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A Cell Autonomous Requirement for the Cell Cycle Regulatory
RB/E2F Pathway in Neuronal Migration

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A CELL AUTONOMOUS REQUIREMENT FOR THE CELL CYCLE REGULATORY RB/E2F PATHWAY IN NEURONAL MIGRATION

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ABSTRACT

While the role of the cell cycle regulatory Rb/E2F pathway is well appreciated as a key regulator of neural precursor proliferation, here a novel role for the Rb pathway in neuronal migration is described. Using cortical slice co-culture assays a cell autonomous requirement was identified for Rb in regulating migration of ventrally derived interneurons in the developing telencephalon. Next, we sought to determine the mechanism by which Rb mediates proliferation and migration in neurogenesis. Members of the E2F transcription factor family are key Rb interacting factors, well known for mediating cell cycle regulation, whose activity is deregulated in the absence of Rb. Using mice with compound null mutations of Rb and E2F1 or E2F3, we asked to which extent either E2F interacts with Rb in neurogenesis. Here, it is reported that E2F1 and E2F3 are both functionally relevant targets in neural precursor proliferation, cell cycle exit, and laminar patterning. Neuronal migration, however, is specifically mediated through E2F3, beyond its role in cell cycle regulation. Through microarray based screening methods neogenin, a member of the neogenin-netrin pathway, was identified as a candidate E2F responsive gene, and through in vitro explant cultures a disrupted netrin mediated migration response was observed in Rb deficient interneurons. Together, these results are the first to establish a requirement for the Rb/E2F pathway beyond cell cycle regulation in nervous system development *in vivo.*

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LIST OF ABBREVIATIONS

3-D	three dimensional
AC-3	active caspase 3
ANOVA	Analysis of Variance
APC	adenomatus polyposis coli
APC/C	anaphase-promoting complex/cyclosome
ApoEr2	apolipoprotein E receptor 2
Arx	Aristaless related homeobox
BDNF	brain derived neurotropic factor
bHLH	basic helix-loop-helix
Bmi-1	B-lymphoma Mo-MLV insertion region 1 protein
BMP	bone morphogenetic protein
b-myb	V-myb myeloblastosis viral oncogene homolog (avian)-like 2
BrdU	Bromodeoxyuridine
Brg-1	brahma related gene 1
BSA	bovine serum albumin
°C	celsius
C. elegans	*Caenorhabditis elegans*
C/EBPα	CCAAT/enhancer-binding protein alpha
CBFA-1	core binding factor alpha1
CCK	cholecystokinin
Ccna2	encoding cyclin A2
Cdc2	cell division cycle 2
Cdh1	also known as fizzy-related/fzr
Cdk	cyclin dependant kinase
CDKI	cyclin dependant kinase inhibitor
CGE	caudal ganglionic eminence
CH	Cortical hem
ChIP	chromation immunoprecipitation
Chip	microarray chip
CIHR	Canadian Institutes of Health Research
Cip/Kip	Cdk interacting protein/ kinase inhibitory protein
c-jun	cellular- ju-nana oncogene
CNS	central nervous system
Cond	conditional
COS	CV-1 (simian) in Origin, and carrying the SV40 genetic material
CP	cortical plate
Cpm	counts per minute
CR	Cajal-Retzius neuron
Cre	cyclization recombination enzyme

CSF	cerebral spinal fluid
CSF-1R	colony stimulating factor-1 receptor
CtBP	carboxy terminal binding protein
C-terminal	carboxy
Cxcl12	cxc chemokine ligand 12
Cxcr4	cxc chemokine receptor 4
D. melanogaster	*Drosophila melanogaster*
Da	Dalton
Dab1	mouse homologue of Drosophila disabled
DCC	deleted in colorectal cancer
DIV	days in vitro
DKO	double knock out
Dlx	distaless related homologue
DNA	deoxyribonucleic acid
DNA pol α	DNA polymerase α
DOC	deoxycholate
DP	DP transcription factor
DTT	1,4-dithiothreitol
E	Embryonic Day
E1A	adenovirus early region 1A
E2F	E2 promoter binding factor
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid,
EMBO	European Molecular Biology Organization
EMSA	electrophoretic mobility shift assay
Emx1,2	empty spiracles (Drosophila) homologue
FGF	fibroblast growth factor
Fig	figure
flox	flanked loxP sites
Foxg1	forkhead box G1
FVB/N	Friend virus B type susceptibility/ NIH mouse
G	gram
G_0	gap 0 (quiescence)
G_1	gap 1 (interphase)
G_2	gap 2 (interphase)
GABA	γ-aminobutyric acid
GCSF	granulocyte colony stimulating factor
GDNF	Glial cell derived neurotrophic factor
GFP	green fluorescent protein
GFRα1	glial cell line derived neurotrophic factor (GDNF) family receptor alpha 1
GTP	guanosine tri-phosphate

h	hour
H4-K20	Histone 4- lysine 20
hBRM	human brahma
HDAC	histine deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hes-1	hairy and enhancer of split-1 homolog
HGF/SF	hepatocyte growth factor/ scatter factor
HP1	heterochromatin protein 1
HPV	Human papilloma virus
HSFC	Heart & Stroke Foundation of Canada
Id2	inhibitor of differentiation 2
Ink4	inhibitors of cdk4
IP	immunoprecipitation
ISH	in situ hybridization
IZ	intermediate zone
Jab-1	c- jun activating binding protein
k	kilo
L	litre
LGE	lateral ganglionic eminence
loxP	locus of X-over P1
Lhx6	LIM homeobox gene 6
LxCxE	lysine- any amino acid- Cysteine- any amino acid- glutamic acid
M	molar
m	micro
M phase	mitosis
m_	milli
_m	metre
μ	micro
MB	marked box domain
Mb	mega base
Mcl-1	myeloid cell leukemia sequence 1
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer family 2
MGE	medial ganglionic eminence
min	minute
mRNA	messenger RNA
MRPs	mitochondrial ribosomal proteins
mut	mutant
myc	myelocytomatosis viral oncogene
MyoD	myogenic determination factor
MZ	marginal zone

n	nano
n=	number
NCAM	neural cell adhesion molecule
NCx	neocortex
NES	nuclear export sequence
NGF	nerve growth factor
Ngn1,2	neurogenin
Nkx2.1	Nk2 homeobox 1
NLS	nuclear localizarion sequence
NP-40	Nonidet P-40
NRC	National research council
NRF-1	nuclear respiratory factor 1
NT-4	neurotrophin-4
N-terminal	amino
Nurr-1	nuclear receptor related protein-1
OB	Olfactory bulb
OGS	Ontario Graduate Scholarship
Otx1	orthodenticle homolog 1 (Drosophila)
P	Probability value
PP	preplate
P19	pluripotent embryonal carcinoma cell line
Pax6	Paired box gene 6
PBS	phosphate buffered saline
PC12	pheochromocytoma cell line
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGC1α	Peroxisome proliferator-activated receptor- coactivator (PGC)-1
pH	potential of hydrogen
PH3	phosphohistone 3
PNS	peripheral nervous system
PPARγ	peroxisome proliferator-activated receptor
PU	purine
PU.1	PU box transcription factor
Rb	retinoblatoma protein
RbAp48	Rb associated protein 48 Kda
RGM	repulsive guidance molecule
RNA	ribonucleic acid
Robo	roundabout protein
RoRb	retinoid-related orphan receptor beta
RT	room temperature
S phase	DNA synthesis

SAC	starburst amacrine
SCF	Skp1-Cullin-F-box protein
SCN	Stem Cell Network
SD	standard deviation
SDF-1/ Cxcl12	stromal cell derived factor-1 / CXC chemokine ligand 12
SEM	standard error of the mean
Sema 3	class 3 semphorin
SGC10	superior cervical ganglion 10
Shh	Sonic Hedgehog
shRNA	short hairpin RNA
siRNA	small interfering RNA
Skp2	S-phase kinase-associated protein 2, the F-box protein of the SCF complex
Smad	small mothers against decepentaplegic
SP	subplate
Suv39h1	suppressor of variegation 3-9 homolog 1 (Drosophila)
SV40	Simian Virus 40
SVZ	subventricular zone
SWI/SNF	switching/sucrose non-fermenting
TAD	transactivation domain
Tbr1	T brain 1
TFE3	transcription factor E3
TGFβ	transforming growth factor β
TrkB	tyrosine receptor kinase B
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP (2'-deoxyuridine 5'-triphosphate) Nick End Labeling
Twist	twist gene homolog 1 (Drosophila)
Unc5h	C. elegans Unc-5 homologue
uPAR	urokinase plasminogen activator receptor
VHL	Von-Hippel Lindau
Vldlr	very low density lipoprotein receptor
VZ	ventricular zone
WM	White matter

CHAPTER 1- INTRODUCTION

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Table 1-1

have been modified with permission from:

McClellan KA & Slack RS. (2006) Novel functions for cell cycle genes in nervous system development. *Cell Cycle*. Jul;5(14):1506-13

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Table 1-3, 1-4, 1-6

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McClellan KA & Slack RS. (2007) Specific in vivo roles for E2Fs in differentiation and development. *Cell Cycle*. Sep;6(23):2917-27

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1.0 NEUROGENESIS—AN OVERVIEW

The mammalian nervous system is comprised of a complex array of cell types and subtypes (reviewed in (Gotz and Huttner, 2005; Guillemot, 2005)). Neurogenesis, the process by which neural precursor cells divide and differentiate to give rise to all the different cell types, occurs in a highly regulated manner (Gotz and Huttner, 2005; Guillemot, 2005). The cortex, a model of neurogenesis, is comprised of a series of layers that form in an inside out manner populated with excitatory projection neurons and inhibitory interneurons (Guillemot, 2005). In both cases, neural precursors divide along the germinal zones lining the ventricles, either symmetrically giving rise to another undifferentiated precursor, or asymmetrically giving rise to a committed progenitor (Gotz and Huttner, 2005). Once committed to a neuronal fate, cells exit the cell cycle and leave the germinal zone to migrate towards their final destination (reviewed in (Gotz and Huttner, 2005; Kriegstein and Noctor, 2004; Marin and Rubenstein, 2001; Marin and Rubenstein, 2003)). Specification and differentiation occur in both spatially and temporally regulated manners. Projection neurons are thought to arise from the dorsal telencephalon where they exit the cell cycle in cohorts and migrate radially to form the cortical layers (Gotz and Huttner, 2005; Marin and Rubenstein, 2003), while interneurons destined for the cortex arise from the ventral telencephalon and migrate along a tangential route (reviewed in (Marin and Rubenstein, 2001; Wonders and Anderson, 2006)). Temporal regulation of precursors underlies the development of layer specific projection neurons (Gotz and Huttner, 2005) and interneuron subtypes (Butt et al., 2005), with earlier born neurons populating deeper cortical layers and later born neurons populating more superficial cortical layers (Gotz and Huttner, 2005; Guillemot, 2005). The fundamental processes of neurogenesis, namely proliferation differentiation, migration, and maturation are controlled by the precise coordination of various genetic pathways (Gotz and Huttner, 2005; Guillemot, 2005). Normal development of the cortex is essential for proper brain function as abnormal development has been hypothesized as an underlying cause in a number of psychiatric disorders including epilepsy, schizophrenia, bipolar disorder, autism, mental retardation, and Tourette syndrome (Benes and Berretta, 2001; Kalanithi et al., 2005; Kato and Dobyns, 2005; Keverne, 1999; Levitt, 2005; Levitt et al., 2004; Lewis and Levitt, 2002; Polleux and Lauder, 2004; Sherr, 2003).

We are beginning to understand the events of neurogenesis at the molecular level. Genetic studies have begun to outline the complex genetic pathways which underlie proliferation, differentiation and neuronal migration during cortical development. While traditionally thought of as a gatekeeper of the G_1/S transition in the cell cycle, new research points towards critical context dependent roles for the retinoblastoma (Rb) tumour suppressor pathway in nervous system development. ***The goal of this introductory chapter is to provide an overview of the events in neurogenesis, focusing on the emerging evidence which exists supporting a role for cell cycle regulation in the process. Attention is then focused on the cell cycle regulatory Rb/E2F pathway, its role in mediating the G_1/S transition of the cell cycle, followed by the evidence which exists suggesting that this pathway is capable of functioning in events beyond traditional cell cycle regulation. The central theme in this thesis is the identification of novel cell autonomous roles for Rb in neurogenesis, which are mediated through cell cycle regulatory E2F interacting factors in a manner beyond traditional cell cycle regulation***.

1.1 SIGNIFICANCE OF CELL CYCLE REGULATION IN NERVOUS SYSTEM DEVELOPMENT

The importance of the cell cycle in regulating neurogenesis and cell fate is well appreciated. Classical [^{3}H] thymidine birthdating studies demonstrated that timing of cell cycle exit is a predictor of laminar destination in the developing cortex (reviewed in (McConnell, 1995)). These studies established that in the development of the six layered cerebral cortex, early born neurons comprise the deeper layers, while later born neurons comprise the superficial layers (Angevine and Sidman, 1961) (reviewed in (McConnell, 1988)) (Figure 1-1). Subsequent studies addressed precisely when within the cell cycle commitment to a particular neuronal fate occurs. Using cell transplantation experiments, early born cortical neurons transplanted to hosts during S-phase adopted the superficial lamina appropriate for the host cell environment, while neurons transplanted during G_2 or M-phases retained the laminar identity of their donor and were found in deeper layers, thus demonstrating that laminar commitment occurs prior to terminal mitosis (McConnell and Kaznowski, 1991). In the retina, the timing between cell cycle exit and neuronal differentiation was estimated using bromodeoxyuridine (BrdU) and RA4, an early ganglion cell marker. In this study, BrdU and RA4 colabeled cells were first detected minutes after detection of BrdU labeled cells that had progressed to M-phase, suggesting that commitment to a neuronal fate occurs during the cell cycle (Wald and McLoon, 1995). These experiments were the first to demonstrate that timing of cell cycle exit influences neuronal fate and that fate is determined within a precise window during the cell cycle. Since the initial studies, great progress has been made in identifying the molecular components of the cell cycle that regulate neurogenesis. Studying mouse models deficient for individual cell cycle genes has revealed that many genes impact on proliferation in the developing nervous system (reviewed in (McClellan and Slack, 2006)). Moreover, through the study of these models, new roles for cell cycle genes in differentiation and development have emerged suggesting that these genes are capable of regulating diverse functions independent from their role in cell cycle control. *Here the key neuronal subtypes in the cortex are introduced, followed by a discussion about proliferation, differentiation, and migration, emphasizing how genes mediating the G_1/S transition are involved.*

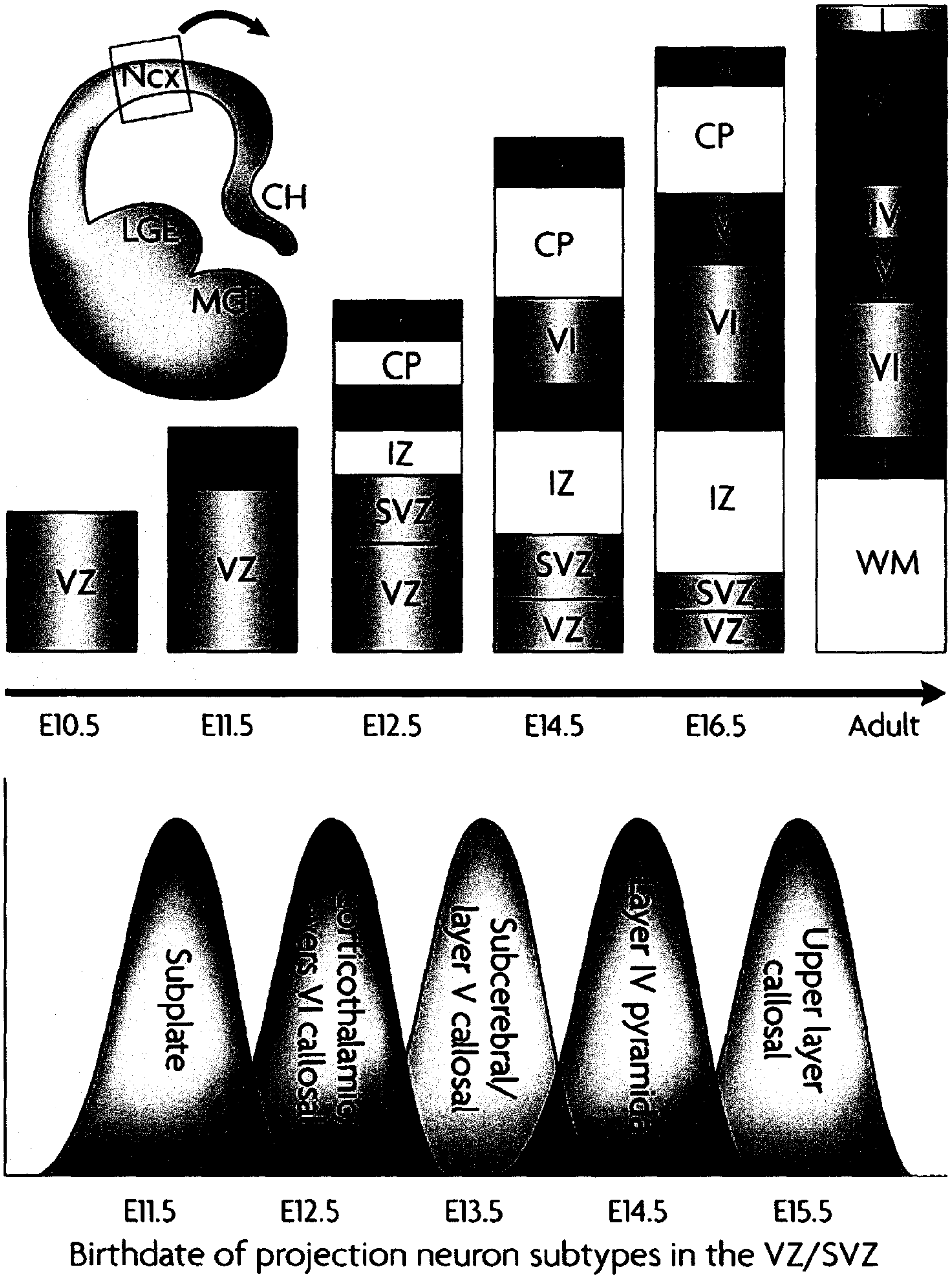
1.2.1 PROJECTION NEURONS—SUBTYPE DIVERSITY

Many of the neurons which comprise the discreet six layer cortical structure belong to a group of neurons known as projection neurons. This large and diverse group of neurons is characterized

Figure 1-1: Development of the cerebral cortex

Projection neurons arising from the dorsal ventricular zone migrate during embryogenesis between E11 and E17. The first neurons to exit the cell cycle form the preplate, which is subsequently split into the marginal zone and subplate, by the arrival of the cortical projection neurons which will ultimately comprise layer VI. Subsequent neuronal cohorts will migrate along radial glial fibres to form the superficial layers V, IV, III, and II. CH= cortical hem, Ncx= neocortex, LGE= lateral ganglionic eminence, MGE= medial ganglionic eminence, PP= preplate, VZ= ventricular zone, MZ= marginal zone, CP= cortical plate, SP= subplate, IZ= intermediate zone, SVZ= subventricular zone, WM= white matter, I =layer 1, II= layer 2, III=layer 3, IV= layer 4, V= layer 5, VI= layer 6.

Modified from: (Molyneaux et al., 2007)



Modified from Molyneaux et al., *Nat Rev Neurosci.*, (2007) 8: 427-37 broadly based on their pyramidal cell bodies, and the excitatory amino acid glutamate (reviewed in (McConnell, 1995; Molyneaux et al., 2007; Steriade, 2004)). Collectively, glutamatergic projection neurons transmit information to multiple brain regions (reviewed in (Molyneaux et al., 2007)). In the adult, projection neurons are classified based on anatomical regions to which they project. For example neurons which project to the striatum or cortex are known as commissural neurons while corticofugal neurons project to the thalamus, brainstem or spinal cord (reviewed in (Molyneaux et al., 2007)). Understanding the diversity of projection neuron subtypes is still an area of active study which likely includes not only anatomical distinctions but also the distinct electrophysiological and functional properties, gene expression, and regional specificity (reviewed in (Molyneaux et al., 2007)).

1.2.2 CAJAL RETZIUS NEURONS

Cajal-Retzius (CR) neurons are among the first population of neurons to appear in the cortex in layer 1, or marginal zone (reviewed in (Soriano and Del Rio, 2005)). They represent a transient population, appearing prior to formation for the cortical layers, and disappearing after neuronal migration is complete (del Rio et al., 1995; Derer and Derer, 1990; Super et al., 1998). Their origins are highly controversial (Bielle et al., 2005; Garcia-Moreno et al., 2007; Yoshida et al., 2006). While it is generally accepted that the cortical hem represents a source from which CR neurons rapidly migrate tangentially to populate layer 1, other controversial sources have been proposed including the septum, as well as ventral sources such as pallial/ subpallial boundary, and anterior preoptic area (Bielle et al., 2005; Garcia-Moreno et al., 2007; Yoshida et al., 2006). Through secretion of reelin, a glycoprotein known to influence development of the laminar structure of the cortex, CR neurons are though to be instrumental in directing cortical development (reviewed in (Soriano and Del Rio, 2005)).

1.2.3 GABAergic INTERNEURONS- SUBTYPE DIVERSITY

Cortical interneurons are key regulators of neuronal function that act by modulating the activity of major excitatory neural circuits (reviewed in (Markram et al., 2004; McBain and Fisahn, 2001)). While they use the neurotransmitter GABA, they exhibit diversity in terms of morphology, immunohistochemical markers, and electophysiological properties (reviewed in (Flames and Marin, 2005; Markram et al., 2004; McBain and Fisahn, 2001; Wonders and Anderson, 2006). In the adult, major subtypes are identified by the calcium binding proteins parvalbumin and calretinin as well as their neuropeptides, such as somatostatin and neuropeptide Y (Flames and Marin, 2005; Wonders and Anderson, 2006). Calbindin is a third calcium binding protein which labels embryonic and adult interneurons, however its expression overlaps with parvalbumin, somatostatin and to a lesser extent calretinin (Kawaguchi and Kubota, 1997; Kubota et al., 1994). While each marker represents a distinct subtype, colocalization has been demonstrated for somatostatin and calretinin as well as somatostatin and neuropeptide Y (DeFelipe, 1997; Kawaguchi and Kubota, 1997; Kubota et al., 1994), creating additional subtypes which can be further subdivided based on morphological and electrophysiological properties(Butt et al., 2005). In the mouse, specification of interneurons takes place between embryonic day (E) E12.5 and birth, with many subtypes evident only postnatally (Flames and Marin, 2005; Wonders and Anderson, 2006). Interneurons destined for the cortex arise in the ventral telencephalon in the medial (MGE), caudal (CGE) and to a lesser extent lateral ganglionic eminences (LGE) (Butt et al., 2005; Nery et al., 2002), with specification being both spatially and temporally regulated. For example parvalbumin and some somatostatin positive interneurons are thought to arise from the MGE, while calretinin positive interneurons are thought to arise primarily from the CGE (Butt et al., 2005).

1.3 NEURONAL PRECURSOR PROLIFERATION

While many genes impact on proliferation and division of neural precursor cells, a review of the literature reveals that cell cycle genes that impact on proliferation in the nervous system are concentrated on those genes that regulate the G_1/S cell cycle transition and, in turn, the Rb signaling pathway (Table 1-1). In models of tissue specific Rb deficiency, ectopic cell division of differentiating precursors was observed in the telencephalon, central (CNS), and peripheral nervous system (PNS), and retina, whereas increased proliferation was observed in the cerebellum. Related Rb family members are also involved in regulating proliferation in the nervous system, often in conjunction with Rb. Indeed, Rb family members p107 and p130 have both been shown to work with Rb in regulating proliferation in the retina as deletion of either p107 or p130 combined with Rb deficiency leads to retinoblastoma in the mouse (Chen et al., 2004a; MacPherson et al., 2004; Robanus-Maandag et al., 1998). In addition, combined loss of Rb and p107 in the cerebellar vermis leads to an exacerbation of the proliferation defects in granule cells that are observed in Rb deficiency alone (Marino et al., 2003) A unique function, however, for p107 has been recently described in regulating the neural precursor population in the nervous system (Vanderluit et al., 2004). The absence of p107 alone leads to an enhanced self-renewal capacity of neural precursors resulting in an expansion of the neural precursor pool. These findings demonstrate that p107 functions to regulate the neural precursor cell number through a mechanism distinct from Rb function (Vanderluit et al., 2004).

Given the importance of Rb in regulating proliferation in the nervous system, it follows that the cyclins and cyclin dependant kinases (Cdk), genes that regulate Rb function, also impact on proliferation in the nervous system. Cyclin D2 deficient mice exhibit smaller cerebella as a result of decreased proliferation and increased apoptosis.(Huard et al., 1999) Additionally, cyclin D2 regulates proliferation in the adult CNS where its absence leads to severely reduced proliferation in the subgranular zone of the hippocampus.(Kowalczyk et al., 2004). New data has recently emerged indicating that Cdk5, a unique member of the Cdk family with sequence homology to Cdc2, previously believed not to be involved in cell cycle regulation, plays an essential role in regulating neuronal cell cycle arrest (Cicero and Herrup, 2005) (reviewed in (Dhavan and Tsai, 2001)). The absence of Cdk5 results in the presence of 'neurons engaged in cell cycle activities' in the cortical plate, similar to what is observed in Rb deficiency in the brain (Cicero and Herrup, 2005; Ferguson et al., 2002; MacPherson et al., 2003). These cells continue to express proliferating cell markers indicating that Cdk5 is also required for regulating cell cycle exit (Cicero and Herrup, 2005). As Cdk5 is capable of phosphorylating Rb both *in vivo* and *in vitro*, these observations lead to the hypothesis that Cdk5 may be acting to regulate Rb function in the nervous system (Cicero and Herrup, 2005).

Similarly, members of the Cip/Kip and Inhibitors of Cdk4 (Ink4) families of cyclin dependant kinase inhibitors (CDKI) that regulate cyclins and Cdk activity have also been shown to regulate neural precursor proliferation. Indeed multiple members of both families have been shown to be negative regulators of proliferation in the nervous system highlighting the importance and complexity of cell cycle regulation at the CDKI level. Loss of $p27^{Kip1}$ leads to increased proliferation of transit amplifying progenitor cells in the adult subventricular zone, while loss of $p57^{Kip2}$ leads to ectopic proliferation of retinal progenitors during development (Doetsch et al., 2002; Dyer and Cepko, 2000).

Thus as disruption of a number of individual cell cycle genes results in proliferation defects in the nervous system, these studies demonstrate that precise cell cycle regulation is a crucial component of proper nervous system development.

1.4 NEURONAL DIFFERENTIATION AND CELL CYCLE EXIT

Differentiation of neural precursors in the cerebral cortex is a precise, orchestrated event and it is clear that cell cycle regulation plays a key role in the overall process. In the mouse, neurons of the cerebral cortex are formed in 11 precise cell cycles over the course of six days during embryonic

Table 1-1: Cell Cycle Genes Impact on Neural Precursor Proliferation

NAME	FUNCTION IN NEURAL PRECURSOR PROLIFERATION	REFERENCE
Rb Family		
Rb	Tissue specific absence of Rb leads to ectopic proliferation in CNS & retina. In telencephalon, ectopically dividing cells are committed to a neuronal fate and initiate differentiation.	(Ferguson et al., 2002) (Chen et al., 2004a; MacPherson et al., 2003)
p107	Absence of p107 leads to increased self renewal of neural precursors	(Vanderluit et al., 2004)
E2F1	Absence of E2F1 leads to decreased neural precursor proliferation in adults	(Cooper-Kuhn et al., 2002)
E2F3	Absence of E2F3 leads to decreased neural precursor proliferation in embryo and adults	(McClellan et al., 2007)
Cyclins and Cdks		
Cyclin D1	Absence of Cyclin D1 leads to reduced proliferation of retinal precursors	(Sicinski et al., 1995)
Cyclin D2	Absence of Cyclin D2 leads to decreased proliferation of neural precursors in the adult	(Kowalczyk et al., 2004)
Cdk 4/6 and Cdk2	Inactivation of Cdk4/6 or Cdk2 activity induces growth arrest in primary neural precursor cells	(Ferguson et al., 2000)
Cdk 5	Absence of Cdk5 leads to ectopic proliferation in cortical plate. Ectopically proliferating cells are also differentiating	(Cicero and Herrup, 2005)
CDKI		
Kip/Cip		
p21^{Cip1}	Absence of p21 leads to increased proliferation of neural precursor cells in the adult forebrain, but exhibit reduced capacity for self-renewal	(Kippin et al., 2005)
p27^{Kip1}	Absence of p27 leads to increased proliferation of transit amplifying progenitors but a decrease in number of migrating neuroblasts (reduced due to apoptosis)	(Doetsch et al., 2002)
p57^{Kip2}	Absence of p57 leads to ectopic proliferation and apoptosis in the embryonic retina	(Dyer and Cepko, 2000)
Ink4		
p16^{Ink4a}	Absence of p16 leads to increased proliferation of neural precursor cells	(Molofsky et al., 2003)
Bmi-1	Absence of Bmi-1 leads to decreased proliferation of neural precursor cells, an effect mediated by suppression of p16^{Ink4a}, p19^{Arf}, and additional pathways	(Molofsky et al., 2005; Molofsky et al., 2003) (Pardal et al., 2003)

development from E11 to E17 (Takahashi et al., 1999; Takahashi et al., 1995; Takahashi et al., 1996). Shortly after precursors exit the cell cycle and leave the germinal zones, a host of internal and external factors continue to direct differentiation. Gradients of extracellular factors including Sonic Hedgehog (Shh), Bone Morphogenetic Proteins (BMP), Fibroblast Growth Factors (FGF), and retinoids, are involved in establishing the initial regional patterning, while intracellularly, genes largely of the basic helix-loop-helix (bHLH) and homeobox transcription factor families function to initiate the specification and differentiation programs (reviewed in (Gotz and Huttner, 2005; Guillemot, 2005; Guillemot, 2007; Molyneaux et al., 2007)). What is less understood, however, is how expression of these individual genes mediating differentiation is initiated. Since timing of cell cycle exit is acknowledged to play a role in differentiation, an emerging area of research is focused on identifying how individual genes mediating cell cycle progression play a more active role in the process. Here, in particular events during G_1, are likely to play a key role as previous experiments have demonstrated that the length of the cell cycle increases as neurogenesis progresses, an effect resulting from an approximate fourfold increase in the length of G_1 (Takahashi et al., 1995). Indeed, recently genes implicated in the G_1/S transition, such as p107, cyclin D2 and $p57^{Kip2}$ have been shown to mediate events including commitment to a neuronal fate and neuronal differentiation (Huard et al., 1999; Vanderluit et al., 2004).

First, recent data has implicated p107, a member of the Rb family of pocket proteins which regulate the G_1/S transition, in neuronal differentiation and commitment to a neuronal fate. Mice deficient for p107 exhibit an increased number of neural precursors as a result of increased precursor cell renewal, however, differentiation is defective as fewer proliferating cells commit to a neuronal fate (Vanderluit et al., 2004; Vanderluit et al., 2007). The mechanism by which p107 mediates these events, however, was hypothesized to be outside of traditional cell cycle regulation as p107 deficient neural precursor cells exhibit increased expression of members of the Notch-Hes signaling pathway, genes which ultimately act to regulate self-renewal by repressing expression of proneural genes. As reduction of Hes levels in a p107 null background restored many aspects of neurogenesis, these data support a model whereby p107 promotes neuronal differentiation by repressing expression of Hes-1 (Vanderluit et al., 2007). This provocative data further supports the concept that cell cycle genes are indeed capable of mediating differentiation events through mechanisms distinct from their roles in cell cycle progression.

Next, it follows that those genes which regulate Rb family activity are also involved in differentiation. Indeed, cerebella from cyclin D2 deficient mice exhibited a reduced number of granule cells and stellate interneurons (Huard et al., 1999). The reduction in granule cell number was hypothesized to reflect the requirement for cyclin D2 in terminal differentiation of these cells, as death occurred in postnatal granule precursor cells undergoing terminal differentiation. Reduction in stellate cell number may also reflect a role for cyclin D2 in regulating differentiation of these cells as stellate cells are thought to arise from a common precursor cell that gives rise to three interneurons subtypes: Golgi, stellate and basket cells. Since Golgi and basket cell numbers are unchanged in cyclin D2 deficiency, this suggests a requirement for cyclin D2 in stellate cell differentiation (Huard et al., 1999).

Solid support for a role for cell cycle genes regulating neuronal differentiation exists for $p57^{Kip2}$ where roles for differentiation have been separated from cell cycle control. A role for $p57^{Kip2}$ in neuronal differentiation has been described for both retinal amacrine cells and midbrain dopaminergic neurons (Dyer and Cepko, 2000; Joseph et al., 2003). In the retina, $p57^{Kip2}$ is expressed in dividing retinal progenitors during embryonic development, but was also found postnatally in a subset of post-mitotic differentiating amacrine neurons. Expression of $p57^{Kip2}$ in these cells was not required for preventing cell cycle re-entry, but rather required for differentiation of calbindin expressing amacrine neurons as $p57^{Kip2}$ deficient retinae exhibited increased numbers of these cells (Dyer and Cepko, 2000) These observations lead to the hypothesis that $p57^{Kip2}$ has a dual function in the retina; first to regulate proliferation in mitotic retinal progenitors, and then, possibly through a distinct mechanism, to regulate the differentiation of a subpopulation of amacrine cells (Dyer and Cepko, 2000). Further support for the idea that $p57^{Kip2}$ can regulate neuronal differentiation independently of cell cycle function came about more recently through the examination of $p57^{Kip2}$ during midbrain development (Joseph et al., 2003). Similar to the retina, $p57^{Kip2}$ was found to be expressed in post-mitotic differentiating dopaminergic neurons of the midbrain, however, mice lacking $p57^{Kip2}$ exhibited fewer dopaminergic neurons (Joseph et al., 2003). Several lines of evidence indicate that the effect of $p57^{Kip2}$ on differentiation occurs independently from cell cycle regulation. First, no defect in proliferation was observed through BrdU incorporation in the ventral midbrain in $p57^{Kip2}$ deficient brains when examined at multiple stages of development (Joseph et al., 2003). Next, $p57^{Kip2}$ expression in these cells was shown to be cell cycle independent, and depended on the expression of Nurr-1, a nuclear orphan receptor expressed in post-mitotic cells that is essential for dopamine neuron development (Joseph et al., 2003). Finally, $p57^{Kip2}$ was found to physically interact with Nurr-1 *in vitro* to promote differentiation of dopaminergic neurons. While depletion of $p57^{Kip2}$ inhibited differentiation, differentiation still occurred, however, with a $p57^{Kip2}$ mutant unable to inhibit Cdk activity, yet still capable of binding Nurr-1. Hence these data support the idea that $p57^{Kip2}$ regulates differentiation through interaction with Nurr-1, independent of cell cycle regulation (Joseph et al., 2003). Thus collectively, these observations advance the concept that cell cycle genes are capable of unique functions in neuronal differentiation.

1.5 NEURONAL MIGRATION

A defining feature of the developing nervous system is that neurons are born in one location but migrate, often along long or convoluted pathways before reaching their final destinations (reviewed in (Corbin et al., 2001; Marin and Rubenstein, 2001; Marin and Rubenstein, 2003; Nadarajah and

Parnavelas, 2002)). Migrating neurons are guided by a series of external cues, and specificity is achieved as only those neurons expressing the correct complement of receptors and intercellular effectors are capable of responding to such cues (reviewed in (Marin and Rubenstein, 2003; Nadarajah and Parnavelas, 2002)). Thus an essential component of proper neuronal migration is continued differentiation of neurons such that the appropriate complement of genes required to respond to guidance cues are expressed. Here a brief overview is presented regarding the mechanisms of radial migration of projection neurons and tangential migration of interneurons.

1.5.1 RADIAL MIGRATION OF PROJECTION NEURONS

Projection neurons arising from the dorsal ventricular zone migrate during embryogenesis between E11 and E17. The first neurons to exit the cell cycle form the preplate, which is subsequently split into the marginal zone and subplate, by the arrival of the cortical projection neurons which will ultimately comprise layer VI. Subsequent neuronal cohorts will migrate along radial glial fibres to form the superficial layers V, IV, III, and II (Figure 1-1). A major external factor thought to guide projection neurons is reelin, a large extracellular glycoprotein, secreted by the Cajal Retzius (CR) neurons in the marginal zone (reviewed in (Rice and Curran, 2001)). The significance of reelin in cortical development was appreciated in so-called *reeler* mouse mutants which lacked functional reelin protein (Caviness, 1982a). In these mice, as well as mice in which reelin receptors Vldlr, or ApoEr2, or downstream intracellular effector Dab1 are dysfunctional, the pattern of cortical layer formation is inverted (Gonzalez et al., 1997; Hoffarth et al., 1995; Howell et al., 1997; Ogawa et al., 1995; Rice et al., 1998; Sheldon et al., 1997; Sheppard and Pearlman, 1997; Sweet et al., 1996; Trommsdorff et al., 1999). Thus by extension, CR neurons, as a source of reelin, have long been considered to be necessary for the radial migration of projection neurons. This hypothesis has been recently challenged, however, as a model of genetic ablation of CR neurons did not result in the inverted cortical layer *reeler* phenotype (Yoshida et al., 2006). In this model other sources of reelin within the developing cortex were thought to direct layer formation. As mentioned, the cell cycle plays a key role in radial migration as timing of cell cycle exit influences the ultimate laminar position. ***A key finding in this thesis is that deficiency of the cell cycle regulatory protein, Rb, results in perturbation of the formation of the cortical laminar structure (Chapter 2).***

1.5.2 TANGENTIAL MIGRATION OF INTERNEURONS

Interneurons originating from the ventral telencephalon migrate during embryogenesis from E11.5 through to birth (reviewed in (Marin and Rubenstein, 2001; Marin and Rubenstein, 2003; Wonders and Anderson, 2006)). Interneurons destined for the cortex depart in a series of waves and migrate along tangential trajectories, with the ultimate destination dependent on their time and place of origin (reviewed in (Marin and Rubenstein, 2001; Marin and Rubenstein, 2003; Wonders and Anderson, 2006)). The majority of interneurons destined for the dorsal cortex originate in the MGE and CGE and migrate along two distinct trajectories, a superficial marginal zone (MZ) trajectory and a deeper intermediate zone (IZ), subventricular zone (SVZ) trajectory (DeDiego et al., 1994; Del Rio et al., 1992; Denaxa et al., 2001; Lavdas et al., 1999; Marin et al., 2001; Wichterle et al., 2001) (reviewed (Marin and Rubenstein, 2001)) (Figure 1-2).

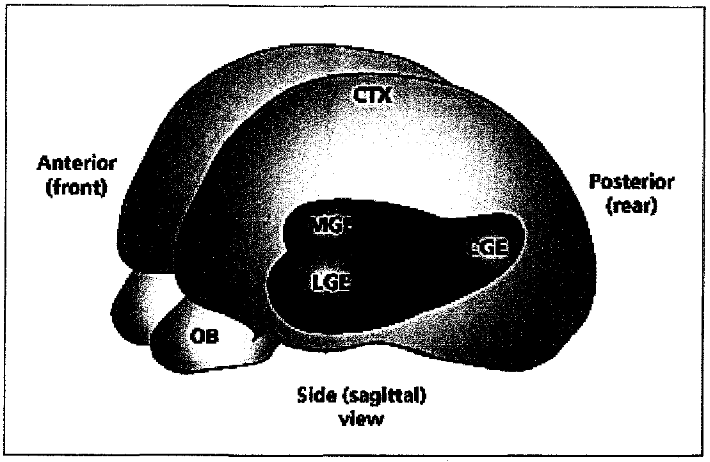
Directing interneuron migration is a group of guidance molecules, the list of which is still growing. While the study of tangential migration is still in its relative infancy, already a long and complex list of regulatory mechanisms have been identified guiding successive steps of interneuron migration as interneurons exit the MGE, travel through the ventral telencephalon and migrate up and into the cortex (Table 1-2).

Figure 1-2: **Origin and migration of cortical interneurons**

(Upper)- Interneurons arise from the ganglionic eminences in the ventral telencephalon during embryonic development. MGE= medial ganglionic eminence, LGE= lateral ganglionic eminence, CGE= caudal ganglionic eminence, OB= olfactory bulb. Modified from Corbin, J.G. et al., (2001) *Nat. Neurosci.*, **4**:1177-82.

(Lower)- Interneurons originating from the MGE migrate along two distinct trajectories (boxed red) to the dorsal cortex: 1) The superficial marginal zone (MZ) route, and 2) The deeper intermediate zone (IZ) route. MZ= marginal zone, IZ= intermediate zone, NCx= neocortex. Modified from Marin and Rubenstein (2001) *Nat Rev Neurosci* **2**:780-90

Ganglionic eminences in the embryonic forebrain



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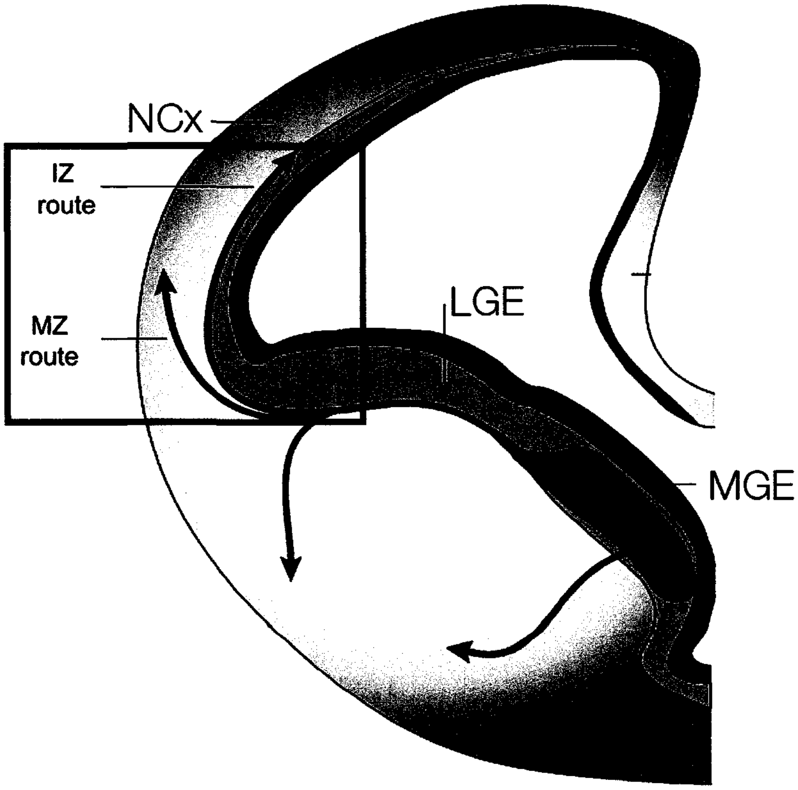


Modified from Marin and Rubenstein (2001) *Nat Rev Neurosci* **2**:780-90

Table 1-2: Select Signaling Pathways Implicated In Regulating Tangential Migration of Cortical Interneurons

FAMILY/ PATHWAY	FUNCTION	EXPRESSION AGE & LOCATION	REFERENCES
Neuropilin/ Semaphorin	Neuropilins direct sorting of striatal vs cortical interneurons	E12.5 to 14.5 (maybe longer) Sema 3A and F in striatum, neuropilins in adjacent regions and developing dorsal telencephalon	(Marin et al., 2001)
Slit/ Robo	Robo-1 mediates migration of interneurons destined to the cortex through the ventral telencephalon	Slit- E13- E17- cortical plate LGE, MGE (some variation between Slit 1, 2, 3) Robo E7-17 axon tracts in developing cortex and corpus callosum	(Andrews et al., 2006; Sundaresan et al., 2004; Yuan et al., 1999)
Netrin /Neogenin	Netrin-1 is repulsive to migrating SVZ cells Neogenin has recently been hypothesized to influence cortical interneuron migrtation	Netrin expressed in ventral VZ as early as E12 DCC expressed in preplate and neogenin is expressed on migrating calbindin and parvalbumin interneurons	(Fitzgerald et al., 2006; Gad et al., 1997; Hamasaki et al., 2001)
HGF/SF	Acts as a motogen for GABAergic interneurons	HGF/SF is expressed in the VZ from E11.5. From E13.5 until postnatal life is expressed in cortical plate	(Powell et al., 2001)
TrkB, BDNF, NT-4	TrkB loss of function mice show reduced interneuron migration into cortex. BDNF and NT-4 stimulate tangential migration *in vitro*.	Migrating cells of the MGE at E14.5 on leading process	(Polleux et al., 2002)
GDNF/ GFRα1	GDNF promotes GABAergic neuron differentiation and acts as chemoattractant for GABAergic interneurons. GDNF and GFRα1 deficient mice exhibit aberrant tangential migration and fewer GABA interneurons	Both expressed in MGE and MZ, CP and SVZ/VZ of developing cortex E12.5- E15.5	(Pozas and Ibanez, 2005)
Cxcl12/Cxcr4	Cxcl12/Cxcr4 signaling regulates interneuron entry into the cortical plate from the marginal zone and deeper intermediate/ subventricular zone trajectories	Cxcl12 is expressed in the meninges and within the SVZ from E13.5 to at least E16.5 Cxcr4 is expressed in migrating cortical interneurons starting from E13.5	(Li et al., 2008; Lopez-Bendito et al., 2008)

Initial studies implicated semaphorin and neuropilins in the sorting of striatal from cortical interneuron populations (Marin et al., 2001). Migrating interneurons destined to the cortex were distinguished from striatal interneurons by neuropilin expression, a receptor absent from interneurons destined for the striatum (Marin et al., 2001). As neuropilin mediates chemorepulsion through semaphorin 3A and 3F, these observations led to the proposed mechanism whereby cortical interneurons are directed to the cortex, in part, as a result of repulsion from the striatum, a source of semaphorin 3A and F (Marin et al., 2001).

Once sorted, interneurons destined to the cortex are directed by a series of molecular guidance pathways. For example, genes in the Hepatocyte Growth Factor (HGF), and GDNF/GFRα1, pathways have been implicated in mediating neuronal migration as ligands have been shown to influence migration interneurons migration *in vitro*, and mice deficient for ligands of receptors have shown aberrant distribution of calbindin labeled cortical interneurons *in vivo* (Polleux et al., 2002; Powell et al., 2001). As migration was not entirely disrupted in these mutants, these factors are likely to mediate migration of subtypes of cortical interneurons.

In addition to the initial identification of pathways influencing migration, many other known families of classical neuronal guidance molecules have been shown to be expressed in the ventral telencephalon and along the route of migration (Table 1-2). New research continues to emerge demonstrating and hypothesizing roles for these genes. First, a role for the Slit/Robo has recently been indicated in influencing the migratory route of cortical interneurons. In the absence of Robo-1 increased calbindin interneurons were observed in ventral regions normally restrictive for cortical interneurons supporting the hypothesis that the Slit/Robo pathway mediates early migration of cortical interneurons through the ventral telencephalon (Andrews et al., 2006). In addition, neogenin has recently been shown to be expressed by the population of calbindin and parvalbumin labeled interneurons and as a result neogenin has been hypothesized to be involved in mediating cortical interneuron migration (Fitzgerald et al., 2006). Finally, well characterized pathways mediating migration in other organ systems have also been implicated in mediating migration of cortical interneurons. The SDF-1/ Cxcl12 chemokine, and its cognate receptor Cxcr4, well known for mediating leukocyte chemotaxis have been implicated in the final stages of cortical interneuron migration (reviewed in (Patrussi and Baldari, 2008)). Indeed, disruption of the Cxcl12/cxcr4 signaling demonstrated that this pathway contributes to proper interneuron positioning in the cortex by directing their exit from their marginal and intermediate zone trajectories (Li et al., 2008; Lopez-Bendito et al., 2008).

Collectively these studies demonstrate that multiple pathways contribute to interneuron migration and our understanding of the mechanisms governing interneuron migration continues to evolve. While the genetic pathways that ultimately regulate expression of guidance molecules are poorly defined (reviewed in (Marin and Rubenstein, 2003)), there is reason to believe, however that the cell cycle influences the migratory capabilities of interneurons. Much like cortical projection neurons, the timing of cell cycle exit is an internal factor which ultimately impacts on final destination as earlier born interneurons have been shown to populate deeper cortical layers, while later born interneurons populate superficial layers (Fairen et al., 1986; Miller, 1985; Peduzzi, 1988).

A central concept in this thesis is the discovery that the cell cycle regulatory Rb tumour suppressor exhibits a cell autonomous role in mediating neuronal migration of a population of cortical interneurons (Chapter 2)

2.0 OVERVIEW- Rb MOLECULAR FUNCTION

Arguably the most important decision a cell makes during development is the decision to divide. The cell cycle regulatory retinoblastoma tumour suppressor protein (Rb) is considered to be a key regulator of the G_1/S transition, which, in essence, represents a stage of commitment for a cell to engage along the division pathway. Our knowledge regarding the molecular function of the cell cycle regulatory Rb gene came about through the study of cancer, and indeed Rb holds a special place in the cancer field as it was the first tumour suppressor identified (reviewed in (Weinberg, 1991)). The approximate seven year period following the discovery of Rb, was characterized by a flurry of research activity aimed at understanding Rb molecular function in the context of cancer. The study of Rb binding partners, both viral and cellular, and regulation of Rb activity, formed the basis of our understanding of the role of Rb, not only in carcinogenesis, but also as a universal cell cycle regulator in normal cellular proliferation. Rb ultimately acts as a gatekeeper through the G_1/S phase of the cell cycle by regulating activity of E2F transcription factors which act to regulate transcription of genes required for S-phase progression (reviewed in (Dyson, 1998)). While inhibition of E2F transcription can come about through simple association with E2F, in addition, Rb has been shown to mediate active transcriptional repression through interaction with chromatin modifying proteins (reviewed in (Harbour and Dean, 2000)). Regulating Rb activity are the cyclins and cyclin dependent kinases, which in turn are regulated by two separate groups of cyclin dependent kinase inhibitors (reviewed in (Besson et al., 2008; Ortega et al., 2002)). The coordinated interaction of these proteins established the model of cell cycle progression through G_1/S in virtually all cells, a model which is still highly relevant today (reviewed in (Weinberg, 1995)) (Figure 1-3). Here the established model of Rb activity is reviewed, in light of its discovery in the context of cancer, followed by a discussion of recent advances which demonstrate that our understanding of Rb function in cell cycle progression continues to evolve and exhibits a greater complexity than the initial model suggested.

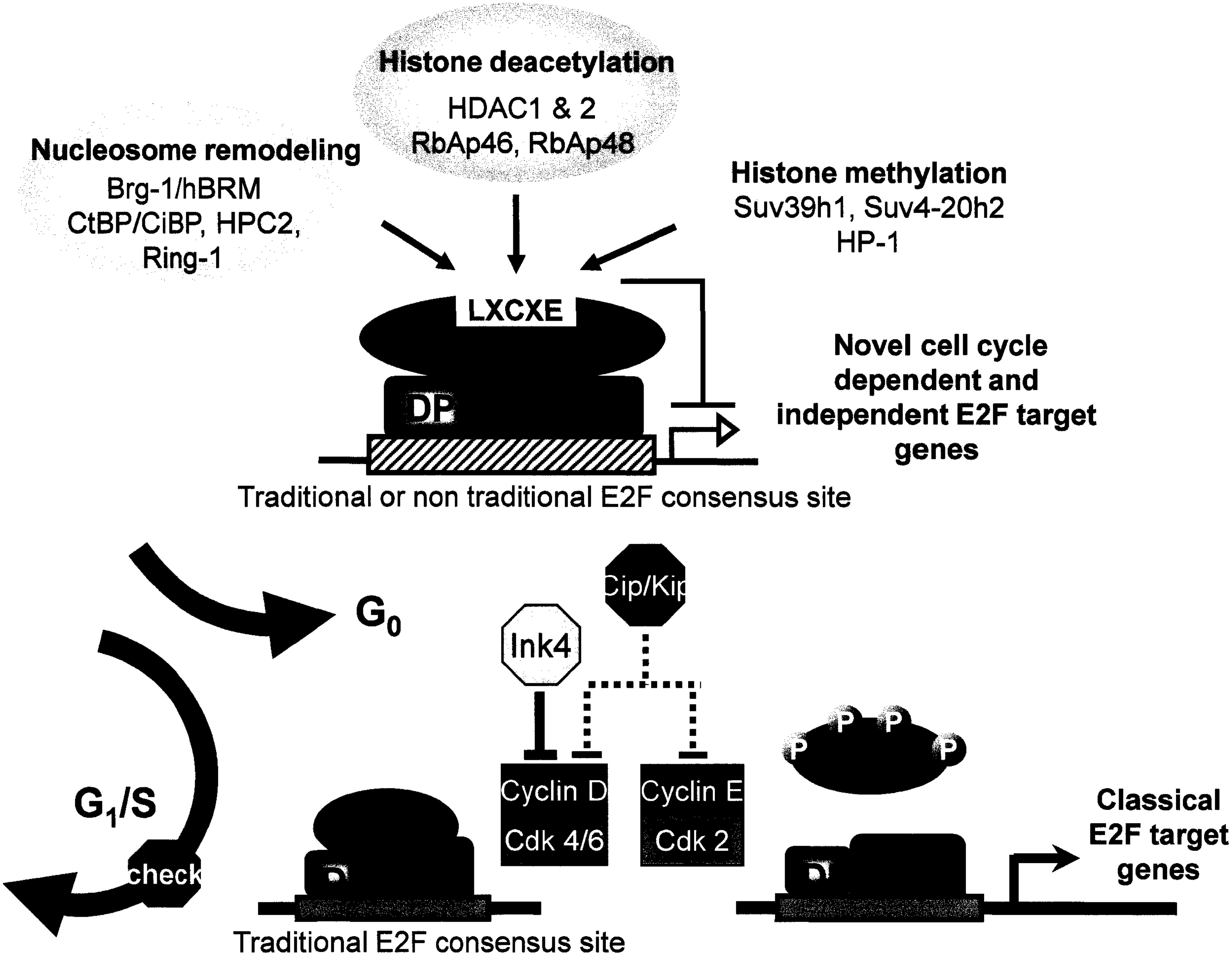
2.1 Rb- IDENTIFICATION OF THE FIRST TUMOUR SUPPRESSOR

Figure 1-3: **Model of Rb molecular function.**

(Lower) Rb/ E2F interaction with the traditional E2F consensus site mediates transcription of classical E2F target genes largely in the context of the G_1/S transition. Regulation of Rb phosphorylation by cyclin dependant kinases (CDK) and CDK Inhibitors mediates Rb association with E2F and thereby transcription of genes required for S-phase.

(Upper) Rb interaction of with chromatin modifying complexes through the LxCxE binding cleft, represents a means of achieving active transcriptional repression of classical E2F target genes. In addition, proteins that bind the LxCxE domain, to modulate chromatin structure, such as genes of the histone deacetylation (blue), histone methylation (pink), and nucleosome remodeling (purple) families, combined with E2F interaction through non traditional consensus sites represents a possible means of achieving specific transcriptional regulation of novel cell cycle dependent and independent genes outside of G_1/S. The two pathways are not mutually exclusive.

Modified from: McClellan & Slack (2007) Cell Cycle 6:2917-27



Nucleosome remodeling
Brg-1/hBRM
CtBP/CiBP, HPC2,
Ring-1

Histone deacetylation
HDAC1 & 2
RbAp46, RbAp48

Histone methylation
Suv39h1, Suv4-20h2
HP-1

LXCXE

DP

Novel cell cycle dependent and independent E2F target genes

Traditional or non traditional E2F consensus site

G_0

G_1/S

check

Ink4

Cip/Kip

Cyclin D
Cdk 4/6

Cyclin E
Cdk 2

P P P P

Classical E2F target genes

Traditional E2F consensus site

Modified from: McClellan & Slack (2007) Cell Cycle 6:2917-27

Interest in what is now referred to as the retinoblastoma protein, Rb, can be traced back to early hypotheses regarding the genetic basis cancer. In the early 1970's, at a time when common cancers were hypothesized to be the result of 3-7 genetic mutations, Alfred Knudson hypothesized that cancer could be the result of as few as two mutational events (Knudson, 1971). Through the study of individual cases of retinoblastoma, a rare childhood eye cancer, Knudson calculated the probability that such tumours arose from two distinct mutational events based on acceptable values of mutation rates by examining the frequency of tumours, and age of onset in cases of both inherited and familial retinoblastoma (Knudson, 1971). These studies provided the basis of the "Knudson two hit hypothesis"; which postulates that in familial cases of retinoblastoma one mutation is inherited from the germline, while the second mutation is somatic (Knudson, 1971). Given the number of cells within the retina, relative to the mutation rate, Knudson calculated that the probability of a second mutation is sufficiently high to account for the dominant pattern of inheritance of susceptibility to retinoblastoma (Knudson, 1971). In cases of sporadic retinoblastoma, however, both mutations are somatic and explain the reduced frequency in cases and frequency of cancerous lesions (Knudson, 1971).

Ultimate proof of Knudson's hypothesis came from subsequent research examining individual cases of retinoblastoma which demonstrated that susceptibility to retinoblastoma could indeed be mapped to a discreet region of the genome- 13q14—the retinoblastoma susceptibility locus, a chromosomal locus mutated or absent within retinoblastomas (Benedict et al., 1983; Cavenee et al., 1983; Cavenee et al., 1985; Godbout et al., 1983). Eventual cloning lead to the identification of the Rb gene as a nuclear phosphoprotein with DNA binding activity whose mRNA or protein was truncated, diminished, or absent in retinoblastoma tumours (Bernards et al., 1989; Dunn et al., 1988; Dunn et al., 1989; Friend et al., 1986; Fung et al., 1987; Hong et al., 1989; Lee et al., 1987a; Lee et al., 1987b). Identification of the Rb gene and its absence in retinoblastoma provided ultimate proof of Knudson's hypothesis (reviewed in (Weinberg, 1991)).

With the identification of Rb gene and its localization to the chromosome 13q locus, Rb activity was subsequently observed to be absent or altered not only in retinoblastoma tumours, but also in small cell lung carcinoma, bladder cancer, breast cancer, prostate cancer, osteosarcoma and cervical cancer; (Bookstein et al., 1990; Dyson et al., 1989; Friend et al., 1986; Fung et al., 1987; Harbour et al., 1988; Horowitz et al., 1990; Lee et al., 1988; T'Ang et al., 1988; zur Hausen, 1991). Thus discovery of Rb and its association with numerous other cancers represented a second important milestone in cancer research-- proof of existence of a hypothesized class of cancer genes now referred to as tumour suppressors (reviewed in (Weinberg, 1991)). Whereas the role of cellular oncogenes, genes whose activation or overactivation contribute to cancer, was well appreciated, discovery of the Rb gene demonstrated the existence of a second class, the so called anti-oncogenes or tumour suppressor, genes whose absence leads to cancer (reviewed in (Weinberg, 1991)).

Thus the initial study of retinoblastoma and discovery of the Rb gene and its association with numerous cancers, in addition to shedding light on the molecular basis of cancer, also established that the retinoblastoma protein is a molecule of critical importance in preventing tumorigenesis, in a broad sense. Indeed, deregulation of Rb pathway has been hypothesized to occur in virtually all human tumours, thus our understanding of the role of Rb as a tumour suppressor and cell cycle regulator has been an important area of research (reviewed in (Nevins, 2001; Sherr, 2000)). As a result of its discovery and association with cancer, however, the caveat which is now evident is that knowledge and understanding of Rb molecular function has been studied almost exclusively in a cancer context.

2.2 Rb PROTEIN STRUCTURE LENDS INSIGHT INTO Rb MOLECULAR FUNCTION

The basis of our understanding of Rb function came about initially through the characterization of Rb structure. The Rb protein is a 110 KDa nuclear phosphoprotein and member of the so-called pocket protein family of cell cycle regulators, comprised of Rb, and related family members p107, and p130 (reviewed in (Classon and Dyson, 2001; Classon and Harlow, 2002)). Homologues of Rb have been observed in many species in addition to humans, including mice, chicken, xenopus, zebrafish, *D. melanogaster*, *C. elegans*, maize, *Arabidopsis* and the single cell algae, *Chlamydomonas* (Bernards et al., 1989; Boehmelt et al., 1994; Destree et al., 1992; Du et al., 1996; Duffy et al., 2005; Feinstein et al., 1994; Grafi et al., 1996; Lu and Horvitz, 1998; Umen and Goodenough, 2001; Vandepoele et al., 2002). Much like the study of retinoblastoma cancer lead to the discovery of the gene and identification of tumour suppressors, early insights about Rb molecular structure and function also came about in the context of cancer research through the study of transforming DNA tumour viruses. Viral oncoproteins such as Adenovirus E1A, human papilloma virus (HPV) E7 protein, and SV40 large T antigen were each known for their potent abilities in transforming primary cells into neoplastic cells (reviewed in (Eckhart, 1969; Frisch and Mymryk, 2002; Galloway and McDougall, 1989)). Subsequent to its cloning and identification as a nuclear protein, Rb was shown to exist in stable complexes with E1A, HPV E7, and SV40 large T antigen in cells transformed with the respective tumour viruses (DeCaprio et al., 1988; Dyson et al., 1989; Munger et al., 1989; Whyte et al., 1988). The significance of these interactions was further appreciated when it was demonstrated that the regions required for transforming function by E1A are the same regions where Rb and E1A interact leading to the hypothesis that Rb in fact was the viral oncoprotein cellular target required for mediating transformation (Whyte et al., 1989).

Through crystal structure and deletion mapping studies, the key functional domains of the Rb protein have been described (Figure 1-4). The A and B domains are two key domains which associate to form the 3-D pocket domain. This pocket has been observed to be highly conserved across all species examined and is also shared with p107 and p130 (reviewed in (Dick, 2007; Lee et al., 1998)). The significance of the pocket domain was appreciated through both biochemical and physiological

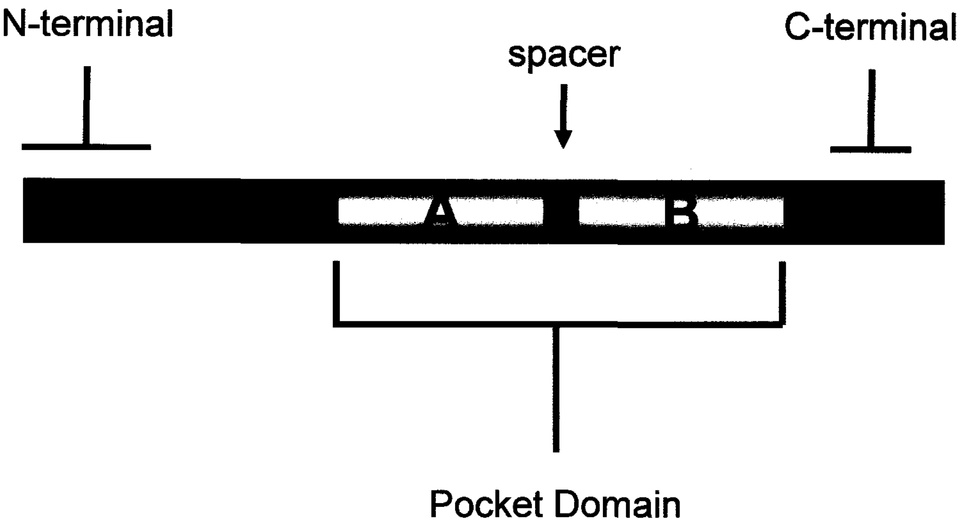
Figure 1-4: **Rb Structural Domains**

(Upper) Select Rb structural domains are depicted. Note the “A” and “B” domains which, together with the non conserved spacer region, comprise the pocket domain.

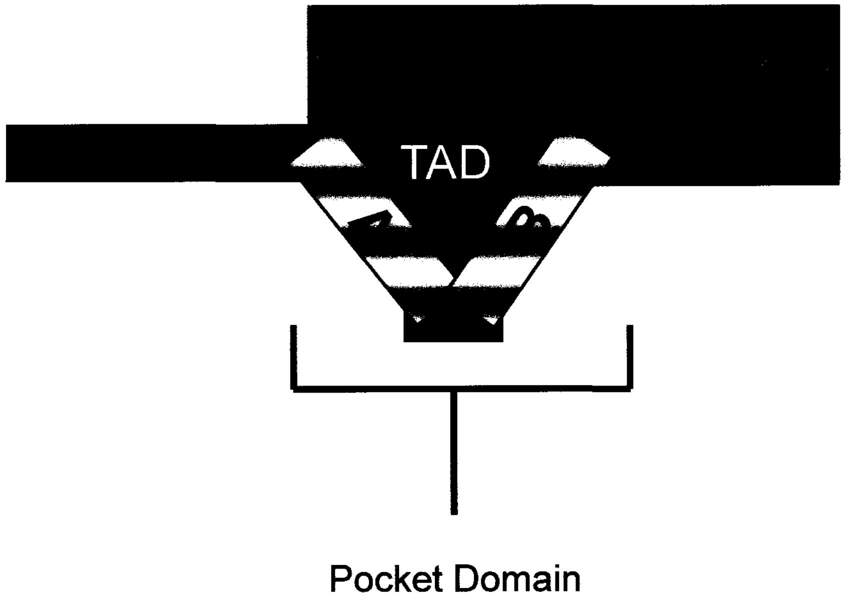
(Lower) General diagram of Rb bound to E2F depicting interaction of the E2F transactivation domain with the Rb pocket. Note: E2F interacts with Rb through both the pocket and C-terminal domain. TAD= transactivation domain.

Modified from: (Dick, 2007)

Rb structure



Rb bound to E2F



observations. Deletions or mutations to either the A or B domain abolished viral oncoprotein interaction with Rb *in vitro* (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990) (Qin et al., 1992). Further, retrospective examination of documented Rb mutations arising in a variety of cancers were observed to occur in the pocket domain (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990; Lee et al., 1998). Deletion and crystal structure studies have shown that viral oncoproteins contain an "LxCxE" peptide motif through which they bind to a conserved groove within the B box (reviewed in (Dick, 2007; Lee et al., 1998)). Finally, while previously overlooked, new data has emerged pointing to the significance of the N-domain (Hassler et al., 2007). Also frequently mutated in cancer (reviewed in (Hassler et al., 2007)), the crystal structure of the N-terminal domain is shown to interact with the pocket supporting a revised model whereby the N- terminal domain is capable of acting as a "gate" and regulating interaction with the pocket (Hassler et al., 2007).

While Rb functional domains were initially described in the context of viral oncoprotein binding, cellular proteins which bind to Rb were shown to do so through interaction with same domains to mediate two major functions: inhibition of transcriptional activation of E2F transcription factors and active transcriptional repression through chromatin binding proteins. Most notably, the E2F transcription factor binds to Rb by interacting with the pocket domain and C- terminal domain to mediate growth suppression (Lee et al., 2002; Qin et al., 1992; Rubin et al., 2005). Further, in addition to viral oncoproteins, several other cellular proteins contain LxCxE domains and are capable of binding to Rb (reviewed in (Dick, 2007; Goodrich, 2006; Morris and Dyson, 2001). Many of these LxCxE containing proteins belong to families of chromatin modifying proteins such as histone deacetylation, histone methylation and nucleosome remodeling (reviewed in (Morris and Dyson, 2001)). Coordinate interaction of these proteins with Rb has been shown to mediate active transcriptional repression through chromatin modification (Fig 1-3).

2.3 MECHANISM OF ACTION 1: INHIBITION OF E2F MEDIATED TRANSCRIPTIONAL ACTIVATION

Much like the identification of the Rb gene and protein structure were examined in the context of cancer research, valuable insight into Rb molecular function came indirectly through the study of the mechanism of activation of adenovirus gene expression by viral oncoproteins. Through the examination of how E1A activates transcription of genes from the E2 promoter, a cellular factor was identified (Kovesdi et al., 1986a; Kovesdi et al., 1986b; La Thangue and Rigby, 1987). This transcription factor, E2F, which exhibited increased DNA binding activity in E1A infected cells, was nevertheless a cellular protein, present in untransformed cells as part of a complex of proteins at the E2 promoter (Kovesdi et al., 1986a; Kovesdi et al., 1986b; La Thangue and Rigby, 1987). The mechanism by which E1A mediates cellular transformation was shown to be indirect whereby E1A disrupts E2F association with cellular proteins thereby liberating it to activate gene expression, and drive cells into S-phase for viral replication (Kovesdi et al., 1986a; Kovesdi et al., 1986b; La Thangue and Rigby, 1987). Subsequent to its discovery, the sequences bound by E2F in the E2 adenoviral promoter were used to identify potential cellular target genes regulated by E2F (Hamel et al., 1992; Hiebert et al., 1989; Mudryj et al., 1990; Thalmeier et al., 1989). E2F sites were found predominantly in the promoter regions of a large number of genes involved in the mechanics of cell division. Genes which are induced by E2F activity can be grouped into genes that regulate cell growth such as dihyrofolate reductase, DNA replication such as DNA polymerase α, thymidine kinase, thymidylate synthase; cell cycle progression such as cyclin E, cyclin A, p107, Rb, b-myb, E2F, and c-myc (reviewed in (Dyson, 1998; Helin, 1998). The observation that these genes are induced in response to serum stimulation established an important link between E2F transcription and cell cycle control (reviewed in (Dyson, 1998; Helin, 1998).

Later the cellular factor shown to interact and inhibit E2F activity was identified as unphosphorylated Rb (Bandara and La Thangue, 1991; Chellappan et al., 1991), with E1A capable of dissociating Rb from E2F (Bandara and La Thangue, 1991; Chellappan et al., 1991). As the sequences of E1A required for dissociating Rb from E2F were the same sequences required for E1A mediated transformation, a model emerged whereby Rb dissociation from E2F represents part of the mechanism by which E1A induces transformation (Bandara and La Thangue, 1991; Chellappan et al., 1991). The significance of this interaction also had implications for the understanding of normal cell division when it was shown that Rb-E2F interaction coincides with inhibition of E2F transcriptional activity and the nature of the interaction varied cyclically with normal cell cycle progression (Chellappan et al., 1991; Hiebert et al., 1992; Weintraub et al., 1992).

Thus combined, these observations of E2F as a transcription factor mediating transcription of cell cycle related genes, and the cyclical association of Rb with E2F which in turn mediates transcriptional activity formed the basis of our understanding of how Rb can act as a regulator of cell proliferation. While initially studied in the context of cancer, these studies highlighted the importance of Rb-E2F in the context of normal cellular proliferation. Indeed, Rb mediated inhibition of E2F activation, is thought to regulate passage trough G_1/S in the majority of proliferating cells, and disruption of this association is thought to underlie the molecular mechanism of Rb mediated tumour suppression (reviewed in (Dyson, 1998; Weinberg, 1995)).

2.4 MECHANISM OF ACTION 2: ACTIVE TRANSCRIPTIONAL REPRESSION

In addition to E2F transcription factors, Rb is capable of interacting with a number of different proteins to mediate gene expression and other diverse cellular events (reviewed in (Morris and Dyson, 2001)). Of these Rb binding proteins, several contain an LxCxE peptide motif and bind to Rb in the same functional binding domain as the transforming viral oncoproteins. Many of these LxCxE containing proteins belong to families of chromatin modifying proteins, such as histone deacetlyases (Brehm et al., 1998; Lai et al., 1999; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), histone methyltransferases (Nielsen et al., 2001), and members of the SWI/SNF ATP-dependent nucleosome remodeling complexes (Dunaief et al., 1994; Strober et al., 1996; Trouche et al., 1997) and have been shown to play important roles in mediating E2F regulated gene expression.

Similar to the direction of the initial work characterizing Rb and E2F function, the functional significance of Rb interaction with chromatin modifying proteins has been investigated largely in the context of cell cycle regulation through *in vitro* experiments. For example, Rb has been shown to interact with histone deacetylase 1 (HDAC1), and Brg1/hBRM, members of the SWI/SNF nucleosome remodeling complex, to repress transcription of classical E2F target genes, most notable cyclin E (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Zhang et al., 2000). This repression required functional deacetylase activity, as inhibiting deacetylase activity relieved Rb mediated repression of cyclin E transcription (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Zhang et al., 2000). In addition, histone methylation represents another example of how Rb interaction with chromatin modifying enzymes can mediate transcriptional repression of E2F genes. Here Rb was shown to exist in a complex with Suv39h1, a histone methyltransferase, and heterochromatin protein 1 (HP1) a methylated histone binding protein (Nielsen et al., 2001). Together, Suv39h1 and HP1 were shown to associate with Rb to repress E2F mediated transcription of cyclin E in fibroblasts *in vitro* through methylation of histones at the cyclin E promoter (Nielsen et al., 2001). Thus collectively, these studies established roles for Rb/E2F in influencing chromatin structure through active transcriptional repression as a way of regulating transcription at the G_1/S transition.

2.5 REGULATION OF RB ACTIVITY THROUGH REGULATED PHOSPHORYLATION

The ubiquitous expression of Rb throughout the mouse embryo, and in a number of adult tissues, in particular, liver, brain, placenta raised the question as to how Rb activity is in turn regulated (Bernards et al., 1989; Lee et al., 1987b). Again, the mechanism by which Rb activity is regulated was initially established largely through the study of Rb interaction with DNA tumour virus proteins. Rb was initially identified as a nuclear phosphoprotein, thus in an effort to understand how the interaction of SV40 T antigen with Rb regulated cell growth, the ability of Rb association with T antigen was examined in relation to Rb phosphorylation status (Ludlow et al., 1989). Despite the cellular coexistence of phosphorylated and unphosphorylated Rb, T antigen was shown to associate, and mediate transformation, through binding unphosphorylated Rb. This led to the hypothesis that Rb phosphorylation status relates to its ability to regulate cell growth (Ludlow et al., 1989).

The significance of Rb phosphorylation in the context of the normal cell cycle was subsequently demonstrated through the following observations. First, Rb was shown to be synthesized throughout the cell cycle with Rb existing in an unphosphorylated state in G_0 and G_1, yet existing in a phosphorylated state through S, G_2 and M-phases (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). Later, the consequences of this cyclical phosphorylation were appreciated when it was shown that only unphosphorylated Rb binds to E2F and in effect, phosphorylation status of Rb dictates E2F transcriptional activity (Chellappan et al., 1991; Hiebert et al., 1992; Weintraub et al., 1995; Weintraub et al., 1992)

Phosphorylation of Rb takes place during mid to late G_1 and is thought to mediate passage through the restriction point (reviewed in (Sherr, 1996; Weinberg, 1995)). As many as 21 different phosphorylation sites have been described, 16 of which are potential cyclin/Cdk sites (reviewed in (Dick, 2007)). Mitogen responsive D type cyclins were the first cyclins shown to associate with Rb and cooperate with Cdk4/6 to induce Rb phosphorylation (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993). While cyclinD- Cdk4/6 phosphorylation is on its own insufficient to induce Rb dissociation from E2F, cyclinD and Cdk4/6 phosphorylation were shown to facilitate cyclinE-Cdk2 phosphorylation within the Rb pocket domain (Harbour et al., 1999; Lundberg and Weinberg, 1998). The sequential phosphorylation by cyclinD-Cdk4/6 followed by cyclinE-Cdk2 results in Rb dissociation from E2F thereby activating gene expression and ultimately driving S phase (reviewed in (Gil and Peters, 2006)). The significance of all 21 sites and whether all require phosphorylation for Rb/E2F dissociation to occur remains unknown.

Regulating Cdk activity and in turn, Rb function, are the intertwined activities of two families of inhibitors, the Cip/Kip family and the INK4 family. The Cip/Kip family, comprised of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, are strong inhibitors of cyclinE- Cdk2 activity (reviewed in (Sherr and Roberts, 1999)). Their inhibition on the cell cycle, however, is complex as upon mitogen stimulation, $p21^{Cip1}$ and $p27^{Kip1}$ are actually found in association with D cyclins and Cdks, where their presence has been shown to promote stability of the cyclinD-Cdk4 complex (Cheng et al., 1999; LaBaer et al., 1997). It is thought that in this context, cyclinD-Cdk4/6 acts as a buffer for Cip/Kip activity, effectively sequestering Cip/Kip from cyclin E, thereby allowing S-phase to proceed (reviewed in (Gil and Peters, 2006)). Actual Cip/Kip mediated inhibition comes about as a consequence of disruption of the cyclinD-Cdk -Cip/Kip complexes (reviewed in (Gil and Peters, 2006)). First, mitogen withdrawl leads to cyclinD degradation resulting in an increase of the levels of free Cip/Kip which are then able to inhibit cyclinE-Cdk2 mediated phorphorylation of Rb, thereby promoting G_1 arrest (reviewed in (Gil and Peters, 2006)). Second, activation of the second family of inhibitors, the INK4 family also serves to mediate cell cycle arrest. The INK4 family, comprised of four members, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, that are activated in response to various growth arrest stimuli (reviewed in (Sherr, 2001)). INK proteins are thought to mediate G_1 arrest by disrupting the cyclinD-Cdk4 complex (reviewed in (Sherr, 2001)). In this conformation, not only is the Cdk-INK4 complex catalytically inactive unable to phosphorylate Rb, this resulting complex causes release of associated Cip/Kip inhibits—which effectively inhibits cyclinE-

Cdk2 mediated Rb phosphorylation (reviewed in (Sherr, 2001)). Indeed a direct link between Ink4-Cdk-Rb pathway is that the ability of Ink4 to arrest cell cycle depends on the integrity of functional Rb (Guan et al., 1994; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995).

2.6 COMPLEXITY OF RB FUNCTION IN CELL CYCLE PROGRESSION

Whereas the above model provides a clear and general model as to how Rb orchestrates progression through the G_1/S transition, what is now emerging is that Rb exhibits far greater complexity and diversity of function in regulating the cell cycle than first appreciated (reviewed in (Goodrich, 2006). Rb has been shown to bind over 150 different proteins (reviewed in (Goodrich, 2006; Morris and Dyson, 2001), a list which continues to grow. While the authenticity of many of these interactions has been questioned as they were observed under artificial conditions, new research has demonstrated that through novel mechanisms, our understanding of Rb function, as a tumour suppressor, in cell cycle regulation, and beyond, is still developing (reviewed (Goodrich, 2006; Morris and Dyson, 2001)). Recent studies addressing new Rb interacting proteins in the context of Rb phosphorylation, the role of Rb in mediating chromatin methylation, and the mechanism by which Rb regulates E2F activity, are three examples which represent clear evidence that challenge the conventional dogma of Rb function in cell cycle progression.

First, two exceptions have emerged suggesting that our understanding of phosphorylation of Rb at the G_1/S transition is incomplete. Namely, kinases other than the cyclin-Cdk family are capable of mediating Rb phosphorylation in the context of G_1/S, and Cdk2, 4, and 6 are dispensable for Rb phosphorylation in early cell division (Dasgupta et al., 2004; Santamaria et al., 2007; Wang et al., 1998). Raf-1 kinase, a member of the Map kinase pathway has been shown to bind and phosphorylate Rb upon mitogenic stimulation, independent of cdk activity (Dasgupta et al., 2004; Wang et al., 1998). This interaction resulted in Rb inactivation and reversal of Rb mediated repression of E2F transcription (Wang et al., 1998). Further, preventing this interaction in proliferating cells prevented subsequent Rb phosphorylation and significantly reduced cell cycle progression *in vitro* (Wang et al., 1998). As Raf-1 association with Rb preceded Rb association with cyclins, it has been proposed that Raf-1 mediated phosphorylation of Rb is required to facilite subsequent cyclin cdk mediated phosphorylation (Dasgupta et al., 2004). Next, while cdk 2, 4, and 6 are thought to be required for relieving Rb mediated repression of E2F transcription factors, the minimum cdk activity required to drive the cell cycle appears to be context dependent as mouse embryos lacking all four interphase Cdks 2,3,4 and 6 are able to survive until midgestation (Santamaria et al., 2007). In this model, Cdk1 alone was capable of phosphorylating Rb and sufficient for driving the cell cycle in early dividing cells supporting the hypothesis that Rb phosphorylation is tissue specific (Santamaria et al., 2007).

Next, in an elegant series of experiments, a novel role for Rb in regulating progress through M phase has been described. In this study, a transgenic mouse was engineered in which the LxCxE binding domain within Rb is mutated (RbΔL), yet Rb/E2F interaction is preserved (Isaac et al., 2006). In these animals, which are viable and fertile, RbΔL expression levels in MEFs are similar to controls, and RbΔL interaction with E2F transcription factors is indistinguishable from wild-type, however, interactions with chromatin modifying enzymes such as HDAC1 and 2; RbAp48, a component of the HDAC complex; and the Brg1/hBRM co-factor CtBP, are defective (Isaac et al., 2006). *In vitro* analysis of MEFs cultured from these animals reveals that in contrast to Rb -/- progress through G1 is normal, however, RbΔL mutant MEFs exhibit an increased proportion of cells in G_2/M and a higher incidence of aneuploid cells(Isaac et al., 2006). The cause of aneuploidy was attributed to reduced H4-K20 trimethylation of pericentric heterochromatin resulting in centromere fusions and chromosome missegregation, events occurring after S-phase (Isaac et al., 2006). Hence these results point to a unique role for Rb in regulating distinct cell cycle events beyond G_1/S in an LxCxE dependent manner.

Finally, while unphosphorylated Rb is thought to prevent passage through G_1/S by remaining bound to E2F—it is emerging that this regulation is more complex. Preliminary studies revealed that re-expression of Rb in Rb deficient cells led to G_1 arrest (Ji et al., 2004). Rather than being the result of repression of E2F target genes, instead arrest depended on early accumulation of $p27^{Kip1}$ and suggested that additional mechanisms are involved (Ji et al., 2004). Indeed, recent studies have shown that Rb interacts directly with the APC/C^{Cdh-1}, a ubiquitin ligase complex that indirectly regulates levels of $p27^{Kip1}$ by ubiquitinating Skp2, a component of the SCF^{Skp2} complex which targets $p27^{Kip1}$ for degradation(Binne et al., 2007). By interacting with APC/C^{Cdh-1}, unphosphorylated Rb actively promotes degradation of Skp2 leading to the inactivation of SCF^{Skp2} complex. The net effect is an increase in $p27^{Kip1}$ activity which, in turn, results in increased levels of unphosphorylated Rb and cell cycle arrest (Binne et al., 2007). Hence in this model, Rb plays more of an active and sophisticated role than previously appreciated by influencing its own activity in turn through regulating the stability of its kinase inhibitor.

These examples together paint an increasingly expanded and complex picture of Rb activity, beyond what the initial model suggested. They also serve to remind us of the dynamic nature of our understanding of Rb function at the molecular level. ***Central to this thesis is the theme that the Rb/E2F pathway exhibits functions beyond what the traditional model of Rb function at the G1/S transition would suggest.***

3.0 OVERVIEW E2F TRANSCRIPTION FACTORS

Like Rb, much of our current understanding regarding E2F function has been shaped largely as a result of its initial discovery and characterization as an adenovirus E2 promoter binding factor

(Kovesdi et al., 1986a; Kovesdi et al., 1987) (reviewed in (Dyson, 1998)). Subsequent to its discovery, the observation that E2F interacts with the cell cycle regulatory and tumour suppressor, Rb protein (Bagchi et al., 1991; Bandara and La Thangue, 1991; Chellappan et al., 1991; Chittenden et al., 1991) led to the search for E2F responsive genes which could be influencing cell cycle progression. This directed search resulted in the characterization of what we now consider to be classical E2F responsive genes, a list which includes genes required for cell cycle progression and DNA synthesis (reviewed in (Dyson, 1998; Trimarchi and Lees, 2002)). Together these observations established, early on, the model of E2Fs as activators or repressors of cell cycle progression whose activity is regulated through interaction with Rb family proteins (reviewed in (Dyson, 1998; Farnham, 2006)). New research, however, has challenged the simple view by highlighting exceptions to the initial classification. Further, in part as a result of the diversity of the E2F family, two new questions have emerged. The first issue is concerned with determining whether despite binding similar recognitions sites in target genes, how are E2Fs able to achieve specificity of function. Also at issue is whether E2Fs, collectively, are indeed capable of regulating expression of genes beyond their classical repertoire of genes related to the mechanics of cell division. Here we provide a brief overview of the E2F family and their general function in transcriptional regulation, followed by a more in depth discussion surrounding the specificity of E2F function and scope of transcriptional target debates that have been examined in *in vitro* contexts. Hence our understanding of E2F transcription factors is expanded in light of both breadth and intricacy of function.

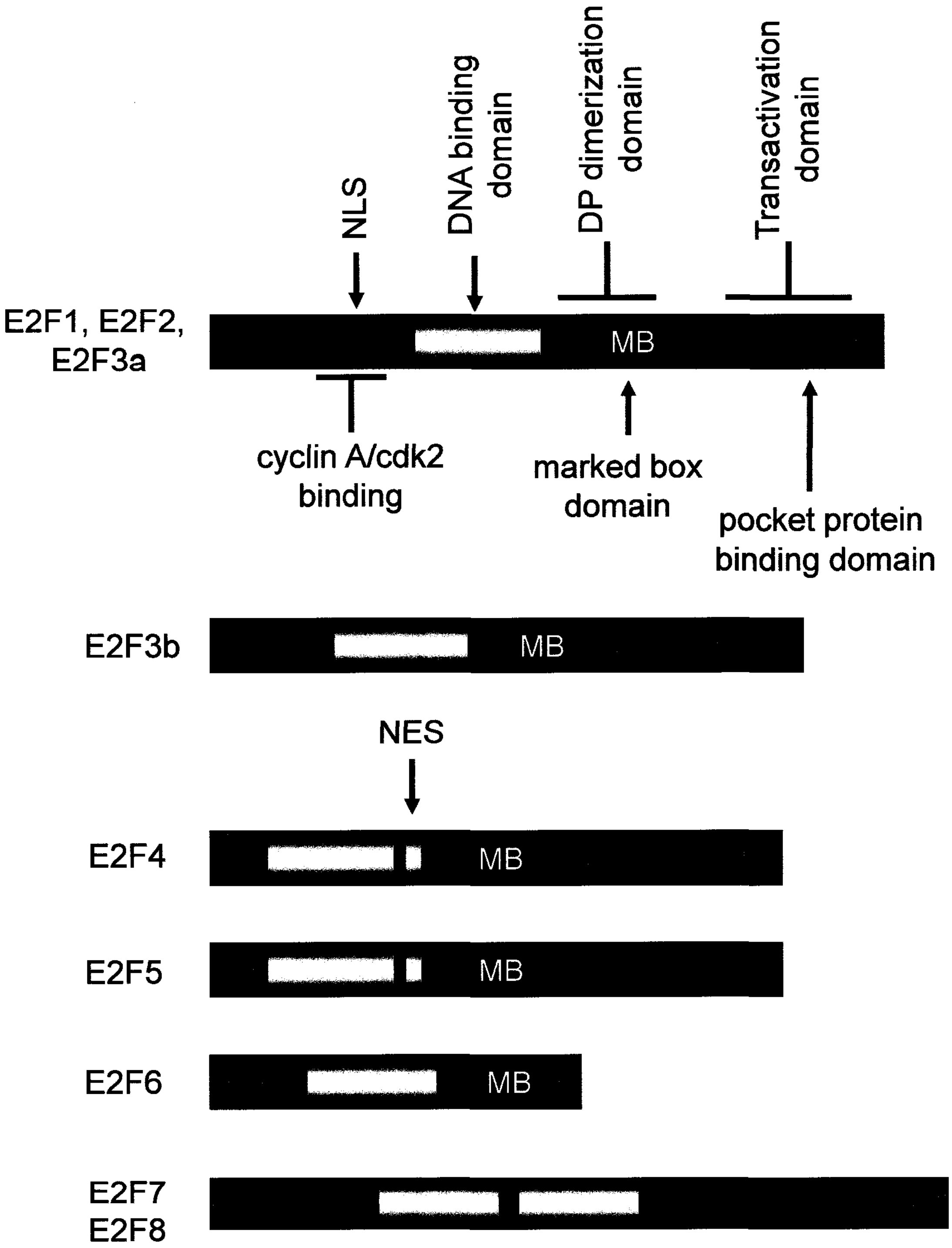
3.1 THE FAMILY OF E2F TRANSCRIPTION FACTORS

E2F collectively refers to a group of eight transcription factors (Fig 1-5) most recognized for their ability to regulate cell cycle progression (Table 1-3). Grouped into categories, E2F1, 2 and 3a act

Figure 1-5: **The E2F transcription factor family**

Select domains of each E2F are depicted. Note that the DNA binding domain is conserved across the family. E2F1-6 each contain the "marked box" domain. E2F1-3 and E2F5 each have nuclear localization sequences, while E2F4 and 5 have nuclear export signals (Apostolova et al., 2002; de la Luna et al., 1996; Gaubatz et al., 2001) reviewed in (Trimarchi and Lees, 2002). Figure modified from (DeGregori and Johnson, 2006; Dick and Dyson, 2006; Hallstrom and Nevins, 2003) NLS = nuclear localization sequence, NES =-nuclear export sequence, MB = marked box domain.

Modified from: McClellan & Slack (2007) Cell Cycle 6:2917-27



Modified from: McClellan & Slack (2007) Cell Cycle 6:2917-27

Table 1-3: Broad roles for E2Fs in proliferation

NAME	BROAD FUNCTION IN PROLIFERATION
E2F1	• Each of E2F1, 2 and 3 are capable of activating transcription of E2F responsive genes (reviewed in (Vanderluit, 2006)) • Expression of E2F1 induces S-phase entry in quiescent fibroblasts and cultured myeloid cells (Lukas et al., 1996; Strom et al., 1998) • Together, E2F1, 2 &3 are absolutely required for proliferation as combined absence of E2F1, 2, & 3 results in fibroblasts unable to enter S-phase (Wu et al., 2001)
E2F2	• Expression of E2F2 alone in quiescent fibroblasts induces S-phase entry (Lukas et al., 1996)
E2F3a&b	• E2F3 is required for S-phase entry as depletion of E2F3 in proliferating cells prevents entry into S-phase and E2F3 -/- fibroblasts exhibit reduced proliferation as a result of reduced DNA synthesis and reduced rate of S-phase progression and mitogen induced cell cycle re-entry (Leone et al., 1998) (Humbert et al., 2000b) • E2F3 regulates centrosome duplication and chromatid separation as loss of E2F3 leads to centrosome amplification and aneuploidy (Saavedra et al., 2003) • E2F3b represses Arf in normal MEFs to regulate cell cycle re-entry as E2F3/Arf DKO rescues the cell cycle re-entry defects present in E2F3-/- (Aslanian et al., 2004) • Required for myc induced S-phase in quiescent fibroblasts (Leone et al., 2001)
E2F4 & E2F5	• Individual loss of E2F4 or loss of E2F5 does not result in cell cycle or proliferation defects in MEFs (Rempel et al., 2000) (Humbert et al., 2000a) • Expression of E2F4&5 together with DP-1 induces quiescent fibroblasts into S-phase (Lukas et al., 1996) • Together with E2F5, required for pocket protein mediated G_1 arrest in response to p16^{INK4a}(Gaubatz et al., 2000) • Either E2F4 or 5 are capable of indirect regulation of cell cycle by acting as Smad co-factor to repress c-myc in response to TGFbeta receptor activations (Chen et al., 2002)
Act independently from pocket proteins	
E2F6	• Represses a subset of E2F responsive cell cycle related genes during S-phase (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998) (Giangrande et al., 2004b)
E2F7 & E2F8	• Acts as a transcriptional repressor for a subset of E2F target genes to slow proliferation & delay cell cycle progression at G_1/S (de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004), (Christensen et al., 2005) (Logan et al., 2005; Maiti et al., 2005)

as transcriptional activators by dimerizing with DP proteins and activating transcription through E2F consensus sites on promoters of a large list of genes required for cell cycle progression, including cyclins A & E, p107, dihydrofolate reductase, thymidine kinase, thymidylate synthetase, DNA polymerase α, and even E2F1 itself (reviewed in (Dick and Dyson, 2006; Vanderluit, 2006)). Several hallmark experiments established early on the importance of each of these genes in regulating proliferation. Their expression is cell cycle regulated with peak expression coinciding with G_1 (He et al., 2000; Hsiao et al., 1994; Johnson et al., 1994; Lees et al., 1993; Leone et al., 1998; Leone et al., 2000; Neuman et al., 1994; Sears et al., 1997). Expression studies demonstrated overlapping functions as deregulated expression of E2F1, E2F2, or E2F3 individually is sufficient to induce quiescent cells to enter S-phase (Lukas et al., 1996), while collective ablation of E2F1, 2, and 3 in mouse fibroblasts completely abolishes entry into S-phase, and as a result, proliferation(Wu et al., 2001). E2F4 and 5, however, are thought to act as transcriptional repressors through pocket protein binding (reviewed in (Dick and Dyson, 2006; Vanderluit, 2006)). In addition to being expressed during the cell cycle, E2F3b, E2F4 and 5 are expressed at significant levels in G_0 (He et al., 2000; Leone et al., 2000; Moberg et al., 1996; Sardet et al., 1995). The activities of both the activating E2Fs and the repressive E2Fs are believed to be regulated by pocket proteins with E2F1, 2 and 3 thought to interact primarily with Rb, and E2F5 primarily through p130, while E2F4 is capable of interacting with all three pocket proteins (Ginsberg et al., 1994; He et al., 2000; Hijmans et al., 1995; Leone et al., 2000; Moberg et al., 1996; Vairo et al., 1995) (reviewed in (Dick and Dyson, 2006; Trimarchi and Lees, 2002; Vanderluit, 2006)).

As the list of E2Fs grows and as our knowledge about their function increases, this simple classification of activators and repressors must be revised. Indeed, E2F6, 7 and 8 do not fit into this category as E2F6 does not interact with pocket proteins, while E2F7 and 8 do not interact with either pocket proteins or DP proteins (Cartwright et al., 1998; Christensen et al., 2005; de Bruin et al., 2003; Di Stefano et al., 2003; Gaubatz et al., 1998; Logan et al., 2004; Logan et al., 2005; Maiti et al., 2005; Trimarchi et al., 1998). Moreover, multiple transcripts introduce a new level of complexity whereby E2F3 is thought to be capable of both activation and repression (He et al., 2000; Leone et al., 2000). Next, individual loss of function models reveal that E2F function in regards to proliferation and cell cycle progression is highly tissue and context specific, capable of both unique and combinatorial function with other E2Fs (Table 1-4). The functions described are often paradoxical given the essential roles E2Fs play in mediating pocket protein activity. For example, as the Rb tumour suppressor is thought to repress the activity of E2F1, it should follow that E2F1, itself, behaves as an oncogene. While the E2F1 loss of function mouse model confirms this hypothesis, surprisingly a broad spectrum of tumours in particular tissues such as the lung and reproductive tract were observed, leading to the additional characterization of E2F1 as a tumour suppressor (Yamasaki et al., 1996).

Perhaps some of these surprising results can be explained through evidence which suggest that traditional E2F activators are capable of transcriptional repression, while E2F repressors can behave as activators. For example, the resulting increased proliferation of T-lymphocytes in the absence of E2F2 is thought to be the result of a loss of E2F2 mediated repression of cell cycle genes such as E2F1 in the lymph node (Murga et al., 2001). A role in transcriptional repression has also been shown for E2F1, where E2F1 itself is capable of direct repression of Mcl-1, an anti-apoptotic Bcl-2 family member (Croxton et al., 2002). The E2F4 transcriptional repressor has also been hypothesized to be capable of transcriptional activation, based largely on ectopic expression studies (Chen et al., 2004b; Deschenes et al., 2004; Ebelt et al., 2005; Gill and Hamel, 2000; Wang et al., 2000). This hypothesis is further supported by new observations from E2F4 loss of function models. For example, while E2F4 is thought to repress expression of Ccna2 in fibroblasts, the absence of

Table 1-4: Tissue Specific Roles for E2Fs in proliferation

NAME	TISSUE SPECIFIC FUNCTION IN PROLIFERATION
E2F1 & E2F2	• Required for myc induced S-phase in quiescent fibroblasts (Leone et al., 2001) • Combined absence of E2F1/2 leads to reduced S-phase progression in bone marrow derived cells (Li et al., 2003) • Capable of repressing proliferation of T-lymphocytes, as absence of E2F2 alone leads to enhanced proliferation of T cells (Murga et al., 2001) • Capable of acting as a repressor of genes required for S-phase in Jurkat T cells (Zhu et al., 2001) • Combined absence of E2F1/2 leads to enhanced proliferation of T cells in response to subthreshold antigen induced stimuli, but defective in S-phase progression for homeostasis driven T cell proliferation (DeRyckere and DeGregori, 2005) • Overexpression in thymus leads to unscheduled division and oncogenic transformation of thymic epithelial cells (Scheijen et al., 2004) • E2F1/2 DKO exhibit increased numbers of replicating cells in postnatal pancreas (Iglesias et al., 2004)
E2F3a&b	• Expression of E2F3 alone induces S-phase entry in fibroblasts but not in cultured myeloid cells (Lukas et al., 1996; Strom et al., 1998) • Expression of E2F3a induces cell cycle re-entry in lens fibre cells (Chen et al., 2004b) • Transgenic expression of E2F3a in the epidermis leads to hyperproliferation (similar to E2F4 & 1) (Paulson et al., 2006) • Positive regulator of neural precursor proliferation as loss of E2F3 leads to reduction of neural precursor cells (McClellan et al., 2007) • Oncogenic in bladder cancer as it is frequently amplified and knockdown of E2F3 in bladder cells reduces proliferation (Oeggerli et al., 2006; Oeggerli et al., 2004) (Olsson et al., 2007)
E2F4	• Presence of E2F4 on promoters of E2F responsive genes in erythroid cells suggests that E2F4 is capable of transactivation of genes required for cell cycle progression (Kinross et al., 2006) • Absence of E2F4 leads to a delay through late S-phase and/or G2 of cell cycle and reduced proliferation of erythroid cells (Kinross et al., 2006)

E2F4 in erythroid cells leads to decreased Ccna2 expression, suggestive of a transcriptional activation relationship (Kinross et al., 2006). Indeed, ChIP studies demonstrate the presence of E2F4 at the Ccna2 promoter in erythroid cells at the time of activation further supporting the hypothesis that E2F4 can function as an activator (Kinross et al., 2006). Hence these data serve not only to challenge the early classification of E2F function, collectively they suggest that E2Fs exhibit far greater complexity and diversity of function than initially hypothesized.

3.2 ARE E2Fs REDUNDANT OR CAPABLE OF UNIQUE FUNCTION?

Given the apparent complexity, a prevalent question is how specificity of function among classical E2F family members (E2F1-5) is achieved. Initially, structural studies revealed no differences in the amino acid sequences among E2Fs and DP proteins within their DNA binding and dimerization domains, suggesting that all are equally capable of recognizing traditional E2F consensus sites within promoters (Zheng et al., 1999). The notion that individual E2Fs did not exhibit preference for individual promoters was further strengthened by initial chromatin immunoprecipitation (ChIP) studies where prevalence of particular E2Fs at given promoters was shown to be dependent on cell cycle stage and pocket protein interaction rather than E2F identity (Takahashi et al., 2000; Wells et al., 2000). Together with the observation that E2Fs exhibit tissue and cell type specific expression patterns during development (Dagnino et al., 1997a; Dagnino et al., 1997b), these data supported a hypothesis that spatial and temporal regulation of individual E2F activity, combined with differential pocket protein interaction determines specificity of function (Dagnino et al., 1997a; Dagnino et al., 1997b).

While elements of this hypothesis still remain true, it is now emerging that individual E2Fs are inherently capable of unique functions, irrespective of time and space regulation. This has been illustrated with E2F1 and 3 at the molecular level where each has been shown to interact with specific factors to mediate either transcriptional activation or regulation of cell death. Specificity among E2Fs in mediating transcription has been shown for E2F3, which interacts with the E-box factor, TFE3, to activate transcription of the p68 subunit of DNA pol α, and ribonucleotide reductase 1 & 2. This effect was observed to be specific to E2F3 as E2F1 was not shown to interact with TFE3 or capable of activating transcription of these genes (Giangrande et al., 2003; Giangrande et al., 2004a). As E2F3 and TFE3 are each capable of activating transcription individually, yet when bound together regulate transcription of a distinct group of genes, this led to the proposal that combinatorial interaction could represent a mechanism for achieving specificity in E2F mediated transcription(Giangrande et al., 2004a). Similarly specific function has been demonstrated for E2F1. Jab-1, a c-jun activation domain binding protein, interacts specifically with E2F1 to mediate cell death-- an interaction not observed with other E2Fs (Hallstrom and Nevins, 2006). Remarkably, in both instances, specificity of function can be isolated to a region within each E2F protein known as the "marked box" domain, a region adjacent to the DNA binding and DP dimerization domains (Fig 1-5). In the case of E2F3, absence of the marked box domain eliminates the ability of E2F3 to interact with TFE3 and synergistically activate transcription (Giangrande et al., 2003). Similarly, the marked box domain of E2F1 is required for induction of apoptosis, as chimeric proteins in which the marked box domain and adjacent region of E2F3 is substituted into E2F1 eliminates the ability of E2F1 to interact with Jab-1 and induce apoptosis (Black et al., 2005; Hallstrom and Nevins, 2006). The importance of the marked box domain in regulating transcription is further illustrated by microarray studies which demonstrate that both E2F1 and 3 exhibit distinct patterns of gene expression when individually expressed in fibroblasts. Similar microarray studies with chimeric proteins confirm that differential gene expression is determined by the identity of the marked domain (Black et al., 2005). Thus from these studies it has emerged that despite their similarities, E2Fs have inherent differences which can serve to impart specificity of function. ***Central to this thesis is the concept that individual E2Fs are capable of unique function in a physiologically relevant context (Chapter 3).***

3.3 E2F- FUNCTIONS BEYOND CELL CYCLE REGULATION

A second debate regarding E2F function centers on whether or not E2Fs are truly capable of cell cycle independent function. Questions as to whether such roles exist and how cell cycle independent functions are achieved are emerging in the literature. While initially hypothesized to have roles outside of cell cycle regulation as a result of phenotypes in loss of function mouse models, this idea has since gathered momentum through microarray studies examining changes in gene expression in response to deregulated E2F expression (Black et al., 2005; Dimova et al., 2003; Ishida et al., 2001; Ma et al., 2002; Muller et al., 2001; Polager et al., 2002; Young et al., 2003). In many of these studies gene expression was examined at fixed time points in cultured cells expressing various E2Fs. Despite the fact that the time points at which gene expression was examined were often chosen to correlate with expression of known cell cycle responsive genes, nevertheless each study identified novel E2F target genes with roles outside of G_1/S cell cycle transition (Black et al., 2005; Dimova et al., 2003; Ishida et al., 2001; Ma et al., 2002; Muller et al., 2001; Polager et al., 2002; Young et al., 2003). This was first observed in cells where E2F1, E2F2, and E2F3 were each expressed acutely in human osteosarcoma cells (Muller et al., 2001). In this study, a large number of genes with known roles in development and differentiation were observed to be deregulated (Muller et al., 2001). This list included genes such as homeobox transcription factors and bone morphogenetic proteins (Muller et al., 2001). Deregulation of a select number of genes involved in new processes, alone, is not sufficient to demonstrate novel functions for E2Fs. By performing a meta-analysis on the list of new genes identified, however, the authors argue that as a result of the disproportionately high number of genes identified with roles in novel processes, E2Fs are likely to be authentically involved in the regulation of these events (Muller et al., 2001). Indeed, this argument can be extended across subsequent studies which have since emerged. When examined collectively, the microarray studies have each identified similar groups of target genes which support the hypothesis that E2Fs regulate transcription of genes in differentiation and development (Black et al., 2005; Dimova et al., 2003; Ishida et al., 2001; Ma et al., 2002; Polager et al., 2002; Young et al., 2003).

While microarray data point towards a role for E2Fs in regulating processes outside of cell cycle regulation, further acceptance of this idea has come from new and sophisticated technologies designed to identify what promoters are occupied by E2Fs on a genomic scale, and whether such interaction is representative of a functional relationship *in vivo*. Still a relatively new technique, two groups have employed a genomic approach that combines chromatin immunoprecipitation with microarray technology, so-called “ChIP-on-chip”, to identify gene promoters occupied by E2F transcription factors (Balciunaite et al., 2005; Bieda et al., 2006; Cam et al., 2004; Jin et al., 2006; Ren et al., 2002; Weinmann et al., 2001; Weinmann et al., 2002). With this technique, it has become apparent that E2Fs bind to an unexpectedly high number of gene promoters, many not through the traditional E2F consensus site (Balciunaite et al., 2005; Weinmann et al., 2002) or through new co-factors (Cam et al., 2004). As a result, new roles for E2Fs have been identified in regulation of genes required for G_2/M, DNA repair (Ren et al., 2002), as well as mitochondrial biogenesis and metabolism (Cam et al., 2004). For example, as a result of the diversity of potential novel E2F regulated targets identified through ChIP-on-chip for E2F4, a motif finding algorithm was employed to determine if binding motifs of other co-factors were overrepresented within the promoters of the newly identified E2F targets (Cam et al., 2004). Through this method, the binding motif of nuclear respiratory factor-1 (NRF-1), an activator of numerous mitochondrial genes, such as cytochrome C and a number of mitochondrial ribosomal proteins (MRPs), was identified (Cam et al., 2004). Subsequent ChIP analysis demonstrated that NRF-1 and E2F4 co-existed in close proximity at both cell cycle and mitochondrial protein promoters. Further, siRNA mediated knockdown of NFR-1 led to a reduction in the RNA levels of target genes (Cam et al., 2004).

As the microarray chips themselves evolve to include increased proportions of gene regulatory and promoter sequences within the genome, the number of E2F target genes is likely to increase. Indeed, through a recent analysis of ChIP-on-chip data performed with arrays representing 30Mb of the human genome, it was suggested that greater than 20% of promoters in the human genome bind E2F1 (Bieda et al., 2006). This is a provocative hypothesis indeed if even a fraction of these sites are functional. Finally, using a distinct approach where genes induced by E2F1 are identified based on subtraction screening, separate groups of genes were identified in response to serum stimulation (Iwanaga et al., 2006). Whereas E2F1 expression coupled with serum stimulation induced genes with roles in cell cycle progression, DNA replication, and apoptosis, E2F1 was also capable of directly inducing genes outside of serum stimulation, suggestive of atypical cell cycle independent targets (Iwanaga et al., 2006). Of these cell cycle independent E2F targets, many have known roles in development and differentiation (Iwanaga et al., 2006).

While together these data provide evidence that our scope of E2F function should be expanded to include regulation of genes beyond the cell cycle, perhaps through atypical means, the caveat remains that that majority of this work has examined E2F function at the *in vitro* level. Thus the physiological significance of these results remains largely unexamined. ***A key hypothesis explored in this thesis relates to the concept of potential E2F mediated roles beyond cell cycle progression in vivo (Chapter 3), possibly through regulation of genes outside of their classical cell cycle targets (Chapter 4).***

4.0 OVERVIEW *IN VIVO* EVIDENCE FOR Rb FUNCTION BEYOND CELL CYCLE PROGRESSION

A common theme which has emerged is that through the study of cancer we learned about the role of the Rb pathway at the molecular level, however, as a result, our initial understanding of Rb function has been shaped almost exclusively in this context. While *in vitro* evidence has supported the general model whereby Rb and E2F interact to mediate cell cycle progression, studies which have examined their roles *in vivo* have called into question the universality of this view. Indeed, individual, *in vivo*, loss of function models for Rb and E2Fs are notable for the wide array of tissue specific abnormalities observed. Since then, through *in vivo* and tissue specific studies, the Rb field has exploded and new functions have been described in survival, genome stability, and senescence (Hernando et al., 2004; Longworth et al., 2008; Narita et al., 2003), (reviewed in (Goodrich, 2006; Liu et al., 2004a)). Here an overview is provided of the *in vivo* models of Rb inactivation, including the controversy surrounding their interpretation, with a focus on differentiation. Next, what has been described in other tissues is presented to highlight examples of both E2F independent and dependent roles in Rb mediated differentiation. Finally a discussion about the cell type specific roles in the nervous system for Rb in cell cycle exit, survival and differentiation are discussed. Together these studies have served to illustrate that far from having a uniform function across all cell types, Rb function is highly tissue and cell type specific.

4.1 *In vivo* evidence for complexity and distinct functions for Rb E2F activity

The initial mouse models of germline Rb deficiency provided the first clues that Rb function *in vivo* exhibits greater complexity than the *in vitro* models initially suggested. Germline Rb deficiency was embryonic lethal as mutant embryos died *in utero* between E13.5 and E15.5 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Defects observed were tissue specific, with embryos exhibiting gross defects in erythropoeisis and nervous system development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The presence of a large number of nucleated red blood cells suggested a block in the final stages of erythrocyte differentiation, while the presence of ectopic mitoses and massive cell death within the hindbrain and spinal ganglia of the developing nervous system suggested requirements in differentiation and survival in these tissues (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). At the time, these observations were considered surprising for a number of reasons (Harlow, 1992). First, the survival of the embryos themselves until mid gestation demonstrated that Rb is not universally required for regulating the passage of G_1/S for all cell cycles (Clarke et al., 1992; Harlow, 1992; Jacks et al., 1992; Lee et al., 1992). Next, the tissue specific nature of the abnormalities suggested that Rb could be playing a role in cell cycle exit or terminal differentiation. Indeed, the abnormalities in the nervous system and erythrocytes, two tissues where cells terminally differentiate early in development, represented the first evidence which led to the suggestion that Rb could be playing a role in terminal differentiation (Harlow, 1992). Subsequent studies with germline Rb deficient mice revealed reduced or absent expression of differentiation markers in the CNS and lens supported this hypothesis (Lee et al., 1994a; Morgenbesser et al., 1994). Observations of increased Rb expression in differentiating P19 cells, as well as expression patterns in differentiating cells in developing embryos further advanced the differentiation hypothesis (Slack et al., 1993; Szekely et al., 1992; Zacksenhaus et al., 1993).

Defining the requirements for Rb *in vivo* have been challenging to reconcile as what has emerged through the study of chimeric and tissue specific loss of function models has revealed that many of the initial phenotypes represent the result of both cell autonomous and non-cell autonomous requirements for Rb. Studies with chimeric mice reported that Rb chimeric mice survive to adulthood where some abnormalities were retained relative to the germline models of Rb deficiency, and others were not. For example, in Rb chimeras, erythropoeisis was largely, but not completely rescued as comparatively fewer nucleated red blood cells were observed relative to germline (Maandag et al., 1994). In both the CNS and lens, chimeric embryos exhibited extensive ectopic S-phase entry, however, unlike germline Rb deficient embryos, these cells did not complete division, but rather were arrested at G_2/M (Lipinski et al., 2001; Maandag et al., 1994). Additionally, Rb deficient cells from chimeras survived and were capable of differentiating into neurons (Lipinski et al., 2001). Thus the differences between germline Rb deficient embryos and Rb chimeric mice called into question the requirement for Rb in neuronal survival and differentiation. This lead to the hypothesis that the role of Rb during CNS development could be separated into a cell autonomous function for Rb in cell cycle exit and non- cell autonomous functions in widespread survival (Lipinski et al., 2001; Maandag et al., 1994; Williams et al., 1994). Indeed, widespread apoptosis in the CNS, and to a large extent the erythroid abnormalities of germline Rb deficient embryos were later found to be a consequence of Rb deficiency in the placenta (de Bruin et al., 2003; Wu et al., 2003). Cell death and ectopic proliferation in the lens, ectopic proliferation in the CNS, as well as a requirement for Rb in skeletal muscle differentiation, however, were not related to Rb deficiency in the placenta (de Bruin et al., 2003; Wu et al., 2003).

Nevertheless these studies pointed towards to a previously unappreciated breadth of function for Rb in cell cycle exit by demonstrating that tissue specific differences do indeed exist. Through the study of Rb inactivation in a tissue specific manner subsequent studies have outlined a number of important roles for Rb in differentiation in multiple tissues including adipose, muscle, erythroid, and nervous tissue (Table 1-5).

4.1.1 Rb MEDIATED DIFFERENTIATION- E2F INDEPENDENT

While the distinction between the role of Rb in cell cycle exit and differentiation can be difficult to reconcile, several examples are noted where Rb regulates differentiation through mechanisms distinct from the regulation of E2F activity. For example, Rb is thought to influence erythropoeisis in the liver by sequestering Id2, a molecule thought to interact with the transcription factor PU.1 to

Table 1-5: Select roles for Rb in differentiation suggested through *in vivo* studies

Tissue	Role	E2F involvement	Reference
Adipogenesis	Directs differentiation of brown adipose tissue from common progenitor by regulation transcription of PGC1α possible through E2F	Hypothesized	(Scime et al., 2005)
	Promotes terminal differentiation of adipocytes by interacting with C/EBPα to promote activation of adipocyte genes in terminally differentiating cells		(Chen et al., 1996; Puigserver et al., 1998)
	Inhibits PPARγ thereby preventing differentiation, through HDAC		(Fajas et al., 2002a)
Erythrogenesis	Rb sequesters Id2 thereby allowing PU.1 to activate transcription of CSF-1R, a gene required for macrophage differentiation		(Iavarone et al., 2004)
	Rb absence leads to an increase in the proporation of late intermediate erythroid progenitors hypothesized to be the result of reduced mitochondrial biogenesis regulated by Rb/E2F interaction	Hypothesized	(Sankaran et al., 2008)
	Specific loss of Rb in erythroid cells results in delay in final stages of erythroid differentiation		(Clark et al., 2004)
Myogenesis	Rb is required for cell cycle exit and induction of differentiation of skeletal muscle, but not for maintenance of differentiated state		(Huh et al., 2004)
	Rb promotes MyoD mediated activation of MEF (myocyte enhancer family) 2 to promote transcription of muscle specific genes		(Novitch et al., 1999)
Osteogenesis	Rb absence leads to reduced late stage osteogenic differentation. Rb interacts with CBFA-1 and possibly Hes -1 to activate transcription of genes involved in osteogenic differntiation such as osteocalcin	E2F independent	(Lee et al., 2006; Thomas et al., 2001)

prevent transcription of CSF-1R, a gene involved in macrophage differentiation. Thus the net effect of Rb interaction with Id2 is an increase in free PU.1 activity which ultimately promotes macrophage differentiation (Iavarone et al., 2004). In myogenic differentiation, Rb has been hypothesized to be mediate differentiation through a domain physically distinct from the E2F binding and transcriptional repression domains. By examining the activity of Rb mutants unable to bind E2F, repress transcription and mediate G_1/S block, it was observed that these mutants, nevertheless are capable of mediating transcriptional activation of genes involved in muscle cell differentiation and inducing a differentiated cell phenotype *in vitro* (Sellers et al., 1998). Indeed Rb has been shown to mediate transcription of markers of muscle differentiation through mediating activity of MyoD and MEF2, two muscle specific transcription factors (Novitch et al., 1999). Hence together, these observations support the hypothesis that proliferation and differentiation are genetically separable events.

4.1.2 E2F INVOLVEMENT IN Rb MEDIATED DIFFERENTIATION

While the above examples demonstrate that Rb is capable of acting independently from E2F to mediate differentiation, the question that follows is whether Rb is capable of interacting with E2F to mediate events beyond cell cycle regulation. This hypothesis is still in its infancy, yet *in vivo* evidence is emerging which points to roles for E2Fs in such events. First, the idea that E2Fs themselves play a role in differentiation is compelling. In addition to the *in vitro* data discussed suggesting that E2Fs are capable of regulating genes beyond the classical cell cycle regulatory target genes, *in vivo* phenotypes of individual and combined E2F deficient mice indicate that E2Fs exhibit a wide array of tissue specific abnormalities indicative of roles for E2Fs differentiation (Table 1-6). While the involvement of Rb in these newly described roles for E2F remains unclear or unexamined, examination of the studies that describe a role for Rb in differentiation points to possible E2F involvement in some instances (Table 1-5). For example the recent report indicating that Rb mediates differentiation of adipocyte progenitors

Table 1-6 Known and hypothesized roles for E2Fs in differentiation and development

NAME	FUNCTION	Shared/ Unique*
E2F1	Regulates transcription of neuropilin -1, a gene with known roles in neuronal migration (Jiang et al., 2007)	
	Blocks GCSF mediated survival and differentiation of granulocyte differentiation (Strom et al., 1998)	Distinct from E2F3
E2F2	Together with E2F1 hypothesized to regulate postnatal differentiation and maturation of pancreatic cells based on abnormalities of pancreas in E2F1/2 DKO mice (Iglesias et al., 2004)	Shared with E2F1
E2F3 a& b	Required for normal embryonic development as lethality begins at E13.5 during development and likely required for proper heart development as surviving animals die from congestive heart failure hypothesized to be a result of a primary heart defect (Humbert et al., 2000b) (Cloud et al., 2002)	Unique
E2F4	Involved in regulation of gut epithelium development and craniofacial development as E2F4 -/- exhibit shortening and broadening of the snout and reduced intestinal crypts and villi (Rempel et al., 2000) (Humbert et al., 2000a)	Unique
	Required to repress adipocyte differentiation, in part through regulation of PPARγ expression. Repression of adipogenesis appears to be separable from any role of E2F4 in cell cycle regulation or pocket protein binding (Fajas et al., 2002b) (Landsberg et al., 2003)	Opposing role with E2F1 and 3
	Required for ventral telencephalic patterning and regulation in the size and self-renewal capacity of neural precursors Both defects appear to be mediated by E2F4 through a genetic interaction with the morphogen Sonic Hedgehog (Ruzhynsky et al., 2007)	Unique
	Promotes NGF induced neuronal differentiation and maintenance of differentiated state of PC12 cells as expression of E2F4 in PC12 cells promotes differentiation and reduction of E2F4 inhibits NGF mediated neurite extension (Persengiev et al., 1999)	Unique
	Required for cell differentiation during development of airway epithelium, as loss of E2F4 prevents formation of ciliated cells early in development and overproduction of mucin producing goblet like cells—defects occur independent of any defects in cell proliferation (Danielian et al., 2007)	Unique

Table 1-6 Known and hypothesized roles for E2Fs in differentiation and development cont'd.

NAME	FUNCTION	Shared/ Unique*
E2F5	Involved in regulating secretion of cerebral spinal fluid as E2F5 -/- exhibit hydrocephalus due to an apparent overproduction of CSF (Lindeman et al., 1998)	Unique
E2F6	Interacts with polycomb group proteins to repress transcription, preferentially in quiescent cells (G_0) (Trimarchi et al., 2001) (Ogawa et al., 2002)	Unique
	Required for normal skeleton development & testicular development as E2F6 -/- exhibit posterior homeotic transformation of the axial skeleton and testicular abnormalities (Storre et al., 2002)	Unique
	Required to maintain male germ cell specific expression of α-tubulin 3 & 7 as E2F6-/- exhibit near ubiquitous expression of these genes in other organs (Pohlers et al., 2005)	Unique

** wherever possible shared and unique functions relative to other E2Fs are pointed out* by regulating levels of PGC-1α, a gene involved in brown adipocyte differentiation, observed an E2F component to this regulation (Scime et al., 2005). While Rb was shown to repress the PGC-1α promoter in luciferase assays, this repression was enhanced in the presence of E2F4, and abolished in the presence of an Rb mutant deficient in E2F binding (Scime et al., 2005). Next while the absence of Rb has been shown to result in a differentiation block in the development of late erythroblasts, the mechanism by which this occurs is thought to be two-fold as aberrantly elevated expression of cell cycle progression genes was observed alongside low levels of genes involved in mitochondrial function of mature erythroblasts (Sankaran et al., 2008). These observations formed the basis of a hypothesis whereby Rb and E2F interact to regulate erythroid differentiation by coupling cell cycle exit with mitochondrial function (Sankaran et al., 2008). Finally, attempts to identify the contribution of E2F to Rb mediated differentiation were recently addressed *in vivo* through the generation of a transgenic mouse in which Rb interaction with E2F1, 2, and 3 is deficient (Sun et al., 2006). By comparing the phenotype of the resulting mutants with original germline Rb deficiency mutants, the authors hypothesized that those phenotypes in common with the original germline Rb mutants could be attributable to deregulated E2Fs, while those phenotypes rescued, would be represent cell type specific E2F independent roles for Rb (Sun et al., 2006). While embryos survived for an additional two days, rescue was partial as mutants still exhibited effects in differentiation in the retina, and erythroid compartments supporting the hypothesis that Rb interacts through E2F to mediate differentiation (Sun et al., 2006). Hence together, these data suggest that E2Fs do, indeed, contribute to Rb mediated differentiation. ***A key concept explored in this thesis is whether Rb mediates events beyond cell cycle regulation in vivo through E2F (Chapter 3).***

4.2 *IN VIVO* ROLES FOR RB IN NERVOUS SYSTEM DEVELOPMENT

Our comprehension of the role of Rb in the nervous system is not as advanced compared to other tissues as early studies were focused on defining the role for Rb in neuronal survival. Our understanding has advanced more recently through the use of tissue specific models of Rb inactivation. What have emerged are very context dependent roles within the nervous system in both proliferation and roles beyond mediating mechanics of cell cycle. Here the role of Rb in mediating neural precursor proliferation is presented, followed by a discussion surrounding the evidence in support of the roles for Rb in neurogenesis beyond cell cycle regulation. What is clear from this discussion is that preliminary evidence does in fact support complex, cell type specific roles for Rb in neurogenesis, beyond its role in cell cycle regulation *in vivo*.

4.2.1 Rb MEDIATED PROLIFERATION IN THE DEVELOPING NERVOUS SYSTEM

The use of tissue specific Rb deficient mice has allowed for a more precise definition of the cell autonomous functions of Rb in the nervous system. Telencephalon specific deletion of Rb resulted in ectopic proliferation of committed neuroblasts that were able to survive and initiate neuronal differentiation (Ferguson et al., 2002). The presence of BrdU labeled cells co-expressing neuronal markers indicated that Rb is required for coupling cell cycle exit with neuronal differentiation (Ferguson et al., 2002). In a separate study, deletion of Rb in nestin positive progenitor cells throughout the CNS and PNS also resulted in inappropriate S-phase entry and cell survival (MacPherson et al., 2003). Similarly, specific deletion of Rb in the developing retina resulted in ectopic division of differentiating precursor cells (Chen et al., 2004a; MacPherson et al., 2004; Zhang et al., 2004). In contrast to the forebrain, where Rb is not required for widespread cell survival, in the retina, Rb is required for survival of three major cell types: ganglion, bipolar and rod photoreceptor cells (Chen et al., 2004a). Finally, specific deletion of Rb in precursor cells in the cerebellar vermis resulted in increased proliferation and apoptosis specific to granule cell precursors (Marino et al., 2003). As these studies have described different cell type specific requirements for Rb in regulating neural precursor proliferation and survival, together they demonstrate a context dependent function for Rb in the developing nervous system. Related Rb family members are also involved in regulating proliferation in the nervous system and are thought to compensate for the loss of Rb. Indeed, Rb family members p107 and p130 have both been shown to work with Rb in regulating proliferation in the retina as deletion of either p107 or p130 combined with Rb deficiency leads to retinoblastoma in the mouse (Chen et al., 2004a; MacPherson et al., 2004; Robanus-Maandag et al., 1998). In addition, combined loss of Rb and p107 in the cerebellar vermis leads to an exacerbation of the proliferation and apoptosis defects in granule cells that are observed in Rb deficiency alone.

4.2.2 Rb MEDIATED DIFFERENTIATION IN THE DEVELOPING NERVOUS SYSTEM

After the initial observation in germline Rb deficient mice, the idea that Rb plays a role in neuronal differentiation gained momentum through the study of Rb expression in the nervous system. *In vitro* studies established that while Rb is expressed in mitotic cells, expression is markedly increased as cells undergo neuronal differentiation (Slack et al., 1993; Zacksenhaus et al., 1993). *In vivo*, Rb was observed expressed in both proliferating and postmitotic cells of the nervous system, however, regional differences were noted. For example, in the developing retina, Rb expression was confined largely to the post-mitotic inner layer and devoid in the mitotically active outer layer, whereas in the telencephalon, Rb is expressed in both proliferating and post-mitotic tissues (Jiang et al., 1997). Beyond noting its expression in differentiating neurons, the requirement for Rb in neuronal determination was precisely defined by examining the expression pattern of Tα1 α-tubulin, a gene expressed as determined neurons undergo cell cycle exit, in Rb mutants (Slack et al., 1998). As expression of Tα1 α-tubulin was observed in the absence of Rb, Rb is not required to initiate differentiation. When cultured *in vitro*, however, these cells underwent cell death shortly thereafter, indicating that while Rb is dispensable for initiation of differentiation, it is required for proper cell cycle exit (Slack et al., 1998).

The role of Rb in differentiation in other tissues and the pattern of Rb expression in differentiating cells of the nervous system support a hypothesis that Rb may contribute to differentiation in the nervous system. Indeed early studies supported a role for Rb in neuronal differentiation in the PNS as germline Rb deficient embryos exhibited reduced expression of differentiation markers in peripheral ganglia (Lee et al., 1994b). *In vivo* studies of Rb inactivation in the peripheral and central nervous system, however, have painted a picture of Rb function that is rich and complex, with variations described region by region throughout the nervous system. For example, in cerebellum and retina, terminal differentiation is thought to be linked with cell cycle exit and survival, however in the forebrain, despite aberrant proliferation, survival remains unaffected. As a result of this complexity, our understanding of the role of Rb in neuronal differentiation is incomplete, and, our knowledge lags behind what is known in other tissues as no specific genetic pathways have been identified.

In the cerebellum, a cell type specific role for Rb in differentiation has been suggested. While cerebellar development is initially unperturbed in the absence of Rb, a progressive reduction of granule, stellate, and basket neurons is observed. The corresponding increase in ectopic cell division and cell death at later time points suggested that that aberrant differentiation and cell cycle exit ultimately resulted in reduced survival of a subset of cerebellar neurons (Marino et al., 2003). Purkinje neurons, however, while exhibiting occasional aberrant morphology and delayed migration, ultimately migrate to the appropriate location and are present in comparable numbers (Marino et al., 2003). Hence, while the exact role of Rb in cerebellar differentiation in either granule precursor or Purkinje neuron differentiation remains ambiguous, nevertheless this study has highlighted the highly cell type specific requirement that exists for Rb in cerebellar development.

In the retina, a role for Rb has been suggested in retinal differentiation; however, observations in separate mouse models support differing interpretations. In one model where Rb is removed from retinal progenitors, rods are absent and remaining rod cells fail to develop (Zhang et al., 2004). While the absence of rod cells was attributed to aberrant differentiation of rod cells (Zhang et al., 2004), this result remains controversial as cell death was not examined (Chen et al., 2004a). Nevertheless the remaining rods in this model do exhibit abnormal structure, suggesting that their differentiation and maturation is perturbed. Further, the failure of rods to develop is thought to ultimately influence maturation of other retinal cell types (Donovan and Dyer, 2004; Johnson et al., 2006). By contrast, in a separate model where Rb is removed from peripheral retina progenitors at E10, an ultimate loss of ganglion, bipolar as well as rod cells is observed. This loss, however, was not attributed to aberrant differentiation, as their precursors appear specified in embryo. Rather this loss was attributed to a requirement for Rb in regulating neuronal survival as an increase in cell death accounted for the subsequent cell absence. Here these results were interpreted as supporting the hypothesis that Rb mediates retinal cell death prior to terminal differentiation (Chen et al., 2004a). Thus much like the cerebellum, in reconciling these two accounts, it emerges that our understanding of the role of Rb in retinal differentiation is cell type specific, and still incomplete.

Despite the differences, the common theme to have emerged from the study of the retina and cerebellum is that failed cell cycle exit or terminal differentiation appears to be associated with corresponding cell death. In the CNS, however, a different picture emerges as neurons that exhibit failed cell cycle exit does not result in cell death. Indeed while cultures of Rb deficient neural progenitors exhibit a delay in cell cycle exit and terminal differentiation, these cells ultimately exit the cell cycle and differentiate into the proper proportion of neurons and glia (Callaghan et al., 1999).

Similar results were observed through models of tissue specific Rb deficiency. Where Rb is deleted from the CNS, PNS and lens, ectopic proliferation in post-mitotic regions is observed in all three regions, however, only within the CNS are the levels of cell death comparable to the level observed in control (MacPherson et al., 2003). The PNS and lens, however, exhibit cell death comparable to that observed in the whole RB knock-out (MacPherson et al., 2003). Similarly where Rb is deleted specifically in telencephalon, a high level of ectopic proliferation is observed. The level of cell death in the telencephalon is significantly reduced relative to germline Rb deficiency yet slightly elevated relative to controls (Ferguson et al., 2002). Consistent with this, conditional mutants exhibited enlarged telencephalic lobes, and enhanced cellularity (Ferguson et al., 2002). Both studies convincingly demonstrate a role for Rb in cell cycle exit and provide clear evidence challenging the notion that failed cell cycle exit is synonymous with cell death.

The issue as to the role that Rb ultimately plays in differentiation still remains a pertinent question. Neither study examined the cell type specific consequences of failed cell cycle exit on neuronal differentiation yet there is reason to believe that a role for Rb exists in post mitotic events. In particular aberrant morphology was observed and it was noted that cycling cells also display markers of differentiation together suggesting that differentiation may in fact be perturbed (Ferguson et al., 2002). The ultimate fate of these cells in context of nervous system development, however, remains unknown. ***Hence the basis of this thesis is to understand the role that Rb/E2F plays in nervous system development by examining the consequences of Rb deficiency in a physiologically relevant context.***

5.0 STATEMENT OF OBJECTIVES

While the roles of Rb and E2F acting as important regulators of the G1/S transition by way of coordinated regulation of genes involved in the mechanics of cell division are well established, *in vitro* and *in vivo* evidence exists pointing towards functions beyond classical cell cycle regulation. In particular, tissue specific models of Rb inactivation in the nervous system have demonstrated that a role for Rb in terminal differentiation exists, however, the ultimate consequences of failed cell cycle exit on differentiation and development, of the nervous system remained unknown. Thus the goals of the present studies are threefold:

1) To define the role of Rb in neuronal differentiation by examining the consequences of failed cell cycle exit on nervous system development in a cell type specific, physiologically relevant context. (Chapter2)

Stemming from the above conclusions,

2) To examine the molecular mechanism through which these cell autonomous requirements are mediated, namely through its cell cycle regulatory partner E2F, and to determine if this regulation occurs in a manner related to, or beyond cell cycle regulation. (Chapter 3)

And,

3) To explore the hypothesis that Rb interacts with E2F3 to mediate migration by influencing gene expression of migration related receptor genes in a physiological context by determining if exposure of Rb mutants to corresponding ligands elicits an aberrant migration response. (Chapter 4)

5.1 Research Objective 1

The failed cell cycle exit and aberrant morphology observed indicated that Rb is required for coupling cell cycle exit with neuronal differentiation (Ferguson et al., 2002). The survival of ectopically proliferating neurons in Rb deficiency, hence, represents an ideal model to understand the result of uncoupling cell cycle regulation and differentiation in a physiologically relevant context.

Hypothesis: Uncoupling cell cycle regulation and differentiation during neurogenesis would result in aberrant nervous system development.

In this study, a number of abnormalities of cortical development including abnormal laminar patterning, and cell type specific cell death of Cajal Retzius neurons were observed. Of particular interest, aberrant migration was observed of a population of calbindin positive GABAergic interneurons that arise from the ventral telencephalon and migrate tangentially to the dorsal cortex. While these cells appear normally specified early in their development, by mid neurogenesis a dramatic and specific reduction of these cells along their migratory trajectories *en route* to the dorsal cortex was observed. Using cortical slice co-culture assays, defective migration was observed among Rb deficient cells migrating in a control environment thereby demonstrating a cell autonomous requirement for Rb in regulating neuronal migration during forebrain development. *This finding represents a novel function for the cell cycle regulatory Rb protein, lending itself to a new area of research to identify both the mechanism of Rb mediated neurogensis and explore a possible role in brain function.*

5.2 Research Objective 2

Having described roles for Rb in neurogenesis which include cell cycle exit, laminar patterning and neuronal migration, the next objective was to determine the Rb interacting partner(s) mediating these effects. E2F1 and E2F3 are well known for their interactions with the Rb tumour suppressor and for their role in regulating expression of genes required for cell cycle progression. In addition, E2F1 and 3 are two E2Fs previously observed to exhibit deregulated expression in neural precursor cells lacking Rb.

Hypothesis: Rb regulates neurogenesis, in part through E2F1 and 3, the deregulated activity of which contributes to the neurogenesis phenotypes observed in Rb deficiency in vivo. (Chapter 4)

In this study E2F1 and E2F3 are both functionally relevant targets in neural precursor proliferation, cell cycle exit, and laminar patterning. Each also partially mediates the Rb requirement for neuronal survival. Neuronal migration, however, is specifically mediated through E2F3, beyond its role in cell cycle regulation. Further, microarray studies of migrating interneurons reveal a number of cell cycle independent genes deregulated within this population with known or hypothesized roles in neuronal migration. *This study not only outlines overlapping and distinct functions for E2Fs in neurogenesis, but is also the first to establish a physiologically relevant role for the Rb/E2F pathway beyond cell cycle regulation in vivo.*

5.3 Research Objective 3

As accumulating *in vitro* evidence points towards roles for E2Fs in differentiation and development, our identification of a role for Rb/E2F3 in mediating neuronal migration represents an attractive model to identify novel cell cycle independent E2F target genes in the context of an *in vivo* physiological function. Given the cell autonomous requirement for Rb in neuronal migration which is mediated through E2F3, we hypothesized that chemotactic receptors represented a candidate class of genes possibly regulated by E2Fs in the context of migration.

Hypothesis: If Rb, through E2F3 regulates migration through transcription regulation of a chemotactic receptor, exposure of migrating cells to the corresponding ligands should elicit an aberrant response.

Here neogenin, a novel E2F regulated target gene and receptor involved in axon guidance and neuronal migration, is specifically deregulated in the ganglionic eminence of cond Rb mutants and restored in compound Rb E2F3 deficient mutants, where neuronal migration is restored. Through *in vitro* explant cultures, a novel role for the neogenin ligand, netrin, is observed in mediating migration from the ganglionic eminences, a response which is defective in Rb deficiency. *By identifying a receptor ligand pathway disrupted in Rb deficiency, these results contribute to the overall hypothesis that Rb interacts through E2F3 to regulate transcription of genes beyond classical E2F targets to mediate physiologically relevant events distinct from cell cycle progression.*

CHAPTER 2

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**These authors contributed equally to this work.*

The experiments were performed jointly by KLF and KAM. JLV provided assistance with slice culture experiments and confocal microscopy. WCM provided technical assistance with genotyping and tissue sectioning. CS and FP provided reagents and technical guidance. KLF wrote the first draft of the manuscript while KAM carried out subsequent experimental additions and text revisions for publication. All authors contributed to critical review of the manuscript. RSS as the principal investigator provided conceptual, technical, and editorial guidance.

A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration

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ABSTRACT

Precise cell cycle regulation is critical for nervous system development. To assess the role of the cell cycle regulator, retinoblastoma (Rb) protein, in forebrain development, we studied mice with telencephalon-specific Rb deletions. We examined the role of Rb in neuronal specification and migration of diverse neuronal populations. While layer specification occurred at the appropriate time in Rb mutants, migration of early-born cortical neurons was perturbed. Consistent with defects in radial migration, neuronal cell death in Rb mutants specifically affected Cajal-Retzius neurons. In the ventral telencephalon, while calbindin and *Lhx6* expressing cortical neurons were generated at embryonic day 12.5, their tangential migration into the neocortex was dramatically and specifically reduced in the mutant marginal zone. Cell transplantation assays revealed that defects in tangential migration arose due to a cell autonomous loss of Rb in migrating interneurons, not due to a defective cortical environment. These results revealed a cell-autonomous role for Rb in regulating the tangential migration of cortical interneurons. Taken together, we reveal a novel requirement for the cell cycle protein, Rb, in the regulation of neuronal migration.

INTRODUCTION

Cell cycle regulation is essential for cortical neurogenesis, ensuring maintenance of the progenitor cell pool, production of the correct proportions of diverse cell types, and coordination of the timing of neuronal differentiation. During corticogenesis, cortical precursor cells located in the germinal zone of the dorsal telencephalon undergo multiple rounds of proliferation, between embryonic days (E) E10 to E17 (Takahashi et al., 1995; Takahashi et al., 1996). 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, which is then split by subsequent neuronal cohorts into the superficial marginal zone (MZ), and the deeper subplate. The MZ, also known as layer I, is a heterogeneous population of neurons that includes Cajal-Retzius neurons, which provide important guidance cues for neuronal migration during CP formation. Cortical layers (lamina) II-VI form between the MZ and subplate in an inside-out pattern, such that earlier generated neurons reside in deep layers, and later born neurons give rise to more superficial layers (Takahashi et al., 1999). As cortical development proceeds, the subplate becomes separated from the germinal zone by the intermediate zone (IZ), a white matter tract containing afferent and efferent projections (Caviness, 1982b; Sidman and Rakic, 1973).

In addition to the excitatory projection neurons that are born in the dorsal telencephalon, interspersed throughout the cortical layers are a population of GABAergic inhibitory interneurons that are generated in the ventral telencephalon and reach the cortex via tangential migration (Anderson et al., 1997; Anderson et al., 2001; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). Tangentially migrating interneurons follow very specific migratory routes, generally avoiding the developing striatum to form two distinct paths- either superficial to or underlying the striatal mantle (Marin et al., 2001). Superficially migrating neurons do not enter the CP, and instead migrate along the MZ, whereas interneurons following the deeper path travel through the lower IZ and subventricular zone (SVZ) (DeDiego et al., 1994; Del Rio et al., 1992; Denaxa et al., 2001; Lavdas et al., 1999; Marin and Rubenstein, 2001; Marin et al., 2001; Wichterle et al., 2001). Once GABAergic interneurons have completed their tangential migration, they switch to a radial mode of migration to enter the CP (Polleux et al., 2002).

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, 1995; McConnell and Kaznowski, 1991). The cell cycle dependence of laminar specification was best shown by a series of heterochronic 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 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 that 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.

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(Cam and Dynlacht, 2003; Classon and Harlow, 2002; Stevaux and Dyson, 2002; Trimarchi and Lees, 2002). Studies with Rb-deficient embryos were the first to show that Rb has 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., 1994a; Lee et al., 1992). More recently, we and others have shown that Rb deficiency does not result in large scale apoptosis in a cell-autonomous manner (Ferguson et al., 2002; Lipinski et al., 2001; MacPherson et al., 2003; Wu et al., 2003). Indeed, 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 zones (Ferguson et al., 2002).

In this study, we examined the impact of deregulated cell cycle regulation resulting from the loss of Rb function in the developing telencephalon. We demonstrate that, despite defective exit from the cell cycle, Rb mutants appear to generate and specify diverse neuronal populations at the appropriate developmental time. Neuronal birthdating experiments, however, reveal that Rb mutants exhibit defective laminar patterning and impaired radial migration. Finally, by slice co-culture assays, we reveal a cell autonomous defect in tangential migration. The results of our studies reveal a novel role for the tumour suppressor protein, Rb, in the regulation of neuronal migration during development.

RESULTS

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

In our initial examination of telencephalon-specific Rb knockouts, we demonstrated that these mutants retained the ectopic mitoses phenotype characteristic of Rb germline knockouts (Ferguson et al., 2002) Supp. Fig. 1b). Confocal analyses showing BrdU and βIII-tubulin co-labeling have previously identified the ectopically dividing cells as early neuroblasts, suggesting the ability to cycle after the initiation of neuronal differentiation (Ferguson et al., 2002). Here we sought to examine the impact of deregulated cell cycle regulation resulting from Rb deficiency on laminar patterning, the timing of neuronal differentiation, and the regulation of distinct neuronal populations.

Laminar patterning is perturbed in the absence of Rb:

Since the precise timing of cell cycle exit is believed to be critical for proper generation of cortical layers (McConnell, 1995; McConnell and Kaznowski, 1991; Takahashi et al., 1999), we questioned whether conditional Rb mutants might display defective laminar patterning. To ask whether layers were appropriately generated in the absence of Rb function, we first assessed the expression of layer-specific markers by *in situ* hybridization (Fig. 1). Sections were examined at E15.5 to coincide with the peak occurrence of ectopic mitoses. At E15.5, only neurons in the deepest cortical layers, V and VI, have differentiated, while layer IV neurons are in the process of being born, and layer II-III neurons are just beginning to withdraw from the cell cycle (Takahashi et al., 1999). *Tbr1,* a T-box transcription factor, is highly expressed in post-mitotic glutamatergic projection neurons in layer VI (Rubenstein et al., 1999). We noted that *Tbr1* was expressed in the Rb mutant CP at E15.5 but unlike control embryos, strong *Tbr1* labeling was also observed within the IZ (Fig. 1c, d). CP

Figure 2-1: **Laminar patterning is perturbed in the absence of Rb.**

In situ hybridization of 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 (arrows) (c, d). Similarly, *SCG10* expression is highly elevated within the mutant IZ (arrows) (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, MZ=marginal zone, CP=cortical plate, IZ=intermediate zone.

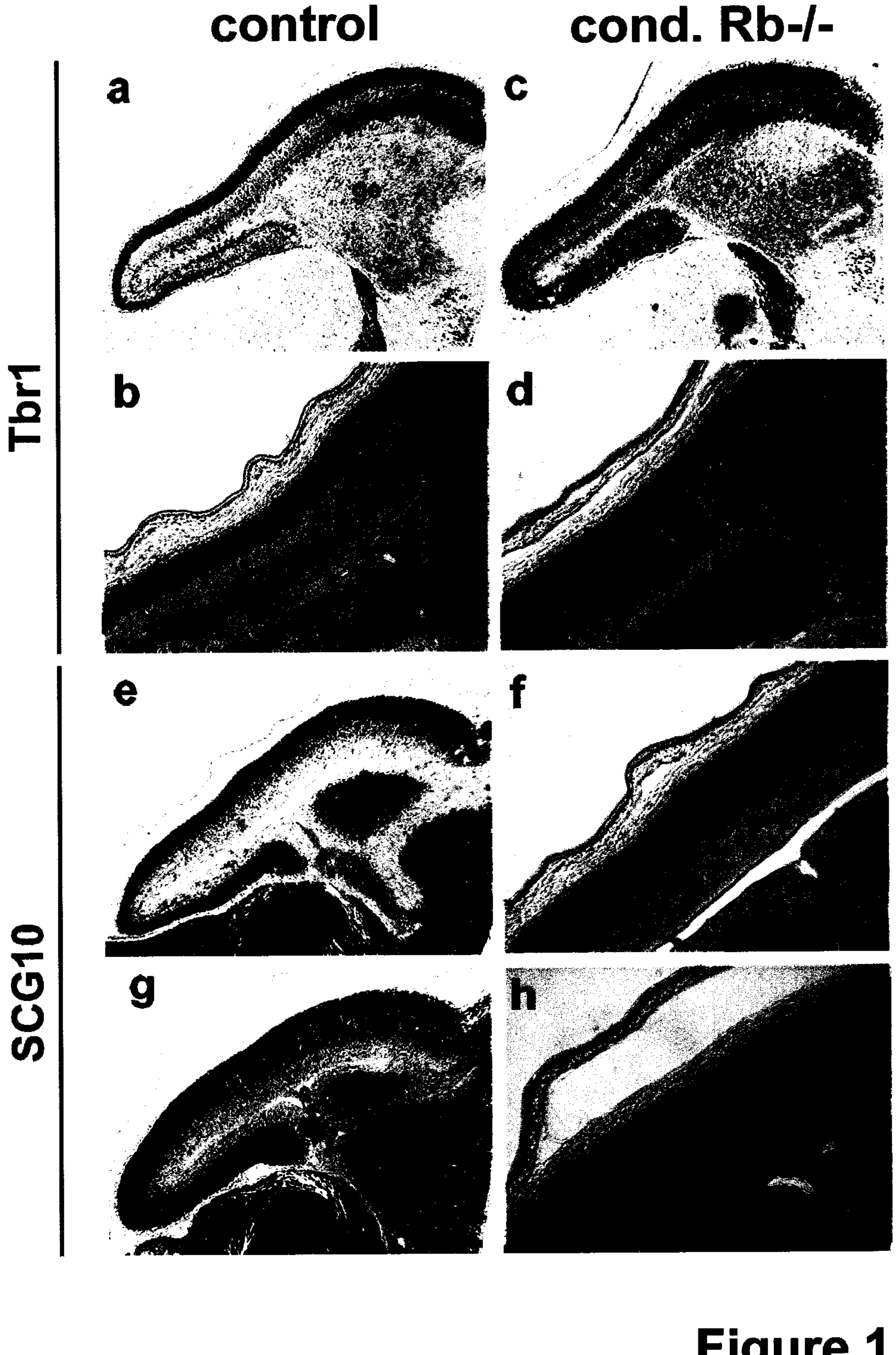


Figure 1

expression of the pan-neuronal marker, *SCG10*, (Gavet et al., 2002; Stein et al., 1988) in Rb mutant cortices was similar to littermate controls, but ectopic *SCG10* expression was also detected within the IZ (Fig. 1g, h), such that the boundary between the mutant CP and IZ lacked the clear definition observed in control embryos. The more dramatic *SCG10* phenotype is likely due to its pan-neuronal expression as opposed to the more restricted expression of *Tbr1* to deep layer neurons. This pattern was further confirmed by *in situ* hybridization with the layer-restricted markers *Id2*, *RORβ*, and *Otx1*, which also displayed increased IZ expression in Rb mutants (data not shown). These results suggest either a requirement for Rb function in the establishment of a cortical laminar structure or in the specification of layer identities.

To distinguish between these possibilities, we examined whether the abnormal lamination in Rb mutants was the result of defective radial migration by conducting neuronal birthdating experiments (Fig. 2). Pregnant females were injected with a single dose of BrdU at 13.5 and embryos were dissected at E18.5, when neurogenesis is complete but neuronal migration is still in progress. Examination of brightly labeled BrdU-positive cells reveals the location of neurons which exited the cell cycle at E13.5, as cells that continued to cycle would dilute the BrdU label. Quantification of BrdU positive cells revealed an aberrant distribution of early-born neurons in the developing cortex of Rb mutants (Fig. 2a, b). The number of BrdU-positive cells within the VZ was not significantly different between control and mutant brains. In control sections, an average of 33.3±9.2 BrdU-labeled cells was counted within the IZ as compared to 158.0±14.3 cells in the Rb mutant (Fig. 2c; $P<0.001$). The substantial increase of BrdU-labeled cells within the mutant IZ was compensated for by a corresponding decrease in labeled cells within the CP (480.2±57.9 cells in controls and only 336.9±30.5 neurons in the Rb mutants) (Fig. 2c; $P<0.05$). In contrast, the number of BrdU-positive

Figure 2-2 **Rb-deficient cortical neurons exhibit delayed radial migration**

Pregnant females at E13.5 of gestation were injected with a single dose of 20 µg/g body weight BrdU. Embryos were removed 5 days later at E18.5, fixed, and subjected to immunohistochemistry for BrdU (a, b). BrdU-labeled cells were counted across a 620 µm-wide section of dorsal cortex. In contrast to the control sections with only 33.3±9.2 cells, 158±14.3 labeled cells were counted within the IZ of Rb mutants, representing an almost 5-fold increase (arrows) (c; $P<0.001$). This dramatic increase was compensated for by a corresponding decrease of labeled cells which had reached the CP. The controls had 480.2±57.9 cells within the CP compared to only 336.9±30.5 in the Rb mutants (c; $P<0.05$). These results indicate that although similar numbers of neurons were generated at E13.5 in control and mutant cortices, Rb-deficient cortical neurons are delayed in reaching their ultimate position within the CP. Error denotes standard error. (n=3 control; 4 cond. Rb-/-) Bar=50 µm. MZ=marginal zone CP=cortical plate, IZ=intermediate zone, VZ= ventricular zone

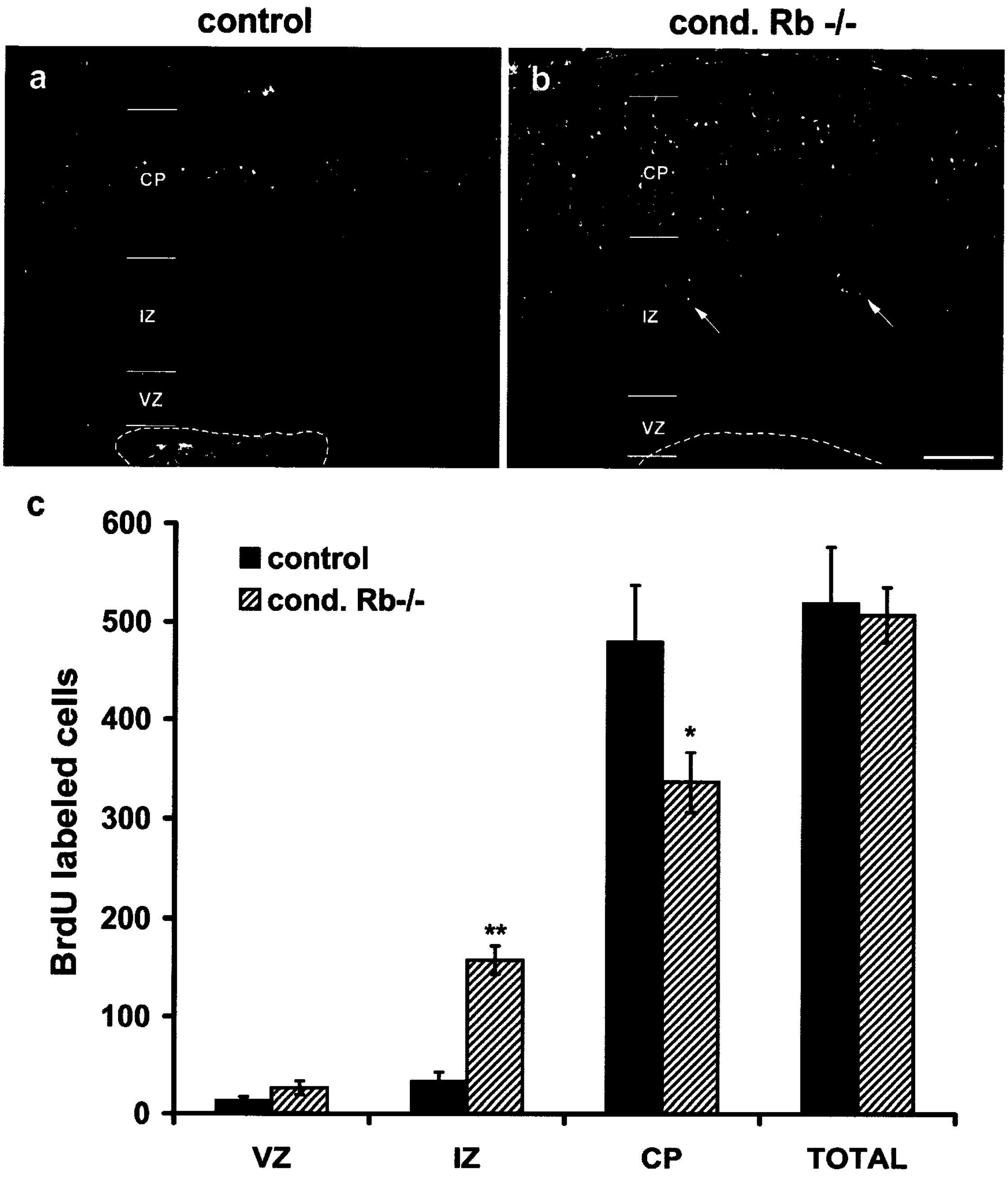

control
cond. Rb -/-
a
CP
IZ
VZ
b
CP
IZ
VZ
c
BrdU labeled cells
600
500
400
300
200
100
0
■ control
▨ cond. Rb-/-
**
*
VZ
IZ
CP
TOTAL

Figure 2

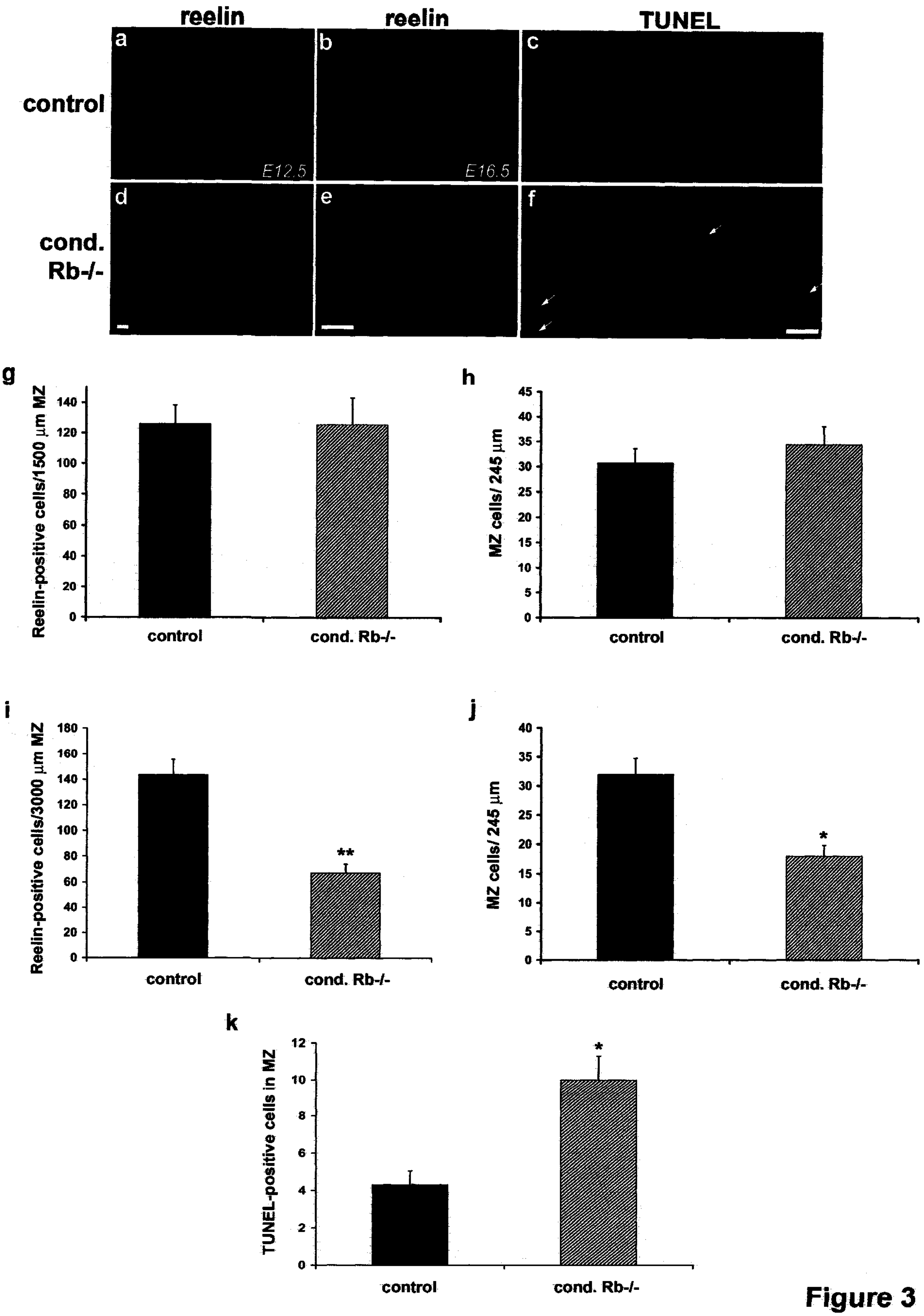
cells within the VZ was not significantly different between control and mutant brains. Consistent with an increase in IZ cell number, total cell counts of E16.5 embryos revealed significantly increased cell numbers within the Rb mutant IZ (Supplemental Fig.1c-e). These results suggest that many of the Rb deficient cells failed to reach their correct laminar destination within the cortex.

Reduced number of Cajal-Retzius neurons in the Rb mutant marginal zone

We next examined whether there were defects in the generation or survival of layer I Cajal-Retzius cells, pioneering neurons born between E11-12 that synthesize and secrete Reelin, and which are essential to guide newly generated cortical neurons along radial glial fibres (Frotscher, 1998; Sarnat and Flores-Sarnat, 2002). To assess Cajal-Retzius cell number, immunohistochemistry with a Reelin (G10) antibody was performed on mutant and control E12.5 and E16.5 embryos (Fig. 3). At E12.5, the number of Reelin-expressing cells in the cortical MZ was similar between mutant and control embryos (Fig. 3a, d), with 126.2±12.3 cells in the control and 126.0±17.8 cells in the Rb mutant MZ (Fig. 3g). In addition, total MZ cell counts at E13.5 demonstrated similar cell numbers between control and mutant littermates (Fig. 3h). In contrast, at E16.5, quantification of Reelin-positive cells revealed a dramatic 50% decrease in Cajal-Retzius neurons in the mutant MZ, with an average of 143.8±11.8 cells in the controls and 67.5±6.9 cells in Rb mutants (Fig. 3b, e, i; $P<0.001$). To confirm that reduced Reelin labeling was due to a decrease in positive cells and not merely a down-regulation of Reelin protein, we counted the total cell number within the MZ. Consistent with Reelin immunohistochemistry, total cell counts revealed that mutant cortices contained nearly 50% fewer MZ cells compared to control embryos (Fig. 3j) (32±6 cells in the control and 18±3 cells in the mutant; $P<0.05$). These results suggest that Rb deficiency does not negatively impact upon the generation of Cajal-Retzius neurons, but does affect the survival of this specific neuronal population.

Figure 2-3 **Rb is required for survival of Cajal-Retzius neurons**

Coronal sections of control and Rb mutant embryos at E12.5 (a, d) and E16.5 (b, e) were subjected to immunohistochemistry 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 E12.5, Reelin expression in the cortical MZ appeared similar between mutant and control embryos (a, d, g) (n=3 controls, 3 cond. Rb-/-). Total MZ cells counted within a 245 μm length of the dorsal cortex of E13.5 embryos confirmed similar cell numbers between mutant and controls at this time (h; n=4 control; 4 cond. Rb-/-). However, by E16.5, Rb mutants contained approximately 50% fewer Reelin-positive neurons as compared to control embryos (b, e, i, $P<0.001$) (n=5 controls, 4 cond. Rb-/-). Total MZ cell number quantified within a 245 μm length of the dorsal cortex of E16.5 embryos resulted in a similar reduction in Rb mutants (j, $P<0.05$) (n=3 control; 4 cond. Rb-/-). 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 MZ. Rb mutants exhibited significantly increased TUNEL-labeling within the MZ (c,f,k, $P<0.05$; arrows point to representative cells) (n= 4 control, 5 cond. Rb-/-) Error denotes standard error. Bar= 25 μm; MZ=marginal zone.


reelin
reelin
TUNEL
control
cond.
Rb-/-
a
b
c
d
e
f
E12.5
E16.5
g
Reelin-positive cells/1500 μm MZ
0
20
40
60
80
100
120
140
control
cond. Rb-/-
h
MZ cells/ 245 μm
0
5
10
15
20
25
30
35
40
45
control
cond. Rb-/-
i
Reelin-positive cells/3000 μm MZ
0
20
40
60
80
100
120
140
160
180
**
control
cond. Rb-/-
j
MZ cells/ 245 μm
0
5
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15
20
25
30
35
40
*
control
cond. Rb-/-
k
TUNEL-positive cells in MZ
0
2
4
6
8
10
12
*
control
cond. Rb-/-

Figure 3

While telencephalon-specific Rb deficiency is not associated with the large scale neuronal death characteristic of Rb germline knockouts, we previously reported a slight increase in TUNEL-positive cells within the mutant telencephalon at E13.5 (Ferguson et al., 2002). Since Cajal-Retzius neurons are lost by E16.5 in Rb mutants, we questioned whether this was due to neuronal-specific apoptosis. To test this, we performed TUNEL labeling on mutant and control E13.5 and E16.5 embryos, and quantified positive cells within the germinal zones, IZ, CP, and along the MZ. At E13.5, control and Rb mutant sections contained similar numbers of TUNEL-positive cells within the germinal zones and IZ/CP (Supplemental Fig. 2a). In contrast, Rb mutant sections showed elevated apoptosis within the MZ, with an average of 10.0±1.3 TUNEL-positive cells, as compared to 4.3±0.7 cells in control sections, representing a 2.3-fold increase (Fig. 3k; $P<0.05$). At E16.5, TUNEL-positive cells in all quantified regions were not significantly different between mutant and control samples (Supplemental Fig. 2b). Therefore, the specific increase in TUNEL labeling at E13.5 within the MZ of Rb mutants suggests a requirement for Rb in the survival of Cajal-Retzius neurons. Due to the important role for Cajal-Retzius neurons in guiding radial migration of newly born cortical neurons, reduction of these cells by mid-neurogenesis may contribute to the aberrant neuronal migration we observed in Rb mutants.

Rb deficiency does not significantly impact neuronal specification

Due to the defective terminal mitosis characteristic of Rb deficiency, we questioned whether the generation and/or specification of distinct neuronal populations may be altered in Rb mutants. We first examined the expression of cortical progenitor markers, including *Ngn1*, *Ngn2*, *Pax6*, and *Emx1*, by *in situ* hybridization at E15.5 in Rb mutant and control embryos. For all markers tested, no difference was observed between mutant and control sections (data not shown). Similarly, examination of ventral progenitor markers such as *Hes1*, *Hes5*, *Nkx2.1*, and *Lhx7* did not reveal any defects in progenitor cell generation or specification in Rb mutants (data not shown). Given that post-mitotic cortical neurons were mis-localized within the Rb mutant cortex (Fig. 1), we investigated whether Rb deficiency might also impact upon the positioning of ventrally-derived interneurons. To test this, we examined expression of GABAergic interneuron markers early in neurogenesis, at E12.5, and at mid-neurogenesis (E15.5/16.5). At E12.5, we did not observe any differences in the examined markers, including calbindin (Fig. 4a, b) and *Lhx6* (Fig. 4c, d) in Rb mutant embryos. In contrast, at mid-neurogenesis, the distribution of calbindin and *Lhx6* were noticeably perturbed in Rb mutant sections. Immunolabeling revealed that, while calbindin expression appeared normal in the ventral telencephalon, labeling was dramatically reduced in the temporal cortical MZ, to such an extent as to be almost absent (Fig. 5b, d). Similarly, *in situ* hybridization with an *Lhx6* riboprobe demonstrated defective expression in mutant embryos (Fig. 5e-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. 5h). Furthermore, *Lhx6* expression was reduced within the Rb mutant CP, suggesting that regardless of the migratory route, fewer *Lhx6* positive interneurons were reaching the CP. In contrast, other interneuron markers were unaltered in Rb mutants, including calretinin, *Lhx7*, and *Nkx2.1* (data not shown).

Possible explanations for the mislocalization of *Lhx6*- and calbindin-expressing interneurons in the Rb mutant cortex are that they failed to properly migrate along their appropriate trajectories or that these neurons were lost by apoptosis. Calbindin-positive neurons were quantified in mutant and control sections along either the MZ or within the deeper IZ migratory route. Rb mutants showed a substantial 2.8-fold reduction in calbindin-positive neurons in the MZ, with 23.2±3.9 cells in the littermate controls and 8.3±1.7 cells in the mutant MZ (Fig. 5i; $P<0.05$). This reduction in MZ neurons in the mutant appears to be accounted for by an increased IZ population. As compared to control sections with 51.6±4.9 cells within the IZ, Rb mutants exhibited significantly more calbindin-positive

Figure 2-4 **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 *in situ* hybridization with an *Lhx6* riboprobe. 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.

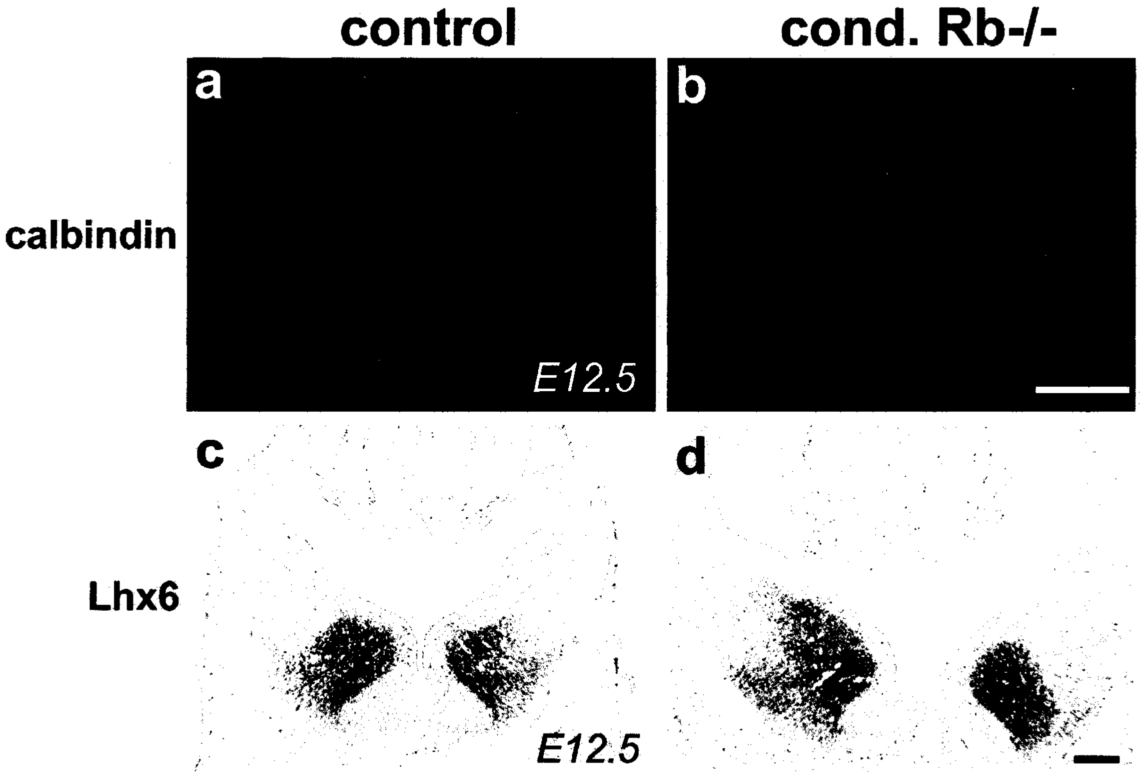


Figure 4

Figure 2-5 **Cortical interneurons are mis-localized in Rb mutants**

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 *in situ* hybridization with an *Lhx6* riboprobe. While calbindin expression appeared normal in other telencephalic regions (a, b), these cells were dramatically reduced in the mutant MZ (c, d). Similarly, *Lhx6* expression was normal along the SVZ/ IZ migratory route, but was substantially reduced in the mutant CP and MZ (g, h; n= 4 control, 5 cond. Rb-/- (E15.5); 5 control, 5 cond. Rb-/- (E16.5). Calbindin-positive cells were quantified either within the MZ or deeper corresponding to the IZ. (Arrows point to MZ route; arrowheads denote IZ population). Although the total number of calbindin-positive cells does not differ, there is an approximately 50% reduction in cell number within the Rb mutant MZ (i, $P<0.05$). The decreased number of calbindin positive neurons within the Rb mutant MZ is associated with a corresponding increase in these neurons within the mutant IZ(i, $P<0.01$). Error denotes standard error. Bar= 100μm (a, b, e-h) 25 μm (c, d). MZ=marginal zone; IZ=intermediate zone.

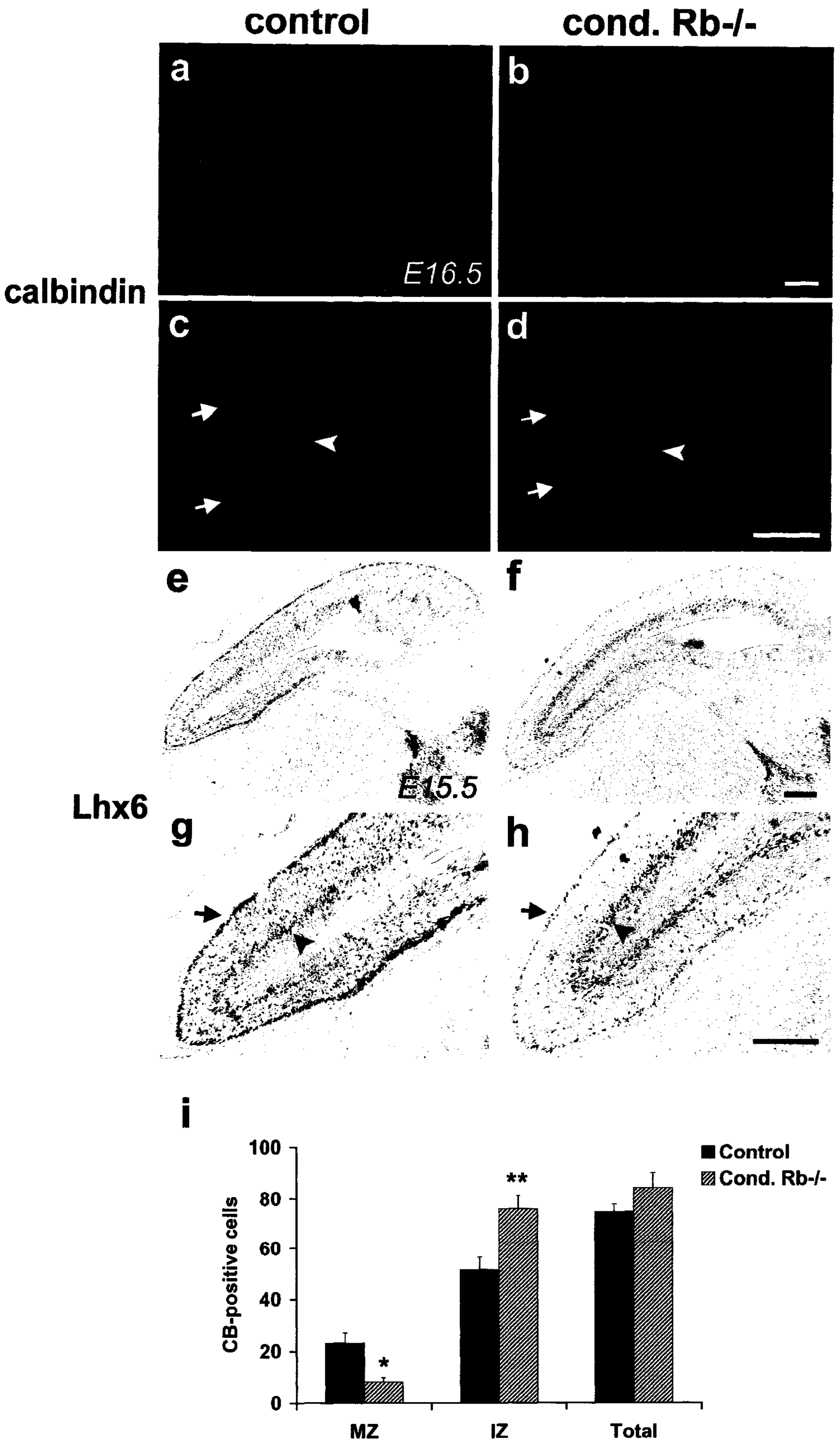

control
cond. Rb-/-
a
b
E16.5
calbindin
c
d
e
f
E15.5
Lhx6
g
h
i
100
80
60
40
20
0
CB-positive cells
**
*
MZ
IZ
Total
■ Control
▨ Cond. Rb-/-

Figure 5

neurons along this migratory route with 76.1±5.1 cells (Fig. 5i, $P<0.01$). Although the total number of calbindin-expressing neurons appeared slightly elevated in Rb mutants, the difference was not statistically significant (74.8±3.1 cells in controls and 84.4±5.6 in mutants). These results indicate that the dramatic reduction of these ventrally-derived interneurons specifically along the MZ migratory route, is not due to selective apoptosis, but instead suggests that these tangentially migrating neurons may be diverted from their normal MZ path into the deeper IZ trajectory.

A cell-autonomous requirement for Rb in interneuron migration

Cortical GABAergic interneurons are derived from the ventral ganglionic eminences (Anderson et al., 1997; Anderson et al., 2001; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Wichterle et al., 2001). These neurons migrate tangentially through the ventral telencephalon to the cortex along distinct routes: a deep trajectory in the lower IZ/ SVZ and a superficial route along the MZ. To determine whether Rb may be required to regulate tangential migration along the MZ route, we performed slice co-culture assays (Fig. 6a). Mice were interbred to generate Rb mutants at a 25% frequency, with one parent additionally expressing GFP such that 50% of embryos would also be GFP-positive. Telencephalons of GFP-negative embryos were sectioned and plated onto coated filter-membrane inserts. Medial ganglionic eminence (MGE) explants were excised from GFP-positive littermates and placed directly on the area of the sections corresponding to the MGE. Co-cultures were grown *in vitro* for 72h prior to fixation and immunolabeling for GFP. It has previously been shown that within 72 hours, explanted cells will readily integrate into the slice and will migrate up to the dorsal cortex, along the appropriate MZ and IZ trajectories (Polleux et al., 2002). We assessed the migratory routes of GFP-positive cells, specifically focusing on the MZ trajectory, and classified each slice hemisphere into one of three categories: "cell integration", "MZ stops early", and "MZ migration". "Cell

Figure 2-6 **Slice co-cultures**

To assess the requirement for Rb function in interneuron migration, we performed slice co-culture assays. a) GFP-negative telencephalon sections were plated onto coated filter-membrane inserts in a 6-well dish. MGE were removed from GFP-positive littermates and equal-sized pieces were placed directly on sections corresponding to the MGE. Co-cultures were grown *in vitro* for 72 h prior to fixation and GFP immunohistochemistry. The migratory routes of the GFP-positive cells, specifically the MZ trajectory, were analyzed and classified as follows: "cell integration", in which GFP-positive cells integrated into the section (arrow) but did not follow a distinct migratory route (arrowhead) or reach the MZ (b, e, h). "MZ stops early" occurred when GFP-positive cells formed a MZ route (arrow) but did not reach the dorsal cortex (arrowhead) (c, f, i) "MZ migration" included sections in which a GFP-positive MZ migratory route reaching the dorsal cortex was observed (arrows) (d, g, j). d, g Bar= 100μm, j Bar= 50μm ; MGE=medial ganglionic eminence, MZ=marginal zone.

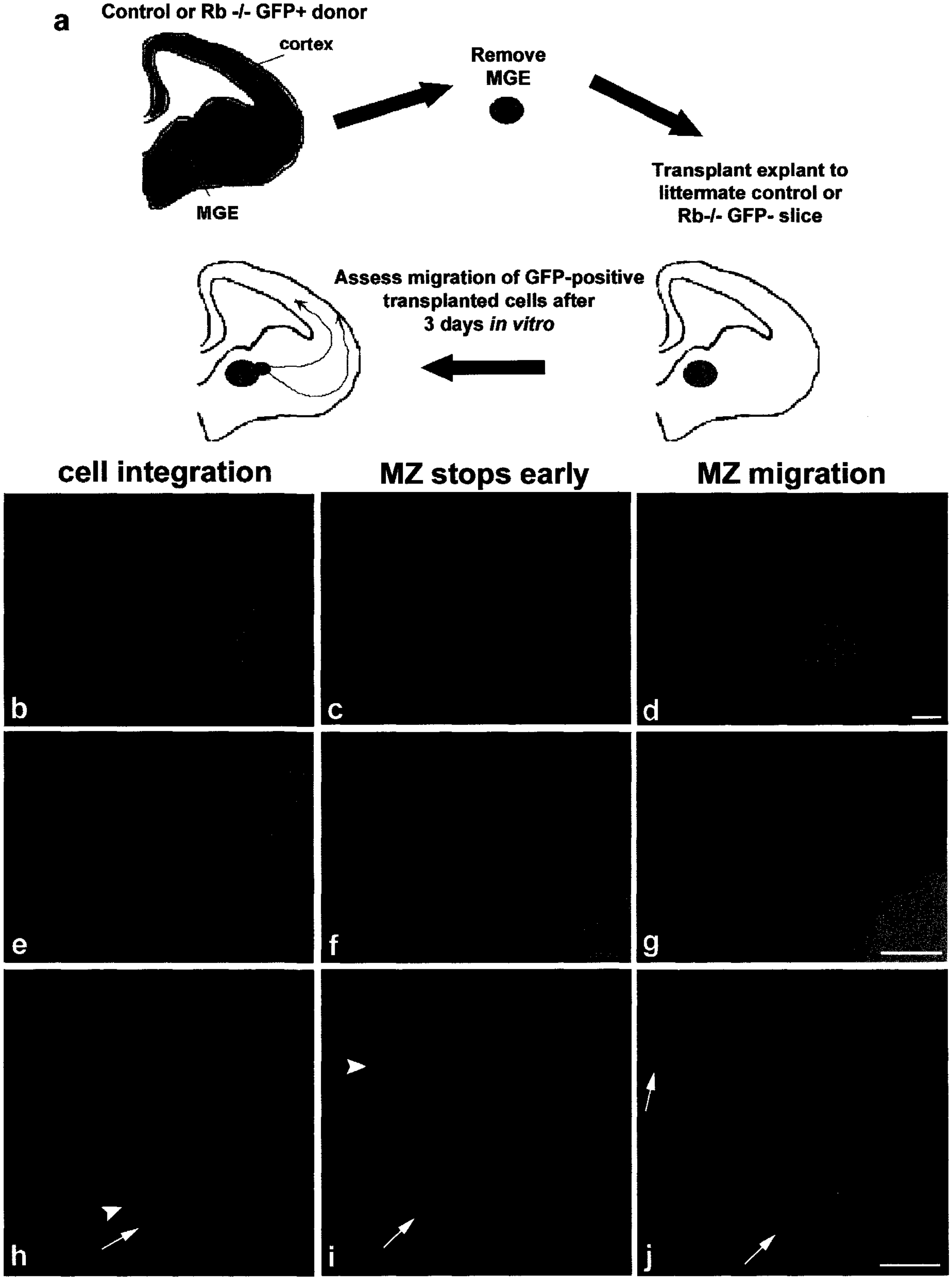

a
Control or Rb -/- GFP+ donor
cortex
MGE
Remove MGE
Transplant explant to littermate control or Rb-/- GFP- slice
Assess migration of GFP-positive transplanted cells after 3 days *in vitro*
cell integration
MZ stops early
MZ migration
b
c
d
e
f
g
h
i
j

Figure 6

integration" refers to the condition in which GFP-positive cells integrated into the section and initiated tangential migration in the appropriate ventrolateral direction, however, these cells failed to follow a distinct migratory route or reach the MZ. This was considered to be the most extreme form of failed migration (Fig. 6b,e,h). The second category, "MZ stops early", was considered to be a more moderate failure to migrate along the MZ and occurred when GFP-positive cells were detected in the MZ route, but did not reach the cortex (Fig. 6c,f.i). The third category, "MZ migration" included all sections in which a GFP-positive migratory route was observed along the MZ, reaching the dorsal cortex (Fig. 6d,g,j).

In addition to guiding radial migration, Cajal-Retzius neurons have recently been suggested to have a potential role in regulating the tangential migration of interneurons from the ventral telencephalon (Hack et al., 2002; Morante-Oria et al., 2003; Shinozaki et al., 2002; Stoykova et al., 2003). Because Rb mutants exhibit a 50% reduction in Cajal-Retzius neurons, we asked whether tangential migration in Rb mutants may be altered due to defective environmental cues. To test this, we examined the MZ migratory routes formed by control MGE explants on either control or Rb mutant slices (Fig. 7). When control explants were placed on control slices, 93.3% of sections revealed complete MZ migration (28/30) while only 7.1% failed to migrate and were classified as "cell integration" (2/30). Similarly, cells derived from control MGE explants placed on Rb mutant slices were able to appropriately migrate along the Rb-deficient MZ. Under control explant: mutant slice conditions, GFP cells displayed complete MZ migration, in all sections examined (19/19). These data demonstrate that, despite the reduced number of Cajal-Retzius neurons in the Rb mutants, control MGE-derived interneurons do not exhibit any defects in migrating along the MZ route in the Rb-null cortex.

We next questioned whether Rb may be required by migrating neurons in order to properly navigate the MZ migratory route. We compared the MZ migration of GFP cells derived from either

Figure 2-7 **A cell-autonomous requirement for Rb in tangential migration**

a) The MZ migratory routes resulting from GFP-positive MGE explants were compared according to the previously described classifications (Fig. 6). Control explants were able to complete MZ migration in 95.9% (47/49) of sections examined, regardless of whether they were placed on mutant or control slices (b). In contrast, only 19.2% (5/26) of mutant explants placed on either mutant or control slices displayed proper MZ migration (c); MZ=marginal zone.

a

Genotype	% cell integration	% MZ Stops Early	% MZ Migration
Control Slice Control Explant	**7.1** (2/30)	**0.0** (0/30)	**93.3** (28/30)
Mutant Slice Control Explant	**0.0** (0/19)	**0.0** (0/19)	**100.0** (19/19)
Control Slice Mutant Explant	**61.9** (13/21)	**14.2** (3/21)	**23.8** (5/21)
Mutant Slice Mutant Explant	**40.0** (2/5)	**60.0** (3/5)	**0.0** (0/5)

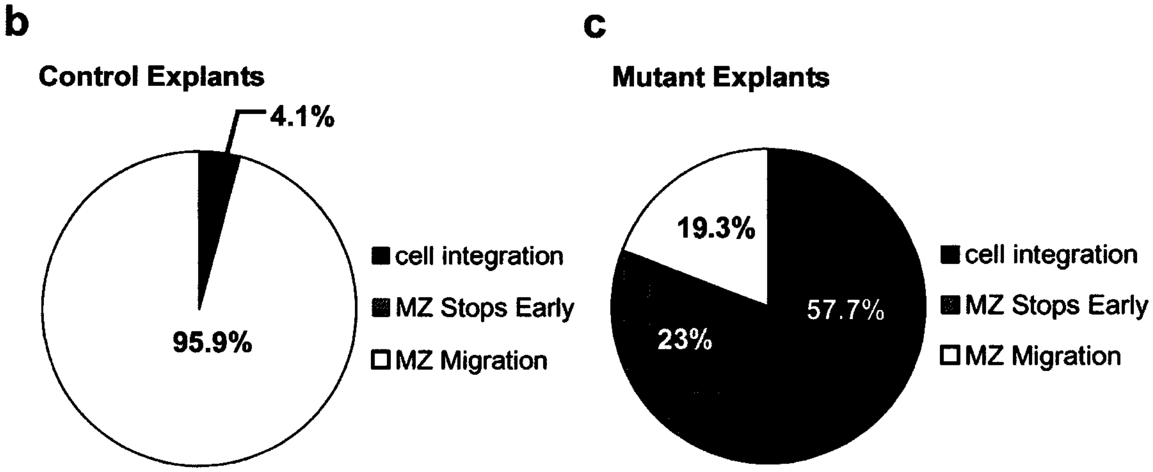


Figure 7

control or mutant MGE explants on control slices. While the large majority of control explants placed on control sections exhibited complete MZ migration (93.3%), Rb-deficient migrating neurons showed a dramatic failure to migrate along the MZ of the control slices. The majority of sections (61.9% or 13/21) exhibited the most severe form of failed migration in the form of "cell integration", while in 14.2% of sections, the MZ route stopped early (3/21), and only 23.8% of sections showed complete MZ migration (5/21). Furthermore, we examined the migratory capacity of Rb-deficient MGE-derived interneurons on Rb mutant slices. Under these conditions, 40% (2/5) of sections exhibited "cell integration", 60% (3/5) exhibited a MZ trajectory that stopped early, and none of the sections underwent complete MZ migration (Fig. 7). These results demonstrate that Rb is essential for the MGE-derived interneurons to complete tangential migration along the MZ route. When pooled together, 95.9% (47/49) of sections with control explants showed complete MZ migration while only 19.2% (5/26) of sections with mutant explants displayed proper MZ migration (Fig. 7). These data comprise the first evidence indicating a cell-autonomous requirement for the cell cycle protein, Rb, in regulating neuronal migration during forebrain development.

DISCUSSION

In this study, we examined the requirement for the cell cycle regulator, Rb, in telencephalic development. Although Rb mutants produced appropriate numbers of correctly specified cortical projection neurons and interneurons, the radial and tangential migration of these neuronal populations, respectively, were perturbed. In particular, neuronal birthdating and marker analyses revealed that the radial migration of early-born neurons within the cortex was impaired in the absence of Rb. Similarly, an examination of cortical interneuron markers and the use of slice co-culture assays revealed that Rb is essential for normal tangential migration. In conclusion, our results demonstrate a novel cell-autonomous function for the Rb tumour suppressor, in regulating neuronal migration during telencephalic development.

Cell cycle and cell fate

Proper timing of cell cycle exit and terminal mitosis is believed to be critical for the generation of specific neuronal cell types in the developing neocortex (McConnell, 1995; McConnell and Kaznowski, 1991). We have previously shown the prevalence of ectopically dividing neuroblasts throughout the IZ and CP of Rb mutants (Ferguson et al., 2002). Despite the fact that Rb deficient mutants exhibit defective terminal mitoses, we show here that cortical neuronal populations do not appear to be mis-specified. First, all dorsal progenitor cell markers examined showed normal expression patterns in Rb mutants. Second, layer-specific markers were appropriately expressed in Rb mutants, with the exception of ectopic expression in the IZ at early developmental stages. While neuronal fate determination has been shown to be tightly coupled with the timing of terminal mitosis (McConnell, 1995; Wald and McLoon, 1995), the Rb deficient mouse represents an anomaly in which these events become aberrantly uncoupled. Although Rb-deficient neurons fail to undergo terminal mitosis at the correct time they are still able to generate the appropriate neuronal populations as in wild type animals. This lack of correlation between neuronal gene expression and terminal mitosis suggests that terminal mitosis may not always be a prerequisite for the specification of the appropriate neuronal population.

Our neuronal birthdating experiments revealed that although similar numbers of early-born cortical neurons were generated in Rb mutants, they failed to reach their ultimate destination within the CP, suggestive of a role for Rb in radial migration. In contrast, later-born neurons were able to migrate to their appropriate layers, raising the possibility that Rb is required for the migration of specific neuronal subpopulations. We cannot, however, rule out the idea that the early-born cortical projection neurons migrate inappropriately because they are born in ectopic locations. Confocal microscopy has revealed that the vast majority of dividing cells in the IZ are committed neuroblasts co-expressing BrdU and the neuronal marker ß-III tubulin (Ferguson et al., 2002). This is consistent with the interpretation that Rb deficient neurons are committed to a specific fate before they leave the VZ but then continue to divide ectopically. At present, therefore, we can not distinguish between whether (a) neurons born in the IZ fail to undergo appropriate radial migration as a consequence of their generation in an inappropriate location; or (b) early-born projection neurons require Rb for their radial migration.

A key question when evaluating radial migration is whether any of the migrating cells, particularly those that fail to find their appropriate destination undergo apoptosis. We have quantified apoptosis in each of the zones including VZ, IZ, CP and MZ and have only found a significant increase in apoptosis in the MZ at E13.5 and no difference in any of these regions at E16.5. This suggests that Rb is not essential for the survival of CP neurons and that neurons born in the IZ do not appear to default to an apoptotic pathway. Our studies reveal however, that Rb is required for the survival of

Cajal Retzius neurons in the MZ. It should be noted that mice carrying the conditional Rb mutation die at birth, hence we cannot comment on the long-term survival of these cortical neurons.

Selective loss of Cajal-Retzius neurons

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 a small but significant 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 dramatically increased within the cortical MZ, specifically affecting the Cajal-Retzius neurons. While the initial generation of Reelin-positive Cajal-Retzius neurons is normal, by E16.5, these cells are reduced in number by nearly 50% in the Rb deficient MZ. This raises the possibility that the reduction in Cajal-Retzius cell numbers could account, at least in part, for the defects in radial and tangential migration. Indeed, Cajal-Retzius neurons are known to be critical for guiding radial migration of newly generated cortical neurons (Frotscher, 1998; Sarnat and Flores-Sarnat, 2002), and the loss of these cells by mid-neurogenesis may be associated with the aberrant radial migration and defective laminar patterning observed in the Rb mutant cortex. A similar reduction of Cajal-Retzius neurons occurs in mice deficient for Emx2, in which the neurons appear to be properly generated but are subsequently lost. These mice exhibit defective radial migration and laminar patterning (Mallamaci et al., 2000; Shinozaki et al., 2002). While a reduction of Cajal-Retzius neurons by mid-neurogenesis would not be expected to have a great effect on the positioning of early-born neurons, the loss may be responsible for more subtle defects.

Rb regulates tangential migration in a cell-autonomous manner

The specification and generation of calbindin- and *Lhx6*-positive neurons was not initially impaired, however, by mid-neurogenesis, expression of these cortical interneuron markers was dramatically reduced along the MZ, while there was a corresponding increase in the IZ. Thus, the total number of calbindin-positive neurons was similar between Rb mutant and control embryos, demonstrating that these interneurons were not lost due to apoptosis or neurogenesis defects. Instead, we found the reduction of cells within the MZ was associated with a corresponding increase in calbindin-positive cells within the IZ. These results indicate that the specific MZ loss of interneurons is due to their failure to properly follow their MZ migratory route, and that these cells instead become re-routed towards the deeper IZ trajectory.

Our data show that control cells from MGE explants migrate equally well on control and mutant sections, indicating that the migratory environment to be relatively inconsequential in terms of the defective migration observed in Rb mutants. Instead, we demonstrate that Rb is essential among certain MGE-derived interneurons in order to migrate along the MZ. Despite the dramatic reduction in MZ migration derived from Rb mutant explants, a small proportion of samples that included Rb-deficient interneurons (23.8%) demonstrated complete MZ migration. This may be explained by the heterogeneity of the MGE population as Rb signaling may only be required in specific interneuron subsets.

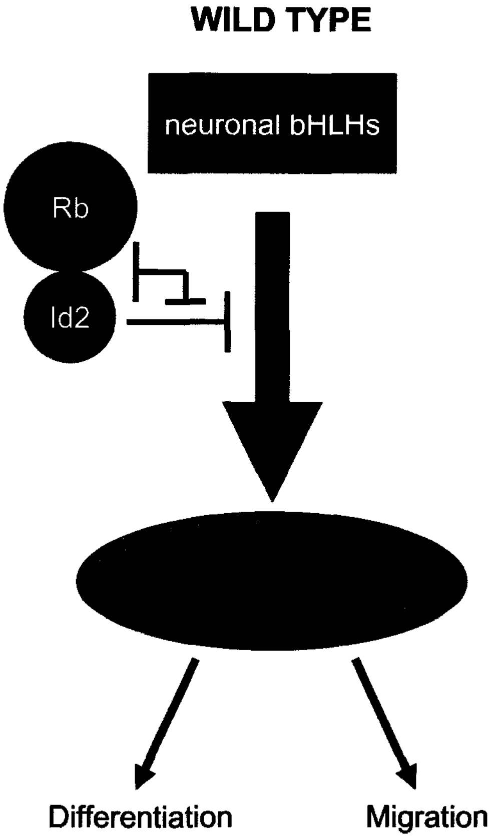
What may account for the mechanism through which Rb could impact neuronal migration? Previous studies have shown that Rb interacts with a number of genes that can regulate differentiation. A well established interaction is with the helix-loop-helix (HLH) family member, Id2, an important negative regulator of neuronal differentiation (Perk et al., 2005). By acting as a dominant inhibitor of proneural bHLH factors, Id2 represses the transcription of neuron specific genes. Rb has been shown to interact with Id2, thereby suppressing its activity (Lasorella et al., 2002). Several studies demonstrate the importance of the Rb interaction with Id2 in neural development. First, the neurological defects found in Rb knockouts are rescued by the absence of Id2 (Lasorella et al., 2000). Second, expression of Id2 in cortical progenitor cells was shown to inhibit the induction of neuron-specific genes, while this inhibition was alleviated by the co-expression of a constitutively active Rb (Toma et al., 2000).

The consequence of increased free Id2 activity in Rb deficiency could lead to inhibition of genes that impact on differentiation and migration. One such example is TrkB which has previously been shown to be substantially reduced in Rb deficient brains (Lee et al., 1994b). In addition to its well known role in neuronal survival (Atwal et al., 2000; Stucky et al., 2002), TrkB has also been shown to regulate radial and tangential neuron migration (Behar et al., 1997; Brunstrom et al., 1997; Medina et al., 2004; Polleux et al., 2002). Recent studies have demonstrated that Id2 can also directly repress TrkB expression in neural cells (Liu et al., 2004c). Thus, we envisage a model whereby the absence of Rb leads to deregulatated Id2 activity causing inhibition of transcription of neuron-specific genes required for differentiation and migration, such as TrkB (Fig. 8). Future studies exploring molecules that regulate neuronal migration in the Rb deficient brain will be required to identify the specific pathways which are dependent on Rb.

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, require Rb for survival. Furthermore, we reveal, for the first time, an essential role for the cell cycle protein, Rb, in regulating neuronal migration during cortical development.

Figure 2-8 **Proposed model of Rb-mediated regulation of neuronal differentiation and migration**

In wildtype cells, Id2 is sequestered by Rb and is unable to inhibit bHLH mediated transcription of specific neuronal genes, such as TrkB, that are required for neuronal differentiation and migration. In the absence of Rb, Id2 activity is deregulated, allowing inhibition of TrkB htranscription, which, in turn, leads to impaired neuronal migration and differentiation.


WILD TYPE
neuronal bHLHs
Rb
Id2
Differentiation
Migration

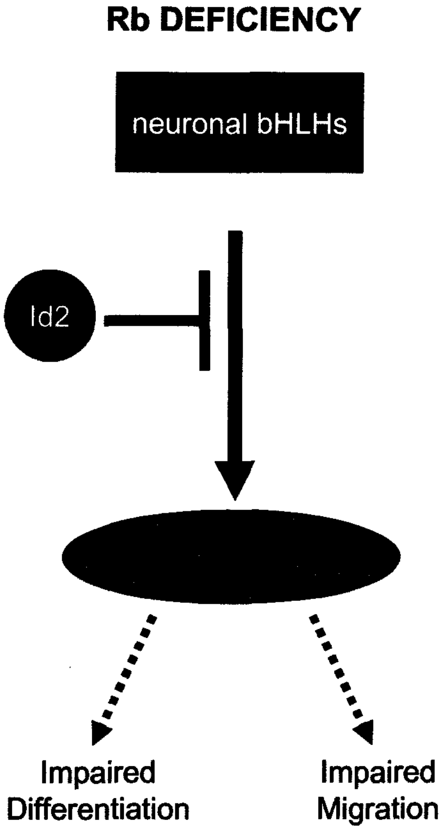

Rb DEFICIENCY
neuronal bHLHs
Id2
Impaired Differentiation
Impaired Migration

Figure 8

MATERIALS AND METHODS

Mice

Telencephalon-specific Rb deficient mice were generated by crossing Rb-F19 (Marino et al., 2000) and Foxg1-cre mice (Hebert and McConnell, 2000), as previously described (Ferguson et al., 2002). 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 subjected to sequential solutions of 12, 16, and 22% 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 on a cryostat as 14 μm sections at -20°C and mounted 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 6 μm thickness. Cresyl violet staining was performed on paraffin sections according to standard protocols.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed on fixed frozen sections with the following primary antibodies: TuJ1 (mouse monoclonal hybridoma supernatant, 1:50, Dr. David Brown, University of Ottawa), 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 immunohistochemistry, 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. For BrdU incorporation analyses, pregnant females were injected intra-peritoneally with 20 or 50 µg BrdU/ g body mass (Boehringer Mannheim #280879) and were processed as previously described. TUNEL staining was performed as previously described (Ferguson et al., 2002). Non-radioactive *in situ* hybridization and digoxygenin 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 with a Zeiss Axioskop 2 fluorescence microscope, and visualized with a Sony Power HAD 3CCD color video camera with Northern Eclipse software.

Slice co-cultures

The conditional Rb mutant mice were bred such that one of the parents was additionally heterozygous for green fluorescent protein (GFP). With this crossing, 25% of embryos would be expected to be Rb deficient while 50% of embryos should express GFP. Heterochronic slice co-cultures were performed on E16.5 litters, as previously described, with some modification (Polleux et al., 2002) (Supplementary information).

ACKNOWLEDGEMENTS

The authors are indebted to Dr. K. Herrup for numerous helpful discussions. We thank J. MacLaurin and L. Jui for excellent technical assistance, and Drs. W. Hendelman, P. Humphries, and L. Maler for advice with anatomical examinations. This work was funded by a CIHR grant to RSS. KLF is a recipient of a CIHR studentship, KAM of OGS and SCN studentships, and JLV is a recipient of a HSFC fellowship.

Supplementary information is available at *The EMBO Journal Online.*

SUPPLEMENTARY INFORMATION

Materials and Methods

Slice co-cultures

The conditional Rb mutant mice were bred such that one of the parents was additionally heterozygous for green fluorescent protein (GFP). With this crossing, 25% of embryos would be expected to be Rb deficient while 50% of embryos should express GFP. Heterochronic slice co-cultures were performed on E16.5 litters, as previously described, with some modifications (Polleux et al., 2002). Briefly, brains were removed from non-GFP-expressing embryos and embedded in low melting point agarose. Agarose-embedded brains were glued onto a platform and coronally sectioned into 250 μm sections on a Leica VT1000S vibratome. Brain sections were collected and plated on poly-*L*-lysine/laminin-coated filter-membrane inserts placed on top of culture media in each well of a 6-well dish. MGE explants were removed from GFP-positive embryos and cut into 4 equal-sized pieces. MGE explant pieces were then placed directly on each section corresponding to the MGE. The co-cultures were grown *in vitro* for 72 h prior to overnight fixation in 4% PFA at 4°C. Slices were rinsed 3 × 30 min in PBS then permeabilized for 2.5 h at RT. Sections were incubated with anti-GFP antibody conjugated to Alexa 488 (Molecular Probes; 1:1000 in permeabilization buffer) for 1 h at RT. Sections were rinsed in PBS, mounted on Fisher Superfrost slides, and analyzed by confocal microscopy.

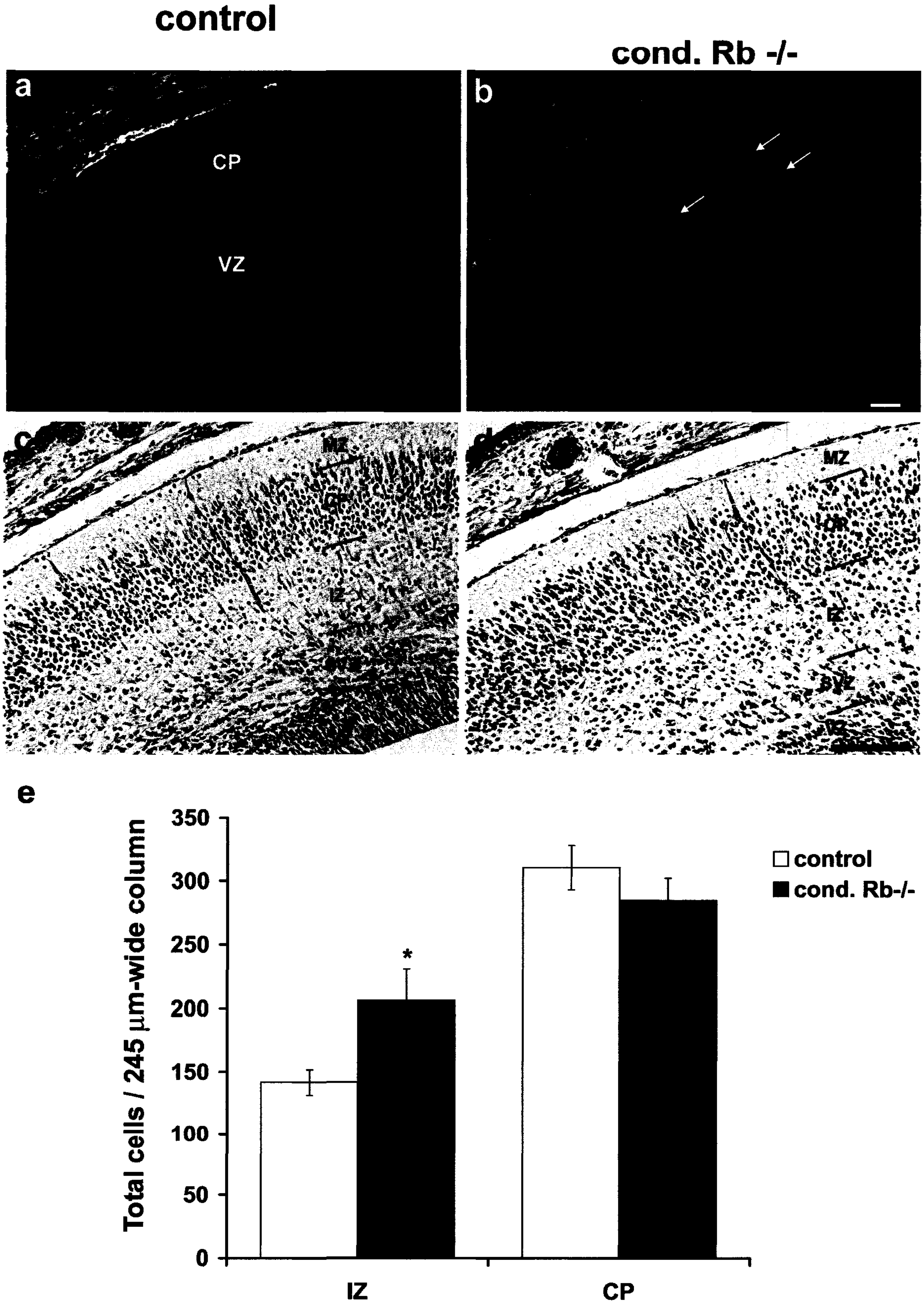
The MZ migratory routes of the GFP-positive cells were classified as either: "cell integration", "MZ stops early", or "MZ migration". "Cell integration" occurred when GFP-positive cells integrated into the section but failed to form a distinct migratory route or to reach the MZ. Sections were classified as "MZ stops early" when GFP-positive cells were detected along the MZ, but did not reach the dorsal cortex. "MZ migration" included all sections in which a GFP-positive migratory route was observed along the MZ, reaching the dorsal cortex.

Cell counting and statistical analyses

For neuronal birthdating, 20 µg/g body weight BrdU was injected interperitoneally at E13.5 and BrdU-positive cells were counted within a 645 µm-wide coronal section of the dorsal cortex at E18.5 from the ventricle up to the pial surface(n=3 controls, 4 cond. Rb-/-. 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 each case, the counting region initiated at the dorsal midline of coronal sections and extended ventrolaterally. For total MZ cell counts, cells were quantified in Cresyl Violet-stained paraffin sections (n=4 control; 4 cond. Rb-/- (E13.5); n=3 control; 4 cond. Rb-/- (E16.5)) within 245 µm-wide columns of the dorsal cortex. To quantify TUNEL labeling, positive cells along the MZ were counted on E13.5 coronal telencephalic sections (n= 4 control, 5 cond. Rb-/-). Calbindin-positive cells were quantified within the temporal cortex either along the MZ, or deeper corresponding to cells migrating along the IZ route (n= 5 control, 5 cond. Rb-/-). In all cases, statistical analyses were conducted with Microsoft Excel software using student's t-test assuming unequal variances.

Supplemental Figure 2-1 **Rb deficient progenitor cells undergo ectopic cell divisions**

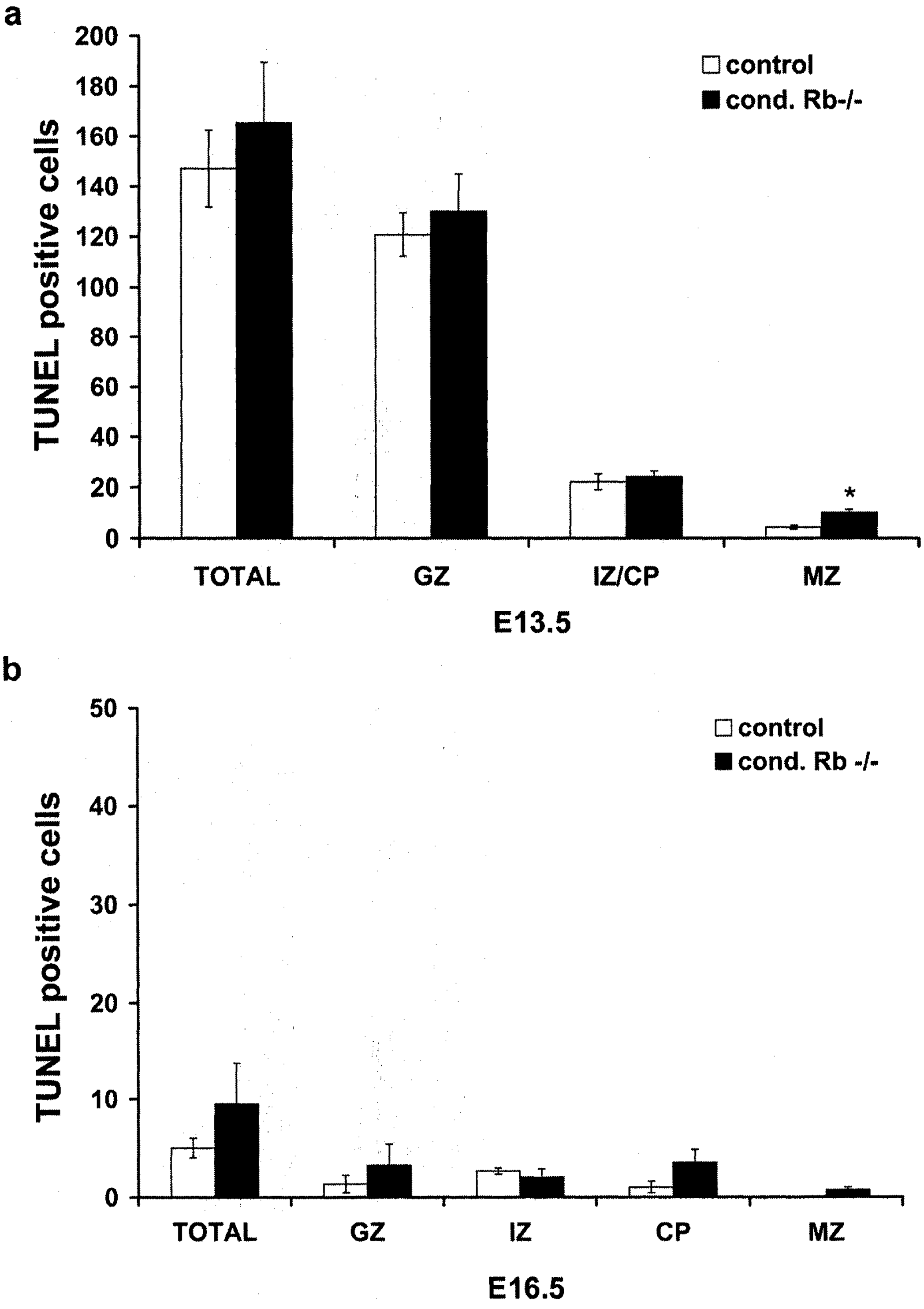
Pregnant females at E15.5 of gestation were injected with 50 µg/g body weight BrdU. Embryos were removed 2 h later, fixed, and subjected to immunohistochemistry for BrdU (green) and TuJ1 for βIII-tubulin (red). In contrast to the controls (a), the conditional Rb mutants exhibited extensive BrdU incorporation outside the VZ (b). These ectopically dividing cells typically contained round nuclei (arrows) characteristic of non-migrating cells. (n=4 control; 4 cond. Rb-/-) Bar= 50 µm. Paraffin sections (6 µm) sections from control and Rb mutant E16.5 embryos were stained with Cresyl Violet (c, d). Quantification of total cell number revealed a significantly increased cell number within the mutant IZ, while CP cell number was similar to control animals (P<0.05, e) (n=3 control; 4 cond. Rb-/-). Error denotes standard error.



Supplemental Figure 1

Supplemental Figure 2-2 **TUNEL labeling in E13.5 and E16.5 cortex**

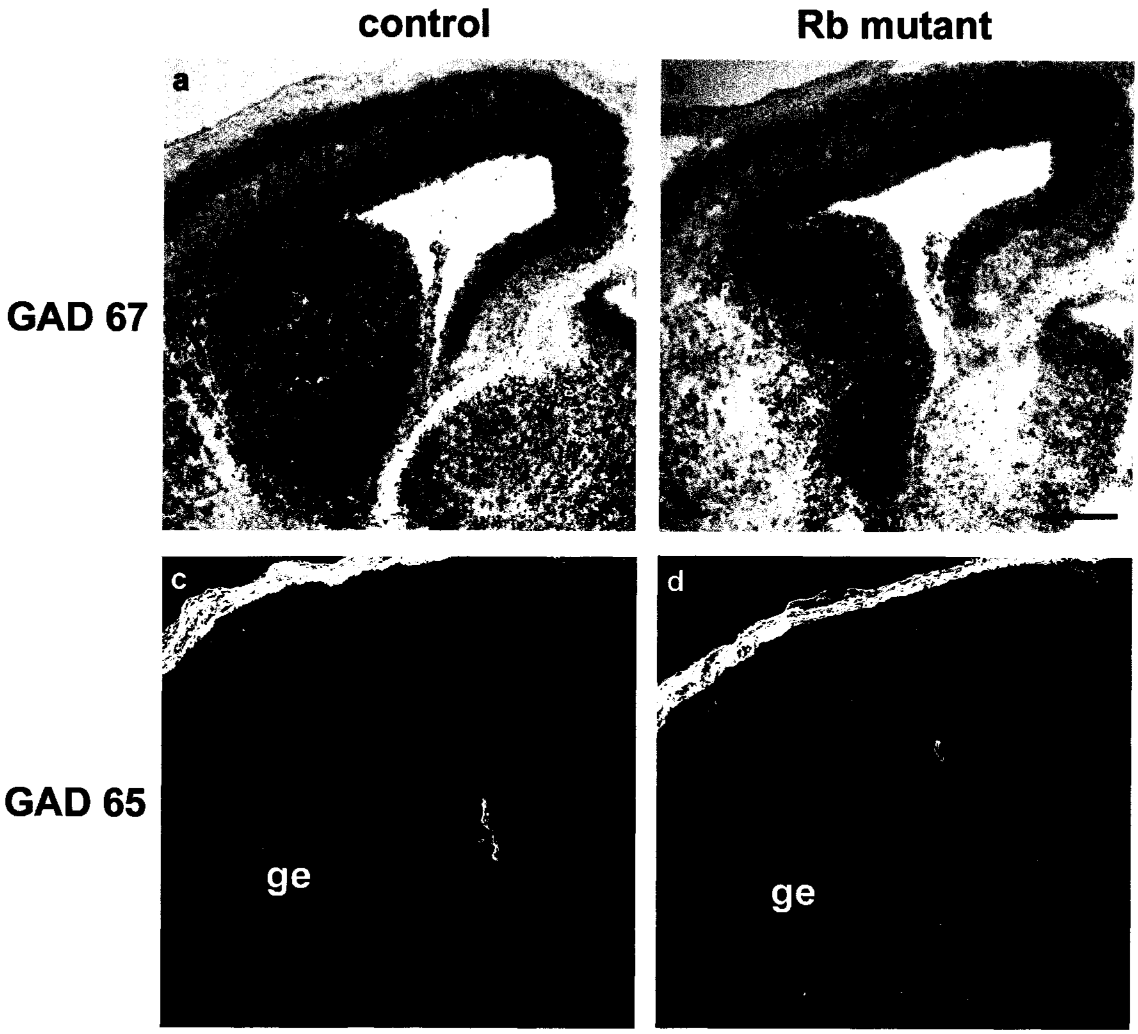
Control and Rb mutant embryos at E13.5 (a) and E16.5 (b) were sectioned and subjected to TUNEL analysis. At E13.5, all regions quantified contained similar numbers of apoptotic cells, except the Rb mutant MZ, which contained an approximately 50% increase in TUNEL-positive cells ($P<0.05$) (n= 4 control, 5 cond. Rb-/-). At E16.5, TUNEL-positive cells were at similar levels in all examined areas (n= 3 control, 3 cond. Rb-/-). Error denotes standard error.



Supplemental Figure 2

Supplemental Figure 2-3 Similar population of GABAergic interneurons

GAD 67 in situ hybridization and GAD 65 immunohistochemistry demonstrate similar levels of GABAergic interneuron markers between control and cond. Rb mutant at E13.3 (a-b) and E14.5 (c-d)



Supplemental Figure 3

REFERENCES

As per the guidelines "Writing a M.Sc. or Ph.D thesis" from the Department of Cellular and Molecular Medicine at the University of Ottawa, Sub-section "Format of a Collection of Articles", references of all sections are listed in an amalgamated list at the end of the thesis.

http://www.intermed.med.uottawa.ca/cellmed/eng/writing.html

CHAPTER 3

McClellan, K.A., V.A. Ruzhynsky, D.N. Douda, J.L. Vanderluit, K.L. Ferguson, D. Chen, R. Bremner, D.S. Park, G. Leone, & R.S. Slack (2007) Overlapping and unique roles for Rb/E2F1 and 3 in regulating neural precursor proliferation and neuronal migration: Evidence for *in vivo* cell cycle independent functions. *Molecular & Cellular Biology* **27**:*4825-43*

The experiments were conceptualized and performed by KAM, with the exception of the in situ hybridization for E2F1 and E2F3, which were performed by VAR. DND contributed to mouse breeding of Rb:E2F1, and assisted with immunohistochemistry for calbindin and reelin. JLV assisted with the E2F3 adult progenitor experiment and KLF provided preliminary data in support of the role of E2F1 in neurogenesis. DC, RB, and GL provided transgenic mice. KAM wrote the first, and subsequently revised drafts of the manuscript. All authors contributed to critical review of the manuscript. RSS as the principal investigator provided conceptual, technical, and editorial guidance.

A unique requirement for Rb/E2F3 in neuronal migration: Evidence for cell cycle independent functions

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Running Title: Rb regulates neuronal migration through E2F3

Word Count for Materials and Methods: 1373
Word Count for Introduction, Results, and Discussion: 6494

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ABSTRACT

The cell cycle regulatory retinoblastoma (Rb) protein is a key regulator of neural precursor proliferation, however, its role has been expanded to include a novel cell autonomous role in mediating neuronal migration. We sought to determine the Rb interacting factors that mediate both the cell cycle and migration defects. E2F1 and 3 are likely Rb interacting candidates that we have shown to be deregulated in the absence of Rb. Using mice with compound null mutations of Rb and E2F1 or E2F3, we asked to which extent either E2F interacts with Rb in neurogenesis. Here, we report that E2F1 and E2F3 are both functionally relevant targets in neural precursor proliferation, cell cycle exit, and laminar patterning. Each also partially mediates the Rb requirement for neuronal survival. Neuronal migration, however, is specifically mediated through E2F3, beyond its role in cell cycle regulation. This study not only outlines overlapping and distinct functions for E2Fs in neurogenesis, but is also the first to establish a physiologically relevant role for the Rb/E2F pathway beyond cell cycle regulation *in vivo*.

Keywords: Cell cycle independent / Retinoblastoma / E2F / Neuronal Migration / Developmental

Subject Category: Cell cycle / Development

INTRODUCTION

Neurogenesis is a highly regulated process by which neural precursors divide and differentiate giving rise to the cells that make up the nervous system (reviewed in (Gotz and Huttner, 2005; Guillemot, 2005)). While the role of cell cycle genes regulating proliferation of neural precursor cells is well appreciated, accumulating data points convincingly to their unique roles in regulating diverse cellular processes, independent of cell cycle regulation (reviewed in (McClellan and Slack, 2006)). The retinoblastoma (Rb) tumor suppressor is a key cell cycle regulator that we and others have shown to play a number of roles in neurodevelopment including proliferation, survival and more recently neuronal migration (Callaghan et al., 1999; Chen et al., 2004a; Clarke et al., 1992; Ferguson et al., 2005; Ferguson et al., 2002; Jacks et al., 1992; Lee et al., 1992; MacPherson et al., 2003). Differentiating Rb deficient neural precursor cells exhibit delayed cell cycle exit, while the absence of Rb in the telencephalon leads to ectopic proliferation of neural precursor cells and enhanced brain size at mid-gestation (Callaghan et al., 1999; Ferguson et al., 2002; MacPherson et al., 2003). In a recent study we described a role for Rb in regulating the survival of discrete neuronal subpopulations, and a novel cell autonomous role for Rb in regulating neuronal migration (Ferguson et al., 2005).

The mechanism by which Rb regulates neurogenesis and the extent to which defects in migration and survival are the result of cell cycle deregulation remains unknown. While Rb is known to interact with numerous proteins (reviewed in (Morris and Dyson, 2001)), many of which are expressed in quiescent cells or have cell cycle independent functions, members of the cell cycle regulatory E2F family are likely targets in neurogenesis. The E2F family of transcription factors are comprised of E2Fs 1-8, however, E2F1, 2 and 3, the so-called activating E2Fs, are key Rb interacting targets best known for their role in promoting cell cycle progression (reviewed in (Trimarchi and Lees, 2002))(Christensen et al., 2005; de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004;

Logan et al., 2005; Maiti et al., 2005). Both E2F1 and E2F3 are likely candidates involved in Rb mediated regulation of neurogenesis. Deficiency of either E2F1 or E2F3 was observed to correct the ectopic proliferation observed in the CNS in germline Rb deficiency alone, and both E2F1 and E2F3 are grossly deregulated in proliferating neural precursors in the absence of Rb (Callaghan et al., 1999; Saavedra et al., 2002; Tsai et al., 1998; Ziebold et al., 2001).

While E2F1 and E2F3 are key regulatory targets in the Rb signaling pathway, the extent to which each contribute to Rb mediated neurogenesis is unknown. Whether E2F1 and 3 are functionally redundant or are capable of unique function is still subject to debate and likely depends on the context examined. Individually, E2F1 is a tumor suppressor and its deficiency results in mice that are viable but develop tumors at an advanced age (Yamasaki et al., 1996). E2F1 expression is cell cycle regulated, with expression peaking at G1-S (reviewed in (Trimarchi and Lees, 2002)). A role for E2F1 in neurogenesis is indicated in the adult where mice deficient for E2F1 exhibit decreased precursor cell division in the proliferative regions of the lateral ventricle and hippocampus (Cooper-Kuhn et al., 2002). By contrast, E2F3 is not known as a tumor suppressor, but mice lacking E2F3 do exhibit a developmental phenotype (Cloud et al., 2002). E2F3 deficient mice survive postnally at a frequency of 25% on a mixed 129/Sv: C57BL/6 genetic background, and no E2F3 deficient mice are born on a pure 129/Sv genetic background (Cloud et al., 2002; Humbert et al., 2000b). Additionally, the E2F3 locus expresses two distinct transcripts- full length E2F3a and N terminal truncated E2F3b transcribed from an intronic promoter within the E2F3 locus (He et al., 2000; Leone et al., 2000). E2F3a expression is cell cycle regulated and is similar to that of E2F1 (He et al., 2000; Leone et al., 2000). E2F3b, however, is expressed equivalently in quiescent and proliferating cells, is a specific partner for Rb in quiescent cells, and thus may have an opposing role to E2F3a in cell cycle control (He et al., 2000; Leone et al., 2000).

As both E2F1 and 3 are expressed in the developing cortex starting from E11.5, and are deregulated in the absence of Rb, (Callaghan et al., 1999; Dagnino et al., 1997a), we sought to determine the extent to which each is a target in Rb mediated neurogenesis. Using mice with compound null mutations of Rb and E2F1 or E2F3, we describe both overlapping and unique functions for each. Here, we report that E2F1 and E2F3 are both functionally relevant targets in neural precursor proliferation, cell cycle exit, and laminar patterning. Each can partially mediate the Rb requirement for neuronal survival. Neuronal migration, however, is specifically mediated through E2F3. This study not only outlines overlapping and distinct functions for E2Fs in neurogenesis, but is also the first to establish a physiologically relevant requirement for the Rb/E2F pathway beyond cell cycle regulation *in vivo*.

MATERIALS AND METHODS

Mice

Germline E2F1 null mice were generated previously (Field et al., 1996), and obtained from the Jackson Laboratory (Bar Habor, ME) and maintained on a C57BL/6 genetic background. Germline E2F3 null mice were generated previously (Leone et al., 2001) and maintained on a mixed 129/ Sv and C57BL/6 genetic background. To generate E2F1 and E2F3 deficient mice, heterozygous (E2F1+/- or E2F3 +/-) mice were crossed. Embryos and animals were genotyped according to standard protocols with previously published primers for E2F1 (Field et al., 1996) and E2F3 (Leone et al., 2001). Telencephalon specific Rb deficient mice were generated by crossing floxed Rb-F19 (Marino et al., 2000; Vooijs et al., 1998) and Foxg1-cre mice (Hebert and McConnell, 2000), and were genotyped according to standard protocols with previously published primers (Ferguson et al., 2005; Ferguson et al., 2002). Telencephalon specific Rb deficient: germline deficient E2F1 (Rb E2F1 DKO) were generated by interbreeding flox Rb: Foxg1-cre mice with flox Rb:germline E2F1 deficient mice, both maintained on an FVBN background. Telencephalon specific Rb deficient: Telencephalon specific E2F3 deficient (Rb E2F3 DKO) were generated by crossing flox Rb: Foxg1-cre mice with flox E2F3 mice (Wu et al., 2001), both maintained on an FVBN genetic background. Mice were genotyped for flox E2F3 according to standard protocols with previously published primers for flox E2F3 (Wu et al., 2001). For embryonic time points, the time of plug identification was counted as embryonic day (E) 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.

Tissue Fixation and Cryoprotection

Pregnant female mice and adult mice were euthanized with a lethal injection of sodium pentobarbitol. Embryos were dissected and fixed overnight in 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS), pH 7.4, cryoprotected in sequential solutions of 12,16 and 22% sucrose in 1x PBS followed by embedding in OCT (TissueTek 4583), and frozen on liquid N_2. Adult mice were perfused with 1x PBS followed by cold 4% PFA and brains removed. Brains were post-fixed overnight in 4% PFA, cryoprotected in 22% sucrose in 1x PBS, and frozen. Sections from either embryos or adults were collected as 14 μm coronal cryosections on Superfrost Plus® slides (Fisher Scientific, 12-550-15).

BrdU labeling, Immunohistochemistry, and In situ hybridization

To assess neural progenitor proliferation in adults (12 weeks), intraperitoneal injections of BrdU (dissolved in 0.007 N NaOH in 0.9% NaCl; 50 mg/kg body mass) (Sigma, B-5002) were given every 2 h over a 10 h period. Mice were euthanized 30 min following the last injection (Tropepe et al., 1997; Vanderluit et al., 2004). BrdU detection was performed with a mouse monoclonal anti-BrdU (1:100, BD Biosciences, 347580) as previously described (Ferguson et al., 2002). BrdU positive cells were counted in the subependyma of the lateral ventricles in every 10th coronal cryosection (14 μm thick) from the most caudal crossing of the corpus callosum to the start of the 3rd ventricle (crossing of the anterior commissure). Two-tailed t-test was performed to compare the mean numbers of BrdU positive cells and significant differences assessed at values of $\alpha=0.05$. To assess neural progenitor proliferation in embryos, pregnant females were injected intra-peritoneally with 50 μg BrdU/ g body mass and processed as above. BrdU labeled cells were quantified over a 650μm region of dorsal cortex with a mimimum of three matched sections counted per embryo. To assess cells in mitotic M-phase, phospho-histone H3 labeling was performed with rabbit polyclonal anti-PH3 (1:100; Upstate

Biotechnology, 06-570) as previously described (Ferguson et al., 2002). To assess cell death, either TUNEL labeling or active caspase-3 (AC-3) immunohistochemistry combined with Hoescht nuclear staining was performed according to standard protocols (Ferguson et al., 2002). (In situ end labeling kit Roche) (AC-31:500; BD Pharmingen, 559565 Rabbit Polyclonal). To quantify cell death specific to the marginal zone, active caspase-3 labeled cells in the marginal zone were counted from the cingulate cortex to the dorsal-ventral boundary. Both hemispheres were quantified and counts expressed as the mean of the two hemispheres from four matched sections per embryo. To quantify cell death in the ventral telencephalon, active caspase-3 labeled cells were counted below the dorsal-ventral boundary from four matched sections per embryo. Reelin and calbindin immunolabeling were performed with the mouse monoclonal anti-Reelin G10 (1:500; Calbiochem, #553731) and rabbit polyclonal anti-calbindin (D-28) (1:1000; Chemicon, AB1778) as previously described (Ferguson et al., 2005). Reelin labeled cells were quantified along a 500μm region of dorsal cortex and temporal cortex from a minimum of four matched sections per embryo. Calbindin labeled cells were quantified within the MZ, or within the same area comprising all cells within the migratory route (TOTAL) of each hemisphere from four matched sections per embryo and expressed as cells per 500μm length. For all immunohistochemistry, secondary antibodies were obtained from Molecular Probes and used at a concentration of 1:500. Cresyl violet staining was performed according to standard protocols and cells in the marginal zone were quantified along a 500 μm region of dorsal cortex and expressed as the mean from a minimum of four matched sections per embryo. Non-radioactive *in situ* hybridization and digoxygenin probe labeling was performed according to previously described protocols (Wallace and Raff, 1999). Tbr1 antisense riboprobe was used, as previously described (Bulfone et al., 1995) and neogenin riboprobes were generous gifts of Dr. Helen Cooper- University of Queensland (Gad et al., 1997) and Dr. Elke Stein- Yale University. E2F1 and E2F3 DIG-labeled riboprobes were generated from pBS-IIKS-E2F1 and pBS-IIKS-E2F3 templates, containing 0.65 kb and 0.72 kb cDNA inserts, respectively, which were amplified by PCR with primers E2F1 (For – ATCGGAATTCTCTCTTTGACTGTGACT; Rev – ATTAAAGCTTCGATCGGAAAACTT), E2F3 (For – ATCGAAGCTTAGACTTGGCTTCTAACAACT; Rev – TGGCAGAATTCCATTCCGTGGTAG) and verified by sequencing.

Microarray Analysis

For microarray analysis, total RNA was extracted from tissue derived from ganglionic eminences at E14.5 from control and cond. Rb mutants using Trizol reagent according to manufacturer's instructions (Invitrogen, San Diego, CA). Samples from n=6 embryos were pooled for each genotype. RNA was sent to the Ottawa Genomics Innovation Centre Microarray Facility where the Affymetrix Mouse genome 430 2.0 Array was used and analyzed.

Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed on total protein extracts from neural precursor cells as described (Callaghan et al., 1999), with the following modifications. Total cell protein was extracted in a lysis buffer (Buffer A) and assayed by the method of Bradford (Bio-Rad Protein Assay Reagent Catalog No. 500-0006). A 20μg aliquot of lysate was incubated with an excess of ^{32}P-labeled double-stranded DNA probe (70 000 c.p.m./0.2 ng of DNA) containing a single E2F-binding site: 5'-GGATTTAAGTTTCGCGCCCTTTCTCAA-3'. The binding reaction (25 μL) was carried out at room temperature for 20 min in binding buffer [20mM HEPES pH7.6, 4% Ficoll, 2.5% $MgCl_2$, 40mM KCl, 0.1mM EGTA, 0.5mg/ml acetylated bovine serum albumin (BSA), 0.5mM DTT]. To control for binding specificity, a 10-fold excess of unlabeled wild type oligonucleotide was added to the binding reaction and incubated for 20 min before the addition of labeled probe. To identify the composition of the complexes, tissue culture supernatant or purified antibody was added to the reaction mixture. Complexes were resolved on a 5.0% gel run for 4 h, dried, and visualized by autoradiography. The tissue culture supernatant containing the monoclonal pRb antibody 21C9 was a gift from David Cobrinik (Templeton and Weinberg, 1991). All other antibodies were purchased from Santa Cruz Biotechnology Inc. E2F1 (sc 193), E2F3 (sc878 and sc878x). IP-EMSA was performed as described (Ikeda et al., 1996) with the following modifications. For immunoprecipitation, 200μg of total protein from neural precursors was incubated with either 2μg of mouse monoclonal anti-human Rb antibody (BD Biosciences cat # 554136), or equivalent mouse serum (Sigma M-5905) conjugated to Protein G sepharose beads (Amersham/ GE Healthcare 17-0618-01) in 1X shift buffer [20 mM HEPES pH7.9, 40mM KCl, 6mM $MgCl_2$, 1mM EGTA, 0.4mM sodium vanadate, 0.4 mM sodium fluoride, 0.1% NP-40, 1mM DTT, and protease inhibitors] for 1hr with gentle rotation. Beads were washed 3-4 times in 1X shift buffer, followed by treatment with 16μL of 0.8% deoxycholate (DOC) for 10 mins on ice to dissociate the E2F complexes. Following neutralization with 4μL of 6% NP-40, 5μL of the supernatant was used for E2F EMSA as described above.

Microscopy

Sections treated for immunohistochemistry were examined by a Zeiss Axioskop 2 microscope with standard fluorescence and brightfield/darkfield settings at X5 0.17 or X20 0.17 NA objectives. Images were captured using a digital black and white camera with Northern Eclipse software. For confocal microscopy, a Zeiss LSM 510 META on an Axiovert 200M inverted microscope was used with images captured through the manufacturer's integrated digital imaging software. Figures were compiled using Adobe Photoshop CS2. Manipulations of brightness and intensity were made equally to all treatment groups.

RESULTS

E2F3 is a positive regulator of neural precursor proliferation

Previous studies have described a role for E2F1 in regulating neural precursor proliferation *in vivo* (Cooper-Kuhn et al., 2002), however, little is known regarding the role of E2F3. As E2F3 is known as a major regulator of cellular proliferation (Humbert et al., 2000b) (reviewed in (Trimarchi and Lees, 2002)), we first asked if E2F3 is also capable of regulating neural precursor proliferation using mice lacking E2F3. As cell cycle time of neural progenitors in the adult brain has been estimated to be 12.7 hours (Morshead and van der Kooy, 1992), we administered a series of bromodeoxyuridine (BrdU) injections over 10.5 hours (Vanderluit et al., 2004). Similar to what has been reported for E2F1, E2F3 -/- mice exhibited 35% fewer neural progenitors lining the lateral ventricles relative to littermate controls (Fig. 1 E2F3 +/+ 662 ± 25.5 cells, E2F3 -/- 434 ± 14.5 cells; n=3 per genotype). To determine whether the decrease in BrdU labeled cells was due to an increase in apoptosis, we performed TUNEL labeling and active caspase 3 staining on E2F3-/- and littermate controls. No difference was observed in the number of dying cells along the lateral ventricles (data not shown). In the embryo, a similar 30% reduction in the number of proliferating cells and no difference in cell death was observed (data not shown). These results indicate that E2F3 is a positive regulator of neural precursor proliferation and point to functional redundancy among E2Fs in regulating neural precursor proliferation.

E2F1 and 3 are physiologically relevant Rb interacting partners *in vivo*

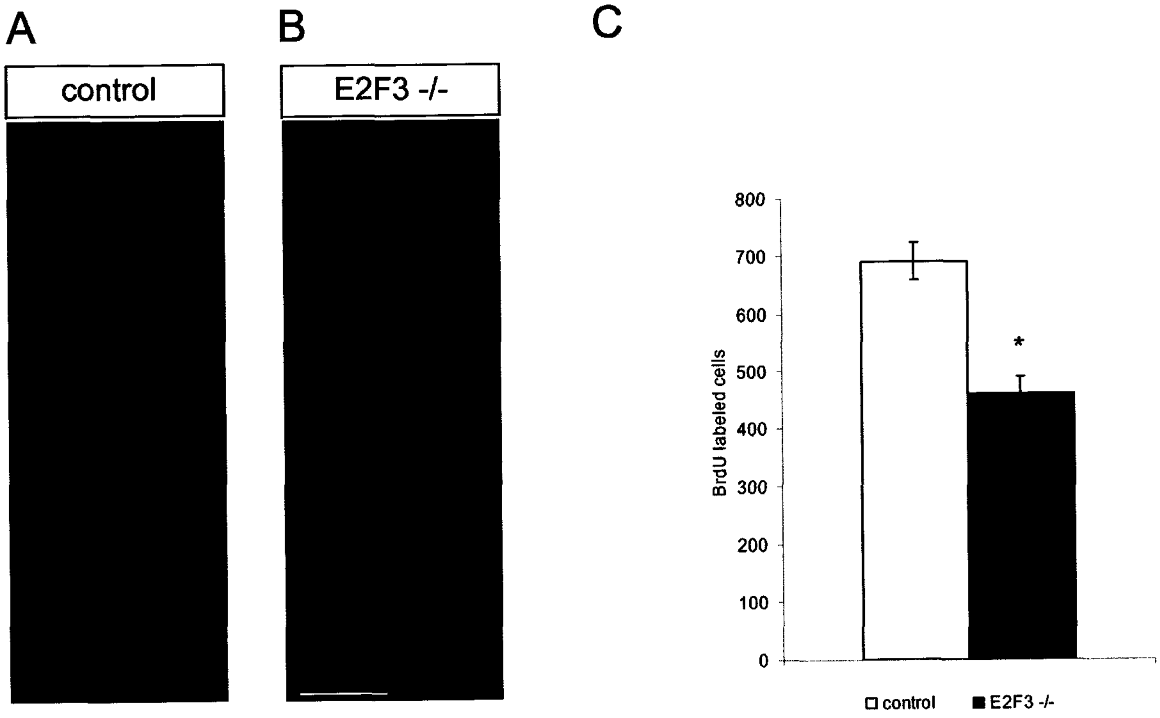

A
B
C
control
E2F3 -/-
800
700
600
500
400
300
200
100
0
BrdU labeled cells
*
☐ control
■ E2F3 -/-

Figure 1

While both E2F1 and 3 function independently in regulating neural precursor proliferation, the context dependent nature of Rb/E2F interaction led us to ask whether E2F1 and 3 are physiologically relevant Rb interacting partners in the developing nervous system. The nature of Rb/E2F complexes was examined in protein extracts of neural precursors derived from embryonic forebrain through electrophoretic mobility shift assay (EMSA) and complexes were identified using antibodies specific to E2F family members (Fig 2a). In wild-type tissue, both free E2F1 and 3 as well as bound Rb/E2F activity are observed (Fig 2a lane 1), indicating that E2F1 and 3 are active in the context of neurodevelopment. Here we also observe that Rb is bound predominantly to E2F3 and E2F1 as antibodies to E2F1 (Fig 2a lane 3) and E2F3 (Fig 2a lane 5) displace the Rb band, however, E2F3 appears to be the more predominant Rb binding partner. To confirm that Rb is indeed binding to both E2F1 and E2F3 in neural precursor cells, we performed an immunoprecipitation for Rb from neural precursor cells, followed by deoxycholate (DOC) treatment to release the associated E2F activity, and subjected this extract to EMSA (Ikeda et al., 1996; Leone et al., 2000) (Fig 2b). Immunoprecipitation for Rb followed by DOC treatment gave rise to a similar pattern of free E2F binding activity (Fig 2b lane 3) as DOC treatment on extracts alone (Fig 2b lane 2). Supershifts of the IP extract with E2F1 and 3 antibodies demonstrate that both E2F1 (Fig 2b lane 4) and E2F3 (Fig 2b lane 5) bind to Rb in the nervous system. We next asked what the consequence of disrupting Rb activity would have on E2F1 and 3 activity. We hypothesized that if E2F1 and 3 are significant Rb binding partners in wild-type tissue, then the absence of Rb should lead to an increase in their free activity. Indeed extracts of brain tissue from Rb mutants exhibit a gross deregulation of E2F1 and 3 activity relative to control (Fig 2a, lane 2). This increase appears specific to free E2F1 and 3 activity as supershifts with E2F1 and 3 antibodies displaces the free E2F1 and 3 band (Fig 2a lanes 4, & 6). Thus together, these data provide biochemical evidence that E2F1 and 3 are physiological Rb interacting factors in the developing nervous system *in vivo.*

Figure 3-2 E2F1 and 3 are physiologically relevant Rb interacting partners *in vivo*

(A) For EMSA experiments, total protein was extracted from proliferating neural precursors in cond Rb mut and controls. Total protein extracts were incubated alone or in the presence of E2F antibodies prior to incubation with double stranded ^{32}P labeled E2F consensus probe. Antibodies used for supershift are indicated above the corresponding lane. In control extracts, Rb is bound to both E2F1 and E2F3 as antibodies to E2F1 and E2F3 both displace the Rb band (lanes 3 & 5). In the absence of Rb an obvious increase in free E2F1 and 3 binding activity is noted compared to control (lane 2, E2F1 & 3 supershifts lanes 4 & 6 respectively). (B) For IP-EMSA experiments, total protein extracts from control proliferating neural precursors were subjected to immunoprecipitation for Rb followed by DOC treatment to release E2F associated with Rb as described in Materials and Methods. The released material was subjected to EMSA (lane 3) and assayed for E2F1 (lane 4) or E2F3 (lane 5) binding activity. As controls, IP-EMSA was repeated using control mouse serum in place of Rb immunoprecipitating antibodies (lane 6), or Rb deficient neural precursor protein extracts instead of control extracts (lane 7). Finally, a sample of protein extract was subject to EMSA (lane 1) or directly treated with DOC and then assayed for E2F binding activity (lane 2).

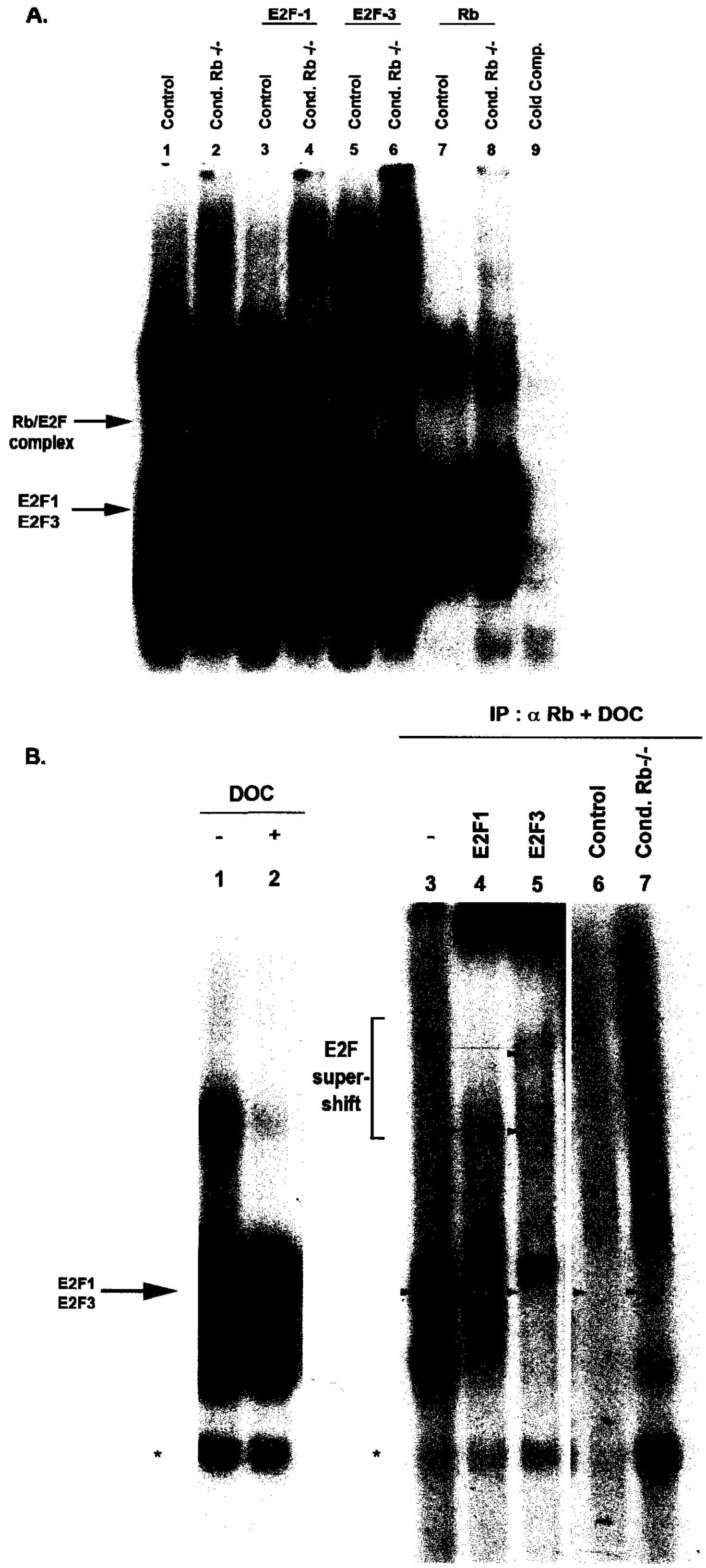

A.
E2F-1
E2F-3
Rb
1 Control
2 Cond. Rb -/-
3 Control
4 Cond. Rb -/-
5 Control
6 Cond. Rb -/-
7 Control
8 Cond. Rb -/-
9 Cold Comp.
Rb/E2F complex
E2F1
E2F3
B.
DOC
-
+
1
2
IP : α Rb + DOC
-
E2F1
E2F3
Control
Cond. Rb-/-
3
4
5
6
7
E2F super-shift
E2F1
E2F3
*
*

Figure 2

E2F1 and E2F3 exhibit overlapping patterns of expression in the developing telencephalon *in vivo*

As E2F1 and E2F3 are both functionally relevant interacting partners in neural precursor cells, we next asked if E2F1 and E2F3 are expressed in the same cell populations in the developing telencephalon. Sections from control tissue at E13.5 and E15.5 were subjected to in situ hybridization for E2F1 and E2F3. At each age, both E2F1 and E2F3 expression is observed in a similar overlapping pattern (Fig 3). This region of robust expression encompasses both the dorsal and ventral ventricular/ subventricular zones where proliferating and newly committed neurons reside (reviewed in (Guillemot, 2005)). In addition, at E15.5, both E2F1 and E2F3 expression is observed throughout the ganglionic eminences, the region which gives rise to migrating populations of interneurons; interneurons that ultimately exhibit aberrant migration in Rb deficiency (Ferguson et al., 2005). Hence these data support the hypothesis that E2F1 and E2F3 could each be functional targets in both Rb mediated proliferation and migration in the developing telencephalon.

Both E2F1 and E2F3 are functional targets in Rb mediated neural precursor proliferation

Our previous studies have demonstrated that Rb deficiency in the telencephalon leads to ectopic proliferation of neural precursor cells without the widespread apoptosis observed in germline Rb deficiency (Ferguson et al., 2002). The gross deregulation of E2F1 and E2F3 binding activity in Rb deficient neural precursors and their overlapping patterns of expression in proliferating neural precursor cells *in vivo* suggests that these E2Fs could be targets in Rb mediated neural precursor proliferation. To ask if the proliferation defect observed in the absence of Rb could be attributed to deregulated E2F1 or E2F3 activity, we generated mice with a) compound null mutations for Rb and E2F3 in the telencephalon (Rb E2F3 DKO) and b) absence of Rb in the telencephalon and whole embryo E2F1 deficiency (Rb E2F1 DKO). Pregnant females were subjected to a BrdU injection 2

Figure 3-3: E2F1 and E2F3 exhibit overlapping patterns of expression in the developing telencephalon *in vivo*

Control E13.5 and E15.5 sections were subjected to in situ hybridization for E2F1 and E2F3 using antisense riboprobes, or sense riboprobes as a control. At both ages, both E2F1 and E2F3 are expressed in overlapping patterns in the developing telencephalon. At E13.5 expression of both E2F1 and E2F3 is largely confined to the developing ventricular and subventricular zones comprised of proliferating and post-mitotic cells lining the lateral ventricles. At E15.5 expression is highest within the ventricular and subventricular zones, but for both E2F1 and E2F3 expression is also observed similarly throughout the ganglionic eminences (n=4 embryos, for each E2F). Note the absence of signal in the sense control for each probe. LGE= lateral ganglionic eminence, MGE= medial ganglionic eminence, ge= ganglionic eminence Bar = 200μm

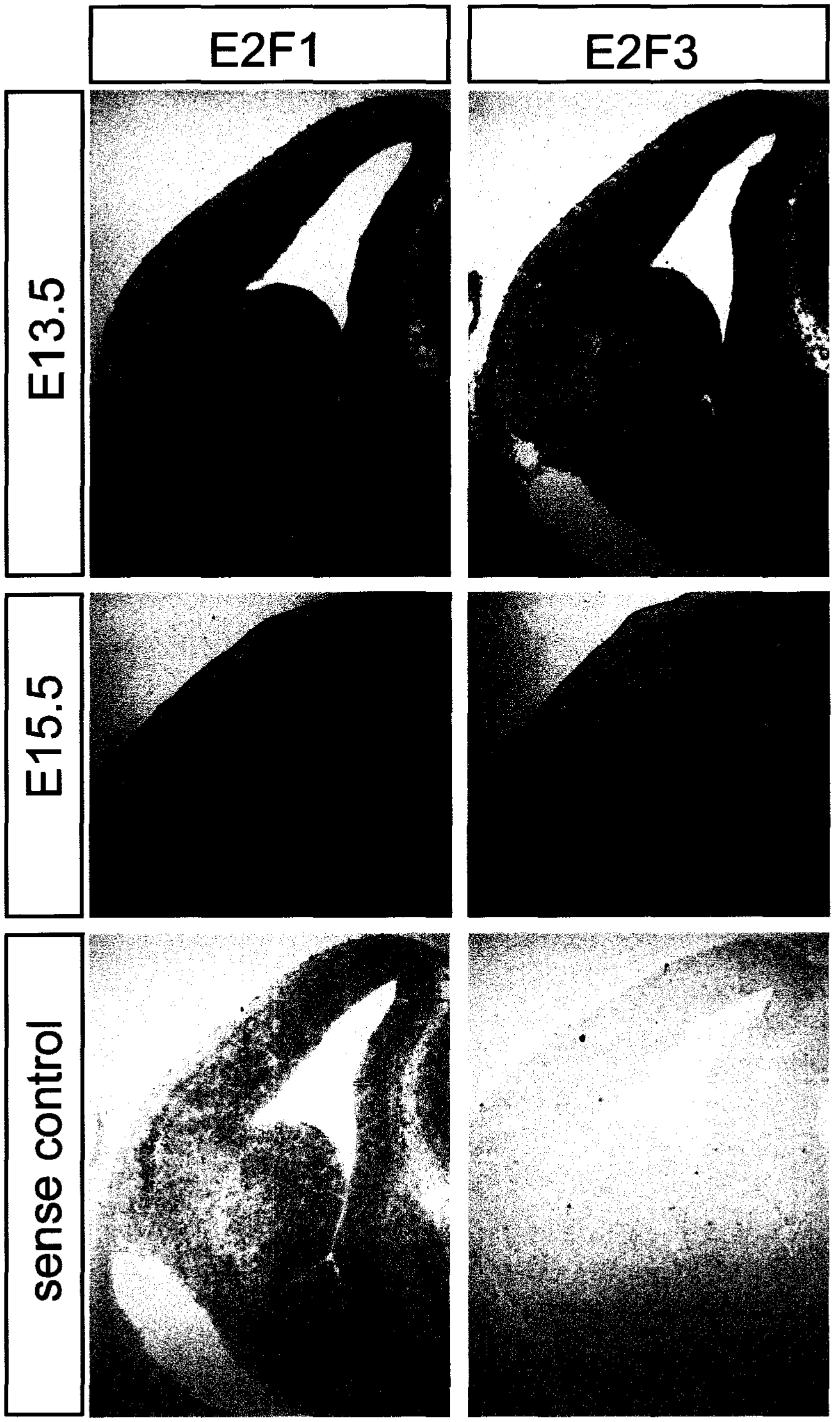


Figure 3

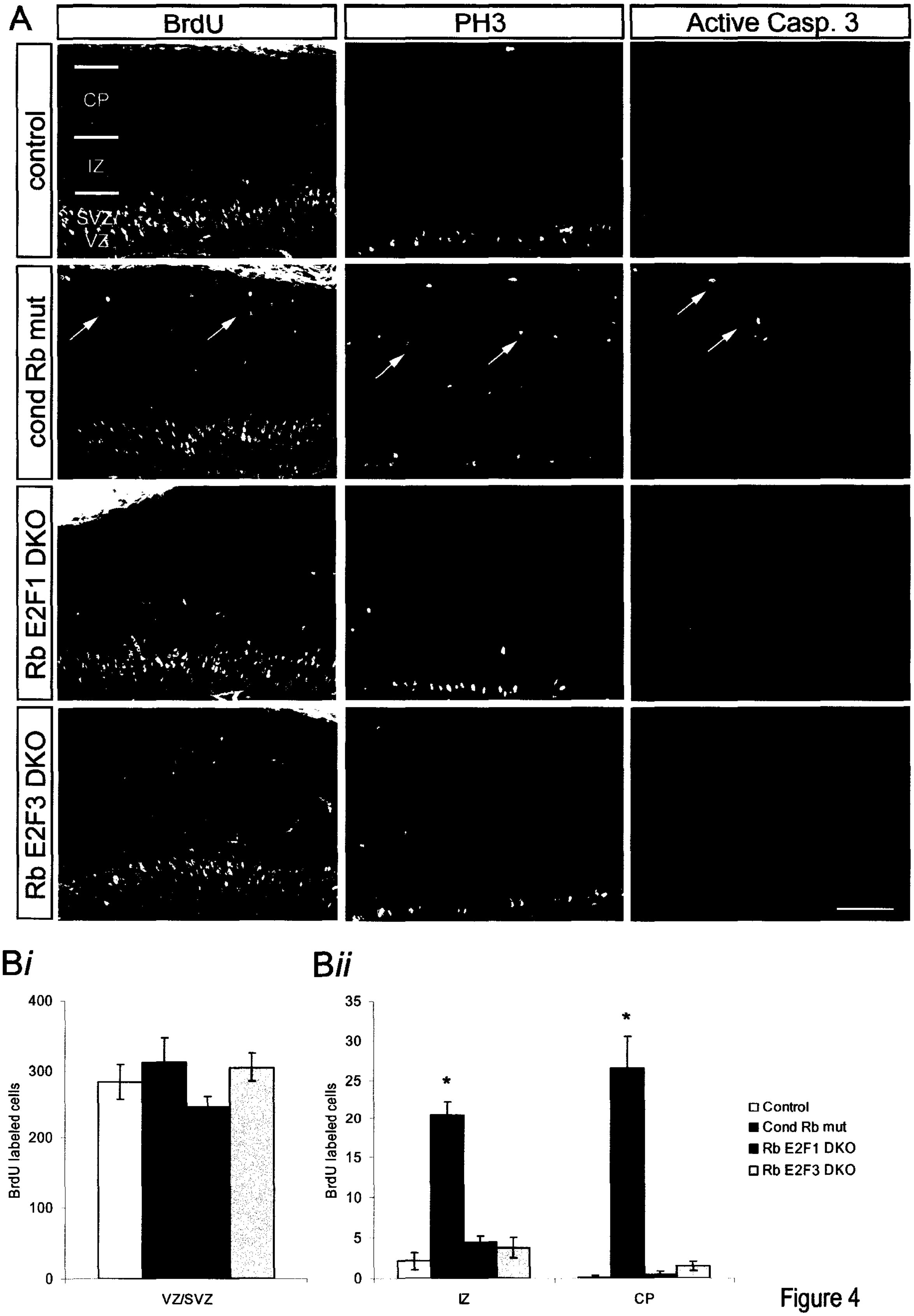
hours prior to sacrifice at E15.5 and embryonic sections were subjected to BrdU immunohistochemistry. In contrast to the cond. Rb mutant where proliferating cells are observed in the ventricular zone and post-mitotic regions, in both Rb E2F1 DKO and Rb E2F3 DKO, BrdU labeled cells were largely confined to the ventricular and subventricular zones with a minor proportion observed in the intermediate zone (Fig 4A). Quantification of BrdU labeled cells confirms that Rb E2F1 DKO and Rb E2F3 DKO leads to a rescue of ectopically proliferating cells caused by Rb deficiency alone (Fig 4B*ii*). Similar observations were made in sections labeled with the M phase marker, phospho-histone H3 (PH3) (Fig 4A). Whereas PH3 labeled cells are observed in the cortical plate, intermediate zone, and subventricular zone in the Rb mutant, mitotically active cells are largely observed in the ventricular and subventricular zones of Rb E2F1 DKO and Rb E2F3 DKO, indicative of a rescue of the failed cell cycle exit in committed neurons. To determine whether the rescue of cell cycle exit was due to an increase in apoptosis specific to the population of ectopically positioned proliferating cells, we performed TUNEL labeling and active caspase 3 staining on Rb E2F1 DKO and Rb E2F3 DKO (Fig 4A). No increase in cell death was observed in Rb E2F1 DKO or Rb E2F3 DKO relative to Rb mutant or control (Fig 4A; data not shown). Together, these data demonstrate that the absence of either E2F1 or E2F3 in Rb deficiency leads to a rescue of the ectopic proliferation. These findings indicate that E2F1 and E2F3 are each functionally relevant targets in Rb mediated regulation of neural precursor proliferation and cell cycle exit.

Rb mediated regulation of radial migration and laminar patterning occurs through interactions with E2F1 and 3

We have recently described that the loss of Rb leads to defective radial migration and laminar patterning in the developing cortex (Ferguson et al., 2005). Specifically, in the cond. Rb mutant we observe the absence of a clear cortical plate/intermediate zone (CP/IZ) boundary in histological

Figure 3-4: Both E2F1 and E2F3 are functional targets in Rb mediated neural precursor proliferation

(A) Sections from E15.5 cond Rb mut, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls were labeled with an antibody to BrdU to label cells in S-phase, phospho-histone H3 (H3) to label cells in M phase, or active caspase-3 to label dying cells. While cond. Rb mutants exhibit BrdU and PH3 labeled cells in the VZ/SVZ, IZ and CP, both Rb E2F1 DKO and Rb E2F3 DKO exhibit BrdU and PH3 labeling confined to the VZ/SVZ. No difference was observed in active caspase-3 labeling between cond Rb mut, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls. (B) BrdU labeled cells were quantified along a 650μm region of dorsal cortex and classified according to zone, from 4 matched sections per embryo. Number of BrdU labeled cells counted was expressed as the mean ± S.E.M. Whereas no difference in the number of labeled cells was observed between cond. Rb mut, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls in the VZ/SVZ (B*i*), significantly fewer BrdU labeled cells were observed in the IZ and CP of Rb E2F1 DKO and Rb E2F3 DKO relative to cond. Rb mutants, but not different relative to their respective controls (B*ii*). Significance was determined using a single factor ANOVA with Tukey post-hoc test. * $P < 0.05$, n=4 all genotypes. VZ= ventricular zone, SVZ= subventricular zone, IZ= intermediate zone, CP= cortical plate. Bar = 100μm


A
BrdU
PH3
Active Casp. 3
control
cond Rb mut
Rb E2F1 DKO
Rb E2F3 DKO
CP
IZ
SVZ
VZ
B*i*
400
300
200
100
0
BrdU labeled cells
VZ/SVZ
B*ii*
35
30
25
20
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10
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0
BrdU labeled cells
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*
IZ
CP
□ Control
■ Cond Rb mut
■ Rb E2F1 DKO
□ Rb E2F3 DKO

Figure 4

sections and expanded expression of cortical plate markers into the IZ. To determine whether radial migration and laminar patterning is mediated through the Rb/E2F cell cycle regulatory pathway we asked, therefore, whether compound Rb E2F1 or Rb E2F3 deficiency is sufficient to correct the laminar patterning defects observed in the Rb mutant. Sections of Rb E2F1 DKO or Rb E2F3 DKO at E15.5 were stained with cresyl violet. In control tissue, a clear boundary exists between the packed cells of the developing cortical plate and the underlying intermediate zone (Fig 5). Compared to the cond. Rb mutant, where the developing cortical plate appeared to be comprised of loosely packed cells that are intermingled with cells of the intermediate zone, both the Rb E2F1 DKO and Rb E2F3 DKO exhibited a cortical plate with a similar structure of densely packed cells and a clear CP/IZ division as the control (Fig 5). No defects in laminar patterning were observed in either E2F1 or E2F3 single deficiency (data not shown). We next asked whether the layer specific defects we observe in the cond. Rb mutant were also restored. Sections from Rb E2F1 DKO and Rb E2F3 DKO were subjected to in situ hybridization with Tbr1, a layer specific marker demarcating the preplate and layer 6 from the intermediate zone (Bulfone et al., 1995; Hevner et al., 2001), that exhibited vivid defects in the cond. Rb mutant (Ferguson et al., 2005). Consistent with the restoration of gross overall morphological structure, sections from Rb E2F1 DKO and Rb E2F3 DKO exhibited Tbr1 expression that was largely confined to the developing cortical plate, and exhibited a clear division between the cortical plate and intermediate zone, similar to the division observed in control sections (Fig 5). This Tbr1 expression pattern in Rb E2F1 DKO and Rb E2F3 DKO is in contrast to the cond. Rb mutant where Tbr1 expression is expanded beyond the confines of the cortical plate into the intermediate zone (Fig 5). These data demonstrate that the compound absence of either Rb E2F1 or Rb E2F3 is sufficient to restore cortical structure and laminar patterning. Furthermore, as both Rb E2F1 DKO and Rb E2F3 DKO are capable of rescuing both the cell cycle and laminar patterning defects, these data

Figure 3-5: Rb mediated radial migration and laminar patterning defects occur through interactions with E2F1 and 3

E15.5 sections of cond. Rb mut, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls were stained with cresyl violet and subjected to in situ hybridization for Tbr1. In cresyl violet stained sections, cond. Rb mutants exhibit the absence of a clear boundary between cortical plate and intermediate zone as compared to control (arrows). This defect appears corrected in both Rb E2F1 DKO and Rb E2F3 DKO. Similarly, whereas Tbr1 expression is expanded beyond the CP and into the IZ in the cond. Rb mutant (arrows), both Rb E2F1 DKO and Rb E2F3 DKO exhibit Tbr1 expression which is confined to the CP, similar to that observed in control. (n=3 per genotype). MZ=marginal zone, CP=cortical plate, IZ=intermediate zone, SVZ= subventricular zone, VZ= ventricular zone. Bar = 100μm

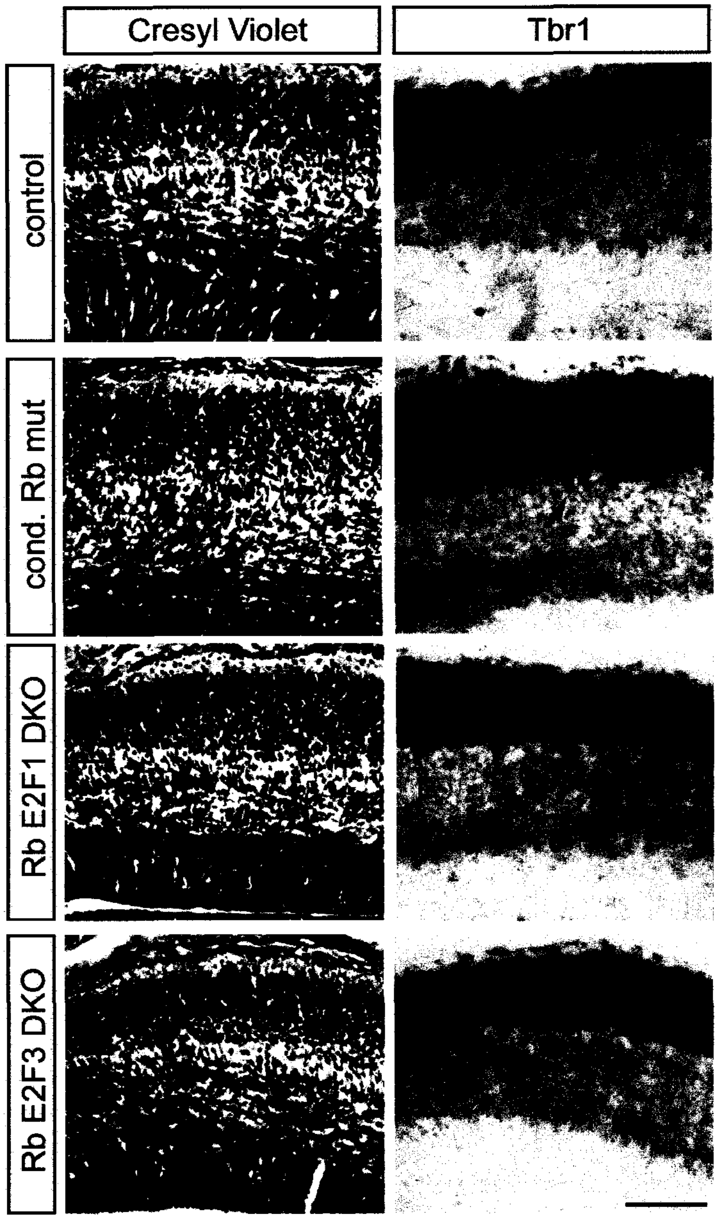
Cresyl Violet
Tbr1
control
cond. Rb mut
Rb E2F1 DKO
Rb E2F3 DKO

Figure 5

are consistent with the interpretation that Rb mediated radial migration and cortical structure occurs as a result of defects in cell cycle regulation.

The Rb mediated requirement for survival of a subset of neurons is partially mediated through the Rb/E2F pathway

While cond. Rb mutants have shown that Rb is not required for widespread neuronal survival, we have recently demonstrated that Rb is required for survival of neurons within the marginal zone of the developing cortex (Ferguson et al., 2005; Ferguson et al., 2002; MacPherson et al., 2003). As E2F1, in particular, is a well known mediator of survival (reviewed in (Matsumura et al., 2003)), we asked to what extent marginal zone neuronal survival could be mediated through Rb interactions with E2F1 or E2F3. Total cells within the marginal zone were quantified from histological sections stained with cresyl violet of cond. Rb mut., Rb E2F1 DKO and Rb E2F3 DKO. Both Rb E2F1 DKO and Rb E2F3 DKO sections exhibited an increased number of marginal zone cells relative to Rb mutants, and no difference relative to control (Fig. 6A). To determine if this increase was the result of increased survival, we examined cell death by quantifying active caspase- 3 labeled cells within the marginal zone. Consistent with the increase in marginal zone cell numbers, both Rb E2F1 DKO and Rb E2F3 DKO exhibited a decrease in the number of active caspase-3 labeled cells relative to cond. Rb mutants, and no difference relative to control (Fig 6B). These data suggest that E2F1 and 3 are both capable of mediating the Rb requirement for neuron survival within the marginal zone.

The marginal zone is a complex layer comprised of a heterogeneous population of cells including Cajal-Retzius (CR) neurons (Marin-Padilla, 1998; Meyer et al., 1999). We have previously demonstrated the cond. Rb mutants exhibit a specific loss of CR neurons, by cell death which contributes to the overall reduction of cells within the MZ (Ferguson et al., 2005). Thus to determine if Rb mediated neuronal survival is also mediated through Rb interactions with E2F1 and 3, we

Figure 3-6: The Rb mediated requirement for survival of Cajal-Retzius neurons is only partially mediated through the Rb/E2F pathway

(A) Marginal zone cells in cresyl violet stained sections of E15.5 cond. Rb mut, Rb E2F1 DKO, Rb E2F3 DKO and their corresponding controls were quantified along a 500μm region of dorsal cortex from a minimum of three matched sections per embryo and expressed as mean ± S.E.M. n= 4 embryos per genotype. Whereas cond. Rb muts exhibit decreased number of cells in MZ, both Rb E2F1 DKO and Rb E2F3 DKO exhibit an increased number of cells in the marginal zone, similar to their respective controls. (B) Active-caspase-3 (AC-3) labeled cells of cond. Rb mut, Rb E2F1 DKO, Rb E2F3 DKO and their corresponding controls were quantified within the marginal zone from the cingulate cortex to the dorsal-ventral boundary. Both hemispheres were quantified and counts expressed as the mean of two hemispheres from 4 matched sections per embryo. Bars represent mean ± S.E.M. n= 8 per genotype. Whereas cond Rb muts exhibit increased number of AC-3 positive cells in MZ, both Rb E2F1 DKO and Rb E2F3 DKO exhibit a decreased number of AC-3 positive cells in the marginal zone, similar to their respective controls. (C) A noticeable increase in reelin labeled cells was observed in Rb E2F1 DKO, Rb E2F3 DKO relative to cond. Rb mut. Reelin labeled cells were quantified along a 500μm region of dorsal cortex and temporal cortex and expressed as the mean from a minimum of four matched sections per embryo. Whereas cond. Rb mut exhibit decreased number of reelin labeled cells in MZ, both Rb E2F1 DKO and Rb E2F3 DKO exhibit an increased number of cells in the marginal zone relative to the cond. Rb mutant, yet still significantly less than that observed in their respective controls. Bars represent mean ± S.E.M. n= 3 control and cond. Rb mutant, n=4 Rb E2F1 DKO, n= 5 Rb E2F3 DKO. In all cases significance was determined using a single factor ANOVA with Tukey post-hoc test. * $P < 0.05$ AC-3 = active caspase-3. MZ= marginal zone. Bar = 100μm

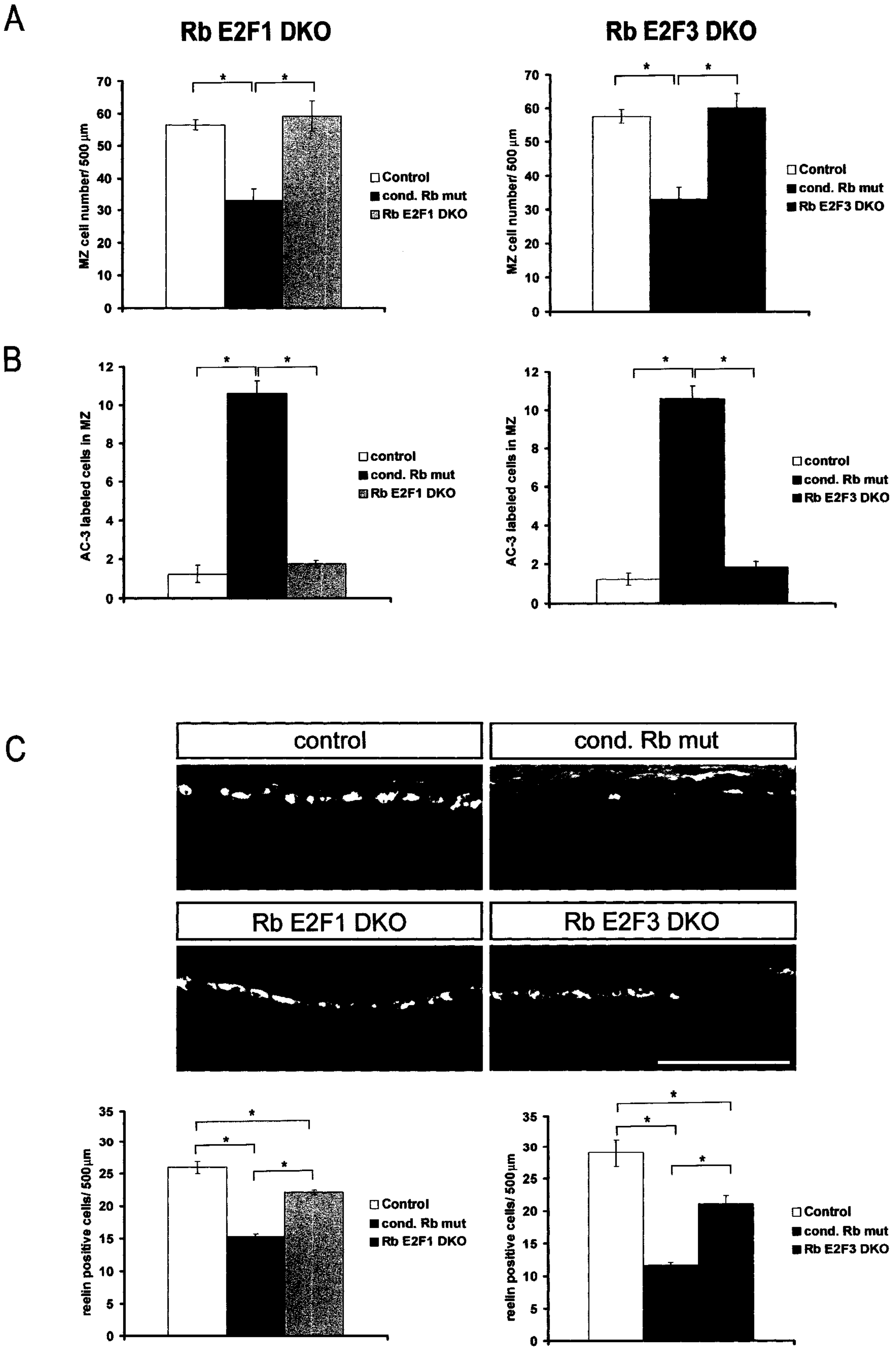
A
Rb E2F1 DKO
Rb E2F3 DKO
MZ cell number/ 500 µm
0
10
20
30
40
50
60
70
*
☐ Control
■ cond. Rb mut
▨ Rb E2F1 DKO
■ Rb E2F3 DKO
B
AC-3 labeled cells in MZ
0
2
4
6
8
10
12
☐ control
■ cond. Rb mut
▨ Rb E2F1 DKO
■ Rb E2F3 DKO
C
control
cond. Rb mut
Rb E2F1 DKO
Rb E2F3 DKO
reelin positive cells/ 500µm
0
5
10
15
20
25
30
35
☐ Control
■ cond. Rb mut
■ Rb E2F1 DKO
■ Rb E2F3 DKO

Figure 6

examined CR neurons within the MZ in Rb E2F1 DKO and Rb E2F3 DKO. Sections were subjected to immunohistochemistry for reelin, a CR neuron specific protein (reviewed in (Meyer et al., 1999)). Whereas both Rb E2F1 DKO and Rb E2F3 DKO sections exhibited increased numbers of reelin positive cells relative to cond. Rb mutants, fewer reelin positive cells were observed relative to control (Fig 6C). These data indicate that E2F1 and 3 only partially mediate the Rb requirement for CR neuron survival. As CR neurons themselves represent a heterogeneous population of cells (Bielle et al., 2005), these data support the hypothesis that the Rb/E2F pathway mediates survival of a subtype of CR neurons.

E2F3 specifically mediates the aberrant tangential migration of interneurons in Rb mutants

Interneurons are key regulators of neuronal function that act by modulating the activity of major excitatory neural circuits (reviewed in (McBain and Fisahn, 2001)). Interneuron dysfunction and/ or aberrant migration of interneurons during development has been implicated in a wide range of neurological disorders including autism, epilepsy, schizophrenia, and bipolar disorder (reviewed in (Benes and Berretta, 2001), (Bentivoglio et al., 2003)). In our recent study, we demonstrated that interneurons arising from the ventral telencephalon exhibit aberrant tangential migration to the dorsal cortex in conditional Rb deficiency. Specifically, calbindin labeled cells, a marker of GABAergic interneurons, are absent in Rb mutants along the marginal zone migratory route that is taken by these cells (Ferguson et al., 2005). As many of the neurodevelopmental defects described in cond. Rb mutants appear to be mediated through both E2F1 and E2F3, and as both are expressed in the ventral ganglionic eminences, where interneurons originate, we questioned whether the E2F pathway could also be mediating migration. To assess the degree to which E2F1 and E2F3 could be contributing to the tangential migration defect in cond. Rb mutants, we examined the calbindin cell population along their migratory route in Rb E2F1 DKO and Rb E2F3 DKO (Fig 7A). At E15.5,

Figure 3-7: E2F3 specifically mediates the aberrant tangential migration of interneurons in Rb mutants

(A) Schematic diagram of superficial marginal zone and deeper intermediate zone routes taken by migrating calbindin labeled interneurons originating from the MGE. Modified from (Marin and Rubenstein, 2003). Box indicates region of magnification in (B). Calbindin labeling was examined in cond. Rb mut, Rb E2F1 DKO, Rb E2F3 DKO and their respective controls along the marginal and intermediate zone migratory routes at E15.5. Calbindin positive cells are reduced or absent along the marginal zone migratory route in Rb E2F1 DKO and are present at a level similar to that observed in the cond. Rb mutant. By contrast, an abundance of calbindin labeled cells is observed in Rb E2F3 DKO along the marginal zone migratory route at the dorsal ventral boundary (arrows). (C) Calbindin-positive cells were quantified within the MZ (indicated by vertical bars) or along the same length within the complete migratory route as demarcated by the marginal zone and external capsule as lateral and medial boundaries [boxed area in (A)] for TOTAL. The TOTAL region was comprised of the marginal zone, as well as the intermediate zone, and cortical plate and labeled cells were counted on each hemisphere from four matched sections per embryo and expressed as cells per 500μm length. Quantification confirms that calbindin cells positioned in the MZ are restored in Rb E2F3 DKO but not in Rb E2F1 DKO, yet the total number of calbindin labeled cells is the same across all groups. Bars represent mean ± S.E.M, n=4 embryos per genotype. Significance was determined using a single factor ANOVA with Tukey post-hoc test. * $P < 0.05$. LGE= lateral ganglionic eminence, MGE= medial ganglionic eminence. Bar = 100μm

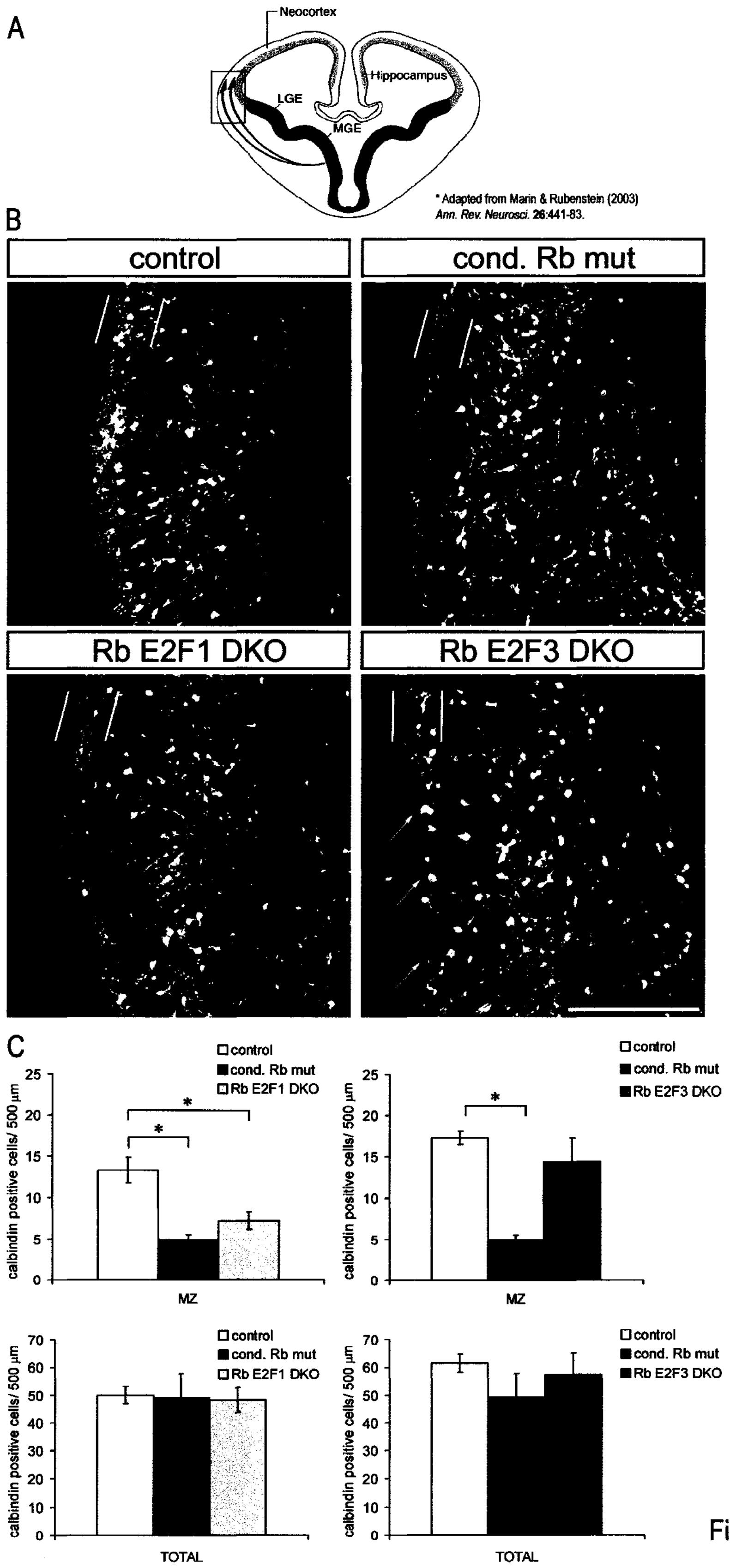
A
Neocortex
Hippocampus
LGE
MGE
* Adapted from Marin & Rubenstein (2003)
Ann. Rev. Neurosci. 26:441-83.
B
control
cond. Rb mut
Rb E2F1 DKO
Rb E2F3 DKO
C
□control
■cond. Rb mut
□Rb E2F1 DKO
■Rb E2F3 DKO
calbindin positive cells/ 500 µm
0
5
10
15
20
25
30
40
50
60
70
*
MZ
TOTAL

Figure 7

calbindin labeled cells are reduced or absent along the marginal zone migratory route in Rb E2F1 DKO, similar to that observed in the cond. Rb mutant (Fig 7B). By contrast, an abundance of calbindin labeled cells is observed in Rb E2F3 DKO along the marginal zone migratory route at the dorsal ventral boundary (Fig 7B). Quantification of the number of calbindin positive cells specifically within the marginal zone indicates that Rb E2F1 DKO exhibited significantly fewer calbindin labeled cells within the marginal zone, similar to that observed in the cond. Rb mutant, while Rb E2F3 DKO exhibited no difference in number of calbindin labeled cells relative to control (Fig 7C). Further, no difference in the distribution of calbindin labeled cells was observed in single E2F3 deficiency at E15.5 (data not shown).

While these results are suggestive of a specific role for Rb acting through E2F3 in mediating neuronal migration, to rule out the possibility that cell death was involved, we examined both the total number of calbindin positive cells within the migrating region and the level of apoptosis within the ventral telencephalon in Rb E2F1 DKO and Rb E2F3 DKO. As we have previously observed (Ferguson et al., 2005), quantification of total calbindin labeled cells revealed no significant differences between control, and cond. Rb mutant. Additionally, we observe no difference between Rb E2F1 DKO or Rb E2F3 DKO and control or cond. Rb mutants in the total number of calbindin labeled cells, suggestive of a population of similar size among all groups. We also quantified cell death as measured by active caspase-3 labeling in the ventral telencephalon including the ventricular zone, where interneurons originate; the marginal zone and cortical plate, along the route of migration, and points in between (Fig 8A). While a low level of cell death was observed overall, consistent with our previous results (Ferguson et al., 2002), we observe a small but significant increase in the overall level of cell death in the cond. Rb mutant relative to control (Fig 8B). Quantification of Rb E2F1 DKO and RbE2F3 DKO both revealed a similar level of cell death between that of control and cond. Rb

Figure 3-8: Rb E2F1 DKO and Rb E2F3 DKO exhibit similar levels of cell death in the ventral telencephalon

E15.5 sections of cond. Rb mut, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls were stained with active caspase-3 and Hoescht to examine cell death in the ventral telencephalon. Labeled cells were counted along the ventricular zone, where interneurons originate; the marginal zone and cortical plate, along the route of migration, and points in between from 4 matched sections per embryo. A similar level of cell death is observed between Rb E2F1 DKO and RbE2F3 DKO. Each exhibited a level of cell death between that of control and cond. Rb mutant. Bars represent the mean of total number of active caspase-3 labeled cells counted ± S.D, n=4 embryos per genotype. Significance was determined using a single factor ANOVA with Tukey post-hoc test. * $P < 0.05$ Bar =50μm

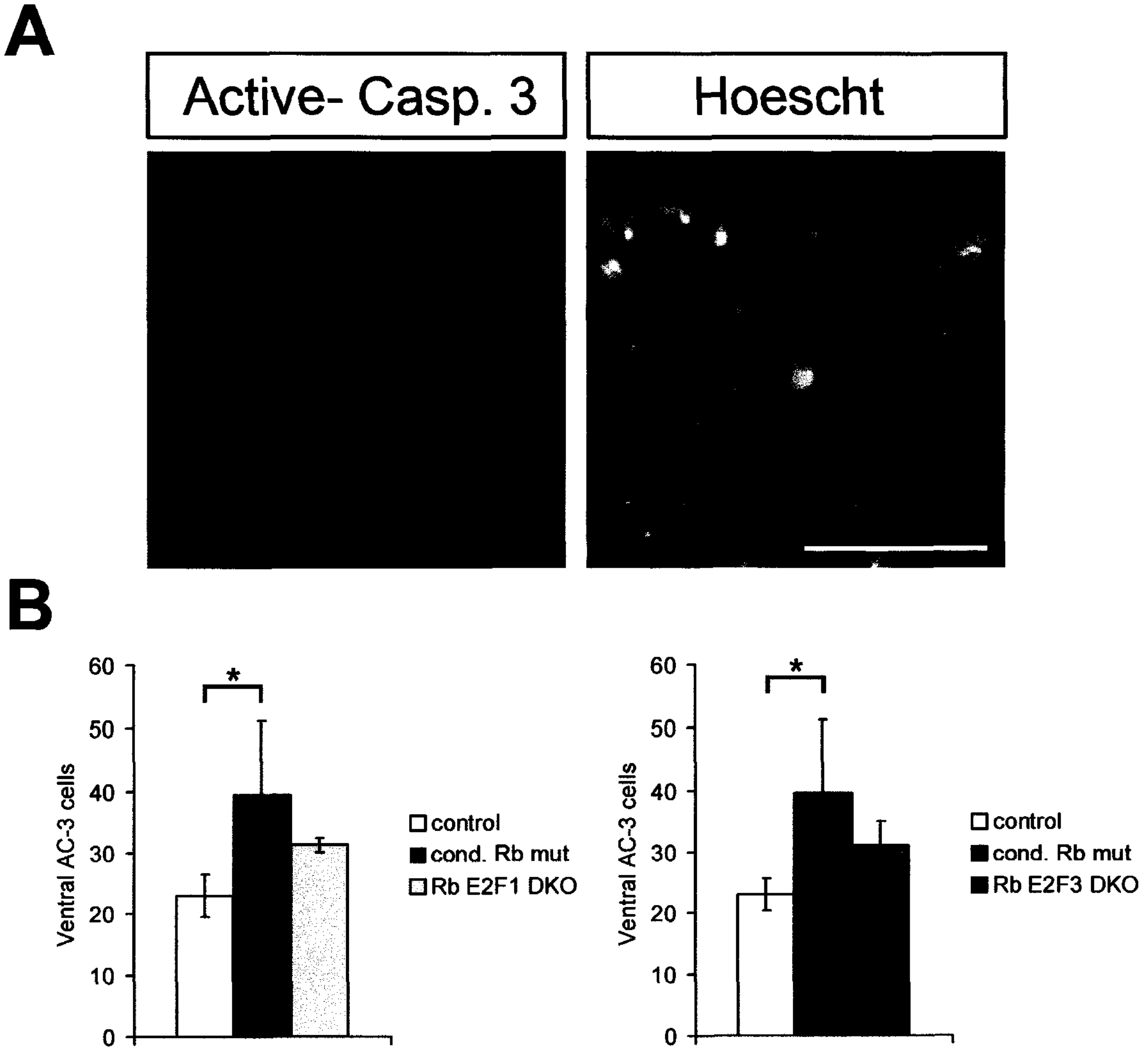
A
Active- Casp. 3
Hoescht
B
Ventral AC-3 cells
60
50
40
30
20
10
0
*
☐ control
■ cond. Rb mut
▨ Rb E2F1 DKO
Ventral AC-3 cells
60
50
40
30
20
10
0
*
☐ control
■ cond. Rb mut
■ Rb E2F3 DKO

Figure 8

mutant (Fig 8B). Together these results support our interpretation that the specific rescue of calbindin labeled cells within the marginal zone of Rb E2F3 DKO represents a rescue of the aberrant migration of calbindin labeled cells in the cond. Rb mutant and is not the result of altered cell death or other defects in the calbindin cell population. Thus these results reveal a unique function for E2F3 as the Rb target that mediates migration, a function distinct from the role of E2F1. Furthermore, these findings indicate that neuronal migration is mediated via an E2F dependent mechanism, and hence point towards novel targets specific for E2F3 mediated transcription.

Rb/E2F3 mediates neuronal migration in a manner beyond cell cycle regulation

Since Rb/E2F interaction is best characterized for its role in cell cycle regulation, our observation that Rb interaction with E2F3 is capable of mediating interneuron migration led us to question whether Rb mediated migration could be the result of defects in cell cycle control. To address this issue, we performed a thorough analysis by confocal microscopy of the location of proliferating and calbindin labeled cells in cond. Rb mutants at two developmental timepoints: at E15.5 when defective migration is observed, and at an earlier developmental timepoint, E13.5. We hypothesized that if the aberrant migration of calbindin interneurons is the result of defects in cell cycle control, then a population of calbindin labeled cells should be double labeled with BrdU after a standard 2hr pulse. In control embryos, at E15.5, at low magnification, proliferating cells are largely confined to the dorsal and ventral ventricular zones (Fig 9A top panel). In the cond. Rb mutants, ectopically proliferating cells appear largely confined to the post mitotic region of the dorsal cortex (Fig 9B 1'). The dorsal cortex region corresponds to the region where aberrant laminar patterning and radial migration is perturbed, thus further supporting the hypothesis that Rb mediated regulation of radial migration and cortical structure occurs as a result of defects in cell cycle signaling. By contrast, migrating calbindin labeled cells are observed in the ventral telencephalon, a distinct neuroanatomical

Figure 3-9 (A-B): Rb/E2F3 mediated tangential migration is not the result of cell cycle deregulation

Sections from cond. Rb mut and control were double labeled with BrdU (red) and calbindin (green) at E15.5 (A-B) and E13.5 (C-D). At E15.5 at low magnification, BrdU labeling is largely observed surrounding the ventricle (A & B- top panel red), whereas calbindin labeled cells are localized largely in the ventral telencephalon (A & B- top panel green). At higher magnification, ectopic proliferation in cond Rb mut is observed largely confined to the dorsal cortex (B1'), while no difference in the low level of BrdU labeling between cond Rb mut and control is observed in the ventral telencephalon (A2, A3 and B2', B3'). Instead, in this region, where BrdU labeling is not detected, an absence of calbindin labeled cells is observed in the marginal zone and the absence of calbindin labeled cells along the marginal zone migratory route in cond Rb mutants is observed (B2') (arrowheads). By confocal microscopy no BrdU calbindin double labeled cells are observed in the telencephalon at any of the three regions examined. Bar =100μm

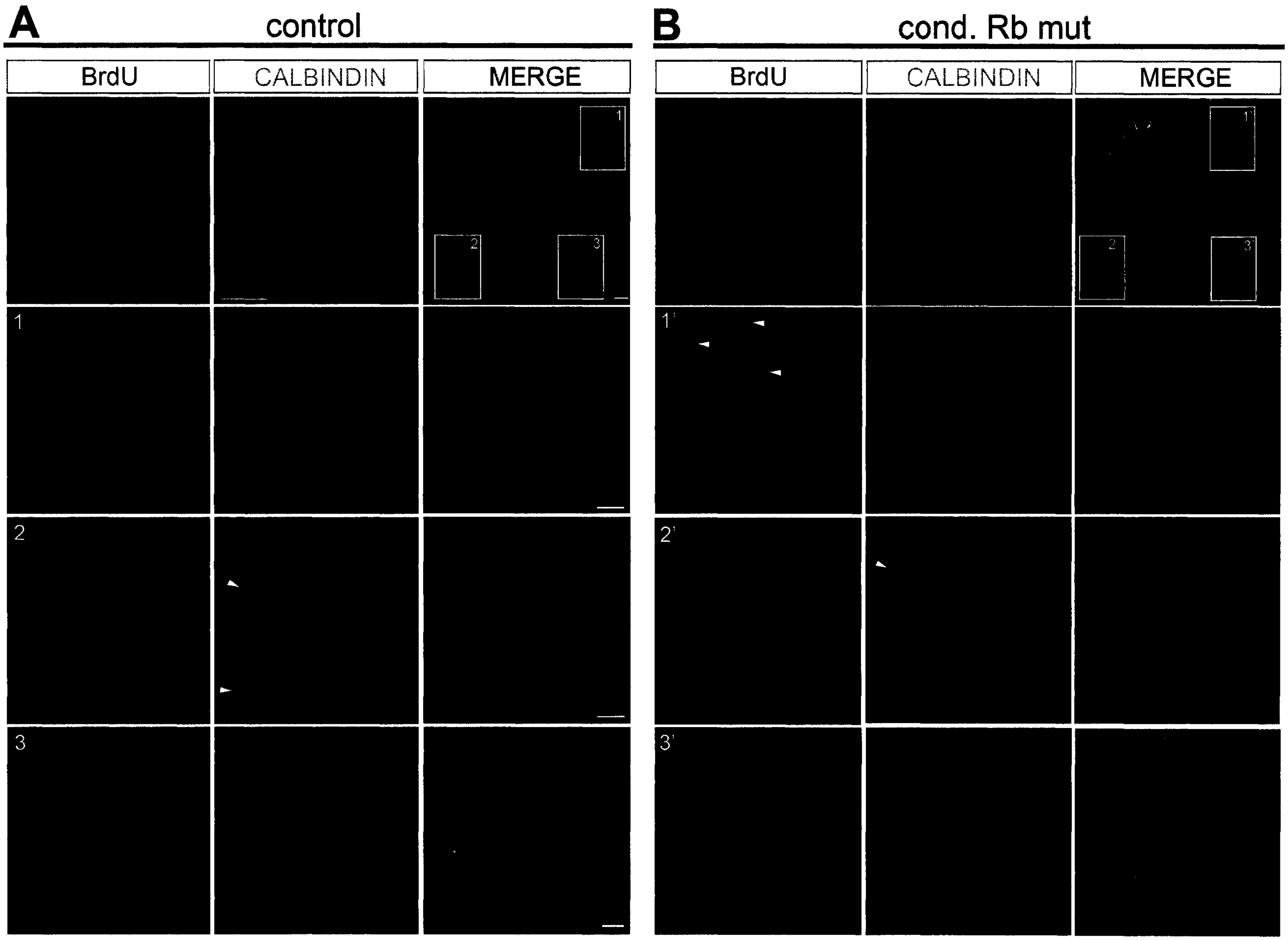
A
control
B
cond. Rb mut
BrdU
CALBINDIN
MERGE
BrdU
CALBINDIN
MERGE
1
2
3
1'
2'
3'
1
2
3
1'
2'
3'
E15.5

Figure 9 A-B

region, beyond the regions of normal and ectopic proliferation (Fig 9A 2, 3 and 9B 2', 3'). Using confocal microscopy we examined three distinct regions to see if calbindin labeled cells were ectopically proliferating: the dorsal cortex (Fig 9A 1, 9B 1'), where ectopically proliferating cells are observed in cond. Rb mutants; the migratory route within the ventral telencephalon (Fig 9A 2, 9B 2'), where calbindin labeled cells are aberrantly localized within the cond. Rb mutants; and the ventral ventricular zone within ganglionic eminences (Fig 9A 3, 9B 3'), where calbindin labeled cells originate. Close inspection from at least three sections from multiple embryos did not reveal BrdU and calbindin double labeled cells in either control or cond. Rb mutants in any of the three regions examined.

To determine if calbindin labeled cells that are migrating at E15.5 were ectopically proliferating at an earlier timepoint, we performed a similar confocal microscopy analysis of BrdU/calbindin double labeling at E13.5 (Fig 9C-D). At this age, we do observe ectopic BrdU labeled cells in the ventral telencephalon of cond. Rb mutants, along the route of migration of calbindin labeled interneurons (Fig 9D 2'). Quantification of ectopically proliferating cells demonstrates a significant increase in ventral ectopic proliferation at this age (Fig 9E), however, while several distinct neuronal subtypes exist in this region (reviewed in(Wonders and Anderson, 2006)), none of these ectopically dividing progenitors were colabeled with calbindin. Specifically, we examined distinct regions at E13.5 to see if calbindin labeled cells were ectopically proliferating, focusing on the ventral regions: the future route of migration route of aberrantly migrating cells within the ventral telencephalon (Fig 9C 2, 9D 2'); and the ventral ventricular zone within medial ganglionic eminence (Fig 9C 1, 9D 1'), where calbindin labeled cells originate. Similar to what we observed at E15.5, at E13.5, using confocal microscopy we did not observe BrdU and calbindin double labeled cells in either control or cond. Rb mutants at either regions examined. While we can't unequivocally rule out that aberrantly migrating cells were once ectopically proliferating, since we did not observe calbindin co-localized with BrdU in any region at either age examined, these data suggest that calbindin labeled cells have successfully exited the cell cycle in

Figure 3-9 (C-E): Rb/E2F3 mediated tangential migration is not the result of cell cycle deregulation

Sections from cond. Rb mut and control were double labeled with BrdU (red) and calbindin (green) at E13.5 (C-D).

At E13.5 ectopic proliferation is prevalent in cond. Rb mutants in the dorsal and ventral telencephalon (D—top panel and 9D2'), however, by confocal microscopy in either control or cond Rb mutant, we do not detect BrdU calbindin double labeling at either the ganglionic eminence, where calbindin cells originate (C1, D1') or at the future ventro-lateral migratory route (C2, D2'). Double labeled cells, however, were occasionally observed within the blood vessel rich pial layer outside of the telencephalon and are likely blood cells. Bar =100μm

(E) Quantification of ectopic proliferation within the ventral telencepahlon at E13.5, comprising the region of migration in control and cond. Rb mutant

BrdU cells within the region were counted in 4 matched sections per embryo. Bars represent mean of the average number of BrdU labeled cells within a 2000μm length along the ventrolateral boundary comprising the region of migration between the outermost edge of the marginal zone and the external capsule as lateral and medial boundaries ± S.D., n= 3 embryos per genotype. Significance was determined using a two-tailed T-test * $P < 0.05$

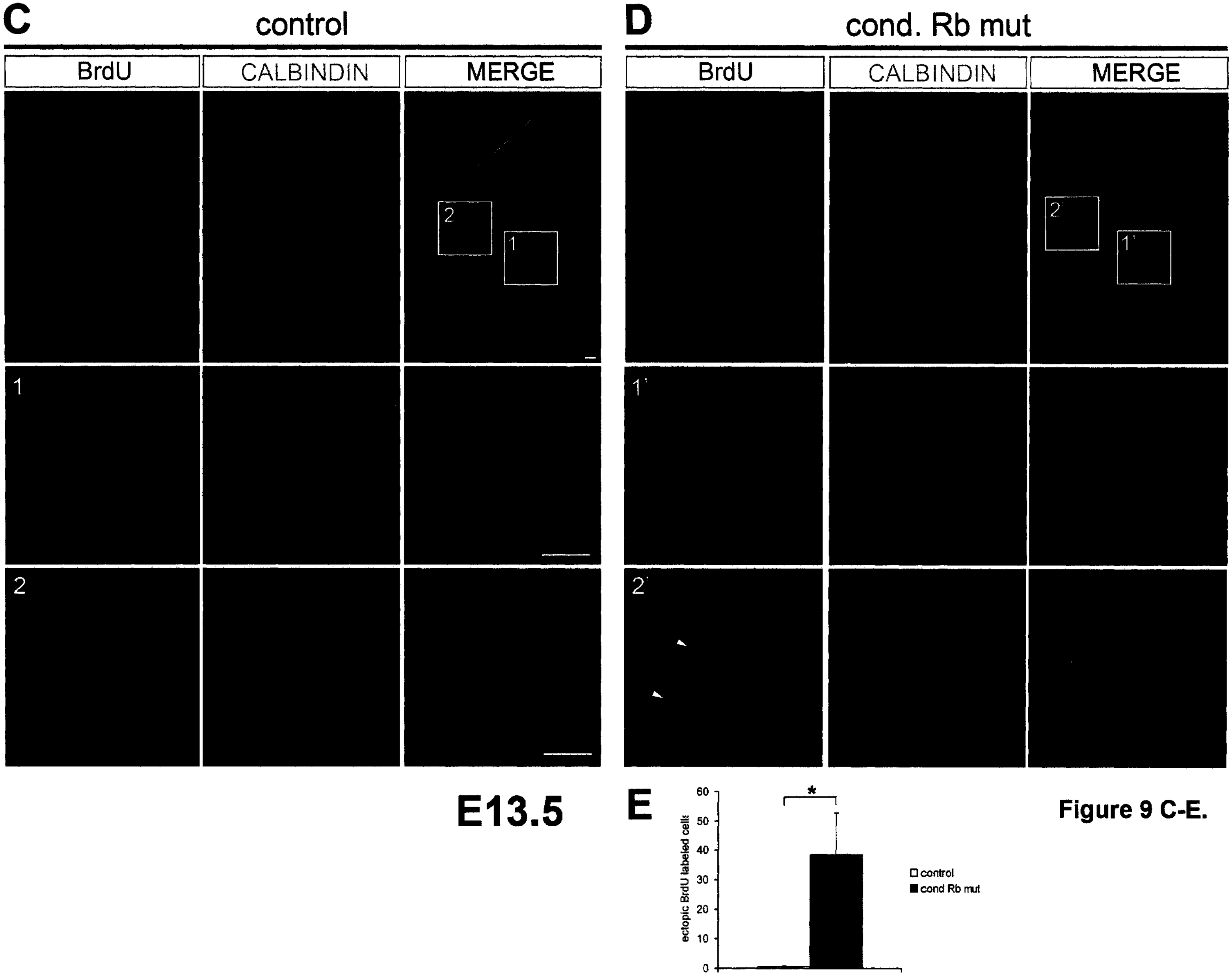
C
control
BrdU
CALBINDIN
MERGE
2
1
1
2
D
cond. Rb mut
BrdU
CALBINDIN
MERGE
2'
1'
1'
2'
E13.5
E
ectopic BrdU labeled cells
60
50
40
30
20
10
0
*
□ control
■ cond Rb mut

Figure 9 C-E.

cond. Rb mutants. Hence our data support the hypothesis that Rb mediates tangential migration through E2F3 in a manner beyond cell cycle regulation.

Rb mediates the expression of genes involved in regulating neuronal migration

Our data demonstrating that Rb mediates migration through E2F3, *in vivo*, represents physiological evidence in support of the hypothesis that Rb/E2F could be regulating the transcription of novel genes unrelated to cell cycle regulation. In an effort to identify candidate genes, we performed microarray analysis on neural precursor cells from control and cond. Rb mutants derived from the medial ganglionic eminences, the region which gives rise to migrating populations of interneurons which ultimately exhibit aberrant migration in Rb deficiency (Ferguson et al., 2005). We hypothesized that if Rb/E2F3 is mediating migration of interneurons in a cell autonomous manner as our previous data demonstrated (Ferguson et al., 2005), then absence of Rb would lead to deregulation of genes required to regulate this process. Through our microarray analysis, we have identified several candidate genes that are deregulated in cond. Rb mutants which have been shown to mediate neuronal migration, including migration of interneurons, such as members of the neogenin/netrin/RGM signaling pathway, the semaphorin/ neuropilin signaling pathway, and the Slit/Robo pathway (Table 3-1). While all candidates remain plausible targets, we have focused our initial attention on neogenin, a cell surface receptor and member of the immunoglobulin superfamily (Vielmetter et al., 1994) with well-known (Rajagopalan et al., 2004; Wilson and Key, 2006) and hypothesized roles in regulating neuronal migration, including interneuron migration (Fitzgerald et al., 2006). By microarray, neogenin expression was increased threefold, consistent with the role of E2F3 as a transcriptional activator. We next confirmed this observation through in situ hybridization for neogenin in cond. Rb mutants (Fig 10). At E13.5, a moderate increase in neogenin expression in the cond. Rb mutant was observed throughout the telencephalon including the ganglionic eminences (Fig

10). At E15.5 this increase in neogenin expression is more pronounced, particularly within the ganglionic eminences, where interneurons originate. Together, these data confirm that absence of Rb leads to deregulation of known genes required for interneuron migration, within the population of migrating cells during the time of migration, and in addition, establishes neogenin as a potential target gene in Rb/E2F mediated interneuron migration.

Table 3-1: Candidate Molecules Identified in Microarray from control and cond. Rb mutant ventral precursor cells

NAME	PATHWAY	FOLD CHANGE**	MIGRATION FUNCTION	REFERENCE
Neogenin	Netrin/Repulsive Guidance Molecule (RGM)	3X increase	Binds netrin and RGM Repels temporal retinal axons through RGM	(Rajagopalan et al., 2004) (Matsunaga et al., 2004)
Sema3d	Neuropilin /semaphorin	Moderate increase	Guides retinal axon along DV axis in zebrafish Can be repulsive or attractive	(Liu et al., 2004b) (Wolman et al., 2004)
VLDLR Very low density lipoprotein receptor	reelin signaling	Moderate increase	Receptors for reelin. VLDLR & ApoE2R KO mice have reeler like phenotype- inverted cortical lamina structure	(Trommsdorff et al., 1999)
ApoE	reelin signaling	2X increase	Could this be out competing reelin for binding to ApoER2?	NA
CCK Cholecystokinin		2.5 X increase	A marker of GABAergic interneurons CCK also reduces migration of GnRH neurons	(Lopez-Bendito et al., 2004) (Giacobini et al., 2004)
TWIST1 Twist neighbour	bHLH transcription factors	Moderate decrease 2X increase	Loss of function mutant leads to defect in neural crest cell migration	(O'Rourke and Tam, 2002)

** increase/decrease in mutant relative to control

Figure 3-10: Neogenin, a microarray identified gene exhibits deregulated expression in cond. Rb mutants

Control and cond. Rb mutant E13.5 and E15.5 sections were subjected to in situ hybridization for neogenin. At both ages, neogenin expression appears increased in the cond. Rb mutant relative to control. At E13.5 expression appears increased overall including in the ganglionic eminences (arrowheads). At E15.5 this increase in neogenin expression appears more pronounced, particularly within the ganglionic eminences, where interneurons originate (n=4 embryos for each genotype). ge= ganglionic eminence Bar = 100μm

NEOGENIN

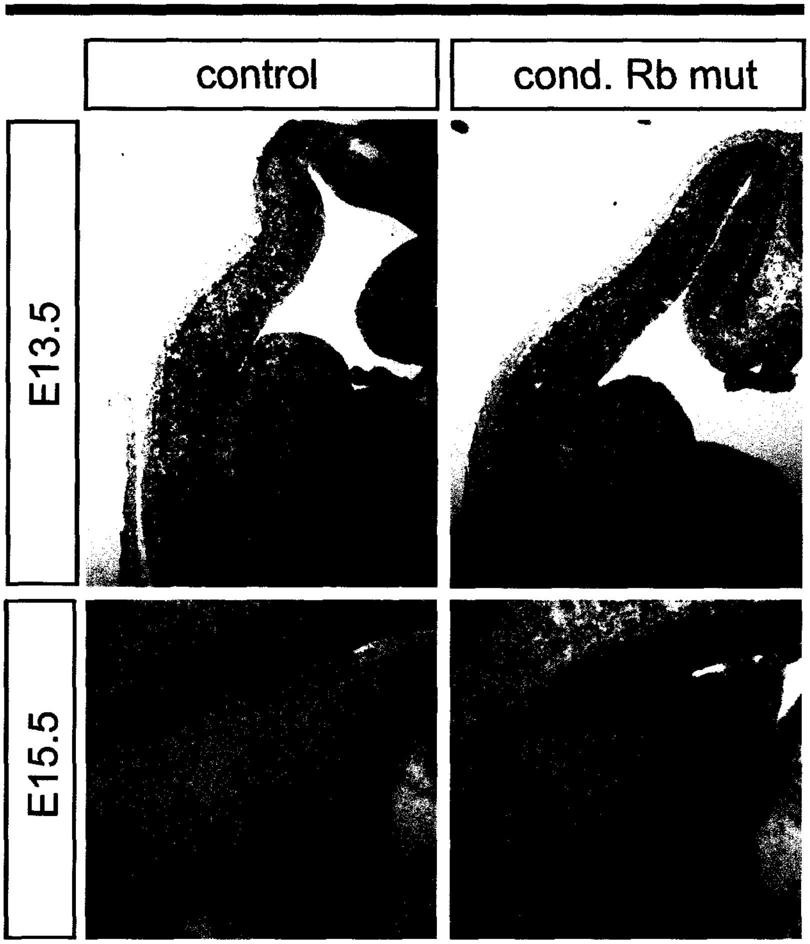

Figure 10

DISCUSSION

The mammalian nervous system is comprised of a complex array of different cell types and subtypes (reviewed in (Gotz and Huttner, 2005; Guillemot, 2005)). Neurogenesis, the process by which neural precursor cells divide and differentiate to give rise to all the different cell types, occurs in a highly regulated manner (reviewed in(Gotz and Huttner, 2005; Guillemot, 2005)). The cortex, a model of neurogenesis, is comprised of a series of layers that form in an inside out manner populated with excitatory projection neurons and inhibitory interneurons (reviewed in (Guillemot, 2005)). In both cases, neural precursors divide along the germinal zone lining the ventricles (reviewed in(Gotz and Huttner, 2005)). Once committed to a neuronal fate, cells exit the cell cycle and leave the germinal zone to migrate towards their final destination with projection neurons migrating radially to form the layers of the cortex and cortical interneurons migrating from the ventral telencephalon along tangential routes into the dorsal cortex (reviewed in (Gotz and Huttner, 2005; Kriegstein and Noctor, 2004; Marin and Rubenstein, 2001; Marin and Rubenstein, 2003)). The fundamental processes of neurogenesis, namely proliferation differentiation, migration, and maturation are controlled by the precise coordination of various genetic pathways (reviewed in (Gotz and Huttner, 2005; Guillemot, 2005). Normal development of the cortex is essential for proper brain function as abnormal development has been hypothesized as an underlying cause in a number of neurological and psychiatric disorders (Benes and Berretta, 2001; Kalanithi et al., 2005; Kato and Dobyns, 2005; Keverne, 1999; Levitt, 2005; Levitt et al., 2004; Lewis and Levitt, 2002; Polleux and Lauder, 2004; Sherr, 2003) Numerous studies have demonstrated the pivotal roles that Rb plays in nervous system development, and in addition, much insight has been gained into Rb function itself by studying its role in the nervous system (Chen et al., 2004a; Clarke et al., 1992; Ferguson et al., 2002; Jacks et al., 1992; Lee et al., 1994a; Lee et al., 1992; Lipinski et al., 2001; Maandag et al., 1994; MacPherson et al., 2003;

MacPherson et al., 2004; Marino et al., 2003; Morgenbesser et al., 1994; Robanus-Maandag et al., 1998; Williams et al., 1994)

In this study we evaluated the *in vivo* contributions of E2Fs as regulatory targets for Rb mediated neurogenesis and our results herein support a number of conclusions. First, this study establishes that E2Fs are indeed major physiological targets in Rb mediated neurogenesis, mediating both cell cycle dependent processes and roles beyond cell cycle regulation. We also demonstrate that functional redundancy exists among E2Fs in regulating cell cycle exit, laminar patterning, and radial migration, as well as cell survival. Finally our data demonstrating that Rb mediates neuronal migration specifically through E2F3 represent the first physiologically relevant requirement for the Rb/E2F pathway beyond cell cycle regulation *in vivo.*

E2Fs are physiological targets in Rb mediated neurogenesis regulating cell cycle dependent processes and mediating roles beyond cell cycle regulation

Rb is known to interact with numerous proteins, many of which are expressed in quiescent cells or have cell cycle independent functions. Thus the search for Rb interacting proteins in neurogenesis represents a potentially long list (reviewed in (Morris and Dyson, 2001)). We hypothesized that members of the cell cycle regulatory E2F family represent functional targets of Rb in neurogenesis *in vivo* for a number of reasons. First, E2F1 and E2F3 have both been established as Rb targets in regulating neural precursor proliferation as each are capable of rescuing the ectopic proliferation in the CNS in germline Rb deficiency (Saavedra et al., 2002; Tsai et al., 1998; Ziebold et al., 2001). While these studies support the hypothesis that E2F1 and 3 are targets in Rb mediated neurogenesis, the use of germline Rb deficient mice limited the interpretation due to the widespread defects that were the result of non-cell autonomous requirements for Rb during development (Ferguson et al., 2002; MacPherson et al., 2003; Wu et al., 2003). To circumvent this issue, we examined the role of E2F1 and 3 as targets in Rb mediated neurogenesis using telencephalon specific Rb deficient mice crossed with E2F1 deficient or telencephalon specific E2F3 deficient mice. It is possible that some of the differences observed between the Rb E2F1 DKO and Rb E2F3 DKO models are due to systemic loss of E2F1 and effects outside the nervous system. The justification for using a telencephalon specific model of E2F3 deficiency, however, was out of necessity due to the vital role of E2F3 during development (Cloud et al., 2002; Humbert et al., 2000b). As E2F1 does not have such a vital role (Yamasaki et al., 1996), no analogous tissue specific model of E2F1 deficiency was available.

Here our results demonstrate that E2F1 and 3 are physiologically relevant Rb targets in neurogenesis *in vivo*. First, we observe that both are expressed in overlapping patterns in the developing telencephalon. *In vitro*, we show that Rb interacts predominantly with E2F1 and 3 in extracts of neural tissue. The significance of this interaction is demonstrated *in vivo* where we show that E2F1 and 3 are targets not only of Rb mediated neural precursor proliferation, but we also establish that E2Fs play a major role as Rb targets in laminar patterning, radial migration, cell survival and tangential migration of interneurons. As Rb E2F1 DKO and Rb E2F3 DKO are both capable of rescuing the proliferation defect, along with the laminar patterning and radial migration defects observed in telencephalon specific Rb deficiency, our data are consistent with the interpretation that the radial migration and laminar patterning defects may occur as the result of cell cycle deregulation. Rb mediated tangential migration, however, appears to be mediated specifically through E2F3. Thus with these results, we establish roles for E2F1 and 3 as the physiological targets in Rb mediated neurogenesis.

Functional redundancy exists among E2F1 and 3 *in vivo*

Our results demonstrate that functional redundancy exists among E2F1 and E2F3 in the context of neural precursor proliferation, cell cycle exit, and survival *in vivo*. First, we show that E2F3 alone is a positive regulator of neural precursor proliferation, similar to what has been reported for E2F1 (Cooper-Kuhn et al., 2002). Next, as both Rb E2F1 DKO and Rb E2F3 DKO are both capable of rescuing the proliferation and survival defects observed in Rb deficiency in the telencephalon, our data support the hypothesis that E2F1 and E2F3 are functionally equivalent targets in Rb mediated cell cycle exit and survival.

The idea that E2Fs are functionally redundant is still debated within the field. *In vitro* studies have indicated that individually, E2F1 and E2F3 are capable of regulating the expression of distinct genes, likely as a result of differences within the marked box domain (Black et al., 2005; Hallstrom and Nevins, 2003). In the context of Rb interaction, however, it remains unresolved as to whether Rb interaction is equivalent with each E2F (Dick and Dyson, 2003; Rubin et al., 2005). *In vivo*, absence of either E2F1 or E2F3 results in individual and distinct phenotypes, yet in the context of Rb interaction, both have been shown to rescue many of the proliferation, apoptosis, and mid-gestational survival defects associated with germline Rb deficiency (Tsai et al., 1998; Ziebold et al., 2001). Specificity, however, has been reported to exist in Rb mediated phenotypes where a unique function for E2F1 has been reported in mediating apoptosis in the Rb deficient lens and retina (Saavedra et al., 2002). Thus the function of E2F1 and 3 as targets in Rb mediated neurogenesis should be viewed as context dependent, even within the central nervous system.

Finally, our observation that E2F1 and 3 only partially mediate the Rb requirement for subtype specific neuronal survival raises a number of questions. First, it is unlikely that the partial rescue in CR neurons in Rb E2F1 DKO or Rb E2F3 DKO is related to the E2F3 mediated rescue of Rb mediated tangential migration. While CR neurons have been hypothesized to have roles in regulating tangential migration of interneurons (Morante-Oria et al., 2003), the decrease in CR neurons observed in cond. Rb deficient embryos is unlikely to influence migration of interneurons as our previous findings demonstrated that the role for regulating Rb is cell autonomous (Ferguson et al., 2005). Rather as CR neurons themselves are a heterogeneous population (Bielle et al., 2005), these data support the hypothesis that the Rb/E2F pathway mediates survival of only a subtype of CR neurons. Two possible explanations are hypothesized. First E2F1 and 3 may mediate survival of non-overlapping populations of CR cells that together mediate survival of the entire population of CR cells that is absent in the cond. Rb mutant. Alternatively, it is also possible that other non-E2F Rb interacting factors are contributing to survival. Id2 is a non-E2F Rb interacting factor that has been shown to mediate many of the neurological defects arising in Rb mutants, including apoptosis (Lasorella et al., 2000), thus it is possible that E2F and Id2 are acting along parallel pathways to regulate CR neuron survival. The latter is a particularly provocative hypothesis as there is little discussion in the literature about a possible E2F independent role for Rb in cell survival.

Unique physiological requirement for Rb/E2F3 beyond cell cycle regulation exists in mediating neuronal migration *in vivo*

Here, we demonstrate that Rb mediated migration of interneurons is indeed mediated through the E2F pathway, specifically through E2F3. These results support a model for specificity among E2Fs in nervous system development. Further it is this specificity of E2F function which underlies the hypothesis that E2F3 specific mediated interneuron migration represents a novel physiologically relevant requirement beyond cell cycle regulation for the Rb/E2F pathway *in vivo*. In support of this hypothesis, we observe that E2F1 and E2F3 are expressed in overlapping patterns within the ganglionic eminences, where interneurons originate and that Rb E2F1 DKO and Rb E2F3 DKO are both capable of rescuing the proliferation defects, yet only Rb E2F3 DKO can rescue the migration defect. In addition, aberrantly migrating interneurons do not incorporate BrdU, either during (E15.5) or before (E13.5) migration. While we can't unequivocally rule out that aberrantly migrating cells were once ectopically proliferating, the absence of double labeled cells suggests that the population of calbindin interneurons have successfully exited the cell cycle in cond. Rb mutants.

Further support for role for E2F3 beyond cell cycle regulation can be inferred from the known function of E2F3 itself. E2F3 is one of the more intriguing E2Fs as the locus expresses two distinct transcripts- full length E2F3a whose expression is cell cycle regulated and acts as a transcriptional activator, and E2F3b which is expressed equivalently in quiescent and proliferating cells, and is a specific partner for Rb in quiescent cells (He et al., 2000; Leone et al., 2000). While it is possible to hypothesize that Rb/E2F3 mediated neuronal migration could be regulated through E2F3b, complementary to our work, it has been shown that a defect in the differentiation of Rb-deficient cholinergic neurons in the retina is mediated through E2F3a (Chen et al., 2007), an effect also shown to be cell cycle independent. Thus together our data suggest a common mechanism through which Rb exhibits roles beyond cell cycle regulation through E2F3 during nervous system development.

Finally, further support for a novel, *in vivo* function for the Rb/E2F pathway beyond cell cycle regulation, comes from our search for novel E2F regulated target genes in the context of neuronal migration. E2F mediated regulation of cell cycle independent genes is an emerging concept. *In vivo*, studies examining individual E2F deficient mice demonstrated vast array of tissue specific defects in development and differentiation suggesting that E2Fs may be regulating non cell cycle related genes (reviewed in (Attwooll et al., 2004; Dimova and Dyson, 2005)). These studies were limited, however, as the individual phenotypes could be the result of E2F acting independently from interactions with Rb. Here, we have performed microarray analysis on neural precursor cells from the medial ganglionic eminences, the region which gives rise to migrating populations of interneurons which ultimately exhibit aberrant migration in Rb deficiency. With this strategy, we identified a number of putative target genes that are deregulated in cond. Rb mutants and which have been shown to mediate target genes that are deregulated in cond. Rb mutants and which have been shown to mediate neuronal migration, including migration of interneurons. Further, we have validated our microarray results for one candidate gene, neogenin. Neogenin is of particular interest, not only because of its known and hypothesized roles in regulating neuronal migration (Fitzgerald et al., 2006; Rajagopalan et al., 2004; Wilson and Key, 2006), but also because it has been identified through other E2F microarray studies (Muller et al., 2001). Even more significant, through sensitive subtractive screening assays, neogenin, was recently shown to be a novel, direct target gene of E2F1, with expression induced in a cell cycle independent manner (Iwanaga et al., 2006). As E2F1 is capable of inducing E2F3 expression, it is possible that these genes may be also induced by E2F3 (reviewed in (Trimarchi and Lees, 2002)). While previous studies have provided evidence in support of roles for Rb/E2F pathway in regulating cell cycle independent functions, evidence for deregulation of such genes *in vivo*, has not been reported. Thus our data demonstrating that Rb interacts specifically through E2F3 to mediate neuronal migration represents the first physiological demonstration that such an *in vivo* role for the Rb/E2F pathway beyond cell cycle regulation exists.

In conclusion, our results demonstrate that both functionally redundant and unique roles exist for E2F1 and 3 in regulating Rb mediated neurogenesis. As Rb mediated migration is mediated specifically through E2F3, our results represent a novel physiologically relevant requirement for the Rb/E2F pathway beyond cell cycle regulation *in vivo*, pointing towards novel targets specific for E2F3 mediated transcription in the context of neuronal migration.

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As per the guidelines "Writing a M.Sc. or Ph.D thesis" from the Department of Cellular and Molecular Medicine at the University of Ottawa, Sub-section "Format of a Collection of Articles", references of all sections are listed in an amalgamated list at the end of the thesis.

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CHAPTER 4

McClellan, K. A., S. Rodrigues, T. E. Kennedy, & R. S. Slack Evidence for novel netrin neogenin interaction in Rb mediated interneuron migration *In preparation*.

The experiments were conceptualized and performed by KAM. SR provided technical training. TEK contributed to project conceptualization and provide technical guidance and reagents. KAM wrote the first, and subsequently revised drafts of the manuscript. RSS as the principal investigator provided conceptual, technical, and editorial guidance.

Evidence for novel netrin neogenin interaction in Rb mediated interneuron migration

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Word Count for Introduction, Results, and Discussion:

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ABSTRACT

Appreciated for its role in regulating cell cycle progression, it is now emerging that the Rb/E2F pathway is capable of mediating physiological events beyond cell cycle regulation. The exact molecular mechanisms through which these roles are achieved remain unknown. As we have described a physiological requirement for Rb interacting through E2F3 to mediate neuronal migration during nervous system development, we hypothesized that chemotactic receptors represent a candidate class of genes possibly regulated by E2Fs in the context of migration. Here we report that neogenin, a novel E2F regulated target gene and receptor involved in axon guidance, is specifically deregulated in the ganglionic eminence of cond Rb mutants, and restored in compound Rb E2F3 mutants, where neuronal migration is restored. Through *in vitro* explant cultures, we observe a novel role for the neogenin ligand, netrin, in mediating migration from the ganglionic eminience, a response which is defective in Rb deficiency. By identifying a receptor ligand pathway disrupted in Rb deficiency, these results contribute to our overall hypothesis that Rb interacts through E2F3 to regulate transcription of genes beyond classical E2F targets to mediate physiologically relevant events distinct from cell cycle progression.

INTRODUCTION

The process by which neural precursors divide and differentiate to give rise to the cells comprising the nervous system is highly regulated by multiple complex genetic pathways (reviewed in (Gotz and Huttner, 2005; Guillemot, 2005)). While the role of cell cycle genes regulating proliferation of neural precursor cells is well appreciated, increasingly, genes of the cell cycle regulatory family have been implicated in a number of cellular processes in nervous system development, which extend beyond their traditional roles in cell cycle regulation (reviewed in (McClellan and Slack, 2006)). The retinoblastoma (Rb) cell cycle pathway is a key pathway that we and others have shown to play a number of roles in neurodevelopment including proliferation and survival (Callaghan et al., 1999; Chen et al., 2004a; Clarke et al., 1992; Ferguson et al., 2005; Ferguson et al., 2002; Jacks et al., 1992; Kim et al., 2007; Lee et al., 1992; MacPherson et al., 2003). More recently, we have demonstrated a cell autonomous role for Rb in regulating neuronal migration, a function that is specifically regulated through interaction with the transcription factor E2F3, in a manner beyond cell cycle regulation (McClellan et al., 2007). While these results indicate a novel physiological function for the Rb/E2F pathway beyond cell cycle regulation, the ultimate molecular mechanism by which Rb/E2F3 regulates neuronal migration remains unknown.

E2Fs are perhaps best characterized for their roles in regulating cell cycle progression through transcriptional regulation of classical target genes, however, accumulating *in vitro* and *in vivo* evidence is emerging to suggest that E2Fs are capable of regulating expression of atypical target genes outside cell cycle regulation (reviewed in (McClellan and Slack, 2007). For example, *in vitro*, several microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well characterized roles in differentiation, development, and migration (Black et al., 2005; Dimova et al., 2003; Ishida et al., 2001; Ma et al., 2002; Muller et al., 2001; Polager et al., 2002; Young et al., 2003).

More recently, ChIP-on-chip studies have localized E2Fs to a number of gene promoters unrelated to cell cycle (Balciunaite et al., 2005; Bieda et al., 2006; Cam et al., 2004; Jin et al., 2006; Ren et al., 2002; Weinmann et al., 2001; Weinmann et al., 2002). Finally, using an approach whereby novel genes induced by E2F1 are identified based on subtraction screening, genes with known roles in differentiation and migration were identified as being directly induced by E2F1 in a manner beyond cell cycle regulation (Iwanaga et al., 2006). Thus together these data provide evidence that our scope of E2F function should be expanded to include the possibility that E2Fs are involved in transcriptional regulation beyond the cell cycle, perhaps through atypical means.

In parallel, studies from *in vivo* models of E2F activity are also emerging which implicate E2Fs in a number of novel processes that appear to be beyond their roles in cell cycle regulation. In the nervous system, both E2F3 and E2F4 have each been implicated in distinct aspects of nervous system development. E2F4 has been shown to regulate development of the ventral telencephalon through a genetic interaction with the Sonic Hedgehog pathway (Ruzhynsky et al., 2007). We have recently shown that specific interaction of Rb with E2F3 mediates migration of a subpopulation of GABAergic interneurons (McClellan et al., 2007). In the same study, we also observed deregulation of a number of genes with known roles in neuronal migration in migratory cell populations lacking Rb, suggesting a role for E2F3 in regulating transcription of novel targets (McClellan et al., 2007). The importance of E2F3 in mediating cell cycle independent functions was further underscored in a subsequent study in which a cell cycle independent role for E2F3a in regulating Rb mediated interneuron differentiation was observed in the retina, however, the target genes involved have yet to be identified (Chen et al., 2007). Thus while the aforementioned *in vitro* data point towards the possibility that E2Fs are capable of regulating transcription of genes outside of cell cycle progression, the *in vivo* data point to the existence of physiological roles for E2Fs beyond cell cycle regulation. What remains to be addressed, however, is the identification of E2F regulated target genes which specifically regulate cell cycle independent, physiological roles for the Rb/E2F pathway in nervous system development.

Our identification of a role for Rb/E2F3 in mediating neuronal migration represents an attractive model to identify novel cell cycle independent E2F target genes in the context of an *in vivo* physiological function(Ferguson et al., 2005; McClellan et al., 2007). Given our previous observations of deregulation of a number of genes in families of known chemotactic ligands and receptors implicated in neuronal migration in the absence of Rb, and the cell autonomous requirement for Rb in neuronal migration, we hypothesized that Rb, through E2F3 regulates migration through transcription regulation of a chemotactic receptor. We focused our efforts on the neogenin receptor family given its independent identification as an E2F regulated gene by ourselves and others (Iwanaga et al., 2006; McClellan et al., 2007). Here we report that neogenin is specifically deregulated among two families of known ligand /receptor pathways mediating neuronal migration from the MGE. Using *in vitro* explant cultures of migrating neurons we establish an *in vitro* system for testing which ligands influence migration. With this technique we observe a previously unidentified role for the neogenin ligand, netrin, in mediating interneuron migration, a response which is defective in MGE derived neurons lacking Rb. By identifying a receptor ligand pathway which appears disrupted in Rb deficiency, these results build on our overall hypothesis that Rb interacts through E2F3 to regulate transcription of genes beyond classical E2F targets to mediate physiologically relevant events distinct from cell cycle progression.

MATERIALS & METHODS

Mice

Telencephalon specific Rb deficient mice were generated by crossing floxed Rb-F19 (Marino et al., 2000; Vooijs et al., 1998) and Foxg1-cre mice (Hebert and McConnell, 2000), and were genotyped according to standard protocols with previously published primers (Ferguson et al., 2005; Ferguson et al., 2002). Telencephalon specific Rb deficient: Telencephalon specific E2F3 deficient (Rb E2F3 DKO) were generated by crossing flox Rb: Foxg1-cre mice with flox E2F3 mice as previously described (McClellan et al., 2007), both maintained on an FVBN genetic background. Mice were genotyped for flox E2F3 according to standard protocols with previously published primers for flox E2F3 (Wu et al., 2001). For embryonic time points, the time of plug identification was counted as embryonic day (E) 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.

Tissue Preparation and In situ hybridization

Tissue was dissected, fixed, cryoprotected, and sectioned as previously described (McClellan et al., 2007). Briefly, brains were dissected from embryos and fixed overnight in 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS), pH 7.4, cryoprotected in sequential solutions of 12,16 and 22% sucrose in 1x PBS followed by embedding in OCT (TissueTek 4583), and frozen on liquid N_2. Sections from either embryos were collected as 14 μm coronal cryosections on Superfrost Plus® slides (Fisher Scientific, 12-550-15).

Non-radioactive *in situ* hybridization and digoxygenin probe labeling was performed according to previously described protocols (Wallace and Raff, 1999). Neogenin, DCC, netrin, RGM, Robo, and

Slit riboprobes were generous gifts of Dr. Helen Cooper- University of Queensland (Gad et al., 1997), Dr. Elke Stein- Yale University, and Dr. Silvia Arber, University of Basel (Niederkofler et al., 2004),

In vitro explant cultures

In vitro explant cultures were performed as described (Colombo et al., 2007; Kennedy et al., 1994; Metin et al., 1997; Pozas and Ibanez, 2005; Pozas et al., 2001) with some modifications. Briefly, brains were removed from E14.5 embryos in L15 (Gibco) media and medial ganglionic eminences (MGE) were dissected as described (Ferguson et al., 2005) followed by dividing explants into pieces approximately 200μm in diameter with sharpened tungsten needles. MGE explants were then transferred into either matrigel (Basement membrane matrix, BD Biosciences Cat No.356234) or collagen (Inamed BioMaterials PureCol™Cat No. 5409) inside culture dishes and allowed to solidify for 40 minutes prior to addition of Neurobasal media supplemented with FBS.

For explants co-cultured with RGM secreting or control cell aggregates, cells were cultured according to previously published protocols (Kennedy et al., 1994; Monnier et al., 2002). RGM secreting cells were cultured under zeocyin antibiotics (Invitrogen, 250μg/mL) to select for RGM secreting cells. RGM expression was confirmed according through detection of myc-tagged RGM by Western Blot analysis of protein extracts from cells grown under selection antibiotics using previously published protocols (Ferguson et al., 2000; Tassew et al., 2008; Vanderluit et al., 2004). Hanging drop aggregates, were prepared as described(Kennedy et al., 1994) with cells at 80% confluence, harvested and resuspended in selection free media at 20 million cells/mL and cultured overnight in 10μL drops on culture dish lids. The following day, aggregates were cut into pieces approximately 200μm and transferred into matrigel at approximately 500μm from MGE explants. For explants cultured in the presence of netrin, purified netrin-1 was added to the culture media at a final concentration of 200ng/mL.

Aggregate explants, or explants alone cultures were grown *in vitro* for 24h at which point images were captured with a Zeiss upright microscope for quantification and analysis. Quantification of cell migration in matrigel was performed following protocols described by others (Colombo et al., 2007; Pozas and Ibanez, 2005). The following two measurements were taken: total area occupied by cells migrating from explants 360 degrees around the explant, and area of migrating cells in proximal and distal explants quadrants relative to secreting aggregate. The latter measurement was used to assess directional influence of RGM based on the ratio of proximal to distal migration. For quantification of cell migration in collagen, total number of cell bodies migrating from explants was quantified. For both collagen and netrin experiments, 2-4 explants per embryo were measured and values averaged. Two-tailed t-tests were performed to compare mean migration between genotype, or treatment groups and significant differences assessed at values of α=0.05.

Microscopy

Sections treated for in situ hybridization and explants cultures were examined by a Zeiss Axioskop 2 microscope with standard fluorescence and brightfield/darkfield settings at X5 0.17 or X20 0.17 NA objectives. Images were captured using a digital black and white camera with Northern Eclipse software. Figures were compiled using Adobe Photoshop CS3. Manipulations of brightness and intensity were made equally to all treatment groups.

RESULTS

Rb deficiency results in specific deregulation of the neogenin

Migration of interneurons during cortical development is a complex process that has been shown to be influenced by numerous receptor ligand families (reviewed in (Andrews et al., 2007; Corbin et al., 2001; Marin and Rubenstein, 2003; Nadarajah and Parnavelas, 2002). Our understanding continues to increase as new genes regulating the process continue to be identified. While we have previously observed that Rb deficiency results in the deregulation of neogenin, a receptor known to be involved in regulating axon guidance in the developing nervous system (McClellan et al., 2007), we wished to examine the specificity of this deregulation by asking if it is unique to the neogenin receptor or extends across the family of neogenin receptors and ligands. To address this question, sections from cond. Rb mutants were subjected to in situ hybridization to examine the expression profiles of neogenin, and related receptor Deleted in Colorectal Cancer (DCC), as well as ligands Repulsive Guidance Molecule (RGM) and netrin (Fig 1). Consistent with our previous microarray and in situ hybridization reports (McClellan et al., 2007), we observed increased neogenin expression in cond. Rb mutants within the MGE, the source of migrating interneurons, as well as along the route of migration. No difference was observed, however, in the expression pattern of DCC, a netrin receptor (Fig 1). Examination of expression patterns of neogenin and DCC receptor ligands, netrin and RGM, also revealed no difference between control and cond. Rb mutants. Thus the difference in neogenin expression we observe in cond Rb mutants is restricted within the neogenin/ DCC receptor ligand family to neogenin itself.

Since interneuron migration is influenced by a number of receptor ligand families, we next sought to determine whether Rb deficiency results in deregulated gene expression among other receptor ligand families known to regulate interneuron migration. As our previous results demonstrated that

Figure 4-1: The neuronal migration receptor neogenin, is specifically deregulated among the netrin receptor ligand family in cond. Rb mutants

Control and cond. Rb mutant E13.5 and E15.5 sections were subjected to in situ hybridization for neuronal migration receptors neogenin and DCC, as well as corresponding ligands repulsive guidance molecule (RGM) and netrin. While neogenin expression appears increased in the cond. Rb mutant relative to control in the ganglionic eminences where interneurons originate, no difference in expression between control and cond. Rb muts is detected in DCC netrin receptor, as well as ligands netrin and RGM (n=3-4 embryos for each genotype). Bar = 200μm

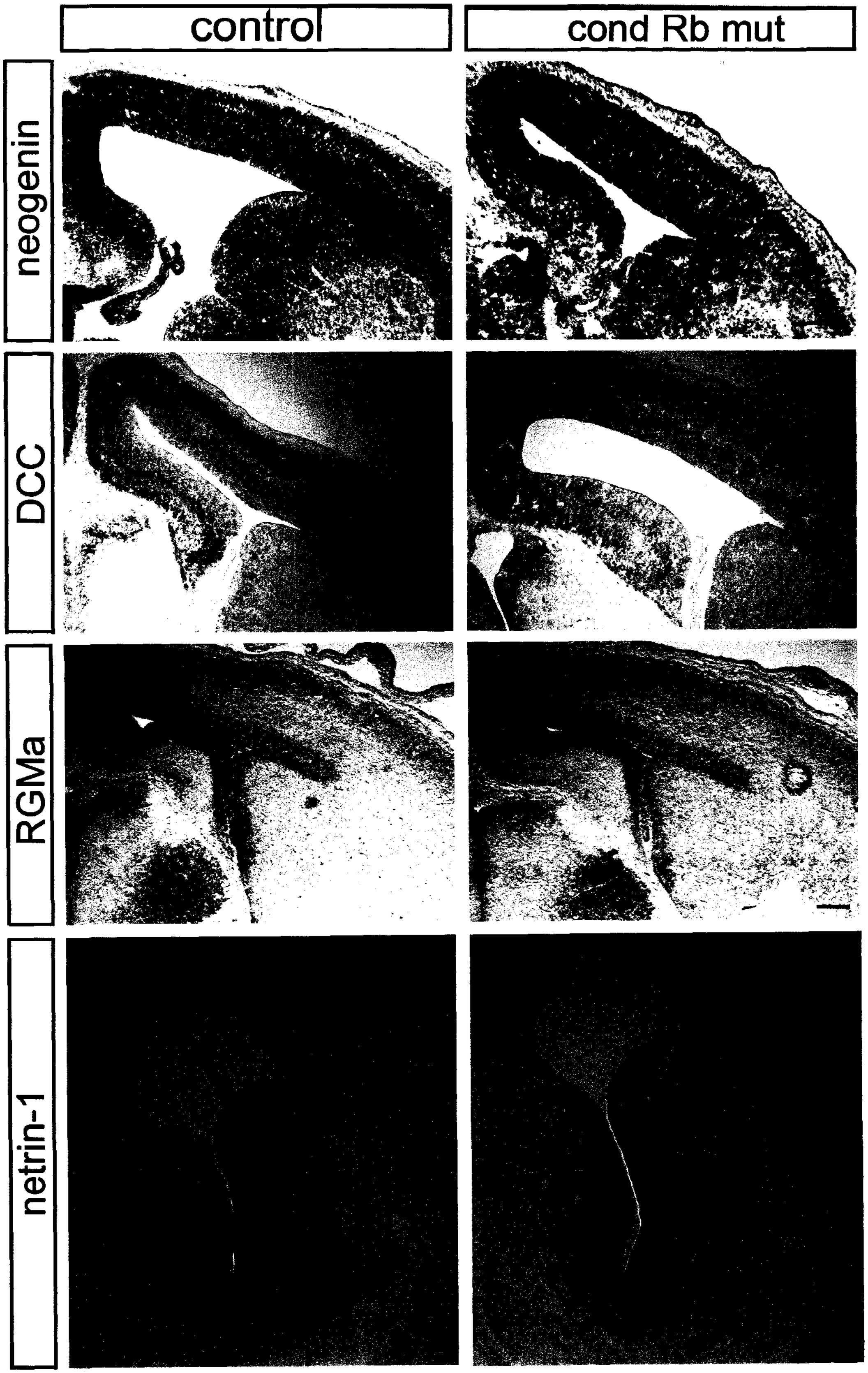


Figure 1

interneurons in the cond. Rb mutant exhibit a defect as they migrate out of the ventral telencephalon, we focused on members of the Slit /Robo family given that this receptor ligand pair influences early migration of interneurons as the migrate through the ventral telencephalon (Andrews et al., 2008; Andrews et al., 2006; Andrews et al., 2007). Through in situ hybridization for Slit receptors Robo-1 and 2, we observed no difference in expression patterns within the ventral telencephalon and along the route of interneuron migration between control and cond Rb mutants between E13.5 and E15.5 (Fig 2). Similarly, we observed no difference in expression patterns for corresponding ligands Slit 1, and 2, in similar regions at either age examined (Fig 2). Hence we conclude that among members of the neogenin receptor ligand family, and among multiple ligands and receptors known to influence neuronal migration from the MGE, neogenin is specifically deregulated in the ventral telencephalon in cond. Rb mutants.

Absence of E2F3 rescues aberrant neogenin expression in cond Rb mutants

Our observation of specific deregulation of neogenin expression in our cond Rb mutants supports a model whereby Rb influences transcription of a particular receptor or receptor(s) which in turn regulate migration of neurons from the MGE. Consistent with this hypothesis, Rb regulates gene transcription through E2F transcription factors whose activity is significantly deregulated in the absence of Rb. As we have recently demonstrated that Rb mediated neuronal migration is mediated specifically through E2F3 (McClellan et al., 2007) and as neogenin has recently been identified as a novel E2F target gene regulated in a cell cycle independent manner (Iwanaga et al., 2006), together this supports a model whereby Rb mediated neuronal migration is mediated in part through E2F regulated transcription of neogenin (Fig 3a). Thus we hypothesized that if increased neogenin expression in Rb mutants is contributing to the aberrant neuronal migration, then a restoration in neogenin expression should be observed in animals lacking both Rb and E2F3 (Rb E2F3 DKO) where neuronal migration is

Figure 4-2: No difference in expression profiles for members of the Slit-Robo family of neuronal migration receptors and ligands

Control and cond. Rb mutant E13.5 and E15.5 sections were subjected to in situ hybridization for neuronal migration receptors Robo-1 and Robo-2, as well as corresponding ligands Slit-1 and Slit-2. No difference in expression among any family member is observed within the ganglionic eminences or along the route of migration between control and cond. Rb mutants (n=3-4 for each genotype). Bar = 200μm

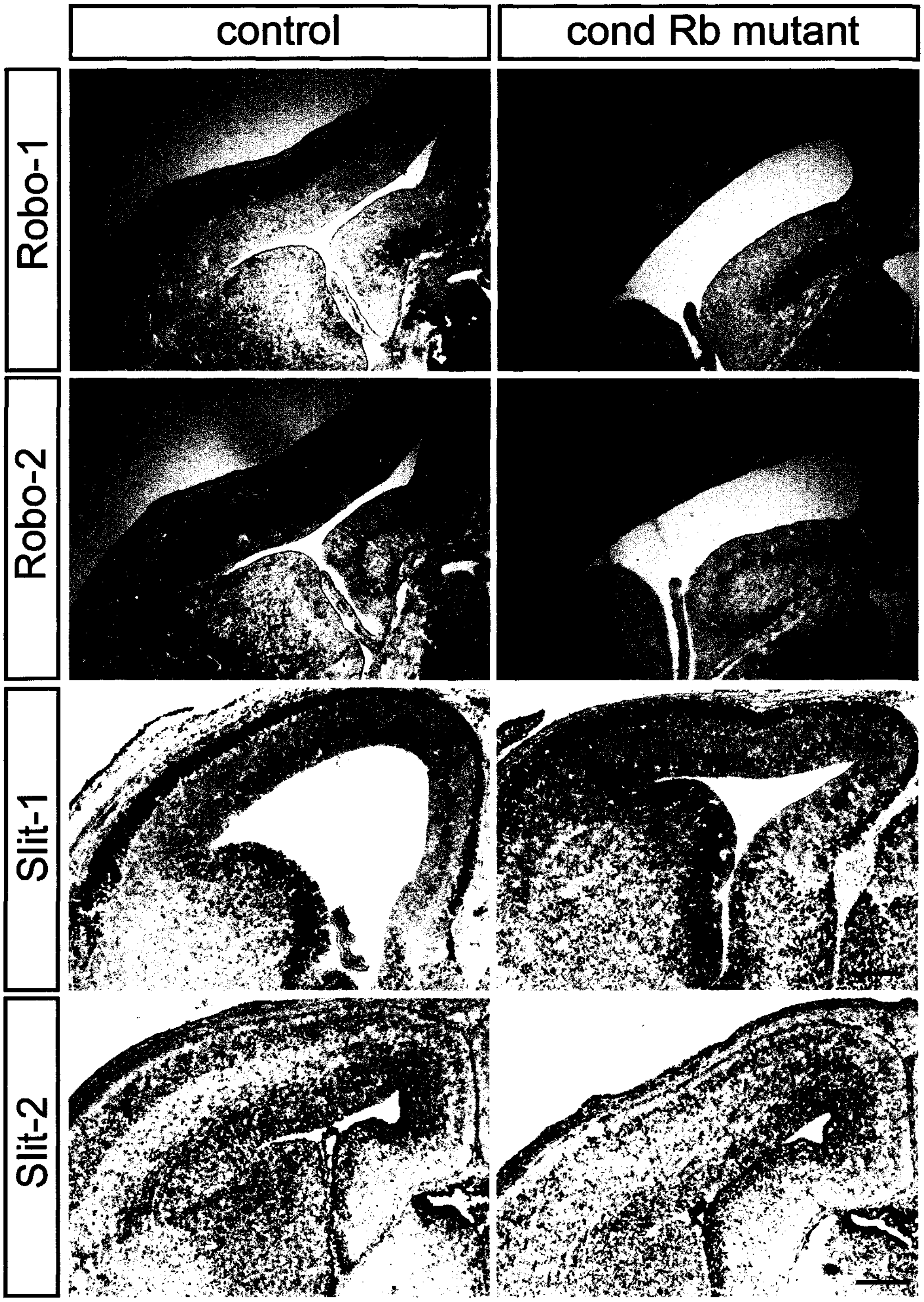


Figure 2

Figure 4-3: Neogenin is a candidate E2F target gene for Rb mediated neuronal migration

(A) Proposed mechanism for Rb E2F3 mediated regulation of neogenin expression. While Rb functions to inhibit E2F3 mediated activation of migration related genes such as neogenin, in the absence of Rb, such genes appear upregulated, whereas in the absence of both Rb and E2F3, no activation is observed.

(B) E15.5 sections of cond. Rb mut, and Rb E2F3 DKO and controls were subjected to in situ hybridization neogenin. Whereas cond. Rb mutants exhibit increased neogenin expression in the ganglionic eminences compared to control (arrows), expression appears corrected, and is similar to control in Rb E2F3 DKO. (n=3-4 for each genotype). ge = ganglionic eminence Bar = 200μm

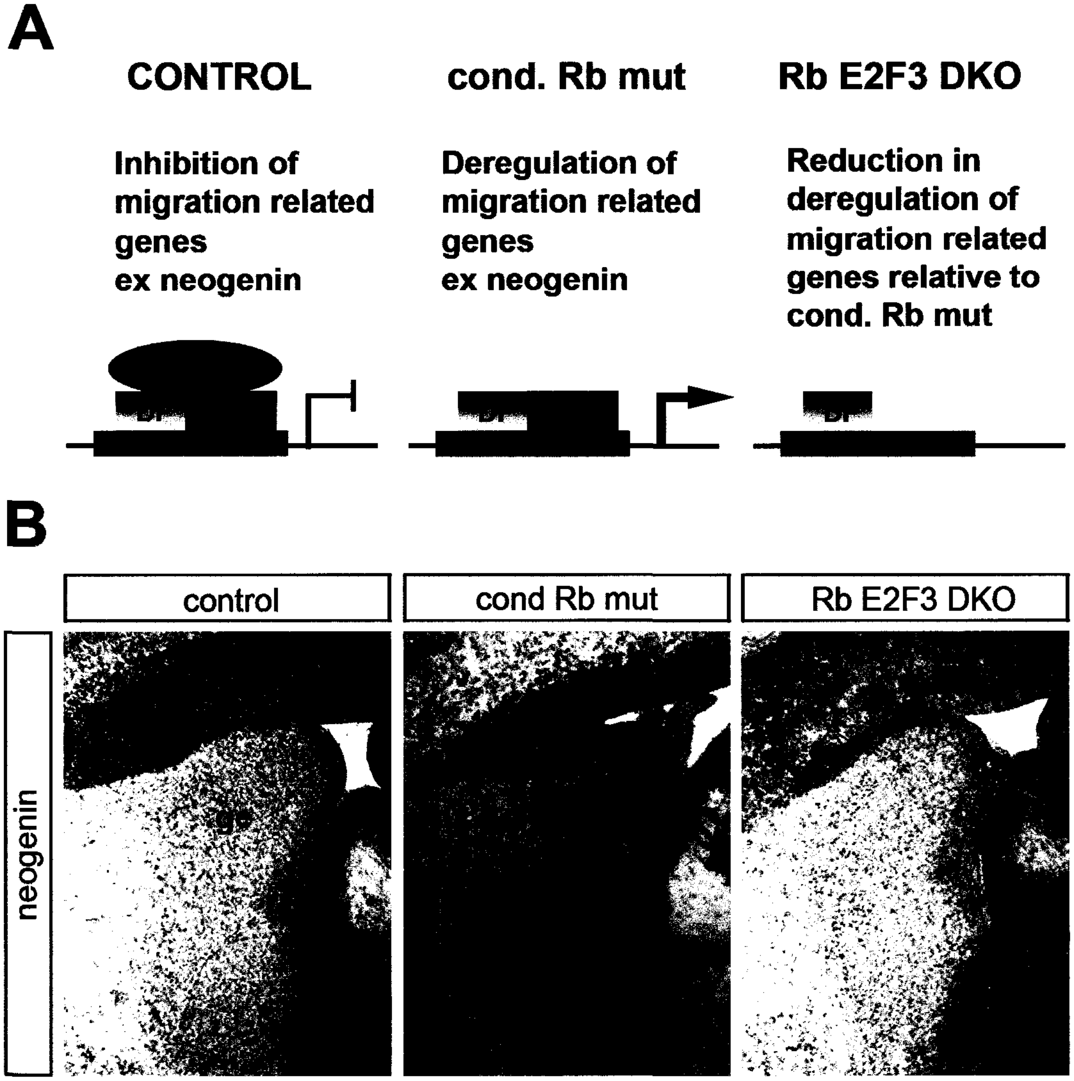

A
CONTROL
cond. Rb mut
Rb E2F3 DKO
Inhibition of migration related genes ex neogenin
Deregulation of migration related genes ex neogenin
Reduction in deregulation of migration related genes relative to cond. Rb mut
B
control
cond Rb mut
Rb E2F3 DKO
neogenin

Figure 3

also restored. Indeed, while cond Rb mutants exhibit increased neogenin mRNA expression in the ganglionic eminences, Rb E2F3 DKO exhibit similar low levels of neogenin in the ganglionic eminence as observed in control (Fig 3b). Thus the rescued neogenin expression observed in Rb E2F3 DKO lends further support to our hypothesis that Rb/E2F mediated neuronal migration represents a physiologically relevant cell cycle independent event regulated through transcription of cell cycle independent genes.

Netrin exerts a chemoattractive response in migration from the MGE

While our data provide evidence supporting the hypothesis that Rb mediated migration is regulated in part through neogenin, the extent to which neogenin is involved in mediating neuronal migration of MGE derived cells under normal/ native circumstances remains unknown. Thus to evaluate whether neogenin could have a physiologically relevant role in mediating neuronal migration from the MGE, we examined neuronal migration from control MGE in response to individual neogenin ligands, RGM and netrin, through an *in vitro* migration assay. In this experiment, MGE explants were isolated from control E14.5 cerebral hemispheres, followed by culture for 24 hours in collagen or matrigel supplemented with netrin or diffusible RGM, after which cell migration from the explant was quantified (Fig 4). To assess the relative contribution of RGM, a ligand known to interact through neogenin to elicit chemorepulsion (Colombo et al., 2007), we co-cultured control MGE explants with control or RGM secreting COS cell aggregates, in matrigel, a matrix suitable for assessing repulsion (Colombo et al., 2007; Pozas et al., 2001). While cells readily migrate into the matrigel in the absence of added factor, we failed to detect a difference in the migratory response of MGE cells in the presence of RGM (Fig 5). To assess the relative contribution of netrin, a well known chemoattractrant in the developing nervous system, we cultured control MGE explants in collagen, a matrix suitable for

Figure 4-4: *In vitro* migration assay

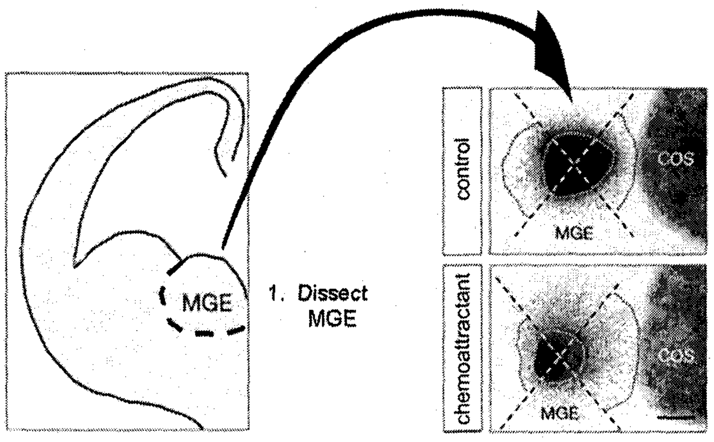
For *in vitro* explant experiments, MGE are dissected from control, or cond. Rb mutants and cut into small 100 μm explants. For netrin experiments, explants are embedded in collagen and cultured with media supplemented with recombinant netrin-1. After 1 DIV, cell bodies migrating away from explants are quantified. For RGM experiments explants are embedded in matrigel and placed next to COS cell aggregates, either control or RGM secreting. After 1 DIV migrating cell bodies are quantified with respect to total cell migration and with respect to aggregate position (as indicated).

In vitro Migration Assay

2. Embed explant in collagen and culture for 1-2 DIV in presence of **NETRIN**
3. Quantify migrating cell bodies from explant

OR

2. Embed explant matrigel, place next to COS cell aggregate secreting **RGM**, and culture for 2 DIV



1. Dissect MGE

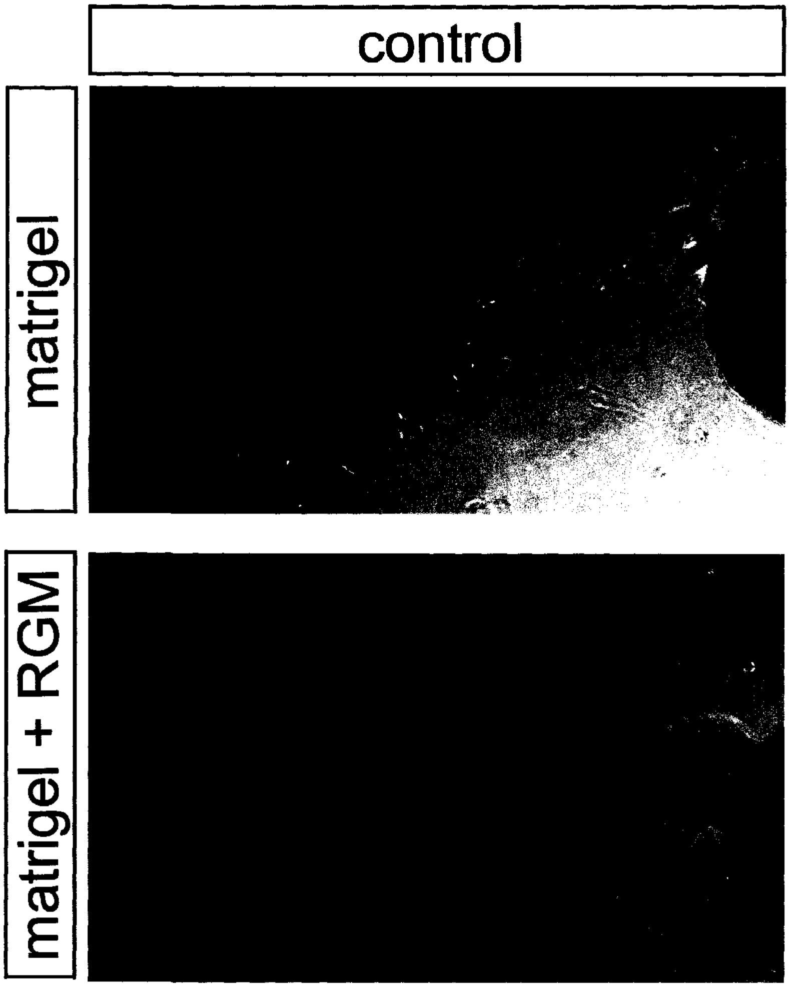
3. Divide explant into quadrants and quantify cells migrating from each quadrant
4. A higher proportion of migrating cells ***TOWARD*** the secreting aggregate indicates a ***CHEMOATTRACTANT*** *(pictured)* while a higher proportion of migrating cells ***AWAY*** from the secreting aggregate indicates a ***CHEMOREPELLANT***

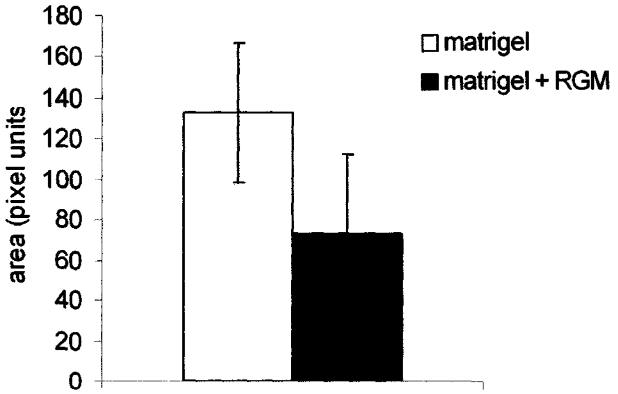
Figure modified from: Pozas & Ibanez et al., *Neuron* **45**: 701-13

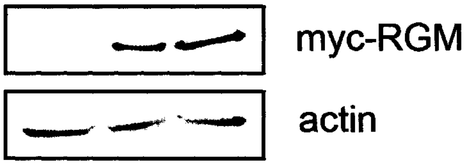
Figure 4

Figure 4-5: No observable migration response of MGE derived neurons in presence of RGM

Control MGE explants were cultured in matrigel in the presence of control, or RGM secreting COS cell aggregates. Total migration was quantified by measuring the area covered by cells migrating from the explants. Bars represent mean of the average area covered by cells migrating from an individual explant ± S.E.M. Significance was examined through a paired two-tailed t-test and no difference in migration response was observed between explants cultured with control or RGM secreting COS cells. (n= 6 embryos per treatment, 2-3 explants examined per embryo). Bar = 100 μm. Western Blot confirms that cell aggregates do indeed secrete RGM as indicated by the absence of myc-RGM in control cells (left most lane) and presence of myc-RGM in two distinct cell preparations (middle lane, and right lane).


control
matrigel
matrigel + RGM


area (pixel units
180
160
140
120
100
80
60
40
20
0
□ matrigel
■ matrigel + RGM


myc-RGM
actin

assessing chemoattraction and cell motility (Kennedy et al., 1994; Metin et al., 1997). Whereas in the absence of netrin, control cells migrated modestly from MGE explants; in the presence of netrin, we observe a robust four-fold increase in the number of cells migrating from the MGE explants (Fig 6). These data, combined with observations that the expression of netrin and neogenin overlap in the ventral telencencephalon during neuronal migration, support a model whereby netrin and neogenin to influence migration of MGE derived neurons *in vivo*. Further, these data support a hypothesis whereby if neogenin is indeed involved in Rb mediated neuronal migration, it is likely to occur through interactions with netrin, the neogenin ligand we show mediating a robust migratory response among MGE derived neurons.

Rb deficiency results in aberrant neuronal migration from MGE in the presence of netrin

Given the profound migratory response we observe in control MGE derived cells in response to netrin exposure, we next sought to determine whether the ability to respond to netrin is intact in cond. Rb mutants. First we examined migration of both control and cond Rb mutant explants in collagen alone and observed modest migration from each with no appreciable difference in their migration. In the presence of netrin, however, a clear difference was observed (Fig 7A). While control explants exhibited a four-fold increase in migration from the MGE in the presence of netrin compared to the absence, no difference in migration was observed between cond Rb mutant explants cultured in the presence and absence of netrin (Fig 7B). Since the migration response was comparable between control and cond Rb mutant explants cultured in collagen alone, these results support our interpretation that the apparent absence of a migratory response in cond Rb mutant explants in the presence of netrin is not the result of an inability of cond Rb mutant explants to migrate in collagen. Rather, as we have demonstrated that control neurons derived from the MGE exhibit a migratory response to netrin, a response not observed in cond Rb mutants, these results suggest that Rb mutants are inherently unable to elicit an appropriate migratory response to netrin itself. Further, these data support the hypothesis that this aberrant response to netrin ultimately contributes to the failed interneuron migration observed in cond Rb mutants.

Figure 4-6: MGE explants exhibit a robust migratory response in the presence of netrin

Control MGE explants were cultured in collagen in the absence or presence of recombinant netrin-1. Migration was quantified by counting the individual cell bodies migrating from each explant. Bars represent mean of the average number of cells migrating from an individual explant ± S.E.M. Note the approximate four-fold increase in migration observed in the presence of netrin relative to control. Significance was determined through a paired two-tailed t-test. * $P<0.05$ (n= 4 embryos per treatment, 2-3 explants examined per embryo) Bar = 100 μm.

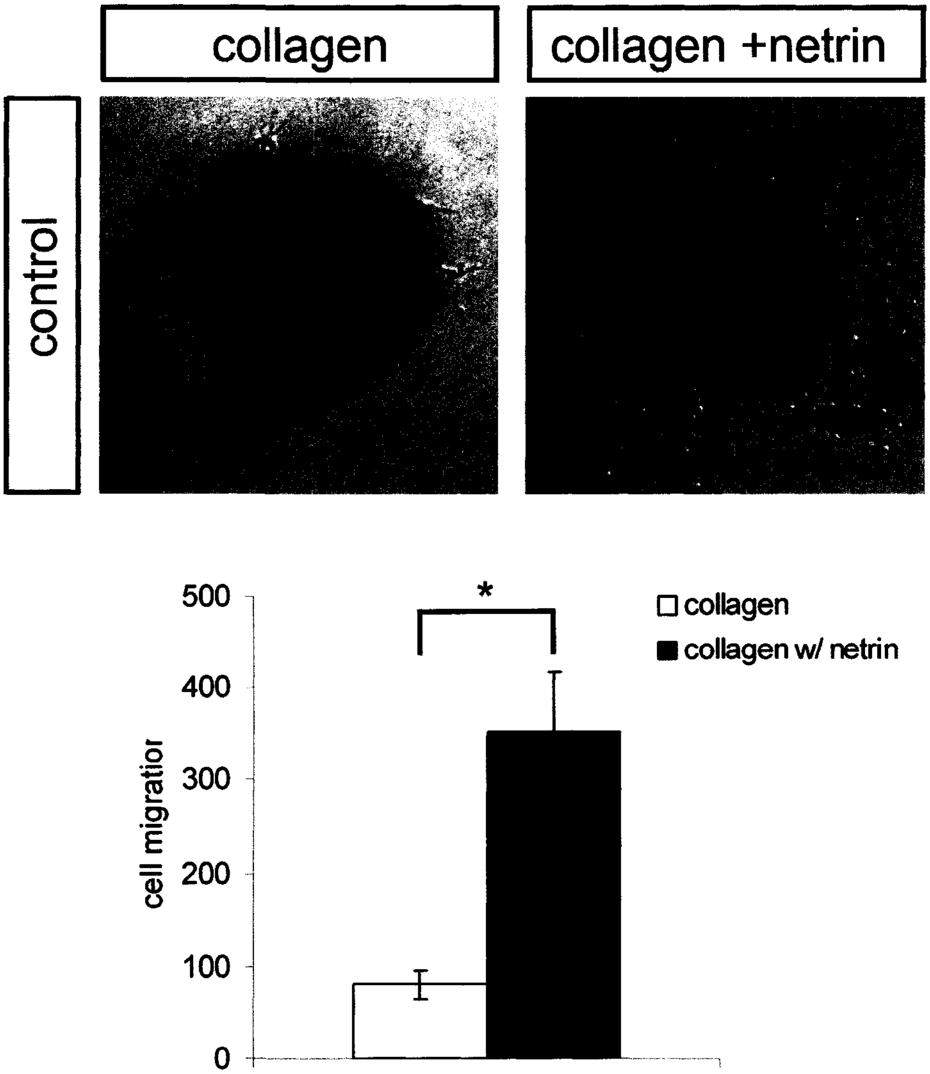

collagen
collagen +netrin
control
500
400
300
200
100
0
cell migratior
*
□ collagen
■ collagen w/ netrin

Figure 6

Figure 4-7: Aberrant migration response from MGE of cond RB mutants in presence of netrin Control and cond Rb mut MGE explants were cultured in collagen in the absence or presence of recombinant netrin-1. Migration was quantified by counting the individual cell bodies migrating from each explant. Bars represent mean of the average number of cells migrating from an individual explant ± SEM. While control cells exhibit a nearly fourfold increase in migration in the presence of netrin, no difference is observed in cond Rb mutants between presence and absence of netrin. Significance was determined through a paired two-tailed T test for explants of the same genotype and a two-tailed t-test for explants of different genotype. * $P<0.05$ (n= 4 embryos per treatment, per genotype, 2-3 explants examined per embryo) Bar = 100 μm.

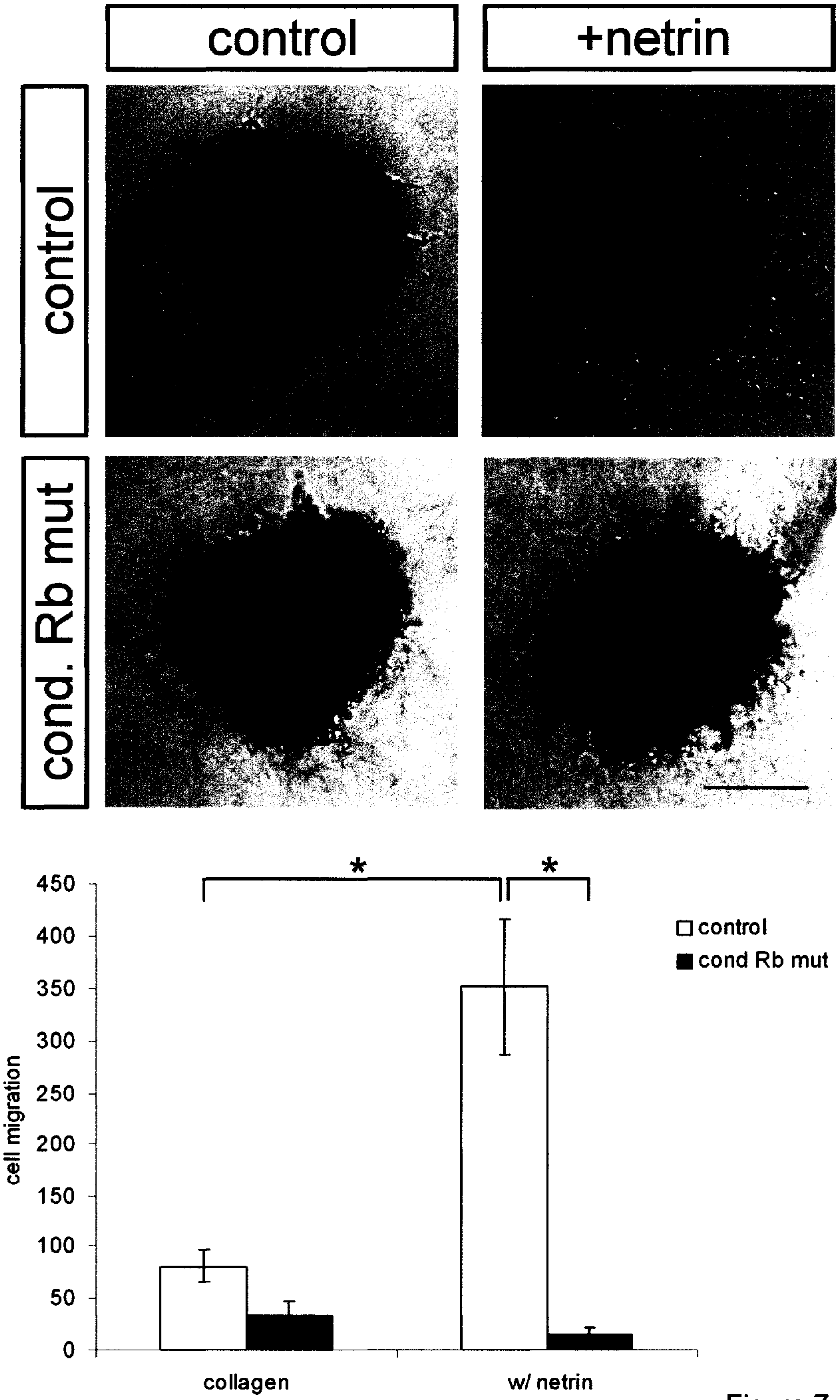

control
+netrin
control
cond. Rb mut
450
400
350
300
250
200
150
100
50
0
cell migration
*
*
□ control
■ cond Rb mut
collagen
w/ netrin

Figure 7

DISCUSSION

In this study we evaluated the potential for neogenin, a previously identified putative target, to contribute to Rb mediated neuronal migration during development. Here we report two key findings. First, among the family of neogenin ligands and receptors, neogenin is specifically upregulated in cond Rb mutants but expression is restored in Rb E2F3 DKO, where Rb mediated migration is also restored. Second, through the use of *in vitro* migration assays we report a novel role for netrin in mediating neuronal migration from the MGE, a response which is defective in Rb deficiency. Further, our results support a model whereby Rb interacts through E2F3 to mediate neogenin expression which ultimately impacts on the pool of migrating interneurons. By identifying a receptor ligand pathway which appears disrupted in Rb deficiency, these results advance our overall hypothesis that Rb interacts through E2F3 to regulate transcription of genes beyond classical E2F targets to mediate physiologically relevant events distinct from cell cycle progression.

Neogenin is a candidate E2F target gene for Rb mediated neuronal migration

In our previous work, we established that Rb mediated neuronal migration occurs specifically through interactions with E2F3 (McClellan et al., 2007). Through microarray analysis of migrating cells we observed upregulation of neogenin, a receptor with known roles in mediating axon guidance in the vertebrate telencephalon (Rajagopalan et al., 2004; Wilson and Key, 2006). In an independent study, neogenin was identified as an atypical E2F target gene with expression induced in a cell cycle independent manner in cultured cells (Iwanaga et al., 2006). E2F expression was also observed to be capable of activating neogenin gene expression *in vitro* (Iwanaga et al., 2006). As our model of Rb/E2F mediated neuronal migration represents a model to examine the physiological existence of cell cycle independent gene expression through E2Fs, we sought to further explore the role that neogenin could be playing in this process. Through examination of expression patterns of neogenin, in addition to related ligands in the neogenin family, as well as another migration related receptor family, we establish that an element of specificity exists in the observed upregulation of neogenin expression in Rb deficiency. In addition, the rescue of neogenin expression that we observe in Rb E2F3 DKO, where migration is restored, is consistent with the premise that deregulated neogenin expression observed in cond Rb mutants is a result of deregulated E2F activity, and this deregulation may contribute to the aberrant neuronal migration in cond Rb mutants. While these data, are not indicative of a direct role for neogenin in neuronal migration, together this data establishes that a physiological relationship between Rb, E2F3 and neogenin exists in the context of MGE development and strengthens our hypothesis that neogenin is a possible E2F regulated target gene involved in Rb mediated neuronal migration.

Neogenin ligand, netrin, mediates neuronal migration of MGE derived neurons

While our data point towards a role for neogenin in Rb mediated neuronal migration, using an *in vitro* migration assay we first sought to establish through which of its two ligands, netrin or RGM, would neogenin be likely mediating the effect. Here we observe that of the two ligands, netrin is uniquely capable of eliciting a robust migratory response in control MGE derived cells. Having observed a migration response in control cells in only one out of two neogenin ligands, netrin, permits us to address the question if Rb mediates migration through E2F regulated transcription of neogenin, then exposing Rb deficient MGE explants to netrin should elicit a differential migratory response. Indeed, the relative lack of migratory response we observe in Rb deficient MGE explants when exposed to netrin is consistent with this hypothesis and provides physiological evidence that the molecular mechanism with which to respond to netrin is defective in MGE derived neurons lacking Rb. Having observed an aberrant migration response to netrin in Rb deficiency, we extend our hypothesis to suggest that this defective response ultimately contributes to the failed interneuron migration observed in cond Rb mutants.

Model of netrin neogenin interaction mediating neuronal migration from the MGE

Thus far we have identified deregulated neogenin expression in cond Rb mutants, expression which appears restored in Rb E2F3 DKO, a model where Rb mediated migration appears corrected. Further we have identified a novel role for the neogenin ligand, netrin in mediating migration of MGE derived cells, a response defective in conditional Rb mutants. By reconciling our observations, we propose the following model to suggest how netrin and neogenin interact to mediate interneuron migration (Fig 8). First as we observe here, and others have noted, the regions of netrin and neogenin expression overlap specifically within the ganglionic eminences where migrating neurons originate, thus leading to the hypothesis that local short range interactions are mediating neuronal migration out of the MGE. Indeed, an emerging theme in netrin function is that short range action is thought to mediate adhesion (reviewed in (Baker et al., 2006)). Further, a well established local interaction exists in the mammary gland where netrin and neogenin interact to mediate clustering and adhesion (Srinivasan et al., 2003). Based on this interaction, we hypothesize that in control MGE, netrin is present on resident cells, or within the local extracellular matrix, where it interacts with neogenin expressed on migrating interneurons (Fitzgerald et al., 2006), to mediate clustering. We hypothesize that the level of neogenin present on individual migrating neurons is such that not all neurons express neogenin at sufficient levels to mediate adhesion (A), or some neurons exist which do not express, or have lost neogenin expression (B). In either scenario, we hypothesize that levels of neogenin present in control cells are insufficiently low for mediating adhesion through netrin among all migrating cells. Hence neurons with low or absent levels of neogenin are those which migrate out of the MGE and towards the cortex. By contrast, in the Rb mutant, where interneuron migration from the MGE is reduced (Ferguson et al., 2005), we hypothesize that the increased neogenin we observe is such that neogenin is expressed at increased levels on individual migrating neurons (A'), or at similar levels among an increased population of migrating neurons (B'). Regardless of the scenario, we hypothesize that increased neogenin expression results in an increased number of cells that interact with netrin to mediate adhesion, ultimately resulting in fewer neurons migrating out of the MGE.

The significance of our hypothesis is twofold. First, our model provides insight into a possible new mechanism through which migration of MGE neurons is regulated. While several ligands and receptors have been implicated in mediating migration of interneurons from the MGE, specific roles for netrin and neogenin have not been described. Netrins are broadly expressed throughout the developing central nervous system (Livesey and Hunt, 1997) and have been shown to mediate a wide range of chemotactic functions (reviewed in (Moore et al., 2007)), most notably attraction of commissural axons to the ventral midline in the vertebrate spinal cord(Kennedy et al., 1994) While netrin is expressed in the MGE, its function has been described as guiding cortical efferent axons (Metin et al., 1997). Similarly, while neogenin has been shown to interact with netrin to mediate axon attraction in the supraoptic tract in Xenopus telencephalon (Wilson and Key, 2006), and with RGM to mediation chemorepulsion of retinal axons, (Rajagopalan et al., 2004) the role of neogenin in mediating interneuron migration remains hypothetical. Neogenin has been shown to be expressed by the population of calbindin and parvalbumin labeled interneurons, and as a result, is hypothesized to

Figure 4-8: Model of netrin-neogenin interaction mediating neuronal migration from the MGE

We suggest that netrin present on resident cells, or within the local extracellular matrix of the MGE interacts with neogenin expressed on migrating interneurons to mediate clustering. In control, not all neurons express neogenin at sufficient levels to mediate adhesion (A), or some neurons exist which do not express, or have lost neogenin expression (B). In either scenario, levels of neogenin present in control cells are insufficiently low for mediating adhesion through netrin among all migrating cells. Hence neurons with low or absent levels of neogenin are those which migrate out of the MGE and towards the cortex. In the cond Rb mutant, neogenin is expressed at increased levels on individual migrating neurons (A'), or at similar levels among an increased population of migrating neurons (B'). Regardless, increased neogenin expression results in an increased number of cells that interact with netrin to mediate adhesion, ultimately resulting in fewer neurons migrating out of the MGE. Green circles= migrating cells, red circles, netrin, blue rectangles= resident cells or extracellular matrix, black Y= neogenin receptor.

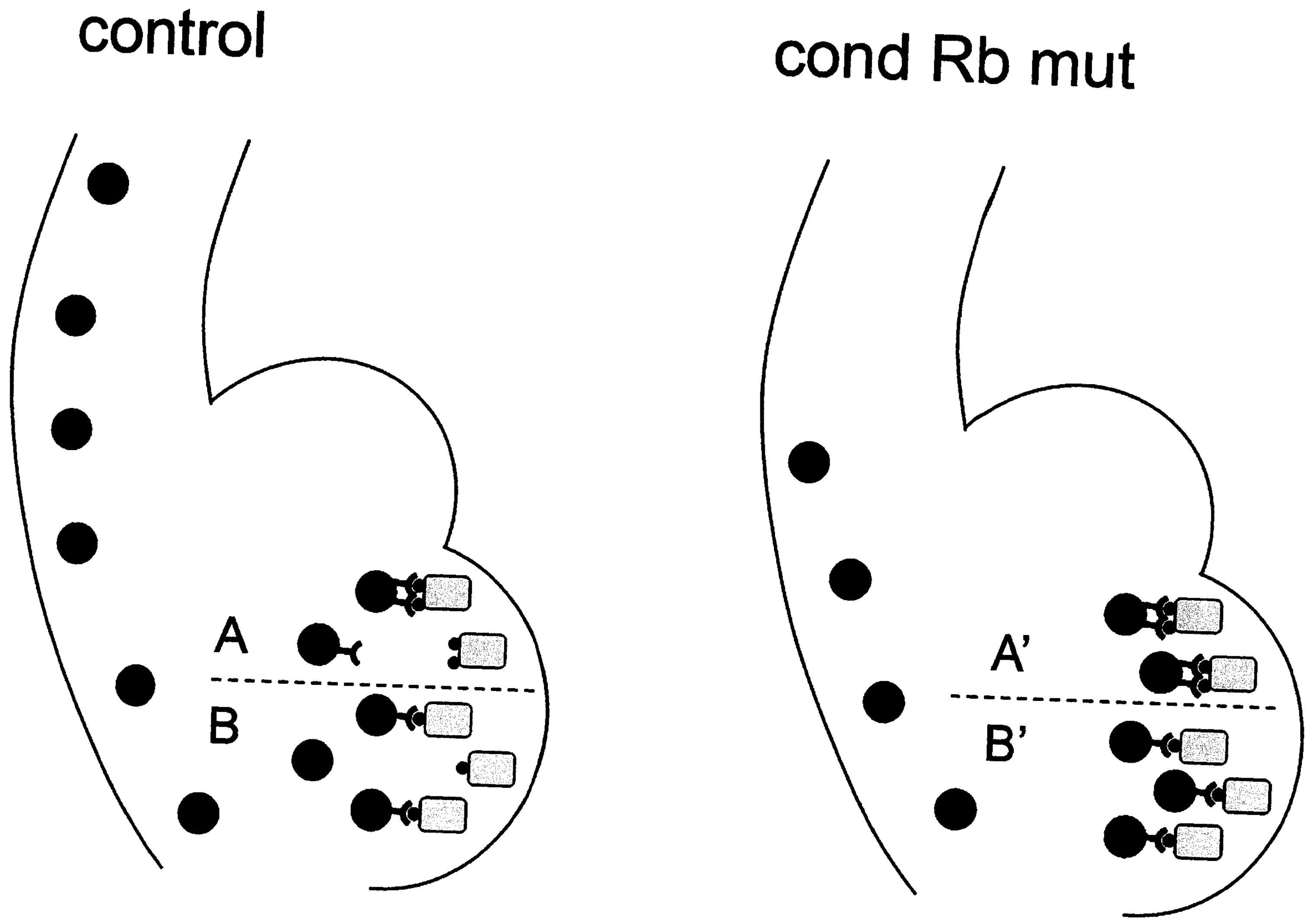


Figure 8

be involved in mediating interneuron migration (Fitzgerald et al., 2006). The mechanism and interacting ligand, however, remains unknown. Hence as we observe netrin and neogenin expression in overlapping patterns in the ventral telencephalon, and as we show that netrin elicits a response in MGE cells, we hypothesize that they interact here. Neogenin and netrin have been shown to interact in the mammary gland to mediate adhesion (Srinivasan et al., 2003), and our observations that excess neogenin leads to reduced migration in the Rb mutant support the hypothesis that they interact through a similar mechanism here.

Next, by addressing how Rb interacts with E2F3 to regulate neogenin expression, we ultimately contribute to our hypothesis that together Rb/E2F regulate gene expression to mediate physiological events distinct from cell cycle progression. Previous studies, including our own, have put forward this hypothesis as a results of physiological phenotypes observed in Rb deficiency that are attributed to deregulated E2F activity (Chen et al., 2007; Ferguson et al., 2005; McClellan et al., 2007). While we previously reported deregulated expression of putative target genes, here we extend our earlier studies by evaluating the role of one target, neogenin, in neuronal migration. Our results provide further evidence supporting a role for neogenin, a cell cycle independent E2F target gene, in mediating neuronal migration, in a manner distinct from cell cycle progression.

While we favour our model as a hypothesis to explain how Rb interacts through E2F to regulate gene expression of cell cycle independent targets that contribute to cell cycle independent physiological processes, it is important to consider other possible explanations. For example, the aberrant neuronal migration we observe in cond Rb mutants could be the result of aberrant proliferation or cell death induced in response to netrin exposure. These possibilities, are not favoured however, as cell death has been shown to increase in the absence rather than the presence of ligand, and absence of Rb has been shown to result in increased rather than decreased proliferation (reviewed in Mehlen and Furne, 2005) (Ferguson et al., 2002). Alternatively the aberrant neuronal migration observed could also be the result of a profound inability for neurons to migrate such that cond Rb mut cells will respond aberrantly to any ligand to which they are exposed. While the similar migration profiles we observe in both control and cond Rb mutants cultured in collagen alone argue against this interpretation, nevertheless, we cannot rule out this possibility. Detecting comparable neuronal migration in cond Rb mutants relative to control in response to other known ligands which positively influence migration would establish the specificity of the aberrant response to netrin. It is important to note, however, that uncovering a ligand which elicits a wild-type like migration response in cond Rb mut could prove difficult as it is possible that multiple factors are involved in mediating the aberrant neuronal migration from the MGE.

Finally, as our studies herein have reported novel observations, and have served, importantly, to extend two hypotheses namely a) deregulated neogenin contributes to the aberrant neuronal migration in Rb deficiency, and b) Rb /E2F regulate expression of novel genes to mediate physiologically relevant events distinct from cell cycle progression, nevertheless, a significant caveat is noted. While we have put forward and provided evidence in support of our hypothesis that a causal relationship exists between the upregulated neogenin expression we observe in Rb deficiency and the aberrant migration of interneurons, substantial studies are required to assess the validity of this hypothesis. To establish a causal relationship, functional studies are required which demonstrate that modulating the level of neogenin expression in the absence of Rb in MGE derived interneurons is ultimately sufficient to correct the aberrant migration of interneurons. Nonetheless, these studies provide essential rationale for the hypothesis, and justify the pursuit of the above proposed experiments.

In conclusion, these results establish a physiological relationship between Rb, E2F3, and neogenin and netrin in the context of MGE development. Further these results contribute to our overall hypothesis that Rb interacts through E2F3 to regulate transcription of genes beyond classical E2F targets to mediate physiologically relevant events distinct from cell cycle progression.

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As per the guidelines "Writing a M.Sc. or Ph.D thesis" from the Department of Cellular and Molecular Medicine at the University of Ottawa, Sub-section "Format of a Collection of Articles", references of all sections are listed in an amalgamated list at the end of the thesis.

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CHAPTER 5- DISCUSSION

The following sections from this chapter:

Section 5.2.1 p.178

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McClellan KA & Slack RS. (2006) Novel functions for cell cycle genes in nervous system development. *Cell Cycle*. Jul;5(14):1506-13

The following sections from this chapter:

5.2.2 p180-2

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McClellan KA & Slack RS. (2007) Specific in vivo roles for E2Fs in differentiation and development. *Cell Cycle.* Sep;6(23):2917-27

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5.0 OVERVIEW

Here a novel cell autonomous role for Rb in mediating neuronal migration during nervous system development has been described; a requirement mediated specifically through E2F3 in a manner beyond cell cycle control. Further, a putative target gene, previously reported to be a novel E2F target gene that may be contributing to this effect has been identified. These data represent the first physiological demonstration of cell cycle independent roles for Rb/E2F in vivo, and advance the hypothesis that Rb through E2F is capable of regulating events beyond the G_1/S transition in vivo. These results support a growing trend which has emerged demonstrating cell cycle independent roles for cell cycle genes and the Rb/E2F pathway in nervous system development. Further, these studies open up the door to new and exciting avenues of Rb/E2F function. Here each study is first discussed in the context of remaining questions and future directions. Next, the significance of these results are discussed in the context of novel cell cycle independent function for cell cycle genes, as well as how these observations could contribute novel insights into our understanding of Rb function as tumour suppressor, and our understanding of the mechanisms that govern development of neural circuits.

5.1 FUTURE DIRECTIONS

The research presented here has supported a number of novel and exciting conclusions about Rb and E2F function in the context of nervous system development. Here, new questions that arise from each study are presented as issues to consider for future studies.

5.1.1 A CELL AUTONOMOUS REQUIREMENT FOR RB IN NEURONAL MIGRATION

In our initial study a role for Rb in mediating neuronal migration is described (Ferguson et al., 2005). The following observations support this interpretation. First quantification of the calbindin interneuron population reveals similar numbers in Rb deficiency relative to control, arguing against a differentiation defect in the initial population (Ferguson et al., 2005). Next, the slice culture experiments which demonstrate that Rb deficient neurons originating from the MGE exhibit reduced migration when cultured atop control coronal slices also support the interpretation of the role of Rb in neuronal migration (Ferguson et al., 2005). Nevertheless, other alternative interpretations are possible. First, an alternative interpretation is possible whereby the reduced migration observed is the result of a failure of the interneuron population to properly differentiate which ultimately is manifested through failed interneuron migration. In this regard, identification of the genetic pathways mediating the aberrant interneuron population would distinguish between differentiation and migration. Next, it is possible that our role for Rb in mediating neuronal migration reflects a combined requirement for Rb in mediating both differentiation and migration. While in the absence of Rb, the calbindin interneuron population appears to be specified in correct numbers, our slice cultures demonstrate a rather profound migratory phenotype. Indeed, as the calbindin interneuron population represents only a subset of interneurons migrating from the MGE, it is possible that Rb mediates differentiation of distinct populations, while mediating migration of other populations. As many populations of interneurons mature postnatally (reviewed in (Wonders and Anderson, 2006)), examining the ultimate fate of the cortical interneuron population, in a model of Rb developmental deficiency which survives to adulthood, would address this possibility.

5.1.2 A UNIQUE REQUIREMENT FOR Rb/E2F3 IN NEURONAL MIGRATION

In this study we describe that Rb mediated neuronal migration occurs through interactions with E2F3 specifically. Observations supporting this interpretation include the restoration of calbindin labeled interneurons along the marginal zone migratory route specifically in mice with compound mutations in Rb and E2F3, and not in mice with compound mutations in Rb and E2F1. Thus a question that can be raised as a result is, does the E2F3 mediated rescue of Rb mediated migration reflect a bona fide requirement for E2F3 itself in interneuron migration? An examination of the data suggests this is not the case. In support of this interpretation, E2F3 itself is not required for interneuron migration as aberrant migration of interneurons is not detected in E2F3 loss of function mouse model (McClellan and Slack, unpublished observations). Rather, the E2F3 mediated rescue of Rb mediated neuronal migration likely reflects correction of the deregulated E2F3 expression known to occur in the absence of Rb. Hence the specific ability of E2F3 and not E2F1 to rescue migration reflects the underlying specificity of E2F3 in regulating distinct aspects of Rb function.

5.1.3 A putative model for Rb/E2F3 mediated neuronal migration

The observations that Rb mediated neuronal migration occurs through specific interactions with E2F3 supports a hypothesis that a transcriptional relationship exists whereby Rb and E2F3 interact to regulate expression of transcriptional target gens mediating migration. The microarray data from migrating neural precursors were used as a guide in the identification of putative target genes. Indeed, through microarray, a number of candidate target genes previously implicated in neuronal migration were identified as deregulated in Rb deficiency. While attention was focused on neogenin as a putative target gene implicated in the process, Rb mediated neuronal migration likely involves multiple target genes and complex regulatory mechanisms. As discussed in the introduction, migration of cortical interneurons is the result of coordinated action of numerous genetic pathways directing migrating neurons. Moreover, each receptor ligand interaction results in activation of distinct intracellular pathways that converge on numerous cellular processes including, but not limited to transcription, cytoskeleton remodeling, or cell death (reviewed in (Bagri and Tessier-Lavigne, 2002; Cirulli and Yebra, 2007; Ghose and Van Vactor, 2002; Hinck, 2004; Mehlen and Furne, 2005)). Thus putative target genes implicated in Rb mediated neuronal migration represent not only receptors for guidance molecules, but also all intracellular effector genes implicated in chemotactic receptor activation. Although the studies here were focused on the possible role of the neogenin receptor, and the observations support a hypothesis that deregulated neogenin contributes to Rb mediated neuronal migration, given the complexity of mechanisms mediating interneuron migration, it is acknowledged that other genetic pathways are likely involved.

Finally, while the data presented in chapter 2 and 3 support a simple hypothesis whereby Rb regulates E2F3 mediated transcription of target genes involved in neuronal migration, it is important to emphasize the complexity of mechanisms mediating Rb activity. As discussed in the introduction, Rb regulation of gene expression occurs both through regulation of E2F activity, and active transcriptional repression through interaction with chromatin modifying proteins. Further it has been shown in the context of cell cycle target genes that both mechanisms are contributing to the regulation of gene expression. Indeed, as multiple protein complexes interact with Rb to mediate regulation of chromatin structure, Rb can be seen as a molecular bridge, linking chromatin remolding complexes to individual gene promoters through E2Fs. The vast number of proteins that exist in these complexes, often with cell type gene expression patterns (reviewed in (McClellan and Slack, 2007)) represents additional mechanisms which could be contributing to the cell type specific aspects of Rb mediated regulation of gene expression. Thus it is likely that the model of Rb/E2F interaction regulating expression of novel target genes may involve more complex mechanisms than those presented. The examination of such mechanisms will form the basis of future studies.

5.2—NOVEL FUNCTIONS FOR CELL CYCLE GENES IN NERVOUS SYSTEM DEVELOPMENT- AN *EMERGING* THEME

5.2.1 ROLES BEYOND CELL CYCLE REGULATION MEDIATED THROUGH DISTINCT MECHANISMS

Our observations regarding a novel cell autonomous requirement for Rb in mediating neuronal migration through E2F in vivo are significant, as they represented the first observations of what is now an emerging trend of cell cycle independent roles for genes regulating the G_1/S transition in nervous system development. Indeed, three subsequent studies have shown that members of the cyclin dependant kinase inhibitor family, in particular $p27^{Kip1}$ play important roles in mediating neuronal migration and differentiation through mechanisms distinct from its role in regulating cell cycle progression.

Evidence for a cell cycle independent role for $p27^{Kip1}$ in neuronal migration first came about through the study of models of $p27^{Kip1}$ loss of function. First, acute reduction of $p27^{Kip1}$ levels through electroporation of $p27^{Kip1}$ short hairpin RNA (shRNA) constructs in the developing cortex resulted in reduced migration of cortical neurons and cells with abnormal process morphology suggesting that $p27^{Kip1}$ may have a role in regulating neuronal migration (Itoh et al., 2007; Kawauchi et al., 2006). Consistent with this hypothesis, $p27^{Kip1}$ was observed in punctate cytoplasmic inclusions in the tips of neuronal processes of post-mitotic cells and colocalized with F-actin (Kawauchi et al., 2006). A series of elegant experiments lead to the conclusion that the effect of $p27^{Kip1}$ on neuronal migration occurs independently from cell cycle regulation (Kawauchi et al., 2006). First, electroporation of $p27^{Kip1}$ shRNA resulted in decreased expression of $p27^{Kip1}$ protein in post-mitotic neuronal cells but had no effect on $p27^{Kip1}$ levels in proliferating cells in the ventricular zone. Reduction of $p27^{Kip1}$ did not result in proliferation defects in transfected cells or proliferating cells of the ventricular zone. Next, the authors characterized a mechanism of action for $p27^{Kip1}$ that is distinct from its role in cell cycle regulation (Kawauchi et al., 2006). Whereas in proliferating cells $p27^{Kip1}$ acts through Cdc2, in this study p27^{Kip1} was shown to be phosphorylated by Cdk5 in post-mitotic neurons. This phosphorylation led to stable levels of p27^{Kip1} which in turn inhibited phosphorylation of cofilin, an actin severing protein active in its unphosphorylated state. Inhibiting Cdk5 activity or p27^{Kip1} activity individually both resulted in increased cofilin phosphorylation in primary neurons. Overexpression of a stable form of p27^{Kip1} together with inhibition of Cdk5 suppressed the increased cofilin phosphorylation that was observed through inhibiting Cdk5 activity alone, indicating that Cdk5 acts upstream of p27^{Kip1} in the same pathway to regulate cofilin activity. Proof that this pathway is involved in regulating neuronal migration was shown by disrupting cofilin phosphorylation which resulted in impaired cortical migration in vivo (Kawauchi et al., 2006).

In the second report, similar to electroporation mediated knockdown of p27^{Kip1}, birthdating studies in p27^{Kip1} deficient mice revealed reduced radial migration of neurons into the developing cortical plate during embryonic development (Nguyen et al., 2006a). Here p27^{Kip1} mediated migration was also observed to occur independently from its role in cell cycle regulation as a p27^{Kip1} mouse model deficient in cyclin/Cdk binding, and cell cycle exit promoting ability exhibited no such migration defect, but required the presence of an intact N-terminal. In this study, migration was attributed to increased activity of RhoA, a small GTPase involved in actin cytoskeleton remodeling, as electroporation of dominant negative RhoA rescued the p27^{Kip1} mediated aberrant neuronal migration (Nguyen et al., 2006a). Indeed this observation supports a hypothesis put forward in the previous studywhere RhoA was suggested to be the p27^{Kip1} target which mediates cofilin phosphorylation through Rho-kinase (Kawauchi et al., 2006; Nguyen et al., 2006a; Nguyen et al., 2006b).

In addition to its role in migration, study of p27^{Kip1} deficient mice, and electroporation mediated knockdown of p27^{Kip1} in vivo also revealed a reduction in neuronal differentiation (Nguyen et al., 2006a). Much like migration, this effect was also determined to occur independently from p27^{Kip1} mediated cell cycle regulation as a p27^{Kip1} mouse model deficient in cyclin/Cdk binding, and cell cycle exit promoting ability exhibited no such aberrant differentiation. This effect, in contrast to what was observed in neuronal migration was shown to require an intact C-terminal domain. The mechanism of $p27^{Kip1}$ mediated neuronal differentiation was suggested to be through its effect on stabilizing levels of neurogenin 2 (Ngn2), a gene involved in specification of glutamatergic projection neurons (Nguyen et al., 2006a). Levels of Ngn2, which are swiftly downregulated as cells leave the VZ/SVZ, were observed to correlate strongly with the levels of $p27^{Kip1}$ expression, with Ngn2 expression increasing as $p27^{Kip1}$ levels increased and decreasing as $p27^{Kip1}$ levels decreased. The role of Ngn2 in $p27^{Kip1}$ mediated neuronal differentiation was causative as increasing levels of Ngn2 in the absence of $p27^{Kip1}$ led to a rescue of the differentiation phenotype (Nguyen et al., 2006a). Hence together these observations indicate that the role of cell cycle genes regulating the G_1/S transition can be broadened to include regulation of events beyond cell cycle function in nervous system development. Through the study of $p27^{Kip1}$ mediated neuronal differentiation and migration we learn that this comes about through distinct structural domains and diverse cellular interacting partners which differ from its mechanism of action in cell cycle regulation. Further, these differentiation and migration functions are distinct not only from cell cycle regulation but also from each other as each function was mediated through a different cellular interacting mechanism and distinct protein domain.

5.2.2 ROLES BEYOND CELL CYCLE REGULATION MEDIATED THROUGH SHARED CELL CYCLE REGULATORY MECHANISMS

While the above studies convincingly demonstrate existence of novel functions, what is notable is that entirely distinct domains and binding partners are used to execute these roles beyond cell cycle regulation. Thus a relevant question that remains for cell cycle genes capable of mediating function beyond cell cycle regulation is, must these functions be carried out through a distinct mechanism? In the studies presented here, this question is addressed as we demonstrate that Rb mediated neuronal migration occurs in a manner beyond cell cycle regulation, yet is mediated through its cell cycle interacting partner, E2F3. Again these observations were quickly corroborated by subsequent studies demonstrating that Rb is capable of regulating neuronal differentiation through E2F3 in the retina, also in a manner distinct from cell cycle regulation.

While absence of Rb in the retina is known to result in inappropriate proliferation of progenitor cells and death of specific cell types, it has recently been observed to result in a defect in differentiation of a subset of amacrine neurons in the retina (Chen et al., 2004a; Chen et al., 2007). In the absence of Rb, starburst amacrine (SAC) neurons exhibit a defect in the advanced stages of differentiation (Chen et al., 2007). Despite the ability of Rb to interact with numerous other molecules, this defect in SAC neuron differentiation is mediated particularly through E2F3a (Chen et al., 2007). When retinal specific Rb deficient mice are interbred with mice deficient for E2F1, E2F2, or E2F3, only Rb:E2F3a deficient retinas exhibit restoration of SAC neuron differentiation, thereby demonstrating specificity of function for E2F3a *in vivo* (Chen et al., 2007). Moreover, the study also provides evidence that E2F3 mediated regulation of SAC neuron differentiation occurs independently of cell cycle regulation. While Rb:E2F3 deficient retinas still exhibit ectopic proliferation suggesting that aberrant differentiation could be the result of defects in proliferation, the results argue otherwise. SAC differentiation was also examined in Rb:E2F1 deficient retinas where ectopic proliferation and apoptosis were both rescued (Chen et al., 2007). As Rb:E2F1 deficient retinas exhibit a similar defect in SAC differentiation in the absence of proliferation defects, these observations demonstrate that in vivo, E2F mediated proliferation can be separated from its effect on differentiation(Chen et al., 2007). Further, these results demonstrate that E2F1 and 3 are capable of specific functions, and that through pocket protein interaction, they are capable of regulating differentiation in a cell type specific manner. Thus together our data suggest a common mechanism through which Rb exhibits roles beyond cell cycle regulation through E2F3 during nervous system development.

A resulting question which emerges is how could Rb and E2F act together to mediate events beyond cell cycle regulation? Two scenarios are envisaged. In the first, it is possible that differentiation occurs as a consequence of cell cycle exit, guided simply through distinct cell type specific mechanisms in Rb mediated gene regulation of cell cycle related E2F target genes. Indeed, support for this hypothesis has recently been shown in the physiological context of neuronal terminal differentiation. In this example isoforms of the methylated histone binding protein HP1 have been shown to exhibit preferential association in the pocket protein histone methyltransferase complex, with preference determined by the stage of neuronal differentiation (Panteleeva et al., 2007). In these complexes, Rb acts as a bridge between E2Fs located at the promoter region of E2F responsive genes, and histone methytransferases and associated binding proteins such as HP1, in effect directing long term gene silencing through regulation of chromatin structure (Nielsen et al., 2001). In early differentiating cerebellar granule neurons, ChIP experiments demonstrate that HP1γ is preferentially located at methylated histone residues in E2F responsive elements within the E2F1 promoter region (Panteleeva et al., 2007). At the early stages of neuronal differentiation, E2F1 is still transcribed despite methylation, likely as a result of increased acetylation relative to methylation (Panteleeva et al., 2007). As neuronal differentiation proceeds, however, HP1γ is replaced with HP1α (Panteleeva et al., 2007). Concomitantly, histones in the E2F responsive elements become increasingly methylated resulting in significant repression of E2F1 (Panteleeva et al., 2007). The significance of this observation was demonstrated *in vitro* where cells fail to express markers of differentiated neurons and downregulate E2F1 expression after HP1α knockdown (Panteleeva et al., 2007). In vivo, HP1α expression is excluded from proliferating cerebellar granule progenitors cells and strongly expressed in post-mitotic migrating neurons (Panteleeva et al., 2007). Together these data support a hypothesis where the temporal shift in HP1 isoform association with methylated histones guides neuronal differentiation (Panteleeva et al., 2007). Further, the authors propose a model whereby HP1γ induces transient repression throughout the cell cycle, in contrast to HP1α, which is proposed to direct longer term gene silencing (Panteleeva et al., 2007). Hence in this example, differentiation occurs as a consequence of Rb/E2F mediated long term silencing of cell cycle related genes.

While it is likely that any mechanism through which Rb and E2F3 regulate neuronal differentiation and migration function will be complex and related to Rb mediated cell cycle regulation, another means through which Rb and E2F could interact to mediate events beyond cell cycle progression is by regulating expression of genes outside of the classical list of E2F target genes. This hypothesis has been put forward largely as a result of in vitro microarray and array based chromatin immunoprecipitation studies where E2Fs were observed to regulate and occupy the promoters of a significant number of genes with roles unrelated to the G_1/S transition (reviewed in (McClellan and Slack, 2007)). Here through our studies of Rb/E2F3 mediated neuronal migration, we provide physiological evidence to advance the hypothesis that E2F transcription factors regulate transcription of non cell cycle related genes in a physiologically relevant setting. Indeed, our microarray studies of Rb deficient migrating explants identified deregulated genes with known roles in neuronal migration (McClellan et al., 2007). One target in particular, neogenin, was identified in an independent study as a novel E2F target gene regulated in manner beyond cell cycle regulation, and consistent with our hypothesis, we observed a rescue of neogenin in Rb E2F3 where migration is also rescued (Iwanaga et al., 2006); McClellan et al., unpublished observations). While more studies are needed to establish that deregulated expression of migration related genes contributes to Rb mediated migration, nevertheless, these data do provide evidence that points towards the existence of E2F mediated regulation of novel target genes in a physiologically relevant context.

5.3—THE ROLE OF Rb AS A TUMOUR SUPPRESSOR

Cancer has been broadly described as the result of deregulation of one or more of six cellular processes: response to growth promoting or growth inhibitory signals, execution of cell death, telomere and genome maintenance, angiogenesis, and attachment to the cellular microenvironment (Hanahan and Weinberg, 2000). As the first identified tumour suppressor, intense interest has been focused on defining the exact molecular mechanism through which Rb mediates tumour suppression. Indeed, the early studies which identified E2F transcription factors as Rb targets mediating cell division, established the model that Rb mediated tumour suppression is the result of its restraint of E2F transcription factors and in turn, the G_1/S transition, and cell division (reviewed in (Weinberg, 1995)). Newer studies, however, that have identified roles for Rb in maintaining genome stability and promoting senescence have broadened the scope and complexity of Rb mediated tumour suppression (Hernando et al., 2004; Longworth et al., 2008; Narita et al., 2003), (reviewed in (Goodrich, 2006; Liu et al., 2004a)). In addition, expanded definitions of the mechanisms through which tumour suppressors act is not restricted to Rb. Similarily, the mechanisms through which other tumour suppressors such as Von-Hippel Lindau (VHL) and adenomatous polyposis coli (APC) act to suppress tumour formation have recently been expanded (reviewed in (Aoki and Taketo, 2007; Frew and Krek, 2007)). Thus having demonstrated a novel role for the Rb/E2F pathway in mediating neuronal migration, the data presented here raise the question as to whether Rb function as a tumour suppressor could be further expanded and contribute to the regulation of other cellular processes involved in cancer beyond regulation of cell division. Here the relationship between genes which regulate neuronal migration and their role in tumourigenesis is discussed to suggest one possible example of an additional mechanism through which Rb could mediate tumour suppression.

Deregulation of the Rb pathway in cancer has been traditionally associated with sustained proliferation, however, Rb mutations are frequently found in metastatic cancers including small cell lung carcinoma and osteosarcoma (reviewed in (Classon and Harlow, 2002)). Metastasis, the process by which tumourigenic cells detach from their resident tissues, migrate, and colonize into new sites, is a complex process mediated through a number of different genetic pathways. Classes of genes which when deregulated contribute to tumour metastasis include adhesion molecules that mediate cell-cell and cell- substrate interactions, and extracellular proteases (Hanahan and Weinberg, 2000). While the above represent classes of genes which mediate the initial stages of metastasis, how tumor cells acquire the capacity to colonize and survive within distinct tissues to form a secondary tumour is an active area of research (reviewed in (Chambers et al., 2002; Hoon et al., 2006)). Although physical factors such as vasculature diameter can facilitate metastasis, increasingly genetic pathways which mediate chemotaxis have been shown to represent an important mechanism underlying organ specific metastasis (reviewed in (Chambers et al., 2002; Hoon et al., 2006)).

Of growing interest is the role that genes regulating neuronal migration during nervous system development have been shown to play in tumourigenesis and metastasis. Three of the major families of genes which mediate neuronal migration, namely the Semaphorin/ Neuropilin, Slit/Robo, and Netrin families, have each been heavily implicated in multiple aspects of cancer and tumorigenesis. Ligands and receptors from each family are frequently found deregulated or lost altogether in numerous cancers (reviewed in (Arakawa, 2004; Chedotal et al., 2005)). Through various mechanisms, roles in metastasis have been demonstrated for members of each family (Table 5-1). For example, the semaphorin receptor, neuropilin-2 which has been shown to repel interneurons during neuronal migration, has recently been shown to mediate tumour cell metastasis (Caunt et al., 2008). Function blocking neuropilin antibodies inhibited proper activation of the neuropilin-2 receptor and led to a

Table 5-1: Select Signaling Pathways Implicated In Neuronal Migration and Possible Roles in Tumour Metastasis

FAMILY	ROLE IN METASTASIS	REFERENCES
Neuropilin/ Semaphorins	Class 3 semaphorins are both downregulated or lost and upregulated in a number of distinct cancers including lung, breast and ovarian	Reviewed in (Chedotal et al., 2005)
	ex Sema 3F- Downregulated or lost in lung cancer and correlates with advanced-stage cancer	(Bielenberg et al., 2004)
	Metastatic tumour cells transfected with Sema 3F have a diminished migratory capacity in vitro and fail to form metastatic tumours when implanted into mice	(Lantuejoul et al., 2003)
	Neuropilin—Upregulated in numerous cancers including glioblastoma and lung cancer	
	Blocking activity of neuropilin-2 inhibits endothelial cell migration and reduces metastasis	(Caunt et al., 2008)
Slit/ Robo	Slits are both upregulated and downregulated in a number of distinct cancers including prostate, breast, lung and small cell lung carcinoma	Reviewed in (Legg et al., 2008)
	ex Slit 1 and 2 are hypermethylated and inactivated in breast and cervical cancers	(Sharma et al., 2007)
	Slit influences CXCL12/CXCR4 induced breast cancer cell chemotaxis and invasion, and based on differential responses to Slit from bone vs brain metastasizing breast cancer cells, is thought to underlie specificity for brain metastasizing breast cancer	(Schmid et al., 2007) (Prasad et al., 2004)
Netrin/ DCC/Neogenin / Unc5h	Netrin -1 downregulated in brain tumours, neuroblastoma, and prostate cancer, but overexpressed in metastatic breast cancer	Reviewed in (Arakawa, 2004; Chedotal et al., 2005)
	Netrin-1 expression induces invasion in vitro and promotes metastasis when implanted into mice. Underlying mechanism is thought to be through an enhanced survival of metastatic cells	(Fitamant et al., 2008; Rodrigues et al., 2007)
	DCC and Unc5h are downregulated in more than half of all colorectal cancers	

reduction of lung cancer metastasis in *in vivo* tumour models as a result of a reduction in lymphatic vessel density (Caunt et al., 2008). Through a separate mechanism, netrin-1 has recently been shown to play a role in both breast and colorectal cancer metastasis (Fitamant et al., 2008; Rodrigues et al., 2007). Netrin-1 overexpression in human breast tumours has been shown to correlate with metastatic breast cancer. Tumour cells expressing netrin adopted a highly invasive phenotype in vitro (Rodrigues et al., 2007). The mechanism through which invasion was thought to occur was through evasion of cell death as reduction in netrin levels, or introducing the netrin receptor DCC resulted in increased cell death. (Fitamant et al., 2008; Rodrigues et al., 2007)

While the mechanisms governing metastasis are varied and complex, it is clear that genes mediating neuronal migration play an appreciable role. Thus, our phenotype demonstrating that Rb exhibits a cell autonomous requirement in neuronal migration which is mediated through E2F3, combined with our observations of deregulated expression of genes belonging to several known neuronal guidance families form the basis of the hypothesis that Rb interacts through E2F to regulate expression of these genes to mediate neuronal migration. Given the emerging importance of these genes in mediating aspects of tumour metastasis, it is possible that mechanisms which regulate expression of migration related genes during development are the same mechanisms which when deregulated, contribute to the development of tumour metastasis. Hence these observations provide a rationale to examine the possibility that deregulated Rb activity can contribute to deregulated expression of neuronal migration related genes which in turn could contribute to the development of metastatic cancer. Further exploration of this hypothesis in the context of cancer could lend new insight into our understanding of the mechanisms of Rb mediated tumour suppression.

5.4—SIGNIFICANCE TO COGNITIVE FUNCTION AND BEHAVIOR

Defects in GABAergic transmission have been postulated to be a major underlying factor that can contribute to the etiology of a number of psychiatric disorders (Benes and Berretta, 2001; Kalanithi et al., 2005; Kato and Dobyns, 2005; Keverne, 1999; Levitt, 2005; Lewis and Levitt, 2002; Polleux and Lauder, 2004; Sherr, 2003). Two major reasons support this hypothesis. First, a reduction in GABAergic interneurons has been observed in post mortem samples in a number of disorders such as epilepsy (DeFelipe, 1999) schizophrenia (Beasley et al., 2002; Benes and Berretta, 2001; Benes et al., 2001; Blum and Mann, 2002; Reynolds et al., 2004; Reynolds et al., 2002), bipolar disorder (Benes et al., 2001; Reynolds et al., 2004), Tourette syndrome (Kalanithi et al., 2005), and mental retardation (Pancoast et al., 2005). In addition, disruption of GABAergic fibres has been noted in autistic patients (Casanova et al., 2003; Polleux and Lauder, 2004; Rubenstein and Merzenich, 2003). Further support of the GABAergic hypothesis comes from the observation that symptoms in epileptic, schizophrenic and bipolar patients have been shown to ameliorate upon administration of mood stabilizers such as valproate, lithium and carbamazepine, drugs that act to modulate GABAergic transmission (reviewed in (Brambilla et al., 2003a; Brambilla et al., 2003b; Czapinski et al., 2005; Hosak and Libiger, 2002)). Many events can lead to an absence of interneurons, however, an emerging hypothesis is that defects that arise during neurodevelopment, in particular defects in neuronal migration can result in the absence or mislocalization of interneurons in adulthood, which in turn leads to behavioural phenotypes. This hypothesis has been put forth for epilepsy, schizophrenia, Tourette syndrome, mental retardation, bipolar disorder, and autism (Benes and Berretta, 2001; DeFelipe, 1999; Kalanithi et al., 2005; Kato and Dobyns, 2005; Keverne, 1999; Levitt, 2005; Levitt et al., 2004; Lewis and Levitt, 2002; Polleux and Lauder, 2004; Sherr, 2003). While the complex nature of these diseases makes testing such a hypothesis challenging, evidence in support of defects in neuronal migration leading to epileptic and behavioural phenotypes has come about through the study of genetic mouse models (Table 5-2). As some genetic models correlate with clinical disorders in

Table 5-2: Genes required for GABAergic interneuron migration and/or differentiation and implicated in regulating brain activity and behaviour

GENE	FUNCTION	GABAERGIC INTERNEURON PHENOTYPE	EPILEPTIC PHENOTYPE	BEHAVIOURAL PHENOTYPE	REFERENCE
uPAR	a key gene involved in the biochemical processing of HGF/SF- a "motogen" required for interneuron migration	Fewer calbindin positive interneurons in embryonic and early post-natal cortex in uPAR-/- Defective interneuron migration and accumulation of interneurons subcortically and fewer parvalbumin labeled interneurons in uPAR-/- adult	uPAR -/- exhibit seizures of varying severity uPAR-/- exhibit increased susceptibility to GABA antagonist induced seizures	uPAR -/- exhibit increased anxiety as exhibited by light-dark and avoidance and elevated plus maze tests	(Powell et al., 2001) (Powell et al., 2003)
Arx	An X-linked homeobox transcription factor regulated by Dlx.	Absence of Arx in mice leads to decreased calbindin positive interneurons in the MZ Arx mutations in humans results in Absence of interneurons in cortex	Arx -/- mice are embryonic lethal Humans with ARX mutations exhibit varying degrees of seizures/ epilepsy	Humans with Arx mutations have been reported to display mental retardation, autism	(Kitamura et al., 2002) Reviewed in (Kato and Dobyns, 2005; Sherr, 2003)
Dlx1	Member of Dlx homeodomain transcription factor family required for interneuron specification and migration of GABAergic interneurons	Reduction of calretinin and somatostatin interneurons in Dlx1 -/- mice older than 1 month	Dlx1 -/- exhibit seizures of varying severity Dlx1 -/- exhibit increase in theta rhythm amplitudes	Not examined	(Cobos et al., 2005)
NCAM	Regulates aspects of axonogenesis, dendritic development and synaptogenesis	Overexpression of extracellular NCAM in mice from late neuronal differentiation through adulthood leads to reduced synaptogenesis of GABergic interneurons	Not examined	Extracellular overexpressing NCAM mice exhibit sensory gating deficits exhibited by decreased prepulse inhibition	(Pillai-Nair et al., 2005)

humans, the term "interneuronopathies" has been proposed to describe phenotypes that arise as a result of aberrant interneuron migration during development (Kato and Dobyns, 2005).

The results presented here which identify a role for Rb and E2F3 in regulating GABAergic interneuron migration during nervous system development support a novel concept whereby this regulation may in turn influence neural circuit formation and cognitive function. While hypothesizing a role for Rb in mediating aspects of higher brain function is hardly intuitive, reports of cognitive impairment and mental retardation in childhood retinoblastoma survivors have, in fact, been reported (Baud et al., 1999; Caselli et al., 2007; Cowell et al., 1989; Moll et al., 1997). Many of these patients exhibit chromosomal abnormalities that are likely contributing to their cognitive impairment. Indeed, the underlying cause has been hypothesized to be the result of mutations in other genes in close proximity to Rb. Nevertheless, some cases have been reported where no other obvious chromosomal or genetic abnormalities are present (Cowell et al., 1989). Thus it remains remotely possible that mutations in Rb itself, may, in part, contribute to the clinical manifestation of cognitive impairment.

While the mechanisms governing cognitive behavior are complex, it is clear that proper neuronal migration of interneuons during development play an appreciable role. Our phenotype demonstrating that Rb regulates migration of a population of GABAergic interneurons which is mediated through E2F3 during development raises the question as to what is the ultimate fate of these mis-migrating neurons and what, if any, are the consequences of aberrant migration during development on adult cognitive functioning. Hence these observations provide a rationale to examine these questions in models of Rb deficiency whereby Rb is absent specifically in migrating interneurons during development, yet animals survive to adulthood. In such a context our model of Rb deficiency could represent an instructive model, lending new insight into our understanding of the role that nervous system development plays in shaping neural circuit formation.

5.5 Summary

Collectively these studies have identified a novel role for Rb tumour suppressor in regulating neuronal migration, a role which is mediated specifically through its cell cycle regulatory partner E2F3. Further, we provide data contributing to the hypothesis that Rb/E2F mediated neuronal migration comes about through regulation of novel transcriptional target genes which fall outside the class of traditional E2F cell cycle targets. Together these results support further examination into conceptually novel directions in Rb/E2F function; namely examining novel cell cycle independent transcriptional targets, and the activity of target genes in context of tumour suppression and higher brain function. While the Rb /E2F pathway is perhaps best characterized for its role as a universal gatekeeper of the G_1/S cell cycle transition, these results are part of an emerging new era for cell cycle genes which challenges this traditional dogma, and through physiological examination, argues for an expanded view of Rb/E2F activity.

CHAPTER 6- REFERENCES CITED

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zur Hausen, H. 1991. Viruses in human cancers. *Science.* 254:1167-73.

APPENDIX A

CV

KELLY A. MCCLELLAN

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EDUCATION

2003- Present

Doctor of Philosophy, Cellular and Molecular Medicine-
Neuroscience program
University of Ottawa, Ottawa Health Research Institute, Ottawa, ON

Thesis Supervisor: Dr. Ruth Slack, PhD
Project Title: *A cell autonomous requirement for the cell cycle regulatory Rb/E2F pathway in neuronal migration*

2000- 2002

Master of Science, Biology
McGill University, Montréal, QC
** Dean's Honour List*

Thesis Supervisors: Dr. Teruko Taketo, PhD & Dr. Roger Gosden, PhD, DSc
Project Title: *Murine oocyte loss occurs during fetal development*

1995-1999

Bachelor of Science, Anatomy and Cell Biology
McGill University, Montréal, QC

Project Supervisor: Dr. Alain Beaudet, MD, PhD
Project Title: *Immunohistochemical localization of delta-opioid receptor in rat central nervous system*

ACADEMIC HONOURS AND RECOGNITION (16)

Academic, Research, and Leadership Recognition

2007	**Ron Worton OHRI Researcher in Training Award** *Recipient of inaugural award given to top trainee at graduate or post-doctoral level for research accomplishments among all Ottawa Health Research Institute (OHRI) trainees (over 200)*	$2,000
2007	**Senior Women Academic Administrators of Canada- Graduate Student Award of Merit** *One of five graduate students within the province of Ontario to receive award for having demonstrated outstanding leadership while maintaining exemplary academic records*	$1,000
2006	**Fisher Scientific- Faculty of Medicine- University of Ottawa Graduate Student Award of Excellence** *Top PhD student in the Neuroscience graduate program*	$500
2005-8	**CIHR- Canada Graduate Scholarship-Doctoral level**	$35,000/ yr
2005	**CIHR - Institute of Aging Recognition Prize for Research in Aging** *An additional award offered to the top ranked Doctoral research award applicant nationally in the CIHR open competition in the field of aging research*	$1,000
2004-6	**Ontario Graduate Scholarship**	$15,000/yr
2005-8	**Heart & Stroke Foundation of Canada- Focus on Stroke**	Declined
2004-6	**Stem Cell Network Trainee Award**	$10,000/yr
2003-8	**University of Ottawa- National Excellence & Graduate Admission Scholarship**	Tuition fees
2004	**Ottawa Health Research Institute 4th Annual Research Day- 2nd place poster prize**	$100
2003-4	**University of Ottawa- Dept. of Cell. & Mol. Med. Outstanding PhD Seminar Award**	N/A
2003	**McGill University Dean's Honour List- Master of Science**	N/A

International Research Conference Recognition

2006	**Cold Spring Harbor Travel Supplement**	$250
2005	**Company of Biologists -- 15th ISDB Meeting**	$1,000
2004	**Eli Lilly CAN Young Neuroscientist Award**	$250
2004	**Gordon Research Conference Travel Award**	$1,000

UNIVERSITY OF OTTAWA COMMUNITY INVOLVEMENT

University of Ottawa

2007-present	University of Ottawa, Presidential Search Committee, Member • Appointed among members of the University of Ottawa Board of Governors
2004-2007	**President**- Cellular & Molecular Medicine/ Neuroscience (CMM/NSC) Graduate Student Council, Faculty of Medicine, University of Ottawa
2005-2007	Board of Governors, University of Ottawa, Member
2005-2007	Board of Governors, Executive Committee, University of Ottawa, Member
2004-2005	Senate, University of Ottawa, Member
2005	Faculty of Medicine Setting Direction Subcommittee- Communicating our Worth
2004-2007	Graduate Student Association (GSAED) council member
2005-2007	GSAED Bulletin Committee- member and contributing author (6 articles)

UNIVERSITY OF OTTAWA TEACHING

Course lecturer

Fall 2007 **Guest lecturer -Health Sciences HSS 3301- Biological Basis of Disease**
University of Ottawa

Contributions to student supervision:

2007 **Dominique Yelle**
University of Ottawa, BSc summer and Honours student- Slack lab
- Providing technical training and supervision for her project resulting in contribution to ongoing project
- Contributing to providing critical feedback on poster and seminar presentations
- Promoting critical thinking through biweekly literature review discussion sessions

2005-2007 **David Douda**
University of Ottawa, MSc student- Slack lab
- Provided regular technical training, guidance, and general technical assistance for his project resulting in contribution to published manuscript (*see publication list McClellan et al., 2007)*
- Contributed to providing critical feedback on poster and seminar presentations, and thesis advisory committee reports

PEER REVIEWED PUBLICATIONS (9 PUBLISHED, 1 IN REVISION)

PhD Degree- *In revision*

8. Vanderluit, J.L., **K.A. McClellan**, D.S. Park, & R.S. Slack (2007) p107 regulates FGF2-mediated proliferation of neural progenitor cells. *Molecular & Cellular Neuroscience. In revision.*

PhD Degree- *Published*

7. **McClellan, K.A** & R.S. Slack (2007) Specific cell cycle independent roles for E2Fs in differentiation and development. *Cell Cycle.* **6**: 2917-27.

6. **McClellan, K.A.**, V.A. Ruzhynsky, D.N. Douda, J.L. Vanderluit, K.L. Ferguson, D. Chen, R. Bremner, D.S. Park, G. Leone, & R.S. Slack (2007) Overlapping and unique roles for Rb/E2F1 and 3 in regulating neural precursor proliferation and neuronal migration: Evidence for in vivo cell cycle independent functions. *Molecular & Cellular Biology* **27**:*4825-43*

5. Ruzhynsky, V.A., **K. A. McClellan**, J. L. Vanderluit, M. Furimsky, D. S. Park, V. A. Wallace & R. S. Slack (2007) Cell cycle regulator E2F4 is a mediator of Shh signaling activity. *Journal of Neuroscience* **27**:5926-35

4. Vanderluit, J.L., C.A. Wylie, **K.A. McClellan**, A. Fortin, S. Callaghan, J G. MacLaurin, & D.S. Park, R. S. Slack (2007) The retinoblastoma family member, p107 regulates the rate of progenitor commitment to a neuronal fate. *Journal of Cell Biology.* **178**:129-39

3. **McClellan, K.A.** & R. S. Slack (2006) Novel functions for cell cycle genes in nervous system development. *Cell Cycle.* **5**:1506-13

2. Cheung, E.C.C, N. Joza, N. A. E Steenaart, **K.A. McClellan**, M. Neuspiel, S. McNamara, J. G. MacLaurin, P. Rippstein, D. S Park, G. C. Shore, H. M. McBride, J. M. Penninger, & R. S. Slack (2006) Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. *EMBO Journal.* **25**:4061-73.

1. Ferguson, K.L.*, **K. A. McClellan***, J.L. Vanderluit, W.C. McIntosh, C. Schuurmans, F. Polleux & R. S. Slack (2005) A cell-autonomous requirement for the Rb protein in neuronal migration. *EMBO Journal.* **24**:4381-91. **These authors contributed equally to this work.*

MSc Degree- *Published*

1. **McClellan, K. A.**, R. G. Gosden, & T. Taketo (2003) Continuous loss of oocytes throughout meiotic prophase in the normal mouse ovary. *Developmental Biology.* 258:334-48.

BSc Degree- *Published*

1. Cahill, C. M., **K. A. McClellan**, A. Morinville, C. Hoffert, D. Hubatsch, D. O'Donnell, & A. Beaudet (2001) Immunohistochemical distribution of delta opioid receptors in the Rat CNS: Evidence for somatodendritic labeling and antigen-specific cellular compartmentalization. *Journal of Comparative Neurology.* 440:65-84.

INVITED PRESENTATIONS (6 SCIENTIFIC, 4 GENERAL)

PhD Degree- *Scientific*

6. 6. A novel requirement for the Rb/E2F cell cycle signaling pathway in regulating neuronal migration Ottawa Health Research Institute- 7th Annual Research Day. Ottawa ON, November 29 2007.

5. A novel requirement for the cell cycle regulatory Rb/ E2F pathway in regulating neuronal migration. Memorial University Medical Sciences Seminar Series. Memorial University, St. John's NL, July 31, 2007. Audience size: (~30)

4. A novel requirement for the Rb/E2F cell cycle signaling pathway in regulating neuronal migration. Stem Cell Network Annual General Meeting. Ottawa, ON. Nov 13 2006. *Trainee Lightening Round Presentation*. Audience size: (~100)

3. A novel requirement for the Rb/E2F cell cycle signaling pathway in regulating neuronal migration. Society for Neuroscience Annual Meeting, Atlanta, GA. October 18, 2006. Audience size: (~150)

2. A novel function for the Rb signaling pathway in regulating neuronal migration during development. Cold Spring Harbor Laboratory Meeting: The Cell Cycle. Cold Spring Harbour, NY May 18th 2006. Audience size: (~300)

1. A novel role for the Rb signaling pathway in regulating neuronal migration. 26th Annual Great Lakes Mammalian Development Meeting. Toronto, ON. March 5th, 2006. Audience size: (~150)

PhD Degree- *General*

4. Perspectives of a senior graduate student on extracurricular involvement and working in a non-academic environment. Invited Panelist Presenter. Stem Cell Network Annual General Meeting. Toronto, ON. November 7 2007

3. Perspectives of a senior graduate student on training in multiple research environments, tips for a successful scholarship application, and working in a non-academic environment. Invited presenter. Memorial University Medical Sciences Trainee Lunch. Memorial University, St. John's NL. August 3 2007

2. CIHR- More important than the money it distributes: A trainee perspective. CIHR Research Division Staff Retreat. Lord Elgin Hotel, Ottawa, ON. January 6, 2006

   **I was honoured with an invitation from Nancy MacLellan, Head, Research Capacity Development at CIHR to speak to ~100 staff of the CIHR research portfolio as a CIHR funded trainee about my research and the importance of CIHR to the research community and the Canadian public.*

1. The role of Rb family proteins in brain development and neural stem cells: Implications for regenerative therapies. University of Ottawa Animal Care and Veterinary Services Continuing Veterinary Education Seminar Series. Ottawa, ON. February 18, 2005.

SELECT ABSTRACTS (17)

PhD Degree

15. **McClellan, K.A.**, V.A. Ruzhynsky, J.L. Vanderluit, A.S. Martens, R. Bremner, D.S. Park, G. Leone, F.A. Dick & R.S. Slack. (2007) A unique requirement for Rb/E2F3 in neuronal migration during development: Evidence for cell cycle independent functions. Stem Cell Network Annual General Meeting. Toronto, ON. November 7-9 2007.
14. Douda, D.N., **K.A. McClellan**, J.G. MacLaurin, & R.S. Slack (2006) Investigating the role of E2F1 as the Rb target in cortical development. Society for Neuroscience Annual Meeting, Atlanta, GA. October 14-18, 2006
13. **McClellan, K.A.**, D.N. Douda, K.L. Ferguson, J.L. Vanderluit, D.S. Park, C. Schuurmans, F. Polleux, & R.S. Slack (2006) A novel function for the Rb/E2F signaling pathway in regulating neuronal migration during cortical development. 16th Biennial meeting of the International Society for Developmental Neuroscience. Banff, AB. August 24-28, 2006.
12. **McClellan, K.A.**, K.L. Ferguson, J.L. Vanderluit, D.S. Park, C. Schuurmans, F. Polleux, & R.S. Slack (2006) A cell-autonomous requirement for the cell cycle regulatory protein Rb in neuronal migration. 3rd Canadian Developmental Biology Conference, 1st Canada Regional SDB Conference Meeting. Mt. Tremblant, QC April 6-9, 2006
11. **McClellan, K.A.**, K.L. Ferguson, J.L. Vanderluit, D.S. Park, C. Schuurmans, F. Polleux, & R.S. Slack (2005) Distinct roles for Rb family proteins in regulating cortical development. Stem Cell Network Annual General Meeting. Calgary, AB. Nov 22-25 2005
10. **McClellan, K.A.**, K.L. Ferguson, J.L. Vanderluit, D.S. Park, C. Schuurmans, F. Polleux, & R.S. Slack (2005) Distinct roles for Rb family proteins in regulating cortical development. OHRI Research Day. Ottawa, ON October 2005
9. **McClellan, K.A.**, K.L. Ferguson, J.L. Vanderluit, D.S. Park, C. Schuurmans, F. Polleux, & R.S. Slack (2005) Distinct roles for Rb family proteins in regulating cortical development. 15th International Society of developmental Biologist's Congress. Sydney, Australia. September 3-7 2005.
8. **McClellan, K.A.**, K.L. Ferguson, J.L. Vanderluit, V. Ruzhynsky, & R.S. Slack (2005) Rb family signalling molecules exhibit distinct roles in neurogenesis. Keystone Symposia, Cancer & Development. Banff, AB. Feb. 5-10, 2005.
7. **McClellan, K.A.**, K.L. Ferguson, V. Ruzhynsky, & R.S. Slack (2004) A role for E2F3 in the regulation of neural precursor cells and neurogenesis. OHRI Research Day. Ottawa, ON. November 3-5, 2004.
6. **McClellan, K.A.**, K.L. Ferguson, V. Ruzhynsky, & R.S. Slack (2004) A role for E2F3 in the regulation of neural precursor cells and neurogenesis. Stem Cell Network Annual General Meeting. Montréal, QC. November 3-5, 2004.
5. **McClellan, K.A.**, K.L. Ferguson, V. Ruzhynsky, & R.S. Slack (2004) A role for E2F3 in the regulation of neural precursor cells and neurogenesis. Society for Neuroscience Meeting. San Diego, CA. October 23-27, 2004.
4. Ferguson, K.L., **K. A. McClellan**, V. Nikoletopoulou, J.L. Vanderluit, C.J. Shuurmans, F. Polleux, & R.S. Slack (2004) Telencephalon- specific Rb knockouts reveal specific marginal zone neuronal loss. Society for Neuroscience Meeting, San Diego, CA. October 23-27, 2004.

SELECT ABSTRACTS CONT'D.

PhD Degree cont'd

3. **McClellan, K.A.**, K.L. Ferguson, V. Ruzhynsky, & R.S. Slack (2004) Rb family signalling molecules exhibit distinct roles in neurogenesis. Gordon Research Conference, Neural Development. Newport, RI. August 15-20, 2004.
2. **McClellan, K.A.**, K.L. Ferguson, V. Nikoletopoulou, J.L. Vanderluit, W.C. McIntosh, J. MacLaurin, D.S. Park, & R.S. Slack. Rb signaling pathway is required for cortical development. Great Lakes Mammalian Development Meeting. Toronto, ON. March 6-7, 2004
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MSc Degree

1. **McClellan, K. A.**, R. G. Gosden, & T. Taketo (2002) Murine Oocyte Loss Occurs During Fetal Development. Cold Spring Harbor Laboratory- Germ Cells Meeting, Cold Spring Harbor, NY. October 9-13, 2002

BSc Degree

1. Morinville, A., C.M. Cahill, **K. A. McClellan,** C. Hoffert, D. Hubatsch, D. O'Donnell, & A. Beaudet (2001) Evidence for Antigen- Specific Cellular Compartmentalization of the Delta Opioid Receptor in the Rat CNS. Great Lakes GPCR [G-Protein Coupled Receptor] 2001 Joint Meeting. Bromont, QC. October 26-28, 2001

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A cell-autonomous requirement for the cell cycle regulatory protein, Rb, in neuronal migration

Kerry L Ferguson[1,4], Kelly A McClellan[1,4], Jacqueline L Vanderluit[1], William C McIntosh[1], Carol Schuurmans[2], Franck Polleux[3] and Ruth S Slack[1,]*

[1]Ottawa Health Research Institute, University of Ottawa, Ottawa, Ontario, Canada, [2]Department of Biochemistry & Molecular Biology, University of Calgary, Health Sciences Centre, Calgary, Alberta, Canada and [3]Department of Pharmacology, Neuroscience Center, University of North Carolina, Chapel Hill, NC, USA

Precise cell cycle regulation is critical for nervous system development. To assess the role of the cell cycle regulator, retinoblastoma (Rb) protein, in forebrain development, we studied mice with telencephalon-specific Rb deletions. We examined the role of Rb in neuronal specification and migration of diverse neuronal populations. Although layer specification occurred at the appropriate time in Rb mutants, migration of early-born cortical neurons was perturbed. Consistent with defects in radial migration, neuronal cell death in Rb mutants specifically affected Cajal–Retzius neurons. In the ventral telencephalon, although calbindin- and *Lhx6*-expressing cortical neurons were generated at embryonic day 12.5, their tangential migration into the neocortex was dramatically and specifically reduced in the mutant marginal zone. Cell transplantation assays revealed that defects in tangential migration arose owing to a cell-autonomous loss of Rb in migrating interneurons and not because of a defective cortical environment. These results revealed a cell-autonomous role for Rb in regulating the tangential migration of cortical interneurons. Taken together, we reveal a novel requirement for the cell cycle protein, Rb, in the regulation of neuronal migration.

The EMBO Journal (2005) **24**, 4381–4391. doi:10.1038/sj.emboj.7600887; Published online 24 November 2005
Subject Categories: development; neuroscience
Keywords: cell cycle; neurogenesis; neuronal differentiation; retinoblastoma; tangential migration

Introduction

Cell cycle regulation is essential for cortical neurogenesis, ensuring maintenance of the progenitor cell pool, production of the correct proportions of diverse cell types, and coordination of the timing of neuronal differentiation. During corticogenesis, cortical precursor cells located in the germinal zone of the dorsal telencephalon undergo multiple rounds of proliferation, between embryonic days (E) E10 and E17 (Takahashi *et al*, 1996). 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, which is then split by subsequent neuronal cohorts into the superficial marginal zone (MZ) and the deeper subplate. The MZ, also known as layer I, is a heterogeneous population of neurons that includes Cajal-Retzius neurons, which provide important guidance cues for neuronal migration during CP formation. Cortical layers (lamina) II–VI form between the MZ and subplate in an inside-out pattern, such that earlier generated neurons reside in deep layers and later-born neurons give rise to more superficial layers (Takahashi *et al*, 1999). As cortical development proceeds, the subplate becomes separated from the germinal zone by the intermediate zone (IZ), a white matter tract containing afferent and efferent projections (Sidman and Rakic, 1973; Caviness, 1982).

In addition to the excitatory projection neurons that are born in the dorsal telencephalon, interspersed throughout the cortical layers are a population of GABAergic inhibitory interneurons that are generated in the ventral telencephalon and reach the cortex via tangential migration (Anderson *et al*, 1997; Lavdas *et al*, 1999; Sussel *et al*, 1999; Wichterle *et al*, 1999). Tangentially migrating interneurons follow very specific migratory routes, generally avoiding the developing striatum to form two distinct paths—either superficial to or underlying the striatal mantle (Marin *et al*, 2001). Superficially migrating neurons do not enter the CP, and instead migrate along the MZ, whereas interneurons following the deeper path travel through the lower IZ and subventricular zone (SVZ) (DeDiego *et al*, 1994; Lavdas *et al*, 1999; Denaxa *et al*, 2001). Once GABAergic interneurons have completed their tangential migration, they switch to a radial mode of migration to enter the CP (Polleux *et al*, 2002).

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). The cell cycle dependence of laminar specification was best shown by a series of heterochronic 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 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 that 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

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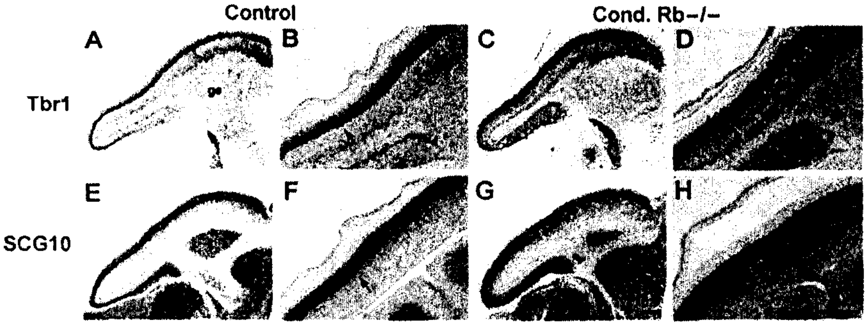

Figure 1 Laminar patterning is perturbed in the absence of Rb. *In situ* hybridization of E15.5 sagittal sections of mutant and control embryos demonstrates enhanced expression of neuronal markers, *Tbr1* and *SCG10*, in the Rb mutants. *Tbr1* labeling is slightly elevated within the mutant CP, and strongly upregulated within the IZ (arrows) (**C, D**), relative to control (**A, B**). Similarly, *SCG10* expression is highly elevated within the mutant IZ (arrows) (**G, H**), relative to control (**E, F**). 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, MZ = marginal zone, CP = cortical plate, IZ = intermediate zone.

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.

The retinoblastoma (Rb) protein is a key cell cycle regulator. First discovered as a tumor suppressor, Rb regulates the G1/S phase restriction point, thereby controlling entry into S phase (reviewed in Trimarchi and Lees, 2002). Studies with Rb-deficient embryos were the first to show that Rb has 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, 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). Indeed, 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 zones (Ferguson *et al*, 2002).

In this study, we examined the impact of deregulated cell cycle regulation resulting from the loss of Rb function in the developing telencephalon. We demonstrate that, despite defective exit from the cell cycle, Rb mutants appear to generate and specify diverse neuronal populations at the appropriate developmental time. Neuronal birthdating experiments, however, reveal that Rb mutants exhibit defective laminar patterning and impaired radial migration. Finally, by slice co-culture assays, we reveal a cell-autonomous defect in tangential migration. The results of our studies reveal a novel role for the tumor suppressor protein, Rb, in the regulation of neuronal migration during development.

Results

Telencephalon-specific Rb-deficient progenitor cells undergo ectopic cell divisions

In our initial examination of telencephalon-specific Rb knockouts, we demonstrated that these mutants retained the ectopic mitoses phenotype characteristic of Rb germline knockouts (Ferguson *et al*, 2002; Supplementary Figure 1B). Confocal analyses showing BrdU and βIII-tubulin co-labeling have previously identified the ectopically dividing cells as early neuroblasts, suggesting the ability to cycle after the initiation of neuronal differentiation (Ferguson *et al*, 2002). Here, we sought to examine the impact of deregulated cell cycle regulation resulting from Rb deficiency on laminar patterning, the timing of neuronal differentiation, and the regulation of distinct neuronal populations.

Laminar patterning is perturbed in the absence of Rb

As the precise timing of cell cycle exit is believed to be critical for proper generation of cortical layers (McConnell and Kaznowski, 1991; Takahashi *et al*, 1999), we questioned whether conditional Rb mutants might display defective laminar patterning. To ask whether layers were appropriately generated in the absence of Rb function, we first assessed the expression of layer-specific markers by *in situ* hybridization (Figure 1). Sections were examined at E15.5 to coincide with the peak occurrence of ectopic mitoses. At E15.5, only neurons in the deepest cortical layers, V and VI, have differentiated, whereas layer IV neurons are in the process of being born and layer II–III neurons are just beginning to withdraw from the cell cycle (Takahashi *et al*, 1999). *Tbr1*, a T-box transcription factor, is highly expressed in postmitotic glutamatergic projection neurons in layer VI (Rubenstein *et al*, 1999). We noted that *Tbr1* was expressed in the Rb mutant CP at E15.5 but, unlike control embryos, strong *Tbr1* labeling was also observed within the IZ (Figure 1C and D). CP expression of the pan-neuronal marker, *SCG10* (Stein *et al*, 1988), in Rb mutant cortices was similar to littermate controls, but ectopic *SCG10* expression was also detected within the IZ (Figure 1G and H), such that the boundary between the mutant CP and IZ lacked the clear definition observed in control embryos. The more dramatic *SCG10* phenotype is likely due to its pan-neuronal expression as opposed to the more restricted expression of *Tbr1* to deep-layer neurons. This pattern was further confirmed by *in situ* hybridization with the layer-restricted markers *Id2*, *RORβ*, and *Otx1*, which also displayed increased IZ expression in Rb mutants (data not shown). These results suggest either a requirement for Rb function in the establishment of a cortical laminar structure or in the specification of layer identities.

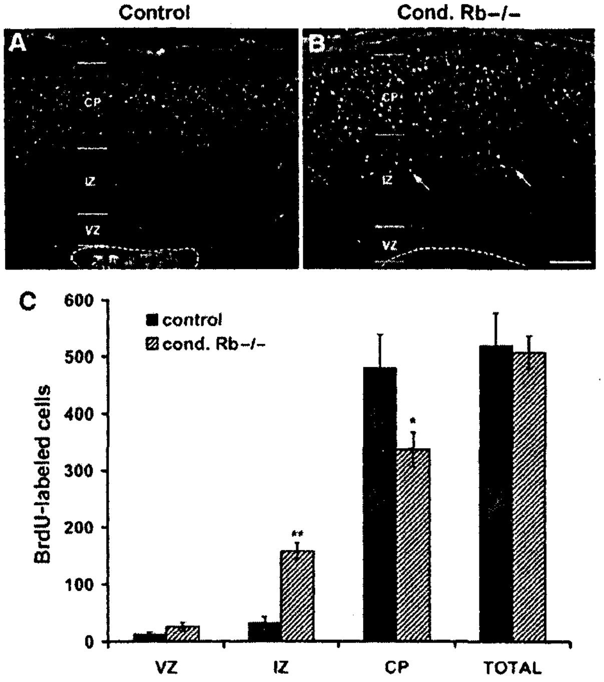

Figure 2 Rb-deficient cortical neurons exhibit delayed radial migration. Pregnant females at E13.5 of gestation were injected with a single dose of 20 µg/g body weight BrdU. Embryos were removed 5 days later at E18.5, fixed, and subjected to immunohistochemistry for BrdU (**A**, **B**). BrdU-labeled cells were counted across a 620-µm-wide section of dorsal cortex. In contrast to the control sections with only 33.3±9.2 cells, 158±14.3 labeled cells were counted within the IZ of Rb mutants, representing an almost five-fold increase (arrows) (**C**; $P<0.001$). This dramatic increase was compensated for by a corresponding decrease of labeled cells that had reached the CP. The controls had 480.2±57.9 cells within the CP compared to only 336.9±30.5 in the Rb mutants (C; $P<0.05$). These results indicate that although similar numbers of neurons were generated at E13.5 in control and mutant cortices, Rb-deficient cortical neurons are delayed in reaching their ultimate position within the CP. Error denotes standard error ($n=3$ control; $n=4$ cond. Rb−/−). Bar: 50 µm. MZ: marginal zone; CP: cortical plate; IZ: intermediate zone; VZ: ventricular zone.

To distinguish between these possibilities, we examined whether the abnormal lamination in Rb mutants was the result of defective radial migration by conducting neuronal birthdating experiments (Figure 2). Pregnant females were injected with a single dose of BrdU at E13.5 and embryos were dissected at E18.5, when neurogenesis is complete but neuronal migration is still in progress. Examination of brightly labeled BrdU-positive cells revealed the location of neurons that exited the cell cycle at E13.5, as cells that continued to cycle would dilute the BrdU label. Quantification of BrdU-positive cells revealed an aberrant distribution of early-born neurons in the developing cortex of Rb mutants (Figure 2A and B). The number of BrdU-positive cells within the VZ was not significantly different between control and mutant brains. In control sections, an average of 33.3±9.2 BrdU-labeled cells was counted within the IZ as compared to 158.0±14.3 cells in the Rb mutant (Figure 2C; $P<0.001$). The substantial increase of BrdU-labeled cells within the mutant IZ was compensated for by a corresponding decrease in labeled cells within the CP (480.2±57.9 cells in controls and only 336.9±30.5 neurons in the Rb mutants) (Figure 2C; $P<0.05$). In contrast, the number of BrdU-positive cells within the VZ was not significantly different between control and mutant brains. Consistent with an increase in IZ cell number, total cell counts of E16.5 embryos revealed significantly increased cell numbers within the Rb mutant IZ (Supplementary Figure 1C–E). These results suggest that many of the Rb-deficient cells failed to reach their correct laminar destination within the cortex.

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

We next examined whether there were defects in the generation or survival of layer I Cajal–Retzius cells, pioneering neurons born between E11 and E12 that synthesize and secrete Reelin, and which are essential to guide newly generated cortical neurons along radial glial fibers (Frotscher, 1998; Sarnat and Flores-Sarnat, 2002). To assess Cajal–Retzius cell number, immunohistochemistry with a Reelin (G10) antibody was performed on mutant and control E12.5 and E16.5 embryos (Figure 3). At E12.5, the number of Reelin-expressing cells in the cortical MZ was similar between mutant and control embryos (Figure 3A and D), with 126.2±12.3 cells in the control and 126.0±17.8 cells in the Rb mutant MZ (Figure 3G). In addition, total MZ cell counts at E13.5 demonstrated similar cell numbers between control and mutant littermates (Figure 3H). In contrast, at E16.5, quantification of Reelin-positive cells revealed a dramatic 50% decrease in Cajal–Retzius neurons in the mutant MZ, with an average of 143.8±11.8 cells in the controls and 67.5±6.9 cells in Rb mutants (Figure 3B, E, and I; $P<0.001$). To confirm that reduced Reelin labeling was due to a decrease in positive cells and not merely a downregulation of Reelin protein, we counted the total cell number within the MZ. Consistent with Reelin immunohistochemistry, total cell counts revealed that mutant cortices contained nearly 50% fewer MZ cells compared to control embryos (Figure 3J) (32±6 cells in the control and 18±3 cells in the mutant; $P<0.05$). These results suggest that Rb deficiency does not negatively impact upon the generation of Cajal–Retzius neurons, but does affect the survival of this specific neuronal population.

Although telencephalon-specific Rb deficiency is not associated with the large-scale neuronal death characteristic of Rb germline knockouts, we previously reported a slight increase in TUNEL-positive cells within the mutant telencephalon at E13.5 (Ferguson *et al*, 2002). As Cajal–Retzius neurons are lost by E16.5 in Rb mutants, we questioned whether this was due to neuronal-specific apoptosis. To test this, we performed TUNEL labeling on mutant and control E13.5 and E16.5 embryos, and quantified positive cells within the germinal zones, IZ, CP, and along the MZ. At E13.5, control and Rb mutant sections contained similar numbers of TUNEL-positive cells within the germinal zones and IZ/CP (Supplementary Figure 2A). In contrast, Rb mutant sections showed elevated apoptosis within the MZ, with an average of 10.0±1.3 TUNEL-positive cells, as compared to 4.3±0.7 cells in control sections, representing a 2.3-fold increase (Figure 3K; $P<0.05$). At E16.5, TUNEL-positive cells in all quantified regions were not significantly different between mutant and control samples (Supplementary Figure 2B). Therefore, the specific increase in TUNEL labeling at E13.5

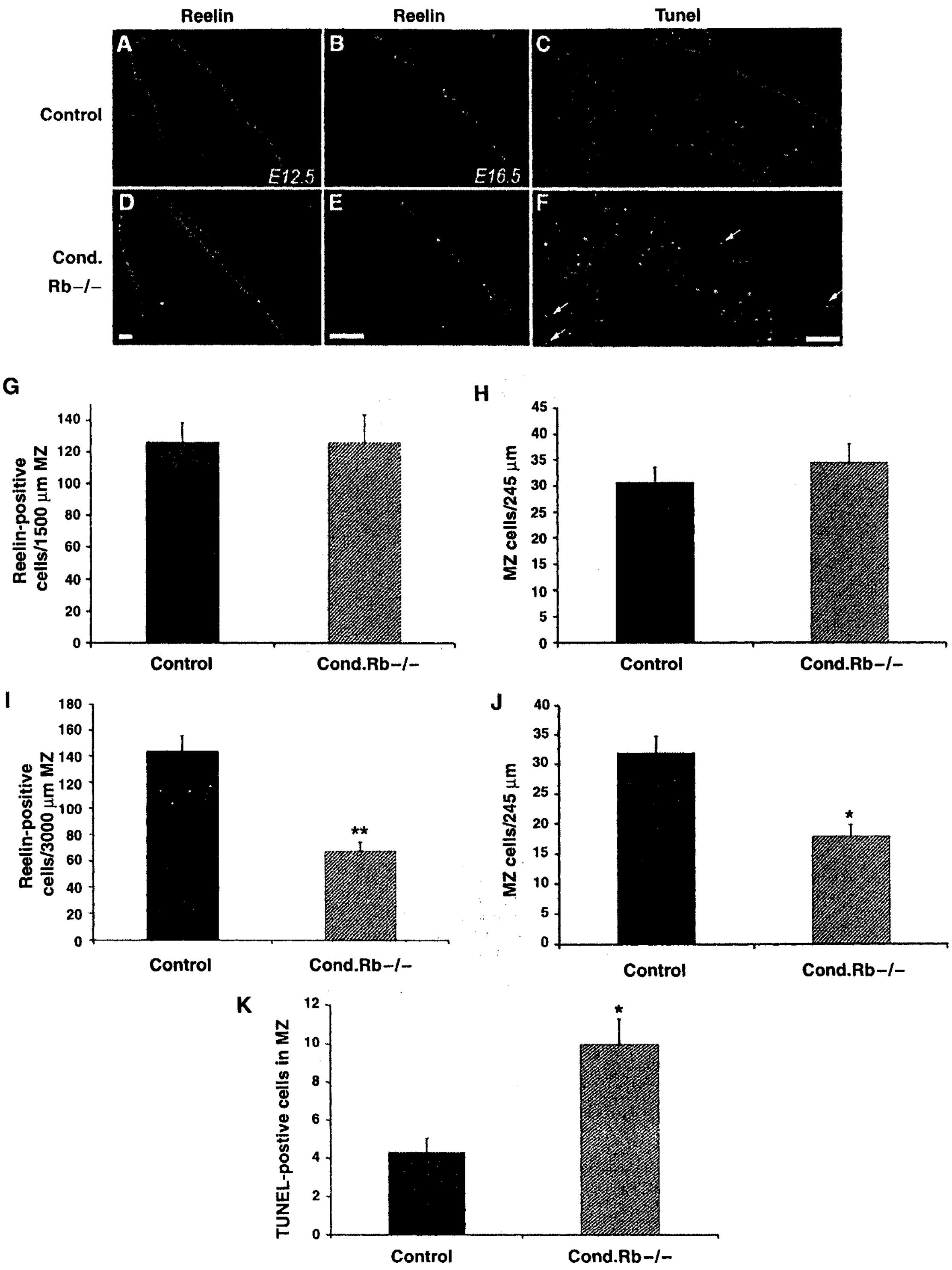

Figure 3 Rb is required for survival of Cajal–Retzius neurons. Coronal sections of control and Rb mutant embryos at E12.5 (**A, D**) and E16.5 (**B, E**) were subjected to immunohistochemistry with a Reelin (G10) antibody. Positive cells in each section were quantified along a 1500 μm (E12.5) or 3000 μm (E16.5) length of the MZ. At E12.5, Reelin expression in the cortical MZ appeared similar between mutant and control embryos (A, D, **G**) ($n=3$ control; $n=3$ cond. Rb−/−). Total MZ cells counted within a 245 μm length of the dorsal cortex of E13.5 embryos confirmed similar cell numbers between mutant and controls at this time (**H**; $n=4$ control; $n=4$ cond. Rb−/−). However, by E16.5, Rb mutants contained approximately 50% fewer Reelin-positive neurons as compared to control embryos (**B, E, I**, $P<0.001$) ($n=5$ controls; $n=4$ cond. Rb−/−). Total MZ cell number quantified within a 245 μm length of the dorsal cortex of E16.5 embryos resulted in a similar reduction in Rb mutants (**J**, $P<0.05$) ($n=3$ control; $n=4$ cond. Rb−/−). 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 MZ. Rb mutants exhibited significantly increased TUNEL labeling within the MZ (**C, F, K**, $P<0.05$; arrows point to representative cells) ($n=4$ control; $n=5$ cond. Rb−/−). Error denotes standard error. Bar: 25 μm. MZ: marginal zone.

within the MZ of Rb mutants suggests a requirement for Rb in the survival of Cajal–Retzius neurons. Owing to the important role of Cajal–Retzius neurons in guiding radial migration of newly born cortical neurons, reduction of these cells by mid-neurogenesis may contribute to the aberrant neuronal migration we observed in Rb mutants.

Rb deficiency does not significantly impact neuronal specification

Owing to the defective terminal mitosis characteristic of Rb deficiency, we questioned whether the generation and/or specification of distinct neuronal populations may be altered in Rb mutants. We first examined the expression of cortical progenitor markers, including *Ngn1*, *Ngn2*, *Pax6*, and *Emx1*, by *in situ* hybridization at E15.5 in Rb mutant and control embryos. For all markers tested, no difference was observed between mutant and control sections (data not shown). Similarly, examination of ventral progenitor markers such as *Hes1*, *Hes5*, *Nkx2.1*, and *Lhx7* did not reveal any defects in progenitor cell generation or specification in Rb mutants (data not shown). Given that post-mitotic cortical neurons were mislocalized within the Rb mutant cortex (Figure 1), we investigated whether Rb deficiency might also impact upon the positioning of ventrally derived interneurons. To test this, we examined expression of GABAergic interneuron markers early in neurogenesis and at mid-neurogenesis. At earlier time points, we did not observe any differences in the ventral populations with the examined markers, including calbindin (Figure 4A and B), *Lhx6* (Figure 4C and D), *GAD65*, and *GAD 67* (Supplementary Figure 3A–D) in Rb mutant embryos. In contrast, at mid-neurogenesis, the distribution of calbindin and *Lhx6* was noticeably perturbed in Rb mutant sections. Immunolabeling revealed that, although calbindin expression appeared normal in the ventral telencephalon, labeling was dramatically reduced in the temporal cortical MZ, to such an extent as to be almost absent (Figure 5B and D). Similarly, *in situ* hybridization with an *Lhx6* riboprobe demonstrated defective expression in mutant embryos (Figure 5E–H). Although expression was similar in mutant and control embryos along the IZ/ SVZ migratory route, there was substantially reduced expression along the cortical MZ (Figure 5H). Furthermore, *Lhx6* expression was reduced within the Rb mutant CP, suggesting that regardless of the migratory route, fewer *Lhx6*-positive interneurons were reaching the CP. In contrast, other interneuron markers were unaltered in Rb mutants, including calretinin, *Lhx7*, and *Nkx2.1* (data not shown).

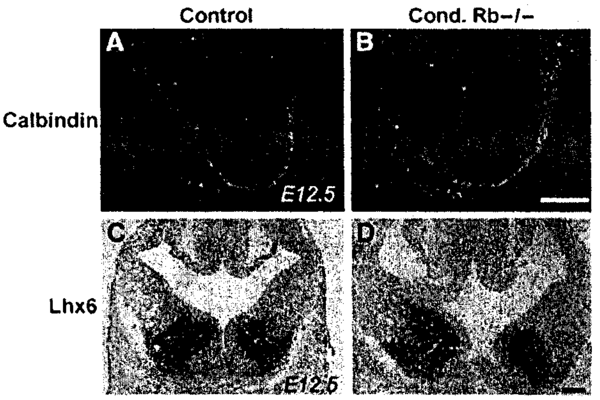

Figure 4 Rb deficiency does not impact interneuron specification or generation. To examine whether interneurons are properly generated in the absence of Rb, mutant and control E12.5 coronal sections were immunolabeled with a calbindin (D-28) antibody or subjected to *in situ* hybridization with an *Lhx6* riboprobe. The generation of calbindin- (**A, B**) and *Lhx6*-positive (**C, D**) progenitors appeared similar in the mutant and control embryos ($n = 3$ control; $n = 3$ cond. Rb−/−). Bar: 50 μm.

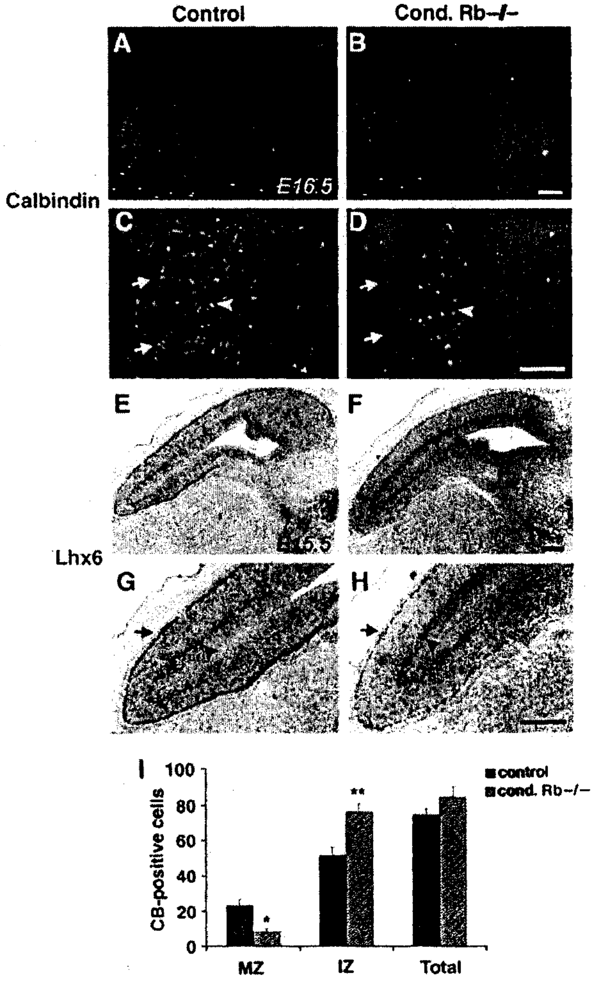

Figure 5 Cortical interneurons are mislocalized in Rb mutants. Rb mutant and control embryo sections were examined at mid-neurogenesis to determine whether specific interneuron populations may be impacted by Rb deficiency. E16.5 sections (coronal) were immunolabeled with a calbindin (D-28) antibody and E15.5 (sagittal) sections were subjected to *in situ* hybridization with an *Lhx6* riboprobe. Although calbindin expression appeared normal in other telencephalic regions (**A, B**), these cells were dramatically reduced in the mutant MZ (**C, D**). Similarly, *Lhx6* expression was normal along the SVZ/IZ migratory route, but was substantially reduced in the mutant CP and MZ (**G, H**; $n = 4$ control, $n = 5$ cond. Rb−/− (E15.5); $n = 5$ control, $n = 5$ cond. Rb−/− (E16.5). Calbindin-positive cells were quantified either within the MZ or deeper corresponding to the IZ (arrows point to MZ route; arrowheads denote IZ population). Although the total number of calbindin-positive cells does not differ, there is an approximately 50% reduction in cell number within the Rb mutant MZ (**I**, $P < 0.05$). The decreased number of calbindin-positive neurons within the Rb mutant MZ is associated with a corresponding increase in these neurons within the mutant IZ (**I**, $P < 0.01$). Error denotes standard error. Bar: 100 μm (A, B, **E–H**), 25 μm (C, D). MZ: marginal zone; IZ: intermediate zone.

Possible explanations for the mislocalization of *Lhx6*- and calbindin-expressing interneurons in the Rb mutant cortex are that they failed to properly migrate along their appropriate trajectories or that these neurons were lost by apoptosis. Calbindin-positive neurons were quantified in mutant and control sections along either the MZ or within the deeper IZ migratory route. Rb mutants showed a substantial 2.8-fold reduction in calbindin-positive neurons in the MZ, with 23.2 ± 3.9 cells in the littermate controls and 8.3 ± 1.7 cells in the mutant MZ (Figure 5I; $P<0.05$). This reduction in MZ neurons in the mutant appears to be accounted for by an increased IZ population. As compared to control sections with 51.6 ± 4.9 cells within the IZ, Rb mutants exhibited significantly more calbindin-positive neurons along this migratory route with 76.1 ± 5.1 cells (Figure 5I; $P<0.01$). Although the total number of calbindin-expressing neurons appeared slightly elevated in Rb mutants, the difference was not statistically significant (74.8 ± 3.1 cells in controls and 84.4 ± 5.6 in mutants). These results indicate that the dramatic reduction of these ventrally derived interneurons, specifically along the MZ migratory route, is not due to selective apoptosis, but instead suggests that these tangentially migrating neurons may be diverted from their normal MZ path into the deeper IZ trajectory.

A cell-autonomous requirement for Rb in interneuron migration

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). These neurons migrate tangentially through the ventral telencephalon to the cortex along distinct routes: a deep trajectory in the lower IZ/SVZ and a superficial route along the MZ. To determine whether Rb may be required to regulate tangential migration along the MZ route, we performed slice co-culture assays (Figure 6A). Mice were interbred to generate Rb mutants at a 25% frequency, with one parent additionally expressing green fluorescent protein (GFP) such that 50% of embryos would also be GFP-positive. Telencephalons of GFP-negative embryos were sectioned and plated onto coated filter-membrane inserts. Medial ganglionic eminence (MGE) explants were excised from GFP-positive littermates and placed directly on the area of the sections corresponding to the MGE. Co-cultures were grown *in vitro* for 72 h prior to fixation and immunolabeling for GFP. It has previously been shown that within 72 h, explanted cells will readily integrate into the slice and will migrate up to the dorsal cortex, along the appropriate MZ and IZ trajectories (Polleux *et al*, 2002). We assessed the migratory routes of GFP-positive cells, specifically focusing on the MZ trajectory, and classified each slice hemisphere into one of three categories: 'cell integration', 'MZ stops early', and 'MZ migration'. 'Cell integration' refers to the condition in which GFP-positive cells integrated into the section and initiated tangential migration in the appropriate ventrolateral direction; however, these cells failed to follow a distinct migratory route or reach the MZ. This was considered to be the most extreme form of failed migration (Figure 6B, E, and H). The second category, 'MZ stops early', was considered to be a more moderate failure to migrate along the MZ and occurred when GFP-positive cells were detected in the MZ route, but did not reach the cortex (Figure 6C, F, and I). The third category, 'MZ migration', included all sections in which a GFP-positive migratory route was observed along the MZ, reaching the dorsal cortex (Figure 6D, G, and J).

In addition to guiding radial migration, Cajal–Retzius neurons have recently been suggested to have a potential role in regulating the tangential migration of interneurons from the ventral telencephalon (Hack *et al*, 2002; Shinozaki *et al*, 2002; Morante-Oria *et al*, 2003; Stoykova *et al*, 2003). Because Rb mutants exhibit a 50% reduction in Cajal–Retzius neurons, we asked whether tangential migration in Rb mutants may be altered owing to defective environmental cues. To test this, we examined the MZ migratory routes formed by control MGE explants on either control or Rb mutant slices (Figure 7). When control explants were placed on control slices, 93.3% of sections revealed complete MZ migration (28/30), whereas only 7.1% failed to migrate and were classified as 'cell integration' (2/30). Similarly, cells derived from control MGE explants placed on Rb mutant slices were able to appropriately migrate along the Rb-deficient MZ. Under control explant:mutant slice conditions, GFP cells displayed complete MZ migration, in all sections examined (19/19). These data demonstrate that, despite the reduced number of Cajal–Retzius neurons in the Rb mutants, control MGE-derived interneurons do not exhibit any defects in migrating along the MZ route in the Rb-null cortex.

We next questioned whether Rb may be required by migrating neurons in order to properly navigate the MZ migratory route. We compared the MZ migration of GFP cells derived from either control or mutant MGE explants on control slices. Although the large majority of control explants placed on control sections exhibited complete MZ migration (93.3%), Rb-deficient migrating neurons showed a dramatic failure to migrate along the MZ of the control slices. The majority of sections (61.9% or 13/21) exhibited the most severe form of failed migration in the form of 'cell integration', whereas in 14.2% of sections, the MZ route stopped early (3/21), and only 23.8% of sections showed complete MZ migration (5/21). Furthermore, we examined the migratory capacity of Rb-deficient MGE-derived interneurons on Rb mutant slices. Under these conditions, 40% (2/5) of sections exhibited 'cell integration', 60% (3/5) exhibited an MZ trajectory that stopped early, and none of the sections underwent complete MZ migration (Figure 7). These results demonstrate that Rb is essential for the MGE-derived interneurons to complete tangential migration along the MZ route. When pooled together, 95.9% (47/49) of sections with control explants showed complete MZ migration, whereas only 19.2% (5/26) of sections with mutant explants displayed proper MZ migration (Figure 7). These data comprise the first evidence indicating a cell-autonomous requirement for the cell cycle protein, Rb, in regulating neuronal migration during forebrain development.

Discussion

In this study, we examined the requirement for the cell cycle regulator, Rb, in telencephalic development. Although Rb mutants produced appropriate numbers of correctly specified cortical projection neurons and interneurons, the radial and tangential migration of these neuronal populations, respectively, were perturbed. In particular, neuronal birthdating and marker analyses revealed that the radial migration of early-

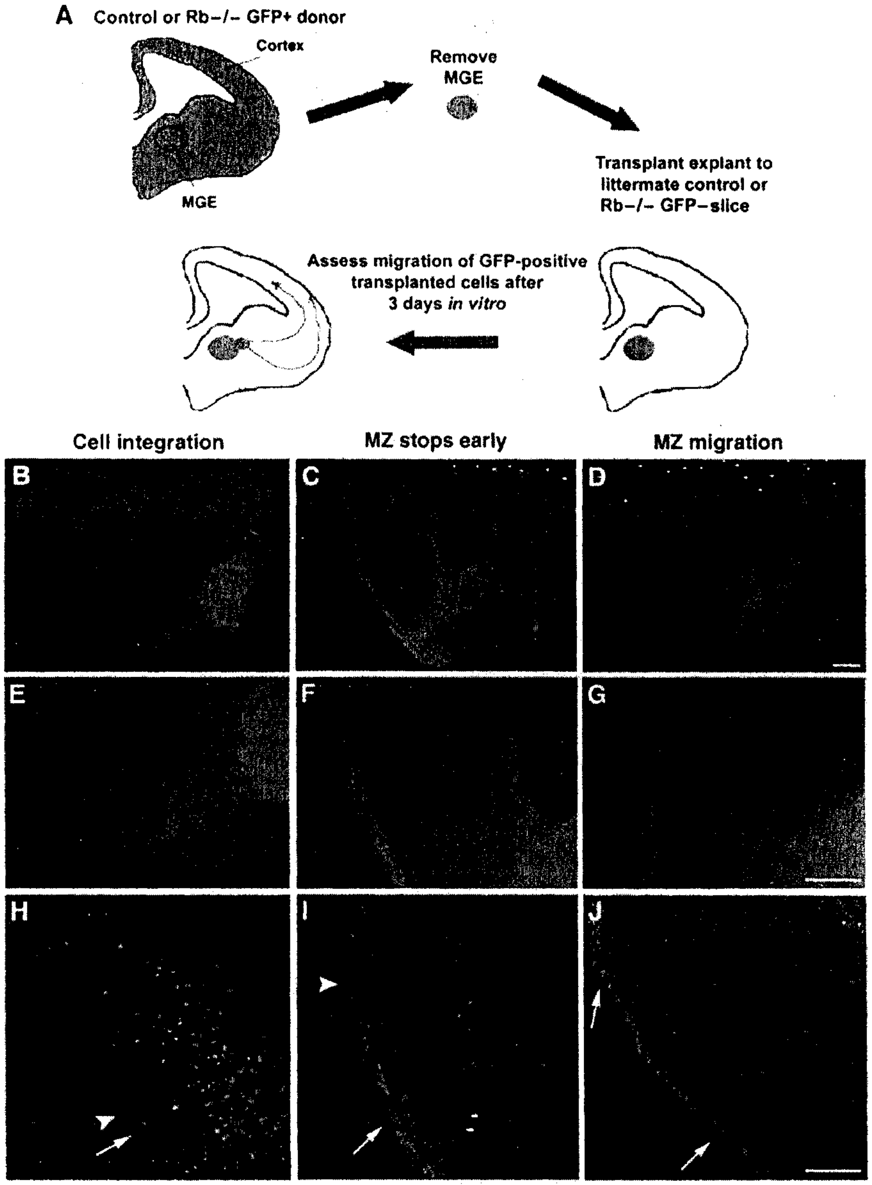

Figure 6 Slice co-cultures. To assess the requirement for Rb function in interneuron migration, we performed slice co-culture assays. (**A**) GFP-negative telencephalon sections were plated onto coated filter-membrane inserts in a six-well dish. MGE were removed from GFP-positive littermates and equal-sized pieces were placed directly on sections corresponding to the MGE. Co-cultures were grown *in vitro* for 72 h prior to fixation and GFP immunohistochemistry. The migratory routes of the GFP-positive cells, specifically the MZ trajectory, were analyzed and classified as follows: 'cell integration', in which GFP-positive cells integrated into the section (arrow) but did not follow a distinct migratory route (arrowhead) or reach the MZ (**B**, **E**, **H**). 'MZ stops early' occurred when GFP-positive cells formed an MZ route (arrow) but did not reach the dorsal cortex (arrowhead) (**C**, **F**, **I**) 'MZ migration' included sections in which a GFP-positive MZ migratory route reaching the dorsal cortex was observed (arrows) (**D**, **G**, **J**). Bar: 100 μm (D, G), 50 μm (J). MGE: medial ganglionic eminence; MZ: marginal zone.

born neurons within the cortex was impaired in the absence of Rb. Similarly, an examination of cortical interneuron markers and the use of slice co-culture assays revealed that Rb is essential for normal tangential migration. In conclusion, our results demonstrate a novel cell-autonomous function for the Rb tumor suppressor, in regulating neuronal migration during telencephalic development.

Cell cycle and cell fate

Proper timing of cell cycle exit and terminal mitosis is believed to be critical for the generation of specific neuronal cell types in the developing neocortex (McConnell and Kaznowski, 1991). We have previously shown the prevalence of ectopically dividing neuroblasts throughout the IZ and CP of Rb mutants (Ferguson *et al*, 2002). Despite the fact that Rb-deficient mutants exhibit defective terminal mitoses, we show here that cortical neuronal populations do not appear to be misspecified. First, all dorsal progenitor cell markers examined showed normal expression patterns in Rb mutants. Second, layer-specific markers were appropriately expressed in Rb mutants, with the exception of ectopic expression in the IZ at early developmental stages. While neuronal fate determination has been shown to be tightly coupled with the timing of terminal mitosis (McConnell, 1995; Waid and

A

Genotype	% cell integration	% MZ stops early	% MZ migration
Control slices Control explant	7.1 (2/30)	0.0 (0/30)	93.3 (28/30)
Mutant slices Control explant	0.0 (0/19)	0.0 (0/19)	100.0 (19/19)
Control slices Mutant explant	61.9 (13/21)	14.2 (3/21)	23.8 (5/21)
Mutant slices Mutant explant	40.0 (2/5)	60.0 (3/5)	0.0 (0/5)

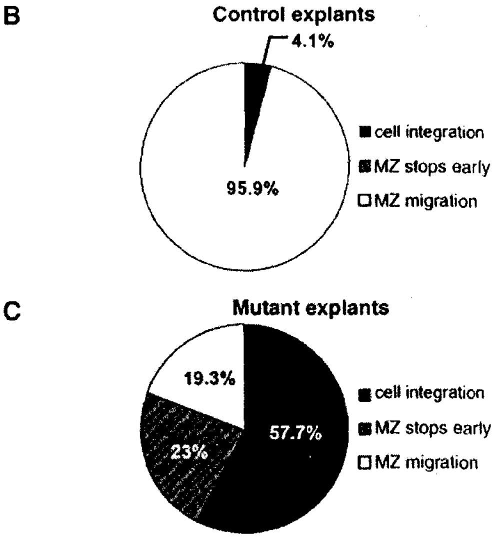

Figure 7 A cell-autonomous requirement for Rb in tangential migration. (**A**) The MZ migratory routes resulting from GFP-positive MGE explants were compared according to the previously described classifications (Figure 6). Control explants were able to complete MZ migration in 95.9% (47/49) of sections examined, regardless of whether they were placed on mutant or control slices (**B**). In contrast, only 19.2% (5/26) of mutant explants placed on either mutant or control slices displayed proper MZ migration (**C**). MZ: marginal zone.

McLoon, 1995), the Rb-deficient mouse represents an anomaly in which these events become aberrantly uncoupled. Although Rb-deficient neurons fail to undergo terminal mitosis at the correct time, they are still able to generate the appropriate neuronal populations as in wild-type animals. This lack of correlation between neuronal gene expression and terminal mitosis suggests that terminal mitosis may not always be a prerequisite for the specification of the appropriate neuronal population.

Our neuronal birthdating experiments revealed that although similar numbers of early-born cortical neurons were generated in Rb mutants, they failed to reach their ultimate destination within the CP, suggestive of a role of Rb in radial migration. In contrast, later-born neurons were able to migrate to their appropriate layers, raising the possibility that Rb is required for the migration of specific neuronal subpopulations. We cannot, however, rule out the idea that the early-born cortical projection neurons migrate inappropriately because they are born in ectopic locations. Confocal microscopy has revealed that the vast majority of dividing cells in the IZ are committed neuroblasts co-expressing BrdU and the neuronal marker β-III tubulin (Ferguson *et al*, 2002). This is consistent with the interpretation that Rb-deficient neurons are committed to a specific fate before they leave the VZ but then continue to divide ectopically. At present, therefore, we cannot distinguish between whether (a) neurons born in the IZ fail to undergo appropriate radial migration as a consequence of their generation in an inappropriate location or (b) early-born projection neurons require Rb for their radial migration.

A key question when evaluating radial migration is whether any of the migrating cells, particularly those that fail to find their appropriate destination, undergo apoptosis. We have quantified apoptosis in each of the zones including VZ, IZ, CP, and MZ and have only found a significant increase in apoptosis in the MZ at E13.5 and no difference in any of these regions at E16.5. This suggests that Rb is not essential for the survival of CP neurons and that neurons born in the IZ do not appear to default to an apoptotic pathway. Our studies reveal however that Rb is required for the survival of Cajal–Retzius neurons in the MZ. It should be noted that mice carrying the conditional Rb mutation die at birth, hence we cannot comment on the long-term survival of these cortical neurons.

Selective loss of Cajal–Retzius neurons

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 a small but significant 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 dramatically increased within the cortical MZ, specifically affecting the Cajal–Retzius neurons. Although the initial generation of Reelin-positive Cajal–Retzius neurons is normal, by E16.5, these cells are reduced in number by nearly 50% in the Rb-deficient MZ. This raises the possibility that the reduction in Cajal–Retzius cell numbers could account, at least in part, for the defects in radial and tangential migration. Indeed, Cajal–Retzius neurons are known to be critical for guiding radial migration of newly generated cortical neurons (Frotscher, 1998; Sarnat and Flores-Sarnat, 2002), and the loss of these cells by mid-neurogenesis may be associated with the aberrant radial migration and defective laminar patterning observed in the Rb mutant cortex. A similar reduction of Cajal–Retzius neurons occurs in mice deficient for Emx2, in which the neurons appear to be properly generated but are subsequently lost. These mice exhibit defective radial migration and laminar patterning (Mallamaci *et al*, 2000; Shinozaki *et al*, 2002). Although a reduction of Cajal–Retzius neurons by mid-neurogenesis would not be expected to have a great effect on the positioning of early-born neurons, the loss may be responsible for more subtle defects.

Rb regulates tangential migration in a cell-autonomous manner

The specification and generation of GABAergic interneurons, specifically calbindin- and *Lhx6*-positive neurons, was not initially impaired; however, by mid-neurogenesis, expression of these cortical interneuron markers was dramatically reduced along the MZ, whereas there was a corresponding increase in the IZ. Thus, the total number of calbindin-positive neurons was similar between Rb mutant and control embryos, demonstrating that these interneurons were not lost owing to apoptosis or neurogenesis defects. Instead, we found that the reduction of cells within the MZ was associated with a corresponding increase in calbindin-positive cells within the IZ. These results support our interpretation that the specific MZ loss of interneurons is due to their failure to properly follow their MZ migratory route, and that these cells instead become re-routed toward the deeper IZ trajectory. We cannot, however, rule out that the impaired migration we observe could be the result of a failure of these cells to express the full complement of genes required for proper migration.

Our data show that control cells from MGE explants migrate equally well on control and mutant sections, indicating the migratory environment to be relatively inconsequential in terms of the defective migration observed in Rb mutants. Instead, we demonstrate that Rb is essential among certain MGE-derived interneurons in order to migrate along the MZ. Despite the dramatic reduction in MZ migration derived from Rb mutant explants, a small proportion of samples that included Rb-deficient interneurons (23.8%) demonstrated complete MZ migration. This may be explained by the heterogeneity of the MGE population, as Rb signaling may only be required in specific interneuron subsets.

What may account for the mechanism through which Rb could impact neuronal migration? Previous studies have shown that Rb interacts with a number of genes that can regulate differentiation. A well-established interaction is with the helix–loop–helix (HLH) family member, Id2, an important negative regulator of neuronal differentiation (Perk *et al*, 2005). By acting as a dominant inhibitor of proneural basic HLH factors, Id2 represses the transcription of neuron-specific genes. Rb has been shown to interact with Id2, thereby suppressing its activity (Lasorella *et al*, 2002). Several studies demonstrate the importance of the Rb interaction with Id2 in neural development. First, the neurological defects found in Rb knockouts are rescued by the absence of Id2 (Lasorella *et al*, 2000). Second, expression of Id2 in cortical progenitor cells was shown to inhibit the induction of neuron-specific genes, while this inhibition was alleviated by the co-expression of a constitutively active Rb (Toma *et al*, 2000).

The consequence of increased free Id2 activity in Rb deficiency could lead to inhibition of genes that impact on differentiation and migration. One such example is TrkB, which has previously been shown to be substantially reduced in Rb-deficient brains (Lee *et al*, 1994). In addition to its well-known role in neuronal survival (Atwal *et al*, 2000; Stucky *et al*, 2002), TrkB has also been shown to regulate radial and tangential neuron migration (Polleux *et al*, 2002; Medina *et al*, 2004). Recent studies have demonstrated that Id2 can also directly repress TrkB expression in neural cells (Liu *et al*, 2004). Thus, we envisage a model whereby the absence of Rb leads to deregulated Id2 activity causing inhibition of transcription of neuron-specific genes required for differentiation and migration, such as TrkB (Figure 8). Future studies exploring molecules that regulate neuronal migration in the Rb-deficient brain will be required to identify the specific pathways that are dependent on Rb.

In conclusion, we demonstrate a cell type-specific requirement for Rb in the regulation of cortical development. Although the majority of cortical neurons survive in the absence of Rb, specific populations, including Reelin-positive Cajal–Retzius neurons, require Rb for survival. Furthermore, we reveal, for the first time, an essential role for the cell cycle protein, Rb, in regulating neuronal migration during cortical development.

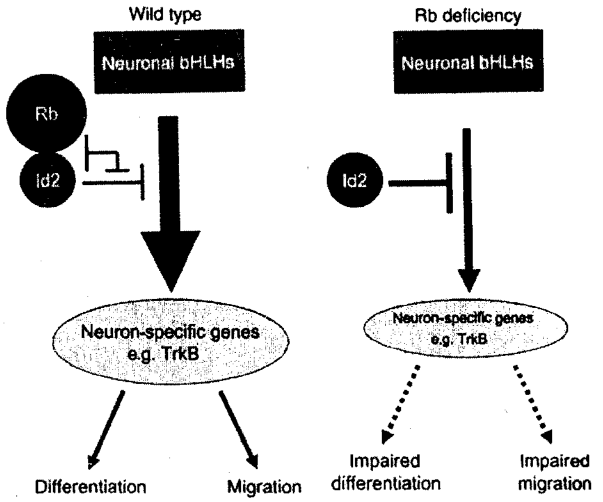

Figure 8 Proposed model of Rb-mediated regulation of neuronal differentiation and migration. In wild-type cells, Id2 is sequestered by Rb and is unable to inhibit basic HLH-mediated transcription of specific neuronal genes, such as TrkB, that are required for neuronal differentiation and migration. In the absence of Rb, Id2 activity is deregulated, allowing inhibition of TrkB transcription, which, in turn, leads to impaired neuronal migration and differentiation.

Materials and methods

Mice

Telencephalon-specific Rb-deficient mice were generated by crossing Rb-F19 (Marino *et al*, 2000) and Foxg1-cre mice (Hebert and McConnell, 2000), as described previously (Ferguson *et al*, 2002). 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 killed by a lethal injection of sodium pentobarbitol and embryos were removed and placed in 1 × PBS. Embryos were fixed in 4% paraformaldehyde/0.1 M phosphate buffer pH 7.4 for 1–2 days at 4°C. For frozen sections, tissue was subjected to sequential solutions of 12, 16, and 22% 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 on a cryostat as 14 μm sections at −20°C and mounted 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 6 μm thickness. Cresyl violet staining was performed on paraffin sections according to standard protocols.

Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed on fixed frozen sections with the following primary antibodies: TuJ1 (mouse monoclonal hybridoma supernatant, 1:50, Dr David Brown, University of Ottawa), mouse monoclonal anti-Reelin G10 (1:500; Calbiochem, #553731), rabbit polyclonal anti-calbindin (D-28) (1:1000; Chemicon, AB1778), and mouse monoclonal anti-GAD 65 (1:100; BD Pharmingen, 559931/69221A). 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 immunohistochemistry, sections were subjected to an antigen retrieval pretreatment: 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. For BrdU incorporation analyses, pregnant females were injected intraperitoneally with 20 or 50 μg BrdU/g body mass (Boehringer Mannheim #280879) and were processed as described previously (Ferguson *et al*, 2002). TUNEL staining was performed as described previously (Ferguson *et al*, 2002). Non-radioactive *in situ* hybridization and digoxygenin probe labeling was performed according to previously described protocols (Wallace and Raff, 1999). The following antisense riboprobes were used, as described previously: *Tbr1*, *SCG10*, *Lhx6*, and *GAD 67*. Riboprobe references are found in Supplementary data. Sections were examined with a Zeiss Axioskop 2 fluorescence microscope and visualized with a Sony Power HAD 3CCD color video camera with Northern Eclipse software.

Slice co-cultures

The conditional Rb mutant mice were bred such that one of the parents was additionally heterozygous for GFP. With this crossing, 25% of embryos would be expected to be Rb deficient while 50% of embryos should express GFP. Heterochronic slice co-cultures were performed on E16.5 litters, as previously described, with some modification (Polleux *et al*, 2002) (Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Unique Requirement for Rb/E2F3 in Neuronal Migration: Evidence for Cell Cycle-Independent Functions[▽]

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The cell cycle regulatory retinoblastoma (Rb) protein is a key regulator of neural precursor proliferation; however, its role has been expanded to include a novel cell-autonomous role in mediating neuronal migration. We sought to determine the Rb-interacting factors that mediate both the cell cycle and migration defects. E2F1 and E2F3 are likely Rb-interacting candidates that we have shown to be deregulated in the absence of Rb. Using mice with compound null mutations of Rb and E2F1 or E2F3, we asked to what extent either E2F1 or E2F3 interacts with Rb in neurogenesis. Here, we report that E2F1 and E2F3 are both functionally relevant targets in neural precursor proliferation, cell cycle exit, and laminar patterning. Each also partially mediates the Rb requirement for neuronal survival. Neuronal migration, however, is specifically mediated through E2F3, beyond its role in cell cycle regulation. This study not only outlines overlapping and distinct functions for E2Fs in neurogenesis but also is the first to establish a physiologically relevant role for the Rb/E2F pathway beyond cell cycle regulation in vivo.

Neurogenesis is a highly regulated process by which neural precursors divide and differentiate, giving rise to the cells that make up the nervous system (reviewed in references 24 and 25). While the role of cell cycle genes in regulating proliferation of neural precursor cells is well appreciated, accumulating data point convincingly to their unique roles in regulating diverse cellular processes, independent of cell cycle regulation (reviewed in reference 63). The retinoblastoma (Rb) tumor suppressor is a key cell cycle regulator that we along with others have shown to play a number of roles in neurodevelopment including proliferation, survival, and, more recently, neuronal migration (7, 8, 10, 18, 19, 33, 40, 52). Differentiating Rb-deficient neural precursor cells exhibit delayed cell cycle exit, while the absence of Rb in the telencephalon leads to ectopic proliferation of neural precursor cells and enhanced brain size at midgestation (7, 19, 52). In a recent study we described a role for Rb in regulating the survival of discrete neuronal subpopulations and a novel cell autonomous role for Rb in regulating neuronal migration (18).

The mechanism by which Rb regulates neurogenesis and the extent to which defects in migration and survival are the result of cell cycle deregulation remain unknown. While Rb is known to interact with numerous proteins (reviewed in reference 67), many of which are expressed in quiescent cells or have cell cycle-independent functions, members of the cell cycle regulatory E2F family are likely targets in neurogenesis. The E2F family of transcription factors is comprised of E2Fs 1 to 8; however, E2F1, E2F2, and E2F3, the so-called activating E2Fs, are key Rb-interacting targets best known for their role in promoting cell cycle progression (9, 14, 17, 48, 49, 54; reviewed in reference78). Both E2F1 and E2F3 are likely candidates involved in Rb-mediated regulation of neurogenesis. Deficiency of either E2F1 or E2F3 was observed to correct the ectopic proliferation observed in the central nervous system (CNS) in germ line Rb deficiency alone, and both E2F1 and E2F3 are grossly deregulated in proliferating neural precursors in the absence of Rb (7, 75, 81, 93).

While E2F1 and E2F3 are key regulatory targets in the Rb signaling pathway, the extent to which each contributes to Rb-mediated neurogenesis is unknown. Whether E2F1 and E2F3 are functionally redundant or are capable of unique function is still subject to debate and likely depends on the context examined. Individually, E2F1 is a tumor suppressor, and its deficiency results in mice that are viable but develop tumors at an advanced age (92). E2F1 expression is cell cycle regulated, with expression peaking at G_1/S (reviewed in reference 78). A role for E2F1 in neurogenesis is indicated in the adult, where mice deficient for E2F1 exhibit decreased precursor cell division in the proliferative regions of the lateral ventricle and hippocampus (12). By contrast, E2F3 is not known as a tumor suppressor, but mice lacking E2F3 do exhibit a developmental phenotype (11). E2F3-deficient mice survive postnatally at a frequency of 25% on a mixed 129/Sv × C57BL/6 genetic background, and no E2F3-deficient mice are born on a pure 129/Sv genetic background (11, 30). Additionally, the

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E2F3 locus expresses two distinct transcripts, full-length E2F3a and N-terminal-truncated E2F3b transcribed from an intronic promoter within the E2F3 locus (27, 41). E2F3a expression is cell cycle regulated and is similar to that of E2F1 (27, 41). E2F3b, however, is expressed equivalently in quiescent and proliferating cells, is a specific partner for Rb in quiescent cells and thus may have an opposing role to E2F3a in cell cycle control (27, 41).

As both E2F1 and E2F3 are expressed in the developing cortex starting from embryonic day 11.5 (E11.5) and are deregulated in the absence of Rb (7, 13), we sought to determine the extent to which each is a target in Rb-mediated neurogenesis. Using mice with compound null mutations of Rb and E2F1 or E2F3, we describe both overlapping and unique functions for each. Here, we report that E2F1 and E2F3 are both functionally relevant targets in neural precursor proliferation, cell cycle exit, and laminar patterning. Each can partially mediate the Rb requirement for neuronal survival. Neuronal migration, however, is specifically mediated through E2F3. This study not only outlines overlapping and distinct functions for E2Fs in neurogenesis but also is the first to establish a physiologically relevant requirement for the Rb/E2F pathway beyond cell cycle regulation in vivo.

MATERIALS AND METHODS

Mice. Germ line E2F1 null mice were generated previously (20) and obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained on a C57BL/6 genetic background. Germ line E2F3 null mice were generated previously (42) and maintained on a mixed 129/Sv and C57BL/6 genetic background. To generate E2F1- and E2F3-deficient mice, heterozygous ($E2F1^{-/-}$ or $E2F3^{+/-}$) mice were crossed. Embryos and animals were genotyped according to standard protocols with previously published primers for E2F1 (20) and E2F3 (42). Telencephalon-specific Rb-deficient mice were generated by crossing floxed Rb-F19 (58, 84) and Foxg1-cre mice (28), and mice were genotyped according to standard protocols with previously published primers (18, 19). Telencephalon-specific Rb-deficient/germ line-deficient E2F1 (Rb E2F1 DKO) mice were generated by interbreeding flox Rb/Foxg1-cre mice with flox Rb/germ line E2F1-deficient mice, both maintained on an FVBN background. Telencephalon-specific Rb-deficient/telencephalon-specific E2F3-deficient (Rb E2F3 DKO) mice were generated by crossing flox Rb/Foxg1-cre mice with flox E2F3 mice (91), both maintained on an FVBN genetic background. Mice were genotyped for flox E2F3 according to standard protocols with previously published primers for flox E2F3 (91). For embryonic time points, the time of plug identification was counted as E0.5. 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.

Tissue fixation and cryoprotection. Pregnant female mice and adult mice were euthanized with a lethal injection of sodium pentobarbital. Embryos were dissected and fixed overnight in 4% paraformaldehyde (PFA) in 1× phosphate-buffered saline (PBS), pH 7.4; cryoprotected in sequential solutions of 12, 16, and 22% sucrose in 1× PBS, followed by embedding in OCT (TissueTek 4583); and frozen on liquid N_2. Adult mice were perfused with 1× PBS followed by cold 4% PFA, and brains were removed. Brains were postfixed overnight in 4% PFA, cryoprotected in 22% sucrose in 1× PBS, and frozen. Sections from either embryos or adults were collected as 14-μm coronal cryosections on Superfrost Plus slides (catalog no. 12-550-15; Fisher Scientific).

BrdU labeling, immunohistochemistry, and in situ hybridization. To assess neural progenitor proliferation in adult mice (12 weeks old), intraperitoneal injections of bromodeoxyuridine ([BrdU] dissolved in 0.007 N NaOH in 0.9% NaCl; 50 mg/kg of body mass) (B-5002; Sigma) were given every 2 h over a 10-h period. Mice were euthanized 30 min after the last injection (80, 82). BrdU detection was performed with a mouse monoclonal anti-BrdU (1:100 dilution; catalog no. 347580; BD Biosciences) as previously described (19). BrdU-positive cells were counted in the subependyma of the lateral ventricles in every 10th coronal cryosection (14 μm thick) from the most caudal crossing of the corpus callosum to the start of the third ventricle (crossing of the anterior commissure). A two-tailed *t* test was performed to compare the mean numbers of BrdU-positive cells, and significant differences were assessed at α values of 0.05. To assess neural progenitor proliferation in embryos, pregnant females were injected intraperitoneally with 50 μg of BrdU/g of body mass and processed as above. BrdU-labeled cells were quantified over a 650-μm region of dorsal cortex with a minimum of three matched sections counted per embryo. To assess cells in mitotic M phase, phospho-histone H3 (PH3) labeling was performed with rabbit polyclonal anti-PH3 (dilution of 1:100; catalog no. 06-570; Upstate Biotechnology) as previously described (19). To assess cell death, either terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) (in situ end labeling kit; Roche) or active caspase-3 ([AC-3] 1:500; 559565 rabbit polyclonal; BD Pharmingen) immunohistochemistry combined with Hoescht nuclear staining was performed according to standard protocols (19). To quantify cell death specific to the marginal zone, AC-3-labeled cells in the marginal zone were counted from the cingulate cortex to the dorsal-ventral boundary. Both hemispheres were quantified, and counts are expressed as the mean of the two hemispheres from four matched sections per embryo. To quantify cell death in the ventral telencephalon, AC-3-labeled cells were counted below the dorsal-ventral boundary from four matched sections per embryo. Reelin and calbindin immunolabeling were performed with the mouse monoclonal anti-reelin G10 (1:500; catalog no. 553731; Calbiochem) and rabbit polyclonal anticalbindin (D-28; 1:1,000) (item AB1778; Chemicon) as previously described (18). Reelin-labeled cells were quantified along a 500-μm region of dorsal cortex and temporal cortex from a minimum of four matched sections per embryo. Calbindin-labeled cells were quantified within the marginal zone or within the same area comprising all cells within the migratory route ("total") of each hemisphere from four matched sections per embryo and expressed as cells per 500-μm length. For all immunohistochemistry, secondary antibodies were obtained from Molecular Probes and used at a concentration of 1:500. Cresyl violet staining was performed according to standard protocols, and cells in the marginal zone were quantified along a 500-μm region of dorsal cortex and expressed as the mean from a minimum of four matched sections per embryo. Nonradioactive in situ hybridization and digoxigenin probe labeling were performed according to previously described protocols (85). Tbr1 antisense riboprobe was used, as previously described (6), and neogenin riboprobes were generous gifts of Helen Cooper, University of Queensland (22), and Elke Stein, Yale University. E2F1 and E2F3 digoxigenin-labeled riboprobes were generated from pBS-IIKS-E2F1 and pBS-IIKS-E2F3 templates, containing 0.65-kb and 0.72-kb cDNA inserts, respectively, which were amplified by PCR with primers E2F1 (Forward, ATCGGAATTCT CTCTTTGACTGTGACT; Reverse, ATTAAAGCTTCGATCGGAAAACTT) and E2F3 (Forward, ATCGAAGCTTAGACTTGGCTTCTAACAACT; Reverse, TGGCAGAATTCCATTCCGTGGTAG) and verified by sequencing.

Microarray analysis. For microarray analysis, total RNA was extracted from tissue derived from ganglionic eminences at E14.5 from control and conditional Rb mutants using Trizol reagent according to the manufacturer's instructions (Invitrogen, San Diego, CA). Samples from embryos ($n = 6$) were pooled for each genotype. RNA was sent to the Ottawa Genomics Innovation Centre Microarray Facility, where the Affymetrix Mouse Genome 430 2.0 Array was used for analysis.

EMSA. Electrophoretic mobility shift assay (EMSAs) were performed on total protein extracts from neural precursor cells as described previously (7), with the following modifications. Total cell protein was extracted in a lysis buffer (buffer A) and assayed by the method of Bradford (Bio-Rad protein assay reagent, catalog no. 500-0006). A 20-μg aliquot of lysate was incubated with an excess of ^{32}P-labeled double-stranded DNA probe (70,000 cpm/0.2 ng of DNA) containing a single E2F-binding site: 5′-GGATTTAAGTTTCGCGCCCTTTCTCAA-3′. The binding reaction (25 μl) was carried out at room temperature 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 bovine serum albumin, 0.5 mM dithiothreitol). To control for binding specificity, a 10-fold excess of unlabeled wild-type oligonucleotide was added to the binding reaction mixture and incubated for 20 min before the addition of labeled probe. To identify the composition of the complexes, tissue culture supernatant or purified antibody was added to the reaction mixture. Complexes were resolved on a 5.0% gel run for 4 h, dried, and visualized by autoradiography. The tissue culture supernatant containing the monoclonal pRb antibody 21C9 was a gift from David Cobrinik (77). All other antibodies were purchased from Santa Cruz Biotechnology Inc. (E2F1, sc 193; E2F3, sc878 and sc878x). Immunoprecipitation-EMSA was performed as described previously (31) with the following modifications. For immunoprecipitation, 200 μg of total protein from neural precursors was incubated with either 2 μg of mouse monoclonal anti-human Rb antibody (catalog no. 554136; BD Biosciences) or equivalent mouse serum (Sigma M-5905) conjugated to protein G-Sepharose beads (17-0618-01; Amersham/GE Healthcare) in 1× shift buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 0.4 mM sodium

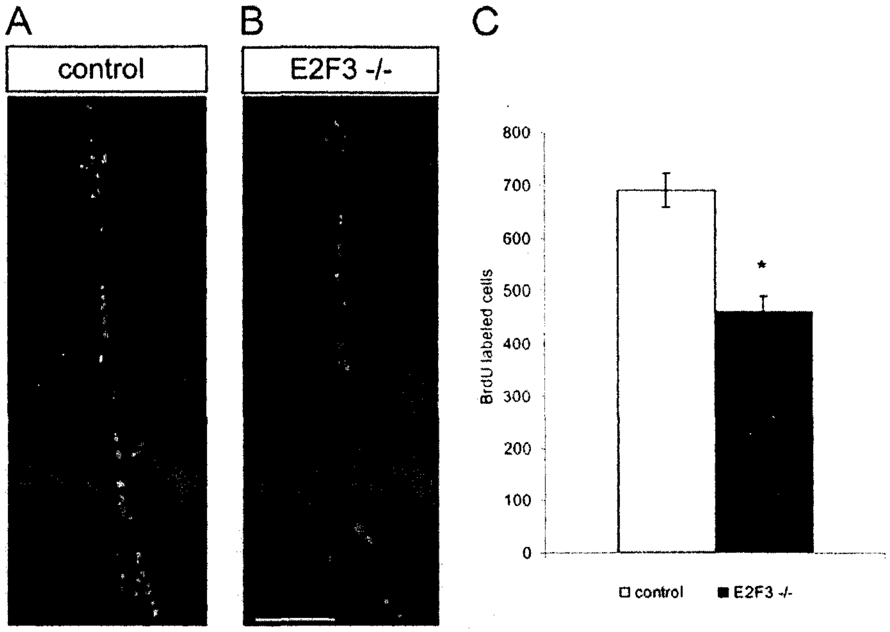


FIG. 1. E2F3 is a positive regulator of neural precursor proliferation. (A and B) BrdU was administered over 10.5 h at 2-h intervals to adult male E2F3$^{-/-}$ and wild-type littermates to label dividing progenitor cells. Sections were labeled with an antibody to BrdU. BrdU-labeled cells lining one lateral ventricle were counted every 10th section between anatomical landmarks, and the total number of BrdU-labeled cells counted was expressed as the mean ± standard error of the mean (C). Approximately one-third fewer BrdU-labeled cells were observed in E2F3$^{-/-}$ compared to wild-type littermates ($n = 3$). Significance was determined using a two-tailed t test. *, $P < 0.05$. Bar, 100 μm.

vanadate, 0.4 mM sodium fluoride, 0.1% NP-40, 1 mM dithiothreitol, and protease inhibitors) for 1 h with gentle rotation. Beads were washed three to four times in 1× shift buffer, followed by treatment with 16 μl of 0.8% deoxycholate (DOC) for 10 min on ice to dissociate the E2F complexes. Following neutralization with 4 μl of 6% NP-40, 5μl of the supernatant was used for E2F EMSA as described above.

Microscopy. Sections treated for immunohistochemistry were examined by a Zeiss Axioskop 2 microscope with standard fluorescence and bright-field or dark-field settings with 5× (numerical aperture, 0.17) or 20× (numerical aperture, 0.17) objectives, respectively. Images were captured using a digital black and white camera with Northern Eclipse software. For confocal microscopy, a Zeiss LSM 510 META on an Axiovert 200 M inverted microscope was used with images captured through the manufacturer's integrated digital imaging software. Figures were compiled using Adobe Photoshop CS2. Manipulations of brightness and intensity were made equally to all treatment groups.

RESULTS

E2F3 is a positive regulator of neural precursor proliferation. Previous studies have described a role for E2F1 in regulating neural precursor proliferation in vivo (12); however, little is known regarding the role of E2F3. As E2F3 is known as a major regulator of cellular proliferation (30; reviewed in reference 78), we first asked if E2F3 is also capable of regulating neural precursor proliferation using mice lacking E2F3. As cell cycle time of neural progenitors in the adult brain has been estimated to be 12.7 h (68), we administered a series of BrdU injections over 10.5 h (82). Similar to what has been reported for E2F1, E2F3$^{-/-}$ mice exhibited 35% fewer neural progenitors lining the lateral ventricles relative to littermate controls (Fig. 1) (E2F3$^{+/+}$, 662 ± 25.5 cells; E2F3$^{-/-}$, 434 ± 14.5 cells; $n = 3$ per genotype). To determine whether the decrease in the number of BrdU-labeled cells was due to an increase in apoptosis, we performed TUNEL and AC-3 staining on E2F3$^{-/-}$ and littermate controls. No difference was observed in the number of dying cells along the lateral ventricles (data not shown). In the embryo, a similar 30% reduction in the number of proliferating cells and no difference in cell death were observed (data not shown). These results indicate that E2F3 is a positive regulator of neural precursor proliferation and point to functional redundancy among E2Fs in regulating neural precursor proliferation.

E2F1 and E2F3 are physiologically relevant Rb-interacting partners in vivo. While both E2F1 and E2F3 function independently in regulating neural precursor proliferation, the context-dependent nature of Rb/E2F interaction led us to ask whether E2F1 and E2F3 are physiologically relevant Rb-interacting partners in the developing nervous system. The nature of Rb/E2F complexes was examined in protein extracts of neural precursors derived from embryonic forebrain through EMSA, and complexes were identified using antibodies specific to E2F family members (Fig. 2A). In wild-type tissue, both free E2F1 and E2F3 as well as bound Rb/E2F activity are observed (Fig. 2A, lane 1), indicating that E2F1 and E2F3 are active in the context of neurodevelopment. Here, we also observe that Rb is bound predominantly to E2F3 and E2F1 as antibodies to E2F1 (Fig. 2A, lane 3) and E2F3 (Fig. 2A, lane 5) displace the Rb band; however, E2F3 appears to be the more predominant Rb binding partner. To confirm that Rb is indeed binding to both E2F1 and E2F3 in neural precursor cells, we performed an immunoprecipitation for Rb from neural precursor cells, followed by DOC treatment to release the associated E2F activity, and subjected this extract to EMSA (31, 41) (Fig. 2B). Immunoprecipitation for Rb followed by DOC treatment gave rise to a pattern of free E2F binding activity (Fig. 2B, lane 3) similar to that of DOC treatment on extracts alone (Fig. 2B, lane 2). Supershifts of the immunoprecipitation extract with E2F1 and E2F3 antibodies demonstrate that both E2F1 (Fig. 2B, lane 4) and E2F3 (Fig. 2B, lane 5) bind to Rb in the nervous system. We next asked what consequences disrupting Rb activity would have on E2F1 and E2F3 activity. We hypothesized that if E2F1 and E2F3 are significant Rb binding partners in wild-type tissue, then the absence of Rb should lead to an increase in their free activity. Indeed, extracts of brain tissue from Rb mutants exhibit a gross deregulation of E2F1 and E2F3 activity relative to the control (Fig. 2A, lane 2). This increase appears specific to free E2F1 and E2F3 activity as supershifts with E2F1 and E2F3 antibodies displace the free E2F1 and E2F3 band (Fig. 2A, lanes 4 and 6). Thus, together these data provide biochemical evidence that E2F1 and E2F3 are physiological Rb-interacting factors in the developing nervous system in vivo.

E2F1 and E2F3 exhibit overlapping patterns of expression in the developing telencephalon in vivo. As E2F1 and E2F3 are both functionally relevant interacting partners in neural precursor cells, we next asked if E2F1 and E2F3 are expressed in the same cell populations in the developing telencephalon. Sections from control tissue at E13.5 and E15.5 were subjected to in situ hybridization for E2F1 and E2F3. At each age, both E2F1 and E2F3 are expressed in similar overlapping patterns (Fig. 3). This region of robust expression encompasses both the dorsal and ventral ventricular/subventricular zones where proliferating and newly committed neurons reside (reviewed in reference 25). In addition, at E15.5, the expression of both E2F1 and E2F3 is observed throughout the ganglionic eminences, the region which gives rise to migrating populations of interneurons, interneurons that ultimately exhibit aberrant migration in Rb deficiency (18). Hence, these data support the hypothesis that E2F1 and E2F3 could each be functional targets in both Rb-mediated proliferation and migration in the developing telencephalon.

Both E2F1 and E2F3 are functional targets in Rb-mediated neural precursor proliferation. Our previous studies have demonstrated that Rb deficiency in the telencephalon leads to ectopic proliferation of neural precursor cells without the widespread apoptosis observed in germ line Rb deficiency (19). The gross deregulation of E2F1 and E2F3 binding activity in Rb-deficient neural precursors and their overlapping patterns

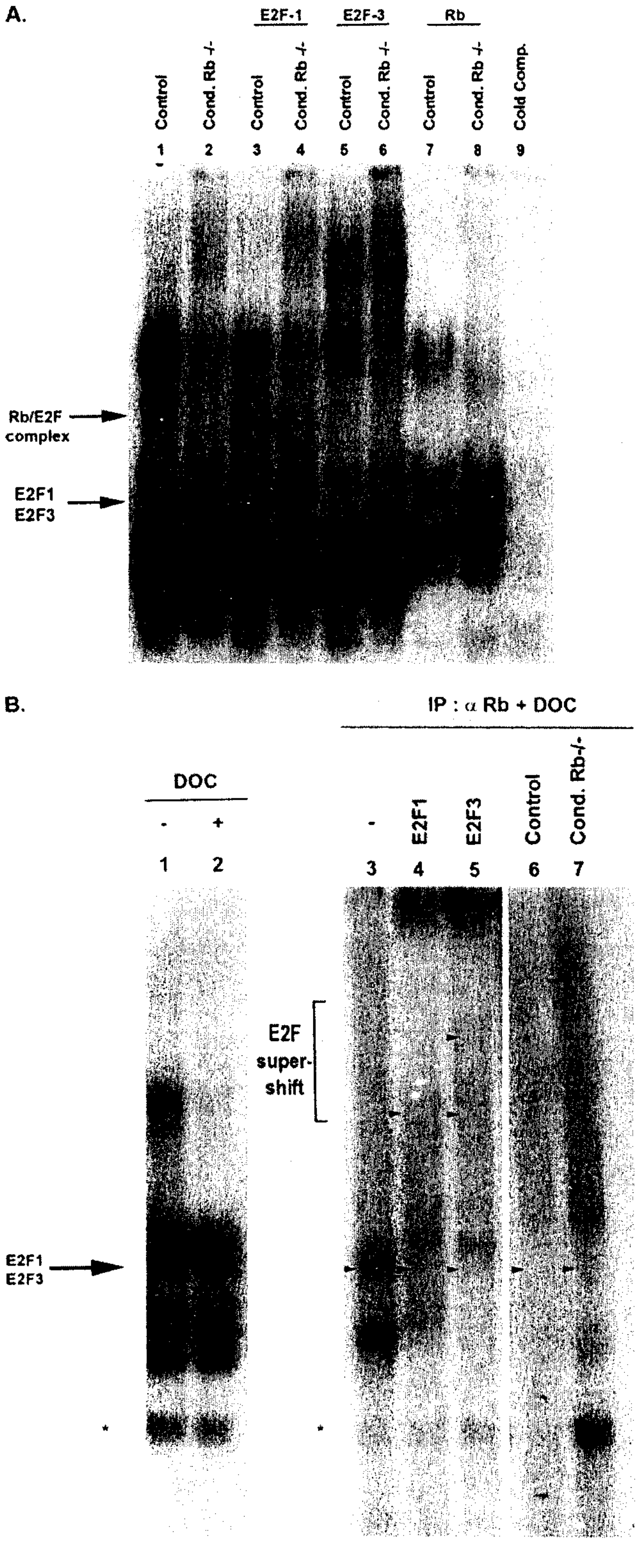


FIG. 2. E2F1 and E2F3 are physiologically relevant Rb-interacting partners in vivo. (A) For EMSA experiments, total protein was extracted from proliferating neural precursors in conditional Rb mutant and controls. Total protein extracts were incubated alone or in the presence of E2F antibodies prior to incubation with double-stranded ^{32}P-labeled E2F consensus probe. Antibodies used for supershift are indicated above the corresponding lane. In control extracts, Rb is bound to both E2F1 and E2F3 as antibodies to both E2F1 and E2F3 displace the Rb band (lanes 3 and 5). In the absence of Rb, an obvious increase in free E2F1 and E2F3 binding activity is noted compared to control (lane 2; E2F1 and E2F3 supershifts are shown in lanes 4 and 6, respectively). (B) For immunoprecipitation (IP)-EMSA experiments, total protein extracts from control proliferating neural precursors were subjected to immunoprecipitation for Rb followed by DOC treatment to release E2F associated with Rb, as described in Materials and Methods. The released material was subjected to EMSA (lane 3) and assayed for E2F1 (lane 4) or E2F3 (lane 5) binding activity. As controls, IP-EMSA was repeated using control mouse serum in place of Rb-immunoprecipitating antibodies (lane 6) or using Rb-deficient neural precursor protein extracts instead of control extracts (lane 7). Finally, a sample of protein extract was subject to EMSA (lane 1) or directly treated with DOC and then assayed for E2F binding activity (lane 2). α, anti; Cond, conditional.

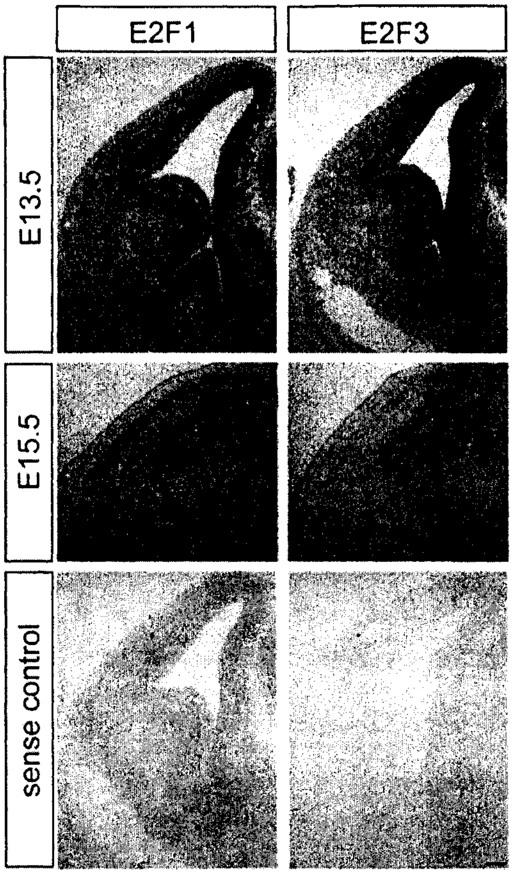


FIG. 3. E2F1 and E2F3 exhibit overlapping patterns of expression in the developing telencephalon in vivo. Control E13.5 and E15.5 sections were subjected to in situ hybridization for E2F1 and E2F3 using antisense riboprobes or sense riboprobes as a control. At both time points, both E2F1 and E2F3 are expressed in overlapping patterns in the developing telencephalon. At E13.5 expression of both E2F1 and E2F3 is largely confined to the developing ventricular and subventricular zones comprised of proliferating and postmitotic cells lining the lateral ventricles. At E15.5 expression is highest within the ventricular and subventricular zones, but for both E2F1 and E2F3 expression is also observed similarly throughout the ganglionic eminences ([ge] $n = 4$ embryos for each E2F). Note the absence of signal in the sense control for each probe. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Bar, 200 μm.

of expression in proliferating neural precursor cells in vivo suggest that these E2Fs could be targets in Rb-mediated neural precursor proliferation. To ask if the proliferation defect observed in the absence of Rb could be attributed to deregulated E2F1 or E2F3 activity, we generated mice with (i) compound null mutations for Rb and E2F3 in the telencephalon (Rb E2F3 DKO) and (ii) an absence of Rb in the telencephalon and whole-embryo E2F1 deficiency (Rb E2F1 DKO). Pregnant females were subjected to a BrdU injection 2 h prior to sacrifice at E15.5, and embryonic sections were subjected to BrdU immunohistochemistry. In contrast to the conditional Rb mutant where proliferating cells are observed in the ventricular zone and postmitotic regions, in both Rb E2F1 DKO and Rb E2F3 DKO sections, BrdU-labeled cells were largely confined to the ventricular and subventricular zones with a minor proportion observed in the intermediate zone (Fig. 4A). Quantification of BrdU-labeled cells confirms that the Rb E2F1 DKO and Rb E2F3 DKO mutations lead to a rescue of ectopically proliferating cells caused by Rb deficiency alone (Fig. 4B graph ii). Similar observations were made in sections labeled with the M phase marker, PH3 (Fig. 4A). Whereas PH3-labeled cells are observed in the cortical plate, intermediate zone, and subventricular zone in the Rb mutant, mitotically active cells are largely observed in the ventricular and subventricular zones of Rb E2F1 DKO and Rb E2F3 DKO cells, indicative of a rescue of the failed cell cycle exit in committed neurons. To determine whether the rescue of cell cycle exit was due to an increase in apoptosis specific to the population of ectopically positioned proliferating cells, we performed a TUNEL assay and AC-3 staining on Rb E2F1 DKO and Rb E2F3 DKO tissues (Fig. 4A). No increase in cell death was observed in Rb E2F1 DKO or Rb E2F3 DKO cells relative to the Rb mutant or control (Fig. 4A; data not shown). Together, these data demonstrate that the absence of either E2F1 or E2F3 in Rb deficiency leads to a rescue of the ectopic proliferation. These findings indicate that E2F1 and E2F3 are each functionally relevant targets in Rb-mediated regulation of neural precursor proliferation and cell cycle exit.

Rb-mediated regulation of radial migration and laminar patterning occurs through interactions with E2F1 and E2F3. We have recently reported that the loss of Rb leads to defective radial migration and laminar patterning in the developing cortex (18). Specifically, in the conditional Rb mutant we observe the absence of a clear cortical plate-intermediate zone boundary in histological sections and expanded expression of cortical plate markers into the intermediate zone. To determine whether radial migration and laminar patterning are mediated through the Rb/E2F cell cycle regulatory pathway, we therefore asked whether compound Rb E2F1 or Rb E2F3 deficiency is sufficient to correct the laminar patterning defects observed in the Rb mutant. Sections of Rb E2F1 DKO or Rb E2F3 DKO tissues at E15.5 were stained with cresyl violet. In control tissue, a clear boundary exists between the packed cells of the developing cortical plate and the underlying intermediate zone (Fig. 5). Compared to the conditional Rb mutant, where the developing cortical plate appeared to be comprised of loosely packed cells that are intermingled with cells of the intermediate zone, both the Rb E2F1 DKO and Rb E2F3 DKO tissues exhibited a cortical plate with a similar structure of densely packed cells and a clear cortical plate-intermediate zone division as the control (Fig. 5). No defects in laminar patterning were observed in either E2F1 or E2F3 single-deficiency tissue (data not shown). We next asked whether the layer-specific defects we observe in the conditional Rb mutant were also restored. Sections from Rb E2F1 DKO and Rb E2F3 DKO tissues were subjected to in situ hybridization with Tbr1, a layer-specific marker demarcating the preplate and layer 6 from the intermediate zone (6, 29), which exhibited vivid defects in the conditional Rb mutant (18). Consistent with the restoration of gross overall morphological structure, sections from Rb E2F1 DKO and Rb E2F3 DKO tissues exhibited Tbr1 expression that was largely confined to the developing cortical plate and exhibited a clear division between the cortical plate and intermediate zone, similar to the division observed in control sections (Fig. 5). This Tbr1 expression pattern in Rb

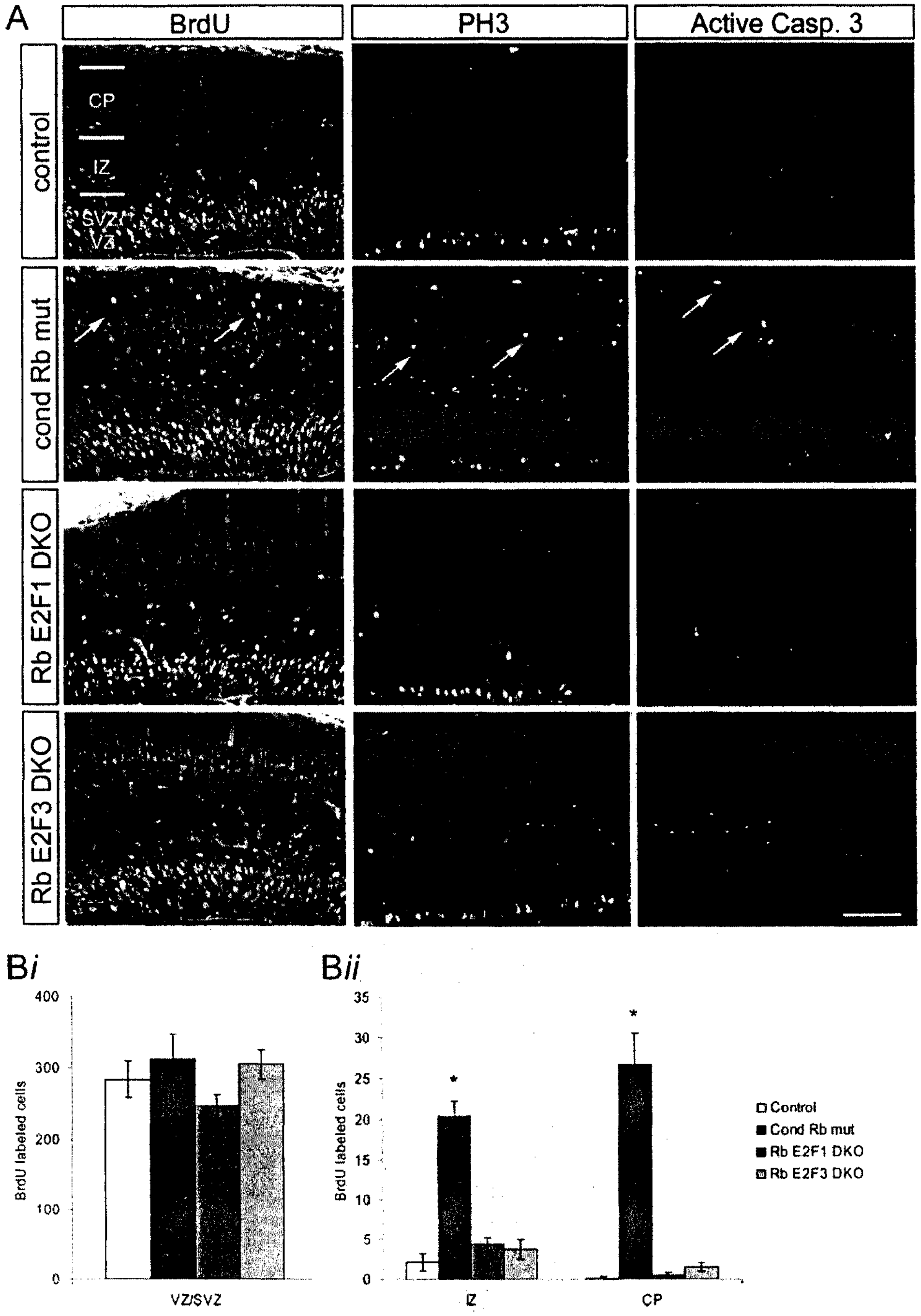


FIG. 4. Both E2F1 and E2F3 are functional targets in Rb-mediated neural precursor proliferation. (A) Sections from E15.5 conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO tissues and their corresponding controls were labeled with an antibody to BrdU to label cells in S phase, PH3 to label cells in M phase, or AC-3 to label dying cells. While conditional Rb mutants exhibit BrdU- and PH3-labeled cells in the ventricular zone/subventricular zone (VZ/SVZ), intermediate zone (IZ), and cortical plate (CP), both Rb E2F1 DKO and Rb E2F3 DKO sections exhibit BrdU and PH3 labeling confined to the VZ/SVZ. No difference was observed in AC-3 labeling between conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls. (B) BrdU-labeled cells were quantified along a 650-μm region of dorsal cortex and classified according to zone, from four matched sections per embryo. The number of BrdU-labeled cells counted was expressed as the mean ± standard error of the mean. Whereas no difference in the number of labeled cells was observed between conditional (cond) Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls in the VZ/SVZ (graph i), significantly fewer BrdU-labeled cells were observed in the IZ and CP of Rb E2F1 DKO and Rb E2F3 DKO sections relative to conditional Rb mutants but not different relative to their respective controls (graph ii). Significance was determined using a single-factor analysis of variance with a Tukey posthoc test. *, $P < 0.05$, $n = 4$ all genotypes. Bar, 100 μm.

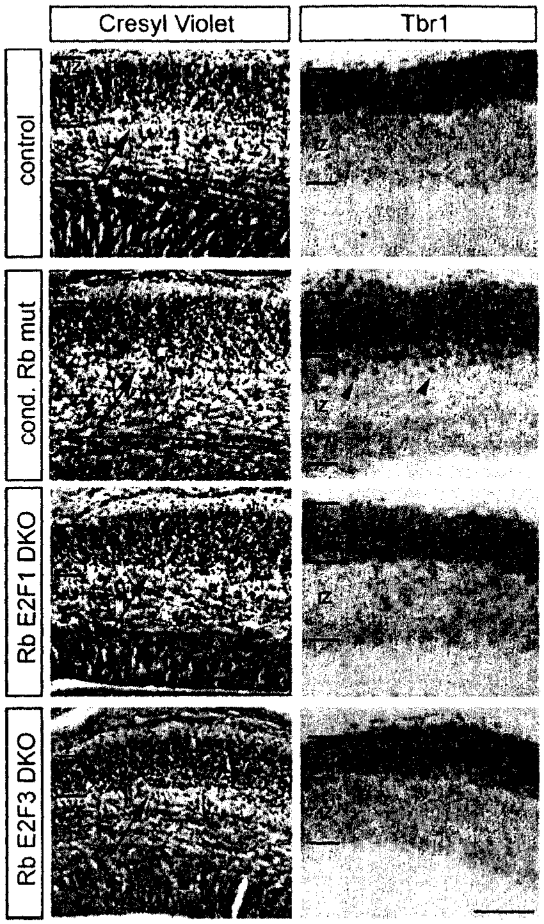


FIG. 5. Rb-mediated radial migration and laminar patterning defects occur through interactions with E2F1 and E2F3. E15.5 sections of conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO tissues and their corresponding controls were stained with cresyl violet and subjected to in situ hybridization for Tbr1. In cresyl violet-stained sections, conditional Rb mutants exhibit the absence of a clear boundary between cortical plate and intermediate zone compared to control (arrows). This defect appears corrected in both Rb E2F1 DKO and Rb E2F3 DKO cells. Similarly, whereas Tbr1 expression is expanded beyond the cortical plate and into the intermediate zone in the conditional Rb mutant (arrows), both Rb E2F1 DKO and Rb E2F3 DKO tissues exhibit Tbr1 expression which is confined to the cortical plate, similar to that observed in the control (n = 3 per genotype). MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; and VZ, ventricular zone. Bar, 100 μm.

E2F1 DKO and Rb E2F3 DKO tissues is in contrast to the conditional Rb mutant, where Tbr1 expression is expanded beyond the confines of the cortical plate into the intermediate zone (Fig. 5). These data demonstrate that the compound absence of either Rb E2F1 or Rb E2F3 is sufficient to restore cortical structure and laminar patterning. Furthermore, as both the Rb E2F1 DKO and Rb E2F3 DKO mutants are capable of rescuing both the cell cycle and laminar patterning defects, these data are consistent with the interpretation that Rb-mediated radial migration and cortical structure occur as a result of defects in cell cycle regulation.

The Rb-mediated requirement for survival of a subset of neurons is partially mediated through the Rb/E2F pathway. While conditional Rb mutants have shown that Rb is not required for widespread neuronal survival, we have recently demonstrated that Rb is required for survival of neurons within the marginal zone of the developing cortex (18, 19, 52). As E2F1, in particular, is a well-known mediator of survival (reviewed in reference 60), we asked to what extent marginal zone neuronal survival could be mediated through Rb interactions with E2F1 or E2F3. Total cells within the marginal zone were quantified from histological sections stained with cresyl violet of conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO tissues. Both Rb E2F1 DKO and Rb E2F3 DKO sections exhibited an increased number of marginal zone cells relative to Rb mutants and no difference relative to the control (Fig. 6A). To determine if this increase was the result of increased survival, we examined cell death by quantifying AC-3-labeled cells within the marginal zone. Consistent with the increase in marginal zone cell numbers, both Rb E2F1 DKO and Rb E2F3 DKO tissues exhibited a decrease in the number of AC-3-labeled cells relative to conditional Rb mutants and no difference relative to the control (Fig. 6B). These data suggest that E2F1 and E2F3 are both capable of mediating the Rb requirement for neuron survival within the marginal zone.

The marginal zone is a complex layer comprised of a heterogeneous population of cells including Cajal-Retzius (CR) neurons (59, 64). We have previously demonstrated that the conditional Rb mutants exhibit a specific loss of CR neurons by cell death which contributes to the overall reduction of cells within the marginal zone (18). Thus, to determine if Rb-mediated neuronal survival is also mediated through Rb interactions with E2F1 and E2F3, we examined CR neurons within the marginal zone in Rb E2F1 DKO and Rb E2F3 DKO tissues. Sections were subjected to immunohistochemistry for reelin, a CR neuron-specific protein (reviewed in reference 64). Whereas both Rb E2F1 DKO and Rb E2F3 DKO sections exhibited increased numbers of reelin-positive cells relative to conditional Rb mutants, fewer reelin-positive cells were observed relative to the control (Fig. 6C). These data indicate that E2F1 and E2F3 only partially mediate the Rb requirement for CR neuron survival. As CR neurons themselves represent a heterogeneous population of cells (4), these data support the hypothesis that the Rb/E2F pathway mediates survival of a subtype of CR neurons.

E2F3 specifically mediates the aberrant tangential migration of interneurons in Rb mutants. Interneurons are key regulators of neuronal function that act by modulating the activity of major excitatory neural circuits (reviewed in reference 62). Interneuron dysfunction and/or aberrant migration of interneurons during development has been implicated in a wide range of neurological disorders including autism, epilepsy, schizophrenia, and bipolar disorder (3; reviewed in reference 2). In a recent study, we demonstrated that interneurons arising from the ventral telencephalon exhibit aberrant tangential migration to the dorsal cortex in conditional Rb deficiency. Specifically, calbindin-labeled cells, a marker of GABAergic interneurons, are absent in Rb mutants along the marginal zone migratory route that is taken by these cells (18). As many of the neurodevelopmental defects described in conditional Rb mutants appear to be mediated through both E2F1 and E2F3 and as both are expressed in the ventral ganglionic eminences, where interneurons originate, we questioned whether

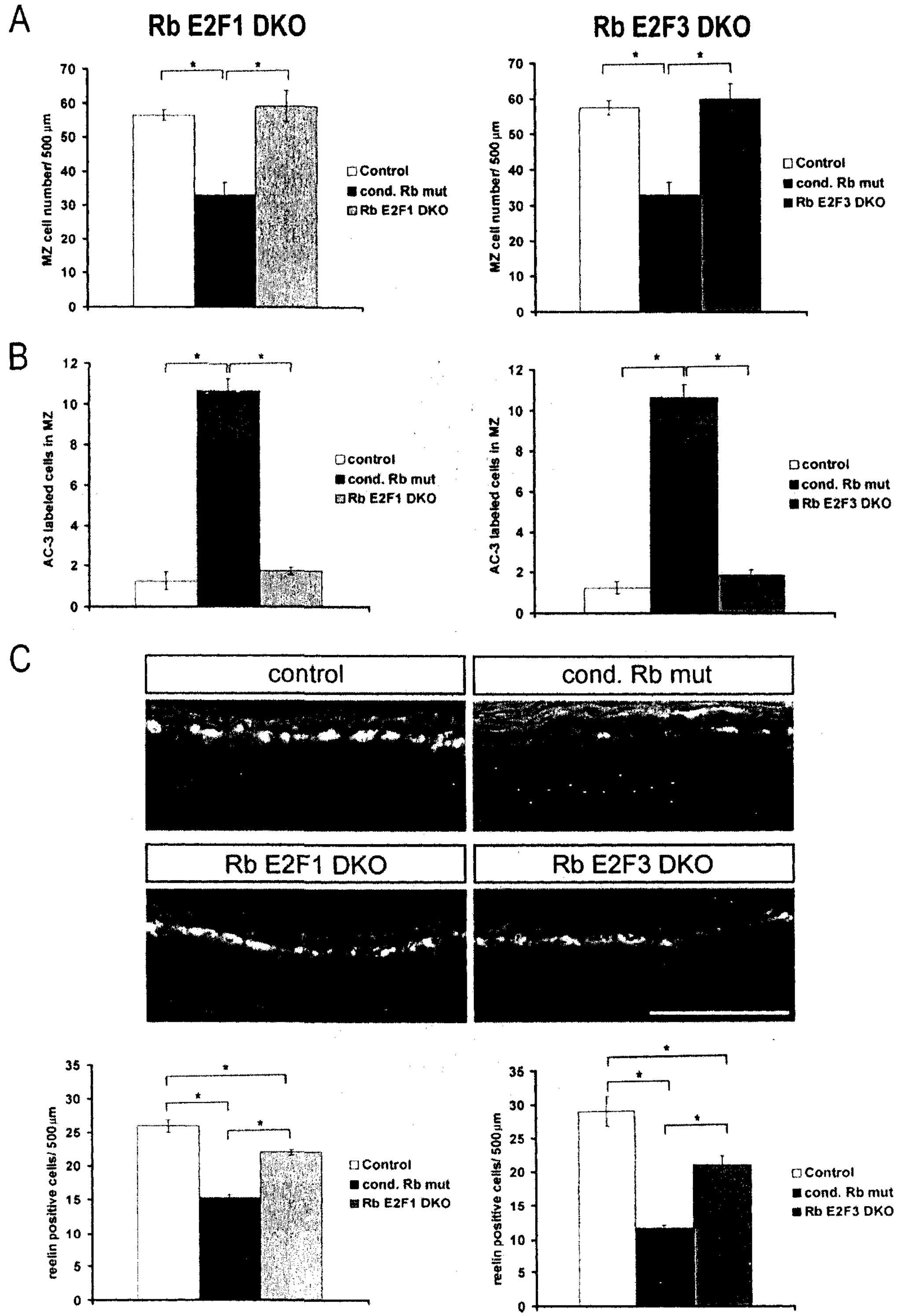


FIG. 6. The Rb-mediated requirement for survival of CR neurons is only partially mediated through the Rb/E2F pathway. (A) Marginal zone cells in cresyl violet-stained sections of E15.5 conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO tissues and their corresponding controls were quantified along a 500-µm region of dorsal cortex from a minimum of three matched sections per embryo and expressed as mean ± standard error of the mean (*n* = 4 embryos per genotype). Whereas conditional Rb mutants exhibit decreased numbers of cells in the marginal zone, both Rb E2F1 DKO and Rb E2F3 DKO sections exhibit an increased number of cells in the marginal zone, similar to that observed in their respective controls. (B) AC-3-labeled cells of conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO tissues and their corresponding controls were quantified within the marginal zone from the cingulate cortex to the dorsal-ventral boundary. Both hemispheres were quantified, and counts are expressed as the mean of the two hemispheres from four matched sections per embryo. Bars represent mean ± standard error of the mean (*n* = 8 per genotype). Whereas conditional Rb mutants exhibit increased numbers of AC-3-positive cells in the marginal zone, both Rb E2F1 DKO and

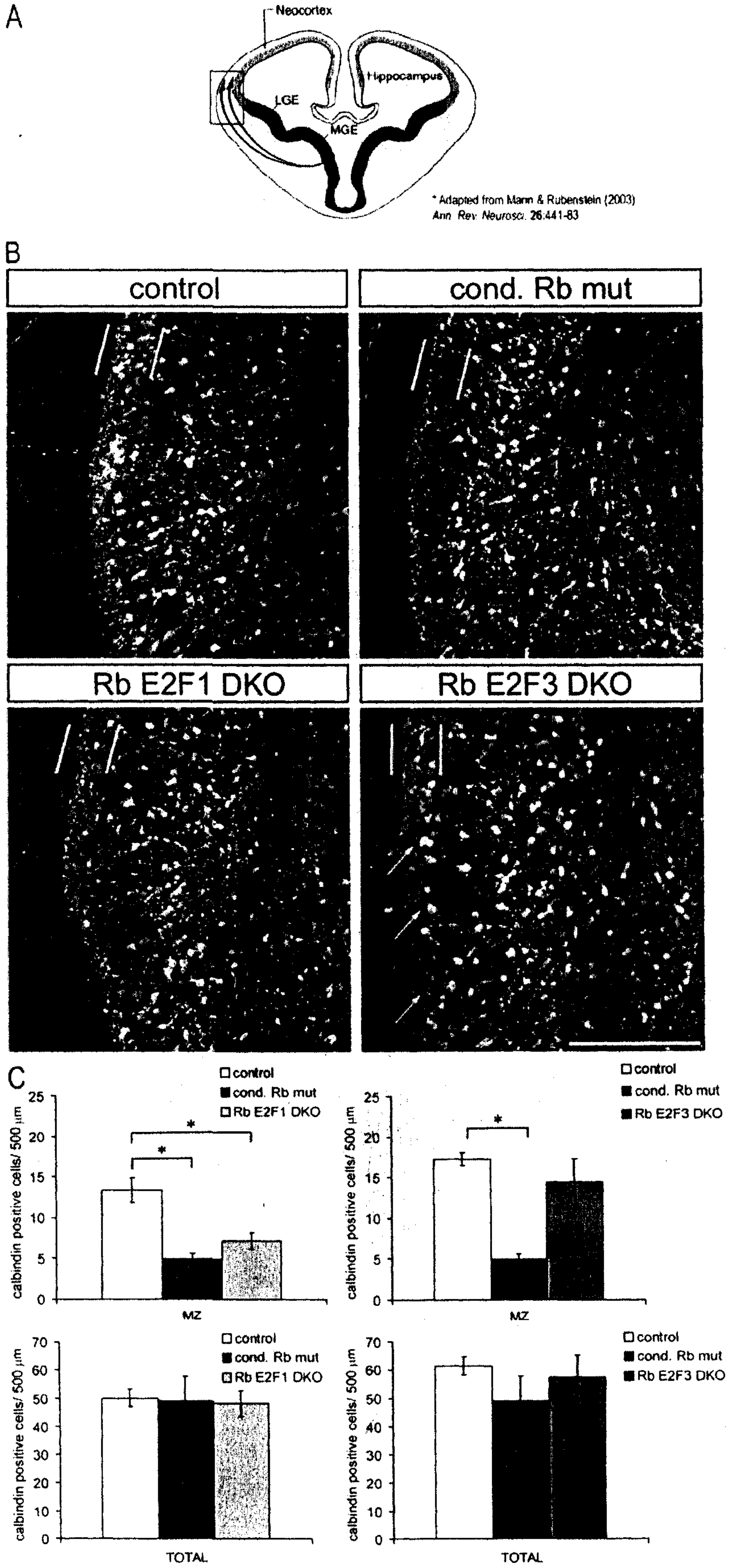
the E2F pathway could also be mediating migration. To assess the degree to which E2F1 and E2F3 could contribute to the tangential migration defect in conditional Rb mutants, we examined the calbindin cell population along its migratory route in Rb E2F1 DKO and Rb E2F3 DKO tissues (Fig. 7A). At E15.5, calbindin-labeled cells are reduced or absent along the marginal zone migratory route in Rb E2F1 DKO sections, similar to that observed in the conditional Rb mutant (Fig. 7B). By contrast, an abundance of calbindin-labeled cells is observed in Rb E2F3 DKO sections along the marginal zone migratory route at the dorsal ventral boundary (Fig. 7B). Quantification of the number of calbindin-positive cells specifically within the marginal zone indicates that Rb E2F1 DKO sections exhibited significantly fewer calbindin-labeled cells within the marginal zone, similar to that observed in the conditional Rb mutant, while Rb E2F3 DKO sections exhibited no difference in number of calbindin-labeled cells relative to the control (Fig. 7C). Further, no difference in the distribution of calbindin-labeled cells was observed in single E2F3 deficiency at E15.5 (data not shown).

While these results are suggestive of a specific role for Rb acting through E2F3 in mediating neuronal migration, to rule out the possibility that cell death was involved, we examined both the total number of calbindin-positive cells within the migrating region and the level of apoptosis within the ventral telencephalon in Rb E2F1 DKO and Rb E2F3 DKO tissues. As we have previously observed (18), quantification of total calbindin-labeled cells revealed no significant differences between the control and conditional Rb mutant. Additionally, we observed no difference between Rb E2F1 DKO or Rb E2F3 DKO embryo and the control or conditional Rb mutants in the total number of calbindin-labeled cells, suggestive of a population of similar size among all groups. We also quantified cell death as measured by AC-3 labeling in the ventral telencephalon including the ventricular zone, where interneurons originate; in the marginal zone and cortical plate, along the route of migration; and points in between (Fig. 8A). While a low level of cell death was observed overall, consistent with our previous results (19), we observed a small but significant increase in the overall level of cell death in the conditional Rb mutant relative to the control (Fig. 8B). Quantification of cell death within the same regions of the ventral telencephalon in Rb E2F1 DKO and RbE2F3 DKO tissues revealed similar levels of cell death for each genotype, which was observed at a level between that of the control and the conditional Rb mutant (Fig. 8B). Together, these results support our interpretation that the specific rescue of calbindin-labeled cells within the marginal zone of Rb E2F3 DKO tissues represents a rescue of the aberrant migration of calbindin-labeled cells in the conditional Rb mutant and is not the result of altered cell death or other defects in the calbindin cell population. Thus, these results reveal a unique function for E2F3 as the Rb target that mediates migration, a function distinct from the role of E2F1. Furthermore, these findings indicate that neuronal migration is mediated via an E2F-dependent mechanism and, hence, point toward novel targets specific for E2F3-mediated transcription.

Rb/E2F3 mediates neuronal migration in a manner beyond cell cycle regulation. Since Rb/E2F interaction is best characterized for its role in cell cycle regulation, our observation that Rb interaction with E2F3 is capable of mediating interneuron migration led us to question whether Rb-mediated migration could be the result of defects in cell cycle control. To address this issue, we performed a thorough analysis by confocal microscopy of the location of proliferating and calbindin-labeled cells in conditional Rb mutants at two developmental time points: at E15.5 when defective migration is observed and at an earlier developmental time point, E13.5. We hypothesized that if the aberrant migration of calbindin interneurons is the result of defects in cell cycle control, then a population of calbindin-labeled cells should be double labeled with BrdU after a standard 2-h pulse. In E15.5 control embryos at low magnification, proliferating cells are seen largely confined to the dorsal and ventral ventricular zones (Fig. 9A, top). In the conditional Rb mutants, ectopically proliferating cells appear largely confined to the postmitotic region of the dorsal cortex (Fig. 9B, 1′). The dorsal cortex region corresponds to the region where aberrant laminar patterning and radial migration are perturbed, thus further supporting the hypothesis that Rb-mediated regulation of radial migration and cortical structure occurs as a result of defects in cell cycle signaling. By contrast, migrating calbindin-labeled cells are observed in the ventral telencephalon, a distinct neuroanatomical region, beyond the regions of normal and ectopic proliferation (Fig. 9A, rows 2 and 3, and B, rows 2′ and 3′). Using confocal microscopy we examined three distinct regions to see if calbindin-labeled cells were ectopically proliferating: the dorsal cortex (Fig. 9A, row 1, and B, row 1′), where ectopically proliferating cells are observed in conditional Rb mutants; the migratory route within the ventral telencephalon (Fig. 9A, row 2, and B, row 2′), where calbindin labeled cells are aberrantly localized within the conditional Rb mutants; and the ventral ventricular zone within ganglionic eminences (Fig. 9A, row 3, and B, row 3′), where calbindin-labeled cells originate. Close inspection from at least three sections from multiple embryos did not reveal BrdU and calbindin double-labeled cells in either control or conditional Rb mutants in any of the three regions examined.

To determine if calbindin-labeled cells that are migrating at E15.5 were ectopically proliferating at an earlier time point, we performed a similar confocal microscopy analysis of BrdU-calbindin double labeling at E13.5 (Fig. 9C and D). At this

Rb E2F3 DKO samples exhibit a decreased number of AC-3-positive cells in the marginal zone, similar to that observed in their respective controls. (C) A noticeable increase in reelin-labeled cells was observed in Rb E2F1 DKO and Rb E2F3 DKO tissues relative to the conditional Rb mutant. Reelin-labeled cells were quantified along a 500-μm region of dorsal cortex and temporal cortex and expressed as the mean from a minimum of four matched sections per embryo. Whereas conditional Rb mutant tissues exhibit decreased numbers of reelin-labeled cells in the marginal zone, both Rb E2F1 DKO and Rb E2F3 DKO sections exhibit an increased number of cells in the marginal zone relative to the conditional Rb mutant yet still significantly less than that observed in their respective controls. Bars represent mean ± standard errors of the mean ($n = 3$ for the control and conditional Rb mutant; $n = 4$ for Rb E2F1 DKO; and $n = 5$ for Rb E2F3 DKO). In all cases significance was determined using a single-factor analysis of variance with a Tukey posthoc test. *, $P < 0.05$. MZ, marginal zone; cond, conditional. Bar, 100 μm.


A
Neocortex
Hippocampus
LGE
MGE
* Adapted from Marin & Rubenstein (2003) Ann Rev Neurosci. 26:441-83
B
control
cond. Rb mut
Rb E2F1 DKO
Rb E2F3 DKO
C
☐ control
■ cond. Rb mut
▨ Rb E2F1 DKO
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25
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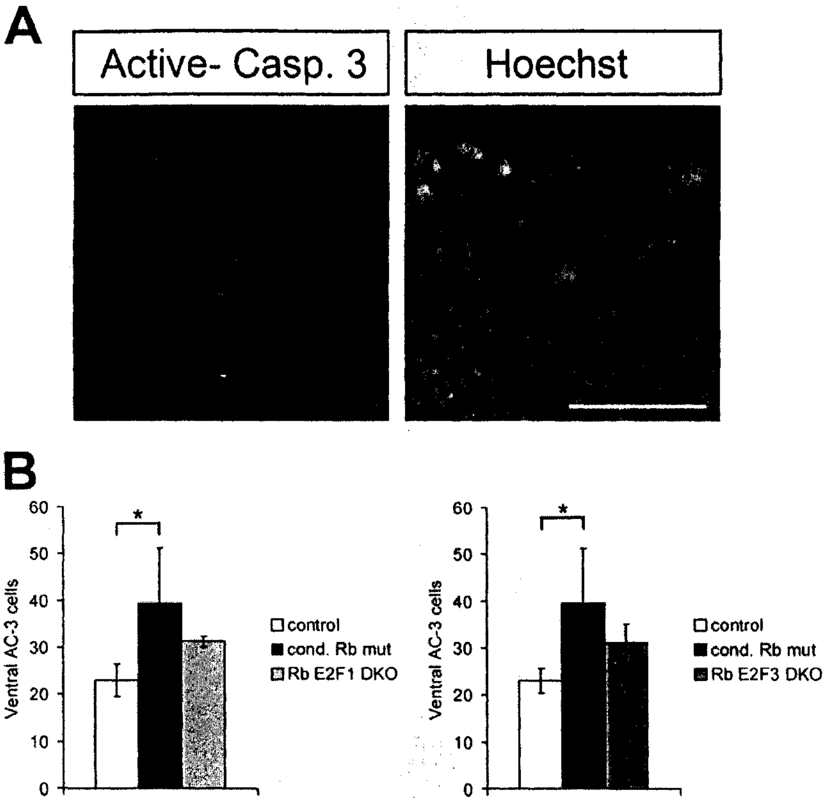
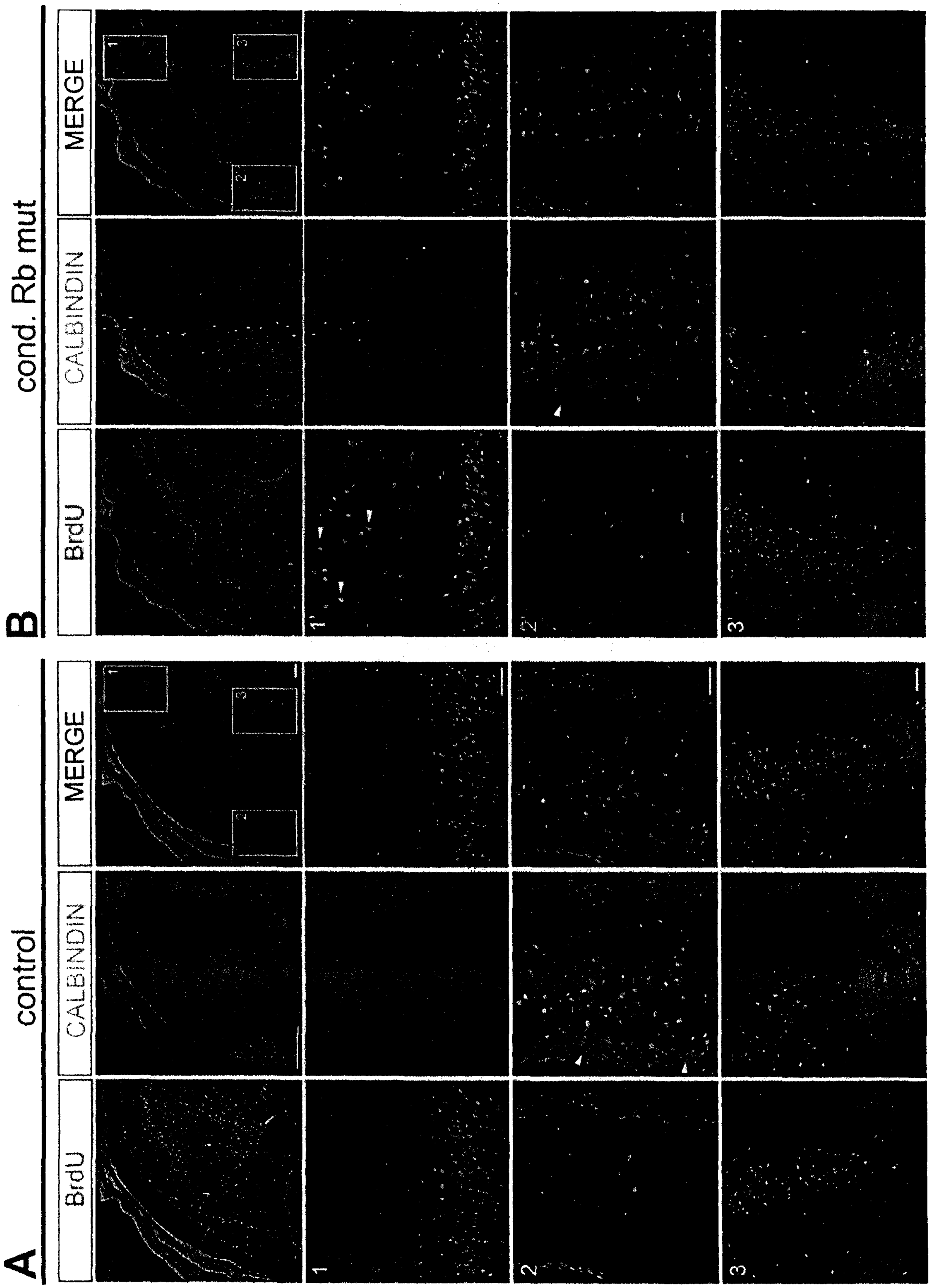


FIG. 8. Rb E2F1 DKO and Rb E2F3 DKO tissues exhibit similar levels of cell death in the ventral telencephalon. E15.5 sections of conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO and their corresponding controls were stained with AC-3 and Hoescht to examine cell death in the ventral telencephalon. Labeled cells were counted along the ventricular zone, where interneurons originate; the marginal zone and cortical plate along the route of migration; and points in between from four matched sections per embryo. A similar level of cell death is observed between Rb E2F1 DKO and Rb E2F3 DKO cells. Each exhibited a level of cell death between that of control and conditional (cond) Rb mutant. Bars represent the mean of total number of AC-3-labeled cells counted ± standard deviation (n = 4 embryos per genotype). Significance was determined using a single-factor analysis of variance with a Tukey posthoc test. *, $P < 0.05$. Bar, 50 μm.

time point, we do observe ectopic BrdU-labeled cells in the ventral telencephalon of conditional Rb mutants along the route of migration of calbindin-labeled interneurons (Fig. 9D, row 2′). Quantification of ectopically proliferating cells demonstrates a significant increase in ventral ectopic proliferation at this age (Fig. 10); however, while several distinct neuronal subtypes exist in this region (reviewed in reference 89), none of these ectopically dividing progenitors was colabeled with calbindin. Specifically, we examined distinct regions at E13.5 to see if calbindin-labeled cells were ectopically proliferating, focusing on the ventral regions: the future route of migration of aberrantly migrating cells within the ventral telencephalon (Fig. 9C, row 2, and D, row 2′); and the ventral ventricular zone within medial ganglionic eminence (Fig. 9C, row 1, and D, row 1′), where calbindin-labeled cells originate. Similar to what we observed at E15.5, at E13.5, using confocal microscopy we did not observe BrdU and calbindin double-labeled cells in either control or conditional Rb mutants at either

FIG. 7. E2F3 specifically mediates the aberrant tangential migration of interneurons in Rb mutants. (A) Schematic diagram of superficial marginal zone and deeper intermediate zone routes taken by migrating calbindin-labeled interneurons originating from the medial ganglionic eminence (modified from reference 56). Box indicates region of magnification in panel B. Calbindin labeling was examined in conditional Rb mutant, Rb E2F1 DKO, and Rb E2F3 DKO tissues and their respective controls along the marginal and intermediate zone migratory routes at E15.5. Calbindin-positive cells are reduced or absent along the marginal zone migratory route in Rb E2F1 DKO and are present at a level similar to that observed in the conditional Rb mutant. By contrast, an abundance of calbindin-labeled cells is observed in Rb E2F3 DKO tissue along the marginal zone migratory route at the dorsal ventral boundary (arrows). (C) Calbindin-positive cells were quantified within the marginal zone (indicated by vertical bars) or along the same length within the complete migratory route as demarcated by the marginal zone and external capsule as lateral and medial boundaries (boxed area in panel A) for the total region. The total region was comprised of the marginal zone, as well as the intermediate zone, and cortical plate and labeled cells were counted on each hemisphere from four matched sections per embryo and expressed as cells per 500-μm length. Quantification confirms that calbindin cells positioned in the marginal zone are restored in Rb E2F3 DKO but not in Rb E2F1 DKO cells, yet the total number of calbindin-labeled cells is the same across all groups. Bars represent mean ± standard error of the mean (n = 4 embryos per genotype). Significance was determined using a single-factor analysis of variance with a Tukey posthoc test. *, $P < 0.05$. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; cond, conditional. Bar, 100 μm.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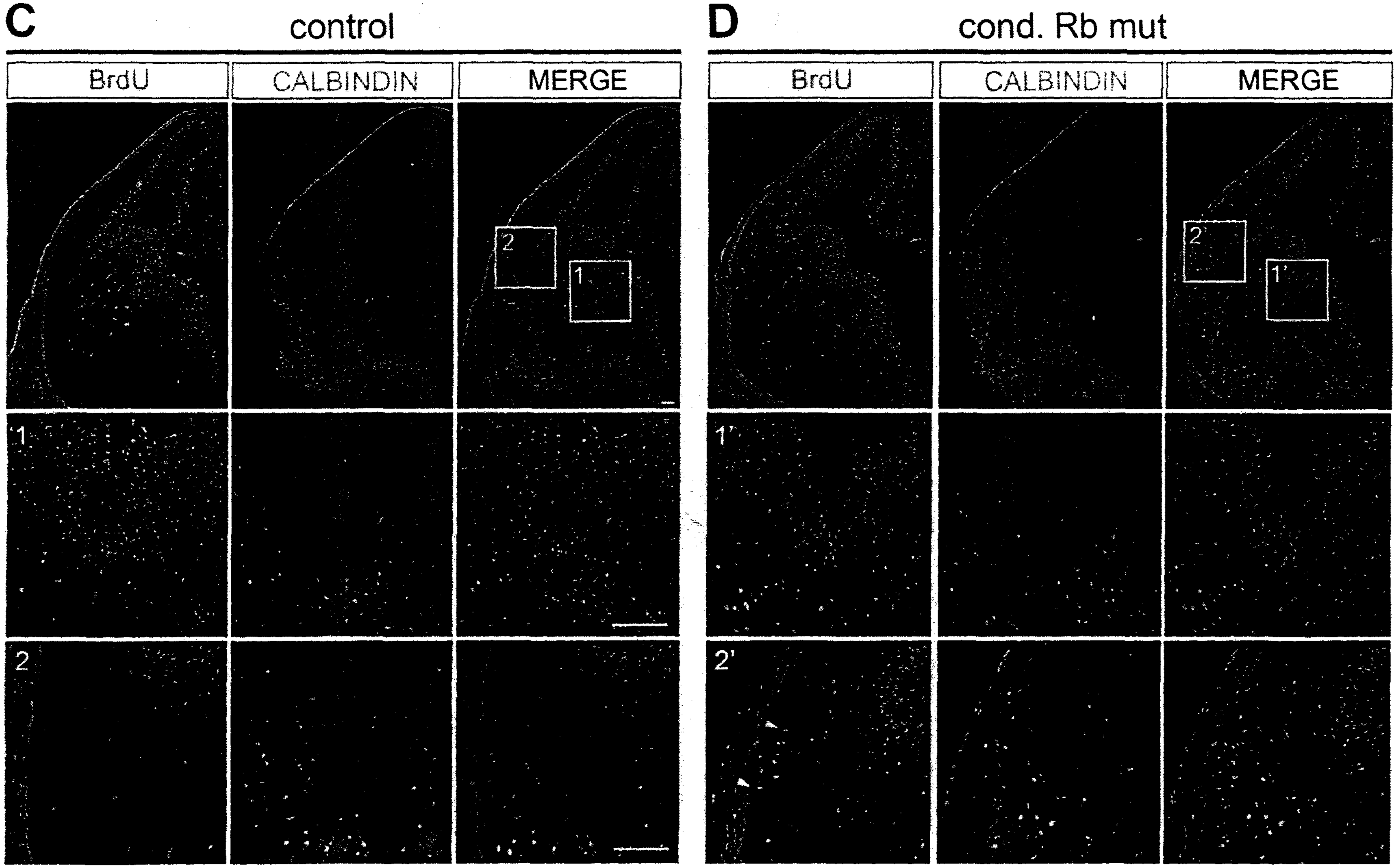

FIG. 9. Rb/E2F3-mediated tangential migration is not the result of cell cycle deregulation. Sections from conditional Rb mutant and control were double labeled with BrdU (red) and calbindin (green) at E15.5 (A and B) and E13.5 (C and D). At E15.5 at low magnification, BrdU labeling is largely observed surrounding the ventricle, whereas calbindin-labeled cells are localized largely in the ventral telencephalon. At higher magnification, ectopic proliferation in conditional (cond) Rb mutant is observed largely confined to the dorsal cortex (B1′), while no difference in the low level of BrdU labeling between the conditional Rb mutant and control is observed in the ventral telencephalon (B2′ and B3′ versus A2 and A3, respectively). Instead, in this region where BrdU labeling is not detected, an absence of calbindin-labeled cells is observed in the marginal zone, and the absence of calbindin-labeled cells along the marginal zone migratory route in conditional Rb mutants is observed (B2′) (arrowheads). By confocal microscopy no BrdU calbindin-double-labeled cells are observed in the telencephalon at any of the three regions examined. At E13.5 ectopic proliferation is prevalent in conditional Rb mutants in the dorsal and ventral telencephalon (D, top panel and 2′); however, by confocal microscopy in either control or the conditional Rb mutant, we do not detect BrdU-calbindin double labeling at either the ganglionic eminence, where calbindin cells originate (C1 and D1′) or at the future ventro-lateral migratory route (C2 and D2′). Double-labeled cells, however, were occasionally observed within the blood vessel-rich pial layer outside of the telencephalon and are likely blood cells. Bar, 100 μm.

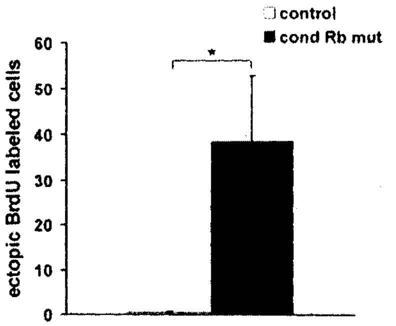

FIG. 10. Quantification of ectopic proliferation within the ventral telencephalon at E13.5, comprising the region of migration in the control and conditional (cond) Rb mutant. BrdU cells within the region were counted in four matched sections per embryo. Bars represent means of the average number of BrdU-labeled cells within a 2,000-μm length along the ventrolateral boundary comprising the region of migration between the outermost edge of the marginal zone and the external capsule as lateral and medial boundaries ± standard deviation (n = 3 embryos per genotype). Significance was determined using a two-tailed t test. *, $P < 0.05$.

region examined. While we cannot unequivocally rule out that aberrantly migrating cells were once ectopically proliferating, since we did not observe calbindin colocalized with BrdU in any region at either time point examined, these data suggest that calbindin-labeled cells have successfully exited the cell cycle in conditional Rb mutants. Hence, our data support the hypothesis that Rb mediates tangential migration through E2F3 in a manner beyond cell cycle regulation.

Rb mediates the expression of genes involved in regulating neuronal migration. Our data demonstrating that Rb mediates migration through E2F3 in vivo represent physiological evidence in support of the hypothesis that Rb/E2F could regulate the transcription of novel genes unrelated to cell cycle regulation. In an effort to identify candidate genes, we performed microarray analyses on neural precursor cells from control and conditional Rb mutants derived from the medial ganglionic eminences, the region which gives rise to migrating populations of interneurons which ultimately exhibit aberrant migration in Rb deficiency (18). We hypothesized that if Rb/E2F3 mediates migration of interneurons in a cell-autonomous manner as our previous data demonstrated (18), then the absence of Rb would lead to deregulation of genes required to regulate this process. Through our microarray analysis, we have identified several candidate genes that are deregulated in conditional Rb mutants which have been shown to mediate neuronal migration, including migration of interneurons, such as members of the neogenin/netrin/repulsive guidance molecule (RGM) signaling pathway, the semaphorin/neuropilin signaling pathway, and the Slit/Robo pathway (Table 1). While all candidates remain plausible targets, we have focused our initial attention on neogenin, a cell surface receptor and member of the immunoglobulin superfamily (83) with well-known (72, 87) and hypothesized roles in regulating neuronal migration, including interneuron migration (21). By microarray, neogenin expression was increased threefold, consistent with the role of E2F3 as a transcriptional activator. We next confirmed this observation through in situ hybridization for neogenin in conditional Rb mutants (Fig. 11). At E13.5, a moderate increase in neogenin expression in the conditional Rb mutant was observed throughout the telencephalon including the ganglionic eminences (Fig. 11). At E15.5 this increase in neogenin expression is more pronounced, particularly within the ganglionic eminences, where interneurons originate. Together, these data confirm that absence of Rb leads to deregulation of known genes required for interneuron migration within the population of migrating cells during the time of migration and, in addition, establishes neogenin as a potential target gene in Rb/E2F-mediated interneuron migration.

DISCUSSION

The mammalian nervous system is comprised of a complex array of different cell types and subtypes (reviewed in references 24 and 25). Neurogenesis, the process by which neural precursor cells divide and differentiate to give rise to all the different cell types, occurs in a highly regulated manner (reviewed in references 24 and 25). The cortex, a model of neurogenesis, is comprised of a series of layers that form in an

TABLE 1. Candidate molecules identified in microarray from control and conditional Rb mutant ventral precursor cells[a]

Molecule name	Pathway	Relative change[b]	Migration function	Reference
Neogenin	Netrin/RGM	3-fold increase	Binds netrin and RGM; repels temporal retinal axons through RGM	72 61
Sema3d	Neuropilin/semaphorin	Moderate increase	Guides retinal axon along DV axis in zebrafish; can be repulsive or attractive	47 88
VLDLR	Reelin signaling	Moderate increase	Receptors for reelin; VLDLR and ApoER2 KO mice have reeler-like phenotype with inverted cortical lamina structure	79
ApoE	Reelin signaling	2-fold increase	Out-competes reelin for binding to ApoER2?	NA
CCK		2.5-fold increase	A marker of GABAergic interneurons; CCK also reduces migration of GnRH neurons	50 23
TWIST1	bHLH transcription factor	Moderate decrease	Loss-of-function mutant leads to defect in neural crest cell migration	70
Twist neighbor	bHLH transcription factor	2-fold increase		

[a] VLDLR, very-low-density lipoprotein receptor; CCK, cholecystokinin; bHLH, basic helix-loop-helix; DV, dorsal-ventral; ApoER2, ApoE receptor 2; NA, not applicable.

[b] Increase/decrease in mutant relative to control.

[c] Twist neighbor.

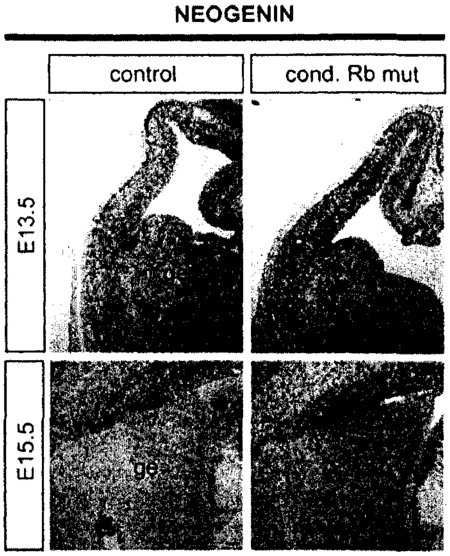

FIG. 11. Neogenin, a microarray-identified gene, exhibits deregulated expression in conditional Rb mutants (cond Rb mut). Control and conditional Rb mutant E13.5 and E15.5 sections were subjected to in situ hybridization for neogenin. At both time points, neogenin expression appears increased in the conditional Rb mutant relative to the control. At E13.5 expression appears increased overall including in the ganglionic eminences (arrowheads). At E15.5 this increase in neogenin expression appears more pronounced, particularly within the ganglionic eminences (ge), where interneurons originate ($n = 4$ embryos for each genotype). Bar, 100 μm.

inside-out manner populated with excitatory projection neurons and inhibitory interneurons (reviewed in reference 25). In both cases, neural precursors divide along the germinal zone lining the ventricles (reviewed in reference 24). Once committed to a neuronal fate, cells exit the cell cycle and leave the germinal zone to migrate toward their final destination with projection neurons migrating radially to form the layers of the cortex and cortical interneurons migrating from the ventral telencephalon along tangential routes into the dorsal cortex (reviewed in references 24, 37, 55, and 56). The fundamental processes of neurogenesis, namely, proliferation differentiation, migration, and maturation, are controlled by the precise coordination of various genetic pathways (reviewed in references 24 and 25). Normal development of the cortex is essential for proper brain function as abnormal development has been hypothesized as an underlying cause in a number of neurological and psychiatric disorders (2, 34–36, 43–45, 71, 76) Numerous studies have demonstrated the pivotal roles that Rb plays in nervous system development, and in addition, much insight has been gained into Rb function itself by studying its role in the nervous system (8, 10, 19, 33, 39, 40, 46, 51–53, 57, 66, 73, 86).

In this study we evaluated the in vivo contributions of E2Fs as regulatory targets for Rb-mediated neurogenesis, and our results herein support a number of conclusions. First, this study establishes that E2Fs are indeed major physiological targets in Rb-mediated neurogenesis, mediating both cell cycle-dependent processes and roles beyond cell cycle regulation. We also demonstrate that functional redundancy exists among E2Fs in regulating cell cycle exit, laminar patterning, and radial migration, as well as cell survival. Finally, our data demonstrating that Rb mediates neuronal migration specifically through E2F3 represent the first physiologically relevant requirement for the Rb/E2F pathway beyond cell cycle regulation in vivo.

E2Fs are physiological targets in Rb-mediated neurogenesis regulating cell cycle-dependent processes and mediating roles beyond cell cycle regulation. Rb is known to interact with numerous proteins, many of which are expressed in quiescent cells or have cell cycle-independent functions. Thus, the search for Rb-interacting proteins in neurogenesis represents a potentially long list (reviewed in reference 67). We hypothesized that members of the cell cycle regulatory E2F family represent functional targets of Rb in neurogenesis in vivo for a number of reasons. First, E2F1 and E2F3 have both been established as Rb targets in regulating neural precursor proliferation as each is capable of rescuing the ectopic proliferation in the CNS in germ line Rb deficiency (75, 81, 93). While these studies support the hypothesis that E2F1 and E2F3 are targets in Rb-mediated neurogenesis, the use of germ line Rb-deficient mice limited the interpretation due to the widespread defects that were the result of non-cell-autonomous requirements for Rb during development (19, 52, 90). To circumvent this issue, we examined the role of E2F1 and E2F3 as targets in Rb-mediated neurogenesis using telencephalon-specific Rb-deficient mice crossed with E2F1-deficient or telencephalon-specific E2F3-deficient mice. It is possible that some of the differences observed between the Rb E2F1 DKO and Rb E2F3 DKO models are due to systemic loss of E2F1 and effects outside the nervous system. The justification for using a telencephalon-specific model of E2F3 deficiency, however, was out of necessity due to the vital role of E2F3 during development (11, 30). As E2F1 does not have such a vital role (92), no analogous tissue-specific model of E2F1 deficiency was available.

Here, our results demonstrate that E2F1 and E2F3 are physiologically relevant Rb targets in neurogenesis in vivo. First, we observed that both are expressed in overlapping patterns in the developing telencephalon. In vitro, we showed that Rb interacts predominantly with E2F1 and E2F3 in extracts of neural tissue. The significance of this interaction is demonstrated in vivo where we not only show that E2F1 and E2F3 are targets of Rb-mediated neural precursor proliferation but also establish that E2Fs play a major role as Rb targets in laminar patterning, radial migration, cell survival, and tangential migration of interneurons. As both Rb E2F1 DKO and Rb E2F3 DKO cells are capable of rescuing the proliferation defect, along with the laminar patterning and radial migration defects observed in telencephalon-specific Rb deficiency, our data are consistent with the interpretation that the radial migration and laminar patterning defects may occur as the result of cell cycle deregulation. Rb-mediated tangential migration, however, appears to be mediated specifically through E2F3. Thus, with these results, we establish roles for E2F1 and E2F3 as the physiological targets in Rb-mediated neurogenesis.

Functional redundancy exists among E2F1 and E2F3 in vivo. Our results demonstrate that functional redundancy exists among E2F1 and E2F3 in the context of neural precursor proliferation, cell cycle exit, and survival in vivo. First, we show that E2F3 alone is a positive regulator of neural precursor proliferation, similar to what has been reported for E2F1 (12). Next, as both Rb E2F1 DKO and Rb E2F3 DKO cells are capable of rescuing the proliferation and survival defects observed in Rb deficiency in the telencephalon, our data support the hypothesis that E2F1 and E2F3 are functionally equivalent targets in Rb-mediated cell cycle exit and survival.

The idea that E2Fs are functionally redundant is still debated within the field. In vitro studies have indicated that, individually, E2F1 and E2F3 are capable of regulating the expression of distinct genes, likely as a result of differences within the marked box domain (5, 26). In the context of Rb interaction, however, it remains unresolved as to whether Rb interaction is equivalent with each E2F (15, 74). In vivo, absence of either E2F1 or E2F3 results in individual and distinct phenotypes, yet in the context of Rb interaction, both have been shown to rescue many of the proliferation, apoptosis, and midgestational survival defects associated with germ line Rb deficiency (81, 93). Specificity, however, has been reported to exist in Rb-mediated phenotypes where a unique function for E2F1 has been reported in mediating apoptosis in the Rb-deficient lens and retina (75). Thus, the function of E2F1 and E2F3 as targets in Rb-mediated neurogenesis should be viewed as context dependent, even within the CNS.

Finally, our observation that E2F1 and E2F3 only partially mediate the Rb requirement for subtype-specific neuronal survival raises a number of questions. First, it is unlikely that the partial rescue in CR neurons in Rb E2F1 DKO or Rb E2F3 DKO cells is related to the E2F3-mediated rescue of Rb-mediated tangential migration. While CR neurons have been hypothesized to have roles in regulating tangential migration of interneurons (65), the decrease in CR neurons observed in conditional Rb-deficient embryos is unlikely to influence migration of interneurons as our previous findings demonstrated that the role for regulating Rb is cell autonomous (18). Rather, as CR neurons themselves are a heterogeneous population (4), these data support the hypothesis that the Rb/E2F pathway mediates survival of only a subtype of CR neurons. Two possible explanations are hypothesized. First E2F1 and E2F3 may mediate survival of nonoverlapping populations of CR cells that together mediate survival of the entire population of CR cells that is absent in the conditional Rb mutant. Alternatively, it is also possible that other non-E2F Rb-interacting factors are contributing to survival. Id2 is a non-E2F Rb-interacting factor that has been shown to mediate many of the neurological defects arising in Rb mutants, including apoptosis (38); thus, it is possible that E2F and Id2 are acting along parallel pathways to regulate CR neuron survival. The latter is a particularly provocative hypothesis as there is little discussion in the literature about a possible E2F-independent role for Rb in cell survival.

Unique physiological requirement for Rb/E2F3 beyond cell cycle regulation exists in mediating neuronal migration in vivo. Here, we demonstrate that Rb-mediated migration of interneurons is indeed mediated through the E2F pathway, specifically, through E2F3. These results support a model for specificity among E2Fs in nervous system development. Further, it is this specificity of E2F function which underlies the hypothesis that E2F3-specific mediated interneuron migration represents a novel physiologically relevant requirement beyond cell cycle regulation for the Rb/E2F pathway in vivo. In support of this hypothesis, we observe that E2F1 and E2F3 are expressed in overlapping patterns within the ganglionic eminences, where interneurons originate, and that both Rb E2F1 DKO and Rb E2F3 DKO cells are capable of rescuing the proliferation defects, yet only Rb E2F3 DKO cells can rescue the migration defect. In addition, aberrantly migrating interneurons do not incorporate BrdU either during (E15.5) or before (E13.5) migration. While we cannot unequivocally rule out that aberrantly migrating cells were once ectopically proliferating, the absence of double-labeled cells suggests that the population of calbindin interneurons has successfully exited the cell cycle in conditional Rb mutants.

Further support for role for E2F3 beyond cell cycle regulation can be inferred from the known function of E2F3 itself. E2F3 is one of the more intriguing E2Fs as the locus expresses two distinct transcripts: full-length E2F3a, whose expression is cell cycle regulated and acts as a transcriptional activator, and E2F3b, which is expressed equivalently in quiescent and proliferating cells and is a specific partner for Rb in quiescent cells (27, 41). While it is possible to hypothesize that Rb/E2F3-mediated neuronal migration could be regulated through E2F3b, complementary to our work, it has been shown that a defect in the differentiation of Rb-deficient cholinergic neurons in the retina is mediated through E2F3a (D. Chen, R. Opaskvy, M. Pacal, N. Tanimoto, P. Wenzel, M. W. Seeliger, G. Legne, and R. Bremner, unpublished data), an effect also shown to be cell cycle independent. Thus, together our data suggest a common mechanism through which Rb exhibits roles beyond cell cycle regulation through E2F3 during nervous system development.

Finally, further support for a novel, in vivo function for the Rb/E2F pathway beyond cell cycle regulation comes from our search for novel E2F-regulated target genes in the context of neuronal migration. E2F-mediated regulation of cell cycle-independent genes is an emerging concept. In vivo, studies examining individual E2F-deficient mice demonstrated a vast array of tissue-specific defects in development and differentiation, suggesting that E2Fs may be regulating non-cell cycle-related genes (reviewed in references 1 and 16). These studies were limited, however, as the individual phenotypes could be the result of E2F acting independently from interactions with Rb. Here, we have performed microarray analysis on neural precursor cells from the medial ganglionic eminences, the region which gives rise to migrating populations of interneurons which ultimately exhibit aberrant migration in Rb deficiency. With this strategy, we identified a number of putative target genes that are deregulated in conditional Rb mutants and that have been shown to mediate neuronal migration, including migration of interneurons. Further, we have validated our microarray results for one candidate gene, neogenin. Neogenin is of particular interest, not only because of its known and hypothesized roles in regulating neuronal migration (21, 72, 87) but also because of its identification through other E2F microarray studies (69). Even more significant, through sensitive subtractive screening assays, neogenin, was recently shown to be a novel, direct target gene of E2F1, with expression induced in a cell cycle-independent manner (32). As E2F1 is capable of inducing E2F3 expression, it is possible that these genes may be also induced by E2F3 (reviewed in reference 78). While previous studies have provided evidence in support of roles for the Rb/E2F pathway in regulating cell cycle-independent functions, evidence for deregulation of such genes in vivo has not been reported. Thus, our data demonstrating that Rb interacts specifically through E2F3 to mediate neuronal migration represent the first physiological demonstration that such an in vivo role for the Rb/E2F pathway beyond cell cycle regulation exists.

In conclusion, our results demonstrate that both functionally redundant and unique roles exist for E2F1 and E2F3 in regulating Rb-mediated neurogenesis. As Rb-mediated migration is mediated specifically through E2F3, our results represent a novel, physiologically relevant requirement for the Rb/E2F pathway beyond cell cycle regulation in vivo, pointing toward novel targets specific for E2F3-mediated transcription in the context of neuronal migration.

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Perspective

Novel Functions for Cell Cycle Genes in Nervous System Development

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ABSTRACT

Many cell cycle genes are known to play important roles in regulating proliferation in the nervous system, however, a growing body of research has proposed that these genes have diverse functions beyond cell cycle regulation. Through the study of new genetic models, cell cycle regulatory genes have been shown to impact on a number of processes during nervous system development including apoptosis, differentiation, and, most recently, neuronal migration. Here we emphasize that the proposed roles for cell cycle genes in neuronal differentiation and migration are not the consequence of deregulated cell cycle, but represent truly novel functions for cell cycle genes.

INTRODUCTION

Neurogenesis is a highly regulated developmental process by which neural precursors divide and differentiate to form the cells that make up the nervous system. The importance of the cell cycle in regulating neurogenesis and cell fate is well appreciated. Classical [^{3}H] thymidine birthdating studies demonstrated that timing of cell cycle exit is a predictor of laminar destination in the developing cortex. These studies established that in the development of the six layered cerebral cortex, early born neurons comprise the deeper layers, while later born neurons comprise the superficial layers[1] (reviewed in ref. 2). Subsequent studies addressed precisely when within the cell cycle commitment to a particular neuronal fate occurs. Using cell transplantation experiments, early born cortical neurons transplanted to hosts during S phase adopted the superficial lamina appropriate for the host cell environment, while neurons transplanted during G_2 or M phases retained the laminar identity of their donor and were found in deeper layers, thus demonstrating that laminar commitment occurs prior to terminal mitosis.[3] In the retina, the timing between cell cycle exit and neuronal differentiation was estimated using bromodeoxyuridine (BrdU) and RA4, an early ganglion cell marker. In this study, BrdU and RA4 colabeled cells were first detected minutes after detection of BrdU labeled cells that had progressed to M-phase, suggesting that commitment to a neuronal fate occurs during the cell cycle.[4] These experiments were the first to demonstrate that timing of cell cycle exit influences neuronal fate and that fate is determined within a precise window during the cell cycle. Since then, great progress has been made in identifying the molecular components of the cell cycle that regulate neurogenesis. Studying mouse models deficient for individual cell cycle genes has revealed that many genes impact on proliferation in the developing nervous system. Moreover, through the study of these models, new roles for cell cycle genes in differentiation and development have emerged suggesting that these genes are capable of regulating diverse functions independent from their role in cell cycle control.

OVERVIEW OF THE CELL CYCLE- THE G_1-S PHASE TRANSITION

Cell division is a complex process that is tightly regulated at multiple levels. For an in depth analysis of the process, readers are referred to a number of excellent reviews.[5,6-11] Here we will present a brief overview of the cell cycle, focusing on the G_1-S phase transition, placing into context those genes that play key roles regulating proliferation in the nervous system.

The cell cycle is comprised of four distinct phases: G_1 or gap phase 1; S phase, where DNA synthesis occurs; G_2 or gap phase 2; and M-phase or mitosis (see Figure 1; reviewed in ref. 11). Cells exiting the cell cycle to differentiate or become quiescent leave the cell cycle and enter into G_0. Commitment to undergo mitosis occurs in late G_1, after cells pass

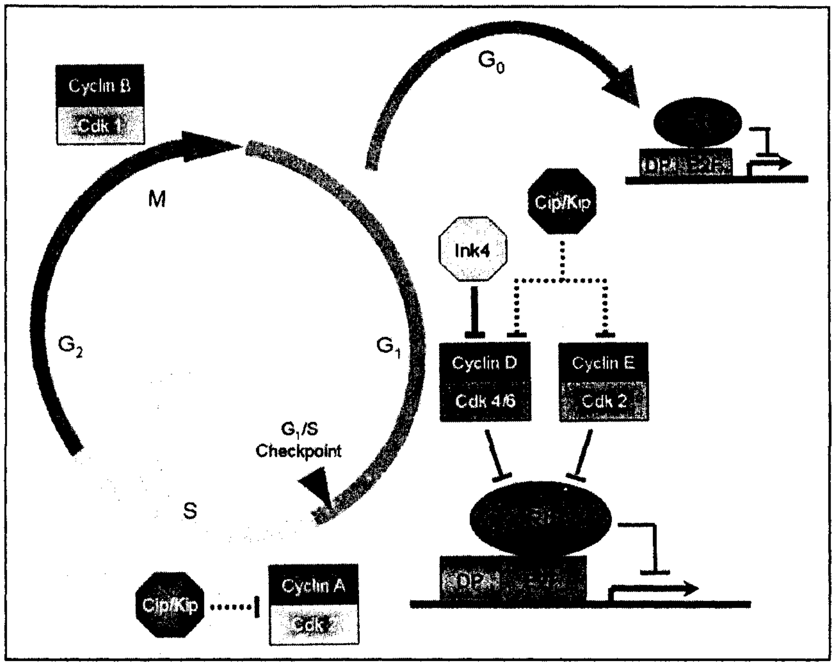

Figure 1. Schematic diagram of cell cycle, focusing on G$_1$-S phase transition. Adapted from reference 10.

the G$_1$-S restriction point. Progression through the restriction point and entry into S-phase is dependent on phosphorylation of the retinoblastoma tumor suppressor protein, Rb. Under non-growth stimulating conditions, hypophosphorylated Rb remains bound to E2F transcription factors preventing E2F mediated gene transcription. At the G$_1$-S transition, Rb phosphorylation by cyclin dependent kinases (Cdks) results in the release of Rb from E2F transcription factors, allowing E2F mediated transcription to occur and driving S-phase progression. E2F refers to a family of eight known transcription factors, at least five of which can bind to Rb. Classical E2F targets include a number of genes required for DNA synthesis such as thymidine kinase, dihydrofolate reductase, and DNA polymerase-α; as well as genes required for subsequent cell cycle regulation such as cdc2, myc, b-myb and cyclins D1, A and E; Rb and Rb related family member p107; E2F1, and recently geminin.[11-15]

Regulation of Rb activity is carried out at two levels: the cyclins and Cdks that associate to phosphorylate Rb and the cyclin dependent kinase inhibitors which, in turn, regulate the activity of cyclin/Cdk complexes. As cells pass through late G$_1$ and into S phase, Rb specific cyclin/Cdk complexes phosphorylate Rb in succession. In early to mid G$_1$, cyclin D/Cdk4/6 complexes are formed and are the first to phosphorylate Rb, followed by cyclin E/Cdk2 complexes in late G$_1$, followed by cyclin A/Cdk2 complexes that are active in the G$_1$-S phase boundary and throughout S phase. Cyclin/Cdk activity is negatively regulated by two families of Cdk inhibitors (CDKIs). The first family, inhibitors of Cdk4 (Ink4), is comprised of p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}, which specifically inhibit cyclin D-associated kinases, Cdk4 and Cdk6. The second family, Cip/Kip, include p27^{Kip1}, p21^{Cip1} and p57^{Kip2} which act generally to regulate cyclin D-, cyclin E- and cyclin A-dependent kinases (reviewed in ref. 11).

Cell cycle genes that impact on proliferation in the nervous system are overwhelmingly concentrated on those genes that regulate the G$_1$-S transition and, in turn, the Rb signaling pathway.

CELL CYCLE GENES AND NEURAL PRECURSOR PROLIFERATION—THE IMPORTANCE OF THE RB SIGNALING PATHWAY

A review of the literature reveals that multiple genes at each level of regulation at the G$_1$-S transition impact on proliferation in the central nervous system (Table 1). Perhaps the most intensely studied is the Rb tumor suppressor. A role for Rb in regulating neural precursor proliferation was first indicated through the study of germline Rb deficient mouse embryos where absence of Rb lead to ectopic proliferation and widespread apoptosis in the central and peripheral nervous system (CNS and PNS).[16-18] Subsequent studies with germline Rb deficient mice revealed reduced or absent expression of differentiation markers in the CNS and lens, leading to the hypothesis that Rb is required not only for cell cycle regulation and survival, but also differentiation.[19,20] Studies with chimeric mice, however, reported that Rb chimeric mice survive to adulthood and do not exhibit widespread apoptosis in the CNS.[21-23] In the CNS, chimeric embryos exhibited extensive ectopic S phase entry, however, unlike germline Rb deficient embryos, these cells did not complete division, but rather were arrested at G$_2$-M. Additionally, Rb deficient cells from chimeras survived and were capable of differentiating into neurons.[23] Thus the differences between germline Rb deficient embryos and Rb chimeric mice called into question the requirement for Rb in neuronal survival and differentiation. This lead to the hypothesis that the role of Rb during CNS development could be separated into a cell autonomous function for Rb in cell cycle exit and non-cell autonomous functions in widespread survival and neuronal differentiation.[21-23] Indeed, widespread apoptosis in the CNS of germline Rb deficient embryos was later found to be a consequence of Rb deficiency in the placenta.[24,25]

The use of tissue specific Rb deficient mice has allowed for a more precise definition of the cell autonomous functions of Rb in the nervous system. Telencephalon specific deletion of Rb resulted in ectopic proliferation of committed neuroblasts that were able to survive and initiate neuronal differentiation.[26] The presence of BrdU labeled cells coexpressing neuronal markers indicated that Rb is required for coupling cell cycle exit with neuronal differentiation.[26] In a separate study, deletion of Rb in nestin positive progenitor cells throughout the CNS and PNS also resulted in inappropriate S-phase entry and cell survival.[27] Similarly, specific deletion of Rb in the developing retina resulted in ectopic division of differentiating precursor cells.[28] In contrast to the forebrain, where Rb is not required for widespread cell survival, in the retina, Rb is required for survival of three major cell types: ganglion, bipolar and rod photoreceptor cells.[28] Finally, specific deletion of Rb in precursor cells in the cerebellar vermis resulted in increased proliferation and apoptosis specific to granule cell precursors.[29] As these studies have described different cell type specific requirements for Rb in regulating neural precursor proliferation and survival, together they demonstrate a context dependent function for Rb in the developing nervous system.

Table 1 **Cell cycle genes impact on neural precursor proliferation**

Name	Function in Neural Precursor Proliferation	Reference
Rb Family		
Rb	Tissue specific absence of Rb leads to ectopic proliferation in CNS and retina. In the telencephalon, ectopically dividing cells are committed to a neuronal fate and initiate differentiation.	26–28
p107	Absence of p107 leads to increased self-renewal of neural precursors	32
E2F1	Absence of E2F1 leads to decreased neural precursor proliferation in adults	67
E2F3	Absence of E2F3 leads to decreased neural precursor proliferation in embryo and adults	McClellan, Slack, unpublished observations
Cyclins and Cdks		
Cyclin D1	Absence of Cyclin D1 leads to reduced proliferation of retinal precursors	68
Cyclin D2	Absence of Cyclin D2 leads to decreased proliferation of neural precursors in the adult	34
Cdk 4/6 and Cdk2	Inactivation of Cdk4/6 or Cdk2 activity induces growth arrest in primary neural precursor cells	69
Cdk 5	Absence of Cdk5 leads to ectopic proliferation in the cortical plate. Ectopically proliferating cells are also differentiating	35
CDKI		
Kip/Cip		
p21^{Cip1}	Absence of p21^{Sip1} leads to increased proliferation of neural precursor cells in the adult forebrain, but exhibit reduced capacity for self-renewal	70
p27^{Kip1}	Absence of p27^{Sip1} leads to increased proliferation of transit amplifying progenitors but a decrease in number of migrating neuroblasts (reduced due to apoptosis)	37
p57^{Kip2}	Absence of p57^{Kip2} leads to ectopic proliferation and apoptosis in the embryonic retina	38
Ink4		
p16^{Ink4a}	Absence of p16 leads to increased proliferation of neural precursor cells	71
Bmi-1	Absence of Bmi-1 leads to decreased proliferation of neural precursor cells, an effect mediated by suppression of p16^{Ink4a}, p19^{Arf}, and additional pathways	71–73

Related Rb family members are also involved in regulating proliferation in the nervous system and are thought to compensate for the loss of Rb. Indeed, Rb family members p107 and p130 have both been shown to work with Rb in regulating proliferation in the retina as deletion of either p107 or p130 combined with Rb deficiency leads to retinoblastoma in the mouse.[28,30,31] In addition, combined loss of Rb and p107 in the cerebellar vermis leads to an exacerbation of the proliferation and apoptosis defects in granule cells that are observed in Rb deficiency alone.[29] A unique function, however, for p107 has been recently described in regulating the neural precursor population in the nervous system.[32] The absence of p107 alone leads to an enhanced self-renewal capacity of neural precursors resulting in an expansion of the neural precursor pool. These findings demonstrate that p107 functions to regulate the neural precursor cell number through a mechanism distinct from Rb function.[32]

Given the importance of Rb in regulating proliferation in the nervous system, it follows that the cyclins and Cdks that regulate Rb phosphorylation also impact on proliferation in the nervous system. Cyclin D2 deficient mice exhibit smaller cerebella as a result of decreased proliferation and increased apoptosis.[33] Additionally, cyclin D2 regulates proliferation in the adult CNS where its absence leads to severely reduced proliferation in the subgranular zone of the hippocampus.[34] New data has recently emerged indicating that Cdk5, a unique member of the Cdk family with sequence homology to Cdc2, previously believed not to be involved in cell cycle regulation, plays an essential role in regulating neuronal cell cycle arrest[35] (reviewed in ref. 36). The absence of Cdk5 results in the presence of 'neurons engaged in cell cycle activities' in the cortical plate, similar to what is observed in Rb deficiency in the brain.[26,27,35] These cells continue to express proliferating cell markers indicating that Cdk5 is also required for regulating cell cycle exit.[35] As Cdk5 is capable of phosphorylating Rb both in vivo and in vitro, these observations lead to the hypothesis that Cdk5 may be acting to regulate Rb function in the nervous system.[35]

Similarly, members of the Cip/Kip and Ink4 families of CDKIs that regulate cyclins and Cdk activity have also be shown to regulate neural precursor proliferation. Indeed multiple members of both families have been shown to be negative regulators of proliferation in the nervous system highlighting the importance and complexity of cell cycle regulation at the CDKI level (Table 1). Loss of p27^{Kip1} leads to increased proliferation of transit amplifying progenitor cells in the adult subventricular zone, while loss of p57^{Kip2} leads to ectopic proliferation of retinal progenitors during development.[37,38]

Finally, the idea that all of these genes contribute to regulating proliferation in the nervous system is reinforced by the phenotype of conditional N-myc deficient mice. Absence of N-myc, a transcription factor known to regulate transcription of genes such as, Cdk4, Cyclin D2, E2Fs, p27^{Kip1} and p21^{Cip1} in neural progenitor cells, leads to a severe reduction of brain mass which comes as a result of reduced proliferation without an increase in apoptosis.[39]

As disruption of a number of individual cell cycle genes results in proliferation defects in the nervous system, these studies demonstrate that precise cell cycle regulation is a crucial component of proper nervous system development.

NEW ROLES FOR CELL CYCLE GENES IN NEURONAL DIFFERENTIATION AND MIGRATION

Recently it has become apparent that in addition to their cell cycle regulatory functions, cell cycle genes are capable of regulating diverse processes in the nervous system including programmed cell death, differentiation, and more recently neuronal migration. Much

Table 2 **Cell cycle genes impact on diverse cellular processes in nervous system development**

Name	Distinct Functions Outside of Proliferation	Reference
Differentiation		
p107	p107$^{-/-}$ results in increased expression of Hes1 and Notch^{-1} in the cerebral ventricular zone suggesting that p107 can repress differentiation through regulation of the Notch signaling pathway	32
Cyclin D2	Cyclin D2 is expressed in post-mitotic neurons following cell cycle exit; Cyclin D2$^{-/-}$ exhibit fewer granule and near absence of stellate neurons in cerebellum suggesting that cyclin D2 regulates differentiation of these neurons	33
p57$^{\mathrm{Kip2}}$	p57 is expressed in post-mitotic differentiating neurons; p57$^{-/-}$ exhibit increased numbers of amacrine neurons in the retina and fewer midbrain dopaminergic neurons; Differentiation of dompaminergic neurons requires physical interaction of p57 with Nurr-1	38,43
Geminin	Inhibition of Geminin results in increased neuronal differentiation, an effect that requires geminin interaction with Brg-1 to inhibit transcription of proneural bHLHs	47,48
Rb/E2F	Absence of Rb leads to a defect in starburst amacrine differentiation in retina— an effect that appears to be mediated through specific interaction with E2F3	Chen et al., unpublished observations
Axon Morphogeneis		
Cdh-Anaphase Promoting Complex (APC)	Cdh is expressed in post-mitotic neurons. In vitro, knockdown of Cdh in cerebellar granule neurons results in increased axonal length in vitro an interaction dependent on association with APC. In vivo, reduction of Cdh levels results in abnormal axon outgrowth and tract formation	74
Migration		
Rb	Conditional Rb -/- exhibit a cell autonomous defect in migration of ventrally derived interneurons	51
p27$^{\mathrm{Kip1}}$	Reduction of p27 levels in post-mitotic cells in vivo results in decreased radial migration in the cortex. Increased levels of p27 lead to increased (activity/ stabilization) of cofilin, an actin severing protein that plays a role in regulating neuronal migration	50
Cdk5	Highest expression and kinase activity in nervous system; phosphorylates numerous substrates thus implicating Cdk5 in regulating a vast number of cellular processes, most notably neuronal migration, but also membrane transport, axon guidance, synaptic structure and cytoskeletal dynamics	reviewed in ref. 36

attention has been given to the role of cell cycle genes regulating programmed cell death both during neurodevelopment and in models of neurodegeneration, and as such will not be discussed here (reviewed in refs. 40–42).

While many cell cycle genes are known to be expressed in post-mitotic neurons, the idea that cell cycle genes can moonlight to regulate neuronal differentiation and migration has developed recently through loss of function studies (Table 2). For example, roles for cyclin D2, p107, p57$^{\mathrm{Kip2}}$, the Rb/E2F target gene Geminin, as well as the Rb/E2F pathway, in neuronal differentiation have been described, while roles for p27$^{\mathrm{Kip1}}$ and Rb have been described for neuronal migration. While it is possible that the resulting phenotypes observed occur as a secondary consequence of cell cycle deregulation, through new and innovative experiments many studies have revealed that these genes can function outside of their cell cycle regulatory roles. The increasing number of such examples argues that cell cycle proteins have novel functions in regulating these processes.

Differentiation. Roles for G$_1$-S transition signaling components, cyclin D2 and p107, in regulating neuronal differentiation have been suggested.[32,33] Cerebella from cyclin D2 deficient mice exhibited a reduced number of granule cells and stellate interneurons.[33] While a reduction in granule cell number is thought to be the result of decreased proliferation and increased cell death, the death occurred in postnatal granule precursor cells undergoing terminal differentiation, supporting the hypothesis that cyclin D2 is required for proper terminal differentiation of these cells. Reduction in stellate cell number may also reflect a role for cyclin D2 in regulating differentiation of these cells as stellate cells are thought to arise from a common precursor cell that gives rise to three interneurons subtypes: Golgi, stellate and basket cells. Since Golgi and basket cell numbers are unchanged in cyclin D2 deficiency, this suggests a requirement for cyclin D2 in stellate cell differentiation.[33] Next, mice deficient for p107 exhibit an increased number of neural precursor cells in the brain.[32] While the mechanism is unknown, p107 deficient neural precursor cells exhibit increased expression of members of the Notch signaling pathway and interaction of p107-E2F complexes with E2F sites on the Notch promoter was observed. Together these data support a provocative hypothesis whereby the increased number of progenitor cells in p107 deficient mice reflects a novel role for p107 in promoting differentiation of neural precursor cells.

More solid support for a role for cell cycle genes regulating neuronal differentiation exists for p57$^{\mathrm{Kip2}}$, Geminin, and the Rb/E2F pathway, where roles for differentiation have been separated from cell cycle control. A role for p57$^{\mathrm{Kip2}}$ in neuronal differentiation has been described for both retinal amacrine cells and midbrain dopaminergic neurons.[38,43] In the retina, p57$^{\mathrm{Kip2}}$ is expressed in dividing retinal progenitors during embryonic development, but was also found postnatally in a subset of post-mitotic differentiating amacrine neurons. Expression of p57$^{\mathrm{Kip2}}$ in these cells was not required for preventing cell cycle reentry, but rather required for differentiation of calbindin expressing amacrine neurons as p57$^{\mathrm{Kip2}}$ deficient retinae exhibited increased numbers of these cells.[37] These observations lead to the hypothesis that p57$^{\mathrm{Kip2}}$ has a dual function in the retina; first to regulate proliferation in mitotic retinal progenitors, and then, possibly through a distinct mechanism, to regulate the differentiation of a subpopulation of amacrine cells.[38] Further support for the idea that p57$^{\mathrm{Kip2}}$ can regulate neuronal differentiation independently of cell cycle function came about more recently through the examination p57$^{\mathrm{Kip2}}$ during midbrain development.[43] Similar to the retina, p57$^{\mathrm{Kip2}}$ was found to be expressed in post-mitotic differentiating dopaminergic neurons of the midbrain, however, mice lacking

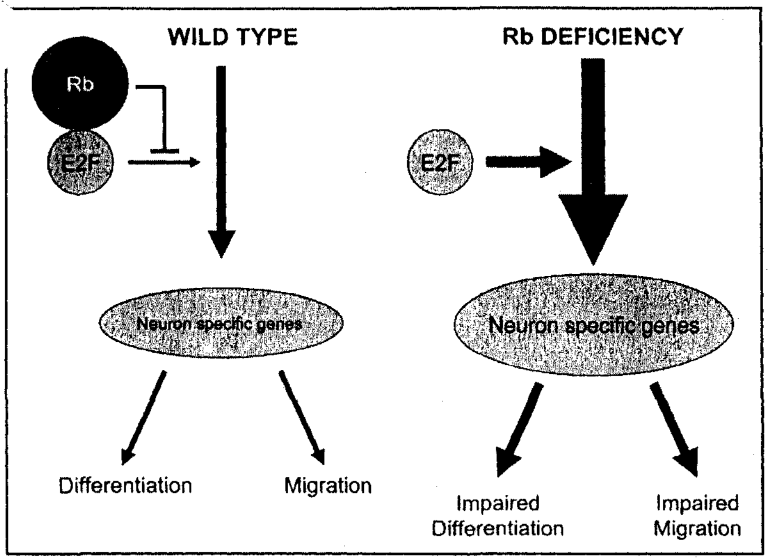

Figure 2. Proposed model of Rb/E2F-mediated regulation of neuronal differentiation and migration. In wildtype cells, E2F activity is inhibited by Rb resulting in repression of E2F transcription of genes involved in neuronal differentiation and migration. In the absence of Rb, E2F activity is deregulated, allowing for transcription of these genes, which, in turn, leads to impaired neuronal migration and differentiation.

$p57^{Kip2}$ exhibited fewer dopaminergic neurons. Several lines of evidence indicate that the effect of $p57^{Kip2}$ on differentiation occurs independently from cell cycle regulation. First, no defect in proliferation was observed through BrdU incorporation in the ventral midbrain in $p57^{Kip2}$ deficient brains when examined at multiple stages of development. Next, $p57^{Kip2}$ expression in these cells was shown to be cell cycle independent, and depended on the expression of Nurr-1, a nuclear orphan receptor expressed in post-mitotic cells that is essential for dopamine neuron development. Finally, $p57^{Kip2}$ was found to physically interact with Nurr-1 in vitro to promote differentiation of dopaminergic neurons. Depletion of $p57^{Kip2}$ inhibited differentiation. Differentiation still occurred, however, with a $p57^{Kip2}$ mutant unable to inhibit Cdk activity, yet still capable of binding Nurr-1. Together this indicates that $p57^{Kip2}$ regulates differentiation through interaction with Nurr-1, independent of cell cycle regulation.[43]

Geminin, a newly discovered cell cycle regulatory molecule,[44] and Rb/E2F target gene,[14,15] has recently been shown to play a role in regulating differentiation, distinct from its role in cell cycle regulation. During S phase and G_2, Geminin prevents rereplication of DNA by binding and sequestering Cdt1, a component of DNA prereplication complexes.[44] Three recent reports, however, have demonstrated novel functions for Geminin in development and differentiation through interaction with other proteins.[45-47] In two-hybrid assays Geminin was found to interact with multiple members of the Hox gene family, Six3, and Brg-1 to regulate early embryonic development, retinal development, and neuronal differentiation. The role for Geminin in regulating neurogenesis was first suggested in the initial characterization of this protein where overexpression of Geminin in early *Xenopus* embryos led to expansion of the neural plate.[48] A subsequent study outlined a mechanism whereby Geminin inhibits neuronal differentiation by binding to Brg-1, a member of the SWI/SNF chromatin remodeling complex, which blocks bHLH mediated transcription of neuron specific genes.[47] In *Xenopus* embryos, overexpression of a Geminin mutant lacking the central domain that binds Cdt and is required to prevent DNA rereplication, lead to an expansion of the neural territory. A decrease in N-tubulin expression, a marker of differentiated neurons, was noted.[47] Similarly, knockdown of Geminin resulted in an increase in neuronal differentiation in *Xenopus* embryos and mammalian cells.[47] Together these data suggest that Geminin can function to inhibit neuronal differentiation. A search for binding partners uncovered Brg-1, which binds the proneural bHLH; Neurogenin, and NeuroD, to promote transcription of neuron specific genes.[47,49] Geminin was found to bind to Brg-1 in multiple cell types, not through the central Cdt binding domain, but rather through its C-terminal domain. This suggests that Geminin regulates neuronal differentiation through a mechanism independent of its cell cycle regulatory function. The biochemical significance of Geminin interaction with Brg-1 was demonstrated in vitro where overexpression of Geminin inhibited the association of Neurogenin and NeuroD with Brg-1. In addition, Geminin inhibited transcription of a bHLH E-box reporter gene; an effect that depended on the presence of the Brg-1 binding domain. Proof that Geminin inhibits neuronal differentiation through interaction with Brg-1 came from binding mutants where expression of Geminin mutants lacking the Brg-1 binding domain in *Xenopus* embryos resulted in increased N-tubulin expression. Together these results demonstrate that Geminin interacts with distinct proteins through separate binding domains to regulate neuronal differentiation versus cell cycle.

Finally, new data has emerged implicating the classical Rb/E2F pathway in regulating neuronal differentiation. In addition to the ectopic proliferation and apoptosis of differentiating cells, specific absence of Rb in the retina results in a defect in differentiation of a specific subset of cholinergic neurons in the retina (Chen, Wenzel, Leone, Bremner, 2006 submitted manuscript). In the absence of Rb, these neurons are specified, initiate differentiation, and survive; however, tracts corresponding to their dendritic processes and synaptic connections were notably absent, indicative of a defect in later stages of differentiation. By interbreeding retinal specific Rb deficient mice with mice deficient for E2F1, E2F2 or E2F3, Chen et al. demonstrate that the defect in cholinergic retinal neuron differentiation is mediated specifically through E2F3 as only Rb:E2F3 deficient retinae exhibit restoration of differentiation. Moreover, these results demonstrate that E2F3 mediated regulation of cholinergic neuron differentiation in the retina occurs independently of cell cycle regulation. The dissociation of these two processes was demonstrated by the finding that Rb:E2F3 deficient retinae still exhibit ectopic proliferation despite rescued differentiation. Cholinergic retinal neuron differentiation was also examined in Rb:E2F1 deficient retinae where ectopic proliferation and apoptosis were rescued. As Rb:E2F1 deficient retinae exhibit a similar defect in cholinergic neuron differentiation in the retina in the absence of proliferation defects, these observations demonstrate that Rb can function to regulate differentiation independently from cell cycle control.

Together these studies demonstrate that numerous roles exist for cell cycle genes in regulating neuronal differentiation. For cyclin D2 and p107, the exact mechanism by which regulation of neuronal differentiation occurs remains unclear. For $p57^{Kip2}$ and Geminin, egulation of neuronal differentiation has been clearly demonstrated o occur through mechanisms distinct from their cell cycle regulatory function. Finally, Rb has been shown to regulate differentiation through the classical E2F pathway suggesting that cell cycle and differentiation can be regulated through similar pathways. Thus regulating neuronal differentiation represents not only novelty of function for cell cycle genes but also diversity in the mechanisms by which these genes impact differentiation.

Migration. New roles for cell cycle genes are not only restricted to neuronal differentiation. Recently two reports emerged demonstrating the involvement of $p27^{Kip1}$ and Rb in regulating neuronal migration.[50,51]

While $p27^{Kip1}$ deficient mice exhibit no obvious defect in migration, reduction of $p27^{Kip1}$ levels through electroporation of $p27^{Kip1}$ short hairpin RNA (shRNA) constructs in the developing cortex resulted in reduced migration of cortical neurons and cells with abnormal process morphology, suggesting that $p27^{Kip1}$ may have a role in regulating neuronal migration.[50] Consistent with this hypothesis, $p27^{Kip1}$ was observed in punctate cytoplasmic inclusions in the tips of neuronal processes of post-mitotic cells and colocalized with F-actin. A series of elegant experiments lead to the conclusion that the effect of $p27^{Kip1}$ on neuronal migration occurs independently from cell cycle regulation. First, electroporation of $p27^{Kip1}$ shRNA resulted in decreased expression of $p27^{Kip1}$ protein in post-mitotic neuronal cells but had no effect on $p27^{Kip1}$ levels in proliferating cells in the ventricular zone. Reduction of $p27^{Kip1}$ did not result in proliferation defects in transfected cells or proliferating cells of the ventricular zone. Next, the authors characterized a mechanism of action for $p27^{Kip1}$ that is distinct from its role in cell cycle regulation. Whereas in proliferating cells $p27^{Kip1}$ acts through Cdc2, in this study $p27^{Kip1}$ was shown to be phosphorylated by Cdk5 in post-mitotic neurons. This phosphorylation led to stable levels of $p27^{Kip1}$ which in turn inhibited phosphorylation of cofilin, an actin severing protein. Inhibiting Cdk5 activity or $p27^{Kip1}$ activity individually both resulted in increased cofilin phosphorylation in primary neurons. Overexpression of a stable form of $p27^{Kip1}$ together with inhibition of Cdk5 suppressed the increased cofilin phosphorylation that was observed through inhibiting Cdk5 activity alone indicating that Cdk5 acts upstream of $p27^{Kip1}$ in the same pathway to regulate cofilin activity. Proof that this pathway is involved in regulating neuronal migration was shown by disrupting cofilin signaling activity which resulted in impaired cortical migration in vivo.[50] While other factors are no doubt involved, the above data provide strong evidence for a cell cycle independent function for $p27^{Kip1}$ in regulating neuronal migration.

Another recent example of a cell cycle gene regulating neuronal migration comes from the study of telencephalon specific Rb deficient mice.[51] In this study we observed aberrant migration of a population of GABAergic interneurons that arise from the ventral telencephalon and migrate to the dorsal cortex. While these cells appear normally specified early in their development, by mid neurogenesis we observe a dramatic and specific reduction of these cells along their migratory trajectories en route to the dorsal cortex, suggesting an apparent requirement for Rb in regulating neuronal migration. To address whether the requirement for Rb exists in the environment or within the cells themselves, we employed cortical slice coculture assays whereby mutant or control explants are placed atop mutant or control cortical slices and cells from the explant are allowed to migrate for 72 hours. We observed a higher proportion of failed migration among Rb mutant explants compared to control explants, thus demonstrating a cell autonomous requirement for Rb in regulating neuronal migration during forebrain development.[51]

While these data indicate a role for Rb in regulating this process, they do not address whether this defect occurs as a consequence of deregulated cell cycle that is present in these mice,[26] or represents a truly novel, cell cycle independent function for Rb. While we can't rule out the former, mounting evidence exists in support of the latter. Rb is known to interact with numerous proteins besides E2F transcription factors, many of which are expressed in quiescent cells or have cell cycle independent functions.[52] One such interacting factor is Id2, a member of the bHLH family of Id transcription factors that inhibit differentiation and DNA binding (reviewed in ref. 53). Id2 not only physically interacts with Rb, it is a functionally relevant target for Rb in the nervous system as combined deletion of Rb and Id2 rescues the inappropriate proliferation and apoptosis activity characteristic of whole embryo Rb deficiency.[54] In relation to Rb mediated regulation of neuronal migration, the model put forth hypothesizes that in the absence of Rb, the increase of deregulated Id2 activity would result in repression of genes required for neuronal migration.[51]

While this remains an attractive model to explain how Rb could be regulating migration independently from cell cycle, it is also possible that Rb interacts through the classical Rb/E2F pathway to regulate other cellular functions such as migration. The idea that E2Fs can regulate developmental genes first came about from the examination of E2F deficient mice (reviewed in refs. 6 and 7). The phenotypes of these mice suggested that these genes play roles in the regulation of other cellular functions. Following this, groups using microarray, or sensitive subtractive screening assays in cells expressing or lacking individual activating E2Fs observed deregulation of a number of genes involved in differentiation, development, and apoptosis, supporting the hypothesis that E2F genes are capable of regulating a number of diverse cellular processes.[55-58] Classes of genes that would be expected to regulate neuronal migration would include genes involved in cytoskeleton remodeling or signaling, guidance molecules and their receptors, and extracellular matrix (ECM) molecules. Indeed, close examination of the data shows many of these studies each identified numerous genes in these categories, some with neuron specific expression profiles or known roles in regulating neuronal migration or axon guidance. Some examples include heparin sulfate 2-O-sulfotransferase which regulates heparin sulfate structure in the ECM, structure that is critical for proper axon guidance, and stathmin, a microtubule destabilizing protein shown to regulate migration of olfactory neurons[59] (reviewed in ref. 60).

While several of these targets remain unverified, together these studies do support the idea that E2Fs are capable of regulating diverse cellular functions beyond cell cycle control. Additionally, select genes have been further characterized as bona fide E2F target genes, some of which represent promising targets in regulating migration. By inactivating dE2F1 in *Drosophila* cells, Dimova et al. observed a downregulation of netrin and neurofibromin-1, an effect also observed in the absence of dDP. Both netrin and neurofibromin-1 have been shown to play roles in neuronal migration. Netrin, a secreted molecule is chemoattractive and chemorepulsive for many cells in the nervous system, while neurofribromin-1 has been shown to regulate migration of Schwann cells in the nervous system.[61,62] While these genes may be regulated by E2Fs in an indirect manner, dE2F1 was shown to be at the promoter of neurofibromin suggestive of a direct relationship.[56] Other novel E2F target genes have been identified through sensitive subtraction screening assays using ectopically expressed E2F1 in mouse embryonic fibroblasts.[58] While a number of new cell cycle dependent genes were identified in this study, a separate group of genes not induced by growth stimulation was also observed. Two genes in this group, neogenin and WASF1, were shown to be cell cycle independent, direct targets of E2F1 through luciferase assays in serum-starved cells. Both neogenin and WASF1 are expressed in the nervous system.[63,64] Neogenin is a cell surface receptor known to regulate migration of axons in the chick nervous system, while WASF1 is a member of the WASP family of cytoskeletal regulatory proteins involved in actin reorganization at the axon growth cone.[65,66] Together these genes make up a group of putative targets and provide evidence in support of roles for Rb/E2F signaling pathway in regulating neuronal migration.

CONCLUSION

Overall, these studies point convincingly to a previously unappreciated breadth of function for cell cycle genes. That cell cycle genes are involved in regulating diverse cellular functions should come as no surprise. Indeed, as the early experiments demonstrated that cell cycle exit is tightly coupled with cell fate it stands to reason that cell cycle genes should be involved in instructing differentiation pathways. What is surprising, however, are the novel and unique mechanisms by which cell cycle genes regulate these processes. Thus with each new function identified, a whole new avenue for discovery emerges and exciting times lie ahead for cell cycle researchers.

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Review

Specific In Vivo Roles for E2Fs in Differentiation and Development

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ABSTRACT

E2Fs have been historically considered as key interacting factors for the retinoblastoma (Rb) family of pocket proteins, acting as universal regulators of cell cycle progression. Often exhibiting overlapping function, deregulated E2F activity is thought to lead to cancer or cell death. While early reports hypothesized that E2Fs may be capable of regulating distinct functions beyond proliferation, several recent reports have characterized increasingly diverse, context dependent functions for different E2Fs in vivo, often in what appears to be a manner beyond traditional cell cycle regulation. Ironically, many of these new functions are still mediated through the classical cell cycle regulatory Rb family of interacting factors. Here we review the recent advances, focusing on differentiation and development, to emphasize that E2F function is likely more complex than the simple model suggests, capable of exhibiting both specificity of function, and roles beyond cell cycle progression in vivo.

INTRODUCTION

Much of our current understanding regarding E2F function has been shaped largely as a result of its initial discovery and characterization as an adenovirus E2 promoter binding factor[1,2] (reviewed in ref. 3). Subsequent to its discovery, the observation that E2F interacts with the cell cycle regulatory and tumor suppressor, retinoblastoma (Rb) protein[4-7] led to the search for E2F responsive genes which could be influencing cell cycle progression. This directed search resulted in the characterization of what we now consider to be classical E2F responsive genes, a list which includes genes required for cell cycle progression and DNA synthesis (reviewed in refs. 3 and 8). Together these observations established, early on, the model of E2Fs as activators or repressors of cell cycle progression whose activity is regulated through interaction with Rb family proteins (reviewed in refs. 3 and 9). This model of Rb/E2F acting as the checkpoint regulator of the G_1/S transition in virtually all cells across species is a model that remains relevant and virtually unchanged today.

Clues that E2F function may be more complex, however, came from the number of different E2Fs that were subsequently cloned and the first in vivo models of E2F inactivation. E2Fs were originally hypothesized to exhibit redundant function resulting from in vitro experiments illustrating that E2Fs exhibit overlapping pocket protein binding capabilities, similar target gene activation through common consensus sites, and similar ability to induce S-phase in quiescent cells (reviewed in refs. 3, 8 and 10). Subsequent studies of in vivo inactivation of individual E2Fs, however, have since questioned the universality of this view. Notably, eight E2Fs have now been identified, with six E2Fs studied through individual models of in vivo inactivation (reviewed in refs. 8 and11–16). Each E2F loss of function model has given rise to unique and often surprising phenotypes. For example, in mice, inactivation of E2F1 led to testicular atrophy as well as the formation of tissue specific tumors, characterizing E2F1 as both an oncogene and tumor suppressor.[17] Inactivation of E2F2, however, led to autoimmune disease,[18] while E2F3 inactivation resulted in partial embryonic lethality but no apparent tumors.[19,20] In addition, the phenotypes of the individual loss of function models were often quite distinct from what had been hypothesized, given the well defined roles of E2Fs as pocket protein interactors.

In part, as a result of these unique phenotypes, recent work into E2F function has been directed in exciting new avenues towards addressing the issues of specificity and context dependent function, as well as cell cycle independent roles. New and sophisticated iochemical means have identified novel E2F targets and non-tradi-onal recognition sites that are specific for individual E2Fs which together suggest that E2Fs are capable of regulating gene expression outside of cell cycle regulation. Such genes represent a diverse group, and as a result are directing E2F research along new avenues in development and differentiation. Moreover, in vivo studies have demonstrated that E2Fs have diverse and complex roles that can be separated from cell cycle control. Here we review the recent advances, focusing on differentiation and development, to emphasize that E2F function is likely more complex than the simple model suggests, capable of exhibiting both specificity of function, and roles beyond cell cycle progression.

E2F TRANSCRIPTION FACTORS AND CELL CYCLE REGULATION

E2F collectively refers to a group of eight transcription factors (Fig. 1) most recognized for their ability to regulate cell cycle progression. Grouped into categories, E2F1, 2 and 3a act as transcriptional activators by dimerizing with DP proteins and activating transcription through E2F consensus sites on promoters of a large list of genes required for cell cycle progression, including cyclins A & E, p107, dihydrofolate reductase, thymidine kinase, thymidylate synthetase, DNA pol α, and even E2F1 itself (reviewed in refs. 21 and 22). Several hallmark experiments established early on the importance of each of these genes in regulating proliferation. Their expression is cell cycle regulated with peak expression coinciding with G_1.[23-30] Expression studies demonstrated deregulated expression of E2F1, E2F2, or E2F3 individually is sufficient to induce quiescent cells to nter S-phase,[31] while collective ablation of E2F1, 2, and 3 in mouse fibroblasts completely abolishes entry into S-phase, and as a result, proliferation.[32] E2F4 and 5, however, are thought to act as transcriptional repressors through pocket protein binding (reviewed in refs. 21 and 22). In addition to being expressed during the cell cycle, E2F3b, E2F4 and 5 are expressed at significant levels in G_0.[29,30,33,34] The activities of both the activating E2Fs and the repressive E2Fs are believed to be regulated by pocket proteins with E2F1, 2 and 3 thought to interact primarily with Rb, and E2F5 primarily through p130, while E2F4 is capable of interacting with all three pocket proteins[29,30,34-37] (reviewed in refs. 8, 21 and 22).

As the list of E2Fs grows and as our knowledge about their function increases (Table 1), this simple classification of activators and repressors must be revised. Indeed, E2F6, 7 and 8 do not fit into this category as E2F6 does not interact with pocket proteins, while E2F7 and 8 do not interact with either pocket proteins or DP proteins.[11-16,38-40] Moreover, multiple transcripts introduce a new level of complexity whereby E2F3 is thought to be capable of both activation and repression.[29,30] Next, individual loss of function models reveal that E2F function in regards to proliferation and cell cycle progression is highly tissue and context specific, capable of both unique and combinatorial function with other E2Fs (Table 1). The functions described are often paradoxical given the essential roles E2Fs play in mediating pocket protein activity. For example, as the Rb tumor suppressor is thought to repress the activity of E2F1, it should follow that E2F1, itself, behaves as an oncogene. While the E2F1 loss of function mouse model confirms this hypothesis, surprisngly a broad spectrum of tumors in particular tissues such as the lung and reproductive tract were observed, leading to the additional characterization of E2F1 as a tumor suppressor.[41] Perhaps some of these surprising results can be explained through evidence which suggest that traditional E2F activators are capable of transcriptional repression, while E2F repressors can behave as activators. For example, the resulting increased proliferation of T-lymphocytes in the absence of E2F2 is thought to be the result of a loss of E2F2 mediated repression of cell cycle genes such as E2F1 in the lymph node.[18] A role in transcriptional repression has also been shown for E2F1, where E2F1 is capable of direct repression of Mcl-1, an anti-apoptotic Bcl-2 family member.[42] The E2F4 transcriptional repressor has also been hypothesized to be capable of transcriptional activation, based largely on ectopic expression studies.[43-47] This hypothesis is further supported by new observations from E2F4 loss of function models. For example, while E2F4 is thought to repress expression of Ccna2 in fibroblasts, the absence of E2F4 in erythroid cells leads to decreased Ccna2 expression, suggestive of a transcriptional activation relationship.[48] Indeed, ChIP studies demonstrate the presence of E2F4 at the Ccna2 promoter in erythroid cells at the time of activation further supporting the hypothesis that E2F4 can function as an activator.[48]

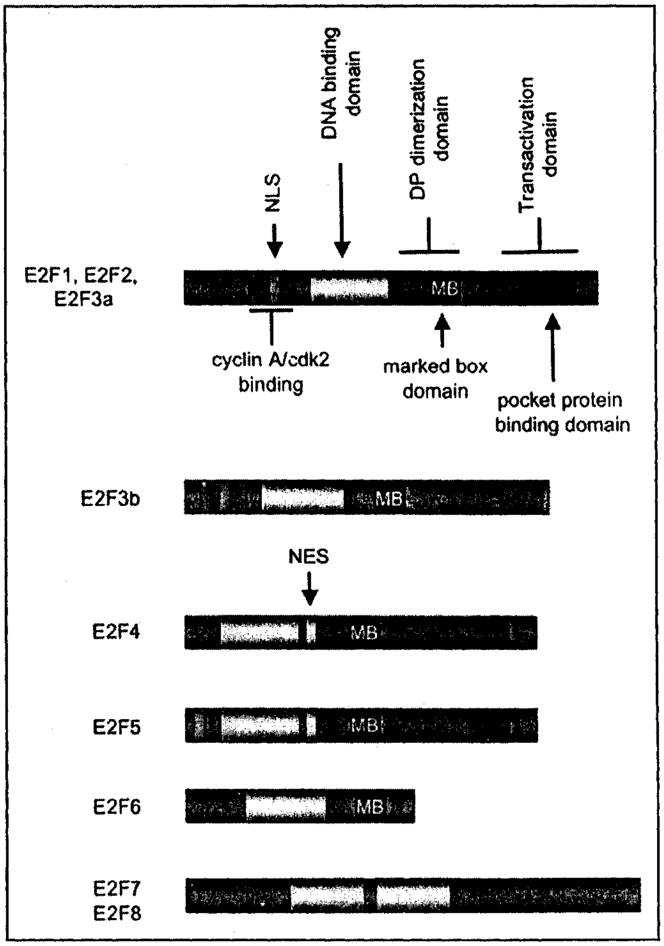

Figure 1. The E2F family-Select domains of each E2F are depicted. Note that the DNA binding domain is conserved across the family. E2F1–6 each contain the "marked box" domain. E2F1–3 and E2F5 each have nuclear localization sequences, while E2F4 and 5 have nuclear export signals[130-132] (reviewed in ref. 8). Figure modified from refs. 22, 10 and 133. NLS, nuclear localization sequence; NES, nuclear export sequence; MB, marked box domain.

ble 1 Known and hypothesized roles for E2Fs in proliferation

Name	Function
E2F1	HYPOTHESIZED UNIVERSAL FUNCTION Capable of activating transcription of E2F responsive genes (reviewed in ref. 21)
	Expression of E2F1 induces S-phase entry in quiescent fibroblasts and cultured myeloid cells[31,105]
	Together, E2F1, 2 and 3 are absolutely required for proliferation as combined absence of E2F1, 2 and 3 results in fibroblasts unable to enter S-phase[32]
E2F2	HYPOTHESIZED UNIVERSAL FUNCTION
	Capable of activating transcription of E2F responsive genes (reviewed in ref. 21)
	Expression of E2F2 alone in quiescent fibroblasts induces S-phase entry[31]
	Together, E2F1, 2 and 3 are absolutely required for proliferation as combined absence of E2F1, 2 and 3 results in fibroblasts unable to enter S-phase[32]
	EXAMPLE OF TISSUE SPECIFIC FUNCTION
	Required for myc induced S-phase in quiescent fibroblasts[106]
	Combined absence of E2F1/2 leads to reduced S-phase progression in bone marrow derived cells[107]
	Capable of repressing proliferation of T-lymphocytes, as absence of E2F2 alone leads to enhanced proliferation of T cells[18]
	Capable of acting as a repressor of genes required for S-phase in Jurkat T cells[108]
	Combined absence of E2F1/2 leads to enhanced proliferation of T cells in response to subthreshold antigen induced stimuli, but defective in S-phase progression for homeostasis driven T cell proliferation[109]
	Overexpression in thymus leads to unscheduled division and oncogenic transformation of thymic epithelial cells[110]
	E2F1/2 DKO exhibit increased numbers of replicating cells in postnatal pancreas[111]
E2F3a&b	HYPOTHESIZED UNIVERSAL FUNCTION
	Capable of activating transcription of E2F responsive genes (reviewed in ref. 21)
	E2F3 is required for S-phase entry as depletion of E2F3 in proliferating cells prevents entry into S-phase and $E2F3^{-/-}$ fibroblasts exhibit reduced proliferation as a result of reduced DNA synthesis and reduced rate of S-phase progression and mitogen induced cell cycle re-entry[28,19]
	Together, E2F1, 2 and 3 are absolutely required for proliferation as combined absence of E2F1, 2 and 3 results in fibroblasts unable to enter S-phase[32]
	E2F3 regulates centrosome duplication and chromatid separation as loss of E2F3 leads to centrosome amplification and aneuploidy[112]
	E2F3b represses Arf in normal MEFs to regulate cell cycle re-entry as E2F3/Arf DKO rescues the cell cycle re-entry defects present in $E2F3^{-/-}$[113]
	Required for myc induced S-phase in quiescent fibroblasts[106]
	EXAMPLE OF TISSUE SPECIFIC FUNCTION
	Expression of E2F3 alone induces S-phase entry in fibroblasts but not in cultured myeloid cells[31,105]
	Expression of E2F3a induces cell cycle re-entry in lens fibre cells[45]
	Transgenic expression of E2F3a in the epidermis leads to hyperproliferation (similar to E2F4 and 1)[114]
	Positive regulator of neural precursor proliferation as loss of E2F3 leads to reduction of neural precursor cells[77]
	Oncogenic in bladder cancer as it is frequently amplified and knockdown of E2F3 in bladder cells reduces proliferation[115-117]
E2F4	HYPOTHESIZED UNIVERSAL FUNCTION
	Alone loss of E2F4 does not result in cell cycle or proliferation defects in MEFs[118,119]
	Expression of E2F4&5 together with DP-1 induces quiescent fibroblasts into S-phase[31]
	Together with E2F5, required for pocket protein mediated G_1 arrest in response to $p16^{INK4a}$[120]
	Capable of indirect regulation of cell cycle by acting as Smad co-factor to repress c-myc in response to TGFbeta receptor activations[121]
	EXAMPLE OF TISSUE SPECIFIC FUNCTION
	Presence of E2F4 on promoters of E2F responsive genes in erythroid cells suggests that E2F4 is capable of transactivation of genes required for cell cycle progression[48]
	Absence of E2F4 leads to a delay through late S-phase and/or G_2 of cell cycle and reduced proliferation[48]
E2F5	HYPOTHESIZED UNIVERSAL FUNCTION
	Alone, loss of E2F5 does not result in proliferative defects[122]
	Expression of E2F4&5 together with DP-1 induces quiescent fibroblasts into S-phase[31]
	Together with E2F4, required for pocket protein mediated G_1 arrest in response to $p16^{INK4a}$[120]
	Capable of indirect regulation of cell cycle by acting as a Smad co-factor to repress c-myc in response to TGFB receptor activation[121]
	Act independently from pocket proteins
.2F6	Represses a subset of E2F responsive cell cycle related genes during S-phase[38-40,123]
E2F7	Acts as a transcriptional repressor for a subset of E2F target genes to slow proliferation & delay cell cycle progression at G_1/S[11-13]
E2F8	Acts as a transcriptional repressor for a subset of E2F target genes to slow proliferation & delay cell cycle progression at G_1/S[14-16]

ble 2 **Known and hypothesized roles for E2F in differentiation and development**

Name	Function	Shared/ Unique*
E2F1	Identified as an activator of genes involved in development in a cell cycle independent manner[71]	
	Regulates transcription of neuropilin -1, a gene with known roles in neuronal migration[124]	
	Blocks GCSF mediated survival and differentiation of granulocyte differentiation[105]	Distinct from E2F3
E2F2	Together with E2F1 hypothesized to regulate postnatal differentiation and maturation of pancreatic cells based on abnormalities of pancreas in E2F1/2 DKO mice[111]	Shared with E2F1
E2F3a& b	Required for normal embryonic development as lethality begins at E13.5 during development and likely required for proper heart development as surviving animals die from congestive heart failure hypothesized to be a result of a primary heart defect[19,20]	Unique
	Mediates neuronal migration and differentiation through Rb[76,77]	Unique
E2F4	Involved in regulation of gut epithelium development and craniofacial development as E2F4 -/- exhibit shortening and broadening of the snout and reduced intestinal crypts and villi[118,119]	Unique
	Required to repress adipocyte differentiation, in part through regulation of PPARγ expression. Repression of adipogenesis appears to be separable from any role of E2F4 in cell cycle regulation or pocket protein binding[72,73]	Opposing role with E2F1 and 3
	Required for ventral telencephalic patterning and regulation in the size and self-renewal capacity of neural precursors, as loss of E2F4 results in specific loss of ventral telencephalic structures and reduction in the size and self-renewal capacity of neural precursors in the embryo. Both defects appear to be mediated by E2F4 through a genetic interaction with the morphogen Sonic Hedgehog[75]	Unique
	Promotes NGF induced neuronal differentiation and maintenance of differentiated state of PC12 cells as expression of E2F4 in PC12 cells promotes differentiation and reduction of E2F4 inhibits NGF mediated neurite extension[125]	Unique
	Required for cell differentiation during development of airway epithelium, as loss of E2F4 prevents formation of ciliated cells early in development and overproduction of mucin producing goblet like cells-defects occur independent of any defects in cell proliferation[74]	Unique
E2F5	Involved in regulating secretion of cerebral spinal fluid as $E2F5^{-/-}$ exhibit hydrocephalus due to an apparent overproduction of CSF[122]	Unique
2F6	Interacts with polycomb group proteins to repress transcription, preferentially in quiescent cells (G_0)[126,127]	Unique
	Required for normal skeleton development & testicular development as $E2F6^{-/-}$ exhibit posterior homeotic transformation of the axial skeleton and testicular abnormalities[128]	Unique
	Required to maintain male germ cell specific expression of α-tubulin 3 and 7 as $E2F6^{-/-}$ exhibit near ubiquitous expression of these genes in other organs[129]	Unique
E2F7	No known role	
E2F8	No known role	

* wherever possible shared and unique functions relative to other E2Fs are pointed out.

Finally, as reviewed in Table 2, models of E2F inactivation also demonstrate that loss of individual E2Fs can result in phenotypes which suggest roles outside of cell cycle regulation in differentiation and development. As our view of E2F function becomes increasingly complex, it can represent a challenge to reconcile the existing data.

IN VITRO EVIDENCE FOR SPECIFICITY OF FUNCTION

Given the apparent complexity, a prevalent question is how specificity of function among classical E2F family members (E2F1–5) is achieved. Initially, structural studies revealed no differences in the amino acid sequences among E2Fs and DP proteins within their DNA binding and dimerization domains, suggesting that all are equally capable of recognizing traditional E2F consensus sites within promoters.[49] The notion that individual E2Fs did not exhibit preference for individual promoters was further strengthened by initial chromatin immunoprecipitation (ChIP) studies where prevalence of articular E2Fs at given promoters was shown to be dependent on cell cycle stage and pocket protein interaction rather than E2F identity.[50,51] Together with the observation that E2Fs exhibit tissue and cell type specific expression patterns during development,[52,53] these data supported a hypothesis that spatial and temporal regulation of individual E2F activity, combined with differential pocket protein interaction determines specificity of function.[52,53]

While elements of this hypothesis still remain true, it is now emerging that individual E2Fs are inherently capable of unique functions, irrespective of time and space regulation. This has been illustrated with E2F1 and 3 at the molecular level where each has been shown to interact with specific factors to mediate either transcriptional activation or regulation of cell death. Specificity among E2Fs in mediating transcription has been shown for E2F3, which interacts with the E-box factor, TFE3, to activate transcription of the p68 subunit of DNA pol α, and ribonucleotide reductase 1 and 2. This effect was observed to be specific to E2F3 as E2F1 was not shown to interact with TFE3 or capable of activating transcription of these genes.[54,55] As E2F3 and TFE3 are each capable of activating transcription individually, yet when bound together regulate transcription of a distinct group of genes, this led to the proposal that combinatorial interaction could represent a mechanism for achieving specificity in E2F mediated transcription.[55] Similarly specific function has been demonstrated for E2F1. Jab-1, c-jun activation domain binding protein, interacts specifically ith E2F1 to mediate cell death—an interaction not observed with other E2Fs.[56] Remarkably, in both instances, specificity of function can be isolated to a region within each E2F protein known as the "marked box" domain, a region adjacent to the DNA binding and DP dimerization domains (Fig. 1). In the case of E2F3, absence of the marked box domain eliminates the ability of E2F3 to interact with TFE3 and synergistically activate transcription.[54] Similarly, the marked box domain of E2F1 is required for induction of apoptosis, as chimeric proteins in which the marked box domain and adjacent region of E2F3 is substituted into E2F1 eliminates the ability of E2F1 to interact with Jab-1 and induce apoptosis.[56,57] The importance of the marked box domain in regulating transcription is further illustrated by microarray studies which demonstrate that both E2F1 and 3 exhibit distinct patterns of gene expression when ectopically expressed in fibroblasts. Similar microarray studies with chimeric proteins confirm that differential gene expression is determined by the identity of the marked domain.[57] Thus from these studies it has emerged that despite their similarities, E2Fs have inherent differences which can serve to impart specificity of function.

IN VITRO EVIDENCE FOR FUNCTION BEYOND CELL CYCLE REGULATION

A second debate regarding E2F function centers on whether or not E2Fs are truly capable of cell cycle independent function. Questions as to whether such roles exist and how cell cycle independent functions are achieved are emerging in the literature. While initially ypothesized to have roles outside of cell cycle regulation as a result of phenotypes in loss of function mouse models, this idea has since gathered momentum through microarray studies examining changes in gene expression in response to deregulated E2F expression.[57-63] In many of these studies gene expression was examined at fixed time points in cultured cells expressing various E2Fs. Despite the fact that the time points at which gene expression was examined were often chosen to correlate with expression of known cell cycle responsive genes, nevertheless each study identified novel E2F target genes with roles outside of the G_1/S cell cycle transition.[57-63] This was first observed in cells where E2F1, E2F2, and E2F3 were each expressed acutely in human osteosarcoma cells.[58] In this study, a large number of genes with known roles in development and differentiation were observed to be deregulated.[58] This list included genes such as homeobox transcription factors and bone morphogenetic proteins.[58] Deregulation of a select number of genes involved in new processes, alone, is not sufficient to demonstrate novel functions for E2Fs. By performing a meta-analysis on the list of new genes identified, however, the authors argue that as a result of the disproportionately high number of genes identified with roles in novel processes, E2Fs are likely to be authentically involved in the regulation of these events.[58] Indeed, this argument can be extended across subsequent studies which have since emerged. When examined collectively, the microarray studies have each identified similar groups of target genes which support the hypothesis that E2Fs regulate transcription of genes in differentiation and development.[57,59-63]

While microarray data point towards a role for E2Fs in regulating processes outside of cell cycle regulation, further acceptance of this idea has come from new and sophisticated technologies designed to identify what promoters are occupied by E2Fs on a genomic scale, and whether such interaction is representative of a functional relationship in vivo. Still a relatively new technique, two groups have employed a genomic approach that combines chromatin immunoprecipitation with microarray technology, so-called "ChIP-on-chip" to identify gene promoters occupied by E2F transcription factors.[64-70] With this technique, it has become apparent that E2Fs bind to an unexpectedly high number of gene promoters, many not through the traditional E2F consensus site[65,68] or through new co-factors.[67] As a result, new roles for E2Fs have been identified in regulation of genes required for G_2/M, DNA repair,[66] as well as mitochondrial biogenesis and metabolism.[67] For example, as a result of the diversity of potential novel E2F regulated targets identified through ChIP-on-chip for E2F4, a motif finding algorithm was employed to determine if binding motifs of other co-factors were overrepresented within the promoters of the newly identified E2F targets.[67] Through this method, the binding motif of nuclear respiratory factor-1 (NRF-1), an activator of numerous mitochondrial genes, such as cytochrome C and a number of mitochondrial ribosomal proteins (MRPs), was identified.[67] Subsequent ChIP analysis demonstrated that NRF-1 and E2F4 co-existed in close proximity at both cell cycle and mitochondrial protein promoters. Further, siRNA mediated knockdown of NFR-1 led to a reduction in the RNA levels of target genes.[67]

As the microarray chips themselves evolve to include increased proportions of gene regulatory and promoter sequences within the genome, the number of E2F target genes is likely to increase. Indeed, through a recent analysis of ChIP-on-chip data performed with arrays representing 30 Mb of the human genome, it was suggested that greater than 20% of promoters in the human genome bind E2F1.[70] This is a provocative hypothesis indeed if even a fraction of these sites are functional. Finally, using a distinct approach where genes induced by E2F1 are identified based on subtraction screening, separate groups of genes were identified in response to serum stimulation.[71] Whereas E2F1 expression coupled with serum stimulation induced genes with roles in cell cycle progression, DNA replication, and apoptosis, E2F1 was also capable of directly inducing genes outside of serum stimulation, suggestive of atypical cell cycle independent targets.[71] Of these cell cycle independent E2F targets, many have known roles in development and differentiation.[71] Thus together these data provide evidence that our scope of E2F function should be expanded to include regulation beyond the cell cycle, perhaps through atypical means.

IN VIVO EVIDENCE FOR SPECIFITY OF FUNCTION AND ROLES BEYOND CELL CYCLE REGULATION

While much of what we know regarding an expanded view of E2F function has been established from in vitro settings, it remains essential to determine whether or not these hypothesized roles represent bona fide E2F regulated processes in a physiological context. Indeed, an increasing number of recent examples demonstrate that E2Fs are capable of regulating specific, cell cycle independent functions in vivo.

One of the first characterized examples of in vivo cell cycle independent function for E2Fs came from the examination of the role of E2F4 in adipocyte differentiation.[72,73] As E2F4 deficient and chimeric MEFs exhibit increased hormone induced adipocyte differentiation, and E2F4 is capable of binding and regulating transcription of PPARγ, a mediator of adipocyte differentiation, E2F4 was hypothesized to act as a negative regulator of adipocyte ifferentiation.[72] Initially, E2F4 mediated adipocyte differentiation was thought to be regulated by pocket protein interaction, namely through p130, as p130 was capable of cooperatively repressing the PPARγ promoter through E2F4.[72] Subsequent studies, however, demonstrated that E2F4 also represses spontaneous adipocyte differentiation, a model distinct from hormone induced differentiation, and this occurs independently of both cell cycle regulation and pocket protein interaction.[73] In support, the increase in spontaneous adipocyte differentiation in E2F4 deficient MEFs occured in the absence of any known defects in cell cycle exit, proliferation, or E2F responsive cell cycle related gene expression in these cells or precursor cells.[73] Further, while some aspects of adipogenesis are regulated through E2F4 interaction with pocket proteins, evidence exists suggesting that it is controlled, at least in part, independently from pocket proteins. Truncated E2F4 proteins lacking the pocket protein binding domain are capable of restoring spontaneous adipocyte differentiation of E2F4 deficient MEFs to wild-type levels suggesting that, in this model, E2F4 can inhibit differentiation independently from interaction with pocket proteins.[73] Together these data revealed an altogether novel aspect of E2F function whereby E2F4 is observed to be capable of regulating a cell cycle independent phenomenon, and this novel function appeared independent from interaction with pocket proteins.

In addition to its role in adipocyte differentiation, two recent studies have demonstrated novel roles for E2F4 through the study of in vivo phenotypes of E2F4 deficient mice.[74,75] As E2F4 deficient mice exhibit a striking respiratory defect, the underlying cause has ecently been examined in greater depth.[74] Analysis of the airway epithelium during development reveals an absence of ciliated cells that are usually present in this region, and instead exhibit an excess of mucin secreting cells.[74] Examination of differentiation markers for ciliated cells and mucin secreting cells reveals that in the absence of E2F4, a significant population of cells stained with a normal respiratory epithelial marker also exhibit aberrant staining for mucins, a component normally restricted to specialized goblet cells.[74] Further analysis demonstrated that the mucin containing cells comprised two types; one population likely representing goblet cells, and a second population of atypical mucin containing cells.[74] These observations, coupled with the observation that there is a complete absence of markers for differentiated ciliated cells are suggestive of defects in cell fate decision and further support the hypothesis that E2F4 plays a role in differentiation within the respiratory epithelium.[74] As no differences are observed in cell proliferation in the airway epithelium in the absence of E2F4, these data further support a cell cycle independent role for E2F4 in differentiation.[74]

While this study does not address the molecular mechanism through which E2F4 regulates cellular differentiation of the airway epithelium, a second recent study examining the E2F4 phenotype describes a novel role for E2F4 in development of the ventral telencephalon and provides tantalizing data indicating that this function is mediated through a genetic interaction with the Sonic Hedgehog morphogenetic pathway.[75] Here, the absence of E2F4 results in a dramatic loss of ventral telencephalic structures, coincident with aberrant expression of homeodomain markers of specified ventral structures and a specific absence of Sonic Hedgehog expression.[75] While proliferation of neural progenitors is unaffected, self-renewal of neural precursor cells is reduced in the absence of E2F4.[75] With such results, it could be hypothesized that E2F4 is mediating these functions as a result of its known role in cell cycle regulation, however, as restoration of Sonic Hedgehog signaling rescues the self-renewal and homeodomain gene expression in vitro, and as E2F4 deficiency specifically reduces expression of a Sonic Hedgehog reporter gene in vivo, these data convincingly demonstrate that E2F4 mediates development of the ventral telencephalon through a novel interaction with the Sonic Hedgehog pathway.

In all three instances, while it is clear that E2F4 is capable of functions beyond cell cycle regulation, whether or not these functions occur independently of pocket protein interaction remains unresolved. In the case of adipocyte differentiation, it is clear that pocket proteins are involved, but a pocket protein independent aspect still exists. Regarding differentiation in the airway epithelium and patterning of the ventral telencephalon, whether such phenomena are occurring through pocket protein interaction is still a matter of significant interest.

While the idea that E2Fs are capable of cell cycle independent function is certainly provocative, perhaps an even more fundamental issue is whether or not such functions are mediated independently of pocket protein interaction. In this regard two recent examples have emerged implicating E2F3 in the regulation of cell cycle independent processes during development; namely, differentiation and migration in the developing nervous system.[76,77] In both instances regulation of these processes is carried out through E2F3 interaction with its cell cycle regulatory partner, Rb.

First, a specific, cell cycle independent role for E2F3 in modulating neuronal differentiation through Rb has been recently described in the retina (ref. 76). While absence of Rb in the retina is known to result in inappropriate proliferation of progenitor cells and death of specific cell types,[78] recently Rb deficiency in the retina has also been observed to result in a defect in differentiation of a subset of amacrine neurons.[76] In the absence of Rb, starburst amacrine (SAC) neurons exhibit a defect in the advanced stages of differentiation.[76] Despite the ability of Rb to interact with numerous other molecules, this defect in SAC neuron differentiation is mediated particularly through E2F3a.[76] When retinal specific Rb deficient mice are interbred with mice deficient for E2F1, E2F2, or E2F3, only Rb:E2F3a deficient retinas exhibit restoration of SAC neuron differentiation, thereby demonstrating specificity of function for E2F3a in vivo.[76] Moreover, the study also provides evidence that E2F3 mediated regulation of SAC neuron differentiation occurs independently of cell cycle regulation. While Rb:E2F3 deficient retinas still exhibit ectopic proliferation suggesting that aberrant differentiation could be the result of defects in proliferation, the results argue otherwise. SAC differentiation was also examined in Rb:E2F1 deficient retinas where ectopic proliferation and apoptosis were both rescued.[76] As Rb:E2F1 deficient retinas exhibit a similar defect in SAC differentiation in the absence of proliferation defects, these observations demonstrate that in vivo, E2F mediated proliferation can be separated from its effect on differentiation. Further, these results demonstrate that E2F1 and 3 are capable of specific functions, and that through pocket protein interaction are capable of regulating differentiation in a cell type specific manner.

The second example of a role for E2F3 in mediating specific cell cycle independent function through its pocket protein interacting partner comes from the study of neuronal migration during forebrain development. We have previously reported that specific absence of Rb in the telencephalon during development results in not only ectopic proliferation of neural precursor cells, but also aberrant migration of a subpopulation of interneurons.[79,80] While these interneurons are specified, they exhibit a cell autonomous defect in migration.[80] In a follow-up study, we examined the role of E2F1 and 3 in mediating the Rb requirement for neuronal migration using mice with compound deficiency for Rb and E2F1 or Rb and E2F3.[77] Similar to SAC neuron differentiation in the retina, we observe that neuronal migration is mediated specifically through E2F3 as only Rb:E2F3 is capable of restoring interneuron migration.[77] Distinct from the retina, however, is the finding that E2F1 and E2F3 are each capable of mediating proliferation of neural precursor cells as both Rb:E2F1 and Rb:E2F3 deficient embryos exhibit a rescue of ectopically proliferating cells.[77] At first glance these results may appear contradictory in interpreting the role of E2F3 in Rb mediated migration as a cell cycle independent phenomenon; however, three lines of evidence support our interpretation that this does indeed represent a cell cycle independent role for Rb/E2F3. First, a comprehensive analysis of ectopically proliferating cells in conditional Rb mutants reveals that they are unlikely to contribute to aberrant migration, as ectopically proliferating cells were not observed to express markers of migrating neurons.[77] Next, we performed microarray analysis, specifically on the population of migrating interneurons in control and Rb deficient tissue to gain insight into which Rb regulated genes could be mediating migration.[77] Much to our surprise, many of the genes identified through the microarray included genes with known or hypothesized roles in neuronal migration, including, neogenin, a gene identified as a novel E2F target gene capable of activation in a cell cycle independent manner.[71] Finally, as we observe that both E2F1 and 3 are capable of regulating neural precursor proliferation through Rb, yet only E2F3 is capable of mediating migration, our data demonstrate that proliferation and migration can be mechanistically dissociated. Thus these data, which demonstrate specific roles for E2F3 in mediating cell cycle independent functions through Rb, represent the first physiological demonstration that in vivo roles for the Rb/E2F pathway beyond cell cycle regulation exist.

POCKET PROTEIN E2F MEDIATED REGULATION OF CHROMATIN STRUCTURE

The question that follows, then, is if E2Fs are capable of specific, cell cycle independent functions in vivo through interaction with their cell cycle regulatory partners, how is such specific regulation of cell cycle independent gene expression achieved? Perhaps the answer lies not with E2F itself, but rather within the pocket protein family. In addition to E2F transcription factors, Rb is capable of interacting with a number of different proteins (reviewed in ref. 81) and has been suggested to mediate cell cycle independent function (reviewed in Ref. 82). While Rb family members bind to E2F through both the pocket domain and the carboxy-terminal region, Rb, along with p107 and p130, are also capable of binding proteins containing an "LXCXE" amino acid sequence through a distinct site within the pocket domain known as the LXCXE binding site[83] (reviewed in refs. 84 and 85). The nature of the interaction is such that E2F binding and inactivation are thought to occur independently from protein interaction through the LXCXE binding site.[86 87,88] The significance of the LXCXE binding site was first highlighted through experiments which demonstrated that the transforming oncoproteins capable of inactivating Rb; namely E1a, SV40 large T antigen, and E7 each contain LXCXE sequences and require an intact LXCXE binding site to inactivate Rb function (reviewed in ref. 89). Subsequent studies have identified numerous endogenous proteins which contain an LXCXE sequence and are capable of binding to pocket proteins through the LXCXE binding site (reviewed in refs. 3, 84, 85 and 90). Many of these LXCXE containing proteins belong to families of chromatin modifying proteins, such as histone deacetlyases,[91-94] histone methyltransferases,[95] and members of the SWI/SNF ATP-dependent nucleosome remodeling complexes[96-98] and have been shown to play important roles in mediating E2F regulated gene expression.

Similar to the direction of the initial work characterizing Rb and E2F function, the functional significance of pocket protein interaction with chromatin modifying proteins has been investigated largely in the context of cell cycle regulation through in vitro experiments. For example, Rb has been shown to interact with histone deacetylase 1 (HDAC1), and Brg1/hBRM, members of the SWI/SNF nucleosome remodeling complex, to repress transcription of classical E2F target genes, most notable cyclin E.[91-93,99] This repression required functional deacetylase activity, as inhibiting deacetylase activity relieved Rb mediated repression of cyclin E transcription.[91,92,99] In addition, histone methylation represents another example of how Rb interaction with chromatin modifying enzymes can mediate transcriptional repression of E2F genes. Here Rb was shown to exist in a complex with Suv39h1, a histone methyltransferase, and heterochromatin protein 1 (HP1) a methylated histone binding protein. Together, Suv39h1 and HP1 were shown to associate with Rb to repress E2F mediated transcription of cyclin E in fibroblasts in vitro through methylation of histones at the cyclin E promoter.[95] Thus collectively, these studies established roles for Rb/E2F in influencing chromatin structure through multiple means as a way of regulating transcription at the G_1/S transition.

Could Rb/E2F mediated regulation of chromatin structure represent a mechanism of regulating cell cycle independent gene expression in vivo? This is an attractive possibility indeed, and preliminary evidence exists in support of this hypothesis.

First, Rb/E2F mediated repression of gene expression through chromatin modifying enzymes such as Brg1/Brm has been shown to require other co-factors, many of which have well defined roles outside of cell cycle regulation. For example, in cultured cells Rb has been shown to interact with Brg-1/hBRM to repress E2F mediated transcription of cyclin A, an event which is HDAC independent, and ultimately impacts on entry from G_2 into mitosis.[99] This event, however, appears to require the presence of CtBP, and polycomb proteins HPC2 and Ring-1 within the Brg1-Rb-E2F complex.[100] What is significant about the presence of CtBP and polycomb proteins within the complex is that each exhibit well known roles in regulating gene expression and cellular events beyond cell cycle regulation. CtBP is a transcriptional co-repressor, originally characterized in Drosophila as a corepressor for genes which are required for establishing the anterior-posterior and dorsal-ventral body axes in early development (reviewed in ref. 101). Ring-1 and HCP2 act as molecular scaffolds for other polycomb group proteins, which together form complexes required for establishing patterns of Hox gene expression during development (reviewed in ref. 102). Thus the establishment of a link between Rb/E2F and polycomb proteins, coupled with emerging observations about the diversity of E2F target genes including Hox genes, lends itself to the hypothesis as to how

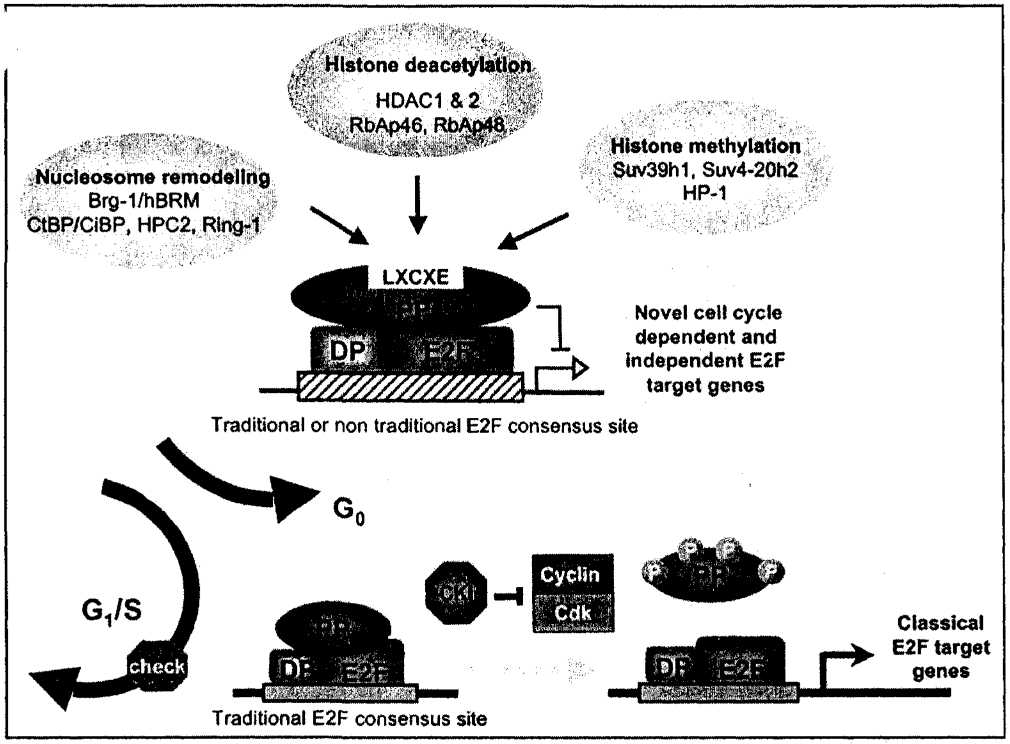

Figure 2. Possible mechanism of chromatin structure mediated regualtion of E2F gene expression through pocket proteins. Regulation of E2F activity through pocket protein interaction with chromatin modifying enzymes or pocket protein phosphorylation. Pocket protein/E2F interaction with the traditional E2F consensus site is well characterized in the context of the G_1/S transition and classical E2F targets (lower). Interaction of pocket proteins through chromatin modifying complexes through the LXCXE binding cleft, combined with E2F interaction through non traditional consensus sites represents a possible means of achieving specific transcriptional regulation of novel cell cycle dependent and independent genes outside of G_1/S (upper). The two pathways are not mutually exclusive. PP, pocket proteins.

Rb/E2F could be regulating gene expression outside of cell cycle regulation. In addition, the subunit or co-factor specificity within nucleosome remodeling complexes provides a model for how Rb mediated regulation of chromatin structure could direct the specificity of E2F mediated gene expression in a cell cycle independent context in vivo.

Indeed, the latter hypothesis is particularly enticing as evidence in support of it has recently been shown in the context of a complex physiological phenomenon: neuronal terminal differentiation. In a recent study, isoforms of the methylated histone binding protein HP1 have been shown to exhibit preferential association in the pocket protein histone methyltransferase complex, with preference determined by the stage of neuronal differentiation.[103] In early differentiating cerebellar granule neurons, ChIP experiments demonstrate that HP1γ is preferentially located at methylated histone residues in E2F responsive elements within the E2F1 promoter region.[103] At the early stages of neuronal differentiation, E2F1 is still transcribed despite methylation, likely as a result of increased acetylation relative to methylation.[103] As neuronal differentiation proceeds, however, HP1γ is replaced with HP1α.[103] Concomitantly, histones in the E2F responsive elements become increasingly methylated resulting in significant repression of E2F1.[103] The significance of this observation was demonstrated in vitro where cells fail to express markers of differentiated neurons and downregulate E2F1 expression after HP1α knockdown.[103] In vivo, HP1α expression is excluded from proliferating cerebellar ganule progenitors cells and strongly expressed in post-mitotic migrating neurons.[103] Together these data support a hypothesis where the temporal shift in HP1 isoform association with methylated histones guides neuronal differentiation.[103] Further, the authors propose a model whereby HP1γ induces transient repression throughout the cell cycle, in contrast to HP1α, which is proposed to direct longer term gene silencing.[103]

While these newly described roles for pocket proteins in regulating chromatin structure have served to expand our view about how they function to regulate gene expression, in both examples only regulation of classical E2F target genes was examined, and both in the context of cell cycle regulation. Thus determining whether pocket protein mediated chromatin remodeling could be involved in regulating events beyond cell cycle regulation still remains a key question (Fig. 2). One possible means of addressing this issue would be to exploit the differential binding of chromatin modifying enzymes to pocket protein-E2F complexes and examine the consequences in a physiological context. As chromatin modifying enzymes interact with pocket proteins largely through the LXCXE binding site, a site distinct from E2F interaction, interfering with the LXCXE site represents a way of examining whether chromatin remodeling could be regulating cell cycle independent function. In an elegant series of experiments, a transgenic mouse was engineered in which the LXCXE binding domain within Rb is mutated (RbΔL), yet Rb/E2F interaction is preserved.[104] In these animals, which are viable and fertile, RbΔL expression levels in MEFs are similar to controls, and RbΔL interaction with E2F transcription factors is indistinguishable from wild-type, however, interactions with chromatin modifying enzymes such as HDAC1 and 2; RbAp48, a component of the HDAC complex; and the Brg1/hBRM co-factor CtBP, are defective.[104] In vitro analysis of MEFs cultured from these animals reveals aberrant methylation of pericentric heterochromatin resulting in an increased proportion of aneuploid cells in RbΔL mutants. What is remarkable about these animals, in relation to cell cycle independent function, however, is their differences in E2F regulated gene expression profiles in proliferating MEFs compared to quiescent MEFs. In the context of typical E2F target gene expression, proliferating RbΔL mutant MEFs exhibit a pattern of E2F regulated gene expression similar to wild type.[104] Consistent with this, passage through G_1/S ppears unaffected in these cells. By contrast, in serum starved MEFs, e pattern of E2F regulated gene expression of RbΔL mutants more closely resembles that of germline Rb deficiency, a situation where E2F activity is grossly deregulated.[104] One interpretation of this data is that collectively, chromatin remodeling through the LXCXE binding site of pocket proteins preferentially influences gene expression in quiescent cells over proliferating cells. Thus the RbΔL mutant could represent an attractive physiological model to examine this hypothesis in vivo in an environment where the G_1/S transition remains intact.

CONCLUSION

Overall, as a result of these many new and exciting directions, our view regarding E2F function has changed dramatically since its initial discovery. The emerging in vitro data regarding novel E2F co-factors and newly hypothesized recognition sites, as well as our understanding about the role that chromatin structure plays in regulating transcription together challenge our view about the possible breadth and specificity of E2F function. Combined with newly described in vivo examples of specific functions beyond cell cycle regulation, a framework is emerging to examine the physiological significance of an expanded view of E2F function.

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APPENDIX D

Co-author publications—first page

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Development/Plasticity/Repair

Cell Cycle Regulator E2F4 Is Essential for the Development of the Ventral Telencephalon

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Early forebrain development is characterized by extensive proliferation of neural precursors coupled with complex structural transformations; however, little is known regarding the mechanisms by which these processes are integrated. Here, we show that deficiency of the cell cycle regulatory protein, E2F4, results in the loss of ventral telencephalic structures and impaired self-renewal of neural precursor cells. The mechanism underlying aberrant ventral patterning lies in a dramatic loss of Sonic hedgehog (Shh) expression specifically in this region. The E2F4-deficient phenotype can be recapitulated by interbreeding mice heterozygous for E2F4 with those lacking one allele of Shh, suggesting a genetic interaction between these pathways. Treatment of E2F4-deficient cells with a Hh agonist rescues stem cell self-renewal and cells expressing the homeodomain proteins that specify the ventral telencephalic structures. Finally, we show that E2F4 deficiency results in impaired activity of Shh forebrain-specific enhancers. In conclusion, these studies establish a novel requirement for the cell cycle regulatory protein, E2F4, in the development of the ventral telencephalon.

Key words: E2F4; cell cycle; Sonic hedgehog; neural precursors; neural patterning; telencephalon

Introduction

Forebrain development is characterized by extensive proliferation of neural precursors combined with complex structural transformations regulated by multiple morphogenic signaling pathways (for review, see Fuccillo et al., 2006). This morphogenetic signaling must be tightly coordinated with cell cycle control to ultimately shape the developing brain. Presently, little is known regarding the mechanisms by which the cell cycle machinery is integrated with these key developmental events.

Studies are emerging demonstrating the importance of cell cycle regulatory proteins in nervous system development. In particular, members of the retinoblastoma (Rb) family of cell cycle genes, and genes which in turn regulate Rb activity, have been shown to have important roles both in cell cycle-dependant and developmental processes that go beyond the mechanics of cell division (for review, see McClellan and Slack, 2006). For example, Rb has been shown to have a critical function in regulating terminal mitosis of neuroblasts in the CNS, PNS, and retina (Chen et al., 2002; Ferguson et al., 2002; MacPherson et al., 2003; Marino et al., 2003). Recently, we and others have shown that Rb exhibits non-cell cycle-dependent functions. For example, Rb has been shown to have a cell-autonomous function in ventral telencephalon development, because telencephalon-specific Rb-deficient mice exhibit a migration defect in ventrally derived interneurons (Ferguson et al., 2005). The closely related Rb family member, p107, has also been shown to be an important regulator of precursor self-renewal (Vanderluit et al., 2004). This regulation comes about through modulation of the activity of the fate-determining Notch signaling pathway. Clearly, cell cycle regulators and, in particular, members of the Rb family have functions beyond the regulation of the cell cycle machinery and play a pivotal role in shaping the developing brain.

Rb family proteins execute the function of cell cycle regulation by regulating the activity of E2F transcription factors (for review, see Trimarchi and Lees, 2002). In addition to regulating genes involved in cell cycle progression, E2Fs are ubiquitously expressed and control an array of genes involved in development, differentiation, and apoptosis (Muller et al., 2001). E2F4, a binding partner for Rb and p107, is believed to be a repressor of transcription (Trimarchi and Lees, 2002; Attwooll et al., 2004) and may be involved in the regulation of differentiation. E2F4-deficient mice exhibit growth retardation and developmental defects, including hematopoietic cell maturation and gut epithelial development (Humbert et al., 2000; Rempel et al., 2000). Although E2F4-deficient mice exhibit defects in craniofacial devel-

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The Retinoblastoma family member p107 regulates the rate of progenitor commitment to a neuronal fate

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The Retinoblastoma protein p107 regulates the neural precursor pool in both the developing and adult brain. As p107-deficient mice exhibit enhanced levels of Hes1, we questioned whether p107 regulates neural precursor self-renewal through the repression of Hes1. p107 represses transcription at the Hes1 promoter. Despite an expanded neural precursor population, p107-null mice exhibit a striking reduction in the number of cortical neurons. Hes1 deficiency rescues neurosphere numbers in p107-null embryos. We find that the loss of a single Hes1 allele in vivo restores the number of neural precursor cells at the ventricular zone. Neuronal birthdating analysis reveals a dramatic reduction in the rate of neurogenesis, demonstrating impairment in p107$^{-/-}$ progenitors to commit to a neuronal fate. The loss of a single Hes1 allele restores the number of newly generated neurons in p107-deficient brains. Together, we identify a novel function for p107 in promoting neural progenitor commitment to a neuronal fate.

Introduction

Cell cycle genes and specifically those genes that regulate the G1/S transition have been shown to play an important role in regulating the neural precursor population. Members of the cyclin-dependent kinase inhibitor (CDKI) family have received much of the attention. CDKIs, p21^{Cip1}, and p27^{Kip1} negatively regulate embryonic and adult neural precursor proliferation (Doetsch et al., 2002; Kippin et al., 2005). Bmi-1 promotes self-renewing cell division in both hematopoeitic and neural precursors through the transcriptional repression of CDKIs, p16^{Ink4a}, and p19^{Arf} (Molofsky et al., 2003, 2005). However, cell cycle regulators impacting the neural precursor population are not only restricted to CDKIs (McClellan and Slack, 2006). We have recently shown that the Retinoblastoma (Rb) family member p107, an inhibitor of the cell cycle G1/S transition, negatively regulates the neural precursor pool in the developing and adult brain by regulating self-renewal (Vanderluit et al., 2004). p107 has been shown to function by interacting with E2F transcription factors (preferentially E2F4) to repress the transcription of genes required for cell cycle progression (Stevaux and Dyson, 2002). Distinct from other Rb family members, p107 is only expressed in cycling neural precursor cells in the ventricular zone (VZ; Jiang et al., 1997).

The Notch–Hes pathway is necessary for self-renewing cell division and, thus, maintenance of the neural precursor population (Ishibashi et al., 1995; Ohtsuka et al., 2001; Hitoshi et al., 2002; Hatakeyama et al., 2004). Whereas the deletion of either Notch1, Hes1, or Hes1 and Hes5 causes premature differentiation of embryonic neural precursors, resulting in their depletion (Ishibashi et al., 1995; Ohtsuka et al., 2001; Hitoshi et al., 2002), the overexpression of activated Notch1 or Hes1 results in an expansion of neural precursor numbers (Ishibashi et al., 1994). Hes1 and Hes5 inhibit differentiation by repressing the expression of the proneural genes *Mash1*, *NeuroD*, and *Math1* (Sasai et al., 1992; Ishibashi et al., 1995). Because the Notch–Hes signaling pathway is crucial for neural precursor self-renewal and inhibition of premature differentiation, we asked whether the cell cycle protein p107 may be regulating the neural precursor population and progenitor differentiation by the repression of Hes1.

In this study, we demonstrate that the p107-mediated regulation of neural precursor number occurs through the repression

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Abbreviations used in this paper: BS, binding site; CDKI, cyclin-dependent kinase inhibitor; IZ, intermediate zone; PCNA, proliferating cell nuclear antigen; PH3, phosphohistone H3; pRb, Rb protein; Rb, Retinoblastoma; SVZ, sub-VZ; VZ, ventricular zone.

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Dissociating the dual roles of apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis

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The mitochondrial protein apoptosis-inducing factor (AIF) translocates to the nucleus and induces apoptosis. Recent studies, however, have indicated the importance of AIF for survival in mitochondria. In the absence of a means to dissociate these two functions, the precise roles of AIF remain unclear. Here, we dissociate these dual roles using mitochondrially anchored AIF that cannot be released during apoptosis. Forebrain-specific AIF null (tel. Aif^{Δ}) mice have defective cortical development and reduced neuronal survival due to defects in mitochondrial respiration. Mitochondria in AIF deficient neurons are fragmented with aberrant cristae, indicating a novel role of AIF in controlling mitochondrial structure. While tel. Aif^{Δ} $Apaf1^{-/-}$ neurons remain sensitive to DNA damage, mitochondrially anchored AIF expression in these cells significantly enhanced survival. AIF mutants that cannot translocate into nucleus failed to induce cell death. These results indicate that the proapoptotic role of AIF can be uncoupled from its physiological function. Cell death induced by AIF is through its proapoptotic activity once it is translocated to the nucleus, not due to the loss of AIF from the mitochondria.

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Introduction

Mitochondria are the central relaying stations for apoptotic signals. After the induction of apoptosis, cytochrome *c* is released from the mitochondria that interacts with Apaf1 and procaspase 9, which in turn activates the caspase cascade (reviewed in Yuan *et al*, 2003; Danial and Korsmeyer, 2004). Apart from the caspase-dependent pathway, mitochondrial factors also initiate a caspase-independent apoptotic signaling cascade (reviewed in Cregan *et al*, 2004; Hong *et al*, 2004). This pathway is initiated by the release of the mitochondrial protein, apoptosis-inducing factor (AIF), which translocates to the nucleus and induces DNA fragmentation through interactions with factors including EndoG in *Caenorhabditis elegans*, CypA in mice, and others such as FEN-1 (Susin *et al*, 1999; Daugas *et al*, 2000; Wang *et al*, 2002; Parrish and Xue, 2003; Cande *et al*, 2004). The significance of these interactions, however, are not yet clear, as EndoG and CypA null animals have no apparent defect in apoptosis (Colgan *et al*, 2000; Irvine *et al*, 2005).

The role of AIF in neuronal cell death was first suggested from the observation that AIF translocates to nucleus after the induction of various types of acute neuronal injury *in vitro* and *in vivo* (Zhang *et al*, 2002; Cao *et al*, 2003; Plesnila *et al*, 2004; Wang *et al*, 2004). Mitochondrial release of AIF has been shown to depend on PARP activity (Yu *et al*, 2002; Wang *et al*, 2004). We have previously demonstrated that AIF translocation following neuronal injury is caspase independent (Cregan *et al*, 2002; Cheung *et al*, 2005). Using $Apaf1^{-/-}$ neurons, we have shown that AIF is translocated to the nucleus on induction of apoptosis, and this can be inhibited by microinjecting AIF neutralizing antibodies (Cregan *et al*, 2002). Depending on the cell type and death stimulus, the release of AIF may also be caspase dependent, as studies using *C. elegans* with BH-3 only protein EGL-1 (Wang *et al*, 2002), HeLa cells with staurosporine (Arnoult *et al*, 2003), and rat cortical neurons (Lang-Rollin *et al*, 2003) have previously shown. We have used *Harlequin* (*Hq*) mice, which exhibit only 20% AIF expression (Klein *et al*, 2002), to directly investigate the role of AIF in various models of neuronal cell death. Using $Hq/Apaf1^{-/-}$ double mutant mice we have shown that reduced levels of AIF, along with inactivation of caspase activity, can sustain neuronal survival after DNA damage and excitotoxic induced cell death. These results revealed that AIF is involved in both Bax dependent and Bax independent mechanisms of cell death (Cheung *et al*, 2005). In mammalian systems, therefore, AIF is a key death inducer that functions in multiple mechanisms of neuronal cell death; thus understanding its mechanism of action is crucial.

Apart from the apoptotic role of AIF, studies with AIF depleted cells have indicated that AIF also has a physiological role in the mitochondria. Studies using *Hq* mice, which exhibit cerebellar degeneration and increased sensitivity to

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