uOttawa

L'Université canadienne
Canada's university

FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTOCTORALES

uOttawa

L'Université canadienne
Canada's university

FACULTY OF GRADUATE AND POSDOCTORAL STUDIES

David Douda

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Neuroscience)

GRADE / DEGREE

Department of Cellular and Molecular Medicine

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Role of E2F1 as Downstream Target for Rb in Cortical Development

TITRE DE LA THÈSE / TITLE OF THESIS

R. Slack

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

David Park

Marie-Andrée Akimenko

Matthew Hogan

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

The role of E2F1 as Downstream Target for Rb in Cortical Development

By David N. Douda

A report submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of Master of Science

Neuroscience Program
Department of Cellular and Molecular Medicine
Faculty of Medicine
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-49193-5
Our file Notre référence
ISBN: 978-0-494-49193-5

Table of Contents

Acknowledgements

I would first like to thank my supervisor, Dr. Ruth Slack for the opportunity to be a part of her great lab. She encouraged me to push myself to aim higher and reach new limits and also provided me with an opportunity to grow in my scientific training, such as by sending me to numerous conferences. My last two years has been an extremely rewarding experience, and for that I am grateful. I feel proud to have been a part of the Slack lab.

I must also express my gratitude to Kelly McClellan, a Ph.D. candidate in our lab. She took me under her wings and generously offered to teach me all the experimental techniques, and guided me in the right direction throughout my project. Specifically, she has contributed to the work presented here in terms of: 1) Rb:E2F1:cre mouse colony initiation and assistance with breeding and colony maintenance; 2) Provision of Rb mutant tissue for all experiments; 3) Performed BrdU immunostaining and photography; 4) For total marginal zone cell number and calbindin cells, cell counts and initial finding; and; 5) Assistance with study design and interpretation. She also encouraged me to join the student council, which helped enrich my experience as a graduate student.

Thanks to Vladimir Ruzhynsky for his technical support while I carried out in *in situ* hybridizations and also carrying out the *in situ* hybridization using Tbr1 riboprobes, and Dr. Jackie Vanderluit for her help in BrdU immunolabeling.

I would also like to thank our lab technicians, Jason MacLaurin for his help in genotyping, plasmid preparations for *in situ* hybridization probes, and any computer related advice, Linda Jui for carrying out tissue sectioning, and Carl McIntosh for his help in tagging and tailing and colony maintenance.

To all members of the Slack lab, past and present, including Dr. Jackie Vanderluit, Kelly McClellan, Eric Cheung, Vladimir Ruzhynsky, Nicole Arbour, Arezu Jahani-asl, Dr. Andre Fortin, Lisa Julian, Nicole LeGrand, Dr. Noel Ghanem, Crystal Wylie, Jason MacLaurin, Carl McIntosh, Linda Jui, Eric Ouellet, William Xu, and Dominique Yelle, thank you for your emotional support and making the lab a fun place to be. I will cherish all the memories we shared in and out of the lab.

To my family, mom, dad, and my brother Michael, I couldn't have done it without your unconditional love and support. You were always there to help me be stronger, and without you guys, I couldn't have done it.

Lastly, to my fiancé, Miyuki, you were always there for me right when I needed you. You supported me through everything, even when things got tough. Thank you for giving me the courage, and making my life brighter.

Abstract

Cell cycle regulation is believed to be crucial to nervous system development. In this study, we investigated whether Rb plays a role in the expression of guidance molecules involved in neuronal migration. We found an ectopic expression of robo2 gene, and an increase in the expression of neogenin gene in the developing cortex in conditional Rb knockout mice, suggesting an involvement for Rb in the expression of these genes. We also investigated whether deregulated activity of E2F1 is involved in the defects observed in Rb mutant mice. Using mice with compound null mutations for Rb and E2F1, we found that ectopic proliferation was rescued, and the aberrant laminar patterning in the cortex was restored, while there was a partial rescue in the survival of Cajal-Retzius neurons. Tangential migration of GABA-ergic interneurons, however, was not rescued. Thus, we have demonstrated that E2F1 is involved in Rb mediated ectopic mitoses, cortical laminar patterning, and partially, for the requirement of Rb in the survival of Cajal-Retzius neurons.

List of Figures

pg

List of Abbreviations

Apaf1	Apoptotic protease activating factor 1
ARF	alternate reading frame (ARF) product of the INK4
ATM	ataxia telangiectasia mutated
BCIP	x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate
Bcl-2	B-cell CLL/lymphoma 2
Bim	Bcl-2-interacting mediator of cell death
BH3	Bcl-2 homology domain 3
Bmi1	B lymphoma Mo-MLV insertion region
bp	base pairs
BrdU	5-bromo-2-deoxyuridine
CDK	cyclin dependent kinase
Chk2	checkpoint kinase 2
CKI	CDK inhibitor
CGE	Caudan ganglionic eminence e
CNS	central nervous system
CP	cortical plate
DCC	Deleted in Colorectal Cancer
DEPC	diethylpyrocarbonate
DKO	double knockout
Dlx	distal-less homeobox
DNA	deoxyribonucleic acid
DP	DRTF1-polypeptide

DP5	death protein 5
E2F	Adenovirus E2 promoter binding factor
EDTA	ethylenediamine tetra-acetic acid
GABA	gamma-aminobutyric acid
HDAC	histone deacetylase
HPV	human papillomavirus
Hrk	harakiri
HSC	hematopoietic stem cells
Id2	inhibitor of differentiation protein 2
IgG	immunoglobulin g
INK4	inhibitors of CDK4
IZ	intermediate zone
Jab1	c-Jun activating binding protein
JMY	junction mediating and regulatory protein
KO	knockout
μL	micro-liter
LGE	Lateral ganglionic eminence
Mdm2	mouse double minute 2
MDT	multi-directional tangential migration
MGE	medial ganglionic eminence
MHC	myosin heavy chain
min	minutes
mM	mili-moles

mRNA	messenger ribonucleic acid
MZ	marginal zone
NBT	4-nitro blue tetrazolium chloride
PBS	phosphate buffered saline
PcG	Polycomb group
PCR	polymerase chain reaction
PFA	paraformaldehyde
PP	primordial plexiform layer/preplate
PUMA	p53 upregulated modulator of apoptosis
PVA	polyvinyl alcohol
Rb	retinoblastoma protein
RGM	repulsive guidance molecule
RMS	rostral migratory stream
ROBO	roundabout
rpm	revolutions per minute
RT	room temperature
RYBP	Ring1 and YY1 binding protein
SCG10	superior cervical ganglia neural-specific 10 protein
sec	seconds
SEM	standard error of mean
SNF	sucrose nonfermenting
SP	subplate
SSC	sodiumchloride/trisodium citrate

SWI	switch
SV40	Simian vacuolating virus
SVZ	subventricular zone
Tbr1	T-box brain gene 1
TE	Tris-EDTA
TP53INP1	tumor protein p53 induced nuclear protein 1
VZ	ventricular zone
WT	wild-type

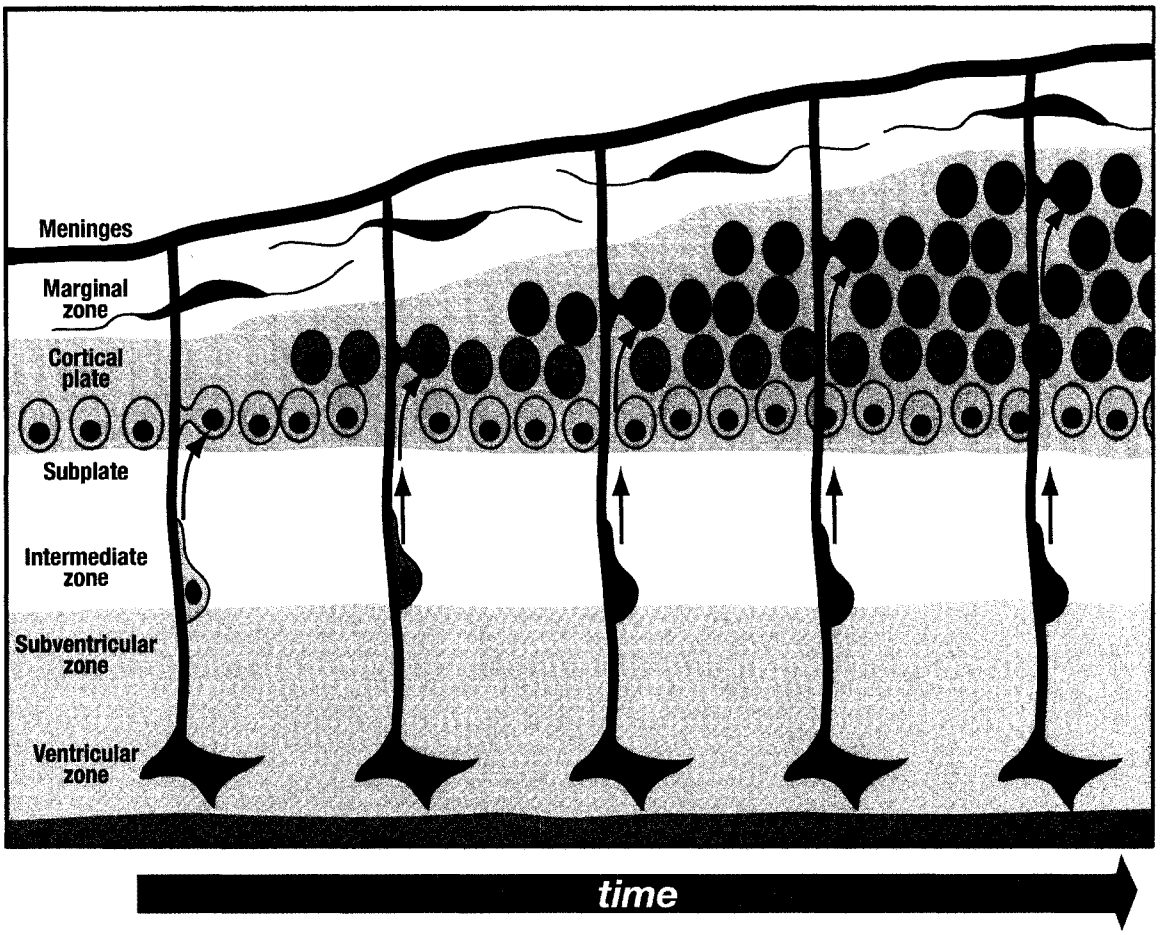
Introduction

I. Cortical Development

The vertebrate telencephalon is the most complex part of the brain unique to mammals, and it is responsible for higher cognitive processes. Telencephalon can be subdivided into two components: the cortex and the basal ganglia. Dorsal half of the developing telencephalon is called the pallium, and it gives rise to the cerebral cortex and the hippocampus. The ventral half called the subpallium gives rise to the globus pallidus and the striatum, which make up the basal ganglia (Lavdas et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). In the developing telencephalon, there are highly proliferative zones called ganglionic eminences in the subpallium. Medial (MGE) and lateral (LGE) ganglionic eminences reside in the rostral region, and caudal ganglionic eminence (CGE) is found just caudal to MGE and LGE. LGE has been found to be the primary source for the development of striatum and also contribute to the neuronal population found in the olfactory bulb (Deacon et al., 1994; Stenman et al., 2003; Wichterle et al., 2001). MGE produces cells found in both pallium and subpallium (Anderson et al., 2001; Olsson et al., 1997; Wichterle et al., 2001). CGE also contributes to the generation of pallium and subpallium, but it is thought to contribute distinct cell types from MGE or LGE (Nery et al., 2002).

Adult neocortex consists of six layers and its generation is accomplished in a highly organized manner (Figure 1). During corticogenesis neural precursor cells proliferate in a specified layer called the ventricular zone (VZ) which lines the wall of the lateral ventricle (Rakic, 1972). These precursors then exit the cell cycle and start the differentiation process. Cortical development begins with the formation of primordial

Figure 1. **The formation of cortical plate.** Cortical projection neurons (green) proliferate in the ventricular zone. Upon terminal mitosis, these neurons radially migrate along radial glia (orange) to generate the cortical plate. The cortical plate is generated in an "inside-out" manner where earlier born neurons generate deeper layers while later born neurons (darker green) generate more superficial layers. The radial migration of these neurons, and thus, the formation of cortical plate is directed by reelin expressing Cajal-Retzius neurons (red) residing in the marginal zone.



Adapted from Bielas et al, 2004. Ann. Rev Cell Dev Biol. 20:593-618

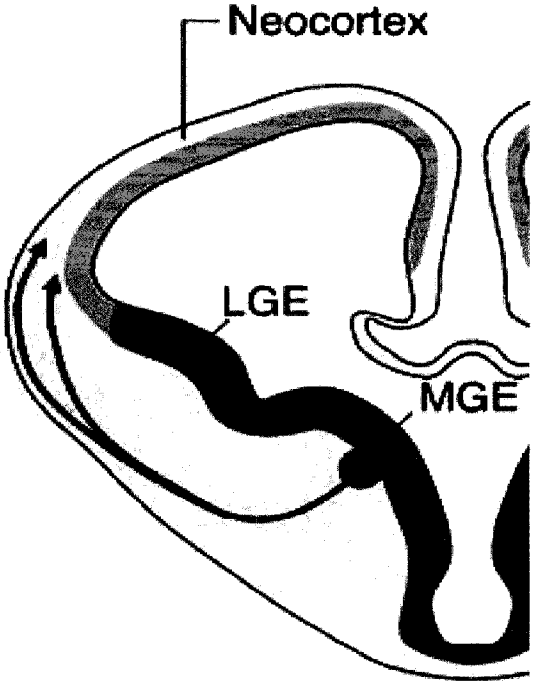
plexiform layer, or preplate (PP) above the ventricular zone (Rickmann and Wolff, 1981). After this point, post mitotic neurons migrate radially from the ventricular zone to form the cortical plate (CP) and split the preplate into superficial marginal zone (MZ) and subplate (SP) which lies beneath the developing CP. The SP then gets separated from the VZ by cell sparse intermediate zone (IZ), which later become the white matter. Later in cortical development, a second proliferative layer called the subventricular zone (SVZ) emerges.

A birth dating study by Angevine and Sidman in the 1960s using tritium (^{3}H) labeled thymidine demonstrated that cortical layer formation is accomplished in an "inside-out" manner (Angevine and Sidman, 1961) (Figure 1). That is, earlier born neurons make up the deeper layers, while later born neurons make up more superficial layers with newly born neurons radially migrating outward. Radially migrating neurons from the pallial VZ are the major contributor of projection neurons found in the CP (Gorski et al., 2002) (Figure 1). Most of the GABA-ergic interneurons, in turn, are derived from cells in the MGE, and arrive to the CP via tangential migration (Anderson et al., 2001; Wichterle et al., 2001) (Figure2). These migrating cells acquire positional cues from guidance molecules and receptors to navigate through the developing cortex. In this section, I will focus on the two modes of migration: radial and tangential migration, and guidance cues that are thought to be important for correct migration pattern.

Radial Migration

There has been a long debate over the mode of radial migration by newly born neurons. The very first model of the radial migration was put forth by Berry and Rogers,

Figure 2. **Tangential migration of GABA-ergic interneurons from MGE.** In the developing telencephalon, there are highly proliferative zones called lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE) in the subpallium. Most, if not all GABA-ergic interneurons destined to the cortical plate are generated in the MGE (Anderson et al., 2001; Wichterle et al., 2001). These interneurons migrate tangentially to their final destination in the neocortex (red arrows). Tangentially migrating interneurons follow two distinct paths, where one group of neurons migrate through the marginal zone while the other group of neurons migrate through the intermediate zone.



Adapted from Marin and Rubenstein, 2003. Annual Review of Neuroscience. 26:441-83

who proposed that just prior to cytokinesis after completing the cell cycle, one of the daughter nuclei leave the ventricular zone and migrate up to the CP (Berry and Rogers, 1965). Through Golgi staining of opossum cortex, Morest later proposed a similar model called the somal translocation (Morest, 1970). In somal translocation, a newly born neuron is bipolar, extending processes to the ventricular and pial surfaces. A cell will commence migration upon detachment and retraction of the process from the ventricular surface and travel to the CP. Subsequently, Rakic proposed yet another mode of migration where migrating cells use thin fibers of radial glia as scaffolds (Rakic, 1972). This was shown by serial sections of electron micrographs of the developing CP and the migrating cells seemed to be maintaining an intimate association with the radial fibers. These conflicting views of the modes of migration were finally resolved by the observation that in fact both modes of migrations exist, but probably at different points during development (Nadarajah et al., 2001). Real time imaging of the migrating neurons revealed that at early stages during cortical development, the predominant form of migration is via somal translocation while at later stages migrating neurons seem to use glia guided locomotion (Nadarajah et al., 2001).

Reelin is thought to be critical for radial migration, and thus, proper formation of cortical layers. It is a secreted protein that is produced by the Cajal-Retzius neurons of the marginal zone. Cortical lamination mutant mouse, *reeler*, lack the expression of reelin (D'Arcangelo et al., 1995; Ogawa et al., 1995). In *reeler* mouse, the cortical layers are formed in a reversed, "outside-in" manner as opposed to the normal "inside-out" fashion (Reviewed in (Caviness and Rakic, 1978). Subsequent studies showed that mice lacking cytoplasmic adaptor protein, disabled 1 (Dab1), and cell surface receptors: very

low density lipoprotein receptor (VLDLR), and apolipoprotein E receptor 2 (ApoER2), show similar phenotype to the *reeler* mouse, suggesting that these genes are involved in a pathway downstream of reelin (Howell et al., 1997; Trommsdorff et al., 1999). It is now known that reelin is a ligand for VLDLR and ApoER2 (D'Arcangelo et al., 1999; Hiesberger et al., 1999) where activation of these receptors by reelin causes tyrosine phosphorylation of Dab1 and the downstream signaling affects cytoskeletal network and cell motility (Hiesberger et al., 1999; Stolt and Bock, 2006). The cytoskeletal rearrangement by reelin signaling seems to be accomplished by the activation of Akt and Src family kinases (Ballif et al., 2003; Bock and Herz, 2003). It is known through yeast-two-hybrid screen that Dab1 interacts with Src and Fyn (Howell et al., 1997). A combined loss of Src and Fyn in mice shows a similar phenotype to the *reeler* mice, providing evidence that these two kinases play crucial roles in reelin signaling (Kuo et al., 2005). When phosphorylated by Fyn, Dab1 is sent for degradation by proteosomes, and this temporal reduction in Dab1 level is thought to be important for transient response to reelin signaling (Arnaud et al., 2003). In addition, mice lacking CDK5, a member of the CDK family exclusively expressed in the post mitotic neurons, and p35, an activator of CDK5, also show reversed cortical lamination pattern similar to *reeler* (Chae et al., 1997; Gilmore et al., 1998). To elucidate the possible relationship between reelin signaling and CDK5/p35 singnaling pathways, mice that have combined defect of reelin and CDK5/p35 were generated (Ohshima et al., 2001). Additional alterations in either pathway resulted in extensive migration defects, suggesting that there is a synergistic contribution to cortical laminar positioning by reelin and CDK5/p35 signaling pathways (Ohshima et al., 2001). A recent study has shown that phosphorylation of

Dab1 by CDK5 modulates interactions between Fyn and Dab1, providing further link between CDK5 and reelin signaling pathways (Ohshima et al., 2007). A recent study in our lab indicated that mice lacking Rb specifically in the telencephalon also has a defective cortical lamination patterning (Ferguson et al., 2005).

Tangential Migration

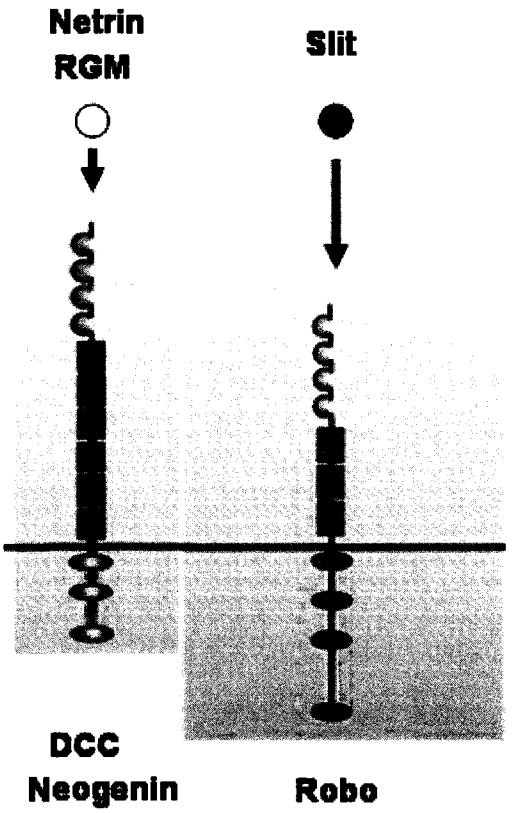
While cortical projection neurons migrate from the ventricular zone radially, most, if not all GABA-ergic interneurons migrate to CP from the MGE and CGE through tangential migration (Anderson et al., 1997; Tan et al., 1998; Xu et al., 2004) (Figure 2). The idea that cells may not only migrate radially to make up the neocortex, but also migrate in the tangential manner came from lineage studies using retroviruses (Austin and Cepko, 1990; Reid et al., 1995; Walsh and Cepko, 1988). These studies showed that clonally related cells not only resided in a simple column as suggested by radial migration, but also existed in a tangentially dispersed manner. These results were further supported by the finding that some cortical neurons disperse tangentially using cortical slices and X-inactivation mosaics to trace clonally related cells (O'Rourke et al., 1992; Tan et al., 1995). Later studies showed that cells originating from subpallium do in fact migrate to the pallium via tangential migration, and that Dlx 1 and 2 were important in this process (Anderson et al., 1997). Dlx1 and 2 are critical for the specification of the cortical interneurons. These GABA-ergic interneurons directed to the neocortex were found to originate from MGE, while LGE gives rise to various cell types in the olfactory bulb and the striatum (Lavdas et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). Recent evidences suggest that diverse subtypes of interneurons have distinct origins (Butt

et al., 2005; Nery et al., 2002; Xu et al., 2004). In vivo fate mapping assay have revealed that interneurons arising from CGE have distinct nuclei from those originated in other regions (Nery et al., 2002). Transplant assays have revealed that parvalbumin and somatostatin positive interneurons arise from the MGE, while calretinin positive interneurons arise from the CGE (Xu et al., 2004). Furthermore, electrophysiological assays have revealed that at E13.5, the MGE gives rise to fast-spiking interneurons, whereas the CGE gives rise to regular-spiking interneurons (Butt et al., 2005). Using fluorescent tracer, DiI, Lavdas and colleagues found that tangentially migrating cells follow two distinct paths (Lavdas et al., 1999). One group of cells migrates through the MZ, while the other group migrates through lower IZ. Before entering the dorsal cortex, it was found through pharmacological experiments that ambient GABA promotes interneurons' entry into the dorsal cortex (Cuzon et al., 2006). Once in the dorsal cortex, these migrating interneurons show multidirectional tangential (MDT) migration where interneurons may migrate rostrocaudally (Tanaka et al., 2006). Studies from our lab indicated a specific population of cells migrating through marginal zone is largely diminished in mice lacking Rb specifically in telencephalon (Ferguson et al., 2005).

Guidance Cues

Tangential migration of GABA-ergic interneurons is thought to be directed by guidance cues classically thought to direct axonal guidance. These guidance cues include Slit-Robo pathway and Netrin-DCC family pathway (Kennedy et al., 1994; Marillat et al., 2002; Rothberg et al., 1988; Serafini et al., 1996; Yuan et al., 1999) (Figure 3). Slit was originally identified in fly as a gene critical for keeping the integrity of longitudinal

Figure 3. **Guidance cues and their receptors**. A schematic representation of guidance cues involved in axon navigation and neuronal migration. DCC mediates the attractive cues from a diffusible ligand, Netrin. While neogenin can also bind Netrin, it interacts with repulsive guidance molecule (RGM) with much higher affinity to mediate its repulsive cue. Robo receptors mediate the repulsive cues from the Slit ligands.



Adapted from Carmeliet and Tessier-Lavigne, Nature Reviews 436:193-200 (2005)

axonal tracts (Rothberg et al., 1988; Rothberg et al., 1990). Later studies revealed that there are three homologs of the *Drosophila Slit* gene in mouse, and they interact with roundabout (ROBO) receptor (Yuan et al., 1999). *Robo* is a transmembrane receptor of immunoglobulin G (IgG) superfamily and mediates Slit's chemorepulsive cue for axonal guidance (Brose et al., 1999; Kidd et al., 1999). To date, three *Slit* genes (*Slit1*, *Slit2*, and *Slit3*) and three *Robo* genes (*Robo1, Robo2,* and *Rig1*) has been identified in mammals (Marillat et al., 2002). Role for Slit/Robo pathway in interneuron migration to the cortex was suggested with an explant assay using ventricular zone explant from the ganglionic eminence (Zhu et al., 1999). The authors found that VZ explant was able to repel GABA-ergic interneurons and blockade of Slit was sufficient to inhibit the repulsive effect. Furthermore, the role for *Slit* genes in migration of interneurons to the olfactory bulb in the rostral migratory stream (RMS), as well as migration of cortical neurons has been well characterized (Hu, 1999; Wu et al., 1999). In adult mice lacking Slit1, the SVZ derived cells that normally migrate rostrally to the olfactory bulb instead migrated caudally to corpus callosum (Nguyen Ba-Charvet et al., 1999). In addition, Sawamoto and coworkers have suggested that concentration gradient of Slit caused by secretion of Slit into the cerebral spinal fluid acts as the directional cue for the RMS (Sawamoto et al., 2006). The involvement of Slit/Robo signaling in interneuron migration, however, have been challenged by studies of mutant mice where normal tangential migration of interneurons from subpallium to pallium was observed in Slit1/Slit2 null mice (Marin et al., 2003). It is possible that Slit/Robo pathway is important for only a subset of migrating interneurons. It was also suggested that Slit/Robo may be important for providing positional cues within CP once the

interneurons reach the pallium (Marin et al., 2003). In a study involving Robo1 knock out mice, it was found that significantly more interneurons migrated to the cortex (Andrews et al., 2006). This phenotype is distinct from Slit knock out mice and the authors suggested that there may be additional ligands, receptors or receptor partners in Slit/Robo pathway (Andrews et al., 2006).

Netrin/DCC is another chemoattractive and chemorepulsive guidance cues involved in axon path finding (Kennedy et al., 1994; Serafini et al., 1996). Although netrin-1 and its receptor, DCC (deleted in colorectal cancer), have been implicated in neuronal migration (Bloch-Gallego et al., 1999; Hamasaki et al., 2001; Yee et al., 1999), it has been suggested that Netrin-1/DCC pathway is not involved in tangential migration of interneurons (Anderson et al., 1999; Marin et al., 2003). Rather, Netrin-1/DCC are critical for ventrally directed tangential migration of guidepost neurons (Kawasaki et al., 2006). Neogenin is a related protein to DCC and it was found to be expressed in neuronal cells during mouse development (Keeling et al., 1997). It was found to mediate Netrin's attractive cue like DCC, but it was recently found that neogenin can also associate with repulsive guidance molecule (RGM) to mediate its repulsive cue for axonal path finding (Rajagopalan et al., 2004). Although both Netrin and RGM bind to neogenin, binding affinity of RGM for neogenin is much higher than that of Netrin (Yamashita et al., 2007). A report by Park and coworkers describes an emerging role for neogenin in cell migration (Park et al., 2004). Park and coworkers found that netrin-neogenin signaling promotes migration of vascular smooth muscle cells during angiogenesis (Park et al., 2004). In addition, neogenin was found to be expressed in both

radially and tangentially migrating neurons, indicating that neogenin may have a role in migration of newly born neurons (Fitzgerald et al., 2006).

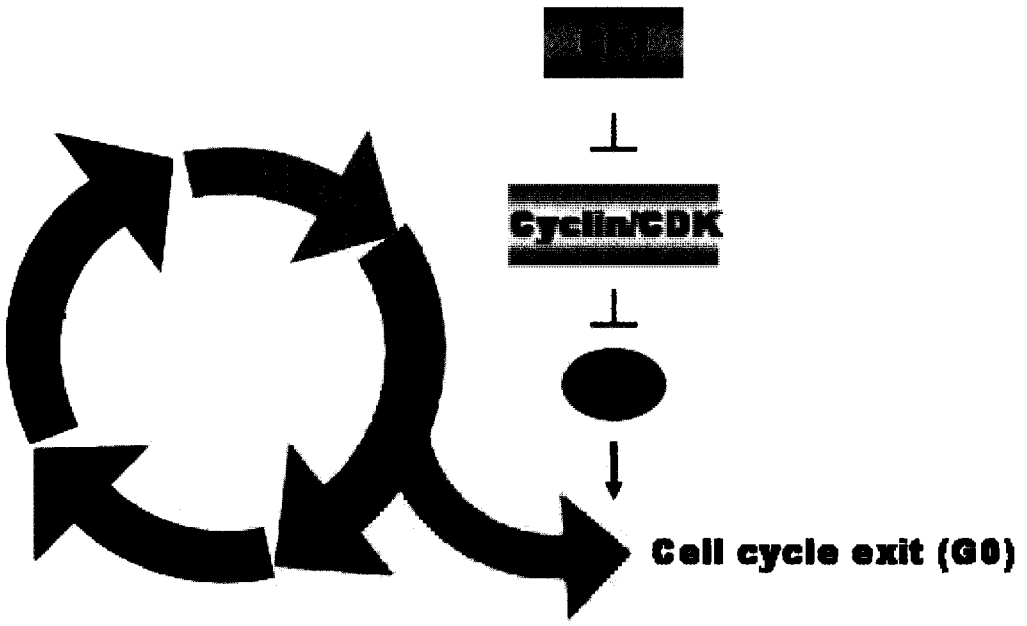
II. Cell cycle

Cell cycle regulation is a key process during development that ensures the maintenance of progenitor pool, generation of correct numbers of various cell types, and correct timing of cell cycle withdrawal for cell differentiation. Retinoblastoma protein (Rb) is considered the regulator of cell cycle, where its activity negatively regulates cell cycle progression (Figure 4). Rb was the first tumor suppressor to be identified and cloned, and acts as a negative regulator of cell cycle. Rb is a phosphoprotein and its activity is regulated through phosphorylation by CDKs (reviewed in (Malumbres and Barbacid, 2005). CDK activities are controlled by cyclins, which activate the CDK upon binding, and CKIs, which inhibit the CDK activity. Hyperphosphorylation of Rb by CDK's results in its inactivation, and allows cells to enter S phase. One of the major targets of Rb are the E2F family of transcription factors. Of those, the primary target of Rb are the so called activating E2Fs.

Regulation of Rb activity in cell cycle

Cyclin dependent kinases (CDK) belong to a family of serine/threonine protein kinases and they regulate Rb activity through phosphorylation (reviewed in (Malumbres and Barbacid, 2005). These CDKs heterodimerize with their activating partners known as cyclins. Best known partners for Rb phosphorylation are cyclin D and cdk4/cdk6, and Cyclin E and cdk2 (Dowdy et al., 1993; Lundberg and Weinberg, 1998). Upon activation

Figure 4. **Cell cycle progression is negatively regulated by Rb.** A schematic representation of cell cycle progression. Rb negatively regulates G1 to S phase transition, and promotes cell cycle withdrawal. The activity of Rb is negatively regulated by cyclin/CDK complexes, and cyclin/CDK, in turn, are negatively regulated by CDK inhibitors (CKI).


CyclinCDK
Cell cycle exit (G0)

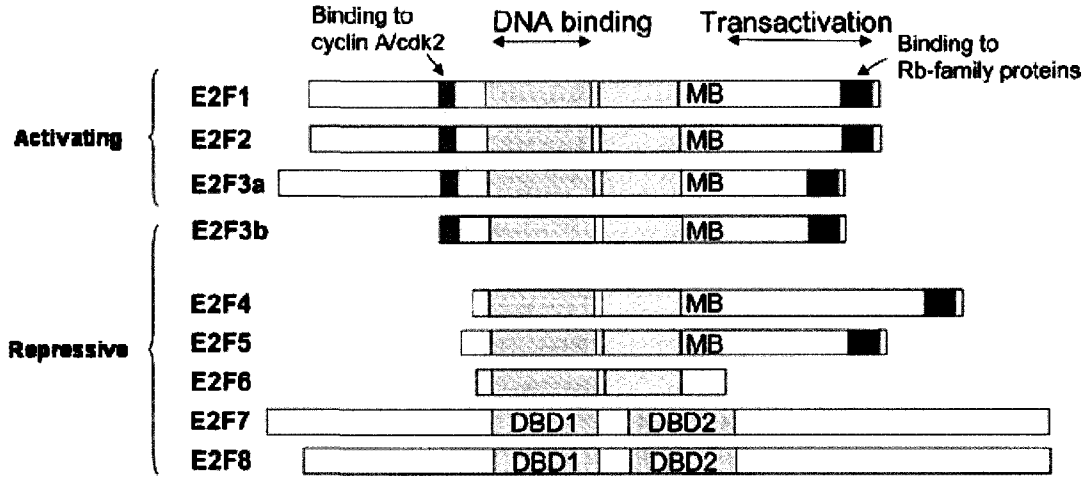
with mitogens, cyclin D/cdk4 or cyclin D/cdk6 complexes phosphorylate Rb in mid G1 phase (Matsushime et al., 1994; Meyerson and Harlow, 1994). Though phosphorylation of Rb by Cyclin D1/cdk4 is an important step in the inactivation of Rb (Connell-Crowley et al., 1997), sequential phosphorylation by Cyclin D1/cdk4 and Cyclin E/cdk2 seem to be required for successful inactivation (Lundberg and Weinberg, 1998). It has also been shown that another cyclin/cdk complex, CyclinC/cdk3, can promote G0 to G1 transition in some human tumor cells (Ren and Rollins, 2004).

While cellular mitogens can activate cyclin/cdk complexes, there are cyclin dependent kinase inhibitors (CKI) that inhibit the activity of the CDK. There are two families of CKIs that inhibit CDK activity. The first family, inhibitors of CDK4 (INK4) family, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ inhibit the activity of cdk4 and cdk6 (Chan et al., 1995; Guan et al., 1994; Hannon and Beach, 1994; Serrano et al., 1993). Immunoprecipitation and *in vitro* phosphorylation experiments revealed that INK4 family specifically binds to and inhibit the activity of cdk4 and cdk6 (Hannon and Beach, 1994; Hirai et al., 1995; Quelle et al., 1995; Serrano et al., 1993). The second family, Cip/Kip family, is comprised of $p27^{Kip1}$ (Polyak et al., 1994), $p21^{Cip1}$ (Harper et al., 1993), and $p57^{Kip2}$ (Lee et al., 1995). Cip/Kip inhibitors are more general inhibitors of cyclins, and they could affect the activities of cyclins A, D, and E (Reviewed in (Sherr and Roberts, 1999). $p21^{Cip1}$ is a potent inhibitor of cyclins A, D, and E, and cdk2 complexes and prevents phosphorylation of Rb by these cyclin/cdk complexes (Harper et al., 1993; Lee et al., 1995; Polyak et al., 1994). Also, overexpression of $p57^{Kip2}$ in mammalian cells was found to completely abolish cell cycle progression (Lee et al., 1995).

E2F transcription factors

The first identified cellular target of Rb was the E2F1 transcription factor (Bandara and La Thangue, 1991; Chellappan et al., 1991; Helin et al., 1993; Shan et al., 1992). E2F was originally discovered as a DNA binding protein capable of activating viral E2 promoter in an E1A dependent manner (Kovesdi et al., 1987). The importance of E2F in cell cycle was highlighted when it was found that overexpression of E2F was sufficient to drive G1 to S phase transition (Johnson et al., 1993). It is now known that E2F binding sites are present not only on E2 promoter, but also on promoters of cell cycle regulators like cyclin A/E, cdc2, Rb, and E2F1, enzymes required for nucleotide synthesis like dihydrofolate reductase, thymidylate synthetase, and thymidine kinase, and proteins involved in DNA replication like cdc6, ORC1, and MCM proteins (reviewed in (Trimarchi and Lees, 2002). The transcriptional activity of E2F1 was found to rely on its obligatory dimerization partner, DP (Krek et al., 1993). Since its first discovery in the early 1990s, there are now 8 E2F members (E2F1-8) that have been identified in the mammalian system (de Bruin et al., 2003a; Di Stefano et al., 2003; Ginsberg et al., 1994; Itoh et al., 1995; Ivey-Hoyle et al., 1993; Lees et al., 1993; Maiti et al., 2005; Trimarchi et al., 1998) (Figure 5). Of those, E2F3, E2F6, and E2F7 loci produce alternatively spliced variants (Dahme et al., 2002; Kherrouche et al., 2004; Leone et al., 2000). E2F3 locus encodes for two E2Fs called E2F3a and E2F3b (Leone et al., 2000), while two and four isoforms of E2F6 have been found in mouse and human, respectively (Dahme et al., 2002; Kherrouche et al., 2004), and two isoforms of E2F7 have been found (Di Stefano et al., 2003). E2F1-6 are among the E2F family members that form heterodimers with their

Figure 5. **The E2F family of transcription factors.** All E2F transcription factors contain DNA binding domain (blue). E2F1, 2, and 3a are known as activating E2Fs while the remaining E2F3b-8 are considered to be repressive E2Fs. E2F1-6 heterodimerize with their obligatory partner, DP, and contain hydrophobic domain required for the dimerization with DP (pink). E2F1-5 contain pocket protein binding domain in their C-terminals, where E2F1-3 solely bind to Rb, E2F4 interact with all three pocket proteins, and E2F5 bind to p130. Marked box domain (MB) is thought to be critical for binding to pocket proteins.



Adapted from DeGregori and Johnson, 2006. 6:739-48

obligatory partner, DP (Wu et al., 1995). In mammals, there are two DP (DP1 and DP2) proteins present, and all possible combinations of E2F/DP exist *in vivo* (Wu et al., 1995).

E2F1-3a are called "activating E2Fs" as they are thought to be crucial for the activation of genes required for G1-S phase transition (Wu et al., 2001). E2F3b-8 are considered as the "repressive E2F" and they are poor activators of E2F promoter and rather, they seem to repress the activation of these promoters. The activating E2Fs are regulated solely by Rb binding as the activation of their target genes is achieved upon the release of E2Fs from Rb (Lees et al., 1993). It has been shown that overexpression of activator E2Fs are capable of driving quiescent cells into S phase (Johnson et al., 1993; Lukas et al., 1996). Although E2F4 associates with Rb, and also has the ability to induce S phase entry in G1 arrested cells upon overexpression, it could only be achieved through simultaneous overexpression of DP1 (Lukas et al., 1996). Conversely, cell proliferation is completely blocked when cells lack all three activating E2Fs (Wu et al., 2001). Recently, it was revealed that removal of the activating E2Fs leads to the activation of p53, the transcriptional activator of $p21^{Cip1}$, causing cell cycle arrest (Sharma et al., 2006; Timmers et al., 2006; Vairo et al., 1995). This evidence suggests that the activating E2Fs are required for the S phase entry. It has been shown that the promoters of E2F responsive genes are occupied by E2F4, p130 and p107 during G0/G1, and they are thought to be repressing the activation of these E2F responsive genes (Takahashi et al., 2000; Vairo et al., 1995). E2F3b associates with Rb in quiescent cells (Leone et al., 2000), and this may explain the role for Rb as transcriptional repressor during quiescence (Adams et al., 2000; Aslanian et al., 2004).

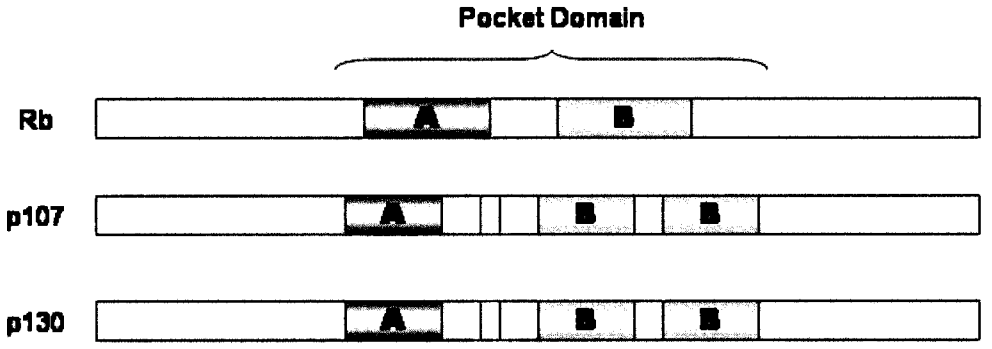
III. Rb

Rb belongs to a family of so called pocket proteins, and the members of this family include Rb, p107, and p130 and they all share two conserved domains called the pocket domains A and B (Reviewed in (Claudio et al., 2002) (Figure 6). Both of the pocket domains are essential for Rb to bind to its target proteins like E2F for repression (Chow and Dean, 1996). The viral oncoproteins and many of the Rb interacting proteins contain a conserved LxCxE motif, through which they bind to Rb (Lee et al., 1998), and Rb interacts with this LxCxE motif through its B domain (Lee et al., 1998). In this pocket domain, there is also an E2F binding site that is required for physical interaction with E2Fs (Flemington et al., 1993; Helin et al., 1993). Rb inactivates E2F activity in two ways: a) it physically inactivates E2F by binding to E2F transactivation domain; b) it recruits HDAC1 (Luo et al., 1998), SWI/SNF factors (Dunaief et al., 1994), PcG (Dahiya et al., 2001), and methyltransferase (Nielsen et al., 2001) to form heterochromatin for repression of E2F responsive genes.

Rb as tumor suppressor

Rb gene was first discovered as the gene mutated in retinoblastoma, a malignant tumor of the retina (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Knudson proposed that retinoblastoma was a result of a two hit model of genetic mutations (Knudson, 1971), and these two mutations leading to retinoblastoma were found to be on human chromosome 13 (Cavenee et al., 1983). The cloning of Rb gene (Friend et al., 1986) and the identification of the mutations in both alleles of Rb gene causing retinoblastoma (Dunn et al., 1988; Fung et al., 1987) supported Knudson's hypothesis.

Figure 6. **The pocket domains of Rb family proteins.** The pocket proteins Rb, p107 and p130 contain pocket domains A and B. The B domain contains E2F binding domain, as well as LxCxE binding domain. p107 and p130 show higher homology to one another than Rb, and they contain two B domains and contain cyclin A/E binding site (yellow) in between A and B.


Pocket Domain
Rb
A
B
p107
A
B
B
p130
A
B
B

Later findings revealed that Rb mutation was common in other forms of cancer like osteosarcoma, lung carcinomas, and bladder, kidney, prostate and breast cancer (Bookstein et al., 1990a; Friend et al., 1986; Fung et al., 1987; Harbour et al., 1988; Horowitz et al., 1990; Lee et al., 1988; T'Ang et al., 1988; Xu et al., 1991). Viral oncoproteins target and inhibit Rb by direct binding. Those include the adenovirus E1A protein, SV40 T antigen (DeCaprio et al., 1988), HPV E7 protein (Dyson et al., 1989), and large T antigen of polymaviruses (Dyson et al., 1990). When introduced into a variety of tumour types, Rb is able to suppress tumourigenicity, arrest cell proliferation, and induce cellular senescence (Bookstein et al., 1990b; Huang et al., 1988; Qin et al., 1992). Together, these findings pointed to Rb as having an important role in cell cycle as a regulator.

The role of Rb in differentiation

Involvement of Rb in differentiation of many cell types was suggested by the finding that there was an increase in mRNA and protein levels of Rb upon induction of differentiation in erythroid cells, muscle cells, neurons, and B-cells (Coppola et al., 1990; Slack et al., 1993). In muscle, Rb was found to directly interact with and required for the activity of MyoD, a basic helix loop helix transcription factor that regulates muscle differentiation (Gu et al., 1993). Using primary fibroblasts lacking Rb, it was found that Rb was not only critical for differentiation, but also for the expression of late differentiation markers such as myosin heavy chain (MHC) (Novitch et al., 1996). A tissue specific knockout for Rb with a use of Myf-5Cre:Rb floxed mice, which specifically knocks out Rb in myofibers showed that these mice die shortly after birth

with almost complete absence of myofibers and increased apoptosis (Huh et al., 2004). Furthermore, removal of Rb after the differentiation of primary myoblasts did not affect the maintenance of their differentiated state (Huh et al., 2004). These studies highlighted that Rb is required for the induction of terminal mitosis, as well as late differentiation genes, but not for the maintenance of differentiated state for muscle fibers.

Rb was suggested to have non-cell autonomous function in erythropoiesis. Studies of the chimeric mice carrying wild type and Rb null cells showed relatively normal development of erythrocyte, with an increase in nucleated erythrocytes only in highly chimeric mice (Maandag et al., 1994). Inhibitor of differentiation protein, Id2, is a known target for Rb, and seems to be critical for erythrocyte maturation. Id2 acts as an antagonist to basic helix loop helix transcription factors involved in differentiation, and binds directly to Rb (Iavarone et al., 1994). Double knock out mice deficient for Rb and Id2 survived to birth and had normal erythrocyte development (Lasorella et al., 2000). A recent study indicated that the defect in erythrocyte development was due to defects in fetal liver macrophages, which are important for the enucleation of erythrocytes upon contact with one another (Iavarone et al., 2004). The authors found that sequestration of Id2 by Rb was required to reverse the inhibitory effect of Id2 on Pu.1, a transcription factor critical for macrophage differentiation (Iavarone et al., 2004). In another report, the role of Rb in the adult hematopoietic stem cells (HSC) was investigated (Walkley and Orkin, 2006). It was found that Rb null HSCs contributed normally to myeloid and lymphoid lineages, providing evidence that Rb is dispensable in terms of lineage specification and self renewal of adult HSC (Walkley and Orkin, 2006).

Role of Rb in Development

As the role for Rb as the cell cycle regulator was becoming apparent, the importance of Rb during mammalian development became clear from the observations that Rb deficient mouse embryos die around E14 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). These Rb-/- mice had defects in erythropoiesis where there were a significant number of immature nucleated erythrocytes compared to the wild type (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Furthermore, these mice exhibited ectopic mitoses and massive cell death in the central nervous system, especially in their hindbrain, and defects in skeletal muscle, erythroid, and lens development. These findings led to investigations for the role of Rb in the development of several tissues. Surprisingly, it was recently reported that many of the defects observed in Rb null mice are not cell autonomous, but rather are secondary effects due to abnormal placenta (de Bruin et al., 2003b; Wu et al., 2003). Rb deficient embryos supplied with wild type placenta were able to survive to term, and apoptosis in the CNS and erythroid development was restored (Wu et al., 2003). In those mice, however, ectopic proliferation persisted in CNS and in developing lens, and increased apoptosis was still observed in the lens (de Bruin et al., 2003b). Additionally, these mice die at birth due to persisting skeletal muscle defects. It was recently found that a specific loss of Rb in trophoblast stem cell population was responsible for the development of abnormal placenta (Wenzel et al., 2007). Together, these studies indicated that there are cell autonomous, and non cell autonomous role for Rb during embryonic development, and also suggests a role for Rb in the maintenance of stem cell population.

Role of Rb in nervous system development

There have been ample evidences which suggest Rb to be an important regulator of neuronal differentiation. As mentioned above, loss of Rb results in massive apoptosis and ectopic mitoses within the nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In addition, Rb is highly expressed from E9.5 during neurogenesis (Jiang et al., 1997). Studies using embryonal carcinoma cells revealed that Rb is up-regulated in these cells when induced to differentiate into neuronal tissues *in vitro* (Slack et al., 1993). In other experiments, Rb was found to be critical for the survival of differentiating cultures of P19 cells, where E1A expression, and thus Rb inactivation, caused induction of apoptosis in these cells (Slack et al., 1995). The importance of Rb in neuronal survival was also highlighted in experiments where induction of apoptosis was seen with expression of SV40 T antigen in cerebellar Purkinje neurons (Feddersen et al., 1995). Similarly, conditional knockout of Rb and p107 in cerebellum caused inappropriate cell cycle entry and increased apoptosis in cerebellar granule cells (Marino et al., 2003). In addition, inactivation of Rb family proteins by E1A expression in cortical progenitor cells induced to differentiate resulted in these neurons undergoing apoptosis (Slack et al., 1998). Furthermore, ablation of Rb in post mitotic neurons did not affect their survival. These studies demonstrated not only that Rb is required for the survival and successful terminal mitosis of the neuron, but there is a specific time at which Rb is required for survival (Slack et al., 1998).

In order to study the role of Rb in retinal development, Rb(lox) gene was selectively inactivated using retroviral vector to introduce Cre recombinase in the retina to study the role of Rb in retinal development. In this study, it was found that Rb is

required in a cell autonomous manner for proper exit from the cell cycle in retinal progenitor cells and for rod development (Zhang et al., 2004). Using a conditional knock out approach, Rb was removed in retinal progenitors in p107 deficient mice. It was found that loss of Rb and p107 does not affect proliferation or precursor specification, but resulted in ectopic proliferation of these precursors (Chen et al., 2004). Another study also using conditional Rb knockout in mouse retina showed inappropriate S-phase entry and elevated apoptosis (MacPherson et al., 2004). During post natal development of the retina in these mice, more widespread apoptosis was observed and the loss of photoreceptors and bipolar cells was observed (MacPherson et al., 2004).

Studies using chimeric mice containing Rb deficient cells also revealed that although ectopic mitoses were observed in the brain, abnormal apoptosis was not observed (Lipinski et al., 2001). Conditional knock out of Rb in the CNS also resulted in inappropriate entry into S phase but no elevated apoptosis (MacPherson et al., 2003). Moreover, neural precursor cells cultured from Rb null mice can differentiate and survive (Callaghan et al., 1999). These results added further evidence that massive apoptosis observed in Rb null mice are due to hypoxia, and not the result of the loss of Rb (Wu et al., 2003). Telencephalon-specific Rb deficient mice generated in our lab has shown survival, ectopic proliferation of neural precursor cells, enlarged brain size, and defects in radial and tangential migration (Ferguson et al., 2005; Ferguson et al., 2002). Telencephalon specific Rb-deficient mice survived until birth and exhibited ectopic mitoses where proliferating neurons were found outside of ventricular zones (Ferguson et al., 2002). Conditional Rb deficient mice also showed neurons expressing Tbr1 and SCG10 appearing in the intermediate zone while it is only be expressed within the CP in

the wild type (Ferguson et al., 2005). BrdU incorporation assay also revealed that conditional Rb knockout showed delayed radial migration compared to the wild type (Ferguson et al., 2005). Conditional Rb deficiency also affected survival of Cajal-Retzius neurons in the marginal zone where there was a significant reduction in number of Cajal-Retzius neurons in Rb deficient mice (Ferguson et al., 2005). Tangential migration was affected in conditional Rb deficient mice, where there was a reduction in the number of GABA-ergic interneurons taking the marginal zone migratory route (Ferguson et al., 2005).

IV. E2F1

While having an important role in cell cycle progression as a member of the activating E2Fs, E2F1 has a unique role in inducing cell death, and functions as a tumor suppressor. E2F1's role as a tumor suppressor was first hinted from the fact that spontaneous cancer formation were observed some tissues in E2F1 deficient mice (i.e. reproductive tract sarcoma, lung tumor, and lymphoid tumor (Field et al., 1996; Yamasaki et al., 1996). The authors also noted that E2F1 was important for causing cell death in T-lymphocytes as increased number of these cells was observed in E2F1 null mice (Field et al., 1996). In addition, experiments involving overexpression of E2F1 in fibroblast cells have been shown to cause cell death (Shan and Lee, 1994; Wu and Levine, 1994). The ability of E2F1 to induce apoptosis is thought to be regulated by Rb as there is an E2F1 specific binding site at C-terminal domain of Rb, and this interaction is thought to be sufficient for the regulation of apoptosis by E2F1 (Dick and Dyson,

2003). Since the discovery of the pro-apoptotic activity of E2F1, many experiments have been done to elucidate the mechanism by which E2F1 induces cell death.

E2F1 dependent cell death

It is now known that E2F1 is capable of inducing cell death in a p53 dependent and independent manner (Holmberg et al., 1998; Wu and Levine, 1994). E2F1 promotes p53 mediated cell death by directly upregulating proapoptotic cofactors of p53 like ASPP1, ASPP2, JMY and TP53INP1 (Hershko et al., 2005). E2F1 can promote p53 stabilization by ARF expression, where ARF inhibits the activity of MDM2, which causes ubiquitination of p53 for its degradation (Bates et al., 1998; Kubbutat et al., 1997). E2F1/p53 mediated cell death can also occur in ARF independent manner. In this case, p53 stabilization is promoted through Chk2 and ATM, both of which are involved in the DNA check point pathway as response to genotoxic effects such as double stranded break (Berkovich and Ginsberg, 2003). p53 independent induction of cell death by E2F1 is thought to be mediated through p73, a p53 homologue (Stiewe and Putzer, 2000). p73 induces cell death by upregulation of proapoptotic gene, PUMA, which causes cytochrome c release via BAX translocation to the mitochondria (Melino et al., 2004). E2F1 can also transactivate apoptosis protease activating factor 1 (Apaf1) as shown by chromatin immunoprecipitation assay (Furukawa et al., 2002). Recent experiments have now shown that in addition to Apaf1, E2F1 is capable of upregulating proapoptotic BH3 proteins PUMA, Noxa, Bim, and Hrk/DP5 by direct translation (Hao et al., 2007; Hershko and Ginsberg, 2004). In a recent report, the induction of apoptosis by E2F1 was shown to require an obligatory binding partner, Jab1, as cells deficient in Jab 1 were unable to undergo E2F1 mediated cell death (Hallstrom and Nevins, 2006).

The ability of E2F1 to cause cell death has been documented in neuronal cells as well (Fortin et al., 2004; Giovanni et al., 2000; O'Hare et al., 2000; Park et al., 2000). It has been shown that proapoptotic protein SIVA is also a direct transcriptional target of E2F1 and p53 in neurons, and is upregulated in stroke injury in mice, as well as DNA damage induced by camptothecin in primary neurons (Fortin et al., 2004). Neuronal cell death caused by DNA damage promotes CDK activity, leading to the inactivation of Rb, and free E2F activity. Blockade of E2F activity by dominant negative DP1 protected cortical neurons against cell death (Park et al., 2000). Neuronal cell death caused by beta amyloid evoked toxicity and potassium deprivation are caused by E2F1 in a p53 independent manner. (Giovanni et al., 2000; O'Hare et al., 2000). E2F1-deficient neurons were significantly protected both against B-Amyloid evoked cell death (Giovanni et al., 2000) and cell death caused by potassium (K+) deprivation(O'Hare et al., 2000). Consistent with the notion that E2F1 is an important factor in neuronal cell death, E2F1 deficient mice have been shown to be protected against focal ischemic insult (MacManus et al., 2003). In terms of regulation by Rb, consistent with the notion that E2F1 has a pro-apoptotic property, it has been shown that additional loss of E2F1 extends survival of Rb null embryos (Tsai et al., 1998). In Rb/E2F1 double null mice, the survival of the embryo was extended to E17 from E15, which was seen in Rb null mice (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Moreover, the up-regulation of p53 was also suppressed in double null mutants (Tsai et al., 1998). These evidences point to E2F1 as a key target for Rb in mouse development.

V. Research Objectives

To date, the role of downstream targets for Rb and Rb interacting partners during cortical development have not been addressed. Therefore, we aimed to: 1) Characterize the guidance molecules involved in neuronal migration to assess whether Rb signaling pathway was involved in their expression. 2) Assess to what extent E2F1 is a downstream target for Rb during cortical development.

Hypothesis 1

Previous work in our lab has demonstrated that Rb deficiency leads to defects in tangential migration of GABA-ergic cortical interneurons (Ferguson et al, 2005). Based on the findings that migration of these interneurons are directed by migration cues, ***we hypothesized that Rb signaling pathway mediates GABA-ergic interneuron migration through regulation of guidance molecules.***

To address this hypothesis, the objective was **to characterize the expression of guidance molecules involved in neuronal migration to determine whether Rb deficiency altered their expression pattern.**

In order to test this, we have generated telencephalon specific Rb knockout mice. We then examined the expression pattern of Netrin-1, DCC, Neogenin, Slit1, Slit2, Slit3, Robo1 and Robo2 in developing telencephalon using *in situ* hybridization.

Hypothesis 2

We have previously reported that telencephalon specific deficiency of Rb leads to ectopic proliferation, defective laminar patterning, defect in survival of Cajal-Retzius

neurons, and defective tangential migration of GABA-ergic interneurons (Ferguson et al, 2002; Ferguson et al, 2005). According to our previous report showing that activities of E2F1 and E2F3 are deregulated in Rb deficient neural precursor cells (Callaghan et al., 1999), ***we hypothesized that Rb regulates cortical development through E2F1 transcription factor.***

To address this hypothesis, the objectives were as follows:

1) To determine whether deregulated activity of E2F1 is responsible for ectopic proliferation. To test this, we have generated a compound knock out of telencephalon specific Rb-/- and E2F1-/- mice. We then used a 2hr BrdU incorporation assay to determine whether newly born neurons undergo ectopic proliferation in the double knock out. If deregulated activity of E2F1 due to Rb deficiency is causing ectopic proliferation, removal of E2F1 in telencephalon specific Rb deficient mice should correct this phenotype.

2) To determine whether deregulated activity of E2F1 is responsible for aberrant laminar patterning. To test this, we used cortical sections of telencephalon specific Rb-/- E2F1-/- double knock out mice for *in situ* hybridization for Tbr1 and cresyl violet staining. If deregulated activity of E2F1 due to Rb deficiency is causing aberrant laminar patterning, removal of E2F1 in telencephalon specific Rb deficient mice should correct this phenotype.

3) To determine whether deregulated activity of E2F1 is responsible for decreased survival of Cajal-Retzius neurons. To test this, we immunostained cortical sections of telencephalon specific Rb-/- E2F1-/- double knock out mice against reelin, a marker for Cajal-Retzius neurons. We then quantified reelin positive cells in the

marginal zone. If deregulated activity of E2F1 due to Rb deficiency is causing the decrease in survival of Cajal-Retzius neurons, removal of E2F1 in telencephalon specific Rb deficient mice should restore the number of reelin positive cells to control level.

4) To determine whether deregulated activity of E2F1 is responsible for defective tangential migration of GABA-ergic interneurons. To test this we immunostained cortical sections of telencephalon specific Rb-/- E2F1-/- double knock out mice against calbindin, which marks a subset of tangentially migrating GABA-ergic interneurons. We then quantified the number of calbindin positive cells migrating through the marginal zone. If deregulated activity of E2F1 due to Rb deficiency is causing defective tangential migration, removal of E2F1 should correct the number of cells migrating through the marginal zone to control level.

Materials and Methods

Mice

Floxed Rb-F19 mice generated previously (Vooijs et al., 1998) were obtained from the Berns Laboratory (The Netherlands Cancer Institute). Germline E2F1 knockout mice were obtained from the Jackson Laboratory (Bar Habor, ME). These germline E2F1 knockout mice were generated previously (Field et al., 1996) and were maintained on a C57Bl/6 background. Mice were backcrossed to FVB/N genetic background over 6 generations and were maintained on this genetic background. Germline E2F1 knockout mice were generated by crossing heterozygous (E2F1+/-) mice. Telencephalon specific Rb knockout mice were generated by crossing Floxed Rb-F19 mice to Foxg-1 Cre mice (Hebert and McConnell, 2000), as reported previously (Ferguson et al, 2002; Ferguson et al, 2005) and were maintained on a FVB/N background. Telencephalon specific Rb: germline E2F1 double knockout mice were generated by crossing Rb flox: germline E2F1 deficient mice (Rbf-/-:E2F1-/-) and mice triple heterozygous for Rb flox:E2F1:Foxg-1 Cre (Rbf+/-:E2F1+/-:Cre+/-). For embryonic time points, the time at which plug had been identified was considered embryonic day (E) 0.5. All experiments were approved by the University of Ottawa Animal Care facility's ethics committee, which adheres to the Guidelines of the Canadian Council on Animal Care.

DNA Extraction

For DNA extraction, tail clippings from adult mice or embryonic tissue was dissolved in 500μL of extraction buffer (100mM Tris, 5mM EDTA, 0.2% SDS, 200mM NaCl dissolved in dH_2O, pH8.0) mixed with 10ng of proteinase K (Gibco, 25530-031)

for overnight at 55°C. Phenol/Chloroform extraction for DNA was then carried out. First, 500μL of phenol: chloroform was added to the dissolved sample and each sample was shaken and incubated at room temperature for 10min. Samples were then centrifuged for 5min at 12000 rpm at room temperature. The upper aqueous layer was transferred to a fresh eppendorf tube and 500μL of ice cold isopropanol was added. The samples were mixed by inversion and centrifuged for 5min at 12000 rpm. The aqueous layer was poured out and the samples were washed with 70% ethanol and dried on a heat block set at 55°C. Isolated DNA samples were dissolved in 50μL of TE buffer (10mM Tris, 1.0mM EDTA, dH_2O, pH8.0) and stored at -20°C.

Polymerase Chain Reaction (PCR)

Mice were genotyped for E2F1 using previously published protocol by Field and colleagues (Field et al, 1996) and genotyped for Rb flox and Cre, according to the previously published protocol by Ferguson and coworkers (Ferguson et al, 2002; 2005). The primers used for E2F1 were: 5'-GGATATGATTCTTGGACTTCTTGG-3' (Forward, WT and mutant), 5'-CTAAATCTGACCACCAAACGC-3' (Reverse, WT), and

5'- CAAGTGCCAGCGGGGCTGCTAAAG-3' (Reverse, mutant). Two separate reactions were carried out to detect wild type gene and mutant gene. The following condition was used for PCR for E2F1 gene: 94°C for 5min, 94°C for 35sec, 57°C for 35sec, 72°C for 1min, 72°C for 5min for 27 cycles. Genotyping for Rbflox and Cre were carried out according to previously published protocol (Ferguson et al, 2002, Ferguson et al, 2005). The primers used for genotyping for Rbflox were: Rb18 5'-

GGCGTGTGCCATCAATG-3' and Rb 19 5'-AACTCAAGGGAGACCTG-3'. The condition used for PCR for Rbflox gene were: 94°C for 5min, 94°C for 30sec, 58°C for 30sec, 72°C for 50sec, 72°C for 10min for 30 cycles. The primers used for genotyping for Cre were: 5'-TGACCAGAGTCATCCTTAGCG-3' and 5'-AATGCTTCTGTCCGTTTGCC-3'. The conditions used for PCR for cre gene were: 94°C for 2min, 94°C for 1min, 56°C for 1min, 72°C for 1min30sec, 72°C for 5min for 30 cycles. PCR products for Rbflox and E2F1/Cre were run on 2% and 1% agarose gel, respectively, containing ethidium bromide (Sigma, E1510-10ML). For E2F1, 170bp band was observed for the wild type allele, and 230bp band was observed for the mutant allele. For Rbflox, 650bp band was observed for wild type allele and 750bp band was observed for the floxed allele. DNA bands were visualized with and AlphaImager 2200 (Alpha Innotech Corporation).

Tissue fixation and cryoprotection

Pregnant females were sacrificed by injection of sodium pentobarbital (900mg/kg body weight). Embryos were dissected out of the pregnant females, and fixed in a 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline (PBS), pH 7.4 overnight. Fixed embryos were then cryoprotected by saturating in 12%, 16% and 22% sucrose dissolved in PBS in a sequential order at 4°C. Tissues were then embedded in OCT (TissueTek 4583) and frozen on dry ice. Frozen tissues were sectioned into 14μm sections at -20°C using a cryostat (Microm, HM500) and collected on Superfrost Plus® slides (Fisher Scientific, 12-550-15) and stored at -80°C.

***In situ* hybridization**

Non-radioactive *in situ* hybridization using digoxygenin probe was carried out as previously described (Wallace and Raff, 1999). Tbr1 (Bulfone et al., 1995), neogenin (Masu and Masu, 1996), DCC, Netrin1 (Serafini et al, 1996), Robo1, Robo2, Slit1, Slit2, and Slit3 probes were obtained from Dr. Elke Stein (Yale University, CT). Probes were labeled using DIG RNA labeling kit (Roche, 1277073). Labeled riboprobes were extracted by adding 2.5µL of 4M LiCl and 75µL of prechilled ethanol. The mixture was then incubated at -80°C for 30min. Samples were then centrifuged for 15min at 13000 x g. After decanting the supernatant, the samples were washed with 50µL of ethanol and centrifuged for 5min at 13000 x g. The pellets were then dried, re-suspended in 50µL of DEPC water and stored at -80°C.

The *in situ* hybridization was carried out over the course of three days. On day 1, probes were diluted in a range of 1:500 to 1:1000 with hybridization buffer (1X SSC (0.15M sodium chloride and 0.015M trisodium citrate), deionized formamide, 10% dextran sulfate, 1mg/mL rRNA, 1X Denhardt's, dH_2O), and denatured for 10min at 70°C. The diluted probes were then applied to each slide and the slides were covered with a cover slip. Slides were incubated in an incubation box lined with Whatman paper soaked with 50% formamide/10X salt solution at 65°C overnight.

On day 2, the slides were washed with a fresh wash buffer (1X SSC, 50% formamide, 0.1% Tween-20, dH_2O) three times for 30min each at 65°C with rocking. Samples were then saturated in 1X MABT (50 mM maleic acid, pH 7.5, 250 mM NaCl. and 0.1% Tween 20) for 4X at 20min at room temperature. Sections were then blocked

with a blocking solution (20% heat-inactivated sheep serum, 2% blocking reagent dissolved in 1X MABT) in a humidified slide box for 1hr. The blocking solution was aspirated and anti-DIG antibody (BM, 1093274) diluted 1:1000 in blocking solution was applied to the slides. The slides were then incubated in a humidified slide box overnight at room temperature.

On day 3, samples were rinsed in MABT for 20min at room temperature with rocking for 5 times. Slides were then washed twice with pre-stain solution (100mM NaCl, 50mM $MgCl_2$, 100mM Tris pH 9.0, 0.1% Tween-20) for 10min at room temperature. Slides were then incubated in staining solution (100mM NaCl, 50mM $MgCl_2$, 100mM Tris pH 9.0, 0.1% Tween-20, 10% PVA, 4.5µL/mL NBT (4-nitro blue tetrazolium chloride), 3.5µL/mL BCIP (x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate), dH_2O) in the dark at room temperature for several hours until the reaction takes place. The staining reaction was stopped by washing the slides with 1X PBS for 10min at room temperature with rocking several times. Cover slips were then mounted with 1:3 glycerol:PBS and sealed with a clear nail polish. Slides were stored at 4°C.

BrdU labeling and Immunohistochemistry

To assess ectopic proliferation, pregnant females were injected with 50µg BrdU/g body weight 2 hours prior to sacrifice. For the detection of BrdU, sections were first rehydrated in 1X PBS for 5min at room temperature. Slides were then incubated in pre-warmed 2N HCl for 10min at 37°C. Samples were then incubated in 0.1M $Na_2B_4O_7$ pH8.5 solution for 10min at room temperature. Slides were then rinsed three times with 1X PBS for 5min at room temperature. Slides were then incubated with mouse

monoclonal anti-BrdU (BD Biosciences, 347580) dissolved 1:50 in 1X PBS overnight at room temperature. The next day, slides were washed three times with 1X PBS for 10min each at room temperature. The slides were then incubated for 1hr in Goat anti-mouse Cy3 (Molecular Probes) dissolved in 1X PBS at 1:500 dilution. Slides were then washed three times with 1X PBS for 10min at room temperature.

For calbindin and reelin, an antigen retrieval was first performed. Sections were rehydrated in 1X PBS for 5min at room temperature. Slides were incubated in the retrieval solution (10mM Sodium Citrate, pH6.0 in dH_2O) and were placed in a microwave for 30seconds, or until boil, and then cooled on ice for 5min. The process was repeated three times. The slides were then washed in 1X PBS three times for 5min at room temperature. Samples were then immunoblocked with a block solution (5% normal goat serum, 0.1% BSA, 0.1% Triton-X 100 in 1X PBS) for 1 hr at room temperature. The samples were then incubated in mouse monoclonal anti-Reelin G10 (1:500, Calbiochem, #553731) or rabbit polyclonal anti-calbindin D28 (1:1000, Chemicon, AB1778) diluted in immunoblock solution overnight in a humidified slide box at room temperature. The next day, samples were washed three times with 1X PBS for 10min at room temperature. Samples were then incubated with secondary antibodies for 1hr at room temperature. The secondary antibodies used were goat anti-rabbit 488 Alexa (Molecular Probes) for calbindin and goat anti-mouse 488 Alexa (Molecular Probes) for reelin. Samples were then washed three times with 1X PBS for 10min at room temperature. For all immunohistochemistry, cover slips were mounted with 1:3 glycerol:PBS and sealed with a clear nail polish.

Cresyl Violet Staining

For cresyl violet staining, samples were incubated in a 1:1 0.2% cresyl violet:dH_2O solution for 10min at room temperature. The slides were then washed under running tap water for 10min. The sections were then dehydrated in sequential manner in dehydration baths (60-100% ethanol) for 30sec each. Finally, the sections were incubated in xylene solution and cover slip was mounted with Permouont (Fisher, SP15-100).

Microscopy

All sections were examined using a Zeiss Axioskop 2 microscope. Standard fluorescence was used for immunohistochemistry and brightfield/darkfield settings were used. Objectives used were X10 0.17 or X20 0.17 NA objectives. Images were obtained using a Sony Power HAD 3CCD camera using a Northern Eclipse software. Figures were assembled using Adobe Photoshop CS2 software and corrections for brightness and contrast were made equally for all images.

Quantification of labeled cells

BrdU labeled cells were counted over 650μm along the dorsal cortex, and expressed as the mean of at least three matched sections per embryo. Quantifications were done in three regions: ventricular/subventricular zone, intermediate zone, and cortical plate. Reelin positive cells as well as total cells residing in the marginal zone were counted from dorsal and temporal cortex, each over 500μm along the marginal

zone. The number of cells were quantified and expressed as the mean of at least four matched sections from an embryo. Calbindin positive cells migrating through the marginal zone were counted from a 500μm along the marginal zone, starting from the dorsal most region where calbindin positive cell has migrated. The number of cells were quantified and expressed as the mean of at least four matched sections from an embryo.

Statistical Analysis

All error values are expressed in standard error of the mean (SEM). Significant difference for numbers of BrdU labeled cells, reelin positive cells, total cells in the marginal zone, and calbindin positive cells were assessed using analysis of variance (ANOVA) with a post hoc Tukey test. The statistical significance was assessed at values of $P<0.05$.

Results

I. Role of Rb in the expression of guidance molecules

Robo2 is ectopically expressed in telencephalon specific Rb null mice

During cortical development, interneurons arising from the medial ganglionic eminence (MGE) tangentially migrate through two distinct paths, where one group of neurons travels through the marginal zone, while the other group travels through the intermediate zone (Lavdas et al., 1999). Previously, our group has reported that tangentially migrating, Calbindin positive interneurons are largely diminished from the marginal zone in the telencephalon specific Rb knockout mice (Ferguson et al, 2005). Using slice co-culture assay, we also reported that the defects in the tangential migration of these interneurons were cell autonomous (Ferguson et al, 2005). A classical axon guidance cue, Slit-Robo pathway, where Slit is the secreted ligand and Robo is the receptor for Slit (Brose et al., 1999; Kidd et al., 1999), has been implicated in directing interneuron migration as mediating repulsive cues (Zhu et al., 1999).

Since there was defective tangential migration in conditional Rb knockout mice, we asked whether Rb plays a role in regulation of the expression of guidance molecules. To address this question, I have performed *in situ* hybridization using DIG-labeled Slit1, Slit2, Slit3, Robo1, and Robo2 riboprobes on cortical slices from telencephalon specific Rb knock out mice and Rb(floxed)+/-:Cre+/- double heterozygous littermate embryos as controls. In developing telencephalon, Slit1 is expressed in cortical plate, as well as MGE and LGE (Yuan et al., 1999). In contrast to Slit1, Slit2 and Slit3 are weakly expressed in the developing telencephalon (Yuan et al, 1999). *In situ* hybridization of Slit1 on cortical slices from E14 embryos showed an expression of Slit1 gene in the

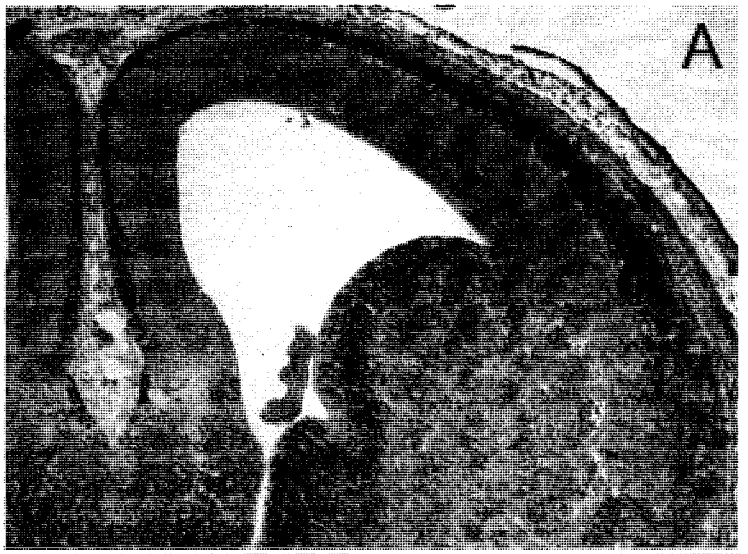
cortical plate, the ventricular zone of the ganglionic eminences, and weakly in the ventricular zone of the developing cortex, as similar to previously reported expression pattern (Yuan et al, 1999) (Figure7). *In situ* hybridization using Slit2 riboprobes on cortical slices from E15 embryos revealed a weak expression of Slit2 gene in the cortical plate and ventricular zone of the developing cortex (Figure 8). *In situ* hybridization using Slit3 riboprobes on cortical slices from E15 embryos revealed a weak expression of Slit3 gene in the cortical plate and ventricular zone of the cortex and ganglionic eminences (Figure 9). There was no difference in the expression of Slit1, Slit2, and Slit3 genes between conditional Rb knock out embryos and littermate control embryos (Figures 7-9 n=3).

Robo1 is expressed in the cortical plate at E15.5 (Yuan et al, 1999). *In situ* hybridization using Robo1 riboprobe on cortical slices from E15.5 embryos showed a similar expression pattern, but with a weak expression in the intermediate zone of the ganglionic eminence (Figure 10). There was no observable difference between the telencephalon specific Rb knock out embryo and littermate control in the expression of Robo1 in the developing telencephalon (Figure 10, n=3). There is no report on the expression pattern of Robo2 in the telencephalon at E15.5; however, it has been reported that Robo2 is expressed in the cortical plate at E17 (Shu and Richards, 2001). In the cortical plate of the E15.5 embryos, reduced expression of Robo2 in the cortical plate, as well as a strong expression in the subventricular zone was seen (Figure 11). In the conditional Rb knock out mice, there was also an ectopic expression of Robo2 transcript in the ventral area of the developing cortical plate compared to the littermate control (Figure 11, arrow, n=3). The results obtained suggest that Rb does not regulate the

Figure 7. **Slit1 expression is not affected in the absence of Rb.** *In situ* hybridization was performed on coronal section from E14 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Slit1 riboprobe. An expression of Slit1 gene was observed in the cortical plate, ventricular zone of ganglionic eminences, and weakly in the ventricular zone of the dorsal cortex (A and B). No difference was observed in the expression of Slit1 transcript between control and conditional Rb knockout (A and B). Similar results were obtained in three independent experiments. Bar: 500μm.

Slit 1

Control



cond. Rb KO

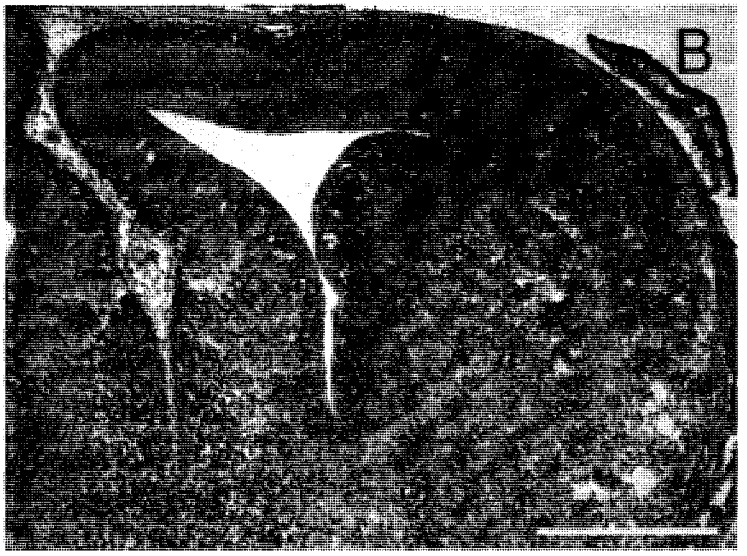
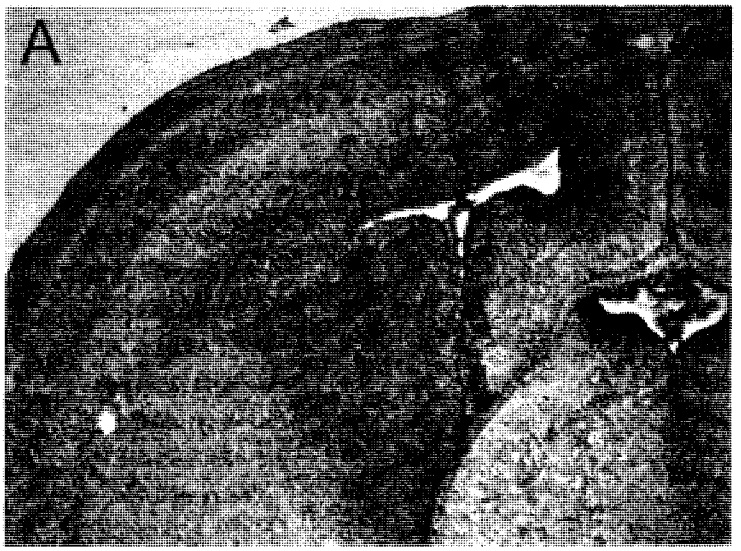


Figure 8. **Slit2 expression is not altered in the absence of Rb.** *In situ* hybridization was performed on coronal section from E15 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Slit2 riboprobe. A weak expression of Slit2 gene was observed in the cortical plate and ventricular zone of the dorsal cortex (A and B). No difference was observed in the expression of Slit2 transcript between control and conditional Rb knockout (A and B). Similar results were obtained in three independent experiments. Bar: 500μm.

Slit 2

Control



cond. Rb KO

Figure 9. **Slit3 expression is not altered in the absence of Rb.** *In situ* hybridization was performed on coronal section from E15 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Slit3 riboprobe. A weak expression of Slit3 gene was observed in the cortical plate, ventricular zone of the dorsal cortex and ganglionic eminences (A and B). There was no difference in the expression of Slit3 transcript between control and conditional Rb knockout. Similar results were obtained in three independent experiments. Bar: 500μm.

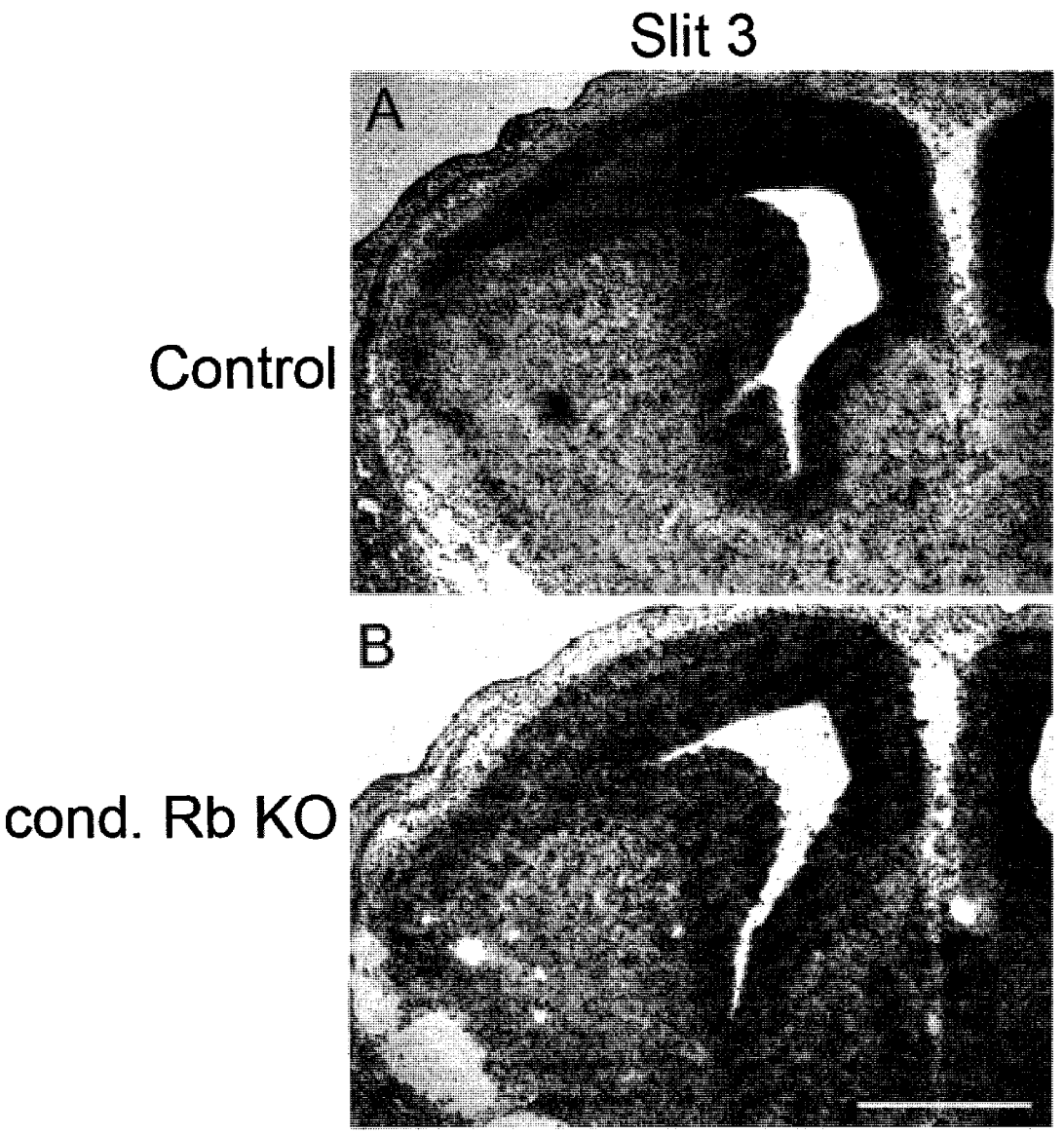

Slit 3
A
Control
B
cond. Rb KO

Figure 10. **Robo1 expression is not altered in the absence of Rb.** *In situ* hybridization was performed on coronal section from E15.5 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Robo1 riboprobe. Expression of Robo1 was seen in the developing cortical plate (A and B). There was no observable difference in the expression of Slit1 transcript between control and conditional Rb knockout. Similar results were obtained in three independent experiments. Bar: 500μm.

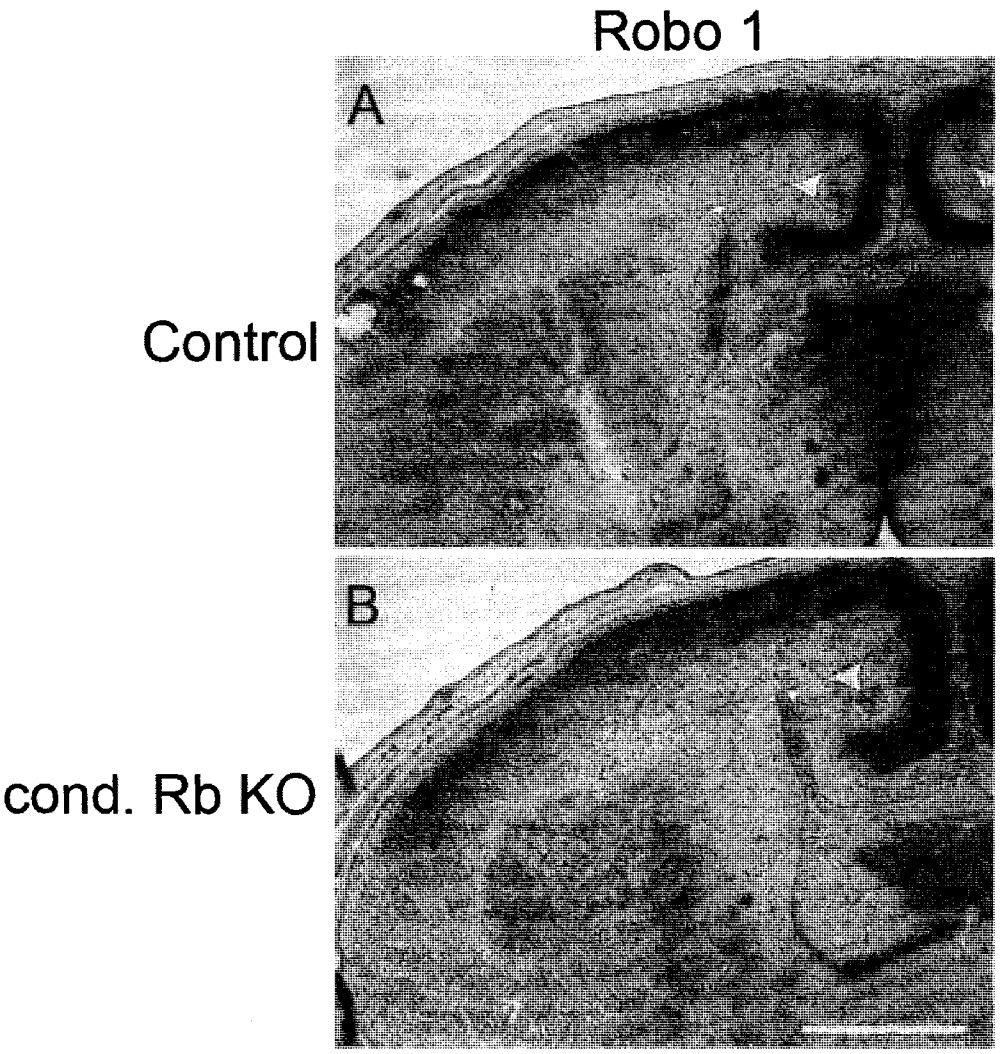
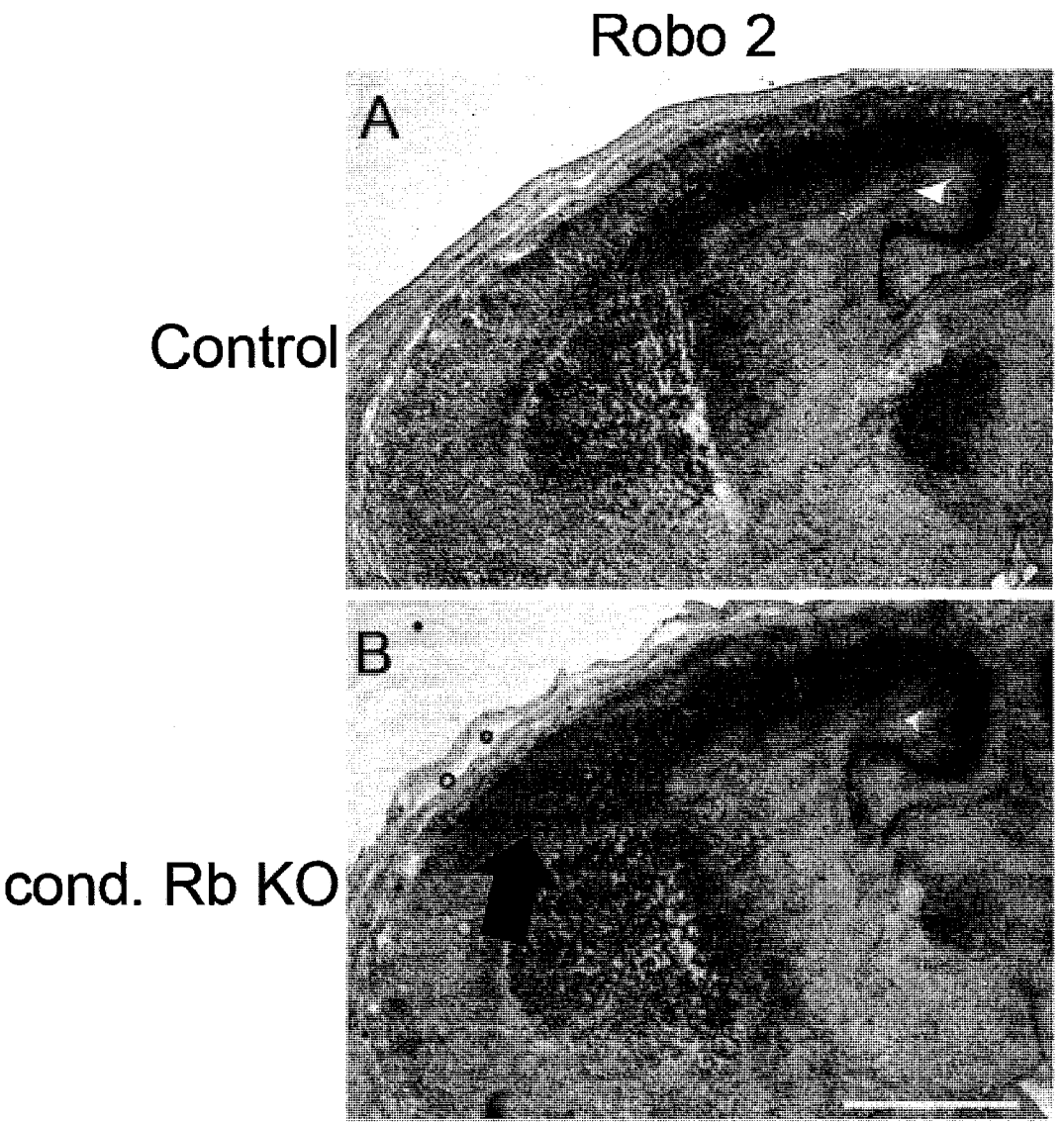

Robo 1
A
Control
B
cond. Rb KO

Figure 11. **Robo2 gene is ectopically expressed in the absence of Rb.** *In situ* hybridization was performed on coronal section from E15.5 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Robo2 riboprobe. An expression of Robo2 was seen in the dorsal most region of developing cortical plate, as well as in the subventricular zone of the dorsal cortex (A and B). In addition, an ectopic expression of Robo2 gene was observed in a ventral region of cortical plate (B, arrow) compared to the control (A). Similar results were obtained in three independent experiments. Bar: 500μm.


Robo 2
A
Control
B
cond. Rb KO

expression of Slit1, Slit2, Slit3, and Robo1, but may regulate the expression of Robo2 in a selected population of cells residing in the dorsal region of developing cortical plate, or a subset of interneurons migrating through that region. This has led us to question whether Rb pathway may be regulating the expression of Netrin-DCC, another pathway involved in axonal path finding and cell migration (Fitzgerald et al., 2006; Kennedy et al., 1994; Park et al., 2004).

Expression of Neogenin is upregulated in telencephalon specific Rb null mice

Netrin-DCC pathway is a classical chemoattractive guidance cue for axonal path finding (Kennedy et al., 1994; Serafini et al., 1996). For this reason, we have also asked whether Rb signaling pathway regulates the expression of Netrin1, DCC, and Neogenin, a related protein to DCC (Keeling et al., 1997). To address this, I performed *in situ* hybridization using DIG-labeled riboprobes for Netrin1, DCC, and neogenin. In developing telencephalon, Netrin-1 is expressed in the ganglionic eminences lining the ventricular surface (Serafini et al, 1996). *In situ* hybridization using Netrin1 riboprobe revealed a similar expression profiles to that in previous reports (Figure 12). At E15, there was no observable difference in the expression of Netrin1 between conditional Rb knockout mice and their littermate controls (Figure 12, n=3). DCC, the receptor for Netrin1, is expressed in regions surrounding the cortical plate in developing cortex, forming a banding pattern (Gad et al., 1997). *In situ* hybridization using DCC riboprobe on cortical sections from E14 Rb knockout mice and their littermate controls revealed an expression of DCC in the cortical plate (Figure 13). There was no observable difference in the expression of DCC between conditional Rb knock out and the littermate control

Figure 12. **Netrin1 expression is not altered in the absence of Rb.** *In situ* hybridization was performed on coronal section from E15 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Netrin1 riboprobe. Expression of Netrin1 gene was observed in the ventricular surface of ganglionic eminences (A and B). There was no observable difference in the expression of Netrin1 between control and conditional Rb knockout. Similar results were obtained in three independent experiments. Bar: 500μm.

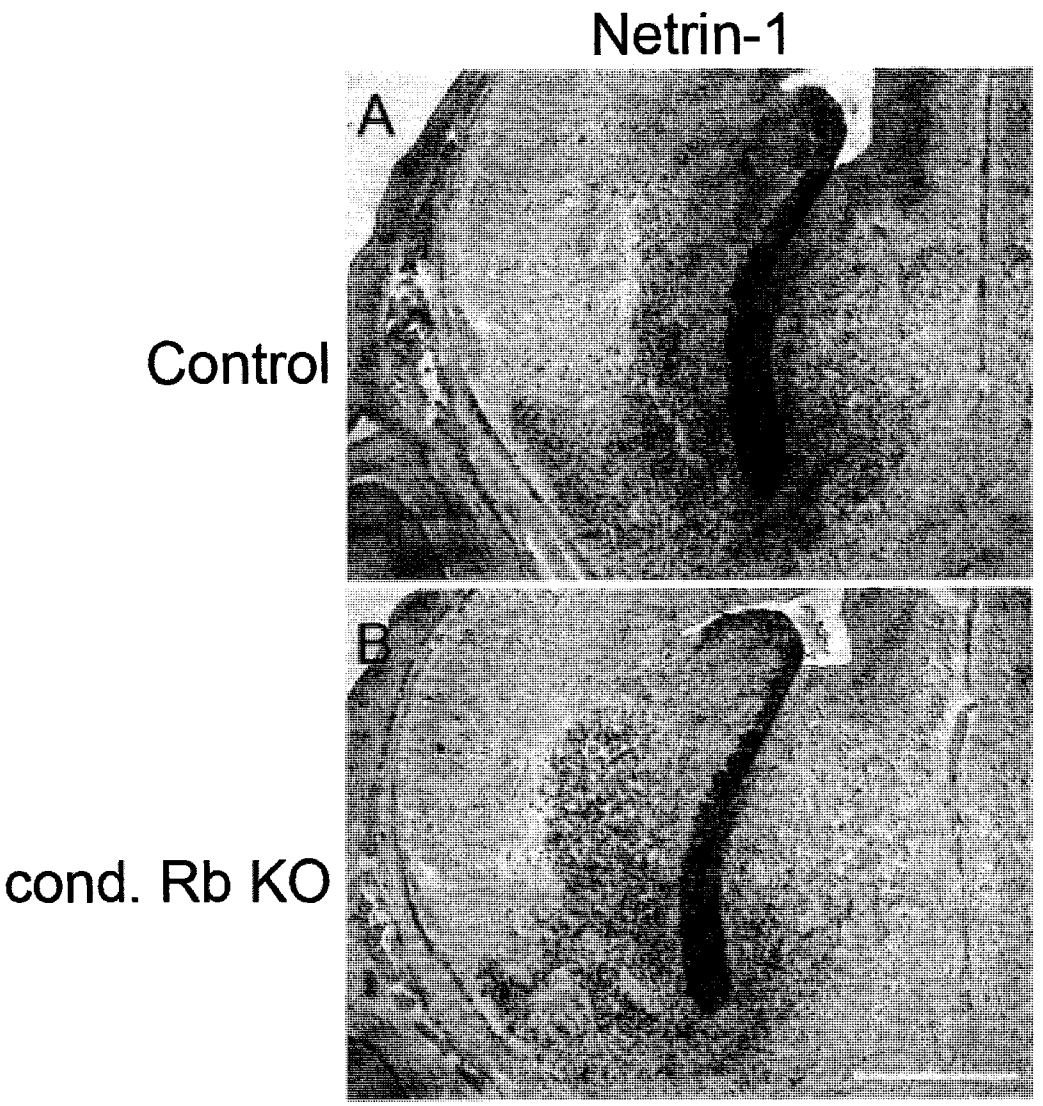
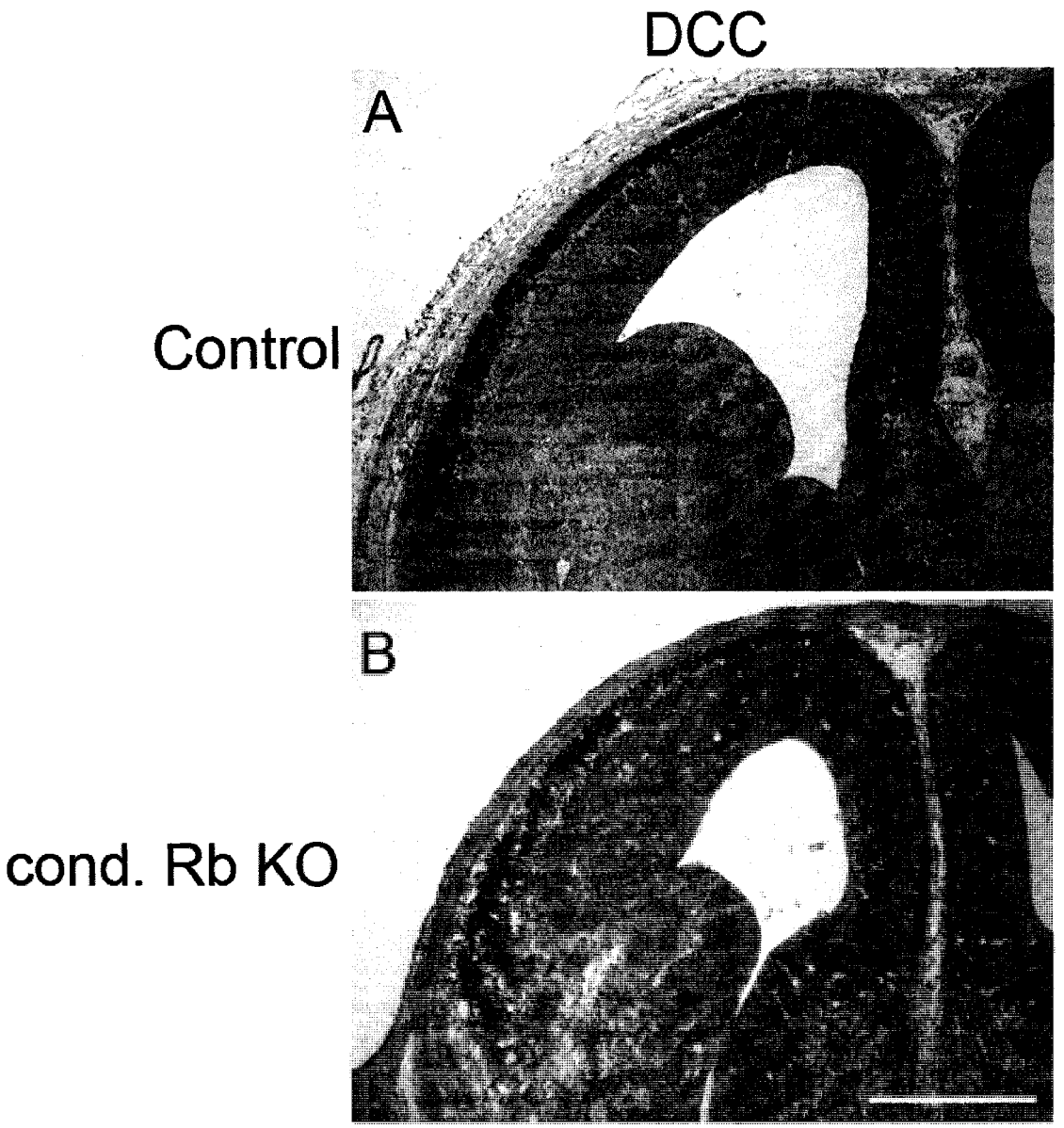

Netrin-1
A
Control
B
cond. Rb KO

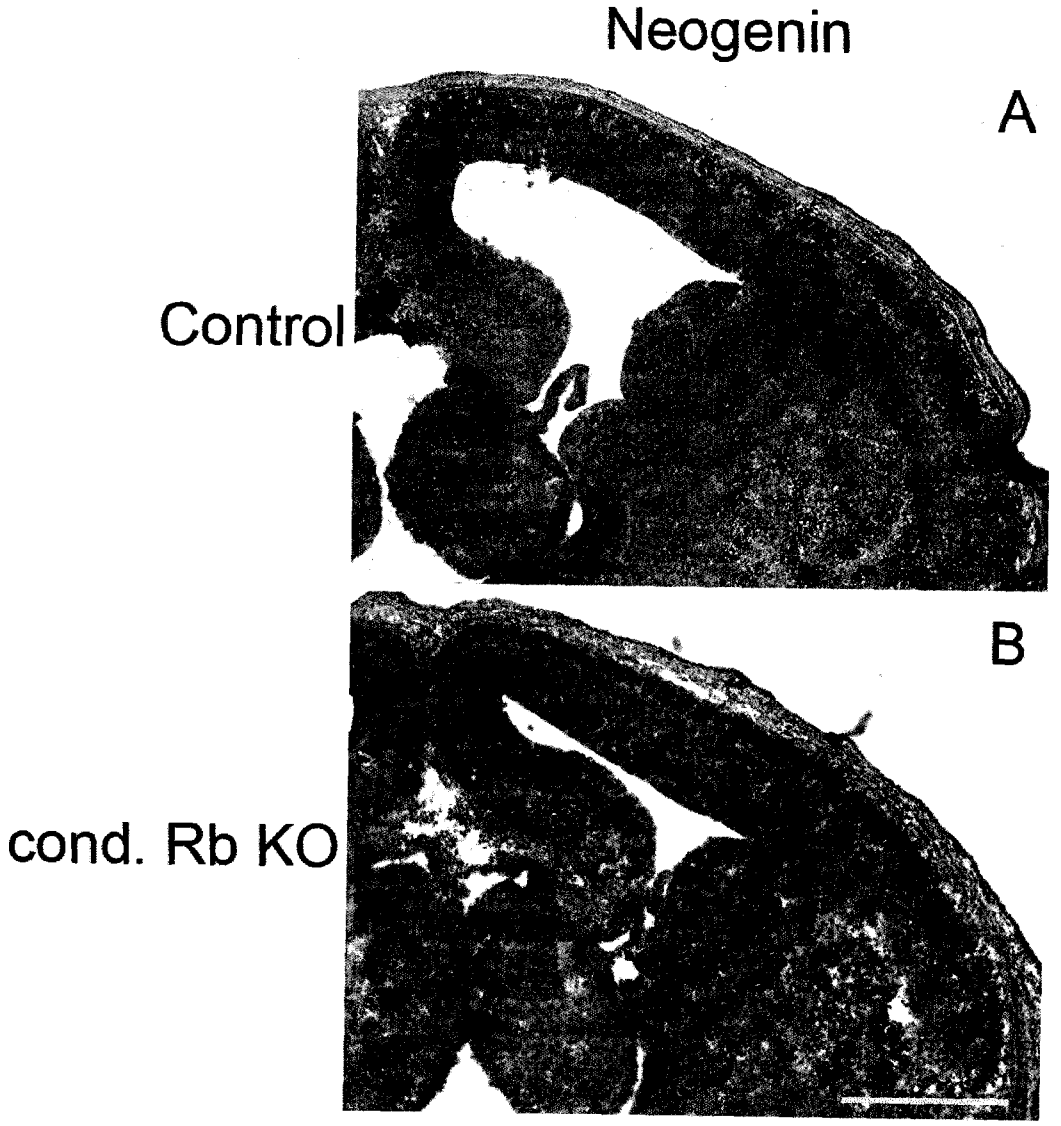
Figure 13. **DCC expression is not altered in the absence of Rb.** *In situ* hybridization was performed on coronal section from E14 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled DCC riboprobe. Expression of DCC gene was observed in the developing cortical plate (A and B). There was no observable difference in the expression of DCC between control and conditional Rb knockout. Similar results were obtained in three independent experiments. Bar: 500μm.


DCC
A
Control
B
cond. Rb KO

(Figure 13, n=3). The results obtained for Netrin1 and its receptor, DCC, are in agreement with the previous report that Netrin1 and DCC are not involved in tangential migration of interneurons, but critical for the tangential migration of guidepost neurons (Anderson et al., 1999; Kawasaki et al., 2006; Marin et al., 2003). Neogenin is a related protein to DCC, and also mediates guidance cues (Keeling et al, 1997). In a recent report, it was found that neogenin was expressed in newly born migrating neurons, implicating neogenin's role in neuronal migration (Fitzgerald et al, 2006). In agreement with this notion, *in situ* hybridization using neogenin riboprobe revealed that at E14.5, there is an increase in the expression of neogenin in the MGE, as well as other regions of conditional Rb knock out mice in comparison to its littermate control (Figure 14, n=3). This result suggests that Rb signaling pathway may regulate the expression of neogenin in interneurons arising from the MGE as well as projection neurons in the cortical plate.

Through *in situ* hybridization using riboprobes against several guidance genes, we have found that Robo2 gene is ectopically expressed in telencephalon-specific Rb knockout mice compared to littermate controls. In addition, we have found that the expression of neogenin gene is upregulated in telencephalon-specific Rb knockout mice compared to littermate controls. These results imply that Rb mediates the transcription of these genes during cortical development.

Figure 14. **Neogenin expression is upregulated in conditional Rb KO**. *In situ* hybridization was performed on coronal section from E14.5 control (Rbfloxed +/-:Cre +/-)(A) and conditional Rb knockout (B) embryos with a DIG-labeled Neogenin riboprobe. Expression of neogenin was seen in the cortical plate and the ventricular zone of dorsal cortex (A and B). There was a significant increase in the expression of neogenin in the conditional Rb knockout compared to the littermate control (B). Similar results were obtained in three independent experiments. Bar: 500μm.


Neogenin
A
Control
B
cond. Rb KO

II. Role of E2F1 in Rb mediated cortical development

Previously, our lab has reported that absence of Rb from mouse telencephalon leads to a number of defects including: a) ectopic mitoses in the developing cortical plate (Ferguson et al, 2002); b) aberrant laminar patterning; c) loss of Cajal-Retzius neurons; and d) defects in tangential migration of GABAergic interneurons (Ferguson et al 2005). It has been reported by Tsai and colleagues that additional loss of E2F1 in Rb deficient mice suppresses ectopic mitoses and extends the survival of these Rb deficient mice (Tsai et al, 1998). We have also reported that in Rb deficient neuronal precursors, there is deregulated activity of E2F1 (Callaghan et al, 1999). Based on these results, we have asked whether deregulated activity of E2F1 was responsible for the defects seen in mice lacking Rb in the telencephalon. To address this question, I have generated Rb:E2F1 double knock out (DKO) mice by crossing telencephalon specific Rb knockout mice and germline E2F1 knockout mice and examined whether the previously reported defects seen in Rb mutants could be rescued (Ferguson et al, 2002; Ferguson et al, 2005).

Deregulated E2F1 activity is responsible for ectopic mitoses

In normal development, neural progenitors proliferate in the ventricular and subventricular zones, and only when they exit the cell cycle, they leave the proliferative zones and commence migration to their final destination (reviewed in (McConnell, 1995). In telencephalon specific Rb knockout mice, migrating neurons exhibit S phase entry in the intermediate and even in the cortical plate as revealed by bromodeoxyuridine (BrdU) incorporation assay (Ferguson et al, 2002). Therefore, we asked whether deregulated E2F1 activity was the cause of ectopic mitoses. To address this question, I performed

BrdU incorporation assay on conditional Rb/E2F1 double knockout mice (DKO) at E15.5 to see if the removal of E2F1 was sufficient to rescue the defect in conditional Rb deficient mice (Figure 15). For this assay, pregnant females were injected with BrdU (50μg/g body weight) 2 hours prior to sacrifice at E15.5. Coronal sections of embryonic brain were then subjected to immunolabeling for BrdU. BrdU positive cells represent the cells entering S-phase during those 2 hours prior to sacrifice. Ectopic proliferation seen in conditional Rb mutant (Figure 15, b) is diminished in DKO (Figure 15, c), and is comparable to the control (Figure 15, a). I then quantified BrdU positive cells in ventricular/subventricular zone, intermediate zone, and cortical plate (Figure 16). In the ventricular zone and subventricular zone, there was no significant difference between the number of BrdU positive cells, where there was 235±26.0 cells in control, 271±13.3 cells in conditional Rb knockout, and 184±12.0 cells in the DKO (Figure 16, a; $P<0.05$, n=3). In the intermediate zone and cortical plate, however, there was a significant reduction ($P<0.05$) in the number of BrdU positive cells in the DKO compared to conditional Rb knockout mice (Figure 16, b; n=3). In the intermediate zone, there were 3.1±0.3 cells in control, 13.3±1.3 cells in the conditional Rb knockout, and 3.6±0.5 cells in the DKO, while in the cortical plate, there were 1.1±0.5 cells in control, 14.3±2.1 cells in the conditional Rb knockout, and 0.8±0.1 cells in the DKO (Figure 16, b). There was no significant difference between control and DKO mice in the numbers of BrdU positive cells in the intermediate zone and cortical plate (Figure 16, b; n=3). These results indicated that absence of E2F1 in Rb mutant mice successfully rescues the ectopic proliferation, and suggests that deregulated activity of E2F1 is involved in ectopic mitoses.

Figure 15. **Deregulated E2F1 activity is involved in ectopic proliferation in Rb mutants.** Pregnant females were subjected to bromodoxyuridine (BrdU) injection (50μg/g body weight) 2hours prior to sacrifice at E15.5 and the sections of embryonic brain were subjected to immunolabeling for BrdU. a) In control, most proliferating cells were present in the ventricular and subventricular zone, with some in the intermediate zone and cortical plate. b) In conditional Rb knockout embryos, there were abundant ectopically proliferating cells in the intermediate zone and the developing cortical plate. c) In the cond. Rb/E2F1 double knockout embryos, there were less ectopically proliferating cells in the intermediate zone and developing cortical plate comparable to the control. Arrows indicate ectopically proliferating cells. Bar: 100μm

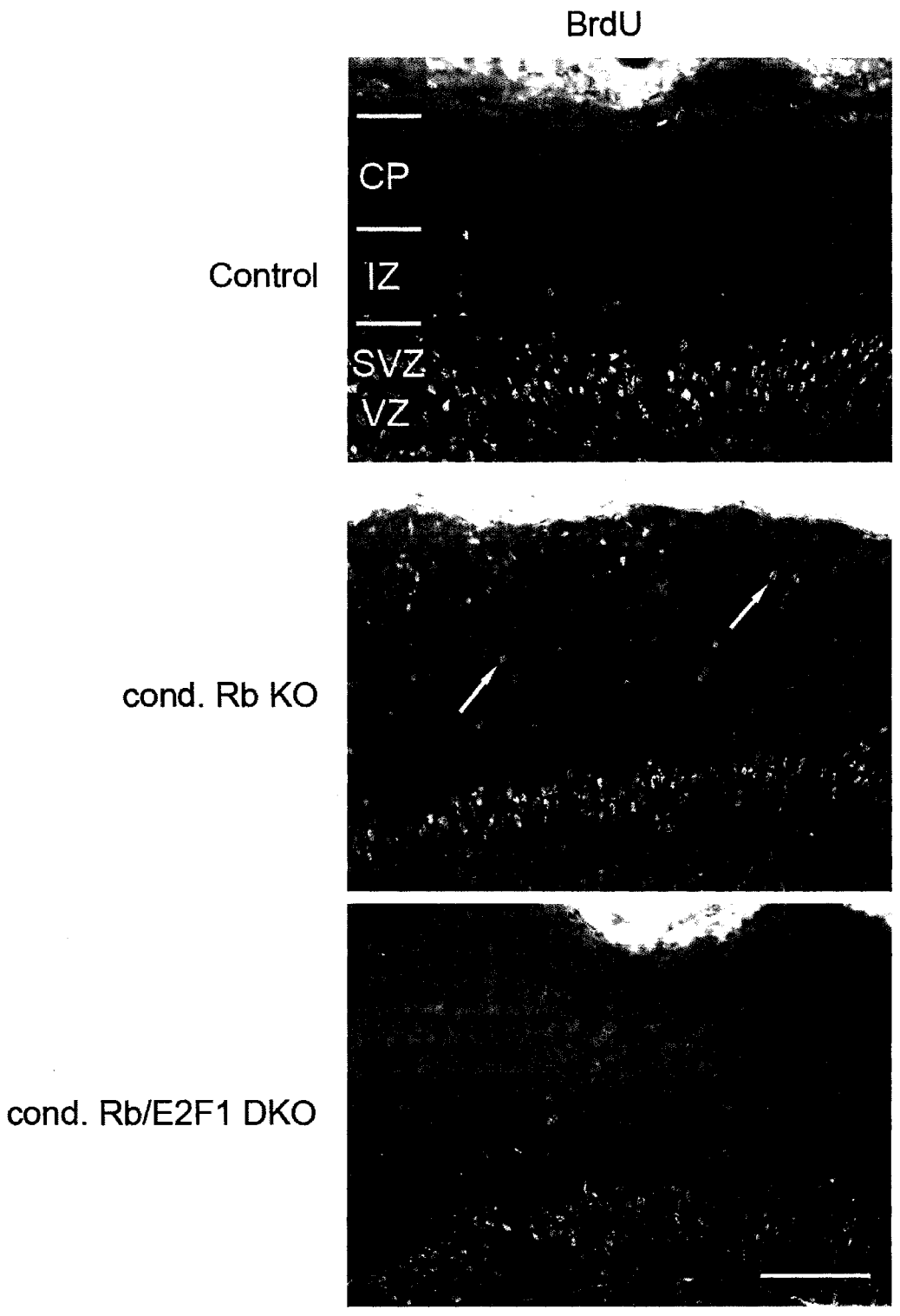
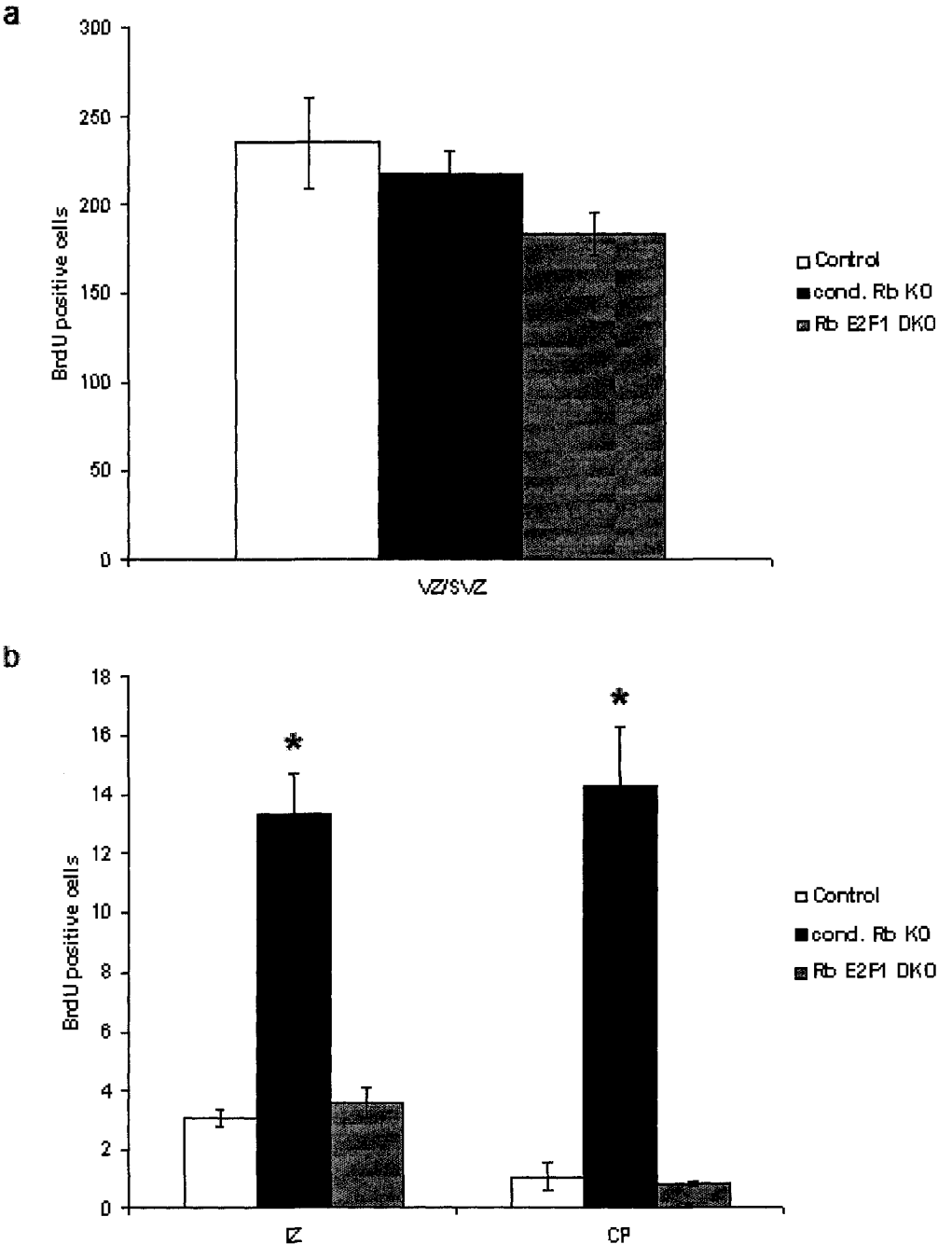

BrdU
CP
IZ
SVZ
VZ
Control
cond. Rb KO
cond. Rb/E2F1 DKO

Figure 16. **Absence of E2F1 rescues ectopic proliferation in Rb KO**. Number of proliferating cells in the BrdU incorporation assay was quantified over 650μm of the developing cortex in three zones: i) ventricular and subventricular zone (VZ/SVZ); ii) intermediate zone (IZ); iii) developing cortical plate (CP). a) There was no significant difference between the number of proliferating cells in the VZ/SVZ. b) In the IZ and CP, the number of ectopically proliferating cells in DKO mice was significantly less than the cond. Rb knockout mice and was comparable to that of control ($P<0.05$, $n=3$).

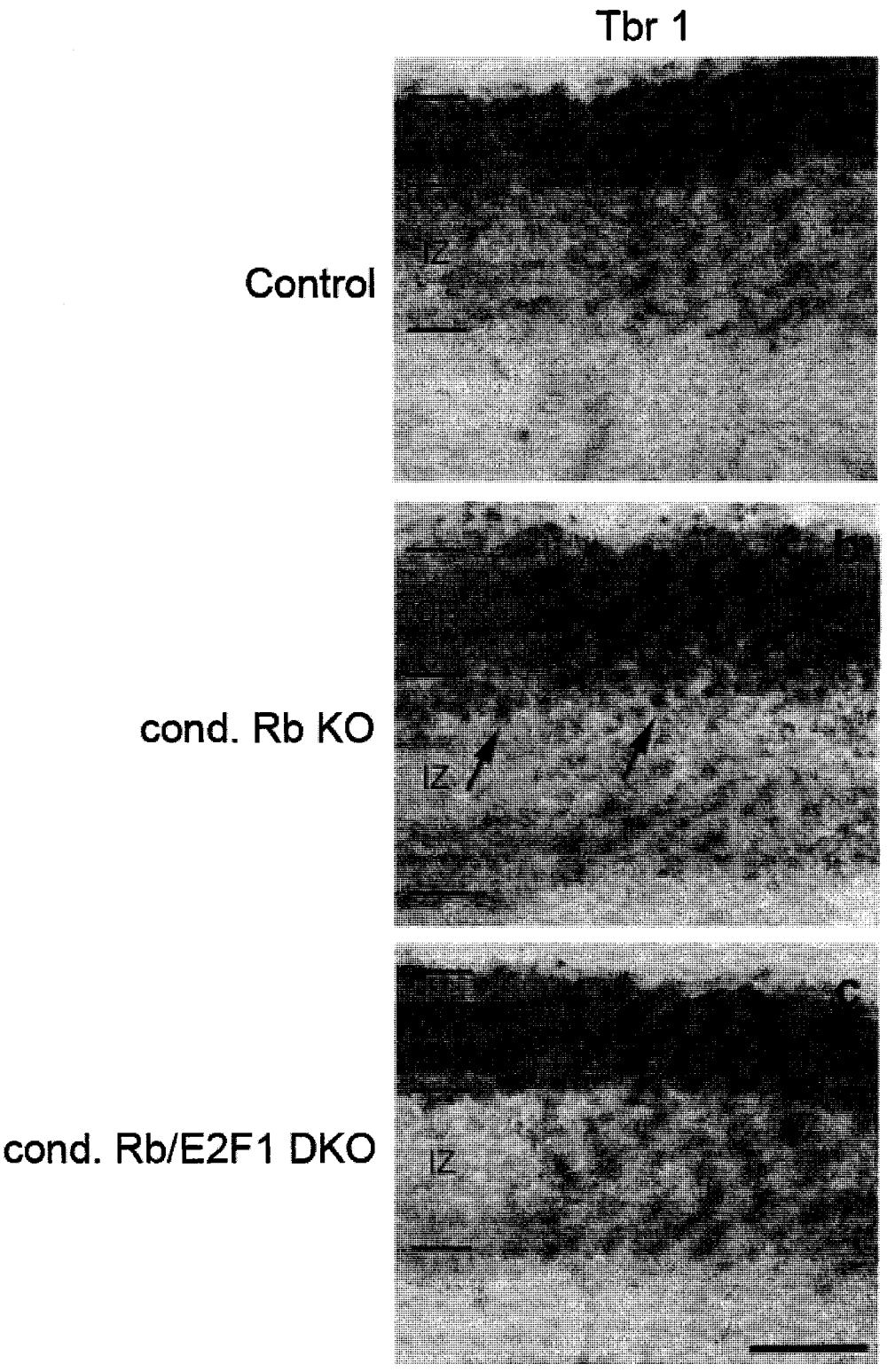

a
300
250
200
150
100
50
0
BrdU positive cells
VZ/SVZ
Control
cond. Rb KO
Rb E2F1 DKO
b
18
16
14
12
10
8
6
4
2
0
BrdU positive cells
*
*
IZ
CP
Control
cond. Rb KO
Rb E2F1 DKO

Deletion of E2F1 in Rb mutants restores laminar patterning in the cortical plates of conditional Rb KO mice

During neural development in mice, neural progenitor cells undergo eleven rounds of cell divisions over 6 day period (E11 to E17) (Takahashi et al., 1995). During this 6 day period, the cortical plate develops in an "inside out" manner where earlier born neurons will reside in deeper layers, while later born neurons will reside in the more superficial layers (Reviewed in (Bielas et al., 2004). At E15.5, layers VI and V have been generated, and layer IV neurons are being born (Takahashi et al., 1999). Previously, our lab has reported that there was an aberrant laminar patterning observed in conditional Rb knockout mice at E15.5 (Ferguson et al, 2005). We therefore asked whether deregulated activity of E2F1 was responsible for this aberrant laminar patterning. To address this question, I have performed *in situ* hybridization on coronal sections of E15.5 embryos using DIG labeled Tbr1 riboprobes. Tbr1 is a T-domain transcription factor, and its expression is up regulated in post mitotic projection neurons in layer V (Bulfone et al., 1995; Rubenstein et al., 1999). I have also examined the morphology of the developing cortex by performing Cresyl Violet stain on coronal sections of E15.5 embryonic brains.

In situ hybridization using DIG labeled Tbr1 riboprobe has revealed a tight laminar patterning in the layer V of the control brain with a clear distinction between the cortical plate and the intermediate zone (Figure 17, a). In contrast, Tbr1 staining was seen in the upper intermediate zone area as well as the cortical plate (layer V) in the conditional Rb knockout brain (Figure 17,b) as previously reported (Ferguson et al,

Figure 17. **E2F1 deficiency corrects defective laminar patterning in Rb mutant mice.** *In situ* hybridization was performed on coronal section from E15.5 embryos of control, conditional Rb knockout mice, and conditional Rb/E2F1 DKO embryos using DIG labeled Tbr1 riboprobe. a) There is a tight laminar pattern in the expression of Tbr1 in the layer V of the cortical plate. b) Tbr1 was aberrantly expressed in the upper layer of the intermediate zone (arrows) in conditional Rb knockout embryo. c) The laminar pattern of Tbr1 expression was restored comparable to control in DKO embryo. n=3. Bar: 100μm



Adapted from McClellan et al, *Mol Cell Biol.* (2007) 27(13):4825-43

2005). In the DKO, Tbr1 expression was confined to the layer V of cortical plate and resembled the expression pattern seen in the control (Figure 17, c) (n=3).

I then performed a cresyl violet staining to examine the morphology of developing cortex. A clear distinction between cell dense developing cortical plate and cell sparse intermediate zone was seen in control (Figure 18, a). On the other hand, this distinct boundary between cortical plate and intermediate zone was lost in the conditional Rb knockout embryos and it appeared more diffuse (Figure 18, b). The morphology in the DKO (Figure 18, c) resembled that of control, where a clear distinction between cell dense cortical plate and cell sparse intermediate zone can be observed (n=3). These results together suggest that the aberrant laminar patterning observed in the conditional Rb knockout mice may be a result of deregulated E2F1 activity.

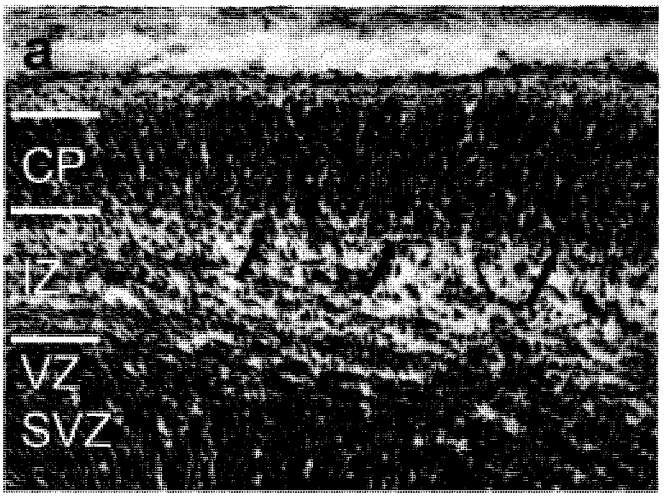
The requirement for Rb in the survival of Cajal-Retzius neurons is partially mediated through E2F1

In the marginal zone of developing cortex, there are two main types of cells present: a) Cajal-Retzius neurons; and b) migrating GABAergic interneurons (Hevner et al., 2003). Both cell types are present throughout corticogenesis (Hevner et al, 2003). Cajal-Retzius neurons release reelin (D'Arcangelo et al., 1995), which acts as a stop signal for radially migrating neurons in the developing cortical plate (Hiesberger et al., 1999; Stolt and Bock, 2006). We have previously reported that there is a significant reduction in the number of Cajal-Retzius neurons in the marginal zone of conditional Rb knockout mice by E16.5 (Ferguson et al, 2005). We therefore asked whether deregulated activity of E2F1 was responsible for the loss of Cajal-Retzius neurons. To address this

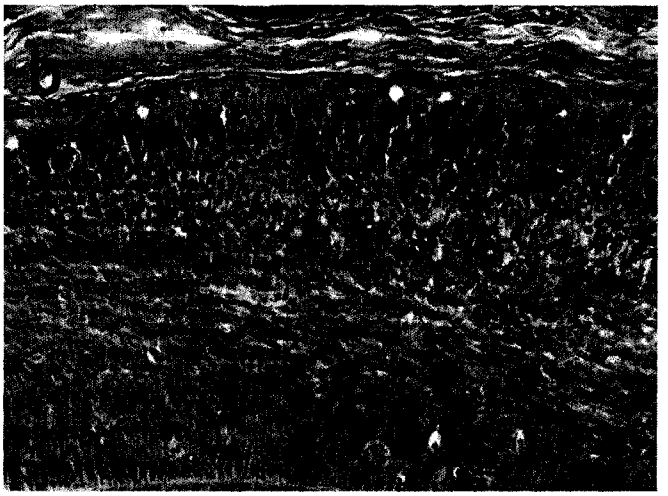
Figure 18. **Rb mediates correct laminar patterning through E2F1.** Cresyl violet staining was performed on coronal sections from E15.5 control, conditional Rb knockout mice, and conditional Rb/E2F1 DKO embryos. a) Morphological staining revealed a clear distinction between the developing cortical plate and the intermediate zone in control. Arrows indicate the boundary between cortical plate and intermediate zone. b) In conditional Rb knockout embryos, the clear distinction had disappeared and it appeared to be more diffuse. c) The morphology was restored to comparable level to that of control. n=3. Bar: 100μm.

Cresyl Violet

Control



cond. Rb KO



cond. Rb/E2F1 DKO

question I performed an immunohistochemistry for Reelin on coronal sections of E15.5 embryos (Figure 19), and quantified reelin positive Cajal-Retzius neurons, as well as the total number of cells residing in the marginal zone. In control, the reelin staining was seen at regular intervals along the marginal zone (Figure 19, a). In the conditional Rb knockout mice, there was less reelin staining in the marginal zone, indicating a loss of Cajal-Retzius neurons in those regions (Figure 19, b). In the DKO, there was an increase in reelin positive cells present compared to the conditional Rb knockout, but there were still regions where there was no reelin positive cell present (Figure 19, c). I then quantified the number of reelin positive cells over 500μm along the marginal zone (Figure 20, a). There was 26±0.9 reelin positive cells (n=3) in the marginal zone of control. In the conditional Rb KO mice, there were 15±0.4 cells (n=4), and this number was significantly less than the control ($P<0.05$). In the DKO, there were 22±0.4 cells (n=4). The number of reelin positive cells in the marginal zone of DKO mice were significantly more than conditional Rb KO mice ($P<0.05$), but was significantly less than the control mice ($P<0.05$). This result suggests that the deregulated activity of E2F1 is partially involved in the loss of Cajal-Retzius neurons. In the previous study, our lab has reported that there was also a significant reduction in the total number of cells in the marginal zone of the conditional Rb knockout mice (Ferguson et al, 2005). I therefore quantified total number of cells present in the marginal zone using cresyl violet staining (Figure 20, b). The total number of cells in the marginal zone of control mice was 64±3.6 cells, while in the conditional Rb knockout there was 40±0.9 cells, and in DKO there was 55±3.3 cells (n=4). The number of cells in the marginal zone in the conditional Rb knockout mice were significantly less than that of control ($P<0.05$) and DKO ($P<0.05$).

Figure 19. **Absence of E2F1 increases reelin staining in conditional Rb KO.** Immunohistochemistry was performed on coronal sections from E15.5 control, conditional Rb knockout mice, and conditional Rb/E2F1 DKO embryos for reelin. a) Cajal-Retzius cells as revealed by reelin immunostaining seemed to be present in the marginal zone at regular intervals in control embryos. b) There was less reelin staining along the marginal zone in conditional Rb knockout embryos, suggesting loss of Cajal-Retzius neurons. c) In conditional Rb/E2F1 DKO embryos, there seemed to be more reelin expressing cells present than the conditional Rb knockouts, however, the absence of reeling positive cells in certain regions still persisted. Arrows indicate reelin positive cells in the marginal zone. n=3 control, n=4 conditional Rb knockout and DKO embryos. Bar: 50μm.

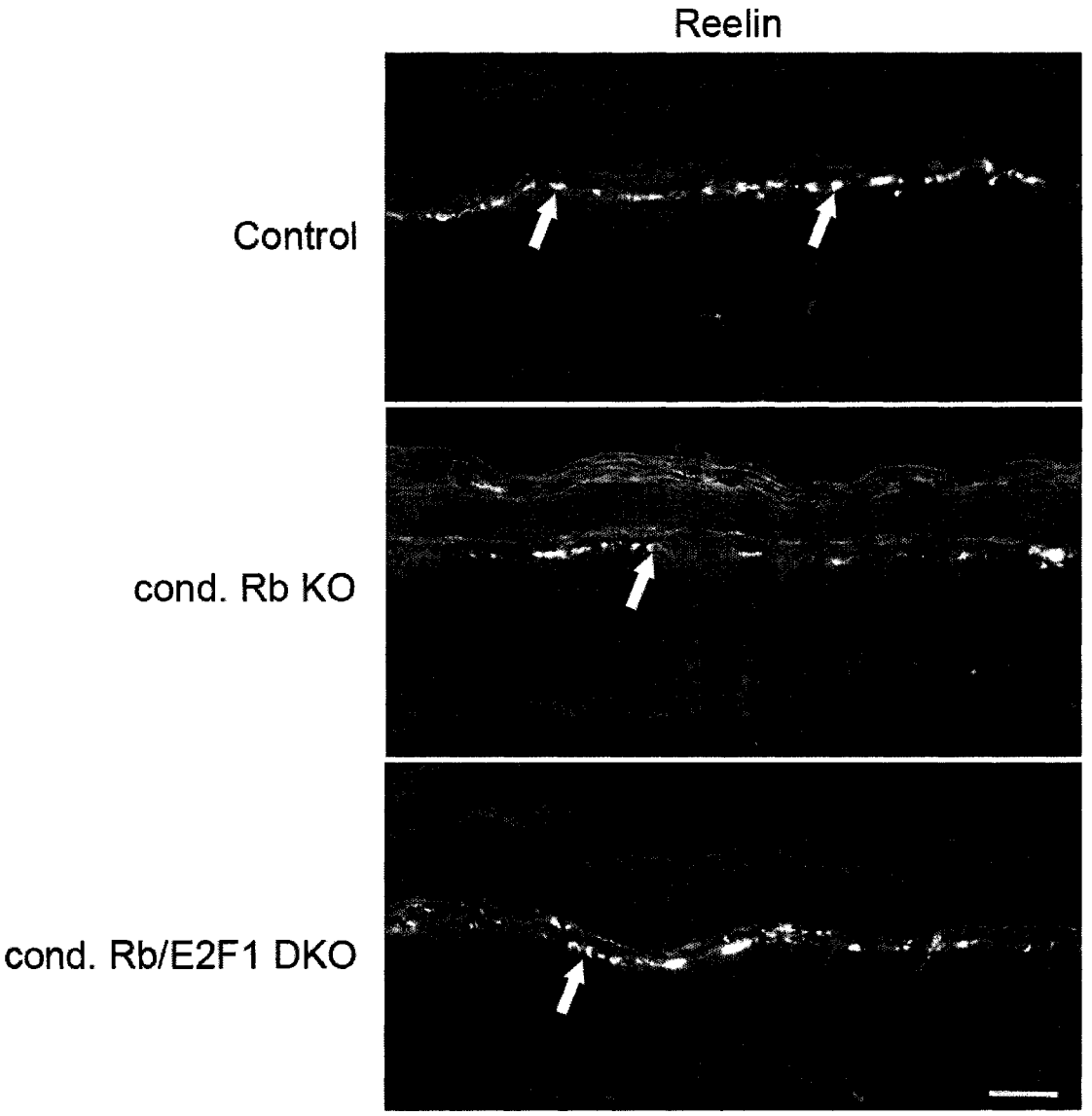
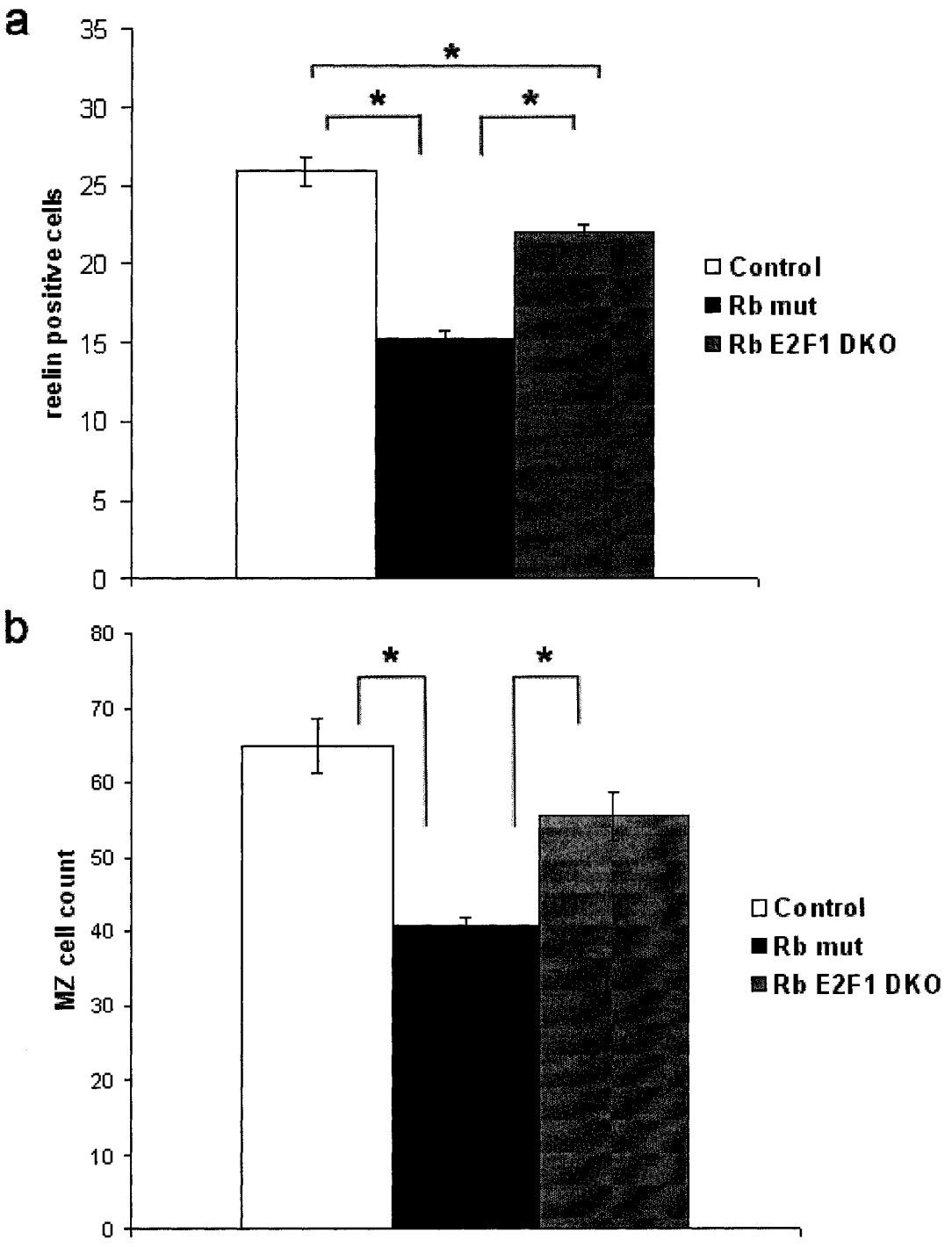

Reelin
Control
cond. Rb KO
cond. Rb/E2F1 DKO

Figure 20. **E2F1 deficiency partially rescues the survival of Cajal-Retzius neurons in conditional Rb KO.** Number of reelin positive cells and total cells within the maginal zone was quantified from dorsal and temporal cortex, each over 500μm along the marginal zone. a) The number of reeling positive cells were significantly higher in the DKO compared to conditional Rb knockout, but was also significantly less than the control ($P<0.05$). b) Cresyl violet staining was performed and the total number of cells in the marginal zone over 500μm along the marginal zone was counted. The number of marginal zone cells in the conditional Rb knockout was significantly less than that of control ($P<0.05$). The number of marginal zone cells in the DKO was significantly higher than the conditional Rb knockout ($P<0.05$), and was not significantly different from the control. n=3.


a
35
30
25
20
15
10
5
0
reelin positive cells
*
*
*
Control
Rb mut
Rb E2F1 DKO
b
80
70
60
50
40
30
20
10
0
MZ cell count
*
*
Control
Rb mut
Rb E2F1 DKO

There was no significant difference between the total number of cells in the marginal zone between control and DKO.

Absence of E2F1 does not correct for aberrant tangential migration in Rb mutant mice.

GABAergic interneurons are thought to arise from the MGE and CGE (Anderson et al., 1997; Tan et al., 1998; Xu et al., 2004). These interneurons migrate through two distinct paths, where one group of cells travel through marginal zone, and the other group travels through the lower intermediate zone (Lavdas et al., 1999). We have previously reported that a subpopulation of interneurons, specifically calbindin positive neurons, was diminished from the marginal zone migratory route (Ferguson et al, 2005). Furthermore, we have reported that this mislocalization arises from a cell autonomous defect in tangential migration, as revealed by a slice co-culture assay (Ferguson et al, 2005). Therefore, we asked whether deregulated E2F1 activity was responsible for this defect. To address this question, I performed immunohistochemistry for calbindin on coronal sections of E15.5 mice (Figure 21). I then quantified calbindin positive cells in the marginal zone over 500μm to compare the number of calbindin positive, migrating interneurons between control, conditional Rb knockout, and DKO mice (Figure 22). In control mice, there were calbindin positive cells present in both the marginal zone and the intermediate zone (Figure 21, a). In the conditional Rb knockout mice, there were calbindin positive cells present in the intermediate zone, and there were very little calbindin staining in the marginal zone (Figure 21, b). In the DKO mice, there were calbindin positive cells present in the intermediate zone, and very little calbindin staining in the marginal zone (Figure 21, c). Quantification of these calbindin positive cells have

Figure 21. **Absence of E2F1 does not rescue aberrant tangential migration in Rb mutant.** Immunohistochemistry was performed on coronal sections from E15.5 control, conditional Rb knockout, and conditional Rb/E2F1 DKO embryos for calbindin. a) The two trajectories taken by calbindin positive GABAergic interneurons are present in marginal zone and intermediate zone in control. b) The trajectory through marginal zone is largely diminished in conditional Rb knockout. c) The diminished trajectory through marginal zone is comparable to conditional Rb knockout in control, suggesting no rescue by removal of E2F1. Arrows indicate calbindin positive cells in the marginal zone. n=4 control. n=3 conditional Rb knockout and DKO. Bar: 250μm

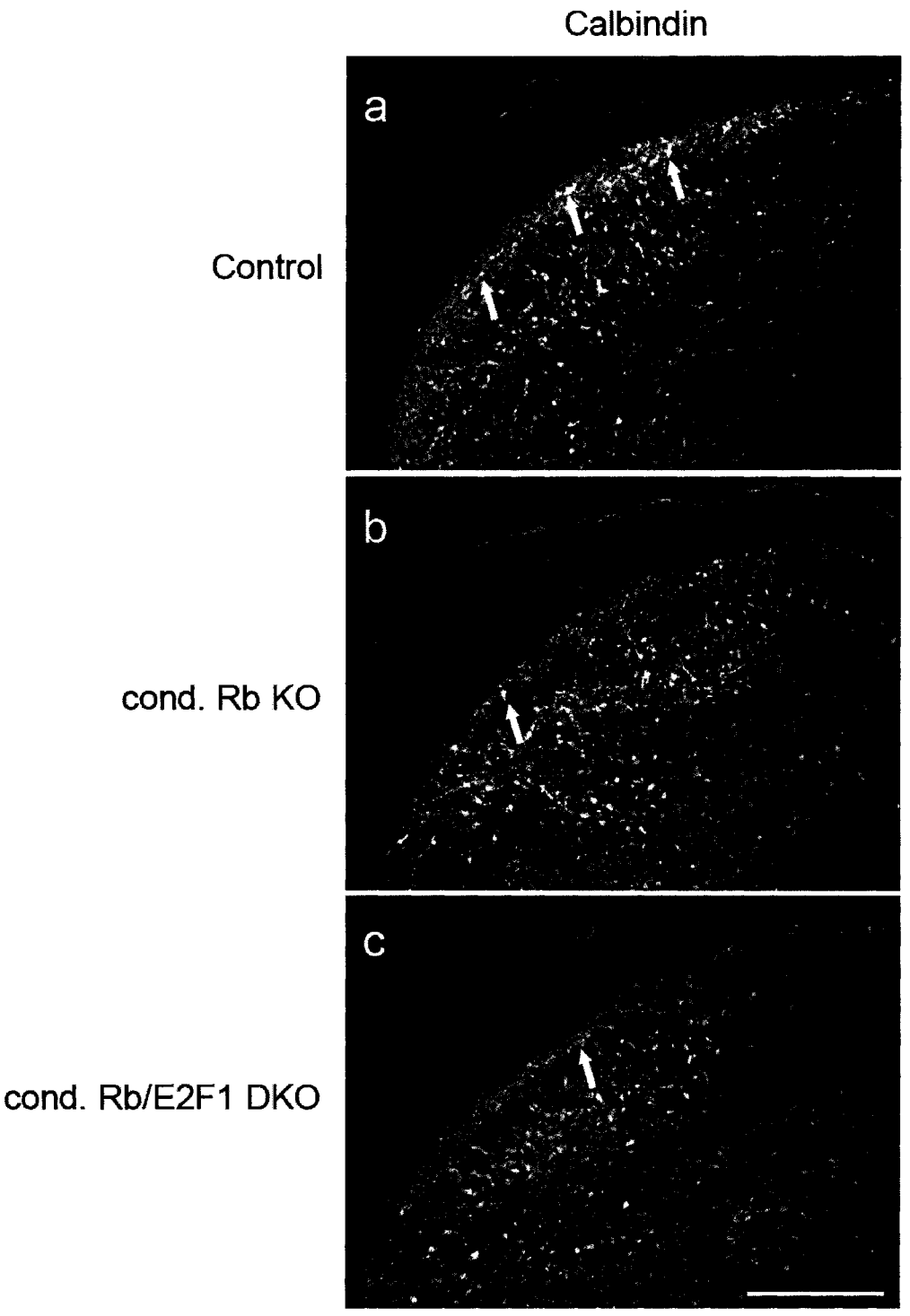
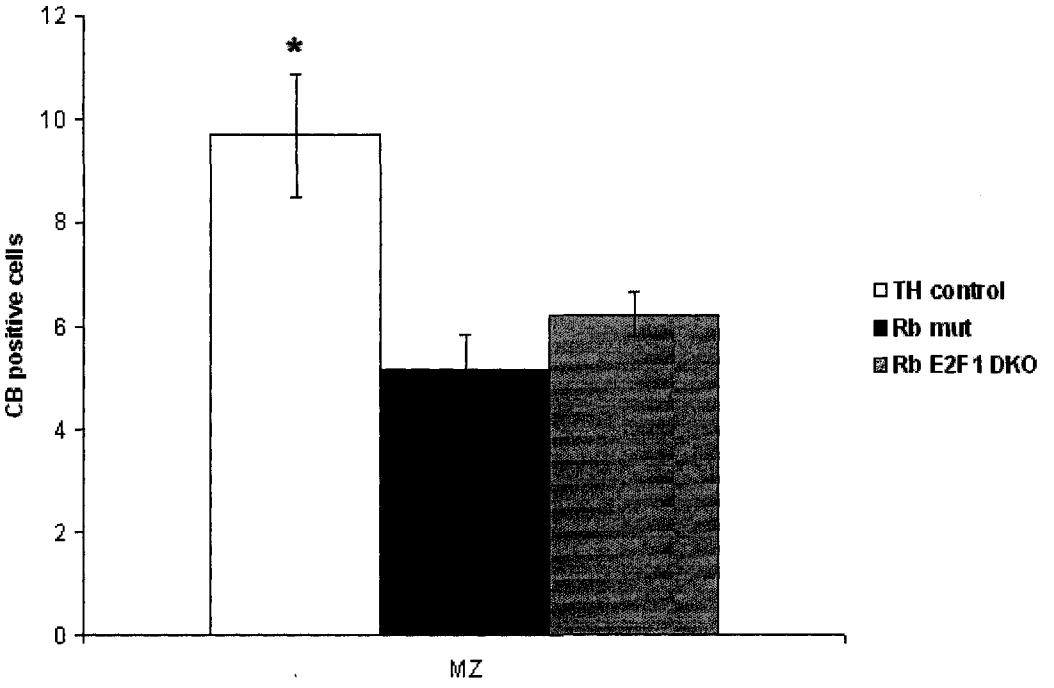

Calbindin
a
b
c
Control
cond. Rb KO
cond. Rb/E2F1 DKO

Figure 22. **Absence of E2F1 does not rescue the number of calbindin positive cells in the marginal zone of Rb mutant mice.** The number of calbindin positive GABAergic interneurons in the marginal zone was quantified over 500μm along the marginal zone. The number of cells in the DKO was significantly less than the control ($P<0.05$), and was comparable to the conditional Rb knockout. n=4 control. n=3 conditional Rb knockout and DKO


12
10
8
6
4
2
0
*
CB positive cells
MZ
TH control
Rb mut
Rb E2F1 DKO

revealed that there were 10±1.2 cells in control mice (n=4), 5±0.7 cells in the conditional Rb knockout mice (n=3), and 6.2±0.4 cells in the DKO mice (n=3). Statistical analysis showed that there was a significant difference between the number of calbindin positive cells in the marginal zone between control and conditional Rb knockout mice ($P<0.05$), as previously reported (Ferguson et al, 2005). Furthermore, the number of calbindin positive cells between the control and DKO mice were also significantly different ($P<0.05$), while there was no significant difference between the conditional Rb knockout mice and the DKO mice (Figure 22). Together, these results suggest that E2F1 may not be involved in Rb mediated tangential migration of GABAergic interneurons.

To investigate the role of E2F1 in Rb mediated terminal mitosis of cortical neurons, laminar patterning, survival of Cajal-Retzius neurons, and tangential migration of GABA-ergic interneurons, we generated telencephalon specific Rb: germline E2F1 double knock out mice (DKO). Through BrdU incorporation assay, we found that the ectopic proliferation was reduced to control level in DKO. Through *in situ* hybridization for Tbr1 and cresyl violet stain, we found that aberrant laminar patterning observed in the telencephalon specific Rb KO was restored in DKO. Through immunohistochemistry, we found that there was a partial rescue for the survival of Cajal-Retzius neurons in DKO. Through immunohistochemistry for calbindin positive GABA-ergic interneurons, we found that the defects in tangential migration of these neurons were not rescued in DKO. In summary, the results of the studies revealed that E2F1 may be involved in Rb mediated terminal mitoses of newly born neurons and correct laminar patterning, and partially in the survival of Cajal-Retzius neurons, but not involved in Rb mediated tangential migration of GABAergic interneurons.

Discussion

The work presented here describes several findings demonstrating the importance of Rb and E2F1 during forebrain development. We hypothesized that *Rb mediates GABA-ergic interneuron migration through regulation of guidance molecules.* Through *in situ* hybridization, I have found that there is an ectopic expression of Robo2 gene in the ventral cortical plate, as well as an upregulation of neogenin gene in the MGE, cortical plate and ventricular zone of dorsal cortex. These results suggest that Rb may be involved in the regulation of transcriptional control of guidance genes, Robo2 and neogenin. We also hypothesized that *Rb regulates cortical development through E2F1 transcription factor.* Through generation of Rb:E2F1 double knock out (DKO) mice by crossing telencephalon specific Rb knockout mice and germline E2F1 knockout mice, I have found that: a) ectopic proliferation of cortical neurons in conditional Rb KO mice is rescued through removal of E2F1; b) aberrant cortical laminar patterning in conditional Rb KO mice is restored through removal of E2F1; c) the loss of Cajal-Retzius neurons in conditional Rb KO mice is partially rescued by removing E2F1 ; and d) Rb mediated migration of calbindin positive GABA-ergic interneurons cannot be rescued through removal of E2F1. These findings suggest that E2F1 is a downstream target for Rb in the regulation of proliferation of cortical neurons, laminar patterning, and partially, the survival of Cajal-Retzius neurons.

Rb mediates expression of Robo2 and neogenin

Our lab has previously reported a cell autonomous defect in tangential migration of GABA-ergic interneurons (Ferguson et al, 2005). GABA-ergic interneurons born in

MGE travel to the dorsal cortex via tangential migration (Anderson et al, 1997; Tan et al, 1998; Xu et al, 2004). Tangential migration of these GABA-ergic interneurons is thought to be directed by Slit-Robo pathway (Zhu et al, 1999; Nguyen-Ba-Charvet et al, 2004; Andrews et al, 2006). Through *in situ* hybridization, we have found that the expression of Slit1, Slit2, Slit3, and Robo1 are not altered in telencephalon specific Rb KO, suggesting that Rb does not mediate the transcription of these genes. Robo2, on the other hand, was found to be ectopically expressed in the telencephalon specific Rb KO mice. Robo2 was ectopically expressed in the ventral region of developing cortical plate in the knockout mice compared to its littermate control. This suggests that Rb may be negatively regulating the expression of Robo2 gene in a subset of these neurons in that region and in the absence of Rb, Robo2 expression is upregulated. The identity of these neurons, however, has not been explored in this study. The ectopic expression of Robo2 gene could also be a result of abnormal re-routing of migrating cells. A recent study by Andrews and coworkers (Andrews et al., 2006) revealed that Robo1 knockout mice had significantly more interneurons migrating to the cortex (Andrews et al, 2006). This phenotype, the authors described, was different from Slit1 null mice, and they suggested a possibility for the existence of additional ligands, receptors, or receptor partners in Slit/Robo pathway. Our results may be consistent with this notion since we did not find alterations in any of the Slit ligand expression, but only the expression of Robo2 was altered in our knockout mice. This may be resulting from Rb regulating unidentified ligands, receptors, or receptor partners which caused Robo2 expressing cells to be re-routed.

Netrin-DCC pathway is another pathway thought to be involved in neuronal migration (Bloch-Gallego et al, 1999; Yee et al, 1999; Hamasaki et al, 2001). Also through *in situ* hybridization, we have found that the expression of Netrin1 and DCC are not altered in the absence of Rb. From this we concluded that Rb does not mediate the transcription of Netrin1 and DCC. Strikingly, neogenin, a transmembrane receptor related to DCC (Keeling et al, 1997) was found to be expressed at a much higher level in the MGE, cortical plate, and ventricular zone of telencephalon specific Rb knockout mice compared to their littermate controls. Recently, it has been demonstrated that neogenin is a direct transcriptional target of E2F1 by ectopically expressing E2F1 in mouse embryonic fibroblast cells and through utilization of sensitive subtraction method to detect E2F1 responsive genes (Iwanaga et al., 2006). This, together with our result suggests that Rb regulates the transcription of neogenin in developing nervous system through E2F. Neogenin is a known receptor for repulsive guidance molecule (RGM) which mediates a repulsive cue (Rajagopalan et al., 2004). It is possible that the lack of GABA-ergic interneurons in MZ in the absence of Rb (Ferguson et al, 2005) is a result of upregulation of neogenin in migrating interneurons, causing the repulsion from MZ migratory route. The finding that genes affected by the loss of Rb are transmembrane protein receptors is in support of the notion that the defect in tangential migration of GABA-ergic interneurons is a cell autonomous defect (Ferguson et al, 2005).

E2F1 is involved in Rb mediated terminal mitosis of cortical neurons and laminar patterning *in vivo*

In the previous report, our lab has described that in the absence of Rb, there are ectopically proliferating neurons in the intermediate zone and cortical plate (Ferguson et al, 2002). Deletion of E2F1 has been shown to suppress inappropriate S-phase entry in the CNS in Rb null mouse embryos (Tsai et al, 1998; Saavedra et al, 2002). In both studies, however, studies were conducted with germline Rb null mice, and thus, there were defects that caused embryonic lethality. It is now clear that embryonic lethality is in fact a result of defects in placenta upon loss of Rb (Wu et al., 2003). Through generation of telencephalon specific Rb: germline E2F1 double knockout (DKO) mice and utilizing a 2hr-BrdU incorporation assay, we have found that removal of E2F1 in the absence of Rb rescues the ectopic proliferation in the CNS. Previously, expression of mutant E2F1 incapable of binding to Rb in rat fibroblasts has been shown to induce enhanced S-phase entry (Shan et al, 1996). This, together with our result suggests an important role for E2F1 as target for Rb in proper cell cycle control. Interestingly, a recent report from our lab suggests that E2F3 is also involved in the ectopic proliferation of cortical neurons (McClellan et al., 2007). Similar to the results obtained from Rb:E2F1 DKO mice presented in this report, results obtained from telencephalon specific Rb:E2F3 DKO mice indicated that removal of E2F3 in conditional Rb KO mice can rescue the ectopic proliferation of cortical neurons (McClellan et al, 2007). E2F3 has also been shown to contribute to the ectopic mitoses in the CNS of Rb null embryos as deficiency of E2F3 is able to suppress inappropriate S-phase entry in those embryos (Ziebold et al, 2001; Saavedra et al, 2002). This suggests that E2F1 and E2F3 contribute equally in Rb

mediated cell cycle exit. It is possible that ectopic mitoses in the absence of Rb requires the deregulated activity of both E2F1 and E2F3, and that combined effects from E2F1 and E2F3 contribute to a total free E2F activity, driving these cortical neurons to undergo inappropriate S-phase entry.

The previous report from our lab also described a defect in cortical laminar patterning due to defective radial migration (Ferguson et al, 2005). Through *in situ* hybridization and cresyl violet staining, we have found that cortical lamination defect is rescued in DKO mice. Similarly to ectopic mitoses, deletion of E2F3 in telencephalon also rescues aberrant laminar patterning in the absence of Rb (McClellan et al, 2007). It has been established that environmental factors play an important role in the laminar fate determination of cortical neurons, and their ability to respond to the environmental cues are dependent of cell cycle phases (McConnell and Kaznowski, 1991). It is likely that migration defects are secondary effect of inappropriate S-phase entry. Recently, Nguyen and coworkers have described a role for yet another cell cycle gene, $p27^{kip1}$, in radial migration of cortical neurons (Nguyen et al., 2006). In their study, they found that $p27^{kip1}$ null mice had defects in radial migration of cortical neurons (Nguyen et al, 2006). $p27^{kip1}$ is considered to be a general inhibitor of cyclins and its activities promote Rb to be active (Polyak et al, 1994; Sherr and Roberts, 1999). This further supports the importance of proper Rb activities in terminal mitoses and radial migration of cortical neurons. In this report, we have demonstrated that E2F1 has important roles in Rb mediated terminal mitoses, radial migration and cortical laminar patterning *in vivo*.

Rb partially mediates survival of Cajal-Retzius neurons through E2F1

Our lab has previously described the requirement for Rb in survival of Cajal-Retzius neurons (Ferguson et al, 2005). In this study, we found that the total number of MZ cells was increased in Rb:E2F1 DKO as compared to the conditional Rb KO. By employing immunohistochemistry for reelin on cortical sections from Rb:E2F1 DKO, we have found that removal of E2F1 in the absence of Rb partially rescues the survival of Cajal-Retzius neurons. This suggests that the requirement for Rb in the survival of Cajal-Retzius neurons is partially mediated by E2F1. Although the induction of apoptosis has in fact been suggested to be specific to E2F1 in some reports (DeGregori et al., 1997; Wang et al., 2000), overexpression of E2F3 has also been shown to be able to induce cell death in other reports (Ebelt et al., 2005; Paulson et al., 2006). Thus, whether the induction of cell death is a function specific to E2F1 is yet to be determined. Moreover, recent report from our laboratory indicates that removal of E2F3 in the absence of Rb is also capable of partially rescuing the survival of Cajal-Retzius neurons (McClellan et al, 2007). From these results, one could speculate that deregulated activities of E2F1 and E2F3 are each involved in the loss of different subtypes of these Cajal-Retzius neurons, and together, both E2F1 and E2F3 mediate the requirement for Rb in the survival of total population of Cajal-Retzius neurons. It has been suggested that E2F3 can induce p53 independent apoptosis, and this induction of apoptosis is dependent on E2F1 (Lazzerini Denchi and Helin, 2005). Interestingly, it has recently been shown that an axon guidance molecule, neuropilin1 is a direct target for E2F1 during cerebral ischemia-induced neuronal death, where upregulation of neuropilin1 by E2F1 causes axonal retraction and

neuronal death (Jiang et al., 2007). This demonstrates a novel way in which E2F1 causes cell death, and adds further support for specificity for E2F1 in causing neuronal death.

Both E2F1 and E2F3 contain E2F responsive elements (Araki et al., 2003; Leone et al., 2000), and the expression of E2F1 has been shown to be directly regulated by E2F1 and E2F3 (Araki et al, 2003). It is then possible that deregulated activity of either E2Fs cause upregulation of E2F1, and thus, inducing cell death in Cajal-Retzius neurons. If E2F-induced apoptosis is solely E2F1 dependent, it does not explain the partial rescue of the survival of Cajal-Retzius neurons that we observed in Rb:E2F1 DKO mice. It is then possible that the requirement for Rb in the survival of a subset of Cajal-Retzius neurons is mediated through non-E2F Rb interacting partner. For example, nuclear ABL tyrosine kinase has been shown to directly interact with Rb, and its proapoptotic activity has been shown to be regulated by Rb (Wang, 2000; Wang, 2005).

Rb does not mediate the tangential migration of GABA-ergic interneurons through interactions with E2F1

Our lab has previously reported a cell autonomous requirement for Rb in tangential migration of GABA-ergic interneurons (Ferguson et al, 2005). Through immunohistochemistry on telencephalon specific Rb: germline E2F1 DKO mice using calbindin as marker for the interneurons, we have found that removal of E2F1 in the absence of Rb does not rescue the defect in tangential migration seen in conditional Rb KO. Thus, this suggests that E2F1 is not involved in the requirement for Rb in proper tangential migration of GABA-ergic interneurons. On the contrary, a recent data from our lab suggests that E2F3 is involved in Rb mediated tangential migration of GABA-

ergic interneurons (McClellan et al, 2007). Through generation of conditional Rb:E2F3 DKO mice, it was shown that removal of E2F3 successfully rescues the aberrant tangential migration of these interneurons (McClellan et al, 2007). These results imply that there are divergent roles within the activating E2Fs. Moreover, it was shown that the migration defect was not due to defects in cell cycle kinetics (i.e. ectopic mitoses), demonstrating a role for Rb and E2F3 outside of the cell cycle role (McClellan et al, 2007). Calbindin positive interneurons only represent a subset of GABA-ergic interneurons arising from the MGE (Reviewed in Metin et al, 2006). There are other populations of cortical interneurons arising from MGE such as parvalbumin and somatostatin expressing neurons (Metin et al., 2006; Valcanis and Tan, 2003; Wichterle et al., 2001). Thus, the data obtained here may be a cell type specific effect. It will be interesting to determine, first, if there are defects in other population of GABA-ergic interneurons in the absence of Rb. If there are defects in the migration of other populations, it is possible that E2F1 may be the interacting partner for Rb in Rb mediated tangential migration in other populations of GABA-ergic interneurons.

E2F1 and E2F3 have been suggested to have unique and overlapping roles in development (Attwooll et al., 2004). While E2F1 deficient mice are viable and fertile (Field et al, 1996; Yamasaki et al, 1996), E2F3 deficiency results in embryonic lethality in most cases, and surviving pups die prematurely (Cloud et al., 2002). These results suggest specificity in the activities of each E2F. For example, tumor formation seems to be specific to E2F1 loss as compound null mutants for E2F1 and E2F3 did not display increased tumor formation. In the lenses of developing mice, E2F1 has been shown to specifically regulate the expression of E2F3a and $p19^{ARF}$ (Hyde and Griep, 2002).

Furthermore, apoptosis in the lenses of Rb deficient mice seem to be specifically mediated by E2F1 as only deletion of E2F1 rescues apoptosis observed in Rb deficient mice (Saavedra et al, 2002). As described above, growing evidence suggest E2F dependent apoptosis to be specifically mediated by E2F1 (DeGregori et al., 1997; Hallstrom and Nevins, 2003; Lazzerini Denchi and Helin, 2005; Wang, 2000). This specific effect of E2F1 in inducing apoptosis has been suggested to be regulated by E2F1 specific binding site at the C-terminal of Rb (Dick and Dyson, 2003; Pennaneach et al., 2004). Surprisingly, a recent report suggests that E2F1 can also protect cells against γ-irradiation induced apoptosis in a context dependent manner in *Drosophila* (Moon et al., 2006). The authors found that dE2F1/dDP seem to protect non-proliferating cells from apoptosis, and proliferating cells were sensitized to apoptosis induced by γ–irradiation (Moon et al, 2006). Thus, not only do different E2Fs have distinct roles, individual E2Fs may have unique roles depending on the cellular context. In this study, we have demonstrated that E2F1 is not involved in Rb mediated tangential migration, while other studies from our lab has demonstrated that E2F3 is involved in this Rb mediated tangential migration (McClellan et al, 2007). These results add further support that E2F1 and E2F3 have distinct roles.

Future Directions

The result obtained for the expression levels of neogenin and Robo2 were from *in situ* hybridization. While it demonstrates the importance of Rb in the expression of these genes, our data do not address whether these genes are direct target for Rb. Although neogenin has been shown to be the direct target for E2F1 (Iwanaga et al, 2006), our data

suggests that in the cortical interneurons, neogenin expression may not be the target for E2F1 since the abnormal tangential migration was not rescued in the DKO. This interpretation is based on the correlation between the expression level of neogenin and the rescue for tangential migration. Thus, it does not completely exclude the possibility that neogenin is in fact a direct target of E2F1 in the cortical interneurons. Interestingly, a data from our lab suggests that E2F3 may be the interacting partner for Rb mediated tangential migration (McClellan et al, 2007). This raises the possibility that neogenin may be the direct transcriptional target for neogenin in cortical interneurons. It will be interesting to see whether increased expression of neogenin can be rescued in Rb:E2F3 DKO, and if so, to see whether neogenin is a direct transcriptional target for E2F3 in cortical interneurons by carrying out promoter analysis and luciferase assay.

We have established that E2F1 is partially involved in the requirement for Rb in the survival of Cajal-Retzius neurons. It will be important to investigate whether there are other Rb interacting partners present that work in parallel with E2F1 to promote the survival of Cajal-Retzius neurons. E2F1's ability to induce cell death has been well established (Berkovich and Ginsberg, 2003; Hershko et al., 2005; Melino et al., 2004; Stiewe and Putzer, 2000). A pro-survival gene, Bis, has recently been shown to be critical for the survival of Cajal-Retzius neurons. In addition, Bcl-2 has been shown to be a target for E2F1 (Eischen et al., 2001; Yang et al., 2000). Thus, investigating whether Rb:E2F1 pathway has a role in the expression of Bis or Bcl-2 may help us enlighten the mechanism by which Rb mediates the survival of Cajal-Retzius neurons. In addition, investigating whether in the absence of Rb, proapoptotic genes such as p53 and p73 (Zamzami and Kroemer, 2005) are altered, and whether E2F1 may have a role in the

expression of these genes will also aid in investigating the mechanism of Cajal-Retzius neuron loss.

Conclusion

The studies presented here show that Rb mediates the expression of guidance molecules Robo2 and neogenin. The studies also show that E2F1 is involved in Rb mediated terminal mitoses and laminar patterning of cortical neurons, and partially involved in the requirement for Rb in the survival of Cajal-Retzius neurons. These studies demonstrate a role for Rb in the expression of guidance molecules and also suggest that E2F1 may be a downstream target for Rb in the regulation of proliferation of cortical neurons, laminar patterning, and partially, the survival of Cajal-Retzius neurons.

References

Adams, M.R., Sears, R., Nuckolls, F., Leone, G. and Nevins, J.R. (2000) Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. *Mol Cell Biol*, **20**, 3633-3639.

Anderson, S., Mione, M., Yun, K. and Rubenstein, J.L. (1999) Differential origins of neocortical projection and local circuit neurons: role of Dlx genes in neocortical interneuronogenesis. *Cereb Cortex*, **9**, 646-654.

Anderson, S.A., Eisenstat, D.D., Shi, L. and Rubenstein, J.L. (1997) Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science*, **278**, 474-476.

Anderson, S.A., Marin, O., Horn, C., Jennings, K. and Rubenstein, J.L. (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development*, **128**, 353-363.

Andrews, W., Liapi, A., Plachez, C., Camurri, L., Zhang, J., Mori, S., Murakami, F., Parnavelas, J.G., Sundaresan, V. and Richards, L.J. (2006) Robo1 regulates the development of major axon tracts and interneuron migration in the forebrain. *Development*, **133**, 2243-2252.

Angevine, J.B. and Sidman, R.L. (1961) Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature*, **192**, 766-768.

Araki, K., Nakajima, Y., Eto, K. and Ikeda, M.A. (2003) Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the E2F1 promoter. *Oncogene*, **22**, 7632-7641.

Arnaud, L., Ballif, B.A., Forster, E. and Cooper, J.A. (2003) Fyn tyrosine kinase is a critical regulator of disabled-1 during brain development. *Curr Biol*, **13**, 9-17.

Aslanian, A., Iaquinta, P.J., Verona, R. and Lees, J.A. (2004) Repression of the Arf tumor suppressor by E2F3 is required for normal cell cycle kinetics. *Genes Dev*, **18**, 1413-1422.

Attwooll, C., Lazzerini Denchi, E. and Helin, K. (2004) The E2F family: specific functions and overlapping interests. *Embo J*, **23**, 4709-4716.

Austin, C.P. and Cepko, C.L. (1990) Cellular migration patterns in the developing mouse cerebral cortex. *Development*, **110**, 713-732.

Ballif, B.A., Arnaud, L. and Cooper, J.A. (2003) Tyrosine phosphorylation of Disabled-1 is essential for Reelin-stimulated activation of Akt and Src family kinases. *Brain Res Mol Brain Res*, **117**, 152-159.

Bandara, L.R. and La Thangue, N.B. (1991) Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature*, **351**, 494-497.

Bates, S., Phillips, A.C., Clark, P.A., Stott, F., Peters, G., Ludwig, R.L. and Vousden, K.H. (1998) p14ARF links the tumour suppressors RB and p53. *Nature*, **395**, 124-125.

Berkovich, E. and Ginsberg, D. (2003) ATM is a target for positive regulation by E2F-1. *Oncogene*, **22**, 161-167.

Berry, M. and Rogers, A.W. (1965) The migration of neuroblasts in the developing cerebral cortex. *J Anat*, **99**, 691-709.

Bielas, S., Higginbotham, H., Koizumi, H., Tanaka, T. and Gleeson, J.G. (2004) Cortical neuronal migration mutants suggest separate but intersecting pathways. *Annu Rev Cell Dev Biol*, **20**, 593-618.

Bloch-Gallego, E., Ezan, F., Tessier-Lavigne, M. and Sotelo, C. (1999) Floor plate and netrin-1 are involved in the migration and survival of inferior olivary neurons. *J Neurosci*, **19**, 4407-4420.

Bock, H.H. and Herz, J. (2003) Reelin activates SRC family tyrosine kinases in neurons. *Curr Biol*, **13**, 18-26.

Bookstein, R., Rio, P., Madreperla, S.A., Hong, F., Allred, C., Grizzle, W.E. and Lee, W.H. (1990a) Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma. *Proc Natl Acad Sci U S A*, **87**, 7762-7766.

Bookstein, R., Shew, J.Y., Chen, P.L., Scully, P. and Lee, W.H. (1990b) Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated RB gene. *Science*, **247**, 712-715.

Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M. and Kidd, T. (1999) Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell*, **96**, 795-806.

Bulfone, A., Smiga, S.M., Shimamura, K., Peterson, A., Puelles, L. and Rubenstein, J.L. (1995) T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron*, **15**, 63-78.

Butt, S.J., Fuccillo, M., Nery, S., Noctor, S., Kriegstein, A., Corbin, J.G. and Fishell, G. (2005) The temporal and spatial origins of cortical interneurons predict their physiological subtype. *Neuron*, **48**, 591-604.

Callaghan, D.A., Dong, L., Callaghan, S.M., Hou, Y.X., Dagnino, L. and Slack, R.S. (1999) Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. *Dev Biol*, **207**, 257-270.

Cavenee, W.K., Dryja, T.P., Phillips, R.A., Benedict, W.F., Godbout, R., Gallie, B.L., Murphree, A.L., Strong, L.C. and White, R.L. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature*, **305**, 779-784.

Caviness, V.S., Jr. and Rakic, P. (1978) Mechanisms of cortical development: a view from mutations in mice. *Annu Rev Neurosci*, **1**, 297-326.

Chae, T., Kwon, Y.T., Bronson, R., Dikkes, P., Li, E. and Tsai, L.H. (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron*, **18**, 29-42.

Chan, F.K., Zhang, J., Cheng, L., Shapiro, D.N. and Winoto, A. (1995) Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol Cell Biol*, **15**, 2682-2688.

Chellappan, S.P., Hiebert, S., Mudryj, M., Horowitz, J.M. and Nevins, J.R. (1991) The E2F transcription factor is a cellular target for the RB protein. *Cell*, **65**, 1053-1061.

Chen, D., Livne-bar, I., Vanderluit, J.L., Slack, R.S., Agochiya, M. and Bremner, R. (2004) Cell-specific effects of RB or RB/p107 loss on retinal development implicate an intrinsically death-resistant cell-of-origin in retinoblastoma. *Cancer Cell*, **5**, 539-551.

Chow, K.N. and Dean, D.C. (1996) Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. *Mol Cell Biol*, **16**, 4862-4868.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A. and te Riele, H. (1992) Requirement for a functional Rb-1 gene in murine development. *Nature*, **359**, 328-330.

Claudio, P.P., Tonini, T. and Giordano, A. (2002) The retinoblastoma family: twins or distant cousins? *Genome Biol*, **3**, reviews3012.

Cloud, J.E., Rogers, C., Reza, T.L., Ziebold, U., Stone, J.R., Picard, M.H., Caron, A.M., Bronson, R.T. and Lees, J.A. (2002) Mutant mouse models reveal the relative roles of E2F1 and E2F3 in vivo. *Mol Cell Biol*, **22**, 2663-2672.

Connell-Crowley, L., Harper, J.W. and Goodrich, D.W. (1997) Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. *Mol Biol Cell*, **8**, 287-301.

Coppola, J.A., Lewis, B.A. and Cole, M.D. (1990) Increased retinoblastoma gene expression is associated with late stages of differentiation in many different cell types. *Oncogene*, **5**, 1731-1733.

Cuzon, V.C., Yeh, P.W., Cheng, Q. and Yeh, H.H. (2006) Ambient GABA promotes cortical entry of tangentially migrating cells derived from the medial ganglionic eminence. *Cereb Cortex*, **16**, 1377-1388.

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D.S., Sheldon, M. and Curran, T. (1999) Reelin is a ligand for lipoprotein receptors. *Neuron*, **24**, 471-479.

D'Arcangelo, G., Miao, G.G., Chen, S.C., Soares, H.D., Morgan, J.I. and Curran, T. (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature*, **374**, 719-723.

Dahiya, A., Wong, S., Gonzalo, S., Gavin, M. and Dean, D.C. (2001) Linking the Rb and polycomb pathways. *Mol Cell*, **8**, 557-569.

Dahme, T., Wood, J., Livingston, D.M. and Gaubatz, S. (2002) Two different E2F6 proteins generated by alternative splicing and internal translation initiation. *Eur J Biochem*, **269**, 5030-5036.

de Bruin, A., Maiti, B., Jakoi, L., Timmers, C., Buerki, R. and Leone, G. (2003a) Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. *J Biol Chem*, **278**, 42041-42049.

de Bruin, A., Wu, L., Saavedra, H.I., Wilson, P., Yang, Y., Rosol, T.J., Weinstein, M., Robinson, M.L. and Leone, G. (2003b) Rb function in extraembryonic lineages suppresses apoptosis in the CNS of Rb-deficient mice. *Proc Natl Acad Sci U S A*, **100**, 6546-6551.

Deacon, T.W., Pakzaban, P. and Isacson, O. (1994) The lateral ganglionic eminence is the origin of cells committed to striatal phenotypes: neural transplantation and developmental evidence. *Brain Res*, **668**, 211-219.

DeCaprio, J.A., Ludlow, J.W., Figge, J., Shew, J.Y., Huang, C.M., Lee, W.H., Marsilio, E., Paucha, E. and Livingston, D.M. (1988) SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell*, **54**, 275-283.

DeGregori, J., Leone, G., Miron, A., Jakoi, L. and Nevins, J.R. (1997) Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc Natl Acad Sci U S A*, **94**, 7245-7250.

Di Stefano, L., Jensen, M.R. and Helin, K. (2003) E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes. *Embo J*, **22**, 6289-6298.

Dick, F.A. and Dyson, N. (2003) pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. *Mol Cell*, **12**, 639-649.

Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A. and Weinberg, R.A. (1993) Physical interaction of the retinoblastoma protein with human D cyclins. *Cell*, **73**, 499-511.

Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R. and Goff, S.P. (1994) The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell*, **79**, 119-130.

Dunn, J.M., Phillips, R.A., Becker, A.J. and Gallie, B.L. (1988) Identification of germline and somatic mutations affecting the retinoblastoma gene. *Science*, **241**, 1797-1800.

Dyson, N., Bernards, R., Friend, S.H., Gooding, L.R., Hassell, J.A., Major, E.O., Pipas, J.M., Vandyke, T. and Harlow, E. (1990) Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. *J Virol*, **64**, 1353-1356.

Dyson, N., Howley, P.M., Munger, K. and Harlow, E. (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science*, **243**, 934-937.

Ebelt, H., Hufnagel, N., Neuhaus, P., Neuhaus, H., Gajawada, P., Simm, A., Muller-Werdan, U., Werdan, K. and Braun, T. (2005) Divergent siblings: E2F2 and E2F4 but not E2F1 and E2F3 induce DNA synthesis in cardiomyocytes without activation of apoptosis. *Circ Res*, **96**, 509-517.

Eischen, C.M., Packham, G., Nip, J., Fee, B.E., Hiebert, S.W., Zambetti, G.P. and Cleveland, J.L. (2001) Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. *Oncogene*, **20**, 6983-6993.

Feddersen, R.M., Clark, H.B., Yunis, W.S. and Orr, H.T. (1995) In vivo viability of postmitotic Purkinje neurons requires pRb family member function. *Mol Cell Neurosci*, **6**, 153-167.

Ferguson, K.L., McClellan, K.A., Vanderluit, J.L., McIntosh, W.C., Schuurmans, C., Polleux, F. and Slack, R.S. (2005) A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration. *Embo J*, **24**, 4381-4391.

Ferguson, K.L., Vanderluit, J.L., Hebert, J.M., McIntosh, W.C., Tibbo, E., MacLaurin, J.G., Park, D.S., Wallace, V.A., Vooijs, M., McConnell, S.K. and Slack, R.S. (2002) Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. *Embo J*, **21**, 3337-3346.

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Jr., Livingston, D.M., Orkin, S.H. and Greenberg, M.E. (1996) E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell*, **85**, 549-561.

Fitzgerald, D.P., Cole, S.J., Hammond, A., Seaman, C. and Cooper, H.M. (2006) Characterization of neogenin-expressing neural progenitor populations and

migrating neuroblasts in the embryonic mouse forebrain. *Neuroscience*, **142**, 703-716.

Flemington, E.K., Speck, S.H. and Kaelin, W.G., Jr. (1993) E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc Natl Acad Sci U S A*, **90**, 6914-6918.

Fortin, A., MacLaurin, J.G., Arbour, N., Cregan, S.P., Kushwaha, N., Callaghan, S.M., Park, D.S., Albert, P.R. and Slack, R.S. (2004) The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. *J Biol Chem*, **279**, 28706-28714.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M. and Dryja, T.P. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature*, **323**, 643-646.

Fung, Y.K., Murphree, A.L., T'Ang, A., Qian, J., Hinrichs, S.H. and Benedict, W.F. (1987) Structural evidence for the authenticity of the human retinoblastoma gene. *Science*, **236**, 1657-1661.

Furukawa, Y., Nishimura, N., Furukawa, Y., Satoh, M., Endo, H., Iwase, S., Yamada, H., Matsuda, M., Kano, Y. and Nakamura, M. (2002) Apaf-1 is a mediator of E2F-1-induced apoptosis. *J Biol Chem*, **277**, 39760-39768.

Gad, J.M., Keeling, S.L., Wilks, A.F., Tan, S.S. and Cooper, H.M. (1997) The expression patterns of guidance receptors, DCC and Neogenin, are spatially and temporally distinct throughout mouse embryogenesis. *Dev Biol*, **192**, 258-273.

Gilmore, E.C., Ohshima, T., Goffinet, A.M., Kulkarni, A.B. and Herrup, K. (1998) Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. *J Neurosci*, **18**, 6370-6377.

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z.X., Xu, G., Wydner, K.L., DeCaprio, J.A., Lawrence, J.B. and Livingston, D.M. (1994) E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev*, **8**, 2665-2679.

Giovanni, A., Keramaris, E., Morris, E.J., Hou, S.T., O'Hare, M., Dyson, N., Robertson, G.S., Slack, R.S. and Park, D.S. (2000) E2F1 mediates death of B-amyloid-treated cortical neurons in a manner independent of p53 and dependent on Bax and caspase 3. *J Biol Chem*, **275**, 11553-11560.

Gorski, J.A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J.L. and Jones, K.R. (2002) Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J Neurosci*, **22**, 6309-6314.

Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V. and Nadal-Ginard, B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell*, **72**, 309-324.

Guan, K.L., Jenkins, C.W., Li, Y., Nichols, M.A., Wu, X., O'Keefe, C.L., Matera, A.G. and Xiong, Y. (1994) Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev*, **8**, 2939-2952.

Hallstrom, T.C. and Nevins, J.R. (2003) Specificity in the activation and control of transcription factor E2F-dependent apoptosis. *Proc Natl Acad Sci U S A*, **100**, 10848-10853.

Hallstrom, T.C. and Nevins, J.R. (2006) Jab1 is a specificity factor for E2F1-induced apoptosis. *Genes Dev*, **20**, 613-623.

Hamasaki, T., Goto, S., Nishikawa, S. and Ushio, Y. (2001) A role of netrin-1 in the formation of the subcortical structure striatum: repulsive action on the migration of late-born striatal neurons. *J Neurosci*, **21**, 4272-4280.

Hannon, G.J. and Beach, D. (1994) p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature*, **371**, 257-261.

Hao, H., Dong, Y., Bowling, M.T., Gomez-Gutierrez, J.G., Zhou, H.S. and McMasters, K.M. (2007) E2F-1 induces melanoma cell apoptosis via PUMA up-regulation and Bax translocation. *BMC Cancer*, **7**, 24.

Harbour, J.W., Lai, S.L., Whang-Peng, J., Gazdar, A.F., Minna, J.D. and Kaye, F.J. (1988) Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science*, **241**, 353-357.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805-816.

Hebert, J.M. and McConnell, S.K. (2000) Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol*, **222**, 296-306.

Helin, K., Harlow, E. and Fattaey, A. (1993) Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol Cell Biol*, **13**, 6501-6508.

Hershko, T., Chaussepied, M., Oren, M. and Ginsberg, D. (2005) Novel link between E2F and p53: proapoptotic cofactors of p53 are transcriptionally upregulated by E2F. *Cell Death Differ*, **12**, 377-383.

Hershko, T. and Ginsberg, D. (2004) Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. *J Biol Chem*, **279**, 8627-8634.

Hevner, R.F., Neogi, T., Englund, C., Daza, R.A. and Fink, A. (2003) Cajal-Retzius cells in the mouse: transcription factors, neurotransmitters, and birthdays suggest a pallial origin. *Brain Res Dev Brain Res*, **141**, 39-53.

Hiesberger, T., Trommsdorff, M., Howell, B.W., Goffinet, A., Mumby, M.C., Cooper, J.A. and Herz, J. (1999) Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron*, **24**, 481-489.

Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A. and Sherr, C.J. (1995) Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol*, **15**, 2672-2681.

Holmberg, C., Helin, K., Sehested, M. and Karlstrom, O. (1998) E2F-1-induced p53-independent apoptosis in transgenic mice. *Oncogene*, **17**, 143-155.

Horowitz, J.M., Park, S.H., Bogenmann, E., Cheng, J.C., Yandell, D.W., Kaye, F.J., Minna, J.D., Dryja, T.P. and Weinberg, R.A. (1990) Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. *Proc Natl Acad Sci U S A*, **87**, 2775-2779.

Howell, B.W., Hawkes, R., Soriano, P. and Cooper, J.A. (1997) Neuronal position in the developing brain is regulated by mouse disabled-1. *Nature*, **389**, 733-737.

Hu, H. (1999) Chemorepulsion of neuronal migration by Slit2 in the developing mammalian forebrain. *Neuron*, **23**, 703-711.

Huang, H.J., Yee, J.K., Shew, J.Y., Chen, P.L., Bookstein, R., Friedmann, T., Lee, E.Y. and Lee, W.H. (1988) Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science*, **242**, 1563-1566.

Huh, M.S., Parker, M.H., Scime, A., Parks, R. and Rudnicki, M.A. (2004) Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. *J Cell Biol*, **166**, 865-876.

Hyde, R.K. and Griep, A.E. (2002) Unique roles for E2F1 in the mouse lens in the absence of functional pRB proteins. *Invest Ophthalmol Vis Sci*, **43**, 1509-1516.

Iavarone, A., Garg, P., Lasorella, A., Hsu, J. and Israel, M.A. (1994) The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev*, **8**, 1270-1284.

Iavarone, A., King, E.R., Dai, X.M., Leone, G., Stanley, E.R. and Lasorella, A. (2004) Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. *Nature*, **432**, 1040-1045.

Itoh, A., Levinson, S.F., Morita, T., Kourembanas, S., Brody, J.S. and Mitsialis, S.A. (1995) Structural characterization and specificity of expression of E2F-5: a new member of the E2F family of transcription factors. *Cell Mol Biol Res*, **41**, 147-154.

Ivey-Hoyle, M., Conroy, R., Huber, H.E., Goodhart, P.J., Oliff, A. and Heimbrook, D.C. (1993) Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. *Mol Cell Biol*, **13**, 7802-7812.

Iwanaga, R., Komori, H., Ishida, S., Okamura, N., Nakayama, K., Nakayama, K.I. and Ohtani, K. (2006) Identification of novel E2F1 target genes regulated in cell cycle-dependent and independent manners. *Oncogene*, **25**, 1786-1798.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A. and Weinberg, R.A. (1992) Effects of an Rb mutation in the mouse. *Nature*, **359**, 295-300.

Jiang, S.X., Sheldrick, M., Desbois, A., Slinn, J. and Hou, S.T. (2007) Neuropilin-1 is a direct target of the transcription factor E2F1 during cerebral ischemia-induced neuronal death in vivo. *Mol Cell Biol*, **27**, 1696-1705.

Jiang, Z., Zacksenhaus, E., Gallie, B.L. and Phillips, R.A. (1997) The retinoblastoma gene family is differentially expressed during embryogenesis. *Oncogene*, **14**, 1789-1797.

Johnson, D.G., Schwarz, J.K., Cress, W.D. and Nevins, J.R. (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature*, **365**, 349-352.

Kawasaki, T., Ito, K. and Hirata, T. (2006) Netrin 1 regulates ventral tangential migration of guidepost neurons in the lateral olfactory tract. *Development*, **133**, 845-853.

Keeling, S.L., Gad, J.M. and Cooper, H.M. (1997) Mouse Neogenin, a DCC-like molecule, has four splice variants and is expressed widely in the adult mouse and during embryogenesis. *Oncogene*, **15**, 691-700.

Kennedy, T.E., Serafini, T., de la Torre, J.R. and Tessier-Lavigne, M. (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell*, **78**, 425-435.

Kherrouche, Z., De Launoit, Y. and Monte, D. (2004) Human E2F6 is alternatively spliced to generate multiple protein isoforms. *Biochem Biophys Res Commun*, **317**, 749-760.

Kidd, T., Bland, K.S. and Goodman, C.S. (1999) Slit is the midline repellent for the robo receptor in Drosophila. *Cell*, **96**, 785-794.

Knudson, A.G., Jr. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A*, **68**, 820-823.

Kovesdi, I., Reichel, R. and Nevins, J.R. (1987) Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. *Proc Natl Acad Sci U S A*, **84**, 2180-2184.

Krek, W., Livingston, D.M. and Shirodkar, S. (1993) Binding to DNA and the retinoblastoma gene product promoted by complex formation of different E2F family members. *Science*, **262**, 1557-1560.

Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) Regulation of p53 stability by Mdm2. *Nature*, **387**, 299-303.

Kuo, G., Arnaud, L., Kronstad-O'Brien, P. and Cooper, J.A. (2005) Absence of Fyn and Src causes a reeler-like phenotype. *J Neurosci*, **25**, 8578-8586.

Lasorella, A., Noseda, M., Beyna, M., Yokota, Y. and Iavarone, A. (2000) Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature*, **407**, 592-598.

Lavdas, A.A., Grigoriou, M., Pachnis, V. and Parnavelas, J.G. (1999) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J Neurosci*, **19**, 7881-7888.

Lazzerini Denchi, E. and Helin, K. (2005) E2F1 is crucial for E2F-dependent apoptosis. *EMBO Rep*, **6**, 661-668.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H. and Bradley, A. (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature*, **359**, 288-294.

Lee, E.Y., To, H., Shew, J.Y., Bookstein, R., Scully, P. and Lee, W.H. (1988) Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science*, **241**, 218-221.

Lee, J.O., Russo, A.A. and Pavletich, N.P. (1998) Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature*, **391**, 859-865.

Lee, M.H., Reynisdottir, I. and Massague, J. (1995) Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev*, **9**, 639-649.

Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y. and Lee, E.Y. (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science*, **235**, 1394-1399.

Lees, J.A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N. and Helin, K. (1993) The retinoblastoma protein binds to a family of E2F transcription factors. *Mol Cell Biol*, **13**, 7813-7825.

Leone, G., Nuckolls, F., Ishida, S., Adams, M., Sears, R., Jakoi, L., Miron, A. and Nevins, J.R. (2000) Identification of a novel E2F3 product suggests a mechanism

for determining specificity of repression by Rb proteins. *Mol Cell Biol*, **20**, 3626-3632.

Lipinski, M.M., Macleod, K.F., Williams, B.O., Mullaney, T.L., Crowley, D. and Jacks, T. (2001) Cell-autonomous and non-cell-autonomous functions of the Rb tumor suppressor in developing central nervous system. *Embo J*, **20**, 3402-3413.

Lukas, J., Petersen, B.O., Holm, K., Bartek, J. and Helin, K. (1996) Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. *Mol Cell Biol*, **16**, 1047-1057.

Lundberg, A.S. and Weinberg, R.A. (1998) Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol*, **18**, 753-761.

Luo, R.X., Postigo, A.A. and Dean, D.C. (1998) Rb interacts with histone deacetylase to repress transcription. *Cell*, **92**, 463-473.

Maandag, E.C., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A. and te Riele, H. (1994) Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *Embo J*, **13**, 4260-4268.

MacManus, J.P., Jian, M., Preston, E., Rasquinha, I., Webster, J. and Zurakowski, B. (2003) Absence of the transcription factor E2F1 attenuates brain injury and improves behavior after focal ischemia in mice. *J Cereb Blood Flow Metab*, **23**, 1020-1028.

MacPherson, D., Sage, J., Crowley, D., Trumpp, A., Bronson, R.T. and Jacks, T. (2003) Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. *Mol Cell Biol*, **23**, 1044-1053.

MacPherson, D., Sage, J., Kim, T., Ho, D., McLaughlin, M.E. and Jacks, T. (2004) Cell type-specific effects of Rb deletion in the murine retina. *Genes Dev*, **18**, 1681-1694.

Maiti, B., Li, J., de Bruin, A., Gordon, F., Timmers, C., Opavsky, R., Patil, K., Tuttle, J., Cleghorn, W. and Leone, G. (2005) Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. *J Biol Chem*, **280**, 18211-18220.

Malumbres, M. and Barbacid, M. (2005) Mammalian cyclin-dependent kinases. *Trends Biochem Sci*, **30**, 630-641.

Marillat, V., Cases, O., Nguyen-Ba-Charvet, K.T., Tessier-Lavigne, M., Sotelo, C. and Chedotal, A. (2002) Spatiotemporal expression patterns of slit and robo genes in the rat brain. *J Comp Neurol*, **442**, 130-155.

Marin, O., Plump, A.S., Flames, N., Sanchez-Camacho, C., Tessier-Lavigne, M. and Rubenstein, J.L. (2003) Directional guidance of interneuron migration to the cerebral cortex relies on subcortical Slit1/2-independent repulsion and cortical attraction. *Development*, **130**, 1889-1901.

Marino, S., Hoogervoorst, D., Brandner, S. and Berns, A. (2003) Rb and p107 are required for normal cerebellar development and granule cell survival but not for Purkinje cell persistence. *Development*, **130**, 3359-3368.

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J. and Kato, J.Y. (1994) D-type cyclin-dependent kinase activity in mammalian cells. *Mol Cell Biol*, **14**, 2066-2076.

McClellan, K.A., Ruzhynsky, V.A., Douda, D.N., Vanderluit, J.L., Ferguson, K.L., Chen, D., Bremner, R., Park, D.S., Leone, G. and Slack, R.S. (2007) A unique requirement for Rb/E2F3 in neuronal migration: Evidence for cell cycle independent functions. *Mol Cell Biol.*

McConnell, S.K. (1995) Constructing the cerebral cortex: neurogenesis and fate determination. *Neuron*, **15**, 761-768.

Melino, G., Bernassola, F., Ranalli, M., Yee, K., Zong, W.X., Corazzari, M., Knight, R.A., Green, D.R., Thompson, C. and Vousden, K.H. (2004) p73 Induces apoptosis via PUMA transactivation and Bax mitochondrial translocation. *J Biol Chem*, **279**, 8076-8083.

Metin, C., Baudoin, J.P., Rakic, S. and Parnavelas, J.G. (2006) Cell and molecular mechanisms involved in the migration of cortical interneurons. *Eur J Neurosci*, **23**, 894-900.

Meyerson, M. and Harlow, E. (1994) Identification of G1 kinase activity for cdk6, a novel cyclin D partner. *Mol Cell Biol*, **14**, 2077-2086.

Moon, N.S., Di Stefano, L. and Dyson, N. (2006) A gradient of epidermal growth factor receptor signaling determines the sensitivity of rbf1 mutant cells to E2F-dependent apoptosis. *Mol Cell Biol*, **26**, 7601-7615.

Morest, D.K. (1970) A study of neurogenesis in the forebrain of opossum pouch young. *Z Anat Entwicklungsgesch*, **130**, 265-305.

Nadarajah, B., Brunstrom, J.E., Grutzendler, J., Wong, R.O. and Pearlman, A.L. (2001) Two modes of radial migration in early development of the cerebral cortex. *Nat Neurosci*, **4**, 143-150.

Nery, S., Fishell, G. and Corbin, J.G. (2002) The caudal ganglionic eminence is a source of distinct cortical and subcortical cell populations. *Nat Neurosci*, **5**, 1279-1287.

Nguyen Ba-Charvet, K.T., Brose, K., Marillat, V., Kidd, T., Goodman, C.S., Tessier-Lavigne, M., Sotelo, C. and Chedotal, A. (1999) Slit2-Mediated chemorepulsion and collapse of developing forebrain axons. *Neuron*, **22**, 463-473.

Nguyen, L., Besson, A., Heng, J.I., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J.M. and Guillemot, F. (2006) p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. *Genes Dev*, **20**, 1511-1524.

Nielsen, S.J., Schneider, R., Bauer, U.M., Bannister, A.J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R.E. and Kouzarides, T. (2001) Rb targets histone H3 methylation and HP1 to promoters. *Nature*, **412**, 561-565.

Novitch, B.G., Mulligan, G.J., Jacks, T. and Lassar, A.B. (1996) Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J Cell Biol*, **135**, 441-456.

O'Hare, M.J., Hou, S.T., Morris, E.J., Cregan, S.P., Xu, Q., Slack, R.S. and Park, D.S. (2000) Induction and modulation of cerebellar granule neuron death by E2F-1. *J Biol Chem*, **275**, 25358-25364.

O'Rourke, N.A., Dailey, M.E., Smith, S.J. and McConnell, S.K. (1992) Diverse migratory pathways in the developing cerebral cortex. *Science*, **258**, 299-302.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H. and Mikoshiba, K. (1995) The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron*, **14**, 899-912.

Ohshima, T., Ogawa, M., Veeranna, Hirasawa, M., Longenecker, G., Ishiguro, K., Pant, H.C., Brady, R.O., Kulkarni, A.B. and Mikoshiba, K. (2001) Synergistic contributions of cyclin-dependant kinase 5/p35 and Reelin/Dab1 to the positioning of cortical neurons in the developing mouse brain. *Proc Natl Acad Sci U S A*, **98**, 2764-2769.

Ohshima, T., Suzuki, H., Morimura, T., Ogawa, M. and Mikoshiba, K. (2007) Modulation of Reelin signaling by Cyclin-dependent kinase 5. *Brain Res*, **1140**, 84-95.

Olsson, M., Campbell, K. and Turnbull, D.H. (1997) Specification of mouse telencephalic and mid-hindbrain progenitors following heterotopic ultrasound-guided embryonic transplantation. *Neuron*, **19**, 761-772.

Park, D.S., Morris, E.J., Bremner, R., Keramaris, E., Padmanabhan, J., Rosenbaum, M., Shelanski, M.L., Geller, H.M. and Greene, L.A. (2000) Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. *J Neurosci*, **20**, 3104-3114.

Park, K.W., Crouse, D., Lee, M., Karnik, S.K., Sorensen, L.K., Murphy, K.J., Kuo, C.J. and Li, D.Y. (2004) The axonal attractant Netrin-1 is an angiogenic factor. *Proc Natl Acad Sci U S A*, **101**, 16210-16215.

Paulson, Q.X., McArthur, M.J. and Johnson, D.G. (2006) E2F3a stimulates proliferation, p53-independent apoptosis and carcinogenesis in a transgenic mouse model. *Cell Cycle*, **5**, 184-190.

Pennaneach, V., Barbier, V., Regazzoni, K., Fotedar, R. and Fotedar, A. (2004) Rb inhibits E2F-1-induced cell death in a LXCXE-dependent manner by active repression. *J Biol Chem*, **279**, 23376-23383.

Polyak, K., Kato, J.Y., Solomon, M.J., Sherr, C.J., Massague, J., Roberts, J.M. and Koff, A. (1994) p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev*, **8**, 9-22.

Qin, X.Q., Chittenden, T., Livingston, D.M. and Kaelin, W.G., Jr. (1992) Identification of a growth suppression domain within the retinoblastoma gene product. *Genes Dev*, **6**, 953-964.

Quelle, D.E., Ashmun, R.A., Hannon, G.J., Rehberger, P.A., Trono, D., Richter, K.H., Walker, C., Beach, D., Sherr, C.J. and Serrano, M. (1995) Cloning and characterization of murine p16INK4a and p15INK4b genes. *Oncogene*, **11**, 635-645.

Rajagopalan, S., Deitinghoff, L., Davis, D., Conrad, S., Skutella, T., Chedotal, A., Mueller, B.K. and Strittmatter, S.M. (2004) Neogenin mediates the action of repulsive guidance molecule. *Nat Cell Biol*, **6**, 756-762.

Rakic, P. (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol*, **145**, 61-83.

Reid, C.B., Liang, I. and Walsh, C. (1995) Systematic widespread clonal organization in cerebral cortex. *Neuron*, **15**, 299-310.

Ren, S. and Rollins, B.J. (2004) Cyclin C/cdk3 promotes Rb-dependent G0 exit. *Cell*, **117**, 239-251.

Rickmann, M. and Wolff, J.R. (1981) Differentiation of 'preplate' neurons in the pallium of the rat. *Bibl Anat*, 142-146.

Rothberg, J.M., Hartley, D.A., Walther, Z. and Artavanis-Tsakonas, S. (1988) slit: an EGF-homologous locus of D. melanogaster involved in the development of the embryonic central nervous system. *Cell*, **55**, 1047-1059.

Rothberg, J.M., Jacobs, J.R., Goodman, C.S. and Artavanis-Tsakonas, S. (1990) slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. *Genes Dev*, **4**, 2169-2187.

Rubenstein, J.L., Anderson, S., Shi, L., Miyashita-Lin, E., Bulfone, A. and Hevner, R. (1999) Genetic control of cortical regionalization and connectivity. *Cereb Cortex*, **9**, 524-532.

Sawamoto, K., Wichterle, H., Gonzalez-Perez, O., Cholfin, J.A., Yamada, M., Spassky, N., Murcia, N.S., Garcia-Verdugo, J.M., Marin, O., Rubenstein, J.L., Tessier-Lavigne, M., Okano, H. and Alvarez-Buylla, A. (2006) New neurons follow the flow of cerebrospinal fluid in the adult brain. *Science*, **311**, 629-632.

Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Beddington, R., Skarnes, W.C. and Tessier-Lavigne, M. (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell*, **87**, 1001-1014.

Serrano, M., Hannon, G.J. and Beach, D. (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704-707.

Shan, B. and Lee, W.H. (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol Cell Biol*, **14**, 8166-8173.

Shan, B., Zhu, X., Chen, P.L., Durfee, T., Yang, Y., Sharp, D. and Lee, W.H. (1992) Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. *Mol Cell Biol*, **12**, 5620-5631.

Sharma, N., Timmers, C., Trikha, P., Saavedra, H.I., Obery, A. and Leone, G. (2006) Control of the p53-p21CIP1 Axis by E2f1, E2f2, and E2f3 is essential for G1/S progression and cellular transformation. *J Biol Chem*, **281**, 36124-36131.

Sherr, C.J. and Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*, **13**, 1501-1512.

Shu, T. and Richards, L.J. (2001) Cortical axon guidance by the glial wedge during the development of the corpus callosum. *J Neurosci*, **21**, 2749-2758.

Slack, R.S., El-Bizri, H., Wong, J., Belliveau, D.J. and Miller, F.D. (1998) A critical temporal requirement for the retinoblastoma protein family during neuronal determination. *J Cell Biol*, **140**, 1497-1509.

Slack, R.S., Hamel, P.A., Bladon, T.S., Gill, R.M. and McBurney, M.W. (1993) Regulated expression of the retinoblastoma gene in differentiating embryonal carcinoma cells. *Oncogene*, **8**, 1585-1591.

Slack, R.S., Skerjanc, I.S., Lach, B., Craig, J., Jardine, K. and McBurney, M.W. (1995) Cells differentiating into neuroectoderm undergo apoptosis in the absence of functional retinoblastoma family proteins. *J Cell Biol*, **129**, 779-788.

Stenman, J., Toresson, H. and Campbell, K. (2003) Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. *J Neurosci*, **23**, 167-174.

Stiewe, T. and Putzer, B.M. (2000) Role of the p53-homologue p73 in E2F1-induced apoptosis. *Nat Genet*, **26**, 464-469.

Stolt, P.C. and Bock, H.H. (2006) Modulation of lipoprotein receptor functions by intracellular adaptor proteins. *Cell Signal*, **18**, 1560-1571.

T'Ang, A., Varley, J.M., Chakraborty, S., Murphree, A.L. and Fung, Y.K. (1988) Structural rearrangement of the retinoblastoma gene in human breast carcinoma. *Science*, **242**, 263-266.

Takahashi, T., Goto, T., Miyama, S., Nowakowski, R.S. and Caviness, V.S., Jr. (1999) Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. *J Neurosci*, **19**, 10357-10371.

Takahashi, T., Nowakowski, R.S. and Caviness, V.S., Jr. (1995) The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J Neurosci*, **15**, 6046-6057.

Takahashi, Y., Rayman, J.B. and Dynlacht, B.D. (2000) Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev*, **14**, 804-816.

Tan, S.S., Faulkner-Jones, B., Breen, S.J., Walsh, M., Bertram, J.F. and Reese, B.E. (1995) Cell dispersion patterns in different cortical regions studied with an X-inactivated transgenic marker. *Development*, **121**, 1029-1039.

Tan, S.S., Kalloniatis, M., Sturm, K., Tam, P.P., Reese, B.E. and Faulkner-Jones, B. (1998) Separate progenitors for radial and tangential cell dispersion during development of the cerebral neocortex. *Neuron*, **21**, 295-304.

Tanaka, D.H., Maekawa, K., Yanagawa, Y., Obata, K. and Murakami, F. (2006) Multidirectional and multizonal tangential migration of GABAergic interneurons in the developing cerebral cortex. *Development*, **133**, 2167-2176.

Timmers, C., Sharma, N., Opavsky, R., Maiti, B., Wu, L., Wu, J., Orringer, D., Trikha, P., Saavedra, H.I. and Leone, G. (2006) E2f1-3 Control E2F-target Expression and Cellular Proliferation via a p53-dependent Negative Feedback Loop. *Mol Cell Biol.*

Trimarchi, J.M., Fairchild, B., Verona, R., Moberg, K., Andon, N. and Lees, J.A. (1998) E2F-6, a member of the E2F family that can behave as a transcriptional repressor. *Proc Natl Acad Sci U S A*, **95**, 2850-2855.

Trimarchi, J.M. and Lees, J.A. (2002) Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol*, **3**, 11-20.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R.E., Richardson, J.A. and Herz, J. (1999) Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell*, **97**, 689-701.

Tsai, K.Y., Hu, Y., Macleod, K.F., Crowley, D., Yamasaki, L. and Jacks, T. (1998) Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. *Mol Cell*, **2**, 293-304.

Vairo, G., Livingston, D.M. and Ginsberg, D. (1995) Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes Dev*, **9**, 869-881.

Valcanis, H. and Tan, S.S. (2003) Layer specification of transplanted interneurons in developing mouse neocortex. *J Neurosci*, **23**, 5113-5122.

Vooijs, M., van der Valk, M., te Riele, H. and Berns, A. (1998) Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse. *Oncogene*, **17**, 1-12.

Walkley, C.R. and Orkin, S.H. (2006) Rb is dispensable for self-renewal and multilineage differentiation of adult hematopoietic stem cells. *Proc Natl Acad Sci U S A*, **103**, 9057-9062.

Wallace, V.A. and Raff, M.C. (1999) A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. *Development*, **126**, 2901-2909.

Walsh, C. and Cepko, C.L. (1988) Clonally related cortical cells show several migration patterns. *Science*, **241**, 1342-1345.

Wang, D., Russell, J.L. and Johnson, D.G. (2000) E2F4 and E2F1 have similar proliferative properties but different apoptotic and oncogenic properties in vivo. *Mol Cell Biol*, **20**, 3417-3424.

Wang, J.Y. (2000) Regulation of cell death by the Abl tyrosine kinase. *Oncogene*, **19**, 5643-5650.

Wang, J.Y. (2005) Nucleo-cytoplasmic communication in apoptotic response to genotoxic and inflammatory stress. *Cell Res*, **15**, 43-48.

Wenzel, P.L., Wu, L., de Bruin, A., Chong, J.L., Chen, W.Y., Dureska, G., Sites, E., Pan, T., Sharma, A., Huang, K., Ridgway, R., Mosaliganti, K., Sharp, R., Machiraju, R., Saltz, J., Yamamoto, H., Cross, J.C., Robinson, M.L. and Leone, G. (2007) Rb is critical in a mammalian tissue stem cell population. *Genes Dev*, **21**, 85-97.

Wichterle, H., Garcia-Verdugo, J.M., Herrera, D.G. and Alvarez-Buylla, A. (1999) Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat Neurosci*, **2**, 461-466.

Wichterle, H., Turnbull, D.H., Nery, S., Fishell, G. and Alvarez-Buylla, A. (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development*, **128**, 3759-3771.

Wu, C.L., Zukerberg, L.R., Ngwu, C., Harlow, E. and Lees, J.A. (1995) In vivo association of E2F and DP family proteins. *Mol Cell Biol*, **15**, 2536-2546.

Wu, L., de Bruin, A., Saavedra, H.I., Starovic, M., Trimboli, A., Yang, Y., Opavska, J., Wilson, P., Thompson, J.C., Ostrowski, M.C., Rosol, T.J., Woollett, L.A., Weinstein, M., Cross, J.C., Robinson, M.L. and Leone, G. (2003) Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature*, **421**, 942-947.

Wu, L., Timmers, C., Maiti, B., Saavedra, H.I., Sang, L., Chong, G.T., Nuckolls, F., Giangrande, P., Wright, F.A., Field, S.J., Greenberg, M.E., Orkin, S., Nevins, J.R., Robinson, M.L. and Leone, G. (2001) The E2F1-3 transcription factors are essential for cellular proliferation. *Nature*, **414**, 457-462.

Wu, W., Wong, K., Chen, J., Jiang, Z., Dupuis, S., Wu, J.Y. and Rao, Y. (1999) Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature*, **400**, 331-336.

Wu, X. and Levine, A.J. (1994) p53 and E2F-1 cooperate to mediate apoptosis. *Proc Natl Acad Sci U S A*, **91**, 3602-3606.

Xu, H.J., Hu, S.X., Cagle, P.T., Moore, G.E. and Benedict, W.F. (1991) Absence of retinoblastoma protein expression in primary non-small cell lung carcinomas. *Cancer Res*, **51**, 2735-2739.

Xu, Q., Cobos, I., De La Cruz, E., Rubenstein, J.L. and Anderson, S.A. (2004) Origins of cortical interneuron subtypes. *J Neurosci*, **24**, 2612-2622.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E. and Dyson, N.J. (1996) Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell*, **85**, 537-548.

Yamashita, T., Mueller, B.K. and Hata, K. (2007) Neogenin and repulsive guidance molecule signaling in the central nervous system. *Curr Opin Neurobiol*, **17**, 29-34.

Yang, H.L., Dong, Y.B., Elliott, M.J., Liu, T.J. and McMasters, K.M. (2000) Caspase activation and changes in Bcl-2 family member protein expression associated with E2F-1-mediated apoptosis in human esophageal cancer cells. *Clin Cancer Res*, **6**, 1579-1589.

Yee, K.T., Simon, H.H., Tessier-Lavigne, M. and O'Leary, D.M. (1999) Extension of long leading processes and neuronal migration in the mammalian brain directed by the chemoattractant netrin-1. *Neuron*, **24**, 607-622.

Yuan, W., Zhou, L., Chen, J.H., Wu, J.Y., Rao, Y. and Ornitz, D.M. (1999) The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. *Dev Biol*, **212**, 290-306.

Zamzami, N. and Kroemer, G. (2005) p53 in apoptosis control: an introduction. *Biochem Biophys Res Commun*, **331**, 685-687.

Zhang, J., Gray, J., Wu, L., Leone, G., Rowan, S., Cepko, C.L., Zhu, X., Craft, C.M. and Dyer, M.A. (2004) Rb regulates proliferation and rod photoreceptor development in the mouse retina. *Nat Genet*, **36**, 351-360.

Zhu, Y., Li, H., Zhou, L., Wu, J.Y. and Rao, Y. (1999) Cellular and molecular guidance of GABAergic neuronal migration from an extracortical origin to the neocortex. *Neuron*, **23**, 473-485.