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The Role of the Retinoblastoma Protein in Cortical Neurogenesis

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ABSTRACT

The retinoblastoma (Rb) protein was known to be a critical cell cycle regulator in tumour cells, however, little was known regarding its role in normal neural development. To determine the importance of the Rb signalling pathway in neural precursor proliferation and differentiation, I first characterized the key cell cycle regulators and examined the requirement for Rb in these processes. In contrast to previous studies, dominant negative CDK4/6 mutants induced mitotic arrest in neural precursors, despite the binding and sequestration of the Cip/Kip CKIs, $p21^{Cip1}$ and $p27^{Kip1}$. The activity of these mutants was Rb-dependent, indicating that activation of the Rb pathway was sufficient to cause cell cycle arrest in neural precursor cells. While the phenotype of Rb germline knockouts indicated a requirement for Rb in neural precursor differentiation and survival, embryonic lethality and contradictions in the literature precluded a definitive answer. To assess the neural requirement for Rb in the absence of pleiotropic defects, I examined a conditional knockout in which Rb was specifically deleted in the developing telencephalon. These mutants survived to birth and exhibited minimal apoptosis. Although Rb-/- progenitor cells divided ectopically, they were able to survive and differentiate. Due to increased neuroblast proliferation, mutant telencephalic lobes were significantly enlarged. These studies demonstrated that cell cycle deregulation during differentiation does not necessitate apoptosis and terminal mitosis may not be required to initiate differentiation. I next examined the impact of Rb deficiency on cortical development and neuronal differentiation. By histological examination and *in situ* analyses, Rb mutants were found to exhibit defective laminar organization and region-specific cellularity. Although the majority of cortical neurons survived in the absence of Rb, specific populations, such as Cajal-Retzius neurons, required Rb for survival. By the selective reduction of calbindin- and Lhx6-positive

interneurons from the cortical MZ, Rb was shown to regulate these tangentially migrating populations. These studies revealed a role for Rb in the development of cortical architecture, neuronal migration and its requirement in diverse neuronal populations. Together, my studies demonstrated critical functions for Rb in the developing cortex, from the regulation of precursor differentiation to cell type-specific requirements in neuronal survival and migration.

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LIST OF ABBREVIATIONS

Ad	adenovirus
ALS	amyotrophic lateral sclerosis
Apaf1	apoptosis associated factor 1
ApoER2	apolipoprotein E receptor 2
ATP	adenosine tri-phosphate
Bcl2	B-cell leukemia 2
BDNF	brain-derived neurotrophic factor
BF-1	brain factor-1
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BrdU	bromo-deoxyuridine
BRG1	brahma-related gene 1
BRM	brahma
BSA	bovine serum albumin
CAA	cortical attractive activity
c-abl	cellular- Abelson murine leukemia virus
CDK	cyclin-dependent kinase
C/EBP	CCAAT/enhancer binding protein
c-fos	cellular- FBJ murine osteosarcoma virus
CGE	caudal ganglionic eminence
CKI	cyclin-dependent kinase inhibitor
CNS	central nervous system

CP	cortical plate
CR	Cajal-Retzius neuron
CXCR4	CXC chemokine receptor 4
DAB1	mouse homologue of *Drosophila* Disabled
DHFR	dihydrofolate reductase
Dlx	Distal-less homeobox gene
DMEM	Dulbecco's Modified Eagle Media
Dn	dominant negative
DNA	deoxyribonucleic acid
DTT	1,4-dithiothreitol
E	embryonic day
E1A	adenovirus early region 1A
E2F	E2 promoter binding factor
Ebi	`shrimp' in Japanese
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acidxd./
Emx2	empty spiracles (drosophila) homologue 2
Foxg1	forkhead box G1
G0	gap 0 (quiescence)
G1	gap1 (interphase)
G2	gap 2 (interphase)
GABA	γ-aminobutyric acid
GE	ganglionic eminence
GPI	glycosylphosphatidylinositol

HCl	hydrochloric acid
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hes1	hairy and Enhancer of split homolog 1
HGF/SF	hepatocyte growth factor/scatter factor
HPV-16	human papillomavirus
HRP	horseradish peroxidase
GFAP	glial fibrillary acidic protein
Id2	inhibitor of differentiation-2
INK4	inhibitors of cdk4
IZ	intermediate zone
LacZ	beta-galactosidase
LDL	low density lipoprotein
LGE	lateral ganglionic eminence
Lhx6	LIM homeobox gene 6
M	mitosis
MAP2	microtubule-associated protein 2
MCK	creatine kinase M
MCM7	minichromosome maintenance 7
MDM-2	murine double minute-2
MGE	medial ganglionic eminence
MOI	multiplicity of infection
MRF4	muscle regulatory factor 4
MyoD	myogenic determination factor

MZ	marginal zone
NGF	nerve growth factor
Npn-1 or 2	neuropilin-1 or -2
NT-3	neurotrophin-3
NT-4	neurotrophin-4
P	post-natal day
PBS	phosphate buffered saline
PC-12	pheochromocytoma-12
PCR	polymerase chain reaction
PFA	paraformaldehyde
PH3	phospho-histone H3
PI-PLC	phosphatidylinositol-specific phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PNS	peripheral nervous system
POa	preoptic area
PP	preplate
Pten	phosphatase and tensin homolog deleted on chromosome ten
Rb	retinoblastoma
RbK	Rb/histone H1 kinase
RBP1	Rb-binding protein
RT	room temperature
S	DNA synthesis
SCG10	superior cervical ganglia-10
SDF-1	stromal cell-derived factor-1

SDS PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sema3A/F	class 3 semaphorin A/F
SP	subplate
SV-40	simian virus-40
SWI/SNF	switching/sucrose nonfermenting
Tag	large T antigen
TAG-1	transient axonal glycoprotein-1
Tbr1	T-brain-1
TPBS	Tris-phosphate buffered saline
TrkA	tyrosine receptor kinase A
TrkB	tyrosine receptor kinase B
TSA	trichostatin A
Ttk88	Tramtrack88
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling
u-PAR	urokinase-type plasminogen activator receptor
VLDLR	very-low-density lipoprotein receptor
VZ	ventricular zone

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PREFACE

In accordance with the guidelines established by the School of Graduate Studies and Research (SGSR) of the University of Ottawa, the experimental findings presented in this thesis are comprised of published or submitted journal articles.

The experimental findings of this thesis are preceded by an overview of the current state of knowledge regarding retinoblastoma (Rb) function, an overview of cortical development, as well as the contradictions and complications in the current understanding of the field (Chapter 1). The subsequent chapters (2, 3, and 4) are three manuscripts examining the requirement of Rb in cortical neurogenesis. These experimental findings are summarized and discussed in the context of the relevant literature in Chapter 5.

Each of the articles included in this thesis is prefaced with a Statement of Contributions of Collaborators, as required by the SGSR (University of Ottawa). Portions of this thesis are reprinted with permission from: The Journal of Biological Chemistry and the EMBO Journal. Copies of official letters granting permission to reproduce the articles are included in Appendix A.

Chapter 1. Introduction to Rb Function

The aim of the introductory chapter of this thesis is to provide the reader with a broad overview of the role of Rb in neural development. In this first section, I will summarize Rb function in cell cycle regulation, focusing on the mechanisms of its activity through phosphorylation, and its ability to repress cell cycle progression by targeting the E2F family of transcription factors.

I. Rb Function in Cell Cycle Regulation

I-I. Overview

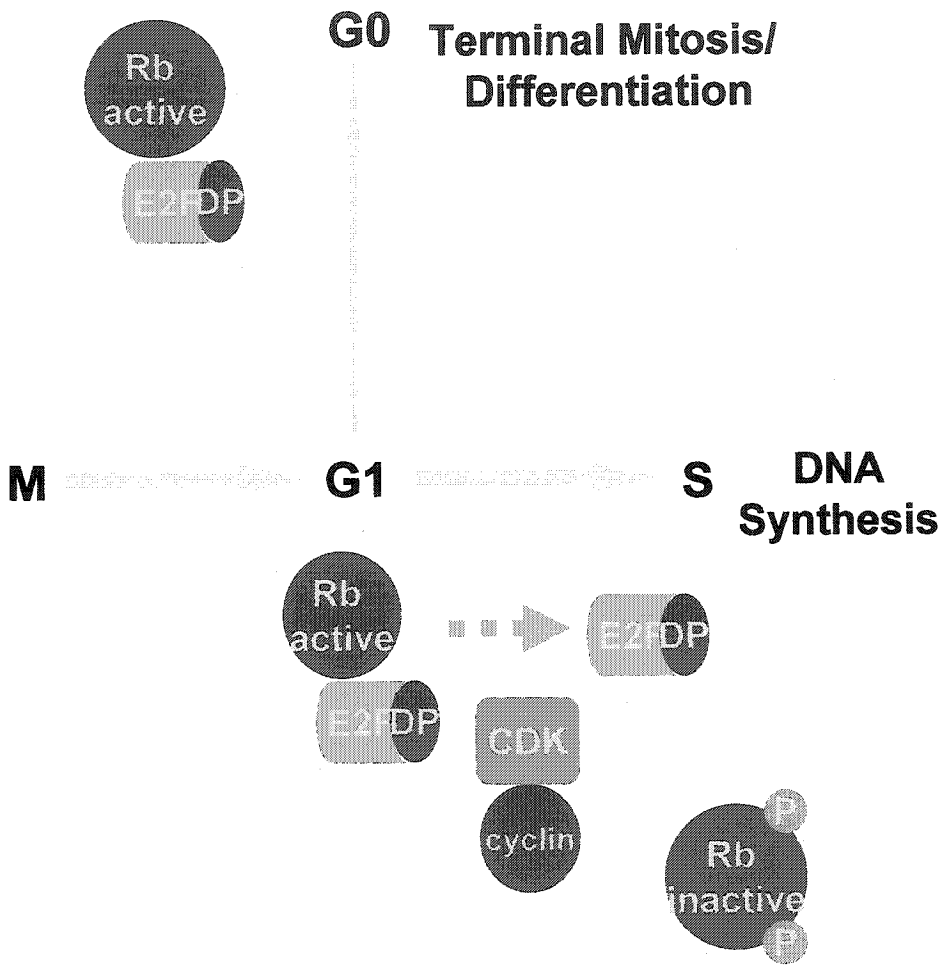
Regulation of cellular division is a complex process. The mammalian cell cycle is divided into four distinct phases, referred to as G1, S, G2, and M phases. The two gap periods, G1 and G2, are growth phases, which are followed, respectively, by DNA synthesis and replication (S phase) and mitosis (M phase) (Sherr, 1994). In late G1, cells pass through the restriction point, at which they commit to complete the cell cycle, independently of further growth factor signalling. Passage through the restriction point and entry into S phase is regulated by Rb phosphorylation by cyclin-dependent kinases (CDKs) (Chen *et al.*, 1989; Hinds *et al.*, 1992; Lundberg and Weinberg, 1998). When underphosphorylated, Rb is active and able to bind to and repress transcription factors which promote proliferation, most notably, the E2F family of transcription factors. Phosphorylation by CDKs inactivates Rb, thereby releasing these transcription factors and driving S phase progression (Buchkovich *et al.*, 1989). CDK activity is, in turn, regulated by 1) the synthesis and binding of specific

regulatory subunits, the cyclins, 2) the association and dissociation of CDK inhibitors (CKIs), and 3) by inhibitory or activating phosphorylation events. See also reviews (Dyson, 1998; Mulligan and Jacks, 1998; Lipinski and Jacks, 1999; Harbour and Dean, 2000; Stevaux and Dyson, 2002) (Fig. 1-1: The Rb pathway in cell cycle regulation).

I-II. Rb as tumour suppressor

The retinoblastoma susceptibility gene (*RB*) gene was the first tumour suppressor gene to be identified. It was initially discovered due to its mutation in the rare pediatric eye tumour, retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987b). Retinoblastoma tumours can occur as sporadic or hereditary cases, and can be used as a paradigm for tumourigenesis through loss-of-function mutations (Weinberg, 1991). In familial cases of retinoblastoma, young children develop bilateral multifocal retinal tumours, such that individuals carrying a germline mutation for one *RB* allele have a 95% chance of developing retinoblastoma (Gallie *et al.*, 1990). By statistical analysis of the affected families, Knudson proposed that the children inherited one defective autosomal allele and the second wildtype allele was lost during retinal development, leading to retinoblastoma (Knudson, 1971). The deletion or mutational inactivation of one *RB* allele is, therefore, the rate limiting step for the development of these retinal tumours (Knudson, 1971; Friend *et al.*, 1986; Lee *et al.*, 1987a; Bookstein *et al.*, 1988; Hong *et al.*, 1989). In hereditary cases, patients have over a 30-fold increased risk of developing a second primary tumour, such as

Fig. 1-1: The Rb pathway in cell cycle regulation. Rb phosphorylation and activity vary according to the phase of the cell cycle. In G0 and early G1, Rb is in its underphosphorylated, active state and is able to bind and repress E2F transcription factors. During G1, Rb becomes phosphorylated by multiple CDK-cyclin complexes. Hyperphosphorylated Rb then releases E2Fs, allowing them to transactivate genes required for DNA synthesis and cell cycle progression.


G0
Terminal Mitosis/
Differentiation
Rb
active
E2F
DP
M
G1
S
DNA
Synthesis
Rb
active
E2F
DP
E2F
DP
CDK
cyclin
P
Rb
inactive
P

osteosarcoma, melanoma, and brain tumours (Eng *et al.*, 1993; Moll *et al.*, 1997). The *RB* gene, which is located on human chromosome 13q, is in an area of the genome frequently lost in sporadic forms of cancer. Mutations are often associated with tumours in several cell types, including sporadic retinoblastoma, osteosarcoma, small cell lung carcinomas, and cancers of the bladder, kidney, prostate and breast (Friend *et al.*, 1986; Fung *et al.*, 1987; Harbour *et al.*, 1988; Lee *et al.*, 1988; T'Ang *et al.*, 1988; Bookstein *et al.*, 1990; Horowitz *et al.*, 1990; Xu *et al.*, 1991).

The *RB* gene product, Rb, was identified as a target of oncoproteins expressed by DNA tumour viruses including the adenovirus E1A protein, the simian virus 40 large T antigen (SV40 Tag) (DeCaprio *et al.*, 1988), human papillomavirus (HPV) E7 protein (Dyson *et al.*, 1989; Munger *et al.*, 1989), and the large T antigen of polyomaviruses (Dyson *et al.*, 1990). As these viral oncoproteins are capable of immortalizing and transforming various cell types, studies examining their properties demonstrated the importance of Rb as a regulator of cell proliferation. By direct binding, these oncoproteins had the capacity to interfere with the growth suppressive functions of Rb. Inactivation of Rb by E1A was essential to drive cells into a proliferative state (Whyte *et al.*, 1988; Egan *et al.*, 1989). Mutations in the Rb binding region of any of these viral oncoproteins abrogated their capacity for viral transformation (Egan *et al.*, 1989).

Rb is a 110 kDa nuclear phosphoprotein (Lee *et al.*, 1987b; Varley *et al.*, 1989; Xu *et al.*, 1989; Horowitz *et al.*, 1990). It is expressed ubiquitously at equivalent levels in all human and mouse cells examined, with the exception of tumour cells in which the *RB* gene is inactivated by mutation or deletion (Lee *et al.*, 1987b; Varley *et al.*, 1989; Xu *et al.*, 1989;

Horowitz *et al.*, 1990). Its status as a tumour suppressor was shown by the fact that re-introduction of Rb into Rb-deficient tumour cells was sufficient to partially block the malignant phenotype (Huang *et al.*, 1988). Further, Rb can be inactivated by constitutive hyperphosphorylation in tumours that do not contain mutations in the retinoblastoma gene (Sherr, 1996). Together, these studies linking Rb disruption to tumour formation and demonstrating Rb to be one of the obligatory cellular targets for viral transformation, have provided strong evidence for Rb as an important regulator of cellular proliferation.

I-III. Rb targets in cell cycle regulation: E2F family of transcription factors

The importance of Rb function in tumour suppression and with viral oncoproteins suggested that Rb may have a role in regulating normal cellular proliferation. It was found that Rb overexpression induced cells to arrest in G1 of the cell cycle (Huang *et al.*, 1988; Goodrich *et al.*, 1991; Qin *et al.*, 1992), whereas cells deficient for Rb had an accelerated G1-S phase transition (Herrera *et al.*, 1996; Hurford *et al.*, 1997; Classon *et al.*, 2000b).

The first identified cellular target of Rb was the E2F1 transcription factor (Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Helin *et al.*, 1992; Kaelin *et al.*, 1992; Shan *et al.*, 1992). E2F1 was first identified as a cellular factor required for the adenovirus early region 1A (E1A)-transforming protein to mediate the transcriptional activity of the viral E2A promoter (Nevins, 1992). The importance of E2F1 in cell proliferation was shown by studies in which E2F1 overexpression was found to be sufficient to promote the G1-S phase transition (Johnson *et al.*, 1993). Further, Rb was able to arrest cells in G1 by inhibiting E2F1

transactivation (Zhu *et al.*, 1993; Qin *et al.*, 1995). Since then, a whole family of E2F transcription factors has been identified, most of which interact with Rb or its closely related family members, p107 and p130.

In mammalian cells, seven E2F family members have now been identified (E2F1-E2F7) and three of their obligate binding partners (DP1-DP3) (Bagchi *et al.*, 1991; Bandara and La Thangue, 1991; Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Helin *et al.*, 1992; Lam and La Thangue, 1994; Buck *et al.*, 1995; Zhang and Chellappan, 1995; de Bruin *et al.*, 2003a; Di Stefano *et al.*, 2003). E2F transcriptional activity arises from the formation of a heterodimer with one member of the E2F family bound to one member of the DP family. All possible combinations of E2F/DP complexes can exist *in vitro*, allowing the potential for a wide array of cellular E2F complexes. E2F consensus binding sites are present not only in the promoter region of E2A, but in many genes required for cell division. Identified E2F targets include cell cycle regulators such as cdc2, *myc*, *b-myb* and cyclins D1, A and E; enzymes required for DNA synthesis such as dihydrofolate reductase (DHFR), thymidine kinase, and DNA polymerase α; the pocket proteins Rb and p107; and E2F1 itself (Hiebert *et al.*, 1989; Thalmeier *et al.*, 1989; Mudryj *et al.*, 1990; Dalton, 1992; Hamel *et al.*, 1992; Lam and Watson, 1993; Shan and Lee, 1994; deGregori *et al.*, 1995; Neuman *et al.*, 1995; Shimizu *et al.*, 1995; Zhu *et al.*, 1995b; Botz *et al.*, 1996; Karlseder *et al.*, 1996; Smith *et al.*, 1996; Estivill-Torrus *et al.*, 2002). *In vitro*, different E2F/DP complexes recognize similar nucleotide sequences (Lees *et al.*, 1993; Buck *et al.*, 1995; Zhang and Chellappan, 1995), however, there is also evidence that some heterodimers preferentially bind to specific E2F

sequences (Tao *et al.*, 1997). It is not yet clear whether specific E2F complexes target preferred E2F-regulated promoters.

The E2F family members can be categorized into four groups based on sequence homology, with functional similarities apparent in each subgroup. For instance, the expression of E2Fs 1-3, is cell-cycle regulated and peaks in late G1 (Lees *et al.*, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994; Neuman *et al.*, 1994; Sears *et al.*, 1997; Leone *et al.*, 1998), while E2Fs 4 and 5 are more uniformly expressed over the cell cycle (Ginsberg *et al.*, 1994; Sardet *et al.*, 1995). The preference with which E2F/DP heterodimers associate with the pocket proteins appears to be specified by the E2F family member: E2Fs 1-3 preferentially bind with Rb, whereas E2Fs 4 and 5 interact predominantly with p107 and p130 (Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Hijmans *et al.*, 1995; Qin *et al.*, 1995) reviewed in (Trimarchi and Lees, 2002; Stevens and La Thangue, 2003).

E2Fs 1-3, which represent the first subgroup, activate E2F-responsive genes and drive cellular proliferation (deGregori *et al.*, 1997). Overexpression of each one is sufficient to induce quiescent cells to re-enter the cell cycle (Johnson *et al.*, 1993; Asano *et al.*, 1996; Lukas *et al.*, 1996; Wang *et al.*, 1998), and expression of a dominant-negative DP mutant has been shown to block S phase entry (Wu *et al.*, 1996). The combined deletion of E2Fs 1, 2 and 3 results in a complete blockage of S phase entry (Wu *et al.*, 2001). Expression of these E2F factors is sufficient to override growth suppression by various CKIs and cell cycle arrest induced by a dominant-negative CDK2 (DeGregori *et al.*, 1995; Lukas *et al.*, 1996; Mann and Jones, 1996). This ability is dependent upon the dimerization, DNA-binding, and

transactivation domains, suggesting that transcription of E2F-regulated genes is required for S phase entry (Johnson *et al.*, 1993; Qin *et al.*, 1994).

In contrast, the second E2F subgroup, E2Fs 4 and 5, can act as repressors of E2F-responsive genes. Expression of these E2Fs has been shown to induce cell cycle withdrawal and differentiation in various cell types, including adipocytes, keratinocytes, and neural precursors (Lindeman *et al.*, 1998; Persengiev *et al.*, 1999; Paramio *et al.*, 2000; D'Souza *et al.*, 2001; Fajas *et al.*, 2002; Landsberg *et al.*, 2003). Their activity may, in part, be due to their subcellular localization. They are predominantly cytoplasmic and require assembly either with a pocket protein or with DP2, which unlike DP1 contains an NLS, for nuclear import (de la Luna *et al.*, 1996; Magae *et al.*, 1996; Allen *et al.*, 1997; Lindeman *et al.*, 1997; Verona *et al.*, 1997). However, significant levels of E2Fs 4 and 5 are present in the nucleus of quiescent (G0) cells (Gaubatz *et al.*, 2001). During G0/G1, significant levels of E2F4/p130 complexes occupy E2F sites, with low levels of local histone acetylation (Vairo *et al.*, 1995; Takahashi *et al.*, 2000). Reduced E2F4 occupancy occurs in late G1, correlating with the timing of gene induction, the appearance of E2Fs 1-3, and increased acetylation of histones H3 and H4. The reduced E2F4 promoter occupancy also occurs at the same time as dissociation of E2F4/p130 complexes and relocation of E2F4 to the cytoplasm (Takahashi *et al.*, 2000). Export of E2F4 from the nucleus has been demonstrated to be an active process, and to prevent its ability to induce cell cycle arrest (Gaubatz *et al.*, 2001). The fact that E2F4- and 5-pocket protein complexes predominate in G0/G1 cells suggests that they may be important for repression of early cell cycle progression (Moberg *et al.*, 1996). Consistent with this, mutation of the E2F binding site in certain E2F-responsive promoters, including B-

myb, cdc2, and E2F1, results in increased expression during G0/G1 (Dalton, 1992; Lam and Watson, 1993; Hsiao *et al.*, 1994).

Subsequent to its initial characterization, the E2F3 locus has been found to encode two protein products: the original E2F3 which is now termed E2F3a, and a novel E2F3b which is encoded by a unique mRNA transcribed from an intronic promoter (Adams *et al.*, 2000; Leone *et al.*, 2000). E2F3b is constitutively expressed throughout the cell cycle, and therefore, differs from E2F3a, which is cell cycle regulated like E2Fs 1 and 2. While the uniform expression of E2F3b is similar to E2Fs 4 and 5, unlike these E2Fs which bind p130, E2F3b maintains preferential association with Rb in G0 cells. These E2F3b/Rb complexes may reflect the previously described role of Rb as a transcriptional repressor in quiescent cells (Adams *et al.*, 2000; Leone *et al.*, 2000).

E2F6 and E2F7 are each categorized in individual subgroups. E2F6 diverges considerably from the other E2F family members. Although the core DNA binding and dimerization domains are conserved, it shares almost no sequence homology with the other family members outside these regions. In addition, E2F6 has truncated C- and N-terminal regions, and therefore, lacks the transactivation and pocket protein binding domains (Morkel *et al.*, 1997). E2F6 acts as a transcriptional repressor by recruitment of multiple cellular factors to form a potent repressor complex, binding to E2F-responsive promoters and inhibiting activation by other E2F members (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998; Trimarchi *et al.*, 2001; Ogawa *et al.*, 2002).

A recently identified member of the E2F family, E2F7, has also been found to act as a transcriptional repressor (de Bruin *et al.*, 2003a; Di Stefano *et al.*, 2003). E2F7 expression is

cell cycle regulated, and localized to the nucleus, where it binds DNA E2F recognition sites with high affinity (de Bruin *et al.*, 2003a; Di Stefano *et al.*, 2003). It differs from the remainder of the E2F family in that it contains two distinct DNA-binding domains but lacks the domains required for dimerization, transcriptional activation and pocket protein binding (de Bruin *et al.*, 2003a). Expression of E2F7 suppressed E2F target genes and induced the accumulation of cells in G1 (de Bruin *et al.*, 2003a; Di Stefano *et al.*, 2003). While E2F7 was shown to bind only a subset of E2F-dependent promoters *in vivo*, the association increased in S phase and resulted in specific derepression of these promoters (Di Stefano *et al.*, 2003). These results suggest that the transcriptional repression of specific E2F target genes by E2F7 may be required for cell cycle progression.

I-IV. Mechanisms of Rb-mediated transcriptional repression

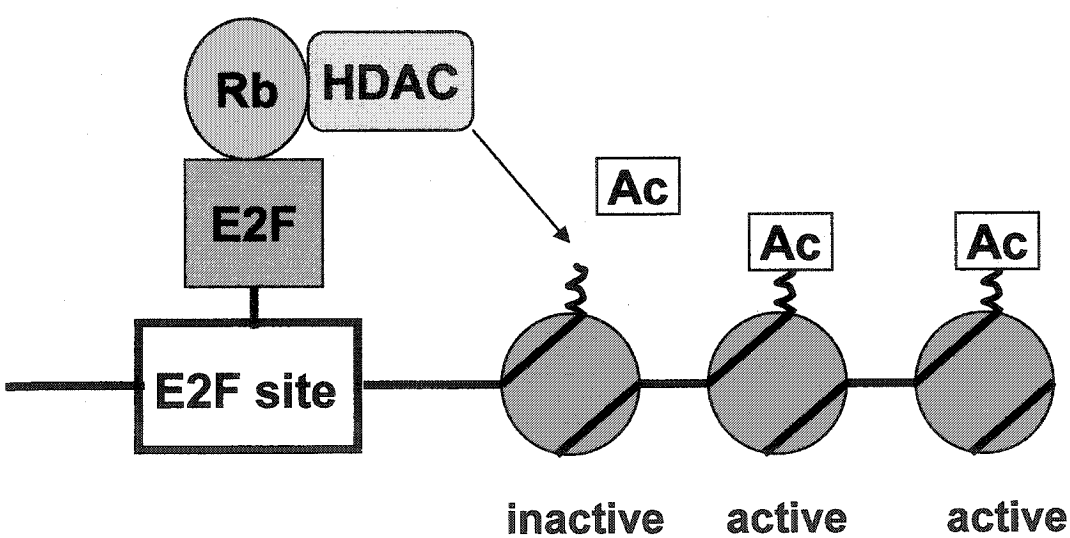
It is now widely believed that Rb regulates the cell cycle through its repression of E2F-dependent gene transactivation. This repression can occur in two ways: First, Rb binds transcription factors such as E2F and represses their ability to activate transcription (Flemington *et al.*, 1993; Helin and Harlow, 1993). Transcription of E2F target genes is activated when an unbound E2F-DP heterodimer binds to the promoter. Since the pocket protein binding domain is integrated within the transactivation domain, Rb can block E2F activity by binding and thereby masking, the E2F transactivation domain (Flemington *et al.*, 1993; Helin and Harlow, 1993). Rb binding has been shown to block the ability of E2F to assemble the basal transcriptional machinery (Ross *et al.*, 1999).

Secondly, Rb can actively repress transcription by recruitment of chromatin remodelling enzymes, to the Rb-E2F repressor complex (Bremner *et al.*, 1995; Sellers *et al.*, 1995; Weintraub *et al.*, 1995). Once targeted to the promoter by E2F, Rb recruits proteins involved in chromatin modification such as histone deacetylases (HDACs), and ATPases of the SWI/SNF complex, brahma (BRM) and brahma-related gene 1 (BRG1) (Dunaief *et al.*, 1994) (Singh *et al.*, 1995). The HDACs remove acetyl groups from lysine residues within the tails of histone octamers, which facilitates the condensation of nucleosomes into inactive chromatin (Harbour and Dean, 2000). The chromatin condensation, in turn, prevents access of transcription factors to the promoter (Kingston and Narlikar, 1999; Kornberg and Lorch, 1999; Wolffe and Hayes, 1999) (Fig. 1-2: Rb-mediated transcriptional repression by chromatin modification).

Rb has also been shown to repress transcription through recruitment of the Rb-binding protein, RBP1 (Lai *et al.*, 1999a; Lai *et al.*, 1999b). RBP1 acts as a corepressor, capable of repressing E2F-mediated transcription via its association with the Rb pocket (Lai *et al.*, 1999b). RBP1 also acts as a bridging molecule by recruiting HDACs with one repression domain and through a second domain, repressing in an HDAC-independent manner (Lai *et al.*, 1999a).

The relative importance of the two methods of repression is not yet clear. *In vitro*, it was shown that Rb could inhibit E2F1 transactivation in the apparent absence of any corepressors (Ross *et al.*, 1999). In contrast, transfection assays suggested that Rb required HDAC to inhibit E2F1 (Brehm *et al.*, 1998; Magnaghi *et al.*, 1998). By altering nucleosomal

Fig. 1-2: Rb-mediated transcriptional repression by chromatin modification. Rb acts to actively repress transcription by recruitment of chromatin remodelling enzymes, such as histone deacetylases (HDAC), to the Rb-E2F repressor complex. By removal of acetyl groups from the tails of histone octamers, the HDACs promote the nucleosomal condensation into inactive chromatin. The chromatin condensation, thereby, prevents access of transcription factors to the promoter. (Modified from Harbour and Dean, 2000).


Rb
HDAC
E2F
E2F site
Ac
Ac
Ac
inactive
active
active

structure and position in an ATP-dependent manner, BRG1 and BRM can affect the ability of transcription factors to access the promoter. Overexpression of BRG1/BRM may facilitate the ability of Rb to repress E2F transactivation (Trouche *et al.*, 1997). However, inhibition of E2F activity did not appear to be affected in BRG1/BRM-deficient cells (Weintraub *et al.*, 1992; Zhu *et al.*, 1993; Zhang *et al.*, 2000).

Rb appears to be able to recruit HDAC and the SWI/SNF complex either separately or together (Zhang *et al.*, 2000). In one study, the Rb-SWI/SNF complex was found to be sufficient to repress the cyclin A and cdc2 genes, while the repression of cyclin E and E2F1 also required HDAC (Zhang *et al.*, 2000). However, a second group demonstrated that Rb-mediated histone deacetylation of cyclin A, cdc2, topoisomerase IIalpha, and thymidylate synthase was HDAC-dependent, since the deacetylation was prevented by the HDAC inhibitor trichostatin A (TSA) (Siddiqui *et al.*, 2003). However, repression of only a specific subset of genes was inhibited by TSA treatment, suggesting that the requirement of HDACs for Rb-mediated transcriptional repression is promoter specific (Siddiqui *et al.*, 2003). Therefore, it appears that distinct types of repressor complexes may regulate different gene sets (Zhang *et al.*, 2000; Siddiqui *et al.*, 2003).

I-V. Rb functional domains

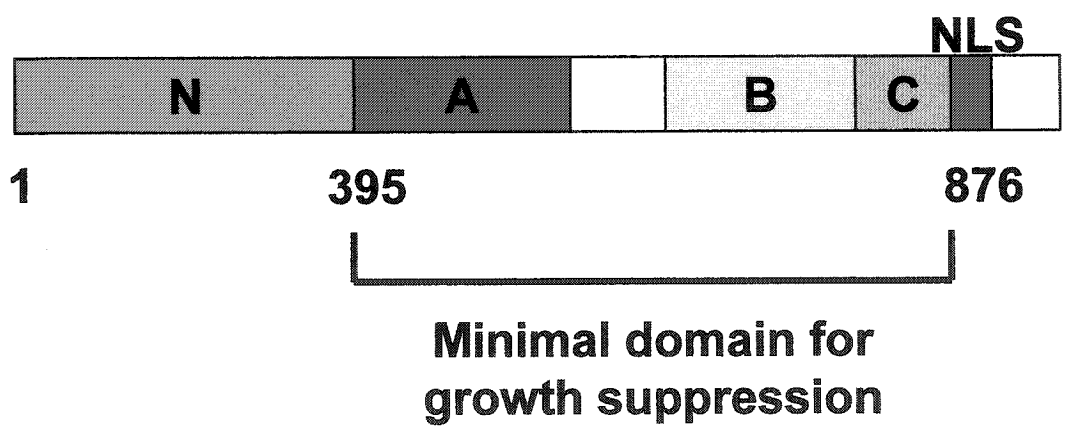
The Rb protein has a globular structure and contains several domains required for its function (Lee *et al.*, 1998). The highly conserved domains A and B, separated by a spacer region, interact to form a central "pocket" structure (Chow and Dean, 1996; Lee *et al.*, 1998).

The pocket is disrupted in most naturally-occurring and tumour-derived mutations in retinoblastoma patients (Horowitz *et al.*, 1990; Harbour, 1998). It is now known that the pocket domain consists of binding sites so that more than one protein can bind simultaneously (Fig. 1-3: Rb protein domains).

Many viral oncoproteins and endogenous Rb-binding proteins contain an LXCXE motif that allows them to bind Rb (Whyte *et al.*, 1988; Dyson *et al.*, 1989; Ludlow *et al.*, 1989; Lee *et al.*, 1998). While the LXCXE binding site is located within a shallow groove of domain B (Lee *et al.*, 1998), domain A is required for the proper conformation of domain B (Kim and Cho, 1997; Lee *et al.*, 1998). Endogenous Rb-binding proteins which contain an LXCXE-like motif include the chromatin remodelling factors HDACs-1 and -2 and BRG1 (Dunaief *et al.*, 1994; Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi *et al.*, 1998). However, the LXCXE motif is not required for binding to the pocket domain. In contrast to HDACs-1 and -2, HDAC-3 does not contain an LXCXE sequence and mutation of the LXCXE binding site in Rb does not inhibit HDAC-3 binding (Chen and Wang, 2000; Dahiya *et al.*, 2000). Further, although BRG1 contains an LXCXE sequence, this sequence does not appear to be required to bind Rb (Zhang *et al.*, 2000).

E2Fs lack an LXCXE sequence and instead contain an Rb-binding motif at their carboxyl terminus (Flemington *et al.*, 1993; Helin *et al.*, 1993). While truncation analyses have shown the Rb pocket domain to be sufficient for stable interaction with E2F1 (Qin *et al.*, 1992), more recent studies have revealed that Rb contains two distinct E2F binding sites (Dick and Dyson, 2003). The first binding site, located within the pocket region, is necessary

Fig. 1-3: Rb protein domains. The highly conserved domains A and B of the Rb protein are separated by a spacer region to form a central "pocket" structure. Rb interacts with the majority of proteins, including those containing an LXCXE sequence, through the pocket domain. E2F binding requires both the central pocket region and the carboxy terminus. The carboxy-terminal region of Rb also contains sites, distinct from the E2F-binding site, for c-abl tyrosine kinase and for MDM-2. In contrast, the amino terminal of Rb does not appear to be involved in growth suppression.


NLS
N
A
B
C
1
395
876
Minimal domain for
growth suppression

for stable association with DNA. The removal of this site inhibits Rb-mediated growth suppression but maintains regulation of E2F1-induced apoptosis. A second site in the carboxy-terminal region of Rb is specific for E2F1. Rb-E2F1 complexes at this site have low affinity for DNA, but are required to regulate E2F1-mediated apoptosis (Dick and Dyson, 2003). The distinct Rb binding sites allow E2Fs to bind simultaneously with other proteins, such as those with an LXCXE sequence (Adams, 2001; Dick and Dyson, 2003).

The carboxy-terminal region of Rb contains binding sites for the cellular homologue of the transforming sequence of Abelson murine leukemia virus (c-abl) tyrosine kinase and murine double minute 2 (MDM-2), which appear to be distinct from the E2F-binding site (Welch and Wang, 1993; Xiao *et al.*, 1995). The tyrosine kinase function of c-abl is blocked when it is bound to Rb, which appears to be important for Rb-mediated growth suppression (Welch and Wang, 1993; Whitaker *et al.*, 1998).

In contrast, the amino-terminal region of Rb seems to be dispensable for growth repression (Qin *et al.*, 1992; Xu *et al.*, 1996; Yang *et al.*, 2002). Overexpression of a truncated Rb mutant which lacked the amino-terminus, retained the ability to suppress the proliferation of tumour cells (Xu *et al.*, 1996). While not necessary for growth regulation, this region may be important for other functions of Rb. Several proteins interact with the Rb amino-terminus including the transcription factor Sp1, minichromosome maintenance 7 (MCM7), a replication licensing factor, and Rb/histone H1 kinase (RbK) which is a G2/M cycle-regulated kinase (Sterner *et al.*, 1995; Udvadia *et al.*, 1995). The region also contains several consensus CDK phosphorylation sites, which may be important for Rb cell-cycle regulation. The amino-terminus has been suggested to be important for Rb function

developmentally by studies in which the embryonic lethality of Rb knockouts was delayed by re-introduction of an Rb transgene with an amino-terminus mutation (Riley *et al.*, 1997).

I-VI. Regulation of Rb by CDK-dependent phosphorylation

The variable phosphorylation state of Rb during the cell cycle acts to regulate its activity. Quiescent cells, or cells in early G1, have mainly hypophosphorylated Rb. As cells progress through late G1 and into S phase, Rb becomes increasingly phosphorylated, and remains this way until it becomes dephosphorylated in late stages of mitosis. Rb is in a hypophosphorylated state in cells exiting the cell cycle to undergo senescence or terminal differentiation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989; Mihara *et al.*, 1989). There are several lines of evidence showing that phosphorylation of Rb by cyclin-CDK complexes in late G1 and S phase is required for its inactivation and cell cycle progression. First, hyperphosphorylation of Rb is correlated with the timing of cell cycle progression. Second, ectopic expression of cyclin-CDK complexes is sufficient to suppress Rb-mediated growth arrest and this is accompanied by Rb hyperphosphorylation (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993). Third, mutants of Rb which are unable to be phosphorylated have been found to be more potent growth suppressors than wildtype Rb (Xiao *et al.*, 1995; Knudsen and Wang, 1997; Lukas *et al.*, 1997).

In early to mid G1, mitogen stimulation induces the synthesis of D cyclins (D1, D2, and D3). These D cyclins then assemble with their catalytic partners to form cyclin D-CDK4/6 complexes, which phosphorylate Rb on distinct sites. Rb becomes further

phosphorylated by cyclin E-CDK2 complexes in late G1 (Dulic *et al.*, 1992; Koff *et al.*, 1992; Ohtsubo and Roberts, 1993). The cyclin E gene is E2F responsive so cyclin E-CDK2 complexes act in a positive feedback loop to facilitate progressive Rb phosphorylation and E2F release (Lundberg and Weinberg, 1998). This feedback loop produces a rapid rise in cyclin E-CDK2 needed to allow cells to enter S phase. Cyclin A-CDK2 complexes become activated at the G1/S phase boundary and throughout S phase (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992). During G2/M, specific phosphatases remove the inactivating phosphate groups from Rb, enabling the binding of E2Fs before the cells enter a new cycle (Chao *et al.*, 1992; Ludlow *et al.*, 1993).

Rb has 16 possible phosphorylation sites. Each cyclin-CDK complex phosphorylates Rb on particular phosphoacceptor sites, although the preferred sites reported differ from study to study (Knudsen and Wang, 1996; Coqueret, 2002). It appears that cyclins may have an important role in the selection of various substrates. For example, cyclin A-cdc2, but not cyclin B-cdc2, phosphorylates p107 (Peeper *et al.*, 1993). The interaction of cyclin A- and cyclin E-CDK2 with many of their substrates, including E2Fs 1-3, p107, and p130, depends upon interaction between an RXL motif in the substrate and a hydrophobic patch in the cyclin (Zhu *et al.*, 1995a; Adams *et al.*, 1996; Saha *et al.*, 1998; Schulman *et al.*, 1998). Disruption of either interaction site abrogates substrate phosphorylation. There are several RXL (or related) motifs within the last 100 residues of Rb which, if deleted, prevent CDK2-dependent phosphorylation (Adams *et al.*, 1999).

Under certain conditions, Rb phosphorylation by cyclin D1-CDK4 also requires an RXL motif in the substrate (Adams *et al.*, 1999). Further, the hydrophobic patch in cyclin A

appears to be conserved in cyclin D1 (Schulman *et al.*, 1998). However, at least *in vitro*, it appears that cyclin D1-CDK4 recognizes Rb by a mechanism distinct from that of cyclin A- and cyclin E-CDK2 complexes. It has been proposed that substrate recognition by cyclin D1-CDK4 depends on an LXCXE-based interaction. While all D-type cyclins contain an LXCXE sequence and initial studies reported the requirement for this sequence for Rb phosphorylation (Ewen *et al.*, 1993), subsequent examination has not been supportive. Mutant versions of cyclins D1 and D2, lacking the LXCXE sequence, can still efficiently phosphorylate Rb *in vitro* and *in vivo* (Horton *et al.*, 1995; Connell-Crowley *et al.*, 1997). Further, growth arrest can still be suppressed by cyclin D-CDK4 kinases in an Rb mutant which is unable to interact with LXCXE-containing proteins (Dick *et al.*, 2000).

Early studies suggested that each cyclin-CDK complex, individually, was capable of inactivating Rb-dependent growth suppression (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993). However, these experiments were conducted by ectopic overexpression of Rb and cyclins in tumour cells, which may have resulted in a loss of specificity. A subsequent study assessed growth arrest in cells following microinjection of Rb which had been phosphorylated *in vitro* with recombinant cyclin-CDK complexes (Connell-Crowley *et al.*, 1997). It was determined that only phosphorylation by cyclin D-CDK4, but not by cyclin A- or cyclin E-CDK2 inactivated Rb, and this required phosphorylation on S795, a cyclin D-CDK4-specific site (Connell-Crowley *et al.*, 1997). Further studies demonstrated that Rb must be phosphorylated by both cyclin D-CDK4 and cyclin E-CDK2 complexes, in order to be inactivated (Ezhevsky *et al.*, 1997; Lundberg and Weinberg, 1998). Because cyclin D-CDK4/6 complexes act first to phosphorylate Rb, followed by cyclin E-CDK2- dependent

phosphorylation, it is often assumed that CDK4/6 are nonessential for Rb inactivation. It is now known that many, if not all of these sites, must be phosphorylated in a sequential manner by different cyclin-CDK complexes, in order to inactivate Rb, reviewed in (Adams, 2001).

Phosphorylation on specific sites regulates Rb function:

Phosphorylation at specific sites appears to regulate distinct Rb functions. For instance, binding of E2F, LXCXE proteins, and c-abl is regulated by distinct sets of phosphorylation sites (Knudsen and Wang, 1996; Knudsen and Wang, 1997). Disruption of E2F-Rb complexes was found to depend upon accumulation of phosphates on several sites, but phosphorylation of specific sites did not appear to be required (Brown *et al.*, 1999). Rb phosphorylation either in the carboxy-terminus or within the spacer may be sufficient to prevent E2F binding (Knudsen and Wang, 1997). Of the seven carboxy-terminal sites, phosphorylation of S780 was found to be sufficient, but not necessary, for disruption of Rb-E2F complexes (Knudsen and Wang, 1997). It was further shown that Rb phosphorylated on S780 *in vivo* failed to bind E2F (Kitagawa *et al.*, 1996). It appears that E2F release may depend on the accumulation of phosphate groups on several sites, and that there may be redundancy between sites. In addition, disruption of the interface between the A and B domains of the Rb pocket, where E2F is believed to bind, was shown following phosphorylation of S567. The disruption of the interface was accompanied by E2F release (Harbour *et al.*, 1999).

CDK inhibitors:

CDK activity is negatively regulated by two families of CDK inhibitors (CKIs). The inhibitors of CDK4 (INK4) family which is comprised of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, specifically inhibit the cyclin D-associated kinases, CDK4 and CDK6 (Ruas and Peters, 1998). The second family, Cip/Kip, include $p27^{Kip1}$, $p21^{Cip1}$, and $p57^{Kip2}$. The Cip/Kip inhibitors act more generally, and affect the activities of cyclin D-, cyclin E- and cyclin A-dependent kinases (Sherr and Roberts, 1999). Both $p27^{Kip1}$ and $p21^{Cip1}$ inhibit cyclin E-CDK2 complexes, but are less effective at blocking the enzymatic activity of cyclin D-CDK4 (Soos *et al.*, 1996; Blain *et al.*, 1997). In contrast, physical association with Cip/Kip subunits has been shown to facilitate the assembly, stability, and nuclear retention of cyclin D1-CDK4 complexes (LaBaer *et al.*, 1997; Cheng *et al.*, 1999; Alt *et al.*, 2002; Muraoka *et al.*, 2002). Cells lacking $p27^{Kip1}$ and $p21^{Cip1}$ show reduced levels of cyclin D-dependent kinase activity following mitogen stimulation (Cheng *et al.*, 1999; Parry *et al.*, 1999). It had been suggested, therefore, that the involvement of cyclin D-CDK4/6 complexes in Rb growth suppression does not depend on phosphorylation of Rb, but rather by their ability to sequester $p27^{Kip1}$and $p21^{Cip1}$, thereby preventing cyclin E-CDK2 repression. This idea has prevailed and CDK4/6 function in Rb phosphorylation became viewed as nonessential but merely facilitative. This model of CDK-dependent Rb phosphorylation can be explained as follows: In quiescent cells, cyclin D levels are low and cyclin D-CDK4/6 complexes do not form. High levels of $p27^{Kip1}$ suppress cyclin E-CDK2 activity. Mitogen stimulation induces D cyclins while sequestration of $p27^{Kip1}$ regulates its assembly with CDK4/6, thereby relieving cyclin E-CDK2 suppression. The $p27^{Kip1}$ remains bound to cyclin D-CDK4/6 while the remainder is

phosphorylated by cyclin E-CDK2, triggering its ubiquitination and degradation (Sheaff *et al.*, 1997; Vlach *et al.*, 1997). In this way, both cyclin D-CDK4/6 and cyclin E-CDK2 complexes cooperate in Rb phosphorylation as cells approach the G1-S phase transition, reviewed in (Sherr and McCormick, 2002) (Fig 1-4. Regulation of G1 phase progression by CDK-dependent Rb phosphorylation).

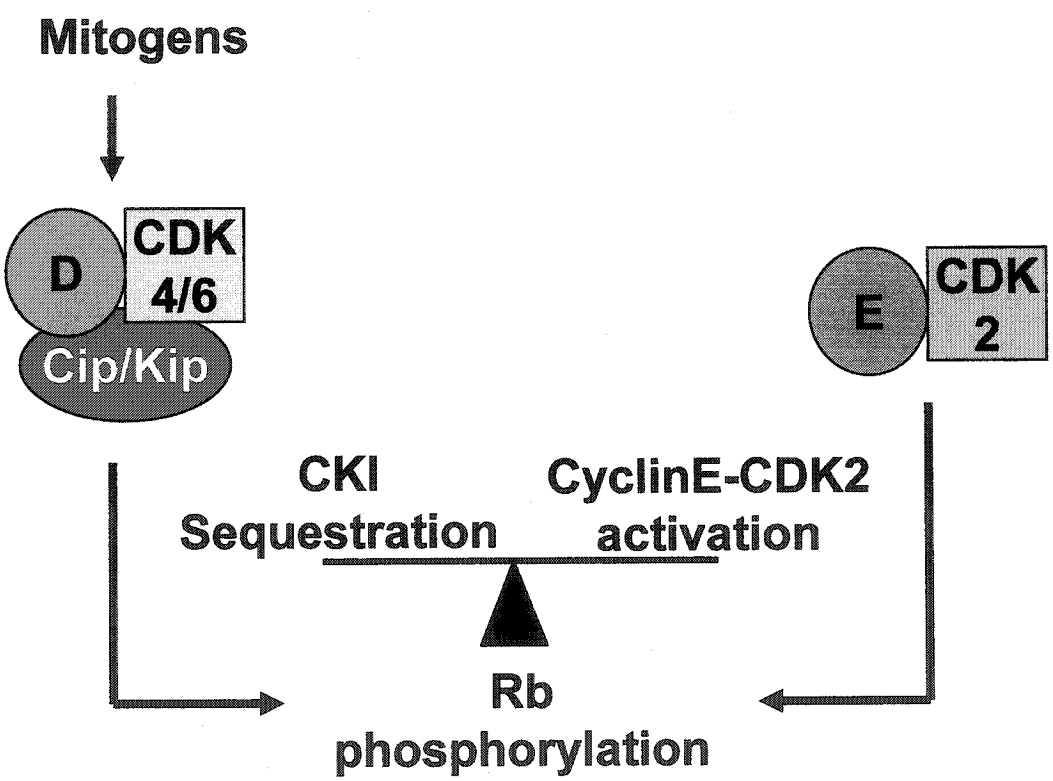
I-VII. Research Objectives (Chapter 2)

While Rb had been extensively studied in the context of tumour suppression, very little was known regarding its function in normal neuronal development. Due to the lack of knowledge in the literature, my objective was first to characterize the various cell cycle regulators of the Rb pathway in the context of neuronal precursor cells. Secondly, I sought to determine the requirement for Rb signalling in regulating growth arrest of dividing neuronal precursor cells.

II. Rb Function in Development

Rb is known to be a critical regulator of cell cycle progression, and genetic mutations are often associated with tumour formation. Through its multiple targets, especially the E2F family of transcription factors, Rb controls cellular proliferation and differentiation. However, its importance differs according to the tissue examined. This variability is due

Fig 1-4: Regulation of G1 phase progression by CDK-dependent Rb phosphorylation. The regulation of the G1-S phase transition is regulated by CDK-dependent Rb phosphorylation. Association of Cip/Kip CKIs has been shown to promote the assembly of cyclin D subunits with CDKs 4 and 6. Unlike CDK2 complexes, these CKIs do not inhibit CDK4/6 activity. In turn, the CKI sequestration by CDK4/6 acts to prevent inhibition of cyclin E-CDK complexes. (Modified from Sherr & Roberts, 1999).


Mitogens
D
CDK
4/6
Cip/Kip
E
CDK
2
CKI
Sequestration
CyclinE-CDK2
activation
Rb
phosphorylation

presumably, to differential expression patterns and to compensation and redundancy by the other Rb family members, p107 and p130. In Part II of this introductory section, I will provide an overview of the developmental requirements for Rb in several tissues, with a particular focus on the nervous system.

II-I. Rb in the developing embryo

Although Rb is ubiquitously expressed in the adult mouse, during embryonic development, levels of Rb mRNA are temporally and spatially controlled. Rb is highly expressed in developing skeletal muscle and lens, as well as the haematopoietic and nervous systems (Jiang *et al.*, 1997; Lipinski and Jacks, 1999; Yoshikawa, 2000). The importance of Rb in various tissues is best shown by examination of the Rb deficient phenotype. Rb-/- die by mid-gestation, between embryonic days 12-15, and exhibit defective erythroid, lens, skeletal muscle and neuronal development (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). The defects are usually associated with enhanced apoptosis, failed differentiation and ectopic cell divisions, or inappropriate cell cycle entry, reviewed in (Lipinski and Jacks, 1999; Classon and Harlow, 2002).

During mouse embryonic development, the major source of haematopoiesis shifts from the yolk sac to the liver at around E12.5 (Dzierzak and Medvinsky, 1995; Palis and Segel, 1998). At this stage, Rb-/- embryos have smaller livers, containing enlarged sinusoids and reduced hepatocytes. In addition, there are increased numbers of immature, nucleated red blood cells in the liver and peripheral circulation (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee

et al., 1992). These defects are associated with severely defective erythrocyte differentiation, such that by E13.5, the Rb mutants are noticeably paler than wildtype littermates (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992).

Increased apoptosis was observed in skeletal muscle precursor cells in germline Rb knockouts, however the mutants died prior to the completion of myogenesis (Jiang *et al.*, 2000; Lasorella *et al.*, 2000). The requirement for Rb in muscle development became evident by the examination of transgenic mice expressing low levels of Rb, driven by an *RB* minigene (Zacksenhaus *et al.*, 1996). The minigene, which consisted of a genomic fragment spanning 1.3kb of the mouse *RB* promoter, with the first exon and intron fused to exons 2 to 27 of the mouse *RB* cDNA, was expressed in Rb-deficient embryos. These mutants survived to birth but exhibited specific skeletal muscle defects, including ectopic proliferation within the myotubes, elevated apoptosis prior to myoblast fusion, shorter myotubes with fewer myofibrils, reduced numbers of muscle fibres, and reduction in expression of the late muscle-specific genes creatine kinase M (MCK) and muscle regulatory factor 4 (MRF4). These results implicated a role for Rb in cell survival and in permanent withdrawal from the cell cycle (Zacksenhaus *et al.*, 1996).

Rb deficiency results in ectopic mitoses and apoptosis throughout the lens, and central (CNS) and peripheral nervous systems (PNS) (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994; Morgenbesser *et al.*, 1994). Lens defects include disorganized histological appearance, cataracts, and defective lens fibre differentiation and elongation (Morgenbesser *et al.*, 1994). In the nervous system, the most severely affected regions were found to be the hindbrain, diencephalon, spinal cord, and dorsal root ganglia,

perhaps due to the fact that these were among the most developmentally advanced regions at the time of lethality (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). Further examination revealed that proliferating neural precursor cells in the ventricular zones appeared normal. However, within the intermediate zones of the developing neural tube, through which newly committed post-mitotic neurons migrate and which do not normally contain dividing cells, there was an abundance of abnormal mitoses and apoptotic death (Lee *et al.*, 1994). Expression of a number of markers of neuronal differentiation, including the neuron-specific βII tubulin, and the neurotrophin receptors TrkA, TrkB, and p75, were all significantly decreased, particularly in dorsal root ganglia (Lee *et al.*, 1994).

Rb chimeric mice have been generated which, in contrast to germline knockouts, survived and exhibited minimal apoptosis (Maandag *et al.*, 1994; Williams *et al.*, 1994b; Lipinski *et al.*, 2001). Although these mice appeared grossly normal, they retained certain defects including cataracts, hyperplasia of the adrenal medulla, enlarged cells in the cerebellum and the liver, and most prevalently, pituitary tumours (Maandag *et al.*, 1994; Williams *et al.*, 1994b). While the apoptotic phenotype was significantly rescued, many defects associated with inappropriate proliferation and failed differentiation persisted. Ectopic proliferation and extensive cell degeneration were observed in the embryonic retina and the development of lens fibres remained defective (Maandag *et al.*, 1994; Williams *et al.*, 1994b). In addition to chimeric analyses, the lens defects also failed to be rescued by introduction of a hypomorphic *RB* minigene (Maandag *et al.*, 1994; Williams *et al.*, 1994b; Zacksenhaus *et al.*, 1996). In the nervous system, Rb-deficient cells in the chimeras exhibited ectopic S phase entry, similar to germline knockouts (Lipinski *et al.*, 2001). However, further

examination revealed that Rb-null cells from chimeric mice arrested in G2, prior to completion of the cell cycle. In contrast, cells from Rb germline knockouts completed ectopic mitoses, followed by apoptosis. It thus appeared that the absence of inappropriate division in chimeric mice may have accounted for their survival. Based on these results, it was proposed that neighbouring wild-type cells could rescue chimeric Rb-deficient cells, possibly by providing survival factors (Lipinski *et al.*, 2001).

Chimeric analyses, as well as transplantation of Rb-/- cells into irradiated recipients, have demonstrated that Rb deficient cells can contribute to all haematopoietic lineages (Maandag *et al.*, 1994; Williams *et al.*, 1994b; LaBaer *et al.*, 1997). However, defective erythropoeisis persisted in the wildtype mice transplanted with Rb deficient liver cells (LaBaer *et al.*, 1997). The reconstituted mice were anaemic and showed increased levels of nucleated red blood cells for up to 6 months. It was, therefore, suggested that the defect in Rb null mice may arise from a defective cell in the haematopoietic lineage and not from defective hepatocyte function (LaBaer *et al.*, 1997).

While in humans, loss of *RB* is rate-limiting to the development of retinoblastoma, *RB* heterozygous mice fail to develop retinal tumours, but primarily form thyroid medullary carcinomas and pituitary adenocarcinomas of the intermediate lobe (Jacks *et al.*, 1992; Hu *et al.*, 1994; Maandag *et al.*, 1994; Williams *et al.*, 1994a). These malignancies usually appear with three months of age while older mice often develop lung metastases (Nikitin *et al.*, 1999). Introduction of a human *RB* transgene was sufficient to repress these malignancies (Nikitin *et al.*, 1999). In addition, Rb chimeric mice fail to develop retinoblastoma, suggesting that Rb loss in the mouse retina is insufficient to induce tumour development

(Maandag *et al.*, 1994; Williams *et al.*, 1994b). The reason why Rb-mediated tumour formation differs between mice and humans is not clear. Is has been suggested to be due to species-specific differences in the number of susceptible cells, the timing of susceptibility, or a difference in mutational requirements (Jacks, 1996; Hooper, 1998). It may be that additional mutations are required for retinoblastoma development in the mouse. While p107 or p130-deficient mice are not predisposed to tumour formation (Cobrinik *et al.*, 1996; Lee *et al.*, 1996), Rb/p107 double null chimeric mice develop retinoblastoma, suggesting that p107 can act as a tumour suppressor in the absence of Rb (Robanus-Maandag *et al.*, 1998), reviewed in (Vooijs and Berns, 1999).

II-II. Rb as a survival factor

Due to the highly elevated levels of apoptosis in Rb-deficient embryos, Rb was concluded to be a critical survival factor in various developing tissues (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). It was thought that the loss of Rb may trigger apoptosis, which acted to protect against cells in which Rb function was deregulated (Morgenbesser *et al.*, 1994). Rb deficiency induces both p53-dependent and -independent apoptotic pathways, in a tissue-specific manner (Lipinski & Jacks, 1999). Protein levels and DNA binding activity of p53 are increased in the brains of Rb knockout embryos, along with up-regulation of the p53 target, $p21^{Cip1}$ (Macleod *et al.*, 1996). In the CNS and lens, apoptosis resulting from Rb deficiency is p53-dependent (Macleod *et al.*, 1996), such that the apoptotic phenotype in these regions is rescued in Rb/p53-null animals (Macleod *et al.*,

1996). However, in the PNS, cell death and $p21^{Cip1}$ expression levels in Rb mutants function independently of p53 (Macleod *et al.*, 1996), reviewed in (Chau and Wang, 2003).

Apoptosis resulting from Rb deficiency is often attributed to deregulation of target E2F transcription factors, and its consequential impact on cell cycle. Consistent with this, protein expression levels of E2F, in both the free and complexed forms, and cyclin E, have been shown to be increased in the CNS of Rb-deficient embryos (Macleod *et al.*, 1996; Callaghan *et al.*, 1999). Deletion of E2F1 in Rb-null mice greatly reduced both ectopic mitoses and apoptosis in the lens and CNS, concomitant with a down-regulation of the p53 pathway (Pan *et al.*, 1998; Tsai *et al.*, 1998; Ziebold *et al.*, 2001; Saavedra *et al.*, 2002). Further, the apoptotic rescue in Rb/E2F1-deficient mice was very similar to that observed in Rb/p53 double knockouts (Morgenbesser *et al.*, 1994; Macleod *et al.*, 1996). These observations led to the conclusion that deregulated E2F1 activity was necessary and sufficient for the p53-mediated apoptosis in Rb deficient embryos (Tsai *et al.*, 1998).

Several lines of evidence suggest that the release of free E2F1 may be responsible for triggering apoptosis. E2F1 overexpression has been shown to be capable of inducing apoptosis (Kowalik *et al.*, 1995; deGregori *et al.*, 1997; Hsieh *et al.*, 1997; O'Hare *et al.*, 2000), and E2F1-deficient neurons were protected from certain apoptotic stimuli (Giovanni *et al.*, 2000; Hou *et al.*, 2000; O'Hare *et al.*, 2000). Furthermore, Rb inactivation by transgenic targeting of the large T antigen induced DNA synthesis and apoptosis of cerebellar Purkinje neurons (Giovanni *et al.*, 2000). This would imply that the release of free E2F resulting from inactivation of Rb was sufficient to result in apoptosis. However, in the same study, overexpressed E2F1 was insufficient to cause cell death in Purkinje neurons, but

accelerated cellular degeneration (Giovanni *et al.*, 2000). The fact that overexpression of E2F1 did not induce apoptosis in Purkinje neurons could be due to a cell type-dependent variability in the sensitivity to deregulated Rb pathway signalling. This is consistent with studies in which Rb was conditionally deleted in the developing cerebellum (Marino *et al.*, 2003). While Rb deficiency resulted in apoptosis of cerebellar granule cells, the Purkinje neurons survived, although they exhibited abnormal cellular morphology (Marino *et al.*, 2003). From these studies, among others, it was concluded that the role of Rb in apoptosis appears to be a protective function, while E2Fs, in particular E2F1, act as inducers of cell death.

It has been suggested that Rb function in apoptosis may function through CDK activity. Cyclin D1 transcripts and CDK4/cdc2 protein levels are increased coincident with death of sympathetic neurons and neuronal PC12 cells following NGF withdrawal (Freeman *et al.*, 1994; Gao and Zelenka, 1995). Protein levels of cyclin D1, cyclin B, and CDK4 are elevated in brains of Alzheimer's disease and stroke patients (Li *et al.*, 1997; McShea *et al.*, 1997; Busser *et al.*, 1998). Stroke injury and DNA damaging agents induced phosphorylation of Rb and p107, followed by loss of Rb and p107 (Park *et al.*, 1998; Osuga *et al.*, 2000; Park *et al.*, 2000). The CDK inhibitor, flavopiridol, led to neuroprotection, as well as a suppression of the Rb and p107 phosphorylation and loss (Park *et al.*, 1998; Osuga *et al.*, 2000; Park *et al.*, 2000). These studies demonstrate the involvement of CDKs in neuronal cell death, through their ability to phosphorylate Rb.

In spite of the fact that E2Fs1-3 are all potent inducers of cell cycle progression, E2F1 is generally believed to be unique in its additional ability to induce apoptosis (Qin *et*

al., 1994; Wu and Levine, 1994; deGregori *et al.*, 1995; Hiebert *et al.*, 1995; deGregori *et al.*, 1997; Hsieh *et al.*, 1997; Phillips *et al.*, 1997; Kowalik *et al.*, 1998). However, the importance of the various E2F factors is highly dependent on the cell type. For instance, deletion of E2Fs 1, 2, or 3 suppressed inappropriate proliferation in the lens of Rb-deficient embryos, with E2F3 having the most pronounced effect, but only E2F1 deficiency rescued the apoptotic phenotype. However, in the CNS, the absence of either E2Fs 1 or 3 was sufficient to repress the ectopic proliferation and apoptosis, while the loss of E2F2 had only a minor effect on proliferation (Saavedra *et al.*, 2002). A second study found E2F3 deficiency to almost completely suppress inappropriate proliferation and apoptosis in the developing lens and CNS, in which apoptosis is known to be p53-dependent, while partially rescuing the defects in the p53-independent PNS (Ziebold *et al.*, 2001). From these studies, it was not clear if E2F deficiency rescued apoptosis because of a pro-apoptotic E2F ability or simply by suppressing the inappropriate proliferation which was thought to induce apoptosis.

These abilities of E2F1 to drive cell cycle progression and to induce apoptosis can be mechanistically dissociated, since mutants that fail to induce S phase are still capable of inducing apoptosis (Phillips *et al.*, 1997). Mutational analysis has revealed that the ability of E2F1 to induce apoptosis requires DNA binding but is independent of its transactivation domain (Hsieh *et al.*, 1997). However, E2F1 has been shown to directly transactivate several pro-apoptotic genes including Apaf1, caspases-3 and -7, and the BH3-only members of the Bcl2 family, Puma, Noxa, Bim and Hrk/DP5 (Moroni *et al.*, 2001; Hershko and Ginsberg, 2003). In addition, disruption of pro-apoptotic genes such as Apaf1 and caspase-3, which have been shown to be regulated by E2F1, can partially rescue defects associated with Rb

deficiency (Guo *et al.*, 2001; Simpson *et al.*, 2001). While E2F1 clearly has a role in mediating apoptosis associated with Rb deficiency, the exact nature of this function remains unclear.

II-III. Role of Rb in differentiation

Many of the defects associated with Rb deficiency during development are associated with failed differentiation. While it appears that the majority of cells which are induced to divide inappropriately subsequently undergo apoptosis, there has also been strong evidence of failed differentiation in the surviving cells. Cells of the erythroid, neural, and lens lineages are able to initiate differentiation, however, they often fail to achieve a fully differentiated state. For example, erythrocytes exhibit inefficient enucleation, and some cells of the lens and CNS have reduced or absent expression of specific late differentiation markers (Lee *et al.*, 1994; Morgenbesser *et al.*, 1994). In chimeric mice, Rb-deficient cells did not contribute to the post-natal retina (Maandag *et al.*, 1994; Robanus-Maandag *et al.*, 1998). Rb deficient cells in both the outer- and the inner-nuclear layers of the retina underwent apoptosis, indicating that Rb may be required at specific stages of retinal differentiation (Maandag *et al.*, 1994; Robanus-Maandag *et al.*, 1998). Rb deficiency is also associated with defective myogenesis. Rb deficient fibroblasts induced to differentiate by MyoD are impaired in the acquisition of late differentiation markers and are unable to maintain the differentiated state (Schneider *et al.*, 1994; Novitch *et al.*, 1996). In addition, Rb-/- fibroblasts exhibit defective

adipocyte differentiation, which is related to a direct interaction between Rb and the CCAAT/enhancer binding protein (C/EBP) (Chen *et al.*, 1996).

While transcriptional repression appears to be the mechanism by which Rb mediates cell cycle arrest, its role in differentiation is thought to function through transcriptional activation (Bremner *et al.*, 1995; Lee *et al.*, 1995; Qin *et al.*, 1995). Transcription factors which have been shown to interact with Rb and induce differentiation include BRG1, c-abl, C/EBP, and the basic helix-loop-helix (bHLH) transcription factor, myogenic determination (MyoD) (Dunaief *et al.*, 1994; Welch and Wang, 1995; Chen *et al.*, 1996; Strobeck *et al.*, 2000) reviewed in (Morris and Dyson, 2001).

The bHLH transcription factors have been shown to have important roles in cell fate determination and differentiation in several systems including myogenesis (Weintraub, 1993), haematopoiesis (Porcher *et al.*, 1996) and neurogenesis (Jan and Jan, 1993). Inhibitor of differentiation (Id) proteins are members of the HLH family and are important negative regulators of differentiation in virtually all tissues (Norton, 2000). Since they lack the basic DNA-binding domain, they act as dominant inhibitors of bHLH factors. Through binding and sequestration of E proteins, the required cofactors for bHLH dimerization and activation, Id proteins inhibit bHLH function (Benezra *et al.*, 1990; Norton, 2000). Of the Id family, Id2 has the additional ability to bind Rb family members, and does so exclusively in their active, hypophosphorylated state (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). By binding to the pocket domain, Id2 abolishes the growth inhibitory functions of Rb (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). Expression of Id2 in cortical progenitor cells was shown to inhibit the induction of neuronal-specific genes, while suppression was eliminated by co-expression of a

constitutively active Rb (Toma *et al.*, 2000). Many of the defects associated with Rb deficiency have been shown to be rescued by the additional deletion of Id2 (Lasorella *et al.*, 2000). In contrast to mid-gestation lethality in Rb-deficient embryos, the Rb/Id2 mutants survived to birth and the haematopoeitic and neurological defects were rescued (Lasorella *et al.*, 2000). Throughout the CNS, there was no evidence of enhanced apoptosis or inappropriate proliferation, including ectopic mitoses (Lasorella *et al.*, 2000). However, the Rb/Id2 mutant mice were born with a severe reduction in muscle mass, as Id2 deficiency was unable to rescue apoptosis in muscle cells (Lasorella *et al.*, 2000). While Id2 appears to have an important role in mediating the Rb deficient phenotype, the mechanism of action remains unknown and may differ greatly depending on the tissue examined.

II-IV. Compensation by Rb family members

The Rb family members, p107 and p130, are also involved in the regulation of cell cycle interactions (Zhu *et al.*, 1993; Claudio *et al.*, 1994). The family shares sequence homology in their A/B pocket, the domain with which they interact with transcription factors and viral oncoproteins- and are thereby termed "pocket proteins" (Ewen *et al.*, 1991; Hannon *et al.*, 1993; Li *et al.*, 1993; Mayol *et al.*, 1993). All three members undergo growth arrest in response to expression of the CKI, $p16^{INK4a}$ (Bruce *et al.*, 2000). Rb itself does not appear to be essential for cell cycle control (Jacks, 1996). Recruitment of HDACs to E2F-responsive promoters in normally cycling fibroblasts is performed mainly by p107 and p130 (Rayman *et al.*, 2002). It has been proposed that Rb may contribute to gene repression only at certain

times, for instance, during differentiation or senescence (Sellers *et al.*, 1998; Lipinski and Jacks, 1999; Thomas *et al.*, 2001).

Rb family members share many overlapping roles and have a partially compensatory ability. In the nervous system, Rb is expressed in cycling and differentiated cells, whereas p107 is present only in proliferating cells, becoming rapidly down-regulated upon differentiation (Bernards *et al.*, 1989; Jiang *et al.*, 1997; Callaghan *et al.*, 1999). In Rb deficient embryos, p107 expression is up-regulated and can partially compensate for the loss of Rb (Callaghan *et al.*, 1999). However, the absence of p107 from differentiating and post-mitotic neurons may explain the partial rescue. The loss of p107 in quiescent Rb-deficient mouse embryonic fibroblasts (MEFs) dramatically increased cell cycle re-entry, indicating that p107 was necessary to maintain the quiescent state in these cells (Sage *et al.*, 2003).

While Rb deficiency results in embryonic lethality at mid-gestation (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992), mice lacking p107 or p130 develop normally on most genetic backgrounds (Cobrinik *et al.*, 1996; Lee *et al.*, 1996; LeCouter *et al.*, 1998a; LeCouter *et al.*, 1998b; Sage *et al.*, 2000). However, on a BalbC genetic background, p107 deficiency results in growth retardation and myeloid hyperplasia (LeCouter *et al.*, 1998a), and p130 loss induces embryonic lethality (LeCouter *et al.*, 1998b). Compound mutations such as Rb/p107 and Rb/p130 double null mice die earlier during embryogenesis and exhibit more pronounced cell cycle defects and apoptosis (Lee *et al.*, 1996; Sage *et al.*, 2000). Mice deficient for p107/p130 die shortly after birth and display defective bone development (Cobrinik *et al.*, 1996). The more severe phenotype of these compound mutant mice implies a partial redundancy in the function of these Rb family members.

al., 2004). Further, in cerebellar development, the requirements for Rb and p107 depend on the cell type examined (Marino *et al.*, 2003). The regulation of cell cycle exit, differentiation, and survival of granule cell precursors was found to be Rb-dependent, and p107 was unable to fully compensate for its deficiency. In contrast, neither Rb nor p107 was required for the differentiation and survival of Purkinje neurons (Marino *et al.*, 2003). Taken together, these studies demonstrate the essential role of these proteins in the control of the G1-S transition and highlight the shared versus distinct roles in the control of cellular processes, such as differentiation.

II-V. Rb Involvement in Neural Development

Several studies have demonstrated that Rb is a key regulator of neuronal differentiation. First, the Rb protein is expressed at high levels from E9.5 onwards in the developing nervous system (Bernards *et al.*, 1989; Jiang *et al.*, 1997). Second, *in vitro* differentiation of embryonal carcinoma cells up-regulate Rb when induced to neural tissues (Slack *et al.*, 1993). Third, Rb-deficient embryos displayed numerous abnormalities in the nervous system, which occur shortly after the time at which Rb would normally be expressed (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). Together, these studies demonstrate an important role for Rb in the developing nervous system.

The temporal requirement for Rb, relative to the neuronal commitment decision, was investigated by introduction of a neuronal marker gene into Rb null mice (Slack *et al.*, 1998). This transgene consisted of the neuron specific Tα1 α-tubulin promoter (Gloster *et al.*, 1994)

driving a β-galactosidase (lacZ) reporter gene (Tα1:nlacZ) that was induced as progenitor cells became committed to a neuronal fate (Gloster *et al.*, 1999). Abnormal neuronal development was detected throughout the nervous system, including the olfactory epithelium, retina, and neocortex. Based on the timing of marker gene expression, it appeared that Rb was required immediately following the neuronal commitment decision. In the absence of Rb, virtually all neuronal populations underwent apoptosis (Slack *et al.*, 1998). These observations suggested that Rb was critical when newly committed neurons attempted to undergo terminal mitosis and initiate differentiation. Further, the inability to undergo terminal mitosis in the absence of Rb was thought to lead to the conflicting signals that ultimately resulted in apoptosis.

The role of Rb in neuronal survival was further established by a series of studies examining Rb inactivation by viral oncoproteins. Expression of the SV40 Tag in cerebellar Purkinje neurons induced apoptosis, in a manner dependent on the Rb-binding domain (Feddersen *et al.*, 1995). In another study, transgenic mice were generated in which the expression of the HPV-16 E7 protein was directed to the developing lens (Pan and Griep, 1994). Mutants exhibited defective lens fibre cell differentiation, ectopic proliferation and apoptosis. However, the lens defects were rescued in transgenics expressing an E7 mutant that was unable to bind Rb/p107 (Pan and Griep, 1994). Further, E1A induction in differentiating P19 embryonal carcinoma cells resulted in apoptosis immediately following commitment to a neuronal fate (Slack *et al.*, 1995). Coincident with neuroectoderm differentiation, there was a dramatic increase in c-fos expression and extensive cell death, which was dependent on the ability of the E1A protein to bind Rb and related proteins (Slack

et al., 1995). Together, the evidence from these studies strongly supported the requirement for Rb in neural development and survival, particularly during neuronal differentiation.

It is widely believed that inappropriate cell cycle signals are sufficient to induce neuronal cell death. For instance, proliferative cues received by a post-mitotic or differentiating neuron may conflict with other cellular or environmental cues, and instead trigger apoptosis. Examination of retinae in mice lacking the CKI $p57^{Kip2}$ revealed both inappropriate S-phase entry and apoptosis (Dyer and Cepko, 2000). Apoptosis induced by kainate treatment in cultured cerebellar granular neurons was associated with the induction of cyclin D1 (Giardina *et al.*, 1998), and neurons were protected from apoptosis by overexpression of $p16^{Ink4a}$, an inhibitor of cyclin D-dependent kinases (Kranenburg *et al.*, 1996). Further, in the absence of neurotrophin-3 (NT-3), sensory precursor cells underwent apoptosis, following uncontrolled S phase entry (ElShamy *et al.*, 1998). This aberrant proliferation and apoptosis could be prevented by a cell cycle blocker (ElShamy *et al.*, 1998). These studies, along with characterization of the Rb-null phenotype in which inappropriate neuronal proliferation is followed by massive neuronal fallout, have firmly entrenched the belief that contradictory growth regulation in differentiating or post-mitotic neurons will trigger an apoptotic response. It has, therefore, been proposed that the deregulated cell cycle regulation associated with Rb deficiency induces apoptosis subsequent to inappropriate cell cycle entry.

Although the neurological phenotype in Rb nullizygous mice is severe, primary neuronal cultures of Rb-deficient cells are able to survive and differentiate (Lee *et al.*, 1994; Slack *et al.*, 1998; Callaghan *et al.*, 1999). For example, Rb-deficient trigeminal and dorsal

root ganglia isolated at E11.5 and E12.5, respectively, were indistinguishable from wildtype cells (Lee *et al.*, 1994). In addition, cortical progenitor cells derived from E12.5 Rb null embryos appeared morphologically identical to controls and were able to differentiate and express the neuronal marker, microtubule-associated protein 2 (MAP2) (Slack *et al.*, 1998). Further studies revealed however, that Rb-deficient cells exhibited delayed terminal differentiation accompanied by a compensatory up-regulation of p107 (Callaghan *et al.*, 1999). However, despite the increased p107 expression, Rb deficiency resulted in reduced DNA-binding activity of E2Fs 1 and 3 and the deregulation of some E2F-inducible genes (Callaghan *et al.*, 1999). Thus, although p107 may be partially compensatory, it is insufficient to fully substitute for the absence of Rb in developing embryos.

II-VI. Research Objectives (Chapter 3)

Although the germline knockouts provided fairly convincing evidence of the role of Rb as a required neuronal survival factor, there was also contradictory evidence. For example, neuronal precursor cells derived from Rb-deficient embryos were able to survive and differentiate in culture (Lee *et al.*, 1994; Slack *et al.*, 1998; Callaghan *et al.*, 1999). However, it could be argued that this result merely represented a tissue culture "artifact", since the cells were provided with all their required growth factors and trophic support, so may not reliably represent the *in vivo* situation. Further studies with Rb chimeric mice provided yet another perspective (Maandag *et al.*, 1994; Williams *et al.*, 1994b; Lipinski *et al.*, 2001). While the majority of Rb deficient cells were able to survive, it was proposed that

the neighbouring wildtype cells were responsible for maintaining their survival, likely through the secretion of survival factors (Lipinski *et al.*, 2001). My objective was to examine the importance of Rb in the developing telencephalon, in the absence of the pleiotropic defects associated with the germline knockouts. By examination of a conditional knockout in which Rb was specifically deleted in the developing telencephalon, I determined the cell autonomous and non-cell-autonomous requirements for Rb in progenitor proliferation, differentiation and neuronal survival.

III. Cell Cycle Regulation in Cortical Development

The generation of neurons during embryogenesis is a complex process. Cell cycle regulation appears to have a critical role in the initial phases of neurogenesis, when a progenitor cell commits to a neuronal fate, withdraws from the cell cycle, and begins to differentiate. This final introductory section will provide an overview of cortical development and examine the requirements therein for proper cell cycle regulation. The third manuscript of this thesis (Chapter 4) will examine the requirement for Rb in the establishment of proper cortical organization.

III-I. Organization of the telencephalon

The mammalian telencephalon, the anterior-most component of the nervous system, is the most complex structure of the brain. It is required for high-order cognitive skills,

including reasoning and language, as well as for planning and coordinating movement and evaluating sensory perceptions. This functional diversity is accomplished by the creation of distinct anteroposterior and dorsoventral progenitor domains, with generally distinct neuronal cell types, projection patterns, gene expression profiles, and functions, reviewed in (Marin and Rubenstein, 2003). Specific transcription factors become induced in different progenitor domains to promote the acquisition of particular neuronal identities. Progenitors are believed to be specified to their eventual neuronal fate prior to the onset of differentiation and migration (Marin and Rubenstein, 2003).

The telencephalon can be broadly subdivided into dorsal (pallial) and ventral (subpallial) domains. The cerebral cortex and hippocampus are the two primary dorsal telencephalic structures, whereas the basal ganglia comprise the main ventral structures. The basal ganglia arise from two bulges in the wall of the lateral ventricle, the medial (MGE) and lateral ganglionic eminences (LGE), which give rise to the globus pallidus and the striatum, respectively (Deacon *et al.*, 1994).

The development of the cerebral cortex involves sequential waves of neurogenesis followed by gliogenesis. The temporal generation of neurons and glia is highly regulated, and a proper sequence of origin of the diverse cell types is crucial for normal laminar structure (McConnell and Kaznowski, 1991; McConnell, 1995). Neural stem cells are multipotent precursors that both self-renew and give rise to neuronal and glial progenitors. It appears that the potential of stem cells to produce diverse progeny varies over developmental time (Qian *et al.*, 2000). In this way, an early stem cell can divide asymmetrically to generate another stem cell and a restricted neuroblast. With age, the neurogenic potential of the stem

cell declines, such that it generates fewer neurons and initiates glioblast development (Qian *et al.*, 2000), reviewed in (Arsenijevic, 2003; Galli *et al.*, 2003; Seaberg and van der Kooy, 2003; Zhong, 2003).

Neural precursors undergo 11 rounds of proliferation, between embryonic days 10 to 17 (E10-17), to generate the developing cortex (Takahashi *et al.*, 1995; Takahashi *et al.*, 1996). The multiple rounds of progenitor proliferation are restricted to the pseudostratified neuroepithelial ventricular zone (VZ). Later in the neurogenic period, the germinal neuroepithelium becomes subdivided into two distinct proliferative regions- the VZ which is just adjacent to the ventricle surface, contains early-arising primary progenitors; and a more superficial subventricular zone (SVZ), which comprises a later-arising population of secondary progenitors (Privat, 1975; Reznikov *et al.*, 1997; Noctor *et al.*, 2004).

III-II. Development of cortical laminar structure

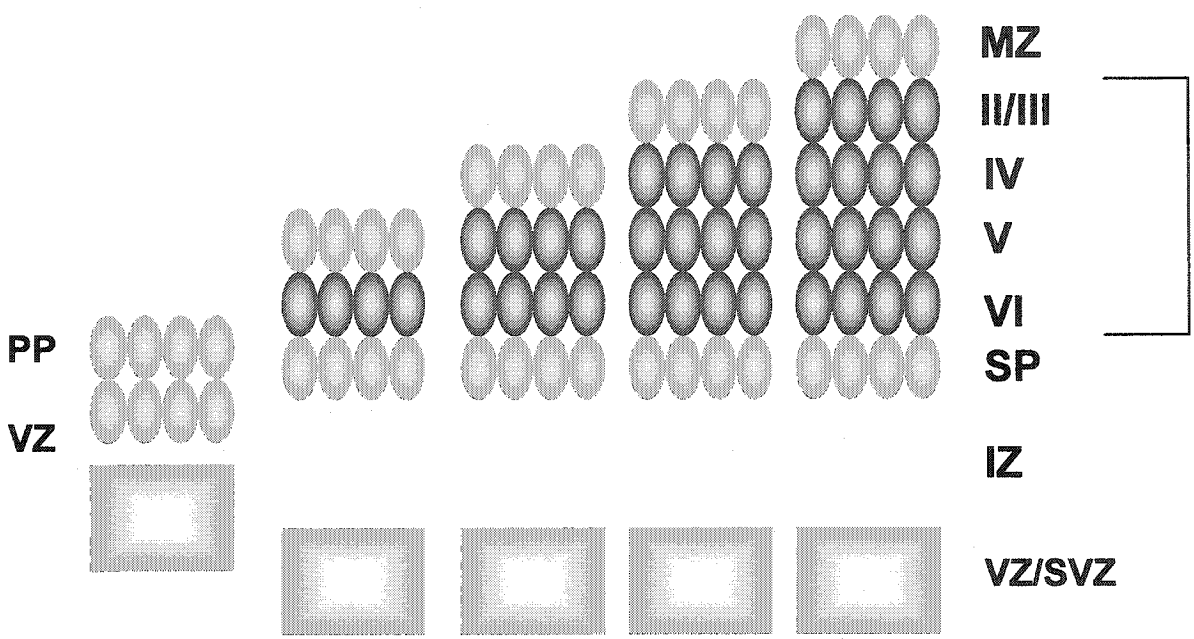
Following cell cycle withdrawal, newly born neurons initiate expression of early neuronal markers and commence migration into the developing cortical plate (CP) (McConnell and Kaznowski, 1991; McConnell, 1995). The first neurons generated, or the "pioneering neurons", are born around E10-11. These neurons give rise to the primordial plexiform layer, or preplate (PP), which is located immediately overlying the progenitor populations in the VZ. The pioneering neurons are followed by the radial migration of a second neuronal cohort destined to reside in the deepest cortical layer, layer VI. This arrival splits the preplate into an overlying marginal zone (MZ), and an underlying subplate (SP).

The MZ contains the future layer I cells, including a transient population of Cajal-Retzius (CR) neurons; the SP also contains some transient cells and contributes others to layer VI. Subsequent radial arrivals migrate past the SP and layer VI neurons, and stop as they reach the MZ. This process continues for the remainder of the cortical neurons, such that layers II-VI are generated in an outside-out manner. Therefore, earlier generated neurons (layers V/VI) come to reside in deep layers, and later born neurons (layers II/III) locate to more superficial positions (Takahashi *et al.*, 1999). As cortical development proceeds, the SP becomes separated from the germinal regions by development of the intermediate zone (IZ), a white matter region containing afferent and efferent projections (Sidman and Rakic, 1973; Caviness, 1982b), reviewed in (Marin-Padilla, 1998) (Fig. 1-5: Development of cortical layers).

III-III. Terminal mitosis and cell identity

Terminal mitosis and terminal differentiation are usually intimately coupled. Cells in the mitotic layer of the developing retina induce expression of a retinal ganglion cell marker within minutes of S phase of terminal mitosis (McLoon and Barnes, 1989; Wald and McLoon, 1995). Neural precursor cells, when transplanted throughout the developing cortex, exhibit appropriate neuronal identity only if they had undergone terminal mitosis following implantation (McConnell and Kaznowski, 1991). The time at which a newly generated neuron undergoes terminal mitosis and exits the cell cycle, or its' "birthdate", correlates highly with eventual laminar fate (McConnell and Kaznowski, 1991; McConnell, 1995). The

Fig. 1-5: Development of cortical layers. The six-layered cortex develops in a sequential manner such that the earliest generated neurons give rise to deep cortical layers, while neurons generated later will migrate to more superficial positions. The first neurons born form the preplate (PP), located adjacent to the VZ. These are followed by a second neuronal cohort (future layer VI), which splits the preplate into an overlying marginal zone (MZ), and an underlying subplate (SP). Subsequent radial arrivals migrate past the SP and layer VI neurons, and stop as they reach the MZ. This process continues for the remainder of the cortical neurons, such that layers II-VI are generated in an outside-out manner. The SP subsequently becomes separated from the germinal regions by development of the intermediate zone (IZ), a white matter region containing afferent and efferent projections. (Modified from Glmore & Herrup, 1997).


MZ
II/III
IV
V
VI
SP
IZ
VZ/SVZ
PP
VZ

cell cycle dependence in neuronal identity was best shown by a series of transplantation studies in the ferret (McConnell and Kaznowski, 1991). Cells isolated at E29, which would normally give rise to layer VI, were [^{3}H] thymidine-labelled *in vitro* and transplanted into post-natal hosts, in which layers II/III were currently being generated. It was found that neurons which completed their current cell cycle prior to transplantation migrated to layer VI, maintaining the laminar identity appropriate for their birthdate. However, when E29 precursors were transplanted during their S phase, about 85% of labelled cells switched fates and migrated to layers II/III, thereby adopting the laminar fate of currently generated host neurons (McConnell and Kaznowski, 1991). These studies demonstrated that laminar identity is decided prior to terminal mitosis, and established the importance for proper cell cycle control in cortical development.

III-IV. Neuronal migration

The locations of mature cortical neurons are distinct from the germinal regions in which they were born. Therefore, to reach their final destination, neurons must migrate from their site of origin to their site of function. This is important not only for the establishment of cortical architecture, but also to provide neuronal heterogeneity (Tanaka *et al.*, 2003). The developing nervous system is topologically a tube, with the germinal zones on the inside lining fluid-filled ventricles. Therefore, the newly born neurons must travel in an inside-out direction, moving from the inner germinal zones out towards the outer surface (or pial

surface) to reach their ultimate destinations. While the most direct route would be a radial trajectory, there have now been found to be more complex migratory pathways.

Radial migration

Historically, it was believed that all cortical neurons were generated within the VZ and underwent radial migration to their ultimate position (Ramón y Cajal, 1891; Rakic, 1972; Rakic, 1988). The columnar organization of the cortex was the basis of the protomap hypothesis which postulated a point-to-point relationship between the VZ of the neural tube and the pial surface (Rakic, 1988). In this way, cortical progenitors born in the dorsal VZ would migrate radially out into the developing CP, using radial glial fibres as a substrate.

Radial glial cells arise during early VZ development. Each cell has its soma in the VZ and extends a long, unbranched, process which spans the width of the cortex to reach the pial surface (Retzius, 1892; Ramón y Cajal, 1911; Schmechel and Rakic, 1979; Gadisseux *et al.*, 1989). Newly born neurons use the radial glial cell processes to guide their migration, termed glial-guided locomotion (Rakic, 1972; Edmondson and Hatten, 1987; Noctor *et al.*, 2001). Once neuronal migration has completed, the radial glial cells retract their ventricular and pial attachments and undergo differentiation into astrocytes (Ramón y Cajal, 1911; Schmechel and Rakic, 1979; Levitt *et al.*, 1981; Gaiano *et al.*, 2000). It has been discovered more recently that radial glial cells do not serve merely a supportive function, but can divide to produce new neurons (Malatesta *et al.*, 2000; Miyata *et al.*, 2001; Noctor *et al.*, 2001; Heins *et al.*, 2002; Noctor *et al.*, 2002).

Migrating neurons are guided by Cajal-Retzius (CR) neurons in the MZ. The CR neurons synthesize and secrete Reelin, a large protein which associates with the extracellular matrix (D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995; Ogawa *et al.*, 1995) and is a high affinity ligand for two members of the low density lipoprotein (LDL) family of lipoprotein receptors- the very-low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999). These receptors are expressed by migrating neurons and their activation induces Reelin internalization and tyrosine phosphorylation of the mouse homologue of *Drosophila* Disabled (DAB1) (D'Arcangelo *et al.*, 1999; Hiesberger *et al.*, 1999; Howell *et al.*, 1999; Howell *et al.*, 2000). DAB1 is a cytoplasmic adapter protein that interacts with the cytoplasmic tails of VLDLR and ApoER2, and is involved in cytoskeletal reorganization (Trommsdorff *et al.*, 1998; Trommsdorff *et al.*, 1999). Reeler mutant mice, which lack the Reelin protein, exhibit a loss of the normal, "inside-out" laminar pattern in the cortex. Although neurons are formed in the correct sequence, they become localized in the cortex in a partially inverted and disorganized "outside-in" pattern (Caviness, 1982a; Hoffarth *et al.*, 1995; Ogawa *et al.*, 1995; Sheppard and Pearlman, 1997; Rice and Curran, 2001). Mice with mutations or deletions in the genes encoding VLDLR, ApoER2, or DAB1 have phenotypes almost identical to that of the reeler mouse, suggesting that these molecules function in a common pathway (Sweet *et al.*, 1996; Gonzalez *et al.*, 1997; Howell *et al.*, 1997; Sheldon *et al.*, 1997; Ware *et al.*, 1997; Yoneshima *et al.*, 1997; Rice *et al.*, 1998; Trommsdorff *et al.*, 1999).

It has been suggested that Reelin may act as a stop signal for migrating neurons (Frotscher, 1997; Sheppard and Pearlman, 1997; Dulabon *et al.*, 2000), since treatment with

full length recombinant Reelin is sufficient to reduce the migratory rate of cells *in vitro* (Dulabon *et al.*, 2000). Alternatively, Reelin may promote neuron detachment from radial glial fibres, since defective detachment of early neuronal cohorts could explain the accumulation of successive populations underneath (Marin and Rubenstein, 2003). A third hypothesis would be that Reelin may directly regulate radial glial function (Super *et al.*, 2000). This is consistent with the observation that the radial glia scaffold fails to form properly in reeler mice (Hunter-Schaedle, 1997; Forster *et al.*, 2002) and that loss of CR neurons induces radial glia to undergo premature astrocytic differentiation (Super *et al.*, 2000).

A second neuronal positioning pathway involves signalling through CDK5. Unlike the remainder of the CDK family, CDK5 is expressed almost exclusively in differentiated neurons and does not appear to have a role in cell cycle regulation (Tsai *et al.*, 1993; Ino *et al.*, 1994). Mice deficient for CDK5 or its activating subunits, p35 and p39, exhibit defective lamination in a manner similar but not identical to the reeler mouse (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Gilmore *et al.*, 1998; Kwon and Tsai, 1998; Ko *et al.*, 2001).

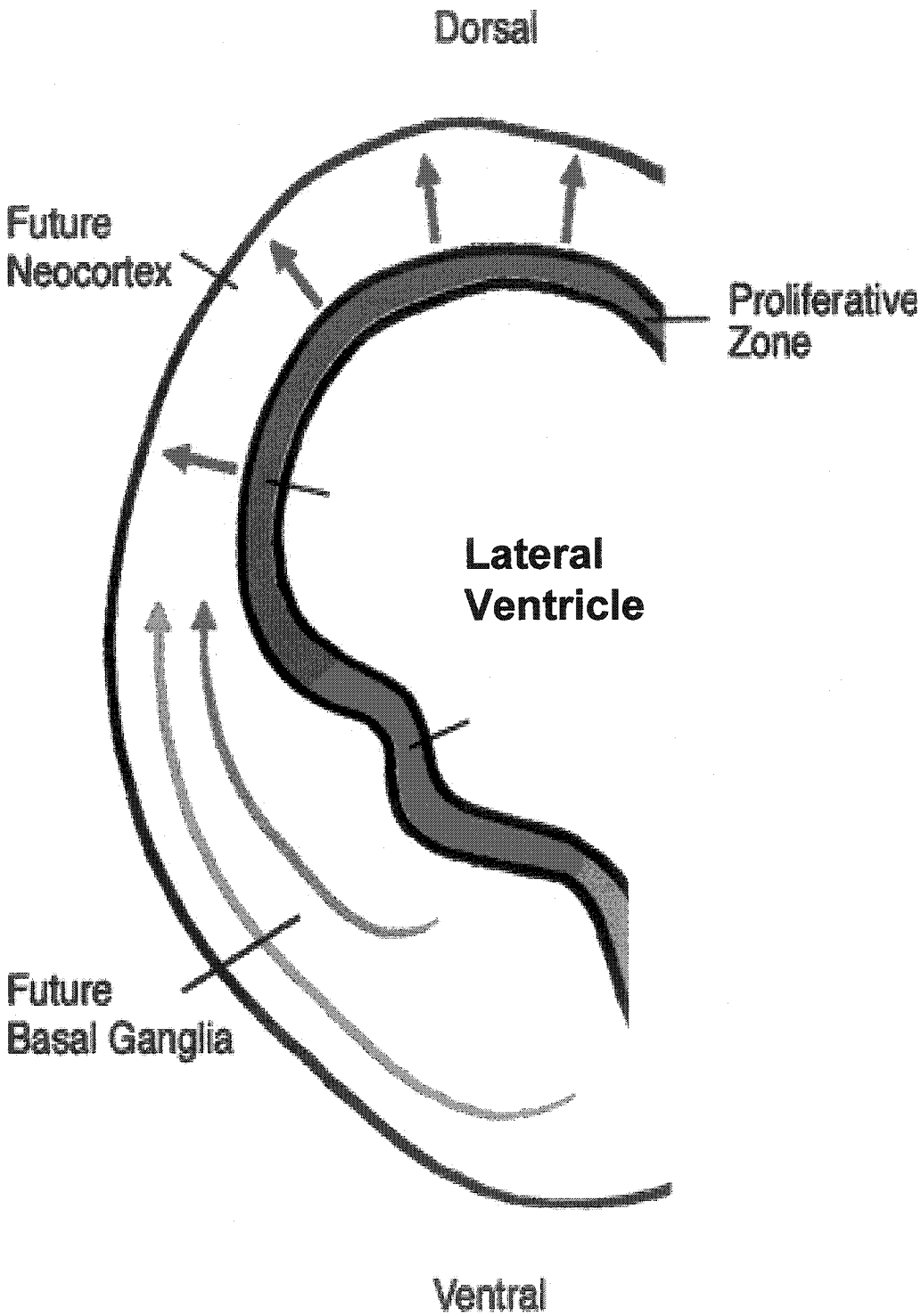
Tangential migration

The first evidence of nonradial migration came from observations of tangential neuronal dispersion during cortical development (Parnavelas *et al.*, 1991; O'Rourke *et al.*, 1992; Fishell *et al.*, 1993; Tan and Breen, 1993; O'Rourke *et al.*, 1995; Tan *et al.*, 1995; de Carlos *et al.*, 1996; O'Rourke *et al.*, 1997). Further support for the idea came from studies of retroviral lineage clones in which some clonally-related neurons were found to be dispersed

over wide lateral ranges (Price and Thurlow, 1988; Walsh and Cepko, 1988; Walsh and Cepko, 1992; Mione *et al.*, 1994; Reid *et al.*, 1995). A striking difference was found between these clonally related cells- radially oriented clones were predominantly pyramidal, glutamatergic, neurons, while the tangentially scattered clones were usually nonpyramidal, GABAergic neurons (Mione *et al.*, 1994). However, the source of these tangentially migrating cells was unknown until it was observed that the *Distal-less* homeobox gene 1 and 2 (*Dlx1/Dlx2)* knockout mice had virtually no GABAergic cortical neurons (Anderson *et al.*, 1997). Since Dlx1 and Dlx2 are expressed in the germinal regions of the GE, it was proposed that GE-derived neurons migrated to the cortex and preferentially gave rise to GABAergic interneurons (Anderson *et al.*, 1997). These studies are consistent with what is now known about the diverse migrating populations: neurons derived from the dorsal VZ which migrate radially in to the cerebral cortex give rise to glutamatergic projection neurons (Parnavelas, 2000; Noctor *et al.*, 2001; Hatanaka and Murakami, 2002; Parnavelas *et al.*, 2002), while cells arising from the ventral telencephalon, mainly the MGE, generate GABAergic interneurons (Anderson *et al.*, 1999; Corbin *et al.*, 2001; Lambert de Rouvroit and Goffinet, 2001; Marin and Rubenstein, 2001; Wichterle *et al.*, 2001; Anderson *et al.*, 2002; Nadarajah and Parnavelas, 2002; Parnavelas *et al.*, 2002; Stuhmer *et al.*, 2002) (Fig. 1-6: Radial and tangential neuronal migration).

LGE-derived neurons migrate ventrally and anteriorly, giving rise to medium spiny neurons in the dorsal and ventral striatum and to interneurons in the olfactory bulb. Proliferating stem cells in the LGE remain in the SVZ where they establish a germinal region that persists into adulthood (Lois and Alvarez-Buylla, 1993). Although progenitors derived

Fig. 1-6: Radial and tangential neuronal migration. Cortical neurons derived from the dorsal VZ (blue) migrate radially into the cortical plate, giving rise to glutamatergic projection neurons. In contrast, cortical interneurons are belived to be almost exclusively generated in the medial and lateral ganglionic eminences (MGE and LGE, respectively) of the ventral telencephalon (orange). Ventrally-derived interneurons migrate tangentially to the cortex along two distinct routes- one deep and the other superficial to the developing striatum (orange and green arrows) (Modified from Ross, Greenberg & Stiles, 2003).


Dorsal
Future
Neocortex
Proliferative
Zone
Lateral
Ventricle
Future
Basal Ganglia
Ventral

from the LGE and caudal ganglionic eminence (CGE) have also been found to be origins of cortical interneurons, the majority are derived from the MGE (Anderson *et al.*, 2001; Nery *et al.*, 2002). In contrast to LGE-derived progenitors, those originating in the MGE migrate dorsally and spread across most of the dorsal forebrain. The main target is the neocortex (Anderson *et al.*, 1997; Lavdas *et al.*, 1999; Sussel *et al.*, 1999; Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Wichterle *et al.*, 2001), but these neurons also migrate to the dorsal striatum (Marin *et al.*, 2000), amygdala, globus pallidus (Wichterle *et al.*, 2001), and hippocampus (Pleasure *et al.*, 2000), however, they do not migrate into neighbouring ventral regions such as the hypothalamus, preoptic area, septum, or olfactory bulb (Wichterle *et al.*, 2001).

Tangentially migrating interneurons follow restricted migratory routes. They generally avoid entering the developing striatum and therefore, form two distinct paths- either superficial or deep to the striatal mantle (Marin *et al.*, 2001). Superficially migrating neurons tend to avoid the cortex, migrating instead along the MZ or the SP, whereas the deep neurons travel through the lower IZ and SVZ (Del Rio *et al.*, 1992; DeDiego *et al.*, 1994; Lavdas *et al.*, 1999; Denaxa *et al.*, 2001; Marin and Rubenstein, 2001; Wichterle *et al.*, 2001). Whichever path they take, once they have migrated the appropriate distance, they can then switch to a radial mode of migration to enter the cortex (Polleux *et al.*, 2002). The particular route traveled may depend on the neuronal subtype and also the time of generation.

While the molecules and substrates involved in guiding radial migration are fairly well understood, those involved in tangential migration are not yet known. Hepatocyte growth factor/scatter factor (HGF/SF), which is expressed in the telencephalon during the

period of tangential migration, has been shown in slice cultures to increase cell migration away from ventral regions (Powell *et al.*, 2001). Further, mice lacking the urokinase-type plasminogen activator receptor (u-PAR), required for HGF/SF activation, exhibit abnormal interneuron migration (Powell *et al.*, 2001). Tangential migration of MGE-derived neurons has been shown to be strongly stimulated by the TrkB ligands, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) (Polleux *et al.*, 2002). Further TrkB-deficient mice have a significant decrease in the number of migrating calbindin-positive GABAergic interneurons (Polleux *et al.*, 2002).

The substratum used by tangentially migrating interneurons is unknown. Unlike in radial migration, these neurons do not associate with radial glial fibres. The transient axonal glycoprotein-1 (TAG-1) adhesion molecule is expressed on corticofugal axons and has been suggested to act as a guidance substrate, since antibodies against TAG-1 reduced interneuron migration in slice culture (Denaxa *et al.*, 2001). However, while the upper IZ is rich in corticofugal axons, the majority of neurons migrate within the axon sparse lower IZ and SVZ (Marin and Rubenstein, 2003). Since TAG-1 is a glycosylphosphatidylinositol (GPI) anchored protein, its requirement as a substratum for migrating interneurons was directly tested by treatment of cortical slices with phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI anchors (Tanaka *et al.*, 2003). While TAG-1 staining was abolished, migration was unaffected by the treatment, demonstrating that tangentially migrating populations do not require association with corticofugal axons (Tanaka *et al.*, 2003).

The general ventral-to-dorsal direction of migrating interneurons seems to be established by cortical chemoattractive cues and by chemorepulsive factors generated in the ventral preoptic area (POa) (Marin *et al.*, 2003; Wichterle *et al.*, 2003). A diffusible cortical attractive activity (CAA) has been identified as a potential chemoattractive source (Marin *et al.*, 2003; Wichterle *et al.*, 2003). In matrigel matrix experiments, MGE cells preferentially migrated towards cortical cells (Marin *et al.*, 2003; Wichterle *et al.*, 2003). Further, in slice cultures, the addition of an ectopic cortex was sufficient to reroute migrating interneurons, in a distance-dependent manner (Marin *et al.*, 2003). Although Emx1 and 2 are co-expressed in the cortical VZ, with only minimal Emx2 present in the LGE (Briata *et al.*, 1996; Mallamaci *et al.*, 1998), tangential migration was greatly reduced in *Emx1/Emx2* mutants (Shinozaki *et al.*, 2002). In these mice, the cortex was severely disorganized, suggesting a requirement for normal cortical function in the guidance of tangentially-derived interneurons (Shinozaki *et al.*, 2002). Recently, stromal cell-derived factor-1 (SDF-1) and its receptor, the CXC chemokine receptor 4 (CXCR4) have been shown to regulate tangential migration into the cortex (Stumm *et al.*, 2003). SDF-1, which is expressed in meningeal cells at the pial surface, acts as a potent chemoattractant for CXCR4, which is expressed in migratory interneurons. Mice with mutations in either SDF-1 or CXCR4 had far fewer interneurons present in superficial cortical layers, with accordingly increased interneurons in deep cortical layers. It was thereby concluded that SDF-1/CXCR4 signalling selectively promotes interneuron migration, by acting on distinct populations, and at later developmental stages (Stumm *et al.*, 2003).

Although the chemorepulsive agent(s) have not yet been identified, the Slits, which are large extracellular matrix molecules (Brose and Tessier-Lavigne, 2000), have been suggested to be involved. Gain-of-function studies have found that Slit1, which is expressed in the GE, may guide GABAergic neurons to the cortex and olfactory bulb (Wu *et al.*, 1999; Zhu *et al.*, 1999). In addition, ectopic Slit1 was capable of reversing the direction of interneurons migrating in culture (Ward *et al.*, 2003). However, *Slit1/Slit2* mutant mice retain the chemorepellant activity in the POa and display normal tangential migration (Marin *et al.*, 2003).

The guidance of interneurons to specific destinations may be mediated by repulsion through neuropilin/semaphorin interactions. Neuropilin-1 (Npn-1) and -2 (Npn-2) are large transmembrane receptors (Raper, 2000), which are expressed by interneurons migrating to the cortex, but not by striatal-bound interneurons (Tamamaki *et al.*, 2003). The neuropilins mediate the repulsive activity of the class 3 semaphorins (Sema3A and Sema3F) (Raper, 2000), which are present on axons within the striatal mantle. Disruption of Npn-1 or Npn-2 results in an increased number of interneurons migrating to the striatum, and consequent decrease in cortical interneurons (Marin *et al.*, 2001). It seems likely that a variety of chemoattractants and repellents as well as motogens and adhesion molecules may be responsible for promoting and guiding the migration for diverse neuronal populations. Similar to radial migration, the final location of tangentially migrating interneurons is not random. The laminar pattern still holds such that earlier generated interneurons tend to reside in deeper cortical layers while those born later will migrate to more superficial locations (Valcanis and Tan, 2003).

III-V. Research Objectives (Chapter 4)

Given the intimate link between precise cell cycle regulation and the generation of diverse neuronal subtypes, the role of Rb function was examined in the developing cortex, within the context of telencephalon-specific deficiency. My objective was to examine the role of Rb in the developing cortex, and determine requirements for proper cortical structure, and the production and migration patterns of specific neuronal subtypes. I assessed whether Rb deficiency affected laminar cortical organization, the expression of various neuronal specification markers, and the generation and localization of diverse neuronal lineages.

IV. Statement of Problems and Objectives

Although there has been extensive research examining Rb-mediated cell cycle regulation, the overwhelming majority of this work has focused on its role as a tumour suppressor, or has used tumour cells which lack functional Rb protein to study cell cycle dynamics. Therefore, in the present studies, I aimed to: 1) Characterize the key cell cycle regulatory molecules involved in neural precursor cell proliferation and differentiation and ascertain whether the Rb signalling pathway was required in this process. 2) Determine the requirement for Rb in the developing cortex, in the absence of the pleiotropic defects associated with the whole embryo Rb knockout; and 3) Examine the role that Rb plays in

determining proper cortical architecture and defining diverse neuronal subtypes. Therefore, the primary objectives of this research were as follows:

1. **To characterize the cell cycle regulators involved in neural precursor cell proliferation and differentiation, and to determine whether Rb was sufficient to induce growth arrest in this context (Chapter 2).**

Given the previous evidence demonstrating the tissue-specific variability, and the fact that there existed very little information regarding Rb function in primary neural precursor cells, I sought to undertake a detailed characterization of the cell cycle regulators of the Rb signalling pathway, in this context. This was conducted by Western analyses examining expression patterns during an *in vitro* time course of differentiating primary neural precursor cells. Further, I aimed to determine the importance of Rb signalling in this process.

I hypothesized that activation of the Rb pathway alone would be sufficient to induce cell cycle arrest in primary neural precursor cells. Further, this would be independent of the proposed function of cyclin-dependent kinases 4/6 (CDK4/6) to sequester the CIP/KIP CDK inhibitors (CKIs) p21 and p27 from CDK2.

To test this, I introduced dominant negative CDK mutants, using adenovirus mediated gene delivery, into primary neural progenitor cells derived from E12.5 wild type and Rb-deficient embryos. I then assessed the growth arrest mediated by each mutant and

whether this ability to induce cell cycle exit was dependent on the presence of Rb. By immunoprecipitation studies, I confirmed that the CDK mutants retained their physiological binding preferences and examined whether the ability to induce growth arrest was independent of the previously proposed ability of CKI sequestration. This study was an important first step in determining the importance of the Rb signalling pathway in neural precursor cell cycle regulation. I then sought to determine the requirement for Rb in the *in vivo* context.

2. To determine the *in vivo* functions of Rb in cortical neurogenesis, in the absence of the pleiotropic defects associated with the germline Rb knockouts (Chapter 3).

The dramatic neural phenotype of germline Rb deficiency strongly suggested an important role for Rb in the developing nervous system, as these mutants exhibited extensive apoptosis throughout the CNS and PNS, as well as ectopic mitoses and defective differentiation (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). It was concluded from these studies that the inappropriate cell cycle regulation resulting from Rb deficiency was responsible for inducing apoptosis. The idea that unrestrained proliferation in a cell attempting to undergo differentiation, or in a terminally differentiated cell, would induce conflicting signals ultimately resulting in apoptosis, became a widely accepted theory. Rb then became regarded as a required neuronal survival factor. However, several inconsistencies existed in the literature. For example, neural precursors derived from Rb-deficient embryos were able to survive and differentiate in culture (Lee *et al.*, 1994;

Slack *et al.*, 1998; Callaghan *et al.*, 1999). Further, Rb deficient cells in chimeric mice also survived and appeared to undergo normal neuronal differentiation (Lipinski *et al.*, 2001). It was proposed that the neighbouring wildtype cells were able to maintain the survival of the Rb deficient cells, possibly by providing a required survival factor (Lipinski *et al.*, 2001). Due to the contradictions in the literature and the fact that Rb-deficient embryos died during mid-neurogenesis, the exact role of Rb in the developing nervous system remained unclear.

I hypothesized that Rb function would be required for terminal mitosis, but not for cell survival.

In order to ascertain the role of Rb in cortical neurogenesis, a conditional Rb mutant was generated in which Rb deficiency was restricted to the developing telencephalon. After confirming the efficiency and specificity of Rb deletion, I examined sections of conditionally deleted Rb mutants with littermate controls for the presence of inappropriate proliferation, apoptosis, and neuronal differentiation. This model provided an excellent opportunity to examine cell-autonomous and non-cell-autonomous functions of Rb in the absence of the pleiotropic defects associated with whole embryo Rb deficiency.

3. **To examine the requirement for Rb in the development of proper cortical architecture, and the generation of diverse neuronal subtypes (Chapter 4).**

While my previous study (Chapter 3) had aimed to establish the requirement for Rb in the developing cortex, in terms of cell cycle regulation, survival and neuronal differentiation, I next wanted to examine the role that Rb played in cortical organization. More specifically, I aimed to identify potential Rb functions in the development of the six-layered cortical structure, and in the generation of specific neuronal subtypes. It was reasoned that since precise cell cycle regulation is believed to be critical for determining the neuronal identity and laminar position of newly generated neurons (McConnell and Kaznowski, 1991; McConnell, 1995), then defective cell cycle regulation, resulting from Rb deficiency, may impair these processes. While it had been believed for some time that proper cell cycle regulation is an important component of cortical development, the underlying mechanisms were virtually unknown. The manner in which the timing of cell cycle exit, or the cell cycle regulators themselves, may impact the developmental process had not been studied. Therefore, I aimed to determine whether Rb itself played a role in the development of cortical organization, and to more precisely define its functions.

I hypothesized that Rb signalling would be essential for the proper development of cortical laminar architecture and for the generation and proper localization of diverse neuronal subtypes.

To test my hypothesis, I further made use of the telencephalon-specific conditional Rb mutants for my investigations. In sections from Rb deficient and littermate control embryos, I examined cortical laminar morphology histologically and quantified the layer-

specific cellularity. I then assessed the expression patterns of specific neuronal markers and specification factors by *in situ* hybridization and immunohistochemical analyses. This final study represented the last step in our examination of Rb function in the developing cortex. My studies started with the characterization of cell cycle molecules involved in neural precursor cell cycle regulation, to discovering the intricacies of Rb regulation in the development of cortical architecture, neuronal migration and the its requirement in diverse neuronal populations.

Ferguson, K. L. *et al* (2000) The Rb-CDK4/6 Signaling Pathway Is Critical in Neural Precursor Cell Cycle Regulation. *J. Biol. Chem.* **275**: 33593-600.

This first manuscript represents a characterization of various cell cycle molecules involved in the Rb signalling pathway. We examined the requirement for the Rb pathway in regulating neural precursor cell proliferation and differentiation.

The experiments in this manuscript were performed by K. L. Ferguson, with the assistance of S. M. Callaghan for some of the Western blots. The adenoviral vectors were constructed and provided by M. J. O'Hare, S. M. Callaghan, and Dr. D. S. Park. Reagents were provided as cited in the text. This manuscript was written by K. L. Ferguson with guidance and editorial assistance from Dr. R. S. Slack.

THE RB-CDK4/6 SIGNALING PATHWAY IS CRITICAL IN NEURAL PRECURSOR CELL CYCLE REGULATION

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ABSTRACT

The tumor suppressor, retinoblastoma (Rb), is involved in both terminal mitosis and neuronal differentiation. We hypothesized that activation of the Rb pathway would induce cell cycle arrest in primary neural precursor cells, independent of the proposed function of CDK4/6 to sequester the CKIs p21 and p27 from CDK2. We expressed dominant negative adenovirus mutants of CDKs 2, 4 and 6 (dnCDK2, 4 and 6) in neural progenitor cells derived from E12.5 wild type and Rb deficient mouse embryos. In contrast to previous studies, our results demonstrate that in addition to dnCDK2, the dnCDK4/6 mutants can induce growth arrest. Moreover, the dnCDK4/6-mediated inhibition is Rb-dependent. The dnCDK2 partially inhibited cell growth in Rb deficient cells, suggesting that CDK2 may have additional targets. A previously proposed function of CDK4/6 is CKI sequestration, thereby preventing the resulting inhibition of CDK2, believed to be the key regulator of cell cycle. However, our immunoprecipitations revealed that the dominant negative CDK mutants could arrest cell growth despite their interaction with p21 and p27. Taken together, our results demonstrate that both CDK2 and CDK4/6 are crucial for cell cycle regulation. Furthermore, our data underscore the importance of the Rb regulatory pathway in neuronal development and cell cycle regulation, independent of CKI sequestration.

INTRODUCTION

During embryogenesis, cycling neural progenitor cells in the ventricular zones commit to a neuronal fate, and as a consequence of that decision undergo terminal mitosis and adopt a neuronal phenotype. A key developmental step in this process is the decision to undergo terminal mitosis. Several lines of evidence indicate that terminal mitosis and terminal differentiation are intimately coupled. First, cells in the mitotic layer of the developing retina induce expression of a retinal ganglion cell marker within minutes of the S phase of terminal mitosis (1, 2). Second, neural precursor cells transplanted throughout the developing cortex exhibit appropriate neuronal identity only if they had undergone terminal mitosis following implantation (3). Third, sensory neurons override the G_1 restriction point and undergo S-phase death in the absence of the neurotrophin, NT-3 (4). The importance of terminal mitosis in neuronal development is underscored by the fact that failure to permanently withdraw from the cell cycle results in impaired differentiation and apoptosis. One key regulator of the cell cycle, the tumor suppressor protein pRb, has recently been implicated in terminal mitosis and neuronal differentiation.

Mice nullizygous for Rb die by E15.5 from haematopoietic and neurological defects attributed to failed terminal differentiation (5-7). By E12.5 onward, ectopic mitoses and massive cell death are observed throughout the developing nervous system. Studies examining neuronal development have revealed reduced expression of pan-neuronal genes as well as the NGF receptor TrkA, suggesting that Rb has an important role in differentiation (8). Examination of the expression of a neuron-specific transgene in Rb-deficient embryos revealed impaired neurogenesis in virtually all neuronal cell types examined (9).

Furthermore, Rb-deficient neural precursor cells exhibited a dramatic delay in their ability to undergo terminal mitosis (10), indicating that Rb plays an important role in regulating the onset of terminal mitosis in progenitor cells.

While Rb is thought to be a key regulator of terminal mitosis in neural precursor cells (reviewed in 11), there is little evidence that activation of the Rb pathway is sufficient to induce cell cycle arrest in this cell type. Furthermore, although it is well established that CDK2, 4 and 6 are all involved in cell cycle regulation, their precise roles and importance are still the subject of controversy. Indeed, previous studies examining the role of cyclin-dependent kinases (CDKs) in cell cycle regulation have indicated that inhibition of CDK4/6, which function through the Rb pathway, is insufficient to induce mitotic arrest in several cell types. Specifically, a dominant negative mutant of CDK2, which sequesters cyclins A and E from endogenous CDKs, could efficiently induce growth arrest, while dominant negative mutants of CDK4/6 which sequester D cyclins, were unable to mediate cell cycle arrest (12). One interpretation of these results is that activation of the Rb pathway alone may not be sufficient to mediate arrest of cell growth. It has recently been suggested that CDK4/6 in its association with D cyclins, may have dual functions: First, these proteins inactivate Rb activity by phosphorylation (13-18). Secondly, CDK4/6 may sequester the CIP/KIP CDK inhibitors (CKIs) p21 and p27, thus rendering them unavailable to bind and inactivate CDK2 (19). Recently, p21 and p27 have been shown to associate with CDK4/6 in a facilitatory CDK-cyclin interaction without inhibition of kinase activity (19-21). In addition, as the INK4 CKIs p15, p16, p18, and p19 become expressed, they displace p21/p27, thereby releasing them to associate with CDK2, an interaction mediating G_1 arrest (19, 22, 23). Thus, it is believed that the inhibition of CDK2/cyclin E activity is the crucial step in cell cycle

regulation. The notion of CDK2 as the major regulator of cell cycle progression, with CDK4/6 being merely facilitative has become quite pervasive (19). While this model is supported by a number of studies, it should be noted that the experiments using dominant negative mutants were conducted entirely on tumor cells and may not reflect the true mechanism of cell cycle regulation in primary cells.

In view of the importance of Rb, as demonstrated by the severe nervous system defects in transgenic mice carrying null mutations for Rb, we hypothesized that activation of the Rb pathway alone would be sufficient to induce cell cycle arrest in primary neural precursor cells, independent of its function in the sequestration of the p21/p27 proteins from CDK2. To address this question, we have used primary neural progenitor cells derived from E12.5 wild type and Rb-deficient embryos. Dominant negative CDK mutants were introduced into these cells using adenovirus mediated gene delivery. In contrast to studies using cell lines, our results demonstrate that dominant negative mutants of CDK4 and 6 can effectively induce mitotic arrest in primary neural precursor cells, independent of the previously proposed binding and sequestration of p21 and p27. The fact that these CDK4/6 mutants are non-functional in cells that lack Rb demonstrates that the activation of the Rb pathway in itself is sufficient to cause cell cycle arrest in primary neural precursor cells.

METHODS

Transgenic Mice

Rb-deficient transgenic mice, originally generated by Jacks *et al.*, (6), were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained on a C57BL6 genetic background. Mice were genotyped by PCR, as previously described (6) using DNA extracted from tails for adults or from remaining embryonic tissue for cell culture studies. For timed pregnancies, mice were bred and the time of plug identification was counted as day 0.5. For cultures of primary neural precursor cells, embryos were removed at E12.5.

Primary Cultures of Cortical Progenitor Cells, Neurons and Neural Stem Cells

Neural progenitor cells were prepared as previously described (24), with some modification (9). Cortices were collected from E12.5 mouse embryos, triturated, and aggregates were plated on poly-*L*-ornithine/laminin coated dishes. The culture medium consisted of Neurobasal medium (Gibco/BRL), 0.5 mM glutamine, 50 units/mL penicillin-streptomycin, and 1% N2 supplement (Gibco/BRL). In addition, bFGF (25 ng/mL, Sigma) was added only upon plating. After 48 hours, medium was replaced with the same medium except 1% N2 supplement was now replaced with 2% B27 (Gibco/BRL). To culture Rb-deficient progenitor cells, the neocortex was removed from embryos at E12.5, triturated, and plated individually. After PCR genotyping, the appropriate wild type and $Rb^{-/-}$ littermates were selected for experimentation on day 2 after plating. Mature postmitotic neurons were prepared from E17 embryos, from which cortices were collected and triturated in culture

medium (Neurobasal medium with 0.5 mM glutamine, 50 units/mL penicillin-streptomycin, 1% N2 supplement and 2% B27).

As a second primary precursor cell population, rapidly proliferating neural stem cells were isolated from the striatum. At E12.5, the striatum was removed from mouse embryos and triturated to produce a single-cell suspension. Stem cells were plated at a density of 4×10^4 cells/mL in DMEM/F12 medium containing 20 ng/mL bFGF as previously described (25). These cells were grown as spheres in suspension and medium was added on day 3 after trituration. To passage, spheres were again triturated to obtain a single-cell suspension and replated at 4×10^4 cells/mL. Stem cells were used for experimentation after at least 2 passages and were not kept beyond 8 passages.

BrdU Incorporation and Immunocytochemistry

For BrdU labeling, cells were incubated in medium containing 10 μM BrdU for 18 hours prior to staining. After a 5 min fixation in -20°C 1:1 methanol-acetone, cells were washed 3 - 5 min in PBS and stained with antibodies directed against lacZ or FLAG. Following 3 subsequent washes, cells were stained with the appropriate secondary antibody conjugated to CY3. Prior to staining with anti-BrdU, cells were treated with 2N HCl for 10 min to denature DNA, and then neutralized with 0.1 M $Na_2B_4O_7$ for another 10 min. Samples were washed 3 × 5 min with PBS containing 0.5% Tween-20 and 1% BSA prior to an overnight incubation at 4°C with the primary anti-BrdU antibody diluted at 1:25. After 3 - 5 min washes, samples were incubated with a goat anti-mouse CY2-conjugated secondary

antibody (Jackson Laboratories) for 1 hour at RT and then washed 3 × 5 min in PBS prior to examination.

Construction of Recombinant Adenovirus Vectors

The cDNA for the dominant negative mutants of CDK2, 4 and 6 were obtained from Dr. Ed Harlow (12), and were epitope-tagged with FLAG at the C-terminus (26). The CDK mutants were subcloned into the Xba1 site of the pAdlox vector (27) that was recombined with the adenoviral backbone DNA and amplified in CRE8 cells as previously described (27). The control vector carrying the *E. coli* lacZ expression cassette, Ad5CA17lacZ (Ad[lacZ]), was constructed by Christina Addison in the laboratory of Dr. Frank Graham (unpublished), McMaster University, Hamilton, Ontario.

Infection of Primary Neural Precursor Cells with Recombinant Adenovirus Vectors

For virus infection, cortical progenitor cells were plated in 4-well Nunc tissue culture dishes coated with poly-*L*-ornithine/laminin in 400 μL of plating medium. After 48 hours, another 400 μL of medium containing the appropriate titre of the adenovirus recombinant vector was added to each well. Eighteen hours following infection, a half medium change was carried out. The multiplicity of infection (MOI) indicates the number of plaque-forming units added per cell. Neural stem cells were infected with adenovirus recombinants 3 days after trituration. The appropriate vector titre was added directly into each dish and incubated for 6 hours after which a complete medium change was carried out. The cells were centrifuged at 1000 × g and the pellet was resuspended in fresh medium. Samples were harvested 72 hours post-infection for immunoprecipitation.

Western Blot Analysis

For Western analysis, protein was harvested in lysis buffer A (50 mM HEPES pH 7.8, 250 mM KCl, 0.1 M EDTA, 0.1 M EGTA, 10% glycerol, 0.1% NP40, 1.0 mM DTT, 0.5 mM PMSF, 5 g/mL aprotinin, 2 g/mL leupeptin, 0.4 mM sodium vanadate) for 20 min on ice, followed by a 10 minute microfuge centrifugation. Protein was separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking overnight at 4°C in 5% skim milk, membranes were incubated in the primary antibody for 1-2 h at RT. After 3 - 5 min washes in TPBS (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 0.5 N NaCl, 0.1% Tween-20), membranes were incubated for 1 hour at RT in the secondary HRP-conjugated antibody (anti-mouse HRP-Biorad Cat. #170-6516; anti-rabbit HRP-Biorad Cat.#170-6515). Blots were developed by chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

Immunoprecipitations

Neural stem cells expressing the dominant negative CDK mutants were harvested 72 hrs postinfection in lysis buffer B (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM CaCl, 1% triton X-100, 1.0 mM DTT, 0.5 mM PMSF, 5 g/mL aprotinin, 2 g/mL leupeptin, and 0.4 mM sodium vanadate) for 20 min on ice. Cell lysates containing 100 μg protein as well as a control sample containing beads in lysis buffer only, were rotated overnight at 4°C with anti-FLAG M2-conjugated agarose beads. The beads were washed in lysis buffer three times and then eluted by boiling for 2 min in SDS PAGE loading buffer. The eluate was then used for Western analysis with antibodies directed against the appropriate cyclins and CKIs as noted in figure legends.

Antibodies

The following primary antibodies were used: anti-β-galactosidase mouse monoclonal antibody (Promega, Z3789), mouse monoclonal M2 anti-FLAG antibody (Sigma, F3165) and beads (Sigma, A1205) biotinylated M2 anti-FLAG antibody (Sigma, F9291), mouse monoclonal anti-p27 (Transduction Laboratories, K25020), mouse monoclonal anti-BrdU (Becton Dickinson, 347580), and mouse monoclonal anti-Rb (Pharmingen, 14001A). All other antibodies were purchased from Santa Cruz Biotechnology Inc.: mouse monoclonal anti-p16 (sc-1661), rabbit polyclonal anti-p21 (sc-0397), rabbit polyclonal anti-CDK2 (sc-163), rabbit polyclonal anti-CDK4 (sc-749), rabbit polyclonal anti-CDK5 (sc-173), rabbit polyclonal anti-CDK6 (sc-177), mouse monoclonal anti-cyclin D1 (sc-6281), rabbit polyclonal anti-cyclin D2 (sc-593), rabbit polyclonal anti-cyclin D3 (sc-182), rabbit polyclonal anti-cyclin A (sc-596), and rabbit polyclonal anti-cyclin E (sc-481).

RESULTS

Regulated expression of CDK, cyclin, and CKI proteins in differentiating cortical progenitor cells

Prior to assessing the role of the Rb pathway in cell cycle regulation of neural precursor cells, Western analysis was conducted to characterize the expression of the cyclin-dependent kinases, cyclins and cyclin-dependent kinase inhibitors during the time course of differentiation. Stem cells, representing a highly proliferative population, and mature postmitotic neurons (E17) consisting of a quiescent population, were included at either end of time courses as a basis for comparison. First, the expression patterns of the CDKs were examined during the differentiation of cortical progenitor cells. As the population undergoes differentiation *in vitro*, CDK2 protein gradually becomes downregulated to very low levels (Fig. 1A). Similarly, CDK4 and 6 (Fig. 1B and D, respectively) are highly expressed in proliferating neural precursor cells and protein levels decrease as neurons develop. In contrast, the expression of CDK5, which is highly expressed in neuronal cells and not believed to function in the regulation of cell cycle (28, 29), is increased during the time course of differentiation, coincident with the appearance of postmitotic neurons in these cultures (Fig. 1C). Thus, in cortical progenitor cells, CDK expression is such that the levels of CDKs2, 4 and 6 become downregulated to low levels while CDK5 is induced as neurons are generated.

We next examined the expression of cyclins during the time course of neurogenesis (Fig. 2). The D cyclins, including cyclins D1, D2 and D3 are all expressed during neuronal

Fig. 1: Cyclin-dependent kinase expression is differentially regulated during neurogenesis. Cortical progenitor cells were cultured from wild type E12.5 embryos and allowed to differentiate over 7 days *in vitro*. Self-renewing neural stem cells (St) and E17 postmitotic cortical neurons (N) cultured for 7 days were included for comparison. After days 0 (initial isolation), 1, 3, 5 and 7, protein was extracted and 30 μg was loaded into each lane. Western blots were probed with antibodies against (A) CDK2, (B) CDK4, (C) CDK5 and (D) CDK6.

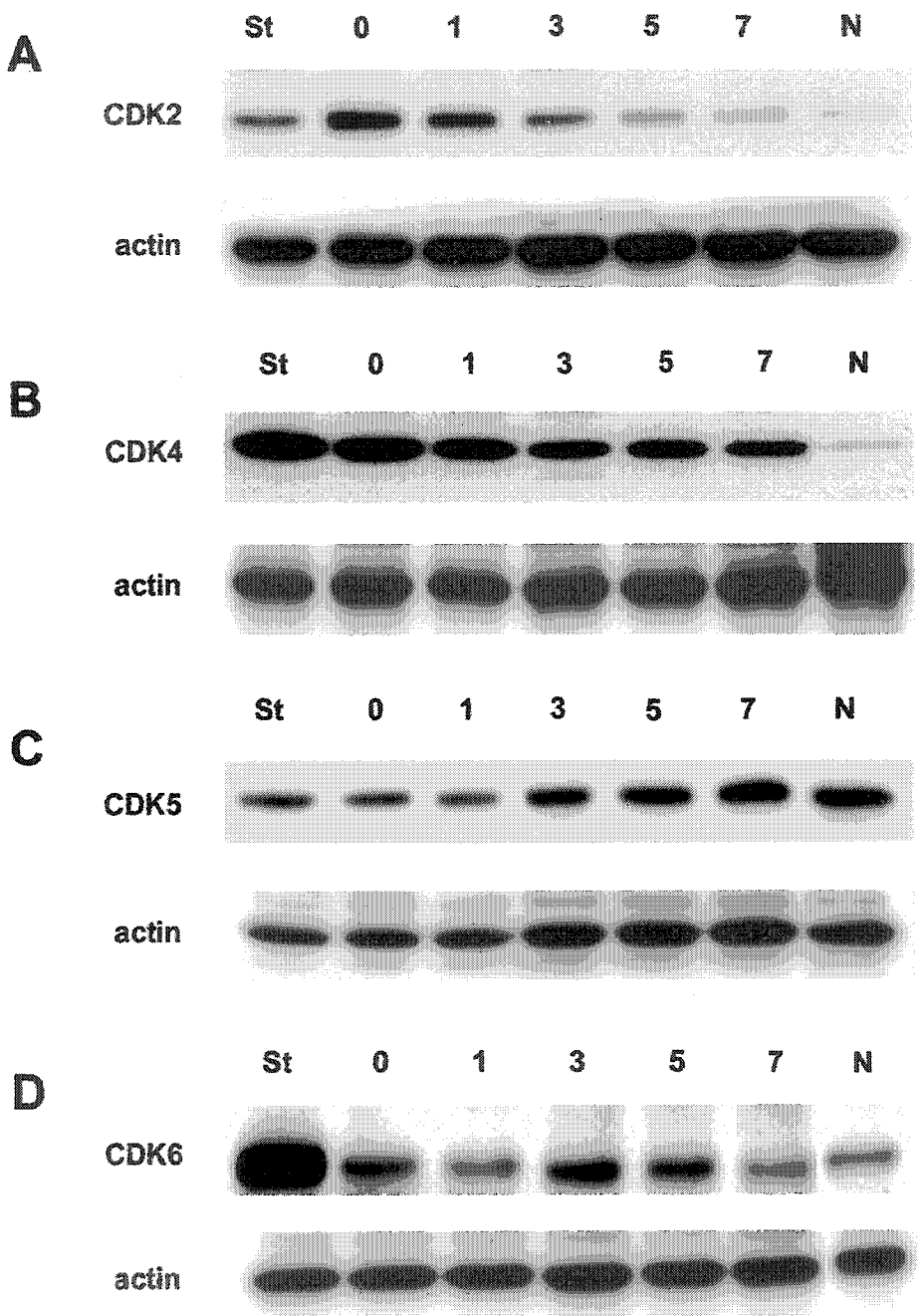
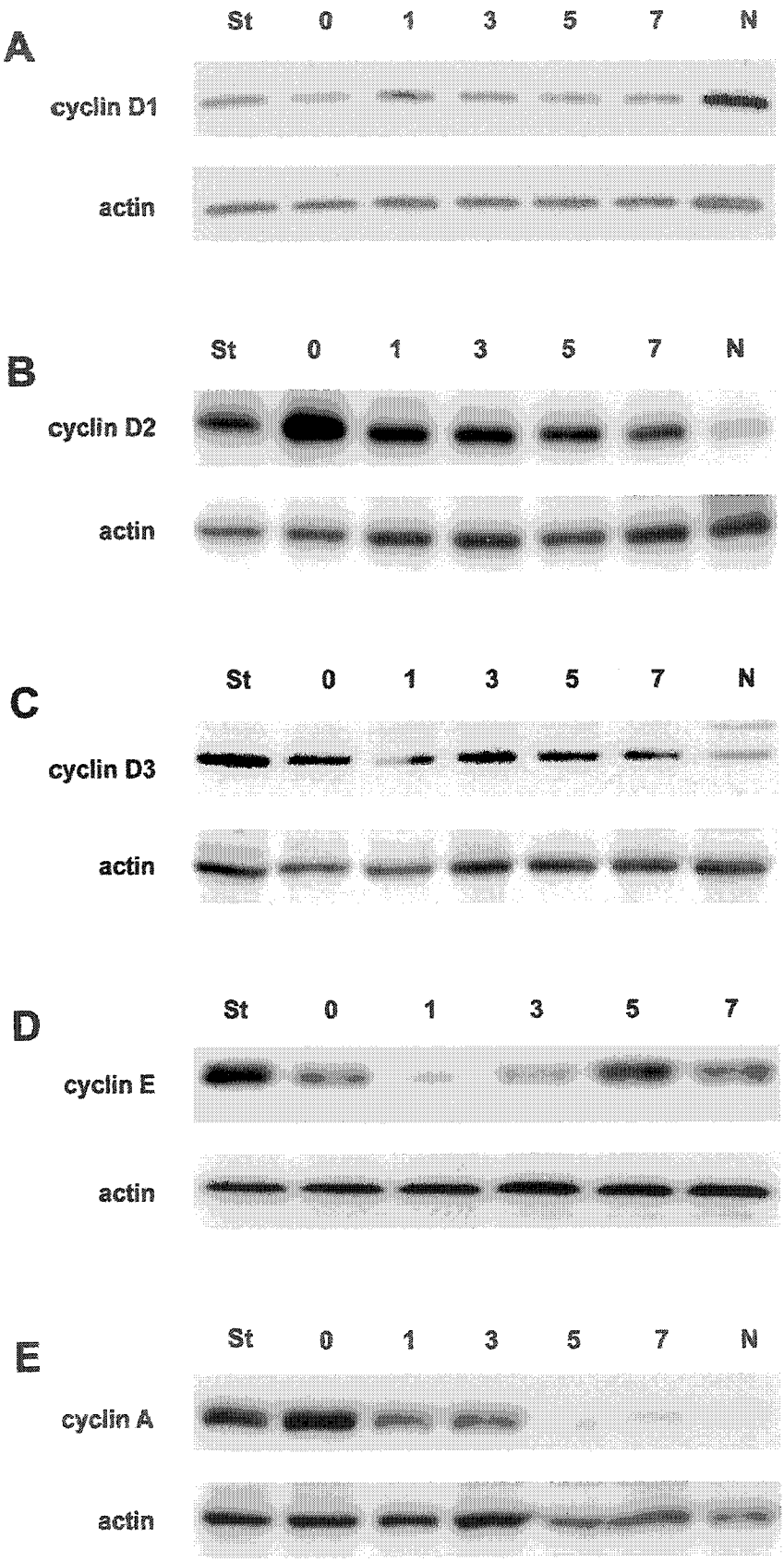

A
St 0 1 3 5 7 N
CDK2
actin
B
St 0 1 3 5 7 N
CDK4
actin
C
St 0 1 3 5 7 N
CDK5
actin
D
St 0 1 3 5 7 N
CDK6
actin

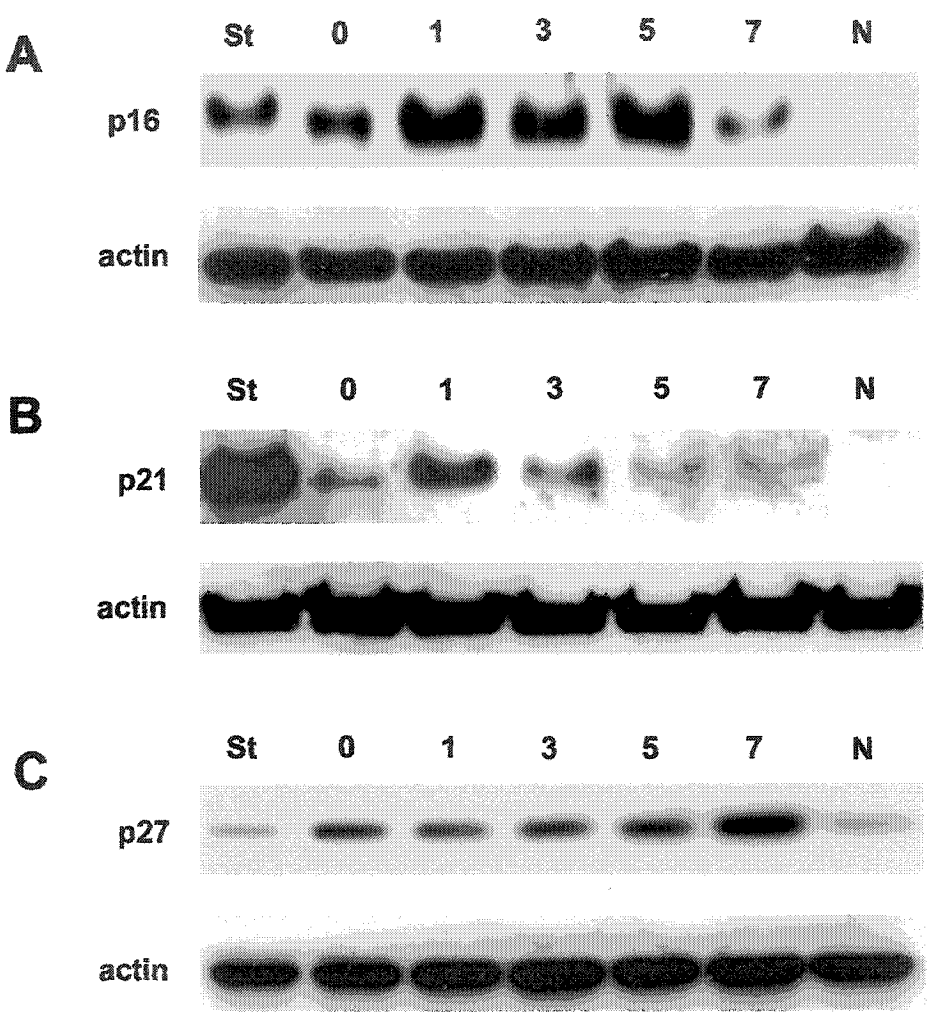
Fig. 2: Cyclin expression during differentiation of cortical progenitor cells. Cortical progenitor cells were cultured from wild type E12.5 embryos and allowed to differentiate over 7 days *in vitro*. Self-renewing neural stem cells (St) and E17 postmitotic cortical neurons (N) cultured for 7 days were included for comparison. After days 0 (initial isolation), 1, 3, 5 and 7, protein was extracted and 30 μg was loaded into each lane. Western blots were probed with antibodies against (A) cyclin D1, (B) cyclin D2, (C) cyclin D3, (D) cyclin E and (E) cyclin A.


A
St 0 1 3 5 7 N
cyclin D1
actin
B
St 0 1 3 5 7 N
cyclin D2
actin
C
St 0 1 3 5 7 N
cyclin D3
actin
D
St 0 1 3 5 7
cyclin E
actin
E
St 0 1 3 5 7 N
cyclin A
actin

differentiation *in vitro*, however, distinct differences in their regulation was apparent. Cyclin D1 is present at relatively low levels in proliferating cells but becomes upregulated as cells undergo differentiation, with highest expression in differentiated, postmitotic neurons (Fig. 2A). In contrast, cyclin D2 is highly expressed in proliferating cells but becomes downregulated during differentiation, although detectable levels remain in neuronal cultures (Fig. 2B). Cyclin D3 is readily detectable both in differentiated and undifferentiated cells (Fig. 2C). Cyclin E, a CDK2 binding partner, is expressed throughout the time course of differentiation (Fig. 2D). However, cyclin A, similar to the pattern observed with its binding partner CDK2, is expressed at high levels in proliferating cells and becomes downregulated as neurons are generated (Fig. 2E).

While CDK activity is regulated at several levels including the differential expression of the cyclin binding partners as well as the expression of the kinase itself, cyclin kinase inhibitors (CKIs) also play key roles in cell cycle regulation during neural differentiation (30, 31). We therefore examined the expression of the INK4 CKI p16 as well as the CIP/KIP kinase inhibitors p21 and p27 (Fig. 3). P16 is expressed in neural precursor cells and is upregulated as cells undergo differentiation (Fig. 3A). P27 exhibits a similar expression pattern, showing low levels in proliferating neural precursor cells that increase as differentiation proceeds (Fig. 3C). In contrast, p21 shows highest levels in rapidly proliferating neural stem cells, which gradually decrease during the time course of differentiation to low or undetectable levels in postmitotic neurons (Fig. 3B). The unexpected pattern of p21 expression, particularly the high levels in neural stem cells is consistent with the suggestion that p21 has functions beyond that of inhibition of cyclin-dependent kinase

Fig. 3: Cyclin-dependent kinase inhibitor protein expression during neuronal differentiation. Cortical progenitor cells were cultured from wild type E12.5 embryos and allowed to differentiate over 7 days *in vitro*. Self-renewing neural stem cells (St) and E17 postmitotic cortical neurons (N) cultured for 7 days were included for comparison. After days 0 (initial isolation), 1, 3, 5 and 7, protein was extracted and 30 μg was loaded into each lane. Western blots were probed with antibodies against (A) p16, (B) p21 and (C) p27.


A
St
0
1
3
5
7
N
p16
actin
B
St
0
1
3
5
7
N
p21
actin
C
St
0
1
3
5
7
N
p27
actin

activity (20, 21). Western analysis indicated that Rb is present at high levels in proliferating neural precursors and decreases as neurogenesis proceeds (Fig. 4).

Dominant inhibitory mutants of CDK2, 4 and 6 induce growth arrest in neural precursor cells

To determine which CDKs are important in the regulation of cell cycle progression in primary neural precursor cells, we constructed recombinant adenovirus vectors carrying FLAG-tagged dominant negative mutants of CDK2, 4 and 6 (dnCDK2, dnCDK4 and dnCDK6). These dominant inhibitory mutants have been previously described (12) and contain a point mutation in the kinase region, thereby rendering them inactive. These mutants retain the ability to bind cyclins, thus they will inactivate the function of endogenous CDKs by sequestering their required cyclins. To verify that adenovirus recombinants could express the CDK mutant proteins, neural progenitor cells were infected at 50 MOI and Western analysis was carried out 72 hours after viral infection. Under these infection conditions, the possibility of viral-mediated effects on cellular function and viability would be minimal (32). Western analysis using antibodies specific for lacZ, CDK2, CDK4 and CDK6 confirmed expression of the appropriate CDK mutants in cortical progenitor cells (Fig. 5). Protein expression was also readily detectable by immunostaining with an anti-FLAG antibody (see below).

Previous studies have shown that the dominant inhibitory mutant of CDK2 was capable of inducing growth arrest in several cell lines, whereas mutants of CDK4/6 were ineffective (12). As these studies were carried out in tumor cell lines, we questioned whether the activities of CDK4/6, which function through the Rb pathway, may be important in cell

Fig. 4: Expression of retinoblastoma protein during neurogenesis. Cortical progenitor cells were cultured from wild type E12.5 embryos and allowed to differentiate over 7 days *in vitro*. Self-renewing neural stem cells (St) and E17 postmitotic cortical neurons (N) cultured for 7 days were included for comparison. After days 0 (initial isolation), 1, 3, 5 and 7, protein was extracted and 30 μg was loaded into each lane. Western blots were probed with antibodies against Rb.

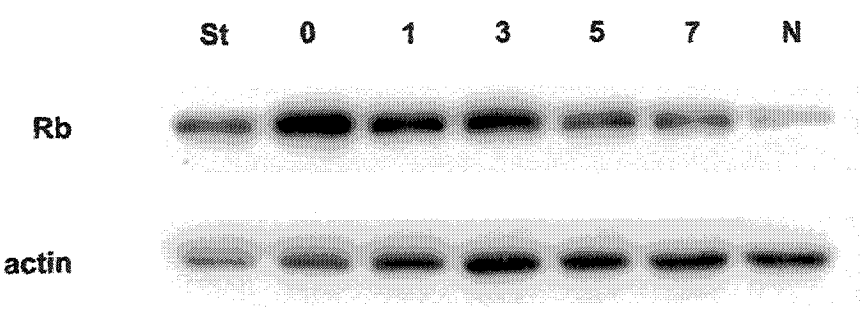
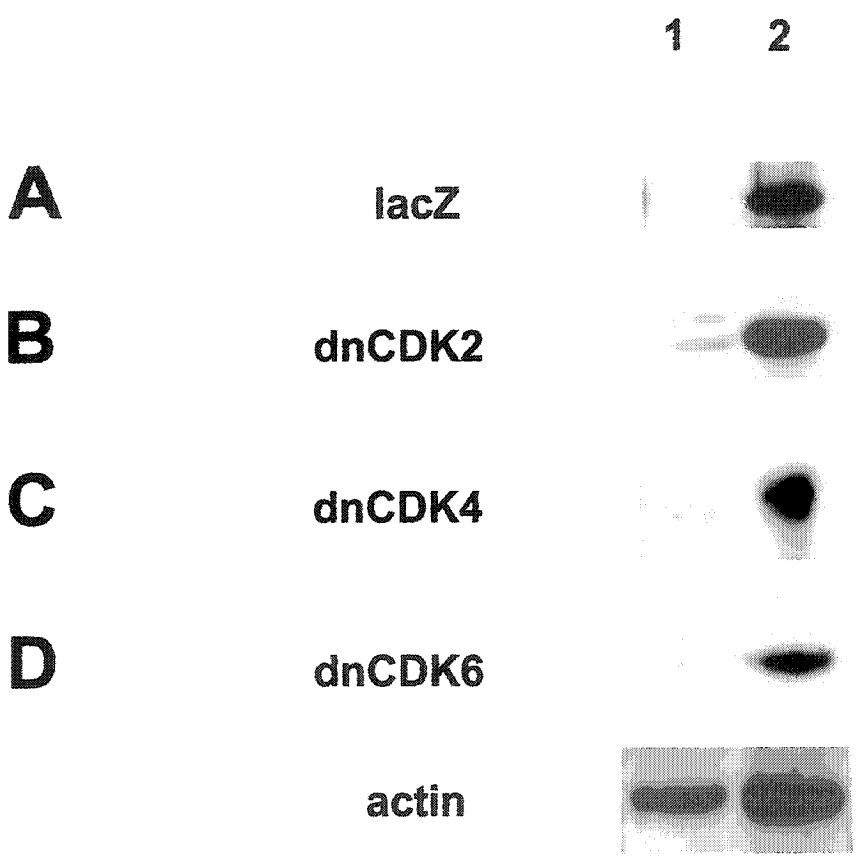

St
0
1
3
5
7
N
Rb
actin

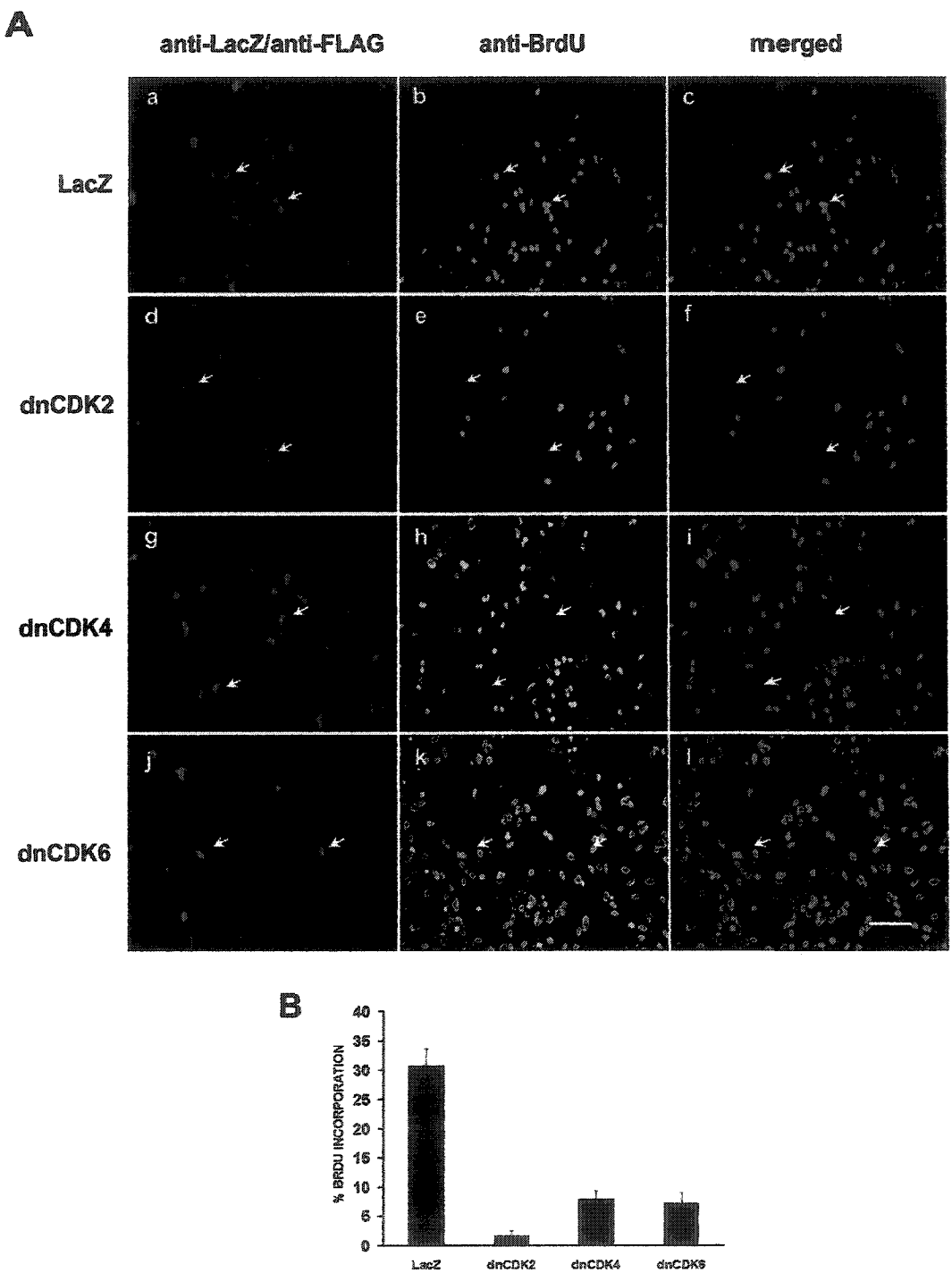
Fig. 5: Overexpression of dominant inhibitory CDK mutants in cortical progenitor cells by adenovirus mediated gene delivery. Cortical progenitor cells cultured were either untreated (Lane 1) or were infected (Lane 2) at 50 MOI with Ad[lacZ], Ad[dnCDK2], Ad[dnCDK4] or Ad[dnCDK6] 2 days after plating. At 72 h following infection, protein was extracted and 30 μg protein was loaded into each lane. Western blots were probed with antibodies against (A) lacZ, (B) CDK2, (C) CDK4 and (D) CDK6.


1
2
A
lacZ
B
dnCDK2
C
dnCDK4
D
dnCDK6
actin

cycle regulation in primary neural progenitor cells. Wild type neural precursor cells were infected after 2 days *in vitro* and BrdU incorporation was measured 72 hours thereafter. Since viral infections at 50 MOI resulted in less than 20% infection, cells were first stained with anti-FLAG to identify expressing cells followed by immunostaining with anti-BrdU (Fig. 6A). The number of FLAG-expressing cells that were BrdU positive was counted and the rate of BrdU incorporation was expressed as a percentage of infected cells. The percent BrdU incorporation was then compared to lacZ-expressing controls (Fig. 6B). In contrast to lacZ-expressing cells, which showed a significant rate of BrdU incorporation (Fig. 6A), there was very little BrdU incorporation in cells expressing dnCDK2 (Fig. 6A). Quantification of these results indicated that 31±3% BrdU incorporation was found in lacZ controls, whereas in dnCDK2 expressing cells, only 2±1% were BrdU-positive. Consistent with previous studies (12), our results indicate that dnCDK2 can efficiently induce growth arrest in cortical progenitor cells.

We next questioned whether dnCDK4 and dnCDK6 could also induce growth arrest in neural precursor cells. Examination of cortical progenitor cells expressing either dnCDK4 or dnCDK6 revealed that the majority of cells transduced were growth arrested and did not enter S phase (Fig. 6). Cell counts revealed that relative to 31±3% in lacZ-expressing controls, cells expressing dnCDK4 and dnCDK6 exhibited only 8±2% and 7±2% BrdU incorporation, respectively (Fig. 6B). These results indicate that dominant negative mutants, which specifically block the activity of CDK4/6, are sufficient to induce growth arrest in neural precursor cells. While no such inhibition was seen in previous studies using cell lines, our results suggest that this is an important growth regulatory pathway, and that CDK4/6 activation is essential for cell cycle progression in primary neural progenitor cells.

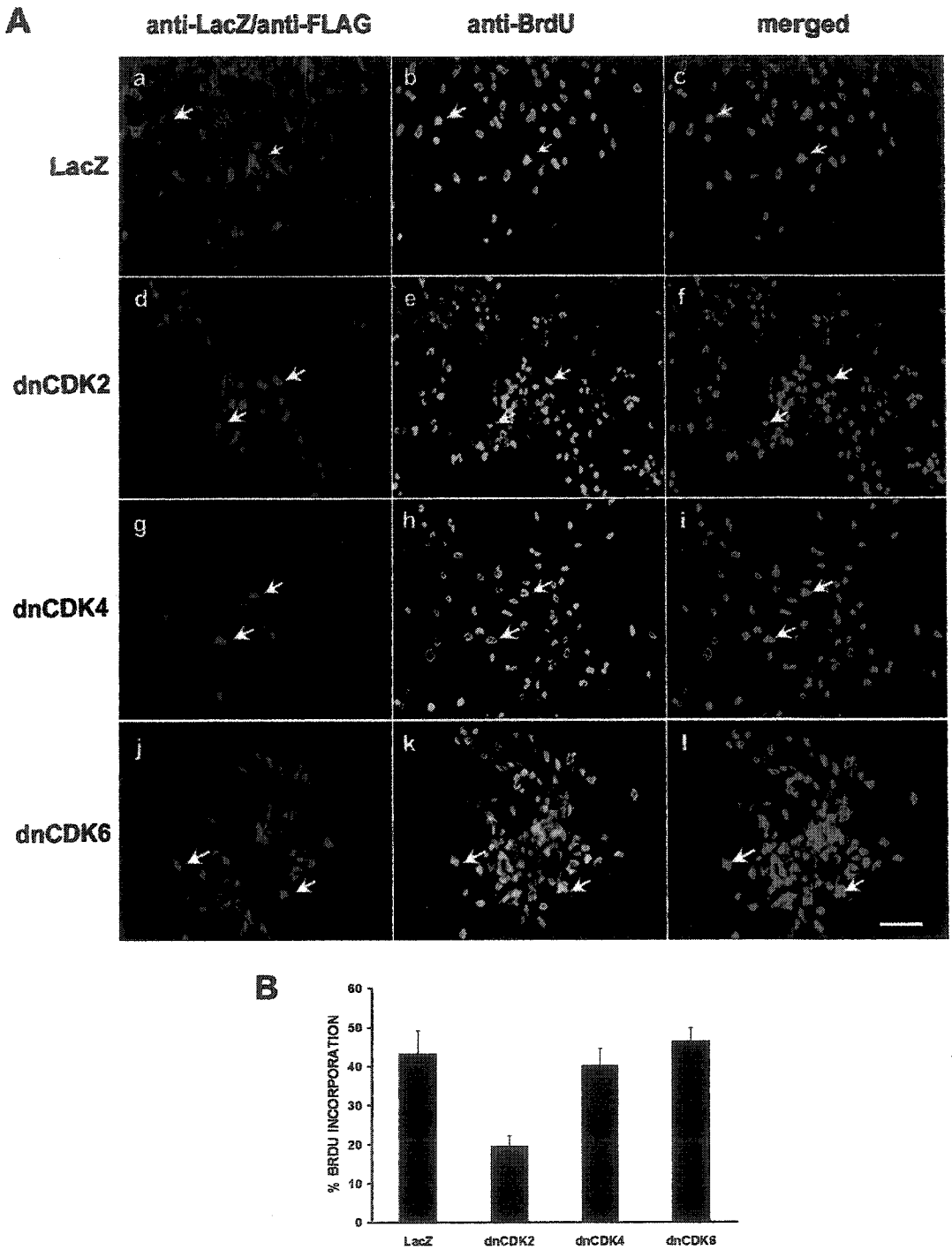
Fig. 6: Dominant negative mutants of CDKs 2,4 and 6 induce growth arrest in cortical progenitor cells. (A) Cortical progenitor cells cultured from wild type E12.5 embryos were infected at 50 MOI with Ad[lacZ] (a, b, c), Ad[dnCDK2] (d, e, f), Ad[dnCDK4] (g, h, i) and Ad[dnCDK6] (j, k, l) 2 days after plating. BrdU was added to the culture medium 18 h prior to fixation. At 72 h following infection, cells were fixed and labeled with anti-lacZ (a) or anti-FLAG (d, g, j) and anti-BrdU (b, e, h, k). Panels displaying lacZ/FLAG and BrdU labeling were superimposed (c, f, i, l). Arrows point to representative cells. Bar = 50 μm. (B) Quantification of BrdU incorporation. Averages were obtained from 8 separate cell cultures (n=8). Error bars denote standard error.


A
anti-LacZ/anti-FLAG
anti-BrdU
merged
LacZ
dnCDK2
dnCDK4
dnCDK6
a
b
c
d
e
f
g
h
i
j
k
l
B
% BRDU INCORPORATION
40
35
30
25
20
15
10
5
0
LacZ
dnCDK2
dnCDK4
dnCDK6

Previous studies have shown that cell cycle regulation by CDK4/6 functions primarily through Rb phosphorylation (16-18, 33); therefore, overexpression of such mutants in Rb deficient cells should have no effect. As a control to verify that the dnCDK4/6 mutants are appropriately targeting Rb, we repeated the above experiment in Rb-deficient cortical progenitor cells (Fig. 7A). In the absence of Rb, cells expressing lacZ incorporated BrdU at a higher rate of 43±6% relative to 31±3% measured in wild type cells (Fig. 7B). Cells expressing dnCDK2 continued to show a decrease in BrdU incorporation despite the absence of Rb (Fig 7A). Quantification of the percent BrdU incorporation at 20±3%, however, indicated that dnCDK2 was less efficient in arresting the growth of Rb-deficient cells (Fig. 7B). The ability of dnCDK2 to decrease BrdU incorporation relative to control cultures was nevertheless statistically significant. This suggests that in neural precursor cells, CDK2 function is partially Rb-dependent, however there are also other targets. Consistent with Rb as the sole target for CDK4/6, the corresponding dominant negative mutants had no effect on cell growth (Fig. 7). While lacZ-expressing cells exhibited 43±6% BrdU incorporation, dnCDK4- and dnCDK6-expressing cells showed 40±4% and 46±3% incorporation, respectively (Fig. 7B). These results indicate that recombinant adenovirus vectors carrying the dnCDK mutants are functioning appropriately when overexpressed in neural precursor cells.

In view of the biological impact of the dominant inhibitory CDK mutants in cortical progenitor cell cycle regulation, we next sought to verify that the CDK mutants were functioning as predicted, and thereby rule out the possibility that overexpression may lead to

Fig. 7: Dominant negative mutants of CDK4/6 mediate growth arrest in cortical progenitor cells in an Rb-dependent manner. (A) Cortical progenitor cells cultured from E12.5 $Rb^{-/-}$ embryos were infected at 50 MOI with Ad[lacZ] (a, b, c), Ad[dnCDK2] (d, e, f), Ad[dnCDK4] (g, h, i) and Ad[dnCDK6] (j, k, l) 2 days after plating. BrdU was added to the culture medium 18 h prior to fixation. At 72 h following infection, cells were fixed and labeled with anti-lacZ (a) or anti-FLAG (d, g, j) and anti-BrdU (b, e, h, k). Panels displaying lacZ/FLAG and BrdU labeling were subsequently superimposed (c, f, i, l). Arrows point to representative cells. Bar = 50 μm. (B) Quantification of BrdU incorporation. Averages were obtained from 6 independent cell cultures including 6 separate knockout embryos and their wild type littermates. Error bars denote standard error.


A
anti-LacZ/anti-FLAG
anti-BrdU
merged
LacZ
dnCDK2
dnCDK4
dnCDK6
a
b
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e
f
g
h
i
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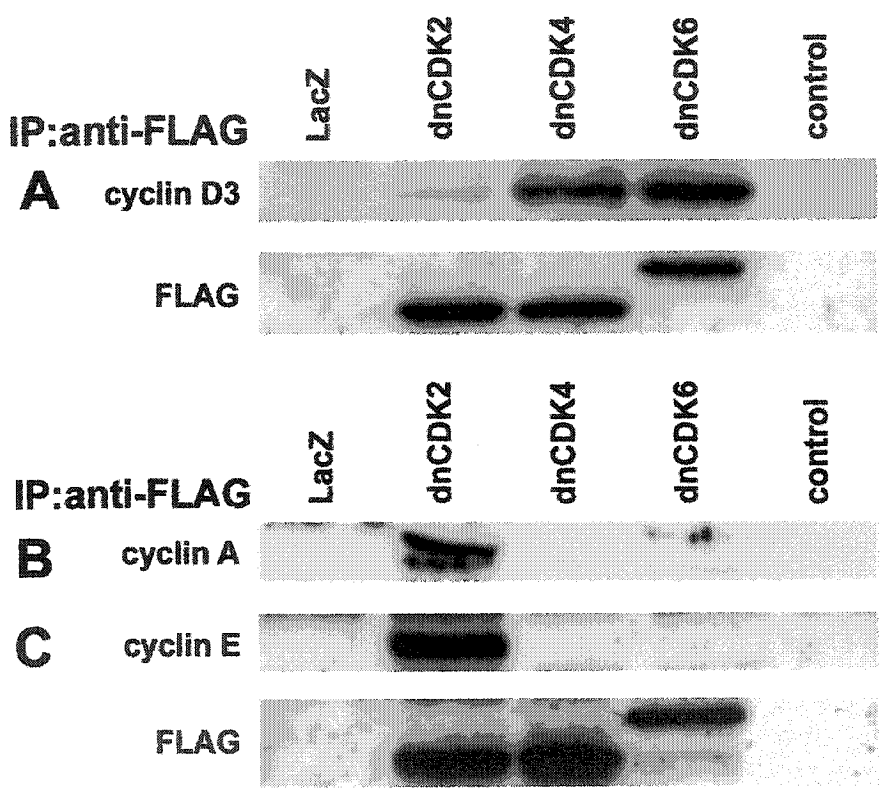
k
l
B
% BRDU INCORPORATION
60
50
40
30
20
10
0
LacZ
dnCDK2
dnCDK4
dnCDK6

binding of inappropriate cyclin partners. The dnCDK mutants were expressed in neural stem cells in order to examine their binding specificities. Stem cells lend themselves readily for immunoprecipitation experiments because they yield high quantities of tissue and can be readily manipulated with adenovirus vectors such that 60-80% of cells express the exogenous protein. Cells were transduced with lacZ, dnCDK2, dnCDK4, or dnCDK6 at 250 MOI and harvested 72 h after infection. Lysates were prepared for immunoprecipitation with anti-FLAG beads to precipitate the mutant CDK complexes. The immunoprecipitates were examined for CDK-associated proteins by Western analysis. The membranes were subsequently probed for cyclin D3, a physiological binding partner for CDK4/6 and cyclins A and E, which bind CDK2 *in vivo*, and all of which are endogenously expressed at high levels in neural stem cells (Fig. 2C, D, E). Cyclin D3 was detected in immunoprecipitates from stem cells expressing dnCDK4/6, but not in cells expressing dnCDK2 or lacZ (Fig. 8A). Similarly, cyclins A and E were associated with their appropriate partner, the dnCDK2 but not dnCDK4/6 (Fig. 8B, C). These results demonstrate that despite their overexpression, the dominant negative mutants interact with their appropriate cyclin partners in neural precursor cells.

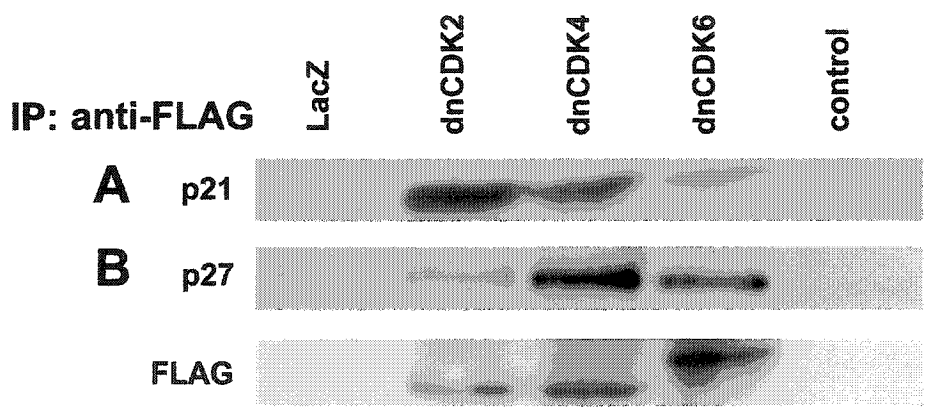
Although it is known that CDK4/6 function through the regulation of Rb activity by phosphorylation, recently studies suggest that these proteins have an additional function in the sequestration of the KIP family of CKIs. These CKIs have been shown to have no inhibitory effects on CDK4/6 activity but merely function to stabilize the CDK/cyclin complex (19-21). One possible explanation for the cell cycle inhibition of CDK4/6 is that they are no longer able to bind p21 or p27 efficiently, thereby rendering them available to inhibit the activity of CDK2. We therefore examined whether or not the CDK mutants could

Fig. 8: Dominant negative CDK mutants retain their physiological binding specificities. Neural stem cells were infected at 250 MOI with Ad[lacZ], Ad[dnCDK2], Ad[dnCDK4] or Ad[dnCDK6] 3 days after plating. At 72 h following infection, cells were extracted and cell lysates (100 μg protein), and beads only in lysis buffer (control), were immunoprecipitated with anti-FLAG M2-conjugated agarose beads. The membranes were probed with antibodies against (A) cyclin D3, (B) cyclin A and (C) cyclin E. Antibodies against FLAG detect FLAG-tagged dnCDK2 (34 kDa), dnCDK4 (34 kDa), and dnCDK6 (37 kDa). As expected, cyclin D3 was present in complexes with dnCDK4/6 but not in complexes with dnCDK2 or lacZ. Similarly, cyclins A and E were detected in complexes with dnCDK2, but not in complexes with dnCDK4/6 or lacZ.


IP:anti-FLAG
LacZ
dnCDK2
dnCDK4
dnCDK6
control
A
cyclin D3
FLAG
IP:anti-FLAG
LacZ
dnCDK2
dnCDK4
dnCDK6
control
B
cyclin A
C
cyclin E
FLAG

interact with the CIP/KIP CKIs, p21 and p27. Immunoprecipitations demonstrated that p21 was present primarily in complexes with dnCDK2, but was also detected in dnCDK4/6 complexes (Fig. 9A). Consistent with previous reports (34, 35), p27 was found almost exclusively in dnCDK4/6 complexes (Fig. 9B). These results indicate that cell cycle arrest was not mediated by the release of these cyclin kinase inhibitors. Our studies demonstrate, therefore, that dnCDKs can efficiently block cell cycle progression despite their interaction with p21 and p27 and that CDK4/6 mediated cell cycle arrest in primary neural precursor cells functions through the regulation of Rb activity.

Fig. 9: Dominant negative CDK mutants are associated with the KIP/CIP inhibitors p21 and p27. Neural stem cells were infected at 250 MOI with Ad[lacZ], Ad[dnCDK2], Ad[dnCDK4] or Ad[dnCDK6] 3 days after plating. At 72 h following infection, cells were extracted and cell lysates (100 μg protein), and beads only in lysis buffer (control) were immunoprecipitated with anti-FLAG M2-conjugated agarose beads. The membranes were probed with antibodies against (A) p21 and (B) p27. Antibodies against FLAG detect FLAG-tagged dnCDK2 (34 kDa), dnCDK4 (34 kDa), and dnCDK6 (37 kDa).


IP: anti-FLAG
LacZ
dnCDK2
dnCDK4
dnCDK6
control
A
p21
B
p27
FLAG

DISCUSSION

The onset of pan-neuronal gene expression is closely tied to terminal mitosis (1-4). Understanding the molecules that regulate cell cycle is key to delineating the mechanisms underlying neurogenesis. While studies with Rb deficient mice have demonstrated the importance of this tumor suppressor in nervous system development (5-7), there is little evidence that Rb or its upstream regulators can induce cell cycle arrest. In the present study, we demonstrate that the Rb pathway is a critical regulator of cell cycle progression in primary neural progenitor cells. In contrast to previous studies (12), we show that dominant negative mutants of CDK4 and 6 can induce mitotic arrest in neural precursor cells, despite the binding and sequestration of the KIP CKIs p21 and p27. The activity of these mutants is dependent on the presence of Rb, indicating that the activation of the Rb pathway is sufficient to cause cell cycle arrest in primary neural precursor cells.

The restriction point of the cell cycle is defined as the time in G_1 after which cells no longer require growth factors to enter S phase (36). Studies indicate that CDK2 and CDK4/6 function at two distinct regulatory time points during the G_1 phase of the cell cycle. CDK4/6 along with D cyclins, function through the inactivation of Rb early in G_1 to regulate the restriction point transition (13-15, 17). The passage through the restriction point and the upregulation of D cyclins is mitogen-dependent and commits the cell to enter S phase (37, 38). CDK2, with cyclins A and E, regulates the cell cycle later in G_1, at which time the cell is committed to undergo the G_1/S phase transition (39, 40). Previous studies have shown that dominant negative mutants of CDK2 but not CDK4/6 result in cell cycle arrest in several cell lines (12). These results are consistent with the interpretation that CDK2 activity may be

crucial for G_1/S phase transition while CDK4/6 may play a more important role in regulating entry into G_0. Indeed it has been suggested that CDKs 4/6 may have dual functions, including the regulation of Rb phosphorylation as well as the sequestration of CKIs p21 and p27 to prevent the inhibition of CDK2 activity. It is believed that this latter function may be a crucial one in the regulation of the G_1/S transition (19-21).

To determine which CDKs are important cell cycle regulators in neural precursor cells, we delivered dominant negative CDK mutants using adenovirus vectors. Delivery of the dnCDK2 mutant kinase results in a striking growth arrest in neural precursor cells, represented by a 94% decrease in BrdU incorporation relative to the lacZ-expressing controls. Unlike previous results with cell lines (12), dnCDK4/6 were also efficient at blocking cell cycle progression, as cells expressing these mutants exhibited a 74% decrease in BrdU incorporation. These results indicate that in addition to the established role for CDK2 in regulating cell cycle progression, CDK4/6 activity is also essential for neural precursor cells to enter S phase. To verify that such mutants behave appropriately, we expressed dnCDK4/6 in Rb deficient progenitor cells and as predicted, our results demonstrated that the cell growth regulation by CDK4/6 activity was entirely dependent on the Rb pathway. In contrast, the expression of dnCDK2 in Rb null progenitor cells could cause a significant reduction in BrdU incorporation relative to lacZ-expressing control cells. This effect, however, did not appear to be as efficient in the absence of Rb, suggesting that CDK2 functions partially through the Rb pathway as well as additional targets. It is unlikely that growth arrest is due to toxic effects resulting from adenovirus infection because cells expressing Ad[lacZ] exhibited BrdU incorporation at a rate typically found in untreated progenitor cells after 5 days *in vitro* (10) and delivery of dnCDK4/6 to Rb-deficient

precursor cells had no effect of the rate of BrdU incorporation. In addition, the low viral titres and short time courses would minimize the likelihood of viral-mediated effects (32). By examining the composition of the CDK complexes we have confirmed that the overexpressed CDK mutants function in the expected manner with respect to retaining their physiological binding specificities to their cyclin partners (Fig. 8). These results suggest that CDK4/6 activity is critical for cell cycle progression in primary neural precursor cells.

Previous studies involving microinjection of cyclin D1 antibodies into primary fibroblasts have demonstrated growth arrest in an Rb-dependent manner (33). Consistent with our results, this suggests that CDK4/6 regulation is critical for cell cycle transition in primary cells. It has been suggested, however, that cyclin D1 antibodies may have also caused the displacement of the CIP/KIP CKIs, thereby leading to CDK2 inhibition which was the key effector in cell growth arrest. As CDK4/6 have been shown to interact with the CIP/KIP family of CKIs, p21 and p27, one possible mechanism for CDK4/6-mediated growth arrest is the aberrant displacement of p21 and p27, rendering them available to inhibit CDK2. Thus, the observed growth arrest could actually result from the indirect inhibition of CDK2 activity. To address this question in our studies, we performed immunoprecipitations of the dnCDK mutants in order to examine whether the ability to sequester these CKIs is retained. Our results demonstrate that both p27 and p21 are highly associated with dnCDK4/6 complexes. This indicates that the dnCDK4/6 mutants retain the ability to bind the CIP/KIP CKIs, and can arrest cell growth despite this continued association. Clearly, inhibition of the CDK4/6 kinase activity is essential for S phase entry and Rb is the target for this activity.

The results of the present study demonstrate that CDK4/6 activity, specifically their ability to phosphorylate Rb, is critical for cell cycle transition in primary neural precursor cells. As previous studies were conducted in tumor cells, which pass through the G_1 phase of the cell cycle in a mitogen-independent manner, the early G_1 phase of the cell cycle may be non-functional in this cell type. In primary cells, it appears that both CDK4/6 and CDK2 activity is required to allow the cells to undergo S phase. The requirement of two independent pathways would seem evolutionarily favorable in primary cells for the protection against neoplastic transformation during their normal development.

REFERENCES

1. McLoon, S. C. and Barnes, R. B. (1989) *J. Neurosci.* **9**, 1424-1432.

2. Waid, D. K. and McLoon, S. C. (1995) *Neuron* **14**, 117-124.

3. McConnell, S. K. and Kaznowski, C. E. (1991) *Science* **254**, 282-285.

4. ElShamy, W. M., Fridvall, L. K. and Ernfors, P. (1998) *Neuron* **21**, 1003-1015.

5. Clarke, A. R., Maandag, E. R., Vam Roon, M., van der Lugt, N. M. T., van der Valk, M., Hooper, M. I., Berns, A. and Te Reile, H. (1992) *Nature* **359**, 328-330.

6. Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A. and Weinberg, R. A. (1992) *Nature* **359**, 295-300.

7. Lee, E. H. P., Chang, C. Y., Hu, N., Wang, Y. C. J., Lai, C. C., Herrup, K., Lee, W. H. and Bradley, A. (1992) *Nature* **359**, 288-294.

8. Lee, E., Hu, N., Yuan, S. S. F., Cox, L. A., Bradley, A., Lee, W., and Herrup, K. (1994) *Genes & Dev.* **8**, 2008-202.

9. Slack, R. S., El-Bizri, H., Wong, J., Belliveau, D. J., and Miller, F. D. (1998) *J. Cell. Biol.* **140**, 1497-1509.

10. Callaghan, D. A., Dong, L., Callaghan, S. M., Hou, Y. X., Dagnino, L., and Slack, R. S. (1999) *Dev. Biol.* **207**, 257-270.

11. Slack, R. S. and Miller, F. D. (1996) *Dev. Genet.* **18**, 81-91.

12. van den Heuvel, S. and Harlow, E. (1993) *Science* **262**, 2050-2054.

13. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. (1992) *Cell* **70**, 993-1006.

14. Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J.-Y., and Livingston, D. M. (1993) *Cell* **73**, 487-497.

15. Kato, J-Y, Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993) *Genes & Dev.* **7**, 331-342.

16. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Masabumi, S., Sherr, C. J., and Kato, J.-Y. (1994) *Mol. Cell Biol.* **14**, 2066-2076.

17. Meyerson, M. and Harlow, E. (1994) *Mol. Cell Biol.* **14**, 2077-2086.

18. Mittnacht, S., Lees, J. A., Desai, D., Harlow, E., Morgan, D. O., and Weinberg, R. A. (1994) *EMBO J.* **13**, 118-127.

19. Sherr, C. J. and Roberts, J. M. (1999) *Genes & Dev.* **13**, 1501-1512.

20. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997) *Genes & Dev.* **11**, 847-862.

21. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999) *EMBO J.* **18**, 1571-1583.

22. Reynisdóttir, I. and Massagué, J. (1997) *Genes & Dev.* **11**, 492-503.

23. Reynisdóttir, I., Polyak, K., Iavarone, A., and Massagué, J. (1995) *Genes & Dev.* **9**, 1831-1845.

24. Ghosh, A. and Greenberg, M. E. (1995) *Neuron* **15**, 89-103.

25. Vescovi, A. L., Reynolds, B. A., Fraser, D. D. and Weiss, S. (1993) *Neuron* **11**, 951-966.

26. Park, D. S., Levine, B., Ferrari, G., and Greene, L. A. (1997) *J. Neurosci.* **17**, 8975-8983.

27. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. (1997) *J. Virol.* **71**, 1842-1849.

28. Ino, H., Ishizuka, T., Chiba, T., and Tatibana, M. (1994) *Brain Res.* **661**, 196-206.

29. Tsai, L. H., Delalle, I., Caviness, V. S., Chae, T., and Harlow, E. (1994) *Nature* **371**, 419-423.

30. Kranenburg, O., Scharnhorst, V., van der Eb, A. J., and Zantema, A. (1995) *J. Cell Biol.* **131**, 227-234.

31. Gill, R. M., Slack, R., Kiess, M., and Hamel, P. (1998) *Exp. Cell Res.* **244**, 157-170.

32. Cregan, S. P., MacLaurin, J., Gendron, T. F., Park, D. S., Parks, R. J., Graham, F. L., Morley, P., and Slack, R. S. in press.

33. Lukas, J., Bartkova, J., Rohde, M., Strauss, M. and Bartek, J. (1995) *Mol. Cell. Biol.* **15**, 2600-2611.

34. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P. and Massagué, J. (1994) *Cell* **78**, 59-66.

35. Toyoshima, H. and Hunter, T. (1994) *Cell* **78**, 67-74.

36. Planas-Silva, M. C. and Weinberg, R. A. (1997) *Curr. Opin. Cell Biol.* **9**, 768-772.

37. Matsushime, H., Roussel, M. F., Ashmun, R. A. and Sherr, C. J. (1991) *Cell* **65**, 701-713.

38. Sewing, A., Burger, C., Brusselbach, S., Schalk, C., Lucibello, F. C., and Müller, R. (1993) *J. Cell Sci.* **104**, 545-555.

39. Dulic, V., Lees, E., and Reed, S. I. (1992) *Science* **257**, 1958-1961.

40. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M. and Pagano, M. (1995) *Mol. Cell Biol.* **15**, 2612-2624.

Chapter 3.

Ferguson K. L. *et al.* (2002) Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. *EMBO J.* **21**: 3337-46.

By generation and examination of telencephalon-specific conditional Rb mutants, this manuscript demonstrated the cell-autonomous and non-cell-autonomous roles for Rb in the developing cortex.

The experiments in this manuscript were conducted by K. L. Ferguson, assisted occasionally by Dr. J. L. Vanderluit and W. C. McIntosh, with the exception of the gel shift experiment which was performed by J. G. MacLaurin. The Rb-F19 mice were constructed and provided by Dr. M. Vooijs in the lab of Dr. A. Berns, and the Foxg1-cre mice were constructed and provided by Dr. J. M. Hebert in the lab of Dr. S. K. McConnell. W. C. McIntosh and E. Tibbo provided valuable technical help with animal breeding, genotyping and histological sectioning. Drs. D. S. Park and V. A. Wallace provided helpful suggestions and critical review of the manuscript. Reagents were provided as cited in the text. This manuscript was written by K. L. Ferguson, under the guidance and editorial assistance of Dr. R. S. Slack.

TELENCEPHALON-SPECIFIC RB KNOCKOUTS REVEAL ENHANCED NEUROGENESIS, SURVIVAL AND ABNORMAL CORTICAL DEVELOPMENT

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ABSTRACT

Correct cell cycle regulation and terminal mitosis are critical for nervous system development. The retinoblastoma (Rb) protein is a key regulator of these processes, as Rb-/- embryos die by E15.5, exhibiting gross hematopoietic and neurological defects. The extensive apoptosis in Rb-/- embryos has been attributed to aberrant S phase entry resulting in conflicting growth control signals in differentiating cells. To assess the role of Rb in cortical development in the absence of other embryonic defects, we examined mice with telencephalon-specific Rb deletions. Animals carrying a floxed Rb allele were interbred with mice in which cre was knocked into the Foxg1 locus. Unlike germline knockouts, mice specifically deleted for Rb in the developing telencephalon survived until birth. In these mutants, Rb-/- progenitor cells divided ectopically, but were able to survive and differentiate. Mutant brains exhibited enhanced cellularity due to increased proliferation of neuroblasts. These studies demonstrate that 1) cell cycle deregulation during differentiation does not necessitate apoptosis, 2) Rb deficient mutants exhibit enhanced neuroblast proliferation, and 3) terminal mitosis may not be required to initiate differentiation.

Keywords: apoptosis/cell cycle/central nervous system/differentiation/retinoblastoma

INTRODUCTION

Neurogenesis is a highly regulated developmental process in which cycling neural precursor cells withdraw from the cell cycle, differentiate into post-mitotic neurons, and commence migration into cortical layers. Proper cell cycle regulation is critical during these initial phases of neuronal development. Precursor cell proliferation is restricted to the ventricular zone, and only following cell cycle withdrawal do these cells begin expressing early neuronal markers and commence migration into cortical layers (McConnell and Kaznowski, 1991; McConnell, 1995). The laminar structure of the cortex is formed by an inside-out pattern of migration, such that the earlier generated neurons form the deeper layers, and the later born neurons give rise to more superficial layers (Rakic, 1988; McConnell, 1995). It is believed that terminal mitosis initiates neuronal differentiation and migration of newly born neurons to their appropriate laminar fate (McConnell and Kaznowski, 1991; McConnell, 1995).

The tumor suppressor retinoblastoma (Rb), is a key regulator of cell cycle and differentiation (Mulligan and Jacks, 1998; Lipinski and Jacks, 1999; Ferguson and Slack, 2001). While Rb interacts with many transcription factors (Morris and Dyson, 2001), the primary targets for Rb are members of the E2F family. When hypophosphorylated, Rb is active and able to bind to and repress E2F activity. Phosphorylation by cyclin-dependent kinases inactivates Rb, resulting in the release of E2Fs, which become able to bind DNA and transactivate genes related to DNA synthesis, differentiation, and apoptosis (Muller et al., 2001), reviewed in (Weinberg, 1995; Dyson, 1998; Nevins, 1998).

The importance of Rb as a regulator of cell cycle and differentiation was shown in Rb knockouts, which die embryonically by E15, exhibiting widespread developmental defects

(Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). The severity of Rb deficiency varied by tissue, with the most prominent defects in the developing liver, lens and nervous system. Ectopic mitoses were observed in several brain regions and massive cell death occurred throughout the central and peripheral nervous systems. Further examination revealed that the proliferating precursors in the ventricular zone appeared normal, but neuronal differentiation was impaired (Lee et al., 1994). Expression of a number of neuronal markers, including βII tubulin, and the neurotrophin receptors TrkA, TrkB, and p75, were significantly decreased (Lee et al., 1994). Based on expression of a neuron specific LacZ reporter gene, it was found that the requirement for Rb was immediately following commitment to a neuronal fate (Slack *et al.*, 1998).

Although Rb null embryos exhibited dramatic neural defects, studies with chimeric animals revealed survival and differentiation of Rb deficient cells in the CNS (Maandag *et al.*, 1994; Lipinski *et al.*, 2001). Further, mutant cells displayed comparable levels of ectopic S phase entry, in both chimeric and knockout embryos. Rb-/- cells from chimeric mice arrested in G2, prior to completion of the cell cycle, unlike cells from knockouts that exhibited ectopic mitoses. The absence of ectopic mitoses in chimeric mice appeared to account for the lack of apoptosis in these cells (Lipinski *et al.*, 2001). In spite of their arrested state, the majority of Rb deficient cells in chimeric mice seemed to differentiate normally (Lipinski *et al.*, 2001). Based on these results, it was proposed that the neighboring wild-type cells could rescue the Rb-/- cells, possibly by providing survival factors (Lipinski *et al.*, 2001). In contrast, Rb null cells from whole embryo knockouts underwent complete ectopic division, followed by apoptosis (Lipinski *et al.*, 2001).

Due to the widespread embryonic defects and early lethality of Rb-/- embryos prior to the completion of neurogenesis, we generated a tissue specific knockout for Rb in the

telencephalon. To produce conditional mutants in which Rb deletion is specific to the telencephalon, we have interbred mice carrying a floxed Rb allele (Marino *et al.*, 2000) with mice in which cre recombinase (cre) was knocked into the Foxg1 locus (Hebert and McConnell, 2000). With these mice, we show 1) that Rb excision is virtually complete throughout the developing telencephalon. 2) Rb deficient brains exhibit ectopic cell divisions at a rate similar to whole embryo knockouts. This is unlike Rb chimeric embryos in which cells become arrested in G2. 3) Rb deficiency specific to the telencephalon is not associated with widespread apoptosis, despite ectopic cell division. Rb null neurons are able to survive and differentiate, unlike whole embryo knockouts in which the majority of neurons are lost (Slack et al., 1998). 4) Ectopically dividing cells express the early neuronal marker, TuJ1, consistent with enhanced neuroblast proliferation in Rb deficient brains. Taken together, these results demonstrate that Rb may be a key regulator of neural proliferation in the developing cortex, but may not be required for neuronal survival or the initiation of differentiation.

RESULTS

Generation of telencephalon-specific Rb knockouts:

Whole-embryo Rb deletion results in embryonic lethality associated with neurological and hematological defects (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). To establish the role of Rb in cortical neurogenesis, it is imperative that the embryos survive during this developmental time period, and that the pleiotropic effects associated with Rb deficiency are eliminated. Conditional mutants therefore, were generated in which Rb deletion is specific to the telencephalon. Animals in which cre was knocked into the Foxg1 locus (Hebert and McConnell, 2000) were interbred with mice carrying the floxed Rb allele (Marino *et al.*, 2000). Foxg1 expression is detectable as early as E8-9, and shows peak levels at E17, restricted to the cerebral cortex, caudate putamen, hippocampus, and dentate gyrus (Tao and Lai, 1992; Shimamura and Rubenstein, 1997). Thus cre-mediated excision of the floxed allele would occur in neural precursors at E9. This model system provides an excellent opportunity to examine the impact of Rb deficiency throughout the timecourse of telencephalon development, beginning at the time at which the first neurons are born.

Rb is completely deleted from the telencephalon of conditional mutants:

To test the efficiency of cre-mediated recombination of a floxed allele, whole mount LacZ analysis was performed on E13.5 embryos generated from Foxg1-cre mice crossed with Rosa26 reporter mice (Soriano, 1999). The Rosa 26R mice contain a transcriptional stop site flanked by loxP sites, located upstream of a β-galactosidase coding region. In the

presence of cre, the stop site is excised, resulting in β-galactosidase transcription (Soriano, 1999). In contrast to control littermates, Foxg1-cre embryos displayed intense LacZ staining in the telencephalon, olfactory bulb, whisker barrels, dorsal midbrain, and eye (Fig. 1A). In the eye, it has previously been shown that cre expression is restricted to the lens and anterior (nasal) retina (Hebert and McConnell, 2000). Cross-sections revealed essentially ubiquitous LacZ expression in the telencephalon (Fig. 1B), showing that cre-mediated recombination was virtually complete.

To assess deletion of the floxed Rb allele, PCR and Western analyses were performed. First, PCR was used to detect the presence of Rb exon 19, using DNA isolated from the telencephalon of mutant and control littermates (Fig. 2A). In the absence of cre (embryos 1 and 2), the floxed and wild-type alleles remain intact, as determined by 283- and 235-bp fragments, respectively. When cre-mediated recombination occurs (embryo 3), the floxed allele is deleted, resulting in a 260-bp band (Marino *et al.*, 2000). The presence of only the middle, 260-bp band in the conditional mutant (embryo 3), suggested complete recombination (Fig. 2A).

Efficient Rb excision was further confirmed by Western analysis. Tissue isolated from E13.5 and 16.5 mutant and control littermate telencephalon was assayed for Rb expression. Rb was undetectable in the mutant telencephalon (Fig. 2B). Based on PCR and Western analyses, our results show that cre-mediated excision of the floxed allele was virtually complete.

It was previously demonstrated that E2F activity is deregulated in whole embryo Rb knockouts (Callaghan et al., 1999; Macleod et al., 1996). To determine whether E2F is deregulated in telencephalon-specific Rb knockouts, electrophoretic mobility shift assays were performed. The telencephalon of E16.5 mutant and control littermates was examined

Fig. 1: Cre expression results in efficient recombination of floxed alleles. A) Embryos from Foxg1-cre mice paired with Rosa26 reporter mice were taken at E13.5, whole-mounted and stained with X-gal to visualize cre expression. **B)** Robust LacZ staining was exhibited in the telencephalon of Foxg1-cre mice (coronal) (n=4). Bar= 50 μm.

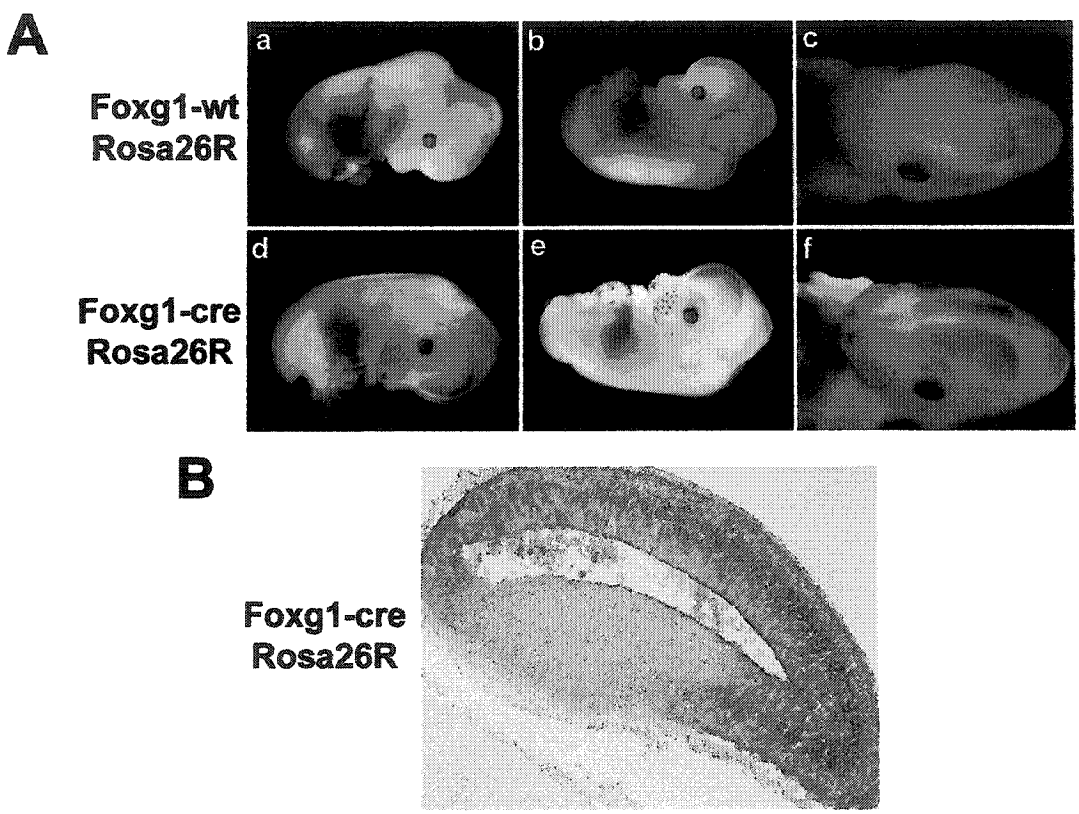
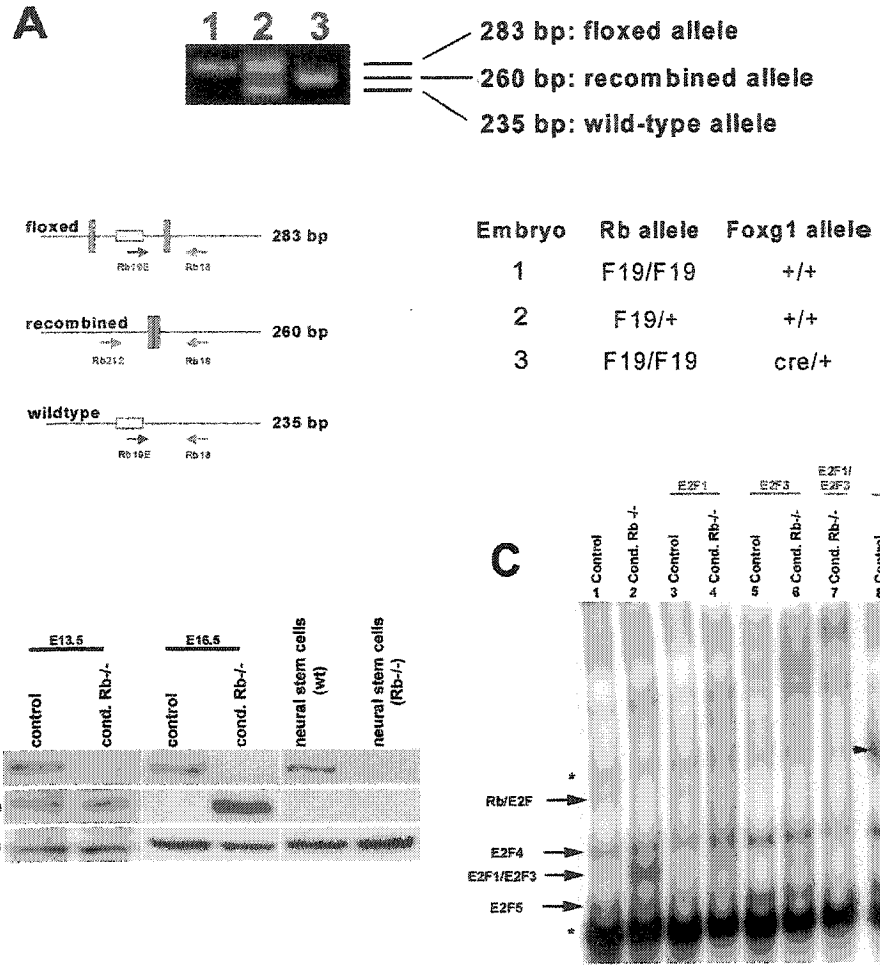

A
Foxg1-wt
Rosa26R
a
b
c
Foxg1-cre
Rosa26R
d
e
f
B
Foxg1-cre
Rosa26R

Fig. 2: Rb is completely deleted from the telencephalon of conditional mutants. **A)** To detect the presence of Rb exon 19, DNA isolated from the telencephalon of E16.5 mutant and control embryos was subjected to PCR analysis. In the absence of cre, the floxed and wild-type alleles remain intact, as determined by 283- and 235-bp fragments, respectively. When cre-mediated recombination occurs, the floxed allele is deleted, resulting in a 260-bp band (Marino *et al.*, 2000). The conditional mutant (embryo 3) exhibited only the middle 260-bp band, indicating that recombination had occurred, with no detection of the intact floxed allele (n=3). **B)** Rb and cre protein expression were assayed at E13.5 and E16.5 in tissue isolated from the telencephalon of mutant (homozygous Rb-F19 and heterozygous for Foxg1-cre) and control (double heterozygous at E13.5 and heterozygous for Rb-F19 at E16.5) embryos. As control, protein was also assayed from neural stem cells derived from wild-type and germline Rb-/- brains. Rb protein was undetectable in the mutant telencephalon (n=4). **C)** Protein was extracted from the telencephalon of E16.5 mutant and control littermates and subjected to electrophoretic mobility shift assay. In contrast to the control (lane 1), the mutant extract exhibited increased levels of free E2F1/3 (lane 2). The uncomplexed E2F band was partially deleted upon the addition of an antibody to E2F1 (lane 4) or supershifted with antibodies specific for E2F3 (lane 6) and was completely deleted when antibodies to both E2Fs 1 and 3 were added (lane 7). In the mutant extract, Rb/E2F complexes were undetectable (lane 2). The addition of the Rb antibody produced a supershift in littermate controls (arrowhead, lane 8) but not in extracts from the conditional Rb mutant (lane 9). Asterisks denote non-specific bands (n=4).


A
1 2 3
283 bp: floxed allele
260 bp: recombined allele
235 bp: wild-type allele
floxed
283 bp
Rb19E
Rb18
recombined
260 bp
Rb21/2
Rb18
wildtype
235 bp
Rb19E
Rb18
Embryo Rb allele Foxg1 allele
1 F19/F19 +/+
2 F19/+ +/+
3 F19/F19 cre/+
B
E13.5
E16.5
control
cond. Rb-/-
control
cond. Rb-/-
neural stem cells (wt)
neural stem cells (Rb-/-)
Rb
cre
actin
C
E2F1
E2F3
E2F1/ E2F3
Rb
Control
Cond. Rb -/-
Control
Cond. Rb-/-
Control
Cond. Rb-/-
Cond. Rb-/-
Control
Cond. Rb-/-
Comp.
1 2 3 4 5 6 7 8 9 10
Rb/E2F
E2F4
E2F1/E2F3
E2F5

for E2F DNA binding at E2F consensus sites (Fig. 2C). Rb deficient extracts exhibited increased levels of free E2Fs1 and 3 (lane 2), which were completely supershifted when antibodies to both E2Fs 1 and 3 were added to the reaction (lane 7). While a supershift for Rb was readily detected in control tissue (lane 8 arrow), there was no Rb supershift in the tissue derived from the conditional knockout (lane 9). Thus, the conditional telencephalon-specific mutants reveal enhanced E2F DNA binding ability consistent with that previously shown in whole embryo Rb deletions (Callaghan et al., 1999; Macleod et al., 1996).

Telencephalon-specific Rb knockouts survive to birth:

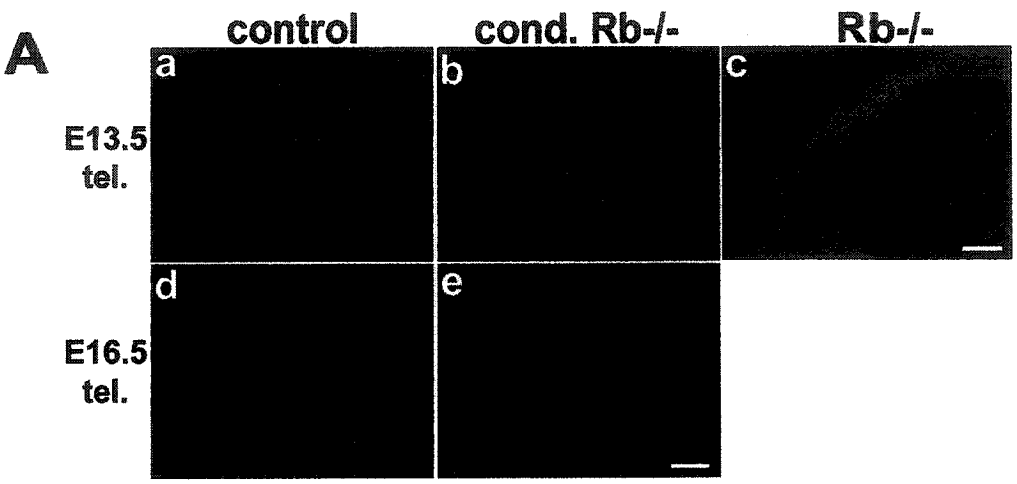
Whole embryo Rb knockouts die by E15.5, associated with extensive developmental defects (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). To determine the survival rate of conditional mutants, mice homozygous for the floxed allele [Rb-F19/F19] were interbred with [Foxg1-cre:Rb-F19+/-] double heterozygous mice. Embryonic survival was assessed in mice from FVB/N and 129SV genetic backgrounds at E13.5, 15.5, 16.5, 17.5, 18.5, 19.5 and P0. During embryogenesis, the frequency of telencephalon-specific mutants was consistent with the expected Mendelian frequencies of 25%, however, conditional mutants died 10-20 minutes after birth, due to respiratory defects (not shown). Thus, unlike animals carrying germline Rb mutations that die during mid-gestation, this conditional Rb knockout will provide the unique opportunity to study the role of Rb and cell cycle regulation throughout the embryonic timecourse of cortical development.

Telencephalon-specific Rb deficiency is compatible with neuronal survival:

Widespread apoptosis throughout the CNS is a hallmark of the Rb deficient embryo, such that by E14.5, the majority of cells expressing neuronal markers are absent (Lee et al., 1994; Slack et al., 1998). This apoptotic phenotype was significantly diminished in Rb-/- chimeric mice and was attributed to rescue by surrounding wild-type cells (Maandag *et al.*, 1994; Lipinski *et al.*, 2001). To determine whether complete deletion of Rb in the telencephalon would affect neuronal survival, we examined the level of apoptosis in the conditional Rb knockouts. Telencephalon-specific mutants and control littermates, as well as germline Rb knockouts, were analyzed at E13.5 for TUNEL labeling (Fig. 3). Unlike the whole embryo knockout, massive apoptosis did not occur in the E13.5 conditional mutant telencephalon. There was however, a slight 1.5-fold increase in TUNEL labeling in the mutant, in which 79.7±14.0 cells/section were TUNEL-positive relative to 51.0±4.7 cells/section in control animals (Fig. 3B). This phenotype is distinct from that observed in the whole embryo knockout in which massive apoptosis is observed in the developing cortex (Fig. 3A-c). Detection of neuronal degeneration by FluoroJade labeling (Schmued *et al.*, 1997; Noraberg *et al.*, 1999) yielded comparable results (not shown). Cell death was further examined in later stage embryos (E15.5, 16.5, 17.5, and 19.5), with no detectable apoptosis over littermate controls (Fig. 3B; 3A-d, e). Consistent with the absence of apoptosis, neurons in the conditional Rb-/- telencephalon were able to differentiate and survive. Thus, in spite of deregulated E2F activity, telencephalon-specific Rb deficiency does not induce the massive neuronal cell death characteristic of germline Rb knockouts.

Newborn Rb conditional mutant pups die perinatally due to respiratory difficulties. Since the pons and the medulla, which are known to function in respiration, revealed some

Fig. 3: Telencephalon-specific Rb deficiency does not produce widespread apoptosis. A) To detect cell death, E13.5 conditional mutant and control littermates, as well as germline Rb knockouts, were sectioned (coronal) and assayed for TUNEL labeling. Unlike whole embryo Rb knockouts (c), conditional Rb mutants (b) did not exhibit massive neuronal apoptosis. However, in the telencephalon, TUNEL-positive cells in the conditional mutant (b) were slightly higher than controls (a), with 79.7±14.0 and 51.0±4.7 cells/section, respectively **(B)**. Cell death was further examined in later stage embryos (E15.5, 16.5, 17.5, and 19.5), with no detectable apoptosis over littermate control (d,e). (n=5). Error bars denote standard error. Bar=100 μm.


A
control
cond. Rb-/-
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a
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E13.5
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E16.5
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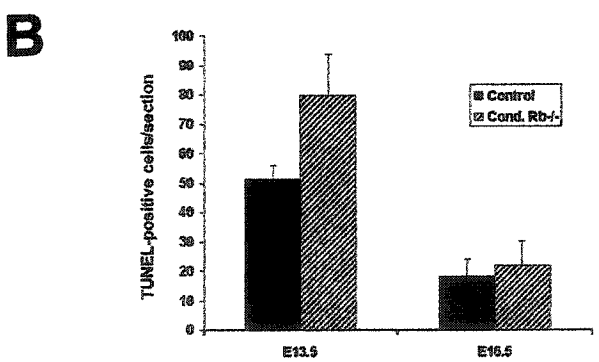
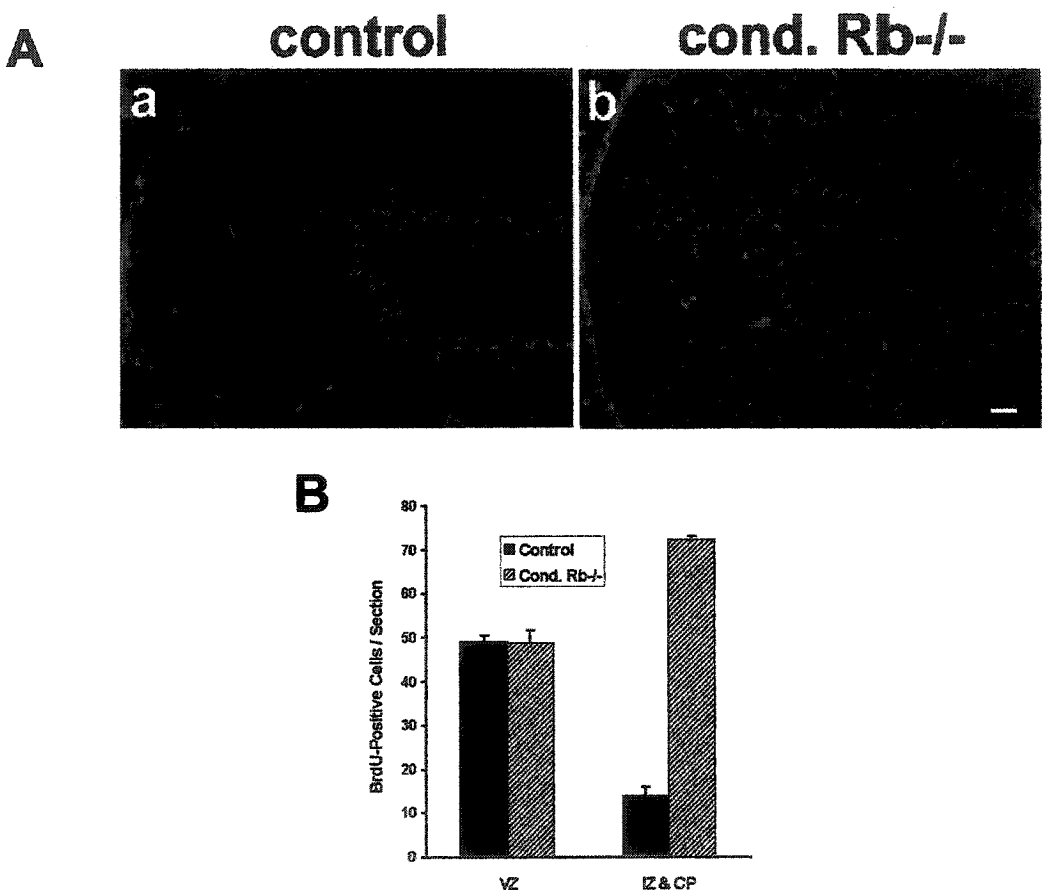

B
TUNEL-positive cells/section
100
90
80
70
60
50
40
30
20
10
0
Control
Cond. Rb-/-
E13.5
E16.5

cre-mediated recombination (Hebert & McConnell, 2000), we examined apoptosis in these regions. Embryos were sectioned at E13.5, 17.5, and 19.5, and assayed with FluoroJade and TUNEL to detect both necrotic and apoptotic forms of cell death. Although no differences were detected at E17.5 or 19.5, intense labeling was observed in the mutant lateral pons at E13.5 (not shown), a region containing many critical nuclei for respiratory regulation (Dick et al., 1994; Jodkowski et al., 1994; Morrison et al., 1994). Cell death in this region was consistent with the perinatal lethality associated with respiratory difficulties in the conditional mutants.

Telencephalon-specific Rb deficient progenitor cells undergo complete cell divisions:

Since ectopic mitoses are characteristic of whole embryo Rb knockouts, whereas chimeric Rb-/- mice exhibit growth arrest at G2, we determined whether telencephalon-specific Rb deficiency would lead to ectopic cell division. Females were injected with BrdU (100 μg/g body weight) at E15.5 of gestation. Embryos were removed 2h later, and mutant and corresponding control littermates were assayed for BrdU incorporation by immunohistochemistry. The conditional mutant exhibited extensive BrdU incorporation, which extended beyond the ventricular zone (Fig. 4A-b). The extent of BrdU-labeling was similar to levels observed in whole embryo Rb knockouts (not shown). At later time points, however (E17.5, 19.5), ectopic proliferation in the conditional Rb mutants was reduced (not shown). Because it was recently shown that cells in Rb chimeric embryos incorporated BrdU but arrested in G2 (Lipinski *et al.*, 2001), we investigated whether the ectopically proliferating cells in the conditional knockouts were actually completing cell division. Sections were labeled with an antibody directed against the M phase marker, phospho-

Fig. 4: Telencephalon-specific Rb deficiency induces ectopic S phase entry. A) Females at E15.5 were injected with BrdU and 2h later, mutant and control embryos were removed and assayed for BrdU incorporation immunohistochemically. The conditional mutants exhibited extensive BrdU incorporation outside the ventricular zone (horizontal). **B)** Quantification revealed that BrdU-positive cells in the control and mutant did not differ within the ventricular zone (VZ), with 48.8±1.6 and 48.8±2.9 positive cells/section, respectively. In the intermediate zone (IZ) and cortical plate (CP), there were only 14.0±1.2 BrdU-positive cells in the control, as compared to 72.1±1.8 positive cells in the mutant. Quantification was performed by counting cells within a superimposed grid. (n=8). Error bars denote standard error. Bar=50 μm.


A
control
cond. Rb-/-
a
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B
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Cond. Rb-/-
BrdU-Positive Cells / Section
0
10
20
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40
50
60
70
80
VZ
IZ & CP

histone H3 (PH3). In contrast to the control, the mutant exhibited abundant mitoses outside the ventricular zone (Fig. 5). Thus, unlike the G2 arrest observed in Rb-/- chimeric mice, the telencephalon-specific knockout revealed progenitor cells undergoing complete cell divisions outside the ventricular zone.

Ectopically dividing Rb-/- cells express early neuronal marker, TuJ1

Although cortical gliogenesis normally begins at E18 (Cameron & Rakic, 1991; Levers et al., 2001), the cell type exhibiting ectopic proliferation was assessed. Tissue was, therefore, double-labeled for BrdU and cell-type specific markers, including nestin (progenitor cells), TuJ1 (early neurons) and GFAP (astrocytes). Confocal microscopy of double labeled cells revealed that cells ectopically incorporating BrdU did not express GFAP (not shown). Several cells expressed nestin (not shown) and the vast majority of ectopic BrdU-positive cells co-expressed TuJ1, an early pan-neuronal gene (Fig. 6). Since TuJ1 labeling is cytoplasmic and BrdU is nuclear, serial confocal images are shown to demonstrate co-labeled cells. These results indicate therefore, that ectopically dividing cells had committed to a neuronal fate and initiated differentiation.

Telencephalon-specific mutants exhibit enhanced neurogenesis and aberrant cortical morphology:

To determine whether the elevated proliferation in the conditional Rb mutant telencephalon would affect cortical development, we compared cortical morphology in mutants and littermate controls by Cresyl Violet staining (Fig. 7). At E16.5, the average area

Fig. 5: Rb deficient cells undergo complete ectopic mitosis. Sections were labeled with an antibody against the M phase marker, phospho histone H3 (PH3) at E16.5. In contrast to the control, the mutant exhibited widespread mitoses outside the ventricular zone (coronal). Boxed areas (a, b) are magnified (c, d) (n=4). Bar=25 μm.

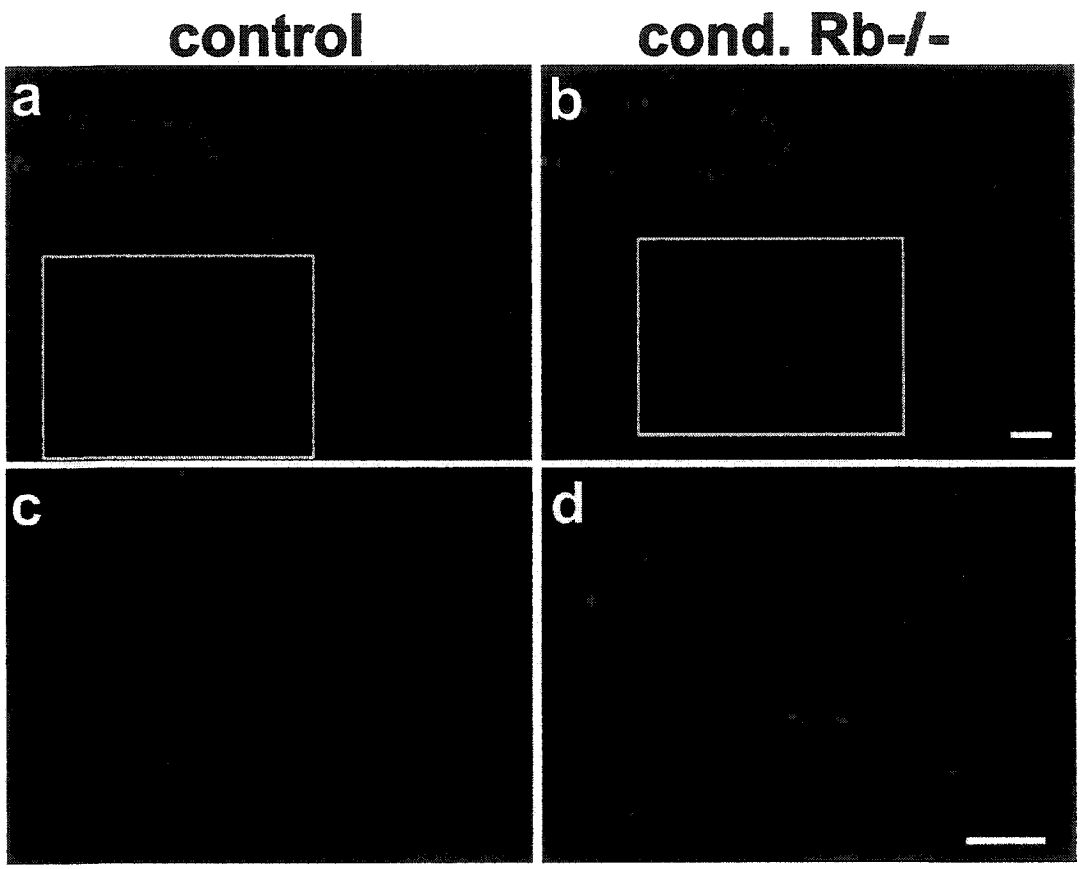

control
cond. Rb-/-
a
b
c
d

Fig. 6: Ectopically dividing Rb-/- cells expressed early neuronal marker, TuJ1. Females at E15.5 were injected with BrdU and 2h later, mutant and control embryos were removed, and double-labeled with cell type specific markers (nestin, GFAP, and TuJ1) and BrdU, then examined by confocal microscopy. Cells that had ectopically incorporated BrdU (green) were negative for GFAP (not shown), but the vast majority of BrdU-positive cells co-expressed TuJ1, an early neuronal marker (red). Arrows point to representative cells. Serial confocal micrographs show sequential 0.7 μm levels through coronal sections, to demonstrate double labeling of cells (n=4). Bar= 12.5 μm.

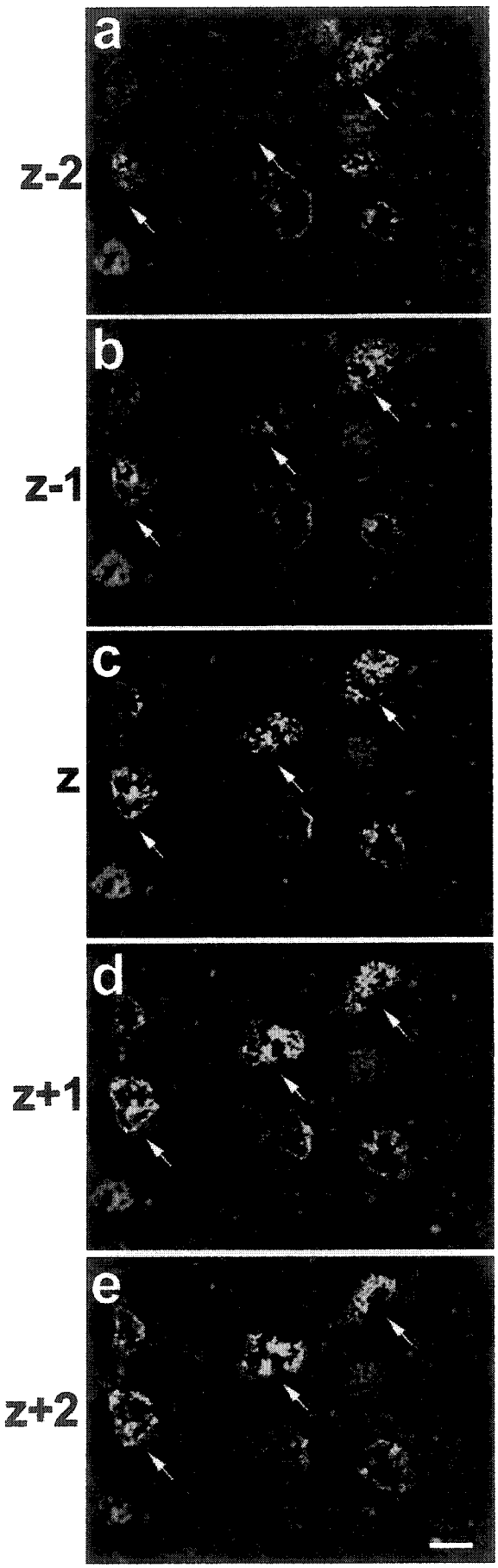
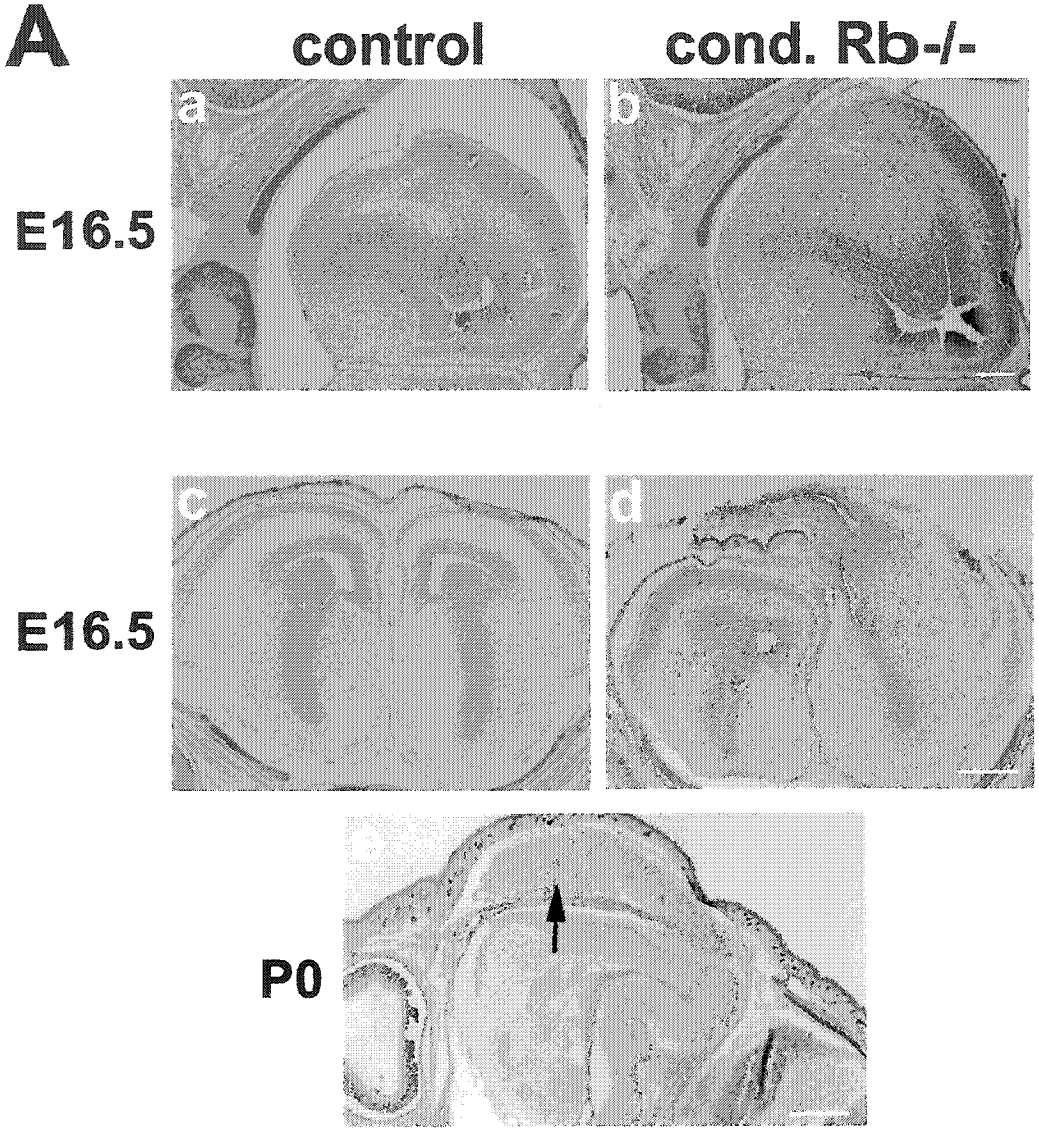
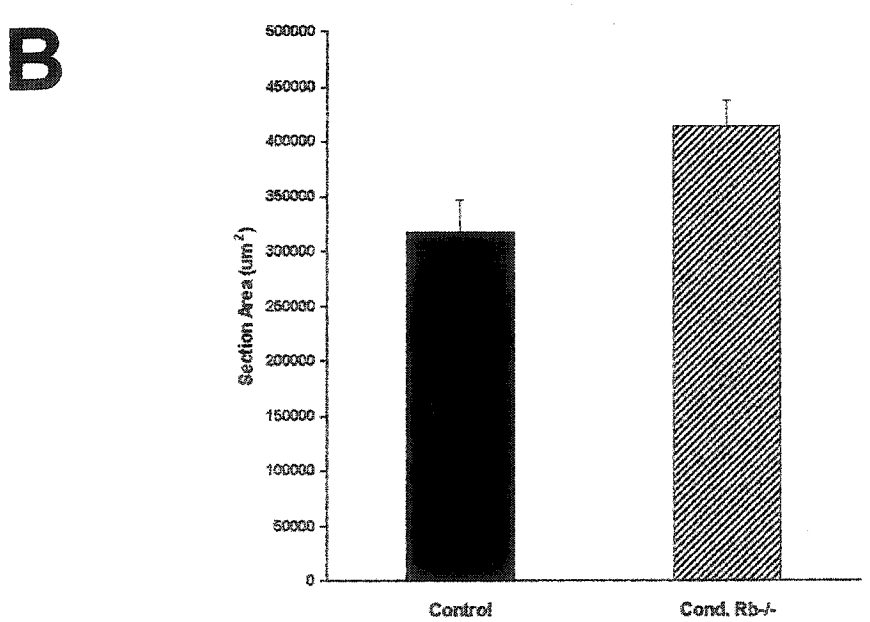

a
z-2
b
z-1
c
z
d
z+1
e
z+2

Fig. 7: Telencephalon-specific mutants exhibit enhanced neurogenesis and aberrant cortical morphology. A) At E16.5, many mutants exhibited enlarged telencephalic lobes compared to controls (coronal) (a, b), averaging to an approximately 30% increase over littermate controls (P>.01) **(B)**. It should be noted that some Rb deficient brains were found with no gross abnormalities but occasionally mutants were observed with severely aberrant cortical development, for example, large, single-hemisphere, telencephalic protrusions were observed at E16.5 (coronal) (Fig. 7A-d) and at P0 (sagittal, arrow points to protrusion) (Fig. 7A-e). E16.5 (n=9), E17.5 (n=7), E18.5 (n=9), E19.5 (n=7), and P0 (n=11). Error bars denote standard error. Bar =300 μm.


A
control
cond. Rb-/-
E16.5
E16.5
P0
a
b
c
d


B
500000
450000
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350000
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100000
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Section Area (um²)
Control
Cond. Rb-/-

of telencephalic sections was measured and compared between Rb mutant and littermate controls. Our results revealed that at E16.5, the conditional mutant telencephalic lobes were on average 30% larger than corresponding littermate controls ($P<.01$) (Fig. 7B). A representative enlarged mutant telencephalon is shown (Fig. 7A-b), however, it should be noted that some mutants did not differ from control littermates. Occasionally, Rb conditional mutants were observed with severely aberrant cortical development, for example, large single-hemisphere telencephalic protrusions were observed at E16.5 (Fig. 7A-d) and P0 (Fig. 7A-e). These results demonstrate that Rb deficiency in the developing telencephalon may result in enhanced neurogenesis and often, excessive cellularity.

In summary, we have generated a conditional knockout, with complete Rb excision throughout the developing telencephalon, that survives until birth. Our results demonstrate that telencephalon-specific Rb mutants exhibit complete ectopic cell division of committed neuroblasts, based on the co-expression of BrdU and the early neuronal marker, βIII-tubulin. Thus, in Rb-deficient cortex, neurons are able to differentiate and survive, consistent with enhanced neurogenesis and increased cellularity. Taken together, these results demonstrate that Rb may be a key regulator of neuroblast proliferation, however, correct cell cycle regulation may not be required for neuronal differentiation and survival.

DISCUSSION

Rb deficiency in whole embryo knockouts has been well characterized, with extensive apoptosis and differentiation defects in many tissues, including the nervous system (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). The Rb null apoptotic phenotype was shown to be substantially rescued in chimeric animals, which survive and show apparently normal cortical development (Maandag *et al.*, 1994; Lipinski *et al.*, 2001). Based on these studies, it was suggested that the Rb-/- cells in the chimeras may have been rescued by surrounding wild-type cells, possibly by a released survival factor (Maandag *et al.*, 1994; Lipinski *et al.*, 2001). The absence of complete cell division implied that survival of Rb deficient cells in chimeras may result from cell cycle arrest at G2, prior to entering mitosis. In contrast, Rb null cells from whole embryo knockouts undergo complete ectopic division, followed by apoptosis (Lipinski *et al.*, 2001).

Using Rb conditional knockouts, we now show that in spite of a virtually full Rb deletion in the telencephalon, neurons were able to survive and initiate differentiation. Telencephalon-specific Rb mutants exhibited increased cell division leading to enhanced neurogenesis and by E16.5, a significant increase in average brain size was observed. While we have not yet examined regional development and lamination in the cortex, ectopic cell division and survival of neurons were characteristic features and were consistently observed in all conditional Rb knockouts. Gross cortical development exhibited phenotypic variability, which could result from enhanced attrition, possibly due to limiting growth factors. This would be consistent with the slight increase in apoptosis observed at early time points (E13.5). In contrast to germline Rb knockouts, however, telencephalon-specific Rb deficiency did not induce the massive neuronal fallout as previously reported in whole embryo knockouts. Instead, brain tissue appeared morphologically normal. Neuronal loss

observed in the CNS of germline knockouts may therefore result from an extrinsic defect, perhaps due to extensive liver degeneration and failed erythropoeisis. It is well established that hypoxia is a potent inducer of apoptosis, particularly in neurons (Gwag *et al.*, 1995; Rosenblum, 1997; Banasiak and Haddad, 1998; Banasiak *et al.*, 2000). Our results show that ectopic cell division in differentiating neuroblasts may not necessarily evoke a default apoptotic pathway due to conflicting signals, but that Rb deficient neuroblasts can survive and initiate differentiation.

Consistent with our findings in conditional Rb mutants, primary neuronal cultures of Rb-deficient cells survive and differentiate, although terminal mitosis is delayed (Lee *et al.*, 1994; Slack *et al.*, 1998; Callaghan *et al.*, 1999). Rb-deficient trigeminal, dorsal root ganglia (Lee et al., 1994), and cortical progenitor cells (Slack *et al.*, 1998) were shown to be morphologically identical to wild-type cells, and were able to differentiate and express the neuronal marker MAP2 (Slack *et al.*, 1998). Although p107 protein levels were up-regulated in Rb-/- cells in a presumably compensatory manner (Callaghan *et al.*, 1999), Rb/p107 double null neural stem cells display neuronal differentiation and survival *in vitro* (Vanderluit & Slack, unpublished data).

Several lines of evidence have attributed the apoptotic phenotype in Rb-deficient mice to increased levels of free E2F and deregulated E2F activity. First, E2F protein levels, in both the free and complexed forms, were increased in the CNS of Rb-deficient embryos (Macleod *et al.*, 1996; Callaghan *et al.*, 1999). Second, E2F1 overexpression was found to induce apoptosis in several neuronal cell types (Azuma-Hara *et al.*, 1999; Hou *et al.*, 2000; O'Hare *et al.*, 2000), and E2F1-deficient neurons were protected from certain apoptotic stimuli (Giovanni *et al.*, 2000; Hou *et al.*, 2000; O'Hare *et al.*, 2000). Finally, the additional absence of E2F1 dramatically reduced p53-dependent apoptosis in Rb-deficient mice,

associated with a down-regulation of the p53 pathway (Pan *et al.*, 1998; Tsai *et al.*, 1998). The role of Rb in apoptosis appears to be a protective function, while E2Fs, in particular E2F1, act as inducers of cell death.

Apoptosis, associated with Rb deficiency, is often explained by E2F's ability to produce conflicting growth control signals. In this model, cell cycle gene transactivation, in the absence of mitogen or in a division-incompetent cell type, may instead trigger apoptosis. However, the dual abilities of E2F1 to induce proliferation and apoptosis have been shown to be separable (Phillips et al., 1997). Through the use of E2F1 deletion mutants, it was found that E2F1 requires transcriptional activity to promote proliferation, but it can initiate apoptosis in the absence of its transactivation domain and without inducing S phase entry (Phillips et al., 1997). In another study, mutation of one allele of E2F3 was able to suppress apoptosis but not ectopic proliferation in Rb knockouts (Ziebold *et al.*, 2001). In addition, we have shown that levels of E2Fs 1 and 3 are elevated in the mutant telencephalon, similar to levels found in whole embryo Rb knockouts. Together, these studies demonstrate that apoptosis is not necessarily induced as a default pathway of deregulated E2F activity.

In conclusion, we describe a conditional knockout with virtually complete Rb excision throughout the developing telencephalon. We demonstrate that telencephalon-specific Rb mutants survive throughout embryogenesis and exhibit neuronal survival, in spite of undergoing complete ectopic cell division. Our results indicate that the widespread cell death observed in the CNS of Rb-/- embryos is not likely due to deregulated cell division but likely occurs secondary to other embryonic defects. Ectopically dividing cells expressed the early neuronal marker, TuJ1, consistent with enhanced neurogenesis and increased cellularity in the developing Rb deficient brain. Taken together, these results demonstrate that Rb is an

important regulator of neuroblast proliferation, but may not be required for neuronal survival or differentiation.

MATERIALS AND METHODS

Mice:

The floxed Rb-F19 mice were generously provided to us by Dr. Anton Berns, of the Netherlands Cancer Institute. To generate the telencephalon-specific Rb conditional knockouts, floxed Rb-F19 nullizygous mice were bred with Foxg1-cre:Rb-F19 double heterozygous mice, to generate mutants (Foxg1-cre:Rb-F19-/-) at a frequency of 25%. Mice were maintained on CD1, F/VBN, and 129SV uniform genetic backgrounds and individual colonies. Mice were genotyped by polymerase chain reaction, as described previously, using DNA extracted from tails or remaining embryonic tissue. For timed pregnancies, mice were bred, and the time of plug identification was counted as day 0.5.

Whole Mount Analysis:

To determine the efficiency of cre-mediated recombination, whole mount LacZ staining was performed by crossing Foxg1-cre mice with Rosa26 reporter mice. At E13.5, embryos were removed and fixed in 4% PFA in 0.1 M NaH_2PO_4, pH 7.3 for 1 h at 4°C. The embryos were rinsed in Wash solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40 in 0.1 M NaH_2PO_4, pH 7.3) for 3 × 30 min, then subjected to Stain solution (1 mg/mL X-gal in DMSO, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% NP-40 in 0.1 M NaH_2PO_4, pH 7.3) for 2-24 h at 37°C. The embryos were subsequently washed 3 × 5 min in 1× PBS and post-fixed for 24 h in 4% PFA in 0.1 M NaH_2PO_4, pH 7.3 at 4°C.

PCR analysis of recombination:

To detect the presence of cre-mediated recombination, PCR analysis of Rb exon 19 was performed (Vooijs *et al.*, 1998; Marino *et al.*, 2000). DNA from the telencephalon of mutant and control embryos was isolated and extracted by phenol-chloroform. Primers for Rb212 (5'-GAAAGGAAAGTCAGGGACATTGGG-3'), Rb18 (5'-GGCGTGTGCATCAATG-3'), and Rb19E (5'-CTCAAGAGCTCAGACTCATGG-3') yielded 283- and 235-bp products for the unrecombined floxed and wild-type alleles, respectively, and a 260-bp band for the recombined floxed allele (Vooijs *et al.*, 1998; Marino *et al.*, 2000).

Western Blot Analysis:

For Western analysis, protein was harvested in lysis buffer A (50 mM HEPES, pH 7.8, 250 mM KCl, 0.1 M EDTA, 0.1 M EGTA, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmthylsulfonyl fluoride, 5 g/mL aprotinin, 2 g/mL leupeptin, 0.4 mM sodium vanadate) for 20 min on ice, followed by a 12 min centrifugation at 14 000 RPM. Protein was separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking overnight at 4°C in 5% skim milk, membranes were incubated in the primary antibody for 1-2 h at RT (mouse monoclonal anti-Rb, 1:250, PharMingen, 14001A or mouse monoclonal anti-cre, 1:500, Babco, MMS-106P). After three 5 min washes in TPBS (100 mM Na_2HPO_4, 100 mM NaH_2PO_4, 0.5 N NaCl, 0.1% Tween 20), membranes were incubated for 1 h at RT in the secondary horseradish peroxidase-conjugated antibody (anti-mouse HRP, Bio-Rad #170-6516). Blots were developed by chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (Gel Shift):

Total cell protein was extracted in lysis buffer (Buffer A). 10 μg of lysate was incubated with an excess of ^{32}P-labeled double stranded DNA probe (60 000 cpm/0.2 ng of DNA) containing a single E2F binding site: 5'-GGATTTAAGTTTCGCGCCCTTTCTCAA-3'. The binding reaction (25 μL) was carried out at RT for 20 min in binding buffer (20 mM HEPES, pH 7.6, 4% Ficoll, 2.5% $MgCl_2$, 40 mM KCl, 0.1 mM EGTA, 0.5 mg/mL acetylated BSA, 0.5 mM DTT, and 100 ng of sonicated herring sperm DNA). To control for binding specificity, a 100-fold excess of wild-type or mutant oligo (5'-GATTTAAGTTTCGATCCCTTTCTCAA-3') was added to the binding reaction and incubated for 20 min prior to the addition of labeled probe. To identify the composition of the complexes, 1-2 μL of purified antibody was added to the reaction mixture. Complexes were resolved on a 5% gel run for 4 h, dried, and visualized by autoradiography.

Tissue Fixation and Cryoprotection:

Pregnant females were sacrificed by a lethal injection of sodium pentobarbitol and embryos were removed and placed in 1× PBS. Embryos were fixed in 4% paraformaldehyde (PFA)/ 0.1 M phosphate buffer pH 7.4 for 1-2 days at 4°C. Tissue was rinsed 3 times in PBS, then subjected to sequential solutions of 12, 16, and 18% sucrose/0.1 M phosphate buffer for 1 day each at 4°C. Embryos were embedded in OCT (TissueTek 4583), frozen on liquid N_2, and cut in 14 μm sections at -20°C on Superfrost slides (Fisher #12-550-15).

BrdU Labeling and Immunohistochemistry:

Pregnant females were injected intra-peritoneally with 100 μg/g body mass BrdU (Boehringer Mannheim #280879). Mice were sacrificed and embryos harvested at different time points, and were fixed and cryoprotected as described above. For BrdU detection, sections were pretreated in 1 N HCl for 10 min at 37°C, 0.2 M $Na_2B_4O_7$ for 10 min at RT, then 3 × 10 min rinses in PBS. Sections were incubated in mouse monoclonal anti-BrdU (1:50, Becton Dickinson) for 1-2 h at RT, rinsed three times in PBS for 10 min then incubated in the appropriate secondary antibody (goat anti-mouse Alexa-488 or Alexa-594, 1:1000, Molecular Probes). Other antibodies used: TuJ1 (mouse monoclonal hybridoma supernatant, 1:50, Dr. David Brown, University of Ottawa), GFAP (rabbit polyclonal, 1:400, DAKO, AB986), nestin (mouse monoclonal, 1:200, RDI, 21714), and phospho histone H3 (rabbit polyclonal, 1:200, Upstate Biotechnology, 06-570).

Determination of Cell Death:

TUNEL staining: Sections were incubated for 1 h at 37°C with 75 μL of mixture (Roche Diagnostics, Mississauga, ON) consisting of 0.5 μL terminal deoxynucleotide transferase (TdT), 0.95 μL biotin-16-dUTP, 6.0 μL $CoCl_2$, 15.0 μL 5× TdT buffer, and 52.55 μL distilled water. After three washes in 4× SSC buffer, sections were incubated with an Alexa 488-streptavidin (1:1000, Molecular Probes) for 1 h at RT.

FluoroJade Staining: To evaluate neuronal degeneration including both apoptotic and necrotic modes of cell death, FluoroJade staining was used (Schmued *et al.*, 1997; Noraberg *et al.*, 1999). Sections were dried on a slide warmer, then dipped in alcoholic formalin, pH 10 for 15 sec. Following a 1 min rinse in dH_2O, sections were treated in 0.06% $KMnO_4$ for 15

min. After a brief rinse in dH_2O, sections were incubated in 0.001% FluoroJade solution in 0.1% acetic acid for 30 min at 4°C. Following three washes in dH_20, sections were air dried and mounted with Permount.

Microscopy:

Sections were examined by a Zeiss Axioskop 2 fluorescence microscope, and visualized by a Sony Power HAD 3CCD color video camera with Northern Eclipse software. Confocal images were generated with a BioRad 1024 confocal microscope using a standard 2-channel configuration and a 60× 1.4 NA oil immersion objective.

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REFERENCES

Azuma-Hara, M., Taniura, H., Uetsuki, T., Niinobe, M. and Yoshikawa, K. (1999) Regulation and deregulation of E2F1 in postmitotic neurons differentiated from embryonal carcinoma P19 cells. *Exp. Cell Res.*, **251**, 442-451.

Banasiak, K.J., Xia, Y. and Haddad, G.G. (2000) Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol*, **62**, 215-249.

Banasiak, K.L. and Haddad, G.G. (1998) Hypoxia-induced apoptosis: effect hypoxic severity and role of p53 in neuronal cell death. *Brain Res.*, **29**, 295-305.

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 E2F1 and 3 activity. *Dev.Biol.*, **207**, 257-270.

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

Dick, T.E., Bellingham, M.C. and Richter, D.W. (1994) Pontine respiratory neurons in anesthetized cats. *Brain Res.*, **636**, 259-269.

Dyson, N. (1998) The regulation of E2F by pRb-family proteins. *Genes Dev.*, **12**, 2245-2262.

Ferguson, K.L. and Slack, R.S. (2001) The Rb pathway in neurogenesis. *Neuroreport*, **12**, 55-62.

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. *J. Biol. Chem.*, **275**, 11553-11560.

Gwag, B.J., Lobner, D., Koh, J.Y., Wie, M.B. and Choi, D.W. (1995) Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro. *Neuroscience*, **68**, 615-619.

Hebert, J. 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.

Hou, S.T., Callaghan, D., Fournier, M.C., Hill, I., Kang, L., Massie, B., Morley, P., Murray, C., Rasquinha, I., Slack, R. and MacManus, J.P. (2000) The transcription factor E2F1 modulates apoptosis of neurons. *J. Neurochem.*, **75**, 91-100.

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.

Jodkowski, J.S., Coles, S.K. and Dick, T.E. (1994) A 'pneumatic centre' in rats. *Neurosci. Lett.*, **172**, 67-72.

Lee, E., Hu, N., Yuan, S.S.F., Cox, L.A., Bradley, A., Lee, W. and Herrup, K. (1994) Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes & Dev.*, **8**, 2008-2021.

Lee, E.H.P., Chang, C.Y., Hu, N., Wang, Y.C.J., 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.

Lipinski, M.M. and Jacks, T. (1999) The retinoblastoma gene family in differentiation and development. *Oncogene*, **18**, 7873-7882.

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 Rb tumor suppressor in developing central nervous system. *EMBO J.*, **20**, 3402-3413.

Maandag, E.R., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A. and te Riele, H.T. (1994) Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *EMBO*, **13**, 4260-4268.

Macleod, K.F., Hu, Y. and Jacks, T. (1996) Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *EMBO J.*, **15**, 6178-6188.

Marino, S., Vooijs, M., van der Gulden, H., Jonkers, J. and Berns, A. (2000) Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev*, **14**, 994-1004.

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

McConnell, S.K. and Kaznowski, C.E. (1991) Cell cycle dependence of laminar determination in developing neocortex. *Science*, **254**, 282-285.

Morris, E.J. and Dyson, N.J. (2001) Retinoblastoma protein partners. *Adv. Cancer Res.*, **82**, 1-54.

Morrison, S.F., Cravo, S.L. and Wilfehrt, H.M. (1994) Pontine lesions produce apneusis in the rat. *Brain Res.*, **652**, 83-86.

Muller, H., Bracken, A.P., Vernell, R., Moroni, M.C., Christians, F., Grassilli, E., Prosperini, E., Vigo, E., Oliner, J.D. and Helin, K. (2001) E2Fs regulate the expression of genes

involved in differentiation, development, proliferation, and apoptosis. *Genes Dev.*, **15**, 267-286.

Mulligan, G. and Jacks, T. (1998) The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet.*, **14**, 223-229.

Nevins, J.R. (1998) Toward an Understanding of the Functional Complexity of the E2F and Retinoblastoma Families. *Cell Growth & Differ.*, **9**, 585-593.

Noraberg, J., Kristensen, B.W. and Zimmer, J. (1999) Markers for neuronal degeneration in organotypic slice cultures. *Brain Res. Brain Res. Protoc.*, **3**, 278-290.

O'Hare, M.J., Hou, S.T., Morris, E.J., P., C.S., Xu, Q., Slack, R.S. and Park, D.S. (2000) Induction and modulation of cerebellar granule neuron death by E2F-1. *J. Biol. Chem.*, **18**, 25358-25364.

Pan, H., Yin, C., Dyson, N.J., Harlow, E., Yamasaki, L. and van Dyke, T. (1998) Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. *Mol. Cell*, **2**, 283-292.

Phillips, A.C., Bates, S., Ryan, K.M., Helin, K. and Vousden, K.H. (1997) Induction of DNA synthesis and apoptosis are separable functions of E2F-1. *Genes Dev.*, **11**, 1853-1863.

Rakic, P. (1988) Specification of cerebral cortical areas. *Science*, **241**, 170-176.

Rosenblum, W.I. (1997) Histopathologic clues to the pathways of neuronal death following ischemia/hypoxia. *J Neurotrauma*, **14**, 313-326.

Schmued, L.C., Albertson, C. and Slikker, W.J. (1997) Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res.*, **751**, 37-46.

Shimamura, K. and Rubenstein, J.L.R. (1997) Inductive interactions direct early regionalization of the mouse forebrain. *Development*, **124**, 2709-2718.

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

Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.*, **21**, 70-71.

Tao, W. and Lai, E. (1992) Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. *Neuron*, **8**, 957-966.

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.

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.

Weinberg, R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323-330.

Ziebold, U., Reza, T., Caron, A. and Lees, J.A. (2001) E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. *Genes Dev.*, **15**, 386-391.

Chapter 4.

Ferguson *et al.* (2004) Telencephalon-specific Rb mutants reveal specific marginal zone neuronal loss through apoptosis and defective tangential migration. (to be submitted)

This manuscript represents a more detailed examination of the telencephalon-specific conditional Rb mutants in the developing cortex. We demonstrate a requirement for Rb in proper cortical organization, neuronal survival and neuronal migration of specific progenitor subtypes. The experiments in this manuscript were conducted by K. L. Ferguson, with assistance from K. McClellan for the *in situ* hybridization, immunohistochemical analyses, and cell counting. This manuscript was written by K. L. Ferguson, with guidance and editorial assistance from Dr. R. S. Slack.

Telencephalon-specific Rb mutants reveal specific marginal zone neuronal loss through apoptosis and defective tangential migration

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ABSTRACT

Precise cell cycle regulation is critical for nervous system development. To assess the role of the key cell cycle regulator, Retinoblastoma (Rb), in cortical development, we examined mice with telencephalon-specific Rb deletions. Animals carrying a floxed Rb allele were interbred with mice in which cre was knocked into the Foxg1 locus. We have previously shown that these conditional Rb mutants survive until birth. Rb-/- progenitor cells divided ectopically, but were able to survive and initiate neuronal differentiation. In this study, we have examined the impact of Rb deficiency on cortical development and neuronal differentiation. Histological examination of cortical morphology revealed defective laminar organization and region-specific cellularity at mid-gestation. Rb mutants exhibited increased intermediate zone cells and a partial loss of the intermediate zone-cortical plate boundary. To assess the expression of the neuronal markers, Tbr1 and SCG10, *in situ* hybridization revealed greatly elevated expression in the mutant intermediate zone. Further, in spite of normal neuronal generation, Rb mutants contained an approximately 50% reduction in total marginal zone cells and Reelin expression, indicating a specific loss in Cajal-Retzius neurons. This loss was confirmed by significantly increased apoptosis within the Rb mutant MZ. Although labeling in other telencephalic regions appeared normal, expression of the interneuron markers, calbindin and Lhx6, was dramatically reduced in the mutant marginal zone, suggesting defective tangential migration. These studies demonstrate cell type-specific requirements for Rb, such that in its deficiency, there is a selective neuronal loss within the mutant marginal zone, resulting from apoptosis and defective tangential migration.

INTRODUCTION

Cell cycle regulation is required for several aspects of cortical neurogenesis including maintaining the progenitor pool, producing the correct proportion of diverse cell types, and coordinating the timing of neuronal differentiation. To give rise to the developing cortex, precursor cells undergo multiple rounds of proliferation, between embryonic days 10 to 17 (E10-17) (Takahashi *et al.*, 1995; Takahashi *et al.*, 1996). Progenitor proliferation is restricted to the pseudostratified neuroepithelial ventricular zone (VZ). Following cell cycle withdrawal, newly-born neurons initiate expression of early neuronal markers and commence migration into the developing cortical plate (CP). The first neurons generated, or the "pioneering neurons", are born around E10-11. Pioneering neurons give rise to the preplate (PP), which is then split by subsequent cohorts into the superficial marginal zone (MZ), and the deeper subplate (SP). The MZ contains the future layer I cells, including a transient population of Cajal-Retzius (CR) neurons. Cortical layers (lamina) II-VI form between the MZ and SP in an inside-out pattern, such that earlier generated neurons reside in deep layers, and later born neurons migrate to more superficial layers (Takahashi *et al.*, 1999). As cortical development proceeds, the SP becomes separated from the VZ by the intermediate zone (IZ), a white matter region containing afferent and efferent projections (Sidman and Rakic, 1973; Caviness, 1982b). In addition to radial neuronal migration, the cortex is also generated by tangential migration of newly generated neurons, in particular, GABAergic interneurons, from the ventral telencephalon (Anderson *et al.*, 1997; Lavdas *et al.*, 1999; Sussel *et al.*, 1999; Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Wichterle *et al.*, 2001).

The time at which a newly generated neuron undergoes terminal mitosis and exits the cell cycle correlates highly with its' eventual laminar fate and neuronal identity (McConnell and Kaznowski, 1991; McConnell, 1995). The cell cycle dependence in neuronal identity was best shown by a series of transplantation studies in the ferret (McConnell and Kaznowski, 1991). Cells isolated at E29, which would normally give rise to layer VI, were [^{3}H] thymidine-labeled *in vitro* and transplanted into post-natal hosts, in which layers II/III were currently being generated. It was shown that the majority of precursors that were transplanted during their S phase, switched fates and migrated to layers II/III, thereby adopting the laminar fate appropriate for their new environment. In contrast, neurons which were in later cell cycle stages at the time of transplantation migrated to layer VI, maintaining the laminar identity appropriate for their birth date (McConnell and Kaznowski, 1991). These studies demonstrated that cells receive their environmental cues for correct laminar identity during terminal mitosis, and established the importance for proper cell cycle control in cortical development. Because of the strong correlation between neuronal subtype and time of generation, it is believed that precise cell cycle regulation and the determination of neuronal identity are intimately connected.

Following neuronal commitment and cell cycle withdrawal, newly born neurons initiate differentiation and migration into the developing cortex. Pioneering studies revealed that cortical neurons were generated within the VZ and underwent radial migration to their ultimate position (Ramón y Cajal, 1891; Rakic, 1972; Rakic, 1988). Radially migrating neurons are guided by CR neurons in the MZ. The CR neurons synthesize and secrete Reelin, a large protein which associates with the extracellular matrix (D'Arcangelo *et al.*, 1995; Hirotsune *et al.*, 1995; Ogawa *et al.*, 1995). More recent evidence suggests that virtually all

cortical GABAergic interneurons are derived from the medial ganglionic eminence (MGE) and undergo tangential migration (Anderson *et al.*, 1999; Corbin *et al.*, 2001; Lambert de Rouvroit and Goffinet, 2001; Marin and Rubenstein, 2001; Wichterle *et al.*, 2001; Anderson *et al.*, 2002; Nadarajah and Parnavelas, 2002; Parnavelas *et al.*, 2002; Stuhmer *et al.*, 2002). Tangentially migrating interneurons follow very specific migratory routes. They generally avoid entering the developing striatum and therefore, form two distinct paths- either superficial or deep to the striatal mantle (Marin *et al.*, 2001). Superficially migrating neurons tend to avoid the CP, migrating instead along the MZ, whereas the deep neurons travel through the lower IZ and SVZ (Del Rio *et al.*, 1992; DeDiego *et al.*, 1994; Lavdas *et al.*, 1999; Denaxa *et al.*, 2001; Marin and Rubenstein, 2001; Wichterle *et al.*, 2001). Once the neurons have migrated the appropriate distance, they can then switch to a radial mode of migration to enter the cortex (Polleux *et al.*, 2002). The particular route traveled may depend on the neuronal subtype and also the time of generation.

The retinoblastoma (Rb) protein is a key cell cycle regulator. First discovered as a tumour suppressor, Rb regulates the G1/S phase restriction point, thereby controlling entry into S phase, reviewed in (Classon and Harlow, 2002; Stevaux and Dyson, 2002; Trimarchi and Lees, 2002; Cam and Dynlacht, 2003). Studies with Rb-deficient embryos were the first to show that Rb had an important role in nervous system development. Rb null mutants died by mid-gestation (E12-15) with massive apoptosis throughout the liver and nervous system, as well as ectopic mitoses (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). More recently, we and others have shown that Rb deficiency does not result in large scale apoptosis in a cell-autonomous manner (Lipinski *et al.*, 2001; Ferguson *et al.*, 2002; MacPherson *et al.*, 2003; Wu *et al.*, 2003). In the developing telencephalon, Rb deficiency is

fully compatible with survival of the majority of neuronal populations (Ferguson *et al.*, 2002). In contrast, Rb regulation of cell proliferation is cell-autonomous because telencephalon-specific Rb deficient mutants exhibit ectopic cell divisions outside the germinal regions (Ferguson *et al.*, 2002).

In the present study, we examine the impact of Rb deficiency on cortical development and neuronal differentiation. We reveal that Rb mutants exhibit defective laminar organization and region-specific cellularity at mid-gestation. While the majority of cortical neurons survive in the absence of Rb, specific populations, including Reelin-positive Cajal-Retzius neurons, may require Rb for survival. The selective reduction of calbindin- and Lhx6-positive interneurons from the cortical MZ indicates an involvement for Rb in the regulaton of tangentially migrating interneurons. Together, these studies demonstrate cell type-specific requirements for Rb, such that in its deficiency, there is a selective neuronal loss within the mutant MZ, resulting from apoptosis and defective tangential migration.

MATERIALS AND METHODS

Mice:

The Rb-F19 (Marino *et al.*, 2000) and Foxg1-cre mice (Hebert and McConnell, 2000) were generously provided by Dr. Anton Berns, of the Netherlands Cancer Institute and Dr. Susan McConnell of Stanford University, respectively. To generate the telencephalon-specific Rb conditional knockouts, floxed Rb-F19 nullizygous mice were bred with Foxg1-cre:Rb-F19 double heterozygous mice, to generate mutants (Foxg1-cre:Rb-F19-/-) at a frequency of 25%. Mice were maintained on an F/VBN/129SV genetic background and were genotyped by polymerase chain reaction (Ferguson *et al.*, 2002), using DNA extracted from tails or remaining embryonic tissue. For timed pregnancies, mice were bred, and the time of plug identification was counted as day 0.5. All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

Histology:

Females at various stages of gestation were sacrificed by a lethal injection of sodium pentobarbitol and embryos were removed and placed in 1× PBS. Embryos were fixed in 4% paraformaldehyde (PFA)/ 0.1 M phosphate buffer, pH 7.4, for 1-2 days at 4°C. For frozen sections, tissue was rinsed 3 times in PBS, and then subjected to sequential solutions of 12, 16, and 18% sucrose/0.1 M phosphate buffer for 1 day each at 4°C. Embryos were embedded in OCT (TissueTek 4583), frozen on liquid N_2, and cut as 14 μm sections at -20°C on Superfrost slides (Fisher #12-550-15). For paraffin sections, fixed embryos were dehydrated

in 60% ethanol for 1-2 days, embedded in paraffin wax, and sectioned at a thickness of 6 μm. Cresyl Violet staining was performed on paraffin sections according to standard protocols.

Immunohistochemistry and in situ hybridization:

Immunohistochemistry was performed on frozen sections with the following primary antibodies: mouse monoclonal anti-Reelin G10 (1:500; Calbiochem, #553731) and rabbit polyclonal anti-calbindin (D-28) (1:1000; Chemicon, AB1778). Sections were incubated in primary antibody overnight at 4°C, rinsed three times for 10 min each in PBS, and then incubated in the appropriate secondary antibody. For Reelin immunochemistry, sections were subjected to an antigen retrieval pre-treatment: sections were brought to a boil in 10 mM sodium citrate buffer, pH 6.0, placed in an ice bath for 5 min, then the process was repeated twice more. Secondary antibodies used include: goat anti-mouse and donkey anti-rabbit CY3 (1:1000, Jackson ImmunoResearch); goat anti-mouse and goat anti-rabbit Alexa-488 (1:1000, Molecular Probes).

For TUNEL staining, sections were incubated for 1 h at 37°C with 75 μL of mixture (Roche Diagnostics) consisting of 0.5 μL terminal deoxynucleotide transferase (TdT), 0.95 μL biotin-16-dUTP, 6.0 μL $CoCl_2$, 15.0 μL 5× TdT buffer, and 52.55 μL distilled water. After three washes in 4× SSC buffer, sections were incubated with an Alexa 488-streptavidin (1:1000, Molecular Probes) for 1 h at RT.

Non-radioactive *in situ* hybridization and digoxigenin probe labeling was performed according to previously described protocols (Wallace and Raff, 1999). The following

antisense riboprobes were used, as previously described: Tbr1 (Bulfone *et al.*, 1995), SCG10 (Stein *et al.*, 1988), and Lhx6 (Grigoriou *et al.*, 1998).

Sections were examined on a Zeiss Axioskop 2 fluorescence microscope, and images were captured with a Sony Power HAD 3CCD color video camera using Northern Eclipse software.

Cell counting and statistical analyses:

Cells in each anatomical region (VZ, SVZ, IZ, CP, MZ) were quantified in Cresyl Violet-stained coronal paraffin sections (n=4 control; 4 cond. Rb-/- (E13.5); n=3 control; 4 cond. Rb-/- (E16.5)) within 245 μm-wide columns, across the entire ventricle-pial range of the dorsal cortex. All sections were at similar anterior-posterior levels. To quantify TUNEL-positive cells, positive cells were counted on E13.5 coronal telencephalic sections and binned according to the following anatomical regions: dorsal and ventral germinal zones, cortical IZ/CP, cortical MZ, and non-germinal regions of the ventral telencephalon (n= 4 control, 5 cond. Rb-/-). Cells counts were expressed as total cells in each anatomical region per telencephalic lobe per section. Reelin-positive cells in each embryo section (n=3 controls, 3 cond. Rb-/- (E12.5); n=5 controls, 4 cond. Rb-/- (E16.5)) were quantified along a 1500 μm (E12.5) or 3000 μm (E16.5) length of the MZ. In all cases, statistical analyses were conducted using one- or two-tailed t-tests.

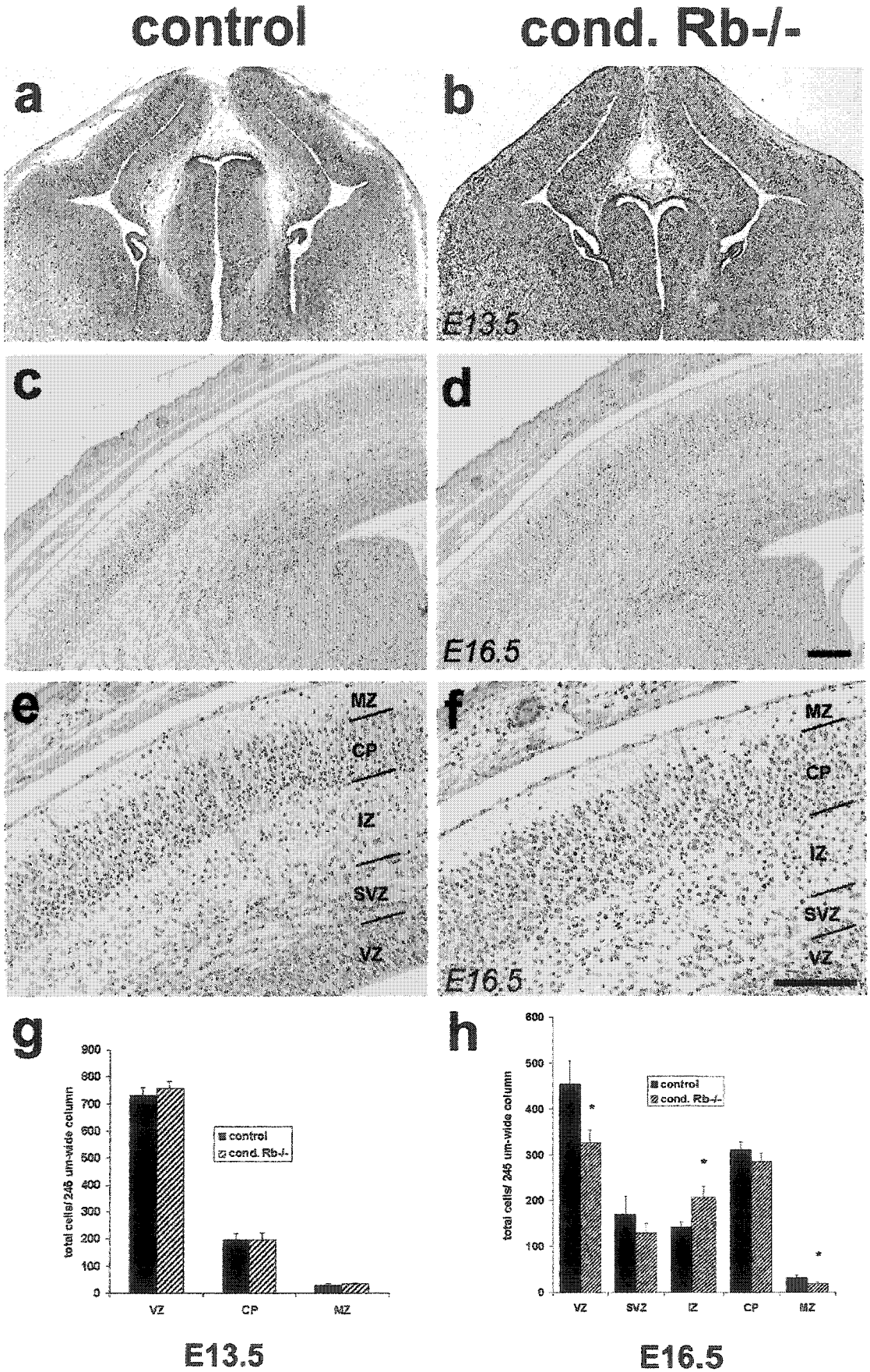
RESULTS

Telencephalon-specific Rb mutants exhibit abnormal cortical morphology:

In our initial examination of the telencephalon-specific Rb knockouts, we demonstrated that these mutants retained the ectopic mitoses phenotype characteristic of Rb germline knockouts. The severe neuronal apoptosis associated with germline deficiency, however, was rescued by conditional Rb deletion (Ferguson *et al.*, 2002). The conditional mutants had enlarged cortices, which occasionally resulted in cortical protrusions (Ferguson *et al.*, 2002). Since the defective cell cycle regulation resulting from Rb deficiency could impact the timing and generation of cortical neurons, cortical morphology was examined at E13.5 and 16.5 (Fig. 1). Cells in each anatomical region were quantified within a 245 μm-wide column, across the entire ventricle-pial range of the dorsal cortex. Early in neurogenesis (E13.5), control and mutant embryos appeared structurally similar and did not exhibit any difference in size or morphology (Fig. 1a, b; n=4 control; 4 cond. Rb-/-). Cell numbers within the VZ, CP and MZ were similar (Fig. 1g).

At E16.5, the Rb mutants exhibited significant differences in cortical morphology and region-specific cellularity (Fig. 1c-f, h; n=3 control; 4 cond. Rb-/-). While the germinal regions (VZ and SVZ) appeared structurally normal, the mutant embryos contained significantly fewer precursor cells. Within the VZ, control embryos had approximately 454±51 cells as compared to 326±27 cells in the mutant ($P<0.05$). There was a similar trend in the SVZ, with 170±38 cells in the control as compared to 129±20 cells in the mutant, however, this failed to reach statistical significance. In contrast, the mutant IZ was more

Fig. 1: Telencephalon-specific Rb mutants exhibit abnormal cortical morphology. Conditional Rb mutant and control littermates were paraffin sectioned coronally and stained with Cresyl Violet at E13.5 and 16.5. Representative control and mutant sections are shown. Cells in each anatomical region were quantified within 245 μm-wide columns, across the entire ventricle-pial range of the dorsal cortex. At E13.5, no differences were observed between control and Rb mutant embryos in relation to morphology or cell number (a, b, g) (n=4 control; 4 cond. Rb-/-). At E16.5, the Rb mutants exhibited aberrant cortical morphology and region-specific cellularity. As compared to the controls, mutant embryos contained significantly fewer precursor cells within the VZ (h, $P<0.05$). In contrast, the mutant IZ was more densely packed and contained significantly more cells than the control embryos (d, f, h, $P<0.05$). While the number of CP cells was similar in controls and mutants (h), the mutant brains lacked the clear IZ-CP boundary that was evident in the controls (d, f). Within the MZ, the mutant cortices contained nearly 50% fewer cells as compared to controls (h, $P<0.05$) (n=3 control; 4 cond. Rb-/-). Asterisk denotes a significance level of $P<0.05$ by one-tailed t-test. Bar= 100 μm.

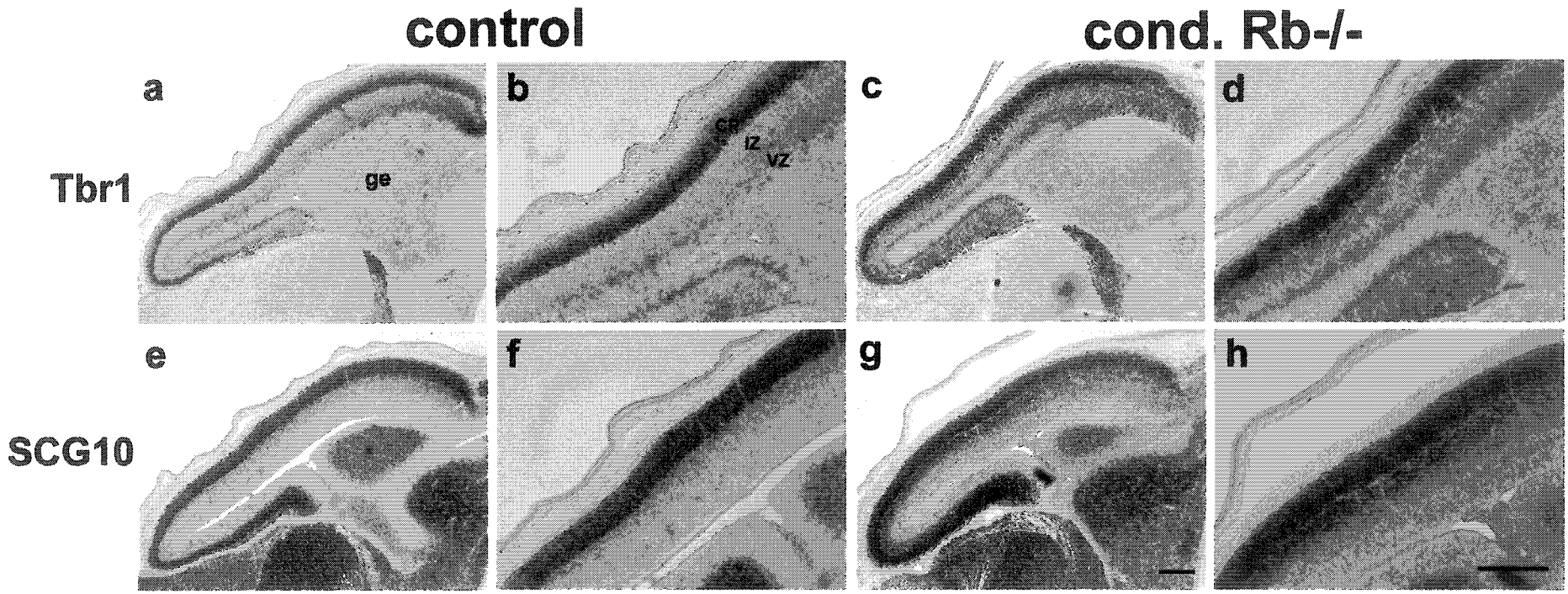

control
cond. Rb-/-
a
b
E13.5
c
d
E16.5
e
MZ
CP
IZ
SVZ
VZ
f
MZ
CP
IZ
SVZ
VZ
E16.5
g
total cells/ 245 um-wide column
900
800
700
600
500
400
300
200
100
0
control
cond. Rb-/-
VZ
CP
MZ
E13.5
h
total cells/ 245 um-wide column
600
500
400
300
200
100
0
control
cond. Rb-/-
VZ
SVZ
IZ
CP
MZ
E16.5

densely packed and contained significantly more cells (206±25) than the control embryos (142±10; $P<0.05$). While the number of CP cells was similar in controls and mutants (311±18 and 284±18, respectively), the mutant brains lacked the clear IZ-CP boundary that was evident in the controls, which could be due to the increased cell number within the mutant IZ. Within the MZ, the mutant cortices contained nearly 50% fewer cells as compared to control embryos (32±6 cells in the control and 18±3 cells in the mutant; $P<0.05$).

Laminar patterning is perturbed in the absence of Rb:

Since Rb is an important regulator of terminal mitosis, and precise timing of cell cycle regulation is believed to be critical for proper generation of cortical layers (McConnell and Kaznowski, 1991; McConnell, 1995; Takahashi *et al.*, 1999), we questioned whether the conditional Rb mutants may show defective laminar patterning. To ask whether appropriate lamination was formed, the expression of layer-specific markers was investigated by *in situ* hybridization (Fig. 2). Sagittal sections were examined at E15.5 to coincide with the aberrant cortical morphology observed mid-neurogenesis. At this time, only the deepest cortical layers, layers V and VI, are formed. Layer IV has recently been generated and the neurons of layers II-III are in the process of withdrawing from the cell cycle and migrating to their laminar positions (Takahashi *et al.*, 1999). Tbr1 is a T-box transcription factor, which is a marker of post-mitotic glutamatergic projection neurons, with high expression in layer VI (Rubenstein *et al.*, 1999; Hevner *et al.*, 2001). Expression of this marker appeared slightly enhanced within the Rb mutant CP. Further, unlike the control embryos, the mutants exhibited strong labeling within the IZ (Fig. 2c, d). A similar pattern was observed with a

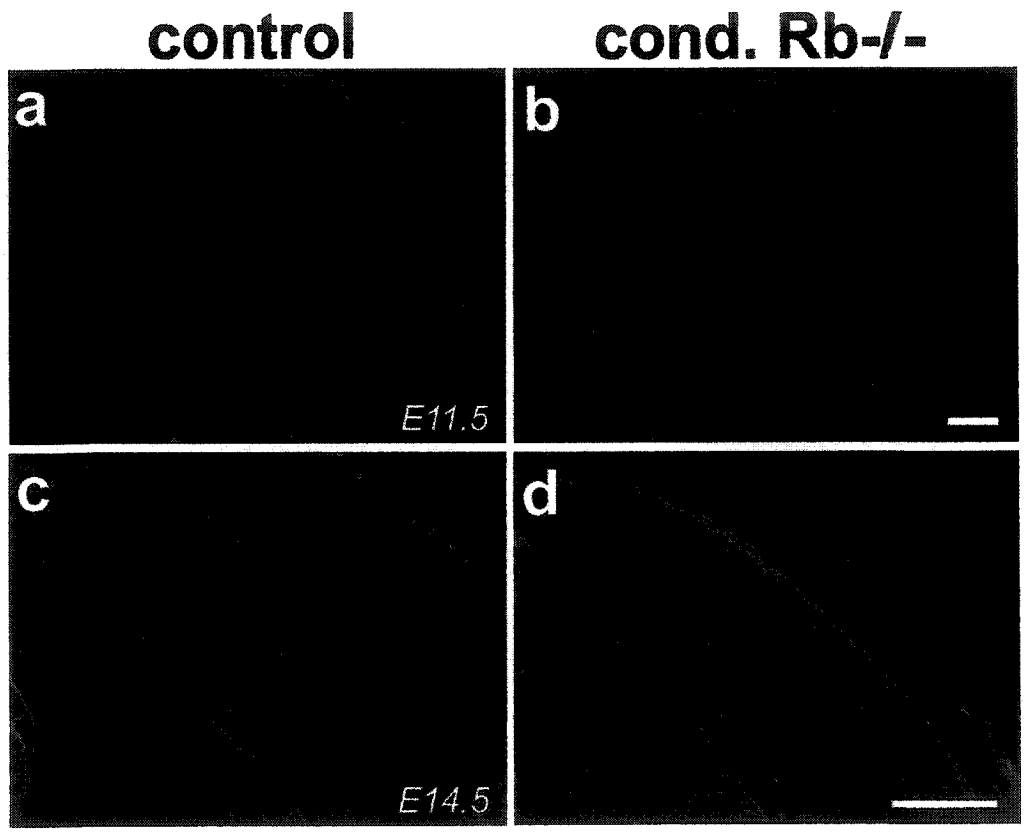
Fig. 2. Laminar patterning is perturbed in the absence of Rb. *In situ* hybridization in E15.5 sagittal sections of mutant and control embryos demonstrates enhanced expression of neuronal markers, Tbr1 and SCG10, in the Rb mutants. Tbr1 labeling is slightly elevated within the mutant CP, and strongly up-regulated within the IZ (c, d). Similarly, SCG10 expression is highly elevated within the mutant IZ (g, h). The boundary between the mutant CP and IZ lacks the clear definition observed in the control embryos (n=4 control; 5 cond. Rb-/-) Bar=100 μm, ge=ganglionic eminence.


control
cond. Rb-/-
a
b
c
d
Tbr1
ge
CP
IZ
VZ
e
f
g
h
SCG10

second marker, SCG10, which is a neuron-specific growth-associated protein, often used as a pan-neuronal marker (Stein *et al.*, 1988; Gavet *et al.*, 2002). While CP expression appeared normal, the mutant embryos exhibited highly elevated SCG10 expression within the IZ (Fig. 2g, h). Once again, the boundary between the mutant CP and IZ lacked the clear definition observed in the control embryos. This pattern was further confirmed by probing with the neuronal markers Id2, RORβ, and Otx1, which also displayed increased IZ expression in the Rb mutant (not shown).

Elevated expression of these CP markers within the mutant IZ could be indicative of precocious neuronal differentiation. Therefore, we sought to determine whether Rb deficiency may impact the timing of neuronal differentiation. Mutant and control sections were immunolabeled with the early pan-neuronal marker, βIII-tubulin, at various embryonic time points (Fig. 3). At E11.5, when the first neurons are being generated, the extent of βIII-tubulin labeling was similar in mutant and control embryos (Fig. 3a, b). Further, no differences were observed at later embryonic ages (E14.5, 16.5, 18.5), indicating that Rb deficiency does not alter the timing of pan-neuronal differentiation (Fig. 3c, d; not shown). In addition, mutant and control sections were labeled with the precursor marker, nestin, with no observed differences (not shown). Therefore, the altered morphology and laminar patterning in the mutant cortex does not appear to be due to premature neuronal differentiation. This abnormal laminar pattern could also be explained by defective neuronal migration. We have demonstrated an increased cell number within the mutant IZ at E16.5, which suggests that Rb deficient cells may be migrating more slowly to reach their ultimate destination within the CP.

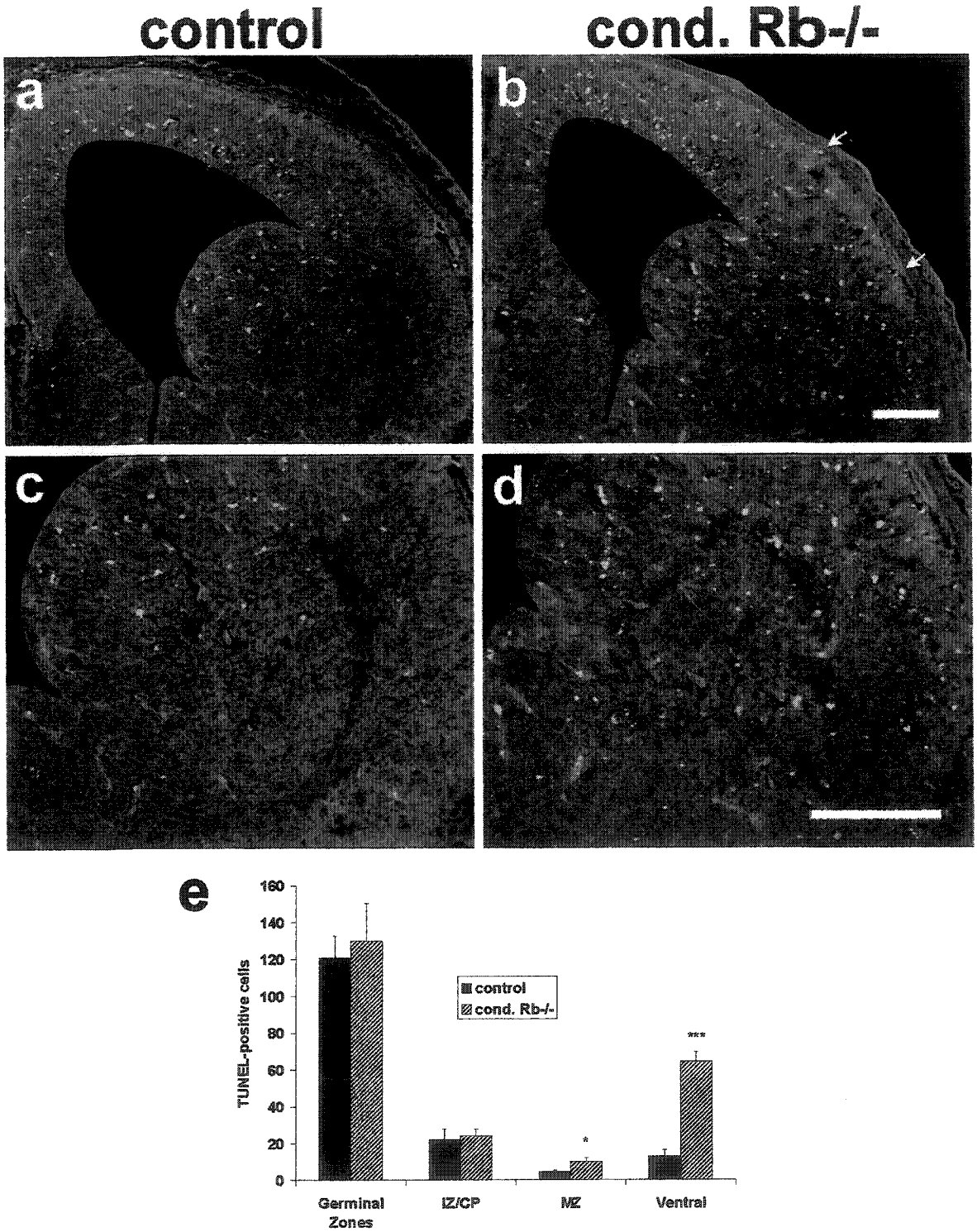
Fig. 3: Initiation of neuronal differentiation in not affected by Rb deficiency. To examine whether neuronal differentiation may occur prematurely in Rb deficiency, coronal sections of control and mutant embryos were immunolabeled with the early neuronal marker, βIII-tubulin (TuJ1), at various embryonic ages. At E11.5 (a, b) and E14.5 (c, d), there were no observed differences in the onset or extent of βIII-tubulin expression (n=3 control, 3 cond. Rb-/- (E11.5); 3 control, 4 cond. Rb-/- (E14.5). Bar=100 μm.


control
cond. Rb-/-
a
E11.5
b
c
E14.5
d

Conditional Rb mutants exhibit selective neuronal apoptosis in the cortical MZ and ventral telencephalon

We have previously reported that telencephalon-specific Rb deficiency is not associated with the large scale neuronal death characteristic of the germline knockouts. While at later time points, no difference between Rb mutants and controls was observed, at E13.5, we reported an approximately 1.5-fold increase in TUNEL-positive cells within the mutant telencephalon (Ferguson *et al.*, 2002). Since the Rb mutants exhibit region-specific differences in cellularity at mid-gestation, one may predict that specific neuronal populations may be undergoing apoptosis. To test this, we performed TUNEL labeling on mutant and control E13.5 embryos, and quantified positive cells within the dorsal and ventral germinal zones, the cortical IZ/CP, the cortical MZ, and the non-germinal regions of the ventral telencephalon (Fig. 4; n= 4 control, 5 cond. Rb-/-). In control embryos, the large majority of TUNEL-positive cells were restricted to the dorsal and ventral germinal regions, with few cells undergoing cell death outside these areas (Fig. 4a, c). Within the germinal regions, the control and mutant embryos had similar numbers of apoptotic cells (120.7±12.3 and 131.0±20.4 cells, respectively) (Fig. 4e). In addition, no difference in cell number was observed in the IZ/CP regions, with 22.1±5.6 cells in the control embryos and 24.6±3.5 cells in the Rb mutants (Fig. 4e). In contrast, the Rb mutant sections contained significantly more TUNEL-positive cells within the cortical MZ (Fig. 4b). Control embryos contained an average of 4.3±0.9 cells in the MZ, as compared to 9.9±2.0 cells in the mutant, representing a 2.3-fold increase (Fig. 4e; $P<0.05$). The elevated TUNEL labeling within the Rb mutant

Fig. 4: Conditional Rb mutants exhibit selective neuronal apoptosis in the cortical MZ and ventral telencephalon. To detect cell death, E13.5 conditional mutant and control littermates were assayed for TUNEL labeling. On each section, positive cells were quantified within the dorsal and ventral germinal zones, the cortical IZ/CP, the cortical MZ, and the non-germinal regions of the ventral telencephalon. Similar levels of apoptosis were observed within the germinal regions and IZ/CP of control and mutant embryos (a, b, e). Rb mutants exhibited significantly increased TUNEL-labeling within the MZ (b, $P<0.05$, one-tailed t-test; arrows point to representative cells) and the non-germinal regions of the ventral telencephalon (e; $P<0.001$, two-tailed t-test). (n= 4 control, 5 cond. Rb-/-). Asterisk denotes significance level (* for $P<0.05$; *** for $P<0.001$) by t-test. Bar= 100 μm.

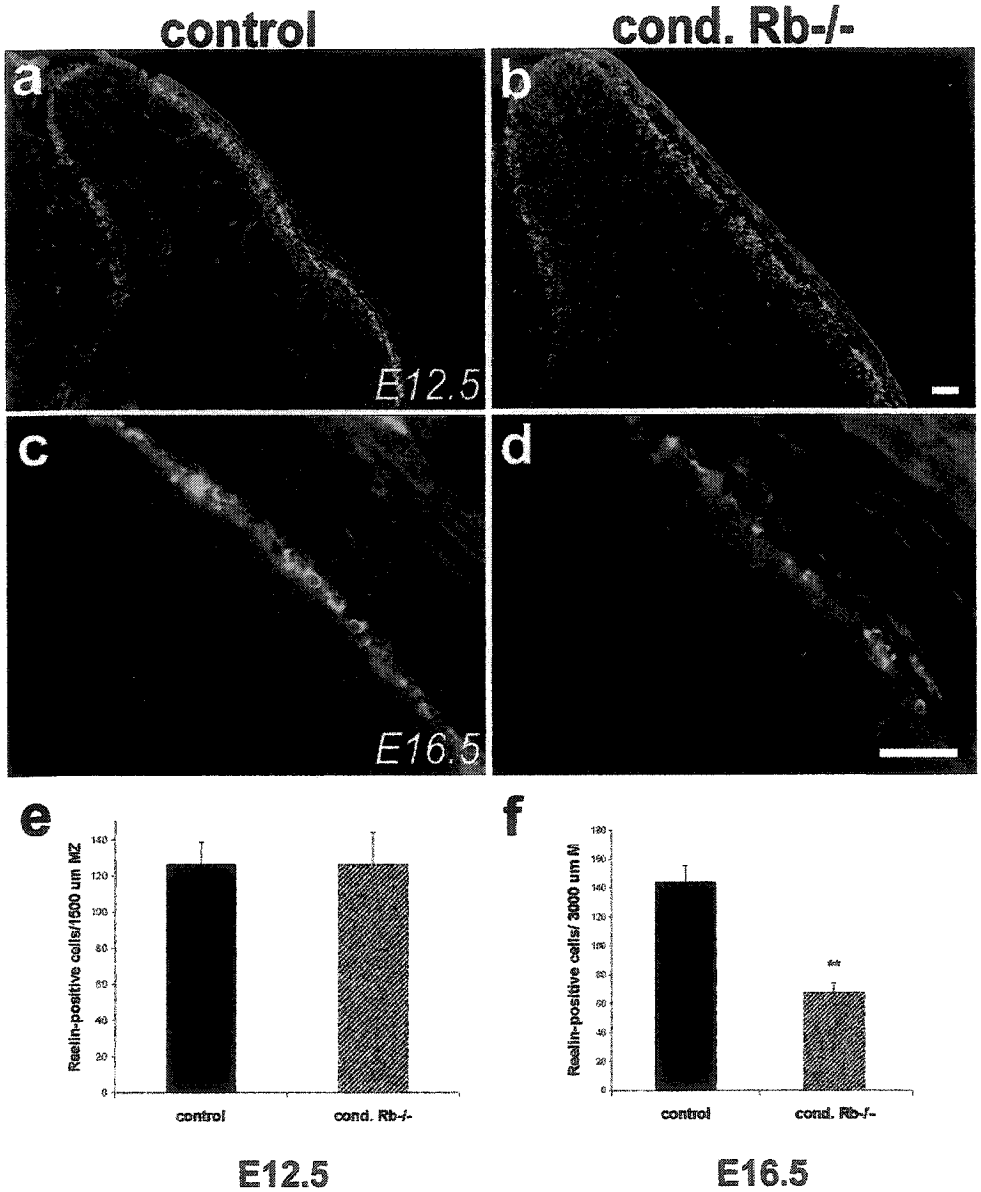

control
cond. Rb-/-
a
b
c
d
e
TUNEL-positive cells
160
140
120
100
80
60
40
20
0
control
cond. Rb-/-
*
***
Germinal Zones
IZ/CP
MZ
Ventral

MZ is consistent with the observed reduction in total MZ cell number (Fig. 1e). Further, the Rb mutant embryos displayed a dramatic increase in apoptotic cells within the non-germinal regions of the ventral telencephalon. While the control embryos contained an average of 12.6±3.4 TUNEL-positive cells, the Rb mutants had 63.9±5.2 apoptotic cells, resulting in a 5-fold increase over controls (Fig. 4e, $P<0.001$). The fact that the Rb mutants exhibited region-specific increases in TUNEL labeling within the MZ and the non-germinal regions of the ventral telencephalon suggests that Rb may be required for the survival of specific neuronal populations.

Reduced number of Cajal-Retzius neurons in the Rb mutant MZ

We have shown that Rb mutants have a substantial reduction in cell number and elevated apoptosis, within the MZ. Due to the aberrant laminar morphology indicative of defective neuronal migration, we hypothesized that Rb deficiency could impact the survival of CR neurons. CR cells, the pioneering neurons born between E11-12, are among the most prevalent cells within the MZ, and are required for proper cortical lamination (Frotscher, 1998; Sarnat and Flores-Sarnat, 2002; Hevner *et al.*, 2003). To assess CR cell number, immunochemistry with a Reelin (G10) antibody was performed on mutant and control E16.5 embryos (Fig. 5c, d). Quantification of positive cells revealed a significant decrease in CR neurons in the mutant MZ, with an average of 143.8±11.8 cells in the controls and 67.5±6.9 cells within the mutant MZ (Fig. 5f). This resulted in a greater than 50% reduction in CR neurons in the Rb mutants (Fig. 5f; $P<0.01$; n=5 controls, 4 cond. Rb-/-). We next questioned whether the reduced number of CR neurons in the Rb mutant was due to

Fig. 5: Reduced number of Cajal-Retzius neurons in the Rb mutant MZ. Coronal sections of control and Rb mutant embryos at E11.5 (not shown), 12.5 (a, b) and 16.5 (c, d) were subjected to immunochemistry with a Reelin (G10) antibody. Positive cells in each section were quantified along a 1500 μm (E12.5) or 3000 μm (E16.5) length of the MZ. At E11.5 (not shown) and E12.5, Reelin expression in the cortical MZ appeared similar between mutant and control embryos (a, b, e). However, by E16.5, Rb mutants contained approximately 50% fewer Reelin-positive neurons as compared to control embryos (f, $P<0.001$) (n=3 controls, 3 cond. Rb-/- (E11.5); 3 controls, 3 cond. Rb-/- (E12.5); 5 controls, 4 cond. Rb-/- (E16.5)). Asterisk denotes a significance level to $P<0.01$, by two-tailed t-test. Bar= 25 μm.


control
cond. Rb-/-
a
b
E12.5
c
d
E16.5
e
Reelin-positive cells/1500 um MZ
control
cond. Rb-/-
E12.5
f
Reelin-positive cells/ 3000 um M
control
cond. Rb-/-
E16.5

defective neuronal generation or alternatively, if these neurons were being subsequently lost. We, therefore, examined the expression of Reelin by immunochemistry in E11.5 and E12.5 embryos. At these time points, Reelin expression in the cortical MZ appeared similar between mutant and control embryos (Fig. 5a, b; and not shown). At E12.5, the number of positive cells did not differ, with 126.2±12.3 cells in the control and 126.0±17.8 cells in the Rb mutant MZ (Fig. 5e; n=3 controls, 3 cond. Rb-/-). These results indicate that Rb deficiency does not impact the generation of CR neurons, but may be required for their survival.

Conditional Rb mutants exhibit reduced cortical interneurons in MZ

The majority of cortical GABAergic interneurons are derived from the ventral ganglionic eminences (Anderson *et al.*, 1997; Lavdas *et al.*, 1999; Sussel *et al.*, 1999; Wichterle *et al.*, 1999; Anderson *et al.*, 2001; Wichterle *et al.*, 2001). These neurons migrate tangentially through the ventral telencephalon to the cortex along distinct routes. To avoid entering the developing striatum, they form two distinct paths- superficial or deep to the striatal mantle (Marin *et al.*, 2001). Once they enter the cortex, they migrate along a deep trajectory in the lower IZ/SVZ and a superficial route along the MZ (Marin *et al.*, 2001). Following tangential migration, neurons can then switch to a radial mode of migration, to reach their final destination in the CP (Polleux *et al.*, 2002). Since the Rb mutants exhibited highly elevated levels of apoptosis within the non-germinal regions of the ventral telencephalon (Fig. 4e), we questioned whether Rb deficiency may affect the survival of ventrally-derived interneurons. Calbindin and Lhx6 have previously been shown to label

specific tangentially migrating interneuron populations (Grigoriou *et al.*, 1998; Lavdas *et al.*, 1999; Jimenez *et al.*, 2002a; Jimenez *et al.*, 2002b; Polleux *et al.*, 2002). We examined mutant and control embryo sections at mid-neurogenesis to determine whether these neuronal populations may be impacted by Rb deficiency (Fig. 6). Immunolabeling revealed that, while calbindin expression appeared normal in most areas of the telencephalon, labeling was dramatically reduced in the temporal cortical MZ, to such an extent as to be almost absent (Fig. 6b, d). Similarly, *in situ* hybridization with an Lhx6 probe demonstrated defective expression in the mutant embryos (Fig. 6e-h). Although Lhx6 expression was similar in mutant and control embryos along the IZ/SVZ migratory route, there was substantially reduced expression along the cortical MZ (Fig. 6h). Further, Lhx6 expression was reduced within the Rb mutant CP, suggesting that regardless of the migratory route traveled, fewer Lhx6 interneurons were reaching the CP. Together, the data demonstrate a requirement for normal Rb function in regulating specific interneuron populations.

The reduced expression of these interneuron markers could be the result of different defects. First, it could indicate that Rb is required for the proper specification and/or generation of these neuronal populations. Second, Rb may be required for the survival of these interneurons. Finally, Rb may be involved in guiding or promoting the migration of these ventrally-derived neurons along their tangential routes. Since neuronal birth date is an important determinant for the generation of diverse neuronal subtypes, we first investigated whether Rb deficiency may impact neuronal fate. At E12.5, the expression of calbindin (Fig. 7a, b) and Lhx6 (Fig. 7c, d) was examined in mutant and control embryo sections. The expression patterns of these interneuron markers within the ganglionic eminences were similar, if not slightly enhanced in the Rb mutant embryos. Therefore, in spite of decreased

Fig. 6: Reduced cortical interneurons in Rb mutant MZ. Rb mutant and control embryo sections were examined at mid-neurogenesis to determine whether specific interneuron populations may be impacted by Rb deficiency. E16.5 sections (coronal) were immunolabeled with a calbindin (D-28) antibody and E15.5 (sagittal) sections were subjected to RNA *in situ* hybridization with an Lhx6 probe. While calbindin expression appeared normal in other telencephalic regions (a, b), these cells were dramatically reduced in the mutant MZ (c, d; arrows point to MZ population). Similarly, Lhx6 expression was normal along the SVZ/IZ migratory route, but was substantially reduced in the mutant CP and MZ (g, h; arrows point to MZ). n= 4 control, 5 cond. Rb-/- (E15.5); 5 control, 5 cond. Rb-/- (E16.5) Bar= 100μm (a, b, e-h) 25 μm (c, d).

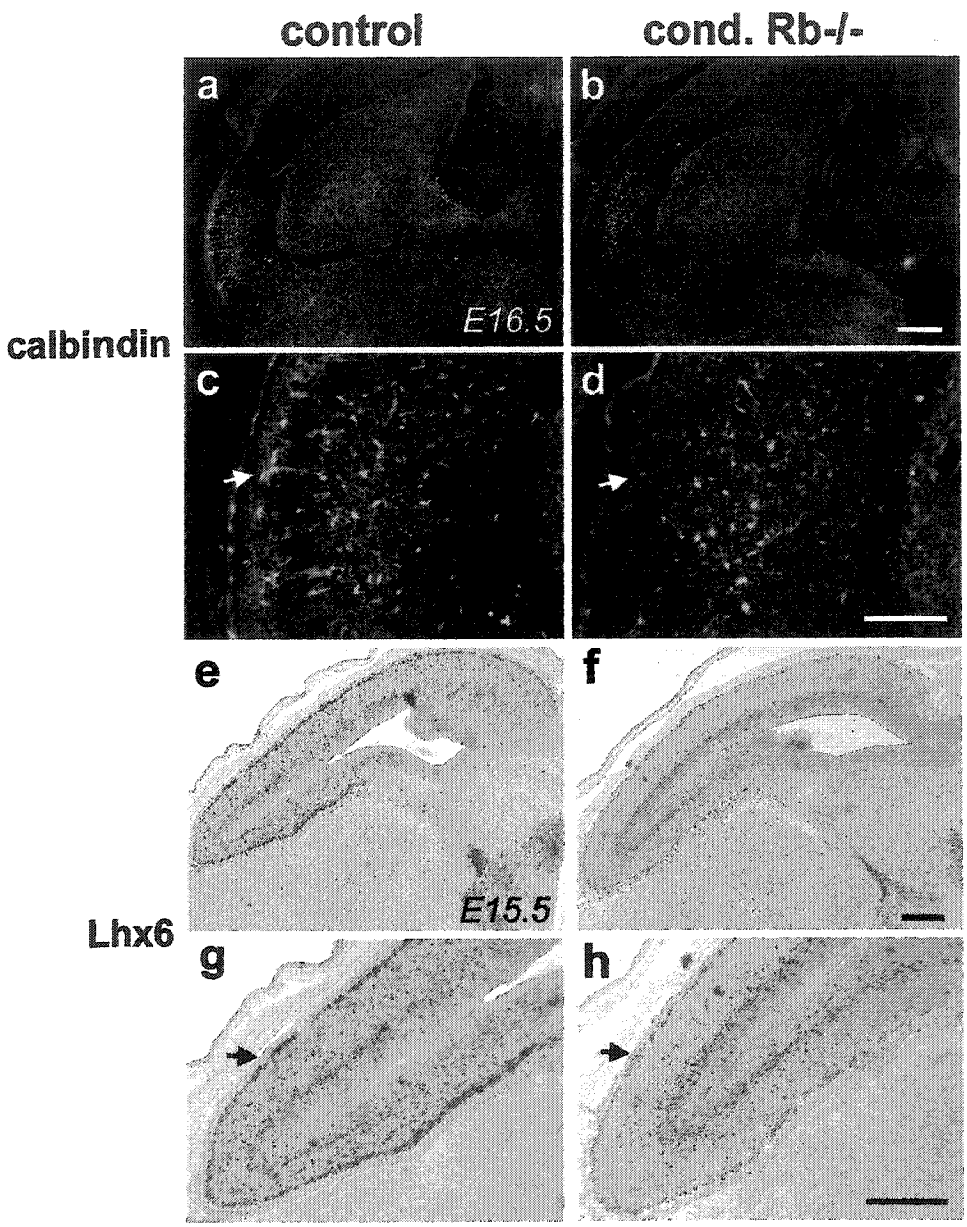
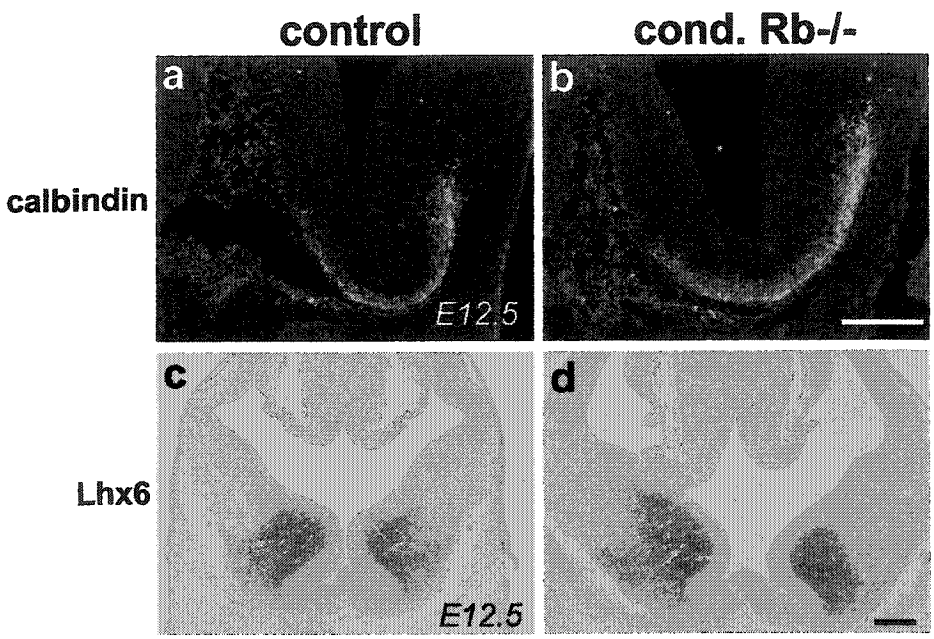

control
cond. Rb-/-
a
b
E16.5
calbindin
c
d
e
f
E15.5
Lhx6
g
h

Fig. 7: Rb deficiency does not impact interneuron specification or generation. To examine whether interneurons are properly generated in the absence of Rb, mutant and control E12.5 coronal sections were immunolabeled with a calbindin (D-28) antibody or subjected to RNA *in situ* hybridization with an Lhx6 probe. The generation of calbindin- (a, b) and Lhx6-positive (c, d) progenitors appeared similar in the mutant and control embryos. n=3 control, 3 cond. Rb-/-. Bar= 50 μm.

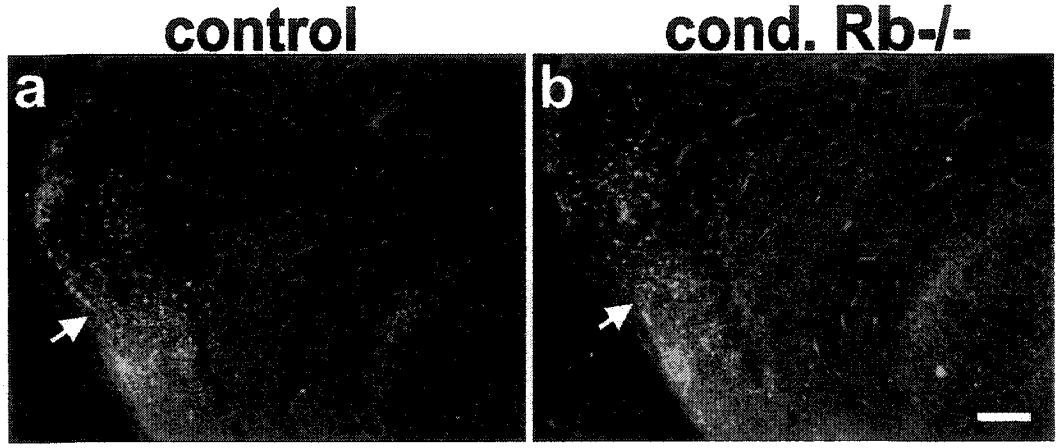

control
cond. Rb-/-
a
b
calbindin
E12.5
c
d
Lhx6
E12.5

MZ expression at mid-neurogenesis, the generation of these neuronal populations does not appear to be impaired by Rb deficiency.

Calbindin-positive interneurons may exhibit defective tangential migration

At E16.5, the expression of calbindin-positive interneurons is dramatically altered such that these cells are virtually absent from the Rb mutant cortical MZ (Fig. 5b, d). While it is possible that this selective neuronal loss is exclusively due to apoptosis, it is unusual that calbindin expression would remain intact in other cortical regions. An alternative explanation is that these interneurons failed to properly follow their MZ migratory route. In control embryos, tangentially migrating populations follow discrete pathways through the cortex, one along the MZ and another along the lower IZ/SVZ (Marin *et al.*, 2001). The origin of these pathways can be discerned in the control embryos, in which the migrating population can be seen to bifurcate at the corticostriatal boundary (arrow) (Fig. 8a). In the Rb mutant, the loss of MZ calbindin neurons appears to begin at the corticostriatal boundary (Fig. 8b). While a few interneurons are observed along the MZ path, the majority of these cells appear scattered across the CP, between the two distinct migratory routes. While it cannot be ruled out that the selective loss of calbindin-positive neurons in the Rb mutant MZ is exclusively due to apoptosis, there is also evidence that interneurons may fail to migrate tangentially along the MZ, resulting in the dramatic loss of these neurons from the cortical MZ.

Fig. 8: Calbindin-positive interneurons exhibit defective tangential migration. At E16.5, mutant and control coronal sections were immunolabeled with a calbindin (D-28) antibody. In contrast to the control in which calbindin-expressing neurons migrate tangentially along the MZ (a), this migratory route appears perturbed in the mutant (b). In contrast to the control embryos, in the Rb mutants, few calbindin-positive neurons are observed along the MZ, with several cells scattered across the CP, between the two distinct migratory routes. This expression pattern is consistent with reduced tangential migration of this subpopulation along the MZ pathway, whereas the IZ/SVZ migratory route remains intact (n=5 control, 5 cond. Rb-/-) Bar=50um.


control
cond. Rb-/-
a
b

DISCUSSION

In the present study, we have examined the requirement for Rb in cortical development and neuronal differentiation. We demonstrate defective laminar organization and region-specific cellularity at mid-gestation in the Rb mutants. In spite of the absence of widespread apoptosis in these conditional mutants, specific neuronal populations, including Reelin-positive Cajal-Retzius neurons, may require Rb for survival. We reveal a selective loss of calbindin- and Lhx6-positive interneurons from the Rb mutant MZ, indicating a role for Rb in guiding tangentially migrating interneurons. Our results demonstrate cell type-specific requirements for Rb, in the regulation of neuronal survival and tangential migration.

Proper timing of cell cycle and terminal mitosis is believed to be critical for the generation of specific neuronal cell types (McConnell and Kaznowski, 1991; McConnell, 1995). We have previously shown the prevalence of ectopically dividing neuroblasts throughout the IZ and CP of the Rb mutant (Ferguson *et al.*, 2002). However, in spite of the fact that Rb deficient mutants exhibit defective terminal mitosis, neuronal populations do not appear to be mis-specified. As demonstrated by immunochemistry with the early neuronal marker, βIII-tubulin, Rb deficiency does not seem to impact the timing of neuronal differentiation (Fig. 3). Therefore, it is probable that Rb deficient cells initially exit the cell cycle at the appropriate time. Even if they are subsequently induced to re-enter the cell cycle, these ectopically dividing cells would have already initiated differentiation and therefore, would be irreversibly committed to a specific neuronal fate. The increased cell number within the Rb mutant IZ and the loss of the discrete CP-IZ boundary is likely due to the accumulation of ectopically dividing cells within these regions. Ectopically dividing cells generally contain round nuclei, unlike the elongated, oval-shaped nuclei characteristic of

migrating cells. Therefore, as they migrate through the IZ, these cells likely pause to divide. Although the initiation of neuronal differentiation appears to be properly timed, these newly generated neurons may take longer to reach the CP, resulting in the enhanced expression of the late neuronal markers, Tbr1 and SCG10, within the mutant IZ (Fig. 2).

Although Rb deficiency specific to the telencephalon does not induce the widespread apoptosis observed in germline knockouts, certain neuronal populations may require Rb for survival. We previously reported an approximately 1.5-fold increase in TUNEL-positive cells within the mutant telencephalon (Ferguson *et al.*, 2002). We now demonstrate that the neuronal loss in the Rb mutants is selective, and is dramatically increased within the cortical MZ and the non-germinal regions of the ventral telencephalon (Fig. 4).

Although the generation of Reelin-positive CR neurons was normal, by E16.5, these cells were reduced by more than 50% in the Rb deficient MZ (Fig. 5). It could be argued that the CR neurons were not lost in the Rb mutant, but instead down-regulated the Reelin protein. However, since the decreased Reelin expression coincided with reduced overall MZ cell number in the mutant, the most obvious explanation would be that Rb is required for the survival of CR neurons. Further, the loss of CR neurons is consistent with the dramatically increased levels of apoptosis observed in the Rb mutant MZ. The requirement for Rb may depend on the neuronal cell type. In cerebellar development, the regulation of cell cycle exit, differentiation, and survival of granule cell precursors was found to be Rb-dependent. In contrast, neither Rb nor the closely-related family member, p107, was required for the differentiation and survival of Purkinje neurons (Marino *et al.*, 2003). The differential requirement for Rb may depend on the expression patterns in diverse cell types and on the degree of compensation by Rb family members, especially p107.

Although the generation of calbindin- and Lhx6-positive neurons was not impaired, by mid-neurogenesis, expression of these interneurons was substantially reduced along the MZ (Fig. 6). While certain ventrally-derived populations appear to undergo apoptosis in the absence of Rb, it is unlikely that this selective neuronal loss of calbindin- and Lhx6-positive interneurons would be exclusively due to apoptosis. If this were the case, it would be expected that the interneurons would be lost throughout the telencephalon, instead of specific reduction within the cortical MZ. In the case of Lhx6, reduced expression was also observed within the CP, suggesting that fewer neurons were reaching the cortex. In the Rb mutant, the loss of MZ calbindin neurons appeared to begin at the corticostriatal boundary. While a few calbindin-positive cells were observed along the MZ route, the majority of these neurons appeared scattered across the CP, between the two distinct migratory pathways. This aberrant migratory pattern suggests that Rb-deficient interneurons may fail to follow the MZ trajectory, resulting in the dramatic loss of these neurons from the cortical MZ.

While the guidance cues regulating tangential migration are not well known, certain molecules have been identified that may selectively target specific interneuron populations. The repulsive activity of class 3 semaphorins (Sema3A and Sema3F) is mediated by the neuropilins (Npn), such that disruption of Npn-1 or Npn-2 disrupts the number of interneurons migrating to the striatum and cortex (Marin *et al.*, 2001). Half of the GE-derived interneurons have been found to be regulated by Sema3A, and the other half by Sema3F, moreover, interneurons which are guided by Sema3A migrate along a slightly different route than neurons regulated by Sema3F (Tamamaki *et al.*, 2003). In addition, signalling by stromal cell-derived factor-1 (SDF-1) and its receptor, the CXC chemokine receptor 4 (CXCR4), may act selectively on distinct interneuron populations, since mice with

mutations in either SDF-1 or CXCR4 had far fewer interneurons present in superficial cortical layers, with accordingly increased interneurons in deep cortical layers (Stumm *et al.*, 2003). The differential regulation of diverse interneuron populations may explain the selective neuronal loss from the Rb-deficient MZ.

While the dramatic reduction in interneurons from the Rb mutant MZ could result from a failure to migrate along the MZ route, an alternative explanation is that these cells may undergo apoptosis coincident with, or as a result of, defective migration. The absence of appropriate substrate or migratory cues may be sufficient to trigger cell death. In addition to its well known role in neuronal survival (Atwal *et al.*, 2000; Stucky *et al.*, 2002), the neurotrophin receptor, TrkB, has also been demonstrated to regulate radial and tangential neuronal migration (Behar *et al.*, 1997; Brunstrom *et al.*, 1997; Polleux *et al.*, 2002). Since it has been previously shown that Rb germline knockouts have reduced TrkB expression (Lee *et al.*, 1994), impaired neurotrophin signalling may underlie both the apoptotic and migratory defects.

In conclusion, we demonstrate a cell type-specific requirement for Rb in the regulation of cortical development. While the majority of cortical neurons survive in the absence of Rb, specific populations, including Reelin-positive Cajal-Retzius neurons, may require Rb for survival. Further, the selective reduction of calbindin- and Lhx6-positive interneurons from the cortical MZ indicates an involvement for Rb in the regulation of tangentially migrating interneurons.

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REFERENCES

Anderson, S., M. Mione, K. Yun and J. L. Rubenstein (1999). Differential origins of neocortical projection and local circuit neurons: role of Dlx genes in neocortical interneuronogenesis. *Cereb Cortex* **9:** 646-54.

Anderson, S. A., D. D. Eisenstat, L. Shi and J. L. Rubenstein (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* **278:** 474-6.

Anderson, S. A., C. E. Kaznowski, C. Horn, J. L. Rubenstein and S. K. McConnell (2002). Distinct origins of neocortical projection neurons and interneurons in vivo. *Cereb Cortex* **12:** 702-9.

Anderson, S. A., O. Marin, C. Horn, K. Jennings and J. L. Rubenstein (2001). Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* **128:** 353-63.

Atwal, J. K., B. Massie, F. D. Miller and D. R. Kaplan (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron* **27:** 265-77.

Behar, T. N., M. M. Dugich-Djordjevic, Y. X. Li, W. Ma, R. Somogyi, X. Wen, E. Brown, C. Scott, R. D. McKay and J. L. Barker (1997). Neurotrophins stimulate chemotaxis of embryonic cortical neurons. *Eur J Neurosci* **9:** 2561-70.

Brunstrom, J. E., M. R. Gray-Swain, P. A. Osborne and A. L. Pearlman (1997). Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. *Neuron* **18:** 505-17.

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

Cam, H. and B. D. Dynlacht (2003). Emerging roles for E2F: Beyond the G1/S transition and DNA replication. *Cancer Cell* **3:** 311-6.

Caviness, V. S., Jr. (1982). Early events of neocortical assembly: experimental studies and human pathology. *Int J Neurol* **16-17:** 102-9.

Clarke, A. R., E. R. Maandag, M. van Roon, N. M. van der Lugt, M. van der Valk, M. L. Hooper, A. Berns and H. te Riele (1992). Requirement for a functional Rb-1 gene in murine development. *Nature* **359:** 328-30.

Classon, M. and E. Harlow (2002). The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* **2:** 910-7.

Corbin, J. G., S. Nery and G. Fishell (2001). Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. *Nat Neurosci* **4 Suppl:** 1177-82.

D'Arcangelo, G., G. G. Miao, S. C. Chen, H. D. Soares, J. I. Morgan and T. Curran (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* **374:** 719-23.

DeDiego, I., A. Smith-Fernandez and A. Fairen (1994). Cortical cells that migrate beyond area boundaries: characterization of an early neuronal population in the lower intermediate zone of prenatal rats. *Eur J Neurosci* **6:** 983-97.

Del Rio, J. A., E. Soriano and I. Ferrer (1992). Development of GABA-immunoreactivity in the neocortex of the mouse. *J Comp Neurol* **326:** 501-26.

Denaxa, M., C. H. Chan, M. Schachner, J. G. Parnavelas and D. Karagogeos (2001). The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. *Development* **128:** 4635-44.

Ferguson, K. L., J. L. Vanderluit, J. M. Hebert, W. C. McIntosh, E. Tibbo, J. G. MacLaurin, D. S. Park, V. A. Wallace, M. Vooijs, S. K. McConnell and R. S. Slack (2002). Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. *Embo J* **21:** 3337-46.

Frotscher, M. (1998). Cajal-Retzius cells, Reelin, and the formation of layers. *Curr Opin Neurobiol* **8:** 570-5.

Gavet, O., S. El Messari, S. Ozon and A. Sobel (2002). Regulation and subcellular localization of the microtubule-destabilizing stathmin family phosphoproteins in cortical neurons. *J Neurosci Res* **68:** 535-50.

Grigoriou, M., A. S. Tucker, P. T. Sharpe and V. Pachnis (1998). Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development* **125:** 2063-74.

Hebert, J. M. and S. K. McConnell (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.

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

Hevner, R. F., L. Shi, N. Justice, Y. Hsueh, M. Sheng, S. Smiga, A. Bulfone, A. M. Goffinet, A. T. Campagnoni and J. L. Rubenstein (2001). Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* **29:** 353-66.

Hirotsune, S., T. Takahara, N. Sasaki, K. Hirose, A. Yoshiki, T. Ohashi, M. Kusakabe, Y. Murakami, M. Muramatsu, S. Watanabe and et al. (1995). The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. *Nat Genet* **10:** 77-83.

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

Jimenez, D., L. Lopez-Mascaraque, J. A. de Carlos and F. Valverde (2002a). Further studies on cortical tangential migration in wild type and Pax-6 mutant mice. *J Neurocytol* **31:** 719-28.

Jimenez, D., L. M. Lopez-Mascaraque, F. Valverde and J. A. De Carlos (2002b). Tangential migration in neocortical development. *Dev Biol* **244:** 155-69.

Lambert de Rouvroit, C. and A. M. Goffinet (2001). Neuronal migration. *Mech Dev* **105:** 47-56.

Lavdas, A. A., M. Grigoriou, V. Pachnis and J. G. Parnavelas (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J Neurosci* **19:** 7881-8.

Lee, E., N. Hu, S. S. F. Yuan, L. A. Cox, A. Bradley, W. Lee and K. Herrup (1994). Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes & Dev.* **8:** 2008-2021.

Lee, E. Y., C. Y. Chang, N. Hu, Y. C. Wang, C. C. Lai, K. Herrup, W. H. Lee and A. Bradley (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359:** 288-94.

Lipinski, M. M., K. F. Macleod, B. O. Williams, T. L. Mullaney, D. Crowley and T. Jacks (2001). Cell-autonomous and non-cell-autonomous functions of the Rb tumor suppressor in developing central nervous system. *Embo J* **20:** 3402-13.

MacPherson, D., J. Sage, D. Crowley, A. Trumpp, R. T. Bronson and T. Jacks (2003). Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. *Mol Cell Biol* **23:** 1044-53.

Marin, O. and J. L. Rubenstein (2001). A long, remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosci* **2:** 780-90.

Marin, O., A. Yaron, A. Bagri, M. Tessier-Lavigne and J. L. Rubenstein (2001). Sorting of striatal and cortical interneurons regulated by semaphorin-neuropilin interactions. *Science* **293:** 872-5.

Marino, S., D. Hoogervoorst, S. Brandner and A. Berns (2003). Rb and p107 are required for normal cerebellar development and granule cell survival but not for Purkinje cell persistence. *Development* **130:** 3359-68.

Marino, S., M. Vooijs, H. van Der Gulden, J. Jonkers and A. Berns (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev* **14:** 994-1004.

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

McConnell, S. K. and C. E. Kaznowski (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254:** 282-285.

Nadarajah, B. and J. G. Parnavelas (2002). Modes of neuronal migration in the developing cerebral cortex. *Nat Rev Neurosci* **3:** 423-32.

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

Parnavelas, J. G., P. Alifragis and B. Nadarajah (2002). The origin and migration of cortical neurons. *Prog Brain Res* **136:** 73-80.

Polleux, F., K. L. Whitford, P. A. Dijkhuizen, T. Vitalis and A. Ghosh (2002). Control of cortical interneuron migration by neurotrophins and PI3-kinase signaling. *Development* **129:** 3147-60.

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

Rakic, P. (1988). Specification of cerebral cortical areas. *Science* **241:** 170-6.

Ramón y Cajal, S. (1891). Sur la structure de l'écorce cerebral de quelques mammiferes. *La Cellule* **7:** 125-178.

Rubenstein, J. L., S. Anderson, L. Shi, E. Miyashita-Lin, A. Bulfone and R. Hevner (1999). Genetic control of cortical regionalization and connectivity. *Cereb Cortex* **9:** 524-32.

Sarnat, H. B. and L. Flores-Sarnat (2002). Cajal-Retzius and subplate neurons: their role in cortical development. *Eur J Paediatr Neurol* **6:** 91-7.

Sidman, R. L. and P. Rakic (1973). Neuronal migration, with special reference to developing human brain: a review. *Brain Res* **62:** 1-35.

Stein, R., N. Mori, K. Matthews, L. C. Lo and D. J. Anderson (1988). The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. *Neuron* **1**: 463-76.

Stevaux, O. and N. J. Dyson (2002). A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol* **14**: 684-91.

Stucky, C. L., J. B. Shin and G. R. Lewin (2002). Neurotrophin-4: a survival factor for adult sensory neurons. *Curr Biol* **12**: 1401-4.

Stuhmer, T., S. A. Anderson, M. Ekker and J. L. Rubenstein (2002). Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. *Development* **129**: 245-52.

Stumm, R. K., C. Zhou, T. Ara, F. Lazarini, M. Dubois-Dalcq, T. Nagasawa, V. Hollt and S. Schulz (2003). CXCR4 regulates interneuron migration in the developing neocortex. *J Neurosci* **23**: 5123-30.

Sussel, L., O. Marin, S. Kimura and J. L. Rubenstein (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**: 3359-70.

Takahashi, T., T. Goto, S. Miyama, R. S. Nowakowski and V. S. Caviness, 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**: 10357-71.

Takahashi, T., R. S. Nowakowski and V. S. Caviness, Jr. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J Neurosci* **15**: 6046-57.

Takahashi, T., R. S. Nowakowski and V. S. Caviness, Jr. (1996). The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. *J Neurosci* **16:** 6183-96.

Tamamaki, N., K. Fujimori, Y. Nojyo, T. Kaneko and R. Takauji (2003). Evidence that Sema3A and Sema3F regulate the migration of GABAergic neurons in the developing neocortex. *J Comp Neurol* **455:** 238-48.

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

Wallace, V. A. and M. C. Raff (1999). A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. *Development* **126:** 2901-9.

Wichterle, H., J. M. Garcia-Verdugo, D. G. Herrera and A. Alvarez-Buylla (1999). Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat Neurosci* **2:** 461-6.

Wichterle, H., D. H. Turnbull, S. Nery, G. Fishell and A. Alvarez-Buylla (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* **128:** 3759-71.

Wu, L., A. de Bruin, H. I. Saavedra, M. Starovic, A. Trimboli, Y. Yang, J. Opavska, P. Wilson, J. C. Thompson, M. C. Ostrowski, T. J. Rosol, L. A. Woollett, M. Weinstein, J. C. Cross, M. L. Robinson and G. Leone (2003). Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature* **421:** 942-7.

Chapter 5. Summary and Discussion

Implications of Reported Findings

Summary of findings

In Chapter 2, I demonstrate that the Rb pathway is a critical regulator of cell cycle progression in primary neural progenitor cells. In contrast to previous studies (van den Heuvel and Harlow, 1993), the dominant negative mutants of CDK4 and CDK6 were found to induce mitotic arrest in neural precursor cells, despite the binding and sequestration of the Cip/Kip CKIs, $p21^{Cip1}$ and $p27^{Kip1}$. The activity of these mutants was dependent on the presence of Rb, indicating that the activation of the Rb pathway is sufficient to cause cell cycle arrest in primary neural precursor cells.

In Chapter 3, I describe the generation and examination of a conditional knockout in which Rb is specifically deleted in the developing telencephalon. In spite of virtually complete Rb excision in the forebrain, these mutants survived to birth and exhibited minimal apoptosis. Precursors did not arrest prior to mitosis, but underwent complete ectopic cell divisions outside the normal proliferative regions. Co-labeling with the early neuronal marker, βIII-tubulin, revealed that ectopically cycling cells had initiated neuronal differentiation. At E16.5, mutant telencephalic lobes were significantly enlarged, and in some cases, mutants developed cortical lobe protrusions. The enhanced neurogenesis observed in these conditional mutants demonstrated that Rb deficiency in the telencephalon was compatible with neuronal survival and differentiation.

In Chapter 4, I examined the impact of Rb deficiency on cortical development and neuronal differentiation. It was revealed that Rb mutants exhibit defective laminar organization and region-specific cellularity at mid-gestation. While the majority of cortical neurons survive in the absence of Rb, specific populations, including Reelin-positive Cajal-

Retzius neurons, were shown to require Rb for survival. The selective reduction of calbindin- and Lhx6-positive interneurons from the cortical MZ indicated an involvement for Rb in the regulation of tangentially migrating interneurons. Together, these studies demonstrated cell type-specific requirements for Rb, such that in its deficiency, there was a selective neuronal loss within the mutant MZ, resulting from apoptosis and defective tangential migration.

Rb is required for neural precursor terminal differentiation

Although the function of Rb had been extensively studied in tumour cell lines, its importance in the regulation of neural precursor proliferation and cell cycle exit was not known. Previous studies had shown that dominant negative mutants of CDK2, but not CDK4/6, resulted in cell cycle arrest in several cell lines (van den Heuvel and Harlow, 1993). These results were consistent with the interpretation that Rb phosphorylation by CDK2 was required for G1/S phase transition, while CDK4/6 may have a more facilitative role. While the Cip/Kip CKIs, $p21^{Cip1}$ and $p27^{Kip1}$, inhibit CDK2-dependent kinase activity, they have been found to act as positive regulators of CDK4/6 activity (LaBaer *et al.*, 1997; Cheng *et al.*, 1999). It has, therefore, been proposed that CDKs 4/6 may serve dual functions (Sherr and Roberts, 1995; Sherr and Roberts, 1999). The most studied role of CDK4/6 is the regulation of Rb phosphorylation early in G1, reviewed in (Sherr and Roberts, 1999). A second suggested function for cyclin D-CDK4/6 complexes is the sequestration of CKIs $p21^{Cip1}$ and $p27^{Kip1}$. In this way, CDK4/6 would act to "titrate" unbound Cip/Kip CKIs away from cyclin E-CDK2 complexes, thereby preventing the inhibition of CDK2 activity (Sherr and Roberts, 1995; Sherr and Roberts, 1999). It was believed that this latter function may be

crucial in the regulation of the G1/S transition (LaBaer *et al.*, 1997; Cheng *et al.*, 1999; Sherr and Roberts, 1999).

In contrast to the studies using tumour cells (van den Heuvel and Harlow, 1993), we demonstrated that the dominant negative mutants of CDK4 and CDK6, in addition to CDK2, were sufficient to induce mitotic arrest in neural precursor cells. These dominant inhibitory mutants contained a point mutation in the kinase region, thereby rendering them inactive (van den Heuvel and Harlow, 1993). However, since they retained the ability to bind cyclins, the function of endogenous CDKs was inactivated by sequestration of their required cyclins. It could be argued that the ability of the dominant negative CDK4/6 mutants to mediate growth arrest was due to the fact that they were unable to efficiently bind $p21^{Cip1}$ and $p27^{Kip1}$, which would effectively eliminate CDK2 relief from CKI constraint. Thus, the observed growth arrest by CDK4/6 complexes could actually result from the indirect inhibition of CDK2 activity. We addressed this question in our studies, by performing immunoprecipitations using the CDK mutants, to examine whether the ability to sequester these CKIs was retained. Our results demonstrated that both $p27^{Kip1}$ and $p21^{Cip1}$ were highly associated with the dominant negative CDK4/6 complexes, indicating that the CDK4/6 mutants retained the ability to bind the Cip/Kip CKIs and could arrest cell growth despite this continued association. These studies demonstrated an important role for CDK4/6 activity, in addition to CDK2, in the regulation of G1-S phase progression in neural precursor cells.

Compensation of Rb deficiency by p107

Rb is known to be a critical regulator of G1-S phase progression and its deficiency results in defective cell cycle kinetics and decreased cell cycle length, in many cell types examined. However, in cortical progenitors, Rb loss does not appear to greatly impact cell cycle progression. While dividing precursor cells appear normal, once these cells undergo terminal mitosis, defects associated with Rb deficiency become apparent. This could be due to compensation by the Rb family member, p107. P107 is expressed at high levels in proliferating neural stem cells and progenitors and has been shown previously to be up-regulated as a result of Rb deficiency (Callahan *et al.*, 1999). However, in the nervous system, Rb is expressed in cycling and differentiated cells, whereas p107 is present only in proliferating cells, becoming rapidly down-regulated upon differentiation (Bernards *et al.*, 1989; Jiang *et al.*, 1997; Callaghan *et al.*, 1999). Therefore, p107 can presumably undertake many of the functions normally carried out by Rb in proliferating cells, but not following terminal differentiation.

Cell-autonomous and non cell-autonomous functions of Rb

Germline Rb deficiency results in mid-gestational lethality (E12-15) and defective erythroid, lens, skeletal muscle and neuronal development (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Throughout the nervous system, abundant ectopic mitoses and apoptoses were observed (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1994). Although proliferating precursors in the ventricular zone appeared normal, abnormal

cell division and apoptosis was observed outside the germinal regions, within the developing intermediate zone and cortical plate (Lee *et al.*, 1994). Several neuronal markers, including βII tubulin, and the neurotrophin receptors TrkA, TrkB, and p75, were significantly decreased, particularly in dorsal root ganglia (Lee *et al.*, 1994).

Rb chimeric mice have been generated which, in contrast to germline knockouts, survived and exhibited minimal apoptosis (Maandag *et al.*, 1994; Williams *et al.*, 1994b; Lipinski *et al.*, 2001). Rb-deficient cells in chimeras exhibited ectopic S phase entry in the developing nervous system, similar to germline knockouts (Lipinski *et al.*, 2001). However, further examination revealed that Rb-null cells from chimeric mice arrested in G2, prior to completion of the cell cycle. In contrast, cells from Rb germline knockouts completed ectopic mitoses, followed by apoptosis. It thus appeared that the absence of inappropriate division in chimeric mice may have accounted for their survival. Based on these results, it was proposed that neighboring wild-type cells could rescue chimeric Rb-deficient cells, possibly by providing survival factors (Lipinski *et al.*, 2001).

To determine the requirement for Rb in the developing cortex, in the absence of the pleiotropic defects associated with germline Rb deficiency, we generated a conditional knockout in which Rb was specifically deleted in the developing telencephalon (Chapter 3). In spite of virtually complete Rb excision in the forebrain, these mutants survived to birth and exhibited minimal apoptosis. Subsequent to our own study, a second group described a conditional Rb mutant in which Rb was deleted from all neural lineages (nestin-cre) (MacPherson *et al.*, 2003). These results were consistent with our own and showed that ectopic cell division in differentiating neuroblasts may not necessarily evoke a default apoptotic pathway due to conflicting signals, but that Rb-deficient neuroblasts can survive

and initiate differentiation. We had concluded that neuronal loss observed in the CNS of germline knockouts may have resulted from an extrinsic defect, perhaps due to extensive liver degeneration and failed erythropoiesis. It is well established that hypoxia is a potent inducer of apoptosis, particularly in neurons (Gwag *et al.*, 1995; Rosenblum, 1997; Banasiak and Haddad, 1998; Banasiak *et al.*, 2000).

Since then, however, views on the origin of lethality resulting from Rb deficiency have been updated. Rb deficient embryos reconstituted with functionally normal placentae, either through tetraploid aggregation or by conditional knockout strategies, survived to term and lacked many of the characteristic defects associated with Rb deficiency (Wu *et al.*, 2003). The liver defects, including increased numbers of nucleated red blood cells and hepatocellular hypoplasia, were almost fully reversed with wild-type placentae (Wu *et al.*, 2003). In the CNS, mutants exhibited similar proliferation to germline Rb knockouts, including ectopic division, but without elevated apoptosis (de Bruin *et al.*, 2003b; Wu *et al.*, 2003). Examination of Rb deficient placentae revealed excessive trophoblast cell proliferation and perturbed architecture of the placental labyrinth, which were suggested to be due to failed trophoblast differentiation. These defects resulted in decreased vascularization and placental transport (Wu *et al.*, 2003). Together, these studies demonstrated the cell autonomous requirement for Rb in cell cycle regulation, and provided strong evidence that Rb deficiency, and the associated cell cycle perturbations, do not necessitate apoptosis.

Rb requirement for neuronal survival

Although Rb deficiency specific to the telencephalon does not induce the widespread apoptosis observed in germline knockouts, certain neuronal populations may require Rb for survival. At E13.5, we reported an approximately 1.5-fold increase in TUNEL-positive cells within the mutant telencephalon. In control embryos, the large majority of TUNEL-positive cells were restricted to the dorsal and ventral germinal regions, with very few cells undergoing cell death outside these areas. In Rb mutants, TUNEL labeling within the germinal zones was similar, however, the mutant embryos additionally contained several cells undergoing apoptosis within the ventral telencephalon and the cortical MZ. The location of the TUNEL-positive cells in the mutant telencephalon often coincided with the routes traveled by tangentially migrating interneurons and with MZ populations.

Although at early time points (E11.5, 12.5), Reelin expression was normal, by E16.5, expression was reduced by more than 50% in the Rb deficient cortex. It could be argued that the CR neurons were not lost in the Rb mutant, but instead down-regulated the Reelin protein. However, since the decreased Reelin expression coincided with reduced overall MZ cell number in the mutant, the most obvious explanation would be that Rb is required for the survival of CR neurons. The loss of CR neurons is also consistent with increased apoptotic levels in the Rb mutant MZ. The majority of CR neurons are transient, and undergo apoptosis following the completion of neurogenesis (Frotscher, 1998; Sarnat and Flores-Sarnat, 2002; Hevner *et al.*, 2003). Perhaps in the case of Rb deficiency, these neurons receive their self-destruction signals prematurely. It is not known what signal triggers their demise, but it is possible that ectopic proliferation and interrupted migration associated with

Rb deficiency could impact their survival. A similar loss of Reelin expression occurs in mice deficient for Emx2, in which the neurons appear to be properly generated but are subsequently lost (Mallamaci *et al.*, 2000). While telecephalon-specific Rb deficiency does not result in the large scale neuronal cell death observed in the germline knockout, Rb appears to be required for the survival of specific neuronal populations.

The requirement for Rb may depend on the neuronal cell type. In the developing cerebellum, Rb was found to be required for the regulation of cell cycle exit, differentiation, and survival of granule cell precursors, and p107 was unable to fully compensate for its deficiency. In contrast, the differentiation and survival of Purkinje neurons was independent of both Rb and p107 (Marino *et al.*, 2003). The differential requirement for Rb may depend on the expression patterns in diverse cell types and on the degree of compensation by Rb family members, especially p107.

It is generally believed that inappropriate cell cycle signals are sufficient to induce neuronal cell death. The Rb-null phenotype in which ectopic neuronal proliferation is followed by massive neuronal fallout, has firmly entrenched the belief that contradictory growth regulation in differentiating or post-mitotic neurons will trigger an apoptotic response. It has, therefore, been proposed that the deregulated cell cycle regulation associated with Rb deficiency induces apoptosis subsequent to inappropriate cell cycle entry. However, we have demonstrated that telencephalon-specific Rb mutants are capable of undergoing complete ectopic cell divisions without necessitating apoptosis. Therefore, it seems that the conflicting signals theory may be an oversimplification. While specific neuronal population may require Rb for survival, as a general rule, this is not the case.

Inappropriate cell cycle re-entry has been shown to be involved in post-mitotic neuronal apoptosis in certain neurological diseases, including Alzheimer's disease (Ogawa *et al.*, 2003a; Ogawa *et al.*, 2003b), Parkinson's disease (Shirvan *et al.*, 1997; Lee *et al.*, 2003) and amyotrophic lateral sclerosis (ALS) (Nguyen *et al.*, 2003; Ranganathan and Bowser, 2003). Further, abnormal cell cycle cues have been found to precede neuronal cell death in several death models. Cyclin D1 transcripts and CDK4/cdc2 protein levels were increased coincident with death of sympathetic neurons and neuronal PC12 cells following NGF withdrawal (Freeman *et al.*, 1994; Gao and Zelenka, 1995). Protein levels of cyclin D1, cyclin B, and CDK4 were elevated in brains of Alzheimer's disease and stroke patients (Li *et al.*, 1997; McShea *et al.*, 1997; Busser *et al.*, 1998). Stroke injury and DNA damaging agents induced phosphorylation of Rb and p107, followed by loss of Rb and p107 (Park *et al.*, 1998; Osuga *et al.*, 2000; Park *et al.*, 2000). The CDK inhibitor, flavopiridol, led to neuroprotection, as well as a suppression of the Rb and p107 phosphorylation and loss (Park *et al.*, 1998; Osuga *et al.*, 2000; Park *et al.*, 2000). These studies suggest that inappropriate proliferative signals in a post-mitotic neuron may be sufficient to induce apoptosis.

Why then do ectopically dividing neuroblasts in the Rb deficient cortex survive? One explanation is that there is a developmental time window, shortly following terminal mitosis, in which a newly born neuron can safely respond to both proliferation and differentiation signals. The cell cycle machinery has not yet fully shut down and the newly generated neuron has not yet completed differentiation. Likely, once a neuron has achieved its fully differentiated and quiescent state, these terminal modifications would be inconsistent with the promotion of cell division.

Ectopic proliferation- more than a cell cycle defect

Although cell cycle exit and the onset of neuronal differentiation are usually tightly coupled, these events may at times become dissociated. A characteristic phenotype of Rb deficiency is ectopic mitoses. Ectopic proliferation implies more than just the division of precursors in an abnormal location. We and others have shown that ectopically dividing cells express early neuronal markers (Lee *et al.*, 1994), implying that Rb deficient cells have the capacity to divide following the initiation of neuronal differentiation and migration. This suggests that Rb deficient cells fail to exit the cell cycle prior to the initiation of these processes. Alternatively, newly generated neurons may be induced to re-enter the cell cycle in the absence of Rb. While the first possibility cannot be ruled out, the latter explanation is more likely. It is known that neurons can be induced to re-enter the cell cycle in certain disease conditions (Shirvan *et al.*, 1997; Lee *et al.*, 2003; Nguyen *et al.*, 2003; Ogawa *et al.*, 2003a; Ogawa *et al.*, 2003b; Ranganathan and Bowser, 2003). In addition, terminally differentiated neurons in several brain regions were induced to re-enter the cell cycle in mice deficient for both the CKIs, $p19^{Ink4d}$ and $p27^{Kip1}$ (Zindy *et al.*, 1999). This explanation would also be consistent with the fact that ectopic cells are only rarely observed adjacent to the germinal regions. Instead, there is usually an almost complete absence of ectopic cells in the lower IZ, appearing as a band surrounding the germinal regions, with ectopic cells observed throughout the upper IZ and CP. This pattern would suggest that newly born Rb deficient neurons begin their migration out of the germinal zones, but soon after, are induced to cycle again. Regardless of the nature of the defect, ectopic division reveals a critical role for Rb in

the regulation of terminal mitosis, such that in its deficiency, differentiating neuroblasts are unable to completely exit the cell cycle.

At mid-neurogenesis (E16.5), Rb mutants exhibited abnormal cortical morphology and region-specific cellularity. We have shown that cortical lobes of Rb deficient mutants were approximately 30% larger than control littermates. This observation was consistent with a subsequent study in which Rb deficient brains were shown to be enlarged by 27% (MacPherson *et al.*, 2003). However, the overall cellularity in Rb deficient cortex was not increased. The mutants contained fewer precursor cells within the VZ and SVZ, and increased cells within the IZ. These differences in region-specific cellularity could account for the increased cortical size of the Rb mutants. Cells within the germinal zones are round and very tightly packed, in contrast to migrating cells within the IZ which are elongated and sparsely distributed.

The fact that the Rb mutants contain fewer precursor cells could suggest that neuronal differentiation occurs prematurely in the Rb mutant. However, the Rb mutant cortices did not contain more CP neurons. Further, immunolabeling with the early neuronal marker, βIII-tubulin, did not reveal precocious differentiation. Another possible explanation would be that Rb mutant progenitors migrate out of the germinal zones, but pause in the IZ before continuing to the CP. We have previously shown extensive ectopic division in Rb mutant cortex, which is most prevalent between E14-16. These ectopically dividing neuroblasts undergo complete ectopic cell divisions with the IZ and CP and co-label with the early neuronal marker, βIII-tubulin. It is likely that these ectopically dividing cells may be interrupted in their migration to undergo cell division. Ectopically dividing cells generally have round nuclei, unlike the elongated, oval-shaped nuclei characteristic of migrating cells.

Therefore, these cells likely pause in their migratory route to divide. Once they have completed division, they would then presumably, continue their migration up to the CP. This could account for the increased cell number within the mutant IZ. Further, mutant cortices exhibit elevated levels of the neuronal markers, Tbr1 and SCG10, within the IZ population. Since it has been previously established that ectopically dividing cells are able to initiate neuronal differentiation while still actively cycling, this aberrant laminar expression pattern could be explained by increased ectopic IZ cells. It is possible that these neurons have initiated expression of these neuronal markers at the appropriate time but, due to the ectopic division and pause within the IZ, they have not yet completed their migration to the CP.

Although Rb shares strong sequence homology and function with its family members, p107 and p130, ectopic division has not been reported in these mutants (Cobrinik *et al.*, 1996; Lee *et al.*, 1996; LeCouter *et al.*, 1998a; LeCouter *et al.*, 1998b). Knockouts of the upstream CKIs, which act to inhibit phosphorylation and inactivation of Rb by CDKs, could also be expected to produce this defect. Mutants of individual CKIs appear to retain coupled cell cycle exit and differentiation (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996; Serrano *et al.*, 1996; Yan *et al.*, 1997; Zhang *et al.*, 1997; Franklin *et al.*, 1998; Latres *et al.*, 2000; Zindy *et al.*, 2000), which is especially striking with $p27^{Kip1}$, since oligodendrocyte precursors derived from knockouts undergo an increased number of divisions (Durand *et al.*, 1998). However, examination of $p19^{Ink4d}/p27^{Kip1}$double knockout mice at post-natal day 18 showed that terminally differentiated neurons in several brain regions were induced to re-enter the cell cycle (Zindy *et al.*, 1999). Multiple CKI deletions may be required to maintain Rb in an inactive, hyperphosphorylated state, thus disabling the ability of Rb to maintain a state of growth

arrest in post-mitotic neurons. In *Drosophila*, mutants of ebi, an enhancer of an over-proliferation phenotype due to elevated E2F activity, exhibited ectopic mitoses and had a reduced capacity to undergo neuronal differentiation (Boulton *et al.*, 2000). The common thread in these instances of ectopic division is that normal Rb function is disrupted. Perhaps Rb activity is normally required to suppress neuronal migration and differentiation, while precursors are actively cycling. Rb may function as a link between cell cycle regulation and promotion of neuronal differentiation, processes which would then become uncoupled in the instance of Rb deficiency.

Apart from Rb deficiency, there are several known instances of cell cycle deregulation. Although cortical abnormalities often arise, these mutants all share a tight regulation of cell cycle shutdown with the onset of differentiation. Neural precursors in knockouts of the tumour suppressor, phosphatase and tensin homolog deleted on chromosome ten (Pten), showed increased proliferation (Groszer *et al.*, 2001). However, these mutants did not exhibit ectopic proliferation or the expression of precocious neuronal markers in cycling cells. Instead, increased division produced a greatly enlarged cortical ventricular zone (Groszer *et al.*, 2001). Neural precursor cells in conditional N-myc mutants underwent premature neuronal differentiation associated with reduced precursor proliferation (Knoepfler *et al.*, 2002). Similarly, transgenic mice lacking DNA-binding activity for BF-1 demonstrated accelerated neuronal differentiation with concomitant cell cycle withdrawal (Hanashima *et al.*, 2002). In spite of mutations affecting their timing, it appears that cell cycle exit and neuronal differentiation remain coupled, provided Rb activity is intact. This implicates a unique role for Rb in the coupling of cell cycle withdrawal and the onset of neuronal differentiation that is distinct from other cell cycle regulating molecules.

In order for a neural precursor to terminally exit the cell cycle and initiate neuronal differentiation, there must be some level of collaboration between cell cycle regulators and factors promoting neuronal gene induction. While the mechanism with which this occurs remains to be elucidated, it seems likely that there would exist some degree of cross-talk between these signaling pathways. Mouse P19 embryonal carcinoma cells were induced to differentiate to a neuronal phenotype by transient transfection with various proneural bHLH factors along with their putative dimerization partner E12 (Farah *et al.*, 2000). Differentiation was preceded by cell cycle withdrawal and increased expression of CKI $p27^{Kip1}$(Farah *et al.*, 2000). Further, suppression of Hes1 expression in human neural stem cells initiated GABAergic neuronal differentiation and stimulated expression of CKI $p21^{Cip1}$ (Kabos *et al.*, 2002). While it is not known whether CKI induction by bHLH factors is direct, at some level, there is likely communication between the HLH and Rb pathways.

Cell cycle regulation and neuronal fate

Proper timing of cell cycle and terminal mitosis is believed to be critical for the generation of specific neuronal cell types (McConnell and Kaznowski, 1991; McConnell, 1995). However, in spite of the fact that Rb deficient mutants exhibit defective terminal mitosis, neuronal populations do not appear to be mis-specified. It is probable that Rb deficient cells initially exit the cell cycle at the appropriate time. Even if they are subsequently induced to re-enter the cell cycle, neuronal identity would presumably be determined by the initial terminal mitosis. These ectopically dividing cells have already

initiated differentiation and therefore, would be irreversibly committed to a neuronal fate. Therefore, in spite of undergoing additional cell division and terminal mitosis, these neurons would not alter their fate. This explanation is consistent with the fact that Rb deficient mutants do not appear to be defective in terms of their initiation or extent of pan-neuronal differentiation.

Rb regulation of cortical migration

At mid-neurogenesis, calbindin- and Lhx6-positive interneurons were substantially reduced along the Rb mutant MZ. Calbindin expression was decreased to such a degree as to be nearly absent from the cortical MZ, although other calbindin-positive populations appeared intact. Similarly, in spite of reduced MZ expression, Lhx6 labeling along the IZ/SVZ was normal. Since it was established that Rb deficiency does not result in the generation of fewer calbindin- or Lhx6-positive interneurons at E12.5, Rb may be required for the survival of these interneurons, and/or Rb may be involved in guiding or promoting the migration of these ventrally-derived neurons along their tangential routes. It seems that both possibilities may be involved in the loss of these interneuron populations from the cortical MZ.

While the level of apoptosis in mutant and control embryos was similar within the germinal regions, Rb mutants exhibited significantly increased TUNEL labeling within the ventral telencephalon and cortical MZ. However, although certain ventrally-derived populations appear to undergo apoptosis in the absence of Rb, cell death is not likely to explain the observed selective neuronal loss among the MZ population. In the Rb mutant,

calbindin-positive neurons were observed to be lost around the juncture of the corticostriatal boundary. Although a few neurons remained along the MZ, it seemed that many calbindin-expressing cells were scattered across the CP, between the two distinct migratory routes. It appeared that in the absence of Rb, calbindin-positive interneurons may have failed to migrate tangentially along the MZ, resulting in the dramatic loss of these neurons from the cortical MZ. Alternatively, the specific loss of MZ interneurons may have occurred due to selective apoptosis within this subpopulation. Perhaps these neurons underwent cell death as a consequence of, or for reasons related to, the defective migration. For example, the neurotrophin receptor, TrkB, has been demonstrated to regulate both neuronal survival (Atwal *et al.*, 2000; Stucky *et al.*, 2002), and radial and tangential neuronal migration (Behar *et al.*, 1997; Brunstrom *et al.*, 1997; Polleux *et al.*, 2002). Rb germline knockouts have previously been shown to contain reduced TrkB expression levels (Lee *et al.*, 1994), therefore, both the apoptotic and migratory defects in Rb conditional mutants may be the result of TrkB deficiency.

Although the MZ includes a heterogeneous neuronal population, the Reelin-positive Cajal-Retzius neurons are the most characterized. The CR neurons are critical for regulating radial migration, but there is also evidence of a role in guiding tangentially-derived neurons (Lavdas *et al.*, 1999; Morante-Oria *et al.*, 2003). At mid-neurogenesis, Reelin expression is dramatically reduced in the Rb mutant. It is not clear whether the loss of Reelin may be responsible for the observed defects in tangential migration. Further, reduced Reelin expression may be the cause of the disorganized cortical structure. While there still may be sufficient Reelin expression to ensure the formation of gross laminar structure, perhaps the reduced levels may impact the "fine-tuning" of cortical organization. For example, although

the layers are all present and are arranged in the appropriate sequence, in the Rb mutants, the boundaries between the IZ and CP are not well defined with extensive cell overlap between these layers.

While the evidence for defective tangential migration in the Rb mutants is intriguing, it warrants further investigation. The use of cortical slice cultures would be a useful means to further examine migration in these mutants. Thick sections of embryonic forebrain can be grown in culture and maintained for up to one week *in vitro* (Polleux *et al.*, 2002). The advantage of cortical slice cultures is that they provide an accurate representation of *in vivo* physiological processes, while retaining the ability to be modulated by various *in vitro* techniques. Using the slice culture system, rescue experiments could be conducted in which Rb-tagged with GFP would be introduced into the Rb-deficient slices. By examining the localization and migratory paths of GFP-positive cells, it could be determined whether Rb deficiency is directly responsible for defective migration.

In conclusion, my studies have focussed on the requirement for the Rb protein in cortical neurogenesis. I began my research by characterizing the key cell cycle regulators involved in regulating neural precursor proliferation and differentiation, and by determining a requirement for Rb signalling in the regulation of these processes. Next, to identify the requirement for Rb in the developing cortex, I examined telencephalon-specific Rb conditional mutants. These mice survived to birth and did not exhibit the widespread neuronal apoptosis characteristic of germline deficiency. Further, due to increased proliferation of neuroblasts, the Rb mutants exhibited significantly enlarged cortices, and in some cases, cortical protrusions. These studies demonstrated that apoptosis is not necessarily a default mechanism of cell cycle deregulation during differentiation and that terminal

mitosis may not be required to initiate differentiation. In my final study, I examined the role of Rb more precisely in the regulation of cortical morphology and neuronal differentiation. Due to the selective loss of specific neuronal populations from the cortical MZ, it was concluded that Rb may have cell-type specific requirements in neuronal survival and interneuron migration. Together, my studies demonstrated critical functions for Rb in the developing cortex, from the regulation of precursor differentiation to the development of cortical architecture, neuronal migration and its requirement in diverse neuronal populations.

References Cited

Adams, M. R., R. Sears, F. Nuckolls, G. Leone and J. R. Nevins (2000). Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. *Mol Cell Biol* **20:** 3633-9.

Adams, P. D. (2001). Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. *Biochim Biophys Acta* **1471:** M123-33.

Adams, P. D., X. Li, W. R. Sellers, K. B. Baker, X. Leng, J. W. Harper, Y. Taya and W. G. Kaelin, Jr. (1999). Retinoblastoma protein contains a C-terminal motif that targets it for phosphorylation by cyclin-cdk complexes. *Mol Cell Biol* **19:** 1068-80.

Adams, P. D., W. R. Sellers, S. K. Sharma, A. D. Wu, C. M. Nalin and W. G. Kaelin, Jr. (1996). Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Mol Cell Biol* **16:** 6623-33.

Allen, K. E., S. de la Luna, R. M. Kerkhoven, R. Bernards and N. B. La Thangue (1997). Distinct mechanisms of nuclear accumulation regulate the functional consequence of E2F transcription factors. *J Cell Sci* **110 (Pt 22):** 2819-31.

Alt, J. R., A. B. Gladden and J. A. Diehl (2002). p21(Cip1) Promotes cyclin D1 nuclear accumulation via direct inhibition of nuclear export. *J Biol Chem* **277:** 8517-23.

Anderson, S., M. Mione, K. Yun and J. L. Rubenstein (1999). Differential origins of neocortical projection and local circuit neurons: role of Dlx genes in neocortical interneuronogenesis. *Cereb Cortex* **9:** 646-54.

Anderson, S. A., D. D. Eisenstat, L. Shi and J. L. Rubenstein (1997). Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science* **278:** 474-6.

Anderson, S. A., C. E. Kaznowski, C. Horn, J. L. Rubenstein and S. K. McConnell (2002). Distinct origins of neocortical projection neurons and interneurons in vivo. *Cereb Cortex* **12:** 702-9.

Anderson, S. A., O. Marin, C. Horn, K. Jennings and J. L. Rubenstein (2001). Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development* **128:** 353-63.

Arsenijevic, Y. (2003). Mammalian neural stem-cell renewal: nature versus nurture. *Mol Neurobiol* **27:** 73-98.

Asano, M., J. R. Nevins and R. P. Wharton (1996). Ectopic E2F expression induces S phase and apoptosis in Drosophila imaginal discs. *Genes Dev* **10:** 1422-32.

Atwal, J. K., B. Massie, F. D. Miller and D. R. Kaplan (2000). The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. *Neuron* **27:** 265-77.

Azuma-Hara, M., H. Taniura, T. Uetsuki, M. Niinobe and K. Yoshikawa (1999). Regulation and deregulation of E2F1 in postmitotic neurons differentiated from embryonal carcinoma P19 cells. *Exp. Cell Res.* **251:** 442-451.

Bagchi, S., R. Weinmann and P. Raychaudhuri (1991). The retinoblastoma protein copurifies with E2F-I, an E1A-regulated inhibitor of the transcription factor E2F. *Cell* **65:** 1063-72.

Banasiak, K. J. and G. G. Haddad (1998). Hypoxia-induced apoptosis: effect of hypoxic severity and role of p53 in neuronal cell death. *Brain Res* **797:** 295-304.

Banasiak, K. J., Y. Xia and G. G. Haddad (2000). Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol* **62:** 215-49.

Bandara, L. R. and N. B. La Thangue (1991). Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature* **351:** 494-497.

Behar, T. N., M. M. Dugich-Djordjevic, Y. X. Li, W. Ma, R. Somogyi, X. Wen, E. Brown, C. Scott, R. D. McKay and J. L. Barker (1997). Neurotrophins stimulate chemotaxis of embryonic cortical neurons. *Eur J Neurosci* **9:** 2561-70.

Beijersbergen, R. L., R. M. Kerkhoven, L. Zhu, L. Carlee, P. M. Voorhoeve and R. Bernards (1994). E2F-4, a new member of the E2F gene family has oncogenic activity and associates with p107 in vivo. *Genes Dev.* **8:** 2680-2690.

Benezra, R., R. L. Davis, D. Lockshon, D. L. Turner and H. Weintraub (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* **61:** 49-59.

Bernards, R., G. M. Schackleford, M. R. Gerber, J. M. Horowitz, S. H. Friend, M. Schartl, E. Bogenmann, J. M. Rapaport, T. McGee, T. P. Dryja and et al. (1989). Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein. *Proc Natl Acad Sci U S A* **86:** 6474-8.

Blain, S. W., E. Montalvo and J. Massague (1997). Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 with cyclin A-Cdk2 and cyclin D2-Cdk4. *J Biol Chem* **272:** 25863-72.

Bookstein, R., E. Y. Lee, H. To, L. J. Young, T. W. Sery, R. C. Hayes, T. Friedmann and W. H. Lee (1988). Human retinoblastoma susceptibility gene: genomic organization and analysis of heterozygous intragenic deletion mutants. *Proc Natl Acad Sci U S A* **85:** 2210-4.

Bookstein, R., P. Rio, S. A. Madreperla, F. Hong, C. Allred, W. E. Grizzle and W. H. Lee (1990). Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma. *Proc Natl Acad Sci U S A* **87:** 7762-6.

Botz, J., K. Zerfass-Thome, D. Spitkovsky, H. Delius, B. Vogt, M. Eilers, A. Hatzigeorgiou and P. Jansen-Durr (1996). Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol Cell Biol* **16:** 3401-9.

Boulton, S. J., A. Brook, K. Staehling-Hampton, P. Heitzler and N. Dyson (2000). A role for Ebi in neuronal cell cycle control. *Embo J* **19:** 5376-86.

Brehm, A., E. A. Miska, D. J. McCance, J. L. Reid, A. J. Bannister and T. Kouzarides (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391:** 597-601.

Bremner, R., B. L. Cohen, M. Sopta, P. A. Hamel, C. J. Ingles, B. L. Gallie and R. A. Phillips (1995). Direct transcriptional repression by pRB and its reversal by specific cyclins. *Mol Cell Biol* **15:** 3256-65.

Briata, P., E. Di Blas, M. Gulisano, A. Mallamaci, R. Iannone, E. Boncinelli and G. Corte (1996). EMX1 homeoprotein is expressed in cell nuclei of the developing cerebral cortex and in the axons of the olfactory sensory neurons. *Mech Dev* **57:** 169-80.

Brose, K. and M. Tessier-Lavigne (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. *Curr Opin Neurobiol* **10:** 95-102.

Brown, V. D., R. A. Phillips and B. L. Gallie (1999). Cumulative effect of phosphorylation of pRB on regulation of E2F activity. *Mol Cell Biol* **19:** 3246-56.

Bruce, J. L., R. K. Hurford, Jr., M. Classon, J. Koh and N. Dyson (2000). Requirements for cell cycle arrest by p16INK4a. *Mol Cell* **6:** 737-42.

Brugarolas, J., C. Chandrasekaran, J. I. Gordon, D. Beach, T. Jacks and G. J. Hannon (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377:** 552-7.

Brunstrom, J. E., M. R. Gray-Swain, P. A. Osborne and A. L. Pearlman (1997). Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. *Neuron* **18:** 505-17.

Buchkovich, K., L. A. Duffy and E. Harlow (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58:** 1097-1105.

Buck, V., K. E. Allen, T. Sorensen, A. Bybee, E. M. Hijmans, P. M. Voorhoeve, R. Bernards and N. B. La Thangue (1995). Molecular and functional characterisation of E2F-5, a new member of the E2F family. *Oncogene* **11:** 31-8.

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

Busser, J., D. S. Geldmacher and K. Herrup (1998). Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. *J Neurosci* **18:** 2801-7.

Callaghan, D. A., L. Dong, S. M. Callaghan, Y. X. Hou, L. Dagnino and R. S. Slack (1999). Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F1 and 3 activity. *Dev.Biol.* **207:** 257-270.

Cam, H. and B. D. Dynlacht (2003). Emerging roles for E2F: Beyond the G1/S transition and DNA replication. *Cancer Cell* **3:** 311-6.

Cartwright, P., H. Muller, C. Wagener, K. Holm and K. Helin (1998). E2F-6: A novel member of the E2F family is an inhibitor of E2F-dependent transcription. *Oncogene* **16:** x.

Caviness, V. S., Jr. (1982a). Development of neocortical afferent systems: studies in the reeler mouse. *Neurosci Res Program Bull* **20:** 560-9.

Caviness, V. S., Jr. (1982b). Early events of neocortical assembly: experimental studies and human pathology. *Int J Neurol* **16-17:** 102-9.

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

Chao, R., W. Khan and Y. A. Hannun (1992). Retinoblastoma protein dephosphorylation induced by D-erythro-sphingosine. *J Biol Chem* **267:** 23459-62.

Chau, B. N. and J. Y. Wang (2003). Coordinated regulation of life and death by RB. *Nat Rev Cancer* **3:** 130-8.

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

Chen, P. L., D. J. Riley, Y. Chen and W. H. Lee (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev* **10:** 2794-804.

Chen, P. L., P. Scully, J. Y. Shew, J. Y. Wang and W. H. Lee (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* **58:** 1193-8.

Chen, T. T. and J. Y. Wang (2000). Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. *Mol Cell Biol* **20:** 5571-80.

Cheng, M., P. Olivier, J. A. Diehl, M. Fero, M. F. Roussel, J. M. Roberts and C. J. Sherr (1999). The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *Embo J* **18:** 1571-83.

Chittenden, T., D. M. Livingston and W. G. Kaelin, Jr. (1991). The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. *Cell* **65:** 1073-82.

Chow, K. N. and D. C. Dean (1996). Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. *Mol Cell Biol* **16:** 4862-8.

Clarke, A. R., E. R. Maandag, M. Vam Roon, N. M. T. Van der Lugt, M. Van der Valk, M. I. Hooper, A. Berns and H. Te Reile (1992). Requirement for a functional Rb-1 gene in murine development. *Nature* **359:** 328-330.

Classon, M. and E. Harlow (2002). The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* **2:** 910-7.

Classon, M., B. K. Kennedy, R. Mulloy and E. Harlow (2000a). Opposing roles of pRB and p107 in adipocyte differentiation. *Proc Natl Acad Sci U S A* **97:** 10826-31.

Classon, M., S. Salama, C. Gorka, R. Mulloy, P. Braun and E. Harlow (2000b). Combinatorial roles for pRB, p107, and p130 in E2F-mediated cell cycle control. *Proc Natl Acad Sci U S A* **97:** 10820-5.

Claudio, P. P., C. M. Howard, A. Baldi, A. De Luca, Y. Fu, G. Condorelli, Y. Sun, N. Colburn, B. Calabretta and A. Giordano (1994). p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. *Cancer Res* **54:** 5556-60.

Cobrinik, D., M. H. Lee, G. Hannon, G. Mulligan, R. T. Bronson, N. Dyson, E. Harlow, D. Beach, R. A. Weinberg and T. Jacks (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* **10:** 1633-44.

Cobrinik, D., P. Whyte, D. S. Peeper, T. Jacks and R. A. Weinberg (1993). Cell cycle - specific association of E2F with the p130 E1A - binding protein. *Genes Dev.* **7:** 2392-2404.

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

Coqueret, O. (2002). Linking cyclins to transcriptional control. *Gene* **299:** 35-55.

Corbin, J. G., S. Nery and G. Fishell (2001). Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. *Nat Neurosci* **4 Suppl:** 1177-82.

Dahiya, A., M. R. Gavin, R. X. Luo and D. C. Dean (2000). Role of the LXCXE binding site in Rb function. *Mol Cell Biol* **20:** 6799-805.

Dalton, S. (1992). Cell Cycle regulation of the human cdc 2 gene. *EMBO Journal* **11:** 1797-1807.

Dannenberg, J. H., A. van Rossum, L. Schuijff and H. te Riele (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev* **14:** 3051-64.

D'Arcangelo, G., R. Homayouni, L. Keshvara, D. S. Rice, M. Sheldon and T. Curran (1999). Reelin is a ligand for lipoprotein receptors. *Neuron* **24:** 471-9.

D'Arcangelo, G., G. G. Miao, S. C. Chen, H. D. Soares, J. I. Morgan and T. Curran (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* **374:** 719-23.

de Bruin, A., B. Maiti, L. Jakoi, C. Timmers, R. Buerki and G. Leone (2003a). Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. *J Biol Chem* **278:** 42041-9.

de Bruin, A., L. Wu, H. I. Saavedra, P. Wilson, Y. Yang, T. J. Rosol, M. Weinstein, M. L. Robinson and G. Leone (2003b). Rb function in extraembryonic lineages suppresses apoptosis in the CNS of Rb-deficient mice. *Proc Natl Acad Sci U S A* **100:** 6546-51.

de Carlos, J. A., L. Lopez-Mascaraque and F. Valverde (1996). Dynamics of cell migration from the lateral ganglionic eminence in the rat. *J Neurosci* **16:** 6146-56.

de la Luna, S., M. J. Burden, C. W. Lee and N. B. La Thangue (1996). Nuclear accumulation of the E2F heterodimer regulated by subunit composition and alternative splicing of a nuclear localization signal. *J Cell Sci* **109 (Pt 10):** 2443-52.

Deacon, T. W., P. Pakzaban and O. Isacson (1994). The lateral ganglionic eminence is the origin of cells committed to striatal phenotypes: neural transplantation and developmental evidence. *Brain Res* **668:** 211-9.

DeCaprio, J. A., J. W. Ludlow, J. Figge, J. Y. Shew, C. M. Huang, W. H. Lee, E. Marsilio, E. Paucha and D. M. Livingston (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54:** 275-83.

DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C. M. Huang and D. M. Livingston (1989). The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58:** 1085-95.

DeDiego, I., A. Smith-Fernandez and A. Fairen (1994). Cortical cells that migrate beyond area boundaries: characterization of an early neuronal population in the lower intermediate zone of prenatal rats. *Eur J Neurosci* **6:** 983-97.

deGregori, J., T. Kowalik and J. R. Nevins (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis and G1/S -regulatory genes. *Mol.Cell.Biol.* **15:** 4215-4224.

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

DeGregori, J., G. Leone, K. Ohtani, A. Miron and J. R. Nevins (1995). E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. *Genes Dev* **9:** 2873-87.

Del Rio, J. A., E. Soriano and I. Ferrer (1992). Development of GABA-immunoreactivity in the neocortex of the mouse. *J Comp Neurol* **326:** 501-26.

Denaxa, M., C. H. Chan, M. Schachner, J. G. Parnavelas and D. Karagogeos (2001). The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. *Development* **128:** 4635-44.

Deng, C., P. Zhang, J. W. Harper, S. J. Elledge and P. Leder (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82:** 675-84.

Di Stefano, L., M. R. Jensen and K. Helin (2003). E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes. *Embo J* **22:** 6289-98.

Dick, F. A. and N. Dyson (2003). pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. *Mol Cell* **12:** 639-49.

Dick, F. A., E. Sailhamer and N. J. Dyson (2000). Mutagenesis of the pRB pocket reveals that cell cycle arrest functions are separable from binding to viral oncoproteins. *Mol Cell Biol* **20:** 3715-27.

Dick, T. E., M. C. Bellingham and D. W. Richter (1994). Pontine respiratory neurons in anesthetized cats. *Brain Res.* **636:** 259-269.

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

D'Souza, S. J., A. Pajak, K. Balazsi and L. Dagnino (2001). Ca2+ and BMP-6 signaling regulate E2F during epidermal keratinocyte differentiation. *J Biol Chem* **276:** 23531-8.

Dulabon, L., E. C. Olson, M. G. Taglienti, S. Eisenhuth, B. McGrath, C. A. Walsh, J. A. Kreidberg and E. S. Anton (2000). Reelin binds alpha3beta1 integrin and inhibits neuronal migration. *Neuron* **27:** 33-44.

Dulic, V., E. Lees and S. I. Reed (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* **257:** 1958-1961.

Dunaief, J. L., B. E. Strober, S. Guha, P. A. Khavari, K. Alin, J. Luban, M. Begemann, G. R. Crabtree and S. P. Goff (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell* **79:** 119-30.

Durand, B., M. L. Fero, J. M. Roberts and M. C. Raff (1998). p27Kip1 alters the response of cells to mitogen and is part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation. *Curr Biol* **8:** 431-40.

Dyer, M. A. and C. L. Cepko (2000). p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. *Development* **127:** 3593-605.

Dyson, N. (1998). The regulation of E2F by pRb-family proteins. *Genes Dev.* **12:** 2245-2262.

Dyson, N., R. Bernards, S. H. Friend, L. R. Gooding, J. A. Hassell, E. O. Major, J. M. Pipas, T. Vandyke and E. Harlow (1990). Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. *J Virol* **64:** 1353-6.

Dyson, N., P. M. Howley, K. Munger and E. Harlow (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243:** 934-7.

Dzierzak, E. and A. Medvinsky (1995). Mouse embryonic hematopoiesis. *Trends Genet* **11:** 359-66.

Edmondson, J. C. and M. E. Hatten (1987). Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. *J Neurosci* **7:** 1928-34.

Egan, C., S. T. Bayley and P. E. Branton (1989). Binding of the Rb1 protein to E1A products is required for adenovirus transformation. *Oncogene* **4:** 383-8.

ElShamy, W. M., L. K. Fridvall and P. Ernfors (1998). Growth arrest failure, G1 restriction point override, and S phase death of sensory precursor cells in the absence of neurotrophin-3. *Neuron* **21:** 1003-1015.

Eng, C., F. P. Li, D. H. Abramson, R. M. Ellsworth, F. L. Wong, M. B. Goldman, J. Seddon, N. Tarbell and J. D. Boice, Jr. (1993). Mortality from second tumors among long-term survivors of retinoblastoma. *J Natl Cancer Inst* **85:** 1121-8.

Estivill-Torrus, G., H. Pearson, V. van Heyningen, D. J. Price and P. Rashbass (2002). Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development* **129:** 455-66.

Ewen, M. E., H. K. Sluss, C. J. Sherr, H. Matsushime, J. Kato and D. M. Livingston (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73:** 487-497.

Ewen, M. E., Y. G. Xing, J. B. Lawrence and D. M. Livingston (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell* **66:** 1155-64.

Ezhevsky, S. A., H. Nagahara, A. M. Vocero-Akbani, D. R. Gius, M. C. Wei and S. F. Dowdy (1997). Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. *Proc Natl Acad Sci U S A* **94:** 10699-704.

Fajas, L., R. L. Landsberg, Y. Huss-Garcia, C. Sardet, J. A. Lees and J. Auwerx (2002). E2Fs regulate adipocyte differentiation. *Dev Cell* **3:** 39-49.

Farah, M. H., J. M. Olson, H. B. Sucic, R. I. Hume, S. J. Tapscott and D. L. Turner (2000). Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* **127:** 693-702.

Feddersen, R. M., H. B. Clark, W. S. Yunis and H. T. Orr (1995). In vivo viability of postmitotic Purkinje neurons requires pRb family member function. *Mol Cell Neurosci* **6:** 153-67.

Ferguson, K. L. and R. S. Slack (2001). The Rb pathway in neurogenesis. *Neuroreport* **12:** 55-62.

Ferguson, K. L., J. L. Vanderluit, J. M. Hebert, W. C. McIntosh, E. Tibbo, J. G. MacLaurin, D. S. Park, V. A. Wallace, M. Vooijs, S. K. McConnell and R. S. Slack (2002). Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival and abnormal cortical development. *Embo J* **21:** 3337-46.

Fero, M. L., M. Rivkin, M. Tasch, P. Porter, C. E. Carow, E. Firpo, K. Polyak, L. H. Tsai, V. Broudy, R. M. Perlmutter, K. Kaushansky and J. M. Roberts (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* **85:** 733-44.

Fishell, G., C. A. Mason and M. E. Hatten (1993). Dispersion of neural progenitors within the germinal zones of the forebrain. *Nature* **362:** 636-8.

Flemington, E. K., S. H. Speck and W. G. Kaelin, 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:** 6914-8.

Forster, E., A. Tielsch, B. Saum, K. H. Weiss, C. Johanssen, D. Graus-Porta, U. Muller and M. Frotscher (2002). Reelin, Disabled 1, and beta 1 integrins are required for the formation of the radial glial scaffold in the hippocampus. *Proc Natl Acad Sci U S A* **99:** 13178-83.

Franklin, D. S., V. L. Godfrey, H. Lee, G. I. Kovalev, R. Schoonhoven, S. Chen-Kiang, L. Su and Y. Xiong (1998). CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* **12:** 2899-911.

Freeman, R. S., S. Estus and E. M. Johnson, Jr. (1994). Analysis of cell cycle-related gene expression in postmitotic neurons: selective induction of Cyclin D1 during programmed cell death. *Neuron* **12:** 343-55.

Friend, S. H., R. Bernards, S. Rogelj, R. A. Weinberg, J. M. Rapaport, D. M. Albert and T. P. Dryja (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323:** 643-6.

Frotscher, M. (1997). Dual role of Cajal-Retzius cells and reelin in cortical development. *Cell Tissue Res* **290:** 315-22.

Frotscher, M. (1998). Cajal-Retzius cells, Reelin, and the formation of layers. *Curr Opin Neurobiol* **8:** 570-5.

Fung, Y. K., A. L. Murphree, A. T'Ang, J. Qian, S. H. Hinrichs and W. F. Benedict (1987). Structural evidence for the authenticity of the human retinoblastoma gene. *Science* **236:** 1657-61.

Gadisseux, J. F., P. Evrard, J. P. Misson and V. S. Caviness (1989). Dynamic structure of the radial glial fiber system of the developing murine cerebral wall. An immunocytochemical analysis. *Brain Res Dev Brain Res* **50:** 55-67.

Gaiano, N., J. S. Nye and G. Fishell (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26:** 395-404.

Galli, R., A. Gritti, L. Bonfanti and A. L. Vescovi (2003). Neural stem cells: an overview. *Circ Res* **92:** 598-608.

Gallie, B. L., J. A. Squire, A. Goddard, J. M. Dunn, M. Canton, D. Hinton, X. P. Zhu and R. A. Phillips (1990). Mechanism of oncogenesis in retinoblastoma. *Lab Invest* **62:** 394-408.

Gao, C. Y. and P. S. Zelenka (1995). Induction of cyclin B and H1 kinase activity in apoptotic PC12 cells. *Exp Cell Res* **219:** 612-8.

Gaubatz, S., J. A. Lees, G. J. Lindeman and D. M. Livingston (2001). E2F4 is exported from the nucleus in a CRM1-dependent manner. *Mol Cell Biol* **21:** 1384-92.

Gaubatz, S., J. G. Wood and D. M. Livingston (1998). Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F-6. *Proc Natl Acad Sci U S A* **95:** 9190-5.

Gavet, O., S. El Messari, S. Ozon and A. Sobel (2002). Regulation and subcellular localization of the microtubule-destabilizing stathmin family phosphoproteins in cortical neurons. *J Neurosci Res* **68:** 535-50.

Giardina, S. F., N. S. Cheung, M. T. Reid and P. M. Beart (1998). Kainate-induced apoptosis in cultured murine cerebellar granule cells elevates expression of the cell cycle gene cyclin D1. *J Neurochem* **71:** 1325-8.

Gilmore, E. C. & K. Herrup (1997) Cortical development: layers of complexity. *Curr. Biol.* 7:R231-4

Gilmore, E. C., T. Ohshima, A. M. Goffinet, A. B. Kulkarni and K. Herrup (1998). Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. *J Neurosci* **18:** 6370-7.

Ginsberg, D., G. Vairo, T. Chittenden, Z. X. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence and D. M. Livingston (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev* **8:** 2665-79.

Giovanni, A., E. Keramaris, E. J. Morris, S. T. Hou, M. O'Hare, N. Dyson, G. S. Robertson, R. S. Slack and D. S. Park (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:** 11553-60.

Girard, F., U. Strausfeld, A. Fernandez and N. J. Lamb (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67:** 1169-79.

Gloster, A., H. El-Bizri, S. X. Bamji, D. Rogers and F. D. Miller (1999). Early induction of Talpha1 alpha-tubulin transcription in neurons of the developing nervous system. *J Comp Neurol* **405:** 45-60.

Gloster, A., W. Wu, A. Speelman, S. Weiss, C. Causing, C. Pozniak, B. Reynolds, E. Chang, J. G. Toma and F. D. Miller (1994). The T alpha 1 alpha-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. *J Neurosci* **14:** 7319-30.

Gonzalez, J. L., C. J. Russo, D. Goldowitz, H. O. Sweet, M. T. Davisson and C. A. Walsh (1997). Birthdate and cell marker analysis of scrambler: a novel mutation affecting cortical development with a reeler-like phenotype. *J Neurosci* **17:** 9204-11.

Goodrich, D. W., N. P. Wang, Y. W. Qian, E. Y. Lee and W. H. Lee (1991). The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67:** 293-302.

Grigoriou, M., A. S. Tucker, P. T. Sharpe and V. Pachnis (1998). Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development. *Development* **125:** 2063-74.

Groszer, M., R. Erickson, D. D. Scripture-Adams, R. Lesche, A. Trumpp, J. A. Zack, H. I. Kornblum, X. Liu and H. Wu (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science* **294:** 2186-9.

Guo, Z., S. Yikang, H. Yoshida, T. W. Mak and E. Zacksenhaus (2001). Inactivation of the retinoblastoma tumor suppressor induces apoptosis protease-activating factor-1 dependent and independent apoptotic pathways during embryogenesis. *Cancer Res* **61:** 8395-400.

Gwag, B. J., D. Lobner, J. Y. Koh, M. B. Wie and D. W. Choi (1995). Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro. *Neuroscience* **68:** 615-9.

Hamel, P. A., R. M. Gill, R. A. Phillips and B. L. Gallie (1992). Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. *Mol Cell Biol* **12:** 3431-8.

Hanashima, C., L. Shen, S. C. Li and E. Lai (2002). Brain factor-1 controls the proliferation and differentiation of neocortical progenitor cells through independent mechanisms. *J Neurosci* **22:** 6526-36.

Hannon, G. J., D. Demetrick and D. Beach (1993). Isolation of the Rb-related p130 through its interaction with Cdk2 and cyclins. *Genes Dev.* **7:** 2378-2391.

Harbour, J. W. (1998). Overview of RB gene mutations in patients with retinoblastoma. Implications for clinical genetic screening. *Ophthalmology* **105:** 1442-7.

Harbour, J. W. and D. C. Dean (2000). The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev* **14:** 2393-409.

Harbour, J. W., S. L. Lai, J. Whang-Peng, A. F. Gazdar, J. D. Minna and F. J. Kaye (1988). Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* **241:** 353-7.

Harbour, J. W., R. X. Luo, A. Dei Santi, A. A. Postigo and D. C. Dean (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* **98:** 859-69.

Hatanaka, Y. and F. Murakami (2002). In vitro analysis of the origin, migratory behavior, and maturation of cortical pyramidal cells. *J Comp Neurol* **454:** 1-14.

Hebert, J. and S. K. McConnell (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.

Heins, N., P. Malatesta, F. Cecconi, M. Nakafuku, K. L. Tucker, M. A. Hack, P. Chapouton, Y. A. Barde and M. Gotz (2002). Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci* **5:** 308-15.

Helin, K. and E. Harlow (1993). The retinoblastoma protein as a transcriptional repressor. *Trends Cell Biol* **3:** 43-45.

Helin, K., E. Harlow and A. Fattaey (1993). Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol Cell Biol* **13:** 6501-8.

Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow and A. Fattaey (1992). A cDNA encoding a pRb-binding protein with properties of transcription factor E2F. *Cell* **70:** 337-350.

Herrera, R. E., V. P. Sah, B. O. Williams, T. P. Makela, R. A. Weinberg and T. Jacks (1996). Altered cell cycle kinetics, gene expression, and G1 restriction point regulation in Rb-deficient fibroblasts. *Mol Cell Biol* **16:** 2402-7.

Hershko, T. and D. Ginsberg (2003). Up-regulation of BH3-only proteins by E2F1: role in apoptosis. *J Biol Chem.*

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

Hevner, R. F., L. Shi, N. Justice, Y. Hsueh, M. Sheng, S. Smiga, A. Bulfone, A. M. Goffinet, A. T. Campagnoni and J. L. Rubenstein (2001). Tbr1 regulates differentiation of the preplate and layer 6. *Neuron* **29:** 353-66.

Hiebert, S. W., M. Lipp and J. R. Nevins (1989). E1A-dependent trans-activation of the human MYC promoter is mediated by the E2F factor. *Proc Natl Acad Sci U S A* **86:** 3594-8.

Hiebert, S. W., G. Packham, D. K. Strom, R. Haffner, M. Oren, G. Zambetti and J. L. Cleveland (1995). E2F-1:DP-1 induces p53 and overrides survival factors to trigger apoptosis. *Mol Cell Biol* **15:** 6864-74.

Hiesberger, T., M. Trommsdorff, B. W. Howell, A. Goffinet, M. C. Mumby, J. A. Cooper and J. Herz (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* **24:** 481-9.

Hijmans, E. M., P. M. Voorhoeve, R. L. Beijersbergen, L. J. van't Veer and R. Bernards (1995). E2F5, a new E2F family member that interacts with p130 in vivo. *Mol.Cell.Biol.* **15:** 3082-3089.

Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed and R. A. Weinberg (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70:** 993-1006.

Hirotsune, S., T. Takahara, N. Sasaki, K. Hirose, A. Yoshiki, T. Ohashi, M. Kusakabe, Y. Murakami, M. Muramatsu, S. Watanabe and et al. (1995). The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. *Nat Genet* **10:** 77-83.

Hoffarth, R. M., J. G. Johnston, L. A. Krushel and D. van der Kooy (1995). The mouse mutation reeler causes increased adhesion within a subpopulation of early postmitotic cortical neurons. *J Neurosci* **15:** 4838-50.

Hong, F. D., H. J. Huang, H. To, L. J. Young, A. Oro, R. Bookstein, E. Y. Lee and W. H. Lee (1989). Structure of the human retinoblastoma gene. *Proc Natl Acad Sci U S A* **86:** 5502-6.

Hooper, M. L. (1998). Tumour suppressor gene mutations in humans and mice: parallels and contrasts. *Embo J* **17:** 6783-9.

Horowitz, J. M., S. H. Park, E. Bogenmann, J. C. Cheng, D. W. Yandell, F. J. Kaye, J. D. Minna, T. P. Dryja and R. A. Weinberg (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:** 2775-9.

Horton, L. E., Y. Qian and D. J. Templeton (1995). G1 cyclins control the retinoblastoma gene product growth regulation activity via upstream mechanisms. *Cell Growth Differ* **6:** 395-407.

Hou, S. T., D. Callaghan, M. C. Fournier, I. Hill, L. Kang, B. Massie, P. Morley, C. Murray, I. Rasquinha, R. Slack and J. P. MacManus (2000). The transcription factor E2F1 modulates apoptosis of neurons. *J Neurochem* **75:** 91-100.

Howell, B. W., R. Hawkes, P. Soriano and J. A. Cooper (1997). Neuronal position in the developing brain is regulated by mouse disabled-1. *Nature* **389:** 733-7.

Howell, B. W., T. M. Herrick and J. A. Cooper (1999). Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning. *Genes Dev* **13:** 643-8.

Howell, B. W., T. M. Herrick, J. D. Hildebrand, Y. Zhang and J. A. Cooper (2000). Dab1 tyrosine phosphorylation sites relay positional signals during mouse brain development. *Curr Biol* **10:** 877-85.

Hsiao, K. M., S. L. McMahon and P. J. Farnham (1994). Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter. *Genes Dev.* **8:** 1526-1537.

Hsieh, J. K., S. Fredersdorf, T. Kouzarides, K. Martin and X. Lu (1997). E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. *Genes Dev.* **11:** 1840-1852.

Hu, N., A. Gutsmann, D. C. Herbert, A. Bradley, W. H. Lee and E. Y. Lee (1994). Heterozygous Rb-1 delta 20/+mice are predisposed to tumors of the pituitary gland with a nearly complete penetrance. *Oncogene* **9:** 1021-7.

Huang, H. J., J. K. Yee, J. Y. Shew, P. L. Chen, R. Bookstein, T. Friedmann, E. Y. Lee and W. H. Lee (1988). Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* **242:** 1563-6.

Hunter-Schaedle, K. E. (1997). Radial glial cell development and transformation are disturbed in reeler forebrain. *J Neurobiol* **33:** 459-72.

Hurford, R. K., D. Cobrinik, M. H. Lee and N. Dyson (1997). pRb and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev.* **11:** 1447-1463.

Iavarone, A., P. Garg, A. Lasorella, J. Hsu and M. A. Israel (1994). The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev* **8:** 1270-84.

Ino, H., T. Ishizuka, T. Chiba and M. Tatibana (1994). Expression of CDK5 (PSSALRE kinase), a neural cdc2-related protein kinase, in the mature and developing mouse central and peripheral nervous systems. *Brain Res* **661:** 196-206.

Jacks, T. (1996). Tumor suppressor gene mutations in mice. *Annu Rev Genet* **30:** 603-36.

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

Jan, Y. N. and L. Y. Jan (1993). HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* **75:** 827-30.

Jiang, Z., P. Liang, R. Leng, Z. Guo, Y. Liu, X. Liu, S. Bubnic, A. Keating, D. Murray, P. Goss and E. Zacksenhaus (2000). E2F1 and p53 are dispensable, whereas p21(Waf1/Cip1) cooperates with Rb to restrict endoreduplication and apoptosis during skeletal myogenesis. *Dev Biol* **227:** 8-41.

Jiang, Z., E. Zacksenhaus, B. L. Gallie and R. A. Phillips (1997). The retinoblastoma gene family is differentially expressed during embryogenesis. *Oncogene* **14:** 1789-97.

Jimenez, D., L. Lopez-Mascaraque, J. A. de Carlos and F. Valverde (2002a). Further studies on cortical tangential migration in wild type and Pax-6 mutant mice. *J Neurocytol* **31:** 719-28.

Jimenez, D., L. M. Lopez-Mascaraque, F. Valverde and J. A. De Carlos (2002b). Tangential migration in neocortical development. *Dev Biol* **244:** 155-69.

Jodkowski, J. S., S. K. Coles and T. E. Dick (1994). A 'pneumatic centre' in rats. *Neurosci. Lett.* **172:** 67-72.

Johnson, D. G., K. Ohtani and J. R. Nevins (1994). Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. *Genes Dev* **8:** 1514-25.

Johnson, D. G., J. K. Schwarz, W. D. Cress and J. R. Nevins (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365:** 349-52.

Kabos, P., A. Kabosova and T. Neuman (2002). Blocking HES1 expression initiates GABAergic differentiation and induces the expression of p21(CIP1/WAF1) in human neural stem cells. *J Biol Chem* **277:** 8763-6.

Kaelin, W. G., Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar and et al. (1992). Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. *Cell* **70:** 351-64.

Karlseder, J., H. Rotheneder and E. Wintersberger (1996). Interaction of Sp1 with the growth- and cell cycle-regulated transcription factor E2F. *Mol Cell Biol* **16:** 1659-67.

Kim, H. Y. and Y. Cho (1997). Structural similarity between the pocket region of retinoblastoma tumour suppressor and the cyclin-box. *Nat Struct Biol* **4:** 390-5.

Kingston, R. E. and G. J. Narlikar (1999). ATP-dependent remodeling and acetylation as regulators of chromatin fluidity. *Genes Dev* **13:** 2339-52.

Kitagawa, M., H. Higashi, H. K. Jung, I. Suzuki-Takahashi, M. Ikeda, K. Tamai, J. Kato, K. Segawa, E. Yoshida, S. Nishimura and Y. Taya (1996). The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *Embo J* **15:** 7060-9.

Kiyokawa, H., R. D. Kineman, K. O. Manova-Todorova, V. C. Soares, E. S. Hoffman, M. Ono, D. Khanam, A. C. Hayday, L. A. Frohman and A. Koff (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* **85:** 721-32.

Knoepfler, P. S., P. F. Cheng and R. N. Eisenman (2002). N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. *Genes Dev* **16:** 2699-712.

Knudsen, E. S. and J. Y. Wang (1996). Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. *J Biol Chem* **271:** 8313-20.

Knudsen, E. S. and J. Y. Wang (1997). Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. *Mol Cell Biol* **17:** 5771-83.

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* **68:** 820-3.

Ko, J., S. Humbert, R. T. Bronson, S. Takahashi, A. B. Kulkarni, E. Li and L. H. Tsai (2001). p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. *J Neurosci* **21:** 6758-71.

Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza and J. M. Roberts (1992). Formation and activation of a

cyclin E-CDK2 complex during the G1 phase of the human cell cycle. *Science* **257:** 1689-1694.

Kornberg, R. D. and Y. Lorch (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98:** 285-94.

Kowalik, T. F., J. DeGregori, G. Leone, L. Jakoi and J. R. Nevins (1998). E2F1-specific induction of apoptosis and p53 accumulation, which is blocked by Mdm2. *Cell Growth Differ* **9:** 113-8.

Kowalik, T. F., J. DeGregori, J. K. Schwarz and J. R. Nevins (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J Virol* **69:** 2491-500.

Kranenburg, O., A. J. van der Eb and A. Zantema (1996). Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *Embo J* **15:** 46-54.

Kwon, Y. T. and L. H. Tsai (1998). A novel disruption of cortical development in p35(-/-) mice distinct from reeler. *J Comp Neurol* **395:** 510-22.

LaBaer, J., M. D. Garrett, L. F. Stevenson, J. M. Slingerland, C. Sandhu, H. S. Chou, A. Fattaey and E. Harlow (1997). New functional activities for the p21 family of CDK inhibitors. *Genes Dev* **11:** 847-62.

Lai, A., J. M. Lee, W. M. Yang, J. A. DeCaprio, W. G. Kaelin, Jr., E. Seto and P. E. Branton (1999a). RBP1 recruits both histone deacetylase-dependent and -independent repression activities to retinoblastoma family proteins. *Mol Cell Biol* **19:** 6632-41.

Lai, A., R. C. Marcellus, H. B. Corbeil and P. E. Branton (1999b). RBP1 induces growth arrest by repression of E2F-dependent transcription. *Oncogene* **18:** 2091-100.

Lam, E. W. and N. B. La Thangue (1994). DP and E2F proteins: coordinating transcription with cell cycle progression. *Curr Opin Cell Biol* **6:** 859-66.

Lam, E. W. F. and R. J. Watson (1993). An E2F-binding site mediates cell cycle regulated repression of mouse B-myb transcription. *EMBO Journal* **12:** 2705-2713.

Lambert de Rouvroit, C. and A. M. Goffinet (2001). Neuronal migration. *Mech Dev* **105:** 47-56.

Landsberg, R. L., J. E. Sero, P. S. Danielian, T. L. Yuan, E. Y. Lee and J. A. Lees (2003). The role of E2F4 in adipogenesis is independent of its cell cycle regulatory activity. *Proc Natl Acad Sci U S A* **100:** 2456-61.

Lasorella, A., A. Iavarone and M. A. Israel (1996). Id2 specifically alters regulation of the cell cycle by tumor suppressor proteins. *Mol Cell Biol* **16:** 2570-8.

Lasorella, A., M. Noseda, M. Beyna, Y. Yokota and A. Iavarone (2000). Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature* **407:** 592-8.

Latres, E., M. Malumbres, R. Sotillo, J. Martin, S. Ortega, J. Martin-Caballero, J. M. Flores, C. Cordon-Cardo and M. Barbacid (2000). Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *Embo J* **19:** 3496-506.

Lavdas, A. A., M. Grigoriou, V. Pachnis and J. G. Parnavelas (1999). The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. *J Neurosci* **19:** 7881-8.

LeCouter, J. E., B. Kablar, W. R. Hardy, C. Ying, L. A. Megeney, L. L. May and M. A. Rudnicki (1998a). Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. *Mol Cell Biol* **18:** 7455-65.

LeCouter, J. E., B. Kablar, P. F. Whyte, C. Ying and M. A. Rudnicki (1998b). Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. *Development* **125:** 4669-79.

Lee, E., N. Hu, S. S. F. Yuan, L. A. Cox, A. Bradley, W. Lee and K. Herrup (1994). Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes & Dev.* **8:** 2008-2021.

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

Lee, E. Y., H. To, J. Y. Shew, R. Bookstein, P. Scully and W. H. Lee (1988). Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* **241:** 218-21.

Lee, J. E., S. M. Hollenberg, L. Snider, D. L. Turner, N. Lipnick and H. Weintraub (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268:** 836-844.

Lee, J. O., A. A. Russo and N. P. Pavletich (1998). Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature* **391:** 859-65.

Lee, M. H., B. O. Williams, G. Mulligan, S. Mukai, R. T. Bronson, N. Dyson, E. Harlow and T. Jacks (1996). Targeted disruption of p107: Functional overlap between p107 and Rb. *Genes Dev.* **10:** 1621-1632.

Lee, S. S., Y. M. Kim, E. Junn, G. Lee, K. H. Park, M. Tanaka, R. D. Ronchetti, M. M. Quezado and M. M. Mouradian (2003). Cell cycle aberrations by alpha-synuclein over-expression and cyclin B immunoreactivity in Lewy bodies. *Neurobiol Aging* **24:** 687-96.

Lee, W. H., R. Bookstein, F. Hong, L. J. Young, J. Y. Shew and E. Y. Lee (1987a). Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235:** 1394-9.

Lee, W. H., J. Y. Shew, F. D. Hong, T. W. Sery, L. A. Donoso, L. J. Young, R. Bookstein and E. Y. Lee (1987b). The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature* **329:** 642-5.

Lees, J. A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson and K. Helin (1993). The retinoblastoma protein binds to a family of E2F transcription factors. *Mol Cell Biol* **13:** 7813-25.

Leone, G., J. DeGregori, Z. Yan, L. Jakoi, S. Ishida, R. S. Williams and J. R. Nevins (1998). E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* **12:** 2120-30.

Leone, G., F. Nuckolls, S. Ishida, M. Adams, R. Sears, L. Jakoi, A. Miron and J. R. Nevins (2000). Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. *Mol Cell Biol* **20:** 3626-32.

Levitt, P., M. L. Cooper and P. Rakic (1981). Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: an ultrastructural immunoperoxidase analysis. *J Neurosci* **1:** 27-39.

Li, Y., M. Chopp, C. Powers and N. Jiang (1997). Immunoreactivity of cyclin D1/cdk4 in neurons and oligodendrocytes after focal cerebral ischemia in rat. *J Cereb Blood Flow Metab* **17:** 846-56.

Li, Y., C. Graham, S. Lacy, A. M. Duncan and P. Whyte (1993). The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes Dev* **7:** 2366-77.

Lindeman, G. J., L. Dagnino, S. Gaubatz, Y. Xu, R. T. Bronson, H. B. Warren and D. M. Livingston (1998). A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes Dev* **12:** 1092-8.

Lindeman, G. J., S. Gaubatz, D. M. Livingston and D. Ginsberg (1997). The subcellular localization of E2F-4 is cell-cycle dependent. *Proc Natl Acad Sci U S A* **94:** 5095-100.

Lipinski, M. M. and T. Jacks (1999). The retinoblastoma gene family in differentiation and development. *Oncogene* **18:** 7873-82.

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

Lois, C. and A. Alvarez-Buylla (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* **90:** 2074-7.

Ludlow, J. W., J. A. DeCaprio, C. M. Huang, W. H. Lee, E. Paucha and D. M. Livingston (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* **56:** 57-65.

Ludlow, J. W., C. L. Glendening, D. M. Livingston and J. A. DeCarprio (1993). Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol Cell Biol* **13:** 367-72.

Lukas, J., T. Herzinger, K. Hansen, M. C. Moroni, D. Resnitzky, K. Helin, S. I. Reed and J. Bartek (1997). Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes Dev* **11:** 1479-92.

Lukas, J., B. O. Petersen, K. Holm, J. Bartek and K. Helin (1996). Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. *Mol Cell Biol* **16:** 1047-57.

Lundberg, A. S. and R. A. Weinberg (1998). Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol* **18:** 753-61.

Luo, R. X., A. A. Postigo and D. C. Dean (1998). Rb interacts with histone deacetylase to repress transcription. *Cell* **92:** 463-73.

Maandag, E. C., M. van der Valk, M. Vlaar, C. Feltkamp, J. O'Brien, M. van Roon, N. van der Lugt, A. Berns and H. te Riele (1994). Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *Embo J* **13:** 4260-8.

Macleod, K. F., Y. Hu and T. Jacks (1996). Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *EMBO J.* **15:** 6178-6188.

MacPherson, D., J. Sage, D. Crowley, A. Trumpp, R. T. Bronson and T. Jacks (2003). Conditional mutation of Rb causes cell cycle defects without apoptosis in the central nervous system. *Mol Cell Biol* **23:** 1044-53.

Magae, J., C. L. Wu, S. Illenye, E. Harlow and N. H. Heintz (1996). Nuclear localization of DP and E2F transcription factors by heterodimeric partners and retinoblastoma protein family members. *J Cell Sci* **109 (Pt 7):** 1717-26.

Magnaghi, J., L., R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J. P. Le Villain, F. Troalen, D. Trouche and A. Harel-Bellan (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391:** 533.

Malatesta, P., E. Hartfuss and M. Gotz (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127:** 5253-63.

Mallamaci, A., R. Iannone, P. Briata, L. Pintonello, S. Mercurio, E. Boncinelli and G. Corte (1998). EMX2 protein in the developing mouse brain and olfactory area. *Mech Dev* **77:** 165-72.

Mallamaci, A., S. Mercurio, L. Muzio, C. Cecchi, C. L. Pardini, P. Gruss and E. Boncinelli (2000). The lack of Emx2 causes impairment of Reelin signaling and defects of neuronal migration in the developing cerebral cortex. *J Neurosci* **20:** 1109-18.

Mann, D. J. and N. C. Jones (1996). E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. *Curr Biol* **6:** 474-83.

Marin, O., S. A. Anderson and J. L. Rubenstein (2000). Origin and molecular specification of striatal interneurons. *J Neurosci* **20:** 6063-76.

Marin, O., A. S. Plump, N. Flames, C. Sanchez-Camacho, M. Tessier-Lavigne and J. L. Rubenstein (2003). Directional guidance of interneuron migration to the cerebral cortex relies on subcortical Slit1/2-independent repulsion and cortical attraction. *Development* **130:** 1889-901.

Marin, O. and J. L. Rubenstein (2001). A long, remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosci* **2:** 780-90.

Marin, O. and J. L. Rubenstein (2003). Cell migration in the forebrain. *Annu Rev Neurosci* **26:** 441-83.

Marin, O., A. Yaron, A. Bagri, M. Tessier-Lavigne and J. L. Rubenstein (2001). Sorting of striatal and cortical interneurons regulated by semaphorin-neuropilin interactions. *Science* **293:** 872-5.

Marino, S., D. Hoogervoorst, S. Brandner and A. Berns (2003). Rb and p107 are required for normal cerebellar development and granule cell survival but not for Purkinje cell persistence. *Development* **130:** 3359-68.

Marino, S., M. Vooijs, H. van Der Gulden, J. Jonkers and A. Berns (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev* **14:** 994-1004.

Marin-Padilla, M. (1998). Cajal-Retzius cells and the development of the neocortex. *Trends Neurosci* **21:** 64-71.

Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu and A. Giordano (1993). Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. *Oncogene* **8:** 2561-2566.

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

McConnell, S. K. and C. E. Kaznowski (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254:** 282-285.

McLoon, S. C. and R. B. Barnes (1989). Early differentiation of retinal ganglion cells: an axonal protein expressed by premigratory and migrating retinal ganglion cells. *J.Neuroscience* **9:** 1424-1432.

McShea, A., P. L. Harris, K. R. Webster, A. F. Wahl and M. A. Smith (1997). Abnormal expression of the cell cycle regulators P16 and CDK4 in Alzheimer's disease. *Am J Pathol* **150:** 1933-9.

Mihara, K., X. R. Cao, A. Yen, S. Chandler, B. Driscoll, A. L. Murphree, A. T'Ang and Y. K. Fung (1989). Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. *Science* **246:** 1300-3.

Mione, M. C., C. Danevic, P. Boardman, B. Harris and J. G. Parnavelas (1994). Lineage analysis reveals neurotransmitter (GABA or glutamate) but not calcium-binding protein homogeneity in clonally related cortical neurons. *J Neurosci* **14:** 107-23.

Miyata, T., A. Kawaguchi, H. Okano and M. Ogawa (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31:** 727-41.

Moberg, K., M. A. Starz and J. A. Lees (1996). E2F-4 switches from p130 to p107 and pRb in response to cell cycle reentry. *Mol.Cell.Biol.* **16:** 1436-1449.

Moll, A. C., S. M. Imhof, L. M. Bouter and K. E. Tan (1997). Second primary tumors in patients with retinoblastoma. A review of the literature. *Ophthalmic Genet* **18:** 27-34.

Morante-Oria, J., A. Carleton, B. Ortino, E. J. Kremer, A. Fairen and P. M. Lledo (2003). Subpallial origin of a population of projecting pioneer neurons during corticogenesis. *Proc Natl Acad Sci U S A* **100:** 12468-73.

Morgenbesser, S. D., B. O. Williams, T. Jacks and R. A. DePinho (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* **371:** 72-4.

Morkel, M., J. Wenkel, A. J. Bannister, T. Kouzarides and C. Hagemeier (1997). An E2F-like repressor of transcription. *Nature* **390:** 567-568.

Moroni, M. C., E. S. Hickman, E. L. Denchi, G. Caprara, E. Colli, F. Cecconi, H. Muller and K. Helin (2001). Apaf-1 is a transcriptional target for E2F and p53. *Nat Cell Biol* **3:** 552-8.

Morris, E. J. and N. J. Dyson (2001). Retinoblastoma protein partners. *Adv Cancer Res* **82:** 1-54.

Morrison, S. F., S. L. Cravo and H. M. Wilfehrt (1994). Pontine lesions produce apneusis in the rat. *Brain Res.* **652:** 83-86.

Mudryj, M., S. W. Hiebert and J. R. Nevins (1990). A role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. *Embo J* **9:** 2179-84.

Muller, H., A. P. Bracken, R. Vernell, M. C. Moroni, F. Christians, E. Grassilli, E. Prosperini, E. Vigo, J. D. Oliner and K. Helin (2001). E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. *Genes Dev.* **15:** 267-286.

Mulligan, G. and T. Jacks (1998). The retinoblastoma gene family: cousins with overlapping interests. *Trends Genet.* **14:** x.

Munger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow and P. M. Howley (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *Embo J* **8:** 4099-105.

Muraoka, R. S., A. E. Lenferink, B. Law, E. Hamilton, D. M. Brantley, L. R. Roebuck and C. L. Arteaga (2002). ErbB2/Neu-induced, cyclin D1-dependent transformation is accelerated in p27-haploinsufficient mammary epithelial cells but impaired in p27-null cells. *Mol Cell Biol* **22:** 2204-19.

Nadarajah, B. and J. G. Parnavelas (2002). Modes of neuronal migration in the developing cerebral cortex. *Nat Rev Neurosci* **3:** 423-32.

Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii and D. Y. Loh (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85:** 707-20.

Nery, S., G. Fishell and J. G. Corbin (2002). The caudal ganglionic eminence is a source of distinct cortical and subcortical cell populations. *Nat Neurosci* **5:** 1279-87.

Neuman, E., E. K. Flemington, W. R. Sellers and W. G. Kaelin, Jr. (1994). Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol Cell Biol* **14:** 6607-15.

Neuman, E., E. K. Flemington, W. R. Sellers and W. G. Kaelin, Jr. (1995). Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol Cell Biol* **15:** 4660.

Nevins, J. R. (1992). E2F: A link between the retinoblastoma tumor suppressor protein and viral oncoproteins. *Science* **258:** 424-429.

Nevins, J. R. (1998). Toward an Understanding of the Functional Complexity of the E2F and Retinoblastoma Families. *Cell Growth & Differ.* **9:** 585-593.

Nguyen, M. D., M. Boudreau, J. Kriz, S. Couillard-Despres, D. R. Kaplan and J. P. Julien (2003). Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1. *J Neurosci* **23:** 2131-40.

Nikitin, A. Y., M. I. Juarez-Perez, S. Li, L. Huang and W. H. Lee (1999). RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/- mice. *Proc Natl Acad Sci U S A* **96:** 3916-21.

Noctor, S. C., A. C. Flint, T. A. Weissman, R. S. Dammerman and A. R. Kriegstein (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409:** 714-20.

Noctor, S. C., A. C. Flint, T. A. Weissman, W. S. Wong, B. K. Clinton and A. R. Kriegstein (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J Neurosci* **22:** 3161-73.

Noctor, S. C., V. Martinez-Cerdeno, L. Ivic and A. R. Kriegstein (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci.*

Noraberg, J., B. W. Kristensen and J. Zimmer (1999). Markers for neuronal degeneration in organotypic slice cultures. *Brain Res. Brain Res. Protoc.* **3:** 278-290.

Norton, J. D. (2000). ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* **113 (Pt 22):** 3897-905.

Novitch, B. G., G. J. Mulligan, T. Jacks and A. B. Lassar (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:** 441-56.

Ogawa, H., K. Ishiguro, S. Gaubatz, D. M. Livingston and Y. Nakatani (2002). A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* **296:** 1132-6.

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

Ogawa, O., H. G. Lee, X. Zhu, A. Raina, P. L. Harris, R. J. Castellani, G. Perry and M. A. Smith (2003a). Increased p27, an essential component of cell cycle control, in Alzheimer's disease. *Aging Cell* **2:** 105-10.

Ogawa, O., X. Zhu, H. G. Lee, A. Raina, M. E. Obrenovich, R. Bowser, H. A. Ghanbari, R. J. Castellani, G. Perry and M. A. Smith (2003b). Ectopic localization of phosphorylated histone H3 in Alzheimer's disease: a mitotic catastrophe? *Acta Neuropathol (Berl)* **105:** 524-8.

O'Hare, M. J., S. T. Hou, E. J. Morris, S. P. Cregan, Q. Xu, R. S. Slack and D. S. Park (2000a). Induction and modulation of cerebellar granule neuron death by E2F-1. *J Biol Chem* **275:** 25358-64.

Ohshima, T., J. M. Ward, C. G. Huh, G. Longenecker, Veeranna, H. C. Pant, R. O. Brady, L. J. Martin and A. B. Kulkarni (1996). Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc Natl Acad Sci U S A* **93:** 11173-8.

Ohtsubo, M. and J. M. Roberts (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science* **259:** 1908-1912.

O'Rourke, N. A., A. Chenn and S. K. McConnell (1997). Postmitotic neurons migrate tangentially in the cortical ventricular zone. *Development* **124:** 997-1005.

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

O'Rourke, N. A., D. P. Sullivan, C. E. Kaznowski, A. A. Jacobs and S. K. McConnell (1995). Tangential migration of neurons in the developing cerebral cortex. *Development* **121:** 2165-76.

Osuga, H., S. Osuga, F. Wang, R. Fetni, M. J. Hogan, R. S. Slack, A. M. Hakim, J. E. Ikeda and D. S. Park (2000). Cyclin-dependent kinases as a therapeutic target for stroke. *Proc Natl Acad Sci U S A* **97:** 10254-9.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorge and G. Draetta (1992). Cyclin A is required at two points in the human cell cycle. *Embo J* **11:** 961-71.

Palis, J. and G. B. Segel (1998). Developmental biology of erythropoiesis. *Blood Rev* **12:** 106-14.

Pan, H. and A. E. Griep (1994). Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. *Genes Dev* **8:** 1285-99.

Pan, H., C. Yin, N. J. Dyson, E. Harlow, L. Yamasaki and T. van Dyke (1998). Key roles for E2F1 in signaling p53-dependent apoptosis and in cell division within developing tumors. *Mol. Cell* **2:** 283-292.

Paramio, J. M., C. Segrelles, M. L. Casanova and J. L. Jorcano (2000). Opposite functions for E2F1 and E2F4 in human epidermal keratinocyte differentiation. *J Biol Chem* **275:** 41219-26.

Park, D. S., E. J. Morris, R. Bremner, E. Keramaris, J. Padmanabhan, M. Rosenbaum, M. L. Shelanski, H. M. Geller and L. A. Greene (2000). Involvement of retinoblastoma family members and E2F/DP complexes in the death of neurons evoked by DNA damage. *J Neurosci* **20:** 3104-14.

Park, D. S., E. J. Morris, J. Padmanabhan, M. L. Shelanski, H. M. Geller and L. A. Greene (1998). Cyclin-dependent kinases participate in death of neurons evoked by DNA-damaging agents. *J Cell Biol* **143:** 457-67.

Parnavelas, J. G. (2000). The origin and migration of cortical neurones: new vistas. *Trends Neurosci* **23:** 126-31.

Parnavelas, J. G., P. Alifragis and B. Nadarajah (2002). The origin and migration of cortical neurons. *Prog Brain Res* **136:** 73-80.

Parnavelas, J. G., J. A. Barfield, E. Franke and M. B. Luskin (1991). Separate progenitor cells give rise to pyramidal and nonpyramidal neurons in the rat telencephalon. *Cereb Cortex* **1:** 463-8.

Parry, D., D. Mahony, K. Wills and E. Lees (1999). Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. *Mol Cell Biol* **19:** 1775-83.

Peeper, D. S., L. L. Parker, M. E. Ewen, M. Toebes, F. L. Hall, M. Xu, A. Zantema, A. J. van der Eb and H. Piwnica-Worms (1993). A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. *Embo J* **12:** 1947-54.

Persengiev, S. P., Kondova, II and D. L. Kilpatrick (1999). E2F4 actively promotes the initiation and maintenance of nerve growth factor-induced cell differentiation. *Mol Cell Biol* **19:** 6048-56.

Phillips, A. C., S. Bates, K. M. Ryan, K. Helin and K. H. Vousden (1997). Induction of DNA synthesis and apoptosis are separable functions of E2F-1. *Genes Dev.* **11:** 1835-1863.

Pleasure, S. J., S. Anderson, R. Hevner, A. Bagri, O. Marin, D. H. Lowenstein and J. L. Rubenstein (2000). Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. *Neuron* **28:** 727-40.

Polleux, F., K. L. Whitford, P. A. Dijkhuizen, T. Vitalis and A. Ghosh (2002). Control of cortical interneuron migration by neurotrophins and PI3-kinase signaling. *Development* **129:** 3147-60.

Porcher, C., W. Swat, K. Rockwell, Y. Fujiwara, F. W. Alt and S. H. Orkin (1996). The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* **86:** 47-57.

Powell, E. M., W. M. Mars and P. Levitt (2001). Hepatocyte growth factor/scatter factor is a motogen for interneurons migrating from the ventral to dorsal telencephalon. *Neuron* **30:** 79-89.

Price, J. and L. Thurlow (1988). Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. *Development* **104:** 473-82.

Privat, A. (1975). Postnatal gliogenesis in the mammalian brain. *Int Rev Cytol* **40:** 281-323.

Qian, X., Q. Shen, S. K. Goderie, W. He, A. Capela, A. A. Davis and S. Temple (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* **28:** 69-80.

Qin, X. Q., T. Chittenden, D. M. Livingston and W. G. Kaelin, Jr. (1992). Identification of a growth suppression domain within the retinoblastoma gene product. *Genes Dev* **6:** 953-64.

Qin, X. Q., D. M. Livingston, M. Ewen, W. R. Sellers, Z. Arany and W. G. Kaelin, Jr. (1995). The transcription factor E2F-1 is a downstream target of RB action. *Mol Cell Biol* **15:** 742-55.

Qin, X. Q., D. M. Livingston, W. G. Kaelin, Jr. and P. D. Adams (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc Natl Acad Sci U S A* **91:** 10918-22.

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

Rakic, P. (1988). Specification of cerebral cortical areas. *Science* **241:** 170-6.

Ramón y Cajal, S. (1891). Sur la structure de l'écorce cerebral de quelques mammiferes. *La Cellule* **7:** 125-178.

Ramón y Cajal, S. (1911). Histologie du Système Nerveus de l'Homme et des Vertébrés. Paris, Maloine.

Ranganathan, S. and R. Bowser (2003). Alterations in G(1) to S phase cell-cycle regulators during amyotrophic lateral sclerosis. *Am J Pathol* **162:** 823-35.

Raper, J. A. (2000). Semaphorins and their receptors in vertebrates and invertebrates. *Curr Opin Neurobiol* **10:** 88-94.

Rayman, J. B., Y. Takahashi, V. B. Indjeian, J. H. Dannenberg, S. Catchpole, R. J. Watson, H. te Riele and B. D. Dynlacht (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. *Genes Dev* **16:** 933-47.

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

Retzius, G. (1892). Die neuroglia des gehirns beim menschen und bei saugerthieren. *Biol. Unters.* **3:** 1-24.

Reznikov, K., S. E. Acklin and D. van der Kooy (1997). Clonal heterogeneity in the early embryonic rodent cortical germinal zone and the separation of subventricular from ventricular zone lineages. *Dev Dyn* **210:** 328-43.

Rice, D. S. and T. Curran (2001). Role of the reelin signaling pathway in central nervous system development. *Annu Rev Neurosci* **24:** 1005-39.

Rice, D. S., M. Sheldon, G. D'Arcangelo, K. Nakajima, D. Goldowitz and T. Curran (1998). Disabled-1 acts downstream of Reelin in a signaling pathway that controls laminar organization in the mammalian brain. *Development* **125:** 3719-29.

Riley, D. J., C. Y. Liu and W. H. Lee (1997). Mutations of N-terminal regions render the retinoblastoma protein insufficient for functions in development and tumor suppression. *Mol Cell Biol* **17:** 7342-52.

Robanus-Maandag, E., M. Dekker, M. Van der Valk, M. L. Carrozza, J. C. Jeanny, J. H. Dannenberg, A. Berns and H. Te Riele (1998). p107 is a suppressor of retinoblastoma development in pRb-deficient mice. *Genes Dev.* **12:** 1599-1609.

Rosenblum, W. I. (1997). Histopathologic clues to the pathways of neuronal death following ischemia/hypoxia. *J Neurotrauma* **14:** 313-26.

Ross, J. F., X. Liu and B. D. Dynlacht (1999). Mechanism of transcriptional repression of E2F by the retinoblastoma tumor suppressor protein. *Mol Cell* **3:** 195-205.

Ross, S. E., M. E. Greenberg an C. D. Stiles (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39:** 13-25.

Ruas, M. and G. Peters (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* **1378:** F115-77.

Rubenstein, J. L., S. Anderson, L. Shi, E. Miyashita-Lin, A. Bulfone and R. Hevner (1999). Genetic control of cortical regionalization and connectivity. *Cereb Cortex* **9:** 524-32.

Saavedra, H. I., L. Wu, A. de Bruin, C. Timmers, T. J. Rosol, M. Weinstein, M. L. Robinson and G. Leone (2002). Specificity of E2F1, E2F2, and E2F3 in mediating phenotypes induced by loss of Rb. *Cell Growth Differ* **13:** 215-25.

Sage, J., A. L. Miller, P. A. Perez-Mancera, J. M. Wysocki and T. Jacks (2003). Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* **424:** 223-8.

Sage, J., G. J. Mulligan, L. D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou and T. Jacks (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev* **14:** 3037-50.

Saha, P., J. Chen, K. C. Thome, S. J. Lawlis, Z. H. Hou, M. Hendricks, J. D. Parvin and A. Dutta (1998). Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol Cell Biol* **18:** 2758-67.

Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen and R. A. Weinberg (1995). E2F-4 and E2F-5, two members of the E2F family members of the E2F family are expressed in the early phases of the cell cycle. *Proc.Natl.Acad.Sci.USA* **92:** 2403-2407.

Sarnat, H. B. and L. Flores-Sarnat (2002). Cajal-Retzius and subplate neurons: their role in cortical development. *Eur J Paediatr Neurol* **6:** 91-7.

Schmechel, D. E. and P. Rakic (1979). A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anat Embryol (Berl)* **156:** 115-52.

Schmued, L. C., C. Albertson and W. J. Slikker (1997). Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. *Brain Res.* **751:** 37-46.

Schneider, J. W., W. Gu, L. Zhu, V. Mahdavi and B. Nadal-Ginard (1994). Reversal of terminal differentiation mediated by p107 in Rb -/- muscle cells. *Science* **264:** 1467-1471.

Schulman, B. A., D. L. Lindstrom and E. Harlow (1998). Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc Natl Acad Sci U S A* **95:** 10453-8.

Seaberg, R. M. and D. van der Kooy (2003). Stem and progenitor cells: the premature desertion of rigorous definitions. *Trends Neurosci* **26:** 125-31.

Sears, R., K. Ohtani and J. R. Nevins (1997). Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. *Mol Cell Biol* **17:** 5227-35.

Sellers, W. R., B. G. Novitch, S. Miyake, A. Heith, G. A. Otterson, F. J. Kaye, A. B. Lassar and W. G. Kaelin (1998). Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. *Genes Dev.* **12:** 95-196.

Sellers, W. R., J. W. Rodgers and W. G. Kaelin, Jr. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc Natl Acad Sci U S A* **92:** 11544-8.

Serrano, M., H. Lee, L. Chin, C. Cordon-Cardo, D. Beach and R. A. DePinho (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85:** 27-37.

Shan, B. and W. H. Lee (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol Cell Biol* **14:** 8166-73.

Shan, B., X. Zhu, P. L. Chen, T. Durfee, Y. Yang, D. Sharp and W. H. Lee (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:** 5620-31.

Sheaff, R., M. Groudine, M. Gordon, J. Roberts and B. Clurman (1997). Cyclin E-CDK2 is a regulator of p27Kip1. *Genes & Dev.* **11:** 1464-1478.

Sheldon, M., D. S. Rice, G. D'Arcangelo, H. Yoneshima, K. Nakajima, K. Mikoshiba, B. W. Howell, J. A. Cooper, D. Goldowitz and T. Curran (1997). Scrambler and yotari disrupt the disabled gene and produce a reeler-like phenotype in mice. *Nature* **389:** 730-3.

Sheppard, A. M. and A. L. Pearlman (1997). Abnormal reorganization of preplate neurons and their associated extracellular matrix: an early manifestation of altered neocortical development in the reeler mutant mouse. *J Comp Neurol* **378:** 173-9.

Sherr, C. J. (1994). G1 phase progression: cycling on cue. *Cell* **79:** 551-555.

Sherr, C. J. (1996). Cancer cell cycles. *Science* **274:** 1672-7.

Sherr, C. J. and F. McCormick (2002). The RB and p53 pathways in cancer. *Cancer Cell* **2:** 103-12.

Sherr, C. J. and J. M. Roberts (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev* **9:** 1149-63.

Sherr, C. J. and J. M. Roberts (1999). CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes & Dev.* **13:** 1501-1512.

Shimamura, K. and J. L. R. Rubenstein (1997). Inductive interactions direct early regionalization of the mouse forebrain. *Development* **124:** 2709-2718.

Shimizu, M., E. Ichikawa, U. Inoue, T. Nakamura, T. Nakajima, H. Nojima, H. Okayama and K. Oda (1995). The G1/S boundary-specific enhancer of the rat cdc2 promoter. *Mol Cell Biol* **15:** 2882-92.

Shinozaki, K., T. Miyagi, M. Yoshida, T. Miyata, M. Ogawa, S. Aizawa and Y. Suda (2002). Absence of Cajal-Retzius cells and subplate neurons associated with defects of tangential cell migration from ganglionic eminence in Emx1/2 double mutant cerebral cortex. *Development* **129:** 3479-92.

Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Morgan, D. M. Livingston and T. Chittenden (1992). The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. *Cell* **68:** 157-66.

Shirvan, A., I. Ziv, A. Barzilai, R. Djaldeti, R. Zilkh-Falb, T. Michlin and E. Melamed (1997). Induction of mitosis-related genes during dopamine-triggered apoptosis in sympathetic neurons. *J Neural Transm Suppl* **50:** 67-78.

Siddiqui, H., D. A. Solomon, R. W. Gunawardena, Y. Wang and E. S. Knudsen (2003). Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition. *Mol Cell Biol* **23:** 7719-31.

Sidman, R. L. and P. Rakic (1973). Neuronal migration, with special reference to developing human brain: a review. *Brain Res* **62:** 1-35.

Simpson, M. T., J. G. MacLaurin, D. Xu, K. L. Ferguson, J. L. Vanderluit, M. A. Davoli, S. Roy, D. W. Nicholson, G. S. Robertson, D. S. Park and R. S. Slack (2001). Caspase 3 deficiency rescues peripheral nervous system defect in retinoblastoma nullizygous mice. *J Neurosci* **21:** 7089-98.

Singh, P., J. Coe and W. Hong (1995). A role for retinoblastoma protein in potentiating transcriptional activation by the glucocorticoid receptor. *Nature* **374:** 562-5.

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

Slack, R. S., P. A. Hamel, T. Bladon, M. Gill and M. W. McBurney (1993). Regulated expression of the retinoblastoma gene in differentiating P19 cells. *Oncogene* **8:** 1585-1591.

Slack, R. S., I. S. Skerjanc, B. Lach, J. Craig, K. Jardine and M. W. McBurney (1995). Cells differentiating into neuroectoderm undergo apoptosis in the absence of functional retinoblastoma family proteins. *J Cell Biol* **129:** 779-88.

Smith, E. J., G. Leone, J. DeGregori, L. Jakoi and J. R. Nevins (1996). The accumulation of an E2F-p130 transcriptional repressor distinguishes a G0 cell state from a G1 cell state. *Mol Cell Biol* **16:** 6965-76.

Soos, T. J., H. Kiyokawa, J. S. Yan, M. S. Rubin, A. Giordano, A. DeBlasio, S. Bottega, B. Wong, J. Mendelsohn and A. Koff (1996). Formation of p27-CDK complexes during the human mitotic cell cycle. *Cell Growth Differ* **7:** 135-46.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21:** 70-71.

Stein, R., N. Mori, K. Matthews, L. C. Lo and D. J. Anderson (1988). The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. *Neuron* **1:** 463-76.

Sterner, J. M., Y. Murata, H. G. Kim, S. B. Kennett, D. J. Templeton and J. M. Horowitz (1995). Detection of a novel cell cycle-regulated kinase activity that associates with the amino terminus of the retinoblastoma protein in G2/M phases. *J Biol Chem* **270:** 9281-8.

Stevaux, O. and N. J. Dyson (2002). A revised picture of the E2F transcriptional network and RB function. *Curr Opin Cell Biol* **14:** 684-91.

Stevens, C. and N. B. La Thangue (2003). E2F and cell cycle control: a double-edged sword. *Arch Biochem Biophys* **412:** 157-69.

Strobeck, M. W., K. E. Knudsen, A. F. Fribourg, M. F. DeCristofaro, B. E. Weissman, A. N. Imbalzano and E. S. Knudsen (2000). BRG-1 is required for RB-mediated cell cycle arrest. *Proc Natl Acad Sci U S A* **97:** 7748-53.

Stucky, C. L., J. B. Shin and G. R. Lewin (2002). Neurotrophin-4: a survival factor for adult sensory neurons. *Curr Biol* **12:** 1401-4.

Stuhmer, T., S. A. Anderson, M. Ekker and J. L. Rubenstein (2002). Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. *Development* **129:** 245-52.

Stumm, R. K., C. Zhou, T. Ara, F. Lazarini, M. Dubois-Dalcq, T. Nagasawa, V. Hollt and S. Schulz (2003). CXCR4 regulates interneuron migration in the developing neocortex. *J Neurosci* **23:** 5123-30.

Super, H., J. A. Del Rio, A. Martinez, P. Perez-Sust and E. Soriano (2000). Disruption of neuronal migration and radial glia in the developing cerebral cortex following ablation of Cajal-Retzius cells. *Cereb Cortex* **10:** 602-13.

Sussel, L., O. Marin, S. Kimura and J. L. Rubenstein (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126:** 3359-70.

Sweet, H. O., R. T. Bronson, K. R. Johnson, S. A. Cook and M. T. Davisson (1996). Scrambler, a new neurological mutation of the mouse with abnormalities of neuronal migration. *Mamm Genome* **7:** 798-802.

Takahashi, T., T. Goto, S. Miyama, R. S. Nowakowski and V. S. Caviness, 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:** 10357-71.

Takahashi, T., R. S. Nowakowski and V. S. Caviness, Jr. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J Neurosci* **15:** 6046-57.

Takahashi, T., R. S. Nowakowski and V. S. Caviness, Jr. (1996). The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neuronogenesis. *J Neurosci* **16:** 6183-96.

Takahashi, Y., J. B. Rayman and B. D. Dynlacht (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes Dev* **14:** 804-16.

Tamamaki, N., K. Fujimori, Y. Nojyo, T. Kaneko and R. Takauji (2003). Evidence that Sema3A and Sema3F regulate the migration of GABAergic neurons in the developing neocortex. *J Comp Neurol* **455:** 238-48.

Tan, S. S. and S. Breen (1993). Radial mosaicism and tangential cell dispersion both contribute to mouse neocortical development. *Nature* **362:** 638-40.

Tan, S. S., B. Faulkner-Jones, S. J. Breen, M. Walsh, J. F. Bertram and B. E. Reese (1995). Cell dispersion patterns in different cortical regions studied with an X-inactivated transgenic marker. *Development* **121:** 1029-39.

Tanaka, D., Y. Nakaya, Y. Yanagawa, K. Obata and F. Murakami (2003). Multimodal tangential migration of neocortical GABAergic neurons independent of GPI-anchored proteins. *Development* **130:** 5803-13.

T'Ang, A., J. M. Varley, S. Chakraborty, A. L. Murphree and Y. K. Fung (1988). Structural rearrangement of the retinoblastoma gene in human breast carcinoma. *Science* **242:** 263-6.

Tao, W. and E. Lai (1992). Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. *Neuron* **8:** 957-966.

Tao, Y., R. F. Kassatly, W. D. Cress and J. M. Horowitz (1997). Subunit composition determines E2F DNA-binding site specificity. *Mol Cell Biol* **17:** 6994-7007.

Thalmeier, K., H. Synovzik, R. Mertz, E. L. Winnacker and M. Lipp (1989). Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human MYC promoter. *Genes Dev* **3:** 527-36.

Thomas, D. M., S. A. Carty, D. M. Piscopo, J. S. Lee, W. F. Wang, W. C. Forrester and P. W. Hinds (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol Cell* **8:** 303-16.

Toma, J. G., H. El-Bizri, F. Barnabe-Heider, R. Aloyz and F. D. Miller (2000). Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. *J Neurosci* **20:** 7648-56.

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

Trimarchi, J. M., B. Fairchild, J. Wen and J. A. Lees (2001). The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. *Proc Natl Acad Sci U S A* **98:** 1519-24.

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

Trommsdorff, M., J. P. Borg, B. Margolis and J. Herz (1998). Interaction of cytosolic adaptor proteins with neuronal apolipoprotein E receptors and the amyloid precursor protein. *J Biol Chem* **273:** 33556-60.

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

Trouche, D., C. Le Chalony, C. Muchardt, M. Yaniv and T. Kouzarides (1997). RB and hbrm cooperate to repress the activation functions of E2F1. *Proc Natl Acad Sci U S A* **94:** 11268-73.

Tsai, K. Y., Y. Hu, K. F. MacLeod, D. Crowley, L. Yamasaki and T. Jacks (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.

Tsai, L. H., T. Takahashi, V. S. Caviness, Jr. and E. Harlow (1993). Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* **119:** 1029-40.

Udvadia, A. J., D. J. Templeton and J. M. Horowitz (1995). Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: superactivation by Rb requires amino acids necessary for growth suppression. *Proc Natl Acad Sci U S A* **92:** 3953-7.

Vairo, G., D. M. Livingston and D. Ginsberg (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 S. S. Tan (2003). Layer specification of transplanted interneurons in developing mouse neocortex. *J Neurosci* **23:** 5113-22.

van den Heuvel, S. and E. Harlow (1993). Distinct Roles for Cyclin-Dependent Kinases in Cell Cycle Control. *Science* **262:** 2050-2054.

Vanderluit, J. L., K. L. Ferguson, S. McNamara, M. A. Rudnicki and R. S. Slack (2004). *submitted.*

Varley, J. M., J. Armour, J. E. Swallow, A. J. Jeffreys, B. A. Ponder, A. T'Ang, Y. K. Fung, W. J. Brammar and R. A. Walker (1989). The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. *Oncogene* **4:** 725-9.

Verona, R., K. Moberg, S. Estes, M. Starz, J. P. Vernon and J. A. Lees (1997). E2F activity is regulated by cell cycle-dependent changes in subcellular localization. *Mol Cell Biol* **17:** 7268-82.

Vlach, J., S. Hennecke and B. Amati (1997). Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. *EMBO J.* **16:** 5334-5344.

Vooijs, M. and A. Berns (1999). Developmental defects and tumor predisposition in Rb mutant mice. *Oncogene* **18:** 5293-303.

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

Wald, D. K. and S. C. McLoon (1995). Immediate differentiation of ganglion cells following mitosis in the developing retina. *Neuron* **14:** 117-124.

Wallace, V. A. and M. C. Raff (1999). A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. *Development* **126:** 2901-9.

Walsh, C. and C. L. Cepko (1988). Clonally related cortical cells show several migration patterns. *Science* **241:** 1342-5.

Walsh, C. and C. L. Cepko (1992). Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science* **255:** 434-40.

Wang, Z. M., H. Yang and D. M. Livingston (1998). Endogenous E2F-1 promotes timely G0 exit of resting mouse embryo fibroblasts. *Proc Natl Acad Sci U S A* **95:** 15583-6.

Ward, M., C. McCann, M. DeWulf, J. Y. Wu and Y. Rao (2003). Distinguishing between directional guidance and motility regulation in neuronal migration. *J Neurosci* **23:** 5170-7.

Ware, M. L., J. W. Fox, J. L. Gonzalez, N. M. Davis, C. Lambert de Rouvroit, C. J. Russo, S. C. Chua, Jr., A. M. Goffinet and C. A. Walsh (1997). Aberrant splicing of a mouse disabled homolog, mdab1, in the scrambler mouse. *Neuron* **19:** 239-49.

Weinberg, R. A. (1991). Tumor suppressor genes. *Science* **254:** 1138-46.

Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell* **81:** 323-330.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell* **75:** 1241-4.

Weintraub, S. J., K. N. Chow, R. X. Luo, S. H. Zhang, S. He and D. C. Dean (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375:** 812-5.

Weintraub, S. J., C. A. Prater and D. C. Dean (1992). Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358:** 259-61.

Welch, P. J. and J. Y. Wang (1993). A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. *Cell* **75:** 779-90.

Welch, P. J. and J. Y. Wang (1995). Abrogation of retinoblastoma protein function by c-Abl through tyrosine kinase-dependent and -independent mechanisms. *Mol Cell Biol* **15:** 5542-51.

Wells, J., K. E. Boyd, C. J. Fry, S. M. Bartley and P. J. Farnham (2000). Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* **20:** 5797-807.

Whitaker, L. L., H. Su, R. Baskaran, E. S. Knudsen and J. Y. Wang (1998). Growth suppression by an E2F-binding-defective retinoblastoma protein (RB): contribution from the RB C pocket. *Mol Cell Biol* **18:** 4032-42.

Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg and E. Harlow (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334:** 124-9.

Wichterle, H., M. Alvarez-Dolado, L. Erskine and A. Alvarez-Buylla (2003). Permissive corridor and diffusible gradients direct medial ganglionic eminence cell migration to the neocortex. *Proc Natl Acad Sci U S A* **100:** 727-32.

Wichterle, H., J. M. Garcia-Verdugo, D. G. Herrera and A. Alvarez-Buylla (1999). Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat Neurosci* **2:** 461-6.

Wichterle, H., D. H. Turnbull, S. Nery, G. Fishell and A. Alvarez-Buylla (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* **128:** 3759-71.

Williams, B. O., L. Remington, D. M. Albert, S. Mukai, R. T. Bronson and T. Jacks (1994a). Cooperative tumorigenic effects of germline mutations in Rb and p53. *Nat Genet* **7:** 480-4.

Williams, B. O., E. M. Schmitt, L. Remington, R. T. Bronson, D. M. Albert, R. A. Weinberg and T. Jacks (1994b). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. *Embo J* **13:** 4251-9.

Wolffe, A. P. and J. J. Hayes (1999). Chromatin disruption and modification. *Nucleic Acids Res* **27:** 711-20.

Wu, C.-L., M. Classon, N. Dyson and E. Harlow (1996). Expression of dominant negative mutant DP-1 block cell cycle progression in G1. *Mol.Cell.Biol.* **16:** 3698-3706.

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

Wu, L., A. de Bruin, H. I. Saavedra, M. Starovic, A. Trimboli, Y. Yang, J. Opavska, P. Wilson, J. C. Thompson, M. C. Ostrowski, T. J. Rosol, L. A. Woollett, M. Weinstein, J. C. Cross, M. L. Robinson and G. Leone (2003). Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature* **421:** 942-7.

Wu, L., C. Timmers, B. Maiti, H. I. Saavedra, L. Sang, G. T. Chong, F. Nuckolls, P. Giangrande, F. A. Wright, S. J. Field, M. E. Greenberg, S. Orkin, J. R. Nevins, M. L. Robinson and G. Leone (2001). The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414:** 457-62.

Wu, W., K. Wong, J. Chen, Z. Jiang, S. Dupuis, J. Y. Wu and Y. Rao (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* **400:** 331-6.

Wu, X. and A. J. Levine (1994). p53 and E2F1 cooperate to mediate apoptosis. *Proc.Natl.Acad.Sci.USA* **91:** 3602-3606.

Xiao, Z. X., J. Chen, A. J. Levine, N. Modjtahedi, J. Xing, W. R. Sellers and D. M. Livingston (1995). Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* **375:** 694-8.

Xu, H. J., S. X. Hu, P. T. Cagle, G. E. Moore and W. F. Benedict (1991). Absence of retinoblastoma protein expression in primary non-small cell lung carcinomas. *Cancer Res* **51:** 2735-9.

Xu, H. J., S. X. Hu, T. Hashimoto, R. Takahashi and W. F. Benedict (1989). The retinoblastoma susceptibility gene product: a characteristic pattern in normal cells and abnormal expression in malignant cells. *Oncogene* **4:** 807-12.

Xu, H. J., Y. Zhou, J. Seigne, G. S. Perng, M. Mixon, C. Zhang, J. Li, W. F. Benedict and S. X. Hu (1996). Enhanced tumor suppressor gene therapy via replication-deficient

adenovirus vectors expressing an N-terminal truncated retinoblastoma protein. *Cancer Res* **56:** 2245-9.

Yan, Y., J. Frisen, M. H. Lee, J. Massague and M. Barbacid (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev* **11:** 973-83.

Yang, H., B. O. Williams, P. W. Hinds, T. S. Shih, T. Jacks, R. T. Bronson and D. M. Livingston (2002). Tumor suppression by a severely truncated species of retinoblastoma protein. *Mol Cell Biol* **22:** 3103-10.

Yoneshima, H., E. Nagata, M. Matsumoto, M. Yamada, K. Nakajima, T. Miyata, M. Ogawa and K. Mikoshiba (1997). A novel neurological mutant mouse, yotari, which exhibits reeler-like phenotype but expresses CR-50 antigen/reelin. *Neurosci Res* **29:** 217-23.

Yoshikawa, K. (2000). Cell cycle regulators in neural stem cells and postmitotic neurons. *Neurosci Res* **37:** 1-14.

Zacksenhaus, E., Z. Jiang, D. Chung, J. D. Marth, R. A. Phillips and B. L. Gallie (1996). pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev* **10:** 3051-64.

Zhang, H. S., M. Gavin, A. Dahiya, A. A. Postigo, D. Ma, R. X. Luo, J. W. Harbour and D. C. Dean (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell* **101:** 79-89.

Zhang, P., N. J. Liegeois, C. Wong, M. Finegold, H. Hou, J. C. Thompson, A. Silverman, J. W. Harper, R. A. DePinho and S. J. Elledge (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* **387:** 151-8.

Zhang, Y. and S. P. Chellappan (1995). Cloning and characterization of human DP2, a novel dimerization partner of E2F. *Oncogene* **10:** 2085-93.

Zhong, W. (2003). Diversifying neural cells through order of birth and asymmetry of division. *Neuron* **37:** 11-4.

Zhu, L., E. Harlow and B. D. Dynlacht (1995a). p107 uses a p21CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. *Genes Dev* **9:** 1740-52.

Zhu, L., S. van den Heuvel, K. Helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson and E. Harlow (1993). Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev* **7:** 1111-25.

Zhu, L., E. Xie and L. S. Chang (1995b). Differential roles of two tandem E2F sites in repression of the human p107 promoter by retinoblastoma and p107 proteins. *Mol Cell Biol* **15:** 3552-62.

Zhu, Y., H. Li, L. Zhou, J. Y. Wu and Y. Rao (1999). Cellular and molecular guidance of GABAergic neuronal migration from an extracortical origin to the neocortex. *Neuron* **23:** 473-85.

Ziebold, U., T. Reza, A. Caron and J. A. Lees (2001). E2F3 contributes both to the inappropriate proliferation and to the apoptosis arising in Rb mutant embryos. *Genes Dev.* **15:** 386-391.

Zindy, F., J. J. Cunningham, C. J. Sherr, S. Jogal, R. J. Smeyne and M. F. Roussel (1999). Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. *Proc Natl Acad Sci U S A* **96:** 13462-7.

Zindy, F., E. Lamas, X. Chenivesse, J. Sobczak, J. Wang, D. Fesquet, B. Henglein and C. Brechot (1992). Cyclin A is required in S phase in normal epithelial cells. *Biochem Biophys Res Commun* **182:** 1144-54.

Zindy, F., J. van Deursen, G. Grosveld, C. J. Sherr and M. F. Roussel (2000). INK4d-deficient mice are fertile despite testicular atrophy. *Mol Cell Biol* **20:** 372-8.

Letters of Permission to Reprint Published Materials

Appendix B

Curriculum Vitae

Publications

Vanderluit, J. L., **K. L. Ferguson**, V. Nikoletopoulou, M. H. Parker, V. Ruzhynsky, T. Alexson, S. M. McNamara, J. G. MacLaurin, W. C. McIntosh, D. S. Park, M. Rudnicki & R. S. Slack (2004) p107 regulates the neural precursor cell population in the brain. (submitted)

Ferguson, K. L. & R. S. Slack (2003) Growth Factors: Can they Promote Neurogenesis? ***TINS*** **26** 283-285 (review).

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

Ferguson, K. L. & R. S. Slack (2001) The Rb Pathway in Neurogenesis. ***Neuroreport*** **12** A55-62 (review).

Simpson, M. T. W., J. G. MacLaurin, D. Xu, **K. L. Ferguson**, M. A. Davoli, D. W. Nicholson, G. S. Robertson, D. Park & R. S. Slack (2001) Caspase 3 deficiency rescues peripheral nervous system defect in retinoblastoma nullizygous mice. ***J. Neurosci.*** **21** 7089-7092.

Ferguson, K. L., S. M. Callaghan, M. J. O'Hare, D. S. Park & R. S. Slack (2000) The Rb-CDK4/6 signaling pathway is critical in neural precursor cell cycle regulation. ***J. Biol. Chem.*** **275** 33593-33600.

Invited Presentations

Ferguson, K. L. (2001) Telencephalon-specific knockout mice survive but exhibit cell cycle defects. *Perkin-Elmer Developmental Biology Social, Soc. Neuroscience Annual Meeting*, San Diego, CA

Ferguson, K. L. (2001) Telencephalon-specific Rb knockout mice survive but exhibit a cell cycle defect. *Seventeenth Annual Meeting on Oncogenes*, Frederick, MA

Abstracts

Ferguson, K. L., V. Nikoletopoulou, J. L. Vanderluit, W. C. McIntosh & Ruth S. Slack (2003). Telencephalon-specific Rb Knockouts Reveal Aberrant Cortical Development. *Soc. Neuroscience Annual Meeting*, New Orleans, LA

Ferguson, K. L., J. L. Vanderluit, V. Nikoletopoulou, W. C. McIntosh & Ruth S. Slack (2003). Telencephalon-specific Rb Knockouts Reveal Aberrant Cortical Development. *International Society for Developmental Biologists Annual Meeting*, Boston, MA

Ferguson, K. L., J. L. Vanderluit, V. Nikoletopoulou, W. C. McIntosh & Ruth S. Slack (2003). Telencephalon-specific Rb Knockouts Reveal Abnormal Cortical Development. *23rd Annual Great Lakes Mammalian Development Meeting*, Toronto, ON

Ferguson, K. L., J. L. Vanderluit, W. C. McIntosh, D. S. Park, M. Vooijs & R. S. Slack (2002) Aberrant neuronal differentiation and cortical development in telencephalon-specific Rb knockouts. *Cancer Genetics and Tumor Suppressor Genes*, Cold Spring Harbor, NY

Ferguson, K. L., J. L. Vanderluit, J. M. Hébert, W. C. McIntosh, E. Tibbo, J. G. MacLaurin, D. S. Park, V. A. Wallace, M. Vooijs, S. K. McConnell & R. S. Slack (2002) Telencephalon-specific Rb knockouts reveal enhanced neurogenesis, survival, and abnormal cortical development. *Canadian Developmental Biology Annual Meeting*, Mont Tremblant, PQ

Ferguson, K. L., E. Tibbo, J. Vanderluit, W. C. McIntosh, V. A. Wallace, J. Hebert, M. Vooijs, D. S. Park, S. K. McConnell, A. Berns & R. S. Slack (2001) Telencephalon-specific knockout mice survive but exhibit cell cycle defects. *Soc. Neuroscience Annual Meeting*, San Diego, CA

Ferguson, K. L., S. M. Callaghan, M. J. O'Hare, D. S. Park & R. S. Slack (2000) The Rb-CDK4/6 signaling pathway is critical in neural precursor cell cycle regulation. *NGF Annual Meeting*, Montreal, PQ

Ferguson, K. L., S. P. Cregan, S. Callaghan & R. S. Slack (2000) The role of E2F1 in neural precursor cell proliferation and differentiation. *Soc. Neuroscience Annual Meeting*, New Orleans, LA

Ferguson, K. L., S. M. Callaghan, J. MacLaurin, M. J. O'Hare, K. Chaundy, D. S. Park & R. S. Slack (1999) E2F transcription factors are critical regulators of neural precursor cell proliferation. *Soc. Neuroscience Annual Meeting*, Miami, FL