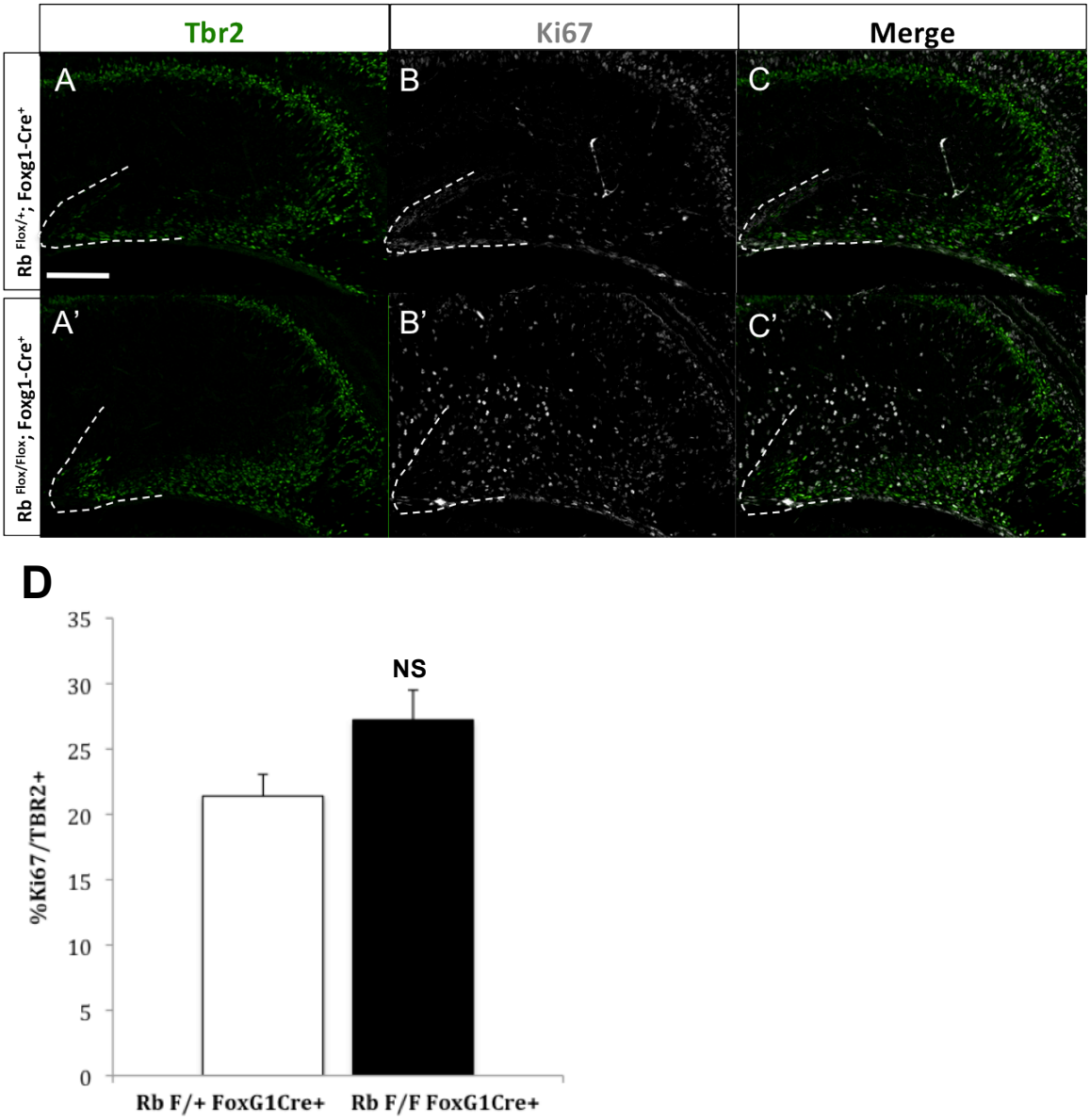
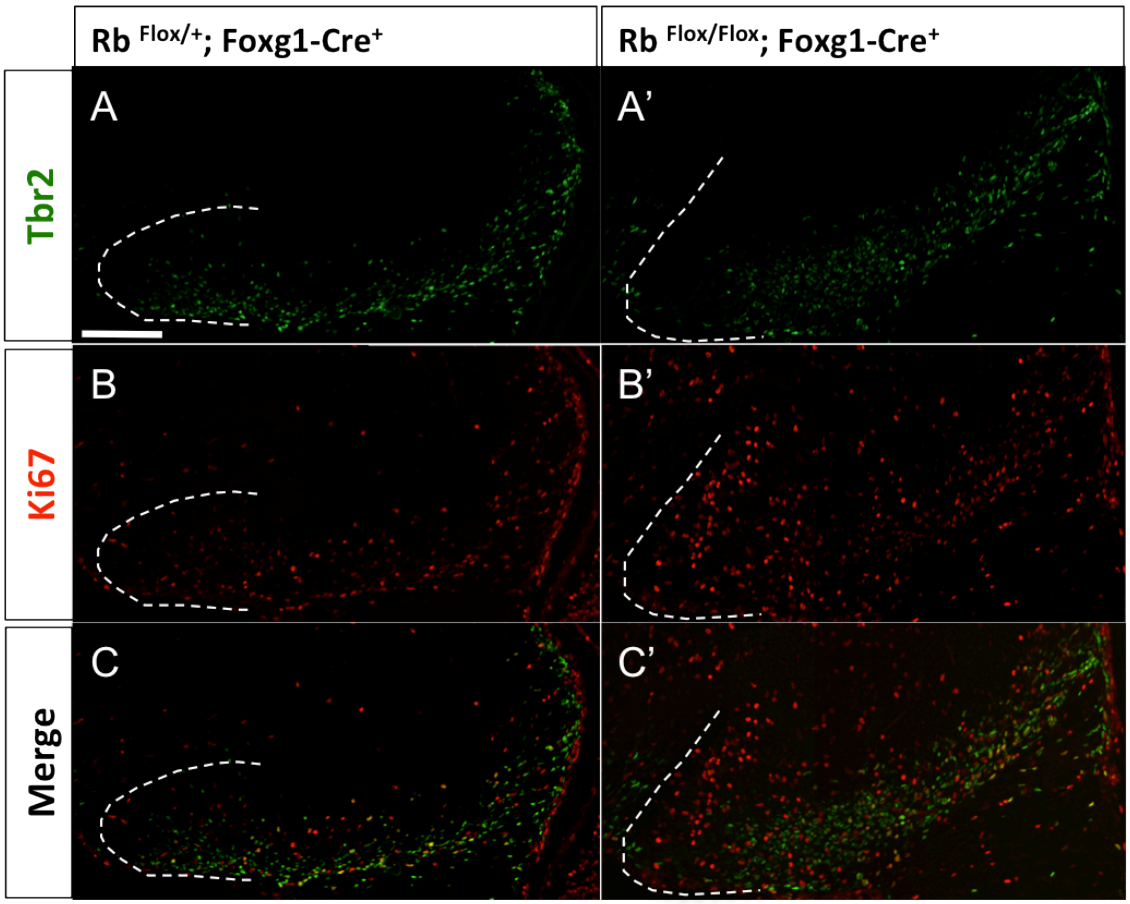


Figure 16: No significant change in the proliferative state of TBR2+ cells at E16.5. TBR2/Ki67 co-labeling experiments, on frozen coronal sections, indicate that Rb deletion (A'-C') does not affect proliferation of the TBR2+ cell population in the DG at E16.5 when compared with littermate control brains (A-C). (D) No significant change in the percent of Ki67+ cells within the TBR2+ population was seen (Mean +/- SEM, Students T-test, n=3, p = 0.11). Dashed line indicates the third matrix of the DG. Images were acquired with a Ziess confocal microscope, 20x objective, and Zen imaging software. The scale bar = 100μm.



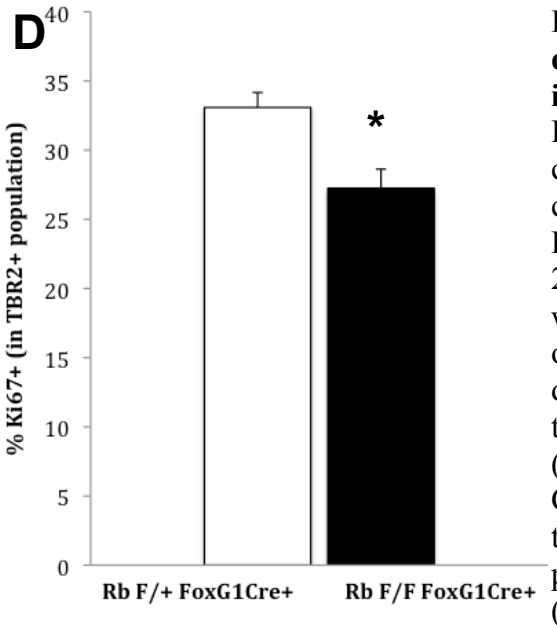


Figure 17: Cell cycle markers are decreased in the INP population at E18.5 in the Rb deleted dentate gyrus.
E18.5 brains from Rb null and littermate control pups were frozen, sectioned coronally and immunostained for Tbr2 and Ki67. A Zeiss confocal microscope with 20x objective and Zen imaging software were used for image acquisition. Analysis of co-labeling of TBR2 and Ki67 indicates a decrease in the expression of Ki67 within the Tbr2 population in the Rb deleted DG (A'-C') when compared with controls (A-C). (D) A slight but significant decrease in the number of Ki67+ cells within the TBR2 population is present in the Rb deleted DG (Mean, +/- SEM, Student's T-test, n = 3, p = 0.03). Dashed lined indicates the third matrix of the DG, scale bar = 100μm.

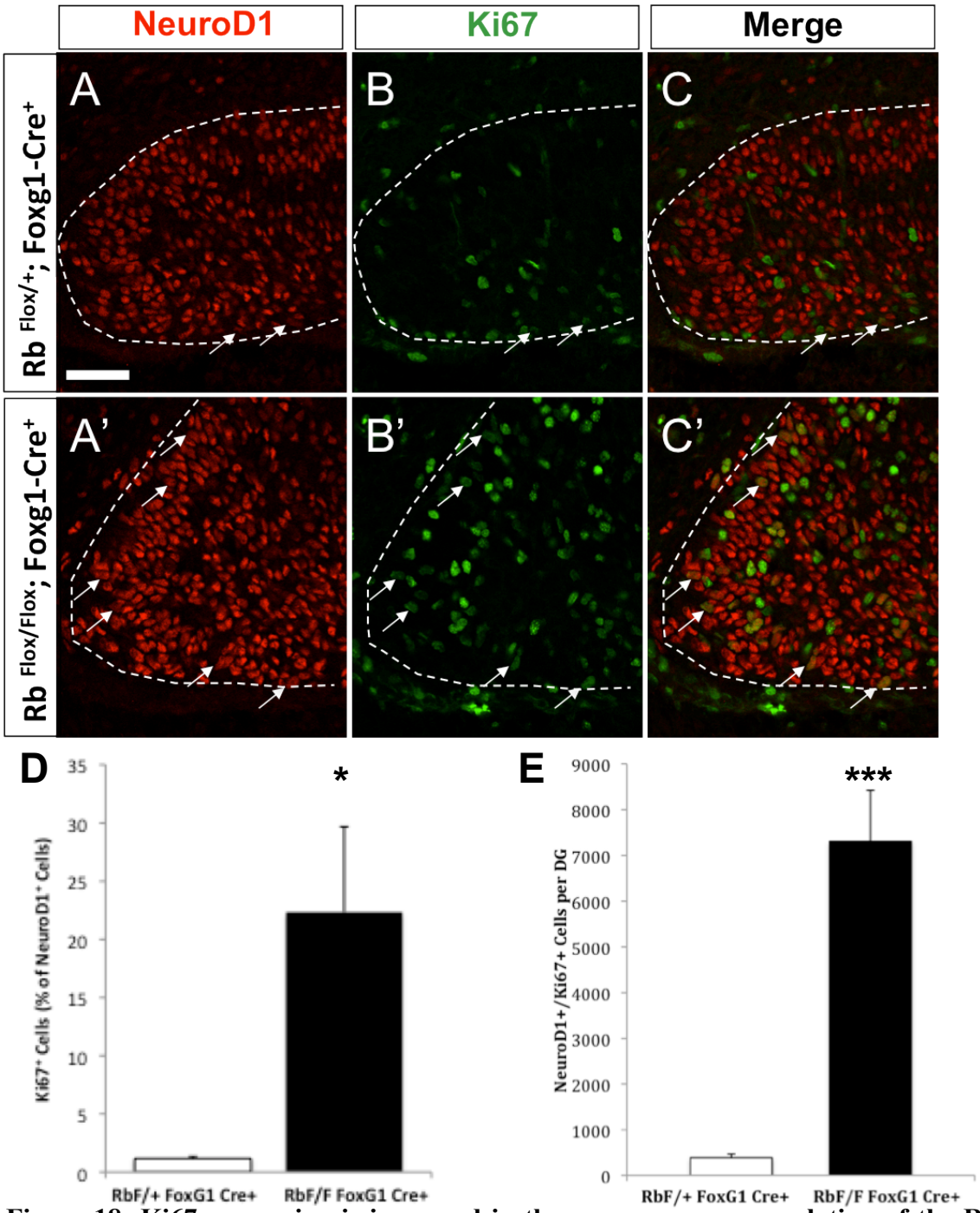


Figure 18: Ki67 expression is increased in the young neuron population of the Rb deleted DG.

Confocal images of immunostaining for NeuroD1 and Ki67 reveal an increase in the expression of Ki67 within the young neuron population of the E18.5 DG of Rb null brains (A'-C') when compared to littermate controls (A-C). The increased expression appears to be located primarily within the dorsal blade of the Rb deleted DG (arrows). (D) Manual quantification reveals a significant 19-fold increase in Ki67+ cells within the NeuroD1 population of the DG (Students T-test, p= 0.04). (E) Quantification of every sixth section throughout the DG also reveals a 19-fold increase in Ki67+/NeuroD1+ cells, validating the previous result (Mean +/- SEM, Students T-test, n=3 for Rb deleted, n=4 for controls, p= 0.003). The dashed line outlines the third matrix of the DG, scale bar = 50μm.

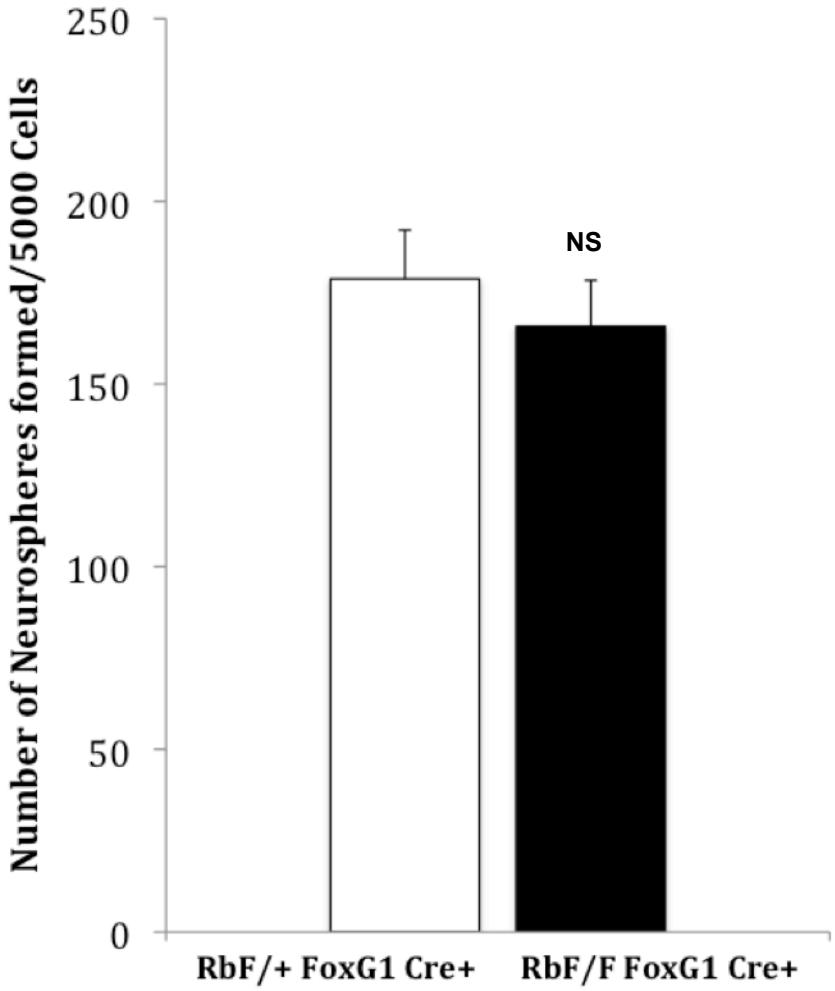


Figure 19: *In vitro* analysis of stem cell population suggests no change in proliferation.
Cells from the E14.5 dentate neuroepithelium were isolated from Rb deleted and littermate control animals. Cells were plated for a neurosphere assay and allowed to proliferate. The mean number of sphere forming cells from Rb deleted and control dentate neuroepithelium indicates no significant change in the sphere forming population (Mean +/- SEM, Student's T-test, n=4, p = 0.5).

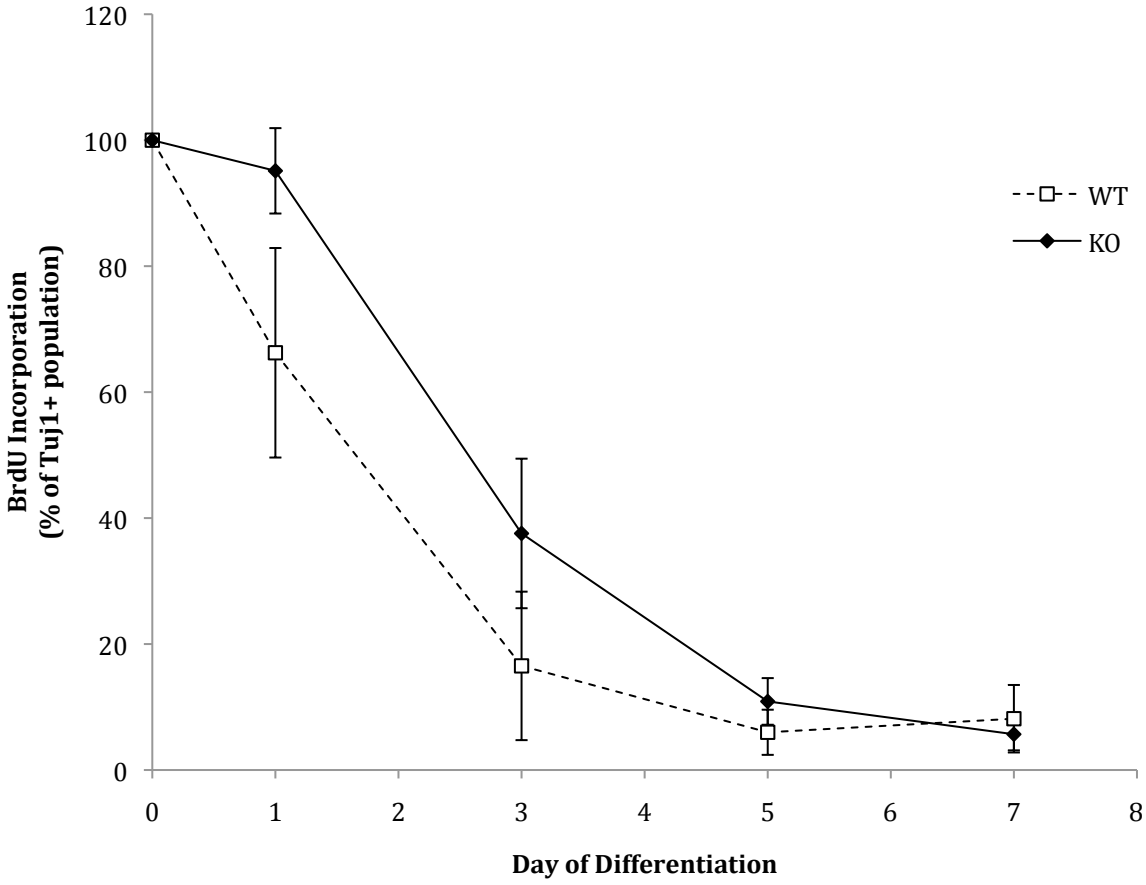


Figure 20: *In vitro* analysis suggests a tendency for delayed differentiation in the absence of Rb.

Cells isolated from the E14.5 DG were plated as a proliferative monolayer, and then differentiated. All cells were differentiated for 7 days with BrdU added to different plates at 0, 1, 3, 5 and 7 days of differentiation. Immunostaining for Tuj1 was used to identify neurons within the cultures. Quantification of Tuj1+/BrdU+ cells throughout differentiation shows a tendency for Rb deleted cells to continue to divide further into a differentiation timeline then controls. In the Rb deleted cells, there is a 1.4-fold increase in BrdU incorporation 1 day into differentiation and a 2.5 fold increase in BrdU incorporation 3 days into differentiation when compared with control cells, though this finding was not significant (Mean, +/- SEM, One-way ANOVA, n=4, $p>0.05$).

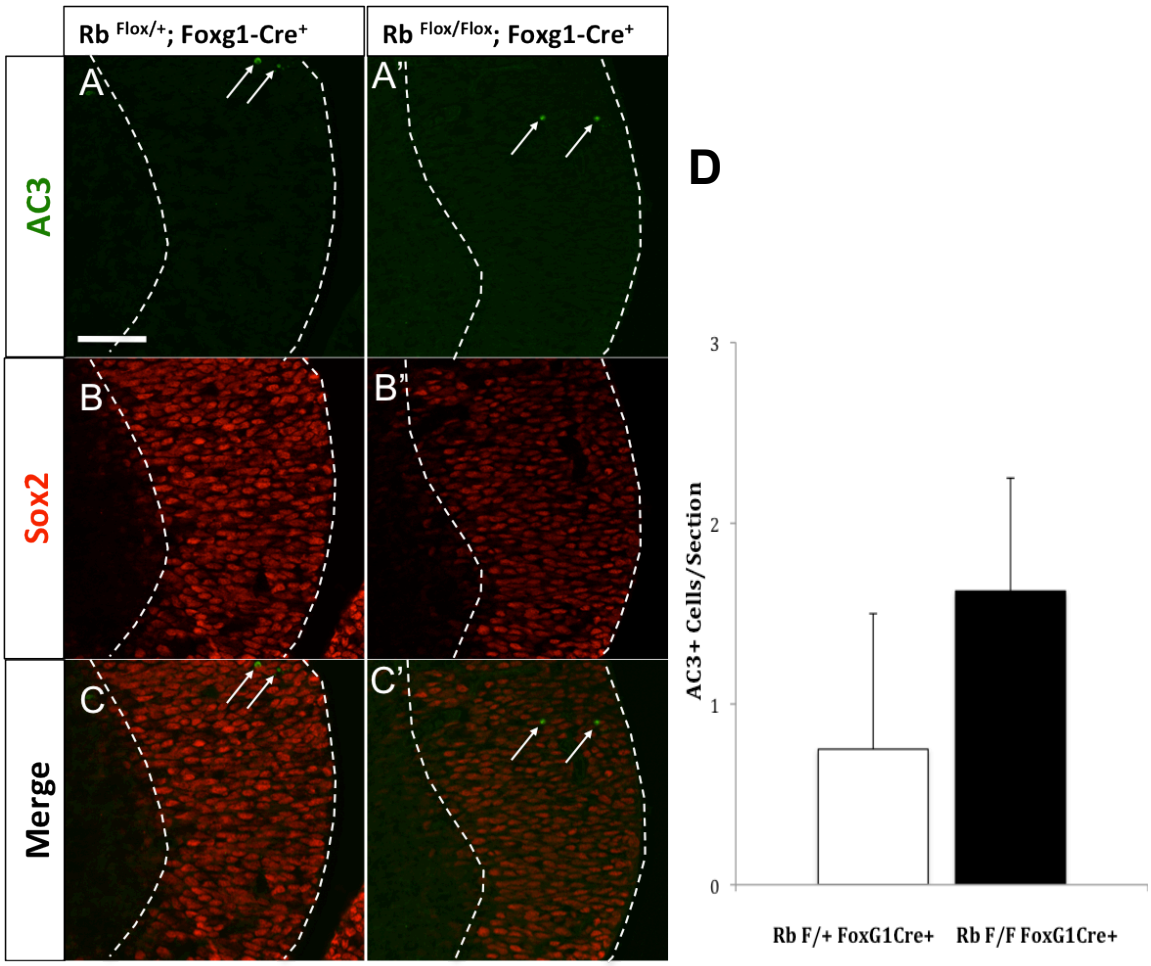


Figure 21: No change in apoptotic cell death at E14.5.
Immunostaining for activated caspase3 (arrows) in the E14.5 Rb null DG (A'-C') showed no change when compared with littermate controls (A-C). Frozen sections were immunostained with Sox2 to mark the stem-like cells in the neuroepithelium in addition to AC3 (D) Manuel quantification of sections leveled rostro-caudally reveals no significant change in the number of AC3 positive cells per section (Student't T-test, n=3, p = 0.46). A Zeiss confocal microscope with a 40x oil objective and Zen imaging software was used to acquire all images. The dashed line outlines dentate neuroepithelium, the lateral ventricle is to the right of the image, the scale bar = 50μm.

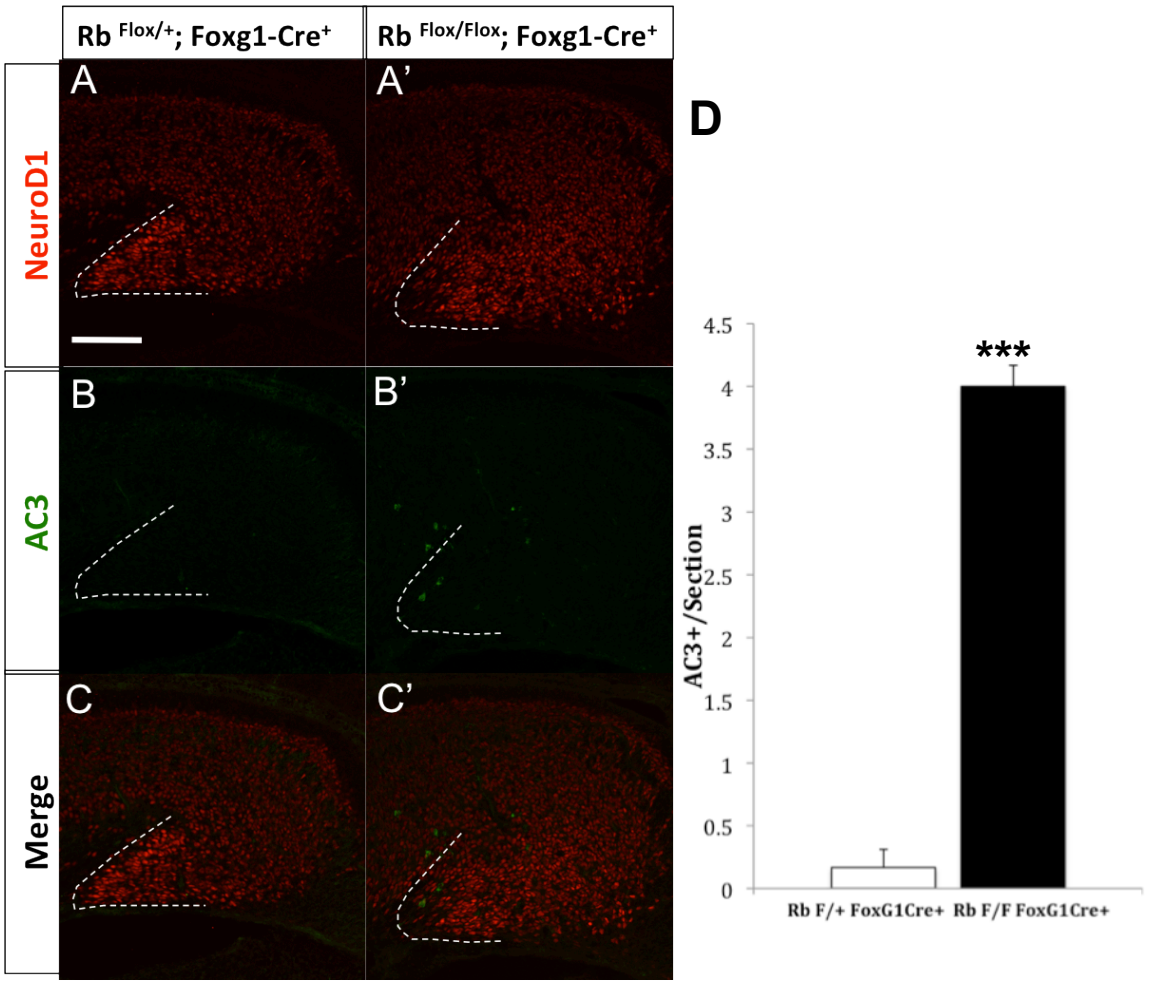


Figure 22: Apoptotic cell death is increased in the E16.5 DG.
Confocal images of NeuroD1 and AC3 immunostaining at E16.5 reveal an increase in cell death in the DG of Rb deleted brains (A'-C') when compared with littermate controls (A-C). (D) A significant increase in the number of AC3+ cells was found when quantified manually by section (Mean, +/- SEM, Student's T-test, n=3, p = 0.53). The dashed line outlines the third matrix of the DG and arrows point to AC3+ staining. Staining was performed on coronal sections; images were acquired with a 20x objective and Zen software. The scale bar = 100μm.

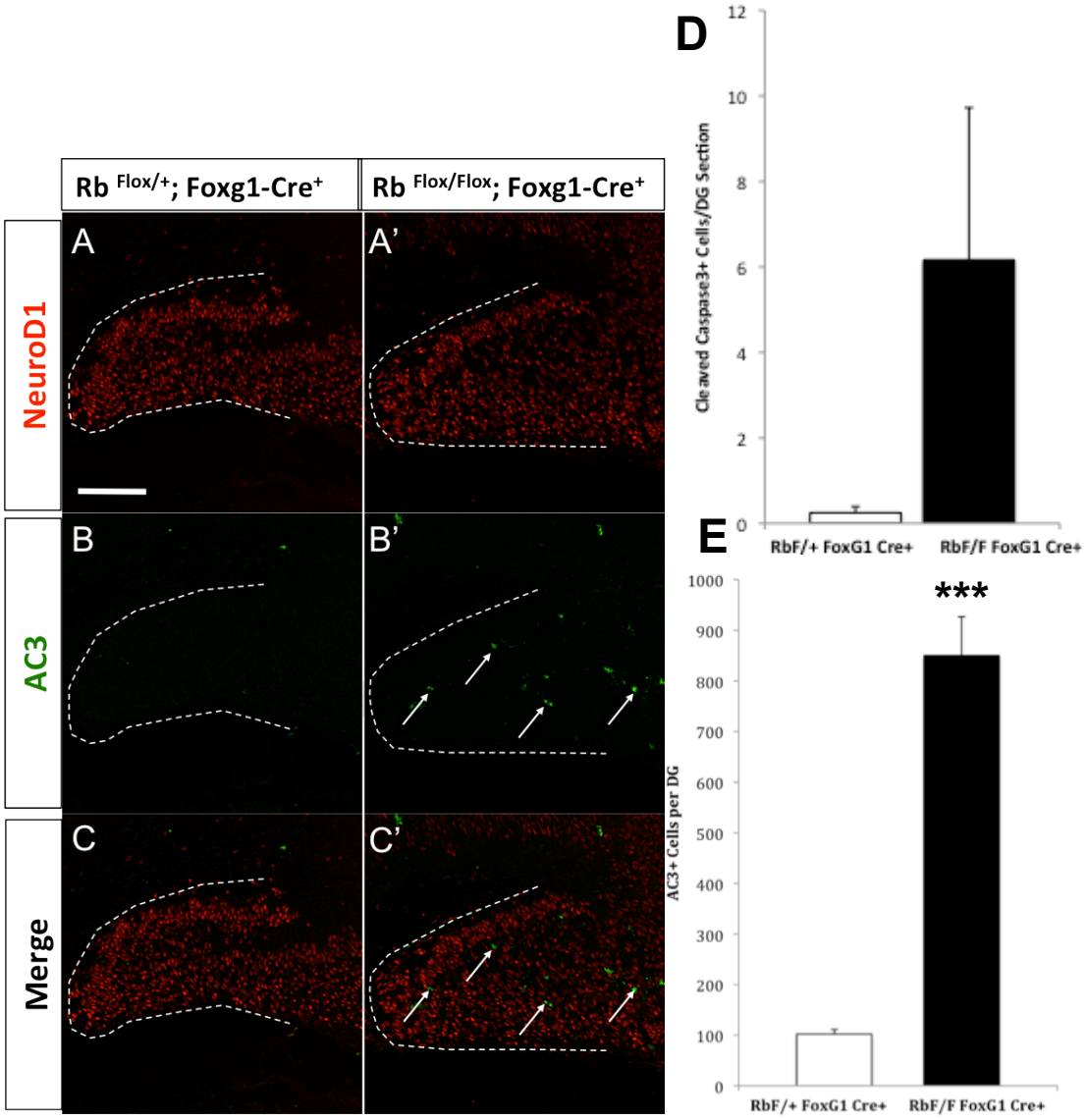


Figure 23: Increased AC3+ Cells in E18.5 DG.
Confocal images of immunostaining with AC3 and NeuroD1 at E18.5 uncovers increased apoptosis in the Rb deleted DG (A'-C') compared to controls (A-C). (D) Manual quantification reveals a 24-fold trending increase in AC3+ cells in the DG of Rb deficient animals when compared to littermate controls (Mean +/- SEM, Studen't T-test, p = 0.08) (E) Estimates, based on counting every sixth section throughout the DG, of total AC3+ cells reveal a significant 8-fold increase in cell death in the absence of Rb (Mean +/- SEM, Student's T-test, n=3 for Rb deleted, n=4 for controls, p = 0.0001). Staining was performed on 14μm, frozen, coronal sections. The dashed line outlines the third matrix of the DG, arrows indicate positive AC3 staining, the scale bar = 100μm.

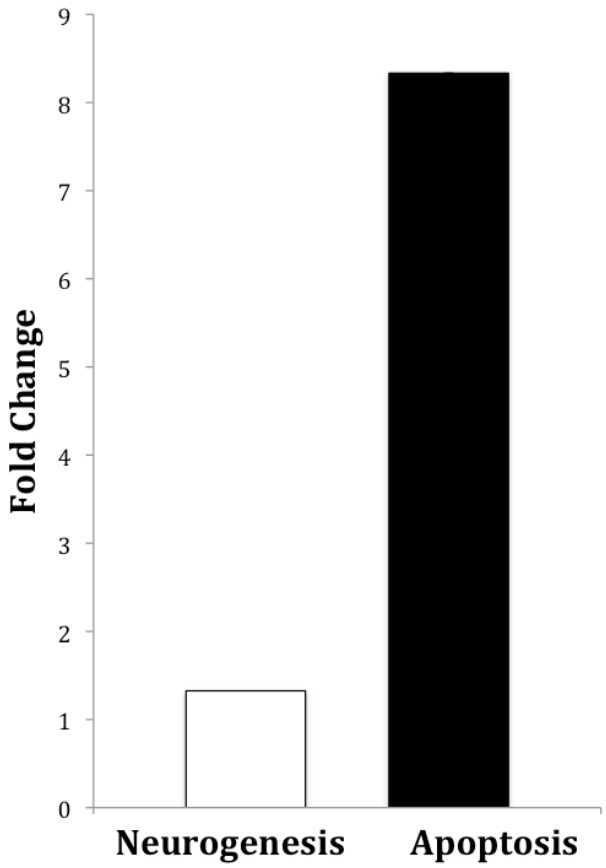


Figure 24: Comparison of fold changes present in neurogenesis and apoptosis.
As Rb deficient animals display both increased neurogenesis and increased cell death, it is possible that the cell death phenotype simply be an artifact of more cells being present in the Rb deleted brain. To address this, analysis of fold increases in the NeuroD1+/Prox1+ population (Neurogenesis) and fold increases in AC3+ cells (Apoptosis) were compared. Comparison revealed a 1.3-fold increase in neurogenesis and an 8-fold increase in cell death, suggesting that the cell death phenotype is not simply due to the presence of more cells.

Aim 3: Migration of DG Cells

Our lab has previously shown that during cortical development as well as olfactory bulb neurogenesis, Rb functions in cell migration (Ferguson et al., 2005; McClellan et al., 2007; Andrusiak et al., 2011). During the analysis of proliferation, it was hypothesized that the observed changes in the dorsal blade could be due to a migration defect. Also, when analyzed at E18.5, the Tbr2+ population appears to build up in the secondary matrix of the developing DG. The inability of cells to migrate properly could be due to a cell intrinsic loss of migration ability or due to incorrect radial glial scaffold formation. During DG development, Reelin signaling from Cajal Retzius cells is crucial to the correct formation of the radial glial scaffold (Weiss et al., 2003) and previous work from our lab has indicated that Rb deficiency results in the loss of these cells in the cortex (Ferguson et al., 2002); therefore it was hypothesized that the morphological defects present could be caused by poor scaffold formation. To address this, the Cajal Retzius cell population was analyzed at E18.5 using Reelin and calretinin, a calcium binding protein expressed in Cajal Retzius cell, double labeling. In addition, the radial glial scaffold was visualized at E16.5 and E18.5, with BLBP, a marker of radial glia.

The results of the Reelin/calretinin co-labeling suggest that there is a significant 65% decrease in the number of Cajal-Retzius cells within the hippocampal fissure (Student's t-test, $p = 0.007$; Fig. 25), and though it cannot be quantified immunohistochemically, there also appears to be a decrease in Reelin secretion in this area.

Since Reelin signaling has been strongly implicated in the formation of a radial glial scaffold during DG develop, BLBP staining was used to visualize the radial glial scaffold. At E16.5, a slight disorganization of the scaffold is present in the Rb null DG, with an apparent decrease in radial glial fibers in the hippocampal fissure (Fig. 26, arrow). By E18.5 this disorganization has become more apparent, with misalignment of fibers throughout CA3 and an apparent decrease in fiber lengths within the hippocampal fissure (Fig. 27, arrows).

In summary, these results suggest that a potential migratory defect could be due to malformations in the radial glial scaffold, likely caused by decreases in the number of Cajal-Retzius cells and thus decreased Reelin signaling. Further study is needed into the cell autonomous ability of DG cells to migrate in the absence of Rb, as previous work has documented cell autonomous roles in migration (Ferguson et al., 2005).

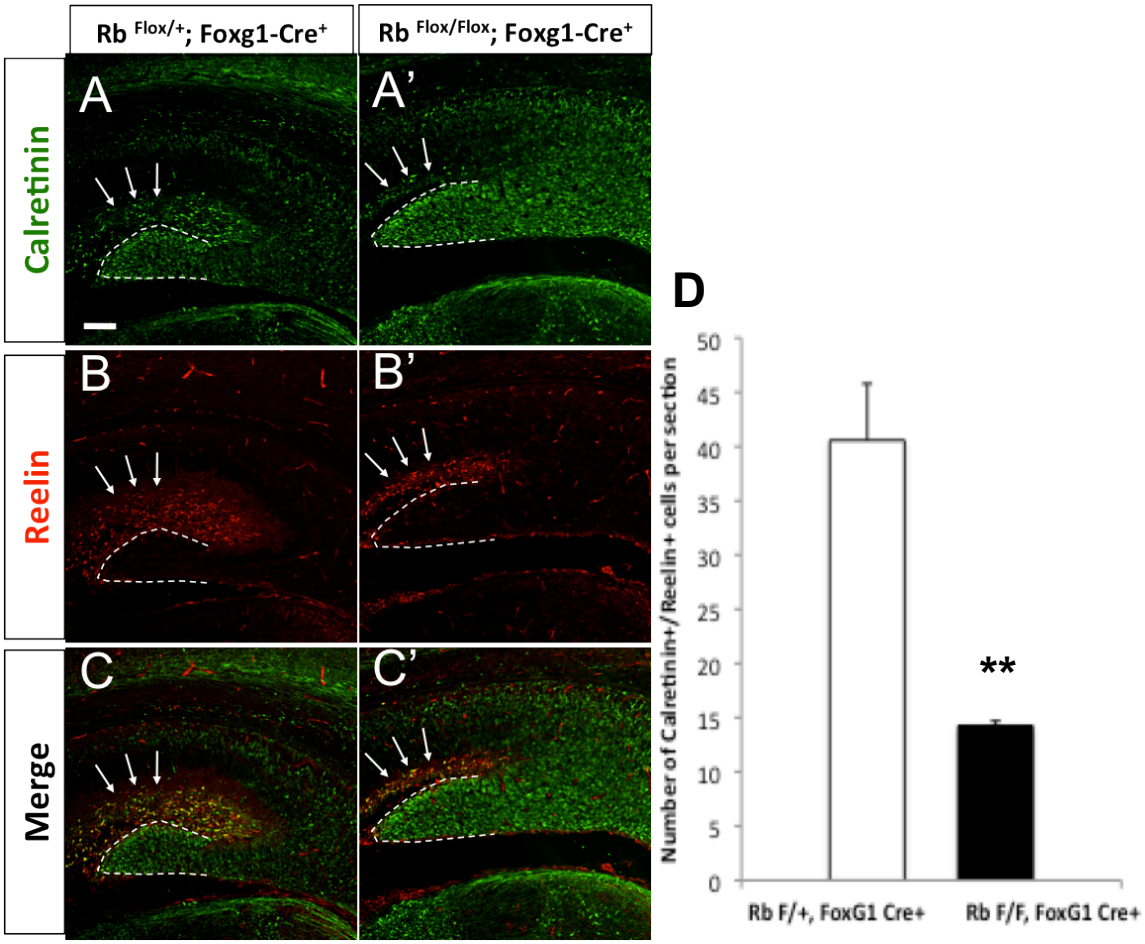


Figure 25: Decrease in Cajal-Retzius cell number in the absence of Rb.
Calretinin/Reelin co-labeling was used to visualize Cajal-Retzius cells within the hippocampal fissure (located above the third matrix, arrows) of control (A-C) and littermate Rb null (A'-C') animals and indicates a decrease in Cajal-Retzius cells in the absence of Rb. (D) Manual quantification of calretinin/Reelin double positive cells, on rostro-caudally level coronal sections, indicates a significant decrease in this population in the absence of Rb (Mean +/- SEM, Student's T-test, n=3, p = 0.007). Confocal images were acquired using a 10x objective and Zen imaging software, the scale bar = 100μm.

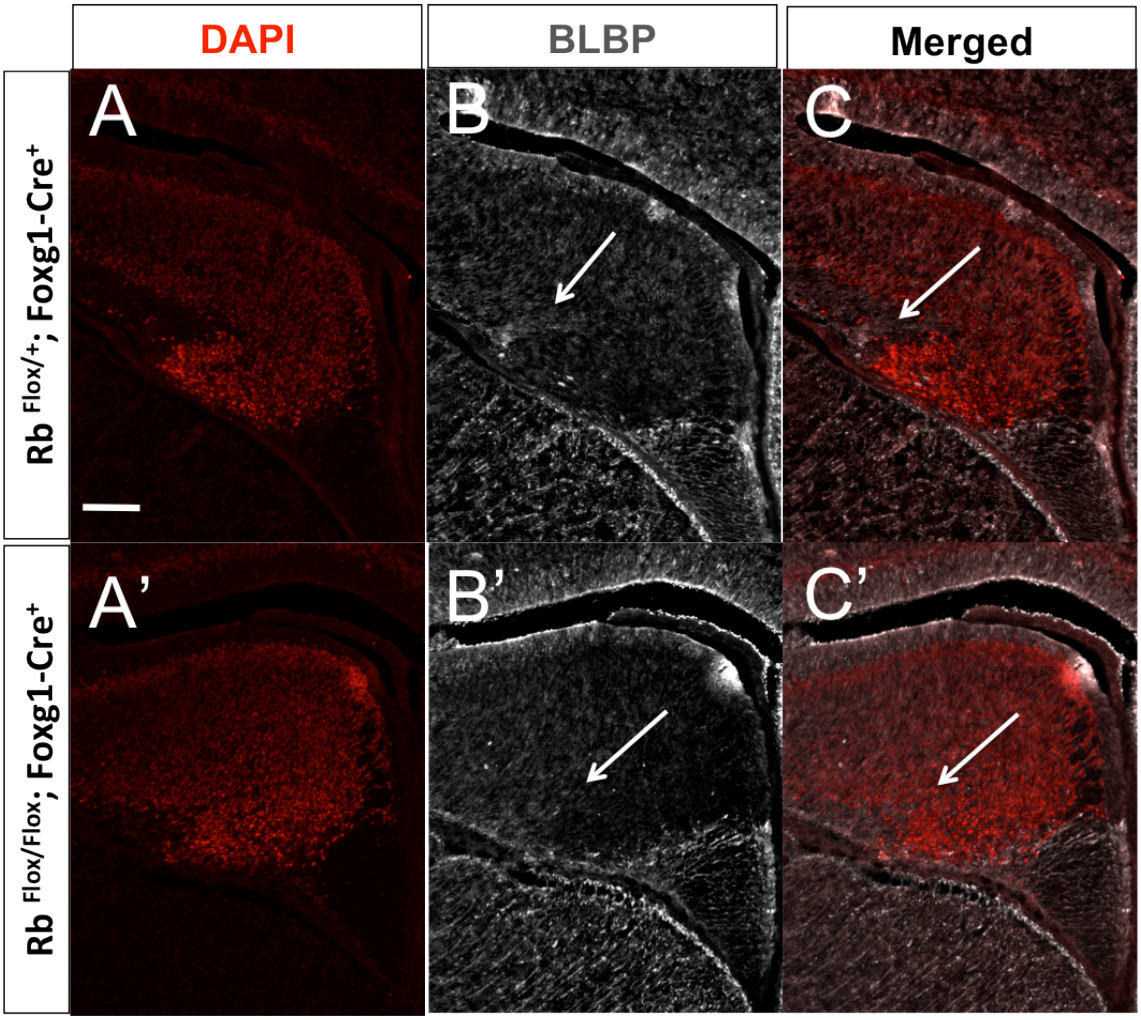


Figure 26: Minor radial glial scaffolding defects present at E16.5.
BLBP immunostaining revealed a decrease in the density of radial glial fibers in the presumptive hippocampal fissure (arrow) at E16.5 when compared to littermate controls. In the control fissure, fibers projecting around the dorsal blade of the DG can be easily seen (A-C). However, in the Rb null hippocampus, these fibers appear to much less dense (A'-C'). Immunohistochemistry was performed on 14μm coronal sections and images were acquired using a Zeiss confocal microscope with a 10x objective, the scale bar = 200μm.

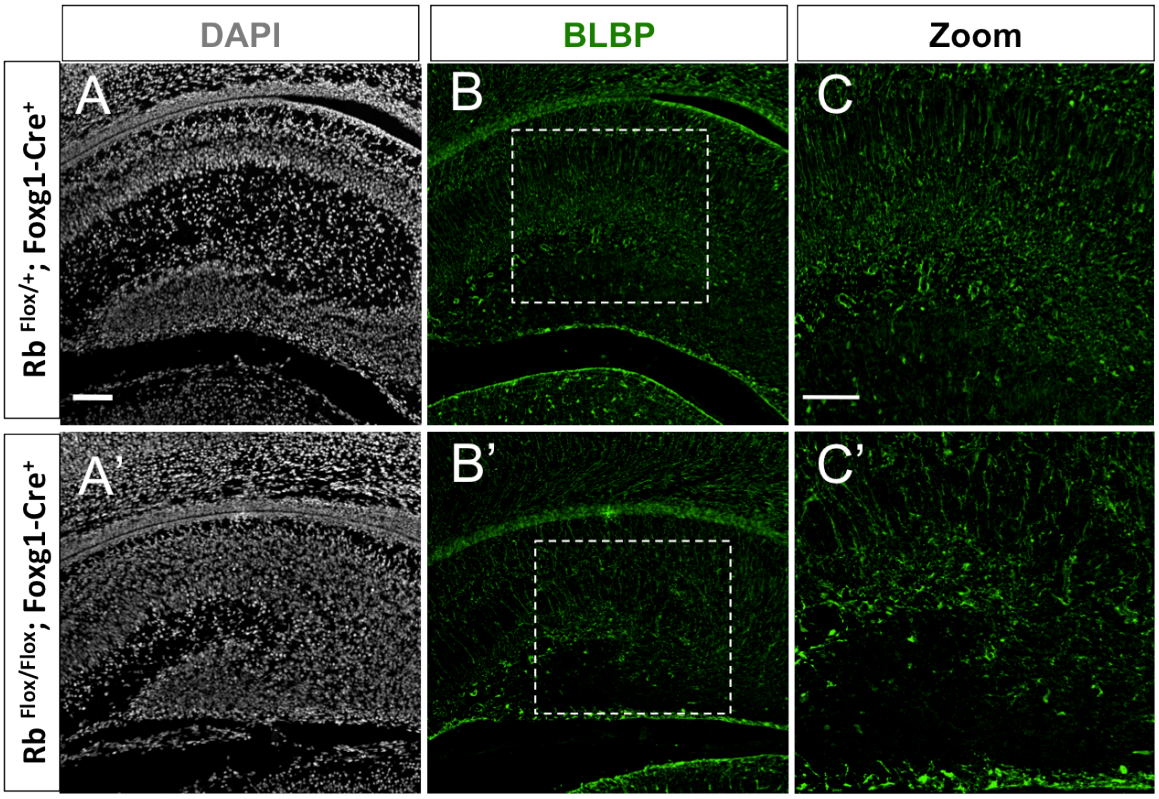


Figure 27: Increased disorganization of the radial glial scaffold is present at E18.5. Confocal images of BLBP immunostaining on E18.5 coronal sections reveals disorganization of the radial scaffold in the hippocampal fissure (square) as well as throughout the CA region in the Rb null hippocampus (A'-C') when compared with littermate controls (A-C). The region in the square is shown enlarged in C, C'. All staining was performed on frozen coronal sections and images were acquired using Zen imaging software, the scale bar = 100μm (A-B), scale bar = 50 μm (C).

Discussion

The purpose of this study was to determine the role of Rb in DG neurogenesis during development, and determine if Rb deletion holds promise to enhance neurogenesis in the adult DG. To address this, the Rb null hippocampus was analyzed at various stages of development and it was found that: 1) Rb is crucial to regulating the size of the granule cell population as well as the correct expression of differentiation markers. 2) Rb is important to regulating the proliferation and survival of young neurons and 3) Rb plays a role in the migration of DG cells, possibly through a non cell-autonomous role in the formation of the radial glial scaffold. Overall my results suggest that modulation of the Rb/E2F pathway may provide a viable approach to enhance dentate gyrus neurogenesis in an adult system.

Regulation of Granule Cell Number

The main finding of this study is that Rb is important to the regulation of granule cell number within the developing DG. In addition, this study shows a potential role for Rb in cell specification, as the absence of Rb generates a population of cells incorrectly expressing markers previously characterized in the developing hippocampus.

Expansion of neural cell populations in the absence of Rb has been documented, and the results of my study fit well with previous findings (Callaghan et al., 1999; Ferguson et al., 2002). Previous work from our lab has indicated that Rb regulates neurogenesis in the cortex and that cortical tissue is able to differentiate in the absence of Rb (Callaghan et al., 1999; Ferguson et al., 2002). In addition, work by Wirt et al., (2010) suggests that neural tissue is able to differentiate in the absence of all pocket proteins (Rb, p107 and p130), further supporting my finding of increased neurogenesis.

However, my study also finds that the fold-increase in cell number appears to decrease with age. At E16.5, there was a1.8-fold increase in the granule cell population, however by E18.5 only a 1.5-fold increase was observed. This reduction could be the result of increased cell death, which will be discussed below. Additionally, though an increase in the granule cell population is present, the functionality of these neurons is not known. A postnatal model would be advantageous to determine the survival of these cells and their capacity to integrate into a functional hippocampal network.

In addition to enhanced neurogenesis, a population of cells incorrectly expressing differentiation markers was generated in the Rb deleted DG. The Prox1+/NeuroD1- population, though present in postnatal and adult mice, is not present in control animals of this age. During embryonic development, the vast majority of granule cells are still expressing the differentiation factor, NeuroD1. To add further complexity, over 90% of this Prox1+/NeuroD1- population were found to be positive for Ki67, a marker of cycling cells, and the location of these cells suggests they are some of the oldest in the DG (Fig. 12). The presence of this population is perplexing as the lack of NeuroD1 expression in a DG cell expressing high levels of Prox1 usually indicates a postmitotic granule cell; however the presence of Ki67 within this population suggests they are cycling and have not undergone terminal mitosis. Whether this population is the result of 1) ectopic Prox1 expression or 2) a decrease in NeuroD1 expression remains to be elucidated but will be discussed further below.

One possible explanation for this population is ectopic Prox1 expression. Work by Wenzel et al., (2011) has shown that the Prox1 locus has an E2F binding site. During lens development in the absence of all activator E2Fs, Prox1 is down regulated,

suggesting that the E2Fs could actively promote Prox1 expression (Wenzel et al., 2011). As E2F transcription factors are regulatory targets for Rb, it is possible that Rb deletion, which causes deregulated E2F activity (Callaghan et al., 1999), could cause ectopic expression of Prox1 (Fig. 28). The possibility of Prox1 acting as an E2F target is interesting, as Prox1 has been documented to affect both proliferation and differentiation depending on tissue type (Dyer et al., 2003; Lavado et al., 2010). A potential link between Rb, a known tumor suppressor, and Prox1 in neural tissue is intriguing as recent research suggests that ectopic Prox1 expression could repress proliferation of neuroblastoma tumor cells (Foskoluo et al., 2012). Investigating the possible regulation of Prox1 by Rb may reveal new insights into the mechanisms regulating cell specification in the DG as well as interactions between tumor suppression pathways that are not yet known.

An alternate explanation of the presence of a Prox1+/NeuroD1- population is a premature decrease in NeuroD1 expression. NeuroD1 is strongly implicated in differentiation and cell cycle exit (Miyata et al., 1999); therefore it is possible that early loss of NeuroD1 could lead to continued cycling of cells that have already begun expressing Prox1 at a high level. NeuroD1 regulation is not completely understood during the development of the hippocampus; however Wnt signaling is crucial to NeuroD1 expression during adult neurogenesis (Kuwabara et al., 2009). Though a direct link between Rb and NeuroD1 expression has not been shown, E2F1 is known to inhibit canonical Wnt signaling (Morris et al., 2008; Wu et al., 2011). It is possible that in the absence of Rb, increased E2F1 activity (Callaghan et al., 1999) leads to an increased inhibition of Wnt signaling, causing a lowering of NeuroD1 expression (Fig. 29).

Though these are possible explanations for this population, a substantial amount of work is needed to determine the regulation of Prox1 and NeuroD1 with regards to the Rb/E2F pathway.

Overall, though erroneous expression of markers exists in the absence of Rb, the vast majority of cells in the Rb null DG appear to express the correct markers at the correct time suggesting that modulation of the Rb/E2F pathway could potentially enhance neurogenesis in the adult system.

Regulation of Terminal Mitosis and Granule Cell Survival

Once an expansion of the granule cell population had been established, I next set out to determine the underlying reason for the increase in granule cell numbers. An increase in Ki67 expression suggested enhanced proliferation (Fig. 14), so I next determined which of the three populations of the developing DG, the stem-like cells, the intermediate neuronal progenitors (INPs) or the young neurons, this proliferation could be attributed to.

Rb had no apparent role in the regulation of stem-like cell proliferation or population size in the developing DG. No morphological or proliferative defects are present in the E14.5 neuroepithelium (Fig. 14). The lack of phenotype present at E14.5 supports previous work from our lab indicating that Rb deletion does not affect proliferation of stem cells within the ventricular zone of the cortex (Ferguson et al., 2002). The *in vitro* data generated using neurosphere assays further supports the finding that Rb deletion has little affect on the size of the stem-like cell population (Fig. 19). Overall, the results of my study fit well with previous findings for the role of Rb in stem-like cells.

Interestingly, though expected results were obtained with the stem-like cell population, the role of Rb in the intermediate neuronal progenitor population appears to be more complex. My results suggest that Rb is important in maintaining proliferation of the progenitor population in the developing DG. Using Tbr2 to label intermediate neuronal progenitors and Ki67 to mark cycling cells; I found that the proliferative state of the intermediate neuronal progenitors was decreased at E18.5 in the absence of Rb (Fig. 17). Though it is possible that Rb is intrinsically required to maintain proliferation of INPs in the developing DG, I hypothesize that this is likely non-cell autonomous and could have occurred for two reasons. First, it is possible that the analysis of the Tbr2+ population was confounded by a migratory defect in the Rb deleted brain. During normal development, cells move from the dentate neuroepithelium to the third matrix (Angevine, 1965; Li and Pleasure, 2005; Li and Pleasure, 2007; Figure 4). In the absence of Rb, the movement of Tbr2+ cells was perturbed such that there appeared to be a buildup of Tbr2+ cells within the secondary matrix of the DG with a notable lack of Tbr2+ cells within the third matrix (Fig. 4 & Fig. 17). It is possible that proliferation in this population was disrupted due to incorrect localization within the migratory stream. Previously, the proliferative state of the Tbr2 population has been shown to be sensitive to location within the DG; in the absence of correct migration, the secondary proliferative zone within the hilus is not established (Li et al., 2009). Second, there could be decreased proliferation in the progenitor population due to the presence of a feedback mechanism from the young neuronal population. It is possible that the enhanced proliferation in the NeuroD1 population is leading to negative feedback within the Tbr2 population, resulting in decreased proliferation in these cells. Previous work in adult

neural stem cell populations suggests that intricate feedback loops exist between the different cell types within the neurogenic niche to maintain the various pools of cell types (For review see: Miller and Gauthier-Fisher, 2009). In summary the decreased proliferation in the Tbr2+ cell population is not fully understood, however more work could reveal a novel role for Rb in the proliferation of progenitor cells or increase our understanding of the feedback loops present during DG development.

Analysis of the young neuron population, with NeuroD1 labeling, within the DG suggests that Rb is imperative to regulating cell division within this population as Rb deletion results in increased proliferation of the NeuroD1+ population (Fig. 18). Further study suggests a potential role for Rb in promoting the cell cycle exit of newly born neurons. *In vivo* analysis showed that in the absence of Rb a significantly higher proportion of NeuroD1+ cells continue to express Ki67, a marker of cycling cells, then in littermate controls (Figure 18). It is also interesting to note that a large increase in proliferation was present in the dorsal blade of the DG (Fig. 14 & Fig. 18), the region corresponding, to the oldest cells in the DG (Angevine, 1965; Li and Pleasure, 2005). The ectopic proliferation present within the oldest cells of the DG could mean that either: 1) there is a delay is cell cycle exit or 2) cells undergo terminal mitosis but cannot maintain quiescence in the absence of Rb. In an effort to address this issue, an *in vitro* differentiation assay was used and determined that cells from the Rb deleted dentate neuroepithelium could have a tendency to undergo terminal mitosis later than control cells. Earlier work from our lab has documented a delay in cell cycle exit of cortical precursors in the absence of Rb (Callaghan et al., 1999), which would fit with our model. The *in vitro* results suggest a trend for delayed cell cycle exit in the absence of Rb,

however this does not rule out the possibility of a delayed terminal mitosis, followed by a later cell cycle re-entry. A role for Rb is the maintenance of quiescence within differentiated cells has been well established (Weber et al., 2008; Guo et al., 2009). Our lab has recently documented that loss of Rb in post-mitotic DG neurons results in cell cycle re-entry followed by cell death (Andrusiak and Vandenbosch, 2012). Overall, the results suggest that Rb is critical to regulate proliferation in young granule neurons, but the mechanism of regulation is not yet known.

Finally, an analysis of cell death in the absence of Rb was performed using an antibody directed against the activated form of caspase3 (AC3), a marker of apoptotic cell death. My results indicate that Rb is required to maintain cell survival, as an increase in apoptotic cells was seen in the absence of Rb at both E16.5 and E18.5 (Fig. 22 & Fig. 23). Previous research has documented a direct role for Rb in cell death as the Rb/E2F pathway has been linked to direct activation the p53 pathway (Qin et al., 1995; Macleod et al., 1996) and has also been linked to expression of Apaf1, a pro-apoptotic protein (Guo et al., 2001). Interestingly, I found that cell death was not present in the stem-like cell population (Fig. 21) and that apoptosis became present with increased gestational age suggesting that the increase in cell death may not be linked directly to Rb deficiency but could instead be linked to a deregulated cell cycle or the inability of these neurons to integrate into a neuronal network.

In summary my results indicate that although Rb is important to DG cell survival, many cells are surviving up to E18.5 in the absence of Rb. Further research into the survival of these cells is crucial to fully understand whether or not neurogenesis is enhanced and maintained throughout life; a model that can be studied postnatally would

be beneficial in this regard. Taken together, my results support the previous findings that Rb deletion results in enhanced proliferation and delayed cell cycle exit (Callaghan et al., 1999; Ferguson et al., 2002).

Non-Autonomous Role and DG Cell Migration

During the study of neurogenesis and proliferation, it was noticed that the dorsal blade of the DG consistently appeared at a greater angle relative to the ventral blade in Rb deleted animals when compared to control animals. In addition, the Tbr2+ population appeared to be impaired in its movement toward the third matrix. These observations lead to further investigation into the formation of the dorsal blade in the absence of Rb.

Previous work from our lab has implicated Rb in both cell autonomous and non-cell autonomous roles in cell migration (Ferguson et al., 2002; Ferguson et al., 2005; McClellan et al., 2007; Andrusiak et al., 2011). We have previously documented the detrimental effect of Rb deletion on the Cajal Retzius cell population within the embryonic cortex (Ferguson et al., 2002). Cajal Retzius cells are early born neurons that secrete the chemoattractant Reelin and are crucial to correct lamination of the cortex as well as formation of the radial glial scaffold in the DG. Using calretinin, a calcium binding protein expressed in Cajal Retzius cells, and Reelin co-labeling, this study was able to confirm the previous findings that Rb is important to the maintenance of the Cajal Retzius cell population (Fig. 25). There was found to be a significant decrease in the number of Cajal Retzius cells within the hippocampal fissure of the Rb deleted brain at E18.5 (Fig. 25), and there also appeared to be a decrease in the level of Reelin secreted from these cells.

As Reelin secretion from Cajal Retzius cells has been shown to be crucial to the correct formation of the radial glial scaffold (Weiss et al., 2003), the scaffold was analyzed using BLBP staining. At E16.5, the fibers of the glial scaffold appear to be denser along the developing hippocampal fissure in the control animals then in the Rb deleted brains (Fig. 26). Analysis of the glial scaffold at E18.5 revealed an overall disorganization of fibers throughout the CA regions as well as the DG (Fig. 27). In addition, though the density of fibers in the hippocampal fissure appears similar between Rb deleted and control animals, the length of both the fiber tract and hippocampal fissure appear to be reduced in the absence of Rb. The presence of a malformed glial scaffold in addition to decreases in the Cajal Retzius cell population could be the cause of a migration defect.

Finally, in addition to malformation of the radial glial scaffold, it is possible that the absence of Rb is causing an intrinsic defect in the migration of cells from the ventricle into the third matrix, as our lab has documented cell autonomous roles for Rb in the cell migration during cortical development (Andrusiak et al., 2011; Ghanem et al., 2012; Ferguson et al., 2005).

In summary, the results of my studies indicate that the morphological defects present in the Rb deleted hippocampus could arise from decreases in the Cajal Retzius cell population, leading to malformation of the radial glial scaffold and thus impaired migration, however further study could reveal a cell autonomous role for Rb in DG cell migration.

Conclusion

In conclusion, this study reveals that Rb is involved in multiple aspects of DG development. Functioning Rb protein is required to maintain the correct number of granule cells in the DG, possibly through aiding the cell cycle exit of young neurons and promoting survival. Though the majority of cells appear normal, we show that Rb is required for proper expression of Prox1 and/or NeuroD1 in a small number of DG cells, though the underlying mechanisms remain unknown. My results suggest that Rb does not play a role in the regulation of the early stem cell population. Also, Rb is required for the proper migration of DG cells into the third matrix.

The results of this study are intriguing as they suggest that modulation of the Rb/E2F pathway could be used to enhance neurogenesis in the adult system. Though an increase in cell death is present in the embryo, the granule cell population is still expanded in the absence of Rb, suggesting that many of the cells are surviving. Also, the cells being generated in the absence of Rb express markers specific to granule neurons and differentiate in culture.

Overall, the Rb deleted DG appears to develop relatively normally with the exception of increased cell number, making it likely that modulation of the Rb/E2F pathway would enhance neurogenesis in the adult.

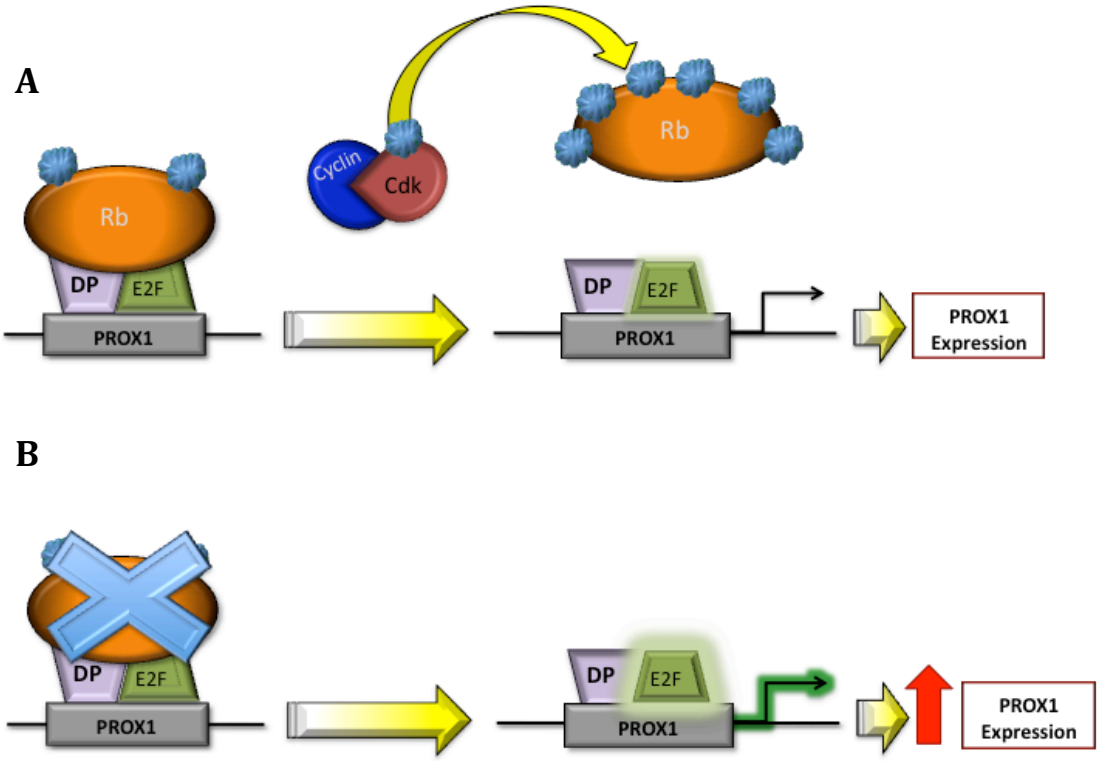


Figure 28: Rb/E2F pathway and Prox1 expression.
A model of Prox1 regulation by Rb/E2F is shown above. A) In the presence of Rb, E2F is correctly regulated leading to normal expression of Prox1. B) In the Rb deleted animal, E2Fs are deregulated leading to increased expression of downstream target genes, one of which could be Prox1.

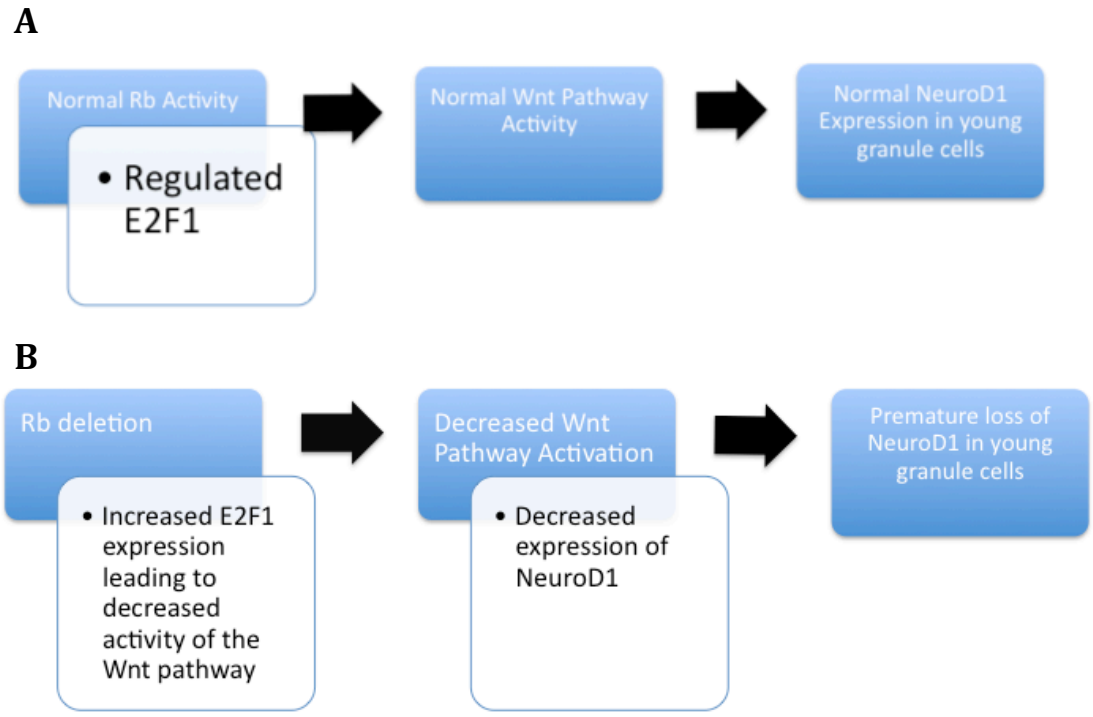


Figure 29: Rb/E2F pathway and NeuroD1 expression.
A model of NeuroD1 regulation by Rb/E2F via the Wnt pathway is shown above. A) In the presence of Rb, E2F is correctly regulated allowing normal activation of the Wnt pathway. Normal Wnt activity leads to normal expression of Wnt target genes, one of which is NeuroD1. B) In the Rb deleted animal, E2Fs are deregulated leading to inhibition of the Wnt pathway, and possibly a decrease in NeuroD1 expression.

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